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Kugelzellen in larval anuran epidermis: an ultrastructural study on tadpoles of *Pelobates cultripes* (Pelobatidae) and *Phyllobates bicolor* (Dendrobatidae)

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Abstract

Prior to hind limb development, tadpoles of the western spadefoot frog *Pelobates cultripes* (Pelobatidae) and dart-arrow frog *Phyllobates bicolor* (Dendrobatidae) possess large clear cells in the basal layer of the epidermis. These cells closely resemble Kugelzellen (KZn) of larval clawed frog, *Xenopus laevis* (Pipidae) and share ultrastructural traits with Leydig cells (LCs) of Caudata and Caecilia. In both species, KZn possess a transparent cytoplasm and a remarkable peripheral cytoskeleton of tonofilaments: in the arrow frog tonofilaments form bands parallel to the cell surface, in the spadefoot frog thin bundles, arranged in a three-dimensional network. KZn combine turgor (resulting from the hydrated cytoplasm) with stiffness (from peripheral cytoskeleton), thus providing structural stability to the larval epidermis.

Contents

Introduction

Large cells characterized by translucent cytoloplasm and involved in secretory or mechanical roles have been described in the epidermis of amphibian larvae (Fox, 1988). Gregarious bufoniid tadpoles possess giant cells (*Riesenzellen*, RZn) with basal-apically arranged organelles and secretory granules resembling the products of serous glands in adult toad skin (Delfino, 1991; Delfino *et al.,* 1995a, b). Giant cells synthesize alarm substances that, once released into the environ (Fox, 1988), trigger fright reaction in tadpoles (Pfeiffer, 1974).

Larval urodeles (Kelly, 1966; Fährmann, 1971a, b; Greven, 1980; Fox, 1988) as well as caecilians (Fox, 1986a, b; Breckenridge *et al.,* 1987; Fox,1988) possess spheroid-ellipsoidal cells (Leydig cells, LCs) that exhibit a complex, peripheral cytoskeleton (Langerhans net) of tonofilaments (Fährmann, 1971a, b; Greven, 1980; Rosenberg *et al.,* 1982; Kantorek and Clemen, 1990). LC cytoplasm exhibits a central-peripheral polarization: the inner, perinuclear region (*Hofcytoplasma*) contains biosynthesis organelles, the outer region granules of various density. These granules may be discharged following LC rupture (Jarial, 1989) or released through merocrine processes (Quagliata *et al.,* 2006).

Similar epidermal cells have been described in tadpoles of the South African clawed frog *Xenopus laevis* and referred to as *Kugelzellen* (KZn or ball cells), on account of their spheroid shape (Fröhlich *et al.,* 1977). KZn have also been described as clear cells (Fox, 1988) or "vacuoles" (Seki *et al.,* 1989) due to their remarkably transparent cytoplasm. KZn do not exhibit any ultrastructural patterns of secretory activity, but possess thick bundles of tonofilaments and a dense layer of peripheral cytoplasm (Fröhlich *et al.,* 1977, Delfino and Malentacchi, 2006; Quagliata *et al.,* 2006). KZn perform a mechanical function (Fröhlich *et al.,* 1977) before undergoing degeneration during metamorphosis.

On account of the remarkable subcellular features outlined above, the present light and transmission electron microscope (LM and TEM) study investigates the larval epidermis of the western spadefoot frog *Pelobates cultripes* (Cuvier, 1829) and dart-arrow frog *Phyllobates bicolor* Duméril and Bibron, 1841, representative of distant branches: families Pelobatidae [97] and Dendrobatidae [462] of the anuran phylogenetic tree (Frost *et al.,* 2006). Although these frogs have been investigated in previous studies on epidermal specialized cell lines (Delfino and Malentacchi, 2006; Quagliata *et al.,* 2006), this is the first comparative report which analyses KZn in their tadpoles.

Material and methods

We analysed limbless tadpoles in the ontogenetic range 23-27, marked by development of operculum and labial teeth (Gosner, 1960). Epidermis maintains a stable structure in these stages, until cutaneous glands develop during toe morphogenesis (Delfino *et al.,* 1991). Tadpoles of *Pelobates cultripes* - six specimens - and *Phyllobates bicolor* - eight specimens - were collected near Ciudad Real (Spain) and Medelin (Colombia), respectively, and sacrificed with 0.1% chlorobutanol. Skin strips, 9 mm^2, were fixed in the Karnovsky (1965) solution, postfixed in 1% OsO_4 (both in cacodylate buffer) and embedded in Epon 812. Semi-thin sections of 1.5 µm thickness (for the LM) were stained with buffered (1%, borax) toluidine blue, ultrathin sections

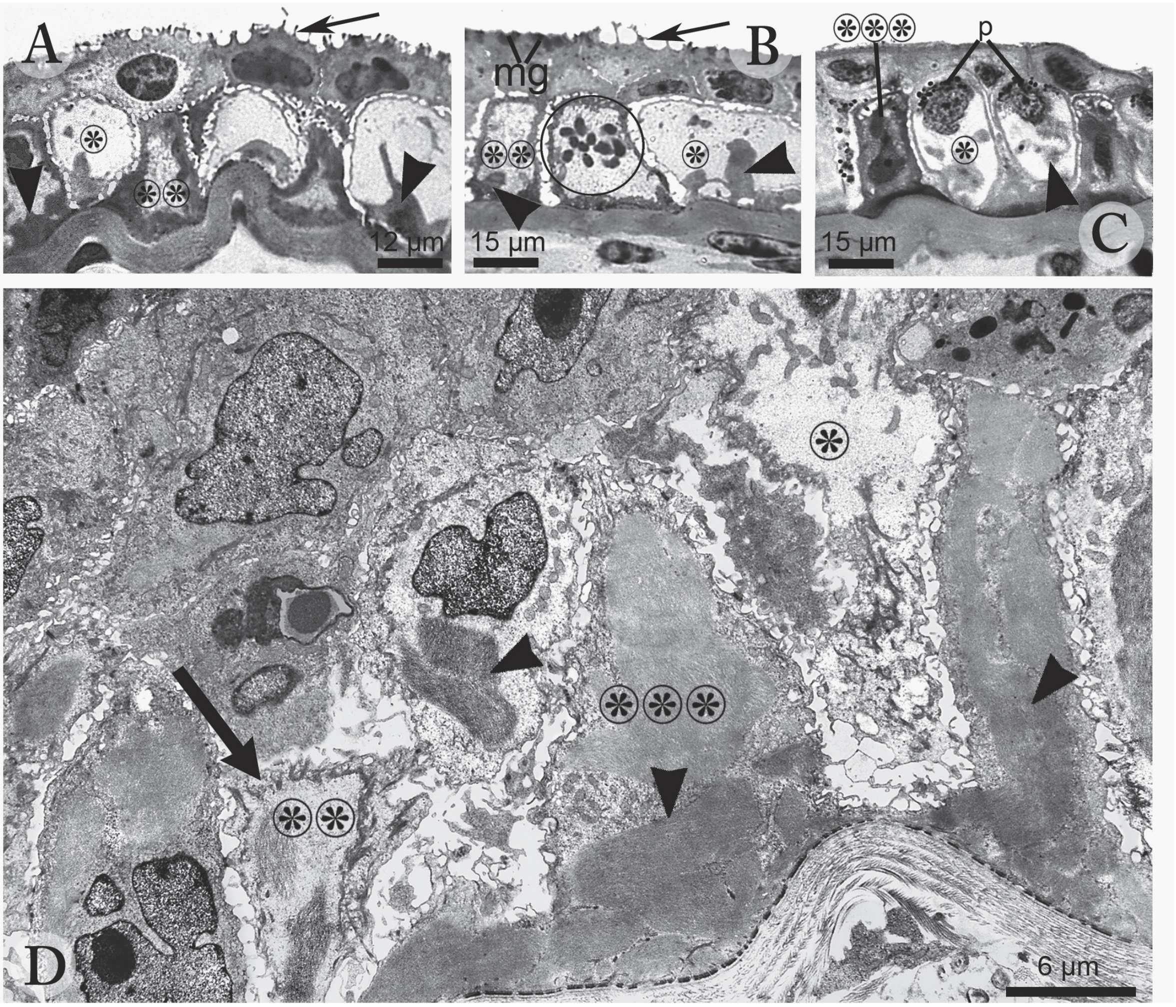

Fig. 1. Epidermis structure in limbless tadpoles of *Phyllobates bicolor* (a-b, LM micrographs) and *Pelobates cultripes* (c, LM micrograph; d, TEM micrograph). **A.** KZn and pavement cells varying in cytoplasm density. **B.** Mitotic pattern (encircled) in KZ and mucous granules (mg) in pavement cell. **C.** KZn with different cytoplasm densities and melanin granules (p). **D.** KZn vary considerably in both cytoplasm density and filament content. (✱)(✱)(✱) ◊ (✱) = decreasing cytoplasm density, small arrows in a and b point to microridges, arrowheads in a-d to basal tonofilament patches, large arrow in d to peripheral cytoskeleton.

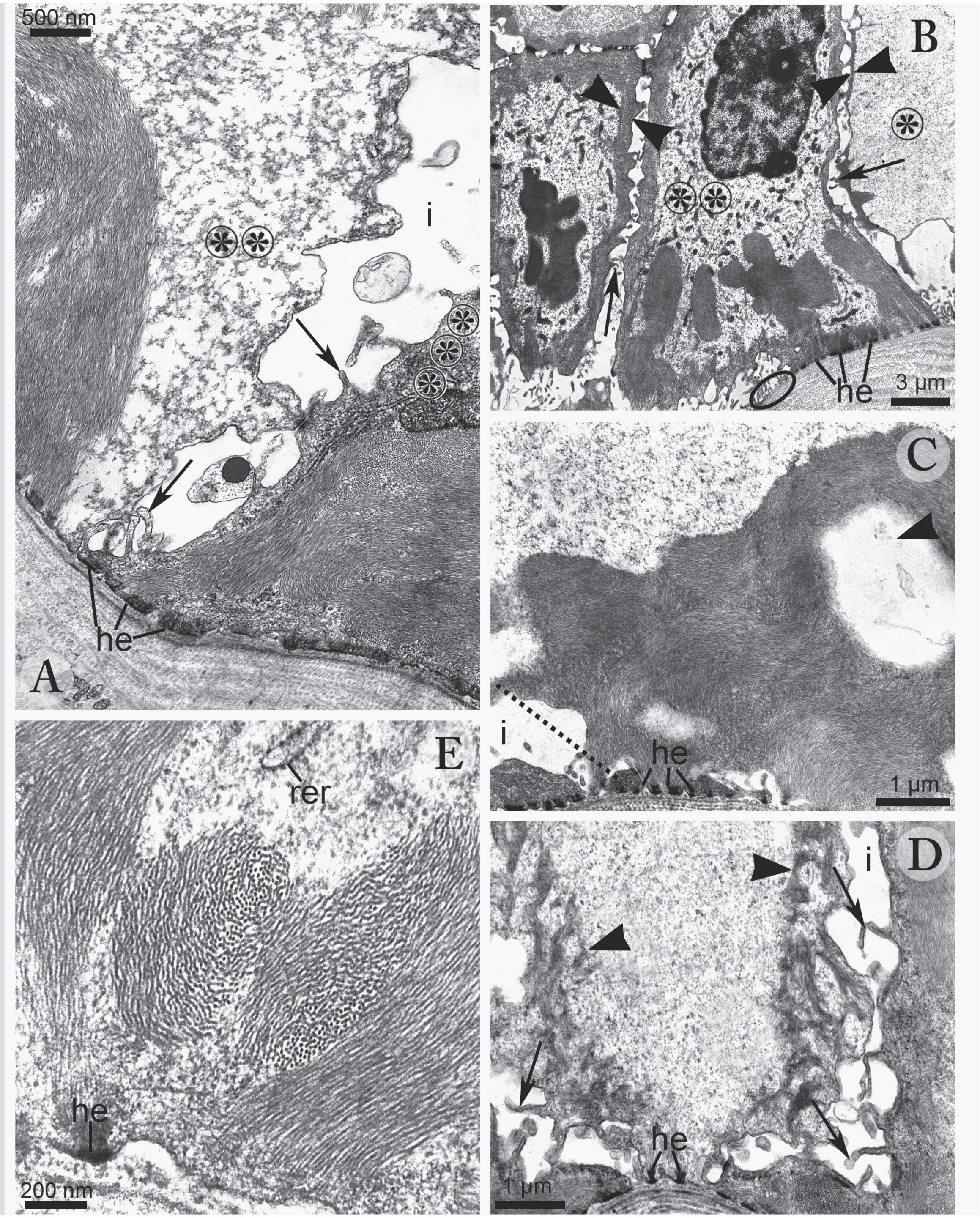

Fig. 2. Basal tonofilament bundles and hemidesmosomes in KZn of *Pelobates cultripes* (a, d) and *Phyllobates bicolor* (b, c, e). **A.** Both submature (✱)(✱) and immature (✱)(✱)(✱) KZn exhibit basal bundles. **B.** Two submature KZn (✱)(✱) and one mature KZ (✱) with thick and thin cytoplasm 'cortices', respectively (opposite arrowheads); ellipse encircles a hemidesmosome-free area at the dermal-epidermal interface. **C.** The light area (arrowhead) is an interstitial space in a semi-tangential cell section, as suggested by the dotted line. **D.** In this mature KZ, tonofilaments form a micro-trabeculation (arrowheads). **E.** Tonofilaments converging towards the dermal side. Arrows point to thin cytoplasm processes, he = hemidesmosomes, i = interstice, rer = rough endoplasmic reticulum.

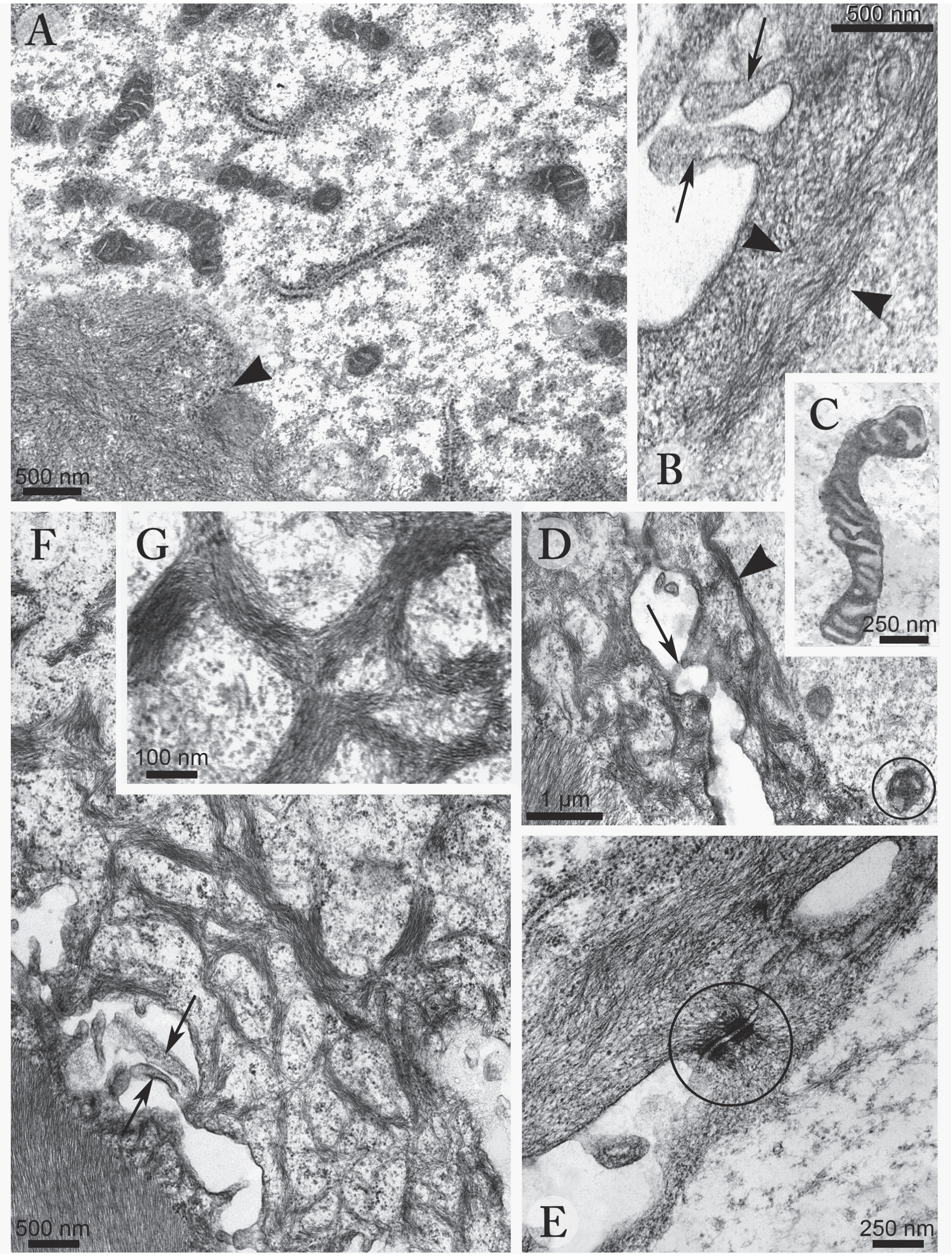

Fig. 3. Representative organelles and lateral portions of KZn. *Pelobates cultripes* (d, f, g) and *Phyllobates bicolor* (a, b, c, e). **A.** Cisterns of rough endoplasmic reticulum and mitochondria with dense matrix; arrowhead points to a tonofilament patch. **B.** Slender processes (arrows) from KZn and peripheral tonofilaments with a regular course (opposite arrowheads). **C.** Mitochondrion with dilated cristae. **D.** Micro-trabeculation (arrowhead) and spheroid mitochondrion with dilated cristae (encircled); arrow points to slender cytoplasm process. **E.** Desmosomes (encircled) and contiguous cell cortices. **F.** Irregular, micro-trabeculation network; arrows point to slender cytoplasm processes. **G.** Detail of micro-trabeculation.

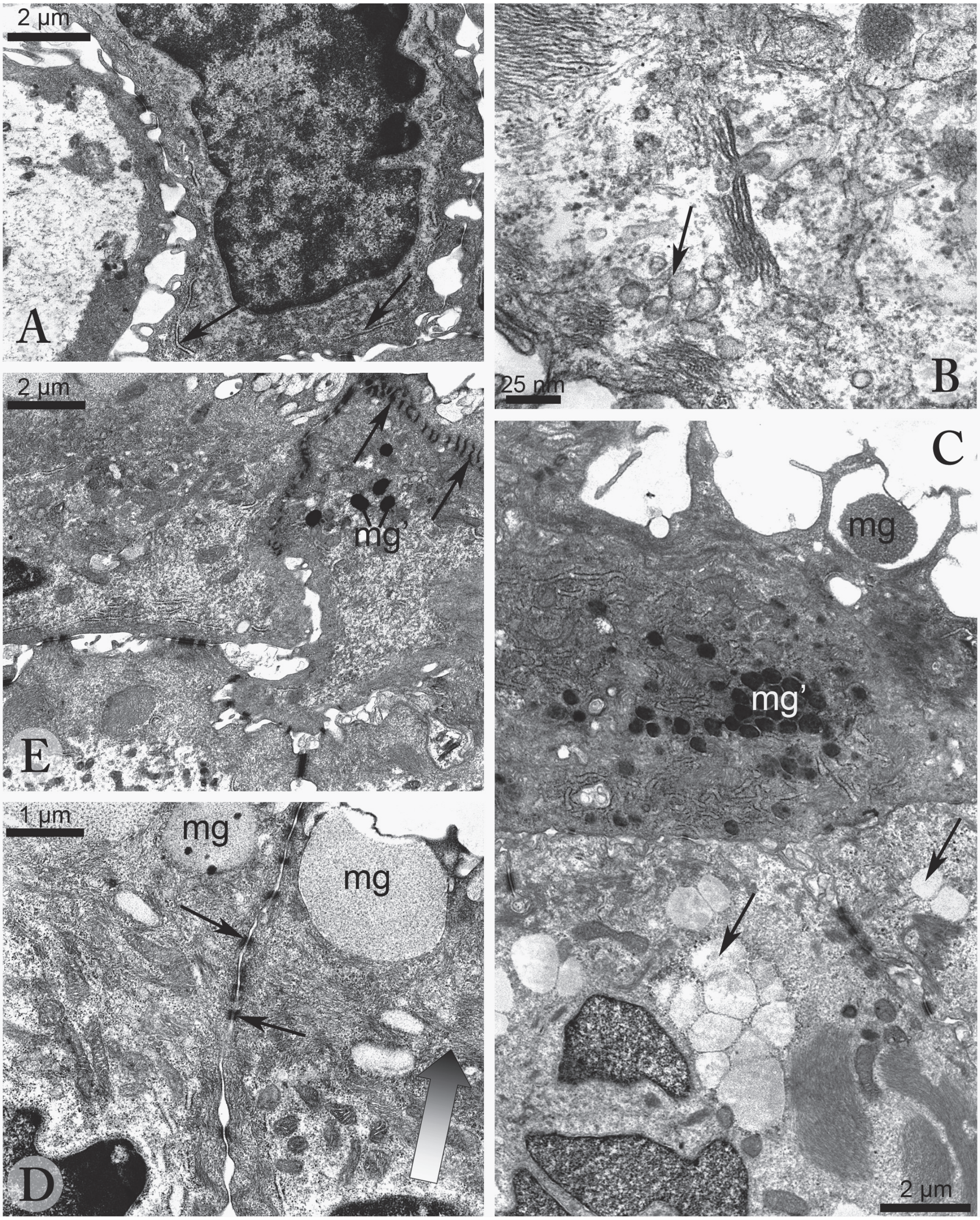

Fig. 4. Biosynthesis machinery in KZn (a, *Phyllobates bicolor*, b and c, *Pelobates cultripes*) and secretory activity in pavement cells (c, *Pelobates cultripes*, d and e, *Phyllobates bicolor*). **A.** Immature KZ with slender rough reticulum cisterns (arrows). **B.** Golgi stack releasing minute vesicles (arrow). **C.** Pavement cell storing small dense granules (mg') and releasing a larger granule (mg), contiguous to immature KZn holding vesicle clusters (arrows). **D.** Serial desmosomes (arrows) and large granules (mg) near the body surface. The large, grey-gradient arrow shows a gradual increase in cytoplasm density. **E.** Small dense granules (mg') and serial desmosomes (arrows).

with a hydroalcoholic saturated solution (25 mg/ml) of uranyl acetate followed by alkaline lead citrate (2 mg/ml). Ultrathin sections were observed (80 KV) with a Siemens 101 TEM.

Results

Since epidermis in *Pelobates cultripes* and *Phyllobates bicolor* tadpoles exhibits similar structural and ultrastructural traits, a common description has been given, unless distinctive features were observed.

LM analysis: Larval epidermis is a bilayered epithelium with inner (basal) and outer (pavement) cells that shows specific morphological traits (Fig. 1A-C). Inner cells have been ascribed to the basal layer since they contact the basement membrane (dermal-epidermal junction), regardless of their derivation from the undifferentiated epidermis cells. Basal cells rest on a relatively thick dense dermis, and are large and tall prismatic to spheroid-ellipsoidal in shape. Pavement cells are smaller and roughly cuboidal or short prismatic in shape. On the dermal side of the basal cells, tonofilaments form dense patch-bands (Fig. 1A-C) in a cytoplasm background that ranges from opaque in prismatic cells to translucent in spheroid-ellipsoidal cells. These clear cells have been regarded as mature KZn, the main target of our research. In *P. bicolor* they possess an obvious peripheral layer of opaque cytoplasm (cortex, Fig. 1A, B), which is thinner in translucent KZn than KZn with a relatively dense cytoplasm, sometimes engaged in mitotic processes (Fig. 1B). Cytoplasm density is also variable in pavement cells (Fig. 1) which contain mucous granules (Fig. 1B) near their microridged free surface (Fig. 1A, B).

TEM analysis: oblique sections and/or partially overlapping of contiguous cells may give the apparence of a multi-layered epidermis (Fig. 1D). A somewhat range of cytoplasm density and tonofilament content is obvious in KZn, suggesting a maturational evolution: as a rule, the denser the cytoplasm the larger the amounts of filaments, accumulating in the infranuclear cytoplasm. Beside basal tonofilament accumulations, translucent KZn contain a distinctive peripheral cytoskeleton (Fig. 1D). All KZn contact the dermal-epidermal junction (Fig. 2A-E), confirming that they belong to the basal cell layer. On the dermal side, the tonofilament bundles arch upwards, approaching to the plasma membrane (Fig. 2E) and contributing to the hemidesmosomes (Fig. 2A-E). These junctions are not found only where KZn reach the epidermal-dermal junction with thin processes (Fig. 2B). Relationships between contiguous KZn are relatively loose on the dermal side, resulting in wide interstices (Fig. 2A, D). In *P. bicolor* KZn, the tonofilaments in the infanuclear cytoplasm form peripheral leaf-like patches (Figs. 2B and 3A) and bands roughly parallel to the cell surface (Fig. 2B). The tonofilament patches contour exiguous compartments of the interstices, contained in invaginations of KZ surface (Fig. 2C). In *P. cultripes* KZn the peripheral cytoskeleton of the infranuclear cytoplasm is less compact, forming a reticular framework (Fig. 2D).

The lateral surfaces of KZn emanate thin processes resembling irregular microvilli (Figs. 2A, B, D and 3B, D, F) as well as larger processes joined by spot desmosomes (maculae adherentes, Fig. 3E). The cytoskeleton retains differential features in the two species: tonofilament bands roughly parallel to the lateral cell surface in *P. bicolor* (Fig. 3B, E), but irregular micro-trabeculation networks in *P. cultripes,* (Fig. 3D, F, G). Submature KZn contain slender rough endoplasmic reticulum (rer) cisterns (Figs. 2E and 3A), as well as small mitochondria with dense matrix (Fig. 3A) and dilated cristae (Fig. 3C, D). In *P. bicolor*, an obvious cell cortex starts to appear in immature KZn (Fig. 4A), assumes its typical appearances as a dense 1-1.5 µm thick cytoplasm layer in intermediate maturational stages (Fig. 2B), and eventually thins out in translucent KZn (Figs. 2B and 3E). High nucleo-plasmatic ratio confirms that KZn with relatively dense cytoplasms are immature cells of this specialised line, although they possess rer cisterns (Fig. 4A). In *P. cultripes*, the biosynthesis machinery includes Golgi stacks, releasing small vesicles with moderately opaque content (Fig. 4B). In the supranuclear cytoplasm, this product forms clusters of larger, translucent vesicles (up to 1.5 µm in diameter, Fig. 4C) contiguous to opaque mucous granules in pavement cells (Fig. 4C). The mucous product displays similar, secretory phase-dependent features in the two species investigated: small (up to 0.5 µm), electron-dense granules during intracytoplasmic storage (Fig. 4C, E), and large (up to 2 µm), moderately opaque granules during release (Fig. 4C, D). Near the body surface, contiguous cells are joined together by serial desmosomes, so that the intercellular spaces are reduced to slender interstices (Fig. 4C-E). The cytoplasm of pavement cells is opaque (Fig. 4C), but with a central-peripheral density gradient (Fig. 4E) towards the skin surface (Fig. 4D).

Discussion

Limbless tadpoles of *Pelobates cultripes* and *Phyllobates bicolor* possess KZn in the basal epidermal layer, namely ephemeral cells (Fröhlich *et al.* 1977), destined to be substituted by typical keratinocytes of the anuran epidermis. In the ontogenetic range observed, KZn show no degeneration features at all, and retain their proliferative potential, at least in intermediate stage of maturation. KZn can regulate the cytoplasm turgor by active transmembranary transport of ions (Fröhlich *et al.,* 1978) and act as pressure-elasticity modules, recalling the notochordal cells in their mechanical properties (Fröhlich *et al.,* 1977). However, the hydrostatic pressure requires a stiffness component to perform a supporting role: this is accomplished by the tonofilament bundles in the peripheral cytoskeleton. Tonofilaments form compact bands in *P. bicolor* KZn, but in *P. cultripes* they are arranged in peculiar micro-trabeculatures, that foreshadow the Langerhans net of Leydig cells (Fährmann, 1971a, b; Greven, 1980; Rosenberg *et al.,* 1982; Kantorek and Clemen, 1990). However, the LC peripheral filaments are assembled in a more solid arrangement, and in Salamandridae, the bundles form a regular network with polygonal meshes (Delfino and Malentacchi, 2006). The patterns of vesicle production described in KZn of *P. cultripes* represent further morpho-functional traits shared with Leydig cells, as suggested by similar features in the organelles involved (Rosenberg *et al.,* 1982; Kantorek and Clemen, 1990). Homology between KZn and LCs is, nevertheless, a matter of debate, involving the three orders of extant amphibians with two possible contradictory meanings: Pflugfelder and Schubert (1965), and Nieuwkoop and Faber (1967) assign these cells to the same line. Fox (1988), on the contrary, maintains that Leydig cells have no homologous counterparts in Anura, while they are shared by urodele and *Ichtyophis* larvae. This suggests a relationship between Caudata and Gymnophiona which may be of phylogenetic significance (Fox, 1986b). In the light of the possible evolution by convergence of KZn and LCs, it is of interest that their typical ultrastructural traits: lucent cytoplasm and a peripheral felt-work of tonofilaments, have also been described in large epidermal cells of bony fishes (Whitear, 1986; Fox, 1989; Yokoya and Tamura, 1992). Possibly, the wide distribution of large clear cells among water-inhabiting vertebrates comes from their adaptive flexibility. Along with the mechanical function, these cells may regulate intraepidermal water flow (Fröhlich *et al.,* 1977) and/or contrast dehydration (Kelly, 1966; Yokoya and Tamura, 1992).

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