Cell Tissue Res (2007) 328:167–183
DOI 10.1007/s00441-006-0306-1

REGULAR ARTICLE

Enameloid/enamel transition through successive tooth replacements in *Pleurodeles waltl* (Lissamphibia, Caudata)

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Received: 27 March 2006 / Accepted: 13 July 2006 / Published online: 19 December 2006

Abstract Study of the evolutionary enameloid/enamel transition suffers from discontinuous data in the fossil record, although a developmental enameloid/enamel transition exists in living caudates, salamanders and newts. The timing and manner in which the enameloid/enamel transition is achieved during caudate ontogeny is of great interest, because the caudate situation could reflect events that have occurred during evolution. Using light and transmission electron microscopy, we have monitored the formation of the upper tooth region in six successive teeth of a tooth family (position I) in *Pleurodeles waltl* from late embryos to young adult. Enameloid has only been identified in embryonic tooth I_1 and in larval teeth I_2 and I_3. A thin layer of enamel is deposited later by ameloblasts on the enameloid surface of these teeth. From post-metamorphic juvenile onwards, teeth are covered with enamel only. The collagen-rich enameloid matrix is deposited by odontoblasts, which subsequently form dentin. Enameloid, like enamel, mineralizes and then matures but ameloblast participation in enameloid matrix deposition has not been established. From tooth I_1 to tooth I_3, the enameloid matrix becomes ever more dense and increasingly comes to resemble the dentin matrix, although it is still subjected to maturation. Our data suggest the absence of an enameloid/enamel transition and, instead, the occurrence of an enameloid/dentin transition, which seems to result from a progressive slowing down of odontoblast activity. As a consequence, the ameloblasts in post-metamorphic teeth appear to synthesize the enamel matrix earlier than in larval teeth.

Keywords Tooth replacement · Enameloid · Enamel · Transmission electron microscopy · Amphibia · *Pleurodeles waltl* (Urodela)

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Introduction

In vertebrates, teeth are protected from wear and injury by either enamel or enameloid (Huysseune and Sire 1998). These hard tissues, although similar when well mineralized, differ in the structure and organization of their organic matrix prior to mineralization and maturation and are differently distributed within vertebrate lineages. Enameloids are present in chondrichthyans (sharks and rays: Sasagawa 2002), actinopterygians (bony fish: Shellis and Miles 1976; Sasagawa 1988) and early larval stages of caudate amphibians (newts and salamanders: Chibon 1970; see review in Davit-Béal et al. 2006a). Prismatic enamels are found in mammals (Koenigswald 1997), whereas prismless enamels exist in reptiles (Sander 2001), adult amphibians (Schmidt 1970; Spinelli and Chibon 1978) including the neotenic axolotl (Bolte and Clemen 1992) and lungfish (Smith 1992; Kemp 2002).

The enamel organic matrix is mainly composed of specific proteins (amelogenin, enamelin and ameloblastin), which are exclusively deposited by epithelial cells, viz. the ameloblasts. In contrast, the organic matrix in enameloids is principally composed of a loose dentin-like collagenous network, which is deposited by mesenchymal cells, viz. the odontoblasts, prior to their involvement in dentin matrix deposition. Enameloid mineralizes more strongly than dentin and a maturation process takes place, which suggests

the active participation of ameloblasts, giving rise to an extremely hard tissue similar to enamel. This mode of formation explains why this protective tissue has previously been called either “durodentin” (Schmidt 1957) or “mesodermal enamel” (Kvam 1960). These confusing terms have subsequently been replaced by "enameloid" (Poole 1967; Ørvig 1967), a name that is widely accepted, although used to include all types of enamel-like tissues that are not true enamel. Structural descriptions and immunohistochemical studies have suggested that ameloblasts play a role in enameloid matrix formation and mineralization, e.g. showing the presence of enamel matrix proteins such as enamelin and/or amelogenin (Herold et al. 1989). Alternatively, ameloblast action might be restricted to the end of mineralization and to the maturation process, during which the organic matrix progressively disappears as for enamel (Sasagawa 2002). However, these data need to be confirmed by using molecular approaches aimed at identifying the expression of enamel protein and matrix metalloproteinase genes in the ameloblasts lining the enameloid.

Enameloids are not well known in comparison with enamels, for which a large amount of data is now available, although studies have mostly been restricted to mammalian enamels. Despite the mode of formation of enameloid being similar in various lineages, the different organization and composition of the organic matrix and the typical mineralization process of enameloids have led to their being split into two types. The term “enameloid” is conserved for actinopterygians and larval caudate enameloids, although “acrodin” was previously proposed for the enameloid of fossil actinopterygians (Ørvig 1978). In chondrichthyans, a subtype of enameloid, called “coronoin”, has been described by Bendix-Almgreen (1983) in a fossil shark and coined “adameloid” in modern chondrichthyans (Sasagawa 2002). The tubular vesicles in which the palisade-type mineral crystals are initialized distinguish adameloid from the other enameloids. In the latter, the initial mineralization is associated with matrix vesicles and then crystallites are oriented along the collagen fibrils (Sasagawa 1988). Nevertheless, aside from these structural differences, osteichthyan enameloids and chondrichthyan adameloid share numerous features that indicate evolutionary relationships. The currently observed differences are related more to parallel evolution during the long geological time separating living chondrichthyans and osteichthyans from their last common ancestor (approximately 450 million years) than to convergency.

Enameloids and enamels are also evolutionary related. The question as to which tissue appeared first in vertebrate evolution has fed a long debate between those authors who consider enamel to be a primitive tissue (e.g. Smith 1992) and those authors who believe enamel to be derived from enameloid (Poole 1967; Reif 1979; Slavkin and Diekwisch 1996). Answering this question is important because some osteichthyan relationships are based on the presence or absence of enameloid (Rosen et al. 1981; Meinke and Thomson 1983; Schultze 1986; Panchen and Smithson 1988). Increasing evidence indicates that enamel has arisen from enameloid (Kawasaki et al. 2005).

However, the story is not that simple. When studying the upper tooth region in detail, a common feature in sharks (Poole 1971; Suga et al. 1978; Clement 1984; Slavkin and Diekwisch 1996), teleost fish (Shellis and Miles 1974; Reif 1982) and larval caudates (Chibon 1970) is the presence of a thin enamel layer on the enameloid/adameloid surface. This suggests that enamel and, as a consequence, enamel-specific proteins appeared soon during vertebrate evolution, at least in crown gnathostomes (*sensu* Donoghue and Sansom 2002). They even might have been present earlier in view of the thin enamel layer shown to be present on the odontodes ornamenting the body surface of the Middle Ordovician jawless vertebrate *Eriptychius* (Smith and Hall 1990). Thus, enamel differentiation has to be traced back deep into vertebrate history, both tissues coexisting in the skeletons of early jawless vertebrates. Indeed, the switch between enameloid and enamel has occurred many times and independently and is interpreted as a result of the dissociation in timing of the secretion of specific matrix proteins by the odontoblasts and the ameloblasts, i.e. heterochrony (Smith 1995). Whatever the origin of enamel in early vertebrates, basal osteichthyans later exhibited enamel that was initially deposited as a thin layer over the enameloid. In actinopterygians, the enamel layer did not thicken except for the ganoin of polypterid and lepisosteid scales (Sire et al. 1987; Sire 1994) and even disappeared in some teleost species (Sasagawa and Ishiyama 1988). In sarcopterygians, the enamel layer became thicker as ameloblasts became more active in the deposition of enamel specific proteins (Smith 1995).

The situation in caudate teeth is particularly interesting in that it might reflect aspects of tooth evolution and might aid our understanding of the enameloid/enamel transition. Caudates, as all non-mammalian taxa, replace their teeth thoughout life. In contrast to other tetrapods (anurans, reptiles and mammals), they possess toothed larvae. Of note, not all caudates have free-living larvae, many species being direct developers with a few being viviparous. We assume that tooth development and replacement in these species are similar to those known in oviparous caudates, although the features of the first-generation teeth might slightly differ. Moreover, many species of caecilians (gymnophiones) have toothed larvae but these have been omitted from this consideration: they simply resemble caudates in the features mentioned.

The teeth of caudates might thus represent an ancestral condition for osteichthyans (Sire et al. 2002). In larval

caudate teeth, enameloid is covered by a thin layer of enamel (Smith and Miles 1971; Chibon 1972; Roux and Chibon 1973; Roux 1973). After metamorphosis, enameloid is no longer recognizable and is replaced by a thick layer of enamel (Chibon et al. 1971; Smith and Miles 1971). However, the timing and manner in which the enameloid/enamel transition is achieved through successive tooth replacements during caudate ontogeny are still unknown. Is enameloid only present in the first-generation teeth and does it disappear suddenly in the following replacement teeth or progressively through successive tooth generations? Is enameloid present in larval teeth until metamorphosis and is it suddenly replaced in the next tooth generation by enamel? Answering these questions is a prerequisite for undertaking studies such as the tracing of enamel matrix protein expression during enameloid matrix formation.

Using light and transmission electron microscopy, we have examined the structure and organization of the tooth tip matrix throughout the ontogeny of *Pleurodeles waltl* with particular attention being paid to the fate of the enameloid layer. With this aim, we have monitored the formation of the upper region in successive teeth of a single tooth family, from the first-generation tooth in late embryos to the sixth tooth generation, in young sexually mature adults. This study is based on our previous description of tooth replacement chronology in *P. waltl* (Davit-Béal et al. 2006b).

Materials and methods

Materials

Pleurodeles waltl Michahelles, 1830 was bred in our laboratory. Eggs were obtained from September to May. Hatching occurred 12 days post-fertilization (dPF) and the larvae took their first prey (*Artemia nauplii*) at 1 week after hatching (19 dPF). Metamorphosis started after a larval period of approximately 100 days and sexual maturity was reached at 18 months. The larvae were staged according to the developmental table published by Gallien and Durocher (1957).

The development of each successive tooth in a family was studied on the right lower jaw (for position I), the position closest to the symphysis. From embryos to young adults, six tooth generations were studied (tooth I_1 to tooth I_6): the embryonic tooth, I_1; two larval teeth, I_2 and I_3; two juvenile teeth (post-metamorphosis), I_4 and I_5; the first adult tooth I_6. These ontogenetic stages and appropriate developmental steps were as defined in a previous study, in which we carefully monitored tooth replacement in a growth series from larvae to young adults (Davit-Béal et al. 2006b) and were useful for selecting each tooth in the family and for monitoring the morphological events occurring in the upper region of the tooth. A total of 26 specimens was used:

- first-generation tooth (five specimens), viz. embryonic stages 33a, 33b and 34 (9, 10 and 11 dPF, i.e. 3, 2 and 1 day before hatching, respectively) and larval stages 35 and 36 (12 and 13 dPF, i.e. at hatching and 1 day later);
- second-generation tooth (four specimens), viz. larval stages 38, 39 (two specimens) and 42 (17, 20 and 28 dPF, respectively);
- third-generation tooth (eight specimens), viz. larval stages 48 to 55a (50 to 90 dPF);
- fourth-generation tooth (four specimens), viz. larval stage 56 (two specimens: 110 dPF) and 4-month-old juveniles (two specimens);
- fifth-generation tooth (two specimens): 5- and 8-month-old juveniles;
- sixth-generation tooth (three specimens): one 12- and two 18-month-old specimens.

All specimens were anaesthetized (MS222), measured and killed by decapitation in accordance with the French law on animal experimentation (no. 87–848, 19 October 1987).

Methods

Depending on size, the specimens were fixed either entirely (embryos and larvae until stage 42) or only the head (larval stages 43–52) or the lower jaw (specimens larger than 25 mm) was fixed. The samples were immersed for 2 h at room temperature in a mixture containing 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). Some samples were decalcified either for 3 days (embryos and larvae until stage 42), 7 days (larval stages 43–51), 15 days (larval stages 52–56 and young juveniles) or 21 days (old juveniles and adults) at 4°C in the same fixative, to which 5% EDTA was added. The decalcifying mixture was changed every 2 days. After being rinsed in the same buffer overnight at 4°C, the samples were postfixed in 1% osmium tetroxide in cacodylate buffer, rapidly rinsed, dehydrated through a graded series of ethanol and embedded in Epon 812 for serial sectioning (1 μm thick) with a diamond knife (Reichert OMU3-Leica). The sections were stained with toluidine blue and examined by light microscopy. In a few samples, the series were interrupted at appropriate locations for ultra-thin sectioning. The thin sections were contrasted with uranyl acetate and lead citrate and viewed in a Philips 201 transmission electron microscope operating at 80 kV.

The jaws of small specimens (embryos and early larvae) were sectioned either transversely or longitudinally to improve the chance of obtaining sections passing through the limited amount of enameloid at the very tip of the tooth.

Results

The present study describes the changes occurring in the upper tooth region during *P. waltl* ontogeny. The events taking place in other regions of the teeth (dentin, dividing zone, pedicel, attachment region and pulp cavity) have been described elsewhere (Davit-Béal et al. 2006b).

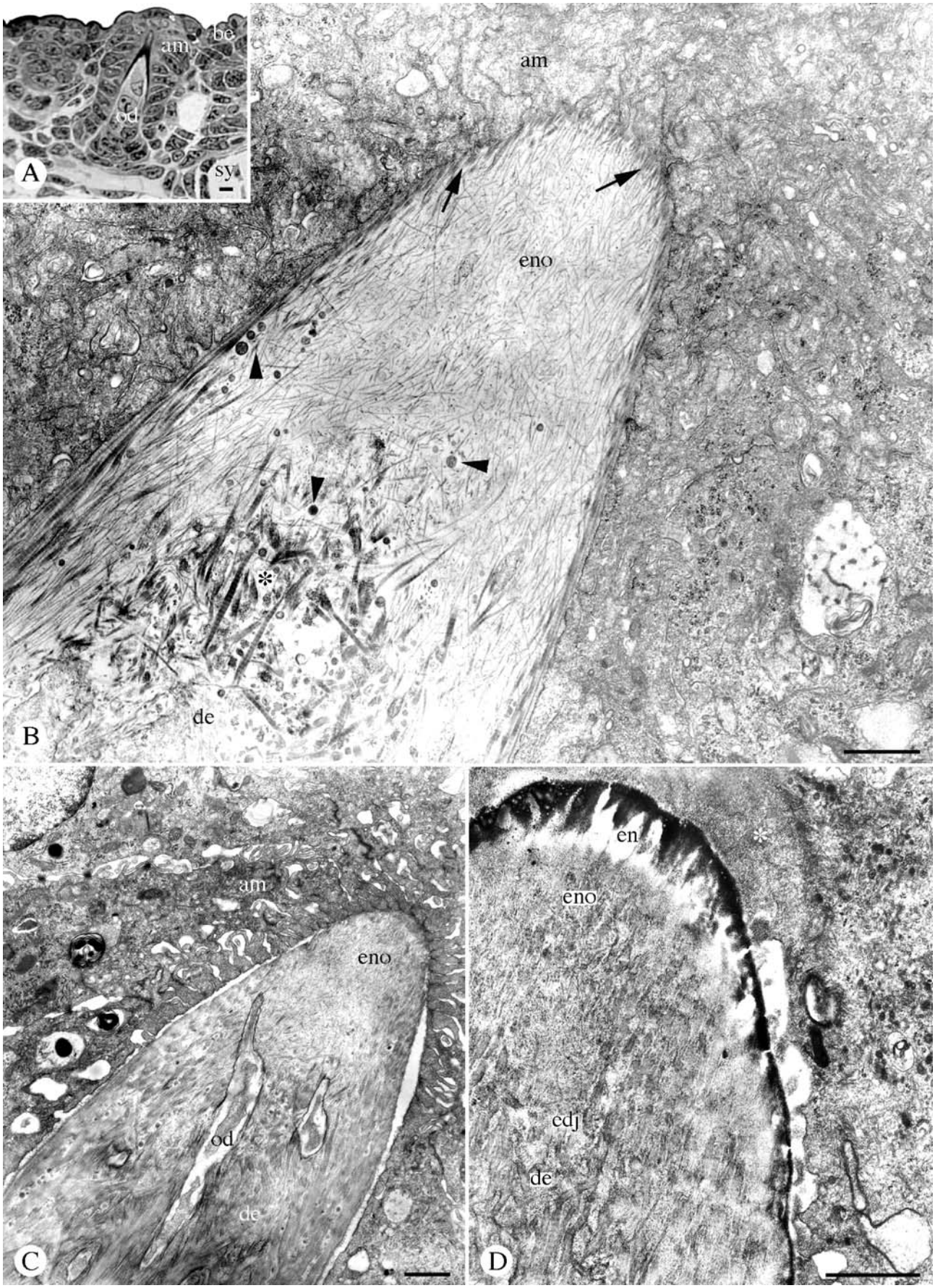
Tooth I_1

This so-called embryonic tooth develops from larval stage 33 (2 days before hatching, 9 dPF) to stage 37 (15 dPF), at which time it attaches to the bone support and becomes functional. Tooth I_1 is conical, monocuspid and small (80 μm high). The largest diameter of the upper region, measured at the limit between enameloid and dentin, is 4–5 μm and the distance from the dentin surface to the tooth tip is 5–6 μm. Sections passing through this region are, therefore, difficult to obtain and the various tissues are hardly distinguishable at the light-microscopical level (Fig. 1a). At the end of enameloid matrix formation, which occurs at stage 34 (11 dPF), the embryonic tooth is surrounded by the enamel organ, which consists of two cell layers: the outer dental epithelium composed of elongated cells hardly visible at the light-microscopical level, and the inner dental epithelium, a well-defined layer composed of large juxtaposed polarized cells (ameloblasts). Such a two-layered enamel organ has been found in all teeth in the series examined, from tooth I_1 to tooth I_6: neither a stratum intermedium nor a stellate reticulum are present in *P. waltl*. The enameloid cap is surrounded by 6–7 well-differentiated ameloblasts with a nucleus located distally. Mitochondria, Golgi apparatus, free ribosomes and rough endoplasmic reticulum (RER) cisternae are mostly located in this region of the cytoplasm (not shown). In the proximal region, close to the enameloid surface, the cytoplasm is electron-dense and contains numerous vesicles of variable shape and diameter (Fig. 1b). Some of these vesicles are filled with an electron-dense granular substance. This region of the ameloblasts located at the tooth tip is also characterized by some membrane folds. No basement membrane can be distinguished between the cell membrane and the enameloid matrix (Fig. 1b).

At the light-microscopical level, the still-unmineralized enameloid and dentin matrices are deeply stained with toluidine blue, whereas the predentin matrix is unstained (Fig. 1a). The enameloid layer is not homogeneous being composed of a central region with loose, randomly organized, thin (10 nm in diameter) collagen fibrils surrounded, towards the periphery, by a narrow region consisting of densely packed, thick (30–40 nm) collagen fibrils (Fig. 1b). These fibrils are oriented parallel to the long axis of the tooth. At the tooth tip, the collagen fibrils exhibit a strong relationship with the plasma membrane of the ameloblast, to which they seem to anchor (Fig. 1b). Some fibrils enter the narrow extracellular spaces created by the membrane folds. In the deep region of the enameloid, numerous cytoplasmic prolongations of the odontoblasts are visible close to the predentin surface. Here, the enameloid matrix is highly heterogeneous with randomly organized, large collagen fibrils and numerous matrix vesicles (Fig. 1b).

Embryonic tooth I_1 is so small that the pulp cavity is entirely filled with only a dozen odontoblasts, with a single odontoblast occupying the tip of the cavity. This single cell first deposits the enameloid matrix and is then involved in the production of the dentin below (Fig. 1a). The nucleus of this odontoblast is located in the distal region of the cytoplasm, whereas the proximal region is occupied by a well-developed RER network, Golgi apparatus, mitochondria and numerous vesicles (not shown). The other odontoblasts are less active, as illustrated by their high nucleo-cytoplasmic ratio. They are involved in the deposition of the predentin matrix of the forming tooth shaft (Fig. 1a). The limit between the enameloid and dentin, the so-called enameloid-dentin junction, is recognizable but not well defined. This is perhaps understandable as the same odontoblast is responsible for the deposition of both matrices in this region (Fig. 1b,c). Instead, the number and diameter of the collagen fibrils increase progressively from the enameloid to predentin layer. This occurs particularly in the peripheral regions in which the features suggest that several thin collagen fibrils aggregate to form the larger fibrils (50–75 nm diameter) of the predentin. Such a progressive transition is less clear in the central region but this might be related to the presence of numerous

Fig. 1 First tooth developing at position I: the embryonic tooth, I_1. ▶ EDTA decalcified (**a–c**) or undecalcified (**d**) samples. **a** Larval stage 36 (13 dPF). Well-formed tooth, shortly before attachment. At the tooth tip, enameloid has started to mature as indicated by the lighter staining. Enameloid and dentin matrices are hardly distinguishible. A single odontoblast (*od*) occupies the tip of the pulp cavity (*am* ameloblasts, *be* buccal epithelium, *sy* symphysis). *Bar* 10 μm. **b** Late embryo, stage 34 (11 dPF). End of enameloid deposition (*am* ameloblast). Enameloid (*eno*) is composed of collagen fibrils (*arrows*) that seem to be anchored to the cell membrane. Note the disorganized aspect of the matrix at the enameloid-dentin junction (*asterisk*) and the presence of matrix vesicles in the dentin (*de*) and enameloid (*arrowheads*). *Bar* 1 μm. **c** Larval stage 35 (12 dPF). Beginning of enameloid maturation. Odontoblast tubules reach the enameloid-dentin junction. Note the characteristic folded aspect of the ameloblast membrane along the tooth tip and the presence of numerous vacuoles in the cytoplasm, indicating that the maturation process has begun (*am* ameloblasts, *de* dentin, *eno* enameloid, *od* odontoblasts). *Bar* 1 μm. **d** Larval stage 36. Oblique section through the upper region of the tooth. End of enameloid maturation (*de* dentin, *edj* enameloid-dentin junction, *en* enamel, *eno* enameloid). A thin electron-dense enamel layer covers the enameloid. The tooth tip is separated from the ameloblasts by a layer of granular unmineralized matrix (*asterisk*). *Bar* 1 μm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odontoblast cytoplasmic prolongations that reach the enameloid layer (Fig. 1b,c). The dentin layer is more electron-dense than the enameloid, indicating that a non-collagenous background subtance, synthesized by the odontoblast, has been added to the collagen network (Fig. 1c).

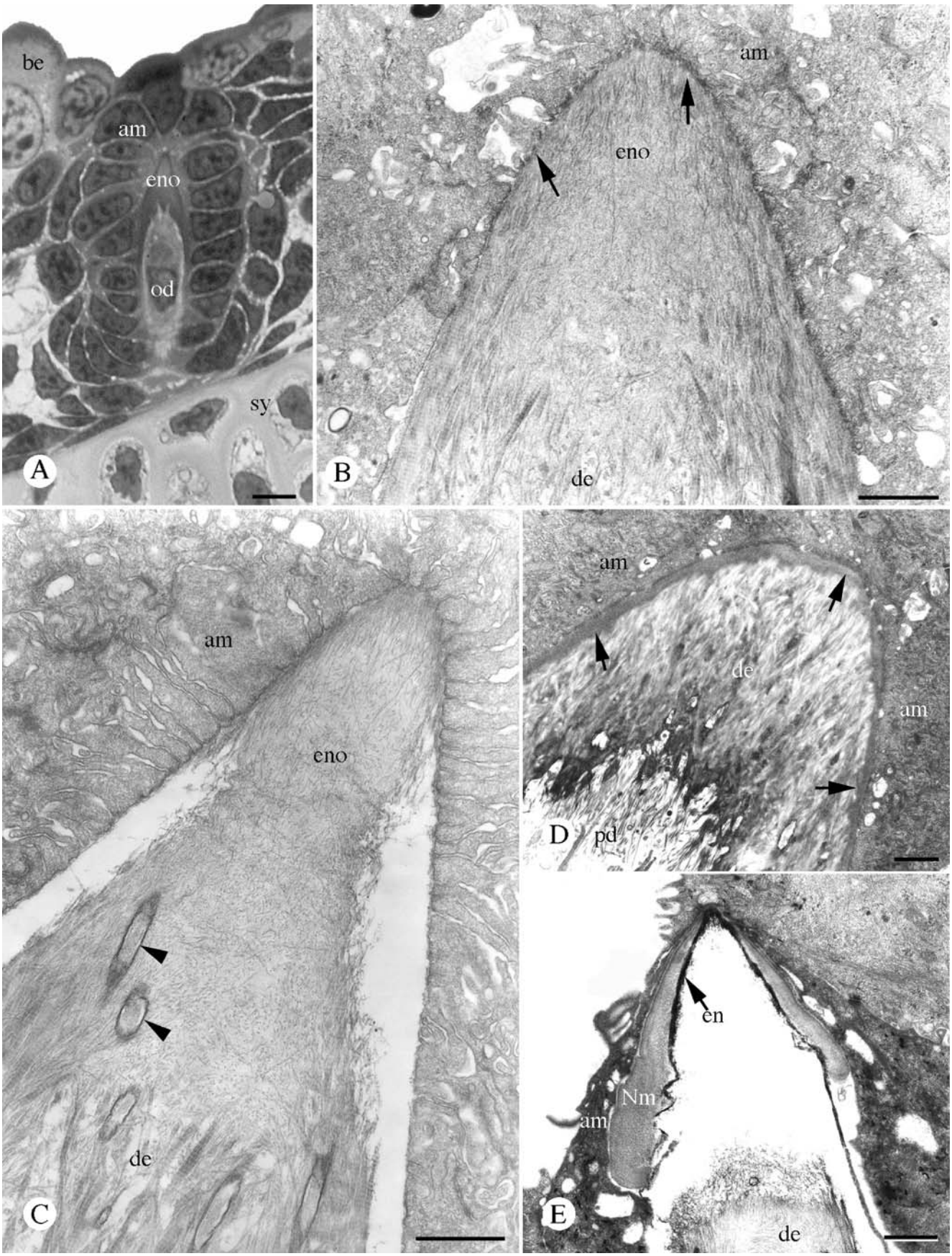
When enameloid and predentin are well formed, mineralization begins in both tissues simultaneously. The ameloblasts facing the mineralising enameloid show a well-developed ruffled border, characterized by extended foldings of the cell membrane and an organelle-free cytoplasmic region. Large extracellular spaces can be seen and numerous vacuoles are located in the cytoplasm (Fig. 1c). In contrast, along the tooth shaft, the ameloblasts have not developed a ruffled border and are separated from the dentin matrix by a basement membrane. At this stage of odontogenesis, the enameloid matrix is still visible after decalcification, suggesting that the maturation process, which involves proteolysis of the organic matrix, is not well advanced. No additional matrix that can be likened to enamel is detectable at the enameloid surface (Fig. 1c).

By the end of the embryonic tooth formation, dentin is well mineralized and the maturation process of the enameloid matrix is well advanced (Fig. 1d). In addition, a highly mineralized, electron-dense, thin (0.1–0.5 μm) layer covers the tooth surface. We interpretate this late-deposited layer as enamel but further analyses are necessary, in particular with respect to identifying the potential presence of enamel-specific proteins in this layer. The finding that this enamel layer is undetectable in previous stages strongly suggests that it is deposited rapidly and at the end of tooth formation. Facing this enamel layer, the ruffled border of the ameloblasts and the large vacuoles have disappeared (Fig. 1d). The cytoplasm is characterized by the presence of a large number of microfilaments and of small vesicles with an electron-dense content. A narrow (maximum: 1 μm) homogeneous unmineralized layer of a thin granular substance is located at the tooth surface (Fig. 1d). This amorphous material, which fills the narrow space located between the enameloid and attachment epithelium at the tooth tip has undoubtedly been deposited by the ameloblasts, the only cells present at the enameloid surface. The composition of this layer remains to be elucidated (either enamel-specific material or basement membrane components). It resembles a membrane through which the epithelium attaches to the tooth surface after completion of tooth formation and prior to eruption. In mammals, a similar membrane covering the tooth surface prior to eruption has been found and is termed Nasmyth's membrane (described by Alexander Nasmyth in 1839 as a "primary enamel cuticle"; Permar 1970); it is considered to be the final product of the degenerating ameloblasts after completion of enamel formation. Given the similarities (structureless and same spatio-temporal location) between these membranes, we consider that the membrane in *P. waltl* is homologous to the mammalian Nasmyth's membrane.

Tooth I_2

This first larval tooth I_2 develops from larval stage 36 (13 dPF) to stage 42 (28 dPF), at which time it becomes functional. Tooth I_2 is larger (250–300 μm high) than embryonic tooth I_1 but both show similar structural and developmental features. At the end of enameloid matrix deposition, which occurs at larval stage 38 (17 dPF), the tooth tip is surrounded by eight to nine ameloblasts, although only the five to six upper ones seem to be active in protein synthesis; the other inner dental epithelial cells located along the future tooth shaft possess a large nucleo-cytoplasmic ratio indicating that terminal differentiation is not completed (Fig. 2a). The tooth tip is 6–7 μm wide at its largest diameter. As described for tooth I_1, a single odontoblast deposits the enameloid matrix and is subsequently involved in predentin matrix formation. The cytoplasm of the ameloblasts facing the tooth tip is electron-dense and houses numerous vesicles of various sizes and free ribosomes. The cell membrane is mostly smooth, except in the region lining the tooth tip where it is folded (Fig. 2b). The central region of the enameloid layer is composed of small diameter (10–15 nm) collagen fibrils. This diameter increases progressively towards the dentin below and towards the ameloblast surface at the periphery. Most collagen fibrils are oriented parallel to the long axis of

Fig. 2 Second tooth developing at position I: the first larval tooth, I2. ▶ EDTA decalcified samples. **a** Larval stage 38 (17 dPF). End of enameloid formation. The tooth base is close to the cartilage surface of the symphysis (*sy*). In the upper tooth region, mineralization has begun as indicated by the central clearer zone. A single odontoblast (*od*) occupies the tip of the pulp cavity (*am* ameloblasts, *be* buccal epithelium, *eno* enameloid). *Bar* 10 μm. **b** Same stage (*am* ameloblasts, *de* dentin, *eno* enameloid). Note the numerous folds of the ameloblast membrane located along the tooth tip (*arrows* interactions of collagen fibrils with the cell membrane). *Bar* 1 μm. **c** Larval stage 39 (20 dPF). Enameloid maturation (*de* dentin). At the tooth tip, membrane folds of the ameloblast (*am*) are well developed. Note the loose aspect of the collagen fibrils of the enameloid (*eno*) facing the folded ameloblasts. The empty spaces along the ameloblast surface are artefactual: cells have detached from the matrix during the fixation process, except around the enameloid region. Odontoblast processes reach the enameloid region (*arrowheads*). *Bar* 1 μm. **d** Larval stage 42 (28 dPF). Slightly oblique section through the upper region of the dentin (*de*) in a recently attached tooth (*am* ameloblast, *pd* predentin). The dentin is covered by a narrow layer of enamel matrix (*arrows*). *Bar* 2 μm. **e** Same stage. Section through the upper tooth region (*de* dentin). The tooth tip is covered by flattened ameloblasts (*am*) and the enamel (*en*) and enameloid matrix have been removed during the maturation process. Mineral crystals have disappeared during decalcification. A thin dense layer of enamel matrix remains at the tooth surface. The tooth tip is covered by the Nasmyth's membrane (*Nm*). *Bar* 1 μm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the tooth and, in the uppermost region, they interact with the cell surface as described for tooth I_1.

By the end of the mineralization process, which occurs at larval stage 39 (19 dPF), the ameloblasts lining the enameloid matrix show features that characterize them as having entered the maturation process (Fig. 2c). The collagen fibrils of the enameloid matrix, from the tooth tip to the dentin region, are still visible but they have lost their characteristic striation. They appear thinner and are more loosely distributed than in the previous stage. These features confirm that the maturation process, which leads to the degradation of the collagen matrix, has begun in this region.

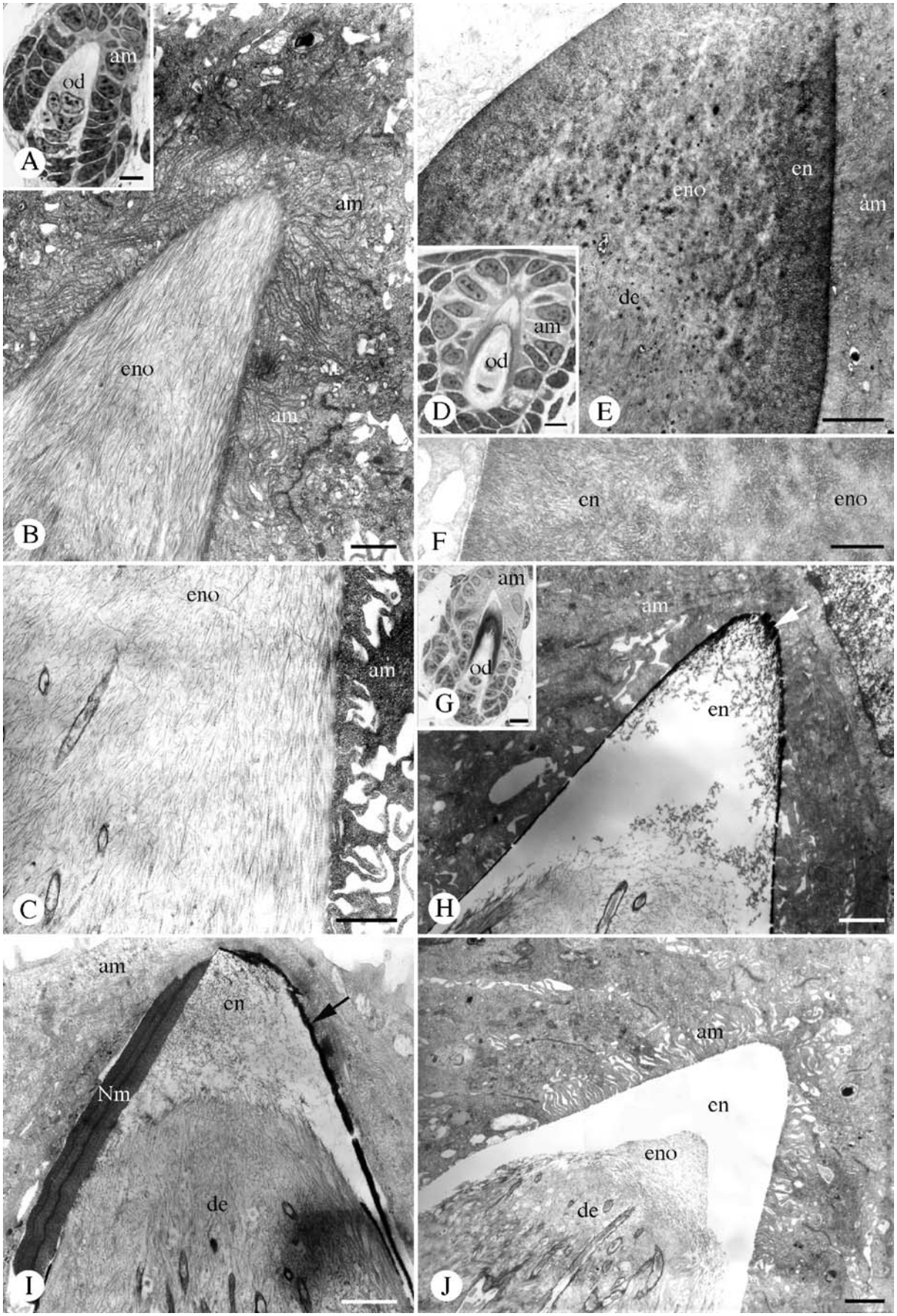
The end of tooth I_2 formation occurs at larval stage 42 (28 dPF). A thin (1 μm) layer of enamel has been deposited by the ameloblasts, both on the enameloid and the dentin surface, along the upper region of the tooth shaft (Fig. 2d). After decalcification, the enamel matrix appears as a thin homogeneous granular electron-dense material. Facing this layer, which is devoid of collagen fibrils, the ameloblasts are still well polarized but their ruffled border has disappeared and the number of vacuoles has diminished. The cytoplasm is more electron-dense than in the previous stage and, in the region facing the tooth surface, it contains numerous bundles of microfilaments, small vesicles and a large number of free ribosomes.

The maturation process of tooth I_2 is completed shortly before tooth eruption. In decalcified samples, most of the organic matrix of the enamel and enameloid has disappeared from the tooth tip to the dentin region, leaving a large empty space (Fig. 2e). A small amount of enameloid matrix persists at the level of the dentin-enameloid junction, which is now easily recognizable. Around the tooth tip, a thin, incompletely mineralized but electron-dense layer delimits the tooth surface from a homogeneous unmineralized granular layer located between the tooth and the ameloblasts. The dense layer is considered as the not-as-yet completely mineralized, or matured, outer region of the enamel layer, which has recently been deposited by the ameloblasts on the enameloid surface. Nasmyth's membrane, composed of amorphous material, covers the tooth surface. At the end of the maturation process, this membrane is still covered by the ameloblasts, which are elongated and show an electron-dense cytoplasm attributable to an increase in the amount of microfilaments and free ribosomes.

Tooth I_3

The second larval tooth I_3 develops in larvae from stage 44 (36 dPF) to stage 55a (90 dPF), at which time it becomes functional, a few days before metamorphosis, which starts at 100 dPF. Tooth I_3 is 400 μm tall and its tip is 10–12 μm in diameter at the level of the enameloid-dentin interface. This junction is ill-defined because (1) the transition between both matrices is progressive and (2) the enameloid matrix is denser than in the preceding teeth in the family, viz. I_1 and I_2. It resembles, therefore, predentin matrix (compare Fig. 3a,b with Figs. 1b, 2b). Enameloid matrix deposition is finished at larval stage 48 (50 dPF). Around the tooth tip, seven to eight of the ameloblasts are well differentiated and polarized, whereas the other ameloblasts located along the tooth shaft are not active, as indicated by their high nucleo-cytoplasmic ratio (Fig. 3a). At this stage, two odontoblasts are located at the tip of pulp cavity. They are responsible for the formation of the enameloid and for the subsequent dentin deposition in this region. In the central region of the enameloid, the collagen fibrils have a larger diameter (20–30 μm) compared with that (10–15 nm) measured in the same region of teeth I_1 and I_2. In the peripheral regions, the diameter of the collagen fibrils progressively increases to reach 50–75 μm close to the ameloblast surface, as described for teeth I_1 and I_2. Most of these fibrils are oriented parallel to the long axis of the

Fig. 3 Third tooth developing at position I: the second larval tooth, I_3. ▶ EDTA decalcified (**a–c**, **g–j**) or undecalcified (**d–f**) samples. **a** Larval stage 48 (50 dPF). End of enameloid formation (*am* ameloblasts). The enameloid matrix has been deposited by two odontoblasts (*od*). *Bar* 10 μm. **b** Same stage. The recently deposited enameloid matrix (*eno*) is denser than in the previous teeth (cf. Figs. 1b, 2b). Note the folded membrane of the ameloblasts (*am*) around the tooth tip. No enamel matrix is visible at the enameloid surface. *Bar* 1 μm. **c** Larval stage 49 (53 dPF). Enameloid mineralization (*am* ameloblasts). Deep region of the enameloid (*eno*). The enameloid-dentin interface is not clearly defined and odontoblast processes are embedded in the enameloid layer. *Bar* 1 μm. **d** Larval stage 51 (61 dPF). Advanced stage of mineralization (*od* odontoblasts). Upper region of the tooth. The mineralized region is not stained. In this region, the ameloblasts (*am*) are well polarized. *Bar* 10 μm. **e** Same stage. Section through the tooth tip (*am* ameloblast). An electron-dense enamel matrix (*en*) covers the enameloid (*eno*) and the upper region of the dentin (*de*) shaft. Patches of enamel matrix are seen within the upper region of the enameloid matrix; this could be considered an enameloid/enamel boundary. *Bar* 2 μm. **f** Detail of **e** showing the enamel crystals perpendicular to the tooth surface (*en* enamel, *eno* enameloid). *Bar* 500 nm. **g** Larval stage 53 (72 dpf). Maturation process in the upper tooth region. This tooth is not yet functional. The enamel and enameloid matrices are degraded and the upper tooth region appears empty after decalcification (*am* ameloblasts, *od* odontoblasts). *Bar* 10 μm. **h** Same stage. The maturation process is not completed (*am* ameloblasts). A little enamel (*en*) and enameloid material is still visible at the tooth tip. Note the presence of a thin layer of uncompletely matured enamel at the tooth surface (*arrow*). *Bar* 1 μm. **i** Larval stage 54 (79 dpf). Advanced maturation (*am* ameloblasts, *de* dentin). Tooth tip, shortly before eruption. Some degraded enamel (*en*) and enameloid material persists. *Left* The multilayered Nasmyth's membrane (*Nm*) has formed at the tooth surface. *Right* A layer of incompletely matured enamel is visible (*arrow*). *Bar* 1 μm. **j** Larval stage 55a (90 dpf). End of maturation process. Slightly oblique section near the enameloid-dentin junction. The enameloid matrix (*eno*) close to the dentin (*de*) is not completely degraded. Note the extremely folded ameloblast (*am*) membranes facing the mature region (*en* enamel). *Bar* 2 μm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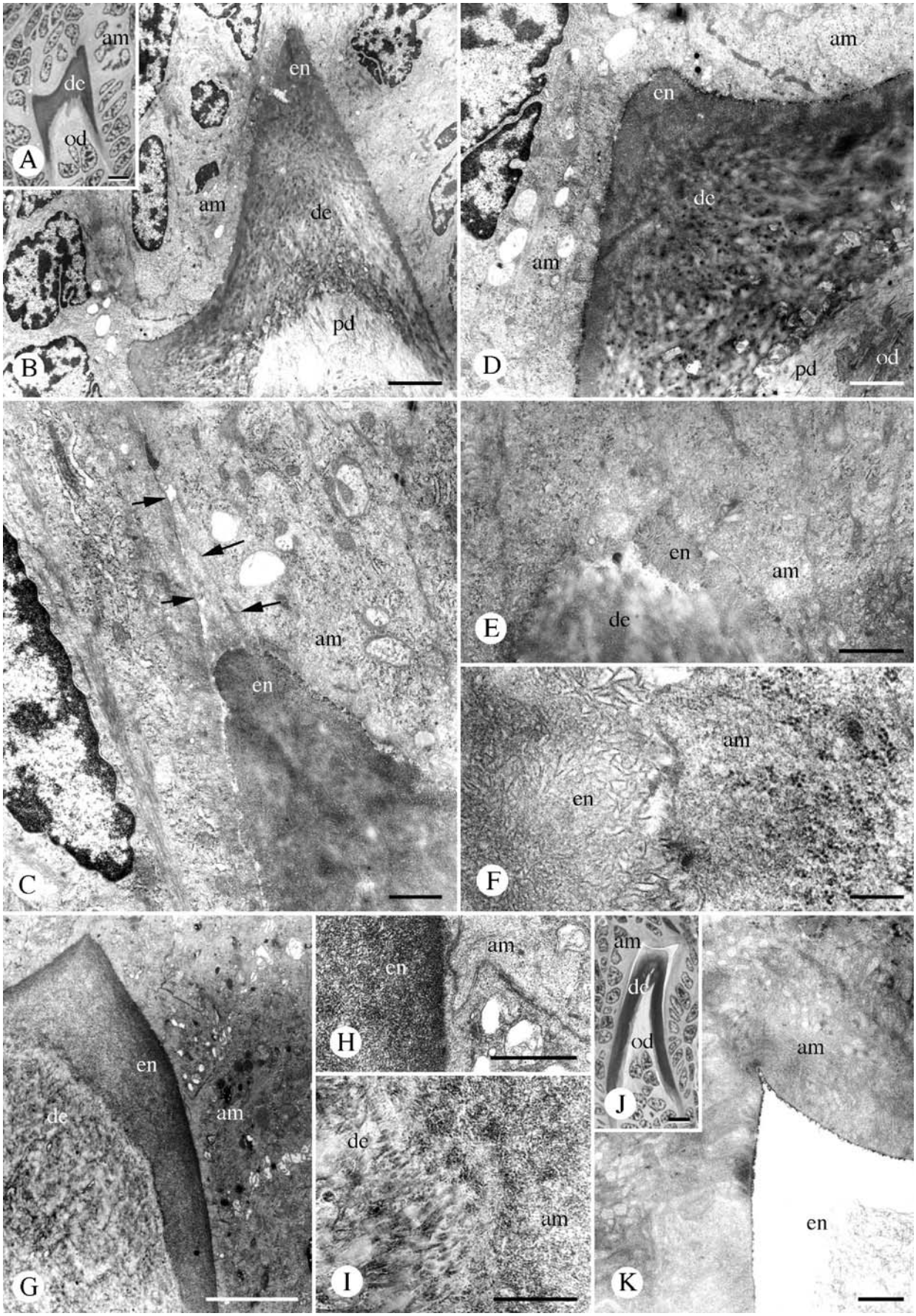
tooth (Fig. 3b). Matrix mineralization and the subsequent maturation process progressively lead to the degradation of the enameloid matrix (Fig. 3c). At this stage, the ameloblasts facing this region show large folds of their membrane and distended extracellular spaces. The result of the proteolytic activity of the ameloblasts is readily visible in the upper tooth region of decalcified samples. Here, both the matrix of the enameloid and of the upper region of the dentin are degraded. The collagen fibrils are short and thin and appear more loosely distributed than at the previous stage (compare Fig. 3b,c). Several odontoblast processes are embedded in the deep region of the enameloid layer. Following enameloid deposition and during the further formation of the dentin by the odontoblasts, mineralization takes place slowly in the upper central region of the tooth and then progresses towards the tooth tip, the peripheral regions and the dentin surface. An enamel layer is deposited by the ameloblasts at the surface of the tooth tip, at the time that the enameloid matrix is mineralising. Mineralization rapidly extends to the depositing enamel layer (Fig. 3d,e). Some patches of enamel crystals are seen within the upper region of the enameloid layer, suggesting that enamel proteins are deposited there before the maturation process takes place; this region composed of mixed matrices might correspond to an enameloid/enamel junction (Fig. 3e). These patches of enamel crystals become more numerous at the enameloid surface and thus a continuous homogeneous layer of enamel forms. In this layer, the crystallites lie parallel to one another and perpendicular to the cell surface (Fig. 3f). During the mineralization and maturation processes, numerous folds of the cell membrane appear and large extracellular spaces form (Fig. 3f). At a further stage of maturation, decalcified samples reveal that most of the enameloid and enamel matrix has been degraded at the tooth tip (Fig. 3g,h). However, some material is still visible in this region, particularly at the very tooth tip and along the tooth surface where the enamel matrix has recently been deposited and is probably not as yet completely mature. This confirms that the maturation process extends from the central region to the tooth surface. Prior to tooth eruption and by the end of the maturation process, Nasmyth's membrane is deposited at the tooth surface by the ameloblasts (Fig. 3i). In contrast to the two previous teeth, this granular electron-dense membrane is multilayered, an organization different from that reported in mammals (Permar 1970) and suggesting that its components are deposited during successive waves. Eventually, the maturation process leads to the complete disappearance of the enameloid and enamel organic matrices, except at the level of the enameloid-dentin junction, where some loose material remains after decalcification (Fig. 3j).

Tooth I_4

The first juvenile tooth I_4 starts to develop at larval stage 55a (90 dPF), shortly before metamorphosis and becomes functional in 5.5-month-old juvenile specimens. Tooth I_4 is a tall (420–450 μm) bicuspid tooth, with a major and minor cusp, located lingually and labially respectively (Fig. 4a). This contrasts with the monocuspid conical teeth I_1, I_2 , and I_3. Another important difference consists in the absence of enameloid matrix located between the dentin and the enamel. The enamel matrix is directly deposited by the ameloblasts onto the dentin surface and the enamel layer is thicker at the level of the cusps (Fig. 4b–e, g–j). The shape of the two cusps is roughly defined when the first dentin matrix has been formed by the odontoblasts, three of them being responsible for this deposit at the upper tooth region (Fig. 4a). The ameloblasts are typically polarized cells along the tooth surface but their orientation changes in the upper tooth region at the level of the cusps. Indeed, in addition to the ameloblasts of the inner dental epithelium located along the tooth shaft, another ameloblast population

Fig. 4 Fourth tooth developing at position I: the first juvenile tooth, I_4. EDTA decalcified samples. **a** Stage 56 (110 dPF, post-metamorphosis). Enamel matrix deposition. Tooth I_4 is bicuspid. The shape of the upper region is nearly completed. A thick layer of dentin (*de*) has been deposited by the odontoblasts (*od*). Note the particular orientation of the ameloblasts (*am*) surrounding the cusps. *Bar* 10 μm. **b** Same tooth, upper region (*am* ameloblasts, *de* dentin, *pd* predentin). A thin layer of enamel (*en*) is visible at the dentin surface. No enameloid can be identified. *Bar* 5 μm. **c** Detail of the major cusp (*right cusp* in **b**). The tip is composed of enamel matrix (*en*) only (*arrows* two upper ameloblasts (*am*) still depositing enamel matrix). Note the presence of numerous organelles. *Bar* 1 μm. **d** Detail of the minor cusp (*left cusp* in **b**). No enameloid is identified between the enamel (*en*) and dentin (*de*). The ameloblasts (*am*) are still depositing enamel material (*pd* predentin, *od* odontoblast). *Bar* 2 μm. **e** Stage 56, second specimen. Early enamel matrix deposition. The dentin layer (*de*) is well formed when enamel material (*en*) is deposited (*am* ameloblast). *Bar* 1 μm. **f** Detail of the first-deposited enamel material (*en*) with typical organization of the organic matrix (*am* ameloblast). *Bar* 250 nm **g** Juvenile (4 months old). Major cusp (*am* ameloblast). End of enamel deposition and beginning of maturation. A thick layer of enamel (*en*) is now present at the dentin surface (*de*). *Bar* 5 μm. **h** Detail of **g** showing the enamel surface. During the maturation process, the enamel matrix (*en*) is progressively degraded, hence the presence of thin granules. The ameloblast (*am*) membrane is folded and numerous large vacuoles are visible. *Bar* 1 μm. **i** Detail of **g** showing the enamel-dentin junction (*am* ameloblasts, *de* dentin). No enameloid is visible. *Bar* 1 μm. **j** Juvenile (4 months old). Tooth I_4 of the second specimen, shortly before attachment. Enamel matrix maturation is well advanced. After decalcification, the enamel layer appears empty as most of the organic matrix has been removed during maturation (*am* ameloblasts, *de* dentin, *od* odontoblasts). *Bar* 10 μm. **k** Detail of the tip of the major cusp shown in **j**. After decalcification, some enamel material remains along the ameloblast surface. Traces of degraded collagen matrix are still visible near the dentin surface (*am* ameloblasts, *en* enamel). *Bar* 1 μm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is present between the two cusps (Fig. 4a,b). The final shape and size of the cusps is elaborated by the collaborative work of these two ameloblast populations. The cells located at the level of the two cusp tips deposit more enamel matrix material than the ameloblasts located between them (Fig. 4c,d). At this stage of enamel matrix formation, the ameloblasts show a typical aspect of cells involved in protein synthesis: numerous mitochondria, RER cisternae, Golgi apparatus and vesicles. The first deposited enamel material is typically composed of a radially oriented, electron-dense fibrillar matrix. Then, when the enamel layer has thickened, the fibrils are oriented perpendicularly to the tooth surface (Fig. 4e,f). Enamel matrix deposition occurs on previously mineralized dentin matrix and the mineralization progresses in the enamel layer, from the deeper region (dentin surface) to the upper region (tooth surface) where the enamel matrix continues to be deposited (Fig. 4g). The maturation processes start rapidly after the enamel layer is completed and well mineralized. The enamel layer is 6 μm thick at the level of the major cusp. Maturation progresses from the central first-deposited region towards the more recent peripheral regions. The matrix is progressively degraded: the fibrils are no longer visible and are replaced by small electron-dense granules (Fig. 4g–i). Facing the maturing enamel region, the ameloblasts show the same features as described for this stage in the previous teeth, i.e. a highly folded membrane, a narrow region of cytoplasm devoid of organelles and large vacuoles. A detailed observation of the enamel-dentin interface does not reveal the presence of enameloid matrix, the enamel matrix lying directly in contact with the large collagen fibrils of the dentin (Fig. 4i). In decalcified samples, the maturation process can be followed as a progressive disappearance of the granular matrix. A few days before eruption, the enamel layer is mature, i.e. no organic matrix is visible after decalcification, except along the ameloblast surface and near the enamel-dentin junction (Fig. 4j,k). Eventually, Nasmyth's membrane forms at the tooth surface (as described for the previous teeth in the family) and the tooth attaches to the dentary bone and becomes functional (not shown).

The second and third juvenile teeth I_5 and I_6 start to develop in 5- and 8-month-old juvenile *P. waltl*, respectively, and become functional during the 8th and 14th month, respectively. Both teeth are similar to tooth I_4 and therefore their formation is not illustrated. In particular, the features described above for amelogenesis of tooth I_4 are identical: dentin forms first and starts to mineralize, after which enamel is deposited, mineralizes rapidly and matures. No enameloid layer has been identified between the dentin and enamel matrices of teeth I_5 and I_6.

Discussion

During *P. waltl* ontogeny, a hard tissue (enameloid), which is well represented in larval teeth, is topologically replaced by another hard tissue (enamel) in juvenile and adult teeth. This replacement, which takes place progressively, has lead previous authors to think that, during ontogeny, enameloid disappears while enamel develops. In monitoring, step by step, the events occurring at the tooth tip of the successive teeth of a tooth family, we conclude that the enameloid matrix does not disappear but is progressively changed into true dentin, whereas enamel progressively thickens. From tooth I_4 onwards, the enameloid matrix can no longer be distinguished from dentin. This phenomenon is concomitant with an increase of enamel deposition by the ameloblasts. Therefore, an event believed to be an enameloid/enamel transition might, instead, be an enameloid/dentin transition. In the following, we discuss these results in the light of the previous interpretations of enameloid structure and fate in caudates, with a particular focus on the roles played by the odontoblasts and ameloblasts. We conclude in proposing the most probable scenario for the "enameloid/enamel" transition during *P. waltl* ontogeny. This scenario is supported by morphological data only and will obviously need to be confirmed by using molecular tools.

Enameloid of larval teeth

The nature of the enamel-like material covering the teeth in larval and adult caudates has long been debated since the pioneer studies by Owen (1845), Leydig (1867) and Hertwig (1874). Levi (1940), Kvam (1946, 1953, 1960) and Kerr (1960) have interpreted the external covering of the monocuspid teeth in larval salamanders as a "mesodermal enamel", i.e. a highly mineralized dentin (durodentin) exclusively deposited by the dental papilla cells. Our results indicate that such a description is valid but might be restricted to the outer covering of the developing embryonic and larval teeth (teeth I_1 to I_3). There is little doubt that the enameloid matrix, which is deposited in the upper region of these teeth, is deposited by a single or, at most, two odontoblasts. In the newt *Triturus pyrrhogaster*, Kogaya (1999) has also shown that the enameloid matrix is entirely formed by the odontoblasts of the dental papilla. However, the question of a possible direct participation of the ameloblasts in enameloid matrix formation remains open (see below).

To our opinion, enameloid is only found in embryonic tooth I_1. Enameloid is a loose network of collagen fibrils (10–15 nm in diameter) deposited by odontoblasts, with no morphologically identifiable background substance, and is subsequently highly mineralized. This tissue could be either

called “durodentin” when refering to the collagen matrix that mineralizes strongly or termed "enameloid" when refering to the result of the maturation process, which leads to an enamel-like tissue. Schmidt (1957, 1958) who first regarded the outer surface of the adult caudate teeth as durodentin, subsequently changed his view and suggested that this layer is an “ectodermal enamel”, i.e. deposited by the cells of the enamel organ (Schmidt 1970). The outer hard protective tissue in the juvenile and adult teeth (I_4, I_5 and I_6) of *P. waltl* is clearly composed of enamel only.

In a first attempt to study amelogenesis in *P. waltl*, Chibon et al. (1971) found no enamel until metamorphosis. Indeed, as shown in the present study, the enamel layer in larval teeth is too thin to be identified at the light-microscopic level. Transmission electron-microscopic observations by Smith and Miles (1971), Chibon (1972), Roux and Chibon (1973) and Roux (1973), however, have revealed its presence at the tooth tip of caudate larval teeth (probably embryonic teeth given the early stages examined). We agree with this interpretation and confirm that the thin enamel cover is present as early as in the first embryonic tooth I_1. However, in the literature, previous references to “larval teeth” should be considered with caution because they might refer to larval tooth I_2 and/or tooth I_3, and not to embryonic tooth I_1.

In embryonic tooth I_1, enameloid is distinguishable from dentin in having small-diameter collagen fibrils and no background substance. In contrast, in larval teeth I_2 and I_3, it is difficult to distinguish enameloid from dentin because the components of both tissues are similar. In particular, enameloid in teeth I_2 and I_3 contains larger-diameter fibrils embedded in an electron-dense background substance. This structural similarity explains the absence of a clear enameloid-dentin junction in these larval teeth, until the beginning of the maturation stage. From tooth I_2 to tooth I_4, the enameloid matrix gradually changes into a dentin-like matrix. Nevertheless, this dentin-like matrix is subsequently changed into enameloid in teeth I_2 and I_3 during the maturation phase. Such a maturation process does not occur in tooth I_4 in which the enameloid matrix entirely changes into dentin. The maturation of enameloid in teeth I_1 to I_3 is under the influence of ameloblasts (see below).

The modifications of the enameloid from tooth I_1 to tooth I_3 supports a progressive transformation of enameloid into dentin, rather than a sudden disappearance of the enameloid. This leads to further comments on the role played by the odontoblasts. Our study clearly shows that the timing of odontoblast functioning does not change dramatically during the odontogenesis of teeth I_1 to I_4. The odontoblasts first deposit enameloid and/or dentin matrix, long before ameloblasts deposit enamel. However, the matrix that is deposited by the odontoblasts changes progressively through successive tooth generations, from true enameloid matrix (as defined for tooth I_1) to true dentin (as observed in tooth I_4). We interpret this transition as the result of a slowing down of odontoblast activity. The upper tooth region is formed within a couple of days for tooth I_1, whereas weeks are required for this region of tooth I_4 (Davit-Béal et al. 2006b). The faster the odontoblasts deposit the matrix, the looser it is, and the looser the matrix is, the more it is mineralized. The clear boundary between the enameloid layer and the dentin in tooth I_1 could be related to a change in odontoblast functioning, viz. from a rapidly deposited matrix (enameloid) to a less rapidly formed matrix (dentin). The slowing down of odontoblast activity leads to this clear boundary and the maturation process is arrested along this frontier. This means that, when the odontoblasts slow down their activity, not only do they deposit a denser collagenous network with larger collagen fibrils, but they also synthesize additional substances embedded in the collagen network. This more structured matrix is protected from the action of metalloproteinases, which are synthesized by the ameloblasts and secreted into the enameloid layer. The progressive increase of the diameter of the collagen fibrils and the deposition of a background substance in the enameloid matrix, from tooth I_1 to tooth I_3, support the hypothesis of a progressive change of enameloid into dentin, culminating in tooth I_4, in which no enameloid can be identified.

In *P. waltl*, we have linked enameloid deposition to fast-growing teeth and the transition from enameloid to dentin to a slowing down of odontoblast activity. However, this does not agree with what is known about enameloid formation in other lineages, e.g. teleost fish (actinopterygians) and sharks and rays (chondrichthyans; Sasagawa 1988, 2002; Sasagawa and Ishiyama 1988). In these taxa, which do not possess enamel, enameloids are deposited in adult specimens in which teeth develop slowly. Although the development of first-generation teeth is similar in larvae (Sire et al. 2002), which suggests a similar origin of enameloid formation, another pathway has been selected during evolution in these lineages, leading to the conservation of enameloid as a protective tissue, whereas enamel has appeared in the tetrapod lineage. As described for *P. waltl* tooth development, the ameloblasts surrounding enameloid are well differentiated in sharks and teleosts but whether they participate in enameloid formation and in what manner remain unknown (but see below).

Do ameloblasts participate in enameloid formation in *P. waltl* larvae?

As early as the onset of the deposition of the enameloid matrix of tooth I_1 by its single odontoblast, the few ameloblasts located around the tooth tip are well differentiated. They show the characteristic features described

classically for ameloblasts during amelogenesis: well-polarized cells with a nucleus located distally in the cytoplasm and surrounded by organelles. In addition, strong morphological relationships are observed between the collagen fibrils and the cell membrane, and no basement membrane separates the cells from the matrix surface. These features, which are also observed in larval teeth I_2 and I_3, suggest that these ameloblasts could be involved in the formation of collagen fibrils, in the production of associated molecules that are incorporated into the upper layer of the enameloid matrix or in both. However, our detailed morphological data do not indicate that the ameloblasts are involved in the formation of the enameloid matrix (i.e. the presence of numerous secretory vesicles) and such an hypothesis has never been proposed in the literature. Several authors have suggested that the features observed in the ameloblasts reveal their possible role during the maturation processes of enameloid (Goto 1978; Nanci et al. 1983). We agree with this view. All the features described during enameloid maturation in the first teeth of *P. waltl* indicate that the ameloblasts are involved in this process and that this function is initiated prior the deposition of the enamel layer.

The main type of collagen described in vertebrate teeth is collagen type I. Although collagen type I secretion by epithelial cells has been described during development of the chick cornea (Hay and Revel 1969), of the skin in a caudate (Hay and Revel 1963) and of the skin in a teleost fish (Le Guellec et al. 2004), collagen type I has not been reported, to our knowledge, to be synthesized by ameloblasts. In addition, the synthesis of collagen type I, for example by odontoblasts, is generally represented by the presence, in the cytoplasm, of large secretory vesicles (Smith and Miles 1971; Roux 1973; Leblond 1989). In *P. waltl* larvae, such vesicles have been observed in the cytoplasm of the odontoblasts but not in the ameloblasts facing the forming enameloid.

In tetrapods, ameloblasts synthesize enamel-specific proteins (amelogenin, ameloblastin and enamelin). In *P. waltl*, it would be of interest to check, in selected stages of embryonic and larval tooth development, whether enamel protein genes are expressed by the ameloblasts prior to and during enameloid deposition. Such data would represent a significant advance in the knowledge of enameloid evolution mediated by changes of surrounding cell function. This goal could be achieved, for example, by using in situ hybridization techniques. Among the candidate proteins, amelogenin seems to be the best choice as it is the major enamel protein (Sire et al. 2006). The amelogenin gene is now well known in mammals (Delgado et al. 2005), in reptiles (Ishiyama et al. 1998; Toyosawa et al. 1998; Delgado et al. 2006) and in a lissamphibian, the pipid anuran *Xenopus laevis* (Toyosawa et al. 1998). We are currently cloning the amelogenin gene in *P. waltl* in order to study its expression during enameloid formation in larval teeth I_1 to I_3. The two other enamel protein genes could similarly be studied. The finding that ameloblasts eventually deposit a layer of true enamel at the enameloid surface indicates that these cells are able to produce enamel proteins, despite enameloid still being present.

Mammalian ameloblasts are known to participate actively in enamel maturation via the synthesis of matrix metalloproteinases, including enamelysin and kallikrein4, which are believed to be the predominant degradative enzymes that clear enamel proteins from the matrix during maturation (Simmer and Hu 2002). The expression of the genes coding for these proteinases (and principally enamelysin, which degrades amelogenin) should be examined by means of in situ hybridization. Enameloid maturation (degradation of the collagen matrix) has been shown to occur in early larvae and the morphological features of the ameloblasts (ruffled border, extended extracellular spaces and large vacuoles) strongly suggest their early involvement in this process (Smith and Miles 1971). The lack of a basement membrane facilitates the deposition of material in the extracellular matrix by the ameloblasts. However, to our knowledge, the gene coding for enamelysin is only known for mammals (Bartlett et al. 1996; Fukae and Tanabe 1998; Bartlett and Simmer 1999). In addition, the question of the specificity of this metalloproteinase remains because enameloid is rich in collagen fibrils. However, enamelysin is also known to work as a collagenase (Simmer and Hu 2002).

Enamel of larval teeth

We have studied numerous serial sections and several stages of odontogenesis in early *P. waltl* larvae in order to discern enamel on the enameloid surface of the embryonic tooth I_1. Such a layer is more easily identified in the second and third generation teeth, I_2 and I_3. This location in tooth I_1 probably corresponds to the descriptions dealing with larval teeth in the literature (Smith and Miles 1971; Chibon et al. 1971; Roux and Chibon 1973; Kogaya 1999). The fast growth of tooth I_1 could be the reason for the problematic identification of the true enamel covering. In the subsequent teeth, which develop more slowly, the enamel matrix is easier to find. To support the identification of this layer as enamel, enamel-specific proteins or the genes encoding them should be revealed by using immunohistochemical and molecular tools, respectively.

In caudate larvae, our study demonstrates that (1) enameloid is always formed first, long before the first enamel material is observed at the tooth surface, which could support the view that enameloid is present before enamel during evolution (see above) and (2) the amelo-

blasts are first involved in enameloid maturation (proteases?) and later in enamel matrix synthesis. This has also been described in the newt *Triturus pyrrhogaster* by Kogaya et al. (1992) and contrasts with aspects known for mammalian tooth development, in which dentinogenesis and amelogenesis start almost simultaneously.

Concluding remarks

Our morphological study answers most of the current questions about the enameloid/enamel transition and the fate of enameloid during *P. waltl* ontogeny. The processes can be summarized in the following hypothetical scenario. The first embryonic tooth I_1 is small and forms rapidly within a few days. When the enamel epithelium and the dental papilla have differentiated, the single odontoblast located at the upper region of the dental papilla rapidly deposits the tooth matrix. Its action results in the formation of a loose network of collagen fibrils devoid of additional substances: enameloid. The activity of the odontoblast then slows down and, subsequently, predentin matrix, which is denser than the enameloid matrix and contains additional non-collagenous material, is deposited. Concomitant with the differentiation of the odontoblasts, the ameloblasts located around the tooth tip differentiate. They synthesize substances, probably proteases, that are deposited in the loose collagenous network of enameloid. We do not know whether enamel proteins are produced at this time. The maturation process leads to the completion of enameloid formation, whereas dentin continues to be deposited by the odontoblasts. A thin enamel layer is formed by the ameloblasts shortly before this first tooth is functional. The ameloblasts also synthesize an unmineralized membrane-like layer.

Teeth I_2 and I_3 form less rapidly but follow the same steps as those described above. The odontoblasts start to deposit the enameloid collagen network, but more slowly than for the first tooth, so that the fibrillar network is denser. In addition, non-collagenous material is deposited in the background material before odontoblasts slow down their activity and deposit predentin matrix. The ameloblasts synthesize various substances, probably still dominated by proteinases, which degrade the collagen fibrils and the associated non-collagenous proteins, leading to enameloid. When enameloid is maturing, the ameloblasts deposit a layer of enamel at the tooth surface. This enamel mineralizes shortly before tooth eruption. A membrane, probably homologous to the mammalian Nasmyth's membrane, is finally deposited on top of the enamel.

The next tooth, I_4, requires several weeks to form. The same odontogenetic steps are followed, but with a slow rhythm. The odontoblasts work slowly and deposit a true predentin matrix instead of the previous dentin-like (enameloid) network.

Our interpretations of enameloid/dentin transition in *P. waltl* larvae and of ameloblast function in enameloid maturation first and in enamel matrix synthesis later have generality not only for other oviparous caudates (e.g. *Ambystoma mexicanum*, see Wistuba et al. 2002), but also for direct developing and viviparous species. In these species, early tooth development is not well known at the histological level but there is no reason to suppose that the successive replacement teeth do not show the same features as those described for *P. waltl* larvae, at least before metamorphosis. Similarly, the features of young developing teeth in caecilians (Gymnophiona: see Wake 1976, 1980) resemble those described for *P. waltl* and so the scenario might be the same. However, other lissamphibians in which teeth develop late and possess only enamel, such as the anurans, exhibit clear differences.

The events described above might also be valid for other vertebrate species that possess enameloid, such as most actinopterygian fishes, because the features of the first-generation teeth in post-embryonic stages (Sire et al. 2002) are similar to those described in *P. waltl*. The only difference (but of importance) is that enameloid is neither changed into dentin nor progressively replaced by enamel in these actinopterygian species. A detailed study of ameloblast function during enameloid formation and its subsequent maturation should be undertaken to determine whether this scenario has generality for teleost fish. Our view that enameloid was present before enamel in evolution suggests similar features occurring in both lineages, at least in the first developing teeth. However, the long evolutionary period (>420 million years) separating these lineages has probably led to some differences, both in spatial-temporal ameloblast functioning and in the molecules involved.

Acknowledgements We are grateful to Prof. Ann Huysseune (Ghent University, Belgium) for critical reading and comments on our manuscript. Transmission electron microscopy was carried out at the Service de Microscopie Electronique de l'IFR de Biologie Intégrative, Université Paris 6-CNRS.

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