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MORPHOLOGY AND CYCLIC ACTIVITY OF THE DIGESTIVE GLAND OF *ZIDONA DUFRESNEI* (CAENOGASTROPODA: VOLUTIDAE)

Mariel Ojeda[1*], Florencia Arrighetti[2] & Juliana Giménez[1,3]

ABSTRACT

Zidona dufresnei is a carnivorous snail that plays an important role in the trophic web as a top predator. It is a threatened species due to high levels of fisheries exploitation. In this study, we describe the morphology of the digestive gland of *Z. dufresnei* from Mar del Plata, Argentina, and discuss the function and the cycle of activity of the different cell types. Histological analysis reveals two types of tubules: type 1 tubules composed of digestive and basophilic cells and type 2 tubules lined by basophilic cells only. Pyramidal basophilic cells contain lipofuscin and a large amount of rough endoplasmic reticulum, which suggests that these cells are responsible for the secretion of digestive enzymes that initiate the extracellular digestion of the food. Columnar digestive cells exhibit large membrane-bound vesicles that contain proteoglycans, neutral glycosaminoglycans and small amounts of lipid and lipofuscin. The presence in digestive cells of many endocytic vesicles and residual lysosomal bodies, as evidenced by the presence of lipofuscin pigments in the apical region, indicate that digestion is completed intracellularly within these cells. The digestive cells are subject to cyclical changes involving four phases: initial, digestion, fragmentation, and disintegration stages.

Key words: Gastropoda, carnivorous snail, digestive cells, basophilic cells, South Atlantic Ocean.

INTRODUCTION

Zidona dufresnei (Donovan, 1823) is a large carnivorous neogastropod endemic to the south-western Atlantic Ocean, inhabiting the region from Rio de Janeiro, Brazil (22°S), to Patagonian waters, San Matías Gulf, Argentina (42°S) (Kaiser, 1977). Reproductive aspects, population dynamics, growth and mortality were studied in *Z. dufresnei* (Giménez & Penchaszadeh, 2002, 2003; Giménez et al., 2004, 2008), but a detailed study on the anatomy, histology and ultrastructure of the digestive system of *Z. dufresnei* is missing. In recent years *Z. dufresnei* became a valuable resource in Argentina and is likely to be seriously endangered by high levels of exploitation (Giménez et al., 2005; Torroglosa & Giménez, 2010).

Most studies of the histology of the molluscan digestive gland have been conducted on bivalves (Owen, 1970; Thompson et al., 1974; Logan et al., 2008) and herbivorous microphagous gastropods (Merdsoy & Farley, 1973; Boghen & Farley, 1974; Reader, 1976; Wigham, 1976; Nelson & Morton, 1979; Taïeb & Vicente, 1999; Lobo-da-Cunha, 1999, 2000; Taïeb, 2001; Baqueiro Cárdenas et al., 2007; Gros et al., 2009; Volland & Gros, 2012). However, a study by Dimitriadis & Andrews (2000) on the neogastropod *Nucella lapillus* (Linnaeus, 1758) has provided the only report of the functional morphology of the digestive gland of a carnivorous caenogastropod.

The main objective of this study is to describe the structure and function of cells of the digestive gland in the carnivorous snail *Z. dufresnei.* We used histochemical tests and ultrastructural analysis to identify the function of the various cell types and their cycles of activity, in order to compare and contrast the digestive glands of herbivorous grazers and predatory carnivores within the Caenogastropoda.

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MATERIALS AND METHODS

A total of 152 adults specimens of *Z. dufresnei* were collected from October 2008 to December 2009 by bottom trawling in Mar del Plata (38°20'S, 57°37'W) at depths of 40 to 60 m. For histological analysis of the digestive gland, individuals were removed from the shell and small pieces of the digestive gland of each were fixed in Carnoy solution for 1 h, 10% formaldehyde for 12 h, and Bouin's solution for 12 h, and then were subsequently stored in 70% alcohol. Tissues were dehydrated using an ascending series of ethanol concentrations and then embedded in resin (Leica Historesin). Sections were cut at 5 µm with an electronic microtome (Leica), stained with hematoxylin and eosin, and observed under a light microscope.

All the samples collected were used for histochemical studies. The following methods were performed and modified for resin: periodic acid Schiff (PAS) for neutral glycosaminoglycans, Alcian Blue (pH 2.5) for proteoglycan (AMPS), Sudan Black for neutral lipids and phospholipids, Von Kossa method for calcium, Schmorl test for lipofuscin and Best´s carmine for glycogen. Sections of positive and negative controls were made for all histochemical studies.

For electron microscopical analysis, small pieces of tissue form 7 individuals were fixed for 3 h at 4°C in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and washed in the same buffer. Subsequently, these tissues were placed in a 1% solution of osmium tetroxide in 0.1 M sodium phosphate buffer for 1.5 h, again buffer-rinsed, ethanol-dehydrated, and embedded in araldite resin. Ultrathin sections were cut using either a Reichert or an LKB IV ultramicrotome and stained with uranyl acetate and lead citrate. Sections were examined and photographed using Zeiss EM 109T, Hitachi 300 and Jeol 1010 transmission electron microscopes operated at 75 to 80 kV.

RESULTS

The digestive gland of *Z. dufresnei* was situated in the distal portion of the coiled visceral hump close to the gonad. It was an array of blind-ending tubules interconnected with large ducts opening into the stomach. Tubules and ducts were surrounded by connective tissue, muscle fibres, and blood vessels, and had different appearances based on histological sections (Fig. 1). Whatever the month in which the animals were collected, the digestive gland showed the same histological features.

Histochemical Tests

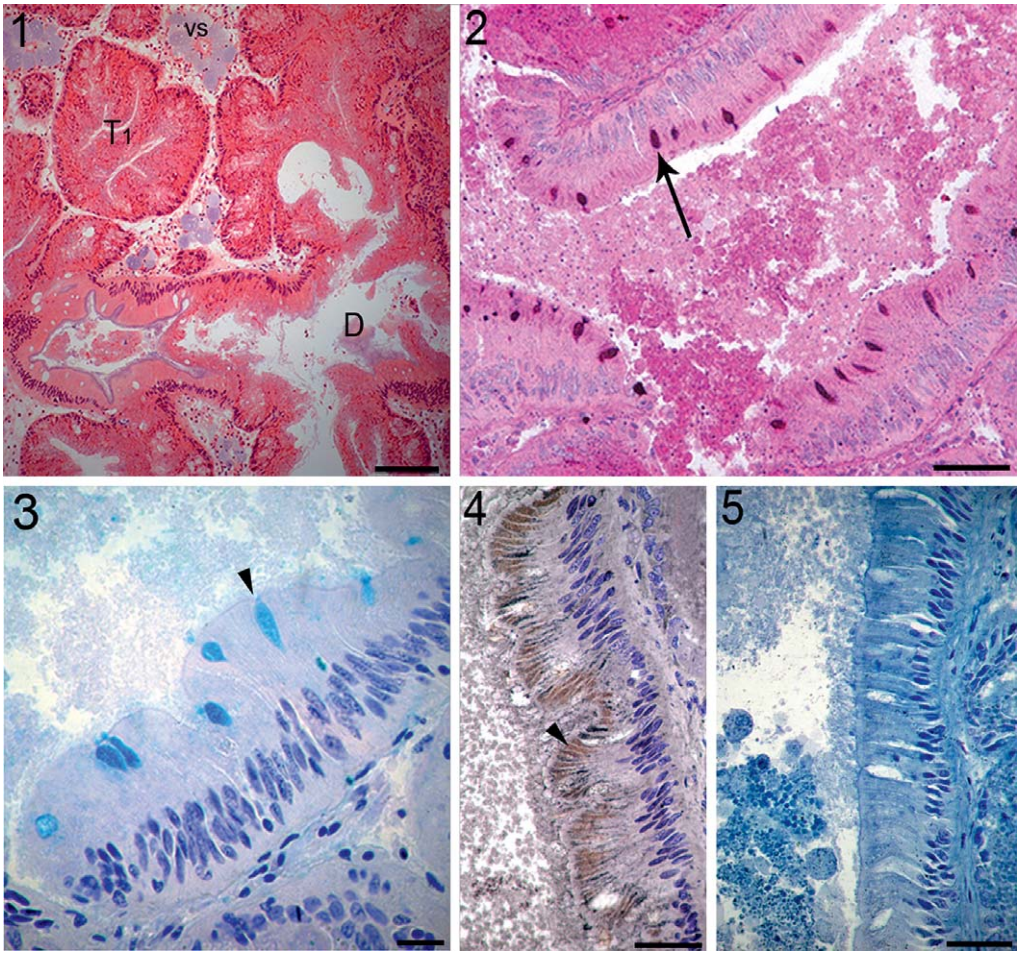
The results of histochemical tests on resin sections are summarized in Table 1. Ducts were characterized by the presence of one type of ciliated, columnar epithelial cells with a basal nucleus (Figs. 2–5). The apical region contained vesicles that reacted positively to the PAS test, showing the presence of neutral glycosaminoglycans (Fig. 2). Lipid vesicles were evidenced by the Sudan Black technique (Fig. 4) and Schmorl reactions showed the presence of lipofuscin (Fig. 5). Secretory cells were interspersed with ciliated cells and stained intensely with the PAS (Fig. 2) and AMPS test (Fig. 3). The lumens of the ducts were usually

TABLE 1. Histochemical test in the *Zidona dufresnei* digestive gland.

	Duct cells		Tubule cells			
	Columnar cells	Secretory cells	Digestive cells			Basophilic cells
			apical	media	basal	
PAS	+	+++	+++	+++	+	-
Alcian Blue pH 2.5	-	+++	+++	+	-	-
Sudan Black	++	-	+++	+	+	+
Schmorl	+	-	+++	-	-	+++
Von Kossa	-	-	-	-	-	-
Best's carmin	-	-	-	-	-	-

filled with an amorphous substance histochemically similar to the contents of the columnar ciliated cells (Figs. 1–5).

In relation to the characteristics of the cells, two types of digestive tubules were observed (Fig. 6): type 1 tubules were lined by digestive and basophilic cells (Fig. 6) while the type 2 tubules contained only clumps of basophilic cells (Fig. 6). The most abundant cell type was the narrow, closely packed columnar digestive cell, which contained a basal nucleus and a variety of membrane-bound vesicles in the cytoplasm (Fig. 7). Granules of neutral glycosaminoglycans (PAS-positive) were frequently observed occupying a large portion of the digestive cells, although sometimes they were restricted to the basal and medial region (Fig. 7). The apical region, and, to a lesser extent, the middle region of the cell, contained vesicles that reacted positively to the AMPS test for proteoglycan



FIGS. 1–5. Histology of *Zidona dufresnei.* FIG. 1: General view of a portion of the digestive gland of *Zidona dufresnei* comprising sections of the ducts (D) and tubules type 1 (T_1), note blood vessel (vs); FIG. 2: Transversal section of a duct with PAS test, a pronounced neutral glycosaminoglycans accumulation is evident in the secretory cells (arrow); FIG. 3: Transversal section of a duct with AMPS test, proteoglycans are accumulated in secretory cells (arrowhead); FIG. 4: Sudan Black technique reveals an accumulation of lipidic vesicles in the apical region of the columnar cells (arrowhead); FIG. 5: Presence of lipofuscin revealed by Schmorl technique. Scale bars: Fig. 1 = 100 µm; Figs. 2, 4, 5 = 50 µm; Fig. 3 = 20 µm.

(Fig. 8), to the Schmorl test for lipofuscin (Fig. 9) and to the Sudan Black technique (Figs. 10, 11). Von Kossa tested positive on some diffuse vascular tissue but not on the digestive cells. There was no positive reaction of the cells with the glycogen reagent Best's carmine.

The pyramidal-shaped basophilic cells were located in crypts of the tubules. The cytoplasm of this cell type contained small vacuoles which reacted negatively to all tests except for the Schmorl reaction for lipofucsin (Fig. 10).

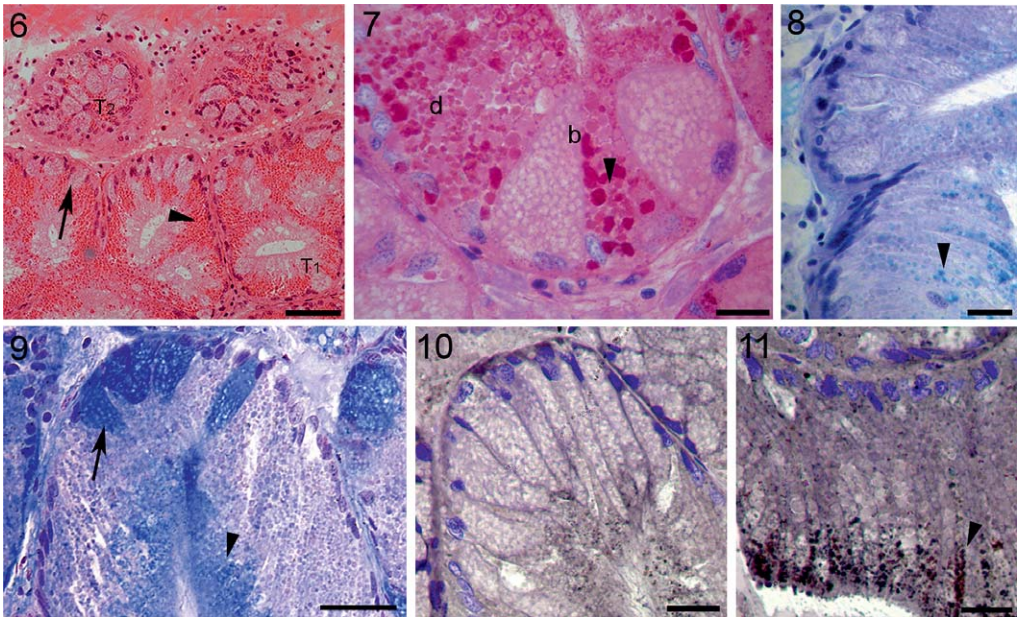
Ultrastructure

The apical surface of the digestive cells was ciliated and bore microvilli (Figs. 12–14). A series of membrane-bound vesicles was present in the cytoplasm (Figs. 12, 13, 15). Pinocytotic vesicles appeared at the base of the microvilli (Fig. 13, inset) followed by vesicles with different contents and electron densities. Type V_1 vesicles were electron transparent (Fig. 12) and of various sizes. Vesicles of type V_2 were frequently found in the mid-region of the cell; they were large and contained granular or fibrillar material of moderate electron density (Fig. 15). Some of these vesicles exhibited an electron-transparent space separating contents and membrane. From the mid-region toward the basal region of the cytoplasm, vesicles containing dense material (type V_3 vesicles) were observed (Fig. 12).

The nucleus in the basophilic cells was situated in the basal region (Figs. 16, 17) and a highly developed rough endoplasmic reticulum, free ribosomes, and abundant secretory granules occupied the cytoplasm (Fig. 17, inset). The secretory granules contained a homogenous electron-dense material (Fig. 17).

Cycle of Activity

Epithelial digestive cells of type 1 tubules displayed four distinct morphological phases,



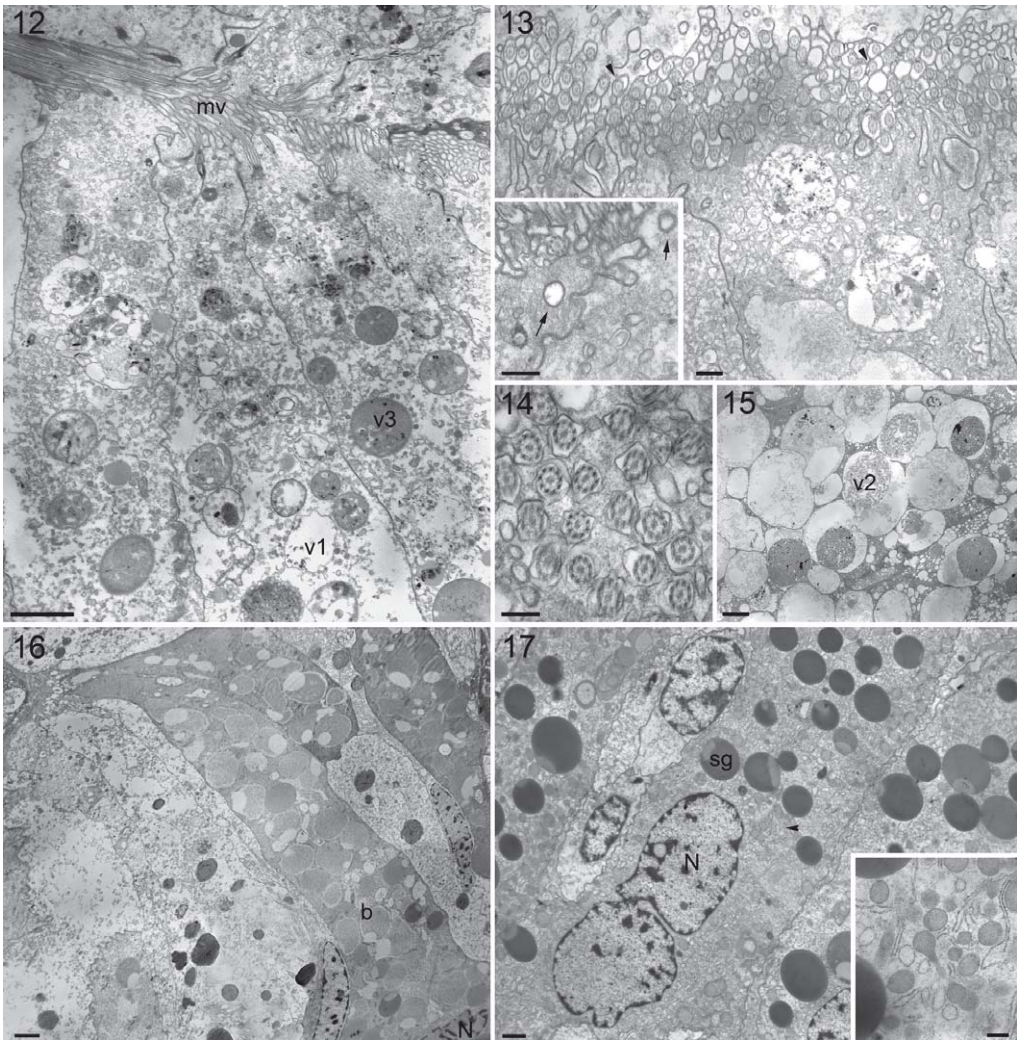
FIGS. 6–11. Histology of the tubules of the digestive gland of *Zidona dufresnei*. FIG. 6: Digestive tubules of type 1 (T_1) are lined by basophilic (arrow) and digestive cells (arrowhead) and tubule of type 2 (T_2) are lined by basophilic cells; FIG. 7: Presence of neutral glycosaminoglycans granules (arrowhead) in the basal and medial region of digestive cells (d) by PAS technique. Note the absence of neutral glycosaminoglycans in the cytoplasm of the basophilic cell (b); FIG. 8: AMPS test showing proteoglycan in the apical and medial region of the digestive cells (arrowhead); FIG. 9: Lipofuscin inclusions in the apical and medial region of digestive cells by Schmorl technique (arrowhead) and in the entire cytoplasm of the basophilic cells (arrow); FIG. 10: Basophilic cells showing the negative reaction to the Sudan Black technique; FIG. 11: Phospholipids and neutral lipids, as evidenced by the Sudan Black technique in the middle and apical region of the digestive cells (arrowhead). Scale bars: Fig. 6 = 50 µm; Figs. 7, 8 = 20 µm; Figs. 9–11 = 50 µm.

but intermediate stages between these four were commonly found:

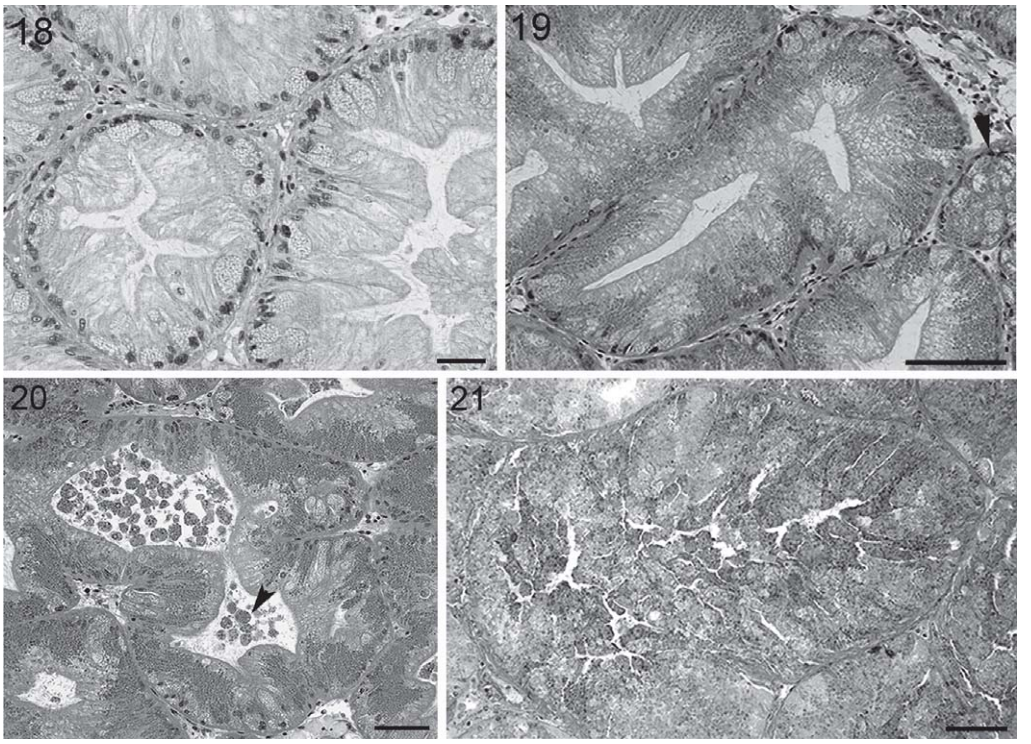
Stage 1. Tubules 1 and 2 were present and the digestive cells were characterized by a homogeneous cytoplasm without granules (Fig. 18).

Stage 2. The digestive cells were characterized by the presence of granules (Fig. 19) in their cytoplasm.

Stage 3. In this stage, the apical region of digestive cells was rounded and the brush border was not observed. The lumen of the tubules



FIGS. 12–17. Ultrastructural aspect of the digestive and basophilic cells of the digestive gland of *Zidona dufresnei*. FIG. 12: General view of the apical surface of the digestive cell showing the presence of microvilli (mv) and different type of vesicles (V_1 and V_3); FIG. 13: General view showing the presence of the cilias (arrowhead). Inset, pinocytotic vesicles in the apex of a digestive cell (arrow); FIG. 14: Detail of the transversal section of the cilias; FIG. 15: Detail of the vesicles (V_2) present in the cytoplasm of a digestive cells; FIG. 16: General view of a basophilic cell (b); FIG. 17: Detail of a basophilic cell showing the cytoplasm full of secretory granules (sg) and rough endoplasmic reticulum (arrowhead). Note the presence of the nucleus (N) in the basal region. Inset, Detail of the well-developed rough endoplasmic reticulum in the cytoplasm of a basophilic cell. Scale bars: Figs. 12, 13 = 1 µm; Fig. 13, inset = 0.2 µm; Fig. 14 = 0.2 µm; Fig. 15 = 1 µm; Figs. 16, 17 = 2 µm; Fig. 17, inset = 1 µm.



FIGS. 18–21. Cycle of activity in the type 1 tubules of the digestive gland of *Zidona dufresnei*. FIG. 18: Digestive cells in the initial stage, with an homogeneous cytoplasm; FIG. 19: Digestive cells in digestive stage, containing numerous granules in the cytoplasm. Note the presence of type 2 tubule (arrowhead); FIG. 20: Digestive cells in fragmentation stage with fragmentation spherules (arrowhead) in the lumen of the digestive tubule; FIG. 21: Disintegration of the epithelium. Scale bars: Figs. 18, 20, 21 = 50 µm; Fig. 19 = 100 µm.

was characterized by the presence of some fragmentation spherules (Fig. 20). Vesicles present in the digestive cells were histochemically similar to the spherules, in that they reacted positively to the AMPS and PAS tests.

Stage 4. The cytoplasm of the digestive cells seemed to be breaking off from the rest of the cell, and the cellular limits were not defined (Fig. 21). The apical region of the digestive cells reacted positively to the PAS test.

DISCUSSION

The general aspect of the digestive gland of *Z. dufresnei* is similar to that found in other gastropod species, although it appeared to be more complex than the digestive gland of microphagous (Wigham, 1976), herbivorous (Owen, 1958; Merdsoy & Farley, 1973) or other carnivorous gastropods (Dimitriadis & Andrews, 2000). The digestive gland of *Z. dufresnei* consisted of a single digestive duct that connected the stomach to the digestive tubules. The epithelium of the digestive tubules was composed of columnar ciliated cells with neutral glycosaminoglycans and lipids in their cytoplasm. Positioned among these cells were the secretory cells with their cytoplasm composed of neutral glycosaminoglycans and proteoglycans. The glycosaminoglycans of both cells, columnar and secretory, were likely responsible for the lubrication of the lumina surface of the digestive tract and seemed to be related to the flow of secretion along the duct caused by ciliary beating. Also, beating of duct cilia probably carried fine food particles from the stomach into the digestive gland tubules.

The tubules of the digestive gland of *Z. dufresnei* shared some characteristics with the digestive tubules in *Aplysia punctata* (Cuvier, 1803) (Taïeb & Vicente, 1999; Taïeb, 2001)

and *Strombus gigas* (Linnaeus, 1758) (Gros et al., 2009) in that two different type of digestive tubules with different cell types were present. In *Z. dufresnei*, tubules of type 1 were formed by an epithelium containing two cell types: digestive and basophilic cells, as in most other gastropods that have been studied (Morton, 1955; Merdsoy & Farley, 1973; Reader, 1976; Wigham, 1976; Nelson & Morton, 1979; Dimitriadis & Andrews, 2000; Baqueiro Cárdenas et al., 2007; Gros et al., 2009; Volland et al., 2012). The type 2 tubules of *Z. dufresnei* are exclusively composed of basophilic cells and different stages are not observed in these cells as in *A. punctata* (Taïeb, 2001).

In some species, digestive cells can collect relatively large food particles by phagocytosis, but in others extracellular digestion occurs and only dissolved substances are captured by small endocytic vesicles (Merdsoy & Farley, 1973; Boghen & Farley, 1974; Lobo-da-Cunha, 2000). Many endocytic vesicles were observed in the apical border of *Z. dufresnei* digestive cells. Basal to this area, there is a zone full of vesicles, morphological and histochemically different.

Type 1 vesicles (V_1) are electron-lucent structures limited by a single membrane and containing only a small amount of material. These vesicles could be formed by the fusion of endocytic vesicles, as was observed in other mollusc species (Owen, 1970; Boghen & Farley, 1974; Lobo-da-Cunha, 2000; Taïeb, 2001). The second type of vesicle (V_2) resembled the heterolysosomes (or phagosomes, or digestive vacuoles, or green granules) reported in other molluscs; these are structures produced by the fusion of endocytic vesicles and enzyme-rich vesicles such as lysosomes (Owen, 1970; Pal, 1972; Dimitriadis & Andrews, 2000). Continuous intracellular digestion results in the accumulation of residual bodies (here called V_3 vesicles). These residual bodies correspond to the granules stained positively with Alcian blue. These "blue granules" are composed of proteoglycan and seemed similar to the large residual bodies described in other molluscan digestive cells (Dimitriadis et al., 2004; Lobo-Da-Cunha, 2000; Taïeb, 2001). Such residual bodies could be secretory products as they were found concentrated in the apical surface of the cell and also in the lumen of the digestive ducts. But some remained in the cytoplasm, becoming lipofuscin granules that stained positively with Schmorl technique. The remnants of undigested material were extruded to the lumen of the digestive gland together with the apical cytoplasm of the cell; the same process has been observed in *Aplysia depilans* (Gmelin, 1791) (Lobo-da-Cunha, 2000) and *Nucella lapillus* (Dimitriadis & Andrews, 2000).

In *N. lapillus* a third cell type full of secretory granules is present in the tubules of the digestive gland epithelium (Dimitriadis & Andrews, 2000). The main function of these small cells was the lubrication and protection of the tubule surface, a function that is analogous to that of secretory cells found in the digestive ducts of the *Z. dufresnei* digestive gland.

The morphology of basophilic cells (also named crypt cells, excretory cells, or secretory cells) found in the digestive tubules of *Z. dufresnei* is similar to the basophilic cells described for other molluscs species (Owen, 1970, 1973; Pal, 1971; Merdsoy & Farley, 1973; Boghen & Farley, 1974; Mathers et al., 1979; Nelson & Morton, 1979; Henry et al., 1991; Lobo-da-Cunha, 1999; Baqueiro Cárdenas et al., 2007; Gros et al., 2009; Volland & Gros, 2012). In *A. punctata*, similar cells containing dense granules of calcium salts, named calcium cells, were found (Taïeb & Vicente, 1999). These cells were not observed in the digestive gland of *Z. dufresnei*. Basophilic cells have a pyramidal shape, large amount of rough endoplasmic reticulum and secretion granules. Some studies reported a positive cytochemical detection for ß-glucuronidase and arylsulphatase in the secretory granules of the basophilic cells (Pipe, 1986; Cajaraville et al., 1995; Lobo-da-Cunha, 1999; Dimitriadis et al., 2004), which enhances the hypothesis concerning the production and secretion of lysosomal enzymes. The presence of a large amount of lipofuscin, an insoluble pigment that is accumulated in lysosome-like structures, in the basophilic cells of *Z. dufresnei* is consistent with this hypothesis. According to this, basophilic cells seem to be responsible for the secretion of digestive enzymes that undertake the extracellular digestion of the food. This statement is supported by the ultrastructural observation of the rough endoplasmic reticulum in the basophilic cells.

Little is known about the cycle of activity present in the tubules of the digestive gland in gastropods. Digestive phases linked to environmental factors such as the tidal cycle have been described in some bivalves (Benninger & Le Pennec, 2006) and gastropods (Merdsoy & Farley, 1973; Boghen & Farley, 1974; Nelson & Morton, 1979). The digestive gland of *Z. dufresnei* has a dynamic state in its morphology, in which most tubules are at the same stage. A cycle of activity is observed in type 1 tubules involving changes in the composition and morphology in

digestive cells. During the first stage, here called initial stage, no digestive activity was observed. The absorption of food material is presumed to take place in the tubules at this stage as was demonstrated in numerous experiments with marker substances (Owen, 1955; Mathers et al., 1979). In the second stage, called the digestion stage, the absorption is less important, and intracellular digestion takes place in digestive cells within the large mid-region vesicles. The end-products of this process are accumulated in residual bodies; this was also observed in *Maoricrypta monoxyla* (Lesson, 1831) (Nelson & Morton, 1979). In stage 3, called fragmentation, the fragmentation spherules observed in the lumen are formed by "nipping off" the apical portion of the digestive cells. The next stage is the disintegration of the epithelium. The new tubules could be formed either by reformation of existing tubules or by disassociation of clumps of basophilic cells, forming type 2 tubules, as has been described for other molluscs (Owen, 1970; Nelson & Morton, 1979; Beninger & Le Pennec, 2006).This is supported by the prevalence of type 2 tubules during the breakdown and disintegration stage.

The duration of a complete digestive cycle was studied in some bivalves and it may range from 12 to 24 hours duration to accommodate feeding during each twelve hour tidal cycle (Mathers et al., 1979). The duration of a complete digestive cycle of a single tubule of *Z. dufresnei* is still not known and some feeding and starvation experiments are necessary to correlate feeding activity and digestive functions with localized variations of environment. Complementary information is necessary for the better understanding of the structure and precise function of the cells, specifically through the use of immunocytochemistry.

Further comparative studies including physiology and functional assessment will be considered to understand the role of the principal cell components of the digestive glands related to the feeding habits of the marine gastropods.

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