

Understanding variation in salamander ionomes: A nutrient balance approach

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1 **Abstract**

2 1. Ecological stoichiometry uses information on three key elements (C, N, and P), which are
3 abundant in biomass to explain ecological patterns. Comparatively less is known however about
4 dynamics of the other essential elements comprising biological tissues (i.e., the ionome) or their
5 roles in growth and development of vertebrate consumers, especially ecologically sensitive taxa
6 such as amphibians.

7 2. In this paper, we report observations of ionic variation in two species of salamander
8 (*Ambystoma opacum* and *A. talpoideum*) across ontogenic stages using specimens from
9 biological collections from two wetlands sampled over a 30-year period. This unique data set
10 allowed us to explore the extent of ionic variation between species and through space and
11 time.

12 3. We found species- and to a lesser extent site-specific differences in traditionally studied
13 stoichiometric elements along with 13 other elements forming salamander ionomes but saw no
14 evidence of temporal changes. Salamander ionic composition was most strongly related to
15 ontogeny with relatively higher concentrations of many elements in adult males (i.e., Ca, P, S,
16 Mg, Zn, and Cu) compared to metamorphic juveniles, which had greater amounts of C, Fe, and
17 Mn.

18 4. In addition to patterns in individual elements, covariance among elements was used to
19 construct multi-elemental nutrient balances, which revealed differences in salamander elemental
20 composition between species and sites and showed systematic changes in elemental proportions
21 across ontogenic development. These multi-elemental balances along with traditional
22 stoichiometric balances also better distinguished among species-site-ontogenic groups than
23 stoichiometric balances only.

24 5. Overall, this study highlights the responsiveness of consumer ionomes to life-history and
25 environmental variation, while reflecting underlying relationships among elements tied to
26 biological function. As such, ionomic studies can provide important insights into factors shaping
27 consumer elemental composition and for predicting how these changes might affect higher-order
28 ecological processes.

29 **Introduction**

30 Elements are the fundamental building blocks of living cells and are involved in all metabolic
31 processes. Organisms must take up all elements from the environment and, despite vast
32 differences in environmental supplies, maintain their body elemental composition within a
33 relatively narrow range (Persson *et al.*, 2010). Elemental content differs considerably among
34 species due to diversity in classical traits (e.g., life-history, morphological, and physiological
35 traits) that are constructed using different elements or the same elements in differing ratios
36 (Jeyasingh, Cothran & Tobler, 2014). Thus, it follows that the elemental composition of an
37 individual is determined by the acquisition, assimilation, and allocation of elements within the
38 organism. This abstraction is useful because information on the elemental content of a species
39 enables the application of mass-balance principles to make predictions about sequestration and
40 excretion of elements, both of which have strong impacts on ecological dynamics (Reiners,
41 1986). This elemental approach has triggered a vibrant area of research integrating data on
42 species elemental composition with cellular processes and organismal life-history to study key
43 ecological phenomena (Sturner & Elser, 2002).

44 In addition to species-level differences, it is apparent that there is considerable
45 intraspecific variation in elemental composition both within and among populations, which often
46 rivals interspecific variation (Jeyasingh *et al.*, 2014; Prater, Wagner & Frost, 2017). For
47 example, Bertram *et al.* (2008) found that intraspecific variation in the phosphorus (P) content of
48 a single species of cricket collected across field populations was as extensive as that observed
49 across several orders of insect taxa (Woods *et al.*, 2004). Further, strong P plasticity among
50 distinct ontogenic stages in metazoans linked to differences in organismal growth and
51 reproduction has commonly been observed (e.g., Villar-argaiz *et al.* 2002, Capps *et al.* 2015,

52 Tiegs et al. 2016). Besides P, recent work also suggests that many additional elements interact at
53 the lowest levels of biological organization whose metabolic relationships have yet to be fully
54 realized (e.g., Baxter 2010, Goos et al. 2017, Jeyasingh et al. 2017). At a time when it is
55 becoming increasingly clear that growth and productivity can be limited by elements in addition
56 to P, or more likely by a combination of elements (Salt, Baxter & Lahner, 2008; Harpole *et al.*,
57 2011; Parent *et al.*, 2013a), determination of the extent and nature of variation in the content of
58 the other 20-odd elements found in biological tissues is needed to establish their connections
59 with organismal metabolism and ecological functions.

60 The ionome is defined as the mineral nutrient and trace element composition of an
61 organism (Salt *et al.*, 2008), which underlies the morphological, anatomical, and physiological
62 state of an organism. Although little is known about the ionomes of phagotrophic metazoans (but
63 see Goos et al. 2017), studies on osmotrophs such as *Saccharomyces cerevisiae* (Eide *et al.*,
64 2005) and *Arabidopsis thaliana* (Baxter *et al.*, 2008) in controlled laboratory conditions clearly
65 show that genetics and resource-supply stoichiometry interact to shape organismal growth and
66 ionic profiles. Evidence for ionome-wide shifts from the field are also available. For example,
67 *Synechococcus* cells collected from regions of the Sargasso Sea that vary in nitrogen (N) and P
68 supply exhibited several-fold cell quota differences in a variety of elements [e.g., manganese
69 (Mn), nickel (Ni), and zinc (Zn); Twining et al. 2010]. Such studies quantifying dynamics in
70 multiple elements have made it clear that considering combinations of elements, regardless of
71 their abundance in biomass, is important for developing a better understanding of the role of
72 elemental limitation in controlling organismal growth and developmental rates (Parent *et al.*,
73 2013a; Baxter, 2015). Despite this growing body of literature however, our knowledge of factors

74 driving elemental variation in ecologically sensitive taxa, such as amphibians, remains limited to
75 a relatively small number of elements.

76 Across life stages, frog and salamander carbon (C) content generally decreases while
77 %N, P, calcium (Ca), and sulfur (S) tend to increase (Capps *et al.*, 2015; Luhring, DeLong &
78 Semlitsch, 2017). These patterns are correlated with developmental and growth rates (Bumpers
79 *et al.* 2015; Stephens *et al.* 2017), arguably due to ontogenic changes in macromolecular
80 demands that differ in stoichiometric composition (e.g., lipids rich in C, proteins rich in N or S,
81 and bones rich in Ca and P; Costello and Michel 2013; Liess *et al.* 2013; Stephens *et al.* 2017). In
82 addition to ontogeny, amphibian C:N:P stoichiometry differs considerably across sites at local
83 scales (Milanovich & Hopton, 2014) due to differences in both bottom-up nutrient supplies
84 (Stephens, Berven & Tiegs, 2013; Bumpers *et al.*, 2015) and top-down predation pressure
85 (Costello & Michel, 2013). Furthermore, abiotic factors such as hydroperiod and temperature
86 have been shown to alter population-level stoichiometry at decadal scales (Capps *et al.* 2015) and
87 might explain regional stoichiometric differences through local adaptation of amphibian
88 populations across latitudinal gradients (Liess *et al.*, 2013).

89 Here, we report observations on ionic variation in two salamander species [marbled
90 salamanders (*Ambystoma opacum*) and mole salamanders (*Ambystoma talpoideum*)] using frozen
91 specimens that were collected at two wetlands over a 30-yr period. Based on stoichiometric
92 theory (Sturner & Elser, 2002), we hypothesized that species ionomes should vary due to
93 differences in life-history and ontogeny, and we sought to identify linkages among elements,
94 including those already known to vary significantly (i.e., C, Ca, N, P, and S; Capps *et al.* 2015,
95 Tiegs *et al.* 2016, Luhring *et al.* 2017). Moreover, because hydroperiod can affect salamander
96 growth and development, we hypothesized that salamander ionomes should differ due to

97 documented differences in hydroperiod between sites. Because hydroperiod varied considerably
98 over the 30-yr time period during which samples were collected (Daszak *et al.*, 2005; Todd *et al.*,
99 2011), we also expected to see temporal changes in salamander ionomes. In addition to
100 examining variation in individual elements, we also constructed nutrient balances (Parent *et al.*,
101 2013a,b) using knowledge of elemental metabolism and multivariate data from 15 elements to
102 see how these basic biological factors influence covariation among suites of elements.

103 **Methods**

104 *Study Sites, Specimen Collection, and Salamander Life-History:* Study animals were collected at
105 the United States Department of Energy's Savannah River Site (SRS) located in South Carolina.
106 Salamanders were sampled at two ephemeral wetlands, Rainbow Bay (RB) and Ginger's Bay
107 (GB), as part of a long-term monitoring program that began in 1978 and 1986 at each wetland,
108 respectively (Scott, 1990; Pechmann *et al.*, 1991). Rainbow Bay and GB are similar in area at
109 full ponding (1-1.5 ha) but occur 14 km apart in different physiographic subregions. Rainbow
110 Bay is located at ~97 m elevation on the Aiken Plateau whereas GB occurs on an old Pleistocene
111 floodplain terrace of the Savannah River (Sunderland Terrace) at 61 m elevation. Both wetlands
112 are surrounded by well-drained sandy loam soils in the Fuquay and Dothan series, but wetlands
113 themselves are located on poorly-drained sandy fine loam soils (Davis & Janecek, 1997).
114 Wetland soils also differ in elemental composition from surrounding upland soils as depressional
115 wetland soils on the SRS tend to have elevated aluminum (Al), barium (Ba), and total N, P, and
116 C levels compared to upland soils (Looney *et al.*, 1990; Dixon *et al.*, 1997). Wetlands on the
117 Sunderland Terrace (GB) tend to hold water for more extended periods (i.e., longer hydroperiod)
118 than Aiken Plateau wetlands (RB), have dissimilar zooplankton communities (Mahoney, Mort &
119 Taylor, 1990), and may have differences in cation concentrations (Schalles *et al.*, 1989). The

120 mean hydroperiod at RB is 129.6 ± 98.5 days (mean \pm 1 SD), and it has never held water from
121 one year to the next; GB in contrast has a longer hydroperiod (D.E. Scott and K.A. Capps
122 *unpublished data*) and remains filled year-round on average once every three years.

123 Each wetland was encircled by a drift fence with paired 19-L pitfall traps positioned at
124 10- m intervals. Individuals entering and leaving the wetlands were censused daily (RB) or
125 periodically (GB) during the breeding season which occurs from late fall through early spring for
126 our study species (Semlitsch *et al.*, 1993; Scott *et al.*, 2013). When mortality occurred during our
127 study (usually due to predation or bucket flooding) we collected the deceased salamanders,
128 identified individuals to species, and sexed terrestrial adults. Predated upon specimens were used
129 for census counts but excluded from our current study. We classified specimens as one of three
130 ontogenic stages: terrestrial adult male (*A. opacum* only), recently emerged metamorph (both
131 species), or recently emerged aquatic adult paedomorph (*A. talpoideum* only; defined below),
132 and stored them in a -70°C freezer until processing (Nunziata, Scott & Lance, 2015). A total of
133 214 organisms were analyzed in our study representing a mix of species, sites, and ontogenic
134 stages collected between 1986-2015 (Supp. Table 1). All study specimens were collected under
135 annual renewals of South Carolina Department of Natural Resources collection permits and
136 triannual renewals of animal capture and handling protocols approved by the University of
137 Georgia Institutional Animal Care and Use Committee.

138 Ambystomatid salamander life-history differs considerably between species. *Ambystoma*
139 *opacum* breeds terrestrially in late summer/autumn (Sept. – Dec.) and oviposits in nests in dry
140 pond beds prior to wetland filling (Scott, 2005). *Ambystoma talpoideum* breeds aquatically
141 during late fall and winter (Nov. - Mar.), and females lay single eggs or small clusters attached
142 to aquatic vegetation (Pechmann et al. 1991, Semlitsch 1987). *Ambystoma talpoideum* eggs

143 typically hatch about 2 months later than *A. opacum*, which may lead to a predatory or
144 competitive advantage for *A. opacum* larvae (Boone, Scott & Niewiarowski, 2002). In wetlands
145 such as GB that occasionally hold water from one year to the next, *A. talpoideum* are
146 facultatively paedomorphic. Rather than metamorphosing and emigrating to terrestrial uplands,
147 these individuals may remain in the wetland, retain their gills, become sexually mature aquatic
148 adults in the winter (Semlitsch, 1987), and usually exit the wetland in spring after one
149 reproductive bout.

150 Ambystomatid salamander life-history varies with environmental conditions including
151 temperature, predation, food quantity/quality, and population density, but appears to be most
152 strongly tied to pond hydroperiod (Semlitsch 1987, Scott 1990, Pechmann et al. 1991, Daszak et
153 al. 2005, Stephens et al. 2017). Yet, despite this extensive phenotypic variation clear differences
154 exist between our study species. Both species appear to have relatively similar larval growth
155 rates (Semlitsch, 1987; Scott, 1990), although *A. talpoideum* typically metamorphoses later and
156 at a larger body size than *A. opacum* (Pechmann, 1995). Reproductive success (i.e., the
157 production of juveniles that metamorphose and emigrate from wetlands) of both species is tied to
158 hydroperiod, but *A. talpoideum* requires a later date of pond drying and more frequently faces
159 inadequate water at RB, which results in catastrophic larval mortality (Daszak *et al.*, 2005).
160 Consequently, both species had stable populations at GB over our study period (Nunziata *et al.*,
161 2015), but *A. opacum* populations increased, and *A. talpoideum* decreased due to a shortened
162 hydroperiod at RB (Daszak *et al.*, 2005). Few mortality events occurred for terrestrial *A.*
163 *talpoideum*, so we collected and analyzed fewer of these species compared to *A. opacum*. This
164 partially explains the uneven sample sizes and lack of adult *A. talpoideum* in our data set (Supp.
165 Table 1).

166 *Sample Processing and Ionomic Profile Generation:* We removed animals from the
167 freezer and placed them individually into trace clean polypropylene grinding vials. Carcasses
168 were then freeze-dried and powdered using a Spex Mill (8000D, Metuchen, NJ) and
169 methacrylate grinding balls, and ground tissues were stored at -20°C. Before elemental analyses,
170 we dried subsamples overnight at 60°C and weighed them to the nearest 0.1 µg. Tissue C and N
171 content was measured using an automated vario MICRO cube analyzer (Elementar Americas
172 Inc., Mt. Laurel, NJ). To measure all other elements, we digested between 3-12 mg of
173 homogenized tissues in a 2:1 v/v solution of trace metal grade nitric acid and hydrogen peroxide
174 (BDH Aristar ® Plus, VWR International, Radnor, PA) for 24h in metal-free polypropylene
175 tubes (VWR International, Radnor, PA) followed by dilution to 10 ml with trace metal grade
176 water. Digested samples were then analyzed through inductively coupled plasma optical
177 emission spectrometry (ICP-OES; Thermo Scientific iCAP 7400, Waltham, MA). Sample
178 nutrient concentrations were determined using standard curves from multi-element standards
179 (CCV stds. 1A&B, CPI International, Santa Rosa, CA) and calibrated using an internal Yttrium
180 standard (Peak Performance Inorganic Y Standard, CPI International, Santa Rosa, CA).

181 We measured a total of 28 elements across all organisms. However, measurements for
182 many trace elements fell below the limits of detection (LOD) for many samples (Supp. Table 2).
183 After omitting all elements with analytical uncertainties, a total of 16 elements: aluminum (Al),
184 barium (Ba), C, Ca, copper (Cu), iron (Fe), potassium (K), lithium (Li), magnesium (Mg), Mn,
185 N, sodium (Na), P, S, strontium (Sr), and Zn were analyzed. For multivariate statistics, any
186 remaining values falling below the LOD's were replaced with ½ of the LOD value calculated for
187 each individual element. All elemental concentrations were then converted to percentages by
188 dividing them by the total sample dry mass (Supp. Table 3).

190 To visualize relationships between individual elements in Euclidian space, we first
191 conducted partial least squares (PLS) regressions using ontogeny, species, site, and year as
192 independent variables and log-transformed elemental percentages as dependent variables. We
193 also calculated variable importance (VIP) scores to compare the relative importance of each
194 predictor variable (Wold, Sjöström & Eriksson, 2001; Eriksson *et al.*, 2013). Plots of PLS
195 weights revealed correlations between elements and predictor variables, which differentially
196 separated across 2 factors (Fig. 1). These correlations reflect biologically relevant differences in
197 elemental signatures among organisms. Therefore, we used these partitions (i.e., elemental
198 position in Euclidian space) to construct nutrient balances to further explore differences in
199 elemental profiles among ontogenic stages, species, sites, and years.

200 To more thoroughly investigate sources of variation in salamander ionomes, we
201 constructed isometric log-ratio balances (hereafter referred to as nutrient or elemental balances).
202 These balances represent unbiased estimates of multivariate relationships between elements,
203 which avoid violating common statistical assumptions and can be used to describe and interpret
204 elemental interactions in organismal tissues (Parent *et al.*, 2013a). Specifically defined in the
205 context of our study, nutrient balances represent orthogonal log contrasts derived from binary
206 partitions of multivariate elemental data projected into Euclidean space. To construct our
207 balances, we first separated all elements into bulk and trace elements based on the classification
208 of Frausto da Silva and Williams (2001). We then constructed one bulk and one trace elemental
209 balance to reflect elemental partitions along each PLS axis using absolute PLS weight values of
210 0.1 as a cutoff for including elements in each balance. Following conventions (Sternner & Elser,

211 2002), we ordered elements from high to low in each balance as a function of their percentage of
212 dry mass. Nutrient balances were then calculated using the equation:

$$213 \text{ Balance} = \text{SQRT} (rs/r+s) \ln [g(c^+)/g(c^-)] \quad (\text{Parent } et \text{ al.}, 2013a)$$

214 where r and s represent the number of elements on the left- and right-hand side of the balance
215 and $g(c^+)$ and $g(c^-)$ minus represent the geometric mean of the elements on the left- and right-
216 hand side of the balance, respectively. In total, we constructed 4 novel balances: bulk [C | Ca, P,
217 S, Mg]; [N | Ca, Na, Mg] and trace [Fe, Mn | Zn, Ba, Cu, Sr, Li]; [Fe, Zn, Al, Ba | Cu, Sr, Li] in
218 addition to two traditional stoichiometric balances [C | P] and [N | P]. These balances represent a
219 combination of 1) knowledge of well-known elemental relationships related to organismal
220 macromolecular composition (C, N, P, Ca) and 2) statistically identified biologically relevant
221 relationships related to organismal ecology. As such their formation represents a blend of
222 metabolically grounded expert knowledge and exploratory biplot analyses (Parent *et al.*, 2013b),
223 which while not exhaustive may serve as a starting point for future functional studies.

224 To compare general differences in elemental balances, we first separated the data into
225 unique species, site, and ontogenic stage groups (n=7) and conducted one-way analysis of
226 variance (ANOVAs) to examine differences across groups. After identifying significant group
227 differences, we designed contrasts to compare differences across species at the same ontogenic
228 stage (metamorphs averaged across sites), sites (separate comparisons of metamorphs and adult
229 males averaged across species), and stages (*A. opacum* males and metamorphs averaged across
230 sites and *A. talpoideum* metamorphs and paedomorphs from GB) using post-hoc Tukey's tests.
231 Specific comparisons were made rather than full factorial comparisons as complete species, site,
232 stage combinations were not available. All *P*-values were adjusted using Bonferroni corrections.

233 In addition to univariate comparisons, we first used Wilks' Lambda tests from
234 multivariate ANOVA (MANOVA) to determine multivariate differences in nutrient balances
235 among each species, ontogenic stage, and site groups. We used similar methods to examine
236 temporal patterns in salamander balances by conducting MANOVAs on salamanders separated
237 into species(site) data sets for each ontogenic stage, which were grouped by decade to
238 compensate for uneven sample collection and inadequate samples sizes in certain years across
239 the 30-year period. Finally, we conducted two separate discriminant analyses including all traits
240 and one including only [C | P] and [N | P] balances to: (a) visualize trait differences across
241 groups, and (b) compare the classification accuracy of isometric log-ratio balances vs. classical
242 stoichiometric traits. Cross-validation of these models was conducted using leave-one-out
243 methods.

244 **Results**

245 *Individual elemental profiles:*

246 Ontogeny was the strongest predictor of elemental variation according to PLS regressions
247 followed by species and site, which were moderate and weak predictors, respectively (Supp. Fig.
248 1). Temporal effects were the weakest by far (VIP < 0.2) indicating that salamander elemental
249 content was relatively invariant through time. Elemental composition was most divergent along
250 the first PLS factor, which separated strongly between terrestrial adult males (hereby referred to
251 as males) and juvenile metamorphic ontogenic stages with aquatic adult paedomorphs showing
252 intermediate phenotypes (Fig. 1). Species and sites also separated out to a lesser extent along this
253 axis with *A. opacum* and individuals from GB falling on the left-hand side and *A. talpoideum* and
254 specimens from RB on the right-hand side. Bulk elemental concentrations on the male side of the
255 axis were generally higher for all elements other than C, which was higher in metamorphs and N,

256 Na, and K, which did not differ greatly along this axis. Trace elements were similarly negatively
257 correlated to the first axis and were higher in males for all elements except for Fe and Mn.
258 Compared to the first factor, factor two explained far less variation but seemed to separate
259 paedo- and metamorphic ontogenic stages along with species and site differences. Relatively
260 fewer bulk elements separated on this axis as N was positively correlated and Ca, Mg, and Na
261 were negatively correlated with factor 2, respectively. In contrast, all trace elements differed
262 along the second axis with higher concentrations of Fe and Zn associated with *A. opacum* and
263 RB and *A. talpoideum* and GB showing higher amounts of Cu.

264 *Nutrient balances:*

265 In addition to individual elements, nutrient balances differed across species, sites, and
266 ontogenic stages (Fig. 2). Metamorphs of *A. opacum* and *A. talpoideum* differed significantly in
267 their body [N | P] and [C | P] but did not differ in other balances (Table 1). In contrast, organisms
268 did not differ in stoichiometric balances among sites, but we found significant site-specific
269 differences for all other multi-element balances in metamorphs and for [N | Ca, Na, Mg] balances
270 in males. All balances differed significantly among *A. opacum* males and metamorphs, but only
271 [N | P] differed between *A. talpoideum* metamorphs and pedomorphs.

272 Balances for unique species-ontogeny-site combinations also differed in multivariate
273 space ($P < 0.001$), but we found no evidence of significant temporal changes ($P > 0.1$). According
274 to the discriminant analysis, male *A. opacum* phenotypes diverged strongly from other groups
275 along the first discriminant axis (Fig. 3), as males showed lower [C | P] and [N | Ca, Na, Mg]
276 values and other stages had higher [C | Ca, P, S, Mg] and [Fe, Mn | Zn, Ba, Cu, Sr, Li] balances.
277 Species differences separated out on the second discriminant axis as *A. opacum* generally
278 showed lower [C | P] and [Fe, Zn, Al, Ba | Cu, Sr, Li] concentrations and *A. talpoideum* had

279 higher [N | P] and [C | Ca, P, S, Mg]. Elemental balances showed relatively greater overlap
280 between sites in *A. talpoideum* but were more negatively correlated with the second discriminant
281 axis for each ontogenic stage in *A. opacum* from RB. Dual balances explained nearly half of the
282 elemental variation among all groups but were inadequate for distinguishing between
283 metamorphs of each species (Table 2). Including additional multi-elemental balances improved
284 classification accuracy from 49 to 65% among these groups.

285 **Discussion**

286 In this study, we found ecologically relevant differences in traditionally studied stoichiometric
287 elements (i.e. C, N, and P) along with a suite of other elements comprising salamander ionomes.
288 In contrast, we saw no significant temporal changes in elemental composition in either species.
289 Salamander ionic composition was most strongly related to ontogeny with relatively higher
290 concentrations of many elements in males (Ca, P, S, Mg, Zn, and Cu) compared to recently
291 emerged metamorphic juveniles, which had greater amounts of C, Fe, and Mn. In addition to
292 identifying correlations among single elements, we also constructed multi-elemental balances to
293 further examine differences among species, site, and ontogenic stages in multivariate space. We
294 found systematic differences in elemental relationships between species and sites and evidence
295 of suites of changes in elemental proportions across ontogenic development. Together, these
296 results demonstrate the power of using ionic approaches for studying environmental and
297 biological sources of consumer elemental variation and their ecological effects.

298 Like previous amphibian studies (Milanovich, Maerz & Rosemond, 2015; Luhring *et al.*,
299 2017), we found strong differences in species elemental composition. Consistent with
300 stoichiometric predictions, the species with the smaller body size and faster developmental rates,
301 *A. opacum*, had significantly higher body P contents than *A. talpoideum*. The larger-bodied *A.*

302 *talpoideum* also had higher body %C, which supports previous work showing positive
303 relationships between body size, juvenile lipid content, and body %C in ambystomatid
304 salamanders from this study region (Scott *et al.*, 2007; Luhring *et al.*, 2017). In addition to
305 differences in these traditional stoichiometric elements, we confirmed species differences in
306 salamander Ca and S content found in previous work (Milanovich *et al.*, 2015; Luhring *et al.*,
307 2017) and identified differences in several other essential elements (Fig. 1). Altogether, these
308 patterns validate the focus on well-studied elements (i.e., C and P) for determining the ecological
309 importance of interspecific variation in body stoichiometry. Our results also suggest that
310 expanding the range of study elements would yield further insights into the ecological roles and
311 nutritional ecology of these species.

312 Compared to species differences, we found relatively weaker evidence of spatio-temporal
313 effects on salamander ionomes. Site was a poor predictor of salamander stoichiometric variation
314 in males and metamorphs despite strong differences in genetic structure for *A. opacum* between
315 these populations (Nunziata *et al.*, 2015). Male multi-elemental balances also varied little, but
316 interestingly male balances differed considerably in metamorphs. These patterns would seem to
317 indicate that while male nutrient uptake (i.e., diet based) appeared to be similar in the
318 surrounding uplands, metamorph elemental acquisition either from dietary sources or epithelial
319 diffusion directly from the water column (e.g., metals or ions; Motais and Garcia-Romeau 1972,
320 Handy *et al.* 2002) likely differed across wetlands separated by only a short distance (~14 km).
321 Furthermore, the extensive annual variation in breeding season temperature and hydroperiod at
322 our study sites (Pechmann *et al.*, 1991; Daszak *et al.*, 2005; Todd *et al.*, 2011) might have
323 resulted in a lack of consistent selection pressures on growth and development obfuscating
324 temporal signatures in our data set. Indeed, previous genetic work examining a subset of animals

325 from our study (Nunziata *et al.*, 2017) along with our phenotypic results would seem to preclude
326 the role of evolutionary change in shaping observed ionic patterns in our study populations.
327 As hydroperiod changes have been tied to shifts in species abundances at RB (Daszak *et al.*,
328 2005), altered hydroperiod could also modify nutrient cycles in these wetlands by causing local
329 species extinctions and shaping salamander elemental profiles. However, these ecological
330 changes almost certainly interact with organismal life-history and ontogeny to ultimately control
331 these processes.

332 Salamander ontogenic stage had the strongest effect on elemental variation in our study
333 animals. Consistent with a previous amphibian study, we found that larger-bodied terrestrial
334 adult males had considerably higher body %P than juvenile metamorphs (Tiegs *et al.*, 2016).
335 Higher %P along with %Ca is typically associated with increased bone development and
336 ossification in adults (Kemp & Hoyt, 1969; Milanovich *et al.*, 2015; Luhring *et al.*, 2017) and
337 may explain the lower [C | P] and [N | Ca, Na, Mg] balances found in our study. In addition to
338 these elements, we measured higher concentrations of many other essential elements in adult
339 tissues (Zn, S, Li, Cu, and Mg). Our results also confirm previous measurements of higher
340 juvenile body %C and no difference in body N content between terrestrial adults and
341 metamorphic amphibians (Tiegs *et al.*, 2016) while showing differences in Fe and Mn
342 concentrations and in related [Fe, Mn | Zn, Ba, Cu, Sr, Li] nutrient balances between these
343 groups for the first time. This suggests that in addition to traditionally examined stoichiometric
344 elements (e.g.; N and P; Stephens *et al.* 2017), ontogenic variation in organismal elemental
345 composition may reflect unique nutritional physiologies and elemental requirements. However, it
346 also appears that environmental nutrient supplies may potentially override these differences as
347 we found substantial overlap in elemental profiles between pond-dwelling *A. talpoideum*

348 metamorphs and paedomorphic adults, which only differed in their %N content out of all
349 elements surveyed (Supp. Table 1). As such, it is clear that we must move beyond studies of
350 individual or pairs of elements and examine integrated changes in suites of metabolically
351 connected elements by developing tools such as nutrient balances (Parent *et al.*, 2013a; Baxter,
352 2015) to better understand the complex relationships between environmental nutrient supplies
353 and organismal life-history traits and ionomes.

354 Traditional stoichiometric ratios relate nutrient interactions to biological functions (e.g.,
355 N:P ratios to ribosomal protein translation; Loladze and Elser 2011), and nutrient balance
356 concepts extend this approach to encompass the entire organismal ionome (Parent *et al.*, 2013a).
357 While it is beyond the scope of this paper to functionally relate changes in salamander balances
358 to specific metabolic pathways and physiological process, our study demonstrates the utility of
359 using nutrient balance principles to examine relationships between key traits and consumer
360 ionic profiles. Similar to work on plants, we found systematic differences in elemental
361 combinations between species and sites (Watanabe *et al.*, 2007; Parent *et al.*, 2013a) and found
362 evidence for novel elemental traits [C | Ca, P, S, Mg] and [Fe, Mn | Zn, Ba, Cu, Sr, Li] that
363 differed across ontogenic development. Previously constructed balances inherently differ from
364 ours due to their focus on specific aspects of plant physiology, but the substantial overlap
365 between elemental relationships in our study and previous research is perhaps indicative of
366 fundamental elemental relationships governed by biological processes operating at the cellular
367 level (Sturner & Elser, 2002; Watanabe *et al.*, 2007; Baxter *et al.*, 2008; Parent *et al.*, 2013a).

368 In this study, we examined correlations between salamander ionic profiles and
369 biological and environmental processes. As previously demonstrated, traditional stoichiometric
370 balances (C | P and N | P) were useful for highlighting species differences in biogeochemically

371 important elements (Sterner & Elser, 2002). However, we also showed that focusing solely on
372 traditional stoichiometric elements alone could neglect other important elements, which might be
373 differentially required for optimal metabolic functioning in terrestrial adult males (Ca, S, Mg,
374 Zn, & Cu) and juveniles (Fe & Mn), respectively. Ionomic data analyzed here using balance
375 techniques could thus be used to generate a greater understanding of the nutritional ecology of
376 amphibian development. It is important to note that elemental correlations and nutrient balances
377 in our data set could contain information attributable to artifacts of high-dimensional analyses,
378 with potentially limited biological relevance. Nevertheless, such data are vital for providing a
379 starting point for designing manipulative experiments to not only better understand the
380 nutritional ecology of threatened vertebrates such as salamanders but also to illuminate the entire
381 suite of complex ecological functions that they might influence.

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527

Table 1. Trait differences between species, sites, and ontogenic stages. Degrees of freedom (*df*), f-ratio of mean squares (F), and *P*-values (*P*) are reported for 1-way main effects ANOVAs and multiple comparisons. Significant differences are shown in bold for *P*-values adjusted using Bonferroni corrections (Meta: 0.05/3= 0.017; Adult: 0.05/2=0.025). Specific contrast details include: averaged across sites¹, averaged across species², *Ambystoma opacum* only³, average across sites *A. opacum* only⁴, *A. talpoideum* from Ginger’s Bay only⁵. All elements are reported in standard scientific notation. Other abbreviations include: metamorph (Meta), paedomorph (Paedo).

Model	<i>df</i>	[C P]		[N P]		[C Ca,P,S,Mg]		[N Ca,Na,Mg]		[Fe,Mn Zn,Ba,Cu,Sr,Li]		[Fe,Zn,Al,Ba Cu,Sr,Li]	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Group	6	44.88	<0.001	34.86	<0.001	53.56	<0.001	19.52	<0.001	64.52	<0.001	27.07	<0.001
R ²		0.565		0.503		0.608		0.361		0.652		0.440	
Contrast	<i>df</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
¹ Species (Meta)	1	7.12	0.008	8.26	0.005	0.85	0.358	0.02	0.877	1.53	0.217	0.00	0.998
² Site (Meta)	1	5.02	0.026	3.36	0.068	6.02	0.015	9.93	0.002	10.40	0.002	20.66	<0.001
³ Site (Male)	1	0.54	0.463	1.21	0.272	2.11	0.148	14.54	<0.001	0.95	0.332	0.49	0.484
⁴ Stage (Male vs. Meta)	1	165.07	<0.001	122.92	<0.001	221.84	<0.001	60.10	<0.001	259.22	<0.001	89.82	<0.001
⁵ Stage (Meta vs. Paedo)	1	0.04	0.838	9.33	0.003	0.01	0.906	3.26	0.072	0.26	0.611	4.60	0.033

Table 2. Comparison of discriminant analysis classification accuracy based on (A) stoichiometric balances only and (B) all isometric log-ratio balances. Cross-validation results are reported as percentages, and percentages correctly assigned to each group are shown in bold. Abbreviations include: *Ambystoma opacum* (Amop), *A. talpoideum* (Amta), metamorph (Meta), pedomorph (Paedo), Ginger’s Bay (GB), and Rainbow Bay (RB).

A. [C|P] & [N|P]

Species	Ontogenic Stage	Site	Group	1	2	3	4	5	6	7	Total
Amop	Male	GB	1	61.5	12.8	0	25.6	0	0	0	49.1
Amop	Male	RB	2	43.2	35.1	0	18.9	0	0	2.7	
Amop	Meta	GB	3	0	0	0	100	0	0	0	
Amop	Meta	RB	4	0	0	0	98.4	0	1.6	0	
Amta	Meta	GB	5	0	5.6	0	83.3	0	11.1	0	
Amta	Meta	RB	6	0	0	0	71.4	0	9.5	19	
Amta	Paedo	GB	7	0	0	0	50	0	0	50	

B. All Traits

Species	Ontogenic Stage	Site	Group	1	2	3	4	5	6	7	Total
Amop	Male	GB	1	76.9	17.9	0	0	2.6	2.6	0	64.5
Amop	Male	RB	2	35.1	62.2	0	2.7	0	0	0	
Amop	Meta	GB	3	0	0	33.3	48.1	11.1	7.4	0	
Amop	Meta	RB	4	0	3.2	8.1	87.1	1.6	0	0	
Amta	Meta	GB	5	0	0	16.7	16.7	38.9	22.2	5.6	
Amta	Meta	RB	6	0	0	4.8	19	23.8	33.3	19	
Amta	Paedo	GB	7	0	0	10	0	10	0	80	

Figure Captions

Figure 1. Relationships between ontogeny, species, site, date, and salamander elemental composition. Partial least squares (PLS) regression loadings plot of variables weights demonstrate correlations between independent (x) and dependent (y) variables where relationships between variables are directly proportional to the sign and distance of all other variables in Euclidian space. Abbreviations of x-variables include: metamorph (meta), paedomorph (Paedo), *Ambystoma opacum* (Amop), *A. talpoideum* (Amta), Ginger's Bay (GB), and Rainbow Bay (RB). All y-variables (elements) are reported in standard scientific notation.

Figure 2. Variation in isometric log-ratio balances between ontogenic stages, species, and sites. Boxplots depict medians, 25th and 75th percentiles (boxes), and 10th and 90th percentiles (error bars) for each balance. Elemental balances differed significantly among ontogenic stages, species (left- and right-hand panels), and sites according to Wilks's lambda scores ($P < 0.001$). Abbreviations include: metamorph (Meta), paedomorph (Paedo), Gingers Bay (GB; Panels A&B), and Rainbow Bay (RB; Panels C&D).

Figure 3. Multivariate relationships among ontogenic stages, species, and sites. Discriminant analysis (DA) loadings plots of centroids and 95% confidence intervals are shown for each group, and the two highest positive and negative standardized canonical discriminant function coefficient scores out of six isometric log-ratio balances are reported for each axis. Elemental balances differed significantly among groups according to Wilks's lambda scores ($P < 0.001$). Abbreviations include: metamorph (Meta), paedomorph (Paedo), *Ambystoma opacum* (Amop), *A. talpoideum* (Amta), Ginger's Bay (GB), and Rainbow Bay (RB).

Figure 1

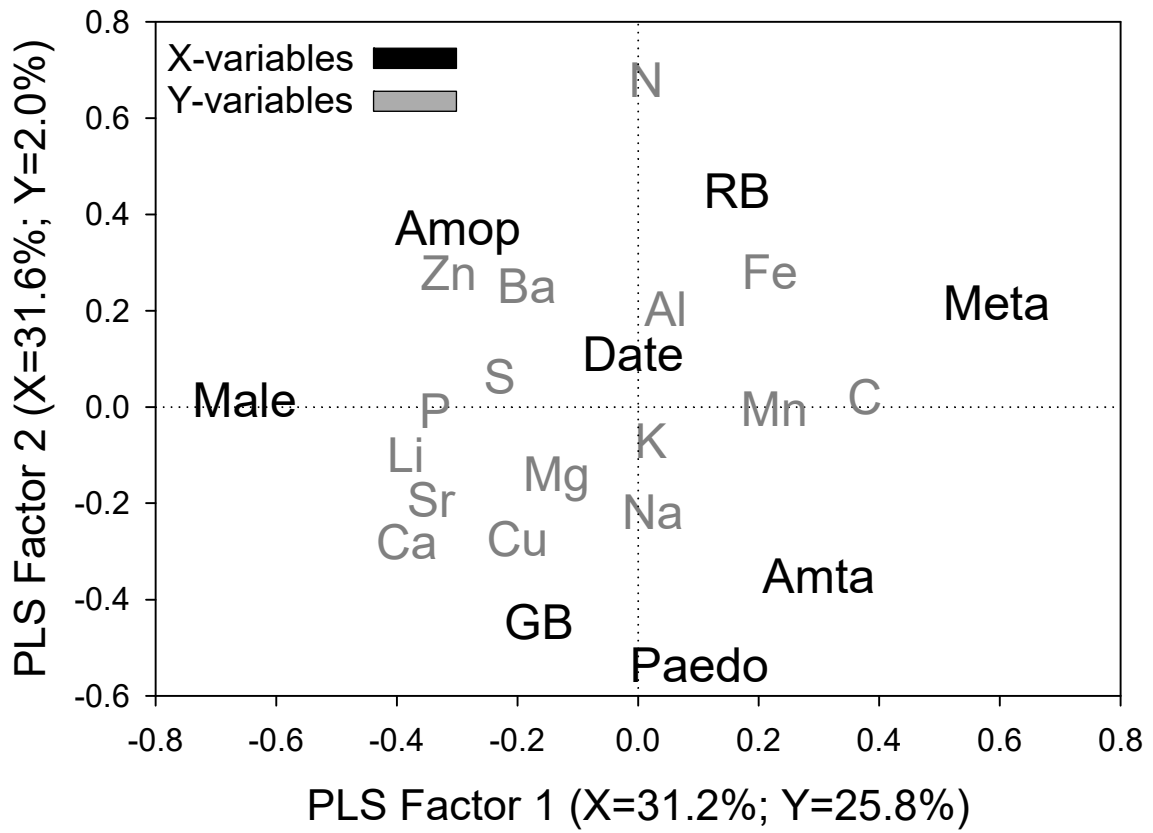


Figure 2

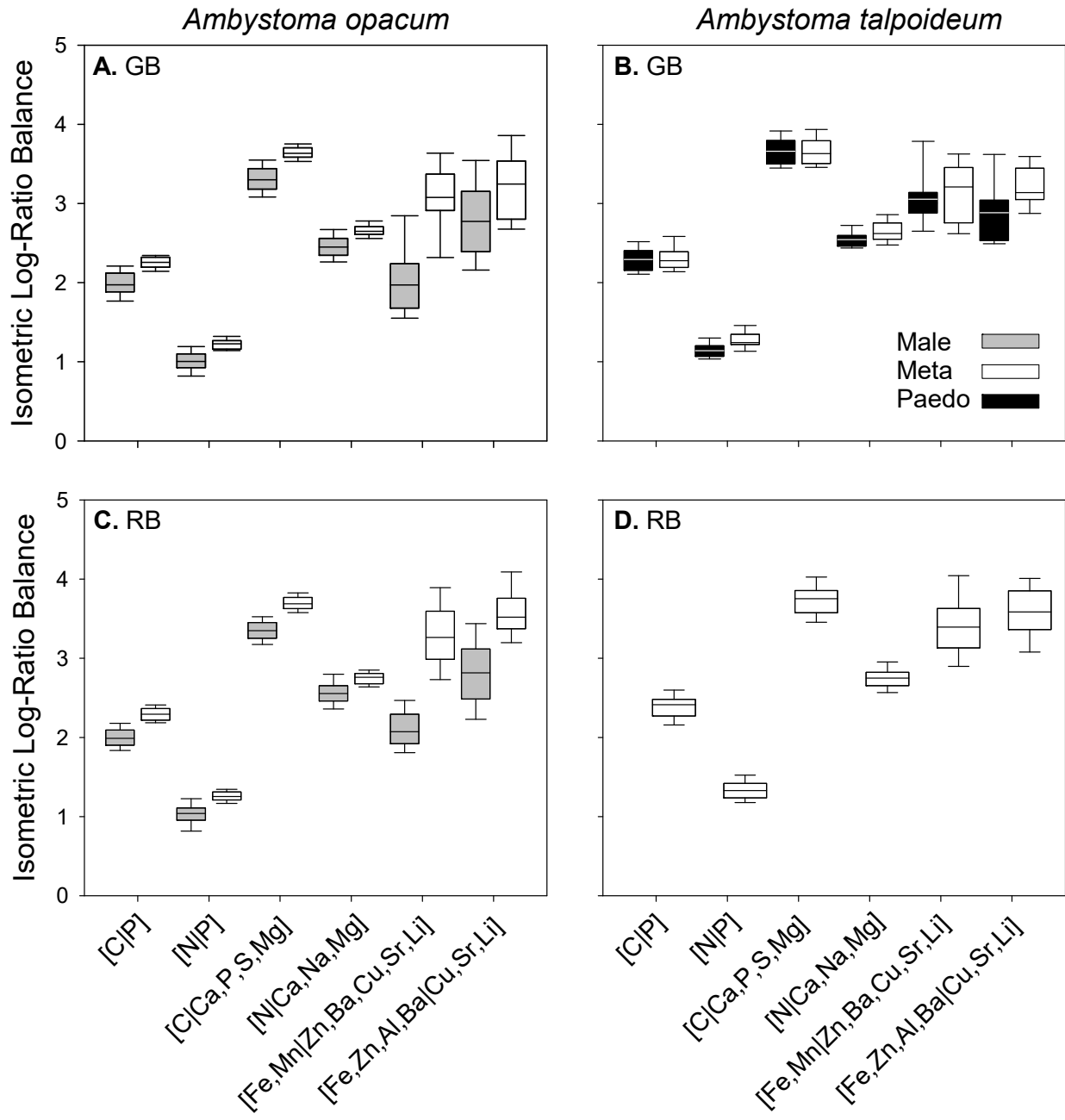
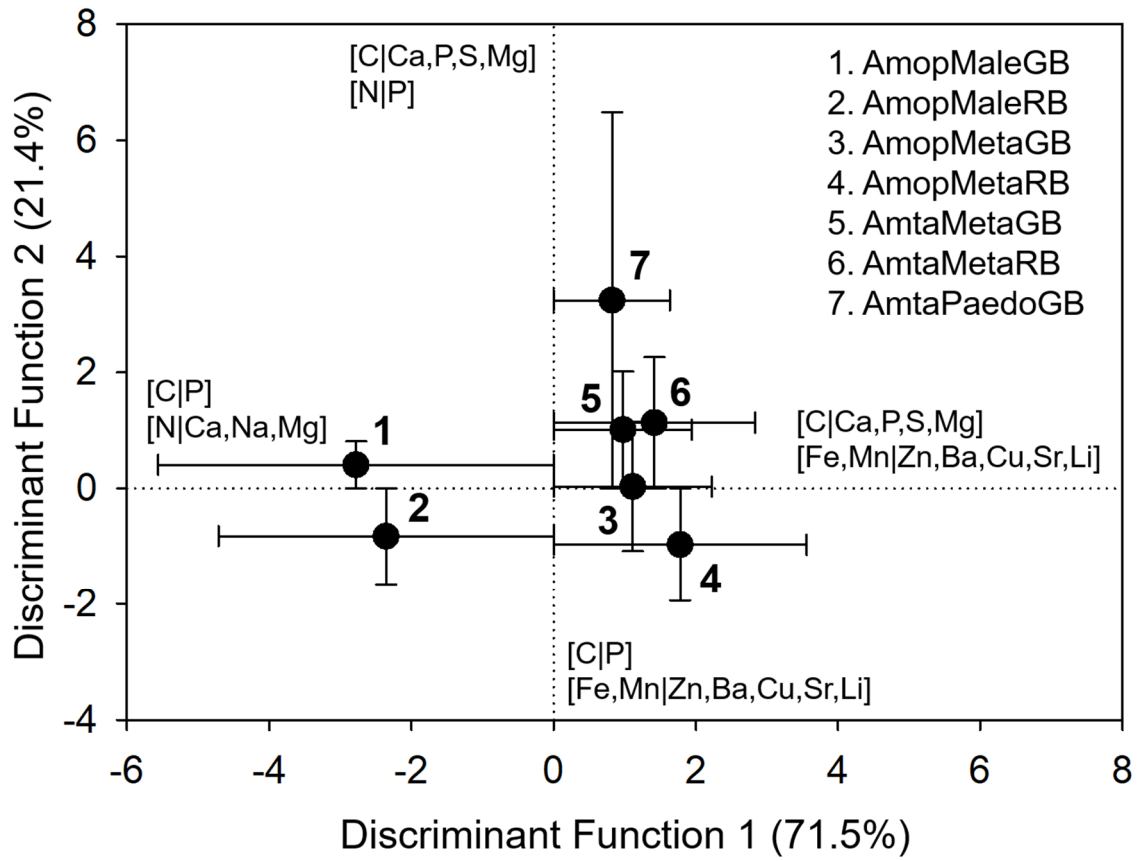


Figure 3



Supplementary Table 1. Sample information for each species, site, and ontogenic stage subgroup.

Species	Site	Ontogenic Stage	# of Specimens	Dates
<i>Ambystoma opacum</i>	Ginger's Bay	Adult Male	39	1987-2007
<i>Ambystoma opacum</i>	Ginger's Bay	Metamorph	28	1994-2010
<i>Ambystoma opacum</i>	Rainbow Bay	Adult Male	37	1986-2013
<i>Ambystoma opacum</i>	Rainbow Bay	Metamorph	61	1984-2015
<i>Ambystoma talpoideum</i>	Ginger's Bay	Metamorph	18	1995-2010
<i>Ambystoma talpoideum</i>	Ginger's Bay	Paedomorph	10	1995-2003
<i>Ambystoma talpoideum</i>	Rainbow Bay	Metamorph	21	1984-2010

Supplementary Table 2. Limits of detection (LOD) for individual elements measured in salamander tissues.

Element	LOD ($\mu\text{g L}^{-1}$)	% of Samples Below LOD
Al	5.18	8.63
As	2.12	85.97
B	2.42	97.48
Ba	0.79	0.00
Be	0.05	87.77
Bi	1.85	96.76
Ca	46.53	0.00
Cd	0.12	83.27
Co	0.26	88.85
Cr	0.72	80.94
Cu	1.60	0.00
Fe	1.84	0.00
K	1.93	0.00
Li	0.04	0.00
Mg	0.57	0.00
Mn	0.13	0.00
Mo	0.75	99.46
Na	1.76	0.00
Ni	0.91	64.03
P	13.74	0.00
Pb	0.93	70.50
S	15.33	0.00
Se	4.10	99.64
Si	6.75	89.93
Sr	0.08	0.00
Tl	16.17	84.17
V	0.85	61.15
Zn	0.67	0.00

Supplementary Table 3. Differences in elemental composition (%) between ontogenic stages for each study species. Significant differences were determined using one-way analysis of variance (ANOVA) on log transformed elemental data, and significant ontogenic differences in mean elemental concentrations for each species are reported in bold font using *P*-values adjusted using Bonferroni corrections (0.05/16= 0.003). All elements are reported in standard scientific notation. Additional abbreviations include: *Ambystoma opacum* (Amop), *A. talpoideum* (Amta), metamorph (Meta), paedomorph (Paedo),

Element	<i>A. opacum</i> (Male)		<i>A. opacum</i> (Meta)		<i>A. talpoideum</i> (Meta)		<i>A. talpoideum</i> (Paedo)	
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
Al	0.00651	0.00669	0.00745	0.00608	0.00574	0.00354	0.00293	0.00265
Ba	0.00362	0.00136	0.00233	0.00080	0.00263	0.00094	0.00213	0.00043
C	43.7329	2.16887	48.5848	1.45480	49.1785	2.15605	49.0310	3.08152
Ca	5.12175	1.26839	2.68337	0.63523	2.75864	0.69731	3.07284	0.41898
Cu	0.00071	0.00022	0.00057	0.00019	0.00054	0.00020	0.00059	0.00014
Fe	0.02386	0.02357	0.05078	0.03454	0.04486	0.03325	0.02741	0.01737
K	0.83393	0.23969	0.87748	0.16619	0.76237	0.11954	0.82354	0.06751
Li	0.00020	0.00005	0.00013	0.00003	0.00012	0.00003	0.00012	0.00001
Mg	0.12586	0.01941	0.11444	0.01935	0.11692	0.01858	0.11543	0.02017
Mn	0.00776	0.00314	0.01095	0.00480	0.01292	0.00537	0.01260	0.00319
N	11.1089	0.67467	11.2169	0.40746	11.2790	0.92505	9.61800	0.91705
Na	0.39286	0.11029	0.40937	0.12755	0.40883	0.10386	0.37721	0.04708
P	2.64180	0.50909	1.96324	0.35281	1.79744	0.32488	1.92948	0.29694
S	0.79403	0.07657	0.71121	0.11401	0.69614	0.09493	0.67393	0.05514
Sr	0.00614	0.00207	0.00344	0.00105	0.00329	0.00086	0.00330	0.00048
Zn	0.01484	0.00422	0.01025	0.00276	0.00899	0.00175	0.00841	0.00094

Supplementary Figure 1. Variable importance predictor scores (VIP) from partial least squares regressions show the relative influence of predictor variables on organismal ionic profiles. Scores between 0.5-0.8 are considered weakly important, those between 0.8-1 are considered moderately important, and scores >1 are considered to be strong predictors. Abbreviations include: metamorph (Meta), paedomorph (Paedo), *Ambystoma opacum* (Amop), *A. talpoideum* (Amta), Gingers Bay (GB), and Rainbow Bay (RB).

