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Associations among elements in freshwater mussel shells (Unionidae) and their relation to morphology and life history

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Abstract

1. Biogeochemical ecology of organisms typically focuses on C, N, and P despite *c.* 25 elements needed for organismal function. Embracing novel suites of elements in biomass is a first step in linking elements to organismal and ecological functions, improving our ability to predict how species interact with their environment. This research area has been fruitful for terrestrial plant ecologists, yet few studies have considered animal ecology within a framework encompassing elements beyond C, N, and P.
2. Freshwater mussels (Unionidae) are highly endangered filter-feeding bivalves that can be important to ecosystem function. Interspecific trait variation influences soft tissue elemental composition that has been linked to ecosystem biogeochemical cycling using traditional C:N:P stoichiometric approaches. However, whether interspecific trait variation influences shell elemental composition is not well studied, especially for elements other than C, N, and P.
3. We quantified B, C, Ca, Cu, Fe, K, Mg, Mn, N, P, and Zn and constructed isometric log-ratios (nutrient balances) for shells of seven species comprising diverse morphologies and two life history strategies to test whether shell elemental composition is influenced by these biological traits. Additionally, we evaluated whether the growth rate hypothesis applies to shell P concentration and elements associated with P in nutrient balances.
4. Bulk and trace elemental composition varied taxonomically and with biological traits. Nutrient balances for [C | P] and [C, Ca | P] were influenced by life history strategy. Shell P composition was negatively related to growth rates. Coincidentally, [C | P] and [C, Ca | P] were greater in the species with the highest growth rate (*Lampsilis ornata*), suggesting greater concentrations of C and Ca relative to P in shells of faster growing mussels. We hypothesise this observed pattern results from greater P allocation to soft tissue in fast growing mussels compared to slow growing mussels studied previously, but explicit tests of this hypothesis in a strict stoichiometric framework are needed.
5. Overall, we demonstrate how quantifying elements beyond C, N, and P, may be useful in uncovering elemental diversity associated with trade-offs in elemental allocation among biological traits. Whether such elemental diversity correlates to evolutionary history or contributes to the biogeochemical template of freshwater habitats remains to be seen but should be explored.

KEYWORDS
biogeochemistry, growth rate hypothesis, nutrient balance, shells, Unionidae

1 | INTRODUCTION

Elements are essential for organisms and their metabolic processes. Organisms acquire elements from their environment to maintain their body elemental composition (Sterner & Elser, 2002), which varies among species due to different traits (e.g. life-history, morphological, and physiological) and evolutionary history (Allgeier et al., 2020; Atkinson et al., 2020). Knowledge of the elemental composition of species allows application of mass-balance principles to make predictions about elemental acquisition, assimilation, allocation, and release that influences ecological and evolutionary dynamics (Atkinson et al., 2017; Jeyasingh & Weider, 2007). Cataloguing elements comprising organisms reflects constraints faced in their environment and contributes to understanding of elemental plasticity and the ability of species to cope with environmental change (Van De Waal et al., 2010; Williamson et al., 2016). Ecological stoichiometry has revealed substantial diversity in elemental composition among taxonomic groups and within populations by focusing on the relative proportions of the three important elements, carbon (C), nitrogen (N), and phosphorus (P) in relation to their physiological functions (Elser et al., 1996). Considering elements in organisms beyond just C, N, and P, is a first step toward understanding links among multiple elements comprising biomass and the abiotic environment (Baxter, 2015a; Kaspari & Powers, 2016).

While much work has focused on the elemental composition of autotrophs such as algae (Cunningham & John, 2017) and terrestrial plants (Huang & Salt, 2016), a lack of studies considering aspects of animal ecology within a framework embracing elements beyond C, N, and P still exists (Yoshida et al., 2014). Animal studies typically use lab- reared specimens or tissues collected shortly after death (Goos et al., 2017; Jeyasingh et al., 2020; Rudman et al., 2019). However, endangered animals that cannot be sacrificed, handled, or reared in labs warrant other means of obtaining elemental data to better understand aspects of their ecology. Bivalve molluscs provide opportunities to study natural elemental variation using relatively accessible and ecologically relevant morphological traits because fresh spent shells can be found in aquatic habitats where they are abundant and substantial shell material exists in museums and research labs.

Freshwater mussels (Family: Unionidae) are a species-rich group (*c*. 300 mussel species in North America) of filter-feeding bivalves. Mussels are long-lived (4–100 years), highly endangered, (Böhm et al., 2020; Strayer & Dudgeon, 2010; Williams et al., 1993) and important to ecosystem productivity and biogeochemical cycling in freshwater (Vaughn & Hoellein, 2018) and adjacent riparian ecosystems (Allen et al., 2012; Lopez et al., 2020). Mussels occur as dense, spatially and temporally stable aggregations where taxonomic diversity is typically high. Within mussel aggregations, densities range from *c*. 10–100 individuals/m^2 (Sansom et al., 2018). Shell production of aggregations typically ranges from 0.1–10 g dry mass m^{-2} $year^{-1}$, but can be as high as *c*. 1,000 g dry mass m^{-2} $year^{-1}$ (Strayer & Malcom, 2007). Spent shells result from natural mortality (Strayer, 2014), predation (van Ee et al., 2020), or through disturbance-driven mass mortality events (DuBose et al., 2019) and are therefore abundant where mussels occur. Spent shell material adds complexity to the benthos (Gutiérrez et al., 2003), engineering habitat for other organisms (Hopper et al., 2019; Spooner & Vaughn, 2006). Furthermore, spent shells may slowly release or directly supply biologically important elements (Strayer, 2014), adding spatial variation in elements that contribute to the abiotic habitat template (Kaspari & Powers, 2016) underlying freshwater systems.

The shape, size, thickness, and colour of shells vary greatly among mussel species, but basic structures and formation appear similar (Checa, 2000). Each valve has three layers. The external layer is the periostracum and varies in colour and colour pattern; it can be entirely smooth, covered in knobs, pustules, spines, or have wrinkles and undulations depending on the species (Haag, 2012; Williams et al., 2008). Periostracum functions as a covering that protects the calcareous internal layers from abrasion and mineral dissolution in acidic waters. Vertical prisms of calcium carbonate form a thin intermediate layer. The third and largest portion of the shell, called the nacre, is made from thin plates of calcium carbonate acquired from ingested food and ambient water (Compere & Bates, 1973; Pynnönen, 1991). Both outer layers are secreted by glands at the margin of the soft mantle tissue and cause areal expansion of the shell, while the nacre is produced from the entire mantle (Checa, 2000). Shell thickness increases by successive deposits of nacre on the entire inner surface. Despite generalities in the calcareous composition and formation, intra- and interspecific differences could arise via variation in elements required by essential or complementary functions during biomineralisation of shell layers that result in aforementioned shell variations (Checa, 2000). Using functional classification systems accounting for morphological variation and performance associated with habitat types (Watters, 1994) may offer a starting point to understand interspecific elemental diversity of mussel shells as it relates to organismal function.

Mussels also exhibit exceptional diversity in life history and physiological traits (Haag, 2012) that may influence shell elemental composition. For instance, mussels span a fast-to-slow life history continuum reflecting *r*-selected to *K*-selected reproductive strategies with end points characterising trade-offs between increased energy investment early in life at the expense of later and lower investment in reproduction and growth (Haag, 2012). The strong and

ubiquitous negative relationship between growth rate and longevity for mussels suggests that growth rate represents species' positions along the fast-to-slow life history continuum (Haag, 2009). Growth rates may influence shell elemental composition due to trade-offs in elemental allocation to growth or to reproductive effort among different life-history strategies. More specifically, the growth rate hypothesis states that the rate of protein synthesis is constrained by allocation of P to ribosomal RNA in cells, resulting in faster growing organisms having higher tissue P concentrations (Elser et al., 2003). Complex physiological adjustments associated with growth rates may shift demand for elements (e.g., P) and interspecific differences in shell elemental composition could arise from responses that impact the processing of suites of elements and associated energy demands required to meet elemental demand. Indeed, growth rates influence mussel soft tissue stoichiometry (Atkinson et al., 2020), but whether this relationship exists for shells has not been addressed. Thus, studying naturally occurring suites of elements making up mussel shells is a first step toward a more informed understanding of links between all elements involved in organismal function (Baxter, 2015b).

While elemental composition of species' shells could be influenced by life-history strategies and morphology based on elemental requirements underlying physiological and stoichiometric trade-offs governing interspecific trait variation (Jeyasingh et al., 2014), possible underlying physiological mechanisms are currently not well understood. As a first step in gaining a deeper understanding of elements in species' biomass, we tested whether elemental composition differs along taxonomic, morphological, and life history axes. Additionally, we constructed nutrient balances using multivariate data to examine how interspecific trait variation influences covariation among elements (Parent et al., 2013). Finally, we tested whether shell P composition and suites of correlated elements were influenced by growth rates. We hypothesised that shell P composition relative to other elements would be negatively related to growth rates and elements used in biomineralisation (e.g., C and Ca) should be positively related to growth rates because mussels should allocate relatively more P to faster growing soft tissues (e.g., mantle tissue) that secrete the shell.

2 | METHODS

2.1 | Shell collections, life history, and morphological classifications

To test the hypothesis that mussels would express interspecific differences in elemental composition we used fresh spent shells (shiny nacre, intact hinge ligament, soft tissue present) of seven species representing four phylogenetic tribes collected from the Sipsey River, Alabama, U.S.A. during 2016 (Figure 1). While contemporary and historical shell material have proven useful for reconstructing historical environmental conditions (Fritts et al., 2017) and been used extensively in pollution studies (Brown et al., 2005; Wilson et al., 2018), shells from the Sipsey River offer a starting point for quantifying the natural elemental composition of shells, as it is relatively undisturbed river with low background nutrient concentrations and has a diverse, intact native mussel community (Atkinson et al., 2019; Haag & Warren, 2010). Because we were interested in whether trade-offs linked with interspecific trait variation influence elemental composition of species, we classified species' life history strategies according to Haag (2012), used published growth rates (*k*) for species in our system (Haag & Rypel, 2011) and shell functional morphology classifications following Watters (1994; Figure 1).

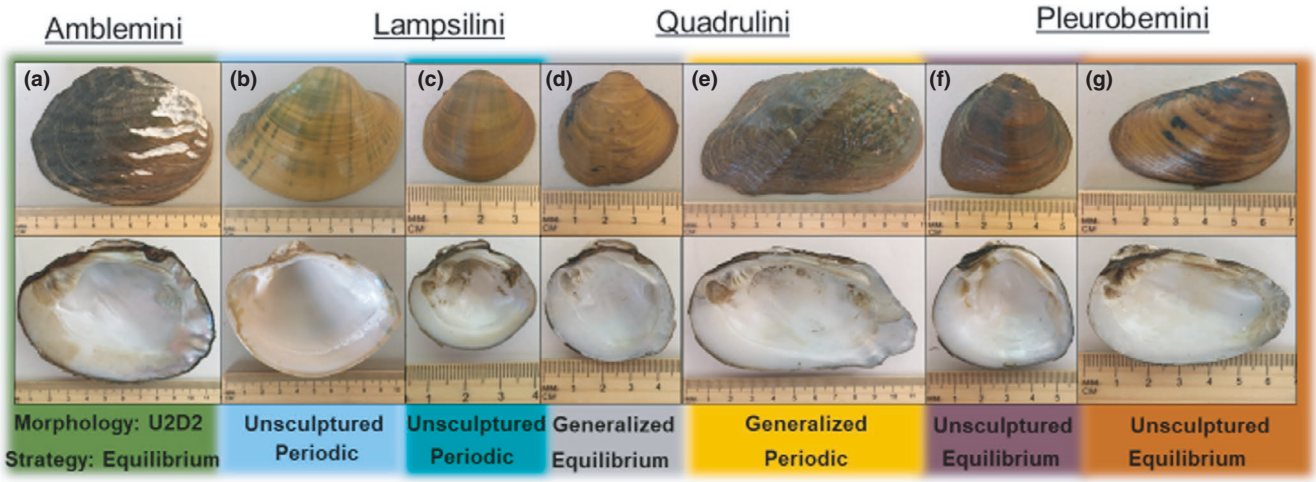

FIGURE 1 Exterior (top) showing interspecific variation of periostracum and interior (bottom) view of representative shells for *Amblema plicata*, $n = 5$ (a), *Lampsilis ornata*, $n = 6$ (b), *Obovaria unicolor*, $n = 5$ (c), *Cyclonaias asperata*, $n = 6$ (d), *Tritigonia verrucosa*, $n = 4$ (e), *Fusconaia cerina*, $n = 5$ (f), and *Pleurobema decisum*, $n = 7$ (g) collected from the Sipsey River, Alabama, U.S.A. Phylogenetic tribes are listed above the top panel. Functional morphological (Watters, 1994) and life history strategy (Haag, 2012) classifications are listed below

2.2 | Shell and water elemental composition

A single valve of each specimen was washed with distilled water and gently scrubbed with a soft-bristled brush to remove adhered sediments and remaining soft tissue. We measured the longest axis of each shell (mm) and then dried them at 60°C for 48 hr. We homogenised dried shell fragments taken from the ventral shell margin using a ball mill grinder (Retsch MM400, Verder Scientific Inc. Newton, PA, U.S.A.) and weighed subsamples to the nearest 0.1 mg for all seven species (Figure 1). Shell fragments included the proteinaceous external layer of the shell (periostracum) given intraspecific variation in colour and textures may result from different elemental composition (Figure 1). These samples should reflect conditions during the final *c*. 2–3 years of life. We measured %C and %N using a Carlo Erba CHNS-O EA1108-Elemental Analyzer (Isomass Scientific Inc., Calgary, Alberta, Canada). We did not separate organic and inorganic C, but organic C can constitute a small percentage (*c*. 0.53%) of bivalve shell mass (Walz, 1979). Next, we sent 0.5 g sub-samples to Waters Agricultural Laboratories, Inc. (Camilla, GA, U.S.A.). Samples were digested in 4 ml of nitric acid and 6 ml of hydrochloric acid before they were diluted to 50 ml with deionised water, then analysed on a Spectro Arcos II ICP-OES. To explore differences between elements in water and shells we collected water samples ($n = 6$) in 2019 and 2020 from near where shells were collected. We used previously published dissolved organic C measurements (Hopper et al., 2021) and measured NH_4–N, NO_3–N, NO_2–N, and soluble reactive phosphorus (PO_4–P) on a SEAL AQ300 Discrete Analyzer (SEAL Analytics, WI, U.S.A.). We combined these parameters as a measure of total inorganic N from water samples collected during 2020. Analyses for all other elements were run at Waters Agricultural Laboratories Inc. as for shells. We measured 11 elements (Figure 2): boron (B), C, calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), N, P, and zinc (Zn). Elemental concentrations for shells were converted and expressed as absolute element mass per sample (mg/g) and water (mg/L).

2.3 | Data analysis and nutrient balance construction

Statistical analyses and data visualisation were performed in R v3.6.0 (R Core Development Team, 2019; Wickham, 2011). Elemental composition of animals can be influenced by ontogeny and sex (Metcalfe-Smith et al., 1996; Prater et al., 2019). We were unable to evaluate influences of body size and species interactions reflecting interspecific ontogenetic shifts in elemental composition because body sizes did not overlap for all species when evaluated using analysis of variance ($F_{6,31} = 30.85$, $p < 0.001$). Associated information of the sex of each mussel was not available. Shell elemental compositional data failed test of multivariate normality (Mardia skewness = 404.49, $p < 0.001$, Mardia kurtosis = 1.92, $p = 0.06$; mvn function; package MVN; Razali et al., 2016). Therefore, we tested for taxonomic, morphological, and life history differences in elemental concentrations using non-parametric permutational multivariate analysis of variance (PERMANOVA; *adonis* function, vegan package Oksanen et al., 2019) and *betadisper* to test for homogeneity of variance (Anderson, 2006; Oksanen et al., 2019). All elemental data were subjected to tests of univariate normality (*shapiro.test* function) and homogeneity of variance (*leveneTest* function; car package Fox et al., 2018). All elements met univariate homogeneity of variance assumptions, but C, Ca, Cu, Mn, and Mg did not meet normality assumptions following log transformation. Therefore, we used non-parametric analysis of variances (ANOVA) on ranks (*dunn.test* function) to test for differences in concentrations of specific elements among groups and assessed pairwise differences using Dunn's tests with Bonferroni adjusted *p*-values. Overall, this provides a conservative estimate of elemental differences among groups. Specific group comparisons were made instead of full factorial comparisons as complete species, life history, and morphological combinations were not included in our dataset.

We constructed isometric log-ratio balances (*ilr*), commonly called nutrient balances, for traditional ecological stoichiometry metrics (C | P, N | P) and novel nutrient balances from principle components analysis of elemental data. Nutrient balances represent orthogonal log contrasts of elements derived from binary partitions of multivariate elemental data projected into Euclidean space (Parent et al., 2013). The *ilr*s provide unbiased estimates of multivariate relationships among elements, avoid violating statistical assumptions, and describe element interactions in biomass (Parent et al., 2013). Moreover, *ilr*s are useful for hypothesis testing using general linear models without inflating critical values due to multiple tests for treatment effects on multiple elements. To construct nutrient balances, we first separated all elements into bulk and trace elements based on the classification of Frausto da Silva and Williams (2001). Next, we visualised relationships among elements and examined variation among species using principal component analysis (PCA; prcomp, *vegan*; Oksanen et al., 2019). Elemental concentrations were log transformed prior to PCA. We retained PCs if their eigenvalues exceeded 1. We included elements with absolute loadings >0.1 on each PC axis to construct bulk and trace *ilr*s for each individual following Parent et al.,(2013) using the equation:

$$ilr = \sqrt{\frac{rs}{r+s}} \ln \frac{g(c^+)}{g(c^-)}$$

where r and s reflect the number of elements on the left- and right-hand side of the balance and $g(c^+)$ and $g(c^-)$ are geometric means of the elements on the left- and right-hand side of the balance, respectively.

We constructed two traditional nutrient balances [C | P], [N | P] and 5 novel nutrient balances for shells of all seven species. We calculated a single *ilr* for PC 1, and bulk and trace *ilr*s for PC 2 and PC 3. The *ilr* for PC 1 was [C, N, P, K, B, Zn, Fe, Cu, Ca | Mn]. The Bulk *ilr* for PC 2 were [C, N | Ca, K] and the Trace *ilr* for PC 2 was [Cu, Mn, Zn | Fe, Mg]. Bulk *ilr* for PC 3 was [C, Ca | P] and the Trace *ilr* for PC 3

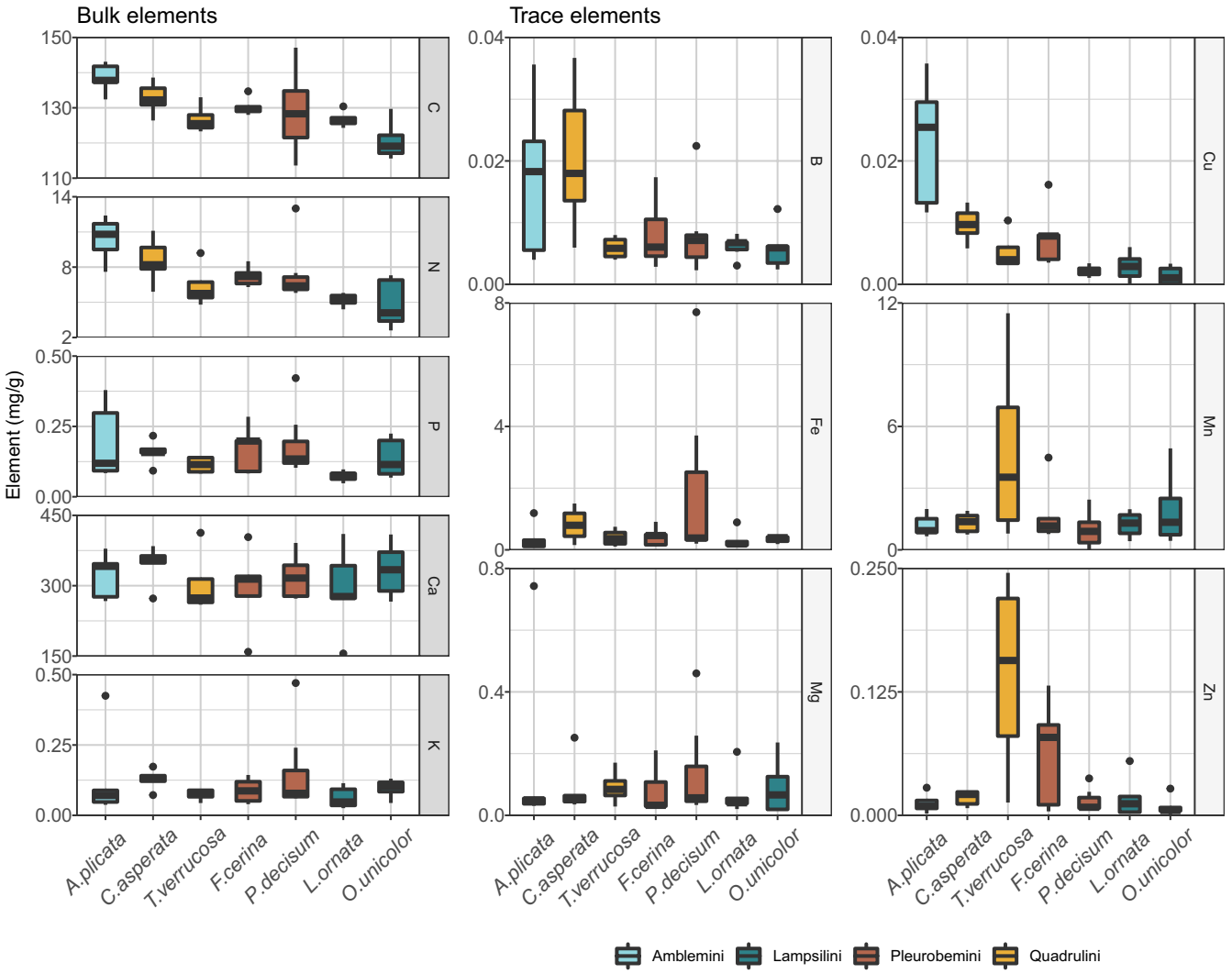

FIGURE 2 Elements in shells of seven freshwater mussel species (Unionidae). Boxes are coloured according to phylogenetic tribe classifications to visualise potential phylogenetic patterns. Boxes cover the first to third quartiles; horizontal black lines within boxes indicate medians. Grey and white panel headings denote bulk and trace elements, respectively. Pairwise differences can be found in Table 2. Note different y axes

was [Cu, Fe, Mn, Zn, | B]. These correlations are considered to reflect biologically relevant differences in elemental composition among organisms (Parent et al., 2013).

First, we assessed differences in all nutrient balances among species, life history strategies, and morphological classification using multivariate analysis of variance (MANOVA). Then, we tested for differences among species, life history strategies, and shell morphological classification with univariate tests (ANOVA) for each *ilr* separately. We followed these tests with pairwise contrasts for groups using Tukey's HSD tests (package emmeans Lenth, 2018).

Finally, to test whether growth rates influenced P composition and associated elements, we regressed growth rates against shell P composition, traditional nutrient balances, and novel nutrient balances containing P given the strong biological link between growth rates and P (Elser et al., 2003). Complimentary univariate and multivariate analyses were intended to quantify potential sources of variance in individual balances (ANOVAs) and multivariate phenotypes (MANOVAs) and reduce the likelihood of statistical artifacts associated with multivariate data analyses. As for elemental composition, we focused on group comparisons over full factorial comparisons because complete species, life history, and morphological combinations were unavailable.

3 | RESULTS

3.1 | Morphological and life history trait diversity

Shells of the seven species were classified into four separate groups. *Amblema plicata* (min. length = 58.61 mm, max. length = 73.40 mm, mean length = 67.08 mm) was the only U2D2 shell type. This shell type consists of undulating ribs aligned obliquely to the burrowing axis that anchor the animal once buried and can be

effective across a range of sediment types (Figure 1a). *Lampsilis ornata* (min. length = 62.05 mm, max. length = 88.51 mm, mean length = 74.72 mm), *Obovaria unicolor* (min. length = 26.61 mm, max. length = 38.66 mm, mean length = 33.33 mm), *Pleurobema decisum* (min. length = 31.15 mm, max. length = 46.31 mm, mean length = 39.43 mm), and *Fusconaia cerina* (min. length = 31.10 mm, max. length = 46.31 mm, mean length = 36.31 mm) had unsculptured shells. Species with unsculptured shells are more mobile and able to re-bury quickly if dislodged and often inhabit mixed sand and fine gravel sediment. *Cyclonaias asperata* (min. length = 28.09 mm, max. length = 38.11 mm, mean length = 33.92 mm) and *Tritogonia verrucosa* (min. length = 45.22 mm, max. length = 69.40 mm, mean length = 59.50 mm) each have generalised shells. Generalised shells have pustules dispersed across their discs that aid in anchoring once the mussel is buried and can be effective in sand and gravel mixtures.

We assigned mussel life history strategies according to Haag (2012). *Amblema plicata*, *F. cerina*, *P. decisum*, and *C. asperata* were classified as equilibrium species because their relatively long lifespan and late maturity coinciding with increasing fecundity. *Lampsilis ornata*, *O. unicolor*, and *T. verrucosa* are periodic strategists and tend toward faster growth rates, shorter life spans, age at maturation, and fecundity.

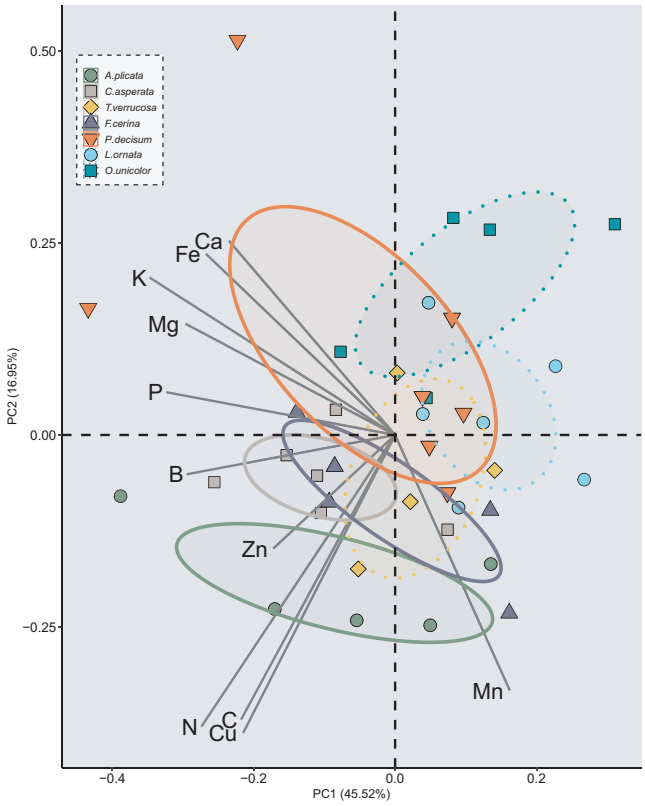

FIGURE 3 Principal components analysis of concentrations (molar) of 11 elements in shells of seven freshwater mussel species collected from the Sipsey River, AL. Ellipses are 95% confidences ellipses. Periodic strategists have dotted ellipses while equilibrium strategists have solid ellipses

3.2 | Patterns in natural shell elemental composition, water, and shell nutrient balances

Elemental composition varied among species (pseudo $F_{6,31} = 1.72$, $p = 0.09$; Figure 3), morphologies (pseudo $F_{2,35} = 1.85$ $p = 0.141$) and between life history strategies (pseudo $F_{1,36} = 3.99$, $p = 0.01$; Figure 3) according to PERMANOVA. Among group dispersion for elemental compositions was not discernible for species ($F_{6,31} = 0.73$, $p = 0.63$), morphology ($F_{2,35} = 0.94$, $p = 0.40$) or life history strategy ($F_{1,36} = 0.36$, $p = 0.55$).

Univariate tests indicated that shell C, Cu, N, and P varied across species (Table 1), while shell C, Cu, and N varied with morphology (Table 1). Shell B, C, Cu, N, and P were all greater in equilibrium species compared to periodic strategists (Tables 1). Pairwise comparisons among species (Table 2) indicated *O. unicolor* had lower shell C than *A. plicata* (z = 3.62, $p = 0.003$) and *C. asperata* (z = 2.82, $p = 0.05$). Cu concentrations were greater in *A. plicata* compared to *L. ornata* (z = 3.31, $p = 0.009$), *O. unicolor* (z = 3.91, $p = 0.001$), and *P. decisum* (z = 3.85, $p = 0.001$), while *C. asperata* had increased Cu concentrations relative to *O. unicolor* (z = 3.19, $p = 0.01$) and *P. decisum* (z = 3.08, $p = 0.02$). Similar to C, *A. plicata* had greater N compared to *L. ornata* (z = 3.76, $p = 0.002$), and *O. unicolor* (z = 3.27, $p = 0.01$), and *C. asperata* had greater N compared to *L. ornata* (z = 3.12, $p = 0.02$). Finally, *L. ornata* has lower concentrations of P compared to *C. asperata* (z = 3.01, $p = 0.03$) and *P. decisum* (z = −2.93, $p = 0.04$). When grouped into morphological classification, pairwise differences in B were not strong enough to separate statistically. U2D2 represented by *A. plicata* only had greater C than the unsculptured group (z = 3.17, $p = 0.002$) that included four species (*F. cerina*, *L. ornata*, *O. unicolor*, *P. decisum*). The U2D2 species, *A. plicata* (z = 3.94, $p < 0.001$), and generalised group, *C. asperata* and *T. verrucosa* (z = −1.51, $p = 0.005$), had greater Cu concentration than the unsculptured group. *Amblema plicata* had greater N compared to the unsculptured group (z = 3.24, $p = 0.002$), and differences in P were not strong enough to separate in pairwise tests.

The ordination illustrated a gradient from shells of equilibrium strategists with greater elemental concentrations (negative loadings) to shells of periodic strategists with lower concentrations (positive loadings) and manganese was the only element to diverge from the others along PC 1 (Figure 3; Table S1). Of the elements in shells, Mg, Ca, C (as dissolved organic C), K, N (as total inorganic N), and Mn were found in water samples, while B, Cu, Fe, P, and Zn were below detection limits (Table S2).

Nutrient balances were influenced by species (approx. $F_{6,31} = 1.78$, $p < 0.008$, Wilk's $\lambda = 0.10$), life history strategy (approx. $F_{1,36} = 4.30$, $p = 0.002$, Wilk's $\lambda = 0.50$) and morphology (approx. $F_{2,35} = 1.91$, $p = 0.04$, Wilk's $\lambda = 0.47$). Subsequent univariate tests indicated the traditional nutrient balance [C | P] and the novel nutrient balance [C, Ca | P] were strongly influenced by life history strategy and [C, Ca | P] differed among species (Table 3,4). Pairwise tests showed periodic strategists had lower [C | P] (z =

TABLE 1 Results of non-parametric analyses of variance examining elemental differences among shells of seven freshwater mussel species (Unionidae) from the Sipsey River, Alabama, U.S.A.

		Bulk									
		C		N		P		K		Ca	
Model	*df*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
Species	6, 31	**18.11**	**0.005**	**22.17**	**0.001**	**12.99**	**0.04**	8.31	0.21	2.93	0.81
Morphology	2, 35	**10.59**	**0.005**	**11.52**	**0.003**	1.5	0.47	2.28	0.32	0.30	0.86
Life history	1, 36	**10.79**	**0.001**	**16.75**	**<0.001**	**9.01**	**0.003**	2.27	0.13	0.49	0.48

		Trace											
		B		Cu		Fe		Mn		Mg		Zn	
Model	*df*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
Species	6, 31	9.47	0.14	**28.7**	**<0.001**	7.94	0.24	4.53	0.60	2.13	0.90	11.31	0.07
Morphology	2, 35	4.95	0.08	**19.89**	**<0.001**	2.38	0.30	1.67	0.43	0.53	0.76	4.97	0.08
Life history	1, 36	**3.82**	**0.05**	**8.05**	**0.005**	2.18	0.13	1.18	0.28	0.09	0.75	0.16	0.67

Note: Results from global permutational multivariate analysis of variance are reported in the text. Bolded fonts are significant at $\alpha = 0.05$.

TABLE 2 Pairwise differences ($p < 0.05$) following non-parametric univariate tests for elements in shells of seven species of freshwater mussels from the Sipsey River, Alabama, U.S.A.

Species	C	N	P	K	Ca	B	Cu	Fe	Mg	Mn	Zn
Amblema plicata	**a**	**a**	**ab**	a	a	a	**a**	a	a	a	a
Cyclonaias asperata	**a**	**ab**	**b**	a	a	a	**ac**	a	a	a	a
Tritogonia verrucosa	**ab**	**a**	**ab**	a	a	a	**a**	a	a	a	a
Fusconaia cerina	**ab**	**a**	**ab**	a	a	a	**a**	a	a	a	a
Pleurobema decisum	**ab**	**a**	**b**	a	a	a	**b**	a	a	a	a
Lampsilis ornata	**ab**	**c**	**a**	a	a	a	**bc**	a	a	a	a
Obovaria unicolor	**b**	**bc**	**ab**	a	a	a	**b**	a	a	a	a

Note: Bolded font highlights significant pairwise test for species.

TABLE 3 Results of analyses of variance examining nutrient balance differences among shells of seven freshwater mussel species (Unionidae) from the Sipsey River, Alabama, U.S.A.

		Traditional ecological stoichiometry balances						Novel nutrient balances							
		[C | P]		[N | P]		All PC 1 [C, Ca, N, P, K, B, Cu, Fe, Mg, Zn, | Mn]		Buk PC 2 [C, N | Ca, K]		Bulk PC 3 [C, Ca, | P]		Trace PC 2 [Cu, Mn, Zn | Fe, Mg]		Trace PC3 [Cu, Fe, Mn, Zn | B]	
Model	*df*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Species	6, 31	2.19	0.07	1.77	0.14	1.7	0.15	0.77	0.6	**2.38**	**0.05**	1.87	0.12	2.27	0.06
Morphology	2, 35	0.28	0.76	0.91	0.41	0.91	0.41	0.30	0.74	0.41	0.67	0.45	0.64	2.21	0.12
Life history	1, 36	**8.15**	**0.007**	0.12	0.73	0.16	0.7	0.15	0.7	**10.5**	**0.003**	3.38	0.07	2.98	0.09

Note: Global multivariate test results are reported in the main text. Bold text is used to highlight significant univariate tests at $\alpha = 0.05$.

−2.73, $p = 0.003$) and [C, Ca | P] ($z = -2.88$, $p = 0.002$) compared to equilibrium strategists. Growth rates predicted shell P composition ($r^2 = 0.13$, $F = 6.73$, $p = 0.01$; Figure 4a), [C | P] ($r^2 = 0.20$, $F = 9.99$, $p = 0.003$; Figure 4b) and [C, Ca | P] ($r^2 = 0.21$, $F = 10.87$, $p = 0.002$; Figure 4c), but not [N | P] ($r^2 = 0.01$, $F = 1.32$, $p = 0.26$). However, each of these relationships was strongly influenced by *L. ornata*, and fell apart when this species was removed (mg/g P: $r^2 = 0.25$, $F = 1.31$, $p = 0.31$; [C|P]: $r^2 = 0.00$, $F = 0.001$, $p = 0.94$; [C, Ca | P]: $r^2 = 0.03$, $F = 0.13$, $p = 0.73$). Thus, there appears to be weak evidence in support of the growth rate hypothesis in the

TABLE 4 Pairwise differences ($p < 0.05$) for nutrient balances for shells of seven species of freshwater mussels from the Sipsey River, Alabama, U.S.A.

Species	[C | P]	[N | P]	[B, C, Ca, Cu, Fe, K, Mg, N, P, Zn | Mn]	[C, N| Ca, K]	[C, Ca | P]	[Cu, Mn, Zn, | Fe, Mg]	[Cu, Fe, Mn, Zn | B].
Amblema plicata	a	a	a	a	**ab**	a	a
Cyclonaias asperata	a	a	a	a	**ab**	a	a
Tritogonia verrucosa	a	a	a	a	**ab**	a	a
Fusconaia cerina	a	a	a	a	**b**	a	a
Pleurobema decisum	a	a	a	a	**b**	a	a
Lampsilis ornata	a	a	a	a	**a**	a	a
Obovaria unicolor	a	a	a	a	**ab**	a	a

Note: Bold entries indicate significant test results at $p < 0.05$.

assemblage we studied. Nevertheless, the species with the highest growth rate had the lowest shell P concentrations.

4 | DISCUSSION

Using key physical traits involved in organismal function, we found elemental diversity within a highly endangered group of filter-feeding bivalves. We quantified divergent patterns of traditionally studied elements (C, N, and P) and two other biologically relevant elements (B, Cu) comprising shell elemental profiles for an assemblage of mussels. Our nutrient balance approach, typically applied to plants, identified relationships among bulk elements that differed across animal species with divergent morphologies and life history strategies in multivariate space. By constructing novel nutrient balances in addition to two balances based on traditional ecological stoichiometry, we provide empirical evidence of species-specific elemental relationships that illustrate associations among elements required for shell biomineralisation and growth. Overall, we highlight a useful approach to quantifying elements beyond C, N, and P for studying biological sources of elemental diversity in animal biomass and add to the growing knowledge of elemental diversity among animals (Allgeier et al., 2015, 2020), and more specifically unionid mussels (Atkinson et al., 2020; Hopper et al., 2021; Vaughn, 2010).

Underlying causes of organismal elemental variation may be linked to constraints imposed by environmental availability or trait adaptation that alter elemental demands (Jeyasingh et al., 2014). Overall, elemental composition of shells was fairly similar within the local assemblage we studied, suggesting some conservatism in elemental recipes for unionid shells. However, shell bulk elemental composition, particularly for classical ecological stoichiometry elements (C, N, and P) was explained by conventional traits and taxonomy. This result indicates that trade-offs might exist for aspects of functional morphology and position along the fast-to-slow life history continuum (Haag, 2012). Whereas our study comprised individuals from only one location, spatial differences in elemental profiles of habitats occupied by mussels across spatial scales (i.e., patches, reaches, catchments) could also influence shell elemental composition, warranting additional research.

We also found weak support for the growth rate hypothesis in mediating elements in unionid shells. Previous work found a positive relationship between growth rates and soft tissue P concentration of co-occurring species that was weakly associated evolutionary history (Atkinson et al., 2020). Using a subset of species (different individuals) from that study, we found that shell P concentrations showed a weak relationship with growth rates; however, the sign of this relationship was negative, such that the faster growing species had comparatively less P. Whether this indicates trade-offs in P allocation to soft and shell tissue remains to be seen and should be further explored by analysing a larger subset of species varying in growth rates. Furthermore, explicit stoichiometric approaches that balance P and suites of elements acquired, assimilated, allocated, and released are needed (Jeyasingh et al., 2020; Prater et al., 2020). Given that mussel soft tissue stoichiometry is constrained by evolutionary history (Atkinson et al., 2020), it seems plausible the same mechanisms may influence proportions of elements in shell material. Further efforts employing comparative phylogenetic approaches with broad taxonomic and body size representation spanning ecological gradients are warranted to test whether elemental composition of different tissue types varies as a function of evolutionary history or local factors.

Shells also contained various concentrations of elements other than traditionally studied C, N, and P. While our study cannot determine whether elements quantified herein are important to mussels themselves, shells could contribute to the biogeochemical template of aquatic ecosystems following mussel deaths. For instance, trace metals that we quantified such as B, Cu, Mn, Fe, and Zn, comprise 0.1% of animal mass but they are functional parts of thousands of known enzymes that maintain various cellular and organismal functions (Bairoch, 2000; Waldron et al., 2009) and all are spatially heterogeneous in ecosystems (John et al., 2007; Leslie et al., 1986). Spent shells might subsidise food webs or biogeochemical cycles with trace elements, but this is not well studied (but see Ilarri et al., 2015; McDowell & Sousa, 2019; Strayer & Malcom, 2007) compared to soft tissue decomposition. For example, elements stored in mussel soft tissue are released quickly via microbial mineralisation following mass die-offs, affecting ecosystem productivity (DuBose et al., 2019). In contrast to soft tissue that decomposes in

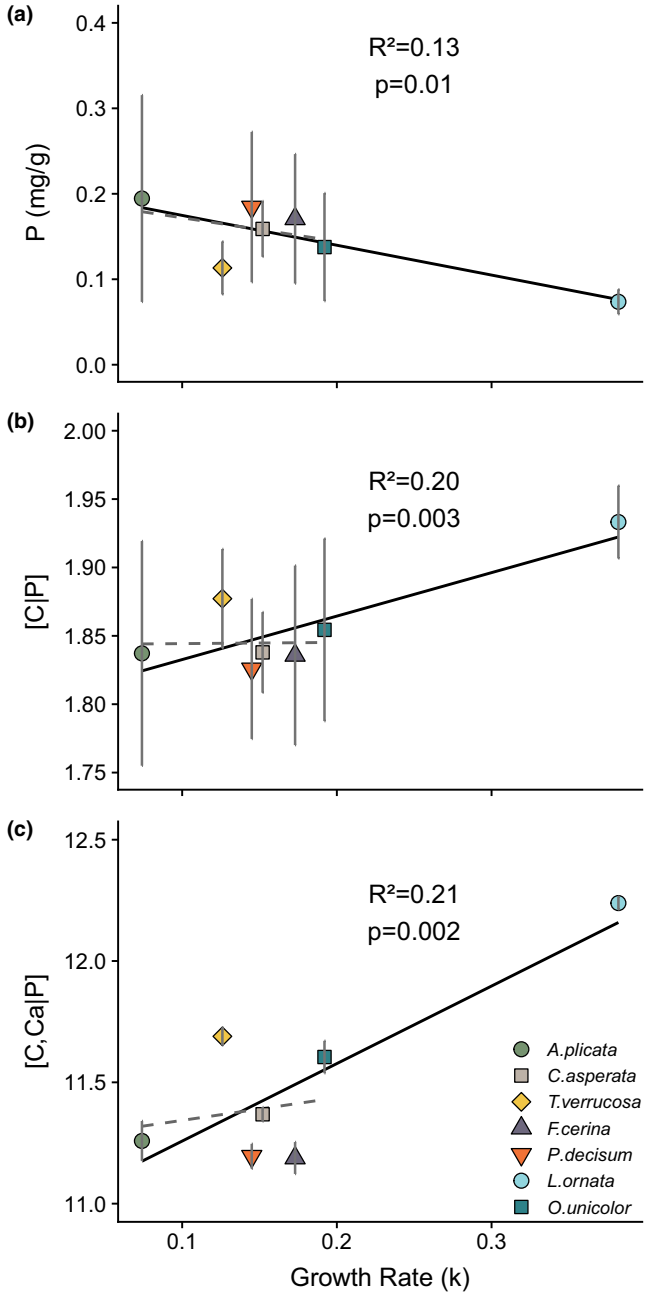

FIGURE 4 Relationship between species-specific growth rates (from Haag & Rypel, 2011) collected from our the Sipsey River, U.S.A. and shell P (a), traditional stoichiometric balance [C | P] (b), and novel balance [C, Ca | P] (c). Points are means ±2 standard error. Gray dashed lines illustrate each relationship excluding *Lampsilis ornata*. Associated r^2 and *p* values are reported in the text

days, shells can linger for a significant time after death, inherently storing coupled bulk and trace elements (Ravera et al., 2003). While naturally occurring spent shells in mussel beds may not represent temporally distinct pulses of elemental subsidies beyond C, N, and P, such as Pacific salmon (*Onchorhyncus spp.*) carcasses following spawning runs (Currier et al., 2020), they are likely to affect elemental heterogeneity in benthic habitats. Because shells consist mostly of $CaCO_3$ (Strayer & Malcom, 2007), most studies assume that spent shells do not serve as slow release nutrient pools that fuel productivity like vertebrate skeletons that are P reservoirs (Subalusky et al., 2017). Indeed, Ca (315.2 mg/g ± 60.2 mean of all species) and C (128.7 mg/g ± 5.1 mean of all species) were dominant elements in shells, but we found eight additional elements, two of which varied among species with different morphologies and life histories. Thus, it seems plausible that shells may serve as spatially patchy sources of trace elements that serve essential or complementary functions to other organisms growing on or near them as they break down (Lukens et al., 2017; Spooner & Vaughn, 2006; Vaughn et al., 2002). Additionally, interspecific and intraspecific variability of Zn, Mg, B, and Cu in shells of some species (e.g., *T. verrucosa*) may reflect elemental profiles driven by microhabitat conditions, but more rigorous tests of this are beyond the scope of this study. Whether such elemental variability is unique to species warrants further investigation of populations dispersed along ecological gradients. Collectively, our cataloguing of shell elemental profiles advances our understanding of phenotypic diversity that may contribute to cycling and storage of biologically important, but understudied bulk and trace elements (Currier et al., 2020). Still, assessing the relative importance of unionid shells in elemental cycling, warrants additional research that incorporates comparative data on water, sediment, and food sources.

Nutrient balances characterise element interactions and have only recently been applied to analyses of animal elemental composition (e.g., Prater et al., 2019, 2020). Life history strategy influenced [C | P] and [C, Ca | P] nutrient balances, suggesting that there may be a link between positions along the life history continuum and shell elements, as reflected by growth rates. Variation was primarily driven by greater [C | P] and [C, Ca | P] balances of *L. ornata* shells relative to other species. Another study found faster growing *L. ornata* had greater soft tissue P, yielding lower C:P and N:P (Atkinson et al., 2020). Growth rates generally control P content of animals (Elser et al., 2003); however, this has primarily been evaluated in soft tissue samples of mussels. Moreover, [C|P] and [C, Ca | P] appear to describe a similar axis of shell elemental composition. When elemental data beyond C, N, and P for unionid shells are unavailable, the classical stoichiometric ratio for C and P is probably a sufficient metric for P relative to the calcareous shell matrix. Combined with previous work, our novel nutrient balance for biologically relevant elements [C, Ca | P] demonstrates that biomineralisation of a key trait for organismal function of unionids (shells) may be driven by inverse allocation of P to soft and shell tissue. Thus, we hypothesise that faster growing mussels prioritise P allocation toward soft tissue growth resulting in relatively reduced P allocation to the shell compared to the $CaCO_3$ matrix. However, more explicit tests of this hypothesis will require balancing elements in paired soft and shell tissue from species covering the full fast-to-slow continuum.

While freshwater mussels, and more generally molluscs, are threatened by many factors, shells are commonplace in habitats where they exist (Gutiérrez et al., 2003) and are stored in museum

collections or malacological research labs. For abundant widespread species, this presents low-risk research avenues exploring elemental variation among populations to understand whether and how elemental profiles are influenced by resource stoichiometry gradients, or temporal comparisons that track responses to global change (Black et al., 2017). As an example, elemental composition of macroinvertebrate communities in headwater streams shifted following dietary N and P enrichment that varied interspecifically (Prater et al., 2020). We anticipate that widespread habitat degradation (Chiba & Roy, 2011; Strayer & Dudgeon, 2010), coupled with the key role of molluscs across all ecosystems (Vaughn & Hoellein, 2018; Zaady et al., 1996), may fundamentally alter cycling of understudied biologically essential elements by changing their relative proportions stored in soft tissue and shells. Thus, we advocate that well preserved shell material is useful in studying novel aspects of mollusc trait diversity as they relate to nutritional and functional ecology, especially in the context of global change. Consideration of elemental diversity of mussels presents a basis for generating experiments to advance our understanding of the ecology of this highly endangered group and, more broadly, animal trait diversity, and how the elemental composition of animals is linked to ecosystem functions.

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AUTHOR CONTRIBUTIONS

G.W.H. and C.L.A. conceived the idea. G.W.H. and G.K.D. collected and prepared the samples. G.W.H. analysed the data and wrote the original draft. All authors contributed to editing and revisions.

DATA AVAILABILITY STATEMENT

Data are available at https://github.com/HopperG311/Hopper_et_al_FWB_Shellements

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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