

EGG HULL MORPHOLOGY IN TWO CHITONS (POLYPLACOPHORA) FROM THE SOUTHWESTERN ATLANTIC OCEAN

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INTRODUCTION

Polyplacophorans with planktotrophic development (free spawners) are known to have eggs with elaborate extra-cellular coverings known as egg hulls (Eernisse & Reynolds, 1994). In these species, hulls are commonly ornamented with projections in the form of cupules, cups, cones, flaps or spines. Members of Lepidochitonidae (*Lepidochitona* spp., now classified as *Cyanoplax* spp.) from the Pacific coast of North America were shown to have reduced, nearly smooth, egg hull sculpturing, which is a reduction of a cone-like hull in free-spawning relatives (Eernisse, 1988). *Leptochiton asellus*, a species of Leptochitonidae (Lepidopleurida) has eggs with smooth jelly-like hulls (Buckland-Nicks & Hodgson, 2000; Buckland-Nicks, 2008). Some brooder species, such as *Chiton nigrovirescens*, have eggs with hulls provided with short spines that serve to maintain eggs packed within the pallial groove (Buckland-Nicks & Brothers, 2008). Thus, the shape and the length of hull projections is indicative of the mode of development, that is, brooders vs. free spawners (Eernisse, 1988).

Variations in the morphology of the egg hulls are known to be of taxonomic value (Eernisse, 1984; Sirenko, 1993; Eernisse & Reynolds, 1994). Recently, the morphology of hull projections was recognized as a valuable character in the study of phylogenetic affinities (Buckland-Nicks, 2006, 2008; Sirenko, 2006).

During oogenesis, follicle cells are probably responsible for the shaping of the egg hulls, acting as molds for the depositing of mucopolysaccharides and proteins that constitute the egg hull layers (Eernisse & Reynolds, 1994). Buckland-Nicks & Reunov (2009) reported the involvement of both oocyte and follicle cells in the formation of the egg hull in a chitonid, *Rhyssoplax tulipa*.

The existence of a close relationship between the shape of egg hulls, morphology of sperm,

and mode of fertilization has been reported (Buckland-Nicks, 1993, 1995, 2006, 2008). All chiton species with complex egg hulls have dart-like sperm with reduced acrosomes, usually at the tip of a nuclear filament of variable length; conversely, lepidopleurid species with smooth egg hulls have sperm with prominent acrosomes (Hodgson et al., 1988; Pashchenko & Drosdov, 1998; Buckland-Nicks, 2006; Buckland-Nicks & Brothers, 2008).

Knowledge of the hull morphology and characteristics of the follicle epithelium of genera and species from the southwestern Atlantic is scant. Sirenko (1993, 2006) provided general sketches of the hull morphology of many chitons, among them, species of the genus *Chaetopleura*, *Plaxiphora* and *Tonicia*. However, details of the morphology of these species are entirely unknown. Buckland-Nicks (2008) and Buckland-Nicks & Brothers (2008) reporting on the fertilization process and evolution in chitons, briefly described and illustrated the hull morphology of species of the genera *Chaetopleura* and *Callochiton* from the Northern Hemisphere.

The present study describes the ultrastructure of the egg hulls of *Chaetopleura isabellei* (d'Orbigny, 1841) and *Plaxiphora aurata* (Spalowsky, 1795), two common species along Argentine shores. A peculiar behavior of the ooplasm during early stages of oogenesis in *P. aurata* is described. This characteristic, to date reported only in species belonging to Acontochitonina, could represent a reproductive trait unique for this clade, reinforcing the phylogenetic coherence derived from morphology. Our results suggest that knowledge of details of the oogenesis process, a dramatic event in defining life history of a species, may be of help in defining phylogenetic relationships among chitons. Morphology of the egg hull of *P. aurata* here reported, strongly suggests the need for a new placement of *Plaxiphora*, outside Mopaliidae.

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MATERIALS AND METHODS

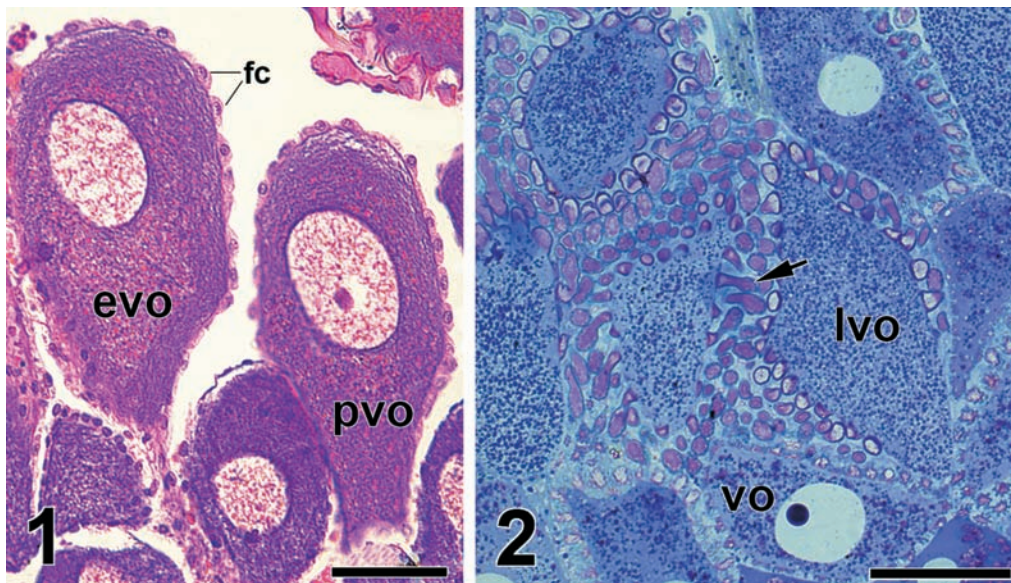
Specimens of *Plaxiphora aurata* were collected on July 2008 from the rocky shore at Punta Cuevas (42°46'46"S, 65°00'06"W), Chubut, Argentina, and processed immediately for histology and scanning electron microscopy (SEM). Specimens of *Chaetopleura isabellei* were collected at Necochea (38°34'S, 58°39'W), Buenos Aires, Argentina, and maintained in aquaria with aerated sea water, exposed to a light/dark (L/D) cycle of 24 h (L/D: 12/12). On January 5, 2009, ripe eggs and sperm coming from spontaneous gamete emissions in the aquaria were collected in small vials and fixed in a 5% formaldehyde solution. After dehydration in ascending ethanol series, in place of crytical point drying, eggs and sperm were treated for 5 min in hexamethyldisilazane, air dried, mounted in stubs and coated for SEM. Female gonads of *P. aurata* and *C. isabellei* were fixed in 4% glutaraldehyde with 5% formaldehyde solution in phosphate buffer (pH 7.1) at 4°C for 20 h. A portion of gonads and free oocytes were dehydrated and treated as previously described for SEM. Gonads for general histology were infiltrated in Histo-resin® (Leica) and sectioned in a rotary motorized

Leica RM 2265 microtome. Sections of 3.5 µm were stained either with haematoxylin-eosin or toluidine blue (pH 9.0).

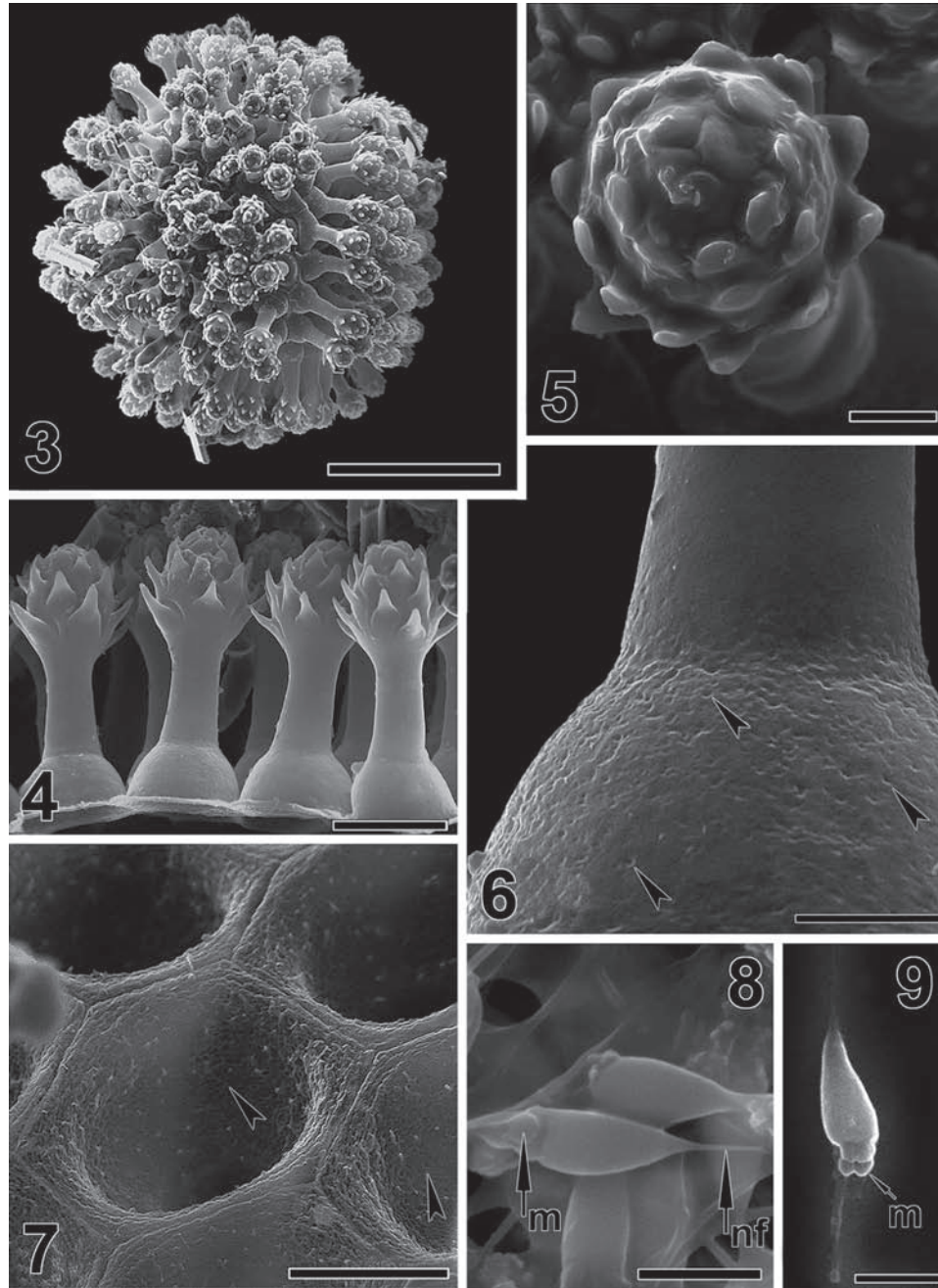
RESULTS

Chaetopleura isabellei

Just before vitellogenesis begins in *Chaetopleura isabellei* (Chaetopleuridae: Chitonina), each oocyte is completely surrounded by a large number of follicle cells (more than 30 per transverse section), closely applied to the plasma membrane (Fig. 1). As oogenesis progresses, the deposition of materials forming the egg hull begins, and the follicular epithelium is progressively pushed away from the oocyte surface (Fig. 2). Under light microscopy, hull projections appear composed of two layers of materials when stained with toluidine blue and viewed with light microscopy; an outer, very thin layer showed an orthochromatic reaction, and an inner layer was metachromatic. These reactions are compatible with neutral and acid mucopolysaccharides, respectively (Fig. 2). Ripe eggs were about 295 µm diameter (after dehydrating for SEM egg diameter was reduced



FIGS. 1, 2. Oogenesis in *Chaetopleura isabellei*. FIG. 1: Previtellogenic oocyte (pvo) and early vitellogenic oocytes (evo) (note acidophilic droplets in the cytoplasm) encompassed by a large number of follicle cells (fc); FIG. 2: Late vitellogenic oocytes (lvo) with egg hull projections (arrow) showing ortho and metachromatic reaction of outer and inner layers. Scale bars Fig. 1 = 50 µm; Fig. 2 = 100 µm.



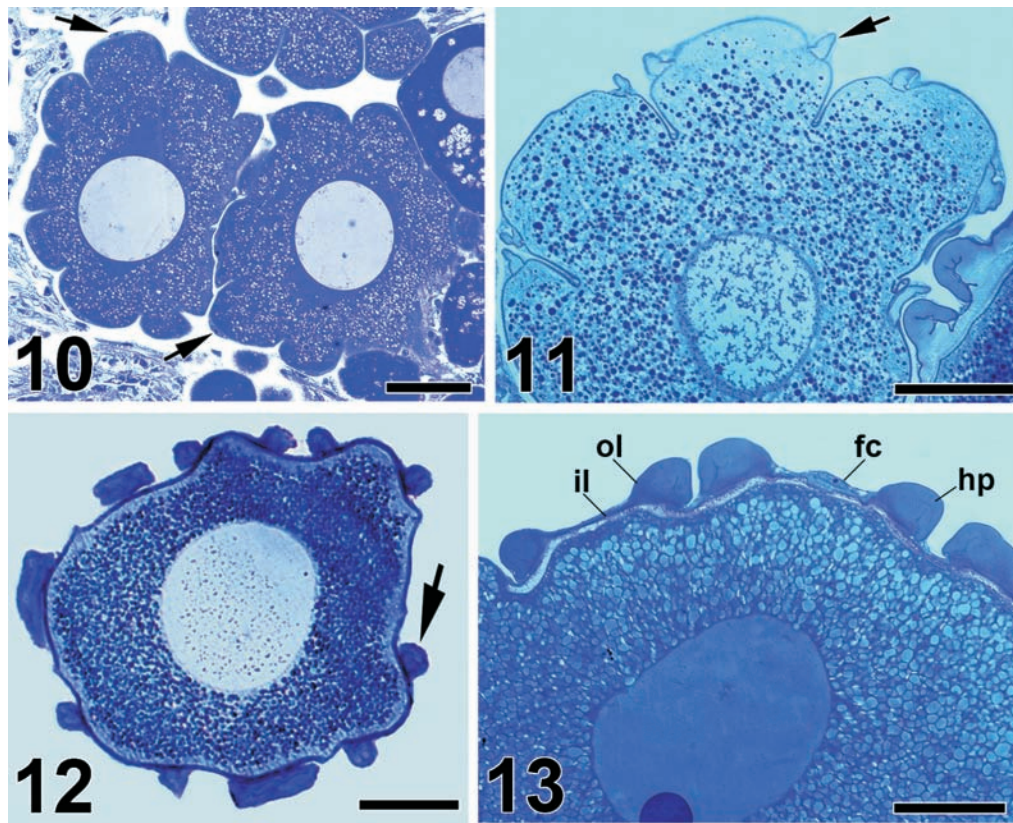
FIGS. 3–9. *Chaetopleura isabellei*. FIGS. 3–7. SEM photographs of egg hull ultrastructure; FIG. 3: General view of an egg; FIG. 4: Close-up of four egg hull spines; FIG. 5: Detail of the arrangement of leaflet-like thorns on the tip of a spine; FIG. 6: Lateral view of dome-shaped base of a spine with micropores (arrow); FIG. 7: Inner view of an egg hull showing the polygonal basal plates with micropores (arrow). FIGS. 8, 9: SEM of sperms. FIG. 8: Lateral view of a sperm showing nuclear filament (nf) and mitochondria (m) at the base of the nucleus; FIG. 9: A sperm showing the four laterally placed mitochondria (m). Scale bars Fig. 3 = 100 μm ; Fig. 4 = 20 μm ; Figs. 5, 6 = 5 μm ; Fig. 7 = 10 μm ; Figs. 8, 9 = 2 μm .

to about 240 μm). Egg hulls were sculpted with hollow projections also referred to as spines, about 50 μm length (Figs. 3, 4). Each projection extends from a dome-shaped hexagonal plate of about 20 μm diameter, which continues in a somewhat short, cylindrical column that ends in a club-shaped closed tip (Figs. 4, 6, 7). The tip is surrounded by four to six whorls of thorn-like projections; successive whorls of thorns were distributed following an alternate pattern (Fig. 5). At the inner and outer surface of the dome-shaped base of each projection, numerous open micropores were observed (Figs. 6, 7).

Sperm of *C. isabellei* showed a bullet-shaped nucleus (about 3 μm length), with an elongate nuclear extension of about 2 μm length (Fig. 8), and four mitochondria, somewhat laterally displaced, at the base of the nucleus (Fig. 9).

Plaxiphora aurata

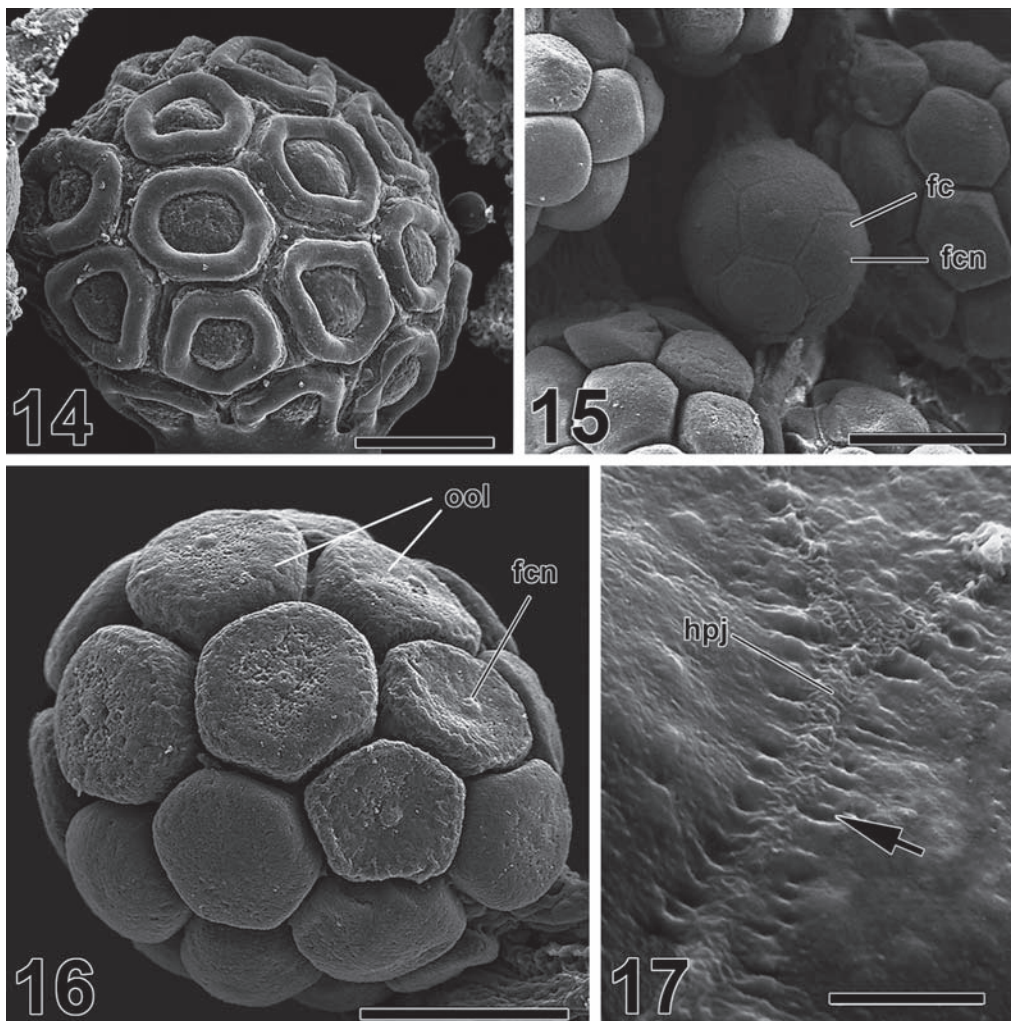
Late previtellogenic or early vitellogenic oocytes of *Plaxiphora aurata* (Mopaliidae (?): Acanthochitonina) are encompassed by a reduced number of follicle cells (less than 10 per transverse section) (Fig. 10), each delimiting a polygonal (pentagonal or hexagonal) space on the surface of oocytes (Fig. 15). At the beginning or just after starting vitellogenesis, the ooplasm showed a series of lobules, which in transverse sections appear as deep incisions, determined by the infolding of the oocyte membrane (Figs. 10, 11, 16). The deepest part of each incision corresponded to the contact zone of two contiguous follicle cells (Fig. 10). As vitellogenesis progressed, the deposition of materials forming the egg hull begins and progressively the ooplasm lobules reduce in size, vanishing in late vitel-



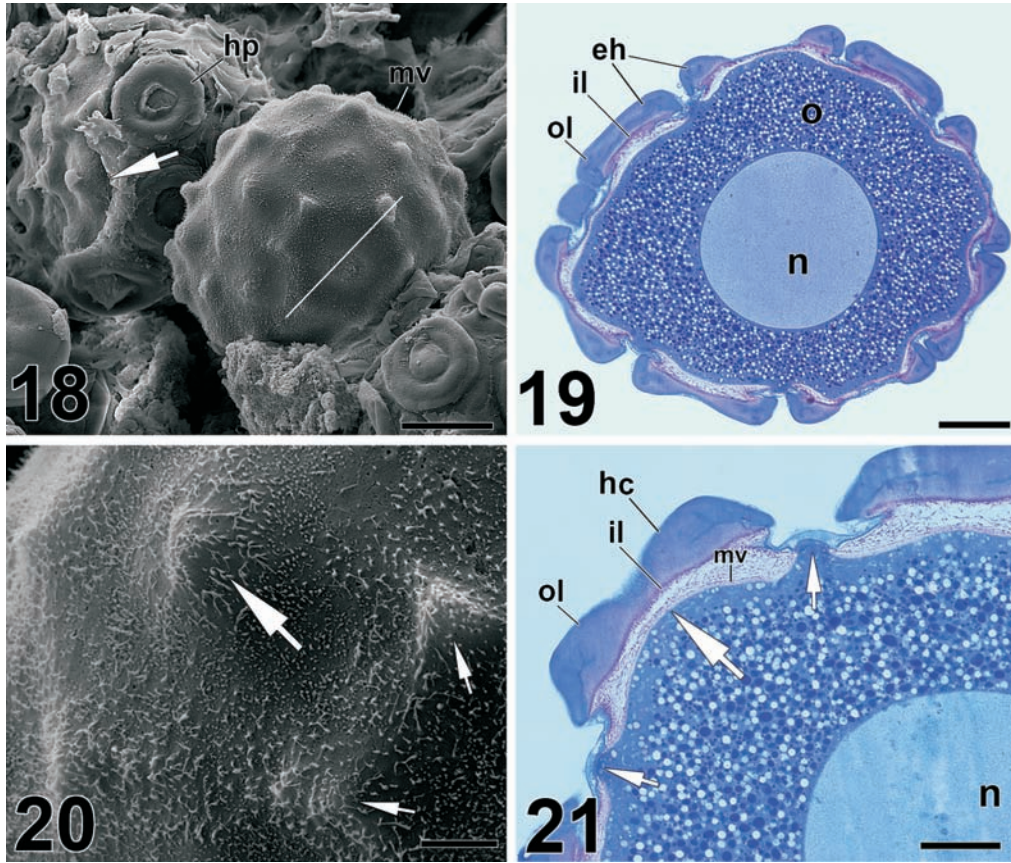
FIGS. 10–13. Oogenesis in *Plaxiphora aurata*. FIG. 10: A late previtellogenic oocyte showing the lobulations of the ooplasm, each one with an associated follicle cell (arrows); FIG. 11: An early vitellogenic oocyte at the initial phase of egg hull formation (arrow); FIG. 12: A mid vitellogenic oocyte with nearly inconspicuous ooplasm lobulations (arrow indicates egg hull); FIG. 13: Late vitellogenic oocyte with the hull in an advanced stage of development showing the ortho and metachromatic reactions of outer (ol) and inner (il) layers of hull wall, and a follicle cell (fc) still associated to a hull projection. Scale bars = 50 μm .

logenic or ripe oocytes (Figs. 11–13, 19). Egg hulls in *P. aurata* are composed of polygonal, usually pentagonal or hexagonal plates, 50 μm in diameter, with low areole-shaped projections; the somewhat inflated border of the areole surrounds a depressed area, slightly elevated in the centre by a very low cupule (Fig. 14). The egg membrane underneath the egg hull shows clearly demarcated polygonal areas, covered with relatively long, uniformly sparse microvilli that reproduce the pattern, and coincide with the polygonal bases of the egg hull projections

(Figs. 18, 20). At each of these areas, the egg surface show a central bulge at the place formerly occupied, during early stages of hull formation, by the nucleus of the associated follicle cell (Figs. 13, 18, 20, 21), and corresponding to the slightly elevated centre of each hull cupule. Five or six peripheral points coincide with each of the angles of the polygonal base of adjacent hull cupules (Figs. 18, 21). Along the perimeter of each polygonal base of hull cupules, rows of somewhat large and regularly spaced open micropores (0.5–0.7 μm diameter) were present



FIGS. 14–17. *Plaxiphora aurata*. FIG. 14: General view of an egg; FIG. 15: Early vitellogenic oocyte (centre of image) showing polygonal shape of follicle cells (fc) with central nucleus (fcn); FIG. 16: SEM of an advanced vitellogenic oocyte showing the infoldings of the plasma membrane and ooplasm lobulations (ool) with centrally located nucleus of follicle cells (fcn); FIG. 17: Micropores (arrow) at the perimeter of contacting basal plates. Scale bars Figs. 14–16 = 50 μm ; Fig. 17 = 5 μm .



FIGS. 18–21. *Plaxiphora aurata*. FIG. 18: SEM of two late vitellogenic oocytes, one (upper left corner) with the egg hull partially removed (arrow) and another (centre) with egg hull completely removed, showing the cups of ooplasm, and plasma membrane with microvilli (white line indicate position of section showed in figure 21); FIG. 19: Transverse section of an egg with egg hull completely developed (eh, egg hull, hp, hull plate, mv, microvilli, n, nucleus, o, ooplasm); FIG. 20: Close-up of figure 18 showing the central bulge of ooplasm beneath a hull plate (large arrow) and minor bulgings (small arrows) coinciding with the hull plate periphery; FIG. 21: Transverse section of an egg showing central (large arrow) and peripheral (small arrows) bulgings of the ooplasm beneath a hull cupule (hc) (mv, microvilli; il, inner hull layer; ol, outer hull layer). Scale bars Figs. 18, 19 = 50 μ m; Fig. 20 = 10 μ m, Fig. 21 = 20 μ m.

(Fig. 17). In transverse sections of fully developed egg hulls stained with toluidine blue, two component layers were distinguished, the outer orthochromatic and the inner metachromatic, evidence of the probable presence of neutral and acid mucopolysaccharides (Figs. 13, 19, 21).

DISCUSSION

According to Buckland-Nicks (2008), *Chaetopleura apiculata* shows hollow complex branching spines, a morphology unknown from any other genera. Finding very similar spines in

another member of *Chaetopleura* implies that such spines are characteristic of this genus and likely homologous. The egg hull of *Chaetopleura isabellei* and *C. apiculata* are similar to those of other members of Chitonina in the general morphology of the spines, extending from a relatively narrow, dome-shaped polygonal base and formed by a short, hollow column with a closed tip. Compared with *C. apiculata*, eggs of *C. isabellei* are smaller (about 20%), the spines longer and the tips are club shaped, bearing a quite peculiar array of thorn-like projections, arranged in several whorls. In *C. apiculata*, the spine branching is mainly on the proximal half in-

stead of at the distal tip, as in *C. isabellei*. As well as in *C. apiculata* (Buckland-Nicks & Brothers, 2008), the projections of *C. isabellei* are hollow, and the hull is perforated at the base of each projection by minute open pores. Despite the lack of detail of Sirenko's (2006) drawing depicting a hull spine of *Chaetopleura angulata*, it looks similar to those of *C. isabellei* here described. The participation of the follicle cells during hull formation seems also similar to that described by Selwood (1970) in Chitonidae, a different family within Chitonina, specifically in *Sypharochiton septentriones* (Ashby, 1924), in which a large number of follicle cells encompasses each developing oocyte, and are progressively pushed apart from the egg membrane while the materials forming the hull are deposited. Even though the fertilization process was not studied here, it seems probable that the micropores in the base of the egg hull spines correspond to those described for *C. apiculata* as the sites for sperm penetration (Buckland-Nicks, 2008).

The general morphology of the egg hull of *Plaxiphora aurata* is similar to that sketched by Sirenko (1993, 2006) for *Plaxiphora kamehamehae* (Ferreira & Bertsch, 1979) and an unidentified *Plaxiphora* species. The reduced number and the peculiar behavior of follicle cells during oogenesis participating in the formation of several lobules of the ooplasm in *P. aurata* is similar to that described by Garnault (1888) and Richter (1986) in *Cyanoplax* (= *Lepidochitona*) *cinerea* (Linnaeus, 1767); however, the shape of the resulting egg hull in both species is quite different. Egg hull morphology of other species of the genus *Cyanoplax* (= *Lepidochitona*) have been studied in detail (Eernisse, 1988), but nothing is known on details of oogenesis in these species. The process of infolding of the oocyte membrane accompanied by follicle cells during early stage of oogenesis, has also been described by Anderson (1969) in *Mopalia muscosa* (Gould, 1846), Barnes (1972) in *Tonicella lineata* (W. Wood, 1815), Nimitz & Giese (1964) in *Katharina tunicata* (W. Wood, 1815), and Tucker (1960) in *Cryptochiton stelleri* (Middendorff, 1847). It is interesting to note that the peculiar behavior of the ooplasm-follicle cell complex during the early stages of oogenesis is only shared by other species of phylogenetically closely-related genera also included within the Chitonida suborder Acanthochitonina (*sensu* Sirenko, 2006). This should be viewed as an additional character reinforcing the monophyly of the Acanthochitonina, relatively well supported by molecular information, morphology of egg hull, sperm morphology, and mode of fertilization (Okusu et al., 2003; Buckland-Nicks, 2008). Based on different sets

of apomorphic characters, two groups among the Acanthochitonina have been recognized (Buckland-Nicks, 2008). The first has egg hulls with closed cupules and fertilization between cupules, represented by the condition reported in *Cyanoplax* and *Acanthochitona*. The second has, among other similarities, egg hulls with open cupules and fertilization inside cupules, and corresponds to that found in *Mopalia*, *Tonicella*, *Cryptochiton*, which were found to be closely related by Kelly & Eernisse (2008). Because the first condition is probably more widespread within Acanthochitonina, it is probably that it is plesiomorphic within Acanthochitonina, and the second condition is derived for a group that includes *Mopalia*, *Tonicella*, and *Cryptochiton*. *Plaxiphora aurata* clearly falls within the first group. From the previous and based on the peculiarities of the egg hull morphology here described, and, considering that this is a significant character in chiton taxonomy (Eernisse, 1984; Sirenko, 1993; Buckland-Nicks, 2008), it seems more adequate to place *Plaxiphora* outside Mopaliidae. Vendrasco et al. (2008), studying the aesthete canal morphology in Mopaliidae, arrived at the same conclusion.

Buckland-Nicks & Schander (2008) pointed out the plesiomorphic condition of such features as the presence of pores in the polygonal bases of the hull and a series of membrane cups just beneath the spine bases, they found in a member of the Chitonina, *Stenosemus albus* (Linnaeus, 1767). This condition, in their opinion, is reminiscent of that observed previously by Buckland-Nicks & Hodgson (2000) in *Callochiton dentatus* (Spengler, 1797), suggesting the position of *S. albus* as basal to the Chitonina. Other Chitonina and Acanthochitonina have pores in the hull (e.g., *Chaetopleura apiculata*, *C. isabellei* and some species of *Chiton*) (Buckland-Nicks, 2008). However, all of them lack membrane cups beneath the spine bases, or at least they have not been reported yet. Interestingly, in *Plaxiphora aurata*, the egg membrane underneath the egg hull showed clearly demarcated areas, that reproduce the pattern, and coincide with the polygonal bases of the egg hull projections, resembling the condition described in *S. albus*. If these areas of the membrane egg in *P. aurata* correspond to the membrane cups described by Buckland-Nicks & Schander (2008), both supposed plesiomorphic characters would be present in *P. aurata* from Patagonia, a species clearly related, in general morphology and peculiar features of oogenesis, to Acanthochitonina. However, the membrane cups in *P. aurata* are slightly different and may not represent the same structure than in *S. albus* and *C. dentatus*.

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