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Skeletal Development in Oviparous and Viviparous Populations of Saiphos equalis.

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Skeletal Development in Oviparous and Viviparous Populations

of *Saiphos equalis (Reptilia: Scincidae)*

by

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Introduction

Saiphos equalis is a burrowing lizard found on the coast and adjacent ranges of New South Wales and the far southeast region of Queensland. This lizard typically is found under ground cover in heathlands, sclerophyll forests, rainforests, and in urban areas near gardens or compost heaps. The dorsal body is brown and the ventral body is pale to bright yellow except the throat which has brown spots or is blackish. The limbs are short with three small subequal digits, providing the common name of the Three-toed Skink (Cogger 1992).

One of the unique attributes of *Saiphos equalis* is that the species exhibits bimodal reproduction, with some populations reproducing by oviparity (egg-laying) and some by viviparity (live birth). It is one of three lizards for which reproductive mode varies geographically (Heulin et al. 1993; Fairbairn et al. 1998; Smith et al. 2001; Surget-Groba et al. 2001, 2006). Females from oviparous populations lay large eggs with advanced embryos that hatch a week or two after oviposition.

In a previous study of this species, Linville et al. (2010) found that the viviparous females are significantly larger than the oviparous females, but that clutch size is not correlated to female size. They also found that hatchlings of the oviparous population have significantly more calcium than neonates of the viviparous population, perhaps in some part because they utilize calcium from both the yolk and eggshell. Eggs from females in oviparous populations have thicker shells than those from viviparous populations, and these thicker shells represent significantly more calcium available to the developing young. The study concludes that there are significant differences in the amount of calcium available between the different modes of reproduction. Because of these differences in calcium availability, the question arises as to whether there is an impact on development of embryos. A test of the possible impact of differences in calcium availability is observation of embryonic development of the skeleton.

Preliminary descriptions of the pattern of ossification in lizards have been produced for *Lacerta vivipara* (Rieppel 1992) and *Lacerta agilis exigua* (Rieppel 1994). Studies on squamate species *(*Rieppel 1992, 1994; Maisano 2001) concluded that ossification is decoupled from chondrification, in that bones do not ossify in the order they appear in cartilage formation. Ossification begins at stage 34 in *Lacerta agilis exigua* with the pterygoid and surangular of the lower jaw. Ossification begins at stage 32 in *Lacerta vivipara* with the pterygoid and the premaxilla of the upper jaw. Rieppel (1992) suggests significant increase in variability within species in the timing of ossification due to environmental factors, such as humidity. However, the pattern of ossification is poorly known and comparative data are needed.

The individual bones of the lizard skull are well documented (Evans 2008). In her review article, Evans (2008) provided an overview of osteocranial development in lizards. From works cited, she concluded that although considerable variation is present, the following observations of ossification in the cranium are consistent among lizards: (1) the pterygoid bone is first to ossify; (2) the second ossification is always a bone of the lower jaw; (3) the lacrimal is the last bone to begin ossification; and (4) by hatching (or birth in viviparous species) all bones are present although not fully ossified in adult form. Evans (2008) review is exhaustive to date, and also serves to highlight that data on skeletal development are available for only a few species (approximately 12 species) of the diverse family Scincidae which contains approximately 1,400 species (Cogger 1992).

Maisano (2001) studied neonates of seven viviparous and five oviparous species and concluded that all viviparous species were skeletally immature compared to the oviparous species in the study. Due to the skeletal immaturity, she suggested that the viviparous populations may have significantly less calcium as neonates and hypothesized a causal relationship between viviparity and skeletally immature neonates. However, her study compared oviparous and viviparous populations of different species. Thus, the results of her study may reflect broader differences in the evolutionary histories among the species rather than differences resulting directly from changing modes of reproduction.

I aim to determine the pattern of ossification during embryonic development of representative bones of the skeleton, especially the skull, and to compare ossification patterns among specimens from oviparous and viviparous populations of *Saiphos equalis*. Based on previous research the viviparous species should be skeletally immature compared to specimens of the oviparous species at the same stages of development. Skeletal development has not been described previously for this species.

Materials and Methods

Female *Saiphos equalis* from oviparous and viviparous populations were collected in New South Wales, Australia. The oviparous population was found in Botany Bay National Park, Forsythe Park, Hurstsville, Royal National Park, and The University of Sydney. The viviparous population was found in Riamukka State Forest in northern New South Wales (Smith and Shine 1997). The animals were transported to The University of Sydney and maintained in either plastic containers with heat tape or glass aquaria with vented lids and a 25W incandescent bulb and 20 mm shredded sphagnum. The skinks were given water *ad libitum* and fed crickets (*Acheta domestica*) and mealworms (*Tenebrio molitor*) dusted with multivitamins and phosphorus-free calcium (Herptivite™ Rep-Cal CA, USA) twice weekly. Females were maintained until oviposition or killed with an overdose of sodium pentobarbital at various times during gestation for sampling of embryos which were fixed in EtOH. Embryos were transported to East Tennessee State University.

Specimens (Table 1) were staged following the outline of Dufaure and Hubert (1961) and then cleared and stained using the process of Hanken and Wassersug (1981). Specimens at or more advanced than stage 38 were skinned, eviscerated, and the eyes removed. Three distilled water washes (1 h, 12 – 24 h, and 12 – 24 h, respectively) were followed by 12-36 hours in alcian blue. Dehydration was accomplished with three washes of 100% EtOH (1h, 12 – 24h, 12 – 24 h, respectively). Hydration followed the dehydration with washes of 70% EtOH, 50% EtOH, 25% EtOH, and three washes of distilled water. Varying concentrations (0.25-0.75g/ 30mL saturated sodium borate and 70mL distilled water) of the digestive enzyme trypsin began to clear the soft tissue. Trypsin was followed by twenty-four hours in alizarin red. Increasing concentrations of KOH/glycerin (90% KOH/ 10%glycerin, 75% KOH/ 25% glycerin, 50%

KOH/50% glycerin, 25% KOH/ 75% glycerin, 100% glycerin) finished the clearing and staining process.

Table 1. ***Number, developmental stage and mode of reproduction of specimens used in this study.***

Stage	N Viviparous	N Oviparous	Total N
34	1	2	3
35	1	2	3
36	0	1	1
37	0	0	0
38	0	0	0
39	0	1	1
40	3	7	10
Hatchling/Neonate	2	2	4

After clearing and staining, specimens were viewed with a Wild M3Z stereodissecting microscope using a light/dark base. Photographs were obtained with Leica Application Suite 2.6.0 image capture system with a Leica camera (DFC420) connected to a laptop computer. The skeleton of each specimen was examined to assess the relative amount of ossification of individual bones. The following bones were assayed: humerus; pelvic girdle (ischium, ilium, and pubis); and in the skull, the nasal, prefrontal, frontal, postfrontal, parietal, and jugal of the skull roof and cheek; the squamosal, supratemporal, prootic, otootic, and quadrate of the temporal and otic regions; the supraoccipital, basioccipital, and exoccipital of the occiput; the vomer, palatine, sphenoid, pterygoid, and epipterygoid of the palate, and the premaxilla, maxilla, dentary, coronoid, surangular, angular, and articular of the jaws (Tables 2-7).

Ossification was described by assessing the appearance and the extent of ossification in individual bones, as compared with the fully formed condition in adults. This subjective classification was not intended for statistical tests, but only to describe the relative progress of ossification in a generalized stepwise manner. The amount of ossification for each bone was recorded as an ossification index ranging from 0—5, where 0 represents no apparent ossification; 1 is slight ossification (~1-10%); 2 is moderate ossification (approximately 30%); 3 is moderate ossification (approximately 50%); 4 is well-formed but incomplete ossification (approximately 70%); and 5 is ossification approximately equal to the adult condition. Data for each specimen were entered into a spreadsheet and averages computed for each bone by developmental stage separately for the oviparous and the viviparous populations.

Results

Embryos prior to stage 35 showed no ossification so they are not reported in the tables here. Ossification data are recorded for stages 35, 36, 39, 40, and hatchling for the oviparous population but are lacking in stages 37 and 38. Ossification data are recorded for stages 35, 40, and neonate for the viviparous population but are lacking for stages 36, 37, and 38.

Limb and Pelvic Girdle. The humerus is first to ossify (Table 2) and is significantly more ossified in the viviparous population at stage 35 than in the oviparous population. There are small differences in averages of ossification of the pelvic bones between the two populations at stage 40 (Table 2). By hatchling/neonate, the specimens of both populations have virtually the same degree of ossification in these elements.

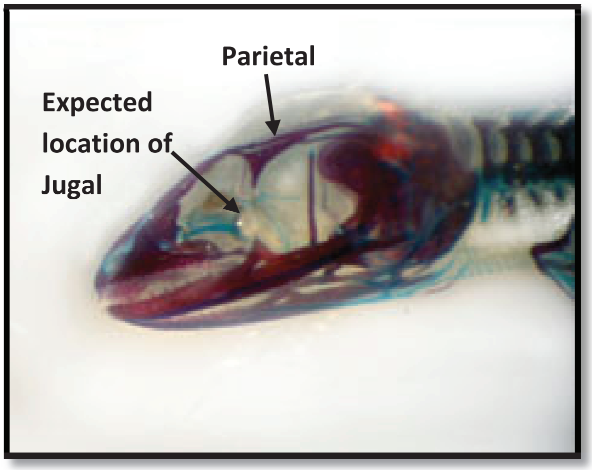
Table 2. ***Ossification averages for the limb and pelvic girdle of viviparous and oviparous populations.***

STAGE	VIVIPAROUS					OVIPAROUS				
	N	Ilium	Ischium	Pubis	Humerus	N	Ilium	Ischium	Pubis	Humerus
35	1	0.0	0.0	0.0	1.0	2	0.0	0.0	0.0	0.0
36	0					1	0.0	0.0	0.0	0.0
39	0					1	3.0	4.0	0.0	5.0
40	3	2.7	4.0	1.7	4.3	7	3.0	3.3	2.1	4.6
Hatch/Neo	2	3.5	5.0	4.0	5.0	2	3.5	5.0	4.0	5.0

Slull Roof and Cheek. In the skull roof (Table 3), the parietal is just beginning to ossify at Stage 35 in embryos from the viviparous population only. Although the parietal (Figures 1 and 2) is one of the first bones to begin ossification in both populations, at hatchling/neonate it remains only slightly ossified (1.0 ossification index). The frontal bone (Figure 3) is slightly more ossified in neonates from the viviparous population, on average (4.5 vs. 4.0), than in hatchlings. The remainder of the bones of the skull roof and cheek show no significant differences between the oviparous and viviparous populations. The jugal is missing (Figures 1 and 2) in three of the oviparous stage 40 specimens and was excluded from averages.

Figure 1. ***Lateral view of skull of a stage 40 embryo from the oviparous population [Specimen 5376-2].***

Figure 2. ***Lateral view of skull of a stage 40 embryo from the viviparous population [Specimen 5382].***



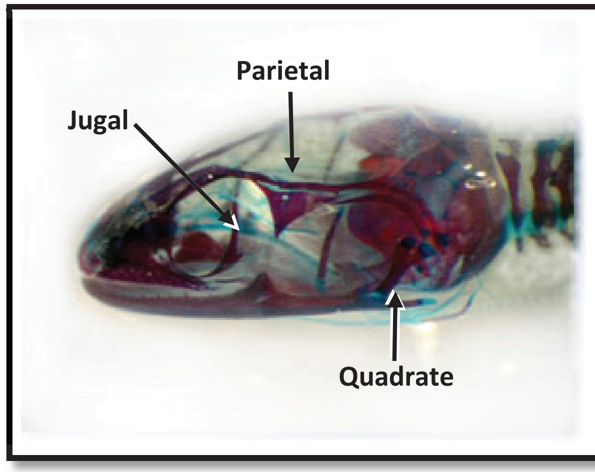


Table 3. ***Ossification averages for the skull roof and cheek of viviparous (upper) and oviparous (lower) populations.***

STAGE		VIVIPAROUS					
	N	Nasal	Prefrontal	Frontal	Parietal	Postfrontal	Jugal
35	1	*0.0*	*0.0*	*0.0*	*1.0*	*0.0*	*0.0*
36	0						
39	0						
40	3	*5.0*	*5.0*	*3.3*	*1.0*	*5.0*	*5.0*
Neonate	2	*5.0*	*5.0*	*4.5*	*1.0*	*5.0*	*5.0*
STAGE		**OVIPAROUS**					
	N	**Nasal**	**Prefrontal**	**Frontal**	**Parietal**	**Postfrontal**	**Jugal**
35	2	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
36	1	*0.0*	*1.0*	*0.0*	*1.0*	*1.0*	*2.0*
39	1	*5.0*	*5.0*	*2.0*	*1.0*	*4.0*	*5.0*
40	7	*5.0*	*5.0*	*3.0*	*1.0*	*4.7*	*4.8*
Hatchling	2	*5.0*	*5.0*	*4.0*	*1.0*	*5.0*	*5.0*

Temporal/Otic Region. In the temporal region (Table 4; Figure 3), the prootic of the viviparous embryos is slightly more ossified (5.0) than oviparous embryos (4.4) at stage 40. There are no other significant differences between the oviparous and viviparous populations in the ossification of the temporal or otic regions of the skull.

Figure 3. ***Dorsal view of the skull of a stage 40 embryo from the oviparous population [Specimen 5398].***

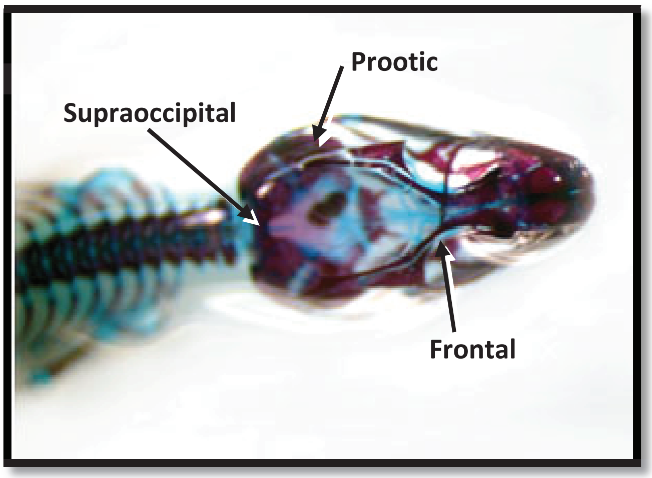


Table 4. ***Ossification averages for the temporal/otic regions of viviparous (upper) and oviparous (lower) populations.***

STAGE	VIVIPAROUS					
	N	Squamosal	Supratemporal	Prootic	Otootic	Quadrate
35	1	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
36	0					
39	0					
40	3	*5.0*	*5.0*	*5.0*	*4.7*	*4.7*
Neonate	2	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*
STAGE	OVIPAROUS					
	N	Squamosal	Supratemporal	Prootic	Otootic	Quadrate
35	2	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
36	1	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
39	1	*5.0*	*5.0*	*1.0*	*1.0*	*3.0*
40	7	*5.0*	*5.0*	*4.4*	*4.4*	*5.0*
Hatchling	2	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*

Occipital Region. In the occipital region of the skull (Table 5), comparison of stage 40 embryos shows ossification of the supraoccipital and exoccipital (Figures 3--5) is somewhat delayed for embryos from the oviparous population. The relative delay in ossification of the exoccipital is also noted in hatchlings (4.5) relative to viviparous neonates (5.0).

Figure 4. ***Ventrolateral view of the skull in a hatchling from the oviparous population [Specimen 5396-1].***

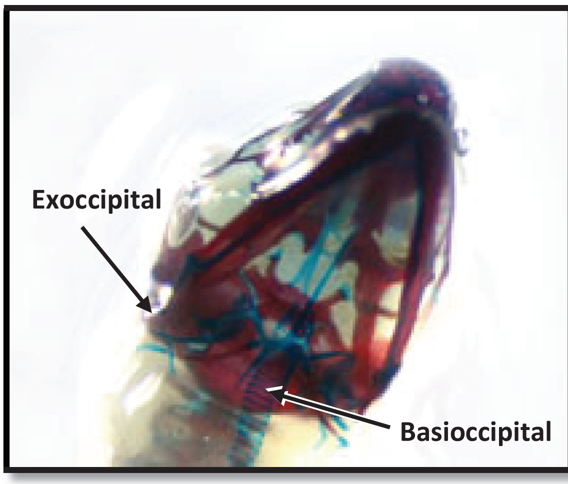


Table 5. ***Ossification averages for the occiput of viviparous (upper) and oviparous (lower) populations.***

STAGE		VIVIPAROUS		
	N	Supraoccipital	Basioccipital	Exoccipital
35	1	*0.0*	*0.0*	*0.0*
36	0			
39	0			
40	3	*3.7*	*3.0*	*4.0*
Neonate	2	*5.0*	*5.0*	*5.0*
STAGE		OVIPAROUS		
	N	Supraoccipital	Basioccipital	Exoccipital
35	2	*0.0*	*0.0*	*0.0*
36	1	*0.0*	*0.0*	*0.0*
39	1	*1.0*	*1.0*	*3.0*
40	7	*3.3*	*3.1*	*3.1*
Hatchling	2	*5.0*	*5.0*	*4.5*

Palate. In the palatal region (Table 6), the pterygoid begins ossification at stage 35 in embryos from the viviparous population but not in embryos from the oviparous population. It is interesting to note that the palatine is less ossified than most skull bones in both hatchlings and neonates, although slightly less so in oviparous (4.0) than viviparous (4.5). This pattern is similar to that observed for the frontal bone. There are no other significant differences between the oviparous and viviparous populations at any stage of development for bones of the palate.

Figure 5. ***Ventral view of skull in a stage 40 embryo from the viviparous population [Specimen 5382].***

Table 6. ***Ossification averages for the palate of viviparous (upper) and oviparous (lower) populations.***

STAGE	VIVIPAROUS					
	N	Vomer	Palatine	Sphenoid	Pterygoid	Epipterygoid
35	1	*0.0*	*0.0*	*0.0*	*1.0*	*0.0*
36	0					
39	0					
40	3	*5.0*	*3.3*	*4.3*	*4.3*	*4.7*
Neonate	2	*5.0*	*4.5*	*5.0*	*5.0*	*5.0*
STAGE	OVIPAROUS					
	N	Vomer	Palatine	Sphenoid	Pterygoid	Epipterygoid
35	2	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
36	1	*0.0*	*0.0*	*0.0*	*1.0*	*0.0*
39	1	*3.0*	*3.0*	*2.0*	*5.0*	*3.0*
40	7	*5.0*	*3.3*	*3.9*	*4.3*	*4.7*
Hatchling	2	*5.0*	*4.0*	*5.0*	*5.0*	*5.0*

Jaw. The dentary is the first bone of the lower jaw to begin ossification in the viviparous population at stage 35 (Table 7). At stage 36, all bones of the lower jaw except the articular show at least some degree of ossification in the oviparous population. There is very little difference between the ossification averages of the oviparous and viviparous populations for all bones of the lower jaw at stage 40 and hatchling/neonate.

Table 7. ***Ossification averages for the jaw of viviparous (upper) and oviparous (lower) populations.***

STAGE	VIVIPAROUS							
	N	Premaxilla	Maxilla	Dentary	Coronoid	Surangular	Angular	Articular
35	1	*0.0*	*0.0*	*1.0*	*0.0*	*0.0*	*0.0*	*0.0*
36	0							
39	0							
40	3	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*	*4.3*
Neonate	2	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*
STAGE	OVIPAROUS							
	N	Premaxilla	Maxilla	Dentary	Coronoid	Surangular	Angular	Articular
35	2	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
36	1	*1.0*	*1.0*	*1.0*	*1.0*	*2.0*	*1.0*	*0.0*
39	1	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*
40	7	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*	*4.4*
Hatchling	2	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*	*5.0*

Discussion

There seems to be little difference in the pattern of ossification between embryos of the viviparous and oviparous populations of *Saiphos equalis*. The beginning of ossification in embryos from the viviparous population was at stage 35 with the pterygoid, dentary, parietal, and humerus each showing approximately 10% ossification (index = 1) (Figures 6 and 7).   In the oviparous population, embryos at Stage 36 showed ossification in the pterygoid, dentary, and parietal as well as the prefrontal, premaxilla, maxilla, coronoid, surangular, angular, jugal, and postfrontal (Figure 8). The humerus appears to begin ossification later in the oviparous than the viviparous population.

Figure 6. ***Ventral view of the skull in a stage 35 embryo from the oviparous population [Specimen 5375-R1].***

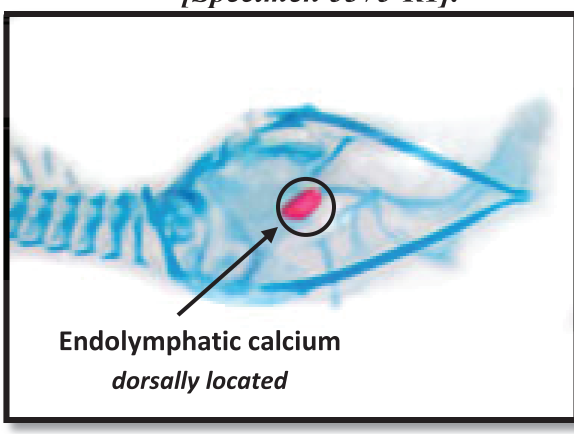


Figure 7. ***Ventral view of the skull in a stage 35 embryo from the viviparous population [Specimen 5359].***

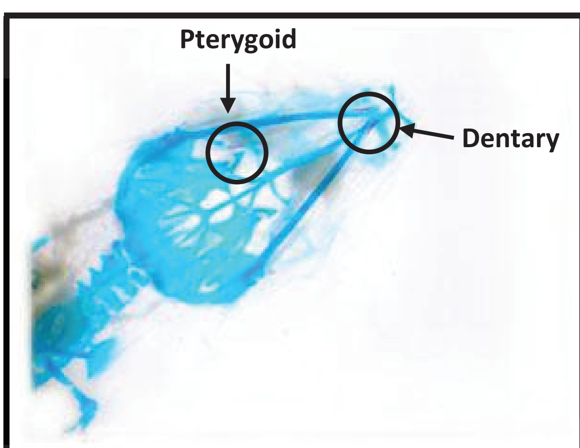
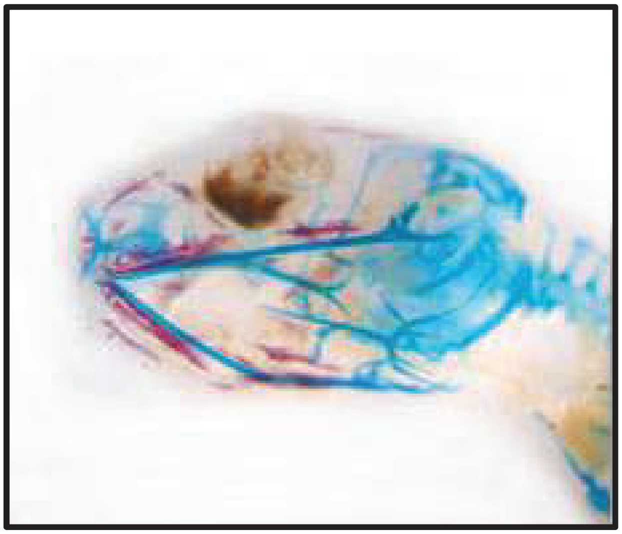


Figure 8. ***Ventrolateral view of the skull in a stage 36 embryo from the viviparous population [Specimen 5373-L]***.



Results here for *Saiphos equalis* are similar to the findings of Rieppel (1994) for *Lacerta agilis exigua. Lacerta agilis* begins ossification at stage 34 with the pterygoid then the surangular. *Saiphos* begins ossification at stage 35 with the pterygoid and dentary, which is also a bone of the lower jaw. However, the findings of Rieppel (1992) on *Lacerta vivipara* conclude that ossification begins at stage 32 with the pterygoid, with the premaxilla showing ossification soon thereafter.

At stage 39, embryos from the oviparous population shows adult-like ossification of the jaw, pterygoid, supratemporal, squamosal, nasal, prefrontal, jugal, and humerus. The supraoccipital, basioccipital, prootic, otootic, parietal, and frontal are just beginning ossification (10-30%, index = 1 or 2). The pubis shows no ossification at this stage. The remaining bones are moderately ossified (a 3 or 4 on the ossification index).

At stage 40, sixteen of the thirty bones assayed seem to show no differences in ossification patterns between embryos from the oviparous and viviparous populations (Figure 9).

There is a small difference (0.5 or 0.6) in average ossification of the palatine, frontal, and prootic bones between the oviparous and viviparous populations (Figure 10). The exoccipital is significantly less ossified in embryos from the oviparous population (average index 3.1 vs. 4.0) at stage 39. The palatine, exoccipital, and frontal bones show small (0.5) differences which could be due to sample size. The majority of the skull is ossified almost to the adult condition, with the exception of the parietal that remains approximately 10% ossified (index = 1) as compared to adults.

Figure 9. ***Bones showing similar ossification at stage 40.***

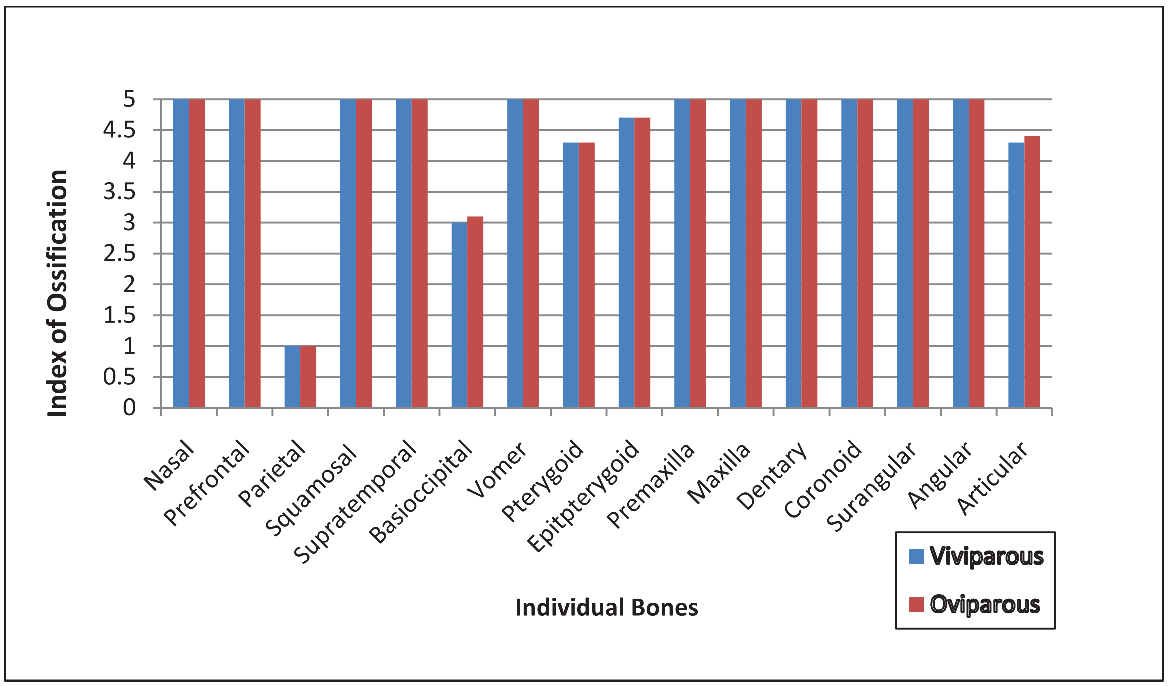
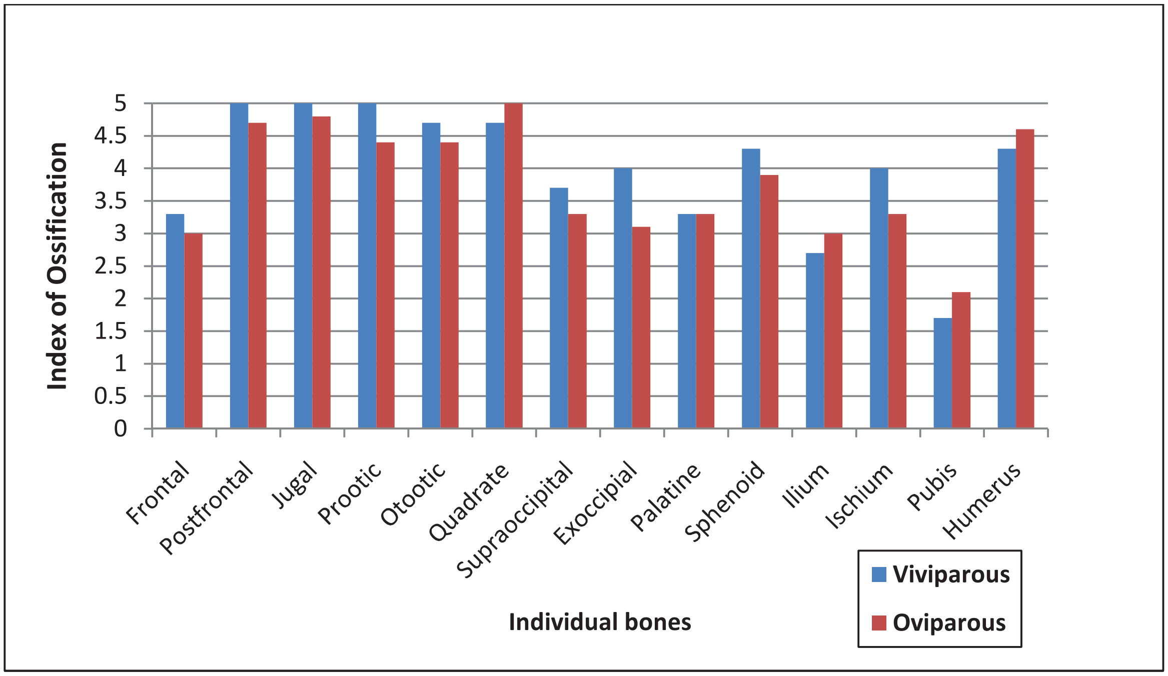


Figure 10. ***Bones showing variable ossification pattern at stage 40.***



There is a high degree of variability in ossification of certain bones within the same stage of development (Table 8). This was particularly evident among the 10 specimens available at stage 40 which had the largest sample size. Within the pelvis, the range in the ossification index among specimens was 1—4 (approximately 10%--70% ossification) for the ilium and ischium, and 0-4 (0—70%) for the pubis. Within the skull, significant variation was present in ossification of the following bones among Stage 40 embryos: supraoccipital (index 0-5, 0—90% ossified; average 3.4); palatine (index 2-4, 30-70% ossified; average 3.3); basioccipital (index 1-5, 10-90% ossified; average 3.1); and the exoccipital (index 3-5, 50-90% ossified; average 3.4). Ossification of the jugal bone was totally absent in three specimens (approximately 30% of total sample). The other samples showed ossification of four or five on the ossification index.

Table 8. ***Ossification index of pelvic girdle at stage 40 showing variation in index scores within both reproductive modes.***

Specimen	Reproductive Mode	Ilium	Ischium	Pubis
5386-1	O	3	4	2
5398	O	1	1	0
5396-2	O	3	3	2
5400	O	3	4	2
5390-3	O	3	3	2
5403-1	O	4	4	4
5376-2	O	4	4	3
5380-L	V	1	4	0
5382	V	2	3	1
5385-4	V	5	5	4

Since there were so many bones ossified at stage 36 in the oviparous population, it is possible that the stage 35 specimens sampled were actually early stage 35 and ossification does not begin until late stage 35. With a larger sample a clearer pattern of ossification could be identified.

The findings of this study are consistent with three of the predictions made by Evans (2008). Her findings predicted that the first bone to ossify should be the pterygoid, and the second should be a bone of the lower jaw. The prediction for the pterygoid holds true for *Saiphos equalis* (this study), *Lacerta vivipara*, and *Lacerta agilis exigua* (Rieppel 1992, 1994). Her second prediction also holds true for *S. equalis* because the second bone to ossify is the dentary of the lower jaw. Rieppel (1994) found that the second bone to ossify in *L. agilis* is the surangular of the lower jaw, but (Rieppel 1992) found that the second bone to ossify in *L. vivipara* was the premaxilla of the upper jaw. The third prediction made by Evans is that all bones will be at least partially ossified by birth/hatching. The data from all three studies support this prediction.

This study showed that the differences in calcium availability as described by Linville et al. (2010) do not necessarily correlate with a difference in the skeletal development, as suggested by Maisano (2001). Maisano (2001) stated that viviparous species are skeletally immature compared to the oviparous species and suggested that differences related directly to differences in calcium availability during development. It is highly possible that the differences observed by Maisano are related to multiple distinctions in the evolutionary histories of the species she examined. The small differences in the ossification between oviparous and viviparous populations of *S. equalis* are most likely due to natural variation between specimens. As such there is little difference between the oviparous and viviparous populations.

It is true that females in the oviparous population of *Saiphos equalis* we sampled lay their eggs only one week prior to hatching. Thus, the time *in utero* varies by approximately only one week between oviparous and viviparous forms. However, the study by Linville et al. (2010) documented significant differences in calcium available from the eggshell and in overall calcium available to embryos between these two populations. This study is a more direct test of the hypothesis that viviparous species will always exhibit delayed ossification of the skeleton and our findings support rejection of this idea as a general observation.

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