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Complex patterns of tooth replacement revealed in the fruit bat 2 (*Eidolon helvum*)

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2 Abstract:

4 How teeth are replaced in normal growth and development has long been an important question for comparative and developmental anatomy. Non-standard 6 model animals have become increasingly popular in this field due to the fact that 8 the canonical model laboratory mammal, the mouse, develops only one generation of teeth (monophyodonty), whereas the majority of mammals possess two generations of teeth (diphyodonty). Here we used the straw-10 coloured fruit bat (Eidolon helvum), an Old World megabat, which has two generations of teeth, in order to observe the development and replacement of 12 tooth germs from initiation up to mineralization stages. Our morphological study uses 3D reconstruction of histological sections to uncover differing 14 arrangements of the first and second generation tooth germs during the process of tooth replacement. We show that both tooth germ generations develop as part 16 of the dental lamina, with the first generation detaching from the lamina, leaving the free edge to give rise to a second generation. This separation was particularly 18 marked at the third premolar locus, where the primary and replacement teeth become positioned side by side, unconnected by a lamina. The position of the 20 replacement tooth, with respect to the primary tooth, varied within the mouth, 22 with replacements forming posterior to or directly lingual to the primary tooth. Development of replacement teeth was arrested at some tooth positions and this 24 appeared to be linked to the timing of tooth initiation and the subsequent rate of development. This study presents an additional species to the growing body of 26 non-model species used in the study of tooth replacement, and offers a new insight into the development of the diphyodont condition. 28 30 32 34 **Keywords:** diphyodont, mammal, tooth replacement, tooth development, dental lamina, fruit bat. 36 38 40 42 44 46

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8 INTRODUCTION

10 Teeth are mineralized appendages found in all vertebrate classes. Although tooth morphology and distribution vary, the initial steps of development are much the

12 same across clades. Reciprocal and sequential interactions between cranial neural crest-derived mesenchyme and ectodermal epithelium (or endodermal in

the case of pharyngeal teeth) lie at the core of tooth morphogenesis (Chai et al. 2000; Rothova et al. 2012; Soukup et al. 2008). During their development, teeth

16 pass through a series of well-characterized stages, defined by the morphology of the dental epithelium: dental lamina, bud, cap and bell (Luckett 1993).

18 Teeth are covered by enamel, which is resistant to abrasion and helps prolong the life of the tooth; however, teeth can be lost, or ground down by tooth wear

20 and need to be replaced. Tooth replacement also allows for changes in the morphology of the dentition as the animal ages, as observed in many fish (Tucker

22 & Fraser 2014; Fraser et al. 2006; Vandenplas et al. 2014; Huysseune 2006; Crucke & Huysseune 2014; Streelman et al. 2003). The ability to replace lost

24 teeth is evolutionarily ancient. The presumed basal character of jawed vertebrates is polyphyodonty, meaning they can continuously replace their

- 26 dentition throughout their lifetime (Tucker & Fraser 2014; Smith et al. 2009; Soukup et al. 2008; Rasch et al. 2016). Tooth replacement capabilities differ
- 28 widely throughout the terrestrial vertebrates: most reptiles are polyphyodonts, while most mammals are diphyodont and possess two sets of generational teeth.
- 30 A subset of mammals and some specialized reptiles are monophyodont, where the teeth are non-replacing. An important aspect that the first two groups (poly-
- 32 and diphyodonts) have in common is the dental successional lamina, a structure crucial for tooth replacement (Jernvall and Thesleff 2012; Tucker and Fraser
- 34 2014; Handrigan and Richman 2010).

In diphyodont mammals, the deciduous dentition develops from the primary dental lamina, which interconnects the teeth along the jaw. The second and final

- generation of teeth develops from a successional dental lamina, an epithelial
 structure connected to the lingual aspect of the deciduous dental organ (Juuri et
- al. 2012). The reduced number of tooth generations in mammals has occurred in
- 40 juxtaposition with an increase in tooth size and complexity (Jernvall & Thesleff 2012). Much of the process of mammalian tooth development has been
- 42 discovered by examination of the house mouse, *Mus musculus* (Lesot et al. 2014; Peterkova et al. 2014). Although the mouse has provided a wealth of valuable
- 44 information on tooth development, its derived rodent monophyodont dentition with only incisors and molars has limited studies on tooth replacement. To
- 46 overcome this problem, other mammalian models with complete heterodont dentitions have been introduced into this field of study. The common shrew and

house shrew (*Sorex araneus, Suncus murinus*) retain a more basal eutherian
dentition and although they do not display full tooth replacement, several
reports have shown that they develop a rudimentary milk dentition. This

- 4 deciduous dentition is suppressed once the second tooth generation initiates
- development and only the permanent teeth erupt (Järvinen et al. 2008;
- 6 Yamanaka et al. 2007; Sasaki, Sato, and Kozawa 2001).

The ferret and minipig are more appropriate models for study of tooth
replacement in mammals as they exhibit diphyodonty as well as heterodonty. All teeth, except for the molars, are replaced once, with the aid of the dental

- 10 successional lamina (Jussila, Crespo Yanez, and Thesleff 2014; Jarvinen,
- Tummers, and Thesleff 2009; Stembírek et al. 2010). Previous studies have suggested that Wnt signaling components (Axin2, beta-catenin) and
- transcription factor Sox2 are involved in the process of tooth replacement as well as cessation of replacement (Juuri et al. 2013; Jarvinen, Tummers, and
 - Thesleff 2009).
- 16
- In the present study, we have analysed the dentition of a series of straw-coloured fruit bat embryos (*Eidolon helvum*) (Figure 1A-I). These mammals belong to the
- order *Chiroptera*, suborder *Megachiroptera* also known as Old World fruit bats and are native to the African continent. Their gestation period lasts a total of nine
- and are native to the Arritan continent. Then gestation period fasts a total of fine months and commences with a long pre-implantation period, followed by the
 actual embryonic development of 4 months (Mutere 1967; Eavenuure & Helstend)
- 22 actual embryonic development of 4 months (Mutere 1967; Fayenuwo & Halstead 1974). Although the lengthy preservation of the specimens prevented us from
- 24 acquiring any molecular data, and given that these animals are not currently available for such studies due to accessibility and near threatened conservation
- 26 status, we provide here a unique and informative analysis of this diphyodont animal with a complete heterodont dentition. Importantly, our histological
- 28 investigation has uncovered new aspects regarding the timing of tooth initiation and the relationship between the deciduous and permanent teeth, and shows
- 30 different modalities of replacement at different regions of the jaw.
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38 MATERIALS AND METHODS

- 40 Pregnant straw-coloured fruit bat females were previously captured and culled in Nigeria in 1972-73 following an approved cull of bats on the campus of Ife
- 42 University (Fayenuwo and Halstead 1974). Uteri were dissected out and a range of stages of eleven fruit bat embryos were fixed in Bouin's fixative solution,
- 44 dehydrated through a series of IMS and stored for long term in 70% IMS. Upon receipt, the embryos were rehydrated using IMS and decalcified with EDTA. In
- this study we divided the eleven embryos into nine stages (Figure 1).

Embryos were photographed under a Leica MZ FLIII stereoscope and

- 2 subsequently dehydrated using a series of increasing concentrations of methanol (30-100%), followed by isopropanol. Heads were embedded in paraffin wax and
- 4 sectioned frontally at a thickness of 8mm. Sections were stained using a trichrome stain (Picro-Sirius Red, haematoxylin and Alcian Blue) and
- 6 photographed in an anterior-to-posterior direction using a Nikon Eclipse 80i microscope.
- 8 Whilst staging is available for a species of New World fruit bat (Cretekos et al. 2005), this is a member of a different suborder (*Microchiroptera*) to Old World
- 10 fruit bats (*Megachiroptera*)(Nowak 1999). Before the current study, embryological staging has not been available for the suborder *Megachiroptera*.
- 12 The lengths of the heads were measured in ImageJ as follows: a square was drawn around the head to include all extremities and a horizontal line parallel to
- 14 the base of the square was drawn from the tip of the nose to the back of the head, the length of which was measured. The average of two different measurements
- 16 was used as the final head length (referred to in Figure 1).
- 18 Three-dimensional reconstructions of dental tissues were generated from histology images in FIJI – ImageJ 1.47v, using the TrackEM2 Plugin (Schindelin et
- 20 al. 2012; Schindelin et al. 2015). 3D objects were processed (smoothening and pseudocolouring) in Blender 2.71.
- 22

24

The adult fruit bat skull is part of the skull collection of the Department of Craniofacial Development and Stem Cell Biology at King's College London.

- 26 **Tooth abbreviation:** upper deciduous dentition: incisors i¹, i²; canines c¹; premolars: pm¹, pm², pm³. Lower deciduous dentition: incisors i₁, i₂; canines c₁;
- 28 premolars: pm₁, pm₂, pm₃. Upper permanent dentition: incisors I¹, I²; canines C¹, premolars: PM², PM³; molars: M¹, M². Lower permanent dentition: incisors I₁, I₂;
- 30 canines C₁, premolars: PM₂, PM₃. molars: M₁, M₂, M₃.
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RESULTS AND DISCUSSION

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Bat embryos were staged according to head size and divided into nine stages (Figure 1 A-I). Over this period the forelimbs developed from hand plates to

large wings with elongated digits and the heads extended from 7.6mm to 16mm
in length. These stages are approximately equivalent to stages 17 to 22 of the

- New World fruit bat species *Carollia perspicillata* (Cretekos et al. 2005), however
- 42 anatomical differences such as lack of leaf nose structures in Eidolon helvum makes direct comparison inaccurate. The straw-coloured fruit bat is heterodont
- 44 and diphyodont, with the deciduous dentition comprising two incisors, one canine and three premolars in each jaw quadrant. The adult dentition exhibits an
- 46 additional two molars on the upper jaw and three molars on the lower jaw (Figure 1J). Here we refer mostly to the upper jaw dentition, however the same
- 48 developmental pattern occurs on the lower jaw.

Development of the fruit bat dentition

4

The fruit bat embryos used in this study presented a wide range of tooth

6 developmental stages, from dental lamina to mineralization stage. The youngest embryo (7.6mm) had already initiated all antemolar first generation teeth, with

- 8 the exception of pm¹, which was still at the dental lamina stage and had no
 mesenchymal condensation visible at this point. There was no evidence of a
- 10 dental lamina in the molar region at this stage. The second incisor i² had developed to the bud stage, showing a sharp inclination toward the lingual side
- 12 of the oral cavity (Figure 2 A1, B1). The tissue containing i¹ for the earliest stage was lost, however inferring from the later stages this tooth germ likely
- 14 developed similarly to i². The incisors only reached the cap stage at 13.3mm and the late differentiation stage was not observed until the oldest embryo, 16.0mm
- 16 (Figure 3 A1-A3, B1-B3). No evidence of a second generation of incisors was apparent at any of the available stages. In contrast, c¹ had already developed to
- 18 early cap stage at 7.6mm, and advanced rapidly through development, with the bud of C¹ being clearly visible by 12.5mm (Figure 2 A1-C4). c¹ commenced the

20 secretion of hard tissues by 13.3mm and by the oldest stage, 16.0mm, C² had already surpassed c¹ in size and reached the late differentiation stage (Figure 3

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22 C1 – C3).
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pm¹ had only reached the dental lamina stage at 7.6mm (Figure 2 D1). Following
dental lamina elongation into the underlying mesenchyme, the early bud stage
was apparent at 10.2mm, with mesenchyme starting to condense immediately

- 28 underneath (Figure 2 D1-D4). A cap stage tooth germ was clearly identified at 13.5mm by the presence of the enamel knot appearing as a very well defined ball
- 30 of cells in the middle of the inner enamel epithelium (Figure 3 D1-D2). The late cap stage was reached in the oldest embryo, however no sign of a dental lamina
- 32 elongation for a second generation was observed at any point (Figure 3 D3). pm² reached the cap stage at 7.6mm, developed further and started to separate
- 34 from the dental lamina at 12.5mm, when PM² began to develop from the dental lamina (Figure 2 E1-E4). Interestingly, although the dental lamina could be
- 36 observed next to pm² as it developed, PM² itself formed immediately posterior to the deciduous tooth, and could be observed in sections after pm² (Figure 3 E,E').
- 38 Mineralization of pm² was apparent at 15.5mm, when PM² was advancing through the cap stage (figure 3 E2, E'2). PM³ lagged slightly behind in

40 development compared to PM², with mineralization not yet present in the oldest specimen. Unlike PM², PM³ developed from the dental lamina directly on the

42 lingual side of the first generation tooth (Figure 2 F1-F4; Figure 3 F1-F3).

- 44 Our histological analysis revealed that overall, the incisors and particularly the first premolar exhibited a slower pace of development compared with the
- 46 canine, second and third premolars. The incisors and first premolars found in the adult fruit bat are remarkably small in size compared to the rest of the dentition
- 48 (Figure 1J). Taking into consideration the lack of visible evidence of a

²⁴

successional lamina next to the incisors and first premolars, it could be argued

- 2 that the fruit bat does not replace its dentition at these three loci.
- 4

In the specimens we had available only the first molar (out of 2 for the upper jaw

- 6 and 3 for the lower jaw) was observed, the other molars presumably forming by successional development at more mature stages; for example, in the mouse the
- 8 third molar does not initiate until after birth (Chlastakova et al. 2011). The M¹ formed as an elongation of the posterior free end of the dental lamina. The bud
- 10 stage was apparent at 10.2mm and by 16mm it had advanced through to the early bell stage, when its connection to the oral epithelium began to break down.
- 12 No extension of the dental lamina was apparent posterior to M1 at this stage (Figure 2 G1-2; Figure 3 G1-G3).
- 14
- Generally in the case of replacing teeth, as the deciduous tooth germ grew in size,it began to detach from the dental lamina, which is then spatially free to generate
- another tooth germ (for example see Figure 2 C1-C4 followed by Figure 3 C1-C3).
- 18 The lingual cervical loops of all antemolar tooth germs developed as part of the dental lamina before the two structures began to separate and detach. This
- 20 aspect was more apparent when viewed in three-dimensional space (Figure 5 upper panel).
- 22 At the early cap stage, the lingual cervical loops of c¹, pm² and pm³ were more elongated than their labial cervical loops, likely representing the extension of the
- 24 dental lamina to give rise to the second generation of teeth (Figure 2 C2, E2, F2). pm² and pm³ exhibited a very thick and short dental stalk (dental lamina
- 26 connecting the tooth to the oral epithelium) when compared to the other teeth, especially the canine, which has a thin and very long dental stalk (Figure 2C, E,
- F). These differences appear to correlate with the tooth length in adult animals: longer canines and larger, shorter premolars (Figure 1J).
- 30

Interestingly, the cervical loops of c¹ pointed towards the labial side, whereas the first generation premolars did not exhibit this bias and developed in a parallel

- direction to the oral-aboral plane (Figure 2 C, E, F; Figure 3 C, E, F). This aspect
 may be related to the degree of separation of the two generations of tooth germs
- at the same dental locus on the oral epithelium later in development: the two generations of canine teeth developed closer together perhaps owing to the early
- tilting of the cervical loops having allowed for sufficient space for the second
- 38 generation to develop; on the other hand, the two successional third premolars became progressively more separated from each other on the oral epithelium,
- 40 possibly to make more space for their further development. At the oldest stage, the two premolar tooth germs appeared almost as if they belonged to two
- 42 different tooth rows (Figure 3 F2-3). Lastly, the first premolar did not show tilting nor different lengths of the cervical loops both aspects could be
- 44 correlated to the lack of replacement at this locus.
- 46 In contrast with C¹ and PM³, which commenced their development on the dental lamina located directly on the lingual side of their deciduous counterparts, this
- 48 process occurred slightly differently at the second premolar locus. The replacement tooth germ developed immediately posterior to the first generation

tooth germ (Figure 3 E, E'; Figure 5), and therefore was not observed in the same plane on frontal sections (Figure 3E1-E3). This could represent another

- 2 plane on frontal sections (Figure 3E1-E3). This could represent another
 adaptation to accommodate the large tooth germ size combined with restricted
 4 spatial availability. This positioning of two generations of tooth germs belonging
- to the same family has also been encountered in other species, such as the short-
- 6 tailed opossum and to some extent in the guinea pig, (van Nievelt and Smith 2005, Popa et al. pers. obs.).
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12

14 Appearance of the dental lamina

16 Classically, the initiation of tooth replacement in diphyodont mammals takes place as the dental lamina extends on the lingual side of the cap stage

- 18 predecessor tooth germ; the lamina appears as an epithelial offshoot elongating at an acute angle and buds at the apical end to give rise to a second generation
- 20 tooth germ (Leche 1895; Jarvinen et al. 2009). However, this phenotype varied to some extent in the fruit bat embryo dentition. The most overtly different
- 22 appearance of the dental lamina of replacing teeth was observed in the lower jaw third premolar; the outer enamel epithelium/dental lamina of the first
- 24 generation tooth became separated from the oral epithelium by a small island of mesenchyme (Figure 4A). More lingually, an inpocketing of the oral epithelium
- 26 re-converged with the dental lamina epithelium, the free end of which extended further into the underlying condensing mesenchyme (Figure 4 A, A1). The
- 28 marked separation of the two successional premolars on the oral epithelium has not been previously described and constitutes a clear divergence from the
- 30 classical description of successive tooth formation in diphyodonts (Buchtová et al. 2012; Stembírek et al. 2010; Jarvinen et al. 2009; Jussila et al. 2014). Variation
- 32 is also present in the morphology of the reptile dental lamina. In the corn snake, ball python, leopard gecko, and Madagascan ground gecko the dental lamina is
- permanently attached to the oral epithelium (Buchtová et al. 2008; Gaete & Tucker 2013; Handrigan et al. 2010; Handrigan & Richman 2010; Zahradnicek et
- 36 al. 2012), whereas in the alligator the dental lamina giving rise to successional teeth loses its connection to the oral epithelium, yet still continues to generate
- 38 successive teeth for the whole lifespan of the animal (Wu et al. 2013). In some species of fish, successional teeth have been shown to develop directly from the
- 40 outer epithelium of the predecessor tooth rather than from a dental lamina (Huysseune & Thesleff 2004; Fraser et al. 2006; Huysseune and Witten 2008;
- 42 Vandenplas, De Clercq, and Huysseune 2014).
- 44 Regression of the dental lamina is an important process for limiting the tooth generation to two in diphyodont animals. In the minipig, the basement
- 46 membrane supporting the dental lamina epithelium breaks down, and the cells undergo a combination of epithelial to mesenchymal transition, migration and
- 48 apoptosis (Buchtová et al. 2012). At 16mm, the bat dental lamina was fragmented at several points along the AP axis of the jaw, with many epithelial

clusters of cells present in the associated mesenchyme (Figure 4B), mirroring the

- 2 situation previously reported in the minipig (Buchtová et al. 2012). A similar epithelial breakdown pattern was also observed in the outer enamel epithelium
- 4 of first generation teeth (figure 4 B, B1), which might indicate loss of the connection with the oral epithelium and from the replacement tooth germ, in
- 6 preparation for eruption.
- 8
- The canine was the first second generation tooth germ to initiate development
 and it advanced through development ahead of the other second generation
 teeth. Interestingly, at 16mm, the second-generation canine exhibited an
- epithelial elongation on its lingual side (Figure 4C, C1). This was reminiscent of the dental lamina appearance during tooth replacement initiation observed in
- 14 the bat at 12.5mm as well as other diphyodont species (Olley et al. 2014; Jarvinen, Tummers, and Thesleff 2009; M. Buchtová et al. 2012). However, the
- 16 canine locus is only replaced once, not twice; the dental lamina epithelium is likely to have lost its potential for tooth replacement by this later stage and
- 18 probably regresses shortly thereafter. A similar occurrence has been observed in the mouse, where a rudiment of the dental lamina protruded on the lingual side
- 20 of the molars during late prenatal stages, after which time it began to reduce in size (Dosedělová et al. 2015). In humans, a primordium of the third dentition has
- 22 been described as an epithelial projection appearing lingual to the permanent tooth germ (Ooë 1981). This indicates that the dental lamina could remain
- 24 competent to generate further teeth, if the correct signals are still operating. The mammalian dental lamina appears to have a limited lifespan and limited
- 26 competence to generate successional teeth as indicated by its regression after the initiation of the second tooth generation in diphyodonts. We propose a
- 28 scenario whereby the earlier the development of the first tooth is initiated, the higher the likelihood the dental lamina will produce more successional teeth.
- 30 This hypothesis could explain the visible attempt to produce a third generation for the canine locus, which is one of the first deciduous teeth (and the first
- 32 permanent tooth) to start developing. It also explains the lack of replacement of the late developing first premolar. Slowly developing tooth germs also appeared
- 34 to be less likely to develop a replacement, as in the case of the incisors which remained at the bud stage for long periods of time, while elsewhere in the jaw
- 36 tooth germs had advanced from early bud to late cap. This timing must be carefully balanced at a molecular level, as crossing this threshold and initiating
- 38 secondary teeth too early could lead to inhibition of the development of the deciduous dentition, as has been proposed to occur in the shrew (Järvinen et al.
- 40 2008). It remains to be investigated what the molecular controls behind the exact timing of tooth initiation are and how this timing may affect the
- 42 diphyodont dental lamina competence to produce successional teeth.
- 44

46 <u>Successional teeth develop in different arrangements</u>

48 We performed three-dimensional reconstructions at the three replacing tooth loci we have described above: canine, second premolar and third premolar in

order to understand the developing relationship between the deciduous and

- 2 replacement tooth at these three loci (Figure 5, upper panel). Following the development of these tooth germs in 3D space we suggest that the first
- 4 generation tooth germs develop with their lingual cervical loops embedded in the dental lamina. As the tooth germs advance in development, they begin to
- 6 cleave from the dental lamina and slowly detached from it, with the epithelial connection between the tooth germ and dental lamina becoming visibly thinner.
- 8 The lamina continued its growth at an angle towards the lingual side and gave rise to the second-generation tooth germ, however this process unfolded slightly
- 10 differently for the three different tooth families: C¹ developed on the lingual side of c¹, whereas PM² developed directly posterior to pm². After initiation of PM³,
- 12 the two successional third premolars detached from each other laterally, and exhibited separate epithelial connections to the oral epithelium.
- 14 Given the morphology of the dental epithelial tissues throughout development, it is likely that the location from which the second tooth generation develops on
- 16 the dental lamina is specified very early on. It has been suggested that signals from the first generation tooth germ are required for the development of a
- 18 second generation, however in dogs with X-linked hypohidrotic ectodermal dysplasia, which lack the deciduous dentition, it was possible to rescue the
- 20 permanent set by administration of recombinant Eda protein, suggesting that the development of the deciduous and permanent dentitions can be independent
- 22 processes (Casal et al. 2007).
- 24
- 26
- 28

30 <u>CONCLUSIONS</u>

- 32 In this study we have given an account of the morphological aspects of tooth development and replacement in a diphyodont animal with a complete dentition.
- We conclude that there are subtle variations to the tooth replacement phenotype when comparing the process at different tooth loci (summarized in Figure 5,
- 36 lower panel). These findings also reiterate the process of tooth replacement in diphyodont animals whereby the second generation tooth forms by growth of
- the dental lamina and not by budding of the deciduous tooth epithelium on the lingual side (Jarvinen, Tummers, and Thesleff 2009; Leche, 1895). Related to this,
- 40 an interesting question to answer would be how the process of separation of first generation tooth germs from the dental lamina occurs in diphyodonts and what
- 42 cellular events are involved in this process. Furthermore, we have shown the first instance of a successional lamina rudiment attached to a second-generation
- 44 tooth in a diphyodont animal. Although this particular species is no longer available for experimental purposes, other more common species of *Chiroptera*
- 46 could be screened for this dental lamina rudiment. Obtaining gene expression data on this structure could lead to a better understanding of why the
- 48 development of a further generation of teeth is suppressed in diphyodonts.

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8	The authors declare no conflict of interest.
10	
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18	obtain part of the data and critically reviewed the manuscript.
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24	
26	
28	
30	
32	
34	<u>References</u>
36	Buchtová, M., Handrigan, G.R., Tucker, A.S., et al. (2008) Initiation and patterning
38	Developmental Biology, 319 (1): 132–145
40	Early Regression of the Dental Lamina Underlies the Development of Diphyodont
42	Casal, M.L., Lewis, J.R., Mauldin, E.A., et al. (2007) Significant correction of disease
44	ectodermal dysplasia. American journal of human genetics, 81 (5): 1050–6
46	during tooth and mandibular morphogenesis. Development, 127(8): 1671–1679
48	Chlastakova, I., Lungova V., Wells K., et al. (2011) Morphogenesis and bone integration of the mouse mandibular third molar. European journal of oral sciences, 119(4): 265–74

Cretekos, C.J., Weatherbee S.D., Chen C., et al. (2005) Embryonic staging system

- for the short-tailed fruit bat, Carollia perspicillata, a model organism for the mammalian order Chiroptera, based upon timed pregnancies in captive-bred
 animala Davalance antal Davaniag 222(2), 721, 720
- animals. Developmental Dynamics, 233(3): 721–738.
 Crucke, J. & Huysseune, A. (2014) Blocking VEGF Signaling Delays Development
 af Paplacement Tooth in Zehrafish, Journal of Dental Passarsh, 04(1): 157–165.
- 6 of Replacement Teeth in Zebrafish. Journal of Dental Research, 94(1): 157–165. Dosedělová, H., Dumková, J., Lesot, H., et al. (2015) Fate of the molar dental
- lamina in the monophyodont mouse. PloS one, 10 (5): e0127543
 Fayenuwo, J.O. and Halstead, L.B. (1974) Breeding Cycle of Straw-Colored Fruit
- 10 Bat, Eidolon helvum, at Ile-Ife, Nigeria. Journal of Mammalogy, 55 (2): 453–454 Fraser, G.J., Berkovitz, B.K., Graham, A., and Smith M.M. (2006) Gene deployment
- for tooth replacement in the rainbow trout (Oncorhynchus mykiss): a developmental model for evolution of the osteichthyan dentition. Evolution & development, 8(5): 446–57.
- Gaete, M. and Tucker, A.S. (2013) Organized Emergence of Multiple-Generations
- 16 of Teeth in Snakes Is Dysregulated by Activation of Wnt/Beta-Catenin Signalling. PLoS ONE, 8 (9): e74484
- 18 Handrigan, G.R., Leung, K.J. and Richman, J.M. (2010) Identification of putative dental epithelial stem cells in a lizard with life-long tooth replacement.
- 20 Development (Cambridge, England), 137 (21): 3545–3549 Handrigan, G.R. and Richman, J.M. (2010) A network of Wnt, hedgehog and BMP
- signaling pathways regulates tooth replacement in snakes. Developmental Biology, 348 (1): 130–141
- 24 Huysseune, A. (2006). Formation of a successional dental lamina in the zebrafish (Danio rerio): support for a local control of replacement tooth initiation. The
- International journal of developmental biology, 50(7): 637–43.
 Huysseune, A. & Thesleff, I. (2004). Continuous tooth replacement: the possible
- 28 involvement of epithelial stem cells. BioEssays : news and reviews in molecular, cellular and developmental biology, 26(6): 665–71.
- 30 Huysseune, A. and Witten, P.E. (2008) An evolutionary view on tooth development and replacement in wild Atlantic salmon (Salmo salar L.). Evolution
- 32 & development, 10 (1): 6–14 Jarvinen, E., Tummers, M. and Thesleff, I. (2009) The role of the dental lamina in
- 34 mammalian tooth replacement. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 312 (4): 281–291
- 36 Järvinen, E., Välimäki, K., Pummila, M., Thesleff, I. and Jernvall, J. (2008) The taming of the shrew milk teeth. Evolution & development, 10 (4): 477–86
- 38 Jernvall, J. and Thesleff, I. (2012) Tooth shape formation and tooth renewal: evolving with the same signals. Development, 139 (19): 3487–3497
- 40 Jussila, M., Crespo Yanez, X. and Thesleff, I. (2014) Initiation of teeth from the dental lamina in the ferret. Differentiation, 87 (1-2): 32–43
- Juuri, E., Jussila, M., Seidel, K., et al. (2013) Sox2 marks epithelial competence to generate teeth in mammals and reptiles. Development (Cambridge, England),
 140 (7): 1424–32
- Juuri, E., Saito, K., Ahtiainen, L., et al. (2012) Sox2+ stem cells contribute to all epithelial lineages of the tooth via Sfrp5+ progenitors. Developmental cell, 23
- 46 epithelial lineages of the tooth via Sfrp5+ progenitors. Developmental cell, 2
 (2): 317–28

Leche W.(1895). Zür Entwicklungsgeschichte des Zahnsystems des Säugethiere,

- 2 zugleich ein Beitrag zur Stammesgeschichte dieser Thiergruppe. I.
 - Ontogenie. Zoologica **6**, 1–160
- 4 Lesot, H., Hovorakova, M., Peterka, M. and Peterkova, R. (2014) Threedimensional analysis of molar development in the mouse from the cap to bell
- 6 stage. Australian Dental Journal, 59 (SUPPL. 1): 81–100
 Luckett, W.P. (1993). Ontogenetic staging of the mammalian dentition, and its
 8 value for assessment of homology and beterochrony. Journal of Mammalian
- 8 value for assessment of homology and heterochrony. Journal of Mammalian Evolution, 1(4): 269–282.
- Mutere, F.A. (2009) The breeding biology of equatorial vertebrates: reproduction in the fruit bat, Eidolon helvum, at latitude 0°20'N. Journal of Zoology, 153 (2):
- 12 153–161 van Nievelt, A.F.H. and Smith, K.K. (2005) To replace or not to replace: the
- 14 significance of reduced functional tooth replacement in marsupial and placental mammals. Paleobiology, 31 (2): 324–346
- 16 Nowak, R.M. (1999). Walker's Mammals of the World, Volume 1 6th ed., Baltimore: Johns Hopkins University Press.
- 18 Olley, R.C., Olley, R., Xavier, G.M., et al. (2014) Expression analysis of candidate genes regulating successional tooth formation in the human embryo. Frontiers in
- 20 physiology, 5: 445
 Ooë, T. (1981) Human tooth and dental arch development. Tokyo: Ishiyaku
 22 Publishers: 217
- Peterkova, R., Hovorakova, M., Peterka, M. and Lesot, H. (2014) Three-
- 24 dimensional analysis of the early development of the dentition. Australian Dental Journal, 59 (SUPPL. 1): 55–80
- Rasch, L.J., Martin K.J., and Cooper R.L., (2016) An ancient dental gene set governs development and continuous regeneration of teeth in sharks.
 Developmental biology.
- Developmental biology.
 Rothova, M., Thompson H., Lickert H., Tucker AS. (2012) Lineage tracing of the
 andoderm during oral development. Developmental Dynamics 241(7): 1183–
- 30 endoderm during oral development. Developmental Dynamics, 241(7): 1183– 1191
- 32 Sasaki, C., Sato, T. and Kozawa, Y. (2001) Apoptosis in regressive deciduous tooth germs of Suncus murinus evaluated by the the TUNEL method and electron
- 34 microscopy. Archives of Oral Biology, 46 (7): 649–660 Schindelin, J., Arganda-Carreras, I., Frise, E., et al. (2012) Fiji: an open-source
- platform for biological-image analysis. Nature methods, 9 (7): 676–82
 Schindelin, J., Rueden, C.T., Hiner, M.C. and Eliceiri, K.W. The ImageJ ecosystem:
- 38 An open platform for biomedical image analysis. Molecular reproduction and development, 82 (7-8): 518–29
- 40 Smith, M.M., Fraser, G.J. and Mitsiadis, T.A., (2009). Dental lamina as source of odontogenic stem cells: Evolutionary origins and developmental control of tooth
- 42 generation in gnathostomes. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 312(4): 260–280.
- 44 Soukup, V., Epperlein HH., Horacek I., Cerny R. (2008) Dual epithelial origin of vertebrate oral teeth. Nature, 455(7214): 795–8
- 46 Stembírek, J., Buchtová, M., Král, T., et al. (2010) Early morphogenesis of heterodont dentition in minipigs. European Journal of Oral Sciences, 118 (6):
- 48 547-558

	Streelman, J.T., Webb JF., Albertson RC., Kocher TD. (2003) The cusp of evolution
2	and development: a model of cichlid tooth shape diversity. Evolution and
	Development, 5(6): 600–608
4	Tucker, A.S. and Fraser, G.J. (2014) Evolution and developmental diversity of tooth regeneration. Seminars in Cell and Developmental Biology, 25-26: 71–80
6	Vandenplas, S., De Clercq, A. and Huysseune, A. (2014) Tooth replacement without a dental lamina: The search for epithelial stem cells in Polypterus
8	senegalus. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution. 322 (5): 281–293
10	Wu, P., Wu, X., Jiang, TX., et al. (2013) Specialized stem cell niche enables repetitive renewal of alligator teeth. Proceedings of the National Academy of
12	Sciences of the United States of America, 110 (22): E2009–18 Yamanaka, A., Yasui, K., Sonomura, T. and Uemura, M. (2007) Development of
14	heterodont dentition in house shrew (Suncus murinus). European Journal of Oral Sciences, 115 (6): 433–440
16	Zahradnicek, O., Horacek, I. and Tucker, A.S. (2012) Tooth development in a model reptile: Functional and null generation teeth in the gecko Paroedura picta.
18	Journal of Anatomy, 221 (3): 195–208
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34	Figure legends
36	Figure 1: Fruit bat embryonic development (A-I) and adult fruit bat skull
38	(J). [A-1] the nine fruit bat embryos used for this study photographed frontally in ascending order of head length. (J) Top half: bottom and top views of the adult
40	upper and lower jaws, respectively. Bottom half: sagittal and frontal views.
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44	Figure 2: Development of the fruit bat dentition from initiation to late cap
46	stage . Frontal histological sections of upper jaw tooth germs, stained with a Picro-Sirius red, Alcian Blue and haematoxylin trichrome stain.
48	(A1-A3) first incisor. (B1-B4) second incisor. (C1-C4) canine. (D1-D4) first premolar. (E1-E4) second premolar. (F1-F4) third premolar. (G1-G) first molar. Asterisks mark the initiation of the second tooth generation.

OE: oral epithelium, OC: oral cavity. Labial and lingual are to the left and right

- 2~ sides of each image, respectively. Scale bars: 100 $\mu m.$
- 4
- Figure 3: Development of the fruit bat dentition from late cap to 6 **mineralization stage.** Frontal histological sections of upper jaw tooth germs, 8 stained with a Picro-Sirius red, Alcian Blue and haematoxylin trichrome stain. (A1-A3) first incisor. (B1-B3) second incisor. (C1-C3 canine) D1-D3 first premolar; arrows point to enamel knot. (E1-E3) deciduous second premolar. DL: 10 dental lamina (E'1-E'3) permanent second premolar. (F1-F3) third premolar. (G1-G3). first molar. OE: oral epithelium, OC: oral cavity. Labial and lingual are to 12 the left and right sides of each image, respectively. Scale bars: 200 μ m. 14 16 Figure 4: Interesting morphological aspects of the dental lamina. (A, A1) 18 Frontal histological sections of lower jaw third premolar at 12.5 mm. (B, B1) upper jaw second premolar at 16 mm; magnification of image in Figure 3 (E3). 20 Black arrows point to fragmenting outer enamel epithelium. Red arrows point to fragmenting dental lamina; (C, C1) upper jaw canine at 16 mm, magnification of 22 image in Figure 3 (C3). Arrowheads point to dental lamina rudiment. OE: oral epithelium, OC: oral cavity. Labial and lingual are to the left and right sides of 24 each image, respectively. All sections were stained with a Picro-Sirius red, Alcian Blue and haematoxylin trichrome stain. Scale bars: 50 μ m. 26 28 Figure 5: Different modalities of tooth replacement throughout the jaw 30 Upper panel: three-dimensional reconstructions of replacing tooth germs during four stages of embryonic fruit bat development. Orientation arrows: La, labial; Li, 32 lingual; A: anterior; P, posterior; O, oral; Ab, Aboral. Ab* and F*: anterior and posterior directions are reversed for better visualisation of both tooth germs. 34 Scale bar: 100 µm. 36 Lower panel: Schematic diagram summarizing the different modalities of replacement tooth germ development in the fruit bat. The second generation 38 tooth germ develops from the dental lamina growing on the lingual side of the first generation tooth germ; depending on the location in the jaw, this may develop directly on the lingual side (canine), directly posterior (second 40 premolar) or may become completely separated from the first generation tooth 42 germ (third premolar). In the case of the first premolar, no second generation tooth germ develops. 44 46
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Figure 2









Figure 4





2 Figure 5

