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Unusual modes of oogenesis and brooding in bivalves: the case of *Gaimardia trapesina* (Mollusca: Gaimardiidae)

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Abstract. I describe an unusual case of follicular oogenesis in a bivalve, *Gaimardia trapesina,* a common marine bivalve from the Magellan Region and adjacent Sub-Antarctic waters, whose members brood their developing larvae. The gonad in *G. trapesina* is an acinus organ that infiltrates the perivisceral connective tissue; the walls of the acini are formed by tall, slender cells with distal nuclei, supported by a thin conjunctive tissue layer. At the onset of vitellogenesis, each developing oocyte becomes surrounded by a one-cell-thick layer of follicle cells, which may originate from the wall of the acinus. The cells form a follicle that completely encompasses single oocytes, except at the basal zone, where oocytes are in contact with the acinus wall. The follicle persists beyond the end of vitellogenesis and spawning. After gamete release, the persistent follicle participates in the attachment of ova and developing embryos to the interfilamental junctions of the inner and outer demibranchs of the gill, where embryos are incubated until hatching as late-stage pediveliger larvae. Ripe eggs are large (~250 μm diameter), suggesting that development is entirely lecithotrophic. The follicle cells that mediate connections between developing embryos and the maternal individual probably have a mechanical role only, providing support and possibly facilitating the accommodation of a large number of embryos to maximize the branchial space available for brooding.

Additional key words: reproduction, follicular oogenesis, brooding

Among molluscs, the presence of a follicle surrounding each developing oocyte is well known in polyplacophorans and cephalopods (Selwood 1968, 1970; Selman & Arnold 1977). In many gastropods, in the early stages of oogenesis, oocytes become associated with a variable number of cells (usually few), currently referred to as "follicle cells," which seal off the oocyte from the acinus "environment" to a variable degree (Taylor & Anderson 1969; Jong-Brink et al. 1983; Pal & Hodgson 2001). After the start of the oocyte growth phase or just at the onset of vitellogenesis, the follicle cells are pushed aside by the growing oocytes, so that the oocytes become exposed to the lumen of the acinus (Pal & Hodgson 2001, 2002). In these cases, the "follicle" surrounding the oocyte constitutes a temporary structure, persisting in some cases up to the late vitellogenic stage (Pal & Hodgson 2002).

Oogenesis in bivalves has largely been known as the solitary type, i.e., without the formation of a follicle surrounding developing oocytes (e.g., Raven 1958; Sastry 1979). In the early stages of oogenesis in the freshwater species *Sphaerium striatinum* Lamarck 1818, *Musculium heterodon* Pilsbry 1895, and *Eupera platensis* Doello-Jurado 1921, a short-term association of developing oocytes with a small number of cells from the acinus wall has been reported (Woods 1931; Okada 1935a; Ituarte 1997). Shortly after the start of the growth phase of the oocyte, these cells of the acinus wall detach from the apex of the oocyte, which then bulges freely into the acinus lumen. Eckelbarger & Davis (1996) reported, in *Crassostrea virginica* Gmelin 1791, a close association of follicle cells and oocytes during the early and middle stages of vitellogenesis, but during later vitellogenic stages the follicle cells are largely confined to the basal stalked region of oocytes. Follicle cells (referred to as "auxiliary cells") have also been reported in Mytilidae (Pipe 1987), Pectinidae (Dorange et al. 1989), and Pinnidae (De Gaulejac et al. 1995), in these cases, in the middle or advanced stages of vitellogenesis, and the follicle cells are always restricted to the stalk region of the oocyte. Motavkine & Varaksine (1983, cited in Dorange et al. 1989) reported, in *Patinopecten yessoensis* Jay 1857 and *Crenomytilus*

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grayana DUNKER 1853, that "auxiliary cells" completely surround oocytes when they are free in the acinus lumen, but further details on this association were not given. *Pseudokellya cardiformis* SMITH 1885 is the only known case among bivalves with oogenesis of a true follicular type (Pelseneer 1903). An article of this unique case of follicular oogenesis in a bivalve appeared in the scientific reports of the Belgium Antarctic Expedition, an article mainly dealing with systematics, and the observation has largely been overlooked. Recently, Zelaya & Ituarte (2009) confirmed this mode of oogenesis for the genus.

Little is known about the biology of members of *Gaimardia trapesina* LAMARCK 1819, a common brooding marine bivalve that lives attached to the giant kelp *Macrocystis pyrifera* LINNAEUS 1771 in the Magellan Region and Sub-Antarctic waters (Pelseneer 1903). Some data on the general reproductive biology in *G. trapesina* were provided by Pelseneer (1903) and Simpson (1977). Ituarte et al. (2001) described tissue reactions in *G. trapesina* against larval digenetic trematodes (Metacercariae), a parasite that does not affect reproduction of the bivalve host.

In the present article, the structural aspects of oogenesis and brooding in *G. trapesina* are described at the light microscope level; preliminary ultrastructural observations on selected stages of oogenesis and brooding are also given.

Methods

Specimens of *Gaimardia trapesina* were hand collected from kelp fronds at Beagle Channel, Ushuaia (54°48′S, 68°19′W), Tierra del Fuego, during December 2007. Specimens were fixed at room temperature either in 10% formalin solution or Bouin' s fluid immediately after collection; adductor muscles were sectioned to facilitate the fixative penetration. Tissues were dehydrated in an ascending ethanol series and embedded in methyl methacrylate resin (Historesin®, Leica Microsystems, Germany) and sectioned at 2–3 μm. Sections were stained with either Mayer's or Groat's hematoxylin and eosin (Gabe 1968). For transmission electron microscopy (TEM), small portions of the visceral mass containing gonad tissue were fixed at 4°C for 10–12 h in a solution of 4% glutaraldehyde and 2% formaldehyde (obtained from paraformaldehyde) in 0.1 mol L^{-1} phosphate buffer (pH 6.9–7). After fixation, tissues were washed in 0.1 mol L^{-1} phosphate buffer and postfixed in 1% osmium tetroxide in the same buffer solution for 90 min. After rinsing in phosphate buffer, tissues were dehydrated in ascending alcohols and infiltrated (via propylene oxide) in Polybed 812® (Polysciences, Warrington, PA, USA). Ultrathin sections were stained with uranyl acetate and lead citrate.

Vouchers, alcohol-preserved specimens, and a series of histological sections were deposited at the Division of Invertebrate Zoology, Museo de La Plata (MLP 5575, 6726), and the Museo Argentino de Ciencias Naturales (MACN-In 37536).

Results

Gaimardia trapesina is a gonochoristic species. Females produce a high number of large yolky eggs (~250 μm diameter) that are brooded in both the inner and the outer demibranchs of the adult gill until an advanced stage of development. Offspring are released as late pediveliger larvae (~0.5 mm length) (Fig. 1A). The ovary in *G. trapesina* is an acinus organ; branched acini ramify throughout the visceral mass (Fig. 1B). The acinus wall consists of a layer of tall columnar, sometimes club-shaped cells (15–20 μm tall) (Fig. 1C,D).

Oogenesis

At the initial stages of development, oogonia, which were rounded or slightly ovate (~10 μm diameter) with a scant cytoplasm, and early previtellogenic oocytes (12–27 μm diameter) with large nuclei (9–19 μm) were scattered along the wall of the acini in close contact with cells of the acinus wall (Fig. 1C,D). Once the previtellogenic oocytes entered the growth phase, they bulged into the lumen of the acinus, remaining connected to the acinus wall by a stalk of egg cytoplasm (Fig. 2A). At the end of the growth phase, previtellogenic oocytes attained ~50–70 μm diameter, with rounded nuclei (~25–28 μm diameter) and conspicuous nucleoli (~13 μm diameter); the ooplasm was still basophilic (Fig. 2A). At this stage, some wandering cells were seen in the lumen of the acini, close to the acinus wall (Fig. 2A,B). Not infrequently, mitosis of cells of the acinus wall was observed (Fig. 2C).

Vitellogenesis and folliclegenesis

At the onset of vitellogenesis, the ooplasm showed very small, rounded acidophilic vitelline droplets (maximum size ~1.9 μm diameter), first visible at the oocyte stalk region (Fig. 2C,D). Early vitellogenic oocytes were ~90–120 μm in diameter (Fig. 2B). At the beginning of follicle formation, usually ten to 30 follicle cells per transverse section were in contact with the oolemma of early vitellogenic oocytes (Fig. 2B,C). At this stage, follicle cells were lenticular or

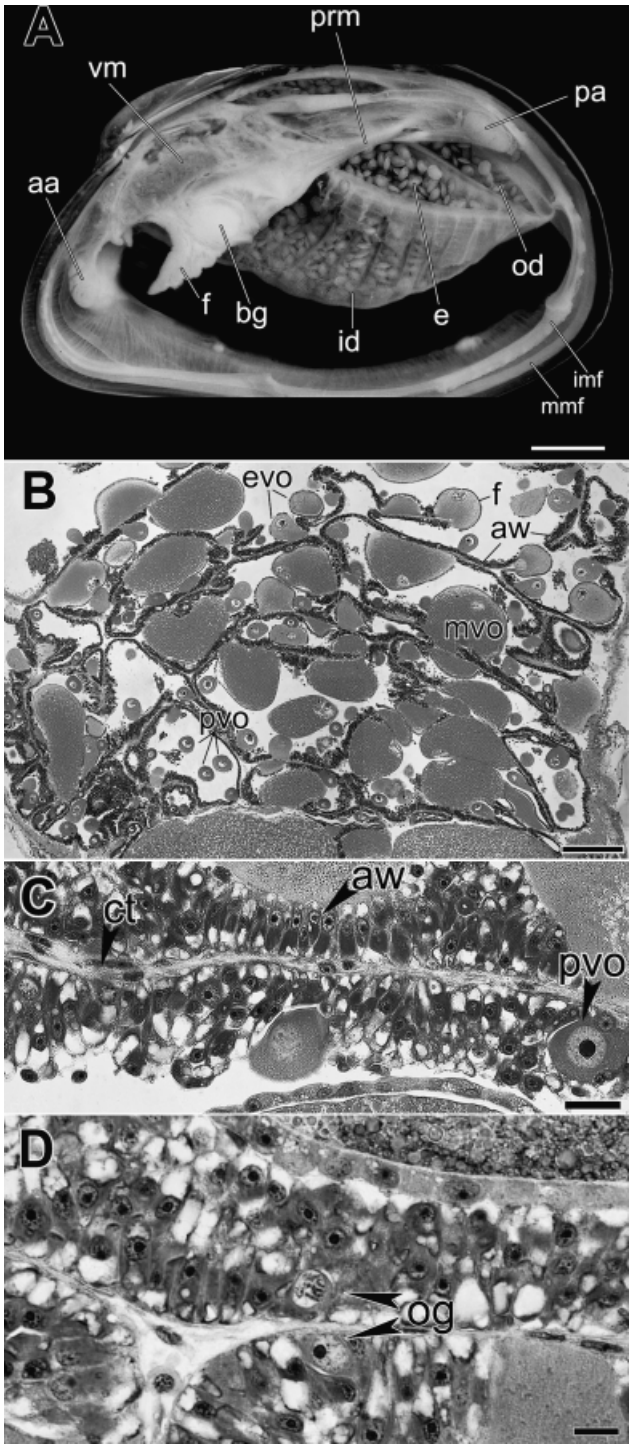

Fig. 1. A. Specimen sectioned through the sagittal plane showing inner and outer demibranchs with brooded embryos (aa, anterior adductor muscle; bg, byssus gland; e, embryos; f, foot; id, inner demibranch; imf, inner mantle fold; mmf, medium mantle fold; od, outer demibranch; pa, posterior adductor muscle; prm, pedal retractor muscle; vm, visceral mass). Scale bar = 2.5 mm. **B.** Gonad structure, transverse section of the gonad with previtellogenic, and early and mid-stage vitellogenic, oocytes (aw, acinus wall; evo, early vitellogenic oocyte; f, follicle; mvo, mid-vitellogenic oocyte; pvo, previtellogenic oocyte). Scale bar = 200 μm. **C.** Detail of the acinus wall (aw) and connective tissue (ct) between adjacent acini (pvo, previtellogenic oocyte). Scale bar = 20 μm. **D.** Detail of the acinus wall with oogonia (og) surrounded by cells of the acinus wall. Scale bar = 10 μm.

squamous-like cells (~8–11.5 μm maximum diameter) with rounded or slightly ovate prominent nuclei (~4.5–5.5 μm diameter) and small nucleoli (~1.5–2 μm); spots of chromatin were scattered on the inner surface of the nuclear membrane or were sparsely distributed within the nucleoplasm; the cytoplasm of follicle cells was scant, showing a discrete vacuolization (Fig. 2C). As vitellogenesis progressed, the number of follicle cells increased. At the end of folliclegenesis, each early vitellogenic oocyte was encompassed by a continuous one-cell-thick follicle epithelium (Fig. 2D,E). In this way, oocytes became ''sealed off'' from the acinus lumen, remaining in contact with the acinus wall at the basal region, the only point where the continuity of the follicle epithelium was interrupted. In mid vitellogenic oocytes, follicle cells became more slender and longer (cytoplasm ~12–17 μm length, nucleus ~5.5–8 μm, nucleolus ~2.3–2.7 μm) and their cytoplasm was vacuolated (Fig. 2E). In the late vitellogenic stages, oocytes reached ~250 μm in diameter and follicle cells further elongated (usually >15 μm length), acquired additional acidophilic inclusions, and their nuclei became more elliptical (~8.6-μm maximum diameter) (Fig. 2F, inset). At the ultrastructural level, the surface of mid vitellogenic oocytes had simple, unbranched microvilli with slightly enlarged tips that supported a fuzzy glycocalyx (Fig. 3A,B).

At the end of vitellogenesis, ripe oocytes detached from the acinus wall and the investing follicle epithelium showed a distinct portion ~160 μm in diameter, which consisted of columnar cells (15–19 μm tall) (Fig. 3C,D). After release, ova were retained within the water tubes of both the inner and the outer demibranchs, where they were fertilized.

Brooding

The follicle, which still encompassed the ova after gamete release, participated in the mechanism that attached the fertilized eggs and embryos to the interfilamental junctions of ascending and descending branchial filaments of both the inner and the outer

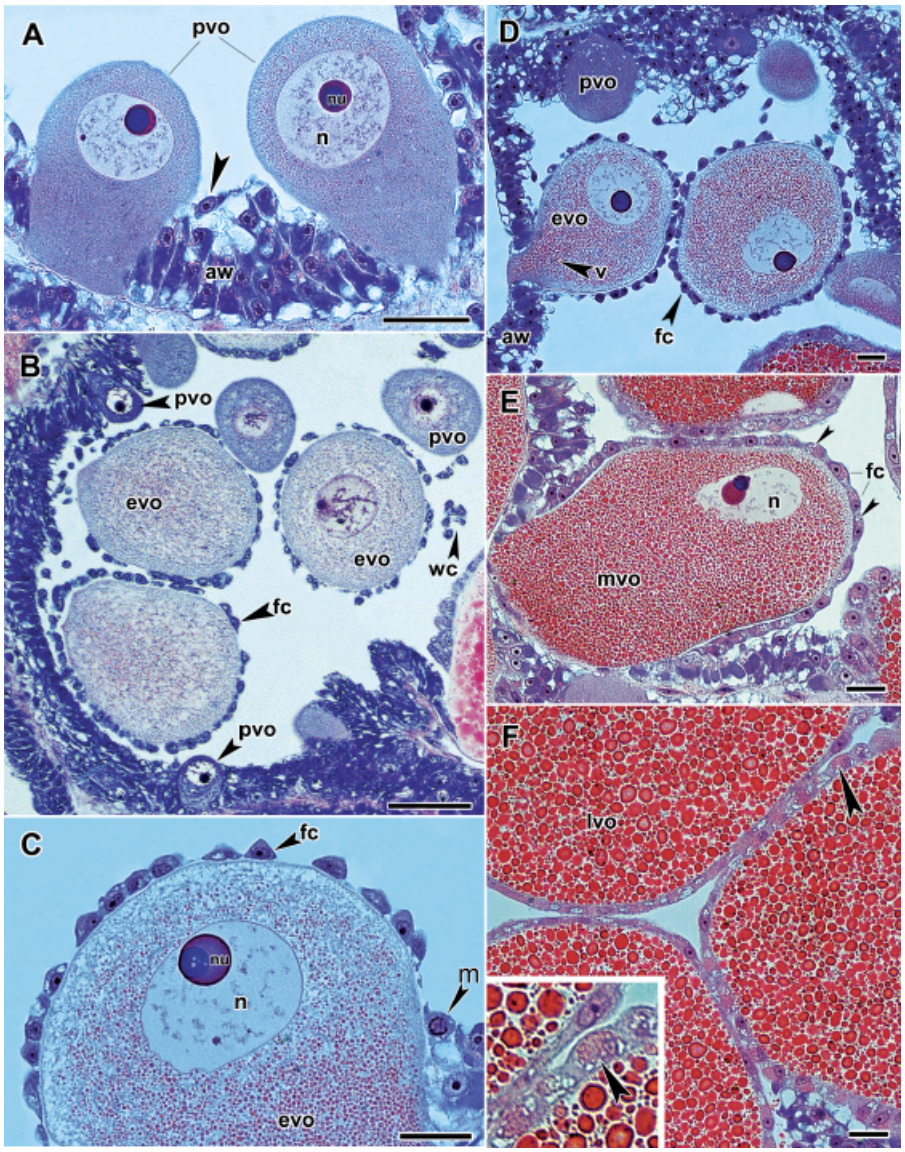

Fig. 2. Stages of oogenesis. **A.** Previtellogenic oocytes (pvo), attached to the acinus wall (aw), with nuclei (n) and prominent nucleoli (nu); the arrow indicates a cell of the acinus wall detached. Scale bar = 10 μm. **B.** Onset of vitellogenesis; early vitellogenic oocytes (evo) are surrounded by follicle cells (fc); some wandering cells (wc) remain in the acinus lumen and close to the acinus wall (pvo, previtellogenic oocytes). Scale bar = 50 μm. **C.** Detail of an early vitellogenic oocyte (evo) showing minute vitelline droplets in the ooplasm; a number of follicle cells (fc) are in contact with the oolemma at the initial stage of the follicle formation; a cell of the acinus wall is undergoing mitosis (m); (n, nucleus; nu, nucleolus). Scale bar = 20 μm. **D.** Two early vitellogenic oocytes (evo) with associated follicle cells (fc), the left one shows that storage of vitelline droplets (v) starts at the oocyte stalk region (aw, acinus wall; pvo, previtellogenic oocyte). Scale bar = 20 μm. **E.** Mid-vitellogenic oocyte (mvo) showing the increase in the ooplasm volume due to vitelline material accumulation; the folliclegenesis is completed; follicle cells (fc) show a subquadrangular shape and a vacuolated cytoplasm (arrows) (n, nucleus). Scale bar = 20 μm. **F.** Three late-stage vitellogenic oocytes (lvo) with the ooplasm filled with gross vitelline droplets; follicle cells show an elongate shape and the cytoplasm with deposits of acidophilic material (arrow and detail in the inset on left down corner). Scale bar = 20 μm.

demibranchs (Fig. 4A). Two or three broods of embryos at different developmental stages usually coexisted within females. Each embryo was secured to the branchial tissues by means of an attachment structure, here called the anchorage plate. The anchorage plate closely resembled the distinctive portion of the follicle epithelium investing ripe oocytes that were free within the gonoducts (Fig. 3C,D). The an-

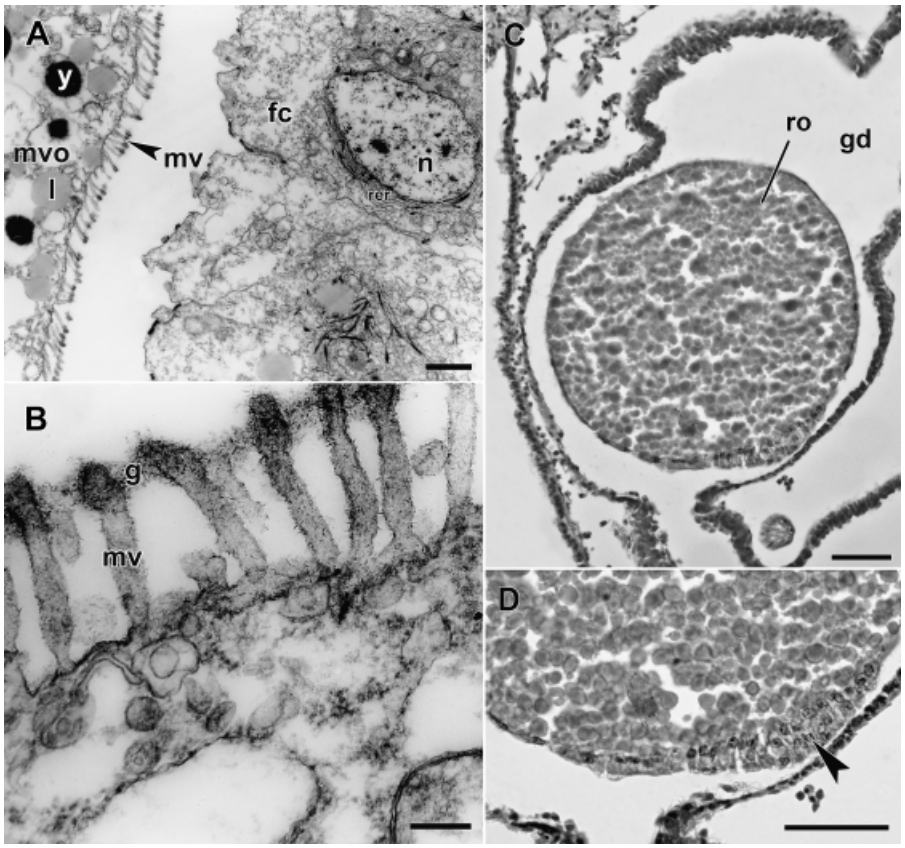

Fig. 3. Oogenesis. **A.** TEM micrograph of a mid-vitellogenic oocyte (mvo) with lipid droplets (l), yolk bodies (y), and unbranched surface microvilli (mv), adjacent follicle cells (fc) with conspicuous nucleus (n), and rough endoplasmic reticulum arrays (rer). Scale bar = 1 μm. **B.** Higher magnification (TEM) showing surface microvilli (mv) with conspicuous glycocalyx (g). Scale bar = 200 nm. **C.** A ripe oocyte (ro) free within the lumen of the gonoduct (gd) showing a differentiated zone of the follicular epithelium corresponding to the anchorage plate. Scale bar = 50 μm. **D.** Detail of the anchorage plate (arrow). Scale bar = 50 μm.

chorage plate had a discoid shape (Fig. 4B,C), being formed by cylindrical or club-shaped cells with rounded nuclei, and the cells were arranged in a radial pattern from the center of the plate. The cytoplasm of the cells forming the anchorage plate was clear, with large vacuolar spaces commonly containing some acidophilic material (Fig. 4B,C). The anchorage plate of each embryo was connected to the branchial filaments at the point of an interfilamental junction.

Specimens collected in December 2007 showed recently released eggs within the water tubes of the outer and inner demibranchs of the gills. A cumulus of sperm was seen in the vicinity of these eggs and the sperm were particularly associated with the anchorage plate (Fig. 4B) of the follicle. Sperm were also commonly seen in the spaces between cells of the anchorage plate (Fig. 4D).

The follicle epithelium persisted throughout the entire embryonic development, and was easily discernible from recently fertilized eggs to late-stage pediveliger larvae that were ready to be released (Fig. 5A,D). At the light microscope level, the follicle that encompassed each egg or embryo appeared closely attached to the anchorage plate (Fig. 4A). At the ultrastructural level, a gap was observed between the anchorage plate and the follicle-embryo (Fig. 5B); the plasma membrane of the cells forming the anchorage plate that faces the follicle of developing embryos appeared profusely expanded in somewhat short digitiform projections, which were simple or bifurcated (Fig. 5B,C).

Discussion

The terminology applied to cells forming the acinus wall and other cells associated more intimately with developing oocytes is confusing; the terms ''acinus'' and ''follicle'' have been used inconsistently to refer to the functional unit of the bivalve gonad. Frequently, cells delimiting the acinus have either been referred to as ''follicle cells'' (Sastry 1979; Jong-Brink et al. 1983; Pipe 1987; Johnson & Le Pennec 1994; Eckelbarger & Davis 1996; Yung Chung 2007), ''auxiliary cells'' (Dorange et al. 1989; De Gaulejac et al. 1995; Yung Chung 2008), or ''ovarian epithelium'' (Okada 1935a). This confusing terminology may have led to a misinterpretation (Pipe 1987; Dorange et al. 1989) of Raven's (1958) statements regarding the ''solitary'' condition of oocyte development in bivalves. It seems clear that Raven (1958) only meant to point out the absence among bivalves of a true

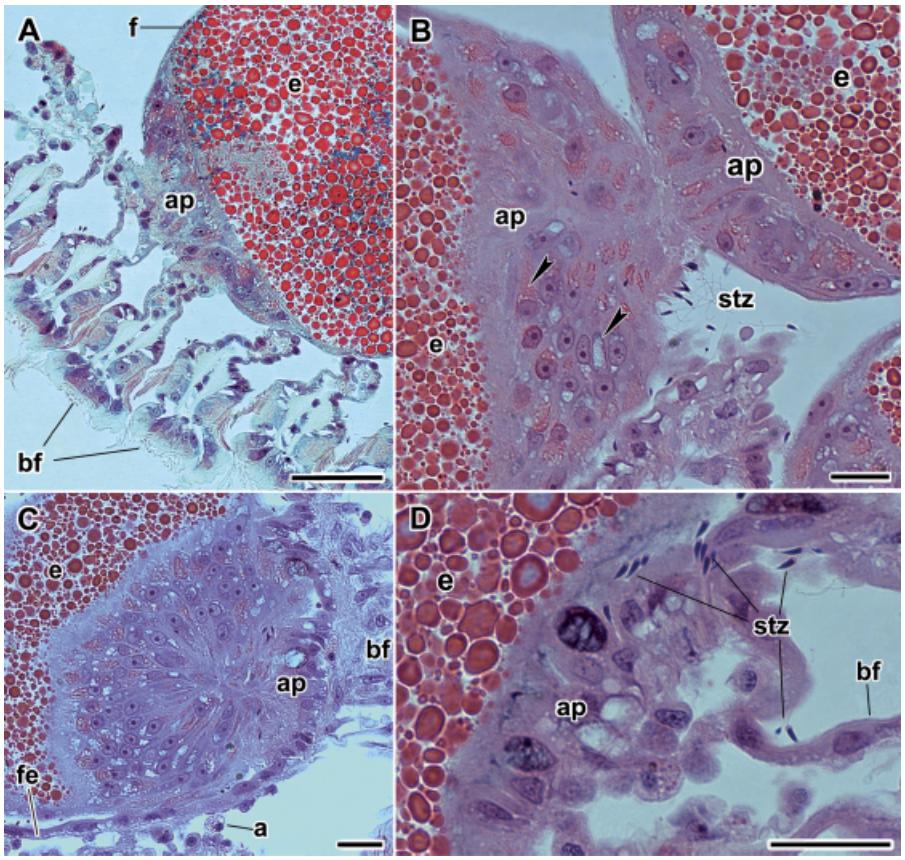

Fig. 4. Brooding. **A.** Still-uncleaved egg (e) attached to interfilamental junctions of branchial filaments (bf) with persistent follicle (f) and anchorage plate (ap). Scale bar = 50 μm. **B.** Sections through two adjacent eggs (e) and a number of sperms (stz) concentrated at the base of anchorage plates (ap) (arrows indicate vacuoles in the cytoplasm of the anchorage plate cells). Scale bar = 20 μm. **C.** Tangential section of an anchorage plate (ap) showing the radial pattern and vacuolated cytoplasm (arrows) of its cells (a, amoebocyte; bf, branchial filament; e, egg; fe, follicle epithelium). Scale bar = 20 μm. **D.** Higher magnification of a transverse section of the anchorage plate (ap) showing sperms (stz) close to the egg cytoplasm (bf, branchial filament; e, egg). Scale bar = 10 μm.

follicle, such as that known for cephalopods (Bottke 1974) and polyplacophorans (Selwood 1968, 1970). A true follicle is one in which individual oocytes are completely encompassed by a continuous layer of follicle cells. In the present article, considering the different functions of each cell type, those elements forming the acinus wall were referred to as "cells of the acinus wall" and the cells that surround entirely each developing oocyte as "follicle cells."

Oogenesis in *Gaimardia trapesina* is quite unusual among bivalves. From the early vitellogenic stages and throughout vitellogenesis, oocytes are completely encompassed by a one-cell-thick follicle; this follicle persists after gamete release and participates in the attachment of each embryo to the branchial tissues, where they develop up to a late-stage shelled pediveliger larva. These peculiar reproductive traits, only shared with species of *Pseudokellya* (Pelseneer 1903; Zelaya & Ituarte 2009), are quite unlike conditions reported in the majority of bivalves (Sastry 1979; Jong-Brink et al. 1983).

In *Pecten maximus* Linnaeus 1758, each developing oocyte is associated with only one follicle cell, referred to as an "auxiliary cell" (Dorange et al. 1989). In the mussel *Mytilus edulis* Linnaeus 1758, Pipe (1987) reported that in the early stages of oogenesis, oocytes are surrounded by a small number of follicle cells but, as oogenesis proceeds, follicle cells are restricted to the stalk region of oocytes. A similar follicle cell–oocyte association was reported by De Gaulejac et al. (1995) in *Pinna nobilis* Linnaeus 1758. In *Patinopecten yessoensis* and *Crenomytilus grayana,* Motavkine & Varaksine (1983, cited in Dorange et al. 1989; Eckelbarger & Davis 1996) reported that "auxiliary cells" completely encompass oocytes that are free in the acinus lumen; however, further details on these cases are unknown.

At the end of vitellogenesis, ripe oocytes detached from the acinus wall and the investing follicle epithelium showed a distinct portion ~160 μm in diameter, the differentiated zone corresponding to the anchorage plate. The origin of the follicle cells in *G. trapesina* was not established with certainty in this study, but two observations suggest that follicle cells may arise from the wall of the acini. Active proliferation of acinar wall cells was indicated by occasionally observed mitotic profiles within these cells. Furthermore, loose cells seen adjacent to late previtellogenic and early vitellogenic oocytes may have originated as cells detached from the acinar wall epithelium. These

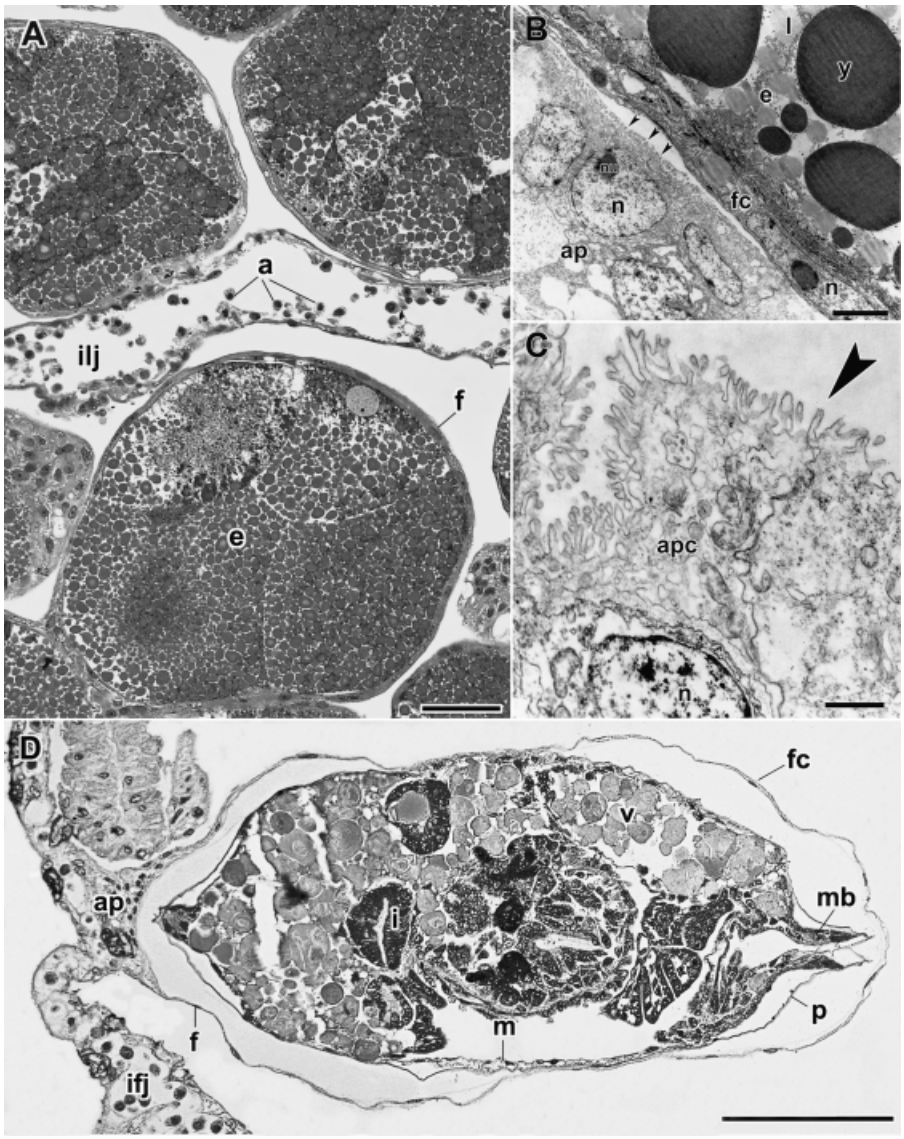

Fig. 5. Brooding. **A.** Eggs in an advanced stage of cleavage (e) with a persistent follicle (f) (a, amebocytes; ilj, interlamellar junction). Scale bar = 50 μm. **B.** TEM micrograph of a developing egg (e) surrounded by follicle cells (fc), arrows indicate digitiform projections of the surface of cells forming the anchorage plate (ap) (l, lipid droplet; n, nucleus; nu, nucleolus; y, yolk body). Scale bar = 100 nm. **C.** Higher magnification (TEM) of cells of the anchorage plate (apc) with surface projections (arrow) (n, nucleus). Scale bar = 50 nm. **D.** A late-stage pediveliger larva attached to the interfilamental junction (ifj) through the anchorage plate (ap), still encompassed by the persistent follicle (f) (fc, follicle cell; i, intestine; m, mantle epithelium; mb, mantle border; p, periostracum; v, vitelline droplets). Scale bar = 100 μm.

cells were cytologically similar to initial follicle cells that began attaching to oocytes at the onset of vitellogenesis (Fig. 2B–F). Similarly, Dorange et al. (1989) described, in *P. maximus,* the migration of "auxiliary cells" from the periphery of the acinus and their attachment to late previtellogenic oocytes (only one auxiliary cell per oocyte in this case).

From the structural point of view, oogenesis in *G. trapesina* also differs from oogenesis in gastropods. In the Viviparidae, *Viviparus viviparus* Linnaeus 1758 (Griffond & Gomot 1979), and in species of Siphonariidae (Pal & Hodgson 2001, 2002), early developing oocytes are completely surrounded by a few follicle cells but, as vitellogenesis progresses, follicle cells become restricted to the stalk region of the oocyte.

The fact that the follicle surrounding individual oocytes formed just at the onset of vitellogenesis might suggest an involvement in vitellogenesis. However, the persistence of the follicular envelope long after gamete release, and its participation in the attachment of developing ova and embryos to branchial tissues, suggest a role in providing mechanical support or protection during embryogenesis. Both nutritive and mechanical roles, among other functions, have been assigned to follicle cells (Eckelbarger & Davis 1996). However, at least at the light microscope level, no evidence for a possible nutritive role for follicle cells has been found.

This article of a bivalve showing follicular oogenesis stands in contrast to the typical situation of solitary oogenesis among bivalves. This finding un-

derlines the importance of further comparative studies, particularly on the many poorly known groups of bivalves, in order to more accurately understand the diversity and evolution of reproductive traits among this group of Molluscs. Further investigations at the ultrastructural level will help to elucidate the role of the follicular epithelium in *G. trapesina,* particularly with respect to the process of vitellogenesis.

Ripe ova remained sealed off from the environment by the surrounding follicle, even after being laid within the water tubes of gills. This fact, along with the presence of sperm in the adjacent anchorage structure and in spaces between cells of the anchorage plate, suggests a possible participation of this structure in the fertilization process. However, further specific studies are needed to clarify this point.

The brooding process in *G. trapesina* is also unique. Among bivalves, different modes of parental care are known: in the Ostreidae and Philobrydae, embryos are protected within the pallial cavity (Brey & Hain 1992); in *Transennella tantilla* GOULD 1853, embryos are brooded within a pouch between the inner demibranch and the visceral mass (Kabat 1985); in some Neoleptonidae, a gelatinous substance glues developing eggs to the anteroventral shell border of females (Zelaya & Ituarte 2004). Branchial incubation also shows considerable structural diversity among bivalves (for a review, see Sellmer 1967; Mackie 1984). In the simplest case, reported for *Lasaea* (Beauchamp 1986), *Eupera, Corbicula,* and *Neocorbicula* (= *Cyanocyclas*) (Ituarte 1984, 1994), embryos are retained within non-modified branchial water tubes. A structurally more complex case is found in *Pisidium* (Burch 1975), in which a brood sac derived from the descending filaments of the inner demibranchs of each gill contains several developing embryos, each one protected within an individual chamber. The greatest complexity has been reported in the species of *Sphaerium* and *Musculium,* in which ≤ 3 sacs, derived from branchial tissues, contain >1 embryo at different stages of development (Okada 1935b; Burch 1975).

In other Gaimardiidae, the brooding of embryos has been reported, but no details on the process have been provided. In *Gaimardia finlayi* POWELL 1933, Morton (1979) described egg incubation within the inner demibranch, the only demibranch present, with the offspring released as shelled juveniles. Benavides & Cancino (1988), in a study dealing with physiology of the little Southwestern Pacific species *Gaimardia bahamondei* OSORIO & ARNAUD 1984, reported the incubation of numerous embryos "adhered to the demibranchs," without providing further details.

The brooding process in *G. trapesina* is unique because each embryo is enclosed in a single protecting sac derived from the persistent follicle. Embryo-containing follicles are attached to the branchial filaments of both the inner and the outer demibranchs of the maternal individual. The physical connection between embryos and maternal tissues is unknown in bivalves other than *G. trapesina* and species of *Pseudokellya*. The large amount of vitellus within ova of *G. trapesina* suggests that development is probably entirely lecithotrophic. No evidence has been found for nutrient transfer from maternal tissues to embryos.

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