Allometric Effects on Clutch Size and Quality in the Panamanian Golden Frog, Atelopus zetek

Micah Christensen
Erik Lindquist
Maryland Zoo in Baltimore

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Allometric Effects on Clutch Size and Quality
in the Panamanian Golden Frog, *Atelopus zeteki*

by

Micah Christensen1*, Erik Lindquist1, and the Maryland Zoo in Baltimore

1Messiah College, Department of Biological Sciences

* Correspondence to: Micah Christensen, 1010 Pross Rd, Lansdale, PA 19446.
E-mail: 1MicahC@gmail.com  Phone: 267-218-4515

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ABSTRACT
This study aimed to determine the relationship between parental phenotypes and egg clutch size and quality in the Panamanian golden frog, *Atelopus zeteki*. This species has not been seen in the wild since 2009, but is maintained through captive breeding programs. Determining factors correlated with egg clutch size and fertility will improve reintroduction models as well as captive breeding programs. Frogs of known lengths and weights were paired and encouraged to mate. However, many of the pairs did not lay eggs even after hormone treatment. Once clutches were laid, they were allowed to develop for five days at which point they were preserved in 10% buffered formalin. For examination, clutches were moved to 70% ethanol and stained with methylene blue solution. A dissection microscope was used to determine and record developmental status of each egg. Independently, female SUL and female pre-lay weight were found to be positively correlated ($R^2$: 0.2992 and 0.2532 respectively) with egg clutch size. In captivity, clutches are far larger (2,570 eggs) than the literature records for in the wild (370 eggs). With the males, phenotypes did not correlate with either egg quantity or fertilization. Non-laying females were found to be significantly younger, shorter (SUL), and lighter-weight suggesting that captive breeding programs should wait until females are at least 4 years old before breeding. Qualitative observations suggest that the eggs may be light-sensitive with light protection positively correlated successful embryo development.

Keywords: reintroduction; fecundity; anuran; fitness; captivity; husbandry

INTRODUCTION
Due to a variety of causes, 32-56% of described amphibian species are in danger of extinction worldwide (IUCN Red List, 2008). The Panamanian Golden frog (*Atelopus zeteki*) is one such amphibian that was thrust into critical endangerment due to over-collection, habitat destruction and disease (Becker et. al, 2014). This small, vibrantly-colored frog was once abundant in the rainforest
and cloud forest streams of central Panama. Unfortunately, A. zeteki populations have declined so severely none have been seen since 2009, and is feared extinct in the wild (Becker et. al, 2014).

Currently, captive populations are maintained in a number of zoos and aquariums in the hopes of future reestablishment in its native habitat (Poole, 2006). In order to maximize the success of these efforts, predictive models (i.e. Vortex Population Viability Analysis software) are needed in order to analyze the feasibility of suggested reintroduction scenarios. The reliability and accuracy of population modeling is tied to the availability of key life history (i.e. reproductive biology) and ecological information on a species. Unfortunately, Panamanian golden frog reproduction was only minimally studied in situ before their decline. Karraker et al. determined the average in situ clutch size to be 370 eggs (Karraker et. al, 2006). However, this figure appears to be at the extreme low end of the species biotic potential, given that clutches from the breeding program at the Maryland Zoo in Baltimore (MZiB) generally contain thousands of eggs per clutch. Yet, the average percentage of fertilized eggs or factors affecting egg clutch size and quality are yet poorly understood for this species.

Studies of other anuran species (Anaxyrus fowleri, Bufo gargarizans, Duttaphrynus melanostictus, and Hyla labialis) have shown larger females produce larger and/or heavier clutches (Fan et. al, 2013; Green, 2015; Lüddecke, 2002). Interestingly, even though anurans exhibit indeterminate growth, age on its own does not correlate with clutch size in Anaxyrus fowleri, Rana dalmatina, or R. temporaria (Green, 2015; Weddeling 2005). Various other characteristics have shown varying results depending on species. For example, Bufo woodhousii exhibited a positive correlation between male size and fertilization efficiency while in Bufo gargarizans and Duttaphrynus melanostictus, male size showed no correlation with fertilization efficiency (Fan et. al, 2013; Sullivan, 1989). Since captive Panamanian golden frogs are unmistakably plumper than their wild counterparts, the results of this study will need to be extrapolated to estimate clutch size and
fertilization efficiency in reestablishment models. In collaboration with MZiB, this study will examine correlation between phenotypic expression and reproductive output (fitness).

METHODS

Ethical Treatment of Animals Statement

Frogs were treated according to the ethical standards of MZiB as certified by Institutional Animal Care and Use Committee (IACUC) and Species Survival Plan (SSP) approvals.

Study Species

In the wild, Panamanian golden frogs breed from November through January which is during the transition between rainy and dry season (Karraker et. al, 2006; Poole 2006). While males live in the streams year-round, the females typically only come to the streams to mate (Poole 2006). During amplexus the female searches for a suitable location to lay her white eggs. A string averaging 370 white eggs is laid underneath rocks or a similarly protected site (Karraker et. al, 2006; Poole 2006). This study examined the Sorá breeding population at MZiB.

Measurements for Population Analysis

Snout-urostyle length (SUL) of each frog was recorded to the nearest 0.01 mm using Vernier calipers (Fisherbrand™ Traceable™ Digital Calipers 14-648-17) and weights were measured to the nearest 0.1 gram using a digital balance (OHAUS Scout SPX 2201 or OHAUS Scout Pro SP2001). For each frog, ZIMS files were obtained to provide information such as age and medical records. Additionally, dorsal and ventral aspects of each frog were photographed using a Canon PowerShot, G15, 12MP digital camera to allow later confirmation of frog identities. Each frog was lightly held within a petri dish to provide immobilization during photography.
Pair Assignments

For this study, 17 pairs of *A. zeteki* from Sorá lineage were provided by the MZiB. Females were divided by SUL into three groups: small, medium, and large. Males were similarly divided by condition into three groups: poor, average, and excellent. Within each of these six groups, frogs were assigned a pair number by drawing slips of paper out of a bowl resulting in nine pairing categories (Small×Poor, Small×Average, Small×Excellent, Medium×Poor, etc). Unfortunately, to account for medical concerns and studbook pairings, the random pairings were drastically adjusted non-randomly.

Breeding

All of the frogs were cared for by zoo staff following standard MZiB husbandry practices with slight modifications (Poole, 2006). Following standard practice, gravidity and weight were recorded before placing pairs into the breeding tank and again after eggs were laid or the pair was removed from the breeding tank. However, each frog was weighed on six occasions to provide a more accurate averaged weight. Individual frogs were weighed six days before, three days before, and the day of transfer to the breeding tank. Each frog was weighed again the day of, three days after, and six days after egg laying or removal from the breeding tank. Only one pair at a time was placed in each breeding tank to avoid mixing the egg clutches. The date and time were recorded when each pair was first observed in amplexus, first observed laying, and first observed finished laying. General oviposition site observations were recorded as well as the breeding tank water pH. The method of separation from amplexus was recorded for each pair as naturally on their own, manually by keepers, or naturally following the death of the female. We hoped that pairs would lay eggs naturally within two weeks of amplexus; however, most of the pairs did not lay eggs within this timeframe. Pairs that did not lay naturally were treated with MZiB’s standard procedure of hormone injections to induce laying, but
even with hormone injections some frogs still did not lay eggs. If pairs did not lay eggs within one week of a second injection of hormones, they were removed from the breeding tank.

**Preservation and Staining**

Breeding tanks were observed once in the morning and once in the afternoon. After eggs were first noticed, the clutch was allowed to develop for five days. The clutch was removed to a vial, fixed in 10% buffered formalin solution, and labelled. After at least twenty-four hours in the formalin solution, the clutch was moved to 70% ethanol. Staining was employed to improve visibility of embryo features. The eggs were stained by soaking in methylene blue solution for at least fifteen minutes followed by rinses of distilled water and 70% ethanol. A fixative was not used so the methylene blue leached into solution over time. Generally, enough of the excess methylene blue was removed by replacing the 70% ethanol in the vial the day after staining.

**Clutch Examination**

Clutches were examined under a dissection microscope. Fiber optic lighting was utilized to display embryo features more clearly. Each clutch of eggs was examined within a petri dish of 70% ethanol. Each egg or embryo was recorded as one of four categories: undeveloped, some development, clearly developed, or damaged/unknown. Clearly developed embryos were those that were developed to the stage where the tail is clearly no longer attached to the yolk. *Atelopus zeteki* embryo stages do not clearly correspond with Gosner stages and there is currently no literature available on *Atelopus* staging, but embryos categorized as ‘clearly developed’ likely correspond to Gosner stage 20.
Preliminary Investigations

Initially MZiB provided 15 clutches of eggs preserved from 2013-2016 in 10% buffered formalin. These clutches of eggs had limited data about the sire and dam and included clutches from both the Sorá and the Mata Ahogado populations. These clutches were stained and examined as described above except only three categories of development were recorded: underdeveloped, clearly developed, and damaged/unknown. The category “underdeveloped” included any egg or embryo not developed to the stage of “clearly developed” as described above. Six of these clutches were fully examined and included in the calculation for average clutch size.

Analysis

Microsoft Excel 2013 was used for all numerical and graphical analyses. All best fit lines and $R^2$ values for graphical analyses assumed a linear relationship. Student t-tests were calculated with Excel’s “T.TEST” function after variances were compared from Excel’s “VAR.S” function. Comparisons to the wild Sorá population were made using field data previously collected by Erik Lindquist, Roberto Ibáñez, and Edgardo Griffith. The degree of light protection each clutch received was retrospectively determined on a five point scale from qualitative records of oviposition site. A ranking of “1” for light protection was given if the clutch was recorded as spread all over the tank and strewn throughout the plants while a ranking of “5” was given if the clutch was recorded as laid entirely or nearly entirely underneath the clay pot. Rankings of “2” through “4” were given to clutches with oviposition records falling between the descriptions for a “1” and “5”.

RESULTS

Female SUL and Female weight each positively correlated with clutch size with $R^2$ values of 0.2992 and 0.2532 respectively (Figure 1.). The phenotypes of the males did not correlate with clutch size or percent of clutch developed.
Clutch size in captive *A. zeteki* populations is extremely large compared to in the wild. The average clutch size from Sorá and Mata Ahogado frogs at MZiB is 2570 eggs (n=14, standard deviation = 1170) while in the wild, clutch size averaged 370 eggs (n=9, standard deviation = 137) (see Table 1.) (Karraker et. al, 2006).

Out of the 17 pairs of frogs, 8 pairs laid eggs; 6 pairs did not lay; and the females from the remaining 3 pairs died. The non-laying females were found to be significantly shorter by SUL (p=0.003868), lighter-weight (p=0.03771), and younger (p=0.01132). The females that did not lay eggs had an average age of 3.4 years while those that did lay eggs had an average age of 5.1 years (see Table 2.). As a part of one necropsy, the eggs within one of the females that died were counted and found to total 3,563 eggs.

Light protection appears to be positively correlated with successful embryo development (see Figure 2.). The percentage of developed embryos graphed linearly against the ranking of light protection produces an $R^2$ value of 0.76.

SUL and weight differ significantly between the wild Sorá population and the captive MZiB Sorá population (see Table 3.). The captive population is slightly shorter by SUL and much heavier. Sorá males in the wild had an average SUL of 41.4 mm and an average weight of 4.77 g while the captive Sorá population at MZiB has an average SUL of 39.8 mm and an average weight of 8.43 g. Females in the wild had an average SUL of 53.5 mm and an average weight of 9.83 g compared to the captive population which has an average SUL of 50.2 and an average weight of 15.56 g.

No clear difference was noticed between clutches laid naturally compared to clutches laid after hormone injection. Three clutches were laid naturally, and five clutches were laid after hormone injection. One frog laid only a tiny clutch of 41 eggs on land after hormone injection. Three of the females laid more eggs after removal from the breeding tank. One of them had laid naturally and the other two had received hormone injections.
None of the phenotypes observed in the parents appear to correlate with a higher percentage of “clearly developed” embryos at five days of development. While pH might affect development rates of embryos, most of the clutches were laid in water with a pH of 7.6. One clutch was laid in water with a pH of 7.4 and the water pH was not recorded for one of the other clutches. The water pH was 7.2 when the one pair laid their eggs on land. Water temperature during embryos development was not recorded.

**DISCUSSION**

Longer SUL females and heavier females were found to lay more eggs as expected with $R^2$ values of 0.2992 and 2532 respectively (see Figure 1.). The $R^2$ values were somewhat low, but size of the female is only one factor in the number of eggs laid. In addition, both $R^2$ values are lowered by the one unexpectedly large clutch of 3,727 eggs from a female measuring 53.4 mm and 19.7 g; a larger sample size would likely provide higher $R^2$ values by balancing out such outliers. Even so, the $R^2$ values are still high enough to support a positive correlation between clutch size and female size measured by either SUL or weight.

Clutch size in captivity is drastically different from that in the wild. In captivity, females lay an average of 2570 eggs per clutch (n=14, standard deviation = 1170) which is 595% larger than the average clutch size in the wild of 370 (n=9, standard deviation = 137) eggs per clutch recorded by Karraker et. al in 2006 (see Table 1.). It’s possible this extreme increase in clutch size is related to the increase in average weight of captive frogs compared to in the wild. Captive Sorá females are on average 58% heavier than their wild counterparts (see Table 3.). Captive females averaged 15.56 g while females in the wild weighed 9.83 g. Similarly, the males on average are likely obese at 77% heavier than their wild counterparts. Captive males averaged 8.43 g while males in the wild weighed 4.77 g. The health of the frogs might be improved by reducing the number of prey items offered or the frequency of feeding. For example, many of the females that die during breeding appear to likely
contain more than the average number of eggs when dissected for necropsy (pers. comm. Kevin Barrett). The increased clutch size, which may be a result of increased weight, might have at least partially led to the death of these females. Strangely, while the females that die during breeding are significantly heavier than their wild counterparts, necropsies have found few fat bodies within these females (pers. comm. Kevin Barrett and Ellen Bronson).

The 14 pairs of frogs that lived through the study exhibited significant differences between females that did lay eggs and those that did not lay eggs (see Table 2.). Length by SUL (p=0.003868), weight (p=0.03771), and age (p=0.01132) were found to significantly differ with the non-laying females being shorter by SUL, lighter-weight, and younger. The non-laying females were likely shorter by SUL and lighter-weight because they were only a few years old and had not had as much time to grow. The females that did not lay eggs had an average age of 3.4 years while those that did lay eggs had an average age of 5.1 years. Captive breeding programs could save time and effort by not selecting females that are too young. However, the average age at which females are likely too young to breed is probably lower than 3.4 years because the sample only included 6 non-laying females and one of them was clearly an outlier at 5.7 years old. The average age of the other 5 non-laying females was 2.9 years old. Females should probably only be considered for breeding if they are at least 4 years old.

Since *Atelopus zeteki* lays white eggs underneath rocks, it has been thought the eggs might be light sensitive. However, this hypothesis has never been tested. The results of this study provide preliminary support for the light sensitivity of *Atelopus zeteki* eggs. The eggs were ranked retrospectively for light protection based on observations of oviposition sites. Ranking of light protection was found to be strongly positively correlated with successful development of embryos with an $R^2$ value of 0.76 (see Figure 2.). However, a more careful study needs to be conducted. The study could be very simplistic. A set of clutches would be protected from light throughout
development and another set of clutches would be exposed to light throughout development with the
expectation that only the clutches protected from light would develop successfully.

The differences in clutches laid naturally compared to clutches laid after hormone injections
remains unclear. The sample size of this study was too small to determine any differences. One of the
females injected with hormones did lay her clutch of only 41 eggs on land. The extremely small size
of her clutch and the abnormal behavior of laying eggs on land might have been a result of the
hormones, but further studies would be necessary. Of the three frogs that laid additional eggs after
removal from the breeding tank, two of the females had been injected with hormones. Hormone
injections could cause females to only lay a portion of their eggs at first. Again though, further studies
are required to determine the effects hormone injections have on the induced egg clutches.

Clutches were not at the same developmental stages after five days of development. This is
not surprising though because temperature was not recorded could have easily differed between
clutches. The water pH was recorded and it remained constant between most of the clutches. Within
clutches, embryos were developed to very different stages as well. This can be explained satisfactorily
by the amount of time it takes between laying the first and last egg. To illustrate, one of the pairs was
found laying eggs at 9:15 AM, and the pair was still laying eggs later that day when the zoo staff left
the building at 3:45 PM. This means the first egg to be laid could begin developing more than 6 hours
before the last egg laid begins development.

CONCLUSIONS

1. In *Atelopus zeteki*, both female weight and SUL are positively correlated with clutch size.
2. Captive *Atelopus zeteki* lay far more eggs per clutch than their wild counterparts.
3. Captive breeding programs may be able to save time and effort by waiting to breed female
   *Atelopus zeteki* until they are 4 years old.
4. Successful development of *Atelopus zeteki* eggs may require protection from light, but further studies are required to confirm this finding.

5. In captivity, *Atelopus zeteki* have a shorter SUL and are far heavier. They may even be obese. Reduced feedings should be considered in captive breeding programs.

6. Embryos within *Atelopus zeteki* clutches exhibit a range of developmental stages because each clutch is laid over a period of many hours.

**ACKNOWLEDGEMENTS**

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http://www.iucnredlist.org/initiatives/amphibians/analysis


TABLE LEGENDS

Table 1. Comparison of Clutch Size in the wild VS Captivity.

Table footnote: Captive data includes clutches from the Sorá and Mata Ahogado populations and has a much higher average clutch size. *data on wild clutches from Karraker et. al, 2006.

Table 2. Factors Correlated with Females Laying Eggs

Table footnote: The average age, SUL, and weight of females that laid eggs (Laid) differed significantly from those that did not lay eggs (Did Not) as shown by the p-values in the t-test row.

Table 3. Differences in SUL and Weight Between the Wild and Captive Sorá Populations.

Table footnote: The captive population has a slightly shorter average SUL and a much higher average weight. Abbreviations—Males (M), Females (F).

Figure 1. Eggs Laid VS Female Size.

A. Eggs Laid VS Female SUL. All eggs laid in water were counted including eggs laid after removal from the breeding tank. B. Eggs Laid VS Female Weight. All eggs laid in water were counted including eggs laid after removal from the breeding tank.

Figure 2. Developmental Success VS Light Protection

The degree of light protection each clutch received was retrospectively determined on a five point scale from qualitative records of oviposition site. A ranking of “1” for light protection was given if the clutch was recorded as spread all over the tank and strewn throughout the plants while a ranking of “5” was given if the clutch was recorded as laid entirely or nearly entirely underneath the clay pot. Rankings of “2” through “4” were given to clutches with oviposition records falling between the descriptions for a “1” and “5”.

Table 1.

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<th>Average Clutch Size (eggs)</th>
<th>Standard Deviation</th>
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<tr>
<td>Wild (n=9)*</td>
<td>370</td>
<td>137</td>
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<tr>
<td>Captive (n=14)</td>
<td>2570</td>
<td>1107</td>
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Table 2.

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<th>Average SUL, mm (Variance)</th>
<th>Average Weight, g (Variance)</th>
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<td>Laid</td>
<td>62 (241)</td>
<td>52 (13.2)</td>
<td>20.6 (23.7)</td>
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<tr>
<td>Did Not</td>
<td>41 (194)</td>
<td>48 (1.94)</td>
<td>16.8 (4.74)</td>
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<td>T-Test</td>
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Table 3.

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</table>
Figure 1.
Christensen 17  Allometric Effects on Egg Clutches

Figure 2.

R² = 0.7559

% Developed

Protection from Light