Getting out of a mammalian egg: the egg tooth and caruncle of the echidna

Fenelon Jane C^{1a}, Bennetts Abbie^{1a}, Anthwal Neal^{2a}, Pyne, Michael³, Johnston Stephen D⁴, Evans Alistair R^{5,6b}, Tucker Abigail S^{2b} and Renfree Marilyn B^{1b}

¹School of BioSciences, The University of Melbourne, Victoria 3010, Australia

²Centre for Craniofacial and Regenerative Biology, King's College London, London, SE1 9RT, UK

³Currumbin Wildlife Sanctuary, Currumbin, Queensland 4223, Australia

⁴School of Agriculture and Food Sciences, The University of Queensland, Gatton, 4343, Australia

⁵School of Biological Sciences, Monash University, Melbourne, Victoria 3800, Australia

⁶Geosciences, Museums Victoria, Melbourne, Victoria 3001, Australia

^aCo-first authors

^bCo-senior authors

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Abstract

In the short-beaked echidna, *Tachyglossus aculeatus*, after an initial period of *in utero* development, the egg is laid in the pouch and incubated for 10 days. During this time, the fetuses develop an egg tooth and caruncle to help them hatch. However, there are only a few historical references that describe the development of the monotreme egg tooth. Using unprecedented access to echidna pre- and post-hatching tissues, the egg tooth and caruncle were assessed by micro-CT, histology and immunofluorescence, to map the changes at the morphological and molecular level. Unlike mammalian tooth germs that develop by invagination of a placode, the echidna egg tooth developed by evagination, similar to that of the first teeth in some reptiles. The egg tooth ankylosed to the premaxilla, rather than forming a mammalian thecodont attachment, with loss of the egg tooth post-hatching associated with high levels of odontoclasts, and apoptosis. The caruncle formed as a separate mineralisation from the adjacent nasal capsule, and as observed in birds and turtles, the nasal region epithelium expressed markers of cornification. Together, this highlights that the monotreme egg tooth shares many similarities with reptilian teeth, suggesting that this tooth is conserved from a common ancestor of mammals and reptiles.

Introduction

The short-beaked echidna, *Tachyglossus aculeatus*, is one of five species in the mammalian subgroup Monotremata. The other living members of the order are the three long-beaked echidnas: *Zaglossus attenboroughi*, *Zaglossus bartoni* and *Zaglossus bruijini*, as well as the platypus, *Ornithorhynchus anatinus*. It is estimated that monotremes diverged from therian mammals (eutherians and marsupials) approximately 184 million years ago (Cúneo et al., 2013; Luo et al., 2011). Whilst the oldest known echidna fossils are 25 million years old, it is believed that echidnas diverged from platypuses between 55 and 70 million years ago (Davit-Béal et al., 2009; Zhou et al., 2021).

The fossil record shows that over time there has been a reduction in the tooth roots of platypus with the development of hardened keratinised palatal spines, which aid in mastication in modern monotremes (Davit-Béal et al., 2009; Musser & Archer, 1998). A number of platypus ancestor fossils have only a few teeth, showing the evolution away from tooth development and towards hardened pads (Archer et al., 1985; Pascual et al., 1992). In contrast, all echidna fossils identified to date are edentulous (Pascual et al., 1992). Since modern platypus nestlings still possess 3 cusped molars which are lost by adulthood, the echidna is assumed to have lost cheek teeth after diverging from the platypus. Monotremes have also lost some of the enamel forming genes over time and echidnas have lost additional enamel genes compared to platypuses, which still have a thin enamel layer on their cheek teeth (Luckett & Zeller, 1989; Zhou et al., 2021). This is consistent with other animals that evolved edentulism, such as pangolins, birds and baleen whales which have lost one or more enamel producing genes over time (Meredith et al., 2013; Meredith et al., 2014).

The only tooth which is retained in modern echidnas is the egg tooth, which is lost shortly after hatching. An ancestral amniote characteristic conserved in monotremes and reptiles that distinguishes them from therian mammals is oviparity. At the time the echidna egg is laid it is 15-

17 mm in diameter and the embryo is at an early somite stage (Griffiths, 1968, 1989; Hughes & Hall, 1998; Semon, 1894). During the short incubation period, the embryo develops into a fetus and the young hatches from the egg after only 10-10.5 days. The monotreme eggshell is leathery, porous and consists of loosely wound keratinous fibres (Griffiths, 1968). Notably, monotremes develop both an egg tooth and a caruncle to escape from their egg (Griffiths, 1968; Hill & De Beer, 1950; Hughes & Hall, 1998).

Reptiles and birds also possess either a caruncle or an egg tooth. Reptiles, turtles, Rhynchocephalia and crocodilians have a caruncle, a thickened keratinised epithelium positioned above the nasal cartilages (Alibardi, 2020). In contrast, squamates have a true tooth that can be either single or paired (Fons et al., 20220). Even viviparous reptiles have an egg tooth, although it is smaller and hidden under a layer of connective tissue (Hermyt et al., 2017). The vast majority of birds also use an egg tooth to hatch out of their egg. However, the avian egg tooth is very similar to the caruncle of turtles and crocodiles, consisting of a sharp, keratinized ‘horn-like projection’ rather than being an actual tooth (Clark, 1961; Kingsbury et al., 1953; Wang et al., 2017). The structure and appearance of the avian egg tooth is incredibly uniform across most of the one hundred avian species examined so far (Wetherbee, 1959). Interestingly, the caruncle in monotremes is supported by a bony protrusion known as an os caruncle, and therefore, is very different from that observed in birds and reptiles (De Beer, 1949; Hill & De Beer, 1950). The relationship of the os caruncle to the premaxilla has been debated as to whether it is a just an extension of the premaxilla or is an independent ossification which fused with the premaxilla (Hill & De Beer, 1950).

Mammalian teeth have remained largely unchanged in structure throughout time (Davit-Béal et al., 2009). Tooth development in therian mammals is initiated by ligand-receptor interactions between the oral epithelium and mesenchyme during embryonic development (Tucker & Sharpe, 2004). The tooth itself forms in different stages called the bud, cap and bell stages (Tucker & Sharpe, 2004). First the oral epithelium thickens, and sinks into the neural crest derived mesenchyme, which condenses around the epithelium and creates the tooth germ; this process relies on both sonic hedgehog (SHH) and fibroblast growth factor (FGF) signals for proliferation and stratification of the dental placode (Li et al., 2016). The epithelium then extends into the mesenchyme, wrapping around the condensing mesenchyme to form the tooth cap (Tucker & Sharpe, 2004). The primary enamel knot forms at the tip of the tooth bud in the cap stage of development and acts as a signalling centre. The bell stage then follows this, where cyto-differentiation occurs and it is here that odontoblasts and ameloblasts form, which produce dentine and enamel respectively (Tucker & Sharpe, 2004).

Odontoblasts are columnar cells that form a uniform layer around the dental pulp cavity. They are differentiated from the dental pulp and odontoblastic secretions form the dentine layer on their outer surface (Balic & Thesleff, 2015). There are different types of dentine involved in tooth development including primary, secondary and tertiary dentine. Osteodentine is a type of dentine, named for its resemblance to bone, and is observed when tertiary dentine develops rapidly, and in doing so, traps odontoblasts and other nearby cells (Avery, 1994). After the development of the dentine layer, ameloblasts mature on the outer surface of the dentine and secrete proteins such as amelogenin leading to enamel formation between the ameloblasts and the dentine layer (Sire et al., 2007). Enamel is the hardest substance in the body and covers the tooth crown. After the

development of this enamel layer the root of the tooth develops and the tooth erupts through the gums (Thesleff & Juuri, 2012).

The mechanism of attachment for most mammals is thecodonty, which refers to the presence of a tooth root being secured in a socket by a periodontal ligament (Spellerberg, 1982). Thecodonty is believed to be the ancestral tooth attachment mode in the most recent common amniote ancestor (Bertin et al., 2018). The mode of tooth attachment is a main point of difference between mammalian and reptilian teeth. The most common modes of tooth attachment in reptile species are acrodont and pleurodont, both of which involve fusion to the bone of attachment (Gans et al., 1985). The overall stages of tooth development and composition have remained largely unchanged throughout time between mammals and reptiles, with enamel knot like structures observed in some reptiles with complex teeth (Sulcova et al., 2020; Zahradnick et al., 2014). In both mammals and reptiles, SHH is expressed in the early dental epithelium and acts to promote initiation of a tooth (Buchtova et al., 2008; Fons et al., 20220; Li et al., 2016).

The overall stages of egg tooth development in reptiles tends to also mirror that of other vertebrate teeth, depicted by a thickening of the oral epithelium and the bud, cap and bell stages, with development starting in the rostral part of the snout (Hermyt et al., 2017). The main difference is that the egg tooth develops earlier than all other dentition and, in the case of some snakes, is larger than any other tooth (Fons et al., 20220; Hermyt et al., 2017). There is, however, no mention of a root for the egg tooth, so the bone attachment may be the only support keeping the tooth in place.

Whilst there has been some research into the platypus egg tooth and caruncle (Green, 1930; Hill & De Beer, 1950), there are little data on how the echidna egg tooth and caruncle develop. The platypus egg tooth has been described as a pulp cavity surrounded by dentine and covered with an outer layer of presumptive enamel (Davit-Béal et al., 2009; Hill & De Beer, 1950), which is lost 2 days after hatching (Manger et al., 1998). In echidnas, the egg tooth is first observed as a median conical papilla protruding from the anterior end of the snout. Similar to the platypus, the echidna egg tooth is described as being attached to the premaxilla and containing a dental pulp covered in dentine (Hill & De Beer, 1950; Seydel, 1899). Here we have, therefore, focused on the echidna egg tooth and caruncle, to understand the potential homology of these structures with their functional equivalents across birds and reptiles, and to address issues such as method of attachment and mechanisms of loss.

Results

The fetal echidna egg tooth forms by evagination of the epithelium

To follow egg tooth development in the echidna post-oviposition fetal samples were imaged and sectioned. Externally, no egg tooth was visible in the day 4 fetus but by day 6 post-oviposition onwards, the egg tooth was clearly visible (Figure 1A-C). This was confirmed with the histology of the echidna fetal heads which showed substantial changes in growth, particularly in the tooth region, between day 4 and day 6 (Figure 1D, G). In the day 4 fetus, the epithelium was thickened and expressed SHH, confirming the egg tooth was at the placode stage of development (Figure 1F). No evidence of invagination of the placode into the underlying mesenchyme was evident but the mesenchyme under the placode had started to condense. At day 6 the egg tooth projected outwards

121 forming a tooth by evagination, in contrast to the invagination observed during eutherian
122 mammalian dental development (Figure 1G-H). SHH remained expressed in the evaginating
123 epithelium, with an additional positive domain in the oral epithelium further back in the mouth
124 (Figure 1I).

125 *The fetal echidna egg tooth forms a dentine layer continuous with the premaxilla bone*

126 In the day 6 fetus, a mineralised layer had begun to form but the dental pulp had only just started
127 to differentiate, and the cells were very densely packed (Figure 1H). By day 7.5, the dentine layer
128 was clearly distinct with odontoblasts lining the inner surface, and the cells of the dental papilla
129 appeared much more loosely packed (Figure 2A, E, I). The epithelium around the egg tooth was
130 thickened at the tip with the formation of polarised cells that had a morphology similar to
131 ameloblasts. However, an enamel layer was not evident at any of the stages examined (Figure 2K,
132 L). The smooth layer of dentine at the tip of the egg tooth gave way to osteodentine at the base,
133 with mesenchymal cells trapped within the dentine matrix (Figure 2I, J). This region appeared
134 continuous with the premaxillary bone, as confirmed by Alizarin red staining (Figure 2D, H). No root
135 was observed at any of the stages examined. The egg tooth is therefore, anchored directly to the
136 bone.

137 *The premaxilla unites the egg tooth and caruncle*

138 The caruncle was first detected in the day 7.5 fetus and was clear in the day 8 fetus as an ossified
139 circle of mesenchyme pressed against the nasal capsule (Figure 3A, B). Unlike the nasal capsule,
140 which expresses high levels of Type II collagen, the caruncle did not express this cartilage marker,
141 and stained with alizarin red, suggesting it is indeed a bone (Figure 3B, D, E, I). The caruncle could
142 be seen to attach directly with the premaxillary bone, which extended upwards over the nasal
143 cartilages, thereby, uniting the egg tooth and caruncle (Figure 3G, H). To confirm the arrangement
144 of these skeletal elements, micro-CT was used to show the tissue in 3D (Figure 3C, F Supplementary
145 Figure 3). The dentine of the egg tooth could be observed at day 8 connecting to the trabecular
146 bone of the premaxilla, with two arms of bone extending up from the premaxilla to contact the
147 caruncle (Figure 3F). The development of the premaxilla was more advanced than the other bones
148 of the head, highlighting the need for this region to develop in advance of the rest of the structures
149 to support the egg tooth and caruncle.

150 The epithelium overlying the osseous caruncle was thickened compared to the epithelium over the
151 rest of the head as has been previously reported (Figure 4A) (Hill & De Beer, 1950). To observe the
152 pattern of differentiation in more detail, markers of the different layers of epithelium were
153 investigated. Keratin 14 is often used as a marker of the basal epithelium and is expressed in
154 mammalian skin from early stages of development as the cells commit to stratification (Koster &
155 Roop, 2004). Around the head, Keratin 14 was expressed in the basal layer of the epithelium,
156 however, in the caruncle it was expressed in the intermediate layer (Figure 4B, C). Keratin 8, another
157 marker of uncommitted epithelium (Liu et al., 2013), was also expressed in the head epithelium but
158 was absent from the caruncle (Figure 4D, E). Finally, Loricrin, a marker of terminal differentiation,
159 was only observed in the caruncle epithelium, in the top layer (Figure 4B), confirming the caruncle
160 epithelial cells had formed a cornified layer.

Post-hatching loss of the egg tooth by apoptosis and odontoclast activity

Loss of the egg tooth was followed by micro-CT post-hatching (Figure 5A-C). The egg tooth, clearly evident on the day of hatching was lost in the day 4 pouch young but was evident in some specimens at day 3 (Figure 5B-C). The mechanism of loss was therefore followed by TRAP staining to identify clast cells and by TUNEL for apoptosis. At the day of hatching very few TRAP positive cells were evident in the egg tooth and surrounding tissue (Figure 5D). In contrast at day 2, just before loss of the tooth, high numbers of TRAP positive cells were evident, particularly within the tooth against the dentine layer (Figure 5E). Thinning and breaks in the dentine were evident, suggesting that the tooth was removed by odontoclasts. High levels of apoptotic cells, as labelled with TUNEL, were evident in the dental pulp, particularly at the border with the premaxilla, highlighting loss of this tissue after hatching (Figure 5F). A combination of detachment of the dentine and death of the cells of the pulp therefore led to its loss. This mode of loss was also observed in the platypus, where breaks in the osteodentine were evident, flanked by multinucleated clast cells (Supplementary Figure 5). Multinucleate clasts cells were also identified in the dental papilla up against the dentine, suggesting that this mode of removal from the inside is conserved in monotremes (Supplementary Figure 5C). In contrast to the egg tooth, no TRAP staining was evident in the echidna caruncle up until day 4 pouch young (data not shown). In keeping with this, micro-CT analysis indicated that the caruncle was still present in a day 11 pouch young but was no longer evident by approximately day 50 (Figure 5 G-I).

Discussion

Echidna fetuses were found to undergo the most significant development with regards to tooth development between day 4 and day 7.5 post-oviposition, with SHH expressed in the thickening oral epithelium at day 4 and with the tooth reaching maturity by day 7.5. The finding of SHH in the egg tooth placode is consistent with the conserved expression of SHH in both mammals and reptiles in defining the position of the dental lamina and promoting tooth development (Balic & Thesleff, 2015; Buchtova et al., 2008). SHH expression was particularly high in the day 6 fetus where the tooth meets the mouth, which may indicate the location of a signalling centre controlling the projection of the tooth or the presence of an additional dental placode that arrests development at this stage.

Interestingly, instead of the placode invaginating into the surrounding mesenchyme, as observed in eutherian mammals, the echidna placode protruded out of the mouth. This evagination is also observed in lizards and crocodilians as part of the development of the first set of teeth (Westergaard & Ferguson, 1990; Zahradnick et al., 2012). Evaginating tooth-like structures have also been observed in avian talpid mutants (Harris et al., 2006). While the later developing teeth in reptiles form from a dental lamina deep within the jaw, the first null generation teeth form superficially and project out of the mouth (Westergaard & Ferguson, 1990; Zahradnick et al., 2012). These early teeth are lost by resorption or are expelled from the mouth and are often made of dentine without an enamel coating (Zahradnick et al., 2012). Interestingly, evaginating teeth develop in some mouse mutants after manipulation of Wnt signalling, highlighting that a simple switch may be able to convert evaginating to invaginating tooth germs (Kim et al., 2021). The similarities in development of the echidna egg tooth and the evaginating early teeth in reptiles and archosaurs,

201 could suggest that they are homologous, with this method of tooth development conserved from
202 the last common amniote ancestor of synapsids and sauropsids.

203 The mature echidna egg tooth at hatching was comprised of layers of dental pulp, odontoblasts and
204 dentine. However, whilst the odontoblasts were comprised of a clearly defined layer, they were
205 more irregular in shape compared to the uniformly columnar odontoblasts typically identified in
206 mammalian teeth. These more rudimentary odontoblasts are likely due to the temporary nature of
207 the tooth. The dentine layer developed rapidly. Whilst the egg tooth was not fully mineralised at
208 day 6, it was at its maximum thickness and mineralised only 1.5. days later. Histological examination
209 of the dentine also revealed that it contained trapped cells at the base of the tooth, which
210 resembled trabecular bone making it difficult to distinguish where the tooth ended and the
211 premaxilla started. We identified this layer as a type of dentine known as osteodentine which can
212 superficially resemble bone due to its rapid formation that can cause cells to be trapped inside it
213 (Avery, 1994). This is consistent with the rapid formation of the echidna egg tooth and the
214 rudimentary development of the odontoblasts.

215 While most of the cell types visible in mammalian teeth were observed in the mature echidna egg
216 tooth, no enamel could be identified in histological sections of the tooth at any stage. Previous
217 platypus egg tooth studies had suggested that there may be a layer of presumptive enamel around
218 the egg tooth and had assumed that the echidna egg tooth had this same layer (Green, 1930; Hill &
219 De Beer, 1950). However, recently it was discovered that both the platypus and echidna have lost a
220 number of genes involved in tooth formation and echidnas have lost additional enamel producing
221 genes compared to the platypus (Zhou et al., 2021). This is unsurprising since whilst both platypus
222 and echidnas possess an egg tooth, once the echidna egg tooth falls out they are edentulous
223 whereas platypuses develop cheek teeth in the nestling stage and these do contain enamel (Davit-
224 Béal et al., 2009). The short-lived nature of the echidna egg tooth and the soft, leathery form of the
225 egg means that a hard enamel layer is unlikely to be necessary for hatching. The presence of enamel
226 may, therefore, depend on the chemical composition of the eggshell, or vice versa.

227 In further contrast to other mammals, the trabecular bone of the echidna and platypus premaxilla
228 was fused to the egg tooth (ankylosed) rather than being held in a socket by a periodontal ligament
229 as occurs in all other mammals examined to date. The rapid projection of the tooth between days 4
230 and 6 and the absence of any invagination of the epithelium, means that a root was not evident as
231 roots form from invaginations of the epithelium (Thesleff & Juuri, 2012). In the absence of
232 invagination in the echidna, the dentine made direct contact with the bone for attachment. A fused
233 tooth is sturdier than a tooth attached via soft tissue (Jenkins & Shaw, 2020). An ankylosed
234 connection can also likely be established faster than a thecodont attachment. This would be an
235 advantage for the echidna since they have only a brief period of time to develop a structure strong
236 enough to allow them to hatch from their egg. Acrodont attachments are also evident in squamate
237 egg teeth, with the dentine attached to the bone via an attachment tissue (Hermyt et al., 2020).
238 Before loss of the egg tooth, large numbers of TRAP positive clast cells were observed within the
239 egg tooth, lining the dentine at the base of the tooth. These cells appeared to be odontoclasts, as
240 they were observed adjacent to regions of thinned dentine, and thereby could break the connection
241 between the tooth and premaxilla. Similar multinucleated cells were observed in the platypus,

242 highlighting a conserved mode of tooth loss in monotremes. Removal of the dental pulp was then
243 completed by programmed cell death.

244 The premaxilla extended up and appeared to fuse with the os caruncle, thereby connecting the egg
245 tooth and caruncle into one functional unit. The premaxilla was one of the first bones in the head
246 to ossify, confirming its important support role for both the egg tooth and caruncle. In addition to
247 the formation of the os caruncle in the mesenchyme, the epithelium overlying this structure was
248 cornified, with a more complex stratified epithelium, with similarities to that observed in birds and
249 turtles (Alibardi, 2020). This suggests that while the mesenchymal os caruncle may be a novelty in
250 monotremes, the epithelial caruncle may be homologous to that of reptiles, suggesting that the
251 common amniote ancestor of reptiles and mammals had both of these characteristics. This would
252 suggest that reptiles without caruncles have lost these as they came to rely more heavily on the egg
253 tooth itself. Alternatively, the thickened epithelial caruncles might have evolved independently in
254 the different lineages (turtles, crocodilians, birds, sphenodon, monotremes). Loss or gain of the
255 caruncle and egg tooth is likely to be closely linked with changes in shell composition.

256 Overall, the results from this study support previous suggestions that the monotreme egg tooth
257 development pathway is conserved through an ancestral type of tooth development. The echidna
258 egg tooth was very similar to the primary teeth of some reptiles. Both are ankylosed teeth that
259 develop rapidly through evagination of the oral epithelium rather than invagination, and both have
260 little to no enamel. Together, these results highlight the similarities between reptiles and
261 monotremes and show conserved traits from a common amniote ancestor over 315 million years
262 ago.

263 [Materials and Methods](#)

264 *Animals*

265 Fetal and pouch young short-beaked echidna (*Tachyglossus aculeatus*) samples were collected from
266 a research breeding colony at Currumbin Wildlife Sanctuary. Fetal samples were dated based on the
267 date of egg laying (oviposition), with samples varying in age from day 4 to day 9 after oviposition
268 (where hatching occurs on day 10) (day 4 n=1, day 6 n=1, day 7.5 n=1, day 8 n=3, day 9 n=2). Pouch
269 young samples were dated based on the day of hatching (day 0 py) (day 0 n=2, day 2 n=2, day3 n=1,
270 day 4, n=1 day 11, n=1, d50 n=1). All samples were fixed in 4% paraformaldehyde overnight, washed
271 twice in phosphate buffered saline and stored in 70% ethanol at 4°C. All samples were approved by
272 the University of Queensland Animal Ethics Committees and followed the Australian National Health
273 and Medical Research Council guidelines (2013). Historic slides of a platypus at day 2 hatching (M44
274 (WW) 16.75mmm collected in 1899) were photographed from the Hill Collection, Museum fur
275 Naturkunde, Leibniz Institute for Research on Evolution and Biodiversity, Berlin.

276 *Micro-CT Scanning and 3-D reconstructions*

277 One of the fetuses had already been collected and processed for histology before the
278 commencement of this project so was unable to be micro-CT (micro computed tomography)
279 scanned. All remaining echidna fetuses and pouch young samples were scanned by micro-CT. Micro-
280 CT scanning of the echidna fetuses and the d11 py was performed with a phoenix nanotom m
281 (Waygate Technologies, Huerth, Germany) operated using xs control and phoenix datos|x

282 acquisition software (Waygate Technologies). An X-ray energy of 35-40 kV and 300 μ A was used.
 283 Scans were conducted using a molybdenum target to maximize contrast from the soft tissue
 284 specimens. The voxel resolution was optimised to the size of the specimen and varied from 3.1 to
 285 11.1 μ m. The number of X-ray projections collected through a full 360-degree rotation was also
 286 optimized to the size of the specimen, varying from 599 to 1798 projections leading to scan times
 287 of 5 to 15 min in a fast scan mode (Table 1). The data was exported as 16-bit volume files for imaging
 288 and analysis. All remaining pouch young samples were scanned using a Scanco micro-CT scanner
 289 and images analysed using Amira (ThermoFisher Scientific, Waltham, MA, USA).

290 Three fetuses were scanned twice, first unstained to detect mineralised tissues and secondly after
 291 being stained with 1% iodine in 100% ethanol overnight to increase the contrast of non-mineralised
 292 tissue. All other fetal samples were scanned once after being previously stained with 1% iodine in
 293 100% ethanol overnight and then cleared with sodium thiosulfate before being stored in 100%
 294 ethanol. After the fetuses were iodine and micro-CT scanned, they were washed in 70% ethanol to
 295 remove any excess iodine stain and then incubated in sodium thiosulfate to clear the iodine
 296 colouration before paraffin embedding and sectioning. Pouch young samples were scanned twice.

297 3D image analysis and segmentation were performed in Avizo v9.7.0 (ThermoFisher Scientific) via
 298 the MASSIVE platform (Goscinski et al., 2014).

299 *Histology*

300 After micro-CT scanning, fetuses were sectioned at a thickness of 5 μ m through the sagittal plane
 301 on a Rotary CUT 4060 microtome (Microtec Laborgerate, GmbH, Walldorf Germany) and dried
 302 overnight in a 40°C oven. Every third slide was then de-waxed and rehydrated through a graded
 303 ethanol series to distilled water (dH₂O) and stained with haematoxylin and eosin (H&E) using
 304 standard techniques. From the H&E-stained slides, the tooth region was identified, and in this
 305 region, every second remaining slide was stained with a Picro-Sirius Red and Alcian blue trichrome
 306 stain for histological examination of bone, cartilage and dentine using standard techniques. The
 307 remaining slides were kept for immunofluorescence

308 *TRAP staining*

309 In order to visualise odontoclasts, dewaxed and rehydrated sections were tartrate-resistant acid
 310 phosphatase (TRAP) stained as previously described (Anthwal et al., 2017). Briefly, rehydrated slides
 311 were incubated in filtered pH 5.32 acetate buffer containing 1 mg/ml Naphthol-AS-TR-phosphate
 312 (Sigma), 100 mM Sodium Tartrate (Sigma-Aldrich, Merck, Darmstadt Germany), and 1 mg/ml Fast
 313 Red TR Salt (Sigma-Aldrich) at 37°C until colour reaction was complete. Slides were then
 314 counterstained with Mayer's Haematoxylin (Sigma) for 10 seconds, and blued in tap water, and
 315 mounted in aqueous mounting medium (Aquatex, Sigma-Aldrich)

316 *Immunofluorescence*

317 Sections used for immunofluorescence were first de-waxed in histolene and rehydrated as above,
 318 with a final wash in dH₂O. To detect protein expression of sonic hedgehog (SHH), collagen type II
 319 (Col2), cytokeratin 14 (K14), cytokeratin 8 (K8), and Loricrin, sections were first placed in the antigen
 320 retrieval buffer 0.01 M Sodium citrate pH10 (w/v) and incubated in a preheated water bath at 95°C
 321 for 20 min before being left to cool at room temperature for 40 min. After cooling, the slides were

322 washed in 1X phosphate buffered saline, (1X PBS) containing 1.37 M NaCl, 27 mM KCl, 18 mM
 323 KH_2PO_4 and 100 mM Na_2HPO_4 . Following the 1x PBS wash, sections were incubated in 0.3% Sudan
 324 Black (w/v, ProSciTech, Qld, Australia) in 70% ethanol for 10 min before being immersed briefly in
 325 fresh 70% ethanol and then washed for 5 min in 1X PBS. Sections were then blocked with 10% (v/v)
 326 normal goat serum in 1x PBS for 1 h at room temperature. After blocking, the sections were
 327 incubated overnight (16 h) in a humid chamber at 4°C with either rabbit SHH antibody (2 µg/ml;
 328 ab19897; Abcam, Cambridge, UK), mouse Col2 antibody (1/50 dilution; II-II6B3; Developmental
 329 Studies Hybridoma Bank, Iowa City, IA, USA), mouse K8 antibody (1/50 dilution; TROMA-1; DSHB),
 330 or co-labelled with mouse anti K14 (0.625µg/ml; ab7800; Abcam) and rabbit Loricrin antibody
 331 (1µg/ml; 905101; BioLegend, San Diego, CA, USA) . Sections were then washed three times in PBS
 332 containing 0.5% Tween 20 (v/v) then incubated for 45 min with goat anti-rabbit Alexa Fluor Plus 555
 333 secondary antibody (A32732, Invitrogen, ThermoFisher Scientific) at either 1:300 dilution (SHH),
 334 goat anti-rabbit Alexa Fluor 568 (A11011, Invitrogen) at 1:400 dilution (Loricrin), or goat anti-mouse
 335 Alexa Fluor 488 (A32723, Invitrogen) at 1:400 (COL2, K8, K14). Nuclei were counterstained with 4',6-
 336 diamidino-2-phenylindole (DAPI; D9542; Sigma-Aldrich) and the slides mounted with media
 337 (homemade, or Fluroshield, Abcam) and kept in the dark until visualisation. Negative control
 338 sections were submitted to the same procedures except that the first antibody was replaced by the
 339 relative isotype control at the same concentration as the primary antibody. The specificity of all
 340 antibodies was confirmed by staining in positive and negative control tissues and lack of staining in
 341 the isotype controls. Images were acquired on a Nikon (Minato City, Japan) A1R spectral confocal
 342 microscope housed at the University of Melbourne, or a Zeiss Apotome fluorescence microscope
 343 housed at King's College London. Visualisation settings were initially optimised to eliminate
 344 background using the isotype control on each slide. These settings were then used to visualise the
 345 remaining positive sections on the slide.

346 Declaration of interest

347 The authors declare no conflict of interests.

348 Statement of Ethics

349 The University of Queensland Animal Experimentation Ethics Committee approved all sampling for
 350 echidnas, in accordance with the National Health and Medical Research Council of Australia
 351 Guidelines (*Australian code for the care and use of animals for scientific purposes*, 2013; 2013).

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355 Author contribution statement

356 JCF, NA, AST, ARE and MBR designed research; JCF, AB and NA performed the research; JCF, SDJ,
 357 MBR and MP collected tissues, JCF, AB, NA, ARE, AST and MBR analysed data; and JCF, AB, NA, AST
 358 and MBR wrote the paper. All authors read and approved the final version of the manuscript.

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Table

Table 1: Micro-CT scan data for 7 echidna fetuses.

Sample description	Scan time (min)	Target	Voltage (kV)	Current (μA)	Resolution (μm)	Projections
d4 unstained	5	Molybdenum	35	300	3.1	599
d4 stained	12	Molybdenum	40	300	3.111	1438
d6 unstained	12	Molybdenum	35	300	4	1438
d6 stained	12	Molybdenum	40	300	3.502	1438
d7.5 stained*	12	Molybdenum	35	300	4	1438
d8 stained*	12	Molybdenum	35	300	4	1438
d8 stained*	13	Molybdenum	35	300	3.1	1558
d8 unstained	13	Molybdenum	35	300	4.001	1558
d8 stained	2x10	Molybdenum	40	300	4	1199
d9 stained*	15	Molybdenum	35	300	4	1798

*previously stained and washed, stored in 70% ethanol

Figures

Figure 1: The echidna egg tooth forms by evagination of the epithelium. A-C: Photographs of post-oviposition echidna fetuses showing changes in external head and tooth morphology over time. A: day 4 fetus with no tooth or caruncle visible, black arrowhead indicates location of eye; B: by day 6.5 the egg tooth (et) is clearly visible; C: day 7 fetus; D: day 4 fetal head through the sagittal plane, the box indicates the magnified region in E; E: morphology of the thickened epithelium in the tooth region, F: SHH expression in the day 4 fetus showing significant expression in the oral thickened epithelium; G: day 6 fetal head through the sagittal plane, the box indicates the magnified region in H; H: morphology of day 6 fetal tooth, I: SHH expression in the day 6 fetus showing high expression in the oral epithelium (arrowhead). Blue staining= DAPI, red staining= SHH. br: brain, et: egg tooth, m: mandible, te: thickened epithelium. Scale bars F&I= 100 μm.

Figure 2: The echidna egg tooth forms a dentine layer continuous with the premaxilla bone. A: day 7.5 fetal head through the sagittal plane, B: day 8 fetal head through the sagittal plane, C: day 9 fetal head through the sagittal plane, D: trichome stained day 9 fetal head through the sagittal plane, E-H: higher power image of A-D showing the continuous dentine layer between the tooth and premaxilla, I-L: high power magnification of the tooth region in E-H. br: brain, dp: dental papilla, d:

dentine, e: epithelium, et: egg tooth, m: mandible, t: tongue, o: osteoblasts. Arrows indicate the developing tooth region. All images H&E stained unless otherwise indicated. Trichrome stained sections: bone and dentine = red; cartilage =blue. Scale bars A-D: = 1 mm, E-H: 200 μ m, I-K: 100 μ m.

Figure 3: The premaxilla unites the egg tooth and the caruncle. A: Trichrome stained egg tooth and caruncle of a day 7.5 fetus; B: The initial calcification of the caruncle; C: Micro-CT volume rendering of the premaxilla and egg-tooth region in the same fetus; D: Trichrome stained egg tooth and caruncle of a day 9 fetus; E: The trabecular bone of the caruncle; F: Frontal plane view of a micro-CT volume rendering of the mineralised structures of the egg tooth, caruncle and premaxilla in a day 8 fetus; G: The snout of a mature fetus showing the egg tooth sharing the same attachment to the premaxilla as the caruncle; H: The connection between the caruncle and the egg tooth via the trabecular bone of the premaxilla; I: Immunofluorescence of collagen type II in the day 2 pouch young. Dotted boxes indicate area of magnified regions in B, E & H. c: caruncle, et: egg tooth, nc: nasal cartilage, pm: premaxilla. Trichrome stained sections: bone and dentine = red; cartilage =blue. Scale bars: A, F, H 200 μ m, B & E: 100 μ m, C: 50 μ m, D & G: 500 μ m. Figure supplement 3 shows the microCT scans of the additional egg tooth structures.

Figure 4: Precocious stratification of the ectoderm over the caruncle. A: DAPI stained Sagittal section through echidna head at day 0 pouch young. Boxes indicate locations shown in B-E. B,C: Expression by immunofluorescence in green of cytokeratin 14 (K14) and in magenta of Loricrin in the epidermal epithelium of the caruncle (B) and the back of the cranium (C). The epithelium at the caruncle is highly stratified compares to that at the back of head. K14 is expressed in the intermediate layer of the stratified epithelium at the caruncle (B), and strongly expressed in the basal layer at the back of the head (C). Loricrin, a marker of terminally differentiated epidermis, is expresses at the caruncle (B), but is absent form the back of the head (C). D,E: Immunofluorescence in green expression of cytokeratin 8 (K8), a marker of immature epidermal epithelium. K8 is not expressed in at the caruncle (D) but is expressed in the epithelium surrounding the back of the head (E). C: caruncle. Scale bars: A= 500 μ m, B-E= 50 μ m

Figure 5: Post-hatching loss of the egg tooth by apoptosis and odontoclast activity. A-C: Soft tissue contrasted micro-CT volume rendering of the mouth region in d0 (A), d3 (B) and d4 (C) pouch young, showing the egg tooth (highlighted in pink) present at d0 and d3, but not at d4. D,E: TRAP staining showing no odontoclast activity in the egg tooth of d0 pouch young (D), whereas at d2 (E) odontoclasts are acting on the inner surface of the egg tooth dentin. Arrow in E shows location of high magnification insert demonstrating odontoclast activity associated with a break in the egg tooth dentin. F: Fluorescence TUNEL staining shows high levels of apoptosis in the pulp of the egg tooth of d2 pouch young. G-I: Mineralised tissue micro-CT volume rendering of the skull in d11 (G-H) and approximately d50 (I) pouch young showing continuation of the caruncle (white arrow), whereas it is gone by d50. Scale bars: A-C = 500 μ m, D-F = 100 μ m, inset E = 10 μ m, G-I = 2mm. Figure Supplement 5 shows the beginnings of similar detachment in the platypus, *Ornithorhynchus anatinus* at 2 days post-hatching.

565 **Figure 3 - figure supplement 3: 3-dimensional models of the echidna egg tooth through the frontal**
 566 **and sagittal planes at different days of development.** Micro-CT volume rendering of the
 567 mineralised tissue. Scale bars= 100 µm.

568 **Figure 5 - figure supplement 5: Platypus (*Ornithorhynchus anatinus*) 2 days post hatching. A-D:**
 569 Frontal views of egg tooth. A,B: The egg tooth has started to detach from the premaxilla with a break
 570 evident on the LHS. C,D: Multinucleated cells are evident inside the dental pulp lining the dentine
 571 (C), and at the border between the tooth and premaxilla in the regions of bone loss (D).

day 7.5 H&E

day 8 H&E

day 9 H&E

day 9 Trichrome

frontal

sagittal

day 7

day 8

day 9