

The conundrum of pharyngeal teeth origin: the role of germ layers, pouches, and gill slits

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ABSTRACT

There are several competing hypotheses on tooth origins, with discussions eventually settling in favour of an ‘outside-in’ scenario, in which internal odontodes (teeth) derived from external odontodes (skin denticles) in jawless vertebrates. The evolution of oral teeth from skin denticles can be intuitively understood from their location at the mouth entrance. However, the basal condition for jawed vertebrates is arguably to possess teeth distributed throughout the oropharynx (i.e. oral and pharyngeal teeth). As skin denticle development requires the presence of ectoderm-derived epithelium and of mesenchyme, it remains to be answered how odontode-forming skin epithelium, or its competence, were ‘transferred’ deep into the endoderm-covered oropharynx. The ‘modified outside-in’ hypothesis for tooth origins proposed that this transfer was accomplished through displacement of odontogenic epithelium, that is ectoderm, not only through the mouth, but also *via* any opening (e.g. gill slits) that connects the ectoderm to the epithelial lining of the pharynx (endoderm). This review explores from an evolutionary and from a developmental perspective whether ectoderm plays a role in (pharyngeal) tooth and denticle formation. Historic and recent studies on tooth development show that the odontogenic epithelium (enamel organ) of oral or pharyngeal teeth can be of ectodermal, endodermal, or of mixed ecto–endodermal origin. Comprehensive data are, however, only available for a few taxa. Interestingly, in these taxa, the enamel organ always develops from the basal layer of a stratified epithelium that is at least bilayered. In zebrafish, a miniaturised teleost that only retains pharyngeal teeth, an epithelial surface layer with ectoderm-like characters is required to initiate the formation of an enamel organ from the basal, endodermal epithelium. In urodele amphibians, the bilayered epithelium is endodermal, but the surface layer acquires ectodermal characters, here termed ‘epidermalised endoderm’. Furthermore, ectoderm–endoderm contacts at pouch–cleft boundaries (i.e. the prospective gill slits) are important for pharyngeal tooth initiation, even if the influx of ectoderm *via* these routes is limited. A balance between sonic hedgehog and retinoic acid signalling could operate to assign tooth-initiating competence to the endoderm at the level of any particular pouch. In summary, three characters are identified as being required for pharyngeal tooth formation: (i) pouch–cleft contact, (ii) a stratified epithelium, of which (iii) the apical layer adopts ectodermal features. These characters delimit the area in which teeth can form, yet cannot alone explain the distribution of teeth over the different pharyngeal arches. The review concludes with a hypothetical evolutionary scenario regarding the persisting influence of ectoderm on pharyngeal tooth formation. Studies on basal osteichthyans with less-specialised types of early embryonic development will provide a crucial test for the potential role of ectoderm in pharyngeal tooth formation and for the ‘modified outside-in’ hypothesis of tooth origins.

Key words: oropharynx, endoderm, ectoderm, periderm, pharyngeal pouches, tooth development, dermal denticles, pharyngeal denticles

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I. INTRODUCTION: GERM LAYERS AND THE EVOLUTIONARY ORIGIN OF TEETH

Teeth play a pivotal role in tracing the evolutionary history of vertebrates (e.g. Janvier, 1996; Ungar, 2010). Not surprisingly, the evolutionary origin of teeth has itself been at the centre of evo-devo research. Based on their composition and development, it was recognised in the 19th century that teeth derive from skin denticles, elements of the ancient vertebrate dermal skeleton (Hertwig, 1874). Both teeth and skin denticles qualify as ‘odontodes’ – isolated structures that consist of a dentine cone, are covered by a hypermineralised layer and have a bony base which serves as an attachment (Reif, 1982; see Table 1). The canonical view that skin denticles invaded the mouth cavity and gave rise to teeth at the dawn of gnathostome evolution, in conjunction with the origin of jaws, dominated our thinking for well over a century (e.g. Romer, 1949; Jollie, 1968; Peyer, 1968; Ørvig, 1977).

In the 1990s, an alternative hypothesis about tooth origins was proposed, based on a revised analysis of palaeontological and neontological data (Smith & Coates, 1998, 2000, 2001; Smith, 2003; Johanson & Smith, 2005). It became known as the ‘inside-out’ hypothesis, to distinguish it from the classical hypothesis that became known as the ‘outside-in’ hypothesis. The arguments were: (i) conodonts were reinterpreted as jawless vertebrates and mineralised conodont structures were accepted as teeth. Since conodonts have no dermal skeleton and the conodont elements were believed to be located inside the animals’ pharynx, it was concluded

that not skin denticles but endoderm-derived pharyngeal structures gave rise to teeth (Smith & Coates, 1998). (ii) The discovery of thelodonts with pharyngeal denticles was argued to provide evidence for an origin of teeth independent of jaws (Smith & Coates, 1998, 2000, 2001). (iii) Patterned denticle arrays were identified on the posterior wall of the pharyngeal cavity, the postbranchial lamina, in placoderms (early jawed vertebrates) (Johanson & Smith, 2005). (iv) It was claimed that transitory stages between teeth and skin denticles cannot be found (Smith & Coates, 1998, 2000, 2001). (v) It was suggested that teeth must always develop from, and be patterned by, a specialised epithelial structure, the dental lamina (Smith & Coates, 1998). That skin denticles develop directly from the interaction between the epithelium and the mesenchyme, without the formation of a dental lamina, was used as an argument against their homology with teeth. The conclusion was that teeth evolved first in the pharynx from the interaction between endoderm and mesenchyme, and not, like skin denticles, from ectoderm and mesenchyme (Smith & Coates, 1998, 2000, 2001; Smith, 2003; Johanson & Smith, 2005; Fraser *et al.*, 2009). This view was further fostered as a result of comparing the patterning of shark skin denticles with that of dentitions (Fraser & Smith, 2011).

The debate extended for over a decade before the pendulum eventually swung back to the earlier, canonical view and the recognition that internal odontodes (oral or pharyngeal teeth or denticles) in jawed vertebrates are evolutionarily derived from the expansion of odontogenic epithelium from the skin into the oropharynx (Witten, Sire & Huysseune, 2014). Key

Table 1. Glossary. In its original concept, the term ‘odontode’ was used to refer to ‘dermal teeth’ – hard tissue units in the corium, corresponding very closely to teeth, composed principally of dentine or dentinous tissue, with or without an outer covering of enameloid, and forming ontogenetically in a single undivided mesenchymal dental papilla (Ørvig, 1967, 1977). Reif (1982) slightly modified the definition of Ørvig (1977) and, importantly, expanded it to encompass both ‘dermal denticles’ and ‘teeth’. In Reif’s view, only those organ systems that are formed by a dental lamina qualify as dentitions, and its components referred to as teeth. Reif’s (1982) definition of an odontode has gained wide acceptance, but the dependence on a dental lamina for teeth is now no longer considered valid: the dental lamina has been seriously challenged as a patterning device for the dentition in early jawed vertebrates (Qu *et al.*, 2013), or is absent altogether in developing teeth in many extant species (see text). Donoghue & Rücklin (2016) found an elegant way to circumvent this problem by referring to external and internal odontodes. While we support this distinction, it does not do justice to the diversity of internal odontodes. Thus, in the current review, we will adopt the terminology shown in this table

Odontode	An isolated superficial structure of the dermal skeleton which consists of a dentinous tissue and a hypermineralised cap of enamel or enameloid either present or absent, and which rests on a base of acellular or cellular bone functioning as an attachment tissue. Developmentally, odontodes form in a single undivided mesenchymal dental papilla, bounded at its surface by an epithelial dental (or enamel) organ (Reif, 1982). Skin (or dermal) denticles, teeth, pharyngeal denticles, and placoid scales all qualify as odontodes.
External odontodes	Odontodes located in the skin, synonymous with skin (or dermal) denticles. Chondrichthyans have external odontodes that are usually called placoid scales. These can be non-growing and replaced, or growing and retained (Reif, 1978 <i>b</i>).
Internal odontodes	Odontodes located within the oropharynx, with their patterning and replacement highly coordinated compared to external odontodes (Doeland <i>et al.</i> , 2019). Two categories of internal odontodes can be distinguished – teeth, and (pharyngeal) denticles – although differences are somewhat blurred as a result of their conserved structure and development.
Teeth	Internal odontodes with an important function in food uptake, transport and other types of food manipulation. Usually large units, and in low numbers. Teeth can be associated with the jaw margins and palate (oral teeth) but also with any of the post-mandibular arches (pharyngeal teeth). In a number of extant teleosts, tooth structures appear in the skin but are phylogenetically discontinuous with skin denticles, and represent the outcome of a tooth developmental program outside the mouth. These have been called ‘extra-oral teeth’ (Sire & Allizard, 2001).
Pharyngeal denticles	Internal odontodes that are topologically related to teeth (i.e. located inside the oropharynx), but that functionally resemble external odontodes, for example in providing the oropharyngeal lining with a rough surface. Usually small units, and in high numbers. Many extant chondrichthyans have internal odontodes that, like external odontodes, are often called ‘placoid scales’ (Nelson, 1970), or ‘mucous membrane denticles’ (Peyer, 1968). In tetrapodomorph fishes and early tetrapods the denticles, as well as the region where they are found, are called ‘branchial’ rather than ‘pharyngeal’ (e.g. Schoch, 2002).
Dental lamina	An epithelial thickening invaginating into the underlying mesenchyme, yielding one or multiple anlagen of enamel organs, and thus tooth germs (Reif, 1978 <i>a</i> , 1982).

elements that led to this insight were: (i) that tooth-like elements such as found in derived conodont taxa are an innovation peculiar to that lineage (Murdock *et al.*, 2013); (ii) that the internal scales in thelodonts fail the test of phylogenetic continuity with teeth of jawed vertebrates (Rücklin *et al.*, 2011); (iii) that the tooth-like structures on the postbranchial lamina in placoderms are in fact focal developments of bone (Rücklin *et al.*, 2012); (iv) the recognition of intermediate stages between external odontodes and teeth (Blais, MacKenzie & Wilson, 2011; King, Marone & Rücklin, 2021), and (v) a reappraisal of the significance of the dental lamina (Huysseune, Sire & Witten, 2009, 2010; Qu *et al.*, 2013; Vandenplas, De Clercq & Huysseune, 2014). The different arguments have been well covered in a series of papers, that also demonstrate that teeth are genuine elements of the dermal skeleton (Sire & Huysseune, 2003; Reif, 2006; Harris *et al.*, 2008; Huysseune *et al.*, 2009, 2010; Turner *et al.*, 2010; Blais *et al.*, 2011; Debiais-Thibaud *et al.*, 2011; Rücklin *et al.*, 2011, 2012; Murdock *et al.*, 2013; Qu *et al.*, 2013; Donoghue & Rücklin, 2016; Haridy *et al.*, 2019; Chen *et al.*, 2020).

Huysseune *et al.* (2009, 2010) proposed a slightly modified version of the canonical view, designated as the ‘modified outside-in’ hypothesis. This proposal followed the recognition that odontodes in the oral and pharyngeal cavity are

serial homologues and to a large extent share genetic developmental pathways (Stock, 2001; Wise & Stock, 2006; Debiais-Thibaud *et al.*, 2007, 2008). Thus, the ‘modified outside-in’ hypothesis was introduced to explain the presence of teeth throughout the mouth and pharynx (collectively called the oropharynx), that is, not just teeth associated with the mandibular arch and the palatal bones (oral teeth), but also teeth developing in association with the more posterior pharyngeal arches, that is the hyoid and the branchial arches *sensu stricto* [pharyngeal teeth (Nelson, 1969; Berkovitz & Shellis, 2016)]. In its original version, the ‘modified outside-in’ hypothesis attached a great deal of importance to open oral and pharyngeal orifices. It suggested that epithelium with odontode-forming competence may have entered the oropharynx *via* these orifices, in particular the gill slits. It was furthermore suggested that an invasion of tooth-competent epithelium into the oropharynx may have occurred independent from (and perhaps even prior to) the presence of jaws (Huysseune *et al.*, 2009, 2010). In this respect, it is interesting to note that several authors have emphasised the disparate evolutionary history of teeth and jaws (McCollum & Sharpe, 2001; Rücklin *et al.*, 2012; Rücklin & Donoghue, 2015).

That the origin of teeth has been placed either in the pharynx or in the skin hints at the crucial role that germ layers have played in the discussions summarised above. Vertebrates, like all bilaterian animals, are built on a three-layered plan, the three layers of embryonic tissue, or germ layers: ectoderm, endoderm and mesoderm. Ectoderm gives rise to the epithelium of the skin, the epidermis, while endoderm forms the epithelial lining of the digestive tube and associated organs. In addition, the ectoderm is at the origin of the neural crest, a cell population that produces diverse tissues and is thus often considered as the fourth germ layer (Hall, 1998, 2000). Vertebrate teeth, as a paradigmatic example of organogenesis, develop through reciprocal interactions between an epithelial component (derived from ectoderm or endoderm, and forming the enamel organ) and the underlying mesenchyme (neural crest-derived, and forming the dental papilla) (Kollar & Baird, 1969; Balic & Thesleff, 2015; Balic, 2019; Yu & Klein, 2020) (Fig. 1A). The enamel organ is responsible, at least in part, for the production of the hypermineralised tooth cap; the dental papilla for the production of dentine.

In the ‘inside-out’ model, the germ layer origin of the epithelium (ectoderm for external odontodes, endoderm for teeth) was considered to impart real differences to the two systems (Smith & Coates, 2001; Smith & Johanson, 2015). With the experimental evidence that the enamel organ can be of ectodermal, endodermal or mixed origin (Soukup *et al.*, 2008), the focus in discussions shifted to the pivotal role of the mesenchyme, and it was proposed that the germ layer origin of the epithelial enamel organ does not matter (Fraser *et al.*, 2010; Calamari, Kuang-Hsien Hu & Klein, 2018). Fraser *et al.* (2010) argued that teeth develop wherever specific signalling networks drive interactions between neural crest-derived mesenchyme and competent epithelium, independent from the germ layer origin of the epithelial layer. This suggestion, labelled as the ‘inside and out’ model, moved the focus away from arguments about primacy of location and/or cell type (Fraser *et al.*, 2010; Fraser & Smith, 2011). In this model the expansion of odontogenic competence from external to internal epithelia is attributed to the primacy of neural crest-derived ectomesenchyme, rather than to the origin of the epithelia (Soukup *et al.*, 2008; Jheon *et al.*, 2012; Donoghue & Rücklin, 2016).

There are nevertheless several arguments to reject the primacy of the ectomesenchyme in odontode induction. From an evolutionary viewpoint, it has been argued that enamel-like (i.e. epithelium-derived) substances may well have been the most primitive vertebrate hard tissues (Maisey, 1988), although the early diversity of vertebrate mineralised tissues makes this a contentious issue (Donoghue & Sansom, 2002; Donoghue, Sansom & Downs, 2006; Kawasaki *et al.*, 2020). From a developmental viewpoint, epithelial primacy in tooth initiation seems well underscored by reports detailing the first molecular evidence of mammalian tooth initiation in the epithelium (Peters *et al.*, 1998; Peters & Balling, 1999; Ohazama *et al.*, 2010; Prochazka *et al.*, 2015; Calamari *et al.*, 2018; Yuan & Chai, 2019) (Fig. 1A). Epithelial primacy appears also evident in the case of intramedullary (also called intraosseous)

tooth formation (Trapani, 2001), as observed in advanced teleost fish [e.g. *Trachinotus teraia* (Frañillon-Vieillot *et al.*, 1994); *Hemichromis bimaculatus* (Huyseune & Thesleff, 2004)]. Here, replacement tooth formation starts with the production of an epithelial strand (the successional dental lamina) branching off from the outer dental epithelium. This strand grows over a large distance, piercing through bone, to reach the medullary cavity. Only then, within the medullary cavity, does mesenchyme start to condense around the tip of the strand, suggesting a later role in tooth development (Fig. 1B–G). The crucial role of the epithelium in initiating tooth formation justifies our focus on the odontogenic epithelium. The germ layer origin of the odontogenic mesenchyme (i.e. neural crest-derived ectomesenchyme) is taken for granted here (see e.g. Raven, 1935; de Beer, 1947; Chibon, 1966, 1967; Chai *et al.*, 2000; Hall & Gillis, 2013).

This review intends to assess, both from an evolutionary and developmental perspective, whether ectoderm plays a role in tooth formation, as proposed in the ‘modified outside-in’ hypothesis, and whether this can explain the presence and distribution of teeth in the oropharynx. The emphasis is on pharyngeal teeth and denticles, as these present a greater challenge to the classical ‘outside-in’ view than oral teeth, which are located close to the mouth entrance.

II. GERM LAYERS CONTRIBUTING TO TEETH AND THEIR ROLE IN TOOTH INDUCTION

Crucial in the discussion about the homology of external odontodes (skin denticles) with internal odontodes (teeth and pharyngeal denticles) is the extent to which the ectoderm penetrates into the oropharynx. According to embryology textbooks, the distribution of ectoderm is limited to the stomodaeal area, whereas the post-mandibular pharyngeal arches are lined by endoderm (Balinsky, 1975; Hildebrand, 1995). Yet, recent studies show that the oral cavity can be covered by endoderm, by ectoderm, or can have a mixed lining, depending on the process of primary mouth formation (Soukup, Horacek & Cerny, 2013; Chen *et al.*, 2017; Minarik *et al.*, 2017) (Fig. 2).

(1) Chondrichthyans

Little attention has been paid to the germ layer origin of chondrichthyan oral teeth. The ectodermal nature of the enamel organ is usually taken for granted, likely because of the close proximity of the dental lamina to the mouth entrance (Hertwig, 1874; Rasch *et al.*, 2016; Fig. 3A, B). At the time the first oral teeth form, the epidermis is clearly stratified, and the basal epithelial layer appears to have a prominent role in early morphogenesis of the enamel organ [illustrated by Rasch *et al.*, 2016 for the spotted catshark (*Scyliorhinus stellaris* and *S. canicula*)].

Few studies have been conducted on the development of pharyngeal denticles in sharks, let alone on their germ layer origin (Fig. 3C, D). Cook & Neal (1921) searched for

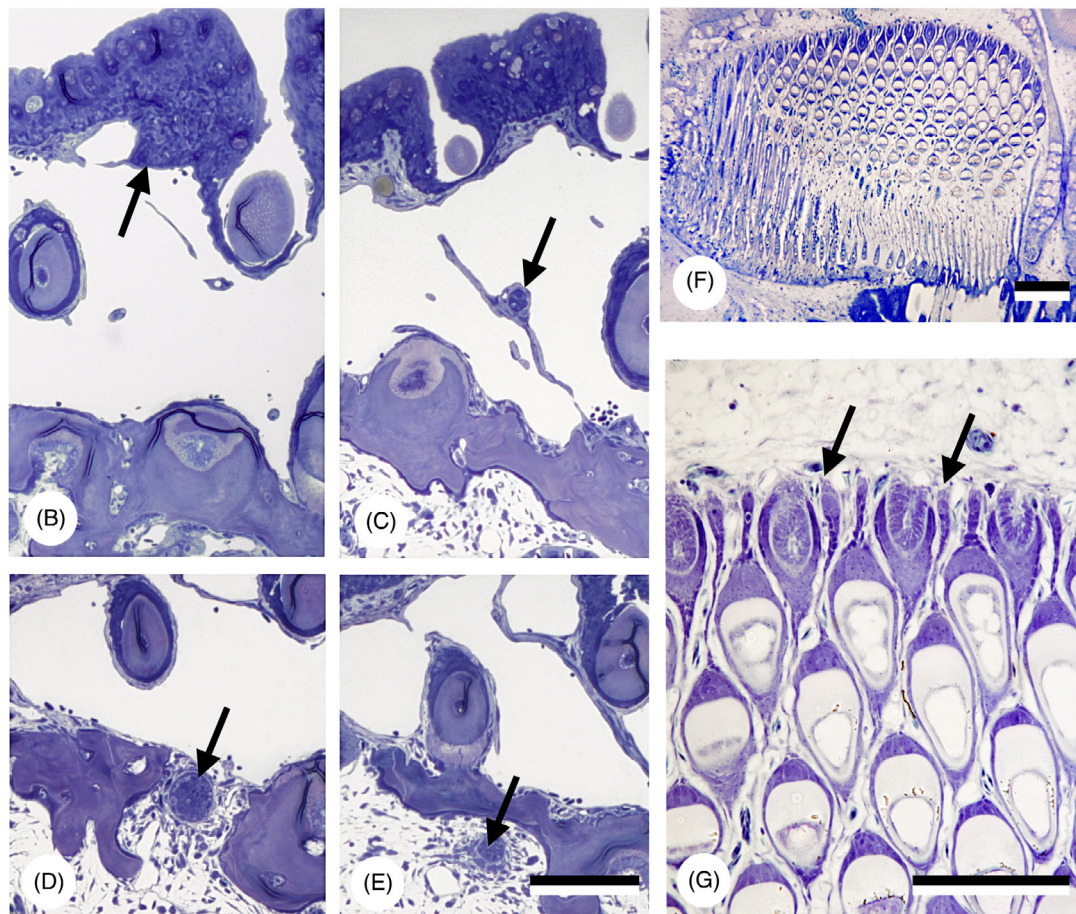
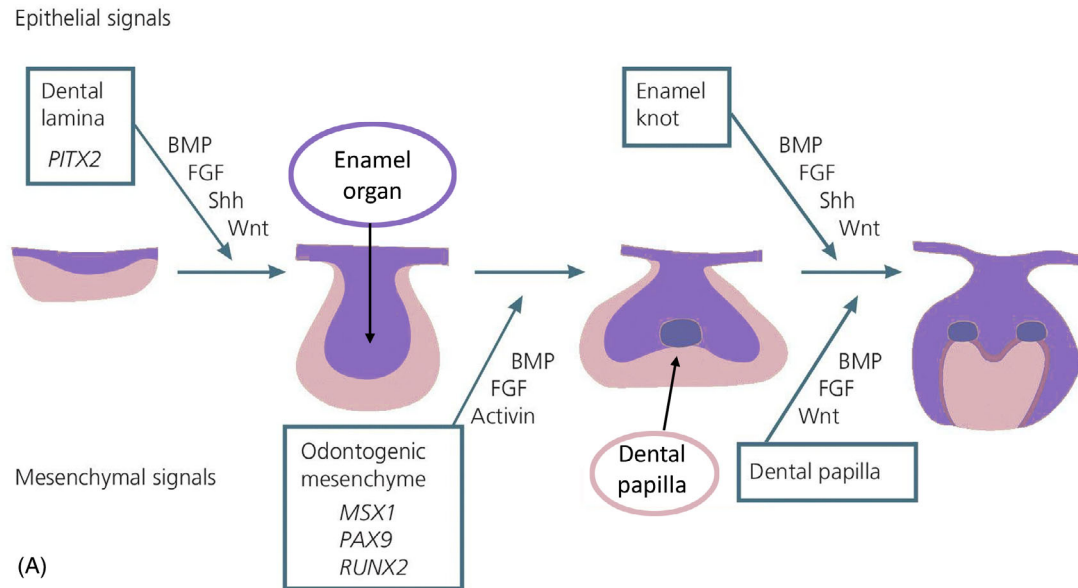


Fig 1. Epithelial primacy in tooth formation. (A) Schematic representation of tooth formation in mammals and transcription factors and signalling molecules involved (modified from Koch, Thesleff & Kreiborg, 2017); purple: epithelium/enamel organ; pink: mesenchyme/dental papilla. BMP, bone morphogenetic protein; FGF, fibroblast growth factor; Shh, sonic hedgehog. (B–E) Intramedullary tooth formation in the lower pharyngeal jaw of the cichlid *Hemichromis bimaculatus*, 28 mm standard length (SL). Successive cross sections through the epithelial strand (arrow) giving rise to the enamel organ of the replacement tooth, showing its origin (B), its course across the large vascular cavity (C), its penetration through the jaw bone (D) and its distal end within the (Figure legend continues on next page.)

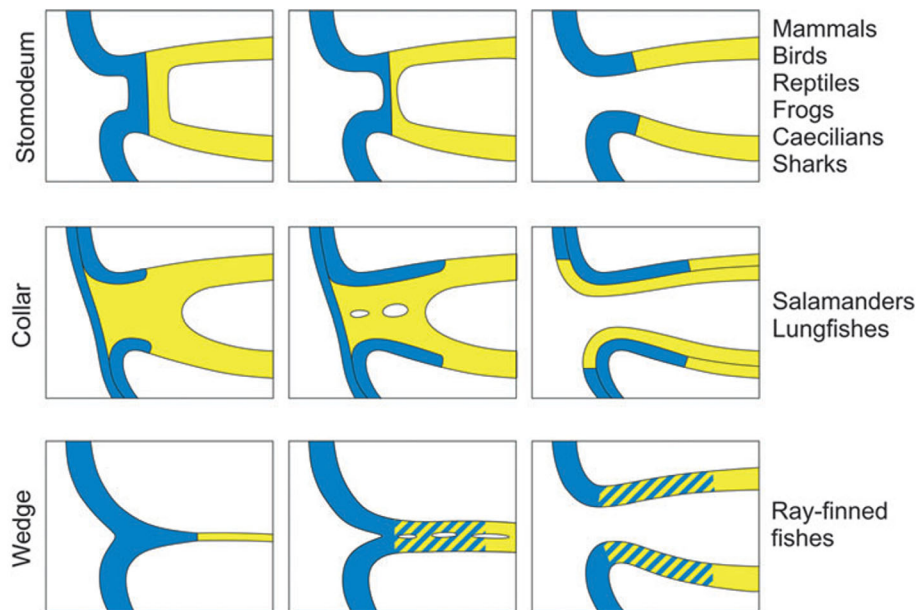


Fig 2. Schematic representation of primary mouth formation in vertebrates [from Soukup *et al.*, 2013, with permission from the authors and publisher]. Yellow, endoderm; blue, ectoderm.

embryological evidence for the origin of the pharyngeal denticles (that they called “pharyngeal placoid scales”) scattered over the surface of the pharynx of the common spiny dogfish (*Squalus acanthias*). Based on histological sections, they concluded that the entire pharyngeal cavity is of endodermal origin. They observed little or no inward migration of ectoderm into the pharynx except in the region of the upper and lower jaws and thus considered pharyngeal denticles to be endodermal (Fig. 3E, F). Interestingly, the early stage of pharyngeal denticle formation is drawn with a multilayered epithelium, an observation whose significance will be discussed later (see Sections II.4 and III). Peyer (1968) illustrated the formation of pharyngeal denticles in the smooth-hound shark (*Mustelus* sp.), insisting on homology with skin denticles. He showed that they form from a stratified epithelium, but did not assign the epithelium to a particular germ layer (Fig. 3G, H). Regrettably the species which, over the past years, has become a popular chondrichthyan model for developmental studies, the lesser spotted dogfish (*Scyliorhinus canicula*), possesses no pharyngeal denticles.

(2) Osteichthyans: actinopterygians

Actinopterygians possess teeth whose distribution can extend deep into the alimentary canal. Teeth can be found associated with the hyoid or any of the branchial arches, even down

into the oesophagus (Gilchrist, 1922; Isokawa *et al.*, 1965). Thus, actinopterygians represent a particularly interesting group to investigate the germ layer origin of the teeth (see e.g. Stock, 2001; Berkovitz & Shellis, 2016).

(a) Non-teleost actinopterygians

While the development of the teeth in basal (non-teleost) actinopterygians has been studied in some detail [e.g. bichir, *Polypterus senegalus* (Sire *et al.*, 2002); bowfin, *Amia calva* (Degener, 1924); gar, *Lepisosteus oculatus* (Sasagawa *et al.*, 2019); Siberian sturgeon, *Acipenser baeri* (Gisbert *et al.*, 1998)], the germ layer origin of the enamel organ has usually not been addressed. Still, endoderm forms substantial parts of the orofacial epithelia in these taxa, and that includes contributions to the teeth (Minarik *et al.*, 2017). It can therefore be expected that the enamel organs of many (if not all) teeth are endoderm-derived. Interestingly, at least in bichir and sturgeon, the odontogenic pharyngeal epithelium is clearly stratified (Fig. 4A, B).

(b) Teleosts

The first detailed study on germ layer participation in teleost teeth comes from Edwards (1929), working on common carp (*Cyprinus carpio*). The carp is a close relative of the zebrafish

(Figure legend continued from previous page.)

medullary cavity of the jaw bone (E). Only few mesenchymal cells have condensed around the distal end. Total length of the epithelial strand is approximately 300 μm . Scale bar for B–E = 100 μm . (F, G) Section through the left premaxilla of a scraping loriciid catfish, *Ancistrus cf. triradiatus*, of 33.5 mm SL. Note virtual absence of mesenchymal cells at the distal end of each successional lamina (arrows). Scale bars: F = 100 μm ; G = 50 μm .

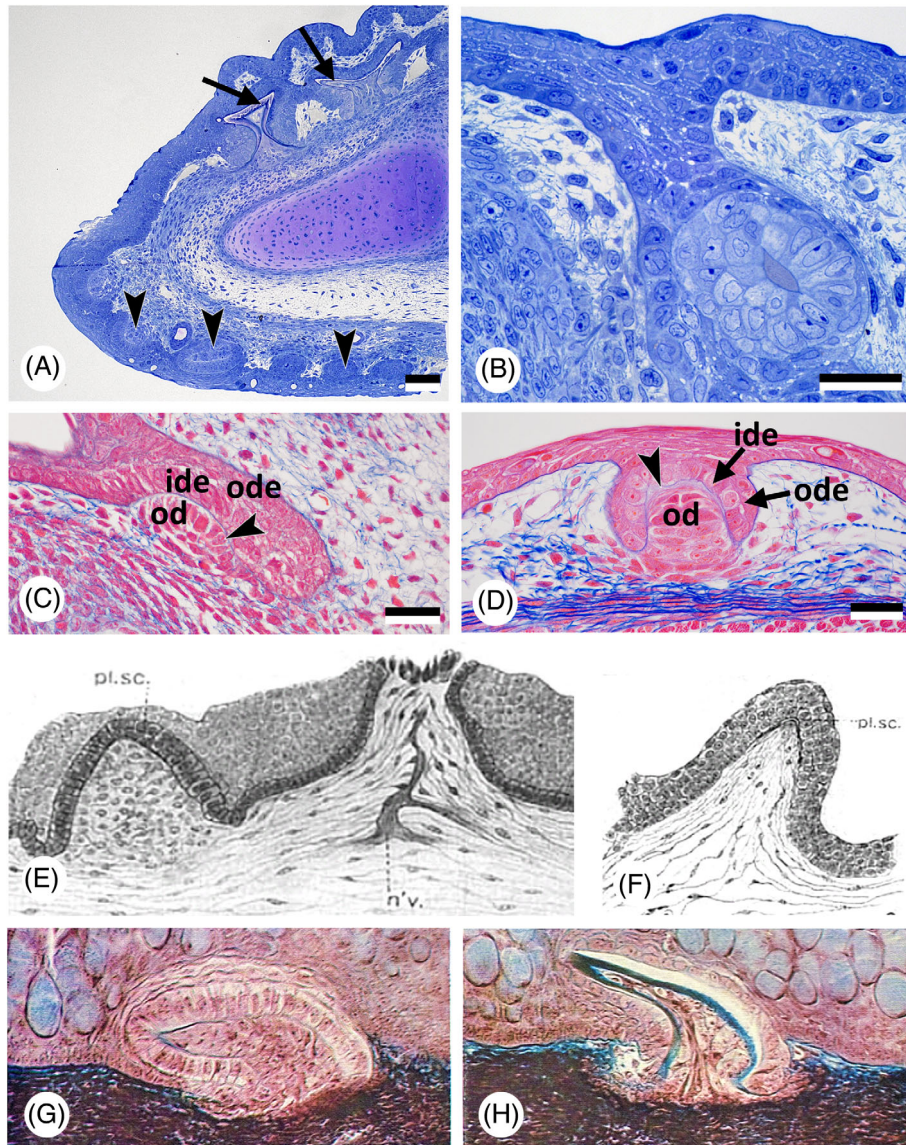


Fig 3. Oropharyngeal epithelium, teeth, and dermal denticles in chondrichthyans. (A) *Scyliorhinus canicula*; sagittal section of the lower jaw of a 85 mm embryo, showing skin (= dermal) denticles (arrowheads), and the dental lamina with developing teeth (arrows). Scale bar = 100 µm. (B) *Scyliorhinus canicula*; larger magnification of the early dental lamina with a developing tooth in a 85 mm embryo. Scale bar = 20 µm. (C, D) *Scyliorhinus canicula*; sagittal section of a tooth anlage in the lower jaw with the invading dental lamina (C) compared to the anlage of a dermal denticle (D) in the same 50 mm embryo. Outer (ode) and inner dental epithelium (ide), the basal lamina of the enamel organ (arrowhead) and the mesenchymal condensation that will give rise to the odontoblasts (od) characterise the anlagen of teeth and dermal denticles. Scale bars = 25 µm. (E, F) *Squalus acanthias*; two stages of development of a 'placoid scale' (from Cook & Neal, 1921). n.v. = nerve; pl. sc. = placoid scale. (G, H) *Mustelus* sp.; two stages of development of a 'mucous membrane denticle' (from Peyer, 1968).

(*Danio rerio*), today a common experimental model for vertebrate development and genetics. As is typical for cyprinids, both species lack oral teeth. Their pharyngeal teeth are restricted to the last (seventh) pharyngeal arch, or fifth branchial (gill) arch. Edwards (1929) drew the following conclusions: (i) the superficial layer of the epidermis invades the gill slits and spreads out over the entire pharynx. Edwards illustrated this for the first gill slit but inferred it to be similar

for the other gill slits. As a consequence, (ii) the pharyngeal epithelium is composed of two layers: a deep, columnar layer, of endodermal origin, and a superficial flattened layer, of ectodermal origin. (iii) The enamel organs of the teeth derive from the deep layer and are therefore of endodermal origin. Nearly a century later, Rosa *et al.* (2019), and Oralová *et al.* (2020), using transgenic zebrafish lines, largely confirmed Edwards' results on carp. The pharyngeal epithelium

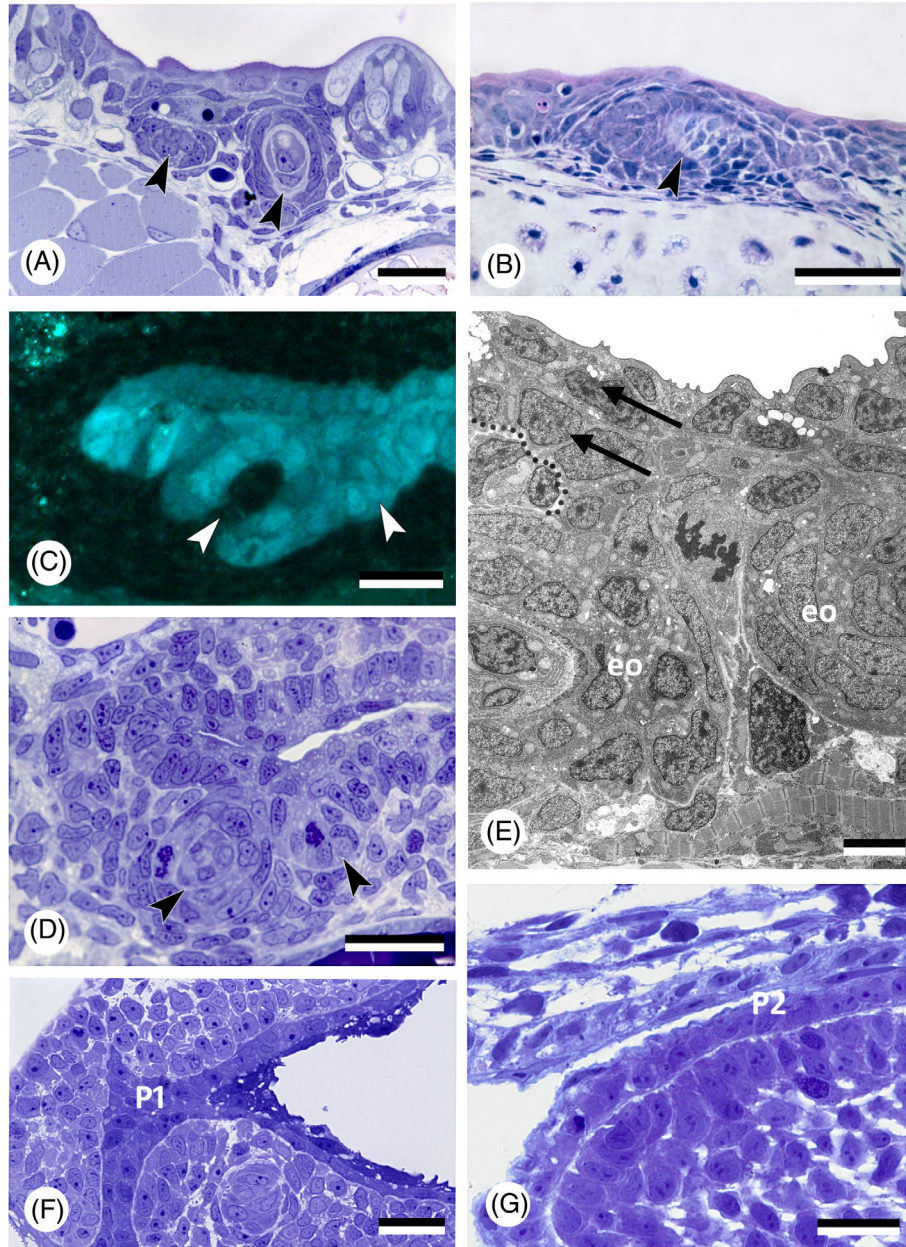


Fig 4. Oropharyngeal epithelium and teeth in actinopterygians. (A) *Polypterus senegalus*; pharyngeal epithelium and forming tooth germs (arrowheads) in an 18 mm larva. Scale bar = 20 μm . (B) *Acipenser ruthenus*; tooth germ (arrowhead) in the buccopharyngeal epithelium in a 15 mm larva. Scale bar = 50 μm . (C, D) *Danio rerio*, cross section of a 56 h post-fertilisation (hpf) embryo at the level of the first forming teeth (arrowheads), shown in a transgenic (*sox17:egfp*) line (C), and corresponding semithin, toluidine blue-stained section of a wildtype fish (D). Scale bars = 20 μm . (E) *Danio rerio*, transmission electron microscopy (TEM) image of the bilayered pharyngeal epithelium (two arrows, delimited by dotted line) of a 72 hpf embryo at the level of the first two teeth (eo, enamel organ). Scale bar = 5 μm . (F) *Hemichromis bimaculatus*, cross section of the oropharynx of a 1 day post-hatching (dph) (4.0 mm total length) specimen at the level of pouch 1 (P1). Scale bar = 20 μm . (G) *Oncorhynchus tshawytscha*, sagittal section of the pharyngeal epithelium in an unhatched embryo 17 days post-fertilisation (dpf), at the level of the (open) pouch 2 (P2). Scale bar = 20 μm .

in zebrafish is bilayered and the enamel organs are derived from the basal layer only, that is endoderm (Fig. 4C–E), observations also fitting with those of Waterman & Kao (1982). Rosa *et al.* (2019) also confirmed the invasion

of the pharyngeal pouches (i.e. the structures in which the gill slits form, see Section III.1) by the superficial epidermal layer, or periderm (for a discussion on the germ layer origin of the periderm, see Section III.3). In contrast to Edwards'

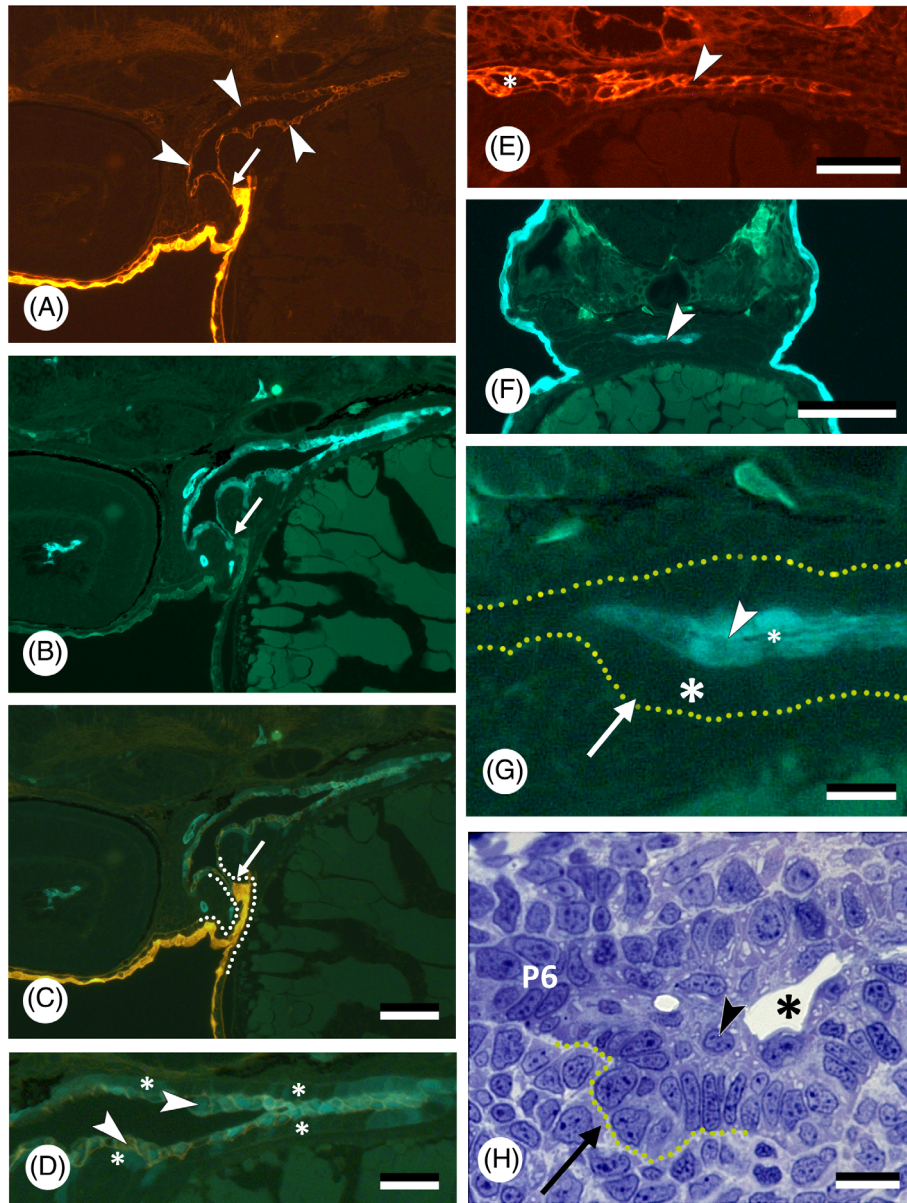


Fig 5. Germ layer contribution to oropharyngeal epithelium and teeth in zebrafish, *Danio rerio*. (A–C) Sagittal section of a double-transgenic (*krt4:tomatoCAAX;sox17:egfp*) 55 h post-fertilisation (hpf) embryo showing periderm having partially invaded pouch 2 (intense orange), and connecting to periderm-like cells investing the oropharynx (red, indicated by white arrowheads, shown also in E); boundary indicated by a small white arrow; both populations do not show further mixing. Micrographs are shown in the red (A) and green channel (B), and as overlay (C). Dotted line in C marks the outline of pouch 2. Scale bar for A–C = 20 μ m. (D) Magnification of the pharynx lining shown in C; periderm-like cells (arrowheads) express both *krt4* and *sox17* and cover endoderm, in which *sox17* is downregulated (asterisks). Scale bar = 20 μ m. (E) Sagittal section of a transgenic (*krt4:tomatoCAAX*) 55 hpf embryo showing periderm-like layer (arrowhead). A lumen has appeared rostrally between the two periderm-like layers (asterisk). Scale bar = 20 μ m. (F) Cross section of a 60 hpf Tg(*krt4:gfp*) embryo at the level of the forming pharyngeal dentition; periderm-like layer indicated by an arrowhead. Scale bar = 100 μ m. (G) Magnification of F, showing the placode of the first pharyngeal tooth (arrow). This develops from the unlabelled basal (endodermal) layer (large asterisk), while being covered by a *krt4*-positive superficial layer (arrowhead). A dotted line delimits the pharyngeal epithelium; a lumen starts to appear between the two layers of periderm-like cells (small asterisk). Scale bar = 10 μ m. (H) Toluidine blue-stained semithin cross section, showing placode stage of first pharyngeal tooth formation corresponding to G (tooth placode indicated by a dotted outline). Note superficial periderm-like layer (arrowhead) with forming pharyngeal lumen (asterisk). Laterally, the pouch (pouch 6, P6) is still bilayered. Scale bar = 10 μ m.

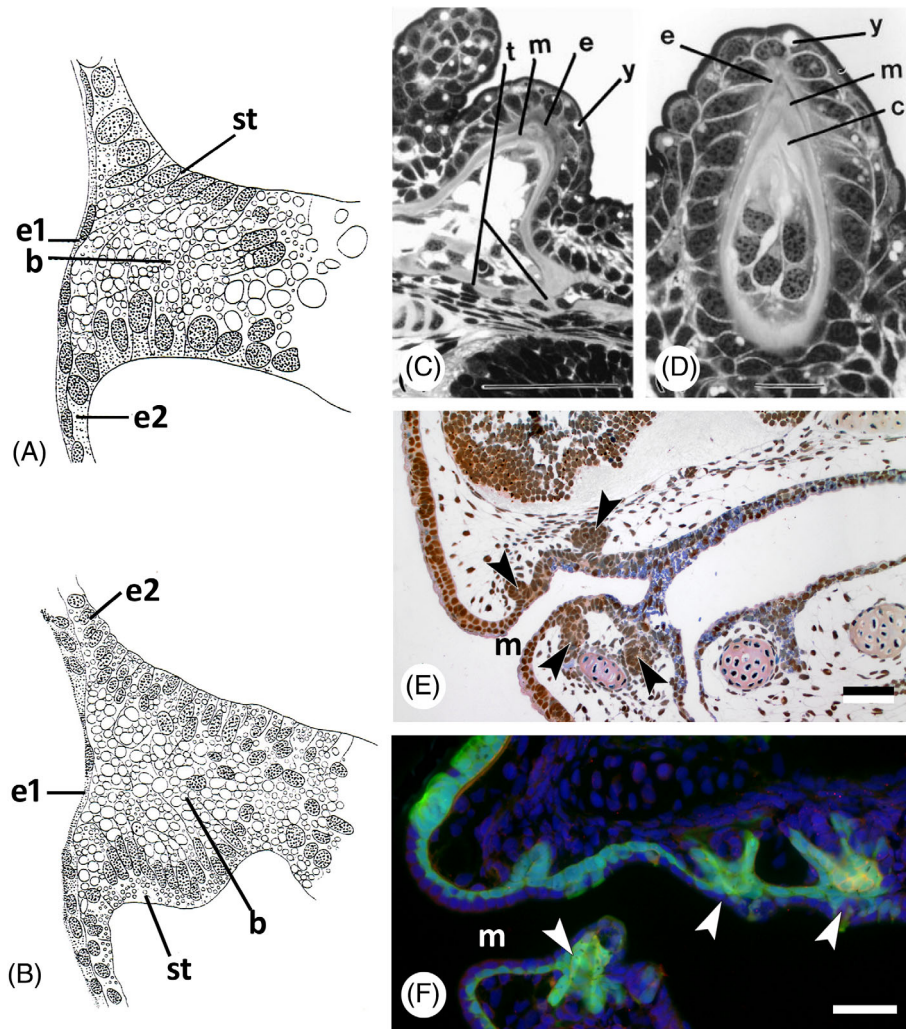


Fig 6. Germ layer contribution to oropharyngeal epithelium and teeth in lungfish and amphibians. (A) *Lepidosiren paradoxa*; drawing of a sagittal section of a stage 30 specimen (after Kerr, 1903); drawing mirrored to show anterior to the left. b, yolk-laden cells in the buccal cavity; e1, superficial ectodermal layer; e2, basal ectodermal layer; st, lining epithelium of buccal cavity arising *in situ*. (B) *Ambystoma mexicanum*; drawing of a similar section to A, embryo of 7.5 mm length (after Kerr, 1903; drawing mirrored to show anterior to the left). (C, D) *Neoceratodus forsteri*, cusp of pterygopalatine tooth plate [from Kemp, 2002a, with permission from the author and publisher]. c, circumdenteonal dentine; e, enamel; m, mantle dentine; t, trabeculae; y, yolk remnants. Scale bars: C = 100 μm ; D = 20 μm . (E) *Ambystoma mexicanum*; sagittal section of a stage 43 embryo; compare with F. Tooth buds indicated by arrowheads. m, mouth opening. Scale bar = 50 μm . (F) *Ambystoma mexicanum*; transgenic embryo of stage 43 with green fluorescent protein (GFP) labelling in the ectoderm [modified from Soukup *et al.*, 2008, courtesy of the authors]. Arrowheads: tooth buds arising in ectoderm; m, mouth opening. Scale bar = 50 μm .

results on carp, however, they established that periderm invasion in zebrafish is arrested halfway into each pouch (Fig. 5A–C). Instead of spreading throughout the oropharynx, the periderm joins another, endogenous population of cells covering the midline endoderm. The latter cells, designated as ‘periderm-like’ cells, are highly similar to the invading periderm cells and cannot be distinguished from these once both populations have joined (Fig. 5A–E). Apart from their similar morphology, both periderm and periderm-like cells (i) express periderm markers (*krt4*, and *zc1044*), (ii) develop microridges that otherwise characterise periderm

cells, and (iii) both cell populations merge imperceptibly, suggesting compatible adhesion; there is no further mixing (Rosa *et al.*, 2019; Fig. 5A–C). The periderm-like cells furthermore display ultrastructural features that are distinctive from the endodermal cells on which they rest. Apart from size and shape, they notably differ from the endodermal cells by their electron-dense cytoplasm (Rosa *et al.*, 2019). The layer of periderm-like cells expands posteriorly along the midline until the oesophageal–intestinal border, where it ends abruptly. Along its way it joins with periderm invading each of the pouches. Like Edwards (1929), Rosa *et al.* (2019)

observed that the presence of periderm or periderm-like cells precedes lumenisation of the pouches and of the pharynx, respectively. Whether zebrafish, as a miniaturised species (Witten *et al.*, 2017), truly differs from carp in that periderm invasion is arrested, and the periderm instead joins a periderm-like layer that expands along the midline, still needs to be established. Significantly, Oralová *et al.* (2020) showed that zebrafish teeth form from the basal, that is endodermal, layer, but only when this is covered by the periderm-like layer. Thus, a layer is required for pharyngeal tooth formation that bears similarities to the periderm, or outer epidermal layer (Fig. 5F–H). Whether the periderm-like layer possesses a signalling function remains unclear (Oralová *et al.*, 2020). A mechanical role should also be considered. In mice, the suprabasal layer of the odontogenic epithelium forms a tensile canopy required for the basal layer of the tooth placode to invaginate (Panousopoulou & Green, 2016). Eventually, elucidating the precise role of the periderm-like cells in tooth induction will only be possible through ablation experiments, using, for example, genetically engineered lines (Chen *et al.*, 2011; Eisenhoffer *et al.*, 2017).

Several other teleost species (both basal and derived) have been shown also to possess a bilayered pharyngeal epithelium early in development, with superficial flattened cells covering basal cuboidal cells (Gonzalez, Blanquez & Rojo, 1996; Rosa *et al.*, 2019) (Fig. 4F, G), suggesting that the bilayered condition could be a common teleost character.

(3) Osteichthyans: sarcopterygians

(a) Dipnoans

Kerr (1903) examined the formation of the mouth cavity in the South American lungfish *Lepidosiren paradoxa*, comparing it with mouth formation in *Ambystoma mexicanum* (Fig. 6A, B). He described that the superficial layer of endoderm transformed into a cell layer with ectoderm-like characters, along with breakdown of its yolk content. In this way, a layer of definite epithelium arose, continuous with the external epiblast anteriorly, and with the yolk-laden endoderm posteriorly. Kerr (1903, p. 423) states:

“It is, as it were, as if an influence were spreading inwards from the external epiblast, gradually transforming the original “endoderm” yolk-laden cells into ectoderm like itself. I find no evidence of an actual bodily involution of ectoderm such as is ordinarily associated with the term stomodaeum.”

He concluded (p. 452) that there is

“no invagination of ectoderm to form a true stomodaeum. The epithelium of the buccal cavity is developed *in situ* from the outer layer of the solid anterior portion of the yolk-laden enteric rudiment. ... The tooth germs appear while the mouth region is still without a lumen.”

Development in the Australian lungfish, *Neoceratodus forsteri*, resembles that in *Lepidosiren*: an endodermal plug makes

contact with the stomodaeal plate, upon which the ectoderm disappears. Once a lumen is formed, the lining of the oral cavity is made up of endoderm (Kemp, 2002a). Up to this point, this resembles the situation in the axolotl (see Section II.3b). Interestingly, as described for *Lepidosiren* (Kerr, 1903), the endodermal oral epithelium in *Neoceratodus* differentiates into two layers. The cells of the superficial layer adopt ectodermal characters, like a brush border, or cilia, while their yolk disappears. Only the basal (proximal) layer of the oral epithelium produces the enamel organs (Kemp, 2002a, 2002b) (Fig. 6C, D).

(b) Lissamphibians

The majority of studies that have attempted to identify germ layer contributions to vertebrate teeth used amphibians, in particular urodeles. Anurans start to develop teeth only at metamorphosis, making them less attractive for experimentation (reviewed in Davit-Béal *et al.*, 2007). The mouth region in urodeles is formed by an endodermal plug surrounded by an ectodermal sleeve, the so-called stomodaeal collar (Adams, 1924). This observation was confirmed with the use of transgenic axolotls (Soukup *et al.*, 2008) (Fig. 6E, F).

In a very detailed account, Adams (1924) described the succession of tooth germs developing on the jaws of the axolotl, *Ambystoma* (then called *Amblystoma*) *punctatum*, all of which appear between stages 37 and 39 (her staging system partly derived from Harrison, 1918). Adams concluded that palatine and splenial (= coronoid) teeth possess endodermal enamel organs, while maxillary (= premaxillary), vomerine, and dentary teeth, with one exception, arise entirely within the ectodermal collar. The median pair of dentary teeth has enamel organs of mixed ectodermal–endodermal composition. Germ layer contributions were identified based on histology, in particular the presence of large yolk granules in the endoderm. Adams (1924) furthermore saw a similarity in the sequence of development of the teeth in the axolotl (first the endodermal, then the ectodermal teeth) and in dipnoans. She attributed this to the presence, of “a thick endodermal component in the oral plate” in both groups (p. 320). Based on the results of extirpation and transplantation experiments, Adams suggested that the formation of an ectodermal collar around the endodermal plug is essential for the development of maxillary, vomerine, and dentary (i.e. ectodermal) teeth. The endodermal splenial and palatine tooth germs were constant in their time of appearance in the stomodaeal regeneration experiments, despite variable appearance of the ectodermal collar (Fig. 7A, B). According to Adams, no tooth germs formed in stomodaeal ectoderm transplanted to the gill region. However, homoplastic ventral body wall ectoderm transplanted to the stomodaeal region could apparently initiate tooth germ development. The conclusion that one can draw from Adams’ studies is that normal ectoderm–endoderm contact (as in a one-layered ectodermal sleeve around an endodermal plug) is required for ectodermal tooth formation. This requirement appears to be less stringent for endodermal teeth, which appeared to develop

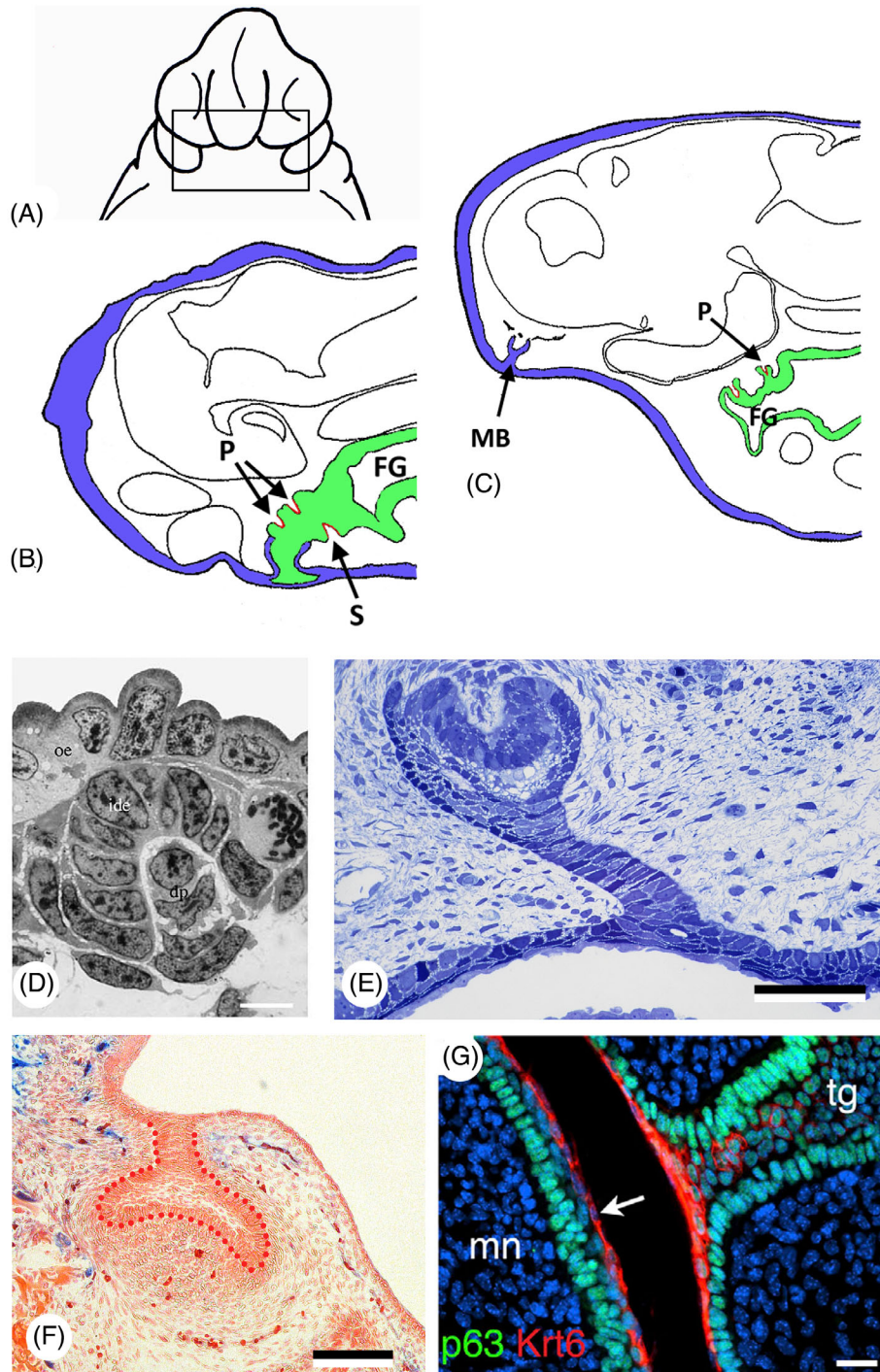


Fig 7. Germ layer contribution to oropharyngeal epithelium and teeth in amphibians and amniotes. (A) *Ambystoma punctatum*, extirpation of the ectoderm of a stage 24 embryo, ventral view (redrawn after Adams, 1924). (B) *Ambystoma punctatum*; sagittal section of a stage 39 embryo after extirpation of the ectoderm; teeth in the ectoderm (blue) are reduced or absent, while endoderm (green) has given rise to splenial (S) and palatine (P) tooth germs (red); FG, foregut (adapted after Adams, 1924). (C) *Ambystoma punctatum*; sagittal section of a stage 45 embryo showing a maxillary bulb (MB) separated from any connection with the foregut; no contact between ectoderm (blue) and endoderm (green), yet a palatine tooth (P) is present (red); FG, foregut (adapted after Adams, 1924). (D) *Pleurodeles waltl*, first-generation tooth, stage 36. dp, dental papilla; ide, inner dental epithelium; oe, oral epithelium (from Davit-Béal *et al.*, 2007, with permission from the publisher). Scale bar = 10 μm . (E) *Chalcides viridanus*; cross section of the dental lamina on the upper jaw of a 30 mm embryo. Scale bar = 50 μm . (F) *Mus musculus*; frontal section through a cap-stage molar tooth germ of a Sox17-2A-iCre/R26R mouse, with the epithelium outlined with red (Figure legend continues on next page.)

more autonomously (Fig. 7B). Adams (1924) nevertheless also reported the presence of ‘maxillary bulbs’, consisting of ectoderm alone, and which contained tooth germs. To her, this indicated some potentiality for independent invagination without endodermal contact (Fig. 7C). In a follow-up study, Adams (1931) removed endoderm from axolotl embryos with closing or closed neural folds (i.e. prospective neural crest mesenchyme). Teeth with endoderm-derived enamel organs were reduced. Their number nevertheless suggested that a re-established ecto–endodermal contact may have promoted the development of such teeth. Teeth with ectoderm-derived enamel organs were also reduced (although no ectoderm was removed), leading Adams to conclude that these teeth need an interaction with the endoderm.

In the years that followed, several authors critically assessed Adams’ results, drawing from their own studies on axolotl. Based on extensive series of experiments, both Ströer (1933) and Holtfreter (1935) concluded that endoderm does not participate in tooth formation, but is nevertheless required. Likewise, Sellman (1946) concluded that all the axolotl enamel organs are most probably ectodermal, a finding we know now to be incorrect (Soukup *et al.*, 2021). More importantly, however, his studies revealed that three tissue layers are required for tooth formation: neural crest, mouth ectoderm and stomodaeal endoderm. Sellman did not rule out the possibility of endoderm-derived enamel organs but insisted that ectoderm would be necessary for endodermal teeth to form (p. 112). Sellman (1946) agreed with Adams (1924) in that extirpation of mouth ectoderm causes tooth reduction, especially of anterior teeth. Still, he contested Adams’ interpretation that the unaffected posterior teeth were endodermal in origin. Sellman also noticed an abnormal formation of the visceral skeleton in these experiments and concluded that tooth reduction was related to disturbance of the neural crest-derived ectomesenchyme. In contrast to Sellman (1946), but in agreement with Adams (1924), de Beer (1947) concluded that enamel organs can be endodermal or ectodermal.

Rather than using transplantation experiments, Wilde (1955) removed parts of the cranial neuroepithelium in *Ambystoma maculatum* prior to the migration of neural crest cells. The epithelium was explanted into a nutrient medium, alone or in combination with stomodaeal ectoderm, foregut endoderm or endomesoderm. He observed teeth only when both stomodaeal ectoderm and foregut endoderm were present in the explants, further supporting the idea that all three tissue layers are required for teeth to be initiated.

From his studies on a urodele, the Spanish newt (*Pleurodeles waltlii*), Chibon (1966) concluded that the neural crest-derived odontoblasts exert an inductive action upon the epithelium,

which can be either ectodermal (in normal conditions) or endodermal. A few years later, Chibon (1970) transplanted the stomodaeal ectoderm of ^3H -thymidine-labelled neurula-stage embryos of *Pleurodeles waltlii* onto non-labelled siblings. Based on 22 embryos that developed normally, he observed that anterior tooth germs are derived from ectoderm, posterior ones from endoderm, while some contained both labelled and non-labelled ameloblasts, which he considered indicative of a mixed origin.

Cassin & Capuron (1979) examined the formation of buccal structures both by intrablastocelic transplantation and by *in vitro* culture, using stage 15 and 18 neurulae of the urodele *Pleurodeles waltlii*. They obtained teeth when neural fold was associated with stomodaeal endoderm, and claimed that stomodaeal ectoderm is incapable on its own of forming teeth, even when associated with neural fold. There are, however, a number of problems associated with this paper, both at the experimental and analytical level. For example, teeth were reported to develop in transplants that had no neural fold (transplants with stomodaeum alone, that is ectoderm and endoderm, with or without tissues underlying the median neural fold). At best, these results are in line with those of Adams (1924), suggesting that endoderm can form teeth alone with neural fold, while ectoderm needs an interaction with endoderm.

To avoid any bias in their study of neural crest potential, Graveson, Smith & Hall (1997) explanted neural fold segments of *Ambystoma mexicanum* along with both stomodaeal ectoderm and pharyngeal endoderm as “inductive epithelia” to elicit tooth formation. In a study primarily aimed at investigating taste buds, Barlow & Northcutt (1995) labelled presumptive cephalic endoderm in *Ambystoma* sp. with the carbocyanine dye, DiI, shortly after the onset of gastrulation and found teeth in areas of labelled cells, suggesting an endodermal origin of the enamel organs.

Soukup *et al.* (2008) re-investigated germ layer origin of enamel organs in the axolotl by means of ectodermal transplantations, taking advantage of transgenic axolotls expressing GFP ubiquitously. The experiments demonstrated conclusively that the enamel organs can be of ectodermal, endodermal, or mixed origin. This confirmed the observations of Adams (1924) and de Beer (1947) based on histology, and of Chibon (1970), based on isotope labelling. Whether these results also apply to other urodele species is currently not known.

In conclusion, while it is now firmly established that the enamel organ in urodeles can be of ectodermal, endodermal, or mixed origin, most studies also indicate that both ectoderm and endoderm are required in conjunction with neural crest-derived mesenchyme to make teeth. Moreover, it has

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dots; there is no contribution of the endoderm (blue) [modified from Rothova *et al.*, 2012, courtesy of the authors]. Scale bar = 100 μm . (G) *Mus musculus*; immunostaining for keratin 6 (Krt6, red) and tumor protein p63 (p63, green) in a E15.5 embryo. Krt6 is expressed uniformly in the periderm superficial to the tooth germ (arrow). mn, mandible; tg, tooth germ (from Peyrard-Janvid *et al.*, 2014, with permission from the publisher). Scale bar = 20 μm .

been recently revealed that the entire oral dentition in the axolotl, comprising both the outer and inner tooth arcs, develop from the common competent zone alongside the ectoderm–endodermal boundary (Soukup *et al.*, 2021). Yet, two reported experiments on axolotl suggest that endoderm can form teeth in the absence of ectoderm. First, the

extirpation of ectoderm did not affect the formation of endodermal teeth (Adams, 1924), and second, transplants or cultures of stomodaeal endoderm alone, together with neural fold, could generate teeth – ectoderm was not required (Cassin & Capuron, 1979). At first sight, this violates the need for ectoderm as stipulated in the ‘modified outside-in’

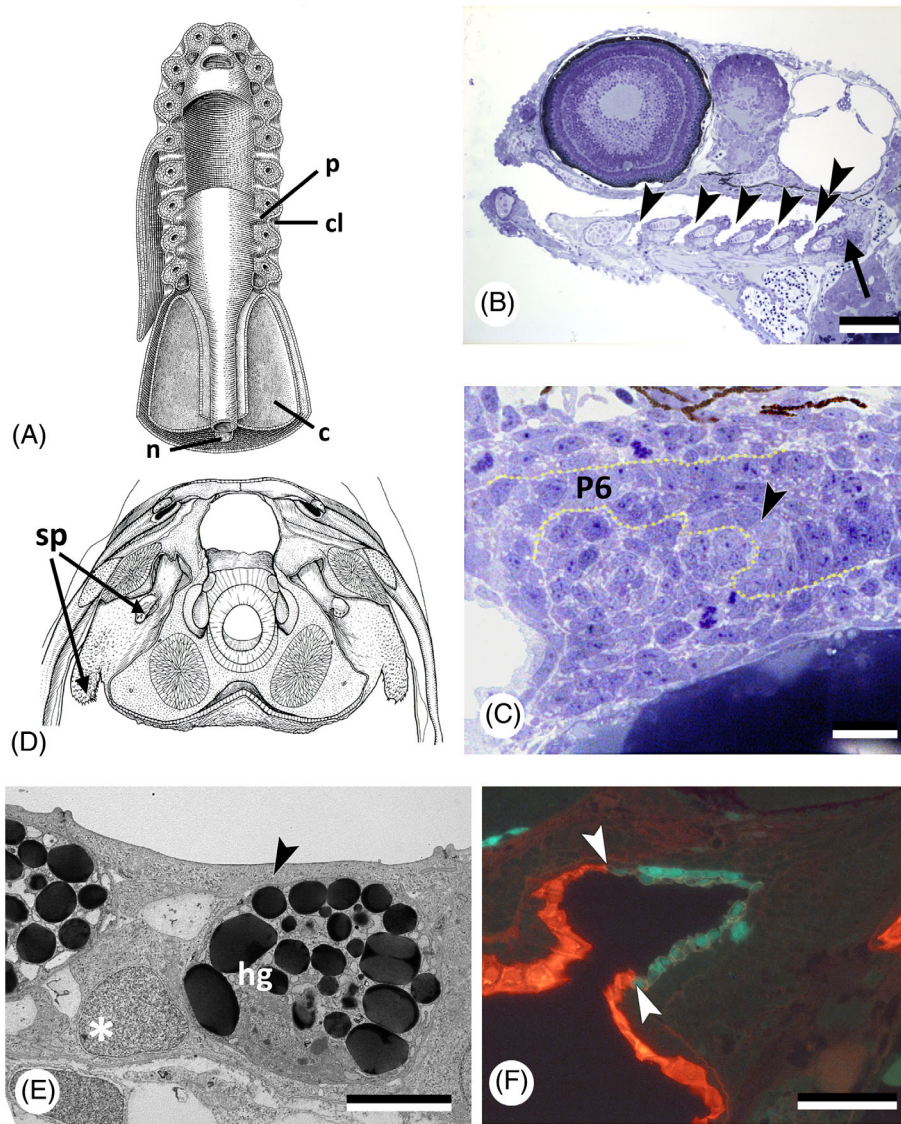
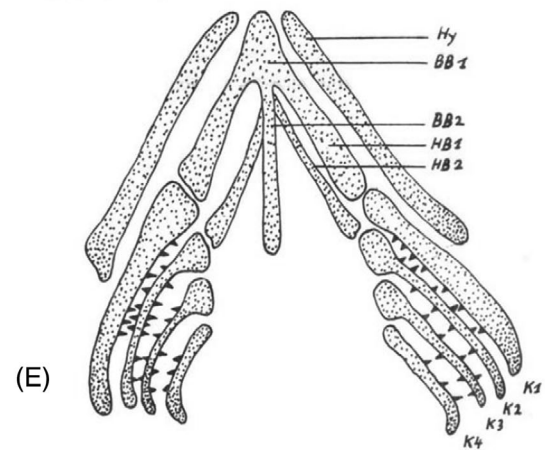
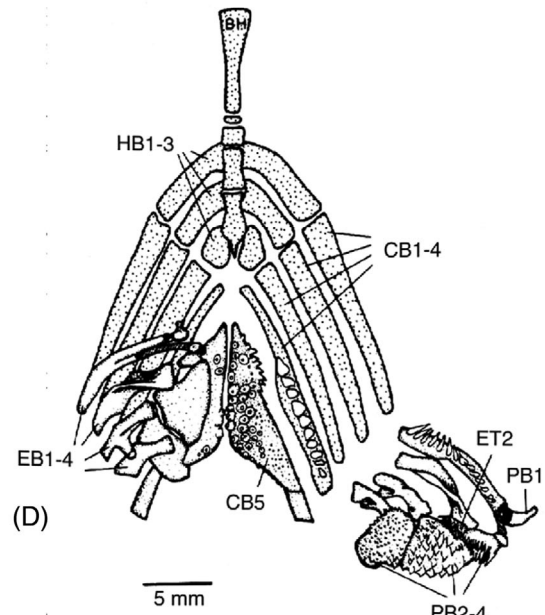
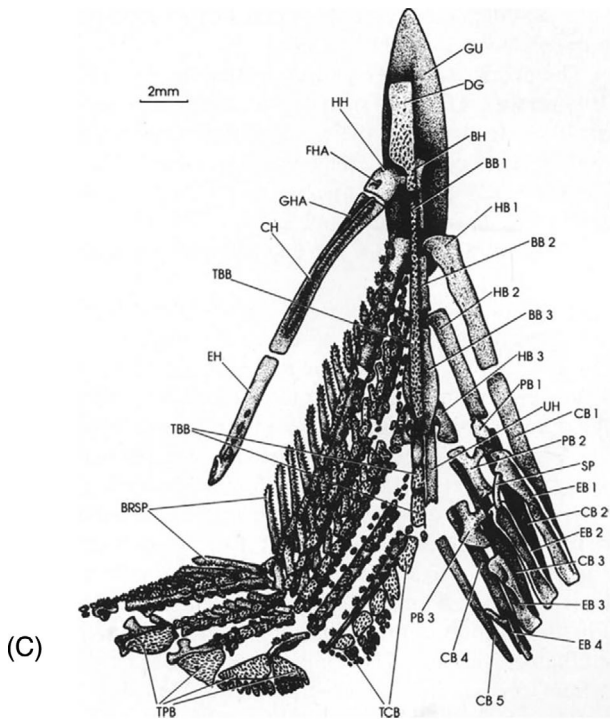
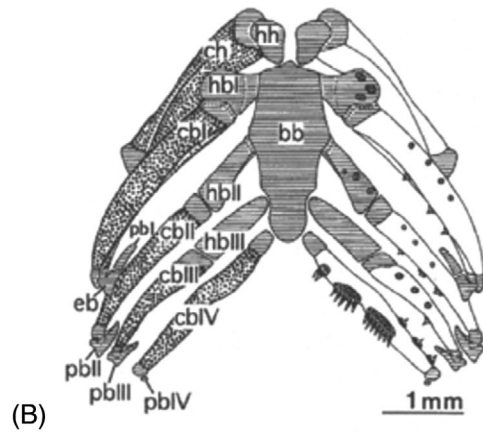
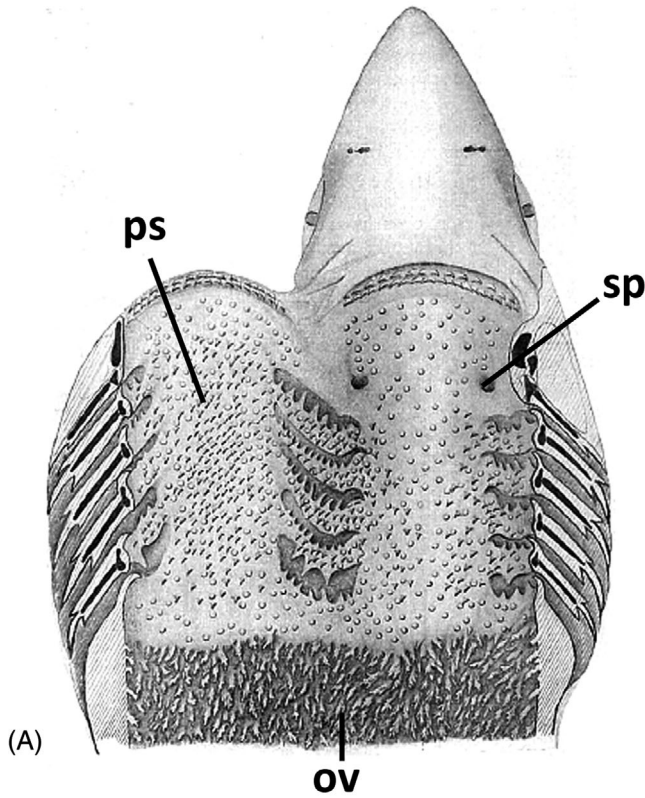


Fig 8. Pouch–cleft contacts and tooth formation. (A) Diagrammatic ventral view of dissected branchial region of an ‘idealised’ primitive craniate. c, coelom; cl, cleft; n, notochord; p, pouch [after Bjerring, 1977, with permission from the author and publisher]. (B) *Danio rerio*; sagittal section showing the open pouches (gill slits) in a 96 h post-fertilisation (hpf) specimen (arrowheads); pharyngeal teeth (arrow) are associated with the last pouch (pouch 6, double arrowhead). Scale bar = 100 µm. (C) *Danio rerio*; cross section at the level of the forming pharyngeal dentition in a 50 hpf specimen. Note connection of the tooth-forming epithelium (arrowhead) with that lining pouch 6 (P6). Dotted line follows basal lamina, delimiting the pharyngeal epithelium. Scale bar = 10 µm. (D) *Latimeria chalumnae*; teeth formed in the spiracular pouch (sp); based on a photograph of a transverse section at the level of the intracranial articulation (modified after Millot & Anthony, 1958, with permission from the publisher). (E) *Oryzias latipes*; transmission electron microscopy (TEM) picture of a cross section of the pharyngeal epithelium in a 7 days post-fertilisation (dpf) embryo, showing that a flattened cell layer (arrowhead) covers the hatching gland cells (hg) and basal endodermal layer (asterisk) of the pharyngeal epithelium. Scale bar = 5 µm. (F) *Danio rerio*; sagittal section through the mouth entrance of a 55 hpf Tg(*krt4:tomatoCAAX*; *sox17:egfp*) embryo, periderm (red) has partially invaded the mouth (boundary indicated by arrowheads); endoderm is green. Scale bar = 50 µm.



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hypothesis. We will return to these findings in Section VI. Importantly, the urodele enamel organ develops from the basal layer of a stratified epithelium (Fig. 7D).

(c) *Sauropsids*

Data on sauropsids are scarce, given that reptiles are not easily accessible for experimental manipulation, and given that all modern birds lack teeth. In the absence of studies using labelling techniques, enamel organs in the different lineages have been assigned to the ectoderm (e.g. snakes; Buchtová *et al.*, 2008), likely because teeth in reptiles are restricted to the oral region (Richman & Handrigan, 2011). Pythons, however, have two distinct types of dental development that appear to be dependent on the degree to which sonic hedgehog (*Shh*) expression is maintained in the oral epithelium (Buchtová *et al.*, 2008). Whether this reflects differences in germ layer origin of these teeth is unknown. Data on the possible stratification of the odontogenic epithelia are even more scarce. At least in the scincid lizard *Chalcides viridanus*, the first tooth placode derives solely from the basal layer of the stratified buccal epithelium (Delgado *et al.*, 2005) (Fig. 7E). Kollar & Mina (1991) transplanted oral epithelium from mice, which is of ectodermal origin (see Section II.3d), onto the mandibular mesenchyme of chickens and generated teeth. Perhaps this experiment showed the undisputed odontogenic potential of oral epithelium of mice rather than a hidden odontogenic potential of chicken. However, Mitsiadis, Caton & Cobourne (2006) transplanted mouse neural folds into chicken and obtained tooth anlagen which suggests a potential of the oral epithelium of chicken to generate an enamel organ.

(d) *Mammals*

It has long been thought that the posterior part of the mammalian mouth is covered with endodermal epithelium, and the anterior part with ectoderm. Thus, incisor and molar teeth could have a different germ layer origin. Imai, Osumi & Eto (1998) used an endodermal cell-tracing system in the rat with a recombinant adenovirus, but could only demonstrate that tooth germs form in ectoderm *adjacent* to labelled endodermal cells (foregut endoderm); there were no functional studies involved. Ohazama *et al.* (2010) showed that the expression of

five endodermally expressed genes extends anterior to the rostral extent of pharyngeal endoderm cells, into what is considered to be oral ectoderm. They concluded that molar (but not incisor) teeth develop from epithelium that shares molecular characteristics with pharyngeal endoderm. The issue was finally settled by Rothova *et al.* (2012). Using Sox17-2A-iCre/Rosa26 reporter mice, their study clearly demonstrated that endoderm does not contribute at any stage to tooth development, including not to molar development. The odontogenic epithelium was identified unequivocally as ectodermal (Rothova *et al.*, 2012) (Fig. 7F). Interestingly, during mouse embryonic development, an initially single-layered ectoderm stratifies to produce an outer layer of flattened periderm cells (Richardson *et al.*, 2014). Whether the periderm has a function in tooth development has not been investigated, but its appearance in mice from E10.5 onwards (Richardson *et al.*, 2014), that is just before the onset of tooth formation, warrants such a study (Fig. 7G).

(4) Conclusion

The general conclusion drawn from the studies reviewed above is that, depending on taxon and position of the teeth, enamel organs can derive from ectoderm, endoderm, or both. Thus, several authors have put the discussion about germ layer origin of the enamel organs to rest (Chibon, 1970; Fraser *et al.*, 2010; Donoghue & Rücklin, 2016; Calamari *et al.*, 2018). Must we then accept that specificity of the germ layers is not a stringent criterion for homology as is sometimes assumed? Hall (1998) argues that homologous structures need not, and often do not, arise from the same germ layer. Coincidentally, teleost scales and fin rays, considered odontode derivatives (Huyseune & Sire, 1998) and, like teeth, components of the dermal skeleton, form a prime example. Elements that cover the postcranial region derive from mesodermal mesenchyme, elements in the head region from neural crest-derived mesenchyme (Lee, Thiery & Carney, 2013; Mongera & Nüsslein-Volhard, 2013). Common to scales, fin rays and odontodes, nevertheless, is their intimate relationship with ectodermal epithelium (Sire & Huyseune, 2003), the latter serving as an important signalling partner for the underlying mesenchyme (e.g. Aman, Fulbright & Parichy, 2018). Thus, it may well be possible that ectoderm is also required in some (hidden) form

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Fig 9. Distribution of pharyngeal teeth. (A) *Squalus acanthias*; adult pharynx. ov, oesophageal villi; ps, 'placoid scales'; sp, spiracle (after Cook & Neal, 1921). (B) *Polypterus senegalus*; visceral skeleton and dentition of pterolarva. bb, basibranchial; cb, ceratobranchial; ch, ceratohyal; eb, epibranchial; hb, hypobranchial; hh, hypohyal; pb, pharyngobranchial (from Wacker *et al.*, 2001, with permission from the publisher). (C) *Elops lacerta*; visceral skeleton and dentition. Tooth plates associated with (amongst others): BB, basibranchials; HB, hypobranchials; CB, ceratobranchials; EB, epibranchials; PB, pharyngobranchials (after Taverne, 1974). (D) *Haemulon sciurus*; visceral skeleton and dentition. BH, branchiohyoideus; CB, ceratobranchial; EB, epibranchial; ET, epibranchial tooth plate; HB, hypobranchial; PB, pharyngobranchial [after Wainwright, 2006, with permission from the author and publisher]. (E) *Triton alpestris*; visceral skeleton and dentition of stage 50, 'Reusenzähnchen', structures considered by the author to be true tooth germs. BB, basibranchial; HB, hypobranchial; Hy, hyoid; K, ceratobranchial [from Wagner, 1955; reproduced with permission from the *Journal of Embryology and Experimental Morphology*].

for the development of internal odontodes, as suggested by the ‘modified outside-in’ hypothesis.

The experimental studies discussed above are rather inconclusive as to whether endoderm can develop autonomously into enamel organs without any ectodermal influence. Yet, a far more significant outcome in our view is the observation that enamel organs always develop from stratified epithelia. In several teleosts, a cell layer with peridermal characters overlies the endoderm prior to the formation of pharyngeal enamel organs. In urodeles, anteriorly positioned teeth develop from the basal (i.e. ectodermal) layer of a double-layered epithelium, with endoderm forming the superficial layer. Even teeth that develop in an exclusive ‘endodermal territory’ develop from a stratified epithelium, with a flattened cell layer that covers the basal epithelial layer [Reisinger, 1933 and references therein; Davit-Béal, Allizard & Sire, 2006; Davit-Béal *et al.*, 2007]. The superficial layer has distinct staining properties and shows apical differentiation. This is not just the case for urodele endodermal teeth but also for dentary teeth in the lungfish *Neoceratodus forsteri* (Kemp, 2002*b*). Because teeth always develop from a stratified epithelium, one must consider the possibility that this epithelium has a composite nature. Whether the superficial layer is ectoderm or an ectoderm equivalent is discussed in the next section.

III. ECTODERMAL INFLUX OR CO-OPTION?

There are various ways in which ectoderm could be part of the composite nature of the oropharyngeal epithelium and, in this way, continue (in evolutionary terms) to exert an influence on tooth formation. One possibility is that odontode-competent ectodermal epithelium effectively invades the oropharynx during development and becomes associated with endoderm. This was originally proposed in the ‘modified outside-in’ hypothesis (Huysseune *et al.*, 2009, 2010). An obvious place for the influx of ectoderm into the oropharynx is where the ectoderm contacts the endoderm, and where an orifice is eventually created, that is at the mouth opening and the gill slits. The ectoderm may also reflect inwards around endoderm without the need for a physical opening, as in the urodele stomodaeal collar. Finally, intraepithelial migration should not be excluded. For example, Prochazka *et al.* (2015) showed that a fibroblast growth factor 8 (Fgf8)-expressing epithelial cell population migrates toward a Shh-expressing region in the mandible, the tooth placode initiation site, and that this is required for tooth development. This cell behaviour is not the only type of intraepithelial migration; diverse examples of intraepithelial cell motility are currently being uncovered (reviewed in Kapsimali, 2017). Motile epithelial cells may ultimately participate in tooth formation, by directly building the enamel organ, as a signalling partner, or by providing the necessary mechanical stimuli.

An alternative hypothesis for the invasion of odontode-competent ectoderm is the co-option of ectodermal characters by endoderm, facilitating the development of odontodes inside the pharynx. Such a co-option event would most likely have

been initiated at the ecto–endoderm contact zone, that is in the mouth, and at the pharyngeal openings, or gill slits. Given our emphasis on pharyngeal teeth, we focus on the latter.

(1) Ectoderm meets endoderm at pouch–cleft contacts

The endodermal epithelium of the developing pharynx produces a series of bilateral outpocketings, the pharyngeal pouches. These extend from the midline endoderm and eventually contact the ectoderm at corresponding clefts (Graham, Okabe & Quinlan, 2005; Grevellec & Tucker, 2010; Graham & Richardson, 2012; Frisdal & Trainor, 2014; Shone & Graham, 2014). [Note that the term ‘cleft’ is used here although there may not necessarily be a visible skin depression]. In chondrichthyans and non-amniote osteichthyans, most pouch–cleft contacts eventually break through to create orifices, called gill slits (Goodrich, 1930; Bjerring, 1977; Kimmel *et al.*, 1995; Shone & Graham, 2014). The pouches, and later gill slits, separate clusters of mesenchymal cells that give rise to, amongst others, cartilaginous pharyngeal arches. Choe & Crump (2015) provide an overview on epithelial morphogenesis at the pouch–cleft contacts, and its molecular control.

Counting the slits behind the first, or hyomandibular, pouch (i.e. pouch 1, P1), there are five in selachians. The exceptions are the sixgill shark *Hexanchus* (six pouches), and the sevengill shark *Heptanchias* (seven pouches), which however do not represent a basal condition. The persistent notion that elasmobranchs primitively had more than five gill slits is unsubstantiated by fossil evidence (Maisey, 2004). Other vertebrates have four or five gill slits beyond the first one (Goodrich, 1930; Brachet, 1935; Graham *et al.*, 2005, 2019). In actinopterygian fishes, commonly six pharyngeal pouches form from anterior to posterior (Fig. 8A). The first pouch (P1) separates the mandibular from the hyoid arch, and is homologous to the first pouch in chondrichthyans, the spiraculum. Basal actinopterygians (e.g. *Polypterus*) and the lobe-finned fish *Eusthenopteron* have spiracular canals, as do anuran tadpoles (Bartsch, Gemballa & Piotrowski, 1997; Haas, 2003; Brazeau & Ahlberg, 2006). Dipnoans on the other hand lack the canal and among the neopterygians, *Lepisosteus* retains only a rudimentary canal (Balfour & Parker, 1882; Goodrich, 1930). Initially the spiraculum may have been used for gas exchange. The stapes of Devonian stem tetrapods traverses a deep and expansive spiracular space homologous with the crown tetrapod middle ear space (Gardiner & Schaeffer, 1989; Brazeau & Ahlberg, 2005). An open spiracular canal is, however, not always present in adults; it became obliterated many times in evolution (Brachet, 1935). As in chondrichthyans, it still gives rise to an opening in species at chondrosteian level (e.g. *Acipenser*) and in *Polypterus* (Brachet, 1935; Bertin, 1958; Kunz, Luer & Kapoor, 2009; Hilton, Grande & Bemis, 2011). By contrast, the spiraculum does not open any longer in neopterygians (e.g. *Lepisosteus*) and in teleosts (Goette, 1901; Goodrich, 1930; Brachet, 1935; Arambourg & Bertin, 1958; Bertin, 1958; Gardiner & Schaeffer, 1989).

The second pouch (P2) separates the hyoid arch from the third pharyngeal arch (also called first branchial or gill arch), pouch 3 (P3) separates pharyngeal arch 3 from 4, and so on. The last pouch (P6) opens anterior to the seventh pharyngeal arch [see Holzschuh *et al.*, 2005, Kopinke *et al.*, 2006, Choe *et al.*, 2013, Rosa *et al.*, 2019 and Oralová *et al.*, 2020 for timing of development of the pouches in zebrafish; Okada *et al.*, 2016 for the Japanese medaka, *Oryzias latipes*; Le Pabic, Stellwag & Scemama, 2009 for Nile tilapia, *Oreochromis niloticus*]. The epithelium from pharyngeal arches 3–6 differentiates into gills; the seventh arch is non-gill bearing. Once the gill slits are open, respiratory water flows over the gills.

How the pouch–cleft contacts eventually perforate varies considerably across vertebrates, although few studies have been devoted to this topic. In zebrafish, separate microscopically small lumina appear within a pouch after invasion of periderm, and subsequently coalesce. Formation of the opening is possibly aided by the secretion of hatching gland cells, at least in rainbow trout, *Oncorhynchus mykiss* (Gonzalez *et al.*, 1996). Alternatively, cell interdigitations of ectoderm and endoderm produce a single cell layer that eventually ruptures, a situation encountered in the branchial membranes of the chick (reviewed in Soukup *et al.*, 2013; Shone & Graham, 2014).

(2) Pharyngeal teeth form at ectoderm–endoderm contacts

If pouch–cleft contacts are the place where ectoderm can invade the pharynx, co-localisation of gill slit openings and internal odontodes can be expected. Explicit statements on their co-localisation are, however, rare. According to Smith & Coates (2001, p. 237), oropharyngeal denticles in the agnathan thelodont *Loganellia* occur “associated with gill pouches and slits” [see also Van der Bruggen & Janvier, 1993; Rücklin *et al.*, 2011]. Jollie (1968) refers to gnathostome teeth that extend dorsally in each pharyngeal pouch. In *Polypterus senegalus*, the position of small tooth plates (with a single tooth each) that cover the inner surfaces of ceratobranchials 1–3 and the outer surfaces of all ceratobranchials corresponds with the position of the gill slits (Wacker, Bartsch & Clemen, 2001). In zebrafish, tooth buds develop from midline endoderm just posterior to where the last (sixth) pouch connects to the ectoderm (Oralová *et al.*, 2020) (Fig. 8B, C). Several actinopterygian fish species possess oesophageal teeth (Isokawa *et al.*, 1965). Gilchrist (1922), who studied oesophageal teeth in members of the deep-sea fish family Stomateidae, noted that these teeth are found in two large saccular outgrowths of the alimentary tract just behind the branchial region, “suggestive of a pair of closed gill-slits” (p. 249). By contrast, we know of no gnathostome species with teeth in the stomach or beyond. An explanation for the occurrence of oesophageal teeth is offered in Section VI.

Among sarcopterygians, the osteolepiform *Eusthenopteron* possessed a spiracular pouch paved with teeth (Bjerring, 1998; Brazeau & Ahlberg, 2006). Spiracular teeth are also found in the extant coelacanth *Latimeria chalumnae*

(Fig. 8D). In this species, the absence of well-defined rows of tooth plates associated with the dorsal parts of the gill arches has been related in part to the virtual absence of the dorsal parts of the gill slits (Nelson, 1969). In temnospondyl amphibians, one of the largest late Palaeozoic tetrapod clades, branchial denticles were common and are considered by palaeontologists as evidence for the presence of open gill slits and an aquatic lifestyle (Schoch, 2001, 2002). Branchial denticles disappeared at metamorphosis (e.g. Boy, 1988). The questionable identification of branchial denticles in extant amphibians, and the distinction between denticles and gill rakers, is discussed in Section IV.1. In amniotes, pouch–cleft contacts may well break through, but pharyngeal teeth are never present.

The observations summarised above suggest that pharyngeal teeth or denticles indeed are closely associated with pharyngeal pouches. If so, does ectoderm use pouch–cleft contact areas (or the orifices formed in them) as passageways to invade the oropharynx and participate in tooth formation? Possibly but not necessarily. The two vertebrate lineages for which details are available to address this question are teleosts and amphibians.

(a) Teleosts

Edwards (1929), studying tooth formation in the common carp, showed that the endodermal pharyngeal folds first come into contact with the basal ectodermal layer (i.e. the layer resting on the basal lamina), which responds by sending inward a wedge-shaped plug of cells. The latter ruptures the apex of the pharyngeal fold which then becomes continuous with the basal ectodermal layer. The surface layer of the ectoderm then grows inwards as rapidly as the cleft-like lumen in the endodermal cells proceeds inward. This results in a lining of the lumen of the pharyngeal cleft and the pharyngeal cavity composed of two cell layers: an apical layer made from flattened (i.e. ectodermal) cells, and a basal (i.e. endodermal) layer of columnar cells. The endodermal layer gives rise to the enamel organs. Rosa *et al.* (2019) demonstrated that in zebrafish the surface layer of the ectoderm (i.e. the periderm) indeed invades each pouch, even prior to orifice formation, but is arrested halfway into the pouch. Moreover, the invasion into pouch 6 (the pouch just anterior to the tooth-forming region) occurs only after the first tooth germ has been initiated (recall that, instead, a periderm-like layer overlies the midline endoderm from which teeth form) (Fig. 5F–H). Pouch–cleft contact is nevertheless required before teeth are initiated (Oralová *et al.*, 2020). This conclusion was based on observations on *vgo* mutants, which carry a mutation in the transcription factor *tbx1* (Kochilas *et al.*, 2003; Piotrowski *et al.*, 2003). These mutants display defective pouch formation and therefore disturbed pouch–cleft contact. The disturbance is often asymmetric, while the medial, unsegmented, pharyngeal endoderm (that is, where teeth normally develop) is less affected (Piotrowski & Nüsslein-Volhard, 2000). Oralová *et al.* (2020) established a significant correlation between the absence of teeth in these mutants and failure of endodermal pouch 6 to contact the

surface ectoderm. Teeth were present if this contact was established. A coincidental, non-causal relationship between pouch–cleft contact and tooth initiation was excluded by the observation of unilateral teeth (i.e. teeth present only on the left or on the right side of the animal) in *ugo* mutants, associated with a normal pouch–cleft contact at that side only. Similar data were obtained on embryos with natural or induced pericardial oedema, which mechanically prevents contact of pouch endoderm with ectoderm. These data strongly suggest that contact of pouch endoderm with skin ectoderm is required for tooth formation. However, since teeth in zebrafish are initiated prior to any invasion of the corresponding pouch–cleft contact by cells from the body surface, a direct participation of the ectoderm in the epithelial enamel organ, *via* this route, appears excluded. Likewise, Atukorala *et al.* (2011) could not see an ectodermal influx into the pouches in Japanese medaka. Unlike Oralová *et al.* (2020), this led them to conclude that the rostral endoderm possesses an intrinsic odontogenic competence. Yet, as in zebrafish, the pharyngeal epithelium in medaka has a superficial layer of flattened cells, similar to the periderm-like layer in zebrafish (Yamamoto, Iuchi & Yamagami, 1979; Fig. 8E). Interestingly, in mice, loss of the transcription factor *Pax9* (Peters *et al.*, 1998) leads not only to pouch defects, but also to defective tooth development, tooth germs being arrested at the bud stage. In zebrafish, *pax9* is expressed in pharyngeal arch mesenchyme and both medial and lateral to the tooth germ in pharyngeal epithelium, but not in the tooth germ itself (Jackman, Draper & Stock, 2004). Morpholino knock-down of *pax9* does not prevent the formation of teeth in zebrafish (Swartz *et al.*, 2011), albeit teeth are reduced in number.

It is unknown whether the pouch–cleft (i.e. endoderm–ectoderm) contact that is needed for pharyngeal tooth development reflects the need for chemical (signalling) factors or for mechanical stimulation. The need for a contact is, however, also apparent from studies on other organs. Balinsky (1975) noted (without referring to a particular taxon) that ectodermal clefts are induced by endodermal pouches when they touch the epidermis but are absent if the endodermal pouch does not reach the epidermis. Holzschuh *et al.* (2005) observed a close correlation between enlarged or reduced contacts made by pouches with the ectoderm and epibranchial defects in *ugo*-mutant zebrafish, and proposed a contact-dependent signal from the endoderm. Specification and patterning of the pharyngeal endoderm is required for ectodermal expression of *gcm2*, a gene necessary for gill filament budding (Hogan *et al.*, 2004). This expression requires Fgf3 and Fgf receptor-like 1a (Fgfr11a) in the pouch endoderm (Hall *et al.*, 2006). Thymic dysgenesis in nude mice that is caused by the failure of third pouch endoderm to contact the ectoderm (Cordier & Haumont, 1980), is reminiscent of the above-described pouch formation defects.

Oralová *et al.* (2020) clearly indicated that a pouch–cleft contact is necessary for tooth initiation, in addition to the presence of a layer of periderm-like cells covering the odontogenic endodermal epithelium. The situation may be different for the oral teeth in teleosts. In cichlid fishes, the start of oral tooth formation consistently follows mouth opening

(Huysseune, 1990). This sequence of events suggests that invasion of the oral cavity by ectoderm or periderm could occur prior to the start of tooth formation. Rosa *et al.* (2019) showed that periderm partially invades the zebrafish mouth cavity (Fig. 8F). However, given the absence of oral teeth in zebrafish (and other cyprinids), nothing can be concluded on the role of periderm or ectoderm in oral tooth formation in this species. It will be interesting to investigate whether the processes observed during zebrafish tooth development represent the general teleost, or even actinopterygian, situation – whether for oral or pharyngeal teeth. Indeed, unlike non-teleost actinopterygians and amphibians, teleosts have a highly derived early development with meroblastic cleavage, and unique patterns of gastrulation, endoderm formation and pouch development (Collazo, Bolker & Keller, 1994). Egg type, cleavage and gastrulation have been proposed to impact the formation of the primary mouth, the other obvious place where ectoderm meets endoderm (Soukup *et al.*, 2013). Clearly, studies on primary mouth and gill slit formation in basal, non-teleost, actinopterygians with holoblastic cleavage are needed to acquire a broader insight into the composition of the oropharyngeal epithelium.

(b) *Lissamphibians*

Extant amphibians, exemplified by urodeles, have teeth along the jaw margins but no pharyngeal teeth. The oral teeth are ectoderm-derived anteriorly, and endoderm-derived posteriorly, some being of mixed origin (see Section II.3b; Fig. 6F). The stomodaeal region is covered by an epithelium consisting of flattened cells on top of cuboidal cells, as described for the urodele *Ambystoma mexicanum* (de Beer, 1947). The epithelial composition in *A. mexicanum* is remarkably similar to the two-layered pharyngeal epithelium in zebrafish. Yet, different from teleosts, the flattened cells are yolk-filled, pigment-free and have been identified as endoderm, while the basal cuboidal cells are yolk-free, pigment-containing cells and represent the ectodermal stomodaeal collar. This was confirmed by Soukup *et al.* (2008) using transgenic axolotls, and by Takahama, Sasaki & Watanabe (1988) for the Tokyo salamander, *Hynobius tonyoensis*. Interestingly, in the latter species, the apical cells of the oral epithelium (and thus the endoderm) contain secretory granules as in the epidermis (Takahama *et al.*, 1988).

Pharyngeal teeth were lost during amphibian evolution, despite the presence of pouch–cleft contacts. Larval and pennibranchiate amphibians retain an open operculum and gill clefts supported by the branchial skeleton (Coates & Clack, 1991). In Section IV.2, we will argue that the absence of teeth despite pouch–cleft contacts does not falsify the hypothesised need for ectoderm–endoderm contact to initiate tooth development.

(3) The possible co-option of endoderm

In zebrafish, the invasion of periderm into the pharyngeal pouches is arrested halfway into the pouches and limited in

extent in the mouth (see Section III.2a). The germ layer origin of the periderm-like layer that expands along the midline of the oropharynx to the end of the oesophagus, is not known (Rosa *et al.*, 2019). Yet, odontogenesis starts only when these periderm-like cells have covered the endoderm-derived prospective odontogenic epithelium. Careful observation suggests that these periderm-like cells in zebrafish may represent a population of endodermal cells that behaves different from the original endoderm. The cells express periderm markers, raising the possibility that these cells have been co-opted to function as a periderm. We use the term ‘co-option’ in the sense of True & Carroll (2002, p. 66) in that “novel expression of just one or a few regulatory genes may result in the redeployment of entire sets of genes in novel contexts”. We propose that conserved ectoderm/periderm gene functions required for tooth development have been redeployed in a subset of endoderm. Thus, the periderm-like cells express the transitory endodermal marker SRY-box transcription factor 17 (*sox17*) but also keratin 4 (*kt4*), an intermediate filament marker expressed in the periderm (Oralová *et al.*, 2020). Redeployment of genes in a novel context is not exceptional. Hogan *et al.* (2004) suggest that a transfer of expression of the transcription factor glial cells missing 2 (*gcm2*) occurred from ectoderm to endoderm at the origin of tetrapods. In non-tetrapod osteichthyans *gcm2* is required for gill filament development, in tetrapods *gcm2* is required for parathyroid development. Thus, the parathyroid gland in tetrapods may have evolved by co-option of ectoderm characters to endoderm, together with the transition from an aquatic to terrestrial environment (reviewed in Graham *et al.*, 2005; Graham & Richardson, 2012).

Why periderm invasion is arrested in zebrafish, and endoderm is possibly co-opted into a periderm-like fate to cover the remaining (medial) part of the oropharynx, remains puzzling. Possibly, this is related to the miniaturisation of zebrafish compared to other cyprinids. Recall that Edwards (1929) saw invasion of the superficial ectodermal layer (i.e. periderm) as deep as the midsagittal plane in the much larger carp (an observation that nevertheless needs to be reassessed). It is known that miniaturisation entails a number of morphological and developmental changes, such as reductions, and structural simplifications but also innovations (Hanken & Wake, 1993). Alternatively, it is possible that the dual origin of the superficial layer of the oropharyngeal epithelium, that is from periderm (in the pouches) and from a periderm-like layer (along the midline), is general for teleosts and relates to their derived mode of early development, as suggested in Section III.2a. At the onset of gastrulation, the outer layer of the embryo, the enveloping layer (EVL), is said to become lineage-restricted and to give rise to the periderm only (Kimmel, Warga & Schilling, 1990), transiently covering the ectoderm. Following this model most authors consider the periderm as extra-embryonic, and not as ectodermal. This view needs to be revised in light of recent findings. First, contrary to what was traditionally assumed, periderm cells persist in the epidermis until at least one month of age (Fukazawa *et al.*, 2010; Fischer *et al.*, 2014; Lee, Asharani & Carney, 2014). Second, recent findings show that the EVL gives rise to other cell types of the organism, notably

to the so-called ‘forerunner cells’ that give rise to Kupffer’s vesicle (whose cells later disperse and assume caudal notochord or muscle fates) (Warga & Kane, 2018). In another example, a study on mouse endoderm has shown that segregation of extra-embryonic and embryonic tissues is not as strict as classically accepted. Indeed, a cell lineage previously considered exclusively extra-embryonic, contributes to the early gut tube of the mouse embryo (Kwon, Viotti & Hadjantonakis, 2008). In contrast to teleosts, non-teleost actinopterygians such as *Polypterus*, *Acipenser* or *Amia*, undergo complete cleavage. A periderm does not form (e.g. *Acipenser*), or is less specialised than in teleosts (as in *Lepisosteus* and *Amia*) (Dettlaff, 1993). In sturgeon, for example, in the absence of a periderm layer, the ectoderm is bilayered from early stages onwards. Thus, it is tempting to consider the teleost periderm as being derived from the outer ectodermal layer in early actinopterygians, due to the peculiar and derived type of embryonic development, a suggestion also made by Warga & Kane (2018) [but see Collazo *et al.*, 1994 for alternative scenarios for the origin of the EVL]. Significantly, in mammals, the periderm is a direct ectodermal derivative.

The ectoderm–periderm relationship outlined above allows us to speculate about a possible evolutionary scenario. In this hypothesis, the outer ectodermal layer penetrates deep into the pharynx in non-teleost actinopterygians. During actinopterygian evolution, substantial changes are known to have taken place in the mode of embryonic development (Collazo *et al.*, 1994). We propose that an invasion of the outer ectodermal layer would then have become more restricted to the entrance of the pouches. Instead, endoderm would have been co-opted to take up the function of the outer ectodermal (now called peridermal) layer. A comparison of miniaturised teleosts such as zebrafish and medaka with non-miniaturised teleost species, and between teleosts and basal actinopterygians that develop a bilayered ectoderm, will be imperative to test these ideas. How this hypothesis then relates to the origin and evolution of internal odontodes (oral and pharyngeal teeth or denticles) is further elaborated upon in Section VI.

IV. GERM LAYERS AND THE DISTRIBUTION OF POST-MANDIBULAR TEETH

(1) The distribution pattern of teeth in the oropharynx

In vertebrates with internal gills such as chondrichthyans, actinopterygians, and sarcopterygian fishes, the pharynx can be completely covered in odontodes. In extant sharks, these are frequently very small and often referred to as (mucous membrane) denticles (Peyer, 1968) (Fig. 9A). Species of batoids (skates and rays) also possess denticles in the pharynx (Atkinson & Collin, 2012). The pattern of distribution of pharyngeal denticles varies widely, from tightly crowded, to widely scattered, depending on the species (Nelson, 1970). The denticles can be distributed throughout the oropharynx, be restricted to its posterior region, or be missing altogether. In selachians they can reach as far back

as the oesophagus (Peyer, 1968). Ratfish, members of the holocephalans (a basal clade among extant chondrichthyans) have tooth plates in the oral cavity (Didier, Stahl & Zangerl, 1994) but there is no report of pharyngeal denticles (Peyer, 1968). Nelson (1969) assumed that primitively the dermal skeleton was represented by small toothed elements distributed in a nearly uniform manner over the surfaces of the oropharyngeal cavity, a condition possibly represented by pharyngeal denticles scattered over the oropharyngeal cavity in elasmobranchs (illustrated for recent forms in Nelson, 1970). Not surprisingly, shark teeth and denticles are the most commonly collected craniate fossil material (Maisey, 2000). There is, however, a discussion as to what extent the condition in early sharks is representative for tooth development in much older early gnathostomes (Reif, 1978*b*; Smith & Coates, 2001; Donoghue & Rücklin, 2016; Haridy *et al.*, 2019; Rücklin *et al.*, 2021). Large tooth-bearing elements occurring in various locations on the gill arches would have arisen from smaller elements by enlargement and/or fusion. This was accompanied by reductions elsewhere in the visceral apparatus (Nelson, 1969).

Extant lineages that are close to the osteichthyan key divergence into ray-finned (actinopterygians) and lobe-finned fishes (sarcopterygians) are represented by bichirs (*Polypterus*), sturgeons (*Acipenser*) and paddlefish (*Polyodon*), and gars (*Lepisosteus*, *Atractosteus*). Pharyngeal teeth in *Polypterus senegalus* are associated with the basibranchial, hypobranchials 1–3, ceratobranchials 1–4, and pharyngobranchials 2 and 3 (Clemen, Bartsch & Wacker, 1998) (Fig. 9B). Adult sturgeons are edentulous (Peyer, 1968; Hilton *et al.*, 2011; Warth *et al.*, 2017) but juvenile sturgeons possess teeth on the dentary, dermopalatine (= maxilla), palatopterygoid, elements of the hyoid arches, hypobranchial 1 and the region around infrapharyngobranchial 1 (= the parasphenoid tooth plate) (Hilton *et al.*, 2011; Warth *et al.*, 2017). Likewise, young American paddlefish (*Polyodon spathula*) carry teeth on upper (dermopalatine and palatopterygoid) and lower (dentary) jaw, as well as on the hyoid and first two branchial arches (hypobranchials, epibranchials and infrapharyngobranchials), but adults are edentulous (Smith *et al.*, 2015). The Cuban gar (*Atractosteus tristoechus*) possesses pharyngeal teeth at least in the posterior pharynx (Comabella *et al.*, 2012). *Amia calva* and *Lepisosteus spatula* have a significantly developed upper pharyngeal dentition (Nelson, 1969).

In teleosts, pharyngeal tooth distribution is extremely variable (Nelson, 1969, 1970; Vandewalle *et al.*, 1994) (Fig. 9C, D). The upper pharyngeal dentition in teleosts consists in part of 1–5 usually paired tooth plates or groups of tooth plates (Nelson, 1969). Across the different lineages of teleosts, there was a – probably independent – loss of tooth plates. The repeated reduction, in particular of the fourth and fifth upper pharyngeal tooth plates, appears to be a general trend towards consolidation of the extensive upper pharyngeal dentition of basal teleosts such as *Elops lacerta* (Fig. 9C), into a single pair of toothed areas (Fig. 9D). This would be formed mainly (if not entirely) by the third infra-pharyngobranchials (Nelson, 1969; Vandewalle *et al.*, 1994).

The evolution of the dentition associated with the ventral parts of the gill arches is more complex. This dentition shows secondary modifications, reductions and losses, as is extensively discussed by Nelson (1969). Again, *Elops* is considered to display the basal teleost condition. Based on what is known about the dermal skeleton of the gill arches, Nelson (1969) assumes that the other toothed surfaces of the buccopharyngeal cavity arose by ‘consolidation’ of tooth plates primitively supported by the visceral endoskeleton (Fig. 9C). Reduction in the pharyngeal dentition took place within the ostariophyans (Nelson, 1969; Vandewalle *et al.*, 1994; Stock, 2007). A dentition restricted to the fifth ceratobranchials [i.e. the last (7th) pharyngeal arch] is characteristic of all families in the order Cypriniformes (including the speciose family Cyprinidae that includes zebrafish). These ventral pharyngeal teeth are the only teeth left as the oral teeth also disappeared (Huysseune, Van der heyden & Sire, 1998; Stock, 2007; Pasco-Viel *et al.*, 2010). Even further reduction took place in another cypriniform family, the Gyriinocheilidae, which lacks pharyngeal teeth (He, Yue & Chen, 1997). A reduction in the extent of distribution of pharyngeal teeth is a general evolutionary trend (Shkil *et al.*, 2010), but it can also be observed within individual lineages, as for example in clupeids (Nelson, 1967).

Little is known about the distribution (and especially reduction) of teeth and denticles at the transition from lobe-finned fishes to tetrapods. *Eusthenopteron* possesses well-developed marginal tooth series, palatal denticle fields as well as large fangs on the palatines, vomers and lower jaw (Smith & Coates, 2000; Doeland *et al.*, 2019). Like *Eusthenopteron*, its Australian relative *Mandageria* has denticulated plates on the anterior basibranchial, hypobranchials and ceratobranchials (Johanson & Ahlberg, 1997). *Latimeria* has an elaborate dentition on upper and lower jaws as well as in the branchial region (Millot & Anthony, 1958; Nelson, 1969). Each branchial arch is ornamented with rows of tooth plates of around 20 teeth, two rows on the inner face, two on the outer. The hyoid arch has one row of tooth plates on its inner surface, and the copula is paved with hundreds of miniature teeth. Moreover, two groups of teeth are placed in the spiracular cavity (Millot & Anthony, 1958; Fig. 8D). *Acanthostega* has palatal bones carrying a range of tooth-like structures, described as shagreen, fangs, toothlets and denticles (Clack, 2002). Palatal teeth appear to have been conserved better than branchial and oropharyngeal tooth plates (Smith & Coates, 2000).

Teeth in extant amphibians can be localised to the lower jaws (dentaries), upper jaws (premaxillae, maxillae) and palate (vomers, palatines). Caecilians possess teeth on the premaxillae, maxillae, vomers, palatines, and dentaries. Salamanders retain teeth on both upper and lower jaws and on the palate, but anurans, with one exception, have lost dentary teeth and have a reduced number of palatal teeth; toads are edentulous (reviewed in Davit-Béal *et al.*, 2007; Berkovitz & Shellis, 2016). Metamorphosis, if present, entails major changes in the dentition. As described below, larval and fetal stages of some urodeles and caecilians have a

dentition very different from that of the adult (Parker & Dunn, 1964).

Many temnospondyl amphibians possessed branchial denticles, considered to be probably homologous to palatal and pharyngeal teeth of actinopterygians (Schoch, 2002). In extant amphibians, denticles disappeared from the pharynx. Exceptionally, teeth have been reported to occur in the pharynx of urodeles. Worthington & Wake (1971, p. 358) mention the frequent presence of a “mineralized gill raker, resembling a tooth crown” on epibranchials 1 and 2 in the ambystomid salamander *Rhyacotriton olympicus*. It remains questionable whether these are teeth and not gill rakers. Stadtmüller (1924) made a study of the development of gill rakers in salamanders, and considered them to be homologous to teeth. Wagner (1955) reported the presence of tooth structures (“*Kiemenzähnechen*”) on the gill arches (but not the hyoid arch) in *Triton alpestris* larvae (and indeed in most urodele larvae). He considered them ‘true teeth’ (“*echte Zahnanlagen*”), that disappear with the closure of the gill slits (Fig. 9E). These teeth consist of a collagenous matrix only, presumably predentine. The matrix does not become mineralised and there is no enamel cap. These structures do not erupt, but eventually degenerate. By contrast, Duellman & Trueb (1986) mention only gill rakers in larval salamanders. Schoch (2001) distinguishes gill rakers from branchial ossicles, tooth-like structures located in the throat and gill pouches. In apparent contradiction to Schoch (2002), Schoch & Witzmann (2011) consider tooth-bearing ossicles (“pharyngeal jaws”) to be not identical and not homologous to teeth and report them to be absent in lissamphibians, but common in temnospondyls. Clearly, more data about gill rakers in actinopterygians and sarcopterygians are required for an unambiguous terminology and to assess their homology to internal odontodes. Interestingly, Schoch & Witzmann (2011) assign gill rakers to the endoderm and ossicles to the ectoderm.

In reptiles, the dentition is restricted to the oral region. There is a single row of marginal teeth on the lower jaw, but a more widely varying pattern on the upper jaw and palate (reviewed in Richman & Handrigan, 2011). The first dental elements are rudimentary papillae called denticles; they consist of dentine but little or no enamel. These teeth never become functional and are resorbed.

(2) The loss of teeth from the post-mandibular arches

Having argued for pouch–cleft contacts (prospective gill slits) as a condition for pharyngeal tooth development, can this explain the distribution of post-mandibular teeth? Yes and no. Yes, because teeth are found near pouch–cleft contacts and prospective orifices, as reviewed above. No, because neither the presence of pouch–cleft contacts *per se*, nor their sequence of formation, coincides with the diverse patterns of pharyngeal tooth distribution across taxa.

How, then, can we reconcile the observation that pouch–cleft contacts are required for tooth formation, with the fact

that there is no one-to-one relationship between pouch–cleft contact and tooth distribution? Arguably, pharyngeal teeth in jawed vertebrates were once widely distributed over all pharyngeal arches. Because of their poor mineralisation and deep location, data on gill skeletons in fossil early gnathostomes are rare (Pradel *et al.*, 2014; Brazeau *et al.*, 2017), and this is even more so for pharyngeal denticles, although advanced imaging techniques may expand our knowledge in the years to come. Within the crown gnathostomes, pharyngeal teeth or denticles are limited to chondrichthyans and osteichthyans (Rücklin & Donoghue, 2019). The basal condition in recent sharks is argued to be denticles covering much or all of the buccopharyngeal surface (Nelson, 1970). In osteichthyans, inference about the evolution of tooth distribution is easier because teeth are attached in some way to the arch skeleton. The basal condition is tooth plates distributed over the different arches (Nelson, 1969; Taverne, 1974; Vandewalle *et al.*, 1994). Thus, rather than arches gaining the capacity to form teeth, one must assume that the capacity to form teeth was lost from some arches (despite the presence of pouch–cleft contacts) but retained on other arches. Stock (2001) discussed potential mechanisms accounting for evolutionary tooth loss: developmental arrest, failure of tooth replacement, or alterations in the factors eliciting tooth initiation or later stages of tooth development. In a follow-up study addressing oral tooth loss in cypriniforms, Jackman & Stock (2006) proposed that changes in trans-acting regulators of the distal-less-like (*Dlx*) genes may have been responsible for loss of teeth associated with the mandibular arch. Fgf signalling was identified as one of the potential upstream regulators of oral *dlx2b* expression (Stock, Jackman & Trapani, 2006). An unanswered question is whether such a mechanism is also responsible for tooth loss on the other pharyngeal arches.

Still, wherever pharyngeal teeth are present, one may expect the sequence of tooth formation to follow the sequence of formation of pouch–cleft contacts. Given the diversity of pharyngeal tooth distribution, teleosts are excellent candidates to test this relationship, provided the species has teeth on more than one pharyngeal arch. In medaka, pharyngeal teeth start to appear around stage 29 on the fourth pharyngobranchial (Debiais-Thibaud *et al.*, 2007) while the corresponding, fifth, pouch forms at stage 27 (Okada *et al.*, 2016) (staging according to Iwamatsu, 2004). In the Nile tilapia, pouch–cleft contacts are established between 30 and 60 h post-fertilisation (hpf), while pharyngeal tooth buds are first reported at 96 hpf (Le Pabic *et al.*, 2009). Obviously, more taxa need to be explored to assess the relationship between timing of pouch–cleft contact, and onset of pharyngeal tooth formation, as well as their dependence on shared genetic regulatory pathways. Should such studies reveal a significant relationship, then both events might well represent an example of co-evolutionary patterning – analogous to what has been suggested for teeth and taste buds (Bloomquist *et al.*, 2015). In the definition of co-evolution adopted here, the evolution

of one entity is closely affected by that of another with which it interacts (Wilkins, 2002). The concept applies to different levels of biological organisation, from molecules to ecosystems, but has rarely been applied to vertebrate organs.

Other than timing and extent of pouches and pouch–cleft contacts, one must also consider molecular regulation of anterior–posterior patterning of the endoderm in determining the sequence of pharyngeal tooth formation. The proximodistal and anteroposterior polarity of the pharyngeal arch skeleton is dictated by the endoderm (reviewed in Frisdal & Trainor, 2014; Graham & Shone, 2019). This is in line with the observation that the polarity of the branchial pouch endoderm is established in the embryo in the absence of neural crest cells (Veitch *et al.*, 1999).

(3) A special identity for pouch 2?

In zebrafish, periderm cells (covering the basal ectodermal layer of the skin) start by invading pouch 2 (the pouch that separates the hyoid from the first branchial arch) at an early developmental stage. Invasion is soon followed by lumenisation of this pouch and opening of the pouch to the exterior. These periderm cells, characterised by *krt4* expression, merge with *krt4*-expressing periderm-like cells located in the pharynx midline at the level of pouch 2. Once these two populations of *krt4*-expressing cells have made contact, *krt4*-expressing cells expand posteriorly towards the gut entrance, as reviewed in Section II.2b (Rosa *et al.*, 2019). A mature pharyngeal epithelium, composed of an endodermal layer covered by a periderm-like layer, is crucial for pharyngeal tooth development in zebrafish.

The apparent involvement of pouch 2 in these events thus raises the question whether pouch 2 plays a key role in the evolutionary origin of the pharyngeal dentition. The early invasion and opening of pouch 2 in zebrafish may be independent of the behaviour of the periderm-like cells. It may simply be related to the sequential maturation of the pouches, given that pouch 1 does not fully open any more in most taxa. Yet, several lines of arguments indicate that this is not the (only) explanation and that pouch 2 has a more significant role. In gnathostomes, pouch 2 separates the jaw–hyoid apparatus, functioning in feeding, from the branchial arches, functioning in respiration (Starck, 1982; Stundl *et al.*, 2019). Thus, the importance of pouch 2 in evolution can be easily understood from a functional viewpoint. Aspects of the development of the first two pharyngeal pouches (and mandibular and hyoid arches) appear to be highly conserved in all vertebrates. The first two pharyngeal pouches form first and concomitantly. The posterior pouches form consecutively along with caudal extension of the pharyngeal endoderm (Graham & Shone, 2019). Their development is controlled by distinct signalling pathways (Graham & Shone, 2019). Consequently, mutations, knockdown of genes, or pharmaceutical inhibition experiments affect mandibular and hyoid arches differently from the posterior arches and pouches [several examples and references in Okada & Takada, 2020; see also Hall *et al.*, 2006; Birkholz

et al., 2009; Schwend & Ahlgren, 2009; Vaccari *et al.*, 2010; Lovely *et al.*, 2016; Duncan *et al.*, 2017]. In all vertebrates, the thyroid develops from pouch 2 whereas other pouch derivatives show larger variation (Graham *et al.*, 2005). In further support of the evolutionary significance of pouch 2 are developmental features in amniotes that reflect the evolutionary ‘memory’ of this pouch, such as an embryonic operculum (although without dermal bones) that persists in amniotes (Wall & Hogan, 1995; Richardson *et al.*, 2011; Graham & Richardson, 2012). In humans, the second (hyoid) arch expands caudally to cover more posterior pouch clefts (as does the operculum in teleosts; Richardson *et al.*, 2011). Second branchial cleft lesions account for 95% of all branchial cleft malformations in humans (Ford *et al.*, 1992; Waldhausen, 2006; Adams *et al.*, 2016).

In conclusion, while all pouches are serial homologues, they do not all appear to be equivalent in terms of potencies. How much this impinges on pharyngeal tooth distribution is largely unexplored. It will be important to assess the relationship between pouch 2 development, its contact with ectoderm, the extent of invading periderm, and the distribution of pharyngeal teeth in selected taxa that cover key steps in vertebrate evolution.

The early opening of pouch 2 before opening of the mouth in zebrafish is remarkable. Possibly this is a general character of actinopterygians as it is also observed in the sturgeon, *Acipenser sturio* and *A. ruthenus* (Goette, 1901; A. Huysseune, personal observations), and in teleosts other than zebrafish, for example in trout, *Trutta fario* (Moroff, 1904), Atlantic salmon, *Salmo salar* (A. Huysseune and P.E. Witten, personal observations), and European seabass, *Dicentrarchus labrax* (Sucré *et al.*, 2009). Early pouch 2 opening also occurs in chondrichthyans, for example in selachians such as *Scyllium* sp. (Brachet, 1935). Stundl *et al.* (2019) showed acceleration of the entire hyoid arch segment in bichirs as a function of the development of external gills, further emphasising the crucial role of this pharyngeal segment.

V. MOLECULAR CONTROL REGULATING THE DISTRIBUTION OF POST-MANDIBULAR TEETH

(1) Genes expressed in pouches and at pouch–cleft contacts

Above, we highlighted the importance of the endodermal pouches and their contact with the ectoderm for pharyngeal tooth formation. Is it possible to identify key genes responsible for tooth development by interfering with genes that are specifically expressed in pouches or at the pouch–cleft contact? In our view, the absence of such genes or their function should compromise the odontogenic competence of the pharyngeal epithelium in species with a pharyngeal dentition. Clearly, we have data about genes that interfere with tooth development in general. However, data specifically related to genes expressed in the pouches are scarce and those available come from zebrafish. Unfortunately, while some studies

report defects in the pouches upon knocking down or knocking out an endodermally expressed gene, the phenotype of the teeth is rarely studied. For example, knockdown of *vgl2a* results in aberrant endodermal pouch morphogenesis, and hypoplastic cranial cartilages, but the phenotype of the teeth was not reported (Johnson *et al.*, 2011). In a rare study considering both teeth and pouches, Jackman *et al.* (2004) mention tooth defects along with aberrant pouch morphology when inhibiting Fgf signalling. Wiweger *et al.* (2012) list several zebrafish mutants based on alizarin red S-stained whole mounts, most of which do not display a tooth phenotype, and none is reported edentulous.

Two caveats should be added when critically reviewing reported tooth phenotypes. One concerns the visualisation of the teeth in minute embryos such as of the zebrafish. First, most studies that examine the pharyngeal dentition rely on alizarin red S-stained specimens, but these reveal only the final steps of odontogenesis. Stages of initiation, morphogenesis and early cytodifferentiation up to early mineralisation of the matrix remain unnoticed. Moreover, first-generation teeth are extremely small, and imaging of the zebrafish dentition often requires sophisticated methods (Bruneel *et al.*, 2015). The second caveat concerns the usefulness, in some cases, of marker genes to assess the presence of teeth. For example, *pitx2* has been reported to be one of the earliest dental epithelial markers for teeth in teleosts (Fraser, Bloomquist & Streebman, 2008). However, blocking Fgf signalling by the receptor tyrosine kinase inhibitor SU5402 in zebrafish, or Shh signalling by cyclopamine A, does not abolish *pitx2* expression, yet tooth germs are no longer morphologically discernable (Jackman *et al.*, 2004; Jackman, Yoo & Stock, 2010).

Signalling between ectoderm and pouches has been shown to be necessary for gill development (Hanaoka *et al.*, 2004; Hogan *et al.*, 2004; Okabe & Graham, 2004), as well as epibranchial placode development in various vertebrates [zebrafish (Holzschuh *et al.*, 2005); mouse (Rizzoti & Lovell-Badge, 2007); reviewed in Graham *et al.*, 2005 and Graham, 2008]. Molecular players, which include Fgfs and bone morphogenetic proteins (Bmps), have been identified. A detailed analysis of signalling at the pouch–cleft contact is nonetheless hindered by the fact that the boundary between pouch endoderm and ectoderm is not easily detected. Also, expression data are usually collected through whole-mount *in situ* hybridisation, preventing precise localisation of the signal (ectoderm or endoderm).

In conclusion, in contrast to gills and epibranchial placodes, no key genes have so far been identified that could act at the pouch–cleft contact to elicit tooth formation. There is nevertheless one candidate molecule, retinoic acid, which has been proposed as a molecular explanation for the distribution of post-mandibular teeth. This will be discussed below.

(2) Tooth distribution: the role of retinoic acid and sonic hedgehog

Tooth development depends on reciprocal epithelial–mesenchymal interactions, which are mediated by the Wnt

(Wingless/integrated), BMP, FGF, Shh (Sonic hedgehog), and Eda (ectodysplasin) pathways (Balic & Thesleff, 2015). Experiments that alter Wnt signalling in teleosts have produced conflicting results. No effects on (pharyngeal) tooth number or distribution were reported in zebrafish (Huyseune, Soenens & Elderweirdt, 2014) while (oral) tooth density decreased by antagonising Wnt signalling in cichlids (Bloomquist *et al.*, 2015). In zebrafish, supernumerary pharyngeal teeth develop when Fgf signalling is up-regulated (Gibert *et al.*, 2010; Jackman *et al.*, 2013), when Bmp signalling is down-regulated (Jackman *et al.*, 2013) or when Eda is overexpressed (Aigler *et al.*, 2014). Supernumerary teeth can also be generated in zebrafish by thyroid hormone level manipulation (Woltmann *et al.*, 2018). Remarkably, in none of these cases do teeth expand over the other (i.e. more anterior) arches. To the best of our knowledge, only manipulation of signalling through retinoic acid expands the dentition to anterior arches (Seritrakul *et al.*, 2012).

Retinoic acid (RA) is a morphogen with a key role in many tissues during several developmental stages. Importantly, RA regulates Hox genes, which play a crucial role in axial patterning (reviewed in Nolte, De Kumar & Krumlauf, 2019). Gibert *et al.* (2010) demonstrated that pharyngeal tooth formation in zebrafish depends on RA. Interestingly, a few years earlier, Kopinke *et al.* (2006) showed that RA signalling is necessary for correct morphogenesis and segmentation of the endodermal pouches. Combining these two observations, one could conceive that tooth formation is only indirectly influenced by RA, namely by depending on proper formation of pouch–cleft contacts. Remarkably, Gibert *et al.* (2010) reported that pharyngeal tooth induction was not compromised by RA inhibition in members of two other lineages of teleosts, the beloniform *Oryzias latipes* (Japanese medaka), and the characiform *Astyanax mexicanus* (Mexican tetra). The authors concluded that in these two species, oral and pharyngeal teeth are induced independently of RA, and proposed that RA-dependent tooth initiation was acquired in the cypriniform lineage. However, Gibert *et al.* (2010) commenced the RA inhibition treatment in medaka at the end of somitogenesis. Since the pharyngeal pouches are formed well before the end of somitogenesis [fifth pouch at stage 27 (Okada *et al.*, 2016); somite completion at stage 32 (Iwamatsu, 2004)], the appropriate signals for tooth formation may have been present prior to the inhibition experiment. Clearly, RA-inhibition experiments, fine-tuned against the timing of pouch–cleft contact, will be required in multiple species to assess the role of pouch–cleft contacts *versus* direct RA signalling. In a follow-up experiment, Seritrakul *et al.* (2012) applied exogenous RA to zebrafish embryos from 24 hpf onwards and demonstrated an expansion of teeth to anterior arches. The authors concluded that changes in the levels of RA may be an evolutionary mechanism controlling tooth distribution, and suggested this could be the result of altered neural crest development. In another set of experiments, Gibert *et al.* (2015) applied exogenous RA to zebrafish later in development (56 hpf and older), and examined adult zebrafish heterozygous for a

mutation in *cyp26b1*, a gene encoding an enzyme involved in RA degradation. This resulted in an additional tooth in the ventral row, anterior to the tooth in position IV. The expansion of the dentition was accompanied by expansion of the mesenchymal markers *dlx2a* and *lhx6* and the dental epithelium marker *pitx2a*. The increase in tooth number was limited to the tooth-bearing arch only, without anterior expansion over the other arches. It was concluded that RA signalling can possibly intervene in the fine-tuning of tooth number at a taxonomically restricted level. Given our focus on the dental epithelium, it is interesting to note that RA regulates the developmental potential of the anterior endoderm (respiratory *versus* pharyngeal) in two other vertebrates, *Xenopus laevis* and mouse (Rankin *et al.*, 2018).

Sonic hedgehog (Shh), a signalling molecule from the Hedgehog family, is another candidate for regulating tooth patterning. In modern elasmobranchs, expression of *shh* marks the prospective loci of tooth initiation within the dental epithelium (Smith *et al.*, 2009; Maisey *et al.*, 2014). In zebrafish, the paralogue *shha* is widely expressed in the pharyngeal epithelium, both in tooth-forming and adjacent, non tooth-forming, regions (Jackman *et al.*, 2010). Its broad expression domain contrasts with the restriction of *Shh* expression to the dental epithelium in the oral teeth of mice (Hardcastle *et al.*, 1998). Tooth development in zebrafish depends on *shha*. However, overactivation of *shha* signalling yields no observable effect on tooth development (Jackman *et al.*, 2010). On the other hand, cyclopamine A, a compound that inhibits hedgehog signalling by binding to the Smoothed receptor, blocks tooth formation on the last (i.e. tooth-bearing) arch when applied early (Jackman *et al.*, 2010).

Recently, El Shahawy *et al.* (2017) reported a highly sensitive balance between SHH and RA signalling. In the mouse tongue, SHH antagonises RA signalling by maintaining expression of two RA catabolic enzymes *Cyp26a1* and *Cyp26c1*. RA, on the other hand, controls *Shh* expression, for example during zebrafish fin regeneration, or in the pharyngeal region of flounder, *Paralichthys olivaceus* (Laforest *et al.*, 1998; Suzuki, Oohara & Kurokawa, 1999; Sehring & Weidinger, 2019). These findings, along with the observation that tooth development in zebrafish can be elicited on normally edentulous arches by the overactivation of RA, hints at a possible epistatic relationship between these pathways. Whether such a proposed balance between RA and SHH plays a role in the distribution of post-mandibular teeth can be tested experimentally.

VI. TOWARDS A UNIFYING CONCEPT FOR GERM LAYER INVOLVEMENT IN TOOTH INITIATION

Odontodes are outstanding examples of vertebrate characters with highly conserved development, structure and composition. The fossil record provides ample evidence for the homology of teeth and external odontodes, including

transitional forms (Blais *et al.*, 2011; Chen *et al.*, 2020), as well as support that internal odontodes evolved through the expansion of odontogenic competence from external to internal epithelia (Rücklin *et al.*, 2011; Donoghue & Rücklin, 2016). External odontode development in early vertebrates undoubtedly required the interaction between ectoderm and mesenchyme. The question is, was an interaction of mesenchyme with ectoderm still required when odontodes started to appear in the oropharyngeal cavity, a tenet of the ‘modified outside-in’ hypothesis? The fossil record is naturally silent about this question (Janvier, 2015). Furthermore, is ectoderm in some form (still) required to allow tooth formation inside the oropharynx? Ongoing studies on oropharynx and tooth development in extant vertebrates can possibly provide answers.

Some of the studies presented in the previous sections appear, at first sight, to contradict the requirement for ectoderm in the formation of internal odontodes (teeth or pharyngeal denticles). For example, the experiments of Adams (1924) and of Cassin & Capuron (1979) in urodele amphibians suggested that endoderm alone can form (oral) teeth in conjunction with neural crest. Challenging these ideas, it was recently shown that pharyngeal teeth in zebrafish develop from endodermal epithelium but only where covered by a superficial layer with periderm-like characters (Oralová *et al.*, 2020).

Below, we propose a hypothesis that attempts to integrate the different and often conflicting results. The observations on lungfish dentitions are inspiring in this context. Here the cells that line the oral cavity are described to be of endodermal origin. Yet, the superficial cells later metabolise their yolk and acquire characters similar to cells of the outer layer of the external epidermis, such as the appearance of microvilli and tight junctions, while the enamel organ differentiates from a basal layer that maintains its yolk as the tooth primordia begin to form (Kerr, 1903; Kemp, 2002a, 2003).

The scenario that we propose for the evolutionary developmental biology of pharyngeal teeth or denticles is described below and depicted in Fig. 10A–D. In the primitive situation, represented by fossil taxa, skin denticles are proposed to have enamel organs derived from the basal layer of a bi- (possibly multi-) layered ectodermal epithelium; endodermal pouches contact the ectoderm (Fig. 10A). At some point, perhaps even before the advent of jawed vertebrates, enamel organs are suggested to have appeared in the endoderm of the pouches, likely as the result of transfer of competence from the ectoderm. However, it is hypothesised that this transfer only happened if and where the endoderm maintained an interaction with ectoderm, resulting from the invasion of the outer ectodermal layer (the periderm) into the oropharyngeal cavity (as in Fig. 10C). The recent study of Chen *et al.* (2020) on the expansion of dermal denticles in the stem osteichthyan *Lophosteus superbus* illustrates how morphogenetic movements of cell layers may play an important role in the evolutionary origin of novel structures.

In chondrichthyans, which can develop an extensive covering of denticles in the oropharynx, the superficial layer of

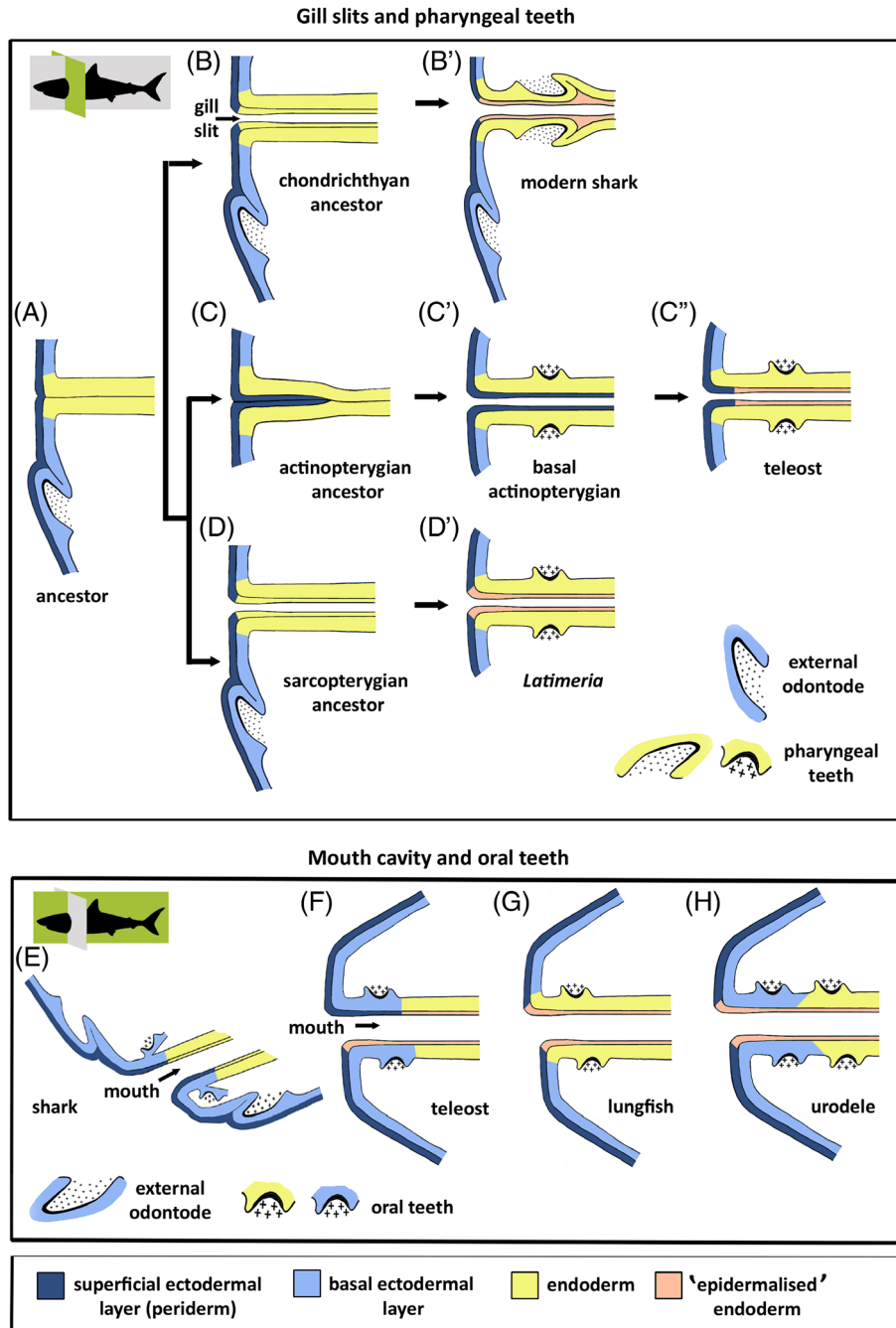


Fig 10. Germ layer contribution to pharyngeal and oral teeth. (A–D') Hypothetical evolutionary scenario for the persisting influence of ectoderm on pharyngeal tooth formation. (A) Primitive situation; external odontode enamel organ derives from basal ectodermal layer; endodermal pouches contact the ectoderm and endoderm partially spreads under the periderm. (B, B') In the chondrichthyan lineage, endoderm of the pouches is stratified and the superficial layer is 'epidermalised'; pharyngeal teeth derive from the basal endodermal layer (modern sharks, B'). (C–C'') In the actinopterygian lineage, periderm invades the pouches, ultimately covering the entire pharyngeal endoderm (basal actinopterygians, C'); in advanced actinopterygians, periderm invasion is restricted to the distal parts of the pouches, while endoderm is 'epidermalised' medially; pharyngeal teeth derive from the basal endodermal layer (teleosts, C''). (D, D') In the sarcopterygian lineage, endoderm of the pouches is stratified and the superficial layer is 'epidermalised'; pharyngeal teeth derive from the basal endodermal layer (*Latimeria chalumnae*, D'). It is possible that basal actinopterygians took path D, leading to the situation in teleosts (i.e. periderm invasion would be a teleost novelty rather than a regressive feature). A study of basal actinopterygians will resolve this issue. (E–H) Schematic representation of germ layer contribution to oral tooth formation in extant vertebrates, as inferred from literature data. (E) Shark; dental lamina derived purely (Figure legend continues on next page.)

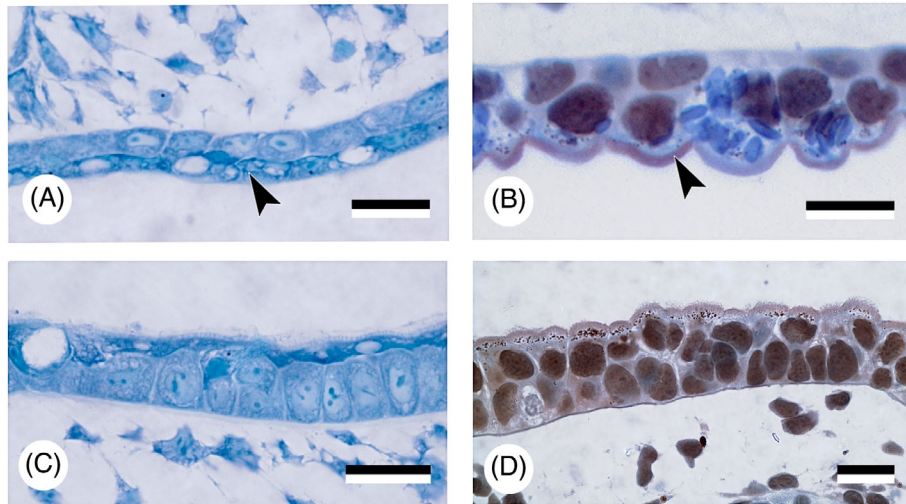


Fig 11. ‘Epidermalised’ endoderm. Detail of the ‘epidermalised’ endodermal epithelium (arrowheads) in the roof of the pharyngeal cavity (A, B) and of the ectodermal epithelium of the skin (C, D) in *Acipenser ruthenus* (A, C) and *Ambystoma mexicanum* (B, D). Note similarities in differentiation of the superficial cells. Scale bars = 25 µm.

endoderm is proposed to have been co-opted into a layer with ectoderm-like characteristics (Fig. 10B–B’). Interestingly, Cook & Neal (1921, p. 45) described the pharyngeal epithelium in *Squalus acanthias* (a shark with pharyngeal denticles) as follows: “the pharyngeal epidermis resembles that of the skin rather than epithelium such as that of the stomach and intestine”. Peyer (1968, p. 72) suggested that the distribution of “mucous membrane” denticles (his terminology) in sharks may be caused by “boundary displacements during embryonic development”, with which he indicated a shift of the posterior limit of the stomodaeum. A possible interpretation for chondrichthyan oral teeth is shown in Fig. 10E.

In teleosts, the pharyngeal epithelium is bilayered in taxa that represent both basal and highly evolved members of the group: members of the anguillids, salmonids, cyprinids, and cichlids (Rosa *et al.*, 2019). Provided that zebrafish can be considered to represent the teleost situation, the basal layer – the layer that produces the enamel organ – is endodermal; it is covered in the oropharynx and oesophagus by a superficial layer with periderm-like characters. This layer covers the endoderm prior to periderm invasion *via* the pouch. Again this possibly represents a case of co-option of endoderm (Fig. 10C’). The extent of the periderm-like layer (down to the oesophagus, but not further) delimits the area from which enamel organs can develop (thus, including the

oesophagus, explaining the oesophageal teeth observed in some teleosts). Interestingly, it coincides with the demarcation between the pharyngeal endoderm anteriorly and the presumptive alimentary canal posteriorly, which develop independently from each other (Ober, Field & Stainier, 2003; Wallace & Pack, 2003; Kobayashi *et al.*, 2006; Sucré *et al.*, 2009). Note, in this context, that extra-oral teeth in teleosts arise in the skin ectoderm only (Sire & Allizard, 2001), never in the gut. Whether a restricted invagination of periderm into the pouches is a teleost-specific regressive feature (Fig. 10C–C’), or whether co-opted endoderm (Fig. 10D–D’) is rooted deep in actinopterygian or even osteichthyan phylogeny, can be tested by studying basal actinopterygians with less-specialised early embryonic development. Unfortunately, these taxa are largely understudied in relation to the composition of ectodermal (skin) and oropharyngeal epithelia. Likewise, teleost species other than cyprinids need to be tested to assess germ layer contributions to oral teeth (Fig. 10F).

With respect to sarcopterygians, in dipnoans (which have oral teeth only), the enamel organs form from endoderm but also here the upper layer adopts features of a superficial ectodermal layer, as commented on in Section II.3a (Kerr, 1903; Kemp, 2002a, 2003) (Fig. 10G). A name that we suggest for this condition is ‘epidermalised endoderm’,

(Figure legend continued from previous page.)

from ectoderm. (F) Teleost; both periderm and periderm-like layer may constitute the superficial layer of the oral epithelium; enamel organs putatively derived from ectoderm. (G) Lungfish; the oral cavity is lined by endoderm, which also gives rise to the enamel organs. However, the superficial cells have acquired characters similar to cells of the outer epidermal layer. (H) Urodele amphibian; the basal ectodermal layer invades the mouth, oral teeth derive from the basal layer (either ectoderm or endoderm) but are covered by ‘epidermalised’ endoderm. Shark profiles show the plane of sectioning (in green): cross sections for gill slits and pharyngeal teeth (upper panels), sagittal sections for mouth cavity and oral teeth (lower panels).

similar to ‘ectodermalised endoderm’, a term that was coined by Hayashi (1972) for the events occurring at opening of the stomodaeum in the newt *Triturus pyrrhogaster* (“ectodermalization of endodermal cells”). In urodele amphibians, the oral teeth can form from ectoderm, from endoderm or have a mixed origin (Adams, 1924; Chibon, 1970; Soukup *et al.*, 2008). Experimental data have shown that endodermal teeth can develop fairly independently from ectoderm, but that, by contrast, ectodermal teeth need endoderm (Adams, 1924, 1931), a situation that seems at odds with our claim of the need for ectoderm. Provided the superficial endodermal layer adopts an ectodermal phenotype (‘epidermalised endoderm’) – and histology suggests that it does (Fig. 11) – this observation is in line with the situation described for lungfish enamel organs (Fig. 10H). In the case of the ectodermal teeth, the enamel organ, forming as a folding of the basal ectodermal layer, is itself covered by a superficial layer of endoderm (or rather, ‘epidermalised endoderm’). The function of this layer is supposedly the same as for the endodermal teeth, that is it acts as a layer co-opted into an ectodermal/peridermal fate. Moreover, in this review it is extensively argued that enamel organs never develop from a monolayer. A co-option of the superficial endodermal layer into an ectodermal/peridermal identity would explain why endodermal teeth can develop fairly independently from the ectodermal collar, but ectodermal teeth need the endodermal plug reaching up to the body surface. This interpretation could also explain the findings of Ohazama *et al.* (2010). These authors reported that mouse teeth possess a dual molecular (ectoderm/endoderm) origin according to position, although we now know that the epithelial components of mouse teeth are strictly ectodermal by origin (Rothova *et al.*, 2012).

The ectoderm–endoderm boundary is often represented as a head-to-tail contact, as for example in the buccopharyngeal membrane, although intermingling of cells has been described [e.g. chick (Waterman & Schoenwolf, 1980); hamster (Waterman, 1977); reviewed in Soukup *et al.*, 2013]. The presence of a periderm-like layer covering the endoderm in zebrafish (Rosa *et al.*, 2019; Oralová *et al.*, 2020), or of ‘epidermalised endoderm’ in axolotls (A. Huysseune, personal observations), strongly suggests that this boundary can also take the form of an interface between two superimposed layers. Different authors have stressed the importance of an ectoderm–endoderm boundary in tooth formation. Smith & Coates (2001) and Smith (2003) suggested that the ectoderm–endoderm boundary is crucial for setting up positional information for patterning of the dentition. Recently, Soukup *et al.* (2021) identified the ectoderm–endoderm boundary in the axolotl as a single tooth-competent zone giving rise to both dental arcades. In the *talpid* chicken mutant, the formation of teeth is coupled with alterations in the specification of the oral/aboral boundary of the jaw (Harris *et al.*, 2006). One might speculate that this novel oral/aboral boundary reflects a shift of the ectoderm–endoderm boundary.

The scenario depicted above supports the role of ectoderm, and aligns with the ‘modified outside-in’ hypothesis

for tooth origins. Indeed, for each of the taxa listed, enamel organs develop from a stratified epithelium with distinctive characters between the basal and superficial layer, and with the superficial layer bearing ectodermal/peridermal characters. The apparent need for stratification is conceivable from a functional viewpoint. In gill-breathing vertebrates the oropharynx is open to the surrounding environment and the superficial layer must maintain a function as a protective covering, with roles as an osmotic, immunological and/or mechanical barrier, whilst the basal layer can differentiate into a particular derivative.

A question that remains is how to explain the sustained need for pouch–cleft contact for pharyngeal tooth formation (as in zebrafish), if it is not required as a channel to allow an influx of ectoderm or periderm. An answer to this question awaits further experimental approaches and dissection of the molecular signalling taking place at these contacts, and especially, examination of both more basal and more derived taxa.

VII. CONCLUSIONS

- (1) Teeth in vertebrates can be distributed, depending on the lineage, in the mouth and on the palate (oral teeth), as well as on any of the post-mandibular arches (pharyngeal teeth). With the evolutionary origin of internal from external odontodes being firmly re-established, the formation of pharyngeal teeth (and denticles), continues to present a challenge to explain how odontode-forming epithelium from the skin could have been ‘transferred’ into the oropharynx to initiate tooth or denticle formation, in conjunction with neural crest-derived mesenchyme.
- (2) The ‘modified outside-in’ hypothesis proposed that this transfer was accomplished through displacement of competent odontogenic epithelium, that is ectoderm, not only through the mouth, but *via* any channel connecting the ectoderm to the epithelial lining of the pharynx (endoderm), such as the gill slits.
- (3) Historic studies based on histology, as well as recent research based on modern cell lineage-tracing techniques, have firmly established that the epithelial component of teeth, the enamel organ – whether oral or pharyngeal – can derive from ectoderm, endoderm, or have a mixed origin. In line with the ‘modified outside-in’ hypothesis, it is argued here that ectoderm continues to be required in some form to initiate tooth formation, even if teeth derive (partially) from endoderm.
- (4) A survey of experimental studies on pharyngeal tooth formation in teleosts and on oral tooth formation in amphibians (the two lineages for which data are available) reveals a seemingly inconclusive picture as to whether only one (either ectoderm or endoderm) or both germ layers are required to initiate tooth

formation. However, upon closer inspection it appears that independent from layer origin, the prospective enamel organ is covered by a layer with characteristics reminiscent of ectoderm. In zebrafish this is a layer resembling the periderm (or outer epidermal layer), and is possibly co-opted from endoderm through evolutionary changes in gene expression. In urodele amphibians, it is the superficial endodermal layer that adopts ectodermal characteristics, and is therefore termed ‘epidermalised endoderm’. Thus, while ectoderm may no longer invade deep into the pharynx, its role may have been taken over through co-option.

- (5) The apparent lack in zebrafish of ectodermal invasion into the pouches prior to tooth initiation, must be considered against the highly derived type of early embryonic development displayed by teleosts, characterised by telolecithal eggs and meroblastic cleavage. There is an urgent need to study basal actinopterygians with holoblastic cleavage to establish whether a bilayered ectoderm is basal for the lineage, whether its superficial layer is homologous to the teleost periderm, whether this layer penetrates into the pharynx, and whether it participates in tooth formation.
- (6) Even though ectoderm, at least in zebrafish, does not penetrate deep into the pharynx *via* gill slits, pouch–cleft contacts (prospective gill slits) are nevertheless required for pharyngeal tooth initiation. A survey of tooth distribution in both actinopterygians and sarcopterygians confirms the close association of pharyngeal teeth with pouches. The nature of the signal at the pouch–cleft contact, whether molecular or mechanical, remains enigmatic.
- (7) The pouch that separates the hyoid from first branchial arch (pouch 2) may well possess a special identity. It constitutes an important borderline separating developmental programs from anterior and posterior pharyngeal arches, as it opens first in many species and serves as passageway for initial invasion of periderm in zebrafish.
- (8) While pouch–cleft contacts are demonstrably important in tooth initiation, this association cannot on its own explain the distribution of post-mandibular teeth. Clearly, teeth are found only near pouch–cleft contacts, but neither the sequence of development of the contacts, nor their number, is indicative for how the patterning of the teeth progresses in ontogeny.
- (9) Because the likely basal condition in extant gnathostomes is to have teeth spread over all arches (Nelson, 1969, 1970), it is safe to assume that, in evolution, the capacity for tooth formation was lost on tooth-bearing arches, rather than gained on edentulous arches. One molecule regulating tooth distribution on the different arches is retinoic acid (RA), whose role is discussed, as well as its possible intersection with sonic hedgehog (SHH) signalling. It is

proposed that a balance between SHH and RA signalling may be operating to assign tooth-initiating competence to the endoderm at any particular pouch–cleft contact.

- (10) It follows from the above description that teeth develop invariably where ectoderm meets endoderm (at pouch–cleft contacts or at the mouth), that teeth always form from the basal layer of a bi- (or multi-) layered epithelium, and that the surface layer appears to adopt ectodermal characteristics (either as periderm, or as ‘epidermalised endoderm’). Ectoderm–endoderm boundaries may thus not necessarily take the shape of head-to-tail contacts, but can also form the interface between two superimposed cell layers, constituting a broad area with odontogenic competence.
- (11) Together, three requirements (pouch–cleft contacts, a stratified epithelium and a surface layer that is ectodermal or epidermalised) appear to limit the distribution of teeth within the oropharynx. The proposed role of ectoderm in tooth initiation, irrespective of the enamel organs’ germ layer origin, is in line with the ‘modified outside-in’ hypothesis of tooth origins.

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