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Oogenesis in *Phragmatopoma* (Polychaeta: Sabellariidae): Evidence for morphological distinction among geographically remote populations

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Abstract

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The Southwest Atlantic Ocean sand-reef building polychaete, *Phragmatopoma lapidosa*, was recently synonymised with *Phragmatopoma caudata* based on morphological characters. This study uses histochemical and ultrastructural procedures to describe oogenesis in *Phragmatopoma caudata* from the Southwest (SW) Atlantic and make a comparison with previously published data for the Northwest Atlantic (NW) forms. In the South American worms, the exposed ovary consists of simple groups of oogonia attached to blood vessels, unlike the NW Atlantic worms in which only the proliferative and previtellogenesis phases of the oocytes are associated with blood vessels. In SW Atlantic worms, the oocytes float in the coelom during the vitellogenic phase. We discovered several heterogeneous features (e.g., cell extensions, amoeboid cells, ovary capsule, active uptake of material from blood vessels and egg envelope) that can be used to distinguish between North and South Hemisphere populations of *P. caudata*. In light of the observed divergence between worms from these separated populations, our findings support reproductive plasticity. The present study reveals biodiversity within sand-reef making sandcastle worms.

Keywords

ultrastructure, histochemistry, reproductive biology, ovary,geographic plasticity, histology, benthic invertebrates, worm reefs.

Introduction

The type-locality of *Phragmatopoma lapidosa* Kinberg, 1866, is Rio de Janeiro, Brazil. The species was synonymised with *P. caudata* Krøyer in Mörch (1863) described from a population in the North Atlantic Ocean (Kirtley, 1994). The same author synonymised *P. moerchi* Kinberg, 1867, *P. digitata* Rioja, 1962 and *P. peruensis* Hartman, 1944 with *P. virgini* Kinberg, 1867 (Kirtley, 1994), demonstrating a different approach to species discrimination in the genus. Systematic studies among *Phragmatopoma* spp. have focused on characters of the anterior region of the body, especially the modified opercular paleae (Amaral, 1987, Hartman, 1944, Kinberg, 1867, Kirtley, 1994, Mörch, 1863). However, there is no concise comparative morphological study on these structures documenting clear variability among and within various *Phragmatopoma* species. Probably because of this, the taxonomy of *Phragmatopoma* spp. is incomplete and imprecise. Several species of sabellariids appear to have been described inadequately and redescription seems to be needed (Kirtley, 1994).

In the past, it was assumed that different strategies of reproductive biology were reliable for delineating species. Therefore, oogenesis and ovary structure in several polychaetes were reviewed, and a summary phylogeny was proposed (Eckelbarger, 1983, 1984, 2005, 2006). Light microscopy and ultrastructural studies of ovary morphology and development were carried out on several species from different polychaete families representing a wide-spectrum sampling of reproductive biology. Among the different species of *Capitella* Blainville, 1828, specific types of yolk precursors and metabolites were uptake by the oocyte during vitellogenesis suggesting variation in the egg envelope. Differentiation of the oocyte occurs

following separation from the follicle cells (Eckelbarger and Grassle, 1983). Studies of oogenesis (Eckelbarger, 1979) and larvae (Eckelbarger, 1976, Eckelbarger and Chia, 1978, McCarthy *et al.,* 2002) in *Phragmatopoma lapidosa* (syn. *P. caudata*) of the Northwest Atlantic Ocean described the regional pattern of reproduction and development. Eckelbarger (1976) observed for North American *Phragmatopoma* the presence of gametes during all months of the year, although seasonal variability in egg number can occur (McCarthy *et al.,* 2003).

Studies of comparative gametogenesis can be helpful in elucidating and testing hypotheses about biogeography and the evolution of a taxon. Studies of oogenesis may be useful for generating phylogenetic hypotheses based on characteristics such as: (1) the presence, number, and location of definable ovaries; (2) the existence of extraovarian versus intraovarian oogenesis; (3) the release of previtellogenic oocytes into the coelom as solitary cells or in clusters; (4) mechanism of vitellogenesis; (5) the presence or absence of accessory cells; (6) the morphology of the egg envelope; and (7) the structure of the yolk pellet (Eckelbarger, 1988, 2006). In the present case, there are 12 genera and over 130 species of sabellariid worms (Read and Fauchald, 2012). Species of sabellariids can be solitary or gregarious and occur from continental shallow waters to continental shelf and slope depths. Morphological studies have elucidated the basal and derived genera within sabellariids (Capa *et al*., 2012, Dales, 1952). Moreover, Capa *et al*., (2012) have not found any phylogenetic relationship between some of the genera that construct colonies and reefs (e.g. *Phragmatopoma*, *Sabellaria*, *Gunnarea*).

Oogenesis has been studied in only a few *Phragmatopoma* and *Sabellaria* species and little progress in studies of sandcastle worm reproductive biology has been achieved in recent years (Culloty *et al.,* 2010). It would be most helpful if information about reproductive biology (Eckelbarger, 1988, 2006) was included in the recent phylogenetic of sabellariids (Capa *et al*., 2012) and further studies should assess whether the pattern of oogenesis is consistent with previous phylogenetic studies published for the family.

The objective of this study is to describe the oogenesis of *Phragmatopoma caudata* from the southeast of the Brazilian coast using the histochemistry and transmission electron microscopy. For the first time, we provide a cytochemical description of oogenesis for Brazilian sabellariids. In addition, we compare consistencies in reproductive characteristics between South and North American *Phragmatopoma* spp. (Eckelbarger, 1979, Eckelbarger and Chia, 1978).

Material and methods

Sampling. *Phragmatopoma caudata* were collected at the Itararé beach at São Vicente, São Paulo State, Brazil (23°58'49"S; 46°22'02"W), during low tide in September 2009. Based on previous fieldwork data, it was known the specimens were carrying gametes in the sampling month, which is the beginning of the spring season (Faroni-Perez pers. obs.). In the laboratory, the specimens were removed from the sand tubes, anaesthetised by thermal shock with cold (4°C) sea-water and sexed. The length of the opercular crown (ventro-dorsal) was measured and only mature females were used, since the size ranged from 1.72 to 2.66 mm (Faroni-Perez, 2014).

Histology. Intact female worms (N=5) were fixed in 4% paraformaldehyde prepared with water from the sampling site containing sodium phosphate buffer 0.2 M (pH 7.2) for 24 hours at 4°C. After fixation, materials were rinsed in the same buffer (twice for 30 min), dehydrated in an ethanol series (70-95%), and embedded in methacrylate resin Leica®. Serial sections of 5 to 8 μm were obtained by Leica RM2252 microtome. Haematoxylin and eosin staining was proceeded according to Junqueira and Junqueira (1983) avoiding ethanol and xylene bath (Sant'Anna *et al*., 2010) and used for traditional histological description. Histological images were acquired by a Leica DM2000 photomicroscope and digitised using the Leica IM50 software.

Cellular measurements. Cell measurements were obtained using the Leica IM50 software with appropriate system calibrations. All oocytes were measured using the 20X objective, and slides were stained by haematoxylin and eosin (HE). The largest cell diameter was obtained using only oocytes showing both a clear nucleus and a prominent nucleolus. Oocyte measurements were performed on five individuals with different operculum lengths and representing adult specimens. For each stage of oogenesis, the average oocyte diameters were obtained from ten cells per individual.

Histochemistry. Xylidine ponceau (Mello and Vidal, 1980) and mercuric-bromophenol blue staining techniques (Pearse, 1985) were used to demonstrate the presence of total protein. The Periodic acid-Schiff (PAS) technique was used to identify neutral polysaccharides with groups 1-2 glycol (Junqueira and Junqueira 1983; Pearse, 1985). The Alcian blue technique (pH 1.0 and 2.5) was chosen to demonstrate acidic polysaccharides (Junqueira and Junqueira, 1983), and Sudan Black B was used to determine the total lipids according Leica® protocol (Zara *et al.,* 2012).

Ultrastructure. For transmission electron microscopy (TEM), individual *Phragmatopoma caudata* (N=5) were fixed in 3% glutaraldehyde and 0.1 M (pH 7.3) sodium cacodylate buffer in filtered seawater (CBSF) for 4 hours and, post-fixed in 1% osmium tetroxide in the same buffer for 1 hour at 4°C. The "en bloc staining" was carried out using 1% aqueous uranyl acetate. The materials were dehydrated in ascending acetone series (50-100%) and embedded in Epon-Araldite resin. The ultrathin sections were stained with uranyl acetate and lead citrate and photographed in the Philips CM100 transmission electron microscopy at 80Kv electron beam.

Results

Histology and Histochemistry. The transverse dimension (ventral to dorsal) of the operculum of the analysed specimens ranged from 1.72 to 2.66 mm. The ovaries were clearly definable and closely associated to blood vessels of the intersegmental septa of *Phragmatopoma caudata* (fig. 1). In the ovary, the oogonia and oocytes were not surrounded by follicle cells (fig. 1). The oogonia, previtellogenic and early vitellogenic oocytes were attached to each other (fig. 1) until they were

released into coelom, where only late vitellogenic oocytes were observed (fig. 2).

The proliferative phase was marked by oogonia during mitosis, with a basophilic cytoplasm and an average diameter of 8.8 ± 2.2 μm. Three growth oocyte stages were classified: including oocytes in previtellogenesis (31.8 ± 5.3 μm) and early vitellogenesis (77.6 ± 9.4 μm), which were attached to the ovary, and late vitellogenesis that occurs in cells free in the coelom (100.4 ± 13.0 μm) (figs. 1 and 2).

The oogonia were smaller than the oocytes which were characterised by a large nucleus showing different stages of meiotic prophase and basophilic cytoplasm. Cell extensions connecting oogonia and blood vessels were noticeable (fig. 1). The previtellogenic oocytes showed a large nucleus and one or more nucleolus. Their cytoplasm was strongly basophilic without granules. Contrasting to the previtellogenic cell, the early vitellogenic oocytes were showed only one nucleolus in their large nucleus and the cytoplasm showed some acidophilic yolk granules (fig. 1). During late vitellogenesis, the mature or late vitellogenic oocytes occupied the entire coelomic cavity without direct contact to blood vessels (fig. 2). These oocytes were large and filled with yolk basophilic granules with less affinity to eosin than those in the previous stage (fig. 2). The basophilic vitelline envelope was clearly observed (fig. 2). There were no additional or follicular cells associated with these germ cells.

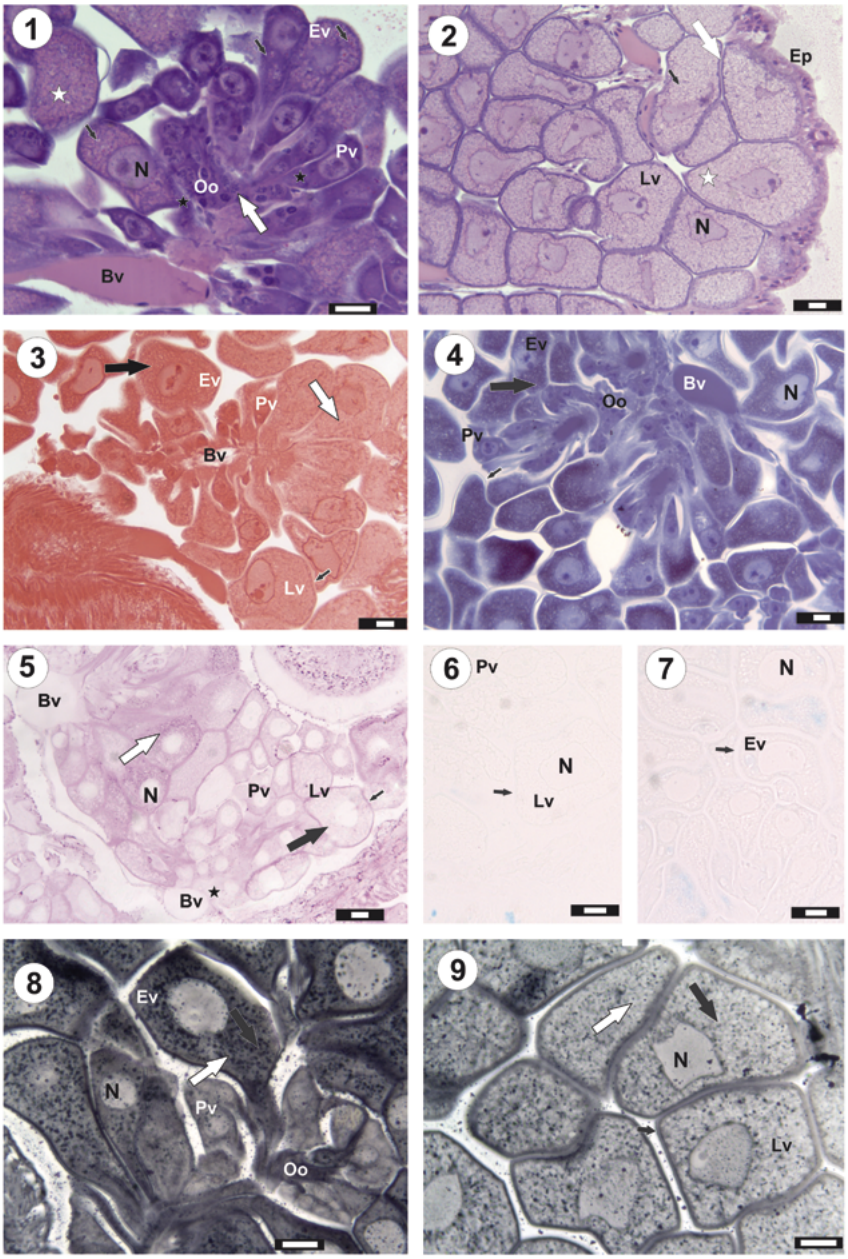
Histochemical analysis using xylidine ponceau (fig. 3) and mercuric-bromophenol blue (fig. 4) revealed that the cytoplasm of oogonia and previtellogenic oocytes were positively stained for proteins. During early vitellogenesis, the oocyte yolk granules, as well the egg envelope, were highly reactive compared to oocytes in late vitellogenesis (figs. 3 and 4). The oogonia were stained slightly for polysaccharides. However, the previtellogenic oocytes exhibited a slight cytoplasmic response to polysaccharides containing 1-2 glycol groups, such as glycogen (fig. 5). The oocytes in early vitellogenesis showed both cytoplasm and yolk granules that were slightly reactive to PAS. The oocytes in late vitellogenesis displayed a negative reaction to yolk granules and positive marks in the egg envelopes, indicating the glycoprotein constitution (fig. 5). The oogonia and oocytes at different stages were negative to Alcian blue tests (pH 1.0 and 2.5) for acidic polysaccharides (figs. 6 and 7). The oogonia were negative for lipid droplets, as stained by Sudan black B (fig. 8). In previtellogenic oocytes, some sparse lipid droplets were stained. The oocytes in early vitellogenesis showed qualitatively more lipid droplets than those in late vitellogenesis, which had several sparse lipid droplets. The yolk granules were negative to Sudan black B (fig. 9).

Ultrastructure

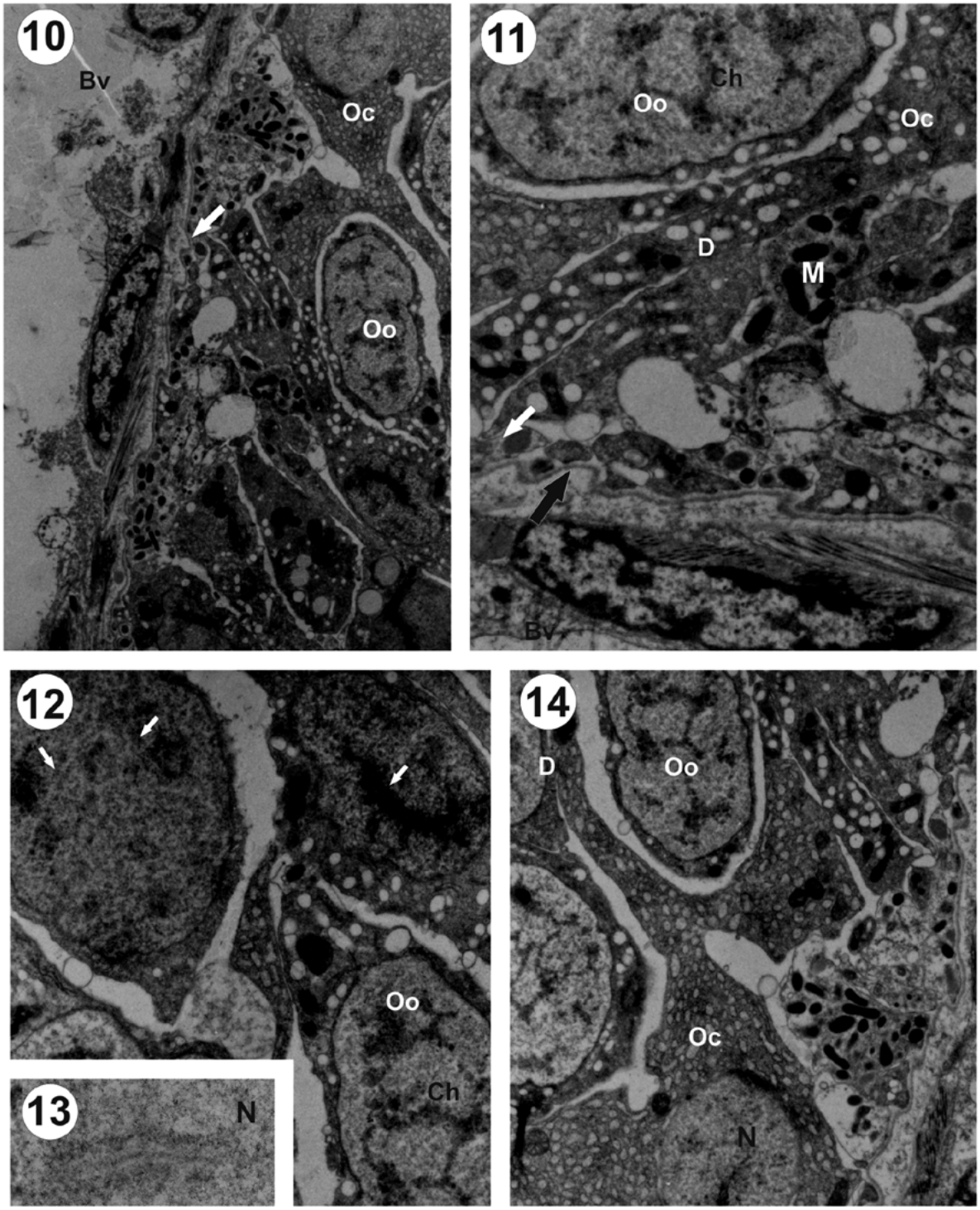
Proliferative phase. The ovary structure showed oogonia and oocytes. Oogonia connected to blood vessels via cellular prolongations to the flat endothelium (fig. 10). Between the germinative cells and the blood vessel occurred by a thin layer of connective tissue (figs. 10 and 11) showing collagen-like fibres contrasting with the electron-dense region of the ovary basal lamina (figs. 11). The oogonia, as well as the oocytes were connected via desmosomes (fig. 11). Differentiation between the oogonia and oocytes was indicated by the presence of mitotic chromosomes on an elliptical oogonia nucleus (figs. 10, 11, 12, and 14). The oocytes showed a rounded nucleolus, and the nucleoplasm contained dispersed chromatin and meiotic synaptonemal complexes (figs. 12-14). The oogonium cytoplasm was narrow and contained electron-dense mitochondria and a few vesicles of rough endoplasmic reticulum (RER) (figs. 10, 11, 12, and 14). The oocytes depicted had the same cytoplasmic characteristic, although cytoplasm was larger than in oogonia and had well-developed vesicular RER (figs. 10, 11, 14).

Growth phase. After meiosis, the previtellogenic oocytes increased both in cytoplasmic and nuclear volumes. The nucleus showed a single, large nucleolus and scattered heterochromatin blocks (figs. 15 and 16). Previtellogenic oocytes were elongated with a rounded, coelomic distal end. The plasma membrane maintained contact with other oocytes in prophase or previtellogenesis near the coelomic distal end (fig. 15). The cytoplasm was filled by RER consisting of parallel lamellae (fig. 16). A few electron-dense mitochondria, with shapes ranging from spherical to ellipitical, were common in the perinuclear cytoplasm (figs. 15-17). Accumulations of electron-dense α-glycogen were scattered in the cytoplasm (fig. 17), in agreement with the PAS-positive stains (fig. 5). The rounded end of the previtellogenic oocytes delineated a free margin in contact with the coelomic cavity characterised by the microvilli and thin egg envelope (figs. 18-21). The cortical cytoplasm showed many Golgi complexes, and several cortical granules were nearby (fig. 18). The cortical granules were filled with fibrous material of different electron densities (figs. 18-21). Among the small microvilli occurs the matrix of the egg membrane, which is granular while the basal region was electron-lucent and forming the wide perivitelline space at this phase (figs. 19-21). The microvilli apex, above the egg envelope, showed an expansion bearing extensive filamentous adornment with an electron-dense central region (figs. 19 and 20). The end of the previtellogenesis was determined by the beginning of endocytotic activity marked by pits in the plasma membrane and presence of coated vesicles in the cortical cytoplasm (figs. 20 and 21) at the same time that the yolk granules arose.

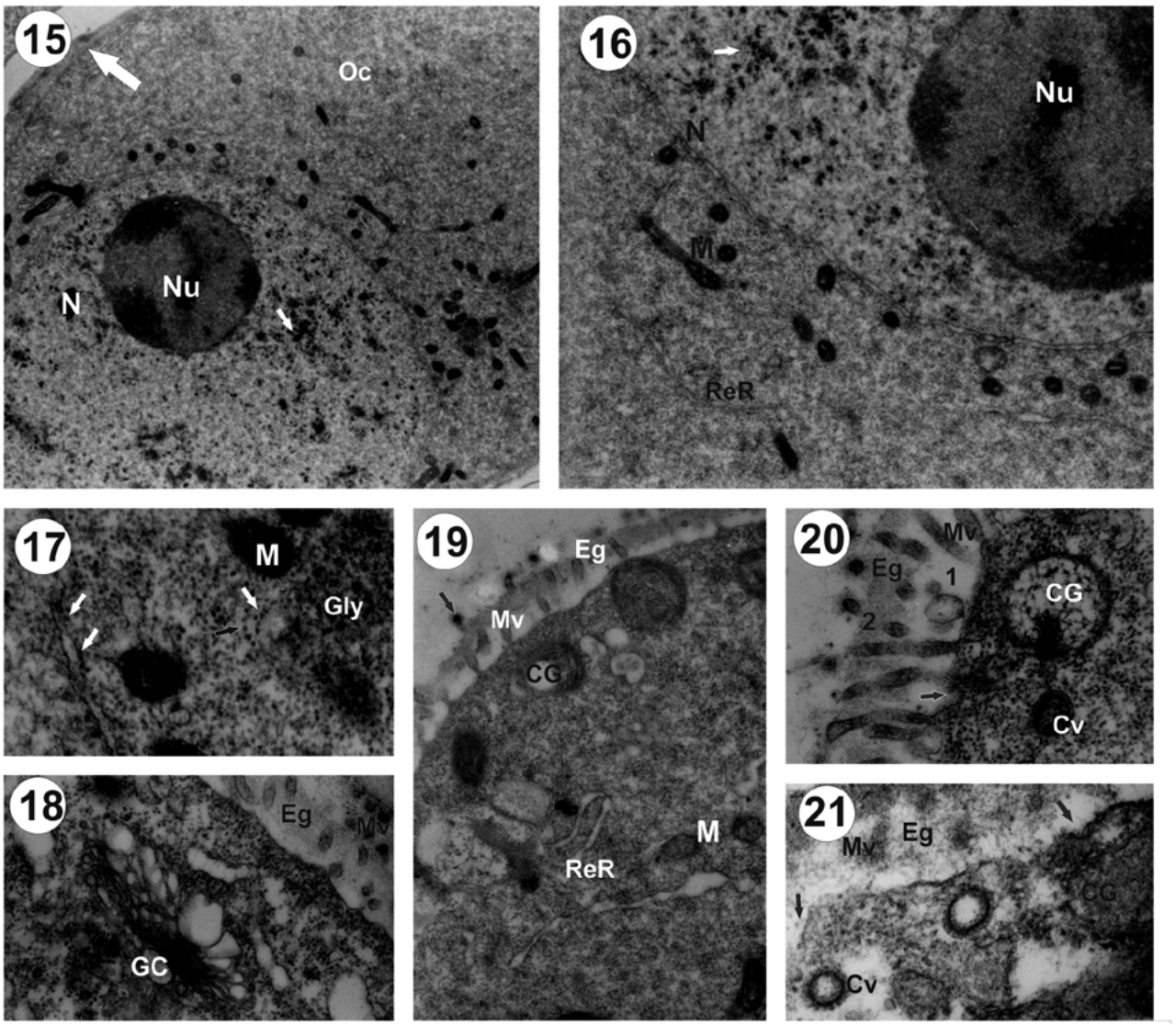
During vitellogenesis or exogenous phase of yolk production, two distinct ultrastructural oocyte stages were observed (*i.e*. oocytes in early and late stage of vitellogenesis). During early vitellogenesis, the oocytes were attached to the ovarian blood vessel and the nucleus showed the same characteristics as the previtellogenic oocytes, with many scattered heterochromatin blocks (figs. 22 and 23). The large number of nuclear pores were indicative of the high nuclear activity during the early vitellogenesis (fig. 23). Clusters of granular electron-dense material, or nuages, were observed next to the nuclear envelope and perinuclear cytoplasm (fig. 23). The cytoplasm was filled with lamellar rough ER, several lipid droplets, and small yolk granules (figs. 22-25). The yolk granules were rounded, compact and showed areas with varied electron densities (figs. 24 and 25). Inside the yolk granules, some electron-lucent spherical areas were visible. (fig. 24). Particles of α-glycogen were adjacent to the yolk granules (fig.



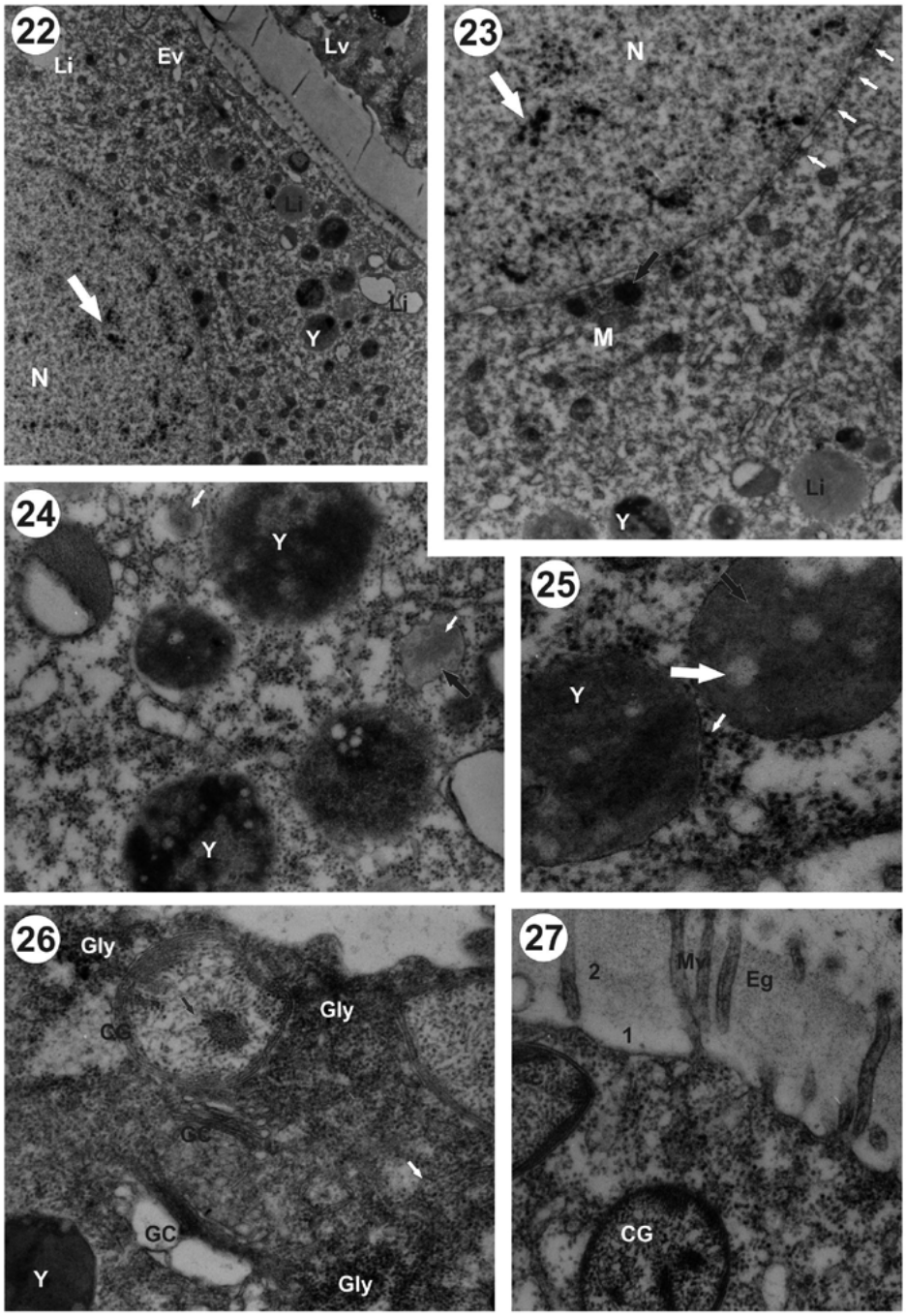
Figures 1–9. Histology and Histochemistry in *Phragmatopoma caudata* of the SW Atlantic. Figures 1 and 2 Oogonia (Oo) and previtellogenic oocytes (Pv) of early vitellogenesis (Ev) associated with the intersegmental blood vessels (BV) for cytoplasmic prolongations (black star). Oocytes in advanced and late vitellogenesis are released into the coelomic cavity (white star). Oocytes in vitellogenesis have intensely acidophilic granules, and the ripe oocytes are less acidophilus (black arrows). Figure 2 depicts oocytes in late vitellogenesis occupying the entire coelom with a basophils vitelline envelope (white arrow). N = nucleus; H&E staining. Scales = 20 μm. **Figures 3 and 4** Techniques used to visualize basic and total proteins, respectively. The previtellogenic and vitellogenesis (Ev) oocytes (Pv) have intense granules (large black arrow), and the ripe in late vitellogenesis (Lv) are less reactive (white arrow). The vitelline envelope is reactive to protein (small arrow). Scales = 20 μm. **Figure 5** Technique for visualizing neutral polysaccharides with positive staining in both the previtellogenic oocyte cytoplasm and the vitellogenic oocyte granules. The reactivity disappears in the vitellogenic granules of ripe oocytes, but surrounding these is a noticeable positive staining (large black arrow). Star = oogonia with little reactivity to neutral polysaccharides. The egg envelope is reactive to PAS (small arrow). Scale = 20 μm. **Figures 6 and 7** Absence of acid polysaccharides (pH 1.0 and 2.5), respectively, in oogonia and oocytes in *P. caudata*. Scales = 20 μm. **Figures 8 and 9** Lipids stained by Sudan Black B. The oogonia are uniformly positive (small white arrow), while the oocytes during previtellogenesis and vitellogenesis have droplets in the cytoplasm (large black arrow). The yolk granules are reactive during vitellogenesis, while the staining is less intensive (large white arrows) in the mature oocytes. The vitelline envelopes have lipids (small black arrow). Scales = 20 μm.



Figures 10–14. Ultrastructure in *Phragmatopoma caudata* of the SW Atlantic. Proliferative Phase. Figures 10 and 11 Oogonia (Oo) and oocytes (Oc) connected via cellular prolongations (white arrow) to the endothelium of the intersegmental blood vessel (Bv). Note that the contact of ovary basal lamina is quite electron dense (black arrow). Desmosomes (D) form the oocyte-oocyte junction (Figure 10, 1,150X; Figure 11, 2,050X). M = mitochondria; Ch = chromosome. **Figure 12** Oogonia with mitotic chromosomes (Ch) and oocytes in prophase with finely granular chromatin and chromosomes united by synaptonemal complexes (arrows) (2,400X). **Figure 13** Synaptonemal complex (33,600X). N = nucleus. **Figure 14** Narrow cytoplasm in oogonia (Oo) androunded nucleus. Oocyte (Oc) with wide cytoplasm and adhesion via desmosomes (D) (2,050X).



Figures 15-21. Ultrastructure in *Phragmatopoma caudata* of the SW Atlantic. Grow Phase. Figures 15 and 16 Previtellogenic oocyte with a large nucleus (N) and nucleolus (Nu), as well as small rough heterochromatin clumps (small arrow). The cytoplasm is filled with rough, lamellar endoplasmic reticulum (RER) and noticeable mitochondria (M) in the perinuclear cytoplasm. The elongated portions of these cells create adhesion with adjacent oocyte (large arrow) (Figure 15, 2,050X. Figure 16, 4,200X). Oc = oocyte. **Figure 17** Cytoplasm showing glycogen α (Gly) greater than the ribosomes (white arrows) attached to the reticulum (black arrow) (13,500X). M = mitochondria. **Figures 18 and 19** Cortical cytoplasm of the rounded surface, showing Golgi complexes (GC) close to the cortical granules (CG), positioned beneath the microvilli (Mv) with the expansion bearing extensive filamentous adornment above the vitelline envelope (Eg) (Figure 18, 10,500x. Figure 19, 5,400X). **Figures 20 and 21** Plasma membrane on a rounded surface showing endocytic depressions (black arrow) and coated vesicles (Cv). The vitelline envelope is composed by means of medium-apical extracellular matrix (2) in relation to microvilli (Mv). The basal region is electron-lucent and forming the beginnings of the perivitelline space (1) (Figure 20. 13,500X. Figure 21, 28,000X). GC = Golgi complexes, Eg = vitelline envelope.



Figures 22-27. Ultrastructure in *Phragmatopoma caudata* of the SW Atlantic. Grow Phase. Figure 22 Oocyte during early vitellogenesis (Ev) with attributes of several yolk granules (Y) and lipid droplets (Li) in the cytoplasm, adjacent to an oocyte in late vitellogenesis (Lv) which vitelline envelope (Eg) thick (1,450X). N = nucleus, arrow = clumps of heterochromatin. **Figure 23** Nucleus showing the heterochromatin clumps (large white arrow) and many complex pores in the nuclear envelope (small white arrows). The perinuclear cytoplasm aspect of granular material accumulations, juxtaposed with the nuclear envelope (black arrow) and next to mitochondria (M) (2,050X). **Figure 24** Small and rounded yolk granules (Y) with different electron-densities and lucid spheres are observed inside. Besides Vesicles containing some electron-dense material (white arrow) also have lucid spheres (black arrow) and resemble nascent yolk granules (8,200X). **Figure 25** Yolk granules (Y) of oocytes at the beginning of vitellogenesis showing varied electron-densities (black arrow) and electron-lucent spheres (large white arrow). Glycogen is also noticed (small white arrow) (21,500X). **Figure 26** Cortical cytoplasm showing Golgi complexes (GC) close to the cortical granules (CG), which are filled with a fibrous material (black arrow). Note the large amount of glycogen (Gly) in the cytoplasm (1,150X). White arrow = glycogen. **Figure 27** Microvilli (Mv) are larger and the egg envelope (Eg) thicker (2), relative to the previous stage. The perivitelline space (1) appears thinner (1500X).

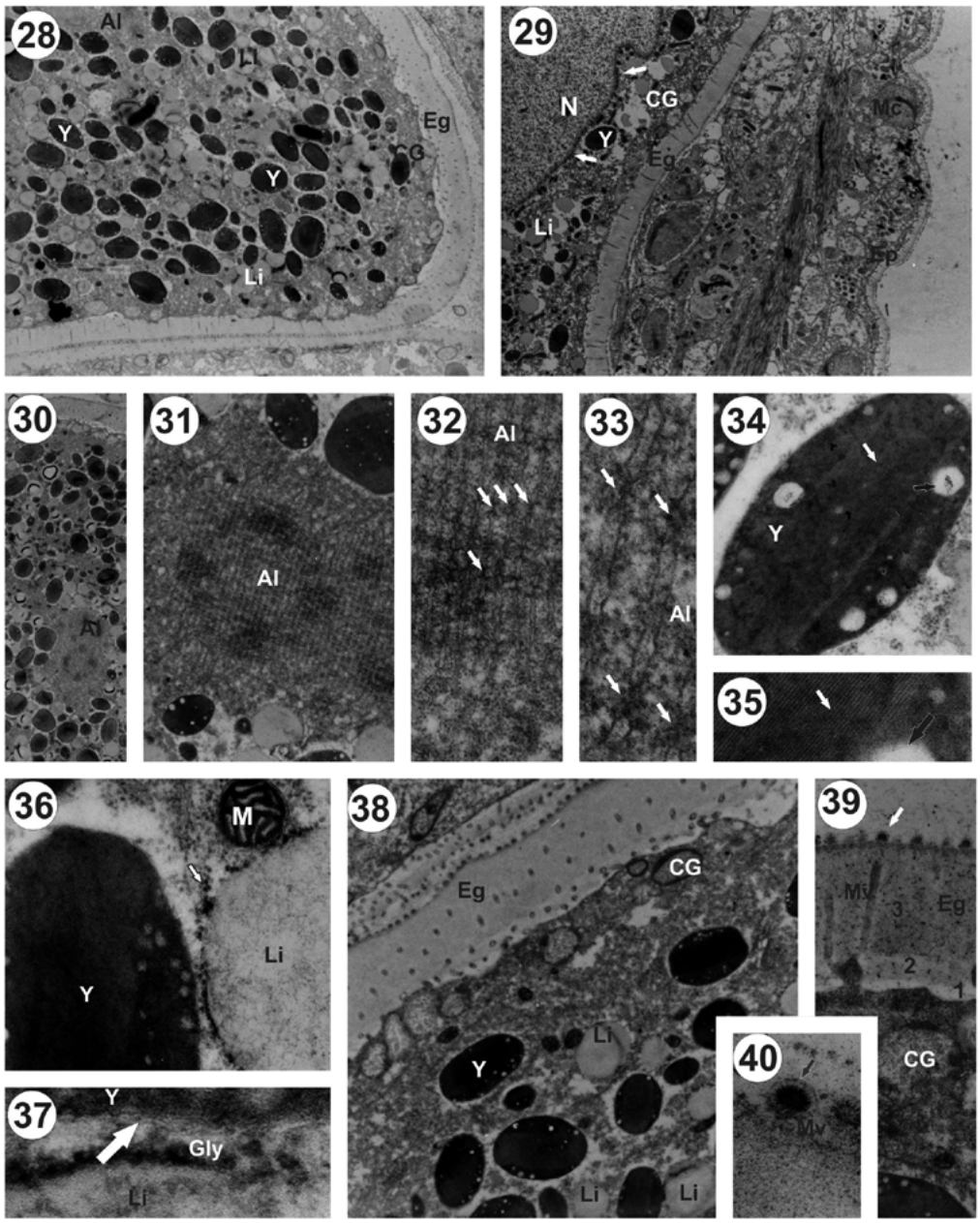
Table 1. Ultrastructural oogenesis in *Phragmatopoma* spp. from Northwest and Southwest Atlantic Ocean.

	NW Atlantic*	**SW Atlantic****
Anterior face of septal blood vessel ciliated	present	present
Oogenesis (type)	intraovarian	intraovarian (until early vitellogenesis) and extraovarian (during late vitellogenesis)
Follicle cells	present	absent
Peritoneal capsule covering ovary during vitellogenesis	present	absent
Asynchronous oogenesis	present	present
Amoeboid cells	present	absent
Mitochondrial cloud locality (in previtellogenic oocytes)	one part of oolema	perinuclear cytoplasm
Golgi complexes	adjacent to oolema where microvilli are formed	close to cortical granules
Golgi complexes (arrangement)	semicircle	parallel
Cortical granules	early vitellogenesis	previtellogenesis
Endocytosis	present	present
Coated vesicles	present	present
Annulate lamellae (coelomic eggs)	present	present
Golgi complexes (coelomic eggs)	few	few
Egg mambrane formation	early vitellogenesis	previtellogenesis
Egg envelope with extracellular matrix (coelomic eggs)	present	present
Microvilli with granular tips	present	present
Intermicrovillar distance change during oogenesis	NA	present
Granules of microvilli changes complexity and number increased during oogenesis	present	present
Autosynthetic crystallized yolk granules (coelomic eggs)	synthesis begin prior to the heterosynthetic yolk granules	synthesis begin after to the heterosynthetic yolk granules
Golgi complexes where occur the synthesis of heterosynthetic yolk granules	absent	present

* Eckelbarger and Chia 1978; Eckelbarger 1979 ** This study. NA: no information available.

25). The glycogen was abundant throughout the cytoplasm, and its size was large compared to the ribosomal particles (fig. 26). The remainder of the cytoplasm had the same characteristics as the previtellogenic stage. The Golgi complexes were more common in the cortical cytoplasm near the cortical granules and a large number of mitochondria were observed (figs. 22-27). The endocytic activity was observed in the plasma membrane, and the larger microvilli maintained the same apical characteristics as observed at the end of previous stage. The perivitelline space (electron-lucent) was smaller and the vitelline envelope thicker than observed for the previtellogenic oocytes (fig. 27).

The oocytes in late vitellogenesis (*i.e.* ripe) were filled with yolk granules, lipid droplets and thick vitelline envelope (fig. 28). These oocytes occupied the entire coelomic cavity toward the body wall (figs. 29). The remarkable cytoplasmic structure was the annulate lamellae that formed patches through the yolk and lipid droplets (fig 28 and 30). The annulate lamellae were characterised by several fenestrated endomembranes with parallel structures (figs. 31-33). The yolk granules were distinct to the early of vitellogenesis forming an elliptical and extremely compact structure. These mature yolk granules showed a medullar crystalline substructure and a cortex with electron-lucent spheres (figs. 34-36). These granules were surrounded



Figures 28-40. Ultrastructure in *Phragmatopoma caudata* of the SW Atlantic. Growth Phase. Figure 28 Oocyte during late vitellogenesis filled with yolk granules (Y) and lipid droplets (Li). Note the cytoplasmic patches where the annulate lamellae (Al) are located and the well-developed egg envelope (Eg) (810x). **Figure 29** Ripe oocytes occupy the entire coelomic cavity toward the body wall (810x). MC = muscle; Ep = epidermis. **Figure 30** General view of the annulate lamellae (Al) between yolk granules and lipid droplets (560X). **Figures 31-33** The annulate lamellae are composed by fenestrated endomembranes (arrows) parallel to each other arranged (Figure 31, 2,900X. Figure 32, 10,500X. Figure 33, 3,100X). **Figure 34** Mature yolk granules with an elliptical shape (Y) showing a medullar crystalline substructure (white arrow) and non-crystalline cortex with electron-lucent spheres (black arrow) (13500X). **Figure 35** Crystallised medullar in a parallel arrangement (white arrow) and a cortical lucid sphere (black arrow) (43,000X). **Figure 36** Yolk granules (Y) and lipid droplets (Li) in the cytoplasm, with the presence of glycogen granules (arrow) close to the droplets (13,500X). M = mitochondria. **Figure 37** Details of yolk granules delimited by the membrane (arrow) next to a lipid droplet (Li) and glycogen (Gly) (61000X). **Figure 38** Cortical cytoplasm filled with yolk granules (Y), lipid droplets (Li) and cortical granules (CG) below the plasma membrane. Notice the thick egg envelope (Eg) (2,050X). **Figure 39** The perivitelline space (1) is narrow and the egg envelope (Eg) is composed of two layers (2 and 3) with different electron-densities. The winding microvilli (Mv) are completely immersed in the egg envelope. Only the apical surface of the microvilli is in contact with the coelomic cavity (arrow) (5,400X). CG = cortical granules. **Figure 40** Microvilli apex showing the expansion bears extensive filamentous adornment with an electron-dense centre (31,000X).

by membrane since the previous phase (fig. 37). The endoplasmic reticulum and a few Golgi complexes were more common at the cortical cytoplasm. The cytoplasm showed fewer glycogen granules, particularly around the yolk granules and lipid droplets (figs. 36 and 37). The oocyte surface had long, sinuous microvilli sharing similar characteristics to the other phases. Additionally, endocytic pits were observed. The microvilli were extensive filamentous adornment structures on the outer surface of oocytes and in the coelomic cavity. The egg envelope displayed two different electron-dense layers and a very thin perivitelline space (figs. 38-40).

We found several differences in oogenesis among the Northwest and Southwest Atlantic Ocean populations, and the concise ultrastructural descriptions are summarised in Table 1.

Discussion

The histochemical results presented here are novel for the Sabellariidae. Oogenesis in *Phragmatopoma caudata* from São Vicente, SP, Brazil, entails a number of new features: 1) a distinct ovarian proliferation characterised by small oogonia clusters that were connected by intercellular extensions to the blood vessel walls; 2) an absence of accessory, or follicular, cells; and 3) a complex vitellogenesis cycle. There were also different stages and mechanisms of yolk formation and yolk precursors from the circulatory system, endocytic activity in previtellogenic oocytes, and the crystallisation in vitellogenic oocytes.

Since oogenesis in *Phragmatopoma* from the southwestern Atlantic Ocean has never been reported, it has not been possible until now to consider intraspecific variation in terms of reproductive characteristics. Oogenesis is different between the worms in the SW Atlantic (present results) from those in the NW Atlantic (Eckelbarger, 1979, 1983, 2005, 2006). The heterogeneity revealed in this study suggests additional molecular analysis should be carried out to investigate the current synonymy based on morphological analyses (Dos Santos *et al*., 2011, Kirtley, 1994). The differences in oogenesis identified between these geographically remote populations may reflect an evolving divergence among the populations.

The nature of oocyte development (*i.e*. from autosynthetically to heterosynthetically) reported in our study differs from those described in NW Atlantic worms by Eckelbarger (1979). Oocyte development in *P. caudata* from the SW Atlantic occurs after the dissemination of the previtellogenic oocytes into the coelom in a free-floating phase. This mechanism of oogenesis is an extraovarian oogenesis process (Eckelbarger, 1983, 1994, 2006). The absence of distinct ovaries in the peritoneum also occurs in some species of nereidids, phyllodocids, and sphaerodorids (Olive, 1983). In these cases, the germ cells, oogonia or very early oocytes, are realesed into the coelom. In addition, in species of pectinariids and sabellids solitary previtellogenic oocytes are released into the coelom where they undergo vitellogenesis (Eckelbarger, 2005).

Both autosynthetic and heterosynthetic mechanisms of yolk production could be observed during vitellogenesis in specimens from the Southern Hemisphere as had previously been described for NW Atlantic specimens (Eckelbarger, 1979). Nevertheless, differences in the cytology of vitellogenic process were observed between worms from the two populations. In NW Atlantic worms, only a single pellet is formed by either mechanism producing two morphotypes of yolk granules (Eckelbarger, 1979). On the other hand, vitellogenesis appeared to be a combination of both processes in the SW Atlantic worms and a single morphological type of yolk granule is formed. In *Phragmatopoma lapidosa* (syn. *P. caudata*), the type II yolk granules, formed heterosynthetically from endocytosis of yolk precursors from the blood vessel, appear much later than type I, which are formed autosynthetically (Eckelbarger, 1979). Although the two morphological types of yolk were described for NW Atlantic worms, the author highlighted that it is possible precursors are sequestered endocytotically from the circulatory system assembly into type I yolk bodies. The convoluted contacts between blood-vessels and developing oocytes (early developing oocytes) increase the surface area (Olive, 1983) and may optimize the uptake of nutrients and yolk precursors from blood vessels. Furthermore, presumably the single type of yolk observed in worms from the SW Atlantic may be the result of the earlier appearance of coated pits besides the oocyte releasing to the coelom before vitellogenesis is completed. In *P. lapidosa* (syn. *P. caudata*) from the NW Atlantic, as well as in *P. caudata* from the SW Atlantic, the ovaries are ephemeral, repeated in a large number of segments, and have the centres of germ cell proliferation connected to blood vessels via the intersegmental septa (Eckelbarger, 1979, 2005, 2006, Eckelbarger and Chia, 1978). The mitotic divisions in *P. caudata* were detected only in the germ cells during the intraovarian growth phase associated with blood vessels.

Follicle cells. In contrast to *Phragmatopoma caudata* from the SW Atlantic population, worms from the NW Atlantic had follicle cells and oocytes connected to the blood vessels during the complete oocyte maturation. Consequently, the vitellogenesis occurred inside the ovaries which, were covered by a peritoneal capsule (Eckelbarger, 1979, Eckelbarger and Chia, 1978). Although lacking physiological evidence that material actually passes from the follicle cells to the developing oocytes, the follicle cells of *Phragmatopoma lapidosa* (syn. *P. caudata*) appear to serve as intermediaries between the oocytes and the surrounding coelomic fluid (Eckelbarger, 1979). Oocytes surrounded by a distinct follicle cells layer as described in *P. lapidosa* (syn. *P. caudata*) species of the NW Atlantic (Eckelbarger, 1979) are absent in *P. caudata* from the SW Atlantic. Thus, the nutrient sources for yolk precursors provided by follicle cells, and amoeboid cells in the NW Atlantic species are directed uptake from the blood vessel (early vitellogenesis) and coelom to proteosythesis for *P. caudata* from the SW Atlantic. In polychaetes, little is known about protein synthesis during oogenesis (Lee, 1988; Song and Lee, 1991). In addition, gene expression connected with oogenesis remains unstudied and therefore nothing is known of which genes code for follicle cells and how differentiation-specific proteins are involved in the formation of the ovarian follicular cells or the timing of oocytes to be released to the coelom among close related species. A prerequisite to further analysis of the role of genes coding for

follicle cells is to find a model to assess how small changes in the genetic structure might affect the adhesion and interrelationships between different cell types. Since current findings reveal morphological variation in the patterns of oogenesis in the two populations described so far, we recommend *Phragmatopoma* as a promising model for further molecular analysis.

Endocytosis and Yolk Synthesis. The presence of coated vesicles in the cortical cytoplasm after the end of previtellogenesis and during the whole vitellogenesis confirms the heterosynthetic process of yolk granules formation. In worms from the SW Atlantic, the large number of coated vesicles observed on the surface of oocyte indicated a common mechanism of substance uptake, primarily the extrinsic vitellogenesis proteins. In addition, the large numbers of coated vesicles indicated the high endocytic activity and relatively short vitellogenesis period (Eckelbarger, 1983). Oogenesis occurs quickly in *P. lapidosa* (syn. *P.caudata*) from the NW Atlantic, and hundreds of coated vesicles were seen for each oocyte during vitellogenesis (Eckelbarger, 1979, 1983) similar to the observations in this study.

Endocytosis is known as a distinct mechanism for incorporating large molecular weight exogenous yolk proteins into the oocyte and there may be some association between the number of endocytotic pits generated along the oocyte oolemma and the length of the vitellogenic phase (Eckelbarger, 1980, 1983). In *Phragmatopoma lapidosa* (syn. *P. caudata*) from the NW Atlantic, precursor molecules for yolk formation via an autosynthetic process may enter the oocyte through the microvillus, that appears just prior to vitellogenesis by means of combined efforts of Golgi complexes and RER assembled into yolk bodies (Eckelbarger, 1979). Even so, the autosynthetic and heterosynthetic processes of yolk synthesis were similar in *Phragmatopoma* worms from both the NW and SW Atlantic Ocean populations. On the other hand, in *Phragmatopoma caudata* from the SW Atlantic, the extraovarian oogenesis occurs during late vitellogenesis without involving follicle cells as reported for other Sabellariidae. Thus, our results provide additional evidence that oogenesis in the populations of the NW and SW Atlantic is clearly dissimilar to previously described (Eckelbarger, 1979, 1983, 1984, 2005). A comparative study has revealed differences in oogenesis among four *Capitella* species (Eckelbarger and Grassle, 1983). The variation in abundance and relative size of specific yolk pellets in the eggs of *Capitella* spp. was apparently related to the quantities of lipid and glycogen stored in the follicle cells. It is plausible that such differences have a significant impact on embryogenesis and larval development.

Cortical Granules. The population from the SW Atlantic Ocean displayed Golgi complex activity, synthesis of cortical granules near the plasma membrane and yolk precursors were uptake from the circulatory system. The cortical granules showed different electron densities and fibrous material similar to that observed in worms from the NW Atlantic (Eckelbarger, 1979, 2005). In *Phragmatopoma lapidosa* (syn. *P. caudata*) from the NW Atlantic the cortical granules appeared early in vitellogenesis, our results showed earlier appearance, during previtellogenesis.

Annulate lamellae. The annulate lamellae are cytomembranes containing pores and are frequently attached or connected to the endoplasmic reticulum. Commonly, ribosomes are attached to the membranes that extend from the annulate lamellae (Kessel, 1989). In polychaetes, both ooplasmic and internuclear annulate lamellae have been described in some species but their function is unknown (Eckelbarger, 1988). In oocytes of *Phragmatopoma caudata* from the NW Atlantic, the annulate lamellae appear during the mid-stage of vitellogenesis. In late-stage, the annulate lamellae are still observed in the ooplasm (Eckelbarger, 1979), similar to this study. Although its function is unclear, in *P. caudata* from the SW Atlantic, the annulate lamellae is closely associated with yolk granules and lipid droplets, and persists until the oocyte is fully-grown.

Egg envelope. Ultrastructural results reveal that the oocyte surfaces in *Phragmatopoma caudata* from the *S*W Atlantic population and in *P. lapidosa* (syn. c*audata*) from the NW Atlantic population (Eckelbarger and Chia, 1978) have a granular extracellular matrix layer. The egg envelope of both *Phragmatopoma* populations exhibited changes during oocytes maturation. In worms from the NW Atlantic, the microvilli appear during the early growth phase, and related granules are continuously produced. The following phase is characterised by an increase in granule formation by the existing microvilli which are no longer being formed (Eckelbarger and Chia, 1978).

In *Phragmatopoma caudata* from the *S*W Atlantic population, the glycoproteinaceous egg envelope, composed only of neutral polysaccharides, is a layer of granules whose complexity and size increase during vitellogenesis and may play a role as a selective barrier for nutrient uptake. Oocytes in contact with coelomic fluid might induce an increase in surface area through elaboration of the oolemma into numerous microvilli showing expansion. This may facilitate the uptake of low molecular weight yolk precursors (Eckelbarger, 1988). However, the differences observed in the formation of the egg membrane between the NW and SW Atlantic Ocean populations may indicate early stages of genetic divergence, or alternatively, may be an adaptive response to the mechanisms underlying the local environment.

Although a recent study supported the synonymy of *Phragmatopoma* species (Dos Santos *et al.,* 2011), this action could be premature (Capa *et al.,* 2012). The plasticity found in oogenesis among the worms from Florida, USA, and São Paulo, Brazil, are considerable (Table 1). In addition we should not assume that the reproductive traits reported in the literature for gregarious intertidal populations of sabellariids would necessarily be accurate for the entire family, including those solitary deep-sea species. Our work demonstrates asynchronous oogenesis in *Phragmatopoma caudata* which was similar to that found in *P. lapidosa* from the NW Atlantic (Eckelbarger, 1979). Thus, different stages of oocyte development can be observed simultaneously within a single organism. This contrasts with *Sabellaria alveolata* in which a gametogenesis is synchronous and all oocyte are in the same stage during the reproductive cycle (Culloty *et al.,* 2010). However, in *S. alveolata* populations from the NE Atlantic, the various gametogenesis stages also occurred simultaneously among

individuals in the population (Culloty *et al.,* 2010). The factors regulating the onset of the reproductive cycle and spawning events are poorly understood in Sabellariidae worms.

In conclusion, oogenesis observed in *Phragmatopoma caudata* of the SW Atlantic is similar to that found in the NW Atlantic indicating that the taxa are closely related and recently separated. However, the diverse aspects of oogenesis documented here give support to the reproductive plasticity among the geographically remote populations. We suggest that the taxonomic status be reviewed incorporating additional traits. Thus, heterogeneity in both oogenesis and oocyte development patterns among the worm populations from the Northern and Southern Hemisphere may indicate (1) different species, and (2) differences in the production of ovarian oocytes due to latitude (i.e. environmental drivers). Further studies, using broad latitudinal comparisons of oogenesis and molecular analyses along with descriptions of the ultrastructure of sperm, are required to determine the number of possible species. It would then be possible to determine if the geographically remote worm populations with their heterogeneous characteristics are the evolutionary products of distinct past tokogenetic events.

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