DOES THE QUANTITY OF RESOURCES IN THE ENVIRONMENT ALTER THE IMPACT OF MULTIPLE PREDATORS ON THEIR PREY?

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Predation is an important biological process affecting prey populations and most prey in nature are exposed to multiple predator species. Much research has revealed that the combined effect of multiple predators on their prey can be substantially different from that which would be expected if the predators operated independently of each other. Some reasons for this discrepancy include interference among predator species and/or anti-predator responses of prey to one predator that alter the effectiveness of the other predator. In systems where one of the predators is an omnivore, an increase in the availability of primary producers via nutrient enrichment could alter the extent to which the combined impact of multiple predators on their prey differs from that which would be predicted if predators were assumed to forage independently of each other. We conducted an experiment in artificial ponds to examine this idea. Our experiment manipulated the occurrence of two common pond predators, omnivorous crayfish (*Procambarus acutus*) and carnivorous dragonfly larvae (*Anax sp.*), in environments that varied in the amount of nutrients available to support algal growth. We measured the mortality, mass at metamorphosis, and larval period of herbivorous tadpoles (*Hyla squirella*).

We saw no statistical difference between observed and predicted proportion of prey consumed in multiple predator treatments. Predicted proportion of prey consumed was derived using a model which assumes the effects of each predator are independent, while observed proportion of prey consumed was the proportion of prey metamorphosed in each multiple predator treatment. Even though there were no statistically significant results, we did observe a trend of prey mortality being 28% less than predicted in high nutrient treatments with both *Anax sp.* and *P. acutus*, compared to low nutrient treatments with both *Anax sp.* and *P. acutus*. We also observed prey mortality being 25% less than predicted in high nutrient treatments with two *Anax sp.* predators and a 20% greater than predicted in low nutrient treatments with two *Anax sp.* present. These differences are rather large and could disrupt ecosystem processes by changing prey abundances. We saw that growth of *H. squirella* varied among predator treatment, but saw little variation depending on nutrient availability. Our experiment shows that multiple predators can have unexpected impacts on regulating prey densities.

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Tyler R. Gelles

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CHAPTER 1

Introduction

Natural scientists have long tried to provide a conceptual understanding of the interactions among species within communities. The relative abundances of different species in a community are strongly influenced by species interactions and are depicted using food webs. While food webs provide a conceptual understanding of community interactions, the power of these depictions is often lost when linkages are viewed as concrete, since species interactions are often flexible in response to environmental variation (Rosenzweig, 1971 Paine, 1980; Polis and Strong, 1996; Chalcraft and Andrews, 1999; Abrams, 2010; Rosenzweig, 1971).

The effects of predators on prey are often controlled by the level of trophic complexity and availability of resources within food webs (Rosenzweig, 1971; Paine, 1980; Polis, 1991; Leibold and Wilbur, 1992; Polis and Strong, 1996; Duffy et al, 2007). Prey can be found in food webs of varying trophic complexity and this complexity can have important consequences for prey (Paine, 1980; Polis, Myers, and Holt, 1989; Polis and Strong, 1996; Carey and Wahl, 2010; Davenport and Chalcraft, 2012). Trophic complexity can range from simple food webs containing 1) only herbivores and primary producers to more complex food webs containing 2) carnivorous, 3) omnivorous, or 4) multiple predator species (Figure 1). The presence of an omnivore increases trophic complexity, in comparison to a carnivore, because omnivores possess the ability to feed at multiple trophic levels thus increasing trophic linkages between trophic levels. Prey are at risk to predation in all food webs that have predators, but increasing trophic complexity by replacing a carnivorous predator with an omnivorous predator or adding multiple predator types can affect both the risk to predation by prey and the intensity of competitive

interactions on prey (Rosenzweig, 1971; Paine, 1980; Diehl, 1993; Polis and Strong, 1996; Nyström, Brönmark, and Granéli, 1996; Cronin et al., 2002; Dorn and Wojdak, 2004; Olsson et al., 2008).

Understanding the effects of multiple predators on prey is an essential part of understanding food web interactions, but historically research had only focused on the effects of a single predator species or one predator species at a time (Sih, Englund, and Wooster, 1998; Relyea and Yurewicz, 2002). However, over the past 20 years studies have examined the effects of multiple predators on prey and have found that the presence of multiple predators can have unexpected impacts on prey mortality. These unexpected impacts have been termed as emergent multiple predator effects and are defined by the amount of prey consumed being different, in the presence of multiple predators, than would be predicted by a model assuming the effects of predators are independent (Sih et al., 1998; Vance-Chalcraft and Soluk, 2005; Vance-Chalcraft et al., 2007).

Emergent multiple predator effects can be described as either prey release or prey suppression. Prey release occurs in a community when prey mortality is less in the presence of multiple predators than was predicted by a model that assumes the effects of each predator are independent. This type of emergent multiple predator effect is often the result of negative interactions between predators or when prey reduce their chance of being consumed through investments in defensive strategies. Negative interactions can be a result of intraguild predation, which occurs when one predator species consumes another predator species that compete for a shared prey resource, or interference competition, which involves aggressive interactions between predators. In contrast, prey suppression occurs in a community when mortality is greater in the presence of multiple predators than was predicted by model that assumes the

effects of each predator are independent. This type of emergent multiple predator effect can be the result of facilitation between predators or when a change in prey behavior, in response to one predator, increases risk to another predator (Polis, Myers, and Holt, 1989; Sih et al., 1998; Vance-Chalcraft and Soluk, 2005; Vance-Chalcraft et al., 2007).

Prey can be regulated by both lethal and non-lethal effects of predators. While lethal effects control prey through direct consumption, non-lethal effects often drive prey to make investments in anti-predatory behaviors to reduce their chance being consumed (West-Eberhard, 1986; McCollum and Van Buskirk, 1996; Werner and Anholt, 1996; Bolnick and Preisser, 2005). Non-lethal effects can be the result of predatory cues and unlike lethal predatory effects, which only effect prey who come in direct contact with predators, effect all prey present within an ecosystem (Bolnick and Preisser, 2005; Peckarsky et al., 2009). An increase in prey resource availability has the ability to disrupt the direct and indirect non-lethal effects of predators on prey (Abrams, 1991; Peacor, 2002; Peacor and Werner, 2004) which can increase prey release (Vance-Chalcraft and Soluk, 2005; Vance-Chalcraft et al., 2007). This can occur because when prey resources are low, prey may not be able to invest in anti-predatory behaviors because prey must avoid starvation (McNamera and Houston, 1987; Lima and Dill, 1990; Abrams, 1991; Relyea, 2001; Bolnick and Preisser, 2005). When prey have invested in anti-predatory behaviors they are not only better able to escape predation in single predator food webs, but may indirectly increase predator activity in multiple predator food webs which can result in a greater risk of interference interactions, such as intraguild predation or interference competition (Soluk and Collins, 1988; Werner and Peacor, 2003; Vance-Chalcraft and Soluk, 2005; Vance-Chalcraft et al., 2007).

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The amount of resources available to primary consumers within a food web is often controlled by primary production, or the amount of energy fixed by autotrophs in an ecosystem (Abrams, 1993; Butzler and Chase, 2009; Rabalais, 2002). The amount of primary production that occurs within an ecosystem is often directly linked and limited by the availability of particular nutrients such as nitrogen and phosphorus (Rabalais, 2002; Elser et al., 2007; Butzler and Chase, 2009). Nitrogen and phosphorus can enter ecosystems through surface water run-off, groundwater flow, atmospheric deposition, or the presence of decaying organic matter. While nitrogen and phosphorus naturally enter ecological systems through these processes, their abundance can be modified through human mediated effects (Polis et al., 1997; Rouse, Bishop, and Struger, 1999; Rabalais, 2002; Moore et al., 2004; Elser et al., 2007). These changes in primary productivity, which stem from nutrient additions, potentially have the ability to alter the effects of multiple predators on prey.

Highly productive environments should have larger effects on prey mortality, than less productive environments, since increased productivity will not only provide more food for prey in predator free environments but should allow for greater investments in defensive strategies and disrupt trophic interactions in more complex food webs (West-Eberhard, 1989; Abrams and Roth, 1994; McCollum and Van Buskirk, 1996; Polis and Strong, 1996; Werner and Anholt, 1996; Werner and Peacor, 2003; Bolnick and Preisser, 2005). While there has been a large amount of research conducted to understand the extent to which emergent multiple predator effects occur within multiple predator food webs (Sokol-Hessner and Schmitz, 2002; Harvey, White, and Nakamoto, 2004; Siddon and Witman, 2004; Griffen, 2006; Vance-Chalcraft and Soluk, 2005; Vance-Chalcraft et al., 2007), there is a lack of information regarding how nutrient addition can disrupt these effects. Future studies need to address how increased primary

productivity, resulting from nutrient addition, can affect predator-prey interactions in food webs that vary in trophic complexity.

Our study will determine whether resource availability can disrupt the extent to which prey mortality is suppressed or released in complex food webs. We expect prey release to occur in environments with multiple predators but the extent of prey release will be greater in nutrient enriched environments that contain multiple predator species rather than multiple individuals of the same species or in environments with fewer nutrients. Prey release will be greater because prey will be able to escape predation through investments in anti-predatory behaviors, which will increase interference interactions between predators and further drive predators to consume less prey. The extent to which prey mortality in environments with multiple predators differ from that expected by a model assuming predator effects are independent will be largest in multiple mixed species predators are present, compared to environments with multiple single species predators, because the risk of intraguild predation will be greater between mixed species predators. We also predict that prey mortality will be the lowest in nutrient enriched environments with no predators because nutrient enrichment will increase the amount of food available to herbivorous prey and prey will not be threatened by predation. However, prey mortality will be the greatest when only carnivorous predators are present because carnivorous predators are restricted to feeding only on herbivorous prey while omnivores can feed on primary producers as well as herbivorous prey.

In addition to our initial questions, we are also interested in determining how a modification in resource availability, through increased nutrient input, affects growth of larval anurans exposed to simple and complex food webs. Many studies have addressed the effects that predation or resource availability can have on controlling growth of larval anurans (Anholt and

Werner, 1995; Werner and Anholt, 1996; Beck, 1997; Kupferberg, 1997; Relyea and Werner, 1999; Eklöv, 2000; Eklöv and Werner, 2000; Van Buskirk, 2001; Relyea, 2001; Relyea, 2003; Wood and Richardson, 2009; Stoler and Relyea, 2011) but have failed to compare the effects of nutrient addition across a complex food web gradient. We further hypothesize that nutrient addition will positively affect growth of prey because prey will have the opportunity to consume greater amounts of food and achieve a larger size. Differences in growth rates of prey will be the largest in nutrient enriched environments with a single predator species present, compared to nutrient enriched environments with mixed predator species, because the threat of predation will be greatest as interference interactions are less likely to occur when a single predator species is present. Regardless of the predator species present, nutrient enrichment will allow prey to better react to non-lethal predator effects since they will not be threatened by the risk of starvation. An increase in lethal predator effects, through direct consumption of prey by predators, will increase prey mortality and increase the amount of food available to remaining prey allowing for greater growth.

<u>Study System</u>

Temporary ponds represent distinct communities that provide a good model system to study predator-prey interactions and how environmental variations can change the strength of species interactions. Artificial ponds are excellent venues to study temporary pond food webs as they allow researchers to replicate environmental conditions, while maintaining independence between experimental units that may not be achievable by using an enclosure experiment, which involves placing screened enclosures in natural ponds (Wilbur 1997; Chalcraft, Binckley, and Resetarits, 2005). Artificial ponds have been particular useful when studying larval anuran

ecology as processes found to be important in artificial ponds have also been found to be important in natural ponds (Petranka, 1989; Scott, 1990; Wilbur, 1997; Resetarits and Fauth, 1998; Rubio and Kiesecker, 2004).

To examine the role that increased resource availability has on aquatic food webs, nitrogen and phosphorus were added to some artificial ponds, while others were left under ambient conditions. Our experimental food webs will expose *Hyla squirella* (squirrel tree frog) tadpoles to two pond predators, *Procambarus acutus* (White River Crayfish) and *Anax sp*. (dragonfly larvae). In order to understand how resources can disrupt the effects of multiple predators on prey, we exposed tadpoles to each predator species alone (*P. acutus* or *Anax sp*.) or together (*P. acutus* and *Anax sp*.). *Procambarus acutus* and *Anax sp.* are good predators to use in this study since they are known to interact in natural ponds within Eastern North Carolina. *Hyla squirella* is a small, locally abundant tree frog that breeds in ephemeral aquatic habitats and makes a good model organism, to study the effects of multiple predators on prey, since they naturally interact with a wide variety of predators.

Both *P. acutus* and *Anax sp.* are locally abundant predators known to coexist in ponds within Eastern North Carolina. *Anax sp.* is a common dragonfly species that spends its larval stage as a voracious aquatic predator. *Procambarus acutus* is an omnivorous crayfish that possesses the ability to forage on consumers, *H. squirella* and *Anax sp.*, and primary producers. Diet preference of crayfish is often influenced by encounter rates and handling time and depends on the relative abundance and quality of the prey item available (Nyström et al., 1996; Cronin et al., 2002; Olsson et al., 2008), which is an essential characteristic to understand how resource quality and availability alters the effects of multiple predators on prey.

<center>Methods</center>

Our experiment consisted of 70 artificial ponds (378 L polyethylene wading pool) at East Carolina University's West Research Campus in Greenville, NC. To effectively evaluate the effects of *P. acutus* and *Anax sp.* on *H. squirella* we used a randomized-block design consisting of 70 artificial ponds in a square array. Before the initiation of the experiment, we identified five clusters of fourteen ponds each as a statistical block. Ponds within each block are closer together and should respond similarly to unknown spatial gradients. We conducted all procedures on a block-by-block basis to minimize variation within a block not attributed to treatment differences. Our experiment consisted of 7 treatments replicated at both high and low nutrient levels, which are summarized in table 1. Two treatments represent single predator treatments, with either one *P. acutus* or one *Anax sp.*, which were necessary in order to test for the direct effects of each predator on the survival of *H. squirella.* Two treatments represent multiple predator treatments of a single predator species, two *P. acutus* or two *Anax sp.*, to evaluate the effects that multiple predator single species treatments have on prey survival. One treatment consists of combined predator species, *P. acutus* and *Anax sp*., to evaluate mixed species multiple predator effects. One treatment consists of *P. acutus* and no prey, which allowed us to estimate algal consumption rates of an omnivorous predator. One treatment represents a control with no predators to determine survival of *H. squirella* in the absence of predators. Each treatment was replicated once at high nutrient conditions and once at low nutrient conditions within each statistical block.

Seven experimental units were randomly assigned a high nutrient level and seven experimental units were randomly assigned a low nutrient level, within each block. Once each tank was assigned an appropriate nutrient level we randomly assigned seven high nutrient

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experimental units a predator treatment and seven low nutrient experimental units a predator treatment, within each block. After all experimental units were assigned a predator treatments we randomly added 275 tadpoles on July 8, 2012 and one or two predators on July 11, 2012, except for two experimental units that had no tadpoles (*P. acutus* only treatments) and two experimental units that had no predators (Tadpole only treatments), to the corresponding treatment within each block. Each experimental unit consisted of aged well water, an initial inoculation of local pond water which included algae and zooplankton, white flagging tape which served as a substrate for periphyton growth, PVC pipe which acted as crayfish refuge, a total of 800g of pine straw to provide bottom coverage, and topped with screen lids to contain individuals and prevent the entrance of unwanted organism. Large invertebrates were filtered out of the initial inoculation of pond water, as they could have resulted in an increased threat of predation.

Our experimental population of *H. squirella* tadpoles was collected on July 3, 2012 in the Northern Croatan National Forest, Craven County N.C. We acquired tadpoles by catching amplexing adults and hatching individual egg masses in controlled laboratory conditions. Newly hatched tadpoles were used because we are interested in estimating survival and growth across larval period. Both species of experimental predators, *P. acutus* and *Anax*, were collected at A Time for Science, Grifton N. C. and blocked by size. *Anax sp.* was block by small sized predators (35mm-50mm total length) and large sized predators (50mm-65mm total length). *Procambarus acutus* was blocked by small sized predators (12mm-20mm carapace length) and large sized predators (20mm-30mm carapace length). Three spatial blocks were assigned small *Anax sp.* and small *P. acutus*, while two spatial blocks were assigned large *Anax sp.* and large *P. acutus*.

To stimulate greater primary productivity an initial dose of nitrogen and phosphorus was added to high nutrient treatments on July 5, 2012, while no additional nutrients were added to low resource treatments. This allowed us to manipulate conditions of ponds that receive a higher input of nutrients. An initial dose of 374.22 mg of crystalline $NaNO_3$ (0.99 mg/l) and 12.474 mg of crystalline NaH_2PO_4 (0.033 mg/l) was used in high nutrient treatments to maintain a 30:1 ratio so that experimental units were not co-limited and ensure that phosphorus will be the limiting nutrient, which is common in freshwater systems (Rabalais, 2002; Butzler and Chase, 2009). These levels were used because they fall within natural ranges of forested ponds in Eastern North Carolina (Chescheir, 2003).

All experimental units were checked daily by walking around each mesocosm a minimum of three times. Checking mesocosms consisted of removing the screen lid, noting any abnormal conditions, and removing visible metamorphs. An individual was considered a metamorph when the emergence of at least one forelimb was detected. Metamorphs were transported to the lab and held until their tail had become fully absorbed, after which they were weighed to determine date and mass at metamorphosis. Subtracting date weighed by date of egg mass laid was used to determine larval period. After each individual was counted and weighed, they were released to their natal pond. On September 15, 2012 each mesocosm was destructively sampled and all remaining tadpoles and predators were counted and weighed. Both tadpoles and predators were released back to their natal pond.

Survival, mass at metamorphosis, and larval period of each metamorph was calculated. Survival was the proportion of *H. squirella* added to each mesocosm that metamorphosed. Since different individuals within each mesocosm are not separate data points, analyses of mass at

metamorphosis and larval period were averaged to obtain individual data points for each mesocosm. It was necessary to use the geometric mean for mass at metamorphosis since the mass of individuals is not normally distributed, while it was appropriate to use the arithmetic mean for larval period since recorded observations are normally distributed. Survival rates of both predators and algal biomass was also calculated for each experimental unit.

Algal biomass was determined by taking phytoplankton and periphyton samples. Phytoplankton samples were taken by removing 550 ml of pond water from the center of each experimental unit. Periphyton strips were removed from the bottom southeast corner of each mesocosm and algae scrapped off and removed from one side. Samples were collected on August 3, 2012 and immediately placed in the dark on ice and brought back to the lab. A recorded volume of phytoplankton and periphyton samples were hand-pumped through glass fiber filters for chlorophyll *a* analysis. Once chlorophyll *a* samples had been acquired, spectrophotometric analysis was done in accordance to Clesceri et al., (1998). Estimates of algal biomass were log transformed.

Statistical Analysis

SAS 4.2 Enterprise Guide statistical software (SAS Institute inc. Cary, NC) was used for statistical analysis. Pending a statistically significant treatment effect or interaction ($p < 0.05$), a Fisher's LSD pairwise comparisons to $p = 0.05$ was performed in all analysis to evaluate which treatments differed from each other. Fisher's LSD may not adequately protect against committing a type 1 error under certain conditions (Kromrey and La Rocca, 1995) and because of this we further adjusted p-values to control for the False Discovery Rate (Benjamini and Hochberg, 1995).

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We tested the hypothesis that predator treatment had no effect on mortality of *H. squirella* at either high or low nutrient levels. To test this hypothesis we used a two-way ANOVA where the dependent variable was instantaneous mortality rate of *H. squirella* and the independent variables were predator treatment, nutrient availability, and a predator treatment x nutrient interaction. Block effects were included in all analysis. Within our two-way ANOVA predator treatment, nutrient availability, and predator treatment x nutrient interaction were fixed effects while block effect, block effect x predator treatment interaction, and block effect x nutrient interaction were random effects. Mortality data was estimated by taking the log of the proportion of individuals surviving in each mesocosm. The two-way ANOVA allowed us to address whether 1) mortality of *H. squirella* varies among predator treatment, 2) if nutrient availability affects mortality, and 3) if the effect of nutrient availability on mortality depended on the specific set of predators present.

After testing for differences in mortality rates, we evaluated whether the observed predation rate for multiple predator species was different from that predicted by the multiplicative risk model. We used the multiplicative risk model ($P_{ab}= P_a+P_b-P_aP_b$) (Soluk and Collins, 1988; Sih et al., 1998; Vance-Chalcraft and Soluk, 2005) to calculate predicted proportion of prey consumed (P_{ab}) in a mesocosm with two predators (a and b). Term P_a represents the probability that *H. squirella* will be captured by predator 1 (*P. acutus*). Term P_b represents the probability that *H. squirella* will be captured by predator 2 (*Anax sp.*). Term P_aP_b in the model accounts for prey removal, since individual prey consumed by one predator is no longer available to other predators. To determine whether multiple predator effects were the result of mixed species

interactions, or an increase in predator density, we calculated predicted values for mixed and single species multiple predator treatments.

To estimate proportion of prey consumed in each multiple predator treatment, survivorship must be corrected for mortality rates when predators are absent. In our experiment, the proportion of *H. squirella* consumed in each high nutrient predator treatment was corrected to mean survivorship in high nutrient tadpole only treatments and the proportion of *H. squirella* consumed in each low nutrient predator treatment was corrected to mean survivorship in low nutrient tadpole only treatments:

$$Z = (X-Y)/X$$

, where X represents the average survivorship of *H. squirella* within all tadpole only treatments, Y represents the observed proportion of prey consumed in particular tank, and Z represents the corrected observed proportion of prey consumed in a particular tank. In two single predator experimental units, *Anax sp.* did not survive and in those cases the data was excluded. In two tadpole only treatments survival of *H. squirella* tadpoles was abnormally low (<25%), due to contamination by *Pantala sp.* larvae (a predatory dragonfly), and in those cases the data was excluded. If P_a or P_b was not available for a tank within a block, predicted proportion of prey consumed (P_{ab}) could not be calculated due to predator block effects and therefore was not included in the analysis.

We tested the hypothesis that there was no difference between the observed and predicted proportion of *H. squirella* consumed in high and low nutrient multiple predator treatments. To test this hypothesis we used a three-way mixed model ANOVA (Vance-Chalcraft and Soluk, 2005) where the dependent variable was mortality rates (that were either observed or predicted

for a tank) and the independent variables were multiple predator treatment (2 x *Anax sp.*, 2 x *P. acutus*, or *Anax sp. & P. acutus*), nutrient level, and whether the response variable represented an observed or predicted value. Independent variables also included all two-way and three-way interactions. Block effects were included in all analysis. Within our three-way ANOVA multiple predator treatment, nutrient level, observed or predicted mortality value, and all two-way and three-way interactions were fixed effects while block effect, block effect x predator treatment interaction, block effect x nutrient interaction, and block effect x observed or predicted mortality value interaction were random effects. Using a three-way ANOVA allowed us to determine 1) if observed proportion of prey consumed differed from the predicted proportion of prey consumed, 2) if the extent to which observed and predicted values differ varied with multiple predator treatment, 3) if the extent to which observed and predicted values differ was dependent on resource levels, and 4) if the extent to which observed and predicted values differ depended on both which predator treatment that is being considered and the amount of nutrients present.

Prey Growth:

To address whether a modification of resource levels affects growth of larval anurans exposed to simple and complex food webs, we tested the hypothesis that there was no difference in growth of *H. squirella* when exposed to increased nutrient availability in food webs of different trophic complexity. We used a two-way multivariate analysis of variance (MANOVA) where the response vector is larval period and mass at metamorphosis. The independent variables were predator treatment, nutrient level, and predator treatment x nutrient interaction. Block effects were included in all analysis. Within our two-way MANOVA predator treatment, nutrient availability, and predator treatment x nutrient interaction were fixed effects while block effect,

block effect x predator treatment interaction, and block effect x nutrient interaction were random effects. A two-way MANOVA allowed us to determine 1) if *H. squirella* growth varies among predator treatment, 2) if nutrient availability effects growth, and 3) if the effect of nutrient availability on growth depended on the specific set of predators present. Within our two-way MANOVA Pillai's Trace was used as the criteria for significance tests as it is more robust than Wilks' lambda to small sample sizes and deviations from multivariate assumptions (Hair et al., 1995). Using a MANOVA helps protect against committing a type I error that might occur if multiple ANOVA's were performed independently (Scheiner, 2001). To understand which growth component varied among treatments, separate univariate mixed model ANOVA's were performed on each growth vector component (mass at metamorphosis and larval period) (Chalcraft and Resetarits, 2003; Butzler and Chase, 2009). The response variables were mass at metamorphosis or larval period and the independent variables were predator treatment, nutrient level, and predator treatment x nutrient interaction. Block effects were included in all analysis. Within each univariate mixed model ANOVA predator treatment, nutrient availability, and predator treatment x nutrient interaction were fixed effects while block effect, block effect x predator treatment interaction, and block effect x nutrient interaction were random effects.

Algae Biomass:

We tested the null hypothesis that increased nutrient availability had no effect on algal biomass in food webs of different trophic complexity. We used a two-way ANOVA on log transformed biomass data where the dependent variable is biomass and the independent variables are predator treatment, nutrient availability, and predator treatment x nutrient interaction. Block effects were included in all analysis. Within our two-way ANOVA predator treatment, nutrient

availability, and predator treatment x nutrient interaction were fixed effects while block effect, block effect x predator treatment interaction, and block effect x nutrient interaction were random effects. To evaluate any differences in algal biomass, we ran a two-way ANOVA for both periphyton biomass and phytoplankton biomass. A two-way ANOVA allowed us to determine 1) if periphyton or phytoplankton biomass varies among predator treatment, 2) if nutrient availability is having an effect on periphyton or phytoplankton biomass, and 3) if the effect of nutrient availability on peripython or phytoplankton biomass depended on the specific set of predators present.

<div align="center">Results</div>

Prey Mortality:

Mortality varied among predator treatments ($F_{5, 20}$=3.58; p=0.0179), but was not affected by nutrient availability ($F_{1, 4}$=0.10; p=0.7730) or predator treatment x nutrient interaction ($F_{5, 16}$=0.84; p=0.5425). Fisher LSD revealed that tadpoles in treatments containing two *Anax sp.* had the highest overall mortality followed by treatments containing a single *Anax sp.* or both *Anax sp* and *P. acutus* (Figure 2). Treatments with two *P. acutus* or a single *P. acutus* had the lowest mortality of all predator treatments, but treatments with tadpoles only had the lowest overall mortality (Figure 2). After adjusting for False Discovery Rate, mortality in treatments with two *Anax sp.* had the greatest mortality followed by treatments with a single *Anax sp.*, two *P. acutus*, a single *P. acutus*, and both *P. acutus* and *Anax sp.* Treatments with tadpoles only had the lowest mortality (Figure 2).

Mortality within multiple predator treatments showed a trend of prey release when two *P. acutus* were present regardless of nutrient availability (High: -29%; Low: -15%) (Figure 3). Prey

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release was also observed in high nutrient treatments with both *P. acutus* and *Anax sp.* (-28%),

but prey release was negligible in low resource treatments with both *P. acutus* and *Anax sp.* (-4%) (Figure 3). In treatments with two *Anax sp.* we actually saw prey suppression occurring in

high nutrient treatments (+25%), while prey release occurred in low nutrient treatments (-20%)

(Figure 3). However, a three-way ANOVA revealed that the differences between observed and

predicted proportion of prey consumed were not statistically different regardless of multiple

predator assemblages, nutrient availability, or a predator treatment x nutrient interaction

(Observed vs. Predicted: $F_{1, 4}$=1.24, p=0.3721; Predator Treatment x Observed vs. Predicted: $F_{2, 18}$=0.54, p=0.5891; Nutrient x Observed vs. Predicted: $F_{1, 18}$=0.01, p=0.9287; Predator Treatment

x Nutrient x Observed vs. Predicted: $F_{2, 18}$=1.24, p=0.3118). The *Anax sp.* predator was not

recovered at the end of the experiment in seven out of the ten mixed species experimental units.

This was a result of intraguild predation as *P. acutus* possesses the ability to consume *Anax sp.*

and in four cases we observed *P. acutus* eating *Anax sp*.

Prey Growth:

   A two-way MANOVA revealed that predator treatment ($F_{10, 38}$= 3.14, p=0.0051) had a

significant effect on *H. squirella* growth, but neither nutrient availability ($F_{2, 3}$=0.02, p=0.9776)

or predator treatment x nutrient interaction ($F_{10, 28}$=1.87, p=0.0939) had an effect on prey growth.

This finding is supported by a univariate ANOVA on mass at metamorphosis (predator

treatment: $F_{5, 19}$=3.17, p=0.0300; nutrient availability: $F_{1, 4}$=0.00, p=0.9829; predator treatment x

nutrient interaction: $F_{5, 14}$=1.78, p=0.1816). Fisher's LSD revealed that individuals in treatments

containing two *P. acutus* metamorphosed at a larger mass, but there were no differences among

other treatments (Figure 4). We did not present differences after adjusting p-values for False Discovery Rate as these differences were the same as reported by Fisher's LSD.

However, when evaluating differences in larval period a univariate ANOVA indicated that predator treatment x nutrient interaction is important ($F_{5,\ 14}$=3.61, p=0.0264), while there was no difference based on predator treatment ($F_{5,\ 19}$=1.94, p=0.1335) or nutrient availability ($F_{1,\ 4}$=0.01, p=0.9347). Fisher's LSD shows that in low nutrient treatments the addition of either one *Anax sp.* or one *P. acutus* or both extended larval period of *H. squirella*, while other predator treatments did not (Figure 5). The addition of *Anax sp.* extended larval period the longest and the addition of *P. acutus* extended larval period the least (Figure 5). The addition of both predators extended larval period an intermediate amount (Figure 5). When comparing high nutrient treatments only the presence of two *Anax sp.* extended larval period relative to all other treatments (Figure 5). When analyzing differences between high and low nutrient treatments the addition of nutrients tended to extend larval period in treatments with two *Anax sp* (Figure 5). Though an increase in nutrient availability did not statistically alter larval period in some predator treatments, when the same predator treatments are compared, there was a change in observed difference when we simultaneously assess how larval period responded to all treatments (Figure 5). Specifically nutrients appear to enhance larval period when two *P. acutus* is present and reduce larval period when either *Anax sp.* or *Anax sp.* and *P. acutus* are present (Figure 5). Increased nutrient availability did not affect larval period when no predators were present (Figure 5). We did not present pairwise differences after adjusting p-values for False Discovery Rate as these differences were the same as reported by Fisher's LSD.

Algal Biomass:

A two-way ANOVA revealed that periphyton biomass was different based on predator treatment ($F_{6, 24}$=3.40, p=0.0143) but not nutrient availability ($F_{1, 4}$=0.05, p=0.8324) or nutrient x predator treatment interaction ($F_{6, 20}$=0.87, p=0.5310). Fisher's LSD showed that there was less periphyton biomass when only *P. acutus* is present then when only tadpoles are present (Figure 6). Combining tadpoles and *P. acutus* together resulted in having as much periphyton present as when only tadpoles were present. Periphyton biomass was reduced relative to treatments with only tadpoles when *Anax sp.* was added, two *P. acutus* were added, or when both *Anax sp.* and *P. acutus* were added (Figure 6).We did not present pairwise differences after adjusting for False Discovery Rate as these differences were the same as reported by Fisher's LSD.

There was also a difference in phytoplankton biomass depending on predator treatment ($F_{6, 24}$=3.18, p=0.0193) and a marginally non-significant trend between nutrient x predator treatment interaction ($F_{6, 20}$=2.37, p=0.0677) (Figure 7). However, we saw no difference based on nutrient availability alone ($F_{1, 4}$=0.04, p=0.8536). Fisher's LSD showed that treatments containing two *Anax sp.* had more phytoplankton biomass but there was no difference in phytoplankton biomass among other treatments (Figure 7). It appears that nutrient enrichment enhanced phytoplankton biomass in treatments with two *Anax sp.* or treatments with tadpoles only, while nutrient enrichment seemed to reduce phytoplankton biomass in treatments with only *Anax sp.* or both *P. acutus* and *Anax sp.* (Figure 7). Nutrient enrichment did not seem to have an effect in other predator treatments (Figure 7). We did not present pairwise difference after adjusting for False Discovery Rate as these differences were the same as reported by Fisher's LSD. Even though there were no statistically significant differences in phytoplankton biomass, based on nutrient input, high nutrient experimental units appeared to have greater phytoplankton growth as visibility was low and the pond water appeared greener than low nutrient experimental units.

This indicates that phytoplankton growth was greater in high nutrient treatments compared to low nutrient treatments.

Discussion

Under the conditions tested, increasing trophic complexity had an effect on regulating prey mortality but nutrient addition did not. When trophic complexity was increased prey mortality decreased. In our experiment, food webs containing only omnivorous predators had a lower mortality of prey than food webs containing only carnivores. This was not surprising since carnivorous predators are restricted to feeding on one trophic level, while omnivores possess the ability to feed at multiple trophic levels (Gherardi et al. 2004; Bondar et al. 2005; Flinders and Magoulick, 2007). However, when multiple predator types were added to a food web prey mortality was equal to mortality rates found in carnivorous only food webs and is possibly a result of foraging efficiency of the predators used.

In our experiment *P. acutus* was as an inefficient predator while *Anax sp*. was much more efficient at consuming large amounts of tadpole prey, which has been seen in other studies (Chovanec, 1992; Werner and McPeek, 1994; Lefcort, 1996; Eklőv & Werner, 2000). We observed a substantial increase in the amount of prey consumed when *Anax sp*. density was increased, but only a marginal difference in the amount of prey consumed when *P. acutus* densities were increased. The large differences seen in prey mortality between multiple *Anax sp*. and multiple *P. acutus* treatments may due to *H. squirella* being better able invest in anti-predatory behaviors in order to avoid predation, since research has shown that prey invest in certain behaviors to reduce their chances of being consumed (West-Eberhard, 1986; McCollum and Van Buskirk, 1996; Werner and Anholt, 1996; Bolnick and Preisser, 2005). When we

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increased densities of *P. acutus*, *H. squirella* tended to metamorphose at a larger mass than in other treatments. Tadpoles metamorphosing at a larger mass indicates that individuals foraged more frequently, which may be due to the risk of predation being less when two *P. acutus* were present than in other predator treatments. Contrary to what was seen when *P. acutus* was present, *Anax sp.* consumed a greater amount of prey than *P. acutus* and may be the result of prey not being able to avoid predation as well as in *P. acutus* treatments. *Anax sp.* acted as a much more efficient predator than *P. acutus* and since the per capita risk of prey being consumed by a predator was greater when *Anax sp.* was present, prey may have foraged less frequently causing *H. squirella* to metamorphose at a smaller mass (West-Eberhard, 1986; Morin, 1998; Anholt and Werner, 1995; Werner and Anholt, 1996; Van Buskirk and Yurewicz, 1998; Relyea, 2002). These differences indicate that the overall risk of predation may have had a stronger direct effect on prey mortality and growth in our experiment than nutrient availability.

While nutrient addition had no direct effect on prey mortality, nutrient addition did seem to regulate the occurrence of emergent multiple predator effects and larval period of *H. squirella* under certain conditions. When nutrients were added to treatments with both *Anax sp.* and *P. acutus*, we observed a 28% difference in the extent of prey release and a six day reduction in larval period of *H. squirella* compared to low nutrient treatments. This large difference in prey release in mixed species treatments may be the result of intraguild predation which would reduce predation events on prey, in comparison to experimental units where intraguild predation did not occur, by directly removing a predator and tended to be more common in high nutrient treatments (Table 2). An increase in nutrient availability may have reduced per capita interaction rates between prey and predators, through increased foraging opportunities, and therefore increased interference interactions between predators (Soluk and Collins, 1988; Werner and

Peacor, 2003). The occurrence of intraguild predation should not be ignored in future experiments as it can have large effects on prey mortality that are different than would be predicted if predators operated independently.

When nutrients were added to multiple *Anax sp.* treatments we observed prey release occurring and larval period being enhanced, compared to low nutrient treatments in which prey suppression occurred. The differences seen in emergent multiple predator effects between high and low nutrient *Anax sp.* treatments is rather surprising and implies that nutrients are causing prey to respond to predation differently under certain conditions. In low nutrient treatments prey may have been forced to forage more frequently to avoid starvation, compared to high nutrient treatments where prey could decrease foraging behavior, thus increasing per capita interaction rates with *Anax sp.* (McNamera and Houston, 1987; Lima and Dill, 1990; Abrams, 1991; Relyea, 2001; Bolnick and Preisser, 2005). Besides per capita interaction rates being greater in low nutrient treatments, compared to high nutrient treatments, the occurrence of cannibalism or *Anax sp.* emergence may also have contributed to differences seen in emergent multiple predator effects. Cannibalism would directly remove a predator and decrease predation events on *H. squirella*, compared to experimental units where cannibalism did not occur. This was a strong possibility in our experiment as *Anax sp.* has been known to become cannibalistic (Van Buskirk, 1992; Hopper, Crowley, and Kielman, 1996; Crumrine, 2010). Crumrine (2010) found that cannibalism actually occurred 78% of the time when individuals varied in size by 1 instar difference or more and 63% of the time when individuals were the same-size (Crumrine, 2010). In multiple experimental units, with two *Anax sp.* present, one *Anax sp.* individual was not recovered and can be attributed to cannibalistic activity (Table 2). This phenomena likely explains some of the variation seen in mortality rates between high and low nutrient treatments,

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as *Anax sp.* of similar size could have become cannibalistic, which would have decreased mortality of *H. squirella* in high nutrient treatments. Another factor that may explain some of this variability in multiple *Anax sp*. treatments is *Anax sp.* emergence. Although we immediately replaced five *Anax sp*. individuals that emerged from five other mesocosms (Table 2), the need to perform these replacements may have added to some of the variability seen in mortality rates of *H. squirella* because *Anax sp.* may have reduced foraging activity when emerging. *Anax sp.* is an efficient predator on tadpole prey and differences in per capita interactions rates or predator removal may explain why prey were consumed in an additive fashion in a low nutrient treatments and non-additive in high nutrient treatments.

Even though increasing nutrient availability caused differences in both mortality and growth rates of prey, nutrient addition only had an effect on phytoplankton production. When nutrients were added to experimental units we saw that phytoplankton was increased in treatments with two *Anax sp.* or treatments with both *Anax sp.* and *P. acutus*, but was not enhanced in any other treatments. It was surprising that periphyton biomass did not differ when nutrients were added and may be a consequence of periphyton and phytoplankton competing for the same resources. Besides phytoplankton and periphyton competing for the same resources, periphyton also serves as a preferred food source for many herbaceous consumers which could further suppress periphyton production. Leibiold and Wilbur (1992) found that nutrient addition greatly increased phytoplankton biomass in the presence of tadpoles, but when tadpoles were absent nutrient addition reduced phytoplankton biomass. In our experiment, both tadpoles and crayfish possess the ability to suppress periphyton production through direct consumption. All of our experimental units contained at least one species of periphyton consumer and likely explains

why there was a difference in phytoplankton biomass based on nutrient availability but no difference in periphyton biomass (Leibold and Wilbur, 1992).

Conclusion

While we predicted that nutrient addition would strongly affect both prey mortality and growth, we found that predation may play a greater role in regulating prey mortality and growth than other biotic or abiotic factors, which has been seen in other experiments (Figiel and Semlitsch, 1990; Gurevitch, Morrison, and Hedges, 2000; Heyer, McDiarmid, and Weigmann, 1975; Relyea and Werner, 1999; Relyea, 2000). Most importantly our experiment shows that multiple predators can have unexpected impacts on regulating prey mortality and has provided a base for future experiments that are interested in investigating how increased nutrient availability can regulate trophic interactions. Even though we saw no statistically significant results between observed and predicted proportion of prey consumed, we did observed large trends that may have important biological implications. Our results have large standard errors, which can be attributed to variability in consumption rates by predators. To account for this variability future experiments should increase the amount of replicates used, which was not possible in our experiment. The biological implications of this study should not be ignored as it provides insight into the effects that increased nutrient availability has on regulating complex trophic interactions within natural food webs.

Table 1: Summary of experimental treatments. Treatment number is listed in the first column where (x5) represents 5 replications of each treatment resulting in a total of 70 experimental units. Predators present in each treatment are listed in the second column. A treatment with "x2" represents the presence of two individual predators in each experimental unit. The number of prey added to each treatment is listed in the third column while nutrient load is represented in the fourth column.

Treatment	Predator	Prey	Nutrient levels	Food Web Complexity
1 (x5)	No predator	275 *H. squirella* tadpoles	Low	Simple
2 (x5)	*P. acutus*	No Prey (crayfish eat algae)	Low	Simple
3 (x5)	*Anax*	275 *H. squirella* tadpoles	Low	Less Simple
4 (x5)	*P. acutus*	275 *H. squirella* tadpoles	Low	More Complex
5 (x5)	*Anax x 2*	275 *H. squirella* tadpoles	Low	Increased Density Less Simple
6 (x5)	*P. acutus* x2	275 *H. squirella* tadpoles	Low	Increased Density More Complex
7 (x5)	*P. acutus & Anax*	275 *H. squirella* tadpoles	Low	Most Complex
8 (x5)	No predator	275 *H. squirella* tadpoles	High	Simple
9 (x5)	*P. acutus*	No Prey (crayfish eat algae)	High	Simple
10 (x5)	*Anax*	275 *H. squirella* tadpoles	High	Less Simple
11 (x5)	*P. acutus*	275 *H. squirella* tadpoles	High	More Complex
12 (x5)	*Anax x 2*	275 *H. squirella* tadpoles	High	Increased Density Less Simple
13 (x5)	*P. acutus* x2	275 *H. squirella* tadpoles	High	Increased Density More Complex
14 (x5)	*P. acutus & Anax*	275 *H. squirella* tadpoles	High	Most Complex

Table 2: Summary of predator survival in each treatment in each block. Nutrient refers to whether treatment was exposed to increased nutrients (High) or ambient conditions (Low). Number in survived column refers to whether each predator present survived. Date in emerged and replaced column refers to when predator emerged and was replaced.

| Block	Predator Treatment	Nutrient	Survived	Emerged & Replaced
A	Anax	Low	1:Yes	7/27/2012
	Anax x2	Low	1 :Yes; 2: Yes	n/a
	Anax & Acutus	Low	No	n/a
	Anax	High	Yes	8/10/2012
	Anax x2	High	1: Yes; 2: Yes	n/a
	Anax & Acutus	High	No	n/a
B	Anax	Low	Yes	n/a
	Anax x2	Low	1: Yes; 2: Yes	n/a
	Anax & Acutus	Low	Yes	n/a
	Anax	High	No	n/a
	Anax x2	High	1: Yes; 2: No	n/a
	Anax & Acutus	High	Yes	n/a
C	Anax	Low	Yes	n/a
	Anax x2	Low	1: Yes; 2: No	n/a
	Anax & Acutus	Low	Yes	n/a
	Anax	High	Yes	n/a
	Anax x2	High	1: Yes; 2: No	n/a
	Anax & Acutus	High	No	n/a
D	Anax	Low	Yes	n/a
	Anax x2	Low	1: Yes; 2: Yes	n/a
	Anax & Acutus	Low	No	n/a
	Anax	High	No	8/7/2012
	Anax x2	High	1: Yes; 2: Yes	n/a
	Anax & Acutus	High	No	n/a
E	Anax	Low	No	n/a
	Anax x2	Low	1:Yes; 2: Yes	n/a
	Anax & Acutus	Low	No	n/a
	Anax	High	Yes	8/7/12
	Anax x2	High	1: Yes; 2: Yes	7/27/2012
	Anax & acutus	High	No	n/a
*All *P. acutus* survived in all treatments in all blocks				
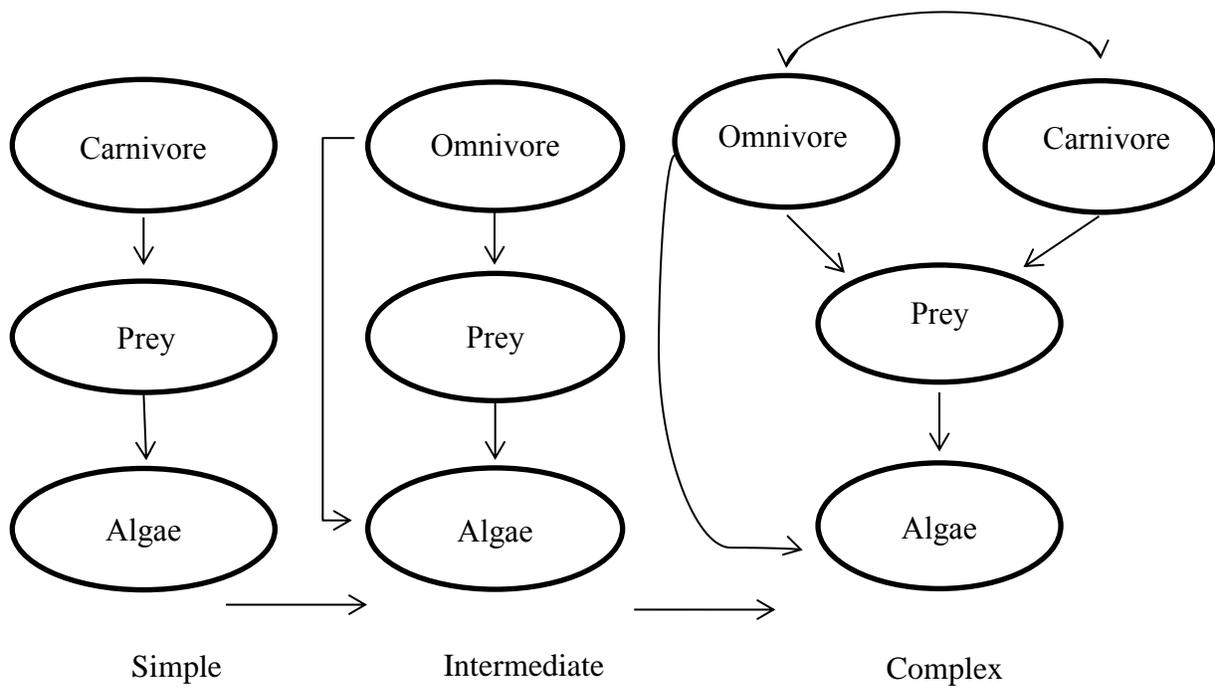
Figure 1. Progression of food web complexity. Most simple food web is on the left. Most complex food web is on the right.
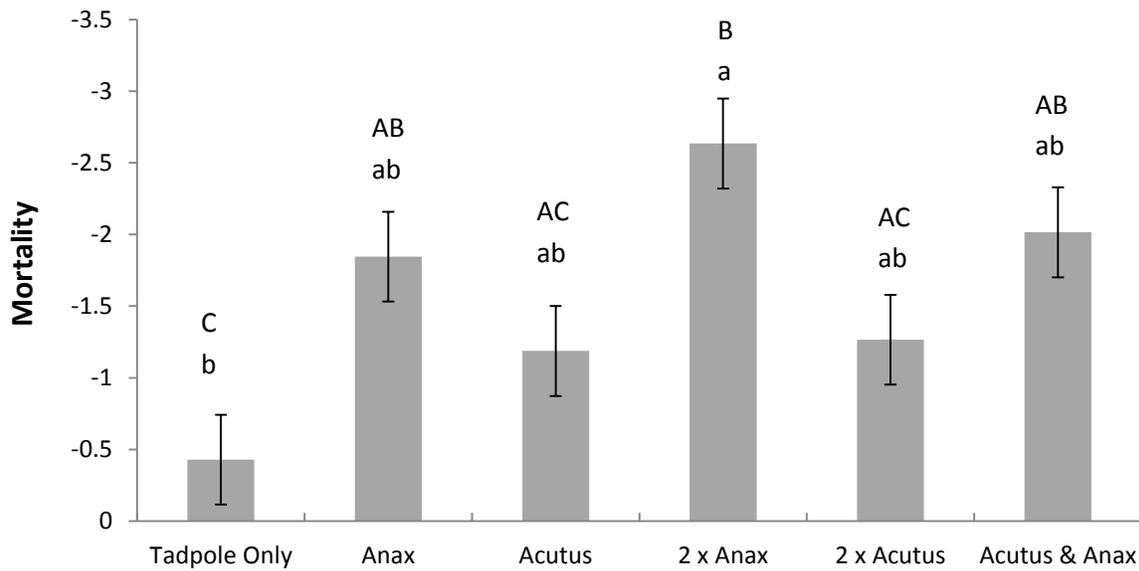
Figure 2: Mortality of *H. squirella*. Data presented as least square mean estimates for responses in predator treatment. Mortality represents the proportion of individuals consumed to the amount of individuals added. Upper case letters indicate treatments that are significantly different based on Fisher's LSD. Lower case letters represent significantly different treatments after adjusting for False Discovery Rate. Bars represent one positive and one negative standard error.
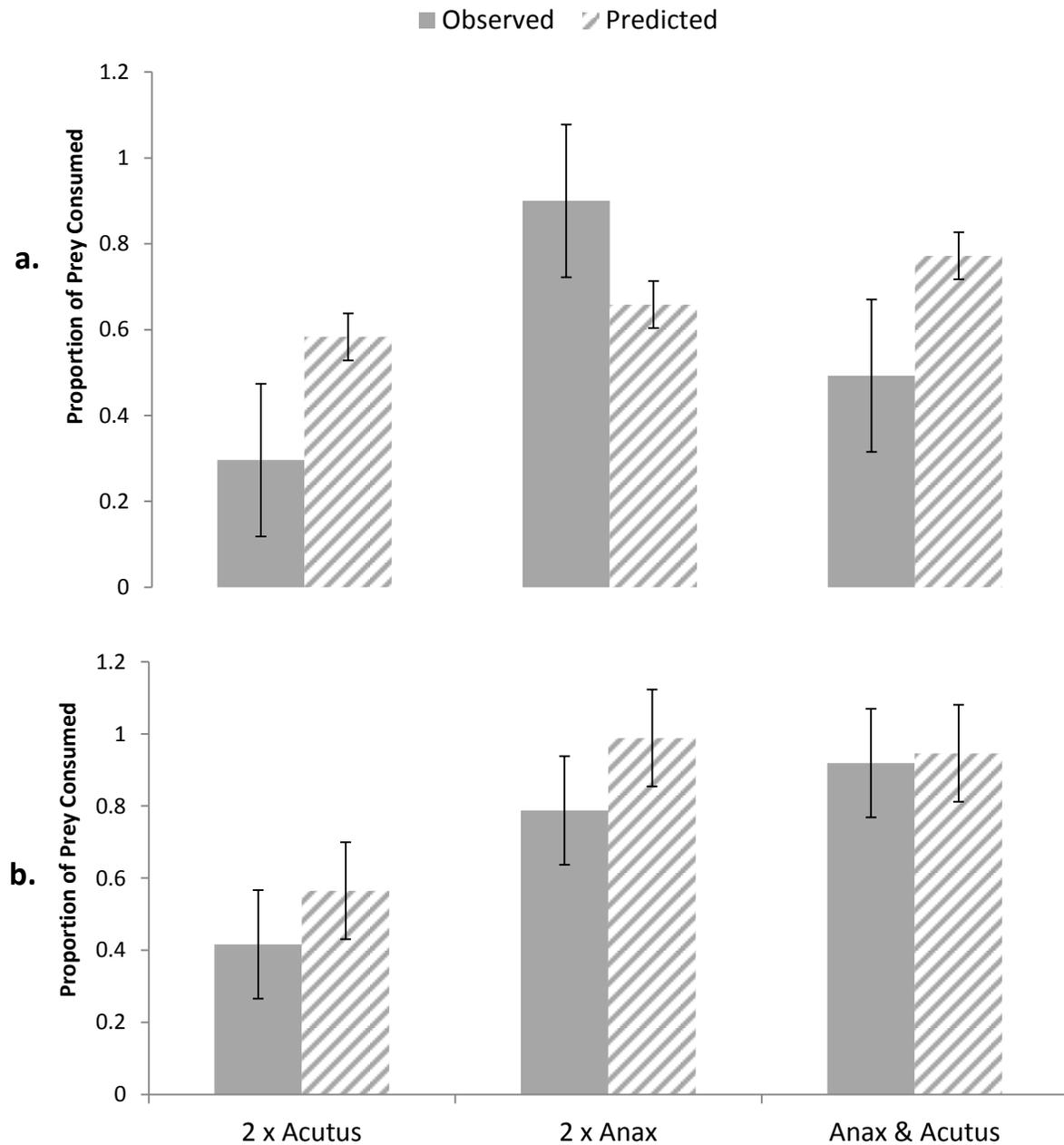
Figure 3: Mean observed and predicted proportion of *H. squirella* consumed in a) low nutrient and b) high nutrient treatments. Data presented as least square mean estimates for responses in predator treatment. "2 x" represents the presence of two individual predators of the respective species within a treatment. Predicted consumption was determined using Soluk and Collins' multiplicative risk model (1988). Bars represent one positive and negative standard error.
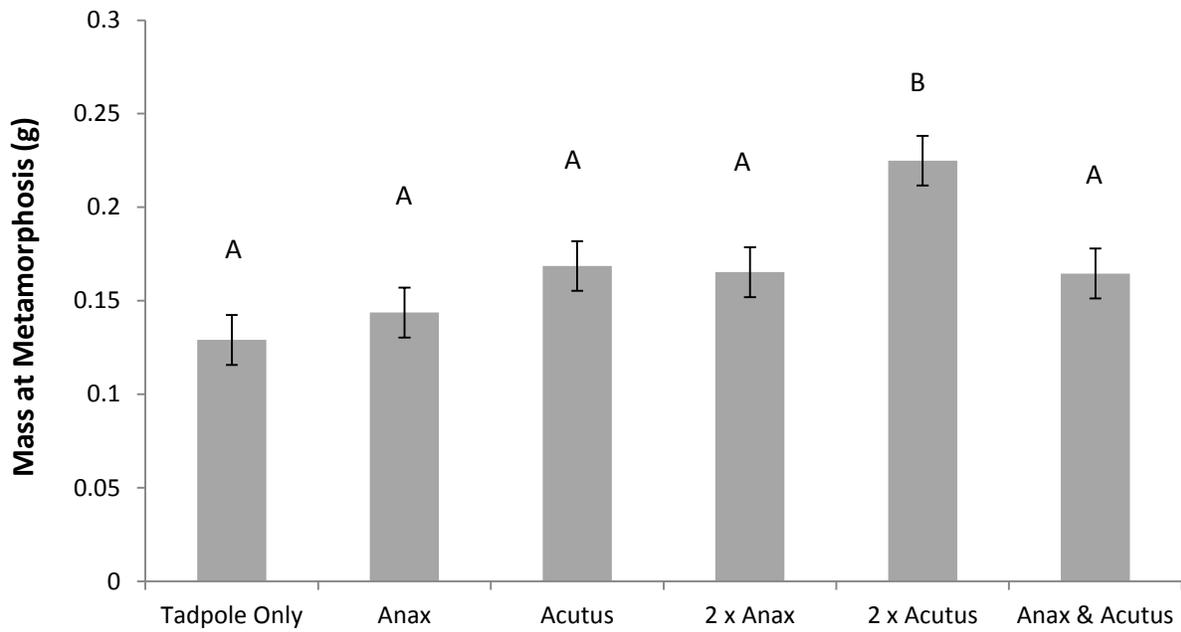
Figure 4: Mass at metamorphosis of *H. squirella*. Data presented as least square mean estimates for responses in predator treatment. Upper case letters indicate treatments that are significantly different based on Fisher's LSD. Bars represent one positive and one negative standard error.

Figure 5: Larval period of *H. squirella.* Data presented as least square mean estimates for responses in predator treatment x nutrient interaction. Upper case letters indicate treatments that are significantly different based on Fisher's LSD. Bars represent one positive and one negative standard error.
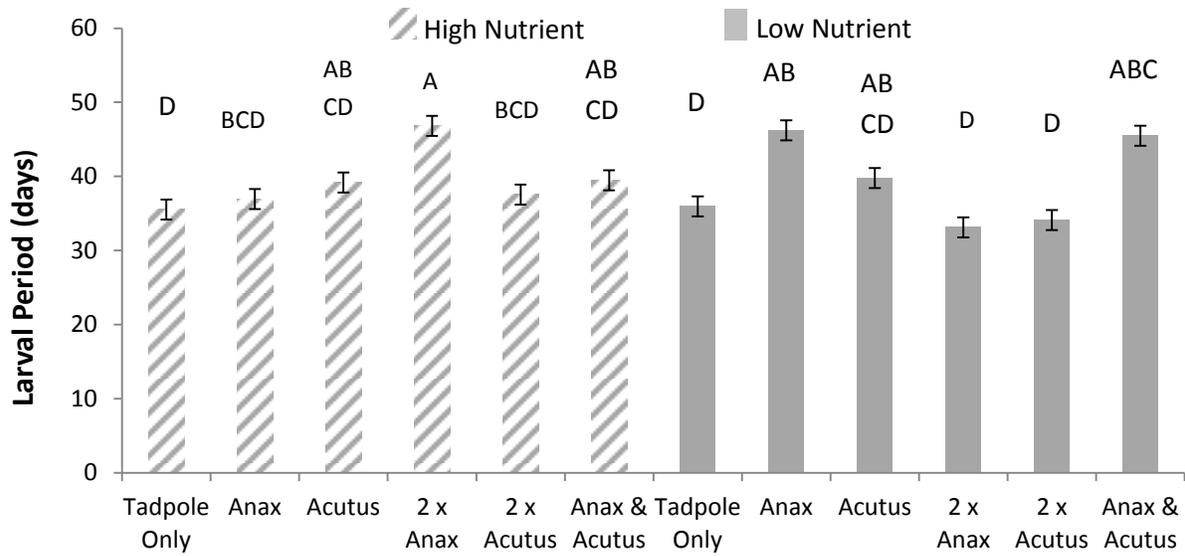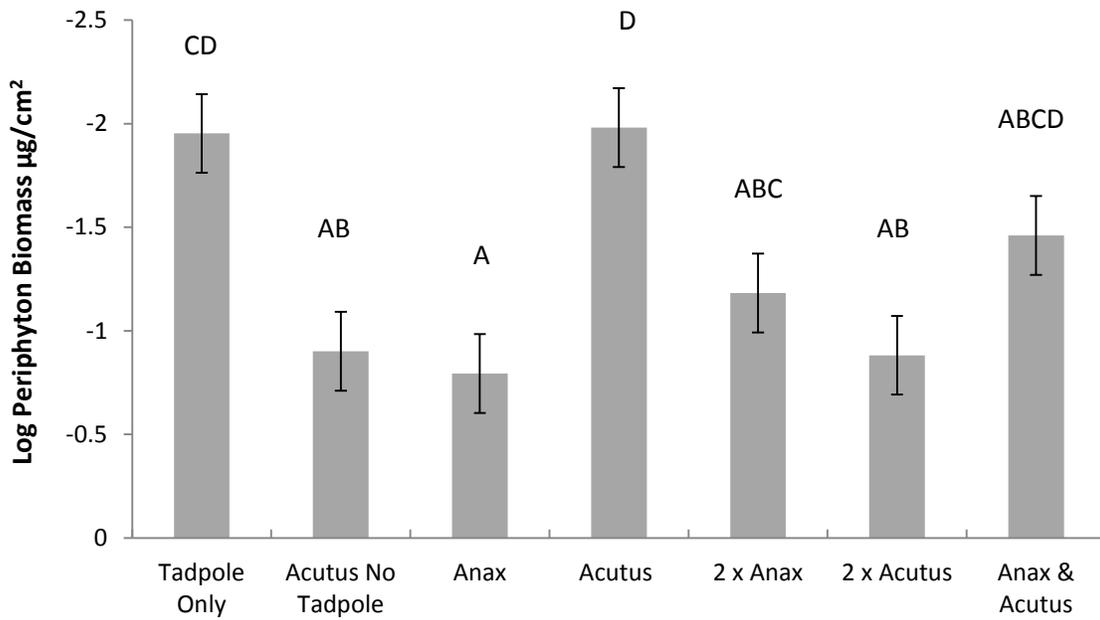
Figure 6: Periphyton biomass. Data presented as least square mean estimates for responses in predator treatment. Upper case letters indicate treatments that are significantly different based on Fisher's LSD. Bars represent one positive and one negative standard error.
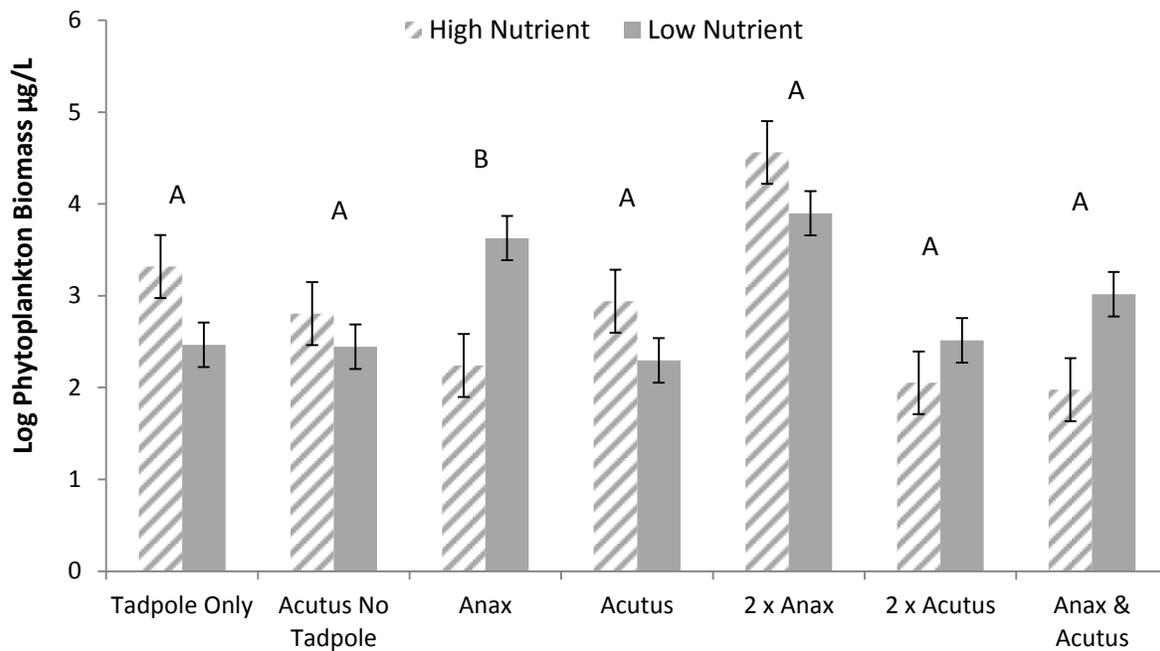
Figure 7: Phytoplankton biomass. Data presented as least square mean estimates for responses in predator treatment. Upper case letters indicate predator treatments that are significantly different based on Fisher's LSD. Bars represent one positive and one negative standard error.

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◫ East Carolina University.

Animal Care and
Use Commitee
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East Carolina University
Greenville, NC 27834

252-744-2436 office
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April 26, 2012

David Chalcraft, Ph.D.
Department of Biology
Howell Science Building
East Carolina University

Dear Dr. Chalcraft:

Your Animal Use Protocol entitled, "Does the Quality of Resources in the Environment Alter the Impact of Multiple Predators on their Prey?" (AUP #D273) was reviewed by this institution's Animal Care and Use Committee on 4/26/12. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

enclosure