Evidence for a prokaryotic origin of intracellular corpuscles in the digestive gland of the queen conch *Lobatus gigas* (Linnaeus, 1758) (Gastropoda: Strombidae)

Federico A. Dellagnola[1,2,3], Israel A. Vega[1,2,3] and Alfredo Castro-Vazquez[1,2,3]

[1]*IHEM, CONICET, Universidad Nacional de Cuyo, Casilla de Correo 33, 5500 Mendoza, Argentina;*
[2]*Instituto de Fisiología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Casilla de Correo 33, 5500 Mendoza, Argentina; and*
[3]*Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Cuyo, Casilla de Correo 33, 5500 Mendoza, Argentina*

Correspondence: I.A. Vega; e-mail: iavega.conicet@gmail.com

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ABSTRACT

Two types of pigmented intracellular bodies have been reported in the digestive glands of several caeno-gastropods, particularly in the families Ampullariidae (Architaenioglossa: Ampullarioidea), Strombidae and Struthiolariidae (Littorinimorpha: Stromboidea). Rounded corpuscles, which are usually light brown, are identified here as C corpuscles, while corpuscles that are oval, darker and larger are identified as K corpuscles. We studied both corpuscular types in *Lobatus gigas* (Strombidae) using (1) differential-interference contrast microscopy, (2) transmission electron microscopy, (3) *in situ* hybridization with a generalized cyanobacterial 16S rRNA probe and (4) autofluorescence before and after lysozyme digestion. Results indicated that C corpuscles were located in the basal regions of columnar cells and the intensity of their pigmentation and alcianophily (indicative of glycosaminoglycans) was variable. They showed an electron-dense wall and contained abundant electron-dense clumps and irregularly arranged membranes, but no thylakoids or nuclei. Hybridization with the 16S rRNA probe varied from none to intense in C corpuscles, indicative of variations in the rRNA content during their life cycles. Their walls were sensitive to lysozyme digestion, which strongly suggests that peptidoglycans are an integral part of this structure. K corpuscles were located within pyramidal cells and were uniformly dark brown but variably alcianophilic. They showed multiple lamellae of moderate electron density, organized around one to three cores, each one containing one or several small spherical bodies. All K corpuscles hybridized with the 16S rRNA probe and were partly digested by lysozyme. Both C and K corpuscles showed red autofluorescence, which suggests the presence of chlorophyll-like pigments. It is concluded that C and K corpuscles in the digestive gland of *L. gigas* may be forms of a prokaryotic symbiont related to the Cyanobacteria.

INTRODUCTION

Pigmented corpuscles in the digestive gland of a gastropod were first documented by Leydig (1850). MacMunn (1883) reported that there were bodies in the digestive gland of several gastropods "which remind one strongly of unicellular algae" and, later (MacMunn, 1900), that a pigment resembling plant chlorophyll was also present in those glands.

More recently, pigmented intracellular corpuscles were reported in *Pila virens* and *Pomacea canaliculata* (Architaenioglossa: Ampullariidae) (Meenakshi, 1955; Andrews, 1965). These observations were later confirmed for *P. canaliculata* and extended to other ampullariids, namely *Pomacea scalaris*, *Pomacea maculata* (as *Pomacea insularum*), *Asolene pulchella* and *Marisa cornuarietis* (Castro-Vazquez *et al.*, 2002; Koch *et al.*, 2006; Vega *et al.*, 2006). There were two corpuscular types, identified as C and K corpuscles: C corpuscles were greenish-brown, round bodies (diameter 14 μm), while K corpuscles were dark brown and oval (length 36 μm, width 14 μm).

Similar corpuscles have also been found in several taxa of Stromboidea (Littorinimorpha), including the genera *Lobatus* (as *Strombus*), *Strombus s. s.*, *Lambis*, *Struthiolaria* and *Pelicaria*.

The present study was prompted by the morphological similarity of pigmented bodies reported in the Stromboidea (e.g. Gros, Frenkiel & Aldana Aranda, 2009) with what had been called C and K corpuscles in species of Ampullariidae (Castro-Vazquez *et al.*, 2002). In the current study, the corpuscles from *L. gigas* were examined using (1) light and transmission electron microscopy (TEM), (2) fluorescent *in situ* hybridization (FISH) with a generalized cyanobacterial 16S rRNA probe and (3) exposure to lysozyme to test for the presence of bacterial peptidoglycans in the corpuscular envelopes.

For ease of comparison with corpuscles found in the Ampullariidae (Castro-Vazquez *et al.*, 2002), the corresponding structures in *L. gigas* will also be identified here as C and K corpuscles (corresponding to the small round "granules" and the large oval "inclusions", respectively, of Gros *et al.*, 2009).

MATERIAL AND METHODS

Animals

Three individuals (two males, 22 cm long, and one female, 20 cm long) were collected in an artificial inlet of the Caribbean Sea (Puerto Aventuras, Quintana Roó State, Yucatán Peninsula, Mexico; 20°30′32.32″N, 87°12′35.49″W) during January 2013. They were photographed and several samples of the digestive gland of each animal were obtained with a razor blade and fixed in 4% paraformaldehyde in seawater.

Light microscopy

Samples of the digestive gland were dehydrated in a graded ethanol series and embedded in a resin-paraffin mixture (Histoplast®). Sections (5 μm thick) were stained with a trichrome stain (Nuclear Fast Red, Alcian Blue 8GX, eosin), in which the nuclei were stained bright red (Nuclear Fast Red), glycosaminoglycans were stained deep blue (Alcian Blue) and cytoplasm was stained from light blue to purple (the superimposition of background Alcian Blue staining and eosin). Micrographs were taken with a Nikon Eclipse 80i (using Nomarski differential-interference contrast (DIC) microscopy) provided with a Nikon DS-Fi1-U3 digital camera.

Abundant C and K corpuscles were the main component in the residue in the vials, where the digestive gland samples had been fixed and those residues were used to observe the pigmentation of corpuscles in unstained preparations, to determine their alcianophily (according to Steedman, 1950) and to determine their sensitivity to lysozyme digestion (see below).

FISH with a generalized cyanobacterial 16S rRNA probe (CYA361)

The hybridization with the digoxigenin-labelled CYA361 probe (Schönhuber *et al.*, 1999) [5′-CCCATTGCGGAAAATTCC-3′] was tested on paraffin-resin sections (prepared as described above). The sections were rehydrated after paraffin removal and were subjected to the following stepwise procedure: (1) incubation in a 2× SSPE hybridization buffer (1× SSPE = 0.15 M NaCl, 0.01 M EDTA, 0.01 M sodium phosphate; pH 7.2) at 70 °C for 20 min; (2) incubation in 0.1 M triethanolamine solution containing 0.25% acetic anhydride for 10 min; (3) exposure to a 2× SSPE hybridization buffer containing herring sperm DNA (0.5 mg/ml), yeast tRNA (0.25 mg/ml) and 5× Denhardt's solution for 60 min at 42 °C; (4) incubation with 100 pmol of the digoxigenin-labelled probe per tissue section, at 37 °C overnight in a humid chamber; (5) sequential washing in decreasing concentrations of the hybridization buffer (SSPE 2×, 1× and 0.5×, 60 min each) at room temperature; (6) incubation in a buffer containing 100 mM Tris (pH 7.5), 150 mM NaCl and 1% goat serum for 5 min. Afterwards, for detection of the digoxigenin-labelled probe, sections were incubated for 5 h in darkness with a 1/4 dilution of a fluorescein-attached antibody against digoxigenin (Roche, catalogue number 11207741910) and were then washed in a buffer containing 100 mM Tris (pH 7.5) and 100 mM NaCl (three times, 10 min each). Finally, the sections were mounted in glycerol-PBS buffer (90:10, v/v) containing 5 mg/ml propyl-gallate (P3130, Sigma) (Longin *et al.*, 1993). Negative controls (i.e. sections exposed to the CYA361 probe, but with no digoxigenin label) were also run. Observations were made with DIC and fluorescence microscopy (excitatory wavelength range = 465–495 nm; emission wavelength range = 515–555 nm).

Transmission electron microscopy

After fixation in 4% paraformaldehyde, the digestive gland samples were stored in 70% ethanol for transfer to the laboratory in Argentina, where the samples were washed in 0.1 M phosphate buffer (pH 7.4) and postfixed in 2.5% glutaraldehyde (dissolved in the same buffer). One day later, tissues were washed three times in phosphate buffer and transferred to 1% osmium tetroxide overnight. Afterwards, they were rinsed in distilled water and treated with an aqueous solution of 2% uranyl acetate for 40 min, gradually dehydrated in a graded ethanol series followed by acetone and finally embedded in Spurr's resin. Ultrathin sections mounted on copper grids were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 900 transmission electron microscope.

Lysozyme digestion

A pool of fixation residues containing C and K corpuscles was used to determine the corpuscular sensitivity to lysozyme digestion. Lysozyme (EC 3.2.1.17) catalyzes the hydrolysis of 1,4-β-linkages between *N*-acetyl muramic acid and *N*-acetyl-D-glucosamine residues in bacterial peptidoglycans (http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/2/1/17.html). The corpuscular suspension was washed three times in mannitol-phosphate buffer (0.14 M, pH 6.0), centrifuged (6,000 rpm, 5 min) and suspended in 250 μl of mannitol-phosphate buffer per aliquot (N = 5). Fifty microlitres of either MiliQ water (control) or lysozyme (Sigma-L3790, 50 μg/μl) were added to each aliquot and the mixtures were incubated for 1 h at room temperature. Drops of each incubate were observed by DIC and fluorescence microscopy (excitation wavelength: 510–560 nm; emission wavelength: ≥590 nm).

RESULTS

Light microscopy

C corpuscles appeared in trichrome sections as round bodies of 7.2 ± 1.2 μm (mean ± SD, N = 100; 26 and 30 measurements were made on each of the males and 44 on the female; since there were no significant differences, the data were pooled for presentation). They were mostly contained within cells of the tubular acini, in the basal regions of columnar cells ("columnar digestive cells" of Gros *et al.*, 2009), although some were free in the glandular lumen as an apocrine emission of columnar cells (Fig. 1A). C corpuscles usually contained a coarse granular material, which was variably alcianophilic, both in tissue sections (Fig. 1A) and in the residue of fixation vials (Fig. 1D). Their light brown pigmentation was also variable in unstained residues of fixation vials (Fig. 1B).

In trichrome-stained sections, K corpuscles were large (26.5 ± 5.1 μm long, 18.2 ± 3.68 μm wide, N = 100 corpuscles; 28 and 33 measurements were made on males and 39 on the female; since there were no significant differences, the data were pooled). They were dark brown and mostly oval bodies (Fig. 1A), contained within pyramidal cells ("crypt cells" of Gros *et al.*, 2009), which can be recognized by their light purple cytoplasm in trichrome-stained sections (Fig. 1A). These corpuscles were also dark brown in unstained preparations of residues and they frequently showed more than one core (as in the upper K corpuscle of Fig. 1C).

Transmission electron microscopy

The granular contents of C corpuscles (Fig. 2A–C) were encased in an electron-dense wall of uniform thickness showing numerous electron-dense clumps in an electron-lucent matrix. Small membrane vesicles and other irregularly arranged membrane stacks were sometimes found in sections, but a thylakoid structure could not be recognized (Fig. 2B). No nuclei were seen. Occasionally, the contents appeared partly detached from the wall (Fig. 2C, white arrowheads) suggesting the existence of a plasma membrane. A finely thread-like material (best seen in Fig. 2C) was often seen in the space around C corpuscles, which may correspond to the glycosaminoglycans detected by Alcian Blue staining under light microscopy.
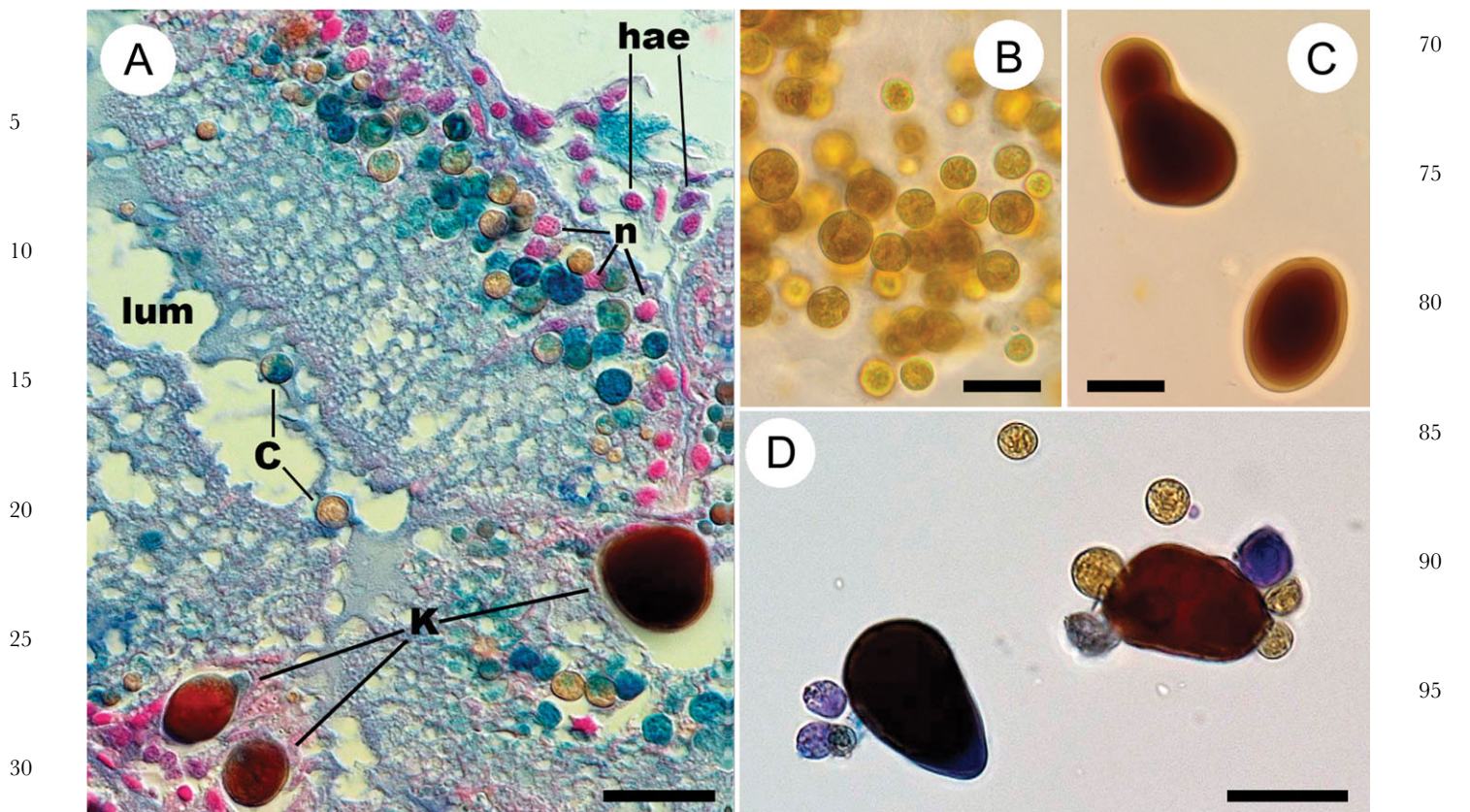
Figure 1. Nomarski differential-interference contrast microscopy of digestive gland of *Lobatus gigas*. **A.** Trichrome-stained sections showing a tubular acinus with numerous C corpuscles in basal epithelial region, which are differentially alcianophilic. Epithelial nuclei are red. Two C corpuscles (one partly alcianophilic) appear free in the lumen. Three K corpuscles are also visible, of which the two smaller ones are surrounded by light purple cytoplasm of pyramidal cells. **B.** Unstained C corpuscles in residue of a fixation vial, showing different degrees of pigmentation and well-defined outer edges that are their walls. **C.** Unstained K corpuscles in residue of a fixation vial; a two- to three-cored (upper left) and a single-cored K corpuscle (lower right) are visible. **D.** Alcian Blue stained C and K corpuscles in residue showing alcianophily in some of them. Abbreviations: C, C corpuscles; hae, haemocytes; K, K corpuscles; lum, lumen; n, epithelial nuclei. Scale bars = 25 μm.

K corpuscles are multilamellar structures, organized around one to three cores, each containing one or more circular arrangements of a fibrogranular material (Fig. 2D, E). The euchromatic nuclei typical of pyramidal cells were frequently found in proximity (Fig. 2E), but no nuclei were seen within K corpuscles. No distinct membranes could be recognized in these corpuscles.

FISH with a generalized cyanobacterial 16S rRNA probe (CYA361)

Sections of the digestive gland exposed to the digoxigenin-labelled CYA361 probe showed fluorescence in K corpuscles and in some of the C corpuscles (usually, but not always, in the smaller and less pigmented ones) (Fig. 3). The cores of some K corpuscles were detached and lost during the hybridization procedure (Fig. 3). Negative controls (exposed to probes that were not labelled with digoxigenin) gave no fluorescence in either types of corpuscles. C corpuscles in these unstained sections showed the same variable brown pigmentation (Fig. 3A) that was visible in trichrome-stained sections (Fig. 1A).

Lysozyme treatment of C and K corpuscles

DIC microscopy of control C and K corpuscles in residues of fixation vials showed red autofluorescence, but emission was less intense and variable in the C corpuscles (Fig. 4A, B). Enzyme treatment resulted in partial degradation of both corpuscle types. Correlative DIC and fluorescence microscopy of treated residues showed that the remaining corpuscles and debris became aggregated in masses held together by an autofluorescent material, likely corresponding to the content of lysed corpuscles (Fig. 4C, D).

DISCUSSION

C and K corpuscles in Lobatus gigas *and* P. canaliculata

Although the occurrence of C and K corpuscles has been shown in several species of Stromboidea and Ampullariidae, most of the information has been gathered in *L. gigas* and *P. canaliculata*, so the discussion will be focused on these species.

C corpuscles are round bodies contained within columnar cells of the digestive gland. They are smaller in *L. gigas* (7.2 ± 0.1 μm wide, this paper) than in *P. canaliculata* (12.3 ± 0.4 μm wide, Koch *et al.*, 2006). Typically, their pigmentation is light brown, but they may stain positively with Alcian Blue; both characters are more variable in *L. gigas* than in *P. canaliculata*. Alcianophily, particularly of the external covers of C corpuscles, is suggestive of Cyanobacteria, because many Cyanobacteria synthesize and excrete glycosaminoglycans (Pereira *et al.*, 2009). In both species, TEM of C corpuscles reveals numerous electron-dense clumps in an electron-lucent matrix, together with small vesicles and irregularly arranged membrane stacks, but lacking a typical thylakoid structure and a nucleus.

The contents of C corpuscles hybridize with a generalized probe for cyanobacterial 16S rRNA in *P. canaliculata*, but only in the less-pigmented corpuscles in *L. gigas*, which many indicate life-cycle
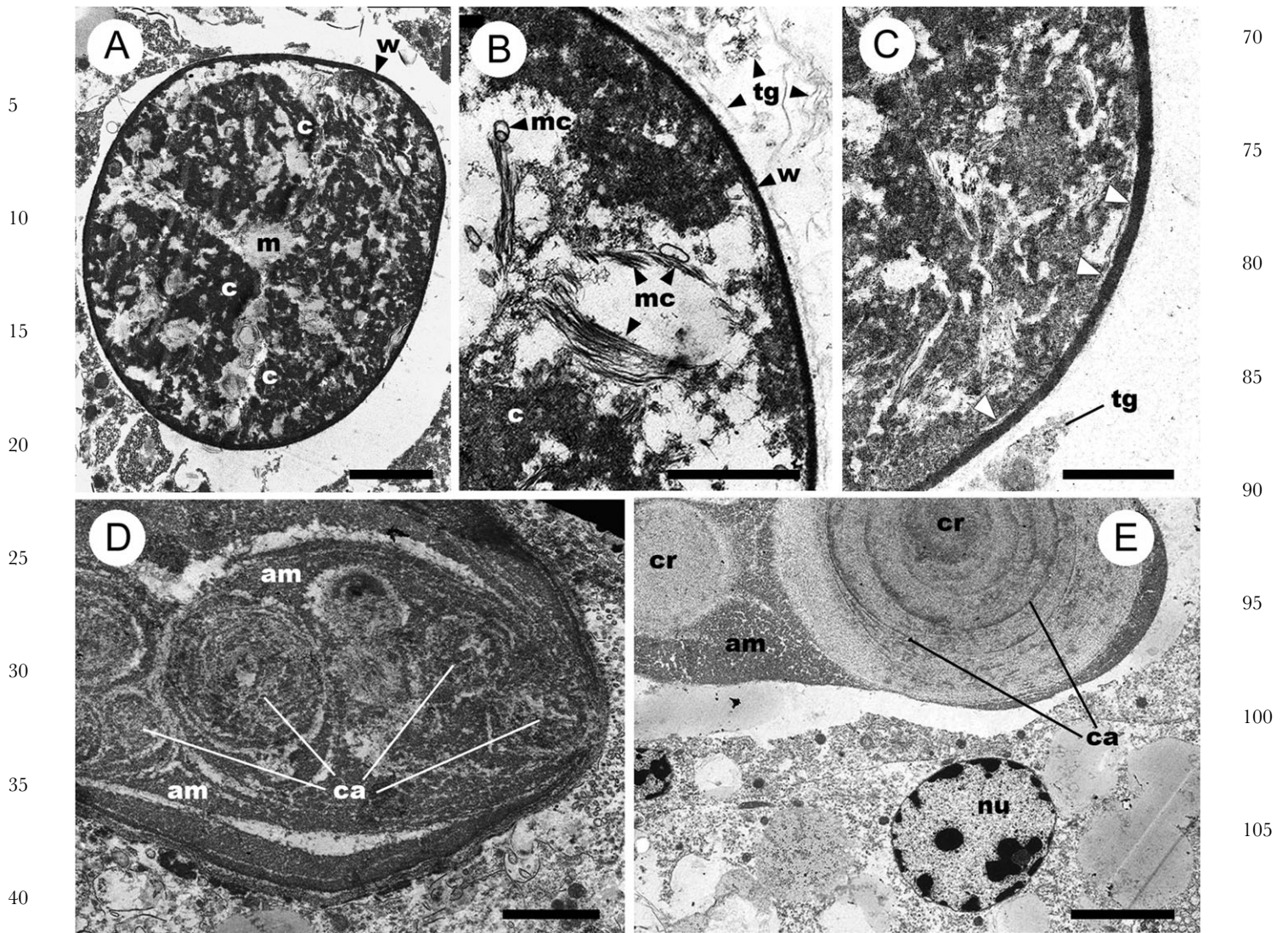
Figure 2. Transmission electron microscopy of digestive gland of *Lobatus gigas*. **A.** General view of C corpuscle showing wall and numerous electron-dense clumps contained within electron-lucent matrix. **B.** View of portion of a C corpuscle showing electron-dense clumps and some irregular membranous complexes, forming stacks and small vesicles. A finely thread-like or microgranular material, interpreted as glycosaminoglycans, can be seen in space around corpuscle. **C.** View of portion of another C corpuscle, showing zones of detachment of outer wall (white arrowheads). **D.** K corpuscle showing circular arrangements of amorphous material. **E.** K corpuscle with two cores lying close to euchromatic nucleus of a pyramidal cell. Abbreviations: am, amorphous material; c, electron-dense clumps; ca, circular arrangements; cr, core; m, electron-lucent matrix; mc, membranous complexes; nu, pyramidal cell nucleus; tg, thread-like and microgranular material; w, electron-dense wall. Scale bars: **A, D** = 2 μm; **B, C** = 1 μm; **E** = 5 μm.

variations of their rRNA content. In both species, the contents of C corpuscles are encased in an electron-dense wall of uniform thickness. In *L. gigas*, the partial degradation of C corpuscles by lysozyme digestion, accompanied by spreading of the corpuscular content, suggests that the electron-dense wall is digested by lysozyme and, thus, that peptidoglycans are an integral part of it, as is the case in the cell wall of Bacteria. Similar results have been obtained in C and K corpuscles from *P. canaliculata* (Dellagnola, 2015).

The contents of C corpuscles of *P. canaliculata* are surrounded by a membrane showing the typical lipid bilayer of plasma membranes (Koch *et al.*, 2006; Fig. 4B). The wall of C corpuscles of *L. gigas* is sometimes detached from their contents, suggesting the existence of a similar membrane, but this could not be clearly shown.

Absorption spectra of acetone extracts have suggested the occurrence of chlorophyll-like pigments in C corpuscles isolated from *P. canaliculata* (Castro-Vazquez *et al.*, 2002) and the main pigments have been identified as modified chlorophylls *a* and *b*,

and with no accompanying phycobilins (Vega *et al.*, 2012b). Similarly, the Prochlorales (now considered a polyphyletic group within the Cyanobacteria; e.g. Komárek, 2016) contain both chlorophylls *a* and *b* and no phycobilins. The thylakoid-lacking cyanobacterium *Gloeobacter violaceus*, which appears close to the Cyanobacteria/chloroplast divergence (Tomitani *et al.*, 1999), may readily integrate chlorophyll *b* in its photosystem I (Araki *et al.*, 2014). However, there is no evidence of the pigment's nature in corpuscles from *L. gigas*, except for their chlorophyll-like autofluorescence.

K corpuscles found in *L. gigas* are also remarkably similar to those described for *P. canaliculata* at the light and electron microscopic levels. They are associated with pyramidal cells, are large and mostly oval (26.5 ± 5.1 μm long), similar in size to those of *P. canaliculata*. Their multiple lamellae are organized around one to three cores in *L. gigas* and other Strombidae (this paper and Volland *et al.*, 2010a), while they are frequently organized around a single core in *P. canaliculata* (Koch *et al.*, 2006). The CYA361
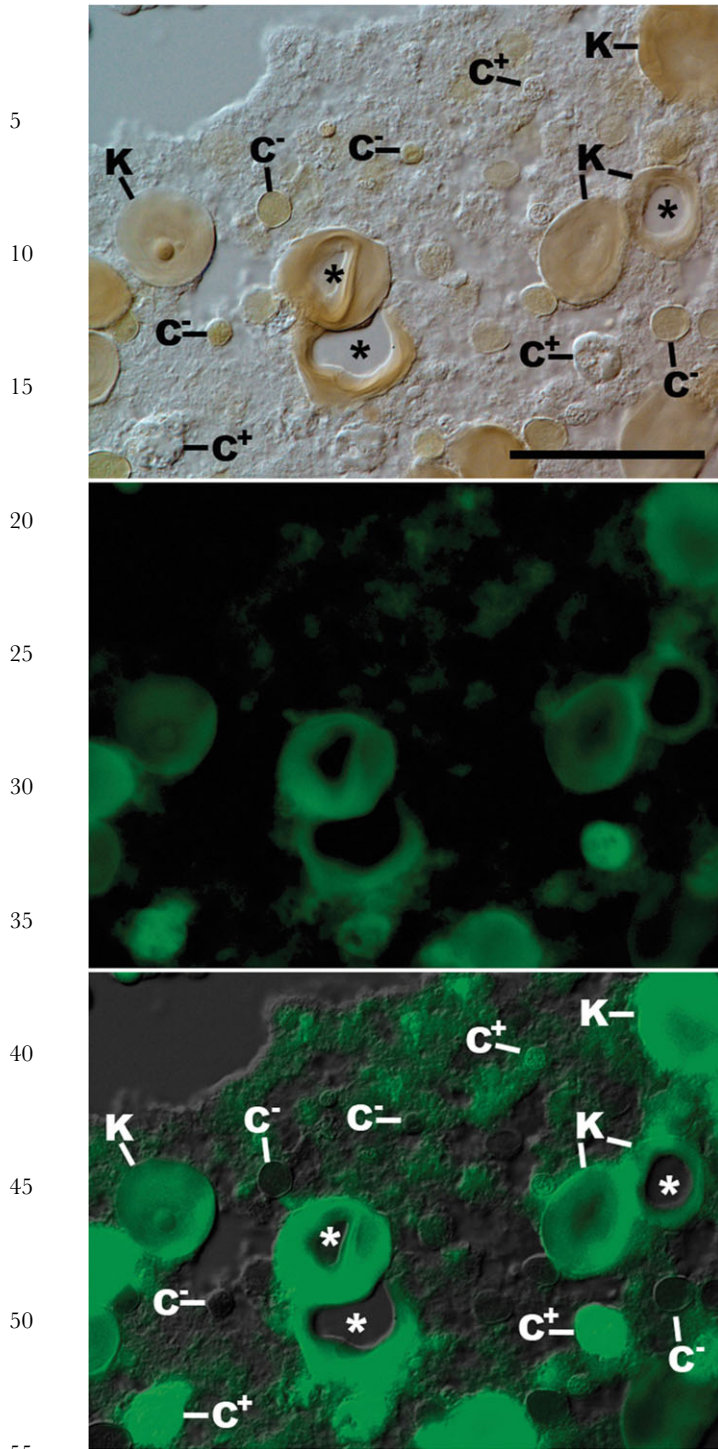
Figure 3. Fluorescent *in situ* hybridization with a generalized cyanobacterial probe for 16S rRNA (CYA361) in C and K corpuscles in a section across the basal region of the glandular epithelium of digestive gland of *Lobatus gigas*. **A**. Differential-interference contrast micrograph for topographic orientation. **B**. Fluorescence micrograph of same section, with representative corpuscles showing either negative (no label) or positive hybridization (green label). **C**. Merged **A** and **B** images. Abbreviations: C$^+$, hybridizing C corpuscles; C$^-$, nonhybridizing C corpuscles; K, hybridizing K corpuscles. Stars indicate where cores of K corpuscles were lost. Scale bar = 50 μm.

probe uniformly hybridizes in K corpuscles in both *L. gigas* (this paper) and *P. canaliculata* (Dellagnola, 2015). K corpuscles from both species are also sensitive to lysozyme digestion, which causes the spreading of the corpuscular contents, suggesting that peptido-glycans are a significant part of the multilamellar structure of K corpuscles. The pigments contained in K corpuscles have not been identified in either species, but acetone extracts of K corpuscles from *P. canaliculata* showed absorption spectra similar to those of chlorophylls (Castro-Vazquez *et al.*, 2002).

Several transitional forms between C and K corpuscles have been observed in *P. canaliculata*, leading to the suggestion that they were stages in the life cycle of the same prokaryotic organism (Castro-Vazquez *et al.*, 2002; Koch *et al.*, 2006). However, our limited material from *L. gigas* did not allow us to ascertain whether these transitional forms also occur in this species.

Identity of the intracellular corpuscles and their relation to the presumptive hosts

Earlier studies of Stromboidea and Ampullariidae considered the pigmented corpuscles as excretory bodies (Morton, 1951; Meenakshi, 1955; Andrews, 1965). More recent studies (e.g., Vega *et al.*, 2006; Gros *et al.*, 2009), however, have hypothesized that they are forms of a 'symbiont' (this term is here used, as in Vega *et al.*, 2006, and Hayes *et al.*, 2015, in the original broad sense of de Bary, 1879, which encompasses parasites, commensals and mutualistic symbionts).

In studies of the Ampullariidae, it has been hypothesized that the C corpuscles are a prokaryote akin to the Cyanobacteria, based on their appearance under light microscopy, the lack of a nucleus and the occurrence of an electron-dense wall (Castro-Vazquez *et al.*, 2002; Koch *et al.*, 2006; Vega *et al.*, 2006).

In studies of the Stromboidea, however, it was hypothesized that they were stages in the life cycle of a eukaryotic symbiont or, more precisely, an apicomplexan parasite (Baqueiro Cárdenas, Frenkiel & Aldana Aranda, 2007a; Baqueiro Cárdenas *et al.*, 2007b; Volland, Aldana Aranda & Gros, 2008; Gros *et al.*, 2009; Aldana Aranda *et al.*, 2010; Volland *et al.*, 2010a, 2010b). These authors later considered that the corpuscles might represent other forms of symbiotic associates (Volland *et al.*, 2008, 2010a, 2010b), mainly based on the fact that C and K corpuscles are present in all individuals of all studied populations of several Stromboidea and that they result in no apparent damage to the individual hosts or their reproduction, as for any mutualistic association.

The same universal presence of the symbiont has also been observed in the studied species of Ampullaridae (Castro-Vazquez *et al.*, 2002; Vega *et al.*, 2006), where no evidence of harm to the host was evident. Indeed, the symbiont of *P. canaliculata* may be serving roles in protein digestion (Godoy, Castro-Vazquez & Vega, 2013) and metal detoxification for the host (Vega *et al.*, 2012a).

The initial interpretation as excretory bodies (Morton, 1951; Meenakshi, 1955; Andrews, 1965) seems unlikely, in view of the electron-dense wall that envelops C corpuscles in both *L. gigas* and *P. canaliculata*. Also, C corpuscles are regular in size and larger than the residual bodies that have been reported in caenogastro-pods (Marigómez *et al.*, 2002; Ojeda, Arrighetti & Giménez, 2015), heterobranchs (Lobo-da-Cunha, 2000; Taïeb, 2001) and bivalves (Marigómez *et al.*, 2002; Dimitriadis, Domouhtsidou & Cajaraville, 2004). Even the largest heterolysosomes reported by Lobo-da-Cunha (2000) in the digestive gland of the heterobranch *Aplysia depilans*, though they approximate the size of C corpuscles of *L. gigas*, lack the electron-dense wall.

The proposed identification of K corpuscles as a member of the Apicomplexa (Baqueiro Cárdenas *et al.*, 2007a, 2007b; Volland *et al.*, 2008, 2010a, 2010b; Gros *et al.*, 2009; Aldana Aranda *et al.*, 2010) is not in agreement with (1) the failure of several TEM stud-ies, both of Ampullariidae (Koch *et al.*, 2006) and Strombidae (Volland *et al.*, 2008, 2010a; Gros *et al.*, 2009) to show nuclei, mitochondria, rough endoplasmic reticulum and the apical
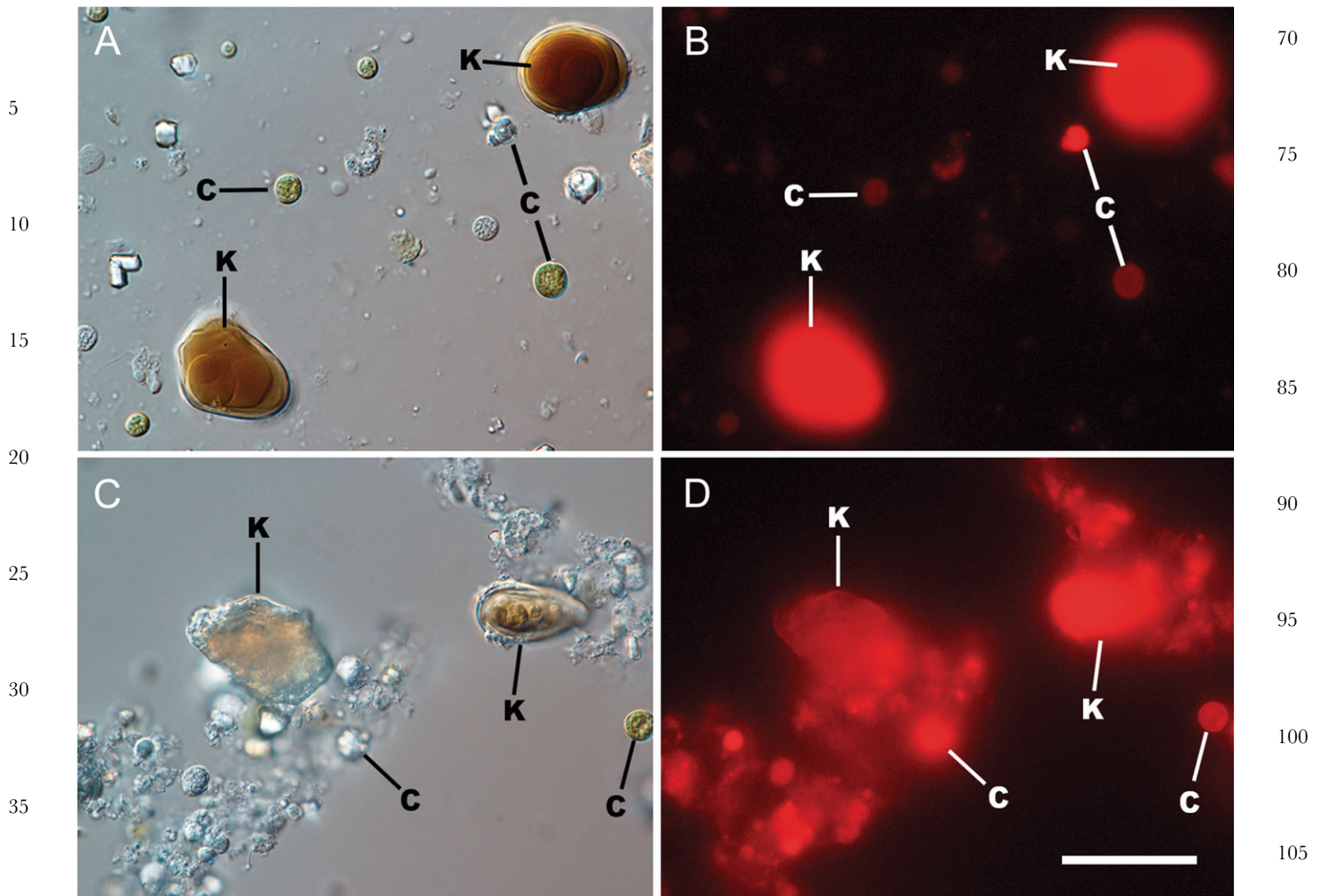
Figure 4. Effect of lysozyme digestion on C and K corpuscles in residue of fixation vials from digestive gland of *Lobatus gigas*. **A**. Suspension of C and K corpuscles that were not exposed to the enzyme (DIC microscopy). **B**. Same field as **A** showing autofluorescence of K corpuscles and weaker and variable autofluorescence of C corpuscles (fluorescence microscopy). **C**. Lysozyme-treated C and K corpuscles, showing partial lysis and aggregation with debris (DIC microscopy). **D**. Same corpuscles and aggregates as **C**, showing spreading of autofluorescent material into the medium (fluorescence microscopy). Abbreviations: C, C corpuscle; K, K corpuscle. Scale bars = 25 μm.

complex organelles, which characterize the Apicomplexa (Hu *et al.*, 2006; Dubremetz & Ferguson, 2009); (2) the *in situ* hybridization of K corpuscles of *L. gigas* with a 16S rRNA generalized cyanobacterial probe (this paper), but not with a generalized eukaryotic18S rRNA probe (Gros *et al.*, 2009); (3) the digestion of the envelope of K corpuscles of *L. gigas* by lysozyme, which agrees with a bacterial rather than an apicomplexan identity and (4) the evidence for chlorophyll-like pigments in K corpuscles from both species (Castro-Vazquez *et al.*, 2002; and this paper).

The apparent segregation of C and K corpuscles in columnar and pyramidal cells, respectively, is intriguing. Both these types of cells have been found in embryos of *M. cornuarietis* and *P. canaliculata*, even before the appearance of C and K corpuscles (Demian & Yousif, 1973; Koch, Winik & Castro-Vazquez, 2009). Further study is needed to determine if the apparent segregation means that two different prokaryote-like organisms are present, or if they represent two different forms or developmental stages of the same microorganism. Another intriguing aspect of the apparent segregation of C and K corpuscles in different gastropod cells is that, at least in *P. canaliculata*, K corpuscles are frequently contained within a membrane-delimited cytoplasmic band, which differs from the cytoplasm loaded with rough endoplasmic reticulum that is typical of the pyramidal cell (Koch *et al.*, 2006; Koch *et al.*, 2009). This clearly needs to be explored further, but it is possible that a K corpuscle develops after a pyramidal cell engulfs a protrusion of another cell, perhaps a columnar cell containing one or more C corpuscles.

CONCLUSIONS AND FUTURE RESEARCH

The evidence provided by this study supports the hypothesis that the symbiont or symbionts found in *L. gigas* are prokaryotic and related to the Cyanobacteria, but their exact phylogenetic position has yet to be determined. Similarly, the phylogenetic position of the symbiont of *P. canaliculata* is still uncertain (Vega *et al.*, 2006, 2012b; Hayes *et al.*, 2015).

Molecular studies in both Strombidae and Ampullariidae should be performed to establish the identity and phylogenetic relationships of the putative symbiont/s. Future research in Strombidae should also parallel the studies made in Ampullariidae regarding the possible functional advantages for the gastropod host (protein digestion, metal detoxification; Vega *et al.*, 2012a; Godoy *et al.*, 2013). Moreover, studies in

other species of Strombidae and Ampullariidae may shed light on functional aspects and the possible coevolution of these symbiotic associations.

REFERENCES

ALDANA ARANDA, D., FRENKIEL, L., BRULÉ, T., MONTERO, J. & BAQUEIRO CÁRDENAS, E. 2010. Occurrence of Apicomplexa-like structures in the digestive gland of *Strombus gigas* throughout the Caribbean. *Journal of Invertebrate Pathology*, **106**: 174–178.

ANDREWS, E.B. 1965. The functional anatomy of the gut of the prosobranch gastropod *Pomacea canaliculata* and of some other pilids. *Journal of Zoology*, **145**: 19–36.

ARAKI, M., AKIMOTO, S., MIMURO, M. & TSUCHIYA, T. 2014. Artificially acquired chlorophyll b is highly acceptable to the thylakoid-lacking cyanobacterium, *Gloeobacter violaceus* PCC 7421. *Plant Physiology and Biochemistry*, **81**: 155–162.

BAQUEIRO CÁRDENAS, E., FRENKIEL, L. & ALDANA ARANDA, D. 2007a. One more threat for the queen conch *Strombus gigas*: coccidian (Apicomplexa) infection of *S. gigas* digestive gland (preliminary results). *Proceedings of the Gulf and Caribbean Fisheries Institute*, **58**: 421–426.

BAQUEIRO CÁRDENAS, E., FRENKIEL, L., ZARATE, A. & ALDANA ARANDA, D. 2007b. Coccidian (Apicomplexa) parasite infecting *Strombus gigas* Linné, 1758 digestive gland. *Journal of Shellfish Research*, **26**: 319–321.

CASTRO-VAZQUEZ, A., ALBRECHT, E.A., VEGA, I.A., KOCH, E. & GAMARRA-LUQUES, C. 2002. Pigmented corpuscles in the midgut gland of *Pomacea canaliculata* and other Neotropical apple-snails (Prosobranchia, Ampullariidae): a possible symbiotic association. *Biocell*, **26**: 101–109.

DE BARY, A. 1879. *Die Erscheinung der Symbiose*. Verlag von Karl J. Trübner, Strasbourg.

DELLAGNOLA, F.A. 2015. *Estudio comparativo de los corpúsculos pigmentarios de la glándula digestiva de tres especies de ampuláridos*. Ph.D. thesis, Universidad Nacional de Cuyo, Mendoza.

DEMIAN, E.S. & YOUSIF, F. 1973. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda, Ampullariidae). II. Development of the alimentary system. *Malacologia*, **12**: 151–174.

DIMITRIADIS, V.K., DOMOUHTSIDOU, G.P. & CAJARAVILLE, M.P. 2004. Cytochemical and histochemical aspects of the digestive gland cells of the mussel *Mytilus galloprovincialis* (L.) in relation to function. *Journal of molecular histology*, **35**: 501–509.

DUBREMETZ, J.F. & FERGUSON, D.J.P. 2009. The role played by electron microscopy in advancing our understanding of *Toxoplasma gondii* and other apicomplexans. *International journal for parasitology*, **39**: 883–893.

GODOY, M.S., CASTRO-VAZQUEZ, A. & VEGA, I.A. 2013. Endosymbiotic and host proteases in the digestive tract of the invasive snail *Pomacea canaliculata*: Diversity, origin and characterization. *PLoS One*, **8**: e66689.

GROS, O., FRENKIEL, L. & ALDANA ARANDA, D. 2009. Structural analysis of the digestive gland of the queen conch *Strombus gigas* Linnaeus, 1758 and its intracellular parasites. *Journal of Molluscan Studies*, **75**: 59–68.

HAYES, K.A., BURKS, R.L., CASTRO-VAZQUEZ, A., DARBY, P.C., HERAS, H., MARTÍN, P.R., QIU, J.-W., THIENGO, S.C., VEGA, I.A., WADA, T., YUSA, Y., BURELA, S., CADIERNO, M.P., CUETO, J.A., DELLAGNOLA, F.A., DREON, M.S., FRASSA, M.V., GIRAUD-BILLOUD, M., GODOY, M.S., ITUARTE, S., KOCH, E., MATSUKURA, K., PASQUEVICH, M.Y., RODRIGUEZ, C., SAVEANU, L., SEUFFERT, M.E., STRONG, E.E., SUN, J.,

TAMBURI, N.E., TIECHER, M.J., TURNER, R.L., VALENTINE-DARBY, P.L. & COWIE, R.H. 2015. Insights from an integrated view of the biology of apple snails (Caenogastropoda: Ampullariidae). *Malacologia*, **58**: 245–302.

HU, K., JOHNSON, J., FLORENS, L., FRAUNHOLZ, M., SURAVAJJALA, S., DILULLO, C., YATES, J., ROOS, D.S. & MURRAY, J.M. 2006. Cytoskeletal components of an invasion machine – the apical complex of *Toxoplasma gondii*. *PLoS Pathogens*, **2**: e13.

KOCH, E., VEGA, I.A., ALBRECHT, E.A., ORTEGA, H. & CASTRO-VAZQUEZ, A. 2006. A light and electron microscopic study of pigmented corpuscles in the midgut gland and feces of *Pomacea canaliculata* (Caenogastropoda: Ampullariidae). *Veliger*, **48**: 17–25.

KOCH, E., WINIK, B.C. & CASTRO-VAZQUEZ, A. 2009. Development beyond the gastrula stage and digestive organogenesis in the apple-snail *Pomacea canaliculata* (Architaenioglossa, Ampullariidae). *Biocell*, **33**: 49–65.

KOMÁREK, J. 2016. Review of the cyanobacterial genera implying planktic species after recent taxonomic revisions according to polyphasic methods: state as of 2014. *Hydrobiologia*, **764**: 259–270.

LEYDIG, F. 1850. Über Paludina vivipara. Ein Beitrag zur näheren Kenntniss dieses Thieres in embryologischer, anatomischer und histologischer Beziehung. *Zeitschrift für wissenschaftliche Zoologie*, **2**: 125–197.

LOBO-DA-CUNHA, A. 2000. The digestive cells of the hepatopancreas in *Aplysia depilans* (Mollusca, Opisthobranchia): ultrastructural and cytochemical study. *Tissue and Cell*, **32**: 49–57.

LONGIN, A., SOUCHIER, C., FFRENCH, M. & BRYON, P.A. 1993. Comparison of anti-fading agents used in fluorescence microscopy: image analysis and laser confocal microscopy study. *Journal of Histochemistry & Cytochemistry*, **41**: 1833–1840.

MACMUNN, C.A. 1883. Observations on the colouring-matters of the so-called bile of Invertebrates, on those of the bile of Vertebrates, and on some unusual Urine Pigments, &c. *Proceedings of the Royal Society of London*, **35**: 370–403.

MARIGÓMEZ, I., SOTO, M., CAJARAVILLE, M.P., ANGULO, E. & GIAMBERINI, L. 2002. Cellular and subcellular distribution of metals in molluscs. *Microscopy research and technique*, **56**: 358–392.

MEENAKSHI, V.R. 1955. The excretory spherioles in the digestive gland of *Pila virens*. *Journal Animal Morphology and Physiology (Bombay)*, **3**: 75–78.

MORTON, J.E. 1951. The ecology and digestive system of the Struthiolariidae (Gastropoda). *Journal of Cell Science*, **3**: 1–25.

OJEDA, M., ARRIGHETTI, F. & GIMÉNEZ, J. 2015. Morphology and cyclic activity of the digestive gland of *Zidona dufresnei* (Caenogastropoda: Volutidae). *Malacologia*, **58**: 157–165.

PEREIRA, S., ZILLE, A., MICHELETTI, E., MORADAS-FERREIRA, P., DE PHILIPPIS, R. & TAMAGNINI, P. 2009. Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. *FEMS Microbiology Reviews*, **33**: 917–941.

SCHÖNHUBER, W., ZARDA, B., EIX, S., RIPPKA, R., HERDMAN, M., LUDWIG, W. & AMANN, R. 1999. In situ identification of Cyanobacteria with horseradish peroxidase-labeled, rRNA-targeted oligonucleotide probes. *Applied and Environmental Microbiology*, **65**: 1259–1267.

STEEDMAN, H.F. 1950. Alcian Blue 8GS: a new stain for mucin. *Quarterly Journal of Microscopical Science*, **3**: 477–479.

TAÏEB, N. 2001. Distribution of digestive tubules and fine structure of digestive cells of *Aplysia punctata* (Cuvier, 1803). *Journal of Molluscan Studies*, **67**: 169–182.

TOMITANI, A., OKADA, K., MIYASHITA, H., MATTHIJS, H.C., OHNO, T. & TANAKA, A. 1999. Chlorophyll b and phycobilins in the common ancestor of cyanobacteria and chloroplasts. *Nature*, **400**: 159–162.

VEGA, I.A., ARRIBÉRE, M.A., ALMONACID, A.V., RIBEIRO GUEVARA, S. & CASTRO-VAZQUEZ, A. 2012a. Apple snails and their endosymbionts bioconcentrate heavy metals and uranium from contaminated drinking water. *Environmental Science and Pollution Research*, **19**: 3307–3316.

VEGA, I.A., DAMBORENEA, M.C., GAMARRA-LUQUES, C., KOCH, E., CUETO, J.A. & CASTRO-VAZQUEZ, A. 2006. Facultative and obligate symbiotic associations of *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). *Biocell*, **30**: 367–375.

VEGA, I.A., DELLAGNOLA, F.A., HURST, J.A., GODOY, M.S. & CASTRO-VAZQUEZ, A. 2012b. A study of chlorophyll-like and phycobilin pigments in the C endosymbiont of the apple-snail *Pomacea canaliculata*. *Biocell*, **36**: 47–55.

VOLLAND, J.-M., ALDANA ARANDA, D. & GROS, O. 2008. Detection of Apicomplexa like parasites in two species belonging to the family Strombidae: *Strombus gallus*, Linnaeus, 1758 and *S. raninus*, Gmelin, 1791. *Proceedings of the Gulf and Caribbean Fisheries Institute*, **61**: 503–505.

VOLLAND, J.-M., FRENKIEL, L., ALDANA ARANDA, D. & GROS, O. 2010a. Occurrence of Sporozoa-like microorganisms in the digestive gland of various species of Strombidae. *Journal of Molluscan Studies*, **76**: 196–198.

VOLLAND, J.-M., GROS, O., FRENKIEL, L. & ALDANA ARANDA, D. 2010b. Apicomplexan parasite in the digestive gland of various species of the family Strombidae: *Strombus costatus*, *S. gigas*, and *S. pugilis*. *Proceedings of the Gulf and Caribbean Fisheries Institute*, **62**: 430–432.