Snout scale development in Alligator mississippiensis and the ancestral condition of keratinized structures in the reptilian face

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Arianna Lord, 28 April 2020
Abstract

The evolution of distinct keratinized structures in reptiles such as beaks, scales, and feathers occurred through specialization of the cornification process. Contrary to the molecular signaling responsible for spatial patterning of reptilian body scales and avian feathers (Di-Poi & Milinkovitch, 2016), scales on the snout of Nile crocodile (*Crocodylus niloticus*) have been described to occur through a stochastic cracking process (Milinkovitch et al., 2013). This cracking process is supposedly unique to the crocodilian face and has not yet been described in any other lineages. Here, the same stochastic cracking process is described in embryos of the American alligator (*Alligator mississippiensis*) and compared to the development of scales on the snout of Squamates and beak formation in Aves and Testudines. Comparative analysis of the morphological changes that occur in the spatial and temporal development of cracks confirms that epidermal patterning on the crocodilian snout is developmentally distinct from other lineages. Crack vertex angle analysis suggests the likelihood of maturation in the process cracking through development. Observations of asynchronous cracking reveals distinct regions correlated with the position of underlying dermal bones and their subsequent ossification and growth. Cracking is initiated at the rostral tip of the snout in conjunction with an inferred local decrease in epidermal elasticity. Rostral epidermal thickening is also involved in the initiation of cornification in the developing beaks of birds and turtles. This process of rostral epidermal thickening that subsequently leads to different morphologies as ontogeny progresses may provide a putative developmental basis for instances of convergent rhamphotheca in some clades but not others.
Introduction

Cornification is the programmed cell death of epidermal keratinocytes to form a resistant outer matrix of hard skin derivatives (Eckhart et al., 2013). In the molecular coordination of this terminal differentiation program waves of gene expression provide instructions for epidermal keratinocytes to replace their intracellular content with a compact proteinaceous cytoskeleton (Eckhart et al., 2013). The evolution of cornification was a critical innovation for the success of amniotes in the terrestrial environment as it helped their common ancestor to confront the challenges of life on land outside of aquatic environment (Tucker & Benton, 1982). In the first animals adapting to fully terrestrial life, the development of a durable, multilayer stratum corneum, resistant to mechanical damage, afford UV protection and prevent water loss was critical to survival and success on land (Chang et al., 2009). Reptilia is characterized by an apomorphic form of corneous proteins, beta keratins, that contribute to the formation of a hard resistant corneous outer epidermis (Alibardi et al., 2009). While production of these proteins increases the strength of the epidermis, it also impedes skin elasticity hence the retention of more flexible alpha keratin in the scale hinges (Alibardi et al., 2009). Reptilian scales and feathers are homologous at the level of embryonic epidermal papilla producing both of them, if not the adult structures produced from them (Chang et al., 2009). Cornification of epidermal appendages starts usually during the later stages of embryogenesis (Alibardi, 2009).

A number of cellular, molecular and mechanical mechanisms are involved in species specific patterning and interactions between epithelial and mesenchymal cells that produce composite embryonic primordia of scales, feathers or beaks. The developmental program of beaks and feathers utilizes mesenchyme as a source of species-specific patterning information (Schneider, 2005). Mesenchymal cells (underlying dermis) in the face and neck arise from cranial neural crest, while epithelial cells arise from the surface ectoderm to form a stratified epidermis, the uppermost layer of which form the stratum corneum (Schneider, 2005). It is this upper most stratum corneum that forms the cornified tissues that comprise scales and the beaks and feathers derived from them. Scales and feathers are the same compositionally (beta keratin), and developmentally (both derived from epidermal papillae), the final product produced in each instance however are not the same.
The Reptilian clade comprises Lepidosauria (squamates and tuatara), Testudines (turtles), and Archosauria (Crocodileans and Aves). The position of Testudines within Reptilian phylogeny has been subject to debate over the years due to its perplexing body plan, however recent genomic and paleontological evidence suggest this clade as sister to Archosauria (fig.1) (Irisarri et al., 2017). For the purpose of this paper, this will be the consensus topology.

![Figure 1. Tree showing phylogenetic relationship among major Reptilian clades.](image)

The evolution and development of keratinized structures in Reptilia is linked to region-specific modifications in dermal-epidermal interactions in specific areas of the skin (Alibardi et al., 2009). Feathers, body scales of crocodilians and shell-scutes of developing turtles from embryonic placodes, alternatively scales in squamates develop from regular dermoepidermal elevations determined by molecular signaling but in the absence of a placode structure, (Di-Poi & Milinkovitch, 2016). While lepidosaurs are peculiar in having superimposed as opposed to juxtaposed alpha and beta keratin zones, epidermal condensation in the initial stages of
development is anatomically common across structures and lineage specific traits develop as pathways become specialized through development (Di-Poi & Milinkovitch, 2016).

Scale development appears to be most commonly studied on the body and limbs of various organisms, this study however focuses on development of scales on the face and snout. While evidence across lineages strongly supports the hypothesis for an anatomical placode-like structure in the common ancestor of all Reptiles, not every part of the body in all lineages demonstrate molecular patterning prior to scalation. Contrary to the regular development of scales on the crocodilian body, the epidermal patterning of the face are random polygonal domains which are suggested to have emerged through a stochastic cracking process (Milinkovitch et al., 2013). These facial scales do not have a placodal origin and it is hypothesized that rapid growth of the face and jaw leads to a mechanical cracking of the overlying, continuous, rigid, keratinized epidermis (Milinkovitch et al., 2013). A thick cornified skin on gator snouts contributes to skull stiffness, so to maintain this stiffness during growth perhaps cracking is preferable to regular scale formation.

Milinkovitch et al. propose that cracking occurs not through shrinkage of the overlying layer but rather growth of the underlying substrate in which bulges in the skin are caused by local cell proliferation propagating in a tension field and causing the overlying layers to crack. They note that the deepest-first-order cracks tend to traverse the width of the face because the head is growing faster longitudinally than in other directions (Milinkovitch et al., 2013).

Following Milinkovitch et al.’s initial paper in 2013, patterning of cracking on the crocodile head was described for a second time as the fragmentation of a thin film over a growing substrate (Qin et al., 2014). Both Milankovitch et al. (2013) and Qin et al. (2014) agree that the patterning of cephalic scales must be caused by the growth of the underlying bones. Qin et al. (2013) discuss the roll of heterogeneous deformation, in that when strain rate (bone growth), is faster in one direction that in the other – a characteristic laddering pattern occurs, as seen on the dorsum side of the alligator snout when longitudinal growth rate exceeds lateral growth rate (Qin et al., 2014). They also noted that fragment size is not only governed by the applied strain but also by the stiffness of the overlying material (Qin et al., 2014).
Further comments on the mechanics behind the patterning of the crocodile snout scales have been made by Nandakishore and Goehring (2016). In their work, they note that as the thickness of a cracking layer increases, the observed crack pattern changes from wavy to ladder-like to isotropic (Nandakishore & Goehring, 2016). Cracking is the effect of differential growth in a brittle layer and its substrate (Nandakishore & Goehring, 2016). The pattern of crocodile snout scales shares the same morphological attributes as isotropic patterning, further implicating the role of epidermal thickness and elasticity in the patterning of scales on the crocodile snout.

Cracks are thus hypothesized to relieve a growing strain in the epidermal layers. In embryonic crocodiles this cracking forms tiles that will eventually mature into scales. As increased cornification makes the outer layer of the epidermis less elastic and more brittle, the first emergence of cracking in the crocodilian snout could thus plausibly indicate regions of initial thickening and increased cornification.

Cracking patterns can generate various angle distributions around the junctions where cracks propagate or join (Milinkovitch et al., 2013). Nonhierarchical cracking occurs when fractures propagate simultaneously and meet at 120-degree angle, this also occurs when a cracking front splits and multiple cracks are propagated from a single spot. Cracks that meet at 90-degree angles occur during laddering – which occurs due to differential growth rates. In an adult crocodile the distribution function of edge angles is bimodal, demonstrating that both hierarchical and nonhierarchical cracking occurs. While they do not describe a developmental series, Milinkovitch et al. propose that this bimodal expression could potentially be caused by maturation of the cracking process through development due to changes in certain variables such as growth rates, cell proliferation, and epidermal elasticity (Milinkovitch et al., 2013).

While the head scales in lizards form in a symmetrical pattern related to the positioning of the underlying bones that is more or less constant across individuals, crocodylian snout-scales do not appear to exhibit any particular pattern. Why these form on the snout and face but not elsewhere on the body, how they develop morphologically and their evolutionary history is not understood.
The crocodilian condition contrasts with the other two major clades in Archosauromorpha, namely Testudines and Aves. The presence of a beak independently evolved multiple times in Testudines and Aves and so therefore must be considered as a derived trait in these groups.

The beak of extant turtles and birds is composed of the Rhamphotheca, a keratinized sheath derived from the epidermis and overlies the rostral facial bones. The transition from a snout to a beak in archosaurs is related to the fusion and elongation of the maxilla during development (Bhullar et al., 2015). While in birds, the bone which predominantly underlies the rhinotheca (the rhamphotheca covering the upper jaw) is the premaxilla, in turtles the rhinotheca also covers the maxillae (Urano et al., 2018).

In the development of the beak and snout, mesenchyme proliferation occurs in the frontonasal primordia, paired maxillary primordia, and paired mandibular primordia (Bhullar et al., 2015; Schneider, 2005). The final shape of the beak or snout is influenced by developmental interactions between mesenchyme, surface ectoderm, vascular epithelium and olfactory epithelium (Schneider, 2005). The frontonasal signaling system is responsible for major morphological differences across the faces of amniotes. Differences in signaling can be used to explain species and clade specific craniofacial features such as the width, length and fusion of facial primordia (Bhullar et al., 2015).

While beaks are all made of the same differentiated materials: bone, keratinized sheath and soft tissues, differential growth rates in the various facial prominences contribute to the relative dimensions and morphologies of the beak (Wu et al., 2006).

The turtle maxillary rhamphotheca develops from two epidermal thickenings or placodes, one located at the rostral front of the snout, and another in the anterior part of the mouth roof (Alibardi, 2016). The bird rhamphotheca develops from a distal epithelial thickening around embryonic day 10, where a caruncle (sometimes called an egg tooth, but not properly a tooth) forms at the dorsal surface of the upper beak atop the position of the premaxilla (Wu et al., 2004). Patterns of epidermal proliferation distinguish the developing caruncle from the surrounding beak region (Shames et al., 1991). Proliferation of basal epidermal cells forms a circular zone of proliferation in the surrounding beak around the caruncle and spreads radially during development to form first
a multilayer epidermis and later a cornified stratum corneum (Shames et al., 1991). The beak as a modified scale

Birds, turtles, and crocodilians all have an analogous but not homologous horny structure that performs a similar function, the caruncle, while in contrast lizards and snakes develop a true egg tooth that is attached to the premaxillary bone in the midline and curves forward in front of the snout (Gottlieb, 1973). Tuataras, while phylogenetically positioned within Lepisodauers display several morphological features that closely resemble those seen in crocodilians or birds (Bogert, 1953). One of these features is the presence of a horny caruncle on the tip of the snout, just as those seen in extant Testudines and Archosaurs (Bogert, 1953). The caruncle or egg-tooth is essential for these animals to break through the egg shell during hatching and is consequently lost shortly after hatching (Gottlieb, 1973).

The beak evolved as a feeding apparatus and evidence from the fossil record suggests that the evolution of the beak occurred concurrently with the loss of teeth in multiple lineages (Louchart & Viriot, 2011). The transition from a toothed jaw to a toothless beak in birds can be seen in transitional fossil taxa such as Archaeopteryx, Ichthyornis and Confuciusornis (Field et al., 2018; Louchart & Viriot, 2011). For example, Ichthyornis fossils show evidence of a small transitional beak at the rostral tip while also displaying teeth more caudally (Field et al., 2018). During the late Jurassic or Cretaceous, dentition reduction occurred independently at least six times in avialans and edentulism was reached independently in four (Louchart and Viriot, 2011).

The final step in the development of beak morphology is the integument of the covering the of the snout, the cornified sheath. The dermis underlying the epidermis induces and regulates the development of beak epidermal structures such as the caruncle, and the cornified beak (Shames et al., 1991). Regional and temporal variations in the inductive capacities of the dermis account for morphological differences in the adjacent epidermal structures (Shames et al., 1991).

While keratin can be preserved in the fossil record, this requires exceptional preservation conditions. The loss of teeth in the development of beaks is well understood due to the preservation of transitional forms in fossil record. In contrast, conclusions on the presence or absence of
rhamphotheca is often reliant on analyzing the textures of the underlying bone. Due to the constraints of the fossil record, to investigate the ancestral state of snout keratinization and the early evolution of the rhamphotheca in Testudines and Archosaurs, a comparative developmental approach is suitable.

**Aim**

While the development of the beak in Testudines and Aves, and scales in Lepidosauria have been studied developmentally, the morphological development of epidermal patterning on the crocodylian snout has not been described within a comparative framework including their closest living relatives. A comparative developmental approach is necessary to best investigate the evolutionary history and developmental basis of the unique epidermal arrangement seen on the crocodilian snout. In order to begin to understand how this unique epidermal arrangement arose, it is crucial to understand how its development is compared to the development of keratinized snout structures seen in the other reptiles.

The aim of this paper is to describe and discuss the morphological development of keratinized structures on the snout of extant reptiles, and discuss how they may or may not relate to one another. An evo-devo approach within a systematic comparative framework is necessary to best investigate the evolutionary history and developmental basis of this unique epidermal patterning seen on the crocodilian snout. It is crucial to understand how it’s development compares to the development of keratinized snout structures seen in other reptile lineages.

Firstly, to confirm that this cracking process occurs not only in the Nile crocodile but is shared across the Crocodylia, the morphological development of cracks and epidermal polygonal tiles in a developmental series of the American alligator (*Alligator mississippiensis*) will be described. Two important variables noted in previous studies on crocodilian snout epidermis are the growth of the underlying bones and the thickness of the skin. On this basis, the positioning and ossification of the underlying bones at each embryological stage will be examined in relation to spatial and temporal distribution of developing epidermis. Thirdly, vertex angles within this layer will be analyzed through development to infer if cracking undergoes a maturation process. In addition,
thickness analysis of the developing epidermal integument will be used to elucidate further insights on the outer most layer of the snout in the developing embryo. These four approaches will be conducted on a representative species for each of the four main reptile clades to allow for comparative analysis.

Methods

Embryo Acquisition, Staging, and Fixation

Developmental series included in this study consist of *Anas platyrhynchos* (three stages, including PO8, PO10, and PO11), *Alligator mississippiensis* (four stages, including FS21, FS22, FS24 and earlier), *Pantherophis guttatus* (four stages, E31, E33, E35, E37), and *Trachemys Scripta* (FS19, FS21 FS22, FS23). These species were selected for comparative analysis as they each represent one of the four main clades of Reptilia: Lepidosauria, Testudines, Crocodylia and Aves, enabling me to assess development in a succession of more inclusive clades of amniotes. Fertile turtle and alligator eggs from were purchased from Bentley Turtle Farm and Rockefeller Wildlife Refuge respectively. Eggs were incubated at Yale at 28°C and 90% humidity. Embryos were collected from eggs opened at different intervals, washed in Phospbate Buffered Saline (PBS), fixed in 4% Paraformaldehyde in PBS for seven days at 4°C, washed four times and stored in 100% Methanol at -20°C. Duck and snake eggs were acquired from external sources, and their embryos treated and prepared in the same way described above.

CT scanning

Embryonic series of *Alligator mississippiensis* (Crocodylia), *Anas platyrhynchos* (Aves), *Pantherophis guttatus* (Lepidosauria) and *Trachemys scripta* (Testudines) were stained in 5% (by mass) phosphomolybdic acid, and CT scanned at Yale University. The CT scans were reconstructed and visualized using the software VGStudio to produce both two-dimensional tomograms and three-dimensional volume renderings of the skin and skull elements.
Capturing Snout Integument patterning
Images of the three-dimensional volume renderings were taken in VGStudio from lateral, dorsal, and ventral angles. Scale, feather and beak pattern and morphology were traced over in Photoshop to create a layer in which the patterns of keratinized features are more clearly visible. The three-dimensional reconstructions of CT scans were positioned in VGStudio to optimize the clarity and magnification of the skin.

Region-of-Interest Extraction
Regions of interest can be extracted from 2D tomograms to form 3D renderings in VGStudio. By adjusting the threshold of the scan individual elements, dermal bone and skin, can clearly be distinguished from one another. Elements were mechanically outlined in each 2D tomography layer. Elements were selected from layers in individual Regions of Interest from three different orientations to ensure comprehensive selection. The region, comprised of multiple layers is then extracted to form a 3D rendering. Regions of skin, ossified dermal bones and cartilage were extracted and identified.

Skin Thickness Analysis
The cross-sectional thickness of the segmented skin and scales were measured using the wall-thickness analysis module in VGStudio and visualized in an inverse rainbow color gradient on a standardized scale (ranging from 0.00-0.6mm thickness with a corresponding color gradient of red to green). To ensure that only the thickness of the skin itself was analyzed, the greyscale histogram for the tomograms were calibrated to the same level for all specimens by selecting most suitable grey values of the background, and material of interest (skin).

Wall thickness analysis can be applied to an extracted Region of Interest of a CT dataset. After extraction, an initial surface determination of the material of interest (skin) must be made. This function identifies the threshold value that distinguishes the skin from the underlying bone, this provides sub-voxel accuracy for surface data. The surface of both internal and external walls is used by the thickness analysis function. The analysis was applied with search tolerances set to the minimum difference between surfaces within a given search angle of 50 degrees. Additionally, the
algorithm was instructed to create a closed surface, and to use high encoding precision. These four settings were standardized across all specimens. Once a focal region’s analysis is completed, each point on the surface is associated with a thickness values which is then represented by a color-coded 3D view. This visual representation allows for easy identification of regional differences and broad patterns.

**Vertex Angle Analysis**

The vertex angles of the cracks and scale vertices, taken from images of CT-scanned embryo, were measured using the protractor function of Onde ruler for Mac (https://www.ondesoft.com/rulers/). The number of scales vary between taxa and through development so the number of scales and nodes measured for each embryo varies. A node is defined as the vertex where two or more of the edges that outline any given scale meet. The angles that comprise each node add to 360 degrees.

To access the hypothesis of hierarchical cracking, node angles were measured in for each stage in the series and then compared. Probability density profiles were created for angles measured from each specimen (sample size of ~40 vertices per specimen) using kernel density estimates in Rstudio. This method was preferred to histograms as it helps to mitigate statistical bias (Martins et al., 2018).
Results

Morphological Development of Snout and Beak Integument

Figure 2. Two-dimensional morphological imaging of the scale, beak and feather development as seen in lateral view in: *Pantherophis guttatus* stages 31, 33, 35, *Trachemys scripta* stages 19, 22, 23, *Alligator mississippiensis* 21, 22, 24, and *Anas platyrhynchos* day 8, 10, 11 developing feathers on the head.
**Pantherophis guttatus**

Superficial pattern of scales has begun to form by stage 33 (fig.2). A series of grooves separate a set of developing scales, this initial pattern closely resembles the pattern of scales seen on in hatchlings. This scale pattern is consistent across individuals and becomes more prominent through development. Beginning at stage 33, scales are very regularly arranged, longitudinal length is typically greater than transversal width. At stage 33, on the ventral side of the head the anterior and posterior chin shields, mental, infralabial and ventral scales are all differentiated. Along the lateral sides of the snout, rectangular-shaped labial scales begin to form along the upper and lower lips. By stage 35 all the labial scales are in place as well as the nasal, ocular, frontal and prefrontal scales (fig.2).

**Trachemys scripta**

At stage 19 no scales are present on the face or jaw, the beak area displays slight creases at the most rostral point and again in the caudal regions of the jaw below the eye. The neck and under side of the jaw do not have any folding or patterning with the exception of slight creasing. It is unclear at this point if creasing correspond to scale hinges. At stage 21 a number of folds begin to form along the neck parallel to one another in a dorsoventral orientation (appendix fig.2). There is increased creasing in skin at caudal region of the lower jaw when viewed laterally (fig.2). Creasing is still evident on rostral tip and the dorsal surface of the beak suggesting that the keratinized rhamphotheca has not yet reached its hatchling morphology. All stages viewed in this series show evidence of a developing rhamphotheca, as suggested by the defined margins indicating the shape of the rhamphotheca caudally on the upper jaw and dorsally on the top of the snout (appendix fig.2).

At stage 22, a number of polygonal scales have formed in the caudal regions of the upper and lower jaw. Increased folding and creasing of the ventral side of the jaw shows the first emergence of a scale like pattern from wrinkles in the skin. In dorsal view, the top of the head and beak are smooth. The caudal edges of the developing rhamphotheca have increased in both height and their caudal extension, extending below and past the caudal edge of the orbit. The angle where the dorsal region of the face meets the developing rhamphotheca is almost at a 90° step; this is a noticeable
difference from a more rounded curvature in this region at earlier stages. By Stage 23, the 90°
“step” at the rostral end of the beak appears fully reinforced with any creases at the rostral point
of the upper beak now flattened out. The polygonal scales on the caudal regions of the upper and
lower jaw have further propagated. The folds under the lower jaw and in the neck have deepened
and the morphology of the skin now resembles a scale like pattern (appendix fig.2).

Alligator mississippiensis (Crocodylia)
Initial cranio-caudal creasing is seen primarily in a caudal to rostral direction along the lateral sides
of the snout at stage 19 (appendix fig.1). Polygonal tiles that resemble a scale pattern form when
propagating grooves meet to form nodes. These nodes form the vertices of adjacent scales. The
first appearance of polygonal tiles occurs at the rostral tip of the snout at stage 21. This epidermal
patternning on the upper jaw (in lateral view) are restricted to the ventrolateral tip of the snout
beneath the presumptive developing caruncle. In the lower jaw it is also localized at the most
rostral tip of the snout.

At stage 22, the formation of polygonal tiles, hereby referred to as epidermal patterning, proceeds
along the margin of the mouth in the upper jaw and up onto the dorso-rostral region of the snout.
A large area of the flat dorsal region of the snout, extending from in line with the orbits over most
of the dorsal snout area is free of any cracks or lines with the exception of a prominent groove
across the entire dorsal region of the snout at the most caudal point of the snout in line with the
antorbital region of the snout (Appendix fig.1). In lateral view, laddering of grooves is evident in
the dorso-lateral regions below the orbit, with a series of parallel grooves forming a region that
extends from the orbit to meet the polygonal tiles at the rostral end of the snout (fig.2, stage 22
Alligator). In the lower jaw in lateral view, the same degree of incomplete polygon formation is
noticeable along its length. At this stage this is no apparent pattern of directional propagation in
cracking as seen in the upper snout, presumptively correlating with thinner, more elastic skin. At
stage 22 a large region of the upper snout was smooth when viewed from dorsal orientation
(appendix, fig.3).

By stage 24 closed polygons are present along the upper and lower jaws. The laddering along the
dorsolateral area of the upper snout have begun to form complete polygonal tiles. This smoothed
area now shows a series of laddering of parallel grooves across the top of the snout. This is rostrally bordered by a U-shaped margin, on the other side of which are closed polygonal tiles. Throughout development this rostral region extends both caudally to the jawline and dorsally, slowly reducing the area of the smooth dorsal region. Scales closer to the jaw line are much smaller and have irregular polygonal contours and are not imbricate.

Epidermal patterning on the upper jaw is asynchronous and can be broken into regions. The rostral region which is where cracking first occurs, expands from a small ventro-lateral region at snout tip. Several distinct, albeit transient, regions of laddering are also seen throughout development. Complete cracking and polygonal tile formation proceeds along the upper jaw line proceeds in a rostral to caudal direction. The U-shaped dorsal region of the snout is the final region to develop the random polygonal epidermal patterning.

Groove shape through the development changes from a series of parallel creases and laddering to another stage in which perpendicular propagations are added, followed by formation of curved and diagonal grooves that “close” the polygons. Expansive areas of complete epidermal polygons only tend to form in the initiation of this final stage.

**Anas platyrhynchos**

The head is mostly covered by feathers instead of scales, the exception being the snout which is covered by a rhamphotheca. Feathers first become superficially visible as their primordia (feather buds) begin to protrude from the skin. At day 8, there are no feather buds present on the duck head, and the caudal margin of the developing rhamphotheca is in-distinguished from the rest of the skin (fig.2). Day 10 epithelial thickenings or feather buds begin to form along the dorsal side of the cranium, around the ears and eyes. These feather buds are organized in discrete tracts, a micro-patterning consistent macro-patterning of feathers tracts present in hatchlings and adults. At day 10 visible buds are absent from the caudal region of the mandible at this stage, however by day 11 they have differentiated further so that the feather shafts are beginning to elongate. The entirety of the jaws lack feather buds. At day 10, the proto beak displays a number of short creases running in rostral – caudal orientation. On the most rostral tip of the beak there is a slight dorsal bump. These creases/grooves are smoothed by day 11 however the positioning of the creasing reflects the
underlying morphology and positions of developing beak bones such as the maxilla (fig.2). On the rostral tip of slight tapers create a visible in a V shape delimiting the dorso-caudal edges of rostral end of the beak.

Development of Keratinized Features Relates to Underlying Bones

![Diagram](image)

Figure 3. *Alligator mississippiensis* (Crocodylia) stage 22 and stage 24 with dermal bones of the snout overlaid by outlines of developing scales. Dermal bones are color coded to match those shown in hatchling skull. Scale development proceeds asynchronously in regions related to underlying dermal bones.

The asynchronous regions of scale development noted in *Alligator mississippiensis* are related to the local development and growth of underlying dermal bones. Isolation and extraction of dermal bones in stage 22 and 24 (fig.3) align with the regionally asynchronous pattern of scalation described above. The rostral most region where cracking begins overlies the premaxillae, this
cracking then proceeds along the ventral margin of the maxillae. At stage 22, the dorso-lateral laddering is above the lengthening of dorsal-caudal area of the maxilla. The U-shaped dorsal region on the top of the snout aligns with the paired nasals. Cracking only proceeds in this region later in the development, in correlation with the late ossification and growth of the nasals.
Integumentary regions also display patterning related to underlying dermal bones in *Trachemys scripta* and *Anas platyrhynchos*. In *Trachemys scripta* the upper rhamphotheca overlies the maxillae and the premaxillae and grows size along with the increasing sizes of these bones (fig.4e,f).

At stage 19 the premaxillae have not ossified, the rostral tip of the upper rhamphotheca displays thickening but is also slightly wrinkled (fig.4a,c). In both stage 19 and 23, the rostral region of the beak is thickest. The 90-degree dorsal-rostral “step” of the snout and beak becomes evident later in development following the ossification of the premaxillae and flattening of the wrinkles present at stage 19 (fig.4f). Facial scalation develops above the caudal edge of the mandible and around the ear. The most notable developmental pattern is the increase in height of rhamphotheca with respect to the dorso-ventral growth of the maxilla (fig.4e,f).

The beak of *Anas platyrhynchos* also exhibits a rostral thickening pattern (fig.5b, fig.7a). At day 11 the rostral region of the upper rhamphotheca that overlies the premaxillae exhibits a dorsal thickening and lateral thickening along the jawline. At this stage, the thickest parts of the rhamphotheca overlie the rostral tip and lateral margins of the premaxilla.
**Scale Vertex Angle Analysis suggests Maturation Process**

Vertex angles at scale nodes have been postulated to indicate progress of the process that leads to polygonal tiles and epidermal patterning (Milinkovitch et al., 2013). Maturation of the cracking process can be observed through changes in probability density profiles of crack angles through the developmental process (Martins et al., 2018). Heterogeneous stress influences both the direction in which cracks propagate as well as the angles at which they intersect (Martins et al., 2018). Density profiles show the distribution of vertex angles at scale nodes on the face and snout for *Trachemys scripta*, *Pantherophis guttatus*, and *Alligator mississippiensis* through three developmental stages for each species (fig.6). Maturation processes are expected as the variables involved in scalation at local levels (epidermal elasticity, cell proliferation, bone growth rates) as well as broader levels (asynchronous scalation) occur throughout development.

Stage 21 *Trachemys scripta* density profile has a notable peak at 173.46°, the sharpness of which indicated by \( P(170° < \theta < 190°) = 0.39 \). However the density profiles in following stages do not display peaks as strong as *Pantherophis guttatus* or *Alligator mississippiensis*. At stage 22 and 23 peaks are located at 115°, the sharpness of these peaks increasing from \( P(110° < \theta < 130°) = 0.19 \) to 0.22, with the proportion of angles around 180 degrees subsequently shifting from 0.22 down to 0.19. These shifts in the shape of the density profiles indicate that there is some sort of maturation process occurring here, with folding or creasing of the keratinized snout skin displaying a preference for junctions forming at 120° increasing through ontogeny.

Density plots for *Pantherophis guttatus* exhibit similar distributions in each of the three developmental stages with peaks for all three stages near 90° (at 91.9°, 90.9°, 92.0° for day 33, 35 and 37 respectively) (fig.6). The development proceeds the peak of the density profiles sharpen. At day 33 \( P(80° < \theta < 100°) = 0.29 \), however this increases to 0.34 by day 35 and 0.41 by day 37. This increase in sharpness of the 90° peak is likely due to the superficial development of dorsal snout scales that occurs between stage 33 and 35 in development.
Figure 6. Density plots show changing distributions of vertex angles at scale nodes through development from: a. *Trachemys scripta* face scales (at caudal end of rhamphotheca on rear of mandible) for stages 21, 22, and 23. b. *Pantherophis guttatus* snout scales for stages 33, 35, and 37. *Alligator mississippiensis* snout scales for stages 21, 22, and 24.

In *Alligator mississippiensis* the density profiles have more than one notable peak. At stage 21 the distribution is bimodal with a peak at 91.8° and 178.3°. The peak at 180° indicates the likely presence of T junctions, where a crack perpendicularly meets another forming two 90° vertices and one at 180°. The bimodal distribution of angles at stage 21 suggests that T junctions are common (fig. 6). However while you may expect to find twice as many vertices at 90° than 180°, this is not the case. While the peak at 180° suggests that three way vertices are common, the overall shape of the density profile indicates that a propagating crack does not always meet another perpendicularly.

At stage 22 the peak close to 90° (90.44°) has sharpened, from \(P(80° < \theta < 100°) = 0.32\) at stage 21 to 0.36 at stage 22. At stage 22 there is an increase of vertices in the realm of 120°, with an increase in the probability of having an angle close to 120°, at stage 21 \(P(110° < \theta < 130°) = 0.09\), and at stage 22 \(P(80° < \theta < 100°) = 0.28\). While 120° angles lag behind 90°
angles, by stage 24 the probability of having a vertex at these angles are almost the same with
\( P(80^\circ < \theta < 100^\circ) = 0.29 \) and \( P(110^\circ < \theta < 130^\circ) = 0.32 \). At stage 24 \( P(170^\circ < \theta < 190^\circ) = 0.15 \), this decreasing from 0.34 and 0.21 at stages 21 and 22 respectively.

Overall these changes in density profile shape suggest that way in which cracking occurs matures,
cracking does not occur in the same way throughout development (fig.6). As the embryo develops,
the cracking process also develops and matures. Initially angles at scale nodes demonstrate a
pattern of parallel and perpendicular cracks, this pattern is lost as polygonal tiles close off and
shapes become more complex due to the stochastic nature epidermal patterning.

**Thickness of Developing Epidermis and Scale Structures**

![Image](Image)

Figure 7. Thickness of developing epidermis in scale development process. a. Stage 21 *Alligator mississippiensis*, upper lateral snout. b. Stage 21 *Trachemys scripta* from under chin. c. Day 10 *Anas platyrhynchos* feather buds on cranium. d. Stage 24 *Alligator mississippiensis* upper lateral snout scales. e. Stage 23 *Trachemys scripta* from under chin. f. Stage 33 *Pantherophis guttatus* lower mandible scales.

Patterns in the thickness of developing skin further illustrate that snout scales of *Alligator mississippiensis* as distinct from feather buds in Aves, lower jaw scales in Lepidosuars and the folds that occur in the soft skinned regions of Testitudines. While it would be best to compare
homologous regions, data pertaining to these regions were unable to be obtained due to COVID-19 preventing access to the lab. In the interim, this comparison displays differences in epidermal morphology across taxa. While the skin prior to folding or cracking in *Alligator mississippiensis* and *Trachemys scripta* exhibit similar thickness and lack of patterning (Fig.7a,b), in later stages the same regions show inverse thicknesses in the morphologies in the thickness of most superficial layers. In *Alligator mississippiensis* the epidermal layer in cracks between the scales are thicker than the epidermal layer which overlies the highest points of the scale.

**Rostral Epidermal Thickening seen in Beaks and Initiation of Alligator Snout Scalation**

While scale formation on the snout of lepidosaurs occurs synchronously and symmetrically, in Testudines and Aves there is a pattern of rostral epidermal thickening in the development of the beak. The first emergence of cracking in the crocodilian snout occurs at the rostral tip. It is hypothesized that the first emergence of cracking could indicate regions of initial thickening and increased cornification. If this is the case, then this rostral cracking indicates rostral epidermal thickening early in development.
Figure 8. Comparison of rostral epidermal integument development. a. Day 8 *Anas platyrhynchos* upper beak, b. Day 11 *Anas platyrhynchos* beak, c. *Trachemys scripta* lateral view upper rhamphotheca at stage 20, d. *Trachemys scripta* front on rostral view of upper rhamphotheca at stage 23, e. Stage 21 *Alligator mississippiensis* lateral view of rostral end of snout, f. Stage 24 *Alligator mississippiensis* lateral view rostral end of snout, g. Stage 33 *Pantherophis guttatus* shout and face, h. Stage 37 *Pantherophis guttatus* snout and face.
Discussion

The epidermal patterning on the alligator snout is distinct from scale formation described in the rest of the body and in other reptilian species. While differing from scale development in other taxa, the rostral initiation of cracking which then proceeds caudally along the snout, follows the same directional (rostral to caudal) development of rhamphotheca in *Anas platyrhynchos* and *Trachemys scripta*. The rostral initiation of the rhamphotheca has also been described in other species of birds and turtles such as *Gallus gallus* (Shames et al., 1991) and *Emydura macquarii* (Alibardi, 2016).

This initial cracking on the rostral tip is not likely to be solely driven by bone growth. In alligator, bone growth tends to result in a laddering pattern in the overlying epidermis, here initial cracking resembles the stochastic pattern seen elsewhere on the snout later in development. Rather than bone growth driving cracking at the rostral tip, loss of epidermal elasticity or local cell proliferation, or a combination of both could be driving this initial cracking. Loss of epidermal elasticity through cornification, the same process that results in a stiff rhamphotheca, is a favorable putative cause of cracking first initiating at the rostral end of the alligator snout.

The ossification and growth of underlying dermal bones is also a variable that contributes to the morphology of propagating cracks on the Alligator snout. Elements of the dermal skeleton develop early in skeletogenesis with most bones initiating ossification prior to mineralization of the endoskeleton (Vickaryous & Hall, 2008). The endoskeleton forms around a cartilaginous primer, the dermal bones of the skull develop through intramembranous ossification (Jheon & Schneider, 2009). Intramembranous ossification occurs as loose mesenchymal cells proliferate and condensate into compact nodules where osteoblast differentiation occurs, with no cartilaginous precursor (Gilbert, 2000). Ossification occurs as a result of calcium binding to a collagen proteoglycan-matrix secreted by osteoblasts, as it proceeds bony spicules radiate from the initial region of bone deposition (Gilbert, 2000). This ossification process early in development is coupled with the development of scales on the snout. Further investigation into the growth rates of individual bones could improve understanding of the regional asynchrony observed in the development of epidermal cracks.
As described, then cracks between epidermal polygons in Alligator mississippiensis are thicker than the epidermal layer which overlies the highest points of the scale. Milinkovitch et al. observed that higher levels of cell proliferation occur in the lower crack regions than on the higher surface of the scales, perhaps providing cellular explanation for the inverse pattern observed here (Milinkovitch et al., 2013). Folded skin in Trachemys scripta, scale in Pantherophis guttatus, and feather buds in Anas platyrhynchos respectively each display greater epidermal thickness in regions of increased height than in hinge or undifferentiated regions.

The results of this study support the development of facial cracks in Alligator as a stochastic process. Milinkovitch et al. suggests that this process is controlled primarily by physical processes but notes that this does not rule out genetically controlled parameters (Milinkovitch et al., 2013). Asynchronous regions of epidermal pattern development suggest that while cracks may form from physical processes, the causes of these are likely under genetic control. The initial rostral cracking, as well as other regional differences exemplifies the underlying genetic control on these processes.

The cracking seen in the developmental series of Alligator mississippiensis results in a similar stochastic pattern of that described in Crocodylus niloticus. In both species scales are random polygonal shapes, distribution across the snout is asymmetric and the distribution in shape and size varies between individuals. Both species also have scales with incomplete cracks propagating into them. Analysis of vertex angles shows another similarity between late stage Alligator and adult Crocodile, both exhibiting a bimodal distribution with peaks near 90° and 120°. While peaks in stage 24 alligator sat near these expected angles, in contrast the peaks for adult Crocodile are wider and tend to center on values less than the expected 90° and 120° (Milinkovitch et al., 2013). This could be due to head growth both during and after cracking, and it reasonable to hypothesize that this distribution pattern may be observed in adult Alligators as well.

In this study four distinct traits of snout integument are described, scales in lepidosuars, cracking in crocodilians, and two instances of convergently evolved beaks in testudines and aves. Regardless of conflicting ideas on reptile phylogeny, as snout integument is unique in each of these groups, as a character trait it can only provide equivocal evidence of relationships within reptilia.
Without an out group showing either the lizard, archosaur or turtle condition to compare these traits to, the data cannot be polarized and so the ancestral condition cannot be determined. Epidermal thickening and cracking in Alligator in ontogeny alone does not provide evidence for a synapomorphic resemblance to the development of the beak in Aves and Testudines.

Paleontological evidence reveals the convergent evolution of a rostral beaks many times over. The biological role of the beak in birds is different from the cropping beaks seen elsewhere in Reptilia. The fossil record displays many instances of convergent rostral rhamphothecas. Permian therapsids (an outgroup to Reptilia), such as Dicynodontia, grow a beak at the tip of their snout (Benoit et al., 2018). If the assumption is made that rostral thickening is the ancestral state, failure to start thickening at the snout tip in lepidosaurs would thereby be a derived apomorphic condition and retention in archosaurs and turtles would be a plesiomorphic resemblance. However, the convergent evolution of the beak in all of these clades impedes any conclusions on the evolutionary history of the epidermal thickening observed in alligators. Furthermore there are examples of ancient crocodilians such as Macelognathus vagans (Holotype YPM 1415) described as being edentulous with a horny beak rostrally, while maintaining teeth caudally (Marsh, 1884). The implication of this being that the rostral rhamphotheca arose convergently within this clade as well. The rostral epidermal thickening that leads to cracking on the snout of Alligator could be convergent itself.

In Aves, the expansion of the rhamphotheca begun at the rostral end of the snout above the premaxillae and is congruent with the hypothesis that the early development of the rhamphotheca started from the caruncle at the rostral tip (Hieronymus & Witmer, 2010; Louchart & Viriot, 2011). However confirming this observation would require studying the development of both the caruncle and the rhamphotheca in ontogeny in multiple species. The presence of the heavily keratinized but non-homologous caruncle in birds, turtles, crocodilians, sphenodons and monotremes, alongside their varied forms of snout integument makes the hypothesis of a caruncle-derived beak challenging to elucidate when relying solely on paleontological data. To investigate the evolutionary history of the caruncle and implications for snout integument it will be necessary to include descriptions of the development the snouts of a wide range of animals both within and outside of Aves.
In addition to this, developmental studies involving immunohistochemical work could aid in describing the cornification process of snouts and beaks in developing embryos across clades. To fully understand the ancestral states and evolutionary history of these characters it will be necessary to conduct further studies on both mammals and a wider variety of reptiles.

In the interim, the spatial and temporal process of cracking on the Crocodilian snout reveals that it is developmentally distinct from any other type of scale or beak development. The laddering pattern described in the development of this integument is likely a distinctive autapomorphy.

**Conclusions**

Epidermal patterning on the snout of *Alligator mississippiensis* develop through a pattern of stochastic cracking. This, plus previous work that suggests stochastic cracking in the snout scales of *Crocodylus niloticus* suggests that this is common across extant Crocodilyans. The cracking involved in the development of alligator snout epidermis undergoes maturation, likely due to changes in variables such as growth rates, localized proliferation and epidermal elasticity through development. Observations of asynchronous scale development reveals distinct regions correlated with the position of underlying dermal bones and their subsequent ossification and growth. Laddering patterns are transitional and form in regions when underlying dermal bone growing or ossifying more rapidly in one direction. Cracking is initiated at the rostral tip of the snout in conjunction with an inferred local decrease in epidermal elasticity. Rostral epidermal thickening is also involved in the initiation of cornification in the developing beaks and caruncles of birds and turtles. This process of rostral epidermal thickening that subsequently leads to different morphologies as ontogeny progresses may be a putative developmental basis for instances of convergent rhamphotheca in some clades but not others. However, while rostral epidermal thickening could provide a developmental basis for the diversity of snout integument across Reptilia, without being able to test this hypothesis with an out-group, a conclusive evolutionary history cannot be described.
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Appendix
Original hand traced images of snout integument development.
CT images of duck, dorsal view.