



Experimental nutrient enrichment of forest streams increases energy flow to predators along greener food-web pathways

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Funding information

NSF, Grant/Award Number: DEB 09818894, DEB 0918904, DEB 0823293

Abstract

1. Nutrient enrichment is a key stressor of lakes and streams globally, affecting the relative availability of important basal resources such as algae and detritus. These effects are controlled by responses of autotrophic and heterotrophic microorganisms that subsequently affect primary consumers and higher level predators. Despite the potential for propagation of these bottom-up effects, few studies have examined how nutrients affect “green” (autotrophic) versus “brown” (heterotrophic) energy pathways to predators via changes in the quantity or type of prey consumed.
2. We studied the pathways by which nutrient enrichment affected two predatory salamander species (*Desmognathus quadramaculatus* and *Eurycea wilderae*) using detailed diet analyses before and during 2-year nutrient additions to five head-water forest streams. The streams were continuously enriched with different concentrations of dissolved nitrogen (N) and phosphorus (P), creating relatively greater N or P concentrations and distinct N:P ratios (2:1, 8:1, 16:1, 32:1 and 128:1) in each stream.
3. Nutrient addition resulted in greater prey number, size and biomass consumed by *D. quadramaculatus*, an effect driven more by P than by N additions. Some of these effects were greater in the second year of enrichment and were greater for larger individuals. Shifts in the prey composition of *D. quadramaculatus* included increases in algivores and decreases in detritivores, tracking observed treatment effects on basal resource quantity (e.g. algivore abundance in guts was related to algal biomass, which increased with enrichment, and detritivore abundance in guts was related to detrital standing stocks, which declined with enrichment). For *E. wilderae* diets, there was limited evidence for increased prey size and number, or for alteration of prey composition with enrichment despite evidence of increased larval growth. We hypothesise that body size differences between the two salamander species partially explain their different dietary responses to enrichment.
4. Our results show that nutrient addition, primarily of P, affected the quantity and composition of predator diets in our nutrient-poor streams. These effects on diet were consistent with concurrent studies showing that P enrichment resulted in faster growth of salamanders and occurred partly via effects on algal biofilm or “green” food-web pathways, despite the dominance of detrital or “brown” resources in our heavily shaded forest stream sites. Thus, nutrient enrichment

can promote algae- versus detritus-based energy-flow pathways in nominally light-limited stream ecosystems, with associated changes in food-web characteristics and function.

KEYWORDS

Desmognathus quadramaculatus, *Eurycea wilderae*, food webs, nutrients, salamanders

1 | INTRODUCTION

Human-mediated increases of nitrogen (N) and phosphorus (P) affect the majority of freshwater ecosystems (Peñuelas et al., 2013; USEPA, 2013). It is now generally established that both N and P alter basal energy resources in streams, stimulating “green” pathways via algal production (Elser et al., 2007) but driving loss of “brown” food-web pathways by accelerating the decomposition and mineralisation of detritus due to enhanced microbial respiration and consumer feeding (Ferreira et al., 2015; Rosemond et al., 2015). Additionally, resource quality, defined here as resource nutrient content (i.e. N and P versus carbon [C] content), is increased by streamwater nutrient availability in both algae-based and detritus-based ecosystems or pathways (Scott et al., 2013; Slavik et al., 2004; Stelzer & Lamberti, 2002; Tant, Rosemond, & First, 2013) (which hereafter are referred to as green and brown pathways). Thus, under nutrient-enriched conditions, consumers are faced with algal resources that typically are higher in nutrient content and quantity (Slavik et al., 2004; Stelzer & Lamberti, 2002) and/or detrital resources that are higher in nutrient content, but lower in quantity (Cross, Wallace, Rosemond, & Eggert, 2006; Danger, Funck, Devin, Heberle, & Felten, 2013; Fuller, Evans-White, & Entrekin, 2015; Kendrick & Benstead, 2013). Growth and production of primary consumers can increase in response to such nutrient-driven shifts in basal resource quality, if resource quantity is sufficient and resources remain edible (Davis, Rosemond, Eggert, Cross, & Wallace, 2010).

Predators may face a similar set of relaxed constraints in nutrient-enriched systems. Predator density and growth may increase if nutrient enrichment leads to increased production of prey that remain edible. Prey quality (e.g. nutrient content) may also change due to nutrient enrichment as a function of altered prey composition or predator selectivity. Increased prey quantity (production or biomass) can lead to increased predator densities and growth rates in terrestrial and aquatic ecosystems with both green and brown resource bases (Chen & Wise, 1999). Changes in the composition of prey assemblages may also have effects on predators through nutritional changes of their diet. However, studies showing that density and growth of predators increase with nutrient enrichment have typically only speculated about specific mechanisms driving the observed responses (Johnston, Perrin, Slaney, & Ward, 1990; Peterson et al., 1993; Johnson, Wallace, Rosemond, & Cross, 2006; but see Collins, Baxter, Marcarelli, & Wipfli, 2016).

As part of a larger study, we found that growth rates of the larvae of two salamander species (*Desmognathus quadramaculatus* and *Eurycea wilderae*) increased up to 40% and 60%, respectively, during

2 years of N and P fertilisation of five detritus-based headwater streams. In that study, we were able to show that streamwater P concentration best explained growth responses of both species (Bumpers, Maerz, Rosemond, & Benstead, 2015). Our goal in this study was to identify the trophic pathways that led to the predator response we observed in order to understand more fully how enrichment affects energy flow and food-web structure in streams.

To achieve this goal, we analysed diets from more than 750 larval salamanders in response to experimental enrichment of whole-stream reaches to determine how prey quantity and composition of larval diets changed in response to nutrient enrichment. We used shifts in patterns of the quantity or type of prey consumed to infer the underlying mechanisms supporting the growth response we observed in our previous study. Two main diet responses could elucidate a growth response. First, we determined whether there was increased prey quantity in salamander guts, testing the hypothesis that salamanders responded to increased production of primary consumers (i.e. prey) due to increased streamwater nutrient concentrations. Second, we determined whether nutrient enrichment altered the composition of prey in salamander diets, which could indicate that nutrient enrichment affected salamander growth via effects on nutritional quality (e.g. based on prey stoichiometry or nutritional content). Determining the true effect of diet shifts that may have occurred due to prey nutrient content would require data on individual prey stoichiometry and other nutritional constituents (e.g. polyunsaturated fatty acids [PUFAs]), which was beyond the scope of our study. However, an observed shift in prey composition is consistent with a prey quality effect and would provide better understanding of the effects of enrichment on trophic pathways. Finally, we tested whether the functional group composition of salamander diets was related to responses in basal resource quantity to our treatments, in order to explore the underlying mechanisms behind changes in diet composition.

2 | METHODS

2.1 | Study site

This study was conducted at the Coweeta Hydrologic Laboratory (Coweeta), a U.S. Forest Service and Long-Term Ecological Research site in Macon County, North Carolina, U.S.A. Coweeta is a heavily forested experimental basin (2,185 ha) in the Blue Ridge physiographic province of the southern Appalachian Mountains (Swank & Crossley, 1988). Forests surrounding our study streams are composed of mixed hardwoods (oak, tulip poplar, maple) and a dense

evergreen understory of *Rhododendron maximum* (Ericaceae) that shades the streams year-round. Stream food webs in Coweeta are primarily based on brown pathways, with green pathways being most significant during warmer spring months before leaf-out (Greenwood & Rosemond, 2005; Hall, Wallace, & Eggert, 2000).

We studied five streams located in the 559-ha Dryman Fork watershed at Coweeta. The five streams had similar chemical and physical characteristics prior to enrichment (i.e. pH, gradient, temperature). The study streams were close to each other (<0.5 km apart) and at approximately 1160 m.a.s.l. Pre-enrichment soluble reactive phosphorus (SRP) concentrations were very low and similar across streams (mean across the five streams: 3 µg/l; range 2.5–3.1 µg/l); ambient nitrogen concentrations were more variable, but still relatively low (NO₃-N mean, 74 µg/l; range 10–179 µg/l; NH₄-N mean, 8 µg/l; range 7–9 µg/l, $n = 17$ measurements per stream per analyte).

2.2 | Focal species

We studied larvae of two plethodontid salamander species common to Coweeta and much of the southern Appalachian region, U.S.A. *Eurycea wilderae* has a larval period of approximately 12 months, metamorphosing at 18–24 mm snout–vent length (SVL; Bruce, 1988). Observations made prior to our study indicated that *E. wilderae* were most commonly found in sandy depositional areas of the study streams (P. M. Bumpers, personal observation). In contrast, *D. quadramaculatus* has a larval stage of 36–48 months and metamorphoses at 40–45 mm snout–vent length (Bruce, Castanet, & Francillon-Vieillot, 2002). Larval *D. quadramaculatus* most commonly inhabit faster flowing riffle areas with cobble substrate, but were regularly found throughout all habitats in the streams. Several studies of *E. wilderae* and their congeners indicate that they most commonly consume chironomids and copepods, though relatively larger aquatic macroinvertebrates can dominate gut biomass (Barrett, Samoray, Helms, & Guyer, 2012; Johnson & Wallace, 2005; Trice, Rosemond, & Maerz, 2015). *Desmognathus quadramaculatus* are reported to be generalist feeders that consume myriad aquatic macroinvertebrate taxa from multiple functional and taxonomic groups (Davic, 1991; Trice et al., 2015). Trice et al. (2015) reported that *E. wilderae* and *D. quadramaculatus* exploit similar macroinvertebrate functional groups and that both derive most of their prey from riffle habitats. However, there is little overlap in the specific taxa consumed by the two species and *D. quadramaculatus* has a wider diet breadth, likely related to its larger size and microhabitat use.

2.3 | Experimental enrichment of five headwater streams

After 1 year of pre-treatment sampling, we conducted a 2-year (July 2011–July 2013) continuous nutrient addition to the five streams. Beginning in July 2011, dissolved nitrogen (21% liquid NH₄NO₃) and phosphorus (85% liquid H₃PO₄) were continuously added to 70-m treatment reaches with solar-powered metering pumps that dosed the nutrient solution at flow-proportional rates based on

instantaneous discharge. The nutrient solution dripped in the streams approximately every 5 m via a gravity-fed irrigation line to ensure mixing in the experimental reach. Increasing concentrations of N were coupled with decreasing concentrations of P to create a gradient of target dissolved N:P ratios (Table 1). Thus, N and P were inversely correlated in our experimental design. Streams were elevated above background concentrations by 2.5–31× and 3–10× for soluble reactive phosphorus (SRP) and dissolved inorganic nitrogen (DIN) respectively (Rosemond et al., 2015). Target enrichment concentrations of both N and P encompassed relatively modest concentrations, up to those that have been shown to be saturating for growth and production of algae and heterotrophic microorganisms. Our experimental design enabled us to test the relative importance of N versus P limitation independently because nutrient concentrations did not increase together. Our experiment and subsequent analyses assume that neither N nor P was added in concentrations that would be inhibitory to organisms in our study streams.

Water samples were taken bi-weekly in the treatment reaches and upstream of the treatment reaches to monitor ambient nutrient concentrations. Briefly, samples were filtered through 0.45-µm nitrocellulose membrane filters (Millipore, Billerica, MA, U.S.A.), transported on ice and frozen until SRP and DIN concentrations were measured spectrophotometrically (SRP) and on an Alpkem Rapid Flow Analyzer 300 (NO₃⁻-N, NH₄⁺-N) respectively. Longitudinal sampling of nutrient concentrations in the treatment reaches indicated no consistent spatial patterns in the distribution of added nutrients and good mixing throughout the treatment reaches (A. D. Rosemond, unpublished data). See Rosemond et al. (2015) for further details regarding the experimental design, stream characteristics and analytical methods.

2.4 | Diet sampling

Salamander larvae were collected for diet analysis before and during experimental enrichment. Larval *D. quadramaculatus* were collected three times during the pre-treatment period: June 2010, April 2011 and July 2011. *Eurycea wilderae* were collected in April 2011 and July 2011 for pre-treatment diet analyses. Collections were made during the spring and summer in each year of enrichment for both species in all streams (Year 1: March 2012, June 2012; Year 2: April 2013, June 2013). All larvae were collected at night when salamanders are most active, using fine-meshed dip nets and turning only loose cover objects. Larvae were too small to use gastric lavage; therefore, lethal sampling was necessary. Larvae were kept on ice, transported back to the laboratory and immediately euthanised in neutral-buffered 0.5% tricaine methanesulfonate (MS-222). We then rinsed larvae with deionised water and then either immediately placed stomachs in Kahle's solution after we dissected them or preserved the entire body in Kahle's solution until later dissection. All stomachs were preserved within 5 hr of capture.

We quantified leaf litter standing stocks monthly from multiple transects in each stream. Briefly, all leaf litter was collected within the wetted portion of eight randomly selected 0.15-m wide transects along the 70-m treatment reaches. Litter was then weighed,

TABLE 1 Mean concentrations (\pm SE) of dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) added to the experimental reaches to achieve target concentrations and molar N:P ratio in each of the five streams (labelled by target N:P ratio)

	Stream				
	2:1	8:1	16:1	32:1	128:1
DIN					
Target	81.3	243.9	365.8	487.7	650.3
YR 1	110 \pm 10.2	232 \pm 18.5	440 \pm 41.2	373 \pm 21.2	428 \pm 30.7
YR 2	82 \pm 5.1	151 \pm 6.7	377 \pm 21.0	381 \pm 10.0	517 \pm 21.7
SRP					
Target	90.0	67.5	50.6	33.8	11.3
YR 1	93 \pm 5.2	52 \pm 4.6	56 \pm 6.2	24 \pm 1.8	10 \pm 0.9
YR 2	77 \pm 4.2	37 \pm 1.5	54 \pm 3.0	28 \pm 0.9	11 \pm 0.4
N:P					
Target	2.0	8.0	16.0	31.9	127.4
YR 1	3 \pm 0.2	11 \pm 1.1	18 \pm 0.6	38 \pm 2.4	104 \pm 3.7
YR 2	3 \pm 0.2	10 \pm 0.9	15 \pm 0.2	31 \pm 0.8	109 \pm 2.4

Concentrations are calculated using the ambient nutrient concentration plus the concentration of nutrients added to stream water that was dripped into the treatment streams via an irrigation line during the 2-year enrichment. Year 1 (YR 1) = July 2011–June 2012, Year 2 (YR 2) = July 2012–June 2013. DIN and SRP are in μ g/l. See Rosemond et al. (2015) for details of the nutrient manipulations.

sub-sampled, oven-dried (60°C), weighed, combusted (500°C) and reweighed to determine ash-free dry mass (AFDM; Rosemond et al., 2015). Fine benthic organic matter (FBOM) was estimated monthly from benthic cores (Benstead et al., 2009). Chlorophyll *a* was quantified by placing eight unglazed tiles at four transects (two per transect) used to measure algal accrual in each stream for 2-month increments. Algal biomass on tiles generally tracked algal biomass on cobbles and is likely a conservative estimate of the response of biofilms to our treatments due to herbivory (A. D. Rosemond unpublished data). Tiles were brushed, filtered onto 0.7- μ m Whatman™ glass fibre filters (Whatman, GE Healthcare Sciences, Pittsburgh, PA, U.S.A.) and chlorophyll *a* was determined spectrophotometrically after being extracted with 90% acetone (Greenwood & Rosemond, 2005).

Stomach contents were identified using a dissecting microscope to the lowest taxonomic level possible (typically genus; Merritt, Cummins, & Berg, 2008), except for the family Chironomidae, which were classified as either non-Tanytopodinae or Tanytopodinae. Prey items were measured to the nearest 0.5-mm using an ocular micrometre or 1-mm graph paper placed on the dissecting microscope stage underneath the prey item. In almost all instances, prey items remained intact as whole organisms. On rare occasions, only a head capsule remained, which we typically were able to identify and measure. Prey biomass (mg AFDM) was then estimated using established length–mass or head width–mass regressions (J. B. Wallace, unpublished data; Benke, Huryn, Smock, & Wallace, 1999).

We estimated mean biomass (EB) for each prey taxon in each treatment stream using the following equation:

$$EB = P[i] \times N \times B$$

where $P[i]$ is the probability of taxa *i* being present in a stomach (composed of the total proportion of stomachs with that taxon multiplied by the total proportion of stomachs containing any prey), *N* is the average number of individuals of that prey in a stomach when present and *B* is the average biomass of that taxon consumed (mg AFDM gut⁻¹). Throughout this study, we refer to this estimated contribution of each taxon to prey biomass as “estimated biomass” and this differs from observed prey biomass in individual stomachs, which we refer to as “prey biomass” and use for analyses with individual salamanders.

2.5 | Statistical analyses

We used linear regression to determine if prey consumption was related to larval body size. Observed prey biomass (salamanders with empty guts were excluded) was combined across all sampling dates and streams and regressed against salamander size (as snout–vent length) for each species of salamander separately. Due to low pre-treatment sample size in the spring, further analyses testing the effect of the treatments were conducted with summer sampling dates only. Patterns in prey consumption (prey number, prey size [length], prey biomass) in response to our experimental treatments were assessed using linear mixed-effects modelling. We used $\log_{10}(x + 1)$ prey biomass to reduce variance among prey items and meet the assumptions of a linear model. We used treatment year (pre-treatment, Year 1, Year 2) to test for differences in prey metrics among years. We included salamander size as a variable in all models and included models that were additive and interactive with size. We then used Akaike's information criterion corrected for small sample size (AIC_c) and a model selection approach (Burnham & Anderson, 2002) to determine whether models that included streamwater N or P concentrations or N:P ratio outperformed the null model. Again,

salamander size was included in all models and we tested for both additive and interactive effects. We also tested for $N \times P$ interactions; however, none were significant so we excluded them from further analyses. All predictor variables were standardised using z scores. Due to the large-scale nature of our experiments, we lacked a control stream and true replication of experiments. Therefore, we included stream as a random effect to control for variation among sites not explained by nutrient concentrations. Nutrient concentrations were calculated from measured streamwater concentrations and therefore reflect the net effects of nutrient addition and uptake. Thus, measured N and P concentrations during the nutrient additions were moderately correlated ($r = -.51$). The measured N concentration was moderately correlated with the measured N:P ratio ($r = .56$), while the measured P concentration was more strongly correlated with the measured N:P ratio ($r = -.85$) during enrichment. Evidence of patterns that emerged across our five streams related more to N or P concentration would be observed as significant N or P effects in models. Because we assumed added concentrations were not inhibitory, we also assumed that negative responses to N were driven by the contrasting P gradient and vice versa, and not by an inhibitory effect of high N or P. All regression analyses were conducted in R version 3.0.2 using the "lme4" package (R Core Team, 2014).

Permutational multivariate analysis of variance (PERMANOVA) was used to test for enrichment effects on prey composition (as estimated biomass) of salamander diets using the "vegan" package in R (Oksanen et al., 2013). The PERMANOVA procedure is a nonparametric analogue to traditional multivariate analysis of variance, but is not constrained by the same assumptions (e.g. multivariate normality; Anderson, 2001). We tested for homogeneity of dispersion of prey composition in ordination space using permutational analysis of multivariate dispersion (PERMDISP). Analyses were conducted separately for each salamander species. We also used PERMANOVA to test for the effects of treatment year and stream on consumer group composition in salamander diets using log-transformed estimated biomass of three consumer groups: detritivores, algivores and carnivores. Classification of the three consumer groups was based on the trophic basis of production (TBP) for macroinvertebrates at Coweeta (Cross, 2004; Demi, 2016) in order to group taxa by the resources they consume and assimilate, instead of by their feeding mode. Briefly, trophic basis of production was estimated based on identified gut contents of invertebrates, assimilation efficiencies of each resource and a net production efficiency (see Cross et al., 2006 for additional details). When trophic bases of production was not available for a specific taxon, we assigned it to a group based on knowledge of the local macroinvertebrate assemblage, as well as classifications in Wallace, Eggert, Meyer, and Webster (1999) and Cross et al. (2006). We classified detritivore taxa as those in which the trophic basis of production was driven primarily ($\geq 50\%$) by coarse particulate organic matter and fungi or by amorphous detritus, which was typically fine particulate organic matter. Algivores were defined as obtaining on average $\geq 5\%$ of their production from algae (range: 5%–93%; Cross, 2004). This low threshold for algivores was based on responses of consumers to increased algae resources reported by Cross (2004) and in this study (Demi, 2016).

The majority of taxa classified as algivores were traditional grazers, but in some cases these classifications deviate from traditional functional feeding groups (*sensu* Merritt et al., 2008) because several collector-gatherers were defined as algivores (e.g. *Serratella*) or carnivores (e.g. *Psilotreta*). Three taxa that are traditionally defined as collector-gatherers and that made up at least 1% of total estimated biomass in any given year were classified as algivores in this study. We classified any taxon obtaining an average of 50% of production from animal material as a carnivore. We acknowledge that this approach is not as robust as using stable isotopes or fatty acids, which was beyond the scope of this study; however, the trophic basis of production approach better reflects the resources that are ultimately supporting salamander production than traditional functional group classifications. Moreover, we observed increases of biomass in salamander guts of both traditional grazer taxa (e.g. *Neophylax*, *Baetis*, *Maccaffertium*) and of those taxa we classified as algivores that are traditionally defined as collectors (e.g. *Serratella*, see Results).

Following PERMANOVA, streams were ordinated by year according to prey taxon and functional group composition in salamander stomachs using non-metric multidimensional scaling (NMDS) in the "vegan" package in R (Oksanen et al., 2013). We used Bray–Curtis dissimilarities as our distance measure. $\log_{10}(x + 1)$ transformations were used to account for large variation in the estimated biomass of prey species (McCune & Grace, 2002). We tested for correlations between diet composition across streams and individual taxon estimated biomass using the "envfit" function in the "vegan" package. We used 1,000 permutations in assessing the significance of fitted vectors to the NMDS (Oksanen et al., 2013).

We tested the degree to which primary consumer group estimated biomass in the guts of *D. quadramaculatus* was related to the quantity of basal resources using linear mixed-effects models. Detritivore estimated biomass was regressed separately against standing stocks of leaf litter and fine benthic organic matter (LLSS and FBOM respectively). Algivore estimated biomass was regressed against algal biomass (chlorophyll *a*). We used mean annual standing stocks and biomass of basal resources in all cases. All models included a random effect for stream.

3 | RESULTS

3.1 | General patterns in larval consumption

We collected 409 *D. quadramaculatus* (mean = 14, min = 9, max = 18 per stream per sampling date) and 349 *E. wilderae* (mean = 13, min = 6, max = 17 per stream per sampling date) from all streams and sampling dates. Prey was found in 373 (91%) and 291 (83%) stomachs for *D. quadramaculatus* and *E. wilderae* respectively. Salamander stomachs with prey contained an average of 4.3 ($SE \pm 0.14$) prey items for *D. quadramaculatus* (1.3 ± 0.11 mg AFDM gut⁻¹) and 6.4 ± 0.33 items per stomach for *E. wilderae* (0.12 ± 0.01 mg AFDM gut⁻¹). Average prey length was 3.1 ± 0.11 and 1.5 ± 0.06 mm for *D. quadramaculatus* and *E. wilderae* respectively. Prey biomass in *D. quadramaculatus* and *E. wilderae* guts was positively related to

salamander size (*D. quadramaculatus*, $R^2 = 0.18$, $p < .001$; *E. wilderae*, $R^2 = 0.22$, $p = .002$). The average length ($R^2 = 0.13$, $p < .001$), but not number of prey items ($R^2 = -0.001$, $p = .4$), in *D. quadramaculatus* stomach was related to salamander size. Conversely, in *E. wilderae*, the number of prey items was weakly and positively correlated to salamander size ($R^2 = 0.07$, $p < .001$), while average prey length was not ($R^2 = 0.008$, $p = .12$).

Collectively, *D. quadramaculatus* consumed 56 prey taxa, while *E. wilderae* consumed 42 prey taxa; 34 taxa were found in diets of both species, resulting in 22 and 8 unique prey taxa found in *D. quadramaculatus* and *E. wilderae* diets respectively. Across all streams and dates for *D. quadramaculatus*, *Tallaperla* (Plecoptera: Peltoperlidae) was the most important prey item in terms of biomass, accounting for 20.7% of estimated prey biomass and 10.3% of abundance, but its contribution was variable across streams and years (Table S1a). *Maccaffertium* (Ephemeroptera: Heptageniidae) and Hydropsychidae (Trichoptera) were the next most important prey items (9.8% and 7.3% estimated biomass; 2.4% and 2.9% abundance respectively). Non-Tanyptodinae chironomids accounted for 22% of all prey items by number in *D. quadramaculatus* stomachs, but were <4% of estimated biomass (Table S1a). The most dominant taxa in stomachs of *E. wilderae* were *Leuctra* (Plecoptera: Leuctridae, 18.3% estimated biomass, 4% abundance; Table S1b), non-Tanyptodinae chironomids (15.2% estimated biomass, 16.3% abundance) and *Serratella* (Ephemeroptera: Ephemerellidae; 8.7% estimated biomass, 1.4% abundance). Copepods accounted for 26% of all prey items by number in *E. wilderae* stomachs, but only 1.1% of estimated biomass (Table S1b).

3.2 | Responses of prey quantity to enrichment

For *D. quadramaculatus*, there was a significant year effect on prey size in both years and mean prey biomass and prey numbers in Year 2 compared to the pre-treatment year (Table S2, Figure 1a–c). Prey biomass was positively related to salamander size and the year effect was greater for larger individuals and was greatest in Year 2 (i.e. there was a significant interaction between salamander size and year). For *E. wilderae*, there was some evidence of a year effect on prey size (Year 2) and prey number, but not on prey biomass. Average number of prey increased significantly in Year 1, and there was an interaction between salamander size and Year 2 resulting in reduced prey numbers (Figure 2a, Table S2). Average prey size in *E. wilderae* guts was significantly larger in Year 2 of enrichment compared to pre- and Year 1 (Figure 2b, Table S2). Prey biomass consumed by *E. wilderae* was not affected by the treatments (Figure 2c, Tables 2 and S2).

Models to explain the patterns in prey number, size (length) and prey biomass in *D. quadramaculatus* guts were largely driven by streamwater P concentrations. The best model explaining prey number included a significant negative effect of N, although the model only explained 2% of the variation (Tables 2 and S3). The top model explaining prey size included a significant effect of salamander size, P concentration and a positive interaction between salamander size and P concentration (Table 2). The increase in prey biomass was best explained by salamander size, a positive effect of P concentration

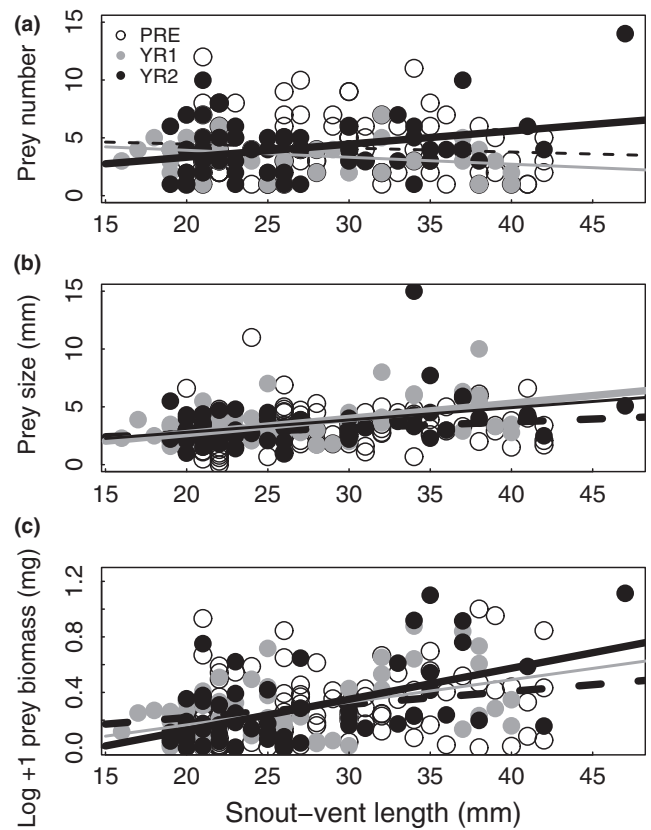


FIGURE 1 Patterns in prey consumption regressed against larval size (snout–vent length) for *Desmognathus quadramaculatus* during summer sampling dates for (a) number of prey consumed, (b) average prey size consumed and (c) total prey biomass. Points represent individuals across all streams in a given year. Lines are based on parameter estimates; dashed lines = pre-treatment, grey lines = Year 1 of enrichment and black lines = Year 2 of enrichment. Thick lines indicate significant regression lines ($p \leq .05$)

and a negative effect of N concentration (Table 2). The remaining models within two AIC_C points included a negative effect of N and N:P (Table S3). The top model for *E. wilderae* prey number included a significant positive effect of N concentration (Table S3). The top model for *E. wilderae* prey size included salamander size and a weak negative effect of N:P ratio (Table 2), suggesting a weak positive effect of P concentration.

3.3 | Prey composition responses to enrichment

Nutrient enrichment altered the diet composition of *D. quadramaculatus* compared to pre-treatment conditions. There was a significant difference among treatment years for diet composition ($F_{2,14} = 1.75$, $p = .046$). This was supported by PERMDISP ($F_{2,12} = 1.43$, $p = .23$), which indicated no heterogeneity—or differences—of variance for diet species composition among years. Diet composition was not different among streams within a given year ($F_{4,14} = 0.49$, $p = .99$). Ordination of prey composition showed clear separation between pre-treatment and enrichment years, but not between Year 1 and Year 2 of enrichment (Figure 3a). Estimated biomass of Hydropsychidae (Trichoptera), *Tallaperla*, *Wormaldia* and detritivores was

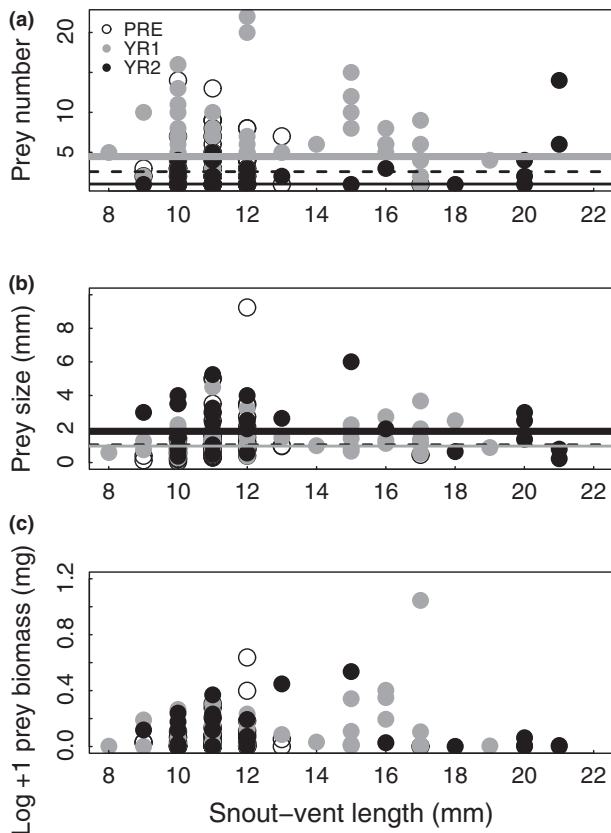


FIGURE 2 Patterns in prey consumption regressed against larval size (snout–vent length) for *Eurycea wilderae* during summer sampling dates for (a) number of prey consumed, (b) average prey size (length) consumed and (c) total prey biomass. Points represent individuals across all streams in a given year. Lines are based on parameter estimates. The relationship with snout–vent length was not significant so lines are plotted according to the intercept for each response. The x-axis is snout–vent length to allow for comparison of response to *Desmognathus quadramaculatus*; dashed lines = pre-treatment, grey lines = Year 1 of enrichment, and black lines = Year 2 of enrichment. Thick lines indicate significant regression lines

associated with pre-treatment dates (Figure 3a), while *Serratella*, *Amphinemura* (Plecoptera: Nemouridae) and algivore estimated biomass in *D. quadramaculatus* stomachs was associated with enriched dates. *Serratella* increased from 0.8% of estimated biomass before enrichment to an average of 9.5% during enrichment. *Amphinemura* accounted for 0.4% and 4.7% of estimated biomass during pre-treatment and enriched conditions respectively. While they were not significant vectors of the ordination, *Baetis* (Ephemeroptera), *Neophylax* (Trichoptera: Uenoidae), *Elmidae* (Coleoptera), *Paraleptophlebia* (Ephemeroptera) and three other caddisfly algivores (together these taxa increased from <0.1% in Pre to 2.9% in Year 2 estimated biomass) all increased during enrichment (Table S1a).

There was a marginally significant difference among treatment years for *E. wilderae* diet composition ($F_{2,14} = 1.83$, $p = .05$); however, there was no clear visual pattern among years on the NMDS plot (Figure 3b). There was no heterogeneity of variances for *E. wilderae* prey composition among years ($F_{2,12} = 1.01$, $p = .39$).

Additionally, there were no prey compositional differences among streams ($F_{4,12} = 1.33$, $p = .17$).

We also analysed diet composition for both salamander species by prey functional groups (defined here as algivore, detritivore or carnivore) to determine whether enrichment affected the pathways of energy flow to salamanders. Ordination indicated some separation of streams among treatment years for *D. quadramaculatus*, with detritivore and carnivore estimated biomass decreasing and algivore estimated biomass increasing; there was a marginally significant change in overall prey functional group composition among years within a stream (PERMANOVA $F_{2,14} = 2.02$, $p = .09$, Figure 4a; NMDS not shown). Prey functional group composition of *E. wilderae* was not significantly different among treatment years ($F_{2,14} = 1.38$, $p = .20$, Figure 4b) or streams ($F_{4,14} = 1.1$, $p = .37$).

3.4 | Relationships between prey groups and basal resources

We found that the quantity of prey functional groups in *D. quadramaculatus* guts was generally related to basal resource quantity and, furthermore, that prey quantity increased for prey dependent on algal resources and decreased for prey dependent on detrital resources—particularly leaf litter—with nutrient enrichment. We assessed these relationships by determining the degree to which estimated biomass of prey functional groups in *D. quadramaculatus* guts was related to availability of their respective dominant food resource across treatment years. Estimated biomass of all prey functional groups in the guts of *D. quadramaculatus* was positively related to their respective food resource (Figure 5). For detritivores, this meant reduced representation in salamander guts due to lower detrital resources in streams during nutrient enrichment. Detritivore estimated biomass was positively related to both leaf litter ($T = 3.09$, Figure 5a) and FPOM standing stocks ($T = 2.7$, Figure 5b), which were both lower in years of nutrient enrichment. Algivore biomass increased with chlorophyll *a* biomass ($T = 2.06$, Figure 5c), which were both higher in years of nutrient enrichment.

4 | DISCUSSION

Our results indicate that nutrient enrichment affected predator diet quantity and composition, reflecting altered energy-flow pathways. Increased prey quantity and changes in diet composition apparently occurred largely via green pathways, despite the dominance of brown resources in the headwater streams we manipulated (see Brett et al., 2017). These results are surprising, given that algae are severely light limited in our study streams, and indicate that green food-web pathways can become disproportionately important due to nutrient enrichment even in highly heterotrophic systems. Enhanced fungal biomass, which increased detrital quality, also likely contributed to increased prey biomass. Nutrient effects on salamander diets were not consistent across two functionally similar species, which supports the conclusions of Trice et al. (2015) that alteration

TABLE 2 Linear mixed-effects model parameter estimates (SE) for the top model explaining each tested diet response in *Desmognathus quadramaculatus* and *Eurycea wilderae*

Parameter	Estimate (SE)	R ²	Parameter	Estimate (SE)	R ²
<i>D. quadramaculatus</i>			<i>E. wilderae</i>		
Size: <i>df</i> = 215, AIC _c = 852.998, Wt = 0.53		0.14	Size: <i>df</i> = 145, AIC _c = 511.06, Wt = 0.28		0.04
Intercept	3.33 (0.11)		Intercept	1.527 (0.11)	
SVL	0.58 (0.11)		SVL	0.219 (0.11)	
SRP	0.025 (0.11)		Stream N:P	−0.191 (0.11)*	
SVL*SRP	0.227 (0.11)				
Number: <i>df</i> = 216, AIC _c = 990.58, Wt = 0.31		0.02	Number: <i>df</i> = 145, AIC _c = 833.8, Wt = 0.47		0.04
Intercept	3.97 (0.15)		Intercept	4.397 (0.307)	
SVL	0.025 (0.15)		SVL	−0.0567 (0.334)	
DIN	−0.307 (0.15)		DIN	0.829 (0.334)	
Biomass: <i>df</i> = 215, AIC _c = −40.32, Wt = 0.40		0.21	Biomass: <i>df</i> = 146, AIC _c = −409.24, Wt = 0.30		0.02
Intercept	0.277 (0.014)		Intercept	0.036 (0.005)	
SVL	0.102 (0.015)		SVL	0.0082 (0.005)	
SRP	0.031 (0.016)				
DIN	−0.054 (0.016)				

SVL, snout-vent length; SRP, soluble-reactive phosphorus; DIN, dissolved inorganic nitrogen.

Parameter estimates are standardised using z-scores and are thus directly comparable. Nutrient concentrations are the measured streamwater concentrations. Bolded numbers indicate parameter estimates with *p* values < 0.05 and those with an asterisk indicate *p* < 0.1.

of stream processes and invertebrate communities may have differential effects on larval salamander species. Consistent with this, our results show that nutrient effects on salamander diets may depend on species and, in some instances, on body size. Finally, the shifts in diet allow us to better understand the mechanisms underlying observed increased growth rates of *D. quadramaculatus* and *E. wilderae* in response to enrichment (Bumpers et al., 2015) and expand our understanding of how nutrient enrichment can affect food-web linkages to higher trophic levels.

4.1 | Prey quantity responses

We observed increased prey quantity in *D. quadramaculatus* diets, with less evidence for prey quantity increases in diets of *E. wilderae*. Many studies have found correlations between predator density and growth responses to the responses of their prey, but have not corroborated those relationships explicitly with diet data (Gratton & Denno, 2003; Johnson et al., 2006; Peterson et al., 1993). In this study, we were able to determine that the amount of food in *D. quadramaculatus* diets increased in response to experimental enrichment. Moreover, two of the three response metrics for *D. quadramaculatus* were positively related to, and best explained by, streamwater P concentration. The relationship we found in this study between prey biomass in *D. quadramaculatus* diets and streamwater P parallels the growth response observed in our previous study, in which we observed that growth rates of both species of salamanders were strongly correlated with streamwater P concentrations (Bumpers et al., 2015). Together, these results suggest that nutrient enrichment alleviated resource limitation in the short term, particularly for *D. quadramaculatus* larvae. We note that both

D. quadramaculatus prey biomass and prey number were negatively correlated with streamwater N in this study. We attribute this to the nature of our experimental design, in which N and P were negatively correlated, and that a positive effect of P, and not an inhibitory effect of N, drove this response.

Salamander growth has been shown to be resource-limited in several studies (Huntsman, Venarsky, Benstead, & Huryn, 2011; Johnson & Wallace, 2005; Johnson et al., 2006). For example, Huntsman et al. (2011) found that *Gyrinophilus palleucus* (Green) had higher consumption and growth rates and larger population size in a cave system receiving greater detrital inputs and thus had higher invertebrate production, compared to a cave receiving fewer detrital inputs. We observed increased prey biomass and increased prey size in *D. quadramaculatus* guts in this study despite reductions in the detrital resource—leaf litter—with enrichment. In our study, increased production of primary consumers would result from increased detrital quality or increased algal biomass. Cross et al. (2006) reported increased secondary production of macroinvertebrates in response to a 2-year experimental enrichment (N:P = 16:1) in a similar forest stream at Coweeta. Preliminary analysis of macroinvertebrate data collected concurrently with this study shows that production of important prey resources (e.g. *Tallaperla*, *Leuctra*) for *D. quadramaculatus* increased during both years of enrichment, as did production of Chironomidae, an important prey taxon for both *D. quadramaculatus* and *E. wilderae* in our study streams (L. M. Demi, unpublished data). Although detritivore biomass did not increase in salamander guts during enrichment years, the slope of the relationship between leaf litter standing stocks and detritivore biomass was steeper during enrichment, suggesting a higher biomass of detritivores per unit leaf litter during enrichment. This likely resulted from enhanced detrital quality

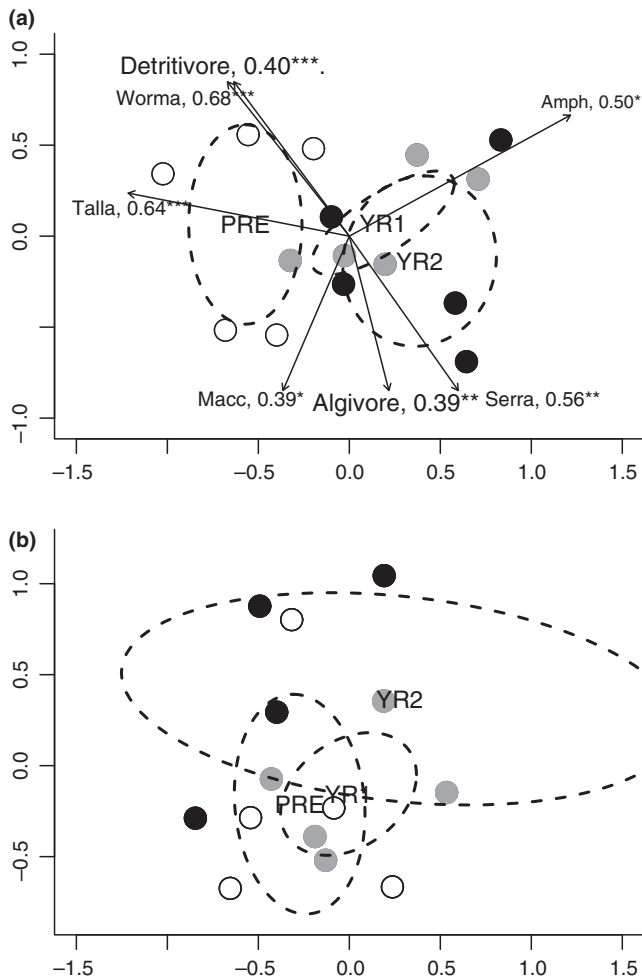


FIGURE 3 Patterns in consumer group composition and biomass for *Desmognathus quadramaculatus* and *Eurycea wilderae*. Non-metric multidimensional scaling ordination plot based on $\log_{10}(x + 1)$ -transformed prey biomass in the stomachs of salamanders. There was clear separation of stomach prey composition between pre-treatment (open) and enriched years, but no distinction between Year 1 (YR1, grey) and Year 2 (YR2, black) of enrichment, based on PERMANOVA and PERMDISP for *D. quadramaculatus* (a). Points are stream averages for each year. There was no distinction among treatment years for *E. wilderae* (b). Vectors show the direction and correlation of individual taxa or the biomass of consumer groups and diet composition within the ordination space. Only vectors with significant correlations are shown ($*p < .1$, $**p < .05$, $***p < .01$). Taxa: Talla = Tallaperla, Hydrops = Hydropsychidae, Worm = Wormaldia, Macc = Maccaffertium, Serra = Serratella, Amph = Amphinemura

due to increased fungal biomass and subsequent increases in the nutrient content of detritus (Manning et al., 2015).

4.2 | Shifts in diet composition

Diet composition shifts of *D. quadramaculatus* included increased incorporation of prey supported via green pathways. Algivores, as defined here, increased in both *D. quadramaculatus* and *E. wilderae* guts in both years of enrichment (although shifts in functional

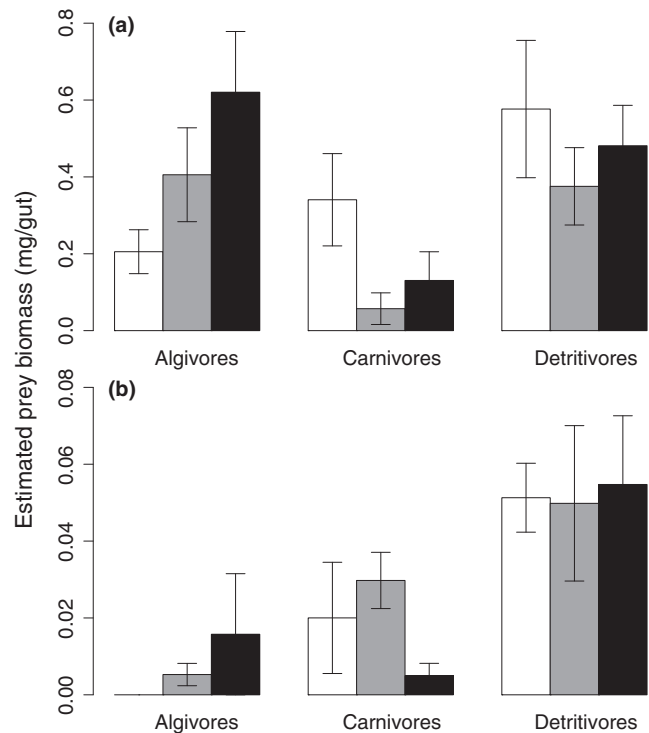


FIGURE 4 Mean estimated prey biomass (\pm SE) of consumer groups from all five streams in *Desmognathus quadramaculatus* (a) and *Eurycea wilderae* (b) stomachs for pre-treatment (white), Year 1 (grey) and Year 2 (black)

group composition were not statistically significant), concurrent with increased algal biomass. Algivores that increased in *D. quadramaculatus* diets, such as *Baetis*, *Neophylax* and *Maccaffertium*, had higher production during enrichment, and the proportion of their production attributed to diatoms increased (Demi, 2016). It is possible that *D. quadramaculatus* fed more selectively on algivores over the course of the experimental enrichment. However, biomass of each functional group in *D. quadramaculatus* diets paralleled responses in basal resource quantity, suggesting that shifts in functional group availability, rather than selectivity by *D. quadramaculatus*, drove changes in diet composition.

These increases are surprising considering the generally low algal biomass in the heavily shaded study streams. Algal biomass increased in response to enrichment; however, even with this increase, algae still made up a very small fraction of the total carbon standing stocks ($<1\%$ of mean annual carbon storage; A. D. Rosemond, unpublished data) and gross primary production did not change with enrichment (Kominoski, Rosemond, Benstead, Gulis, & Manning, 2017). In addition, many of the taxa that consume algae in our study streams have diets that are dominated by detrital resources, such that our findings of more reliance on these taxa should be assessed conservatively. That said, algae have been shown to fuel primary consumer production disproportionately relative to their availability (e.g. McCutchan & Lewis, 2002). Algae may have been most important in the summer months, which coincided with the lowest availability of detrital carbon (Rosemond et al., 2015). Finally, we note that apparent increased reliance

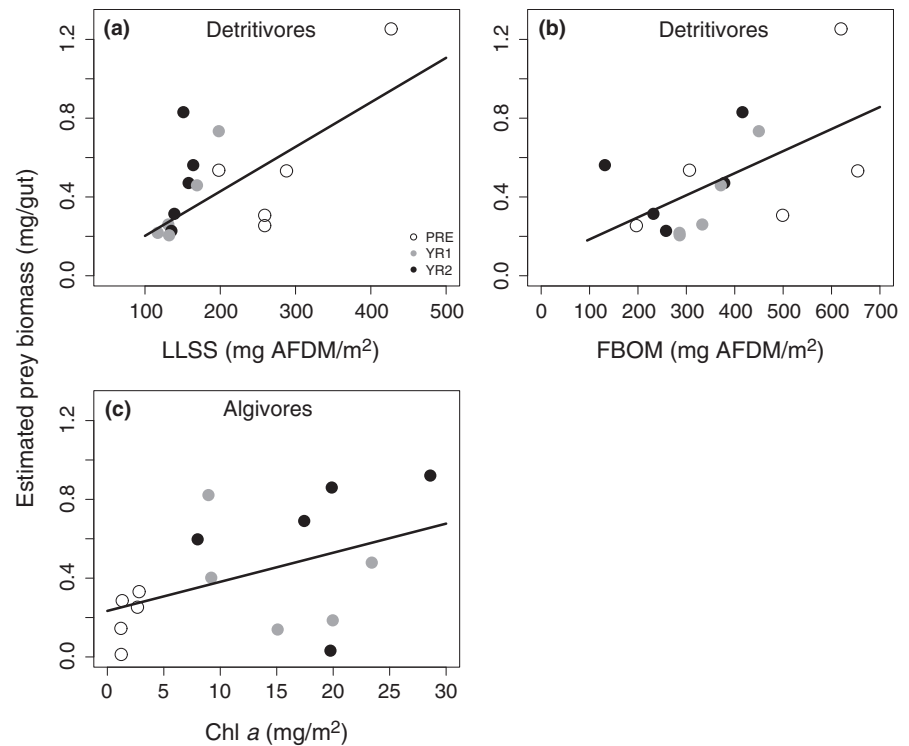


FIGURE 5 Patterns of estimated biomass among consumer groups in *Desmognathus quadramaculatus* stomachs related to basal resource mass (mg AFDM m⁻²). Prey taxa were categorised according to three dominant food resources: leaf litter for detritivores (a), fine benthic organic matter for detritivores (b), and biofilm mass for algivores (c). Line equations are: (a) $y = 0.0019x - 0.09$, $R^2_{\text{marginal}} = 0.38$, $R^2_{\text{conditional}} = 0.49$, $T = 3.09$; (b) $y = 0.001x + 0.07$, $R^2_{\text{marginal}} = 0.34$, $R^2_{\text{conditional}} = 0.35$, $T = 2.7$; (c) $y = 0.014x + 0.23 = 15$, $R^2_{\text{marginal}} = 0.21$, $R^2_{\text{conditional}} = 0.21$, $T = 1.93$. LLSS, leaf litter standing stocks

on algivores (as defined here) could also be driven in part by increased availability of heterotrophic biofilms on leaf litter, which increased concomitantly with algal biofilms. A detailed isotopic or fatty acid study in conjunction with diet analysis would be required to elucidate which basal resource was driving salamander growth. Regardless, the relationship we found in this study between algivore biomass and chlorophyll *a*, in conjunction with increased invertebrate production attributed to diatoms, suggests that algae became more important for salamander growth after nutrient enrichment.

A shift in diet to more algal-supported prey may have implications for energetic or nutritional constraints on predator growth (Dickman, Newell, Gonzalez, & Vanni, 2008; Johnson & Wallace, 2005). Under low-nutrient conditions, biofilm consumers (e.g. scraper and collector–gatherer functional feeding groups) are generally higher in nutrient content than are shredder taxa, although this pattern is highly variable (Cross, Benstead, Rosemond, & Wallace, 2003; Frost et al., 2006). Cross et al. (2003) found that the body C:P of the average scraper and collector–gatherer in a nearby Coweeta stream was 369 and ~250, respectively, compared to c. 500 for shredders. The threshold elemental ratio for C:P in larval salamanders, or the point at which growth limitation switches from carbon to phosphorus, is likely less than 140 (Bumpers et al., 2015). Increased biomass of higher nutrient content prey (i.e. with body C:P closer to 140) could therefore reduce stoichiometric constraints on salamander growth. Moreover, increases in algae-based prey could lead to increased intake of essential fatty acids—which are important for consumer growth—because algae are the primary source of fatty acids in aquatic food webs (Ballantyne, Brett, & Schindler, 2003; Brett & Muller-Navarra, 1997). This could be of particular importance in shaded streams receiving nutrient inputs like those in our

study as fatty acids can increase in response to shading and nutrient addition (Guo, Kainz, Sheldon, & Bunn, 2016).

4.3 | Differences between *Desmognathus quadramaculatus* and *Eurycea wilderae* responses

In our study, it is possible that differences in body size between *D. quadramaculatus* and *E. wilderae* may have contributed to their different responses to enrichment. Larval salamanders are gape-limited suction feeders, and *E. wilderae* are much smaller than *D. quadramaculatus* and consume smaller bodied prey. Therefore, *E. wilderae* may have been less likely to consume taxa that increased in size and so exceeded its gape limit. Size also played a role in the response of *D. quadramaculatus*, because larger larvae had a greater increase in prey biomass in their guts than did smaller larvae. The lack of a clear response of prey biomass in *E. wilderae* could suggest that different mechanisms that we did not test for in this study were responsible for the increased growth rates we previously reported. If *E. wilderae* prey biomass did not increase, it is possible that the nutritional quality of *E. wilderae* prey changed or that the consumption rate increased in response to enrichment. Our approach to diet sampling may not have been sensitive enough to measure an increase in consumption rate because our gut samples represent short-term “snapshots” of individual diets.

The limited evidence for diet shifts in *E. wilderae*, coupled with the clear growth responses shown in our previous study (Bumpers et al., 2015), are similar to the results of Johnson and Wallace (2005), which assessed growth and diet of *E. wilderae* in response to experimental detrital reductions in a Coweeta stream. Johnson and Wallace (2005) found reduced growth of *E. wilderae*, but no changes in prey biomass consumed. Such similar responses to different perturbations,

coupled with the lack of diet flexibility in this species, indicate that the impacts of perturbation on growth of gape-limited predators may be nuanced and depend on prey availability and edibility (e.g. see Davis et al., 2010 for macroinvertebrate predator response).

4.4 | Implications and conclusions

Phosphorus effects on prey quantity and composition were the likely drivers of increased growth of the larger salamander species, *D. quadramaculatus*, observed concurrently with this study (Bumpers et al., 2015). Phosphorus effects propagated to these vertebrate predators through increased production of prey that was strongly associated with changes in the nutrient content (specifically P content) of basal resources (L. M. Demi, unpublished data). Thus, P effects on predators were manifested by altered basal resource quantity (reduced for detrital resources, increased for biofilms) and quality (increased for both brown and green resources) that subsequently resulted in increased prey production.

Our study demonstrates that the effects of nutrient enrichment on basal resources can lead to altered food availability to predators. Additionally, it revealed some mechanisms by which salamanders exhibited increased growth rates in response to nutrient enrichment. The ubiquity of anthropogenic nutrient enrichment is likely transforming stream food-web structure, with potentially widespread impacts on predator life history. We found a shift towards a more algal-based diet, which suggests that trophic transfer efficiency of energy and nutrients (and possibly fatty acids) to predators is likely altered as a result of nutrient enrichment—even in heavily shaded headwater streams—because algae are a more nutrient-rich resource than detritus (Cebrian et al., 2009; Dickman et al., 2008). This may have implications for ecosystem function and consumer-mediated effects on nutrient dynamics. Finally, we demonstrate that closely related, sympatric predators may be affected by nutrient enrichment through subtly different mechanisms, likely mediated by their different microhabitat use or diets.

ACKNOWLEDGMENTS

Funding for this research was supported by NSF grants DEB 09818894 to ADR and JCM and DEB 0918904 to JPB. We thank John Kominoski, Jason Coombs, David Manning, Kristen Cecala, Katie Norris, Kaitlin Farrell, Keith Kinek, Brian Crawford, Sean Sterrett, Todd Pierson, Rachel Mahan, Courtney Allen, Amy Trice, Jenna Martin and Sophia Bonjour for assistance collecting salamanders. Lee Demi provided the FBOM data. We also thank David Plank and The Andersons Inc. for donating the ammonium nitrate, and Rob Case, Daniel Hutcherson and Kevin Simpson of YSI Integrated Systems and Services for engineering the experimental infrastructure. The manuscript was improved by comments from Mary Freeman and David Manning. This research leveraged logistical support from the Coweeta LTER Program at the University of Georgia, which is supported by NSF award (DEB 0823293) from the Long Term Ecological Research Program. This

project was approved by University of Georgia IACUC # A2011 10-019-Y3-A2.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Bumpers PM, Rosemond AD, Maerz JC, Benstead JP. Experimental nutrient enrichment of forest streams increases energy flow to predators along greener food-web pathways. *Freshw Biol.* 2017;62:1794–1805. <https://doi.org/10.1111/fwb.12992>