Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 26(2): 277 – 288 (2022)
www.ejabf.journals.ekb.eg

Histological and histochemical characterization of the ink gland from *Aplysia argus* (Mollusca: Heterobranchia: Aplysiida), Red Sea

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ARTICLE INFO

Article History:
Received: March 12, 2022
Accepted: April 2, 2022
Online: April 6, 2022

Keywords:
Aplysia argus,
Granulated cells,
Adipocytes,
Mucopolysaccharides,
Ink gland,
Red Sea

ABSTRACT

This study aimed to investigate the histological and histochemical characterizations of the ink gland of the sea hare "*Aplysia argus*".This gland is composed of two covering epithelium and an inner matrix. The covering epithelium involved the cubodial facing the mantle cavity and the columnar facing the mantle shelf. Underneath the covering cuboidal epithelium, there are bundles of collagenous fibers permeated by two types of granulated cells. The columnar covering epithelium underlies an adipose connective tissue that includes dispersed cells between adipocytes. The inner matrix of the ink gland possesses three types of secretory vesicles; namely, dark-Red, granulated and transparent. Histochemical investigation indicated that the granulated cells content included neutral mucopolysaccharides and protein, while they were devoid of acid mucopolysaccharides. The dark-red vesicles possess a mixture of acid and neutral mucopolysaccharides; whereas, the granulated vesicles contain acid mucopolysaccharides only. The dark-red and granulated vesicles contain protein contents. The histological characteristics of the ink gland of *Aplysia argus* corroborated the general organization of aplysiids. To the best of our knowledge, new data were added in the current work (for the first time) about the two different types of granulated cells and adipose connective tissue, in addition to the investigation of the histochemical contents of granulated cells, secretory vesicles and dispersed cells.

INTRODUCTION

Aplysiids or "sea hares" are a well-known group of reduced shell heterobranch sea slugs (Mollusca: Heterobranchia: Aplysiida). They use a distinctive defensive chemical weapon, "ink fluid" against predators, which is a complex chemical mixture released from two different glands: ink and opaline **(Nolen *et al.*, 1995; Johnson & Willows, 1999; Kicklighter *et al.*, 2005)**. The ink gland lies in the mantle cavity, from which sea slugs secret a purple-colored ink. However, some species simultaneously release white or white and purple ink, whereas others produce none **(Johnson & Willows, 1999)**. Chemically, ink is a complex mixture of phycoerythrobilin "purple

pigment" protein and small amounts of low molecular mass components **(Troxler *et al.*, 1981; Prince *et al.*, 1988)**.

The chemistry, function, behavior, and biological activities of the ink gland have attracted considerable research interest **(Yamazaki *et al.*, 1986; 1989; Melo *et al.*, 2000, 2002; Johnson, 2002; Kicklighter & Derby, 2006; Cherif *et al.*, 2015)**. However, little attention has been focused on the structure of the gland itself. While, **Hyman (1967)** described secretory vesicle structures of glands from *Aplysia californica*; gland structures were later characterized in the study of **Prince *et al.* (1998)** who postulated that, they included three vesicle types (dark-red-purple, amber and clear), embedded in a matrix of collagen, muscle, with two types of cells, rough endoplasmic reticulum (RER) and granulated cells.

Bezerra *et al.* (2004) described the relationship between purple ink production and green and red algae consumed by *Aplysia dactylomela*. In other work, **Moustafa (2005)** investigated ink gland structures from *Aplysia oculifera*. However, **Bezerra *et al.* (2006)**, in a detailed histological and histochemical study of *A. dactylomela* ink glands, distinguished the cuboidal epithelium facing the mantle cavity and the columnar epithelium facing the mantle shelf. In addition, they investigated muscle, collagenous fiber, dispersed cell, vesicle and duct distribution. **Prince and Johnson (2006)** compared purple ink gland ultrastructure in *A. californica* with the inkless glands of *Dolabrifera dolabrifera*. Additionally, a comparative investigation at the ultrastructure level documented ink glands from four sea hares; *Aplysia. californica*, *Aplysia parvula*, *Aplysia juliana* and *Dolabrifera dolabrifera*, and identified ink protein synthesis and storage sites (**Prince & Johnson, 2015**).

Aplysia argus Rüppell & Leuckart, 1830 is a common species found in Red Sea shallow waters off the Egyptian coast. The species is native to the Indo-Pacific region and was previously identified as *Aplysia dactylomela*, a species native to the Atlantic Ocean (**Valdes *et al.*, 2013; Chandran *et al.*, 2016**, A. Moustafa Per. Commun.). In this study, by using the histological and histochemical analyses, the morphology and chemical composition of the ink gland from *A. argus* from the Egyptian Red Sea coast were described in detail.

MATERIALS AND METHODS

Animal collection

Ten adult *A. argus* specimens, with wet mass of approximately 180–210g and lengths ranging from 20–25cm (for relaxed specimens), were collected from shallow water, 14 km south of Qusair City (25°59`57.69"N: 34°20`6. 92"E), Red Sea governorate, Egypt. Specimens of *A. argus* were sent to California State Polytechnic University (Pomona, USA), and identification was confirmed by Dr. Angle Valdes (A. Moustafa, Per. Commun.).

Histological and histochemical methods

For light microscopy, five specimens were relaxed using menthol crystals in sea water. Then, samples were dissected to isolate the ink glands, fixed in 5% formaldehyde in seawater, and subsequently transformed into 70% ethanol. Specimens were dehydrated in an ascending ethanol series and embedded in paraffin. Serial transverse sections were cut at 5µm and stained in hematoxylin & eosin for general histology.

For histochemical studies, Masson's trichrome was used to stain collagenous fibers, Orcein for elastic fibers, Periodic acid-Schiff stain (PAS) for general carbohydrates, Alcian blue pH 2.5 for acid mucopolysaccharides, combined Alcian blue-PAS to differentiate neutral and acid mucopolyssachrides, and mercury bromophenol blue for total protein. Selected sections were photographed under light microscopy (Axio Lab. A1, Carl ZEISS, Germany) using an AxioCamERc5s Camera. Photoshop (Version CS6) was used to process images.

Transmission electron microscopy (TEM)

Small ink gland samples were fixed in 3% glutaraldehyde buffered in cacodylate buffer, after which post-fixation was performed in 1% osmium tetroxide cacodylate buffer. Specimens were dehydrated in ethanol, treated with propylene oxide, and embedded in epon epoxy resin. Semi-thin sections (0.5-1 µm) were stained in toluidine blue and photographed under light microscopy. Ultrathin sections (60-80 nm) were stained with uranyl acetate and lead citrate and examined by TEM (JEOL TEM 100 CXII) at 80 KV, at Assiut University, Assiut, Egypt.

RESULTS

Histological and histochemical studies

A. argus ink glands were composed of an outer epithelium covering and an inner matrix (Fig. 1A). The epithelium covering facing the mantle cavity comprised simple cuboidal epithelium with rounded and central nuclei (Fig. 1B). However, facing the mantle shelf, we identified simple columnar epithelium with oval shaped nuclei and a basophilic cytoplasm (Fig. 1C).

Beneath the cuboidal epithelium, bundles of collagenous fibers permeated by granulated cells (Figs. 1B, E, F) were observed. These cells were differentiated into two types according to shape: type I cells were more common with rounded or oval shapes and a granular appearance; they were either individual cells or aggregated in groups (Figs. 1B, F). Type II cells were less common, club or rod shaped, and were embedded between cuboidal epithelial cells or underneath in some regions (Fig. 1F).

The covering columnar epithelium was lined by adipose connective tissue, with dispersed cells between fat cells (adipocytes) (Figs. 1C, G). Clusters of smooth muscle fibers, surrounded by collagenous fibers, were observed beneath this connective tissue (Fig. 1G).

The inner matrix of the ink gland contained secretory vesicles of different shapes and sizes, differentiated into three types: 1) dark-red vesicles full of dark-red homogenous staining material, 2) granulated vesicles containing tiny, fine faint granules, and 3) transparent clear vesicles (Fig. 1A). These vesicles were surrounded by a thin layer of smooth muscle and collagenous fibers (Fig. 1E). Secretory vesicle ducts were connected to the gland surface, facing the mantle cavity. These structures represented passages from which secretions were discharged from the gland (Fig. 1D). Bundles of smooth muscle fibers and collagenous fibers were dispersed in several directions within the matrix (Fig. 1E). Elastic fibers were also documented between the different structures in the gland (Figs. 1H, I, J). Superficial pits were presented on the gland surface, facing the mantle cavity and were formed by cuboidal epithelium invaginations (Fig. 1D).

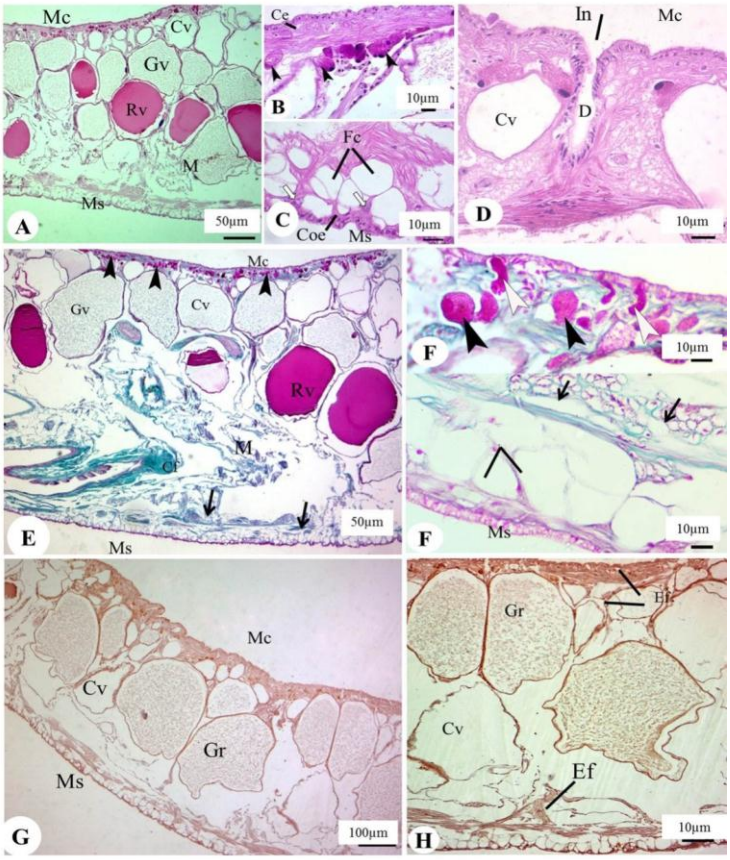

Fig. 1. Transverse sections of the ink gland of *Aplysia argus* stained with H&E **(A-D)** and Masson trichrome stain **(E-G)** showing **(A)**The outer covering epithelium facing the mantle cavity (Mc), other one facing mantle shelf (Ms)and an inner matrix (M) of the gland containing dark red vesicles (Rv), granulated vesicles (Gv), and clear vesicles (cv). **(B)**The cubodial covering epithelium (Ce) followed by fibers and secretory cells (head arrows). **(C)**The columnar covering epithelium(Coe) followed by fat cells (Fc) with dispersed cells (white arrows). **(D)** Invagination of the outer epithelium forming a superficial pit. **(E-G)** Deposition of collagen (Cf) in matrix and localized in clusters around longitudinal muscle fibers (black arrows), type I of secretory cells (black head arrows), type II of secretory cells (white head arrows) and fat cells (Fc). **(H-J)** localization of elastic fibers in different regions of the gland.

From histochemical studies, PAS reactions showed that granulated cells (type I and II) were positively stained for carbohydrates (Figs. 2A, B). Nonetheless, both cell types had negative reactions with Alcian blue (Fig. 2D) and positive reactions with mercury bromophenol blue (Figs. 2H and I), confirming the presence of protein, but not acid mucopolysaccharides.

In terms of secretory vesicles, dark-red and granulated vesicles showed weak reactions with both PAS and Alcian blue (pH 2.5) (Figs. 2A, D). Combined Alcian blue and PAS staining showed that the dark-red material contained a mixture of acid and neutral mucopolysaccharides; however, granulated vesicles contained only acid mucopolysaccharides (Fig. 2E). Both dark-red and granulated vesicles contained protein as they were positively stained with bromophenol blue (Fig. 2H). On the other hand, transparent vesicles generated had negative reactions with all staining techniques, suggesting these vesicles had previously ejected their contents (Fig. 2).

Dispersed cells showed a strong positive reaction to PAS (Fig. 2C), Alcian blue pH 2.5 (Fig. 2D) and combined Alcian blue/PAS staining (Fig. 2G), indicating the presence of carbohydrates and acid mucopolysaccharides. Nevertheless, they were negatively stained with bromophenol blue illustrating the non- presence of protein (Fig. 2H).

In toluidine, blue granulated cells appeared intensely stained dark blue, which indicates the production of neutral mucopolysaccharides (Figs. 3A, B & Table 1), whereas dispersed cells were orthochromatic (Fig. 3C).

Transmission electron microscopy (TEM)

Electron micrographs of ink glands showed gland surfaces facing the mantle cavity, composed of cuboidal cells, with irregular apical surfaces and a slight opaque cytoplasm containing electron dense granules and vacuoles (rarified area of cytoplasm). Euchromatic nuclei, with an irregular rim of thin electron dense peripheral chromatin and an eccentric dense nucleolus were also observed (Fig. 4A). The gland matrix appeared electron lucent, contained elongated, irregular dense collagenous fibers and parts of the cell cytoplasm (Figs. 4A, B).

Type II granulated cells were observed either between the cuboidal covering epithelium (Fig. 4A) or beneath (Fig. 4B). They appeared as club shaped (or elongated) cells containing a slightly dense heterogeneous cytoplasm, with 12–15 electron dense secretory granules and some vacuoles (Fig. 4A and B). Type I secretory cells were large with irregular boundaries, containing a heterogeneous electron dense cytoplasm, with 85–90 electron dense secretory granules. These cells had rounded to oval shaped euchromatic nuclei, with electron dense chromatin (Fig. 4C). Small ovoid cells were also identified close to type I secretory cells; they contained a slightly electron dense heterogeneous cytoplasm with euchromatic nuclei (Fig. 4C).

Adipocytes had irregular boundaries and contained an electron lucent cytoplasm with many fine dark granules and lipid droplets on the gland surface facing the mantle shelf, lined with adipose connective tissue (Fig. 4D).

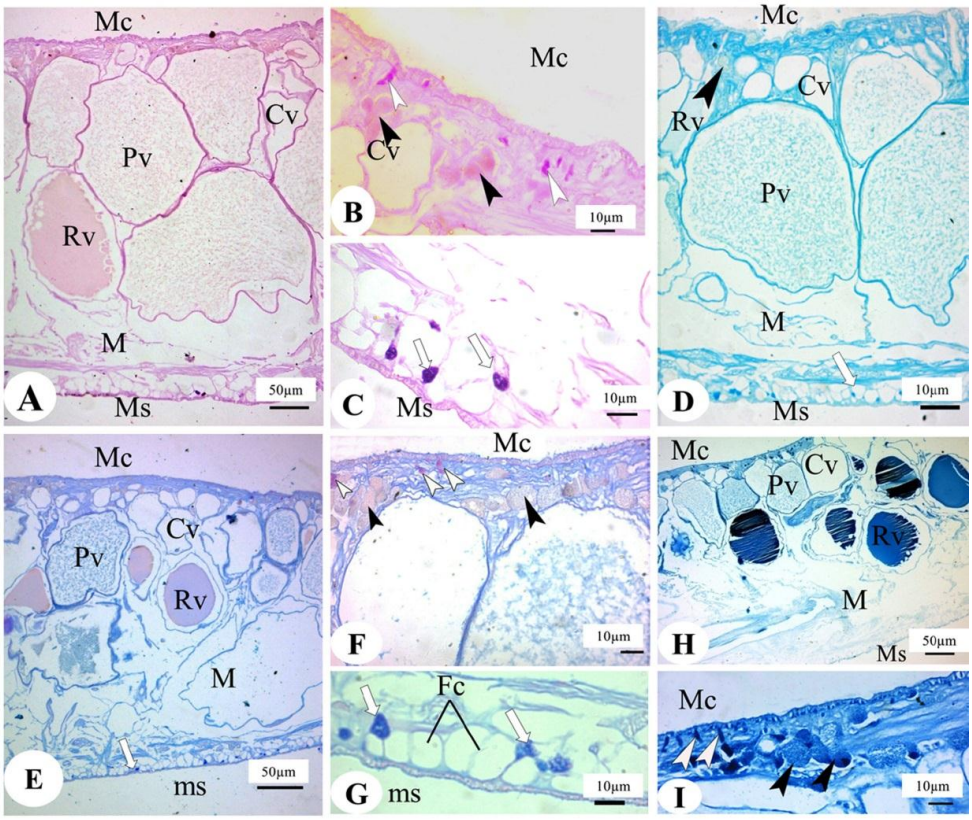

Fig. 2. Transverse sections of the ink gland of *Aplysia argus* stained with PAS **(A-C)** showing deposition of carbohyrates in dark-Red vesicles (Rv) and granulated vesicles (Gv), type I (black head arrows), type II (white head arrows) of secretory cells and the dispersed cells (white arrows). Alcian blue pH2.5 **(D)**:showed the presence of acid mucopolysaccharidesinin Red vesicles (Rv), granulated vesicles (Gv) and the dispersed cells (white arrows). Combined alcian blue-PAS (**E-G)**, showing the neutral mucopolysaccharides contents in Red vesicles (Rv), Type I (black head arrows), Type II (white head arrows) of secretory cells, dispersed cells (white arrows) and the presence of acid mucopolysaccharides in granulated vesicles (Gv).**(H , I)** Mercury bromophenol blue, showing protein contents in dark-Red (Rv) vesicles, granulated vesicles (Gv), type I (black head arrows) and type II (white head arrows) secretory cells.

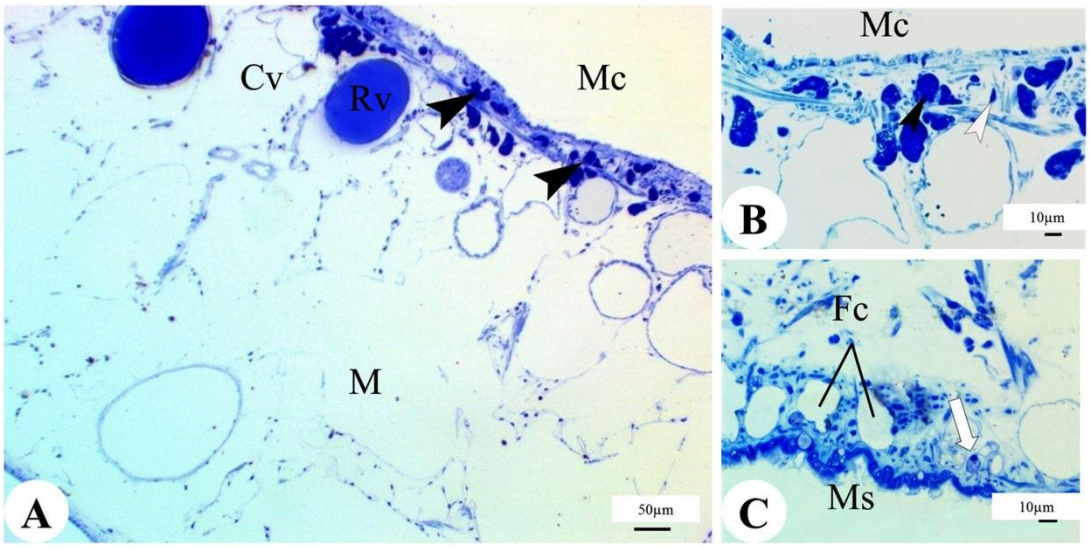

Fig. 3. Semi-thin section of the ink gland (stained with toludine blue) showing: **(A)** The different component of the gland. **(B)** Enlarged portion from the surface facing the mantle cavity (Mc). **(C)** Enlarged portion from the surface facing the mantle shelf (Ms), showing acid mucopolysaccharides in dark red vesicles (Rv), type I (Black head arrows), type II (white head arrows) showing secretory cells and the dispersed cells (white arrows).

Table1. Histochemical staining characters of different cell types in the ink glandof *Aplysiaargus*

Cell Types		PAS	Alcian blue pH2.5	Alcian blue & PAS	Bromophenol blue	Toludine blue
granulate cells	**Type I**	+	-	+	+++	Dark blue
	Type II	+	-	+++	+++	Dark blue
Secretory vesicles	**Dark- Red**	+	+	+	+++	Dark blue
	Granulated	+	+	+	+	Dark blue
	Clear	-	-	-	-	-
Dispersed cells		+	+++	+	-	blue

(–)Negatively stained, (+) weakly stained,(++) moderately stained,(+++) strongly stained.

Triangular shaped dispersed cells were observed between adipocytes; they had tiny invaginations on the outer membrane leading to tiny caves, suggesting material transport through the cell membrane. The cytoplasm was divided into two regions, an outer electron lucent containing rarified area and an inner perinucleus area containing the main cell organelles, including different shaped electron dense granules, mitochondria and the RER (Fig. 4E). The RER organelle was observed in the gland matrix and was highly abundant (Fig. 4F).

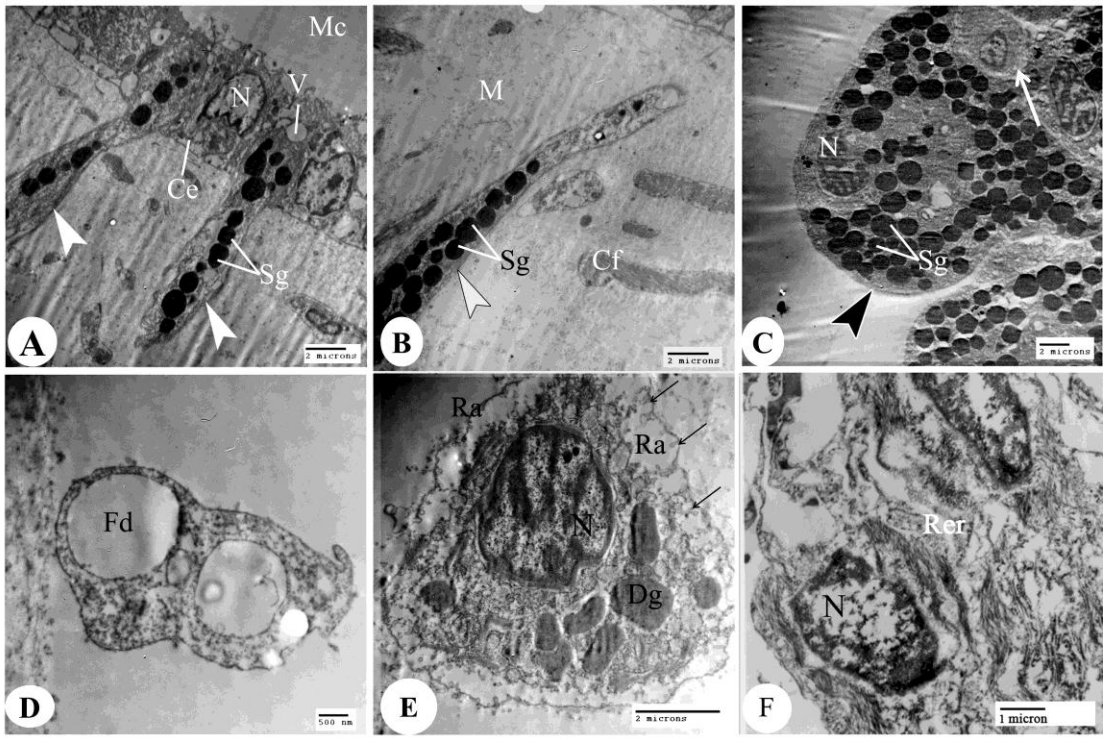

Fig. 4. Transmission electron micrographs of ink gland showing (**A**-**B**) cuboidal cells with irregular apical surface containing vacuoles (V), rarified area (Ra) of cytoplasm and euchromaticnuclei (N), club shape secretory cells (white arrows head) full of electron dense secretory granules (Sg). **(C**) Type II, Large secretory cells containing slightly dense cytoplasm and large number of electron dense secretary granules (Sg) with ovoid euchromatic nucleus (N), small ovoid cells scattered between other secretory cells and contain slightly electron dense heterogenous cytoplasm with euchromatic nucleus (White head arrow). **(D**) Adipocyte with fat droplets (fd). **(E)** Triangle shape dispersed cells with tiny invagination of outer membrane (black arrows head), rarified areas of cytoplasm, dense granules (Dg)euchromatic nucleus with dense elongated chromatin island and thick peripheral ones (arrows). **(F**) The rough endoplasmic reticulum cells (Rer) are dispersed in the matrix of the gland.

DISCUSSION

In the current work, the researchers described the histological and histochemical structures of the ink gland of *A.argus* in comparison with other aplysiids. Glands were composed of two covering epithelium; a cubodial structure facing the mantle cavity and a

columnar structure facing the mantle shelf, in addition to an inner matrix. These structures confirmed the presence of a covering epithelium, which was described in *A. dactylomea* in the study of **Bezerra *et al.* (2006)**.

For the first time to the best of our knowledge, two different granulated cell types were addressed beneath the cuboidal epithelium. Type I cells were club shaped with 12–15 electron dense secretory granules, while type II were large cells containing 85–90 electron dense secretory granules. Only one type of granulate cell was previously described (**Prince *et al.*, 1998; Bezerra *et al.*, 2006**). According to **Prince *et al.* (1998)**, granulate cells from red algal-feeding aplysiids contained 4–14 vacuoles of electron dense material. The present histochemical investigations indicated both granulated cell types containing carbohydrates and protein; however, they lacked acid mucopolysaccharides. Unfortunately, there is no information in literature on the histochemical content of granulated cells.

Superficial pits were detected on the gland surface facing the mantle cavity, which were formed by the invaginations of the cubodial covering epithelium. This observation agrees with that of **Bezerra *et al.* (2006)** with respect to *A. dactylomela*. The previuos authors suggested these invaginations gave rise to ducts, possibly connected to secretory vesicles to the exterior, and could be the route through which ink was released. **Prince and Johnson (2006)** reported those superficial pits in the ink gland of *D. dolabrifera*; they observed transparent vesicles filled with mucous and opened on the gland surface by pores.

The ink discharge mechanism may occur as follows: when predator attacks, ink motor neurons are stimulated, activating muscles which surround ink vesicles, and thus they squeeze and release their contents into the mantle cavity through the vesicle ducts. The ink is exteriorly expelled through the siphon **(Carew & Kandel, 1977; Walters & Erickson, 1986; Prince *et al.*, 1998)**.

Different vesicle types were described; namely, colored (purple), granulated, protein (white and amber) and transparent (clear) vesicles (**Prince *et al.*, 1998; Bezerra *et al.*, 2006; Prince & Johnson, 2015**).In the present study, only three vesicle types were recorded: dark-purple, granulated and transparent. The current observations coincide with those of **Bezerra *et al.* (2006)** who examined *A. dactylomela* and found that those vesicles were present in Red Sea weed-fed aplysiids. However, green-sea weed-fed aplysiids had many transparent, without dark-red-purple vesicles (**Prince *et al.*,1998; Bezerra *et al.*, 2006**). Generally, these vesicles were surrounded by a thin layer of smooth muscle and collagenous fibers, which facilitated releasing ink from vesicles **(Prince *et al.*, 1998)**.

The previous authors described three types of secretory vesicle in *A. californica,* fed on red seaweed, viz. dark-red-purple, protein and transparent vesicles. However, in animals that secrete their ink, these authors observed an additional vesicle type, the light red-purple vesicles. **Prince and Johnson (2015)** reported that protein synthesized in RER

cells and stored in protein vesicles were white and amber vesicles. **Bezerra *et al.* (2006)** postulated that, the chemical nature in ink vesicles in the red-seaweed-fed aplysiid, *A. dactylomela* contains carbohydrates and proteins or glycoproteins, without mucopolysaccharides. Nevertheless, when fed green-seaweed, *A. dactylomela* lacks all ink chemical components.

Furthermore, and for the first time, an adipose connective tissue was recorded beneath the columnar covering epithelium which intermingled with dispersed cells between adipocytes. Such ink gland structures have not been reported before. Therefore, we provide new information on the general configuration of the ink gland in sea hares, in agreement with the studies of **Prince *et al.* (1998)** and **Bezerra *et al.* (2006)**. The main function of fat cells is energy storage **(Church *et al.*, 2012)**. In invertebrates, fat cells are involved in growth, reproduction and immune mediation **(Liu *et al.*, 2009; Choi & Hyun, 2012; Azeez *et al.*, 2014)**. The question now remains, why does this gland contain adipose connective tissue? Are these structures present in all sea hares, or they are only present in species feeding on both green and red algae to use this energy in feeding shift? This hypothesis could be applied to *A. argus*, which feeds on both red and green algae (**Switzer-Dunlap & Hadfield, 1977; Carefoot, 1987**). Similar to other aplysiids, they must consume red seaweed to secrete purple ink (**Prince *et al.*, 1998**). Further studies are required to investigate the ink gland structures secreted from different species.

The current histochemical investigations showed that dispersed cells contained carbohydrates and acid mucopolysaccharides, but no proteinaceous material (Table 1).

CONCLUSION

In conclusion, the present histological characterization of the ink gland of *A. argus* corroborated general gland organization as described in the study of **Prince *et al.* (1998)**. This data confirmed the observations of **Bezerra *et al.* (2006)** on the presence of a covering epithelium. Also, for the first time to the best of our knowledge, we provide new data on two different granulated cell types and adipose connective tissue. Similarly, the histochemical characterization provides new insights on granulated cell, secretory vesicle and dispersed cell content.

Acknowledgements

The senior author expresses his sincere thanks to Dr. Angle Valdés California State Polytechnic University, Pomona, USA for his help in identification.

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