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Impact of Reproductive Mode on Skeletal Development in a Reproductively Bimodal Squamate Species

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Impact of Reproductive Mode on Skeletal Development in a Reproductively Bimodal Squamate Species

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by

Amanda C. Tedder

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Viviparity, skeletal development, squamates

ABSTRACT

Impact of Reproductive Mode on Skeletal Development in a Reproductively Bimodal Squamate Species

by

Amanda C. Tedder

Viviparity has evolved multiple times within squamates. Eggshells are reduced in viviparous forms, which reduces calcium available to embryos during development. This study tested the hypothesis that reduced calcium impacts neonates of viviparous forms. Developmental series from oviparous and viviparous populations of *Zootoca vivipara* (reproductively bimodal) were cleared & stained to reveal bone development. Photographs (high magnification, calibrated to size) were used to obtain measurements of lengths of the body, humerus, femur, skull and lower jaw, and of ossified portions of limb bones. Percent ossification was scored for targeted skull bones. Results were analyzed using general linear models and revealed no differences in ossification in either limbs or skull. Overall size of oviparous neonates was significantly larger. Findings do not support our hypothesis and indicate that reduction in eggshell calcium in embryos of viviparous populations does not negatively impact limb or skull ossification during development but may influence overall size.

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

A key characteristic of all living organisms is the ability to reproduce and pass on genetic

information from one generation to the next. A major distinction in reproductive modes

among vertebrates is oviparity and viviparity. Oviparous females reproduce by laying eggs with

calcified shells that develop in the external environment, whereas viviparous females retain

fertilized eggs that have a reduced or absent calcareous exterior and give birth to live young

(Blackburn 1999). Modern views of these modes recognize matrotrophy (placenta supplies

additional nutrition) as a separate phenomenon coupled with viviparity (Blackburn 1992).

Viviparity has evolved independently in several lineages of vertebrates, such as cartilaginous

fish, sharks and rays, bony fish, reptiles and mammals.

Among reptiles, oviparity represents the ancestral form of reproduction, a finding

supported by the presence of an egg-tooth even in viviparous embryos. Although oviparity is

also the ancestral mode for the squamate lineage, snakes and lizards, they have remarkably

evolved viviparity independently over 100 times, which is more than all other vertebrates

combined (Blackburn 2000). Within squamates, viviparity has evolved at low taxonomic levels

and in recent geological times, making squamates an ideal organism to study the evolution of

viviparity (Blackburn 2006).

Viviparity has evolved so frequently within the squamate lineage that benefits and costs

of both modes of reproduction have been in question. A recent study focused on the relative

costs of reproduction in the European common lizard (*Zootoca vivipara*), which is

reproductively bimodal with some populations oviparous and others viviparous (Foucart et al. 2014). This study demonstrated that the costs of reproduction in metabolic expense in *Zootoca vivipara* is similar between the two reproductive modes until oviposition (egg-laying). By retaining eggs through the exponential growth stages of development, viviparous females have a greater rise in metabolic rate over a prolonged gestation period. During pregnancy, viviparous females change thermoregulatory behavior by displaying an increase in half-basking behavior (only basking part of the female's body) and a higher weight gain than oviparous females. This behavior is known to be a compromise between allocating more time to basking, while also avoiding predation. During pregnancy, both oviparous and viviparous females are slower in mobility and increase their chances of predation (Bleu et al. 2012). Half-basking behavior is more common in viviparous populations than oviparous during egg retention. This behavioral change is one possible mechanism viviparous females use to save energy early in pregnancy that can later be allotted to the cost of a longer gestation period. Thermal preferences between oviparous and viviparous females reflect optimal temperatures for embryonic development, not maternal preferences, and are dependent on maternal body condition (Carretero et al. 2005).

Another trade-off that has been demonstrated in viviparous *Zootoca* is an adjustment of reproductive investment, where reproductive success is positively correlated with amount of rainfall and female body size (Bleu et al. 2012). During years of less rainfall (i.e., food availability), individual offspring mass is reduced while litter size is preserved in both oviparous and viviparous lizards (Bleu et al. 2013).

One hypothesis for the transition to viviparity in lizards is a gradualistic evolutionary model. This model states that the evolution of viviparity occurs due to a gradual and consistent change (Blackburn 1995). In squamates this change would occur in three steps: a gradual increase in egg retention, a reduction in thickness of eggshell (enabling the formation of a simple placenta) and a progressive reliance on placenta for nutrient and gas exchange (Blackburn 1995). This model predicts a long chain of intermediates would occur between reproductive types. Support for this gradualistic approach in lizards is found in the cold-climate model--- extended egg retention at higher altitudes would reduce egg mortality as well as speed up development by keeping eggs at optimal temperatures inside the female, avoiding cold nest temperatures (Andrews 2000).

Support for the cold-climate hypothesis has been demonstrated in freeze tolerance differences between oviparous and viviparous populations of *Zootoca vivipara.* Viviparous populations can tolerate sub-freezing temperatures for up to 24 hours, while oviparous populations can only tolerate only five hours of sub-freezing temperatures. Although, both modes of reproduction can tolerate 50% of their body water freezing, oviparous lizards cannot stabilize ice accumulation as effectively as the viviparous lizards (Voituron et al. 2004). Due to the nature of the moist habitats coupled with higher altitudes, viviparous populations experience more freezing temperatures than the oviparous, lowland populations. Further support for the cold climate model within this species is demonstrated in oviparous *Z. vivipara* at different elevations. Embryos from higher elevations are oviposited (laid) at later stages of development and complete external incubation quicker than oviparous populations at lower elevations (Rodriquez-Dias & Brana 2012)**.**

An alternative explanation to the gradualistic model for the evolution of viviparity in squamates is the punctuated equilibrium model. This model suggests that oviparity and viviparity may represent periods of stasis. Regions of rapid change can occur by mutations or by significant environmental change in a shorter duration of time than the gradualistic model. This model predicts that few intermediate forms may exist if they are not selected against. Prolonged egg retention could represent an intermediate between the two modes (Blackburn 1995). Another alternative hypothesis to the gradualistic model of viviparity is the saltation model. This model hypothesizes that, due to a mutation, some females within an oviparous population should exhibit longer periods of egg-retention, giving birth to fully developed young and leading to speciation (Blackburn 1995). This model predicts that no intermediates between reproductive type should exist.

Within the squamate lineage, lizards of the monophyletic family Lacertidae have been divided by DNA analysis into two subfamilies: Gallotiinae and Lacertinae (Fu 2000). The Lacertinae is divided further into Eurasian and African groups. Among the Eurasian clade of lacertine lizards is our species of interest, *Zootoca vivipara*, which is reproductively bimodal, with both oviparous and viviparous populations. This natural bimodal condition enables a direct study of differences in skeletal development possible within a single species. Recent phylogenetic analysis of *Z. vivipara* concluded the most parsimonious scenario was that the ancestor of *Z. vivipara* was oviparous, and that viviparity evolved one time with one reversal back to oviparity in the Western oviparous clade (Surget-Groba et al. 2006). Oviparous specimens used for this study belong to this Western oviparous clade. An alternative hypothesis is that viviparity evolved in three distinct lineages, which is plausible but not most

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parsimonious. It is worth noting that every attempt at construction of the squamate and Lacertidae phylogeny has noted the difficulty in creating an accurate phylogeny even when using DNA sequence analysis.

Zootoca vivipara, the European Common Lizard, is found from Central France to the British Isles up to Scandinavia and into Eastern Russia (Heulin et al. 1999). The life history of *Z. vivipara* is an important consideration when comparing the two modes of parity. Females of both reproductive modes become mature between the ages of one and two years old, while the males become mature once they have reached a minimum length of 40--42mm SVL (snout-vent length), usually between one and three years of age. In viviparous populations, females only produce one clutch annually. However, in oviparous populations the number of clutches per year depends on the size of the individual female. Large females start reproducing early in the breeding season and produce two to three clutches a year, whereas smaller females start later in the season and produce only one clutch (Bauwens 1999).

Food availability plays a primary role in life-history of *Zootoca vivipara*. Males and females in viviparous populations have similar flexibility for energy reallocation during times of low food availability. In the presence of predator cues, male lizards will lower thermoregulatory efficiency to avoid predators, however post-partum females will not lower thermoregulation and will maintain overall higher activity than males. This response difference to predator cues in times of low food availability may be a means to regain body condition before the next reproduction cycle in females (Herczeg et al. 2008). Within viviparous populations, the effect of food deprivation has a stronger impact on adults than on yearlings; however large yearlings are more affected by competition with older conspecifics due to niche overlapping (Mugabo et al.

2011). Spatial distribution of these lizards within their populations has been attributed to the presence of adult conspecifics. Although biotic interactions may play a role in habitat selection for these lizards, the presence of the potential mates determines their distribution (Penalver-Alcazar et al. 2016).

Oviparous (egg-laying) females oviposit eggs with thick eggshells composed of a mat of fibrous protein overlain by a layer of calcium carbonate (Huelin et al. 2005). Developing embryos depend on calcium-rich yolk as a primary source of nutrients for development, but also rely on calcium extracted from the eggshell as an important secondary source of calcium (Stewart et al. 2009). Among squamates, yolk can provide 19--86% of calcium to the embryo (Stewart & Ecay 2010). Of species sampled, snake embryos extract 72--86% of hatchling calcium from yolk, while lizards range from 19--78%. For oviparous species, the remaining calcium is extracted from the eggshell. *Zootoca vivipara* have calcium-poor yolk among both viviparous and oviparous populations. However, the oviparous populations extract a higher percentage of their calcium from eggshell than most other lizards (Stewart et al. 2009). An oviparous population of *Z. vivipara* from Louvie has eggshells that supply most of the calcium to developing embryos; 81% of calcium mass in hatchlings is extracted from the eggshell. Thus, there is a wide range in utilization of yolk calcium and shell calcium extraction among squamates during development.

Viviparous embryos are retained inside the female oviduct and eggs either lack or have a reduced calcareous layer of the shell. As a result, the chorioallantoic membrane is in close proximity with the uterine epithelium forming a simple placenta (Blackburn 1993). The absence or reduction of the crystallized calcium on the eggshell in viviparous eggs results in a

considerable decrease in the amount of calcium available to embryos, as compared with oviparous (Stewart et al. 2009). Viviparous populations of *Z. vivipara* are predominately lecithotrophic, receiving most of their nutrition from the yolk. However, hatchlings from the viviparous Paimpont population obtain 76% of their calcium from placental transfer, which is unusually high for lecithotrophic lizards. In viviparous species that do not form the calcareous eggshell layer, embryos can be totally dependent on yolk calcium, or can rely on calcium supplemented by placental transfer. In a few highly specialized viviparous species, such as *Mabuya*, yolk calcium is reduced or absent and calcium is provided almost entirely by placental transfer (Ramirez-Pinilla 2006).

Calcium can be delivered to developing embryos once their tissues are able to transport calcium to circulation. Once this function has developed, embryos can obtain calcium from the eggshell or by placental transfer, depending on reproductive mode. Calcium mobilization requires regulated transport across a boundary layer of maternal cells and mobilization can occur by passive or active diffusion (Stewart & Ecay 2010). The mechanism for transcellular transport of calcium is highly conserved between the two reproductive modes (Stewart et al. 2011).

Developing embryos rely on calcium for many functions including skeletal ossification. Calcium can be found in the form of calcium carbonate present on the outer layer of the eggshell in oviparous lizards. It can also be found as a free ionic calcium, used in placental transport to developing embryos, as well as bound to yolk proteins and lipids (Stewart & Ecay, 2010). Calcium is also found in the form of calcium phosphate which is responsible for the formation of hydroxyapatite laid down during skeletal ossification.

Within different taxa, skeletal ossification has been demonstrated to follow predictable patterns. A possible correlation between viviparity and less skeletally mature hatchlings was proposed by Maisano (2001); within the 21 species of lizards she sampled, the six least skeletally mature were all viviparous. Two types of reduction in skeletal ossification could be occurring within these viviparous species---reduction in chondrification (cartilage) or reduction in ossification of either endochondral or dermal bones (Rieppel 1992). Heterochrony (shifts in rate or timing in development) could also play a role in skeletal reduction. Reduction of adult bones in snakes has been shown to result from significant differences in the timing of development (Werenburg & Sanchez-Villagara 2014).

In viviparous populations of *Zootoca vivipara,* the first ossification in the skeleton has been reported to appear in the skull followed by ossification of the limbs, vertebrae, associated ribs and haemal arches, in an anteroposterior sequence (Rieppel 1992). Dermal ossification in the pelvic and pectoral girdle appears to precede endochondral ossifications, but chondral ossification in the pectoral girdle occurs after the pelvic girdle. Ossification of tarsal (hind foot) elements also precede the carpal (forefoot) elements but are the last to ossify (Rieppel 1992).

Based on known calcium provisioning and eggshell content within the two modes of parity the following question was addressed: Does the reduction in eggshell calcium associated with viviparity versus oviparity result in different developmental patterns in the reproductively bimodal species *Zootoca vivipara*? Specifically, I tested the hypothesis that viviparous neonates will be more skeletally immature than oviparous hatchlings (Maisano 2001).

To test this hypothesis skeletal ossification during embryonic development in one oviparous and one viviparous population of *Z. vivipara* was measured and sequenced. If my hypothesis is supported, there will be reduced ossification at birth caused by either a difference in the rate or timing of ossification in viviparous lizards. If the hypothesis is not supported, there will be no difference in skeletal ossification between oviparous and viviparous populations because calcium either is not limited during development or any deficiency from a reduced eggshell is replaced by placental transfer.

CHAPTER 2

METHODS

Pregnant female *Zootoca vivipara* were collected in September 2007 from southern

France; oviparous females were collected from Louvie (43°06'N, 0°23'W; elevation 370m) and

viviparous from Paimpont (48°N, 2°W; elevation 150m). These pregnant females then were

sent to ETSU and maintained in an animal care facility [UCAC P070202] under the same

conditions. Oviposited eggs were incubated at 25°C while viviparous embryos were subject to

fluctuations in maternal temperatures (Stewart et al. 2009). Embryos were sampled at

different time intervals from both viviparous females and egg clutches from oviparous females

and then fixed in phosphate-buffered formalin. Embryos were identified to developmental

stage with an embryonic staging guide specifically developed for *Z. vivipara* (Dufaure & Hubert

1961).

For this study, embryos spanning different developmental stages (35, 36, 37, 39, 40 and

neonates) were used as samples from each population (six oviparous and six viviparous per

stage, except stage 37 with only four embryos from the viviparous population). Embryos were

then processed to reveal changes in skeletal ossification throughout development. Before

formalin-fixed specimens were processed, each embryo was photographed with a ruler for size

reference using a Cannon EOS 70D microscope camera in conjunction with a Leica MZ9 5

microscope. Total length measurements before processing were taken from photographs (using

iSolutionLite® software) by calibrating the measurement tool to the reference ruler in each

photograph.

Embryos were skinned, eviscerated (in stages when organs were more developed) and prepared by clearing and staining (C&S; Hanken & Wassersug 1981; as modified following Mathis 2016) to reveal the developing skeleton, with cartilage stained by Alcian blue and bone by Alizarin red (Figure 1). After C&S, total length was re-measured (using the same photography procedure and iSolutionLite ® software) to determine if the solutions used in C&S impact overall size of the specimens due to acidic ingredients. Due to availability of specimens at certain developmental stages, several stage samples used siblings (more than one embryo from the same maternal source).
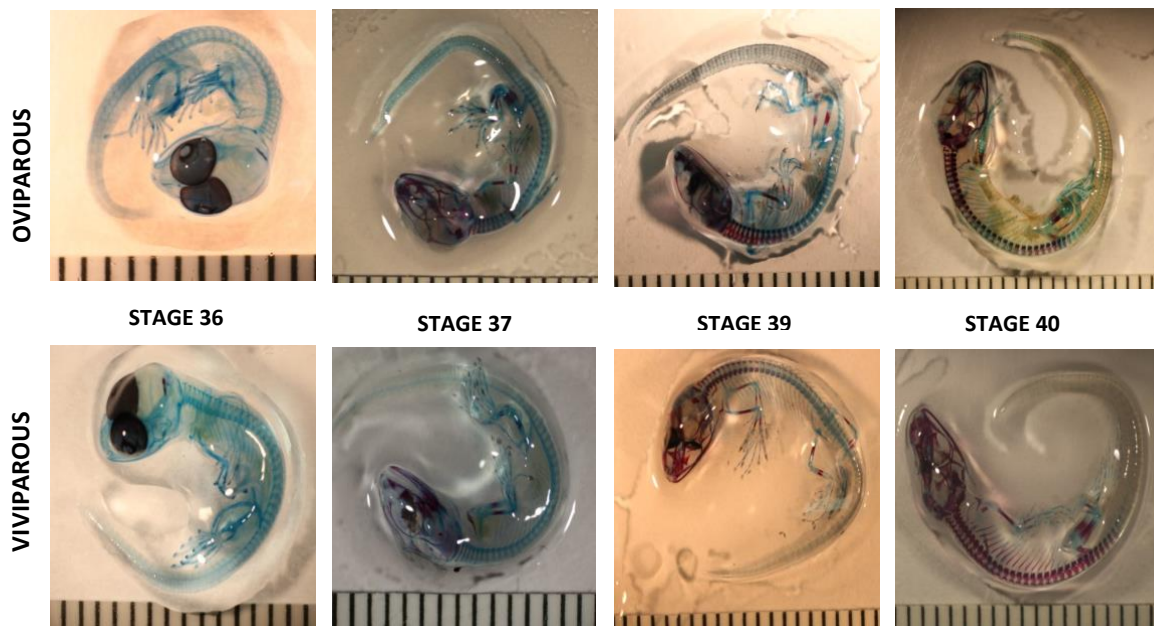

Figure 1: Cleared & stained embryos at various developmental stages. Stage 40 embryos are just prior to hatching/birth. Top row = embryos from oviparous females; Bottom = embryos from viviparous

The following measurements were used to estimate extent of ossification: total length of the femur, length of ossification region within the femur, total length of humerus and the

length of the ossification region within it. In addition, total skull length and length of Meckel's cartilage were obtained as correlates of overall size. All measurements were made on the specimen's right side to control for any asymmetry among individuals. To reduce any further error, all measurements of the long bones were taken on the medial edge of the bone and every measurement was calibrated by reference to the inner edges of the mm marks on the reference ruler (Figure 2). Each measurement on each individual was taken three independent times to test consistency of the measurement system.

Figure 2: Measurements taken on the long bones of embryos (stage 40) from an oviparous female (left) and a viviparous female (right). Left images are humerus and right images are femur. The bars indicate edges measured for all specimens; total length and length of ossification center
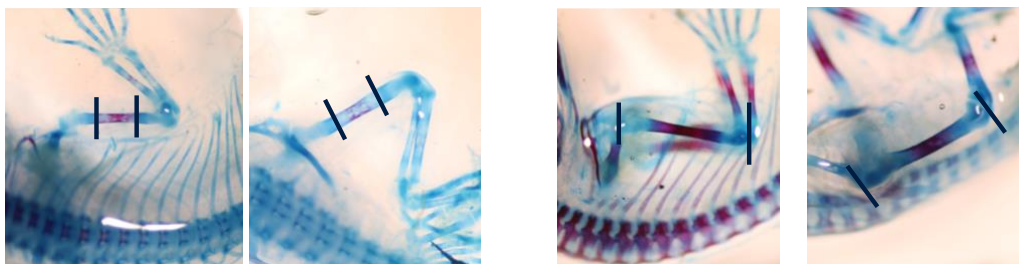
In addition to measurements, each C&S embryo was observed (WILD M3Z stereomicroscope, LEICA MC 190 HD microscope camera) for the appearance of ossification of the following individual skull bones: pterygoid, palatine, vomer, quadrate, jugal, maxilla, nasal, exoccipital and parietal. In an effort to represent progression of ossification in these skull bones, individual bones were scored as follows: 0 = no ossification; 1= 0--50%, 2 = 50--95%, 3 = > 95% (i.e., fully ossified). Full ossification was determined by comparison with the fully ossified skulls of neonates.

Statistical Analysis

In order to determine if independent measurements were consistent (i.e., not different), triplicate measurements of each individual were analyzed using a univariate GLM (SPSS Statistics 25) with individual i.d. number as a fixed variable. The efficacy of overall size measurements was determined by correlation analyses among the measurements of Before C&S Length, After C&S Length, Skull Length and Meckel's Cartilage Length with measurements reflecting extent of ossification of limb bones. Once the measurement for overall size of embryos was determined, that measurement and extent of ossifications was compared among developmental stages and between reproductive types (populations) by ANOVA (SPSS Statistics 25) with type, developmental stage, and maternal identification considered.

CHAPTER 3

RESULTS

Independent triplicate measurements were not different (ANOVA; *P* > 0.15) for each

individual; therefore, a least squares mean of the triplicate measurements was used to

represent each individual (Tables 1, 2) in all further analyses. To determine which measure of

to use for overall size, correlation analyses revealed that the "After C&S" overall length was

more highly correlated with other measurements as compared to "Before C&S", Skull Length

and Length of Meckel's cartilage (Table 3).

Table 1: Means and standard deviations for measurements from oviparous sample of
***Zootoca vivipara* at each developmental stage. Sample size is N = 6 unless indicated (*).**

Stage	36	37	39	40	Hatchling
Before C&S Length	41.37 ± 2.16	48.36 ± 3.63	50.64 ± 3.53	52.52 ± 5.58	62.7 ± 2.97
After C&S Length	37.38 ± 2.58*	46.31 ± 3.02*	47.93 ± 2.18	51.93 ± 3.33	64.15 ± 2.45
Humerus Total	1.69 ± 0.19	2.43 ± 0.22	2.54 ± 0.16	2.86 ± 0.24	2.87 ± 0.24
Humerus Ossification	0	0.59 ± 0.14	0.79 ± 0.22	1.08 ± 0.14	2.13 ± 0.19
Femur Total	1.67 ± 0.22	2.35 ± 0.22	2.5 ± 0.16	2.84 ± 0.09	3.19 ± 0.30
Femur Ossification	0	0.56 ± 0.19	0.80 ± 0.17	1.02 ± 0.30	2.74 ± 0.32
Skull Length	5.81 ± 0.34	6.27 ± 0.36	5.87 ± 0.15	6.89 ± 0.48	7.43 ± 0.41
Length of Meckels	*NA*	5.02 ± 0.41	4.91 ± 0.31	4.25 ± 1.0	6.07 ± 0.35

*N = 5

Table 2: Means and standard deviations for measurements from viviparous sample of *Zootoca vivipara* at each developmental stage. Sample size is N = 6 unless indicated (*).

Stage	36	37**	39	40	Hatchling
Before C&S Length	41.85 ± 3.16	45.39 ± 1.18	50.36 ± 2.0	52.67 ± 5.39	57.94 ± 6.21***
After C&S Length	37.11 ± 2.30*	42.46 ± 1.06	48.13 ± 2.47	49.75 ± 2.90	56.63 ± 4.30***
Humerus Total	1.8 ± 0.18	2.19 ± 0.09	2.59 ± 0.08	2.74 ± 0.18	3.17 ± 0.36
Humerus Ossification	0	0.46 ± 0.06	0.92 ± 0.09	1.3 ± 0.22	2.32 ± 0.35
Femur Total	1.75 ± 0.11	2.24 ± 0.12	2.45 ± 0.14	2.92 ± 0.10	3.15 ± 0.26
Femur Ossification	0	0.41 ± 0.05	0.81 ± 0.11	1.39 ± 0.24	2.71 ± 0.27
Skull Length	5.77 ± 0.34	6.04 ± 0.26	6.06 ± 0.46	6.63 ± 0.50	7.14 ± 0.37
Length of Meckels	*NA*	4.86 ± 0.20	5.0 ± 0.13	5.31 ± 0.12	5.94 0.24

* N = 3   *** N = 4*   **** N = 5*

Table 3: Correlation analyses coefficients

Measurement	Before C&S	After C&S	Skull Length
Humerus Total Length	0.810	0.810	0.710
Humerus Ossification	0.852	0.887	0.766
Femur Total Length	0.840	0.868	0.736
Femur Ossification	0.852	0.898	0.771
Skull Length	0.722	0.785	xxx
Length of Meckels	0.465	0.526	0.459

Ossification in the humerus and in the femur were not significantly different between

reproductive types (oviparous & viviparous) at any stage of development ($F = 2.95$, $P > 0.093$

and $F = 0.553$, $P > 0.461$, respectively; Table 4). However, both measurements differed

significantly among stages of development (Table 4) for both reproductive types.

Measurement of ossification of the femur showed a significant interaction of developmental

stage and population/reproductive type ($F = 2.59$, $P = 0.048$; Table 4), whereas ossification of

the humerus did not show any significant interaction effect (F=1.788, $P = 0.147$; Table 4). Total

"After C&S" length measurements were significant for stage, reproductive type and interaction

between the two ($P < 0.0001$, Table 4). Humerus and femur lengths differed significantly among

stages ($P < 0.0001$) but not between reproductive type (($F = 0.165$, $P > 0.686$ and $F = 0.025$, $P >$

0.874, Table 4). Humerus length indicated a significant interaction between stage and type ($F =$

2.725, $P < 0.05$), while femur total length did not (($F = 0.592$, $P > 0.670$, table 4). Total skull

length was not significant between type or interaction ($F = 1.524$, $P > 0.223$ and $F = 8.124$, $P =$

0.524), but was significant between reproductive stages ($F = 30.728$, $P < 0.001$, Table 4).

Table 4: Significance values for each measurement taken comparing developmental stages, reproductive type and interaction between them. Asterisks represent significant _P_ values.

Measurement	d.f.	_F_	_P_
Humerus Ossified Length			
Stage	4	257.3	<0.001**
Type	1	2.95	0.093
Stage*Type	4	1.788	0.147
Femur Ossified Length			
Stage	4	304.5	<0.001**
Type	1	.553	0.461
Stage*Type	4	2.595	0.048*
Humerus Total Length			
Stage	4	65.920	<0.001**
Type	1	0.165	0.686
Stage*Type	4	2.725	0.040*
Femur Total Length			
Stage	4	107.1	<0.001**
Type	1	0.025	0.874
Stage*Type	4	0.592	0.670
Total After Length			
Stage	4	84.833	<0.001**
Type	1	11.591	0.001**
Stage*Type	4	3.252	0.021**
Total Skull Length			
Stage	4	30.728	<0.001**
Type	1	1.524	0.223
Stage*Type	4	0.811	0.524

Zootoca vivipara embryos from both reproductive types had no ossification of the skull in any specimens of developmental stage 35. Skull ossification begins with the pterygoid; one individual from each reproductive type showed signs of ossification (<50%) in stage 36 (total length = 37mm). By stage 37, all oviparous and viviparous embryos show more than 50% ossification of the pterygoid. One individual from the oviparous reproductive mode still showed no signs of ossification during stage 39, while all others showed more than 50%. During stage 40, several embryos exhibited advanced ossification (>95%; Fig 3).

Figure 3: Comparison of first cranial bones to show ossification during development in oviparous (O) and viviparous (V) *Zootoca vivipara*. Columns represent number of specimens in each sample at each developmental stage; colors represent amount of ossification (e.g., 0% through >95%, as shown in legend).
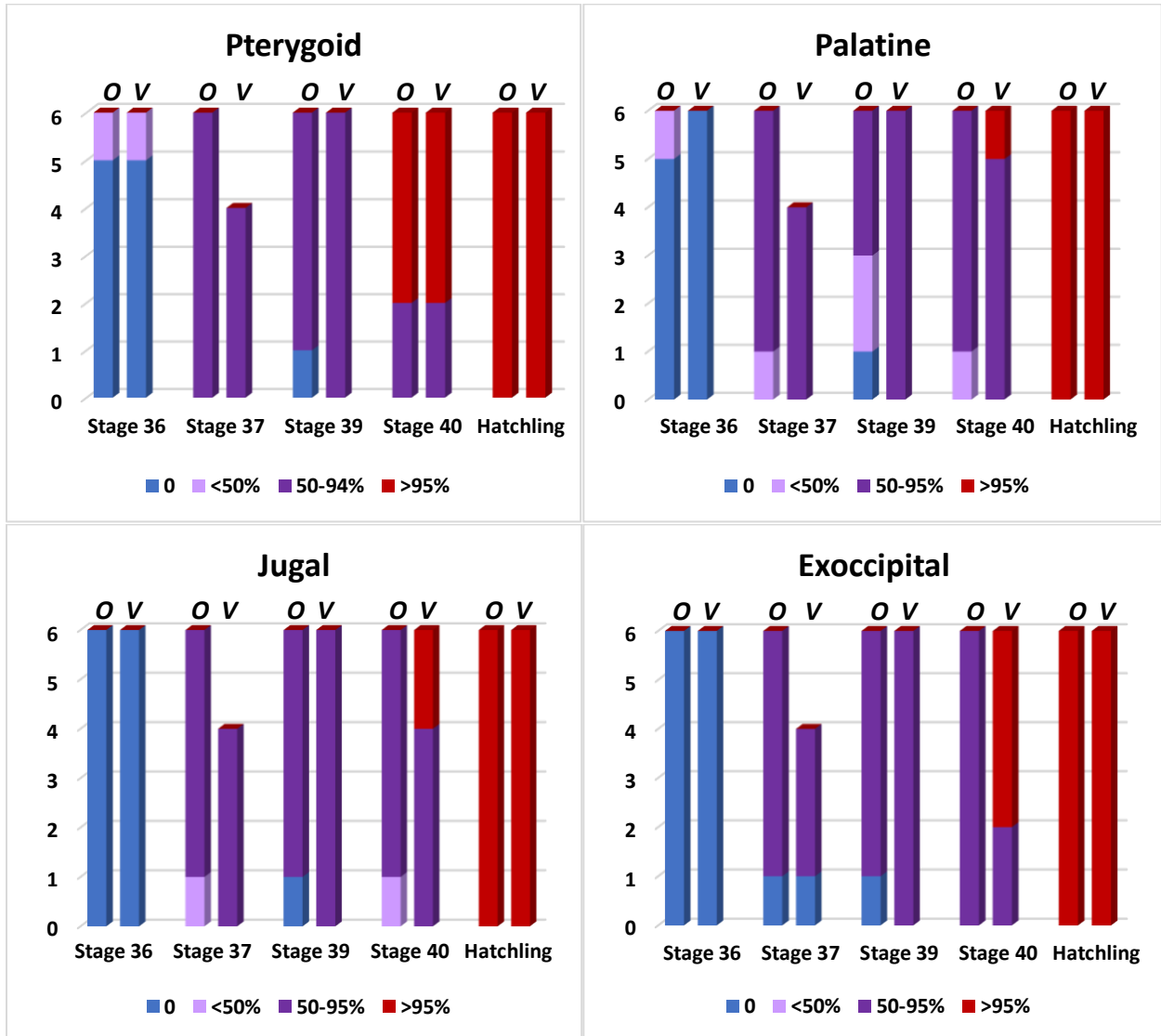
The palatine, jugal and exoccipital bones were next to ossify, showing little to no ossification before developmental stage 37 (total length = 37mm). During stage 37, the majority of embryos from both reproductive types showed more than 50% ossification with few

individuals exhibiting less than 50% ossification (palatine and jugal) and some with no ossification (exoccipital). Embryos in stage 39 (approximately 47mm) show variation in ossification from 0% to <95% among individuals in all three of these bones (Fig. 3). In most stages, more embryos from viviparous females showed a greater degree of ossification of these skull bones, with the single exception being the exoccipital bone at stage 37 (Fig 3).

Ossifications of the angular, vomer, maxilla and quadrate have a similar onset and progression during development but varied considerably among stages in these samples. During stage 37, most embryos from each reproductive type exhibit <50% ossification of these bones (Fig 4), although some individuals still show 0% ossification (vomer, quadrate and maxilla) and some individuals show more than 50% ossification in all three bones. Ossification in stage 39 is more variable; few individuals show no ossification while others have more than 50% (Fig 4). Again, ossification of these bones in embryos from the viviparous population is slightly more advanced in stage 37 than those from the oviparous population, with the exception of the maxilla and quadrate. However, after stage 37 ossification is noticeably more advanced in the viviparous sample; embryos from the viviparous type show advanced ossification in stage 40 (vomer and quadrate). Ossification of the articular is later than other skull bones; ossification occurs in a few viviparous individuals during stage 39, while the majority of embryos show ossification up to 95% during stage 40 (Fig 5). For embryos of both reproductive types, ossification of the parietals and nasal bones is not seen until stage 40, making it the last of the skull elements to ossify.

Figure 4: Comparison of cranial development sequence following onset of ossification in samples of reproductive mode oviparous (O) and viviparous (V). Columns represent number of specimens in each sample at each developmental stage; colors represent amount of ossification (e.g., 0% through >95%, as shown in legend).
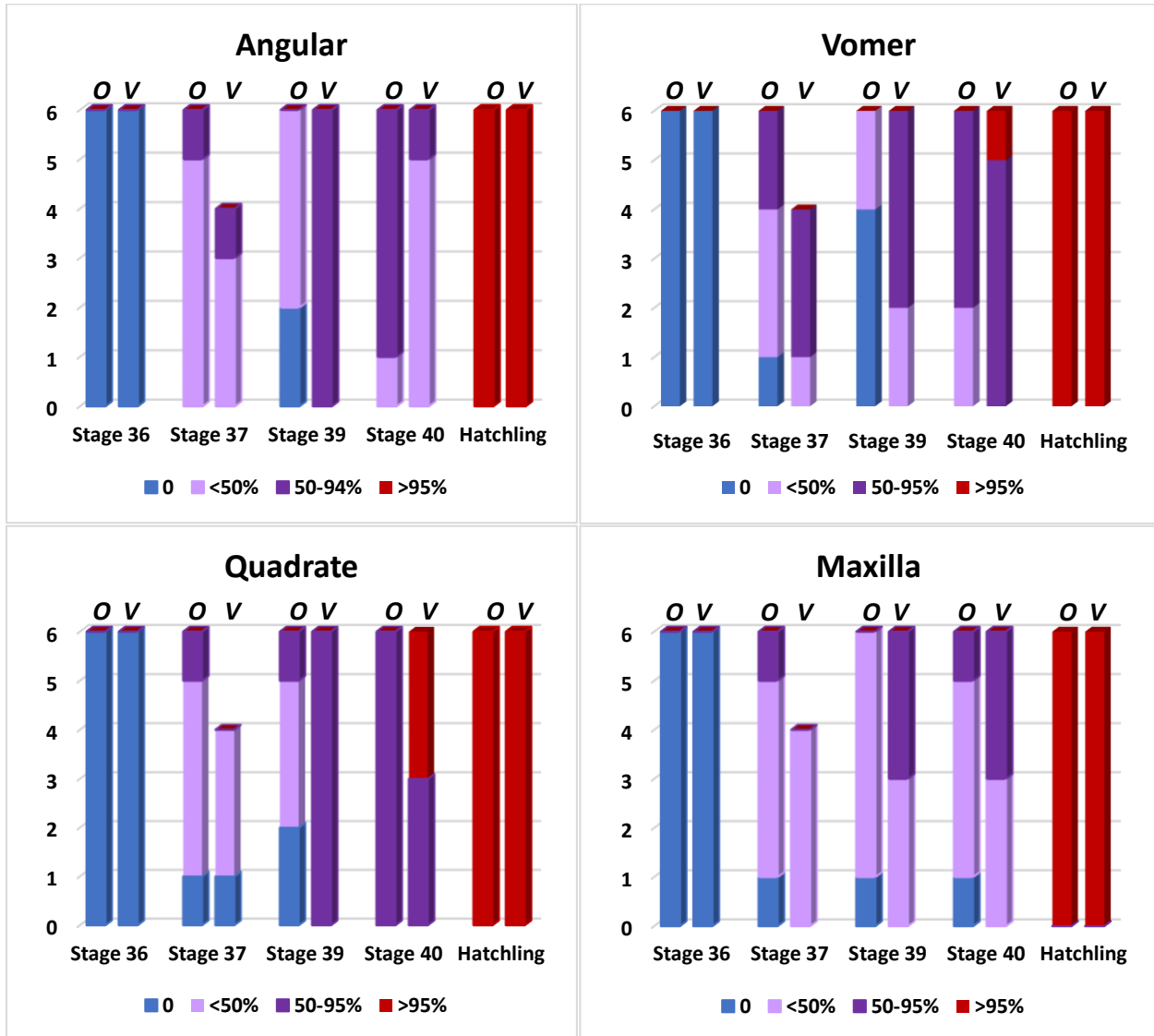
Figure 5: Comparison of developmental reproductive types oviparous (O) and viviparous (V) in the Articular, a cranial bone with delayed onset of ossification. Columns represent number of specimens in each sample at each developmental stage; colors represent amount of ossification (e.g., 0% through >95%, as shown in legend).

Figure 6: Comparison of developmental reproductive types oviparous (O) and viviparous (V) in Nasal and Parietals, with the most delayed onset of ossification. Columns represent number of specimens in each sample at each developmental stage; colors represent amount of ossification (e.g., 0% through >95%, as shown in legend).
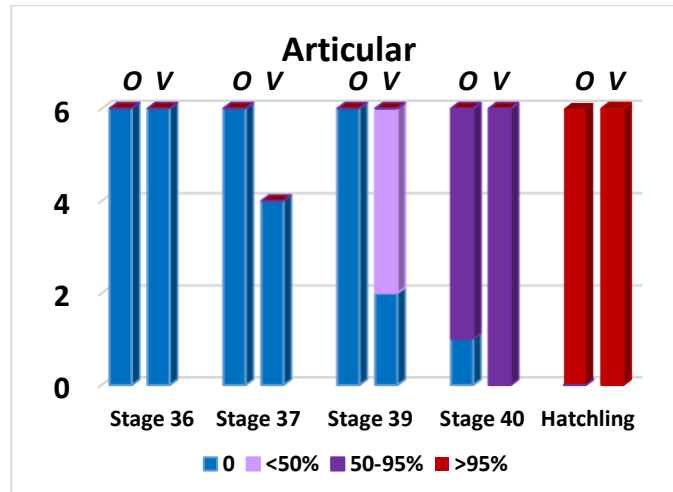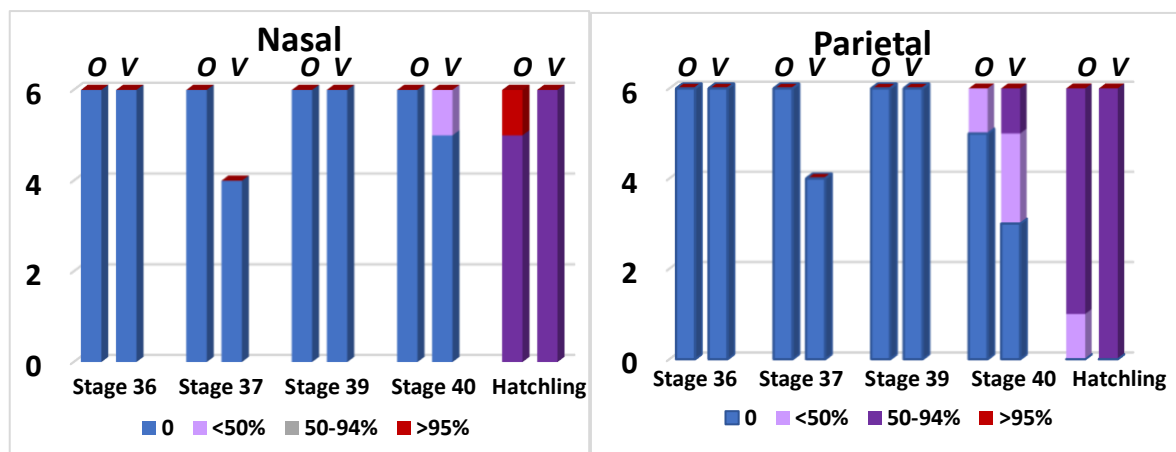
CHAPTER 4

DISCUSSION

Previous research on developmental patterns in several squamates has suggested that

viviparous neonates may be less skeletally mature than oviparous hatchlings. Using a sample of

14 oviparous and seven viviparous squamate species, Maisano (2001) evaluated and compared

the state of skeletal ossification in presumed neonates/hatchlings. Her analysis concluded that

variation in ossification was not related to many life history variables, including size,

incubation/gestation period, growth rate to maturity or age and size at maturity.   She did

conclude that her data, especially limb morphology, suggested a possible correlation between

skeletally "immature" neonates and viviparity. However, this finding was tentative because her

sample of viviparous species was limited to three clades (Xantusiidae, Scincidae, Anguidae) and

the scincid *Chalcides* did not exhibit a delayed skeletal ossification.

The current study was designed to test her hypothesis using *Zootoca vivipara*, one of the

few reproductively bimodal lizards. This lizard species is represented by oviparity in some

populations and viviparity in others. Oviparous and viviparous populations used in this study

are reproductively isolated (Heulin & Guillaume 1989), which contributed to the strength of

comparisons of skeletal ossification between reproductive types. In addition, pregnant females

of both populations were maintained in similar laboratory situations, which allowed for

consistent sampling of embryos at timed intervals that produced developmental series.

Viviparous embryos were subject to temperature fluctuations experienced by the females (8 h

per day heat lamps) with warm days and cool nights, while oviparous eggs were maintained at

constant temperatures (Stewart et al. 2009). Differences in incubation temperatures could have

a possible effect on skeletogenesis.   Measurements of total length, limb lengths and ossification centers were obtained under high magnification and in triplicate to reduce potential error.

As expected, developmental stage was significant for each measurement (Fig. 1), reflecting growth during development. However, embryos from oviparous and viviparous populations did not differ significantly in limb size, extent of limb ossification, nor in ossification sequences of skull bones observed (Table 4; Figs. 3-6).   Thus, results of this study do not support Maisano's suggestion (2001) that viviparity is associated with reduced skeletal ossification during development and in hatchlings or neonates.

The progress of limb ossification is represented in Figures 7 and 8 as percent of ossification relative to total limb lengths. The percent of ossification within the humerus and femur did not differ between reproductive type (F = 2.789, P = 0.101; F= 1.047, P = 0.311, respectively). Ossification of both humerus and femur follow a very similar trend throughout development (Figs. 7,8). We do note that the ossification of the humerus in hatchlings/neonates is roughly 10% less than femur.

Figure 7: Comparison of percent ossification in the humerus during embryonic development (Stages 37-neonate) between oviparous and viviparous. The line represents means by stage and reproductive type.
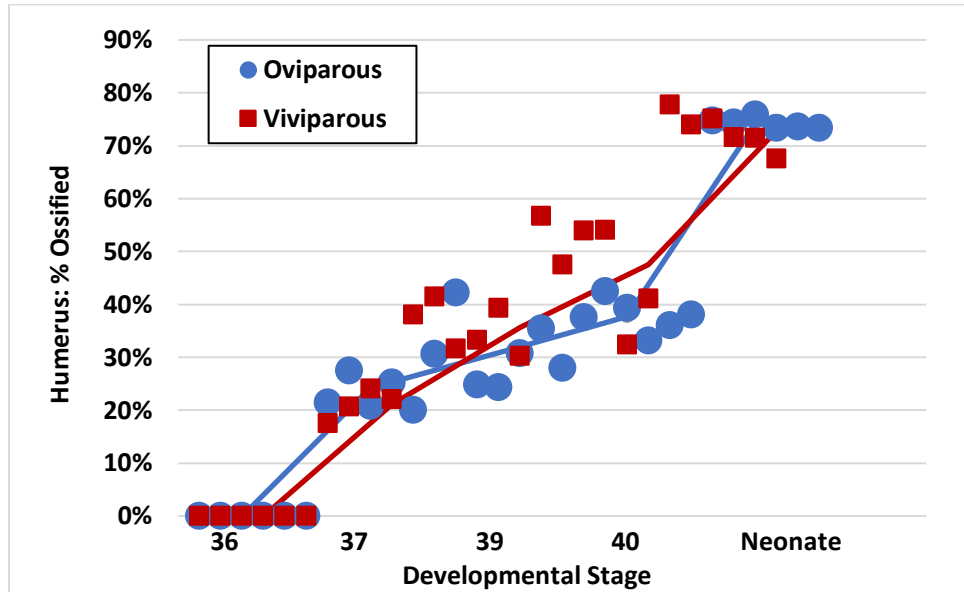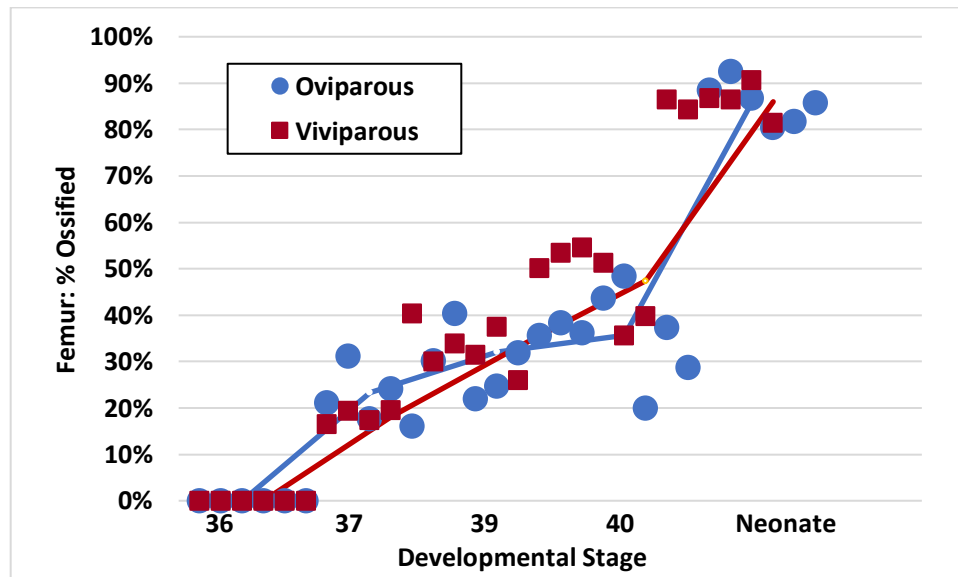

Figure 8: Comparison of percent ossification in the femur during embryonic development (Stages 37-neonate) between oviparous and viviparous. The line represents means by stage and reproductive type.

Previous studies have described patterns of skeletal ossification during embryonic development for *Zootoca vivipara*. Using eight late-stage embryos, Rieppel (1992) evaluated the order of skeletal ossification. He concluded that degree of ossification is not closely related to overall body size, but that skeletal ossification progresses in an anteroposterior fashion, beginning with the skull followed by clavicle, humerus, femur, vertebral column and concluding with carpals and tarsals at neonate stage. In his samples, Rieppel (1992) reported that ossifications in skull elements began first with the pterygoid, followed by the palatine and vomer, and then shortly after by the angular, maxilla, jugal and quadrate. Some of the final elements to ossify in the skull were the nasal and parietal bones. Rieppel (1992) also reported that limb ossification begins concurrently with the pterygoid, which was the first skull element to begin ossification.

Results of this study are generally consistent with Rieppel's overall sequence of skull ossification (Table 5; Fig. 9), but only if his measurements of overall length of embryos are ignored. Rieppel's findings (1992) are reported relative to snout-vent length instead of developmental stage; his specimens were borrowed from museums and some were already cleared and stained, so he was unable to determine developmental stage. Limb and skull ossification (pterygoid) in his study were identified in a specimen identified as 7mm in snout-vent length, which would approximate 14--17mm total body length. In this study, snout-vent length of neonates is approximately 40--50% of total body length. My results show first limb ossification during stage 37, representing 42--46mm in total length (approximately 18--23mm snout-vent length), which is more than a two-fold size discrepancy. Thus, Reippel's data (1992) represents body lengths that are substantially smaller than the ones recorded in this study.

Due to these size discrepancies, we could not directly compare ossification data by

developmental stages, so we compared overall pattern (Table 5). We also note that Reippel

(1992) did not identify whether his samples were taken from oviparous or viviparous

populations.

Table 5: The general pattern of ossification in skull elements of *Zootoca vivipara*
in this study and that of Reippel, 1992 (* = elements not reported).

	First group	Second Group	Final group
Reippel, 1992	Pterygoid Palatine Vomer	Angular Maxilla Jugal Quadrate	Nasal Parietals
This Study	Pterygoid Palatine	Vomer Angular Maxilla Jugal Quadrate *Exoccipital	Nasal Parietals * Articular

Figure 9: Comparison of skull photographs of cleared and stained embryos from oviparous and viviparous populations at stage 37 (top row) and stage 40 (bottom row).
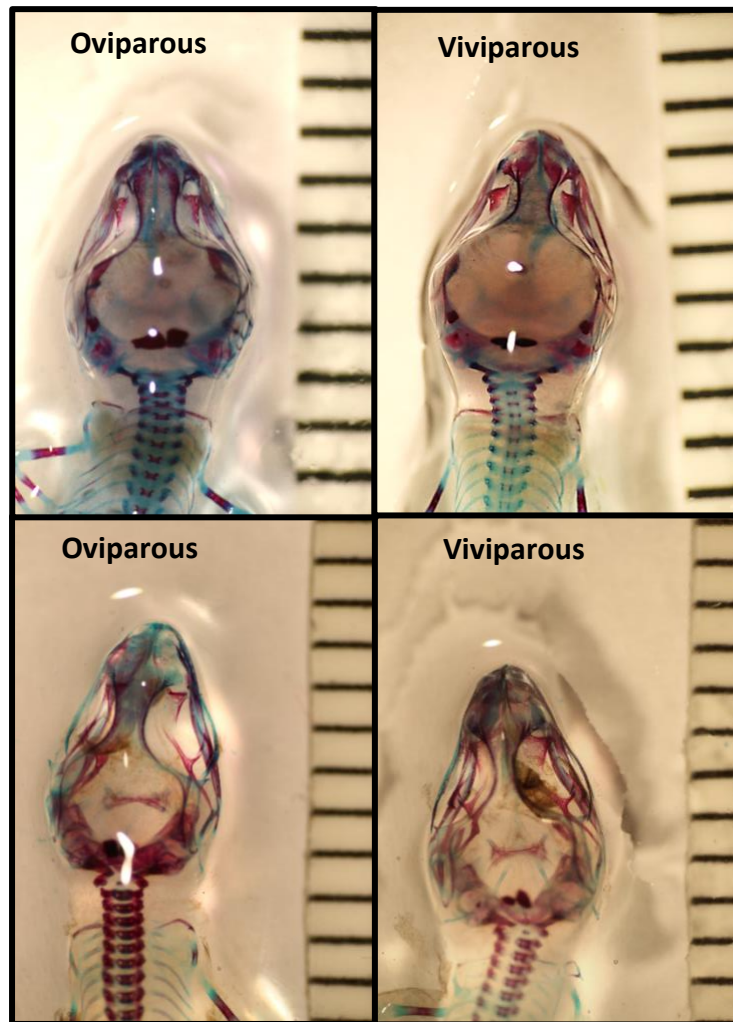
Identification of the pterygoid as the first element of the skull to ossify is consistent with

several different studies (Rieppel 1992, *Zootoca vivipara*; Hernandez-Jaimes et al. 2012,

Ptychoglossus bicolor; Baker 2011, *Saiphos equalis*). In our sample, one individual also showed

ossification in the palatine during stage 36, when the pterygoid was starting to ossify. Several

skull bones (second group, Table 5, Fig. 9) as well as limbs started to ossify in stage 37.

However, the jugal and exoccipital show more advanced ossification than the angular, vomer,

quadrate and maxilla. Onset of ossification in the articular is delayed in our samples (stage 39), consistent with *Liolaemus quilmes* (Abdala et al. 1996). The final elements in the skull to begin ossification are the nasal and parietals in Stage 40 (Fig. 9), the final stage before hatching or birth. This finding is consistent with Reippel (1992), although he indicated onset of ossification of these bones much earlier in development.

Although skull length, limb length and limb ossification do not differ between reproductive types, oviparous neonates are larger than viviparous neonates. Total length "After C&S" is similar between reproductive types during earlier developmental stages, but overall the oviparous hatchlings are larger than viviparous neonates (Fig. 10). Our total length ("After C&S") was more highly correlated with other measurements (Table 3) and determined it was the representation of overall size. It is likely that the nature of processing specimens in acidic conditions and the resulting ease of measurement (specimens are less rigid) reduced error in this measure of overall length. Previous studies (e.g., Roitberg et al. 2013) on *Zootoca vivipara* found that oviparous hatchlings had higher mass than viviparous neonates. The finding that viviparous neonates are smaller in overall size is also consistent with findings from studies on corn snakes (Stewart & Ecay 2013; Mathis 2016). In these two studies, embryos with mechanically reduced eggshell calcium were smaller in overall length and mass than hatchlings with normal eggshells. At sexual maturity, viviparous females are larger than oviparous females but are also older (Bleu 2012), suggesting that an overall smaller size in viviparous neonates may impact life history by causing them to reach sexual maturity later than oviparous females.

Figure 10: Comparison of means of overall body length during embryonic development (Stages 37--neonate) between reproductive types oviparous (OV) and viviparous (V). Error bars represent standard deviation of least squares means.
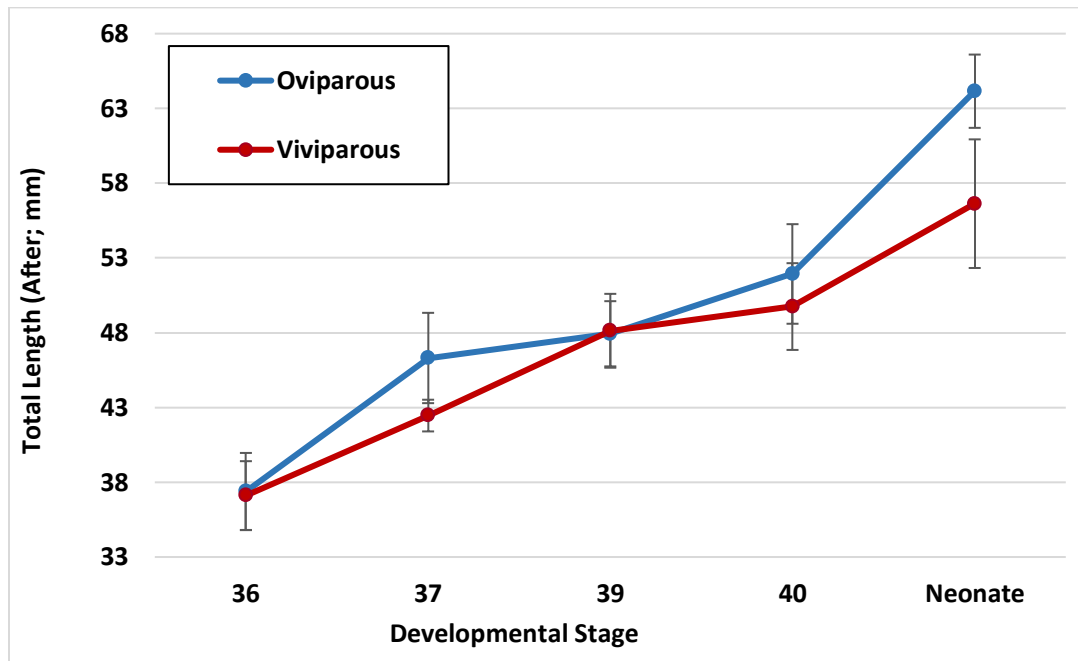
Overall, reproductive type did not affect limb ossification in *Zootoca vivipara*. These results suggest that calcium available to developing embryos is either not a limiting factor for ossification or calcium from eggshells in oviparous females is compensated by placental transfer of calcium in viviparous females. Viviparous *Z. vivipara* receive nearly 76% of embryonic calcium from sources other than yolk (Stewart et al. 2009) suggesting that reduction in eggshell calcium is compensated by some degree of matrotrophy. Reproductive type does impact overall size (this study) and mass of neonates (Roitberg 2013), but not limb ossification. In *Zootoca vivipara* there is no significant relationship between climate and offspring size, challenging predictions of the cold-climate model (Roitberg 2013). Results of this study support

Blackburn's (1995) prediction for both saltation and punctuated equilibrium models that there should be little to no phenotypic variation in closely related species with different reproductive modes. *Zootoca vivipara* from both oviparous and viviparous populations show little difference in phenotypic variation of skeletal development, other than overall size. This size discrepancy at birth may relate to delay of sexual maturity in viviparous females.

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