The development of the osteocranium in the snake *Psammophis sibilans* (Serpentes: Lamprophiidae)

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Abstract

Non-avian reptiles are good models to investigate structural and developmental differences between amniotes. Investigations of craniofacial development in a complete series of embryos from oviposition up to hatching are still relatively rare. Consideration of a complete series can reveal developmental events that were previously missed, and thus correct or confirm theories about developmental events. The Egyptian Sand snake, *Psammophis sibilans*, has been a key species in descriptions of the snake skull development. However, published work was based on a limited sample of specimens collected from the wild. Here we supplement previous descriptions with an illustrated account of skull development in *P. sibilans* based on a staged series of embryos and histological sections. Our findings largely agree with those of previous authors, although we record differences in developmental timing, confirm the presence of an egg tooth in this species. We add further observations on the enigmatic fenestra X, showing that it closes rather than merging with the prootic notch. Our observations revealed the likely contribution of the tectum posterius to the occipital roof, the presence of an internal carotid foramen (possibly transitory or variable), and the formation of the initial laterosphenoid pillar.

Introduction

Non-avian reptiles including lizards and snakes are good models to investigate structural and developmental differences between amniotes (e.g., Chang et al., 2009; Nomura et al., 2013). They are easily bred and provide accessible embryos at different stages of development. Some previous studies of craniofacial development in snakes were based on specimens collected directly from the field (Parker, 1879; Kamal and Hammouda, 1965a–c). Investigations of craniofacial development in a complete series of embryos from oviposition up to hatching are still relatively rare (e.g. Jackson, 2002; Boughner et al., 2007; Boback et al., 2012; Khannoon and Evans, 2015; Polachowski and Werneberg 2015), but can reveal developmental events that were previously missed, and thus correct or confirm theories about developmental events (Khannoon and Evans, 2015).

In a previous work (Khannoon and Evans 2015), we provided an account of ontogeny in the Egyptian cobra (Elapidae), *Naja haje*. Detailed description of the development of the chondrocranium and osteocranium at an accurate level depended primarily on the collection of a complete embryonic series. Comparison of embryonic tables for *Naja haje* (Khannoon and Evans, 2014) and the Sand snake, *Psammophis sibilans* (Khannoon and Zahradnicek, 2017), showed that they have slightly different incubation periods despite being incubated under the same conditions and temperature. This raised the question as to whether, and to what degree, the rate and pattern of craniofacial development would also differ between the two species.

Comparative analysis of osteocranium development in squamates, including snakes, adds to our general understanding of the evolutionary biology of skull development in vertebrates. Additionally it shows the critical events leading to the unique characters of snake skull, and may help to resolve issues that remain contentious. Two such issues in the braincase are the presence or absence of epiotic centres of ossification and the mode of development of the posterior roof of the braincase. In *Naja haje* embryos (Khannoon and Evans, 2015), we were able to establish the presence of separate epiotic centres on the otic capsules as well as the major contribution of the tectum posterius to the intracapsular plate (contra de Beer, 1937; Bellairs and Kamal, 1981). However, a broader sample of taxa is needed to determine whether there is phylogenetically relevant variation in these features between snakes.

Psammophis sibilans is a common colubroid snake (family Lamprophiidae) distributed across Egypt and parts of north-eastern Africa including Ethiopia, Somalia and Eritrea (The Reptile Database, accessed December 2018). It is a back-fanged (opisthoglyphous) venomous snake but is not considered dangerous to humans (e.g. Vidal, 2002). This snake has been popular in developmental biology as it is well distributed around the River Nile, and is easily kept in the laboratory (Baha El Din, 2006). Additionally, *Psammophis* is not dangerous to humans, and it produces a reasonable number of eggs, with a high percentage of incubation success (Khannoon and Zahradnicek, 2017), facilitating laboratory work. *Psammophis* development was originally described in a series of papers by Kamal and Hammouda (1965a-c). They provided a detailed account of the development of the chondrocranium (1965a,b), based on serial sectioning. This account formed the main basis for the description of snake skull development in Bellairs and Kamal (1981). However, for the osteocranium, Kamal and Hammouda (1965c) described one late embryonic stage, with only limited information on earlier stages.

A detailed embryonic staging series for *Psammophis*, based on external features, was published recently by Khannoon and Zahradnicek (2017). In the current study we describe important stages in the development of the osteocranium up to hatching, but also comment on other features where relevant. The descriptive terminology used is mainly that of Cundall and Irish (2008). We then compare our findings with those of Kamal and Hammouda (1965a-c) and Bellairs and Kamal (1981).

Materials and Methods

Adult gravid females of *Psammophis sibilans* were collected directly from the field in the Nile Delta region of Egypt. They were kept in the laboratory under suitable conditions at ambient temperature of approximately 30-32 °C daytime and 25-27 °C at night, in glass cages of 100 x 70 x 70 cm (length x width x height) and were fed with lizards and mice until their eggs were laid. The average clutch size was 7–12.

Oviposited eggs (N=67) were placed in plastic boxes filled with perlite in an incubator (at 85–90% moisture) at a constant incubation temperature of 30±0.5 °C. The embryos hatched in 49–50 days postoviposition (dpo). This is a much shorter incubation period than the 65 days reported by Kamal and Hammouda (1965a). They did not provide an incubation temperature for their embryos, recording only that the eggs were incubated at 'room temperature' in damp soil. In the current study, embryos were sampled every day for the first half of the incubation period and every two days in the second half of the embryonic period. The animal collection and research protocol were approved and permitted by Fayoum University, Faculty of Science.

Embryos were extracted in phosphate-buffered saline (PBS) and excised from the surrounding extraembryonic membranes. Embryonic stages were identified according to the embryonic table by Khannoon and Zahradnicek (2017). Eggs were sampled to represent all stages of osteocranium development, and the embryos preserved in 4% paraformaldehyde and 10 % buffered formalin. The heads were processed and dehydrated in ethanol up to 95% ethanol for 5-7 days, and then stored in acetone for a further three days. Samples were then stained using an ethanol-KOH-Glycerol Alizarin red-Alcian blue staining protocol (Hanken and Wassersug, 1981) for three days, transferred into 1% KOH, and taken through a series of glycerol:KOH ratios. The stained heads were stored in 100% glycerol. Images were captured using an Olympus SZH10 stereo-microscope with a Rebiga 2000R camera attachment.

In addition to the cleared and stained specimens, heads of embryos representing different embryonic stages of *P. sibilans* were excised and fixed at 4%PFA or 10% formalin. At least two heads from each stage were processed. These were washed, and dehydrated in ascending series of ethyl alcohol (Sigma-Aldrich). After dehydration samples were cleared in xylene (Sigma-Aldrich), infiltrated and embedded in paraplast (Sigma-Aldrich). Samples were sectioned either transversely or sagittally at 7-9 µm thickness using a Slee Cut 5062 microtome. Paraffin sections were deparaffinized, hydrated and stained either in Hematoxylin and Eosin or Masson Trichrome Stain (Humason, 1979). Sections were mounted in DPX (Sigma-Aldrich), coverslipped, and imaged using an AmScope Stereo Microscope adapted with AmScope digital camera (Fig.1). For higher magnifications, a Nikon light microscope was used.

Results

The earliest embryo to be studied was opened on the day of oviposition (0 dpo), a stage 1 embryo (based on Khannoon and Zahradnicek 2017). Kamal and Hammouda (1965a,b) provided a detailed account of the chondrocranium based on serial sections. For that reason, we have focused mainly on osteocranial development starting from stage 5 (20dpo), the first stage at which mineralizations could be observed, noting key aspects of chondrocranial morphology as relevant or where our findings differ from those of previous authors. The descriptions are based mainly on the cleared and stained specimens, with additional detail from histological sections if relevant.

Stage 5, 20dpo (Fig. 1A,B)

This stage is roughly equivalent to the late stage III (chondrocranium) of Kamal and Hammouda (1965a,b). The skeletal double staining showed little detail other than the chondrocranial components. However, histological serial sectioning provided evidence of early mineralization around Meckel's cartilage (MC) to form the compound bone of the lower jaw (i.e. fused surangular, prearticular, and articular), and in the palate, the supratemporal, and ventral flanges of the parietals (Fig. 1A,B). This is earlier that revealed by alizarin staining in the whole mount embryos.

Stage 6, 23dpo. (Fig.2)

The main parts of the chondrocranium are well developed at this stage, with prominent nasal capsules (Fig. 2A) and chondrified otic capsules (Fig. 2B). As in all snakes, the trabeculae cranii are separate between the orbits (platytrabic condition, Fig. 2C) and form an angle of approximately 30-40° with the nasal capsule. Anteriorly, the trabeculae are fused and contribute to the nasal septum. Medially, behind the orbits, the pituitary (hypophyseal) fenestra is visible as a wide space flanked by the trabeculae (Fig.2C). Posterior to the pituitary fenestra, and separated from it by the crista sellaris, the basicranial fenestra is large (Fig.2C).

The notochord is visible as a weak thickening along the dorsal midline of the basal plate posterior to the basicranial fenestra (also visible in Fig. 1B). In the otic region, the facial foramen is already extracapsular and is separated from the prootic notch (incisura prootica) anterior to it by the prefacial commissure. The prootic notch is open anteriorly and there is no trace of ossification in its margins. Ventral to both foramina is a large comma-shaped opening that corresponds to the fenestra X of previous authors (e.g. Kamal and Hammouda, 1965b; Bellairs and Kamal, 1981). It reportedly forms by breakdown of the cartilage in this area (Bellairs and Kamal, 1981) and nothing passes through it. The occipital roof, between the otic capsules, is unstained by Alcian blue suggesting that this region may be procartilage.

At 23dpo, the ventral part of the maxilla is visible as a narrow blue strip below the eye (Fig. 2A,C), with a slight development of the facial process (discontinuous). Slender mesenchymal primordia of the premaxilla, prefrontal and frontal are also visible anterior/anterodorsal to the eye, and a thin condensation above the quadrate (Fig. 2D) may be the precursor of the supratemporal, consistent with the histological sections from the 20dpo embryo (Fig.1B). Each ventrolateral parietal flange appears as a diffuse condensation between the eye and the otic capsule, and there is weak alizarin staining ventrally. Palate development has also begun with weak ossification along the length of the skull (Fig.2A). In the lower jaw, Meckel's cartilage is enveloped in mesenchymal anlagen of the dentary and other jaw elements, and ossification is visible in the compound bone. The ventral parts of the occipital arches show the beginning of ossification.

Meckel's cartilage is well developed on each side. A vertical quadrate cartilage, expanded dorsally and tapering ventrally, meets Meckel's cartilage at a right angle (Fig.2B). The quadrate is vertical in orientation and adjacent to the posterior one third of the otic capsule, perichondrial ossification is beginning in the shaft. The posterior extension of Meckel's cartilage behind the quadrato-mandibular joint will form the retroarticular process of the articular. The hyoid appears as an inverted Y-shape, with an anterior lingual process and paired ceratohyals.

The endolymphatic sacs are stained red, and possible saccular and utricular maculae are also visible as blue/purple spots within the vestibular chamber of the ear (Fig.2A).

Stage 6, 24dpo (Fig.3)

A well-defined crescent-like condensation (unossified) is visible in the posterodorsal margin of the orbit. The homologies of this element, as postfrontal or postorbital, remain contentious. Here we refer to it as the postorbital, reflecting the usage of Cundall and Irish (2008). The ventrolateral flanges of the parietal between the eye and otic capsule show further islands of ossification (Fig.3A), and patches of pink alizarin staining, indicating mineralization, are also visible in the dentary, maxilla, premaxilla, prefrontal, and ectopterygoid. Ossification of the compound bone and pterygoid is more extensive, as is the ossification in the quadrate shaft. The internal process is well chondrified but is continuous with the stapedial shaft.

The otic capsule remains mainly cartilaginous (Fig.3B). Anterodorsally, the medial corners of the otic capsules are now joined by a cartilage (Alcian blue stained) intercapsular bridge. At the posterior edge of the occipital roof, the two occipital arches (stained by Alcian blue) extend to the dorsal surface and meet, or nearly meet in the midline. They are separated from the anterior intercapsular bridge by a clear (unstained) zone (Fig.3C). The basicranial fenestra is large and open (Fig.3C). There is ossification in the prefacial commissure that extends into the posterior part of the cartilage bar separating the prootic notch from fenestra X (Fig.3B). Ossification is also beginning in the crista sellaris. At the junction of the crista sellaris and the trabeculae, on each side, there is a foramen which we interpret as being for the internal carotid artery (Fig.3C).

Stage 7, 26dpo (Fig.4) and 29dpo (Fig. 5)

This stage is roughly equivalent to stage IV (chondrocranium) of Kamal and Hammouda (1965a,b). At 26dpo, ossification has progressed in those dermal skull components identified in previous stages (Fig.4A). However, the braincase appears relatively unchanged and is slightly less ossified than at 24dpo (Fig.4B). There remains an internal carotid foramen at the junction of the trabeculae and crista sellaris on both sides. In the occipital roof, the two occipital arches are slightly further separated in the midline (Fig. 4C). In the 29dpo embryo (Fig.5) there is further ossification in some of the dermal elements (e.g. parietal flanges, compound bone), with others are seen clearly for the first time (premaxilla, frontal plate, Fig.5A,B); and the egg tooth shows some mineralization. As in *Naja haje*, the prefrontal seems to have dorsal and ventral centres of ossification (Fig.5B). The internal process of the quadrate is now separated from the stapedial shaft (Fig.5C). However, this embryo also seems to show a delay in development of braincase characters in that there is no ossification in the prefacial commissure and no chondrified intercapsular bridge. The occipital arches are ossified in the posterolateral portions, with the anterodorsal and dorsomedial regions chondrified but separated in the midline by the unstained occipital roof.

Stages 7-8, 31dpo & 32dpo (Figs 6 and 7)

This stage is roughly equivalent to stage V (chondrocranium) of Kamal and Hammouda (1965a,b). In the embryos from 31dpo (Fig.6A-D) to 32 dpo (Fig.7A-D), there is increased maturation of cranial elements including the premaxilla, prefrontal, postorbital, supratemporal, maxilla, quadrate, pterygoid and ectopterygoid, and the first appearance of the septomaxillae (seen in Fig.1C, 35dpo) and vomers. The ventrolateral flanges of the parietal form a more consolidated sheet with ossification having progressed posteriorly over the curve of the otic capsule. Ossification is present in the orbital margins of the frontals at 31 dpo (Fig.6A), and this is more extensive in the 32dpo specimen (Fig.7A). In the lower jaw, the splenial and angular are clearly visible and form an intramandibular joint (Figs 6C, 7C), the compound bone is further ossified, and the quadrate is adjacent to the posterior margin of the otic capsule. The anterior ends of Meckel's cartilage extend medially from the tips of the dentaries (Fig.6C). In the braincase, the basicranium is ossifying and the crista sellaris is bone. However, the lateral margins of the basicranial fenestra remain cartilaginous (31dpo and 32dpo). The prootic (trigeminal) notch is undivided but a small process extending ventrally from the curve of the anterior semicircular canal in the 31dpo embryo partly closes the anterior margin (Fig. 6D). This process, an early stage in the formation of the laterosphenoid, is stained with alizarin, as is a thin strip along the ventral floor of the anterior semicircular canal. The process therefore seems to be a membranous ossification rather than ossification into the cartilage strut that forms the dorsal margin of fenestra X, although there is a small dorsal elevation of the cartilage supporting the ventral part of the bar. However, a detailed histological study would be needed to confirm this. The process is further developed in the 32dpo embryo where it almost meets a second process extending upward from the ventral margin of the prootic notch (Fig.7A). Here the dorsal part of the developing pillar is about twice the length of the ventral component. Fenestra X is still open but its dorsal margin is mostly ossified in the 32 dpo embryo and the ossification seems to be extending over the dorsal part of the fenestra. Histological sections of a 35dpo embryo are consistent with the above interpretation as they show dermal bone forming the laterosphenoid and covering fenestra X (Fig.1D). The footplate of the stapes has ossified by 32dpo, as is most of the adjacent shaft but there is a small area of persistent cartilage at the junction of footplate and shaft. In the otic capsule, there are centres of ossification in the prootic and opisthotic, separated by thick unossified regions of cartilage. In the occipital roof at 31dpo (Fig.6B), the anterior intercapsular bridge is ossifying, with a strong band of Alcian stained cartilage across its posterior edge. The laminae of the occipital arches are also ossified. They do not meet in the midline but there is a strip of Alcian stained cartilage across their anterior margins that is unbroken across the midline. This is further consolidated in the 32dpo embryo (Fig.7D), but the deeply Alcian stained anterior edges of the occipital arch laminae no longer meet in the midline. In the supraoccipital, we could not confirm the presence or absence of separate (epiotic) centres bilaterally, although in the dorsolateral corners of the supraoccipital at 32dpo, there are islands of bone surrounded by cartilage (Fig.7D).

There is a thickening of glandular tissue in the labial margins, although this is still unstained, and also below the parietal flanges where Duvernoy's gland is developing (31dpo). Mineralised tooth caps are visible in association with the upper and lower jaws at 32 dpo (Fig.7A).

Stage 9, 38dpo & 39dpo (Figs 8 and 9)

Two embryos were sampled at this stage and they show different levels of development, particularly in some braincase features. In one specimen, the occipital roof is still a combination of stained cartilage and unstained areas, but in the second the supraoccipital and exoccipitals (=occipital arches) are fully ossified and separated only by narrow strips of cartilage. The specimens also differ in the extent of ossification of the crista sellaris and the rim of the trigeminal foramen (closed prootic notch). However, the nasals and frontals are more fully developed in both specimens, and both specimens show a thickening of the laterosphenoid and intramembranous ossification covers fenestra X. At 39dpo, the frontals are fully ossified, meet in the midline and, with the ossified prefrontal and postorbital, encircle the eyes of the embryo (Fig.9A,C). The nasals form a rhomboid cover over the nasal capsule (Fig. 9E). The facial process of the maxilla has deepened and the teeth are clearly visible on the maxilla and dentary but are not implanted (Fig.9A). The parietal flanges are well ossified and obscure deeper structures but the parietal remains undeveloped dorsally, leaving the brain visible under transparent skin. The remains of the endolymphatic sacs are visible but they are empty. The quadrate is almost fully ossified except at the proximal and distal ends and is angled posteriorly (Fig.9B); the stapedial process is complete. The components of the otic capsule are well ossified but still separated by zones of cartilage. In the occipital roof, only a thin line of cartilage separates the supraoccipital from the dorsal laminae of the exoccipitals (=occipital arches). The latter are not yet fused to the opisthotics. Fenestra X is closed by intramembranous ossification and an intramembranous laterosphenoid ossification extends into the trigeminal foramen from its ventral margin, dividing it anterior (maxillary) and posterior (mandibular) nerve foramina. In the region of the fenestra vestibuli, there appears to be some extension of the posterior margin of the prootic as an incipient crista circumfenestralis but the ventral margin of the fenestra, between the prootic and opisthotic ossifications, remains cartilaginous. In the opisthotic, there are two foramina posterior to the fenestra vestibuli. We interpret the more anterior of these to be the lateral opening of the recessus scala tympani, separated from the fenestra vestibuli by a narrow interfenestral bar, and the second as the vagus foramen.

Stage 10, 46dpo (Fig.10)

This stage is equivalent to stage VI (chondrocranium) of Kamal and Hammouda (1965a,b). Most of the skull and lower jaw elements are well ossified (Fig.10A-D), although the otic capsule components remain separate (including exoccipitals and opisthotics) and the parietal does not yet cover the brain dorsally (Fig.10B). The teeth are more fully developed and heterodont (e.g. four large anterior teeth on mandible with smaller teeth behind) and the laterosphenoid ossification seems to be complete. In the ear region, the ventral margin of the fenestra vestibuli (junction of prootic and opisthotic) is now almost completely ossified, and there appears to be some development of the posterior lamina of the crista circumfenestralis. The head is now snake-like in shape. Supralabial and infralabial glands are visible (having picking up stain), and there is a clear mass ventral to the parietal flange representing the developing venom gland (Duvernoy's gland). A shiny collagenous band extends from the quadrate into the capsule of the gland (Fig.10A).

Prehatching (late stage 10), 49dpo (Fig.11A-D) & 50dpo (Fig.12A-C)

At this stage, the frontals are complete. Ossification is progressing from lateral to medial in the parietal, but the posteromedial part of the skull roof remains incomplete up to hatching. Thin lines of cartilage still separate the otic capsule components. The margins of the footplate are less obvious, suggesting that the circumfenestral crest has begun to develop. The teeth are now implanted and show size differentiation with enlarged maxillary teeth in the anterior midsection of the bone. The supra- and infralabial glands are well developed and Duvernoy's gland is more pronounced.

Discussion

Kamal and Hammouda described head development in *Psammophis sibilans* in a series of papers published in 1965. These papers formed the basis of the basic account of the developing snake chondrocranium in Bellairs and Kamal (1981). We have not sought to add to the description of the chondrocranium, which is very detailed in the above works, but did find some points of discrepancy with respect to developmental timing and some aspects of osteocranial development.

As noted above, Kamal and Hammouda (1965a) reported that the incubation time for the embryos they studied (from wild caught pregnant females) was 65 days at 'room temperature'. Without knowing what that temperature was, nor how much it fluctuated between day and night, it is difficult to explain why it took roughly 15 days longer for their eggs to develop than ours. However, if their eggs were developing more slowly, it would explain many of the discrepancies in developmental timing whereby our embryos are generally 10-15 or more days in advance for any given stage. Thus Kamal and Hammouda (1965a,b), for example, show the basicranial fenestra as absent at 22dpo and state that it begins to form around 26dpo by secondary breakdown of the cartilage in the anterior basal plate. In our 17 dpo embryo, the fenestra is already large. Kamal and Hammouda (1965c) reported tooth rudiments to be first visible at 58 dpo, in conjunction with the formation of the angular-splenial joint, whereas we observed them both at 32 dpo.

In addition, Kamal and Hammouda (1965c) gave a detailed account of the osteocranium in only one late stage (58dpo) embryo, although they mention a small number of earlier ossification events in their paper. According to these authors, the membrane bones of the skull first appear in their 35dpo embryo (contra 20dpo in our study), shortly before the first appearance of ossification in the chondrocranium (24dpo in our study). They also recorded that the maxilla and pterygoid appear slightly before other elements, which agrees with our observations although we also observed ossification in the compound bone of the lower jaw in the same embryo. However, Kamal and Hammouda (1965c) reported that the frontals were incompletely ossified dorsally even for some time after hatching, whereas we found the frontals to be largely complete in our prehatching stage with only the parietal roof open.

Rather surprisingly, Kamal and Hammouda (1965a) reported the absence of a tooth on the premaxilla in *Psammophis*, although they noted the presence of a median egg tooth in other snake embryos. In fact, a median egg tooth is present on the premaxilla in our *Psammophis* embryos (first seen as a mineralized element at 31 dpo) – presumably Kamal and Hammouda (1965a) either missed it or it was lost in preparation in their specimens.

No extant snake possesses a separate lacrimal bone, and it is also unrecorded in any early/primitive fossil snake (e.g. Rieppel et al. 2003; Scanlon, 2006; Zaher & Rieppel, 2012). However, the bone is present in most lizards and may, therefore, have been present in the ancestry of snakes. In *Naja haje* (Khannoon and Evans, 2015), the prefrontal bone developed from two separate centres of ossification, raising the possibility that the more ventral of these represented a lacrimal primordium. Kamal and Hammouda (1965c) discounted the presence of a lacrimal in *Psammophis*, but at 29dpo, our *Psammophis* embryos show dorsal and ventral ossification centres like those of *Naja haje* (Figs 3A and 4A).

In reptiles, the posterior roof of the braincase (intercapsular plate in Khannoon and Evans, 2015) may be formed either from the tectum synoticum (derived from the otic capsules) or a combination of the tectum synoticum and the tectum posterius (derived from the dorsal laminae of the occipital arches)(Bellairs and Kamal, 1981). According to many authors (e.g. De Beer 1939; Bellairs and Kamal 1981; Rieppel and Zaher 2001), the occipital roof in snakes is primarily (or exclusively) derived from the tectum synoticum because the occipital arches fail to meet in the midline. However, El Toubi and Kamal (1965) reviewed the literature on snake skull development and found considerable variation in the literature with respect to the tectal contributions in different snake species. Their view was supported by our study of the Egyptian cobra, *Naja haje* (Khannoon and Evans, 2015) that indicated that the tectum posterius formed the major component of the occipital roof in this species. De Beer (1937, p.393) also commented on the difficulty of distinguishing these components in some embryos.

Kamal and Hammouda (1965a,b), followed by Bellairs and Kamal (1981), reported that the braincase roof in *Psammophis* formed solely from the tectum synoticum, and that the dorsal laminae of the occipital arches fail to meet in the midline to form a tectum posterius. Our observations on *Naja haje* prompted us to re-examine the development of this region in *Psammophis*. At 23 dpo, the anterior, intercapsular, region of the braincase roof is clear (unstained by Alcian blue), but there is deep blue staining posterolaterally in the dorsal laminae of the occipital arches, although these do not meet in the midline. At 24 dpo, the otic capsules are joined anteriorly by a short intercapsular bridge of stained cartilage, with an unstained region separating the anterior bridge from the dorsal laminae of the occipital arches. These laminae meet, or almost meet, in the dorsal midline forming a second Alcian stained bar across the posterior edge of the occipital roof. At 31dpo, ossification extends through the intercapsular bridge and also through the laminae of the occipital arches, but these structures remain separated by three bands of tissue, all of which are continuous across the midline: a band of Alcian stained cartilage along the posterior margin of the developing supraoccipital, an unstained area, and a narrow band of Alcian stained cartilage along the anterior margins of the ossifying dorsal laminae of the occipital arches. The morphology is very similar at 32dpo and 38dpo, except the posterior cartilage band is no longer continuous. In our 39dpo embryo, the unstained region is no longer present and there remains only a thin, continuous strip of cartilage between supraoccipital and occipital arches. This persists until hatching. The dorsal lamina of the fully ossified exoccipitals approach closely in the midline but are not confluent.

Kamal and Hammouda (1965a,b,c) reported the presence of a rudimentary tectum synoticum at 35dpo but rejected the possibility of a contribution to the occipital roof by tectum posterius in *Psammophis* because the occipital arches fail to meet in the midline. This was repeated by Bellairs and Kamal (1981) in their review of the snake chondrocranium. However, because Kamal and Hammouda (1965a,b,c) sampled only a limited number of specimens, they may have missed stages like those demonstrated by our 24dpo and 31dpo embryos where a posterior cartilage band, derived from the occipital arches and therefore equivalent to the tectum posterius, extends across the occipital roof posterior to the area of the tectum synoticum. We therefore interpret the occipital roof in *Psammophis* to be a combination of tectum synoticum and tectum posterius. However, the condition in *Psammophis* does appear to differ from that in *Naje haje* where the dorsal laminae of the occipital arches extended forward between the otic capsules and seem to have contributed the majority of the occipital roof.

A second point of contention with respect to the supraoccipital in snakes is the presence or absence of separate, epiotic, centres of ossification in the dorsal part of the otic capsules. We found evidence of these centres in *N. haje* (Khannoon and Evans, 2015) but were unable to confirm their presence in *Psammophis*.

In the basicranium, we were able to trace the fate of fenestra X. This somewhat enigmatic foramen has not been recorded in lizards but does appear in many snakes (Bellairs and Kamal, 1981). According to Kamal and Hammouda (1965b), the fenestra is closed by a membrane and nothing passes through it. They did not discuss its subsequent fate. Bellairs and Kamal (1981), however, stated that the fenestra may become continuous with the prootic notch. This does appear to be correct if only the fate of the Alcian stained cartilage is followed, but this is because the cartilage bar that separates the fenestra from the prootic notch above it is replaced by bone, with ossification extending into (or from) the prefacial commissure. In later embryos, ossification then extends into the membrane covering the fenestra so that it is closed and incorporated into the ventrolateral wall of the basicranium.

The prootic notch (future trigeminal foramen) lies immediately above fenestra X. In the early embryonic stages (Stages 5,6), the notch is open anteriorly, but the 31dpo embryo (Stage 7) shows the beginning of formation of the laterosphenoid element that closes the notch anteriorly and then extends posteriorly around the exits of the maxillary and mandibular divisions of the trigeminal nerve. The initial closure of the notch involves small dorsal and ventral processes growing towards one another and making contact (between 32 and 38 days in our embryos). The dorsal process extends from the ventral curve of the anterior semicircular canal and forms the major part of this initial laterosphenoid pillar. The ventral process extends from the bar of cartilage forming the upper margin of fenestra X. Neither process appears to have a cartilage precursor, and this is confirmed by the histological sections. This conclusion is consistent with the view of Bellairs and Kamal (1981) that the laterosphenoid in *Psammophis* is fully intramembranous. However, whereas the account and figures (fig.68, 69) of Bellairs and Kamal (1981) suggest the ventral process forms most of the initial pillar, we found that it was actually the dorsal component that predominated in *Psammophis*, with a second sheet then arising from the ventral margin to divide the trigeminal foramen.

One final point concerns the course of the internal carotid artery in relation to the pituitary (hypophysial) fenestra. According to Bellairs and Kamal (1981), the internal carotid arteries pass from dorsal to ventral at the posterolateral corners of the pituitary fenestra where the trabeculae cranii meet the crista sellaris. In some snakes (e.g. *Natrix*, *Lamprophis*), the artery is enclosed within a discrete foramen (Parker, 1879) whereas in others (including *Psammophis*) it merely passes through the corner of the fenestra. However, we identified paired foramina in our 26dpo *Psammophis* embryos (less certainly at 24dpo) that correspond in position to those in described in *Natrix*. The feature may be therefore be variable.

Conclusions

Psammophis has long been one of the key genera in the understanding of skeletal development in colubroid snakes, and was the main exemplar used for colubroid snake development in the review of Bellairs and Kamal (1981). Although our observations largely agree with the descriptions of Kamal and Hammouda (1965a-c), the more complete embryonic series has permitted a fuller account of skull development in this species. It has revealed the likely contribution of the tectum posterius to the occipital roof, the presence of an internal carotid foramen (possibly transitory or variable), and the formation of the initial laterosphenoid pillar. We were able to confirm that *Psammophis* has an egg tooth like other snakes, contra Kamal and Hammouda (1965c), and also add further observations on the enigmatic fenestra X, showing that it closes rather than merging with the prootic notch as suggested by Bellairs and Kamal (1981). Our embryos developed more quickly than those of Kamal and Hammouda (1965c), reaching equivalent stages 10-15 days earlier. Equally, even within our own sample, we found that some embryos were more or less advanced than others. This presumably reflects differences between clutches, and emphasizes the need for researchers to monitor and record clutch and incubation conditions in studies of this kind.

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Abbreviations used in the figures

An – angular
Asc – anterior semicircular canal
Bc.f – basicranial fenestra
bp – basal plate
c.a – clear region of occipital roof
Col.bd – collagenous band
Com – compound bone
CN5 – trigeminal nerve
Cr.s – crista sellaris
D – dentary
dvl- dorsal and ventral processes of laterosphenoid
Ect – ectopterygoid
Eg.tth – egg tooth
En.s – endolymphatic sacs
F.f – facial nerve foramen
FenX – fenestra X
Fr – frontal or frontal primordium
Hy – hyoid
icb – intercapsular bridge (tectum synoticum)
icf – internal carotid foramen
I.l.g - infralabial glands
in.p – internal process of quadrate
Jj – jaw joint
J.o – Jacobson's organ
Lsph - laterosphenoid
Ma – mandible
Mc - macula
Mk.c – Meckel's cartilage
Mx – maxilla
Mx.f – maxillary fangs
N - nasal
N.cp – nasal capsule
no – notochord embedded in basal plate
Occ.a – occipital arch (tectum posterius)
Occ.r – Occipital roof
Ot.c – otic capsule
Op – opisthotic
oss – ossification
Pa – parietal
Pa.fl – parietal flange
Pal - palatine
Pa.pr – palatine process of maxilla
Pi.f – pituitary fenestra
Pmx - premaxilla
Po – postorbital
Prfr – prefrontal
Pfc – prefacial commissure
PrO - prootic
PrOn – prootic notch
Pt – pterygoid
Qu – quadrate bone/cartilage
Qu-A – quadrate articular joint
Rap – retroarticular process
Smx – septomaxilla
Soc - supraoccipital
Sp – splenial
St – supratemporal
Sta – stapes
S.l.g – supra labial glands
Tr - trachea
Tr.c – trabeculae cranii
Tth – teeth or developing teeth
U.o.r – unossified area in parietal roof
Ve.g – venom gland

Figure captions

Fig. 1 Histological sections through different stages of *Psammophis*. A, 20dpo embryo showing ossification around Meckel's cartilage in the lower jaw and also within the palate. B, Stage 5, 20 dpo (days post oviposition), embryo showing supratemporal ossification above the head of the quadrate, as well as the notochord embedded in the dorsal surface of the basal plate. C, 35 dpo embryo showing the development of Jacobson's organ and septomaxilla. D, 35 dpo embryo showing development of the laterosphenoid lateral to the trigeminal ganglion and thin sheet of dermal bone over the position of Fenestra X. Scale bar 1 mm

Fig. 2. Stage 6, 23 dpo, embryo in A, left lateral, B, left posterolateral, C, right ventrolateral, and D, left dorsolateral views, showing chondrocranium and early stages of ossification in the jaw and palate. Scale bar 1 mm

Fig. 3. Stage 6, 24 dpo embryo, in A, right lateral, B, right lateral enlargement of otic capsule, C, posterodorsal views, showing increased dermal ossification and the early stages of supraoccipital formation. (*) This U-shaped edge is the underlying edge of the basicranial fenestra. Scale bar 1 mm

Fig. 4. Stage 7, 26 dpo embryo, in A, right lateral, B, left lateral otic capsule, and C, posterodorsal views. Scale bar 1 mm

Fig. 5. Stage 7, 29 dpo embryo, in A, right lateral, B, right anteroventrolateral, and C, posterior dorsal views. Scale bar 1 mm

Fig. 6. Stage 7/8, 31 dpo embryo, in A, right lateral, B, posterior dorsal, C, right ventrolateral, and D, right posterolateral views. The dotted oval in D encloses the dorsal and ventral components of the developing laterosphenoid. Scale bar 1 mm

Fig. 7. Stage 7/8, 32 dpo embryo, in A, left lateral, B, left lateral otic capsule, C, right ventrolateral, and D, posterior dorsal views. Scale bar 1 mm

Fig. 8. Stage 9, 38 dpo embryo in A, ventral, B, anterior dorsal, and C, posterodorsal views. Scale bar 1 mm

Fig. 9. Stage 9, 39 dpo embryo in A, left lateral, B, dorsal, C, left anterior lateral, D, left ventrolateral, and E, left dorsolateral views. Scale bar 1 mm

Fig. 10. Stage 10, 46 dpo embryo in A, right lateral, B, left dorsolateral of posterior skull, C, left ventrolateral of anterior skull, and D, right dorsolateral of anterior skull. Scale bar 1 mm

Fig. 11. Late stage 10, 49 dpo embryo in A, left dorsolateral, B, supraoccipital region, dorsolateral view, C, left anterodorsolateral view, D, right ventrolateral view showing development of labial glands. Scale bar 1 mm

Fig. 12. Late stage 10, pre-hatching, 50 dpo embryos. A, embryo 1 in right lateral view, and B-C, embryo 2 in B, dorsal and C, right ventrolateral views. Scale bar 1 mm