Stoichiometry and estimates of nutrient standing stocks of larval salamanders in Appalachian headwater streams

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SUMMARY

1. Because of their longevity and skeletal phosphorus demand, vertebrates can have distinct influences on the uptake, storage and recycling of nutrients in ecosystems. Quantification of body stoichiometry, combined with estimates of abundance or biomass, can provide insights into the effect of vertebrates on nutrient cycling.

2. We measured the nutrient content and estimated the abundance and biomass of the larvae of three salamander species to characterise the factors that influence larval salamander stoichiometry and estimate the contribution of larval salamanders to nutrient standing stock and recycling in five southern Appalachian headwaters.

3. The proportion and ratios of carbon (C), nitrogen (N), phosphorus (P) and calcium (Ca) in larval salamanders varied with size within and among species. We found negative relationships between body size and whole-body %N, C : P and N : P and positive relationships between body size and whole-body %P and C : N. Mean estimated larval salamander density was 58 m^{-2} and mean estimated dry biomass was 2813 mg m^{-2}. Estimated standing stock of N, P and Ca were 332, 81 and 103 mg m^{-2}, respectively, which was 1.1 $\times$ and 4.6 $\times$ greater than estimated amounts of N and P reported for macroinvertebrate taxa in similar streams within the region and was higher than in many other consumers in freshwater habitats. Using data from previous studies, we estimate that the mean ingestion rate of N and P by plethodontids is 0.000001 and 0.00001 g^{-1} day^{-1}, respectively, while excretion rates of both are <0.00001 g day^{-1}.

4. Salamanders are the only abundant vertebrates within headwater Appalachian streams, and our results show that larval salamanders represent a large standing stock of N, P and Ca in these streams. These findings complement other recent research demonstrating the effects of larval stream salamanders on macroinvertebrate abundance and seasonal nutrient supply in Appalachian headwaters and stress the importance of plethodontid salamanders to headwater stream processes.

Keywords: biomass, density, ecological role, plethodontid, standing stock

Introduction

Forested headwater streams generally have low concentrations of potentially limiting nutrients, such as nitrogen (N) and phosphorus (P), which are driven primarily by catchment characteristics and inputs (Swank & Vose, 1997; Webster & Meyer, 1997). The influence of consumers on nutrient cycles can be especially strong in low-productivity ecosystems, particularly abundant pre-datory taxa that consume large amounts of secondary production (Wallace *et al.*, 1997). Within headwater streams, we know little about the potential influence of many animals on ecosystem processes, including nutrient dynamics. This knowledge gap occurs at a time when forested hillslopes and associated headwaters are undergoing rapid development in regions of high stream biodiversity (Hansen *et al.*, 2005; Radeloff *et al.*, 2010; Kirk, Bolstad & Manson, 2012). To understand how sys-

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tems may change due to reductions in the abundance of key organisms, their influence on ecosystems processes needs to be quantified.

There are a number of factors that determine how consumers influence nutrient cycling in freshwater ecosystems. Through consumption, assimilation, excretion and decomposition, consumers can alter the fate and ratios of nutrients (Vanni, 2002; Schmitz, Hawlena & Trussell, 2010). In lotic systems, nutrients that are transferred into abundant consumers may become immobilised until remineralised, which will affect the rate at which a nutrient atom travels downstream (Small, Helton & Kazanci, 2009). Species that are trophic specialists, abundant, long-lived, have low metabolic rates or near the top of the food web may be particularly influential in the long-term retention of nutrients in lotic systems. Because of their bony skeleton, vertebrates have relatively high demand for limited nutrients such as P and calcium (Ca) and, when combined with their mobility, high biomass and longevity (Vanni, 2002; Hood, Vanni & Flecker, 2005; Pilati & Vanni, 2007), some freshwater vertebrates may have distinct influences on freshwater nutrient dynamics (Hall, 1972; Kitchell *et al.*, 1979; Vanni *et al.*, 2002; Small *et al.*, 2009). Estimates of the influence of vertebrate consumers on freshwater nutrient cycles are available for freshwater fish in lakes and tropical and temperate streams (Table 1), but little is known about the influence of other vertebrate consumers that occupy freshwater habitats, particularly taxa that occupy headwaters where fish are generally absent (but see Keitzer & Goforth, 2013a; Munshaw *et al.*, 2013).

In forested headwater streams of the eastern and north-western United States, plethodontid salamanders are often the only abundant vertebrates (Peterman, Crawford & Semlitsch, 2008; see Davic & Welsh, 2004; for a review). While salamanders comprise only ~10% of known amphibian species globally, they account for 50–60% of amphibians in North America with over 100 species occurring in the Appalachian Mountains (~17% of global salamander diversity). Appalachian headwater streams are hotspots of diversity for biphasic (those species with aquatic larvae) plethodontids, with seven or more species often occupying a single stream at densities averaging 60 m^{-2} (Peterman & Truslow, 2008). In this regard, the diversity and abundance of plethodontid salamanders distinguishes forested headwater streams of North America from similar systems in other parts of the world. Although salamanders are well studied, we know remarkably little about the influence of plethodontid salamanders on stream ecosystem processes (Davic

& Welsh, 2004). Based on their abundance, it is speculated that larval plethodontids are important to energy flow and nutrient storage in low-order streams (Burton & Likens, 1975a; Davic & Welsh, 2004), and their life cycle may contribute to nutrient fluxes between streams and the adjacent forest. Evidence suggests that plethodontid larvae can affect stream macroinvertebrate abundance and composition (Davic, 1983; Keitzer & Goforth, 2013b) and be seasonally and spatially important in supplying stream nitrogen demand (Keitzer & Goforth, 2013a). Plethodontid diversity and abundance is affected by development throughout the southern Appalachian Mountains. Declines in larval stream salamander abundance have been linked to the loss of riparian forest (Crawford & Semlitsch, 2008; Cecala, 2012), and some species are potentially vulnerable to projected regional climate change (Bernardo & Spotila, 2006; Bernardo *et al.*, 2007; Milanovich *et al.*, 2010). Knowledge of the influence of salamanders on ecosystem processes will lead to a more complete understanding of the potential consequences of their decline.

Ecological stoichiometry provides a framework for predicting the effects of species on nutrient dynamics. Stoichiometry combines the elemental composition of organisms and estimates of organism biomass to provide estimates of the ecological effects of species on nutrient dynamics (see review by Sardans, Rivas-Ubach & Peñuelas, 2011). The objectives of this study were to measure the elemental stoichiometry of an assemblage of larval salamanders and combine those measures with estimates of density and biomass to estimate the contribution of larval salamanders to the standing stock of N, P and Ca in five southern Appalachian headwater streams. Because they are vertebrates, we hypothesised that larval plethodontids would have a high P and Ca content and therefore constitute a large standing stock of P and Ca in headwater streams. Studies of other freshwater vertebrates suggest that P content is positively correlated with body size, which is related to the amount of bony skeleton (McIntyre & Flecker, 2010), and therefore, we hypothesised that variation in P content within and among species would be positively correlated with body size and Ca content. For context, we compare our results with estimates of other vertebrate of N and P in other freshwater systems and with previously quantified estimates of macroinvertebrate standing stocks of N and P for Appalachian headwater streams. We also address the potential contributions of larval salamanders to N and P recycling and stream demand for these limiting nutrients.

Table 1 Estimated biomass [dry mass mg m^{-2} (standard deviation); wet mass value[b]; AFDM value[c]] and standing stock of C, N, P and Ca [mg m^{-2} (SD)] of the three species of salamanders in this study, and macroinvertebrates and fish from stream and lake systems

Taxon or taxa	Biomass	C	N	P	Ca	Habitat	Reference
D. ocoee	12 (2)[c]	6 (1)	1.4 (0.2)	0.2 (0.05)	0.3 (0.1)	Appalachian stream	This study
E. wilderae	196 (147)[c]	107 (80)	25 (18)	3 (2)	5 (4)	Appalachian stream	This study
D. quadramaculatus	2605 (632)[c]	1288 (306)	306 (77)	78 (24)	98 (19)	Appalachian stream	This study
All salamanders	2813[c]	1480	332	81	103	Appalachian stream	This study
Stream macroinvertebrates	3–4300[c]		0.3–503	–	–	Appalachian stream	Mulholland et al. (2000)
	4–180[c]	–	–	0.1–18	–	Appalachian stream	Newbold et al. (1983)
	128–220	–	17–28	–	–	Appalachian stream	Tank et al. (2000)
	–	~700	~300	~20	–	Appalachian stream	Cross et al. (2005)
	0.2–315[c]	–	0.02–40	–	–	Tropical stream	Merriam et al. (2002)
	2–496	–	1.3–45	–	–	Prairie U.S. stream	Dodds et al. (2000)
	20–440[c]	–	2–52	–	–	Pacific U.S. stream	Ashkenas et al. (2004)
	340–1930[c]	–	34–196	–	–	Midwestern U.S. stream	Hamilton et al. (2001)
	350–9620	–	31–840	–	–	Desert U.S. stream	Grimm (1987)
	52–795	–	–	–	–	North-east U.S. stream	Hall, Likens & Malcom (2001)
	35 450	–	4300	–	–	Rocky Mtn. U.S. stream	Hall, Tank & Dybdahl (2003)
	–	–	72	–	–	Rocky Mountain U.S. stream	Hall et al. (2009)
	–	–	~280	~35	–	South-western England stream	Lauridsen et al. (2012)
Stream fish	63[c]	–	9	–	–	Appalachian stream	Tank et al. (2000)
	10–20	–	1–1.4	–	–	Prairie U.S. stream	Dodds et al. (2000)
	90–580	–	15–80	–	–	Pacific U.S. stream	Ashkenas et al. (2004)
	830	–	74	–	–	Midwestern U.S. stream	Hamilton et al. (2001)
	–	–	270	–	–	Desert U.S. stream	Grimm (1987)
	–	–	~400	~60	–	South-western England stream	Lauridsen et al. (2012)
Stream salamander	10–160[c]	–	–	2.7	–	Appalachian stream	Newbold et al. (1983)
	80[c]	–	9.2	–	–	Appalachian stream	Mulholland et al. (2000)
	1250	–	177	–	–	Pacific U.S. stream	Ashkenas et al. (2004)
	–	–	–	1	–	North-east U.S. stream	Burton & Likens (1975a,b)
Lake macroinvertebrates	7050[b]	–	–	11.3	–	Various	Griffiths (2006)
Lake fish	300–111 000[b]	–	–	–	–	Various	Cyr & Peters (1996)
	6730[b]	–	–	34.1	–	Various	Griffiths (2006)

Dq, *Desmognathus quadramaculatus*; Do, *Desmognathus ocoee*, Ew, *Eurycea wilderae*.

Methods

The study was conducted in five streams located within the Coweeta Hydrological Laboratory in Macon County, North Carolina. Coweeta is a 2185-ha basin located within the Blue Ridge physiological province within the southern Appalachian highlands (Swank & Crossley, 1988). The basin is dominated by mixed hardwoods and a dense understorey of *Rhododendron*. The five headwater streams drain Coweeta basin catchments 21, 22, 34 and two unnamed catchments just west of 22 (Swank & Crossley, 1988), which we subsequently designated here

as streams 1–5, with stream 1 representing the stream directly east of catchment 27, stream 2 representing the stream directly west of catchment 22 and streams 3, 4 and 5 representing streams that drain catchments 22, 21 and 34, respectively. Headwater streams at Coweeta are heterotrophic and detritus based, in which up to 90% of the energy for microbial and macroinvertebrate production is derived from detritus (Wallace *et al.*, 1997; Hall, Wallace & Eggert, 2000). Headwater streams at Coweeta are generally fishless and the dominant predatory vertebrates are the larvae of plethodontid salamanders (up to seven species of plethodontids in four genera). The five most common species are *Desmognathus quadramaculatus* (*Dq*), *Desmognathus ocoee* (*Do*), *Desmognathus monticola* (*Dm*), *Eurycea wilderae* (*Ew*) and *Gyrinophilus porphyriticus* (*Gp*). Numerically, streams are dominated by *Ew*, *Do* and *Dq*, the last of which composes the overwhelming majority of larval salamander biomass.

Salamander stoichiometry

We analysed the stoichiometry of the larvae of three plethodontid species that varied in size and length of larval period (Table 2). From 1 June–30 August 2008, we collected 13–15 *Dq*, *Do* and *Ew* from each of the five streams, at a time when larval *Do* were close to metamorphosis, whereas those of *Ew* were newly hatched and of *Dq* ranged from recently hatched to 3 or 4 years of age (Table 2). We killed larvae by immersion in a 0.5% solution of neutral pH-buffered MS-222 (ethyl m-amino-benzoate methanesulphonate), after which animals were thoroughly rinsed with deionised water. We measured the snout–vent length (SVL: from the tip of the snout to the posterior portion of the vent to the nearest millimetre) and wet mass (to the nearest 0.1 mg), dissected the animal to remove the stomach and intestinal tract and froze the remaining body. Later, whole bodies were oven-dried at 60 °C to a constant mass and analysed for whole-body %C and %N with a Carlo Erba NA 1500 CHN analyser (Carlo Erba, Milan, Italy). For P and

Ca analysis, samples were acid-digested (*aqua regia* double acid; Jones, Wolff & Mills, 1991) and analysed spectrophotometrically (ascorbic acid method). For each sample of *Ew*, two individuals of similar size were ground together to obtain enough tissue for sampling. Whole-body %Ca was only measured for 15 *Ew*, 15 *Do* and 20 *Dq*. Because *Dq* larvae represented animals across a 3- to 4-year larval lifespan, for %Ca analysis, we randomly sampled six to eight *Dq* from each of three size classes: SVL 18–25, 26–33 and 33–40 mm. All data are presented either as %C, %N, %P or %Ca of dry mass or as molar ratios.

Estimating salamander densities, biomass and standing stock of nutrients

To estimate the standing stock of nutrients in stream plethodontids, we combined estimates of biomass and whole-body elemental stoichiometry. We used a combination of leaf litter bags and dip netting to estimate the density of larval stream salamanders (Peterman & Truslow, 2008). From 10 May to 28 July 2008, larval and newly metamorphosed salamanders were sampled on three consecutive days (secondary sampling occasions) every 14 days, for a total of six primary sampling periods. Within each stream, we used 10, 1-m-long plots that were 10 m apart. Plots were arranged approximately 20 m upstream of a weir or road crossing. Within each plot, we placed one mesh bag (55 × 25 cm made with 1.3 cm^2 mesh) filled with deciduous litter (collected from the adjacent riparian area) in a wetted portion of the stream channel. We placed a rock from the streambed on top of the mesh bag to prevent it dislodging. We followed the methods of Peterman & Truslow (2008) and Nowakowski & Maerz (2009) to remove contents from each bag. In addition to checking traps, we thoroughly dip-netted the 1-m^2 plot by dislodging substratum particles across the width of the stream. Animals were identified, weighed and measured (SVL).

Table 2 Life history characteristics of larval *Ew* (n = 75) and *Dq* (n = 200) and larval/juvenile *Do* (n = 25) from Coweeta. Mean SVL (SD; range), mass (SD) and ash-free dry mass (AFDM; SD) were taken from abundance data set in this study. Data from previous studies were used for length of larval period and hatching date (Bruce, 1982, 1985, 1988, 1989; Camp & Tilley, 2005; Sever, 2005; Watson, Pauley & Camp, 2005)

Species	Mean SVL (mm)	Mean wet mass (mg)	AFDM mg^{-1}	Wet biomass mg m^{-2}	Larval period length (months)	Typical hatching date
Dq	30.13 (6.01; 18–40)	890 (550)	92 (63)	22 220 (2390)	36–48	July–November
Do	15.35 (3.54; 9–20)	100 (40)	8 (3)	130 (37)	9–10	August–November
Ew	12.60 (1.32; 9–21)	60 (30)	4 (1)	1650 (1110)	12–24	June–August

Dq, *Desmognathus quadramaculatus*; *Do*, *Desmognathus ocoee*, *Ew*, *Eurycea wilderae*.

We used zero-inflated abundance models based on models developed by Wenger & Freeman (2008) to estimate abundance of larval and newly metamorphosed plethodontids in each stream across six sampling periods using count data (see Milanovich, 2010 for details on statistical methods and models). The Wenger & Freeman (2008) population model combines N-mixture models (Royle, 2004; Royle, Nichols & Kery, 2005) and zero-inflated binomial occupancy models of MacKenzie *et al.* (2002) and thus carries the assumptions of both abundance (Royle, 2004; Royle *et al.*, 2005) and occupancy (MacKenzie *et al.*, 2002) models, such as: (i) the sample population is closed to immigration, emigration and state change between samples, and (ii) individuals are independent and equally available for capture. If these assumptions are violated, estimates can be inflated, due to reduced estimates of detection or increased variation in counts at a particular site. Since the probability of detection, and estimates of the abundance of plethodontids, may vary seasonally or geographically (by stream), we modelled the abundance and presence as a function of stream, sampling period or both. We ran 110 000 iterations, discarded the first 50 000 as a burn-in phase and thinned the remaining 60 000 by 150. The best-supported model for each species was then selected from multiple competing models using Akaike's information criterion adjusted for small sample size (Burnham & Anderson, 2002; see Appendix S1 in Supporting Information). Abundance estimates provided are for those individuals that were available for capture (at the surface) and the capture probability represents unconditional capture probability. The surface area (1 m^2) of each plot was combined with estimates of abundance and measures of wet and dry mass to estimate density and biomass m^{-2}.

Standing stock of nutrients for each individual of each species across all streams was determined by multiplying the whole-body %C, %N and %P of each individual by their ash-free dry mass (AFDM mg). Salamander biomass was calculated by the product of mean AFDM of individuals used in stoichiometric analysis and density estimates derived from N-mixture models for each stream. We then calculated the product of mean %C, %N or %P and salamander biomass (AFDM m^{-2}) for each species within each stream to estimate standing stock of C, N and P within the larval salamander assemblage. Since individuals within each species were subsampled to quantify %Ca values, standing stock of Ca was calculated as the product of mean %Ca for each species and salamander biomass with samples combined within a single species, not individual streams. Numbers were

compared to established values of stream litter (fine particulate organic matter [FPOM] and coarse particulate organic matter [CPOM]), macroinvertebrate and fish standing stocks of C, N and P reported by several studies. We estimated dry mass of individual *Ew* larvae using a power function (dry mass = 0.000004*SVL$^{2.991}$; $r^2 = 0.786$) generated between SVL, wet mass and dry mass from five independent data sets composed of 495 larval *Ew* and 154 larval *E. cirrigera*. Next, we used the estimated dry mass of *Ew* and actual dry mass values of *Do* and *Dq* (from specimens used in stoichiometric analysis) to calculate AFDM by assuming the per cent of AFDM to dry mass in our *Eurycea* and *Desmognathus* species was similar to that found for *Eurycea bislineata* (13%) and *Desmognathus fuscus* (15%) in a New Hampshire stream (derived from Burton & Likens, 1975a); thus, 13% of *Ew* dry mass and 15% of *Do* and *Dq* dry mass were AFDM.

Statistical analysis

We used separate general linear models (GLM) for each dependent variable to test for the effects of species (categorical variable) on whole-body %C, %N, %P and %Ca (logit-transformed; Warton & Hui, 2011) and elemental molar ratios of C : N, C : P and N : P (log-transformed). Stream was included in the model as a categorical (blocking) variable to account for differences among streams. Body size of consumers has been found to influence elemental composition of consumers in a number of studies; therefore, we tested whether body size could be a predictor of any significant stream blocking effects using a one-way ANOVA to test for differences in body size of the salamander assemblage across streams. If body size was significantly different across streams, it would warrant inclusion into our existing model. Tukey's honestly significant difference ($\alpha < 0.05$) was used as a *post hoc* test to make pairwise comparisons between streams and species.

We used linear regression to examine the effect of body size (whole-body dry mass) on whole-body %C, %N and %P and elemental ratios of C : N, C : P and N : P. To remain consistent with other studies, these regressions were not conducted with AFDM values, rather raw dry mass (mg) of whole bodies. We assumed that variation in whole-body %Ca would largely reflect differences in amount of bone among individual salamanders; therefore, we used a GLM to regress whole-body %P (logit-transformed) on whole-body %Ca (logit-transformed) to determine whether variation in %P among individual salamanders was potentially related

to bone content (Hendrixson, Sterner & Kay, 2007; Pilati & Vanni, 2007). We included species as categorical predictor variable to determine whether there were differences in %P among individuals and species and whether those differences were a function of variation in the amount of bone content among species.

A two-way analysis of variance (ANOVA) was used to test for the effects of stream and species on the standing stock of C, N, P and Ca using standing stock of C, N, P and Ca (e.g. gC m^2) as dependent variables and stream and species as categorical variables. Tukey's honestly significant difference ($\alpha < 0.05$) was used as a *post hoc* test to make pairwise comparisons between streams and species. Statistical analyses were conducted in STATISTICA 8.0 (Statsoft, Inc., Tulsa, OK).

Results

Salamander stoichiometry

Consistent with our predictions, there were interspecific differences in stoichiometry (Table 3; Appendix S2) related to body size (Figs 1 & 2). *Desmognathus quadramaculatus*, which was the largest species, had the highest mean whole-body %P and %Ca and lowest mean whole-body %N and %C (Table S1). *Eurycea wilderae* had the highest mean whole-body %C and mean whole-body %N, while *Do* was intermediate in both size and stoichiometry for C, N, P and Ca (Table S1). We used stream as a statistically significant blocking variable in all pairwise species comparisons to examine any effect of the stream on dependent variables. The interaction between species and stream was significant for C : N, N and Ca (Table 3). Any differences in mean salamander elemental composition and ratios between streams were generally

small and limited to specific streams (Table S1). For example, C and N content of larvae generally varied by <7% across streams. Content of P across all larvae varied but *Ew* %P accounted for the majority of the variation across streams (Table S1). Body size of the salamander assemblage was not significantly different across streams (ANOVA, $F_{4, 215} = 1.577$, $P = 0.182$). Allometric patterns were observed between whole-body elemental composition and body size for N, P, N : P, C : N and C : P for all species (Figs 1 & 2). We found a negative relationship between body size and whole-body %N, C : P and N : P, and a positive relationship between body size and whole-body %P and C : N (Figs 1 & 2). These patterns were largely driven by ontogenetic variation within species. Although we found only a weak relationship between body size and body %C, %C and %N generally decreased, while %P increased, with salamander size.

Differences in whole-body %Ca, which we used as a proxy for the amount of bone within a salamander, reflected the differences in the amount of P between salamanders. The model with species and whole-body %Ca explained 48% of variation in whole-body %P; whole-body %P was positively correlated with whole-body %Ca (d.f. = 1, MS = 0.416, $F = 4.322$, $P = 0.043$), and the relationship between %Ca and %P was similar among species (species × %Ca interaction: d.f. = 2, MS = 0.092, $F = 0.961$, $P = 0.390$). Once we accounted for %Ca, we found that %P was not different among species (d.f. = 2, MS = 0.159, $F = 1.652$, $P = 0.203$). Linear regression showed that, for all species combined, whole-body %Ca increased significantly with increasing salamander dry mass [body size] ($r^2 = 0.25$; $P < 0.001$, whole-body %Ca = 2.608 + 0.010*dry mass) and whole-body %P ($r^2 = 0.37$; $P < 0.0001$; whole-body %P = 0.916 + 0.432*whole-body %Ca).

Table 3 Results from general linear model tests of mean differences between proportions and molar elemental ratios of C, N, P and Ca; C : N, C : P and N : P among streams and species. Per cent C, N, P and Ca values were logit-transformed and elemental molar ratios were log-transformed

Effect	d.f.	%C MS	F	P	%N MS	F	P	%P MS	F	P	d.f.	%Ca MS	F	P
Stream	4	0.058	7.094	<0.001	0.023	4.700	<0.001	0.333	4.420	0.002	2	0.156	1.405	0.252
Species	2	0.302	36.727	<0.001	0.161	33.700	<0.0001	5.434	72.110	<0.0001	4	1.185	10.673	<0.001
Stream × Species	8	0.013	1.618	0.121	0.010	2.000	0.051	0.096	1.270	0.259	8	0.261	2.348	0.039
Error	207	0.008	–	–	0.005	–	–	0.075	–	–	35	0.111	–	–
		C : N			C : P			N : P						
Stream	4	0.024	20.000	<0.0001	0.297	3.930	0.004	0.288	3.71	0.006	–	–	–	–
Species	2	0.015	12.400	<0.0001	6.666	88.070	<0.0001	6.737	86.790	<0.0001	–	–	–	–
Stream × Species	8	0.003	2.600	0.010	0.110	1.460	0.175	0.104	1.340	0.224	–	–	–	–
Error	207	0.001	–	–	0.076	–	–	0.078	–	–	–	–	–	–
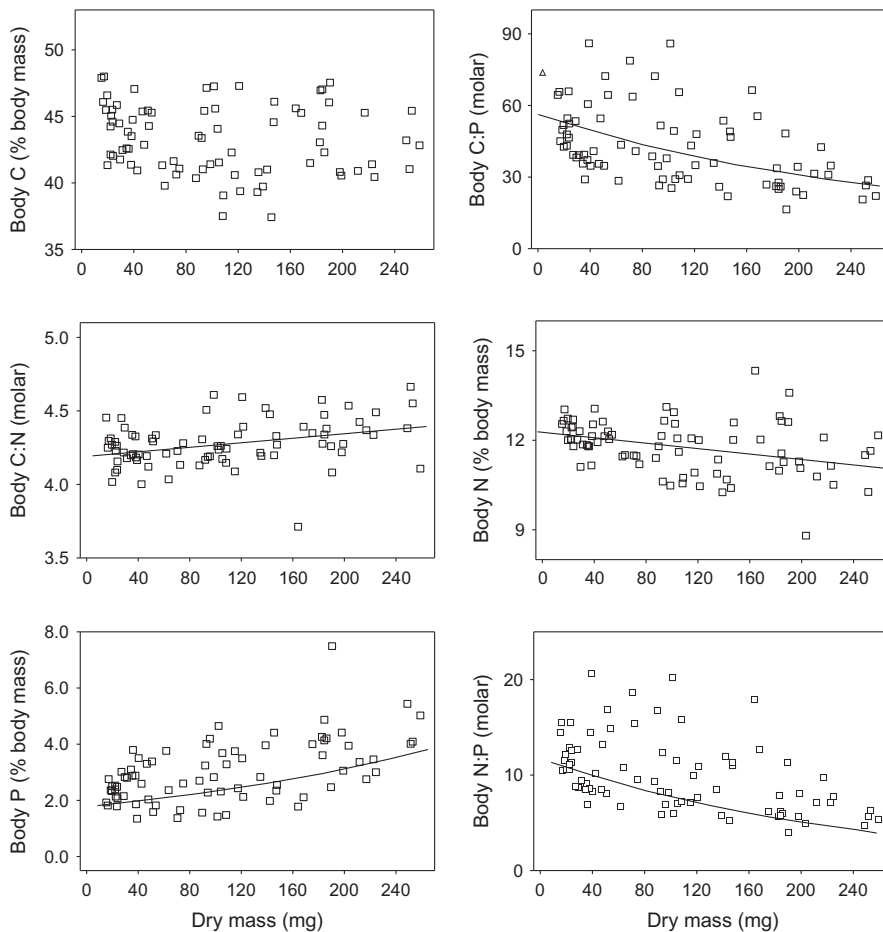
Fig. 1 %C, %N, %P and elemental C : N, C : P and N : P versus body size (dry mass) for *Desmognathus quadramaculatus*. Linear equations for the relationships between untransformed data for C, N, P, C : N, C : P and N : P and body dry mass are as follows: carbon, body % C = 4343.944–0.008 dry mass, $r = -0.197$, $P < 0.081$; nitrogen, body %N = 12.193–0.004 dry mass, $r = -0.318$, $P < 0.004$; phosphorus, body %P = 22.128 + 0.008 dry mass, $r = 0.538$, $P < 0.0001$; C : N, body C : N = 4.199 + 0.01 dry mass, $r = 0.337$, $P = 0.002$; C : P, body C : P = 5454.084–0.109109 dry mass, $r = -0.489$, $P < 0.0001$; N : P, body N : P = 1212.641–0.026026 dry mass, $r = -0.480$, $P < 0.0001$. Linear regression lines of fit were only drawn for relationships significant at $P \leq 0.05$.

## *Plethodontid density, biomass and standing stock of nutrients*

Density of larval salamanders differed among species and across streams. Stream 3 had the highest estimated total larval density at 85 m^{-2}, while stream 1 had the lowest estimated larval density of 37 m^{-2}. Across all streams, mean *Dq* and *Ew* larval densities were 29 and 27 m^{-2}, respectively, while *Do* mean density was <2 larvae m^{-2} (Appendix S3). The density of *Ew* varied the most and the density of *Dq* varied the least among streams (Table S3). Although mean estimates of *Ew* and *Dq* density were similar, because of its significantly larger size, mean estimated biomass of *Dq* was 1,229% higher than *Ew* and 26 600% higher than *Do*.

Standing stock of nutrients for all larvae combined was 1480 mg C m^{-2}, 332 mg N m^{-2}, 81 mg P m^{-2} and 103 mg Ca m^{-2} (Table 1). Standing stocks of C, N, P and Ca varied significantly between *Dq* and the other species (Tables 1, 4 & 5), but did not vary significantly between *Do* and *Ew* (Table 4). Across all species, mean standing stocks of C and N were lowest in *Do* and highest in *Dq*; while standing stocks of P and Ca were highest in *Dq* and lowest in *Do* (Tables 1 & 5). Mean estimated standing stock of C, N and P in the larval salamander assemblage was 7.1% less, 12% greater and 363% greater than the reported standing stock of C, N and P in the stream macroinvertebrate assemblage for similar streams within the same basin (Cross *et al.*, 2005) and was higher than in many other stream and lake consumers (Table 1). Based on published values of macroinvertebrate and leaf litter standing stocks from Coweeta (Cross *et al.*, 2005), we estimated that larval salamanders constituted 0.13, 1.8 and 11.5% to the total pools of C, N and P in headwater streams.

Discussion

Salamander stoichiometry was largely driven by body size and, based on the correlation with Ca content, the allocation of P to bone, with larger species having proportionately greater P and proportionately less C and N (Figs 1 & 2). Whole-body %Ca explained nearly 50% of the variation in whole-body %P among larval salaman-
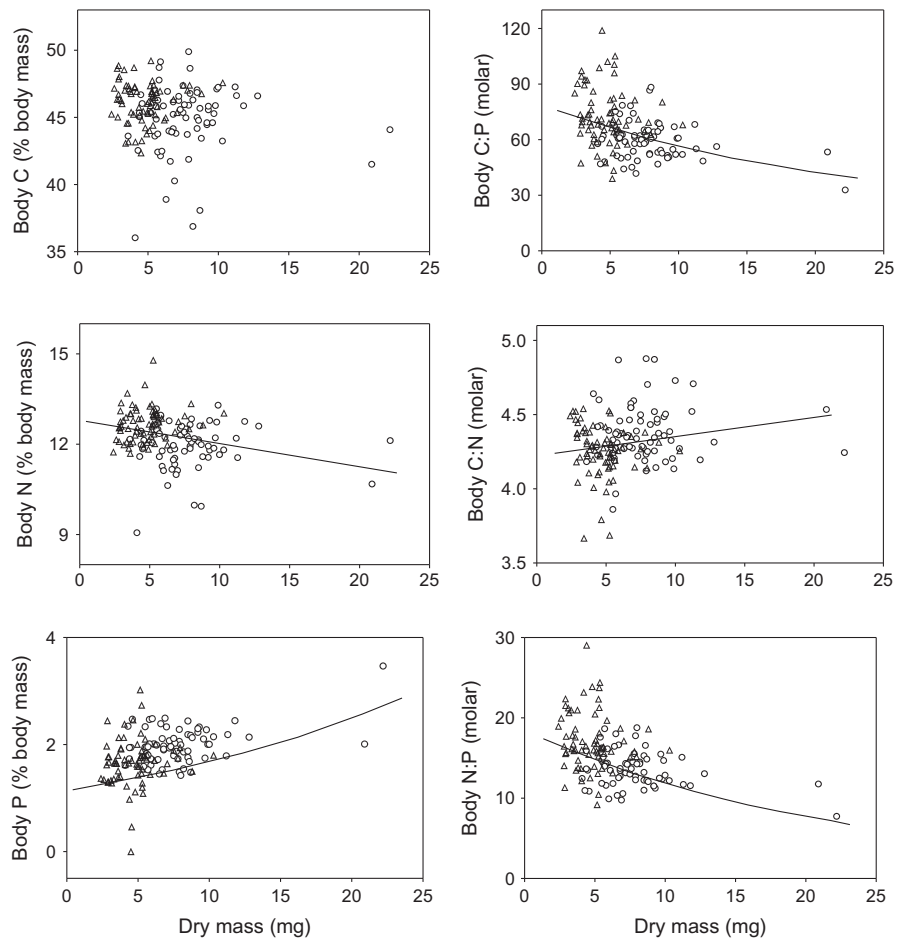
Fig. 2 %C, %N, %P and elemental C : N, C : P and N : P versus body size (dry mass) for *Desmognathus ocoee* (circles) and *Eurycea wilderae* (triangles). Linear equations for the relationships between untransformed data for C, N, P, C : N, C : P and N : P and body dry mass are as follows: carbon, body %C = 46.230–0.118 dry mass, $r = -0.152$, $P < 0.070$; nitrogen, body %N = 12.705–0.063 dry, mass, $r = -0.241$, $P < 0.004$; phosphorus, body %P = 1.452 + 0.061 dry mass, $r = 0.407$, $P < 0.0001$; C : N, body C : N = 4.248 + 0.011 dry mass, $r = 0.163$, $P = 0.052$; C : P, body C:P = 78.709–2.013 dry mass, $r = -0.397$, $P < 0.0001$; N:P, body N:P = 18.484–0.504 dry mass, $r = -0.420$, $P < 0.0001$. Linear regression lines of fit were only drawn for relationships significant at $P \leq 0.05$.

Table 4 Mean magnitude of difference (%) of %C, %N, %P and %Ca and standing stock (mg m^{-2}) of C, N, P and Ca of larval salamanders between each species (mean across all streams)

	Magnitude of difference (%)							
	Carbon		Nitrogen		Phosphorus		Calcium	
Species	*D. ocoee*	*E. wilderae*	*D. ocoee*	*E. wilderae*	*D. ocoee*	*E. wilderae*	*D. ocoee*	*E. wilderae*
D. quadramaculatus	*3.9	*7.3	2.0	*8.0	*43.7	*72.1	*59.3	*61.6
E. wilderae	*3.2	–	*5.8	–	*19.8	–	1.5	–
	Standing stock of C		Standing stock of N		Standing stock of P		Standing stock of Ca	
	D. ocoee	*E. wilderae*	*D. ocoee*	*E. wilderae*	*D. ocoee*	*E. wilderae*	*D. ocoee*	*E. wilderae*
D. quadramaculatus	*21 366	*1103	*21 757	*1124	*38 900	*2500	*32 566	*1860
E. wilderae	1683	–	1685	–	1400	–	1567	–

Dq, *Desmognathus quadramaculatus*; Do, *Desmognathus ocoee*, Ew, *Eurycea wilderae*.
*Significant differences between species across all streams (Tukey's HSD).

ders, suggesting that higher mineral skeleton among larger larvae drives much of the variation in P content within and among larval plethodontid species. The moderate variability in larval phosphorus content, independent of species or larval size, indicates that larvae are either not truly homeostatic with regard to P content

(exhibit plasticity with regard to P stoichiometry) or there is individual variation in P content. These results are consistent with other studies of vertebrates (see review by McIntyre & Flecker, 2010). As with freshwater fish, larval plethodontids also show negative relationships between whole-body N, C : P and N : P relative to

Table 5 Results from two-way analysis of variance tests investigating the differences in mean standing stock of nutrients across three salamander species among five Appalachian headwater streams.

Effect	d.f.	Carbon			Nitrogen			Phosphorus			Calcium		
		MS	F	P	MS	F	P	MS	F	P	MS	F	P
Species	2	2 538 683	70.888	$\leq$0.001	144 127	65.205	$\leq$0.001	9679.047	49.633	$\leq$0.001	15 195.656	132.846	$\leq$0.001
Stream	4	28 196	0.787	0.565	1781	0.806	0.555	181.266	0.930	0.493	145.420	1.273	0.357
Error	8	35 812	–	–	2210	–	–	195.010	–	–	114.385	–	–

body size (e.g. Pilati & Vanni, 2007) and positive relationships between P and body size. Larval salamanders in our study, in general, had higher N:P and C:P ratios (higher %N and lower %P) compared with freshwater fish (e.g. Vanni *et al.*, 2002). Although differences within salamander taxa among streams occurred, these differences were small compared to elemental variation across species (Tables 3, 5 & S1). In total, these results suggest larval plethodontid stoichiometry and effects on nutrient dynamics can be predicted by factors similar to other freshwater vertebrates, such as body size.

Variation in species density and species-specific differences in elemental composition largely determined the amount and variation in standing stock of nutrients among streams. The estimated standing stock of nutrients in the stream salamander assemblage could represent a significant reservoir of key nutrients in headwater streams, particularly P and Ca. Because salamander larvae occur at high densities and allocate P and Ca to bone as they grow, salamanders represent a large standing stock of P and Ca in headwater streams. Our estimates suggest that the standing stock of N and P in larval salamanders was 332 and 81 mg m^{-2}, respectively. This standing stock is 1.1 and 4.6 $\times$ larger than the estimated N and P standing stocks within the macroinvertebrate assemblages reported for Coweeta streams (Table 1) and is greater than similar reports for other stream consumers (Table 1). Concentrations of Ca in macroinvertebrates, stream detritus and litter (mg Ca g^{-1}) show that larval salamanders, collectively, have Ca concentrations 13 to 6665% greater than macroinvertebrates and 1050 to 2070% greater than stream detritus in similar streams (Woodall & Wallace, 1975), thus suggesting (when combined with density estimates) that salamanders in headwater streams are significant standing stocks of Ca as well as P (Woodall & Wallace, 1975). Our measures of standing stock of nutrients were derived from modelled estimates of density and stoichiometric data taken from a subset of individuals. Our use of mean estimated density and stoichiometric values could lead to a conservative or overinflated community-level estimate of nutrient standing stocks; however, we believe our mean values represent a reasonable estimate of nutrient standing stocks within the plethodontid salamander assemblage.

Changes in the abundance of salamanders are likely to alter nutrient spiralling rates and the availability of nutrients, which can determine fungal and bacterial production and leaf litter breakdown rates within heterotrophic headwater streams (Tank & Dodds, 2003; Greenwood *et al.*, 2007; Suberkropp *et al.*, 2010). Independent of effects via direct consumption of secondary consumers (e.g. shredders), tertiary consumers such as larval salamanders can affect the downstream spiralling of nutrients in several key ways. First, metabolic rate and tissue demand will determine rates of nutrient turnover and excretion. Long-lived consumers with high demands for particular elements should slow down the velocity at which nutrients travel downstream by retaining those elements within biotic pools for longer periods of time (Small *et al.*, 2009). In the case of vertebrates, particularly juveniles that are growing and producing skeletal mass, the allocation of P and Ca to bone could dramatically slow the recycling of those elements (however, see Sterrett, Maerz & Katz, 2015). Second, the forms in which organisms excrete elements can affect the uptake rate and therefore the velocity at which elements spiral downstream. For example, fish excrete ammonium, which has a longer uptake distance compared to organic forms of nitrogen, such as urea, that are commonly excreted by amphibians (Brookshire *et al.*, 2005).

Plethodontid salamanders are among the most metabolically efficient vertebrate species known (Fitzpatrick, 1973a,b; Feder, 1976, 1983), and we propose that they are important for the retention of nutrients in headwater streams as both large, metabolically stable standing stocks and through the resupply of highly biologically available forms of N. Mean ingestion rates of N and P of larval *Ew* and *Dq* (accounting for assimilation efficiency) are between 0.01 to 0.1, and 0.001 to 0.01 mg^{-1} day^{-1}, respectively (Bumpers, 2014), and assimilation rates range between 86 and 97% for both species (Fitzpatrick, 1973a,b). Using larval growth rates of *Ew* and *Dq* at

Coweeta (Beachy, 1997; Johnson *et al.*, 2006) and N and P standing stock of larval plethodontids measured in this study, salamander production should result in uptake rates of 0.90 mg N m^{-2} d^{-1} and 0.22 mg P m^{-2} d^{-1}. Areal uptake rates of N and P in Coweeta headwater streams are approximately 28.3 mg N m^{-2} d^{-1} and 8.6 mg P m^{-2} d^{-1} (Webster *et al.*, 2001; Valett *et al.*, 2008), suggesting that approximately 3.2 and 2.5% of N and P areal uptake eventually fluxes into larval plethodontid growth. Mean stoichiometry (N : P and C : P) of the prey of larval plethodontids at Coweeta is 73 and 446, Cross *et al.*, 2003 which is on average 5.3–7.8 times greater than their whole-body stoichiometry, respectively (Bumpers, 2014; Table S1), suggesting that larval salamanders excrete N disproportionately highly compared to ingestion. Excretion rates of *Eurycea* and *Desmognathus* are <0.00001 g N or g P day^{-1} (Milanovich, 2010; Keitzer & Goforth, 2013a), and Keitzer & Goforth (2013a) show that larval Ew and Dq excretion of ammonium was sufficient to supply up 10% of N demand in headwater streams seasonally. However, the mean density estimates in our study were 2.7 times greater than those reported by Keitzer & Goforth (2013a) and salamanders excrete proportionately greater amounts (up to 75%) of urea than ammonium (Milanovich & Hopton, in press), which has a 40% shorter uptake rate (Brookshire *et al.*, 2005). This suggests that larval salamanders could in fact provide far more than 10% of headwater N demand while simultaneously slowing the velocity of N spiralling and increasing retention of N, P and Ca. Combining our density estimates with reported excretion of NH_4-N by Dq and Ew from Keitzer & Goforth (2013a) suggests that larval salamanders could provide minimally 28% of headwater N demand seasonally. These estimates are similar to estimated excretion rates and contributions to N uptake by Pacific giant salamanders (*Dicamptodon tenebrosus*) in northern California streams. By comparison, McManamay *et al.* (2010) report N and P excretion from macroinvertebrates and fish at Coweeta represent 1.5 to 2, and 12 to 119% of N and P stream uptake, respectively. Collectively, these studies indicate a potentially significant influence of larval salamander assemblages to nutrient cycling within headwater streams.

Our estimates and conclusions differ from those of Burton & Likens (1975a) and, to some extent, Newbold *et al.* (1983) who concluded that stream salamanders store very little N and P and have little effect on nutrient cycling at the Hubbard Brook Experimental Forest in New Hampshire and a stream in Tennessee. Using values from Burton & Likens (1975a,b), we estimate the standing stock of N, P and Ca within the stream salamander assemblage (larvae and adults) at Hubbard Brook was 4.9 mg N m^{-2}, 1.2 mg P m^{-2} and 1.6 mg Ca m^{-2}, respectively. Our estimates for streams at Coweeta are between 64 and 68 times greater. Although Burton & Likens (1975a) and Newbold *et al.* (1983) studied different species at a different location, they studied closely related congeners to our focal taxa. Therefore, we do not believe taxonomic or geographic effects account for the differences among studies. The most likely explanation for the differences between studies relates to methods used to estimate salamander abundance and biomass. Burton & Likens (1975a) used surface counts to estimate population density, with the unrealistic assumption of high (near perfect) capture probability, which significantly underestimate larval salamander abundance (Bailey, Simons & Pollock, 2004; Peterman & Truslow, 2008; Nowakowski & Maerz, 2009). We suggest that a re-examination of population estimates from Burton & Likens (1975a) and Newbold *et al.* (1983), using more contemporary methods that incorporate detection, would result in larger estimates of nutrient standing stocks more similar to our estimates.

Knowing that plethodontids can be significant standing stocks and influence the availability of key limiting nutrients within heterotrophic headwater systems means that natural or anthropogenic factors that drive shifts in salamander community composition or abundance could alter nutrient retention and dynamics within headwaters. We estimated that *D. quadramaculatus*, through its dominance of larval biomass and high concentration of limiting nutrients, is the most influential species with regard to larval retention of nutrients within headwater streams. Cecala (2012) sampled 37 first-order streams in the Upper Little Tennessee River catchment surrounding Coweeta and found that riparian forest loss from agriculture or residential hillslope development was associated with declines in the abundance of larval *D. quadramaculatus*. Between 1973 and 2012, riparian forest cover has decreased in the region by 12%, with a projected further loss of 12% by 2030 (Cecala, 2012). If this is realised, the abundance of larval salamanders in streams may decline to 60% of mean abundance in fully forested catchments (Cecala, 2012). Moreover, physiological data suggest that *D. quadramaculatus* in the southern Appalachians exist near the thermal limits of their range and, in combination with evidence from niche models, are identified as a species vulnerable to decline in the southern portion of the species range (Bernardo & Spotila, 2006; Bernardo *et al.*, 2007; Milanovich *et al.*, 2010). Without compensatory replacement by another salaman-

der or invertebrate, our results and those of Keitzer & Goforth (2013a,b) suggest that declines or local extinction of *D. quadramaculatus* could reduce the standing stocks of N and P in the salamander assemblage by up to 96% and alter seasonal N supply to other stream processes.

The southern Appalachian Mountains are a global hotspot for plethodontid salamander diversity, and our analysis helps illuminate the roles these organisms play in the nutrient cycles of headwater ecosystems. We have established that larval plethodontids are a significant standing stock and potentially influential in the recycling and supply of N and P, and probably of Ca. Our results differ from earlier estimates using less rigorous methods of estimating population density. We have established that variation in the composition of N and P within and among larval stages of plethodontid species is consistent with an increase in skeletal investment with increasing size, which is consistent with patterns for other vertebrates in other freshwater systems and allows for predicting how variation in salamander assemblage composition may affect nutrient dynamics. Nonetheless, our understanding of stream salamander effects on stream nutrient dynamics remains incomplete. We need better estimates of larval salamander production, and we do not yet have measures of terrestrial nutrient subsidies derived from salamander consumption of terrestrial invertebrates that are transferred to headwater streams via egg deposition. Although they do not account for large standing stocks of nutrients within headwater streams, species such as *D. ocoee* and *E. wilderae* that are numerous and move between the stream and surrounding forest may be particularly important in the transfer of terrestrial nutrients (Petranka & Murray, 2001). With evidence of declines associated with land-use change and projected declines associated with climate change, emerging knowledge on the influence of plethodontid salamanders on headwater stream communities and processes can inform management and conservation decisions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Mean, standard deviation, and range of values for percentages and molar elemental ratios C, N, P, and Ca for *D. ocoee* (*Do*; $n = 72$ across all streams), *D. quadramaculatus* (*Dq*; $n = 78$ across all streams) and *E. wilderae* (*Ew*; $n = 72$ across all streams), and macroinvertebrate prey items (Cross *et al.* 2003).

Table S2. Composite models depicting the AIC_c, Δ_i, w_i, and the percent of each model to the model with the highest w_i salamander data.

Table S3. Density estimates and estimates of standing stocks.

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