Spring 2016

Comparison of Growth Rates in two Captive Bred Species of Atelopus (Anura; BUFONIDAE), at El Valle Amphibian Conservation Center

Cecile Avery
SIT Graduate Institute - Study Abroad

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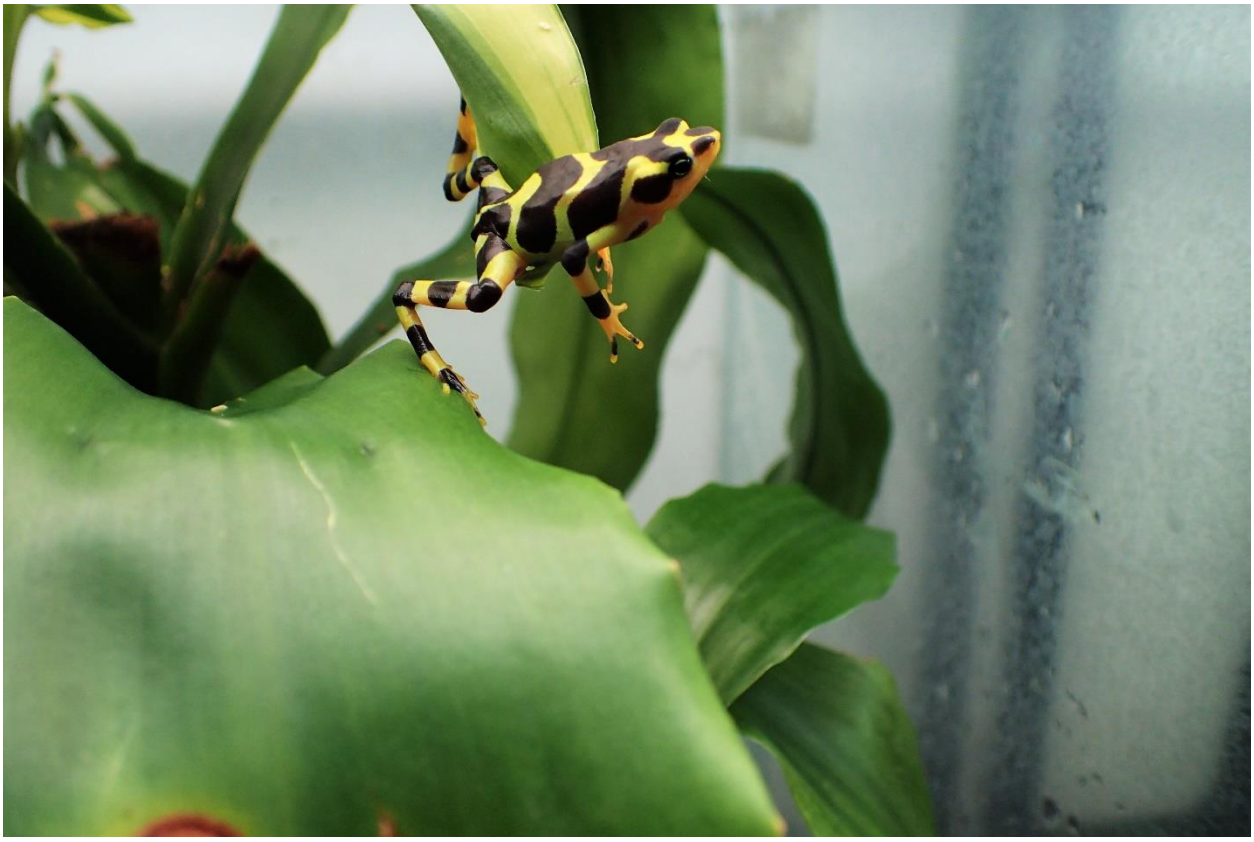

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SIT Panama: Spring 2016

Abstract

Since their evolution, amphibians have managed to survive four mass extinctions. But today's amphibians are now facing severe decline due to a plethora of causes including habitat destruction, climate change, pathogens, and pollution. Of all the possible causes of decline and extinction of amphibian populations, one of the most startling has been the effect of *Batrachochytrium dendrobatidis* (Bd), or chytrid fungus. It was decided that rapid action was needed to preserve the amphibian populations in the area, since it was clear that current *in situ* conservation methods were ineffective against Bd. EVACC, the El Valle Amphibian Conservation Center, was created to act as an ark of sorts for imperiled amphibians in the area. EVACC currently houses populations of *A. varius* and *A. zeteki*, two species particularly vulnerable to Bd. Recently, a difference in size between the juveniles of *A. varius* and *A. zeteki* has been noticed. The SVL and mass of the juveniles of interest were taken to find that there is in fact a statistically significant difference in size. However, growth rate post-metamorphosis between the species was discovered to be essentially equal. Because of the stable conditions in which the hatches were raised, the most likely explanation for the observed difference is the genetic lineages of the parents. More study is required to determine if this is a trend between all juveniles of *A. varius* and *A. zeteki*.

Acknowledgements

First and foremost, I would like to thank Eric Flores for putting me in contact with Edgardo Griffith, who has shared his passion, not only for his project, EVACC, but for the conservation and study of amphibians with me. Thank you for entrusting me with this project, seeing as it involves the handling of The Panamanian Golden Frog. I feel incredibly lucky to have been able to work hands-on with this national and global treasure. I would also like to extend my gratitude to Heidi Ross for allowing me to work in her space, and giving me a chance to contribute to the inner workings of EVACC. I hope that my work proves useful in the future. All of this would have never been done were it not for Aly Dagang, who encouraged me to pursue my passion.

Introduction

Over the course of life history, there have been a recorded five mass extinctions. The first extinction event pre-dated the planet's first terrestrial vertebrates, amphibians. But since their evolution, amphibians have managed to survive the next four mass extinctions (Wake et al. 2008). However, amphibians are now facing severe decline due to a plethora of causes including habitat destruction, climate change, pathogens, and pollution (Murphy et al.2013). This decline was first noticed in the 1980s-1990s (Storfer 2009) and of the causes mentioned, the rapid rate of habitat destruction is the most detrimental (Gardner 2007; Wake et al. 2008). The current extinction rate is approximately 211 times higher than the background extinction rate, and if all threatened species go extinct, the rate would increase as high as 25,000-45,000 times higher than the background extinction rate (Wake et al. 2008). There's no doubt that amphibians are one of the most imperiled classes of organisms (Lotters 2007; Murphy et al. 2013).

Batrachochytrium dendrobatidis

Of all the possible causes of decline and extinction of amphibian populations, one of the most startling has been the effect of *Batrachochytrium dendrobatidis* (Bd), or chytrid fungus. In Costa Rica, chytrid fungus was first identified as a threat when the amphibian populations in a protected area plummeted. Almost simultaneously, the amphibian populations in the forests of Australia followed the same trend, indicating a worldwide phenomenon (Wake et al. 2008). Since then, there has been a noticeable shift in the focus of research from that of habitat loss to the effects of climate change and disease (Gardner 2007).

In the Western Hemisphere, amphibian population decline is concentrated in the montane areas of the tropics, but a study of Costa Rican frog samples show that Bd is present even at lower altitudes (Puschendorf et al. 2016). Chytrid fungus has been present in Costa Rica as early as 1986, and recent climate change has lent to large-scale warming and changes in cloud cover that create an environment that fosters the development of chytridiomycosis and its spread (Puschendorf et al. 2016). The effects of Bd have been devastating, and researchers began to report declines in amphibian communities in Central America from 1987 and on, tracking its spread (Lips et al. 2005). As high disease-induced mortality swept through Central America, it was noted that some species were effected less than others. Hope exists that in studying the microbiota of Bd resistant amphibians, Bd naïve populations can be treated, enabling them to coexist with the fungus *in situ* (Flechas et al. 2012).

Atelopus zeteki has proved to be a species particularly vulnerable to Bd. The combined threats of global warming and Bd have led the entire genus heading towards extinction (Lotters 2007). Studies of *A. zeteki* have shown that previous exposure to Bd does not aid the immune response in fighting back against a second exposure, making their conservation ever more challenging (Ellison et al. 2014; DiRenzo 2014). Bd is able to mount a high infection intensity in *A. zeteki*, leading to host immunosuppression and rapid mortality (Ellison et al. 2014; DiRenzo 2014). Considering this, *Atelopus* as a whole has become of particular interest for studies of mass loss of biodiversity (Lotters 2007).

Methods of Conservation

The first cases of Bd in Panama were recorded in 2004 in Santa Fe and El Cope. It was feared that the forests of El Valle, Panama would be next. It was decided that rapid action was needed to rescue the amphibian populations in the area, since it was clear that current *in situ* conservation methods were ineffective against Bd (Gagliardo 2008). The El Valle Amphibian Conservation Center (EVACC) was created as an alternative to capturing and shipping frogs to conservation centers in the United States. EVACC was to act as a "repository to prevent extinction of the amphibians of El Valle" and to foster a population to later be reintroduced into the wild (Gagliardo 2008). *Ex situ* methods of conservation are generally less favorable to *in situ* methods, but Bd is particularly harmful for tropical frogs because of the multispecies communities they create, thus endangering an exponential number of tropical frog species (Wake et al. 2008). This necessitated dramatic and immediate action.

Amphibian conservation has proven challenging, and different causes of decline call for different solutions. Installation of microclimates and microhabitats in affected habitats, enhancement and restoration of breeding sites, and manipulation of water levels at breeding sites have been suggested as potential short term *in situ* methods (Shoo et al. 2011). It has also been suggested that urban ponds can be a means of amphibian conservation in areas of heavy human traffic (Garcia-Gonzalez 2012). But in cases where *in situ* conservation are not effective, breeding programs at facilities like EVACC occasionally utilize molecular methods to manage their populations. DNA barcoding has been useful in identifying cryptic species in captive populations to improve breeding initiatives (Crawford et al. 2013), and short nucleotide polymorphisms (SNPs) have been used to analyze population distinctiveness, and population diversity (Storfer 2009). Other useful molecular methods exist, but there is a need to develop techniques for these methods so that they can be applied to non-model organisms in order to guide effective management and conservation of threatened species (Storfer 2009).

Captive Breeding and Reintroduction- *ex situ*

The challenge with recently established captive populations is a general lack of knowledge about the husbandry and nutrition of species that have never before been kept in captivity (Michaels et al. 2014; Pessier et al. 2014). Publication bias and impracticality of study has led to 25% of amphibian species to be listed as data deficient by the IUCN (Michaels et al 2014). Without natural history and environmental parameter data, captive environments and protocol design will be lacking, negatively impacting the progress and effectiveness of captive breeding facilities. Unfortunately, it is likely that the species most in need of *ex situ* intervention fall within the aforementioned 25% (Michaels et al. 2014). The effectiveness of this method of conservation is dependent on the health and reproductive success of the founder population-individuals collected from the wild to be bred in captivity. It is critical that *ex situ* conditions match *in situ* conditions as closely as possible in order to prevent genetic and epigenetic shifts that may lead to reduced fitness in later generations reintroduced into the wild (Michaels et al. 2014). Ideally, an assurance colony, a small population of a critically endangered species raised in captivity to prevent complete species extinction, is composed of a high number of founders to preserve genetic diversity, the husbandry of the animal is well understood to ensure reproductive success, and the threat of disease well managed to prevent colony collapse (Pessier et al. 2014).

The establishment of an assurance colony is only half the battle. Typically, the long term goal of these projects is successful reintroduction of species into the wild. This requires long-term commitment as most projects run for at least a decade before any measure of success (Griffiths and Pavajeau 2008). This can be problematic, as such a length in captivity inevitably brings about genetic changes in younger generations as a result of the removal of natural selection, inbreeding depression, and adaptation to captivity (Griffiths and Pavajeau 2008). Studies have also shown that the microbiota of the skin, a vital symbiotic relationship between bacteria and amphibians, is significantly altered in captivity (Antwis et al. 2014; Becker et al. 2014). This is especially problematic, being that Bd and other pathogens present *in situ* may make captive individuals unsuitable for reintroduction (Antwis et al. 2014). However, amphibians generally lend themselves to success for this method of conservation due to their high fecundity, small size (therefore more cost effective in terms of space and resource use). Amphibians are also unlikely to develop behavioral changes in response to captivity (Griffiths and Pavajeau 2008).

Some controversy surrounds captive breeding and reintroduction (CBR) projects, but most conservation scientists agree that the current peril of amphibians renders this method useful (Griffiths and Pavajeau 2008; Bowkett 2009). But removing species from their ecosystems is not ideal, considering the potential negative impact on ecosystem dynamics post-removal and the challenge created upon reintroduction of said removed species. CBR is not meant to replace habitat preservation initiatives, and should also not be the first plan of action for endangered species (Bowkett 2009).

Atelopus zeteki and *Atelopus varius*

Two amphibian species of particular interest is the Panamanian Golden Frog, *Atelopus zeteki*, and the closely related *Atelopus varius*. *A. zeteki* has been listed as critically endangered because of a population decline estimated at over 80% in the past three generations, a decline largely credited to the spread of Bd (Pounds et al. 2010). Unfortunately, this decline is not isolated to *A. zeteki*; 74% of *Atelopus* species in Central America have faced severe decline in response to Bd (Perez et al. 2014). EVACC currently houses assurance colonies of *A. zeteki* and *A. varius* with the hopes of being able to release them into the wild in the future. A recent study has found surviving populations of *A. varius* in western Panama where Bd is now enzootic, but no members of the especially vulnerable *A. zeteki* were discovered (Perez et al. 2014). While the last observation of *A. zeteki* in the wild was made in 2009 around El Valle, there is a high probability that the species will be declared extinct in the wild within the next five years (E. Griffith, pers. comm. 2016). These recent surveys are crucial to guiding conservation efforts of facilities like EVACC, so that the appropriate measures can be taken with the existing assurance colonies.

Interestingly, the species status of *A. zeteki* and *A. varius* has been contested in the past. *A. varius* and *A. zeteki* occupy the same habitats, their distribution overlaps, and have near identical patterning and coloration (Savage 1972; Lindquist and Hetherington 1998). But based on current molecular data, the two have been kept as separate species (Richards and Knowles 2007). The

data collected from this study may hint at a subtle defining characteristic between *A. varius* and *A. zeteki*.

The captive breeding program for *A. zeteki* and *A. varius* is well underway at EVACC. However, there has been an observable difference in size between the two species, despite being the same age. *A. varius* and *A. zeteki* clutches are raised under the same artificially controlled conditions and fed the same diet, so it is possible that the difference in size stems from the genetic lineages of the parents. This study aims to determine if this interspecies difference in size is statistically significant, and compare their relative mass increase. Quantifying these differences, or lack thereof, will allow for further investigation in the topic, and better the understanding of the life history of these species.

Research Question

Is there a statistically significant difference in snout-vent length and body mass between juvenile captive bred hatches of *Atelopus varius* and *Atelopus zeteki* of the same age at El Valle Amphibian Conservation Center?

Methods

This study was conducted within the El Valle Amphibian Conservation Center, located inside El Nispero Zoo at N 08˚37.013' W 080˚07.573' UTM, in El Valle de Anton, Cocle, Panama. At the time of data collection, individuals from two hatches of highland *A. zeteki* and one hatch of highland *A. varius* were large enough to be studied without causing undue stress on the animal. The average ages of the animals considered for this study were between 2-3 months old. During the sampling process, a new set of vinyl gloves was used and all equipment cleaned with 70% ethanol solution between measuring animals from a different enclosure. Only animals that appeared to be in healthy condition were included in the study.

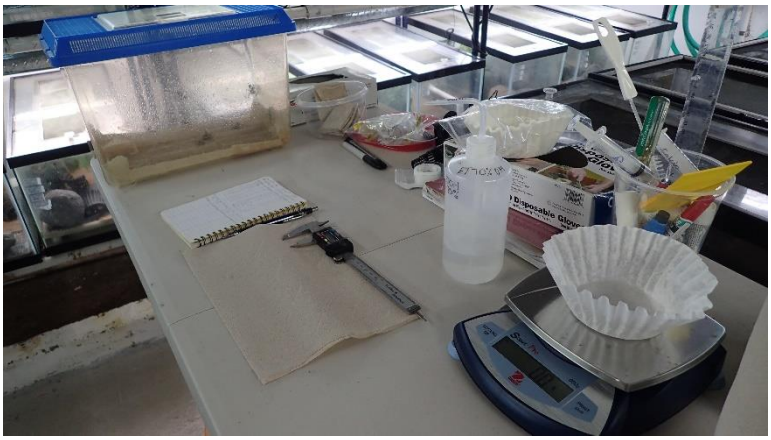


Figure 1. Equipment set-up including temporary enclosure, non-bleached paper towel, caliper, 70% ethanol solution, and scale.

For each enclosure studied, the ID (which indicates the generation, group number, and ID of the parents) and age were recorded. To prevent the double counting of individuals, all animals were first moved to a small temporary tank and moved back to their enclosure as they were processed. Each enclosure housed 9-10 individuals. The

Figure 2. Animal and caliper positioning for measuring SVL

snout-vent length (SVL) was collected by taking the animal's left arm in-between the thumb and forefinger and allowing the frog to rest on the other fingers. A Tool Shop 6 in. digital caliper was then used to measure the length from the tip of the snout to the cloaca (see figure 1). SVL was measured to the nearest 0.01 mm. A Scout Pro SP601 digital analytical scale was used to determine the animal's mass by placing the specimen in a coffee filter on the tared scale. Mass was measured to the nearest 0.1 g.

The mass and SVL recorded from the individuals from each enclosure was averaged and then grouped by hatch and species. The overall averages for each hatch was then calculated, as well as the species average mass and SVL. A standard deviation for each hatch separately and then species was calculated. Based off of the species averages, a percent difference in size was found so that the data may be more easily visualized.

Statistical Analysis

Microsoft Excel was used to run t-tests on the data from the enclosure averages between the two different hatches *A. zeteki* to determine if the two hatches of the same species were significantly different from one another. Another series of t-tests was performed using the enclosure averages of SVL and mass between *A. zeteki* and *A. varius*.

Data from previous months was incorporated into the study to determine if the two species appeared to be growing at the same rate. Selecting enclosures of *A. varius* and *A. zeteki* with the most data available, the average masses for each month were determined. The difference in mass between the months was used to determine the percent increase in body mass. The average percent increase in mass for both species was then calculated. A t-test was run between the percent increases in mass of *A. zeteki* and *A. varius* enclosures to determine if the difference between the two was statistically significant. The alpha level for this study was set to 0.05.

Results

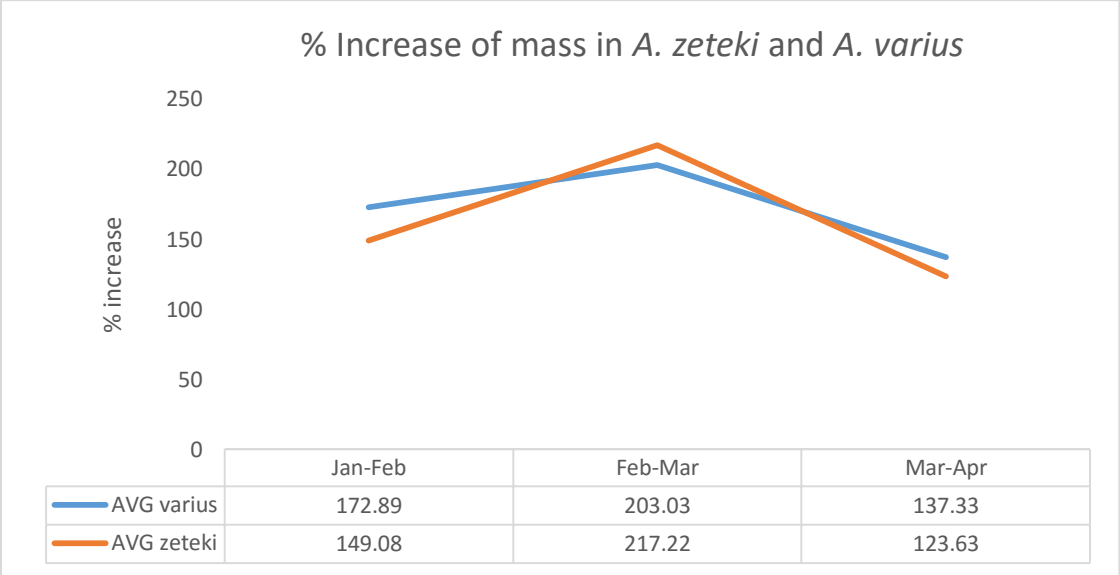
Of the two hatches of *A. zeteki*, 128 individuals were measured from hatch "X" and 31 from "Y" of *A. zeteki*. 192 individuals from hatch "A" of A. varius was measured for a total of 351 *Atelopus*. Only two were omitted from the study due to deformities (one was found in both *A. zeteki* and *A. varius*), for a total of 349 individuals considered for the study. Hatch A is of the F1 generation, and hatches X and Y of the F2 generation.

	Average SVL (mm)	**Average mass (g)**
A. zeteki (X)	18.46 ± 2.51	0.69 ± 0.22
A. zeteki (Y)	16.49 ± 4.26	0.63 ± 0.40
A. zeteki (X and Y)	18.09 ± 2.84	0.68 ± 0.25
A. varius (A)	22.93 ± 2.60	1.11 ± 0.30

Table 1. The average SVL and mass for each hatch of *Atelopus* is reported above, along with the overall average between hatch X and Y of *A. zeteki*.

The two hatches X and Y were compared using a two-tailed t-test. Due to the inequality of data between the two hatches X and Y, data for the t-test was selected from hatch X that came from enclosures of the same age as those from Y. The SVL between X and Y yielded a p-value

greater than 0.05 (p=0.14). The mass between X and Y also yielded a p-value greater than 0.05 (p=0.76). As determined by the results of the t-tests, between X and Y, neither SVL nor mass showed statistically significant difference. A two-tailed t-test was also performed on the data between *A. zeteki* (X) and *A. varius* (A). Data from hatch A that fell outside the age range of X was omitted for the purpose of the t-test. Between the two species, the SVL returned a p-value less than 0.05 ($p= 9.69 \times 10^{-6}$). The mass also returned a p-value less than 0.05 ($p= 3.07 \times 10^{-6}$). These p-values reveal a statistically significant difference between the SVL and mass between the two species. No t-test was run between the SVL and mass of hatch Y and hatch A because of the disparity in the amount of data between the two. A direct comparison of the SVL and mass between the species shows that the SVL of *A. varius* is approximately 26.71% longer, and the mass approximately 63% greater than that of *A. zeteki* at the same age.



Graph 1. Illustrates the percent increase of body mass between *A. zeteki* and *A. varius*. The percent increase of body mass is labeled on the y-axis, and the values are reported below the x-axis. The months listed on the x-axis correspond to the increase in mass between data collection between those two months.

Four enclosures of each species were analyzed, based on availability of data, to estimate growth rate. For *A. varius*, data from hatch A was analyzed. For *A. zeteki*, data from both hatch X and Y were included. These enclosures were of the same approximate age. Graph 1 shows the trends in the data. A t-test revealed that between the two species, between each month of data collection, no significant difference in the growth rate (p-value greater than 0.05); January-February (p=0.25), February-March (p=0.83), March-April (p=0.13).

Discussion

The juvenile stage of life is a period in which an organism prepares for reproduction. It involves many evolutionary trade-offs, since larger size at adulthood generally entails higher fitness but then requires a longer period of vulnerability. As a result, much variation exists in age and size at maturity, and growth patterns (Gotthard 2001). Although this study does not seek to

explain why differences in size exist between the species of interest, it does confirm that differences are present.

In comparing the sizes of the two species, the sample size for *A. zeteki* was smaller relative to *A. varius*. But because two unrelated hatches of the same species were available for comparison, the possibility that either hatch of *A. zeteki* was abnormally small or large could be rejected. Table 1 shows that both hatch X and hatch Y are very similar in size, and the t-test confirmed that any minute difference in size between the two as negligible. Additionally, the average size of the hatches of juvenile *A. zeteki* fell within the range of other juvenile *A. zeteki* found in Parque Nacional Altos de Campana, Panama, Panama (Lindquist and Hetherington 1998). Ideally, more than one hatch of *A. varius* would be studied, but the relatively larger sample size of hatch A may compensate for this factor. And had a more sensitive scale been used, the data of some younger individuals could have been collected, increasing the scope of the study and improving its accuracy.

During data collection, it was clear that *A. varius* was slightly larger than *A. zeteki*, but quantifying this difference reveals by what amount the two species differ. The t-test determined that the two species (hatch X of *A. zeteki* and hatch A of *A. varius*) were of statistically significant different masses and snout-vent lengths. Had the sample size for hatch Y been larger, a direct comparison of hatch Y would have been made to hatch A. However, if hatch X and hatch Y were of the same size, it seems likely that a comparison of hatch Y and hatch A would have reached the same conclusion. It is possible that this hatch of *A. varius* is larger than average, but at maturity, *A. varius* tends to be slightly larger than other members of its genus (E. Griffith, pers. comm. 2016).

The SVL and mass of *A. varius* was over 25% and 60% larger, respectively, than that of *A. zeteki*, despite being raised under the same conditions. The benefit of studying two species of *Atelopus* in a laboratory setting is that all environmental factors such as temperature, food availability, and predator pressure are either constant or non-existent. Controlled studies exist in which temperature and food were toggled in a laboratory setting to determine the effects on juvenile development of anurans, finding that differences in morphology result (Tejedo et al. 2010, Gomez-Mestre et al. 2010). Juvenile development is a very plastic response that is sensitive to conditions during the larval stage, all in order to maximize chances of survival to sexual maturity (Gotthard 2001; Tejedo et al. 2010; Gomez-Mestre et al 2010). So, it is intriguing that this significant difference exists between the two closely related species under identical circumstances. Perhaps the subtle progression of the sympatric speciation of *A. zeteki* and *A. varius* is evidenced in this data.

Because the two hatches are the same age, it was first presumed that the growth rate must differ between the two species. However, based off of the mass data from previous months on the oldest and similarly aged groups of *A. zeteki* and *A. varius*, the growth rate was not significantly different. This may be as expected, since growth rate is largely a product of the environment (Gotthard 2001). Graph 1 shows that the two species have very similar growth trajectories, with a small peak in growth between the second and third month of data collection.

A future study may focus on this pattern and determine if at any point leading up to sexual maturity the two species diverge.

If the growth rate between the species is essentially equal, *A. varius* must be larger than *A. zeteki* upon metamorphosis. The use of more sensitive equipment and ample measurements of newly metamorphosed individuals may verify this. Size at metamorphosis is impacted by a variety of environmental factors such as temperature, food availability, larval density, and desiccation risk during the larval period (Tejedo et al. 2010; Gomez-Mestre 2010). Of course, all of these conditions were equal in this laboratory setting. However, it has been suggested that species may have opposite post-metamorphic phenotypes as a result of similar pressures during the larval stage. For example, a lengthened larval period could result in larger individuals for one species, and stunted individuals in another. It should be noted that the larval period of even very distantly related species is effected similarly by environmental pressures (Gomez-Mestre 2010). Outside of environment, genetic factors, which have been shaped by natural selection to optimize survival to sexual maturity, also play a role in development (Gotthard 2001). The differences observed in this study can be most likely attributed to this.

Conclusion

The life cycle of amphibians is complex, and in terms of study, much more literature exists concerning larval development up to the juvenile stage than juvenile growth itself. This study provides basic information on the early juvenile growth of two species of *Atelopus*, a genus in which life history data is lacking (Karraker et al. 2006; Lotters 2007). Understanding the life history of species can further knowledge of its evolution and aids in the development of conservation strategies (Gotthard 2001; Karraker et al. 2006). This study confirms that a significant difference in mass and SVL exists between the young juveniles of *A. zeteki* and *A. varius* housed within EVACC, while their growth rates remain equal. There is in substantiate evidence to claim that this is true between all individuals of the species due to the small number of hatches studied, and genetic variance present in geographically separate populations (Richards 2007).

Because of the conditions in which the hatches were raised, environmental variance that could affect size are not likely a factor. More plausibly, the difference in their genetic lineages have resulted in slightly larger juveniles in one species compared to the other. Continued data collection for these hatches, as well as new ones, should be carried out to determine if this pattern continues. Also, continued tracking of growth rate could reveal a divergence between the two species before adulthood.

Within facilities like EVACC, monitoring size can be useful in regrouping individuals of enclosures to reduce food competition thus increasing the health and number of individuals that reach adulthood. This study also invites closer investigation of individuals throughout their development. It is possible that relative size at a young age can be indicators of sex far sooner than other characteristics may.

The study of juveniles in their natural environments can prove difficult since they are generally elusive (Lindquist and Hetherington 1998). But should juveniles be collected, the data

from this study could aid in the estimation their age. This could be crucial to conservation efforts, since their age and presence could give clues to the health and reproductive status of a population- the most important information for assurance populations such as the one studied.

As more is learned about the behavior, development, and dietary needs of *Atelopus* and other endangered amphibians, and molecular methods further developed, the hope is that the success of *ex situ* methods is improved so that more *in situ* methods can be used in its place.

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