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LETTER

Ionome and elemental transport kinetics shaped by parallel evolution in threespine stickleback

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Abstract

Evidence that organisms evolve rapidly enough to alter ecological dynamics necessitates investigation of the reciprocal links between ecology and evolution. Data that link genotype to phenotype to ecology are needed to understand both the process and ecological consequences of rapid evolution. Here, we quantified the suite of elements in individuals (i.e., ionome) and differences in the fluxes of key nutrients across populations of threespine stickleback. We find that allelic variation associated with freshwater adaptation that controls bony plating is associated with changes in the ionome and nutrient recycling. More broadly, we find that adaptation of marine stickleback to freshwater conditions shifts the ionomes of natural populations and populations raised in common gardens. In both cases ionomic divergence between populations was primarily driven by differences in trace elements rather than elements typically associated with bone. These findings demonstrate the utility of ecological stoichiometry and the importance of ionome-wide data in understanding eco-evolutionary dynamics.

Keywords

Eco-evolutionary dynamics, ecological stoichiometry, genes-to-ecosystems, ionomics, rapid evolution, parallel evolution.

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INTRODUCTION

Integrating evolutionary biology and ecosystem science is considered a crucial frontier for conceptual unification in biology (Holt 1995; Levin 1998; Elser & Hamilton 2007; Matthews *et al*. 2011). Empirical findings of rapid evolution across many taxa (Hendry & Kinnison 1999) has led to a renewed integration of ecology and evolutionary biology through the study of eco-evolutionary dynamics (Hairston *et al*. 2005; Fussmann *et al*. 2007). Research in eco-evolutionary dynamics has demonstrated that evolution can occur rapidly enough to alter ecological processes, including ecosystem functions like decomposition and nutrient availability (Hendry 2017; Rudman *et al*. 2017). Using the framework of ecological stoichiometry (ES), which considers the balance of energy and materials in ecological interactions and processes (Sterner & Elser 2002), to study eco-evolutionary dynamics has tremendous potential to advance understanding of both ecology and evolutionary biology from genes to ecosystems (Elser *et al*. 2000; Jeyasingh *et al*. 2014; Leal *et al*. 2017b). Key parameters in stoichiometric models include elemental uptake, organismal elemental composition and elemental excretion (Sterner & Elser 2002). Such stoichiometric variables underlie classically studied morphological phenotypes (e.g., skeletal variation) but unlike many aspects of gross morphology they inherently link the organism and the environment. As such, employing tools from ES to study rapid evolution has the potential to uncover links between the selective environment, phenotypic change and the ecological consequences of evolution (Elser *et al*. 2000; Elser 2006; Leal *et al*. 2017b).

To date, most research examining phenotypic variation in an elemental context has focused on three elements: carbon [C], nitrogen [N] and phosphorus [P], which are known for their importance in both biochemical and ecological processes (Sterner & Elser 2002; Elser & Hamilton 2007), but few studies have also assessed other elements (e.g. Snell-Rood *et al*. 2014; Tobler *et al*. 2016). Using only a few elements to describe an organism results in a limited view of phenotypic variation, potentially ignoring important interactions among elements that underlie traits (Salt *et al*. 2008; Jeyasingh *et al*. 2014, 2017). There are roughly 25 elements with documented roles in fundamental biological processes, including physiological processes and structural traits (da Silva & Williams 2001). Measuring the ionome, defined as the mineral nutrient and trace elemental composition of an organism (Salt *et al*. 2008), should capture the ecological roles of an organism at a higher resolution, illuminating mechanisms operating at higher levels of organization (Kaspari & Powers 2016; Leal *et al*. 2017b). Increased elemental resolution provides an enhanced ability to identify the axes of ecologically relevant variation among populations, which could be useful in identifying the phenotypic underpinnings of local adaptation (Huang & Salt 2016; Goos *et al*. 2017). Ionome-wide information is also important when studying the consequences of evolution on the flow of

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nutrients between organisms and their environment, as the availability, uptake, and utilization of specific macronutrients is often dependent on the dynamics of trace elements (Lind & Jeyasingh 2018; Liu *et al.* 2018).

Applications of ES to study trait evolution have largely utilized biochemical information about a trait (e.g., apatite in bone, $Ca_5[PO_4]_3[F,Cl,OH]$) to make predictions about demand for, and excretion of, elements underlying that trait (Kay *et al.* 2005; Jeyasingh & Weider 2007; Snell-Rood *et al.* 2015). Such approaches have been useful in understanding the causes and consequences of trait evolution particularly in the context of environmental variation in the supply of relevant elements (Frisch *et al.* 2014; El-Sabaawi *et al.* 2016; Roy Chowdhury & Jeyasingh 2016; Leal *et al.* 2017b). Nevertheless, attention to only a limited set of elements involved in one or few traits of a taxon that expresses multiple other traits is prone to miss potentially important trade-offs in the handling of other elements, and thus its evolutionary significance or ecological relevance.

Threespine stickleback (*Gasterosteus aculeatus*, hereafter stickleback) is an ecological model system in which employing ionomics could advance our understanding of the process of adaptation and provide a way to link changes in genotype directly to effects on ecological parameters [i.e., extended phenotypes (Whitham *et al.* 2003)]. One particularly well-studied aspect of stickleback evolution is variation in conspicuous bony armour plates that cover the flanks (Heuts 1947; Bell 1981; Colosimo *et al.* 2005; Barrett *et al.* 2008). A single linkage group centred around the gene *Eda* controls much of the variation in plate number and the complete allele ('C') is dominant over the low allele ('L') (Heuts 1947; Colosimo *et al.* 2005). In British Columbia, Canada, there are freshwater populations in neighbouring lakes that exhibit complete- and low-plated phenotypes and even rare populations that have stable polymorphisms for lateral plates. Bony plate evolution is also one of the prominent components of the adaptation of marine stickleback populations to freshwater (Bell & Foster 1994; Bell *et al.* 2004; Barrett *et al.* 2008; Lescak *et al.* 2015). Following the end of the last ice age, marine stickleback colonized freshwater environments hundreds of times independently (Bell & Foster 1994). In addition to evolution in bony plates, marine stickleback adapting to freshwater environments underwent parallel evolution in traits related to pigmentation, defense, and resource acquisition (Bell & Foster 1994; Bell *et al.* 2004; Aguirre & Bell 2012). Although adaptation of marine stickleback to freshwater is amongst the best studied cases of repeated evolution in nature, the majority of studies examining the phenotypic basis of this adaptation focus on gross morphological characters. Comparative genomic work on adaptation to freshwater has uncovered more than 200 unique haplotypes under selection, including several that contain genes involved in ion transport, suggesting that many biological pathways may be involved in adaptation (Jones *et al.* 2012).

Repeated evolution in stickleback presents an opportunity to examine how ecological stoichiometry shapes adaptation and how adaptation shapes nutrient recycling. Stickleback evolution has been shown to alter both community structure and ecosystem functions (Harmon *et al.* 2009; Des Roches *et al.* 2013; Matthews *et al.* 2016; Rudman & Schluter 2016; Best *et al.* 2017), including associations between stickleback genotype and nutrient availability in mesocosms (Harmon *et al.* 2009; Rudman *et al.* 2015; Matthews *et al.* 2016). Previous research has also demonstrated that variation in stickleback armour plating is associated with increased phosphorus content in wild-caught populations (Durston & El-Sabaawi 2017; Leal *et al.* 2017a; Paccard *et al.* 2018) and that armour phenotypes can influence nutrient excretion (El-Sabaawi *et al.* 2016). Yet, many aspects of the relationship between stickleback evolution and ES, both at *Eda* and genome-wide, have not been explored. Notably, the role of adaptation in shaping the ionome, elemental assimilation and examination of the role of genotype-by-environment interactions in shaping ES remain uncertain.

Here, we examine two well-studied cases of evolution in stickleback using the ecological stoichiometry measures of elemental uptake, the ionome, and nutrient recycling. First, we assess how variation at the *Eda* locus shapes the ionome, elemental assimilation, and nutrient recycling. Based on the difference in bone content between *Eda* genotypes, we predict that high-plated stickleback will have higher assimilation of phosphorus (a bone-associated element), that differences in the ionome between high- and low-plated individuals would stem primarily from content of bone-associated elements, and that high-plated individuals would exhibit reduced excretion of phosphorus (i.e., have higher retention). Second, we measured elemental uptake and the ionome to assess the stoichiometric basis of adaptation of marine stickleback, which have extensive bony armour, to freshwater conditions, where stickleback populations have reduced bony armour. We measured the ionome of wild-caught, freshwater common garden, and saltwater common garden individuals from freshwater and marine populations. Based on differences in bone, both in plating and in other defensive structures (Bell & Foster 1994), we expected differences between marine and freshwater fish to largely stem from differing concentrations of bone associated elements. We predicted that these differences would be stable across two rearing salinities and would be caused by differences in assimilation efficiencies.

METHODS

Links between *Eda*-genotype, elemental phenotype and nutrient recycling in the polymorphic Kennedy Lake population:

Collection and breeding of Eda *polymorphic stickleback*

To explicitly address the role of variation in bony plates on elemental phenotypes we collected wild sticklebacks from Kennedy Lake (Vancouver Island, British Columbia, Canada). The stickleback population in Kennedy Lake is polymorphic at the *Eda* locus and hence has individuals varying in plate phenotype co-occurring in the same microhabitat (Marchinko *et al.* 2014). The distribution of lateral plates in Kennedy Lake stickleback is largely bi-modal and we considered any fish with < 8 plates to be 'low-plated' and any fish with > 25 plates to be 'high-plated' (Marchinko *et al.* 2014). Less than 3% of the fish that we captured had intermediate plate counts (i.e. between 8 and 25) and we did not include them in our study. The Kennedy Lake stickleback population shows no evidence of genome-wide differences associated with *Eda*

genotypes and the stickleback of Kennedy Lake are a single genetic population (Marchinko *et al*. 2014). As such, the Kennedy Lake population is ideal to test for the effects of variation at the *Eda* locus on the elemental phenotype. *Eda* genotype affects morphology and diet, with low-plated individuals having smaller heads and more littoral ^{13}C isotopic signatures (Marchinko *et al*. 2014). To determine whether any differences in the elemental phenotype stem from changes in diet or assimilation we both collected wild-caught individuals in Kennedy lake and made lab crosses that were fed a consistent diet. Crosses were made between low-plated (e.g. L/L x L/L) and high-plated (C/- x C/-) individuals (at least two families per genotype). Lab crosses were initially fed *Artemia* brine shrimp twice daily and were switched to a once daily chironomid diet as they matured. Tanks were kept at 18 °C and were kept on a seasonally variable photoperiod (12L:12D in summer, 10L:14D in winter). We sacrificed 8 low-plated individuals and 12 high-plated individuals from field caught fish and 6 low-plated and 12 high-plated from lab reared fish.

Generating ionomes of Eda *locus variants*

To generate ionomic data, fish were sacrificed, viscera were removed, and the fish were dried at 60 °C until they reached a constant dry mass (3–5 days). Once dry, each whole fish was weighed and then digested whole in individual trace-metal free 15 mL polypropylene centrifuge tubes. The digestion solution consisted of a 2 : 1 mix of trace metal grade 67–70% HNO_3 and trace metal grade 30–32% H_2O_2, respectively. The volume of the digestion solutions was determined based on the dry mass of the fish, where 2.5 mL of HNO_3 was used per 1 g of fish dry mass. This mass-specific digestion volume ensured full digestion of all fish, regardless of size. Each digested sample was diluted with Type 1 ultrapure water to a final concentration of 6% v/v of acid. All samples were analysed for 29 elements using an inductively coupled plasma optical emission spectrometer (ICP-OES; Thermo Scientific iCAP 7400). To validate and calibrate the analysis, we used aqueous multielement standard reference solutions (CCV Standard 1A & B, CPI International), as well as an in-line internal standard of yttrium (Peak Performance Inorganic Y Standard) to correct for instrument drift and matrix effects. Digestion blanks consisting of diluted digestion solutions without fish tissue were also run, and all sample concentrations were corrected for background concentrations. Concentrations of elements within our fish samples that were within range of the standard deviations for the blank controls were excluded from further analysis, as these values are indicative of concentrations near or below the limit of detection of the ICP-OES. We plotted the concentrations of each elemental measurement for each stickleback population (Fig. S1).

Nutrient recycling associated with Eda *locus measured in the field*

We conducted nutrient excretion trials on field caught fish from Kennedy Lake to examine the effect of bony plating on nutrient recycling. Adult fish were caught by a combination of hand-netting and minnow trapping. Minnow traps were unbaited and were checked every 3 h to minimize the effects of holding. Following capture, each adult fish was placed in a container with 220 mL of filtered water taken from Kennedy Lake and connected to an air bubbler. Fish were not starved prior to the assay. A water sample was taken 5 min after introducing each fish and a second sample was taken after 4 h. Fish were then sacrificed and weighed. We measured the concentration of nitrogen (as total ammonia) and phosphorus (as soluble reactive phosphorus) in each sample (Hach DR 2800, Loveland, CO, USA) following the manufacturer's instructions. For the analysis of nutrient excretion data between the high and low plate morphs we calculated the change in concentration during the trial (i.e. final-initial) and calculated mass-normalized excretion rates following Torres & Vanni (2007) before significance testing.

Differences in calcium uptake of Eda *variants*

Calcium is a key element in bone, so we assessed calcium uptake rates in high- and low-plated lab-reared families from Kennedy Lake. Calcium uptake rates were determined in stickleback using radio-isotopic techniques. Fish were introduced to exposure chambers with 1.5 L of dechlorinated Vancouver tap water (41 μM Ca) under gentle aeration. After acclimating for 10 min, 20 μCi of ^{45}Ca was introduced to the chamber. Water samples for measurement of total Ca and ^{45}Ca were collected at the beginning and end of the 4 h exposure period to calculate specific activity. At the end of the exposure, fish were triple rinsed in 10 mM $CaCl_2$ to displace loosely bound ^{45}Ca, euthanized by an overdose of MS-222, and frozen. Subsequently, the intestinal tracts of fish were dissected out while still frozen and the remaining carcass was assayed for ^{45}Ca. Fish were first digested in 1M HNO_3 (5:1 v:w) at 70 °C. Digests were centrifuged at 1500 rpm and the overlying supernatant collected. Acid compatible scintillation fluid was added to digests (9:1 v:v) and then samples were counted on a beta counter with appropriate quench correction. In addition to assaying high- and low-plated individuals from Kennedy Lake, we assayed two marine (Oyster and Muddy Lagoons) and two freshwater low plated (Trout and Klein lakes) populations with at least six individuals for each population (Fig. S2).

Examining local adaptation in the elemental phenotypes of marine and freshwater stickleback

Collection and rearing of marine and freshwater stickleback populations

To assess how adaptation of marine stickleback to freshwater environments influenced the ionome we collected wild individuals from four freshwater lakes with distinct populations. Of the four lakes we sampled, North and Ruby Lakes contain stickleback populations that have retained a high-plated phenotype and Trout and Klein Lakes have stickleback with the derived low-plated phenotype most common in freshwater. All four lakes are located on the Sechelt Peninsula (British Columbia, Canada). We also collected high-plated marine stickleback from two locations off of the Sechelt peninsula (British Columbia, Canada).

We produced lab crosses to directly assess the effects of genetic differences between populations and test for interactive effects between genotype and salinity on ES. To test for

heritable differences between a freshwater and marine population, we reared families of marine stickleback (Oyster Lagoon) and a low-plated freshwater population (Trout Lake) in the lab. We produced five families of marine and four families of freshwater stickleback that were held in dechlorinated Vancouver tap water. We also produced six marine and four freshwater families that were held in saltwater (20 ppt, made using Instant Ocean® sea salt). Genetic crosses from wild-caught adults were done at two different salinities (0ppt and 20ppt) immediately after collecting the fish at each location. Marine and freshwater stickleback crosses have high viability in either salinity (Marchinko & Schluter 2007; Gibbons *et al.* 2016). Tanks were kept at 18 °C on a seasonally variable photoperiod, were held at similar densities, and allowed to grow for *c.* 300 days post hatch before being sacrificed (see Gibbons *et al.* 2017 for care of marine/freshwater crosses).

Following the same protocol outlined above, we generated ionomic data for six individuals from each of two wild-caught marine and four wild-caught freshwater populations (two high-plated and two low-plated) (Fig. S3). In addition, we used our factorial lab-rearing common garden to generate ionomic data for six individuals from each treatment (i.e., marine fish-freshwater, marine fish-saltwater, freshwater fish-freshwater, freshwater fish-saltwater).

Phosphorus assimilation efficiency

Phosphorus is a key limiting nutrient in freshwater and is also a component of bone. As such, we measured the assimilation efficiency of one marine (Oyster Lagoon) and one low-plated freshwater (Trout Lake) population using standard radio-isotopic pulse-chase techniques (Wang & Fisher 1999). Live oligochaetes (*Lumbriculus variegatus*) were used as the diet in these experiments. Worms were held for 24 h in 100 mL of Vancouver tap water with 10 mL of mashed sweet potato slurry spiked with 10 μCi of ^{33}P. After exposure, 5 sub-samples of *c.* 100 mg (wet weight) of worms were dissolved in 1 mL of Solvable (Perkin Elmer). Then, 3 mL of scintillation fluid was added to 500 mL of digest and counted on a beta counter to determine the ^{33}P concentration in the diet.

Fish used in assimilation experiments were preweighed and allowed to acclimatize overnight in a container with 200 mL of dechlorinated Vancouver tap water with gentle aeration. The following morning, fish were fed up to 5% body weight of ^{33}P labelled oligochaetes. Fish were allowed 30 min to feed after which uneaten worms were removed and weighed to determine the mass of the ingested meal. Preliminary experiments indicated feeding did not release significant concentrations of ^{33}P into the water. After 2 h, fish were again fed to satiation with unlabeled oligochaetes to 'chase' the labelled food from their digestive tract. After 24 h, fish were euthanized by an overdose of MS-222, frozen and the fish were then dissected and analysed as described in the Ca uptake experiments but for measurement of whole body ^{33}P.

Data analysis

Our analysis focused on assessing the effects of plate phenotypes and the adaptation of marine populations to freshwater on elemental uptake, ionomes and excretion of nutrients. Ionomic data yielded information on the relative concentrations of 26 elements across populations of stickleback (Fig. S1). First, to fully describe the differences in elemental phenotype associated with rapid evolution and changes in environment we used all elements in our analysis. In addition, we conducted separate analyses using only elements involved in growing bone (Ba, Ca, Mg, P, Si, and Sr (Morgulis 1931; Goldberg 1962) as evolution in bony structures is a crucial component of repeated evolution in stickleback in both the plate polymorphic and marine-freshwater contrasts. For each contrast of interest we ran a principal components analysis and extracted all PC axes with eigenvalues > 1. We then used MANOVA to assess differences between groups using these PC axes [R version 3.3.2 (R Core Development Team 2015)]. We used this unconstrained approach with the goal of first identifying the major axes of elemental variation and then determining whether evolved differences between populations influences elemental variation across these axes. For the analysis of nutrient excretion data between the high and low plate morphs, we calculated the change in concentration during the trial (i.e. final – initial), calculated mass-normalized excretion following Torres & Vanni (2007), and tested for the effect of plate phenotype using a *t*-test. We also tested for the effects of stickleback ecotype on Ca influx using ANOVA models and *t*-tests. Our analysis of P assimilation efficiency used an ANOVA model with population (i.e. marine and freshwater) and stickleback mass as fixed effects.

RESULTS

Effects of allelic variation at *Eda* on stoichiometric traits

We used a population of stickleback that exhibits polymorphism in bony armour (Kennedy Lake) to assess how changes in armour phenotype alter elemental composition. When reared under laboratory conditions, we found differences in the ionome associated with variation at *Eda* both when we assessed all elements (Fig. 1a, $F_{1,11} = 4.19$, $P = 0.019$) and only bone-associated elements ($F_{1,14} = 3.45$, $P = 0.046$). When we included all elements, the axis which primarily differentiated between armour morphs (PC2, Fig. 1a, Fig. S4) was strongly influenced by concentrations of selenium, zinc, vanadium and potassium. Field-collected fish showed no differences between high- and low-plated ecotypes when we considered all elements ($F_{1,12} = 1.40$, $P = 0.29$) or only bone associated elements ($F_{1,15} = 0.31$, $P = 0.74$).

We measured whether high- and low-plated fish differed in their rate of phosphorus excretion in the field. There was no difference in mass between high (1.22 g ± 0.25) and low (1.24 g ± 0.25) plated individuals. The high plated ecotype excreted 38% more mass normalized soluble reactive phosphorus than the low plated ecotype ($F_{1,30} = 4.75$, $p = 0.037$) (Fig. 1b). We also found a nonsignificant trend towards increased excretion of total ammonia from the high plated ecotype ($t = 1.93$, d.f. = 33.90, $p = 0.062$) with high plated fish excreting 21% more total ammonia than low plated individuals on average (Fig. 1c). The plate polymorphism in Kennedy Lake was also associated with differences in Ca uptake rates

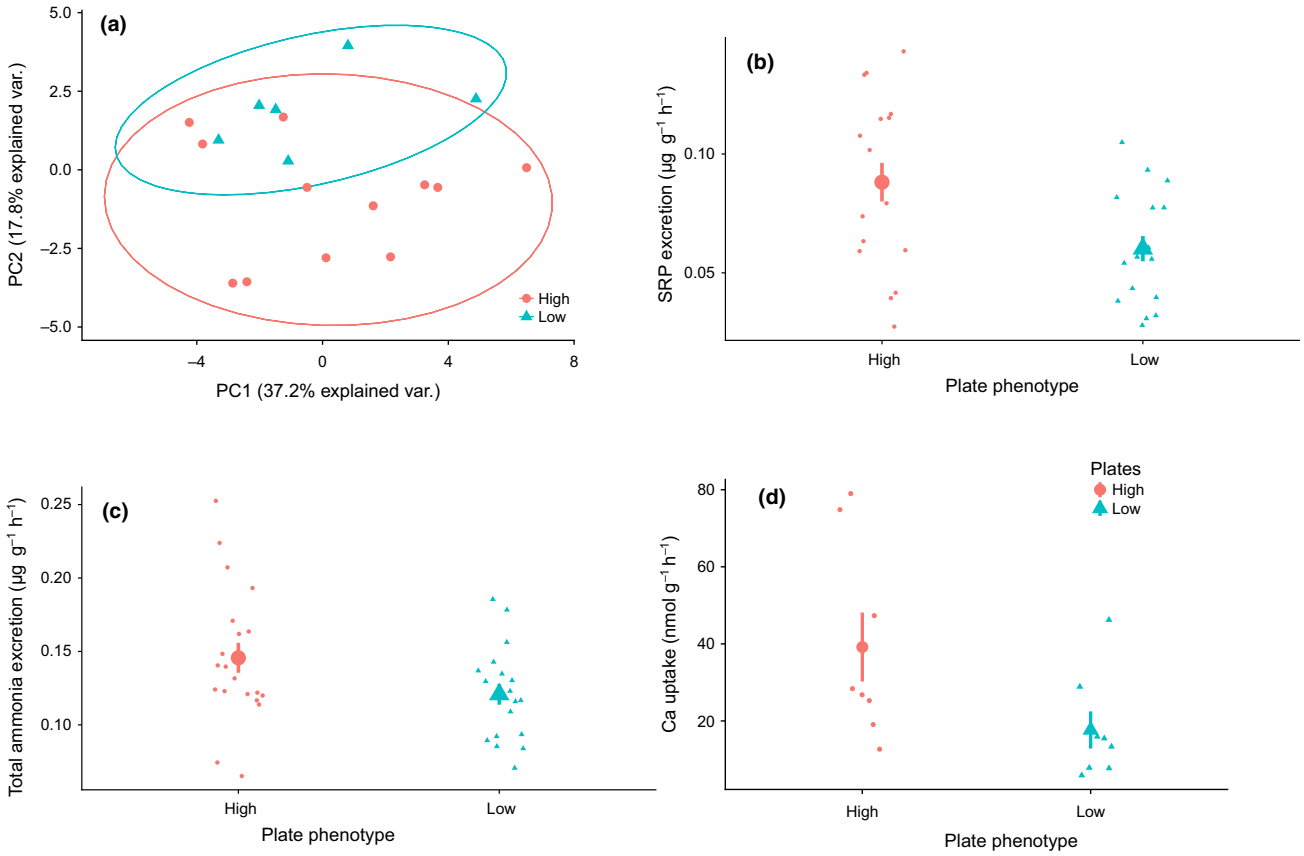

Figure 1 The effect of armour plating polymorphism on: (a) elemental composition based on all 26 elements from lab reared fish with high and low plate phenotypes (top five elements contributing to PC2 are Se, Zn, V, K and Li), (b) Soluble reactive phosphorus (SRP) excretion rate from wild caught fish, (c) Total ammonia excretion rate in wild caught fish, and (d) Ca uptake rate (nmol g^{-1} h^{-1}) for lab-acclimated (> 2 weeks) wild caught fish. Points represent individual stickleback, with triangles representing low-plated individuals and circles representing high-plated individuals. The ellipse in panel (a) represents the 95% normal probability for the elemental composition of each plate morph. Large points in panels (b), (c) and (d) are means ± SEM.

($F_{1,13}$ = 6.12, p = 0.028), as high-plated fish showed a 120% increase compared to the low-plated fish (Fig. 1d).

Elemental phenotypes of marine and freshwater stickleback populations

We found widespread differences in the ionome between wild-collected populations of marine and freshwater stickleback when assessing across all elements ($F_{1,47}$ = 127.35, p < 0.0001; Fig. 2a). PC2, the axis which most strongly differentiated wild-caught marine and freshwater populations, had high loading contributions from strontium, arsenic, barium, lithium and manganese (Fig. S4). When we considered only the subset of elements associated with bone we also found pronounced differences in the ionome of wild-caught marine and freshwater populations ($F_{1,51}$ = 576.62, p < 0.0001).

When we compared the ionome of marine and freshwater stickleback reared in the lab we found significant effects of stickleback ecotype ($F_{1,15}$ = 21.24, P < 0.0001), rearing salinity ($F_{1,15}$ = 78.22, P < 0.0001), and an interaction between ecotype and salinity ($F_{1,15}$ = 4.20, p = 0.014). Marine and freshwater stickleback differed in their ionomes when reared in saltwater ($F_{1,8}$ = 35.35, P < 0.0001; Fig. 2b) and the axis that differentiated between them (PC2, Fig. 2b) was most strongly shaped by boron, cadmium, barium, and manganese content (Fig. S4). When reared in freshwater we also observed differences in composition ($F_{1,5}$ = 68.89, P = 0.0001; Fig. 2c), driven largely by sodium, potassium, barium and magnesium (Fig. S4).

While ionomic data revealed differences between marine and freshwater populations in P content (Fig. S1), we also observed differences in P assimilation efficiency of the marine and freshwater fish with the freshwater population showing 31% greater assimilation ($F_{1,18}$ = 4.90, P = 0.040; Fig. 2d). We detected an interaction between population and fish mass with the freshwater stickleback exhibiting highest assimilation efficiencies at smallest sizes and little effect of mass observed on the assimilation of P in the marine population ($F_{1,18}$ = 13.73, P = 0.0016; Fig. S4).

DISCUSSION

Effect of allelic variation on ionome and nutrient cycling

The conspicuous variation in armour plating between stickleback populations is an exceptionally well-characterized phenotypic polymorphism (Heuts 1947; Bell 1981; Bell & Foster 1994; Colosimo *et al.* 2005). As a Mendelian trait it presents a

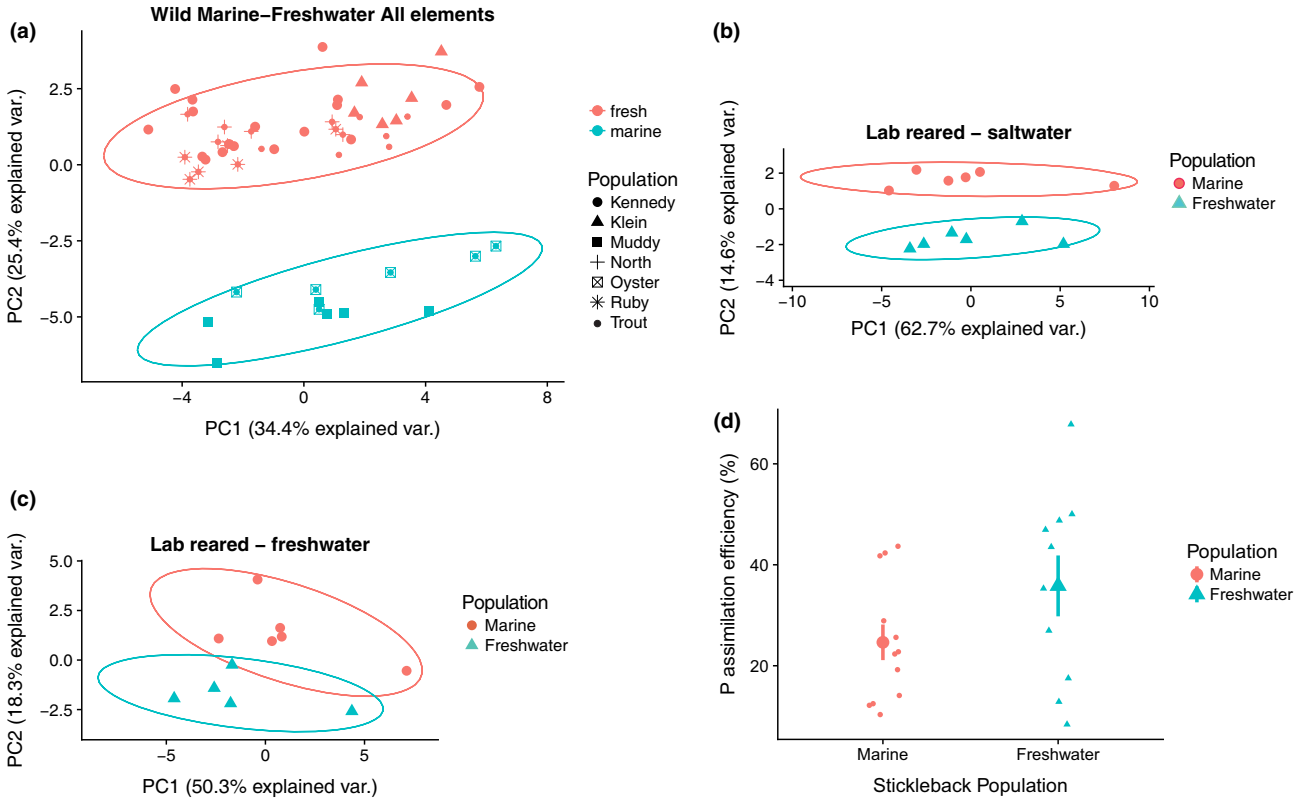

Figure 2 Differences between marine and freshwater populations in: (a) ionome displayed using a PC of elemental variation between wild-caught populations of marine (two populations) and freshwater stickleback (five populations) (top 5 elements contributing to PC2 are Sr, As, Ba, Li and Mn) (b) ionome of marine and freshwater stickleback reared in salt water (20ppt) (top five elements contributing to PC2 are B, Cd, Ba, Mn and K) (c) ionome of marine and freshwater stickleback reared in fresh water (0ppt) (top five elements contributing to PC2 are Na, K, Ba, Ni and Mg) (d) P assimilation efficiency for freshwater and marine stickleback reared in the lab at natal salinities (plotted as means ± SEM).

rare case where allelic shifts at a single locus has observable morphological and fitness consequences (Barrett *et al.* 2008; Marchinko *et al.* 2014). Variation at the *Eda* locus has previously been shown to influence N:P ratio, with low-plated individuals having higher N:P, and plate phenotype is a significant predictor of %P in stickleback both within polymorphic populations and across populations (Durston & El-Sabaawi 2017; Paccard *et al.* 2018). Here we use a population with a stable polymorphism at the *Eda* locus that shows no evidence of population structure linked to *Eda* genotype (Marchinko *et al.* 2014). We uncover effects of *Eda* on the ionome when stickleback were reared in a common garden (Fig. 1a). Yet, there was no detectable effect of *Eda* genotype on the ionome of wild-caught fish, suggesting a genotype-by-environment interaction shaping the ionome. The differences we observed between genotypes in a common garden was primarily driven by variation in trace metal concentrations (e.g. Se, V, Zn) and not by elements that are major components in bone. Trace metals are largely used as cofactors of enzymes catalyzing a variety of pathways and are often tightly regulated as they can become limiting or toxic with comparatively small changes in concentration (Nikinmaa 2014). *Eda* has profound effects on lateral plate phenotype but is also known to have pleiotropic effects (Mills *et al.* 2014; Sadier *et al.* 2014). Moreover, selection for the low-plated *Eda* allele is stronger in freshwater than selection for reduced lateral plate phenotype suggesting that *Eda* genotype may influence additional phenotypes that are adaptive for freshwater (Rennison *et al.* 2015). *Eda* genotype influences the diet of stickleback, as high-plated fish have a ^{13}C signature that is significantly shifted towards littoral carbon relative to the more pelagic carbon signature found in low-plated individuals (Marchinko *et al.* 2014). High-plated fish likely have a higher proportion of benthic invertebrates in their diet (Arnegard *et al.* 2014). Our results suggest that the effects of the *Eda* locus manifest differences in the content of trace elements, more so than in P and Ca (Durston & El-Sabaawi 2017; Paccard *et al.* 2018), but that life history differences found between *Eda* genotypes in nature obscure these effects.

We also observed differences in nutrient excretion between stickleback differing in *Eda* genotype. Previous work has demonstrated that rapid evolution in stickleback can have strong and predictable ecological consequences (Harmon *et al.* 2009; Rudman *et al.* 2015; Matthews *et al.* 2016; Rudman & Schluter 2016), including work demonstrating that population-level differences can affect nutrient recycling (El-Sabaawi *et al.* 2016). Yet, this link between allelic variation at a single locus and an extended phenotype that alters ecology represents a rare example of a genotype-to-phenotype-to-ecology linkage (Whitham *et al.* 2008; Crutsinger *et al.* 2014; Hendry 2017). Stickleback lateral plates contain *c.* 22% of the whole-body P pool and the plates on low-plated individuals contain only *c.*

33% of the P found in the plates of high-plated individuals (Durston & El-Sabaawi 2017). We found differences in P excretion associated with plate phenotype despite detecting only modest differences in P content (Fig. S1), a disconnect between content and recycling that has previously been noted in stickleback (Leal *et al.* 2017a). Moreover, we found increased P excretion in high-plated individuals, which also had higher %P content. This suggests that the diet of high-plated fish is sufficiently enriched for P or that high-plated individuals have a higher P use efficiency that leads to relative increases in both content and excretion. El-Sabaawi *et al. (2016)* found that stickleback with higher %P also exhibited a higher P excretion rate, perhaps owing to higher activity and metabolic rates (Tudorache *et al.* 2007). Given that plate phenotype and the *Eda* locus are under strong selection in the polymorphic population we measured (Marchinko *et al.* 2014), selection that shapes allele frequency at *Eda* could produce an 'extended phenotype' (Whitham *et al.* 2003) that shapes the environment in which the fish occur. Experimental work to assess whether selection on *Eda* alters nutrient cycling at a magnitude sufficient to drive changes in productivity would further illuminate the link between changes in genotype frequencies and ecosystem function.

Elemental basis of marine fish adapting to freshwater

The broader adaptation of marine stickleback to freshwater has been well-studied with morphological, physiological, and genomic data (Bell & Foster 1994; Bell *et al.* 2004; Barrett *et al.* 2008; Jones *et al.* 2012; Gibbons *et al.* 2017). This research has demonstrated that adaptation of marine fish to freshwater produces parallel shifts across populations in a range of traits. We found that adaptation to freshwater lead to significantly more efficient assimilation of P (Fig. 2d). This difference could be driven by body size effects on P use (Gillooly *et al.* 2005) and differential selection for efficient P use between marine and freshwater environments (Kilham & Hecky 1988) and might have important feedbacks to ecology (Rudman *et al.* 2015; El-Sabaawi *et al.* 2016; Paccard *et al.* 2018).

Ionomic data clearly show strong divergence between marine and freshwater stickleback in the concentration of multiple elements. Differences in the ionomes of wild-caught marine and freshwater stickleback were particularly strong, likely stemming from differences in both environment and genotype. A 2×2 factorial common garden demonstrates that evolved differences between a marine and freshwater stickleback population shape the ionome. Differences in the ionome of marine and freshwater stickleback were observed regardless of the salinity of their rearing environment. This suggests that the genetic differences between these populations is sufficiently strong to alter the elemental phenotype across a range of environmental contexts. The elements that vary most strongly between the marine and freshwater populations we assayed differ based on rearing salinity, with only barium contributing substantially to the axis that differentiates these fish across both salinities.

Barium availability varies strongly with salinity, as freshwater environments have significantly higher concentrations of barium (Coffey *et al.* 1997; Neff 2002). Barium concentrations are also reliably higher in freshwater fish, to the extent that Ba:Ca ratio is used to identify freshwater occupancy in migratory fish (Elsdon & Gillanders 2005; Tabouret *et al.* 2010). In our study, barium concentration consistently differed between marine and freshwater stickleback populations, regardless of rearing environment (Fig. S1). One putative explanation is that marine stickleback have a higher uptake (via water and/or diet) efficiency of barium owing to lower environmental availability. This is supported by the finding that marine stickleback had lower barium content than freshwater stickleback in nature but had higher barium content than freshwater stickleback when reared in a freshwater common garden. This pattern suggests that barium could be limiting for marine stickleback in saltwater and that high uptake efficiencies or retention for barium may be lost once fish colonize freshwater environments where barium is more abundant.

Ionomic data also suggest that differences in the local availability of elements do not always lead to evolved differences in uptake efficiency in predicted directions. Strontium concentrations in seawater are approximately an order of magnitude higher than those found in freshwater (Tabouret *et al.* 2010). This is reflected in the strontium content of wild-caught marine stickleback, which exceeds that of wild-caught freshwater stickleback. When reared in a saltwater common garden both marine and freshwater stickleback show high strontium concentrations, demonstrating a strong effect of environmental availability (Fig. S1). Interestingly, marine fish raised in freshwater exhibit high strontium content comparable to that of wild-caught marine fish and greater than that observed in freshwater individuals. Hence, despite strontium being relatively abundant in their native environment, marine fish appear to a have higher uptake efficiency than freshwater stickleback, suggesting strontium is not involved in adaptation to freshwater.

CONCLUSION

Taken together, these data illustrate that adaptation involves ionome-wide adjustments which are functions of rapidly evolving physiological processes such as nutrient assimilation and ionoregulation. The integrative framework of ecological stoichiometry is useful in both uncovering likely agents of selection driving adaptation and in examining the ecological consequences of evolution. An ionomic approach, which measures many biologically active elements, is an unbiased diagnostic tool to discover the physiological and ecological relevance of evolutionary change.

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AUTHOR CONTRIBUTIONS

SMR, KVB, CJB and PDJ conceived of the study. All authors contributed to collecting fish, rearing fish and running experiments. SMR did the initial analysis and wrote the first draft of the manuscript. All authors contributed to editing the manuscript and preparing it for publication.

DATA ACCESSIBILITY STATEMENT

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