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Morphological characterization of the venom secretory epidermal cells in the stinger of marine and freshwater stingrays

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Abstract

Marine and freshwater stingrays are characterized by the presence of one to three mineralized serrated stingers on the tail, which are covered by epidermal cells secreting venom. When these animals are dorsally touched, the stinger can be introduced into the aggressor by a whip reflex mechanism of the tail, causing severe mechanical injuries and inoculating the venom. Accidents in humans are frequent causing intense local pain, oedema and erythema. Bacterial secondary infection is also common. In addition, injuries involving freshwater stingrays frequently cause a persistent cutaneous necrosis. The exact localization of the venom secretory epidermal cells in the stinger is controversial, but it is known that it is preferentially located in the ventrolateral grooves. A comparative morphological analysis of the stinger epidermal tissue of different marine and freshwater Brazilian stingray species was carried out. The results indicate that in freshwater species there is a larger number of protein secretory cells, of two different types, spread over the whole stinger epidermis, while in marine species the protein secretory cells are located only around or inside the stinger ventrolateral grooves. These differences between the stingers of the two groups can justify the more severe envenomation accidents with the freshwater species when compared with the marine species.

Keywords: Stingrays; Venoms; *Dasyatis*; *Aetobatus*; *Potamotrygon*

1. Introduction

Marine stingrays are elasmobranchs found along the Brazilian coast where they are represented by different genera, especially by *Dasyatis*. Besides the marine stingrays, there are three Potamotrygonidae

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genera living in freshwater environment. *Potamotrygon* is well represented in rivers of the North, Central West and Southern Brazilian regions (Rosa, 1985) and, in the last decades, is invading rivers of the Southeast due to hydrographical basin changes caused by the construction of hydroelectric plants (Garrone Neto et al., 2007). Stingrays are characterized by the presence of one to three mineralized stingers (modified barbed spines) in the tail (Fig. 1), which is covered by epidermal cells secreting venom (Halstead, 1970; Charvet-Almeida et al., 2002; Carvalho et al., 2003; Haddad Jr. et al., 2004). The exact localization of this tissue is controversial, but it is known that it is preferentially located in the stinger ventrolateral grooves (Halstead, 1970). Because stingrays are commonly found in the sea or river shores, hidden in the sand, they frequently cause accidents, mainly in the feet and ankles, when people step on them. Hands can also be affected, mainly of fishermen when manipulating fishing nets (Haddad Jr. et al., 2004; Brisset et al., 2006). Accidents are caused by a defensive whip reflex mechanism of the tail in which the stinger is introduced into the aggressor, causing severe mechanical injuries and, at the same time, liberating the venom (Halstead, 1970; Haddad Jr. et al., 2004). There is no specific serum therapy for these accidents (Haddad Jr. et al., 2004). The patients face intense local pain, oedema and erythema. Bacterial secondary infection is also common. In addition, accidents involving freshwater stingrays frequently cause a persistent cutaneous necrosis (Haddad 2000; Haddad Jr. et al., 2004; Magalhães et al., 2006), which is related to differences observed in the biological activities of the venom of marine and freshwater stingrays (Barbaro et al., 2007). Although the toxinology of stingrays is a relevant subject due to the high frequency of accidents, the literature about the morphology of the venom apparatus of these animals is very scarce (Halstead, 1970; Smith et al., 1974, 1981; Liu et al., 2001).

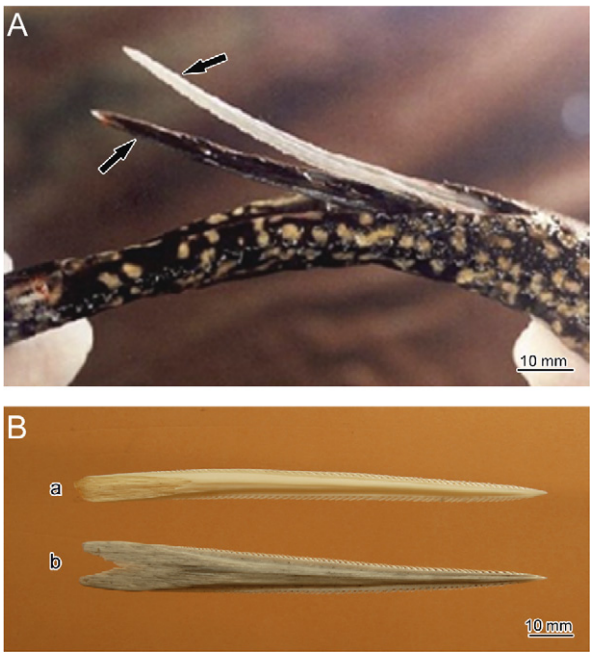


Fig. 1. (A) Tail of *Potamotrygon falkneri* with two stingers (arrows). (B) Mineralized portion of the stingers of *Dasyatis guttata* (a) and *Potamotrygon falkneri* (b). The stingers are flattened dorso-ventrally, with the lateral edges formed by two lines of smaller stingers pointed to the caudal extremity.

In this paper, a comparative morphological analysis of the stinger epidermal tissue of Brazilian marine and freshwater stingrays of different species was carried out in an attempt to verify characteristic patterns in the distribution of venom secretory epidermal cells in these two groups. The comparative analysis of the results indicates significant differences between the stingers of marine species and freshwater species of the *Potamotrygon* genus. These differences can justify the more severe envenomation accidents caused by specimens belonging to this genus when compared with accidents caused by the marine species.

2. Material and methods

2.1. Animals

The stingers were collected from three specimens of each one of the following Brazilian species: *Dasyatis guttata* and *Aetobatus narinari*, from Ubatuba, SP, *Potamotrygon falkneri*, from Três Lagoas, MS, *P. orbignyi*, from Colares, PA, and *P. leopoldi*, from Altamira, PA.

2.2. Histology

After collection, the stingers were immediately immersed in Karnowsky fixative (Karnowsky, 1965) and brought to the Laboratory of Cellular Biology of Instituto Butantan. After 48 h in the fixative, they were submitted to decalcification in 4% EDTA. Pieces of three different regions (apical, medial and basal regions) were then dehydrated and embedded

in paraffin and glycol methacrylate in a transversal orientation.

For general study of the tissues, 2 μm historesin sections were stained with toluidine–fuchsin (Junqueira, 1995).

Samples of the dorsal and ventral skin of *P. falkneri* from both the disc and the tail were embedded in paraffin as described above.

2.3. Histochemistry

The sections were submitted to the following histochemical methods: periodic-acid Schiff (PAS) and Alcian blue, pH 2.5, for the identification of neutral and acidic mucosubstances, respectively, and bromophenol blue, for protein identification (Bancroft and Stevens, 1996).

2.4. Photomicrography

Photomicrographs were obtained with an Olympus BX51 microscope and with an Olympus SZ stereomicroscope, equipped with a digital camera and Image-Pro Express software (MediaCybernetics).

2.5. Transmission electron microscopy

Small fragments of the fixed epidermis of the stinger were taken with the aid of a razor blade from the stinger ventrolateral groove of *D. guttata* and from different spots around the stinger of *P. falkneri*. The samples were post-fixed in 1% osmium tetroxide, contrasted in 1% uranyl acetate, dehydrated and embedded in epoxy resin. Ultrathin sections were obtained in a Sorvall MT6000 ultramicrotome, contrasted in 2% uranyl acetate and lead citrate, and examined in a LEO 906E.

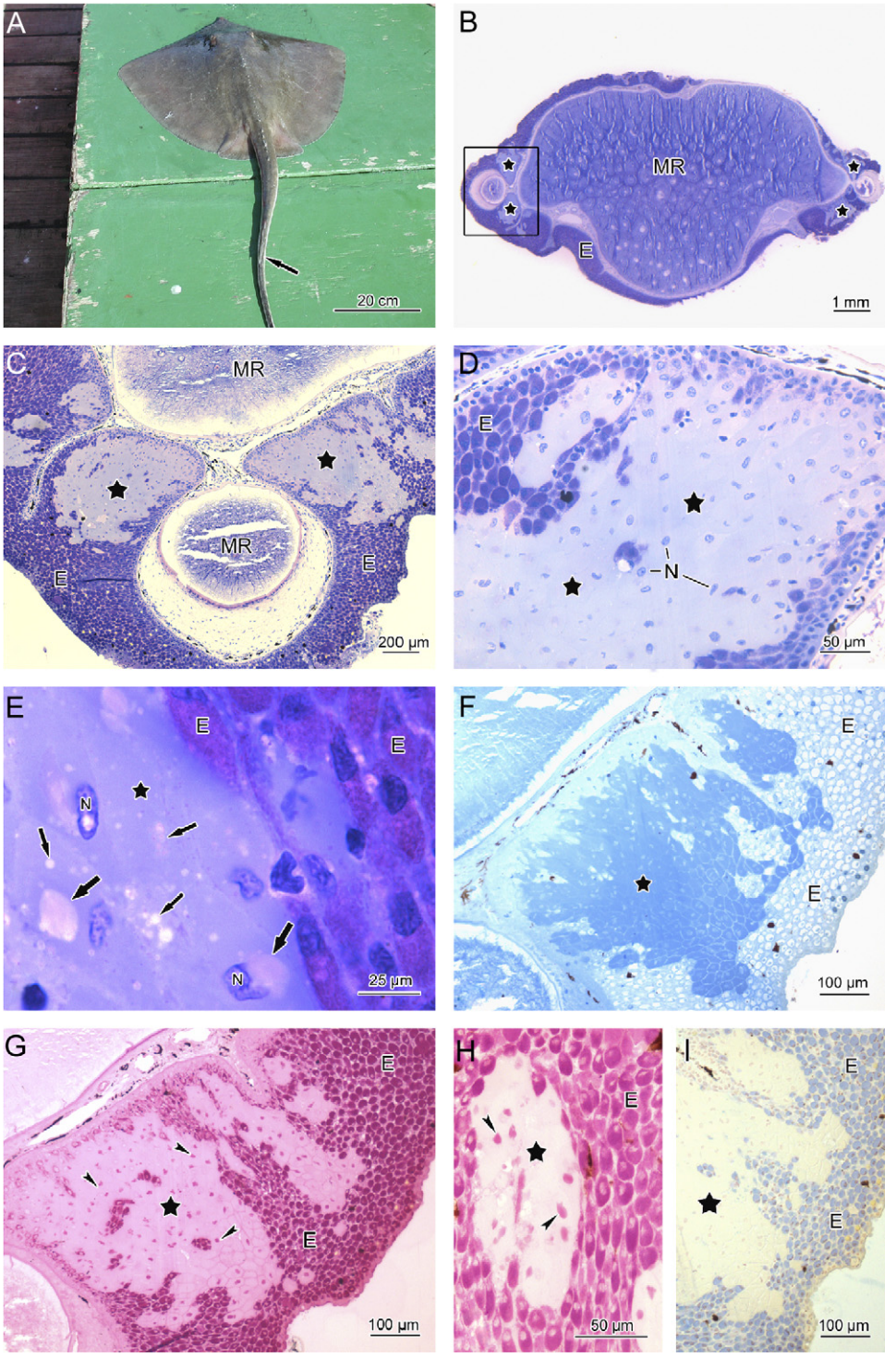
3. Results

3.1. General morphology

The results showed that in the whole stinger of *D. guttata* (Fig. 2A), the epidermis presents specialized cells, which are located preferentially within and/or around the stinger ventrolateral grooves. The rest of the epidermis is formed by spherical cells with a cytoplasm very rich in secretion granules (Fig. 2B–D). The same occurs with the stinger of *A. narinari* (Fig. 3A), although in this species the area occupied by the specialized cells seems to be restricted to the grooves (Figs. 3B and C). In both species, the specialized cells are club-shaped or elliptical and form aggregates without a defined form that can be distributed from the basis of the epidermis to the outermost cellular layers. Their clear homogeneous cytoplasm is characterized by the presence of peculiar vesicles with a filamentous content (Figs. 2E and 3C).

In *P. falkneri* (Fig. 4A), as well as in other species of *Potamotrygon* (Figs. 5A and 6A), the specialized cells form characteristic intermediate layers in the epidermis surrounding the whole stinger. The outermost epidermal layers, as well as the basal internal layers are formed by rounded cells that are similar to the cells composing most part of the epidermis of the marine species, presenting a cytoplasm full of secretion granules (Figs. 4B–D, 5B, C, 6B, C). The specialized layers are more prominent in the region of the ventrolateral grooves and, similar to what was observed in the marine species, their cells are characterized by the same type of fusiform cytoplasmic vesicles (Fig. 4E). Besides these specialized cell layers, it was observed that in the apical epidermal region, there are large flask-shaped secretory cells (Figs. 4D and 5C), which are connected to the exterior. These secretory cells are

Fig. 2. *Dasyatis guttata*. (A) Photo of a specimen. The arrow points to the tip of the stinger. (B) Transversal section of the stinger, indicating the specialized venom secretory cells (*) in the epidermis (E), next to the ventrolateral groove. MR—mineralized region. Toluidine blue-fuchsin. (C) Higher magnification of the ventrolateral region of the stinger, signed by the rectangle in (B), showing the disposition of the specialized cells (*). Toluidine blue-fuchsin. (D) Higher magnification of the specialized cells characterized by the clear homogeneous cytoplasm and irregular nuclei (N). Toluidine blue-fuchsin. (E) Detail of the cytoplasm of the specialized cells (*). The cytoplasm presents many vesicles of different sizes (large and small arrows). Note the morphological difference of these cells with the common epidermal cells (E). N-nuclei. Toluidine blue-fuchsin. (F) Stinger epidermis submitted to bromophenol blue reaction with positive result for the specialized cells (*) indicating protein content, and negative result for the common epidermal cells (E). (G) Stinger epidermis submitted to PAS reaction with negative result for the specialized cells, indicating absence of mucous substances. Such result is the opposite for the common epidermal cells (E), except the PAS-positive result in the vesicles of specialized cells (arrowheads). (H) Higher magnification of the PAS-positive vesicles (arrowheads) in the cytoplasm of the specialized cells (*). (I) Stinger epidermis submitted to Alcian Blue reaction, pH 2.5, with negative result for the specialized cells, also indicating absence of mucous substances. Such a result is the opposite for the common epidermal cells (E).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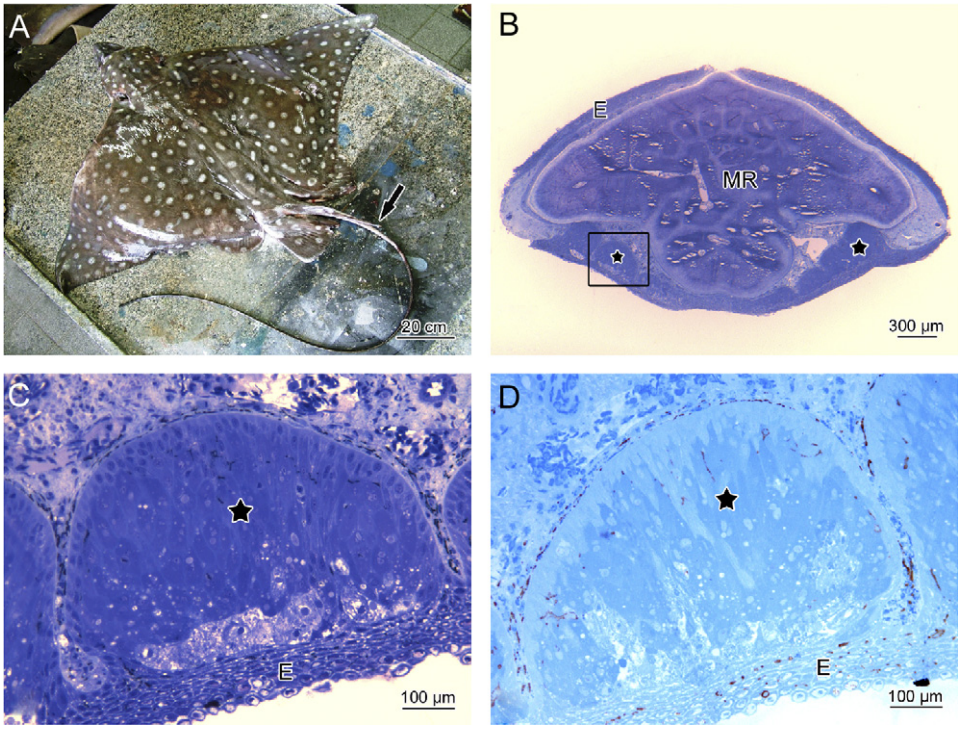

Fig. 3. *Aetobatus narinari*. (A) Photo of a specimen. The arrow points to the tip of the stinger. (B) Transversal section of the stinger indicating the specialized venom secretory cells (*) in the epidermis (E), in a disposition similar to what is observed in *Dasyatis guttata*. MR—mineralized region. Toluidine blue-fuchsin. (C) Higher magnification of the ventrolateral region of the stinger, signed by the rectangle in (B), showing the specialized cells (*) next to the ventrolateral groove. E—epidermis. Toluidine Blue-fuchsin. (D) Stinger epidermis submitted to bromophenol blue reaction with positive result for the specialized cells (*) indicating protein content, and negative result for the common epidermal cells (E).

also present in the epidermis of other parts of the body of the animals, especially in the dorsal skin, where they appear in large numbers (not shown).

3.2. Histochemistry

Histochemical reactions of the specialized cells in marine and freshwater species showed that their cytoplasm is positive to bromophenol blue (Figs. 2F, 3D, 4F, 5D, 6D) and negative to PAS (Figs. 2G, 2H, 4G, 5E, 6E) and Alcian blue (Figs. 2I, 4H), which is opposite to the result observed in the rest of the rounded epidermal cells in which the small granules are strongly positive both to PAS (Figs. 2G, 2H, 4G, 5E, 6E) and Alcian blue (Figs. 2I, 4H). Nevertheless, the characteristic fusiform vesicles of the specialized cells, contrasting to the rest of the cytoplasm, are strongly positive to PAS (Figs. 2G, 2H, 4G, 5E, 6E).

The large flask-shaped secretory cells observed in *Potamotrygon* species, similar to what was observed in the specialized cells, are positive to bromophenol blue (Figs. 4F and 5D).

3.3. Electron microscopy

The specialized cells show a very homogeneous and electrondense cytoplasmic matrix, where, except for a great number of free ribosomes, organelles are hardly distinguishable. The nucleus is irregular (Figs. 7A and B). In the cytoplasm, there are a number of vacuoles packed with tubular structures disposed more or less in the same direction, with cross-section aspect similar to microtubules (Figs. 7B and C).

4. Discussion

The comparative analyses of the results indicate that, in all species studied, there is a specialized type of cells in the epidermis, which covers the stinger. In the stingers of the two marine species, *D. guttata* and *A. narinari*, the specialized cells are present only in the areas inside or around the lateral grooves. On the other hand, in the three species of *Potamotrygon* a larger number of these cells are organized as intermediate epidermal layers covering

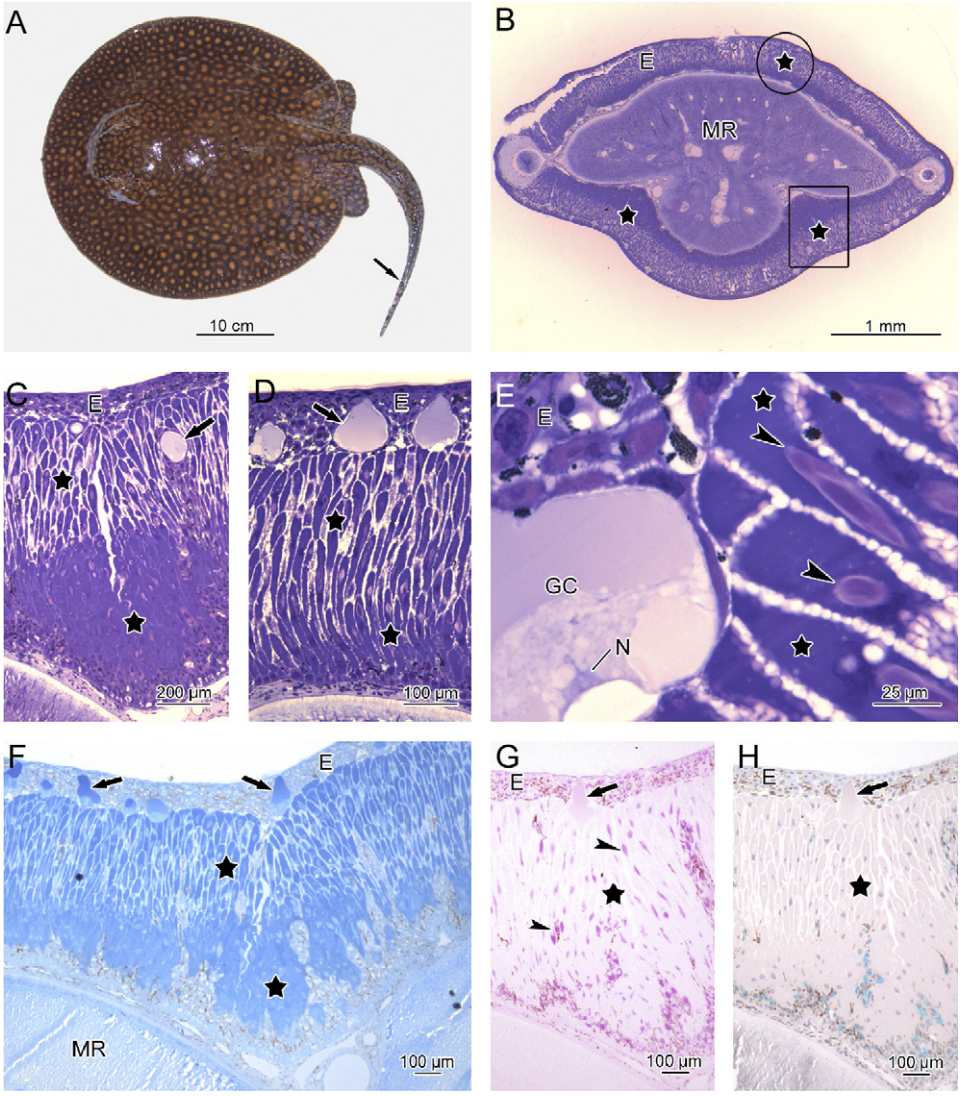

Fig. 4. *Potamotrygon falkneri.* (A) Photo of a specimen. The arrow points to the tip of the stinger. (B) Transversal section of the stinger indicating the specialized venom secretory cells (*) in the epidermis (E), around the whole stinger. MR—mineralized region. Toluidine blue-fuchsin. (C) Higher magnification of the ventrolateral region of the stinger, signed by the rectangle in (B), showing that the specialized cells (*) are spread in the form of several layers. The arrow points to a glandular cell in apical position, among the common epidermal cells (E). Toluidine blue-fuchsin. (D) Higher magnification of the dorsal epidermis of the stinger, signed by the circle in (B), showing the presence of the specialized cells (*) also forming several layers. The arrow points to a glandular cell in apical position, among the common epidermal cells (E). Toluidine blue-fuchsin. (E) Detail of the three types of cells composing the epidermis of the stinger: common epidermal cells (E), glandular cell (GC) and specialized venomous cells (*), characterized by the fusiform vesicles (arrowheads). N—nucleus. Toluidine blue-fuchsin. (F) Stinger epidermis submitted to bromophenol blue reaction with positive result for the specialized cells (*) and the glandular cells (arrows), indicating protein content, and negative result for the common epidermal cells (E). MR—mineralized region. (G) Stinger epidermis submitted to PAS reaction with positive result for the common epidermal cells (E), and negative result for the specialized cells [except the PAS-positive result in the fusiform vesicles (arrowheads)]. The glandular cells (arrow) are weakly positive to the method. (H) Stinger epidermis submitted to Alcian Blue reaction, pH 2.5, with positive result for the common epidermal cells (E) and negative result for the specialized cells (*) and glandular cells (arrow), also indicating absence of mucous substances.

the whole stinger. In all species analysed, the regular epidermal cells are characterized as rounded mucous cells with the cytoplasm full of small granules, evidenced by the positive result to PAS and Alcian blue. These mucous cells compose the whole epidermis of the animals and their morphology is similar to the mucous epidermal cells of fish in general (Whitear, 1986).

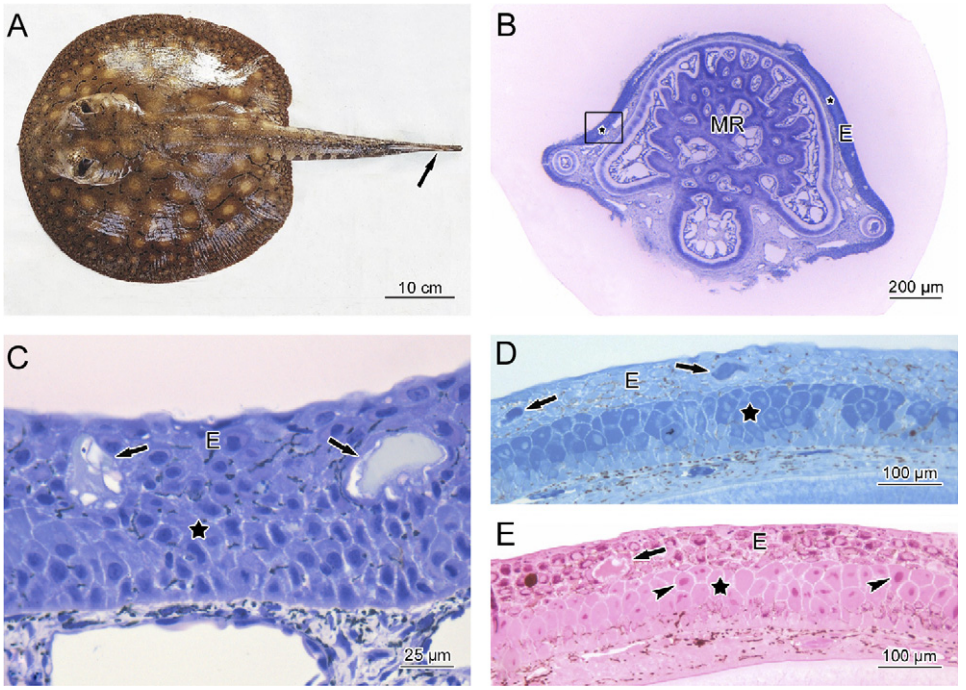

Fig. 5. *Potamotrygon orbignyi*. (A) Photo of a specimen. The arrow points to the tip of the stinger. (B) Transversal section of the stinger indicating the specialized venom secretory cells (*) in the epidermis (E), around the whole stinger. MR—mineralized region. Toluidine blue-fuchsin. (C) Higher magnification of part of the epidermis, signed by the rectangle in (B), showing the specialized cells (*) and the glandular cells (arows). E—epidermis. Toluidine blue-fuchsin. (D) Stinger epidermis submitted to bromophenol blue reaction with positive result for the specialized cells (*), indicating protein content, and negative result for the common epidermal cells (E). (E) Stinger epidermis submitted to PAS reaction with positive result for the common epidermal cells (E), and negative result for the specialized cells (except the PAS positive result in the fusiform vesicles (arrowheads), indicating absence of mucous substances. The glandular cells (arrow) are weakly positive to the method.

Besides the specialized type of cells described above, in the three species of *Potamotrygon*, another type of secretory cell was observed in the epidermis of the stinger, localized in the most superficial layers among the common mucous epidermal cells and directly connected to the epidermal surface. These bottle-shaped cells are much larger than the other cells and their cytoplasm is full of a hyaline protein secretion, demonstrated by the positive result to bromophenol blue. The observation of the epidermis of the disc and of the tail of *P. falkneri* showed that these glandular cells are not present exclusively in the stinger but are also found in the epidermis of other parts of the body, similar to what was observed in some species of catfish (Al-Hassan et al., 1987).

The specialized cells as well as the flask-shaped cells are positive to bromophenol blue, demonstrating that they are rich in protein content. This finding is in accordance with the biochemical analysis of the venom of *D. guttata* and different species of the genus *Potamotrygon*, which indicated a large number of different proteins (Magalhães et al., 2006; Barbaro et al., 2007). A number of these components demonstrated enzymatic activity, which is very common in animal venoms, probably breaking down extracellular matrix or serving as a spreading factor of the venom in the victim's tissue (Tan and Ponnudurai, 1992; Haddad Jr. et al., 2004; Lira et al., 2007; Barbaro et al., 2007).

Although the relevance of stingray toxinology, due to the frequency of human accident caused by them, both in marine and fluvial environments, the literature about the morphology of the stingray venom apparatus is very scarce. Besides the monographic paper of Halstead (1970), which describes the anatomy and histology of stinger of many different species, there are only a few ultrastructural papers about the venom secretory cells (Liu et al., 2001; Smith et al., 1974, 1981).

Halstead (1970) presents transversal sections of the stingers of several species of stingrays, including *Potamotrygon*, and affirms that in all of them the venom secretory cells are always localized inside the ventrolateral grooves or next to them, in ventral

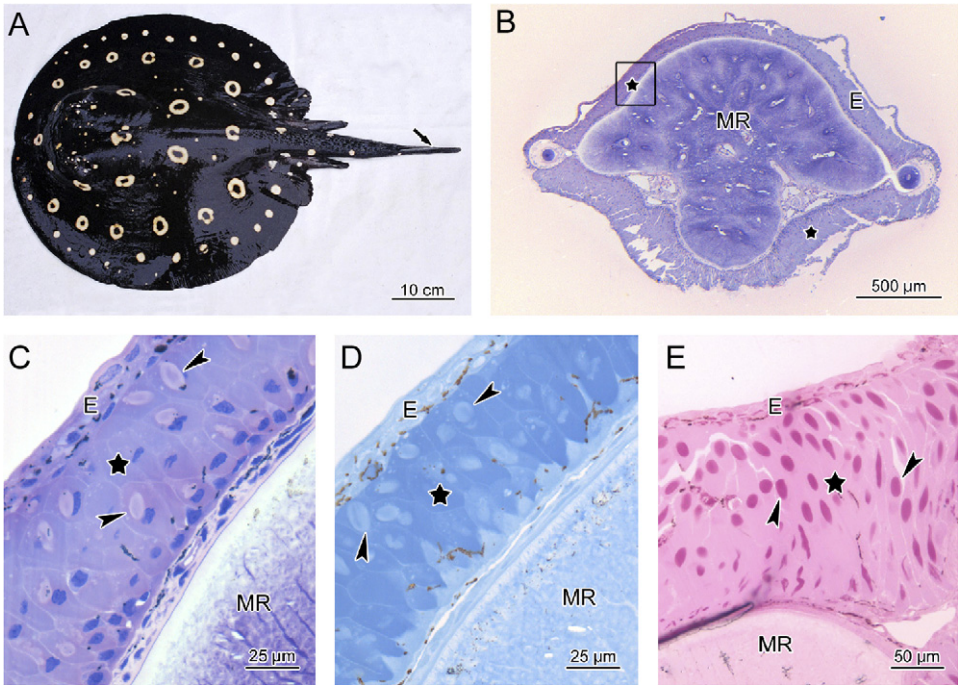

Fig. 6. *Potamotrygon leopoldi*. (A) Photo of a specimen. The arrow points to the tip of the stinger. (B) Transversal section of the stinger indicating the specialized venom secretory cells (*) in the epidermis (E), around the whole stinger. MR—mineralized region. Toluidine blue-fuchsin. (C) Higher magnification of part of the epidermis, signed by the rectangle in (B), showing the specialized cells (*). The arrowheads point to fusiform vesicles. E—epidermis. Toluidine Blue-fuchsin. (D) Stinger epidermis submitted to bromophenol blue reaction with positive result for the specialized cells (*), indicating protein content, and negative result for the common epidermal cells (E). The arrowheads point to fusiform vesicles. (E) Stinger epidermis submitted to PAS reaction with positive result for the common epidermal cells (E), and negative result for the specialized cells [except the PAS positive result in the fusiform vesicles (arrowheads)], indicating absence of mucous substances.

position. Our observations in three species of *Potamotrygon* contrast with the results of Halstead (1970), demonstrating that there are clear continuous layers of cells producing protein material around the whole stinger, which were evidenced by the use of cytochemical methods.

Halstead (1970) comments that the specialized epidermal secretory cells would be of holocrine type, in which the accumulation of the secretion in the cytoplasm ends in the auto-destruction of the cell and liberation of the venomous content. The ultrastructural results indicate that, in fact, these cells synthesize proteins mainly for internal utilization: the presence of a very electrondense cytoplasm full of polysomes and the absence of secretory vacuoles are evidences of a non-exporting secretory activity. Nevertheless, Smith et al. (1974) affirm that no evidence of holocrine secretion was observed in the stinger of *D. sabina*. Our morphological results show that the cells are localized in the intermediate layers of the epidermis and, different from the bottle-shaped glandular cells, are directly connected to the skin surface, there is no evidence of a liberation mechanism of the secretion to the exterior. On the other hand, it is most probable that, in case of accident, the venom would act through the direct contact of the stinger epidermal tissue with the victim's injury caused by mechanical perforation. The delicate skin covering the stinger is certainly dilacerated the moment that the stinger is introduced into the victim's body, liberating the epidermal venom content.

In all analysed species, the specialized epidermal secretory cells show the presence of fusiform vacuoles in the cytoplasm that are especially evidenced in the light microscope by the PAS reaction, or by the ultrastructural observation of microtubule-like structures inside them. These structures were already observed by Smith et al. (1974, 1981) in the specialized epidermal cells of *D. sabina*, and were identified as a peculiar type of "microtubule" that is smaller in diameter when compared with the normal tubulin-composed cytoplasmic microtubules. These authors suggest that these "microtubules" may

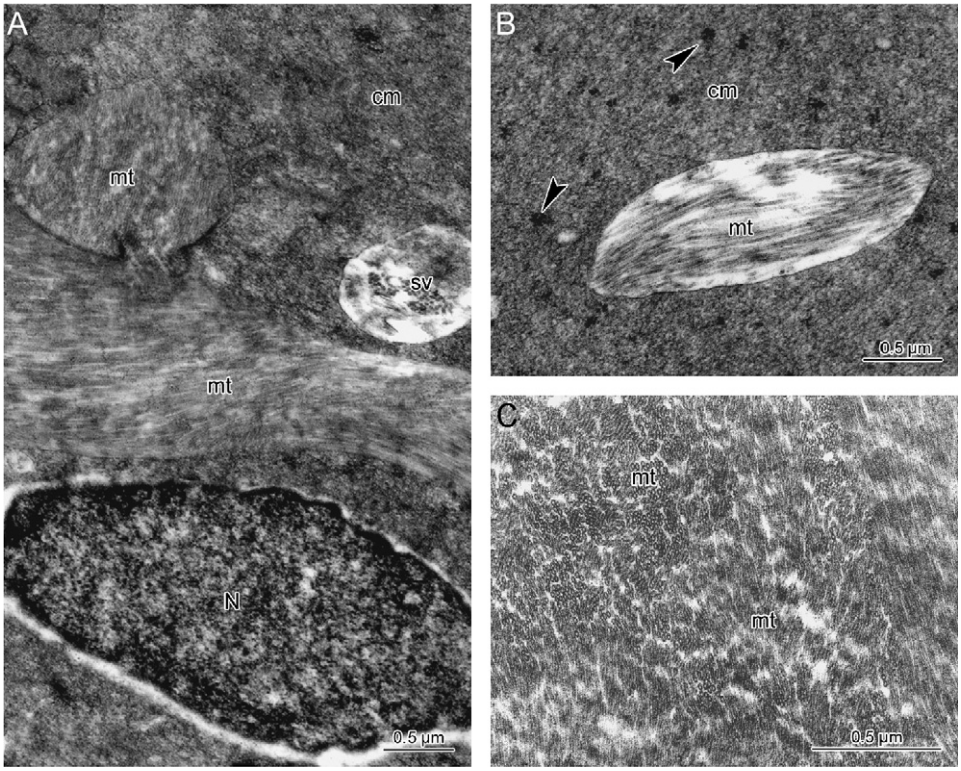

Fig. 7. *Potamotrygon falkneri*, electronmicrographs of the epidermal specialized cells in the stinger. (A) Part of the cytoplasm of a cell showing the nucleus (N), the electrondense cytoplasmic matrix (cm), and the vesicles full of "microtubules" (mt). sv—small vesicle. (B) Higher magnification of a vesicle, with the typical fusiform aspect and the content of "microtubules" (mt) which are longitudinally observed. In the dense cytoplasmic matrix (cm) there are many polysomes (arrowheads). (C) Detail of the microtubules in the interior of a vesicle, sectioned in different planes (left, transversal, and right, oblique or longitudinal).

represent a protein polymer component of the venom that, for some reason, must be kept isolated from the rest of the cytoplasm. Although the chemical nature and function of these structures still remain unknown, the results showed in this paper strongly suggest that they are present in the epidermal cells secreting venom of all species of stingrays and may be used to identify this type of cells.

Accidents with fluvial stingrays have demonstrated to be more severe when compared with the ones caused by marine stingrays (Haddad Jr. et al., 2004). This fact was confirmed by the enzymatic and toxic characterization of the stinger venom in both groups (Barbaro et al., 2007). The great number of specialized epidermal cells covering the whole extension of the stingers in the species of *Potamotrygon* is probably a significant factor in the potentialization of the activity of the venomous secretion of these rays. The protein content of the bottle-shaped cells may also have a role in the envenomation caused by them.

Besides the clinical aspect for human toxinology, from the zoological viewpoint, the morphological differences observed in the disposition of the stinger venomous cells of marine and freshwater stingray species seem to compose well-defined distinct patterns of cellular organization in these two groups, which can have some phylogenetical significance deserving further detailed investigation.

Acknowledgements

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