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# **IMPACT OF REDUCED CALCIUM DURING DEVELOPMENT IN SNAKES**

Thesis submitted in partial fulfillment of Honors

By

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## Abstract

In squamate reptiles, the evolutionary transition from oviparity to viviparity is accompanied by loss of the calcareous outer eggshell, which suggests significant implications for the role of calcium during embryonic development (Packard et al., 1977). An experiment was designed to evaluate the impact of reduced calcium availability during development in the oviparous corn snake, *Pantherophis guttatus* (Stewart and Ecaj, 2013). Results from that study showed significant decreases in the mass and length of hatchling corn snakes when the outer calcareous eggshell layer was removed during development. In vertebrates, variation in total body length reflects skeletal differences---primarily differences in the number or sizes of vertebrae and/or differences in length of the skull. Skeletal components obviously are affected by the availability of calcium during development. My study was designed to determine the anatomical and developmental bases for the smaller size observed in hatchling snakes subjected to reduction of eggshell calcium during embryonic development.

My hypotheses were, as follows: 1) Differences in mass reflected differences in overall length of hatchlings; 2) Differences in length resulted from decreased size of vertebrae---rather than decreased number---and/or decreased length of skulls in hatchlings with reduced calcium. Hatchlings were prepared for skeletal analysis via clearing-and-staining. Vertebrae of each hatchling were counted and size measurements obtained for separate spinal regions (cervical, thoracic, and caudal). Results demonstrate a significant treatment effect on size of vertebrae in the thoracic and anterior caudal regions, as well as length of the skull. These findings suggest that reduced developmental calcium, comparable to the condition in viviparous species, impacts ossification and growth of skeletal elements in late development.

## Introduction

In 1977, Packard et al. proposed that the evolutionary transition from oviparity to viviparity in squamate reptiles had significant implications for the role of calcium. Studies have shown that 14—36% of embryonic calcium is mobilized from the calcareous eggshell during development in oviparous snakes (Stewart and Ecaj, 2010). However, viviparous squamates greatly reduce or eliminate the calcareous eggshell layer, which allows for maternal/embryonic exchange of organic and inorganic nutrients. As a consequence, data for viviparous squamate embryos show that no calcium is mobilized from the eggshell during development (Stewart and Ecaj, 2010).

If embryos are dependent on eggshell calcium, the loss of this calcium source may affect development in viviparous species. To test the potential impact of reduced calcium during development, experiments were designed to reduce the availability of embryonic eggshell calcium in an oviparous reptile, the corn snake *Pantherophis guttatus*. The results of these experiments showed decreases in the mass and length of hatchling corn snakes when the outer calcareous eggshell layer was removed during development (Stewart and Ecaj, 2013). The study reported here was designed to investigate the morphological basis for the decreased size in hatchlings with reduced developmental calcium.

Vertebrate species vary in length, due primarily to morphological differences in the axial skeleton and, in particular, to differences in the vertebral column (i.e., both the number and size of vertebrae). Among vertebrates, there is a large range of variation in vertebral numbers; e.g., frogs have 6-10 vertebrae, and snakes may have

over 300 (Gomez et al., 2008). Within snakes, differences in length found among related species primarily reflects differences in number of vertebrae. However, adults of some snake species may differ in number of vertebrae across populations or even between genders (Shine, 2000). Variation in vertebral number may also reflect environmental conditions; e.g., within some fish species, vertebral number is affected by differences in water temperature during development (Fowler, 1970). In general, increased number of vertebrae is highly correlated with increased body length in both snakes and fishes---a correlation referred as pleomerism (Lindell, 1994; Gomez, 2008). However, in snakes, for any individual undergoing growth (post-hatching), increased length of the vertebral column results from growth and not from addition of new vertebrae (Polly et al., 2001).

Understanding the processes by which vertebrae are formed is essential to determine how reduction in developmental calcium could cause either variation in vertebral size or number. The first major developmental process involved in the production of vertebrae is somitogenesis. This process establishes the initial somites or body segmentation and directly reflects the number of vertebrae formed during development (Gomez et al., 2008; Gomez and Pourquie, 2009; Polly et al., 2001).

After somites have been formed, further tissue differentiation ensues, chondrification begins, and ossification completes the formation of the final vertebral structure. Chondrification first converts the mesenchymal somites into cartilaginous blocks that are immediate precursors to the ossified vertebrae.

Ossification breaks down and replaces the cartilaginous precursor with the hydroxyapatite matrix of bone (Reipel, 1994).

Therefore, if hatchlings with reduced developmental calcium were found to have fewer vertebrae, one could suggest that reduction in developmental calcium impacted the process of somitogenesis and segmentation. However, a study defining the developmental timing of calcium mobilization for corn snake embryos found that greater amounts of calcium are pulled from the eggshell later in development (Stewart et. al., 2004). The timing of somitogenesis in snakes has not been reported by age or description of developmental stage. I made a comparison of two studies on snakes, one describing developmental stages (Zehr, 1962) and a second describing somitogenesis (Gomez, 2007). Based on these studies, I concluded that the process of somitogenesis begins prior to Stage 16 and continues until at least Stage 27. Oviposition of eggs in *Pantherophis guttatus* occurs when embryos are typically Stage 27--29 (Zehr, 1962). When the timing of somitogenesis is compared to the time at which eggs were peeled (Stage 28), I hypothesized that reduction in developmental calcium by peeling should have no impact on the number of vertebrae.

A previous study based on specimens from a similar experiment with corn snakes showed that reduction in developmental calcium had no impact on the pattern or timing of ossification by late development (i.e., Stage 36; Trotter-Ross et al., 2010). It was found that not only the vertebrae, but also skull bones, commenced and completed ossification at the same rate in both control and experimental embryos (Trotter-Ross et al., 2010). However, this study was

concerned with determining the developmental stage at which bones began ossification and the pace of that ossification. The study was confined to embryonic development of vertebrae and skull bones, and did not assess differences in numbers or sizes of vertebrae. Therefore, it is entirely plausible that differences in number of vertebrae, or even continued vertebral growth after initial ossification, could account for possible variation in the mass and lengths of treatment and control hatchlings.

The amount of calcium that squamate embryos recover from the yolk compared to the eggshell is generally correlated with the relative abundance of calcium in each (Stewart and Ecaj, 2010). For corn snakes, the majority of calcium the embryo takes up during development (72%) will come from the egg yolk. Thus, corn snake embryos rely on the eggshell for roughly 28% of overall developmental calcium (Stewart and Ecaj, 2010). Removal of the calcareous layer of eggshell significantly reduced the overall calcium available during embryonic development, which resulted in decreased hatchling length and mass in these experiments. However, the timing of the removal of the eggshell calcium is critical to understanding the developmental basis of reduced size in hatchlings.

In the previous experiments, the calcareous layer of eggshell was removed shortly after oviposition, and embryos continued development with only calcium from yolk available for uptake. The timing of eggshell manipulation is significant for two reasons. The first is that, as previously mentioned, the process of somitogenesis is mostly complete prior to oviposition. Because somitogenesis determines vertebral number, it is unlikely that reduced calcium availability after somitogenesis

would impact number of vertebrae. Secondly, previous studies have shown that the majority of calcium mobilized from eggshell occurs later in development (Stage 34--Hatch; Stewart et. al., 2004). Chondrification and ossification are developmental processes that occur after oviposition and thus, are candidates for disruption of normal development by reduced calcium.

The study described here was designed to test the hypothesis that the decreased size of hatchlings subjected to reduced calcium availability during development resulted from formation of smaller vertebrae and skulls—as opposed to fewer number of vertebrae. To test this idea, data on both sizes and numbers of skeletal elements were obtained.

### **Methods & Materials**

Forty-five hatchling corn snakes obtained from a colony in ETSU's animal care facility were examined (JR Stewart, Protocol #P100201, University Committee on Animal Care). Because maternal factors impact hatchling size, peeled and intact groups included representative sibling hatchlings from each of fifteen different mothers. Twenty-two hatchlings developed from eggs whose outer calcium-rich layer was manually peeled away (treatment group), while twenty-three hatchlings developed from intact eggs (control group). Eggs were peeled by wetting the outer calcareous eggshell layer and gently



**Figure 1: Manual removal of outer calcareous eggshell layer.**

removing with forceps (as shown in Figure 1). Peeling occurred between a range of



embryonic stages (24-31) with average being stage 28. Embryonic stages were determined by sacrificing an embryo from each clutch at the time of peeling and matching its morphological features to a staging chart (Zher, 1962). Initial egg mass was recorded at oviposition; hatchling mass, snout-vent length (SVL), and tail length (Tail) were recorded at hatching (Table 1). SVL and Tail measurements were summed to obtain the total length of each specimen (Total). Skull length also was measured, but from cleared and stained specimens using the same method for vertebral measurements (described below).

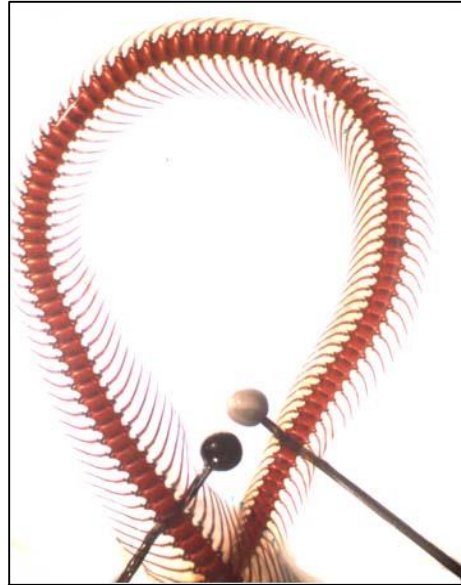
**Table 1: Means and ranges for initial mass and length data in intact vs. peeled hatchling specimens of corn snakes used in this study.**

<b>Treat-ment</b>	<b>N</b>	<b>SVL (mm)</b>	<b>Tail (mm)</b>	<b>Total (mm)</b>	<b>Skull (mm)</b>	<b>Mass (g)</b>	<b>Initial Egg Mass (g)</b>
Intact	23	244.4 193--287	48.4 38--54	292.8 231--334	12.20 10.67--13.49	7.34 3.8--10.5	9.69 6.28--13.58
Peeled	22	229.6 203--274	45.6 42--55	275.3 245--324	12.06 11.01--12.82	6.3 4.7--10.2	9.20 6.63--11.9

Hatchlings were prepared using a clearing and staining protocol (Hanken and Wassersug, 1981). Variations to protocol are detailed in Appendix (See Appendix I: Modified Clearing and Staining Protocol). Data were recorded for size and number of vertebrae present in each hatchling. For accurate vertebral counts, photographs were taken of each cleared and stained specimen using a Leica DFC 420 camera attached to a Wild M3Z Type-S dissecting scope. Dissecting pins were placed between intercostal spaces to serve as landmarks among the multiple photos for each specimen. Most specimens required four to five separate photographs to

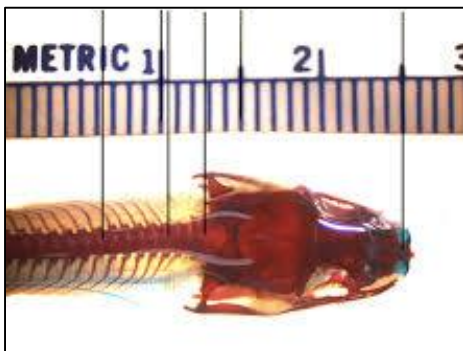
capture images of all vertebrae. An example of one of the photographs used for counting vertebrae can be seen in Figure 2.

Magnifications for each photo varied to capture a clearer image, especially for the smaller sized caudal vertebrae. Vertebral counts were made directly from grayscale printouts of these photos. Counts for cervical, thoracic, and caudal regions were noted to compare regional differences.



**Figure 2: Example of photograph used to count vertebrae. Dissecting pins placed as landmarks between photos.**

Photographs used for size measurements were obtained separately but with the same equipment. Each size photograph included a ruler in the field of view to provide measurement scale. Measurements were made of the skull; the three



**Figure 3: Example of photograph used to measure vertebral regions. Lines drawn indicate where measurements were taken. Ruler in background is present for scale.**

cervical; first five and last five thoracic; and the first five and last five caudal vertebrae.

Measurements were chosen to account for variation resulting from anterior to posterior development in snakes. Measurements were obtained using Adobe Premier Photoshop 12 to create a pixel-to-millimeter ratio with the ruler in each photo for scale. This ratio was then used

to compute actual size (mm) of vertebrae based on how many pixels were between the distances marked on the skeletal structures. Data were recorded for both the

number of pixels for each vertebral and skull measurement and for the pixel:millimeter ratio. An example of the photographs used to collect measurement data can be seen in Figure 3.

Count and measurement data were analyzed using a general linear model procedure (IBM SPSS, GLM Univariate). Analyses were run with Maternal ID as a random effect to check for possible maternal influences on hatchling size and treatment effect. Correlations of mass and body length data were analyzed as Pearson Correlation (IBM SPSS).

## **Results**

### Hatchling Size

Analyses of initial mass and length data confirm previous findings (Stewart and Eca, 2013) for impact of reduced developmental calcium (Table 2). Both treatment and maternal source data shows significant impact on snout-vent length and total length of hatchlings.

Initial egg and hatchling mass data were analyzed (Table 3). As expected, treatment had no effect on initial egg mass measured at oviposition. However, there was a strong maternal effect on initial egg mass. Hatchling mass shows a significant treatment and maternal effect, as well as an extremely high (92.9%) correlation with total hatchling length.

**Table 2: Comparison of hatchling size between control (N=23) and treatment (N=22) groups based on our samples from 15 females.**

	Source	Sum of Squares (Type III)	df	Mean Square	F	P
Snout-Vent Length (mm)	Treatment	1783.220	1	1783.220	12.039	<b>.003</b>
	Maternal ID	11263.823	14	804.559	5.388	<b>.002</b>
Tail Length (mm)	Treatment	56.235	1	56.235	4.444	.051
	Maternal ID	215.427	14	15.388	1.211	.365
Total Length (mm)	Treatment	2472.793	1	2472.793	11.802	<b>.003</b>
	Maternal ID	13701.357	14	978.668	4.664	<b>.004</b>

**Table 3: Comparison of egg and hatchling mass between control (N=23) and treatment (N=22) groups based on our samples from 15 females.**

	Source	Sum of Squares (Type III)	df	Mean Square	F	P
Initial Egg Mass (g)	Treatment	1.217	1	1.217	1.667	.210
	Maternal ID	87.177	14	6.227	9.805	<b>.000</b>
Hatchling Mass (g)	Treatment	9.449	1	9.449	10.773	<b>.004</b>
	Maternal ID	55.649	14	3.975	4.682	<b>.004</b>

Skull and vertebral sizes were obtained from prepared cleared and stained specimens. Skull length showed significant treatment and maternal effects (Table 4).

**Table 4: Comparison of skull size between control (N=23) and treatment (N=22) groups based on our samples from 15 females.**

	Source	Sum of Squares (Type III)	df	Mean Square	F	P
Skull Length (mm)	Treatment	.711	1	.711	5.890	<b>.027</b>
	Maternal ID	10.541	14	.753	6.338	<b>.001</b>

#### Vertebral Number

Vertebral numbers of each region (cervical, thoracic, or caudal; Table 5) were compared separately. Absolute number of cervical vertebrae did not differ between intact and peeled hatchlings. Slight differences in absolute counts for numbers of thoracic, caudal, and total vertebrae were found, but values were not significantly different between treatments.

**Table 5: Means and ranges for vertebral number in control (N=23) and treatment (N=22) groups based on our samples from 15 females.**

Treatment	Cervical #	Thoracic #	Caudal #	Total #
<b>Intact</b>	3	224.6 210--243	71.5* 54--78	299.1* 285--317
<b>Peeled</b>	3	223.5 213--256	68.8 55--78	295.0 279--329

\*N =21 for intact Caudal and Total numbers, owing to damage to tips of tails (n=3).

Numbers of vertebrae were not significantly different between control and treatment groups for either total or regional counts. Numbers of cervical vertebrae did not differ; means of total, thoracic and caudal vertebrae did not differ significantly between groups (Total:  $P=0.054$ ; Thoracic:  $P=0.648$ ; Caudal:  $P=0.150$ ).

### Vertebral Size

Means and ranges for vertebral size data are shown in Table 6. Except for last five caudal, means for vertebral size were larger in intact (control) hatchlings.

**Table 6: Means and ranges for vertebral size (mm) by region in control (N=23) and experimental (N=22) groups based on our samples from 15 females.**

Treatment	Cervical (mm)	1st Five Thoracic (mm)	Last 5 Thoracic (mm)	1st 5 Caudal (mm)	Last 5 Caudal (mm)
Intact	2.450 1.923-2.880	4.027 3.404-4.946	3.953 3.121-4.615	3.762 3.121-4.423	1.928* 0.938-3.217
Peeled	2.364 2.0-2.857	3.857 3.35-4.516	3.806 3.491-4.24	3.631 3.226-3.96	2.056 0.838-3.182

\*N =21 for intact Caudal number, owing to damage to tips of tails.

When analyzed, three vertebral regions (1st-5th Thoracic; Last 5 Thoracic; 1st-5th Caudal; Table 7) showed size differences between control and treatment groups due to a treatment effect. Only one region (1st-5th Thoracic) showed any maternal impact on vertebral size.

**Table 7: Comparison of vertebral size between control (N=23) and experimental (N=22) groups based on our samples from 15 females.**

	Source	Sum of Squares (Type III)	df	Mean Square	F	P
Cervical (mm)	Treatment	.053	1	.053	1.166	.296
	Maternal ID	1.182	14	.084	1.829	.139
1st-5th Thoracic (mm)	Treatment	.334	1	.334	5.305	<b>.034</b>
	Maternal ID	2.430	14	.174	2.848	<b>.031</b>
Last 5 Thoracic (mm)	Treatment	.443	1	.443	6.895	<b>.017</b>
	Maternal ID	1.984	14	.142	2.269	.070
1st-5th Caudal (mm)	Treatment	.365	1	.365	5.394	<b>.035</b>
	Maternal ID	1.815	14	.130	1.811	.144
*Last 5 Caudal (mm)	Treatment	558.396	1	558.396	4.335	.054
	Maternal ID	2319.936	14	165.710	1.255	.341

\*N=43 for Last 5 Caudal measurement, owing to damage to tips of tails.

### Maternal Effect

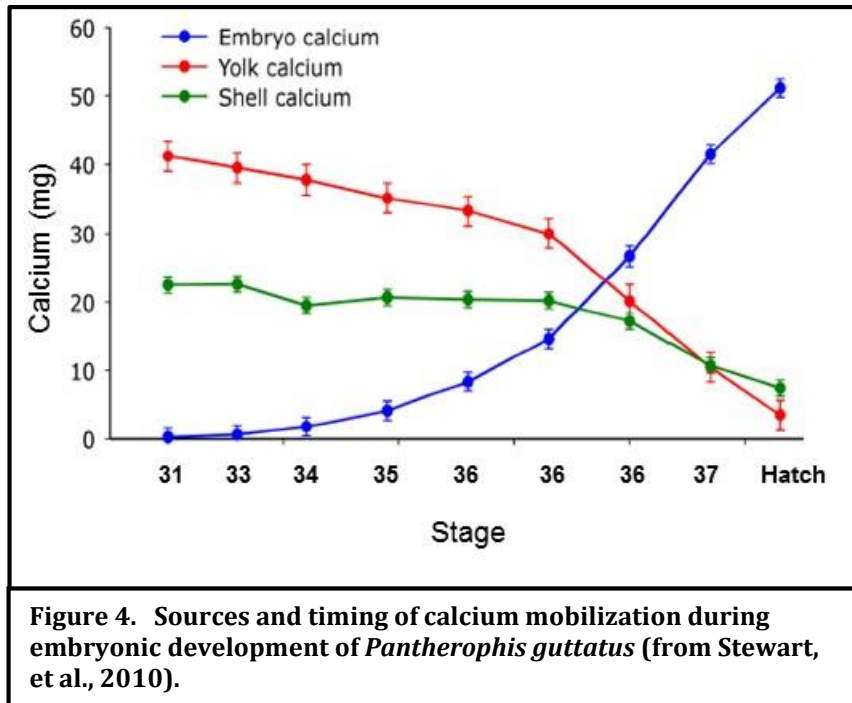
Maternal ID was used to identify all offspring that originated from each female, regardless of date of birth. All analyses tested for effects from Treatment, from Maternal ID, and any interaction between those factors. The results showed no significant interaction between Maternal ID and treatment for any measurements of vertebral number or size. However, maternal effect on offspring had a significant independent impact on vertebral sizes in two regions (Skull, 1<sup>st</sup>-5<sup>th</sup> Thoracic, and; Tables 4, 7). No maternal effect on vertebral number was found.

## Discussion

Results from this study confirm findings of previous studies (Stewart and Ecay, 2013) that reduction in developmental calcium causes a decrease in overall hatchling size (Tables 2, 3), as reflected in both mass and length of hatchling corn snakes. Length differences in elongated vertebrates, such as snakes, may result from differences in either vertebral number or size (and/or skull length) or some combination of these factors. To impact vertebral number, calcium reduction by eggshell peeling would have to affect the process of somitogenesis; conversely, a reduction of vertebral size would result from an impact on the chondrification and/or ossification processes, both of which occur later in development.

Vertebral number did not differ between peeled and intact eggs. This finding fits in with the currently understood model of vertebral development in snakes. Because somitogenesis determines the number of vertebrae formed, an impact on somitogenesis would be necessary to decrease vertebral number. Since corn snake embryos do not begin to mobilize significant amounts of calcium from eggshell until after somitogenesis is complete (Stage 34 or beyond; see Fig. 4, Stewart and Ecay, 2010), removal of eggshell calcium should not affect the process of somitogenesis. In the experiments described here, the calcareous eggshell was removed from eggs of embryos in developmental stages 27-31, whereas the process of somitogenesis is almost entirely completed by stage 27 (Zehr 1962).





However, reduced developmental calcium did impact sizes of vertebrae in three regions (1st-5th Thoracic; Last 5 Thoracic; 1st-5th Caudal; Table 7). Vertebral size was significantly decreased by peeling (Table 7). Interestingly, the length of the skull was also significantly decreased by reducing eggshell calcium (Table 4). Again, these findings fit very well with the current understanding of skeletal development and calcium mobilization in corn snakes (Stewart and Ecaj 2010). Because the processes of chondrification and ossification occur after oviposition, they would coincide with the timing of the majority of calcium uptake by the developing embryo. A significant increase in mobilization of calcium begins around stage 34 and continues until hatching (Fig. 4, Stewart and Ecaj, 2010). Knowing this, it is more likely that reducing developmental calcium at oviposition would impact growth processes occurring later in development. Therefore, when considering

smaller vertebrae along with developmental timing of calcium uptake by the embryo, the most likely cause of the decrease in hatchling length due to reduction of developmental calcium is an impact on chondrification, ossification, or both.

A previous study investigated the pattern of skeletal ossification in corn snake embryos whose developmental calcium was reduced (same treatment regime as this study). Trotter-Ross et al. (2010) found that reducing the amount of developmental calcium had no impact on the timing (beginning of ossification) or patterns (progress) of ossification in hatchlings from eggs with reduced calcium (Trotter-Ross et al., 2010). Those findings, along with the results of this study, strongly suggest that the differences in size of hatchling cornsnakes arises from continued growth of vertebrae (i.e., after initial full ossification) enabled by a full complement of developmental calcium. Cornsnake embryos that develop with decreased amounts of calcium available for uptake are not able to increase their vertebral size, nor add growth to skull elements, late in development. Conversely, embryos with access to the normal amounts of calcium from the eggshell are able to continue to grow and enlarge skeletal elements after initial development and ossification. It appears that reduction of developmental calcium decreases the ability of embryos to continue to grow their skeleton late in development.

Our experimental design showed that the treatment of reducing eggshell calcium was significantly associated with reduced hatchling size as compared to our controls. But our experimental design using siblings from the same females also allowed us to test for maternal effects from the same experiments. Results showed (Tables 3, 4, 7) that significant differences in sizes of some thoracic vertebrae

showed a significant, independent (i.e., no interaction) maternal effect. Maternal nutrient provisioning also plays a role in the impact on skeletal size and growth. Maternal source (i.e., same mother) did not impact the numbers of vertebrae in hatchlings, regardless of treatment. These results parallel our findings for direct effect of treatments on size of skeletal elements.

The underlying genetic and/or metabolic causes for this maternal impact on overall hatchling size and on skeletal size, in particular, is not fully understood. However, in oviparous squamates such as the corn snake used in this study, all calcium required for development is packaged into the yolk and calcareous eggshell prior to oviposition (Stewart and Ecaj, 2010). Therefore, the maternal impact on hatchling and skeletal size may be attributed to the fact that some mothers packaged more calcium (and possibly other nutrients) into the eggs prior to oviposition than others. Thus, hatchlings in both control and treatment groups had access to differing amounts of calcium owing to maternal provisioning prior to oviposition. This possible explanation is consistent with our findings of significant but independent effects of treatment and of maternal source on hatchling size.

Future studies that could strengthen the findings of this experiment could include allowing hatchlings to grow to mature adults. If the impact of reduction in developmental calcium is just a retardation of late embryonic growth, then the difference in length could potentially be overcome post-hatching by growth during juvenile ontogeny. Another potential avenue of investigation would be to investigate developmental series of corn snakes in greater detail to determine the time/stage at which vertebral growth is slowed or reduced when eggshell calcium is

eliminated. If this time point could be determined, it would further strengthen the conclusion that reduction in developmental calcium impacts continued ossification or growth in late development.

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## **Appendix: Modified Clearing and Staining Protocol**

This clearing and staining technique is a modified version of the one outlined by Hanken and Wassersug (1981). Due to the subjective nature of clearing and staining, this protocol was developed through experimentation with practice specimens of the same size, age, and species as the hatchling specimens used in the actual study to obtain optimum results. Formulae for stains and enzyme solutions follow this stepwise description.

1. Specimens were stored in 10% neutral-buffered formalin from hatch date until they were subjected to the clearing and staining process.
2. Specimens were skinned, and eyes and viscera were removed.
3. Specimens were washed in three separate changes of distilled water over the course of two days. The specimens were left in the first distilled water change for 2-4 hours, and for 12-24 hours in the second and third distilled water changes. The volume of distilled water used was the maximum amount the specimen jars would hold (approximately 500mL) in order to most efficiently and thoroughly flush the 10% formalin out of the specimens' tissues.
4. Specimens were placed in Alcian Blue stain (collagen/cartilage) for approximately 24 hours. Leaving specimens in Alcian Blue stain for any length of time over 36 hours made clearing the stain in later steps much more difficult and time consuming, so great care was taken to ensure each specimen stayed in Alcian Blue for the minimum time required to achieve sufficient cartilage staining.

5. Specimens were placed in a quick-change (2-4 hours) solution of absolute ethanol prior to being placed in two more baths of absolute ethanol for a minimum of 24 hours each. This step facilitates de-staining of the Alcian Blue.
6. Specimens were transferred through a series of decreasing concentrations of ethanol solutions (75%, 50%, and 25%) with each step lasting at least two hours. After the 25% solution, the specimens were transferred through two more changes of distilled water (at least 2 hours for each change).
7. Specimens were placed in a Trypsin solution until soft tissue had degraded to leave little to no stain visible. Another indication of readiness is a specimen that is easily pliable when gently manipulated with forceps, which indicates a complete breakdown of muscle fibers. Muscle striation should no longer be visible when viewed under a dissecting scope. Timing of this step was very unpredictable, but depending on concentration of trypsin used, specimens were ready around the 4-day mark. During practice runs, 1.0 g Trypsin/100 ml solution was used; however, this concentration proved ineffective in degrading soft tissue sufficiently (or quickly). For specimens used to collect data, 1.5-2.0 g Trypsin/100 ml of solution was used. If specimens were left in the Trypsin solution too long, connective tissues would completely degrade, skeletons disintegrate and become wholly unusable.
8. Specimens were transferred to Alizarin Red Stain for a minimum of 24 hours. Special care was taken to remove them fairly close to the exact 24-hour mark because prolonged exposure to the stain led to difficulties sufficiently clearing enough of the stain for optimum vertebral visibility.



9. Specimens were removed from Alizarin Red Stain and put through a graded series of Glycerin/KOH solutions (25%, 50%, 75% glycerin,) for 24 hours each, ending with the specimens being placed in 100% Glycerin for storage. If specimens were particularly dark coming out of the Alizarin Red Stain, an extra step of placing them into 100% KOH for 24 hours prior to the 25% glycerin/KOH helped remove more of the stain. Another technique used was to add 3 drops of 3% hydrogen peroxide to the 25% Glycerin/KOH solution. The peroxide also aids in bleaching some of the stain. Placing the specimen jars on a windowsill in direct sunlight while in the 25% plus 3% hydrogen peroxide solution enhanced this bleaching effect. A disadvantage of using hydrogen peroxide, however, was the formation of bubbles in the specimens, which could make clear observation and photography more challenging. The formation of bubbles was combatted somewhat effectively by using a vacuum oven to pull the bubbles out, but the glycerin in the solutions made this difficult. For these reasons, clearing without the aid of hydrogen peroxide was preferred in lieu of reliance on good timing in previous steps involving staining and tissue digestions.
10. After 24 hours in the original 100% glycerin solution, specimens were transferred to fresh glycerin in order to ensure all KOH had been removed from the solution. Long-term storage in KOH will cause specimens to degrade and deteriorate. A few crystals of phenol or thymol could be added to specimens in 100% Glycerin solution to prevent spoilage during long-term storage.

## STAIN & ENZYME FORMULAE

### 10% Neutral-Buffered Formalin:

- 1000 mL 10% Formalin (37% Formaldehyde)
- 4.0 g Sodium Acid Phosphate ( $\text{Na}_2\text{H}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ )
- 6.5 g Anhydrous Disodium Phosphate ( $\text{Na}_2\text{HPO}_4$ )

### Alcian Blue Stain:

- 10 mg Alcian Blue 8GX
- 70 mL absolute ethanol
- 30 mL glacial acetic acid

### Stock Saturate Aqueous Sodium Borate:

- Add sodium borate to distilled water until precipitate forms and does not dissolve

### Trypsin:

- 1.5-2.0 g trypsin
- 30 mL saturated sodium borate
- 70 mL distilled water

### Stock 0.5% KOH Solution:

- 0.5 g Potassium Hydroxide (KOH)
- 100 mL distilled water

### Alizarin Red Stain:

- 0.1 g Alizarin Red S
- 100 mL of 0.5% KOH solution

### Glycerin/KOH Solutions:

- 25%: 25 mL glycerin/ 75 mL 0.5% KOH
- 50%: 50 mL glycerin/ 50 mL 0.5% KOH
- 75%: 75 mL glycerin/ 25 mL 0.5% KOH
- 100%: 100 mL glycerin

\*\*Optional bleaching in 25% solution: Add 2-3 drops of 3% hydrogen peroxide to the 25% Glycerin/KOH Solution

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