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# Structural study of the ovary, oogenesis and brooding in *Tonicia lebruni* (Polyplacophora Chitonidae) from Patagonia

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**Abstract**

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*Tonicia lebruni*, a common, lower intertidal and subtidal chiton inhabiting Patagonian rocky shores, is a gonochoristic iteroparous species producing large eggs (≈ 400 μm in diameter), which are fertilized and brooded within the pallial groves until released as juveniles. A free larval stage is absent, despite this, *T. lebruni* is widely distributed along the south-western Atlantic. At Puerto Deseado, *T. lebruni* has a marked seasonality in the reproductive cycle, reproducing only once a year. The reproductive period is quite short and defined in time: spawning and brooding take place during the late austral winter and beginning of spring. Recovery of the female gonad starts very soon after spawning. Oogenesis takes about 10–11 months for completion. Brood size is correlated with length of maternal individual. The number of embryos per brood varied between 785 and 5945. Extensive resorption of abortive eggs is viewed as related to limitation of space available for brooding. The egg hull is formed by a large number of minute pentagonal or hexagonal plates each one bearing a short spine bent onto the egg surface. The morphology and the surface of the hull could contribute to the cohesiveness of the brooded egg mass within the pallial grooves.

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## Introduction

*Tonicia lebruni* (Rochebrune, 1884) (Chitonidae) is a common low intertidal and shallow subtidal species (living from 1 to 36 m depth) occurring along the rocky shores of Patagonia, being reported from Punta Ninfas (42°59′12″S 64°20′29″W), Chubut to Tierra del Fuego on the Atlantic coast, Dawson Island (Magellan Strait), Isla de los Estados Archipelago and Malvinas (Falkland) Islands (Schwabe *et al.* 2006; Sirenko 2006a).

Knowledge on the biology of chitons from the south-western Atlantic is scant. López Gappa and Tablado (1997) reported on the aspects of the population dynamics of *Plaxiphora aurata*, Scarano and Ituarte (2009) reported a case of occasional hermaphroditism in *P. aurata*, Ituarte *et al.* (2010) described the structure of the egg hull morphology of *Chaetopleura isabellei* and *P. aurata*, and recently, Liuzzi and Zelaya (2013) reported on the egg hull morphology and brooding traits of *Ischnochiton stramineus* from the Magellan Strait, Isla de los Estados and Malvinas (Falkland) Islands.

The number of known brooder chiton species showed a steady increase since the first compilation by Pearse (1979), who listed a total of 19 species. Strack (1987) in a report on polyplacophorans from the Gran Canaria Island reviewed the extant information on brooding species, reporting eight species that brood their eggs at least to the trochophore larval stage and 24 species that brood their embryos through the final stage of metamorphosis including the development of shell plates. Sirenko (2006a) listed 26 brooding species and reported, for the first time, brooding in two species: *Ischnochiton stramineus* and *T. lebruni* from the Magellan Strait (560 Km south to the locality of this study), without giving details about the extension of the period of brooding in the latter. Liuzzi and Zelaya (2013) listed 36 chiton species in which

 

brooding has been reported, giving details on the extension of brooding in each case. Sirenko (2015) added four species to the list, raising the number to 41 brooding species.

It has been long recognized (Eernisse 1988, Sirenko 1993; Buckland-Nicks 1995) the importance and relevance of the egg hull structure (as well as the sperm morphology and the alternatives of the unique mechanism of fertilization (Buckland-Nicks 2006, 2008)) as one of the most important morphological characters in the definition of the major clades within Polyplacophora: the Lepidopleurida, with smooth eggs, and the Chitonida with two different types of elaborate egg hull processes, cupules in Acanthochitonina and long spine projections in Chitonina. More recent molecular-based phylogenies interested in the refining of the definition and relative position of major chiton lineages showed an overall agreement with morphology-based studies – including egg hull structure as a valuable informative source (Okusu *et al.* 2003; Sigwart *et al.* 2013; Irisarri *et al.* 2014). However, some aspects of the taxonomic classification at higher levels as for example the relative phylogenetic position of the basal clade Lepidopleurida with respect to the proposed suborders of Chitonida or the position of specific genera (e.g. the position of *Callochiton* within or not in the Acanthochitonina, or *Plaxiphora* within the proposed superfamilies Cryptoplacoidea or Mopalioidea) remain unsettled or are still controversial (Okusu *et al.* 2003; Sigwart *et al.* 2013).

Regarding the formation and structure of the egg hull, the follicle cells (FCs) that surround the developing oocytes are known to be probably responsible for the shaping of the egg hull projections, acting as moulds for the deposition of mucopolysaccharides and proteins that constitute the egg hull layers (Eernisse and Reynolds 1994; Buckland-Nicks and Reunov 2009). Buckland-Nicks and Reunov (2009, 2010) and Buckland-Nicks (2014) reported that in *Rhyssoplax tulipa*, *Callochiton dentatus* and *Acanthochitona garnoti* the materials for the egg hull are released by microapocrine secretion, directly from the surface of the oocyte, a mechanism that does not involve the intervention of the membrane complex of the cell. In *C. dentatus*, microapocrine secretions from the follicle cells also contribute materials for the egg hull (Buckland-Nicks and Reunov 2010).

Taking into account the limited biological information on south-western Atlantic chitons summarized above, we focused this study in describing the architecture of the oogenesis (not dynamic processes such as vitellogenesis) of *T. lebruni*, the ultrastructure of the egg hull and details of the brooding process, based upon a 2-year period of sampling at Puerto Deseado, Argentina.

## Materials and Methods

Specimens for this study were haphazardly collected during maximum low-tide periods at the lowest intertidal level on the rocky littoral at Puerto Deseado (47°45′S 65°55′W), Santa Cruz province, Argentina. Samples were obtained at irregular intervals from April to September 2009 and February 2010 to December 2011. The number of specimens collected in each sampling event was usually low (<20) owing to the restrictions imposed by extreme weather conditions that occur in lower latitudes.

Specimens for light microscopy (LM) and scanning electron microscopy (SEM) were immediately processed after collection. To avoid curling of specimens while fixing, prior to the immersion in the fixative, each specimen was secured to a microscope slide by wrapping it with a fine thread, and immersed in a 1 : 10 dilution of 4% formalin for 2 min; after this, an incision along the roof of the pallial groove was made and the foot was removed. The partly dissected specimens were immersed in the fixative for 60 min after which the gonads were dissected and immersed in fresh fixative. Gonads for general histology (LM) were fixed in Bouin's fixative, dehydrated in ethanol series, embedded in Historesin™ (Leica®) and sectioned with a rotary motorized Leica® RM 2255 microtome. Sections of 3.5–4 μm were stained either with haematoxylin–eosin or with a solution of alkaline toluidine blue (1% toluidine blue and 2% borax in distilled water, pH 9). For transmission electron microscopy (TEM), portions of gonads and eggs obtained from spontaneous spawns were fixed in 4% glutaraldehyde with 5% formaldehyde (from paraformaldehyde) solution in phosphate buffer (pH 7.1) at 4 °C for 20 h. For SEM, portions of gonad and eggs, fixed as previously described for TEM, were critical point dried, coated with gold and mounted on SEM stubs. Spontaneous gamete emissions were obtained in the laboratory from groups of five specimens (assuming male and females were present) maintained overnight at room temperature (10 °C) in 15-cm-diameter Petri dishes. After spawning, ova were collected from the female pallial groove with a plastic pipette and immediately transferred to the fixative.

As a source of information on the seasonality of reproduction in *T. lebruni*, the shifts in the mean size of oocytes throughout time along the period studied were followed. For this, about 70 oocytes from six to seven females per sample were measured from histological sections with a micrometre ocular. The oocyte size was estimated as the average oocyte diameter (maximum diameter + minimum diameter/2); measurements were obtained only in sections of oocytes in which the nucleus was visible. Mean and standard deviation of oocyte sizes corresponding to each sample were calculated, and data were plotted against time.

For the evaluation of brood size, the eggs or embryos contained in the pallial grooves of six gravid females (with sizes between 2.1 mm and 3.2 mm length) were removed with the aid of thin needles and by repeated water flushes made with a small syringe and brought to 50 mL volume of 5% formalin in sea water. Counting of eggs or embryos was performed using a 5-mL Bogorov chamber. Length of gravid females was measured with a calliper to nearest 0.1 mm as the distance between anterior and posterior margin of nuchal and anal plates. To evaluate the existence or not of a relationship

 

between the mother size and the number of brooding embryos, the Pearson's coefficient was calculated. Line charts in Fig. 1A,B were obtained with a free online chart generator (http://jtblin.github.io/angular-chart.js/), with aim to make visually evident the overall trend of each variable considered.

## Results

### *Gonad architecture and oogenesis*

The information gathered during a 2-year study of the reproductive cycle of *T. lebruni* confirmed that the species is gonochoristic and iteroparous. The gonad, an elongated single sac approximately circular in transverse section, depressed along the mid dorsal region, lies over the intestine and digestive gland and ventral to the kidney and dorsal aorta, immediately below the roof of the visceral mass. The ovarian wall is relatively thick; below the germinal epithelium is an epithelium of elongated cells with a flat nucleus, supported by connective tissue with muscle fibres (Fig. 2A–C). A number of hollow, blind-ended tissue trabeculae (also referred to as 'tissue plates') formed by a monolayer of low columnar cells delimiting a haemocoelic space extend from the ventral gonad wall to the lumen of the gonad, not reaching the dorsal wall (Fig. 3A, B). The inner (luminal) surface of the dorsal and laterodorsal wall of the ovary is formed by low columnar or cuboidal cells with long and dense ciliature (Fig. 3B–D). At the posterior third of the gonad sac, near the origin of the gonoducts, the ciliated epithelium of the dorsal wall is thrown inwards into a series of hollow tubular projections ventrally directed. Each one, hanging from the roof of the gonad, is accompanied by a blood sinus originated in the dorsal aorta (Fig. 3C,D); the epithelium of the dorsal aorta extends for a short length to line the inner surface of the tubular projections (Fig. 3C) but soon disappears, and the blood space is only limited by the projection of the ciliated dorsal wall of the gonad (Fig. 3D). No connection of the tubular projections of the dorsal wall with the tissue trabeculae arising from the ventral wall was observed.

The germinal epithelium develops on the lower 2/3 of the inner surface of the gonad wall (Fig. 3A), where the oogonia (about 20 μm in diameter) are widely attached to the gonad wall and accompanied by one or two follicle cells (FCs) (Fig. 2A) divide by mitosis to form small clusters of about ten cells in transverse section (Figs 2B,C and 3B). After the proliferative phase of oogonia, the oocytes entered the growing phase, which is coincident with the instalment of the follicular

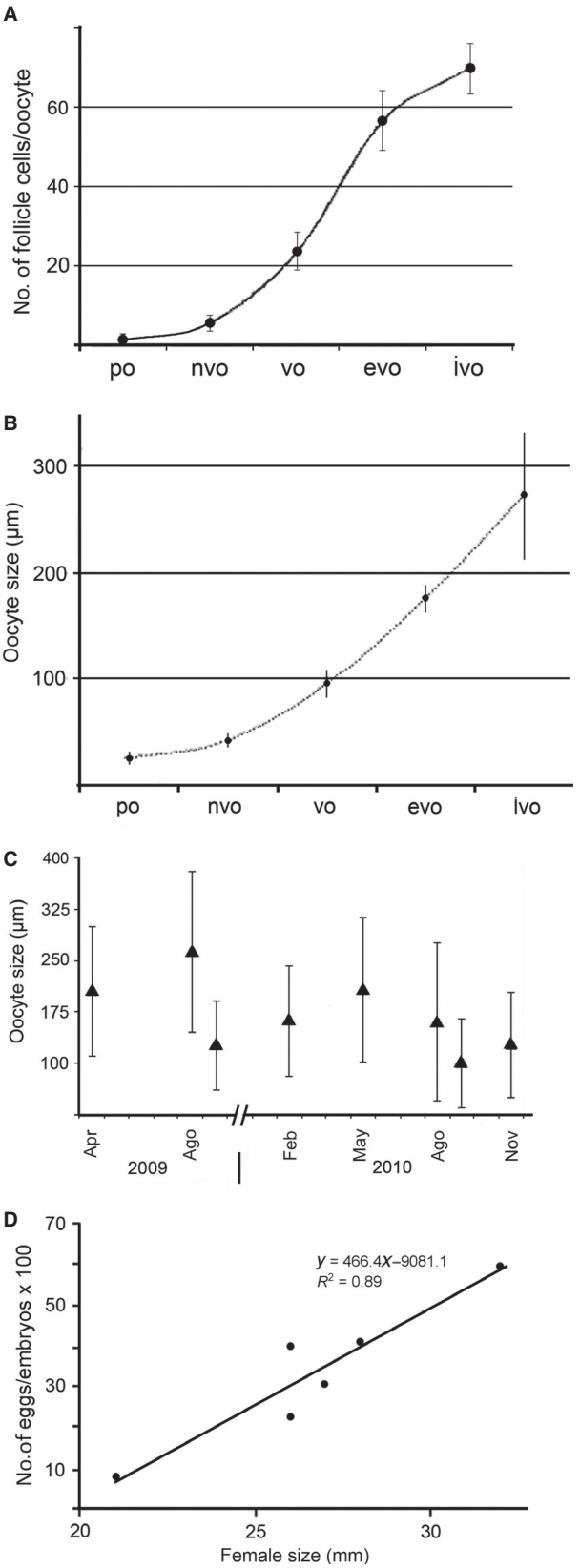


**Fig. 1**—**A.** Plot of the number of follicle cells per oocyte type. —**B.** Increase in the oocyte diameter throughout oogenesis. —**C.** Seasonal variation in mean oocyte size (mean value ± SD). —**D.** Relationship between number of brooding oocytes/embryos and maternal size (length) (data in Fig A–C obtained from histological sections). *Abbreviations*: po. primary oocyte; nvo. oocyte with 'non-vacuolated' cytoplasm; vo. oocyte cytoplasm with 'vacuole-like' electron-translucent areas; evo. early vitellogenic oocyte; lvo. late vitellogenic oocyte.

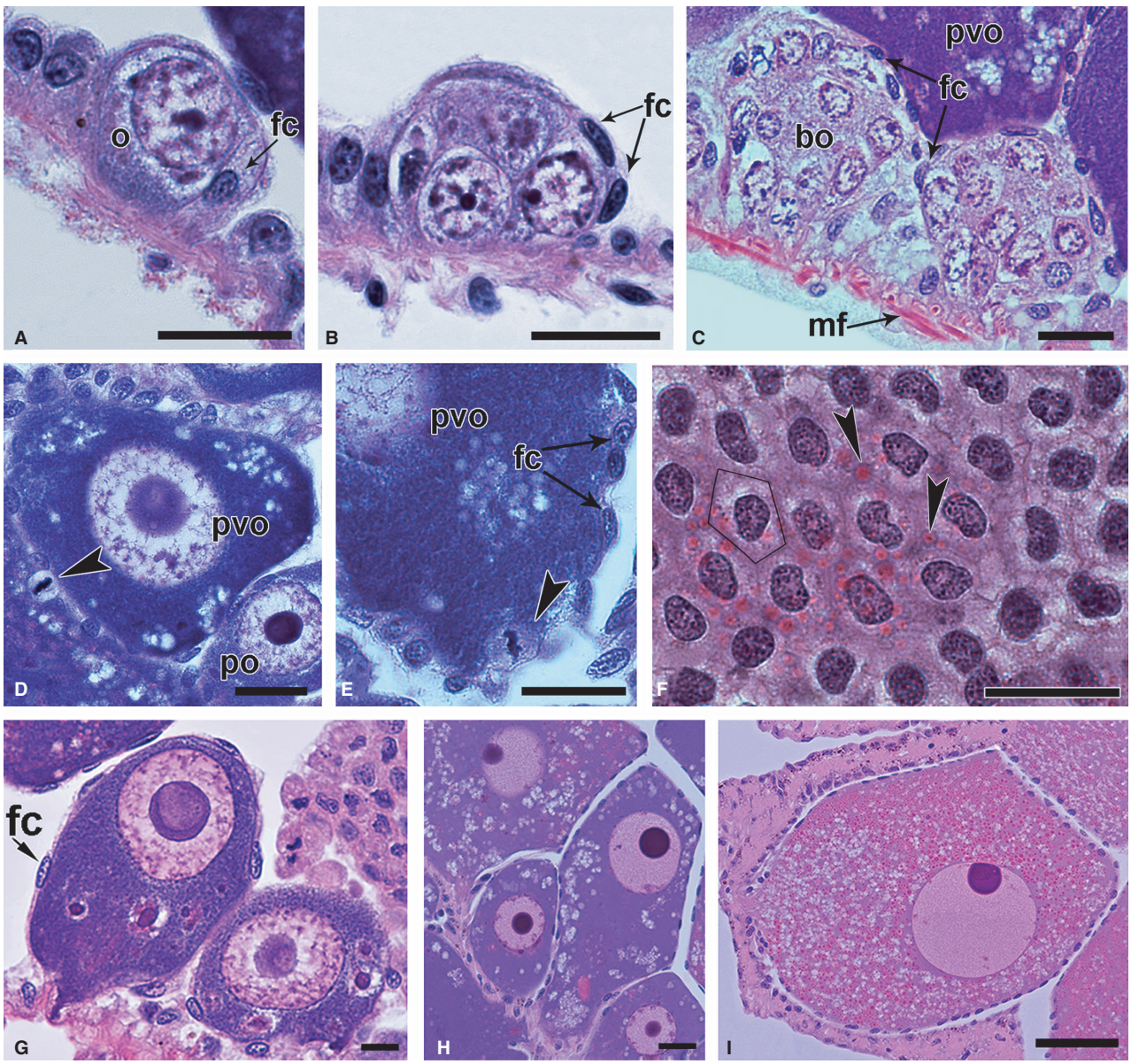


**Fig. 2**—*Tonicia lebruni*: Development of the follicular epithelium. —**A**—**B.** FCs accompanying oogonia in proliferative phase. —**C.** Cluster of oogonia surrounded by FCs. —**D.** Mitosis of a follicle cell (arrow). —**E.** Detail of a metaphase (arrow) of a follicle cell. —**F.** Tangential section showing a completely formed follicular epithelium (the line drawing depicts the polygonal contour of follicular cell cytoplasm) surrounding an early vitellogenic oocyte. Vitellus droplets (arrow head) are seen throughout the follicular epithelium. —**G.** Primary oocyte in the growing phase. —**H.** Previtellogenic oocyte with 'vacuole-like' spaces in the cytoplasm. —**I.** Early vitellogenic oocyte with follicular epithelium completely formed. *Abbreviations*: fc. follicle cell; mf. muscle fibres of the gonad wall; o. oogonia; po. primary oocyte; pvo. previtellogenic oocyte. Scale bars: A–F, I = 20 μm; G = 10 μm, H = 50 μm.

epithelium, formed by a monolayer of FCs that divide by mitosis 'in situ' (Fig. 2D,E) and progressively surrounds each single oocyte. At the end of the process, each oocyte is encompassed by a continuous epithelium of small flat polygonal cells (about 15 μm diameter) each with a flattened nucleus (Figs 2F, and 4A–C). The FCs are held together with each other and with the developing oocytes, by desmosome-like cellular junctions (Fig. 4A–C,E). Throughout oogenesis, FCs change shape from squamous with a flat nucleus in previtellogenic oocytes to cuboidal with a rounded nucleus in vitellogenic oocytes (Fig. 4A,D). Developing oocytes attach to the tissue trabeculae; this relationship persists throughout vitellogenesis (Fig. 2G–I and 5A) and even at the end of vitellogenesis in ripe oocytes (Fig. 6C). Primary oocytes of about

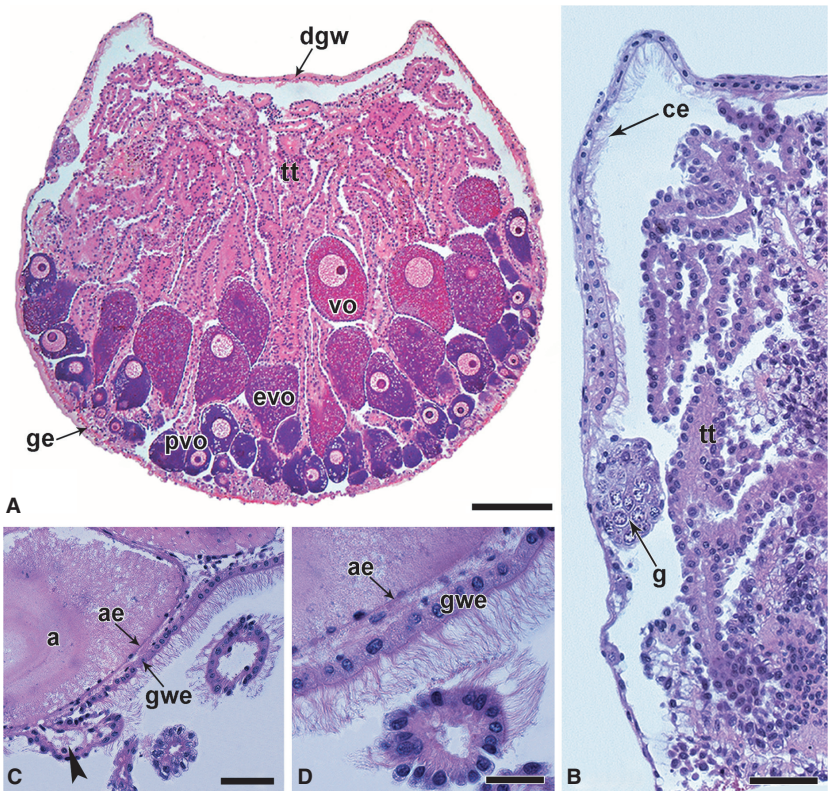


**Fig. 3**—*Tonicia lebruni*: Gonad structure. —**A.** Transverse section of the female gonad. —**B.** Detail of lateral gonad wall. —**C.** Relationship of aorta and gonad showing infolds of gonad dorsal wall (arrow). —**D.** Detail of the aorta epithelium lining the initial portion of an infold of the gonad dorsal wall. *Abbreviations*: a. aorta; ae. aorta epithelium; ce. ciliated epithelium; dgo. dorsal gonad wall; evo. early vitellogenic oocyte; g. gonia; ge. germinal epithelium; gwe. gonad wall epithelium; pvo. previtellogenic oocytes; vo. vitellogenic oocyte; tt. tissue trabeculae. Scale bars: A = 500 μm, B = 100 μm, C = 50 μm, D = 20 μm.

42 μm diameter were surrounded, in transverse section, by about six FCs, this number increased up to about 70 FCs in late vitellogenic oocytes (Figs 1A, 2G–I, and 6C). Just before the onset of vitellogenesis, an increase in the cytoplasmic volume was accompanied by the progressive formation of numerous 'vacuole-like' electron-translucent spaces (not unlikely to be lipid partially extracted during processing of the material for histology or possibly early yolk material) uniformly sparse in the cytoplasm (Figs 2H and 4A). Vitellogenesis started in oocytes of about 100 μm in diameter (Fig. 2H). From the early (Fig. 2I) to late vitellogenic stage (Fig. 6C), the oocyte diameter increased up to 300 μm (Fig. 1B). Ripe and fresh spawned eggs (Fig. 6A,C) were about 386 μm diameter ($n$ = 20, SD = 13.1) (measured from serial semi-thin sections for TEM). The diameter of freshly spawned eggs is about 400 μm.

Approaching the gonad maturity and near the spawning season, a number of large yolky oocytes break down; the oocyte membrane and the follicular epithelium break and the vitellus content is liberated into the gonad lumen (Fig. 7A,B). The resorption of the materials from abortive oocytes is carried out by the epithelial cells of the nearby tissue trabeculae, whose cytoplasm appears full of vitelline droplets (Fig. 7C, D). Often, haemocytes were also seen among cells of the epithelium of tissue trabeculae, contributing to the resorption of vitelline materials (Fig. 7D).

Those gonads in which mid- and late vitellogenic oocytes are predominant always had at the base of the germinal epithelium a number of oogonia, primary and previtellogenic oocytes that are probably arrested in their development (Fig. 7E). Thus, usually two cohorts of oocytes are easily recognized in a gonad from mid- to late vitellogenesis. At a population level, after a brief spawning period that usually spans 1 or 2 months (see below), recovery of gonads for a new reproductive cycle proceeds rapidly from the clusters of oogonia and oocytes that arrested their development during vitellogenesis of the previous cohort of gametes (Fig. 7F). Information from the temporal variation in the oocyte size (Fig. 1C) and the histology of the gonads corresponding to each sampling episode showed that vitellogenesis in *T. lebruni* is a slow and long process; its completion usually takes 9–10 months.

A definite after-spawning resting stage was not observed. The recovery of female gonads for a new reproductive period started soon after spawning. Usually by November each year, the gonads showed an active proliferation of gonia, and oocytes in growing, pre- and vitellogenic phases (Fig. 7F).

### *Egg hull formation and morphology*

Under light microscopy, the egg hull appears to formed by two layers, each composed by materials of different chemical natures; the outer layer is orthochromatic with Toluidine blue,

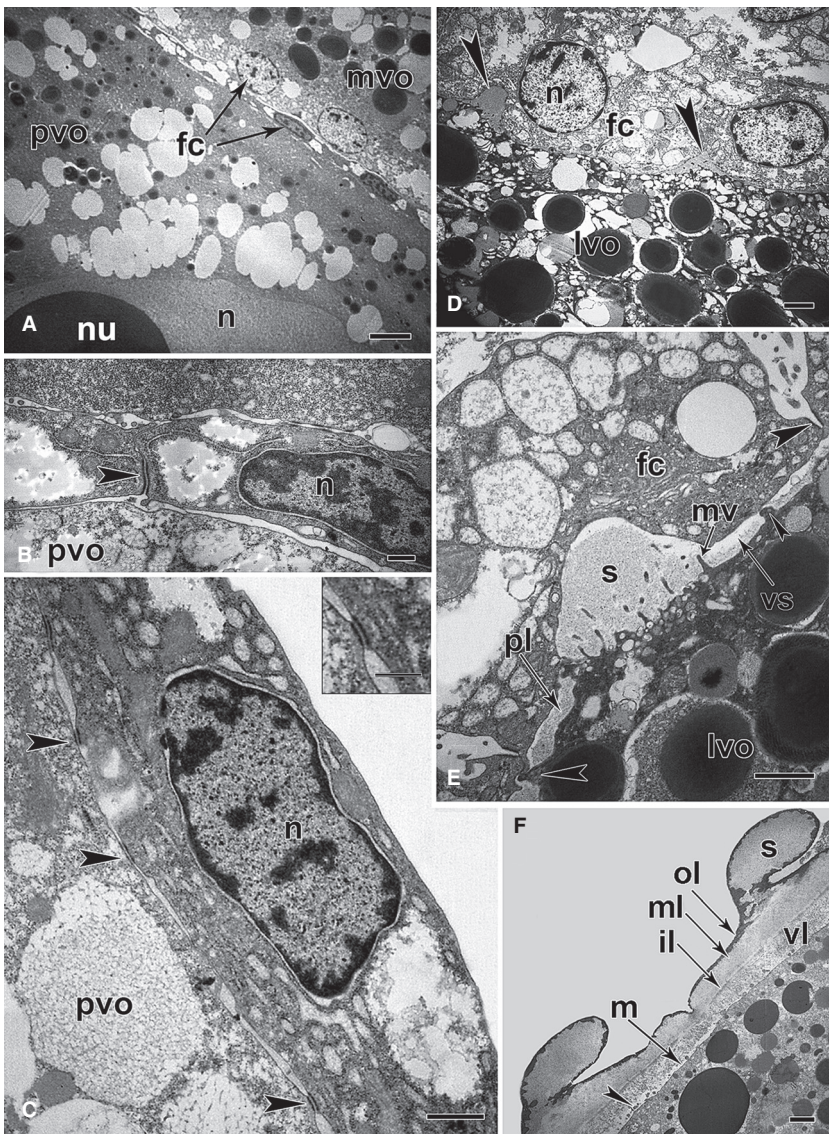


**Fig. 4**—*Tonicia lebruni*. Oocyte/follicle cell relationship and egg hull formation, TEM micrographs. —**A.** Follicle cells accompanying previtellogenic and mid-vitellogenic oocytes (note the electron-translucent areas). —**B.** Cell junction (arrowhead) between follicle cells. —**C.** Cell junctions between oocyte and follicle cell (insert: detail of a cell junction). —**D.** Initial phase of deposition of egg hull materials (arrowheads). —**E.** A more advanced stage of egg hull deposition, note the microvilli-like projections from the oocyte surface, intercellular space and cytoplasmic bridges between oocyte and FC (arrowheads). —**F.** Two adjacent plates and spines of a spawned egg with egg hull completely formed; note the vesicular secretions below vitelline layer (arrowhead). *Abbreviations*: fc. follicle cell; il. inner layer; lvo. late vitellogenic oocyte; mL. intermediate layer; mvo. mid-vitellogenic oocyte. mv. microvilli. mvo. mid-vitellogenic oocyte; n. nucleus; nu. nucleolus; ol. outer layer; pvo. previtellogenic oocyte; s. spine; vl. vitelline layer; vs. vacuolar space. Scale bars: A = 4 μm, B, insert in C = 0.5 μm, C, E = 1 μm, D, F = 2 μm.

while the inner layer is metachromatic, the latter a stain reaction compatible with acid mucopolysaccharides (Fig. 6A,B). When viewed under TEM, a third thin, irregular, electron-dense outermost layer is evident (Fig. 4F).

At the start of vitellogenesis, the deposition of materials for the egg hull becomes evident. At this time, the oocyte surface is covered by a large number of small polygonal flat cells of about 10 μm diameter, each one related to a future hull spine (Fig. 2F and 4A–C). At the end of its development, each hull plate bears a short spine abated towards the egg surface (Figs 4F, 5C,D and 6A,B,D,E). At the intermediate stages of egg hull formation, the SEM images show the surface of vitellogenic oocytes encompassed by a complete sheet of mitre-shaped FCs (Fig. 5B); this peculiar shape is owing to the pushing of material progressively accumulated beneath each FC in the process of egg hull formation (Fig. 4D,E; 5B). The TEM images of the initial stages of hull formation show, after the formation of intercellular spaces between FCs and oocytes, a progressive accumulation of material beneath the follicular epithelium. The FCs are at this stage sharply cuboidal, and the cellular junctions with the oocyte still persist as bridges limiting the spaces between oocyte and FCs (Fig. 4E). Some projections like microvilli extending from the cell membrane of the oocyte are seen, being possibly responsible for contributing material for the egg hull (Fig. 4E). These images are compatible with a mechanism of apocrine secretion of material from the oocyte cytoplasm.

Ripe oocytes and spawned eggs showed a completely formed egg hull (Figs 5C and 6D, E) with the surface bristled by short spines bent to the cell surface; after spawning, the follicular epithelium disappears (Fig. 4F). At the sides of the line of suture between plates of the egg hull, a relatively low number of pores (6–10 at each side of the polygonal plates) were observed (Fig. 5E).

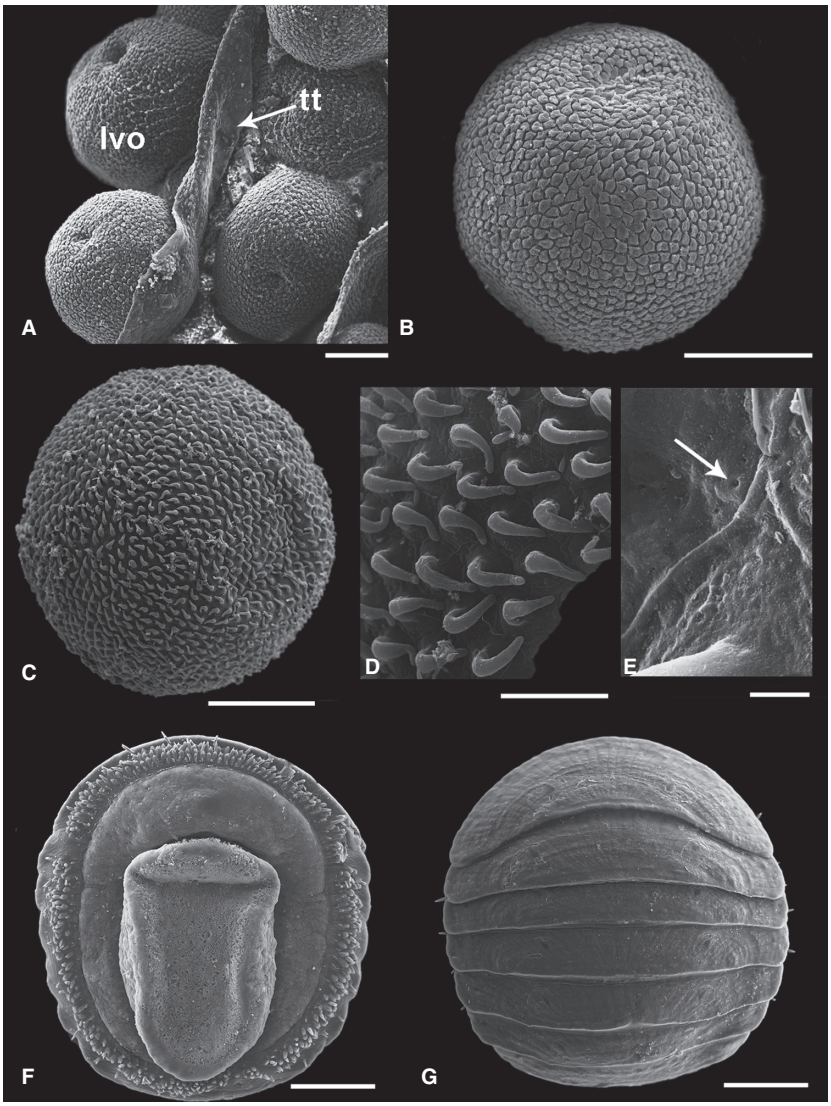


**Fig. 5**—A–G. Scanning electron microscopy. —**A.** Late vitellogenic oocytes attached to a tissue trabecula. —**B.** A vitellogenic oocyte showing mitre-shaped FCs at an intermediate stage of hull formation. —**C.** Spawned egg with completely formed egg hull. —**D.** Detail of egg hull spines. —**E.** Micropores (arrow) at sides of two adjacent egg hull plates. —**F**, —**G**. Fully developed juveniles showed in ventral (F) and dorsal (G) views. *Abbreviations*: lvo. late vitellogenic oocyte; tt. tissue trabecula. Scale bars: A–C, F, G = 100 μm, D = 20 μm, E = 2 μm.

*Brooding and seasonality of reproduction*

*Tonicia lebruni* brood their eggs to an advanced stage of development; a free larval stage is entirely absent; eggs are fertilized and brooded within the pallial groves, being released from the maternal individual as juveniles with a fully developed foot, perinotus ornamentation of spines and 8-plate shell (Fig. 5F, G). From the very early stages of the shell formation, the embryos appear within the pallial grooves devoid of the egg hull for which, despite not having made specific observations on embryonic development, it is likely that hatching takes place at early stages of larval development. The incubation period was relatively short; females with eggs or embryos in their pallial grooves were collected only in August–September 2009 (late austral winter–early spring) and September–October 2010 (early–mid-austral spring), and non-brooding female were collected in July 2009 and November 2010. Coincidently, between August and September of 2009 and 2010, and despite the relatively wide dispersion of values, a consistent lowering in the mean size of oocytes was observed (Fig. 1C).

The minimum and maximum sizes at which a female was found brooding embryos were 2.1 and 3.2 mm length, respectively. The number of eggs/embryos per female ($n$ = 6) varied between 785.5 ± 57 and 5945 ± 218. The clutch size was positively correlated with the female size (Pearson's coefficient $r$ = 0.94 $P$ < 0.01) (Fig. 1D). Embryos in an advanced stage of development appeared densely packed and immersed in a matrix of unknown origin that may help to hold the embryos together within the space of the pallial groove. The brooding egg mass can occupy nearly the entire space of the pallial groove, extending from the ad anal region to the level of the pre oral veil. As a result, the free volume of the pallial groove is restricted and the relatively large volume of the brood pushed the gills up towards the roof of the pallial groove.

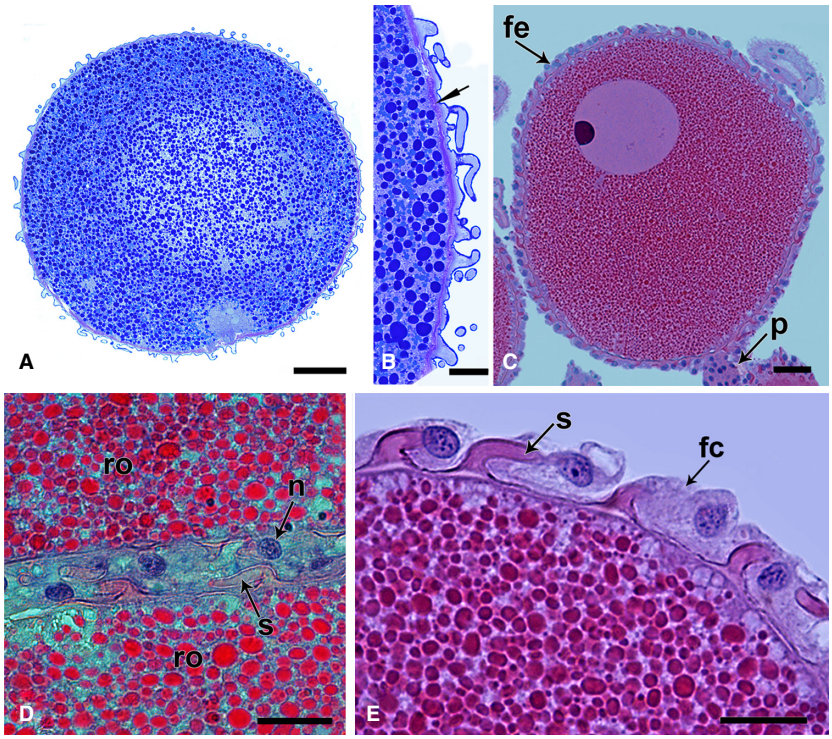


**Fig. 6**—*Tonicia lebruni*: egg hull and follicular epithelium. —**A.** Spawned egg (semi-thin section). —**B.** Detail of Figure A showing the positive metachromatic reaction of the inner layer of the egg hull (arrow). —**C.** Ripe oocyte with its complete follicular epithelium, being still attached to a tissue trabecula. —**D.** Two adjacent ripe eggs showing details of the FCs and the egg hull completely formed. —**E.** Ripe egg surface showing the follicular epithelium just before its detachment. *Abbreviations*: fc. follicle cell; fe. follicular epithelium; n. nucleus of FC; p. peduncle; ro. ripe oocyte; s. spine. Scale bars: A–C = 50 μm, D = 10 μm, E = 20 μm.

## Discussion

Most chitons reproduce by free spawning of gametes followed by indirect development throughout free-swimming trochophore larvae; however, species are known that retain the fertilized eggs in the pallial grooves protecting them for a variable time lapse (Liuzzi and Zelaya 2013; Sirenko 2015). Those which brood their eggs might retain eggs either only to the stage of trochophore larvae or for a longer period, to the last stage of metamorphosis. Twelve or thirteen species have been reported as brooding eggs that hatch as trochophore larvae, which are released and spend a short time as free swimmers. Twenty-seven species (*T. lebruni* among them, according to the results of the present study) brood embryos to the last stage of metamorphosis until develop an eight-shell valve, being released from the maternal individual as fully developed juveniles (except for *T. lebruni*, information from Sirenko 2015).

*Tonicia lebruni*, is a medium-sized species, reaching a maximum length of about 40 mm, and produces large eggs (about 400 μm diameter) that are retained and brooded within the pallial groove until an advanced stage of development, being released from the maternal individual as crawling juveniles of about 500 μm length, with a completely formed shell. Sirenko (2006a) reported for the first time the finding in a museum collection of several brooding females of *T. lebruni* from the Magellan Strait collected at the end of May 2000. Those specimens showed eggs in both pallial grooves, not allowing knowing further details of the extension of the brooding process. Later, Sirenko (2015), without giving new information, include *T. lebruni* among the species that brood their eggs only to the trochophore larval stage. Here, we showed that brooding in *T. lebruni* ends with the release of fully developed juveniles.

When the occurrence of brooding in Polyplacophora is analysed in a taxonomic context, according to the high-level systematic arrangement currently recognized (Sirenko 2006b) among the order Lepidopleurida (a basal clade in the phylogeny of chitons), six species of only two of the nine extant genera (five species of *Leptochiton* and one of *Hanleyella*) have been reported as brooding eggs to the last stage of metamorphosis; no species has been reported as brooding embryos only to the trochophore stage. Regarding the order Chitonida, the more speciose clade of Polyplacophora, the suborder Acanthochitonina, include 11 brooder species (six to the trochophore larvae and five to the last stage of metamorphosis), most of them are species of *Lepidochitona*, a genus in which the brood to the trochophore larva is predominant. The suborder Chitonina comprises 24 brooder species (nine to trochophore and 15 to the last stage of metamorphosis), most of them correspond to the families Chitonidae and Ischnochitonidae (namely the genera *Ischnochiton*, eight species, and *Chiton*, five species) (data gathered from Liuzzi and Zelaya 2013; Sirenko 2015). In this context, the wide range of taxa in which brooding is present strongly suggests that the reproductive pattern that includes the brooding of eggs and/or embryos is a life history trait that appeared several times in the evolutionary history of Polyplacophora, as it was early proposed by Pearse (1979).

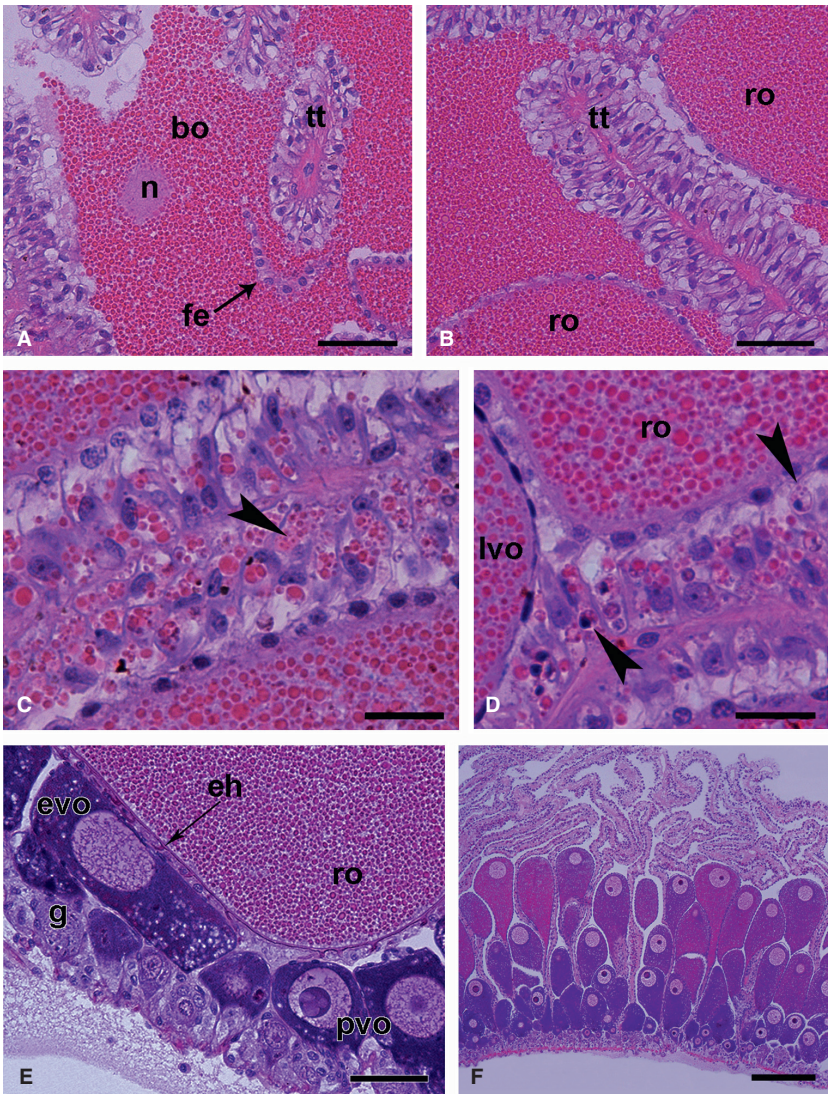


**Fig. 7**—*Tonicia lebruni*: ova breakdown, resorption and gonad recovery. —**A.** Aborted oocyte showing remains of the broken follicular epithelium and cytoplasmic content filling the space between tissue trabeculae. —**B.** Vitelline material of a broken oocyte contacting the epithelium of a tissue trabecula. —**C.** Reaction of the epithelium of a tissue trabecula after having engulfed vitelline droplets (arrow). —**D.** Detail of a portion of epithelium of a tissue trabecula active in resorption, and haemocytes with engulfed vitellus droplets (arrows) accompanying the resorption process. —**E.** Detail of a prespawning mature gonad showing a ripe oocyte and, below, the germinal epithelium ready to restart a new cycle of oogenesis after ova evacuation. —**F.** A restored gonad epithelium about 30 days after spawning. *Abbreviations*: bo. broken oocyte; eh. egg hull; evo. early vitellogenic oocyte; fe. follicular epithelium; g. gonia; tt. tissue trabecula; lvo. late vitellogenic oocyte; n. nucleus; pvo. previtellogenic oocytes; ro. ripe oocyte. Scale bars: A, B = 50 μm, C, D = 20 μm, E = 50 μm, F = 250 μm.

Regarding the adult size of brooding species, it has been argued on the existence of a relationship between adult size and brooding in marine invertebrates: brooders often have relatively small sizes (Strathmann and Strathmann 1982). Pearse (1979) and Eernisse (1988) documented the link between brooding and small adult size in chitons, as a general trend. According to the most recent compilation of information (Sirenko 2015: tables 2, 3), except for few extreme cases such as *Eudoxochiton inornatus* (100 mm length), *Placiphorella borealis* (56 mm length), *Ischnochiton subviridis* (37–50 mm length) and *Callochiton crocinus* (17–50 mm length), the body length of brooding species is below 25 mm (data gathered from Sirenko 2015). The case of *T. lebruni*, with an adult size of 27 mm (maximum length is about 40 mm), is slightly above this general tendency (we found brooding females between 2.1 mm and 3.2 mm length). A definite pattern for a relationship between adult size, number of eggs and stage of development to which eggs are brooded is not evident. Chitons that brood their eggs within the female pallial grooves to the trochophore stage range from 4.7 to 100 mm, and the number of brooded eggs varied (in the relatively few cases in which this was possibly to be determined) between 100 and 700. Length of species that brood their eggs to the last stage of metamorphosis range between 4 and 56 mm length and reported brood size varied from 4 to 3000 eggs (see latest update by Sirenko 2015: tables 2, 3). However, in the latter case, excluding the five species of the genus *Leptochiton*, the adult size surpasses the 10 mm length.

At the moment of making comparisons to uncover general trends or patterns to describe the different characteristics of the phenomenon of brooding in a global taxonomic context, it is important to take into account the limitations imposed by the information at hand, which is rather scant, based in many cases on few observations, often partial (obtained from a few

specimens preserved in collections), or coming from incidental observations, for which the information is not always comparable. This shows the importance of the present study, for a species that was originally identified as brooding from museum specimens (Sirenko 2006a) and can now be understood in detail from field and laboratory observations.

The occurrence of parental care in a medium- or small-sized species by keeping embryos up to a juvenile stage of 500 μm length restricts the number of eggs that can be protected in the limited space of the pallial grooves. The case of *T. lebruni* is particularly interesting because, being a medium-sized species, females are able to brood up to nearly 6,000 eggs/embryos, while the maximum reported number of brooding embryos is 3,000 in *Ornithochiton neglectus* a species with an adult size of 10–13 mm length (Sirenko 2015: table 3). Such limitation for space imposes an indirect restriction to fecundity, a fact that may be in the origin of the intense prespawning resorption processes observed throughout this study. The resorption of surplus ova seems to be an effective way to avoid the significant loss of energy that would represent the spawning of ova containing large amounts of vitelline material, without possibility of being brooded. The involvement of the epithelium of the tissue trabeculae in the resorption of the vitelline material from aborted late vitellogenic or ripe oocytes is here reported for the first time in chitons. Creese (1986) found in *Onithochiton neglectus*, a species smaller than *T. lebruni* (<30 mm length), a similar limited egg production (550–3,000 eggs per brooder); however, information on oogenesis or the possible occurrence of ova abortion and resorption processes was not described. Resorption of abortive female gametes in different developmental stages was reported in other molluscs (for a review, see Jong-Brink *et al.* 1983). However, a phenomenon as observed in *T. lebruni* was only described in the bivalve *Eupera platensis* (Sphaeriidae), a small species that produces large eggs, facing severe limitations of space for brooding in the reduced volume of the interlamellar space of inner demibranchs; in *E. platensis*, the resorption is undertaken by the cells of the acinus wall (referred therein as follicle cells) (Ituarte 1997).

The number of brooded egg/embryos and the size (length) of the maternal individual showed a positive relationship. The positive relationship between clutch size and mother size is congruent with the relationship between an expected increase in fecundity with size (age) of females and the need of increase in the brooding space. Despite the fact that, in the limited number of cases studied, a linear relationship between mother length and number of brooded embryos was found, it is likely that the volume of the pallial groove, the brood space, increases according to a relationship other than linear (cubic?) with respect to the mother length, as the gonad volume does. Thus, there would be a relatively balanced relationship between the volume of gametes emitted at different ages of a female and the space available for incubation. If this is so, the explanation of the intense resorption of abortive ova approaching the reproductive season should be other than the constraint imposed by the restrictions in space for brooding.

Eernisse and Reynolds (1994) claimed that compared with other invertebrate groups, the egg size of brooding chitons does not differ greatly from those of free spawner species. However, the egg size of *T. lebruni*, about 400 μm, is comparatively larger when compared with other brooding and non-brooding chiton species; only as examples, egg size reported for other brooders is *I. stramineus*: about 200 μm (Liuzzi and Zelaya 2013; fig. 1f), *Ischnochiton mayi*: 300–400 μm (Cochran 1986), *Lepidochitona stroemfelti*: 200–230 μm (Strack 1987), *Eudoxaplax inornata*: 200 μm (Turner 1978), *Schizoplax brandtii*: 400 μm (Pearse 1979: table VI), *Cyanoplax fernaldi* and *Cyanoplax thomasi*: 260–280 μm, and *Cyanoplax caverna*: 220–240 μm (Eernisse 1988). Among free spawners, species for which egg size is known are, for example, *Cyanoplax berryana*, 220–240 μm; *Cyanoplax dentiens* and *Cyanoplax hartwegii*: 200–220 μm (Eernisse 1988); *Chaetopleura isabellei* and *Plaxiphora aurata* about 300 μm (Ituarte *et al.* 2010). The differences in egg sizes reported may be due, at least in part, to the condition of the material (fresh spawned eggs, variation in fixation and preparation, etc.) from which measurements were obtained. Reports of brooder species with very large-sized brooded embryos (not egg size) are found in the reviews by Pearse (1979: table VI) and Sirenko (2015: table 3): *Hemiarthrum setulosum* from subantarctic and Antarctic waters, and *Ischnochiton mayi* from Tasmania, about 800 μm; *Chiton nigroviriscens* from South Africa, 700–800 μm, 950 μm according to Sirenko (2015).

Regarding the mechanism of hull formation in *T. lebruni*, our information is still partial and provisional; however, there is some evidence that indicates that the mechanism of secreting materials would be similar to that reported by Buckland-Nicks and Reunov (2009) in *Rhyssoplax tulipa*, a process that comprises the formation of intercellular spaces between FCs and the oocyte and the apocrine secretion of materials for the hull in the form of extensions of the oocyte cytoplasm like microvilli that break along their length releasing their content as a part of materials for the egg hull. Microapocrine secretion also has been reported as the mechanism of secretion of products to build the hull in oocytes of *Callochiton dentatus* (Buckland-Nicks and Reunov 2010) and *Acanthochitona garnoti* (Buckland-Nicks 2014). Further, TEM studies would allow describing this process in detail.

The egg hull structure has been recognized as a relevant feature with significant importance for systematics (Sirenko 1993, 2006b; Buckland-Nicks 2006, 2008; Okusu *et al.* 2003). At high taxonomic level, there is a clear general pattern in egg hull structure: the order Lepidopleurida has smooth jelly-like hulls, while representatives of the order Chitonida have elaborated hull surfaces (with few cupules or cone-like projections arising from wide bases (50–90 μm) in Acanthochitonina, or numerous long and thin spines with narrow bases (5–30 μm) in Chitonina). Most genera in the family

 

Chitonidae have egg hulls with spines usually ending in petaloid tips (Sirenko 1993: figure 6). The spine morphology in *T. lebruni*, short with pointed tips, and bent onto the egg surface, seems to be unique among Chitonidae, and it is likely related to the brooding habit. Furthermore, the spine morphology of *T. lebruni* is quite different to that of other known species of the genus. *Tonicia chilensis* and *Tonicia* (*Lucilina*) sp., as other Chitonidae, have spines with petaloid tips (Sirenko 1993: figure 6 A,B,E).

According to Eernisse (1988); Buckland-Nicks (2006) and Buckland-Nicks and Brothers (2008), in many brooding chitons, there is a tendency to the reduction in the spines or cupules, that is to have less projecting egg hulls, a fact that is interpreted as a mean to achieve a better packing and storage of a high number of eggs in the limited space of the pallial grooves, pointing on exceptions such as the case of *Radsia nigrovirescens* that retained long hooked spines that could aid to the eggs interlock. The ornamentation of the egg hull in *T. lebruni*, with a high number of short spines abated to the egg surface, may be viewed as a morphologic trait that would favour accommodation and cohesiveness of the numerous eggs within the limited space of the pallial groove. Liuzzi and Zelaya (2013) also interpreted the low number of curled projections of hulls in *Ischnochiton stramineus* as an effective interlocking device facilitating the aggregation of the eggs in a mass within the pallial grooves.

Creese (1986) reported that eggs in *Onithochiton neglectus* are held together by mucous secretions, the same was reported in *Cyanoplax thomasi* and *Cyanoplax fernaldi* (Eernisse 1988). In *T. lebruni*, the freshly spawned eggs masses lodged within the pallial grooves may be easily disaggregated, as was observed in *Cyanoplax caverna* in which the eggs are held loosely (Eernisse 1988). However, in *T. lebruni*, brooding embryos in a more advanced stage of development and even juveniles were immersed in a mass of unknown origin (probably debris of the decayed egg hulls, as it has been observed in egg masses containing recently hatched larvae), which could contribute to hold together the embryos within the widely open space of the pallial groove, but a glandular region in the proximal portion of oviducts reported by Pearse (1979) was not observed. This glandular epithelium probably produces, according to the review by Pearse (1979), the mucus that envelopes the spawned eggs in species such as *Katarina tunicata*, *Mopalia ciliata and Tonicella lineata*.

It has been reported before that brooding chitons do not show a well-defined seasonal period of reproduction as most free-spawning species do. Creese (1986) and Creese and O'Neill (1987) reported for *Onithochiton neglectus* and *Chiton aorangi* an extended brooding period along the year, except for winter months. Eernisse (1988) found in three small brooding species of *Cyanoplax* (*C. fernaldi*, *C. thomasi* and *C. caverna*) (all of them <17 mm length) that they 'brood for much or all of the year'. Liuzzi and Zelaya (2013) found brooding specimens of *Ischnochiton stramineus* from Magellan waters, in the austral winter and summer seasons. In contrast, the population of *T. lebruni* studied at Puerto Deseado do not follow this pattern, showing a restricted period of spawn; females brooding eggs or embryos were found in 2009 and 2010 reproductive seasons only in August–September (late winter–early spring) or September–October (austral spring), respectively. After this well-defined period of reproduction, ovaries showed a quick postreproductive recovery, reprising oogenesis soon after spawning. However, vitellogenesis appears as a slow process that takes about 10 months for completion; mature females with ovaries containing oocytes that have completed vitellogenesis were observed only by June–July each year.

Despite the restriction to the dispersal capacity of a species imposed by the suppression of a free-swimming larval stage, *T. lebruni* has a relatively wide distribution range, being present along rocky shores over 1500 km on the western Atlantic Ocean for which the existence of some unknown historical mechanism of dispersal (e.g. kelp rafting, some kind of migration) would be expected.

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