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Histological description of the ink gland of the tropical sea hare *Aplysia dactylomela* Rang, 1828

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Abstract

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This work describes for the first time the ink gland of *Aplysia dactylomela* Rang, 1828 using light microscopy and histochemical tests. The results reveal that this organ is covered by a layer of simple epithelium and that there are some differences between the epithelium facing the mantle shelf and that facing the mantle cavity. The former consists of columnar cells, which may have a secretory function, whereas the latter is a cuboidal epithelium. Underneath the epithelium and along the whole gland there are fibres of smooth muscle and collagen, organized in groups of parallel bundles. There are vesicles of different diameters, apparently with similar morphology. Some are filled with ink, whereas others are either granular or clear. The ink is released through a duct formed by invaginations of the cuboidal epithelium. Tests with bromophenol blue and periodic acid Schiff indicated that the ink and granulated vesicles contain proteins and carbohydrates or maybe glycoproteins. Between the fibre bundles and the vesicles there are dispersed cells. A diagram is presented emphasizing the covering epithelium, the distribution of muscle and collagen fibres, the dispersed cells, vesicles and ducts. This organization is similar to that of the other *Aplysia* species studied to date, *A. californica*.

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Introduction

In the Gastropod subclass Opisthobranchia there has been a general trend towards loss of the shell with many lineages evolving from snails into sea slugs. During the evolutionary loss of the shell, opisthobranchs have progressively substituted the mechanical protection of the shell with chemical defence strategies (Faulkner and Ghiselin 1983).

In sea hares, a thin and poorly calcified internal shell is present and when physically disturbed most sea hares release purple ink from a gland (called the ink, purple, or Blochmann's gland) located on the edge of its mantle shelf (Hyman 1967).

The ink has been viewed by some biologists as a defence against predators; however, several hypotheses have been proposed for the function of ink in *Aplysia*: (1) it acts as a method to rid the animal of unwanted bile pigments consumed in its diet (Chapman and Fox 1969); (2) it acts as a 'smoke-screen' on release, thus shielding the sea hare from visual predators (Eales 1921; Hyman 1967); (3) it is distasteful, causing the sea hare to be unpalatable and thus acting as an 'anti-feedant' (DiMatteo 1981, 1982; Pennings 1994; Nolen *et al.* 1995); (4) it functions as a warning signal (Ambrose *et al.* 1979); (5) it acts as a signal alarm to conspecifics (Fiorito and Gherardi 1990) and (6) it has a metabolic inhibitory effect on other organisms (Carefoot *et al.* 1999; Johnson and Willows 1999).

Despite many published reports on different aspects of sea hare ink, the ink gland has received much less attention. The most complete study on the ink gland was performed by Prince *et al.* (1998) in *Aplysia californica*. These authors showed that the ink gland contains three types of vesicles (dark red-purple, amber and clear) embedded in a matrix of collagen, muscle and two different types of cells, the rough endoplasmic reticulum and granulate cells.

In addition they have shown that when the sea hares were fed red seaweeds (e.g. *Gracilaria*), most of the vesicles were dark red-purple, other vesicles were either amber-coloured or clear. When de-inked at an early age and then placed on a diet of green seaweed (e.g. *Ulva*) the sea hares showed many clear vesicles as well as some amber and small light red-purple vesicles but no dark red-purple ones. Nowadays it is known that the purple-red and amber colours of the ink gland vesicles of *A. californica* are the result, respectively, of the presence of red seaweed-derived chromophores (Chapman and Fox 1969) and of L-amino-oxidases, which utilize flavin adenine dinucleotide as a prosthetic group, which is responsible for a yellowish/orange colour (Johnson *et al.* 2006).

Bezerra *et al.* (2004) have also shown by light microscopic techniques the relationship between seaweed diet and purple ink production in *A. dactylomela*. These authors emphasized that the animals fed green seaweeds have many clear vesicles but no dark red-purple ones, as had been previously described in *A. californica* (Prince *et al.* 1998). The present work aims to extend the current knowledge of the structure of the sea hare ink gland in two ways beyond the current view, which is that of Prince *et al.* (1998). It focuses on a different species, *A. dactylomela*, and offers some morphological details not described previously.

Materials and methods

Sea hares

Ten specimens of *A. dactylomela* Rang, 1828, weighing 150–200 g, were collected during low tide on Flexeiras Beach, north-eastern Brazil (Ceará State) (39°25′48″ W and 3°22′24″ S), where they fed mainly on red seaweeds. The sea hares were transported to the laboratory in a container with seawater. One group (n = 7) was anaesthetized by storage in a freezer immediately upon collection, handled carefully to avoid ink release and then dissected carefully to remove the ink gland. Another group of specimens (n = 3) was de-inked several times by squeezing them gently for a few minutes outside the water and then kept in recirculating seawater tanks at 25–27 °C under a 12 h : 12 h light : dark photoperiod. The sea hares were fed for 15 days a diet of the green seaweed *Ulva fasciata* offered *ad libitum* every other day. At the end of this period, sea hares were anaesthetized by storage in a freezer and dissected carefully to remove the ink gland.

Histological analysis of the ink gland

For light microscopic analysis, 10 ink gland samples were fixed with Bouin mixture (saturated picric acid solution, 40% formaldehyde, glacial acetic acid – 15 : 5 : 1 v/v), dehydrated in ethanol and embedded in paraffin. Tissue sections (5 μm) were stained with haematoxylin & eosin (H&E) and histochemical techniques were applied, such as bromophenol blue test for the detection of proteins, alcian blue staining for mucopolysaccharides and periodic acid Schiff (PAS) for carbohydrates. All these techniques were based on the procedures described by Pearse (1980). In addition, the Gomori method was used to visualize cellular features of collagen and muscle fibres following the methodology described by Luna (1968). After visualizing the slides, the sections were photographed on a Zeiss IV photomicroscope (Germany).

Results

The ink gland of the *A. dactylomela* is a structure that can be visualized by the naked eye. It is located at the free edge of the shell, being continuous with the mantle over the gills. The ink gland is constituted by a covering epithelium, under which there are vesicles of several diameters, permeated by dispersed cells and fibres that run in several directions. To improve our understanding of the gland architecture, each component is described separately below.

Covering epithelium

Histological staining revealed that the ink gland is covered by a layer of simple epithelium. Some differences were observed between the epithelium facing the mantle cavity and that facing the mantle shelf. The former is cuboidal showing basophilic nuclei in the middle portion of the cells and the nucleoli are rather evident (Fig. 1A). The epithelium facing the mantle shelf is columnar or prismatic with nuclei located in the basal portion of the cells (Fig. 1B).

In some areas the cuboidal epithelium invaginates, originating a vesicle duct (Fig. 1C).

Fibres

Underneath the epithelium, and along the whole extension of the ink gland, there are fibres running in several directions and surrounding the vesicles. Dispersed nuclei are observed among the fibres. The fibres are organized in groups of parallel bundles and in some portions are characteristic of smooth muscle, in which all fibres and the associated nuclei have approximately the same size and orientation (Fig. 1D,E). In other regions the fibres observed are characteristic of collagen, which resembles smooth muscle in being fibrous and eosinophilic, but can be readily distinguished by its wavy aspect (Fig. 2A). The reaction of both fibre types was positive to the alcian blue, eosin and PAS and the Gomori method helped to distinguish between these two fibre types, collagen being stained green and smooth muscle red/orange.

Dispersed cells

Between the fibrous bundles and the vesicles there are many dispersed cells of which the shape is not quite spherical (Fig. 2B). The whole cell was stained by H&E; however, the

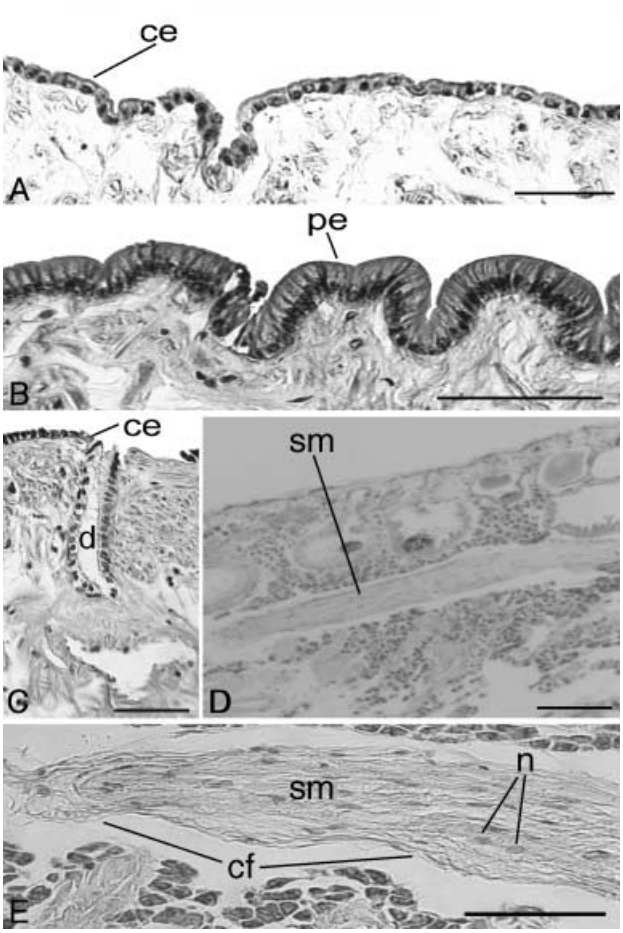

Fig. 1—Light microscopy observations of the ink gland of *Aplysia dactylomela* in paraffin sections. —**A**. The cuboidal epithelium (ce) is observed after staining with haematoxylin & eosin (H&E). Scale bar: 100 μm. —**B**. The columnar or prismatic epithelium (pe) stained with H&E. Scale bar: 100 μm. —**C**. Invaginations of the cuboidal epithelium (ce) giving rise to a duct (d) in sections of ink gland stained with H&E. Scale bar: 100 μm. —**D**. Smooth muscle (sm) visualized after staining with PAS. Scale bar: 100 μm. —**E**. Collagen fibres (cf) surrounding smooth muscle (sm) which were distinguished from each other by Gomori staining; smooth muscle nuclei (n) are easily identified. Scale bar: 50 μm.

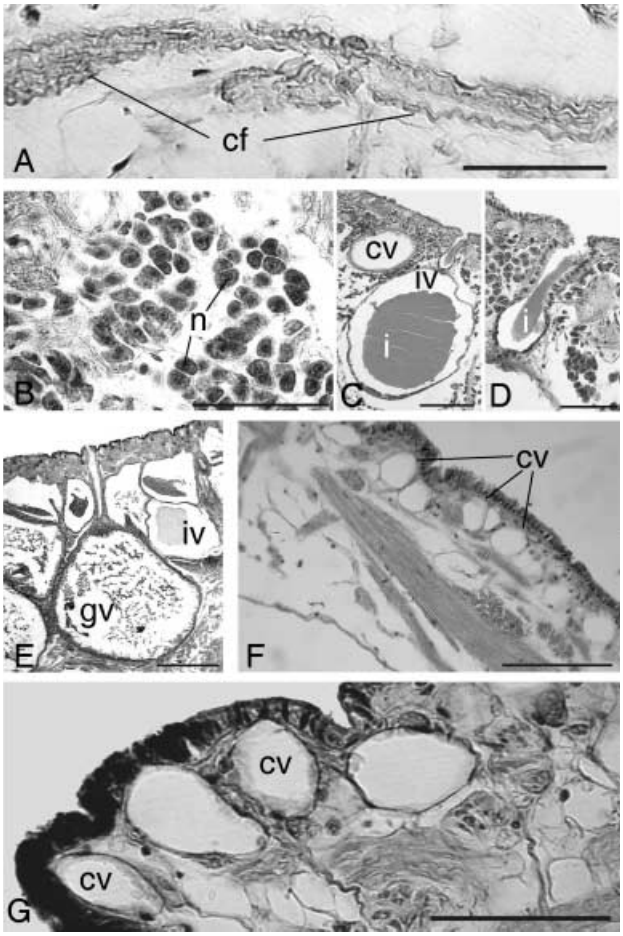

Fig. 2—Light microscopy observations of the ink gland of *Aplysia dactylomela* in paraffin sections. —**A**. The wavy aspect of PAS-stained collagen fibres (cf). Scale bar: 50 μm. —**B**. Cells of the ink gland stained with H&E showing strongly basophilic nuclei (n). Scale bar: 50 μm. —**C**. An ink vesicle (iv) and a clear vesicle (cv) are observed in H&E-stained sections. Scale bar: 100 μm. —**D**. Detail of a releasing duct filled with the ink (i), H&E. Scale bar: 50 μm. —**E**. Vesicles with granular aspect (gv) and vesicles filled with the ink (iv) are visualized in H&E-stained sections. Scale bar: 100 μm. —**F**. Ink gland sections of green-seaweed-fed sea hares showing clear vesicles and reduced number of cells, H&E. Scale bar: 100 μm. —**G**. detail of clear vesicles (cv) in glands of green-seaweed-fed sea hares stained with bromophenol blue. Scale bar: 50 μm.

nucleus was more basophilic than the cytoplasm. In those sea hares fed the red seaweeds the dispersed cells were more numerous and much bigger than those of sea hares fed the green seaweed *U. fasciata* (Fig. 2B,F).

Ink vesicles

The ink gland has many vesicles of different diameters but apparently similar morphology. In the red-seaweed-fed animals most of the vesicles are purple fluid-filled. Other vesicles are either filled with granular material or clear (Fig. 2C,E). The covering of each vesicle is rather thin, suggestive of it being a fine epithelium, which has a positive reaction to the alcian blue, bromophenol blue, H&E and PAS. In the ink-filled vesicles, a duct is observed ending in the covering gland epithelium, which suggests that it is a route for ink release (Fig. 2D). PAS and bromophenol blue tests indicated that the material present in these vesicles consists of carbohydrates and proteins or maybe glycoproteins. Nevertheless, no reaction of this secretion with alcian blue could be observed,

indicating the absence of mucopolysaccharides. Sea hares fed green seaweed have many clear vesicles and no positive reaction was detected to any of the staining tests performed (Fig. 2F,G).

Discussion

The literature contains only two works describing the detailed structure of the ink gland of *Aplysia* species. The first one by Hyman (1967) on *Aplysia* sp. describes basically the structure of the secretory vesicle with purple ink instead of the whole gland. The second study, by Prince *et al.* (1998), describes with details the gland of *A. californica,* showing three types of vesicles embedded in a matrix of collagen, muscle and two different types of cells. When the sea hares were fed red seaweed, most of the vesicles were dark-red purple. They also observed amber-coloured or clear vesicles. On the other hand, green-seaweed-fed sea hares had many clear vesicles as well as some amber and small light red-purple vesicles. In these works, however, no reference is made to the epithelium covering the ink gland.

In the present work conventional light microscopy and classical histological methods were used to describe the morphology and chemical composition of the ink glands, comparing those of animals fed on red and green seaweeds in an attempt to add new aspects to the descriptions by Prince *et al.* (1998). These techniques, although they do not provide great ultrastructural detail, can show different reactions based upon colours. During fixation with Bouin mixture the gland is unstained and thus it is possible to detect the positive reaction with different probes.

The ink gland of the *A. dactylomela* is characterized by cuboidal epithelium facing the mantle cavity and a columnar epithelium, facing the mantle shelf. The presence of a columnar epithelium suggests a secretory function because this tissue is often associated with secretion or absorption (David 2001). The invagination of the cuboidal epithelium forms the vesicle ducts, which connect the vesicles with the external medium, thus providing a route for ink release. The gland possesses numerous fibres, disposed in several directions, some showing a wavy aspect, characteristic of collagen, whereas others are rather straight, distinctive of smooth muscle (Ross *et al.* 1993). Prince *et al.* (1998) have also described the presence of collagenous and muscular fibres in the ink gland of *A. californica* with the muscular fibres surrounding the vesicles, having a role in facilitating ink release from the vesicles. Following a predator attack the ink motor neurones are stimulated to activate the smooth muscle surrounding the ink-release vesicles, which squeezes some of the ink through the vesicle's valve and into a duct that empties into the mantle cavity. The ink is then directed by the siphon and pumped out of the mantle (Carew and Kandel 1977; Walters and Erickson 1986; Prince *et al.* 1998).

Numerous dispersed cells were observed in the ink gland of *A. dactylomela*, although no distinction could be detected among them. Prince *et al.* (1998) described two types of cells in the ink gland of *A. californica*, the granulate cells and the rough endoplasmic reticulum cells. The former are less common and bigger than the rough endoplasmic reticulum cells, which are probably the site for synthesis of high molecular mass protein of secreted ink.

In the red-seaweed-fed animals most of the vesicles are ink-filled. Other vesicles are either filled with granular material or clear. In the ink-filled vesicles, a duct is observed ending in the cuboidal epithelium, demonstrating it to be the route for ink release. PAS and bromophenol blue tests indicated that the material present in these vesicles consists of carbohydrates and proteins or maybe glycoproteins. In addition, no reaction of this secretion with alcian blue could be observed, indicating the absence of mucopolysaccharides.

All the vesicles of green-seaweed-fed sea hares were clear, indicating them to be devoid of ink as previously described by Bezerra *et al.* (2004), and presenting no positive reaction to any of the staining tests performed: proteins and carbohydrates/mucopolysaccharides. An intriguing observation was the clear-cut reduction in the number of cells in the ink gland in these sea hares (see Fig. 2F,G). Although we do not have any quantitative data to substantiate this fact, this might be related to the greater susceptibility of these animals to predators. In fact, sea hares deprived of red algae do not secrete ink and do not escape from predators as successfully as those fed red algae (Nolen *et al.* 1995). Our results showed that green-seaweed-fed sea hares have vesicles devoid of any ink component – pigments, proteins or carbohydrates/mucopolysaccharides. Besides, a reduced number of cells was observed in the ink gland of these animals reinforcing the idea that components of the seaweed and the snail itself must be necessary for defence against predators (Troxler *et al.* 1981; Nolen *et al.* 1995).

Prince *et al.* (1998) had already described vesicles of different types in the ink gland of *A. californica*. Some of those vesicles were filled with acellular material that was stained by bromophenol blue, indicating the presence of proteins. They have also shown that the vesicles originate from vacuoles formed by invaginations of the plasma membrane. Those vacuoles contained a material similar to that observed in the ink vesicles, and in sea hares fed with green algae those vacuoles were empty. Prince *et al.* (1998) analysing the ultrastructure of the gland of *A. californica*, affirmed that vesicles with apparently different aspects and sizes are, in reality, vesicles at different stages of maturation and not different types of vesicles as Hyman (1967) had described previously.

The comparative analysis of the ink glands of *A. dactylomela* and *A. californica* showed that although there was no description of a secretory epithelium in *A. californica*, apparently the sea hares have a similar basic structural organization, showing different types of cells, and vesicles with ducts in addition to muscular and collagenous fibres.

To summarize the organization of the ink gland of *Aplysia* spp. we updated the diagram presented by Prince *et al.* (1998), emphasizing the covering epithelium, which is columnar or

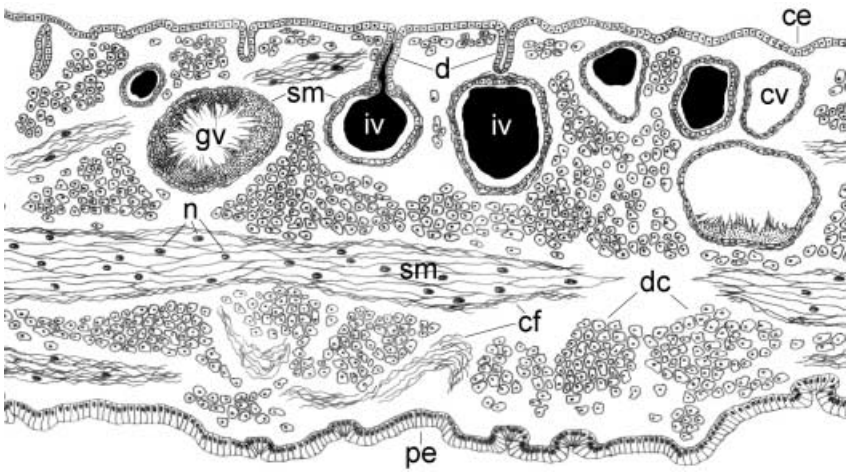

Fig. 3—The ink gland organization of *Aplysia dactylomela*. Diagram of a transverse section of the ink gland of *Aplysia dactylomela* showing the cuboidal epithelium (ce); the columnar or prismatic epithelium (pe); smooth muscle fibres (sm) and nuclei (n); collagen fibres (cf); ink vesicles (iv); granular vesicles (gv); clear vesicles (cv); ducts (d) and dispersed cells (dc). Upper side: mantle cavity; lower side: mantle shelf.

prismatic at the region facing the mantle shelf and cuboidal at the region facing the mantle cavity of the gland, and the distribution of muscle and collagen fibres, cells, vesicles and ducts (Fig. 3).

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