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Canopy cover and anurans: nutrients are the most important predictor of growth and development

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Abstract: Bottom-up effects such as light and nutrient availability can have large impacts on primary producer quantity and quality, which is then translated into the growth and development of consumers. The use of ‘canopy cover’ as a bottom-up predictive factor is a broad categorization, as canopy cover controls both the amount of light allowed into a pond and the nutrient load through leaf litter. To test how light and nutrients influence pond ecosystems, we manipulated inorganic nutrients and light in a 2 x 3 full-factorial, large-scale mesocosm experiment. Larval bullfrogs were reared for six weeks at low densities and then assessed for development, growth, and survival at the end of the experiment. We also collected weekly samples of potential food resources (phytoplankton and periphyton) for the estimation of algal production and stoichiometric quality (carbon:nitrogen:phosphorus). Light had strong effects on food resource quality; however, resource quality did not significantly predict tadpole growth or development. Instead, nutrients seemed to be the most important factor as a stimulator of total algal primary production and some unknown pathway, which in turn affected tadpole development. Ours is the first study to investigate canopy cover using a comprehensive causal model, and our results suggest in regards to tadpole growth and development, canopy cover is important mainly as a source of nutrients to ponds.

Key words: tadpoles, ecological stoichiometry, structural equation modeling, canopy cover, bottom-up effects, *Rana*, *Lithobates*

Introduction

Freshwater ponds are common landscape features. In some regions like the glaciated northern U.S. and Canada they comprise a major portion of all freshwater ecosystems (e.g. vernal pools; Calhoun and DeMaynadier 2007; Colburn 2004). These ecosystems have a high degree of contact between benthic and pelagic food chains and support diverse food webs consisting of phytoplankton, periphyton, microbes, macrophytes, macroinvertebrates, and vertebrates. In fact, the cyclical nature of freshwater ponds creates habitat that supports species either not found in other habitat types or that attain their greatest population sizes in ponds (Williams 1997). Ponds can function as an abundant source of energy to terrestrial environments (Gibbons et al. 2006) or receive energy from terrestrial sources (Regeister et al. 2006), thereby linking aquatic and terrestrial ecosystems. Habitat modifications near ponds (e.g. canopy loss) have strong potential to reduce vertebrate and invertebrate diversity (Findlay and Houlihan 1997; Colburn et al. 2008).

In freshwater systems, loss of consumer diversity (especially sensitive species, e.g., dragonflies and amphibians) is occurring rapidly and with large ecological consequences (Calhoun and DeMaynadier 2007). Amphibian larvae are dominant consumers in freshwater ponds (Wilbur 1980), reach high densities (Gibbons et al. 2006), strongly influence ecosystem properties (Iwai et al. 2012; Seale 1980), serve as important prey for other species (Wells 2010), affect species interactions and community structure (Morin 1983; Wilbur 1997), and can represent a substantial export of biomass to terrestrial systems (Gibbons et al. 2006). Alterations in forests affect canopy cover around and over ponds, and therefore inputs of light and leaf litter, the base energy sources for pond food webs (Colburn et al. 2008). There is increasing evidence that open canopy ponds better support most amphibian and invertebrate communities.

For tadpole primary consumers, open canopy generally results in greater species diversity and survival (Skelly et al. 2002; Werner et al. 2007), presumably because higher light intensity leads to increased energy in the green (algal-based) pathway (Skelly et al. 2002). Indeed, mechanistic studies on tadpoles have suggested that decreased food quality and availability are major factors in reducing amphibian performance (Maerz et al. 2010; Skelly and Golon 2003). Food supplementation in closed canopy ponds has increased growth rate of tadpoles (Schiesari 2006; Skelly et al. 2002; Werner and Glennemeier 1999), and other studies have found a strong positive relationship between the amount of periphyton and performance of tadpoles (Hocking and Semlitsch 2008).

Both light and nutrient availability are affected by pond canopy cover, and can alter the elemental quality of algae for herbivores (Sterner et al. 1997). The light:nutrient hypothesis predicts that primary producers receiving a high supply of light relative to nitrogen (N) and phosphorus (P) are nutrient-poor (high carbon:nutrient ratio, i.e., C:N and C:P). In contrast, algae receiving a low supply of light and a high supply of N and P are nutrient-rich (i.e., low C:N and C:P ratios; Sterner and Elser 2002; Sterner et al. 1997). Algal C:nutrient content determines elemental food quality for herbivores; when consuming food with high C relative to N or P, herbivores may be limited by these nutrients rather than C (energy), and herbivore growth may be lower compared to when consuming food with low C:N or C:P. Thus, the balance of light and nutrients can affect energy flow at the algae-herbivore link (Sterner et al. 1998; Sterner and Elser 2002).

There is a paucity of research on how food quality and quantity affects amphibians. Some controlled diet studies have demonstrated that tadpoles fed diets rich in proteins and lipids developed faster and were larger than tadpoles that were fed carbohydrate-rich diets (Álvarez

and Nicieza 2002; Kupferberg 1997) and that tadpoles fed food enriched in N and P grow faster (Liess et al. 2013). Two recent studies have documented the impacts of different leaf C:nutrient ratios on anuran development times and morphology. Stoler and Relyea (2013) found that differences in C:N leaf quality induced changes that were as large or larger than resource quantity effects in wood frogs (*Lithobates sylvaticus*), while Stephens et al. (2013) found that in the same species, mass at metamorphosis was best explained by leaf litter quality (C:N and C:P) and primary producer biomass. Using leaf litter as a nutrient source can be problematic because leaves can also release toxic secondary compounds (Earl et al. 2012). To the best of our knowledge, no one has directly examined the role of food resource C:nutrient quality in larval amphibian growth and development in the absence of leaf litter (but see Liess et al. 2013). Ponds receive nutrients in both inorganic form (e.g., via runoff) and organic form. Although leaf litter nutrients enter aquatic ecosystems in organic form, previous work has shown that leaf litter input can alter dissolved nutrient supply (Earl and Semlitsch 2013; Stephens et al. 2015), therefore studies on how inorganic nutrients affect amphibian growth are needed.

We sought to separate the effect of canopy cover into its distinct bottom-up mechanisms, light and nutrients. Thus we tested the role of light and inorganic nutrient supply on anuran tadpole development and growth in a rigorous, replicated design. Our study was part of a larger project (Rowland et al. 2015) that examined how light and nutrient variation interactively affect algal quality and quantity, and thereby food chain efficiency in pelagic (phytoplankton to larval fish) and benthic food chains (periphyton to tadpoles). For this study, we sought to determine how the two main components of canopy cover (light and nutrients) separately influence pond resource quantity and quality, and through this pathway the development and growth of tadpoles. Few previous studies have created a predictive model for the effects of canopy cover using such

specific data. We hypothesized that nutrients were most important to tadpoles through stimulating primary production, but that the balance of light and nutrients would be equally important in determining resource quality. Further, we predicted that if food quantity was not a limiting factor, then food quality would be a significant predictor of anuran growth and development. As the tadpoles were reared at very low densities, we expected weak intraspecific competition, and predicted that the treatments with the best food quality would have the highest rates of tadpole growth and development.

Materials and Methods

Experimental design

This experiment was conducted at Miami University's Ecology Research Center (ERC), using outdoor, high-density polyethylene mesocosms (5000 L, 2.25 m diameter, 1.4 m depth) equipped with airlift pumps to constantly circulate water. We used a 2x3 full-factorial design with low and high levels of dissolved inorganic nutrient supply (LN, HN), and low, intermediate, and high levels of light intensity (LL, IL, HL) for a total of six treatments, each with four replicates.

We filled all mesocosms on 23-24 May 2008 with water from an oligotrophic pond, pre-screened through a 150 μm mesh to remove large invertebrates. We then inoculated with phytoplankton and zooplankton obtained from the nearby eutrophic Acton Lake, to provide a diverse plankton assemblage (as documented in Dickman et al. 2008). On 24 May 2008, all high nutrient treatment mesocosms received an initial nutrient pulse of 150 $\mu\text{g N}\cdot\text{L}^{-1}$ as NH_4NO_3 and 15 $\mu\text{g P}\cdot\text{L}^{-1}$ as $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$. This high initial nutrient spike was designed to jump start primary producer growth and minimize local extinctions of Acton Lake species accustomed to high

nutrient supply. Subsequently, we added $50 \mu\text{g N}\cdot\text{L}^{-1}$ and $5 \mu\text{g P}\cdot\text{L}^{-1}$ three times per week to the high nutrient treatments. The fertilization rate after the initial pulse is comparable to inputs received by Acton Lake from its agricultural watershed (Vanni et al. 2001). No additional nutrients were added to the low nutrient treatments. We manipulated light supply by covering mesocosms with shade cloth, yielding light intensities equal to 10, 43 and 75% of ambient light as measured by a LI-COR 1400 meter with a spherical quantum sensor and a deck cell.

We chose the American bullfrog (*Lithobates catesbeianus*) as our larval amphibian species because it is abundant, hardy, widespread, and typically coexists with fish (R.D. Semlitsch, *personal communication*). We collected egg masses from the ERC supply pond on 27 May 2008. Subsamples from six different clutches of similar laying date were pooled to ensure genetic diversity. Eggs were placed into a 1000 L holding tank filled with well water and leaf litter that had been inoculated with plankton two weeks prior. To reduce mortality of fragile organisms, we stocked tadpoles into mesocosms only after they developed to Gosner Stage 25 (Gosner 1960), at which time they reached the free-swimming stage and absorbed their external gills. On 9 June 2008, 50 tadpoles (mean body length = $12.6 \text{ mm} \pm 1.2 \text{ SD}$, $n = 25$) were added to each mesocosm, equivalent to $0.01 \text{ tadpoles}\cdot\text{L}^{-1}$ or $0.179 \text{ mg wet mass}\cdot\text{L}^{-1}$. Our densities were consistent with low natural densities (Cecil and Just 1979), as well as the lowest density in a mesocosm study that yielded the highest rate of development and shortest time to metamorphosis (Provenzano and Boone 2009). Low tadpole density allowed us to quantify production under weak intraspecific competition.

We also added 225 larval gizzard shad (*Dorosoma cepedianum*, mean length $11.1 \pm 2.3 \text{ mm SD}$) to each mesocosm, equivalent to $0.045 \text{ fish}\cdot\text{L}^{-1}$ or $0.180 \text{ mg wet mass}\cdot\text{L}^{-1}$, based on densities observed in Ohio reservoirs (Bremigan and Stein 2001) and long-term data collected on

Acton Lake (M. J. González, *unpublished data*). The fish were part of a larger study and should not have interfered with tadpole development, as bullfrogs are known to be distasteful to predators (Kruse and Francis 1977) and readily coexist with fish. Larval gizzard shad are obligate zooplanktivores (Drenner et al. 1982) and should therefore pose no predation or competition threat to tadpoles. However, all mesocosms were stocked with fish and so we are unable to assess any behavioral or developmental differences directly. Instead, we measured fish carbon standing stock ($\text{mg C} \cdot \text{m}^{-3}$) to use as an estimate of indirect fish effects in our causal model. See Rowland et al. (2015) for details on standing carbon stock estimates. All work was conducted according to Miami University Institutional Animal Care and Use Committee Protocol No. 747.

Sampling and nutrient measurements

We sampled mesocosms for phytoplankton biomass, primary production, and nutrients before fish and tadpoles were added but after the initial nutrient addition and light manipulations (30 May 2008); periphyton biomass was too low to quantify at that time. After fish and tadpoles were added, we sampled mesocosms weekly from 11 June to 16 July 2008 for algal-based parameters. We measured photosynthetically available radiation (PAR) just underneath the surface and at 1 m using a LI-COR 1400 meter (LI-COR, Lincoln, Nebraska USA) with a spherical quantum sensor and a deck cell, which was used to estimate vertical light extinction coefficients (k) that are needed for primary production calculations (see below). We also measured temperature and dissolved oxygen using a YSI model 58 meter (YSI, Yellow Springs, Ohio USA) just below the surface and at 1 m. Temperature and oxygen were very similar among all treatments during the experiment.

We collected integrated (0-1 m) water samples to determine phytoplankton (seston) biomass as chlorophyll *a* (chl *a*), and seston C, N, and P content. Periphyton was sampled using eight polyethylene strips that spanned the vertical water column, affixed to mesocosm walls (in each cardinal direction) with waterproof tape. Each week, we removed one strip from the same cardinal direction in each mesocosm and scrubbed off the periphyton into 2 L of filtered mesocosm water (Pall A/E, 1 μm nominal pore size), which was collected on the same day. This slurry was used to estimate periphyton primary production, chl *a*, C, N and P. We screened seston samples for C, N, and P through a 63- μm mesh to remove most zooplankton, then filtered the samples onto a pre-ashed Pall A/E glass fiber filter. It was not possible to screen periphyton samples due to its filamentous nature, but we removed visible macroinvertebrates from the samples during filtration. We froze chlorophyll samples in the dark, extracted them in acetone at 4°C, and then used a Turner TD-700 fluorometer (Turner Designs, Sunnydale, California USA) to get phaeophytin-corrected chl *a*.

We estimated phytoplankton and periphyton primary production rates (PPr) weekly using ^{14}C uptake, following the methods of Dickman et al. (2008). We added $\text{NaH}^{14}\text{CO}_3$ to mesocosm water samples and incubated them at a range of eight light levels and one dark bottle in the lab to generate chlorophyll-specific photosynthetic-irradiance (PI) curves. Using the PI curve, ambient light, estimated *k*, and chl *a* data, we estimated phytoplankton and periphyton PPr ($\text{mg C} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$) in each mesocosm using the programs PSPARMS and YPHOTO (Fee 1990). The latter interpolates PPr in between sampling dates using incident PAR, which we obtained from the meteorological station at the ERC (www.epa.gov/castnet) and adjusted for shading treatments. Since this process is very labor intensive, we only analyzed 2 of the 4 replicate mesocosms in each treatment on alternate weeks. We used a Perkin Elmer 2400 Series II CHN analyzer (Perkin

Elmer, Boston, USA) to analyze C and N content of phytoplankton and periphyton. To determine P content, we digested samples with HCl to convert particulate P to soluble reactive phosphorus (Stainton et al. 1977), and then quantified them using a Lachat QC 8000 FIA autoanalyzer (Lachat Instruments, Loveland, Colorado USA).

We weighted stoichiometric ratios to determine overall food quality for tadpoles because stable isotope analysis revealed no difference in isotopic signature between seston, periphyton, and tadpoles from the most disparate treatments (Rowland et al. 2015). Therefore, we calculated weighted C:N and C:P ratios using the relative contribution of phytoplankton and periphyton to total PPr. All stoichiometric ratios are presented in molar units. We recognize that ‘phytoplankton’ and ‘periphyton’ samples contain not only algae but also bacteria, detritus, and even small invertebrates, and that these other components can be consumed by tadpoles (Schiesari et al. 2009; Whiles et al. 2010). However, nearly all food available to tadpoles was derived from algal-based production (i.e., there were no inputs of organic matter). Therefore, algal quantity and quality likely determined, directly and indirectly, food quantity and quality for tadpoles.

Tadpole measurements

After six weeks, we ended the experiment and measured all tadpoles using dial calipers over a two-day period. We removed guts, dried and weighed at least twelve tadpoles per mesocosm to obtain a length-dry mass regression to use for tadpoles that were not weighed:

$$W = 0.0022 * e^{0.0669 * L} \quad (1)$$

where W is gutted dry mass (g), and L is total length (mm) ($r^2 = 0.93$, $N = 207$). Half of the surviving tadpoles were immediately frozen for nutrient analyses, so we were only able to assess them for developmental stage (Gosner 1960).

Data analysis

We performed all statistical analyses in R on data transformed to meet the assumption of normality. We also measured body length and width, tail length and width, and gut length on six tadpoles per mesocosm. However upon analysis, we found no significant differences among treatments in tadpole morphology, so instead we focused on size and development. We tested for the influence of light and nutrients on algal parameters using a two-way analysis of variance (ANOVA) on mesocosm means. Survival in each mesocosm was logit-transformed and analyzed using a two-way ANOVA ($n = 24$). We separately analyzed Gosner developmental stage ($n = 317$), mass ($n = 438$), and length ($n = 1057$) data after log-transformation using mixed models, with light and nutrients as fixed factors and mesocosm as a random factor in the *nmle* package (Pinheiro et al. 2013). All tadpoles were measured, but we only weighed the frozen tadpoles and only assessed Gosner stage in tadpoles preserved in ethanol. Thus the differing sample sizes can be attributed to sample processing.

To estimate the underlying mechanisms, we utilized structural equation modeling (SEM) in the *lavaan* package in R (Rosseel 2012). We used SEM as a method of exploring the relative importance of light (shading: 25, 53 or 90%), nutrients (high = 1, low = 0), fish production (as carbon biomass gain over the experiment), algal production, and algal resource stoichiometric quality on tadpole growth and development. Weighted seston and periphyton C:N and C:P were highly correlated ($r > 0.85$), so we included them in the model as measures of the latent variable 'resource quality.' 'Development' was included as a latent variable measured by Gosner stage, and 'size' was a latent variable measured using total length and total biomass at the end of the experiment in each mesocosm. Size and development were highly correlated ($r > 0.90$), so we modeled these responses as covariates. We included fish in the model, even though they did not

compete with tadpoles for resources and were present in all mesocosms, to assess their indirect effect on tadpoles.

We developed our causal model *a priori* based on predicted direct and indirect relationships. Light and nutrients were included as exogenous (independent) predictors. Although our original model looked only at light and nutrient effects as mediated through algae, through examining modification indices we found that an indirect link between nutrients and tadpole size was significant and improved model fit. All data included in the model were log-transformed mesocosm means, with the exception of shading, which was mean centered and scaled. We utilized a χ^2 statistic to test whether the covariance matrix generated by the model differed significantly from the data. A p-value of > 0.05 would indicate that the observed and predicted models were not significantly different, and that the fit for our model was adequate (Shipley 2000). In addition to the χ^2 statistic, we also assessed model fit using root mean square error of approximation (RMSEA) and the Comparative Fit Index (CFI), all of which are relatively insensitive to sample sizes (Fan et al. 1999).

Results

Algal production and stoichiometry

Light, nutrients, and the light x nutrient interaction all significantly affected total PPr (Fig 1a, Table 2). High nutrient conditions increased total PPr relative to low nutrient treatments (Fig 1a). Under intermediate and high light conditions only nutrients limited PPr, but at low light both light and nutrients limited PPr. Weighted seston and periphyton C:N was significantly affected by light and nutrients, but not the light x nutrient interaction (Fig. 1b, Table 1). Adding nutrients significantly decreased weighted C:N. Weighted seston and periphyton C:P was significantly

affected by light, nutrients, and the light x nutrient interaction (Fig 1c, Table 2); nutrients decreased C:P ratio only under low light conditions.

Treatment effects on tadpoles

Tadpoles reared under high nutrient conditions were significantly larger and more developed than those in low nutrient conditions (Fig. 2, Table 2). None of the tadpoles reached metamorphic stages (Gosner developmental stage > 42) during the course of our experiment, and some tadpoles never developed past stage 25, the developmental stage at addition. The most developed tadpoles were found in the intermediate light, high nutrient treatment and least developed in the high light, low nutrient treatment (Fig. 2). There were no significant differences in survival among treatments (Table 2).

Structural equation modeling (SEM)

Our model was a good fit for the data ($\chi^2 = 21.72$, d.f. = 17, $n = 24$, $p = 0.196$; RMSEA = 0.108 and CFI = 0.985). Light and nutrients both significantly predicted all algal parameters (Fig. 3). Increased shading and nutrients positively affected algal resource quality (low C:nutrient; $R^2 = 0.883$). Light and nutrient treatments explained most of the variation in PPr ($R^2 = 0.859$). Shading was a 3.5 times stronger predictor of algal quality than nutrients, but nutrients were almost twice as important as shading in predicting PPr (Fig. 3). Average tadpole size at the end of the experiment was significantly predicted by the indirect effects of nutrients ($p < 0.001$) and fish production ($p = 0.001$), but not algal quantity ($p = 0.447$) nor quality ($p = 0.286$). Development (Gosner stage) was equally predicted by PPr ($p < 0.001$) and fish production ($p < 0.001$), but also not by algal quality ($p = 0.931$). Overall, the model was a good predictor of tadpole size ($R^2 = 0.915$) and development ($R^2 = 0.815$).

Discussion

Algal production and stoichiometry

Our data suggest canopy cover is an important predictor of quantity and quality of algae in ponds. Both light and nutrients were important predictors of primary production. Nutrient addition always increased PPr, and high light treatments had significantly higher PPr than low light treatments. Our data concur with other studies that find increases in canopy cover lead to decreases in both phytoplankton and periphyton production in artificial enclosures (Mokany et al. 2008), natural ponds (Palik et al. 2001; Skelly et al. 2002) and small lakes (Sand-Jensen and Staehr 2009). Our data also supported the light:nutrient hypothesis (Sterner et al. 1997), which predicts that low light and high nutrient conditions result in algae of better nutritional quality. We saw a significant increase in food quality as light decreased, especially under high nutrient conditions. This suggests that although closed canopy ponds have less overall primary production, the food quality is higher for primary consumers.

Treatment effects on tadpoles

We found that tadpole total length, mass, and Gosner developmental stage were all primarily related to nutrient addition, but not light. Survival was not related to light, nutrients, or the light x nutrient interaction. Light was not significantly related to any tadpole parameter, highlighting the importance of the nutrient subsidy that leaf litter provides.

Our hypothesis that food quality would be important for tadpole growth and development, if food quantity was not a limiting factor, was not supported by our mixed models or by our SEM. Even among treatments with high algal biomass, there was no apparent effect of higher food quality (i.e., low algal C:nutrient ratio). For example, we did not see any difference between low light/high nutrient and high light/high nutrient tadpoles, in terms of growth and development,

despite the large differences in algal quality between these treatments. Algal resource quality was not a significant predictor of growth or development. Instead, primary production was the only algal parameter that affected tadpoles in our causal model.

The fact that resource quality was not a significant predictor of tadpole growth or development was surprising given the research on other aquatic organisms showing that food quality is strongly associated with consumer growth and development (Dickman et al. 2008; Schoo et al. 2012; Sterner 1993). Our species is an open-canopy specialist, and previous work has found that open-canopy specialists tend to be more sensitive to variations in resource stoichiometry (Schiesari 2006). We may have found more of an effect of food quality using a quicker developing species. Bullfrogs are a slow developing species with a larval stage of up to two years (Cecil and Just 1979), so food quality differences may not impact their development until they are closer to metamorphosis. Alternatively, it could be that food quantity is the single most important predictor of larval amphibian performance. Interestingly, we observed relatively strong treatment effects on tadpole body nutrients (Rowland et al. 2015). Treatments with the best resource quality resulted in higher N and P concentrations in tadpoles. Presumably these differences were mediated by variation in algal stoichiometry as found in previous studies with diet C:nutrient manipulations (Liess et al. 2013). Differences in tadpole body nutrients could be important for overwinter survival or for supporting rapidly developing limbs at metamorphosis.

The relative insensitivity of bullfrog tadpoles to stoichiometric food quality could also relate to selective foraging. Tadpoles consumed a mix of periphyton and phytoplankton and this likely alleviated food quality differences among treatments. Alternatively, periphyton communities are a complex mix of diatoms, green and blue-green algae, bacteria, and sometimes fungi that exhibit a patchy species distribution on the surfaces of where they grow (Hall and

Meyer 1998; Lock et al. 1984). It is likely that certain areas of the mesocosms had higher food quality, and perhaps the highly mobile tadpoles compensated for overall lower food quality by preferentially grazing on high quality patches, as they have in previous experiments (Kupferberg 1997). It would be interesting to assess whether tadpoles actively graze in the shaded areas where periphyton food quality would be better. On the opposite extreme, tadpoles exposed to fish chemical cues spend significantly more time in refuges to reduce predation risk (Petranka et al. 1987). The bullfrogs in our enclosures may have reduced their activity and foraged less, making them less selective and more vulnerable to quantity and quality effects. This seems unlikely as larval bullfrogs have strong predation deterrents (Kruse and Francis 1977) and often coexist with fish, and so our experimental conditions would not be unusual for this species. Further, our SEM suggested that fish had a positive effect on bullfrog tadpoles. Thus fish did not seem to adversely affect foraging in our experiment.

Although no other studies have looked at the response of anurans to natural variation in resource quantity and stoichiometric quality in the absence of leaf litter, four studies also found a positive effect of nutrients on larval anurans. Lower C:N of leaf litter resulted in quicker growth and development of larval wood frogs (*L. sylvaticus*), and significantly lower body C:N (Stephens et al. 2013; Stoler and Relyea 2013) and lower C:N and C:P of controlled diets resulted in higher growth, faster development and higher body nutrient contents of the common frog (*Rana temporaria*; Liess et al. 2013). In addition, Schiesari (2006) found foregut content stoichiometric quality was positively correlated with growth rate in *L. sylvaticus* and northern leopard frogs (*L. pipiens*). All of these frogs have much shorter developmental periods than our study organism, and so perhaps bullfrog tadpoles may be more affected by variations in resource stoichiometry later in life. We only examined a portion of the bullfrog larval period, and we may

have seen an effect of food quality had the bullfrogs been allowed to grow to metamorphosis. The absence of leaf litter in our study eliminated release of toxic secondary compounds (Earl et al. 2012), but also likely reduced dissolved organic carbon supply that normally would have been released from litter (F. Rowland, *unpublished data*). Our study provides compelling evidence that nutrients released from litter can positively influence resource quality in ponds, but does not account for other changes in water quality induced by increased leaf litter in ponds.

Most previous work has found that tadpole growth and development is increased in open-canopy ponds (Schiesari 2006; Werner and Glennemeier 1999). Even closed-canopy specialists such as wood frogs grew faster in open-canopy ponds (Werner and Glennemeier 1999). Schiesari (2006) suggested that it is food quality and not quantity that depresses open-canopy species performance, and found a decrease in resource stoichiometric quality in the foreguts of tadpoles from closed-canopy environments. Leaf litter adds N and P to the pond, but also carbon. We did not account for this additional carbon in our experiment and this may explain the discrepancies between our study and previous work, and this deserves further investigation. Additionally, pond canopy cover can affect inter- and intraspecific competition. Open-canopy ponds tend not only have higher species diversity (Werner et al. 2007) and therefore higher interspecific competition, but will dry at different rates than closed-canopy ponds, which will alter density. Our finding that resource quantity mattered more than quality would be even more pronounced under the higher competition of multiple species or drying ponds.

SEM model results

Both our mixed and SEM models indicated a strong and significant nutrient effect on size and developmental. Our SEM indicated that nutrients were twice as important as light in

determining total primary production. The model also showed that nutrients were the strongest predictor of tadpole development as mediated through primary production. Nutrients were important in determining size of tadpoles through primary production, but also through an unknown pathway. This strong, indirect effect of nutrients on tadpole size was especially striking. Higher nutrients could have benefitted tadpoles through detrital pathways (Moore et al. 2004), or could suggest an interactive effect of quantity and quality. That is, that size could be highest when both quantity and quality are high. This deserves more investigation. Furthermore, the indirect effect of nutrients outside of the algal pathway was not detected in our other more common statistical techniques. Using the mixed models alone, we would have presumed that because nutrients were a strong predictor of algal quantity and quality, that algal differences must have been the reason for increased size in high nutrient treatments.

Further, by adding in fish production as an exogenous predictor, we were able to detect a positive effect of fish standing stock on size and development of tadpoles. Although all mesocosms were stocked at the same animal density, fish biomass differed at the end of the experiment (Rowland et al. 2015). Our model indicates fish were equally as important as primary production in predicting development, and half as important as nutrients in predicting size of tadpoles. Previous research has found fish indirectly benefit bullfrog tadpoles by reducing predatory macroinvertebrate densities (Adams et al. 2003). Others have found similar bullfrog facilitation by fish in the absence of invertebrate predators (Boone and Semlitsch 2003), but the mechanisms are poorly understood. Fish presence changes zooplankton communities (Dickman et al. 2008), perhaps in ways favorable for bullfrogs. Fish can also increase nutrient cycling (Vanni 2002). Future work should further investigate the mechanisms by which fish facilitate bullfrog tadpoles. The use of SEM in our experiment allowed us to test multiple causal pathways

simultaneously and detect food web patterns we would not have only using mixed models. Using causal networks like SEM to explain food web effects is a powerful tool for describing data.

Our results indicated that nutrients were primarily important as a stimulator of primary production, and that food quantity, not quality, is most important to tadpole development in the early larval stages. The SEM indicated that light was more important than nutrients in predicting algal quality. More light decreased food quality (higher C:nutrient), while nutrient addition increased it (low C:nutrient). Higher food quality had no significant effects on tadpole size and development, but food quantity was an important predictor of development in tadpoles. The effect of light on the food web through mediating algal stoichiometry was relatively less important than the role of nutrients as mediated through primary production.

Conclusions

The effect of canopy cover on anuran growth and development, in terms of light and nutrient availability is still unclear. Skelly et al. (2014) showed that an experimentally circumscribed reduction in forest canopy over vernal pools increased amphibian richness and abundance. This would greatly increase light availability, likely with little or no impact on leaf nutrient subsidies from the surrounding forest, and points to light as the most important factor in the growth of amphibian populations. However, several recent experiments have highlighted the positive relationship between higher nutrient content in leaf litter and larval amphibian growth under controlled light regimes (Earl et al. 2011; Stephens et al. 2013; Stoler and Relyea 2013). Furthermore, Williams et al. (2008) found shading was not as important as litter type in determining survival, time to metamorphosis, and size at metamorphosis.

In natural ponds and wetlands, amphibian abundance and richness tend to decrease with increasing canopy cover (Hocking and Semlitsch 2007; Skelly et al. 2005; Werner et al. 2007).

Interestingly, while many amphibian species seem relatively intolerant to closed canopy conditions (Skelly et al. 2005; Skelly et al. 1999), salamanders tend to develop better in closed canopy environments (Earl et al. 2011; Werner et al. 2007). This suggests food quality may be more important for salamanders as secondary consumers than for anurans as primary consumers, which deserves further investigation.

Light had strong effects on algal quality, but algal quality did not predict tadpole growth or development. Instead, nutrients seemed to be most important factor as a stimulator of total algal primary production and through some unknown non-algal pathway. Overall, our results suggest that canopy cover is important mainly as a source of nutrients to ponds and highlights the need for more research into the mechanisms of how forest cover changes can affect adjacent ecosystems such as ponds.

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Figure legends

Figure 1. Overall experimental means of (a) algal C:N (molar); (b) algal C:P (molar) weighted by algal group contribution to all primary production (PPr); and (c) total primary production (seston plus periphyton). Each point represents the mean $\pm$ SE of four replicate mesocosms. Solid symbols represent high nutrient and open symbols low nutrient treatments. Light level is denoted as high, intermediate (int.), or low.

Figure 2. Averages of tadpole growth (in total length) and development (as Gosner stage) at the end of the experiment. Each point presents the mean $\pm$ SE of four replicates. HLHN = high light, high nutrients; HLLN = high light, low nutrients; ILHN = intermediate light, high nutrients; ILLN = intermediate light, low nutrients; LLHN = low light, high nutrients; and LLLN = low light, low nutrients. The dotted line represents the developmental stage of the tadpoles at the start of the experiment. All high nutrient treatments were significantly different from low nutrient treatments in post-hoc Tukey tests.

Figure 3. SEM with shading, nutrients and fish production as exogenous predictors, and five measured endogenous variables (means per mesocosm of weighted seston and periphyton [resource] C:N and C:P, average primary production (PPr), Gosner stage at the end of the experiment, average end tadpole length, and biomass in each mesocosm). Latent variables (resource quality, development and size) are represented with ovals and directly measured variables with rectangles. Significant paths ($p < 0.05$) are shown as solid arrows with widths proportional to the standardized path coefficient. Non-significant paths are represented with

dashed lines and double-headed arrows show covariates. Numbers on paths represent the standardized path coefficient.

Draft

Table 1. Analysis of variance of the effects of light and nutrients on average experimental primary production (PPr), and algal molar C:N and C:P ratios, weighted by the relative contribution of phytoplankton and periphyton to overall primary production.

Response	<u>Light</u>			<u>Nutrients</u>			<u>Light x nutrients</u>		
	F	d.f.	<i>P</i>	F	d.f.	<i>P</i>	F	d.f.	<i>P</i>
Total PPr	19.26	2, 18	<0.001	132.20	1, 18	<0.001	4.14	2, 18	0.033
Weighted C:N	53.24	2, 18	<0.001	9.98	1, 18	0.005	1.40	2, 18	0.271
Weighted C:P	138.65	2, 18	<0.001	13.25	1, 18	0.002	21.34	2, 18	<0.001

Draft

Table 2. Mixed models testing for total length, mass, and Gosner stage of development with light and nutrients as fixed effects, and mesocosm as a random effect. Survival was analyzed with a two-way ANOVA with logit-transformed proportion of tadpoles surviving to the end of the experiment as the response.

Response	<u>Light</u>			<u>Nutrients</u>			<u>Light x nutrients</u>		
	F	d.f.	<i>P</i>	F	d.f.	<i>P</i>	F	d.f.	<i>P</i>
Total length	2.30	2, 18	0.129	134.87	1, 18	<0.001	0.73	2, 18	0.496
Mass	0.32	2, 18	0.570	151.06	1, 18	<0.001	2.25	2, 18	0.134
Gosner	1.50	2, 18	0.258	56.60	1, 18	<0.001	1.20	2, 18	0.313
Survival	0.04	2, 18	0.848	0.62	1, 18	0.440	0.38	2, 18	0.545

Draft

Figure 1. Overall experimental means of (a) algal C:N (molar); (b) algal C:P (molar) weighted by algal group contribution to all primary production (PPr); and (c) total primary production (seston plus periphyton). Each point represents the mean ± SE of four replicate mesocosms. Solid symbols represent high nutrient and open symbols low nutrient treatments. Light level is denoted as high, intermediate (int.), or low.

111x257mm (300 x 300 DPI)

Figure 2. Averages of tadpole growth (in total length) and development (as Gosner stage) at the end of the experiment. Each point presents the mean $\pm$ SE of four replicates. HLHN = high light, high nutrients; HLLN = high light, low nutrients; ILHN = intermediate light, high nutrients; ILLN = intermediate light, low nutrients; LLHN = low light, high nutrients; and LLLN = low light, low nutrients. The dotted line represents the developmental stage of the tadpoles at the start of the experiment. All high nutrient treatments were significantly different from low nutrient treatments in post-hoc Tukey tests.

140x135mm (300 x 300 DPI)

Figure 3. SEM with shading, nutrients and fish production as exogenous predictors, and five measured endogenous variables (means per mesocosm of weighted seston and periphyton [resource] C:N and C:P, average primary production (PPr), Gosner stage at the end of the experiment, average end tadpole length, and biomass in each mesocosm). Latent variables (resource quality, development and size) are represented with ovals and directly measured variables with rectangles. Significant paths ($p < 0.05$) are shown as solid arrows with widths proportional to the standardized path coefficient. Non-significant paths are represented with dashed lines and double-headed arrows show covariates. Numbers on paths represent the standardized path coefficient.

361x270mm (72 x 72 DPI)