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Developing Tadpoles Exhibit Metabolic and Organ Size Plasticity in Competitive Rearing Environments

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DEVELOPING TADPOLES EXHIBIT METABOLIC AND ORGAN SIZE PLASTICITY IN COMPETITIVE REARING ENVIRONMENTS

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Abstract

Plasticity is the ability of an organism to respond to environmental variation by expressing different phenotypes. In Red-eyed treefrog tadpoles, *Agalychnis callidryas*, competitive environments induce long guts and short tails. Despite having a larger gut, tadpoles reared with competition do not increase intake when food becomes available. Pilot data suggest that this is because they have lower metabolic rates. The ability to maintain a larger gut with a depressed metabolic rate is confusing because guts are energetically expensive, and suggests that another energetic trade-off is taking place. The purpose of this study was to investigate the effect of intraspecific competition on metabolic rate and organ size plasticity in *Agalychnis callidryas* tadpoles to determine if differences in metabolic rate are associated with differences in organ size. *A. callidryas* tadpoles were reared at low and high density in outdoor mesocosms in Gamboa, Panama. Once they reached a standard size, we measured their metabolic rate and dissected and weighed their guts, livers, pancreases, and brains. Additionally, we analyzed effects on the following brain components: forebrain, optic tectum, and medulla oblongata. We also conducted a comparative study of American toads, *Anaxryus americanus*, which were reared at low, high, and extra high density. We measured metabolic rates and organs as in the Red-eyed treefrog study. Additionally, to understand differences in the brain at the cellular level, we determined brain cell nuclear density and size. For both species, we predicted that competition would decrease growth and metabolic rates, increase gut mass, and decrease overall brain mass. We also predicted that high-density tadpoles would have larger optic tecta and smaller medulla oblongatas and forebrains. Competition induced lower metabolic rates in *A. callidryas*. This was associated with smaller livers, pancreases, and brains, but not differences in gut mass. Competition did not induce lower metabolic rates or smaller livers and brains in *A. americanus*, but extra-high densities did. Competition induced smaller optic tecta and forebrains in both species, but smaller medulla oblongatas were only observed in *A. callidyras*. There were no differences observed in *A. americanus* brain nuclei density or nuclei diameter across treatments. We concluded that *A. callidryas* exhibits a stronger metabolic response to competition than *A. americanus*, and that this response may be facilitated by changes in organ size. The naturally higher larval schooling densities that *A. americanus* have relative to *A. callidryas* may explain the differences between species.

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Introduction

Plasticity is the ability of one genotype to express different phenotypes in response to varied environmental conditions (Fox et al. 2019). This ability is well-documented across taxa and is diverse in presentation. For example, intraspecific competition causes mothers to alter their brood sizes in the bryozoan *Bugula neritina* (Allen et al. 2008), diet quality affects the degree of aggressive behavior exhibited by *Salamandra salamandra* (Manenti et al. 2018), temperature influences sex-determination in reptile and fish species (Valenzuela et al. 2019; Shen and Wang, 2014), predation risk alters nest incubation behavior in *Ficedula hypoleuca* (Morosinotto et al. 2013), and a variety of mammals, including humans, demonstrate some level of neural plasticity (La Rosa & Bonfanti, 2018; Papadakakis et al. 2019). This capability to change within their own lifetime, rather than generationally, can be highly advantageous for organisms because it allows them to better fit their variable environment. For example, *Rana sylvatica* tadpoles faced with intraspecific competition exhibit increased levels of activity (Reylea, 2002). This behavioral plasticity allows the *Rana sylvatica* tadpoles living in a resource-limited environment to cover more ground and collect the maximal amount of resources possible (Reylea, 2002).

Plastic responses provide many advantages; however, these must be weighed against their costs. Often, the ability to display the most advantageous form of one trait in a given environment causes trade-offs with other traits that may weaken the overall fitness of an organism (Gervasi and Foufopoulos, 2008). These consequences may come in the form of depressed growth, inefficient feeding, decreased opportunity to find a mate or increased exposure to a predator (Callahan et al. 2008). For example, in comparison with low density populations, moose *(Alces alces)* and white-tailed deer *(Odocoileus virginianus)* from high density populations suffer body mass losses due to resources being limited (Gringas et al. 2014; Ayotte et al. 2019). These constraints induce high density female *A. alces* and *O. virginianus* to take on a conservative reproductive strategy where they sacrifice litter size in order to conserve energy and increase the probability of producing offspring annually (Gringas et al. 2014; Ayotte

et al. 2019). Thus, the benefit of saving energy comes at the cost of producing fewer offspring. Small organisms undergo trade-offs due to plasticity as well. Wood frog tadpoles (*Rana sylvaticus*) experience gut length plasticity in response to different environmental pressures (Reylea et al. 2004). When reared with high levels of intraspecific and interspecific competition, gut length increases allowing for a more efficient nutrient digestibility. However, when *R. sylvaticus* tadpoles are reared with predation, gut length and growth rate decreases and energy is allocated toward developing a larger tail. When predation is the only selective pressure, the larger tail phenotype is clearly the better option over the longer gut phenotype as it allows tadpoles to better escape predators. Yet, the better phenotype becomes less clear when tadpoles are faced with both competition and predation. Experimentally, *R. sylvaticus* tadpoles opt to develop a larger tail and grow more slowly at the cost of having a more efficient gut when faced with both pressures (Reylea et al. 2004).

It is particularly interesting to study amphibian plasticity because of their complex life cycle. After hatching from a jellylike egg, most amphibians enter the world and grow as aquatic larvae until they metamorphose into terrestrial adults (O'Rourke, 2007). Anurans demonstrate a wide range of plasticity at each of these life stages. Remarkably, some frog embryos exhibit hatching plasticity in response to danger-indicating stimuli in their environment. Physical disturbance, such as that created by a predator, induces embryos of *Agalychnis callidyras, Hyalinobatrachium pulveratum,* and *Limnonectes arathooni* to hatch from their eggs sooner at a less developed stage (Warkentin, 2011). Several studies have documented that high-density (competitive) rearing environments induce plasticity in tadpoles. In *Rana sylvaticus* and *Hyla versicolor*, intraspecific competition induces lower growth rates, larger bodies, and smaller tails (Reylea, 2002; Reylea and Hoverman, 2003). Conversely, predation causes tadpoles to develop deeper tails and shorter bodies (Reylea, 2002; Reylea and Hoverman, 2003). Responses to larval rearing environments also have important implications on juvenile frogs. Low density (uncompetitive) rearing environments increase the likelihood of survivorship in juvenile *Hyla versicolor* relative to those from high density environments (Reylea and Hoverman, 2003).

Red-eyed tree frog tadpoles, *Agalychnis callidryas*, respond plastically to competition. Specifically, *A. callidryas* tadpoles develop longer guts and shorter tails when reared in high density environments (Bouchard et al. 2015). One would expect that if you moved these tadpoles to a more favorable environment with more food that their intake rate would increase and they would fill their larger guts. However, they continue to eat at a low level. Pilot data suggest that this is due to metabolic depression. A lowered metabolic rate can provide fitness advantages to an organism facing adverse conditions as it allows them to allocate more energy towards growth and reproduction by lowering their maintenance costs (Burton et al. 2011). For example, brown juvenile trout with the lowest metabolic rates grew the fastest in food limited environments (Auer et al. 2015). However, the ability to maintain a larger gut with a depressed metabolic rate is confusing because guts are metabolically costly. This combination of traits suggest that another energetic trade-off is taking place.

One possible tradeoff that could offset the costs of a larger gut is brain size. The brain is known as a plastic and metabolically costly organ (Eifert et al. 2015). In common frog tadpoles, *Rana temporaria,* tadpoles reared at high densities exhibited changes in brain morphology while maintaining the same brain size as tadpoles reared in low density environments (Gonda et al. 2010). High density tadpoles developed larger optic tecta and smaller medulla oblongata when compared with those raised at low densities. Vision plays an important role in competing for resources, so it may be advantageous for tadpoles in high density environments to develop larger optic tecta, with a decrease in medulla oblongata size being a trade-off for this (Butler and Hodos, 2005). On the other hand, increasing medulla oblongata size may hold benefits that only tadpoles reared in favorable environments can afford (Gonda et al. 2010). Interestingly, differences in the optic tecta persisted after metamorphosis while changes in medulla oblongata did not (Trokovic et al. 2011). Brain differences may also occur at the cellular level. Differences in brain cell density and nuclear size can indicate variations in brain function (Kubke et al. 2014; Keller et al. 2018). Though it has not been widely used in anuran species outside of *Xenopus sp*., DAPI (4′,6-diamidino-2-

phenylindole) is a blue fluorescent stain that has demonstrated successful nuclei visualization and may reveal further answers about differences in brain morphologies (Peunova et al. 2001).

The purpose of this study was to confirm that competitive rearing environments depress metabolic rates and determine if changes in organ sizes could explain this effect in *A. callidryas* and *A. americanus* tadpoles. We reared *A. callidyras* tadpoles at low and high densities in outdoor mesocosms in Gamboa Panama. We measured the growth rates of all tadpoles before measuring the metabolic rates of a subset. We also dissected and weighed guts, livers, pancreases and brains of those individuals. In order to better understand brain morphologies, we measured the following brain parts by photographing brains and analyzing them using ImageJ software: forebrain, optic tecta, and medulla oblongata. We performed a separate comparative study on *A. americanus* tadpoles preserved from a previous metabolic study. An Otterbein alum, Justin McCurdy, reared these tadpoles at low, high, and extra high densities and measured their growth and metabolic rates (Pers comm McCurdy, 2018). We removed these tadpoles from preservative and dissected similarly to *A. callidryas*. We further examined *A. anaxryus* brain plasticity at the cellular level by quantifying the density and size of brain cell nuclei.

For both species, we predicted that tadpoles reared in high density environments would exhibit lower metabolic rates, larger guts, and smaller brains that allow more energy to be allocated to the gut. We also predicted that these high-density tadpoles would develop proportionally larger optic tecta, smaller medulla oblongata, and unchanged forebrain size based on previous findings in another anuran species. Lastly, we predicted *A. anaxryus* tadpoles from extra-high density rearing environments would have either lower brain cell nuclei density or have smaller-sized brain cell nuclei as their resource limitations would impede development at the cellular level. This study is important for increasing what is known about larval anuran responses to competition in addition to opening new directions in the understudied anuran brain plasticity field with a novel study in *A. anaxryus*.

Methodology

Study 1 *Agalychnis callidryas*

This experiment was conducted at the Smithsonian Tropical Research Institute in Gamboa, Panama from July 6, 2018 – August 8, 2018, during the *A. callidryas* breeding season. We collected 9 clutches of eggs that were laid on the same night from the Experimental Pond at the field station and maintained them in the laboratory until hatching. On the day of hatching, 6 days after oviposition, we combined tadpoles from all clutches. Then, we haphazardly selected hatchlings for the study and transferred them to outdoor mesocosms.

Mesocosms were located at the edge of the rainforest and held 30-L of an aged tap water and rainwater mixture. To mimic a pond-like environment, we added 10 large, approximately equally-sized leaves from the Experimental pond to each mesocosm. We covered mesocosms with a secured screen to prevent colonization by other organisms and predation on tadpoles. We had 14 mesocosms in total. Half of those mesocosms were low density (N = 7) while the other half were high density (N = 7). We raised 5 tadpoles in each low-density mesocosm and 15 individuals in each high-density mesocosm. High densities are known to produce competitive environments and induce longer guts (Bouchard et al. 2016). We supplied each low and high density mesocosm with a resource supplement of 1.5g and 0.5g of Sera Micron every 5 days, respectively. These supplement amounts were selected because they provided a resource-abundant environment for low-density tadpoles and a resource-limited environment for high-density tadpoles. The excess of Sera Micron was visible as low density mesocosms always had a green hue.

We measured tadpole growth rate by measuring the change in total body length over a one-week period. Tadpoles were haphazardly assigned to a mesocosm and placed into a shallow basin of water with their respective group. Prior to releasing the tadpoles into the outdoor mesocosms, we took initial photos of each group to establish an approximately uniform initial size. After tadpoles had been in the outdoor mesocosms for one week, we dip-netted them out and placed them in a shallow basin of water from their

own mesocosm. We took photos of each group and analyzed the total body lengths of tadpoles using ImageJ image processing software. The mean of the initial and one-week total body length values were plotted graphically, and standard error was calculated and represented as error bars.

Metabolic Rate Measurements

We measured the metabolic rates of tadpoles once they reached approximately 4.0 cm to standardize for body size across treatments. We fasted the tadpoles prior to testing by removing them from their mesocosm the evening before their test day and placing them in a specialized cup container (Figure 1). We created this specialized cup by first cutting the bottom off of one cup and securing a new mesh bottom. Then, we placed the mesh bottom cup inside a normal cup and filled it with water. With this design, the feces were able to fall through the mesh to the bottom where the tadpole could not eat it.

We measured the metabolic rates of 41 tadpoles over the course of a week. Of these, 20 tadpoles were from low-density treatments and 21 tadpoles were from high-density treatments. We collected three tadpoles per mesocosm, with the exception of three low density mesocosms. Two of these mesocosms were represented by two tadpoles each and the third mesocosm had four tadpoles sampled from it. One tadpole was measured at a time.

To measure the metabolic rates of experimental tadpoles, we measured their oxygen consumption over time with a Witrox 1 oxygen analyzer from Loligo Systems (Figure 2; Svendsen et al. 2016). The tadpole was maintained in a sealed chamber within a water bath. The horizontal, tube-shaped glass chamber we used had an interior diameter of 18.5 millimeters and was 40 millimeters long with a volume of 10.47 ml (Loligo Systems, Mini chamber horizontal, #CH20230). The chamber comes with two ground glass joint stoppers that ensure a one hundred percent gas and watertight chamber (Loligo Systems, Mini chamber horizontal, #CH20230). We ensured the water in the water bath was well oxygenated and placed a temperature probe connected to the Witrox 1 oxygen analyzer into the bath. Average water temperature was 26.06 ± 0.72 °C. We submerged the chamber in the water bath taking

care not to introduce any bubbles. Once all air bubbles were removed from the chamber, we inserted one stopper into the chamber. We then put our experimental tadpole into the water bath and gave it time to settle down, approximately five minutes per tadpole. Once the tadpole was calm and still, we gently corralled the tadpole into the open end of the chamber using a piece of nylon aquatic netting. With the tadpole inside, we closed the chamber with the second stopper. We then aligned the fiber optic oxygen probe connected to the Witrox 1 oxygen analyzer with the oxygen sensor on the metabolic chamber. Once the level of oxygen was registered by the computer and the tadpole was given a few moments to settle down again, we started running the metabolic measurement. Each measurement took approximately 30-60 minutes. Once the oxygen level reading approached 0.005 milligrams of oxygen, we ended the metabolic measurement and immediately removed the tadpole from the chamber.

After having their metabolic rates measured, two tadpoles per tank, or 28 tadpoles total, were euthanized in order to examine their organ plasticity. The remaining 13 tadpoles were released back into the Experimental Pond.

A total of 28 tadpoles from the metabolic study were dissected. Of these tadpoles, there were 14 low-density and 14 high-density individuals. I measured the head-body length, tail length, total length, mass of formalin-fixed tadpoles before dissection. I also staged each tadpole based on the development of their back feet using a visual stage rubric (Gosner, 1960). Brains, guts, livers, pancreases, and fat bodies were dissected and weighed. Brains were photographed dorsally and ventrally, and the length and area of different brain parts was measured using ImageJ. Specifically, I used the ventral view of the brain to determine brain length, medulla oblongata length, and medulla oblongata area. I used dorsal views to determine optic tecta and forebrain area. Different views were used because they allowed for better visualization of brain parts (Figure 3).

Statistical analyses

Unless otherwise stated, we used linear mixed effect models in which density was the fixed effect, mesocosm was the random effect, and body length was the covariate. This allowed us to evaluate for treatment effects while controlling for the non-independence of multiple individuals from a single mesocosm (Bouchard et al. 2015). We reported significant data points with p-values less than 0.05. We analyzed *A. callidryas* data with R programming and *A. americanus* data with Statistical Package for the Social Sciences (SPSS).

Study 2 *Anaxyrus americanus*

This study used *A. americanus* tadpoles preserved from a 2018 metabolic study conducted at Otterbein University in Westerville, Ohio that followed similar methodologies to my red-eyed tree frog study. However, for that study, there were three density treatments: low (5 tadpoles), high (20 tadpoles), and extra high (40 tadpoles) densities. Tadpoles were reared in the lab in 12 L of aged tap water in 15 L plastic Sterlite containers (42.5 x 30.2 x 17.8 cm). In this study, I dissected tadpoles preserved from the 2018 study. The initial experiment ran from April 17, 2018 to June 29, 2018 (Pers comm McCurdy, 2018). I performed dissections on the preserved tadpoles from March 13, 2019 to August 24, 2019.

A total of 40 *Anaxyrus americanus* tadpoles were used for analyses. Of these tadpoles, 16 belonged to low density treatments, 12 to high density treatments, and 12 extra high-density treatments. I measured and dissected *A. americanus* tadpole organs using the exact methodology used in my *A. callidryas* study.

To determine the number and size of brain nuclei from the brains of the experimental *A. americanus* tadpoles, we freed cell nuclei using detergents, buffers, and mechanical dissociation of the brains. For all further brain methodology, we followed a methodology paper by Herculano-Houzel (2005). Each brain was rinsed in 1 mL of dissociation solution (1% Triton X-100 in 40 millimolar sodium citrate and

distilled water) to cleanse of remaining preservative. We transferred each brain and 1 mL of fresh dissociation solution directly into the bottom of the homogenizer using a pipette. We mechanically dissociated each brain with the pistol for approximately five minutes to free nuclei from their cells and make the solution homogenous. The homogenized solution was transferred into a 15-mL centrifuge tube by pipette. The walls and the piston were washed with three 0.25 mL quantities of dissociation solution (0.75 mL total) and added to the total volume in the 15mL centrifuge tube. The nuclei solution was centrifuged for three minutes at 4000 revolutions per minute. The supernatant was pipetted and discarded to leave the pellet undisturbed. I resuspended the pellet in 30% sucrose solution (30% sucrose in phosphate buffer solution) by gently pipetting the solution up and down. This solution was refrigerated for ≥ 2 hours. After the refrigeration period, the sample was prepared for fluorescent staining.

To visualize nuclei under a fluorescent microscope, we used a fluorescent stain called DAPI (4′,6-diamidino-2-phenylindole) that strongly adheres to thymine-adenine dense areas on double-stranded deoxyribonucleic acid (DNA). DAPI is excited by violet light with an excitation maximum of 358nm and emits a blue color with an emission maximum of 461 nm in its excited state (Thermo Fisher Scientific). To prepare nuclei for staining, we centrifuged and resuspended them in 500 μL of phosphate buffer solution (PBS). Then, 0.5 μL of 300 μM DAPI stain was added and incubated for five minutes. We transferred a 10 μL aliquot onto a hemocytometer and covered it with a glass coverslip. We counted nuclei per quadrat under a fluorescent microscope (100x magnification). We counted nuclei that touched the top or right ruling as “in” and nuclei that touched the bottom or left ruling as “out.” The average of these four samples provided the nuclei density for each individual. The coefficient of variation for each average value was calculated (standard deviation ÷ average). Photos were taken in order to calculate the nucleus diameter using DP72 Camera Software measuring tools (Olympus). After all analyses were complete, we equilibrated each sample in 30% sucrose solution for ≥ 2 hours, centrifuged and resuspended in antifreeze solution (30% glycerol and 30% ethylene glycol in PBS) and stored them in the freezer for any future reanalysis.

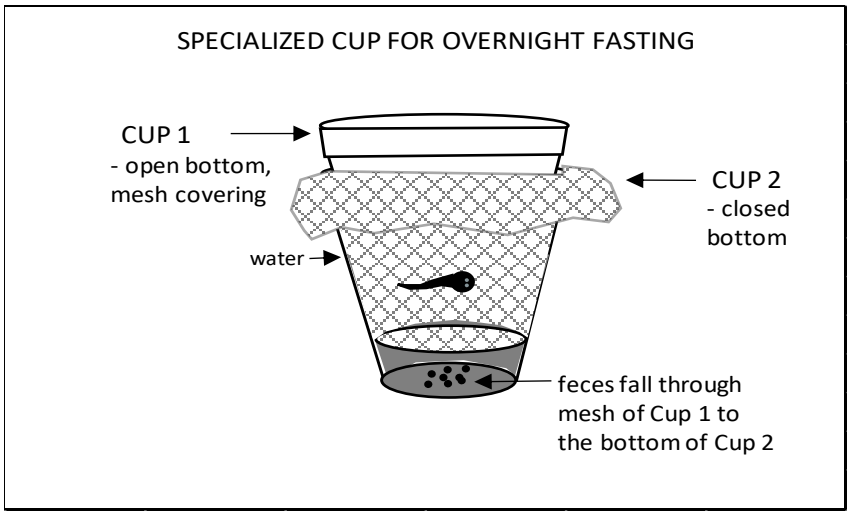

FIGURE 1: Specialized cup design for fasting tadpoles overnight. All tadpoles were fasted in a specialized cup the night prior to having their metabolic rates measured. The purpose of this design was to separate the feces from the swimming space so the tadpoles would not eat them.

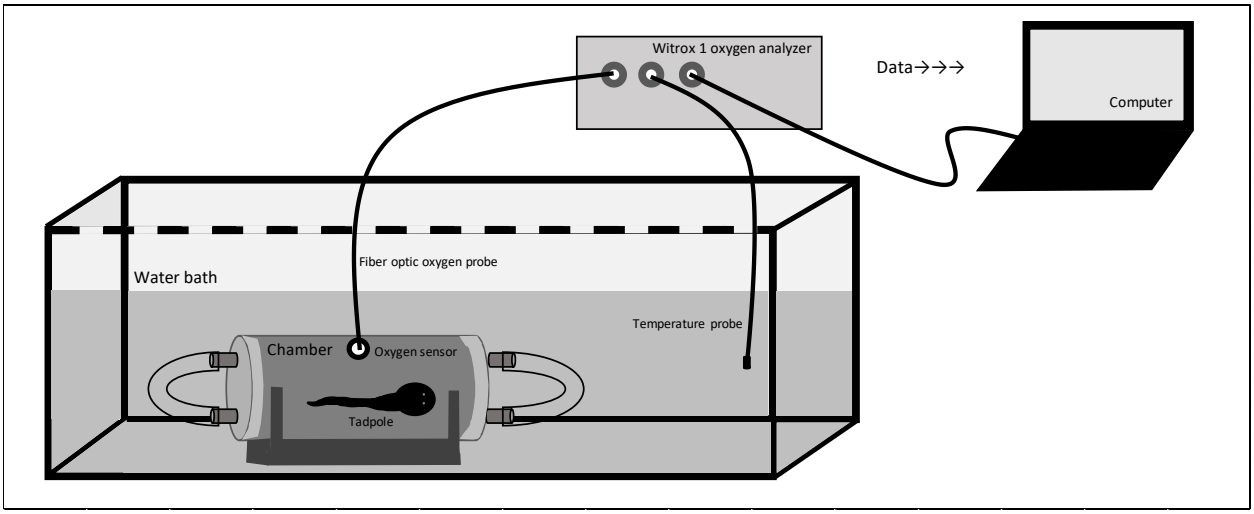

FIGURE 2: Metabolic chamber set-up. A metabolic chamber is placed in a water bath filled with aerated, aged rainwater. The oxygenation of the water within the chamber is measured when the fiber optic oxygen probe is aligned properly with the oxygen sensor. A temperature probe continuously records water temperature throughout each metabolic trial. Once standard conditions for each measurement are met, tadpoles are sealed in the watertight metabolic chamber and the trial begins. The decline of oxygen overtime is measured by the Witrox 1 oxygen analyzer and recorded by the computer.

FIGURE 3: Ventral and dorsal views of brain parts. In image A, the tadpole brain is shown ventrally, and the medulla oblongata area is outlined. In images B and C, the brains are shown dorsally, and the optic tecta and forebrain, respectively, are outlined.

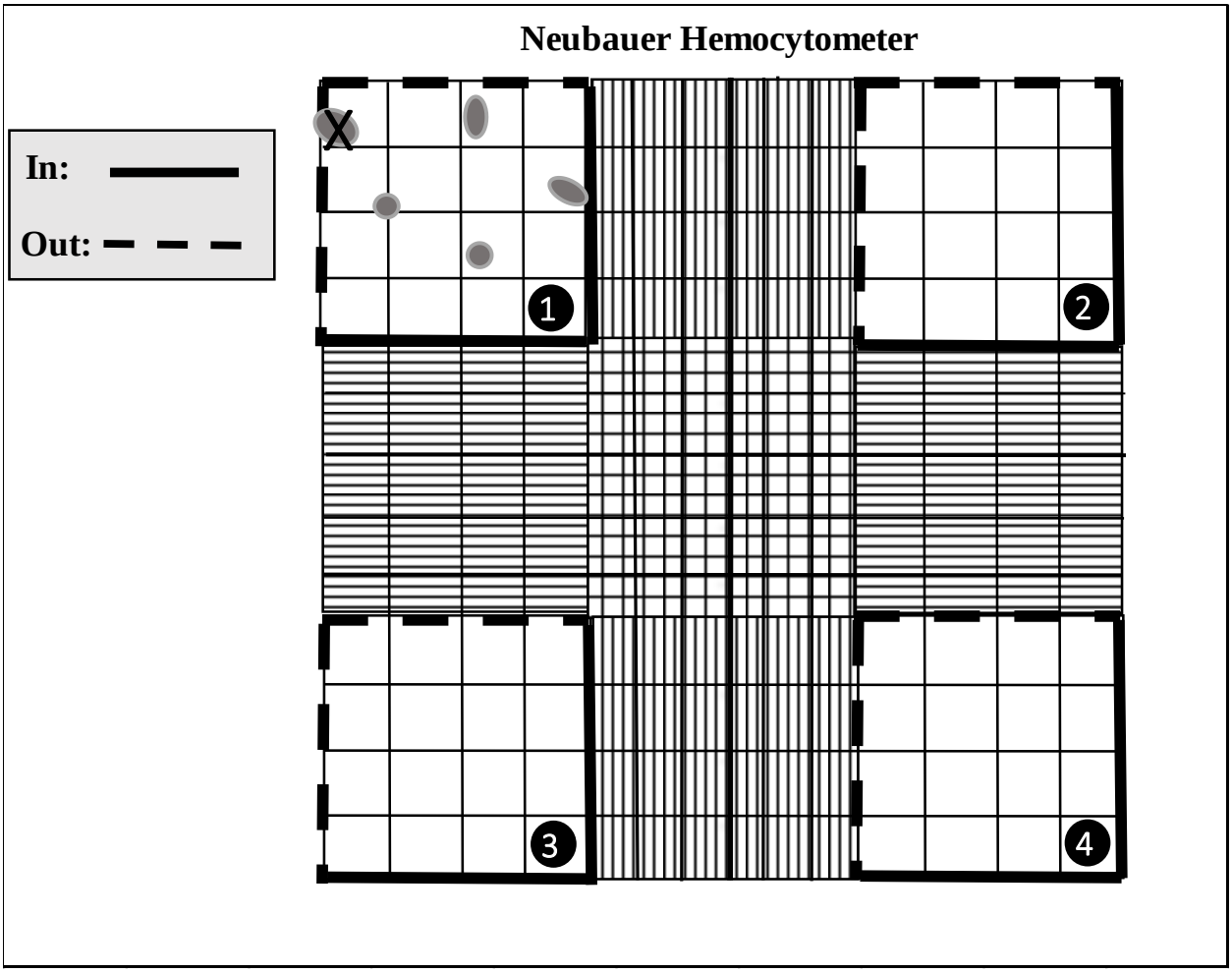

FIGURE 4: Counting method for brain cell nuclei. Pictured are four quadrats on the Neubauer hemocytometer. Gray circles and ovals represent DAPI-stained nuclei. Nuclei that touched the bottom and right side ruling of each quadrat indicated by solid lines were counted. Nuclei that touched the top and left side ruling indicated by dashed lines were not counted.

Results

Agalychnis callidryas

Low density tadpoles grew significantly faster than high density tadpoles over one week (Figure 4A, $X^2 = 26.044$, $p < 0.0001$). Competition also demonstrated a very strong effect on metabolic rate. High density tadpoles had significantly lower metabolic rates than low density tadpoles (Figure 6A, $X^2 = 27.826$, $p < 0.0001$).

While no significant difference was found between the gut mass between treatments, the other digestive organs did demonstrate an effect of competition (Figure 7A, gut; $X^2 = 1.1908$, $p = 0.2752$). Low density tadpoles developed significantly heavier livers and pancreases than high density tadpoles (Figure 7A, liver; $X^2 = 4.5294$, $p = 0.0333$ and pancreas; $X^2 = 8.3777$, $p = 0.0038$). We found that for a given tadpole body length, brains were significantly longer and heavier in low density tadpoles (Figure 8A, brain length: $X^2 = 7.57$, $p = 0.006$, brain mass: $X^2 = 5.289$, $p = 0.0215$). Additionally, for a given brain length, brain mass was significantly heavier in tadpoles from low density rearing environments (Figure 8A, $X^2 = 24.969$, $p < 0.0001$).

We analyzed the size of brain parts using body length as the covariate. We found that for a given body length, the medulla oblongata in the brains of low-density tadpoles were significantly longer and larger in area than in the brains of high-density tadpoles (Figure 9A, medulla length; $X^2 = 7.480$, $p = 0.0062$, medulla oblongata area; $X^2 = 12.524$, $p = 0.0004$). Further, optic tecta area and forebrain area were significantly larger in brains from low-density tadpoles than those from high-density tadpoles (Figure 9A, optic tecta area; $X^2 = 25.677$, $p < 0.0001$, and forebrain area; $X^2 = 13.838$, $p = 0.0001$). We also analyzed brain part sizes using brain length as the covariate. Not only are brain parts larger for low density tadpoles for a given tadpole body length, but they are also significantly larger for a given brain length (Figure 10A, medulla oblongata length; $X^2 = 5.3958$, $p = 0.0466$, medulla oblongata area; $X^2 = 10.554$, $p = 0.0011$, optic tecta area; $X^2 = 26.286$, $p < 0.0001$, forebrain area; $X^2 = 4.734$, $p = 0.0295$).

Anaxyrus americanus

After a one-week period, growth rates were lower for tadpoles raised at high- and extra-high density than at low density (Pers comm Justin McCurdy, Figure 5B, $F_{2, 27.10} = 120.036$, $p < 0.0001$). The metabolic rates of low- and high-density tadpoles did not differ (Figure 6B, Post hoc test, LD=HD; $p = 0.91$, HD=EHD; $p = 0.052$). However, extra high-density tadpoles had significantly lower metabolic rates (Figure 6B, $F_{2, 41.30} = 5.945$, $p = 0.005$).

Livers were significantly heavier in tadpoles from low density environments than from extra high-density environments (Figure 7B, liver; $F_{2, 30.98} = 10.013$, $p = 0.0004$). However, livers from high density tadpoles were not different from either low or extra high densities. Gut mass and pancreas mass were not significantly different across treatments (Figure 7B, gut; $F_{2, 29.24} = 2.066$, $p = 0.145$, pancreas; $F_{2, 28.97} = 1.858$, $p = 0.174$). No difference was found for brain mass or brain length for a given tadpole body length (Figure 8B, brain mass; $F_{2, 32.44} = 2.571$, brain length; $p = 0.092$; $F = {}_{2, 29.66,}$ $p = 0.407$). However, for a given brain length, brains were heavier in low density treatments (Figure 8B, $F_{2, 29.54} = 9.457$, $p = 0.001$).

We analyzed the size of brain parts using body length as the covariate. Across all three treatments, there were no differences in the size of brain parts (Figure 9B, medulla oblongata length, $F_{2, 33.36} = 0.202$, $p = 0.818$, medulla oblongata area, $F_{2, 33.99} = 0.396$, $p = 0.676$, optic tecta area, $F_{2, 32.05} = 1.293$, $p = 0.288$, forebrain area, $F_{2, 28.09} = 2.862$, $p = 0.074$). We also analyzed brain part sizes using brain length as the covariate. For a given brain length, we found no difference in medulla oblongata size across the treatments (Figure 10B, medulla oblongata length; $F_{2, 33.14} = 0.187$, $p = 0.831$, medulla oblongata area; $F_{2, 32.34} = 2.276$, $p = 0.119$). However, optic tecta and forebrains were significantly larger in low density tadpoles than in high- and extra high-density tadpoles (Figure 10B, optic tecta area; $F_{2, 33.62} = 10.479$, $p = 0.0002$, forebrain area; $F_{2, 33.64} = 7.009$, $p = 0.003$).

A sample of fifteen of the thirty-nine tadpole brains were examined to determine the average nuclei diameter and nuclei density per sample (N = 5 per treatment). Due to this small sample size, we

used a Kruskal-Wallis test to perform statistical analyses. The average nuclei diameters ± standard error for low, high, and extra high treatments were 9.86 ± 0.210 micrometers, 9.83 ± 0.370 micrometers, and 9.23 ± 0.367 micrometers, respectively. These values were not statistically significant (Figure 11B, $p = 0.403$). Before calculating the average nuclei density, we excluded four brains that did not have a coefficient of variation less than 0.25. The remaining eleven brains included three low density tadpoles, four high density tadpoles, and four extra high-density tadpoles. The average nuclei densities per treatment ± standard error were 91 ± 12.709 for low density, 100 ± 28.411 for high density, and 122 ± 32.767 for extra-high density. These values were not found to be significantly different (Figure 11A, $p = 0.694$).

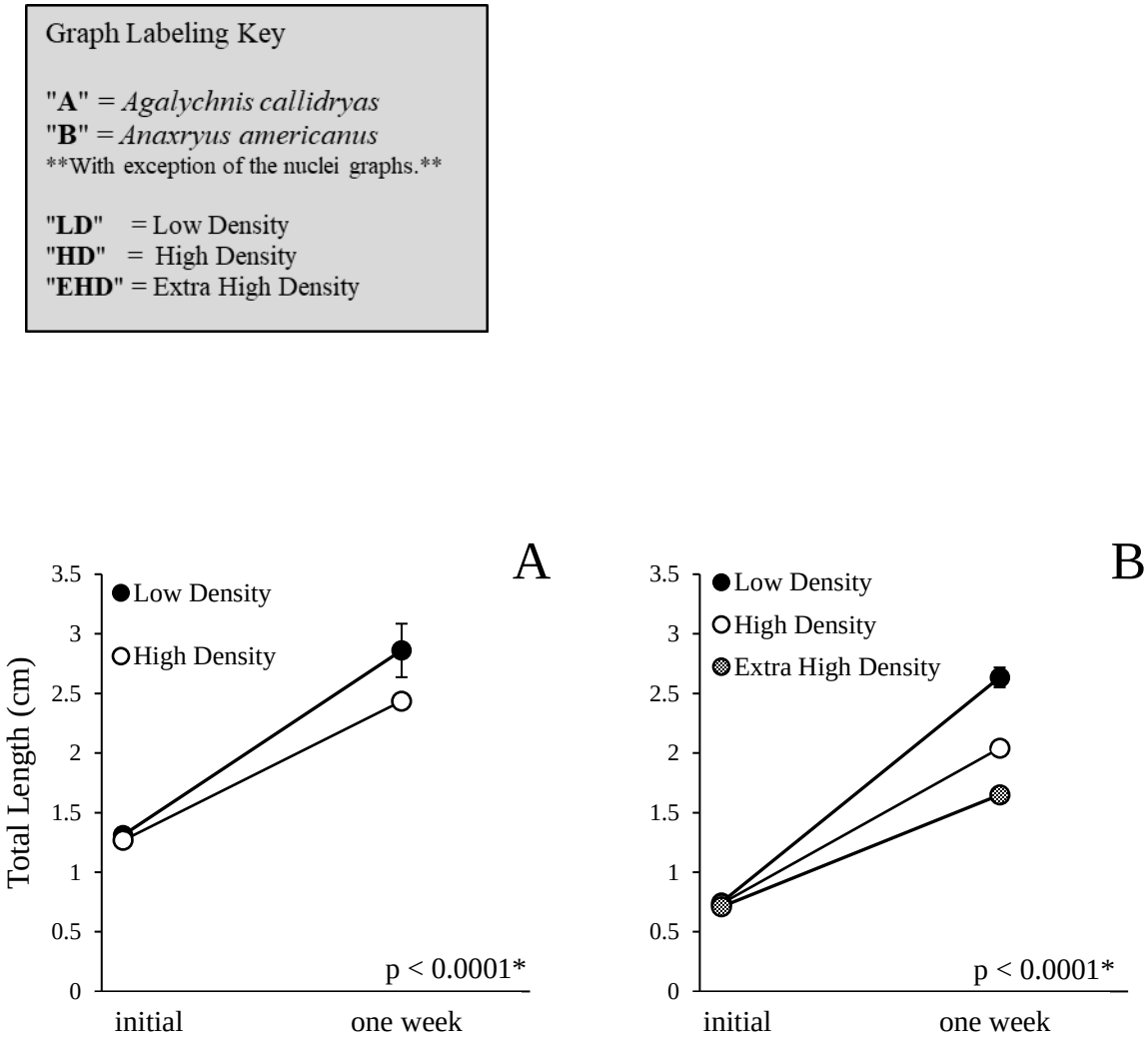

FIGURE 5: Tadpole growth. The increase in total body length over a one-week period was used to determine growth rates of A) *A. agalychnis* (X^2 = 26.044, p < 0.0001) and B) *A. anaxryus*, tadpoles ($F_{2, 27.10,}$ p < 0.0001). Data for *A. anaxryus* was collected by (Pers comm McCurdy, 2018). Data are means ± standard error. Some error bars are very small and not visible.

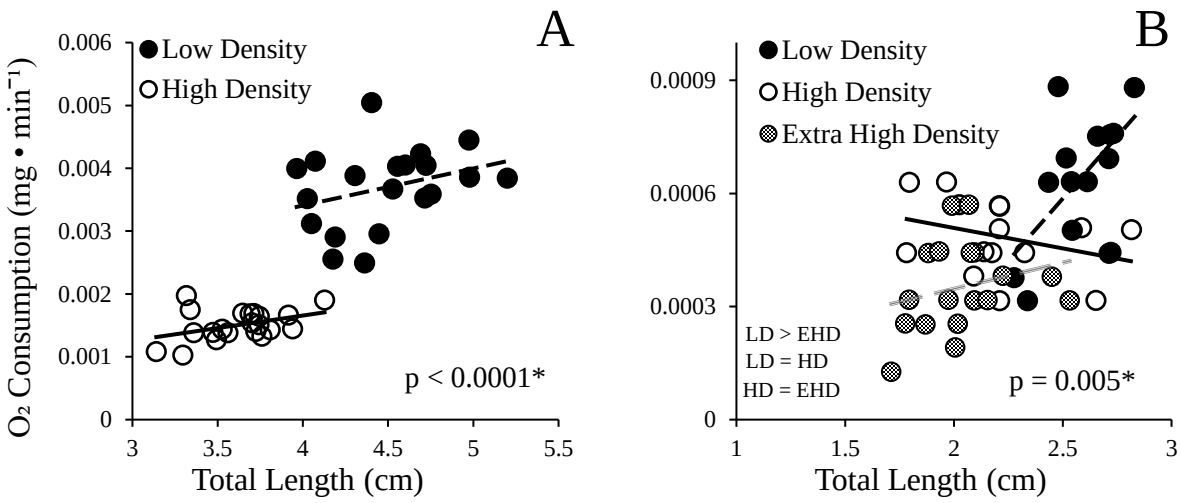

FIGURE 6: Effect of competition on metabolic rate in A) *A. agalychnis* and B) *A. anaxryus* tadpoles. *A. agalychnis* tadpoles reared in high-density environments had significantly lower metabolic rates than those raised in low density ones (X^2 = 27.826, p < 0.0001). Only *A. anaxryus* tadpoles raised in extra high-density environments demonstrated lowered metabolic rates from those of low-density tadpoles ($F_{2, 41.30}$ = 5.945, p = 0.005). Data for *A. anaxryus* was collected by (Pers comm McCurdy, 2018).

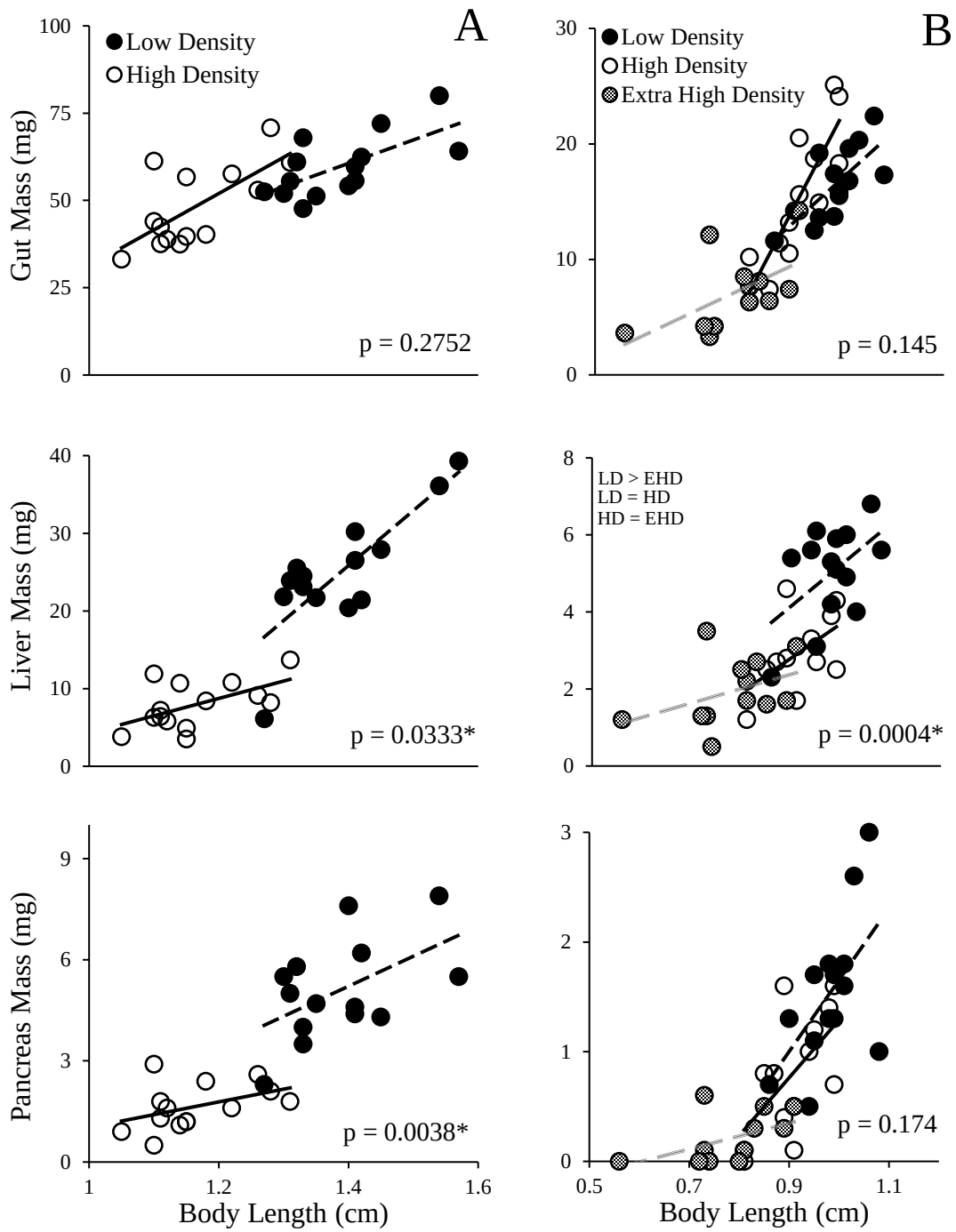

FIGURE 7: **Effect of competition on digestive organ mass** in A) *A. agalychnis* and B) *A. anaxryus* tadpoles. The livers and pancreases of low-density tadpoles in *A. agalychnis* were significantly heavier (liver; $X^2 = 4.5294$, p = 0.0333 and pancreas; $X^2 = 8.3777$, p = 0.0038). In *A. anaxryus* tadpoles, livers of low density tadpoles were only significantly heavier when compared with livers from extra high density tadpoles ($F_{2, 30.98} = 10.013$, p = 0.0004).

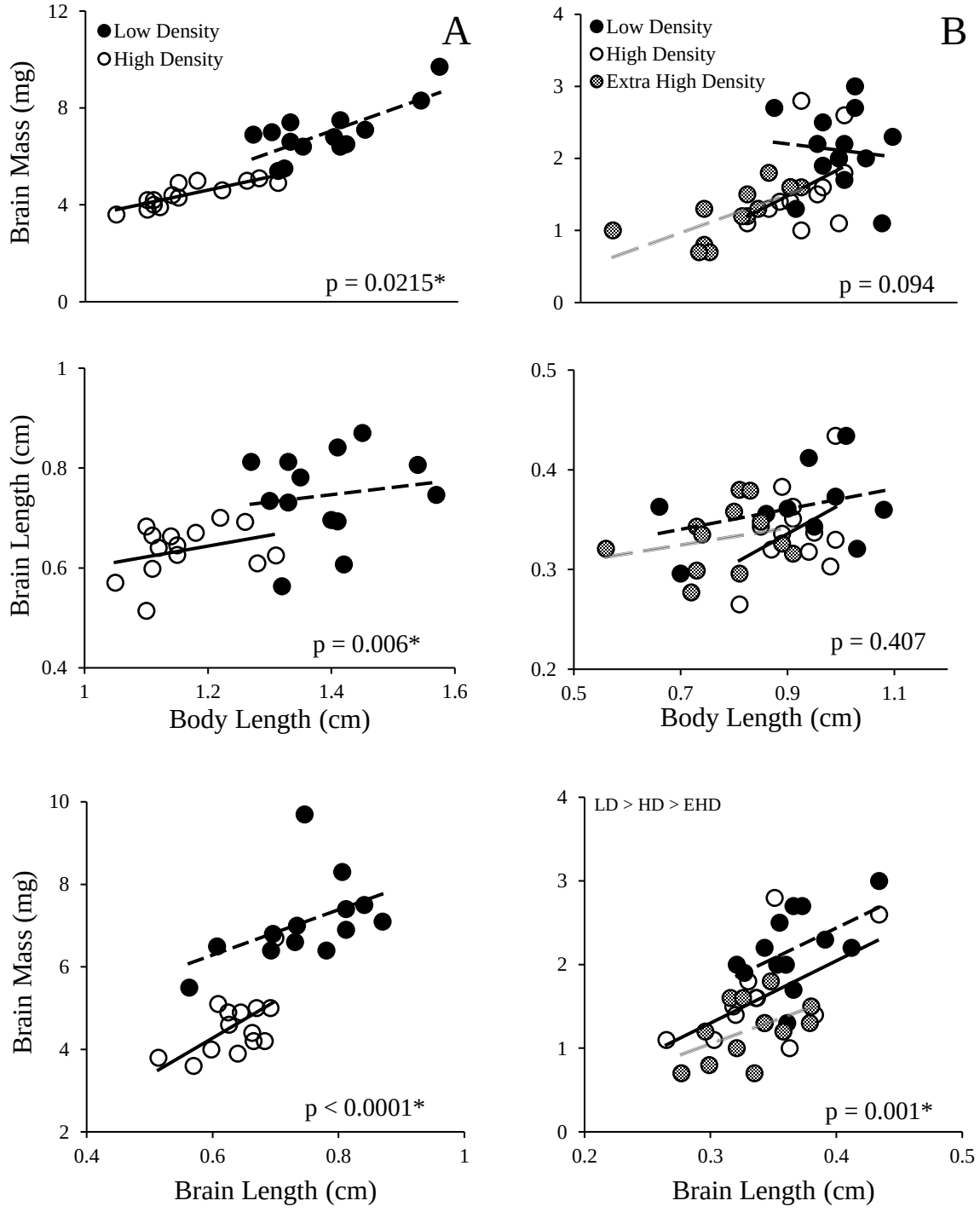

FIGURE 8: Brain mass for a given body length and brain length of A) *A. agalychnis* and B) *A. anaxryus* tadpoles. For a given body length, brain mass and brain length were significantly different in only *A. agalychnis* tadpoles (brain mass: $X^2 = 5.289$, p = 0.0215, brain length: $X^2 = 7.57$, p = 0.006). In both species, however, for a given brain length the brains of tadpoles reared at low density had significantly greater mass (*A. agalychnis:* F = 24. 969, p < 0.0001, *A. anaxryus:* $F_{2, 29.54} = 9.457$, p = 0.001).

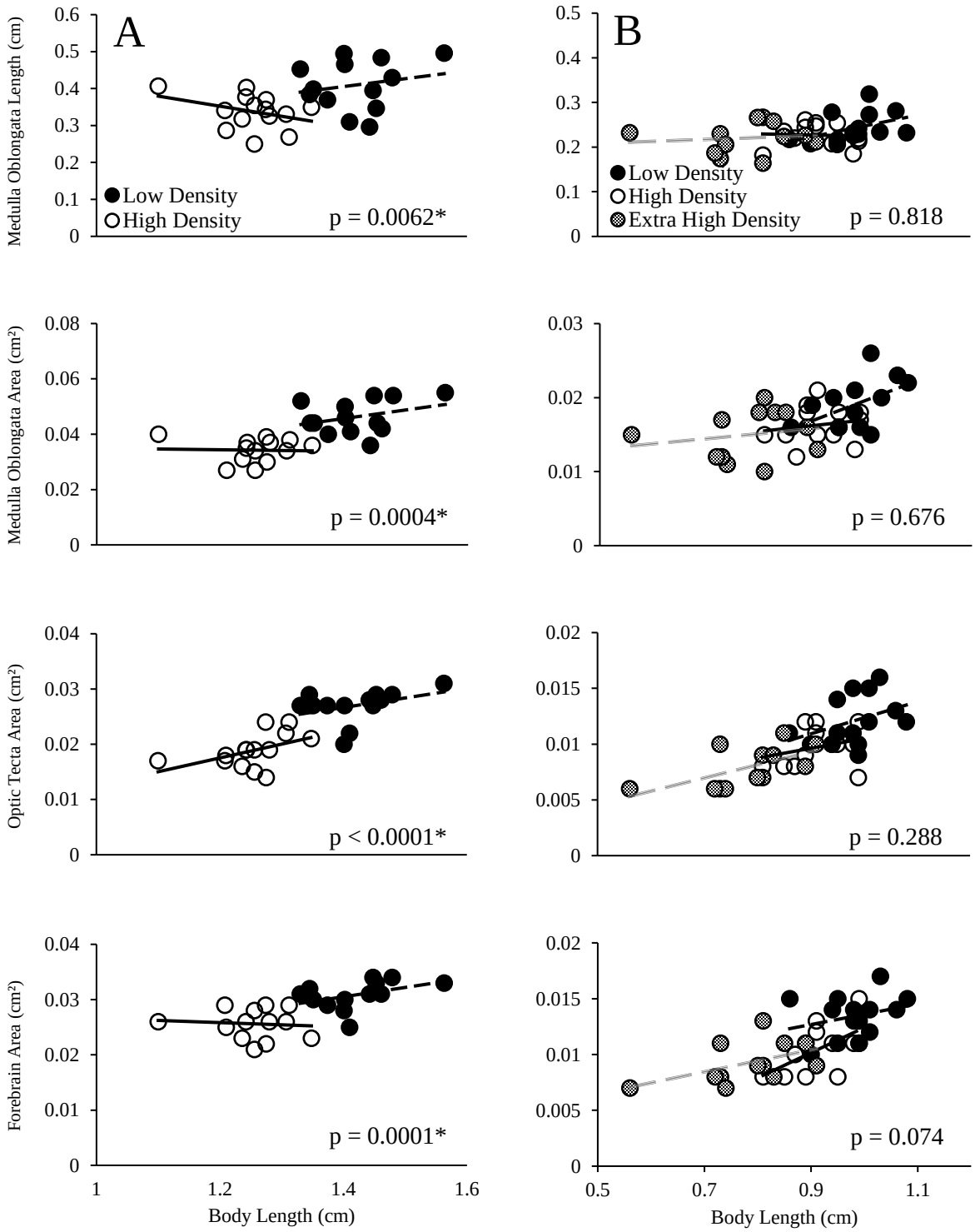

FIGURE 9: Brain part size. For a given body length, low density A) *A. agalychnis* tadpoles produced larger medulla oblongata, optic tecta, and forebrains (medulla oblongata length: F = 7.480, p = 0.0062, medulla oblongata area: F = 12.524, p = 0.0004, optic tecta area: F = 25.677, p < 0.0001, and forebrain area: F = 13.838, p = 0.0002).

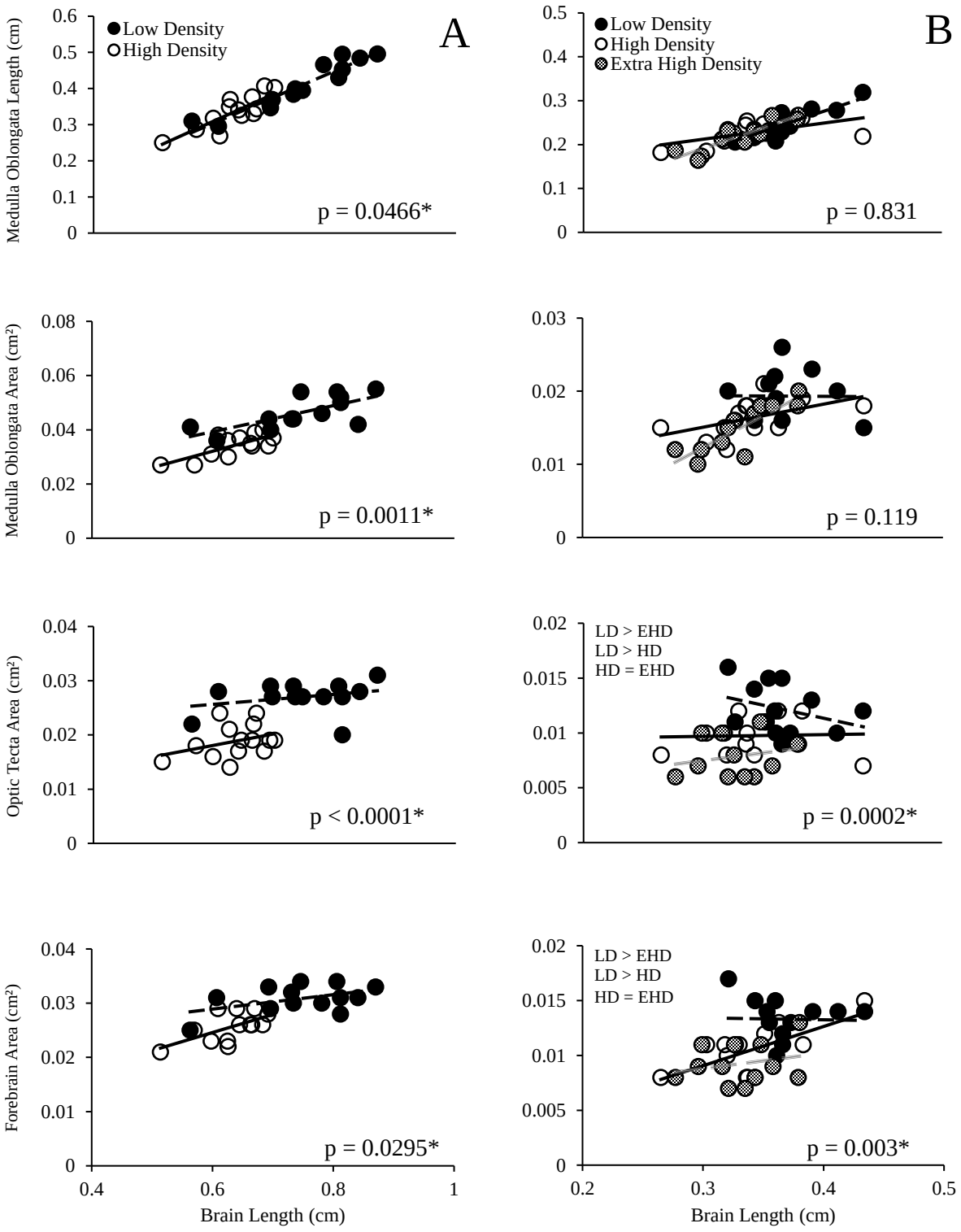

FIGURE 10: Brain part size. For a given brain length, low density A) *A. agalychnis* tadpoles had significantly larger medulla oblongata, optic tecta, and forebrain (medulla oblongata length: $F = 3.958$, $p = 0.0466$, medulla oblongata area: $F = 10.554$, $p = 0.0011$, optic tecta area: $F = 26.286$, $p < 0.0001$, forebrain area: $F = 4.734$, $p = 0.0295$). Low density B) *A. americanus* tadpoles had significantly larger optic tecta and forebrains for a given brain length (optic tecta area: $F_{2, 33.62} = 10.479$, $p = 0.0002$, forebrain area: $F_{2, 33.64} = 7.009$, $p = 0.003$).

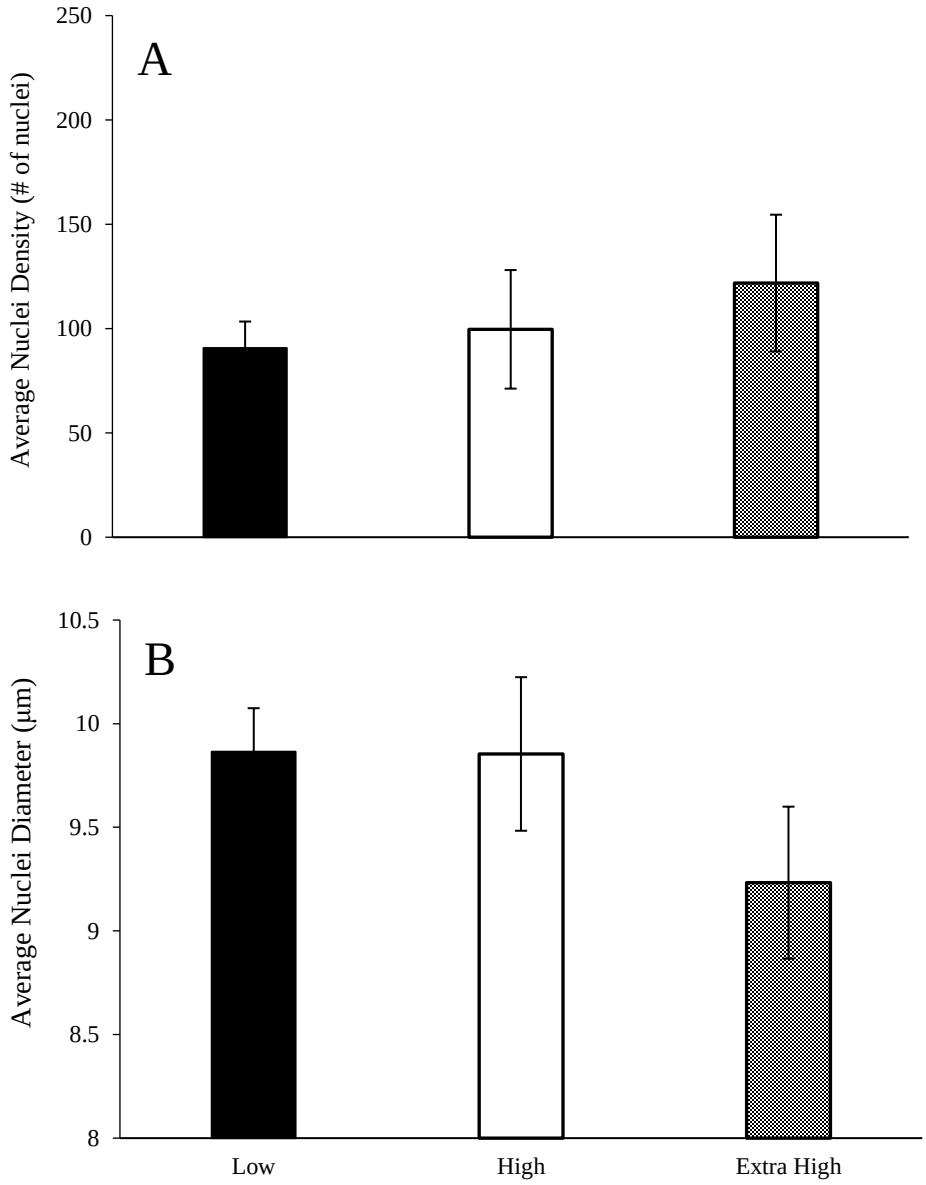

FIGURE 11: Nuclei composition in *Anaxyrus americanus.* There were no significant differences in the density of nuclei nor the size of nuclei in tadpole brains from different treatments. Data are means ± standard error.

Discussion

Agalychnis callidryas tadpoles exhibited high levels of plasticity in response to intraspecific competition. Tadpoles from high density environments experienced decreased growth rates in comparison to low-density tadpoles. This finding was consistent with those found in *Rana sylvatica, Hyla versicolor*, and *A. callidryas* tadpoles reared under similar density conditions (Reylea, 2002; Reylea and Hoverman, 2003; Bouchard et al. 2015). We found that high density environments also produced tadpoles with drastically lowered metabolic rates. This finding confirms pilot data and supports our hypothesis. A reason for this decreased metabolic rate may be to conserve energy under limited food conditions. Metabolic depression in response to environment stressors such as food deprivation is widely documented across a variety of species and is viewed as adaptive lowering (Guppy and Withers, 1999). Alternatively, the stress of living under high competition for food-resources may have increased stress hormone levels that negatively impacted the metabolism of high-density tadpoles (Burraco and Gomez-Mestre, 2016). We believe this difference in metabolic rate is correlated with changes in organ size.

Based upon pilot data and other studies, we predicted *A. callidryas* high density tadpoles to have larger guts than low density tadpoles. However, gut mass was not different across low- and high-density treatments. This result is interesting because other studies have found that high-density environments produce tadpoles with longer (Relyea et al. 2002, Bouchard et al. 2015) and heavier guts (Bouchard et al. 2015). A reason for this disagreement could be because the tadpoles in our study were fasted overnight before metabolic rates measurements and euthanasia. Conversely, the tadpoles in the other studies were not fasted prior to euthanasia. In fact, the tadpoles in one of the experiments were part of an intake study and fed *ab lithium* before being euthanized, which ensured their guts were full (Bouchard et al. 2015). The differences in mass found in the previous studies could have occurred because longer guts can hold more food. It is possible that the increase in gut length found in previous studies is due to the redistribution of resources to form a longer gut with a thinner gut wall, but the same overall mass. This could allow for increased digestive processing with minimal increase in metabolic cost. It is not likely

that the increase in gut length is only due to differences in gut fill because high tadpole densities produce *A. callidryas* froglets with proportionately longer guts even when empty (Bouchard et al. 2016)

Decreases in metabolic rate are correlated with decreases in liver and pancreas size. One explanation for this is that tadpoles in resource abundant (low density) environments are able to devote excess food to developing larger organs. As larger organs require more energy maintenance than smaller ones, it could be possible that low density tadpole metabolic rates are driven higher in order to support these more metabolically costly organs (Steyermark et al. 2005; Burton et al. 2011). Additionally, a study examining the relationship of organ size with metabolic rate variation in a population of leopard frogs (*Rana pipiens)* found that higher metabolic rates correlated with higher mitochondria content in the liver (Steyermark et al. 2005). Therefore, tadpoles in low density environments may experience increases in liver mass due to elevated metabolic activity. Alternatively, it could be that low-density tadpole organ sizes are the baseline size. Due to resource-limitations, high density tadpoles cannot not afford to develop organs as large as those of low-density tadpoles. Smaller livers and pancreases have less tissue that can be activated for metabolic and digestive purposes, which may be the cause for metabolic depression in high-density tadpoles. While small organs and low metabolic rates seem to provide tadpoles faced with adverse conditions with an energy conserving strategy, the consequence of this is slower growth in the larval stage, which may hold negative implications on chances of survival and body size at metamorphosis (Steyermark et al. 2005; Burton et al. 2011; Auer et al. 2015).

Our findings also indicate that changes in brain size correlate with differences in metabolic rate. For a given body size, *A. agalychnis* brains were heavier and longer in low-density tadpoles than high density tadpoles. This result disagrees the study in *Rana temporaria* that found no change in overall brain size in low versus high density tadpoles (Gonda et al. 2010). However, this study did find that when faced with both competition and predation, that low density tadpoles reared with predation developed smaller brains. These differing results may indicate that anuran species perceive various environmental pressures

differently. In general, the brain is an energetically costly organ, so only tadpoles reared in optimal conditions may be able to develop a metabolic rate high enough to support its development (Steyermark et al. 2015; Auer et al. 2015; Eifert et al. 2015). Conversely, tadpoles living at high-density suppress their metabolic rate to conserve energy and produce smaller brains due to these functional and physical constraints. Although smaller brains likely conserve energy, there must be costs associated with it. One study examined the ability of different amphibian species to successfully reestablish after being translocated by humans (Amiel et al. 2011). They found that larger relative brain size correlated with higher level of function as these individuals were more successful at reestablishing in a new area. Although this study investigated brains interspecifically, the same might be inferred in regard to bigger brains permitting higher functional capacity which directly relates to higher levels fitness.

Though *A. callidryas* high-density tadpoles sacrifice overall brain size, we predicted that they would still prioritize optic tectum development at the cost of medulla oblongata and forebrain size. However, we observed a decrease in the size of all three of these brain morphologies. *Rana temporaria* tadpoles reared at high density increase optic tecta size , decrease medulla oblongata size, and maintain telencephalon and diencephalon size relative to those reared at low density (Gonda et al. 2010). We believe the decrease in medulla oblongata size observed in both studies may be a cost of limited resources. The medulla plays instrumental roles in the lateral line system, a mechanoreceptor sensory system in aquatic vertebrates (Butler and Hodos, 2005; Gonda et al. 2010). Developing this system should equivalate to increased awareness of the environment and hold fitness advantages for tadpoles. Therefore, low density tadpoles under no competition stress should maintain development of the medulla oblongata while high density tadpoles may be unable due to energy constraints.

The forebrain is composed of the telencephalon and diencephalon; therefore, we can compare the changes to *R. temporaria* telencephalon and diencephalon directly with *A. callidryas* forebrain. We contend that the forebrains of high density tadpoles in our study decreased because high density tadpoles, as with medulla size, cannot afford to develop a brain part that does not provide them an immediate

advantage. It is not clear why the brains of these two species would respond differently. The forebrain is associated with olfactory and auditory functions in anuran species (Walkowiak et al. 1999). As the senses associated with olfactory and auditory function seem to benefit anurans more in terrestrial life, we may not expect to see increased development of the forebrain until after metamorphosis.

For tadpoles living in high density environments, there is an increase in visual stimulation due to a greater number of moving competitors. Since the optic tecta is the brain part most associated with vision, the demands on the optic tecta should be high and result in more development than the other brain parts (Butler and Hodos, 2005; Gonda et al. 2010). Instead, we found that high density tadpoles had smaller optic tecta than tadpoles reared at low density. This result was surprising because it was the opposite of what was found in *Rana temporaria* reared at high density (Gonda et al. 2010). However, it is possible that these species are affected by competition differently. While *R. temporaria* develop larger optic tecta but are able to retain overall brain size, *A. callidryas* seem to be affected more severely by competition as evidenced by their overall reduction in brain size and decrease in size of all brains parts. Considering that less energy is allocated to brain size alone may be an indication that development of each brain part is also suppressed. Their optic tecta may be unable to respond to increased stimuli in their environment. Further, tadpoles have been documented to alter their feeding strategy in the way that most minimizes the threats around them and maximizes their ability to obtain food and grow fast (Petranka, 1989). Enlarged optic tecta should translate to enhanced visual function and could provide *R. temporaria* with advantageous foraging abilities. If this were true, it could be that *R. temporaria* rely more on their visual capabilities to compete for resources while *A. callidryas* may maintain a different foraging strategy, such as one that depends more on olfactory function.

Anaxryus americanus responded less strongly to competition. Although high-density tadpoles did exhibit lower growth rates, there was no difference between the metabolic rates of low- and high-density tadpoles. Further, neither gut, liver, pancreas nor brain size were different between tadpoles from low and high densities. However, *A. americanus* tadpoles reared in extra high-density treatments did demonstrate

metabolic and organ differences when compared with low-density tadpoles. The extra high-density tadpoles exhibited lower metabolic rates and smaller livers. Their brains responded with minimal plasticity. Extra high-density tadpole brain mass was smaller for a given brain length, but not for a given body length as in high density *A. callidryas* tadpoles. These findings suggest that *A. americanus* must be raised at extra-high densities to experience the same pressure of competition that *A. callidryas* experiences at only high-density.

We believe the reason for this contrast in response to competition level is due to differences in natural larval densities of these two species. The number of eggs that females of each species lay can be used as a relative indication of natural larval density. *Agalychnis callidryas* lay roughly 200 eggs over 25-50 clutches, while *A. americanus* lay approximately 3,000-14,000 eggs over one or two clutches (Kruse, 1981; Funk & Wagnalls, 2018). *Agalychnis callidryas* can be found living in habitats with low and high tadpole densities. The behavior of *A. americanus* is markedly different as they form large, cohesive groups of tadpoles called aggregates (Beiswenger, 1975). These aggregates are characterized by swimming and butting behaviors. They therefore may be more adapted to living under high competition conditions than *A. callidryas.*

As the brain is such an energetically costly organ and thus holds much potential for providing a source for an energetic trade-off, we wanted to determine if there were changes in the parts of the brain taking place. *Anaxryus americanus* did not experience any change in medulla size, but this result is not surprising regarding their lack of change in metabolism and organ size. However, tadpoles at extra high density developed smaller optic tecta and forebrains in response to competition, contrasting with the finding in *Rana temporaria*, but supporting the result in high density *A. callidryas*. This lack morphological brain plasticity made us curious about the existence of changes at the cellular level.

This is first study to our knowledge to ever visualize *Anaxryus americanus* brain cell nuclei using DAPI fluorescent staining. Although DAPI staining has been used in *Xenopus laevis* to stain brain cell nuclei to visualize specific brain parts (Peunova et al. 2001), we could not identify any anuran studies that

focused on measuring the brain cell nuclei themselves. Our experiment appears to be the first to use DAPI fluorescent staining to examine changes in the density and size of brain cell nuclei in response to different rearing densities of larval anurans. We did not find any statistically significant differences in nuclei density or nuclei size in brain samples across density treatments. One explanation for this could be that *A. americanus* does not respond largely to competition and in turn does not exhibit brain cell nuclei plasticity to this pressure. Another reason could be that our small sample size may have limited the ability to detect variation between treatments. Now that we have established a protocol for preparing and staining brain samples, future studies should reexamine brains with a larger sample size using our technique. Further, it would be interesting to evaluate the brains of very plastic species such as *A. callidryas*, as well as to investigate the effect other environmental pressures, such as predation, have on brains at the cellular level.

Conclusion

We confirmed that *A. callidryas* tadpoles reared in high density environments exhibit metabolic depression. We believe this lowering may be adaptive as it allows tadpoles faced with adverse conditions to conserve energy. Lowered metabolic rates correlated with smaller organs. It is unclear if limited resource availability constrains organ size which results in decreased metabolism, or if larger organs developed by low density tadpoles require them to maintain a high metabolic rate. Brain size and brain morphologies were smaller in high density tadpoles than in low density tadpoles, which may be another energy saving strategy. From our comparative study, we learned that *A. americanus* tadpoles did not respond plastically to high density rearing environments as *A. callidryas* did. The fact that high-density *A. americanus* tadpoles did not exhibit differences in organ size and also did not have depressed metabolic rates suggests that changes in organ size account for changes in metabolic rate. While we were not surprised that *A. americanus* tadpoles expressed little plasticity in regard to brain morphology, we predicted we would observe changes at the cellular level. While differences were not observed in brain cell nuclei density or size per sample, we were able to successfully establish a novel protocol for

visualizing brain cell nuclei in tadpoles using DAPI-fluorescent stain. This study contributes to the pool of literature documenting that anurans respond plastically to larval rearing environments and opens a new direction in the field of anuran brain plasticity.

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