Understanding variation in salamander ionomes: A nutrient balance approach

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Keywords: Amphibians, *Ambystoma*, ecological stoichiometry, ionomics, and ontogeny.

Running Title: Salamander ionomic variation

Journal: *Freshwater Biology*

Type of manuscript: Original Research

Summary

1. Ecological stoichiometry uses information on a few key biological elements (C, N, and P) to explain complex ecological patterns. Although factors driving variation in these elements are well-established, expanding stoichiometric principles to explore dynamics of the many other essential elements comprising biological tissues (i.e., the ionome) is needed to determine their metabolic relationships and better understand biological control of elemental flows through ecosystems.

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

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

4. In addition to patterns of individual elements, covariance among elements was used to construct multi-elemental nutrient balances, which revealed differences in salamander elemental composition between species and sites and changes in elemental proportions across ontogenic development. These multi-elemental balances distinguished among species-site-ontogenic stage groups better than using only C, N, and P.

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

Introduction

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

In addition to species-level differences, it is apparent that there is considerable intraspecific variation in organismal elemental composition (Jeyasingh *et al.*, 2014; Prater, Wagner & Frost, 2017). For example, Bertram et al. (2008) found that variation in the phosphorus (P) content of a single cricket species collected across field populations was as extensive as that observed across several orders of insect taxa (Woods *et al.*, 2004). Further, strong P plasticity among distinct ontogenic stages in metazoans linked to differences in organismal growth and reproduction has commonly been observed (e.g., Villar-argaiz et al. 2002, Capps et al. 2015, Tiegs et al. 2016). Besides P, recent work also highlights the potential ecological importance of variation in other elements within organismal tissues, although metabolic causes of this variation are not always well-known (e.g., Baxter 2010; Jeyasingh et al. 2017). At a time when it is becoming increasingly clear that growth and productivity can be limited by combinations of elements (Salt, Baxter & Lahner, 2008; Harpole *et al.*, 2011; Parent *et al.*, 2013a), determination of the extent and nature of variation in the content of the other ~20 essential elements found in biological tissues is needed to establish their connections with organismal metabolism and ecological functions.

The ionome is defined as the mineral nutrient and trace element composition of an organism (Salt, Baxter & Lahner, 2008), which underlies its morphological, anatomical, and physiological state. Although little is known about the ionomes of phagotrophic metazoans (but see Goos et al. 2017), studies on osmotrophs such as *Saccharomyces cerevisiae* (Eide *et al.*, 2005) and *Arabidopsis thaliana* (Baxter *et al.*, 2008) in controlled laboratory conditions clearly show that genetics and resource-supply stoichiometry interact to shape organismal growth and ionomic profiles. Evidence for ionome-wide shifts from the field are also available. For example, *Synechococcus* cells collected from regions of the Sargasso Sea that vary in nitrogen (N) and P supplies exhibited several-fold cell quota differences in a variety of elements [e.g., manganese (Mn), nickel (Ni), and zinc (Zn); Twining et al. 2010]. These studies depict a strong correlative network among elements likely underpinned by a complex web of metabolic connections (Parent *et al.*, 2013a; Baxter, 2015). However, our knowledge of factors related to elemental variation in vertebrate consumers, particularly in ecologically sensitive taxa such as amphibians, remains limited to a relatively small number of elements.

Throughout ontogeny, frog and salamander carbon (C) content generally decreases while %N, P, calcium (Ca), and sulfur (S) tend to increase (Capps *et al.*, 2015; Luhring, DeLong &

Semlitsch, 2017). These patterns are correlated with developmental and growth rates (Bumpers et al. 2015; Stephens et al. 2017), arguably due to ontogenic changes in macromolecular demands that differ in stoichiometric composition (e.g., lipids rich in C, proteins rich in N or S, and bones rich in Ca and P; Costello and Michel 2013; Liess et al. 2013; Stephens et al. 2017). In addition to ontogeny, amphibian C:N:P stoichiometry differs considerably across sites at local scales (Milanovich & Hopton, 2014) due to differences in both bottom-up nutrient supplies (Stephens, Berven & Tiegs, 2013; Bumpers *et al.*, 2015) and top-down predation pressure (Costello & Michel, 2013). Furthermore, abiotic factors such as hydroperiod and temperature have been shown to alter population-level stoichiometry at decadal scales (Capps et al. 2015) and might explain regional stoichiometric differences through local adaptation of amphibian populations across latitudinal gradients (Liess *et al.*, 2013). Overall, amphibian composition of a handful of elements appears to be highly responsive to many ecological factors, and these changes could result in shifts of other metabolically connected elements ultimately influencing biogeochemical fluxes through aquatic environments.

Here, we report observations on ionomic variation in two Ambystomatid salamander species [marbled salamanders (*Ambystoma opacum)* and mole salamanders (*Ambystoma talpoideum*)] using specimens that were collected at two wetlands over a 30-yr period. Based on stoichiometric theory, we hypothesized that the ionomes of each species should vary due to differences in life-history and ontogeny, and we sought to identify linkages among elements, including those already known to vary significantly (i.e., C, Ca, N, P, and S; Capps et al. 2015, Tiegs et al. 2016, Luhring et al. 2017). Moreover, because hydroperiod can affect salamander growth and development, we hypothesized that salamander ionomes should vary due to documented differences in hydroperiod between sites. Since hydroperiod differed considerably over the 30-yr time period during which samples were collected (Daszak *et al.*, 2005; Todd *et al.*, 2011), we also expected to see temporal changes in salamander ionomes. In addition to examining variation in individual elements, we also constructed nutrient balances (Parent *et al.*, 2013a,b) using knowledge of elemental metabolism and multivariate data from 15 elements to see how these basic biological factors influence covariation among suites of elements.

Methods

Study Sites, Specimen Collection, and Salamander Life-History: Study animals were collected at the United States Department of Energy's Savannah River Site (SRS) in South Carolina. Salamanders were sampled at two ephemeral wetlands, Rainbow Bay (RB: 33.315696; -81.773643) and Ginger's Bay (GB: 33.260035; -81.631320), as part of a long-term monitoring program that began in 1978 and 1986 at each wetland, respectively (Scott, 1990; Pechmann *et al.*, 1991). Rainbow Bay and GB are similar in area at full ponding (1-1.5 ha) but are located 14 km apart in different physiographic subregions. Rainbow Bay is found at ~97 m elevation on the Aiken Plateau, whereas GB occurs on an old Pleistocene floodplain terrace of the Savannah River (Sunderland Terrace) at 61 m elevation. Both wetlands are surrounded by well-drained sandy loam soils in the Fuquay and Dothan series, but wetlands themselves are located on poorly-drained sandy fine loam soils (Davis & Janecek, 1997). Wetland soils also differ in elemental composition from surrounding upland soils as depressional wetland soils on the SRS tend to have elevated aluminum (Al) and barium (Ba) concentrations and total N, P, and C levels compared to upland soils (Looney *et al.*, 1990; Dixon *et al.*, 1997). Wetlands on the Sunderland Terrace (GB) usually hold water for more extended periods (i.e., longer hydroperiod) than Aiken Plateu wetlands (RB), have dissimilar zooplankton communities (Mahoney, Mort & Taylor, 1990), and may have differences in cation concentrations (Schalles *et al.*, 1989). The mean hydroperiod at RB is 129.6 ± 98.5 days (mean ± 1 SD), and it has never held water from one year to the next; GB in contrast has a longer hydroperiod (D.E. Scott and K.A. Capps *unpublished data*) and remains filled year-round on average once every three years.

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

Ambystomatid salamander life-history differs considerably between species. *Ambystoma opacum* breeds terrestrially in late summer/autumn (Sept. – Dec.) and oviposits in nests in dry pond beds prior to wetland filling (Scott, 2005). *Ambystoma talpoideum* breeds aquatically during late fall and winter (Nov. - Mar.), and females lay single eggs or small chusters attached to aquatic vegetation (Pechmann et al. 1991, Semlitsch 1987). *Ambystoma talpoideum* eggs typically hatch about two months later than *A. opacum*, which may lead to a predatory or competitive advantage for *A. opacum* larvae (Boone, Scott & Niewiarowski, 2002). In wetlands such as GB that occasionally hold water from one year to the next, *A. talpoideum* are facultatively paedomorphic. Rather than metamorphosing and emigrating to terrestrial uplands, these individuals may remain in the wetland, retain their gills, become sexually mature aquatic adults in the winter (Semlitsch, 1987), and usually exit the wetland in spring after one reproductive bout.

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

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

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

Statistical Analyses and Formulation of Elemental Balances:

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

To more thoroughly investigate sources of variation in salamander ionomes, we constructed a series of isometric log-ratio balances. These balances represent unbiased estimates of multivariate relationships between elements, which avoid violating common statistical assumptions and can be used to describe and interpret elemental interactions in organismal tissues (Parent *et al.*, 2013a). Specifically defined in the context of our study and mathematically defined below, nutrient balances represent orthogonal log contrasts of elements derived from binary partitions of multivariate elemental data projected into Euclidean space. To construct these balances (hereafter referred to as nutrient or elemental balances), we first separated all elements into bulk and trace elements based on the classification of Frausto da Silva and Williams (2001). Then we constructed one bulk and one trace elemental balance to reflect elemental partitions along each PLS axis using absolute PLS weight values of 0.1 as a cutoff for including elements in each balance. Following conventions (Sterner & Elser, 2002), we ordered elements from high to low in each balance as a function of their percentage of dry mass. Nutrient balances were then calculated using the equation:

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

where r and s represent the number of elements on the left- and right-hand side of the balance and $g(c^{+})$ and $g(c^{-})$ minus represent the geometric mean of elemental percentages on the left- and right-hand side of the balance, respectively. To calculate a [C | Ca, P] balance for an adult salamander with an elemental composition of 43% C, 5% Ca, and 3% P for example, we would use the formula [C | Ca, P]= SQRT [(1x2)/(1+2)] ln [(43/g(5,3)]= 1.97. Balances constructed in this way can then be used to be used to examine proportional differences in elemental composition between groups of organisms such as adults and juveniles that are related to underlying biological factors. For instance, we would expect that [C | Ca, P] balance differences between these two ontogenic stages should reflect proportionally higher investment in C-rich lipids in juveniles and higher Ca and P investment into bone for adults resulting in relatively higher juvenile values (Fig. 2).

In total, we constructed 4 novel balances: bulk [C | Ca, P, S, Mg]; [N | Ca, Na, Mg] and trace [Fe, Mn | Zn, Ba, Cu, Sr, Li]; [Fe, Zn, Al, Ba | Cu, Sr, Li] in addition to two traditional stoichiometric balances [C | P] and [N | P]. These balances represent a combination of 1) knowledge of well-known elemental relationships related to organismal macromolecular composition (C, N, P, Ca) and 2) statistically identified biologically relevant relationships related to our study variables (ontogeny, species, site, and time). As such their formation represents a blend of metabolically grounded expert knowledge and exploratory biplot analyses (Parent *et al.*, 2013b), which while not exhaustive may serve as a starting point for future functional studies.

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

In addition to univariate comparisons, we first used Wilks' Lambda tests from multivariate ANOVA (MANOVA) to determine multivariate differences in nutrient balances among each species, ontogenic stage, and site groups. We used similar methods to examine temporal patterns in salamander balances by conducting MANOVAs on salamanders separated into species(site) data sets for each ontogenic stage, which were grouped by decade to compensate for uneven sample collection and inadequate samples sizes in certain years across the 30-year period. Finally, we conducted two separate discriminant analyses including all balances and one including only [C | P] and [N | P] balances to: a) visualize multivariate trait differences across groups and b) examine the classification accuracy of classical stoichiometric balances vs. all elemental balances. Cross-validation of these models was conducted using leave-one-out methods. Overall, these complimentary univariate and multivariate analyses were designed to quantify ecological sources of variance in individual balances (ANOVAs) and multivariate phenotypes (MANOVAs & discriminant analyses) and reduce the likelihood of statistical artifacts associated with multivariate data analyses.

Results

Individual Elemental Profiles:

Ontogeny was the strongest predictor of elemental variation according to PLS regressions followed by species and site, which were moderate and weak predictors, respectively (Fig. 1A). Temporal effects were the weakest by far (VIP< 0.2) indicating that salamander elemental content was relatively invariant through time. Elemental composition was most divergent along the first PLS factor, which separated strongly between terrestrial adult males (hereby referred to as males) and juvenile metamorphic ontogenic stages with aquatic adult paeodomorphs showing intermediate phenotypes (Fig. 1B). Species and sites also separated out to a lesser extent along this axis with *A. opacum* and individuals from GB falling on the left-hand side and *A. talpoideum* and specimens from RB located on the right. Bulk elemental concentrations on the male side of the axis were generally higher for all elements other than C, which was higher in metamorphs and N, Na, and K, which did not differ greatly along this axis. Trace elements were were also higher in males for all elements except for Fe and Mn. Compared to the first factor, factor two explained far less variation but seemed to separate paedo- and metamorphic ontogenic stages along with species and site differences. Relatively fewer bulk elements separated on this axis as N was positively correlated and Ca, Mg, and Na were negatively correlated with factor two, respectively. In contrast, all trace elements differed along the second axis with higher concentrations of Fe and Zn associated with *A. opacum* and RB and *A. talpoideum* and salamanders from GB showing higher amounts of Cu.

Nutrient Balances:

In addition to individual elements, nutrient balances differed across species, sites, and ontogenic stages (Fig. 3). Metamorphs of *A. opacum* and *A. talpoideum* differed significantly in their body [N | P] and [C | P] but did not differ in other balances (Table 1). In contrast, salamander stoichiometric balances did not differ between sites, but we found significant site-specific differences for all other multi-element balances in metamorphs and for [N | Ca, Na, Mg] balances in males. All balances differed significantly among *A. opacum* males and metamorphs, but only [N | P] differed between *A. talpoideum* metamorphs and paedomorphs.

Balances for unique species-site-ontogenic stage group combinations also differed in multivariate space ($P<0.001$), but we found no evidence of significant temporal changes ($P>0.1$). According to the discriminant analysis, male *A. opacum* phenotypes diverged strongly from other groups along the first discriminant axis (Fig. 4), as males showed lower [C | P] and [N | Ca, Na, Mg] values and other stages had higher [C | Ca, P, S, Mg] and [Fe, Mn | Zn, Ba, Cu, Sr, Li] balances. Species differences separated out on the second discriminant axis as *A. opacum* generally showed lower [C | P] and [Fe, Zn, Al, Ba | Cu, Sr, Li] concentrations and *A. talpoideum* had higher [N | P] and [C | Ca, P, S, Mg]. Elemental balances showed relatively greater overlap between sites in *A. talpoideum* but were more negatively correlated with the second discriminant axis for each ontogenic stage in *A. opacum* from RB. Dual balances explained nearly half of the elemental variation among all groups but were inadequate for distinguishing between metamorphs of each species (Table 2). Including additional multi-elemental balances improved classification accuracy from 49 to 65% among all groups. Despite our conservative analyses, it is important to note that elemental correlations and nutrient balances in our data set could still possibly contain information attributable to artifacts of high-dimensional analyses. Although we cannot quantify the potential for these artifacts, consistency between uni- and multivariate statistics suggests that their likelihood of influencing our findings was minimal.

Discussion

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

Similar to previous amphibian studies (Milanovich, Maerz & Rosemond, 2015; Luhring *et al.*, 2017), we found strong differences in the elemental composition of our study species. Consistent with stoichiometric predictions, the species with the smaller body size and faster developmental rates, *A. opacum,* had higher body P content than *A. talpoideum*. The larger-bodied *A. talpoideum* also had higher body %C, which supports previous work showing positive relationships between body size, juvenile lipid content, and body %C in ambystomatid salamanders from this study region (Scott *et al.*, 2007; Luhring *et al.*, 2017). In addition to these traditional stoichiometric elements, we also confirmed species differences in salamander Ca and

S content found in previous work (Milanovich *et al.*, 2015; Luhring *et al.*, 2017) and identified differences in several other essential elements. Altogether, these patterns validate the focus on well-studied elements (i.e., C and P) for determining the ecological importance of interspecific variation in body stoichiometry. Our results also suggest that expanding the range of study elements could yield further insights into the ecological roles and nutritional ecology of these species.

Compared to species, we found relatively weaker evidence of spatio-temporal effects on salamander ionomes. Site was a poor predictor of stoichiometric variation in males and metamorphs despite strong differences in genetic structure for *A. opacum* between these populations (Nunziata *et al.*, 2015). Male multi-elemental balances were also similar across sites, but metamorphs showed strong contrasts between RB and GB. These patterns seem to indicate that while male nutrient uptake (i.e., diet based) appeared to be similar in the surrounding uplands, metamorph elemental acquisition either from dietary sources or epithelial diffusion directly from the water column (e.g., metals or ions; Motais and Garcia-Romeau 1972, Handy et al. 2002) likely differed across wetlands separated by only a short distance (~14 km). In addition to differences between sites, there was also extensive annual variation in breeding season temperature and hydroperiod within each site (Pechmann *et al.*, 1991; Daszak *et al.*, 2005; Todd *et al.*, 2011). This variation may have limited our ability to identify temporal changes in salamander elemental profiles and likely resulted in a lack of consistent selection pressures on salamander life-history traits tied to their elemental composition. Indeed, previous genetic work examining a subset of animals from our study (Nunziata *et al.*, 2017) along with our phenotypic results seem to preclude the role of evolutionary change in shaping observed ionomic patterns in our study populations. However, as longer-term hydroperiod trends have been tied to shifts in species abundances at RB (Daszak *et al.*, 2005), hydroperiod could nevertheless still modify nutrient cycles in these wetlands by causing local species extinctions and thus shaping the elemental profiles of the remaining amphibian communities. However, these ecological changes would almost certainly interact with organismal life-history and ontogeny to ultimately control these dynamics.

Salamander ontogenic stage had the strongest effect on elemental variation in our study animals. Consistent with a previous amphibian study, we found that larger-bodied terrestrial adult males had considerably higher body %P than juvenile metamorphs (Tiegs *et al.*, 2016). Higher %P along with %Ca is typically associated with increased bone development and ossification in adults (Kemp & Hoyt, 1969; Milanovich *et al.*, 2015; Luhring *et al.*, 2017) and may explain the lower adult [C | P] and [N | Ca, Na, Mg] balances found in our study. In addition to these elements, we measured higher concentrations of many other essential elements in adult tissues (Zn, S, Li, Cu, and Mg). Our results also confirm previous measurements of higher juvenile body %C and no difference in body N content between terrestrial adults and metamorphic amphibians (Tiegs *et al.*, 2016) while showing differences in Fe and Mn concentrations and in related [Fe, Mn | Zn, Ba, Cu, Sr, Li] nutrient balances between these groups for the first time. This suggests that in addition to traditionally examined stoichiometric elements (e.g., N and P; Stephens et al. 2017), ontogenic variation in organismal elemental composition may reflect unique stage-specific nutritional physiologies and elemental requirements. However, it also appears that environmental nutrient supplies may potentially override these differences as we found substantial overlap in elemental profiles between pond-dwelling *A. talpoideum* metamorphs and paedomorphic adults in GB, which only differed in their %N content out of all elements surveyed (Supp. Table 3). As such, it is clear that we should consider moving beyond studies of individual or pairs of elements and examine integrated changes in suites of metabolically connected elements by refining tools such as nutrient balances (Parent *et al.*, 2013a; Baxter, 2015) to better understand the complex relationships between environmental nutrient supplies and organismal life-history traits and ionomes.

Traditional stoichiometric ratios relate nutrient interactions to biological functions (e.g., N:P ratios to ribosomal protein translation; Loladze and Elser 2011), and nutrient balance concepts extend this approach to encompass the entire organismal ionome (Parent *et al.*, 2013a). While it is beyond the scope of this paper to functionally relate changes in salamander balances to specific metabolic pathways and physiological process, our study demonstrates the potential of using nutrient balance principles to further our understanding of relationships between consumer ecology and elemental composition. We found evidence for novel nutrient balances [C | Ca, P, S, Mg] and [Fe, Mn | Zn, Ba, Cu, Sr, Li] that differed across ontogenic stages, and similar to plant-based studies, we documented systematic differences in elemental combinations between species and sites (Watanabe *et al.*, 2007; Parent *et al.*, 2013a). Previously constructed balances inherently differ from ours due to their focus on specific aspects of plant physiology, but the substantial overlap between elemental relationships in our study and previous research is perhaps indicative of fundamental elemental relationships governed by biological processes operating at the cellular level (Sterner & Elser, 2002; Watanabe *et al.*, 2007; Baxter *et al.*, 2008; Parent *et al.*, 2013a).

In this study, we examined correlations between salamander ionomic profiles and biological and environmental variables. As previously demonstrated, traditional stoichiometric balances (C | P and N | P) were useful for highlighting species differences in biogeochemically important elements (Sterner & Elser, 2002). However, we also showed that focusing solely on traditional stoichiometric elements alone could neglect other important elements, which might be differentially required for optimal metabolic functioning in terrestrial adult males (Ca, S, Mg, Zn, & Cu) and juveniles (Fe & Mn), respectively. Ionomic data analyzed here using balance techniques could thus be used to generate a greater understanding of the nutritional ecology of amphibian development. Such data are vital for providing a starting point for designing manipulative experiments to not only better understand the nutritional ecology of threatened vertebrates such as salamanders but also to illuminate the entire suite of ecological functions that they might influence.

Acknowledgements

Many people have assisted with drift fence checks and specimen collection over the 39-yr Rainbow Bay study, and we especially thank A. Chazal, A. Dancewicz-Helmers, R. Estes, J. Greene-McLeod, R. Semlitsch, J. Caldwell, L. Vitt, and W. Gibbons. We would also like to thank P. Lind and J. Goos for assistance with elemental analyses. This research was partially supported by U. S. Department of Energy under Award Number DE-FC09-07SR22506 to the University of Georgia Research Foundation and was also made possible the long-term protection of Rainbow Bay and Ginger's Bay by the DOE's Set Aside Program, as well as the designation of the SRS as a DOE National Environmental Research Park (NERP). Our study was also partially supported by the DOE and National Nuclear Security Administration under award numbers: DE-EM0004391 to the University of Georgia Research Foundation.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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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 sites[1], averaged across species[2], *Ambystoma opacum* only[3], average across sites *A. opacum* only[4], and *A. talpoideum* from Ginger's Bay only[5]. All elements are reported in standard scientific notation. Other abbreviations include: metamorph (Meta) and paedomorph (Paedo).

		[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]	
Model	*df*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
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	*df*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
[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 elemental 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), paedomorph (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 Balances

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

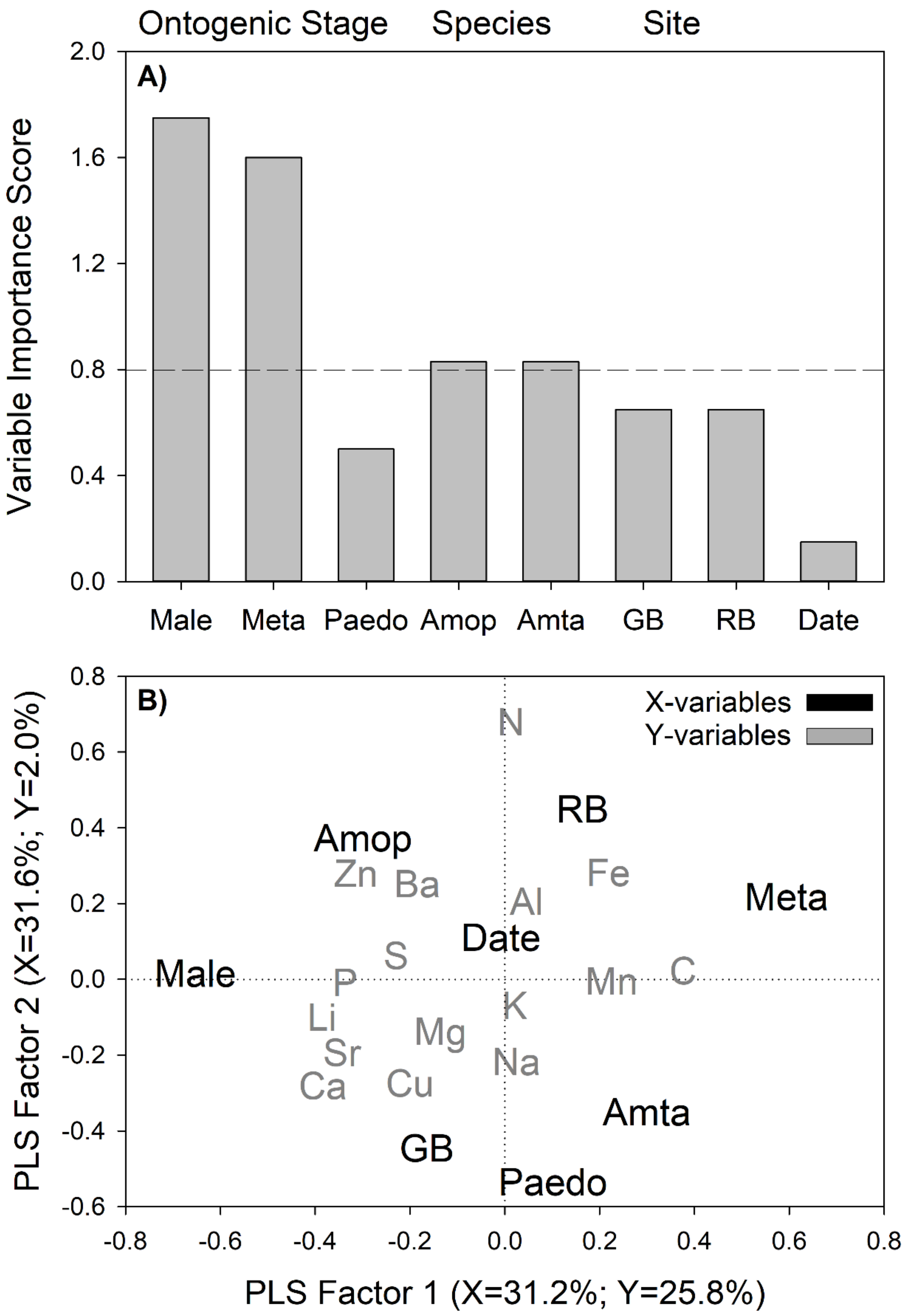
Figure 1. Relationships between ontogeny, species, site, date, and salamander elemental composition. A) Variable importance predictor scores show the relative influence of predictor variables on organismal ionomic profiles. Scores <0.8 are considered weakly important, those between 0.8-1 are moderately important, and scores >1 are strong predictors. B) Partial least squares regression (PLS) loadings plot of variables weights demonstrates 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).

Figure 2. Hypothetical differences in salamander elemental composition between adult and juvenile salamanders. Panel A) depicts univariate differences in body elemental composition between an adult and a juvenile salamander. Panel B) represents a mobile-and-fulcrum plot (*sensu* Parent et al. 2013) showing multivariate differences in [C | Ca, P] balances between these two ontogenic stages. The black circle represents an equilibrium point where salamander C and [Ca, P] content is equal for a given specimen, and the other circles correspond to balance points for each stage.

Figure 3. Variation in nutrient balances among species, sites, and ontogenic stages. Boxplots depict medians, 25th and 75th percentiles (boxes), and 10th and 90th percentiles (error bars) for each balance. All balances differed significantly among species (left- and right-hand panels), sites, and ontogenic stages according to Wilks's lambda scores ($P < 0.001$). Abbreviations include: metamorph (Meta), paedomorph (Paedo), Ginger's Bay (GB; Panels A&B), and Rainbow Bay (RB; Panels C&D).

Figure 4. Multivariate relationships among species, sites, and ontogenic stages. 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 nutrient balances are reported for each axis. All 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



Ontogenic Stage
Species
Site
A)
Variable Importance Score
2.0
1.6
1.2
0.8
0.4
0.0
Male
Meta
Paedo
Amop
Amta
GB
RB
Date
B)
X-variables
Y-variables
N
RB
Amop
Zn
Ba
Fe
Al
Meta
Date
S
Male
P
Mn
C
K
Li
Mg
Sr
Na
Ca
Cu
Amta
GB
Paedo
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
PLS Factor 2 (X=31.6%; Y=2.0%)
PLS Factor 1 (X=31.2%; Y=25.8%)

Figure 2

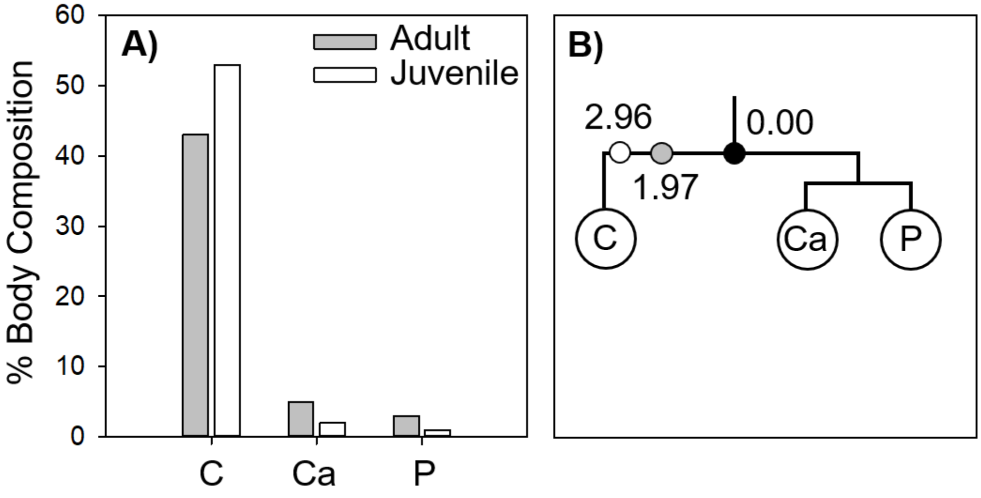
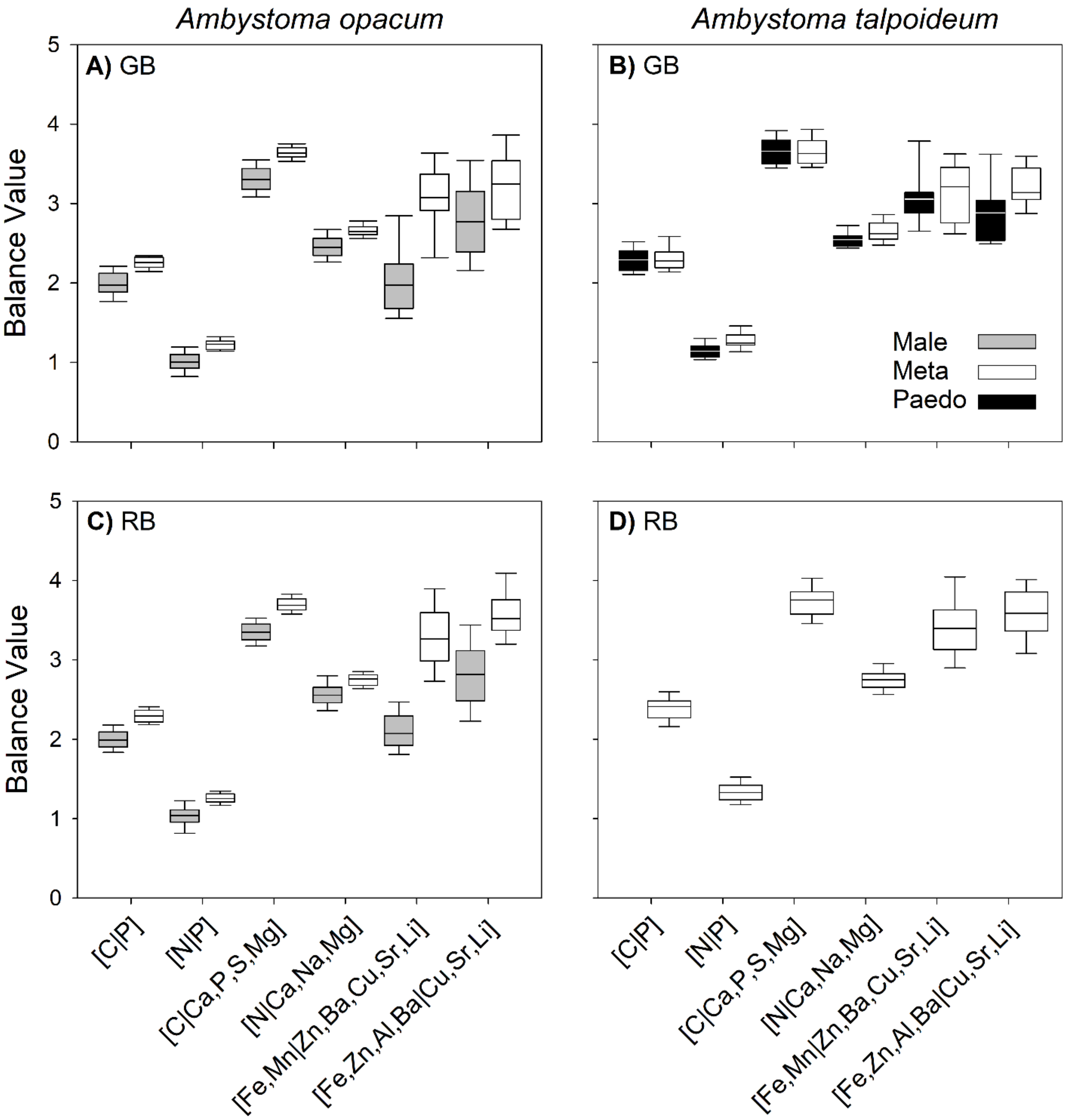
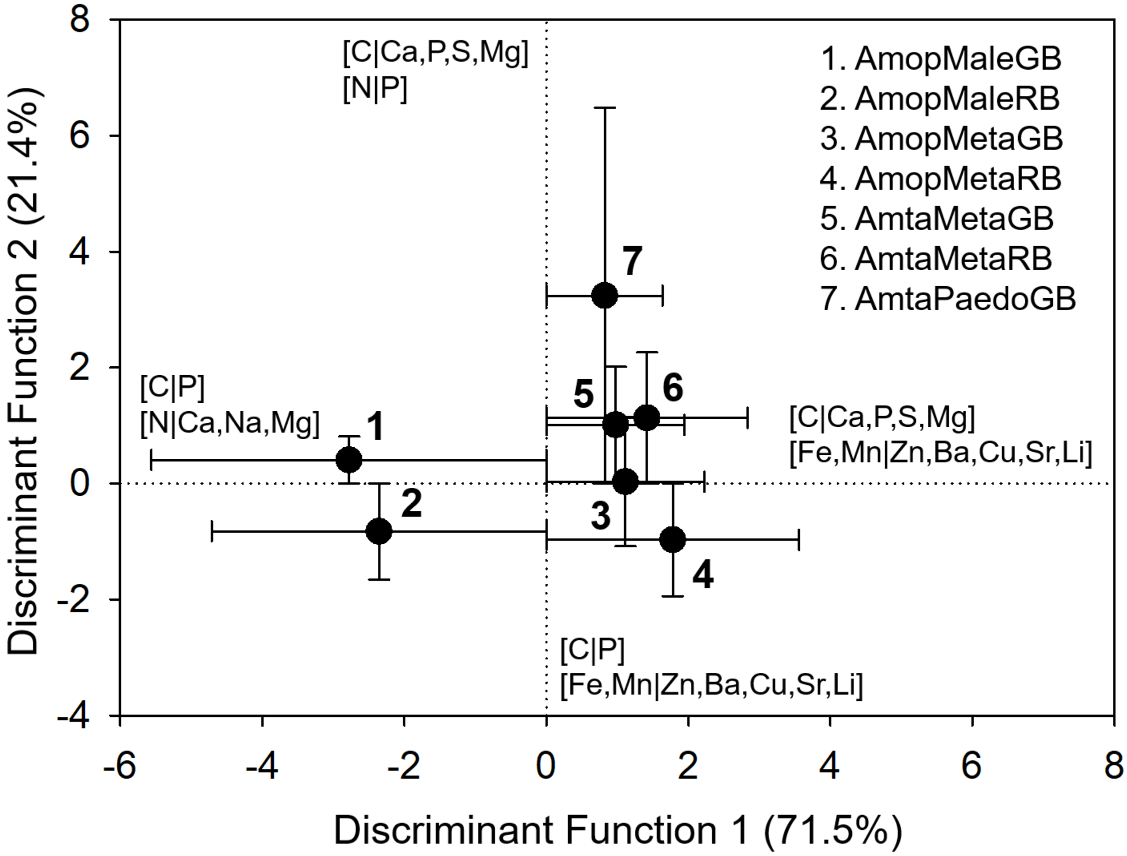

A)
Adult
Juvenile
% Body Composition
60
50
40
30
20
10
0
C
Ca
P
B)
2.96
0.00
1.97
C
Ca
P

Figure 3



Ambystoma opacum
Ambystoma talpoideum
A) GB
B) GB
C) RB
D) RB
Balance Value
Balance Value
0
1
2
3
4
5
Male
Meta
Paedo
[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]

Figure 4


8
6
4
2
0
-2
-4
-6
-4
-2
0
2
4
6
8
Discriminant Function 2 (21.4%)
Discriminant Function 1 (71.5%)
[C|Ca,P,S,Mg]
[N|P]
[C|P]
[N|Ca,Na,Mg]
[C|Ca,P,S,Mg]
[Fe,Mn|Zn,Ba,Cu,Sr,Li]
[C|P]
[Fe,Mn|Zn,Ba,Cu,Sr,Li]
1
2
3
4
5
6
7
1. AmopMaleGB
2. AmopMaleRB
3. AmopMetaGB
4. AmopMetaRB
5. AmtaMetaGB
6. AmtaMetaRB
7. AmtaPaedoGB