Getting out of an egg: merging of tooth germs to create an egg tooth in the snake

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Abstract:

Background:

The egg tooth is a vital structure allowing hatchlings to escape from the egg. In squamates (snakes and lizards), the egg tooth is a real tooth that develops within the oral cavity at the top of the upper jaw. Most squamates have a single large midline egg tooth at hatching, but a few families, such as Gekkonidae, have two egg teeth. In snakes the egg tooth is significantly larger than the rest of the dentition and is one of the first teeth to develop.

Results:

We follow the development of the egg tooth in four snake species, and show that the single egg tooth is formed by two tooth germs. These two tooth germs are united at the midline and grow together to produce a single tooth. In culture, this merging can be perturbed to give rise to separate smaller teeth, confirming the potential of the developing egg tooth to form two teeth.

Conclusions:

Our data agrees with previous hypotheses that during evolution one potential mechanism to generate a large tooth is through congrescence of multiple tooth germs, and suggests that the ancestors of snakes could have had two egg teeth.

INTRODUCTION

The egg tooth is an essential tool for shell rupture, allowing an animal to break out of an egg at hatching. As such, it is found in birds, reptiles and monotremes. In many of these animals, the egg tooth is not a true tooth but instead a caruncle formed from a thickening of the epidermis (turtles, crocodiles, birds) (De Beer, 1949). Monotremes, egg-laying mammalian, represent a peculiar case, as they possess both a caruncle, and a true egg tooth (Hall & De Beer, 1950). The caruncle in this case, unlike that of that observed in birds, is formed by an expansion of the underlying bone rather than the surface epithelium, which is known as an os caruncle (De Beer, 1949, Hall & De Beer 1950; Manger et al, 1998). In squamates, the largest order of reptiles including lizards and snakes, the egg tooth is a real tooth forming dentine and enamel (De Beer, 1949). A few squamates, such as geckos (gekkonids) and dibamids have paired egg teeth, while most other lizards and all snakes have only one egg tooth at hatching, this egg tooth being lost within a few weeks once redundant (De Beer, 1949; Edmund, 1969; Greer, 1985; Vidal and Hedges, 2005; Smith, 1952; Schnabel and Herschel, 1955; Edmund, 1969). The number of egg teeth is significant as the presence of a single or paired egg tooth has been used to aid classification of squamates and infer relationships (Vidal and Hedges, 2005; Underwood and Lee, 2000).

Two egg teeth are often referred to as the basal state in squamates (De Beer, 1949; Edmund, 1969; Greer, 1985; Vidal and Hedges, 2005). In some lizards, two developing egg teeth have been recorded, with one tooth degenerating to leave one remaining tooth germ which goes on to form the final egg tooth. For example in *Lacerta*, the egg tooth is derived from a single right hand tooth germ (De Beer, 1949). In the skink, *Lygosoma*, however, the single egg tooth appears to develop from a single medially developing tooth

germ (De Beer, 1949). In snakes, the single egg tooth was predicted to represent complete dominance of the right tooth germ and entire suppression of the left hand tooth germ (De Beer, 1949; Smith, 1952; Edmund, 1969). This view was supported by descriptions of vestigial left hand tooth germs in the European adder, *Vipera berus* (discussed in Smith, 1952). Alternatively, some authors have suggested that only a single midline tooth germ ever forms, for example in the grass snake *Natrix natrix* (Schnabel, 1955; Schnabel and Herschel, 1955; Hermyt et al., 2017).

We wished to further study formation of the egg tooth in snakes, as the egg tooth is significantly larger than the rest of the dentition, and allows one to address how such a large tooth is generated, while providing insight into its evolutionary origin. We have concentrated on two snake species the corn snake, *Pantherophis guttatus* and Burmese python (*Python molurus bivittatus*). Pythons (Pythonidae family) are considered basal snakes compared to corn snakes (Colubridae family). These two species are non-poisonous and use constriction to immobilise their prey. The embryonic development of the egg tooth was studied, followed by culture of the egg tooth in *Pantherophis guttatus* to investigate the potential of the egg tooth primordia to form one or two teeth.

RESULTS

The single snake egg tooth has a distinct morphology and projects forward out of the mouth

In the newborn corn snake, the single tooth is clearly observed and is significantly larger than the adjacent dentition on the maxilla and palatine (Fig. 1A, B, D). It projects forward

from the premaxilla so that it juts out of the upper jaw, compared to the other teeth that curve backwards (Fig. 1D,E). In the corn snake, as in many snakes, there are no functional teeth associated with the premaxilla, and therefore a gap is observed between the egg tooth and the maxillary dentition (Fig. 1D). The egg tooth is lost post-hatching and is only evident as a slight scar after three weeks (Fig. 1C). The large egg tooth with its distinctive square-shape could be observed by bright field as early as 22 dpo (days post oviposition) (stage 4) (Figure 1F), and 3D reconstruction of soft tissue scans at 23 dpo showed a single placode at this stage (Fig. 1G-I).

Two midline tooth buds are evident during early development in the corn snake

To understand how this single large tooth formed, we analysed its formation during early development in frontal section (see Fig. 1G for plane of section). In the corn snake the egg tooth is formed early in development, prior to the rest of the functional dentition. We observed that at 12 dpo (stage 2) two closely positioned tooth germs were visible developing at the tip of the upper jaw during early development (Fig. 2A). In the same specimen, however, as one moved more posteriorly into the mouth these two distinct tooth germs merged into a single placode (Fig. 2 B-D). A similar pattern was observed at 15 dpo (Fig. 2E-H). To get a better picture of the developing egg tooth at this stage, upper jaws were stained for laminin, to outline the epithelium, and E-Cadherin, to highlight the epithelial cells. Tracking through the tooth from anterior (tip of the snout) to the posterior revealed a U shape (Fig. 2I-L) with two epithelial structures present anteriorly (Fig. 2I,J), which merged into one more posteriorly (Fig. 2K,L). At 25 dpo (stage 4), the compound tooth had lost all trace of its dual origin with the two arms of epithelium enclosing the central dental pulp (Fig. 2M-P). From this stage onwards a

single tooth was observed in the mid line (Fig. 2Q-R), with differentiation of ameloblasts and odontoblasts and dentine deposition (Fig. 2S).

Merging of Shh and Edar expression domains during egg tooth development

To investigate further the process of merger we carried out immunofluorescence for Shh and Edar, two genes that are expressed in the mammalian enamel knot during tooth morphogenesis and associated with early tooth placode formation (Sadier et al., 2019). E-cadherin was used to outline the developing epithelium. At 12 dpo two distinct regions of Edar and Shh expression were present in the corn snake epithelium anteriorly, confirming the apperance of two tooth germs in this region (Fig. 3A-E). However, moving more posteriorly only a single expression domain was observed (Fig. 3F-J). In contrast at 15 dpo a single large broad domain of Shh and Edar was observed in both anterior and posterior parts of the egg tooth, suggesting that by this time the tooth germ primordia had joined to form a single large tooth germ (Fig. 3K-S).

Conserved presence of two tooth germs in other snake species:

To identify whether fusion of two midline tooth germs is conserved across other snake species we investigated early egg tooth development in the Burmese python (*Python molurus bivittatus*) (Fig. 3A-H). As with the corn snake two thickenings, with two distinct Shh domains were clear at very early stages (Fig. 4A-C). Interestingly the Shh areas were associated with areas of low proliferation, similar to the relationship between Shh and proliferation in mammalian enamel knots (Fig. 4F) (Vaahtokari et al., 1996). Complete fusion of the tooth germs, in the Burmese python occurred later in development

compared to the corn snake with twin teeth enclosed within the same Shh expressing epithelium (Fig. 4H-I). Unlike the corn snake, posterior sections showed two tooth germs early on (Fig. 4H), while the more anterior sections showed a united tooth in the same specimen (Fig. 4G), suggesting the tooth germs intiate and fuse in subtly different patterns. A single, Shh expressing, tooth germ was evident from 30 dpo (stage 6) (Fig. 4J-L), suggesting merger of the two tooth germs had completed by this stage. To further address how conserved this mechanism is we investigated two more snake species. The African rock python (*Python sebae*) and Egyptian cobra (Naja haje), which both have a single egg tooth at hatching. In both unrelated snake species we saw evidence of the initiation of two tooth germs closely developing in the mid line, with distinct domains of Shh expression in the cobra (Fig. 4M-O). The central tooth in the snake species investigated is therefore created not by the asymmetrical growth of a single tooth bud but by the fusion of two connected tooth germs.

Potential of the egg tooth to form two distinct tooth germs in culture

In order to study the development of the egg tooth in more detail, we turned to culture, using *Pantherophis guttatus* to investigate the potential of the egg tooth primordium to form two teeth. The upper jaw was sliced into 250um thick live sections and anterior and posterior slices at the front of the jaw were selected for culture (Fig. 5A,B). The posterior slices formed a single midline egg tooth over a period of 10 days (N = 3/3)(Fig. 5C-F). The egg tooth was flanked by two rudimentary premaxilla tooth germs, which extended out on either side of the much larger egg tooth (Fig. 5F). Such premaxillary teeth are vestigal in *Pantherophis guttatus* and disappear during later development (data not

shown). In contrast, the anterior slices developed two smaller symmetrical tooth germs on either side of the midline (N = 3/3) (Fig. 5G-J). When DiI was used to label small areas of mesenchyme above the epithelial thickenings in the posterior slices, the label ended up within the dental papilla of both tooth germs (Fig. 5G-J). As a second method to cause disruption to the fusion event we sliced the upper jaw a 400 microns to create thick slices containing the whole egg tooth primordium. We then dissected the primordium down the midline and cultured the halves (N = 3) (Fig. 6). Here a single small tooth formed at the midline, next to the cut surface, confirming that the egg tooth primordium has the potential to form two independent teeth if the midline connection is disrupted.

DISCUSSION

A single forward facing egg tooth was observed in the corn snake at birth, however surprisingly this single egg tooth was formed from a fusion of two partially distinct tooth germs. Anteriorly (near the snout) the two tooth germs had distinct expression domains of Shh and Edar, which merged posteriorly. The potential to develop two separate teeth from these early tooth germs was clearly demonstrated in our slice cultures, where two small egg teeth developed instead of one large tooth.

The presence of two tooth germs during early development was observed in three families of snake, the Colubridae, Pythonidae and Elapidae, with two domains of Shh, a marker for early placodes and epithelial signalling centres in the tooth. This therefore suggests that fusion of tooth germs is a conserved mechanism used by a wide variety of snakes to create a single large tooth. Interestingly, however, no evidence for two tooth germs have been described in the grass snake *Natrix natrix* (Schnabel & Herschel, 1955;

Schnabel, 1956; Hermyt et al., 2017). This suggests that this process if not a universal feature of snakes and that large egg teeth can be created using different mechanisms. Alternatively an early stage with two tooth germs might have been missed in the previous analysis, as these papers predominantly used sagittal sections, and reconstructions of the epithelium at later stages when fusion might have occurred. It would therefore be interesting to re-assess early egg tooth development in *Natrix natrix* using molecular tools.

Our data may help to explain some observations in *Vipera berus* where in a couple of specimens the egg tooth was bifurcated, with separate tips but united bases (Smith et al., 1952). A late viper embryo with two separate egg teeth was also described by Schmüdderich (discussed in Smith et al., 1952). This double egg-toothed snake was originally interpreted by Schmüdderich as having formed from a normally vestigial left hand egg tooth germ, however, our data suggests that the second tooth could have formed by a failure of two tooth germs to merge completely. This is supported by the fact that the double egg teeth observed by Schmüdderich were of the same size, as in our cultures.

In the corn snake and Burmese python the two tooth germs were not completely separated at any stage of development but were joined at the midline, either anteriorly or posteriorly. However, two tooth germs could be created in cultures of corn snake tissue by severing this connection. The early tooth germs therefore have the potential to form independent teeth. The consequence of this fusion is the formation of a single tooth that is much larger than the normal dentition of the snake. This single large tooth is more robust and broader than the slender teeth in the rest of the mouth. While the teeth found in adult snakes are adapted to puncture and retain prey in the jaw, with

inward curving morphologies to prevent prey escaping, the forward facing broad egg tooth needs to cut through the egg shell. For this action a single wide tooth may be more effective at making cuts through the leathery shell than two pointed teeth. The cutting dynamics would be interesting to test using controlled puncture experiments (Anderson et al., 2016). The development of the egg tooth in the snakes investigated here supports the congrescence theory (also known as integrated development) for the formation of large teeth. This theory proposes that large teeth could have evolved from the fusion of multiple tooth germs (Peterkova et al., 2014). Evidence for this is found in mouse teeth, where the large first molar forms from a fusion of the molar placode with a more anterior vestigal diastema placode, known as R2 (Prochazka et al. 2010). In transgenic and mutant mice where R2 development is rescued, presence of this tooth is associated with a smaller sized first molar (Klein et al., 2006; Grüneberg, 1966). In keeping with this data when the two tooth germs were isolated in the corn snake they formed small teeth, compared to the usually large egg tooth. The egg tooth, in at least some snakes, is therefore a good example of how combining tooth germs can increase the size, and possible complexity, of a tooth.

EXPERIMENTAL PROCEDURES:

Embryo collection and staging:

Pantherophis guttatus embryos and newborn specimens were collected from the Tucker colony at King's College London. Embryos were collected from day 0 to day 49 of development post lay, at hatching (day 60-80), and three weeks after hatching. During this period, the eggs were incubated at 28°C. At day 0, the embryos had already

developed defined pharyngeal arches and other head structures, which are comparable in early development with mouse and chick (Depew and Simpson, 2006).

African rock python (*Python sebae*) embryos were obtained and staged as previously reported (Boughner et al., 2007; Buchtova et al, 2007) and incubated at a temperature of 26°C (day) to 20°C (night). Burmese python embryos (*Python molurus bivittatus*) were obtained from a private breeder (Prague, Czech Republic) and incubated at a temperature of 29°C. Egyptian cobra (Naja haje) embryos were incubated at 30°C.

In order to be able to compare tooth development in our snake embryos, the specimens were staged according to external and internal morphology, putting particular emphasis on the stage of tooth development using the staging system published for the African rock python (Boughner et al., 2007; Buchtova et al., 2007).

Histology:

Embryos were fixed in 4% Paraformaldehyde, dehydrated through an Ethanol series and embedded in wax for sectioning. Sections were cut at 8μm and split over several slides. One sample of each was stained with either Haemotoxylin and Eosin, for those embryos prior to skeletal tissue formation, or Picosirrius red and Alcian blue for the older embryos.

Skeletal prep:

Specimens at hatching were fixed in 95% EtOH for 5 days, then skinned. Heads were treated in acetone for 3 days then 3 days in staining solution made up of 0.3% Alcian Blue 8GS and 0.1% Alizarin red in 70% Ethanol and 5% Glacial acetic acid. After staining specimens were washed in water and cleared in 1% KOH before being moved through a glycerol series to 100% glycerol for storage.

Immunofluorescence:

Immunofluorescence was performed in whole mount for slices and on wax sections as previously described (Gaete, 2013). Primary antibodies and dilutions used were: Anti-laminin 1:200 (Sigma L9393); E-cadherin 1:200 (Abcam Ab76055); Anti-Shh 1:200 (Santa Cruz sc-9024); Anti-Edar 1:200 (Santa Cruz, sc-15289) and Anti-PCNA 1:50 (Agilant Dako M0879). Secondary antibodies Alexa Fluor ® 647 donkey anti mouse, Alexa Fluor® 568 donkey anti rabbit and Alexa Fluor® 488 donkey anti rabbit were used 1:500. Nuclei were stained with DAPI. Alexa Fluor ® 488 phalloidin (Invitrogen A12379) was used at 1/100. Immunofluorescence was imaged with a confocal microscope (TC5 SP5, Leica).

Micro Computerised Tomography for soft tissue 3D Reconstruction:

23dpo snake heads were dehydrate in 30%, 50% ,70% EtOH for 2hr each step (N= 2). Samples were soak in 1% Phosphotungstic acid (PTA) in 70% EtOH for 7days and prepared for microCT.

Image analysis was performed in Amira. The egg tooth epithelium was segmented out for reconstruction and the surface view module was used for visualization.
Volume rendering was used to visualise the whole head.

Culture of *Pantherophis guttatus* egg tooth slices:

Frontal slices were cut through the upper jaw of corn snake embryos at E12 at 250 microns using a McIllwen Tissue chopper (Buchtova et al., 2008; Gaete et al., 2013). See Alfaqeeh et al., 2013 for a full description of the slice culture method for tooth germs. The chopper cut the egg tooth primordial in two, although the precise position of the cut was random. Slices containing the egg tooth primordia were selected for culture and divided into anterior and posterior sections (N = 3). In some cultures DiI (Cell tracker dissolved in 100% EtOH) was injected using a mouth pipette to create labeled areas in the mesenchyme, which could be followed as a reference throughout the culture period. Alternatively thick (400 microns) slices were made, to include the whole egg tooth primordium, which was then bisected into two halves to separate the two tooth germs (N = 3). The slices were then cultured in Advanced DMEM/F12 medium using a modified Trowel method (Sanchez et al., 2018) at 30 degrees C. Slices were photographed over a series of days under a Leica dissecting microscope and then fixed after 9-10 days of culture.

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Conflict of interest:

The authors confirm no conflict of interest.

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Figure legends:

Figure 1:

A single egg tooth is observed at hatching in the corn snake.

(A) Upper jaw in a fixed newborn corn snake, ventral view. The egg tooth is observed jutting out from the upper jaw (arrows). (B) Close up of egg tooth (arrow). (C) Upper jaw in a three week old corn snake, ventral view. The egg tooth has fallen off by this stage and its previous position is visible as a slight scar (arrow). (D,E) Skeletal preparation of specimen in A. (D) Ventral view, showing egg tooth (arrow) separated from the maxillary dentition by a toothless gap. The palatine tooth row and start of the pterygoid tooth row can be observed forming an inner tooth row. (E) Close up of egg tooth dorsal view. The egg tooth juts forward and is closely associated with the premaxillary bone. (F) Egg tooth at 22dpo viewed under the confocal microscope under brightfield. (G-I) Soft tissue counterstained microCT at 23 dpo. (G) Ventral view. Egg tooth epithelium segmented out and coloured red. White line shows plane of section for histology images. Anterior is next to the snout and posterior is moving towards the back of the mouth. (H) Isolated segmented egg tooth epithelium. (I) Side view egg tooth primordium.

Scale bars: A, C, D = 5mm. B, E = 2.5mm. F, H = 50 microns. G, I = 300 microns.

Figure 2:

Two linked early tooth germs merge to form a single tooth in the corn snake.

(A-H, M-P) Frontal sections through the corn snake egg tooth stained with a trichrome stain. Cartilage in blue. Bone in red. (A-D) 12 dpo frontal section. Two tooth germs are evident positioned next to each other in the midline in the anterior while only a single placode is evident posteriorly. (E-H) 15 dpo frontal section. Two tooth germs are evident positioned next to each other in the midline in the anterior while only a single placode is evident posteriorly. (I-L) Confocal projections through the developing egg tooth at E15, highlighting a U shaped structure from anterior (I) to posterior (L). Red = laminin, green = E-Cadherin, DAPI stained nuclei = blue. (M-P) 25 dpo frontal section. A single tooth germ is evident in the midline in the anterior and posterior. (Q) Single egg tooth at 31 dpo. (R) Single egg tooth at 37 dpo. (S) Differentiation of ameloblasts and odontoblasts and dentin deposition (red) at 49 dpo.

Scale bars: A, E, M = 100 microns. Same scale in B-D, F-H and N-P. Scale bar in I = 100 microns, same scale in J-L. Scale bar in Q, R = 500 microns. S = 100 microns.

Figure 3:

Two distinct tooth germs in the anterior of the developing egg tooth primordium revealed by Shh and Edar expression.

(A-J) 12 dpo. (K-T) 15 dpo. (A-E and K-O) Anterior part of tooth germ. (F-J and P-T) Posterior part of tooth germ. Tooth germ epithelium outlined with E-Cadherin. (A-E) Two distinct tooth germs expressing Shh and Edar are observed in the anterior region at 12 dpo. (F-J) Posteriorly these tooth germs merge into a single expressing placode. (K-O) by 15 dpo, the two domains of Shh and Edar in the anterior have merged into one

domain, similar to that found posteriorly (P-T). Scale bar in A = 100 microns. Same scale in all other images.

Figure 4:

Merging of two tooth germs evident in other snakes.

(A-C,F) Frontal sections of haematoxylin and Eosin stained Burmese python embryos. 10 dpo (stage 3), (D,E) E15 (stage 4), (G,H,I) E25 (stage 6), (GJ,K,L) E30, stage 7. Anterior (A,D,G,J,L) and posterior (B,E,H,I,K) sections. (C,I,L) Shh immunofluorescence (green). (F) PCNA. Brown cells are positive for proliferation. Arrows in C and F point to serial sections showing two domains of Shh expression corresponding to regions that are non-proliferative. Two distinct thickening are observed posteriorly at 10 dpo (B) with two fused tooth germs evident at 25 dpo (H). (M,N) Frontal sections stained for eosin and sirrus red in the African rock python. (M) At stage 4 two distinct buds were evident in the midline. (N) At stage 6 two late cap stage tooth germs were still evident, united at the midline and dentine deposition (red) is observed adjacent to the inner enamel epithelium. (O) Naja Haje frontal section. Immunofluorescence for Shh (green) and Ecadherin (red). Two clear buds are evident at 18 dpo.

Scale bars: A = 50 microns, same scale in B, D, E, G, H, J, K. C, F, I, L, O = 100 microns

Figure 5:

Isolation of the anterior egg tooth primordium leads to the formation of two teeth.

(A,B) Schematic showing U shaped egg tooth primordium, spliced into anterior and posterior pieces using a tissue chopper at 12 dpo. (C-J) Images follow posterior (C-F) and anterior (G-J) slices over 10 days of development. (C,G) Day 0. (D,H) Day 4 of culture, (E,I) Day 7 of culture. (F,J) Day 10 of culture. (C-F) Posterior slices develop into a single large central egg tooth, flanked on either side by smaller premaxilary teeth (F). Anterior slices form two distinct tooth germs (J). DiI dots (red) in G-J follow the position of the mesenchyme above the epithelial thickenings.

Scale bar in C = 200 microns, same scale in all other images.

Figure 6:

One half of the egg tooth primordium can form an independent tooth.

(A-B) Day 0 at 15 dpo. Uncut (A) and cut (B). Schematic of division of egg tooth primordium (C). Slice contains half the egg tooth primordium and the premaxillary tooth. Same slice cultures for 2 days (D), 5 days (E), 7 days (F) and 9 days (G). (H,I) Confocal imaging of boxed area in G. (H) Phalloidin stain (green) for F-actin highlighting tooth structure. (I) Dapi (blue) for nuclei. Scale bar A = 200 microns. Same scale in B-G. Scale bar in H = 50 microns. Same scale in I.

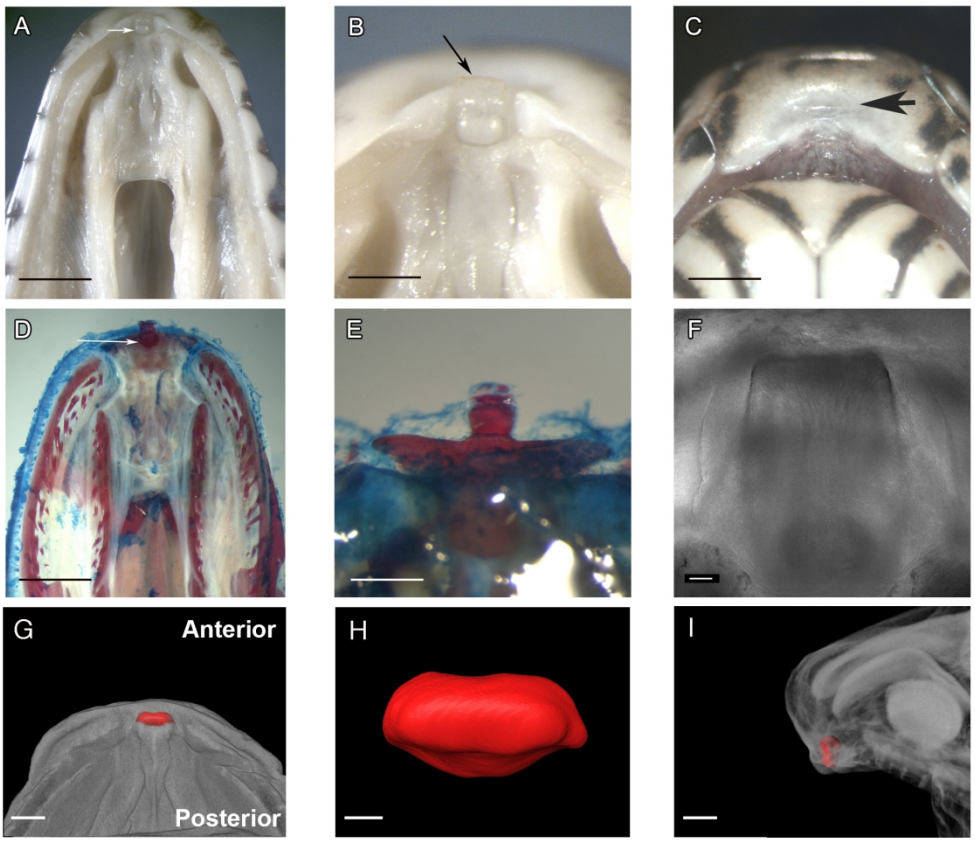

Figure 1

175x146mm (300 x 300 DPI)

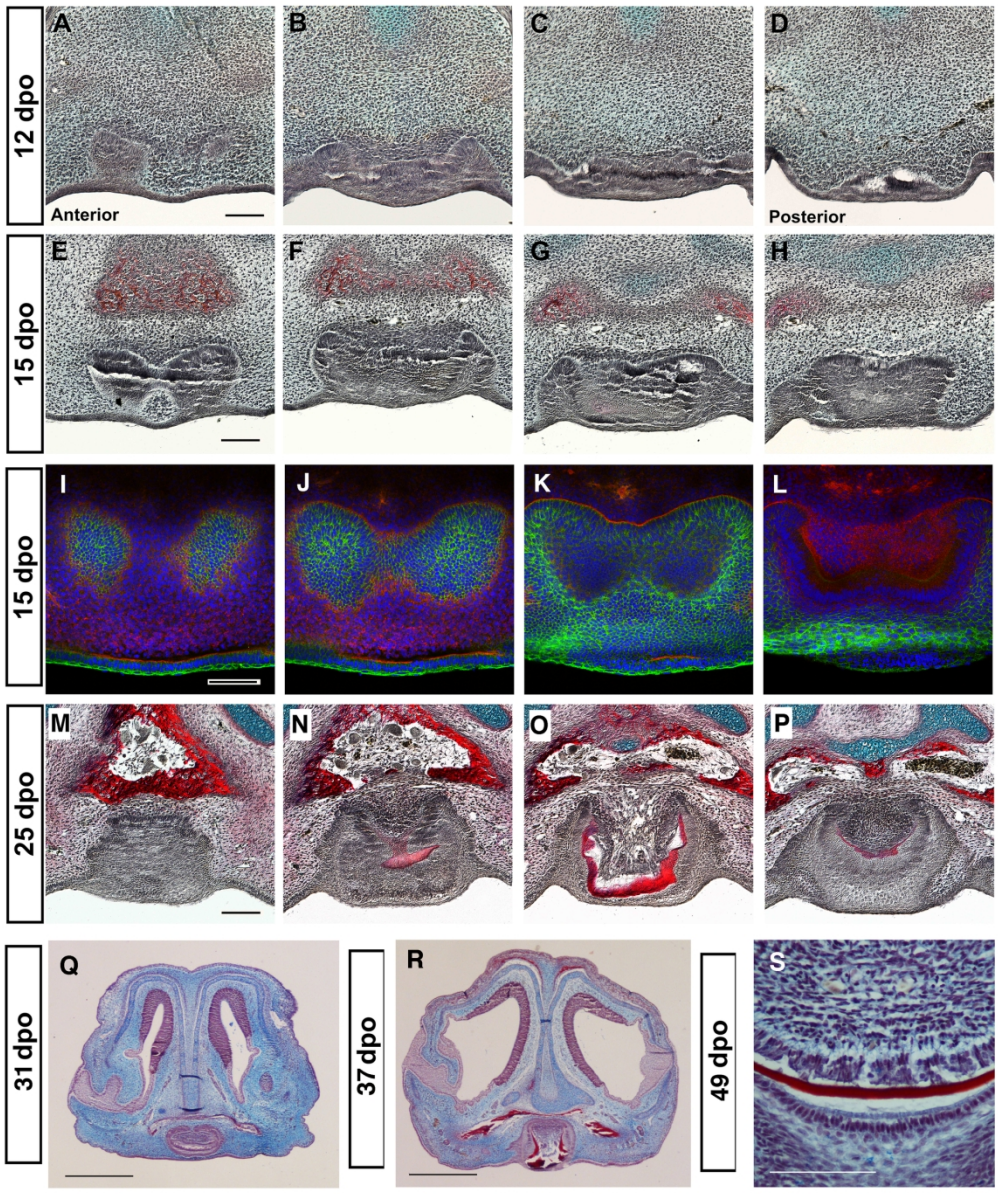

Figure 2

175x210mm (300 x 300 DPI)

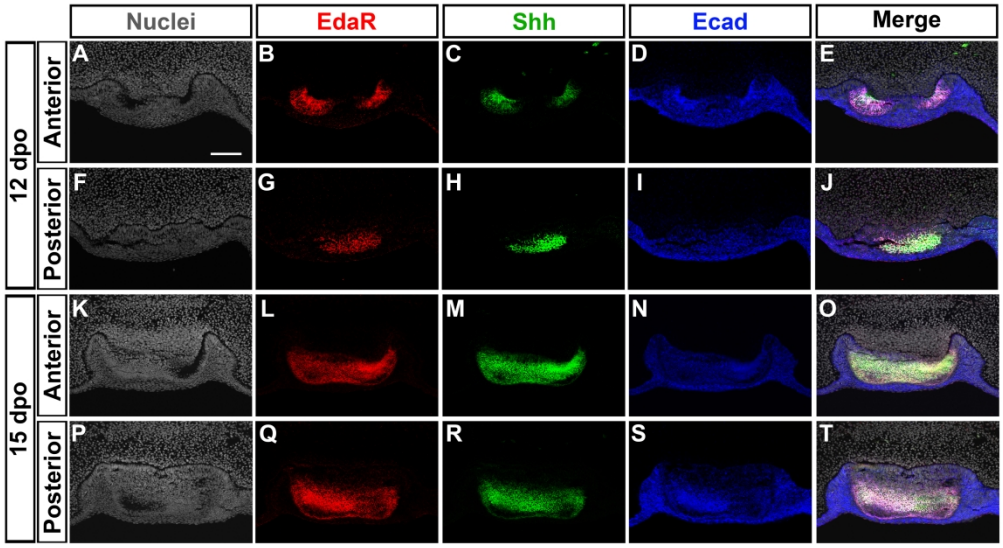

Figure 3

175x95mm (600 x 600 DPI)

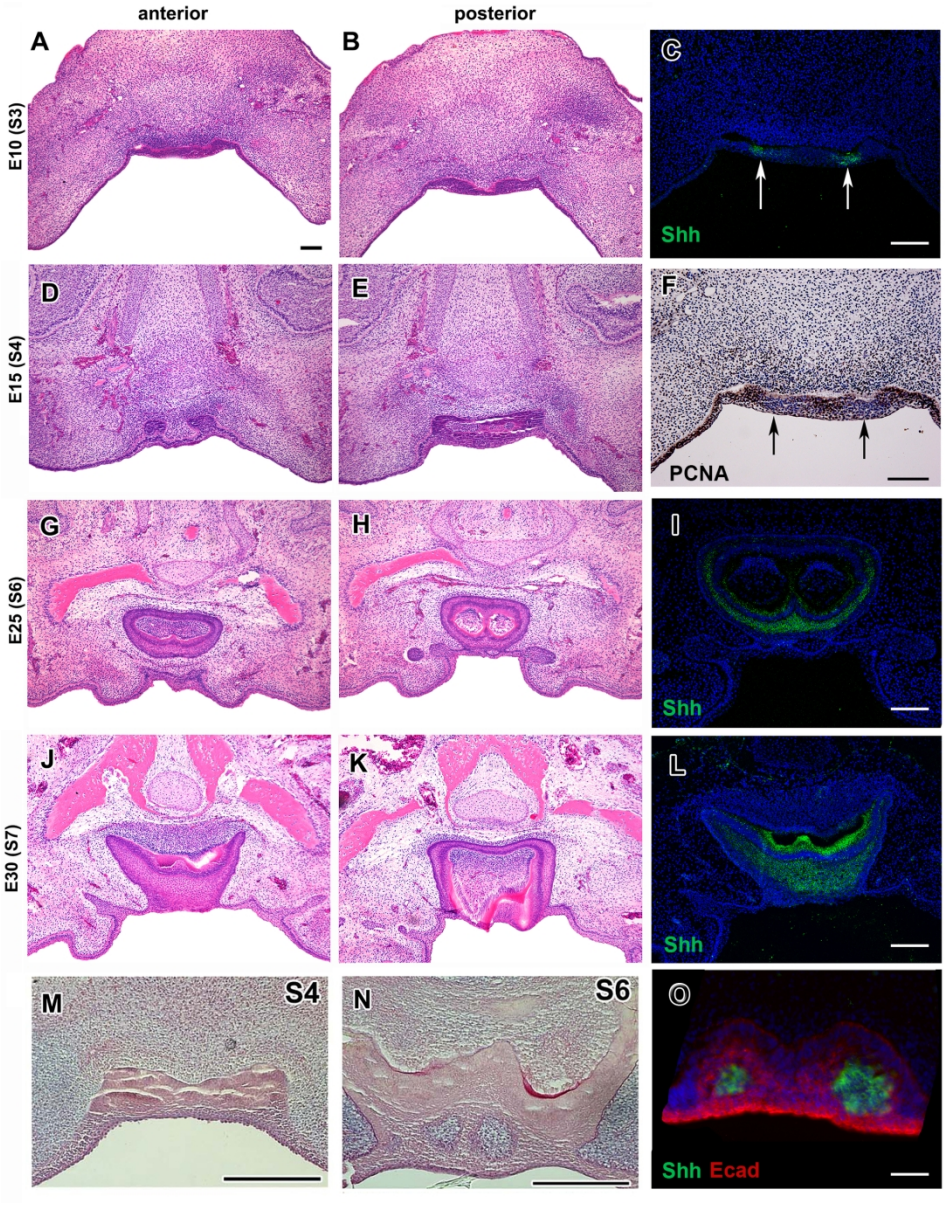

Figure 4

175x220mm (300 x 300 DPI)

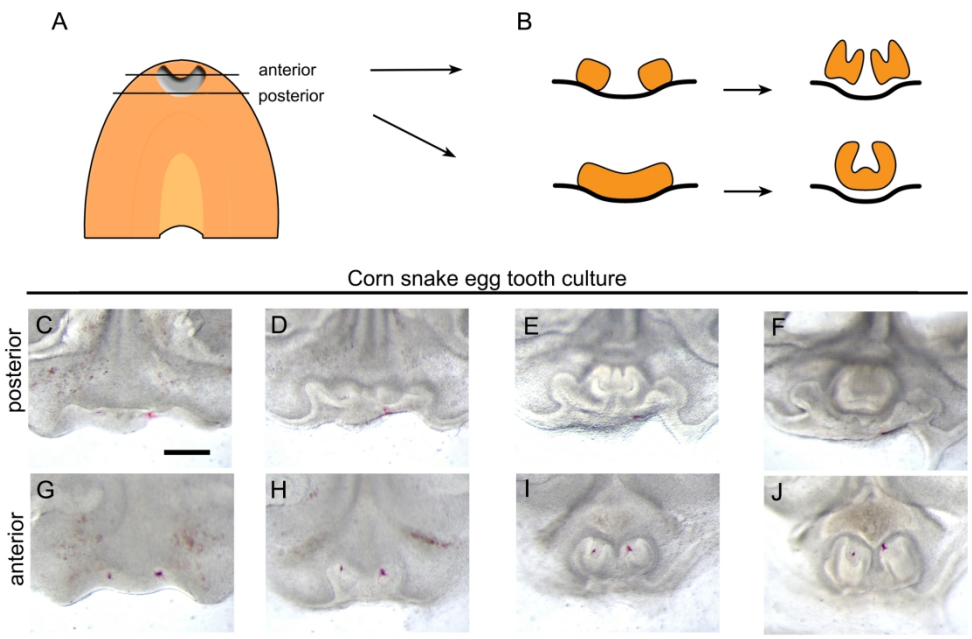

Figure 5

175x112mm (300 x 300 DPI)

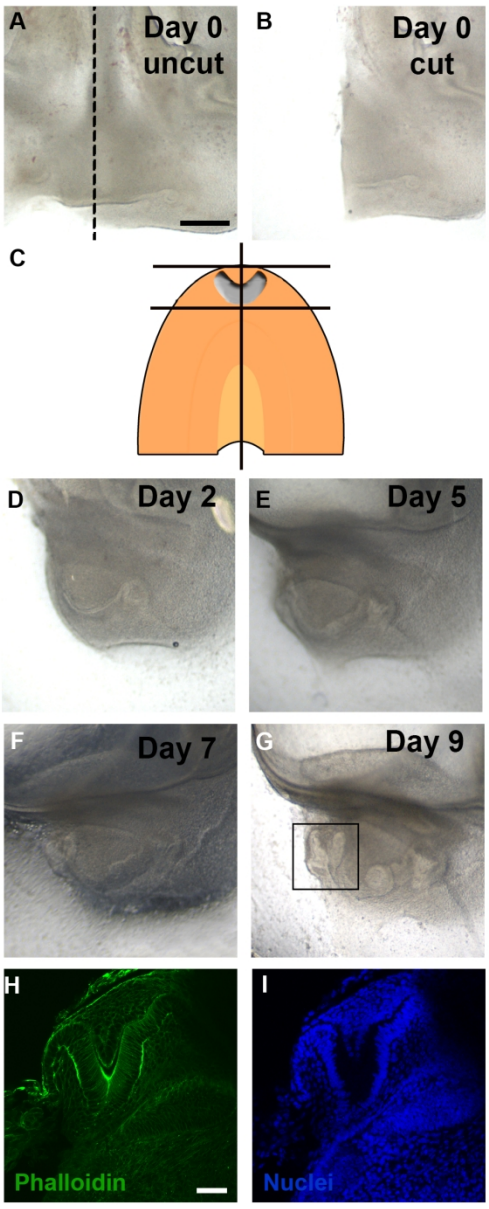
A
Day 0
uncut
B
Day 0
cut
C
D
Day 2
E
Day 5
F
Day 7
G
Day 9
H
Phalloidin
I
Nuclei

Figure 6