THE MECHANISMS OF CONTINUOUS TOOTH REPLACEMENT IN THE NILE CROCODILE

(*CROCODYLUS NILOTICUS*)

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I, Cleopatra Thomadakis declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signed: …….on **21** day of May **2015**

For my children
Eleni, Niko and Demitra

ABSTRACT

It is recognised that tooth loss as a consequence of oral diseases affects quality of life in humans. This has directed studies towards biological tooth replacement *in vivo.* In humans and other mammals, tooth replacement occurs only once (diphyodonty) as opposed to non-mammalian vertebrates where tooth replacement continues throughout life (polyphyodonty). Detailed knowledge of tooth initiation, development and morphology amongst vertebrates and especially amniotes, is necessary to understand the tooth replacement process. Crocodilians provide an interesting model for tooth replacement studies as they also exhibit thecodonty. Regulation of polyphyodonty has not been genetically defined, and it is uncertain whether the molecular mechanisms of continuous tooth replacement are similar to those involved in the primary dentition. **The aim of this study was therefore to analyse crocodilian odontogenesis in detail, with the aid of light microscopy and CT scans, in order to provide a structural framework for molecular processes regulating polyphyodonty.** Crocodile probes to *bmp4* and *pitx2* were designed, generated and labelled for use in *in situ* hybridisation. The expression patterns of *pitx2* and *bmp4* in embryos and hatchlings of the polyphyodont Nile crocodile (*Crocodylus niloticus*) were examined at different stages of tooth development. Histologically crocodilian tooth development appears similar to mammals. Interesting variations include the initiation of odontogenesis in the ectomesenchyme, the presence of dental placodes, the 'null generation teeth', the two different bell-stage tooth germs and the tooth-family organisation. A direct 1:1 relationship between the status of the erupted tooth and the developmental phase of the replacement tooth was not seen. However in more mature teeth, the replacement tooth germs were at a more advanced developmental stage than those associated with less mature teeth. Molecular data revealed that *pitx2* was expressed in the oral epithelium and the dental placode. *Bmp4* expression was not evident in the dental placode, but was localised in the odontoblasts of early bell stage tooth germs.

Pitx2 and *bmp4* were expressed in both the odontoblast and ameloblast layers in late bell stage tooth germs. Expression of *pitx2* and *bmp4* is conserved across vertebrates and *pitx2* may play a role in initiation of primary and successional teeth.

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1. INTRODUCTION

The structural and functional diversity of teeth among the vertebrates and specifically the amniotes, is a characteristic of adaptive evolution. The structure of a tooth is indicative of the ecological habitat which the animal occupies, influences its natural history, and interprets its evolutionary pathway. The path of the evolution of amniotes has included several significant modifications related to the dentition of organisms. These modifications include diversities in relation to tooth number, shape and tooth replacement (Butler, 1995; Line, 2003). Gradual evolutionary modifications involve region-specific tooth loss, regional alteration of tooth shape and the disappearance of a polyphyodont mode of dentition (Stock, 2007). It is possible that unravelling gene expression through modern molecular techniques may enable an understanding of the diversity of dental characteristics in extant amniotes and will also allow for a comparison with shared ancestral traits applicable to human health.

1.1 Dental regenerative biology

Regenerative biology has emerged as a field with increasing possibilities for clinical applications. Investigations on the regulation of tooth development have advanced, partly due to molecular investigations, but also because of increased interest in the field. This interest is largely motivated by the fact that clinical loss or damage to teeth seriously affects mastication, articulation, facial aesthetics and psychological health (Matalova *et al*., 2004; Koussoulakou *et al*., 2009). Tooth loss is a serious problem as approximately 35% of the world's population is edentulous by the age of 65 (Koussoulakou *et al*., 2009). Dental pathological conditions involve enamel and dentine erosion, as well as damage to the living tissues such as the pulp and periodontium. Even though the enamel of the crown is hard and durable, the ingestion of harmful substances still cause significant

damage and contribute to the deterioration of the enamel integrity. Stomatopathosis, therefore, is a frequent human pathology that may ultimately lead to tooth loss if left untreated. In addition to tooth decay, attrition or trauma, the list of tooth related diseases is supplemented by congenital abnormalities including anodontia, amelogenesis imperfecta and supernumerary teeth (Bailleul-Forestier *et al*., 2008; Kapadia *et al*., 2007; Line, 2003).

Treatment for the replacement or loss of teeth includes allotrasplantation, autotransplantation, dental implants and artificial dentures. The effects of these procedures are not often satisfactory, and include lack of osteointegration, damage to surrounding tissues and lack of compatibility between implants and human tissues (Ferreira *et al*., 2007). Dental therapeutic interventions often have unpleasant side effects (Thesleff and Tummers, 2003; Christensen, 2005), fail frequently and have considerable costs. The answers to treating tooth loss therefore, seem to lie in trying to reproduce biological tooth replacement *in vivo* and *in vitro* (Chai and Slavkin, 2003; Thesleff and Tummers, 2003; Bluteau *et al*., 2008).

A detailed understanding of the mechanisms of spontaneous tooth replacement, and knowledge of the genes which control this, may provide insight into how mammalian diversification occurred and whether the regenerative potential of human teeth may be reactivated under specific conditions (Saulnier *et al*., 2005; Gurtner *et al.*, 2008). A biologically orientated therapeutic approach which imitates the physiological mechanisms of tooth initiation and morphogenesis thus seems appropriate.

The multidisciplinary research field of tissue and organ regeneration has grown with hopeful clinical prospects for several tissues and organs including teeth (Stocum and Zupanc, 2008; Weaver and Garry, 2008). Specific culture methods are available to be used with a series of biocompatible biodegradable three-

dimensional support materials (Yu *et al*., 2008). The induction signals of tooth morphogenesis are known. Numerous growth factors and signaling molecules have been identified, but what is not known presently is how tooth size, shape and replacement is controlled (Yu *et al*., 2008). Yen and Sharpe (2008) showed that a bio-engineered bio-tooth germ may be implanted into a prepared alveolar socket under conditions which allow for tooth development, periodontal ligament attachment and osteointegration. These bioengineered tooth germs showed some growth, morphogenesis and eruption (Komine *et al*., 2007; Stock, 2007).

Physiological regeneration or replacing a tooth *in situ* therefore, appears to be an ideal option to treat tooth loss. Tooth forming cells or even stem cells may be instructed to differentiate or transdifferentiate to repeat initial odontogenesis (Marais *et al*., 2011; Cobourne and Sharpe, 2013). In humans, most of the permanent teeth form from the dental lamina of the preceding deciduous tooth. As a rule, in humans, this process only happens once and the competency diminishes with the dental lamina. However, non-mammalian vertebrates undergo tooth replacement throughout life. Simplistically, the teeth of these vertebrates are replaced in a manner similar to the human deciduous tooth replacement situation (Stock, 2007). Therefore, it would be interesting to examine the processes and mechanisms of successional tooth formation in non-mammalian vertebrates in order to understand the factors which may allow the induction of a third generation of teeth in human dental patients in the future.

1.2 Histology of tooth development in the Crocodilia

Crocodilians exhibit a primitive form of dentition within the extant tetrapods, and contain simple, conical teeth in distinct sockets that are continuously being replaced. This makes them an ideal model for studies on dental evolution and development.

Little appears in the literature regarding the detailed histological study of odontogenesis in the crocodile from initiation of the first tooth to hatching and to replacement. The sequence of tooth development has been described in crocodilians in the early literature (Bolk, 1912; Woerdeman, 1921) while Poole (1961) and Edmund (1969), studied crocodile tooth morphology in relation to tooth replacement patterns. The main histological description of tooth development in the Crocodilia, was related to the American alligator (*Alligator mississippiensis*) (Westergaard and Ferguson, 1986; 1987). More recently, the general morphology of the oral cavity of the Nile crocodile was described (Putterill and Soley, 2003). Detailed histological assessment of odontogenesis in the Nile crocodile is thus necessary to compare with and add to the data available on the alligator, where detailed histological studies have been carried out (Westergaard and Ferguson, 1986, 1987; Wu *et al.*, 2013). Examination of odontogenesis in several species in an order, for example the Crocodilia, and the variations and similarities thereof may provide a framework for evolutionary and comparative studies with other reptiles. Gene expression studies require a sound knowledge of the histological structures and their variations between species to determine the phylogenetic framework. Also, a comprehensive comparative atlas of crocodilian dental development may provide a basis to investigate as to how the mammalian cusped dentition, loss of tooth replacement and the edentulous state in birds may have evolved from a crocodilian-like ancestral dental situation.

1.3 Polyphyodonty – continuous tooth replacement

Mammals, unlike some of the other vertebrates, have a restricted ability for tooth renewal. In muroid rodents, bats and shrews tooth replacement does not occur and their dentition is classified as monophyodont-one set of teeth (van Nievelt and Smith, 2005). The early mammalian fossil record reveals a steady decrease in tooth replacement, due to the evolution of occlusion which required an increase in dental complexity (Fig. 1). The central factors in occlusal evolution are related to

diet and mastication (Koussoulakou *et al.*, 2009). There is a strong correlation between tooth form and feeding habits. The evolution of the mammalian jaw and teeth created occlusal surfaces adapted for a variety of foods. Computational tools in association with genetic tools may determine properties including cell adhesion, proliferation and migration in an attempt to unravel the detailed regulation of tooth shape, cusp size and shape which are all significant in correct occlusion.

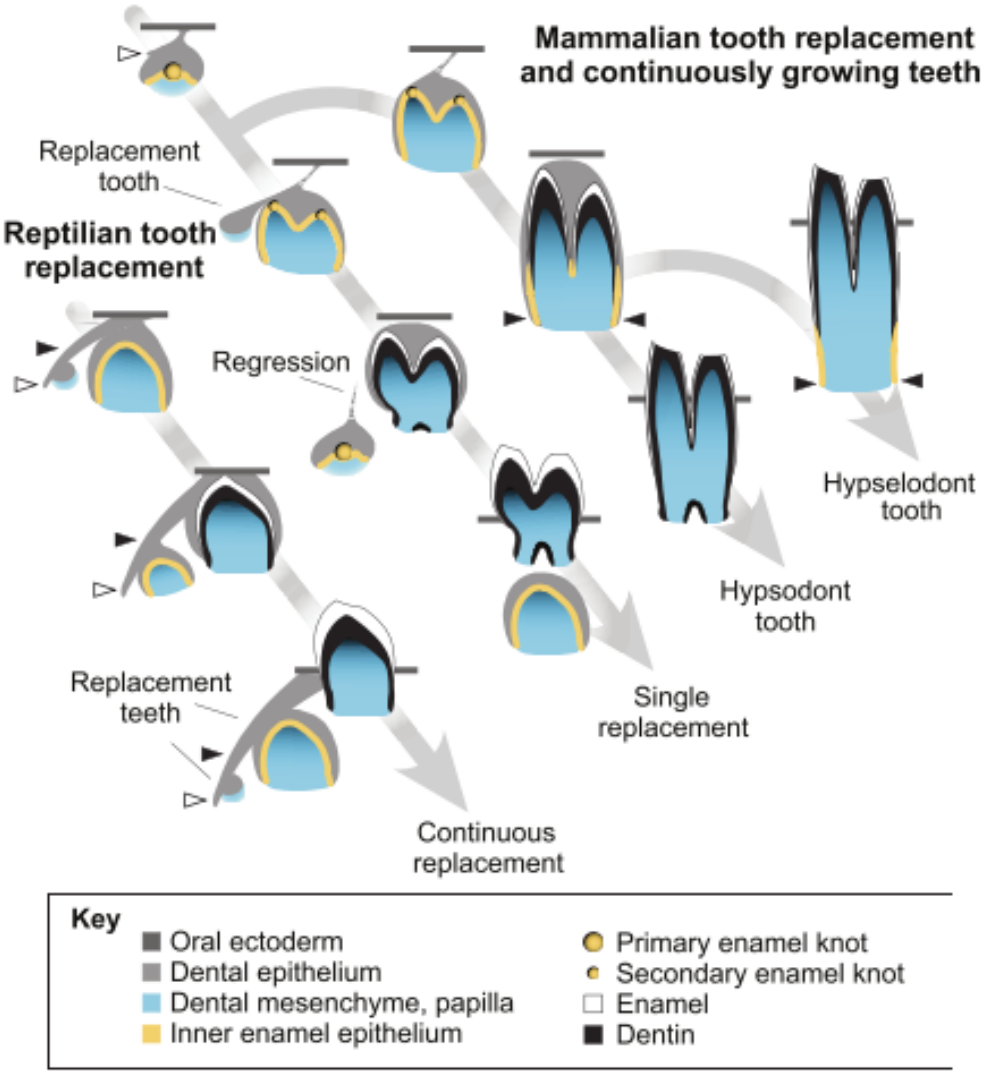


Figure 1. Tooth replacement is variable amongst species. Continuous tooth replacement in reptiles includes a dental lamina that continues to grow to develop a successional lamina (white arrowheads). A stem cell population is thought to occur in the dental lamina (black arrowheads). This method of replacement is prevalent in several fish species. The replacement tooth germ in mammals however, originates from the successional lamina as in the reptilian situation, but the dental lamina degenerates and further tooth replacement ceases. In some species of mammals, the presence of taller teeth (hypsodont) delays tooth formation, and in others like rodent molars, a stem cell niche at the tooth base ensures tooth growth which is continuous throughout life (hypselodont)(from Jernvall and Thesleff, 2012).

An unusual tooth replacement example is evident in the rock wallaby (*Peradorcas concinna),* the manatee (*Trichechus*) and the silvery mole rat (*Heliophobius argenteocinereus*). These animals develop new molars distally behind their last molar (Rodrigues *et al.,* 2011). The first molars are worn, discarded and the remaining teeth move anteriorly to create space for the new molars posteriorly. The evolution of taller or hypsodont teeth (Fig. 1) is another adaptation to overcome tooth loss caused by tooth wear. Hypsodont molars occur in horses, cows, and some rodents. Hypselodonty (a variation of hypsodonty) exists in the molars of rodents such as hares and voles. In these latter animals the teeth retain stem cells at their base and continue to grow throughout the life of the animal (Fig. 1).

The dentitions of vertebrates have been studied extensively due to their tremendous diversity and their significance from an evolutionary perspective. Crocodilians originated from a stock of diapsid reptiles, the Archosauria, which gave rise to dinosaurs, pterosaurs and birds (Toyosawa *et al.,* 1999). In the Triassic period, archosaurs deviated from other reptiles, flourished in the Mesozoic, but declined 65 million years ago at the start of the Cenozoic. Crocodilians are the only remaining archosaurs, from a group known as Thecodontia, which expanded throughout the Mesozoic, and declined rapidly 65 million years ago, at the start of the Cenozoic (Toyosawa *et al*., 1999). In the class Reptilia, Ferguson (1981) emphasised the significance of the order Crocodilia from an evolutionary perspective, as they are the only surviving archosaurs which are separate from the reptilian branch that gave rise to mammals. Irrespective of their different origin, crocodilians display several ‘non-reptilian’ features that are common with the mammalian situation. Recent studies on tooth development in various vertebrate species have indicated similarities in molecular pathways associated with tooth shape and replacement. Also, these pathways appear to be retained throughout evolution (Jernvall and Thesleff, 2012). The evolutionary

changes include the refinement of the spatial and temporal use of the shared signaling pathways.

Studies on the dentitions of vertebrates with multiple sets of replacement teeth may provide insight into the control of tooth replacement. Investigations into replacement of teeth have been carried out on the rainbow trout (Berkowitz and Moore, 1974; 1975; Berkowitz, 1977; 1978; 2000), the lizard (Osborn 1971, 1973b; Delgado *et al*. 2003; 2006), the alligator (Westergaard and Ferguson 1986; 1987) and the Nile crocodile (Poole, 1961). However, the unanswered question in all of these studies is the relationship or pattern between the first generation teeth and their replacements. This relationship is important as it will allow researchers to evaluate the theories explaining tooth replacement and also determine the relationship of these tooth patterns to the early arrangement of tooth development (Huysseune and Witten, 2006).

The continuously replacing reptilian dentition displays several characteristics with some mammalian dentitions, for example the primates, which have two generations of teeth, a condition known as diphyodonty. Also, reptiles are said to be more closely related to mammals than are fish, one of the main models currently used in tooth replacement studies (Huysseune and Thesleff, 2004; Fraser *et al*., 2006b; Huysseune, 2006). In order to comprehend the mechanisms and significance of continuous tooth replacement it would be beneficial to study these mechanisms in a vertebrate that exhibits polyphyodonty or several sets of teeth. Ferguson (1981) emphasized the value of the Crocodilia as a model for odontogenesis as they possess several morphological features which uncharacteristic of reptiles, including a mammal–like secondary palate, a four-chambered heart and a true cerebral cortex. Also, crocodilians are unique among the reptilia in that they exhibit thecodonty ie. their teeth lie in distinct sockets as in the mammalian situation (Osborn, 1971). Crocodiles are an oviparous species and access to their eggs and embryos can be obtained from several crocodile

farms in South Africa. A necessary characteristic of the crocodilian dentition is the ability to retain and compensate teeth for wear, develop teeth during growth or undergo tooth replacement in a coordinated and regulated manner (Smith, 2003). These properties are fundamental for survival. Smith (2003) emphasized that addition of teeth during growth and replacement throughout life in a polyphyodont animal, results from a quantitative, qualitative and temporal gene expression regulation which originated in ontogeny.

The value of studying the crocodile is that it undergoes continuous tooth replacement, which is evident in all regions of the maxilla and the mandible. Also the teeth are generally homodont which allows one to study tooth initiation without the variable of shape as in the heterodont situation. There is no indication in the crocodile, that tooth replacement occurs in response to wear or injury of individual teeth (Osborn, 1971). On the contrary, each tooth is replaced as part of a regular pattern affecting the entire dentition. Polyphyodonty is essential for reptiles since at hatching, the jaw is relatively small and must increase in size many times before maturity. During this period there is no lactation or other maternal feeding which would permit an edentulous juvenile stage. Also, because of the simple structure of reptilian teeth, frequent replacement is necessary to ensure proper crown shape and sharpness. Generally reptiles lack the mammalian modifications of complex cusp patterns, enamel infoldings and continuously growing, open-rooted teeth (Edmund, 1960). The rate of tooth replacement in *Alligator mississippiensis* which was determined radiographically by Edmund (1962) showed that the rate of replacement becomes slower with age, but that the oldest specimen still showed active replacement. Tooth replacement commences early during reptilian embryogenesis thus allowing the investigation of gene function during tooth initiation and replacement. In the crocodile as in mammals, after initiation, teeth undergo bud, cap, early and late bell stages of tooth development. The patterns of tooth development are established early in the form

of an odontogenic plan that is essential for the survival of the animal (Jernvall and Thesleff, 2012).

Amongst living amniotes, extant crocodilians possess the most basic tooth structure (Fig. 2). Several studies of *Alligator mississippiensis* (Westergaard and Ferguson, 1987; Westergaard and Ferguson, 1990) showed that the dentition of the alligator consisted of conical teeth in sockets undergoing continuous tooth replacement. Data from high resolution computerised tomography (CT) scans of fossil and extant taxa (Fig. 2) revealed that crocodilians have a primitive homodont and thecodont dentition with open roots such as those of stem amniotes and other primitive members of both the reptilian and mammalian lineage (Fig. 2). Crocodilian dentition differs from the ancestral amniotes in that their sockets are slightly deeper and the ankylosis reduced (Fig. 2). There is an absence of the medial wall of the tooth socket in Squamates and the teeth of mammals have a specialised and modified morphology with specific cusp pattern and a closed root system (Weeks *et al*., 2013).

Due to the unicuspid nature of the reptilian dentition, it is generally considered as homodont (Osborn and Crompton, 1973). However certain elements of heterodonty have been described within and between many members of the Reptilia (Edmund, 1960; Kieser *et al.,* 1993). Ferguson (1981) suggested that alligator teeth were arranged in a "pseudoheterodont pattern". Due to the different modes of feeding, the different areas of the jaw become specialized in many non-mammalian vertebrates, so that their teeth are of different sizes or shapes i.e. heterodont. Occasionally the morphology of the teeth in a specific region of the jaw may change during the life of an individual. The cheek teeth of the Nile monitor (*Varanus niloticus)* for instance, are conical and fairly sharp-pointed during much of their lives. However as the animal reaches maturity, they become broader and stouter. A similar ontogenetic change occurs in the posterior teeth of crocodilians. Edmund (1969) suggested that in crocodilians there were two areas

in the upper jaw and one in the lower jaw with teeth of increased size. In a study on crocodilian embryos using three-dimensional reconstructions of serial sections, Kieser *et al*. (1993) suggested that the Nile crocodile had a heterodont-type of dentition. In the maxilla, there were four canines, which preceded a group of five teeth in ascending order of size, and which were followed by a group of six similarly sized shorter and more squat teeth. The mandible had a similar arrangement.

Osborn (1978) showed that all replacement teeth located at specific regions on the dental lamina of reptiles constitute a "tooth family". The term "intra-family heterodonty" was used to describe tooth shape in early Triassic mammal-like reptiles such as Thrinaxodon and Diademodon, where the successional tooth replacement of the post-canine dentition developed with different morphological features. Also, Osborn and Crompton (1973) suggested that the steady decrease in the complex structure of replacement teeth at post-canine tooth family positions in Thrinaxodon and in mammals was due to the aging of the dental lamina. The authors hypothesized that if this process was accelerated, the dental lamina would lose its ability to initiate a new tooth and the change from polyphyodonty to diphyodonty would occur.

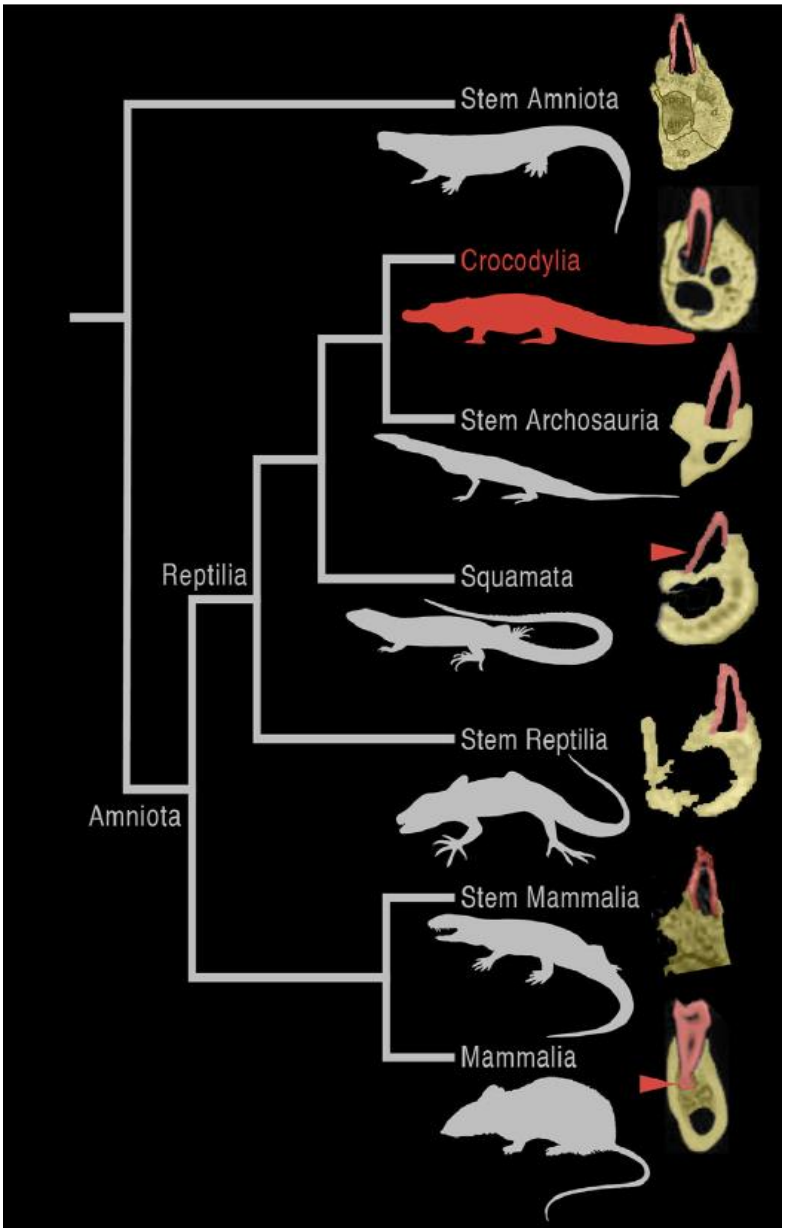


Figure 2. A diagram demonstrating the phylogeny of amniotes with CT-scan-based transverse sections of their dentition to indicate implantation. Crocodilians have single cusped teeth with an open root system similar to stem amniotes and primitive members of both the reptilian and mammalian lineage. In Squamates the medial wall of the tooth socket is absent and mammalian teeth have several functional modifications evident with a complex cuspal pattern and closed roots (arrowheads) (From Weeks *et al*., 2013).

1.4 Theories on the control of tooth replacement

Tooth replacement patterns, in several vertebrate groups, have been studied in detail and have described the arrangement of the initial first formed teeth and the replacement patterns of the successional teeth. However no consensus has emerged. Historically three main theories were proposed to describe the mechanisms and regulation of replacement teeth.

The first theory was the *Zahnreihen* theory. This was based on early work on pre- and post-hatching crocodiles (Röse, 1893a), chameleons (Röse, 1893b) and tuataras (Harrison, 1901) and showed that in a reptilian tooth replacement model (Fig. 3), tooth positions 1, 3, 5 are most similar in size and developmental stage to tooth positions 2, 4, 6 and so on (Fig. 3). In an effort to explain previous observations, Woerdeman (1921) deduced that teeth at various locations along the jaw were initiated autonomously. He referred to this wave-like replacement as *Zahnreihen* highlighting that a group of neighboring teeth from other tooth families were at a similar developmental stage (Fig 3). The *Zahnreihen* formed, ensured that if several teeth were lost or exfoliated simultaneously, other teeth would be almost fully developed to replace the lost ones. Edmund (1960) confirmed that continuous tooth replacement occurred on a regular schedule that varied between animals but was also not directly linked to tooth wear, damage or loss of teeth. Elaborating on the original *Zahnreihen* suggestion, Edmund (1960) hypothesised that an extrinsic 'wave stimulus' whose origin was the anterior ectomesenchyme and was transmitted posteriorly through the series of odontogenic structures, initiated tooth growth as it passed. The stimulus or signal would also decrease in intensity or concentration as it passed along the jaws, thus leaving some teeth at a less developed stage. Successive wave stimuli percolating through the jaws were responsible for successional generations of teeth. Edmund's (1960) hypothesis was founded on two main observations: i) the first tooth to develop was the most anterior tooth and ii) teeth are initiated in order along the length of a tooth row. The *Zahnreihen* theory was initially widely

accepted (Cooper *et al.*, 1970; Hopson, 1971; Ziegler, 1971). However, subsequent research on lizards (Osborn, 1970; Osborn, 1978) and alligators (Westergaard and Ferguson, 1987; Westergaard and Ferguson, 1990) disproved both assumptions. Recently Buchtová *et al.* (2012) in a study on odontogenesis in the chameleon, showed that tooth initiation in this reptilian model, was not in an ordered antero-postero direction, but occurred in areas of increased jaw growth.

In 1939, Butler proposed the "field theory". This theory proposed that heterodonty occurred due to variable concentrations of unknown morphogens. In this theory, each dental quadrant was divided into three subfields: incisors, canines and molariform. Each tooth therefore developed according to its location in the field. Teeth in the same field appear similar if they developed at the same distance from the field. The first human molar has the highest concentration of molar morphogen and therefore is the most stable tooth in the molar field (Butler, 1939). The third human molars are the most variable tooth in structure as they are located at a position where the morphogenetic field is tapering. The field model also hypothesised that mammalian multicusped teeth evolved as a result the union of many single reptilian tooth germs (Koussoulakou *et al*., 2009). Butler (1939) also emphasised that teeth were metameric structures evolved as part of an organ system as opposed to isolated units.

Due to the criticism of the *Zahnreihen* and field models, Osborn (1971) proposed the clonal theory. The timing and spacing of replacement teeth was regulated by proliferation of cells also know as an ectomesenchymal "clone" (Osborn, 1971). In a study on tooth replacement in the lizard, *Lacerta vivipara*, Osborn (1971) proposed that the odontogenic ectomesenchyme controlled tooth replacement and not an external source or morphogen. The tooth germ released a signal into the adjacent ectomesenchyme that inhibited the development of additional teeth within a specific radius around it, creating a zone of inhibition (Fig. 4). As soon as the tooth expressing the inhibitor had erupted, the adjacent dental laminae were no

longer inhibited and tooth development continued. This would cause an alternating pattern of development founded on the sequence of initiation (Osborn, 1971).

The candidate molecules that act as either inhibitors or activators of tooth initiation have not been conclusively identified. However, studies on the arrangement of other ectodermal organs, such as feathers have implicated *Wnt*, *Fgf*, *Eda* and the *Bmp* pathways (Chang *et al*., 2004; Drew *et al*., 2007; Lin *et al*., 2009). Understanding activators and inhibitors would be of benefit to the human dentition in developing treatments to re-stimulate odontogenesis. Zones of inhibition may prevent crowding and may decrease defects in the crown or roots of developing teeth due to crowding. Also, the delaying of tooth development in various teeth may be helpful until jaw size has increased to accommodate them.

Recent evidence on the role of sigalling molecules and expression of homeobox genes during odontogenesis indicate that field and clone models may not be mutually exclusive concepts but may complement each other (Mitsiadis and Smith, 2006). The odontogenic homeobox code shows that odontogenic patterning may occur due to the variable expression domains of homeobox genes in the ectomesenchyme (Mitsiadis and Smith, 2006). Cobourne and Mitsiadis (2006) described how an "inter-mixing" of genes expessed in the ectomesenchyme of the first branchial arch results in the development of different morphogenetic fields. Specific ectodermal signals induce specific domains of homeobox gene expression in the ectomesenchyme. The ectomesenchymal cells then attain a 'memory' of the domains and therefore house the information related to tooth shape (Cobourne and Mitsiadis, 2006).

Figure 3. Diagram illustrating the first seven tooth families in a hypothetical reptile jaw. Diagonal lines connect teeth in decreasing stages of development or *Zahnreihen* can be drawn across tooth families (blue) illustrating waves of replacement. The dashed lines represent a series in a tooth family. (From Handrigan and Richman, 2011).

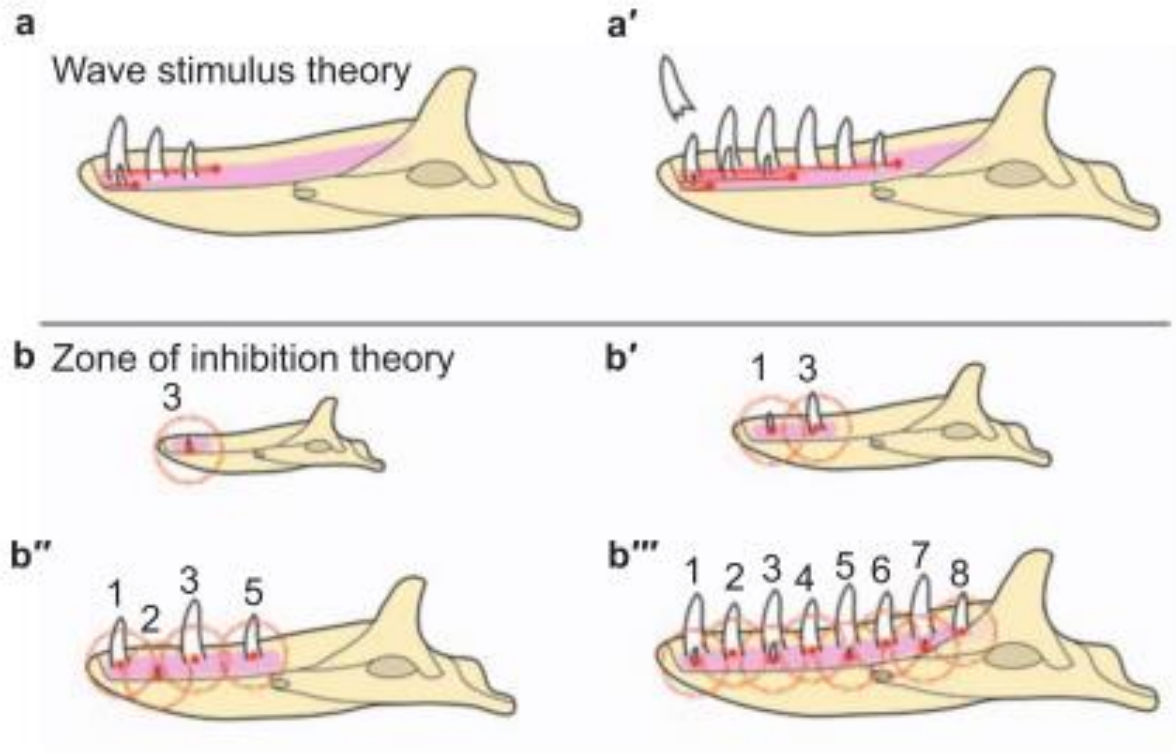


Figure 4. Schematic representation of the two main theories that explain the pattern of tooth replacement observed in reptiles. **a-a':** Edmund's wave theory involves an external chemical stimulus, which originates adjacent to the first tooth position and diffuses distally along the odontogenic anlage, inducing the initiation of tooth development as it passes. In **a,** the first wave stimulus has just passed tooth position 3, resulting in the development of the first three teeth. The second wave stimulus, after passing tooth position 1, stimulates a secondary wave of tooth development. In **a',** the first two wave stimuli have continued distally, and a third stimulus has just passed the first tooth position, where an erupted tooth has just been lost. **b-b':** Osborn's zone of inhibition theory. In **b**, the first tooth to develop (future tooth position 3) generates an unidentified signal preventing tooth development (red dot) in all directions. **b-b'''** indicate how growth of the jaw influences dental spacing as well as the distance between the Zone of Inhibition (ZOI). (From Handrigan and Richman, 2011).

1.5 The evolutionary loss of polyphyodonty in mammals

During evolution, a reduction in the number of teeth has occurred in the pathway from fish to reptiles to mammals (polyodonty to oligodonty) (Line, 2003; Salazar-Ciudad and Jernvall, 2004; Koussoulakou *et al*., 2009). In addition, the number of generations of teeth have also been reduced (polyphyodonty to di- and/or monophyodonty (Stock, 2007; Line, 2003; Koussoulakou *et al*., 2009). The latter authors suggest that "understanding the laws of polyphyodonty would support tooth regenerative efforts" which are important from a human clinical perspective. Also during evolution, there is a transition from the simple or homodont dentition of some osteichthyan fish to the complex morphological structure of mammalian teeth which exhibit a heterodont dentition. Variations in tooth number and shape are therefore significant in the diversification of mammals (Stock, 2007; Line, 2003; Koussoulakou *et al*., 2009).

Richman and Handrigan (2011) questioned the loss of the ability to replace teeth in mammals, since early mammal-like reptiles, like pelycosaurs, could undergo tooth replacement. The possible explanations are: Firstly, a successional lamina does not develop from the enamel organ of the permanent dentition in mammals, and secondly, that the successional lamina does develop, but remains vestigial (Richman and Handrigan, 2011). Studies on the rodent dentition have shown that the continuously erupting incisors and molars that do not develop from a successional dental lamina, may not provide adequate information to elucidate the loss of tooth replacement in mammals. The mouse molar and the human molar are homologous but are not considered successional teeth (Cobourne and Sharpe, 2010). In humans, the third molars originate from a continuation of the dental lamina that extends distally into the ectomesenchyme supporting the oral epithelium (Ooë, 1981). Permanent molars do not have deciduous predecessors. In the murine model, the molars do not originate from a successional lamina, but are derived directly from a molar placode. Supernumerary teeth, in mice, may occur due to the existence of vestigial tooth buds in the diastema (Peterkova,

1983; Klein *et al*., 2006; Ohazama *et al*., 2009). These supernumerary teeth, however, arise from disturbances of signaling pathways such as BMP (Bone Morphogenetic Proteins) and *Wnt* (Wingless Integrade -1) which are involved in tooth formation and development (Richman and Handrigan, 2011).

Although as early as 1912, Bolk dismissed the idea of continuous tooth replacement in diphyodont mammals, Ooë (1981) showed some morphological indication of a rudimentary successional lamina in bell stage permanent human incisors. The recurrent appearance of supernumerary teeth in the incisor and premolar positions of the human jaws, lends support to the presence of a vestigial successional lamina in humans (Gardiner, 1961; Solares and Romero, 2004). The clinical features supporting the fact that supernumerary teeth in humans are derived from a successional lamina associated with the permanent tooth are that they occur on the lingual aspect of the permanent tooth and are at a more immature developmental stage than the permanent tooth (Gardiner, 1961).

In certain reptiles species there is clear evidence of a vestigial dental lamina. Some dentate reptiles for example, the agamids such as the bearded dragon, do not show continuous tooth replacement (Cooper *et al*., 1970). Bearded dragons, in the pre-hatched stage, have a single fully formed set of teeth associated with a successional lamina on their lingual aspect. This lamina is transient and disappears after hatching. The degradation of the successional lamina is thought to be due to increased apoptosis. Also, an inhibition of expression of the *Wnt* pathway, which is prevalent in the successional lamina of other polyphyodont species, may have caused the cessation of its development (Handrigan and Richman, 2010b). Adontia, a condition occurring in turtles, birds and toothless whales, is considered a secondary phenomenon, due to the fact that initially the embryos have tooth germs that degenerate due to apoptotic mechanisms before birth (Koussoulakou *et al.*, 2009). It remains a consideration therefore that since a

reptile can have a vestigial successional lamina, the same may be possible in the mammalian situation.

Understanding the intricate mechanisms of polyphyodonty should assist scientists in enhancing regenerative efforts in relation to teeth. Also understanding why some teeth were lost during the evolution of vertebrates and recurred in an atavistic sense (Kurtén, 1963), therefore contradicting the "law" of irreversibility in evolution, should provide some answers on the spontaneous re-acquisition of lost properties. Tooth loss in specific regions is a common trend in vertebrate evolution. This occurred possibly due to a loss of a specific tooth related initiation signal (Koussoulakou *et al*., 2009) or a decrease in an inductive/inhibitory signal or a decline in the concentration of the necessary proteins. Decreased *PAX9* expression was shown to be related to the absence of canines and premolars in the mouse upper diastema (Turecková *et al*., 1995; Stock, 2007). Another common cause of loss of various teeth is the result of mutations of tooth-specific genes. Rodents lack lateral incisors, canines and premolars and sheep have lost their upper incisors and canines. A study on mutant mice phenotypes clearly demonstrated that specific mutations (eg *GLI2-/-, GLI3+/-)* caused phenotypes resembling the dentition of several ungulates with a lack of all the upper incisors (Stock, 2007; Line, 2003). An interesting phenomenon related to placental mammals is that their teeth disappeared during evolution in the opposite order to that of their eruption (Imai *et al.,* 1998).

1.6 The dental lamina – comparative studies

The dental lamina has been examined in several species of vertebrates and begins as an invagination of the oral epithelium, which extends into the ectomesenchyme (Järviven *et al*., 2009; Fraser and Smith, 2011). Several structural variations of the dental lamina are evident during its development in monophyodont, diphyodont and polyphyodont species (Buchtová *et al*., 2012), which are associated with its

ability to be involved in continuous tooth replacement. The monophyodont mouse, with only one generation of teeth, is the main animal model for odontogenic studies. The mouse dental lamina is short, the teeth develop on its oral surface and there is no indication of a successional lamina. In the pig, ferret and human, which are diphyodont species, the successional lamina is evident on the lingual aspect of the deciduous tooth, when it is in the late bell stage of tooth development (Järvinen *et al*., 2009; Stembirek *et al*., 2010). A second generation of teeth then develops from the successional lamina which then degrades by apoptosis and no further generations of teeth are formed (Moskow and Bloom, 1983; Stembirek *et al.*, 2010). In humans, the incomplete degradation of the dental lamina, has been associated with the formation of epithelial pearls, which may develop into oral cysts or tumours (Moskow and Bloom, 1983; Eversole, 1999).

The dental lamina, in several vertebrate classes is variable in location, position and with respect to primary and secondary tooth initiation (Fraser *et al*., 2006b). Tooth replacement in crocodilians as in mammals, amphibians and other reptiles (Edmund, 1960; Delgado *et al*., 2003; Ooë, 1981; Westergaard, 1988; Sire *et al*., 2002; Järvinen et al., 2009; Smith *et al.*, 2009b; Handrigan and Richman, 2010a) begins with the formation of a dental lamina. The reptilian dental lamina is a multi-layered epithelial ribbon-like structure that runs along the length of the upper and lower jaws (Westergaard and Ferguson, 1986). In alligators tooth germs originate from the lingual surface of the dental lamina, progress through several developmental stages to form a simple unicuspid tooth (Westergaard and Ferguson, 1986). Once the predecessor tooth bud develops, a successional lamina develops from the outer enamel epithelium on the lingual aspect of the tooth. This is the beginning of a new generation of teeth in the crocodilians, as well as in other reptiles and in diphyodont mammals (Järvinen *et al.*, 2008; Järvinen *et al.*, 2009; Ooë, 1981).

In cichlid fish, Vandervennet and Huysseune (2005) showed that the replacement teeth originate from the oral epithelium without the presence of a dental lamina. In the trout and salmon, replacement teeth develop from the outer enamel epithelium of the unerupted predecessor tooth (Fraser *et al*., 2006a; Huysseune and Witten, 2006; Smith *et al*., 2009a). In the zebrafish, the tooth replacement occurs from a successional dental lamina which is an outgrowth from the crypt epithelium of the functional predecessor (Huysseune, 2006). The successional lamina of the zebrafish can persist for a certain period of time without initiating a replacement tooth. This has highlighted the fact that successional dental lamina formation as well as replacement tooth formation are under different molecular controls (Huysseune, 2006). In the zebrafish, each tooth has a successional dental lamina which is distinctly different from the continuous and persisting dental lamina in reptilies (Fraser *et al.*, 2006b). In reptiles, the successional dental lamina originates from the outer enamel epithelium of the previous tooth (Handrigan and Richman, 2010b). This successional lamina elongates and generates a replacement tooth at its tip. The successional teeth develop in sequence from the successional dental lamina (Westergaard and Ferguson, 1987). This typical reptilian tooth replacement pattern was examined in snakes and geckos (Handrigan and Richman, 2010a; Handrigan and Richman, 2010b; Richman and Handrigan, 2011). Both *Wnt* activity as well as BMP expression are evident in the gecko dental lamina (Handrigan *et al*., 2010). Also in the gecko (*Eublepharis macularius*) putative stem cells have been described in the lingual aspect of dental lamina, but were not evident in the proliferative successional lamina (Handrigan *et al*., 2010). These stem cells express stem cell marker including Lgr5, DKK1 and Igfp5 (Handrigan *et al.*, 2010).

1.7 Tooth initiation and development in mammals

The major features of tooth morphogenesis appear to have been conserved throughout evolution. Teeth develop from the the surface oral epithelium and the underlying neural crest-derived ectomesenchyme. Interactions between the oral epithelium and the ectomesenchyme, are mediated by conserved signalling pathways that control epithelial morphogenesis and cellular differentiation (Tummers and Thesleff, 2009). Mammalian tooth development consists of a highly regulated series of reciprocal signals between the epithelium and the ectomesenchyme. In all vertebrates, the first sign of tooth development is the formation of an epithelial primary dental lamina. This lamina is a horseshoe shaped epithelial thickening that exhibits a localized band of gene expression delineating the future tooth forming areas (Fraser *et al*., 2006b). The structure and development of teeth is particularly well known in mammalian models such as mice and rats where they have long served as a model to study epithelial-mesenchymal interactions and have been used in *in vitro* developmental studies (Thesleff and Nieminen, 1996; Thesleff and Sharpe, 1997). The principal stages of tooth development in mammals are demonstrated in Figure 5. Before tooth development the dental lamina, develops from the oral epithelium. Development of individual teeth is then initiated in specific areas of the lamina giving rise to placodes (Fig. 5). During the bud stage, proliferation of the oral epithelium causes it to invaginate into the neural crest-derived ectomesenchyme which condenses around the epithelium to form a tooth bud. As development progresses, the epithelium extends further into the condensed ectomesenchyme, forming the cap stage of dental development. Following the cap stage is the bell stage (Fig. 5) where species-specific cusp patterns emerge. In unicusped teeth, a primary enamel knot, which is apparent at the cap stage, is associated with the development of the tip of the crown. In multi-cusped mammalian teeth, secondary enamel knots are evident in the position of future cusps of the developing teeth (Fig. 5). This stage is followed by growth and matrix secretion, where the ectomesenchymal cells differentiate into odontoblasts and secrete dentine and the adjacent cells of the

inner enamel epithelium differentiate into ameloblasts and secrete enamel matrix (Fig.5). The dental hard tissues, namely enamel, cementum and dentine have similar compositions in all mammals with enamel consisting of up to 98% hydroxyapatite.

The developing tooth ectomesenchyme acquires the ability of inducing tooth organogenesis even when combined with non-dental epithelium (Kollar and Baird, 1970; Kollar and Fisher, 1980; Mina and Kollar, 1987). Original tissue recombination experiments demonstrated that initially inductive signals for tooth initiation originate in the embryonic oral ectoderm. During the early bud stage of tooth development, the tooth inductive potential shifts from the ectoderm to the ectomesenchyme. Signals from the ectomesenchyme induce the formation of an epithelial primary enamel knot, which serves as a signalling centre and which in turn steers the tooth through the cap and bell stages of tooth development. Many signalling molecules, together with members of the BMP, FGF (Fibroblast Growth Factor), SHH (Sonic Hedgehog) and *Wnt* families are expressed during early tooth development (Jernvall and Thesleff, 2000; Tucker and Sharpe, 2004; Zhang *et al*., 2005).

The bone morphogenetic proteins are a group of molecules related to the transforming growth factor–β superfamily (Massagué *et al*., 2000). In mouse tooth development, *bmp4* transcripts have been shown in early development of the tooth, in the presumptive dental epithelium from the thickening of the oral epithelium to the early bud stage (Vainio *et al*., 1993; Winnier *et al*., 1995). *Bmp4* expression occurs for a short period both in the epithelial cells and in the underlying ectomesenchymal cells (Vainio *et al*., 1993). The expression of *bmp4* then shifts to the ectomesenchyme, which corresponds to the transfer of odontogenic potential from epithelium to the ectomesenchyme (Bennett *et al*., 1995; Keränen *et al*., 1998). Preodontoblasts and odontoblasts in the final stage of differentiation express *bmp4* in the mouse (Vainio *et al*., 1993).

The underlying signalling mechanisms of *bmp4* associated with tooth development have primarily been observed in studies in mouse embryos with limited comparative data from other vertebrates (Torres *et al*., 2007). Gene expression patterns have been correlated with morphological characteristics of dentition. Using the example of the molar pattern in mice and voles, Torres *et al*. (2007) suggested that minor changes in gene expression in the species resulted in the position of the secondary cusp. In the same way, variations in gene expression of *bmp4* may be indicative of morphological differences in the dentition.

Bmp4 signaling triggers expression of the Msx1 (Msx homeobox 1) transcription factor which occurs in the developing tooth ectomesenchyme (Tucker *et al*., 1998). In mice lacking Msx1 gene function, the expression of *bmp4* mRNA was down-regulated in the dental ectomesenchyme and tooth development was arrested in the bud stage (Satokata and Maas, 1994; Chen *et al*., 1996).

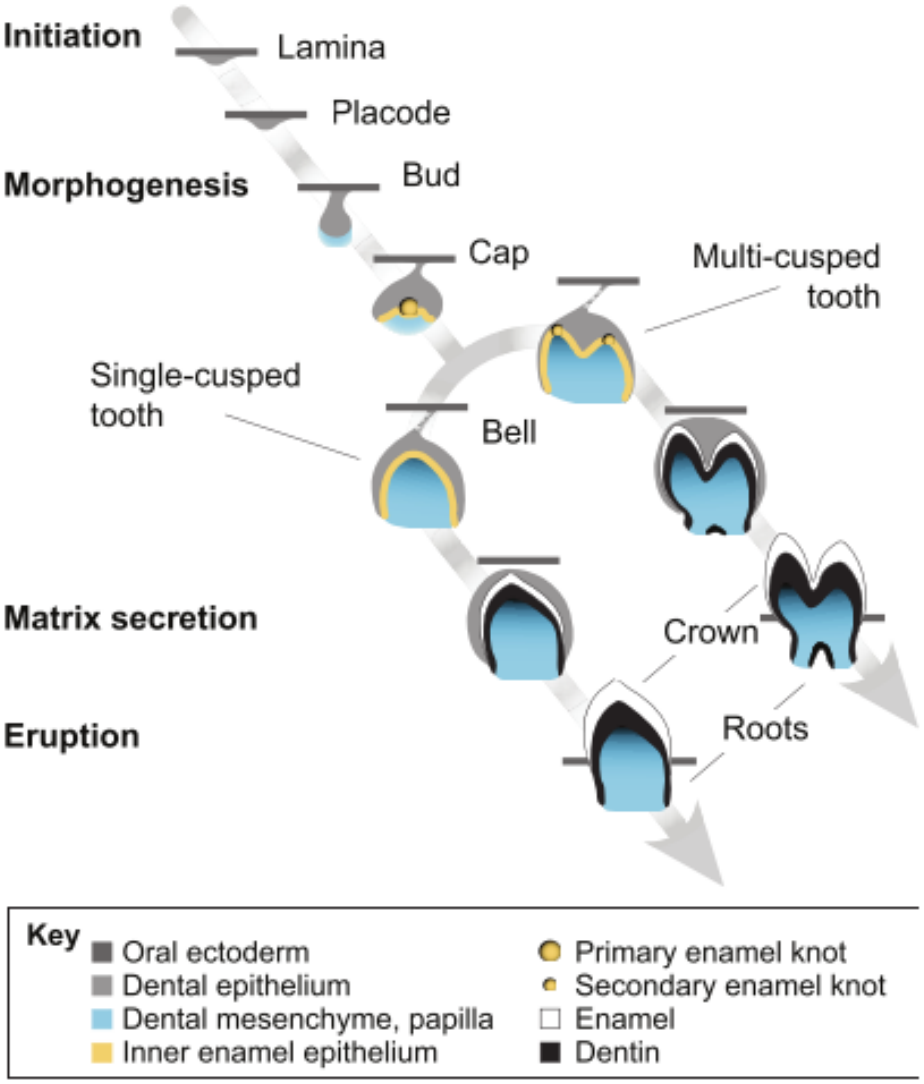


Figure 5. The main stages of mammalian tooth formation. The dental lamina is evident within the oral epithelium. Development of individual teeth, the dental placodes, is then initiated in specific regions of the lamina. At the bud stage, the oral epithelium invaginates into the ectomesenchyme. Then, the cap stage is evident which is followed by the bell stage, during which specific cusp patterns emerge: in a single-cusped tooth, a primary enamel knot, apparent at the cap stage, gives rise to the tip of the crown. In multicusped mammalian teeth, secondary enamel knots develop in the area of the future cusps. This stage is then followed by further growth and matrix secretion. Roots continue to develop during eruption. (From Jernvall and Thesleff, 2012).

In the mammalian situation, teeth develop sequentially in an antero-postero direction but the molecular mechanisms that regulate sequential tooth formation remain evasive. *Bmp4,* in mice lacking the Osr2 (odd skipped related 2) gene, formed supernumerary teeth on the lingual aspect of the molar teeth which was accompanied by lingual expression of *bmp4* in the ectomesenchyme.

Analysis of gene expression patterns may enhance knowledge on patterning of the dentition and could be used to re-examine the old theories of patterning which were regulated either by "morphogens" (field theory) from an external source (Butler, 1939) or "waves" (*Zahnreihen*) of initiation (Edmund, 1960). These theories of Butler (1939) and Edmund (1960) did not investigate morphogens such as retinoic acid and to date, the effects of a morphogen have not been tested. Osborn (1970) disputed the field theory and proposed that information related to patterning came from odontogenic ectomesenchyme which controlled both variation in shape as well as timing of initiation. This lends support to a model invoking autonomous regulation by differential gene expression in either the ectomesenchyme or the oral epithelium. Smith (2003) proposed that the ectomesenchyme was initially naive and that pattern information is carried by the epithelium until it is conveyed to the ectomesenchyme.

There is an interactive genetic cascade with crucial timing that occurs in the ectomesenchyme and oral epithelium in both primary and replacement tooth formation (Fraser *et al.,* 2004). In a study on the rainbow trout (*Oncorhynchus mykiss),* Fraser *et al.* (2004), found that the same three genes involved in tooth initiation in mice (*pitx2, bmp4* and *shh)* were also expressed in the marginal dentition of the trout. These results highlighted the conservation of selected genes common to odontogenesis in the two vertebrate groups. The need therefore, to analyse tooth related genes and their signalling pathways in several vertebrate species is necesssary for the understanding of the genetic basis of evolution related to tooth number, shape and replacement.

Cobourne and Mitsiadis (2006) have reviewed the subject of primacy of the oral epithelium and ectomesenchyme and expressed a restricted time window of opportunity to confer patterns of information to the ectomesenchyme. After this opportunity, particular domains of homeobox gene expression cause a cessation of tooth production (Cobourne and Mitsiadis, 2006). These developmental studies were undertaken using experimental tissue recombinations between mouse and toothless avian embryos. The epithelium in embryonic chicks could be activated when combined with mouse ectomesenchyme to produce chimeric tooth structures during a critical time window (Mitsiadis and Smith, 2006). *Pitx2* and *fgf8* were expressed in the epithelium, but *bmp4* and *shh* were not (Mitsiadis *et al*., 2006). The authors highlighted therefore that *bmp4* and *shh* may be activated by or depend on appropriate signals from the mouse ectomesenchyme. The signalling ability of the chick ectomesenchyme to induce odontogenesis has therefore been lost in the evolutionary process (Mitsiadis *et al.*, 2006).

Harris *et al.* (2006) in a study on the development of rudimentary teeth in the chick talpid mutant suggested that the development of simple conical structures, reminiscent of reptilian teeth, form in the area marked by expression of *pitx2* and *shh*. The authors showed that it was the displacement of the oral/aboral site in the epithelium that determined the novel expression of teeth, as it became associated with competent ectomesenchyme (Harris *et al*., 2006).

A linear band of oral epithelium expressing *shh* and *pitx2* has also been described in the trout (Fraser *et al.*, 2004) in the talpid chick (Harris *et al.*, 2006), in the snake (Buchtová *et al*., 2008; Vonk *et al*., 2008) and in the mouse (Mucchielli *et al.*, 1997; Mitsiadis *et al*., 1998; Cobourne *et al.*, 2004). This expression band is thought to be a site involved in the initiation for tooth development and is considered as the true dental lamina. It is suggested that this site of gene expression imitates the cascade of genes regulating time appearance and position

of all succeeding teeth. Replacement sets of teeth are regulated from these first teeth (Fraser *et al*., 2006b). Each taxon has a unique spatio-temporal pattern of initiation of teeth and this may also differ within species for each component of the dentition (Fraser *et al*., 2006b).

Pitx2 therefore is a gene involved in the initiation of both primary and secondary dentitions in the mammalian model (Mucchielli *et al*., 1997; Mitsiadis *et al*., 1998) suggesting it has an important function as a regulator in odontogenesis.

The same set of genes, in general, function in the mouse and man during the process of tooth development, with some minor differences (Koussoulakou *et al*., 2009). In tooth developmental studies, due to the availability of knock-out strains to determine gene function, the murine model is effective. However, in the vertebrate class the murine model is an exception in that it does not have replacement teeth. Since these animal models only form a single generation of teeth, they fail to answer questions related to the mechanisms of continuous tooth replacement or polyphyodonty which is exhibited by several non-mammalian vertebrates, the study of which may provide valuable insights into tooth replacement. Edmund (1969) emphasized that reptilian teeth are not replaced in "sets", but that many teeth in different parts of the mouth are undergoing replacement at any one time. It is unquestionable that this replacement process is not random but relies on growth parameters, space availability and a complex regulated gene pattern (Fraser *et al*., 2006b).

In order to gain insight into the molecular and developmental foundations of dental development in crocodilians, the present study wished to examine the expression of two genes, *bmp4* and *pitx2,* in odontogenesis. The ability to clone genes, demonstrate their spatio-temporal expression patterns, especially in non-mammalian vertebrates, should shed some light on the molecular control of tooth initiation. This data may highlight the differences between local control of tooth number and spacing and regional influences controlling morphodifferentiation.

In addition to *bmp4* and *pitx2*, a housekeeping gene, *gapdh*, was selected which is known to play key roles during vertebrate odontogenesis. Housekeeping genes are commonly considered to be constitutive genes necessary for preservation of basic cellular function and are expressed in almost all the cells of an organism (Zhu *et al.*, 2008). *Gapdh* has been commonly used as an internal control in mRNA protein expression studies. It is a highly versatile enzyme of -37kDal. The role of *gapdh* includes glycolysis, DNA repair, tRNA export, membrane fusion and transport, cytoskeletal dynamics and programmed cell death (Tristan *et al*., 2011). The multifunctional nature of *gapdh* is reflected by its localization in distinct domains of the cytoplasm, vesicles, mitochondria and the nucleus (Tristan *et al*., 2011; Lin *et al*., 2012). Selection of an optimal gene or genes for gene expression normalization required prior evaluation with a reference gene. It was therefore thought to be useful to identify *gapdh* in the crocodilians to allow accurate comparison of gene expression levels among different samples, by removing potential artefacts caused by sample preparation and detection procedures.

The aim of the present study was therefore to examine tooth replacement in the polyphyodont Nile crocodile (*Crocodylus niloticus*), firstly by examining the detailed histological structure and development of crocodile teeth and their replacements, and secondly, by investigating specific tooth initiating factors known to be associated with continuous tooth replacement.

The objectives of the present study were:

a) to examine in detail the histological and morphological development of the dentition in the Nile crocodile (*Crocodylus niloticus*), from the earliest teeth of embryos to those displaying eruption in a series of carefully staged specimens in order to compare those to the mammalian situation,

b) to determine whether all the erupted teeth are associated with a replacement tooth germ,

c) to examine whether there is a correlation between the state of functionality of a tooth and the state of its replacement and

d) to gain insight into the molecular and developmental foundations of tooth initiation in crocodiles by examining the expression of two genes, *bmp4* and *pitx2* known to play key roles during vertebrate odontogenesis and a housekeeping gene, *gapdh*. A comparison of these genes with the mammalian situation may shed some light on aspects of the origin of dental diversity in amniotes.

2 MATERIALS AND METHODS

2.1 Specimen collection

Crocodile (*Crocodylus niloticus*) eggs (Fig. 6) were collected from the

Figure 6. Photograph of the Nile crocodile eggs collected

Thaba Kwena Crocodile farm in the Limpopo Province, South Africa. Permits for collection and use were obtained from the Department of Environmental Affairs, Limpopo Province (Appendix A). Animal Clearance was obtained from the Animal Ethics Committee, University of the Witwatersrand (2006/79).

Only eggs laid by middle-aged females were used in this study as
these are consistent with low spontaneous malformation rates (Ferguson, 1984). The time of egg laying was estimated from the external appearance of the egg by examining the presence or absence of a mucous and/or an opaque spot on candling (Ferguson, 1984). The total incubation period for crocodiles is approximately 80 days. The incubation temperature of the eggs collected was 30^{o}C and the humidity was 80%. Six eggs were selected from different females

between days 14-31, and six eggs were selected from different females between days 32-65. In addition, six hatched specimens and six specimens at 10-11 days post-hatching were collected.

All specimens were weighed and the body weight was recorded in grams. As no staging method was found for crocodiles at the time of initiation of this study and collection of the eggs, the time intervals selected are according to Ferguson (1984) for alligators.

Embryos and hatchlings were carefully removed from the eggs and staged by morphological features in relation to the criteria of Ferguson (1984). Subsequently during the research period of this study an article was published by Peterka *et al*. (2010) in which prenatal characteristics of the Nile crocodile (*Crocodylus niloticus)* were described during embryonic/incubation days 9-70 only. The article described external morphology, head morphometry and wet body weight before fixation. The results indicated that body weight is the most representative parameter for crocodile staging. It was not possible to compare the data from the Peterka *et al*., (2010) study as the weights of their specimens were very different to the weights of specimens collected in this study at similar incubation times. Therefore the staging according to Ferguson (1984) was retained (even though the alligator has a shorter incubation time, 70 days, than the crocodile).

To remove the embryos from the eggs, the eggs were candled and a window was cut on one side of the opaque area on the upper surface of the eggs, to determine the position of the embryos. The shell and shell membrane were then cut and the embryos were removed. The embryos and hatchlings were then euthenased with an intraperitoneal injection of ketamine (100mg/ml, *Ketelar*). The external morphology was examined and recorded. They were then decapitated and prepared for histological analysis.

2.2 Terminology

Certain terms pertaining to tooth structure are frequently and conveniently used in the description of the tooth surface. In this study, the terms **labial** ('buccal' is usually applied to mammals and 'labial' is used for other vertebrates), **lingual, mesial** and **distal** will be used when discussing the **axes of teeth and/or the dental lamina.** The terms **anterior, posterior**, **medial** and **lateral** will be used when discussing **the relationship of the teeth to the maxilla and mandible**. The oral-aboral axis will refer to the third axis passing through the vertical axis of the dental lamina or teeth. The crown or top of the tooth is the occlusal surface, even in the case of the teeth of lower vertebrates and mammalian incisors, where they do not actually occlude. Clinically, the neck of a tooth in man is the narrow zone that borders against the gingiva, and is also the boundary between the enamel crown and the cementum that covers the dentine of the root. With regard to the external form, the term tooth neck applies only if the diameter of this area is smaller than that of the crown. This rarely occurs in reptiles, except possibly in the crocodilians. The root of a non-mammalian tooth is that part of the tooth that becomes attached to supporting tissues. The tip of the root of the tooth is referred to the as the apex.

2.3 Histological preparation

Crocodile heads were halved in the sagittal plane. The skin was removed. For histology, the left side of the upper and lower jaw was fixed in 10% buffered formal saline for 48 hours. The right side was fixed with 4% paraformaldehyde in PBS (pH 7.4) (Appendix B) overnight at 4^{o}C. Specimens were decalcified for 2-3 weeks in 5% EDTA. The decalcification process was determined by endpoint titration. Specimens were then dehydrated through a graded series of alcohols, cleared in xylene and embedded in paraffin wax. Variation in dental development

is negligible between the right and left sides of the same specimen, between specimens of similar stage and gender (Westergaard and Ferguson, 1986).
Serial histological sections of the left and right side of the crocodile heads were cut on a microtome at 7µm thickness in the sagittal (longitudinal) plane and mounted on glass slides. After routine hydration, sections from the left side were stained with Harris's iron haematoxylin for 1 minute, counterstained in eosin for 30 seconds, dehydrated through a graded series of alcohols and coverslipped with Entellan. Serial sections of the left side of the head were then examined with light microscopy.

2.4 Histological analysis

The sections were initially viewed with a Nikon SE compound microscope to determine the position and developmental stage of first generation and replacement teeth. Sections were photographed on a Zeiss Axsioscope microscope with a digital camera. In addition some sections were scanned with the OlyVIA (Olympus Virtual Slide System BX61VS microscope using u-plan SAPO lenses, Japan). The term 'tooth germ' represents all stages of developing (or resorbing) non-erupted teeth. Serial sections from the right side of each head were stored to be used in *in situ* hybridisation.

In order to determine whether the formation of the replacement tooth is linked to the functional stage of its predecessor, serial haematoxylin and eosin stained sections were screened initially to identify functional teeth using histological characteristics. The functional teeth, were assigned to one of the following categories as described by Huysseune (2006): attaching, young, mature or, in resorption.

Serial sections were also examined to determine the stage of development of the replacement tooth associated with a particular functional tooth. Replacement teeth

were assigned to one of the following stages: successional lamina, morphogenesis, cytodifferentiation (Huysseune, 2006).

All observations of teeth for each category of functional teeth, were pooled and percentages were calculated for the different stages of tooth germs associated with these teeth.

2.5 Micro CT-Scan analysis

Micro CT scan analysis of whole heads of a pre-hatched and post-hatched crocodile specimen (due to specimen availability, as all other specimens were serially sectioned) was carried out at the MIXRAD facility at NECSA (Pelindaba, North West Province, South Africa). The crocodile heads were individually fixed on the sample manipulator in the microfocus X-ray cabinet to ensure no movement of the sample during scanning. CT-Pro reconstruction software then transforms 2D projections into 3D images which can be directly imported in VGStudioMax visualization software that allows and enables the researcher to distinguish between different regions of the sample. The three dimensional nature of the CT scan allows one to confirm relationships seen in histological sectioning. The software for the analysis used was VG studio max version 2.2. The MIXRAD X-ray machine has a detector pixel size of 0.2mm.

The micro CT-Scan analysis involved the examination of a tooth structure and its replacement in a pre-hatched and post-hatched crocodilian specimen. A selected area was examined in lateral, coronal, sagittal and inferior views to examine the functional and replacement teeth from various perspectives.

2.6 *In situ* hybridisation

2.6.1 Specimen collection for *in situ* hybridisation

In addition to the other crocodile specimens collected, an additional one month-old crocodile (*Crocodylus niloticus*) hatchling was collected from Croc City Fourways, Gauteng for DNA analysis. This specimen was therefore collected seperately as specific specimen preparation is required for DNA preservation. Permits for collection and use were obtained from the Department of Environmental Affairs, Limpopo Province. Animal Clearance was obtained from the Animal Ethics Committee, University of the Witwatersrand (2006/79). The hatchling was euthanased with an intraperitoneal injection of ketamine (100mg/ml, Ketalar®). The maxilla and mandible were separated from the rest of the skull, cut into small fragments, weighed, snap frozen in liquid nitrogen, and stored at -70°C until use. Segments of the tail of the hatchling were also weighed, frozen in liquid nitrogen, and stored at -70^{o}C.

2.6.2 Extraction of genomic DNA

DNA was extracted from the crocodile hatchling tail using a basic salting out method modified from the Animal Genomics Laboratory, Liverpool, UK (Miller *et al*., 1988). Crocodile tail muscle tissue (25mg) was ground in liquid nitrogen with a pestle and mortar. The ground tissue was transferred to 1.5 ml micro-centrifuge tubes and the crocodile DNA extracted according to the protocol (Appendix B) (Miller *et al*., 1988). The samples were initially digested with 20mg/ml Proteinase K and incubated overnight at 50^{o}C in a waterbath. This was followed by the addition of 6M NaCl. The tubes were then centrifuged in a M-24A Boeco (Germany) centrifuge at full speed (12000g) and the supernatant was transferred to new 1.5 ml tubes. An equal volume of cold 100% ethanol was added to the tubes. Following agitation, the DNA precipitated out of solution.

Tubes were then centrifuged again at full speed (12000g). A DNA pellet was formed after centrifugation and the supernatant was discarded. The DNA pellet was washed with 70% ethanol to remove salt, following which the ethanol was removed manually. The samples were then left to air dry and the DNA was re-suspended in water and stored at -20^{o}C. The concentration of each DNA sample was determined by spectrophotometry using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). The purity of the samples was assessed by calculating the OD260/OD280 ratio.

2.6.3 Primer Design

Primers were designed using the web-based design programme Primerquest (http://eu.idtdna.com/PrimerQuest/Home/Index). Orthologs used were: mouse, rat, human, frog and a partial alligator sequence (available for *bmp4* only). Primers (Tables 1-3) were designed using *Gallus gallus.* Glyceraldehyde–3–phosphate dehydrogenase (*gapdh*) was amplified using primers designed off the human genome. The expression of the housekeeping gene, *gapdh*, was employed as an internal control to determine the of the technique.

Table 1. Primers for *Bmp4*

Forward Primer

Primer Sequence:	TAATCTCAGCAGCATCCCAGAGGA		
Primer Start Position:	3	Primer Length:	24
Primer T_M:	59.2 ºC	Primer Self Any:	3.0
Primer GC %:	50.0 %	Primer Self End:	3.0
Primer End Stability:	5.72	Primer Penalty:	0.82

Reverse Primer

Primer Sequence:	TGTCCGTTTCGTCCAGGTAAAGCA		
Primer Start Position:	742	Primer Length:	24
Primer T_M:	60.3 ºC	Primer Self Any:	3.0
Primer GC %:	50.0 %	Primer Self End:	2.0
Primer End Stability:	5.97	Primer Penalty:	0.28

Primer Pair/Product

Primer Pair Penalty:	1.10	Primer Pair Comp Any:	3.0
Primer Product Size:	740	Primer Pair Comp End:	0.0

Table 2. Primers for *pitx2*

Forward Primer

Primer Sequence:	CAGCGGCAGATACGAAGGAAAGAT		
Primer Start Position:	87	Primer Length:	24
Primer T_M:	58.8 ºC	Primer Self Any:	3.0
Primer GC %:	50.0 %	Primer Self End:	2.0
Primer End Stability:	4.46	Primer Penalty:	1.24

Reverse Primer

Primer Sequence:	CTGGTTGGATGTTGCTGGCAAAGA		
Primer Start Position:	793	Primer Length:	24
Primer T_M:	59.9 ºC	Primer Self Any:	6.0
Primer GC %:	50.0 %	Primer Self End:	2.0
Primer End Stability:	4.58	Primer Penalty:	0.11

Primer Pair/Product

Primer Pair Penalty:	1.36	Primer Pair Comp Any:	4.0
Primer Product Size:	707	Primer Pair Comp End:	1.0

Table 3. Primers for *gapdh*

Forward Primer

Primer Sequence:	TGGATGTGCACACGGTTTCAGACA		
Primer Start Position:	5	Primer Length:	24
Primer T_M:	60.7 ºC	Primer Self Any:	8.0
Primer GC %:	50.0 %	Primer Self End:	3.0
Primer End Stability:	5.47	Primer Penalty:	0.74

Reverse Primer

Primer Sequence:	ATTCTGCACGCTCGCGTATCCAAA		
Primer Start Position:	762	Primer Length:	24
Primer T_M:	60.9 ºC	Primer Self Any:	6.0
Primer GC %:	50.0 %	Primer Self End:	2.0
Primer End Stability:	5.29	Primer Penalty:	0.94

Primer Pair/Product

Primer Pair Penalty:	1.68	Primer Pair Comp Any:	5.0
Primer Product Size:	758	Primer Pair Comp End:	3.0

Purified DNA was used as a template for the amplification of a portion of the following crocodile genes:
Glyceraldehyde– 3 – phosphate dehydrogenase (*gapdh*)
Bone morhogenetic protein 4 (*bmp4*) and
Paired-like homeodomain 2 (*pitx2*)

2.6.4 PCR Amplification

Purified crocodile DNA (2.5 µl) was added to a 25 µl mixture containing 0,5 U of BIOTAQ™ DNA polymerase (Bioline Ltd, Luckenwalde, Germany), 50µM of each deoxynucleotide triphosphate (dNTP), 500 nM of each primer (Tables 1-3), 50 mM KCl, 10 mM Tris-HCl (pH 8.8) and 1,5 mM $MgCl_2$.

Amplification was performed in a Mini Personal Thermal Cycler thermocycler (BIORAD) as follows: For ***gapdh*****:** denaturation at 94°C held for 60s, annealing at 52°C held for 30s and elongation at 72°C held for 90s, for a total of 30 cycles followed by a final extension at 72°C held for 10 min. For ***bmp4*** **and** ***pitx2***: denaturation at 94°C held for 60s, annealing at 58°C held for 30s and elongation at 72°C held for 90s for a total of 30 cycles, followed by a final extension at 72°C held for 10 min.

For each PCR experiment, negative controls were included by replacing the crocodile DNA template with water, to check for cross over contamination.

2.6.5 Agarose gel electrophoresis

Amplified products were resolved on 1% agarose gel (Appendix C). Once completely solidified, the agarose gel was immersed fully in an electrophoresis tank containing 1xTris Borate EDTA (TBE) buffer (Appendix C). The samples were prepared by adding 0,4 volumes 6x Blue/Orange Loading Dye (15% Ficoll®

400, 0,03% bromophenol blue, 0,03% xylene cyanol FF, 0,4% orange G, 10mM Tris-HCl)(pH 7.5) and 50 mM EDTA) (Promega) and mixed thoroughly. A sample volume of 1µl was loaded on to the agarose gel and the gel electrophoresed at 8 volts/cm until the bromophenol blue tracking dye front had migrated ~75% of the length of the gel. An image of the gel was recorded using the Gel Doc XR Imaging System (Bio-Rad).

2.6.6 Cloning of PCR products into PGEM cloning vector

PCR amplicons were isolated by resolving them on an ethidium bromide agarose gel. The fragments of interest were excised and extracted using the Zymogel DNA Recovery Kit (Zymo, Inqaba, Biotech) according to the manufacturer's instructions. The extracted DNA was eluted in 15 µl best quality water (Sabax) and 1-3µl of this gel purified product was used for cloning.

Ligation reactions were carried out with the pGEM-T Easy Vector System Kit (Promega,USA) according to the manufacturer's instructions. The insert:vector molar ratio can have a significant effect on the outcome of a ligation and subsequent transformation step. The vector molar ratios used for *gapdh, pitx2* and *bmp4* were 1:1 and 3:1 to insure maximum ligation efficiency. The ligations were incubated overnight at 4^{o}C.

2.6.7 Transformation reactions

The transformation protocol involved adding 2µl of plasmid DNA from the ligation reaction to 50 µl of chemically competent DH5-α cells (Appendix C) on ice. After an incubation period of 30 minutes, the cells were heat shocked in a water bath (42^{o}C) for 30 seconds. The cells were then returned to ice for 2 minutes. SOC medium (950µl) (Appendix C) at room temperature was added to the cells and the tubes were incubated in an Orbital Shaker Incubator YIH DER

(12000g) at 37^{o}C for an hour. The cells were centrifuged briefly, 750µl of supernatant was removed and the cells resuspended in the remaining medium.

Three different volumes of transformation reaction (50µl, 100µl and 150µl) were spread on to prewarmed Luria-Bertani (LB) agar plates (Appendix C) containing 50µ/ml ampicillin and incubated overnight at 37^{o}C.

2.6.8 Mini-preparation of plasmid DNA

Individual colonies from specific clones carrying plasmids for *bmp4*, *gapdh* and *pitx2*, were picked out with a sterile toothpick and used to inoculate 15 ml tubes containing 5ml of liquid LB and ampicillin. The 50 ml tubes were cultured at 37^{o}C in a Orbital Shaker Incubator (YIH DER) at 250rpm overnight. After incubation, the growth of bacterial colonies in the tube was characterised by a cloudy haze, as opposed to the clear control tubes which had not been inoculated, but had been subjected to the same culture conditions.

2.6.9 Plasmid DNA purification

DNA extracts were purified using a Qiagen Q1amp DNA mini kit (QIAGEN) according to the manufacturer's instructions. The samples were incubated with QIAGEN Proteinase K (600 mAU/ml) and a lysis buffer overnight at 56 °C on a rocking platform to ensure complete lysis. The following day, the lysed sample was transferred to a QIAamp® spin column, washed twice and eluted in 50 µl best quality water (Sabax, Adcock Ingram Critical Care Ltd., Johannesburg, South Africa). The concentration of each DNA sample was determined by spectrophotometry using a NanoDrop ND-1000 Spectrophotometer.

2.6.10 Restriction mapping of purified plasmid

A 40 µl aliquot of extracted plasmid DNA was restricted at 37°C for 3 hours with 20µl PstI (Thermo Scientific) in 1x Buffer PstI, comprising 50 mM NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$ and 0.1mg/ml BSA pH7.5. A 45 µl aliquot of extracted plasmid DNA was restricted at 37^{o}C for 2 hours with 15µl SacII (Thermo Scientific) in 1x Buffer SacII, comprising of 10mM Tris-HCl, 10mM $MgCl_2$ and 0.1mg/ml BSA pH7.5.

The restricted products were resolved on a 1% agarose gel together with a 1 kb DNA ladder (Promega) to determine whether or not the plasmids contained the fragment of interest

2.6.11 Sequencing of amplified product

Confirmed positive clones were sequenced by Inqaba Biotechnical Industries Ltd (Pretoria, South Africa) using an ABI 3500XL Genetic Analyser. Chromatograms of the sequences generated were inspected with Chromas software (version 1.45; Technelysium Pty. Ltd., Helensvale, Queensland, Australia).

Sequences were subjected to a standard nucleotide-nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/Blast.cgi) to ensure that the amplicons belonged to the family Reptilia, rather than being the result of a non-specific amplification of genomic DNA.

2.6.12 Midi-preparation of plasmid DNA

To obtain sufficient quantity of plasmid DNA to use in the generation of riboprobes for the *in situ* hybridisation a larger scale plasmid DNA preparation method was adopted. Plasmid DNA was isolated from volume (must be a larger

starting volume) the bacterial cultures using the Genejet Promega midipreperation kit according to the manufacturer's instructions. The final elution volume was 600µl. The concentration of each DNA sample was determined by spectrophotometry using a NanoDrop ND-1000 Spectrophotometer.

2.6.13 Purification of Plasmid DNA using Phenol Chloroform Extraction

This protocol is a modification of that described by Sykes (1983). One µg DNA midiprep samples (20µl) for crocodile *gapdh, bmp4* and *pitx2* were initially made up to 100µl with water. One hundred and twenty five microlitres of tris-saturated phenol, (pH7) (SIGMA, Sigma-Aldrich Inc., St Louis, Mo.) and 125µl chloroform: iso-amyl alcohol (26:1) (Appendix C) was added to each sample. The tubes were capped and the contents mixed thoroughly by inversion. The samples were centrifuged at 14000 x g for 5 minutes at room temperature to separate the phases. The aqueous (upper) phase, which contained the DNA, was transferred to a clean 1.5ml tube and 250µl of chloroform: iso-amyl alcohol (26:1) was added to each tube. The samples were then centrifuged again at 14000 x g for 5 minutes and the aqueous phase was once again removed and transferred to a new 1.5ml tube. Twenty five microlitres of 3M sodium acetate and 200µl ice cold 70% ethanol was added to each tube. The samples were then stored at -20^{o}C overnight to precipitate the DNA. The following day, the precipitate of DNA was collected by centrifugation at maximum speed (14000 x g) for 15 min at 4^{o}C. The supernatant was carefully decanted and discarded and the DNA pellet resuspended in 20µl of sterile water and the DNA was stored at –70^{o}C in suitable aliquots. One microlitre of each DNA sample was resolved on a gel with a ladder of known concentration to estimate the concentration.

2.6.14 Digoxigenin (Dig) labelling of DNA probes

Dig labelling was carried out using the DIG RNA Labelling Kit (SP6/T7) (Roche Applied Science, Mannheim, Germany). The DIG-labelled RNAs were transcribed in both the sense and the antisense orientations using SP6 with SacII and T7 with PstI RNA polymerases. Labelling was performed following the manufacturer's directions.

The DIG-labelled probe was purified by passing it through Micro Bio-Spin 30 Chromatography Columns (BIO-RAD Laboratories, Inc), as per the manufacturers instructions, to remove any unincorporated nucleotides.

2.6.15 Determining the efficiency of DIG-labelling

The yield of DIG-labelling (spot test) was estimated in the following way:
A dilution series (diluted in RNA dilution buffer – Appendix C) of the labelling reaction, in this case DIG-labelled crocodile *gapdh*, *bmp4* and *pitx2*, and dilutions of appropriate standards (DIG labelled Control RNA- DIG Labelling Kit, Roche), were spotted on to an appropriately-labelled nucleic acid blotting membrane (Hybond n+, Amersham). The location of each probe and control spot was marked with a pencil.

The membrane was then processed in a short detection procedure. It was initially baked at 80°C for two hours. After a washing step in maleic acid buffer (pH 7.5) (Appendix C), the membrane was incubated for 30 minutes in a 1% blocking solution (Roche). It was incubated in 1:5000 Anti-DIG alkaline phosphatase antibody solution (Roche) for 30 minutes. After a further two washes in maleic acid buffer (pH7.5), the membrane was equilibrated in detection buffer (Roche) for 5 minutes. The membrane was then immersed in nitro-blue tetrazolium and 5-bromo-4-chloro3indolyphosphate (NBT/BCIP) (Roche) solution, in the dark, to allow colour development. When the spots appeared in sufficient intensity, the

reaction was stopped by washing the membrane with TE buffer (Appendix C) for five minutes. The spot intensities of the control and experimental dilutions were used to estimate the concentration of the experimental probe (Fig. 7).

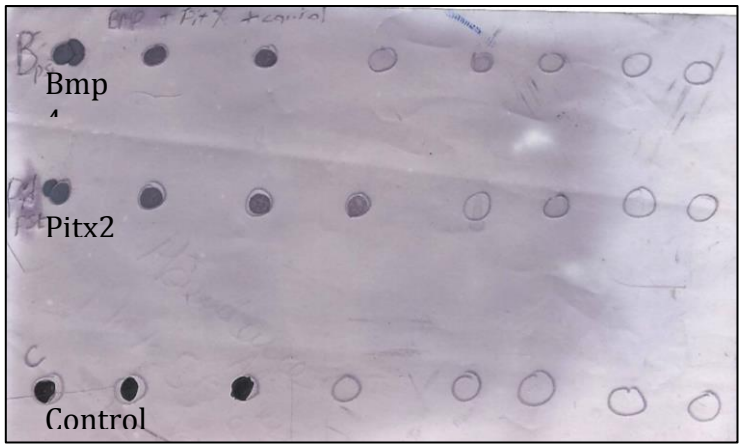


Figure 7. Spot test for *bmp4*, *pitx2* and a control RNA sample

2.7 *In situ* hybridisation technique

Serial sections of the maxilla and the mandible from all age groups were initially dewaxed with xylene, rehydrated through a series of graded alcohols and rinsed in DEPC-treated water (Appendix B).

2.7.1 Pre-hybridisation

Rehydrated sections were washed twice in DEPC-treated PBS for 5 minutes, followed by PBS containing 0.3% Triton-X 100 (Appendix C) for 10 minutes. Sections were then permeabilised with ready-to-use proteinase K (Dako, California USA) for 15 minutes at 37°C and then post- fixed for 5 minutes in 4% paraformaldehyde in PBS at 4°C. After two washes in PBS, sections were acetylated by 2x 5 minute washes in 0.1M triethanolamine (TEA) buffer (pH 8.0) (Appendix C) with 0.25% acetic anhydride (Sigma) to reduce non-specific signal. Due to its instability in solution, acetic anhydride was added to each wash

immediately prior to use. Slides were then rinsed in PBS, and pre-hybridised for 10 minutes in pre-hybridisation buffer (Appendix C) at 37°C.

Prior to hybridisation, the pre-hybridisation buffer was drained from the sections. Sections were then covered with 50 µl of the relevant DIG-labelled RNA probe diluted in fresh hybridisation buffer (Appendix C) at a concentration determined by optimisation: *gapdh* (5ng/µl) , *bmp4* and *pitx2* (10ng/µl) respectively. Prior to application, probes were denatured at 95°C for 5 minutes and then cooled on ice for 5 minutes. After the application of the probes, sections were covered with coverslips and incubated for 12-16 h (overnight) at the optimised annealing temperatures: *gapdh* (44^{o}C), *bmp4* and *pitx2* (56^{o}C) respectively.

2.7.2 Post hybridisation washes and RNAse treatment

Following hybridisation, the coverslips were gently removed by washing the slides in 2x saline sodium citrate buffer (SSC) (Appendix B) at room temperature. This was followed by two 15 minute washing steps in 2xSSC and 1xSSC buffer respectively. In order to digest any single-stranded (unbound) RNA probe, sections were incubated for 30 minutes at 37^{o}C in NTE buffer (Appendix B) containing 20µg/ml RNase A (Sigma). The sections were then washed in 0.1xS**SC** buffer for 30 minutes.

2.7.3 Immunological signal detection

Sections were covered with blocking solution (Sigma) diluted in buffer 1 (Appendix C) for 30 minutes. The blocking solution was decanted and sections were then incubated with a 1:200 dilution of sheep anti-DIG-alkaline phosphatase (Roche) in buffer 2 (Appendix C) at 37°C in a humid chamber, for 2 hours. Sections were then washed consecutively in buffer 1 and 2 for 10 minutes each.

2.7.4 NBT/BCIP colour detection

The colour substrate NBT/BCIP (Roche) was prepared at 1:50 dilution in buffer 2. Sections were covered with 200µl of the colour solution and incubated in a humid chamber for 24 hours in the dark. When the staining was optimal the colour reaction was stopped by incubating the sections in buffer 3 (Appendix C). Sections were then washed in tap water and mounted with an aqueous mounting solution.

2.7.5 Controls for *in situ* hybridisation

The following controls were used to determine the specificity of the *in situ* hybridisation:

a) The tissue was hybridised with both sense and antisense probes in parallel. The antisense probe detects the target mRNA and the sense control probe shows non-specific probe binding due to chemical properties.

b) The sheep anti-DIG- alkaline phosphatase antibody was replaced with buffer and

c) the anti-sense probe was replaced with buffer.

Sections from all age groups of the crocodile specimens were selected and *in situ* hybridization was performed for *gapdh*, *bmp4* and *pitx2*. The results were analyzed by light microscopy for localisation analysis with a Zeiss Axsioscope microscope with a digital camera.

2.8 Phylogenetic analysis

The phylogenetic relationship of three *Crocodylus niloticus* DNA sequences and their translated protein sequences were investigated:

(a) *Crocodylus niloticus* paired-like homeodomain transcription factor (p*itx2)* gene, partial cds., GenBank acc. no. JX978720

(b) *Crocodylus niloticus* bone morphogenetic protein 4 (*bmp4*) gene, partial cds., GenBank acc. no. JX978721

(c) *Crocodylus niloticus* glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene, partial cds., GenBank acc. no. JX978722

2.8.1 Sequence data and alignment

The crocodile sequences were manually aligned using the related sequences as determined by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and sequences of the same gene as published on GenBank. Gaps were introduced by keeping codons together. (Table. 4). The DNA sequences were translated into protein sequences. The final alignments had the following sizes:

(a) *pitx2* gene: 21 taxa, DNA: 516 nucleotides, protein: 172 amino acids.

(2) *bmp4* gene: 23 taxa, DNA: 687 nucleotides, protein: 229 amino acids.

(3) *gapdh* gene: 23 taxa, DNA: 585 nucleotides, protein: 195 amino acids.

Table 4. Taxon sampling for DNA sequence analysis

Taxonomic Group	**Scientific name (Common name)**	**Acc.no. *ptx2*-gene**	**Acc.no. *bmp4*-gene**	**Acc.no. *gapdh*-gene**
Bone fishes - Actinopterygii	*Cyprinus carpio* (common carp)	EF051103	JX446582	AJ870982
	Danio rerio (zebrafish)	AF156906	D49972	BC095386
	Salmo salar (Atlantic salmon)	NM_001123680	NM_001139844	BT050045
Coelacanth	*Latimeria chalumnae* (coelacanth)	XM_005997321	XM_005986478	XM_006004701
Amphibia	*Xenopus laevis* (African clawed frog)	NM_001088287 NM_001088288	BC169549	BC043972
Reptilia	*Alligator mississippiensis* (American alligator)	XM_006268979	XM_006278429	XM_006258364
	Alligator sinensis (Chinese alligator)	XM_006025541	XM_006037481	XM_006030320
	Chrysemys picta bellii (western painted turtle)	XM_005287930	XM_005295477	XM_005291304
	Crocodylus niloticus (Nile crocodile)	JX978720	JX978721	JX978722
	Pelodiscus sinensis (Chinese soft-shelled turtle)	XM_006119272	AB181138	NM_001286927 XM_006124190
Aves	*Anas platyrhynchos* (mallard)	XM_005018647	EF540750	XM_005016745
	Columba livia (rock pigeon)	XM_005515213	XM_005510285	NM_001282835 XM_005506273
	Falco peregrinus (peregrine falcon)	XM_005238072	XM_005243001	XM_005235383
	Gallus gallus (chicken)	AF077092	NM_205237 XR_140157	NM_204305
	Geospiza fortis (medium ground-finch)	XM_005418157	XM_005417969	XM_005420667
	Melopsittacus undulatus (budgerigar)	XM_005148621	XM_005152336	XM_005146692
Mammalia	*Bos taurus*	XM_005207601	GU233961	U85042

	(cattle)			
	Didelphis albiventris (white-eared opossum)	-	DQ192517	-
	Didelphis virginiana (North American opossum)	-	-	DQ403044
	Homo sapiens (human)	NM_153427	BC020546	J02642
	Macaca fascicularis (crab-eating macaque)		XM_005561288	XM_005569913
	Monodelphis domestica (gray short-tailed opossum)		XM_001362554	DQ403045
	Mus musculus (house mouse)		X56848	M32599
	Sarcophilus harrisii (Tasmanian devil)		XM_003758831	XM_003767659

2.8.2 Nucleotide divergence

Nucleotide divergence values were calculated by counting the number of differences between two sequences. This value was divided by the length of the alignment. Unresolved nucleotides were not counted as a difference. Missing nucleotides (gaps) were counted as a difference.

2.8.3 Phylogenetic trees

For each DNA alignment the best-fit model of nucleotide substitution was found using jModelTest Ver. 2.1.4 (Posada and Crandall, 1998; Darriba *et al*., 2012; Guindon & Gascuel, 2003). Neighbor-Joining (NJ), weighted Maximum Parsimony (MP) and Maximum-Likelihood (ML) analyses were carried out using PAUP Ver. 4 beta 10 (Swofford, 2003). For all analyses a bootstrap analysis using 1000 pseudo-replicates were used. The resulting optimized ML-trees were demonstrated as phylograms. For final tree visualization and combination of consensus trees the software TreeMe was used. The phylograms showed an optimized maximum-likelihood tree and the support values on the branches are the bootstrap values for a NJ-, a weighted MP-analysis and a ML-analysis. The value triplets on the branches are the support values for the cluster right of that branch as calculated by a NJ/MP/ML-analyses using a bootstrap analysis with 1000 pseudo-replicates each. The thickness of the branches leading to clusters is proportional to the number of methods supporting that cluster.

2.9 Limitations of cloning and *in situ* hybridisation

It was difficult to clone cDNA fragments of *Crocodylus niloticus* as there were no commercially available primers and therefore primer design was based on reptilian and other sequences available on GenBank. Even after detailed sequence

alignment, several sets of new primers had to be redesigned, as cloning was initially unsuccessful. Primer design, involving reptilian sequences, requires a high CG (Cytosine, Guanine) content and this affects annealing and hybridisation temperatures. Crocodilian probes were particularly sensitive to temperature fluctuations, and were successful only at specific and not at a range of temperatures.

3 RESULTS

3.1 Micro CT scan analysis

In the pre- and post-hatched crocodile specimens (Figs. 8, 9), distinct interdigitation of the teeth was evident. In both the maxilla and the mandible the most anterior teeth were simple and conical (homodont) in shape and curved slightly posteriorly. The posterior teeth were shorter and stouter, with a distinct constriction separating the apex and the base of the tooth. The fourth tooth of the mandible overlapped the maxilla. There appeared to be a gradual transition between the anterior taller conical teeth and the shorter stouter posterior teeth. In the lateral views of the hatched and post-hatched specimens (Figs. 8, 9), there were a series of four anterior 'incisor' type teeth, followed in the upper jaw by five teeth of ascending size. In the lower jaw, the 'incisor' type teeth were followed by five dagger shaped teeth. Teeth with coronal constrictions followed distally in both jaws.

In the coronal views of both the maxilla and the mandible (Figs. 10, 11) the thecodont nature of the crocodile teeth is evident as the teeth are situated in bony alveolar sockets. These sockets house both functional and replacement teeth (Fig. 10). On both the right and left side of the maxilla a functional tooth with a replacement tooth was observed on the lingual aspect. Posteriorly, the teeth of the maxilla, appeared to be at a different developmental stage to the tooth structures in the mandible. The mandibular teeth were developmentally immature, which was evident due to their smaller size (Fig. 10). Occasionally the socket appeared to have immature teeth or perhaps remnants of functional teeth. This was apparent due to the decreased density of the enamel in the smaller teeth (Fig. 10). In both pre and post-hatched specimens, the large open root structure of crocodilian teeth was verified. The coronal constriction seperating the apical and basal aspect of the tooth was also evident.

The simple conical crocodilian teeth in the ventral views of both the pre- and post-hatched specimens (Figs. 12, 13) appeared as a rounded structures in transverse section, highlighting the tubular and hollow nature of crocodilian teeth. There was also a distinct variation in the intensity of the enamel in these specimens where in the same tooth structures of similar size the enamel appeared thicker and more prominent. This was indicative of the various developmental stages with variable mineralisation.

Tooth replacement was evident in the sagittal views of both the pre- and post-hatched specimens (Figs. 14, 15) with a replacement tooth germ developing on the lingual aspect and towards the base of the functional tooth.

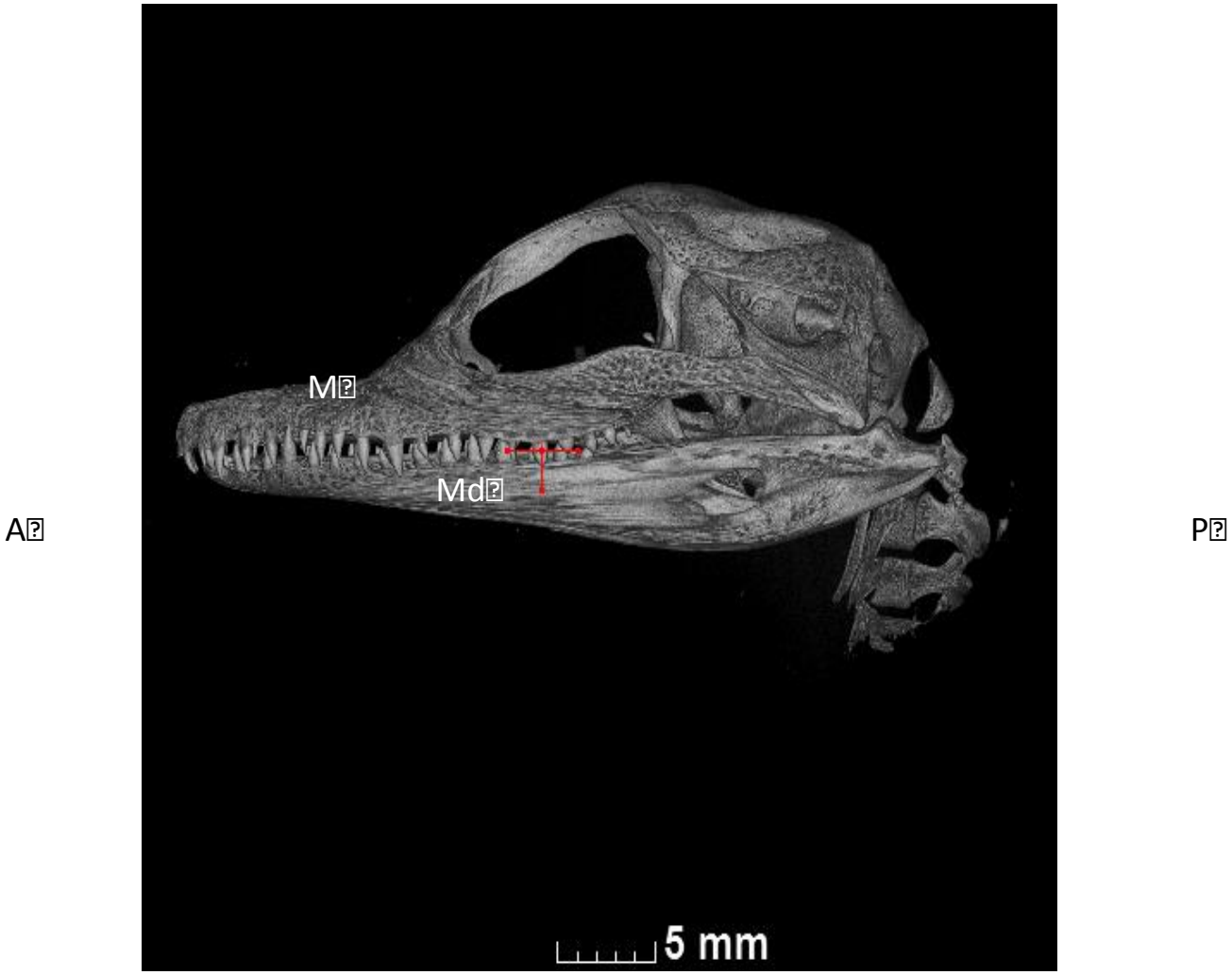


Figure 8. Micro CT scan section showing the lateral view of a **pre-hatched** crocodile specimen. The red T indicates the level of the section used in Figures 10, 12 and 14. M-Maxilla, Md -mandible.

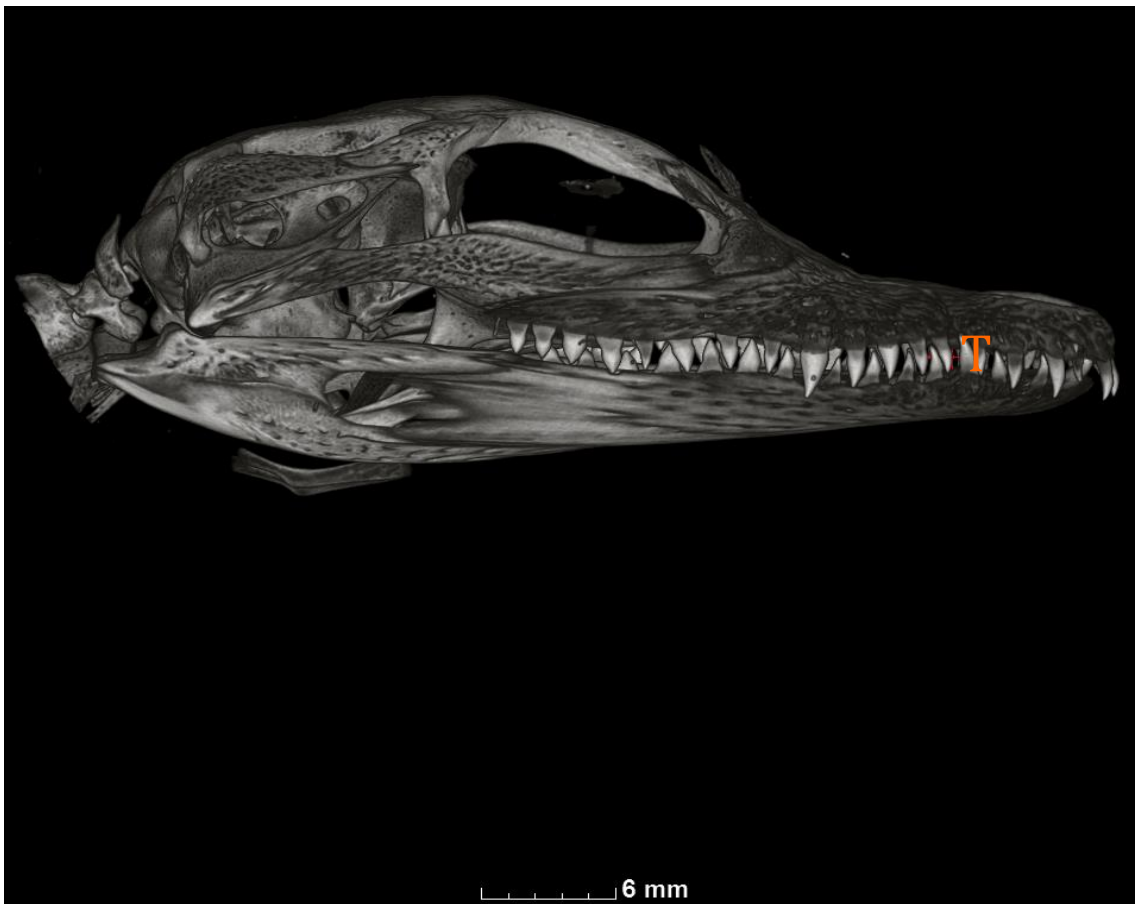
P 

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Figure 9. Micro CT scan section showing the lateral view of a **post-hatched** crocodile specimen. The red T indicates the level of the section used in Figures 11, 13 and 15. M-Maxilla, Md -mandible

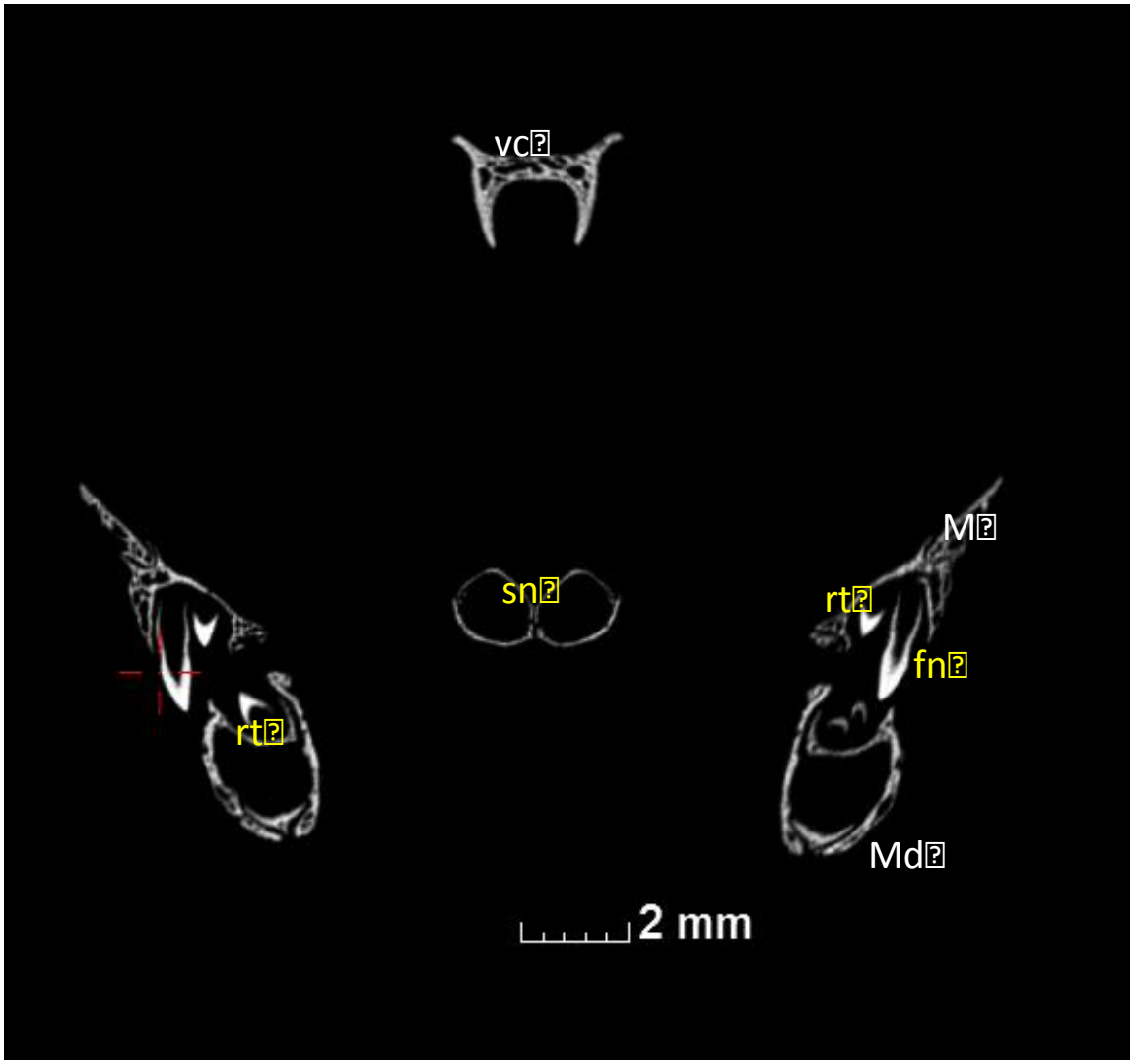


Figure 10. Micro CT scan section showing a coronal view of a **pre-hatched** crocodile embryo. Note the functional (fn) teeth and replacement teeth (rt) in the maxilla (M) and the mandible (Md). vc-vertebral column, sn-sinuses

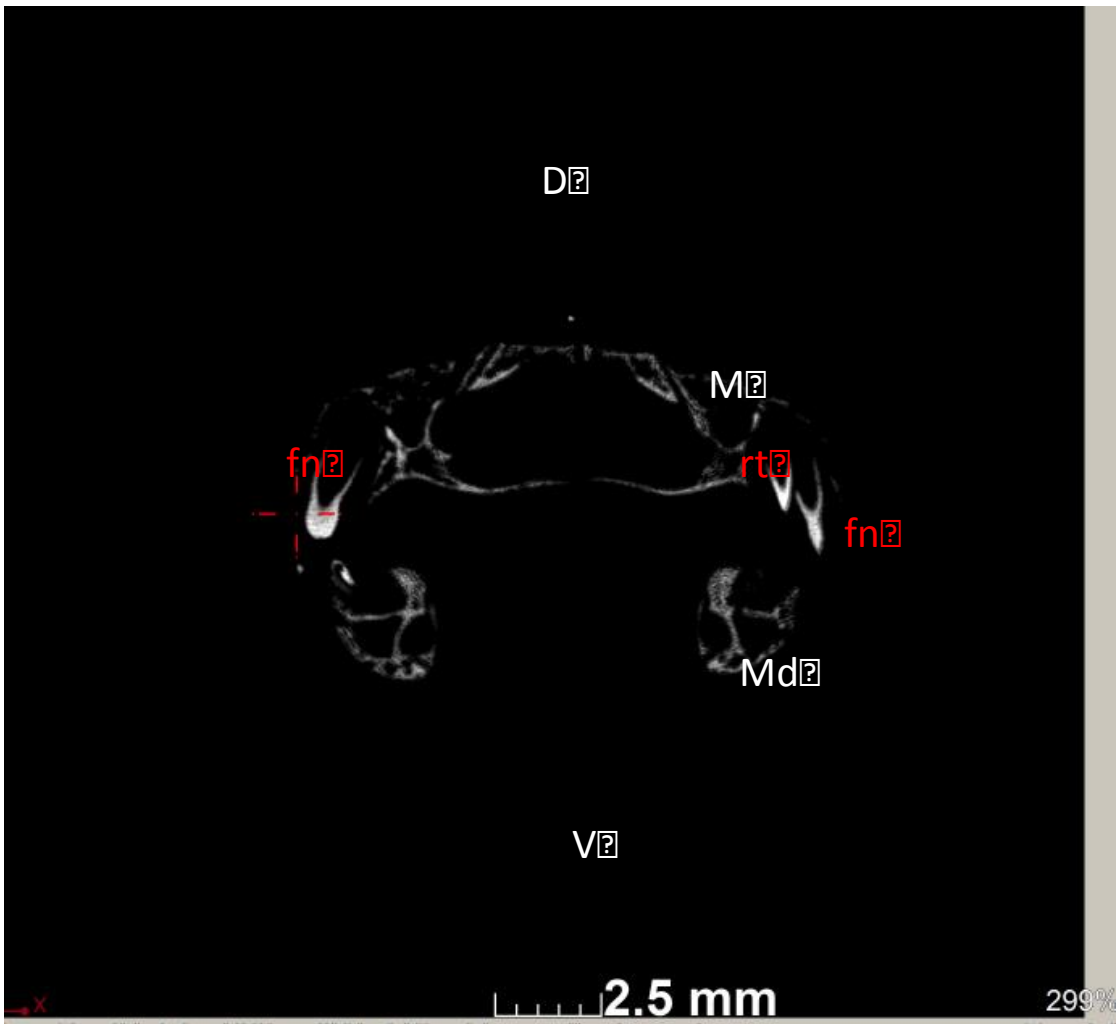
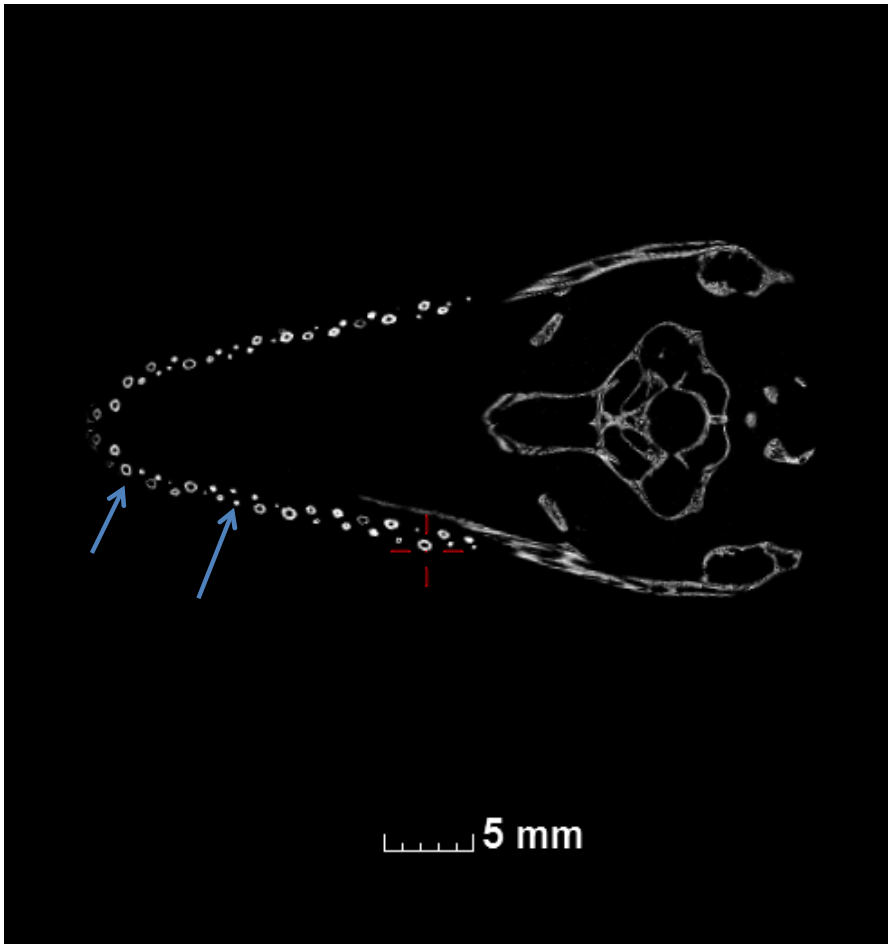


Figure 11. Micro CT scan section showing a coronal view of a **post-hatched** crocodile embryo. Note the functional (fn) teeth and replacement teeth (rt) on the lingual aspect of the functional tooth. M-maxilla, Md - mandible.

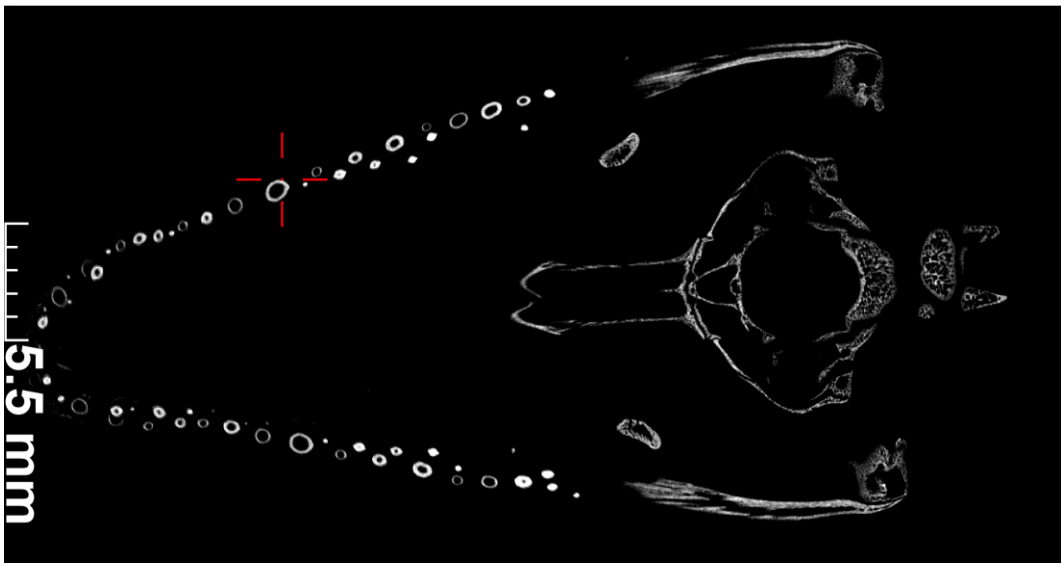
A



P

Figure 12. Micro CT scan showing a section of the ventral view of a **pre-hatched** crocodile specimen. The red cross is indicative of the same position on the jaws denoted in Figure 8. Note teeth (arrows), of various sizes.

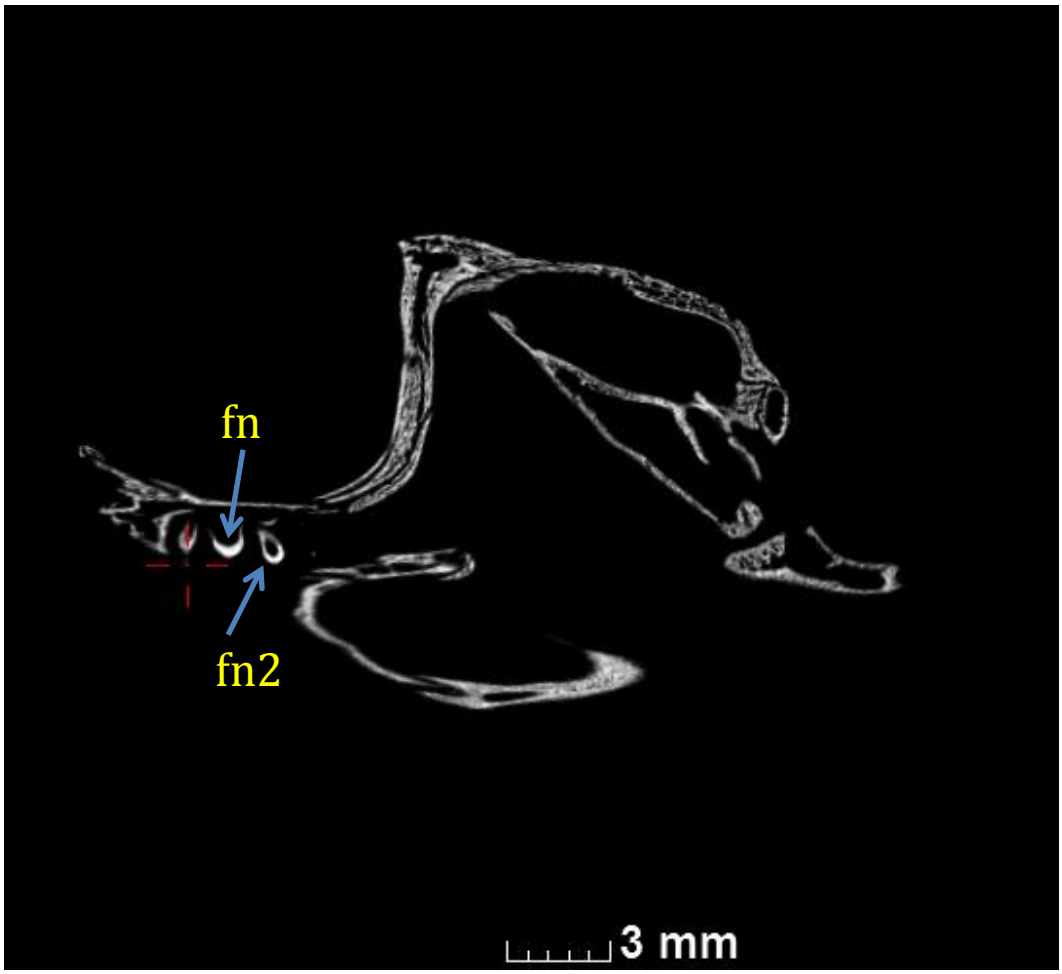
A



P

Figure 13. Micro CT scan showing a section of the ventral view of a **post-hatched** crocodile specimen. The red cross is indicative of the same position on the jaws denoted in Figure 9. Note transverse sections of teeth of various sizes due to various developmental stages.

A



P

Figure 14. Micro CT scan of a section showing a sagittal view of pre-hatched crocodile specimen. The red cross indicates the level of the section as denoted in Figure 1. In this section the functional tooth (fn) of the maxilla interdigitates with a functional tooth (fn2) in the mandible. At this level, no replacement teeth are evident.

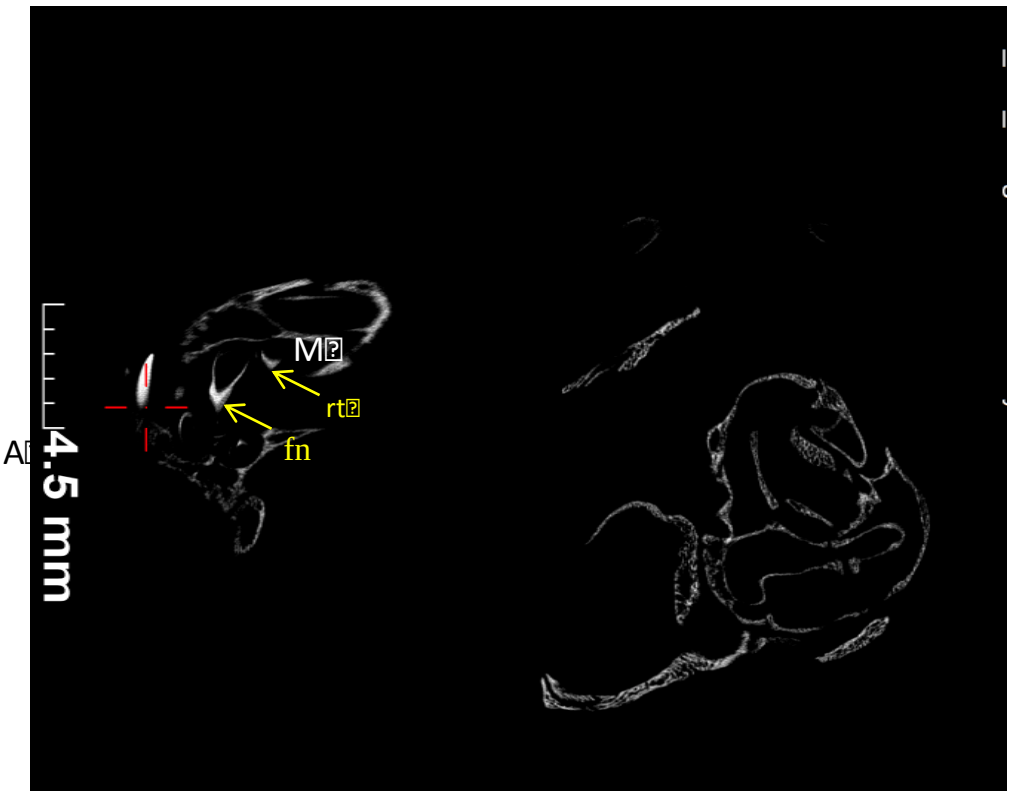


Figure 15. Micro CT scan of a section showing a sagittal view of a **post-hatched** crocodile specimen. The red cross indicates the level of the section as denoted in Figure 9. In this section the functional tooth (fn) of the maxilla (M) is associated with a small replacement tooth (rt).

3.2 Histological analysis

The histological descriptions that follow were confirmed by the examination of serial sections of each specimen.

3.2.1 14-31 day-old specimens

In the earliest specimens investigated, (14-31 days of embryonic life), the oral surface of the maxilla and the mandible were lined by a smooth oral epithelium (Fig. 16a), which was 3-4 cell layers thick (Figs. 16b-d). The oral epithelium was separated from the underlying ectomesenchyme by a distinct basement membrane (Fig. 16d). The ectomesenchyme was composed of scattered spindle-shaped nuclei in a pale, eosinophilic, fibrous matrix (Figs. 16b-d).

The earliest indication of tooth development was seen as equidistant bulging of the oral epithelium of the maxilla (Fig. 16b) and the mandible (Fig. 16c). These thickenings extended throughout the length of the maxilla and the mandible, but were closer together in the anterior aspect of the upper and lower jaws. Posteriorly, although this was not quantitated, larger stretches of oral epithelium occurred between the thickenings in the maxilla and the mandible. The thickenings were evident due to the condensed ectomesenchymal cells causing the oral mucosa to bulge slightly into the oral cavity (Fig. 16b, c, d). A basement membrane separated the oral epithelium from the ectomesenchymal condensation (Fig. 16d). The condensed ectomesenchyme appeared distinct from the surrounding ectomesenchyme due to the less dense matrix of the latter (Fig. 16d). Thin walled blood vessels containing nucleated erythrocytes were present in the ectomesenchyme, parallel to the length of the maxilla and the mandible (Fig. 16c). In addition, developing eosinophilic spicules of bone, lying parallel to the long axis of the jaws, were present (Fig. 16b). These spicules contained both osteocytes in lacunae as well as basophilic osteoblasts on the developing surface of the

spicule. Meckel's cartilage, identified as a basophilic bar of hyaline cartilage, was present in the youngest specimens examined (Fig. 16a).

Examination of serial sections of these young specimens revealed various stages of tooth development in the maxilla and the mandible reminiscent of those in mammalian embryos. These include the placode, bud and cap stages. In addition to these stages, some degenerating tooth structures, described as 'null' generation teeth were also identified. A detailed description of these stages follows.

The **placode stage** of tooth development was identified as invaginations of the oral epithelium into the ectomesenchyme, to form rounded structures (Figs. 17a, b). These placodes were composed of 6-8 layers of cuboidal epithelial cells, compared to the oral epithelium which was only 3-4 cell layers thick. Often the ectomesenchyme appeared to have further condensed at the narrower portion of the invaginations (Fig. 17a). It was more basophilic in this region as opposed to the surrounding ectomesenchyme, which stained eosinophilically (Fig. 17a). Associated with some of the placodes, were eosinophilic dentine fragments, lying in the ectomesenchyme (Fig. 17b). These were identified by the presence of dentinal tubules and were surrounded by a discontinuous layer of cuboidal cells (Fig. 17b). In some parts of these dentine fragments, multinucleated cells were associated with the dentine surface (Fig. 17b).

The next stage of tooth development identified, was the **bud stage** (Fig. 18a), which was reminiscent of bud stage tooth germs during mammalian odontogenesis. The bud stage was not seen commonly in the sections examined. At this stage, a multicellular epithelial dental lamina penetrated into the ectomesenchyme of both the maxilla (Fig. 18a) and the mandible. Its terminal, dilated end was surrounded by a concentric condensation of ectomesenchyme (Fig. 18a). The downgrowth of the dental lamina occurred at an acute angle to the oral epithelium on the buccal aspect of the lamina (Fig. 18b). The

ectomesenchyme was more condensed at this angle (Figs. 18a, b). Developing bone spicules were seen in close proximity (Fig. 18a).

Cap-shaped tooth germs were also evident in the 14-31 day old specimens, indicating the subsequent stage of tooth development. They were located along the length of the maxilla and the mandible but a pattern of development was not identified, as the teeth were not all at the same stage of development. The tooth germs in the cap stage of development appeared mammalian-like (Fig. 18b), consisting of an indented cap-shaped enamel organ connected to the oral epithelium via a dental lamina. At the periphery of the enamel organ, the cells assumed a low cuboidal shape to form a single layer, the outer enamel epithelium. The inner cells of the enamel organ, bordering the dental papilla assumed a low columnar shape forming a single layer of cells, the inner enamel epithelium (Fig. 18b). The inner enamel epithelium cells created a concave surface adjacent to the dental papilla. The entire enamel organ was separated from the dental papilla and dental follicle by a basement membrane. The inner and outer enamel epithelium met at the rim of the enamel organ forming the cervical loop. The cervical loop of these teeth appeared to develop asymmetrically, with the buccal loop being larger than the lingual (Figs. 18b, 19). The inner enamel epithelium was associated with a mass of ectomesenchyme, identified as the dental papilla (Fig. 18b). Successional tooth germs were often seen in association with developing cap stage tooth germs (Fig. 19). Developing eosinophilic alveolar bone spicules were seen in the ectomesenchyme of the 14-31 day embryos. These contained osteocytes in lacunae and were delineated with basophilic osteoblast nuclei at their periphery (Fig. 19).

In addition to normal developing teeth, which were identified in the 14-31day-old specimens, small, apparently degenerating, tooth-like structures were also observed (Fig. 20). These degenerating teeth were sometimes closely associated with the oral epithelium and in other cases were found lying deep in the

ectomesenchyme (Fig. 20). These were identified as ‘null generation’ teeth. These structures were either oval (Fig. 20a), round (Fig. 20b) or crescentric (Fig. 20c). The different shapes were associated with different regions of the jaw. The oval-shaped ‘null generation’, teeth were localised in the anterior aspect of the maxilla and mandible, whereas the round and crescent-shaped ‘null generation’ teeth were found more posteriorly. The round ‘null generation’ teeth consisted of a double layer of cuboidal cells surrounding a mass of irregularly-shaped eosinophilic dentine (Fig. 20b). The dentine was identified by the presence of dentinal tubules. On occasion, odontoclastic or other multinucleate phagocytic-type cells were seen associated with the dentine layer (Fig. 20b). In the crescent shaped ‘null generation’ teeth, a paler eosinophilic pre-dentine layer was discernable associated with the ‘null generation’ teeth (Fig. 20c). While no distinct columnar odontoblasts were identified, the dentine layer was supported by a mass of dental papilla-type cells (Fig. 20c). Ameloblasts and/or enamel were not evident in these structures. However, a layer, reminiscent of the inner enamel epithelium, was occasionally present (Fig. 20c). The oval type of ‘null generation’ teeth were seen to have a more recognisable enamel organ, with an inner and outer enamel epithelium, stellate reticulum and associated condensed ectomesenchyme (Fig. 20a). The oval ‘null generation’ were associated with a dental lamina and a developing tooth germ, either in the bud, cap or early bell stage of tooth development (Fig. 20a). The dental lamina was discontinuous and was made up of clusters of epithelial cells. Its connection to the oral epithelium was at an angle (Fig. 20a). Commonly, the terminal end of the dental lamina was dilated and was associated with a developing tooth germ (Fig. 20a). The oval ‘null generation’ tooth’ and the developing tooth germ appeared to co-occupy a slightly paler cylindrical mass or area of ectomesenchyme (Fig. 20a), which was distinctly different and less eosinophilic than the remainder of the ectomesenchyme that surrounded both these structures.

3.2.2 32-65 day-old specimens

In the 32-65 day embryos, the oral epithelium of both the maxilla and the mandible followed the contours of the underlying submerged developing teeth and appeared to be undulating over the positions of the tooth germs (Fig. 21a). The oral epithelium was 3-4 cell layers in thickness and rested on a prominent basement membrane (Fig. 21b). The maxillary tooth germs were present throughout the length of the maxilla, whereas mandibular tooth germs were developing only in the anterior aspect of the jaw (Fig. 21a). Once again, numerous thin-walled blood vessels, lying close to the oral epithelium, were found in the ectomesenchyme of the maxilla and the mandible and appeared to follow the contour of the epithelium (Fig. 21b). Extensive vascular networks were also seen, branching and radiating in several directions, in the deeper regions of the ectomesenchyme (Fig. 21b). Various stages of tooth development were observed along the jaws, the most prominent being the bell stage (Figs. 21a, c). In this age group most tooth germs appeared to have lost their connection to the oral epithelium. The dental lamina was reduced to a continuous deep lying, dental epithelial string which connected the tooth germs to each other (Figs. 22a, b) and also followed the contours of the oral epithelium. At higher magnification, this string consisted of a double layer of cuboidal epithelial cells (Fig. 22c). It was interesting to note that there was a difference in the staining intensity between the more eosinophilic ectomesenchyme of the oral surface of the dental lamina (surface closest to the oral epithelium) as opposed to the aboral surface which was paler and less dense (Figs. 22a,b). The dental lamina, in the 32-65 day old specimens, had tooth germs developing on both its aboral (Fig. 23a) and oral surfaces (Fig. 23b). Around these developing tooth germs, as well as the surface of the dental lamina away from the tooth germs, a distinct basophilic ectomesenchymal condensation was evident (Fig. 23).

On careful examination of all the specimens, as well as adjacent sections, there appeared to be two ‘types’ of **bell stage** tooth germs in this developmental group

of embryos. The first ‘type’ were reminiscent of mammalian bell stage tooth germs. They had a well differentiated enamel organ with a low cuboidal to squamous outer enamel epithelium (Fig. 24a). The inner enamel epithelium began at the point where the outer enamel epithelium bent to form the concavity filled with the condensed ectomesenchymal cells of the dental papilla. The inner enamel epithelium was differentiated into a layer of columnar ameloblasts (Figs. 24a,b, 25), with nuclei polarised away from the basement membrane (Fig. 24b). A slightly thinner layer of enamel as compared to dentine, was laid down against the dentine surface, but no distinct Tomes’ processes were identified at the peripheral region of the enamel layer, although it appeared uneven. On the outer surface of the inner enamel epithelium, two to three layers of flattened spindle-shaped nuclei, with their long axis perpendicular to the nuclei of the ameloblasts were identified as the stratum intermedium (Figs. 24, 25). Between the stratum intermedium and the outer enamel epithelium, interlinked star-shaped cells, with large intercellular spaces were identified as the stellate reticulum (Figs. 24a, 25). The stellate reticulum and a distinct stratum intermedium were typical of a mammalian single cusped tooth. The first type of bell stage tooth germs had a distinct palisading odontoblastic layer as well as definitive pre-dentine and dentine layers (Figs. 24, 25) with striations identified as dentinal tubules. The inner and outer enamel epithelium met at a cervical loop, which delineated a wide apical root with a dense condensation of ectomesenchyme (Figs. 26a, b). There was an abrupt transition at the cervical loop from the cuboidal cells of the inner enamel epithelium to the squamous flattened cells of the outer enamel epithelium (Fig. 26b). The highly cellular dental papilla had a rich blood supply with a distinct sub-odontoblastic capillary network in the future crown aspect of the tooth (Fig. 26a) as well as in the central region of the pulp (Fig. 26a). A less dense condensation of ectomesenchyme, the dental follicle, surrounded the enamel organ (Figs. 26a, b). The squamous cells of the outer enamel epithelium, in early bell stage tooth germs, formed a funnel shaped structure, filled with stellate reticulum-type tissue, which was linked to the bilaminar dental lamina (Figs. 27a,

b). Numerous capillaries were associated with the funnel shaped structure (Fig. 27b).

The second type of bell stage tooth germ identified in these embryos, had an elongated enamel organ with a cuboidal outer and columnar inner enamel epithelium and stellate reticulum interposed (Figs. 28a, b). The enamel organ was connected to the dental lamina (Figs. 28a, b). Ameloblasts and enamel matrix were not evident in these tooth germs. Some dentine deposition was evident, but a definable layer of odontoblasts was not distinguishable. Adjacent to the inner enamel epithelium, was a highly cellular and condensed dental papilla (Fig. 28b). An enamel knot-type structure was sometimes evident and was associated with the inner enamel epithelium (Fig. 28b). These bell stage structures were generally associated with a dental lamina whose dilated terminal end was identified as a replacement tooth germ in the bud stage of tooth development (Figs. 28a, b).

All tooth germs in this group, were separated from each other by the cellular ectomesenchyme. No distinct alveolar bone sockets were identified at these stages although bone development was evident.

3.2.3 Hatched and post-hatched specimens

Ten to twelve erupted teeth were evident in the maxilla and in the mandible of the hatched and 11-days post-hatched specimens. In three specimens of this group there were more erupted teeth in the upper jaw when compared to the lower jaw. The oral epithelium was lined by a stratified squamous keratinized epithelium (Figs. 29a, b). In the hatched and post-hatched specimens, erupted or functional teeth were seen extending into the oral cavity. The teeth were separated by dense connective tissue and sockets of alveolar bone (Fig. 29b). The functional teeth were conical and had a homodont appearance (Fig. 29a, b). In the crown of the tooth, the enamel had been dissolved during tissue preparation, leaving behind an

enamel space (Fig. 29b). At the neck of the tooth, the enamel layer joined the thin basophilic layer of cementum at the cemento-enamel junction (Fig. 29c). The cementum was a thin acellular layer with its thickness increasing slightly toward the apical region of the tooth (Fig. 29c). Pre-dentine and dentine layers were evident. These had distinct dentinal tubules (Fig. 30) and surrounded a large pulp cavity lined by a pseudostratified layer of odontoblasts at its periphery (Figs. 29c). The odontoblast processes were evident extending from the apical surface of the odontoblasts into the predentine layer (Fig. 30). The thickness of the dentine consisted of radiating dentinal tubules. In some areas of the dentine the tubules had several branching processes (Fig. 30). The dentine layer was continuous from the crown to the apex, narrowing slightly apically (Fig. 29b). The pulp in the erupted, functional teeth, conformed to the general shape of the crown, and was less cellular and more fibrous than that in the unerupted teeth. The pulp had scattered fibroblast-type cells and numerous capillaries especially in the peripheral aspect (Fig. 29c). The periodontal ligament (Figs. 29c, 31a) was highly cellular with fibres arranged in several orientations. Pigmented macrophage-like cells with numerous radiating processes as well as spindle-shaped fibroblast type cells were evident in the ligament (Fig. 31b). Several capillaries and nerve bundles were also evident in the ligament (Figs. 31 a,b). A distinct root sheath was not identified in any of the specimens examined.

The spicules of alveolar bone, forming part of the bony socket had osteocytes in lacunae, basophilic osteoblasts limiting the periphery and spindle shaped fibroblast-like cells associated with the connective tissue periosteum (Fig. 31a).

In several alveolar sockets of the hatched and post-hatched specimens, three types of tooth elements were identified forming a “tooth family”:

a) The remnants of a previously functional tooth or degenerating tooth (tooth 1) (Figs. 32a, 33a, 34). These teeth were identified by their dentine shells,

which were submerged in the ectomesenchyme (Figs. 32a, 33a, 34). These dentine remnants showed characteristics on their periphery which were indicative of resorption (Figs. 33a,b, 34). This included an uneven border with basophilic nuclei housed in lacunae-type structures (Fig. 33b). These lacunae were often embedded within the dentine matrix, making their appearance characteristically similar to bone (Fig. 33b). The apical region of the remnant dentine had a radial arrangement of ectomesenchymal cells associated with the root tip (Fig. 32c). The dentine of the crown (Fig. 34) also exhibited nuclei in lacunae within the dentine matrix. The dentine surface was more eosinophilic and uneven with scattered cells in lacunae (Fig. 34). The ectomesenchymal fibrous tissue was arranged perpendicular to the surface of the dentine in wavy bundles with numerous basophilic nuclei between the fibres (Fig. 34). However, the surface of the dentine facing the pulp cavity did not show signs of resorption (Fig. 34). The peripheral layer of the pulp cavity, had scattered rounded nuclei which were remnants of the odontoblast layer (Fig. 34). The pulp itself was more fibrous and less cellular than the successional tooth (Figs. 32a, 33a, 34).

b) The second member of the tooth family was a replacement tooth germ either in the early or late bell stage of tooth development (tooth 2) (Figs. 32a, 33a). These tooth germs were often the ‘dominant’ structure in the socket (Fig. 32a, 33a).

c) The third structure of the tooth family present in the alveolar socket, was a newly developing successional tooth (tooth 3), associated with the dental lamina on its lingual aspect, either in the bud or cap stage of tooth development (Figs. 32b, 33a, 35a, b). In this group of specimens, the developing tooth germs in the cap stage showed signs of a bulge of cells associated with the inner enamel epithelium, possibly an enamel-knot type structure (Fig. 32b).

In the hatched and post-hatched specimens, several tooth families were identified along the length of the maxilla (Fig. 37a,b) and mandible. After examination of serial sections it was confirmed that development and replacement of teeth, progressed in an antero-posterior direction. This was evident due to the fact that more mature tooth structures constituting a tooth family (Fig. 37a) occurred anteriorly, whereas less developed tooth families were evident in the posterior aspect of the maxilla and mandible (Fig. 37a,b). In tooth families lying more posteriorly (Fig. 37a,b), tooth germs were associated with replacement teeth that were only slightly more advanced than their successors (Fig 37a,b). In the anterior aspect of the maxilla and the mandible, tooth families were housed in bony alveolar sockets (Fig. 37a). Posteriorly however, distinct sockets were not evident and the dental lamina was continuous between the developing tooth germs (Fig. 37a,b). The cellular concentration of the ectomesenchyme, in the more posterior tooth families, appeared diffuse around some tooth germs for e.g. t2 in tooth family 4 (Fig. 37b) and more concentrated around others, as in t2 in tooth family 3 (Fig. 37b).

Erupting and functional teeth had developing replacement tooth germs on their lingual aspect, near the root of the functional tooth. (Figs. 38a,b, 39, 40a, 41a). A schematic representation of this process is illustrated in Figure 38d. A highly cellular pulp cavity surrounded by tall columnar odontoblasts was evident in the replacement teeth, while that in the erupting teeth appeared less cellular (Figs. 38a, 41a) and more fibrous. In the apical region of the functional or erupted teeth and the cervical loop of the replacement teeth, a process of resorption appeared to be occurring due to the presence of several osteoclast-type cells that were identified (Figs. 38a,b; 40a,b, 41a,b).

Osteoclastic activity was identified in relation to signs of remodeling of the alveolar sockets during the tooth replacement process (Fig. 42). Distinct lacunae were evident at the surface of the alveolar bone associated with the apical aspect

of the developing tooth germ. The lacunae were in close proximity to multinucleate eosinophilic cells, with uneven borders, identified as osteoclast-type cells (Fig.42).

Prior to eruption, the well developed tooth crown was covered by an eosinophilic layer of cells with small nuclei which differed from the keratinised oral epithelium (Fig. 39b). This layer was identified as the reduced enamel epithelium. The superficial layers of the reduced enamel epithelium appeared to be desquamating, similar to the surrounding keratinised oral epithelium. In one erupted tooth a rounded mass of nuclei was seen to be associated with the reduced enamel epithelium overlying the erupted tooth. This structure was thought to be the remnant of a 'null generation' tooth that was being pushed orally as the functional tooth which formed below it, erupted (Fig. 39a).

In addition to the general crocodilian conical teeth, molariform-tooth germs in the late bell stage of tooth development were seen in four hatched specimens (Figs. 36a, b). These developing teeth had two cusp-like structures on their coronal surface. The odontoblast layer, the dentine, enamel and ameloblast layers followed the contour of the molariform-type tooth. The outer enamel epithelium surrounding these molariform-type teeth did not appear collapsed (Fig. 36a).

The main features of crocodilian odontogenesis that appear different to the mammalian situation are highlighted below:

- First sign of tooth development is evident as a thickening in the ectomesenchyme
- Dental placodes are present
- The dental lamina is angulated with the oral epithelium with distinct ectomesenchymal thickenings at the angle between oral epithelium and the dental lamina

- Presence of different types of ‘null generation’ teeth
- Two types of bell stage tooth germs, some degenerating
- The dental lamina appears as a deep lying epithelial string in the ectomesenchyme, connecting the tooth germs. The ectomesenchyme superior to the oral surface of the dental lamina stains more eosinophilically than the ectomesenchyme inferior to the dental lamina.
- ‘funnel’ shaped structures, lined by the outer enamel epithelium and filled with stellate reticulum-type tissue were often associated with bell stage tooth germs.
- The successional lamina originates from the outer enamel epithelium on the lingual side of the predecessor
- No cellular cementum
- Tooth families occur in a socket
- Molariform-type teeth evident in the ectomesenchyme.

3.3 The relationship between functional teeth and replacement teeth

The relationship between the state of functionality of the functional (erupted) teeth and the state of differentiation of the replacement teeth was examined in the older specimens i.e. 11 days post-hatching. The functional (erupted) teeth were assigned to one of the following categories: attached, young, mature or in resorption (according to Huysseune, 2006).

Most of the functional teeth were associated with a developing replacement tooth. In some instances, a successional lamina was not apparent in association with an erupted tooth. Erupted teeth in the process of ankylosis were mostly associated with a replacement tooth in morphogenesis (33%) or cytodifferentiation (50%) (Table 5). Fewer (8-9%) less developed replacement teeth were associated with functional teeth that were mature or in resorption (Table 5). Seventy five percent of functional teeth undergoing resorption had replacement teeth in the

cytodifferentiation stage of development (Table 5) (Fig. 43). Young, mature functional teeth and teeth in resorption were not seen to be associated with a successional lamina.

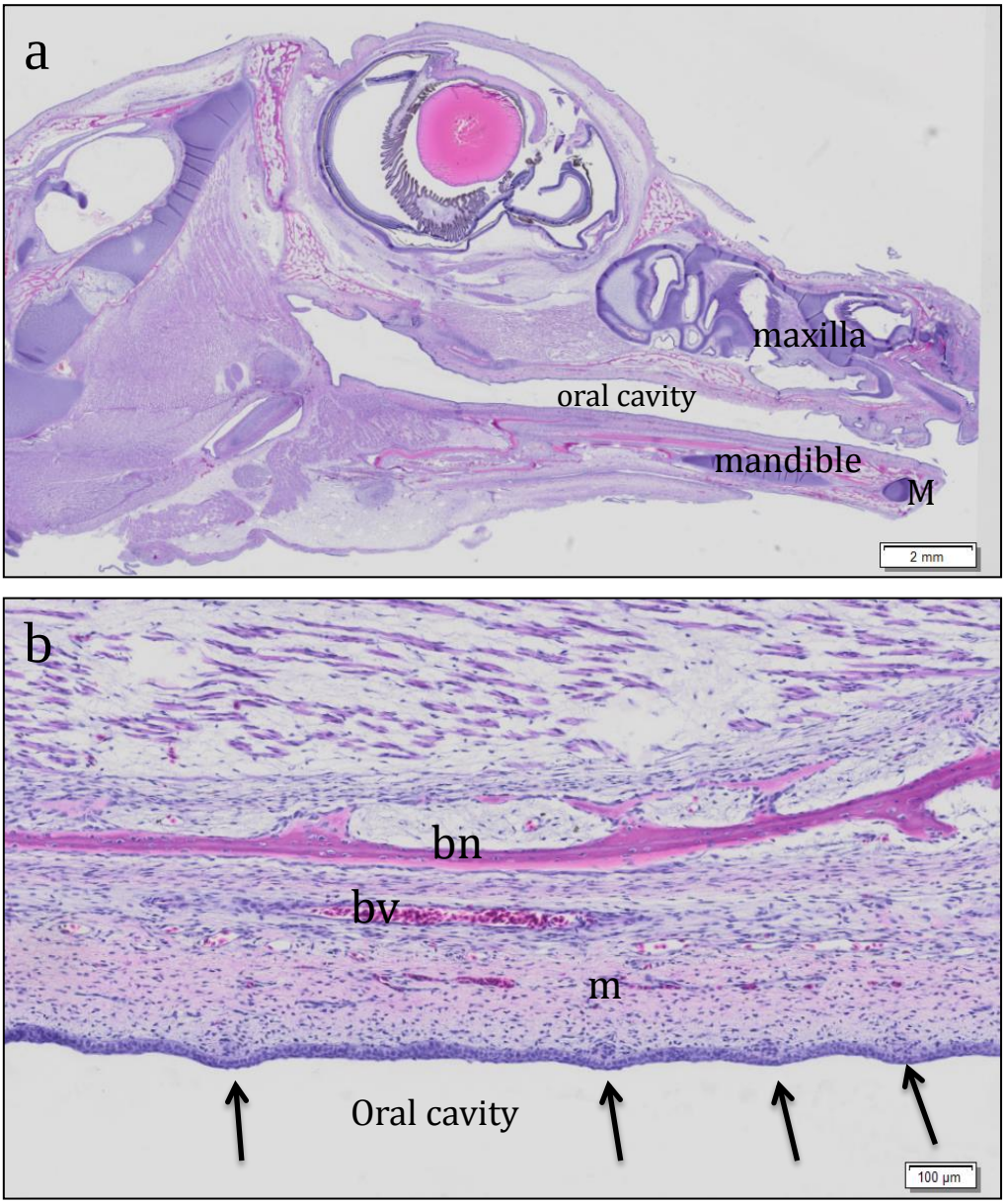


Figure 16 a and b. First signs of odontogenesis a: Sagittal section of a crocodile embryo representative of the 14-31 day group. b: Higher magnification of the oral epithelium of the maxilla showed the thickenings (arrows) indicating the first sign of tooth development. In the ectomesenchyme (m), numerous blood vessels (bv) were evident filled with nucleated erythrocytes. Spicules of alveolar bone (bn) were present.
M-Meckel's cartilage. Haematoxylin and eosin

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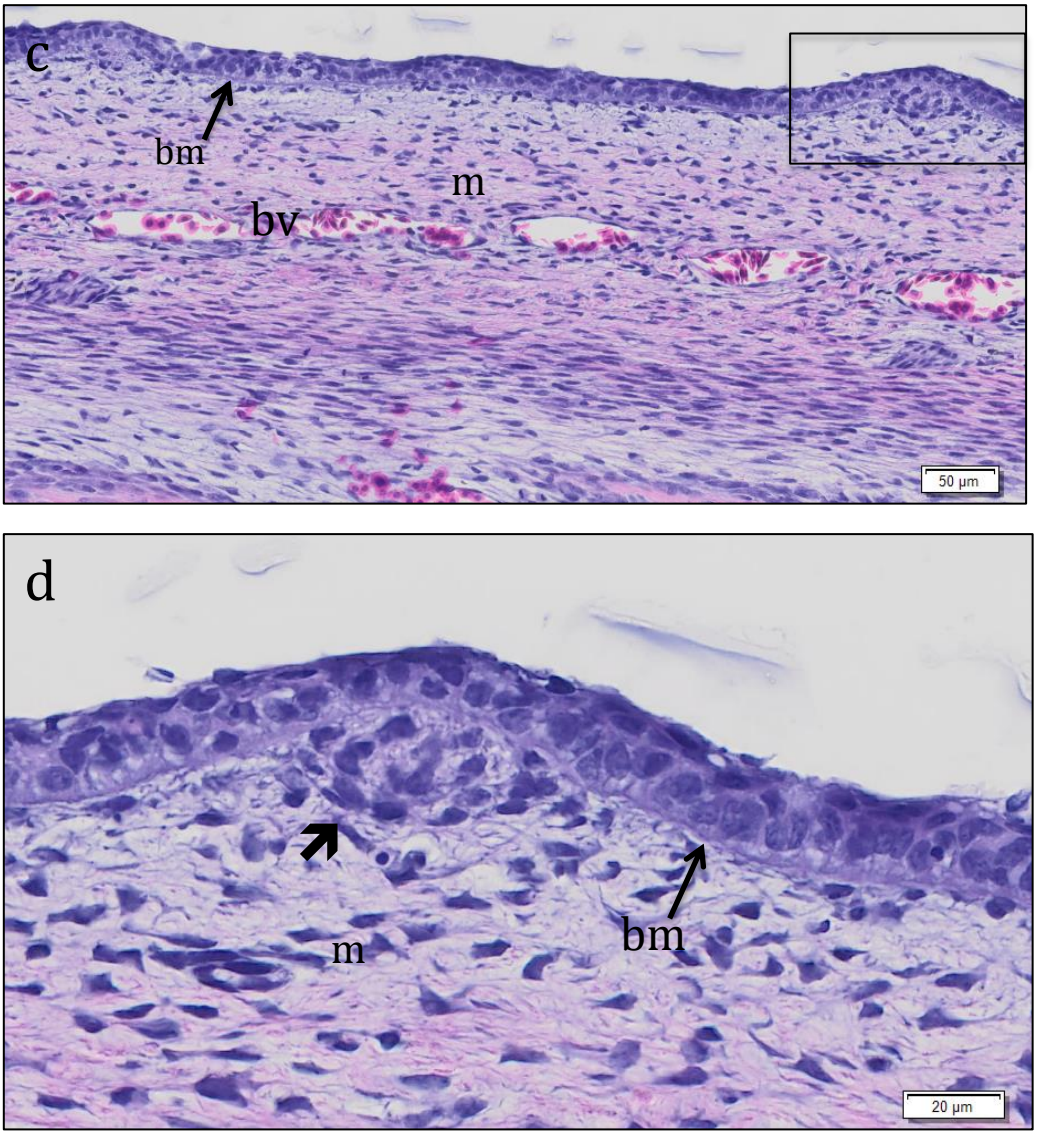

Figure 16 c and d. Photomicrograph showing early signs of tooth development in th 14-31 day-old embryos. c-d: Mandibular thickenings involving the oral epithelium and underlying ectomesenchyme (m) were evident. Numerous small blood vessels (bv) were evident in the ectomesenchyme lying parallel to the long axis of the mandible. d shows the rectangular area in **16c** at higher magnification. Note the distinct basement membrane (bm) supporting the oral epithelium. Haematoxylin and eosin.

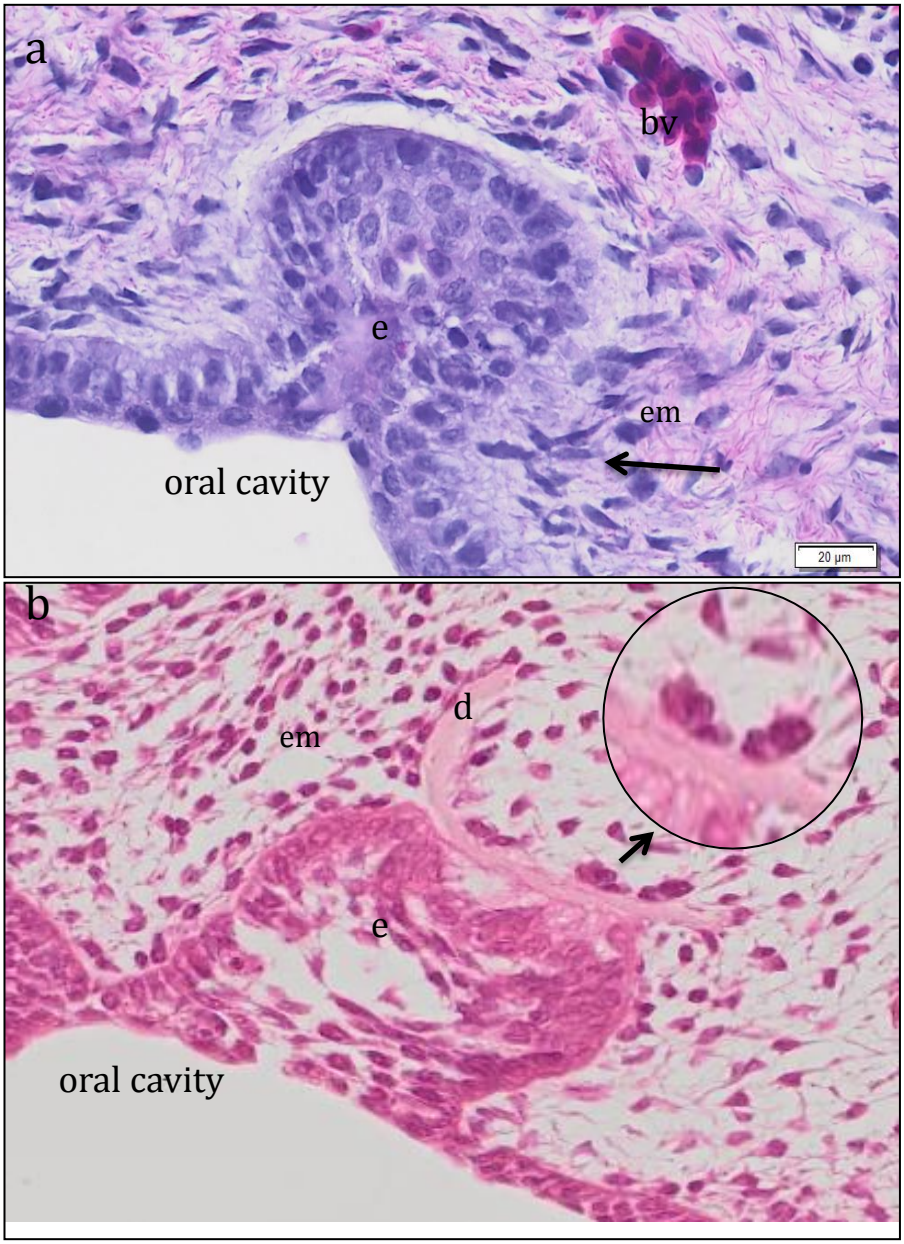

Figure 17 a and b. Dental placodes in the maxilla of the 14-31 day-old specimens. a: Photomicrographic representation of a dental placode in the maxilla. This was composed of an ingrowth of oral epithelial cells (e) into the ectomesenchyme (m). Ectomesenchyme condensation (black arrow) is evident at the lateral aspects of the placode. Blood vessels (bv) were also evident near the placode. b: Photomicrograph depicting a dental placode composed of epithelial cells (e) associated with a segment of dentine (d). Note multinucleate cells adjacent to the dentine fragment (black arrow shows magnified insert with multinucleate cells)Haematoxylin and eosin. Scale bar: 20µm

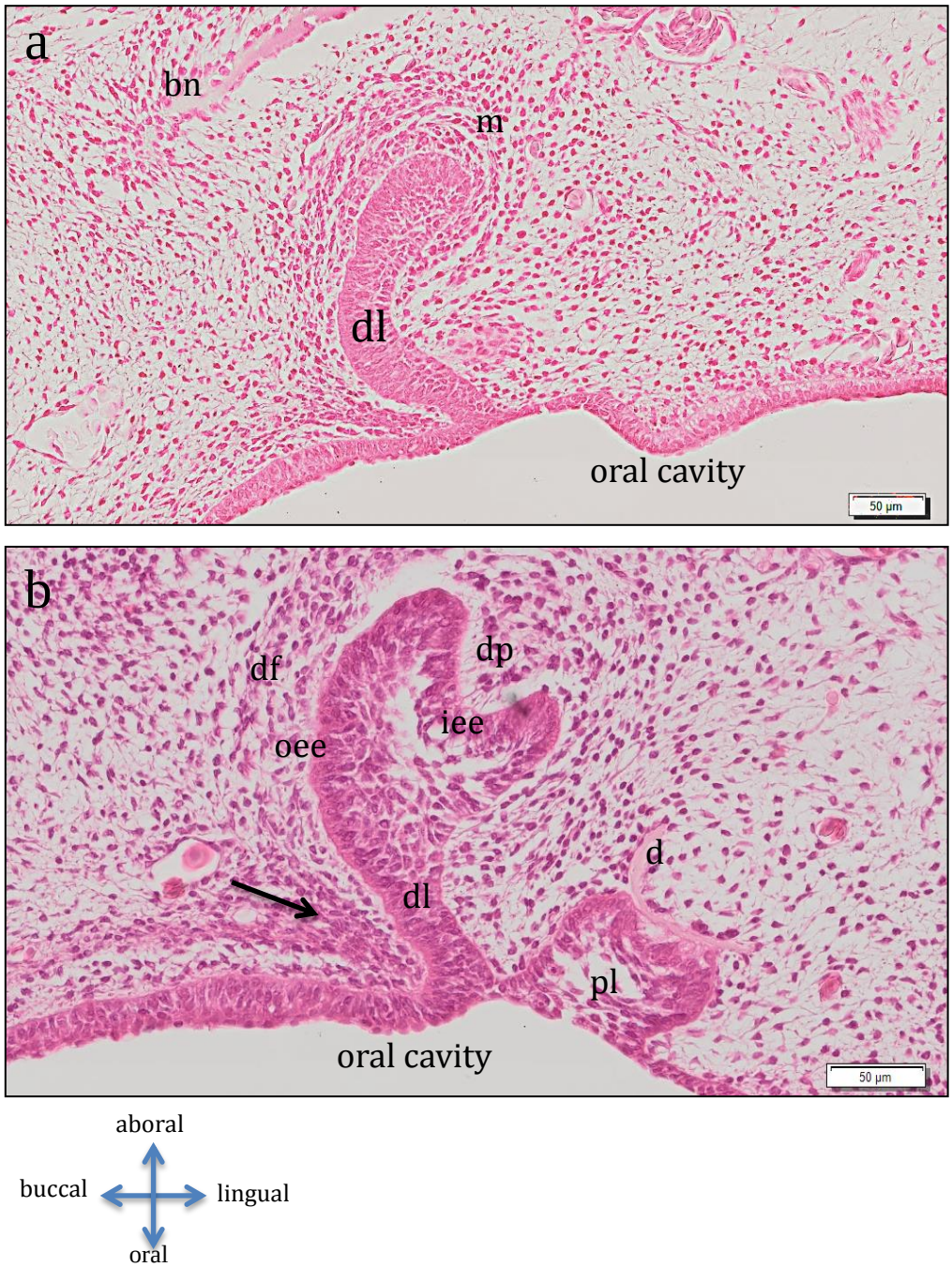

Figure 18 a and b. Bud and cap stage of tooth development in the maxilla of 14-31 day-old crocodiles. a: Representative photomicrograph showing the bud stage of tooth development in 14-31day old specimens. The dilated end of the dental lamina (dl) had a condensation of ectomesenchyme (em). Note bn-bone spicules. b: Photomicrographic representation of a cap stage tooth germ with an inner (iee) and outer (oee) enamel epithelium, a dental papilla (dp) and a dental follicle (df) which was connected to the oral epithelium via a dental lamina (dl). On the buccal aspect, there was a marked condensation of ectomesenchyme at an acute angle between the dental lamina and the oral epithelium (arrow). On the lingual aspect of the cap stage tooth germ was a dental placode (pl) in the ectomesenchyme associated with a dentine fragment (d) (Fig. 17b). Haematoxylin and eosin.

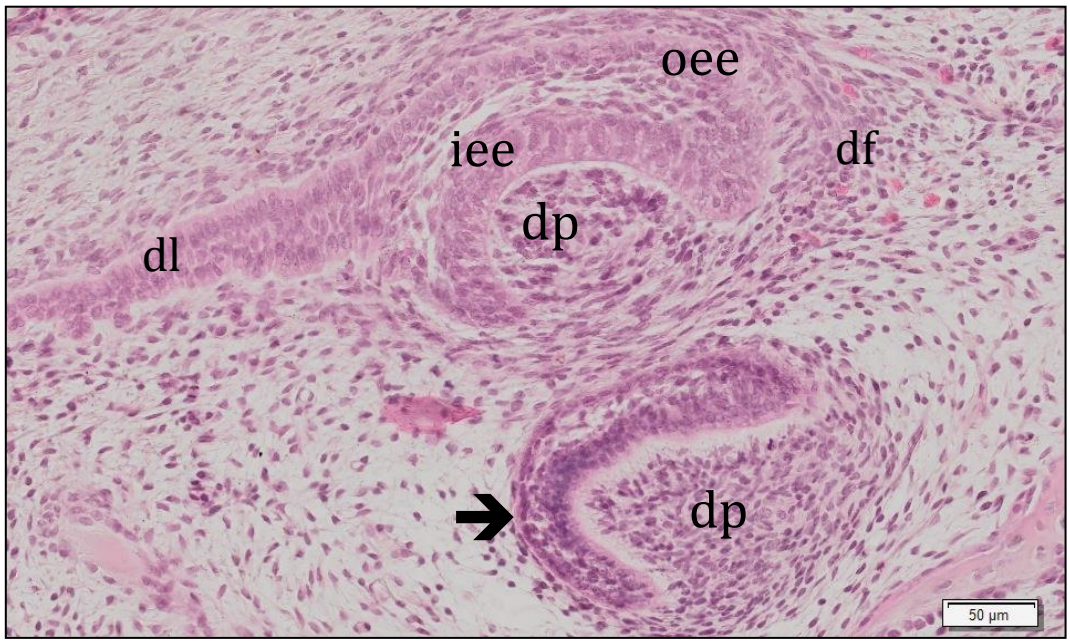

Figure 19. Representative photomicrograph of a sagittal section through the mandible of an embryo from the 32-65 day-old group. Both a cap stage tooth germ (arrow) and a replacement tooth, which is also in the cap stage of tooth development are depicted. dl-dental lamina; iee-inner enamel epithelium; oee-outer enamel epithelium; dp-dental papilla; df-dental follicle; bn-alveolar bone. Haematoxylin and eosin.

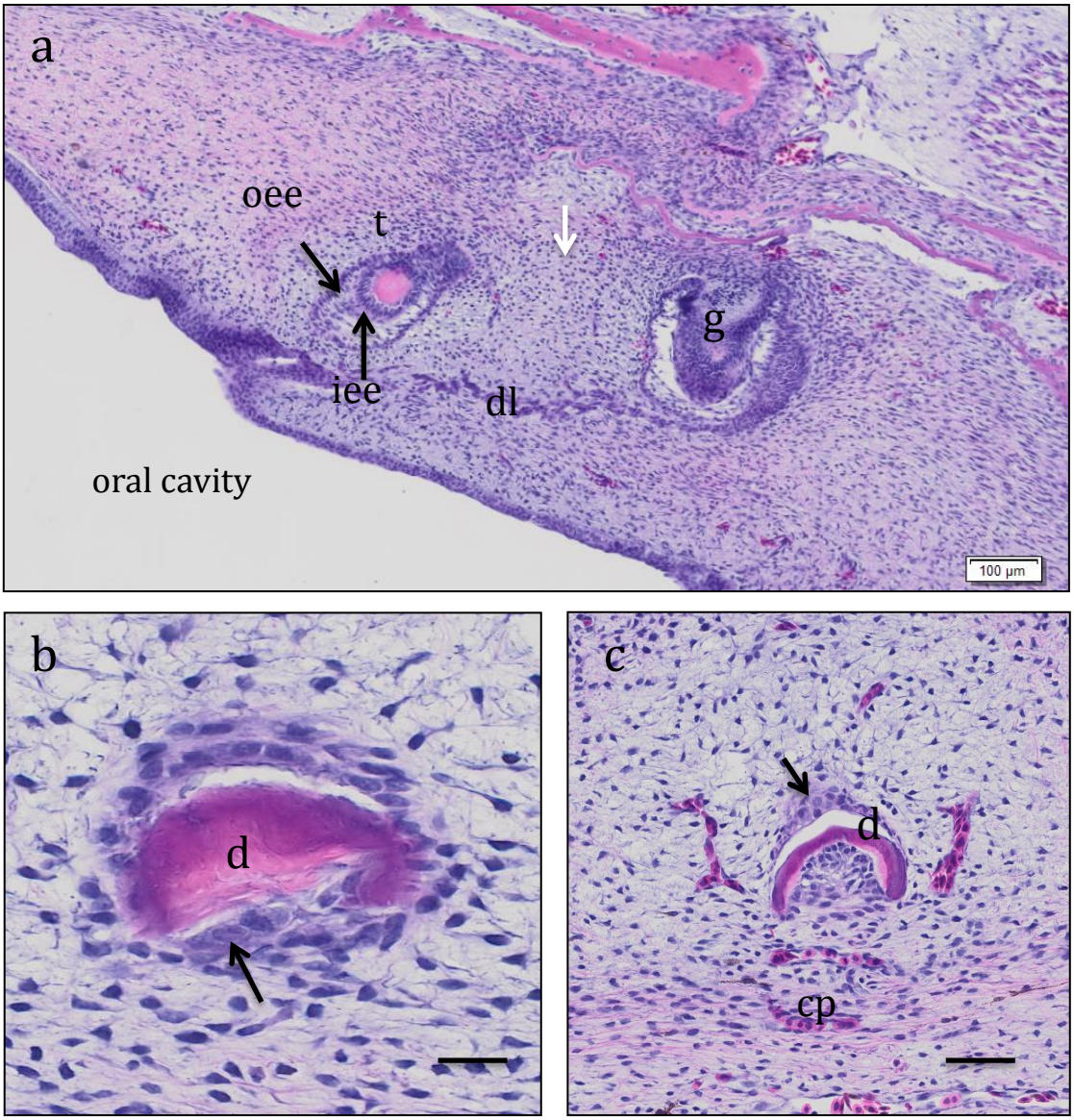

Figure 20 a-c. Representative photomicrograph of 'null' generation teeth in the 14-31 day-old embryo group. a: Oval shaped null generation tooth (t) showing an inner (iee) and outer (oee) enamel epithelium and a mass of eosinophilic dentine. No connection between the dental lamina (dl) and the oral epithelium was found in serial sections. The 'null generation' tooth was associated with a developing tooth germ (g). An oval shaped area (white arrow) around the null generation tooth and tooth germ showed paler staining as compared to the surrounding ectomesenchyme. b: A rounded null generation tooth structure with a mass of eosinophilic dentine (d) surrounded by a double layer of cuboidal cells. c: A half-moon shaped dentine fragment (d) with a pre-dentine layer associated with a mass of ectomesenchymal cells. Numerous capillaries (cp) were evident surrounding the 'null' generation tooth. Arrow indicates a mass of cells in the area of the iee and associated enamel space. Haematoxylin and eosin. Scale bar b,c: 50µm

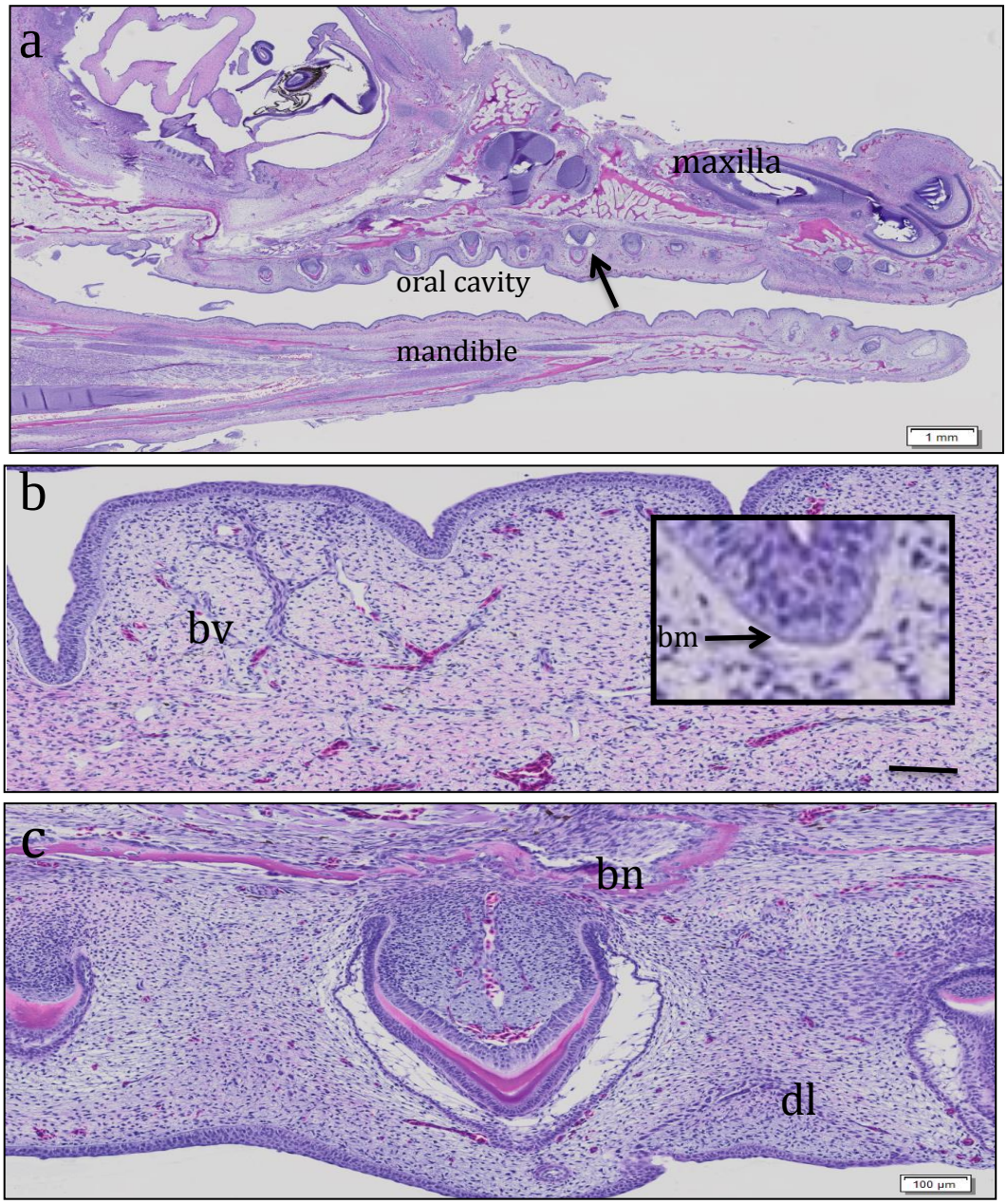

Figure 21 a-c. Representative photomicrographs from 32-65 day-old embryo group. a: Sagittal section through the maxilla and the mandible of a 38 day-old embryo. The maxilla displayed tooth germs, which were mainly in the bell stage of tooth development, with some teeth showing replacements (arrow). In the mandible, fewer tooth germs were visible and were particularly evident in the anterior aspect. b: The undulating oral epithelium was supported by an ectomesenchyme which had an extensive network of blood vessels (bv). Insert shows the basement membrane (bm). c: A maxillary tooth germ in the late bell stage of tooth development. Note the dental lamina (dl) extending between tooth germs. Haematoxylin and eosin. Scale bar: 100µm

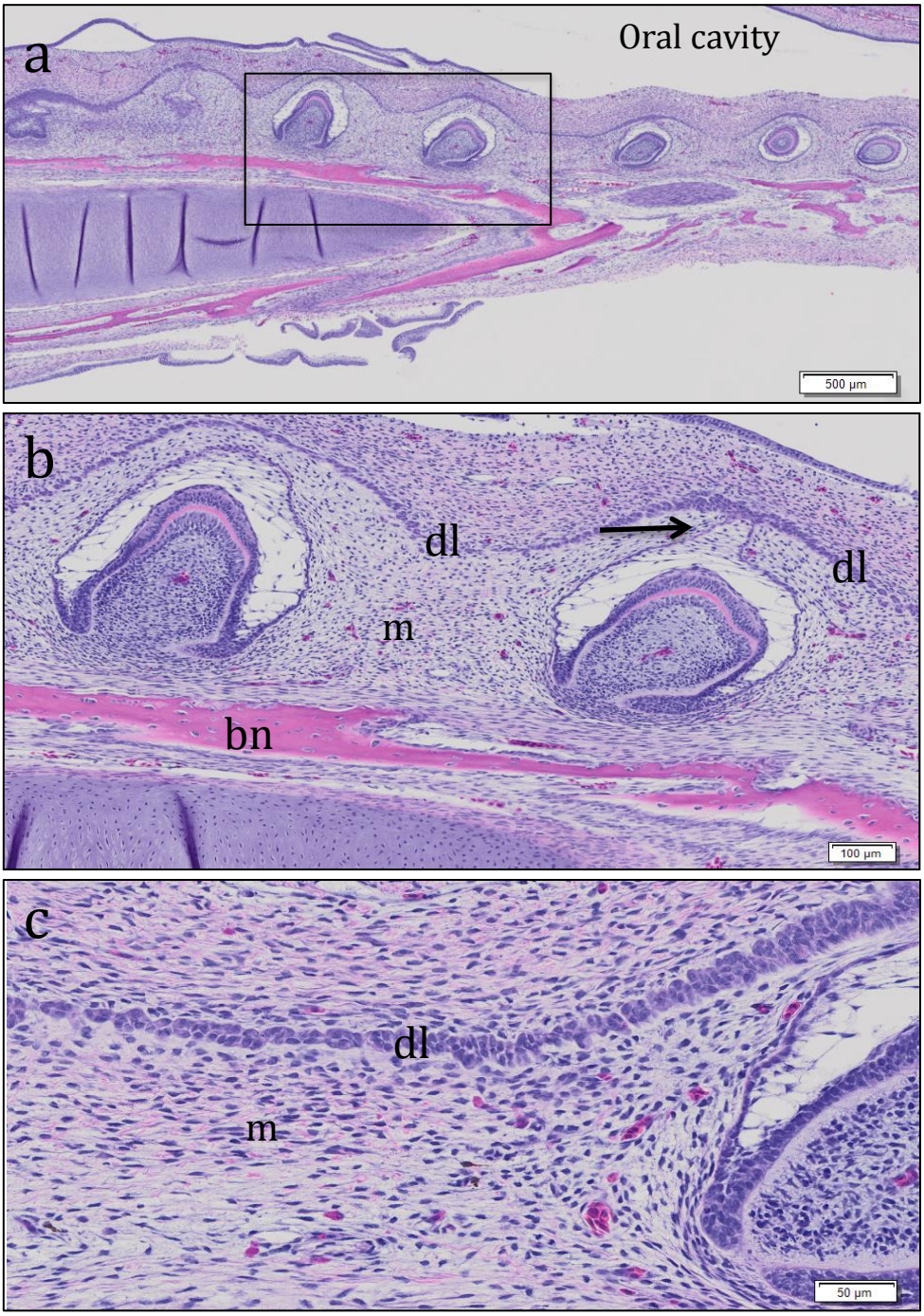

Figure 22a-c. Photomicrographs depicting representative sections of the mandible of 32-65 day-old embryo group. a: The dental lamina (dl) was evident as an epithelial 'string' which joined the tooth germs. b: In a higher magnification of two tooth germs depicted in the rectangle in Figure **22a**, the bilaminar dental lamina (dl) was connected to the one tooth germ by an epithelial lined stalk filled with stellate reticulum (arrow). Note the staining intensity of the ectomesenchyme (m) was different in the oral and aboral surface of the dental lamina. Alveolar bone (bn) was evident along the long axis of the mandible. c: Higher magnification of the bilaminar dental lamina (dl). Haematoxylin and eosin.

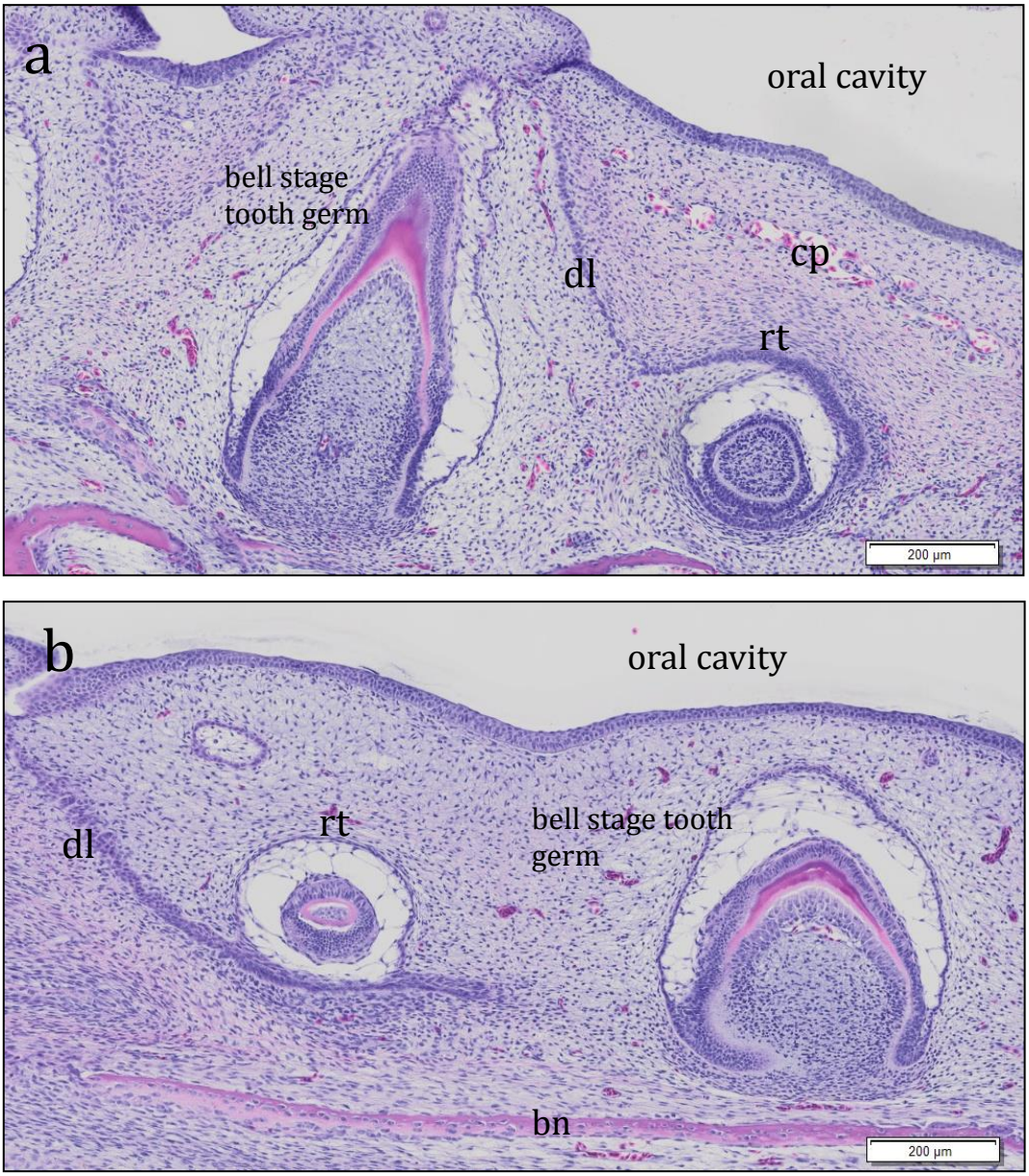

Figure 23 a and b. Photomicrographic representation of the dental lamina in the mandible of an embryo specimen in the 35-65 day-old group. a: The dental lamina (dl) associated with a bell stage tooth germ had a replacement tooth (rt) germ on its aboral surface. There was a distinct ectomesenchymal condensation around the replacement tooth germ. The late bell stage tooth germ and the dental lamina are closely associated by a downgrowth of the oral epithelium. Numerous capillaries (cp) were seen lying parallel to the long axis of the mandible. b: in this specimen, the dental lamina (dl) had a developing tooth germ on its oral surface (rt), while the ectomesenchyme was condensed on the aboral surface of the dental lamina. bn-alveolar bone. Haematoxylin and eosin.

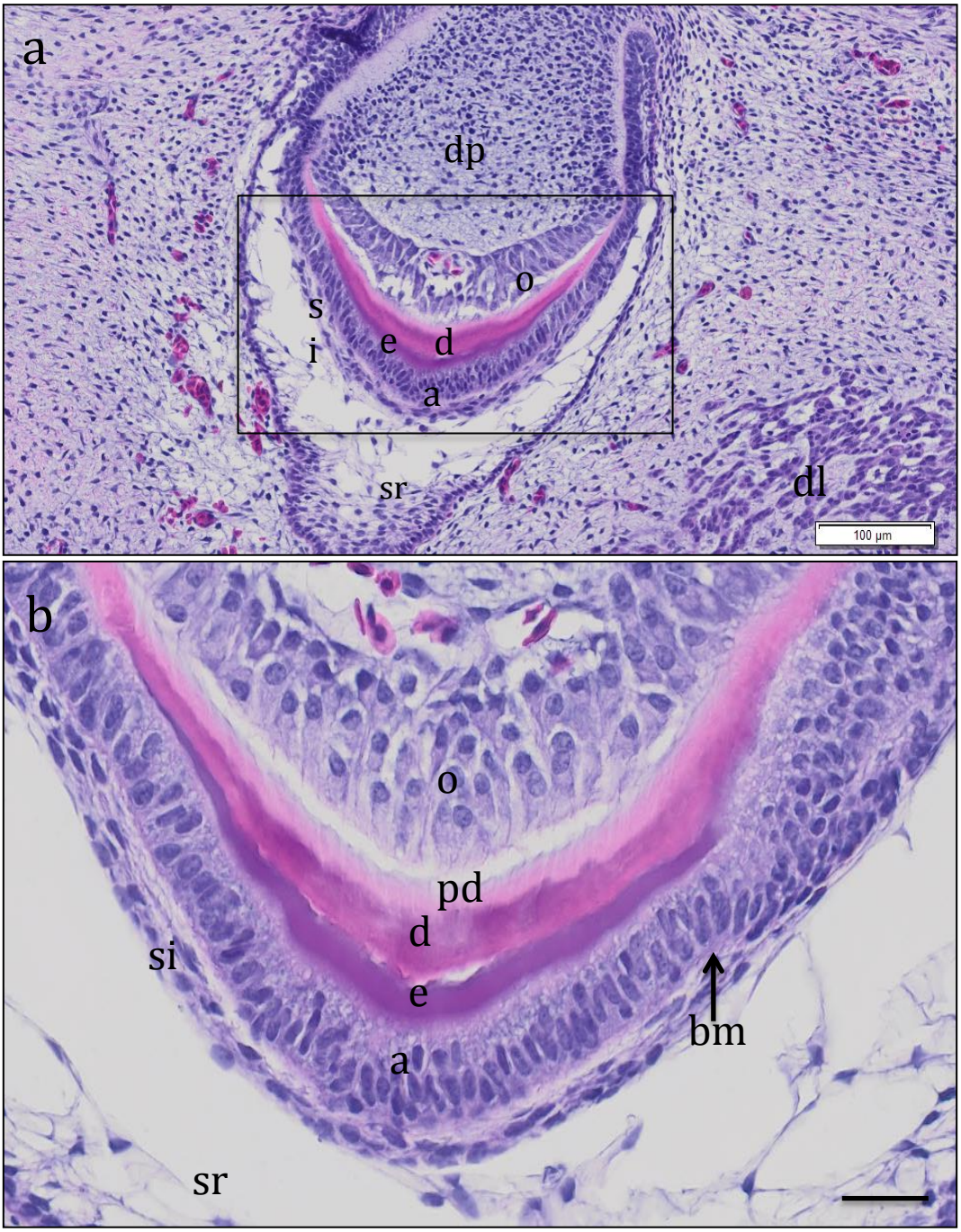

Figure 24a and b. Photomicrograph of a maxillary bell stage tooth germ in an embryo of the 30-65 day-old group. a: Note dp-dental papilla; o-odontoblasts; pd-predentine; d-dentine; e-enamel; si-stratum intermedium; sr-stellate reticulum; dl-dental lamina. b: High power photomicrograph of the rectangular area in **a**, showing the future crown area. Note the p odontoblasts (o) and the ameloblasts (a) resting on a basement membrane (bm). Haematoxylin and eosin. Scale bar: 20µm.

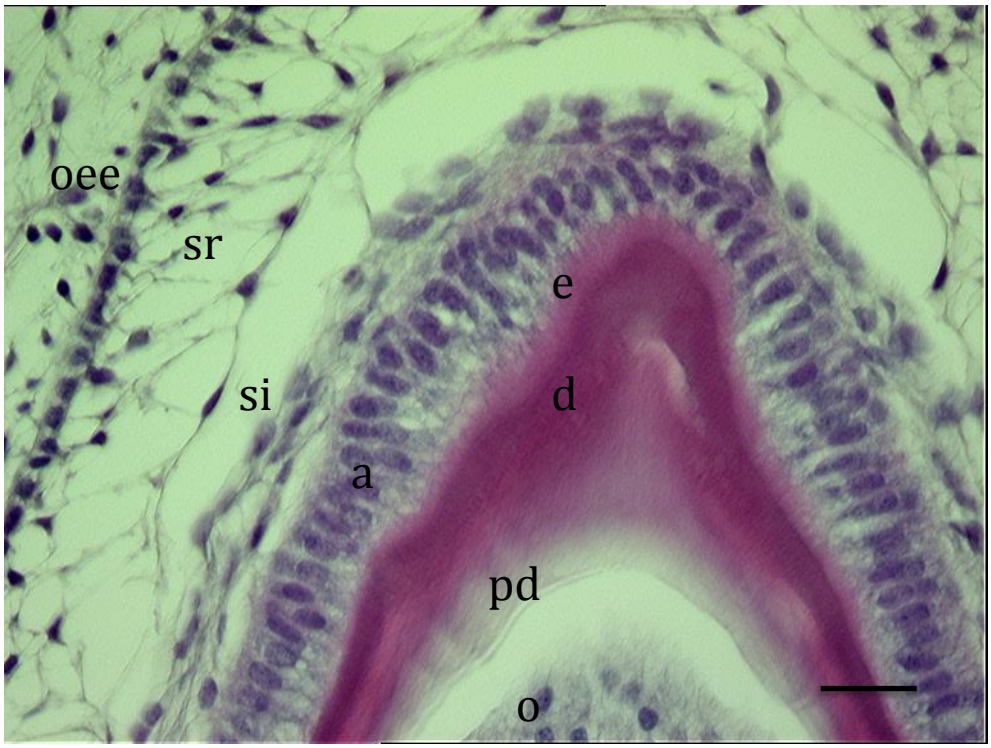

Figure 25. Photomicrograph of the crown of a late bell stage tooth germ indicating the single-cusped nature of the tooth. Note the distinct cuboidal outer enamel epithelium (oee), stellate reticulum (sr), stratum intermedium (si) and polarised ameloblasts (a) with their nuclei away from the adjacent enamel layer (e). Also note the distinct eosinophilic dentine (d) layer next to the paler predentine (pd) layer. o-odontoblasts. Haematoxylin and eosin. Scale bar: 20μm

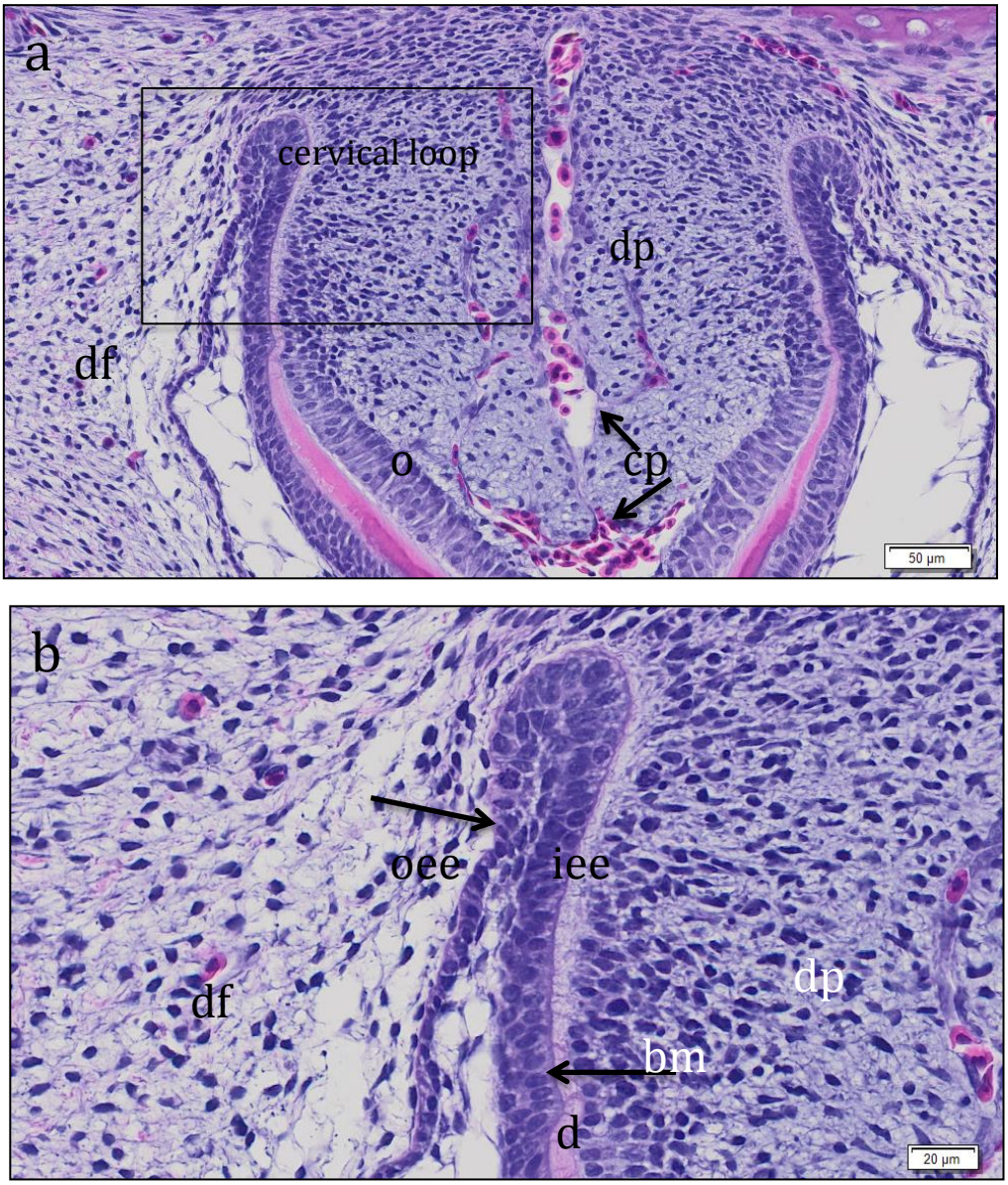

Figure 26 a-b. Photomicrograph of the apical region of a maxillary bell stage tooth germ in the 30-65 day old embryo group showing the cervical loop. a: The cervical loop is seen surrounding a wide apical foramen containing a dense condensation of ectomesenchyme. The dental papilla (dp) has numerous capillaries (cp) as well as a distinct subodontoblastic capillary plexus. b: The rectangular area in A is shown at higher magnification in **26b**. The inner (iee) and outer enamel epithelium (oee) meet at the cervical loop. There is a distinct area where the simple squamous oee changes to a simple cuboidal epithelium (arrow). The iee rests on a distinct basement membrane (bm). A less dense ectomesenchymal dental follicle (df) is evident at the apical aspect of the tooth germ. Haematoxylin and eosin.

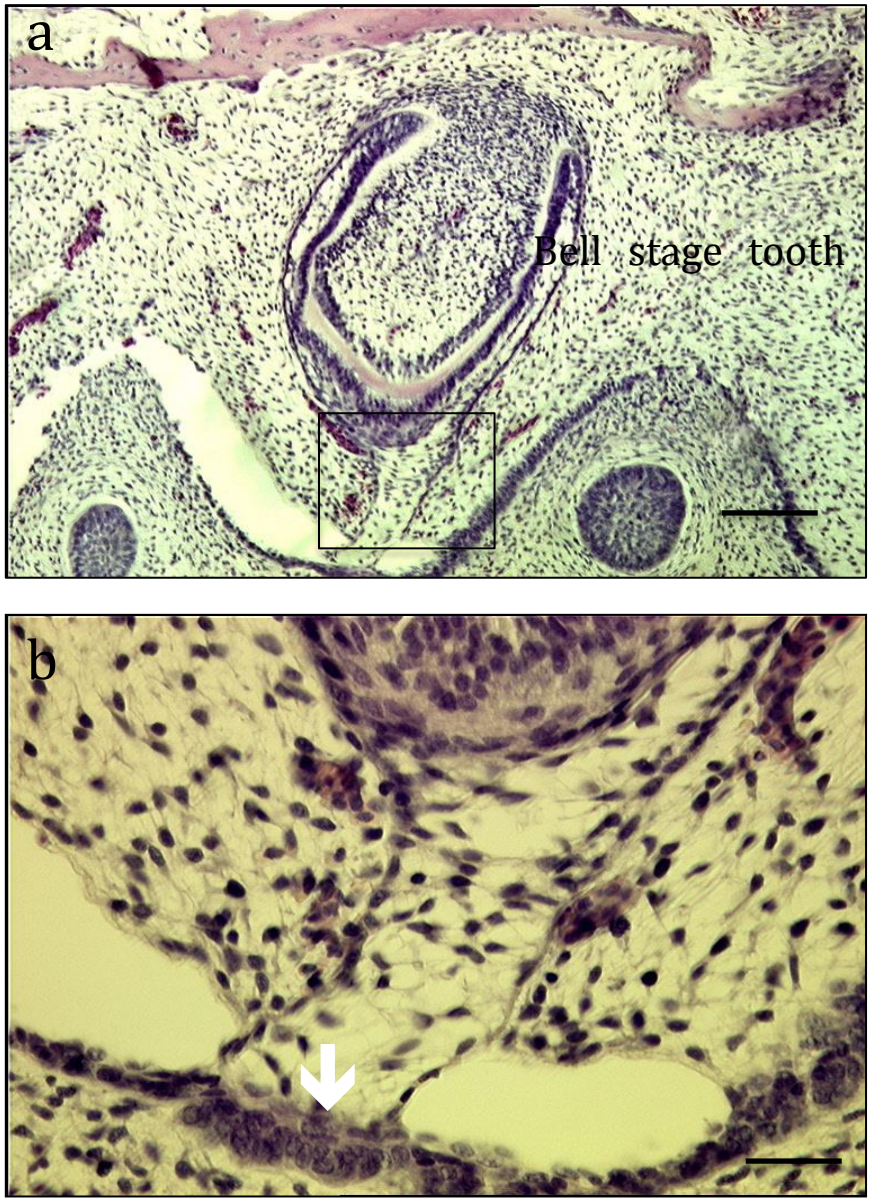

Figure 27 a and b. Photomicrographs depicting a funnel shaped structure associated with bell stage tooth germs a. Bell stage maxillary tooth germ in the 32-65 day-old embryo group showing some dentine but no enamel deposition. In the future cusp area, an epithelial lined funnel-shaped structure was attached to the dental lamina. b: The area in 27**a** was magnified depicting the funnel shaped structure associated with the dental lamina (arrow). The funnel shaped structure was lined by a simple squamous epithelium and was filled with stellate reticulum type tissue. Haematoxylin and eosin. Scale bar: a- 100µm, b-50µm

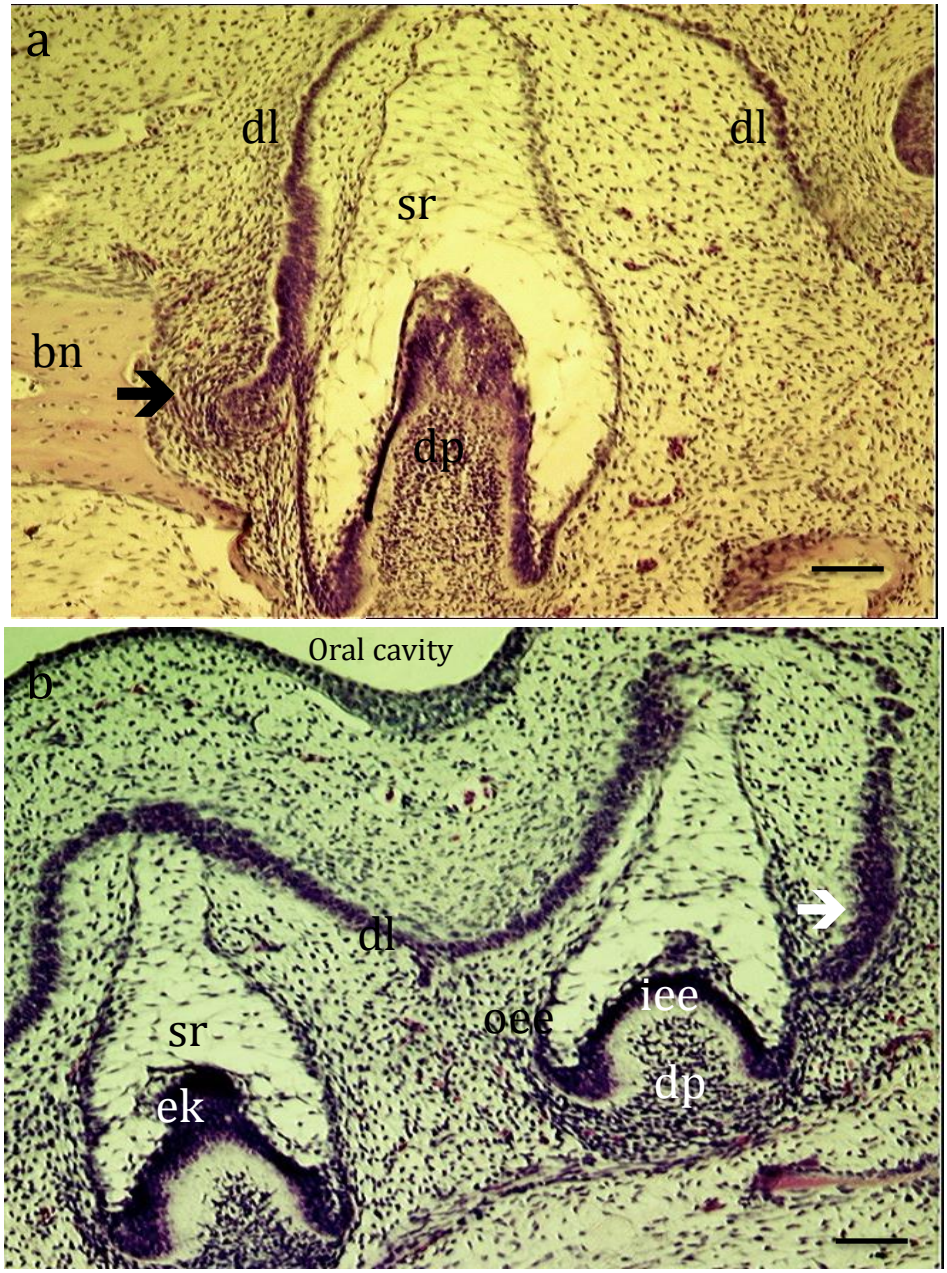

Figure 28 a and b. Photomicrographs depicting degenerating bell stage tooth germs in 32-65 day-old embryo group. a-b: Sections showing mandibular bell stage tooth germs joined by the dental lamina (dl). These tooth germs had an inner (iee) and outer enamel epithelium (oee), a well developed dental papilla (dp) and some dentine deposition, although a distinct odontoblastic layer was not identified. The bell stage tooth germs were often associated with a replacement tooth germ in the bud stage (arrows) of tooth development which was surrounded by a condensation of ectomesenchymal cells. The stellate reticulum (sr) as well as an enamel knot (ek) type structure was also identified. bn-alveolar bone. Haematoxylin and eosin. Scale bar: 50µm.

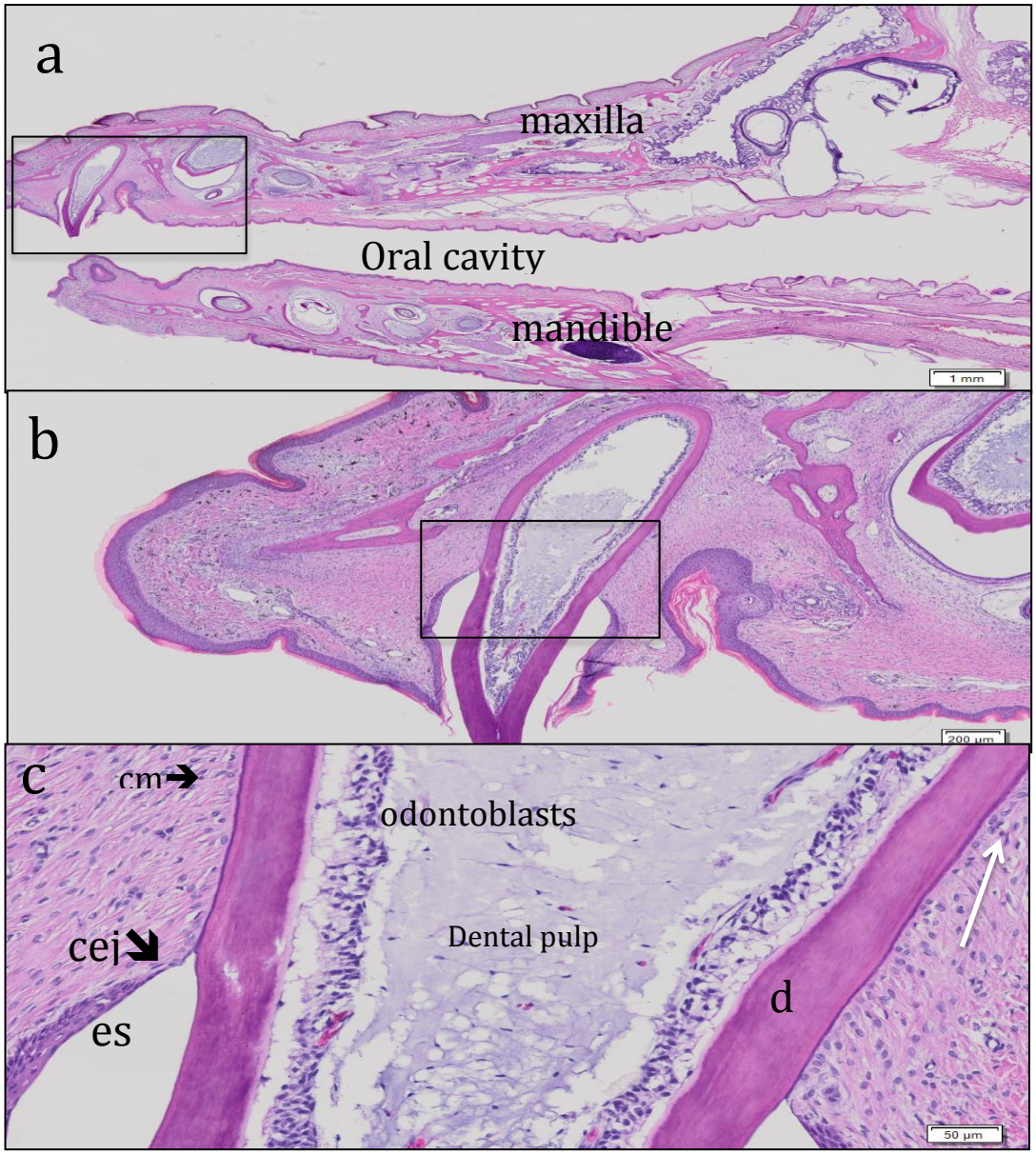

Figure 29 a-c. Representative histological sections of hatched and post-hatched specimens. a: Sagittal section showing the maxilla and the mandible of a crocodile hatchling. Note an erupted, functional maxillary tooth (rectangle). b: Higher magnification of the delineated area in **29a** showing the cemento-enamel junction. c: Increased magnification of the rectangular area in b showing the dental pulp (dp), odontoblasts (o), the dentine layer (d), the enamel space (es), the cementum (cm), and the cemento-enamel junction (cej). Also note the regular arrangement of the periodontal ligament fibres inserting into the cementum. The cementum thickness increased slightly towards the apex of the tooth (white arrow). Haematoxylin and eosin.

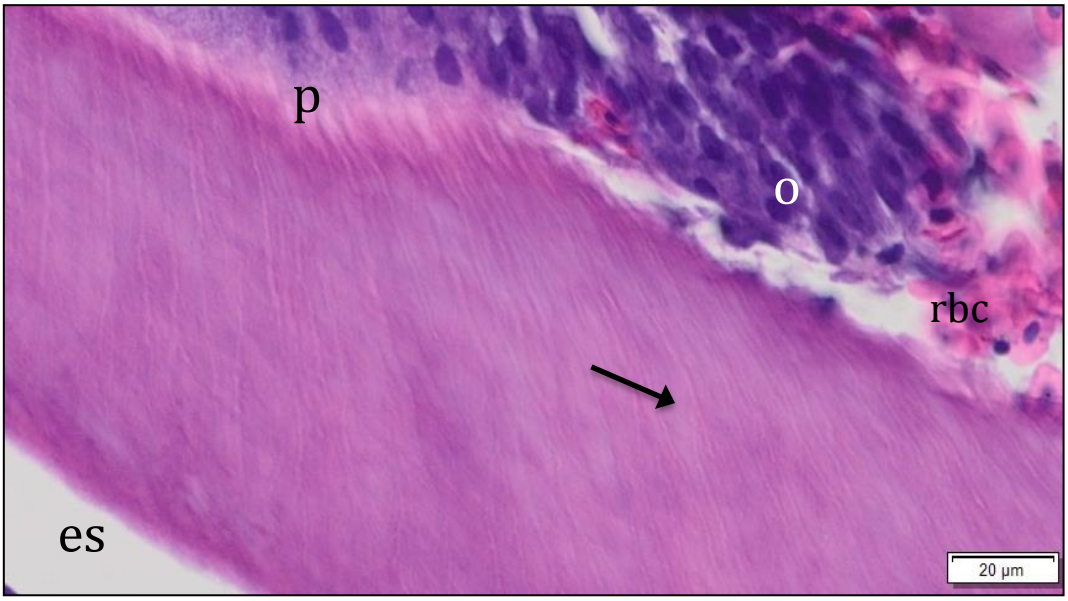

Figure 30. Representative photomicrograph showing dentinal tubules from a specimen of the hatched and post-hatched groups. The tubular nature of the dentine is evident throughout its thickness with some tubules branching (arrow). The odontoblasts (o) have odotoblastic processes which are evident in the paler predentine (p) layer. es-enamel space, rbc-red blood cells

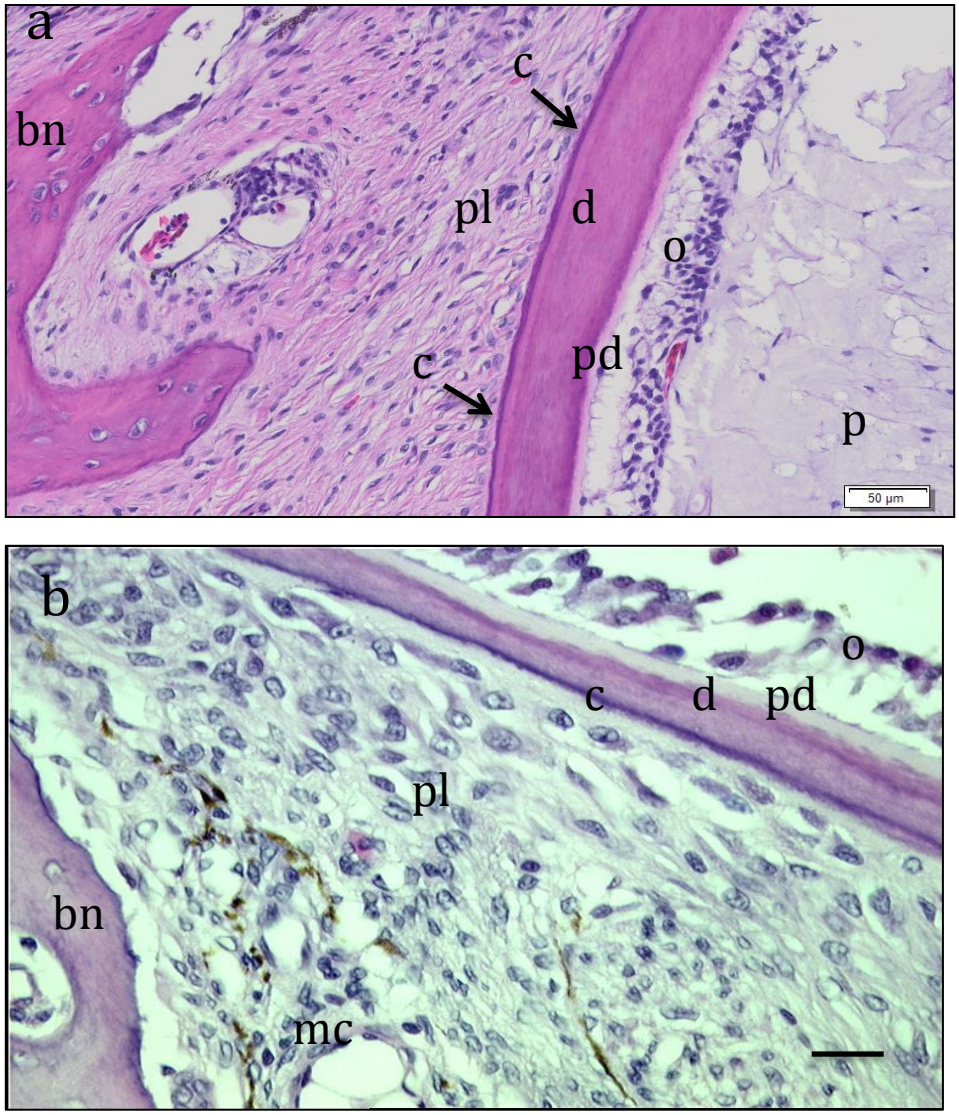

Figure 31a-b. Representative sections of the crocodile periodontium of hatched and post-hatched specimens. Note the bundles of periodontal ligament fibres. p-pulp; o-odontoblasts; pd-predentine;d-dentine; c-cementum; pl-periodontal ligament; bn-alveolar bone, n-nerve bundle, cp- capillaries. In **31b** note the dark staining phagocytic type cells (mc). Haematoxylin and eosin. Scale bar: 50µm.

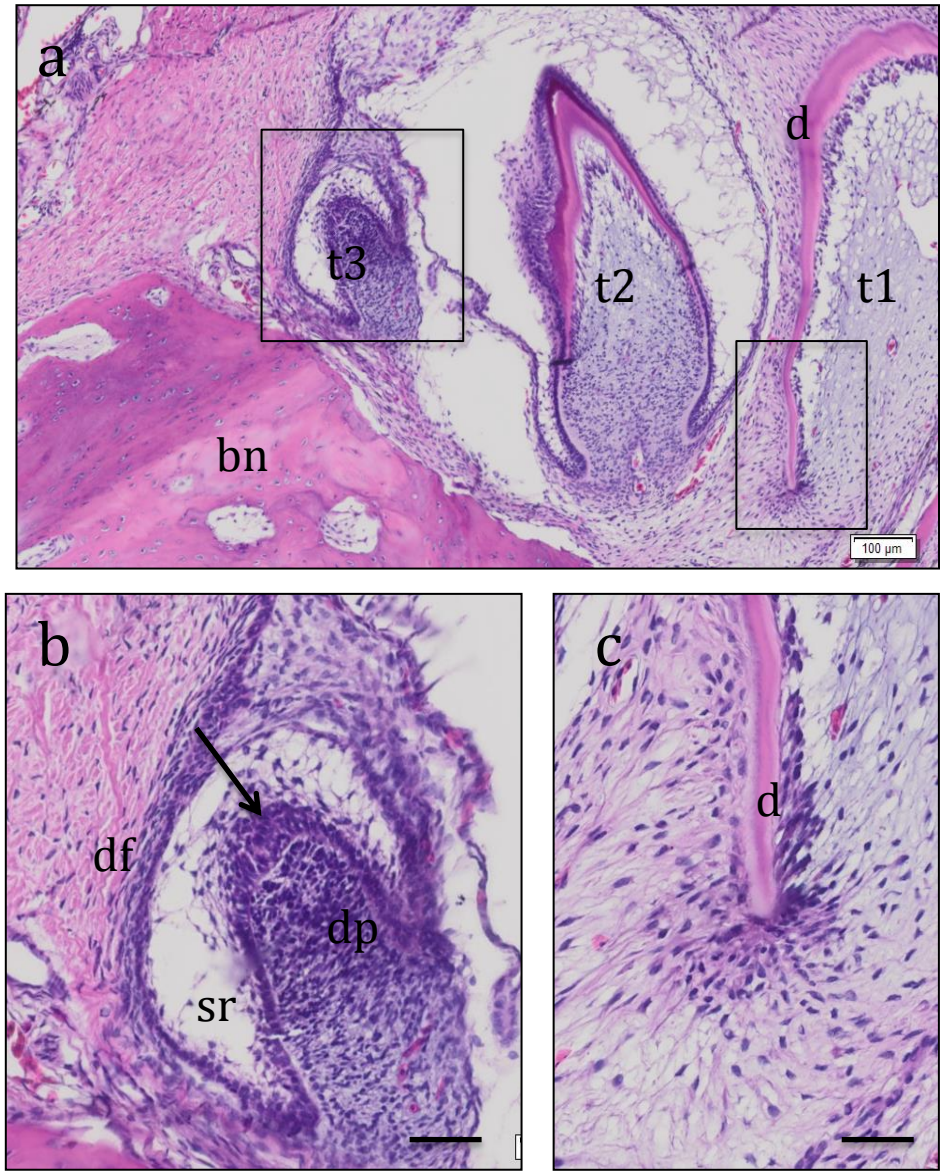

Figure 32a-c. Histological sections showing a "tooth family" in a socket of the hatched and post-hatched group. a: A section showing three tooth elements in a socket: t1-tooth undergoing resorption; t2-the replacement tooth of t1; t3-cap stage tooth germ which is the replacement tooth for tooth 2. bn-alveolar bone; d-dentine. b: Higher magnification of the cap-shaped tooth germ showing a highly condensed dental papilla (dp), a dental follicle (df) surrounding the enamel organ and a mass of cells that are associated with the inner enamel epithelium, indicating an enamel knot type structure (arrow); sr-stellate reticulum. c: Higher magnification of the apical end of tooth 1, showing a 'radial' arrangement of ectomesenchymal cells at the root tip d-dentine. Haematoxylin and eosin. Scale bar: 50µm.

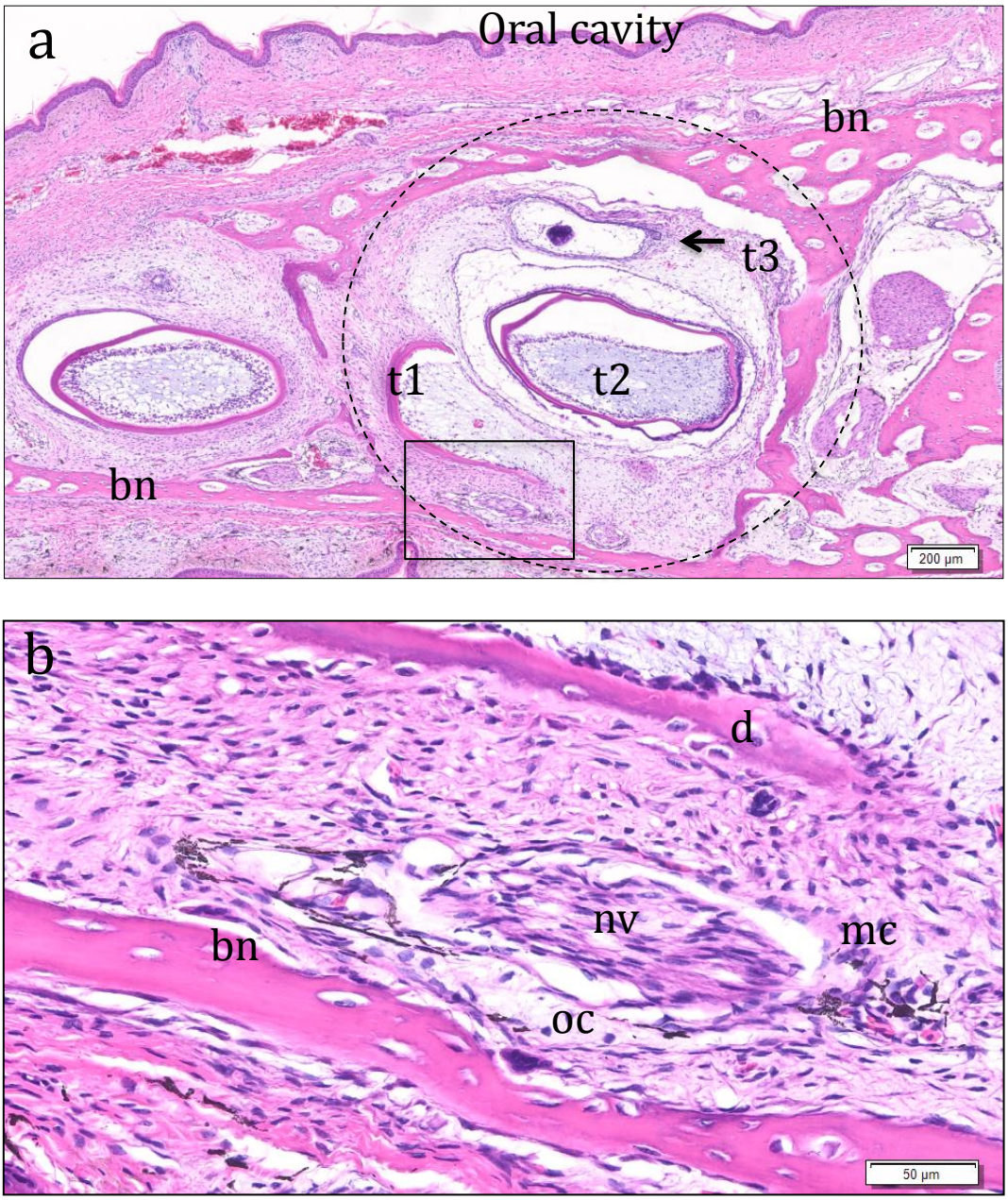

Figure 33 a-c. Photomicrographs depicting tooth elements in and around a tooth socket of specimens from the hatched and post-hatched group. a: Sagittal histological section of the mandible showing a tooth socket (dotted circle) formed by alveolar bone (bn) and housing a tooth family: t1 represents a degenerating tooth, t2 is a bell stage tooth germ, replacing tooth t1 and t3 shows the dental lamina with a dilated terminal end, indicative of the bud stage of tooth development. b: Higher magnification of the rectangular area in figure **33a** showing the dentine (d) of the degenerating tooth (dt), the alveolar bone (bn) of the socket, the periodontal ligament which contains a nerve bundle (nv) and macrophage-type cells (mc). Osteoclast type cells (oc) were also evident in the lacunae of the bone. Haematoxylin and eosin.

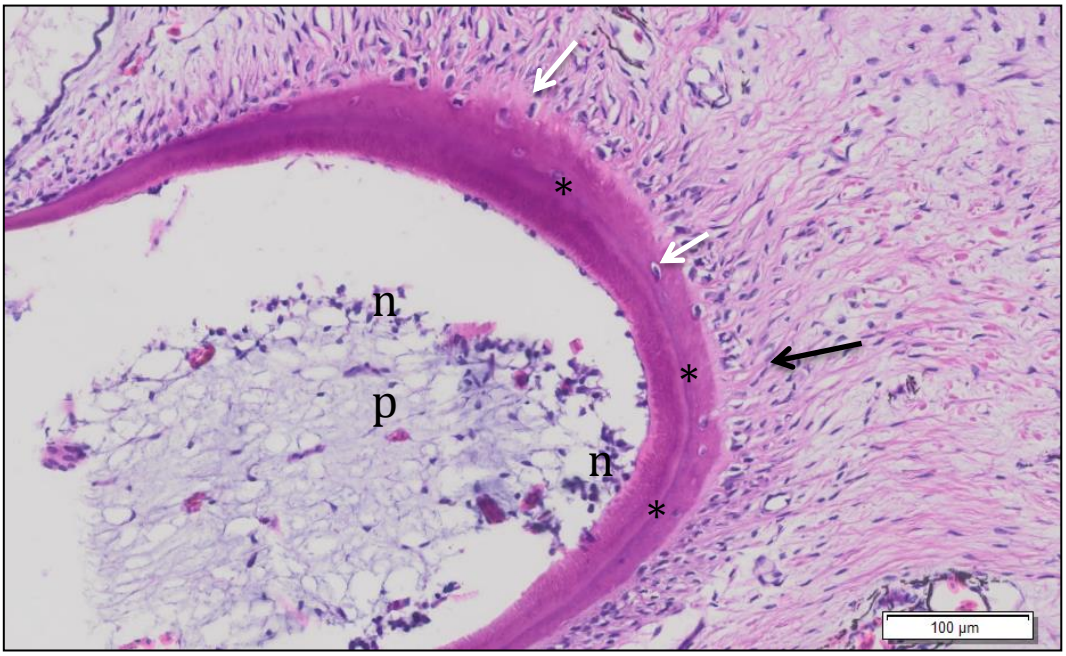

Figure 34. Photomicrograph showing a remnant of dentine of a tooth crown in the ectomesenchyme. Note the fibrous pulp (p) with scattered rounded nuclei at its periphery (n). Note also the incremental nature of the dentine (*) and the lacunae with nuclei (white arrows) as well as the wavy arrangement of the collagen-type fibres on the surface of the dentine (black arrow). Haematoxylin and eosin.

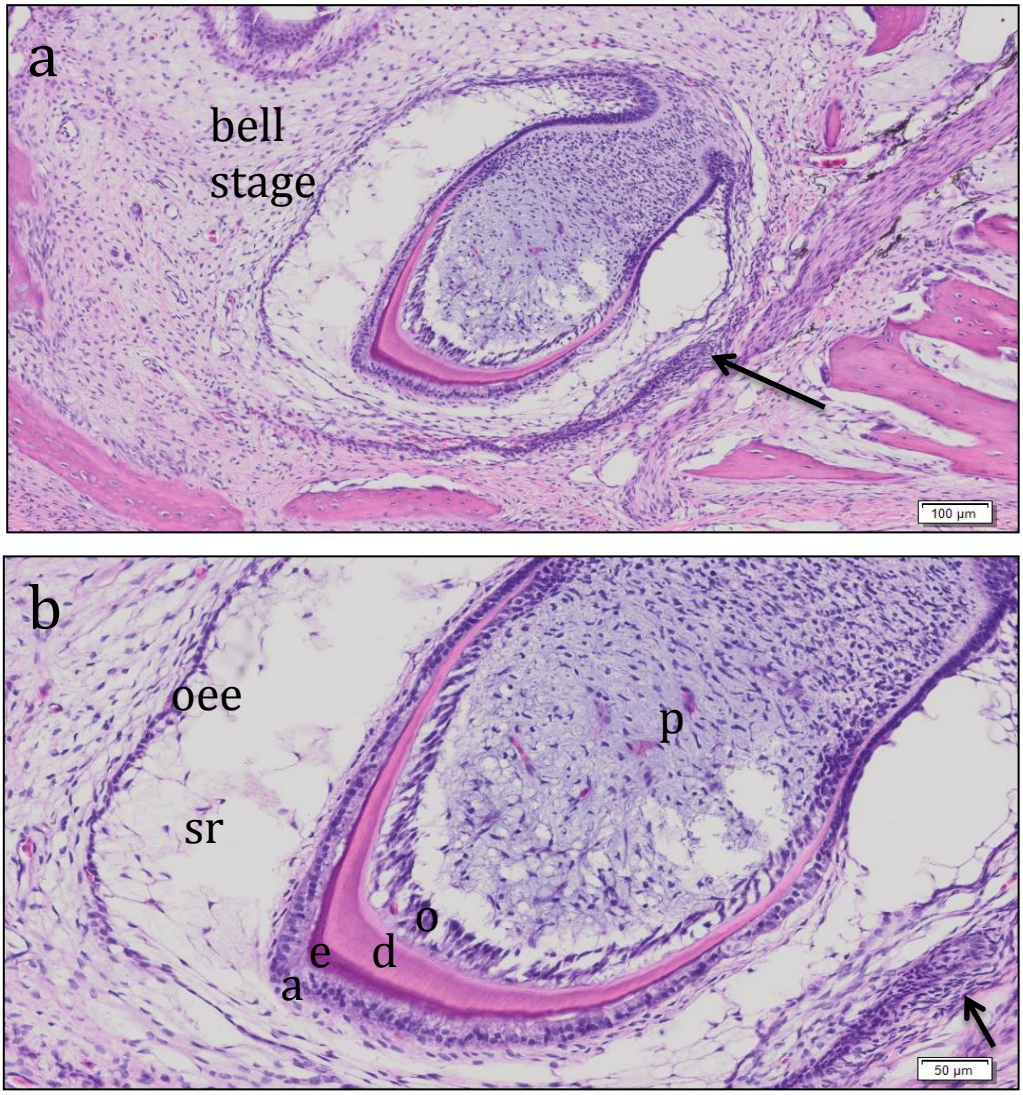

Figure 35 a-b. Photomicrographic representations of a bell stage tooth germ associated with a replacement tooth in the bud stage of tooth development.
a: Maxillary tooth in the bell stage with distinct enamel (e) and dentine (d) layers. Note replacement tooth in the bud stage (arrow) of tooth development. b: Higher magnification of the tooth germ in **35a** where the dental lamina was evident adjacent to the bell stage tooth germ, with a dilated terminal end (arrow). oee-outer enamel epithelium; o-odontoblasts; a-ameloblasts; sr-stellate reticulum; p-pulp. Haematoxylin and eosin.

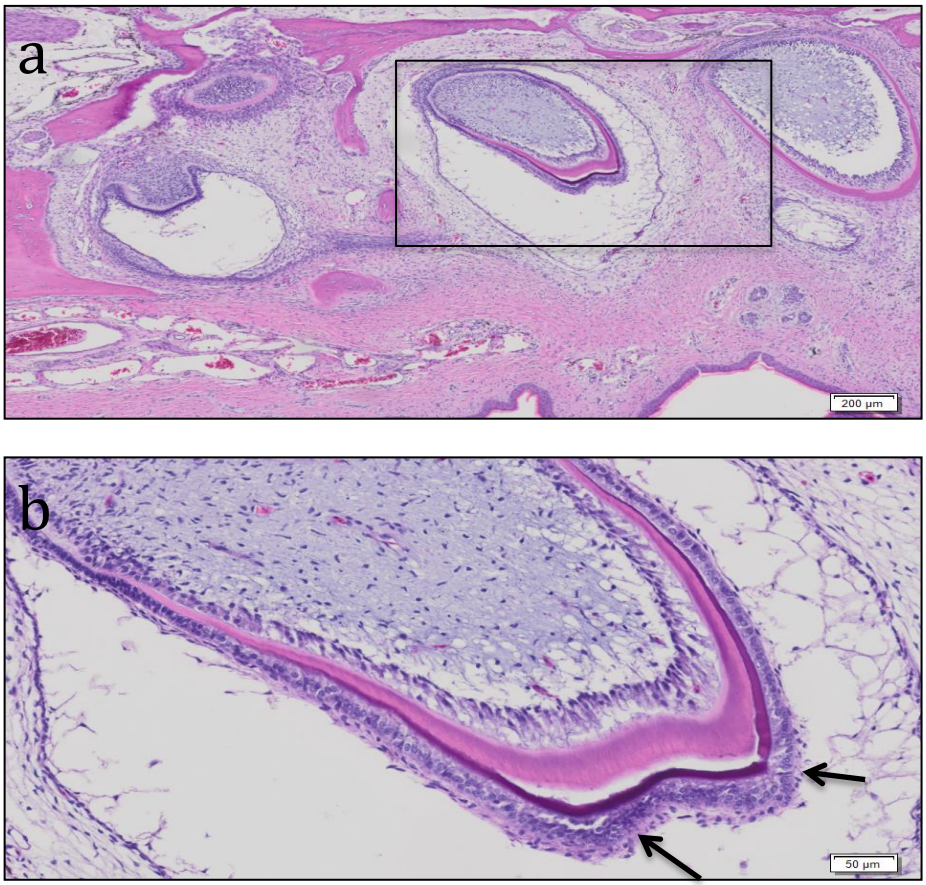

Figure 36 a-b. Bell stage tooth germ with a molariform appearance. a: Maxillary bell stage tooth germ, in an alveolar socket, showing two cusp-like structures on the coronal surface. b: Higher magnification of the rectangular area in **36a** showing the cusp-like structures (arrows) of the molariform tooth germ. Haematoxylin and eosin.

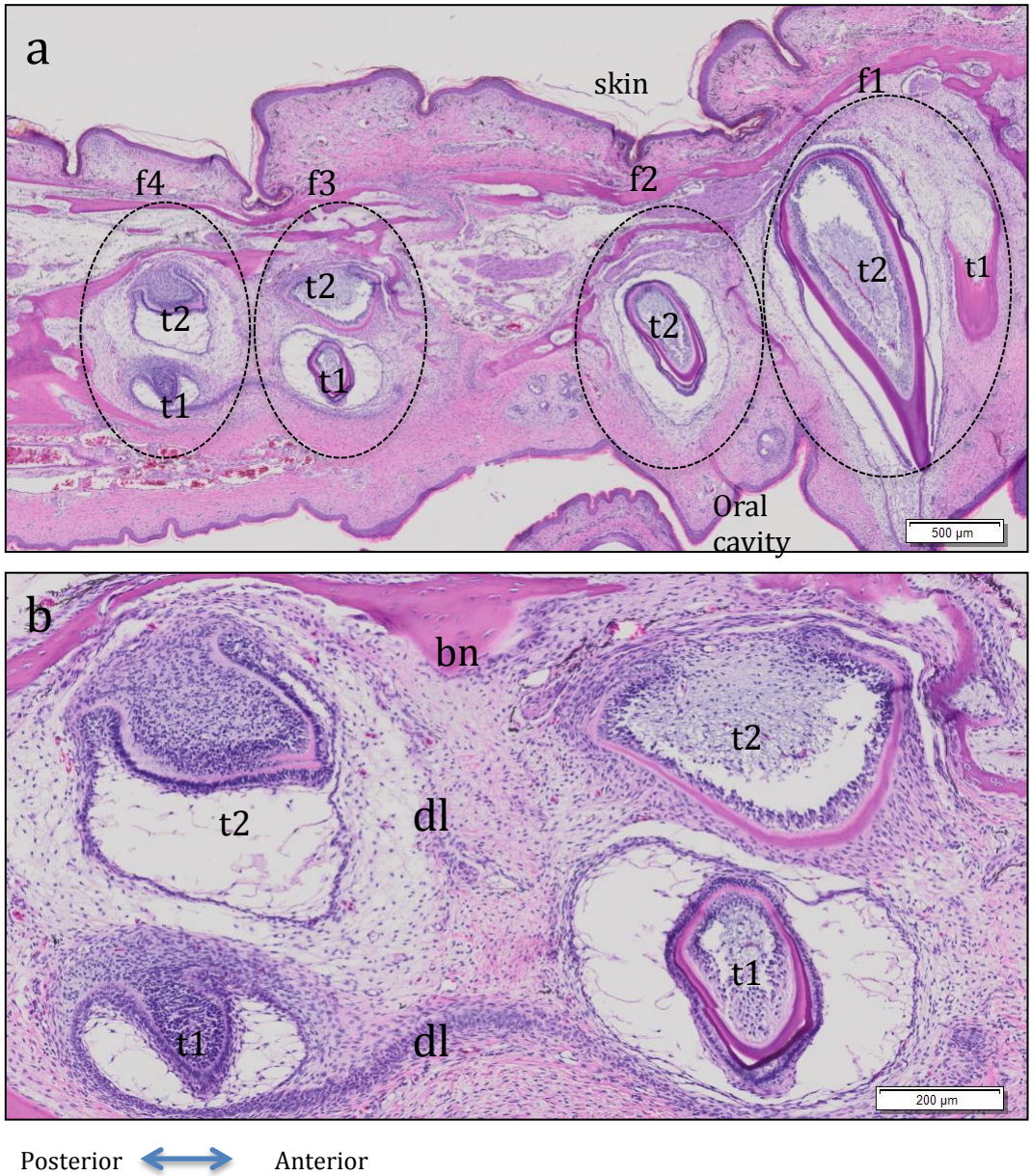

Figure 37a and b. Tooth replacement along the length of the maxilla in a specimen from the hatched and post-hatched group a: Photomicrograph showing a sagittal section of the maxilla. From anterior to posterior, four tooth families (f1-f4) were evident along the length of the maxilla. In f1, t1 (the degenerating tooth) was evident with its successor (t2). In f2 only one member of this tooth family was evident- t2 (confirmed by examination of serial sections). In the more posterior tooth families f3 and f4, t1 and t2 were evident respectively. b: High magnification photomicrograph of tooth families f3 and f4. Adjacent tooth families are still connected via a dental lamina (dl). It is interesting to note that t1 and t2 of both f1 and f2, are at similar developmental age. bn-alveolar bone. Haematoxylin and eosin.

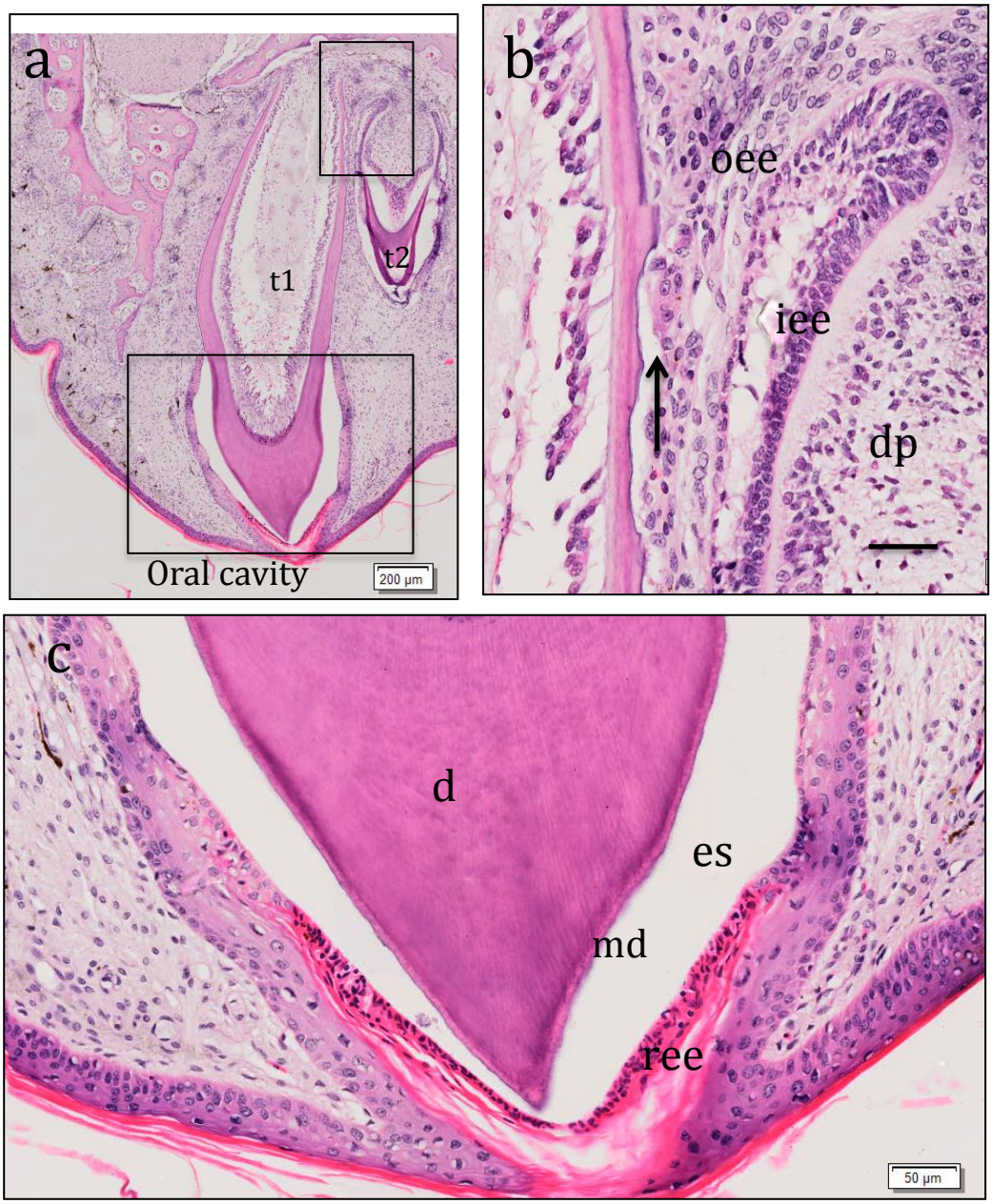

Figure 38 a-c. Tooth eruption and root resorption in the hatched and post-hatched crocodile specimens. a: Photomicrograph showing a well developed pre-eruptive tooth (t1) with a bell stage replacement tooth germ (t2) at its apical end. b: Higher magnification of the rectangular area at the apical aspect of the tooth in 23**a**. Root resorption of the dentine and cementum was evident due to the presence of resorption lacunae. Multinucleate eosinophilic osteoclast type cells (arrow) were present adjacent to the lacunae (arrow). Ooe-outer enamel epithelium; iee-inner enamel epithelium; dp-dental papilla. c: Higher magnification of the rectangle delineating the crown aspect of the tooth in 23**a.** Note dentine (d) with radiating dentinal tubules. The initial layer of 'mantle'dentine (md), adjacent to the enamel space, appeared as a distinct eosinophilic layer. The cusp of the tooth was covered by the reduced enamel epithelium (ree). es-enamel space. Haematoxylin and eosin. Scale bar: 50µm.

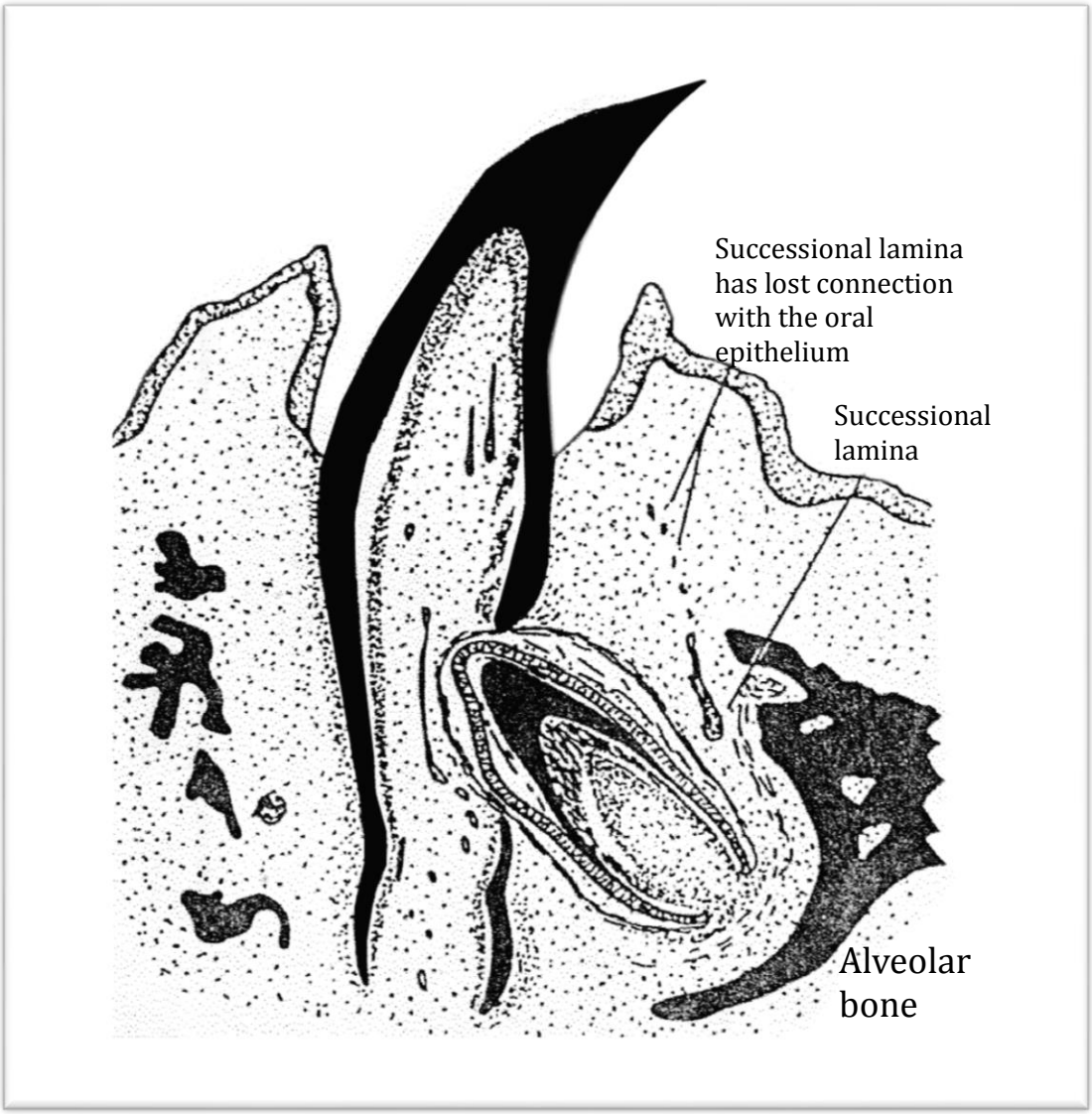

Figure 38d. Schematic representation of a crocodilian mandibular functional tooth with a replacement tooth germ on its lingual aspect. Both structures are housed in an alveolar bony socket, highlighting the thecodont nature of the socket (Adapted from Edmund, 1962)

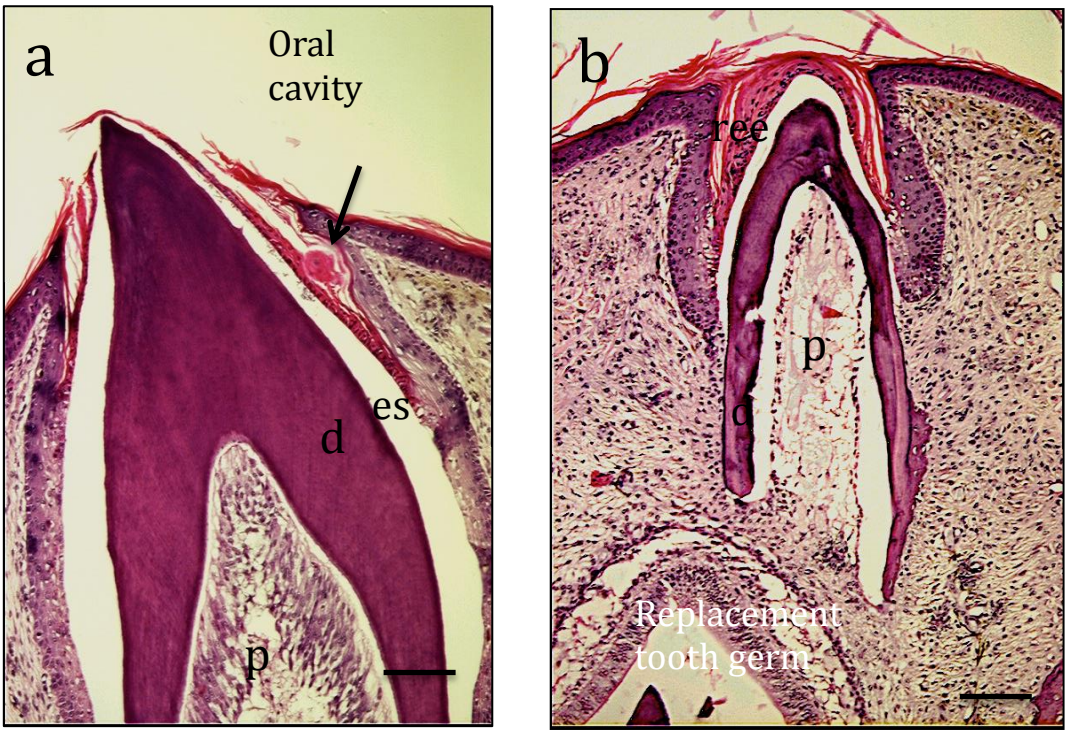

Figure 39 a and b. Eruption of crocodile teeth in specimens from the hatched and post-hatched group. a: An erupted well-developed conical tooth where the crown was already exposed into the oral cavity. Remnants of the reduced enamel epithelium were evident on the lateral surface of the crown. A rounded multinucleate eosinophilic structure (arrow) was evident the reduced enamel epithelium. d-dentine with radiating dentinal tubules: es-enamel space; p-pulp. b: A replacement tooth germ was evident inferior to a well developed mandibular tooth which appeared to be degenerating, prior to eruption. The reduced enamel epithelium (ree) overlying the tooth was distinct. p-pulp cavity; es- enamel space; d-dentine. Haematoxylin and eosin.
Scale bar: a - 50µm b -100µm.

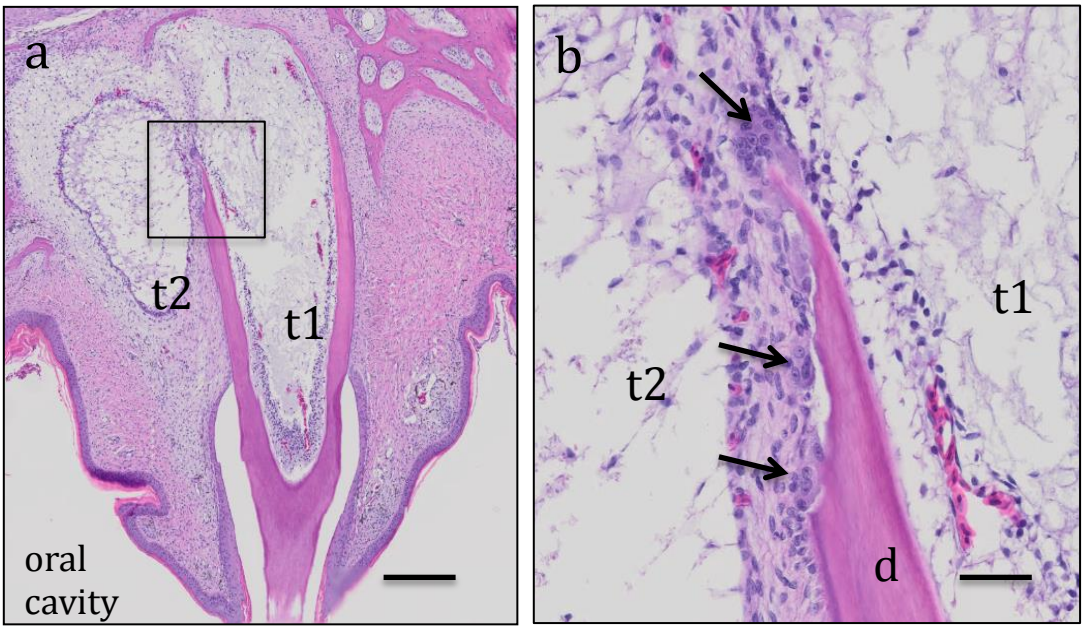

Figure 40 a and b. Photomicrographs showing a portion of a replacement tooth and osteoclast–type cells at the apical region of a functional tooth from a specimen of the hatched post-hatched group. a: Sagittal section of maxillary functional tooth (t1) and replacement tooth germ (t2). b: Higher magnification of the square in **40a.** Note the area in between the functional tooth (t1) and replacement tooth (t2) where osteoclastic activity was evident. The dentine (d) had lacunae associated with osteoclast-type cells (arrows). Haematoxylin and eosin. Scale bar: a-200µm b-50µm

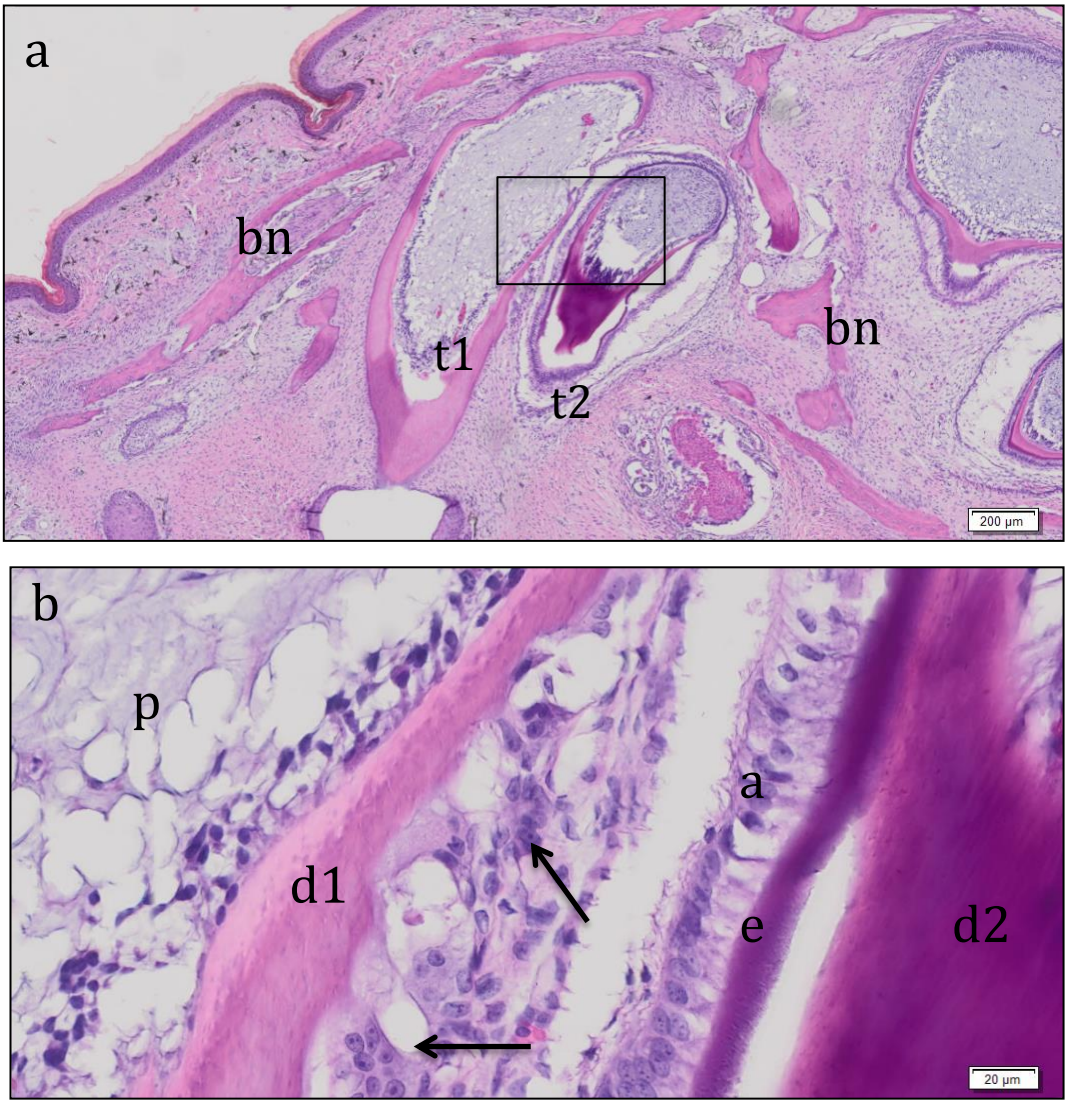

Figure 41 a and b. Photomicrographs showing a well-developed functional tooth with an associated replacement bell stage tooth germ of the hatched and post-hatched group. a: Sagittal section through functional maxillary tooth (1) and replacement tooth (2), surrounded by a bony socket (bn). The replacement tooth was closely associated with the dentine layer of the functional tooth. b: Higher magnification of the rectangular area in **41a**. Note the vacuolated appearance of the pulp (p), the predentine and dentine of the functional tooth (d1), the osteoclast-type cells (arrows) associated with the uneven surface of d1, the ameloblasts (a) and enamel layer (e) and the dentine layer of the replacement tooth (d2). Haematoxylin and eosin.

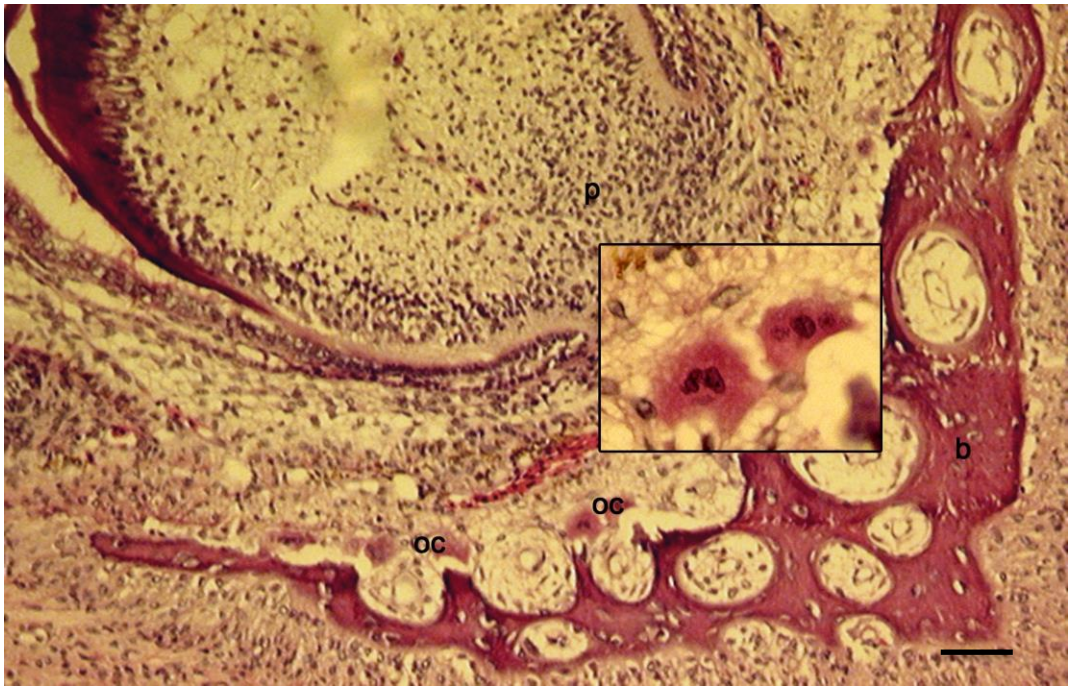

Figure 42. Osteoclast-type cells associated with bone spicules around the apex of a developing tooth. Note the deep lacunae reminiscent of Howship's lacunae, in the bone spicule (b). Insert shows higher magnification of multinucleate osteoclast type cells (oc). These cells were eosinophilic and had an uneven shape. p-pulp. Haematoxylin and Eosin. Scale bar: 100μm

Table 5. The relationship between the state of functionality of erupted teeth and the state of differentiation of the replacement teeth.

Functionality of erupted tooth (ET)	Number of erupted teeth	Stage of associated replacement tooth	Number of replacement teeth	Percentage %
Attached ET	25	Successional lamina	4	16
		morphogenesis	8	32
		cytodifferentiation	13	52
Young ET	25	morphogenesis	7	28
		cytodifferentiation	18	72
		absent		0
Mature ET	25	absent		0
		morphogenesis	9	36
		cytodifferentiation	16	62
In Resorption	25	absent		0
		morphogenesis	6	25
		cytodifferentiation	19	75

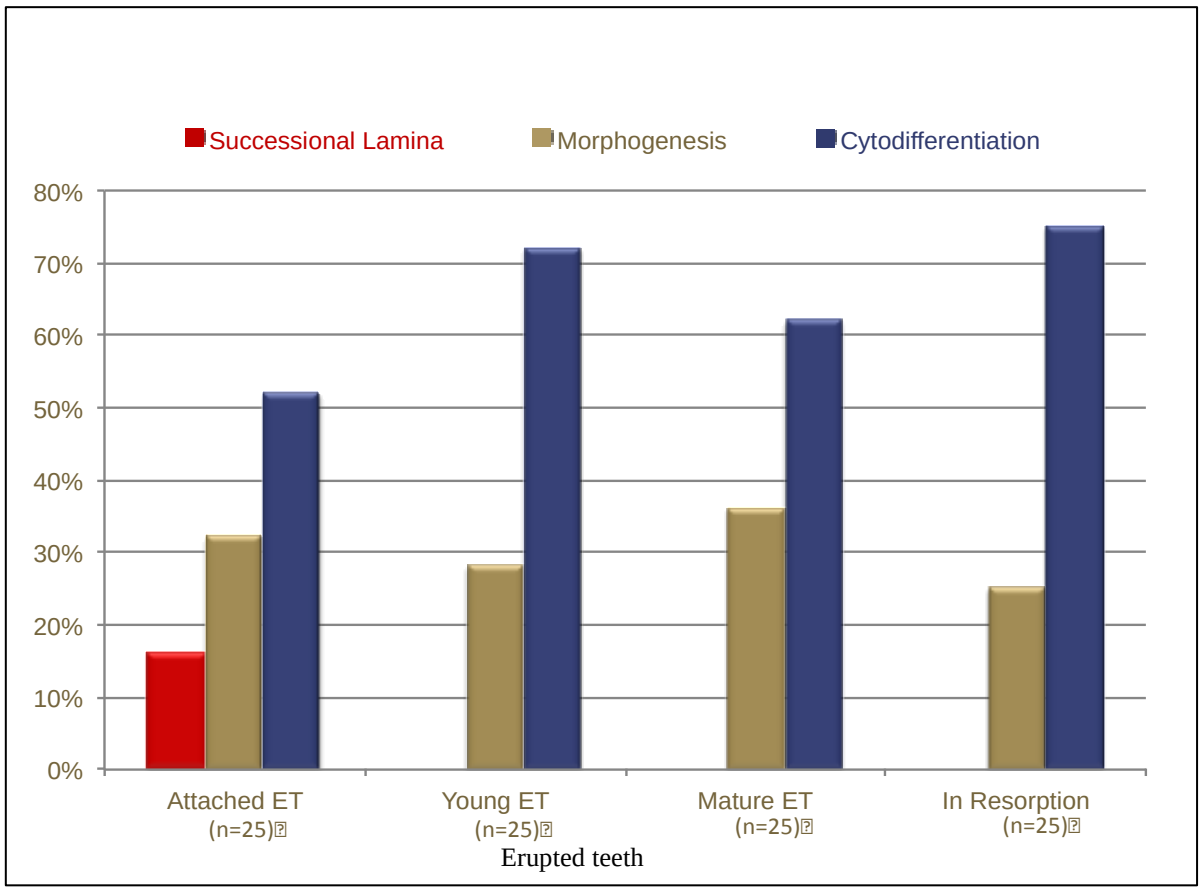

Figure 43. The relationship between the state of functionality of erupted teeth (ET) and the state of differentiation of the replacement teeth in hatched and post-hatched specimens.

3.4 In situ hybridisation

3.4.1 PCR amplification of *gapdh*, *bmp4* and *pitx2*

The PCR, using the relevant primers, revealed three sequences via agarose gel electrophoresis: *gapdh* (568 base pairs) (Fig. 44), *bmp4* (630 base pairs) (Fig. 45) and *pitx2* (650 base pairs) (Fig. 45). The primers produced consistent results for all three sequences. The sequences were published in GenBank :

(a) *Crocodylus niloticus* glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene, partial coding sequences (cds), GenBank acc. no. JX978722

(b) *Crocodylus niloticus* bone morphogenetic protein 4 (*bmp4*) gene, partial cds., GenBank acc. no. JX978721 and

(c) *Crocodylus niloticus* paired-like homeodomain transcription factor (p*itx2)* gene, partial cds., GenBank acc. no. JX978720

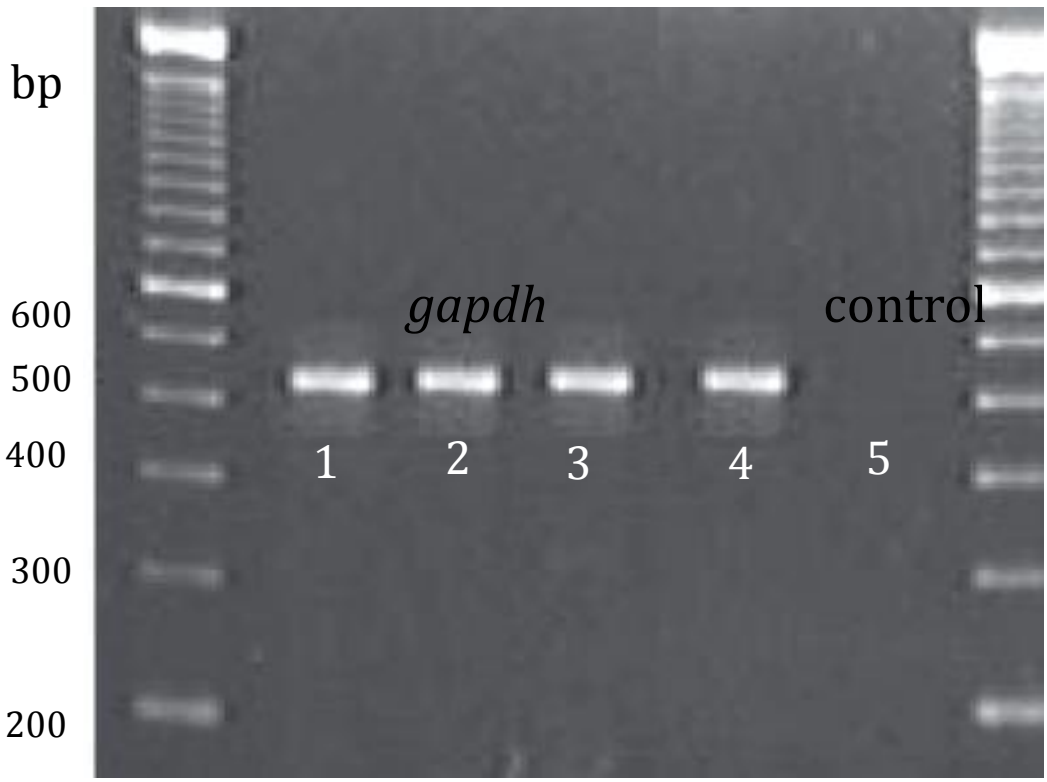

Figure 44. PCR amplification of *gapdh* gene to assess mRNA quality. A ~560 bp region of the *gapdh* gene was amplified and resolved on a 1% EtBr agarose gel. The amplification was evident for all four samples (lanes 1-4) of *gapdh* with no amplification in the negative control (lane 5).

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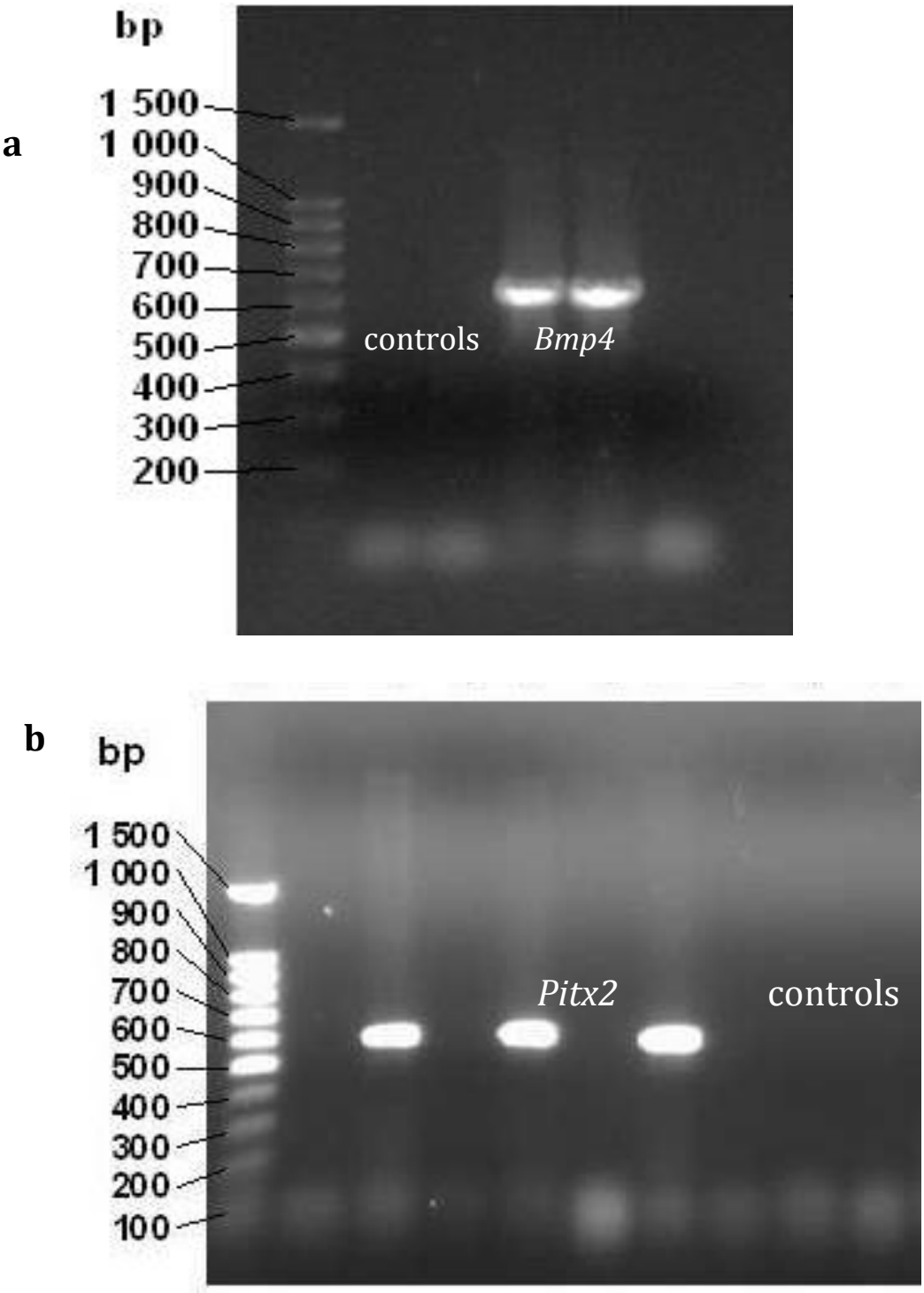

Figure 45 a-b. PCR amplification of *bmp4 and pitx2* genes to assess mRNA quality. a: A ~630 bp region of the *bmp4* gene was amplified and resolved on a 1% EtBr agarose gel. The amplification was evident for both samples of *bmp4* with no amplification in any of the negative controls. b: A ~650 bp region of the *pitx2* gene was amplified.

3.4.2 Controls for *gapdh, bmp4* and pitx2

The expression of *gapdh, bmp4* and *pitx2* was examined in the crocodile specimens of all age groups. Three types of controls were carried out to determine the specificity of the *in situ* hybridisation of *gapdh*, *bmp4* and *pitx2*. In the first control, selected serial sections of the crocodile specimens from all the age groups were hybridised with *gapdh, bmp4* or *pitx2* sense and anti-sense probes respectively. The anti-sense probes for *gapdh, bmp4 or pitx2* were used to detect the target mRNA with sequence specific binding and the sense probes served as the control probes. The sections hybridised with the sense probes for *gapdh*, did not show *gapdh* expression (Fig. 46d). This was also true for *bmp4* (Figs. 48c, 51c, 56d) and *pitx2* (Figs. 62c, 63b) indicating that any expression evident was the anti-sense mRNA binding to the corresponding region in the tissue.

In the second type of control carried out to indicate the specificity of the antibody used in the detection procedure, the sheep anti-DIG alkaline phosphatase antibody was replaced with buffer. These control sections also showed no expression of *gapdh* (Fig. 47c), *bmp4* (Figs. 53c) or *pitx2* (Figs. 60d).

The third control, applied on adjacent serial sections, involved replacing the anti-sense probe with buffer to indicate that any expression evident was due specifically to the anti-sense probe and similarly this control did not reveal any expression of *bmp4* (Fig. 52c) or *pitx2* (Fig. 64c, 65c).

3.4.3 *In situ* hybridisation - *gapdh*

Gapdh, the housekeeping gene, has been widely used in mammalian gene expression studies. Expression of *gapdh* in the sections of the crocodile heads, of all ages examined, was apparent in several tissue types throughout the head region. Specifically, *gapdh* expression in the 14-31 day-old embryos was uniformly expressed in the gray and white matter of the cerebral cortex. However,

expression of *gapdh,* in the gray matter, in the 32-65 day, hatched and post-hatched embryos was expressed more intensely in some clusters of cells (Fig. 46a) as compared to the surrounding cells, but a pattern was not discernable between the age groups. In the white matter, in all the age groups examined, *gapdh* expression was evident in the nerve-type fibres, with some expression in the nuclei associated with the nerve-type fibres (Fig. 46b). Intensified expression of *gapdh,* was detected in the bundles of nerve-type fibres lying within the white matter (Fig. 46b). These were identified by their wavy course. This occurred in all the specimens examined.

*Gapdh e*xpression was consistent and present in all the layers of the retina of the eye (Fig. 46c), except for the pigmented layer of the retina, in all the specimens of all the age groups. The pigmented layer of the retina as well as the pigmented cells of the connective tissue supporting the retina, did not show *gapdh* expression but were distinguished due to the presence of the intense brown pigment. Also, *gapdh* expression was evident in the cartilage plate supporting the eyeball (Fig. 46c), as well as in the collagen-type fibres and associated nuclei of the connective tissue (Fig. 46c) of the head region. Distinctive expression of *gapdh* was seen in striated skeletal muscle in both cross (Figs. 47a,b) and longitudinal (Figs. 47a,b) sections. In cross section, intense expression was visible in the striated skeletal muscle fibres, with no observed expression in the connective tissue perimysium between the striated muscle fascicles (Fig. 47a). The connective tissue ectomysium, around a large mass of skeletal muscle fibres, displayed some expression, as did the surrounding connective tissue (Fig. 47a). In the pre-hatched groups *gapdh* expression was evident uniformly throughout the muscle fibres, but in the hatched and post-hatched specimens, expression was confined to only specific areas of the muscle fibres, with other areas showing little or no expression (Fig. 47b). No distinct pattern was discernable in specific regions of the muscle fibres but expression was evident in the pyknotic nuclei at the periphery of the

fibres (Fig. 47b). Also, the skeletal muscle striations strongly expressed *gapdh*, making the band-type arrangement of striated muscle identifiable (Fig. 47b).

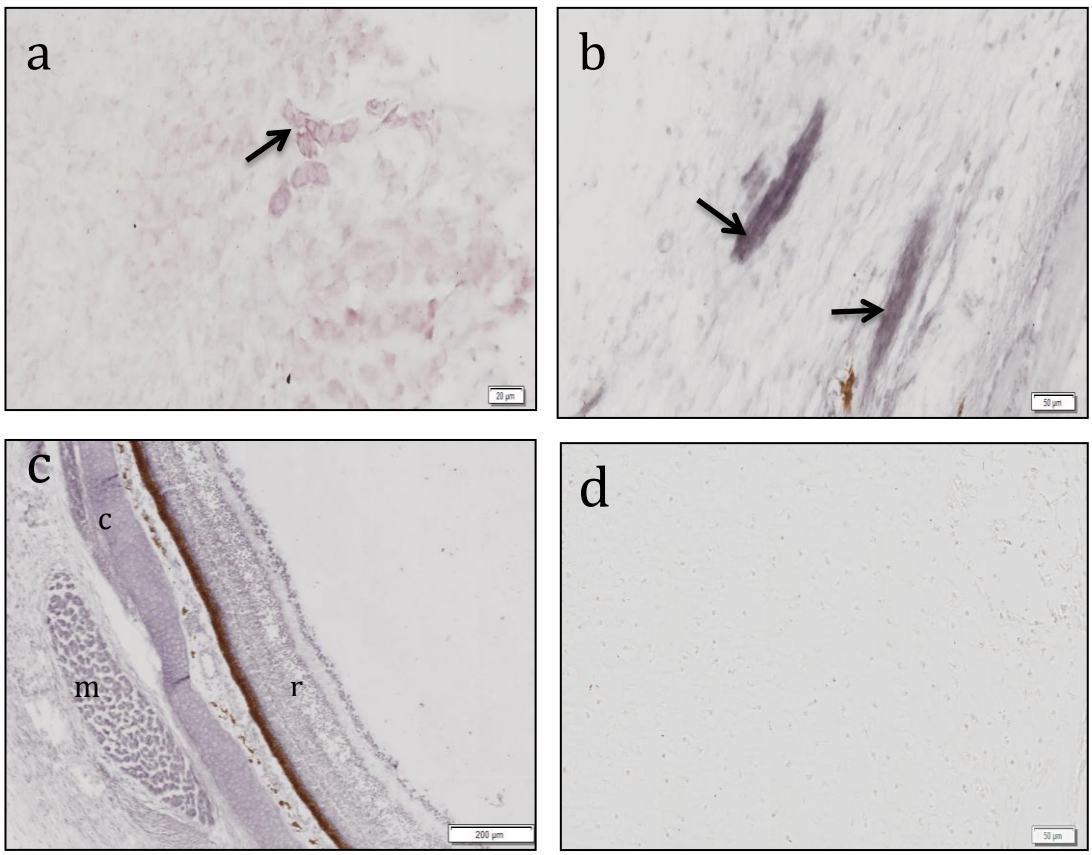

Figure 46 a-d. Representative sections showing g*apdh* expression in the cerebral cortex and the eye of a specimen from the 32-65 day-old hatched and post-hatched group. a: *Gapdh* expression in the cells of the cerebral cortex (arrow). b: *Gapdh* expression in the white matter of the cerebral cortex. Some groups of fibres forming bundles, had intense expression of *gapdh* (arrows). c: *Gapdh* expression was evident in the layers of the retina (r) of the eye and in the cartilage supporting the eye (Note the pigment (p) layer is indicated by the heavy brown pigment. d: Control section serial to **46a**, hybridised with sense probe showing no *gapdh* expression.

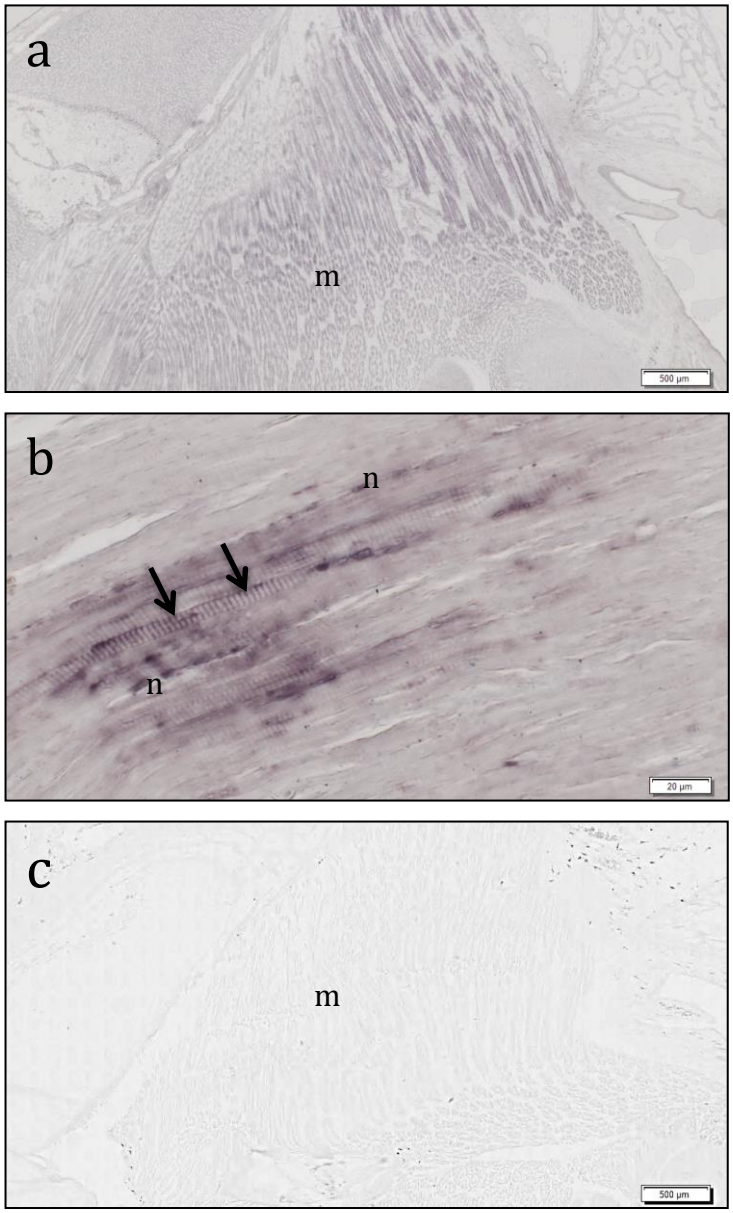

Figure 47 a and b. Representative photomicrographs demonstrating *gapdh* expression in the striated muscle in the crocodile head. a: Skeletal muscle (m) in longitudinal and cross section showing *gapdh* expression. b: High power representative section showing *gapdh* expression in skeletal muscle fibres. Note distinct nuclei (n) at the periphery of the muscle fibres and the distinct and regular striations (arrows). c: Control serial section to **45a** for *gapdh*, where the sheep anti-DIG alkaline phosphatase antibody was replaced with buffer, showing no expression.

3.4.4 In situ hybridisation –*bmp4*

Bmp4 expression was not apparent in the oral epithelium in any of the age groups examined. Also, no expression of *bmp4* was evident in any of the components of the dental placode, bud or cap stage of tooth development in any of the age groups examined.

In the 14-31day-old embryos, *bmp4* was expressed in early bell stage tooth germs, particularly in the odontoblast layer (Figs. 48a, b). The density of expression of the *bmp4* was variable in this layer, with lighter and darker areas of expression (Fig. 48a, b). *Bmp4* expression was also prominent in the alveolar bone spicules of the 14-31 day-old specimens, specifically in the osteoblasts delineating the peripheral surface of the spicules (Fig. 48a). The expression was variable between spicules, some showing more intensity of *bmp4* labelling than others (Fig. 48a).

In the 32-65 day-old specimens (Fig. 49a) while *bmp4* was evident in the bell stage tooth germs there was slight variability in the intensity of expression between these tooth germs. A distinct pattern of variability however, was not discerned. In the younger specimens of this group, *bmp4* expression was confined to the differentiated cells or odontoblasts adjacent to the ectosenchyme of the dental papilla, at the future tip of the cusp (Figs. 49b, 50b). Examination of expression along the length of the jaws, in the younger specimens of this group, (Figs. 49b, 50b) showed the presence of pale *bmp4* transcripts in the differentiated cells of the dental papilla/odontoblasts, specifically at the future cusp region (Figs. 49b, 50b) of the tooth. The osteoblasts outlining the bony spicules and the chondroblasts in the cartilaginous plates of the mandible also expressed *bmp4* (Figs. 49b, 50b). The cartilage matrix showed variability in expression with darker and lighter patches of expression (Figs. 49b, 50b).

In the older specimens of the 32-65 day old age group (Fig. 51a), a distinct band of expression was seen in the odontoblasts at the future tip of the cusp and extending laterally along the cusp slopes (Fig. 51b). No *bmp4* expression was evident in the outer enamel epithelium, inner enamel epithelium, the dental papilla nor in the dentine layer (Fig. 51a).

In the more advanced bell stage tooth germs where a distinct columnar inner enamel epithelium was discernable (Figs. 52, 53), the expression of *bmp4* was still isolated to the odontoblasts and the predentine layer at the future tip of the cusp and slopes of the developing tooth (Figs. 52a, b, 53a, b). The remainder of the dentine layer, identified by the branched dentinal tubules did not express *bmp4* (Fig. 5b). Some areas in the ectomesenchyme surrounding the tooth germs, also showed isolated areas of expression (Fig. 52a). Once again expression of *bmp4* was seen in the osteoblasts and osteocytes of the alveolar bone at theses stages (Fig. 52a).

In the hatched and post-hatched specimens, in which replacement tooth germs in the bell stage of tooth development occupied the same alveolar socket with the functional or pre-eruptive teeth. *Bmp4* was strongly expressed in the odontoblasts (Figs. 54 a-c). In addition, expression was also detected in the apical surface of the inner enamel epithelium (Figs. 54 a-c). There was no expression in the dental papilla nor in the stellate reticulum (Figs. 54 a-c).

In the hatched and post-hatched specimens, *bmp4* was often expressed simultaneously in two developing tooth germs lying in one socket (Figs. 55a, b). Expression in the younger bell stage tooth germ was isolated to the odontoblast layer at the future cusp tip and partially down the cusp slopes, whereas in more mature teeth, expression in the odontoblast layer, extended to the apical area at the tapering tip of the root (Figs. 56b, c). A summary of the expression of *bmp4* in the early and late bell stage of the crocodile specimens is shown in Figure 57.

In the hatched and post-hatched specimens, the alveolar bone and surrounding connective tissue, strongly expressed *bmp4.* The bone spicules expressed *bmp4* in the osteoblasts at the periphery (Figs. 55 a, b) and the osteocytes in the lacunae isolated and in nests and in the bone matrix (Figs. 55a, b). Bars of hyaline cartilage also had a very distinct *bmp4* signal, specifically the cartilaginous matrix (Fig. 55a), which had a stronger expression of *bmp4* than did the bone. Chondrocytes were not easily discernable, but were identified by their lacunae (Fig. 55a). Surrounding the cartilaginous plates, *bmp4*-expressing chondroblasts were identified at the periphery (Fig. 55a). The neighbouring alveolar sockets contained tooth germs showing no expression of *bmp4* (Fig. 56a). The alveolar bone and the surrounding connective tissue fibres had persistent *bmp4* expression in all the hatched and post-hatched specimens (Fig. 56a).

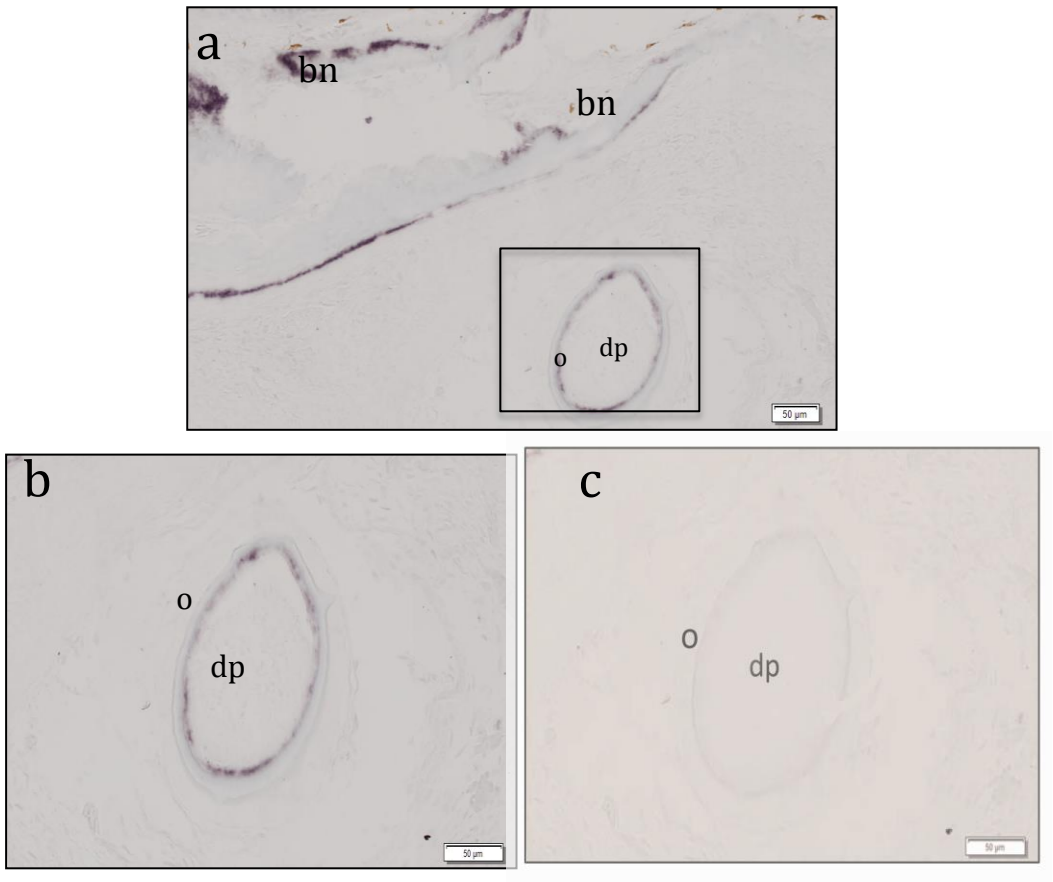

Figure 48 a-c. Representative sections of *bmp4* localised in the odontoblast layer in specimens of the 14-31day-old crocodile group. a: *In situ* hybridisation showing expression of *bmp4* mRNA in the odontoblast (o) layer of an early bell stage tooth germ. Localisation is also present in the osteoblasts lining the bony spicules (bn), dp-dental papilla. Note difference in intensity of *bmp4* in the bony spicules. b: Higher magnification of the rectangular area in **48a**. c: Serial section depicting sense control for *bmp4*.

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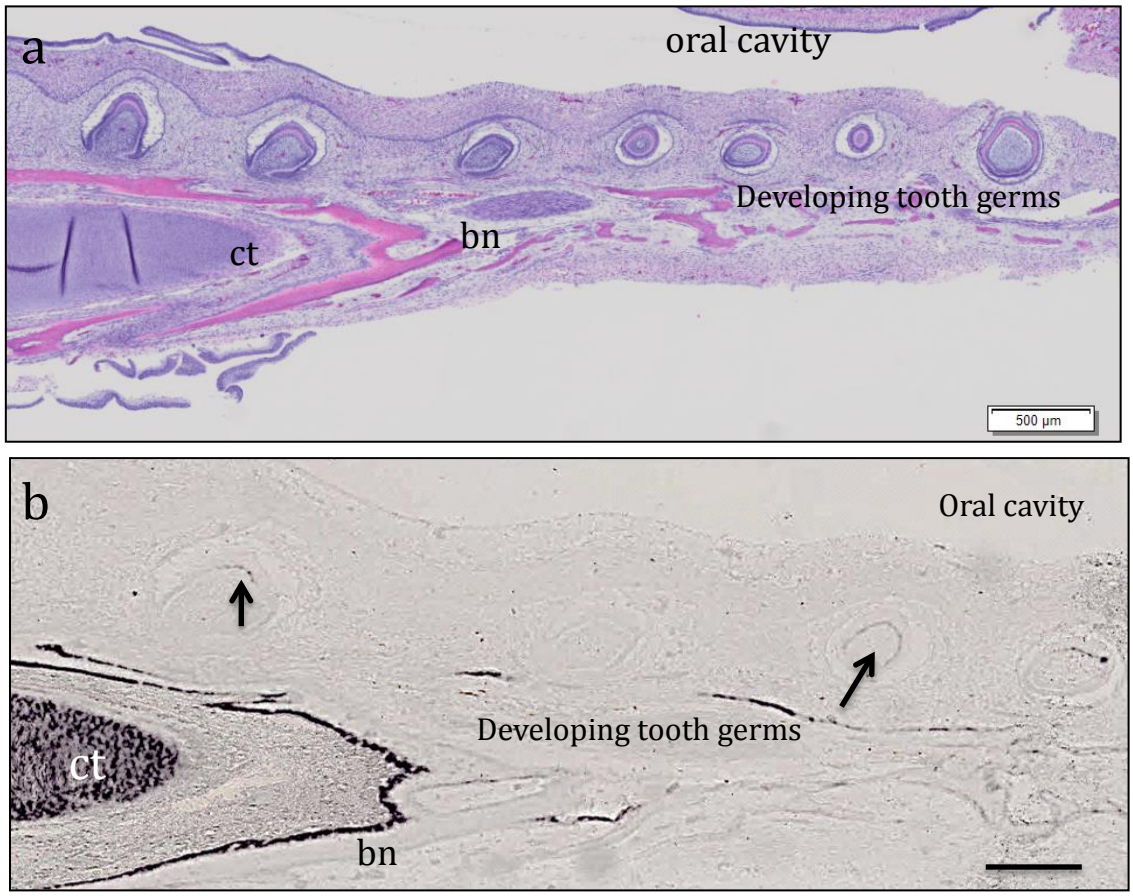


Figure 49 a and b. Photomicrographs showing a sagittal section through the mandible of a specimen from the 32-65 day-old embryo group to determine *bmp4* expression. a: Numerous tooth germs are evident along the length of the mandible. bn-alveolar bone, ct-cartilage. Haematoxylin and eosin. b: *Bmp4* expression in an adjacent serial section from the 32-65 day old crocodile group. Feint expression of *bmp4* was evident in the developing bell stage tooth germs (arrows) and distinct expression was present in the osteoblasts delineating the bone spicule (bn). ct-cartilage. Scale bar: 500µm

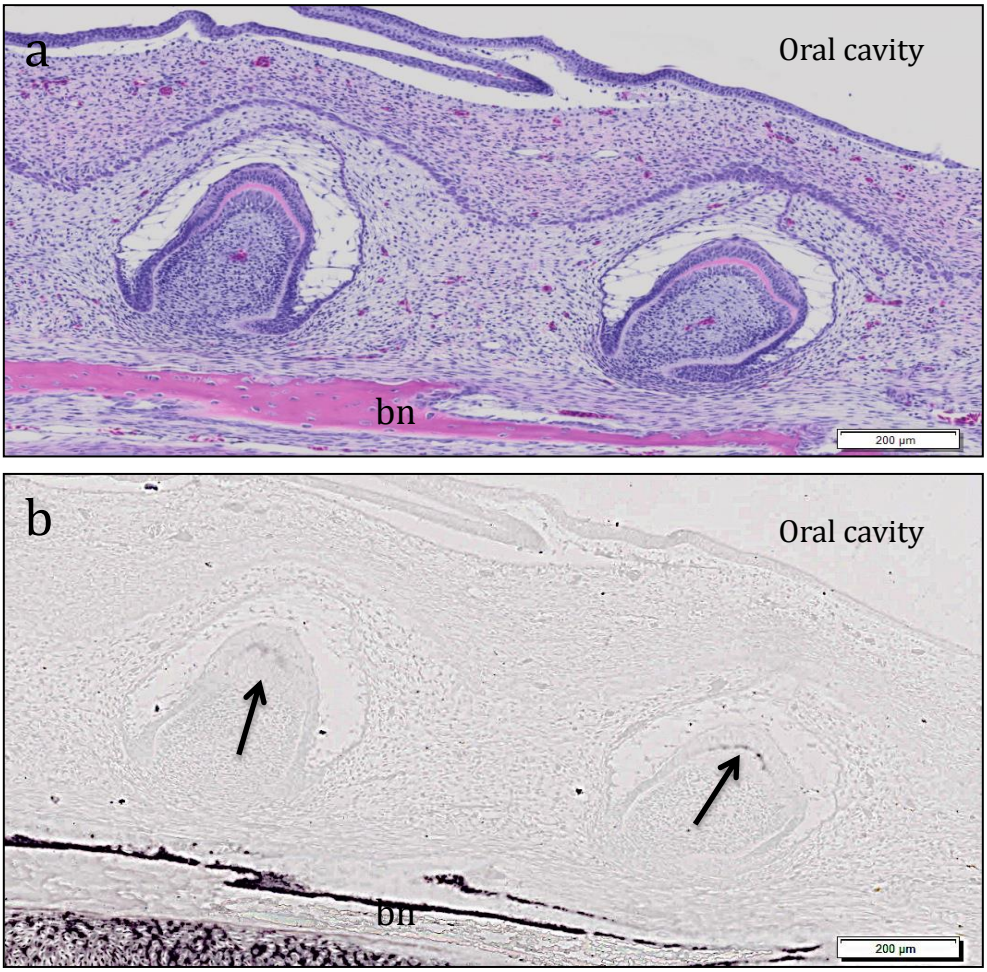


Figure 50 a and b. Expression of *bmp4* in bell stage tooth germs in a representative of the 32-65 day-old embryo group. a: Photomicrograph showing two bell stage tooth germs in the mandible of a. Haematoxylin and eosin. b: Adjacent serial section following the section in 3a, showing feint expression of *bmp4* in future tip of the cusp (arrows) in the tooth germs. bn-bone spicule showing distinct expression for *bmp4.*

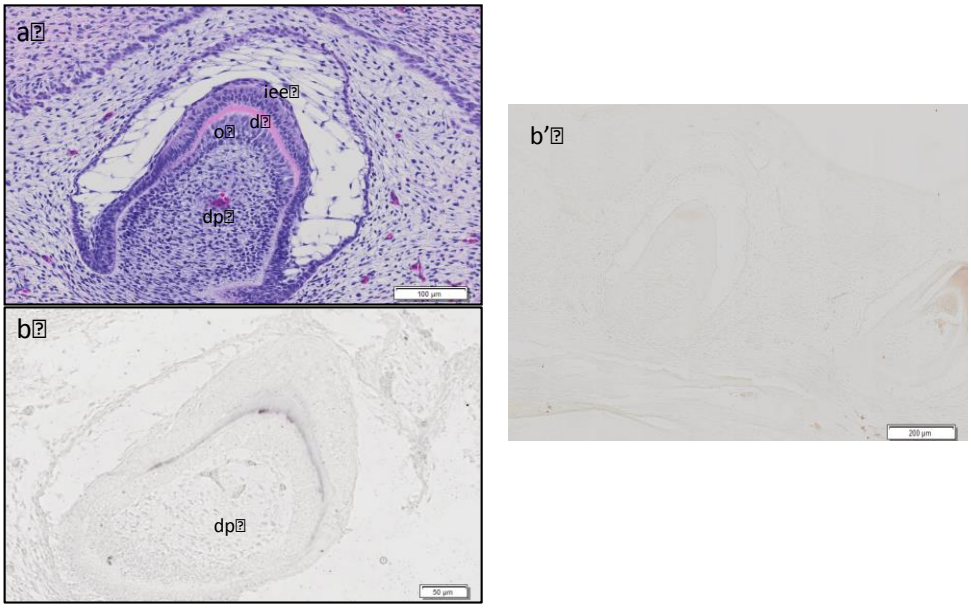


Figure 51 a,b,b'. Expression of *bmp4* in the future cusp tip and slopes of a bell stage mandibular tooth germ in a specimen from the 32-65 day-old group. a: Photomicrograph of bell stage tooth germ. iee-inner enamel epithelium, dp-dental papilla, o-odontoblasts, d-dentine. Haematoxylin and eosin. b: Serial section adjacent to section in **51a** indicating *bmp4* expression (arrows). b': A control section serial to **51b**, using the sense probe, showing no expression of *bmp4.*

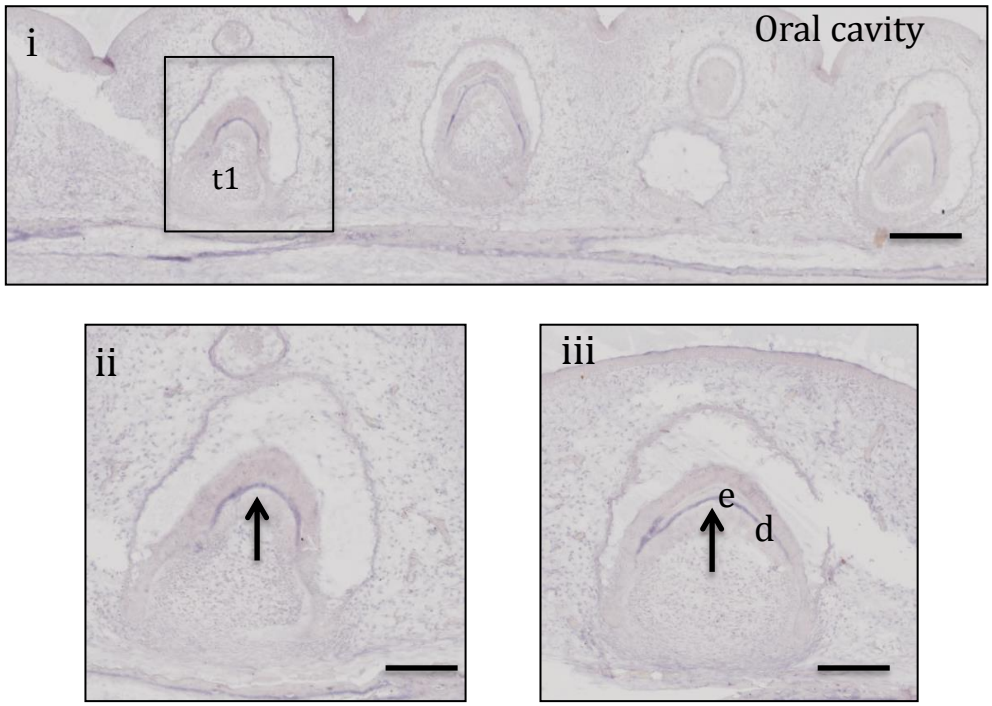


Figure 51c. *Bmp4* expression in the future crown aspect of the tooth germs in embryos of the 32-65 day-old group. i: A series of mandibular early bell stage tooth germs showing *bmp4* expression in the dentine and apical surface of the odontoblasts. ii: High power view of bell stage tooth germ t1 showing *bmp4* expression in the future cusp region (arrow). iii: Later bell stage tooth germ with dentine (d) and enamel (e) layers, showing *bmp4* expression in the dentine layer (arrow) and on the cuspal slopes some expression is evident on the apical surface of the ameloblasts. Scale bar: i 100µm, ii, iii - 50µm

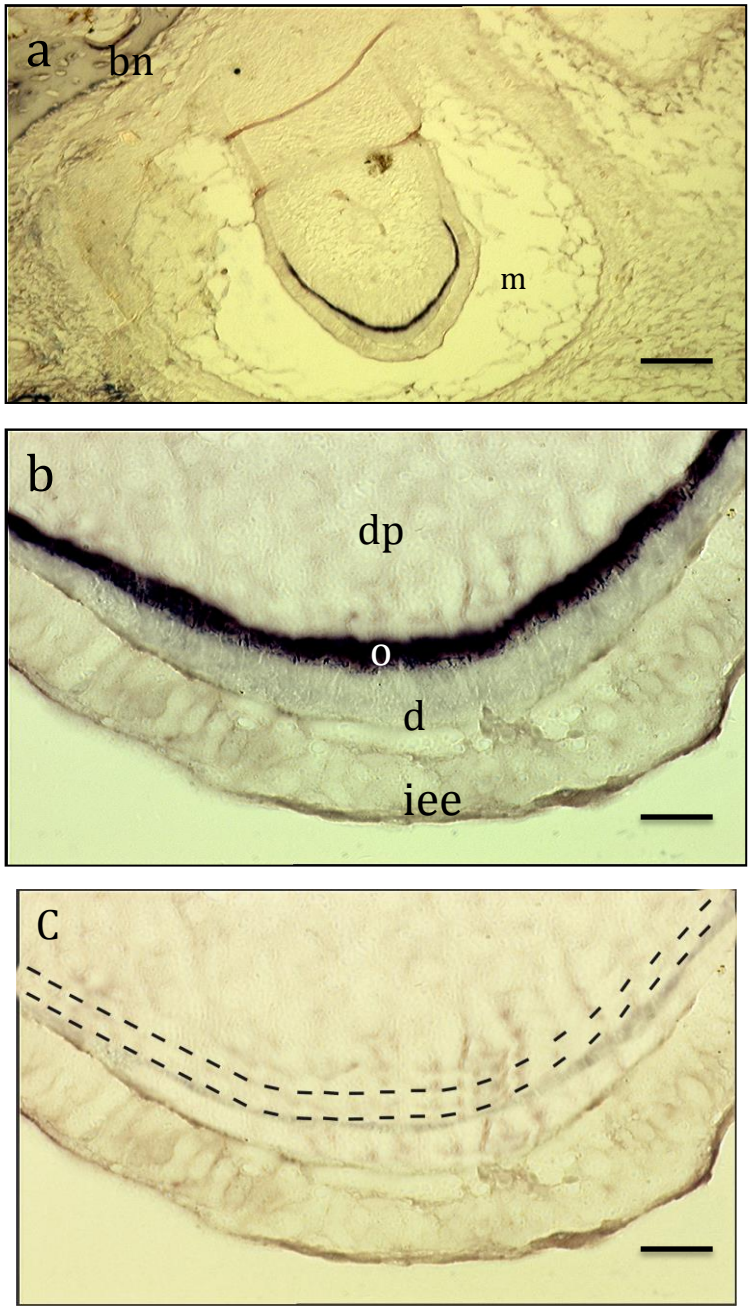


Figure 52a-c. *Bmp4* expression is restricted to the crown aspect of the tooth in embryos of the 32-65 day-old group. a-b: Low and higher power sections showing expression of *bmp4* in the odontoblasts (o) of a bell stage maxillary tooth germ. Note some expression evident in the alveolar bone (bn). *Bmp4* is not expressed in the apical region of the tooth nor in the dentine (d), dental papilla (dp) and inner enamel epithelium (iee) at this stage. c: Control section replacing the anti-sense with buffer, serial to section **52b,** showing no *bmp4* expression. Dashed line indicates region of staining in section 5b. Scale bar: a - 100µm b- 50µm c-50µm

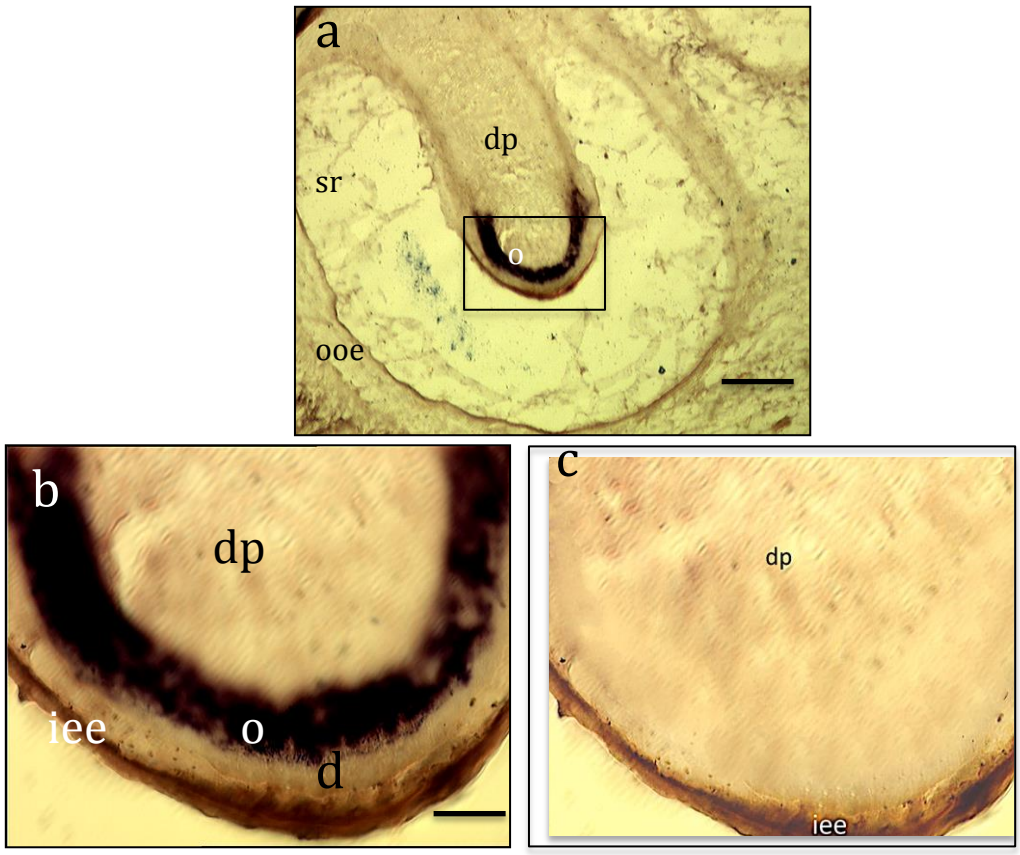


Figure 53 a-c. *Bmp4* expression in the odontoblastic layer in the early bell stage of tooth development in a representative tooth from the 32-65 day- old group. a: ISH showed strong *bmp4* expression in the odontoblasts (o) at the future crown aspect of the tooth. Paler expression occurred in the inner enamel epithelium (iee). No expression was evident in the dental papilla (dp) nor in the dentine layer (d). sr-stellate reticulum, oee- outer enamel epithelium. b: Higher magnification of the rectangular area in **53a**. c: Control section replacing the anti-DIG antibody with buffer, serial to **53b,** showing no specific expression. Scale bar : a-100µm b-20µm c-20µm

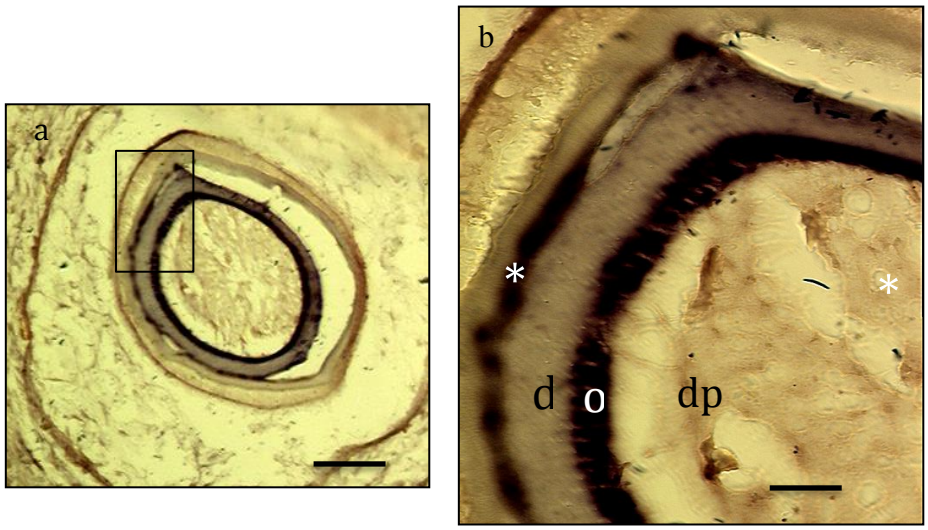


Figure 54 a and b. ISH of *bmp4* in a bell stage replacement tooth germ from the hatched and post-hatched specimen group. a: Low power sections of a cross-section through the bell stage tooth germ. b-c: High power section of the rectangular area in **54a**. *Bmp4* expression was distinct in the odontoblast layer. Expression was also evident in the nuclei of the inner enamel epithelium(*). dp-dental papilla. Scale bar: a-100μm b-50μm

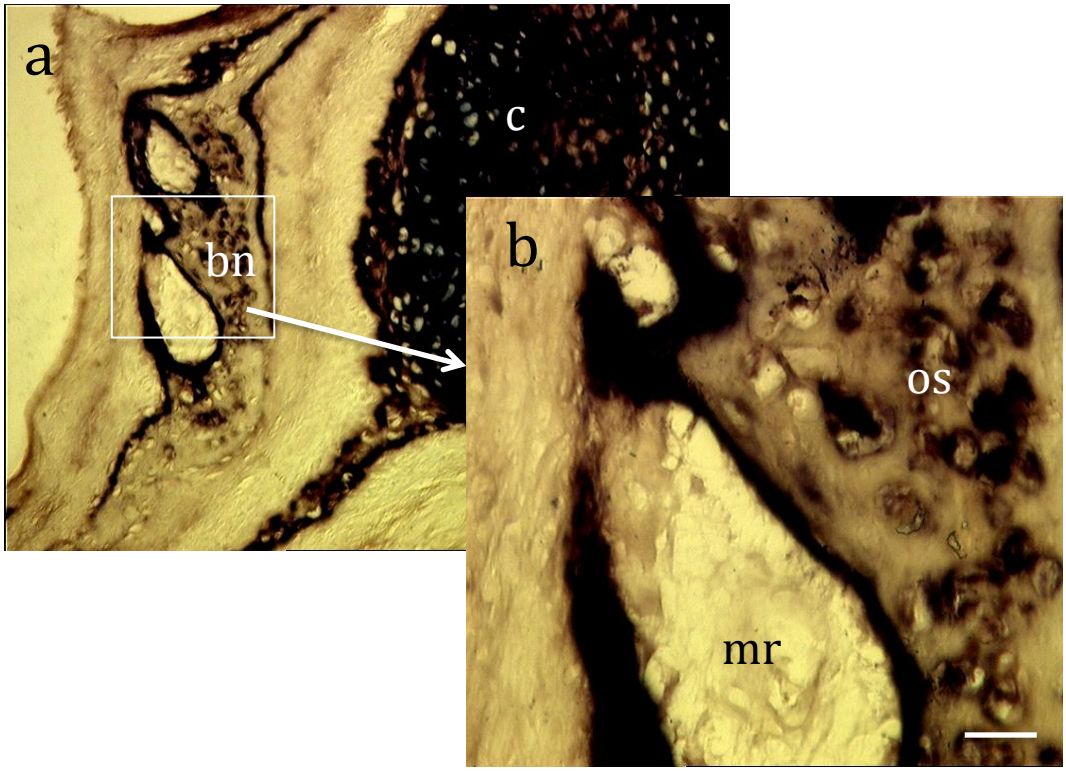


Figure 55 a and b. *Bmp4* expression in the alveolar bone of hatched and post-hatched specimens. a-b: Low and high power sections showing distinct expression of *bmp4* in the bone and cartilage. At the periphery of the bony spicule (bn), strong expression occurs due to the presence of osteoblasts. Osteocytes (os) were present in the centre of the bone. Scale bar: 100μm

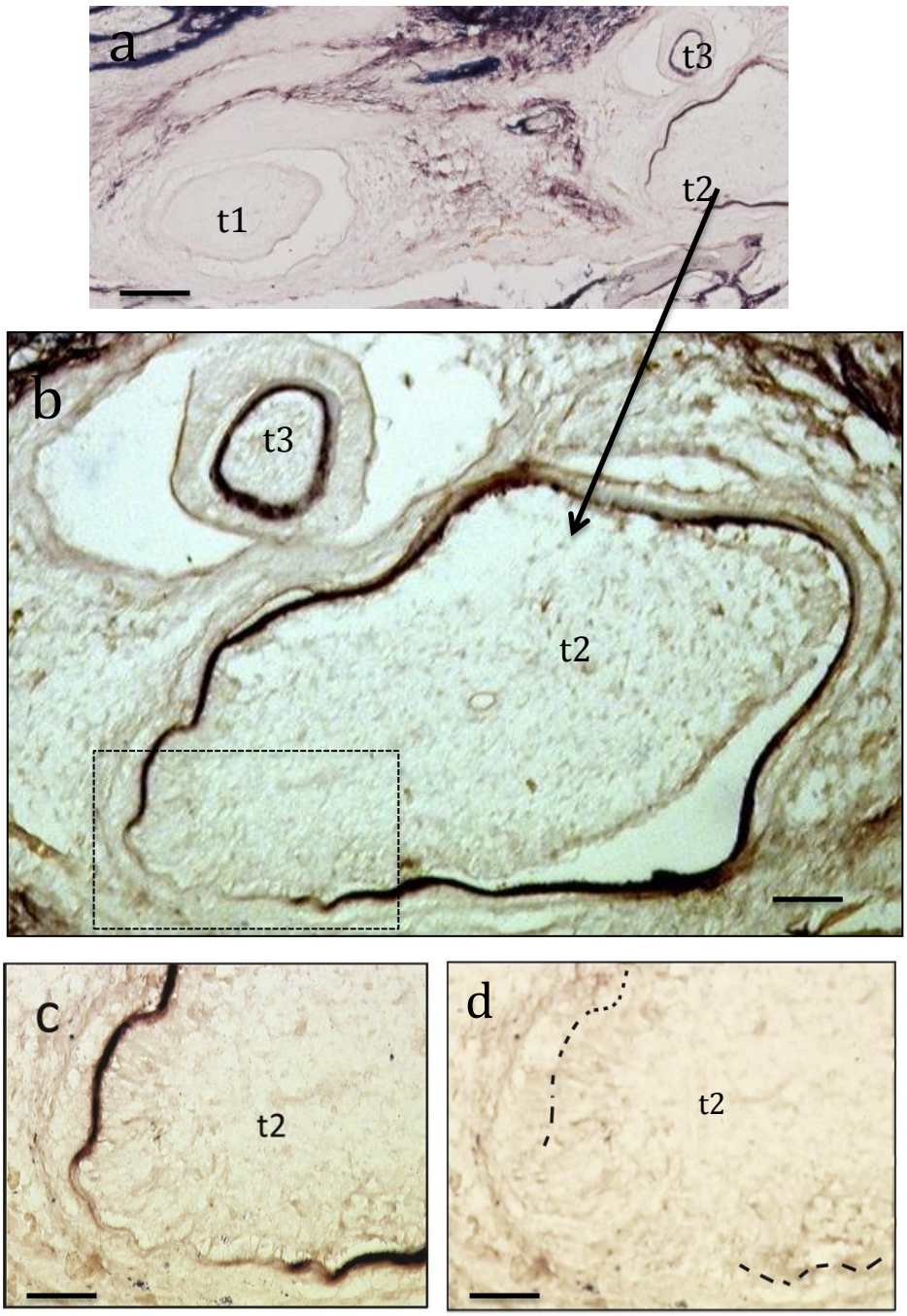


Figure 56 a-d. *Bmp4* expression in a hatched specimen. a: ISH section showing *bmp4* expression in tooth germs t2 and t3, but not t1. Also distinct expression in the alveolar bone and connective tissue. b: *bmp4* expression in the odontoblasts in t2 and t3, extending from the crown to the apical dentine. c: High power view of the rectangle in 9b, showing the tapering root of t2 with *bmp4* expresssion in the odontoblast layer. d: Sense control serial section of **56c**. Dashed lines in area of expression in **56c**. Scale bar: a-100µm, b-50 µm

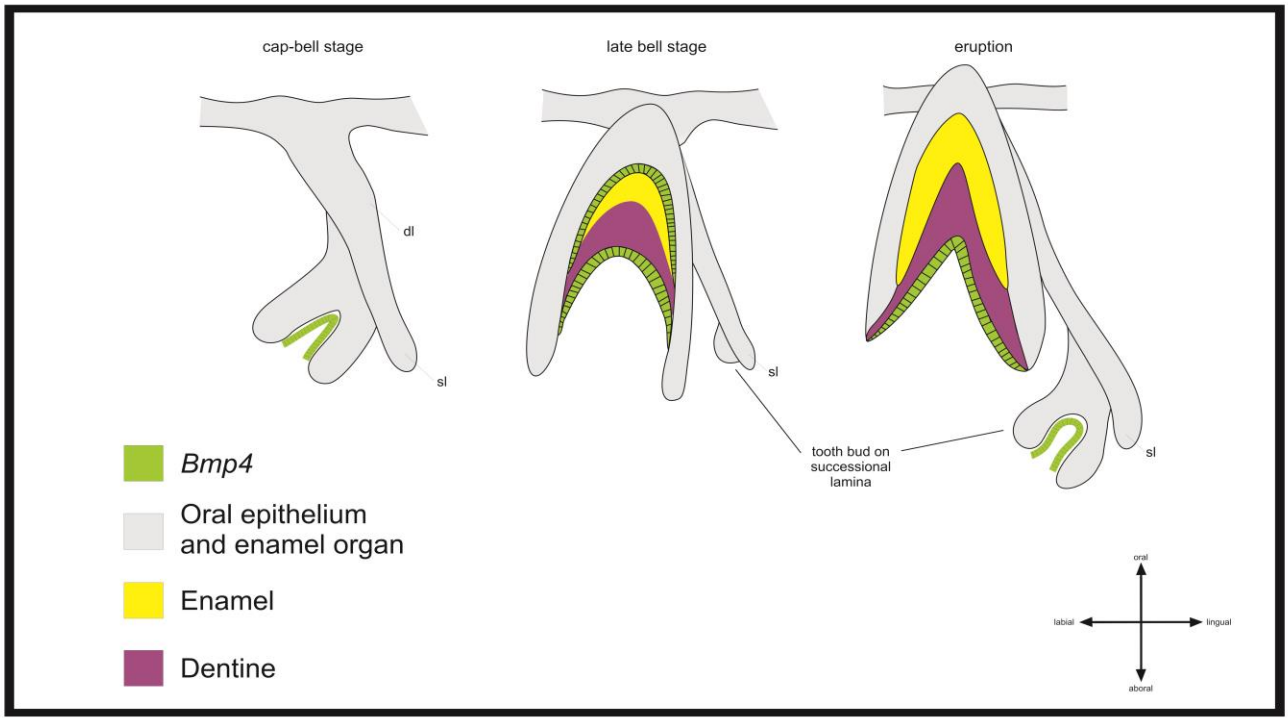


Figure 57. An illustration showing the expression of *bmp4* in primary and replacement teeth in the Nile crocodile. Note dl-dental lamina, iee-inner enamel epithelium, sl-successional lamina

3.4.5 In situ hybridisation for *pitx2*

In the 14-31 day-old crocodile embryos, when comparing haematoxylin and eosin stained sections (Fig. 58a) with adjacent sections hybridised with the probe to *pitx2*, expression of *pitx2* was detected throughout the length of the oral epithelium lining the oral cavity of both the maxilla and the mandible (Fig. 58b).

Pitx2 was also expressed in the dental placodes (Fig. 59a) in the 14-31 day-old embryos. The intensity of expression was uneven in parts of the placode with more distinct expression in some of the basal cells of the placode (Fig. 59a). The oral epithelium, on either side of placodes, displayed similar diffuse but distinct *pitx2* expression. Some expression of *pitx2* was also evident scattered throughout the ectomesenchyme (Fig. 59a), but was most concentrated around small blood vessels (Fig. 59a). Also, in the 14-31 day-old specimens, *pitx2* expression was restricted to the peripheral osteoblasts lining the bony spicules located in the ectomesenchyme (Fig. 58b).

In the 32-65 day old embryos, *pitx2* expression was evident in the inner enamel epithelium at the prospective cusp area of the early bell stage tooth germs (Fig. 60a, b). This expression was more stippled as opposed to a distinct layer (Fig. 60b). In the region of the cervical loop, *pitx2* expression was often more prominent on one side of the enamel organ (Fig. 60a, b). The thinner outer enamel epithelium, dental papilla and dental follicle did not show *pitx2* expression in the early bell stage of tooth development (Fig. 60a). Distinct *pitx2* expression was however detected throughout the length of the successional dental lamina (Fig. 60 a,c) and was also intensely expressed in its terminal dilated portion, reminiscent of a bud stage tooth germ (Figs. 60a). In some tooth germs in this age group, the successional dental lamina showed *pitx2* expression, but its dilated terminal end did not. No expression of *pitx2* was evident in the dental papilla or dental follicle. A summary of *pitx2* expression is demonstrated schematically in Figure 61 for this age group as the expression was variable in some specimens specifically at

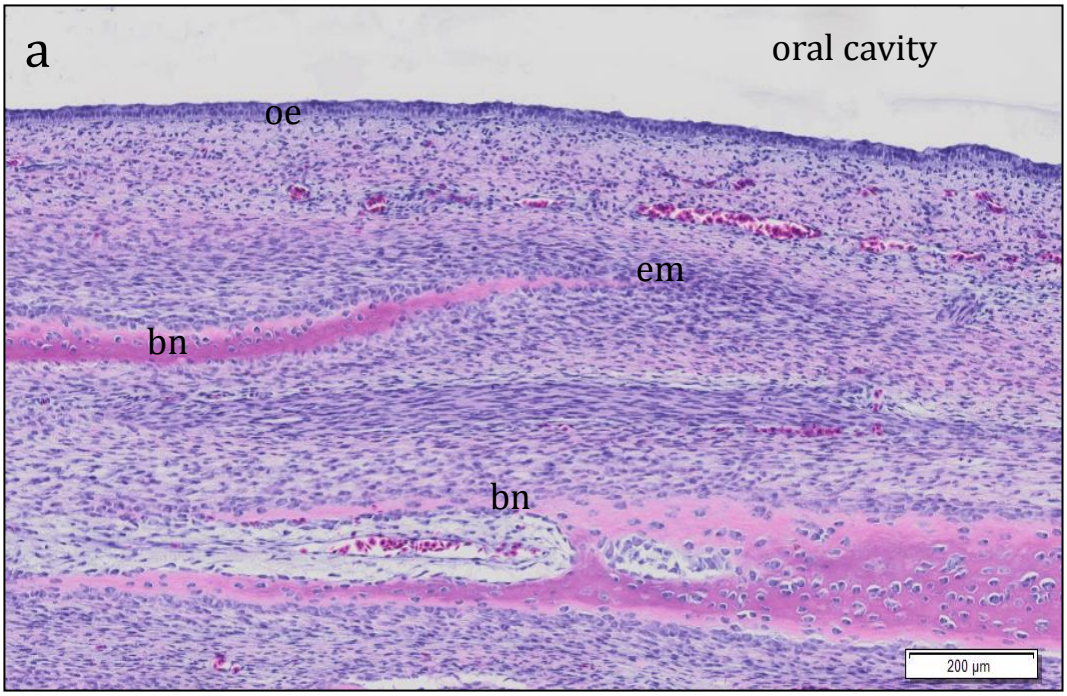
the tip of the successional lamina and in the region of the inner enamel epithelium.

There was also uneven *pitx2* expression displayed in the developing early bell stage tooth germs in the 32-65 day-old crocodile tooth germs (Fig 62a). *Pitx2* expression was more concentrated in the more anterior tooth germs (Fig. 62a). This was also true for the undulating dental lamina overlying the tooth germs (Fig. 62a). This expression was diffuse with scattered cells expressing *pitx2. Pitx2* expression was evident in the inner enamel epithelium, the odontoblast layer and diffuse expression was evident at the peripheral aspect of the dental papilla (Fig. 5b). The outer enamel epithelium showed no *pitx2* expression. In some sections, from the 32-65 day old embryos, *pitx2* expression was distinct in the dental papilla and the dental follicle of the bell stage tooth germs (Fig. 63a). The undulating continuous dental lamina showed expression in its peripheral cells and the signal was more intense where it occurred adjacent to a tooth germ, as opposed to between tooth germs (Fig. 63a). *Pitx2* expression in the late bell stage of tooth development, was distinct in the ameloblast layer, adjacent to the enamel (Figs. 64a, b). The expression was concentrated the apical surface of the ameloblasts and often extended into the enamel layer (Fig. 64b). Diffuse expression of *pitx2* was also present in the basal area of the ameloblasts. *Pitx2* expression was also localised in a diffuse manner in the odontoblastic layer in late bell stage tooth gerns (Fig. 64b). In some tooth germs there was scattered distribution of *pitx2* in the columnar cells of the inner enamel epithelial layer (Fig. 64a), prior to enamel deposition. This expression appeared more concentrated in the lateral inner epithelial cells, as opposed to those at the future cusp region.

In the hatched and post-hatched specimens, *pitx2* expression was evident in the bell stage replacement tooth germs associated with functional and pre-erupting teeth (Fig. 68a). In these tooth germs, expression was detected in the dental papilla, the odontoblasts and the ameloblasts (Fig. 65a). Strong expression was

also evident in the alveolar bone, specifically the osteoblasts at the periphery, but also in the osteocytes in lacunae (Fig. 65a). The bone matrix showed no expression. Late bell stage tooth germs had noticeable expression of *pitx2* at the apical aspect specifically in the cervical loop and the ectomesenchymal condensation at the apical foramen (Fig. 65b). It was also interesting to note that teeth which were degenerating and were in the process of being replaced, displayed *pitx2* expression in the marginal layer of the dentine (Fig. 65b).

In a comparison of *pitx2* and *bmp4* expression in the 32-65 day old embryos (Figs. 66, 67), it was evident that *pitx2* expression was more diffuse and particulate, as opposed to *bmp4* expression which was more distinct and localised. It was interesting also to note that the expression for both *pitx2* and *bmp4* was not even and more concentrated on one side of the developing tooth germs (Figs. 66b,c; 67b,c). A summary of the expression of *pitx2* in crocodilian tooth development is depicted in Figure 68.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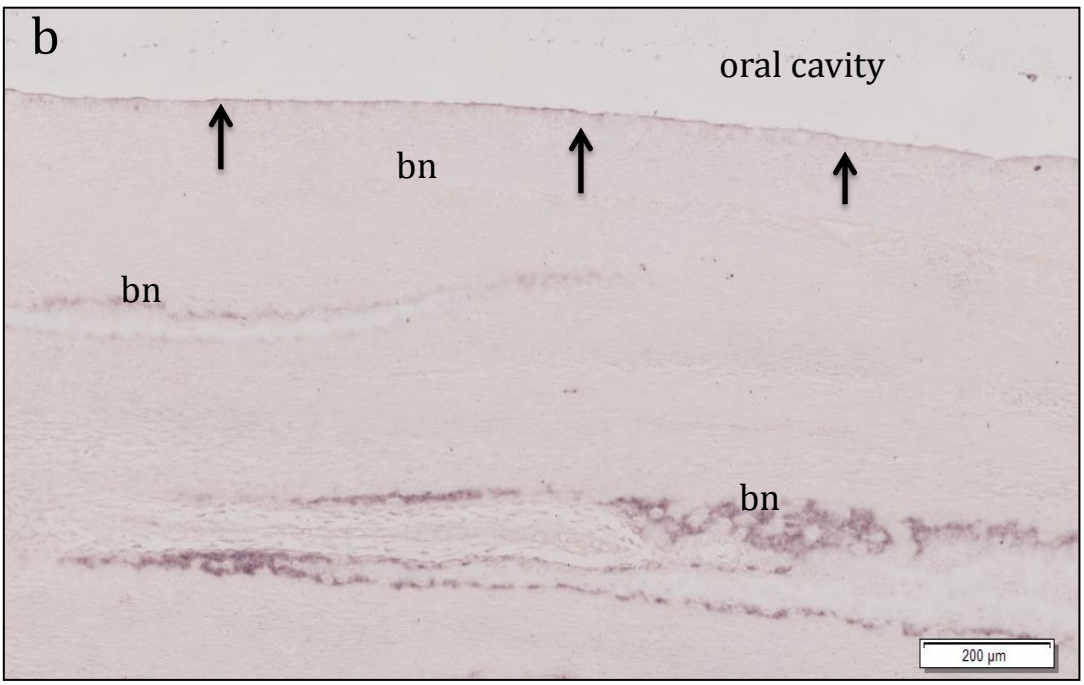
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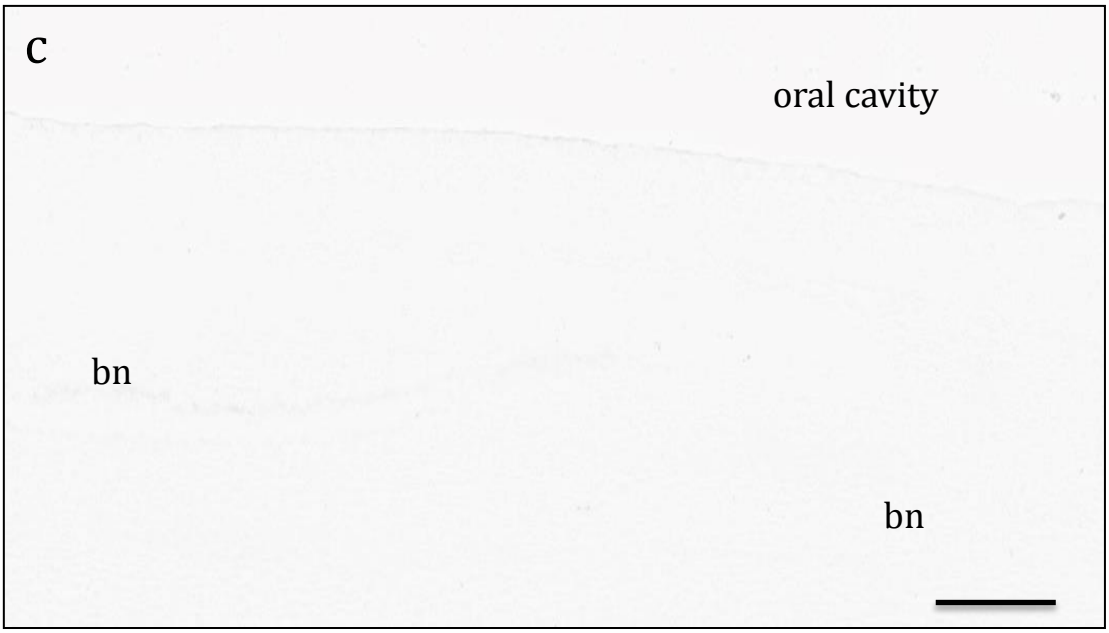


Figure 58a-c. *Pitx2* expression in the oral epithelium prior to the appearance of any tooth germs in the 14-31 day old embryo. a: Representative photomicrograph showing a sagittal section through the mandible of the 14-31 day old specimens. Note the oral epithelium (oe), underlying ectomesenchyme (em) and alveolar bone (bn). Haematoxylin and eosin. b: *Pitx2* expression, in the consecutive section to **58a**, was evident throughout the length of the oral epithelium (arrows). Strong *pitx2* expression was also present in the osteoblasts lining the bone (bn) spicules. c: Sense control on a serial section to that in **58b** showing no expression of *pitx2*. Scale bar: 200μm

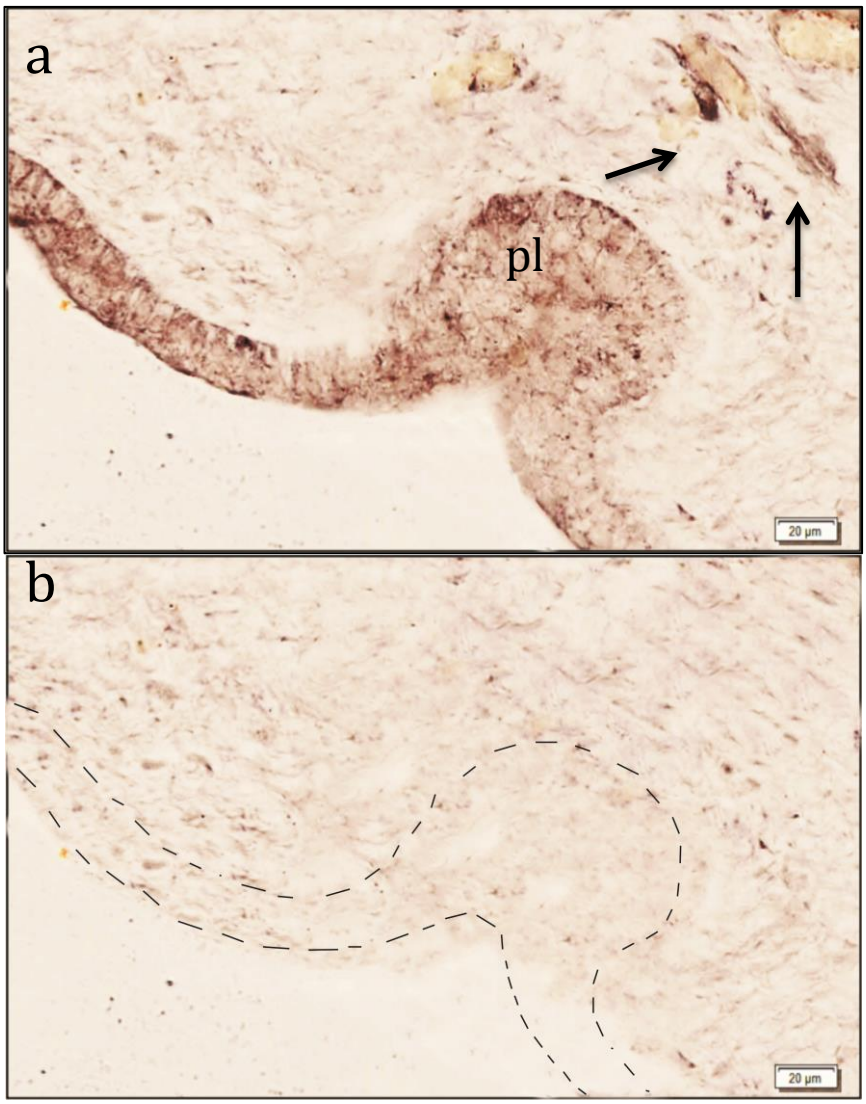


Figure 59 a and b. Expression of *pitx2* in the oral epithelium and dental placode in the 14-31 day old crocodile embryos. a: *pitx2* localisation extends throughout the length of the oral epithelium. It is expressed in the dental placode (pl) of the crocodile maxilla. Some expression occurred in the ectomesenchyme (arrow). b: Control serial to section in **59a**. In this instance the anti-DIG antibody was replaced with buffer.

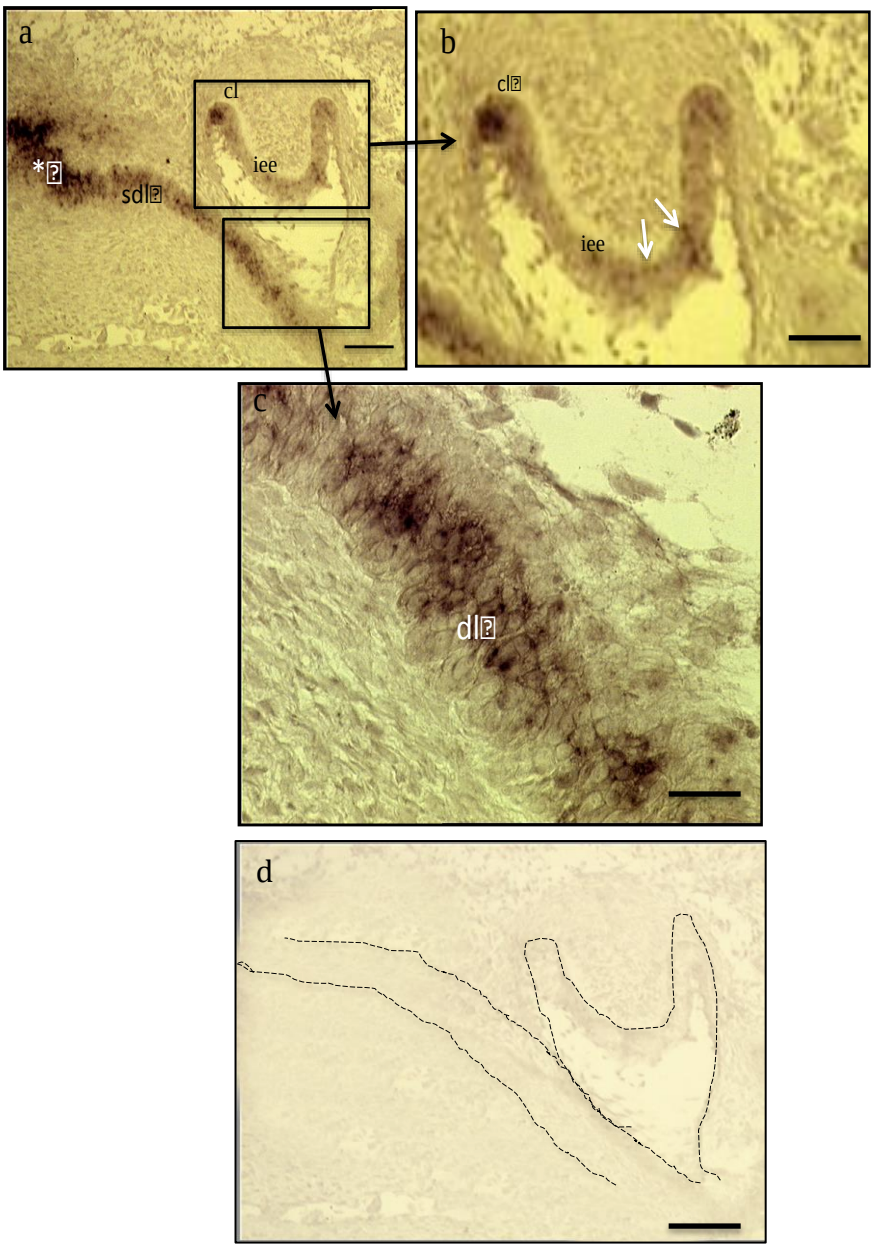


Figure 60 a-d. Expression of *pitx2* in an early bell stage tooth germ in the 32-65 day-old crocodile embryo group showing the successional dental lamina. a: *Pitx2* expression occurred in the inner enamel epithelium (iee) of the maxillary tooth germ. Increased signal was evident in the cervical loop (cl) on one side. Intense *pitx2* expression was seen in the successional dental lamina (sdl), especially at its dilated terminal end (*). b: Higher magnification of the area depicted in the larger rectangle in a. Note expression of *pitx2* in the iee (white arrows) and the cl. c: Higher magnification of the segment of the dental lamina shown in the smaller rectangle in a. d: Control serial section following that of section in **60a,** the anti-DIG antibody was replaced with buffer, showing no expression of *pitx2*. Scale bar a: 100µm, b:

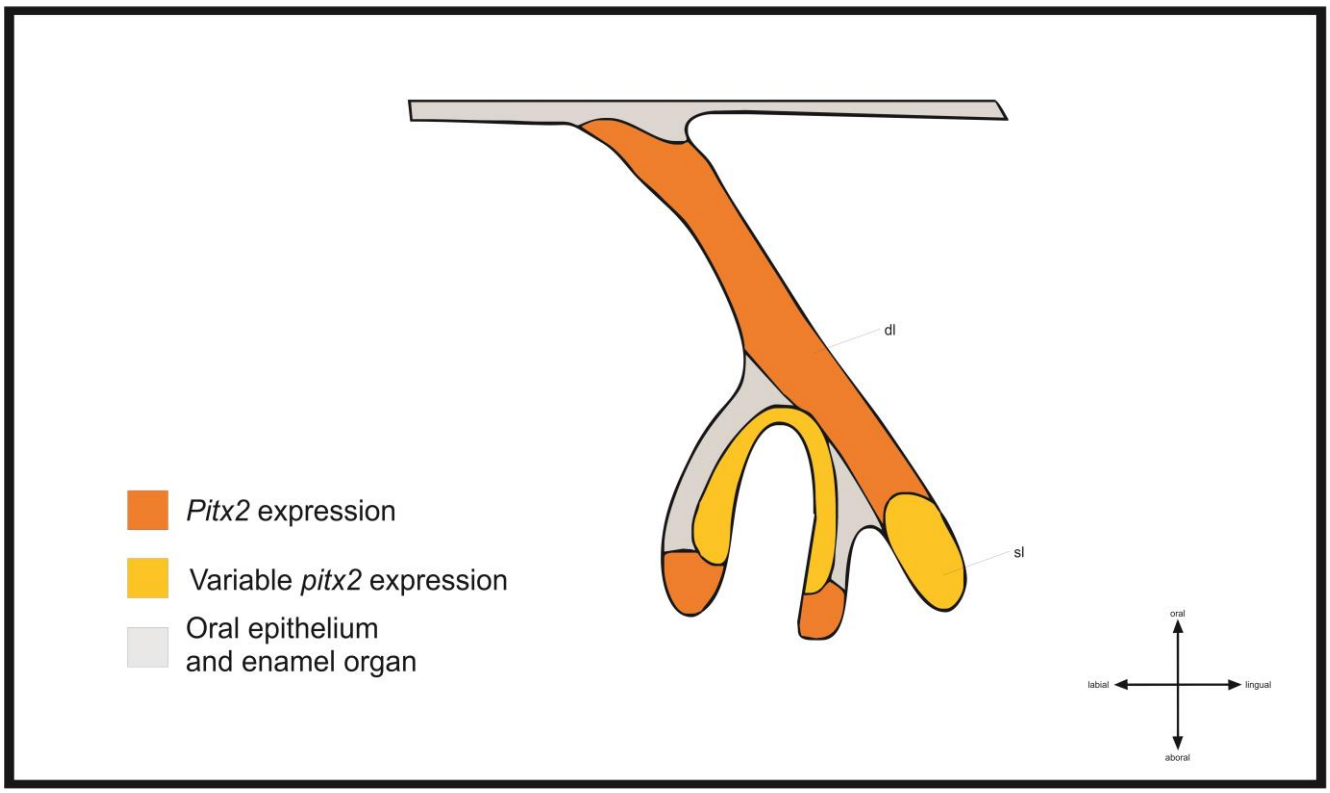


Figure 61. A summary of pitx2 expression in an early bell stage mandibular tooth germ, with its associated dental lamina. note: dl-dental lamina, sl-successional lamina.

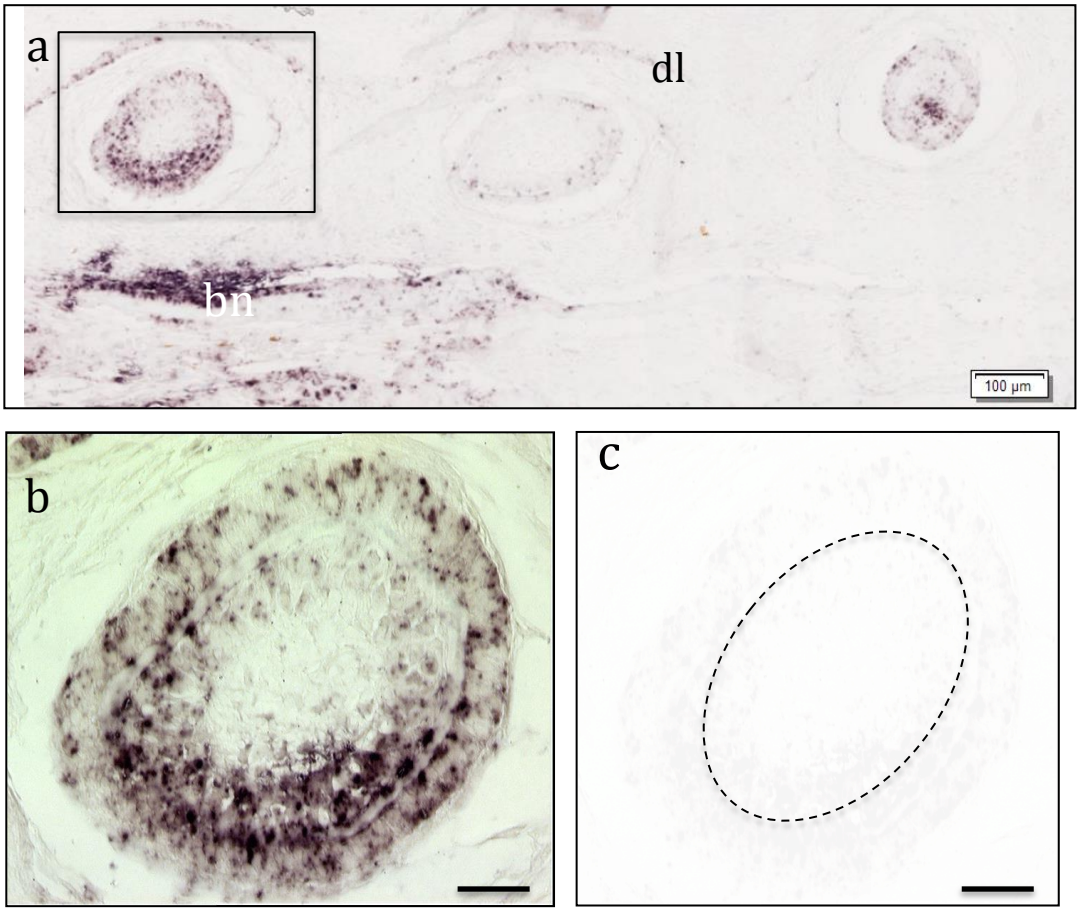


Figure 62a-c. Cross sections of a bell stage tooth germ in the 32-65 day- old embryo group showing expression of *pitx2*. a: Expression of *pitx2* along the length of the crocodile maxilla, showed variability in intensity of expression. The tooth germ in **62a** in the rectangle had more intense expression of *pitx2* than the other tooth germs in the section. Also, the alveolar bone (bn), in close proximity to the tooth germ, showed *pitx2* expression b: High power representation of the tooth germ in the rectangular area in **62**. c: Control slide, using the sense probe, serial to **62b** showing no expression. Scale bar: 50µm.

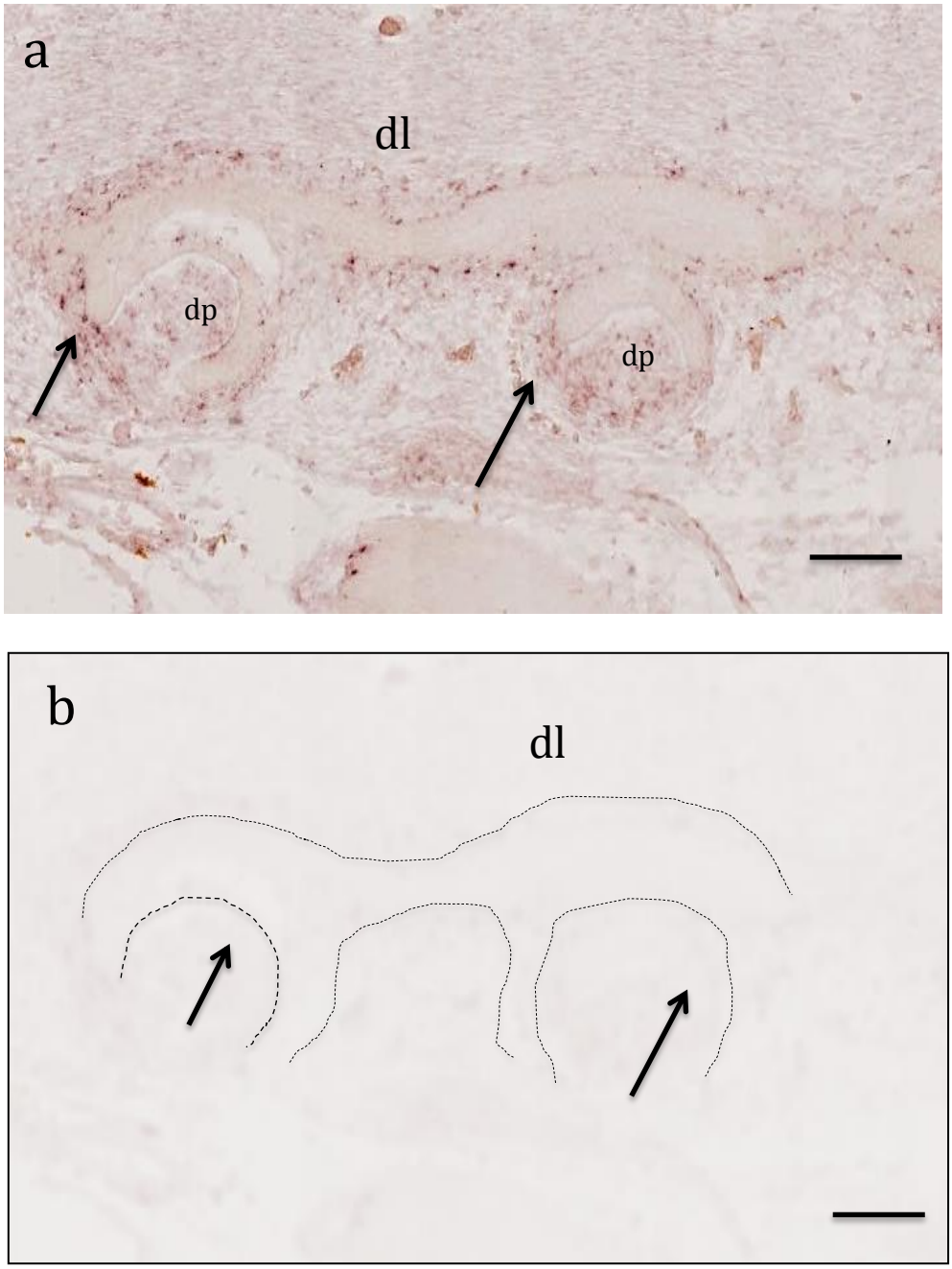
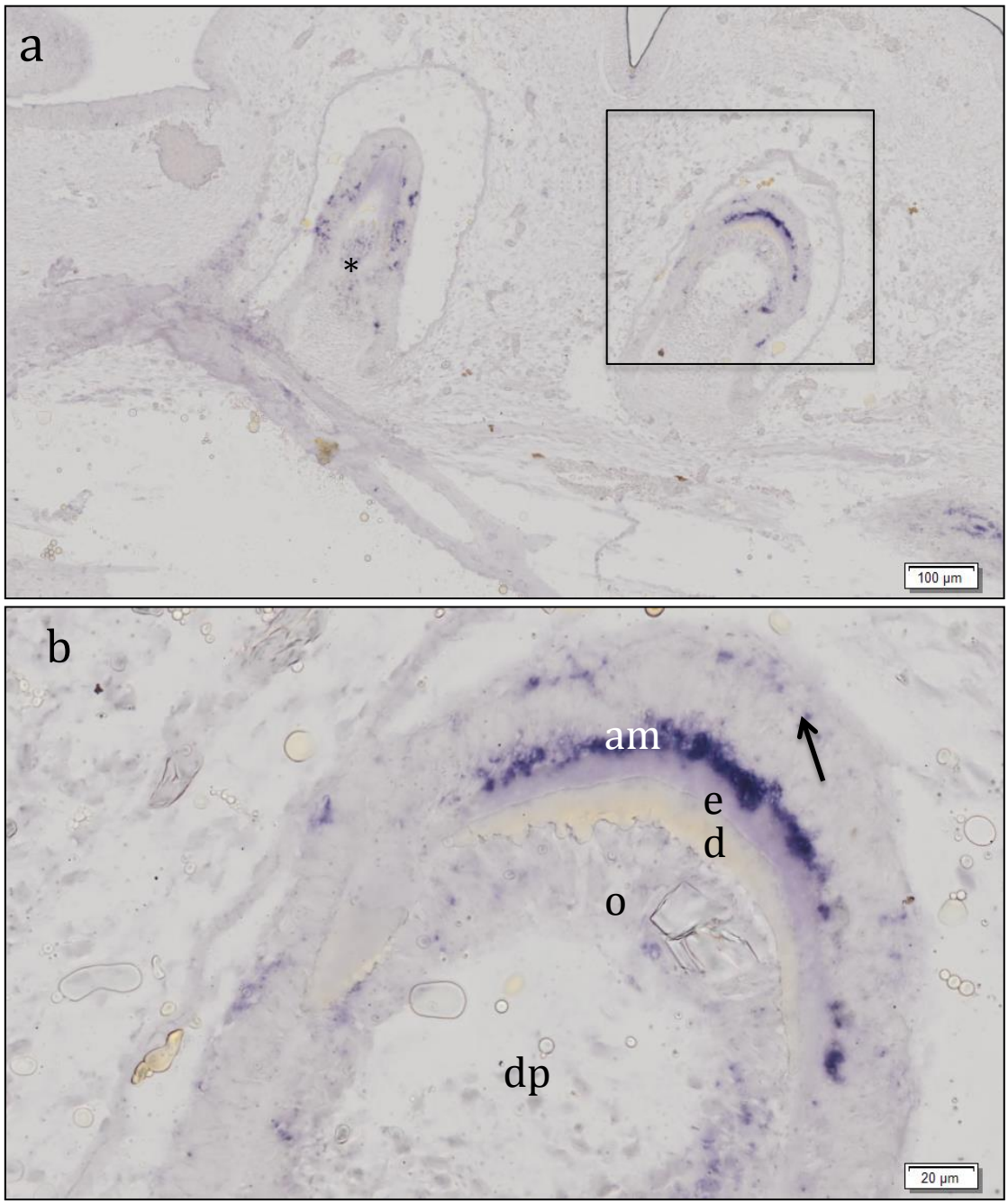


Figure 63 a and b. ISH showing expression of *pitx2* expression in the mandible of a specimen from the 32-65 day-old crocodile embryo group. Expression was evident on the lining cells of the dental lamina (dl) as well as the dental follicle (arrows) and dental papilla.(dp). b: Control section, using the sense probe, serial to **63a** with no expression evident. Scale bar:100μm.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20 µm

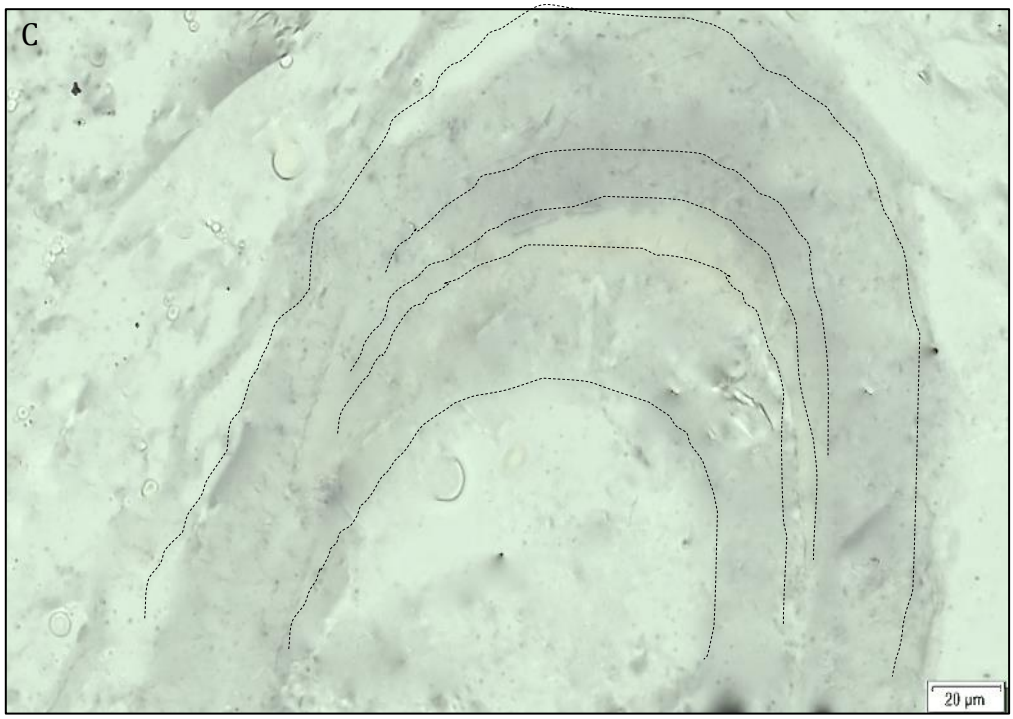

Figure 64 a-c. *Pitx2* expression in late bell stage tooth germs in the mandible of a representative from the 32-65 day old embryo group. a: Low power view showing ISH of *pitx2* in mandibular tooth germs. Expression was evident in the apical region of the polarized ameloblasts. Also in other tooth germs expression was seen scattered within the cells of the ameloblast layer. Diffuse expression was seen in some late bell stage tooth germs in the dental papilla (*). b: High power view of the region in **7a** showing expression of *pitx2* in the apical region of the ameloblast layer–am . Some expression is evident at the basal surface of the ameloblasts (arrow). dp-dental papilla; o-odontoblasts; d-dentine, e-enamel. c: Control serial section showing no *pitx2* expression.

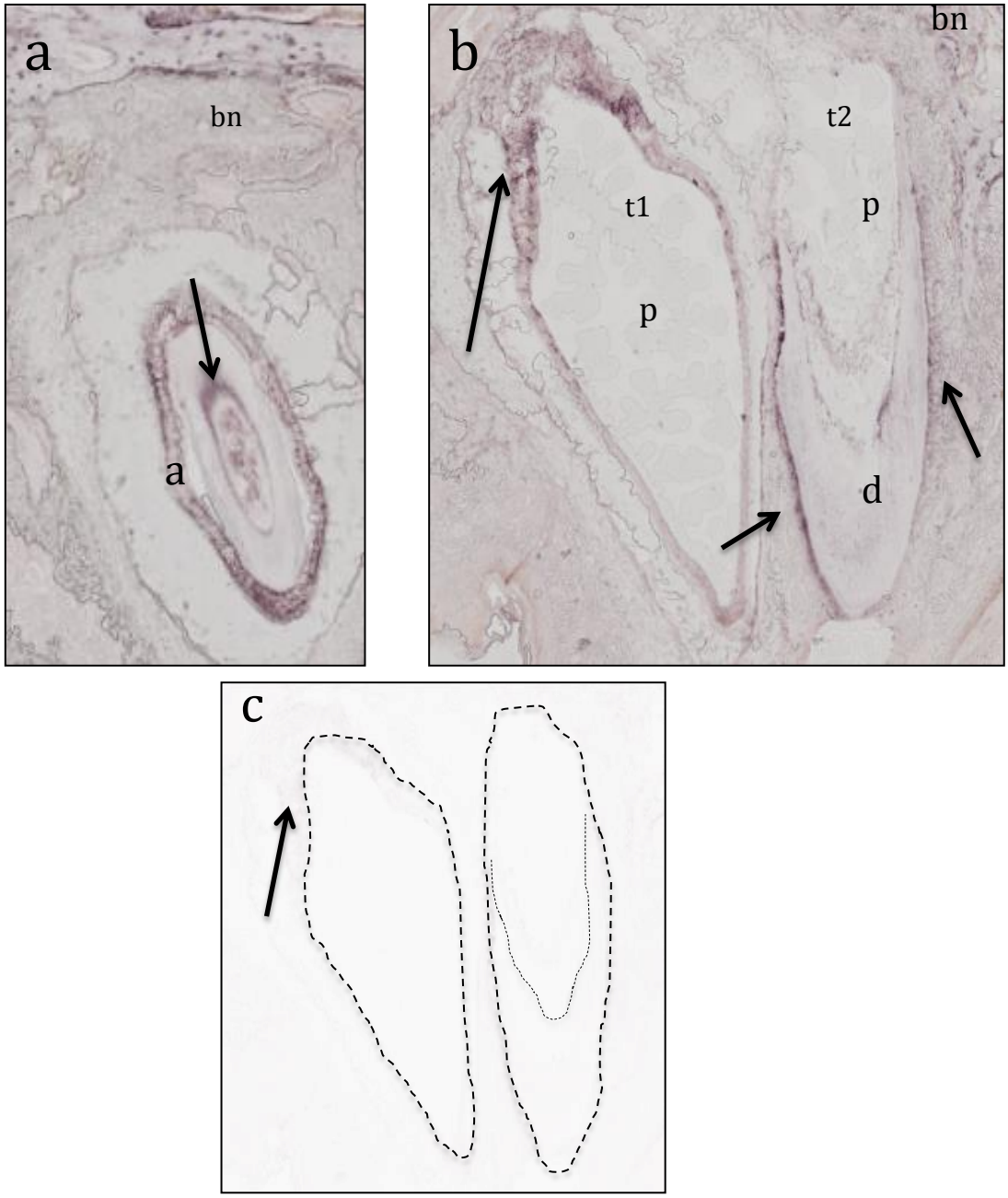

Figure 65a-c. Representative section showing *pitx2* expression in the maxilla of a specimen from the hatched and post hatched group. a: Cross section of a bell stage replacement tooth showing *pitx2* expression in the dental papilla and odontoblast layer predominantly on one side (arrow). The ameloblast layer (a) also showed expression, as well as the alveolar bone (bn). b: *pitx2* expression in maxillary tooth germs was evident on the lateral surfaces of tooth 2 (t2), (arrows) which was the degenerating tooth to be replaced by tooth 1 (t1). Marked expression was also evident at the apical area of tooth 1 (arrow) and the alveolar bone. c: Control serial section to **65b** showed no expression. Scale bar: 100μm

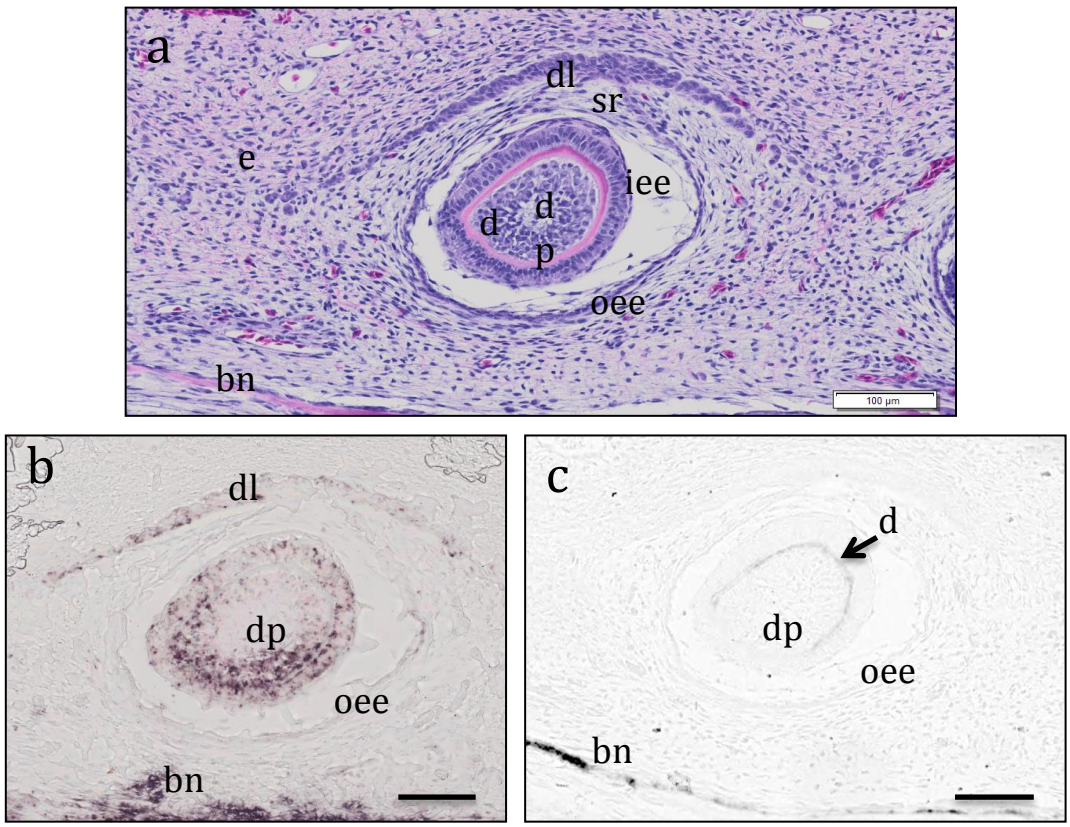

Figure 66a-c. Developing tooth germ in the 14-31 day old embryos showing expression of *pitx2* and *bmp4.* A: Photomicrograph depicting a cross-section of a developing tooth germ. Note dl-dental lamina, e-ectomesenchyme, oee-outer enamel epithelium, iee-inner enamel epithelium, sr-stellate reticulum, d-dentine, bn-alveolar bone. Haematoxylin and eosin. B: Photomicrograph showing expression of *pitx2* in the tooth germ in A. Weak expression was evident in the dental lamina (dl). Expression was also evident in the odontoblasts, the dentine and the inner enamel epithelium. The dental papilla (dp) appeared not to express *pitx2.* The alveolar bone (bn) expressed *pitx2.* c: Photomicrograph showing expression of *bmp4* in the tooth germ in **66a**. Weak expression of *bmp4* is evident in the dentine layer (d) only. Variation in expression was evident in the alveolar bone (bn). Scale bar: 100µm

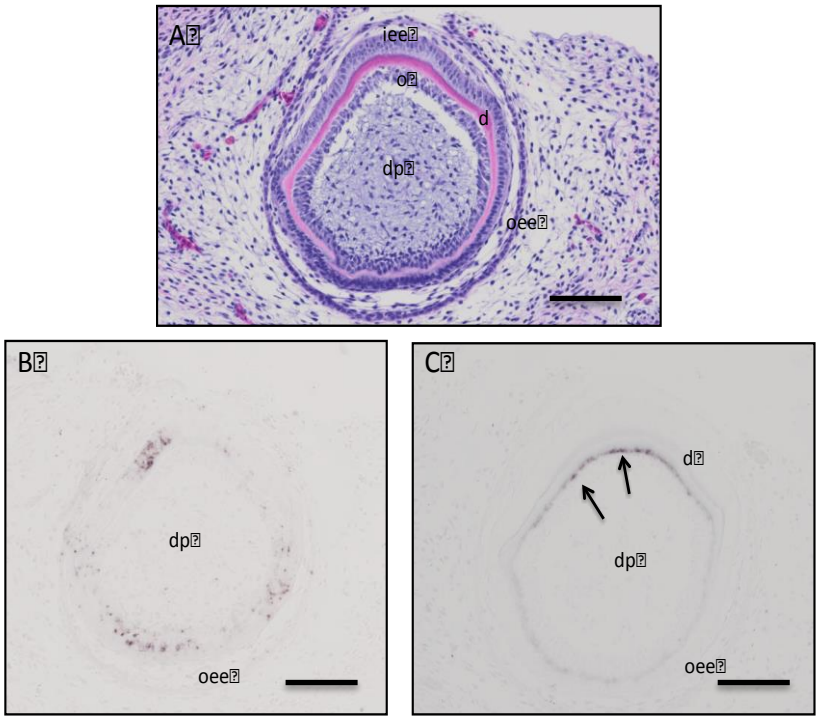

Figure 67a-c. Developing tooth germ in the 14-31 day old embryo group showing expression of *pitx2* and *bmp4*. a: Photomicrograph depicting a cross-section of a developing tooth germ. Note the oee-outer enamel epithelium, iee-inner enamel epithelium, d-dentine, dp-dental papilla. Haematoxylin and eosin. b: Photomicrograph showing expression of *pitx2* in the tooth germ in **67a**. Diffuse expression was evident in the odontoblasts. The dental papilla (dp) appeared not to express *pitx2*. c: Photomicrograph showing expression of *bmp4* in the tooth germ in **67a**. Expression of bmp4 was evident in the odontoblast layer (arrows). Scale bar :100 μm

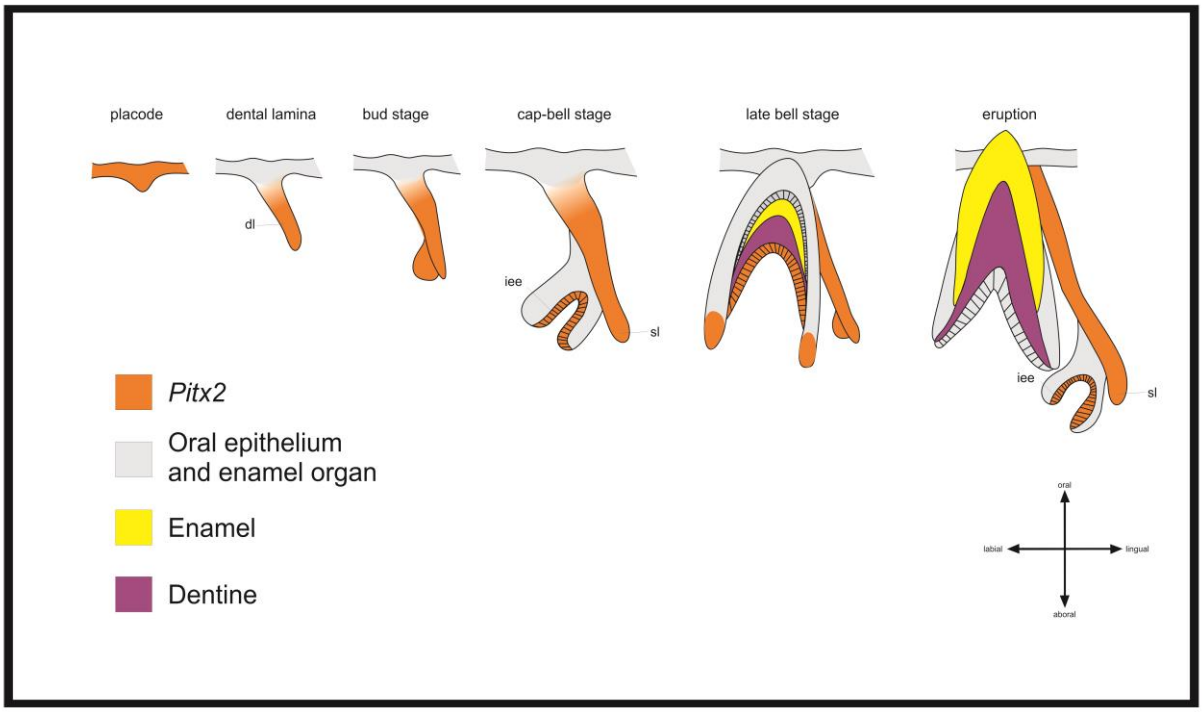

Figure 68. An illustration showing the expression of *pitx2* in primary and replacement teeth in the Nile crocodile. Note dl-dental lamina, iee-inner enamel epithelium, sl-successional lamina.

3.5 Phylogenetic Analysis

Genome sequences from several vertebrate taxa (Table 4) were compared phylogenetically for *gapdh, bmp4 and pitx2* crocodile genes. The mean ± SD intra- and inter-nucleotide divergence between the sequences are indicated in Tables 6, 7 and 8.

3.5.1 Gapdh

The nucleotide intragroup divergences of *gapdh* were similar in reptiles (0.061) and birds (0.059) (Table 6), showing little nucleotide variation between the species. It appeared from this series of taxa that the bony fish had the highest intragroup divergence (0.168). The *gapdh* nucleotide divergence between the amphibians and reptiles (0.181) was decreased as compared to the bony fish and reptiles (0.214) indicating less uniformity in the selected sequences in the bony fish as compared to the reptilian sequences. The *gapdh* sequence in the Amphibia, was therefore slightly more closely related to the sequences in reptiles as opposed to reptiles as compared to the fish (0.214).

3.5.2 Bmp4

In the *bmp4* nucleotide divergence data (Table 7) it was noted that, in the specific taxon sampling of the present study, the intragroup nucleotide divergence in birds (0.039) was lower than in reptiles (0.101), fish (0.232) and mammals (0.140) indicating the similarity of the *bmp4* sequences within the selected bird species.
The intergroup divergence for *bmp4* showed that the nucleotide divergence decreases between birds and reptiles (0.155) as compared to reptiles and a) amphibians (0.286) b) bony fish (0.299) and c) mammals (0.254). This highlighted the similarity of the reptilian *bmp4* sequences with the avian *bmp4* sequences in this series of taxa.

3.5.3 Pitx2

The intra-group vertebrate sequences for *pitx2* (Table 8) showed that within the reptile group (0.048) the sequences were more conserved than within the mammals (0.084), birds (0.098) and bony fish (0.102). However, it was interesting to note that the nucleotide divergence within the reptiles (0.048) was similar to the nucleotide divergence between amphibians and reptiles (0.049). This would be valid only for the taxa included in the present study. Also, an increase in nucleotide divergence was evident in the *pitx2* gene sequence between reptiles and fish (0.196) indicating less uniformity between the sequences in these vertebrate classes.

In the phylogenetic dendrograms of *pitx2*, *bmp4* and *gapdh*, the crocodilian sequences of the present study were closely associated with the alligator sequences which was confirmed with high bootstrap values (>90). In all the sequences, there was a close relationship between the crocodile and birds as well as the crocodile and other reptiles (Figs. 69, 70, 71).

Table 6. *Gapdh* gene mean + SD intergroup and intragroup divergences between the DNA sequences of different taxonomic groups. Nucleotide divergence values were calculated by counting the number of differences between two sequences and dividing this value by the length of the alignment.

	Bony fish	Coelacanth	Amphibia	Reptiles	Birds	Mammals
Bony fish	0,168 ±0,076					
Coelacanth	0,244 ±0,017	n.c.				
Amphibia	0,218 ±0,001	n.c.	n.c.			
Reptiles	0,214 ±0,017	0,185 ±0,008	0,181 ±0,010	0,061 ±0,036		
Birds	0,206 ±0,019	0,193 ±0,006	0,194 ±0,009	0,117 ±0,013	0,059 ±0,014	
Mammals	0,220 ±0,017	0,211 ±0,012	0,209 ±0,011	0,168 ±0,009	0,156 ±0,009	0,116 ±0,035

Table 7. *Bmp4* gene mean + SD intergroup and intragroup divergences between the DNA sequences of different taxonomic groups. Nucleotide divergence values were calculated by counting the number of differences between two sequences and dividing this value by the length of the alignment. n.c. = no calculation possible for a single sample.

	Bony fish	Coelacanth	Amphibia	Reptiles	Birds	Mammals
Bony fish	0,232 ±0,051					
Coelacanth	0,361 ±0,019	n.c.				
Amphibia	0,361 ±0,018	n.c.	n.c.			
Reptiles	0,299 ±0,036	0,283 ±0,017	0,286 ±0,017	0,101 ±0,062		
Birds	0,329 ±0,029	0,305 ±0,010	0,301 ±0,005	0,155 ±0,011	0,039 ±0,013	
Mammals	0,371 ±0,029	0,299 ±0,006	0,291 ±0,009	0,254 ±0,027	0,254 ±0,024	0,140 ±0,062

Table 8. *Pitx2* gene mean + SD intergroup and intragroup divergences between the DNA sequences of different taxonomic groups. Nucleotide divergence values were calculated by counting the number of differences between two sequences and dividing this value by the length of the alignment. n.c. = no calculation possible for a single sample.

	Bone fish	Coelacanth	Amphibia	Reptiles	Birds	Mammals
Bony fish	0,102 ±0,048					
Coelacanth	0,208 ±0,011	n.c.				
Amphibia	0,194 ±0,010	n.c.	n.c.			
Reptiles	0,196 ±0,007	0,186 ±0,003	0,049 ±0,013	0,048 ±0,033		
Birds	0,186 ±0,008	0,179 ±0,005	0,099 ±0,036	0,092 ±0,040	0,098 ±0,055	
Mammals	0,190 ±0,009	0,202 ±0,002	0,157 ±0,005	0,156 ±0,007	0,146 ±0,025	0,084 ±0,032

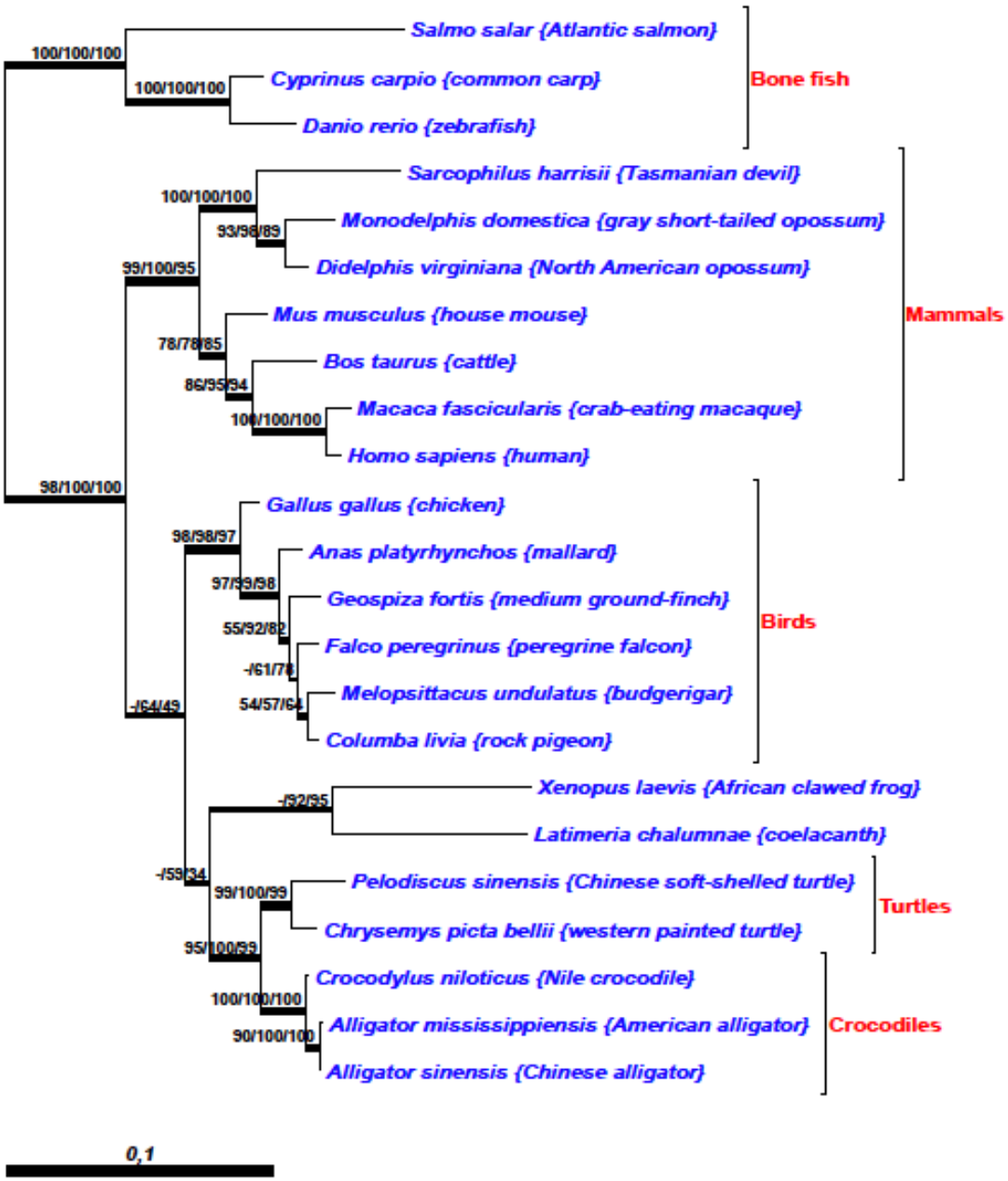

Figure 69. Phylogenetic relationship of *gapdh* gene DNA sequences. Optimised ML phylogram with support values: NJ/ MP/ML

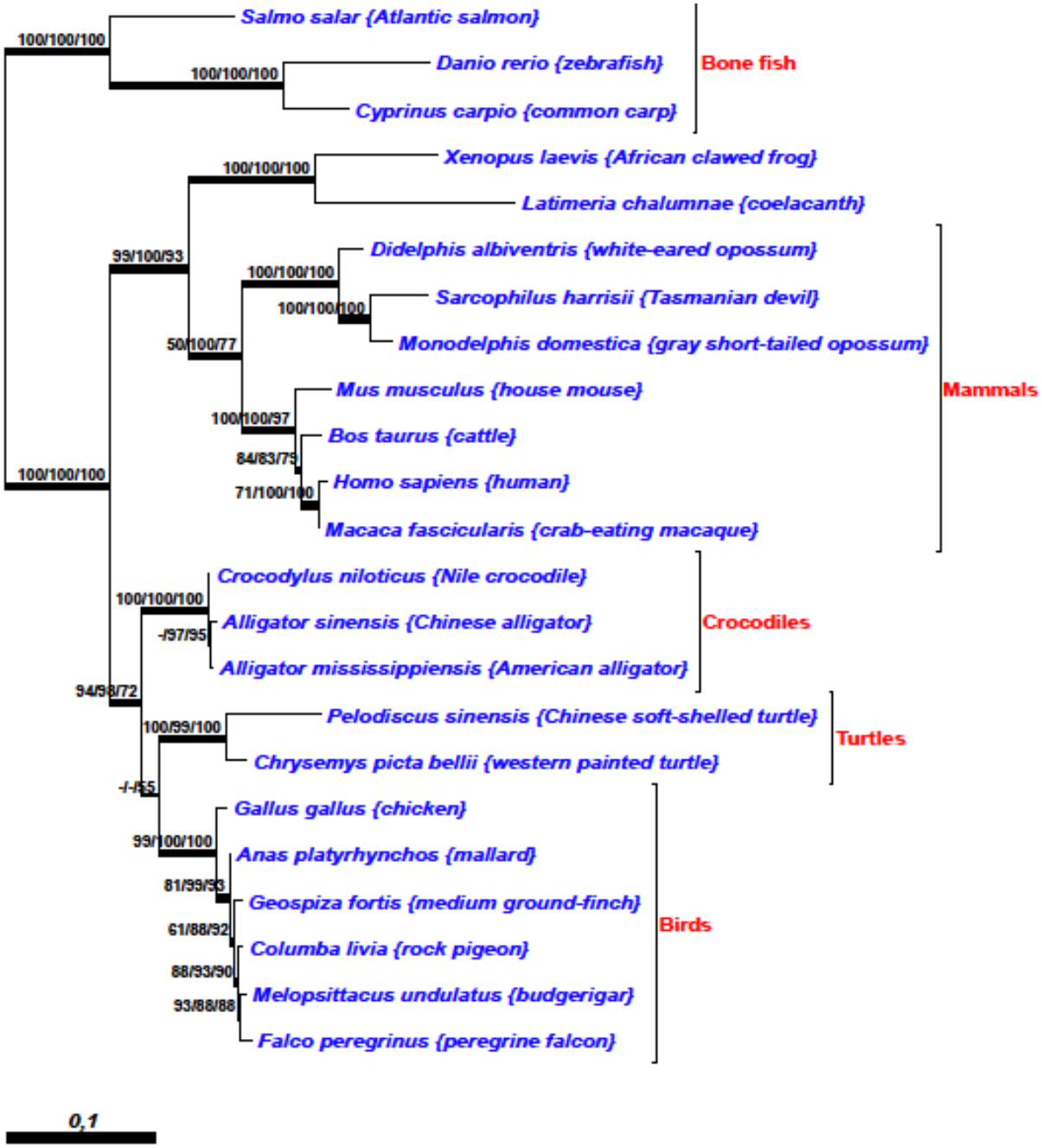

Figure 70. Phylogenetic relationship of *bmp4* DNA sequences. Optimised ML phylogram with support values: NJ/ MP/ML

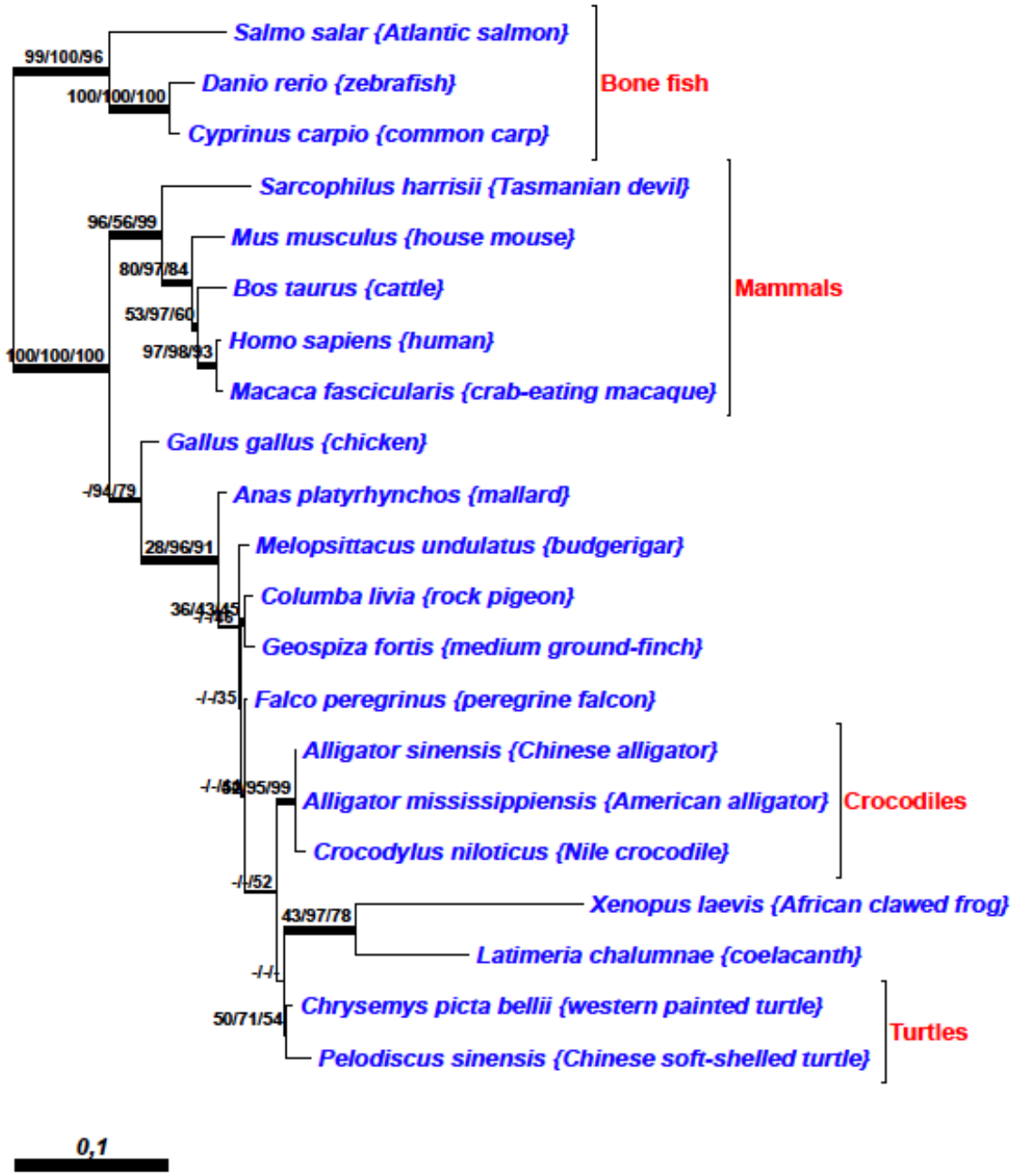

Figure 71. Phylogenetic relationship of *pitx2* gene DNA sequences. Optimised ML phylogram with support values: NJ/ MP/ML

4 DISCUSSION

The detailed histological account of odontogenesis in the Nile crocodile, which has not been previously described, revealed that both histology and development were similar to the mammalian condition. However in the early developmental period in the crocodile, epithelial placodes were found, which degenerate in some mammals and remain as remnant structures in others. Also the bud stage of tooth development in the crocodile, unlike the mammalian situation appeared to be transitory and not prominent. An enamel knot-type structure was identified, but did not express *bmp4* or *pitx2*. Distinct tooth families were noted in the older specimens. Some replacement teeth exhibited a molariform structure, even though the functional dentition of the crocodile exhibits simple conical and unicusped teeth. *Pitx2* expression was evident in the youngest specimens in the oral epithelium and the dental placode. *Bmp4* expression was not evident in the dental placode, but was localised in the odontoblasts of early bell stage tooth germs. *Pitx2* and *bmp4* were both expressed in the odontoblast and ameloblast layers in late bell stage tooth germs.

4.1 Housekeeping gene – *gapdh*

In the crocodile, the housekeeping gene, *gapdh*, showed variation of expression in different tissues possibly revealing the role of *gapdh* in the cell. *Gapdh* in humans catalyzes reactions in the glycolytic pathway (Barber *et al*., 2005). *Gapdh* catalyses the reversible oxidative phosphorulation of glyceraldehyde-3-phosphate via a thioester intermediate. This reaction is regulated by nicotinamide adenine dinucleotide (NAD). Since NAD^+ must be replenished for glycolysis to occur, consequently it is not surprising that *gapdh* expression is not the same in all tissues, and therefore will be higher in tissues with higher energy requirements. In the present study therefore expression of *gapdh* was highest in skeletal muscle and regions of the brain.

4.2 Previous histological studies

Histological studies focused on the development of the reptilian dentition have been previously described in several species such as in *Lacerta vivipara* (common lizard) (Osborn, 1971), *Sphenodon punctatus* (tuatara), *Cnemapsis kandiana* (Kandyan gecko), *Lacerta agilis* (sand lizard) and *Angius fragilis* (slow worm) by Westergaard (1988) and in *Alligator mississippiensis* (alligator) by Westergaard and Ferguson (1986). More recently, Sire *et al*. (2002) examined the first generation teeth in *Chalchides viridanus* (West Canary skink) and *Chalchides sexlineatus* (Grand Canaria skink) and the dentition and replacement patterns of these skinks was also examined by Delgado *et al.* (2003). Tooth development as well as enamel formation was also examined by Delgado *et al*. (2005) in the lizard. A detailed histological analysis on tooth development and replacement in the Nile crocodile, has however not been found in the literature.

4.3 Tooth initiation

The first sign of tooth initiation in the Nile crocodile was evident in the 14-31 day-old embryos as equidistant thickenings in the ectomesenchyme. These thickenings represented the initial site of tooth development, but did not exclude the existence of odontogenic potential over a larger area of the ectomesenchyme (Westergaard, 1988). In earlier studies on alligators and various lepidosaurian species, evidence of a clear condensation of ectomesenchyme was delayed until a distinct epithelial thickening was identified (Westergaard, 1988; Westergaard and Ferguson, 1986). Sometimes tooth initiation occurs with no accumulation in the ectomesenchyme suggesting that the condensation of ectomesenchymal cells is not always required to induce the oral epithelium. It is possible that the signal/s which is/are involved in inducing the odontogenic potential of the oral epithelium and condensation of ectomesenchyme are probably different (Lumsden, 1988; Westergaard and Fergusson, 1990).

The initiation of teeth at intermittent positions along the length of the jaws in the crocodile, suggests that there are molecular controls that regulate the arrangement of teeth along the jaws. Osborn (1973a) proposed a clonal theory which implied that morphologically similar teeth, develop from a clone of cells whose origin is the previously formed tooth, moving along the jaws from anterior to posterior. Therefore a collection of teeth of similar shape are derivatives of a specific clone. As a new tooth develops, it is surrounded by a zone of inhibition, allowing for the equidistant spacing between teeth (Osborn, 1973a; 1978; 1998). Even though the localised condensation is firstly evident in the ectomesenchyme, the original stimulus and inhibition process may originate and disperse in the oral epithelium. The oral epithelium may then induce the underlying ectomesenchyme resulting in its condensation. Osborn's (1978; 1984) clonal model highlights that tooth initiation occurs due to developing clones of ectomesenchymal cells.

In situ hybridization in the youngest specimens collected in the present study, revealed a continuous band of *pitx2* expression along the length of the oral epithelium of the maxilla and the mandible that preceded any epithelial thickenings or dental lamina. This is similar to findings on the expression of *pitx2* in the oral ectoderm of both trout (Fraser *et al*., 2006a) and python (Handrigan and Richman, 2010b) embryos. Perhaps a continuous band of *pitx2* expression assists in the lengthening of the dental lamina. Before the initiation of tooth development, an odontogenic band or dental lamina appears in the oral epithelium as a thickening which is indicative of the tooth-forming area. The exact molecular networks for dental lamina formation are relatively unknown, but numerous studies have revealed the expression of *pitx2* and *shh* in the dental lamina of several vertebrates (Buchtová *et al*., 2007; 2008; Fraser *et al*., 2006b; Keränen *et al*., 1999; Smith *et al*., 2009b; Vonk *et al*., 2008). Teeth are initiated in the lamina, by a gradual restriction in the expression of *pitx2* and *shh t*o specific structures referred to as dental placodes (Jernvall and Thesleff, 2012). This was not evident in the present study where *pitx2* expression remained in the oral epithelium and

the placodes. In gnathostomes, birds and snakes (Fraser *et al.*, 2004; Fraser *et al*., 2006a; Vonk *et al*., 2008; Buchtová *et al*., 2008) the odontogenic band is characterised by expression of *shh*. Thus expression of *pitx2* in the dental lamina in crocodiles may be used to determine the position of the dental lamina within the ectoderm of the facial prominences. As the data of the present study suggests, *pitx2* is also probably required for growth of the dental lamina and the placode into the ectomesenchyme. The lingering expression of *pitx2* in the odontogenic band may be nececssary for retention of the odontogenic band and to preserve competence for the epithelial cells to form more primary or first generation teeth. In some rodents, there are discrete areas of *pitx2* expression (Miyado *et al*., 2007) rather than the continuous band that occurs in crocodiles. *Pitx2* is known as one of the earliest epithelial markers of mouse tooth development (Mucchielli *et al.*, 1997) and has an important role to play in the genetic basis of pattern formation and epithelial differentiation in the tooth (Lin *et al*., 2007; Lu *et al*., 1999; Hjalt *et al*., 2000).

Pitx2 expression is detectable early in mouse embryonic development. It is initially highly expressed in the oral epithelium and the invagination into the ectomesenchyme and then gradually becomes limited to the dental placode, the dental lamina and the enamel knot (St Amand *et al*., 2000). Fraser *et al*. (2004) in a study on cyclid fish described *pitx2* as a putative "odontogenic commissioning gene". *Pitx2* expression marks the whole extent of the dental competent oral epithelium that includes both the developing tooth germ and the inter-tooth region. In the python, *pitx2* expression during tooth initiation occurs mainly around the distal end of the dental lamina and then the successional lamina later in development (Handigan and Richman, 2010b). It appears therefore that a similar molecular pattern regulates initiation and replacement in snakes. Additionally it suggests that the molecular control of successional lamina initiation, in snakes, is specified during the early stages of odontogenesis. Cell-lineage experiments may

confirm the validity of this hypothesis. It seems that the successional initiation during the early stages of odontogenesis is also true of crocodiles.

In the present study, *bmp4* expression was not evident in the oral epithelium in the youngest specimens examined. However, in mice, *bmp4* is expressed in the primary odontogenic band (Bitgood and McMahon, 1995). In mutant mice however, with a phenotype produced to drive the expression of Noggin, a *bmp4* antagonist, placode-like thickenings were evident in the epithelium, but the mandibular molars did not develop (Plikus *et al*., 2005). This showed that several other genes must be investigated in the primary odontogenic band.

While approximately 300 tooth-related genes and approximately 100 growth and transcription factors have been identified as being important in mammalian tooth development (Jernvall and Thesleff, 2000) further advancement studies are necessary to unravel the cellular and molecular mechanisms controlling tooth initiation. Mechanisms involved in tooth development are complex, involving genes that control positional information, cell adhesion, cytoskeletal changes, growth factors and reciprocal epithelial-mesenchymal interactions. In the present study, initiation of tooth development appears to involve the ectomesenchyme as the first sign of tooth formation as evidenced by the thickenings in the 14-31 day-old specimens.

4.4 Dental placodes

After initiation, the continuation of odontogenesis in the crocodile was evident by the development of the dental placode, which occurred in the youngest specimens at stage 14-31 of incubation, prior to the bud stage of tooth development. The placode stage is referred to as the "dental determinant" (Osborn, 1973b) and is the first distinctive phase of tooth development in the crocodile. The surrounding

ectomesenchyme did not always appear condensed. Westergaard and Fergusson (1990) in a study of odontogenesis in the alligator showed that sometimes tooth initiation occurred with no aggregation in the ectomesenchyme. The signal/s which is/are responsible for competence of the epithelium and the ectomesenchyme probably differ. As in several other integumental systems including scales, feathers and hairs, the first sign of development is found in the epithelium (Tanaka and Kato, 1983). With the development of the epithelial placode, cell division ceases in the placode but remains in the interplacodal areas (Tanaka and Kato, 1983). In the alligator, Westergaard and Ferguson (1986; 1987) showed that the first generation teeth evaginate from the oral ectoderm and then sink into the ectomesenchyme forming the dental placodes. In *Ikka* mutant mice, *Ikka* being a critical regulator of keratinocyte differentiation, abnormalities in molar cusp shape, palatal shelf fusion and epithelial cell adhesion were evident (Blackburn *et al.,* 2012). The incisor tooth epithelium in *Ikka* mutant mice, evaginated instead of invaginated into the oral cavity, suggesting *Ikka* has a regulatory role in the orientation of developing tooth germs (Blackburn *et al.*, 2012). Evaginating tooth structures were not evident in the present study,

The placodal teeth in the present study appeared to be poorly differentiated. These rudimentary teeth did not have a fully differentiated inner enamel epithelium. It is possible that the early maxillary and mandibular epithelium is not competent to differentiate into enamel forming ameloblasts at this stage. Also immature odontoblast-type cells and their extracellular secretory products may be adequately different from well differentiated odontoblasts, in that they fail to provide the inductive stimulus required for differentiation of ameloblasts. In the future, experimental investigations using recombinations of dental papillae ectomesenchyme and maxillary and mandibular epithelia at early stages of crocodile embryos may provide some answers as to why some poorly developed teeth develop and later appear to be degenerating. The question as to what causes the initiation of the placode teeth in reptiles and what importance they may play in

tooth development has caused some controversy. Edmund (1960) proposed a theory for reptilian tooth initiation and development. He described the presence of a ‘stimulus generator’ at the anterior tip of the jaws that emitted chemical signals posteriorly through the jaws at regular intervals, initiating tooth germs at each potential tooth position. This theory was criticised by Osborn (1970, 1971) due to insufficient embryological evidence. Osborn (1970) suggested instead, that neural crest cells migrate into the jaws and initiate odontogenesis as soon as they have come into contact with the competent oral epithelium. The tooth germs then establish and are surrounded by a zone of inhibition, which prevents tooth initiation in the immediate area surrounding the developing germ. As the developing dental lamina elongates adequately and passes the inhibition zone of the newly formed tooth germ replacement teeth were initiated from the dental lamina, thus establishing tooth families. Westergaard (1988) specified that tooth initiation in the alligator and other reptiles is interrelated with differential jaw growth. Variation in jaw growth may facilitate the establishment or removal of tooth positions anywhere in the tooth-forming area. New teeth would then be initiated further away from initial teeth. This distance at the point where new teeth are initiated correlates with the sizes and developmental stages of the approximate established teeth, thus lending support to the zone of inhibition (Westergaard, 1988).

Thus crocodile odontogenesis appears to begin by the initiation of the first tooth, the dental determinant or placode (Osborn, 1973b). These placodal embryonic teeth are resorbed, but their presence may indicate a developmental process of importance in establishing the functional dentition. It is interesting to note that the placode teeth develop directly from the oral epithelium, without the presence of a dental lamina. This also occurs in the zebrafish (Huysseune *et al.,* 1998; Laurenti *et al*., 2004) as well as in other non-mammalian tertapods (Huysseune and Sire, 1997; Sire *et al.*, 2002). In the alligator, the first teeth develop from the odontogenic (oral) surface epithelium (dental placode) before the formation of a

dental lamina occurs (Westergaard and Ferguson, 1990). In the pharyngeal dentition of the zebrafish (*Danio rario*) and the cichlid (*Astatotilapia elegans*) (Huysseune, 1983), the first generation teeth are initiated in adjacent positions with one tooth preceding all others. In the salmon (*Salmo salar*) (Fraser *et al*., 2004) and the rainbow trout (*Salmo gairdneri*) (Berkovitz and Moore, 1975) a limited number of first generation teeth are initiated in alternating positions, followed by the positions in between. This was also evident in the mandible of the lizard (*Lacerta vivipara*) (Osborn, 1971). The sequence therefore in which first generation teeth appear in various species suggests there may be some patterning mechanism (Huysseune and Witten, 2006). Smith (2003) suggested that this is a "waveform of reaction activity" and indicated it was a "dynamic field-type process". Westergaard and Ferguson (1986) suggest that in the developing alligator dentition, new tooth germs that occur in the space between two existing teeth always develop slightly nearer to the more developed tooth of the two. Interestingly, Westergaard and Ferguson (1987) showed that field models may not only cause a concentration gradient of a morphogen, but can also interfere with cell-cell or cell-matrix communications or multicomponent gradients. Alternatively, the development of a series of first generation teeth could be the result of the activation of a segment polarity gene. A candidate may be *pitx2*. In the crocodile, in the present study, *pitx2* was expressed prior to placode formation. This is also evident in the zebrafish (Jackman *et al*., 2004) and the rainbow trout (Fraser *et al*., 2004), where *pitx2* expression is first evident in the pharyngeal epithelium prior to the individual tooth formation. Thus it appears that *pitx2* may indicate odontogenic competence. However, a non-tooth forming epithelium would need to be studied in order to determine if this is so.

4.5 Null generation teeth

Other tooth-like structures that were identified in this study appeared rudimentary and were termed 'null generation' tooth germs. 'Null generation' tooth germs

have been described in archosaurs (the most recent common ancestor of birds and crocodiles) and lepidosaurs (reptiles with overlapping scales like lizards and snakes) (Osborn, 1971; Westergaard and Ferguson, 1986; Sire *et al*., 2002). These non-functional teeth were also observed in the gecko (*Paroedura picta*) and were thought to represent remnants of a functional dentition which has been lost (Zahradnicek *et al.*, 2012). These latter authors suggested that non-mammalian species which develop rudimentary teeth have an extended gestation period and an absent larval dentition. These earliest degenerating teeth in crocodiles may be similar to the situation in sharks where the initial first generation teeth never become functional. However, these "rudimentary initiator teeth" define specific tooth loci along the jaws for the development of potential clonal families of functional teeth. In a clonal family the tooth primordial become competent to control tooth shape, size and polarity (Smith, 2003). In the present study, the 'null generation teeth developed before the formation of the dental lamina, thus suggesting that the dental lamina is not necessary for initial tooth development.

The significance of the rounded and oval 'null generation' teeth towards the most posterior aspects of the jaws may be attributed to the change in morphology that occurs in the posterior teeth of the crocodile. An increased gestation period is said to reduce the necessity for a set of early, small rapidly developing tooth germs, making way for a larger set of functional teeth. 'Null generation' teeth in the Nile crocodile were smaller and simpler as compared to the teeth of later generations in this animal. This is also evident in the gecko (Zahradnicek *et al.*, 2012) and in the first generation teeth of the alligator (Westergaard and Ferguson, 1987).

In the present study, some 'null generation' teeth appeared more advanced in structure than others suggesting that these structures may be under spatio-temporal control due to differences in the presence of signaling molecules or transcription factors. A decrease in the expression of *shh* in the bearded dragon (*Pogon barbata)* is said to be the primary factor for development of superficial

teeth which degenerate (Handrigan and Richman, 2010a). Variation in expression of various factors during evolution may have caused early tooth structures to be non-functional. However the presence of these rudimentary tooth structures may provide a framework for the patterning of the later functional dentition. In the gecko (*Paroedura picta*), Zahradnicek *et al.* (2012) described 'null generation' teeth as the dental determinants which play a role in the future dentition of numerous reptile species. This role would identify the reason as to why the 'null generation' teeth are common within the reptile species (Handrigan and Richman, 2010a). In the mouse, rudimentary tooth structures are present in the diastema which occurs distal to the first molar. These underdeveloped tooth germs may represent remnants of ancestral premolars. Premolars are not found in mice and are seen to degenerate at the bud stage (Zahradnicek *et al.*, 2012). It has been suggested that the 'null generation teeth' in the mouse have a patterning role, initiating the sequence of development of the molars (Prochazka *et al*., 2010). In the shrew (*Sorex araneu*), the deciduous tooth germs in the same tooth loci degenerate before they become functional (Järvinen *et al.*, 2008). These authors suggested that the structures present of the later-developing functional dentition, albeit in an immature state, prevent further development of the first generation teeth and that this may also be the situation in reptiles.

In archosaurs the 'null generation' teeth were either expelled into the oral cavity or resorbed (Westergaard and Ferguson, 1986; Westergaard and Ferguson, 1990). Alligator 'null generation' teeth persist, then disintegrate in the ectomesenchyme and their dentine is resorbed (Westergaard and Ferguson, 1986). In the gecko, the fate of the 'null generation' teeth appears to be site dependent. Those closer to the surface epithelium of the oral cavity are expelled into it, whereas those developing deeper in the ectomesenchyme, were resorbed (Zahradnicek *et al.*, 2012). Westergaard and Ferguson (1986) in their detailed study of alligator teeth described osteoclasts in the dental papilla of 'null generation' teeth. Osteoclast-like cells were also seen at the periphery of the 'null generation' teeth in the gecko

(Zahradnicek *et al.*, 2012). In the present study on the crocodile, clusters of multinucleate cells were often associated with the dentine remnants of the 'null generation teeth' indicating possible odontoclastic activity. Also, rounded structures associated with the reduced enamel epithelium in hatched and post-hatched crocodile specimens indicate that some 'null generation' teeth may be released into the oral cavity.

In the alligator, placodal teeth appear before the development of a true dental lamina. After initiation of these teeth, the dental lamina forms and the first formed teeth remain as degenerating rudimentary structures within the ectomesenchyme (Westergaard and Ferguson, 1987; 1990). This appears to be the process in the crocodile as well. Alligator embryos also develop several different tooth structures before hatching, some of which are lost before they become functional (Westergaard and Ferguson, 1987; 1990). In the present study, the 'null generation' teeth are reminiscent of these early rudimentary structures described by Westergaard and Ferguson (1987; 1990). It is interesting to note that these initial rudimentary tooth germs of variable size and shape in crocodilian embryos are in contrast to the relatively homogeneous dentition that occurs in the adult (Westergaard and Ferguson, 1986). Mature vestigial teeth and vestigial tooth germs have been identified in reptiles and various mammalian species ranging from rodents to whales (Witter *et al*., 2005; Handrigan and Richman, 2010a). The phylogenetic implications of these vestigial teeth is undecided. Due to the variable morphological structure of the vestigial teeth it has been proposed that the variation may be due to decreased selective pressure imposed on these teeth (Dayan *et al*., 2002).

The variability of initial tooth structure was also described by Sire *et al*. (2002) in a study on the first generation of teeth in several reptilian lineages. Two types of first formed functional teeth were identified. The first type (Type 1) is characterised by small conical-shaped crowns. The dentine matrix is secreted

mainly parallel to the long axis of the tooth and lacks odontoblastic processes. The dentine tip is covered by a highly mineralised enameloid. The narrow pulp cavity is devoid of nerve endings and capillaries. Examples of Type 1 first generation teeth include the actinopterygians (ray finned fish), some species of lung fish and newts (Sire *et al*., 2002). The second type of first generation tooth described by Sire *et al*. (2002) were found in the Nile crocodile, catshark, and Canaria skink. These organisms have large conical shaped teeth and the mineralised collagenous matrix of the dentine contain odontoblastic processes throughout the extent of the dentine layer. The pulp cavity is large and contains blood vessels. Sire *et al.* (2002) emphasised that in the Type 1 group, the absence of a complex shape of the first generation teeth could be explained from an embryological perspective, as these structures had a decreased cell number which did not allow the complex arrangement of the enamel organ to develop. Sire *et al.* (2002) also highlighted that the Type 1 teeth were the most economical way to form a tooth. The thinner dentine walls did not require odontoblastic processes or an extensive blood supply. However, the atubular dentine in the Type 1 species was considered as an odontogenic precursor to true dentine or orthodentine, characteristic of Type 2 organisms. Type 1 organisms usually have a short embryonic period, whereas the Type 2 group have extended embryonic development. Suppression of odontogenesis during the embryonic period in the form of rudimentary teeth has been associated with Type 2 organisms which include lizards (Osborn, 1971) and alligators (Westergaard and Ferguson, 1986). The crocodiles examined in the present study would be classified as Type 2 organisms according to the criteria by Sire *et al*. (2002). The answer to the question on how several vertebrates start with a simple tooth type, yet ultimately develop a complex dental structure that is unique to their adaptation, lies in the complex molecular mechanisms associated with polyphyodonty (Sire *et al*., 2002).

Peterkova *et al*. (2006) in a study on the phylogenetic memory of developing mammalian dentitions explained that certain structures suppressed during evolution could be re-traced to atavisms and vestiges. Atavism is defined as the exceptional reappearance of a lost ancestral character. Vestiges are rudimentary structures and are defined as a remnant of an ancestral organism, suppressed during evolution and which occurs frequently in members of a present species (Peterkova *et al*., 2006). For example the appendix and the coccyx in humans are two examples of vestiges. Tooth vestiges are useful in verifying the sequential homology of teeth and clarifying evolutionary relations between species (Luckett, 1993). Peterkova *et al*. (2006) identified the presence of transient dental placodes in the mouse incisor and diastema regions that were reminiscent of tooth primordia in reptiles. A pair of large vestigial buds arise anterior to the prospective first molar (in the diastema region) and probably corresponds to the premolars that have disappeared along the pathway of mouse evolution. The posterior premolar vestige is incorporated into the mandibular first molar indicating the process of evolutionary loss of the last premolar in mice. In a study on mutant mice, abnormal odontogenesis leads to the revivification of atavistic supernumerary teeth (Peterkova *et al.*, 2006).

4.6 The dental lamina

After the placode stage in the crocodile, the dental lamina is initiated. Tooth replacement in crocodiles as in mammals, amphibians and other reptiles (Edmund, 1960; Ooë, 1981; Westergaard, 1988; Sire *et al*., 2002; Järvinen *et al*., 2009; Delgado *et al*., 2005, Smith *et al*., 2009b; Handrigan and Richman, 2010a) begins with the formation of a dental lamina. The dental lamina is a ribbon-like epithelial structure that extends along the length of the maxilla and the mandible (Westergaard and Ferguson, 1986). In alligators, tooth germs bud from the labial surface of the dental lamina, progress through a series of stages and form a homodont, unicuspid tooth (Westergaard and Ferguson, 1986). This was also

confirmed in the present study. Once the predecessor tooth bud differentiates, the successional lamina develops from the outer enamel epithelium on the lingual side of the differentiating tooth germ. The development of the successional lamina signifies the beginning of a new generation of teeth in the alligator (Westergaard and Ferguson, 1986) as well as in other reptiles (Sire *et al*., 2002; Delgado *et al*., 2005; Buchtová *et al*., 2008; Handrigan and Richman, 2010b) and in diphyodont mammals (Ooë, 1981; Järvinen *et al*., 2008; Järvinen *et al*., 2009).

It is possible that signals from the ectomesenchyme specify tooth formation at specific locations along the dental lamina. Likewise it is also equally possible that inhibitory signals act on the inter-tooth areas where no tooth formation occurs.
An interesting result of the present study was that in the maxilla and the mandible, teeth form all along the dental lamina and are seen connected to each other. However it is possible to differentiate inter-dental regions between two families in the crocodile. This may indicate that there must be some molecular interactions that determine where the teeth are positioned from anterior to posterior along the jaws. *Pitx2* does not appear to be a candidate for the process of specifying tooth locations in crocodilian embryos due to the lack of differential expression along the dental lamina prior to tooth initiation.

In the early crocodile specimens, the dental lamina lost contact with the oral epithelium, but persisted beneath the epithelium as a continuous epithelial string which formed the successional lamina, along the entire jaw half until hatching. This string appears to be a pathway for tooth initiation along the jaws (Edmund 1962, 1969). Polyphyodont species have a permanent continuous dental lamina. In diphyodont species, fragmentation of the dental lamina occurs and regresses after the initiation of the second tooth generation. Buchtová *et al*. (2012) in a study on dental lamina degradation in the diphyodont minipig, showed that the process involved a combination of cell migration, cell-fate transformation and apoptosis. Also, uncovering the processes of dental lamina degradation allows for

some clarification on the evolutionary mechanisms leading to diphyodonty and provides a mechanism of future manipulations on the number of tooth generations. In contrast to pigs and humans, the dental lamina in the crocodile, loses its connection with the oral epithelium, but persists, linking the developing tooth germs and providing further generations of tooth germs from its leading edge (Buchtová *et al*., 2012). The first sign of dental lamina degradation in snakes was associated with the presence of blood vessels near the lamina, which may stimulate the transformation of dental lamina cells (Buchtová *et al*., 2012). Also, dental lamina cells became enlarged, formed rounded clusters of cells that appeared detached from the lamina. Buchtová *et al*. (2012) emphasised that a signal from the developing tooth or surrounding ectomesenchyme adjacent to the dental lamina may induce its regression. This has significant implications as controlling the signals responsible for dental lamina degradation it may be possible to maintain the lamina and produce additional tooth generations to replace missing damaged or lost teeth. It is therefore important to investigate what causes regression of the dental lamina in a diphyodont species and what factors inhibit its regression as in the case of polyphyodont species. In the snake, *bmp4*, *shh*, and *Wnt* have all been implicated in the persistence of the dental lamina and overexpression of *Wnt* was implicated in dental lamina elongation (Jarvinen *et al.*, 2006). *Bmp4* expression was not evident in the dental lamina in the present study.

4.7 Cap stage of tooth development and the enamel knot

As in the mammalian situation, after the development of the dental lamina in the crocodile, individual teeth were identified as bud, cap, early and late bell stages. These stages appear to be essential for survival of the crocodile as the correct developmental pattern of the teeth depend almost entirely on the patterns established during early odontogenesis (Jernvall and Thesleff, 2012). In the 32-65 day-old specimens the crocodile embryo presented a diversity of tooth stages along the jaw. This, according to Westergaard and Ferguson (1987) is not a

random arrangement, but rather a progression of tooth types (Westergaard and Ferguson, 1987; 1990). The ability of individual teeth to follow separate fates, for example to become rudimentary structures reinforces the theory that each tooth or tooth wave represents an independent structure. Weeks *et al.* (2013) highlighted that adjacent teeth can display unique gene expression patterns and have distinct fates suggesting that the anterior-posterior axis of the jaw has a semi-autonomous dental capacity (Weeks *et al.*, 2013).

The cap stage tooth germs in the crocodiles appeared similar in structure to the mammalian situation, however a distinct enamel knot-type structure, which plays a significant role in mammalian odontogenesis was not discernable. Although a distinct enamel knot was not identified in the crocodile specimens in this study, an enamel-knot type of structure was evident in some bell stage tooth germs suggesting a possible transitional type of structure occurring in crocodiles, distinct from the enamel knot that has been so extensively studied in mammals. In other reptiles, such as snakes and lizards (squamates) enamel knots have not been identified (Handrigan and Richman, 2010a) but *shh* and *bmp4* are expressed in the inner enamel epithelium of the developing tooth germs in snakes and lizards.

In mammals the enamel knot consists of a transient population of non-mitotic epithelial cells that becomes evident during the cap stage of tooth development at the site of the future primary tooth cusps in mammals (Cobourne, 1999). It is thought that the enamel knot acts as a signaling centre which is involved in directing cellular proliferation and cuspal morphogenesis in the developing enamel organ in mammals (Vaahtokari *et al*., 1996). The primary enamel knot is incorporated into the inner enamel epithelium of cap-stage teeth. The enamel knot is a rounded mass of thickened epithelial cells which can be distinguished morphologically. The other distinctive characteristics of the mammalian enamel knot are the absence of proliferation and increased apoptotic activity (Jernvall *et al*., 1994; Matalova *et al*., 2004).

The dominant theory is that the primary enamel knot serves three important functions. Firstly, the enamel knot releases mitotic factors that are involved in stimulating the inner epithelium and cervical loop. Secondly, the dental papilla is induced by the primary enamel knot of the bud-stage teeth and thirdly, the primary enamel knot induces the formation of secondary enamel knots, which are involved in the formation of additional cusps and induce odontoblast development in the adjacent dental papilla (Richman and Handrigan, 2011). The deposition of the dentine matrix therefore begins at the cusp tips. The mouse enamel knot expresses many signaling molecules including BMPs, FGFs, *shh* and *Wnt* families. In a study on lizards and snakes, Buchtová *et al.* (2008) showed *shh* expression in the inner enamel epithelium in cap-stage tooth germs, and this expression was no longer evident at the future cusp tip, as ameloblast polarisation occurred. In some squamates, *Bmp4* transcripts are also transiently expressed in early cap stage tooth germs. However expression diminishes with the differentiation of the inner enamel epithelium (Handrigan and Richman, 2010b). Deletion of several of the signalling molecules in the enamel knot results in disruption of tooth development at the bud or early cap-stage. The precise evolutionary conservation of primary and secondary enamel knots is uncertain. The location of enamel knots in sharks (Smith *et al.*, 2009), fish or amphibians, solely on the basis of morphology, is difficult. A similar situation occurs in squamates where the localized thickening in the inner enamel epithelium is not evident. In the Crocodilia, Westergaard and Ferguson (1986) reported seeing a slightly thickened enamel knot in the American alligator.

In mammalian molar teeth, secondary enamel knots are evident at the future secondary cusp tips, probably under the influence of the primary knot. Jernvall *et al*. (1994) proposed that the cellular proliferation of the enamel organ and the lack of cell division in the enamel knot contributes to the folding of the enamel organ. The proposed signaling function of the enamel knot indicates that a structure

derived from the epithelium, may be involved in the later stages of tooth development. Cobourne (1999) suggested that the enamel knot is required for morphogenesis of the tooth germ to progress from the bud to the cap stage of tooth development.

Bmp4 and *pitx2* were not seen in the 'enamel knot' type structures that were evident in the present study. Handrigan and Richman (2011) suggested that squamates have no enamel knot and that the enamel knot signals originate from the inner enamel epithelium. In a study on the alligator no distinct enamel knot was identified (Weeks *et al*., 2013). Perhaps the lack of an enamel knot structure in crocodilians and squamates suggests that the common ancestor of extant reptiles did not have an enamel knot. Weeks *et al*., (2013) highlighted that the expression of enamel knot genes from the inner enamel epithelium in alligators and squamates occurred due to gradual spatial restriction of genes in the inner enamel epithelium. This restricted expression may affect cell migration, cell-cell adhesion or even cell-survival. Alteration to a specific gene like *Eda* (Ectodysplasin A) is capable of producing changes in tooth number, tooth shape and position. Non-independence of gene signaling in the developing crocodilian tooth could therefore, on the same basis, allow a single gene alteration to cause limited expression of other genes, guiding enamel knot evolution (Kangas *et al*., 2004). It has been shown with certainty that fish have distinct enamel knots or formation of signaling-type centres that cause additional cusp development (Jernvall and Thesleff, 2012). A study on cichlid fish showed that first generation teeth are replaced with two or three cuspid teeth (Streelman *et al*., 2003). The cellular dynamics of the cichlid teeth showed similarities with the molecular cascade found in the regulation of enamel knots in mammalian teeth. *Shh* is expressed in the inner enamel epithelium of cap-shaped tooth germs but expression is "diffuse" with no distinct enamel-knot domain (Handrigan and Richman, 2010a; Handrigan and Richman, 2010b; Handrigan and Richman, 2011). Studies involving cyclopamine, a *shh* signaling inhibitor, (Buchtová *et al*.,

2008; Handrigan and Richman, 2010a) show that tooth morphogenesis does not occur. The molecular networks expressed in the inner enamel epithelium of snake and lizard teeth for example *Wnt6, Wnt7a* and *bmp4* are similar to the expression seen in mammalian tooth development (Handrigan and Richman, 2010b). Recently it has been shown that each mammalian secondary enamel knot, gives rise to individual cusps which express the same set of genes (Jernvall and Thesleff, 2012). In mammals the specialized expression of the numerous factors in the secondary enamel knots indicate a distinctive well regulated cusp-pattern which is a pre-requisite for exact occlusion (Jernvall and Thesleff, 2012).

In a study on the role of *bmp4* during tooth morphogenesis and sequential tooth formation, Shihai *et al.* (2013) concluded that *bmp4* in the ectomesenchyme is responsible for and is required for driving molar tooth morphogenesis from the bud to the cap stage of tooth development. In future work, the *bmp* pathway modulators will contribute to the *shh-bmp-Wnt* signalling cascade and also show other pathways (e.g. *fgf*) converge on the *Wnt* pathway to regulate tooth replacement in crocodile. During the cap stage of tooth development *pitx2* is expressed in the inner enamel epithelium and the length of the successional lamina from which replacement teeth ultimately form. Also the expression of *pitx2* in the replacement teeth mimic expression in the initial tooth developmental process. *Bmp4* expression was also evident in the inner enamel epithelium in the cap stage tooth germs. The same expression of *bmp4* occurred in the replacement tooth germs. This suggests that *bmp4* expression in the crocodile enamel organ may be involved in the differentiation of pre-ameloblasts into secretory ameloblasts. It is also possible that *bmp4* expression, as there is no distinct enamel knot structure in the crocodile, may be involved in cusp formation. In relation to the mouse and man, it is of significance that there was no *bmp4* expression in the crocodile in the dental papilla or dental follicle, in the cap and early bell stages of tooth development. Torres *et al*. (2007) in a study on the opposum, indicated that *bmp4* is considered an important inducer of odontoblast differentiation and

extracellular matrix protein expression. This appears to be a possibility in the crocodile where *bmp4* expression was evident in the odontoblast layer and in the dentine of the cap and early bell stage tooth gerrms. It is interesting in the crocodile, that initially *bmp4* is expressed in the odontoblasts, in the cap stage, and in the late bell stage the expression occurs in the odontoblast and ameloblast layers. This apparent transfer of bmp4 expression from an epithelial to a mesenchymal tissue may coincide with the odontogenic potential in the same direction.

4.8 The bell stage of tooth development

The next stage of tooth development in the crocodile, as in the mammalian situation, was the bell stage of tooth development. In the 32-65 day crocodile specimens, there appeared to be two types of bell stage tooth germs. Some tooth germs in the bell stage of tooth development were undergoing resorption, while some appeared to be developing as the future functional tooth. These bell-type tooth germs produced dentine, but did not form enamel. The degeneration of some resorptive or transitional teeth situated near well developed replacement teeth, may suggest competition or inhibition between developing teeth. The capacity to alter individual teeth with seemingly no consequences for neighbouring teeth, or even to remove a subset of dental structures, is likely made possible by the decoupling of the development of individual teeth from one another at one or many developmental stages (Weeks *et al.*, 2013). Weeks *et al.* (2013) suggested that the presence of an “alligator-like” grand plan in the amniote ancestors may explain the different tooth types and tooth replacement mechanisms which occur.

In the early bell stage tooth germs identified in the 32-65 day-old crocodile embryos, the layer between the ameloblasts and the stellate reticulum, as in mammals was identified as the stratum intermedium, which appeared similar in histological structure to the stratum intermedium in mammals. This layer is said to

play a role in enamel mineralization (Nakamura *et al.,* 1994). The stratum intermedium has also been identified in alligators and geckos (Zahradnicek *et al.*, 2012). However, this layer persists during the later mineralization phase in crocodiles as opposed to mammals where the stratum intermedium cells are evident until the differentiation of ameloblasts occurs (Koyama *et al.,* 2001). In the gecko, the stratum intermedium and the formation of enamel was correlated suggesting that it plays a role in the deposition of enamel. The stratum intermedium has been reported in mammals, fish and reptiles and could signify the plesiomorphic nature of jawed vertebrates. Crocodiles have aprismatic enamel. Crocodilian teeth appear not to have Tomes processes which are a specialized functional structure present during enamel formation in mammals. In a study on the Calcium-ATPase activity in embryonic American alligators, the authors suggested that calcium accumulation and enamel mineralization was also similar to the mammalian situation (Sato *et al*., 1993). In *Chalchides viridanus* (West Canary skink), Delgado *et al*. (2005) showed that the cellular changes of the ameloblasts, even though there are no Tomes processes appear similar to the mammalian situation with distinct secretory and maturing phases. The aprismatic enamel has been described in lizards and other squamates (Delgado *et al.,* 2005). The stellate reticulum in the crocodile persisted until late mineralization and appeared very similar to the mammalian situation. In geckos (Zahradnicek *et al.*, 2012) and snakes (Buchtová *et al.*, 2008) the stellate reticulum is thinner than in mammals and it has been suggested that a thin stellate reticulum is associated with narrow simple conical teeth (Handrigan and Richman, 2010a). Also in the bell stage tooth germs, the function of the ‘funnel-shaped’ structure was uncertain but it may be involved in mediating tooth eruption into the oral cavity and/or the shedding of non-functional teeth and attachment of newly formed replacement teeth.

In the Nile crocodile in the cap-bell transition stage, the present study revealed that in the Nile crocodile, *bmp 4* was present in the odontoblasts and ameloblasts

in the cap-bell transition stage. This was also seen in a study of the alligator (*Alligator mississippiensis*) (Weeks *et al.*, 2013). *Bmp4* produced by the odontoblasts in the murine model, induces ameloblast differentiation (Wang *et al*., 2004). The terminal differentiation of ameloblasts proposed to involve several signals produced by the odontoblasts (Thesleff and Hurmerinta, 1981). Tooth developmental studies on the ball python (*Python regius*) did not show expression of *bmp4* in the odontoblasts or ameloblasts during the cap or bell stage of development. This lack of *bmp4* expression suggests that odontoblasts and ameloblasts of crocodilians and squamates have a different combination of *Wnt* signalling cues (Weeks *et al*., 2013). Handrigan and Richman (2010) in a study on the python showed that *bmp4* was expressed in the condensed ectomesenchyme underlying the epithelial thickening in tooth initiation. As in the mouse and vole, *bmp4* expression in the python was strongest on the labial side of the epithelial thickening (Bitgood and Mc Mahon, 1995; Aberg *et al*., 1997; Keränen *et al*., 1998; Tucker *et al*., 1998). Handrigan and Richman (2010) showed that in the python *bmp4* signalling occurs in a paracrine manner from the ectomesenchyme across the basement membrane to the tip of the dental and successional lamina. This highlighted the expression of *bmp4* in tooth initiation and replacement. Several studies have shown that activation of *Wnt* causes significant changes in tooth number in mammals (Jarvinen *et al*., 2006; Wang *et al*., 2009). Handrigan and Richman (2010) in their study on the python, showed active *Wnt* pathways at the tip of the oral epithelium, from initiation through consecutive rounds of replacement implying that the same molecular network controls tooth initiation and replacement in snakes. *Bmp4* and *pitx2* expression in crocodiles appears the same as the situation in snakes.

Asymmetrical expression of *pitx2* was evident in the dental lamina of bell stage teeth in the 32-65 day old embryos. This was also evident in the well-developed late bell stage tooth germs of the hatched and post-hatched specimens. Asymmetrical expression of *pitx2* has also been described in the dental lamina of

the polyphyodont actinopterygian fish (Fraser *et al*., 2006a). However in the monophyodont mouse *pitx2* was expressed throughout the dental lamina with no asymmetry (Weeks *et al*., 2013). The asymmetric expression of *pitx2* in the crocodile may be important to distinguish the dental lamina epithelial cells from tooth-forming epithelial cells. *Pitx2* has been shown to be involved in left-right asymmetry in organs derived from the mesoderm (Logan *et al*., 1998).

In the bell stage in mice, *pitx2* expression is most intense in the cervical loop and pre-ameloblasts (St Amand *et al*., 2000). Less intense *pitx2* expression is detected in the transitional and secretory ameloblasts. Recently, it has been shown that *pitx2* activates amelogenin expression during normal murine tooth development (Sharp *et al*., 2014). *Pitx2* mutations therefore, in the mouse, lead to a decreased amelogenin expression as well as decreased amelogenin deposition and enamel development (Sharp *et al*., 2014). In humans, *pitx2* mutations cause Axenfeld-Rieger Syndrome, a genetic disease that causes several dental defects including hypodontia and enamel hypoplasia. A comparison of *bmp4* and *pitx2* expression in mammals and in the crocodile is depicted in Table 9. It is evident that *bmp4* expression in the crocodile, although not prominent in initiation (in the specimens examined in the present study) is present early on in the odontoblasts as compared to the mammalian situation where *bmp4* is expressed in the differentiating odontoblasts only at the late bell stage of tooth development. *Bmp4* expression also occurs earlier in the ameloblasts.

Table 9. A comparison of *bmp4* and *pitx2* expression in tooth development between mammals and crocodiles.

	bmp4		*pitx2*	
Stage of tooth development	**Mammal**	**Crocodile**	**Mammal**	**Crocodile**
Initiation	Oral epithelium and ectomesenchyme	none	Dental epithelium	Oral epithelium placode
Bud stage	bud	none	bud	bud
Cap stage	Dental follicle	Odontoblasts, dentine	IEE OEE	IEE
Early bell stage	Dental papilla	Odontoblasts, dentine	IEE OEE	IEE cervical loop
Late bell stage	Dental papilla, Pre-odontoblasts	Odontoblasts ameloblasts ectomesenchyme	IEE OEE	IEE cervical loop
Secretory stage	Ameloblasts	Odontoblasts, ameloblasts ectomesenchyme	_____	_____

IEE – Inner Enamel Epithelium
OEE – Outer Enamel Epithelium

Possibly *bmp4* expression in the enamel organ of crocodiles occurs earlier so that the signaling events can occur earlier. This would be advantageous for a polyphyodont dentition where another tooth germ may be required in rapid succession. *Pitx2* expression play a prominent role in early odontogenesis and then remains restricted to the inner enamel epithelium and cervical loop. It is also expressed earlier in the inner enamel epithelium/pre-ameloblast layer in crocodiles as compared to mammals. Since *pitx2* is an odontogenic commissioning gene perhaps its presence in the inner enamel epithelium allows for 'constant' epithelial competence to induce induction of the mesenchyme when required.

4.9 Tooth families

In the hatched and post-hatched specimens tooth family units were evident. In a study on alligators, the dental lamina becomes discontinuous from the oral epithelium and tooth family units are divided by alveolar bone (Wu *et al.*, (2013). In immature and developing tooth families in alligators, tooth family units were interconnected by the dental lamina string. This was identified as an interfamily dental lamina. This was also confirmed in the present study on the crocodile. In their study on the alligator, based on dental lamina morphology, Wu *et al*. (2013) defined three developmental phases related to tooth family units. The first stage is defined as pre-initiation. In this phase the tooth family unit includes a functional tooth with a resorbed lingual root, a replacement tooth and a dental lamina. In the second phase, the initiation phase, the replacement tooth develops at the tip of the dental lamina and forming a structure comparable to the cap-stage tooth germ of

the mouse. When the tooth germ reaches the bell stage, the tooth family unit is referred to as been in the growth stage (Wu *et al*., 2013). The dental lamina in alligators shows added structural specialization and in juvenile and adult animals the dental lamina does not have a connection with the oral epithelium (Wu *et al.*, 2013*).* In the pre-initiation phase, there is a large cluster of cells known as the dental lamina bulge which houses odontogenic stem cells in alligator tooth development, these cells remain latent, but with specific stimuli, the cells are stimulated and the dental lamina bulge will transform into a bud. The bud then undergoes morphogenesis to the cap and bell stages and then differentiates into the components of the tooth in the growth phase (Wu *et al*., 2013). In order to maintain tooth replacement, the outer enamel epithelium on the lingual aspect of the youngest replacement tooth splits to form a successional dental lamina and a stem cell niche which is retained to initiate the replacement tooth generation. Only some stem cells were retained during initiation and growth (Wu *et al*., 2013). It would be interesting to study the presence of stem cell niches in crocodile odontogenesis. In the gecko it has been suggested that the canonical *Wnt* pathway induces quiescent stem cells to divide and become transit-amplifying cells (Richman and Handrigan, 2011). The question that remains however is what other signals control *Wnt* activity. Future work using crocodile jaw explants, will aim at describing gene expression patterns along the entire length of the dental lamina. Also, localized expression of pathway inhibitors such as secreted frizzled protein 2 (*Wnt* pathway), Dickkopf (*Wnt* pathway), Noggin (*Bmp* pathway) and Sprouty (*Fgf* pathway) will be examined in and around the dental lamina. It is also important to determine whether crocodile dental lamina cells are *de facto* stem cells which can be cultured, stimulated and followed into the next generation of tooth buds.

In the crocodile embryos and hatchlings examined, we attempted to reconcile the expression of *bmp4* and *pitx2*, along the length of the jaws, with the clonal theory or zone of inhibition theory (Osborn, 1978). In the hatched and post-hatched

specimens, in the present study, certain tooth families did not show expression of *pitx2,* while the tooth families on either side showed various degrees of expression, reminiscent of a wave-like form of expression. The significance of future work will be to examine gene expression of pathway inhibitors, such as Noggin (BMP pathway) and secreted fizzled protein 2 (*Wnt* pathway) in the odontogenic areas to support or refute the old tooth replacement pattern theories.

4.10 Aspects of the periodontium

Crocodilians and mammals have a thecodont mode of attachment via a gomphosis, whereas other reptiles and amphibians have a pleurodont mode of attachment via an ankylosis (Mc Intosh *et al., 2002).* The major difference between a crocodilian and mammalian gomphosis, is that crocodilians have teeth with open roots, while mammalian teeth roots become narrow during root development to create the apical foramen (McIntosh *et al.,* 2002). From examination of the hatched and post-hatched specimens it was evident that crocodilians possess distinct sockets into which rooted teeth are attached in a structure that is histologically consistent with a thecodont condition. The alveolus appears to remain intact through multiple tooth replacements and only partial resorption and remodelling of the alveolar bone was evident. The traditional classification of tooth attachments into three types – acrodont, pleurodont and thecodont is said not to fully reflect the biological variations encountered (Caldwell *et al.,* 2003). These different support mechanisms play an important role in tooth function. A wide variety of direct union of tooth to bone as well as fibrous tooth attachment are detectable in the classes osteichthyes, amphibia, reptilia and mammalia. Cementum serves to anchor the fibres of the periodontal ligament to the tooth thus playing a significant role in tooth eruption. A layer of acellular cementum separating the dentine from the cellular cementum is well known in mammals. The absence of cellular cementum was not unexpected in the crocodilian embryos and hatchlings that were examined. A study on Cleidocranial

dysostosis in humans, a disorder associated with delayed eruption of permanent teeth and multiple impacted supernumerary teeth, revealed the absence of cellular cementum at the root apex (Manjunath *et al*., 2008). It appears that in cases of supernumerary teeth cellular cementum is not always apparent.

The Nile crocodile periodontium is an intermediary between the non-mineralized periodontal ligament in the mouse and the ankylosed tooth attachment apparatus of the lizard, which is devoid of periodontal ligament fibres. The tooth attachment by cementum appears similar to mammalian condition, but the teeth are not in persistent sockets and the cylindrical roots have a wide apical foramen (Shimada *et al*., 1992). The cementun in alligator teeth is hypertrophied and the thickness differs from that of mammals. The hypertrophied cementum is evident on the aspect of the root which does not show signs of resorption. The attachment surface of cementum is variable depending on the stage of development and aging (Westergaard and Ferguson, 1987). In a study on the cementum of the American alligator, Shimada *et al.* (1992) emphasised that the eruption of the replacement tooth presses the functional tooth towards the labial side. Resorption occurs on the labial and buccal regions of the bone, and it resembles the situation in mammals.

In the present study, the crocodile periodontium had a well developed periodontal ligament. Most of the periodontal ligament fibre bundles were randomly arranged, although some fibres had a more horizontal orientation. The arrangement of the fibre bundles in embryos, may be related to the multidirectional shear stress forces applied on the teeth during crocodilian feeding (McIntosh *et al*., 2002). Osborn (1984) proposed that the presence of a distinct periodontal ligament around the apices of teeth in mammal-like reptiles signifies an important aspect in the evolution of a gomphosis. The evolution of the periodontal ligament occurred as a response to large axial loads imposed on acrodont ankylosed teeth common to the majority of reptiles (Osborn, 1984). Osborn (1984) described the thecodont gomphosis of alligators and crocodiles comparing it to the mammalian situation.

The distinguishing criterion is that in the crocodilian situation, new teeth develop in and erupt from the socket of the preceding tooth. In mammals on the other hand the deciduous tooth socket walls are probably entirely replaced and remodeled by new bone with the eruption of the replacement tooth.

With the aid of histological serial sections, the present study demonstrated that a tooth family unit at each tooth position is generally comprised of three components at different developmental stages. The most mature tooth was the buccal functional tooth followed by a lingual replacement (or successional tooth) and this tooth was associated with a lingual dental lamina, the latter being the third tooth structure of the tooth family unit. Tooth family units arranged in this configuration therefore, would have a smooth transition from dislodgment to replacement. The tooth family unit may be formed during embryonic development by molecular asymmetries, which separate a single tooth primordium into three constituents (Wu *et al., 2013).* This would allow one tooth germ to become multiple structures, controlled by several signaling centres. However, the molecules or factors produced by replacement tooth, or by the surrounding ectomesenchyme that can activate a pre-initiation stage dental lamina to differentiate, remain unknown. Also, in the alligator the dental lamina was discontinuous with the oral epithelium and the individual tooth family units are separated from each other by alveolar bone (Westergaard and Ferguson, 1986).

In hatchling leopard geckos (*Eublepharis macularius*), each tooth family consists of three generations of teeth connected by the dental lamina (Handrigan *et al*., 2010). Replacement teeth develop in the lingual aspect of the erupted functional tooth and increase in size until they dislodge the predecessor (Handrigan *et al*., 2010). All the replacement teeth originate from the successional lamina which occurs as an outgrowth of the outer enamel epithelium of the predecessor tooth (Handrigan *et al*., 2010). Secondary initiation (in contrast to primary) is defined as the initiation of a replacement tooth occurring at a specific position. The presence

of epithelial-mesenchymal interactions that occur prior to tooth development is suggested by a thickening of the distal end of the dental lamina resulting from increased cell division, as well as an accumulation of ectomesenchymal cells surrounding this. The distal region of the dental lamina, and specifically its labial side, is involved in the primary steps of tooth morphogenesis and differentiation as seen in the crocodile embryos in the present study. It has been postulated that the successional lamina of the leopard gecko and other squamates houses a population of proliferative cells, possibly stem cells (Handrigan *et al*., 2010). It has been shown that the *Wnt* pathway induces dormant stem cells to develop into transit-amplifying cells in the mouse intestine and hair follicle (Lowry *et al*., 2005; Haegebarth and Clevers, 2009). In an attempt therefore, to determine whether the dental lamina of the leopard gecko was *Wnt* active, *Tcf3* and *Tcf4*, which are two targets in the *Wnt* pathway, were examined (Handrigan *et al*., 2010). Their results showed that *Tcf3* probably maintains dental lamina stem cells, whereas *Tcf4* might induces their proliferation. The successional dental lamina is derived from "proliferative descendant stem cells", but the stem cells themselves remain fixed in their position and remain sheltered from the molecular signals until they are required. These hypotheses need to be verified by cell lineage analysis.

In the hatched and post-hatched specimens of this study, as part of a tooth family, molariform-type teeth were evident. Initially these structures were assumed to be an artifact, due to histological processing. However, in several sections examined, no other sign of tissue disturbance was evident. It is thus suggested that these molariform type teeth developed and probably degenerated and were replaced as no functional molariform-type teeth were seen. The question of multicusped development in reptiles has been examined in a study by Handrigan and Richman (2011) on the bicuspid teeth of the leopard gecko. They suggested that the bicuspid appearance was due to variable enamel deposition and not the folding of the inner enamel epithelium that occurs in the mammalian situation. It is

interesting to note however, that some extinct crocodilians exhibit both heterodonty and multicusped molariform teeth (O'Connor *et al*., 2010). Harris *et al*. (2006), described specific patterns of *shh* expression along alligator jaws, suggesting that, unlike snakes and lizards, modern day crocodilians may have the ability to from mammal-like secondary enamel knots. This was not verified in the present study. Some developing bell stage tooth germs in the hatched and post hatched specimens showed an asymmetrical deposition of dentine and enamel over the crown, typical of a molar type tooth germ. This contrasts with mammalian teeth where both cusps develop by folding of the inner enamel epithelium prior to enamel and dentine deposition. Handrigan and Richman (2011) in a study of the leopard gecko (*Eublepharis macularius*) examined small bicuspid teeth in an effort to determine whether multicusped teeth could develop in reptiles. In the bicuspid teeth of the gecko, the inner enamel epithelium did not fold as occurs in the mammalian situation. Instead, differential deposition of the enamel matrix gave rise to the cusps. Expression of *bmp2* is said to inhibit enamel deposition centrally, whereas on either side enamel formation continued, resulting in bicuspid formation (Richman and Handrigan, 2011). No living crocodilians exhibit multicusped teeth, but Westergaard and Ferguson (1987) described an enamel-knot type structure in the alligator. In the extinct terrestrial notosuchian crocodilians, heterodonty and molariform teeth are present (Jernvall and Thesleff, 2012). Röse (1893a), in a study of the crocodilian dentition, maintained that the first formed tooth germs had a bicuspid appearance. Also, with a series of wax reconstructions of crocodile teeth, Woerdeman (1921) concluded that early resorbing teeth were triconodont. However, early multicusped teeth were not confirmed in the studies on the alligator (Westergaard and Ferguson, 1986).

4.11 The relationship between the state of the functional tooth and the state of the replacement tooth.

In the present study, the data to determine whether the formation of the replacement tooth is linked to the functional state of the predecessor in the crocodile showed that in more mature teeth or teeth undergoing resorption, the replacement tooth germ was at a more advanced stage. A direct 1:1 relationship between the status of the erupted tooth and the developmental phase of the replacement tooth was not seen. There appeared to be a developmental link however, between eruption of a tooth, and the presence of a replacement tooth in a fairly developed state. This was also noted by Huysseune (2006) in a study of the zebrafish in which the author suggested that during tooth eruption the displaced epithelium covering the crown of the tooth comes into contact with a new ectomesenchymal environment which may induce the epithelium to develop a new downgrowth. The eruption of a tooth and the formation of the successional dental lamina may be two separate outcomes for one event. It is necessary therefore to search for the genes involved in these events. Perhaps, examination of other cycling structures, like hair, which also develop from epithelial-mesenchymal interactions, may provide some information regarding the molecular cascade governing tooth initiation and replacement. Jamora *et al.* (2003) showed that downregulation of E-cadherin in hair, through activation of *Wnt* signalling, caused epithelial cells to detach and make a new bud. Huysseune and Thesleff (2004), in a study on the zebrafish, suggested that the same scenario could occur on the epithelial layer covering the tip of the crown. In the case of the crocodilia, however, the successional lamina formed well before its predecessor has erupted, making a link between eruption and formation of the successional lamina unlikely. Also, the successional lamina in crocodilians, unlike the zebrafish, is quite long and evident over an extended period of time during reptilian tooth formation.

The successional dental lamina associated with an erupted functional tooth, often showed no sign of differentiation at its distal end to form a tooth germ suggesting that possibly the successional lamina can remain dormant until stimulated. A trigger therefore, other than the state of the predecessor, appears to be responsible for further development of the replacement tooth. The nature of the trigger is of importance due to the parallels associated with the human dentition. A successional lamina in humans develops on the lingual side of each enamel organ. In the deciduous dentition, these lingual downgrowths will give rise to the permanent successors (incisors, canines, premolars). Berkowitz and Hollard (1992) showed that lingual downgrowths are also produced from enamel organs of permanent teeth, but ultimately disappear. Therefore, as in the zebrafish model, there is an uncoupling between the development of a successional lamina and the proliferation of the cells at its distal end giving rise to a tooth germ. Also the three permanent molars, in the human dentition which do not have deciduous predecessors, originate from the distal extension of the primary dental lamina, but can remain dormant for several years. This dental lamina functions for a limited period before differentiation and disintegration. In mice, the transcription factor *Runx2* inhibits secondary tooth formation by preventing the formation of an extension of the dental lamina (Wang *et al*., 2005). Humans affected by cleidocranial dysplasia (caused by lack of expression of one allele of Runx2) develop supernumerary teeth, sometimes forming a third dentition (Jensen and Kreiborg, 1990). The relationship between the eruption of a tooth and the formation of the successional lamina strongly indicates a local control of tooth formation, as opposed to generalised control associated with replacement teeth for the entire dentition. The *Wnt*/β-catenin signaling pathway may play a role. In a study on alligator mandibular explant cultures, as a transgenic option is not available, Wang *et al.* (2013) showed that overexpression of *Wnt* induced increased cell division in the dental lamina. However, the addition of soluble frizzled-related protein 1, an antagonist of the *Wnt*/β-catenin signaling pathway, resulted in a smaller replacement tooth structures. Their results suggested that

Wnt and soluble frizzled-related protein 1, regulate dental lamina proliferation by promoting or inhibiting putative stem cells (Wang *et al*., 2013). The presence of morphogens or field substances percolating the jaws, and initiating tooth formation in successive positions, has been presented to explain the tooth replacement patterns in non-mammalian vertebrates (Berkowitz, 2000; van der Heyden *et al*. 2001).

In the present study however, no distinct pattern of replacement was discerned suggesting that if replacement was under the control of a general field event, there would not be such a variation in the order of replacement. It seems probable therefore that two major phases, acting independently, are involved in the formation of a replacement tooth. The first phase consists of successional lamina formation and the second phase encompasses the initiation of morphodifferentiation and histodifferentiation of the tooth germ proper, which may or may not immediately follow the formation of the successional lamina (Huysseune, 2006). The trigger which is responsible for the successional lamina to develop further is still elusive and appears unrelated to the state of the erupted tooth. Berkovitz *et al.* (1992) in a study in humans showed that successional laminae are also produced from the enamel organs of permanent teeth, but eventually disappear. Moreover, the three permanent molars that do not have deciduous predecessors, develop from a distal extension of the primary dental lamina in a period which extends over several years. The relationship therefore between an erupted tooth and formation of a successional lamina points to local control over replacement tooth formation. The general control that would explain the formation of replacement teeth along the whole jaw, in the form of a field substance or a morphogen, has been extensively debated (Edmund 1960; 1969; Osborn 1970; 1978; Westergaard and Fergusson 1986). However, tooth replacement patterns are extremely variable and a general field effect could not cause such variability (Huysseune, 2006). Epithelial stem cells may be involved,

or a pool of pluripotent progenitor cells or even a dedifferentiation of epithelial cells may occur (Huysseune, 2006).

Few studies are available on tooth replacement in mammals, due to the fact that the main model for odontogenetic studies is the mouse which does not undergo tooth replacement. In some mammalian species, the secondary or replacement teeth were evident originating from the free end of the dental lamina on the lingual aspect of the deciduous predecessors (Berkovitz, 1973; Luckett, 1993). This process resembles the reptilian situation. Järvinen *et al*. (2009) in a study on the ferret highlight the presence of a successional dental lamina in this mammal, which is reminiscent of the reptilian situation. The successional lamina elongates and forms a tooth bud at its tip, the replacement tooth. There was a difference in the timing of lamina elongation and bud initiation between different teeth (Järvinen *et al*., 2009). It appears that there are probably different mechanisms controlling initiation of the successional lamina and the initiation of replacement tooth morphogenesis. Molecular analysis in the replacement teeth of the ferret showed expression of *Wnt* and *bmp*, but not *shh*. It was suggested that *Wnt* signaling may "unlock" tooth replacement which had been lost in the evolutionary process.

4.12 Phylogenetic analysis

The knowledge of the gene coding for *bmp4*, *pitx2* and *gapdh* is mainly based on mammalian sequences. In order to determine whether the Nile crocodile sequence of these genes can be comparable to other vertebrates as well as to the mammalian situation, phylogenetic analysis was carried out. All sequences were analysed and phylogenetic analysis revealed how the sequences were related to other reptilian and alligator sequences. This is important because mammals and birds have mainly been used as representatives of amniotes in molecular studies of odontogenesis. It would be useful to have a range of additional amniote models

for comparative and evolutionary studies. It has to be highlighted, that the samples are small and the phylogenetic analysis would present differently if many more sequences were to be included. The sequences were selected depending on availability on GenBank. In order to compare the genes of interest, sequences were selected from similar vertebrate classes for all three genes of interest. The crocodile sequences of *bmp4*, *pitx2* and *gapdh* showed numerous conserved regions, specifically in the N- and C-terminal regions of the gene. *Pitx2* was highly conserved within the crocodilians, although the sample was small. The *pitx2* gene sequences had a strong phylogenetic relationship with the mammalian sequences specifically the human and primate sequences. Also *pitx2* had a strong phylogenetic relationship to the bony fish. Despite some gene specific divergences it was noted that *pitx2* expression exhibits a highly conserved overall pattern, suggesting that it serves a significant function in development, in the vertebrates.

Bmp4 also appeared to be conserved phylogenetically across most vertebrate classes. In birds, the most well-conserved species was *Gallus gallus.* Nucleotide similarity is expected between birds and crocodiles as they are both terminal taxa of lineages that separated 250 million years ago (Hedges, 2002). It appears that *bmp4* was functionally significant in the lineage leading to modern birds for about 150 million years after the division of bird and crocodile lineages and until their tooth loss (Hedges, 2002). This has also been shown in phylogenetic comparison of enamelin, a crucial protein for enamel formation and mineralisation, that confirmed that tooth loss in modern birds led to the disappearance of enamel for genes which were translocated in an ancestor of the sauropsid lineage (Al-Hashimi *et al*., 2010).

The *gapdh* gene was also highly conserved amongst the various vertebrate species. Interestingly, the least conserved sequences of *gapdh* were the various birds species, which are the closest relatives to the reptilian class. Perhaps due to

the fact that *gapdh* is expressed in almost all cells of an organism (Zhu et al., 2008), a small number of taxa will reveal differences which would not have been as distinct in a larger sample.

The mean intergroup divergences are a measure for the uniformness or non-uniformness between the groups of vertebrates. Nucleotide divergence of *pitx2* showed that they were greater differences in the DNA sequences between reptiles and bony fish as opposed to reptiles and amphibia. The nucleotide divergence in bmp4 was similar between bony fish amphibians and reptiles. *Gapdh* nucleotide divergence showed a higher divergence of reptiles compared to bony fish, as opposed to reptiles and amphibians. Again a comparative analysis of more sequences in various representative species is necessary in order to comprehend how these genes have evolved in the various vertebrate classes. The numerous conserved residues in the genes of interest in the present study may indicate an important function or may indicate a slow evolutionary rate. This may be related to important functional constraints. Alignment and inclusion of several more sequences would provide more precise information. On the phylogenetic trees, based on the available sequences used, the relationship of the sequences for *bmp4*, *pitx2* and *gapdh* are located at the reptilian taxonomical position, thus highlighting their similarity to other reptilian sequences.

4.13 Tooth replacement in vertebrates

The question as to why some vertebrates have a regular replacement pattern, that is not present in others may be partially answered by indicating that the regular replacement pattern would be hindered in an organism that has tooth structures in sockets (Huysseune and Witten, 2006). In the case of the crocodiles, because of their thecodont mode of attachment, which allows drifting of teeth, a regular replacement pattern may be obscured and also may explain why a new theory, the tooth position theory (Westergaard and Ferguson, 1987) was suggested to explain

crocodilian tooth replacement. Also the lack of distinct replacement patterns suggests that the development of a replacement tooth is under local control, rather than regulated by a general field effect. Edmund (1962) showed that any regular replacement pattern becomes less regular with increasing age when teeth in individual loci have undergone several replacement cycles. Local control may also explain why bilateral symmetry is not evident in several species (Huysseune and Witten, 2006).

Tooth replacement patterns in fish are extremely variable (Jernvall and Thesleff, 2012). Cichlid tooth replacement occurs with tooth buds developing directly from the oral epithelium. In the salmon and trout, the replacement teeth develop from a thickening in the outer enamel epithelium of the unerupted predecessor (Fraser *et al*., 2006a; Huysseune and Witten, 2006; Smith *et al*., 2009b). Fraser *et al*. (2006a) showed that *pitx2* and *bmp4* are expressed during tooth development in the trout. Huysseune 2006, in a study on tooth replacement in the zebrafish showed that the pharyngeal teeth of the zebrafish develop a successional lamina when they are functional. This lamina develops from the epithelium lining the socket of the functional tooth. The lamina then increases in length and the replacement tooth develops at its tip. Huysseune (2006) indicated that due to the fact that the dental lamina often persists without developing a replacement tooth, the development of the dental lamina and subsequent development of the replacement tooth are under separate molecular control. Every tooth in the zebrafish develops a distinct successional lamina, distinguishing this successional lamina from the continuous lamina of reptiles. *Pitx2* and *bmp4* expression has been demonstrated in trout during the tooth replacement process

As in the crocodile, amphibians also exhibit polyphyodonty (Gillette, 1955; Davit-Béal *et al*., 2007). They have conical shaped teeth which are generally homodont and are restricted to the oral cavity. Amphibian teeth have several conserved features such as a pulp cavity surrounded by a cone of dentine and

covered by an enamel or enameloid crown. The crown and the supporting dentine pedicel separated by a dividing zone (Davit-Béal *et al*., 2007). The ameloblasts in amphibians are devoid of Tomes processes, which are known to be involved in enamel crystal orientation (Davit-Béal *et al*., 2007). Also the enamel of amphibians differs from mammalian enamel in that it is aprismatic (Davit-Béal *et al*., 2007). In amphibians the first generation teeth develop directly from the oral epithelium. The dental lamina consists of a bilaminar epithelial invagination growing into the ectomesenchyme (Davit-Béal *et al.*, 2007). The basal cells of the lamina adjacent to the ectomesenchyme differentiate into placodes and the ectomesenchyme appears condensed in the region of the placodes. The process of tooth replacement and resorption in amphibians occurs in a similar manner to the processes in Crocodilia. The replacement tooth germ develops as a bud on the lingual aspect of the functional tooth between the enamel organ and the dental lamina of the functional tooth. The bud then elongates to form the successional lamina which will develop into the replacement tooth. The contact of the new replacement germ with the functional tooth stimulates the recruitment of osteoclasts possibly as a reaction to pressure on the external surface of the tooth (Davit-Béal *et al.*, 2007). As in crocodiles, the first generation teeth of amphibians are small and often retained in the jaws of young larvae.

4.14 Stem cells

In osteichthyan fish, Harada and Oshima (2004) showed that a stem cell niche is located in the apical region of the dental epithelium, which is associated with a progenitor cell population in the mesenchyme. A stem cell-like population in the periphery of the dental papilla, as occurs in the dental papilla of mammals, could also have progenitor characteristics (Harada and Oshima, 2004). They therefore questioned which population of cells is involved in continuous tooth replacement. The successional lamina of the gecko and other squamates houses a population of stem cells (Handrigan and Richman, 2010a). These authors suggested that the lingual dental lamina houses the stem cells in order to isolate them from signals

that may stimulate them to proliferate or differentiate (Handrigan and Richman, 2010a). Although epithelial stem cells are a prerequisite for tooth renewal in the gecko, cell lineage analyses would be required to confirm their results. Whether stem cells or not, it is important to determine whether tooth replacement is a conserved feature of all vertebrates and why this capacity has been reduced in the mammalian situation. In Squamates the dental laminae are continuous, plate-like and connected to the oral epithelium (Handrigan *et al*., 2010)a. A cluster of stem cells is found and located on the lingual portion of the dental lamina. Complex compartmentalization of putative stem cells at the distal tip of the dental lamina occurs in alligators (Wu *et al.*, 2013). This cluster of stem cells during alligator tooth replacement may maintain a niche for continual tooth renewal (Handrigan *et al*., 2010).

4.15 Conclusions

Crocodilian odontogenesis, except for a few differences, exhibits several similarities reported for mammalian tooth morphogenesis and cytodifferentiation. At the histological level, the development of 'null generation' teeth may represent an ancestral character in vertebrate dental evolution. *In situ* hybridization has revealed that *bmp4* and *pitx2* expression play a role in odontogenesis in the Nile crocodile. However the relationship between these genes and others like Shh at the molecular level, remains unknown. The significance of the dental lamina and the successional lamina has also been emphasised, with specific reference to the lingual surface of the dental lamina which is probably involved in setting up the replacement pattern. What the present study has also highlighted is the highly conserved phylogenetic relationship of *bmp4* and *pitx2* between the reptilian lineages as well as between reptiles and mammals.

Research in the past 20 years has revealed that development of both the individual teeth and the whole dentition in mammals is controlled by the coordinated actions of a few conserved intercellular pathways. *Bmp4*, *Fgf*, *Shh* and *Wnt* signalling

pathways are used throughout tooth organogenesis and are regulated by a number of activators and inhibitors (Shihai *et al*., 2013). Also, the findings of O'Connell *et al*. (2012) and Wang *et al*. (2009) have revealed that the activation of the canonical *Wnt* signalling pathway in the dental epithelium is able to induce supernumerary tooth development in mice lacking Msx1 or Pax9. Several of the molecular mechanisms mediating the coordination and integration of the signalling pathways and gene regulatory networks in tooth organogenesis remain to be elucidated. However the investigation and understanding of these processes in an organism undergoing continuous tooth initiation and organogenesis, has great implications for development of therapeutic strategies for tooth developmental abnormalities and for congenitally missing or hypoplastic teeth.

The present study with a combination of histological analysis and *in situ* hybridization undertaken at various specific stages of crocodilian tooth development identified the tooth initiating factors *bmp4* and *pitx2.* The key findings of the present study suggests that the processes of tooth morphogenesis and replacement in the crocodile have several common pathways with the mammalian situation. The understanding of the biological processes governing tooth replacement in several vertebrate classes may serve as a foundation for future design and development of regenerated teeth *in vivo.* Natural processes like tooth replacement may be used to generate new therapies. Also, analysis of the molecular loops that regulate the process of successional tooth formation will extend our knowledge on the epithelial-mesenchymal interactions thus allowing the opportunity of tooth restoration in and tooth regeneration *in vivo.* Appropriate culture methods, biocompatible and biodegradable support materials are available. The inductive signals are fairly understood but the questions that remain are the source of easily accessible human somatic stem cells, the quantities of cells and growth factors that need to be combined as well as how tooth initiation shape and size are controlled.

Ferguson (1981) highlighted the significance of studying the crocodilians, as they are the only surviving archosaurs, which are distinct from the reptilian branch that produced mammals. Their status as 'living fossils' makes them an important group to study tooth evolution. Crocodilians are appropriate animal models for such research because their tooth structure is similar to mammals, they have the ability to continuously replace their dentition, while maintaining tooth position and shape. An understanding, therefore, of the mechanisms of polyphyodonty may shed some light on tooth regenerative efforts both *in vitro* and *in vivo*.

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6 APPENDIX A

CPB6
No N 10136

PREMIER VAN DIE PROVINSIE
GAUTENG
(DIREKTORAAT NATUURBEWARING)

PERMIT

(OM 'N LEWENDE WILDE DIER, DOOIE WILD OF 'N PRODUK VAN WILD IN DIE PROVINSIE IN TE VOER)

(Uitgereik ingevolge die bepalings van die Ordonnansie op Natuurbewaring 12 van 1983)

PREMIER OF THE PROVINCE OF
GAUTENG
(DIRECTORATE NATURE CONSERVATION)

PERMIT

(TO IMPORT INTO THE PROVINCE A LIVE WILD ANIMAL, DEAD GAME OR A PRODUCT OF GAME)

(Issued in terms of the provisions of the Nature Conservation Ordinance 12 of 1983)

DEPARTMENT OF AGRICULTURE, CONSERVATION, ENVIRONMENT AND LAND AFFAIRS
DATUMSTEMPEL / DATE STAMP
2006 -11- 03
DIRECTORATE OF CONSERVATION
PERMITS OFFICE
P.O.BOX 8769, JOHANNESBURG 2000

Naam en woonadres van houer van permit: Mrs Cleo Thomadakis
Name and residential address of holder of permit: 41 St Christopher Rd
Senderwood
Bedfordview 2008

BESONDERHEDE VAN PERMIT See attached conditions
DETAILS OF PERMIT

Vanaf From	Besonderhede van invoer Details of import	Bestemming Destination	Werklik ontvang Actually received
Thaba Khuena	Nile crocodile (Crocodylus niloticus)		
Crocodile Farm:	-10 (ten) eggs between incubation days 14-31		
Buiskantein	- 10 (ten) eggs between incubation days 32-65		
Gedeelte 24	-10 (ten) hatchling specimens		
Bela - Bela	-10 (ten) specimens 10-11 days Post Hatching		
Limpopo Province			

Datum en handtekening van houer van permit moet hier aangebring word
Date and signature of permit holder to be appended here

Ingevolge en behoudens die bepalings van die Ordonnansie op Natuurbewaring, 1983 (Ordonansie No 12 van 1983) en die regulasies wat ingevolge daarvan opgestel is, word bogenoemde persoon hierby gemagtig om, onderworpe aan die voorwaardes en vereistes wat op hierdie permit verskyn, die lewende wilde dier/e, dooie wild of produk/te van wild hierbo genoem gedurende die geldigheidsduur van hierdie permit vanaf die plek hierbo genoem in die Provinsie in te voer en dit met die kortste roete na die bestemming hierbo genoem, te vervoer.

In terms of and subject to the provisions of the Nature Conservation Ordinance, 1983 (Ordinance No 12 of 1983) and the regulations framed thereunder, the above-mentioned person is hereby authorized, subject to the conditions and requirements appearing on this permit, to import into the Province the live wild animal/s, dead game or product/s of game referred to above from the place referred to above during the period of validity of this permit and to convey it by the shortest route to the destination referred to above.

Geldigheidsduur van permit: Van die uitreikingsdatum tot
Period of validity of permit: From the date of issue to 02/01/2007

Vir/For PREMIER

Handtekening van houer van permit
Signature of holder of permit

(Sien voorwaardes en vereistes op keersy) – (See conditions and requirements on reverse side)

(V&R Printing Works (012) 328-7180 (5303)

7 APPENDIX B

4% paraformaldehyde in PBS

Heat 90 ml of DEPC treated water (see below) to ~60 ºC (can be put in 56 ºC water bath).
Add 100 µl of 2 N NaOH.
Add 4.0 g of paraformaldehyde and stir.
Allow the solution to clear. Add 10 ml of 10X PBS (see below) (DEPC treated)
Allow the solution to reach room temperature and then adjust the pH to 7.4 with 1N HCl.
Filter using a 0.2 µm syringe filter.
Prepare 5 ml aliquots in polypropylene tubes and store at -20 ºC.

Diethyl pyrocarbonate (DEPC) treated water
Prepare a 0,1% solution of DEPC in distilled water.
Stir overnight at room temperature.
Sterilize by autoclaving and store at room temperature.

Phosphate Buffered Saline (PBS) (pH 7.4)
8 g Sodium chloride
0,2 g Potassium chloride
1,44 g Na2HPO4 (Di-sodium hydrogen orthophosphate)
0,24 g KH2PO4 (Potassium di-hydrogen orthophosphate)
800 ml Distilled water
Adjust pH to 7.4 with hydrochloric acid.
Adjust volume to 1000 ml with distilled water.
Sterilise by autoclaving and store at room temperature

8 APPENDIX C

1% Agarose gel

1 g (1%) Agarose
100 ml 1 x TBE (see below) buffer
Dissolve agarose in 1 x TBE buffer by heating in a microwave oven for ~4 minutes, swirling occasionally, until all the agarose particles arecompletely dissolved. Care must be taken that especially the highpercentage gels do not boil over.
Cool to ~60 °C.
Add 10µl mg / ml Ethidium Bromide and swirl to mix.
Pour into a prepared casting tray and allow to solidify completely at room temperature.

10 x TBE buffer
108 g Tris base
55 g Boric acid
9,3 g EDTA
Adjust volume to 1000 ml with distilled water.
Sterilise by autoclaving and store at room temperature.

1 x TBE buffer
100 ml 10 x TBE Buffer
900 ml Distilled water
Mix thoroughly

DH5-α competent cells

Day 1

1. Streak DH5α for isolation on LB plate (no ampicillin).
2. Grow at 37 ˚C overnight.

Day 2

3. At the end of the day, add a single colony to a 4-16 ml (4 ml for 1X, 16 ml for 4X) SOB media aliquot for overnight culture at 37 ˚C and 250 rpm.

Day 3

4. Add 4.0 mls overnight culture to 400 ml SOB in 2L flask. Grow at 37 ˚C and 250 rpm until OD_{550} = 0.4-05 (2-3 hours). Transfer to conical 250 ml tubes.
5. Chill 5-10 min on ice.
6. Spin at 3-4k for 10 min. Pour off sup.
7. Resuspend cell pellet by pipetting in 150 ml TFB I. Ice for 5 min
8. Spin at 3-4k for 10 min at 4 ˚C. Pour off sup.
9. Gently resuspend in 15 ml TFB II (if > 1X pool together, e.g. 60 ml for 4X). Ice for 15 min

Aliquot 50 ul to prechille, sterile 0.5 ml microcentrifuge tubes). Snap freeze in matrix of dry ice crushed to powder and store in -80°C

SOC Medium

Reagent	Final concentration
Tryptone	2% (w/v)
Yeast extract	0.5% (w/v)
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
Glucose	20 mM

Prepare a solution containing the first four reagents, sterilize at 121 °C, and then add sterile $MgCl_2$ and glucose.

LB agar plates

10 g Tryptone
5 g Yeast
10 g Sodium chloride
15 g Bacteriological agar
Adjust volume to 1000ml with distilled water.
Sterilize by autoclaving.
Cool to approximately 50 °C before adding 1 ml of either 50 mg/ml ampicillin or 50 mg/ml kanamycin as required.
After pouring, allow plates to solidify completely at room temperature before storing at 4 °C.

5 x Maleic Acid Buffer

58g Maleic Acid
43.5g NaCl
55g Tween-20
900ml water
Bring the pH to 7.5 by adding Tris base. About 100g will be required. Bring final volume to 1 liter

24:1 Chloroform: isoamyl alcohol

To a sterile bottle, add:
48 ml Chloroform
2 ml Isoamyl alcohol
Mix thoroughly before using and store at room temperature.

0,5 M Ethylene diamine tetra-acetic acid (EDTA) (pH 8.0)

18,61 g Ethylene diamine tetra-acetic acid
80 ml Distilled water
Stir vigorously on a magnetic stirrer.
Adjust pH to 8.0 with 10 M Sodium hydroxide.
Adjust volume to 100ml with distilled water.
Sterilize by autoclaving and store at room temperature.

Luria-Bertani (LB) medium

10 g Tryptone
5 g Yeast
10 g Sodium chloride
Adjust volume to 1000ml with distilled water.
Sterilize by autoclaving and store at 4 °C.

Phenol chloroform extraction lysing buffer

7 M Urea
0,3 M Sodium chloride
10 mM EDTA
10 mM Tris base
Sterilise by autoclaving and store at room temperature.
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49:1 Chloroform: isoamyl alcohol

To a RNase free, sterile bottle, add:
49 ml Chloroform
1 ml Isoamyl alcohol
Mix thoroughly before using and store at room temperature**.**

RNA dilution buffer

DMPC-treated H_2O, 20X SSC and formaldehyde, mixed in a volume ratio of 5 + 3 + 2.

TE buffer

10 mM Tris-HCl, 0.1 mM EDTA; pH 8.0 (20°C)

TE Buffer (pH 8)

2 ml 0,5 M EDTA
10 ml 1 M Tris-HCl (pH 8.0)
800 ml Distilled water

TEA buffer

0.1 M triethanolamine (TEA) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride (Sigma) (pH 8.0)

Pre-hybridisation buffer

Incubate sections at 37°C for at least 10 min with prehybridization buffer (4X SSC buffer) containing 50% (v/v) deionized formamide.

Hybridisation buffer

Prepare hybridization buffer containing:
2X~ SSC.
1X~ Denhardt's solution [0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2 mg/ml RNasefree
bovine serum albumin].
10% dextran sulfate.
50 mM phosphate buffer (pH 7.0).
50 mM DTT.
250 µg/ml yeast t-RNA.
5 µg/ml polydeoxyadenylic acid.
100 µg/ml polyadenylic acid.
500 µg/ml denatured and sheared salmon sperm DNA.
Probe at relevant concentration

10 mg/ml Salmon sperm DNA

100 mg Salmon sperm DNA
Adjust volume to 10 ml with TE buffer (pH 8.0).
Dissolve overnight.
Sonicate 10 times (2 minutes on, 30 seconds off) and denature by boiling for 10 minutes.
Store frozen at -20 °C in 1 ml aliquots.

75% Ethanol

To a RNase free, sterile bottle, add:
75 ml Absolute ethanol
25 ml DEPC treated water
Store at -20 □C.

2 x Saline sodium citrate (SSC)
100 ml 20 x SSC
800 ml Distilled water
Mix thoroughly.

20 x SSC
88,23 g Tri-sodium citrate
175,32 g Sodium Chloride
800 ml Distilled water
Adjust pH to between 7.0 and 8.0 with concentrated HCl.
Adjust volume to 1000 ml with distilled water.
Sterilise by autoclaving and store at room temperature

NTE buffer

500 mM NaCl,
10 mM Tris,
1 mM EDTA,
pH 8.0

RNA gel loading buffer
5 ml Glycerol
20 µl 0,5 M EDTA (pH 8.0)
0,04 g Bromophenol Blue
0,04 g Xylene Cyanol
Adjust volume to 10 ml with DEPC treated water.
Store in 1 ml aliquots at room temperature.

Buffer 1

100mM Tris HCl 7.5
150mM NaCl
pH 7.5

Buffer 2

100mM Tris HCl
100mM NaCl
50mM $MgCl_2$

Buffer 3
10mM Tris HCl
1mM EDTA
(pH 8.1)