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RESEARCH PAPER

On the enameloid microstructure of Archaeobatidae (Neoselachii, Chondrichthyes)

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Abstract

Purpose In this study, we present new data regarding the enameloid microstructure of the oldest batoid family, Archaeobatidae.

Methods First, all the teeth were etched superficially with HCl 10% for 5 s and photographed in the SEM. Afterwards, the same teeth were embedded in Canadian Balsam, polished and etched again with HCl 10% in order to reveal the enameloid microstructure before being photographed a second time.

Results The enameloid layer of Archaeobatidae consists on a superficial single crystallite enameloid (SCE) with a parallel bundled enameloid (PBE) in all the taxa studied, but only in *Toarcibatis* and *Cristabatis* there exists a tangled bundled enameloid (TBE) under it.

Conclusions The structural complexity and diversity found in Archaeobatidae are comparable to that recently described in others fossil batoids. Our data suggest a general trend to "simplification" in batoid enameloid up till the homogeneous single crystallite enameloid that is present in the majority of current batoids; which contrasts with the increasing structural complexity present in selachimorphs.

Keywords Batoids · Enameloid · Archaeobatidae · Electron microscopy

Resumen

Objetivo En el presente trabajo presentamos nuevos datos concernientes a la microestructura del esmaltoide de los dientes de la familia de batoideos más antigua, Archaeobatidae.

Metodología Primero, todos los dientes fueron atacados superficialmente con HCl al 10% durante 5 s y fotografiados con el SEM. Posteriormente, los mismos dientes fueron incluidos en Bálsamo de Canadá, pulidos y atacados con HCl al 10% durante 5–10 s para ser fotografiados una segunda vez.

Resultados La microestructura del esmaltoide en Archaeobatidae consiste en una capa de SCE con una PBE debajo de esta; pero solo en dos de los taxones estudiados (*Toarcibati*s y *Cristabatis*) encontramos una TBE.

Conclusiones La complejidad y diversidad structural encontrada en el esmaltoide de Archaeobatidae es comparable con la descrita para otros batoideos fósiles. Nuetros datos sugieren un patrón de simplificación en la estructura del esmaltoide hasta llegar a la SCE que se encuentra actualmente en la mayoría de los batoideos; lo que contrasta con el incremento en la complejidad del esmaltoide presente en selachimorfos.

Palabras clave Batoideos · Esmaltoide · Archaeobatidae · Microscopía electronica

1 Introduction

Since pioneer studies of Reif (1973a, b, c) the analyses of chondrichthyan tooth enameloid have been of particular interest to zoologists and paleontologists, proving to be a helpful tool in taxonomic and phylogenetical studies, and providing important information for the understanding of

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the evolutionary history of the group (e.g. Thies 1993; Cuny et al. 2001; Cuny and Risnes 2005; Gillis and Donoghue 2007; Botella et al. 2009, b; Guinot and Cappetta 2011; Andreev and Cuny 2012; Enault et al. 2013, 2015; Manzanares et al. 2016, 2017). During last years, these studies have spread to other fields of research, such physics (Enax et al. 2012), geochemistry (Fischer et al. 2013), chemistry or archaeology (Drew, Philipp and Westneat 2013) in part due to their potential in the development of novel biomimetic materials. A comprehensive historical background on the evolution of studies on shark enameloid microstructure has been recently published (Enault et al. 2015). They also modified the terminology used in the study of chondrichthyan enameloid, unifying and creating new terms to describe its microstructural features, arrangement and diversity. So far, the distinguished two units in the enameloid layer of chondrichthyans: a "Single Crystallite Enameloid" (SCE) unit made of crystallites randomly oriented and the "Bundled Crystallite Enameloid" (BCE) where the crystallites are arranged into bundles with different size and orientation. The orientation of the bundles in this BCE can be parallel to each other ["Parallel Bundle Enameloid" (PBE)] or they can be less organized or "tangled" ["Tangled Bundled Enameloid" (TBE)]. A third possible component of the BCE is the radial bundled enameloid (RBE), where the enameloid crystallites form bundles that originated in the SCE, go perpendicularly through the other BCE components until they reach the enameloid-dentine junction (EDJ).

Several of the most recent studies focused on batoid fishes, filling to some degree a previous lack of information regarding the enameloid microstructure in this group (Enault et al. 2013; Manzanares et al. 2016). These studies demonstrate a great microstructural diversity in the group dismissing earlier assumptions considering batoid enameloid exclusively composed of a single layer of TBE (Gillis and Donoghue 2007) or of SCE (Cuny et al. 2009). Thus, Enault et al. (2015) demonstrate the presence of a complex bundled enameloid organized in different units in several fossil batoids (i.e. *Pytchotrygon* sp., in the 'rhinobatoid' *Belemnobatis* sp. or in *Parapalaeobates* cf. *atlanticus*) whereas a SCE is present in other taxa (i.e. the 'rhinobatoid' *Hypsobatis weileri* Cappetta, 1992 and in some Myliobatiforms). More recently Manzanares et al. (2016) studied the enameloid microstructure through extant batoids phylogeny and found that a homogenous SCE monolayer lacking microstructural differentiation is the most widespread condition, present in all taxa they studied—i.e. in the Rajoidei (*Raja clavata, Atlantoraja platama, Sympterygia acuta* and *Rioraja agassizi*), Platyrhinoidei (*Platyrhina triseriata*), Rhinobatoidei (*Rhinobatos productus*), Torpedinoidei (*Torpedo marmonata*) and Myliobatoidei—with the unique exception the Rhinoidei *Rhyna ancylostoma* where a double-layered enameloid consisting in an outer layer of SCE and an inner layer of bundles with variable orientation was found.

The high variability and complexity found within batoid dental enameloid, especially among fossil taxa unclear the plesiomorphic condition for the whole group, including stem lineages, and the understanding of the evolution of enameloid microstructure in batoids. Clarifying this question consequently requires further investigation with special focus on the enameloid microstructure in earliest batoids taxa (Enault et al. 2015; Manzanares et al. 2016).

In the present work, we study the enameloid microstructure in Archaeobatidae, widely accepted as the oldest batomorph family (Underwood 2006; Cuny et al. 2009; Aschilman 2011; Aschliman et al. 2012; Cappetta 2012; Enault et al. 2013, 2015). The family Archaeobatidae was erected by Delsate and Candoni (2001) to include the genera *Doliobatis*, *Cristabatis* and *Toarcibatis*, all of them represented by isolated teeth found in Toarcian sediments from French, Belgian and Luxembourg localities in the Paris Basin. They were assigned to batoids, based on morphological traits: crushing type crowns, presence of a prominent uvula, hemiaulacorhize roots. The assignment of Archaeobatidae to batoids has been largely followed by posterior authors (e.g. Underwood 2006; Cuny et al. 2009; Aschilman 2011; Aschliman et al. 2012; Cappetta 2012; Enault et al. 2013, 2015; although Cappetta 2012 removed *Doliabatis* from Archaeobatidae and placed it within the family Rhinobatidae).

2 Material

We studied 5 isolated teeth (Table 1): 2 *Toarcibatis elongata* and 2 *Cristabatis crescentiformis* from Halanzy (HLZ) Locality, 1 *Doliobatis weisi* from Ginzebierg (GZB) locality, both locality dated as Toarcian (Fig. 1). The village of Halanzy (Aubange) is situated in the South-East of Belgium, near the French border (Coordinates: East 5º45′10″ North 49º38′23″) (Delsate 1990). The material was collected from a 15–30 cm thick, brown, marly horizon rich in macroinvertebrates

Table 1 List of specimens investigated, with information on their locality, age and details of the SEM analysis

Taxon	Family	Age	Locality	Specimen number	SEM	Voltage (kV)
Toarcibatis	Archaeobatidae	Toarcian	Halanzy	MGUV-36104	S-4100 Hitachii	10
Cristabatis	Archaeobatidae	Toarcian	Halanzy	MGUV-36106	S-4100 Hitachii	10
Doliobatis	Archaeobatidae	Toarcian	Ginzebierg	MGUV-36108	S-4100 Hitachii	10

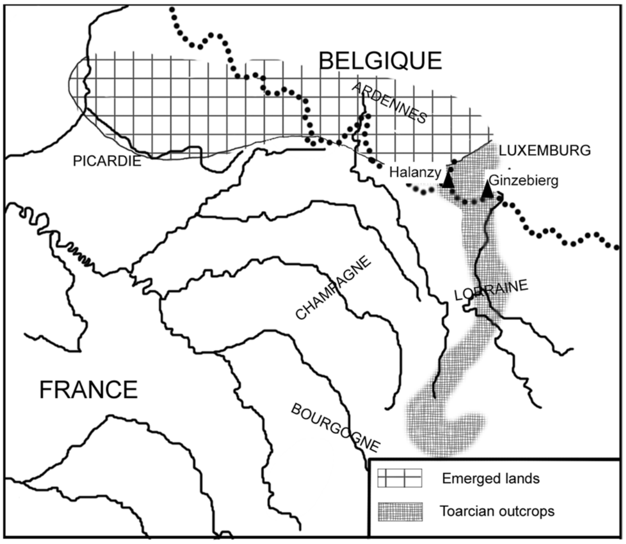

Fig. 1 Situation of the localities of Halanzy (HLZ) and Ginzebierg (GZB) in the Paris Basin. Both localities have been dated as Toarcian. Modified from Delsate and Candoni (2001)

(belemnites, ammonites, gasteropods) and phosphatic centimetric "pebbles". The 'Crassum layer' is characterized as a thin conglomeratic level with changes in lateral facies, containing reworked ammonite faunas of the Bifrons and Variabilis zones. A preliminary analysis of cephalopod (ammonites and belemnites) and shark (micro teeth) faunas provided new data on the biostratigraphic and paleogeographic distribution of these groups in the framework of the Paris basin.

The locality of Ginzebierg (GZB) is situated in the South Grand Duchy of Luxembourg, near Dudelange, at the border between Luxembourg and France (Toarcian *Levesquei* Zone). The material was collected from a laminated coquina marl on top of the "Grès supraliasique" (top of Toarcian). The impressive abundance of bivalves and their fragments determined the choice of this level in the search of vertebrate predators. Cephalopods are *Mesotheuthis rhenana* Oppel 1856 and *Brevibelus breviformis* Voltz 1830, and the ammonite *Pleydellia subcompta* (Branco 1879), which dates the sediments of the Aalensis subZone (top of the *Levesquei* Zone).

All the teeth and preparations are deposited in the Museum of Geology of the University of València with catalogue numbers MGUV-36104 to MGUV-36108.

3 Methods

3.1 Surface study

Due to the scarcity of the material for study, the manipulation and preparation of the teeth were challenging. To optimize the information that could be obtained, studies of the surface and the internal enameloid structure were carried out on each tooth. After each treatment, the teeth were coated in a gold–palladium alloy and photographed on a Hitachi S-4800 scanning electron microscope at the Microscope Service of the Universitat de València. The terminology used in this work is modified from Fischer et al. (2011) (Fig. 2a, b).

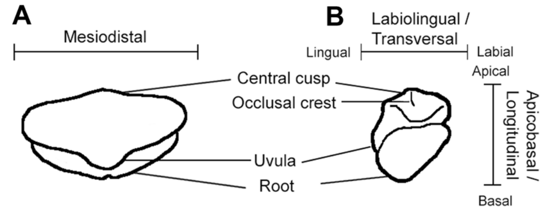

Fig. 2 Sketched tooth of *Toarcibatis* with the descriptive terminology used in this work. **a** Lingual and **b** lateral views

This method consists of etching the exterior of the tooth with HCl diluted at 10% with a duration of 5–10 s (Reif 1973a, b, c1977, 1978, 1979; Cuny 1998; Cuny and Benton 1999; Cuny et al. 2001; Duffin 1980; Guinot and Cappetta 2011; Andreev and Cuny 2012). This allows the acid to penetrate the superficial layers of enameloid and expose its structure, especially the SCE and, depending on how much the acid has penetrated, the PBE that lies beneath it when this layer is present (Andreev and Cuny 2012).

3.2 Section study

This method shows the complete structure of the enameloid layer (Gillis and Donoghue 2007; Guinot and Cappetta 2011; Andreev and Cuny 2012), while the study of the surface does not allow to observe the TBE and the enameloid/dentine junction. Once the superficial analysis was carried out, the same teeth were embedded in Canadian Balsam at 120 °C for two hours before being grounded until the desired plane of section (longitudinal in this work) was reached, then etched with HCl 10% from 5 to 10 s.

4 Results

4.1 *Toarcibatis elongata* (Fig. 3a–l)

The small tooth of *Toarcibatis* presents a well-defined occlusal crest with a small cusp that shows clear signs of wear (Fig. 3a). The longitudinal section reveals a continuous enameloid cover, with a maximum thickness of 78 µm in its central part that becomes thinner distally (Fig. 3e). This tooth shows the most complex microstructure of all the three taxa.

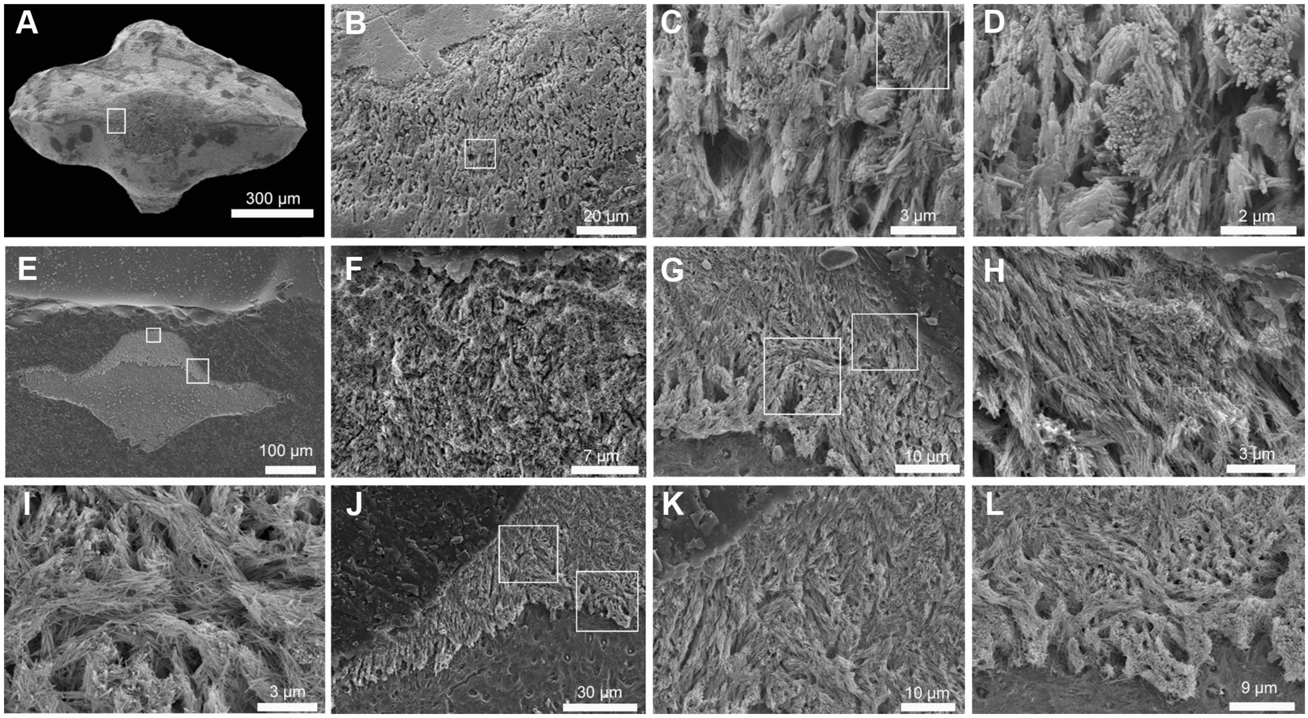

Fig. 3 Tooth of *Toarcibatis* MGUV-36104, **a**–**d** surface study, **e**–**l** sectioned study. **a** General view of *Toarcibatis* tooth. **b** Superficial etched surface of the tooth showing the SCE with randomly hydroxyapatite crystallites and the PBE under it, etched for 5 s in HCl 10%. **c** Detail of the PBE with bundles parallel to the crown surface and with some perpendicular to it. **d** Detail of one of the bundles of the PBE normal to the crown surface. **e** Embedded and grounded longitudinal section of the same tooth showing the totality of the enameloid layer, etched in 10% HCl for 10 s. **f** Central part showing bundles of enameloid with no organization. **g** Detail of the enameloid layer showing the PBE and the transition to the TBE that lays underneath it. **h** Close up of the loose bundles of the PBE, with almost of the hydroxyapatite crystallites of each bundles parallel to each other. **i** Bundles of the TBE showing no organization. **j** Detail of the lateral zone of the enameloid layer, showing how the bundles lose their organization in the central part of the tooth. **k** Close up of **j**. **l** Detail of the TBE and the irregular EDJ. The rectangles show the zones where more detailed pictures were taken

The surface study (Fig. 3a–d) shows the presence of a SCE (Fig. 3b) covering the surface of the crown, with a PBE layer beneath it (Fig. 3b–d). In the SCE, the crystallites are randomly oriented whereas in the PBE the crystallites are arranged into well-defined bundles. These bundles are parallel to the crown surface (Fig. 3b–d, h, i). Additionally, some wider bundles (cross-sectioned in surface study) are perpendicular to the occlusal surface, cross the PBE, then reach the SCE (Fig. 3c, d), which will suggest the presence of a RBE. Section studies evidence a TBE (Fig. 3g, i, l) overlying a very irregular but well defined enameloid/dentine junction (EDJ) (Fig. 3e, g, j, l). In the TBE, the bundles are in general wider and less compacted than the bundles of the PBE (Fig. 3g–l). However, the boundary between these components is diffuse and the PBE component is absent in the lateralmost parts of the teeth (Fig. 3g, j, j).

The central part of the tooth, where the enameloid layer reaches its maximum thickness, displays the most complex microstructure. The bundles of the PBE lose their orientation and cross each other, defining a zone with a compacted interwoven structure that is different from the TBE, which is still clearly distinguishable beneath (Fig. 3f, j–k). The hydroxyapatite crystallites are elongated and measure 2 µm length in the entire enameloid layer.

4.2 *Cristabatis crescentiformis* (Fig. 4a–h)

As in *Toarcibatis*, the tooth of *Cristabatis* presents an occlusal crest with a small central cusp (Fig. 4a). Its enameloid microstructure is organized into a SCE with randomly oriented crystallites covering the surface of the crown (Fig. 4b–c), a PBE and a TBE beneath it (Fig. 3f–g). Surface study shows that bundles of the PBE are oriented parallel to the surface and change their direction near the occlusal crest (Fig. 4c–d) and the section shows that these bundles continue to be parallel to the crown surface (Fig. 4f). Beneath it, the bundles lose this arrangement near the EDJ, where they are less compacted and show the typical woven texture of the TBE (Fig. 4g). In some part of the crown, the enameloid layer shows radial bundles (Fig. 4h) that originated in the SCE and go to the EDJ. The EDJ is irregular but

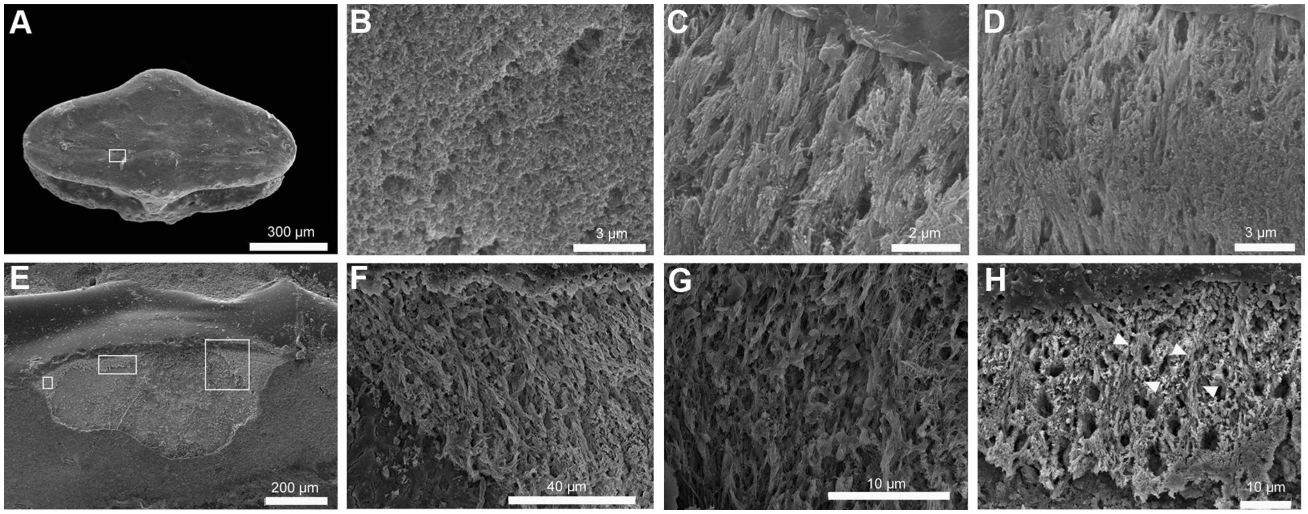

Fig. 4 Tooth of *Cristabatis*, MGUV-36106, **a**–**d** surface study, **e**–h section study. **a** General view of *Cristabatis* tooth, etched for 5 s in HCl 10%. **b** Detail of the etched surface of the crown with the enameloid crystallites of the SCE randomly arranged. **c** Detail of the SCE covering the bundles of the PBE in the occlusal crest, note how the bundles are parallel to the surface and change their orientation to follow the crown surface. **d** Detail of the bundles. **e** Same tooth embedded, sectioned, polished and etched in 10% HCL for 10 s. **f** Parallel bundles of enameloid crystallites. **g** Interwoven bundles of the TBE, showing less degree of organization than in the PBE. **h** Detail of the enameloid layer near the center of the tooth, note how some radial bundles (arrows) that originated in the SCE run perpendicular to the surface until the reach the EDJ. The rectangles show the zones where more detailed pictures were taken

well defined and the enameloid crystallites are elongate and measure ~ 2 µm in length.

4.3 *Doliobatis weisi* (Fig. 5a–h)

This tooth is poorly preserved, showing evidences of boring organisms, especially the inner part of the dentine. The surface of the tooth is worn at the level of the central cusp, where the enameloid layer has disappeared completely and exposes the dentine below (Fig. 5a). The interpretation of its microstructure relies mainly on the superficial study which allows for the identification of a SCE with crystallites randomly oriented at the crown surface (Fig. 5b), and a PBE beneath it. The bundles in the PBE are well defined

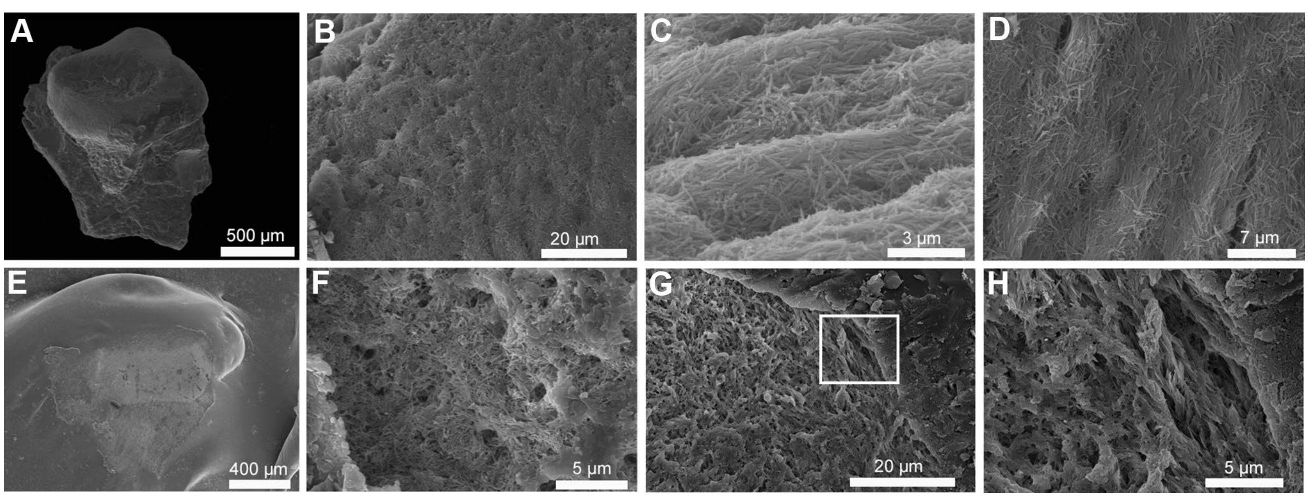

Fig. 5 Tooth of *Doliobatis*, MGUV- 36108. **a** General view of the tooth. **b** Etched surface showing the bundles parallel to the crown surface. **c**–**d** Close up of some bundles showing the crystallites arranged parallel to each other in each bundles. **e** Embedded and sections tooth. **f** Detail of the enameloid layer showing the loss of the organization in the bundles, etched 10 s in HCL 10%. **g** Detail showing the chaotic organization of the bundles. **h** Detail of the highlighted area of **g**, showing some bundles parallel to the crown surface. The rectangles show the zones where more detailed pictures were taken

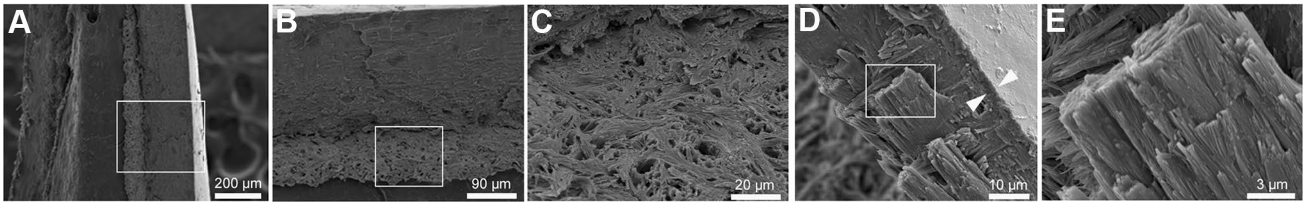

Fig. 6 Sectioned tooth of *Carcharhinus brachyurus* etched in HCl 10% for 5–10 s. **a** Longitudinal section of the tooth showing the complete enameloid layer and the dentine below. **b** Close up of the enameloid layer, where it is possible to differenciate between the PBE and the TBE. **c** Detail of the interwoven bundles of the TBE. **d** Detail of the bundles of the PBE, the arrows indicate the SCE. **e** Close up of the enameloid crystallites inside bundles of the PBE, showing how close and parallel they are organized

and parallel to the crown surface in the superficial study (Fig. 5c–d), but the embedded and polished section shows that the structure of the bundles is less compacted and defined than the bundles of the other two taxa (Fig. 5e–h). The presence of a TBE layer (present in the other two taxa) can neither be confirmed—nor rejected—in *Doliobatis*, due to the poor results obtained in section studies (Fig. 5g, h). The hydroxyapatite crystallites are elongated and measure around 2 µm in length.

5 Discussion and conclusions

Previous analysis of the enameloid microstructure of Archaeobatidae are limited to the species *Doliabatis weisi*, where Delsate (2003) described a double layered enameloid with outer SCE and inner TBE. This interpretation was posteriorly questioned by Cuny et al. (2009) who consider that the tissue interpreted as a TBE is the underlying dentine, and that only a SCE is present in the species *Doliabatis weisi*. In fact, the poor quality and resolution of SEM images provided by Delsate (2003; pl 3, Figs. 3, 4, 5) do not allow for a good characterization of the enameloid microstructure. More recently, Enault et al. (2015) noted (as Enault, pers. com.) that Archaeobatidae possess a complex dental histology, but neither SEM images nor descriptions are provided to demonstrate it.

Our SEM analysis demonstrates the presence of an enameloid layer with a high complex microstructural differentiation in the teeth of all three genus *Doliabati*s, *Toarcibatis* and *Cristabatis*. In general, the enameloid layer is composed of two clear distinct units; (1) a SCE outermost unit consisting of well individualized randomly oriented crystallites. It appears to cap the complete surface of the tooth crown although the dental wear of the studied specimens does not allow us to assert it with total certainty and (2) an inner bundled layer BCE, where TBE, PBE and RBE components can be identified clearly at least in *Cristabatis* and *Toarcibatis.* The poor preservation of the studied tooth of *Doliobatis* only allows for a definitive identification of a SCE outer layer covering a PBE component. However, as said above, a TBE layer has previously been claimed to be present in teeth of *Doliobatis weisi* (Delsate 2003).

The structural complexity and diversity found in Archaeobatidae are comparable to that recently described in other fossil batoids (Enault et al. 2015). Thus, a highly complex enameloid with two well-defined units (SCE + BCE) is present in *Ptychotrygon* sp. (Sclerorhynchoidei), *Belemnobatis* and *Parapalaeobates* cf. *atlanticus*. Sclerorhynchoidei and (*Spathobatis* + *Belemnobatis*) are recovered as successively sister groups to all other batoids (see Claeson et al. 2013; Underwood et al. 1999) and *Parapalaeobates* is still in unclear phylogenetic position. The bundles oriented parallel to the apical surface of the crown and crossed by wider radial bundles found in *Cristabatis* and *Toarcibatis* appears very similar to the enameloid microstructure observed in *Ptychotrygon* sp. and *Parapalaeobates* cf. *atlanticus* (Enault et al. 2015; Fig. 5h–l). In addition, the complex microstructure described at the level of the cusp and occlusal crest in *Belemnobatis* by Enault et al. (2015; Fig. 5f, g), exhibits close similarities with the microstructure found here in the central part of *Toarcibatis* tooth (and probably in *Cristabatis*, but not sectioned at that level) with an outer SCE, a PBE in both lingual and labial sides teeth and a central zone with a compacted interwoven structure and an inner TBE.

The SCE + BCE units identified in Archaeobatidae differ from the triple layered enameloid of modern sharks in both the compaction of the bundles (being more compacted and well-defined in neoselachian sharks) and in the arrangement of the PBE, RBE and TBE components of the BCE (Fig. 6). However, the presence of a complex bundled enameloid in Archaeobatidae, the oldest known batomorph family, and many other ancient batomorphs (Cappetta 2012; Enault et al. 2015) suggests the evolution of a complex layered bundled tooth enameloid prior to the dichotomy between Batomorphii and Selachimorpha (considering a sister-group relationship between them; e.g. Douady et al. 2003; McEachran and Ashliman 2004; Maisey et al. 2004; Aschliman et al. 2012). Thus, the new findings indicate that the amalgamation of individual crystals into bundles forming a layered enameloid would not mark the appearance of Selachimorphii as suggested in Andreev and Cuny (2012), and particularly the

PBE, long time considered typical of non-batoid neoselachians (e.g. Reif 1977; Delsate 2003; Andreev and Cuny 2012) is more widespread among chondrichthyans than previously considered, including even ctenacanth and Synechodontiformes teeth (i.e. *Neosaivodus flagstaffensis*; Guinot et al. 2013; see also Thies et al. 2014). Therefore, the placement within selachians of tooth-based species, some of them previously assigned to batoids (e.g. Hoffman et al. 2016) or even to hybodonts (e.g. Reif 1977; Cuny and Risnes 2005; Andreev and Cuny 2012); based only on of the presence of PBE, must be reconsidered.

However, it is important to note that most of the findings of complex bundled enameloid in non-selachians taxa came from the analysis of fossil isolated teeth. Paleontologists know the limitations of these remains for phylogenetical interpretation. Therefore, studies on the enameloid microstructure of teeth taken from articulated specimens, when available, together with phylogenic analysis on the affinities of Jurassic and Cretaceous batoids will provide more definitive information for the understanding of the diversity and evolution of the enameloid in batoid fishes. Anyway, available data suggest a general trend to "simplification" in batoid enameloid from the high complex bundled enameloid present in Archaeobatidae and several other ancient batomorphs (Enault et al. 2015 and here) to the homogenous SCE layer present in many other fossil taxa as well as in most of recent lineages (Manzanares et al. 2016). This contrasts with the increasing structural complexity present in selachimorphs (Andreev and Cuny 2012; Enault et al. 2015).

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