

Embryonic development of verongid demosponges supports the independent acquisition of spongin skeletons as an alternative to the siliceous skeleton of sponges

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Approximately 85% of extant sponges (phylum Porifera) belong to the class Demospongiae, which contains 14 taxonomic orders. In the orders Verongida, Dictyoceratida, and Dendroceratida, jointly referred to as 'keratose demosponges', the skeleton does not contain siliceous spicules but only spongin fibres. This shared trait has encouraged placement of these orders together within Demospongiae, although their relationships remain uncertain. The present study documents for the first time embryo development in the order Verongida (*Aplysina aerophoba*), providing some clues for phylogenetic inference. Spawned eggs were enveloped by a follicle of maternal cells. Embryos and larvae were chimeric organisms, the blastocoel of which was filled with symbionts and maternal cells migrated from the follicle. The ultrastructure of epithelial larval cells revealed: (1) a basal apparatus characterized by a peculiar, angling accessory centriole; (2) a pear-shaped nucleus with a protruding beak connected to the rootlets of the basal body; and (3) a distinctive Golgi apparatus encircling the nuclear apex. Developmental and ultrastructural findings support the concept, in congruence with recent molecular studies, that Verongida are more closely related to Halisarcida (askeletal sponges) and Chondrosida (askeletal sponges + sponges with spongin + spiculate sponges) than to the remaining 'keratose' orders, making a monophyletic 'supra-ordinal unit' equivalent to a subclass (Myxospongia, new subclass). Hence, spongin skeletons have evolved at least twice in Demospongiae. Independent acquisition of 'corneous' materials as an alternative to silica could have been stimulated by the radiation of diatoms at the Cretaceous–Tertiary boundary (approximately 65 Mya), which depleted silicon in the photic zone of the world's ocean. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, 97, 427–447.

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INTRODUCTION

The sponges (Porifera) constitute a phylum containing approximately 8216 extant species (World Porifera Database; <http://www.marinespecies.org/porifera>) distributed into three classes: Calcarea, Hexactinellida, and Demospongiae. The demosponges represent approximately 85% of living sponges, which are traditionally classified into three subclasses (Homoscleromorpha, Tetractinomorpha, and Ceractinomorpha) and 14 taxonomic orders (Table 1).

Members of most demosponge orders possess an internal inorganic skeleton of siliceous spicules. Nevertheless, in some demosponges, an organic skeleton made of one or more collagen derivatives (i.e. collagen fibrils, spongin filaments, and/or spongin fibres) serves as the entire skeleton without siliceous elements. The phylogenetic relationships between these spicule-lacking demosponges and the remaining demosponge groups remain obscure, as does our understanding of the major pathways in the skeletal evolution of Demospongiae.

Early spongiologists initially regarded the sponges lacking the mineral skeleton to comprise a group

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Table 1. Current distribution of demosponge orders (including the polyphyletic, order-like group 'Lithistida') in subclasses, as outlined in the *Systema Porifera* (Hooper & van Soest, 2002)

Subclass (Lévi 1956 + Bergquist 1978)	Order
Homoscleromorpha Micro-spicules, internal development, cinctoblastula-cinctogastrula larva	Homosclerophorida
Tetractinomorpha Mostly oviparous, clavablastula or hoplitomella larva, skeletal spongin very scarce to absent	Spirophorida Astrophorida Hadromerida Chondrosida 'Lithistida'
Ceractinomorpha Mostly viviparous, parenchymella or dispherula larva, skeletal spongin well developed	Poecilosclerida Halichondrida Agelasida Haplosclerida Dictyoceratida
'Keratose sponges'	Dendroceratida Verongida Halisarcida

Elimination of the subclass rank from the poriferan classification is currently under discussion because Tetractinomorpha and Ceractinomorpha are perceived as polyphyletic subclasses by both morphological and molecular approaches (see Discussion). The three orders having skeletons of spongin fibres and no silica spicules are informally referred to as 'keratose sponges'.

apart from the 'true' demosponges, which were diagnosed as those bearing siliceous spicules (Bowerbank, 1874; Ridley & Dendy, 1887). Before long, progressive understanding of the poriferan anatomy aroused suspicions that spicule-lacking sponges were indeed demosponges that either had not yet acquired their siliceous skeleton or had lost it during the course of evolution (Haeckel, 1889). By the boundary of the 19th and 20th Centuries, it was implicitly assumed that all sponges possessing spongin fibres and lacking siliceous spicules were demosponges (Lendenfeld, 1889; Topsent, 1894) and soon these were formally grouped into the demosponge subclass Ceratellida (Topsent, 1928). The idea of subclass rank was soon abandoned in favour of the view that they better formed an order, the Keratosa (de Laubenfels, 1936, 1948), a name credited to Grant (1861). Initially, the order Keratosa included not only sponges in which spongin fibres were present instead of a skeleton of siliceous spicules (i.e. the former Ceratellida), but also a genus lacking both spicules and spongin fibres (i.e. the genus *Halisarca*; de Laubenfels, 1948; Vacelet,

1959). Because some of those Keratosa had their fibres growing with dendritic pattern, whereas others had them anastomosed to form a reticulum, the order Keratosa¹ was formally split by Bergquist & Hartman (1969) into two orders: Dendroceratida (dendritic fibres) and Dictyoceratida (anastomosing fibres). The skeleton-lacking genus *Halisarca* was initially maintained within the new order Dendroceratida, but subsequently transferred to an order of its own (*Halisarcida*) under claims that it was histologically and chemically different from either group among the 'keratose' sponges (Bergquist, 1996).

After splitting Keratosa into Dictyoceratida and Dendroceratida, Bergquist (1978) also emphasized that, even when all dictyoceratids had skeletons of anastomosing fibres, two major models in fibre structure could be recognized within the group. One of them characterized by fibres showing, in cross section, a peripheral bark made of concentric laminae and a central pith of granular material. The sponges possessing that fibre structure also shared two other distinct features: (1) rapid shift of their bright colours into deep purple to black when exposed to air due to oxidation of characteristic bromotyrosines and (2) oviparous reproduction with external development, which strongly contrasted with the internal fertilization and embryo brooding shown by the remaining dictyoceratids. Consequently, those sponges were set apart from the remaining Dictyoceratida and distinguished as the order Verongida (Bergquist, 1978). This order has conventionally been placed within the subclass Ceractinomorpha in association with the orders Dictyoceratida and Dendroceratida (Table 1) because they all have a 'soft' skeleton made exclusively of spongin. Nevertheless, Bergquist (1978: 178) warned about the artificial nature of such an association in the very original diagnosis of the order Verongida by stating that 'electron microscopy studies have revealed the verongids to have a complex histology with many cell types which are quite different from those of other Ceractinomorpha'. She also realized that the oviparous nature of Verongida was contrary to its inclusion in Ceractinomorpha, but adopted a practical, timely solution by arguing that 'until a complete account of their development becomes available, they are retained in this subclass'.

The recent advent of molecular techniques has revived discussion on the phylogenetic significance of aspiculate demosponges. An analysis of the relation-

¹After the split of the order Keratosa into the orders Dictyoceratida and Dendroceratida, the name 'Keratosa' was invalidated. Nevertheless, the use of this term as an adjective (i.e. keratose) has persisted in the literature to informally refer to those sponges characterized by a skeleton of spongin fibres exclusively. Given the corneous nature of the spongin fibre skeletons, 'keratose' sponges have also been referred to as 'horny sponges' throughout history.

ships of 13 orders of Demospongiae based on 18S, and the C1, D1, and C2 domains of 28S rRNA, suggested the verongids to be more related to the tetractinomorphic order Chondrosida (*Chondrilla* and *Chondrosia*) than to the 'keratose' orders Dictyoceratida and Dendroceratida (Borchiellini *et al.*, 2004). A 28S rRNA analysis in that same study also suggested a relationship between Verongida and Halisarcida (Borchiellini *et al.*, 2004). The clade including Chondrosida, Halisarcida and Verongida was given the old but recently used name 'Myxospongiae' (Borchiellini *et al.*, 2004). A subsequent study using partial data from the large subunit rDNA (LSU) and cytochrome oxidase subunit I (CO I) revealed that, whereas the LSU data set suggested the verongids to be more related to Chondrosida than to Dictyoceratida and Dendroceratida, a monophyletic 'Myxospongiae' clade was not supported by the CO I data (Nichols, 2005). A more recent analysis involving complete mitochondrial genomes gave moderate support to this clade (Lavrov, Wang & Kelly, 2008), lowering the traditional levels of conflict between the phylogenetic signal provided by the nuclear rDNA and the mitochondrial genomes. In this context, there are still three aspects generating some uncertainty relative to the putative relationships between Verongida, Chondrosida, and Halisarcida: (1) the available 28S rDNA and mitochondrial (mt)DNA analyses have failed to confirm the monophyly of Chondrosida (Erpenbeck & Wörheide, 2007); (2) the order Halisarcida, a former 'keratose' member that may represent a crucial stage in the evolution of the spicule-lacking sponges, was not represented in either the 28S rRNA data set analysed by Borchiellini *et al.* (2004) or any of the analyses by Nichols (2005); and (3) a set of morphological shared traits refuting the putative relationships suggested by the molecular approaches has not been identified.

In the present study, new information is provided that may help to clarify the controversial phylogenetic relationships of the 'keratose' assemblage by describing for the first time development in a verongid sponge. To date, very little was known about the reproductive biology of verongids. The few members investigated histologically were thought to be gonochoric (Gallissian & Vacelet, 1976; Tsurumi & Reiswig, 1997; Bergquist & Cook, 2002), except for a to-be-revised report indicating occurrence of a few hermaphroditic individuals in a population of *Aplysina aerophoba* (Scalera-Liaci *et al.*, 1971). Similarly, observations of sperm release by three Caribbean verongids also suggested gonochorism, although this is not fully corroborated (Reiswig, 1970, 1976, 1983). Development was assumed to occur in the water column because embryos or larvae have never been found in histological sections of this sponge group (Maldonado, 2006). By monitoring histologically

gametogenesis in a local Mediterranean populations of the common verongid *A. aerophoba* Schmidt, 1862, gamete release was predicted and gametes were manipulated to fertilize released eggs, obtaining larvae in the laboratory. The present study reports on aspects of fertilization, embryo development, and larval histology, with an emphasis on information that may be of interest for a better understanding of not only reproduction in Demospongiae, but also the phylogenetic relationships of verongid demosponges.

MATERIAL AND METHODS

The studied population of *A. aerophoba* Nardo, 1843 was located in a shallow bay (10 m depth) on the Catalan coast (42°17'35N; 3°17'22E; Mediterranean Sea, Spain). Male and female gametes were collected upon spawning using 60-mL sterile syringes and mixed immediately during a scuba dive. In a 4-h trip, syringes were transported to the laboratory in refrigerated conditions (14 °C) for further observations made by dissecting and compound microscopes. The contents of the syringes were decanted and transferred to sterile polystyrene Petri dishes (5 cm in diameter) filled with 0.4-µm filtered seawater. Culturing of gametes to larvae took place at 24 °C, under a 16 : 8 h light/dark cycle, without agitation or aeration. Embryos, larvae, and settlers in the plates were periodically monitored during the next 3 weeks through an inverted microscope connected to a colour video camera and a time-lapse digital video recorder. Embryos and larvae were periodically collected from the dishes and fixed for transmission electron microscopy (TEM) in accordance with previously described protocols (Maldonado, 2007). Ultrathin sections were studied using a JEOL 1010 transmission electron microscope operating at 80 Kv and provided with a Gatan module for acquisition of digital images. Larvae were also processed for scanning electron microscopy (SEM) as described by Maldonado *et al.* (1999).

RESULTS

FERTILIZATION AND EMBRYO DEVELOPMENT

Upon arrival at the laboratory, observations on syringe contents revealed that almost all oocytes (98%) had been fertilized and started cleavage in the 4-h period elapsed after gamete collection (Fig. 1A, B, C). Hundreds of motile spermatozoa still concentrated around early blastula (Fig. 1A, B). Only rarely were spermatozoa seen swimming across the Petri dishes, a behaviour suggesting that they were strongly attracted (possibly chemically) towards oocytes. Light microscopy observations of fixed eggs and early

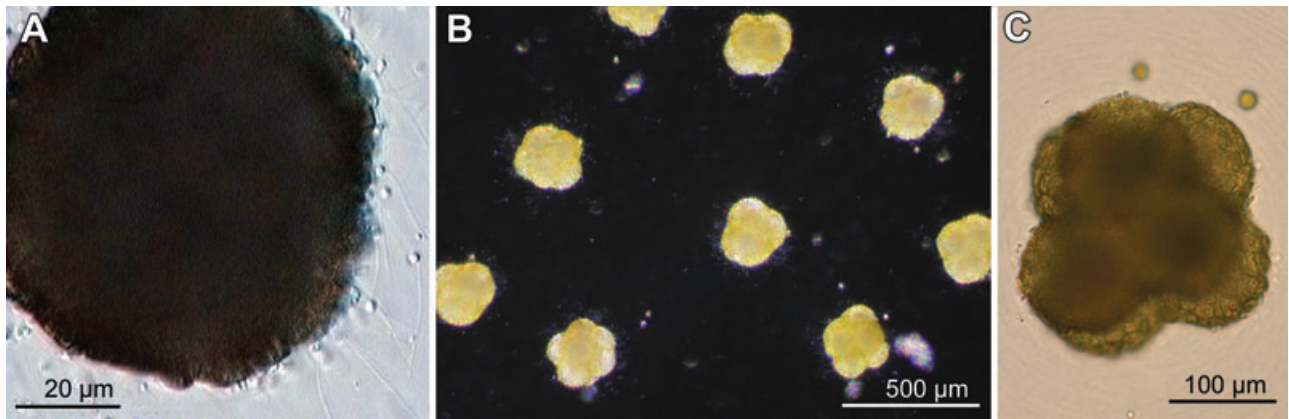


Fig. 1. A, egg surrounded by numerous spermatozoa 4 h after spawning. B, eight-cell blastulae synchronously developing 5 h after spawning. At this stage, all morulae were still surrounded by swimming spermatozoa. C, detail of an eight-cell blastula. Note that the follicle-like envelope was not visible *in vivo*.

blastulae revealed that they all were enclosed within a follicle-like envelope (Fig. 2A, B, C). The envelope was not evident during *in vivo* observations of spawned eggs and early blastulae (Fig. 1A, B, C), probably because it was tightly attached *in situ* and became detached only after chemical fixation. This envelope started forming around mid-size oocytes several weeks before spawning (Fig. 3A). Approximately 5 h after gamete release, most embryos were at two- to eight-cell stages, with approximately equal blastomeres (Figs 1B, C, 2A, B, C). Blastulae grew during early cleavage, with two-cell stages being approximately half the volume of eight or 16 cell stages (Fig. 2A). A tiny intercellular space, which became evident as early as the two-cell stage (Fig. 2B, C), anticipated that the blastula was to develop a blastocoel.

TEM observations revealed that the follicle consisted of a thin peripheral layer of collagen fibrils limiting a lax accumulation of two types of maternal cells: spherulous cells and microgranular cells (Fig. 3B, C). Spherulous cells were relatively abundant, measuring 5–10 µm in their largest diameter (Fig. 3C, D). They were charged with membrane-bound inclusions (i.e. spherules) containing numerous granules in diverse degrees of compaction (Fig. 3D). Microgranular cells, measuring 4–8 µm in their largest diameter, occurred intermingled with the spherulous cells (Fig. 3C, D). Their electron-clear cytoplasm was characteristically filled with small, ovate, membrane-bound, electron-dense inclusions (0.2–0.3 × 0.4–0.5 µm), which exhibited a paracrystalline, striated nature, possibly comprising some type of protein-rich nutritional storage (Fig. 3E, F). During cleavage, both spherulous cells and microgranular cells entered the cleavage furrows to reach the nascent blastocoel (Figs 2C, 4A, B, C, D). At these

early blastula stages, the blastomeres emitted pseudopodia, both within the lumen of cleavage furrows (Fig. 4B, C) and towards the follicular space (Fig. 5A, B, C). Phagocytosis of entire spherulous cells or microgranular cells by blastomeres was never observed. Nevertheless, blastomeres were noted to incorporate diverse inclusions apparently exocytosed by the spherulous cells (Fig. 5B, C, D, E, F). A few free bacteria were observed within the follicle space around the blastomeres. Nevertheless, hundreds of bacteria, but not cyanobacteria, occurred in the peripheral cytoplasm of both eggs and blastomeres, always within membrane-bound vesicles (Figs 3C, 6A, B). These intracellular bacteria, which had been engulfed at the early-stage oocyte, showed no evident sign of digestion within the vesicles. The peripheral distribution of the bacteria within the cytoplasm of blastomeres strongly contrasted with the distribution of yolk bodies, which concentrated in the perinuclear region (Figs 2C, 3C). Yolk bodies (0.4–0.7 µm in diameter) were membrane-bound and showed a heterogeneous structure, incorporating very electron-dense material (probably proteinaceous), glycogen granules, and lipid droplets (Fig. 6B, C).

After the 16-cell stage, cleavage was very rapid and light microscopy observations were obscured by the dense content of blastomeres. Time-lapse monitoring revealed that, approximately 14 h after gamete release, blastulae already contained hundreds of tiny blastomeres, and those at the future posterior pole of the larva started differentiating a cilium. During this phase, there was intensive mitotic activity at the blastula wall and the embryo increased slightly in volume, mostly because the blastocoel enlarged. At no time did the blastulae show a convoluted epithelium similar to the that characterizing the period of intense mitosis during embryo development in other

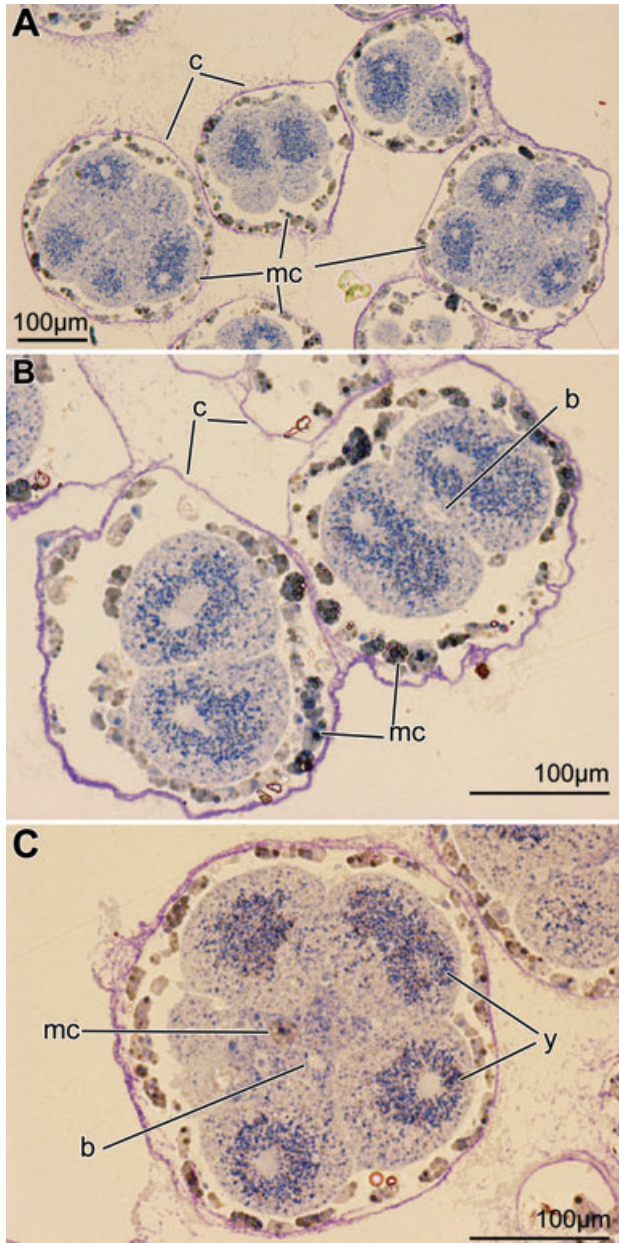


Fig. 2. A, sections of two- to eight-cell morulae, showing the collagen layer (c) and the envelope of maternal cells (mc). Note size differences between two- and eight-cell morulae. B, detail of two-cell morulae, in which a nascent blastocoel is visible (b). C, detail of a eight-cell morulae showing migration of a maternal cell (mc) from the follicle to the nascent blastocoel (b). Note concentration of yolk bodies (y) in the perinuclear region of blastomere nucleus.

demosponges. Approximately 22 h after gamete release, the ciliated blastulae that had been lying at the bottom of the Petri dishes became free swimming. Upon leaving, the remains of the collagen envelope and some follicle cells that had not been incorporated

into the larva remained on the bottom of the dishes.

BEHAVIOUR OF FREE-SWIMMING LARVAE

The transition between ciliated blastulae and early-stage larvae occurred sometime between 22 h and 59 h after gamete spawning. By approximately 60 h, all ciliated embryos had become free-swimming larvae and some of them were already settling down on the plates. Interestingly, many larvae kept swimming for an additional 7 days before settling, indicating substantial variability in the duration of the dispersal phase, at least in laboratory conditions. Larvae appeared brownish under the transmitted light of the compound microscope and bright yellow under the reflecting light of the dissecting microscope, with a darker posterior pole in both cases. The body shape of larvae was relatively plastic. Initially, all larvae were ovate, with an anterior pole slightly narrower than the posterior one (Fig. 7A). They measured 136–160 µm in length and 100–115 in maximum diameter. Nevertheless, late-stage larvae (i.e. those swimming longer than 7 days) lengthened slightly, revealing a globate anterior pole and a narrower posterior pole (Fig. 7B). When these larvae transiently explored the substrate, they flattened against the bottom of Petri dishes and crawled for several minutes to hours, resuming swimming and ovate shape later on (Fig. 7C). Larvae were entirely ciliated. *In vivo* light microscopy and SEM observations indicated that cilia in the cells of the posterior larval pole were longer (22–30 µm in length) and sparser than those on the remaining larval surface (10–15 µm in length). The shorter cilia of the body were bent towards the posterior pole and beat metachronally. The posterior cilia were not bent, but straight (Fig. 7D). They did not beat metachronally, but as independent undulating cilia.

The larva of *A. aerophoba* rotated around their longitudinal axis when swimming, as do larvae of most other demosponges. However, it was surprising that these larvae showed either clockwise or counter-clockwise rotation and that they also had the ability to alternate instantaneously their direction of rotation. Furthermore, larvae transiently ceased rotation to explore the bottom by crawling, resuming swimming, and then subsequent rotation. At no time did the short cilia of the body or the posterior cilia show light-guided beating patterns. Rather, larvae showed no marked positive or photonegative response during the swimming phase in the plates, even though light conditions were manipulated in preliminary attempts to determine whether further phototactic experiments were warranted.

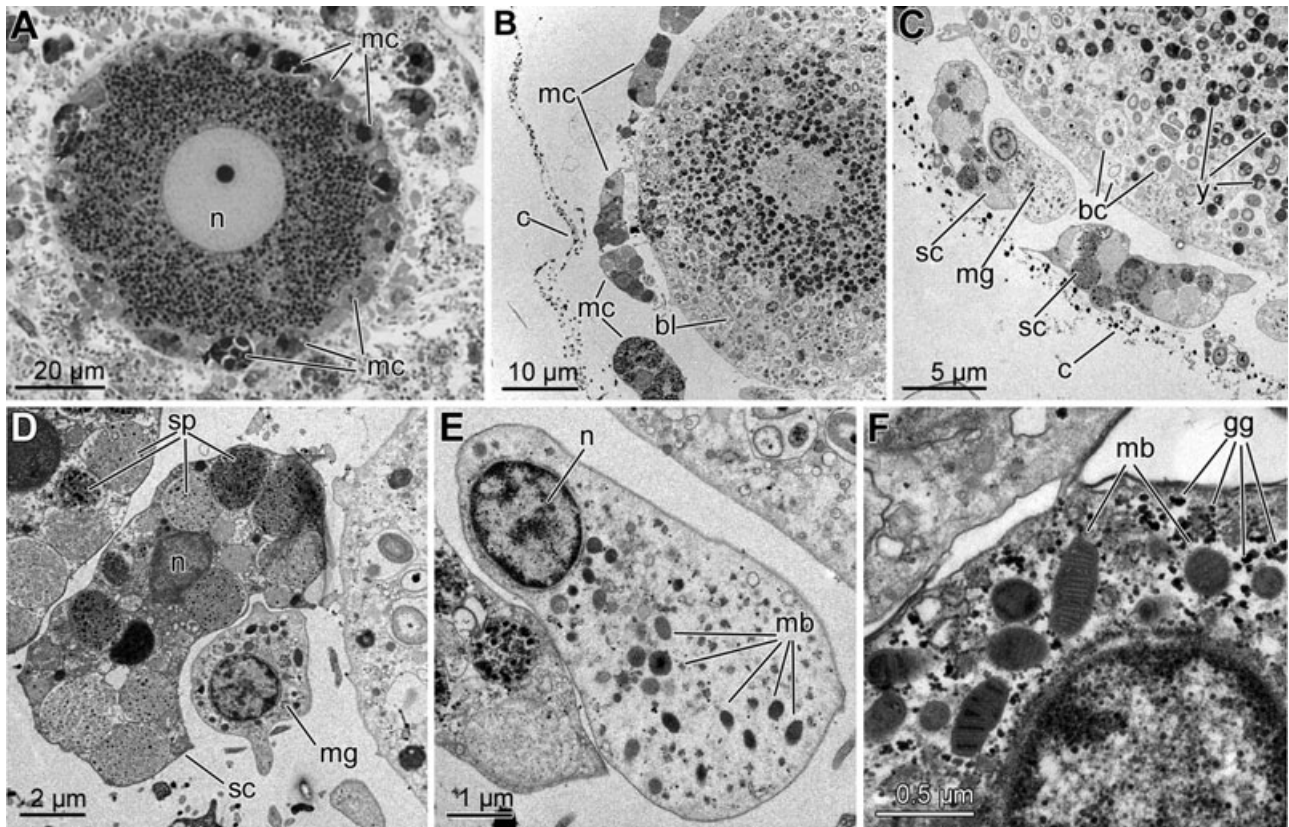


Fig. 3. A, section of a mid-size oocyte nested in the female mesohyl 3 weeks before spawning. The oocyte shows a nucleolate nucleus (n) and an envelope of maternal cells (mc), which occurs in close association with the oocyte membrane. B, details of early blastula showing the lax peripheral collagen layer (c) limiting the follicle-like layer of maternal cells (mc) around the blastomere (bl). C, detail of the follicle showing the collagen layer and two types of cells, microgranular cells (mg) and spherulous cells (sc). Note concentration of bacteria (bc) in the peripheral cytoplasm of blastomeres and yolk bodies (y) at inner regions. D, general comparative view of a spherulous cell (sc) and a microgranular cell (mg). Spherulous cells show a non-nucleolate nucleus (n) and mutigranular, membrane bound inclusions or 'spherules' (sp), the content of which varies in compaction degree. E, detail of a microgranular cell, characterized by a non-nucleolate nucleus (n) and abundance of small electron-dense, membrane-bound bodies (mb). F, detail of membrane-bound bodies (mb) of microgranular cells, the content of which show a striated pattern in transversal section. These cells are also rich in glycogen granules (gg).

ULTRASTRUCTURE OF LARVAE

Under the compound microscope, living larvae were relatively translucent, containing subepithelial cells charged with refringent bodies (Fig. 7E). These refringent bodies showed autofluorescence in the dark (Fig. 7F). Electron microscopy corroborated that larvae consisted of just three cell types (monociliated epithelial cells, spherulous cells, and microgranular cells) and contained abundant symbiotic bacteria in their blastocoel (Figs 8–10).

Epithelial cells were pseudo-cylindrical at most of the larval surface (Figs 8A, 9B, 10A), measuring from $14 \times 3 \mu\text{m}$ to $19 \times 4 \mu\text{m}$. At the posterior pole, these cells were not cylindrical, but T-shaped (Figs 8B, 9C). Epithelial cells contacted each other through their

distal-lateral surfaces by means of specialized junctions (Fig. 8C, D, E), and through lateral pseudopodia (Fig. 9D). Junctions looked like zonula adherens, but poor fixation of the plasmalemma proteins makes this interpretation only tentative. Epithelial cells also emitted numerous short pseudopodia from their distal surface and were monociliated (Figs 8A, B, C, 9C, F, G, 10B). The distribution of cytoplasmic organelles reinforced the strongly polarized organization of these cells (Fig. 10A). The distal region was characterized, besides the presence of pseudopodia and the cilium, by abundant small electron-clear vesicles (Figs 8C, 10A, B). The nucleus was located immediately below the basal apparatus and was pear-shaped (Fig. 10A, B), with a marked beak-like distal protrusion (apex). The cell portion below the nucleus contained abun-

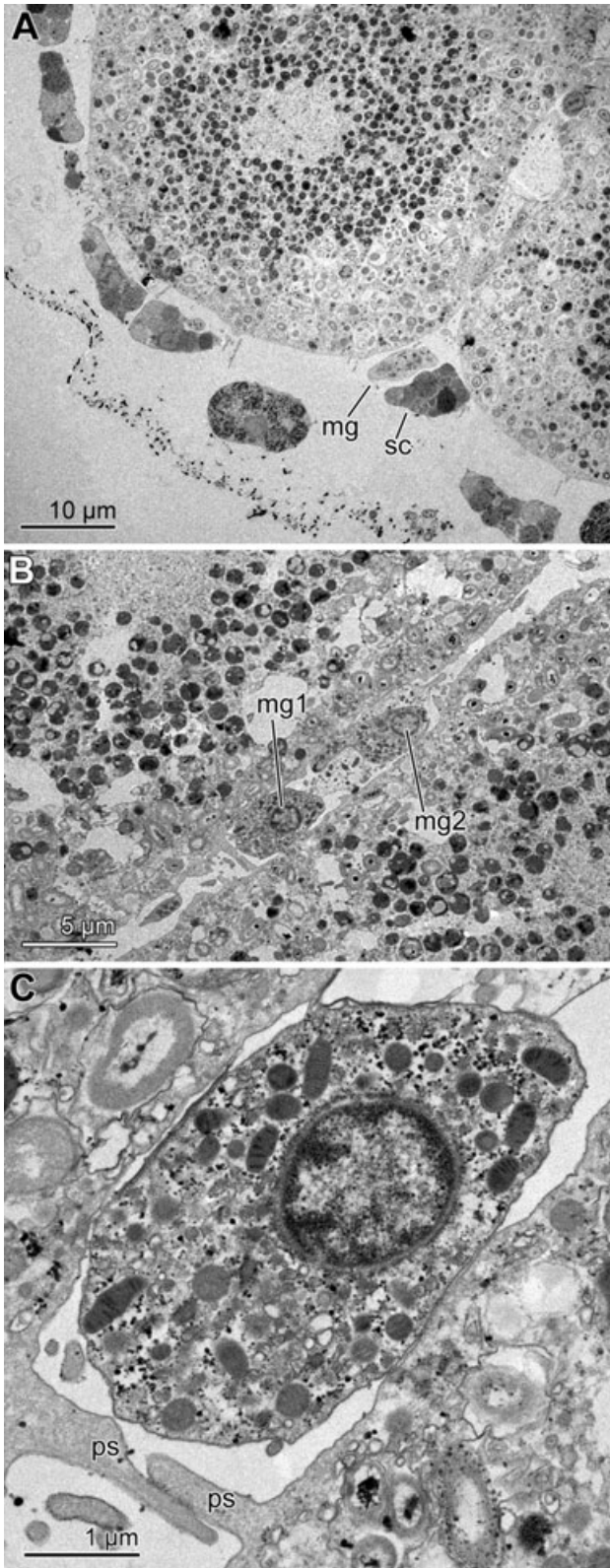


Fig. 4. A, detail of a two-cell blastula in which a spherulous cell (sc) and a microgranular cell (mg) appear to be about to enter the cleavage furrow between the blastomeres. B, detail of two microgranular cells ('mg1' and 'mg2') migrating through the cleavage furrows to reach the nascent blastocoel. C, detail of migrating microgranular cell 'mg1' in the above picture. Note the pseudopodia (ps) emitted by the blastomeres towards the cleavage furrow, probably involved in regulating internalization of maternal cells.

dant heterogeneous membrane-bound yolk bodies at various stages of assimilation (Fig. 10A, B), which were similar to those formerly occurring in the blastomeres.

The cilium and its basal apparatus showed noticeable features. The axoneme was surrounded by a glycocalyx-like enveloping structure (Fig. 10B, D, E), which connected to the ciliary membrane by just thin, radial strands (Fig. 10D). Although some longitudinal sections suggested a typical 9+2 microtubule organization in the ciliary axoneme (Figs 10E, 11A), it was difficult to infer microtubule organization in some cross sections, probably due to some distortion of microtubules during fixation (Fig. 10D). The cilium of each anterior pole cell emerged from a pit at the distal cell surface (Fig. 10C), whereas pits did not occur in cells of other larval regions. The basal apparatus consisted of a principal centriole (or basal body) with a simple basal foot from which microtubules arose (Fig. 11A). Short rootlets projected from the distal edge of the principal centriole (Fig. 11A, B, C, D) to contact the nuclear beak (Fig. 11B, C). There was an accessory centriole located slightly below the level of the basal body (Fig. 11A, B). More importantly, the accessory centriole was not perpendicular to the basal body, but angling by approximately 45°, an extremely uncommon arrangement in Porifera, which may contain phylogenetic information (see Discussion). Another remarkable feature was the occurrence of a large Golgi apparatus encircling the nuclear apex (Fig. 11F, G).

Although spherulous cells were lodged in the intercellular spaces of the larval epithelium at anterior, lateral, and posterior larval regions, only epithelial cells at the posterior larval pole became T-shaped (Figs 8A, B, 12A, B). These T-shaped cells were also monociliated (Fig. 12C) and provided with distal pseudopodia (Fig. 9C, E, F, G). Because T-shaped cells expanded their distal region (Figs 8B, 9A, 12A), between-cilium distances became larger, accounting for the low density of cilia noticed at the posterior larval pole. Although these posterior cilia were longer than those elsewhere on the larva, no distinct structure was noticed in their axonemes (data not shown).

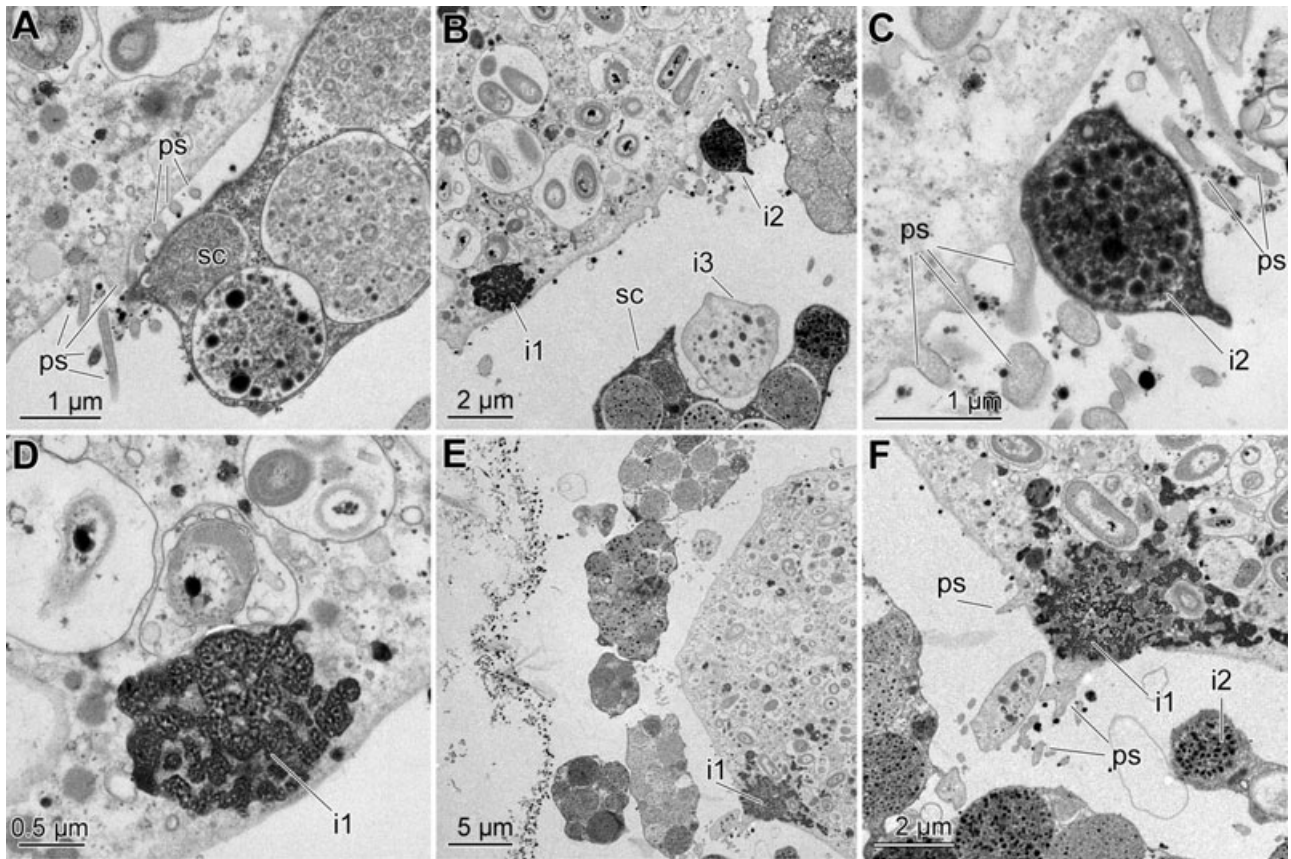


Fig. 5. A, pseudopodia (ps) emitted by the blastomeres at the contact point with a spherulous cell (sc). B, blastomere incorporating an inclusion (i2) exocytosed by a spherulous cell. Note rapid degradation in the blastomere peripheral cytoplasm of an engulfed inclusion (i1). Note also a third inclusion (i3) being exocytosed by the spherulous cell. C, close up of inclusion 'i2' and the surrounding pseudopodia (ps) emitted by the blastomeres. D, close up of engulfed inclusion 'i1', which is being rapidly degraded. E, general view of a blastomere that had recently engulfed an inclusion (i1). F, detail of previous micrograph, showing the incorporated inclusion (i1), the blastomere pseudopodia (ps) still unretracted, and an adjacent inclusion (i2) exocytosed by a spherulous cell.

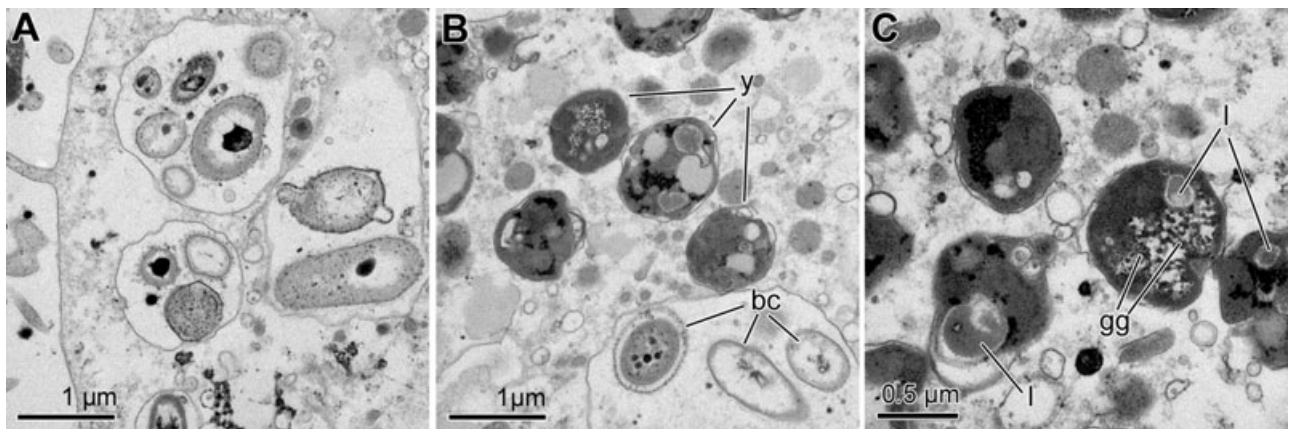


Fig. 6. A, diversity of symbiotic bacteria incorporated into vesicles in the peripheral cytoplasm of the blastomeres. B, comparative view of membrane-bound bacteria (bc) and unbound yolk bodies (y). C, detail of yolk bodies showing a mixture of electron dense (probably proteinaceous) material, along with lipid droplets (l) and glycogen (gg).

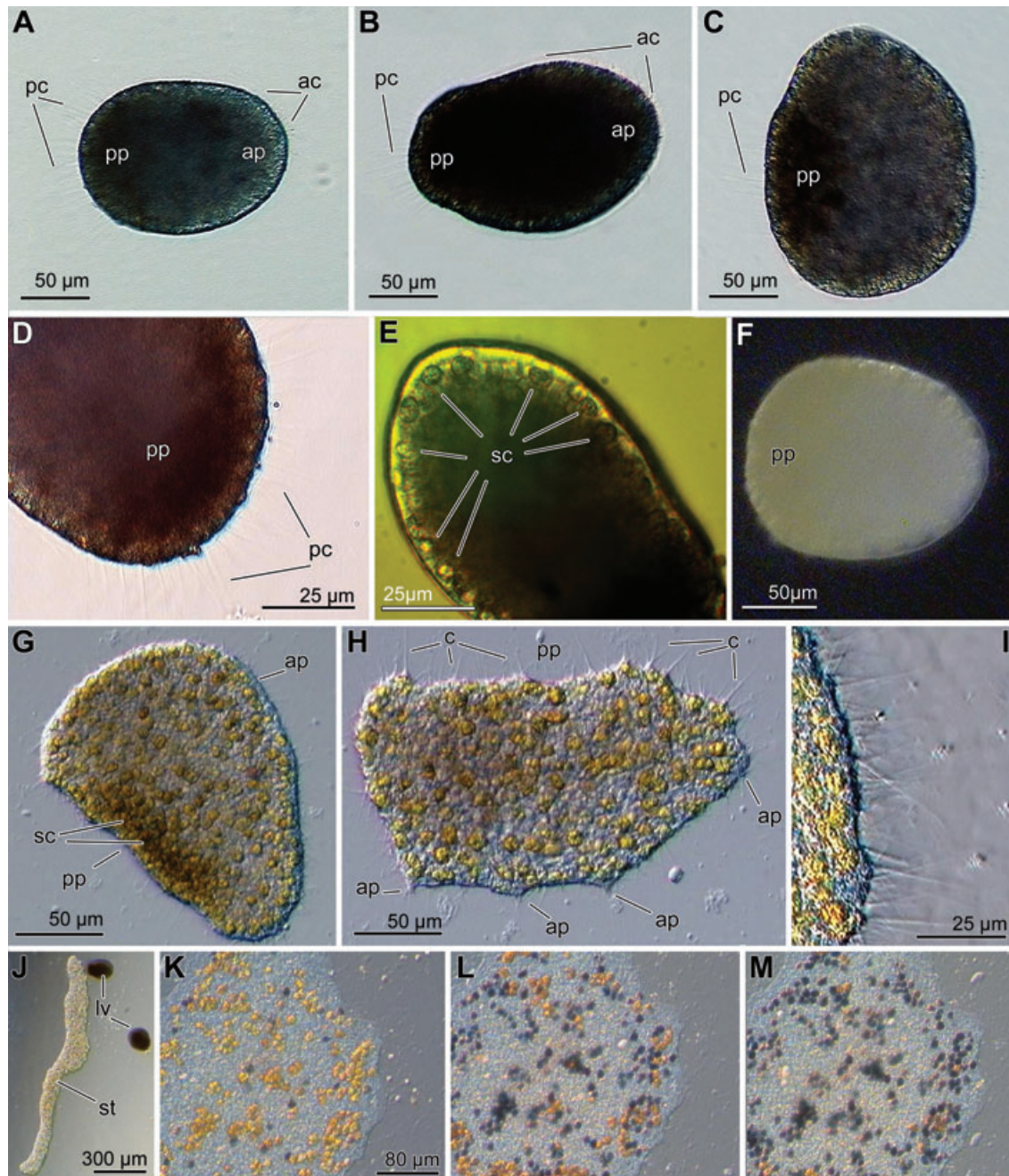


Fig. 7. A, one-day old larva showing a posterior pole (pp) wider than the anterior pole (ap). Cilia are longer at the posterior pole (pc) than at the remaining larval surface (ac). B, three-day old lengthened larva showing a posterior pole (pp) narrower than the anterior one (ap). Note longer cilia at the posterior pole (pc) than at the remaining larval surface (ac). C, five-day old larva exploring the bottom by crawling. Note plasticity of the larval body, in which the longer cilia (pc) indicate the posterior pole (pp). D, detail of a living larva showing the straightened, sparse posterior cilia (pc). E, detail of the anterior region of a living larva showing the maternal spherulous cells (sc) underlying the larval epithelium. F, larval autofluorescence derived from spherulous cells, mostly concentrated under the epithelium at the posterior pole (pp). G, larvae crawling while exploring the bottom for settlement. Note accumulation of spherulous cells at the posterior pole (pp). H, larva attaching to the bottom through several points (ap) of the anterior-lateral region. Note the very obvious cilia (c) of posterior pole (pp). I, detail of straightened cilia at a lateral body region of an attaching larva. J, comparative view of a lengthened, 3-day old settler (st) and two free-swimming larvae (lv) by the side. K, L, M, time-lapse video frames of a settler at 20, 23, and 24 days after fertilization. Note the process of darkening of the spherulous cells, as seen through the translucent epithelium.

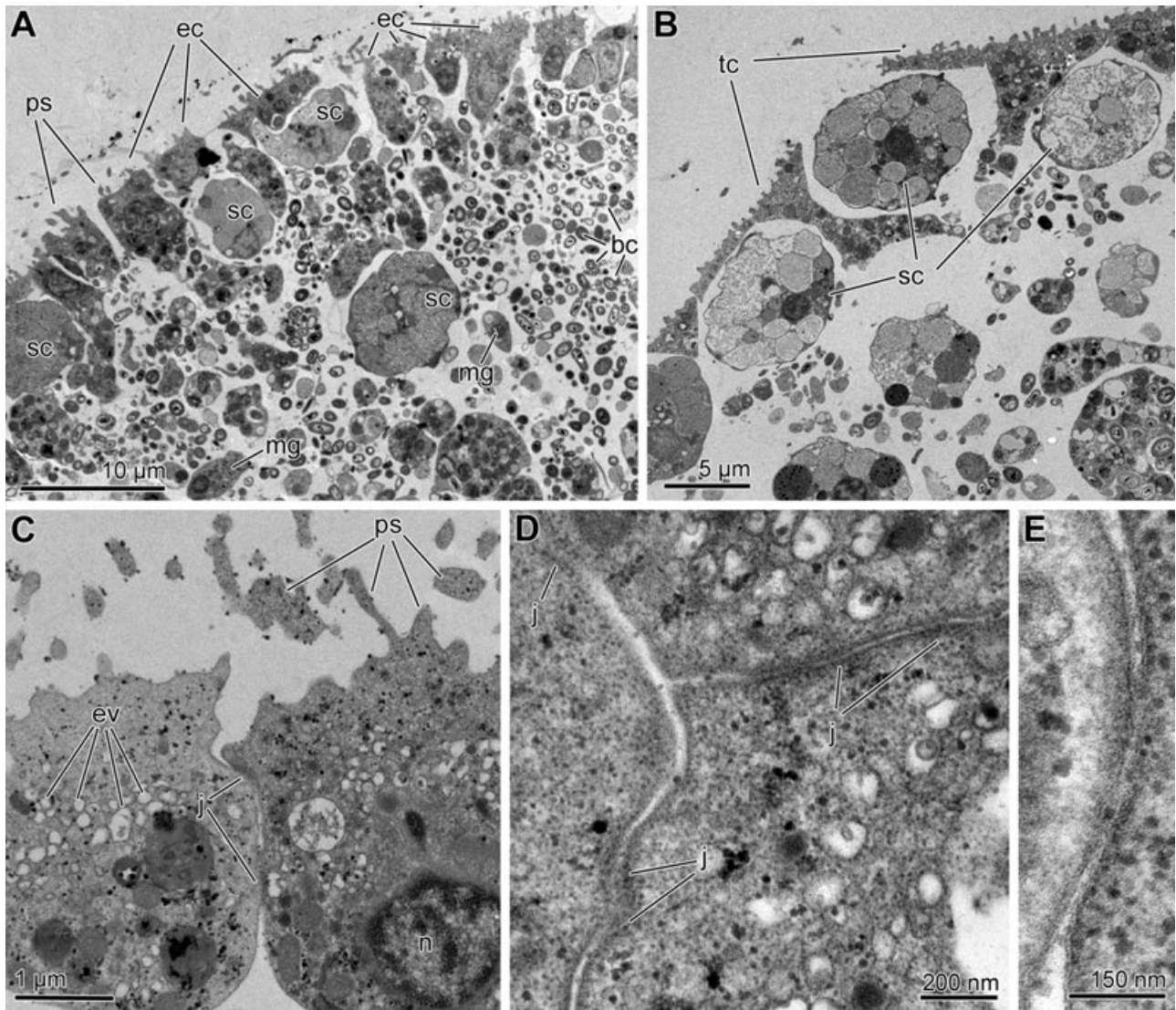


Fig. 8. A, section of the larval body at a lateral region showing the epithelial cells (ec), subepithelial maternal spherulous cells (sc), maternal microgranular cells (mg), and abundant symbiotic bacteria (bc). Note pseudopodia emitted by the epithelial cells (ps). B, section of the body at the posterior larval pole, showing T-shaped epithelial cells (tc) and some maternal spherulous cells (sc) lodged between the bodies of the epithelial cells. C, longitudinal section of the distal region of two epithelial cells showing numerous pseudopodia (ps), abundant, small electron-clear vesicles (ev), intercellular junctions (j), and the nucleus (n). D, cross-section at the distal cell pole showing specialized junctions (j) formed at the convergence of three epithelial cells. E, detail of junction.

A major difference between T-shaped and pseudocylindrical epithelial cells was that the cilium inserted eccentrically on the distal surface of T-shaped cells, so that the basal apparatus was not closely associated with the nucleus and the Golgi apparatus (Fig. 12C, D).

The spherulous cells were fairly abundant immediately below the epithelium, but rare at the centre of the larval blastocoel (Fig. 8A, 12A). At the posterior larval pole, spherulous cells protruded among the T-shaped epithelial cells and were extruded from the

larva through pore-like spaces occurring between the epithelial cells (Fig. 9E, F, G). Their cytology was similar to that of the spherulous cells described around developing oocytes and spawned eggs both in the present study as well as elsewhere (Gallissian & Vacelet, 1976). In brief, they measured $6.6 \times 4.2 \mu\text{m}$ to $10.5 \times 6.2 \mu\text{m}$, being ovate to pseudo-spherical and containing numerous membrane-bound multigranular inclusions or 'spherules' (Fig. 12E, F). Although different spherules within a cell contained minute membrane-bound granules at different stages of

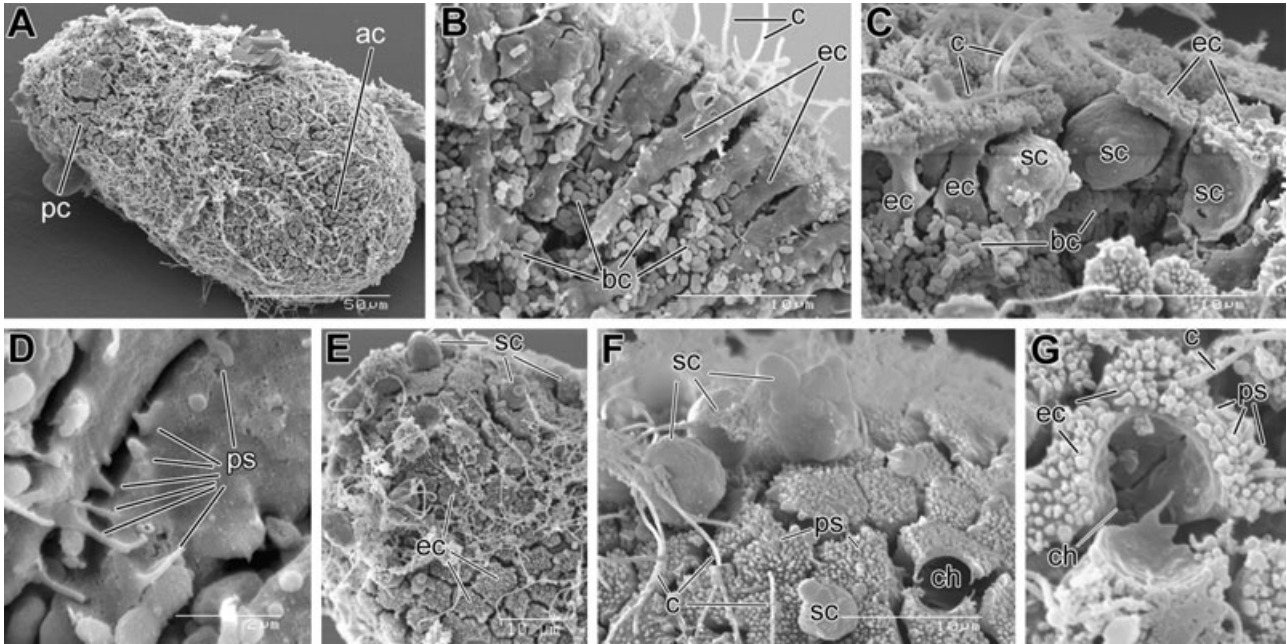


Fig. 9. A, general view of a 3-day old larvae. The exposed surface of cells at the posterior pole (pc) is clearly larger than that of cells (ac) at the anterior pole. B, C, fracture of the larval epithelium at the anterior larval pole and the posterior larval pole. D, detail of contacts between adjacent epithelial cells by lateral pseudopodia (ps). E, detail of the posterior larval pole, showing extrusion of spherulous cells (sc). F, detail of spherulous cells (sc) being extruded through channels (ch) among the epithelial cells (ec). G, detail of an intercellular channel (ch) formed among three T-shaped epithelial cells (ec) for extrusion of spherulous cells. bc, symbiotic bacteria; c, cilium; ec, epithelial cell; ps, pseudopodia.

either degradation or formation (Fig. 12E, F, G, H), all inclusions within each spherule were at a similar stage (Fig. 12G, H). In addition to the spherules, occasional electron-dense multilamellate bodies were found in the cytoplasm of these cells (Fig. 12G, I). Unlike epithelial larval cells, spherulous cells contained neither yolk bodies nor other organelles suggesting their differentiation from blastomeres. A few microgranular cells (Fig. 13A, B), which were identical to those of the oocyte envelope, including a cytoplasm filled with striated inclusions (Fig. 12C), occupied the central portion of the blastocoel, along with abundant bacteria belonging to many different morphological categories (Fig. 13A, B, D, F). These microgranular cells also lacked yolk bodies. It is noteworthy that, although bacteria occurred by the hundreds in the larval blastocoel, only three cyanobacteria were found after intensive search (Fig. 13D, E).

SETTLEMENT

Prior to settlement, larvae rotated with their anterior pole in contact with the bottom of Petri dishes for some time (1–3 h), then flattened the body against the substratum, ceased rotation, and crawled on for several hours. During this period, cilia were held

straight outward, showing reduced motility instead of the usual metachronal beating (Fig. 7G). Attachment took place at anterior–lateral areas (Fig. 7H). Cilia were visible at the surface of early settlers for a few hours after attachment (Fig. 7H, I). Settlement in contact with previous settlers never produced body fusion. Such settlers either reshaped or crawled away to avoid extended between-settler contacts. From 1–2 days after attachment, settlers consistently became remarkably thin and long, serpentine (Fig. 7J). The refringent spherulous cells were very obvious through the translucent epithelium of the early settlers (Fig. 7K). These cells became less obvious after several days, in many cases becoming dark brown to black (Fig. 7L, M), as if they were either degenerating or trans-differentiating into other cell types. The first choanocyte chambers appeared approximately 1 month after larval settlement. At no time during these observations did settlers form appreciable oscular tubes.

DISCUSSION

PHYLOGENETIC AND TAXONOMIC IMPLICATIONS OF FINDINGS

Many traits of the developmental process in *A. aerophoba* strongly suggest that the verongids, tradition-

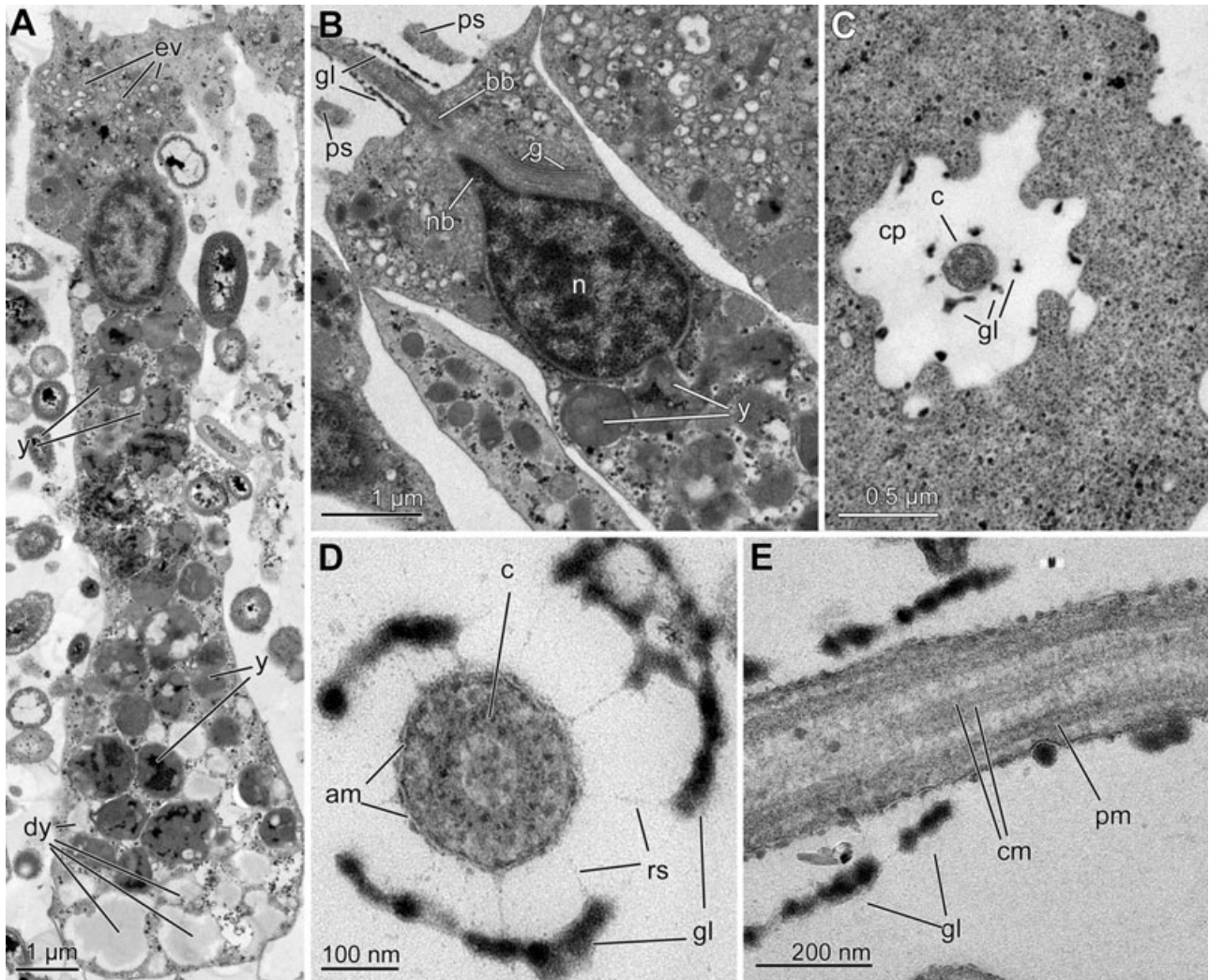


Fig. 10. A, longitudinal section of an epithelial cell of the anterior larval region showing a marked distal–proximal gradient in the distribution of electron clear vesicles (ev), unused yolk bodies (y), and degraded yolk bodies (dy). B, epithelial cell showing the glycocalyx (gl) of the flagellum, the pseudopodia (ps) of the distal surface, the basal body (bb), the Golgi apparatus (g), the beak or ‘apex’ (nb) of the nucleus (n), and yolk bodies (y). C, cross section at the distal cell pole showing the ciliary pit (cp), the cilium (c) and its glycocalyx (gl). D, cross section of the cilium (cl) showing the enveloping glycocalyx (gl) connected to the axonemal membrane (am) by radial strands (rs). E, longitudinal section of the cilium showing central (cm) and peripheral microtubules (pm) and the glycocalyx (gl).

ally classified in the subclass Ceractinomorpha, are related to some demosponges traditionally included in the subclass Tetractinomorpha. Embryo development in *A. aerophoba* occurred externally, as is typical in most Tetractinomorpha. More importantly, late-stage oocytes developed a surrounding follicle-like envelope of collagen and maternal cells, which was maintained around spawned eggs and blastulae. This envelope was astonishingly similar to the one described around late-stage oocytes, spawned eggs, and externally developing morulae of the chondrosid genus *Chondrosia* (Lévi & Lévi, 1976). Furthermore, as in *A. aerophoba*, the envelope of *Chondrosia reniformis* was

also formed by two maternal cell types: (1) pseudopodia-emitting, amoeboid cells charged with abundant symbiotic bacteria (i.e. bacteriocytes) and (2) microgranular cells very similar to the one described here in *A. aerophoba*. As in *A. aerophoba*, cleavage in *C. reniformis* was equal and total and the embryo differentiated monociliated cells approximately 22–30 h after fertilization. At that stage, the cellular follicle enveloping the embryo also disappeared (Lévi & Lévi, 1976), as in *A. aerophoba*.

The larva of *A. aerophoba*, which was an entirely ciliated coeloblastula with the blastocoel filled with maternal cells and symbiotic bacteria, clearly fits

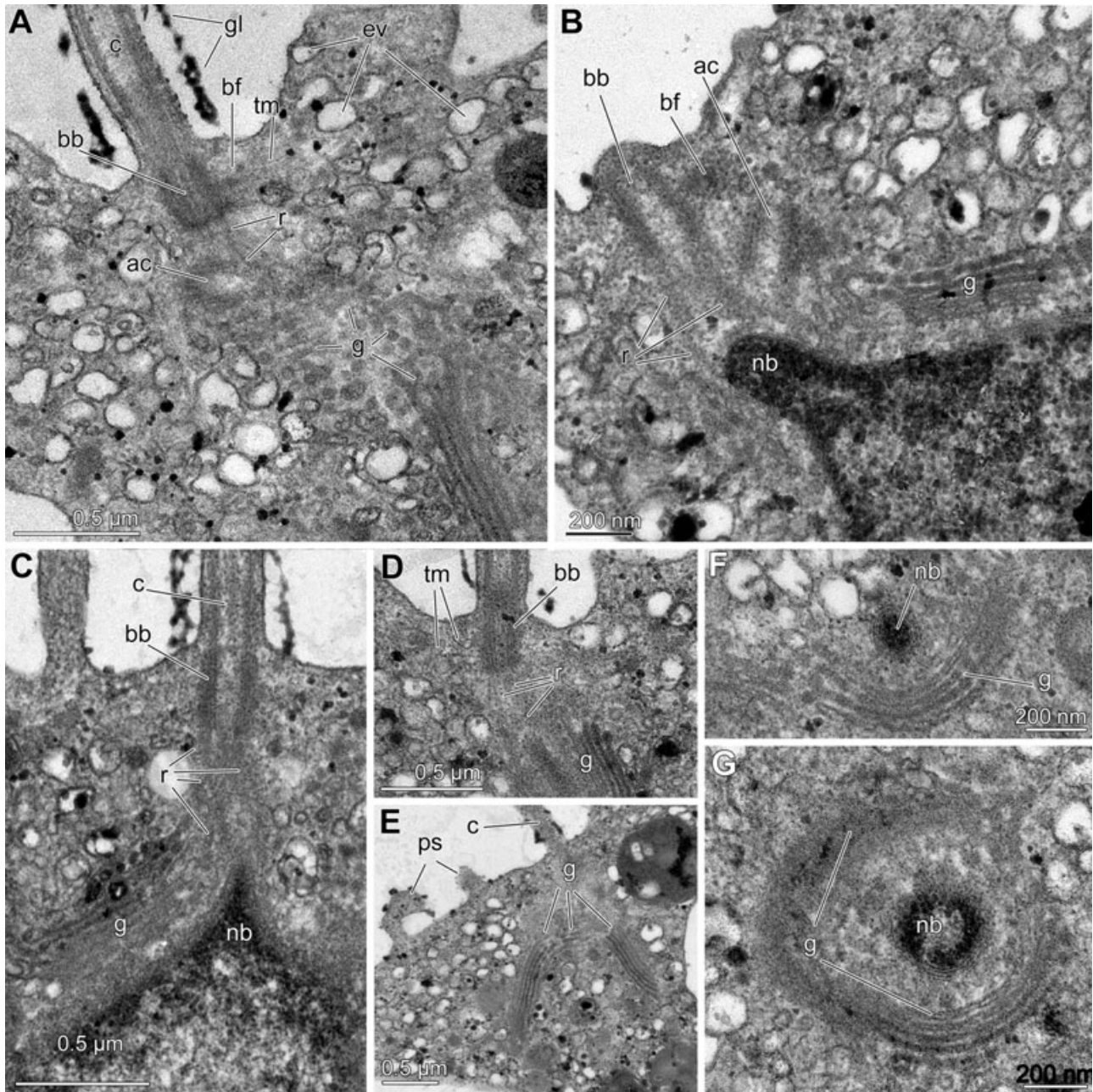


Fig. 11. Longitudinal (A–E) and transversal sections (F–G) at the distal pole of the epithelial cells to show the organelles of the basal apparatus and their relationship between them and the Golgi apparatus and the nucleus, as described in the main text. c, cilium; ps, pseudopodium; gl, glycocalyx; bb, basal body; r, rootlets; bf, basal foot; tm, transverse microtubules; ac, angling accessory centriole; ev, electron-clear vesicle; g, Golgi apparatus; nb, nuclear beak.

within the category of clavablastula (*sensu* Maldonado & Bergquist, 2002) and strongly resembles that previously described for *Chondrosia* and *Chondrilla*. The larva of *A. aerophoba* shows the strongest resemblance in organization with respect to the larva of *C. reniformis*, with just a small difference between the two. Although the blastocoel of *C. reniformis* contains maternal microgranular cells and maternal

bacteriocytes that will exocytose the symbiotic microbes to the larval blastocoel for intergenerational transmission, the blastocoel of *A. aerophoba* contains maternal microgranular cells and spherulose cells but no bacteriocytes. These two larvae also share some noticeable ultrastructural traits. They are both entirely ciliated, with two types of monociliated cells: pseudo-cylindrical in the anterior region and

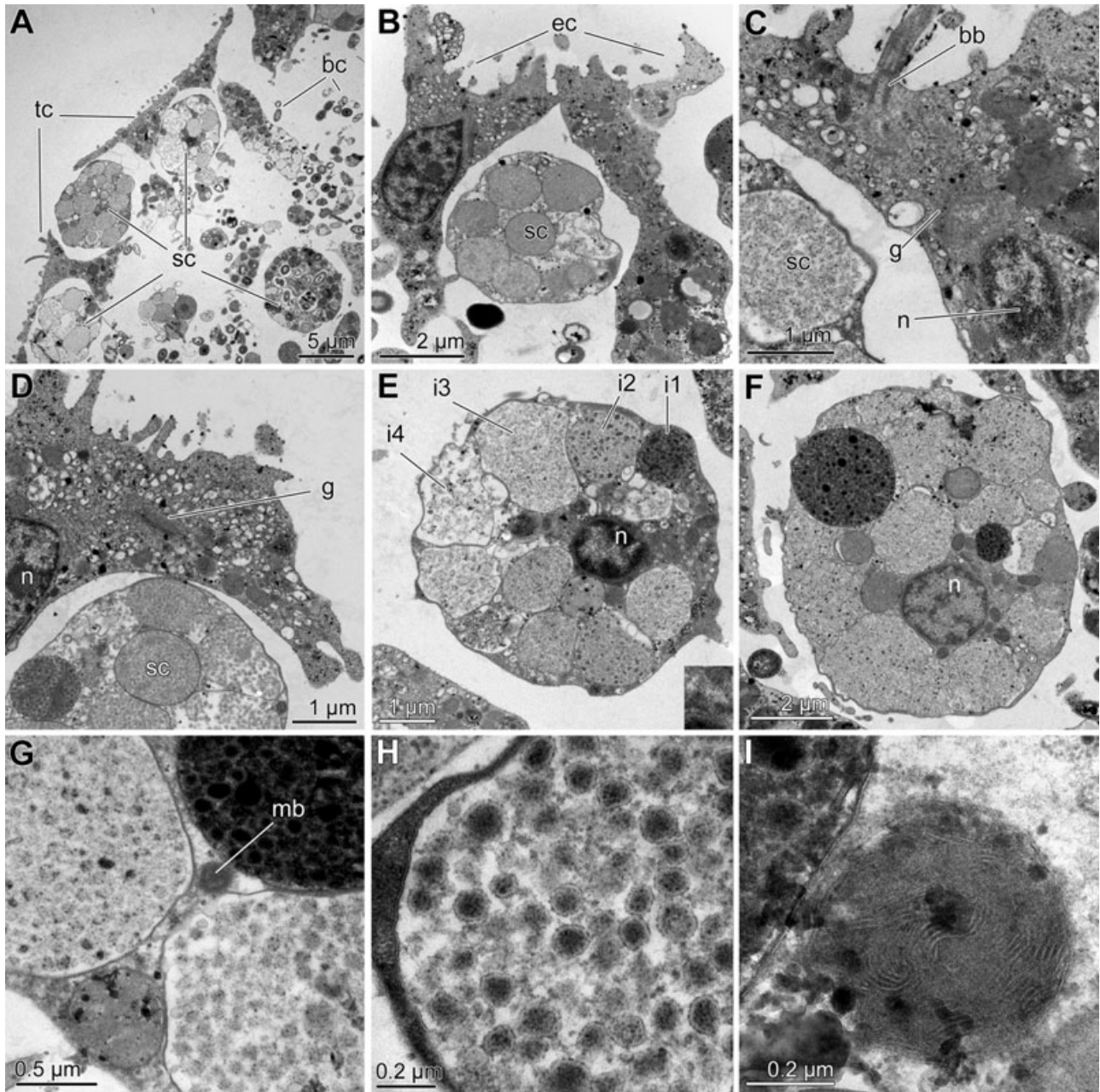


Fig. 12. A, section at the posterior larval pole showing occurrence of maternal spherulous cells (sc) between the body of t-shaped epithelial cells (tc). Some isolated spherulous cells (sc) and abundant symbiotic bacteria (bc) are seen deeper in the blastocoel. B, section at the anterior larval pole showing that the epithelial cells (ec) are not T-shaped despite the occurrence of spherulous cells (sc) between their bodies. C, D, T-shaped cells showing that, unlike non-T-shaped epithelial cells, the basal body (bb), the Golgi apparatus (g) and the nucleus (n) are not in close association. E, F, detail of spherulous cell showing the anucleolate nucleus (n) and marked differences in the level of integrity of some of its inclusions (see 'i1' to 'i4'). G, comparative view of the content of 3 adjacent spherules and a multilayered body (mb) within the cytoplasm of a spherulous cell. H, detail of the structure of granules within a spherule. Granules appear to be bound by a simple membrane. I, detail of one of the multilayered bodies occurring along with the spherules in the spherulous cells.

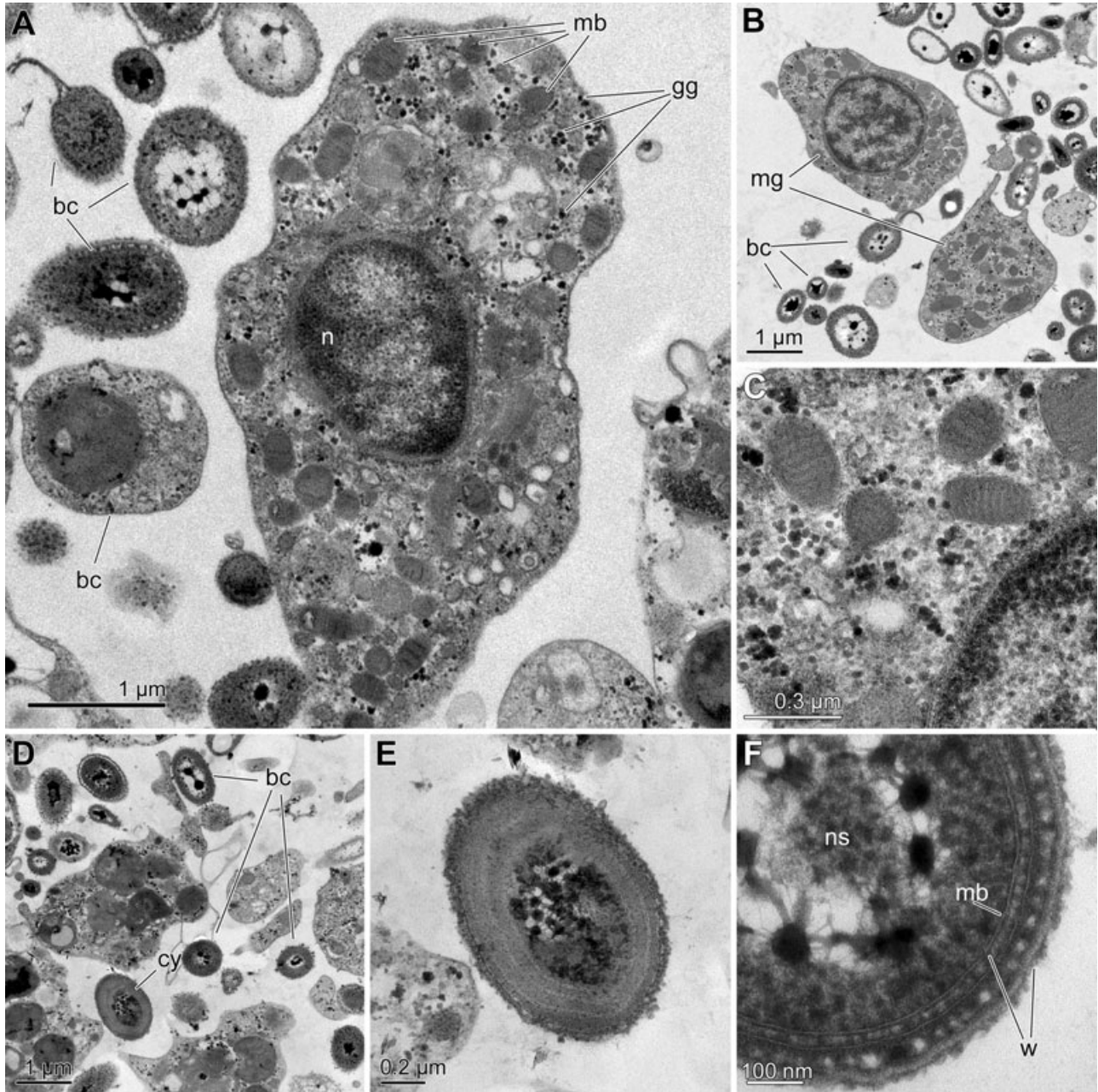


Fig. 13. A, maternal microgranular cell in the larval blastocoel showing the nucleus (n), and a cytoplasm filled with characteristic striated inclusions (mb) and abundant glycogen granules (gg). It is surrounded by symbiotic bacteria. B, abundance of maternal microgranular cells (mg) and symbiotic bacteria (bc) in a deep region of the blastocoel. C, detail of striate inclusions of microgranular cells. D, comparative view of abundance of bacteria (bc) and cyanobacteria (cy) in the blastocoel. E, detail of one of the scarce cyanobacteria. F, detail of the nucleosome (ns), cell membrane (cm) and thick complex wall (w) in a peculiar gram negative symbiotic bacteria.

flattened at the posterior pole. The nucleus of the epithelial larval cells of *C. reniformis* was also reported to be pyriform and, although there was no detailed description of the ultrastructure, illustrations and preliminary description by Lévi & Lévi

(1976) strongly suggest the nucleus to be closely associated with the cilium basal apparatus and the Golgi apparatus, as in *A. aerophoba*.

The general organization of the free-swimming larva of *A. aerophoba* is also similar to that of *Chon-*

drilla australiensis (Usher & Ereskovsky, 2005). The larvae of *C. australiensis* were described as small and ovate, with posterior cilia being sparser and longer than those of the remaining larval surface, with monociliated cells being pseudo-cylindrical except for flattened cells at the posterior pole, and with a larval blastocoel filled with symbiotic bacteria transferred via the blastomeres, as in *A. aerophoba*. More interestingly, ultrastructural peculiarities of the ciliated cells of the *C. australiensis* larva strongly matched some of the ones reported in the present study for *A. aerophoba*: (1) the nucleolated nucleus was pyriform, showing an apex closely associated with the basal apparatus of the cilium, the short rootlet, and the Golgi apparatus and (2) there was also a secondary centriole that was not perpendicular to the basal body but angled at about 60°. Published micrographs (i.e. Usher & Ereskovsky, 2005) suggest that, according to the position of the Golgi apparatus, it could also be encircling the nuclear apex as in *A. aerophoba*. Nevertheless, this detail was not mentioned by those authors and requires re-examination using serial sections.

The striking cytological and ultrastructural similarities in development and larval organization leave little doubt that the orders Verongida and Chondrosida are closely related. The occurrence of these shared distinctive traits comes into complete agreement with the outcome of rRNA and mtDNA analyses, which had also suggested affinity between these two orders (Borchiellini *et al.*, 2004; Nichols, 2005; Lavrov *et al.*, 2008). Although former claims that verongids and chondrosids made a monophyletic clade could initially have been received skeptically in the absence of morphological evidence pointing in the same direction, the congruence found in the present study between developmental and molecular data is far more conclusive. The Chondrosida are a very small order, containing only four genera. It was included in the subclass Tetractinomorpha but had unclear affinities with other demosponge orders (Boury-Esnault, 2002). It is also noteworthy that three of the four chondrosid genera (*Chondrosia*, *Thymosia*, *Thymosiopsis*) lack siliceous spicules and have important development of collagen fibrils in the mesohyl and cortex. Similar collagen development occurs in the fourth genus, *Chondrilla*, but with presence of small, aster-like siliceous spicules. Although, for most of the 20th Century, the Chondrosida had been diagnosed in many, but not all, studies as lacking spongin fibres, recent analyses of 28S rRNA sequences revealed that the monotypic genus *Thymosia*, which is characterized by small, nodule-like spongin fibres, clustered with *Chondrosia* (Vacelet *et al.*, 2000), confirming earlier suspicions of relationship (Topsent, 1895; Boury-Esnault & Lopès, 1985). The nodule-like fibres of *Thymosia* are only approxi-

mately 100 µm in length and show a dendritic arrangement with occasional anastomoses. They have an externally verrucose appearance, which makes them apparently quite different from the diverse fibres found in the order Verongida, Dendroceratida, and Dictyoceratida. Nevertheless, in cross section, they show a laminar bark and granular pith (Topsent, 1895; Boury-Esnault, 2002), resembling the fibre structure in Verongida. This resemblance was also noticed by de Laubenfels (1948), who transferred *Thymosia* to his 'keratose' subfamily Verongiinae from the family Chondrosiidae in which Topsent (1895) had originally placed the genus. In light of the ultrastructural findings reported in the present study, it is instructive to note that both Topsent and de Laubenfels were correct in their interpretations, and that their once-confronting opinions now emerge as complementary because chondrosids and verongids are related.

Interestingly, the analyses of 28S rRNA sequences and mitochondrial genomes have also suggested that Halisarcida are also phylogenetically related to Chondrosida and Verongida (Borchiellini *et al.*, 2004; Lavrov *et al.*, 2008). Similar to the order Chondrosida, the Halisarcida is a very small order containing only the genus *Halisarca*. A complete absence of both mineral and spongin skeletons has extraordinarily complicated the taxonomic allocation of *Halisarca* throughout history. Halisarcida has been stranded within Demospongiae subsequent to its erection by Bergquist (1996), because robust evidence to support affinity to any of the other demosponge orders has not been found (Bergquist, 1996; Bergquist & Cook, 2002). Recent ultrastructural studies of the larva of *Halisarca dujardini* have revealed that monociliated larval cells and choanocytes possess a pyriform nucleus with a marked apex, as well as a basal apparatus bearing an accessory centriole, which is not perpendicular to the basal body, but angling by approximately 45° (Gonoboleva, 2007). In addition, a system of short rootlets arose from the proximal edge of the basal body and run longitudinally to contact the nucleus apex. The accessory centriole, the rootlet, and the nucleus apex are encircled by a large Golgi apparatus, exactly as described in the present study for *A. aerophoba*.

Consequently, in addition to shared features in rRNA and mtDNA organization, three solid apomorphies appear to be consistently shared by the epithelial cells of the larva in the orders Chondrosida, Verongida, and Halisarcida: (1) an angling (nonperpendicular) orientation of the accessory centriole relative to the basal body; (2) a protruding nuclear apex; and (3) a Golgi apparatus encircling the nuclear apex and part of the organelles of the basal apparatus. Furthermore, these three features also occur in at

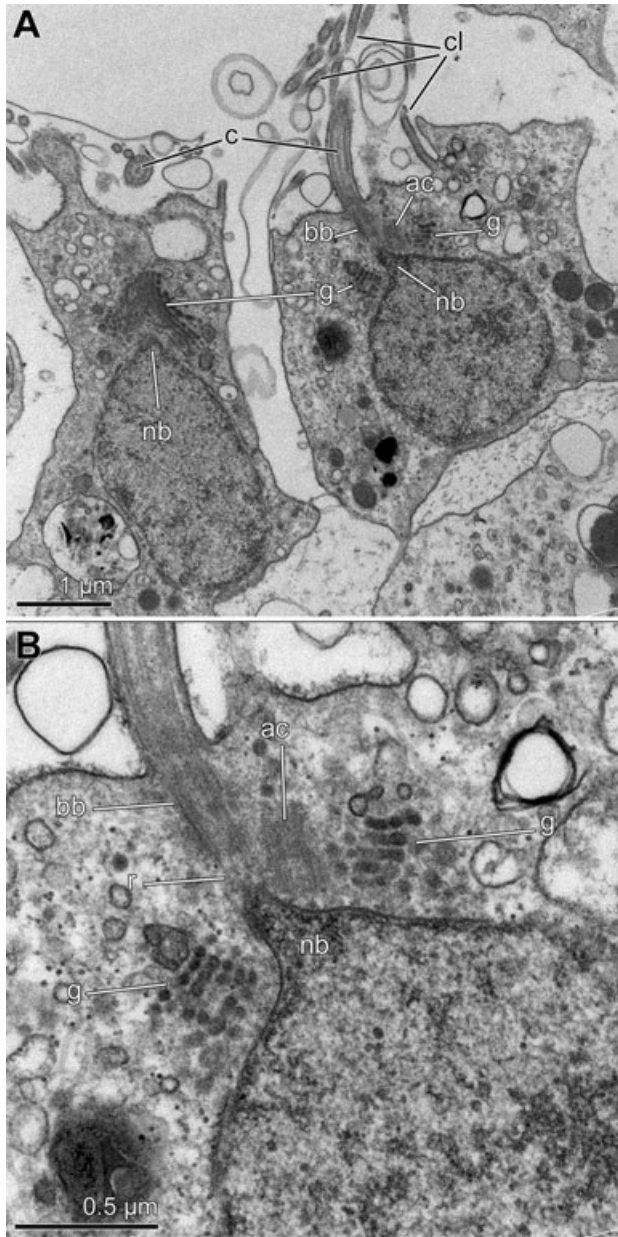


Fig. 14. A, B, longitudinal sections of choanocytes in an adult male sponge. ac, accessory centriole; bb, basal body; c, cilium; cl, collar microvilli; g, Golgi apparatus; nb, nuclear beak; r, rootlets.

least the choanocytes of *Halisarca* (Gonobobleva & Maldonado, 2009) and *A. aerophoba* (Fig. 14).

Regarding embryology, there is some internal heterogeneity within the 'Chondrosida–Verongida–Halisarcida' clade. The viviparous Halisarcida have a distinctive brooded larva produced through a process of internal development essentially different from that described in the oviparous Chondrosida and Verongida (Lévi, 1956; Ereskovsky & Gonobobleva,

2000; Maldonado & Bergquist, 2002). For this reason, this three-order clade does not fit properly into either the subclass Tetractinomorpha (initially erected for oviparous demosponges) or the Ceractinomorpha (originally erected for viviparous sponges). Rather, heterogeneity in development mode within a well-supported 'Chondrosida–Verongida–Halisarcida' clade provides additional evidence that the subclasses Tetractinomorpha and Ceractinomorpha cannot be maintained on embryological criteria any longer. It is widely understood that, although Tetractinomorpha and Ceractinomorpha remain formally accepted taxonomic subclasses (Table 1), their entities have been challenged by both traditional taxonomic discussion (Hooper & Van Soest, 2002) and several recent molecular studies (Borchiellini *et al.*, 2004; Nichols, 2005; Lavrov *et al.*, 2008). The latter studies concluded these subclasses to be polyphyletic assemblages of orders and indicated the need to undertake a high-rank reorganization of the Demospongiae classification.

A first step towards re-organization may be to erect a new subclass to harbour Verongida, Chondrosida, and Halisarcida, which will also reflect taxonomically the phylogenetic closeness between these three orders. For this purpose, the old name Myxospongia (new subclass) is resuscitated because it was recently used by Borchiellini *et al.* (2004) to refer to the clade consisting of 'Chondrosida + Halisarcida + Verongida', as revealed by their 28S rRNA analyses. The name can be traced back until Haeckel (1866: XXIX), who used it at the order level (as 'Myxospongiae') to harbour sponges lacking both mineral and fibre skeleton. Although Haeckel's Myxospongiae originally included the genus *Halisarca* only, genera such as *Chondrosia* and *Oscarella* were added by others later, fostering a non-natural group based on the absence of skeleton. This concept pervaded through the first half of the 20th Century, until Lévi (1957) efficiently argued that the loss of skeleton had been an independent, parallel process in different sponges lineages, and dismantled this and other high-rank groups of that time to establish his subclass system based on embryological features.

Some studies have recently suggested abandoning the subclass rank in the classification system of Porifera. For example, based on examples of mitochondrial genetic divergence within a single family of Demospongiae being approximately three-fold greater than that between two orders, Lavrov *et al.* (2008) recently argued that the traditional taxonomic ranks in demosponge systematics are artificial, proposing that these ranks should be abandoned. However, their arguments connecting mtDNA distances and taxonomic ranks may not be straightforward. It is well established that different genomic regions have expe-

rienced substantially different evolutionary histories and that taxa with high rates of mtDNA change are often taxa with low rates of rRNA evolution. Therefore, the argument by Lavrov *et al.* (2008) would only be valid in the case that genetic distances were calculated from complete genomes. With the current state of knowledge, disparity of evolutionary rates in only the mitochondrial genomes should never be interpreted as a 'lineal' measure of taxonomic closeness among high-level taxa, and not in the absence of complete genomes and without a sensible understanding of the selective pressures behind the evolution of mitochondrial and ribosomal genomes. Given that both molecular and morphological phylogenetic analyses show that the taxonomic orders of the class Demospongiae cluster in groups, those groups must be interpreted taxonomically as subclasses, whenever we understand the class Demospongiae as a monophyletic unit. Hence, rather than eliminating the subclass rank, the challenge is to refine our current understanding of the ordinal relationships so that the taxonomic subclasses reflect natural clades that can be confirmed by both morphological and molecular data, as appears to be the case of Myxospongia. A reliable global re-organization of the Porifera high-rank taxonomy will require extensive discussion on the relative strengths of the phylogenetic signal in a variety of molecular, histological, and reproductive characters.

SPONGIN VERSUS SILICA SKELETONS

The developmental and ultrastructural data gathered in the present study strongly support the hypothesis that spongin skeletons have evolved independently at least twice within Demospongiae. This view is also consistent with the demosponge phylogenies proposed from recent molecular studies (Borchiellini *et al.*, 2004; Nichols, 2005). Loss of spicules followed by development of spongin fibres appears to have occurred at least once within a 'lineage' characterized by aster-like spicules (i.e. Chondrosida–Verongida), and also once through a 'linage' probably characterized by monaxonic spicules (i.e. Haplosclerida 'in part' – Dictyoceratida – Dendroceratida). Independent 'evolutionary assays' to acquire spongin skeletons as an alternative to silica skeletons could have been favoured by major environmental changes at the Cretaceous–Tertiary boundary (approximately 65 Mya). It has been postulated that the evolutionary expansion of diatoms at that time caused a drastic decrease in silicate concentration in the photic zone of the world's ocean, a process suspected to have had a great impact on the skeletal evolution and bathymetric distribution of modern siliceous sponges (Maldonado *et al.*, 1999). During that silicate-

impoverished Late-Cretaceous environment, many siliceous sponge species were extinguished, others migrated down to nonphotic waters richer in silicate, and others remained in silica-impoverished shallow waters at the cost of weakening their siliceous skeletons. In this latter group, and under the increasing environmental pressure of decreasing silicate availability in seawater, any fortuitous production of materials (e.g. collagen derivatives = spongins) that could somehow complement or replace the weakening silica skeletons could have been favoured by natural selection. Therefore, the hypothesis is proposed that spongin skeletons could have been acquired simultaneously, but independently, by different lineages of siliceous demosponges in response to the Cretaceous silicate crisis caused by the expansion of diatoms. Interestingly, this hypothesis is also consistent with the fact that Verongida and Chondrosida, as well as Dictyoceratida and Dendroceratida, are all orders containing mostly shallow water species in temperate and tropical latitudes. Only few species are able to live in relatively cold waters at either high latitudes or bathyal depths (Lévi & Lévi, 1983; Vacelet, 1988; Ilan, Ben-Eliahu & Galil, 1994; McClintock, Amsler & Baker, 2005). Experimental evidence indicates that adults can survive in waters colder than those of their natural habitats, although, in such a case, their sexual reproduction appears to be inhibited (Maldonado & Young, 1998). All this information is consistent with the previously suggested idea of 'corneous' sponges having appeared in shallow waters of temperate or tropical latitudes (Maldonado & Young, 1998). Unfortunately, the fossil record is of little help in this regard because aspiculate sponges rarely leave many obvious traces. The best known fossils regarded as 'keratose' belong to the family Vauxiidae (Walcott, 1920), dating from the middle Cambrian Burgess Shale (520–512 Mya). Such an ancient origin for a 'keratose' sponge would definitively disprove the hypothesis of a Cretaceous origin triggered by the silicate crisis. Nevertheless, there are reasonable doubts that Vauxiidae are fossils characterized by a true spongin skeleton. Their hexagonal skeletal network was originally interpreted by Walcott (1920) as belonging to the class Hexactinellida; subsequently, it was considered to be a lithistid skeleton (Rigby, 1980) and, more recently, putatively reinterpreted as a 'keratose' skeleton (Rigby, 1986). The amazing 'taxonomic journey' from one end to the other of Porifera classification illustrates the enormous uncertainty surrounding any unequivocal interpretation of these ancient fossils. It advises caution before considering *Vauxia* as refutable evidence that the Cretaceous silicate crisis was unrelated to the emergence of at least some forms of aspiculate demosponges. Likewise, it cannot be ruled out that the

Late-Cretaceous silicate crisis also favoured attempts to replace the increasingly scarce silicon for the overabundant calcium carbonate to build the sponge skeletons, giving rise to some particular forms of 'coralline' sponges. Although there is unequivocal fossil evidence that the emergence of most 'coralline lineages' predated the Cretaceous, the case could be different for *Vaceletia*, a hypercalcified sponge recently demonstrated by rDNA analyses to be a dictyoceratid (Wörheide, 2008).

CHIMERISM AND TRANSFER OF MICROBIAL SYMBIONTS

Development in *A. aerophoba* was shown in the present study to be a relatively sophisticated process. Oogenesis involved not only events in the oocyte, but also selective migration of microgranular and spherulous maternal cells to the oocyte vicinity, as well as coupled secretion of an enveloping collagen layer by the oocyte to produce a 'nesting follicle'. It remains unresolved from the present study whether the follicle had a role in either transference of the spermatozoon to the oocytes or in providing a physical barrier to prevent polyspermy. Spherulous cells occur in adults of *A. aerophoba* in a wide range of sizes (up to $12.6 \times 7.0 \mu\text{m}$, measured in the present study), but only those in a mid-size category ($6.6 \times 4.2 \mu\text{m}$ to $10.5 \times 6.2 \mu\text{m}$) were used to build the oocyte follicle. These mid-size cells were previously described in *Aplysina cavernicola* by Vacelet (1967). During oogenesis and also early cleavage, the spherulous cells of the follicle exocytosed some of their multigranular inclusions for subsequent incorporation by both oocytes and blastomeres. Transference of nutrients from the follicle cells had not been detected in a previous study of *Aplysina* oocytes (Gallissian & Vacelet, 1976). Likewise, microgranular cells, which were comparatively scarce in the follicles, were unreported in that same study (Gallissian & Vacelet, 1976).

At no stage of development was there evidence to support that microgranular and spherulous cells in the larva derived from either the blastomeres or epithelial larval cells. Both spherulous and microgranular cells of the larval blastocoel consistently lacked yolk bodies and any other recognizable remains of the cytological organization of blastomeres. By contrast, spherulous and microgranular cells of the follicle were seen to invade the cleavage furrows of early blastulae, strongly supporting the idea that these cells are not proper larval cells derived from blastomeres but maternal cells migrated from the follicle. Migration of both microgranular and spherulous cells from the follicle to the nascent blastocoel of embryos provided a striking example of production of a chi-

meric organism, in which two types of maternal cells co-exit with cells of the newly-produced individual during the embryonic stage, the larval stage, and in early settlers at least. 'Chimeric' larvae incorporating maternal cells have also been described in *Chondrosia* (Lévi & Lévi, 1976) and *Cliona* (Warburton, 1961). *In vivo* light microscopy indicated that many of the maternal spherulous cells occurring in the mesohyl of the *A. aerophoba* settlers started darkening after approximately 3.5 weeks in the laboratory cultures (Fig. 7K, L, M). It remains unresolved whether they were experiencing an apoptotic process to reinstate the nonchimeric condition in the juvenile or just trans-differentiating into other cell types. If it was apoptosis, it is hard to determine whether it was a natural process or it was triggered by culturing conditions because, after 1 month in the laboratory, the surviving juveniles looked unwell, possibly due to non-ideal conditions, compared to those in the ocean, in terms of oxygen supply, light quality, food quality, micronutrients, etc. Therefore, the possibility that the chimeric condition of the juvenile reaches the adult stage cannot be discarded from the present study.

Spherulous cells are the primary cell compartment in which bromotyrosine derivatives are produced and temporarily stored in the sponge (Turon, Becerro & Uriz, 2000). These compounds, with demonstrated antibacterial activity, are postulated to be used in population control of bacterial symbionts. Symbiotic bacteria are engulfed from the maternal mesohyl by the oocyte during early oogenesis. They remain undigested in membrane-bound vesicles, being subsequently transferred to the cytoplasm of blastomeres and finally exocytosed by those to the nascent larval blastocoel of developing larvae. Because a large amount of free symbiotic bacteria are vertically transmitted within the larva blastocoel, coupled transference of spherulous cells could have evolved to make possible the control of transmitted microbial populations during those life-history stages prior to differentiation of a new generation of functional spherulous cells. Extrusion of spherulous cell through the posterior larval pole remains an enigmatic process. Extrusion of spherulous cells through the epithelia of the aquiferous canals of adults had also been documented by Vacelet (1967), who suggested that it could be a way to eliminate excretory products.

Cyanobacteria are vertically transferred along with other symbiotic microbes via the cytoplasm of eggs and sperm in many demosponges (Usher *et al.*, 2001; Maldonado *et al.*, 2005; Oren, Steindler & Ilan, 2005). Therefore, it is remarkable that inter-generational transference of symbiotic cyanobacteria does not appear to be the case in *A. aerophoba*. Although hundreds of bacteria ready for inter-generational transference were seen within oocytes, cyanobacteria were

never detected. After an intense TEM search, just three cyanobacteria were found, and not in eggs but within the blastocoel of a 3-day old free-swimming larva (Fig. 13D, E), which suggests that they had recently been incorporated from the ambient water. The case is similar to the one recently described for *Petrosia ficiformis*, in which neither cyanobacteria nor bacteria were vertically transferred to the progeny (Maldonado, 2007). Hence, they have to be acquired from ambient water at each generation.

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