

## ABSTRACT

### INVESTIGATING THE EFFECTS OF LARVAL PHENOTYPES ON TRAITS EXHIBITED AT A CRUCIAL LIFE-HISTORY SWITCH POINT

by

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In organisms with complex life histories, phenotypes exhibited in non-reproductive life stages are often correlated with adult performance and fitness. For example, in anurans (frogs and toads), age and size at metamorphosis is commonly correlated with age at maturity, fecundity, predator avoidance and competitive ability. However, the mechanisms that lead to variation in fitness-associated traits at metamorphosis are less well understood. This study investigated how hatchling body size and hatchling activity level affected fitness-associated traits at metamorphosis in squirrel treefrogs (*Hyla squirella*), and whether the effects of hatchling phenotypes varied with risk of predation. I demonstrate how hatchling phenotypes, predation risk, and interactions of the two, affected *H. squirella* larval survival as well as fitness-associated traits at metamorphosis. Without predation, initially small and less active hatchlings emerged with traits of better quality at metamorphosis. With predation, initially large, small and active

hatchlings emerged with increased trait quality at metamorphosis, while initially less active hatchling trait quality reduced at metamorphosis. This study provides an important step towards improving our understanding of how mechanisms carry over to affect variation in fitness-associated traits at metamorphosis.



INVESTIGATING THE EFFECTS OF LARVAL PHENOTYPES ON TRAITS EXHIBITED  
AT A CRUCIAL LIFE-HISTORY SWITCH POINT

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## INTRODUCTION

Phenotypic variation exists at all levels of biological organization: among species, populations of the same species, genotypes within populations, individuals within genotypes, and within particular individuals (Westneat *et al.* 2014). Phenotypic variation among individuals can arise through multiple underlying intrinsic and extrinsic processes, including heritable genetic variation (Warne *et al.* 2013), phenotypic plasticity in response to spatial or temporal variation in environmental conditions (Relyea 2001; Warne *et al.* 2013; Van Buskirk 2017), maternal effects (such as oviposition site selection and/or an uneven allocation of resources to offspring; Marshall *et al.* 2007), and dispersal of individuals to different habitats (Garant *et al.* 2006). In addition, among individual variation can often be sustained through rare beneficial alleles that remain in a population (negative frequency-dependent selection), multiple traits that yield similar fitness (balancing selection) and selectively neutral genetic drift (Turelli *et al.* 2004; Charlesworth 2006). Regardless of the underlying mechanisms, phenotypic and genetic variation can be important for populations to adapt and persist in variable environmental conditions (Chevin *et al.* 2010; Westneat *et al.* 2014).

Understanding the processes that generate and maintain phenotypic variation in organisms with complex life-histories is important because phenotypes exhibited during one stage can have carry over effects that influence performance in other life stages (McDiarmid *et al.* 1999; Johansson *et al.* 2010; O'Conner *et al.* 2014). Such carry over effects can manifest in a variety of traits, ranging from morphological and developmental traits to ecological and behavioral traits (Touchon *et al.* 2015). Much work has focused on quantifying phenotypic variation among individuals at metamorphosis (Mangel *et al.* 2001) and how these affect fitness-

associated traits in the adult life stage, such as age at maturation, fecundity, adult survival and competitive ability (Travis 1980; Semlitsch *et al.* 1988). However, the extent to which phenotypic variation at metamorphosis is driven by variation during earlier life stages is not well understood. In this study, we take an important step towards improving our understanding of how the phenotypes of hatchlings carry over to affect variation in fitness-associated traits at metamorphosis.

Being the only tetrapods to undergo metamorphosis (Wilson *et al.* 2012), amphibians have long been model systems for investigations on phenotypic variation at life stage transitions, and how these traits affect individual performance. For example, wood frogs (*Lithobates sylvatica*) that were larger at metamorphosis became larger juveniles and matured earlier as larger, reproductive adults (Berven 1990). Individuals with a larger body size are also often better competitors for limited resources in high-density situations (Asquith *et al.* 2012). In frogs, larger individuals can also have higher breeding success as adults (Smith 1987). Larger males project calls that are more attractive to females than smaller males (McClelland *et al.* 1996), and larger females can lay more eggs per clutch or allocate more resources towards each egg compared to smaller females (McGinley 1989; Marshall and Uller 2007).

Variation in phenotypic traits, such as body size, is important during life stages as well as life stage switch points. While the mechanisms that generate among individual variation are not well understood, we know that even within a sibship, individuals can vary greatly in body size, and that this variation increases through time (Brooks *et al.* 2013). One hypothesis for why variation increases through time is that slight differences in either initial body size or individual activity are exacerbated through time due to autocorrelation in processes such as foraging rates. For example, tadpoles that hatch with larger body sizes are expected to outcompete smaller

tadpoles for food resources (Wilbur & Collins 1973; Steinwascher 1978), and to develop more quickly allowing earlier maturation (Collins 1979; Travis 1980). These initially large individuals are also expected to emerge with larger body sizes and better body conditions (higher body fat mass) (Steinwascher 1978) compared to individuals that hatch with initially small body sizes. Alternatively, newly hatched tadpoles can vary in their intrinsic levels of activity, such as time spent swimming or foraging for food (Sih *et al.* 2004; Herde and Eccard 2013). For example, red-eyed treefrogs (*A. callidryas*) that hatched later were found to have higher activity levels relative to those that hatched earlier (Warkentin 1999). Individuals with higher activity levels often forage for resources more than individuals with less active phenotypes (Skelly 1994). These acquired resources can be allocated towards larval growth (Warne *et al.* 2003), which becomes important when exposed to predation because active individuals can achieve a size refuge and reduce cumulative risk to gape-limited predators (Osenberg & Mittelbach 1989; McCoy and Bolker 2008, McCoy *et al.* 2011). Resources acquired by active individuals can also be allocated towards faster development to permit a quicker emergence for an earlier maturation onset (McCoy *et al.* 2007). Thus, quickly emerging, active tadpoles can also escape their predator-ridden aquatic environment (Relyea 2007). Therefore, hatchlings displaying higher activity levels are expected to emerge quickly as larger metamorphs with better body conditions.

Another important source of phenotypic variation is environmentally-induced adaptive phenotypic plasticity in response to stressors, such as predation, which can also carry over across life stages (Cressler *et al.* 2010; English *et al.* 2016). For example, American toad (*Anaxyrus americanus*) tadpole activity decreased by 41% in the presence of *Anax* dragonfly nymph predator chemical cues compared to when cues were absent (Skelly *et al.* 1990). This induced decrease in activity is immediately advantageous for larval survival (Skelly 1994; Peacor 2002),

but may lead to a longer duration of the larval period (Relyea 2002; Altwegg 2002). However, some studies have found that larvae raised with lethal predators have accelerated development and metamorphose earlier allowing them to escape aquatic predators faster (Relyea 2007). Predation can also induce plastic morphological responses. For example, predator-induced decreases in activity level often occur in concert with an increase in tadpole tail fin musculature (Van Buskirk *et al.* 1998). However, the generation of anti-predator phenotypes often come at the cost of competitive ability and decreased larval and metamorph growth and developmental rates (Relyea 2002). Therefore, predator-induced plasticity can affect both the age and size of individuals at metamorphosis. Moreover, differences among individuals in ability to detect or respond to predation risk can generate additional phenotypic variation among individuals.

For organisms that undergo complex life-histories, many traits exhibited by recently emerged metamorphs, such as body size and body condition, drive fitness-associated traits in the adult life stage. However, less is known about how the initial phenotypes displayed by newly hatched larvae affect the phenotypes exhibited at metamorphosis. Even less is known about how environmental selective pressures, such as predation, may shift the carry over effects of initial hatchling phenotypes on traits displayed at metamorphosis. The goal of this study was to investigate: 1) whether the body sizes or activity levels of hatchlings are important for driving the phenotypes displayed at metamorphosis and 2) if predation dampens or facilitates the carry over effects of initial phenotypes on traits displayed at metamorphosis. In this study, I partitioned individuals of squirrel treefrog (*Hyla squirella*) hatchlings into categories based on initial differences in activity levels and body sizes, and reared them under different predation conditions to determine how the fitness-related traits of metamorphs are influenced. By decoupling hatchling traits, I test hypotheses about the independent effects of activity level and



body size on survival, age, body size and condition at metamorphosis. Specifically, if initial phenotypes of hatchlings carry over to influence metamorphic traits, then more active and larger individuals might be expected to have higher performance in predator free environments, but lower performance in the presence of predators. However, if frequency dependence or stochastic processes maintain phenotypic variation, then we might expect cohorts of similarly sized or active individuals to have similar or lower performance than natural mixtures of phenotypes.

## MATERIALS AND METHODS

### Tadpole Collection

*Hyla squirella* amplexed pairs were collected on June 16<sup>th</sup>, 2016 at East Carolina University's West Research Campus (35°37'51"N 77°29'9"W). Ten pairs were collected using visual surveys, were placed into 2.1L buckets containing a shallow layer of water from the collection site, and left overnight to complete oviposition. All adults were released the following morning. Egg clutches from all 10 pairs of frogs were transported back to East Carolina University where they were maintained in aquaria containing dechlorinated tap water until hatching.

The tadpoles hatched from their eggs, on average, four days post-oviposition. Waste products, such as ammonia, can accumulate to toxic levels in the water during hatching (Jofre *et al.* 1999), so newly hatched larvae were transported to fresh, dechlorinated tap water via transfer pipette. Each clutch from the cohort was combined to eliminate sibling bias prior to any sorting into the experimental treatments (Bridges 1999; Peacor *et al.* 2006). All tadpoles were kept in a laboratory environment of 22-25°C, with a light:dark photoperiod of 12:12 until yolk resorption was completed (Uller *et al.* 2002; Wright 2002), and individuals were ready for phenotype partitioning.

### Tadpole Partitioning

The aim of partitioning the newly hatched tadpoles by specific phenotypes was to determine if traits observed early in life (hereafter termed initial phenotypes) affect among individual variation in growth and developmental rates more than predation or stochastic processes. Specifically, we generated 5 phenotypic groups: initially large, initially small, active, less active and mixed. Each phenotype treatment was placed into a treatment either with or without predation, which yielded 10 total experimental treatments. For an ecologically relevant comparison, tadpoles of varying age-specific body sizes and activity levels were haphazardly removed from the initial cohort. This mixture of phenotypes (Hereafter, known as the mixed treatment) reflected what we might expect in a natural population, and was removed from the initial cohort before the size or activity level partitioning procedures took place.

For the size partitioning, a random selection of hatchlings was sorted into one of the two size classes: initially large or initially small. Size partitioning was executed without consideration of activity levels. To sort the newly hatched tadpoles into size groups, approximately half of the tadpole cohort (~1,800 individuals) were randomly removed and separated out into groups of 20. Each group of 20 was placed in  $\frac{1}{2}$  L glass observation bowls (Carolina Culture dish  $4\frac{1}{2}$  diameter) that were filled with dechlorinated tap water and placed over 8x8mm grid paper. Individuals with the longest body length relative to other individuals were removed from the observation bowls, and were placed into 2.1L holding tanks. The smallest individuals were also removed and placed in a separate 2.1L holding tank. This partitioning procedure yielded 700 initially large and 700 initially small individuals. The remaining intermediately sized individuals were not used in the experiment, and were immediately released at the site of collection.

The remaining half of the tadpole cohort was partitioned for one of two activity level treatments: active or less active. Activity level partitioning was carried out without consideration of individual body size. Twenty individuals were randomly placed into ½ L glass observation bowls filled with de-chlorinated tap water. The tadpoles were allowed to acclimate to the bowls for an hour before behavioral partitioning took place. Once the acclimation period was completed, individuals in each bowl were observed for two, one-minute intervals. Within that time frame, individuals were sorted based on their activity level using an ethogram developed through visual observation of the initial tadpole cohort. Specifically, active individuals were defined as those that performed the following actions: tail flicking, swimming, swirling, nose bumping into another individual, and twitching. If an individual did not perform any of those behaviors during the observation periods, then they were classified as less active. All active individuals were removed from the observation bowls via transfer pipette, and were placed in the appropriate 2.1L holding tanks. The remaining less active individuals were placed into separate holding tanks. Tadpoles transferred from the observation bowls to holding tanks took place only after the observation periods were completed to refrain from artificially inducing an escape response.

This process was repeated until 700 active individuals and 700 less active individuals were identified. Behavioral partitioning was completed during the late morning and early afternoon on a single day to keep circadian rhythms constant (Zavorka *et al.* 2016). We measured our phenotype groups to confirm that our partitioning methods successfully distinguished among body sizes (Supplement 1).

## Experimental Procedures

Experimental mesocosms were created in fifty 568 L Rubbermaid stock tanks. Each was prepared with a mixture of rain water and aged tap water, an initial inoculation of 5mL of dechlorinator, 1L of Premier Sphagnum Peat Moss, a 1L inoculation of concentrated zooplankton collected from an artificial drainage pond, and about 150g of leaf litter (Touchon *et al.* 2015). The leaf litter was collected in 13-gallon trash bags and was haphazardly mixed to assure a heterogeneous mixture of organic matter at the bottom of each tank to provide a nutrient base along with the assorted community of microbes (Van Buskirk and Schmidt 2000). We allowed the zooplankton and algal populations to establish for 30 days before the treatments were initiated (Peacor and Pfister 2006). These ingredients permitted the tadpoles to persist in the tanks without having to be manually fed during the experiment (Van Buskirk 2002). To achieve 3-dimensional complexity, two 30x 60cm pieces of weighted, plastic chicken wire fencing were placed inside each end of each mesocosm. These structures along with the drainage pipe provided sufficient surface area to facilitate algal growth for tadpole nutrition as well as shelter. Each mesocosm was covered with a 60% shade cloth screen to prevent oviposition or colonization by unwanted organisms (Peacor and Pfister 2006).

Fifty total mesocosms were separated into five spatial blocks of ten tanks each oriented together in an open field. Each of the ten treatments [Table 1] were randomly assigned to an experimental tank within a spatial block. The 3,500 partitioned ten-day-old tadpoles (700 per each of the five tadpole treatments) were placed into the appropriate mesocosm treatments, resulting in 70 individuals per tank. For each of the predator treatments, *Belostoma flumineum* (giant water bugs) were placed in the designated tanks.

*Belostoma flumineum* are natural predators to most Eastern North Carolina anuran species. The presence of *B. flumineum* was meant to produce consumptive effects via tadpole mortality as well as potential non-consumptive effects by provoking predator-induced behavioral, morphological and physiological responses (Werner & Gilliam 1983). As sit-and-wait ambush predators, these individuals either hide among leaf litter or hover at the surface of the water. Fifty *B. flumineum* were collected from an artificial drainage pond adjacent to a Lowe's Hardware store in Greenville, North Carolina. Two water bugs were placed in each tank to approximate natural predator densities while minimizing risk of cannibalism.

The tadpoles as well as the water bugs were placed into the mesocosms on June 28<sup>th</sup>, 2017, and were allowed to remain in the respective treatments until natural metamorph emergence occurred (Touchon *et al.* 2015). Each mesocosm was monitored daily for emerging individuals, and additional size and behavioral assays were performed until the metamorphs began emerging from the mesocosms. Each surviving metamorph was collected and placed into individual 162mL plastic cups (Diamond Daily Multi-Purpose Snack Cups) to be transported back to the laboratory at East Carolina University. Each individual was measured for snout-vent length, tail length and total body length using calipers as well as dry mass using a GeneMate digital scale to the precision of a thousandth of a gram. Body condition was assessed by an allometric regression of body mass and body size. The time it took (in days) for each individual to emerge from the water was also recorded along with the survivorship in each treatment. The metamorphs were maintained in the laboratory in their individual plastic cups until tail resorption was completed (Gosner stage 46; Gosner 1960), and they became juveniles. The juveniles were released at the site of egg clutch collection.

## Statistical Analysis

I analyzed the effects of *H. squirella* larval phenotypes at hatching and predation on survivorship to metamorphic emergence, metamorph emergence rate and body size as well as body condition. All analyses were carried out in the R statistical Programming Environment Version 1.0.136 (R Core Team 2015). I used generalized linear mixed models (GLMM) using the lme4 package (Bates, Maechler, Bolker & Walker 2015). For each analysis, I included initial phenotype and predation as fixed effects and block as random effect. For analyses on individual-level endpoints, I specified tank nested within block as my random effect. Model diagnostics were performed via visual inspection of residual and quantile-quantile (QQ) plots. I also inspected the distribution of the conditional nodes for the random effects included in each model. Inferences from GLMMs are based on Likelihood Ratio Tests (LRTs) by comparing full models to reduced models that excluded parameters of interest. Inferences about differences among levels of main effects are based on 95% confidence intervals for treatments being compared.

I examine the effects of activity level and body size independently since I had no *a priori* expectations for comparing across these phenotypic traits. Both data sets included the mixed treatments for comparison because it was assumed to best represent the activity levels and body size distributions of the natural population. Survivorship data were analyzed with a GLMM with a binomial error distribution with a logit link. For emergence rate, I analyzed the proportion of total surviving tadpoles that emerged per day post-hatching. For this analysis, I assumed a binomial error distribution with log link. This analysis included interaction terms between the fixed effects of Days (since being placed into the mesocosms), initial phenotype and predation.

Body size and body condition data were analyzed with a Gaussian error distribution with a log link. These models included an interaction term between initial phenotype and predation.

All analyses excluded the same seven of the 50 tanks due to accidental introduction and oviposition by the narrow-mouth frog (*Gastrophryne carolinensis*). The treatments excluded from the experiment included: three tanks of mixed with predation, two tanks of mixed without predation, one tank of initially small without predation and one tank of less active with predation. Furthermore, in one tank of active tadpoles, one of the water bug predators died during the experiment.



## RESULTS

### Survival and Time to Metamorphosis

Survival was defined as the proportion of larval individuals that survived to metamorphosis out of the total amount initially placed in each mesocosm treatment. Across all initial phenotype and predation treatments, approximately 86% ( $n=3,010$ ) of tadpoles from all treatments survived to metamorphosis. Survivorship was affected differently by initial tadpole body sizes, by initial tadpole activity levels and by predation.

Time to emergence was indicative of how quickly or slowly metamorphic development occurred for individuals that survived the larval life stage. The first metamorphs emerged from the water 20 days after being placed into the mesocosms, which is considered a relatively rapid emergence rate. The final metamorphs were collected 55 days after being placed into the mesocosms, which is considered a slower emergence rate. Time to emergence was affected differently by initial tadpole body sizes, by initial tadpole activity levels and by predation.

There was a significant effect of the interaction between initial tadpole body sizes and predation on survival to metamorphosis ( $\chi^2= 7.65$ ,  $DF= 7$ ,  $P = 0.033$ ). In particular, predation reduced the survival of initially large tadpoles by 16.5% more than initially small tadpoles. However, initially large tadpoles still had a higher survivorship compared to initially small tadpoles. There was no significant effect of predators on survival in the mixed treatment (Figure 1).

There was also a significant effect of the interaction between initial tadpole body sizes and predation on the time to metamorph emergence ( $\chi^2= 18.9$ ,  $DF= 13$ ,  $P = 0.002$ ). Predation

exposure slowed the emergence rate of initially large individuals. However, initially small tadpoles emerged quickly regardless of predation, where individuals completed metamorphic emergence 30 days after being placed into the mesocosms (Figure 3). Tadpoles from the mixed cohort had shorter times to metamorphosis in the presence of predation.

There was a significant effect of the interaction between initial tadpole activity level and predation on survival to metamorphosis ( $\chi^2= 92.2$ ,  $DF= 7$ ,  $P = 2.2 \times 10^{-16}$ ). Specifically, predation reduced the survival of initially less active tadpoles by 50.2%. However, predation increased the survival of initially active tadpoles by 5.77%. Initially active tadpoles had a higher survival compared to initially less active tadpoles regardless of predation (Figure 2).

There was a significant effect of the interaction between initial tadpole activity levels and predation on the time to metamorph emergence ( $\chi^2= 14.1$ ,  $DF= 13$ ,  $P = 0.014$ ). Predation exposure slowed the emergence rate of less active individuals. However, initially active tadpoles emerged at an intermediate rate regardless of predation, where individuals completed metamorphic emergence 37 days after being placed into the mesocosms (Figure 4).

### Metamorph Body Size and Condition

Metamorph body size and body condition was assessed for the individuals that survived the larval life stage to metamorphic emergence. Metamorph body size and condition was affected differently by initial tadpole body size, by initial tadpole activity levels and by predation.

There was not a significant effect of initial tadpole body sizes or predation on metamorph body size ( $\chi^2= 0.151$ ,  $DF= 9$ ,  $P = 0.927$ ). Still, predation increased the body size of initially large individuals by just 1.23%, initially small individuals by 4.19% and mixed individuals by 2.69%.

Regardless, initially large tadpoles still emerged as smaller metamorphs compared to initially small individuals (Figure 5).

There was a significant effect of the interaction between initial tadpole body size and predation on metamorph body condition ( $\chi^2= 10.3$ ,  $DF= 8$ ,  $P = 0.036$ ). Predation reduced the body condition of initially small metamorphs by 12.8%, but only increased that of initially large metamorphs by 2.0%. Initially large and initially small tadpoles emerged with better body conditions than the mixed treatment regardless of predation (Figure 7). Yet, predation increased the body condition of individuals from the mixed treatment by 12.2%.

There was not a significant effect of initial tadpole activity level or predation on metamorph body size ( $\chi^2= 2.76$ ,  $DF= 8$ ,  $P = 0.252$ ) or body condition ( $\chi^2= 0.78$ ,  $DF= 8$ ,  $P = 0.678$ ). Still, predation reduced the body size of initially less active metamorphs by 7.60% but increased their body condition by 10.2%. Predation also increased the body size of initially active metamorphs by 1.50% but reduced their body condition by 1.32% (Figures 7 and 8). Without predation, initially active individuals emerged with 5.80% smaller body sizes and 12.9% worse body conditions compared to initially less active individuals.

## DISCUSSION

In organisms with complex life histories, phenotypes exhibited during one life stage can have carry over effects that influence performance in successive life stages (McDiarmid *et al.* 1999; Johansson *et al.* 2010; O’Conner *et al.* 2014), and these phenotypes are often affected by selective pressures, such as predation (Van Buskirk *et al.* 1998; Cressler *et al.* 2010). This study demonstrated how the initial phenotypes of hatchlings, predation, and interactions of phenotype and predation, affected *H. squirella* larval survival and fitness-associated traits at metamorphosis. Additionally, this study showed that cohorts of similar phenotypes demonstrated traits of higher quality at metamorphosis compared to cohorts of mixed phenotypes, where fitness-associated traits at metamorphosis were unexpectedly decoupled. Therefore, not only are interactions between the phenotypes of hatchlings and predation important for driving fitness-associated traits at metamorphosis, but also phenotypic variation among hatchling individuals in a cohort also drives individual *H. squirella* fitness performance prospects.

### Initial Body Size

We found that *H. squirella* tadpoles with initially larger body sizes showed higher survival to metamorphosis, but smaller body size at metamorphosis than individuals with initially smaller body sizes, indicating that initially larger body sizes might be beneficial for survival, but negatively carry over to the next life stage. Findings of similar studies are somewhat inconsistent in the literature. For example, one study on water frogs (*Rana lessonae*

and *R. esculenta*) found a positive relationship between large tadpole body sizes, survival to metamorphosis, and body size at metamorphosis (Altwegg and Reyer 2003). However, another study on American toad tadpoles (*Anaxyrus americanus*) found size at metamorphosis was inversely correlated with conspecific density (Wilbur 1977). It is possible that high survival increased competition among initially large individuals, resulting in reduced growth (Crossland *et al.* 2009). We also found that tadpoles with initially smaller body sizes showed lower survival to metamorphosis, but larger body size at metamorphosis than initially large individuals, both in the presence and absence of predators. This growth pattern was found in a study on spadefoot toads (*Pelobates fuscus*) where smaller larval conspecifics increased their growth rates to attain larger body sizes at metamorphosis (Schmidt *et al.* 2012). However, the literature did not parallel our findings that initially small individuals have low survival rates in the larval life stage. For example, a study performed on wood frogs (*Lithobates sylvaticus*) found that the allocation of resources towards fast growth may trade-off with allocation to immune function, which could affect long-term survival, but not necessarily survival at the larval life stage (Warne *et al.* 2013). Therefore, body size at hatching may be important for important physiological attributes, including immune function, that carry over to affect traits at metamorphosis as well as additional successive life stages.

Although the mechanisms described above were independent of predation, we found that predation interacted with each decoupled initial body size to enhance the body size and condition at metamorphosis (Figures 5 & 7). We also found that predation reduced the survival of only initially large individuals, while the survival and emergence timing of initially small individuals was unaffected (Figure 1 & 3). It is possible that the predator-induced reduction of initially large individuals could have decreased competition for food resources, which permitted growth to

larger body sizes at emergence for surviving individuals (Crossland *et al.* 2009). However, all other results are inconsistent with previous studies on tadpole predation. Previous work suggests that predation reduces larval survival (Travis *et al.* 1985), activity levels (Skelly *et al.* 1990; Peacor 2002) and growth and developmental rates (Relyea 2002; Altwegg 2002). One explanation for this mismatch is that the effects of predation may have been dampened by predator mortality during the experiment. We did observe *Belostoma* mortality from one mesocosm, but other predator deaths may not have been observed. Similarly, our simulated predator density in each mesocosm may have been too low to observe the predation effects from previous studies. However, it is also possible that initially small individuals grew fast enough to reach a size refuge from predation, which may have offset reductions in survival (McCoy & Bolker 2008; McCoy *et al.* 2011).

### Initial Activity Level

We found that initially active individuals demonstrated much higher survival compared to initially less active individuals without the risk imposed by predation (Figure 2), which is consistent with previous work (Sih *et al.* 2004; Herde and Eccard 2013). This could be due to the relationship between initial activity level, foraging and growth rates, or that lower initial activity levels are due to lethargy associated with illness or poor development *in ovo*. Although many studies point out that tadpole activity levels are often reduced by introductions to pesticides (Relyea *et al.* 2001), UV-B radiation (Hatch *et al.* 2000) and endocrine-disrupting chemicals (Clotfelter *et al.* 2004), which could also lead to lower survival (Blaustein *et al.* 2003), the link

between development and activity levels has not been tested, and could be another instance of phenotypic carry over between the life stages of organisms with complex life histories.

Although tadpoles with higher activity had higher survival rates than initially less active tadpoles (Figure 2), the surviving initially less active metamorphs emerged sooner with larger body sizes and better body conditions than initially active metamorphs (Figures 4, 6 & 8). These findings were consistent with previous work on wood frog tadpoles (*Lithobates sylvaticus*) that alter their activity levels in response to changes in per capita food levels and larval density to achieve optimal body size (Relyea 2002). Therefore, initially less active hatchlings may have increased activity levels in response to reduced intraspecific competition to produce the observed larger body sizes and better body conditions at metamorphosis. Testing for interactions between intraspecific competition and activity levels at hatching on traits at metamorphosis may have further implications for understanding mechanisms driving trait variation at metamorphosis.

We found that predation plays an important role in determining the metamorphic traits of individuals with differing activity levels, where surviving initially less individuals postponed metamorphosis and emerged with smaller body sizes and lower body conditions (Figures 4, 6 & 8). These findings are consistent with those in the literature. Previous work found that predators typically induce reductions in activity and foraging levels for prey individuals in order to decrease the chance of prey mortality (Laurila *et al.* 1998; Peacor 2002). Furthermore, lower activity levels in larval anurans are often associated with slower growth rates (Anholt and Werner 1995). Therefore, it is likely that less active hatchlings exposed to predation remained less active in order to increase the likelihood of survival at the cost of fitness-associated traits at metamorphosis. However, we found that predation enhanced the quality of fitness-associated traits of surviving initially active individuals, where these metamorphs emerged with larger body

sizes with exposure to predation without compromising survival (Figure 2 & 6). Again, this may be due to multiple nuances, such as predator mortality or low predator density in each mesocosm. However, there may be particular traits about *H. squirella* that permit increased fitness potential with predation that other anuran species of previous focus do not display. Therefore, comparison of predation effects on different species of anurans partitioned for body size and activity levels may be of interest.

### Mixed Cohort Performance

One of the surprising results of our study was the performance of the mixed treatment relative to the isolated phenotype treatments. We found that these metamorphs had smaller body sizes, lower body conditions and later times to emergence compared to individuals partitioned for body size and activity levels (Figures 3-8). This treatment was presumed to better represent the body size and activity level distributions found in natural populations of *H. squirella* tadpoles. However, it is possible that the haphazard removal of mixed hatchlings from the initial tadpole cohort may have incorporated a bulk of individuals with poor behavioral or developmental phenotypes. On the other hand, previous work suggests that populations of tadpoles reared in cohorts of non-relatives display slower growth and developmental rates to metamorphosis (Jasienski 1988). Therefore, it is possible that the mixed cohort may have been compromised of non-kin, which stunted larval growth and development and affected fitness-associated traits at metamorphosis. Even so, it is possible that the carry over effects of isolated phenotypes may be altered by phenotypic variation among larval individuals, where the



phenotypes displayed at metamorphosis are more dependent on the phenotype regime of neighboring hatchlings, rather than on the phenotypes of a single individual at hatching.

Many other recent studies have demonstrated that behaviors in tadpoles are consistent and repeatable across life stages and environmental contexts (Wilson *et al.* 2012), which should maintain phenotypic variation among individuals. There is a large body of literature that suggests consistency of behavioral traits within individuals is common throughout the animal Kingdom. Some studies even propose that behavioral consistencies are genetically determined and heritable (Smith and Blumstein 2008; Dochtermann and Dingemanse 2013). Behavioral consistencies may have substantial impacts on not only survivorship, but also on growth and developmental rates in current life stages (Reale *et al.* 2010) and on the cumulative fitness of individuals in later life stages (Niemela *et al.* 2012). The next step for future studies requires testing for behavioral consistency in activity levels across life stages to bring greater insight to how larval activity levels at hatching affect fitness-associated traits of metamorphs. Insights might also be gained by examining the effects of other potentially autocorrelated behaviors, such as boldness, aggressiveness and choosiness (Sih *et al.* 2004; Reale *et al.* 2010), on traits at metamorphosis.

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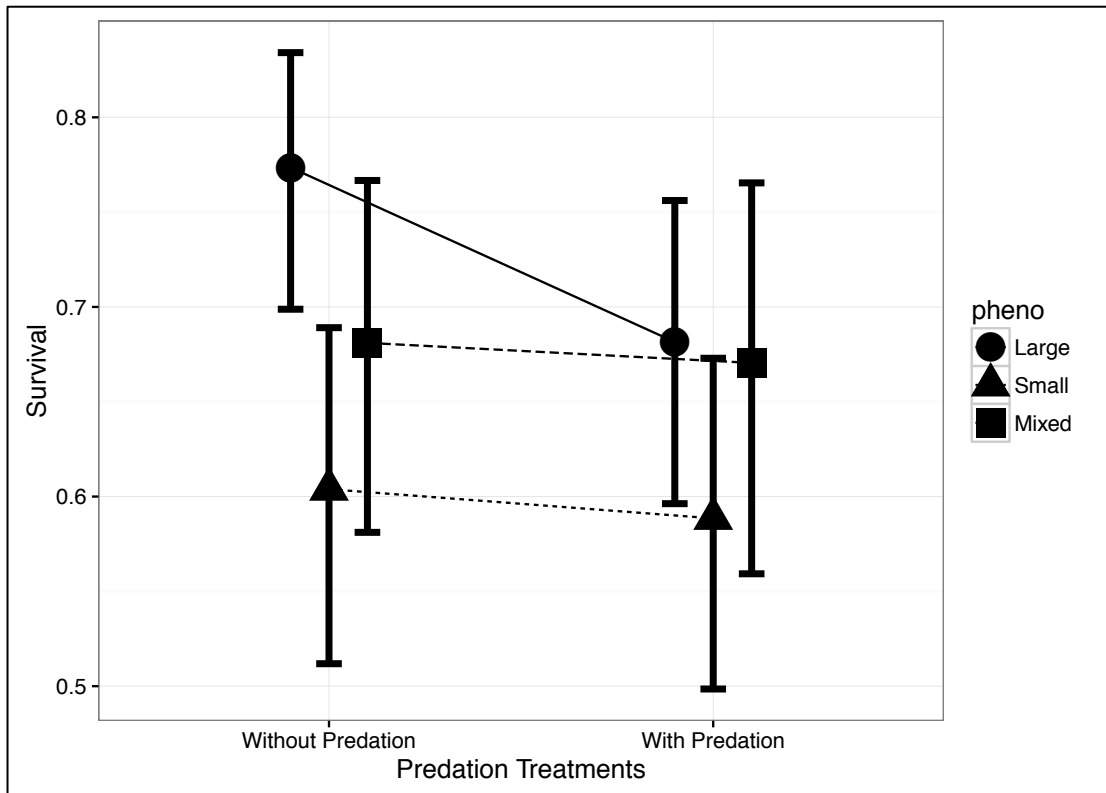
## SUPPLEMENT 1

### Partitioned Tadpole Body Size

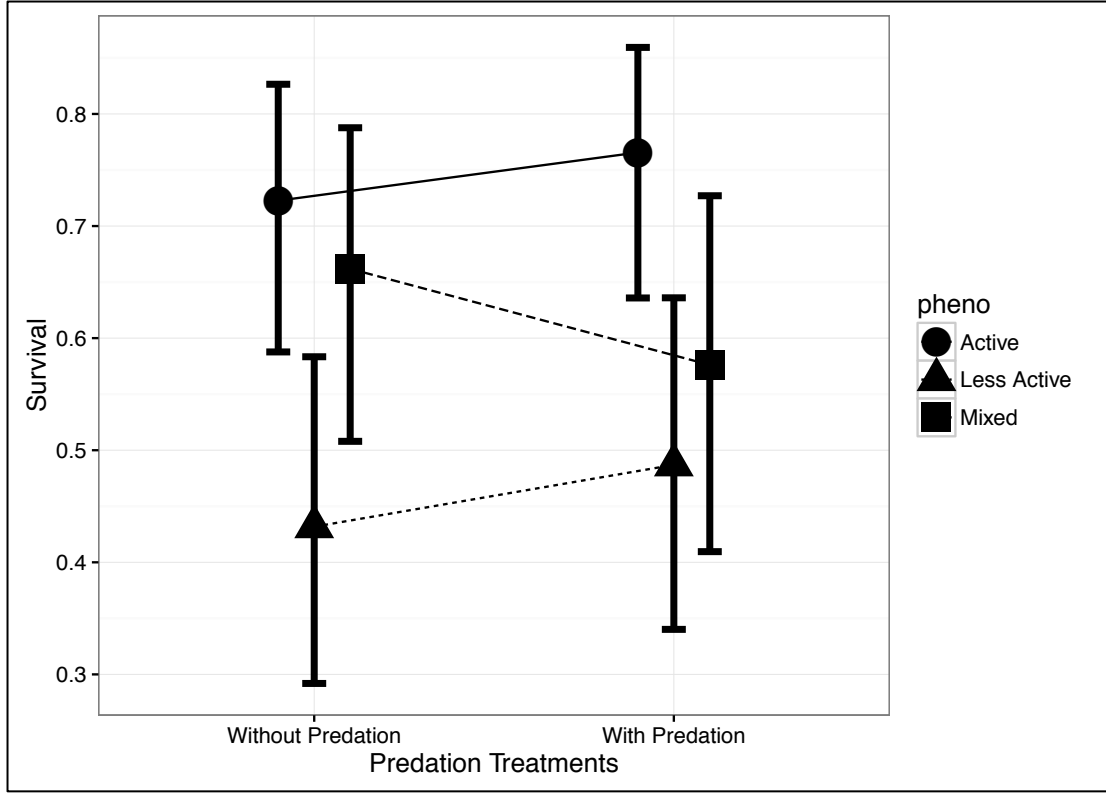
The cohort of tadpoles collected was partitioned for either initial body size or initial activity level (See Methods: Tadpole Partitioning). Initial body size partitioning ( $X^2= 85.2$ ,  $DF= 5$ ,  $P < 2.2 \times 10^{-16}$ ) and initial activity level partitioning ( $X^2= 8.09$ ,  $DF= 5$ ,  $P = 0.004$ ) both significantly affected tadpole body size. In particular, initially small tadpoles were smaller than initially large tadpoles by 27.9% (S1 Figure 1). Also, initially less active tadpoles were smaller than initially active tadpoles by only 6.08% (S1 Figure 2). However, initially small tadpoles were still much smaller than initially less active tadpoles by 15.5%. Mixed tadpoles best represented the body size distribution sampled from the population.

*Table 1: Initial phenotype and predation treatments.*

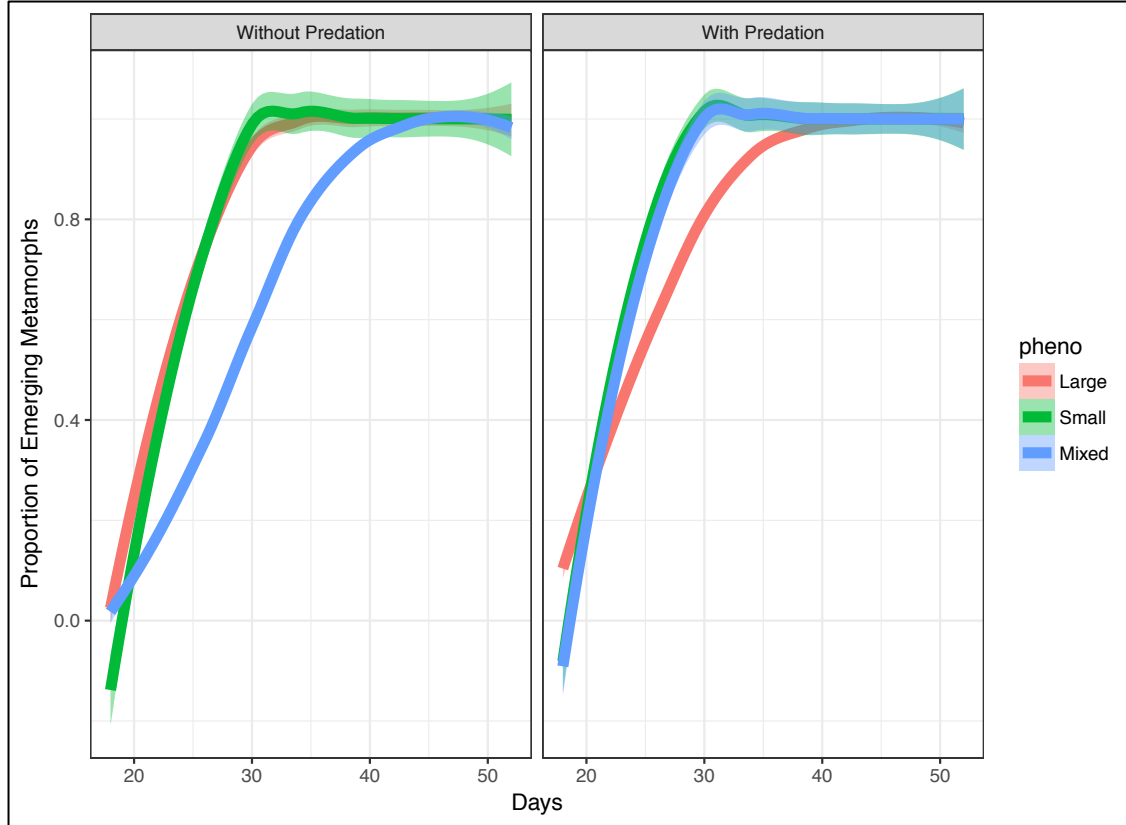
<b>Predation</b>	<b>No Predation</b>
1 – Active	6 - Active
2 – Less Active	7 – Less Active
3 – Initially Large	8 – Initially Large
4 – Initially Small	9 – Initially Small
5 – Mixed	10 – Mixed



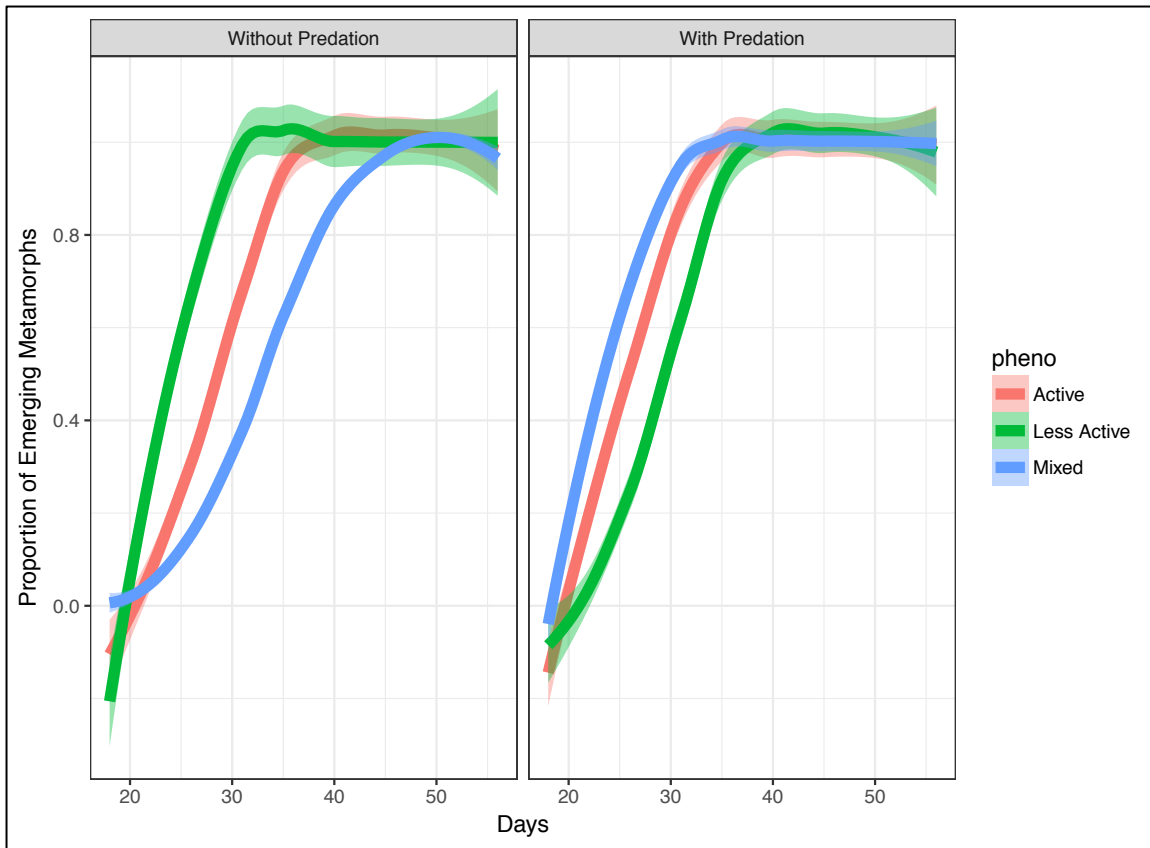
**Figure 1:** Effects of hatchling body size and predation on *H. squirella* larval survival to metamorphosis. Data shown are means values with 95% confidence intervals for each treatment, controlling for random effect of block. Line segments show the interactions between predation and each phenotype treatment.



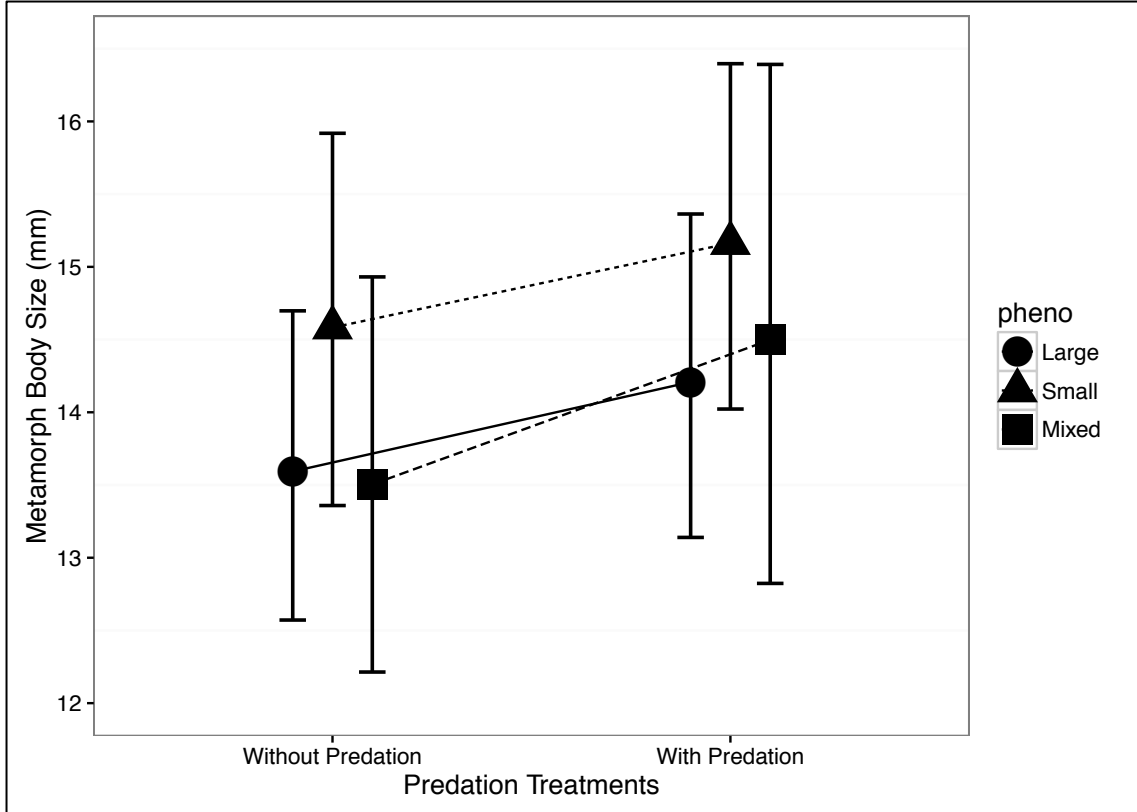
**Figure 2:** Effects of hatchling activity level and predation on *H. squirella* larval survival to metamorphosis. Data shown are means values with 95% confidence intervals for each treatment, controlling for random effect of block. Line segments show the interactions between predation and each phenotype treatment.



**Figure 3:** Effects of time in days, hatchling body size and predation on the proportion of *H. squirella* individuals surviving to metamorphic emergence. Regression curves represent the average proportion of individuals that emerged over time (rate of emergence) with 95% confidence intervals for each treatment. Red colors represent initially large hatchlings, green colors represent initially small hatchlings and blue colors represent mixed phenotype hatchlings.

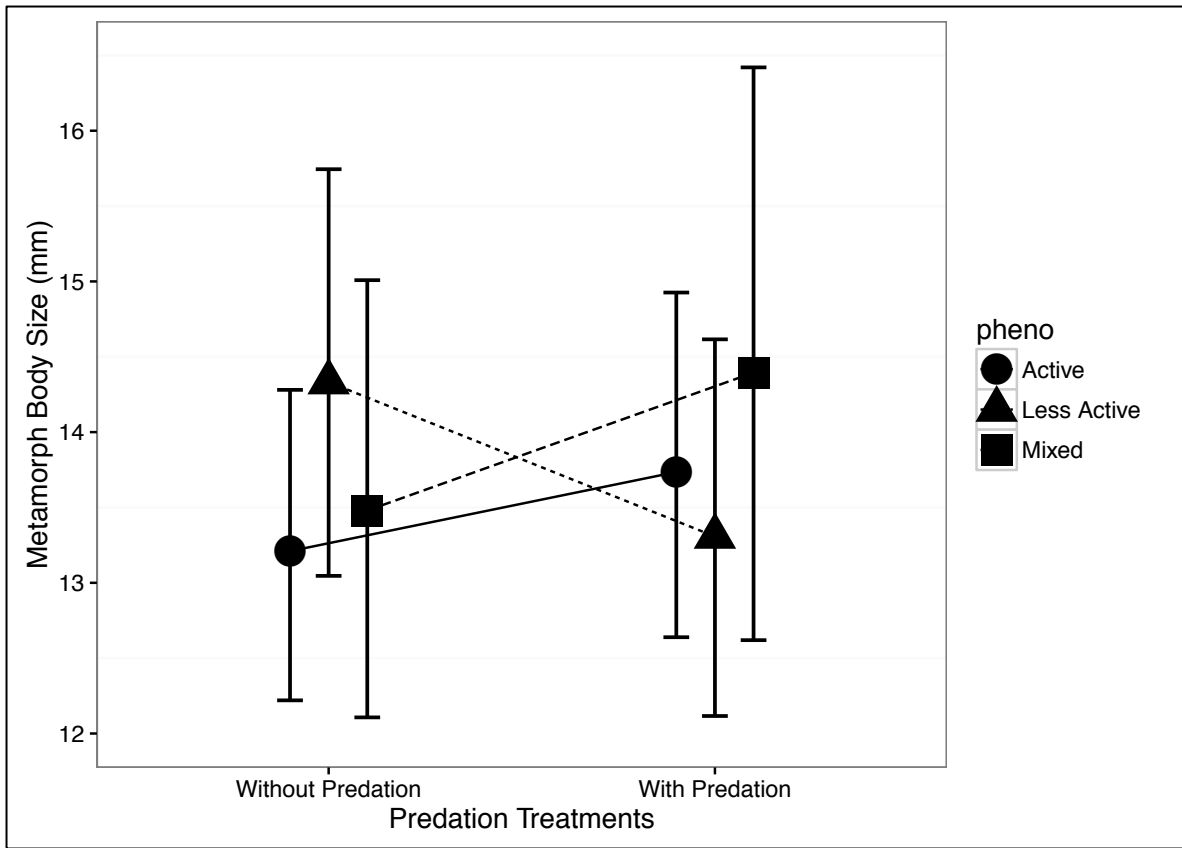


**Figure 4:** Effects of time in days, hatchling activity level and predation on the proportion of *H. squirella* individuals surviving to metamorphic emergence. Regression curves represent the average proportion of individuals that emerged over time (rate of emergence) with 95% confidence intervals for each treatment. Red colors represent active hatchlings, green colors represent less active hatchlings and blue colors represent mixed phenotype hatchlings.

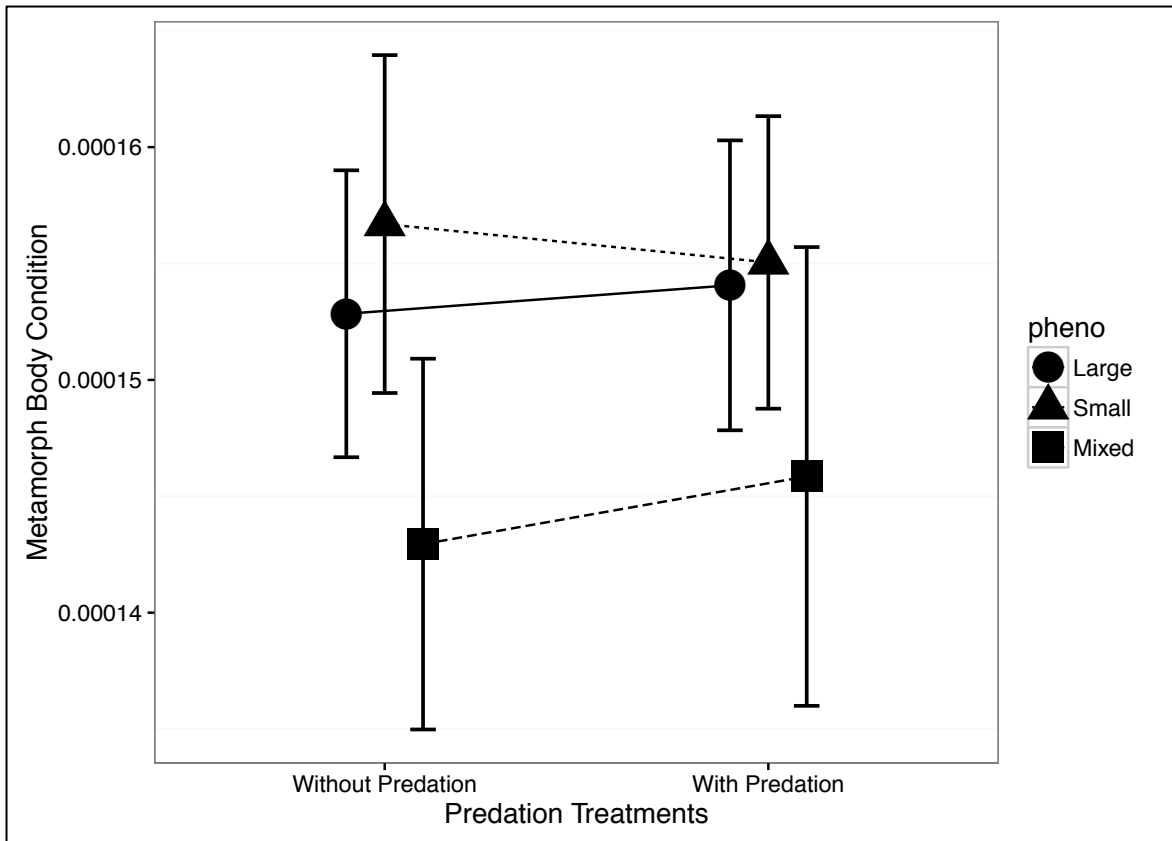


**Figure 5:** Effects of hatchling body size and predation on *H. squirella* metamorph body size (mm) after emergence. Data shown are means values with 95% confidence intervals for each treatment, controlling for random effect of block. Line segments show the interactions between predation and each phenotype treatment.

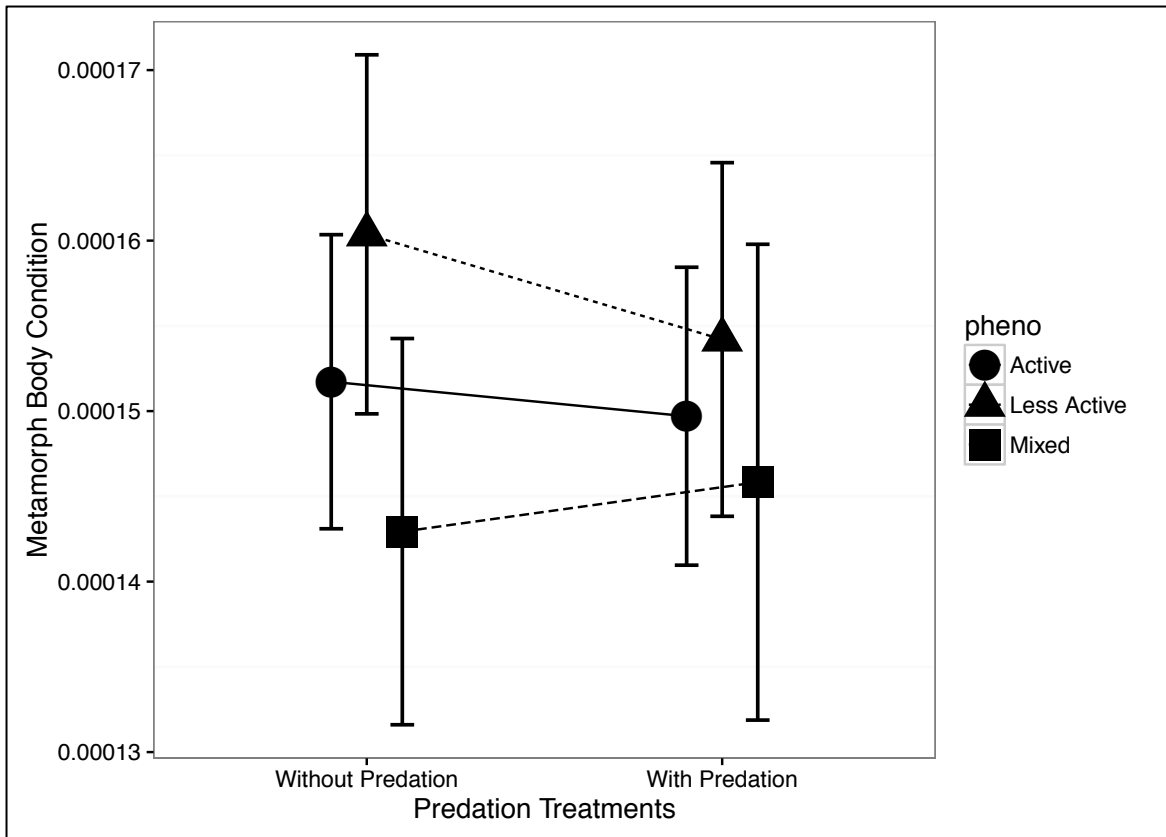




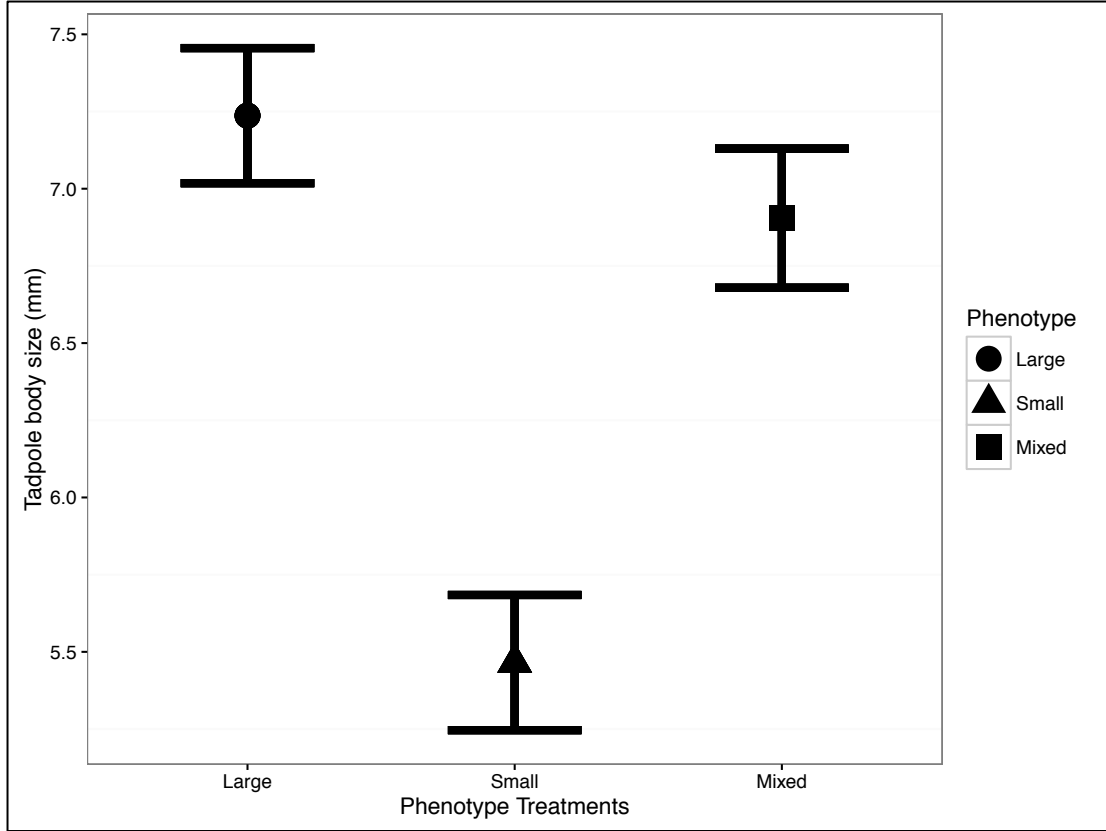
**Figure 6:** Effects of hatchling activity level and predation on *H. squirella* metamorph body size (mm) after emergence. Data shown are means values with 95% confidence intervals for each treatment, controlling for random effect of block. Line segments show the interactions between predation and each phenotype treatment.



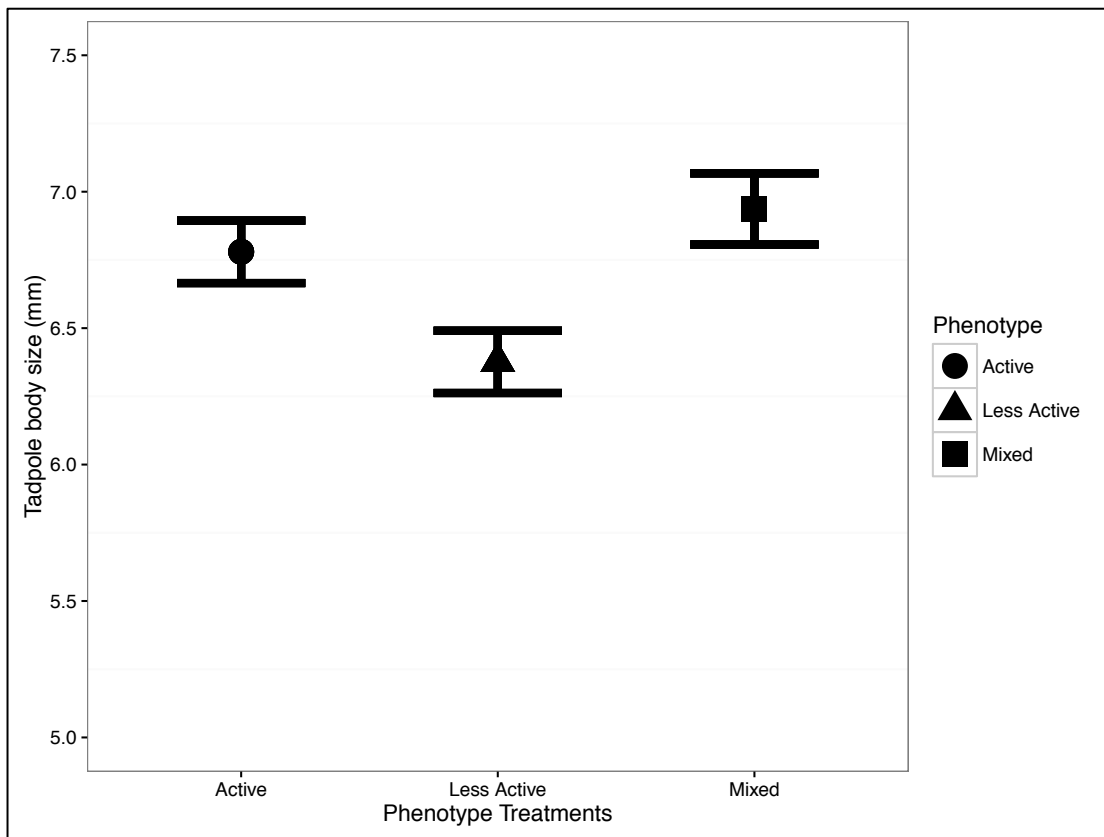
**Figure 7:** Effects of hatchling body size and predation on *H. squirella* metamorph body condition after emergence. Data shown are means values with 95% confidence intervals for each treatment, controlling for random effect of block. Line segments show the interactions between predation and each phenotype treatment.



**Figure 8:** Effects of hatchling activity level and predation on *H. squirella* metamorph body condition after emergence. Data shown are means values with 95% confidence intervals for each treatment, controlling for random effect of block. Line segments show the interactions between predation and each phenotype treatment.



**S1 Figure 1:** Effects of tadpole body size partitioning on *H. sirella* tadpole body size (mm) before experimental onset. Data shown are means values with standard deviation error bars for each treatment.



**S1 Figure 2:** Effects of tadpole activity level partitioning on *H. squirella* tadpole body size (mm) before experimental onset. Data shown are means values with standard deviation error bars for each treatment.



# East Carolina University

**Animal Care and  
Use Committee**

212 Ed Warren Life  
Sciences Building  
East Carolina University  
Greenville, NC 27834

252-744-2436 office  
252-744-2355 fax

April 13, 2016

Michael McCoy, Ph.D.  
Department of Biology  
Howell Science Complex  
East Carolina University

Dear Dr. McCoy:

Your Animal Use Protocol entitled, "Investigating the Co-Variance Between Behavioral and Physiological Trade-Offs" (AUP #D336) was reviewed by this institution's Animal Care and Use Committee on 4/13/16. 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. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

A handwritten signature in blue ink that reads "Eddie Johnson".

Eddie Johnson  
Interim Chair, Animal Care and Use Committee

EJ/jd

Enclosure