Salamander growth rates increase along an experimental stream phosphorus gradient

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Abstract. Nutrient-driven perturbations to the resource base of food webs are predicted to attenuate with trophic distance, so it is unclear whether higher-level consumers will generally respond to anthropogenic nutrient loading. Few studies have tested whether nutrient (specifically, nitrogen [N] and phosphorus [P]) enrichment of aquatic ecosystems propagates through multiple trophic levels to affect predators, or whether N vs. P is relatively more important in driving effects on food webs. We conducted two-year whole-stream N and P additions to five streams to generate gradients in N and P concentration and N:P ratio (target N:P = 2, 8, 16, 32, 128). Larval salamanders are vertebrate predators of primary and secondary macroinvertebrate consumers in many heterotrophic headwater streams in which the basal resources are detritus and associated microorganisms. We determined the effects of N and P on the growth rates of caged and free-roaming larval *Desmognathus quadramaculatus* and the average body size of larval *Eurycea wilderae*. Growth rates and average body size increased by up to 40% and 60%, respectively, with P concentration and were negatively related to N:P ratio. These findings were consistent across both species of salamanders using different methodologies (cage vs. free-roaming) and at different temporal scales (3 months vs. 2 yr). Nitrogen concentration was not significantly related to increased growth rate or body size of the salamander species tested. Our findings suggest that salamander growth responds to the relaxation of ecosystem-level P limitation and that moderate P enrichment can have relatively large effects on vertebrate predators in detritus-based food webs.

Key words: *bottom-up;* Desmognathus; *detritus; ecological stoichiometry;* Eurycea; *larval salamanders; limitation; nitrogen; nutrient enrichment; predator.*

INTRODUCTION

Effective ecosystem management requires understanding of how perturbations affect ecosystem structure and function (Palmer and Febria 2012), including how they propagate through food webs. Excessive nutrient inputs are a major source of impairment to streams and rivers due to nutrient-intensive land use (Woodward et al. 2012, USEPA 2013). Nutrient enrichment has negative consequences for most aquatic ecosystems, and its effects on autotrophic pathways (e.g., algal biomass) are well known (Smith and Schindler 2009). Increased nutrient availability has been shown to propagate through some algal-based food webs and affect vertebrate predators, such as arctic grayling (Slavik et al. 2004); however, nutrient enrichment may not consistently lead to significant growth and productivity responses at higher trophic levels (Borer et al. 2006).

Bottom-up effects of nutrient enrichment may be more likely to attenuate between adjacent trophic levels, compared to top-down effects such as predation (Brett

and Goldman 1997, Borer et al. 2006). Reduced resource diversity and palatability, as well as increased dominance of predator-resistant prey, contribute to such attenuation of bottom-up forces (Brett and Goldman 1997, Borer et al. 2006, Davis et al. 2010). Nonetheless, many secondary and tertiary consumers are resource limited and some exhibit responses to altered basal resource quality due to enrichment (Gratton and Denno 2003, Malzahn et al. 2007, Boersma et al. 2008). Understanding the ecosystem characteristics that promote bottom-up propagation of nutrient enrichment is important for identifying ecosystems that may be most sensitive to anthropogenic alterations of resource availability or quality.

Organismal growth, reproduction, and maintenance are often limited by a scarcity of either energy or elements in the environment (Frost et al. 2005). The balance of elements is of particular importance to animals, as they often face unbalanced diets with regards to nutrients, particularly nitrogen (N), and phosphorus (P) (Sterner and Elser 2002), as well as other dietary constituents (e.g., fatty acids, lipids; Brett and Muller-Navarra 1997, Wilder et al. 2013). Changes in nutrient availability can alter the imbalances that consumers face (Cross et al. 2007, Danger et al. 2013) and these effects

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are predicted to be greater for primary consumers than higher-order consumers due to the variation and plasticity in nutrient content of basal resources. Stoichiometric imbalances faced by secondary consumers are less likely to change in response to nutrient enrichment because prey (i.e., primary consumers) are more homeostatic in nutrient content than are basal resources (Sterner and Elser 2002). However, consumers can instead be energy (carbon) limited. Thus, effects of nutrient enrichment on predators can be a function of changes in the quantity and/or quality (e.g., nutrient content) of basal resources that lead to increased prey production and/or altered prey quality.

The extent to which higher-order consumers may be N or P limited depends on their elemental constraints, as well as those of their prey. Consumers with high N demand (i.e., high body N:P) may be limited by N over P; however, consumers with high body-P demand, such as fast-growing species and vertebrates with bony skeletons, may be more prone to P limitation (Sterner and Elser 2002, Benstead et al. 2014). Watershed land use often disproportionately alters nutrient delivery ratios in aquatic ecosystems (Downing and McCauley 1992), but it is not known to what extent different supply ratios of N vs. P propagate to higher-level consumers.

Here, we report results from two-year experimental enrichments in which we added N and P at different N:P ratios to five detritus-based headwater streams. As part of a large, integrated effort examining effects of enrichment at every trophic level, we measured the response of two common larval salamander species to the experimental N:P gradient. Larval salamanders are abundant predators in many temperate forest streams and may be the only vertebrates in low-order streams where fish are absent (Davic and Welsh 2004, Peterman et al. 2008). Larval salamanders can occur in high densities in streams, acting as important predators of aquatic macroinvertebrates and affecting the storage and cycling of nutrients (Davic 1983, Keitzer and Goforth 2013a, b, Milanovich et al. 2015). Thus, larval salamanders likely integrate the response to nutrient enrichment of lower trophic levels within stream food webs. Because increased prey production was observed in a previous study due to experimental N and P enrichment of a single stream near our study sites (Cross et al. 2006), we predicted that larval salamander predators would respond to nutrient enrichment. We also predicted that because their body N:P ratio is lower than that of their prey (Bumpers 2014, Milanovich et al. 2015), salamanders were likely more P than N limited, making it likely that they would benefit from increased P supply from prey via changes in physiological P storage or prey community composition and production.

Methods

Study site

We conducted this study in five adjacent, first-order streams at the United States Department of Agriculture Forest Service Coweeta Hydrologic Laboratory, a Long-Term Ecological Research site in Macon County, North Carolina, USA. Coweeta is a heavily forested 2185-ha basin in the Blue Ridge physiographic province of the southern Appalachian Mountains (Swank and Crossley 1988). Forests surrounding our study streams were dominated by oak, maple, and poplar, with a dense understory of *Rhododendron maximum* shading the streams year-round. The five streams used in this study were located in the 559-ha Dryman Fork watershed (35°01′44″ N, 83°27′04″ W). Streams were similar with respect to aspect (four streams had east-facing slopes, one northeast), elevation (~1160 m above sea level), pH (circumneutral), gradient (mean across five streams 17 cm/m; range 12–32 cm/m), and temperature (Table 1) and were near each other (<0.5 km). Ambient soluble reactive phosphorus (SRP) concentrations were very low (mean across five streams = 3 µg/L). Ambient dissolved inorganic nitrogen (DIN) concentrations were more variable but still relatively low (NO_3^--N mean, 74 µg/L; range, 10–179 µg/L; NH_4^+-N mean, 8 µg/L; range, 7–9 µg/L), which resulted in a range in mean ambient dissolved N:P of 15:1 to 138:1.

Experimental enrichment

We performed a two-year (July 2011–July 2013; YR1, YR2) continuous nutrient addition experiment in our five study streams following one year of pre-treatment sampling (June 2010–June 2011, PRE). Beginning in July 2011, N (21% aqueous NH_4NO_3) and P (85% H_3PO_4) were continuously added to the experimental streams. Streamwater pH was unaffected by our treatments (A. D. Rosemond, *unpublished data*). Each of the five streams received different added concentrations of N and P to create a crossed gradient in streamwater nutrient concentrations and molar N:P (Table 1, Appendix A: Table A1; hereafter streams will be referenced by their target N:P ratio: 2:1, 8:1, 16:1, 32:1, 128:1). As a result, the highest N concentrations were coupled with the lowest P concentrations and vice versa. The added N and P concentrations mimicked low-to-moderate nutrient concentrations associated with land-use change in the southern Appalachians (Scott et al. 2002) and elsewhere. Using solar-powered metering pumps, (LME Milton Roy, Ivyland, Pennsylvania, USA) nutrients were mixed with ambient stream water in gravity-fed irrigation lines at flow-proportional rates based on instantaneous discharge using a CR800 datalogger (Campbell Scientific, Logan, Utah, USA) connected to a Nanolevel pressure transducer (Keller America, Newport News, Virginia, USA). The irrigation lines ran the length of each 70-m experimental reach and the nutrient solution dripped through valves approximately every 5 m to ensure a well-mixed treatment (see Appendix B: Fig. B1a for a photograph of the treatment apparatus). Water samples were taken every two weeks at three to five points along the 70-m reaches to confirm consistent nutrient concentrations throughout the treat-

TABLE 1. Measured streamwater dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) concentrations in the five treatment streams located in Dryman Fork watershed, Coweeta Hydrologic Laboratory, North Carolina, USA, determined from water samples taken once every two weeks.

Parameter	Stream				
	2:1	8:1	16:1	32:1	128:1
DIN (µg/L)					
Target	81.3	243.9	365.8	487.7	650.3
PRE	18 ± 1.5	112 ± 17.3	37 ± 5.7	189 ± 14.4	57 ± 7.8
YR1	100 ± 9.1	251 ± 14.3	382 ± 35.4	511 ± 37.3	364 ± 29.2
YR2	66 ± 6.2	145 ± 10.4	277 ± 32.9	216 ± 11.5	254 ± 25.3
SRP (µg/L)					
Target	90.0	67.5	50.6	33.8	11.3
PRE	3 ± 0.2	3 ± 0.2	3 ± 0.5	3 ± 0.3	3 ± 0.2
YR1	42 ± 3.1	78 ± 5.3	41 ± 2.6	30 ± 2.1	8 ± 0.6
YR2	53 ± 5.2	32 ± 3.9	30 ± 3.1	14 ± 1.1	6 ± 0.5
N:P					
Target	2.0	8.0	16.0	31.9	127.4
PRE	15 ± 1.8	95 ± 16.3	30 ± 4.5	138 ± 10.8	49 ± 7.1
YR1	8 ± 1.1	19 ± 3.5	26 ± 3.3	54 ± 7.5	160 ± 15.1
YR2	6 ± 1.5	37 ± 8.7	24 ± 3.5	53 ± 7.9	113 ± 13.5
T (°C)					
PRE	11 ± 0.29	10 ± 0.20	10 ± 0.26	10 ± 0.26	10 ± 0.29
YR1	11 ± 0.23	11 ± 0.16	11 ± 0.20	11 ± 0.20	11 ± 0.23
YR2	11 ± 0.22	10 ± 0.15	10 ± 0.21	10 ± 0.19	10 ± 0.23

Notes: Target is the targeted concentration in each treatment. Nutrient concentrations for PRE are the measured ambient stream concentrations in the year prior to enrichment. Year 1 (YR1) and year 2 (YR2) are the measured streamwater concentrations during the experimental enrichment (July 2011–July 2013). Also reported is the annual stream temperature (T) for each stream and year. All values are means ± SE.

ment reaches and from one point upstream of the nutrient release sites to monitor ambient concentrations. Water samples were filtered using a 0.45-µm nitrocellulose membrane filter (Millipore, Billerica, Massachusetts, USA) for NO_3^--N, NH_4^+-N, and SRP, and frozen until analysis with an Alpkem Rapid Flow Autoanalyzer 300 (DIN; Alpkem, College Station, Texas, USA) at the University of Georgia Analytical Chemistry Laboratory (Athens, Georgia, USA), or spectrophotometrically for SRP using the ascorbic acid method (APHA 1998; Shimadzu UV-1700, Tokyo, Japan).

Focal species

Eurycea wilderae Dunn (Ew) and *Desmognathus quadramaculatus* Holbrook (Dq) dominate abundance and biomass, respectively, of salamander assemblages in streams in the study area (Peterman et al. 2008, Milanovich et al. 2015). *Eurycea wilderae* has a larval period of approximately 12 months and metamorphoses at 18–24 mm snout–vent length (SVL; Bruce 1988). In contrast, Dq has a larval stage of 36–48 months and metamorphoses at approximately 40–45 mm SVL (Bruce et al. 2002). Both salamanders are secondary to tertiary consumers of aquatic invertebrates, with Dq able to consume larger predatory macroinvertebrates (Davic 1991, Johnson and Wallace 2005). Due to their investment in bone, larvae are moderately P rich (~1.5–3.5% body P; Bumpers 2014, Milanovich et al. 2015),

comparable in percent P to that reported for many fish species (Frost et al. 2006, Benstead et al. 2014).

Estimating growth rates of salamanders in response to experimental nutrient enrichment

Three separate methods were used to estimate growth responses of Dq and Ew to experimental nutrient additions. We used enclosures and capture–mark–recapture (CMR) of free-roaming individuals to estimate growth of Dq. We used a modified CMR method for Ew because Ew was too small to use in enclosure studies or to mark for a CMR study. However, all larval Ew present in a stream at a given time are members of a single annual cohort. Therefore, we could compare mean SVL among streams at two time points as a proxy for growth rates.

Enclosure studies: Dq

Growth rates of Dq were determined from enclosure studies in the spring (March–June, Spring 2012) and fall of 2012 (July–October, Fall 2012), and during the spring of 2013 (April–July, Spring 2013). For each of the three enclosure studies, plastic-framed mesh enclosures (~0.52 m²) were placed in streams such that the bottom of the enclosures were submerged and lined with cobble and detritus from the streambed (Appendix B: Fig. B1b). Mesh size (~1.5 mm) was small enough to prevent larvae from escaping but allowed water and macroinvertebrates in the size classes consumed by salamanders

to pass freely. Two enclosures were placed in each of the five treatment streams and two or four reference enclosures were used in each study period ($n = 10$ treatment and 2 reference enclosures Spring 2012, Fall 2012; $n = 10$ treatment and 4 reference enclosures Spring 2013).

In each of the three enclosure studies, seven young-of-the-year larval Dq were hand-captured, measured to the nearest mm (SVL), weighed, uniquely marked with a visual implant elastomer tag (VIE, Northwest Marine Technologies, Shaw Island, Washington, USA), and released in each enclosure. This resulted in initial salamander density in the enclosures of 13 individuals/ m², which is within the range of naturally occurring densities for Dq in nearby streams (Keitzer and Goforth 2013a, Milanovich et al. 2015). Approximately three months after each stocking, surviving larvae were measured and weighed again to obtain growth rates.

Placement and number of reference enclosures was different between the enclosure studies. Two enclosures were placed upstream of the nutrient release point in ambient conditions in stream 32:1 in both Spring 2012 and Fall 2012 ($n = 2$ reference enclosures per study). In Spring 2013, two ambient enclosures were placed above the nutrient release points in both streams 32:1 and 8:1 ($n = 4$ reference enclosures). Reference enclosures were not placed upstream of the treatments in every stream for logistical reasons; however, because all streams were physically and chemically similar, the reference enclosures in the 32:1 and 8:1 streams likely represented ambient conditions in all study streams.

CMR study: Dq

Growth of free-roaming Dq individuals was determined using CMR beginning in July 2011 and ending in July 2013. Salamanders were sampled using litter bags deployed in the streams (Nowakowski and Maerz 2009, Cecala 2012). Bags (40×20 cm made with 2.25-cm² plastic netting) were filled with leaf litter from the riparian area and placed approximately every five meters in the wetted portion of the 70-m treatment reaches with a large rock on top to prevent dislodgement ($n = 15$ sample locations per stream, 2 bags per location). Litterbags were deployed at least 48 h prior to each sampling. We sampled salamanders on 11 occasions within the active growing season (generally March–October) at approximately monthly intervals.

To collect salamanders from each litterbag, we quickly lifted the bag into a fine-mesh dip net (~1 mm) and transferred it to a bucket of water. We agitated the bag in the bucket and then redeployed the bag in the stream. Contents of the bucket were filtered through the dip net, and any captured Dq individuals were weighed, measured (SVL), and uniquely marked with a VIE tag. We released salamanders on the upstream side of the bags to prevent downstream drift. Salamanders moved freely in and out of the litterbags between sampling times.

Modified CMR study: Ew

Eurycea wilderae larvae were sampled using a modified capture technique described for the Dq CMR study. When larvae were captured from litterbags they were measured (SVL) and weighed as for Dq and then released. Ew larvae were not marked with a VIE tag when captured because they were too small and sensitive to mark during CMR. Because Ew larvae are present in streams as single annual cohorts we were able to compare mean changes in SVL among streams at two time points. Free-roaming Ew larvae were captured in September 2010 (PRE) and July 2011 and 2012 (YR1, YR2) just after larvae hatched in the streams. A second capture of individuals from the same cohort was made the following May during each year of the study (2011 [PRE], 2012 [YR1], 2013 [YR2]), approximately one month prior to metamorphosis.

Estimation of growth rates

Growth rates for Dq were estimated from changes in SVL between initial capture and subsequent recaptures for both the enclosure and CMR larvae. To estimate changes in mass, SVL was converted to dry mass (DM) using a length-mass regression derived for Dq in Coweeta streams (J. R. Milanovich and J. C. Maerz, *unpublished data*):

$$M = 0.0014L^{3.26}$$

($P < 0.001$, $R^2 = 0.84$, $n = 79$) where M is larval DM (mg) and L is SVL (mm). Individual daily growth rate (g) was then calculated using the following equation (Johnson et al. 2006):

$$g = (\ln W_{fn} - \ln W_{in})/t$$

where W_{in} is the initial DM (mg) and W_{fn} is the final DM (mg) of a salamander, and t is time interval (d).

Growth rate of body length was inversely related to initial length of an individual, with smaller individuals typically having higher growth rates than larger individuals (enclosure larvae, this study $F_{1,192} = 167.70$, $P < 0.001$, $R^2 = 0.46$, $n = 194$; CMR larvae, this study $F_{1,96} = 50.84$, $P < 0.001$, $R^2 = 0.35$, $n = 98$). Because of this, we standardized growth rates with respect to initial SVL (SVL_i) by regressing the relationship of SVL_i against growth rate for larvae used in the enclosure studies (both ambient and treatment individuals together) and from the CMR studies. The following equations were then used to determine predicted growth rate (PGR) for a given SVL_i for enclosure and free-roaming (CMR) individuals, respectively

$$PGR = -0.0142 \ln(SVL_i) + 0.0474$$

$$PGR = -0.0049 \ln(SVL_i) + 0.0189.$$

The SVL_i-adjusted growth rate (hereafter referred to as residual growth or growth rate) was calculated by subtracting the predicted growth rate (PGR) from the

observed growth rate (g); positive residual growth rate values indicate a higher growth rate than that predicted by initial size. The initial sizes of larvae placed in the enclosures were not different among streams within each study season (ANOVA, $F_{1, 191} = 0.33$, $P = 0.57$), nor were the initial sizes of free-roaming CMR individuals across streams ($F_{1, 100} = 0.71$, $P = 0.40$). Therefore, the use of residual growth in subsequent statistical tests was not biased by initial sizes of larvae (Freckleton 2002) and allowed for the comparison of deviation from expected growth among treatments. We limited the calculation of growth rates for CMR individuals to those recaptured a minimum of two weeks apart to minimize the influence of imprecision of body size measurements on growth rate estimates (median recapture interval was over 90 d; see *Results*). Additionally, we used ANOVA to determine if there were differences in CMR recapture intervals among streams in the CMR study; the time intervals over which growth rates were calculated were not different among streams ($F_{1, 100} = 0.93$, $P = 0.34$).

Because Ew larvae were too small and sensitive to mark with a VIE tag we could not estimate growth rates using the same methods as for Dq. Therefore, we compared mean SVL among streams at the two time points to approximate differences in growth. Snout-vent length was averaged for individuals from each stream just after hatching from the July (September for PRE) capture event and just before metamorphosis from the May capture event (hereafter, May SVL) to approximate changes in average body size.

Statistical analyses

Enclosure and CMR growth rate of Dq and Ew body size data were tested for normality with a Shapiro-Wilks test and exhibited a normal distribution (all P values >0.5). Prior to statistical analyses we determined if final densities within enclosures differed among streams, which could influence density-dependent effects on growth rates, by comparing the number of surviving larvae among streams within a respective season using ANOVA. All statistical analyses were performed in R statistical software version 3.0.3 (R Development Core Team 2014).

We used linear regression and model selection to test for the effects of N and P concentrations and N:P ratio on salamander growth rates and body size. Using R package AIC$_C$modavg (Mazerolle 2013), Akaike's Information Criterion corrected for small sample size (AICc) was used to determine the most parsimonious models for growth or body size (Burnham and Anderson 2002). We developed specific models to test whether individual nutrient concentrations (N or P) or N:P ratio were better predictors of growth rate and body size. In model selection we used mean nutrient concentrations and ratios that corresponded to the timing of each salamander growth study. We included two measures of concentrations and ratios: N or P nutrient concentrations that were experimentally added ("added," based on our recorded mass of nutrients added plus ambient concentrations) and nutrient concentrations that were measured in the streams ("measured," which were lower than added because of stream uptake; Tables 1 and A1). Added nutrient concentrations were included in our models because they better characterized our nutrient treatments due to concentration-dependent uptake in the treatment streams (A. D. Rosemond, *unpublished data*). We included measured concentrations as candidate predictor variables in models so that our results could be compared to other studies in which measured stream-water concentrations are typically used. Nutrient ratios were ln-transformed to meet the assumptions of a linear model.

Using mean annual (which was specific to each time period) concentrations and ratio (measured and added; N, P, N:P individually) we developed seven hypothesized models to explain Dq enclosure growth (Appendix A: Table A2). All three time periods (Spring 2012, Fall 2012, Spring 2013) were analyzed together; growth rates were averaged within a stream for a given time period and compared across streams. Because growth was different between seasons (spring vs. fall), season was included as a covariate in each nutrient or ratio model and was also included as a single predictor variable in one model (i.e., a model included only season as a predictor variable). In the case of growth estimates from the reference enclosures placed in un-enriched stream conditions we used the ambient measured nutrient concentrations to represent both the measured and added concentrations in our models because there were no added concentrations. Therefore, in models that included the added treatment concentrations, ambient concentrations measured upstream of our treatments were paired with the reference growth estimates.

CMR models were analyzed using stream-averaged residual growth rate for the two-year treatment period. Again, both added and measured concentrations were used to build models but in this case we used the two-year average nutrient concentration or ratio. We tested six hypothesized models (N, P, and N:P ratio using measured and added values) to explain Dq CMR growth (Appendix A: Table A3).

We used ANOVA to test if pre-enrichment May SVL was different among streams. We tested for the effects of nutrient enrichment on treatment May SVL using the model selection approach described above; mean annual concentrations and ratios were used to build models using either measured or added concentrations (N, P, N:P ratio). We included year as a covariate in models with nutrients or ratio, and one model included only year; thus seven total models were evaluated (Appendix A: Table A4).

Effectiveness of enrichment

The nutrient addition treatments successfully created gradients of both streamwater N and P concentrations and ratios (Table 1, Appendix A: Table A1). Measured streamwater concentrations and ratios varied by year, but were generally reflective of targets (Table 1). Measured SRP concentrations were elevated 2.5–31× and DIN concentrations were elevated 3–10× above background (Table 1). Measured streamwater N:P ratios were always higher than added ratios (the ratio of the solution dripped into the stream), indicating preferential uptake or sorption of P, even in the lowest N:P stream. Throughout the study, water temperatures ranged from 1°C to 19.5°C (annual mean ~10.5°C, Table 1) and were similar among streams. Annual discharge (mean ± SE) was 11.2 ± 2.2 L/s, 5.8 ± 1.6 L/s, and 6.9 ± 1.4 L/s for PRE, YR1, and YR2, respectively, across all streams. Annual mean discharge among streams also varied each year, ranging from 5.1–16.8 L/s, 2.7–12.2 L/s, and 4.9–12.5 L/s for PRE, YR1, and YR2, respectively.

Dq growth in enclosures

Growth was determined from 196 larval Dq from the three enclosure studies (Appendix A: Table A5). We recovered 75 (89%), 50 (60%), and 71 (72%) larvae for Spring 2012, Fall 2012, and Spring 2013, respectively (Appendix A: Table A5). The number of surviving larvae was not different across streams within a respective season ($F_{6,12} = 0.59$, $P = 0.77$), indicating that final densities in the enclosures did not bias growth estimates. The top AIC model included added P concentration (Fig. 1a, Appendix A: Table A2; $F_{2,16} = 15.26$, $P < 0.001$ adjusted $R^2 = 0.61$) and was followed by measured N:P ratio ($F_{2,16} = 15.23$, $P < 0.001$ adjusted $R^2 = 0.61$) and added N:P ratio (Fig. 1b, $F_{2,16} = 13.37$, $P < 0.001$ adjusted $R^2 = 0.58$), and measured P concentration ($F_{2,16} = 12.29$, $P < 0.001$ adjusted $R^2 = 0.56$; Appendix A: Table A2). Average growth rates were always higher in the enrichment compared to the ambient enclosures for a given time period (Fig. 1). Growth rates were positively correlated with P concentration and negatively correlated with N:P ratio (Fig. 1b). Growth rates in the highest P treatment (2:1) represented a ~30% and ~40% increase over ambient growth in spring and fall, respectively. Nitrogen concentration was not an important predictor in enclosure residual growth and was not included in the 95% confidence set of models (i.e., models within 95% of cumulative AIC$_c$ weight; Fig. 1c, Appendix A: Table A2). Season was a significant predictor in all models tested (Appendix A: Table A2). Among all streams, growth rates were higher in spring seasons compared to those measured in the fall. Differences in growth among treatments were most pronounced in the fall (Fig. 1).

Dq growth in CMR study

Over the two-year enrichment period, growth rates were calculated on 102 free-roaming Dq individuals across all five streams (Appendix A: Table A5). Intervals between capture and recaptures varied from 19 days to 425 days (mean ± SE = 153 ± 12 d; median = 95 d). The top model for CMR growth included measured streamwater P concentration, which was significantly correlated with growth (Fig. 2a, Appendix A: Table A3; $F_{1,3} = 12.44$, $P = 0.04$, adjusted $R^2 = 0.74$). Growth was also positively correlated with added P concentration, but was not statistically significant (Fig. 2b, $F_{1,3} = 0.60$, $P = 0.5$, $R^2 = 0.17$). Growth rates were not significantly correlated with any other variables (Appendix A: Table A3).

Ew difference in body size in modified CMR study

We captured 482 free-roaming Ew individuals across all years and streams (Appendix A: Table A6). In all three years (PRE, YR1, YR2), there was no difference in average hatchling (July/September) SVL among streams ($F_{4,10} = 0.13$, $P = 0.97$). May (near metamorphic) SVL was not different across the five streams during our pre-treatment year ($F_{4,55} = 0.78$, $P = 0.54$). The top models (based on AIC) for explaining variation in mean May SVL (metamorphic body size) among streams during our two enrichment years were all related to P enrichment (added N:P, measured N:P, or added P) and included year as a factor. The three top models made up 99% of the cumulative weight in the suite of models analyzed (Appendix A: Table A4). Average May (metamorph) SVL was negatively and significantly correlated with added N:P ratio by year (Fig. 2c, $F_{2,7} = 31.69$, $P < 0.001$, adjusted $R^2 = 0.87$) and measured N:P ratio by year (Fig. 2b, $F_{2,7} = 29.05$, $P < 0.001$, adjusted $R^2 = 0.86$). Added P concentration was positively and significantly correlated with Ew May SVL by year (Appendix A: Table A4, Fig. A1a; $F_{2,7} = 26.81$, $P < 0.001$, adjusted $R^2 = 0.85$); measured P concentration was also positively and significantly (Fig. A1b, $F_{2,7} = 6.99$, $P = 0.02$, adjusted $R^2 = 0.57$) related to May SVL but did not receive any AIC weight (Appendix A: Table A4). Added N concentration was negatively correlated with body size across years (Appendix A: Table A4). Because we had low sample size ($n = 2$, Appendix A: Table A6) for two streams we also analyzed the data aggregated across years. The relationship between May SVL and added N:P ratio became slightly weaker ($F_{1,3} = 15.66$, $P = 0.02$, adjusted $R^2 = 0.79$), while the correlation with added P concentration became stronger ($F_{1,3} = 48.9$, $P = 0.006$, adjusted $R^2 = 0.92$, data not shown). Among streams and both years, mean May (metamorph) SVL of Ew increased by 1.29× compared to the pre-enrichment year (range 1.08–1.45×). Mean May SVL increased the most (1.66×) in YR2 in stream 2:1 compared to the pre-treatment year.
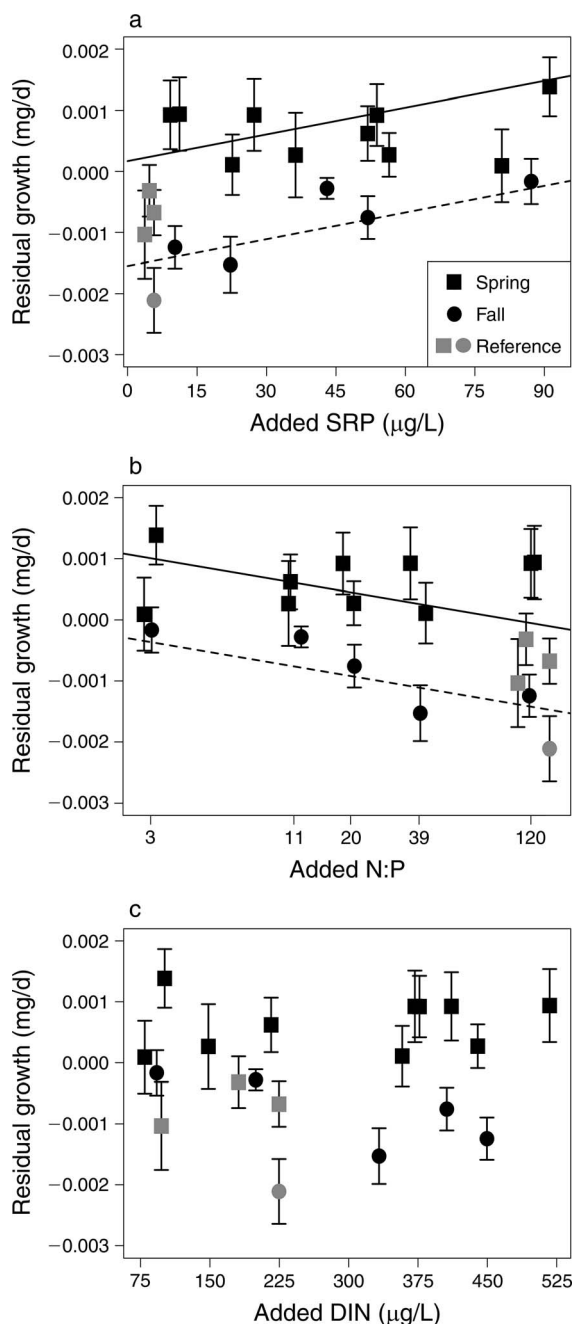
FIG. 1. Initial size-adjusted growth rates (residual growth) for *Desmognathus quadramaculatus* larvae in enclosures from five streams enriched with dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) correlated with (a) added SRP concentration, (b) added N:P ratio, and (c) added DIN concentration at Coweeta Hydrologic Laboratory, North Carolina, USA. Added concentrations represent the ambient stream concentration plus the concentration added to streams to achieve experimental target concentrations. Because reference enclosures did not receive added nutrients, they are plotted using measured nutrient concentrations. Salamander larvae were left in enclosures for approximately 90 days. Regression lines are separated by season (combined spring, solid lines; fall, dashed lines) and indicate a significant overall effect and are derived from models of season and P or N:P, respectively.

DISCUSSION

We found that larval salamander growth in headwater streams was limited by P and responsive to the relaxation of ecosystem-level P limitation. Here, largely single-nutrient effects propagated up food webs from dissolved nutrients through detrital basal resources and macroinvertebrate consumers to two vertebrate predators. Increased predator growth likely occurred through a combination of relaxation of consumer–food imbalances and increased prey production. In our study system, this effect on predator growth was propagated via three to four trophic levels, involving the responses of heterotrophic microorganisms associated with detrital resources and the intermediate macroinvertebrate consumers that constitute salamander prey (Davic 1991, Bumpers 2014).

Phosphorus drives increased growth rates of salamanders

Our results revealed strong responses in vertebrate predator growth to our experimental gradient in P, and no response to N. Though we cannot solely attribute the salamander growth response to P addition alone, because some N was added to all streams, the significant relationships between P and growth, even at low measured DIN, attest to the overriding importance of P in determining salamander responses. The apparent negative relationships between salamander growth and body size and both N:P ratio and N concentration reflected the nature of our experimental design: N and P were inversely related. Therefore, the most parsimonious explanation for N:P ratio being a good predictor of growth is that N:P was driven by the experimental gradient in P concentration. Moreover in all three study components, growth was positively correlated with both measured and added P concentration. Though the importance of different measures of SRP ("added" vs. "measured") differed slightly among analyses of different study components, this was likely an artifact of imprecision of salamander growth and measured SRP and not likely related to process. Generally, all responses were concordant. Our results suggest that prior research demonstrating positive salamander growth in response to dual N and P enrichment (at 16:1 molar N:P ratio; Johnson et al. 2006) was likely due to ecosystem-level responses to both nutrients, but may have been primarily driven by relaxation of ecosystem-level P limitation. In our experiments, if N had also released the food web from limitation similarly to P, we would have seen

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There are two spring time periods (2012 and 2013) and one fall season (2012). Reference enclosures were placed upstream of the treatment reach in stream 32:1 for Spring 2012 and Fall 2012 and in stream 32:1 and 8:1 in Spring 2013. Residual growth rates are means per stream per season. Error bars are ±SE and represent variation within a given stream for that season. Note: N:P ratios were ln-transformed for analysis, but are shown here on a natural-log axis labeled with actual N:P ratio values.
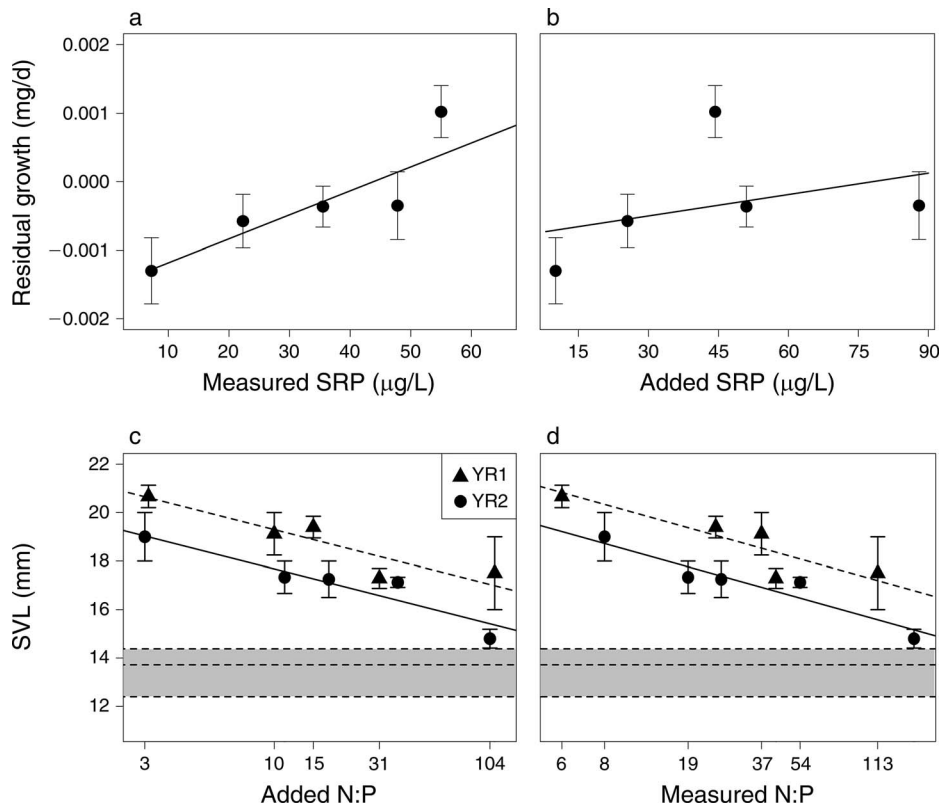
FIG. 2. Growth and body size (mean ± SE) of (a, b) free-roaming *Desmognathus quadramaculatus* (Dq) and (c, d) *Eurycea wilderae* (Ew) in response to experimental nutrient enrichment of five streams at Coweeta Hydrologic Laboratory, North Carolina, USA. Average body-size-adjusted growth rates (residual growth) for free-roaming Dq larvae were best explained by measured streamwater soluble reactive phosphorus (SRP) concentration, with stream 8:1 having the highest residual growth (a). Added SRP was also positively correlated with residual growth (b). Values are the mean from each stream determined from recaptures over two years of enrichment. Average snout–vent length (SVL) of Ew for YR1 (dashed line) and YR2 (solid line) of enrichment was best explained by added (c) or measured (d) N:P ratio (see Fig. 1 legend). Values are the mean body size of free-roaming larvae captured in May of their respective treatment years just before metamorphosis. Body sizes were different among years (i.e., a year effect) and the regression lines are derived from the parameter estimates for each model (see Appendix A: Table A4). The gray bar represents the range in body size (SVL) measured in May, pre-treatment, just before metamorphosing. The dotted line within the shaded bar is the average pre-treatment SVL across all five streams.

approximately equal responses of salamander growth across our treatments. Thus, in streams that have low ambient nutrient concentrations (particularly P), enrichment of N alone may not stimulate productivity of higher-level consumers. For example, nutrient effects on basal resources in streams receiving agricultural or urban runoff, which often have elevated concentrations of both N and P, may propagate to higher consumers more than in streams receiving only enrichment of N (e.g., as occurs with atmospheric N deposition in high-elevation or island ecosystems; Vitousek et al. 1997). Therefore, variation in the delivery of N vs. P may have important consequences for the response of stream food webs, particularly at upper trophic levels.

Potential mechanisms of nutrient effects on salamander growth

The effects of nutrients likely propagated to salamanders through stimulated salamander prey production or increased prey quality mediated by enhanced detrital nutrient content. Salamander growth rates have been correlated with prey biomass (Johnson and Wallace 2005, Huntsman et al. 2011). In our study, it is likely that prey production increased due to enrichment (Cross et al. 2006). Preliminary results indicate that production of some important prey taxa for *D. quadramaculatus* increased in all streams during both years of enrichment (e.g., *Tallaperla*, *Leuctra*; L. M. Demi and J. P. Benstead, *unpublished data*). Additionally, biomass of *Chironomidae*, which are consistently the most important prey resource reported for larval *E. wilderae* and their congeners (Johnson and Wallace 2005, Barrett et al. 2012) increased during at least the first year of enrichment (L. M. Demi and J. P. Benstead, *unpublished data*). However, we note that in earlier studies macroinvertebrate predator production decoupled from macroinvertebrate prey production in response to nutrient enrichment due to increased dominance of large-bodied

primary consumers that were largely invulnerable to predation (Davis et al. 2010). This finding is relevant because larval salamanders are gape-limited predators. That we saw a relatively strong growth response suggests that either dominant prey did not outgrow salamander gape limitation or increases in the number of alternative prey or quality of prey offset any gape limitation.

Salamander responses to P may also have been due to lowering of elemental constraints on individual growth (i.e., reduced nutrient limitation). Threshold elemental ratios (TER), the ratio at which limitation shifts between one element and another, provide one approach to predicting how consumers respond to stoichiometric changes in their prey (Frost et al. 2006). Both salamander taxa in our study have body C:P ratios of ~50–60 (Bumpers 2014, Milanovich et al. 2015). Multiplying body C:P by 2.4 (the average factor for determination of organismal $TER_{C:P}$), equates to a $TER_{C:P}$ of ~120–144 (Frost et al. 2006). This $TER_{C:P}$ is low relative to prey C:P measured from nearby streams (~200–800; Cross et al. 2003), suggesting that if prey quantity is not limiting then larval salamanders are limited by P. Experimental enrichment has been shown to alter the nutrient content of primary consumer macroinvertebrates, likely via microbially enhanced nutrient content of detrital food resources (Cross et al. 2003). Additionally, several laboratory studies show that release from elemental limitation can travel up the food web to increase larval fish condition (Malzahn et al. 2007, Boersma et al. 2008, Dickman et al. 2008). Future analyses examining TERs and the potential role of shifts in prey stoichiometry (either via physiological modification or changes in community structure) may reveal mechanisms by which stoichiometric imbalances contribute to increased growth rates in response to nutrient enrichment.

Nutrient enrichment of detritus- vs. algal-based ecosystems

Our research indicates that predators in detritus- and autotrophic-based systems respond similarly to nutrient enrichment when resources remain palatable, despite fundamentally different responses of the respective primary basal resource (i.e., decreased detrital standing stocks vs. increased algal biomass). Because algal production is primarily light-limited in forest headwater streams, nutrient enrichment leads to a net depletion of basal resources (Rosemond et al. 2015). The availability of detrital resources has been strongly linked to secondary production of aquatic invertebrates (Wallace et al. 1997, Walther and Whiles 2011) and larval salamanders (Huntsman et al. 2011). Therefore, long-term enrichment may have net negative effects on salamander prey production via resource limitation, despite enhanced resource quality, since forested streams will experience little increase in autotrophic C relative to open-canopy streams (Greenwood and Rosemond 2005). Thus, we caution extrapolating the relatively

short-term (i.e., three months to two years) growth responses found here to effects of chronic nutrient loading.

To our knowledge, our study is the first to manipulate N and P availability separately in multiple natural streams and measure the effects on vertebrate predators. Despite bottom-up effects generally having limited propagation to higher-level consumers in many systems, we saw significant and relatively rapid responses in vertebrate predator growth to nutrient enrichment. Our highest P concentrations led to 40% increases in growth rate (Dq) and 66% increases in body size at metamorphosis (Ew). These effects occurred at low to moderate concentrations of N and P relative to those that are observed across landscapes in the U.S. and Europe (Woodward et al. 2012, USEPA 2013). Thus, similarly significant shifts in life history characteristics of animals and food web structure may be widespread. This study provides evidence that nutrient perturbations of stream food webs can affect higher trophic levels (Slavik et al. 2004), and suggests that P may be more important than N in regulating vertebrate predator responses.

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SUPPLEMENTAL MATERIAL

Ecological Archives

Appendices A and B are available online: http://dx.doi.org/10.1890/14-1772.1.sm