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ECOSYSTEM ECOLOGY – ORIGINAL RESEARCH

Ontogenetic changes in sensitivity to nutrient limitation of tadpole growth

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Abstract According to ecological stoichiometry (ES), the growth of a consumer with abundant resources should increase as body and resource stoichiometry become more similar. However, for organisms with complex life cycles involving distinct changes in biology, nutrient demands might change in response to ontogenetic changes in body stoichiometry. Tadpole growth and development has been found to be largely nitrogen (N) limited, as predicted for organisms developing N-rich tissues like muscle. However, tadpole metamorphosis includes periods of rapid development of phosphorus (P)-rich bones in preparation for a terrestrial lifestyle. We hypothesized that tadpole growth and development will exhibit variable nutrient demands during different stages of ontogeny, due to predictable changes in body tissue stoichiometry. To test this, we raised tadpoles on four diets with varying N:P ratios and assessed growth and developmental rates. Specifically, we predicted that tadpoles would be sensitive to N limitation throughout ontogeny (consistent with previous studies), but also sensitive to P limitation during the process of long-bone ossification. Consistent with our prediction, tadpole growth rates and development were sensitive to N limitation throughout ontogeny. Increased dietary N led to a shorter time to metamorphosis and a larger mass at metamorphosis. Also as predicted, growth rates were sensitive to both N and P during the period of peak bone ossification, indicative of co-limitation. These results indicate that P limitation changes through tadpole ontogeny consistent with, and can be predicted by, shifts in body tissue stoichiometry. Future studies should investigate whether ontogenetic shifts in tadpole P limitation lead to seasonal shifts in wetland nutrient cycling.

Keywords Ecological stoichiometry · Amphibian · Homeostasis · Rheostasis · Ossification

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Introduction

The theory of ecological stoichiometry (ES) provides ecologists with a quantitative framework to predict consumer nutrient limitation, based on similarity of stoichiometric ratios in their body tissues relative to their food resource (Sterner and Elser 2002; Elser and Hamilton 2007; Aalto et al. 2015). Many consumers maintain constant elemental ratios in their body tissues despite variation in resource stoichiometry (Persson et al. 2010), through a process of selective absorption and excretion of nutrients (Sterner and Elser 2002). Although mature animals are generally homeostatic (Vanni 2002), some fish and invertebrates undergo shifts in their body stoichiometry as they progress from one life stage to another [e.g., juvenile to adult (Main et al. 1997; Pilati and Vanni 2007)]. Such shifts represent changes in the regulated set point for body stoichiometry, a process referred to as rheostasis. Specifically, rheostasis is defined as a condition in which homeostatic control mechanisms are

in place; however, the level of the parameter controlled may change through time. In the case of stoichiometry, it could be thought of as special case of "strict homeostasis" in which the stoichiometric set point for homeostasis changes through time (Mrosovsky 1990; Villar-Argaiz et al. 2002). Currently, ES theory has not incorporated rheostasis into models of organismal growth and metabolism (Sterner and Elser 2002). To achieve this, an important first step is to understand how rheostasis interacts with resource nutrient limitation.

Rheostatic control of body stoichiometry might be important for vertebrates with complex life cycles with free-living larvae, due to distinct periods of bone ossification (Kemp and Hoyt 1969; Miura et al. 2008). Bone is P rich relative to soft tissues like muscle, which tend to be N rich (Elser et al. 1996; Hendrixson et al. 2007). It is, therefore, likely that vertebrate stoichiometry becomes P enriched during periods of rapid bone ossification, especially for terrestrial vertebrates that require robust appendicular skeletons (Reynolds 1977; Altig 1999). In support of this idea, Tiegs et al. (2016) found that body %P increased through larval wood frog (*Lithobates sylvaticus*) tadpole ontogeny, which corresponded with a period of increased bone growth and ossification, as noted by Kemp and Hoyt (1969). Unless resources somehow become more P rich during periods of bone ossification, this shift in body stoichiometry is likely to increase P demand in developing vertebrates.

Amphibians provide ideal systems for testing this idea because their larvae are free-living consumers whose resources vary greatly in nutritional quality (Feder and Burggren 1992; Altig 1999). This makes it possible to conduct experiments testing nutritional limitation during this period of ontogeny. In contrast, most other vertebrates develop bone in ovo or in utero, which makes the study of nutrient limitation logistically challenging. Typically, tadpoles have been classified as herbivorous and/or detritivorous organisms relying on food sources that vary in nutritional quality by orders of magnitude (Altig 1999). For example, studies have found that individual species of tadpoles are capable of consuming high-quality biofilms and algae, as well as low-quality leaf litter (Altig et al. 2007; Stephens et al. 2013, 2015; Stoler and Relyea 2016).

Prior studies of tadpoles consuming natural food sources (e.g., algae, leaf litter) revealed that rates of growth and development are strongly influenced by the food's nutritional content (Kupferberg 1997b; Altig et al. 2007; Liess et al. 2013; Pinto et al. 2015; Stephens et al. 2015), with several studies finding that N content was the best predictor of tadpole growth rates (Stephens et al. 2013; Stoler and Relyea 2013). N limitation is expected to persist throughout tadpole ontogeny (Stephens et al. 2013) because most developing tissue contains N-rich muscle and connective tissue (Elser et al. 1996). However, N and P tend to co-vary within major groups of resources (Cornwell et al. 2008; Stephens et al. 2013), making it difficult to distinguish between N and P limitation by conducting experiments with natural food sources. Furthermore, the N:P ratio of resources can range dramatically among resource types, such that P limitation might become more important depending on the resource consumed. For example, average N:P (by dry mass) of leaf litter is ~7 (Ostrofsky 1997), whereas periphyton is ~30 (Liess and Hillebrand 2005). Hence, it is possible that tadpoles might be either N or P limited depending on their resource at a particular stage of development. Additionally, it is also possible that N and P have differential effects on growth and development (Stephens et al. 2013).

To better understand how resource stoichiometry relates to tadpole growth, we independently manipulated the N and P content of tadpole diets throughout their larval ontogeny, and examined effects on growth and development. We hypothesized that tadpoles would exhibit rheostatic control of their tissue stoichiometry, as exhibited by variable sensitivity to N and P limitation through ontogeny. Specifically, we predicted that tadpoles would be sensitive to N limitation throughout the larval period due to the continued growth of N-rich soft tissues (e.g., muscle) but that they would also be sensitive to P limitation during the stages of development when the larger bones of the appendicular skeleton undergo ossification (i.e., Gosner stages 34–40; Fig. 1; Gosner 1960). To test this, we fed developing wood frogs controlled diets that varied only in N or P content (i.e., holding all other dietary elements constant) and monitored growth and development.

Methods

Amphibian collection and breeding

We collected ten pairs of adult wood frogs (not amplected) during their breeding migration in the Saginaw Forest (Ann Arbor, Michigan) during the spring of 2014. We transferred the pairs to out-lab at Oakland University where we allowed them to amplect and lay eggs. We separated the egg masses and allowed eggs to hatch individually in pans of aerated tap water. After hatching, we pooled all tadpoles together and allowed them to develop until they reached feeding stage (Gosner stage 26). We then haphazardly distributed individuals across all four treatments. We weighed and staged a subset of 20 tadpoles to determine the initial mass and Gosner stage, which were 0.43 mg (dry mass) and 26, respectively (Gosner 1960).

Experimental design

We used a full-factorial experiment crossing two levels of N (6.36 and 1.06 % dry mass) with two levels of P (0.6 and

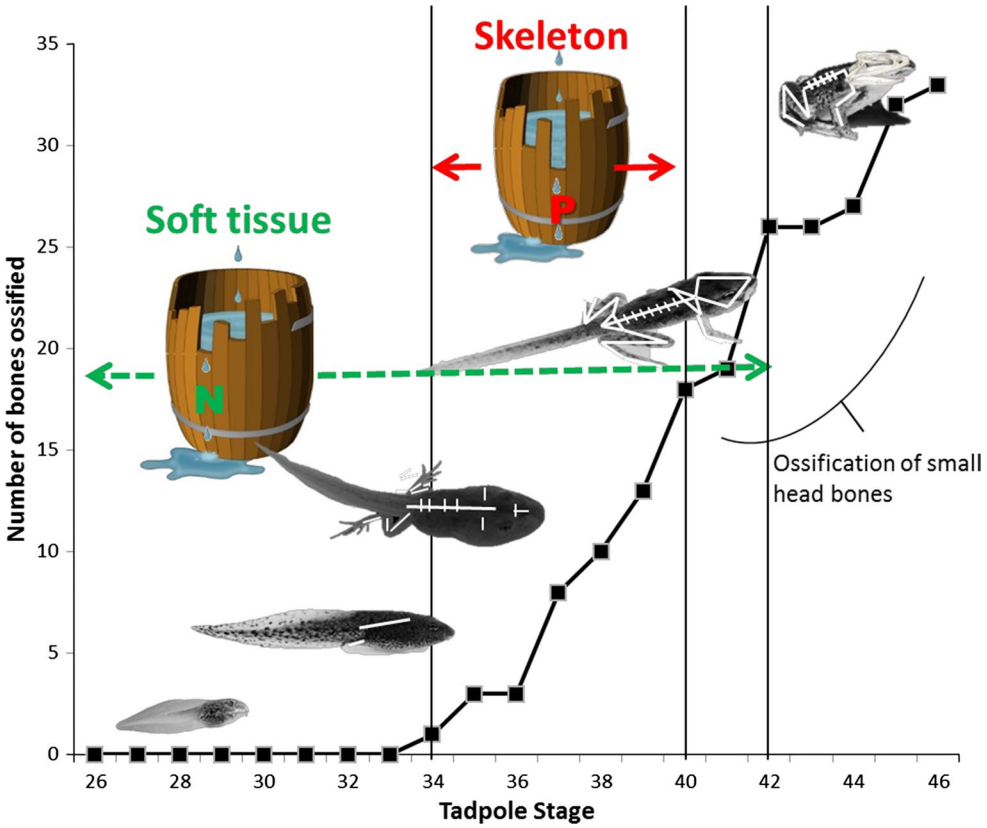

Fig. 1 Depicts the number of different bones ossified at each developmental stage of *Ranid* ontogeny. These results were compiled from Kemp and Hoyt (1969) who characterized the ossification sequence of developing leopard frogs *Rana pipiens*. We also provide a rough pictorial representation of ossification. White lines represent ossified structures. These lines are estimates of the location and size of the various ossified structures. The vertical line at Gosner stage 42 represents metamorphic climax when the upper limbs emerge from the body. Additionally, we included a pictorial representation of our hypothesized N and P limitation through ontogeny using Liebig's Barrel as an analogy. The shortest stave in the barrel corresponds to the most limiting element for growth during the specified time interval

0.17 % dry mass) in artificial tadpole diets. Hence, there were a total of four treatments, including high nitrogen/high phosphorous (HNHP, C:N = 6.7, C:P = 71), high nitrogen/low phosphorous (HNLP, C:N = 6.7, C:P = 253), low nitrogen/high phosphorus (LNHP, C:N = 37, C:P = 65), and low nitrogen/low phosphorous (LNLP, C:N = 37, C:P = 232). Diets were created by TestDiet (Richmond, IN, USA) by manipulating concentration of whey protein (added for high N) and potassium phosphate (added for high P) such that the concentrations of all mineral elements other than N and P were held constant. The test diets were based on Laboratory Diet 5321 (TestDiet; Richmond, IN, USA). This diet is very similar to rabbit chow used in mesocosm experiments (Stoler and Relyea 2011), and is also similar to diets used for feeding tadpoles in laboratory trials (Paden et al. 2011). For example, senescent leaf litter %N and %P (dry mass) ranged from 0.38 to 2.89 and 0.065 to 0.615 %, respectively, among 48 species examined (Ostrofsky 1997). Tadpole algal resources tend to have a higher nutrient content; N ranging between 0.94 and 5.5 % dry mass (Kupferberg et al. 1994) and P ranging between 0.13 and 2.17 % dry mass (Sundbom and Vrede 1997), whereas animal tissue (e.g., zooplankton) can contain upwards of 12 % N and 1.8 % P (Andersen and Hessen 1991).

We raised individual wood frog tadpoles in plastic cups containing artificial spring water (Cohen et al. 1980) assigned to one of the four diet treatments. At the initiation of the experiment, each diet had 60 replicates containing 250 ml of water and a single tadpole. We began the experiment immediately after tadpoles reached free swimming stage and resorbed their gills (Gosner stage 26). We fed tadpoles at a rate of 15 % of their body weight throughout the entire experiment as determined by recording the blot-dry mass of a subset of tadpoles from each food treatment before feeding. Every two days, we changed the water and fed each replicate. On several instances throughout the study, we increased the volume of water in all remaining replicates up to 500 ml to accommodate the increasing size of the tadpoles. Every three to five days, we randomly and destructively sampled a subset of four replicates from each treatment for developmental staging. Due to some mortality, actual sample sizes ranged from two to four replicates per staging event. After staging, we euthanized tadpoles by placing them in a −20 °C freezer. We later removed, thawed, and dried tadpoles at 60 °C for 24 h to determine their final dry mass. The same individual tadpoles were used for both types of analysis as described below.

Analysis of the full time series: non-linear growth and development

To test how growth rate (i.e., mass gain through time) and development rate (i.e., progression of development

stages through time) were affected by resource N and P, we employed regression analysis. First we tested for non-linear effects of time on tadpole mass and developmental stage using polynomial regression. We found that mass and developmental stage were non-linear functions of time based on significant third-order polynomial effects of time (mass–time^3: $F_{1,228} = 63.1$, $P < 0.001$; stage–time^3: $F_{1,228} = 26.197$, $P < 0.001$). We also analyzed mass as a function of developmental stage to provide an alternative view of how mass changes through ontogeny. This relationship was also non-linear (mass–stage^3: $F_{1,228} = 16.519$, $P < 0.001$). Therefore, we analyzed the full time series of each relationship (mass–time, stage–time, and mass–stage) using a modified form of a logarithmic growth function [Eqs. 1, 2 and 3 (Larson and Edwards 2009)].

$$M(t) = \frac{M_C \cdot M_0 e^{gt}}{M_C + M_0 \cdot (e^{gt} - 1)} \quad (1)$$

$$S(t) = \frac{(S_C - 25)(S_0 - 25)e^{dt}}{(S_C - 25) + (S_0 - 25)\left(e^{dt} - 1\right)} + 25 \quad (2)$$

$$M(x - 25) = \frac{M_C \cdot M_0 e^{g(x-25)}}{M_C + M_0 \cdot \left(e^{g(x-25)} - 1\right)} \quad (3)$$

In these models, $M(t)$ represents changes in mass over time (i.e., growth rate), $S(t)$ represents changes in developmental stage over time (i.e., development rate), and $M(x)$ represents changes in mass as a function of developmental stage. M_C and S_C are mass and stage at metamorphic climax, respectively. As determined by our analysis of a subset of 20 tadpoles prior to the experiment, we set $M_0 = 0.43$mg as the initial tadpole mass and $S_0 = 26$ as the initial Gosner stage. To model development as a function of time (Eq. 2), we assumed that $S_C = 42$, the stage at which tadpoles produce visible forelimbs, stop feeding, and begin to resorb their tails (Duellman 1986). The value of $S_C = 42$ is also very close to the fitted value of this parameter (41.999 ± 0.493) as supported by model optimization (Appendix A: Fig. S1a). For Eqs. 1 and 2, $t = 0$ was defined as the first day measurements were taken. For Eqs. 2 and 3, we subtracted 25 from all Gosner stages to make the starting stage $x = 26 - 25 =$ Taylor–Kollros stage 1 (Taylor and Kollros 1946), to ensure that each model approximated exponential development/growth at the beginning of larval development.

Within the framework of each model (Eqs. 1, 2, and 3), g and d are the growth and developmental rate parameters, respectively, t represents time (in days), and x represents the Gosner stage (in the model of growth as a function of development). We defined parameters g, d, and M_C as linear functions of the two experimental treatments and their interaction (N level, P level, and N × P) where:

$$g \sim \mu_0 + \mu_1 \mathrm{N} + \mu_2 \mathrm{P} + \mu_3 (\mathrm{N} \times \mathrm{P}) \quad (4)$$

$$M_C \sim \gamma_0 + \gamma_1 \mathrm{N} + \gamma_2 \mathrm{P} + \gamma_3 (\mathrm{N} \times \mathrm{P}) \quad (5)$$

$$d \sim \sigma_0 + \sigma_1 \mathrm{N} + \sigma_2 \mathrm{P} + \sigma_3 (\mathrm{N} \times \mathrm{P}) \quad (6)$$

to test whether these predictors had main or interactive effects on the fitted value of each parameter. Parameters μ, γ, and σ represent coefficients for the intercept, main and interactive effects of Eqs. 4, 5, and 6, respectively.

We assessed the contribution of each predictor variable by comparing Akaike Information Criterion (AIC) values for nested models with or without each predictor, using a procedure analogous to Type II sums of squares to assess the contributions of main and interactive effects (See Eqs. 4–6). In this analysis, we started with the most complex model (Eq. 4–6) and used standard backward selection procedure to simplify each model, removing predictors at each step if their removal decreased the model AIC. We always retained main effects if they were marginal to an interaction term that improved the model AIC (Nelder 1977). We used function "nls" (non-linear least squares) from the based package of program R (R Core Team 2014) for model optimization.

Analysis of discrete developmental periods: linear models

Although the non-linear models provided good descriptions of the full time series of tadpole growth and development, they assumed the rate parameters were constant throughout ontogeny. This made the non-linear models inappropriate for testing hypothesized changes in growth and developmental rates during specific periods of ontogeny. Changes in mass or developmental stage were approximately linear within each period of interest, so we used general linear models (as described below) to test for effects of dietary N and P within each discrete developmental period. Specifically, we ran three separate analyses of N and P effects on mass and stage during three discrete periods of tadpole development, corresponding to days 1–25, 31–51, and 54–68. For all treatments, these time windows approximately corresponded to Gosner stages 26–33, 34–40, and 41–42, respectively. During the initial development period (~Gosner stages 26–33), the tadpoles are primarily putting on muscle mass and growing cartilage, without ossification (Duellman 1986). The second time period (~Gosner stages 34–40) is when tadpoles grow most of their large limb bones and ossify cartilaginous structures in both the axial and appendicular skeleton (Fig. 1). The third time period (~Gosner stages 41–42) is characterized by metamorphic

climax and the ossification of smaller bones of the head (Kemp and Hoyt 1969). In each of the three analyses, we generated two models that tested for main and interactive effects of N and P on mass or stage, including time as a covariate. Similar to the non-linear analyses, we also generated a third model that analyzed mass as a function of developmental stage for each developmental period. We used a type II sums of squares procedure to assess predictor significance. The coefficient for each main effect variable was derived from the full model used to assess its significance, to ensure that it correctly indicated whether the effect was positive or negative.

Results

Full time series: non-linear models

For tadpole mass as a function of time, the best model included significant positive effects of dietary P on the growth rate parameter g and of dietary N on mass at metamorphosis climax M_C (Table 1; Fig. 2a, Appendix A: Table S1, Figs. S1c, d and S2a, b). There was no effect of dietary N on the growth rate parameter or of dietary P on mass at metamorphosis. For tadpole development as a function of time, the best model included significant positive effects of dietary N and P on the developmental rate parameter d (Table 1; Fig. 2b, Appendix A: Table S2 and Fig. S1b). For tadpole mass as a function of developmental stage, the best model included a significant negative effect of dietary N on the growth rate parameter and a significant positive effect of dietary N on mass at metamorphosis (Table 1, Appendix A: Table S1, Fig. S2d). The negative effect of dietary N on the per-stage growth rate parameter was likely due to faster development of tadpoles in the HN treatments, which led to a slower rate of growth per stage early in development. However, tadpoles under LN reached their mass asymptote at an earlier stage and at a much smaller size relative to HN, leading to overall slower growth rates per stage in the LN treatment late in development (Table 1, Appendix A: Fig. S2d).

Discrete developmental periods: linear models

Depending on the developmental period examined, there were differential effects of N and P on mass and stage (Table 2). As expected, there was a positive effect of time in all models for the first two developmental periods, when tadpoles experienced the fastest rates of growth and development (Table 2; Fig. 2).

During the first period of larval development (days 1–25), we detected no significant main or interactive effects of N or P on tadpole mass (Fig. 3a; Table 2). In contrast, we found an effect of N and time on stage, but there were no other main or interactive effects (Fig. 3b; Table 2). Exploration of our data revealed that there was greater

Table 1 Final model parameter estimates and summary statistics (±standard error)

Parameter	Estimate	Standard error	$F_{1,228}$	P
Model: mass–time				
$\mu_2 P$	0.019	0.009	4.557	0.034
μ_0	0.120	0.004	595.230	<0.001
γ_1 N	0.008	0.001	146.590	<0.001
γ_0	0.044	0.002	278.130	<0.001
Parameter	Estimate	Standard error	$F_{1,230}$	P
Model: stage–time				
σ_1 N	0.002	0.001	49.488	<0.001
σ_1 P	0.008	0.004	4.173	0.042
σ_0	0.283	0.003	1642.300	<0.001
Parameter	Estimate	Standard error	$F_{1,228}$	P
Model: mass–stage				
$\mu_1 N$	−0.010	0.005	4.601	0.033
μ_0	0.533	0.025	848.340	<0.001
γ_1 N	0.006	0.001	85.635	<0.001
γ_0	0.048	0.002	325.580	<0.001

The F statistic and P value for each parameter was calculated using a procedure analogous to Type II sums of squares, in which the final "full" model was compared to a "reduced" model from which the parameter had been removed (i.e., set equal to 0). Parameters are defined in Eqs. 4, 5, and 6

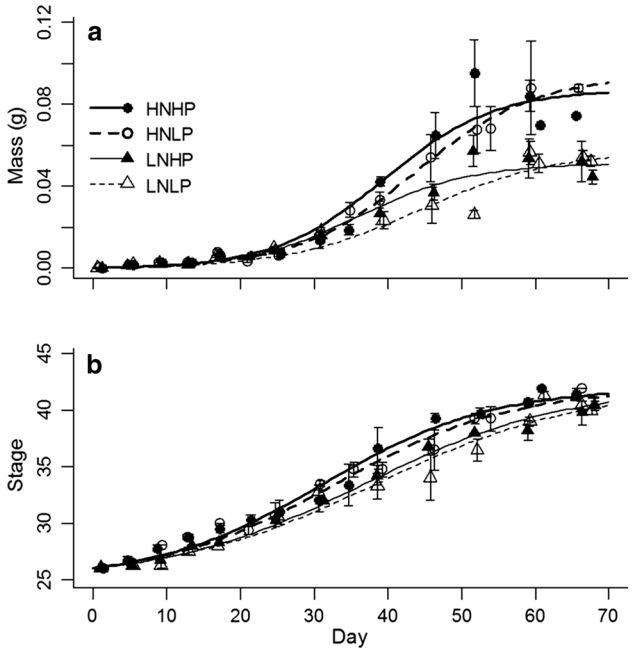

Fig. 2 Logistic growth models fit to each of the food treatments for tadpole mass (**a**) and developmental stage (**b**) through time. Each point represents the mean (±SE) tadpole mass or stage for that particular sampling period. Analysis used each individual as the unit of replication

early development in the high-N treatment. Because of the faster development with high N with no apparent effects on growth, tadpoles with higher dietary N were actually slightly smaller at any given stage during this developmental period (Appendix A: Table S1, Fig. S3a).

During the second developmental period (days 31–51), we found significant and positive effects of dietary N, P, and time on tadpole mass, and also interactions of both N and P with time (Table 2; Fig. 3c). Regarding effects on stage, we found significant positive effects of dietary N and time, as well as a P × time interaction (Table 2; Fig. 3d). Dietary N also had a positive main effect (and N × stage effects) on the mass per stage during this developmental window (Appendix A: Table S1, Fig. S3b).

During the last developmental period (days 54-68), we found a positive main effect of N on tadpole mass, and a significant interaction between N and time (Table 2). This interaction indicates a positive effect on tadpole growth through this time period, which slowed as tadpoles approached metamorphosis (Table 2, Fig. 3e, Appendix A: Table S1, Fig. S3c). Tadpoles in both LN treatments appear to have reached an asymptotic size during this period. We also found positive main effects of dietary N and time on tadpole stage during this period (Table 2; Fig. 3f,), indicating a shorter time to metamorphosis in the HN treatments.

Discussion

Our results indicate that the sensitivity to nutrient limitation of N and P on growth and development of wood frogs changes as a consequence of ontogeny. Specifically, early in development, the rate of development appears to have been most sensitive to N limitation, but there were no effects of dietary P. In contrast, both growth and development were sensitive to both N and P during the middle stages of ontogeny (i.e., Gosner 34–40), indicating co-limitation by both nutrients during this period. During the third and final period of tadpole development, tadpole growth and development were once again most sensitive to N limitation, with no evidence for P limitation. It is worth noting that the lack of P limitation during the first and third stages might be a result of phosphate leaching from food. However, this would not render the P biologically unavailable, as tadpoles are able to graze biofilms that would absorb the P following leaching. In addition, the presence of co-limitation during the second developmental stage indicates that much of the added P was still available for tadpole consumption.

The results of both our non-linear and linear models support our hypothesis that tadpole growth and development were likely co-limited by and N and P during the second developmental period due to the increased ossification of bony structures. This was evident by the positive affect of P on the growth rate parameter in the non-linear model for mass as a function of time, and the N × time and P × time interactions in the linear models for mass during the second time period. While the results of the non-linear models indicate joint limitation of N and P through larval development, the linear models elucidate the specific limitations of N and P through the different time intervals. Furthermore, the non-linear models could be used by future investigators to generate quantitative predictions of tadpole growth and development rates feeding on different nutrient levels.

High dietary N led to greater mass at metamorphosis and faster developmental rates in this study, consistent with previous findings that tadpoles fed N-rich leaf litter metamorphose in less time with greater mass (Maerz et al. 2010; Cohen et al. 2012; Stephens et al. 2013; Stoler and Relyea 2013). Recently, Stephens et al. (2015) demonstrated that these effects of litter N on larval wood frog growth can be quantitatively predicted using an empirical model derived from ES theory, using tadpole body stoichiometry and rates of consumption and excretion. These consistencies among studies demonstrate that dietary N is an important driver of the size at amphibian metamorphosis. Larger size at metamorphosis and earlier time to metamorphosis improve terrestrial survival and reproductive success in amphibians (Semlitsch et al. 1988; Berven 1990). Dietary N could, therefore, have important consequences for amphibian population dynamics.

Based on the results of our linear models, the temporal availability of N and P within temperate wetlands might be as important to tadpoles as their overall availability. Litter detritus, a low-nutrient food source, is readily available early in the season in closed-canopy wetlands (Rubbo et al. 2006), whereas more nutritious foods like algae and bacterial biofilms are likely to be in relatively short supply until later in spring (Rosemond 1994). For early-breeding species like wood frogs, phosphorus limitation might have potentially damaging effects on their morphology. Indeed, we observed that many recently metamorphosed tadpoles from LNLP treatments had thin-looking emaciated legs relative to high-P treatments (J.P.S. personal observation). Such morphological change is not surprising given that other studies have found substantial morphological plasticity among tadpole and metamorphic wood frogs in response to food quality manipulation (Stoler and Relyea 2013; Stoler et al. 2015). Hence, future studies should assess the effects of tadpole diet quality on metamorphic morphology and the potential fitness consequences of these changes.

Tadpoles might meet rheostatic shifts in nutrient demands by changing foraging behavior in response to shifts in nutritional requirements. For example, wood frog tadpoles might change their food preferences as their nutritional needs change throughout ontogeny (Schriever and Williams 2013). Indeed, tadpoles can supplement their diet with P-rich foods like animal protein (Crump 1983; Babbitt et al. 2000), and might do so during the height of bone ossification. Most North American tadpoles consume primarily low-quality food items like detritus and algae (Altig 1999; Altig et al. 2007), and wood frogs appear to be adapted to handle especially poor quality food (Schiesari 2004, 2006; Stephens et al. 2015). However, wood frog tadpoles are observed to consume dead conspecifics, a very high-N and high-P food source, if resources are scarce (Jefferson et al.

Table 2 Results of linear models through tadpole development broken up into three time intervals (days 1–25, 31–51, and 54–68), corresponding roughly to periods of tadpole development (Gosner stages 26–33, 34–40, and 41–42)

Mass				Stage		
Days 1–25	Coefs (II)	$F_{1,92}$	P	Coefs (II)	$F_{1,92}$	P
P	0.274	0.105	0.746	0.284	0.609	0.4373
N	−0.009	0.013	0.908	0.158	23.987	**<0.001**
Time	0.375	184.525	**<0.001**	0.196	388.680	**<0.001**
P × time	0.086	0.635	0.428	0.010	0.045	0.833
N × time	−0.017	3.148	0.079	0.004	1.184	0.279
P × N	0.153	0.194	0.661	−0.049	0.106	0.746
P × N × time	0.033	0.544	0.463	0.009	0.236	0.629
Days 31–51	Coefs (II)	$F_{1,58}$	P	Coefs (II)	$F_{1,58}$	P
P	21.190	7.207	**0.009**	1.974	3.449	0.068
N	3.706	28.648	**<0.001**	0.355	14.501	**<0.001**
Time	2.182	97.893	**<0.001**	0.269	82.092	**<0.001**
P × time	3.578	12.021	**<0.001**	0.292	4.421	**0.039**
N × time	0.419	21.571	**<0.001**	0.017	2.161	0.147
P × N	−0.162	0.003	0.960	−0.238	0.297	0.587
P × N × time	0.071	0.028	0.867	−0.001	0.000	0.985
Days 54–68	Coefs (II)	$F_{1,58}$	P	Coefs (II)	$F_{1,58}$	P
P	−11.799	1.804	0.185	−0.279	0.112	0.738
N	5.546	25.897	**<0.001**	0.330	10.230	**0.002**
Time	0.271	0.304	0.584	0.146	9.848	**0.003**
P × time	−1.519	0.435	0.512	0.190	0.759	0.387
N × time	0.421	4.235	**0.044**	0.012	0.389	0.535
P × N	−3.135	0.380	0.540	0.587	1.486	0.228
P × N × time	−0.006	0.000	0.995	−0.145	2.575	0.114

We present the models for tadpole mass and stage as a function of time, N, P, and their interactions. Model structure is the same for both mass and stage. To provide directionality for model parameters, we report coefficients from type II sums of squares used to generate F and P values.

Bold values indicates significant P-values ($P < 0.05$)

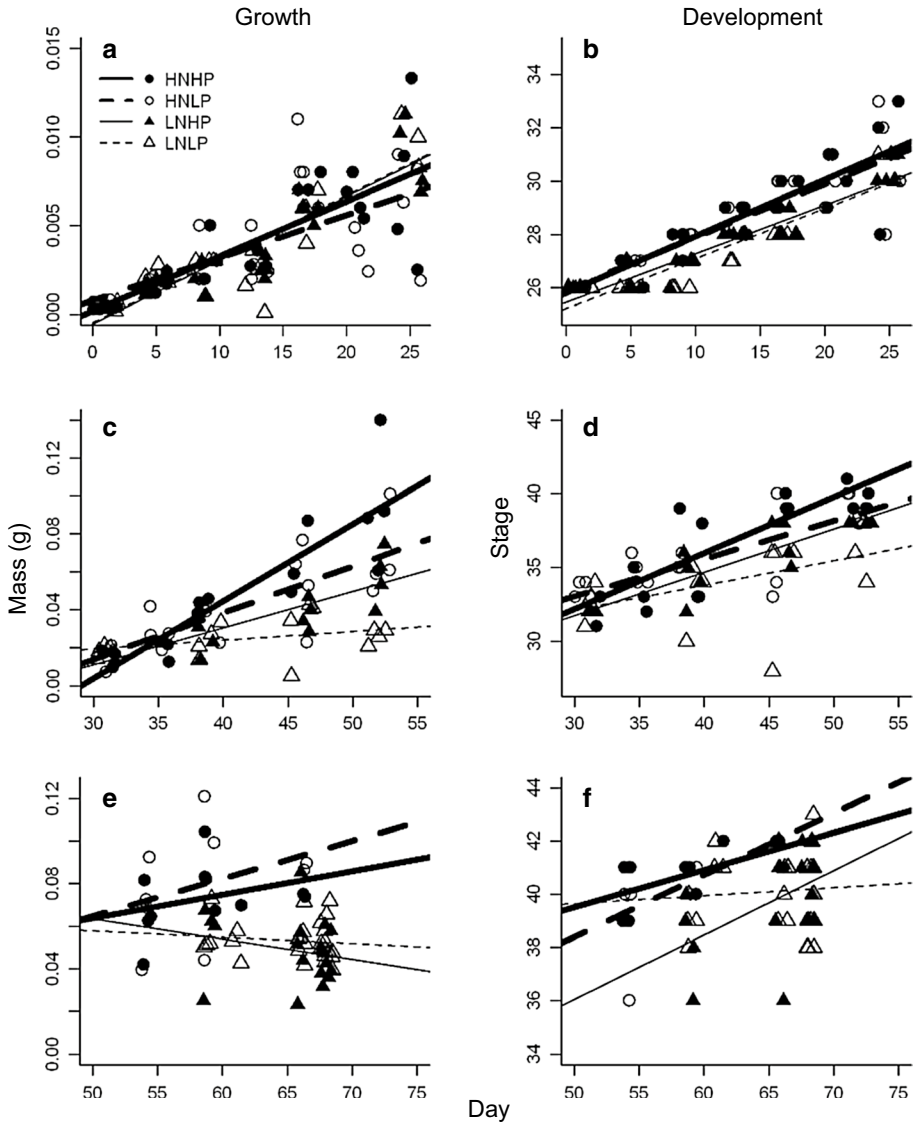

Fig. 3 The effects of N and P on tadpole growth (**a**, **c**, **e**) and development (**b**, **d**, **f**) decomposed into three developmental periods: days 1–25 (**a**, **b**), 31–51 (**c**, **d**), and 54–68 (**e**, **f**). Each data point represents the value of one individual sampled at that time point. Analyses used each individual as the unit of replication

2014a, b). It would be interesting to determine if rates of tadpole cannibalism increase during periods of rapid bone development.

N and P are important elements for vertebrate growth and development, but they might not be the only limiting nutrients in tadpole diets. For example, inadequate calcium (Ca) during vertebrate development can significantly reduce the mineralization and tensile strength of bones, sometimes resulting in decreased overall growth (Shapiro and Heaney 2003). Ca ions can be absorbed through tadpole gills, so Ca content in food is likely to be non-limiting for tadpole growth and development in wetlands with high dissolved Ca (Baldwin and Bentley 1980). However, dissolved Ca levels vary dramatically and might serve as a limiting nutrient in Ca-poor wetlands (Joniak et al. 2007). This is a potentially important context dependency, and it would be interesting to measure tadpole growth and development with artificial Ca-poor diets to determine whether shifts in Ca-limitation during ontogeny parallel observed shifts in P limitation. Additionally, as Sperfeld et al. (2012) points out, studies addressing co-limitation are lacking for consumers relative to autotrophs. In fact, it has been found that *Daphnia* do not strictly follow Liebig's minimum rule under situations of co-limitation, which is often applied to autotroph growth (Sperfeld et al. 2012).

Consequences for ES theory

The concept of the homeostatic consumer, whereby consumers maintain constant body stoichiometry despite feeding on resources of varying quality, has been a fundamental tenet of ES theory (Sterner and Elser 2002). Because many classic models of growth in ES theory hinge on constant consumer body stoichiometry (e.g., the "minimal model"),

the phenomenon of rheostasis has not been incorporated into the ES framework. However, our study demonstrates that wood frog tadpoles are rheostatic, maintaining variable stoichiometric relationships relative to their resources as they pass through ontogenetic stages with different levels of bone ossification. Ossification of connective tissue and cartilage is unique among vertebrates and can be influenced by both life history [aquatic vs. terrestrial lifestyle (Wall 1983)] and environmental factors [e.g., temperature, food availability, competition (Gomez-Mestre et al. 2010)]. Prior studies have identified situations in which consumers are not strictly homeostatic, changing their body stoichiometry in response to variation in food quality (Persson et al. 2010; Wang et al. 2012). Other studies have shown that consumers can change body stoichiometry in response to environmental changes (Small and Pringle 2010; Feijoó et al. 2014) or as a consequence of ontogeny (Tiegs et al. 2016). The results of this study highlight rheostasis as an important framework for understanding changes in stoichiometry and nutrient limitation during early vertebrate development, and the need for similar studies of vertebrates with different life history strategies and from different environments.

Consequences for amphibian metamorphosis and nutrient cycling

Amphibian metamorphosis is a deeply studied phenomenon because it has acted as an important model for general vertebrate development (Smith-Gill and Berven 1979; Wilbur 1980; Travis 1984; Denver et al. 2002). Prior studies have highlighted the role of the endocrine system in this process, demonstrating the importance of growth hormone (Brown and Frye 1969), thyroid hormone (Yaoita and Brown 1990), and corticosterone (Denver 1997a) in mediating the transformation from tadpole to frog (Denver 1997b). Dietary N has been shown to influence pituitary development and hormone synthesis (Harel and Tannenbaum 1993), which in turn affects metamorphic traits in amphibians (Coady et al. 2010; Liess et al. 2013). Thus, the positive effects of N on tadpole development in this study might have been mediated by changes in hormone synthesis. However, less is known about how dietary P influences hormone production, and future studies addressing the influence of N and P on tadpole hormone production would be valuable to determine the proximate mechanism for P effects on tadpole development.

Aquatic vertebrates can supply significant amounts of inorganic N and P to primary producers via nutrient excretion, sometimes creating hot spots of productivity (Vanni et al. 2002; McIntyre et al. 2008; Capps and Flecker 2013; Wheeler et al. 2015). Tadpole excretion can have similar effects in ponds because their densities are often very high (Kupferberg 1997a; Capps et al. 2014). Ontogenic variation in N and P growth limitation might have temporal effects on nutrient cycling (Schindler and Eby 1997; Pilati and Vanni 2007) in wetlands inhabited by wood frogs, because this species has a short breeding season leading to synchronized tadpole development (Berven 1990, 2009). Our results show that tadpole growth becomes more P limited during these middle developmental stages, which suggests that they are likely retaining more P in their bodies and, therefore, excreting less P. Consistent with this hypothesis, Tiegs et al. (2016) found increased body P and decreased mass-specific P excretion during this period of tadpole development. These results are most likely driven by increased P deposition in ossifying bones. Consequently, this might lead to a mid-spring period of increased P limitation for primary producers.

A potential limitation of our experimental design was that there could be carry over effects of nutrient storage from one developmental window to the next. For example, it is possible that tadpoles raised in high-P treatments could have stored extra P during the initial growth window to be used during the ossification window. However, we think it is unlikely that early-stage tadpoles can store excess P for later use. Although they might be able to accumulate N as stores of protein within cells, there are no similar or substantial stores of P within tadpoles prior to bone development. Furthermore, Tiegs et al. (2016) showed that tadpoles actually excreted both N and P at higher rates prior to the ossification window, suggesting that excess nutrients are excreted rather than stored during this early period of ontogeny.

Conclusion

Understanding the functional role of organisms within their environment is a fundamental aspect of ecosystem ecology. Ecological stoichiometry has given the field a powerful tool to help us better understand nutrient and carbon dynamics within ecosystems (Sterner and Elser 2002). However, ecological stoichiometry studies rarely focus on changes in nutrient demand, allocation, and availability through time. Consequently, understanding how tadpole body stoichiometry and nutrient demands change through ontogeny will give researchers a better understanding of their functional roles within these systems (i.e., nutrient cyclers or nutrient sinks). This study indicates that tadpoles have differential nutrient requirements through development, and as a consequence, likely alter the rates at which they sequester or excrete nutrients depending on their stage. Because of this, we point out that rheostasis is a more precise physiological term to describe physiologically the nutrient demands and limitations through tadpole ontogeny. Finally, temporary forested wetlands remain a relatively understudied body of

water relative to rivers and lakes; however, they can contain large numbers of organisms, and are important to overall amphibian population dynamics. Thus, understanding tadpole stoichiometry and nutrient demands may give us a better understanding of temporary forested wetland nutrient dynamics in general.

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Author contribution statement JPS, ABS, KAB, SDT, and TRR conceived and designed the experiment. JPS, JS, AF, and TRR analyzed the data. JPS, ABS, KAB, and AF ran the experiment. JPS wrote the manuscript; all other authors provided editorial advice.

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