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Bony traits and genetics drive intraspecific variation in vertebrate elemental composition

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Abstract

1. Interspecific variation in elemental composition is well known and often leads to predictable differences in ecosystem interactions, but little is known about the extent, causes and importance of intraspecific variation in elemental composition. If intraspecific variation is substantial and has a genetic basis, it may underlie an important mechanism of evolutionary interplay with ecology as individuals compensate for evolutionary changes in elemental demand.
2. To investigate the extent and causes of intraspecific elemental variation in vertebrates, we sampled evolutionary model species *Gasterosteus aculeatus* (Threespine Stickleback) from 12 locations in British Columbia, Canada. Fish were phenotyped, genotyped for *Eda* alleles underlying lateral plate variation and assayed for elemental content (C, N, P).
3. We found stickleback vary widely in elemental composition (2.2%–6.5% P; 3.0–9.4: 1 N:P). Phenotypic models explained the majority of this variation using bony armour traits (pelvis length, lateral plate count), bone mineralization, body size and condition.
4. Subsequent genetic models found allelic variation at *Eda* generates a 7%–14% change in whole organism N:P. As *Eda* allele frequencies are commonly changed through strong natural selection in freshwater habitats, we infer that stickleback elemental composition can evolve rapidly.
5. Further, as genetics are known to drive variation in many of the other influential traits, we conclude that genetic variation constitutes a major source of variation in the elemental composition of *Gasterosteus aculeatus*. As such, we find that elemental composition has a large evolutionary potential which may underlie important evo-eco interactions.

KEYWORDS
eco-evolutionary interactions, ecological stoichiometry, Ectodysplasin, elemental phenotype, *Gasterosteus aculeatus*, phosphorus, stoichiometric trait, Threespine Stickleback

1 | INTRODUCTION

A central tenet of ecosystem ecology is that different species can have unique and predictable effects on their abiotic environments (Sterner & Elser, 2002; Tilman, 1982). These interspecific differences have been well studied, but we know little about intraspecific differences in ecosystem effects (Jeyasingh, Cothran, & Tobler, 2014; Matthews et al., 2011). Recent work has found ecosystem effects can vary substantially within species, but the causes, magnitude and mechanisms by which this occurs are largely unknown (Bassar et al., 2010; El-Sabaawi

et al., 2015; Harmon et al., 2009; Rudman et al., 2015). Differences in ecosystem effects arising from genetic variation rather than plasticity may be especially important, as natural selection here could generate lasting ecological change (Jeyasingh et al., 2014; Matthews et al., 2011). Thus, a mechanistic understanding of intraspecific variation in ecosystem effects is needed for a higher resolution understanding of ecosystem function, and to predict the ecosystem consequences of evolutionary change.

An important mechanism by which species alter ecosystem functions arises from differences in the elemental resources required to build their bodies, leading to differences in resource acquisition and/or release (Sterner & Elser, 2002; Vanni, 2002). For invertebrates, RNA intensive traits (e.g. growth rate) and exoskeletal traits have large influences on phosphorus and nitrogen requirements respectively, while for vertebrates, the skeletal system is the major pool of phosphorus and thus variation in bony traits may be a major source of variation in phosphorus content (Boros, Sály, & Vanni, 2015; El-Sabaawi, Warbanski, Rudman, Hovel, & Matthews, 2016; Hendrixson, Sterner, & Kay, 2007; Sterner & Elser, 2002). Previous work has shown that interspecific differences in elemental composition result in differences in elemental demand, with commensurate effects on the standing stocks and cycling rates of important nutrients (Vanni, 2002; Vanni, Layne, & Arnott, 1997). If within species variation in elemental composition and demand is also substantial, it too may underlie a widespread mechanism whereby phenotypic variation interacts with ecosystem processes (Leal, Seehausen, & Matthews, 2017; Matthews et al., 2011). Thus, understanding the magnitude of intraspecific variation in elemental composition and linking this with its causes and consequences may be an important step towards understanding the variation and evolution of ecosystem effects (Jeyasingh et al., 2014; Leal et al., 2017).

Elemental composition has been shown to vary substantially within species as a result of environmental and ontogenetic factors (Boros et al., 2015; Cross, Hood, Benstead, Huryn, & Nelson, 2015; González, Fariña, Kay, Pinto, & Marquet, 2011; Vrede et al., 2011). Conversely, genetic variation is less studied but could be important if genotypic differences or gene by environment (G × E) interactions alter resource intensive traits (Jeyasingh et al., 2014). Prior work studying genetic variation in elemental composition has been limited to rearing multiple lineages within controlled environments to detect persistent differences (Liess, Rowe, Guo, Thomsson, & Lind, 2013; Roy Chowdhury, Lopez, Weider, Colbourne, & Jeyasingh, 2014; Tobler, Alba, Arias-Rodríguez, & Jeyasingh, 2016). While this work has confirmed that genetic differences do exist, it has not provided links between compositional variation and specific traits or genes. Further, the importance of genetics to compositional variation is unknown, as prior studies have not evaluated the effects of genetics against a background of 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) Is variation in the important traits driven by genetics? and (4) Can one or a few 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 ecology and evolution (Barrett, 2010; Bell & Foster, 1994). This small fish varies phenotypically in numerous physical traits including potentially phosphorus rich bony traits (Hagen & Gilbertson, 1972; Klepaker, Ostbye, & Bell, 2013). As phosphorus can be an important limiting element for freshwater ecosystem processes, such as primary production and nutrient budgets, this bony trait variation may carry with it meaningful ecological consequences for phosphorus dynamics and phosphorus dependent ecosystem functions (Dodds & Whiles, 2010; 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 (Figure 1). Both of these traits vary dramatically based on known genetics (Colosimo et al., 2004; Cresko et al., 2004; Shapiro et al., 2004), enabling these traits to be frequently reduced in freshwater habitats through natural selection (Barrett, 2010; Klepaker et al., 2013). In particular, natural selection in freshwater environments commonly increases the frequency of the low plating allele at the Ectodysplasin locus (*Eda*; Barrett, 2010). Similarly, pelvis reduction and loss occurs often through positive selection on regulatory mutations at the *Pitx1* gene (Chan et al., 2010; Cresko et al., 2004; Shapiro et al., 2004). It is likely that evolutionary reductions in these bony traits also decrease the phosphorus content of stickleback, altering its phosphorus demand and related resource interactions.

While these armour traits may be major determinants of stickleback elemental composition, there may also be trade-offs with other phosphorus intensive traits, such as bone mineralization, which mitigate change in composition. Additionally, traits such as lipid stores, muscle content and body size might influence composition (Boros et al., 2015; Sterner & Elser, 2002). Carbon rich lipids and nitrogen rich muscle contain little phosphorus, so gains here would dilute whole body phosphorus proportions, while body size could alter composition

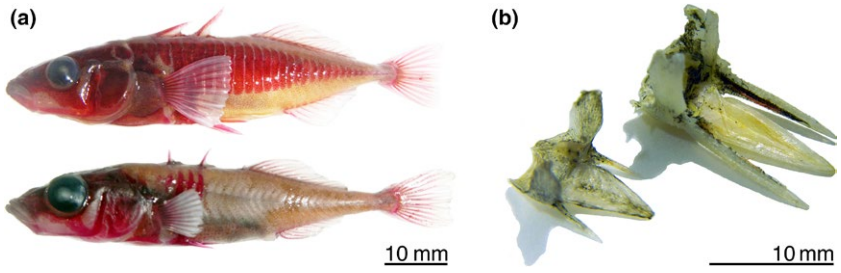

FIGURE 1 Phenotypic variation in lateral plates (a) and pelvic girdle (b). (a) Fish are from Oyster Lagoon (top) and Trout Lake (bottom). Fish are stained with alizarin red which binds to bone calcium. In (b), pelvic girdles were removed from similar sized fish (60–62 mm standard length) to show differences in relative pelvis length, thickness and spine size

though skeletal allometry since bone increases as a proportion of body mass with body size (Casadevall, Casinos, Viladiu, & Ontanon, 1990; Sterner & Elser, 2002).

To investigate links between elemental composition, phenotypic traits and genetics, we first compared fish within Kennedy Lake and Miami River—two populations unusually rich in phenotypic and genetic diversity (hereafter Kennedy and Miami). Both of these sites contain all three genotypes for *Eda*, allowing an intra-population study that avoids the confounded environmental and genetic differences present in a comparison between populations. Fish from both Kennedy and Miami were collected, characterized phenotypically, genotyped for *Eda* alleles and assayed for elemental composition (C, N & P). For each location, elemental variation was modelled against phenotypic traits and genetics.

Additionally, we sampled 10 more stickleback populations representing a diverse range of phenotypes and environments to gain further insight into the extent and causes of intraspecific variation in composition (see Table S1, Figure S1). We investigated which traits and genetics explain variation in elemental composition across these sites to see whether trait-composition and genotype-composition relationships are consistent across diverse environments or overwhelmed by location specific factors.

We expected that bony traits and their underlying genotypes would be major predictors of elemental composition, with less bony individuals 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 (as standard length) would be positively related to phosphorus content through skeletal allometry, and bone mineralization would negatively correlate with other bony traits as a consequence of trade-offs in investment. Overall, we expected genetics (*Eda*) and bony traits with a genetic basis, would explain a large portion of the total intraspecific variation in composition, such that natural selection can meaningfully alter stickleback elemental composition.

2 | MATERIALS AND METHODS

During May–July 2015 we collected 375 Threespine Stickleback (*Gasterosteus aculeatus*) from 12 locations across southwestern British Columbia, Canada (Figure S1). We sampled 22–25 fish from each location except for Cranby Lake (16), North Lake (18), Oyster Lagoon (27), Trout Lake (37), Miami River (61) and Kennedy Lake (71). Phenotypic and environmental details for each study site is provided in the supporting information. All fish were collected using cheddar cheese baited minnow traps deployed for 3 hr and then 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).

We counted the number of lateral plates on the left side of each fish and measured standard length, head length, body depth and pelvis length, as shown in Figure S2. We also removed the seventh lateral armour plate (based on position per Reimchen, 1983), and used this plate to measure bone mineralization (as bone %P), where pure mineral bone (Hydroxyapatite or $Ca_{10}(PO_4)_6(OH)_2$) is 18.5% P by mass. All plates were removed from the left side of each fish except in rare cases where this plate was absent, in which case we used plate seven from the right side.

We discarded all digestive and reproductive tissues prior to elemental analysis as standard procedure (El-Sabaawi et al., 2012). Samples were then dried for 72 hr using a LABCONCO 77545-00-J freeze dryer and each specimen was ground with a Retsch MM400 mixer mill after recording dry mass. We determined phosphorus content (%P) as the mean of two 9–11 mg subsamples of the whole body ground tissue. These samples were ashed at 550°C for 8 hr and digested with 1 N HCl at 105°C for 2 hr before assay with a Mandel UVmini-1240 spectrophotometer using an acid molybdate method (Boros & Mozsár, 2015; Murphy & Riley, 1962). The mean coefficient of variance was <1% between fish replicates and extraction efficiency was >99% for bonemeal (NIST 1486) and spinach (NIST 1570a) standards.

We further assayed each specimen for %C and %N using a 1 mg subsample of whole fish 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. Whole body C:N was used as a measure of condition rather than length-mass residuals, as variation in bony traits also influences mass, making C:N a more reliable proxy for lipid stores (Wilder, Raubenheimer, & Simpson, 2016).

DNA was extracted using Promega Wizard SV96 kits. Stn382 and IDH primers were used to target *Eda* and sex respectively for PCR (Colosimo et al., 2005; Peichel et al., 2004). Amplified DNA was run via electrophoresis with ethidium bromide on 2% agarose gel using a 100 bp ladder. *Eda* alleles were classified as either L (low) or C (complete).

In addition to the aforementioned samples, we took a further sample of 10 adult (61–65 mm) complete plated marine stickleback from Oyster Lagoon to investigate intra-individual phosphorus allocation. We manipulated the armour phenotype of each fish to isolate the effects of armour variation on phosphorus content. From each fish, we individually removed each lateral plate and the pelvic girdle. These components and the remaining body were dried, weighed and individually assayed for phosphorus content via the previously described methods. In our focal populations, LL genotypes are most commonly reduced to a 7 plate phenotype (plate positions 2 through 8, Figure S2), so we used the individual component data to calculate the phosphorus content of this low (2–8) plate phenotype and the complete plate phenotype for each fish.

Prior to statistical analysis, we size adjusted the pelvis length, head length and body depth of each fish to account for allometric correlations with body size within the 12 location dataset. For each trait, we took residuals off the common-within groups relationship from all 12 sites and added these to the mean trait value to allometrically adjust each trait value to the mean body size (as standard length; Reist, 1986). Details of these methods are described elsewhere (Kaeuffer, Peichel, Bolnick, & Hendry, 2012; Wund, Singh, Geiselman, & Bell, 2016) and explained in the supporting information. For size adjustment within

the Kennedy and Miami population datasets, we adjusted the same traits to the mean body size for that population using location specific coefficients of $\log_{10}$ transformed traits against $\log_{10}$ transformed standard length.

Data analysis was done in R (R Core Team, 2016). First, we constructed "location-specific" general linear models (GLMs) to investigate relationships between phenotypic traits and composition within either the Kennedy or Miami populations, and "full dataset" general linear mixed effects models (GLMMs) to investigate compositional variation across all locations, with the latter including location as a random effect. For both model types, we investigated %P and log transformed N:P as response variables. We performed model searches using MUMIN from a global model containing candidate phenotypic traits (Bartoń, 2016). Top models were selected based on corrected akaike information criterion (AICc) after checking all model terms for collinearity via variance inflation factor scores (Fox et al., 2016; Grueber, Nakagawa, Laws, & Jamieson, 2011). Global models contained 8 candidate main effects: standard length, condition (C:N), head length, body depth, pelvis length, sex, bone mineralization and lateral plate count. A correlation matrix for these traits is provided in Figure S3. For the location-specific models, the best model was selected based on AICc and effect sizes were based on partial η^2 (Navarro, 2015), with thresholds of >0.01 (small effect), >0.06 (medium effect) and >0.14 (large effect; Richardson, 2011). For all models, main effects were standardized to a mean of 0 and a *SD* of 0.5 to enable comparison of the coefficients (including categorical variables) as an effect size measure in GLMMs where partial η^2 is unavailable (Gelman, 2008). We averaged the top ranking models (ΔAICc < 5) from the full dataset searches using the MUMIN package (Bartoń, 2016). Lastly, we repeated the model selection process after replacing lateral plate count with *Eda* genotype in the global models (see Figure S4 for lateral plate phenotype vs. *Eda* genotype relationship). For this, we used GLMs for the same three datasets (Kennedy, Miami, full dataset) and investigated only N:P as the response variable. Figures were developed with the VISREG package (Breheny & Burchett, 2016).

3 | RESULTS

At both Kennedy and Miami we observed substantial phenotypic variation in lateral plating, with plate counts ranging from 5/6 plates to 35/36 plates (Kennedy/Miami; Table S2). These populations also varied in standard length (36/38 mm to 62/56 mm), size adjusted pelvis length (10.5/9.2 to 13.7/13.4 mm) and bone mineralization (9.8/10.1% to 11.9/11.9% P; Table S2). The additional 10 populations present in the full dataset contained a wider range of variation in standard length (32–72 mm), bone mineralization (9.2%–12.4% P), lateral plate count (2–36 plates) and size adjusted pelvis length (6.8–13.9 mm).

Composition of *G. aculeatus* was highly variable within the Miami and Kennedy populations, with phosphorus content 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 S3). Across all 12 locations, composition varied even more widely, with phosphorus, nitrogen and carbon spanning ranges of 2.2%–6.5%, 7.3%–12.2% and 30.8%–48.5% respectively (among individuals) and 3.3%–5.0%, 8.1%–11.4% and 35.2%–41.7% (among population means; Table S3). For the full dataset, molar N:P ranged 3× among all individuals (3.0–9.4:1) and 2× among population means (3.9–7.7:1; Table S3). Phosphorus was consistently the most variable element with a coefficient of variation of 18.8% across all populations, compared to 11.0% for nitrogen and 9.5% for carbon (Table S3).

3.1 | Location specific phenotypic models

The best models for %P at both Kennedy and Miami explained most of the variation with 5–7 phenotypic traits (R^2_{Adj} > .76; Table 1). These models found condition had the largest effect on %P (Partial η^2 = 0.46–0.51), with lateral plating, pelvis length, bone mineralization, sex and standard length also having medium to large effects (Partial η^2 = 0.07–0.30; Table 1). Percent phosphorus declined with condition and increased with standard length, pelvis length, lateral plate count and bone mineralization (Figure 2). Sex had differing effects at each site, with males higher in %P at Miami and lower at Kennedy.

Best models for N:P at Kennedy and Miami also explained most of the variation (R^2_{Adj} > .66) but condition had a much reduced effect (Partial η^2 = 0.07–0.11; Table 1). Instead standard length, lateral plate count and bone mineralization were larger effects at both sites (Partial η^2 = 0.13–0.42). Additionally, pelvis length had a medium effect at Kennedy (Partial η^2 = 0.13), while head length had a large effect at Miami (Partial η^2 = 0.15). At Kennedy only, males were higher in N:P than females. In all cases, N:P declined with increases in armour traits, standard length and bone mineralization (Table 1).

3.2 | Full dataset phenotype models

The GLMMs for all 12 locations 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 pelvis length and bone mineralization (e.g. Dougan and Trout; Table S2).

For %P, top GLMMs explained most of the variation (R^2_{Marg} of .70–.75) with 6–8 traits, which were similar to the location-specific models (Table 2). In the averaged model, the most important traits were condition, standard length, lateral plates, pelvis length and bone mineralization, where %P declined with condition and increased with all the others (Figure S5). Of these, condition had the largest effect with an averaged standardized coefficient of −1.13 compared to averaged coefficients of 0.28–0.53 for the others (Table 2).

Top GLMMs for log transformed N:P also explained most of the variation (R^2_{Marg} = .49–.58; Table 2) using similar traits as the %P models. In all top models, we found N:P decreased with standard length, pelvis length, lateral plate count and bone mineralization, while increasing with condition (all p < .001; Figure 3). Effect sizes for the averaged model (as standardized coefficients) were more similar than %P models. Pelvis length (−0.057) and condition (0.057) had the

TABLE 1 Best models based on AICc for %P and N:P at Kennedy and Miami. N:P was $\log_{10}$ transformed prior to modelling. Body depth, pelvis length and head length were size adjusted to the mean population standard length

	Kennedy Lake			Miami River		
Term	**Est.**	***p*-value**	**Par. η^2**	**Est.**	***p*-value**	**Par. η^2**
%P model	R^2_{Adj} = .76			R^2_{Adj} = .81		
Standard length	0.46	<.001	0.30	0.34	.002	0.17
Condition (C:N)	−0.81	<.001	0.51	−1.00	<.001	0.46
Sex (male)	−0.29	.004	0.13	0.34	.013	0.11
Body depth	−0.21	.033	0.07			
Pelvis length	0.30	.004	0.13	0.27	.010	0.12
Lateral plate count	0.26	.005	0.12	0.19	.045	0.07
Bone mineralization	0.54	<.001	0.30	0.33	.006	0.13
N:P model	R^2_{Adj} = .70			R^2_{Adj} = .66		
Standard length	−0.055	<.001	0.39	−0.036	<.001	0.13
Condition (C:N)	0.021	.033	0.07	0.044	.014	0.11
Sex (Male)	0.029	.003	0.13			
Body depth	0.017	.082	0.05			
Head length				−0.036	.003	0.15
Pelvis length	−0.030	.003	0.13			
Lateral plate count	−0.029	.002	0.14	−0.041	<.001	0.23
Bone mineralization	−0.068	<.001	0.42	−0.043	.003	0.15

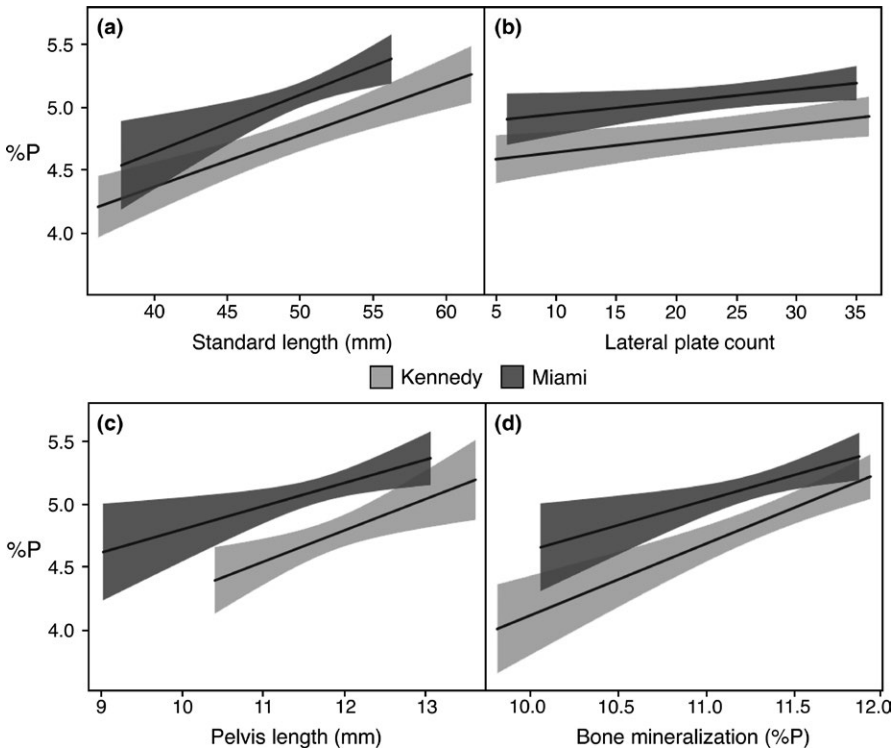

FIGURE 2 Relationships between phenotypic traits and %P at Kennedy and Miami. Plots are outputs from the location specific GLMs (Table 1). Percent P rises significantly with standard length (a), lateral plate count (b), size adjusted pelvis length (c) and bone mineralization (d). Shaded regions depict 95% confidence ranges

largest effects, with the observed range of variation in these traits corresponding to a shift in untransformed N:P from 4.4:1 to 5.7:1. The effects of lateral plating (−0.037), standard length (−0.051) and bone mineralization (−0.044) were nearly as large, and also represented large shifts in untransformed N:P (<4.7:1 to >5.4:1). Sex and head length had smaller (−0.004 to −0.015) and non-significant effects (Table 2).

3.3 | Genetic 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 S4). *Eda* had the largest effect on N:P of all model terms at Miami (p < .001, Partial η^2 = 0.24), with LL genotypes 14.3% higher in N:P than CC genotypes

TABLE 2 Top GLMMs for %P and log transformed N:P with location as a random effect. Top models were selected based on ΔAICc <5 from best model (lowest AICc). Averaged coefficients are full model averages. Model terms are standard length (SL), condition (Cond.), size adjusted body depth (BD), size adjusted head length (HL), sex, size adjusted pelvis length (PL), lateral plate count (LPC) and bone mineralization (BM)

%P top models				Coefficients							
Rank	ΔAICc	R^2_{Marg}	Weight	SL	Cond.	BD	HL	Sex (M)	PL	LPC	BM
1	0	.74	0.32	0.39	−1.14			0.13	0.56	0.27	0.33
2	0.7	.70	0.23	0.38	−1.13		0.12		0.48	0.28	0.33
3	1.1	.72	0.19	0.39	−1.14	−0.12	0.14		0.58	0.27	0.30
4	1.5	.70	0.16	0.38	−1.10				0.44	0.30	0.35
5	3.1	.75	0.07	0.40	−1.15	−0.09		0.13	0.63	0.27	0.32
6	4.5	.72	0.04	0.38	−1.10	−0.09			0.51	0.29	0.34
%P averaged model				**SL**	**Cond.**	**BD**	**HL**	**Sex (M)**	**PL**	**LPC**	**BM**
Importance				1.00	1.00	0.29	0.42	0.39	1.00	1.00	1.00
Coefficient				0.39	−1.13	−0.03	0.05	0.05	0.53	0.28	0.33
Adj. *SE*				0.04	0.05	0.06	0.07	0.07	0.10	0.07	0.05
Significance				<0.001	<0.001	0.57	0.44	0.47	<0.001	<0.001	<0.001
N:P top models				**Coefficients**							
Rank	**ΔAICc**	R^2_{Marg}	**Weight**	**SL**	**Cond.**	**HL**	**Sex (M)**	**PL**	**LPC**	**BM**	
1	0	.49	0.73	−0.051	0.056	−0.021		−0.054	−0.037	−0.044	
2	2.5	.58	0.21	−0.052	0.057		−0.020	−0.065	−0.035	−0.044	
3	5.0	.51	0.06	−0.050	0.051			−0.046	−0.039	−0.048	
N:P averaged model				**SL**	**Cond.**	**HL**	**Sex (M)**	**PL**	**LPC**	**BM**	
Importance				1.00	1.00	0.73	0.21	1.00	1.00	1.00	
Coefficient				−0.051	0.057	−0.015	−0.004	−0.057	−0.037	−0.044	
Adj. *SE*				0.005	0.006	0.011	0.009	0.012	0.008	0.006	
Significance				<0.001	<0.001	0.142	0.618	<0.001	<0.001	<0.001	

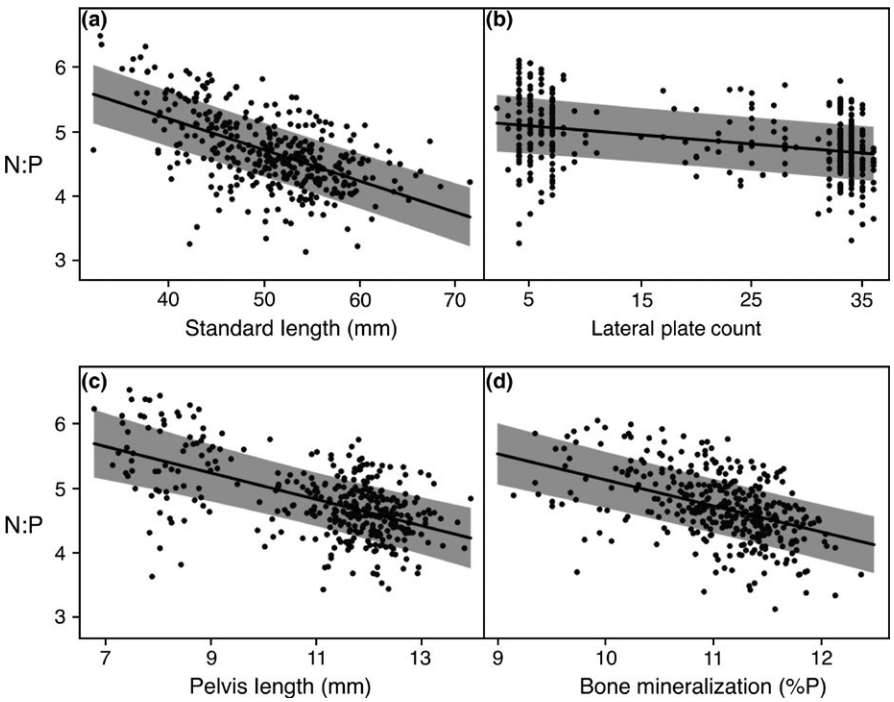

FIGURE 3 Relationships between bony traits and N:P from the best full dataset GLMM (Table 2). N:P is untransformed for visualization. N:P declined significantly ($p < .001$) with standard length (a), size adjusted pelvis length (b), bone mineralization (c) and lateral plate count (d). Shaded regions depict 95% confidence ranges

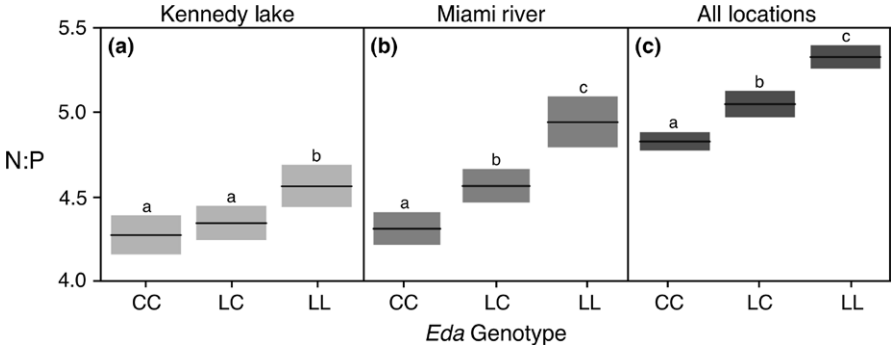

FIGURE 4 Mean untransformed N:P by *Eda* genotype from the best GLM/GLMM models for Kennedy Lake (a), Miami River (b) and the full dataset (c). Genotypes not sharing a letter are significantly different (see Table S4). CC genotypes were significantly lower in N:P than LL genotypes in all cases and were significantly different from heterozygotes (LC) at Miami and all locations. Shaded regions depict ±1 *SE*

(Table S4). We also found *Eda* had a medium sized effect at Kennedy (p = .014, Partial η^2 = 0.10) and across all locations (p < .001, Partial η^2 = 0.09). The best model for the full dataset found a 10.1% increase in N:P for LL vs. CC genotypes (5.3:1 vs. 4.8:1, p < .001), with LC genotypes significantly intermediate to the LL and CC genotypes (Figure 4). Effect sizes and significance for the other model terms were similar to phenotype only models (Table S4).

3.4 | Intra-individual phosphorus allocation

In our sample of 10 complete plated marine fish, we found the lateral plates contained 22.3% of the whole organism phosphorus pool (range of 18.8%–24.1%), while the pelvic girdle contained a further 14.5% (range of 13.0%–16.5%). When comparing the phosphorus content for phenotypes with 2–8 plates vs. complete plating, we found the former set of plates contains 33.6% as much phosphorus as the complete set, such that the removal of all plates except 2–8 caused a 14.8% mean decline in whole body phosphorus (from 3.8% down to 3.3% P). As bone mineral (hydroxyapatite) contains no nitrogen, this magnitude of a reduction in phosphorus content corresponds with a theoretical increase in stickleback N:P of 17.6% (molar ratio).

4 | DISCUSSION

We found the elemental composition of stickleback is highly variable, with elemental ranges (C, N, P) similar to those reported across diverse sets of fish taxa (Benstead et al., 2014; Hendrixson et al., 2007; Vanni, Flecker, Hood, & Headworth, 2002). In %P models, condition (C:N) had by far the largest effect (Tables 1 and 2). These observed declines in %P with increased condition are likely the result of dilution, where gains in carbon rich lipids increase body mass and thus reduce phosphorus as a percentage. Since this effect of condition on %P does not alter the mass of phosphorus (and therefore phosphorus demand), percentage analyses such as this give problematic insight into the variability and basis of an organism's elemental requirements. Clearer insight can be had by evaluating elements of interest in ratios with other important or invariable elements, as we have done here with N:P (Sterner & Elser, 2002). As lipids contain relatively little nitrogen and phosphorus, organism N:P should be decoupled from fluctuations in lipid stores. Furthermore, because nitrogen content is relatively stable (Table S3), N:P models predominantly give insight into variation in phosphorus content.

As expected, the effect of condition in N:P models was much reduced, such that bony traits (bone mineralization, lateral plating, pelvis length) and standard length were the major drivers of variation in N:P (Table 2). Standard length likely influences N:P through skeletal allometry (Casadevall et al., 1990), with similar size-composition relationships having been observed in several other teleost species (Boros et al., 2015; Davis & Boyd, 1978; Hendrixson et al., 2007; Pilati & Vanni, 2007). Condition was still an important predictor of stickleback N:P, but such a pattern is likely the result of the high energetic expense of bone investment, where individuals investing less in bone are higher in condition (Barrett, 2010; Giles, 1983; Marchinko & Schluter, 2007). Thus, condition is positively correlated with stickleback N:P but this is likely a confounded byproduct of the relationship between bony traits and N:P, such that the contribution of bony traits to N:P variation is underestimated even here. Regardless, it is clear that bony traits are the major cause of variation in the phosphorus content (N:P) of Threespine Stickleback.

The surprising variation in stickleback bone mineralization, and its large effect in our elemental models, reveals that whichever factors shape bone mineralization could have a major influence on vertebrate composition and related ecosystem interactions. Variation in mineralization arising from genetic differences would substantially alter resource demand, while plastic variation potentially buffers imbalances between nutrient intake, demand and release. Studies using other fish species have observed plastic reductions in bone mineralization from severely calcium and phosphorus deficient diets, but these studies did not consider genetic variation and the magnitude of these experimental deficiencies may be greater than those encountered by natural populations (Nwanna & Schwarz, 2007; Ye et al., 2006). Similarly, our study was not designed to parse genetic and plastic contributions to variation in bone mineralization and other phenotypic traits. However, if resource constraints do generate plasticity in stickleback bone mineralization, it is possible that we would observe trade-offs in investment between bone mineralization and armour traits within phenotypically diverse populations. However, we found no such correlations (R^2 < .02) between bone mineralization and armour traits (pelvis length, lateral plate count) within the Kennedy and Miami populations.

We do, however, find positive correlations among population means (Table S2) for bone mineralization and lateral plate count (R^2 = .33), as well as pelvis length (R^2 = .30). The result is an interesting, but limited sample size pattern, where populations with the strongest evolutionary reductions in other bony traits also have the lowest bone mineralization (Table S2). This pattern is consistent with a hypothesis of phosphorus

limitation at low armour sites, but phosphorus limitation does not appear likely as these fish have evolutionarily reduced phosphorus demand while occupying littoral habitats where stickleback consume relatively phosphorus rich prey, such as chironomids (Schluter, 1993; Schluter & McPhail, 1992). It is more likely that the observed population level reductions in bone mineralization result from the same selection pressures that reduce other armour traits in these populations, such as improved flight from predators enabled by reduced mass, and resource constraints from energy and/or calcium (Barrett, Rogers, & Schluter, 2009; Bell, Orti, Walker, & Koenings, 1993; Giles, 1983; Marchinko & Schluter, 2007; Reimchen, Bergstrom, & Nosil, 2013). Such adaptation would require genetic variation in bone mineralization, which is largely uninvestigated in fish, but other vertebrate studies have found high levels of heritability (Prentice, 2001; Tse, Macias, Meyer, & Hargens, 2009). Further study into the variation and potential evolution of bone mineralization is needed and may yield important insights into vertebrate composition, how this interacts with nutrient dynamics, and the loss of bone mineralization observed in many vertebrate families (Cohen et al., 2012).

Another notable finding is the strong influence of *Eda* genotype on composition, demonstrating that a single genetic difference can have a large effect on the elemental composition of conspecific individuals. Consistently, we find *Eda* has a medium to large effect on stickleback N:P, with LL genotypes higher than CC genotypes in all models (Table S4), and heterozygotes intermediate between these two (Figure 4). The 10.1% difference in N:P (4.8 vs. 5.3:1) observed across all locations represents 7.7% of the total observed range in N:P (3.0–9.4:1). Thus even against a background of intentionally high environmental variability, we find that a single genetic difference contributes a meaningful change in resource requirements.

The effect of *Eda* on stickleback N:P likely arises as a consequence of its strong influence on lateral plating, but *Eda* also affects other traits via pleiotropy—making causality difficult to ascribe. Most notably, *Eda* has been linked with changes in body shape and growth rate, both of which potentially influence elemental composition (Albert et al., 2008; Barrett et al., 2009). If the effect of *Eda* on elemental composition occurs primarily through changes in lateral plating, we should find a similar difference in N:P between CC and LL genotypes, as we find when completely plated fish are manually reduced to a low plate phenotype. When we did this manual plate removal we found an increase in stickleback N:P of 17.8%, which is similar to the genotypic difference at Miami (14.3%) but larger than the observed genotypic effect at Kennedy (6.5%; Figure 4). This smaller effect at Kennedy could be the result of offsetting pleiotropy, but non-pleiotropic changes in other traits could also reduce the effect of *Eda* by reducing the bone content of all lateral plates (e.g. plates that are shorter, thinner or lower density; Klepaker, 1993; Song et al., 2010; Wiig, Reseland, Østbye, Haugen, & Vøllestad, 2016). As our models take body size into account, we do not think pleiotropic effects on body size underlie the smaller effect at Kennedy, but we are unable to parse the rest of these potential causes. We conclude that *Eda* can have a large influence on composition through changes in lateral plating alone, but additional effects from pleiotropy or changes in related bony traits cannot be ruled out.

A remaining question is how important are genetics in context of the observed natural variation in elemental composition. In addition to the effect of *Eda*, variation in pelvis length—another important predictor of N:P—can have substantial genetic basis (Peichel & Marques, 2017). Within marine populations, variation in pelvis length has been shown to be largely heritable, and deletions in the regulatory regions for the *Pitx1* gene are known to be responsible for some of the major pelvic reductions in freshwater populations (Chan et al., 2010; Leinonen, Cano, & Merilä, 2011; Shapiro et al., 2004). Here we find the pelvic girdle contains 14.5% of the whole body phosphorus pool in marine stickleback, and the observed phenotypic variation in pelvis length in our study populations had an effect on N:P equal to 8.4% of the observed range (Figures 2 and 3). Thus, while we are unaware of the causes of pelvic variation in our study individuals, it is clear that genetic changes can alter pelvis phenotypes and these changes should have large effects on elemental composition (Klepaker et al., 2013). Of the other traits with large effects on N:P, variation in standard length has also been shown to have a large genetic component and natural selection on this variation has achieved more than two fold differences in mean adult standard length between populations (Bell & Foster, 1994; Leinonen et al., 2011; Peichel & Marques, 2017; Reimchen et al., 2013). Thus, despite the uncertain nature of variation in bone mineralization, it is clear that variation in most of the traits important to elemental composition can have a genetic basis, such that natural selection can meaningfully alter stickleback composition.

5 | CONCLUSIONS

Our work demonstrates that elemental composition in Threespine Stickleback is highly variable as a result of genetic and phenotypic variation in a small set of osteological traits. As variation in many of these bony traits is often under strong selection, we conclude that the elemental composition of this species can and has evolved substantially. This genetic variation in elemental composition potentially forms an important part of how ecology and evolution interact. Ecology could influence evolution through selection against individuals with unsuitably high elemental demands, whereas the evolution of elemental composition for any reason—whether directly on elemental requirements or indirectly through other selection affecting bony traits—may affect ecology as individuals with differences in elemental demand compensate through resource interactions (Leal et al., 2017). Whether these eco-evolutionary interactions are abundant, in what form they occur, and to what extent they drive meaningful change remain important areas of future study.

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AUTHORS' CONTRIBUTIONS

R.E. and D.D. designed the study; D.D. collected and analysed the data, and wrote the first draft; R.E. and D.D. contributed substantially to revisions and gave final approval for publication.

DATA ACCESSIBILITY

Data deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.v577v (Durston & El-Sabaawi, 2017).

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SUPPORTING INFORMATION

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