8-2021

The Influence of Incubation Conditions on Oxygen Consumption During the Development of Pantherophis guttatus

Celeste Gallardo
East Tennessee State University

The Influence of Incubation Conditions on Oxygen Consumption During the Development of *Pantherophis guttatus*

A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Biology

by

Celeste R. Gallardo

August 2021

Dr. Joseph Bidwell, Chair

Dr. James Stewart

Dr. Thomas Ecay

Keywords: *Pantherophis guttatus*; embryonic metabolism; calcium; oxygen consumption; shell

ABSTRACT

The Influence of Incubation Conditions on Oxygen Consumption During the Development of *Pantherophis guttatus*

by

Celeste R. Gallardo

The flexible shell of some oviparous reptiles has led to differences in nutrient mobilization and water relations when compared to their rigid-shelled counterparts. Flexible-shelled eggs gain more water during development, and because of the shell structure, yolk calcium content is higher than that of eggshells. When water availability is altered for reptiles with flexible-shelled eggs, differences in both energy and nutrient utilization are observed. This study was designed to determine the baseline metabolic rate during development for the snake, *Pantherophis guttatus* under normal conditions, and to observe how changes in eggshell calcium and water availability impact embryonic oxygen consumption. Eggshell calcium was decreased while water uptake was increased by removing the outer calcareous eggshell layer from some eggs. When eggs are left intact, an exponential increase in oxygen consumption is observed, supporting previous studies, while removal of the eggshell produces no effect on metabolism.

DEDICATION

To my late Father, Terry Gallardo, wonderful Mother, Janet Gallardo, and beloved friend, Travis.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Gregory Bishop from the Department of Chemistry for aiding me with my bomb calorimetry experiments and providing me access to the chemistry laboratory. Furthermore, I would like to thank Dr. Daniel Connors for his early contributions to my respirometry chamber design. I am also thankful for my laboratory colleagues who encouraged through every step of my project. Lastly, I gratefully acknowledge my advisors and my committee members, Dr. Joseph Bidwell, Dr. James Stewart, and Dr. Thomas Ecay for providing me with continual support, guidance, encouragement, and patience that enabled me to complete this project. I am thankful that I was able to work with each of you, and I view you as mentors who helped me academically, personally, and professionally. Thank you for teaching me how to perform good research, this would not have been possible without you.

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION

Eggshell Structure & Calcium in Oviparous Reptiles

The eggshell is a multifunctional structure shared amongst oviparous reptiles. It allows for organisms to be adapted for terrestrial life by providing a protective, nutrient rich habitat for development (Packard and Packard 1984). One of the primary nutrients that can be mobilized from the eggshell is the inorganic compound, calcium. Calcium is utilized for successful hatchling growth by supporting proper skeletal ossification during embryogenesis (Packard and Packard 1984). This nutrient is deposited in the yolk and eggshell; but the quantity stored, and the mobilization method of this element varies amongst the reptile groups. Calcium storage and movement during incubation corresponds with the phylogeny. Birds and crocodiles rely on calcium stored in the eggshell the heaviest, with over 60% of calcium originating from the eggshell in crocodiles, and 72-92% total calcium from the shell within bird species (Packard 1994). Oviparous turtles and lizards differ, exhibiting a split storage of calcium; partitioned somewhat equally amongst the yolk and the eggshell (Packard and Packard 1984; Packard 1994). Snapping turtles, *Chelydra serpentina,* obtain 56% of their calcium from the eggshell, and 44% from the yolk, and because of this, successful development would not occur without the presence of the shell (Packard et. al 1984). The lizard species, *Eumeces fasciatus*, allocates its calcium sources differently, with 39% originating from the eggshell, and 61% from the yolk (Shadrix et al. 1994). Snakes are unusual, obtaining much of their calcium during development from the yolk (Packard 1994). Over half of the calcium content in snake hatchlings has been reported to originate from the yolk, and successful development is possible without utilization of eggshell calcium (Stewart and Ecay 2010; Stewart et. al 2019).

The deposition of calcium differs markedly across reptile groups, with birds and crocodiles displaying the most diverse patterns. Both groups deposit calcium from the shell into the yolk during embryogenesis; this is not exhibited by turtles, lizards, or snakes (Packard 1994). Embryos of developing birds and crocodiles rely on the yolk to supply calcium for the first half of incubation, and once the growth phase is reached, the rapid depletion of calcium from the eggshell begins (Packard 1994). Both birds and crocodiles contain calcium within their residual yolks, but differences exist between bird species based on the developmental mode exhibited (Packard 1994). Altricial birds deposit more calcium in their yolks, when compared to precocial species, allowing for the continued growth of altricial young after hatching (Packard and Packard 1991). Turtles and lizards show little changes in calcium content during the earlier stages of development, once embryos begin growing, calcium stores within the yolk decrease while quantities within the embryo increase (Packard 1994; Shadrix et al. 1994). For the last ~30% of incubation, much of the calcium deposited into the embryo originates from the eggshell (Packard 1994). Snake embryos mobilize most of their calcium from the yolk steadily throughout development, but when growth increases, mobilization of the calcium increases (Jenkins and Simkiss 1968; Packard 1994; Stewart et al 2019). Eggshell calcium of snakes remains as a secondary source of the nutrient, and its extraction from this compartment does not occur until the later stages of development (Stewart et al. 2019). Snake embryos deplete the yolk of almost all its calcium, indicating that residual yolk cannot be used to support skeletogenesis or growth after hatching, which differs markedly from birds and crocodiles (Packard 1994). Instead, the residual yolk can only be used for maintenance of tissues, and calcium will need to be retrieved from the diet for growth to continue (Packard 1994).

Patterns of calcium mobilization and deposition within the Reptilia may have a connection to the evolution of viviparity. Packard et al. (1977) hypothesized that the dependence on eggshell calcium observed within birds, crocodiles, and some turtles, creates a restriction on this evolution. Use of yolk as the primary source for calcium could be an important precursor because embryos would not be dependent on an external shell. The dependence on yolk calcium as well as the evolution of viviparity numerous times within Squamata supports this hypothesis (Packard et al. 1977). However, in the lizard, *Lacerta vivipara*, where populations of oviparous and viviparous individuals exist, eggs of the oviparous populations contain calcium rich eggshells but calcium poor yolk (Stewart et al. 2009). This implies that the evolution of viviparity is not necessarily constrained by the dependance of yolk-calcium.

Water Relations & Interactions with Eggs

The eggshell can be rigid or parchment-like depending on the organism. This distinction can alter interactions with water. A shell with a hard-exterior leads to low water permeability, parchment-shelled eggs, on the other hand, are flexible and have more room to accommodate water uptake (Packard 1991). These differences observed in water uptake and loss are partially determined by the water vapor transport coefficient, k_v (Oftedal 2002). The value, *kv*, can be used to calculate how much water will be lost by an egg in certain environmental conditions (Ackerman 1991; Oftedal 2002). Parchment-shelled eggs have a high k_v, and due to this, any small changes in water availability, humidity, or temperature can cause the egg to lose water to its surrounding environment, which can lead to a decrease in overall egg mass (Morris et. al. 1983; Packard et al 1987; Oftedal 2002). In contrast, rigid-shelled eggs have considerably lower k_v values, indicating environmental conditions that impact water availability have less of an effect of the eggs existing water content (Ackerman 1991; Oftedal 2002).

Water is necessary for the successful development of embryos from parchment-shelled eggs, as it aids with respiration and nutrient utilization. Embryos incubated on wet substrates grow faster, incubate longer, metabolize yolk quicker, and hatchlings are larger. (Morris et al. 1983). An increase in water uptake also correlates with heightened metabolism during embryonic development, but researchers hypothesized this to be caused by increased tissue maintenance of larger embryos (Miller and Packard 1992).

Metabolism During Development in Oviparous Reptiles

Information regarding the patterns of metabolism between different species of developing birds, crocodilians, and lizards has previously been recorded (Vleck et al. 1979; Vleck et al. 1980; Thompson and Stewart 1997). Birds have largely defined this area of research with numerous studies completed across a wide range of species. These studies have displayed the presence of two patterns of metabolism during development. The first pattern described is a sigmoidal curve in oxygen consumption, that slows towards hatching, often truncating in several species (Figure 1.1, Panel A). The second pattern displays an exponential increase in oxygen consumption throughout the course of incubation (Figure 1.1, Panel B) (Vleck and Hoyt 1991). These patterns have been well documented within crocodiles and turtles, both groups exhibit sigmoidal patterns of metabolism with different degrees of truncations (Table 1.1) (Ackerman 1981; Morris et al. 1983; Webb et al. 1986; Packard et al. 1987; Thompson 1989; Crawford et al. 2015). Remaining reptile groups, including snakes and lizards, are continually underrepresented in this type of research, with studies completed in just six lizard and seven snake species (Table 1.1). This lack of research can create ambiguity when hypothesizing about the phylogenetic placement of these patterns within the Squamata lineage. This is observed in the lizard groups, where both sigmoidal and exponential patterns of metabolism have been reported for a single

species (Wang et al. 1989; Wang and Ji 1997). Previously, papers have reported that lizards only exhibit sigmoidal patterns, but the presence of these results can change conclusions, and this uncertainty needs to be corrected with further research (Thompson 1989; Vleck and Hoyt 1991; Thompson and Stewart 1997). Patterns of metabolism documented within snakes have remained consistent, with all researched species displaying exponential increases in oxygen consumption (Clark 1935; Zarrow and Pomerat 1937; Dmi'el 1970; Dmi'el and Borut 1972). Despite this, information for patterns of metabolism exists for only three families within the Serpentes lineage, and further sampling is required.

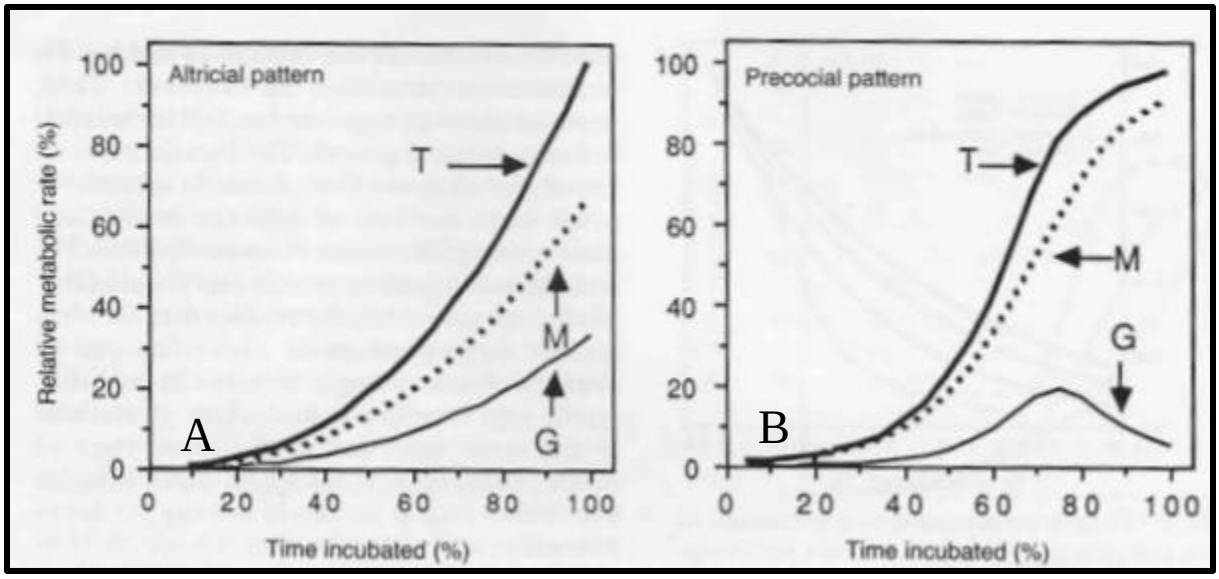


Figure 1.1. Patterns of embryonic metabolism in sigmoidal and altricial birds. Panel 'A' displays the sigmoidal patterns of metabolism exhibited throughout the course of incubation by precocial birds. This pattern is also reported in crocodilians, turtles, and lizards. Panel 'B' displays the exponential pattern of metabolism exhibited throughout the course of development by altricial birds. This pattern is also reported in lizards and snakes. The letters on the graphs represent the metabolism devoted to growth (G) and maintenance (M), these two values are added together to obtain total metabolic rate (T) (Vleck and Hoyt 1991).

Table 1.1. Patterns of metabolism in different snake and lizard species. References: 1. Dmi'el 1970. 2. Clark 1952. 3. Zarrow and Pomerat 1937. 4. Thompson and Russell 1999. 5. Thompson and Stewart 1997. 6. Wang and Xi 1989. 7. Wang and Xi 1997.

Species	**Pattern of Metabolism Exhibited During Development**	**Reference**
Snakes		
Natrix tessellate	Exponential	1
Spalerosophis cliffordi	Exponential	1
Vipera xanthina palaestinae	Exponential	1
Echis colorata	Exponential	1
Cerastes cerastes	Exponential	1
Coluber constrictor	Exponential	2
Python bivitattus	Exponential	3
Lizards		
Morethia boulengeri	Sigmoidal	4
Morethia adelaidensis	Sigmoidal	4
Plestiodon fasciatus	Sigmoidal	5
Plestiodon anthracinus	Sigmoidal	5
Takydromus septentrionalis	Sigmoidal & Exponential *	6 & 7
Gekko japonicus	Exponential	7

The Effects of Incubation Conditions on Metabolism

Developmental metabolism can be altered by different external and internal variables. Many of these variables, incorporate different physiological properties previously discussed in this paper. As mentioned, an increase in water has been shown to intensify the metabolism of many turtle species, but it is unclear whether this is due to increased tissue maintenance or the presence of water itself, which could cause osmotic stress. (Packard and Packard 1986; Miller and Packard 1992; Seymour et al. 1996). Parchment-shelled eggs are sensitive to water availability, and too much can lead to anoxic conditions, a rupturing of shell membranes, and

embryo death (Ewert 1985; Packard and Packard 1988; Seymour et al. 1996). In other instances, increased water uptake is correlated with larger hatchlings and better nutrient utilization, which is thought to enhance the survival of young after abandoning the egg (Packard et al. 1980; Packard et al. 1982; Packard et al. 1987). Snakes display similar trends, with some species exhibiting increases in mass and longer incubation periods when nested on wet substrates versus dry, while others show no differences in the growth and metabolism on the same conditions (Seymour 1984; Gutzke and Packard 1987; Packard and Packard 1987; Ji and Du 2001a; Ji and Du 2001b). Comparable to patterns of metabolism during development, the variations of responses observed amongst these animals may be due to phylogenetic differences, but further analyses are necessary to make this argument. Additionally, water is not the only factor that may be affecting embryonic metabolism (Stewart et al. 2019). During recent experiments utilizing eggs from *Pantherophis guttatus*, calcium uptake was altered when the outer layer of the eggshell was peeled. The results indicate that by removing the outer layer of the eggshell, embryos and hatchlings are smaller in size with lower calcium levels. Despite this, successful development was exhibited, implying that the eggshell is a secondary source that is not necessary for embryos to reach hatching. By peeling the eggshells, water uptake was inadvertently increased, and egg wet mass was significantly different in eggs missing the outer shell membrane (Stewart 2019). This leads to an interesting question; are the embryos from the peeled eggs smaller due to a lack of calcium? Or is an increase in water inhibiting their growth? In earlier stages of development, water uptake by the embryos of oviparous reptiles is a hydraulic, passive process (Warner et al. 2011). If embryos lack physiological mechanisms to regulate increased water availability, osmotic stress could become an issue, and embryos may be forced to dedicate more energy to sustaining physiological processes to remain alive, rather than towards tissue

growth, ultimately resulting in smaller body size. The loss of calcium could elicit a similar response, because although calcium mobilization is a regulated process, loss of this nutrient could result in altered functions of metabolism to compensate for the depletion and ensure survival.

Goals of the Study

The goal of the initial experiment was to determine a developmental curve, showing oxygen consumption throughout incubation in the red corn snake, *P. guttatus*, under standard conditions. It was hypothesized that embryos of *P. guttatus* would exhibit exponential increases of metabolism during development. We also wanted to utilize these results for comparisons with other snake and reptile species. We aimed to use samples obtained from recently oviposited eggs and hatchlings to determine an energy budget for the development of *P. guttatus* embryos. We additionally wanted to compare the energy requirements across reptile groups. For the second experiment, we became interested in data obtained from previous research by Stewart et al. (2019) with *P. guttatus*. In these preceding experiments, eggs of the red corn snake were peeled or left intact to determine whether eggshell calcium was required for successful embryo development (Stewart et al. 2019). While performing this, it was noted that hatchlings from intact eggs were larger when compared to those from the peeled treatment. We were intrigued by this difference and wanted to devise an experiment that tested whether differences in metabolism were present between the treatment groups. To complete this, we divided eggs into three treatment groups (peeled, half-peeled, & intact), and measured oxygen consumption at the latest stage of embryonic development (Stage 37). We wanted to determine whether differences in size are due to an increase in water, causing osmotic stress or a lack of eggshell calcium, leading to smaller embryos requiring less energy for tissue maintenance. Knowing what is causing the

differences in growth will aid in our understanding of how factors, like calcium and water, may influence the energy budget of a developing, parchment-shelled embryo.

CHAPTER 2. OXYGEN CONSUMPTION DURING DEVELOPMENT IN EMBRYOS OF THE OVIPAROUS SNAKE, *PANTHEROPHIS GUTTATUS*

Celeste R. Gallardo[a*], James R. Stewart[a*], Joseph R. Bidwell[a*]

[a]*Biological Sciences, East Tennessee State University, Johnson City, TN, United States*

ABSTRACT

Oxygen Consumption During Development in Embryos of the Oviparous Snake, *Pantherophis guttatus*

by

Celeste Gallardo

In the amniotic egg of oviparous reptiles, all sources of energy required for embryonic development are available within the egg, making interactions with the environment limited to the flux of water, oxygen, and carbon dioxide. Variation in the metabolic patterns among different species of embryonic birds has been well described. These studies have recognized two patterns of oxygen consumption during development: a sigmoidal curve and an exponential relationship between metabolism and incubation time. A sigmoidal curve has been reported for turtles, crocodiles, and most lizards. Snakes are unique, with all studied species exhibiting an exponential curve for metabolic rate during development. The aim of this study was to generate an oxygen consumption curve for the corn snake, *Pantherophis guttatus*, during development to test the hypothesis that embryos will exhibit an exponential increase in oxygen consumption over time. Oviposited eggs were placed in respirometry chambers for 24-hour periods and percent oxygen was recorded throughout development. Embryos were staged and the carcass and yolk were weighed separately. An energy budget was derived and compared for recently oviposited eggs and hatchlings using bomb calorimetry. Results indicated a steady incline in oxygen consumption during the earlier stages of development, with a rapid increase as hatching approached. Our data support the hypothesis that the developmental pattern of oxygen consumption in snakes differs from most other members of the Reptilia. Total energy required for development and metabolism was determined to be 37.03 kJ, comparable to other snake species.

Keywords: *Pantherophis guttatus*, oxygen consumption, development, egg, growth

Introduction

Energetic requirements vary throughout embryogenesis, reflecting the needs of the growing organism (Koch and Wieser 1983). During early stages of development, demands remain moderately low, but as incubation continues, metabolic rates increase due to heightened physiological processes and tissue maintenance. This trend is apparent in the developmental curves of embryos during incubation. Among reptiles, ontogenetic metabolic rates have been primarily documented in birds (Vleck et al. 1979; Vleck and Vleck 1980) which exhibit two major patterns of metabolism: exponential and sigmoidal (Vleck and Hoyt 1991; Cannon et al 1986). Avian species display two developmental modes, altricial and precocial, and the patterns of metabolism exhibited by the embryos are dependent on the developmental mode of the bird (Vleck et al. 1979). Altricial birds require extensive care after hatching. The hatchlings are blind, lack the ability to thermoregulate, and cannot eat without help from the parents. During embryogenesis, altricial species exhibit exponential developmental curves (Vleck et al. 1979). Precocial species are less dependent on the parents. Hatchlings are often mobile, able to search for food, and have some vision. The embryos of this group display sigmoidal curves that drop off rapidly prior to pipping (Vleck et al 1979; Vleck and Vleck 1996). Ratites are a precocial species that show a modified sigmoidal pattern. Their pattern is differentiated from others by the presence of a truncation prior to hatching (Hoyt et al. 1978). Other reptiles exhibit either exponential or sigmoidal patterns of metabolic rate during development (Thompson 1989; Whitehead and Seymour 1990; Thompson and Stewart 1997; Thompson and Russell 1999). Turtles (*Emydura macquarii*, *Chelydra serpentina*, *Carettochelys insculpta*, *Caretta caretta*, & *Chelonia mydas*) and crocodilians (*Crocodylus johnstoni*, *Crocodylus porosus*, *& Alligator mississippians*) display sigmoidal patterns of oxygen consumption (Ackerman 1980; Gettinger et

al. 1984; Webb et al. 1986; Thompson 1987; Thompson 1989; Whitehead and Seymour 1989). Although some turtle species demonstrate a truncated peaked pattern, like ratites. This adjustment is most prevalent in large, marine turtles (Ackerman 1981; Webb et al. 1986). The pattern in lizards is complex, with several species (*Sceloporus virgatus, Morethia boulengeri, Morethia adelaidensis, Eumeces fasciatus, & Eumeces anthracinus)* presenting sigmoidal patterns (Wang et al. 1989; Vleck and Hoyt 1991; Thompson and Stewart 1997; Thompson and Russell 1999). But one study has suggested the presence of an exponential pattern within two lizard species (*T. septentrionalis* and *G. japonicus*; Wang and Ji 1997). In contrast, all snake species that have been examined (*Natrix tessellate, Spalerosophis cliffordi, Vipera xanthina palaestinae, Echis colorata, Cerastes cerastes, Coluber constrictor,* & *Python bivitattus*) show exponential patterns of development, making them distinct within Squamata (Zarrow and Pomerat 1937; Clark 1953; Dmi'el 1970; Dmi'el and Borut 1972).

In this study, we analyzed oxygen consumption in embryos of the red corn snake, *Pantherophis guttatus*, to determine their developmental pattern beginning at oviposition and lasting until hatching. Additionally, we used egg components obtained from recently oviposited embryos and hatchlings to establish an energy budget for their development.

Materials & Methods

Incubation

Recently oviposited eggs were collected from ten female *P. guttatus* housed in the animal care facilities at East Tennessee State University and Trinity College over one reproductive cycle (2019, 10 clutches; mean=17 eggs per clutch). The eggs were numbered and wet mass in grams was recorded (0.01g) using an electronic balance (Mettler Toledo, BB240, Ohio, USA). One live

embryo was sampled from each clutch to determine embryonic stage prior to incubation (Zher 1962). Eggs were placed in plastic containers filled with 2 parts water and one part vermiculite. These containers were placed in an incubator at 26º C (Precision Model 818; Union, New Jersey, USA). Incubation temperatures were monitored and kept in a range (26-30 ºC) that supported normal development. All animals were kept under appropriate conditions as described by the East Tennessee State University Animal Care and Use Protocol (Protocol #: P181201). The containers holding the eggs were opened on a weekly basis to refresh the air. Eggs were weighed using an electronic balance (0.01 g), and water was added on a weekly basis to ensure consistent water potential (Mettler Toledo, BB240, Ohio, USA). Under these conditions, eggs hatched in 68-80 days. Once pipped, the snake hatchlings stay in the shell until yolk sacs are internalized (approximately 1-2 days). Pipped eggs were checked regularly. After yolk sacs were absorbed and the shell was abandoned, hatchlings were sacrificed using a mixture of the anesthetic powder, tricaine mesylate (MS-222). The dosage for this mixture included 250 mg/kg sodium bicarbonate buffered solution followed by 0.5 mL unbuffered 50 % solution (Conroy et al. 2009). After euthanization, compartments (yolk sac, shell, and hatchling) were separated and frozen for future use.

Oxygen Consumption Trials

To measure oxygen consumption, 1-2 incubated eggs from each clutch were weighed (0.01 g) and placed in respirometry chambers at 7-day intervals (Mettler Toledo, BB240, Ohio, USA). The respirometry chambers had a volume of 90 mL and were made of plastic tubing. The tubes had a thickness of 0.3 cm, and lids to the chambers were air-tight with a 1.0 cm thickness. Chambers were attached to fiberoptic oxygen sensors to measure percent oxygen (Fire StingTM, Pyro Science, Aechen, Germany). These sensors were inserted into the chambers with an air-

tight cable gland (Sealcon LLC, Colorado, USA). The sensors were recalibrated to the current incubator temperature before every trial. The chambers and oxygen sensors were placed in the incubator, keeping humidity and temperature controlled. Eggs from each clutch were placed in the chambers and given a one-hour acclimation period. Percent oxygen was then recorded in the chambers every 60 seconds for 24 hours. A peristaltic pump was attached to the chambers and periodically flushed fresh air from the incubator to maintain oxygen concentrations above 18% following Parker and Andrews (2006). The frequency of air circulation was adjusted to match the oxygen consumption rates of the embryos.

After trials, developmental stage of the embryos was determined. The compartments of the eggs (yolk, shell, and embryo) were separated, weighed (0.0001 g), and frozen (-4 ºC) (Mettler Toledo, AL54, Ohio, USA). The frozen compartments of the eggs were placed in a lyophilizer to obtain dry masses (Labconco Freeze Dryer Model 77500; Kansas City, USA). These trial processes continued throughout incubation with the final measurements taken a few days prior to hatching. Twenty-one hatchlings were euthanized after the yolk-sac was internalized with an overdose of MS-222. The carcass, yolk sac, and shell of the hatchlings were separated, weighed (0.0001 g), and frozen for lyophilizing (Mettler Toledo, AL54, Ohio, USA).

Oxygen Conversion from Percent O_2 to Milligrams of O_2

Data were converted from percent oxygen to milligrams of oxygen. The formula used incorporates the ideal gas law, PV=nRT, where P is pressure, v is volume of the respirometry chamber, n is milligrams of oxygen, R is the ideal gas constant, and T is temperature in Kelvin). First, pressure was estimated using the barometric pressure for Johnson City, TN. Uncorrected pressure was estimated by taking the pressure of our research location (Johnson City, TN, USA) and subtracting it by the altitude of our location (Johnson City, TN, USA). This was applied to

calculate oxygen pressure and was then multiplied by the volume of the headspace in the chambers. Second, temperature of the incubator was converted into Kelvin and multiplied by the R constant. The value obtained from the first calculation was divided by the number calculated in the second to solve for milligrams of oxygen. This formula was completed for each embryo that underwent a trial. Slopes, average slopes, and average total oxygen consumption in milligrams was also calculated for each individual.

Bomb Calorimetry

Lyophilized samples of hatchlings and yolk sacs were stored in the freezer (- 4^{o} C). The stored samples from five clutches were then subjected to a flash freeze using liquid nitrogen to ensure complete dryness. Samples were then immediately weighed (0.0001 g) and homogenized using a mortar and pestle (Mettler Toledo, AL54, Ohio, USA). Pellets, with masses that ranged from 0.2-3.0 grams were formed and ignited in an oxygen bomb calorimeter (1341 Plain Jacket Bomb Calorimeter, Parr Instrument Co., IL, USA). Before pellets underwent calorimetry, several pellets of benzoic acid were made and ignited to ensure that the bomb calorimeter was standardized.

Statistical Analyses & Data Reduction

Graphs and associated formulas showing incubation time/embryonic stage, oxygen consumption, and embryo mass over time were created in SigmaPlot (Systat Software Inc., Version 12). Yolk mass was compared to embryonic stage with an estimated marginal means graph, this was plotted, and a regression equation was derived using SPSS (IBM® SPSS Statistics 27). Differences in oxygen consumption between embryonic stages were statistically analyzed using an ANCOVA, with the dependent variable being total oxygen consumption (mg

O_2), the random factor was clutch, and embryo mass was a covariate (IBM® SPSS Statistics 27). Significance was accepted if $P < 0.05$.

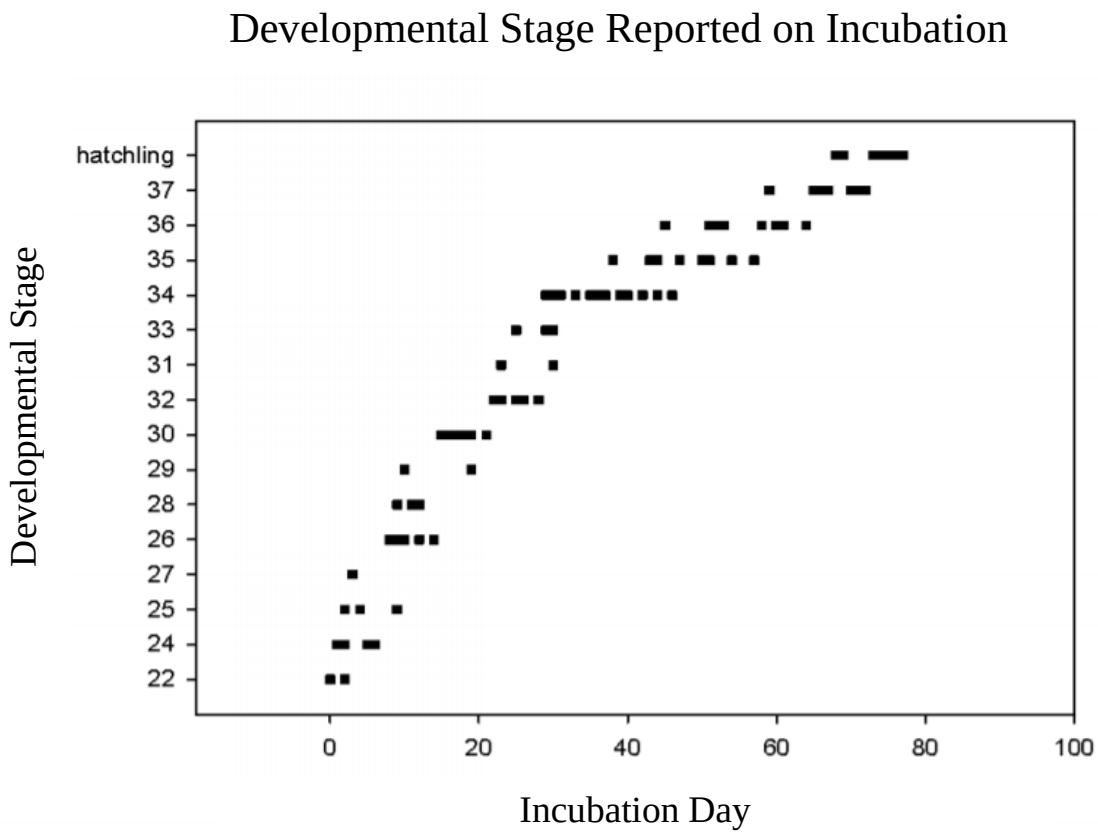
Energetic results from the yolk of recently oviposited eggs, hatchling, and yolk sac samples were converted from kilocalories to kilojoules. These numbers were used to estimate the value of kJ found in each gram of the sample. The total kJ of each sample was averaged to obtain a mean energetic output for the different compartments. These averages were used to calculate total energy used for development of the embryos. Total oxygen consumption during development was determined by calculating the rea under the curve (AOC) from the exponential curve of total oxygen consumption during development. The integral from the AOC was used to calculate the total kJ used for metabolism during development.

Results

Oviposition & Incubation

At oviposition, eggs of *P. guttatus*, collected from the East Tennessee State University colony had a mean wet mass of 8.1 ± .13 g (7 clutches; n = 116 eggs; mean=17 eggs per clutch) and contained embryos of stage 22-24 (Mean = 23, Median = 22, Mode = 22). Four additional clutches were obtained from Trinity College but were not collected until 6-9 days passed after oviposition. Stages at oviposition were not determined for these clutches. Embryonic stage at the introduction of the eggs into the trials ranged from 22-26 (Mean = 25, Median = 25, Mode = 24), and matched closely with embryonic stages available at oviposition. Eggs from the East Tennessee State University colony took 68-78 days to hatch, and those from Trinity College colony took 74-80 days to hatch. Across all stages, there was little variability in embryonic stage relative to day of incubation apart from 34-36 (Figure 2.1). This is most likely due to the length

of the stages, while most earlier stages are no more than a few days long, later stages of development can last beyond a week (Zher 1962).



.

Figure 2.1. Incubation day and the embryonic stage of *P. guttatus* until hatching from ten clutches. Little variability existed during the staging of the embryos until stages 34-37, but these developmental stages last longer than earlier ones

Oxygen Consumption, Mass, & Yolk Utilization

Embryos of *P. guttatus* showed a pattern of change in oxygen consumption that increased exponentially throughout embryonic development. Oxygen consumption remained markedly low for 67% of incubation, with the final 33% of incubation characterized by a rapid increase in the

amount of oxygen consumed by the embryos (Figure 2.2). Rates of oxygen consumption during stages 24-34 were not significantly different from each other ($F = 123.4086$, 11 *df*, $P > 0.05$). But measurements from the later stages of development, 34-37, were significantly different from each other ($F = 123.4086$, 11 *df*, $P < 0.0001$), and stages 24-34 were significantly different from stages 35-37 ($F = 123.4086$, 11 *df*, $P < 0.05$). Embryo mass had a significant influence on oxygen consumption ($F = 51.105$, 1 *df*, $P < 0.0001$), and clutch had no effect on oxygen consumption ($F = 0.685$, 9 *df*, $P > 0.05$). Pipping occurred in one clutch, one day after the last oxygen trial, while others varied from 2-6 days.

The equation relating oxygen consumption (mg O_2) and incubation time (d) was derived with an exponential trendline for *P. guttatus:*

$$y = (1.4364E\text{-}0.07)e^{0.3473x}$$

($r^2 = 0.9746$)

Where 'y' represents oxygen consumption (mg O_2), and 'x' is incubation day. This formula was derived from a graph created in SigmaPlot (Systat Software Inc., Version 12).

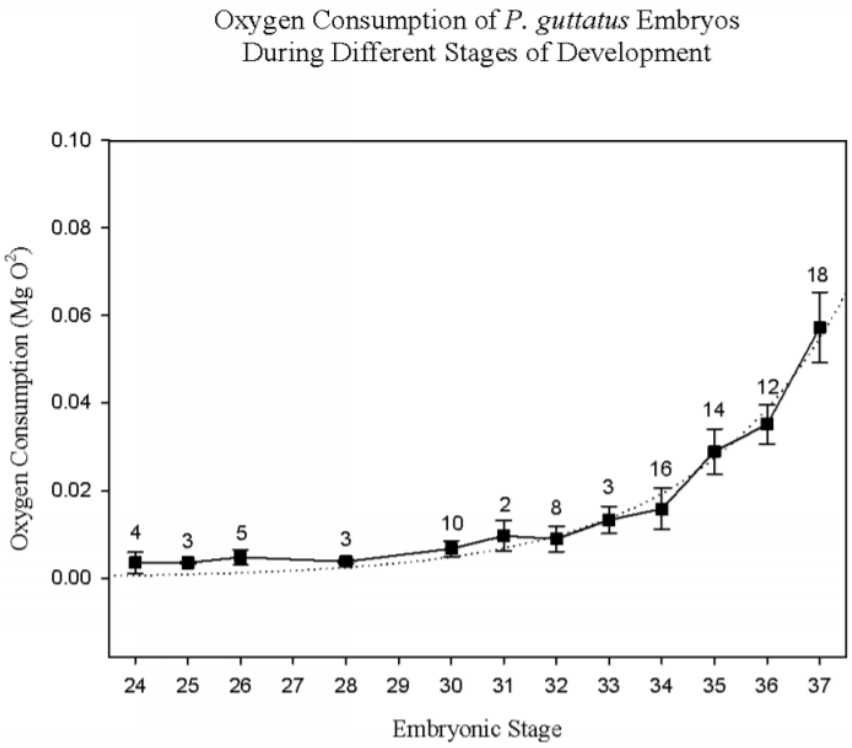


Figure 2.2. Oxygen consumption (mg O^2) of *P. guttatus* during different stages of embryonic development. Embryos experience an exponential pattern of oxygen consumption. Numbers above data points are sample sizes and signify individual embryos. Means are presented ±SD. The exponential equation derived from this data is: y = (1.6345E-0.007)E0.347x , where 'y' represents mg O^2, and 'x' represents embryonic stage.

Intake of oxygen closely followed the growth of the embryos (Fig 2.3). Dry mass of the embryo increased exponentially, similar to oxygen consumption. An exponential equation was derived to show the relationship between incubation day and embryo dry mass (g), where 'y' is embryonic dry mass (g), and 'x' is incubation day. This formula was derived from a graph created in SigmaPlot (Systat Software Inc., Version 12).

$y = 0.0246e^{0.0567x}$

($r^2 = 0.8895$)

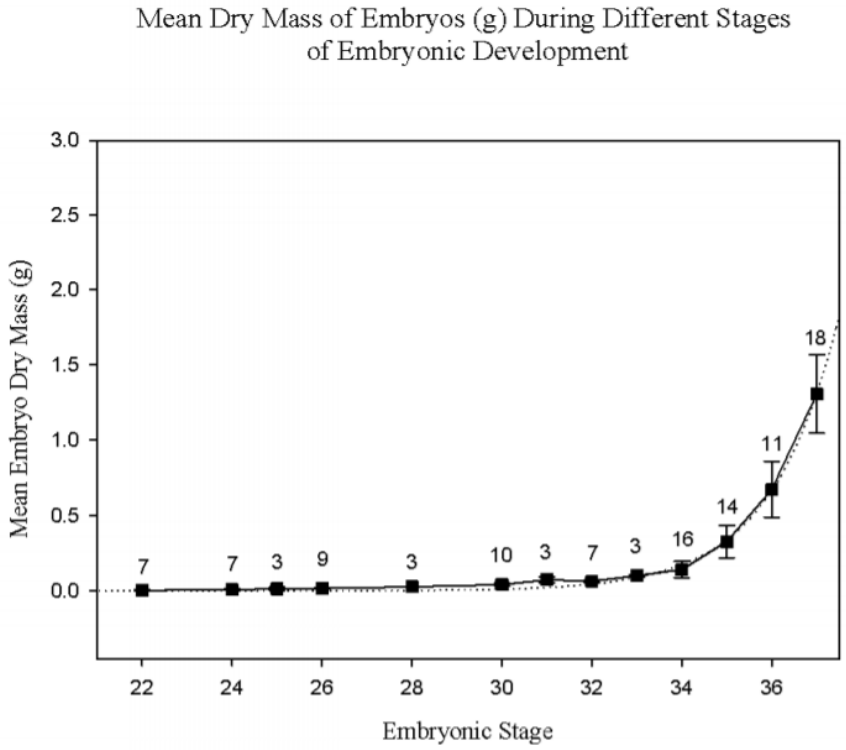


Figure 2.3. Embryonic dry mass (g) of *P. guttatus during* different stages of embryonic development. Embryonic dry mass increases exponentially in a pattern that is similar to the oxygen consumption of embryos (Fig. 1). Means are presented ±SD . Numbers above data points are sample sizes and signify individual embryos. The exponential equation derived from this data is: y = (0.0346E0.0567(x))

Mean dry mass of yolk associated with the embryos decreased linearly over time (Fig 2.4). A linear regression equation was derived from these data. In this equation, 'y' is yolk dry mass and 'x' is incubation day. This formula was derived from a graph created in SPSS (IBM SPSS; Armonk, NY, USA).

$y = -0.0084 x + 0.4863$

($r^2 = 0.4628$, $F = 9.478$, $P = <0.05$)

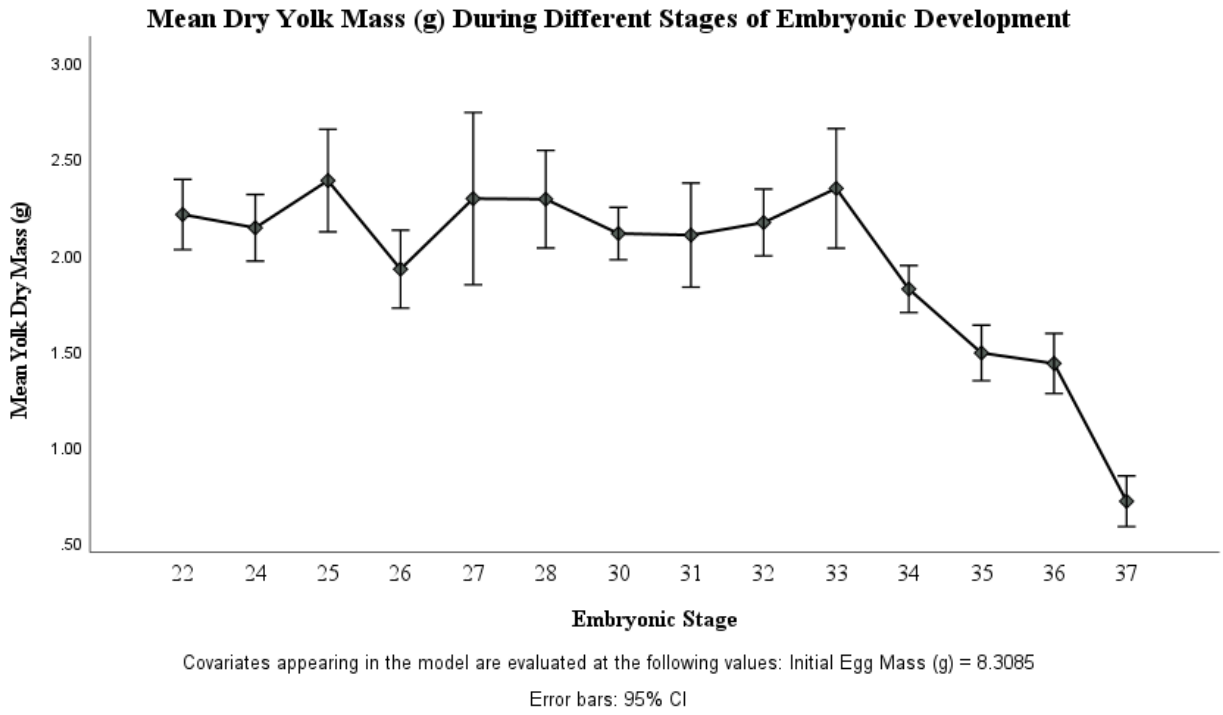


Figure 2.4. Mean yolk mass (g) across different stages of embryonic development. The graph shows the estimated marginal means of dry yolk mass (g) corrected for initial egg mass, over embryonic stage. Yolk utilization in P. guttatus embryos occurs steadily throughout incubation, with a rapid decline prior to pipping. The linear equation derived from this data is: $y = -0.0084 x + 0.4863$; where 'y' is dry yolk mass (g), and 'x' is embryonic stage.

Energy Budget

Yolk samples were collected and sampled from recently oviposited eggs and across various stages of embryonic development of *P. guttatus* to calculate energy requirements for incubation (n=5 clutches). The stages from recently oviposited eggs included stage 22 (n=4),

stage 24 (n=1) and stage 25 (n=1). Yolk from recently oviposited samples had a mean caloric value of 9,633 ± 430 calories, or 40.304 ± 4.41 kilojoules (n=6 yolk samples; five clutches), these samples also contained the greatest energetic yield. Hatchlings contained the second most with 26.599 ± 6.22 kilojoules (n=5 hatchlings; five clutches), and internal yolk sacs (n=5 internal yolk sacs; five clutches) held the least with 7.3270 ± 4.00 kilojoules. Dividing dry masses of the yolk by the caloric output gives kJ/gram for each sample. Within recently oviposited eggs, there was mean of 20.709 ± 0.226 kilojoules per gram of yolk. Embryos of the oviposited eggs ranged from 0.01-0.03 grams; they were too small for our calorimetry equipment and could not be analyzed. In addition to this, hatchlings (n=5) and yolk sacs (n=5) were randomly picked from the same clutches used in the yolk oviposition analysis mentioned earlier. Upon combustion, hatchlings had a mean energy output of 26.599 ± 2.782 kilojoules or 17.440 ± 0.685 kJ/g of tissue. Examining the residual yolk sacs shows an average production of 7.327 ± 1.790 kilojoules or 17.034 ± 0.745 kJ/g. The amount of energy per unit of wet mass was estimated for the hatchlings to be 0.194 kJ/g.

This data indicated that a total of 37.026 kJ of energy was catabolized during the development of *P. guttatus*. The energetic yields of the hatchlings and internal yolk sacs was compared to that of the yolk from recently oviposited eggs to determine the amount of energy required to convert yolk into embryo tissue. Based on the data listed above, 66% of the available yolk energy reserves at oviposition went in the formation of the hatchling body, and 18.1% of the yolk was retained in the yolk sac for hatchling use. These two compartments comprise 33.9 kJ of the available energy. Using the area under the curve, the total amount of energy portioned for metabolism was determined to be 3.1 kJ, implying that 7.7% of the available energy was

allocated for metabolism. This leaves 3.3 kJ, or 8.2 % of available energy, reserved for other physiological processes.

Discussion

Patterns of Oxygen Consumption During Embryogenesis

A limited number of studies focusing on embryonic metabolism have been performed that include snakes in their analyses. These studies have spanned across three different families within Serpentes, and the distribution of this oxygen pattern across these different groups indicates that exponential increases in oxygen consumption during development is characteristic of the Serpentes lineage. Embryos of the black snake (*Pantherophis obsoletus*), smooth green snake (*Opheodrys vernalis*), Burmese python (*Python bivittatus*), and five desert snakes (*Natrix tessellata, Spalerosophis diadema, Daboia palaestinae, Echis coloratus, & Cerastes cerastes*) have shown an exponential pattern of oxygen consumed throughout development (Zarrow and Pomerat 1937; Clark 1952; Dmi'el 1969; Dmi'el 1970; Dmi'el and Borut 1972; Black et al 1984). Our results indicate the presence of this exponential pattern in *P. guttatus* throughout embryogenesis. Between developmental stages 24-33, *P. guttatus* embryos showed no significant differences in oxygen consumption. Consumption was highest during stages 34-37; this is most likely due to an increase of tissue maintenance and organogenesis (Thompson 1989). Weight of the embryos exponentially increased, following a pattern that reflects that of oxygen consumption. The correlation between embryonic weight and oxygen consumption forms a similar pattern to what is observed in species of altricial birds (Vleck and Hoyt 1991).

Comparison with other Reptiles

The sigmoidal pattern is well distributed amongst Testudines, with studies documenting its appearance in five families (Chelydridae, Cheloniidae, Emydidae, Trionychidae, and Chelidae (Ackerman 1981; Morris et al. 1983; Webb et al. 1986; Packard et al. 1987; Thompson 1989; Crawford et al. 2015). Species of the representative families demonstrate various degrees of truncations in oxygen consumption prior to pipping (Ackerman 1981; Morris et al. 1983; Webb et al. 1986; Packard et al. 1987; Thompson 1989). Within crocodiles, oxygen consumption has been determined for three species (*Alligator mississippiensis*, *Crocodylus johnstoni*, and *Crocodylus porosus*) (Thompson 1989; Whitehead and Seymour 1990). All embryos of these experiments showed a sigmoidal pattern with a small truncation towards hatching (Thompson 1989; Whitehead and Seymour 1990). This truncation is comparable to the pattern observed in ratites. (Green et al. 2014). Within the Aves lineages, the precocial and altricial modes of development, which are correlated with their embryonic metabolism, have been mapped onto their phylogeny, providing a relatively clear picture of how patterns differ across their clades (Chen et al. 2019). Altricial patterns of development and exponential increases of oxygen consumption during embryogenesis have evolved independently in multiple lineages within Aves (Vleck et al. 1979; Vleck et al. 1980; Vleck and Vleck 1980; Thompson 1989; Vleck and Hoyt 1991; Vleck and Vleck 1996; Chen et al. 2019).

Lepidosauria is comprised of tuatara, lizards, and snakes, with tuatara occupying the order, Rhynchocephalia (Spenodontia), and lizards and snakes present in the much larger order, Squamata (Gemmell et al. 2020). The tuatara lineage consists of one living member: *Sphenodon punctatus* (Gemmell et al. 2020). A single study regarding embryonic metabolism was completed on *S. punctatus* and showed a sigmoidal pattern of oxygen consumption (Thompson 1989). In

lizards, studies have been completed on five different species (*Sceloporus virgatus, Morethia boulengeri, Morethia adelaidensis, Plestiodon fasciatus, & Plestiodon anthracinus),* showing a peaked oxygen pattern with a small truncation during development (Vleck and Hoyt 1991; Thompson and Stewart 1997; Robert and Thompson 2000). But the presence of an exponential pattern has been reported in two species of lizard (*Takydromus septentrionalis* and *Gekko japonicus)* (Wang and Ji 1997). However, prior to this publishing, the same researchers issued a separate paper indicating a sigmoidal pattern in *T. septentrionalis* (Wang et al. 1989). As previously mentioned, all species of snakes with embryonic metabolism studies completed, exhibit an exponential pattern of oxygen consumption (Clark 1935; Zarrow and Pomerat 1937; Dmi'el 1970; Dmi'el and Borut 1972). Most studies refer to snakes as being unusual among squamates because of their exponential pattern of metabolism, but if some species of lizards also possess this pattern, it is possible that this trait has independently evolved in other lineages of Squamata.

The appearance of these two different forms of metabolism during development across reptilian lineages uncovers two potential patterns, one where exponential patterns of oxygen consumption evolved independently within snakes and in five lineages of birds. Or, one where this pattern evolved within some lizard lineages, retained in snakes, and evolved independently in five lineages of birds. To fully understand the appearance of this trait across reptiles, additional studies need to be completed, particularly within Squamata.

The Ontogeny of Embryonic Metabolism within the Reptilia

The pattern of embryonic oxygen consumption of snakes is convergent on five lineages of birds. Altricial birds hatch with limited vision, an inability to thermoregulate or move, and

they are unable to feed themselves. Parental care in snakes is generally restricted to nest site selection and the protection of recently oviposited eggs (Greene et al. 2002). These behaviors are beneficial, as a study has shown that brooding by children's pythons, *Antaresia childreni,* aids with water balance, which promotes successful embryogenesis (Lourdaies et al. 2007). Despite this brooding behavior exhibited by some snake species, this form of parental care is different than what is observed amongst altricial birds. The distribution of the exponential pattern of metabolism across the bird lineages indicates this is a derived state. Other reports have strongly indicated this patterns presence within Squamata, yet the question arises: why do snakes, and potentially some lizards, exhibit the same developmental pattern as altricial birds without similar parental requirements?

The evolution of this pattern within some lineages is not entirely understood and could differ amongst the reptile groups. Within birds, it is recognized that the pattern exhibited is related to the developmental mode. Precocial species are well developed prior to hatching, and growth stops, with declines in metabolic rate mimicking this. In altricial birds, development is not complete at hatching, and consequently metabolism continues to increase prior to pipping as the embryo is still growing (Vleck and Vleck 1987). This creates an interesting observation, because snakes and potentially some lizards display exponential patterns in development, with growth and oxygen consumption continuously rising to the time of hatching. Determining the reason why some lizard and all reported snake species have an exponential pattern of development is complicated, and it could be attributed to many different factors of embryogenesis. It has been postulated that the sigmoidal oxygen consumption patterns experienced by some reptiles, are due to the maturation of neuromuscular, sensory, and thermoregulatory systems during the truncation period (Vince and Chinn 1971; Thompson

1989). Other papers have suggested snakes exhibit maturation of these systems throughout the course of development, rather than towards the end, implying that a plateau prior to pipping would be unnecessary (Thompson 1989). The timing for the formation of the nervous and other sensory systems within lizards is not as well described, but if similar, could account for the presence of exponential patterns within those species. Similarly, the differences observed between the oxygen consumption of sigmoidal and altricial patterns can be simply due to contrasting growth rates (Vleck and Vleck 1996). In birds and snakes where exponential increases of metabolic rate are observed, embryo mass is also continuously increasing. Conversely, in reptiles exhibiting sigmoidal patterns of development, embryos stop gaining mass prior to pipping. This can lead to declines in growth costs, which can decrease metabolic rate (Vleck and Vleck 1996).

For many reptiles, having a specific pattern may help with survival in certain environments. Synchronous hatching is reported across reptile groups (birds, turtles, crocodilians, lizards, & snakes). Within bird and crocodilian species, embryos are cued to hatch by the vocalizations of their siblings (Woolf et al. 1976; Vergne and Mathevon 2008). In snakes, vibrations caused by the heartbeats of neighboring siblings' aid with hatching synchrony (Aubret et al. 2016). Similarly, lizards also use environmental cues, like vibrations caused by disturbances to their nests (Doody and Paull 2013). Turtles build shallow nests, which can produce temperature differences amongst the eggs (Thompson 1989). This makes development inconsistent across embryos, as eggs closer to the surface are likely to be impacted by environmental temperatures, these embryos often develop slower than those oviposited deeper within the nest (Thompson 1989). Because of this, the large truncation prior to pipping can be utilized as a 'catch-up' period for synchronous hatching (Thompson 1989). This behavior

increases the survivability of the hatchlings, as it promotes group emergence, which helps limit predation on individuals by overwhelming potential predators (Colbert et al. 2010).

Energy Budget

Bomb calorimetry indicated that an average of 37.03 kilojoules was catabolized during embryonic development of *P. guttatus*. This analysis was performed on eggs from five out of the ten clutches utilized in our respiration studies. We were unable to perform bomb calorimetry on recently oviposited samples of embryos. This was because the dry mass of samples weighed 0.01-0.03g and were too small to form pellets with our equipment. But the caloric range of pellets with a weight this small, is likely to be within the scope of variability in the measurements of the yolk at oviposition.

When comparing energy values of different snakes, the energy value of 37.03 kJ was most similar to that of *Spalerosophis diadema*, which was estimated to expend 26.16 kJ during incubation (Vleck and Hoyt, 1991). Comparisons to other species, including *Vipera xanthina* and *Cerastes cerastes*, showed that the total energy metabolized during development was much higher in *P. guttatus*. *Vipera xanthina* has an energy requirement of 11.39 kJ for development, but also has a shorter incubation period. *C. cerastes* is similar in hatchling size but also has a shorter incubation period (62 days), then what we reported in *P. guttatus* (75-80 days). *C. cerastes* has an energy requirement of 17.04 kJ. Using the wet mass reported, its estimated that *C. cerastes* utilizes 0.380 kJ/g of tissue during development, which is higher than the 0.194 kJ/g of tissue required for *P. guttatus.* This would impact the amount of energy utilized for tissue maintenance and growth. There could be a phylogenetic effect causing the differences observed in energy requirements for these species. *P. guttatus* and S. *diadema* are colubrid snakes, possessing higher energetic requirements, while the two viperids reported lower requirements.

Perhaps viperids exhibit a more energetically efficient developmental process. Ultimately, pathways that help achieve this will be selected for in all living organisms, as energetic improvements are often drivers for evolution (Yun et al. 2006). Additionally, these metabolism experiments were performed on eggs incubated at 29-31℃, the differences in temperature could be influencing incubation length (Vleck and Vleck 1991). This could be contributing to some of the different energetic requirements reported between these species.

Data for total energy utilization is available for six lizard species (*Lampropholis guichenoti, Lampropholis delicata, Plestiodon fasciatus, Plestiodon anthracinus, & Sceloporus virgatus*) (Thompson and Stewart 1997; Thompson and Russel, 1999; Vleck and Hoyt 1991;). The energy per unit of wet mass used by these species ranges from 0.236-0.709 kJ/g of tissue. Similar to the comparisons made with other snake species, the demands calculated for *P. guttatus* (0.194 kJ/g) are smaller than what is reported for these lizards.

The crocodilians have the greatest energy requirements for development, but also the longest incubation periods and largest eggs (Vleck and Hoyt 1991). The order Chelonia, has a lot of variation when comparing energy usage across species. Several species have comparable egg masses and incubation periods to that of *P. guttatus*, but smaller hatchling masses and lower energy requirements (Vleck and Hoyt 1991). Aves differ from all other reptile groups; their incubation periods are greatly reduced, and their energy requirements differ based on developmental mode. Altricial embryos require an average of 20 kJ less than what is necessary for precocial embryos (Vleck and Hoyt 1991). But parental care after hatching will increase the energy requirements significantly for each of these organisms.

Summary

The data collected in this study shows the presence of an exponential pattern in oxygen consumption throughout embryogenesis in the red corn snake, *Pantherophis guttatus*, and confirms previous studies indicating that snakes have a derived pattern of embryonic metabolism. Analyses of the wet masses of different egg compartments show an energetically efficient process, with less energy required per gram of tissue when compared to other squamates. To fully understand why exponential patterns of oxygen consumption are present within Squamata, further sampling needs to occur, particularly within lizards.

REFERENCES

Ackerman, RA. 1980. Physiological and ecological aspects of gas exchange by sea turtle eggs. *American Zoologist*, *20*(3), 575–583.

Aubret, F, Blanvillain, G, Bignon, F, Kok, PJR. 2016. Heartbeat, embryo communication and hatching synchrony in snake eggs. *Scientific Reports*, *6*(23519), 1-5.

Black, CP, Birchard, GF, Schuett, GW, & Black, VD. 1984. Influence of incubation water content on oxygen consumption in embryos of the Burmese python (*Python molurus bioittatus*). In: Seymour RS. (eds), *Respiration and Metabolism of Embryonic Vertebrates. Perspectives in vertebrate science*, Dordecht: Springer,*3*, 137–145.

Cannon, ME, Carpenter, RE, & Ackerman, RA. 1986. Synchronous hatching and oxygen consumption of Darwin's rhea eggs (*Pterocnemia pennata*). *Physiological Zoology*, *59*, 95–108.

Chen, CK, Chuang, HF, Wu, SM, & Li, WH. 2019. Feather evolution from precocial to altricial birds. *Zoological Studies*, *58*(24), 1-12.

Clark, H. 1935. Metabolism of the black snake embryo I. Nitrogen excretion. *Journal of Experimental Biology*, *30*, 492–501.

Crawford, NG, Parham, JF, Sellas, AB, Faircloth, BC, Glenn, TC, Papenfuss, TJ, Simison, B W et al. 2015. A phylogenomic analysis of turtles. *Molecular Phylogenetics and Evolution*, *83*, 250–257.

Dmi'el, R. 1969. Circadian rhythm of oxygen consumption in snake embryos. *Life Sciences*, 8(24), 1333-1341.

Dmi'el, R. 1970. Growth and metabolism in snake embryos. *Journal of Embryology and Experimental Morphology*, *23*(3), 761–772.

Dmi'el, R, & Borut, A. 1972. Thermal behavior, heat exchange, and metabolism in the desert snake *Spalerosophis cliffordi*. *Physiological Zoology*, *45*(1), 78–94.

Doody, JS, Paull, P. 2013. Hitting the ground running: Environmentally cued hatching in a lizard. *Copeia*, *2013*(1), 160-165.

Gemmell, NJ, Rutherford, K, Prost, S. et al. 2020. The tuatara genome reveals ancient features of amniote evolution. *Nature*, *584*, 403-409.

Green, RE, Braun, EL, Armstrong, J, Dent, E, Nguyen, N, Hickey, G, & Vandewege, M. 2014. Three crocodilian genomes reveal ancestral patterns of evolution among archosaurs. *Science*, *346*(6215), 1335-1342.

Greene, HW, May, P, Hardy, D, Scitturo, J, & Farrell, T. 2002. Parental behavior by vipers In Schuett, GW, Höggren, M, Douglas, ME, and Greene, HW. (eds), *Biology of the Vipers.* Eagle Mountain (UT): Eagle Mountain Publishing, 179–206.

Gettinger, RD, Paukstis, GL, & Gutzke, WH. 1984. Influence of hydric environment on oxygen consumption by embryonic turtles *Chelydra serpentina* and *Trionyx spiniferus*. *Physiological Zoology*, *57*(4), 468–473.

Hoyt, DF, Vleck, D, & Vleck, CM. 1978. Metabolism of avian embryos: ontogeny and temperature effects in the ostrich. *The Condor*, *80*, 265–271.

Koch, F, & Wieser, W. 1983. Partitioning of energy in fish: Can reduction of swimming activity compensate for the cost of production? *Journal of Experimental Biology*, *107*(1), 141–146.

Lourdais, O, Hoffman, TCM, & Dale, F. 2007. Maternal brooding in the children's python (*Antaresia childreni*) promotes egg water balance. *Journal of Comparative Physiology B*, *177*, 569–577.

Morris, KA, Packard, GC, Boardman, TJ, & Packard, MJ. 1983. Effect of the hydric environment on growth of embryonic snapping turtles (*Chelydra serpentina*). *Herpetologica*, *39*(3), 272–285.

Packard, GC, Packard, MJ, Miller, K, & Boardman, TJ. 1987. Influence of moisture, temperature, and substrate on snapping turtle eggs and embryos. *Ecology*, *68*(4), 983–993.

Parker, SL, & Andrews, RM. 2006. Evolution of viviparity in Sceloporine lizards: In utero PO_2 as a developmental constraint during egg retention. *Physiological and Biochemical Zoology*, *79*(3), 581-592.

Robert, KA, & Thompson, MB. 2000. Energy consumption by embryos of a viviparous lizard, *Eulamprus tympanum*, during development. *Biochemistry*, *127*(4), 481–486.

Thompson, MB. 1989. Patterns of metabolism in embryonic reptiles. *Respiration Physiology*, *76*(2), 243–255.

Thompson, MB, & Russell, KJ. 1999. Embryonic energetics in eggs of two species of Australian skink, *Morethia boulengeri* and *Morethia adelaidensis*. *Journal of Herpetology*, *33*(2), 291–297.

Thompson, MB, & Stewart, JR. 1997. Embryonic metabolism and growth in lizards of the genus *Eumeces*. *Comparative Biochemistry and Physiology*, *118*(3), 647–654.

Vergne, AL, Mathevon, N. 2008. Crocodile egg sounds signal hatching time. *Current Biology*, *18*(12), R513–R514.

Vince, MA, & Chinn, S. 1971. Effects of accelerated hatching on the initiation of standing and walking in the Japanese quail. *Animal Behavior*, *19*(1), 62–66.

Vleck, CM, & Hoyt, DF. 1991. Metabolism and energetics of reptilian and avian embryos. In Deeming, DC, and Ferguson, MJ. (eds), *Egg incubation: Its effects on embryonic development in birds and reptiles*, Cambridge: Cambridge University Press, 285-304.

Vleck, CM, Hoyt, DF, & Vleck, D. 1979. Metabolism of avian embryos: Patterns in altricial and precocial birds. *Physiological Zoology*, *52*(3), 363–377.

Vleck, CM, & Vleck, D. 1980. Patterns of metabolism and growth in avian embryos. *American Zoologist*, *20*(2), 405–416.

Vleck, CM, & Vleck, D. 1996. Embryonic energetics. In C. Carey (eds), *Avian Energetics and Nutritional Ecology*. Boston, MA: Springer, 417-454.

Vleck, CM, & Vleck, D. 1987. Metabolism and energetics of avian embryos. *Journal of Experimental Zoology*, *1*, 111–125.

Vleck, D, Vleck, CM, & Hoyt, DF. 1980. Metabolism of avian embryos: Ontogeny of oxygen consumption in the rhea and emu. *Physiological Zoology*, *53*(2).

Wang, PC, & Ji, X. 1997. A comparison of embryonic metabolic rates in two lizards. *Asiatic Herpetological Research*, 7, 147–152.

Wang, PC., Ji, X, & Ma, W. 1989. Oxygen consumption of *Takydromus septentrionalis* eggs and hatchlings. *Current Herpetology in East Asia: proceedings of the second Japan-China Herpetological Symposium, Kyoto*, 49–53.

Webb, GH, Choquenot, D, & Whitehead, P. 1986. Nests, eggs and embryonic development of *Carettochelys insculpta* (Chelonia: Carettochelidae) from northern Australia. *Journal of Zoology, 1*(3), 521–550.

Whitehead, P, & Seymour, RS. 1990. Patterns of metabolic rate in embryonic crocodilians *Crocodylus johnstoni* and *Crocodylus porosus*. *Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology, 63*(2), 334–352.

Woolf, NK, Bixby, JL, Capranica, RR. 1976. Prenatal experience and avian development: brief auditory stimulation accelerates the hatching of Japanese Quail. *Science 194*(4628), 959–960.

Yun, AJ, Lee, PY, Doux, JD, & Conley, BR. 2006. A general theory of evolution based on energy efficiency: Its implications for diseases. *Medical Hypotheses*, *66*(3), 664–670.

Zarrow, MX, & Pomerat, CM. 1937. Respiration of the egg and young of the smooth green snake, *Liopeltis vernalis* (Harlan). *Growth, 1*(3), 103–110.

Zehr, DW. 1962. Stages in the normal development of the common garter snake, *Thamnophis sirtalis sirtalis*. *Copeia, 1962*(2), 322-329.

CHAPTER 3. INFLUENCE OF CALCIUM AND WATER AVAILABILITY ON OXYGEN CONSUMPTION DURING THE DEVELOPMENT OF *PANTHEROPHIS GUTATTUS*

Celeste R. Gallardo[a*], James R. Stewart[a*], Joseph R. Bidwell[a*], Thomas W. Ecay

---

[a]*Biological Sciences, East Tennessee State University, Johnson City, TN, United States*

[b]*Biomedical Sciences, James H. Quillen College of Medicine, Johnson City, TN, United States*

ABSTRACT

Influence of Calcium and Water Availability on Oxygen Consumption During the Development of *Pantherophis guttatus*

by

Celeste Gallardo

The parchment-shelled eggs of oviparous reptiles are affected by water availability during incubation. Similarly, their eggshell structure influences the storage and mobilization of calcium to developing embryos, a nutrient integral for proper skeletogenesis. Previous studies with parchment-shelled reptiles have demonstrated that increased water uptake is correlated with larger embryos, increased yolk utilization, longer incubation periods, and higher oxygen consumption. Additionally, in embryos of the red corn snake, *Pantherophis guttatus*, it has been shown that decreasing calcium availability to developing embryos by removing the outer fibrous membrane of the shell results in hatchlings reduced in size. Removal of the shell promotes water uptake, increasing the wet mass of the egg. The purpose of this study was to analyze whether there were differences in metabolic rate between *P. guttatus* embryos with intact eggs and those with eggshell calcium manually removed. These results will help determine if size differences between the two treatment types were the result of higher energy demands caused by reduced calcium availability and/or osmotic stress due to increased water uptake by the peeled eggs. Oviposited eggs were separated into three treatment groups with the outer eggshell intact, half peeled, or fully peeled. Oxygen consumption was then measured in stage 37 embryos, when metabolic rate is greatest. Results indicate no differences in metabolic rate between the treatment groups, and embryos did not differ in mass or yolk utilization. Furthermore, increased water uptake of the peeled eggs is not negatively influencing development. These data indicate that reductions in size found in similar previous studies may have been due to the reduction of calcium associated with the shell manipulation.

Introduction

Oviparous animals are specialized when compared to their viviparous counterparts, as development occurs without direct nutrient supply from the mother. Sources of energy and inorganic ions are deposited within the egg structure, and exchange with the environment is limited to the flux of water, oxygen, and carbon dioxide between the nesting site and the egg. Calcium is a primary component for the development of oviparous embryos and is integral for proper skeletogenesis (Simkiss 1967). This nutrient is localized within the yolk and eggshell, but the amount mobilized from these two compartments is influenced by phylogeny. Birds and crocodilians are most dependent on eggshell calcium (Packard 1994). This is dissimilar to turtles and lizards who generally use equal amounts of eggshell and yolk calcium for development (Packard 1994; Stewart and Ecay 2010). Snake embryos rely on eggshell calcium the least and use this compartment as a secondary source of the nutrient, as they can successfully develop without the eggshell (Packard 1994; Stewart et al. 2019). Patterns of calcium mobilization within these compartments are generally determined by eggshell type. Parchment-shelled eggs contain lower amounts of the crystalline calcium component, requiring a reliance on the yolk to compensate for this decreased nutrient source (Packard et al. 1982; Packard 1994).

Similarly, water conductance and sensitivity to hydric conditions in reptile eggs is linked to eggshell type. Because of the structure of parchment-shelled eggs, they are more impacted by changes in water conditions during incubation than are rigid eggshells (Packard et al. 1982). Increasing water availability for parchment-shelled eggs during incubation is correlated with higher egg mass, hatchling mass, and longer incubation periods (Morris et al. 1983; Packard et al. 1987; Miller and Packard 1992). Longer incubation periods could increase metabolic requirements, as tissue maintenance would be necessary for longer periods of time. Additionally,

increased substrate hydration leads to faster conversions of yolk into embryonic tissue (Miller and Packard 1992). Similar results have been recorded within several parchment-shelled lizards (*Plestiodon septentrionalis, Iguana iguana*, & *Sceloporus undulatus*), snakes (*Elaphe carinata, Pituophis melanoleucus,* & *Naja atra*) and turtles (*Caretta caretta, Chrysemys picta, Chelydra serpentina, Graptemys ouachitensis,* & *Graptemys pseudogeographica*) (Gutzke and Packard 1987; Gutzke et al. 1987; Somma 1989; Janzen et al. 1995; McGehee 1990; Packard et al. 1992; Ji and Du 2001a; Ji and Du 2001b).

Differences in metabolism between parchment-shelled embryos incubated on hydric and dry substrates have been reported, with embryos in wet environments exhibiting higher amounts of oxygen consumption when compared to those on dry substrates (Miller and Packard 1992). However, this is hypothesized to be caused by the increase in size exhibited by embryos incubated on wet substrates, rather than an increased metabolic rate triggered by heightened water availability (Miller and Packard 1992).

In this study, an experimental model was devised to test the effects of water uptake and calcium availability on embryonic oxygen consumption and growth. It has been previously reported that reducing calcium availability in the red corn snake, *Pantherophis guttatus*, by removal of the outer fibrous layer of the eggshell, causes smaller hatchlings (Stewart et al. 2019). But embryos from eggs with their outer eggshells peeled, exhibit a greater uptake of water than eggs that are left intact (Stewart et al. 2019). We tested the hypothesis that embryos from intact eggs would exhibit increased oxygen consumption when compared to those from the peeled eggs by performing metabolic experiments at embryonic stage 37 when oxygen consumption of the embryos is highest.

Materials & Methods

Incubation

Recently oviposited eggs were collected from 6 female *Pantherophis guttatus* housed in the animal care facility at East Tennessee State University over one reproductive cycle (2020, 7 clutches; mean= 15 eggs per clutch). The eggs were numbered and wet mass in grams was recorded (0.01 g) using an electronic balance (Mettler Toledo, BB240, Ohio, USA). One live embryo was sampled from each clutch to determine embryonic stage prior to incubation (Zher, 1962). Eggs were sorted by similar masses within their perspective clutch and grouped together. To alter calcium and water availability, the outer fibrous shell membrane was manually removed in 26% of the available eggs (n=24). In 29% of the eggs (n=27), half of the fibrous shell was removed. The remaining 45%, eggshells were left intact (n=42).

Eggs were placed in plastic containers filled with 2 parts water and one part vermiculite. Peeled, half-peeled, and intact eggs were aligned alternately within the incubation containers. These containers were placed in an incubator at 26 º C (Precision Model 818; Union, New Jersey, USA). Incubation temperatures were monitored and kept in range (26-30 ºC) that supported normal development. All animals were kept under appropriate conditions as described by the East Tennessee State University Animal Care and Use Protocol (Protocol #: P181201). The containers holding the eggs were opened on a weekly basis to refresh the air. Eggs were weighed using an electronic balance (0.01 g), and water was added on a weekly basis to ensure consistent water potential (Mettler Toledo, BB240, Ohio, USA).

Oxygen Consumption Trials

Using the embryonic stage at oviposition and previous data, estimates were made for the dates that embryos would reach stage 37. On those estimated dates, 3-4 eggs from the prospective clutches were weighed (0.01 g) and placed in respirometry chambers (Mettler Toledo, BB240, Ohio, USA). The respirometry chambers had a volume of 90 mL and were made of plastic tubing. The tubes had a thickness of 0.3 cm, and lids to the chamber were air-tight 1.0 cm thickness and were sealed with an O-ring. Chambers were attached to fiberoptic oxygen sensors to measure percent oxygen (Fire Sting™, Pyro Science, Aechen, Germany). These sensors were inserted into the chambers with an air-tight cable gland (Sealcon LLC, Colorado, USA). The sensors were recalibrated to the current incubator temperature before every trial. The chambers and oxygen sensors were placed in the incubator, keeping humidity and temperature controlled. Eggs were placed in the chambers and given a one-hour acclimation period. Percent oxygen was recorded in the chambers every 60 seconds for 6 hours. A peristaltic pump was attached to the chambers and flushed fresh air from the incubator into the chambers every hour and half, this was to maintain oxygen concentrations above 18% following Parker and Andrews (2006). Oxygen trials continued until embryos pipped.

After trials, developmental stage of the embryos was determined. The separate compartments of the eggs (yolk, shell, and embryo) were separated, weighed (0.0001 g), and frozen (-4 ºC) (Mettler Toledo, AL54, Ohio, USA). The separate, frozen compartments of the eggs were placed in a lyophilizer to obtain dry masses (Labconco Freeze Dryer Model 77500; Kansas City, USA).

Oxygen Conversion from Percent O_2 to Milligrams of O_2

Data were converted from percent oxygen to milligrams of oxygen. The formula used incorporates the ideal gas law (PV=nRT; where P is pressure, v is volume of the respirometry chamber, n is milligrams of oxygen, R is the ideal gas constant, and T is temperature in Kelvin). First, pressure was estimated using the barometric pressure for Johnson City, TN. Uncorrected pressure was estimated by taking the pressure of our research location (Johnson City, TN) and subtracting it by the altitude of our location (Johnson City, TN). This was applied to calculate oxygen pressure and was then multiplied by the volume of the headspace in the chambers. Second, temperature of the incubator was converted into Kelvin and multiplied by the R constant. The value obtained from the first calculation was divided by the number calculate

ed in the second to solve for milligrams of oxygen. This formula was completed for each embryo that underwent a trial. Slopes, average slopes, and average total oxygen consumption in milligrams was also calculated for each individual.

Statistical Analyses & Data Reduction

Mortality analysis was completed with a chi-square table (Microsoft Excel 2010). Treatment (peeled, half-peeled, or intact) was placed in a table with total number of eggs that survived or died. Results were significant, and individual chi-square tables were made to compare significance between each treatment (peeled vs intact, peeled vs half-peeled, half-peeled vs intact) (Microsoft Excel 2010). Embryonic dry and wet mass were analyzed for treatment effects using a general linear model univariate procedure. Embryonic dry or wet mass were the dependent variables, clutch was inputted as a random factor, treatment (peeled, half-peeled, or intact) was a fixed factor, and initial egg mass was a covariate (IBM SPSS; Armonk,

NY, USA). This procedure was also performed for yolk dry mass. Oxygen consumption was analyzed for treatment effects using a general linear model univariate procedure. Total calculated oxygen consumption was the dependent variable, clutch was inputted as a random factor, treatment was a fixed factor, and dry mass was a covariate (IBM SPSS; Armonk, NY, USA). Differences between treatments in egg mass variation during incubation were analyzed with a repeated measures ANCOVA with initial egg mass as a covariate, and clutch used as a random factor. (IBM SPSS; Armonk, NY, USA). All graphs were created with SPSS (IBM SPSS; Armonk, NY, USA). Significance was accepted in all statistical analyses if $P < 0.05$.

Results

Oviposition & Incubation

At oviposition, eggs of *P. guttatus* had a mean wet mass of 8.3 ± 0.15 g (7 clutches; n = 106 eggs; mean = 15 eggs per clutch) and contained embryos of stage 16-25 (Mean = 22, Median = 24, Mode = 24). Egg mortality significantly differed across treatments ($X^2 = 10.55$; $P < 0.05$), with intact eggs experiencing 14 egg deaths, half-peeled having 3, and peeled having 1. Intact egg mortality counts were significantly different when compared to those from the half-peeled ($X^2 = 4.54$; $P < 0.05$) and peeled ($X^2 = 7.95$; $P < 0.05$) groups. However, there was no significant difference of mortality counts between the half-peeled and peeled treatment groups ($X^2 = 0.921$; $P > 0.05$). Intact eggs outnumbered the other treatments, nearly all were extras, as two females oviposited a second round of eggs several days after the initial oviposition. These eggs were presumably infertile since they died quickly after oviposition, and this most likely caused the disparity observed between mortality and treatments.

Water Relations with Egg, Embryo, & Yolk Mass

Viable eggs from all clutches incurred net increases of water during incubation. Eggs of the intact treatment group had an average increase of 9.9 ± 0.51 g in wet mass throughout incubation. The half-peeled treatment group had an average increase of 10.5 ± 0.79 g and the peeled group had the greatest average increase of 11.2 ± 0.71 g in wet mass. Wet mass differed most between the peeled and intact eggs later in incubation, and the half peeled, and peeled eggs weighed significantly more than the intact eggs on day 77 (Figure 3.1). Despite this, the wet mass of embryos was not significantly different across treatment groups (Figure 3.2; $F = 0.0529$; $P > 0.05$). Yolk utilization was not significantly different between embryos of different treatment groups (Figure 3.3; $F = 0.498$; $P > 0.05$). Similarly, embryo dry mass was not significantly different between intact, half-peeled, and peeled eggs (Figure 3.4; $F = 0.105$; $P > 0.05$).

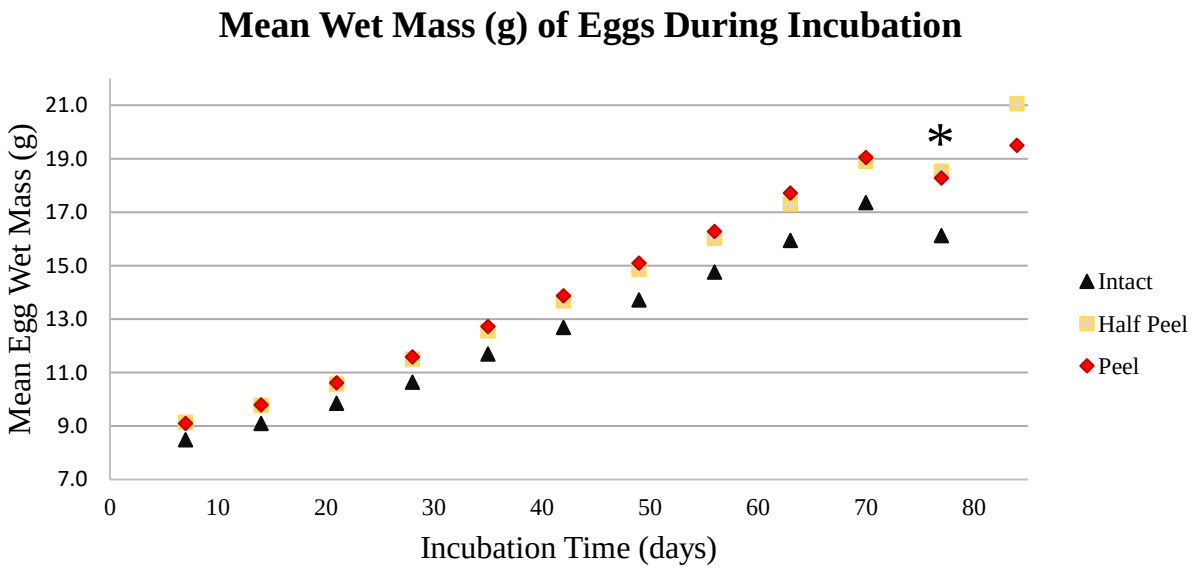


Figure 3.1. Average wet mass (g) over incubation time (days). Peeled and half peeled eggs have a significantly greater wet mass (g) later in incubation. (Intact n=42; Half Peel n=27; Peel n=24) P values less than 0.05 are summarized with one asterisk.

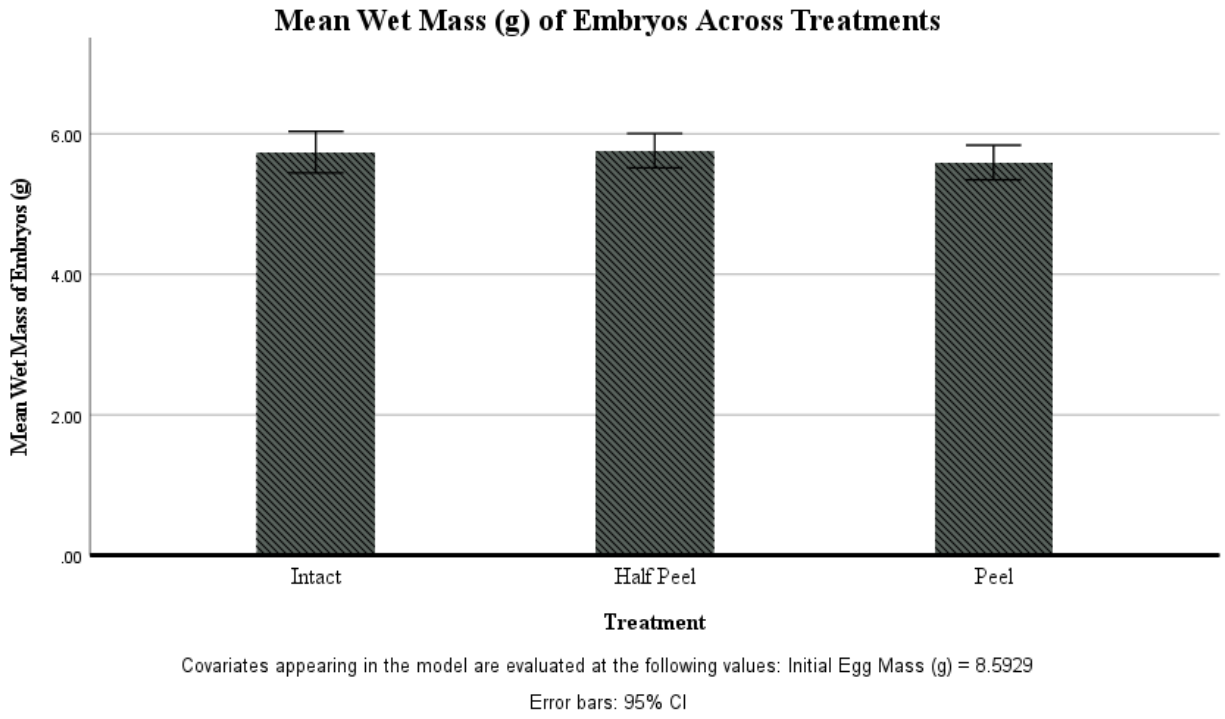


Figure 3.2. Mean wet mass (g) of embryos across the different treatment groups (intact, half peeled, or peeled). There is no significant difference between treatment groups and wet mass (g) of stage 37 embryos ($P > 0.05$; $F = 0.585$).

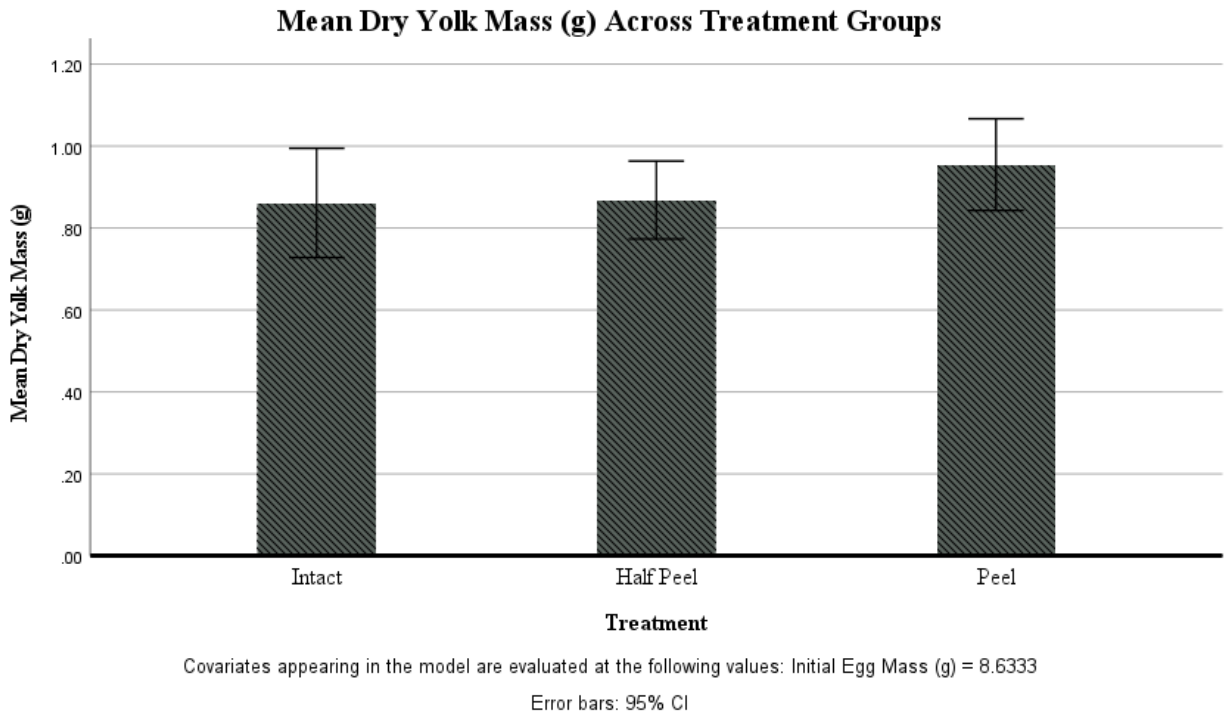


Figure 3.3. Mean dry mass (g) of yolk across the different treatment groups (intact, half peeled, & peeled). There is no significant difference between treatment groups and dry yolk mass (g) of stage 37 embryos ($P > 0.05$; $F = 4.598$).

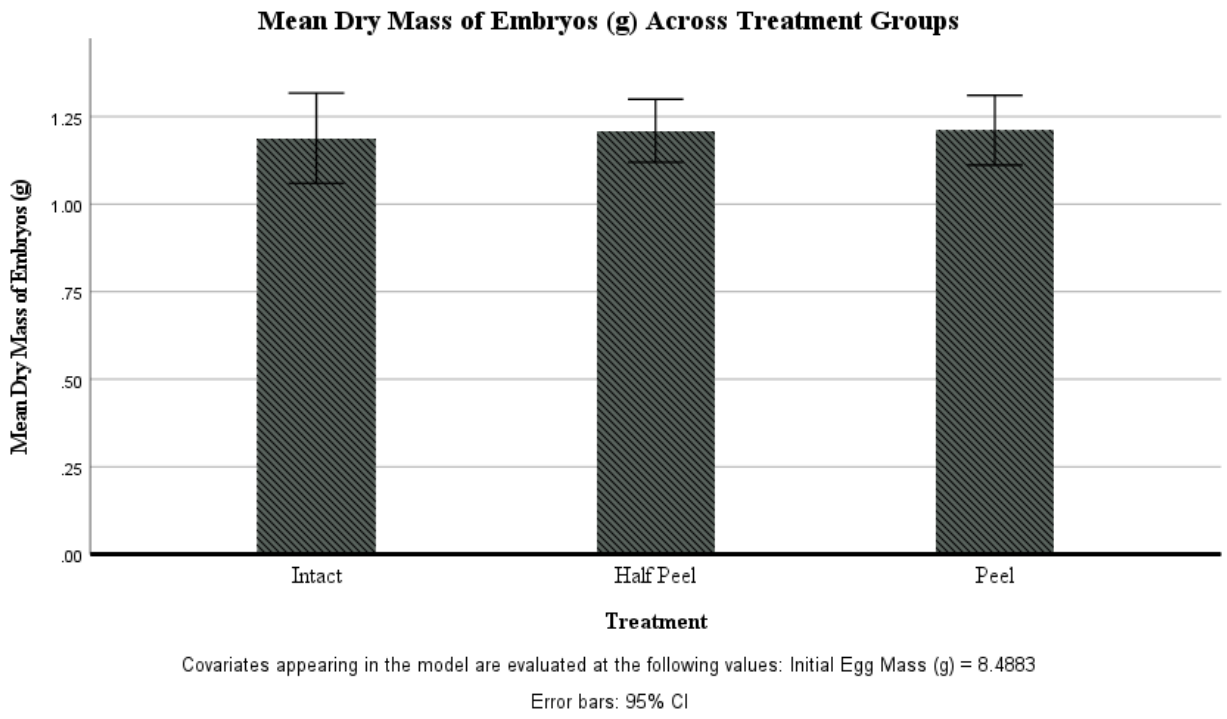


Figure 3.4. Mean dry mass (g) of embryos across the different treatment groups(intact, half peeled, & peeled). There is no significant difference between treatment groups and dry mass (g) of stage 37 embryos ($P > 0.05$; $F = 0.729$).

Oxygen Consumption

The total oxygen consumed by stage 37 embryos was not significantly different between intact, half peeled, or peeled eggs (Figure 3.5; $F = 0.713$; $P > 0.05$). The average total oxygen consumed by the intact group was 0.267 ± 0.011 mg/O_2 ($n = 16$) over the six-hour period. The average for the peeled group was 0.260 ± 0.027 mg/O_2 ($n = 17$) and the half-peeled group 0.261 ± 0.014 mg/O_2 ($n = 15$) total oxygen consumed. There was a significant effect caused by embryo dry mass ($F = 17.024$; $P < 0.01$) and clutch ($F = 4.387$; $P < 0.01$) on the oxygen uptake by the embryos.

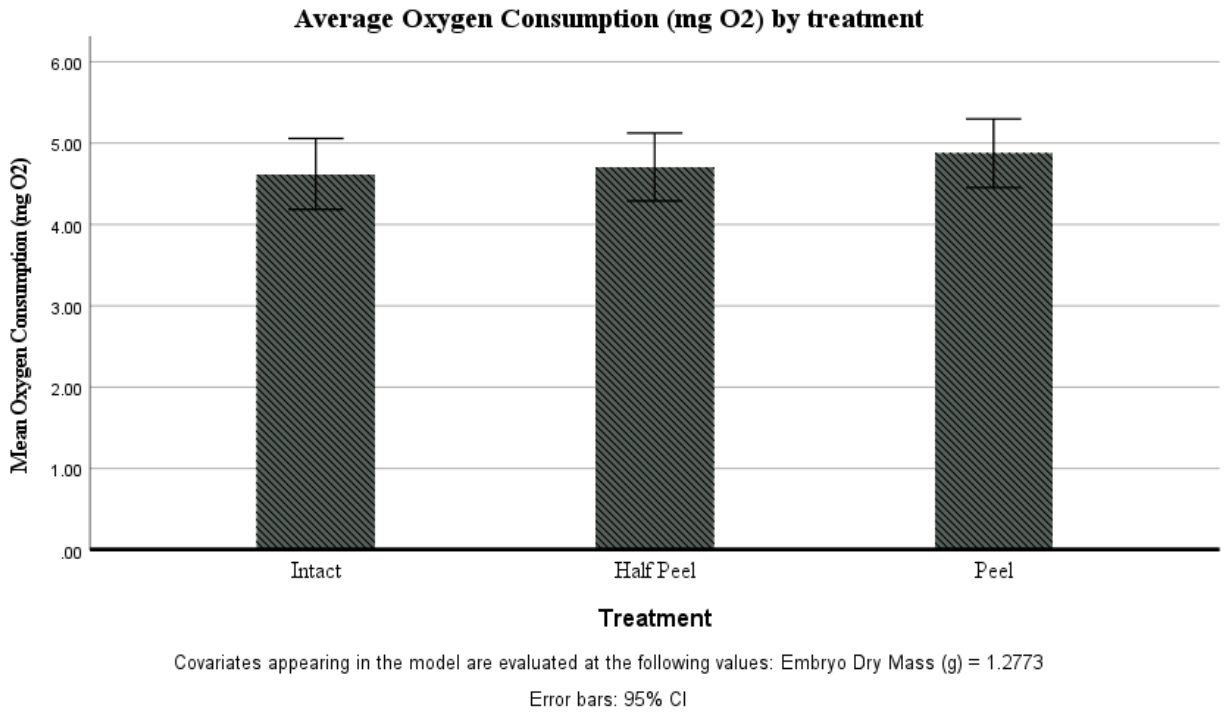


Figure 3.5. Mean dry mass (g) of yolk across the different treatment groups (intact, half peeled, & peeled). There is no significant difference between treatment groups and dry yolk mass (g) of stage 37 embryos ($P > 0.05$; $F = 4.598$).

Discussion

Embryo Sizes Between Treatment Groups

A key driver for the present study was previous work by Stewart et al. (2019) who found that *Pantherophis guttatus* hatchlings from eggs that had been completely peeled were significantly smaller than those from control eggs that had not been manipulated. These observed differences in size were initially attributed to the lack of eggshell calcium available for peeled eggs (Stewart et al. 2019). Snake embryos extract calcium from the eggshell in the later stages of

embryonic development, leading to increased growth during these stages (Packard et al. 1984; Packard 1994; Stewart et al. 2004). Additionally, results indicated no differences in yolk utilization or residual yolk quantities between treatment groups. This could indicate an energetic trade-off in the developing embryos from manipulated eggs. Reduced calcium availability may be causing increased maintenance costs for the embryo, leaving less energy available for growth. It is also possible that the increased water uptake exhibited by peeled eggs is causing osmotic stress, leading to higher metabolic rates with smaller hatchings (Stewart et al. 2019).

However, our results contradicted those of Stewart et al. 2019 in that we found no difference in embryo sizes between treatments. Because the extraction of eggshell calcium occurs towards the end of development, and residual yolk contains no calcium, any differences in growth resulting in larger hatchlings would be observed in the final embryonic stage (Packard et al. 1984; Packard 1994; Stewart et al. 2004; Stewart et al. 2019). After hatching, calcium is required to support the continued growth and skeletogenesis of neonates, and since residual yolk does not contain any of the nutrient, it must be obtained from the diet (Packard 1994). Growth will not occur if the hatchling cannot obtain calcium. For this study, we were unable to collect hatchling data, as we attempted to conserve all eggs for respirometry trials, but due to the patterns of calcium mobilization elicited by snakes, it is unlikely we would have observed differences in sizes of our hatchlings. If growth were not different between treatment groups at the last stage of development, the hatchlings would not differ in size, since no calcium would remain in the residual yolk. Our results may not have followed that of Stewart et al. 2019 because of differences in sample sizes. Experiments were performed over two reproductive seasons, which doubled their sample sizes, this was not possible for the current study. Perhaps,

obtaining more clutches and completing multiple seasons would rear stronger results in the future.

Influence of Water on Metabolic Rate

The lack of difference in oxygen consumption between treatment groups, allows us to conclude that water is not causing negative effects on growth. This is supported by previous studies, which have investigated the potential difference of metabolic rates in embryos from parchment-shelled eggs incubated on wet and dry substrates. In the lizard, *Lampropholis guichenoti,* no difference in phenotypic traits were observed when eggs were incubated on different moisture contents (Du and Shine 2008). Within snakes, differences in oxygen consumption were not recognized in the Burmese python, *Python bivittatus,* when embryos were incubated on substrates with different water contents (Black et al. 1984). Conversely, some studies do report increases in metabolic rate and growth (Gutzke and Packard 1987; Gutzke et al. 1987; Somma 1989; McGehee 1990; Packard et al. 1992; Janzen et al. 1995; Ji and Du 2001a; Ji and Du 2001b). These disparities between results could be due to the use of different water potentials, substrates, or methods for measuring metabolic rate. Yet, these conclusions support the generalization that in proper amounts, water will not impede success of embryos from parchment-shelled eggs. It should be noted that, too much water can be detrimental, as oxygen flow into the egg may be hindered (Ewert 1985; Packard and Packard 1988; Seymour et al. 1996). Mold growth can additionally occur which can kill the embryo (McGehee 1990). It would be beneficial to analyze metabolic rates and determine at what water potential osmotic stress is reached.

Summary

In this study, calcium and water availability were manipulated in eggs of the red corn snake, *Pantherophis guttatus*, by peeling the outer fibrous membrane of the eggshell. Previous work by Stewart et al. 2019, indicated hatchlings from peeled eggs were significantly smaller in size then those from intact eggs. We aimed to determine whether the differences observed in sizes resulted from metabolic rate differences, caused by higher energy demands from reduced calcium availability or osmotic stress from increased water uptake by peeled eggs. Our results differed from that of Stewart et al. 2019, we saw no differences in the wet or dry mass of embryos across the treatment groups and saw no differences in metabolic rate. Despite this, eggs from both the peeled and half peeled eggs took in significantly more water when compared to intact eggs during late incubation. With our data, it can be concluded that water is not causing osmotic stress, hindering development within the embryo, since differences in oxygen consumption were not observed when water uptake was increased. Future studies should incorporate larger sample sizes and include hatchling data.

REFERENCES

Ackerman, RA. 1980. Physiological and ecological aspects of gas exchange by sea turtle eggs. *American Zoologist*, *20*(3), 575–583.

Ackerman, RA. 1991. Physical factors affecting the water exchange of buried reptile eggs. In Deeming, DC, and Ferguson, MJ. (eds), *Egg incubation: Its effects on embryonic development in birds and reptiles*, Cambridge: Cambridge University Press, 193–212.

Aubret, F, Blanvillain, G, Bignon, F, Kok, PJR. 2016. Heartbeat, embryo communication and hatching synchrony in snake eggs. *Scientific Reports*, *6*(23519), 1-5.

Black, CP, Birchard, GF, Schuett, GW, & Black, VD. 1984. Influence of incubation water content on oxygen consumption in embryos of the Burmese python (*Python molurus bioittatus*). In: Seymour RS. (eds), *Respiration and Metabolism of Embryonic Vertebrates. Perspectives in vertebrate science*, Dordecht: Springer,*3*, 137–145.

Cannon, ME, Carpenter, RE, & Ackerman, RA. 1986. Synchronous hatching and oxygen consumption of Darwin's rhea eggs (*Pterocnemia pennata*). *Physiological Zoology*, *59*, 95–108.

Chen, CK, Chuang, HF, Wu, SM, & Li, WH. 2019. Feather evolution from precocial to altricial birds. *Zoological Studies*, *58*(24), 1-12.

Clark, H. 1935. Metabolism of the black snake embryo I. Nitrogen excretion. *Journal of Experimental Biology*, *30*, 492–501.

Crawford, NG, Parham, JF, Sellas, AB, Faircloth, BC., Glenn, TC, Papenfuss, TJ, … Simison, BW. 2015. A phylogenomic analysis of turtles. *Molecular Phylogenetics and Evolution*, *83*, 250–257.

Dmi'el, R. 1969. Circadian rhythm of oxygen consumption in snake embryos. *Life Sciences*, 8(24), 1333-1341.

Dmi'el, R. 1970. Growth and metabolism in snake embryos. *Journal of Embryology and Experimental Morphology*, *23*(3), 761–772.

Dmi'el, R, & Borut, A. 1972. Thermal behavior, heat exchange, and metabolism in the desert snake *Spalerosophis cliffordi*. *Physiological Zoology*, *45*(1), 78–94.

Doody, JS, Paull, P. 2013. Hitting the ground running: Environmentally cued hatching in a lizard. *Copeia*, *2013*(1), 160-165.

Du, W, & Shine, R. 2008. The influence of hydric environments during egg incubation on embryonic heart rates and offspring phenotypes in a scincid lizard (*Lampropholis guichenoti*). *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*, *151*(1), 102–107.

Ewert, MA. 1985. The embryo and its egg: Development and natural history. In Harless, M and Morlock, H (eds), *Turtles: Perspectives and Research*. New York: Wiley, 333-413.

Gemmell, NJ, Rutherford, K., Prost, S et al. 2020. The tuatara genome reveals ancient features of amniote evolution. *Nature*, *584*, 403–409.

Gettinger, RD, Paukstis, GL, & Gutzke, WH. 1984. Influence of hydric environment on oxygen consumption by embryonic turtles *Chelydra serpentina* and *Trionyx spiniferus*. *Physiological Zoology*, *57*(4), 468–473.

Green, RE, Braun, EL, Armstrong, J, Dent, E, Nguyen, N, Hickey, G, & Vandewege, M. 2014. Three crocodilian genomes reveal ancestral patterns of evolution among archosaurs. *Science*, *346*(6215), 1335-1342.

Greene, HW, May, P, Hardy, D, Scitturo, J, & Farrell, T. 2002. Parental behavior by vipers In Schuett, GW, Höggren, M, Douglas, ME, and Greene, HW. (eds), *Biology of the Vipers.* Eagle Mountain (UT): Eagle Mountain Publishing, 179–206.

Gutzke, WH, & Packard, GC. 1987. Influence of the hydric and thermal environments on eggs and hatchlings of bull snakes *Pituophis melanoleucus*. *Physiological and Biochemical Zoology*, *60*(1), 9–17.

Gutzke, WH, Packard, GC, Packard, MJ, & Boardman, TJ. 1987. Influence of the hydric and thermal environments on eggs and hatchlings of painted turtles (*Chrysemys picta*). *Herpetologica*, *43*(4), 393–404.

Hoyt, DF, Vleck, D, & Vleck, CM. 1978. Metabolism of avian embryos: ontogeny and temperature effects in the ostrich. *The Condor*, *80*, 265–271.

Janzen, FJ, Ast, JC, & Paukstis, GL. 1995. Influence of the hydric environment and clutch on eggs and embryos of two sympatric map turtles. *Functional Ecology*, *9*(6), 913–922.

Jenkins, NK, & Simkiss, K. 1968. The calcium and phosphate metabolism of reproducing reptiles with particular reference to the adder (*Vipera berus*). *Comparative Biochemistry and Physiology*, *26*, 865–876.

Ji, X, & Du, W. 2001a. Effects of thermal and hydric environments on incubating eggs and hatchling traits in the cobra, *Naja naja atra. Journal of Herpetology*, *35*(2), 186–194.

Ji, X, & Du, W. 2001b. The effects of thermal and hydric environments on hatching success, embryonic use of energy and hatchling traits in a colubrid snake, *Elaphe carinata*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *129*(2– 3), 461–471.

Koch, F, & Wieser, W. 1983. Partitioning of energy in fish: Can reduction of swimming activity compensate for the cost of production? *Journal of Experimental Biology*, *107*(1), 141–146.

Lourdais, O, Hoffman, TCM, & Dale, F. 2007. Maternal brooding in the children's python (*Antaresia childreni*) promotes egg water balance. *Journal of Comparative Physiology B, 177*, 569–577.

McGehee, MA. 1990. Effects of moisture on eggs and hatchlings of loggerhead sea turtles (*Caretta caretta*). *Herpetologica*, *46*(3), 251–258.

Miller, K, & Packard, GC. 1992. The influence of substrate water potential during incubation on the metabolism of embryonic snapping turtles (*Chelydra serpentina*). *Physiological Zoology*, *65*(1), 172–187.

Morris, KA, Packard, GC, Boardman, TJ., & Packard, M.J. 1983. Effect of the hydric environment on growth of embryonic snapping turtles (*Chelydra serpentina*). *Herpetologica*, *39*(3), 272–285.

Oftedal, OT. 2002. The origin of lactation as a water source for parchment-shelled eggs. *Journal of Mammary Gland Biology and Neoplasia*, *7*(3), 253–266.

Packard, GC, & Packard, MJ. 1980. Evolution of the cleidoic egg among reptilian antecedents of birds. *American Zoologist*, *20*(2), 351–362.

Packard, GC, & Packard, MJ. 1986. Effect of water balance on growth and calcium mobilization of embryonic painted turtles (*Chrysemys picta*). *Physiolical Zoology*, *58*(4), 398–405.

Packard, GC, & Packard, MJ. 1987. Water relations and nitrogen excretion in embryos of the oviparous snake *Coluber constrictor*. *Copeia*, *1987*(2), 395–406.

Packard, GC, & Packard, MJ. 1988. The physiological ecology of reptilian eggs and embryos. In Gans, C and RB (eds), *Biology of the Reptilia*, *16*, 523-605.

Packard, GC, Packard, MJ, Miller, K, & Boardman, TJ. 1987. Influence of moisture, temperature, and substrate on snapping turtle eggs and embryos. *Ecology, 68*(4), 983–993.

Packard, GC, Tracy, CR, & Roth, JJ. 1977. The physiological ecology of reptilian eggs and embryos, and the evolution of viviparity within the class Reptilia. *Biological Reviews.*, *52*, 71–105.

Packard, MJ. 1994. Patterns of mobilization and deposition of calcium in embryos of oviparous, amniotic vertebrates. *Israel Journal of Zoology*, *40*(3-4), 481–492.

Packard, MJ, & Packard, GC. 1991. Patterns of mobilization of calcium, magnesium, and phosphorus by embryonic yellow-headed blackbirds (*Xanthocephalus xanthocephalus*). *Journal of Comparative Physiology B, 160*(6), 649–654.

Packard, MJ, & Packard, GC. 1984. Comparative aspects of calcium metabolism in embryonic reptiles and birds. In Seymour, RS (eds), *Perspectives in vertebrate science*.

Packard, MJ, Packard, GC, & Boardman, TJ. 1982. Structure of eggshells and water relations of reptilian eggs. *Herpetologica, 38*(1), 136–155.

Packard, MJ, Packard, GC, & Gutzke, WH. 1984. Calcium metabolism in embryos of the oviparous snake *Coluber constrictor*. *Journal of Experimental Biology*, *110*(1), 99–112.

Parker, SL, & Andrews, RM. 2006. Evolution of viviparity in Sceloporine lizards: In utero PO_2 as a developmental constraint during egg retention. *Physiological and Biochemical Zoology*, *79*(3), 581-592.

Packard, MJ, Phillips, JA, & Packard, GC. 1992. Sources of mineral for green iguanas (*Iguana iguana*) developing in eggs exposed to different hydric environments. *Copeia, 1992*(3), 851–858.

Robert, KA, & Thompson, MB. 2000. Energy consumption by embryos of a viviparous lizard, *Eulamprus tympanum*, during development. *Biochemistry*, *127*(4), 481–486.

Seymour, RS, Kennett, R, & Christian, K. 1996. Osmotic balance in the eggs of the turtle *Chelodina rugosa* during developmental arrest under water. *Physiological Zoology*, *70*(3), 301–306.

Shadrix, CA, Crotzer, DR, McKinney, SL, & Stewart, JR. 1994. Embryonic growth and calcium mobilization in oviposited eggs of the scincid lizard. *Copeia*, *1994*(2), 493–498.

Simkiss, K. 1967. Calcium in Reproductive Physiology. In *A Comparative Study of Vertebrates*. London: Chapman and Hall Ltd., Reinhold Publishing, New York.

Somma, LA. 1989. Influence of substrate water content on neonate size in the prairie skink, *Eumeces septentrionalis*. *The Great Basin Naturalist*, *49*(2), 198–200.

Stewart, JR, & Ecay, TW. 2010. Patterns of maternal provision and embryonic mobilization of calcium in oviparous and viviparous squamate reptiles. *Herpetological Conservation and Biology*, *5*, 341–359.

Stewart, JR, Ecay, TW, & Blackburn, DG. 2004. Sources and timing of calcium mobilization during embryonic development of the corn snake, *Pantherophis guttatus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *139*(3), 335–341.

Stewart, JR, Ecay, TW, Heulin, B. 2009. Calcium provision to oviparous and viviparous embryos of the reproductively bimodal lizard *Lacerta* (*Zootoca*) *vivipara*. *Journal of Experimental Biology*, *212*(16), 2520-2524.Stewart, JR, Pyles, RA, Mathis, KA, &

Ecay, TW. 2019. Facultative mobilization of eggshell calcium promotes embryonic growth in an oviparous snake. *Journal of Experimental Biology*, *222*(3), 1-7.

Thompson, MB. 1989. Patterns of metabolism in embryonic reptiles. *Respiration Physiology*, *76*(2), 243–255.

Thompson, MB, & Russell, KJ. 1999. Embryonic energetics in eggs of two species of Australian skink, *Morethia boulengeri* and *Morethia adelaidensis*. *Journal of Herpetology*, *33*(2), 291–297.

Thompson, MB, & Stewart, JR. 1997. Embryonic metabolism and growth in lizards of the genus *Eumeces*. *Comparative Biochemistry and Physiology*, *118*(3), 647–654.

Vergne, AL, Mathevon, N. 2008. Crocodile egg sounds signal hatching time. *Current Biology*, *18*(12), R513–R514.

Vince, MA, & Chinn, S. 1971. Effects of accelerated hatching on the initiation of standing and walking in the Japanese quail. *Animal Behavior*, *19*(1), 62–66.

Vleck, CM, & Hoyt, D.F. 1991. Metabolism and energetics of reptilian and avian embryos. In Deeming, DC & Ferguson, MJ (eds), *Egg incubation: Its effects on embryonic development in birds and reptiles*, Cambridge: Cambridge University Press, 285-304.

Vleck, CM, Hoyt, DF, & Vleck, D. 1979. Metabolism of avian embryos: Patterns in altricial and precocial birds. *Physiological Zoology*, *52*(3), 363–377.

Vleck, CM, & Vleck, D. 1980. Patterns of metabolism and growth in avian embryos. *American Zoologist*, *20*(2), 405–416.

Vleck, CM, & Vleck, D. 1996. Embryonic energetics. In C. Carey (eds), *Avian Energetics and Nutritional Ecology*. Boston, MA: Springer, 417-454.

Vleck, CM, & Vleck, D. 1987. Metabolism and energetics of avian embryos. *Journal of Experimental Zoology*, *1*, 111–125.

Vleck, D, Vleck, CM, & Hoyt, DF. 1980. Metabolism of avian embryos: Ontogeny of oxygen consumption in the rhea and emu. *Physiological Zoology*, *53*(2).

Wang, PC, & Ji, X. 1997. A comparison of embryonic metabolic rates in two lizards. *Asiatic Herpetological Research*, 7, 147–152.

Wang, PC, Ji, X, & Ma, W. 1989. Oxygen consumption of *Takydromus septentrionalis* eggs and hatchlings. *Current Herpetology in East Asia: proceedings of the second Japan-China Herpetological Symposium, Kyoto*, 49–53.

Warner, D, Moody, M, & Telemeco, R. 2011. Is water uptake by reptilian eggs regulated by physiological processes of embryos or a passive hydraulic response to developmental environments? *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *160*(3), 421–425.

Webb, GH, Choquenot, D, & Whitehead, P. 1986. Nests, eggs and embryonic development of *Carettochelys insculpta* (Chelonia: Carettochelidae) from northern Australia. *Journal of Zoology*, *1*(3), 521–550.

Whitehead, P, & Seymour, RS. 1990. Patterns of metabolic rate in embryonic crocodilians *Crocodylus johnstoni* and *Crocodylus porosus*. *Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology*, *63*(2), 334–352.

Woolf, NK, Bixby, JL, Capranica, RR. 1976. Prenatal experience and avian development: brief auditory stimulation accelerates the hatching of Japanese Quail. *Science 194*(4628), 959–960.

Yun, AJ, Lee, PY, Doux, JD, & Conley, BR. 2006. A general theory of evolution based on energy efficiency: Its implications for diseases. *Medical Hypotheses*, *66*(3), 664–670.

Zarrow, MX, & Pomerat, CM. 1937. Respiration of the egg and young of the smooth green snake, *Liopeltis vernalis* (Harlan). *Growth, 1*(3), 103–110.

Zehr, DW. 1962. Stages in the normal development of the common garter snake, *Thamnophis sirtalis sirtalis*. *Copeia, 1962*(2), 322-329.

VITA

CELESTE GALLARDO

Education: M.S. Biology, East Tennessee State University, Johnson City, Tennessee, 2021

B.S. Biology, East Tennessee State University, Johnson City, Tennessee, 2018

Public Schools, Northville, Michigan & Oak Ridge, Tennessee

Professional Experience: Graduate Assistant, East Tennessee State University, College of Arts and Sciences, 2019-2021

Pest Survey Specialist, USDA/East Tennessee State University, College of Arts and Sciences, Summer 2020

Specimen Accessioner, Lab Corp, UT Medical Center, Knoxville, Tennessee, 2018-2019

Field Assistant, Gray Fossil Site, Gray, Tennessee, Summer 2017

Lab Technician, Gray Fossil Site, Gray, Tennessee, 2016-2018

Presentations: March 2021. Influence of calcium and water availability on oxygen consumption in the oviparous snake, *Pantherophis guttatus*. ETSU Biological Sciences Seminar. Johnson City, TN.

February 2021: Embryonic metabolism in the embryos of the oviparous snake, *Pantherophis guttatus.* Society for Integrative and Comparative Biology. Virtual.

September 2019. Oxygen consumption in the embryos of the oviparous snake, *Pantherophis guttatus.* Tennessee

Herpetology Conference. Johnson City, TN.

November 2019. Influence of Incubation Conditions on Embryonic Oxygen Consumption in the Oviparous Snake, *Pantherophis guttatus.* ETSU Biological Sciences Seminar. Johnson City, TN.

Honors and Awards: Dean's List, East Tennessee State University, Spring 2017

Dean's List, East Tennessee State University, Fall 2017

Dean's List, East Tennessee State University, Spring 2018