Evolutionary Ecology Research, 2019, **20**: 193–211

The utility of stoichiometric and metabolic theory for understanding the foraging habitat and excretion of threespine stickleback (*Gasterosteus aculeatus*)

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ABSTRACT

Background: Recent extensions to ecological stoichiometry theory propose that the evolution of an organism's elemental content plays an important role in shaping the structure and function of ecosystems. Other work has shown that the elemental content of sticklebacks is meaningfully altered by genetic differences in armour.

Questions: Does genetic-based intraspecific variation in threespine stickleback elemental content explain intraspecific variation in foraging habitat and elemental excretion rates? Are there theoretical effects of intraspecific variation in elemental demand on the community and ecosystem ecology of stickleback populations?

Hypotheses: High phosphorus (low N : P) sticklebacks containing phosphorus-rich traits and genetics will preferentially target phosphorus-rich prey types and exhibit lower rates of phosphorus excretion.

Methods: We studied ten wild populations of threespine stickleback, including two focal populations naturally diverse in individual stoichiometry, related traits, and genetics. Individuals from these populations were captured, excreted, and sacrificed to assess morphology, stoichiometry, genetics, and foraging habitat (using $\delta^{13}C$ stable isotopes).

Results: The N : P ratio of stickleback explained much of the intraspecific variation in $\delta^{13}C$ isotopes within populations; high N : P individuals and *Eda* genotypes were associated with high N : P diet types. But elemental excretion rates and ratios were not correlated with stickleback N : P; instead, they varied in relation to metabolic factors and to reproductive investment. In particular, females investing in low phosphorus reproductive tissues showed high rates of phosphorus excretion.

Conclusions: Genetic-based differences in elemental demand can influence how individuals forage, and highlight the importance of considering sex and reproductive investment when evaluating the ecosystem effects of phenotypic variation. Overall, our results provide partial support for ecological stoichiometry theory as a useful framework for understanding the interplay between intraspecific variation and ecology, and we suggest that evolution can influence ecology through the evolution of elementally expensive traits.

Keywords: eco-evolutionary interactions, ecological stoichiometry, *Ectodysplasin*, elemental phenotype, intraspecific variation, metabolic theory of ecology, phosphorus, stable isotopes.

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INTRODUCTION

Recent studies have accentuated the importance of intraspecific variation in ecology for its contribution to biodiversity, ability to drive change in ecological processes, and integral role in eco-evolutionary dynamics (Bolnick *et al.*, 2011; Roches *et al.*, 2018). While these studies have demonstrated that genetic and phenotypic intraspecific variability can have important effects on ecosystem structure and function (Harmon *et al.*, 2009; Bassar *et al.*, 2010; El-Sabaawi *et al.*, 2015; Rudman *et al.*, 2015), we lack a general understanding of the mechanisms underlying these ecological changes and their relative importance (Schoener, 2011; Jeyasingh *et al.*, 2014). As such, we recognize the importance of intraspecific variation but further study is needed to connect this with its ecological consequences (Bolnick *et al.*, 2011; Leal *et al.*, 2017a; Roches *et al.*, 2018).

In pursuit of this goal, we investigated intraspecific variation in two important ecological interactions – elemental excretion rates and ratios, and foraging habitat – in an attempt to understand the traits, mechanisms, and theories governing these interactions. Variation in elemental excretion rates and ratios is known to be highly important in aquatic ecology due to potential impacts on nutrient cycles (Jeyasingh *et al.*, 2014; Atkinson *et al.*, 2017), while differences in foraging habitat (e.g. benthic vs. pelagic) can alter community structure and rates of nutrient translocation, which provide important links between food webs (Glaholt and Vanni, 2005; Vanni *et al.*, 2005; Atkinson *et al.*, 2017). Recent work has suggested that these ecological interactions may be linked and mediated through intraspecific variation in elemental demand, as predicted by ecological stoichiometry (ES) theory (Matthews *et al.*, 2011; Jeyasingh *et al.*, 2014; Leal *et al.*, 2017a).

Ecological stoichiometry abstracts both animals and resource compartments (diet, waste) into stoichiometric ratios, and then applies mass balance accounting to predict how change in one area will be reciprocated (Sterner and Elser, 2002). Under an ES framework, phenotypic- and genetic-driven variation in elementally expensive traits should alter an individual's organismal stoichiometry (hereafter OS, such as organism N : P) (Durston and El-Sabaawi, 2017; Leal *et al.*, 2017b), and result in compensatory responses in how that animal acquires and/or releases resources (Jeyasingh *et al.*, 2014; Atkinson *et al.*, 2017; Leal *et al.*, 2017b). This reductionist approach has provided numerous insights within the realm of interspecific variation (Vanni *et al.*, 2002; Cross *et al.*, 2005), but its ability to explain intraspecific variation in ecological interactions is unclear (Stephens *et al.*, 2015; Tobler *et al.*, 2016; Tuckett *et al.*, 2016). Alternatively, factors unrelated to nutrient demand – such as differences in morphology or factors governed by the metabolic theory of ecology (MTE) – might instead drive these interactions (Allgeier *et al.*, 2015; Vanni and McIntyre, 2016). The MTE predicts positive effects on metabolic rates from increases in body size and temperature that extend to excretion, since excretion rates are linked with metabolic rates (Allgeier *et al.*, 2015; Vanni and McIntyre, 2016). The effects of MTE on foraging behaviour are less well established but variation in metabolic rates can affect foraging behaviour through a variety of pathways related to bioenergetics and energy budgets (Pecquerie *et al.*, 2010; Humphries and McCann, 2014). As such, we evaluated stoichiometric predictors of excretion rates and foraging habitat alongside a wide range of other factors to discern the most important predictors and theories governing intraspecific variation in these ecological interactions (Bolnick *et al.*, 2011; Leal *et al.*, 2017a).

Our work relied on an evolutionary model species, the threespine stickleback (*Gasterosteus aculeatus*) (Bell and Foster, 1994; Barrett, 2010). Stickleback vary widely in elemental excretion rates but the genetic, phenotypic, and/or stoichiometric drivers of this variation

are unknown (El-Sabaawi *et al.*, 2016; Leal *et al.*, 2017b). Stickleback also vary widely in foraging habitat (Schluter, 1993; Reimchen *et al.*, 2008; Matthews *et al.*, 2010) and individual OS (as N : P) as a result of phenotypic- and genetic-driven variation in phosphorus-rich bony traits (Durston and El-Sabaawi, 2017; Leal *et al.*, 2017b). Most notably, stickleback possess conspicuous variation in bony lateral armour plating as a consequence of allelic variation at the *Ectodysplasin* (*Eda*) locus (Colosimo *et al.*, 2004). These plates are often reduced in freshwater environments (Hagen and Gilbertson, 1972), which meaningfully lowers the whole-body phosphorus content of stickleback while increasing whole-body N : P (Durston and El-Sabaawi, 2017).

We investigated a central prediction from ES: that intraspecific variation in OS alters individual excretion stoichiometry such that stickleback with higher elemental demand (e.g. phosphorus-rich *Eda* genotypes) will retain more and release less of an element (Jeyasingh *et al.*, 2014; Leal *et al.*, 2017a). Alternatively, OS and excretion might be decoupled through variation in components of a stickleback's nutrient budget not considered by ES (Vanni and McIntyre, 2016; Leal *et al.*, 2017a). One decoupling mechanism that has been hypothesized but not previously tested is that female investment into low phosphorus reproductive tissues, rather than high phosphorus somatic tissues, could result in sex being an important predictor of phosphorus excretion rates and excretion stoichiometry (El-Sabaawi *et al.*, 2016). During the breeding season, female stickleback allocate energy almost exclusively into eggs (Bell and Foster, 1994), which are low in phosphorus [1.1% P (El-Sabaawi *et al.*, 2016)] relative to somatic tissues [2.2–6.6% P (Durston and El-Sabaawi, 2017)]. However, factors unrelated to OS may still be more important drivers of intraspecific variation in excretion rates and stoichiometry, such as mass and temperature predictors from MTE, and these may obscure any effect of ES (Allgeier *et al.*, 2015; Moody *et al.*, 2015). We investigated all of these possibilities.

Additionally, we investigated whether predictors from ES (stickleback N : P) alongside morphological and genetic predictors explain intraspecific variation in foraging habitat (as $\delta^{13}C$ stable isotopes). Wide variation in stickleback habitat use has been previously observed, and linked with differences in armour traits, sex, and genetics (Schluter, 1993; Reimchen *et al.*, 2008, 2013; Matthews *et al.*, 2010); however, the explanatory power has typically been low. Any influence from stickleback OS has not been investigated but if stickleback have access to habitats that differ in diet quality, then genetic-based differences in stickleback OS may drive intraspecific variation in foraging habitat. In freshwater lakes, stickleback do have access to phosphorus-rich invertebrates in littoral habitats, such as chironomids and ostracods (Frost *et al.*, 2006; Martinson *et al.*, 2008), along with phosphorus-poor calanoid copepods in pelagic habitats (Lavin and McPhail, 1986; Schluter, 1993; Hall *et al.*, 2004). Thus, if individual nutritional needs drive foraging behaviour, stickleback OS may explain intraspecific variation in foraging habitat. However, other factors besides OS, such as body size, sex, armour phenotype, and trophic morphology, may be more important drivers of foraging habitat through mechanisms such as an improved individual fit with different foraging niches (Reimchen *et al.*, 2008; Matthews *et al.*, 2010).

We began by studying the foraging habitat and excretion stoichiometry of threespine stickleback within two wild populations in British Columbia, at Kennedy Lake and Miami River (Fig. 1). These populations contain natural intra-population variation in *Eda* genotype, thus providing an opportunity to test whether genotypic and/or variation in OS explains intraspecific variation in excretion or foraging interactions within these common environments. In a previous study, we characterized the genetic and phenotypic drivers of OS in these populations and reported for the first time an association between allelic variation (*Eda*) and OS in vertebrates (Durston and El-Sabaawi, 2017). Here, we build on that work

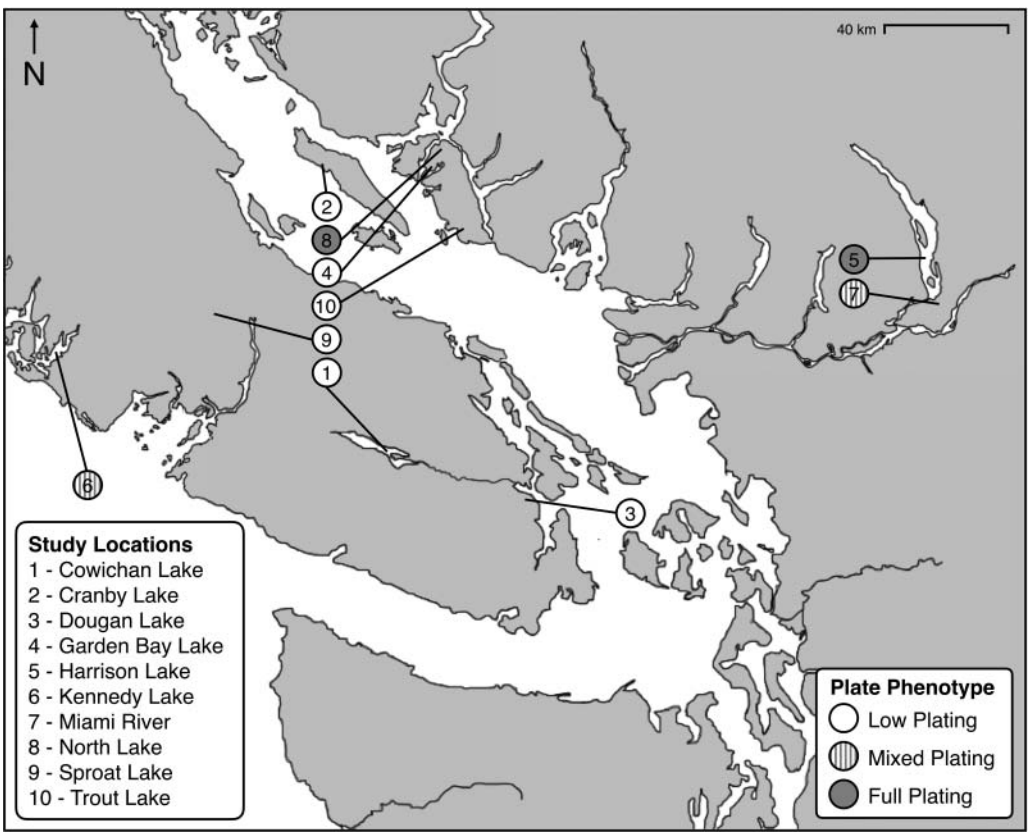

Fig. 1. Map of 10 freshwater study populations in Southwestern British Columbia, Canada sampled in spring and summer 2015. Kennedy Lake and Miami River were mixed plating sites with the full range of variation in plate phenotype (low, partial, full) and *Eda* genotype (LL, LC, CC).

by examining variation in excretion (nitrogen and phosphorus) and foraging habitat (as $\delta^{13}C$ stable isotopes) for these same individuals. Previous stickleback research has demonstrated that $\delta^{13}C$ stable isotopes are strong indicators of stickleback foraging habitat, as pelagic prey are more depleted in ^{13}C than littoral prey in lentic systems (France, 1995; Post, 2002; Bolnick *et al.*, 2008; Matthews *et al.*, 2010; Marchinko *et al.*, 2014). Additionally, we sampled eight other stickleback populations to capture an even wider range of variation in stickleback OS not found within single populations (Fig. 1), and to evaluate whether any patterns observed within the Kennedy and Miami populations were also present across a diverse range of populations and environments (see evolutionary-ecology.com/data/3154Appendix.pdf, Tables S1 and S2).

METHODS

We sampled 311 threespine stickleback (*Gasterosteus aculeatus*) from 10 freshwater locations in British Columbia, Canada during May–July 2015 (Table S2). We took larger samples from two focal locations that were diverse in genetics and OS (71 fish at Kennedy Lake, 61 fish at Miami River), and smaller samples of 21–25 fish from the other eight locations, except for Garden Bay Lake (13), Cranby Lake (16), North Lake (18), and Trout Lake (37). While sharing diversity in *Eda* genotypes and OS, our two focal populations occupy very different environments. Kennedy Lake is a large (6542 ha), oligotrophic lake, while Miami River is hydrologically more similar to a slough than a river, and is both small (6.8 ha) and eutrophic (Table S2).

For all individuals, data on morphology, OS, and *Eda* genotype were available from previous research, where its collection has been described (Durston and El-Sabaawi, 2017). This data includes measures of standard length, head length, dry mass, outer jaw length, body depth, pelvis length, eye diameter, bone mineralization (defined as the percent phosphorus of the bony plates), macroparasite presence/absence, egg presence/absence (in females), condition (as the carbon to nitrogen ratio, C : N) (Wilder *et al.*, 2016), sex, and lateral plate count. *Eda* genotypes were determined by Stn382 primers and electrophoresis on 2% agarose gel using a 100 bp ladder (Colosimo *et al.*, 2005; Durston and El-Sabaawi, 2017). *Eda* alleles were classified as either L (low) or C (complete). Stoichiometry data includes carbon, nitrogen, and phosphorus percentages and ratios (Table S3) (Durston and El-Sabaawi, 2017). Additionally, we calculated gut fullness using residuals from size-adjusted gut length–mass relationships.

Excretion

Fish were captured using minnow traps deployed for 3 hours with cheddar cheese bait (enclosed inside a tea bag to prevent consumption) in accordance with our animal collection (BC MFLNRO) and care permits (University of Victoria). Upon collection, fish were transferred to 550 mL clear plastic containers for 2 hours to observe excretion rates. Each container contained 500 mL of filtered (0.2 μm) source water which was temperature-controlled ±1°C to the source habitat. Water samples were withdrawn at 0, 40, 80, and 120 minutes after gently mixing the water to homogenize nutrient content.

Excretion samples were assayed for phosphorus and nitrogen content using spectrophotometry and fluorometry, respectively (Murphy and Riley, 1962; Holmes *et al.*, 1999). Initially, excretion rates were calculated separately for three periods (0–40 min, 40–80 min, and 80–120 min) to check for any effects of stress and fasting (Whiles *et al.*, 2009). We found that nitrogen excretion rates were stable over the three periods, while phosphorus rates showed small but consistent declines over each period. As such, we combined the data from all three periods to determine the excretion rates and molar ratios.

Isotopic analysis

Fish tissues were analysed for $\delta^{13}C$ using 1 mg subsamples of whole-body ground tissue run on a Finnigan Delta Plus Advantage mass spectrometer at the University of Victoria with a dogfish muscle standard (NRC Canada DORM-4). As lipids can be more depleted in ^{13}C than other tissues, we lipid-corrected the $\delta^{13}C$ values using the same methodology as other stickleback studies (Post *et al.*, 2007; Kaeuffer *et al.*, 2012).

Size standardization

Before statistical analysis, we size-standardized several traits to account for covariance and allometry with body size (as standard length). These traits were head length, pelvis length, gut length, eye diameter, jaw length, body depth, and gut fullness. For analysis within single populations, we used population-specific coefficients from $\log_{10}$-transformed trait–size relationships to eliminate all intra-population trait–size correlations. For analyses across multiple populations, we used common within-group relationships from analysis of covariance (ANCOVA) of $\log_{10}$-transformed traits against standard length similar to some other studies (Reist, 1986; Kaeuffer *et al.*, 2012; Wund *et al.*, 2016).

Data analysis

All data analysis was done in R (R Core Team, 2018). We began by investigating intraspecific variation in elemental excretion rates and ratios using 'location-specific' global linear models for the Kennedy and Miami datasets individually and 'full-dataset' global linear mixed effects (LME) models for all the locations collectively (with location as a random effect). For each of these three datasets, we investigated three response variables: phosphorus excretion rate, nitrogen excretion rate, and log-transformed excretion N : P. We conducted an initial round of model selection using stickleback N : P alongside nine other candidate main effects: dry mass, gut fullness, condition (as C : N), sex, microparasite presence (Y/N), size-standardized eye diameter, size-standardized outer jaw length, size-standardized body depth, temperature (in full dataset models only) or $\delta^{13}C$ (location-specific models only to investigate any influence of diet on excretion). All continuous main effects were statistically standardized to a mean of 0 and a standard deviation of 0.5 to allow comparison of coefficients among both continuous and categorical variables as a measure of effect size for LME models where partial η^2 effect sizes were unavailable (Gelman, 2008). Otherwise, partial η^2 was used as an effect size measure with thresholds of >0.01 (small effect), >0.06 (medium effect), and >0.14 (large effect) (Richardson, 2011; Navarro, 2015). All global models were exhaustively searched and the best models were selected based on the corrected Akaike information criterion (AICc) after checking for collinearity via variance inflation factor (VIF) scores, which were always less than 3 (Bartoń, 2016). We then repeated this analysis with a modified collection of candidate main effects. Specifically, stickleback N : P was replaced by three factors known to drive variation in stickleback N : P: *Eda* genotype, size-standardized pelvis length, and bone mineralization (Durston and El-Sabaawi, 2017).

Next, we used a similar approach to analyse intraspecific variation in foraging habitat (as proxied by $\delta^{13}C$). We elected to model $\delta^{13}C$ directly, rather than an alpha value calculated from $\delta^{13}C$ and baseline values (pelagic, littoral), because we were interested in explaining relative differences rather than parsing the specific contributions of pelagic and littoral sources (Post, 2002). Numerous previous studies that also used the stickleback model system, including several at the same locations as the present study, have shown that $\delta^{13}C$ is a strong proxy for the relative consumption of pelagic vs. littoral prey (Bolnick *et al.*, 2008; Reimchen *et al.*, 2008; Matthews *et al.*, 2010; Marchinko *et al.*, 2014). For the full dataset model, our inclusion of location as a random effect is a statistically robust approach to eliminating the possibility of baseline differences among locations generating relationships between the main effects and $\delta^{13}C$. Furthermore, the single population models from Kennedy Lake and Miami River provide an analysis entirely unaffiliated with differences in baseline.

As with excretion models, global models for foraging habitat were constructed for the Kennedy Lake and Miami River populations individually ('location-specific' global linear models) and across all locations collectively ('full-dataset' global LME models), with the full dataset global LME models again including location as a random effect. For each of these three datasets, we again created two sets of candidate main effects (six global $\delta^{13}C$ models in total). We used the same candidate main effects as we did for $\delta^{13}C$ global models, except (1) standard length was used in place of dry mass, as these traits are highly correlated and standard length is more commonly used in foraging habitat studies (Matthews *et al.*, 2010), and (2) we omitted gut fullness, temperature, and condition (as C : N) as candidate main effects, since these factors were more likely to be influenced by foraging habitat, rather than be drivers of it. All global models were exhaustively searched, best models selected based on AICc and checked for collinearity via VIF scores, which were always less than 2 (Bartoń, 2016). As with the excretion models, we used partial η^2 as a measure of effect size for linear models (Richardson, 2011; Navarro, 2015), and coefficients as an measure of effect size for LME models (Gelman, 2008). All figures were developed using the visreg and ggplot2 packages (Breheny and Burchett, 2016; Wickham *et al.*, 2016).

RESULTS

Excretion

Phosphorus excretion rates varied ten-fold within Kennedy Lake (0.05–0.51 μg/min) and six-fold within Miami River (0.08–0.48 μg/min), with even wider variation observed across all 10 locations (0.02–0.66 μg/min; Table S4). Nitrogen excretion was consistently higher, with ranges of 0.4–2.3 μg/min (Kennedy), 0.6–1.9 μg/min (Miami), and 0.2–2.4 μg/min (all locations). Excretion N : P varied widely from 3 : 1 to 45 : 1 across all locations, with median N : P ratios of 10 : 1 (Kennedy), 13 : 1 (Miami), and 10 : 1 (all locations) (Table S4).

The best excretion models explained a large proportion of the intraspecific variation in phosphorus and nitrogen excretion within our focal locations individually (R^2_{adj} = 0.48–0.58; Table 1), and across all ten locations collectively (R^2_{marg} = 0.55–0.56; Table 1). These models revealed significant increases in nitrogen and phosphorus excretion rates with increasing dry mass (all models except nitrogen at Miami; Table 1). Transforming the nutrient–mass terms from the 'all-locations' models to log-log values, yielded excretion mass scaling coefficients of 0.65 for nitrogen (95% CI = 0.57–0.73) and 0.74 for phosphorus (95% CI = 0.63–0.85). Sex was also an important predictor of phosphorus excretion, with significantly higher phosphorus excretion by females ($P < 0.001$ in all models). Nitrogen excretion was unaffiliated with sex in all cases, but did increase with temperature across all locations ($P = 0.008$). This modelled relationship between nitrogen excretion and temperature represents a 27% increase in nitrogen excretion with a 10°C rise in temperature (Q_{10} = 1.27, 95% CI = 1.25–1.29) over the range of observed temperatures (13.5–27.7°C). Stickleback N : P as a predictor of nitrogen and/or phosphorus excretion rates was only retained in excretion models at Miami, where it had a non-significant relationship with phosphorus and a significant negative correlation with nitrogen excretion. Other factors that were significant in some excretion rate models were gut fullness (all models except phosphorus at Kennedy), body depth, jaw length, and condition (Table 1).

Models for excretion N : P explained only a modest proportion of the total variation ($R^2_{adj/marg}$ = 0.11–0.27; Table 1). The only result that was consistent and significant across

Table 1. Best models for excretion rates (nitrogen, phosphorus) and ratios (N : P) at Kennedy Lake, Miami River, and all 10 locations combined

	Kennedy Lake ($n = 62$)			Miami River ($n = 43$)			All locations ($n = 253$)	
Term	Est.	*P*-value	Partial η^2	Est.	*P*-value	Partial η^2	Est.	*P*-value
Phosphorus excretion		$R^2_{adj} = 0.58$			$R^2_{adj} = 0.52$			$R^2_{marg} = 0.55$
Dry mass	0.138	<0.001	0.52	0.042	0.085	0.08	0.138	<0.001
Sex (male)	−0.068	<0.001	0.22	−0.130	<0.001	0.27	−0.076	<0.001
Eye diameter				0.065	0.037	0.11		
Gut fullness				0.100	<0.001	0.40	0.038	<0.001
Condition	−0.061	0.004	0.14				−0.060	<0.001
Body depth	0.045	0.009	0.11				0.028	0.017
Fish N : P				−0.047	0.066	0.09		
Nitrogen excretion		$R^2_{adj} = 0.48$			$R^2_{adj} = 0.51$			$R^2_{marg} = 0.56$
Dry mass	0.372	<0.001	0.29				0.527	<0.001
Gut fullness	0.226	0.003	0.15	0.339	<0.001	0.31	0.167	<0.001
Jaw length							−0.069	0.044
Parasitized (Y)	0.243	0.069	0.06					
Condition	−0.257	0.006	0.13				−0.344	<0.001
Body depth	0.181	0.020	0.09				0.153	<0.001
Fish N : P				−0.527	<0.001	0.49		
Temperature							0.137	0.008
N : P excretion		$R^2_{adj} = 0.24$			$R^2_{adj} = 0.27$			$R^2_{marg} = 0.11$
Dry mass	−0.134	<0.001	0.21	−0.082	0.116	0.06	−0.036	0.076
Sex (male)	0.112	0.002	0.16	0.319	<0.001	0.32	0.152	<0.001
Eye diameter				−0.171	0.012	0.15	−0.033	0.144
Gut fullness	0.051	0.117	0.04					
Fish N : P				−0.142	0.010	0.16		
Temperature							0.047	0.118

Note: Excretion N : P is log-transformed.

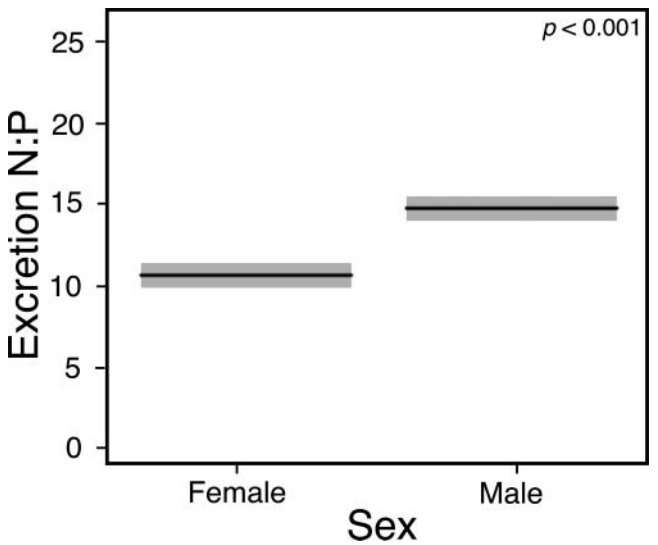

Fig. 2. Male stickleback excrete at significantly higher molar N : P ratios than females (14.7 : 1 vs. 10.7 : 1; $P < 0.001$) as a result of significantly lower phosphorus excretion rates by females (Table 1). Plot is the modelled output of the 'all-locations' LME model for stickleback N : P (Table 1). Shaded regions depict ±1 standard error.

all models was the finding that males release higher N : P excreta (Fig. 2). This relationship had a large partial η^2 effect size at both Kennedy and Miami, and was also the largest effect across all locations (based on standardized coefficients). Across all locations, male stickleback had a mean excretion N : P of 14.7 : 1, whereas that of females was 10.7 : 1 (Fig. 2).

Finally, we report the results of our excretion rates and ratios models where fish stoichiometry (N : P) was replaced by related phenotypic traits (bone mineralization, pelvis length) and genotypes (*Eda*) as candidate effects (Table S5). Here, bone mineralization had a small but significant negative correlation with excretion N : P ($P = 0.047$). We also found a significant effect of *Eda* genotype on nitrogen excretion, but only at Miami, where heterozygotes released significantly more nitrogen than completely-plated (CC) homozygotes ($P = 0.019$), while low-plated (LL) homozygotes were not significantly different (Table S5).

Foraging habitat

Significant relationships between stickleback N : P and foraging habitat (as $\delta^{13}C$) were found in the best models for Kennedy Lake, Miami River, and all locations (Fig. 3, Table S6). Consistently, high N : P (low phosphorus) fish were more depleted in $\delta^{13}C$, indicating a higher proportion of pelagic dietary carbon.

In the best location-specific models for $\delta^{13}C$, stickleback N : P was consistently the largest effect term, with large partial η^2 effect sizes at Kennedy (0.23) and Miami (0.17; Table S6). The best $\delta^{13}C$ model at Kennedy also retained body depth, while the best Miami model also included standard length as a significant but smaller effect (Table S6). Across all

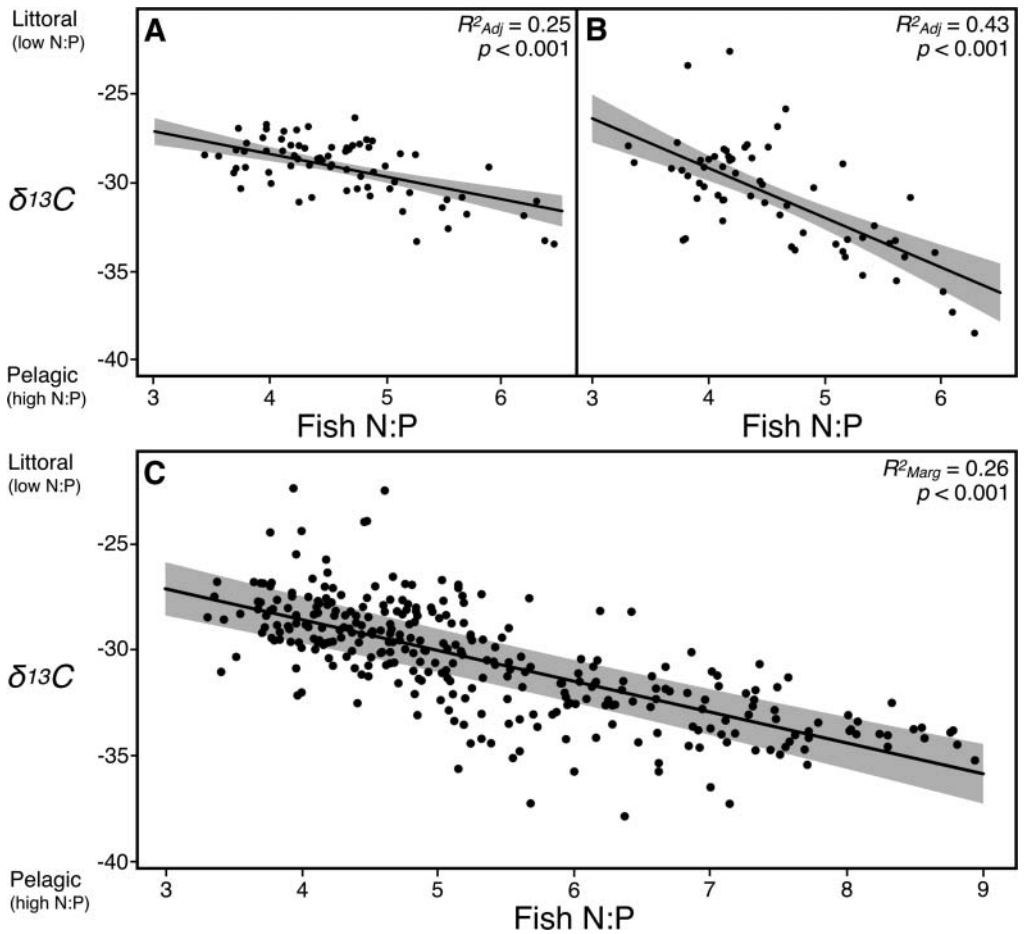

Fig. 3. Relationships between $\delta^{13}C$ and organismal stoichiometry (N : P) at Kennedy Lake (A), Miami River (B), and across all 10 locations combined (C). Fish with high N : P ratios were significantly more depleted in $\delta^{13}C$ in all cases. Plots show the modelled relationships from the best models for each of these three datasets. Shaded areas depict 95% confidence ranges.

locations, the best model retained stickleback N : P as the top – and only – predictor of $\delta^{13}C$ ($P < 0.001$) (Fig. 3, Table S6).

When N : P influencing traits and genotypes were substituted in place of fish stoichiometry in our global models, the best models found significant relationships between $\delta^{13}C$ and two of these factors: bone mineralization and *Eda* genotype (Table 2). Most notably, low-plated (LL) *Eda* genotypes were more depleted in $\delta^{13}C$ than completely-plated (CC) *Eda* genotypes within both Kennedy Lake ($P = 0.006$) and Miami River ($P = 0.014$), as well as across all locations ($P < 0.001$; Fig. 4). Thus, high N : P (LL) genotypes were associated with more pelagic diets than low N : P (CC) genotypes in all models (Table 2). Additionally, a significant association between bone mineralization and $\delta^{13}C$ was observed at Kennedy only, with low bone mineralization (high N : P) phenotypes associated with a more pelagic

Table 2. Best models for $\delta^{13}C$ using N:P-influencing traits and genotypes in place of fish N:P at Kennedy Lake, Miami River, and all 10 locations combined

		Kennedy Lake ($n = 71$)			Miami River ($n = 61$)			All locations ($n = 273$)	
Term		Est.	*P*-value	Partial η^2	Est.	*P*-value	Partial η^2	Est.	*P*-value
$\delta^{13}C$ model			$R^2_{adj} = 0.25$			$R^2_{adj} = 0.42$			$R^2_{marg} = 0.09$
Standard length		1.00	0.004	0.12	2.86	<0.001	0.35	0.79	0.001
Eda	LC genotype	−0.25	0.556	0.13	−1.00	0.081	0.11	−0.76	0.034
	LL genotype	−1.20	0.006		−1.98	0.014		−1.87	<0.001
Eye diameter					0.82	0.131	0.04	0.86	0.001
Sex (male)								−0.69	0.007
Body depth		–0.51	0.127	0.04					
Bone mineralization		0.68	0.048	0.06					

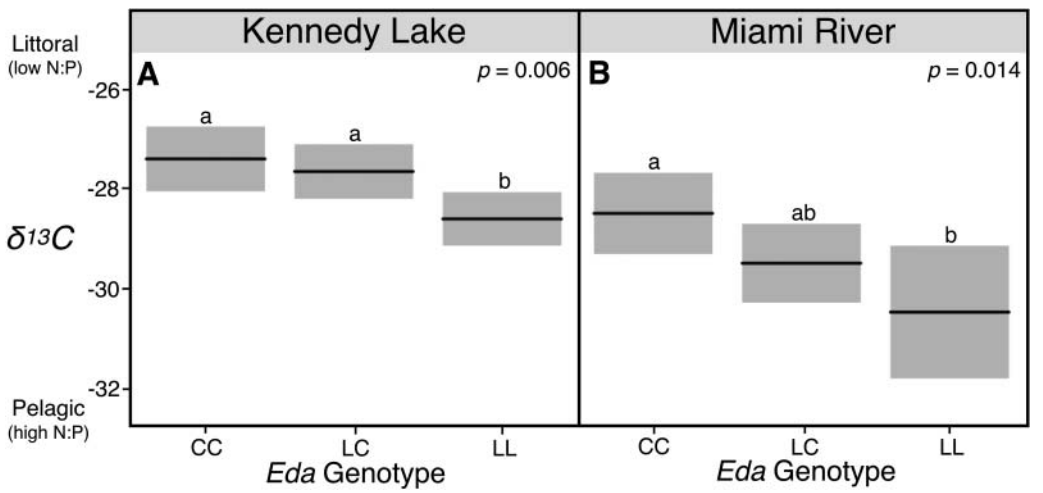

Fig. 4. Modelled relationships between $\delta^{13}C$ and *Eda* genotypes. Plots are the outputs of the best linear model for $\delta^{13}C$ at each focal location (Table 2). Low armour genotypes (LL) were significantly more depleted in $\delta^{13}C$ than full armour genotypes (CC) at both Kennedy Lake ($P = 0.006$) and Miami River ($P = 0.014$). Shaded areas depict 95% confidence regions.

$\delta^{13}C$ signature (Table 2). Significant relationships between $\delta^{13}C$ and standard length, sex, and eye diameter were also found in some models (Table 2).

DISCUSSION

With a growing appreciation of the ubiquity and importance of intraspecific variation in ecological and eco-evo interactions, investigating the mechanistic drivers of this variation has become an important next step (Evangelista *et al.*, 2017; Leal *et al.*, 2017a). In the present study, we investigated intraspecific variation in two important ecosystem interactions, excretion and foraging habitat, which are often implicated as important processes in eco-evo studies (El-Sabaawi, 2017). We did so to compare the relative strength of a wide range of genetic and phenotypic predictors, and relate these to the theories and mechanisms that may govern these interactions (Leal *et al.*, 2017a). We find two key results: a large effect of sex on excretion stoichiometry, and a strong correlation between individual OS and foraging habitat.

For excretion, we observed over an order of magnitude of variation in excretion rates and ratios – often within a single population. Our models explained this variation well (Tables 1 and S5), but interestingly, neither stickleback stoichiometry (N : P) nor related genetics (*Eda*) were consistent predictors of excretion stoichiometry. These terms were only retained in some models at Miami River (Tables 1 and S5), and any effects were insignificant or inconsistent [e.g. *Eda* was retained for nitrogen excretion, but effects of *Eda* on nitrogen excretion were not expected based on ecological stoichiometry (ES), and *Eda* heterozygotes were not intermediate between CC and LL homozygotes]. Thus, even within populations containing unusually high amounts of genetics-influenced variation in stickleback OS (Durston and El-Sabaawi, 2017), we find that excretion stoichiometry is largely decoupled from OS, which is counter to the predictions of ES (Sterner and Elser, 2002; Matthews *et al.*, 2011). This

finding, along with similar results from other recent studies (Tobler *et al.*, 2016; Tuckett *et al.*, 2016; Leal *et al.*, 2017b), demonstrates that excretion stoichiometry is either not substantially governed by ES, or additional factors need to be considered within stoichiometric excretion models, such as consumption rates and diet stoichiometry (Leal *et al.*, 2017b; Moody *et al.*, 2018).

One such factor that could decouple somatic OS from excretion stoichiometry is the timing and stoichiometry of reproductive investment, as the elemental content of reproductive tissues can differ widely from that of somatic tissues (El-Sabaawi *et al.*, 2016). Here we note a key finding that is consistent with this idea: stickleback sex was consistently a large effect predictor of phosphorous excretion rates and excretion stoichiometry (N : P) (Table 1). In every phosphorus excretion and excretion N : P model, we observed a large and significant sex-based effect, with females releasing 25–40% more phosphorus than males without differing in nitrogen excretion rates, such that female excretion N : P (10.7 : 1) was much lower than that of males (14.7 : 1) (Fig. 2). While there are a variety of plausible explanations for this result, we think reproductive investment is the most likely, as high phosphorus excretion is stoichiometrically consistent with female investment into low phosphorus reproductive tissues (Durston and El-Sabaawi, 2017), and this reproductive investment was occurring when our field observations were made (eggs were observed in 147 of 159 females). Previous modelling efforts have predicted this reproductive-based decoupling of individual OS and excretion, but it has not been empirically demonstrated (El-Sabaawi *et al.*, 2016). Alternative explanations include sex-based differences in other factors related to nutrition, such as foraging habitat and consumption rate, but these hypotheses are not supported by our foraging habitat and gut fullness data (Table 2). In any case, it is clear that sex can have a large effect on phosphorus excretion and excretion N : P, such that sex ratios and the timing of reproductive investment should be considered when evaluating the ecosystem-level effects of phenotypic variation.

The relative importance of excretion predictors from the metabolic theory of ecology (MTE) and ES is a fundamental question that has been investigated in interspecific work, but similar research is only beginning at the intraspecific scale (Allgeier *et al.*, 2015; Vanni and McIntyre, 2016). Our intraspecific excretion rate models find mass scaling coefficients for nitrogen and phosphorus of 0.63 and 0.74 respectively, which are similar to the 0.75 coefficients predicted by MTE (Brown *et al.*, 2004). In addition, temperature was a significant predictor of nitrogen (but not phosphorus) excretion rates in the 'all-locations' model where temperature was considered. Conversely, OS (as stickleback N : P) was generally not retained in the top models for nitrogen and phosphorus excretion rates. The lone exception were models at Miami River, where stickleback N : P was retained as a non-significant predictor of phosphorus excretion and a significant predictor of nitrogen excretion (Table 1). Overall, MTE predictors were clearly more important than those from ES in excretion rate models, but a caveat is warranted since the large effect of sex in phosphorus models may relate to the stoichiometry of reproductive investment. Thus while current predictors from ES did little to improve excretion models, expansion of ES theory to include reproductive investment may improve its explanatory power (El-Sabaawi *et al.*, 2016; Vanni and McIntyre, 2016). If so, any effect of ES would likely be particularly important for nutrient ratios, rather than rates, as metabolic predictors had much smaller and often non-significant effects in N : P models, while sex retained its large effect (Table 1). In general, MTE clearly dominates our intraspecific models for excretion rates with the potential for smaller effects from stoichiometry, whereas excretion ratios were much less influenced by metabolic factors and may be governed by ES.

In contrast to our excretion models, intraspecific variation in OS (stickleback N : P) was strongly associated with foraging habitat (Table S6). We observed that low N : P stickleback known to be investing more in phosphorus-rich bony traits were associated with more littoral diets (Durston and El-Sabaawi, 2017). We observed this within the populations at Miami River ($R^2_{adj} = 0.43$, $P < 0.001$) and Kennedy Lake ($R^2_{adj} = 0.25$, $P < 0.001$), as well as across all ten populations ($R^2_{marg} = 0.26$, $P < 0.001$) (Fig. 3). When stickleback N : P was considered alongside other phenotypic candidate effects, the best models consistently returned stickleback N : P as the largest effect with no other terms having large effects or being consistently retained across models (Table S6).

This finding adds to our understanding of intraspecific variation in stickleback foraging habitat, as previous studies have found this variation difficult to explain (Reimchen *et al.*, 2008; Matthews *et al.*, 2010; Marchinko *et al.*, 2014). Previous work has found associations between stickleback armour and habitat use, with more heavily armoured individuals being found in pelagic environments (Reimchen *et al.*, 2008, 2013). However, these patterns have largely been observed among populations as a consequence of natural selection in these environments against less armoured individuals (Reimchen *et al.*, 2013). Previous work has found variation in foraging habitat within populations more difficult to explain, but the limited evidence suggests that different mechanisms are involved, with heavily armoured individuals found to be associated with littoral habitats (Matthews *et al.*, 2010; Marchinko *et al.*, 2014). Our results build on this finding to suggest that nutritional differences resulting from differences in armour may underlie this association between more armoured phenotypes and littoral habitat use.

When phenotypic and genetic factors known to drive variation in OS were used in place of stickleback N : P in foraging habitat models, we found that more bony (low N : P) traits and genotypes were associated with more littoral diets (Table 2, Fig. 4). At both Kennedy Lake and Miami River, we found significant differences in foraging habitat between *Eda* genotypes, with completely-plated genotypes less depleted in $\delta^{13}C$ than low-plated genotypes. Such an association between *Eda* and $\delta^{13}C$ has been observed previously also at Kennedy Lake (Marchinko *et al.*, 2014), but our results reveal this pattern across multiple populations and extend this finding by showing that other N : P influential factors besides *Eda* are also associated with $\delta^{13}C$. Specifically, we find that individuals with higher bone mineralization consumed a higher proportion of littoral prey at Kennedy, while individuals at both Kennedy and Miami with longer standard length [which increases bone and N : P through allometry (Casadevall *et al.*, 1990)] were again associated with littoral foraging habitats. Overall, the consistent direction of these patterns, the greater explanatory power of stickleback N : P than these individual factors combined (based on effect sizes; Tables 2 and S5), and the match between the observed OS–diet correlations and those expected from stoichiometric predictions, suggest that individuals are compensating for differences in OS through differences in diet quality. More specifically, it appears that low N : P stickleback (high phosphorus content) are satisfying that elevated demand through the consumption of phosphorus-rich littoral prey (Andersen and Hessen, 1991; Cross *et al.*, 2003; Frost *et al.*, 2006). Such a possibility is intriguing, and suggest a potential mechanism by which natural selection can alter ecosystem function. This compensatory foraging also provides an explanation for our observation that stickleback excretion is largely decoupled from OS. Such dietary compensation has been observed in a wide range of prior work in response to variation in diet quality (Buck *et al.*, 2003; Berner *et al.*, 2005; Raubenheimer and Jones, 2006; Cease *et al.*, 2016), and similar compensation may be employed in response to variation in elemental demand (from intraspecific variation in stoichiometric investment).

However, there are many alternative explanations for the observed relationship between stickleback OS and foraging habitat. It may be that some other factor correlated with stickleback N:P produces this relationship, such as differences in swimming performance arising from differences in mass because bony individuals are more dense, or differences in foraging habitat driven through pleiotropy by the same genetics that affect OS. Another alternative explanation is that diet quality affects individual OS through plasticity. Such plasticity is known to occur (Liess *et al.*, 2013) and may certainly contribute to this correlation. However, it is well established that genetics (*Eda*) cause meaningful changes in the phosphorus-rich bony traits of stickleback and this underlies much of the variation in stickleback OS (Barrett *et al.*, 2008; Durston and El-Sabaawi, 2017), so it is unlikely that the observed correlation between foraging habitat and OS arises wholly from plasticity. A final alternative explanation is that bony tissues may exhibit differential rates of isotope fractionation, such that bone is correlated with isotopic signatures even without differences in diet (Matthews and Mazumder, 2004). We think this explanation is untenable because bone mineral [hydroxyapatite, $Ca_5(PO_4)_3(OH)$] does not contain carbon and thus has no ability to affect whole-body $\delta^{13}C$. Furthermore, prior work by others also at Kennedy Lake using only muscle tissue samples (rather than whole-body samples) has observed a similar relationship between *Eda* genotype and $\delta^{13}C$, ruling out tissue-specific fractionation in this case (Marchinko *et al.*, 2014). Overall, we think OS-driven differences in diet is the strongest explanation but a variety of explanations are possible and we encourage further study here.

CONCLUSIONS

Our work has focused on understanding the physiological and ecological mechanisms underlying intraspecific differences in ecological function, which are critical for investigating eco-evolutionary dynamics. We have investigated how theories such as ecological stoichiometry and metabolic ecology (ES, MTE) and other factors might explain intraspecific variation in excretion rates and ratios, and foraging habitat. We find that intraspecific variation in excretion rates is governed by MTE more so than ES. Conversely, excretion ratios are strongly affiliated with sex rather than OS or MTE. While sex is ignored in most work on excretion, this result demonstrates the importance of including sex and reproductive phenology in excretion and ecosystem models. In addition, this result supports integrating reproductive investment alongside somatic investment in ES theory as part of a total investment budget, or more broadly, working towards a tighter integration of ES theory with dynamic energy budget theory (Kooijman and Kooijman, 2010). Our study of intraspecific variation in foraging habitat finds that OS is a much more powerful predictor than more widely studied traits such as trophic morphology. Organismal stoichiometry explains large portions of intra- and inter-population variation in foraging habitat, and unifies weaker effects linking bone-related traits and genetics with differences in foraging habitat. These findings on foraging habitat are consistent with the predictions of ES, and yet, its lack of predictive power for excretion stoichiometry suggests this framework is incomplete. These results add to a growing body of evidence that other factors need to be considered within ES, specifically, the stoichiometry of all investment types (somatic and reproductive), the timing of these investments (e.g. growth rates, reproductive phenology), and potential variation/compensation in diet quality and quantity (Vanni and McIntyre, 2016; Moody *et al.*, 2018). Through a more comprehensive assessment of elemental investment rates and ratios, we think it is likely that advances will continue to be made into the theories,

mechanisms, and eco-evolutionary dynamics of intraspecific variation in ecosystem effects.

ACKNOWLEDGEMENTS

This project is supported by an NSERC Discovery grant to R. El-Sabaawi. D. Durston is supported by NSERC and the University of Victoria. The authors thank Misha Warbanski, Nova Hanson, Laura Kennedy, Therese Frauendorf, and Elsabet Lapointe for their assistance in the field and lab. We are grateful to Katie Peichel for her assistance with genotyping, as well as Tom Reimchen and Blake Matthews for their assistance and feedback throughout the project. We also thank the reviewers and editors whose comments improved this manuscript.

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