The Enameloid Microstructure of Euselachian (Chondrichthyes) Scales[1]

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Abstract—The enameloid microstucture of chondrichthyan teeth has been studied for decades and it has proven to be a useful taxonomic tool. Changes in enameloid organization have been related to the emergence of new trophic strategies and Mesozoic radiation of the neoselachian crown group. However, in contrast to the abundance of these data on tooth enameloid, descriptions of chondrichthyan scale enameloid are almost nonexistent. The topology and microstructure of scale enameloid in particular euselachian groups: fossil Mesozoic Hybodontiformes and living neoselachians, including batoids and sharks, are described. It is shown that a thick layer of *single crystallite enameloid* (SCE) covers all studied scales. Although the enameloid of scales clearly does not reach high levels of microstructural differentiation present in the dental enameloid of some neoselachians, we found some degree of organization, such as oriented crystallites, differentiation into sublayers, and the presence of poorly structured sets of densely arranged parallel crystallites. As scales lack feeding functions of teeth, we suggest that the emergence of microstructural organization/differentiation of chondrichthyan enameloid can be understood as consequence of a self-organizing process rather than adaptive pressure.

Keywords: enameloid, chondrichthyan scales, microstucture, SCE

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INTRODUCTION

Enameloid is a hypermineralized tissue that forms a thin layer covering the outer tooth surface of Chondrichthyes, Actinopterygii, larval stages of some caudate amphibians, and other extra-oral elements of the integumentary skeleton of chondrichthyans and several lineages of extinct jawless vertebrates (see Janvier, 1996; Donoghue et al., 2006; Sire et al., 2009).

The enameloid microstructure of chondrichthyan teeth has repeatedly been studied since the early work of Reif (1973), focusing principally on euselachian sharks (Gillis and Donoghue, 2007 and references therein), and emerged as a useful tool for taxonomic studies of the group (e.g., Reif, 1977, 1978; Duffin, 1980; Cuny et al., 1998; Cuny and Risnes, 2005; Botella et al., 2009a; Guinot and Cappetta, 2011). In fact, changes in the organization of tooth enameloid can be related to the evolutionary history of Chondrichthyes. Thus, the multicuspidate teeth of many Paleozoic chondrichthyans adapted exclusively for grasping and swallowing predation, i.e., *Leonodus*, Xenacanthiformes, Phoebodontiformes, Cladose-

lachiformes, Ctenacanthiformes, and Symmoriiformes, possess a homogeneous layer of single crystallite enameloid (SCE) lacking any microstructural differentiation within the layer. Individual hydroxy (fluor-) apatite crystallites are generally discernable and arranged randomly with no preferred orientation relative to the outer enameloid surface (Gillis and Donoghue, 2007; Botella et al., 2009b). In contrast, teeth of all nonbatoid neoselachians, i.e., modern sharks, show a triple-layered enameloid, consisting of an inner layer of tangled-bundled enameloid (TBE), intermediate layer of parallel-bundled enameloid (PBE), and the outermost shiny-layered enameloid (SLE) with crystallites not arranged in bundles (Reif, 1973, 1977; see also Cuny and Risnes, 2005). This microstructural differentiation has been linked to the emergence of new trophic strategies, especially of the cutting and gouging strategy, increasing tensile strength, and resistance to compressive forces that arise from these feeding habits (Preuschoft et al., 1974; Reif, 1978,1979; Thies and Reif, 1985; Cappetta, 1986). Some of these "novel" feeding strategies characteristic of neoselachians also evolved independently in a number of non-neoselachian lineages, which lack a triple-layered enameloid, although, interestingly,

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they show some degree of organization in their SCE. Thus, the crushing teeth of the hybodontiforms *Acrodus* and *Polyacrodus* have a two-layered SCE, with a compact outer layer and a bundled inner layer (Cuny et al., 2001). On the other hand, the cutting dentitions of *Carcharopsis, Priohybodus, Thaiodus*, and *Pseudodalatias* exhibit a very compact single-layered SCE (Reif, 1978; Duffin and Cuny, 2008; Botella et al, 2009a). This compaction of crystallites building up the enameloid has been linked with the necessity of increasing resistance to the tensile stresses induced by these modes of feeding (Duffin and Cuny, 2008).

In contrast to available information regarding the tooth enameloid microstructure, there are virtually no data on the organization of enameloid in chondrichthyan scales. The scale enameloid microstructure in chondrichthyans has never been described in detail and, although it is generally assumed that a thin layer of enameloid covers the scales of euselachians and that of all other groups of extinct chondrichthyans (see Janvier, 1996; Donoghue et al., 2006), this has not been confirmed systematically.

In this study, we attempt to complete this lack of information concerning the microstructure and organization of enameloid in the dermal skeleton elements of euselachians. We describe the scale enameloid layer in certain euselachians: some fossil Mesozoic Hybodontiforms and living neoselachians, including both batoids and sharks in terms of topology and microstructure. As the scale enameloid microstructure is not subject to the same selective pressure due to mechanical stresses derived from new feeding practices of neoselachians, our data can be compared independently with those available for euselachian tooth enameloid. The aims of our analysis are (i) to confirm the presence of enameloid layer in the scales of the examined chondrichthyan taxa and (ii) if so, to describe its microstructure and organization.

MATERIALS AND METHODS

For this purpose polished sections of scales were prepared. Scales were embedded in a transparent polyester resin at 120°C for two hours prior to polishing with a mix of carborundum (800 and 1200 µm) and water until the desired part of the fossil was reached. Afterwards, the sections were etched for 5 to 10 s in 10% HCl or for 1 to 5 minutes in 0.5% orthophosphoric acid. Each sample was repolished and etched as many times as necessary to elucidate the enameloid microstructure. Furthermore, some scales were broken for direct observation of a fresh fracture. The broken surfaces were etched for 2 to 5 s in 10% HCl. Analysis and photography of ground sections were done on a Hitachi S-4100 scanning electron microscope of the Microscope Service of the University of València. Before SEM analysis, scales were coated with gold and palladium alloy. The scales of hybodontiform sharks were collected in the Bugarra

section, Ladinian (Middle Triassic), located in the Iberian Ranges, Spain (see Plá et al., 2009, 2011). Plá et al. (2011) distinguished 13 different morphotypes, following the classification proposed by Johns et al. (1997). For the present work, we studied the ultrastructure of six most abundant morphotypes (*Lobaticorona, Undulaticorona, Parvidiabolus, Coniunctio, Glabrisubcorona*, and *Parviscapha* morphotypes sensu Plá et al., 2011). Scales of the living neoselachians *Raja clavata* (Rajiformes) and *Scyliorhinus canicula* (Carcharhiniformes) were removed from the skin of specimens captured by commercial fisheries at the Mediterranean coast of Spain. All the material studied is deposited in the Museum of Geology in University of València. Referred materials: scales MGUV-26.092 to MGUV-26.100, MGUV-27.191, and MGUV-27.192.

RESULTS

Hybodontiformes

As a rule, a thick (25 to 40 µm) capping enameloid layer, with a well-defined enameloid−dentin junction and many dentine tubules that cross the junction and extend into the capping tissue, is present in all different morphologies of Hybodontiformes scales studied (Fig. 1). Individual hydroxyapatite crystallites are discernible, elongate in shape, varying from 0.5 to 1 µm in length (Figs. 1b, 1f, 1j, 1m). Crystallites usually lack any kind of orientation (e.g., Fig. 1g); although in some areas of the scale morphotypes *Lobaticorona* and *Glabrisubcorona*, crystallites seem to be arranged in a preferred orientation perpendicular to their surfaces (e.g., Figs. 1c, 1j). The parts of the scales where crystallites show this preferred orientation correspond to inter-ridges, whereas in the ridges, the crystallites are randomly oriented (Figs. 1k, 1m). Finally, in the scales of the *Coniunctio* morphotype (Figs. 1h, 1j), crystallites of the inner sublayer are densely arranged in incipient sets and perpendicular to the enameloid−dentine junction (Figs. 1i, 1j).

R e m a r k s. According to Reif (1978), the hybodontid morphogenetic scale type compensates for the growth of the animal by both addition of new scales in the skin and growth of some scales by addition of consecutive odontodes (sensu Ørvig, 1967; see also Karatajūtė-Talimaa, 1998). Thus, the squamation of hybodontids contains nongrowing (single odontode) scales and compound growing scales (following terminology of Reif, 1978; see also Karatajūtė-Talimaa, 1992,1998). Both of them have been found in the material studied. In addition, Reif (1978, textfigs. 2D, 3D) has described scales that represent an intermediate situation between growing and nongrowing ones, in which a pair of odontodes was formed almost at the same time and connected by a neck canal. These types of scales are also present in our material (Fig. 1h).
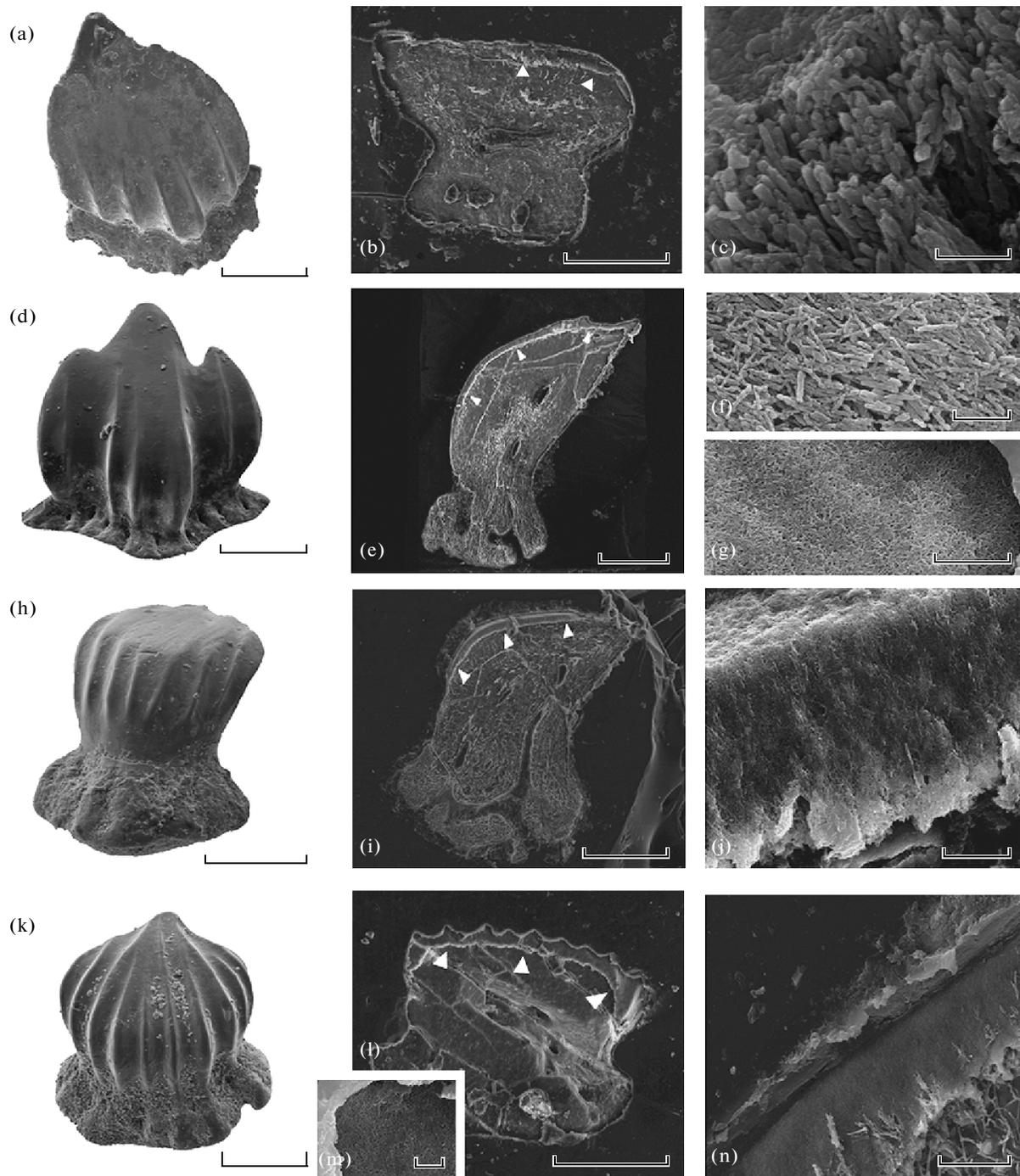
Fig. 1. Different morphologies of hybodontiform scales from the Middle Triassic of Spain: (a) *Glabrisubcorona* type scale (MGUV-26.092); scale bar, 200 μm. (b) Overview of the embedded scale MGUV-26.092 in longitudinal section where the enameloid layer is discernible; etched 5 s in HCl 10%; scale bar, 200 μm. (c) Detail of the SCE layer; etched 5 s in HCl 10%; scale bar 3 μm. (d) *Parvidiabolus* type scale (MGUV-26.093); scale bar, 700 μm. (e) General view of the embedded scale MGUV-26.093 with an enameloid layer that covers the crown surface; etched 5s in HCl 10%; scale bar, 200 μm. (f) High resolution picture of the enameloid crystallites in surface view; etched 5 s in HCl 10%; scale bar 800 nm. (g) Detail of the whole length of the monolayer of SCE randomly arranged; etched 5 s in HCl 10%; scale bar, 6 μm. (h) *Coniunctio* type scale (MGUV-26.094); scale bar, 200 μm. (i) Overview of the embedded scale MGUV-26.094 in which it is possible to distinguish clearly the enameloid across the crown surface and the underlying dentine core; 5 s in HCl 10%; scale bar, 200 μm. (j) Detail of the SCE with an inner part where crystallites are grouped into packets perpendicular to the crown surface; etched 5 s in HCl 10%; scale bar, 7 μm. (k) *Undulaticorona* type scale (MGUV-26.095); scale bar, 200 μm. (1) General view of the embedded scale MGUV-26.095 showing the same pattern as the other morphologies: a cap of enameloid on the crown surface with a dentine layer below; 5 s in HCl 10%; scale bar, 200 μm. (m) Detail of one cusplet in which the SCE are randomly arranged; etched 5 s in HCl 10%; scale bar, 3 μm. (n) Detail of the enameloid-dentine junction with dentine tubules that penetrate into the enameloid layer; etched 5 s in HCl 10%; scale bar, 20 μm. The arrows show the enameloid-dentine junction.

Although scales from Bugarra are clearly of hybodontid type; they cannot be identified at the generic or familial level. Isolated teeth of the genera *Paleobates, Hybodus, Lissodus*, "*Polyacrodus*" (order Hybodontiformes), and *Pseudodalatias* (order *incertae sedis*) occur along with scales (Plá et al., 2013) hampering definite generic identification of scales. However, based upon similar stratigraphical occurrence and comparison with articulated specimens, Plá et al. (2011) suggested association of scales of *Lobaticorona* morphotype with teeth of *Lissodus* and the *Parvidiabolus* morphotype with teeth of *Hybodus*.

Neoselachians

*Raja clavata (*Rajiformes*)*

Scales of *Raja clavata* (Figs. 2a–2f) were selected to represent Batoidea. The morphology of *Raja clavata* scales varies depending on the position they occupy in the body. Scales from the anterior and dorsal parts of the body are analyzed here. In both cases, an enameloid cap of around 15 μm thick is present on top of each scale, where crystallites show a different arrangement between the outer and inner parts of the enameloid layer (Figs. 2b, 2c). In all scales, the crystallites of the outer part of the layer are randomly oriented but very compact. In the inner part of the enameloid layer, the hydroxyapatite crystallites are densely arranged and mainly perpendicular to the surface (Figs. 2b, 2c, 2f). The compaction of crystallites is more evident in some areas of scales, where interwoven incipient "sets" of crystallites appear with a high level of compaction (Fig. 2f). Crystallites of the same set are arranged in parallel, showing the same orientation (Figs. 2e, 2f). Close to the enameloid–dentine junction, the organization of the hydroxyapatite crystallites is less evident and they seem to be randomly oriented (Fig. 2b). In all scales, the hydroxyapatite crystallites are at most 1 μm long; and they are elongated (Figs. 2c, 2e, 2f).

Scyliorhinus canicula (Carcharhiniformes)

Scyliorhinus canicula scales (Figs. 2g–2i) bear an enameloid layer approximately 15 μm thick on the crown surface. Its microstructural organization is somewhat similar to that of *Raja* scales. Two "sublayers" are found in the scales of this carcharhiniform shark; the inner sublayer, with crystals showing a preferred orientation perpendicular to the enameloid–dentine junction, and a thin outer sublayer, with more compact crystallites (Figs. 2h, 2i). In some areas of the scales, crystallites appear densely arranged. As in *Raja clavata*, the hydroxyapatite crystallites are 1 μm long and they are elongate.

R e m a r k s. According to Reif (1978), all neoselachians possess the *Heterodontus* morphogenetic type. Their "placoid" scales, i.e., simple nongrowing scales containing only a single wide pulp cavity, do not grow and are continuously replaced. The growth of the animal is compensated by addition of new scales and by the increasing size of replacement scales. As a result, the number of scales increases throughout ontogeny.

DISCUSSION

Our study demonstrates the existence of a layer of hypermineralized tissue, different from the dentine layer, covering all scales. In all specimens, this layer is composed of individually discernible single elongate crystallites. The location of this hypermineralized layer in the outer surface, the presence of a distinct border between this layer and the underlying dentine, the presence of numerous dentine tubules extending across this junction zone, and the presence of individual hydroxyapatite crystallites suggest its interpretation as single crystallite enameloid (SCE).

Although euselachian scales clearly do not reach the high degree of microstructural differentiation present in the tooth enameloid of some neoselachians with distinct individual bundles, they exhibit some microstructural organization of the crystallites, such as different organization in two "sublayers", preferred orientation and occurrence of poorly structured sets of densely arranged parallel crystallites. Thus, enameloid found in several scales of hybodontiforms and neoselachians demonstrates the presence of an outer part of compact SCE and the inner part of SCE, with some densely arranged crystallites parallel to the scale surface (Figs. 1j, 2i). The tightly compacted enameloid with parallel crystallites aligned perpendicular to the enameloid–dentine junction is comparable to that described in various non-neoselachian shark teeth (Duffin and Cuny, 2008). Finally, the arrangement of crystallites within groups of parallel, more densely arranged crystallites found in scales of rajiformes (Figs. 2e, 2f) resemble in some way the assembly of crystallites in the loosened enameloid bundles of stem selachiomorphs (Johns et al., 1997; Andreev and Cuny, 2012), although it is clearly different from the highly structured enameloid of crown selachiomorphs (compare with Figs. 2k, 2l, where preparation allows identification of crystallites forming the bundles of the PBE of a neoselachian tooth).

As noted above, the emergence of microstructural differentiation in the enameloid of chondrichthyan teeth has usually been interpreted as an adaptational process, preventing crack propagation and increasing resistance to tensile forces (Preuschoft et al., 1974) related to the emergence of new feeding strategies in the group (see Gillis and Donoghue, 2007; Duffin and Cuny, 2008). However, we show here that the enameloid of scales exhibits some degree of microstructural organization. Taking into account that scales lack feeding functions of teeth (and, hence, there is no need to assemble enameloid crystallites in order to resist any compressive or tensile force during feeding),
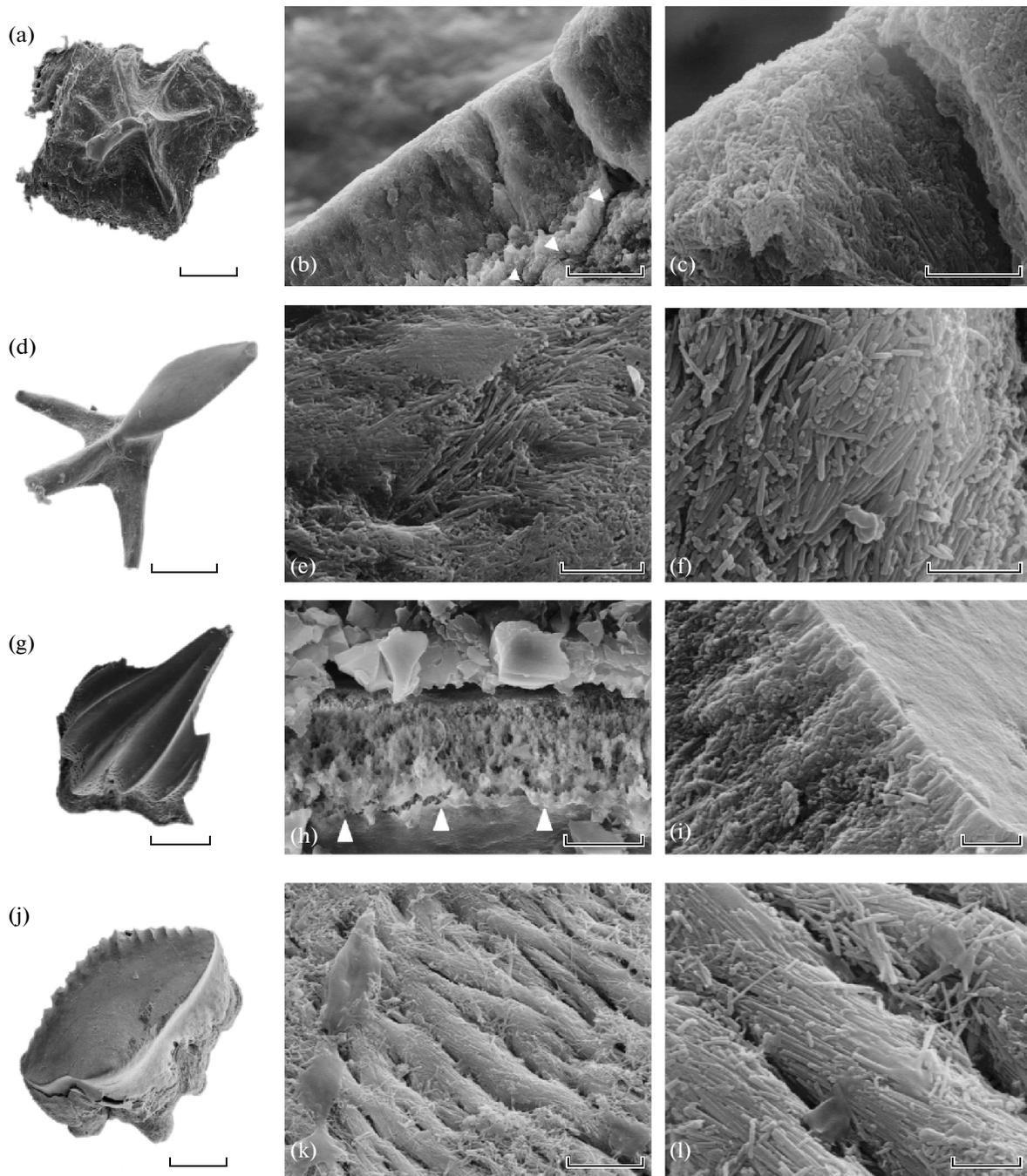
Fig. 2. (a) Body scale of *Raja clavata* (MGUV-26.096); scale bar, 500 μm. (b) Detail of the enameloid layer in a fresh fracture on the crown surface of the scale. The arrows show the enameloid-dentine junction; etched in 0.5% orthophosphoric acid for 2 min; scale bar, 8 μm. (c) SCE with crystallites arranged into two sublayers: the inner one with crystallites aligned perpendicular to the crown surface and the outer one with crystallites arranged more parallel to the crown surface; etched in 0.5% orthophosphoric acid for 2 min; scale bar, 2 μm. (d) Snout scale of *Raja clavata* (MGUV-26.097); scale bar, 200 μm. (e) Detail of the surface in MGUV-26.097, showing the densely arranged crystallites; etched in 0.5% orthophosphoric acid 2 for min; scale bar, 5 μm. (f) Detail of the embedded scale MGUV-27.191 showing the densely arranged crystallites perpendicular to the surface of the scale; etched 5s in HCl 10%; scale bar, 1 μm. (g) Scale of *Scyliorhinus canicula* (MGUV-26.098); scale bar, 200 μm. (h) Embedded and etched scale (MGUV-26.099) showing the enameloid layer (arrows show the enameloid-dentine junction); etched 10s in HCl 10%; scale bar 4 μm. (i) Detail of a fresh fracture in MGUV-27.192, in which, two sublayers are discernible: the thick inner one with the individual crystallites perpendicular to the surface and the thin outer one with high compacted crystallites; etched 10s in HCl 10%; scale bar 1 μm. (j) Tooth of *Mustelus mustelus* (MGUV-26.100); scale bar 300 μm. (k) Detail of the etched tooth surface where the crystallites forming the fibers of the parallel fibered enameloid (PFE) layers are still clearly discernible; etched 10s in HCl 10%; scale bar 7 μm. (1) Detail of the (k); etched 10 s in HCl 10%; scale bar 1 μm.

the occurrence of incipient microstructural organization/differentiation in scale enameloid (i.e., preferred orientation of crystallites, or "sublayers") cannot be linked with any obvious adaptive function and it could be understood as a consequence of self-organizing processes. Self-assembly of hydroxyapatite crystallites into fibers (or enamel prismlike structures) has been reproduced "in vitro" (e.g., Chen et al., 2005; Wang et al., 2008) and self-organizing processes of growing crystallites, along with interactions with amelogenins, have been proposed to play a major role in establishing structural patterns in amniote enamel (Sander, 2000; Margolis et al, 2006 and references therein). It is possible that the self-organization process of enameloid crystallites and proteins also occurred in the evolution of chondrichthyan teeth irrespective of adaptive pressure. Emerging mechanical properties of the microstructural organization/differentiation of the enameloid would make possible its subsequent co-option for new purposes (i.e., new feeding strategies). This supports: (1) the convergent evolution of cutting dentitions in several Paleozoic and Early Mesozoic nonneoselachian chondrichthyans that demonstrate an SCE with crystallites that are densely packed and oriented perpendicular to the tooth surface (for example, in Paleozoic *Carcharopsis*, or Mesozoic *Priohybodus, Thaiodus* (hybodonts), and *Pseudodalatias*: Duffin and Cuny, 2008; Botella et al., 2009a); and (2) the fact that microstructural differentiation of the triple-layered enameloid in neoselachians phylogenetically precedes the emergence of cutting and gouging feeding strategies in the group (see Gillis and Donoghue, 2007). Thus, we suggest that an initial incipient microstructural organization due to self-organizing processes of crystallites could provide an appropriate "substrate" for selective pressure, which led to acquisition of a triple-layered organization in neoselachian teeth. The recognition of several levels of increasing architectural complexity in the enameloid crystallite bundles of stem selachiomorphs (Andreev and Cuny, 2012) suggest that the acquisition of the triple-layered enameloid progressed gradually, step-by-step through several phases, from a plesiomorphic single crystallite state.

A plausible scenario for the increased complexity of the enameloid in neoselachian sharks was proposed by Gillis and Donoghue (2007). The ancestral teeth of gnathostomes, evolutionarily derived from placoid scales, would have had a capping layer of SCE, resulting from a late differentiation of ameloblasts (ectodermal origin) during odontogenesis. Then, the enameloid matrix would be rich in ameloblastic cell secretions and deficient in odontoblast (ectomesenchimal origin) cell products. The acquisition of the triple-layered enameloid in neoselachian (non batoid) lineage would be concordant with heterochronic processes in ameloblast differentiation during odontogenesis with an increase in odontoblast-derived tubular vesicles in the enameloid matrix considering that a mixed matrix composed of both ameloblast cell secretions and odontoblast-derived tubular vesicles is critical for the development of higher order enameloid structures (Gillis and Donoghue, 2007). This underlies the idea that thin layer of SCE overlying the PBE of neoselachians (i.e., SLE produced in complete isolation from odontoblast secretion due to its outer enameloid position and, therefore, derived almost exclusively from ameloblast products: Reif, 1979 in Gillis and Donoghue, 2007) is reminiscent of the plesiomorphic SCE. However, other authors suggested that the SLE (being of ectodermal origin, see above) of neoselachians is a late addition during the evolution of enameloid in Elasmobranchii and that primitive chondrichthyan enameloid would have been a pure mesodermal product (Cuny and Risnes, 2005). Nevertheless, more studies are required before one of these scenarios (or others) can be definitively accepted.

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