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Continuous tooth replacement: what can teleost fish teach us?

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ABSTRACT

Most tooth-bearing non-mammalian vertebrates have the capacity to replace their teeth throughout life. This capacity was lost in mammals, which replace their teeth only once at most. Not surprisingly, continuous tooth replacement has attracted much attention. Classical morphological studies (e.g. to analyse patterns of replacement) are now being complemented by molecular studies that investigate the expression of genes involved in tooth formation. This review focuses on ray-finned fish (actinopterygians), which have teeth often distributed throughout the mouth and pharynx, and more specifically on teleost fish, the largest group of extant vertebrates. First we highlight the diversity in tooth distribution and in tooth replacement patterns. Replacement tooth formation can start from a distinct (usually discontinuous and transient) dental lamina, but also in the absence of a successional lamina, e.g. from the surface epithelium of the oropharynx or from the outer dental epithelium of a predecessor tooth. The relationship of a replacement tooth to its predecessor is closely related to whether replacement is the result of a prepattern or occurs on demand. As replacement teeth do not necessarily have the same molecular signature as first-generation teeth, the question of the actual trigger for tooth replacement is discussed. Much emphasis has been laid in the past on the potential role of epithelial stem cells in initiating tooth replacement. The outcome of such studies has been equivocal, possibly related to the taxa investigated, and the permanent or transient nature of the dental lamina. Alternatively, replacement may result from local proliferation of undifferentiated progenitors, stimulated by hitherto unknown, perhaps mesenchymal, factors. So far, the role of the neurovascular link in continuous tooth replacement has been poorly investigated, despite the presence of a rich vascularisation surrounding actinopterygian (as well as chondrichthyan) teeth and despite a complete arrest of tooth replacement after nerve resection. Lastly, tooth replacement is possibly co-opted as a process to expand the number of teeth in a dentition ontogenetically whilst conserving features of the primary dentition. That neither a dental lamina, nor stem cells appear to be required for tooth replacement places teleosts in an advantageous position as models for tooth regeneration in humans, where the dental lamina regresses and epithelial stem cells are considered lost.

Key words: tooth replacement, dental lamina, tooth patterning, tooth addition, tooth regeneration, neurovascular link, epithelial stem cells, oropharynx, Teleostei, dentition.

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I. INTRODUCTION

Most tooth-bearing non-mammalian vertebrates have the capacity to replace their teeth throughout life, a condition called polyphyodonty. Continuous renewal is a way to ensure that animals with indeterminate growth have a dentition that matches their body size throughout life. It further enables replacement of teeth that are lost by wear or by impact. Moreover, it allows for tooth shape changes during ontogeny, with new generations of teeth adapted to changes in the diet, either as a regular part of the life cycle, or as an expression of phenotypic plasticity or of genetic polymorphism (e.g. Meyer, 1990; Huysseune, 1995; Vandervennet *et al.*, 2006; Gunter *et al.*, 2013; Berkovitz & Shellis, 2016). Some authors use the term 'regeneration' for the process that we and others (e.g. Reif, 1982; Jussila & Thesleff, 2012; Balic, 2019) call 'replacement'; some use both terms interchangeably (Thesleff & Tummers, 2009; Tucker & Fraser, 2014). Regeneration refers to the reactivation of development to restore missing tissues (e.g. amputated body parts, non-functioning organs, etc.) (Gilbert, 2010). While teeth comply with this definition, they also differ from other teleost body structures such as elasmoid scales or fin rays, or from mammalian hairs. Teeth are produced in advance of need ['prefabricated' in Reif's (1982) terminology] as part of normal, physiological turnover. By contrast, elasmoid scales or fin rays are newly formed only in the case of loss, justifying the term 'regeneration'. Interestingly, successional teeth are called 'replacement' – not 'regenerated' – teeth, even by those using the term 'regeneration' for tooth renewal (e.g. Square *et al.*, 2021). The process of replacement (creating a successive generation of teeth at a particular locus) should furthermore be distinguished from the process of tooth addition (creating the founder tooth generation at a new locus).

Classical studies about tooth replacement are mainly interested in the question of how patterns are set up (timing and sequence of formation of first-generation teeth), whether these patterns are maintained, and how fast the teeth cycle. These studies utilised traditional approaches, such as examination of preserved material (Motta, 1984), wax impressions (Berkovitz & Moore, 1974, 1975), serial sections and two-dimensional or three-dimensional reconstructions (Berkovitz, 1977*b*; Berkovitz & Shellis, 1978; Abduweli *et al.*, 2014), cleared and stained preparations (Lawson & Manly, 1973; Van der heyden, Wautier & Huysseune, 2001; Trapani, Yamamoto & Stock, 2005; de Azevedo *et al.*, 2021), scanning electron micrographs (Motta, 1984), radiographs (Berkovitz, 1975; Berkovitz & Shellis, 1978; Witten, Hall & Huysseune, 2005; Huysseune, Hall & Witten, 2007), and injection of fluorochromes (Bergot, 1975; Huysseune, 1989; Abduweli *et al.*, 2014; Ellis *et al.*, 2015) (Fig. 1A–D). These techniques, used either as a single method or in combination, have been supplemented more recently with microcomputed tomography (Bemis & Bemis, 2015; Bemis *et al.*, 2017, 2019; Kolmann *et al.*, 2019; Hulsey, Meyer & Streelman, 2020*b*; Leuzinger *et al.*, 2020; Stuart *et al.*, 2021; Williams, Evans & Simons, 2022), and synchrotron microtomography (Chen *et al.*, 2020) (Fig. 1E, F). In addition to being non-destructive, these two techniques allow demonstration of the presence of replacement teeth, often removed during sample preparation (Huysseune *et al.*, 2007), and examination of rare specimens, including fossils (e.g. Pasco-Viel *et al.*, 2010; Bemis *et al.*, 2017). Bemis & Bemis (2015) present a nice example of how 'iconic' fish species, as they call them, have been the subject of historic studies since the 18th century, reflecting the shifts in techniques applied over time.

With the advent of the molecular era, attention started to shift towards the question of which molecular mechanisms are responsible for continuous replacement. The idea to be

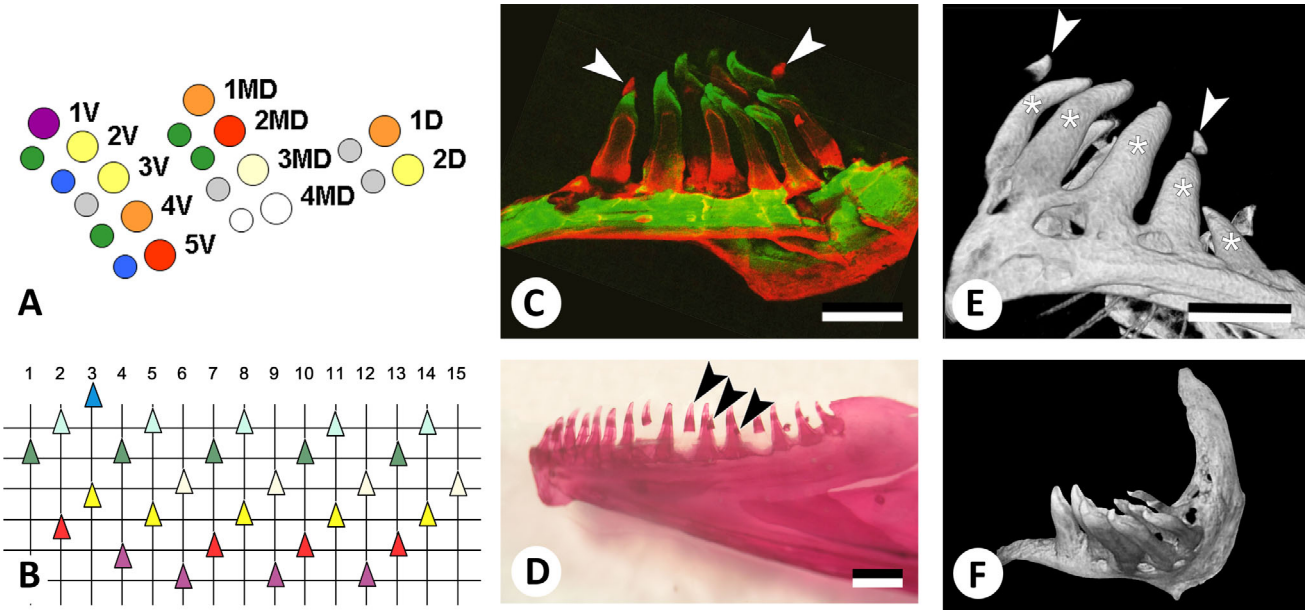

Fig. 1. Imaging methods for teleost dentitions. (A) Graphical representation of the dentition on the right pharyngeal jaw of a 20 mm standard length (SL) zebrafish *Danio rerio* with anterior to the top and medial to the left. Three rows of functional teeth (large circles; ventral, mediodorsal and dorsal row) with their replacement teeth (small circles). Various colours define different developmental stages. 1V–5V = five ventral teeth; 1MD–4MD = four mediodorsal teeth; 1D–2D = two dorsal teeth. Replacement teeth develop medially from their predecessors (for additional explanation and other examples, see Huysseune, 2006). (B) Graphical representation of the mandibular dentition of a parr-stage Atlantic salmon *Salmo salar*; vertical lines (numbered) represent tooth positions (= tooth families) from anterior (left) to posterior (right); horizontal lines connect teeth at a similar stage, with the most advanced stages at the bottom of the chart (labial side of the dentary), and the youngest stages at the top (lingual side of the dentary). Various colours define different developmental stages [reproduced from Huysseune *et al.* (2007) with permission from the publisher]. (C) Double labelling of zebrafish dentition with calceine, followed 35 days later by alizarin red S; note calceine (green) is still present in the tips of functional teeth, replacement tooth tips are labelled with alizarin (red, arrowheads). Scale bar = 250 μm. (D) Left dentary of a male Atlantic salmon at parr stage (108.5 mm fork length, FL), cleared and stained with alizarin red S; a set of three replacement teeth of decreasing developmental stage is indicated with black arrowheads (modified from Huysseune *et al.*, 2007). Scale bar = 1 mm. (E) Volume-rendering pictures of micro-computed tomography (μCT) of adult zebrafish pharyngeal teeth showing both functional teeth (asterisks) and replacement teeth (arrowheads) (modified from Kague *et al.*, 2018). Scale bar = 100 μm. (F) Synchrotron microtomography scan of the dentition of a 2 cm total length (TL) *Danio margaritatus*. Largest tooth is 300 μm tall (courtesy of E. Pasco-Viel; modified from Bruneel *et al.*, 2015).

able to revive the lost capacity of lifelong tooth renewal in mammals presented a strong incentive for such studies. With advancing technology, the methods used shifted accordingly from elucidating expression patterns through *in situ* hybridisation, revealing gene function through loss-of-function (LOF) and gain-of-function (GOF) approaches, to transcriptomic and single-cell analyses.

Herein an overview of studies on tooth replacement is presented, which illustrates the shift from classical morphological approaches to investigations into the molecular control of this process, but also shows that eventually both types of investigation are required to understand tooth replacement. The focus is on ray-finned fish (actinopterygians), with particular attention to the most abundant group of actinopterygians, and the largest group of extant vertebrates: the teleost fishes. Despite being extremely speciose, only a handful of teleost species has been used to expand our knowledge on tooth replacement beyond elucidating the tooth replacement pattern. Not surprisingly, these include common model species such as zebrafish *Danio rerio* (Van der heyden & Huysseune, 2000; Van der heyden, Huysseune & Sire, 2000; Stock, 2007), salmonids (Berkovitz 1977*a*,*b*; Huysseune *et al.*, 2007; Huysseune & Witten, 2008), characids (Trapani *et al.*, 2005), Japanese medaka *Oryzias latipes* (Debiais-Thibaud *et al.*, 2007), cichlids (Huysseune, Rüber & Verheyen, 1999; Streelman *et al.*, 2003; Streelman & Albertson, 2006; Fraser, Bloomquist & Streelman, 2013; Le Pabic, Stellwag & Scemama, 2009), and, more recently, threespine stickleback *Gasterosteus aculeatus* (Ellis, Donde & Miller, 2016).

II. PATTERNS OF TOOTH REPLACEMENT ACROSS TELEOSTS

(1) Diversity of tooth distribution

Teleosts are extremely diverse in terms of tooth distribution in the oropharynx (reviewed in Berkovitz & Shellis, 2016;

Huysseune, Cerny & Witten, 2022*a*). Teleost species belonging to stemward lineages can carry teeth or tooth plates on all pharyngeal arches (e.g. elopomorphs); yet most teleosts have teeth restricted to the mandibular arch and palate (oral teeth) and to specific pharyngeal arches (pharyngeal teeth) (e.g. salmonids, cichlids). Cyprinids, representing the most species-rich family of freshwater fish, and including carp and zebrafish, have lost their oral teeth and possess pharyngeal teeth only (Stock, 2007). Some teleosts lose their dentition during ontogeny [e.g. armoured catfish from the genera *Corydoras* and *Hoplosternum* (Huysseune & Sire, 1997) or the Mekong giant catfish *Pangasianodon gigas* (Kakizawa & Meenakarn, 2003)]; others modify tooth replacement to produce a beak (e.g. several species of pufferfishes; Thiery *et al.*, 2017); still other species are permanently edentulous (e.g. syngnathids; Qu *et al.*, 2021).

In addition to mandibular, palate, and pharyngeal arches, some teleost species also have minute 'free-floating' teeth, attached to the soft tissue that covers the bones of the oral cavity, rather than to the bones themselves. An example is the patch covering the mesopterygoid of the carangid *Parona signata* (Smith-Vaniz & Staiger, 1973).

(2) Diversity of replacement patterns

Despite the broad distribution of teeth within the teleost oropharynx, almost all studies concentrating on replacement tooth patterns concern oral teeth (reviewed in Berkovitz, 2000; Berkovitz & Shellis, 2016). Traditionally, replacement patterns in non-mammalian vertebrates are described as alternating waves sweeping from the back to the front (Fig. 1B, D). This is for example the case in the bluefish *Pomatomus saltatrix* (Bemis, Giuliano & Mcguire, 2005), or in the rainbow trout *Oncorhynchus mykiss* lower jaw (Berkovitz & Moore, 1974) (Table 1). In the king mackerel *Scomberomorus cavalla*, replacement occurs in overlapping waves in alternate tooth loci, but replacement waves may be flat, progress from rear to front, or progress from front to rear (Morgan & King, 1983). Trapani *et al.* (2005) listed many instances of replacement patterns deviating from the wave-like pattern considered to be typical for non-mammalian vertebrates, from haphazard, to fitting a completely different pattern. For example, highly synchronised replacement, with all teeth erupting at the same time in one jaw quadrant, is observed in piranhas *Serrasalmus rhombeus* and other characiforms (e.g. Roberts, 1967, 1975; Shellis & Berkovitz, 1976; Machado-Allison & Garcia, 1986; Gagiano, Steyn & du Preez, 1996) (Table 1), independent of diet (herbivorous or carnivorous; Kolmann *et al.*, 2019). Yet, the predaceous neotropical characoid *Ctenolucius huieta* shows tooth replacement involving replacement waves which pass from the back to the front of the jaws (Lawson & Manly, 1973). The oral jaws of adult Atlantic wolffish *Anarhichas lupus* are partly covered by molariform teeth fitting closely together in a space-filling pattern; teeth are lost and subsequently replaced all at once (Bemis & Bemis, 2015). Grouped replacement is also reported in other advanced teleosts such as the Pacific leaping blenny *Alticus arnoldorum* (Williams *et al.*, 2022) and the tripletails *Lobotes surinamensis* and *Datnioides quadrifasciatus* (Hilton & Bemis, 2005). However, the leaping blenny presents extraosseous replacement, the two tripletail species intraosseous replacement (see Section III.3). In the European barracuda *Sphyraena vulgaris*, large teeth are replaced in a regular and synchronous way, but smaller teeth show no such regular replacement (Levi, 1939*a*). The numerous teeth of the northern pike *Esox lucius* have no regular replacement either (Levi, 1939*a*). Unusual types of replacement are found convergently in rock-scraping species including the percoid opaleye *Girella nigricans*, the blenniid large-banded blenny *Ophioblennius steindachneri* and the pomacentrid bumphead damselfish *Microspathodon bairdii*. Here, multiple replacement teeth form in a deep trough on the premaxillary and dentary bones and move posteriorly into place along the alveolar margins of the jaws (Norris & Prescott, 1959). In the upper jaw of the gobiid fish *Sicyopterus japonicus*, up to 40 rows of replacement teeth are present (Mochizuki & Fukui, 1983). Lawson & Manly (1973) assumed that replacement of relatively unimportant small teeth on the maxillary of the characoid fish *Ctenolucius hujeta* may occur much more infrequently. On the other hand, it is very reasonable to assume that development of a large tooth may not be synchronised with that of a small tooth. Both size differences and distinctive functions of the teeth may indeed result in a very complex pattern of replacement.

The fragmentary nature of the data on oral replacement patterns in teleosts complicates their reading in a phylogenetic context. More focused studies on related taxa will be needed to assess whether specific replacement patterns hold a phylogenetic signal, or are related to form and/or function of the dentigerous bones.

The replacement pattern of pharyngeal teeth has been examined almost exclusively in cyprinids, evidently because cyprinids possess pharyngeal teeth only. Alternating waves sweeping from the back to the front have been described in various cyprinid species: *Gnathopogon coerulescens* (Nakajima, 1979), *Rhodeus ocellatus ocellatus* (Nakajima *et al.*, 1981), *Tribolodon hakonensis* (Nakajima *et al.*, 1983), and *Mylopharyngodon piceus* (Yue & Nakajima, 1995). In carps from the genus *Carassius*, however, the tooth germs develop in order from anterior to posterior (Nakajima *et al.*, 1986). In other cyprinids (Evans & Deubler, 1955), as well as in the zebrafish (Van der heyden *et al.*, 2001), tooth replacement does not strictly adhere to alternating waves sweeping cephalad. Rather, in zebrafish the pattern shows substantial variation, different from the highly predictable pattern set up by the first-generation teeth (Van der heyden & Huysseune, 2000) (Fig. 1A; Table 1). Needless to say, this complicates interpretations of experimental manipulations targeting the tooth replacement process.

(3) Length of the replacement cycle

As can be assumed from the widely divergent sizes of the teeth, the length of the replacement cycle varies greatly. In

Table 1. Character states for variable features of tooth replacement in the most prominent teleost model species. Except for zebrafish and medaka, data refer to the lower jaw teeth, for which most data are available. The zebrafish has pharyngeal teeth only. As medaka is often used as a counterpart to zebrafish, the table refers to pharyngeal teeth also in this species. The data furthermore exclude first-generation teeth, and relate to well-established dentitions showing regular turnover.

Species	Family	One-for-one (O) or many-for-one (M) replacement[1]	Extraosseous (E) or intraosseous (I) replacement[2]	Relative location of successor: lingual (Li) or labial (La)[3]	Successional dental lamina absent (A) or present (P)[4]	Replacement pattern[5]	References
Salmo salar (Atlantic salmon)	Salmonidae	O	E	Li	A	Waves affecting every 3rd position	Huysseune *et al.* (2007)
Oncorhynchus mykiss (rainbow trout)	Salmonidae	O	E	Li	A	Alternating waves sweeping from the back to the front	Berkovitz & Moore (1974); Berkovitz (1977*a*)
Danio rerio (zebrafish)	Cyprinidae	O	E	Medial-ventral	P	From alternate pattern to three odontogenic waves[6]	Van der heyden & Huysseune (2000); Van der heyden *et al.* (2000, 2001)
Astyanax mexicanus (Mexican tetra)	Characidae	O	I[7]	Li?	P	Simultaneous within a jaw quadrant[7]	Atukorala & Franz-Odendaal (2014); Trapani *et al.* (2005)
Oryzias latipes (Japanese medaka)	Adrianichthyidae	M	?[8]	Medial-caudal	Indistinct	No synchronisation between tooth families	Abduweli *et al.* (2014)
Hemichromis bimaculatus (jewel cichlid)	Cichlidae	O	I	La	P	Changes from alternate to irregular	Huysseune & Witten (2006)
Gasterosteus aculeatus (threespine stickleback)	Gasterosteidae	O	I?[9]	Li	Indistinct	Unknown	Ellis *et al.* (2016); Square *et al.* (2021)

[1]'One-for-one' or 'many-for-one' relates to the number of replacement teeth (one or multiple) within a single tooth family (Tucker & Fraser, 2014). Note that in some cases (e.g. zebrafish; Van der heyden & Huysseune, 2000) 'many-for-one' changes to 'one-for-one' between early and later life stages.
[2]'Extraosseous' *versus* 'intraosseous' relates to the place where the replacement tooth forms (on the surface of the dentigerous bone, or within its medullary cavity, respectively). Note that all first-generation teeth develop extraosseously, but this can change to intraosseously in later tooth generations, mostly in advanced teleosts; see Trapani (2001) for a review.
[3]The term 'lingual' or 'labial' is relevant for lower jaw teeth only; for pharyngeal teeth another description is applicable.
[4]Teleosts commonly have a discontinuous dental lamina, that is created anew for each replacement event, and is therefore called 'successional dental lamina'.
[5]For a review on tooth replacement patterns, see Berkovitz & Shellis (2016).
[6]This pattern applies to the ventral tooth row only.
[7]This pertains to the rostral portion of the lower jaws only, after the first few tooth generations.
[8]*Oryzias* has a type of replacement that is intermediate between extra- and intraosseous.
[9]Pharyngeal replacement teeth form intraosseously, although not completely encased in bone.

the lower jaw of rainbow trout, the average generation time varies between 8 and 13 weeks, but on the basihyal, which has the largest teeth, the average generation time is longer: between 12 and 16 weeks (Berkovitz & Moore, 1974). In the upper jaws, the average generation time differs for particular bones and varies between 7 and 14 weeks (Berkovitz & Moore, 1975). In piranhas, the functional lifetime of a tooth (which is shorter than the average generation time) varies between 65 and 130 days (Berkovitz & Shellis, 1978). However, the time between exfoliation of one tooth row and attachment of the succeeding row is of ∼5 days only (Berkovitz & Shellis, 1978). In the cichlid *Astatotilapia elegans*, the generation time of pharyngeal teeth varies between 1 and 1.5 months (Huysseune, 1989). It is important to keep in mind that the length of the cycle is dependent on the size of the tooth, and thus also the body size of the animal. In zebrafish, the initial four first-generation teeth cycle in around 10–16 days, but the same tooth loci in the adult have a cycle that exceeds 5 weeks (Fig. 1C). In adult threespine stickleback pharyngeal dentitions, the cycle exceeds 2 weeks (Ellis *et al.*, 2015). In the opaleye, the youngest teeth visible beneath the lips require from 22 to 32 days to be replaced and shed (Norris & Prescott, 1959). In several species the replacement cycle revolves around 1 month: an average of 27 days in the Pacific lingcod *Ophiodon elongatus* [total length (TL) 134–151 mm] (Carr, Summers & Cohen, 2021), around 40 days in the Mexican tetra *Astyanax mexicanus* (four tooth generations during 163 days of study; Trapani *et al.*, 2005), and ∼4 weeks in the Japanese medaka (Abduweli *et al.*, 2014).

Probably for most species, but demonstrated for a few species only, the rate of tooth replacement is also a function of age. In piranhas the rate of replacement reduces with age (Berkovitz & Shellis, 1978; Berkovitz, 1980). A similar phenomenon is found in rainbow trout. On the basihyal and dentary of this species, generation time (i.e. the time interval between the first appearance of a tooth in the oral cavity and the appearance of its successor) appears to be related to tooth size, which itself seems to be related to body size (Berkovitz & Moore, 1974) (recall that, unlike mammals, teleost fish have indeterminate growth). However, the authors agree that experimental conditions may have an effect on these data.

III. THE MICRO-ANATOMICAL SETTING OF TELEOST TOOTH REPLACEMENT

(1) Anatomical position of the successor

Teleosts display extreme diversity concerning the number of bones in the oropharynx that are toothed. Likewise, numbers, arrangement and size of the teeth can vary widely across species. Moreover, replacement teeth (henceforth 'successors') vary in the location with respect to the tooth that is being replaced (henceforth 'predecessor'). Depending on the species, replacement can occur both at the labial (lateral) and lingual (medial) side of the predecessor (Table 1). For example, cyprinodonts (Franz & Villwock, 1972), eretmodine cichlids (Huysseune *et al.*, 1999) and sticklebacks (Square *et al.*, 2021) replace their oral teeth labially. Nevertheless, replacement of oral teeth lingually/medially appears to be more common [piranhas (Berkovitz, 1975; Berkovitz & Shellis, 1978); Northern pike (Herold & Landino, 1970); *Lophius* (Kerebel, Le Cabellec & Geistdoerfer, 1979); rainbow trout (Berkovitz, 1977*a*); sea trout *Salmo fario* (Bergot, 1975)]. Likewise, pharyngeal teeth in cyprinids are replaced medially, as in the cypriniform species *Gnathopogon caerulescens* (Nakajima, 1979), or in the zebrafish (Van der heyden *et al.*, 2000). Some species display oral jaw tooth replacement laterally as well as medially, for example in the millet butterflyfish *Chaetodon miliaris* (Motta, 1984). In the scombriform bluefish, tooth germs originate from the lingual (dentary) or labial (premaxillary) epithelium (Bemis *et al.*, 2005). Likewise, in Atlantic wolffish, replacement pores for the developing teeth occur on both the labial and lingual sides of the bones (Bemis & Bemis, 2015). In the sawtail *Prionurus microlepidotus*, tooth germs are initiated on the lingual and labial side of the functioning teeth in an alternating pattern (Wakita, Itoh & Kobayashi, 1977).

(2) Connection between predecessor and successor

Adding to the diversity pictured above, there is no single way in which successors are connected to their predecessor, yet this knowledge is important for studies aiming to elucidate the mechanism of continuous tooth renewal. Reif (1982) defined the concept of tooth families (a functional tooth and all of its successors) and of the dental lamina. The dental lamina is a common epithelial structure that connects all the members of a tooth family. In some non-mammalian lineages, the dental lamina is continuous (i.e. one epithelial infolding stretching all along the tooth-bearing element; Reif, 1982). In this case it is also a permanent structure (the prototype would be found in sharks or salamanders). A permanent dental lamina is rather rare among teleosts. The gobiid fish *Sicyopterus japonicus* has numerous permanent, yet discontinuous, plate-like dental laminae coinciding with the number of functional tooth positions (Moriyama *et al.*, 2010). In discontinuous dental laminae, each tooth family is formed by its own epithelial invagination (Reif, 1982). In teleosts, a discontinuous dental lamina is common, but unlike the example of *Sicyopterus japonicus*, it is usually transient. The dental lamina is created anew for each replacement event, and therefore also called successional dental lamina (Fig. 2; Table 1). Species showing such a transient successional lamina include sticklebacks (Ellis *et al.*, 2016), cichlids (Huysseune & Thesleff, 2004), and piranhas (Berkovitz, 1975). The successional lamina can be very short (e.g. in zebrafish) or extremely long [e.g. in the carangiform species *Trachinotus teraia* (Françillon-Vieillot *et al.*, 1994) or in the loricariid *Ancistrus* (Geerinckx, De Poorter & Adriaens, 2007)], and the length can also depend on age. For example, first-generation teeth in zebrafish bud off directly from the enamel organ of their predecessor (Fig. 2B), but in juveniles and adults, a short

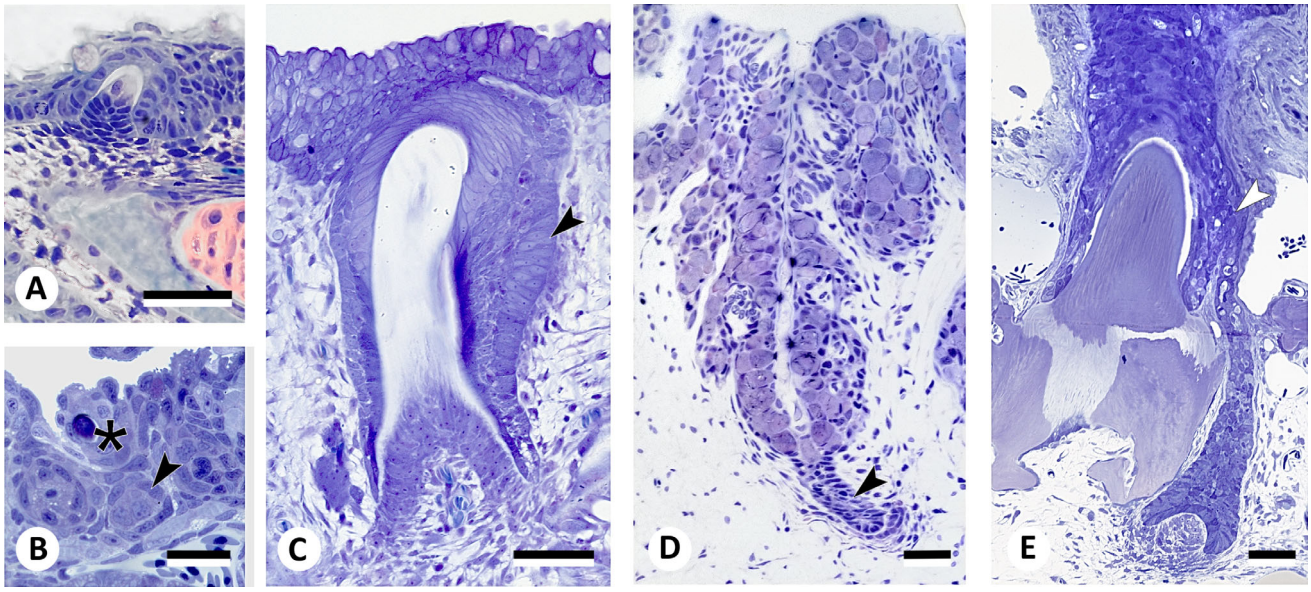

Fig. 2. Epithelial connections between predecessor and successor. Semithin cross sections of plastic-embedded jaws, stained with toluidine blue. (A) Formation of a first-generation tooth from the surface epithelium in Atlantic salmon *Salmo salar* (modified from Huysseune & Witten, 2008). Scale bar = 50 μm. (B) First replacement tooth ($4V^2$; arrowhead) in a zebrafish *Danio rerio*; note direct connection to the predecessor (asterisk) without successional dental lamina. Scale bar = 20 μm. (C) Replacement tooth developing from a thickening of the outer dental epithelium of the predecessor (arrowhead) in Atlantic salmon; compare with the development of a first-generation tooth shown in A (modified from Huysseune & Witten, 2008). Scale bar = 50 μm. (D) Successional dental lamina (arrowhead) forming the start of a replacement tooth germ in an adult zebrafish; compare with the development of the first replacement tooth in this position shown in B. Scale bar = 20 μm. (E) Long epithelial downgrowth (successional dental lamina) in the pharyngeal dentition of the jewel cichlid *Hemichromis bimaculatus*, issuing from the reduced enamel epithelium of the predecessor (arrowhead) and penetrating through a canal into the medullary cavity of the dentigerous bone; development of the tooth germ has started at the proximal tip. Scale bar = 20 μm.

yet distinct lamina develops (Fig. 2D). The location from which the successional lamina buds off is also variable. The prospective new tooth germ can bud off directly from the outer dental epithelium of the predecessor, in which case there is no successional dental lamina at all [e.g. in the most basal extant actinopterygian *Polypterus senegalus*, and in salmonids of the genus *Oncorhynchus*, the rainbow trout (Berkovitz, 1977*a*; Fraser *et al.*, 2006) and *Salmo*, the Atlantic salmon *Salmo salar* (Huysseune & Witten, 2008)] (Fig. 2C). Alternatively, the new tooth can bud off from the boundary between the enamel organ of the predecessor and the surface epithelium, or directly from the oropharyngeal epithelium without clear relationship to any predecessor, for example in a gadid, the Atlantic cod *Gadus callarius* (Holmbakken & Fosse, 1973). In sticklebacks, replacement teeth share an epithelium with the tooth they will replace, but this epithelium extends directly to the luminal pharyngeal epithelium (Ellis *et al.*, 2016). Different types of connections between predecessor and successor can occur simultaneously within a single species such as in the Northern pike (Herold & Landino, 1970), in the European barracuda (Levi, 1939*a*) and in the red bandfish *Cepola rubescens* (Levi, 1939*b*). Conversely, closely related species can differ in the presence and extent of a successional lamina, as in Anguilliformes (De Schepper, 2007). The above examples illustrate the fact that a dental lamina as a discrete structure is not required for tooth replacement, despite claims to the contrary (Hulsey *et al.*, 2020*a*). On the other hand, direct development from the surface epithelium, as is observed in Atlantic cod, and also in medaka (Abduweli *et al.*, 2014; Tan *et al.*, 2017), raises the question whether this can be truly considered as 'replacement', or should rather qualify as 'tooth addition', an issue that is discussed further in Section VIII.

(3) Extraosseous *versus* intraosseous replacement

The above considerations are inextricably linked to the position of the teeth relative to the bone to which they eventually attach (Fig. 3). In teleosts, replacement teeth can develop and attach to the surface of the bone, so-called extraosseous or extramedullary replacement (Fig. 3A, C), or develop within the medullary cavity of the bone, so-called intraosseous or intramedullary replacement (Fig. 3B, D, E; Table 1). Intraosseous replacement is typically a character of advanced teleosts (Figs 2E and 3B, D, E) [see Trapani (2001) for an extensive review]. As always in biology, not everything is black or white. Some species display a type of tooth replacement that cannot be clearly assigned to one of the two types (e.g. medaka; Trapani, 2001). In sticklebacks, replacement teeth form intraosseously but are not completely encased in

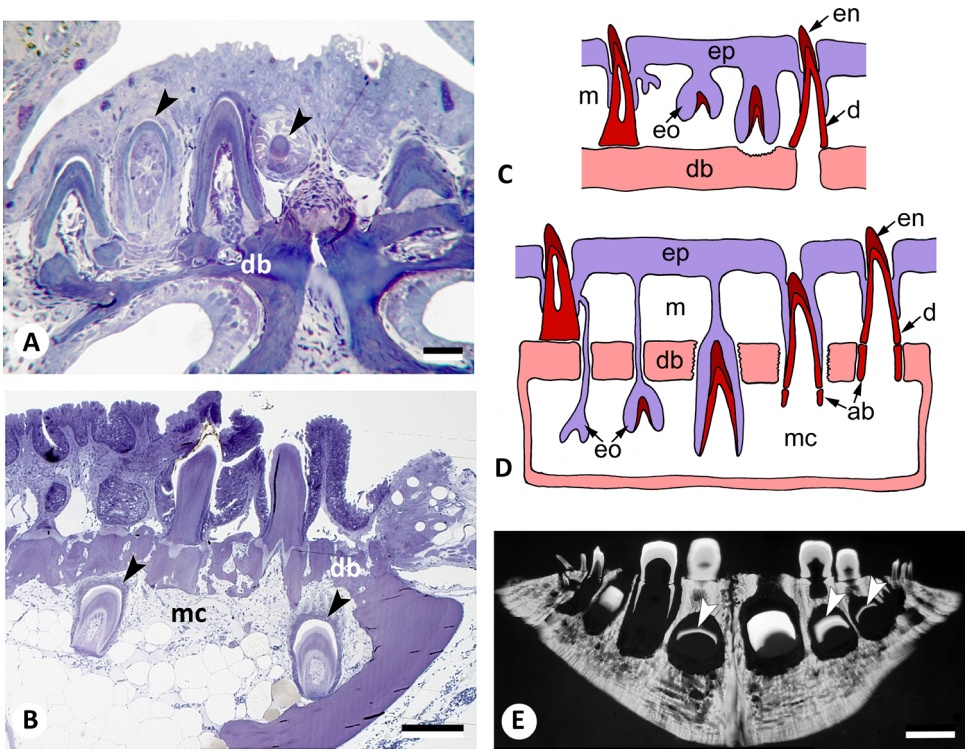

Fig. 3. Extra- and intraosseous tooth replacement. (A, B) Semithin cross sections of plastic-embedded jaws, stained with toluidine blue. (A) Oral jaw of *Anguilla anguilla*; tooth germs (arrowheads) form extraosseously. Scale bar = 100 μm. (B) Pharyngeal jaw of *Hemichromis bimaculatus*; tooth germs (arrowheads) form intraosseously. Scale bar = 100 μm. (C, D) Schematic representations of extraosseous (C) and intraosseous (D) replacement tooth formation [reproduced from Huysseune & Thesleff (2004) with permission from the publisher]. (E) Microradiograph of ground section of pharyngeal jaws of the cichlid *Astatoreochromis alluaudi*, showing several replacement teeth (arrowheads) encased inside the jaw bone. Scale bar = 1 mm. ab, attachment bone; d, dentine; db, dentigerous bone; en, enameloid; eo, enamel organ; ep, epithelium; m, mesenchyme; mc, medullary cavity.

bone (Ellis *et al.*, 2016). [Note that Trapani (2001) used a conservative definition of the term 'intraosseous', in that the teeth should be completely encased by bone.] Different modes of intraosseous replacement can also occur within a single species, depending on the location of the tooth in the jaw (Bemis *et al.*, 2019). Intramedullary replacement requires the formation of an, often very long, epithelial downgrowth (sometimes called a gubernacular strand) that needs to penetrate through a canal in the bone to reach the marrow cavity (Fig. 2E). The proximal tip of the strand then interacts with a local population of mesenchymal cells within the medullary cavity, and starts morphogenesis, cytodifferentiation, and matrix production in conjunction with the mesenchyme. In order for the growing tooth to erupt, the bone must be resorbed to allow passage of the now enlarged tooth germ (Witten & Huysseune, 2009). Intriguingly, in some species, replacement teeth in the medullary cavity start their development 'upside down' and must achieve a 180° rotation before migrating into a functional position. This is the case in *Scheenstia* sp., a fossil lepisosteiform (non-teleost actinopterygian) (Leuzinger *et al.*, 2020), but also in some extant teleost species, such as in the dentary of a muraenid, *Enchelycore nigricans*, in some characiforms (Trapani, 2001), and for the premaxillary fangs of the Atlantic cutlassfish, *Trichiurus lepturus* [see Bemis *et al.* (2019) who also list more examples of rotation during replacement, both intra- and extraosseous].

As far as has been studied, first-generation teeth in teleosts always develop extraosseously (Sire *et al.*, 2002); replacement teeth in evolutionarily less advanced teleosts usually also develop extraosseously. By contrast, advanced teleosts generally display intraosseous tooth replacement (Trapani, 2001). In the Mexican tetra, the switch to intraosseous tooth replacement coincides with a change from unicuspid to multicuspid teeth, and with a change of replacement pattern from haphazard to simultaneous within a jaw quadrant (Trapani *et al.*, 2005). Extraosseous development of replacement teeth is the primitive state in osteichthyans (Trapani, 2001; Chen *et al.*, 2016). Doeland *et al.* (2019) provide further support for the hypothesis that extraosseous tooth replacement is an ancestral condition for crown Osteichthyes.

(4) Number of tooth family members

Many teleosts have a one-for-one replacement (a single replacement tooth being formed at any one time for a single tooth position; Tucker & Fraser, 2014), unlike sharks which have a many-for-one replacement (Reif, 1984).

However, this is by no means universal across teleosts (reviewed in Berkovitz & Shellis, 2016; Tucker & Fraser, 2014). A one-for-one replacement has been described, for example in zebrafish (Van der heyden *et al.*, 2001), trout (Fraser *et al.*, 2006), Atlantic salmon (Huysseune *et al.*, 2007) and stickleback (Ellis *et al.*, 2016). The number of species reported to have a one-for-one replacement may be overestimated due to the techniques used to observe the dentition. For example, young tooth germs in initiation, morphogenesis or early cytodifferentiation stage are overlooked by any method that visualises mineralised structures only. In the sawtail *Prionurus microlepidotus*, each functional tooth on the premaxillae and dentaries is associated with two successive tooth germs, in different developmental stages, located on the lingual and labial sides of their predecessor (Wakita *et al.*, 1977). In medaka, like zebrafish an important biomedical model, tooth families comprise up to five generations of teeth and successional tooth germs (Abduweli *et al.*, 2014). In the northern pike, three germs may be developing at one locus, each germ developing from the enamel organ of a more mature tooth (Levi, 1939*a*). Multiple replacement teeth constitute one tooth family in the loricariid fish *Ancistrus triradiatus* (Geerinckx *et al.*, 2007).

From the above, it is clear that the timing of initiation of the tooth germ with respect to the maturation or functional stage of the predecessor can differ widely. Initiation of the successor often immediately follows the movement of the mature replacement tooth into a functional position (e.g. in the gar characin *Ctenolucius hujeta*; Lawson & Manly, 1973), or the attachment and eruption of the predecessor (e.g. in zebrafish; Huysseune, 2006). Mantoku *et al.* (2016, p. 370) went as far as claiming that 'the medaka attachment bone provides the model to understand the cellular mechanism for tooth replacement'. However, data on *sp7*-mutant zebrafish have shown that attachment and eruption are not required for sustained replacement (Kague *et al.*, 2018). In zebrafish, a time difference can occur between successional dental lamina formation and initiation of the new tooth germ. This suggests that these two processes are uncoupled and are possibly under different control (Huysseune, 2006).

Finally, a puzzling question is how to explain tooth replacement where one large replacement tooth replaces two or more smaller ones, as is observed in species with large molariform teeth [e.g. in the cichlid *Astatoreochromis alluaudi* and the sea bream *Sparus aurata* (Huysseune, 1995; de Azevedo *et al.*, 2021)]. We currently do not know if replacement is repressed in one predecessor, if one germ develops at the expense of the other, or if both predecessors are replaced nevertheless and teeth simply occupy an enlarged surface due to expansion of the bone surface.

(5) Shedding of the functional tooth

At the end of its functional lifetime, the tooth is shed, usually when a replacement tooth is ready to take its position. Yet, in a few loci in the zebrafish dentition, teeth of the three first tooth generations are maintained in place simultaneously, forming a little whorl, before the oldest one is finally shed (Van der heyden & Huysseune, 2000). Shedding frequently involves the action of osteoclasts/odontoclasts (Witten & Huysseune, 2009). However, in Atlantic salmon, the tooth is actively broken down inside the oral epithelium but not shed (Witten *et al.*, 2005). Likewise, in the gobioid *Sicyopterus japonicus*, worn-out teeth are engulfed by the oral epithelium, and resorbed/degraded completely by numerous multinucleated, foreign body giant cells (Sahara *et al.*, 2018). Defective tooth resorption has no influence on tooth development. In a transgenic medaka with dysfunctional tooth resorption, supernumerary teeth develop and attach to the jawbones in-between the predecessor teeth (To *et al.*, 2015).

IV. IS REPLACEMENT PREPATTERNED OR ON DEMAND?

(1) Field and clone models

Replacement patterns have been studied in a number of species, and have often been discussed in the light of one of two alternative hypotheses on the control of jaw tooth replacement in polyphyodont vertebrates: the Zahnreihe hypothesis, a field model favouring the hypothesis of stimuli moving along the dental lamina (Edmund, 1960), and the clone model of Osborn (1971), favouring the hypothesis of an inhibitory mechanism preventing adjacent teeth from developing and controlling turnover of teeth in each 'autonomous' tooth family (Fig. 4). The two hypotheses have been discussed repeatedly in the light of replacement patterns observed in various teleost taxa [extensively reviewed in Berkovitz & Shellis (2016), who concluded that there is insufficient evidence to support the Zahnreihe hypothesis]. The field model was nevertheless revived recently when highlighting the role of one tooth as an initiator tooth to form a tooth row in zebrafish (Sadier *et al.*, 2020). Clearly, a distinction needs to be made when invoking either of these hypotheses for explaining the sequence of development of first-generation teeth *versus* replacement teeth.

The field model suggests that the pattern of replacement proceeds according to preset variables. This is contradicted by a number of observations that suggest that local control over replacement is possible, allowing tooth loci to respond to unexpected events, such as accidental loss or sudden heavy wear. A valid test for local control would be the capacity at a specific tooth locus to accelerate the development of the replacement tooth after extraction of the predecessor. Such an experiment requires teeth that are large enough and easily accessible (oral, rather than pharyngeal teeth) and a highly predictable pattern of replacement in case the extraction site cannot be labelled appropriately (Huysseune *et al.*, 2012). Detailed analyses of teleost dentitions have led to the suggestion that general control sets up the initial pattern (consistent with a field model, *cf.* Sadier *et al.*, 2020), followed by local control for the replacement of individual teeth

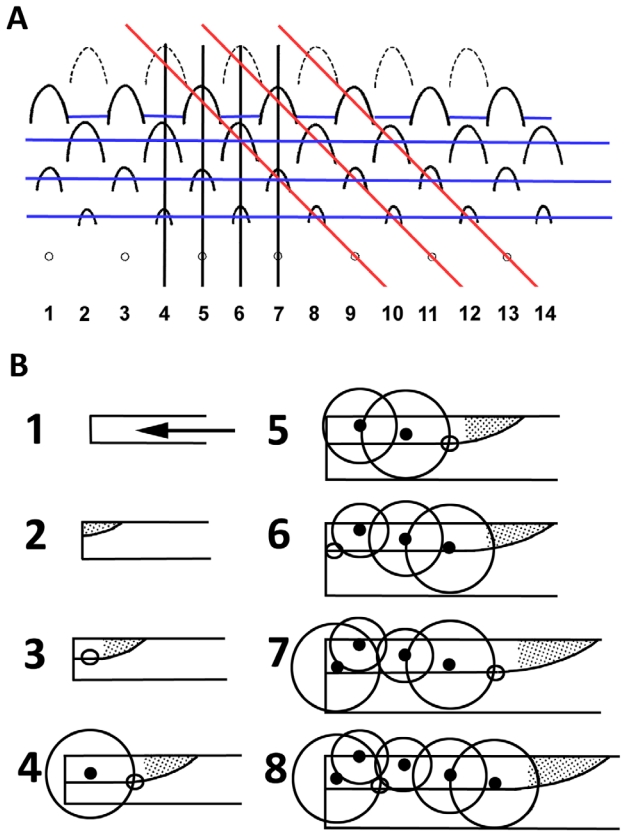

Fig. 4. The field and clone models used to explain tooth replacement patterns. (A) Edmund's model of Zahnreihen. Alternate teeth are replaced at the same time; interrupted lines represent teeth that have just been lost; the size of developing teeth reflects their developmental stage. Blue lines connect rows of teeth and are called odontostichi; red lines represent Zahnreihen; black lines represent tooth families. The pattern is generated by a signal periodically emitted by a transmitter anteriorly that travels back along the jaw; regularly spaced receivers pick up the signal and initiate a tooth germ. Rows of teeth are thus initiated from the front to the back, but the replacement waves resulting from such an arrangement sweep at alternate positions from the back to the front (modified from Osborn, 1971). (B) Osborn's clone model. Ectomesenchyme spreads anteriorly (arrowed in 1) and the oral ectoderm produces a rudimentary dental lamina (stippled area in 2); this becomes competent to initiate a first tooth (small open circle in 3). The dental lamina continues to grow backwards (3–8). As the first tooth grows (black circle in 4) it produces an agent which spreads out and inhibits tooth development (large circle in 4). The growing dental lamina is out of range of the sphere of inhibition and a second new tooth begins to develop (small open circle in 4). A third new tooth is produced in 5. The jaw has been growing anteriorly (1–5) and the sphere of inhibition surrounding the first tooth has been decreasing in size (4–6) so that a new tooth is now developed at the anterior end of the jaw (small open circle in 6) (modified from Osborn, 1971).

(Berkovitz, 1980; Huysseune, 2006; Huysseune & Witten, 2006; Huysseune *et al.*, 2007). Remarkably, Carr *et al.* (2021) conclude that dental wear in the marine species *Ophiodon elongatus* does not control the replacement rate and therefore support the hypothesis that replacement is probably maintained by a spatially and temporally driven developmental network. Obviously, such contradictory conclusions do not facilitate answering the question as to what triggers the start of development of a replacement tooth germ.

(2) Other models

The sequential addition model (SAM) was introduced by Smith (2003) to explain how dentitions are generated in a bilateral initiation pattern from a putative single dental determinant, or primordial tooth-signalling centre, followed by initiation of tooth primordia progressing in a unidirectional linear pattern, as space becomes available through jaw growth. This model incorporates both field and clone hypotheses: initiation of the dentition as a field effect, and replacement tooth germs formed by the clonal mechanism. The ectoderm–endoderm boundary plays a key role to set up the initial pattern according to the SAM model. This is considered by Huysseune *et al.* (2022*a*) to reflect the (ancestral) need for an ectodermal influence in oropharyngeal tooth formation, irrespective of the epithelial source of the enamel organs. Data gathered on salmonids appear to corroborate the dual mechanism of initiation of the dentition as a field effect, and tooth replacement as a clonal mechanism. Both in Atlantic salmon (Huysseune *et al.*, 2007) and in sea trout (Bergot, 1975), deviations of the alternate pattern, or absence of synchronisation in the tooth replacement, were observed and considered evidence for a local control over tooth replacement. Interestingly, in wild Atlantic salmon individuals that do not migrate back to the sea after spawning but spend the winter in the river, the regular patterning of tooth replacement is lost during this dormancy period but re-established when the animals return to the sea the following spring (Witten *et al.*, 2005).

Streelman *et al.* (2003) proposed that tooth formation is controlled by antagonistic actions between factors of competence, which define the field in which teeth can develop, and inhibiting factors that antagonise the factors of competence. The factors of competence are located throughout the odontogenic region while inhibiting signals are located in foci. The concentration of inhibitory signals from these foci subsequently controls the development and position of the nearby teeth. A high concentration of inhibitory signals results in widely spaced tooth positions and unicuspid teeth while a lower concentration results in more tightly spaced teeth with more cusps. The model, which was used to explain both tooth shape and spacing, was further elaborated upon in a follow-up paper (Streelman & Albertson, 2006). Surprisingly, *Bmp4*, proposed as a candidate gene with a role in tooth initiation and morphogenesis, was already expressed differently during early development in three species with distinct adult tooth shape, despite all three species sharing unicuspid teeth at this early stage. Jackman *et al.* (2013) nevertheless found further support for an integrated control of tooth and cusp number.

By upregulating fibroblast growth factor (Fgf) signalling or by downregulating bone morphogenetic protein (Bmp) signalling, they could generate multicuspid teeth along with the appearance of supernumerary teeth in both zebrafish and Mexican tetra. Along the same lines, Jernvall & Thesleff (2012) emphasised that many of the same molecular pathways appear to be involved, across vertebrates, in the determination of both tooth shape and tooth renewal.

The inhibitory cascade model, which is based on work with mammalian (and mostly non-replacing) dentitions (Kavanagh, Evans & Jernvall, 2007), links the sequence of tooth development on the jaw with tooth size, and is therefore applicable to first-generation teeth. The model is less suited to explain replacement tooth formation.

V. THE MOLECULAR TRIGGER FOR TOOTH REPLACEMENT

The homology of oral and pharyngeal teeth in teleosts is well supported by morphological, cellular and molecular data (e.g. Debiais-Thibaud *et al.*, 2007; Ellis *et al.*, 2016). The finding, from transcriptomic analysis of tooth-bearing jaws of teleosts, that only about half of the recovered genes are shared between oral and pharyngeal toothed components has been ascribed at least in part to subfunctionalisation between paralogues (Hulsey, Fraser & Meyer, 2016). Thus, except for specific cases, we make no distinction when presenting data on either oral or pharyngeal teeth. This choice is also justified considering the ancestral condition with teeth on all pharyngeal arches.

(1) A different molecular signature for first-generation *versus* replacement teeth

The search for 'a', or perhaps 'the' gene(s) responsible for initiating tooth replacement has been largely triggered by the human desire to be able to replace lost permanent teeth. The search for a mechanism has been limited by what is known for tooth development in the mouse, the most frequently used model for research in odontogenesis. Yet, the mouse dentition is evolutionarily extremely derived, with only one tooth generation, composed of three molars that are not replaced, and continuously growing (non-replacing) incisors. This limitation is especially relevant considering that replacement teeth in teleosts do not have the same molecular signature as first-generation teeth. For example, in zebrafish, the even-skipped gene *eve1* is expressed in the placode of the first tooth in the dentition and in the dental epithelium throughout morphogenesis and differentiation stages, but only in differentiating ameloblasts in its successor (Laurenti *et al.*, 2004). Six out of the eight Dlx genes [homeobox genes homologous to the *distal-less* (Dll) gene of *Drosophila*] are expressed during morphogenesis and/or cytodifferentiation phases of the first developing teeth in zebrafish ($4V^1$, $3V^1$, and $5V^1$) but only some of them during development of the first replacement tooth $4V^2$, and no expression is detected in the developing replacement teeth $3V^2$ and $5V^2$ (Borday-Birraux *et al.*, 2006). Signal strength and distribution of E-cadherin differs between first-generation and replacement teeth (Verstraeten *et al.*, 2010). Expression of *shh* is not detected during initiation of replacement teeth in trout, while it is expressed in the initiation stage of first-generation teeth (Fraser *et al.*, 2006). Expression of *eda* shifts from mesenchyme in cichlid first-generation teeth to epithelium in replacement teeth (Fraser *et al.*, 2013). Data obtained more recently from human embryos likewise show differential expression patterns in the successional lamina (replacement tooth formation) *versus* the primary dental lamina (development of first-generation teeth) (Olley *et al.*, 2014).

(2) Molecular signature of replacement tooth formation

In mammals that do replace their teeth once, successional laminae express *Foxi3* and *Pitx2* throughout, but the tooth-forming capacity of the interdental lamina is inhibited by *Sostdc1* (reviewed in Balic, 2019). *Pitx2* is expressed in the epithelium of first-generation teeth, as well as in sites of prospective replacement tooth development, both in rainbow trout and Malawi cichlids (Fraser, Graham & Smith, 2004; Fraser *et al.*, 2006; Fraser, Bloomquist & Streelman, 2008). In zebrafish, *pitx2* is strongly expressed in the pharyngeal epithelium well before the first tooth anlagen become morphologically distinguishable (Jackman, Draper & Stock, 2004), but the latter study does not cover the stages of tooth replacement.

Significantly, patients suffering from cleidocranial dysplasia display supernumerary teeth, formed by reactivation of remnants of the dental lamina. The condition is caused by loss-of-function mutations in the *RUNX2* gene (Mundlos *et al.*, 1997; Wang & Fan, 2011; Kreiborg & Jensen, 2018), belonging to the runt domain transcription factors. *Runx2* heterozygote mice show the beginning of successional tooth development (Wang *et al.*, 2005). Thus, both in mice and man, RUNX2 protein appears to prevent budding of successional laminae. A model has been proposed whereby *Runx2* in the dental mesenchyme blocks inhibitors of Wnt (including *Axin2* and *Drapc1*) and thereby suppresses sequential tooth formation (Järvinen *et al.*, 2018). Teleosts possess two *runx2* paralogues, and while their expression and function has been examined in zebrafish skeletal development (Flores *et al.*, 2004, 2006), their expression and role in (replacement) tooth formation have not yet been determined. Yet, an *sp7* (osterix) mutation, coding for a transcription factor downstream of *runx2*, does not initiate the development of supernumerary teeth in zebrafish (Kague *et al.*, 2018).

Aside from transcription factors, the role of growth factors in mammalian replacement tooth formation is slowly being uncovered (Olley *et al.*, 2014; Järvinen *et al.*, 2018; Balic, 2019). These include FGFs, sonic hedgehog (SHH), transforming growth factors (including the BMPs), Wnts

and Notch signals. While the role of many of these factors has been tested in craniofacial development of zebrafish, observations do not usually cover the dentition let alone the formation of replacement teeth. Fraser *et al.* (2013) studied expression of genes of the above listed five signalling pathways in Malawi cichlids and used small molecules to manipulate these pathways. The authors observed that inhibition of BMP or Notch signalling, but not hedgehog (Hh) signalling, disrupted tooth replacement, whereas activation of Wnt/β-catenin signalling only mildly affected replacement. Bloomquist *et al.* (2015), on the other hand, found that inhibition in Malawi cichlids of either Wnt, Shh or BMP signalling led to reduced tooth density, but how far these effects were specific for replacement teeth rather than causing loss of functional teeth is not clear from this study. By contrast, inhibition of expression of Shh signalling pathway components in mice revitalises the rudimentary successional dental lamina and leads to supernumerary teeth (Mao *et al.*, 2022). On the other hand, supernumerary teeth have been obtained in zebrafish when upregulating Fgf signalling (Gibert *et al.*, 2010; Jackman *et al.*, 2013), overexpressing ectodysplasin (Eda) signalling (Aigler *et al.*, 2014), downregulating Bmp signalling (Jackman *et al.*, 2013), or administering retinoic acid (Seritrakul *et al.*, 2012). However, these manipulations resulted in ectopic formation of teeth, and thus disturbances related to patterning, not replacement.

(3) Wnt signalling and replacement tooth formation

Wnts are a large group of paracrine factors that signal through canonical and non-canonical pathways. Because Wnt signalling has been shown to be able to regulate tooth number in mammals (Järvinen *et al.*, 2006, 2018), this pathway became an obvious candidate to test in teleost tooth replacement. By antagonising Wnt signalling in cichlid jaws, Bloomquist *et al.* (2015) observed a decrease in tooth density. However, the analysis of cleared and stained whole-mount specimens used for this study would not allow clear identification of intramedullary replacement tooth germs, especially when not yet mineralised. In a recent paper, Shim *et al.* (2019) investigated the role of Wnt/β-catenin signalling in zebrafish tooth development and found that conditional activation of the pathway inhibited the development of 3V. However, the misidentification of 3V as a successor of 4V, or the presence and location of a successional lamina connected to 4V, are features not supported by the many (ultra)structural and expression studies done on the zebrafish dentition (e.g. Huysseune, Van der heyden & Sire, 1998; Van der heyden & Huysseune, 2000; Van der heyden *et al.*, 2000; Laurenti *et al.*, 2004; Borday-Birraux *et al.*, 2006; Huysseune, 2006). Such flaws illustrate the necessity for high-resolution approaches for proper interpretation of the dentition, especially in miniaturised vertebrates such as zebrafish (Verstraeten, Sanders & Huysseune, 2012; Bruneel *et al.*, 2015; Huysseune *et al.*, 2022*b*). Unlike the results of Shim *et al.* (2019), Huysseune, Soenens & Elderweirdt (2014) could not influence tooth replacement in the zebrafish with either Wnt stimulators or inhibitors, nor was a phenotype observed in mutants mimicking Wnt overexpression (Fig. 5A, B), despite the important Wnt regulator, *dkk1* being expressed in a dynamic pattern (Fig. 5C). Wnts act through a cascade of biochemical steps as the signal transduces from the cell membrane to the nucleus, each of which can interfere with final activation of the pathway and be responsible for the seeming lack of a replacement tooth phenotype. One example concerns *lef1*, a direct target of Wnt signalling. Adult zebrafish *lef1*$^{-/-}$ mutants show a severe reduction of teeth (McGraw *et al.*, 2011), but the single tooth present has a normal adult tooth size that matches the size of the jaw (Wautier, Van der heyden & Huysseune, 2001), suggesting that it must have undergone replacement (Fig. 5D). R-spondins (Rspo1 to −4) are a small family of four secreted growth factors, which in addition to Wnts potently activate β-catenin signalling (Cruciat & Niehrs, 2013). In adult zebrafish teeth, *rspo3* is broadly expressed in the dental pulp, in odontoblasts of newly formed replacement teeth, and in the crypt epithelium, while *rspo2* expression is highest in the enamel epithelium (Alhazmi *et al.*, 2021). Analysis of adult *rspo3*$^{-/-}$ zebrafish showed a few teeth missing, leading the authors to suggest that *rspo3* is required for adult teeth maintenance, rather than for development of first-generation teeth (Alhazmi *et al.*, 2021). However, as in the case of *lef1* mutants, the normal tooth size suggests that this is a patterning rather than a replacement defect. In addition, the early analysis of *rspo3*$^{-/-}$ was done at a time before the full complement of teeth is present, and patterning defects were observed in double *rspo3*$^{-/-}$/*rspo2*$^{\Delta}$ mutants [*rspo2*$^{\Delta}$ is *rspo3*$^{-/-}$ injected at the one-cell stage with guide RNAs (gRNAs) targeting *rspo2*].

(4) Patterning *versus* replacement defects

While some candidate genes may appear crucial for tooth development, their mutants, or knockdown morphants (knockouts became possible only more recently) have rarely led to a complete failure of tooth replacement. Rather, patterning seems to be disturbed, leading to changes in tooth number, but not necessarily preventing replacement from happening. One example, mentioned above, is the severe form of hypodontia displayed by *lef1*$^{-/-}$ mutant zebrafish (McGraw *et al.*, 2011). Likewise, homozygous *scpp5*$^{-/-}$ zebrafish exhibit a marked reduction in the number of teeth (Qu *et al.*, 2021) (Fig. 5E). Here too, the functional teeth that are present are of a size proportional to the size of the jaw, suggesting replacement must have occurred at least for these teeth. A tentative conclusion one can draw from these few examples is that, in teleosts replacement likely does not happen unless a first-generation tooth has been formed. This conclusion is also inspired by the tight epithelial linkage between the replacement tooth and its predecessor. Conversely, the presence of a first-generation tooth is not a guarantee that replacement will occur, as is demonstrated by zebrafish *edar*$^{-/-}$ mutants (Harris *et al.*, 2008). Here, the first

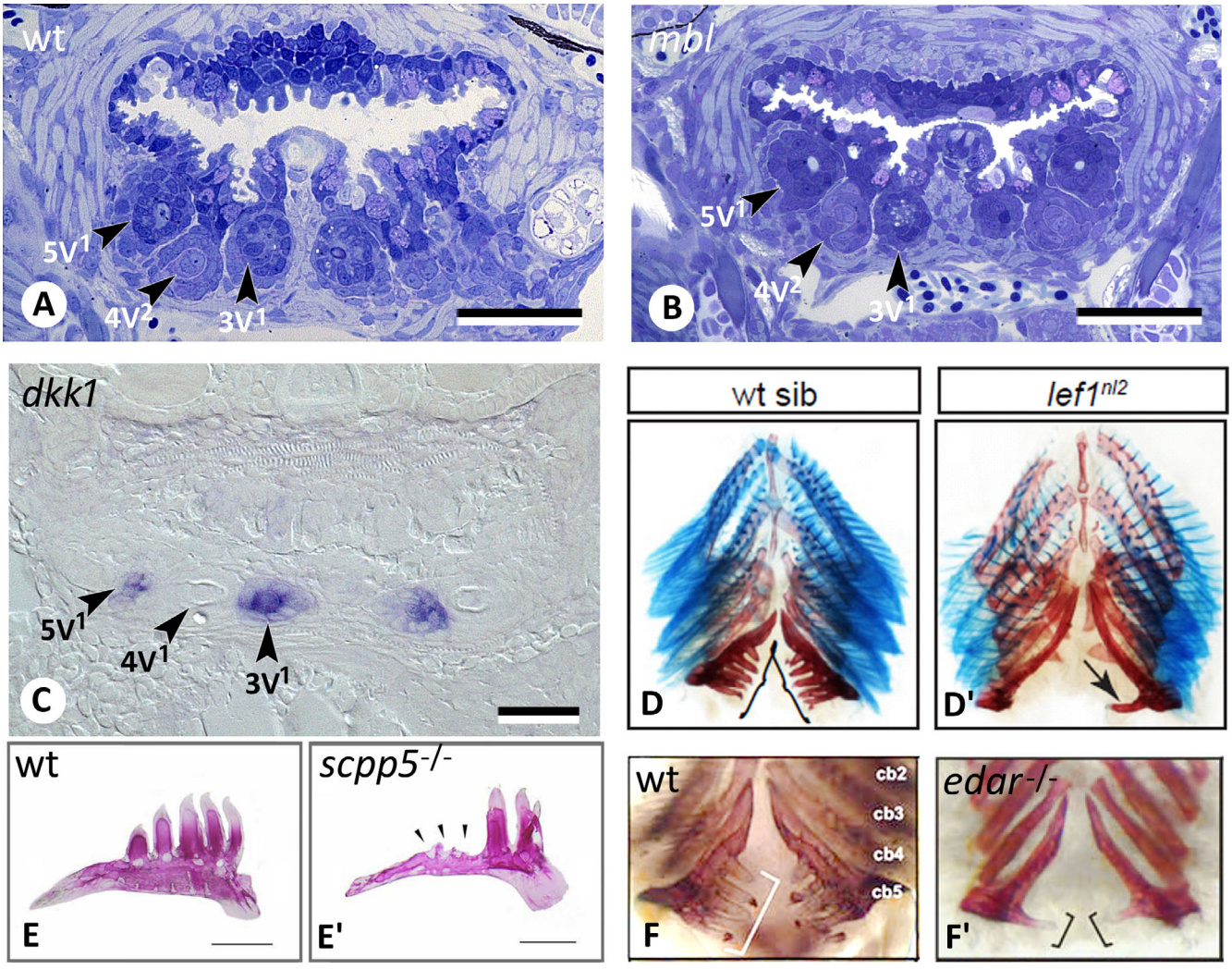

Fig. 5. Molecular control of replacement tooth formation. (A, B) Semithin cross sections of plastic-embedded wildtype (wt) and *mbl/axin1*-mutant (*mbl*) zebrafish *Danio rerio*, displaying the normal set of three first-generation teeth ($3V^1$ and $5V^1$ visible on the section) as well as the first replacement tooth ($4V^2$) (modified from Huysseune *et al.*, 2014). Scale bars = 50 μm. (C) Expression of the soluble Wnt inhibitor *dkk1* in the first-generation dentition of zebrafish (cross section of hybridised specimen; modified from Huysseune *et al.*, 2014). Scale bar = 50 μm. (D–F) Examples of wildtype (wt) zebrafish pharyngeal jaws with normal complement of teeth, and (D'–F') three different mutants, all displaying a reduced number of teeth, yet the teeth present are proportional in size to jaw size. (D') *lef1*$^{-/-}$, reproduced from McGraw *et al.* (2011) with permission of the publisher. (E') *scpp5*$^{-/-}$, reproduced from Qu *et al.* (2021), *Science Advances* 7(34), © The Authors, some rights reserved; exclusive licensee AAAS. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC). (F') *fls/edar*$^{-/-}$, fang allele, modified from Harris *et al.* (2008); cb2-cb5: ceratobranchial 2–5. Scale bar in E, E' = 500 μm.

tooth to form in the zebrafish dentition, $4V^1$, is normal, but it fails to be replaced (Fig. 5F).

(5) New avenues to investigate the molecular control over tooth replacement

Hulsey *et al.* (2016) performed a transcriptome analysis of oral and pharyngeal jaws of the cichlid *Herichthys minckleyi*, yielding some remarkable results. They recovered 284 genes also involved in mouse tooth development, which they argued should mostly be attributed to replacement rather than first-generation teeth, given the age of the fish from which the samples were taken. Interestingly, an additional 57 genes thought to play a role in developing mice teeth were not recovered from the transcriptomes, including several *fgfs*, *bmps*, and *wnt* paralogues. Aside from possible technical reasons, the authors attributed their absence to the predominance of replacement teeth in the samples analysed, and/or to species differences – the mouse dentition representing an extremely derived type of dentition, as mentioned above. Clearly, such data form an interesting basis to dissect further the role of specific genes in replacement tooth formation.

Square *et al.* (2021) took the high diversity of dental replacement systems as a starting point to investigate the molecular signature of tooth replacement in two species with a different predecessor–successor relationship: zebrafish, with a successional dental lamina, and threespine stickleback, which lacks a morphologically distinct successional dental lamina (note that not all positions in sticklebacks have a detectable dental lamina at a given time point; Ellis *et al.*, 2016). Square *et al.* (2021) found a suite of genes expressed in common between naïve dental epithelial cells in both fish species, and suggest that a conserved epithelial progenitor cell type underlies tooth renewal, which they refer to as the 'successional dental epithelium' (SDE). This similarity should not be surprising, since zebrafish, at the stages studied, has no successional lamina yet and highly resembles the stickleback situation (compare their Fig. 1B, from stickleback, with Fig. 2B herein from zebrafish).

One very promising avenue to dissect the genetic circuitry controlling tooth replacement in teleosts comes from studies on threespine stickleback (Cleves *et al.*, 2014, 2018; Ellis *et al.*, 2015; Erickson *et al.*, 2015). Freshwater populations of sticklebacks have a late-developing increase in tooth number compared to marine populations, associated with an accelerated tooth replacement rate (Ellis *et al.*, 2015). Cleves *et al.* (2018) discovered a modular *cis*-regulatory architecture whereby different Bmp6 enhancers drive partially non-overlapping expression patterns in the epithelial and mesenchymal component of developing teeth. Two enhancers of *Bmp4*, a transforming growth factor-β (TGF-β)-responsive 5′ enhancer (Erickson *et al.*, 2015) and an intron 4 enhancer (Cleves *et al.*, 2018), were shown to drive similar mesenchymal expression at early stages of tooth development, and the 5′ enhancer but not the intron 4 enhancer drove strong epithelial expression at these stages (Cleves *et al.*, 2018). Using transcription activator-like effector nucleases (TALEN) to create loss-of-function mutations, they further demonstrated that *Bmp6* is required for specifying tooth number and the size of the tooth field. Intriguingly however, Bmp6 dosage has stronger effects on ventral than on dorsal pharyngeal tooth number. Based on RNA sequencing (RNA-seq) results, *Bmp6* appeared to regulate TGF-β signalling positively in stickleback tooth plate tissue. By contrast, none of seven other signalling pathways usually associated with tooth development (BMP, FGF, SHH, Wnt, Activin, Notch, and EDA) had significant expression differences. Likewise, they found no significant differences in *Sox2* expression between *Bmp6* wild-type and mutant fish. The gene *sox2* has been frequently associated with the presence of stem cells, a topic that is discussed in the Section VI.

VI. REPLACEMENT FROM STEM CELLS?

(1) Chondrichthyans *versus* actinopterygians

The question of the molecular control of tooth replacement is inextricably linked to the question of whether or not stem cells are concerned in the process. Traditionally, stem cells have been defined as slow-cycling cells with the capacity for lifelong self-renewal and an ability to reconstitute appropriate lineages *via* proliferation and differentiation (Harrington, 2004). Nearly 20 years ago, a hypothesis was raised that adult epithelial stem cells may be involved in the process of continuous tooth replacement (Huysseune & Thesleff, 2004). This hypothesis was largely inspired by work of Jamora *et al.* (2003), on the hair stem cell niche, called the 'bulge'. These authors demonstrated that Wnt activation in the bulge leads to downregulation of E-cadherin and reduced cell adhesion, required for the epithelial cells to produce a bud. Several groups subsequently set out to test the epithelial stem cell hypothesis, using a range of species, including sharks (lesser spotted catshark *Scyliorhinus canicula*), several actinopterygians (Atlantic salmon, cichlids, but also the non-teleost Senegal bichir *Polypterus senegalus*), as well as sarcopterygian representatives, including crocodiles. Tests included the use of bromodeoxyuridine (BrdU) pulse–chase experiments to investigate potential label retention, then still considered a hallmark of stemness, and assessing expression of genes considered to be stem cell markers. Importantly, the species listed possess very different types of dental lamina. Whereas sharks have a permanent dental lamina that is also continuous between tooth families, with alternating dental and interdental regions along the dental arcade, bichir and salmon lack a successional dental lamina (Fig. 6). In the latter two species, the successor tooth forms directly from a thickening of the outer dental epithelium of the predecessor (Huysseune & Witten, 2008; Vandenplas *et al.*, 2016*b*). Furthermore, shark dentitions display multiple teeth within one tooth family, while salmon and bichir have just one replacement tooth in each family. Interestingly, bichir and salmon also share the presence of an epithelial tier that we have called the 'middle dental epithelium' (Huysseune & Witten, 2008; Vandenplas, De Clercq & Huysseune, 2014). [Although intermediate in position between outer and inner dental epithelium, the term 'middle dental epithelium' was preferred in order to avoid confusion with the stellate reticulum and the stratum intermedium in mammalian teeth.] The middle dental epithelium is enveloped on the labial side by the inner dental epithelium of the predecessor, and on the lingual side by the outer dental epithelium of the successor tooth. The transition between this outer dental epithelium and the oral epithelium is called the outer dental epithelium transition zone (ODE transition zone). Using BrdU pulse–chase experiments with long chase times, label-retaining cells (possibly epithelial stem cells) were found in the lingual part of the dental lamina in sharks (Vandenplas *et al.*, 2016*a*), but not in the bichir or in salmon (Vandenplas *et al.*, 2014, 2016*b*) (Fig. 6). In the bichir, the last labelled cells disappeared after 4 weeks.

The apparently conflicting results between sharks and actinopterygians may be explained not just by the separate evolutionary history of the two lineages (modern sharks and teleost ancestors diverged ∼400 million years ago), but also by the cellular environment in which replacement takes

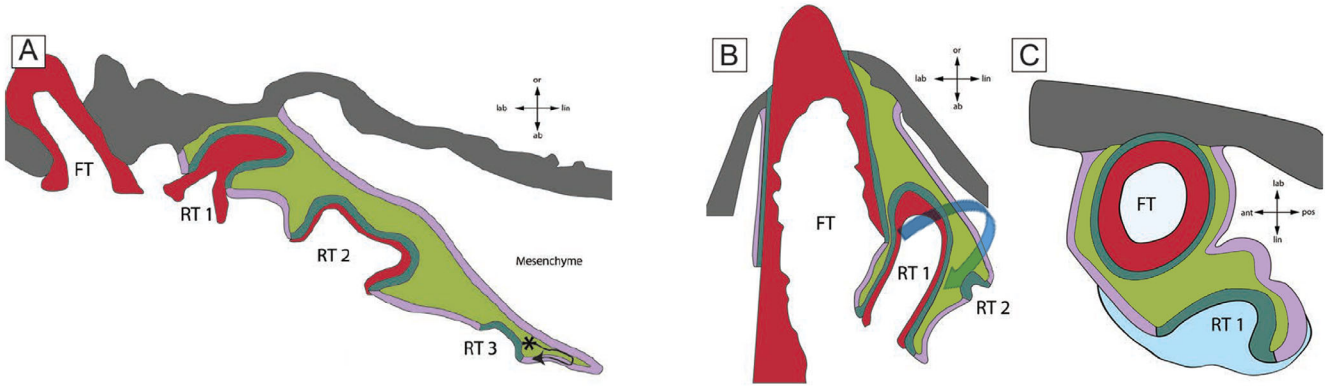

Fig. 6. Schematic representation of tooth replacement. (A) A chondrichthyan, the lesser spotted catshark *Scyliorhinus canicula*; a putative stem cell niche (asterisk) may be present in the middle dental epithelium of the deep part of the dental lamina (modified from Vandenplas *et al.*, 2016*a*). (B) A non-teleost actinopterygian, the Senegal bichir *Polypterus senegalus*; in the absence of a dental lamina, putative stem cells in the outer dental epithelium of the predecessor would need to migrate 180° around the successor to give rise to another tooth generation (indicated by a transparent arrow) (modified from Vandenplas *et al.*, 2014). (C) As in *Polypterus*, the new tooth germ in the teleost Atlantic salmon *Salmo salar* originates directly from the outer dental epithelium of the predecessor and the dental lamina is virtually non-existent (modified from Vandenplas *et al.*, 2016*b*). Grey, oral epithelium; red, tooth matrix; dark green, inner dental epithelium; light green, middle dental epithelium; purple, outer dental epithelium; light blue, mesenchyme. ab, aboral; ant, anterior; FT, functional tooth (predecessor); lab, labial; lin, lingual; or, oral; pos, posterior; RT, replacement tooth (successor).

place: a permanent and continuous dental lamina in sharks *versus* absence of a successional dental lamina in the bony fish species examined. Moreover, the development of a replacement tooth in salmon is initiated from the lingual side of the tooth, which becomes the labial side of the successor. Thus, securing the next tooth generation would involve the unlikely displacement of a putative stem cell niche from labial to lingual at each replacement cycle (Fig. 6B). The presence of a quiescent stem cell niche in the outer dental epithelium of the predecessor (giving rise to the successional lamina) is even more unlikely in intraosseous replacement, typical of advanced teleosts. Here, displacement of stem cells would be needed not just from labial to lingual, but also from outside to inside the bone over a considerable distance. In a discussion about possible stem cell involvement in the formation and regeneration of odontode-derived dermal skeletal elements, it is perhaps also important to consider two studies that show that no stem cells are involved in zebrafish dermal fin ray regeneration. Instead, resident cells de-differentiate and re-differentiate to form dermal fin ray segments (Knopf *et al.*, 2011; Sousa *et al.*, 2011).

(2) Dental stem cells in teleosts

In cichlids, *sox2*, often considered as a stem cell marker, gives a strong signal in the taste buds within the superficial epithelium lining the oral cavity, and a much weaker expression in the dental lamina, either by immunolocalisation or *in situ* hybridisation (Fraser *et al.*, 2013). A strong signal is also observed in the taste buds in bichir and salmon, as well as a strong signal in the ODE transition zone in bichir. However, should this correspond to stem cells, then proliferation would be expected to expand from this area, something that was not observed in the pulsed specimens. Therefore, this layer probably contributes to the oral epithelium and/or the taste buds, but not to tooth cycling. Abduweli *et al.* (2014), using BrdU pulse–chase experiments in medaka, found clusters of label-retaining epithelial cells at the posterior end of each tooth family, coinciding with areas of expression of *sox2*. These results stand in sharp contrast with those of Tan *et al.* (2017), who examined expression and distribution of telomerase reverse transcriptase (Tert), the catalytic unit of telomerase, in medaka. Because stem cells need to support lifelong replenishment of tissues, it has been proposed that their DNA must be protected from telomere shortening, making Tert a potential stem cell marker. Tan *et al.* (2017) noted the absence of Tert-positive cells in the epithelial compartment of early tooth germs, underscored by the absence of expression of the pluripotency markers *oct4* and *lgr5*. By contrast, cells expressing both *oct4* and *lgr5* were found to be evenly distributed in the entire proliferating oral epithelium without specific connection to tooth replacement.

The co-localisation of teeth and taste buds in cichlids has led to the proposal that they are tightly co-patterned, under common genetic and developmental control (Streelman, Bloomquist & Fowler, 2015; Bloomquist *et al.*, 2015, 2019). The origin of the successional lamina from the outer dental epithelium close to where the tooth attaches, i.e. in the deep part of the crypt, in contrast to the localisation of the taste buds on the surface epithelium between adjacent crypts, makes a causative link hard to understand. Moreover, both are separated by differentiated epithelium with many mucus cells. On the other hand, there is little doubt about the plasticity of the fast-proliferating oropharyngeal epithelium. This is supported by the potential, in zebrafish, of normally non-tooth-forming pharyngeal epithelium to form teeth upon exogenous retinoic acid administration (Seritrakul *et al.*, 2012), or the plasticity, in cichlids, to form both tooth and taste-like cell types upon BMP inhibition (Bloomquist *et al.*, 2019). This plasticity stretches throughout

the entire alimentary canal (except that there is no tooth formation possible in the intestine), as demonstrated in zebrafish by a massive change of gut epithelium into a secretory fate upon Notch signalling inhibition (Crosnier *et al.*, 2005).

The seemingly conflicting evidence regarding the presence of stem cells, at least in teleosts, may be resolved when considering that epithelial 'stem' cells in teleost tooth replacement may not correspond to the classical paradigm of stem cells, that is: slow-cycling, giving rise to transit-amplifying cells, which subsequently differentiate into specific epithelial lineages. Instead, renewal may depend on the activation, in the epithelium, of undifferentiated progenitor cells (i.e. multipotent cells not displaying self-renewal). This activation could be part of a Turing-type mechanism involving both activators and repressors, similar to the mechanism proposed to explain budding of second molar teeth from the caudal end of the first molar in the mouse (Järvinen *et al.*, 2018). Recent studies in the mouse incisor, the classical paradigm for dental stem cells, have indeed indicated that renewal capacity can be distributed over a large population of actively dividing progenitors that are moreover quite heterogeneous, as demonstrated in other types of organ renewal (Sharir *et al.*, 2019). These authors also highlighted that putative stem cell markers such as *Sox2*, *Bmi1*, *Gli1* and *Lrig1*, are broadly expressed throughout both the quiescent and proliferating regions. This is also true for the ubiquitous distribution of the putative stem cell marker *Sox2* in the oral epithelium of mammals, as shown by Juuri *et al.* (2013, their Figs 2 and 3). Using single-cell RNA sequencing (scRNA-seq), genetic-lineage tracing and injury-repair studies, Sharir *et al.* (2019) uncovered a highly dynamic stem cell model, distinct from the traditional view of stem cells in the mouse incisor.

Alternatively, although not mutually exclusive with the previous suggestion, the formation of a replacement tooth may be seen as a branching event of the epithelium, similar to the branching of other organs from the anterior alimentary tract epithelium, albeit strictly regulated in time and space. The comparison to other branching events would require not just investigations into potential activators and inhibitors controlling the branching, but especially into the signals that stop branching. Additionally, an increasing body of evidence points to the role of mechanical factors for epithelial invagination (reviewed in Calamari, Kuang-Hsien Hu & Klein, 2018). Studies looking into the question of what drives epithelial invagination, either for budding from a permanent dental lamina, for development of a transient successional lamina, or for the folding of the outer dental epithelium, could take advantage of organotypic cultures in serum-free conditions, which can successfully sustain the development of teleost replacement teeth (Van der heyden *et al.*, 2005).

VII. THE NEUROVASCULAR LINK AND TOOTH REPLACEMENT

Irrespective of whether local progenitors or stem cells are activated, or whether formation of teeth is some form of budding, a factor that is rarely taken into account in studies of teleost tooth replacement is the vascular and neural environment, which are interconnected in what is commonly denominated the 'neurovascular link'. This is surprising, for at least two reasons: the abundant vascularisation that is present around the teeth in the actinopterygian as well as in the chondrichthyan species examined; and a study that showed arrest of tooth replacement as a result of nerve resectioning.

(1) Vasculature

Several species that we have investigated (catshark, bichir and zebrafish) possess an elaborate network of large blood vessels that encircle the teeth (Fig. 7A, B). In zebrafish, the vasculature that serves the pharyngeal dentition originates from the hypobranchial artery, reaches the pharyngeal jaw at the time of formation of the first replacement tooth (Crucke & Huysseune, 2013), and has offshoots exactly where the replacement tooth will form. This intriguing observation led Crucke & Huysseune (2015) to investigate the role of the vasculature in zebrafish tooth replacement. Preventing angiogenesis by inhibition of vascular endothelial growth factor (VEGF) receptor in juvenile zebrafish did not prevent the formation of replacement teeth, but there was a clear delay in their development. Since VEGF inhibition did not remove existing blood vessels, the exact role of the vasculature remains elusive. More specifically, it remains to be seen if the vasculature provides more than just metabolic sustenance (nutrients and oxygen), i.e. specific cues for organ development. The latter has been demonstrated for a (mammalian) endodermal 'appendage', the pancreas (reviewed in Villasenor & Cleaver, 2012).

(2) Innervation

The importance of nerve-derived signalling for correct regeneration has been the topic of research for more than 100 years. The neurotrophic factor(s) hypothesis states that trophic factors produced by the nerves are required for proper regeneration (Pirotte *et al.*, 2016). Studies investigating the role of nerves in tooth replacement in teleosts are very rare. Tuisku & Hildebrand (1994) unilaterally transected the ramus alveolaris of the trigeminal nerve in a cichlid, *Tilapia mariae*, and observed an arrest of tooth replacement after about 100 days, on the denervated side (Fig. 7C, D). At the time, the authors could not make any firm statement about the exact nature of the relationship between nerves and tooth formation. To our knowledge, such denervation experiments have not been performed in other teleosts. Genetically interfering with peripheral nervous system development has been done in zebrafish but the outcome is usually studied in very early stages only, and never reported for the teeth. The findings of Tuisku & Hildebrand (1994) are even more important considering recent studies showing that peripheral nerve-associated glia is an important source of mesenchymal stem cells in mouse incisors (Kaukua *et al.*, 2014). Lineage tracing

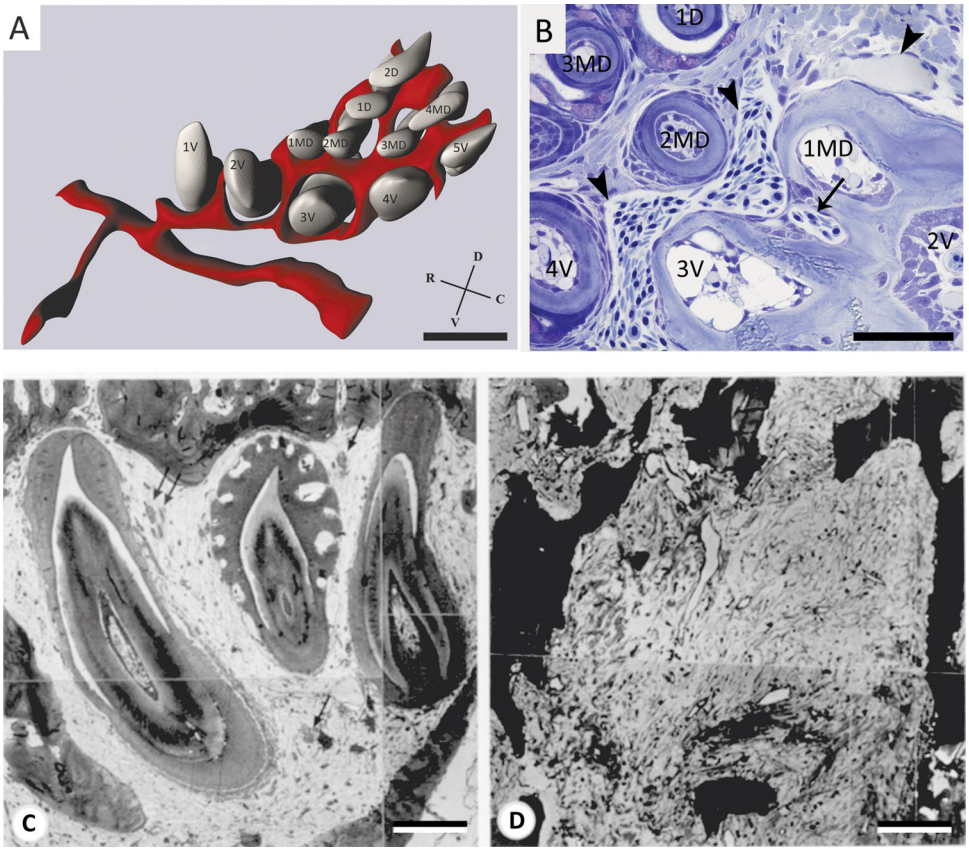

Fig. 7. Vasculature and innervation of teleost dentitions. (A) Three-dimensional reconstruction of the teeth and the surrounding vasculature in a juvenile zebrafish *Danio rerio* [standard length (SL) = 8.0 mm]. Teeth are labelled according to their position as ventral (1V–5V), mediodorsal (1MD–4MD) or dorsal (1D, 2D) teeth [reproduced from Crucke & Huysseune (2013) with permission of the publisher]. Scale bar = 50 μm. (B) Sagittal, toluidine blue-stained semithin section of the dentition of a juvenile zebrafish (SL = 9.5 mm) showing the elaborate network of blood vessels (arrowheads) surrounding the functional teeth (labelled as in A). An arrow indicates a branch supplying the functional tooth at position 2 V [reproduced from Crucke & Huysseune (2013) with permission of the publisher]. Scale bar = 50 μm. (C) Rostral part of an innervated jaw cavity in the cichlid *Tilapia mariae*, with three large tooth germs in the medullary cavity, and some small nerve bundles (arrows) [reproduced from Tuisku & Hildebrand (1994) with permission of the publisher]. (D) Rostral part of the denervated jaw cavity 300 days after neurectomy of the ramus alveolaris of the trigeminal nerve. Note absence of tooth germs [reproduced from Tuisku & Hildebrand (1994) with permission of the publisher]. Scale bars in C and D = 100 μm.

of mesenchymal cells during development, renewal, and repair of the mouse incisor indicated that glial cells generate multipotent mesenchymal stem cells that produce pulp cells and odontoblasts (Kaukua *et al.*, 2014). Berkovitz & Shellis (2016) also highlighted the potentially important role of nerves in both supporting stem cells and providing a source of stem cells for the tooth. Clearly, a wide field remains open to potential investigations.

VIII. TOOTH ADDITION *VERSUS* TOOTH REPLACEMENT

Some teleost species develop a fixed number of teeth early in the establishment of the dentition, after which no new teeth are added and teeth only cycle, although increasing in size with each replacement. This is the case, for example, in zebrafish (Wautier *et al.*, 2001). Other species steadily expand their dentition with a growing number of teeth, concomitant with the growth of the jaw bone, while the teeth already present continue cycling, as for example in the jewel cichlid (*Hemichromis bimaculatus*), the Atlantic salmon (Huysseune *et al.*, 2007), the characoid *Ctenolucius huieta* (Lawson & Manly, 1973), or the bluefish (Bemis *et al.*, 2005) (reviewed in Berkovitz & Shellis, 2016). It is important to distinguish tooth replacement from tooth addition, i.e. the addition of new teeth as space is provided by the growth of the underlying jaw (Chen *et al.*, 2016; Collins & Underwood, 2021). The question is, how is an increase in tooth number achieved during growth? Do teeth in new positions form as first-generation teeth, independent from the adjacent tooth (typically the last tooth in the row)? Or do teeth in a new position form as replacement teeth, which subsequently become

independent from the predecessor? The first scenario would suggest the recapitulation of an embryonic process; the replacement tooth scenario could be considered as co-option of the replacement process. In the absence of molecular markers capable of distinguishing between these two processes, characters that can be used to distinguish between both scenarios are (*i*) tooth shape, (*ii*) tooth location and (*iii*) the epithelial connection of the tooth germ. (*i*) In species with an elaborate (e.g. multicuspid) adult tooth shape, small conical teeth that appear later in life may be indicative of a first-generation tooth, for instance in certain cichlid species (Huysseune *et al.*, 1999; Fig. 8). (*ii*) First-generation teeth always develop extraosseously, even in species where adult teeth develop intraosseously. (*iii*) An epithelial connection of the enamel organ of the new tooth to the enamel organ of a functional tooth (either directly or *via* a successional lamina) is a strong indication that the new tooth starts to develop as a replacement tooth.

Distinguishing tooth addition from tooth replacement is not always straightforward. This is also clear from a study on two fossil pycnodontiform fish species, where at specific positions several smaller teeth appear to have 'replaced' a single large tooth. Collins & Underwood (2021) argue that, in the absence of what they call 'conventional one-for-one replacement', small teeth would be added *via* gap-filling tooth addition. They suggest that the oral epithelium retained an initiatory competence throughout life. Carr *et al.* (2021) used the term 'fated' for an individual tooth that is destined to replace a functional tooth at a specific location, i.e. true replacement. By contrast, 'non-fated' indicates when the identity of a tooth is difficult to relate to a functional tooth, i.e. tooth addition. Interestingly, in humans, both replacement and addition are present: replacement generates the secondary teeth, while serial addition generates the posterior molars. Both modes of tooth initiation are treated as sequential tooth formation, resembling each other morphologically and molecularly (Järvinen *et al.*, 2018).

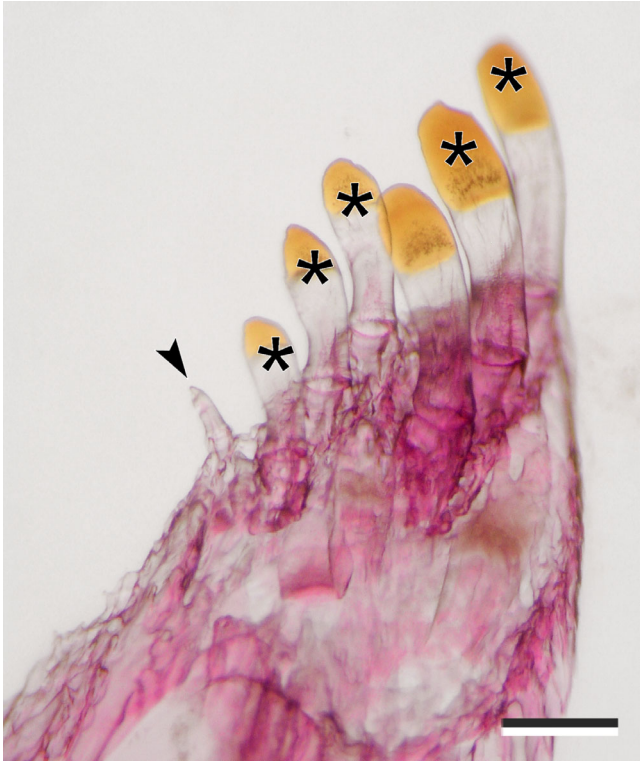

Fig. 8. Tooth addition *versus* tooth replacement. Right dentary of the cichlid *Eretmodus cyanostictus* [standard length (SL) = 23 mm]; cycling teeth are large and spatula-shaped (indicated by asterisks) and have tooth germs below their base inside the medullary cavity (intraosseous replacement). The tooth indicated by an arrowhead is small and conical shaped, similar to a first-generation tooth. A replacement tooth germ is not visible in this position. Scale bar = 500 μm.

An interesting case is the development of supernumerary teeth in the pharyngeal dentition of cyprinids. This can be the result of thyroid hormone deficiency, as shown in *Barbus intermedius* (Shkil *et al.*, 2010) and zebrafish (Woltmann *et al.*, 2018). In both cases, an accessory (fourth) tooth row is formed instead of the normal three tooth rows. Conversely, an increase in thyroid hormone levels reduces the number of tooth rows from three to two (Smirnov & Levin, 2007). Supernumerary teeth also occur in adult zebrafish heterozygous for a mutation in exostosin2 (*ext2*), a gene that encodes a glycosyltransferase crucial for the polymerisation of heparan sulphate (Wiweger *et al.*, 2012). Moreover, supernumerary teeth occur in adult zebrafish heterozygous for the *cyp26b1* mutant. The gene *cyp26b1* codes for an enzyme able to degrade retinoic acid (Gibert *et al.*, 2015). Ahnelt, Herdina & Metscher (2015) suggested that a fourth 'row' in natural populations of zebrafish might occur through a shift in position of one of the two teeth of the external row. On the other hand, Eastman & Underhill (1973) propose that faulty tooth replacement may well contribute to some of the intraspecific variation in the pharyngeal tooth formulae of cyprinid species.

IX. CONCLUSIONS

(1) With about 30,000 species, teleosts present an astonishing diversity in tooth replacement characters that is unmatched by any other group of vertebrates. This diversity relates to which bones are toothed, the anatomical setting of the replacement teeth (e.g. lingual or labial replacement, origin of the tooth bud from the superficial epithelium or from the predecessor, presence or absence of a dental lamina, extra- or intraosseous replacement), sequence and timing of replacement, length of the replacement cycle, mechanism of tooth shedding, etc. Thus, data from different species may not be interchangeable, clearly not facilitating investigations into the mechanism of tooth replacement. The use of teleost species with a well-known and highly predictable pattern of first-generation and subsequent replacement teeth,

such as zebrafish (Van der heyden & Huysseune, 2000) can therefore offer substantial advantages.

(2) Our own studies on bichir and Atlantic salmon, and a survey of the literature available, leads to the conclusion that a dental lamina is not required for tooth replacement. The many examples of species where the replacement tooth forms directly from the outer dental epithelium of the predecessor, or the finding that successional lamina formation is uncoupled from development of the replacement tooth proper, supports this view. The idea that reactivation of a competent dental lamina is key for replacement tooth formation is largely taken from studies on chondrichthyans, reptiles, and diphyodont mammals (Järvinen, Tummers & Thesleff, 2009), which together form a minority of species compared to the number of teleost species.

(3) The field model has recently been revived to explain the formation of a tooth row from an 'initiator tooth' (Sadier *et al.*, 2020). It may well be that the order and sequence of replacement tooth formation, by default, follows the initial setting up of the pattern of first-generation teeth. However, a growing body of evidence supports the idea that replacement is under local control.

(4) The molecular signals that trigger tooth replacement in teleosts remain to be uncovered. Despite its formidable status as a model organism, many studies in zebrafish are performed on embryos and early post-embryonic stages up to 5 days post-fertilisation, a time point when only the first replacement tooth ($4V^2$) is present and mineralised (Borday-Birraux *et al.*, 2006). Conditional knockouts could provide more insights, but, while many studies of such lines exist, the tooth phenotype is usually ignored. Thus, we still know very little about genes possibly controlling tooth replacement. Clearly there is a vast field awaiting exploration. Karagic *et al.* (2020) performed a transcriptomic analysis using RNA-seq in a cichlid to compare toothed first and seventh pharyngeal arches (oral and pharyngeal jaws) with a toothless (sixth) pharyngeal arch. Using a similar approach but comparing an early arch with first-generation teeth with a similar arch at a later time point, with multiple replacement teeth, may uncover genes previously documented to be involved in tooth development, as well as genes without previously known function, possibly specifically related to replacement tooth development.

(5) Nerves and blood vessels are located in the vicinity of replacement tooth initiation sites in salmon, zebrafish and cichlids. Yet, aside from isolated studies, one showing delay of tooth replacement following inhibition of angiogenesis, and a single study demonstrating replacement arrest after denervation, we have no functional data yet demonstrating the importance of the neurovascular link in tooth replacement. Clearly, a vast field awaits exploration.

(6) Replacement in teleosts that develop a transient dental lamina may not rely on stem cells, defined as quiescent slow-cycling cells displaying self-renewal. Instead, replacement may depend on the activation of undifferentiated progenitors by hitherto unknown (possibly mesenchymal) signals. That neither a dental lamina, nor stem cells appear to be required for tooth replacement places teleosts in an advantageous position as models for tooth regeneration in humans, where the dental lamina regresses and epithelial stem cells are lost (Buchtová *et al.*, 2012; Binder *et al.*, 2020).

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