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Ultrastructure and embryonic development of a syconoid calcareous sponge

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Abstract. Recent molecular data suggest that the Porifera is paraphyletic (Calcarea+Silicea) and that the Calcarea is more closely related to the Metazoa than to other sponge groups, thereby implying that a sponge-like animal gave rise to other metazoans. One ramification of these data is that calcareous sponges could provide clues as to what features are shared among this ancestral metazoan and higher animals. Recent studies describing detailed morphology in the Calcarea are lacking. We have used a combination of microscopy techniques to study the fine structure of *Sycon coactum* URBAN 1905, a cosmopolitan calcareous sponge. The sponge has a distinct polarity, consisting of a single tube with an apically opening osculum. Finger-like chambers, several hundred micrometers in length, form the sides of the tube. The inner and outer layers of the chamber wall are formed by epithelia characterized by apical–basal polarity and occluding junctions between cells. The outer layer—the pinacoderm—and atrial cavity are lined by plate-like cells (pinacocytes), and the inner choanoderm is lined by a continuous sheet of choanocytes. Incurrent openings of the sponge are formed by porocytes, tubular cells that join the pinacoderm to the choanoderm. Between these two layers lies a collagenous mesohyl that houses sclerocytes, spicules, amoeboid cells, and a progression of embryonic stages. The morphology of choanocytes and porocytes is plastic. Ostia were closed in sponges that were vigorously shaken and in sponges left in still water for over 30 min. Choanocytes, and in particular collar microvilli, varied in size and shape, depending on their location in the choanocyte chamber. Although some of the odd shapes of choanocytes and their collars can be explained by the development of large embryos first beneath and later on top of the choanocytes, the presence of many fused collar microvilli on choanocytes may reflect peculiarities of the hydrodynamics in large syconoid choanocyte chambers. The unusual formation of a hollow blastula larva and its inversion through the choanocyte epithelium are suggestive of epithelial rather than mesenchymal cell movements. These details illustrate that calcareous sponges have characteristics that allow comparison with other metazoans—one of the reasons they have long been the focus of studies of evolution and development.

Additional key words: Porifera, evolution, development, embryo, Metazoa

The Calcarea are a group of delicate, often inconspicuous sponges that make up less than 5% of all described Porifera. Yet, these animals have had a greater impact on the general understanding of sponge structure and function, and on the ideas concerning the evolution and systematics of the Metazoa, than any other sponge group (Haeckel 1874; Lévi 1963; Tuzet 1963; Borojevic 1970; Borchiellini et al. 2001; Manuel et al. 2003). Calcareous sponges likely have made their mark because they are the only group within the Porifera in which all three types of body organization—asconoid, syconoid, and leuconoid—are found. These categories are described in most texts as morphological grades of evolutionary complexity in all sponges, from "primitive," simple single tubes

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to "advanced," complex, convoluted channels and chambers. However, this diversity of body organization is not found in the Demospongiae or the Hexactinellida, which together include 95% of sponges (e.g., Ruppert et al. 2004). Haeckel (1872) first used ascon, sycon, and leucon body types as families within the Calcarea, but the scheme was quickly found to be artificial and abandoned (see review by Manuel et al. 2002). The three grades of organization are found in different families of the Calcarea where they nonetheless are still proposed to represent an evolutionary lineage from simple to complex (see Borojevic et al. 2002).

Recent molecular data suggest that the Porifera are paraphyletic, i.e., calcareous sponges are more closely related to other metazoans than to other sponges, thereby implying that a sponge-like animal gave rise to other metazoans (Zrzavy et al. 1998; Borchiellini et al. 2001; Peterson & Butterfield 2005). While the molecular data are not yet conclusive (Borchiellini et al. 1998; Manuel et al. 2003), the proposition taught us to consider what features calcareous sponges—seen as an endpoint in one experiment in the evolution of multicellular animals—may possess that reflect the shared common ancestor of all metazoans. Immediately it becomes apparent, however, that a detailed understanding of the morphology of these sponges is lacking.

We examined the ultrastructure of *Sycon coactum,* one of the genera that featured importantly in early evolutionary developmental studies (Haeckel 1872; Schulze 1875, 1878; Hammer 1908). Our goal was to provide a clearer understanding of the syconoid body plan, calcareous sponge tissue organization, and embryonic development, building on the work of Duboscq & Tuzet (1935), Tuzet (1947, 1963), Gallissian (1980, 1983, 1988), Franzen (1988), and most recently Anakina (1997). We have shown that, although slightly built, the sponge has three distinct cellular regions, including well-sealed inner and outer epithelia and a collagenous mesohyl containing both skeletogenic cells and embryos. Our results have illustrated that the feeding chambers are vast structures and choanocytes are highly irregular, suggesting that filtration mechanisms may differ from leuconoid sponges. Our examination of fixed specimens has shown that embryonic development proceeds through a hollow cup to form a near-blastula that inverts to form the larva. Here, we also present evidence that this sponge exhibits distinct apical–basal polarity and polarized epithelia, two characters that could reflect the shared common ancestry of multicellular animals.

Methods

Specimens of the calcareous sponge *Sycon coactum*[1] Urban 1905 (Austin & Ott 1987; Manuel et al. 2002) were collected between May and mid-August 2002–2005 at 10-m depth from pilings and ropes under the docks at the Bamfield Marine Sciences Center (BMSC), Bamfield, BC, Canada. Plastic substrates, gardening pots suspended upside down, to which sponges were attached were lifted to the surface where sponges were removed, while still submerged. The sponges were placed in opaque plastic tubes underwater, and the tubes were floated in 10°C seawater while being transported to the laboratory. In the laboratory, the sponges were cut with a sharp scalpel and forceps into small squares $\sim 5 \times 5$ mm; these were placed directly into a 2-mL cryotube (Sarstedt, Nümbrecht, Germany) containing a cocktail fixative of 1% OsO_4, 2% glutaraldehyde in 0.45 mol L^{-1} sodium acetate buffer, pH 6.4, with 10% sucrose in the final volume. Samples were fixed on ice for 30 min, after which fresh fixative was added and samples were left at 4°C overnight.

Preliminary results using the above fixative showed great variation in the morphology of choanocyte collars in the flagellated chambers. To rule out the possibility that the speed of fixation and/or the type of fixative could affect preservation of choanocytes, an extremely rapid fixation of specimens immediately after collection was used and six other fixation protocols were tested (Table 1). In addition, to evaluate the effect of handling on tissue morphology, sponges were fixed using the primary cocktail fixative (above) after incubation in relaxants (0.3 mol L^{-1} $MgCl_2$ or menthol crystals) and after 5-min agitation. Specimens were also fixed in the presence of alcian blue and ruthenium red after the procedure of Gonobobleva & Ereskovsky (2004) and with lanthanum nitrate (Humason 1979) to highlight cell junctions and test the permeability of epithelia.

[1] The Northeast Pacific species of Calcarea have not been formally revised. According to Austin & Ott (1987) and to the keys of C. Smecher ⟨http://www.interchg.ubc.ca/csmecher/scypha/raphanus.htm⟩ the sponge is closest to that identified by de Laubenfels (1961) as *Sycon raphanus* Schmidt 1862. Other North Pacific species have been variously identified by Franzen (1988) and Kozloff (1987) as *Scypha ciliatum,* which is not in the keys of the region. The genus *Scypha* has been synonomized with *Sycon* (Manuel et al. 2002). The formal identification of our study species by H. Tore Rapp, University of Bergen, is *Sycon coactum* Urban 1905. In previous work (see Leys & Eerkes-Medrano 2005), we have referred to the sponge *Sycon* cf. *raphanus*.

Table 1. Fixation protocols tested on the morphology of *Sycon coactum*. RT, room temperature. Sources: 1, adapted from Harris & Shaw (1984); 2–5, adapted from Ereskovsky & Gonobobleva (2000); 6, adapted from Lethias et al. (1983); 7, adapted from E. Balser, pers. comm.; *adapted from Hayat (2000: p. 27).

Source	Primary fixative	Secondary fixative
1	Cocktail—1% OsO_4, 2% glutaraldehyde, 0.45 mol L^{-1} NaAc buffer, pH 6.4, 12 h, 4°C (both with and without 1% alcian blue or 1 mg mL^{-1} ruthenium red)	
2	1% OsO_4 phosphate buffer* 1.5 h, RT	2.5% glutaraldehyde with 1% alcian blue in phosphate buffer, 1.5 h, RT
3	1% OsO_4 in 0.1 mol L^{-1} cacodylate buffer, pH 7.4, 1.5 h, RT	2.5% glutaraldehyde with 1 mg mL^{-1} ruthenium red in 0.1 mol L^{-1} cacodylate buffer, pH 7.4, 1.5 h, RT
4	2.5% glutaraldehyde with 1% alcian blue, 1.5 h, RT	1% OsO_4 with 1% lanthanum nitrate in 0.1 mol L^{-1} cacodylate buffer, pH 7.4, 1.5 h, RT
5	2.5% glutaraldehyde in 0.1 mol L^{-1} cacodylate buffer, pH 7.4, 1.5 h, RT	1% OsO_4 with 1% lanthanum nitrate in 0.1 mol L^{-1} cacodylate buffer, pH 7.4, 1.5 h, RT
6	0.4% glutaraldehyde in 0.1 mol L^{-1} cacodylate buffer, pH 7.4, 1 h, 4°C	1% OsO_4 in 0.1 mol L^{-1} cacodylate buffer, pH 7.4, 1 h, 4°C
7	2.5% glutaraldehyde in Millonigs phosphate buffer and 0.6 mol L^{-1} NaCl, 1 h, 4°C	1% OsO_4 in phosphate buffer in Millonigs phosphate buffer with 0.75 mol L^{-1} NaCl

For these experiments, using SCUBA, individual sponges were removed from the pilings together with a small piece of the substratum to which they were attached (usually wood or barnacle carapaces) and floated into a plastic bag. The bag was brought to the surface within 2 min of collection and instantly placed in an insulated container filled with 10°C seawater. Rapidly, individual sponges were tipped out of the bag while still underwater and, holding each sponge gently by its substrate, it was cut in half lengthwise (to ensure that the fixative had immediate contact with the interior of the sponge) and placed in a submerged 10 mL plastic tube. The seawater was removed from the tube until the pieces of sponge were just covered ($\sim$2 mL), and the fixative was added. The entire procedure from underwater collection to the addition of the fixative took <10 min and specimens were not removed from cold seawater at any time. The samples were placed on ice and carried to the laboratory where, within 0.5 h, the fixative was renewed. Samples were left in fixative overnight at 4°C.

Fixation was followed by rinsing the samples in filtered seawater and distilled water 3 × for 10–30 min each. Samples were decalcified for 2 h in a 5% solution of ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA). Decalcified samples were rinsed twice for 15 min with distilled water, dehydrated to 70% ethanol, and stored in 70% ethanol for transport to the University of Alberta.

For scanning electron microscopy, the tissue samples were dehydrated to 100% ethanol and fractured in liquid nitrogen. Tissue samples were critical-point dried and 7–8 pieces were mounted on aluminum stubs using clear nail polish as an adhesive. Specimens were coated with gold and viewed with a JEOL 6301F field emission scanning electron microscope. For thin sections, specimens were stained en bloc in uranyl acetate; 80-nm sections were cut on a Leica-Ultracut-T microtome, stained with lead citrate, and examined with a Phillips (FEI, Morgagni) transmission electron microscope.

Other specimens were fixed directly in Bouin fixative fluid for 12–24 h, after which they were transferred to 70% ethanol for transport to the University of Alberta. Pieces were rehydrated and stained en bloc in hematoxylin for 2–3 min. Hand sections of chambers were cut with a razor blade, dehydrated to 100% ethanol, and mounted in Canada Balsam on glass slides for light microscopy. Sections and whole mounts were viewed with a Zeiss Axioskop microscope and images were captured using a QiCam camera with Northern Eclipse software. Images of whole specimens were taken with a Nikonos 5 underwater camera.

Results

Gross morphology

The observed specimens of *Sycon coactum* formed delicate ivory-colored tubes, 10–20 cm in length and 1–2 cm in diameter, which arise from a small stalk ($\sim$0.5 cm long) at the base of the sponge (Fig. 1A). The wall of the tube was 0.5–1.2 mm thick and a fringe of spicules (2 mm long) lined the osculum. The

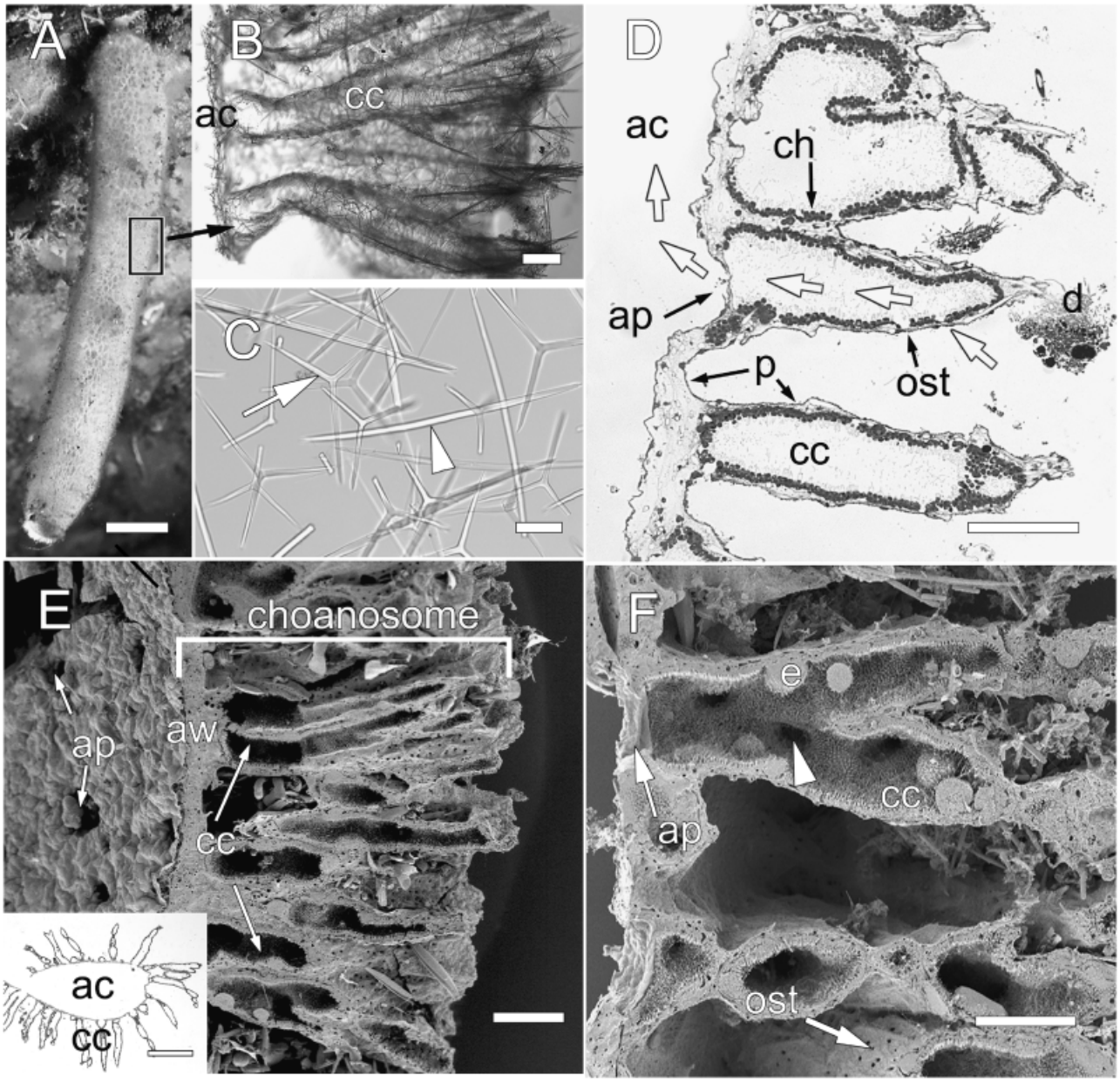


Fig. 1. General anatomy of *Sycon coactum*. **A.** Image of a specimen photographed *in situ* attached to a rock wall. The osculum is facing down, opening toward the left. A region equivalent to that in the box is shown in B and D. Scale bar, 1 cm. **B.** Light micrograph of a thick, hand section of the sponge cleared in xylene to show the spicules supporting choanocyte chambers (cc) to the right of the atrial cavity (ac). Triaxon spicules line the chamber walls, and oxeas are at the distal tips of the chambers. Scale bar, 200 μm. **C.** Light micrograph of triaxon (arrow) and oxea (arrowhead) spicules from the sponge. Scale bar, 100 μm. **D.** Light micrograph of a semi-thin longitudinal section through several choanocyte chambers (cc) and the atrial cavity (ac). The inner surface of the chambers is lined by choanocytes (ch) and the outer surface is lined by pinacocytes (p). The path of water flow through the sponge is indicated by white arrows. Water enters the sponge through ostia (ost) that lead into the choanocyte chambers. From the choanocyte chambers water enters the atrial cavity (ac) via a large opening, the apopyle (ap). d, diatoms. Scale bar, 100 μm. **E**, **F.** Scanning electron micrographs of sponges fractured in liquid nitrogen. **E.** A fracture showing a portion of a cross section of the sponge. The choanosome comprises the region from the distal tip of the choanocyte chambers to the atrial wall (aw) on the left where water exits the choanocyte chambers via apopyles (ap). (Inset shows a light micrograph of a semi-thin cross section for orientation.) Scale bar, 100 μm; inset, 500 μm. **F.** A fracture showing a choanocyte chamber (cc) with several branches (one is indicated by the arrowhead), the apopyle (ap), ostia (ost), and developing embryos (e). Scale bar, 100 μm.

sponges generally occurred singly, but clumps of 2–3 sponges sometimes arose from a single base. Tubes increased in length and diameter over the course of the summer from 1–2 cm long and 0.2–0.3 cm in diameter in May to 5–20 cm long and 0.6–1.3 cm in diameter in August. By mid-August many of the sponges were in various stages of decomposition, and by late August most specimens had died. Numerous epiphytes were among the ridges and spicules on the outside of the sponge; these included diatoms, foraminiferans, ciliates, flatworms, polychaetes, ostracods, caprellids, isopods, and bryozoans.

The gross anatomy of the sponge is illustrated in Fig. 1. The tube wall—the choanosome—was composed of long finger-like choanocyte chambers lying perpendicular to the atrial cavity. Triaxon spicules lined the wall of chambers, and a tuft of oxea spicules projected from the distal tip of each chamber, giving the sponge a hispid, or bushy, appearance (Fig. 1B,C). Adjacent chambers were fused to each other at one or more places; these chambers shared a common outer wall and sometimes a common choanocyte chamber (Fig. 1D–F). Other chambers branched such that up to 4 interconnected chambers opened via a single large (30-μm diameter) apopyle into the atrial cavity (Fig. 1F).

The wall of each choanocyte chamber had three discrete layers: an outer epithelium, the pinacoderm, a central region with ameboid cells, developing embryos and sclerocytes within a loose collagenous mesenchyme, the mesohyl, and an inner choanocyte epithelium, the choanoderm (Fig. 2).

Pinacoderm epithelium

The pinacoderm was formed by square, pentagonal, or hexagonal cells (pinacocytes) that created

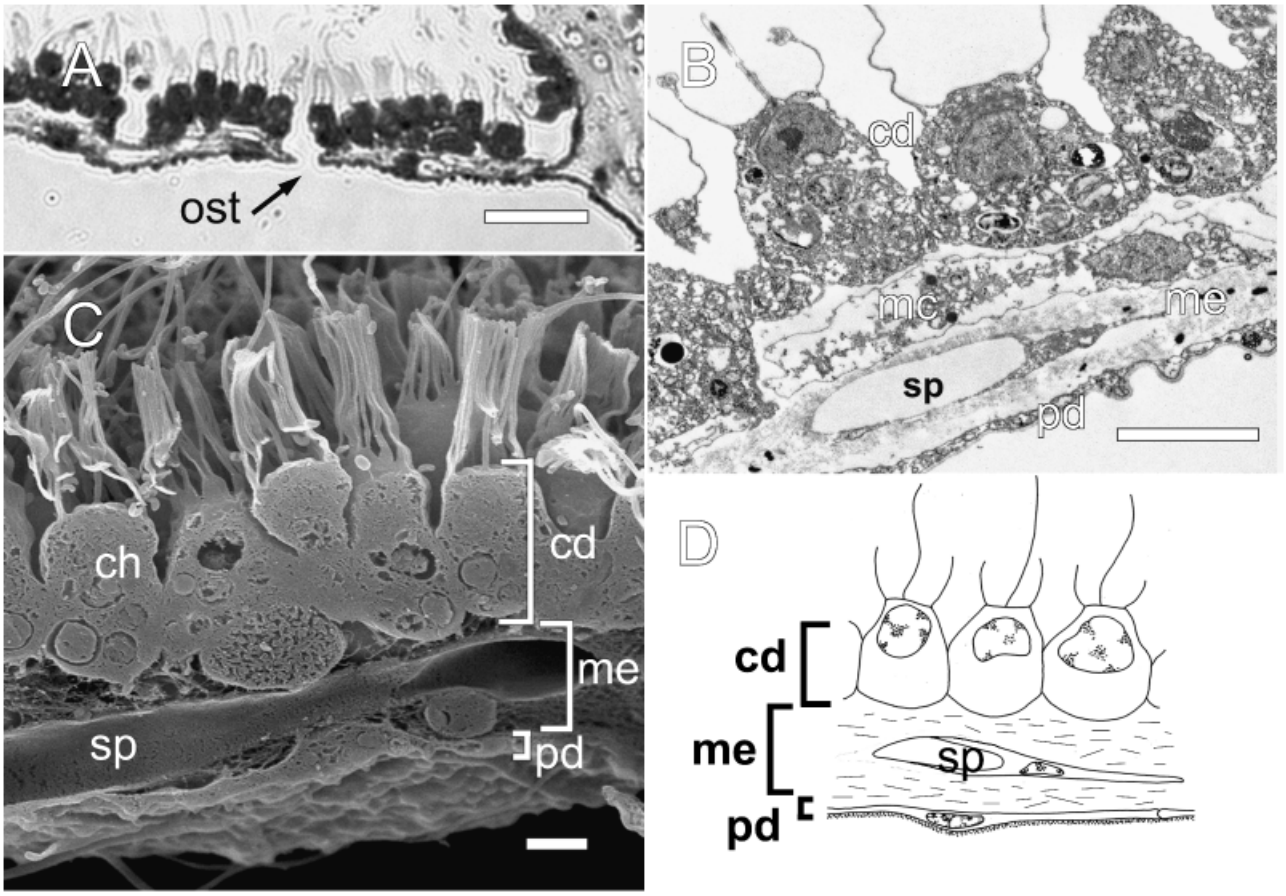


Fig. 2. The wall of choanocyte chambers in *Sycon coactum*. **A.** Light micrograph of a semi-thin plastic section showing an ostium (ost) leading into the choanocyte chamber. Scale bar, 20 μm. **B**, **C.** Transmission and scanning electron micrographs (respectively) of a section and fracture through the choanocyte chamber wall. The choanoderm (cd), composed of choanocytes (ch), and pinacoderm (pd) epithelia sandwich a thin collagenous mesohyl (me) that houses spicules (sp), developing embryos (not shown), and ameboid mesohyl cells (me). Scale bars, B, 5 μm; C, 2 μm. **D.** Sketch illustrating the relationship of cells in each zone.

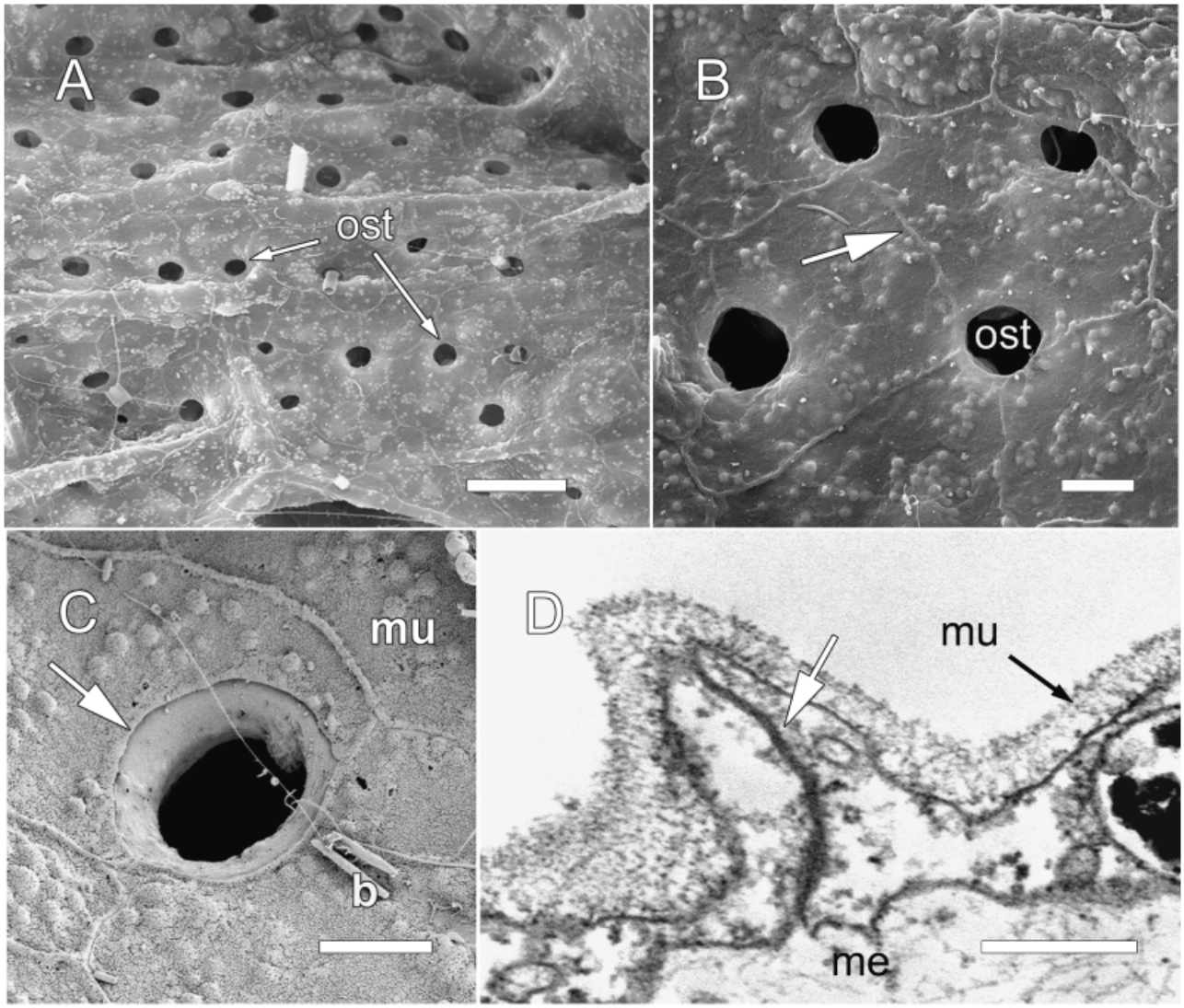


Fig. 3. The pinacoderm, viewed by scanning (A, B, C) and transmission (D) electron microscopy. **A**, **B.** The outer surface of the sponge is formed by an epithelium of square, pentagonal, and hexagonal cells, pinacocytes, that form a ridge (arrow) where they join adjacent cells. The epithelium is littered with 3–5-μm-diameter incurrent openings, ostia (ost). The ostia lie at the junction of two to four pinacocytes, but the pore itself is formed by a tubular cell, the porocyte (shown in detail in Fig. 4). Scale bars, A, 20 μm; B, 5 μm. **C.** The pinacoderm is coated with a mucoid layer (mu) that stops abruptly at the junction of the pinacocytes and porocyte (arrow). Bacteria (b) are frequently caught in the mucoid layer. Scale bar, 5 μm. **D.** A cross section of the pinacoderm shows that pinacocytes form a continuous sheet of cells with sealing junctions between cells (as shown by lanthanum nitrate staining, arrow). Cells are polarized by having a mucous layer (mu) on the apical (external) side and collagen in the mesohyl (me) at the basal side. Scale bar, 0.5 μm.

a cohesive sheet; similar pinacocytes lined the atrial surface of the sponge (Figs. 1D, 3). Pinacocytes were 7–23 μm in diameter (mean 14 μm, $n = 15$) but only 0.5–1 μm thick, and had a granular appearance due to the presence of numerous 1–4-μm-diameter spherical organelles within the cell. The outer surface of the pinacoderm was coated by a mucoid layer that, in some specimens, trapped rod-shaped bacteria (Fig. 3C,D). Ostia were scattered throughout the pinacoderm at the junction of two or more pinacocytes (Figs. 3A–C, 4). The ostium itself was a 1–6-μm-diameter pore (mean 3 μm, $n = 19$) formed by a tubular cell (porocyte) that spanned the body wall of the choanocyte chamber, from the outer pinacoderm to the inner choanoderm (Fig. 4A–D), and which formed dense, osmiophilic junctions with the adjacent pinacocytes (Fig. 4A,B).

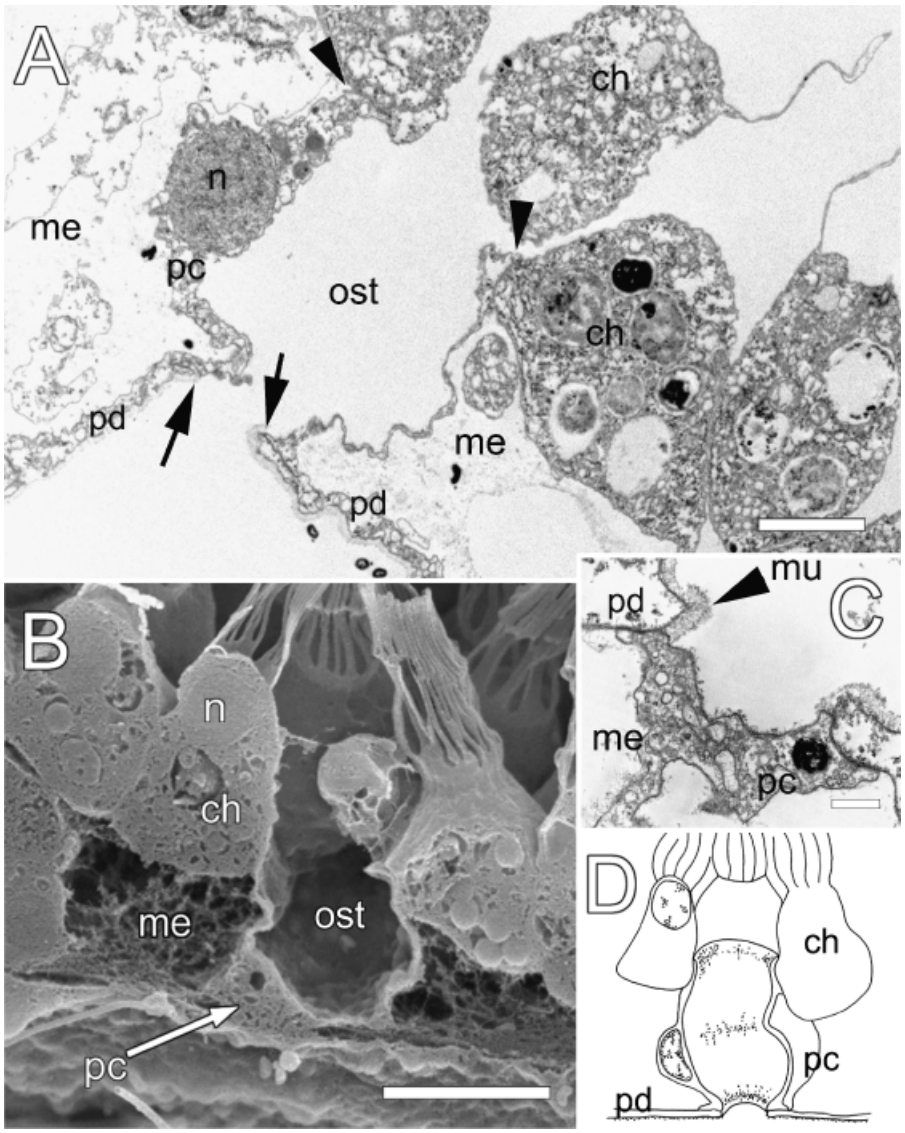


Fig. 4. Structure of incurrent openings (ostia) and porocytes demonstrated with transmission (A, C) and scanning (B) electron microscopy. **A**, **B.** A section and fracture plane (respectively) through the choanocyte chamber wall at the site of an ostium (ost). The ostium is formed by the porocyte (pc) that joins the outer pinacoderm epithelium (pd) (black arrows; equivalent region enlarged in C) to the inner choanocyte (ch) epithelium (black arrowheads). The porocyte is a tubular cell that traverses the mesohyl (me) and has a nucleus (n) midway between the two epithelia. **C.** The junctions formed by the porocyte and pinacocytes are electron dense but have no visible septae. Scale bars, A, 2 μm; B, 5 μm; C, 0.5 μm. **D.** Diagram of the porocyte (pc) in B showing its attachment to the pinacoderm (pd) and choanocytes (ch).

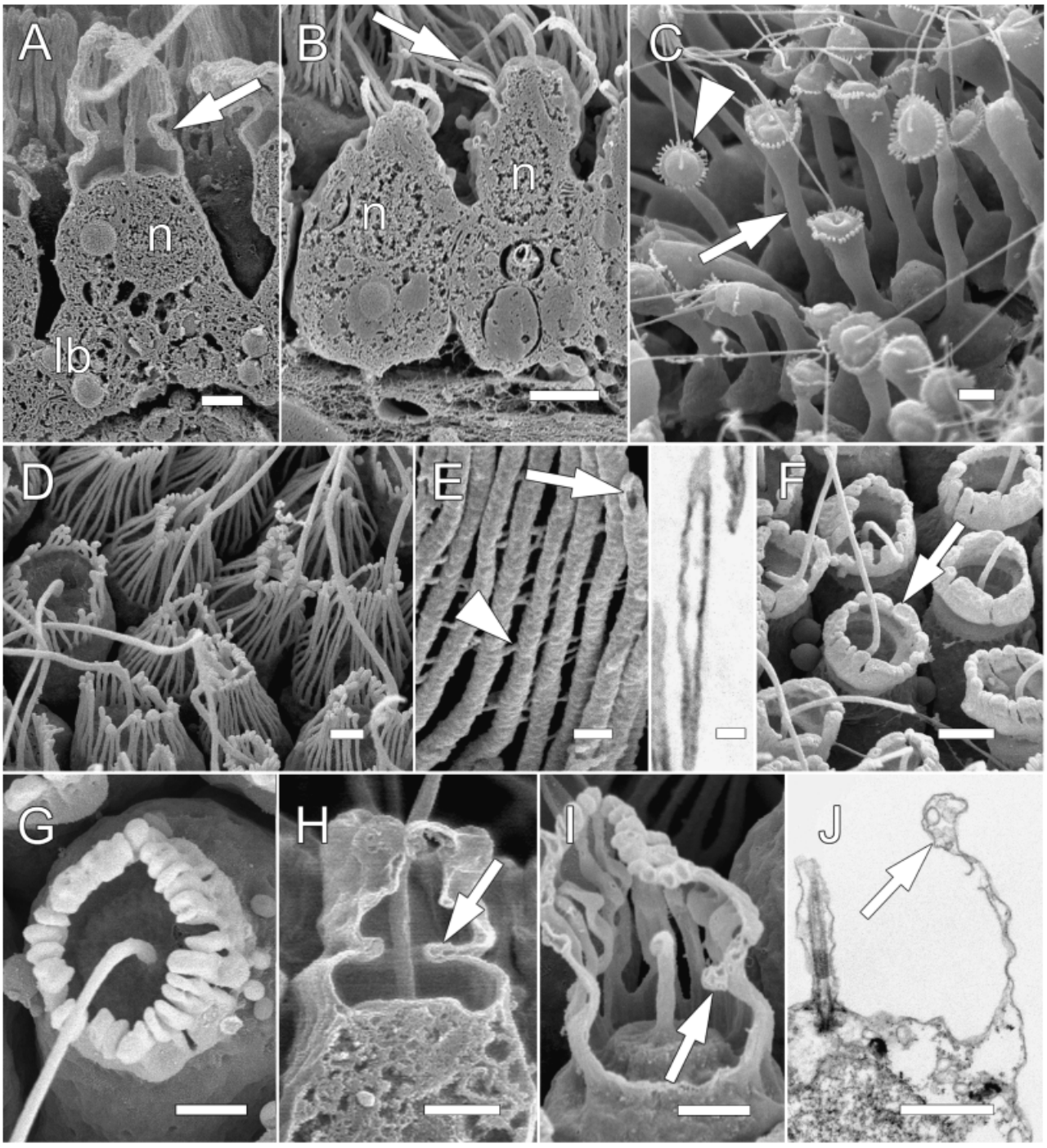
Choanoderm epithelium

The choanoderm was composed of choanocytes, vase-shaped cells that had a round base or cell body and a neck that supported a collar of microvilli surrounding a long flagellum (Fig. 5). Choanocytes varied greatly in size and shape, depending on their location in a choanocyte chamber. Choanocyte cell bodies ranged 2.6–9.2 μm wide (mean 4.1 μm, $n = 7$) and 6.8–15.7 μm long (mean 9.7 μm, $n = 7$) (Fig. 5A,B). While in many choanocytes the collar of microvilli arose directly from the cell body (Fig. 5A,B,D,G,H), in others a neck with microvilli at its apical end extended far into the chamber (Fig. 5C); it was not uncommon to see necks as long as the cell body. The microvilli of the collars also varied in length, number, and shape irrespective of the length of the cell body or neck. Some collars consisted of many long individual microvilli, linked by fine connections forming a 0.2–0.3-μm mesh (Fig. 5D,E). In other collars, the microvilli were separate but were no more than short buds surrounding the central flagellum (Fig. 5C,G). In yet others, the membranes of the microvilli were fused to varying degrees, and the fused villi formed a constriction midway up the collar (Fig. 5A,F,H).

The choanocytes were tightly arranged with their bulbous bases resting on the mesohyl (Fig. 6). The membranes of adjacent cells were tightly juxtaposed (Fig. 6A,B) and, in preparations treated with ruthenium red, slight septae were evident between cells in thin sections (Fig. 6C). In fractured specimens, tightly apposing cell membranes were visible (Fig. 6D) and cells that had pulled apart

had a ridge midway down the basal portion where adjoining cells had made close contact with each other, and in some places specialized junctions between adjacent cell membranes were visible (Fig. 6E, arrows).

Of the seven different fixation protocols used to test for the effect of fixative on cell preservation, the cocktail fixative gave the best results for both scanning and transmission electron microscopy. Alcian blue stained the mucous coat on the outer surface of the sponge and darkened the junctions between pinacocytes. Fixation with lanthanum nitrate highlighted fine threadlike processes between choanocytes, but did not reveal distinct septae. The use of 0.3 mol L^{-1} $MgCl_2$ and menthol crystals as relaxants before fixation gave very poor results. The former appeared



to damage the cell surface and the latter caused the porocytes to constrict but otherwise did not affect the cell structure. Specimens that were mechanically agitated before fixation, or that were left in stagnant water for over 30 min, had very few open ostia, and those that were open were half the diameter of those in untreated sponges (Fig. 7A,B,E). The morphology of collar microvilli was completely unaffected by chemical treatment type, by leaving tissues for 30 min in still water, or by agitation (Fig. 7C,F).

Mesohyl

The mesohyl of the sponge consisted of a 3–14-μm-thick region (mean 8.14 μm, $n = 20$) containing very few cells (Fig. 2B,C). Of these, spicule-secreting cells, sclerocytes, were the least common, but where present they were adjacent to sheaths surrounding spaces left by the decalcification of spicules (Fig. 2B). Spherical cells and amoeboid cells with long extensions were the other principal cells in the mesohyl (Fig. 7C). Neither have a known function, although the close association of both with choanocytes suggests a possible role in nutrient transport.

Oocytes and developing embryos were by far the most common constituent of the mesohyl in specimens collected between June and August. Oocytes were abundant in all regions of the choanocyte chamber wall in June and July; developing embryos were most abundant in July and August. Oocytes, 25–30 μm in diameter, lay directly between the pinacoderm and choanoderm epithelia, and in most cases the choanoderm bulged into the choanocyte chamber to accommodate the oocyte in the narrow mesohyl space (Fig. 8A,B). Cleavage and embryonic development could readily be seen in whole mounts prepared for light microscopy and in specimens fractured for scanning electron microscopy. The first two cleavages were meridional (perpendicular to the choanocyte epithelium) and holoblastic, producing four equal-sized cells (Fig. 8A–C). The third cleavage was oblique, producing a tier of cells displaced laterally (Fig. 8D). Further cleavages were also oblique, lifting the subsequent tier above the last, on the rim of the developing saclike embryo with a small opening still remaining at the side of the choanoderm. Cleavage in cells directly beneath the choanoderm slowed, while those near the pinacoderm continued so as to produce an embryo with a hemisphere of small cells (against the pinacoderm) and a hemisphere of large cells (beneath the choanoderm) (Fig. 8E). Cilia differentiated from the apical surface of the small cells and projected into the blastocoel (center of the embryo). At this stage, embryos appeared as flattened blastulas lying within the mesohyl. The large cells of the embryo then formed junctions with the choanocytes above them, opening a passage through to the choanocyte chamber; this stage is traditionally known as the stomoblastula (Fig. 8F,G). In the next stage the hemisphere of small ciliated cells buckled up through the opening in the choanoderm, thereby turning the embryo inside out as it moved into the choanocyte chamber (Fig. 8H). Fully inverted embryos remained in contact with the choanoderm while they continued differentiation into the larva (Fig. 8I).

Choanocytes directly over the developing oocytes changed size with the growth of the embryo. Over the oocyte, choanocytes were smaller than elsewhere in the flagellated chambers. They had longer necks (3.0–6.7 μm) and shorter bases (1.5–4.4 μm). However, as the embryos progressed to the stomoblastula stage within the mesohyl, overlying choanocytes (both base and neck) became shorter and wider (5.0–9.4 μm, mean 7.6 μm). Choanocytes that lined the rim of the opening between the embryo and the choanocyte chamber lost their vase shape entirely and became round cells measuring 3–4 μm. When the mature larva was released from the sponge, the choanocytes

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Fig. 5. Scanning electron microscopy (A–I) and transmission electron micrographs (E inset, J) illustrating the varied shapes of choanocytes in the choanoderm. **A**, **B.** Fractures of choanocytes showing an apical nucleus (n) and fingerlike microvilli that form a collar (arrow) around the flagellum. In A, the collar arises directly from the cell body and is partially fused at the base, but separates into individual microvilli after the constriction indicated by the arrow. One-mm-diameter latex beads (lb) have been engulfed by the choanocyte. In B, the choanocyte on the right has a "neck region" (arrow). Scale bars, A, 1 μm; B, 2 μm. **C.** "Hourglass" choanocytes with very long necks (arrow) and short microvilli (arrowhead) that barely form a collar. A single flagellum extends from the center of the apex of the neck. Scale bar, 5 μm. **D.** Collars formed by long thin microvilli. Scale bar, 1 μm. **E.** Thin microvilli are linked by a fine mesh of glycocalyx (arrowhead). Note that the narrow microvilli appear to be hollow (arrow), as seen in transmission electron microscopy (inset). Scale bars, 200 nm. **F**, **G.** Choanocytes with collars formed by short microvilli. Some collars appear to form a continuous sheet surrounding the flagellum (F, arrow). Scale bars, F, 2 μm; G, 1 μm. **H**, **I.** Collar microvilli are frequently fused at a constriction near the cell surface ("Minchin's ring") (H, arrow). In I, the fracture plane has gone through the microvilli (arrow). Scale bars H, I, 1 μm. **J.** Section through a collar whose microvilli have clublike tips (arrow). Scale bar, 0.5 μm.

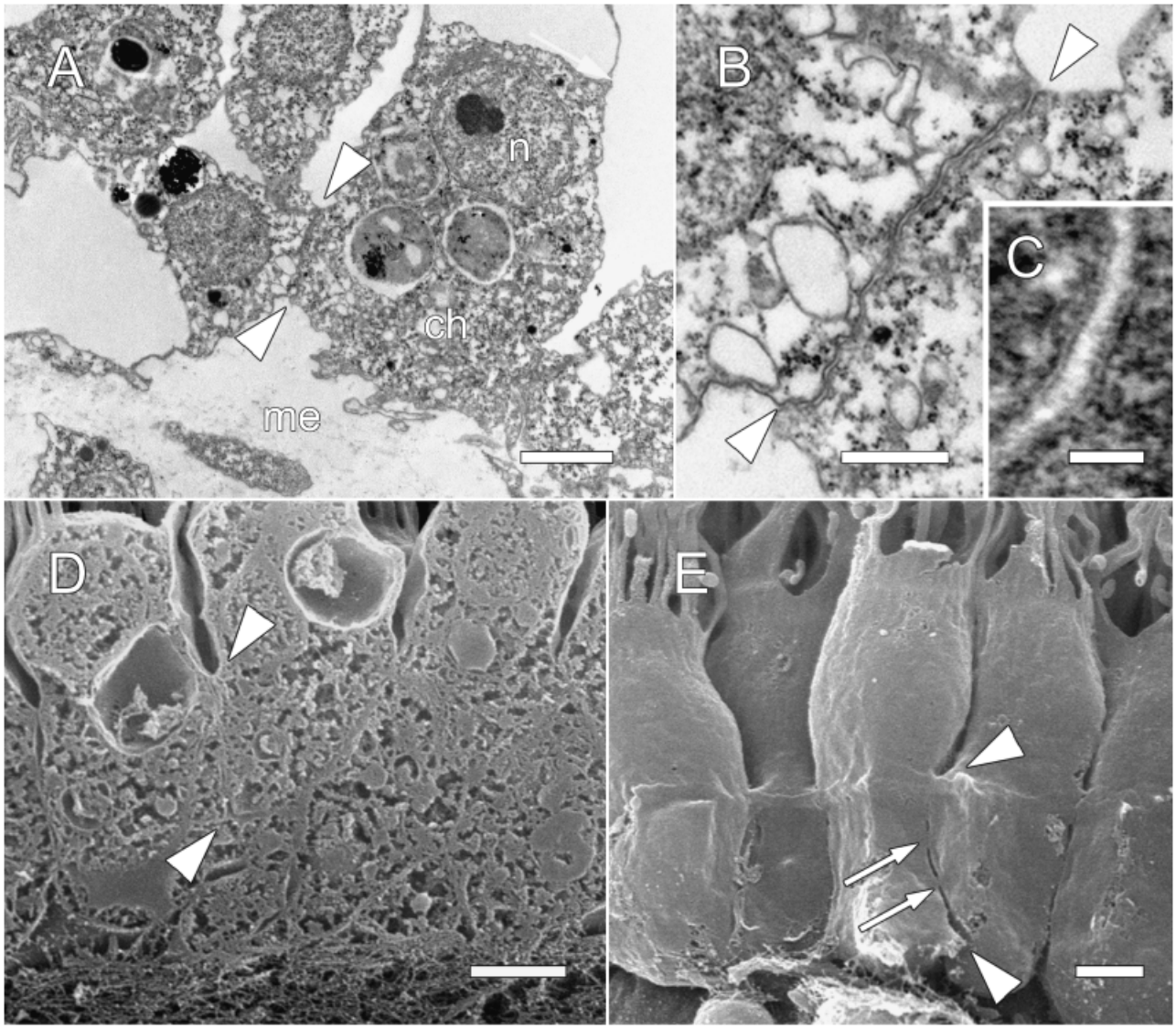

Fig. 6. Cell junctions between choanocytes. **A.** Transmission electron micrograph (TEM) of the choanoderm showing that the basal portions of neighboring choanocytes (ch) are in close contact (arrowheads). Scale bar, 2 μm. Nucleus (n), mesohyl (me). **B**, **C.** Higher magnification TEM showing that choanocytes are tightly juxtaposed (arrowheads), but junctions do not appear to have distinct septae. Scale bars, B, 0.5 μm; C, 50 nm. **D**, **E.** Scanning electron micrographs of the choanoderm also suggest it is tightly sealed (arrowheads), but in addition preparations where cells in the choanoderm have torn away (E) show specialized points of contact (arrows). Scale bar, 2 μm.

appeared to regain a collar. The only choanocytes that lacked collars were those overlying the later stages of developing embryos or those underlying newly inverted embryos.

Discussion

Sponge body plan

Sponges are morphologically versatile animals with unusual body plans that defy direct comparisons with other Metazoa. General assumptions of the cellular organization of sponges and the simplicity within the Calcarea, in particular, are based on a great deal less information than is available for their closest basal metazoan relatives, cnidarians and flatworms. While such generalizations are suitable for overviews of all animal body plans in the context of an introductory treatment, they can be confusing if a precise grasp of relationships between basal metazoan body plans is required. For example, this is the first report on the general ultrastructure of the syconoid calcareous sponge *Sycon,* a remarkable fact

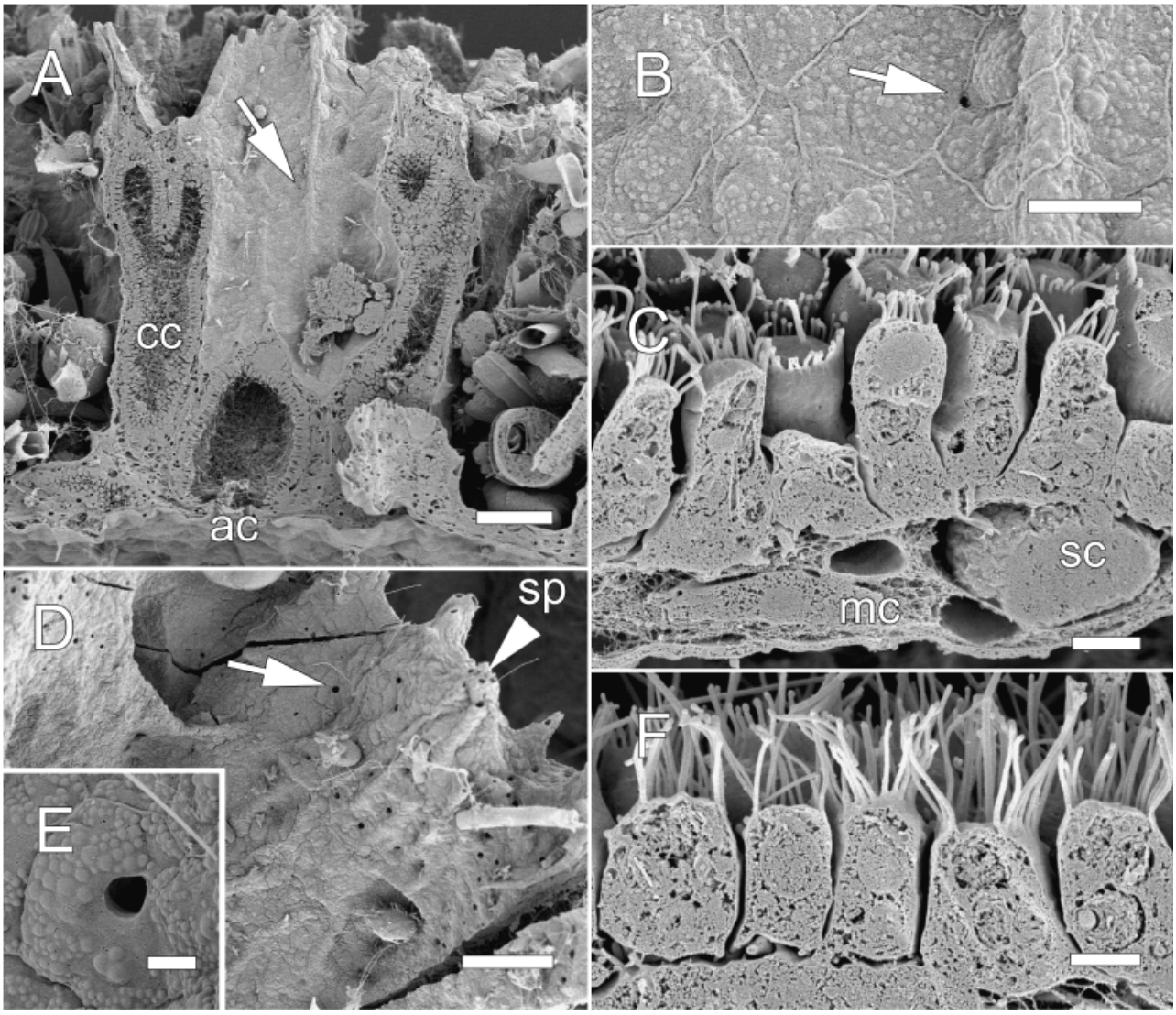

Fig. 7. Scanning electron micrographs showing the effect of chemical and mechanical treatment to the sponge. **A–C.** 30 min in seawater with menthol crystals caused ostia to close (A, B, arrows), but did not affect the shape of collars (C). Scale bars, A, 50 μm; B, 10 μm; C, 2 μm. **D–F.** A 5-min agitation of the sponge caused most ostia to close (D arrow, E), but had little effect on the choanocyte and collar shape (F). Scale bars, D, 50 μm; E, 4 μm; F, 2 μm. ac, atrial cavity; cc, choanocyte chamber; mc, mesohyl cell; sc, spherical cell; sp, spicule space (with arrowhead).

considering the frequent use of this sponge as a model for teaching in courses on animal diversity and invertebrate zoology.

The ascon and sycon body plans of Calcarea have long been considered evidence of the simplicity and hence evolutionary primitiveness of the Calcarea within the Porifera (Tuzet 1973; Borojevic 1979; Manuel et al. 2002). Yet molecular data now suggest that the Calcarea are the sister group of cnidarians and other metazoans (Kruse et al. 1998; Zrzavy et al. 1998; Borchiellini et al. 2001). Thus, assuming that sponges share a common ancestor, either hexactinellid and demosponges have both lost ascon and sycon body plans or Calcarea have derived these *de novo,* i.e., they are secondarily simplified from a leuconoid plan. The ascon and syconoid type of body organization does appear scant in comparison with sponges with a thick collagenous mesohyl, but a close examination of the tissue reveals well-organized layers that can be construed as epithelia. Furthermore, cellular processes that take place during embryogenesis in *Sycon* clearly involve epithelial movements—cells retain junctions with neighbors and move as a sheet—in contrast to equivalent processes in most other sponges where mesenchymal movements dominate (reviewed in Leys & Ereskovsky 2006).

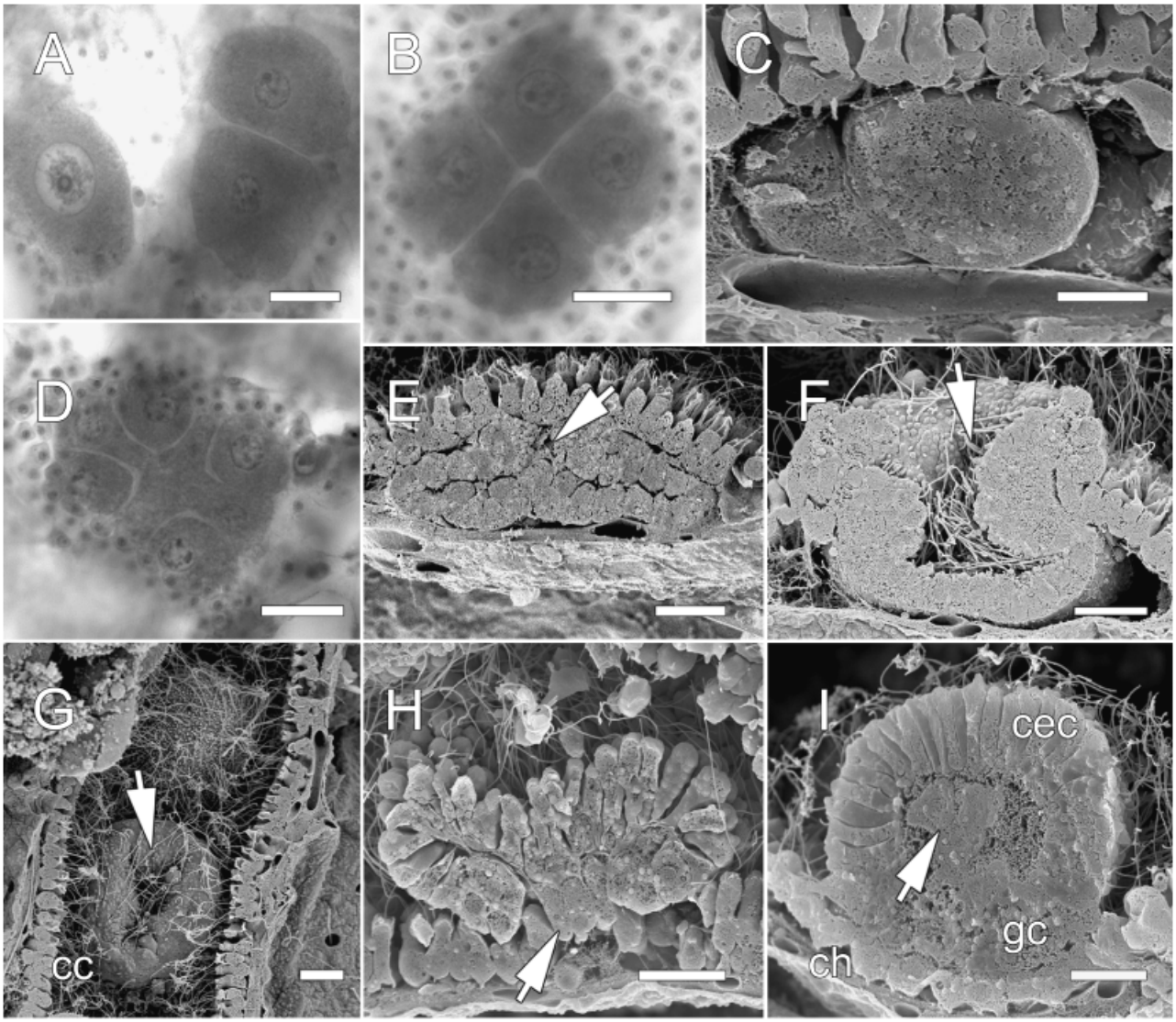

Fig. 8. Embryogenic stages in the mesohyl and during inversion into the choanocyte chamber. A, B, D. Light micrographs of whole mounts; C, E–I. Scanning electron micrographs. **A.** Oocyte (left) and two-cell embryo (right). Scale bar, 20 μm. **B**, **C.** Four-cell embryos showing meridional cleavage (perpendicular to the choanocyte epithelium). Scale bars, B, 20 μm; C, 10 μm. **D.** Eight-cell embryo, lying just beneath the choanoderm. Scale bar, 20 μm. **E.** Blastula with cilia facing into the blastocoel; an opening remains at the side nearest the choanoderm (arrow). Scale bar, 10 μm. **F.** Stomoblastula. A fracture directly through the opening (arrow) between the embryo and the choanocyte chamber. Scale bar, 10 μm. **G.** View of the opening of the stomoblastula (arrow) from the choanocyte chamber. **H.** Embryo inverting into the narrow choanocyte chamber. The ciliated cells of the embryo which before inversion faced inward, into the blastocoel, are now facing outward into the chamber. The smallest cells of the embryo which were previously closest to the pinacoderm are now farthest from the pinacoderm; the larger cells of the embryo remain in contact with the choanocytes. The arrow indicates the last cells to push through the opening. Scale bar, 10 μm. **I.** A fully inverted embryo lying on the choanoderm (ch). The anterior hemisphere of the larva is formed of columnar ciliated epithelial cells (cec); the posterior will eventually be composed of some 8–10 granular cells (gc). Cells in the center of the developing larva (arrow) are presumed to have migrated in from the parent together with bacteria (see Gallissian 1983). Scale bar, 20 μm.

Epithelia: polarity and cell junctions

The absence of extensive septate junctions and basement membranes are the principal reasons why sponges are not considered to possess "true tissues." Generally, sponge tissues are fragile. However, although many electron micrographs show that cell contacts are tenuous, good fixation techniques have

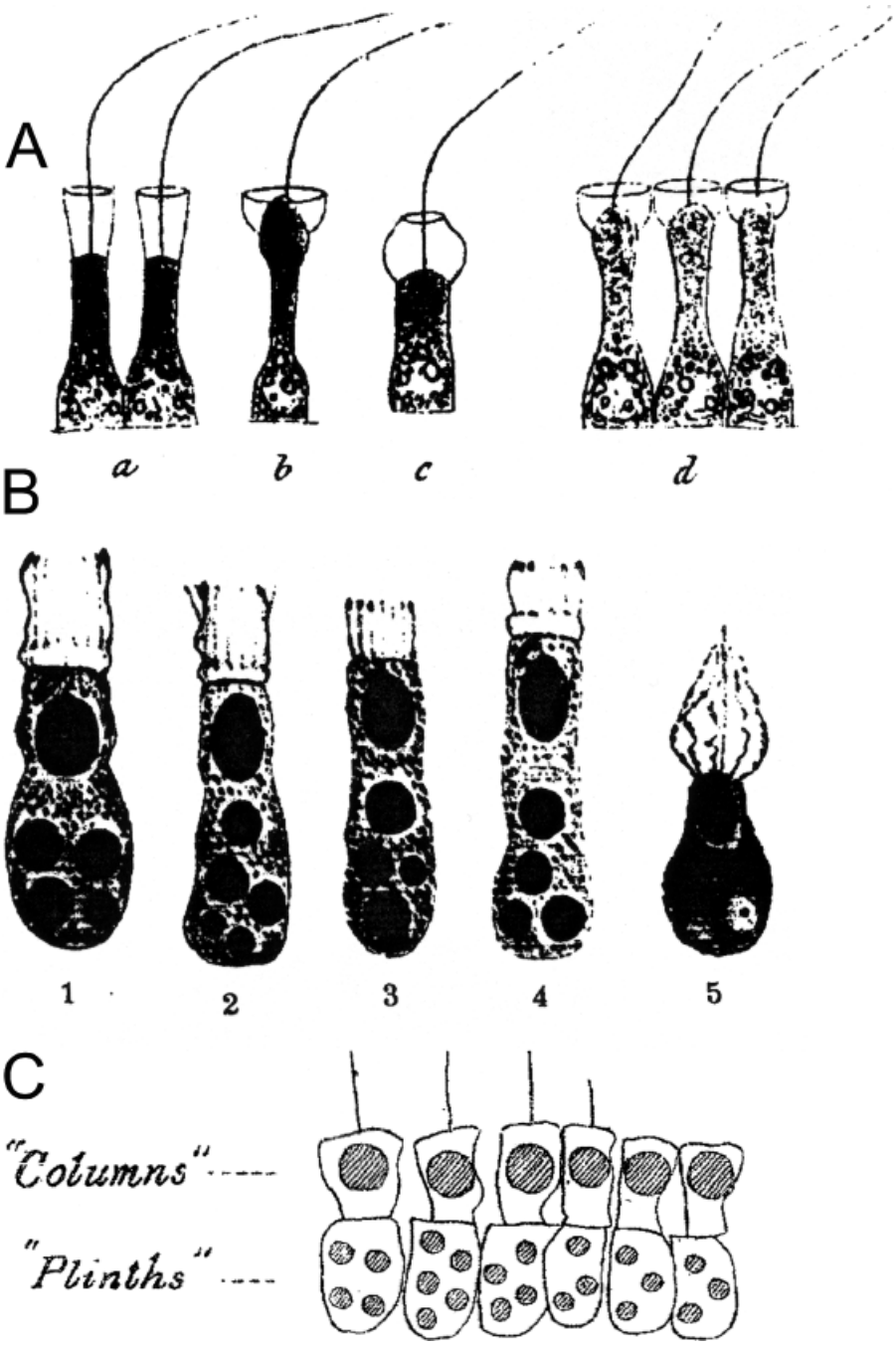

Fig. 9. Drawings of choanocytes in *Sycon* (*Sycandra*) *raphanus* (electronically modified copies of the originals). **A.** Source: adapted from Schulze (1875). **B.** Source: adapted from Duboscq & Tuzet (1939). **C.** Source: adapted from Bidder (1892).

illustrated considerable cell–cell interactions as well as a number of cell junction complexes that may, on further examination, turn out to contain components of septate junctions as in other invertebrates (Pavans de Ceccatty et al. 1970; Garrone et al. 1980; Lethias et al. 1983; Boury-Esnault et al. 2003).

True epithelia have been defined as cells that "share an aligned polarity … are joined by belt-form junctions … and associate with extracellular matrix only on their basal and apical sides" (Tyler 2003). Images of *Sycon coactum* in cross section show distinct, albeit slim, inner and outer layers that form continuous well-sealed epithelia. Pinacocytes are flattened cells that abut a collagenous mesohyl on their basal surface and possess a mucous layer on their apical surface. That the cells are tightly joined together is clear from staining with a lanthanum tracer, but it does not appear that the junctions between pinacocytes form distinct septae. Cells of the choanoderm are clearly joined to one

another by junctions that partition the cell into basal and apical portions; fine electron-dense strands (''almost septae'') and distinct regions of membrane apposition can be seen between choanocytes in *S. coactum* (Fig. 6), and true septate junctions have been shown between choanocytes of another calcareous sponge *Clathrina* sp. GRAY 1867 (Green & Bergquist 1979) as well as between sclerocytes in *S. ciliatum* FABRICIUS 1780, where they are thought to create a unique ionic environment for spicule secretion (Ledger 1975).

To the best of our knowledge, there are only three other reports of junctions with septae in sponges. Larvae of homoscleromorph sponges have an osmiophilic region with septae that bridge the gap between the apical sides of juxtaposed ciliated epithelial cells. These were called desmosomes, but appear to be very like septate junctions (Boury-Esnault et al. 2003). Similar osmiophilic regions and bridging septae link the membrane of collar bodies to the adjacent trabecular reticulum (epithelium) in glass sponges (Hexactinellida) (Mackie & Singla 1983). Cell–cell junctions with septae, with electron-dense regions at sites of membrane apposition, and even examples of exchange of material in vesicles between two cells, have been shown clearly in *Hippospongia communis* LAMARCK 1816 (Pavans de Ceccatty et al. 1970). The term ''desmosome'' has been used to refer to the plugged junction of glass sponges (Boury-Esnault et al. 2003), but this junction has been clearly shown to be an intrasyncytial ''plug'' (not an intercellular junction) that allows aqueous communication between two specialized regions of the syncytial tissues (see Mackie & Singla 1983; Leys 2003). Thus, if care is taken in fixation, septate junctions can possibly be found in all poriferan groups.

The tightly juxtaposed membranes (without septae) of adjacent cells in *S. coactum* are a feature of all cellular sponges and are considered to have an occluding and possibly also an anchoring function (Green & Bergquist 1982). Although these junctions undoubtedly function well for the sponge in its habitat, they are apparently exceptionally fragile in comparison with those seen in animals that are motile. Thus, instances in which sponge epithelia appear leaky in electron micrographs (e.g., Uriz et al. 2001; Rützler et al. 2003) likely reflect either difficulty with fixation of the tissue or problems with infiltration of viscous embedding media which must force cells apart. The infiltration of very small particles (0.1-μm latex beads) between pinacocytes shown in the freshwater sponge *Ephydatia* (e.g., Willenz & van de Vyver 1982) was interpreted as an alternative means of particle uptake, although where particles would go to in the mesohyl of the sponge is unclear. The result could also be due to the pressure exerted by the embedding media on the pinacoderm during specimen preparation, causing junctions to break and allowing the beads to push between cells (S.P. Leys, unpubl. data). In general, the sponge mesohyl is not open to the external medium as has occasionally been suggested (e.g., see Tyler 2003).

Although few studies have investigated components of sponge extracellular matrix (ECM) or sought possible basal laminae in sponges, there is good evidence for a distinct basement membrane that contains type IV collagen in homoscleromorph sponges (Boute et al. 1996; Boury-Esnault et al. 2003). A distinct ECM layer was also found beneath the columnar ciliated epithelium in certain demosponge larvae (Maldonado 2004), and it is likely that other demosponge larvae also have this layer. In *S. coactum,* no distinct basement membrane was observed at the base of either inner or outer epithelia, but the loose collagenous mesohyl presumably provides polarity to the epithelial cells, one of the prime functions of the basement membrane in other metazoan tissues (Engvall 1995).

Thus the continuity and polarity of both the inner and outer cell layers suggest that they could be considered true epithelia. Certainly, an exhaustive search for ECM features that resemble basal laminae, and a detailed study of the composition of intercellular junctions in sponges, should be the focus of future research.

Porocytes and choanocytes

Pinacocytes in *S. coactum* are approximately the same shape and size as pinacocytes in the well-studied freshwater demosponges *Ephydatia* and *Spongilla,* but porocytes in specimens of *S. coactum* differ slightly from those in demosponges (Weissenfels 1980). Porocytes in demosponges are plate-like cells with a central hole that can open and close in a sphincter-like manner (Weissenfels 1980; Willenz & van de Vyver 1982). The margins of these flat cells are sandwiched between pinacocytes of the outer (exo) and inner (endo) pinacoderms. In *S. coactum,* the porocyte is a tubular cell that forms a narrow channel between the pinacoderm (outer) and choanoderm (inner) layers. The calcareous sponge porocyte forms distinct junctions with cells of each layer. Our experiments confirm that, like the demosponge porocyte, the tubular calcareous sponge porocyte contracts in response to strong mechanical agitation (Haeckel 1872; Bidder 1937; Jones 1966) and also when left

in stagnant water. Use of chemical anesthetics also caused the ostia to close. Anesthetics are not generally used with sponges (which lack muscle or nerves), and other cytoskeletal components, such as microvilli, do not usually contract rapidly enough to be a problem in direct fixation protocols. The fact that both chemical relaxants had no effect on the collar microvilli, but did affect other parts of the cell surface and cause contraction of porocytes, suggests that these chemicals are ineffective in relaxing sponges and do not explicitly affect the actin cytoskeleton. The sensitivity of porocytes to menthol crystals and to mechanical agitation is not surprising, given the demonstrated presence of so-called neurochemicals in non-neuronal cells of calcareous sponges (Lentz 1966).

Choanocytes in specimens of *S. coactum* are quite unlike those in demosponges, which typically have a squat cell body and discrete long microvilli that surround a long flagellum (Weissenfels 1982). In *S. coactum,* choanocytes are tall and have an apically located nucleus, a characteristic that distinguishes the subclass Calcaronea from the Calcinea (Manuel et al. 2002). But their overall morphology appears to be variable. From the earliest descriptions of calcareous sponges, authors have noted the unusual length of the apical region of choanocytes, referring to it as the rostrum (Carter 1871), neck (Haeckel 1872; Schulze 1875), and column (Bidder 1892; Duboscq & Tuzet 1939) (Fig. 9). Bidder (1892) initially proposed that the length of the choanocyte reflected feeding state: a fully fed choanocyte becomes narrow and tall, the basal part detaches and moves into the mesohyl and joins the pinacoderm to excrete waste, while the apical portion resumes normal feeding. Bidder also suggested that the choanocytes extend their neck region in conditions of anoxia. This explanation could account for choanocytes with a peculiar hourglass shape, choanocytes ''en sablier'' of Duboscq & Tuzet (1939), which we found in preparations in which the porocytes were contracted and ostia were invisible. But as this type of choanocyte was also found in preparations fixed immediately after collection in which ostia were wide open, anoxia cannot fully explain the long necks.

Some of the variability in shape can be attributed to the growth of oocytes and embryos beneath the choanocyte epithelium as suggested by Duboscq & Tuzet (1938). As oocytes and embryos develop, choanocytes overlying them tend to become shorter and rounder, until the embryo emerges into the choanocyte chamber, at which time they either lose their collar and flagellum entirely, or grow a new tall neck around the edge of the newly emerged embryo. The embryos frequently occupy a large proportion of the volume of choanocyte chambers and the choanocytes possibly change shape in response to changes in flow in the chamber.

Although variability in collar shape was noted by these early researchers, an explanation is lacking. Duboscq & Tuzet (1939) found that many collars were ''retracted,'' with microvilli that bulge into clubs at their tips. They also found many collars that had a constriction just above the cell surface, which they termed ''Minchin's ring'' after that author's description. Our scanning electron micrographs of animals fixed immediately after collection, without exposure to air at any time, and in large volumes of fixative, confirm these observations (Fig. 5H), suggesting that these features of collars are not artifacts. Bidder (1892) proposed that collars contract as the cell elongates when sated but, in the specimens we observed, many short choanocytes also had negligible collars.

An alternative explanation is that the collars are not always used to filter food, but may be used in feeding by phagocytosis. We propose this after finding that, in sponges fed latex microspheres, choanocytes ingested the beads using short pseudopodia and also with long extensions of the apical membrane that sometimes include the collar microvilli (S.P. Leys & D.I. Eerkes-Medrano, in press). These long pseudopodia are likely what de Saedeleer (1929) observed arising from the apical surface of choanoflagellates and sponge choanocytes, and are possibly what Tuzet (1973) called the ''inner collar'' on choanocytes of the calcareous sponge *Grantia compressa* Fabricius 1780. We propose that the variability in morphology of choanocytes and of the collars in *S. coactum* can be attributed to differences in the hydrodynamic flow and mode of feeding. Regions of chambers where flow is strong may have short choanocytes with long microvillar collars that filter, while regions where flow is less consistent may develop choanocytes with longer apical regions and shorter collars. Thus choanocytes may adjust their height in relation to flow in the chamber, as has been shown for individuals in a colony of filter feeders (Larsen & Riisgard 2001).

Embryogenesis

Embryogenesis in the Calcarea is unusual (Tuzet 1973). Oocytes and developing embryos are prominent features of many calcareous sponges during summer months, yet clear images of the early embryo and ''classical'' inversion stage have never been published. Only three papers describe all the stages in embryogenesis, two from the late 1800s and one more

recent. According to Schulze (1875) and Metschnikoff (1874), the first three cleavages are meridional while the fourth is equatorial to produce two layers of eight cells, which with subsequent divisions expand to form a hollow blastula (see Leys & Eerkes-Medrano 2005). Quite recently, Anakina (1997) re-examined development in a related species, *Leucosolenia complicata* MONTAGU 1818, and was able to determine that whereas the first two cleavages are meridional, subsequent divisions are oblique with lateral displacement of the daughter blastomeres above the cleavage plane of the previous tier. She recognized this cleavage to be "table palyntomy," an unusual pattern known from *Volvox carteri* f. *nagariensis* (Green & Kirk 1981).

We found that the same set of cleavage planes occurs in *S. coactum*. The result of this type of cleavage is a cup-shaped embryo lying within the mesohyl that retains an opening toward the overlying choanoderm, hence the term stomoblastula (in *Volvox* the opening is called the phyalopore). Granular cells surrounding the opening form junctions with choanocytes, a channel forms between the center of the embryo and the choanocyte chamber, and the ciliated cells push up into the chamber (as seen in Fig. 8H). Quickly (so presumed because very few embryos are found in this stage), the embryo inverts. In some sponge genera, inversion occurs in a placental membrane (follicular cell lining) (Lufty 1957), and then the embryo is released into the choanocyte chamber.

There are two principal points of interest with respect to inversion of the embryo. Tuzet (1970) suggests that the oocyte of calcaronean sponges is polarized before fertilization, like the frog oocyte, with the animal–vegetal axis perpendicular to the choanocyte epithelium. According to Tuzet, the distinct planes of cleavage and subsequent inversion of the embryo put the animal pole of the oocyte at the posterior pole of the larva. Upon invagination of the anterior pole at metamorphosis, the posterior hemisphere forms the external layer of the juvenile (Metschnikoff 1874; Leys & Eerkes-Medrano 2005). Thus, the fate of the animal pole of the oocyte to become the ectoderm of the adult is claimed to be similar to its fate in other metazoans (Gilbert & Raunio 1997). This would make calcaronean sponges especially tractable models for studying the evolution of germ layers; however, cell-tracing studies are needed to confirm that the cytoplasm of the animal hemisphere in fact follows this route.

The other point of interest concerns the early formation of what appears to be a continuous epithelium in the blastula and the mechanical "turning inside out" that is nearly identical to inversion known from *Volvox* (Kirk 2003). Inversion in *Volvox* has often been compared to the tissue movements that occur during gastrulation (Cole & Reedy 2003). In *Volvox,* a kinesin homolog has been found to be responsible for shifting cytoplasmic bridges from one end of cells to the other, in the process changing the shape of the cells and turning the alga inside out (Nishii et al. 2003). This complex act seems to keep the epithelium intact while inverting the polarity of the cells.

In *S. coactum,* initial stages of inversion do not have bottle-shaped cells (e.g., Fig. 8F), although later stages do (e.g., Fig. 8H). The cells of the embryo are not connected by cytoplasmic bridges (as they are in *Volvox*), but they must nonetheless form junctions to hold the epithelium together as they push through the opening to the choanocyte chamber. Some demosponge larvae develop an extensive convoluted ciliated epithelium as they develop in the restricted space of a follicular epithelium (Leys & Degnan 2002; reviewed in Leys & Ereskovsky 2006), and in at least some individuals of *Halisarca dujardini* JOHNSTON 1842, one or two of the invaginations of this epithelium can become internalized as a sphere of ciliated cells (Ereskovsky & Gonobobleva 2000). However, the retention of a continuous sheet of cells during development, and later during invagination of the larva at metamorphosis (Leys & Eerkes-Medrano 2005), is unique to the Calcaronea. Epithelia and cell junctions deserve a more in-depth exploration in this group.

Embryogenesis in *Sycon* was intensely studied in the late 1800s and early 1900s, but few modern investigations have been conducted and precise details of the fine structure of the adult and of specifics of embryogenesis have been lacking. The details provided here show that this calcareous sponge exhibits fundamental characteristics of metazoan construction, such as polarity of the adult, suggested polarity of the embryo, and morphogenesis of epithelia during larval development and metamorphosis. These features are not always clear in other Porifera. The proposed paraphyly of Porifera and the close relationship of calcareous sponges with other metazoans, coupled with these intriguing morphological features of embryo and adult, suggest that this animal is a good candidate for future genome studies.

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