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Embryogenesis and metamorphosis in a haplosclerid demosponge: gastrulation and transdifferentiation of larval ciliated cells to choanocytes

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Abstract. Early development and metamorphosis of *Reniera* sp., a haplosclerid demosponge, have been examined to determine how gastrulation occurs in this species, and whether there is an inversion of the primary germ layers at metamorphosis. Embryogenesis occurs by unequal cleavage of blastomeres to form a solid blastula consisting micro- and macromeres; multipolar migration of the micromeres to the surface of the embryo results in a bi-layered embryo and is interpreted as gastrulation. Polarity of the embryo is determined by the movement of pigment-containing micromeres to one pole of the embryo; this pole later becomes the posterior pole of the swimming larva. The bi-layered larva has a fully differentiated monociliated outer cell layer, and a solid interior of various cell types surrounded by dense collagen. The pigmented cells at the posterior pole give rise to long cilia that are capable of responding to environmental stimuli. Larvae settle on their anterior pole. Fluorescent labeling of the monociliated outer cell layer with a cell-lineage marker (CMFDA) demonstrates that the monociliated cells resorb their cilia, migrate inwards, and transdifferentiate into the choanocytes of the juvenile sponge, and into other amoeboid cells. The development of the flagellated choanocytes and other cells in the juvenile from the monociliated outer layer of this sponge's larva is interpreted as the dedifferentiation of fully differentiated larval cells—a process seen during the metamorphosis of other ciliated invertebrate larvae—not as inversion of the primary germ layers. These results suggest that the sequences of development in this haplosclerid demosponge are not very different than those observed in many cnidarians.

Additional key words: Porifera, sponges, *Reniera*

Over a century of research on sponge development has identified a heterogeneous mix of morphogenetic patterns during the embryonic, larval, and metamorphic stages. Nevertheless, interpretations of these patterns within conventional paradigms for metazoan development have posed some enigmatic problems. In particular, although gastrulation is recognized as a crucial event in the developmental construction of metazoan body plans, the question of when and how gastrulation occurs in sponges, if it occurs at all, is controversial.

In many respects, uncertainties about the process of gastrulation in sponges stem from the novel body plan of this group. Many would argue that as adult sponges lack a gut, they cannot undergo gastrulation, which by this definition is the formation of the gastrodermis of adult metazoans (Ereskovsky & Korotkova 1997; Ereskovsky 2000). Others counter that the flagellated feeding cells, the choanocytes, are the gut epithelium (gastrodermis) of the adult sponge, and these cells differentiate from the outer ciliated layer of the larva at metamorphosis ("inversion of layers"). Under this interpretation, gastrulation occurs at metamorphosis (Brien 1967; Fell 1974; Simpson 1984). A third school equates gastrulation with the cell migrations that occur during embryogenesis to produce the primary germ layers of a diploblastic larva. They conclude that, because sponges "invert layers" at metamorphosis, sponges are turned inside out relative to eumetazoans (Delage 1892; Lévi 1956; Brien 1967). Yet another school agrees that the cell migrations during embryogenesis comprise gastrulation, but regards the "inversion of layers" at metamorphosis as a reorganization of already differentiated cellular material and therefore as phylogenetically unimportant (Borojevic 1970; Efremova 1997).

All except this last view of gastrulation inevitably

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makes it difficult to include sponges in the Metazoa on the basis of developmental data. However, considering the wealth of recent molecular data that support the monophyly of the Metazoa, few biologists today would separate the Porifera from all other multicellular animals (Garrone 1995; Müller 1995; Cavalier-Smith et al. 1996; Borchiellini et al. 1998, 2001; Collins 1998; Schütze et al. 1999). The problem of gastrulation in sponges has gained new importance in light of evidence for the highly conserved roles of a range of genes for metazoan-specific transcription factors (e.g., *Hox* and *Pax*) and signaling pathways (e.g., *wnt* and *TGF*-β) in the establishment of metazoan body plans (Slack et al. 1993; Finnerty 1998; Finnerty & Martindale 1998; Masuda-Nakagawa et al. 2000; Peterson & Davidson 2000; Carroll et al. 2001; Davidson 2001; Hayward et al. 2001). While sponge genomes possess members of a number of transcription factor gene families that regulate major aspects of bilaterian development (e.g., Degnan et al. 1993, 1995; Seimiya et al. 1994; Manuel & Le Parco 2000), we do not know if these genes are expressed in sponge development. If we wish to determine the ancestral roles of these genes in metazoans, it is important to understand when the primary germ layers and the various cell types evolved, and what features of larval and adult body plans of basal metazoans might be considered homologous to those shared by bilaterians.

Four main larval types are found in the Porifera: the coeloblastula, a ciliated hollow blastula-like larva (Lévi 1963; Amano & Hori 2001); the amphiblastula, a hollow blastula-like larva with ciliated cells confined to one hemisphere (Tuzet 1973); the parenchymella, a ciliated egg-shaped larva that is typically solid, but may have internal ciliated chambers or cavities (Brien 1973); and the trichimella of hexactinellid sponges, which has multiciliated cells in a skirt around the middle, and syncytial tissues like the adults (Boury-Esnault & Vacelet 1994).

The majority of sponge larvae are of the parenchymella type, and are not unlike the cnidarian planula. It is accepted that cnidarian planulae settle on the anterior pole; retain, resorb, or discharge their locomotory cilia (Martin et al. 1983; Stricker 1985); and undergo continued cellular differentiation and reorganization to form a polyp in which the oral pole, where the mouth appears, is the former posterior pole of the larva. Sponge larvae also typically settle on the anterior pole. Numerous researchers have reported that larvae of the amphiblastula and parenchymella type undergo an inversion of layers at metamorphosis. During this process, the anterior and ciliated cells move inwards to become the choanocytes of the flagellated chambers, and the inner and posterior amoeboid cells migrate outwards to form the pinacoderm and mesohyl (Delage 1892; Minchin 1896; Dubosq & Tuzet 1937; Lévi 1956; Borojevic 1970; Boury-Esnault 1976; Amano & Hori 1993, 1996; see Efremova 1997 for review).

However, whether layers really are reversed at metamorphosis has been questioned, as studies have demonstrated that some sponges already have flagellated chambers at the time of settlement (e.g., Brien & Meewis 1938; Wielspütz & Saller 1990), and others discharge their locomotory cilia at settlement (Bergquist & Green 1977). Using a radioactive cell tracer to follow the ciliated epithelial cells during metamorphosis, Misevic et al. (1990) found that the label did not end up in choanocytes in the juvenile sponge. In contrast, two studies that traced a natural marker in the larval ciliated cells found that these cells did transform into choanocytes (Amano & Hori 1996), and into choanocytes and grey cells (Boury-Esnault 1976). Studies of the development or reconstitution of sponges from dissociated larvae provide good evidence for both outcomes: the isolated ciliated epithelium is only able to differentiate into choanocytes (Borojevic & Lévi 1965; Borojevic 1966), yet a purified archaeocyte fraction of dissociated adult sponges is able to form choanocytes (Buscema et al. 1980).

Tracking cells during sponge embryogenesis and development is difficult as most sponges brood their embryos. Embryogenesis has been studied only in a few oviparous species. In *Tetilla,* the egg undergoes direct development through to a morula, after which the inner cells migrate out to form a pinacoderm (Watanabe 1978); it is not clear which cells form the choanocytes. In *Polymastia* and *Chondrosia,* the egg undergoes equal cleavage to form a morula, which then develops into a hollow, ciliated blastula larva (Borojevic 1967; Lévi & Lévi 1976). The migration of cells during metamorphosis has not been followed.

Advances in the technology of fluorescent cell markers have provided a new way of addressing the problem of following cell movements through settlement and metamorphosis of sponge larvae. In the present study a fluorescent cell marker that remains locked inside the cells for several days was used to determine the locations of the ciliated and non-ciliated epithelial cells of the larva of *Reniera* sp. (Demospongiae, Haplosclerida, Chalinidae) during settlement and metamorphosis. The reorganization of cellular material during metamorphosis is compared with the cellular movements that occur during embryogenesis and larval development in this haplosclerid demosponge.

Methods

Collection of adult sponges and larvae

Adult demosponges of *Reniera* sp. were collected on pieces of coral rubble in 0.2 m depth (at low tide)

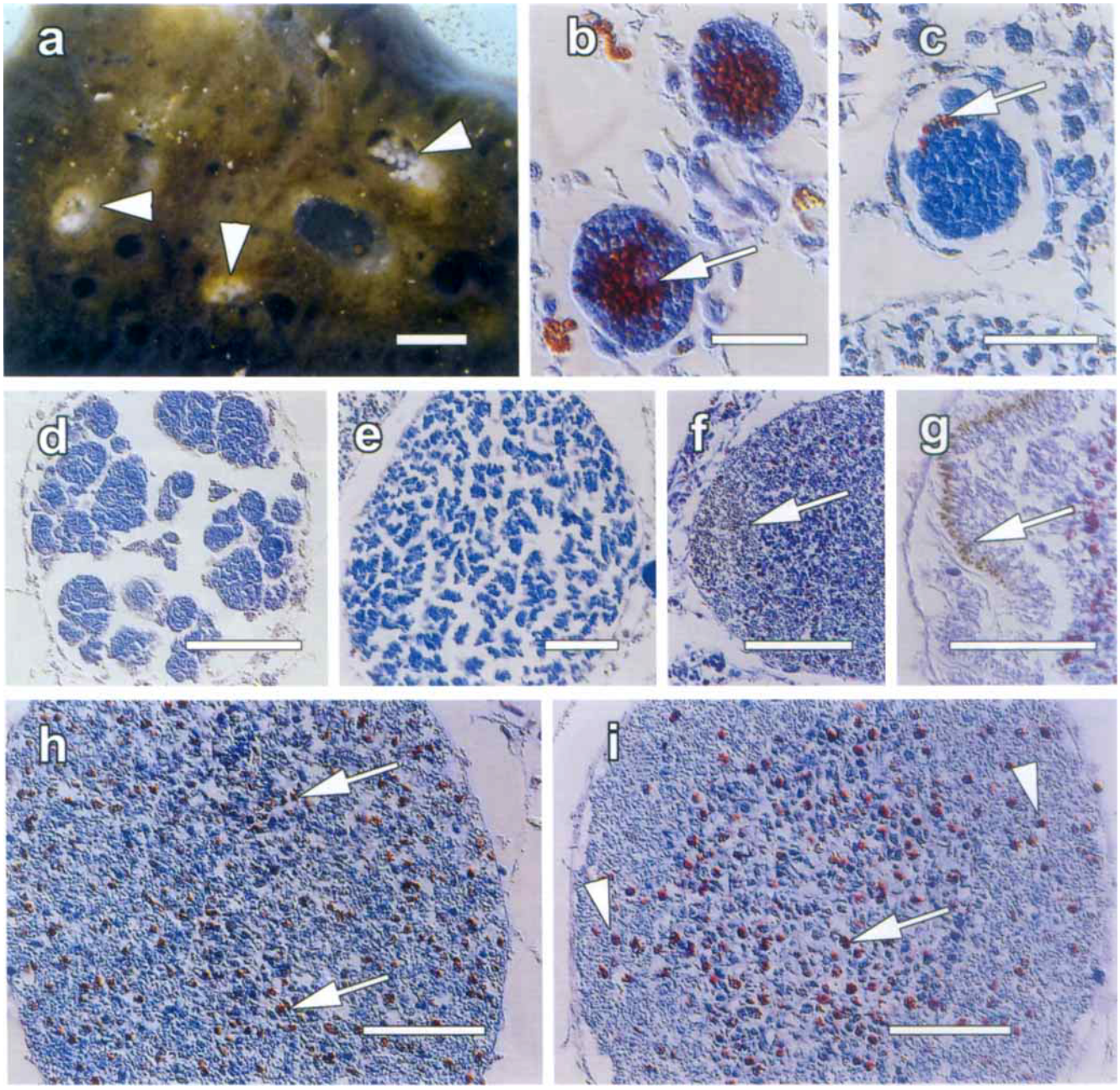

Fig. 1. Stages of embryogenesis in *Reniera* sp. (**a**) Adult sponge cut open to expose the brood chambers (arrowheads), which contain all stages of embryos. Scale bar, 1 cm. Light microscopy (LM) of Mallory's-stained sections (b–i): (**b, c**) Oocytes lie in the maternal tissue at the edge of a brood chamber. The red-stained cells (arrows) are presumed to be spherulous cells or nurse cells from the adult sponge. Scale bars, b, 25 μm; c, 30 μm. (**d**) Early blastula. (**e**) Late blastula. Scale bars for d,e, 100 μm. (**f**) Pigmented micromeres (arrow) at one side of an early gastrula. (**g**) Pigmented micromeres (arrow) at the posterior pole of a late-stage embryo. Scale bars for f,g, 50 μm. (**h**) Early gastrula: red-staining spherulous cells are throughout the embryo (arrows). (**i**) Late gastrula: spherulous cells (arrow) dominate the inner region, and are sparse in the outer region (arrowheads); the outer region consists mainly of ciliated micromeres. Scale bars for h,i, 100 μm.

from Shark Bay on Heron Island Reef, Great Barrier Reef, Australia (23°26′S, 151°03′E). For details on the habitat and general morphology of *Reniera* sp., see Leys & Degnan (2001).

Some sponges were transferred to the laboratory without removal from seawater and cut open with a sharp scalpel while still submerged, to remove the brood chambers. Other sponges on brick-sized pieces

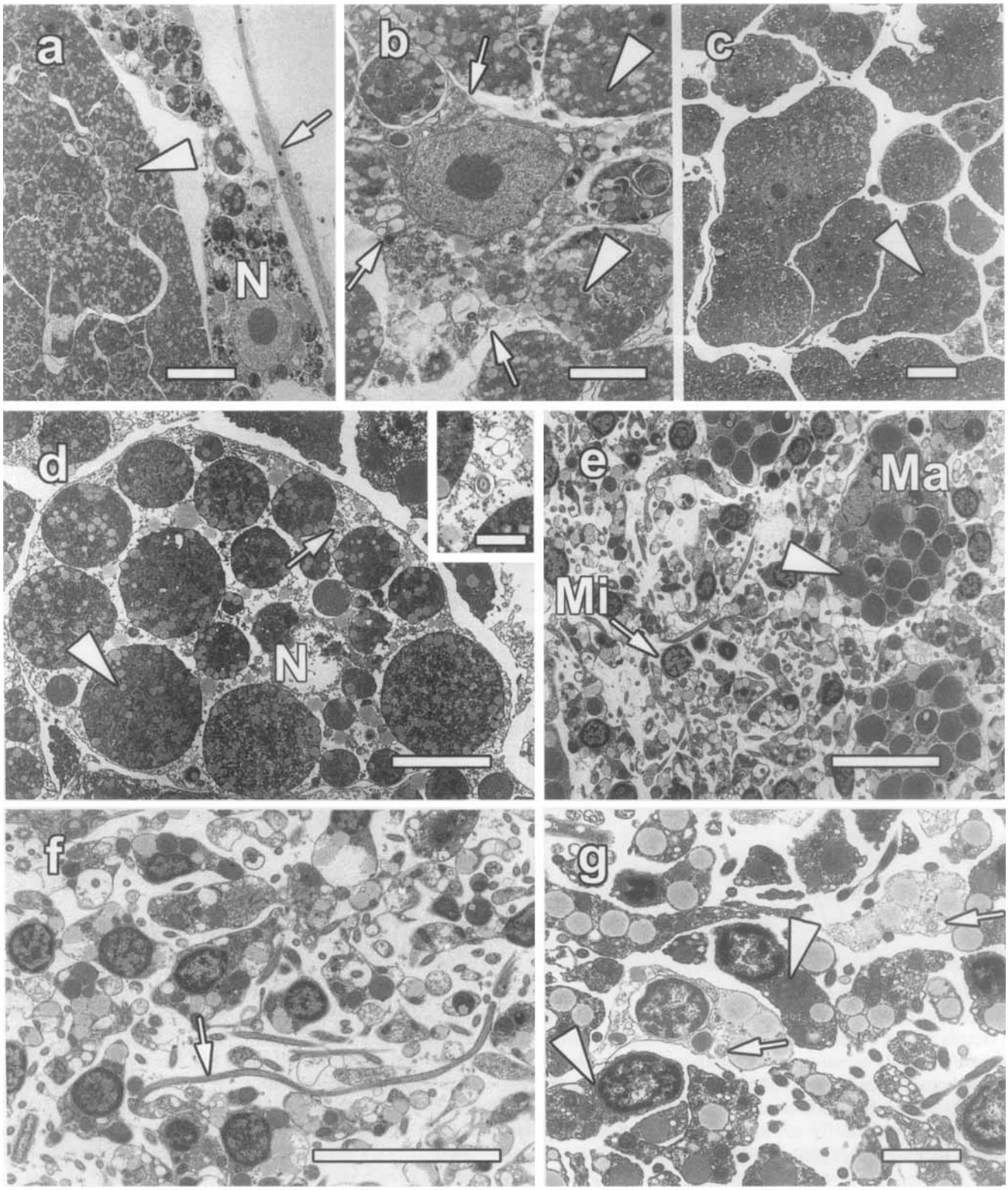

Fig. 2. Oocytes and cleavage. TEM. (**a**) Section through the edge of an oocyte illustrating the dense yolk-filled center (arrowhead) surrounded by a thin band of oocyte cytoplasm containing the nucleus (N). Maternal oocyte follicle (arrow). Scale bar, 5 μm. (**b**) Early blastula. The scant cytoplasm of the blastomere (arrow) is wrapped around a vast mass of yolk-filled inclusions (arrowheads). Scale bar, 2 μm. (**c**) Blastula. Cells are of very different sizes and are densely packed with yolk (arrowhead) from the former nurse cells. Scale bar, 5 μm. (**d**) Late blastula. Cilia (arrow and inset) in blastomeres

of coral rubble were quickly transferred to large black plastic containers (0.8 × 0.3 × 0.3 m deep) of seawater on the shore. The containers were covered and after several hours larvae released from the sponges were siphoned into a collection vial, and the sponges on the coral rubble were returned to the reef flat. In this manner several hundred larvae could be collected daily from the same sponges for up to three weeks without detriment to the sponges. As these sponges have brood chambers and release larvae year round, the same sponges were collected for harvesting larvae several times during the year.

Light microscopy

Brood chambers were fixed in 4% paraformaldehyde in seawater for up to several days at 4°C. Embryos were extracted from the brood chambers after fixation. Whole chambers and individual embryos were then dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Sections (6 μm thick) were stained with Mallory's trichromic stain (Humason 1979) and viewed with a Leitz Aristoplan compound microscope.

Whole larvae were observed and photographed during settlement and metamorphosis with an Olympus SZH dissecting microscope equipped with an Olympus C35AD4 camera.

Fluorescence microscopy

A 10 mM stock solution of CMFDA (Cell Tracker Green, Molecular Probes, Eugene, Oregon) was made in dimethylsulfoxide (DMSO) and diluted to a working concentration of 10 μM in 0.2-μm filtered seawater (FSW). CMFDA is a chloromethyl derivative containing fluorescein that freely diffuses through the membranes into live cells and becomes membrane impermeable once inside. The dye remains locked inside cells for several days after loading.

Newly released larvae were added to FSW containing 10 μM CMFDA in a petri dish. After 30 min the larvae were washed in 10 ml FSW and transferred to a new petri dish containing FSW and a glass coverslip on which the larvae attached and settled. Control larvae were incubated in DMSO for the same period of time and transferred to FSW as above. One larva from each experimental and control batch was examined with an Olympus compound microscope equipped with epifluorescence after the 30-min incubation period, to determine whether the label was absorbed. These larvae were fixed immediately in 4% paraformaldehyde in seawater to confirm whether the dye was able to penetrate past the ciliated epithelial cells during this period. Larvae settled and metamorphosed into juvenile sponges 2–3 days after treatment. Larvae, postlarvae (larvae that had begun to undergo metamorphosis but had not yet formed an aquiferous system), and juveniles attached to coverslips were fixed in 4% parafomaldehyde in seawater at 0, 2, 4, 6, and 8 days after release from the parent, and transferred while still in fixative to the University of Victoria. Specimens were washed 3 times in phosphate buffered saline (PBS) with 0.1% Triton X 100 (TX100) on a rotary shaker, blocked for 1 h in PBS-TX100 with 10% goat serum, and incubated in Alexa-488 anti-fluorescein (Molecular Probes, Eugene, Oregon) at a concentration of 1:1000 for 4 h to enhance the fluorescent signal. Preparations were washed 3 times in PBS-TX100, mounted in glycerol, and viewed in a Zeiss LSM 410 confocal microscope or a Zeiss Axioskop compound microscope equipped with epi-fluorescence.

Electron microscopy

Brood chambers were fixed whole in a cocktail fixative of 1% OsO_4, and 2% glutaraldehyde in sodium acetate buffer (pH 6.4) with 10% sucrose in the final volume.

Larvae were allowed to settle and attach in glass and plastic dishes containing pieces of algae. Newly settled postlarvae were gently pipetted into a 2-ml vial containing the cocktail fixative. Postlarvae attached to coverslips or algae were placed with their substrate directly into a vial of fixative. The exact age of settled postlarvae and juveniles was impossible to determine as most recently settled larvae would resume swimming if disturbed by movement of the dish or by shadows over the dish. At least 50 postlarvae were fixed as soon as it was determined that they had settled and could not swim again. This method ensured that all stages of metamorphosis were obtained.

Brood chambers, larvae, and settled postlarvae and

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that still contain large yolk-filled inclusions (arrowhead) indicate the onset of cellular differentiation. Scale bar, 5 μm; inset, 1 μm. (**e**) Macromeres (Ma) and micromeres (Mi) in a late blastula. Macromeres contain dark spherulous inclusions (arrowhead) that no longer resemble the yolk from nurse cells. Scale bar, 6 μm. (**f**) Micromeres with long cilia (arrow). Scale bar, 6 μm. (**g**) Two types of micromeres can be identified: one has lighter staining cytoplasm and cilia (arrows); the other has cytoplasm that stains more heavily and appears to lack cilia (arrowheads). Scale bar, 2 μm.

juveniles were fixed for 2–4 h at 4°C. Fresh fixative was added after 30 min. After fixation, tissues were rinsed twice in FSW and stored in 70% ethanol for transport to the University of Victoria. Embryos were removed from the brood chambers while in ethanol for further processing.

For transmission electron microscopy (TEM), embryos and settled larvae were desilicified in 4% hydrofluoric acid in 70% ethanol overnight, stained "en bloc" in 0.5% uranyl acetate in 70% ethanol overnight, dehydrated in 100% ethanol, and embedded in epoxy resin (Taab 812). Thick sections were cut with glass knives, stained in Richardson's (Richardson et al. 1960), and viewed in a Zeiss Axioskop compound microscope. Thin sections were cut with a diamond knife, stained with lead citrate, and viewed in a Hitachi H-7000 electron microscope.

For scanning electron microscopy (SEM), embryos were dehydrated in 100% ethanol and fractured in liquid nitrogen while in a vial of ethanol. Embryos and settled postlarvae were critical-point dried and coated with gold in an Edwards sputter coater. Four to five embryos or postlarvae were mounted on specimen stubs with clear nail polish and viewed in a Hitachi S-3500N SEM.

Results

Brood chambers with 20–150 embryos measuring 300–500 μm in diameter were located in the lower portions of the sponge tissue all year round (Fig. 1a). The sponge is hermaphroditic, although only two spermatocysts were found in more than 100 sponges that were collected throughout the year. Fertilization was not observed but presumably is an ongoing event, as many stages of development were found in each brood chamber.

As it was not possible to separate the embryos in the brood chambers by their different stages of development, the following stages of oogenesis and embryogenesis were interpreted from the static images of light and electron microscopy.

Oogenesis and cleavage

Oocytes measuring 30–50 μm in diameter, are found in the maternal tissue at the edges of the brood chamber (Fig. 1b,c). Oocytes that are 30 μm in diameter have large red-stained spherulous cells—presumed to be nurse cells—associated with them. Oocytes are filled with a dense yolky material and have very little peripheral cytoplasm (Fig. 2a). Nurse cell yolk is probably incorporated into the oocyte before fertilization, because oocytes are 250–300 μm in diameter when the first cleavage stages are observed (Fig. 1d). The cytoplasm of the oocyte is so full of yolk that the first planes of cleavage are difficult to determine. However, early embryos clearly have irregularly shaped blastomeres, some 20 μm in diameter, that are filled with small inclusions presumed to be yolk from the nurse cells (Figs. 1d, 2b,c). At later cleavage stages the embryos consist of smaller blastomeres, and the inclusions are contained within membrane-bound vesicles (Figs. 1e, 2c,d). The diameter of the embryo does not change substantially during these first cell divisions.

Cellular differentiation

Cellular differentiation is first apparent with the formation of cilia in blastomeres when they are still largely filled with yolky inclusions (Fig. 2d and inset). The next cell divisions produce cells of two different sizes containing spherical, osmiophilic inclusions that differ in size and staining from the previous yolky inclusions (Figs. 1h, 2e). The larger cells, here termed macromeres, are irregularly shaped and 6–12 μm in diameter, with a nucleus 2 μm in diameter (Fig. 2e). The cytoplasm contains many osmiophilic inclusions 1.5 μm in diameter. Interspersed among the macromeres throughout the embryo are at least two types of smaller cells, here termed micromeres. These cells are 1–3 μm in diameter with a nucleus 2 μm in diameter, and contain pale spherulous inclusions 0.5 μm in diameter. One type of micromere possesses a cilium and has pale cytoplasm; another has dark cytoplasm and apparently no cilium (Fig. 2f,g).

Cell migrations

The first indication of cellular migrations within embryos is the appearance of a light brown hue on the outside of the embryo (Fig. 3a) that eventually condenses into a dense spot of pigment at one pole (Fig. 3b–d), and finally becomes organized into a ring of pigmented cells at that pole (Fig. 3e–h).

The migration of pigmented ciliated micromeres to one pole is evident in both paraffin sections (Fig. 1f,g) and epoxy sections (Fig. 4a). In all subsequent embryonic stages, micromeres are at the periphery and macromeres remain in the center (Fig. 4b). The ciliated micromeres become aligned, with the nuclei located basally, at the periphery of the embryo (Fig. 4d). Cells identified as sclerocytes by the fibrous core left in an intracellular vacuole after desilicification of the embryos are among the cells found at the periphery of the 2-layered embryo (Fig. 4b–d). It is not clear whether the macromeres move to the center or are left there by the peripheral migration of micromeres. At one stage, red-stained cells with inclusions are abundant

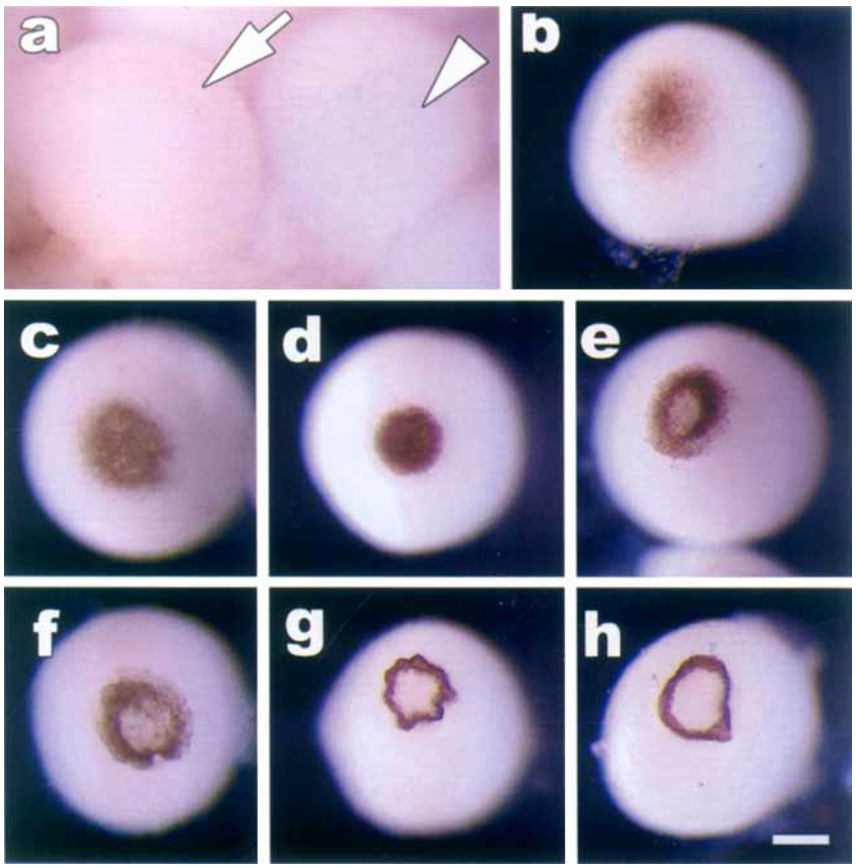

Fig. 3. Development of pigmentation in the outer cell layer of embryos. Light microscopy. (**a**) Late blastulas have a light brown hue on the outer layer (arrow) compared to pale coloured oocytes and early blastulas (arrowhead). (**b–d**) The pigmented cells migrate towards one hemisphere of the embryo and coalesce into a dense spot. (**e–h**) Eventually these cells form a ring of dense pigment at the posterior pole of the future larva. Scale bar, 50 μm.

throughout the embryo (Fig. 1h), whereas at another stage, interpreted as later, these cells are primarily in the central region (Fig. 1g,i). Micromeres dominate the outer region of the 2-layered embryo (Fig. 1i).

Late-stage embryos have a distinctly bi-layered appearance. The center of the embryo consists of numerous amoeboid cells in a dense extracellular matrix of collagen, whereas the outer layer is composed of columnar epithelial cells that give rise to cilia 20 μm long (Fig. 5a,c,d). Between the inner and outer layers is a thin layer of cells that lie perpendicular to the columnar epithelial layer and form a circumferential sheath around the whole larva except at the posterior pole (Fig. 5d). The morphological similarity of these cells to the red-stained spherulous cells in Mallory's-stained sections suggests that they differentiate from micromeres prior to gastrulation, migrate with the ciliated micromeres to the periphery of the embryo during gastrulation, and become aligned in their final position at the base of the columnar ciliated epithelium after gastrulation. The outer layer of columnar ciliated cells is at first folded (Fig. 5a,b). When the embryo elongates prior to release from the brood chamber, the folds in the outer epithelial layer disappear (Fig. 5c,d). At this point, the embryo has all the features of the larva. It is a solid parenchymella with a monociliated epithelial layer covering all but the anterior and posterior poles. The anterior pole has a 15–20 μm diameter region of non-ciliated cells. The posterior pole also has a non-ciliated region some 50–80 μm in diameter that is circumscribed by pigment cells with cilia 120–150 μm long (Fig. 5d).

Details of larval morphology and swimming behaviour are described elsewhere (see Leys & Degnan 2001). Briefly, swimming larvae rotate clockwise through the water (as seen from the posterior pole). The rotations and propulsion for swimming are provided by the beating of short cilia (20 μm long) that extend from the outer monociliated epithelial layer. The larvae are negatively phototactic until 12 h after release. The direction of swimming is controlled by longer cilia (120–150 μm), which arise from the posterior ring of pigmented cells. These cilia straighten or bend in response to abrupt changes in light intensity (Leys & Degnan 2001).

Metamorphosis

Larvae of *Reniera* sp. can swim for up to a week, but most larvae settle between 2 and 48 h after release

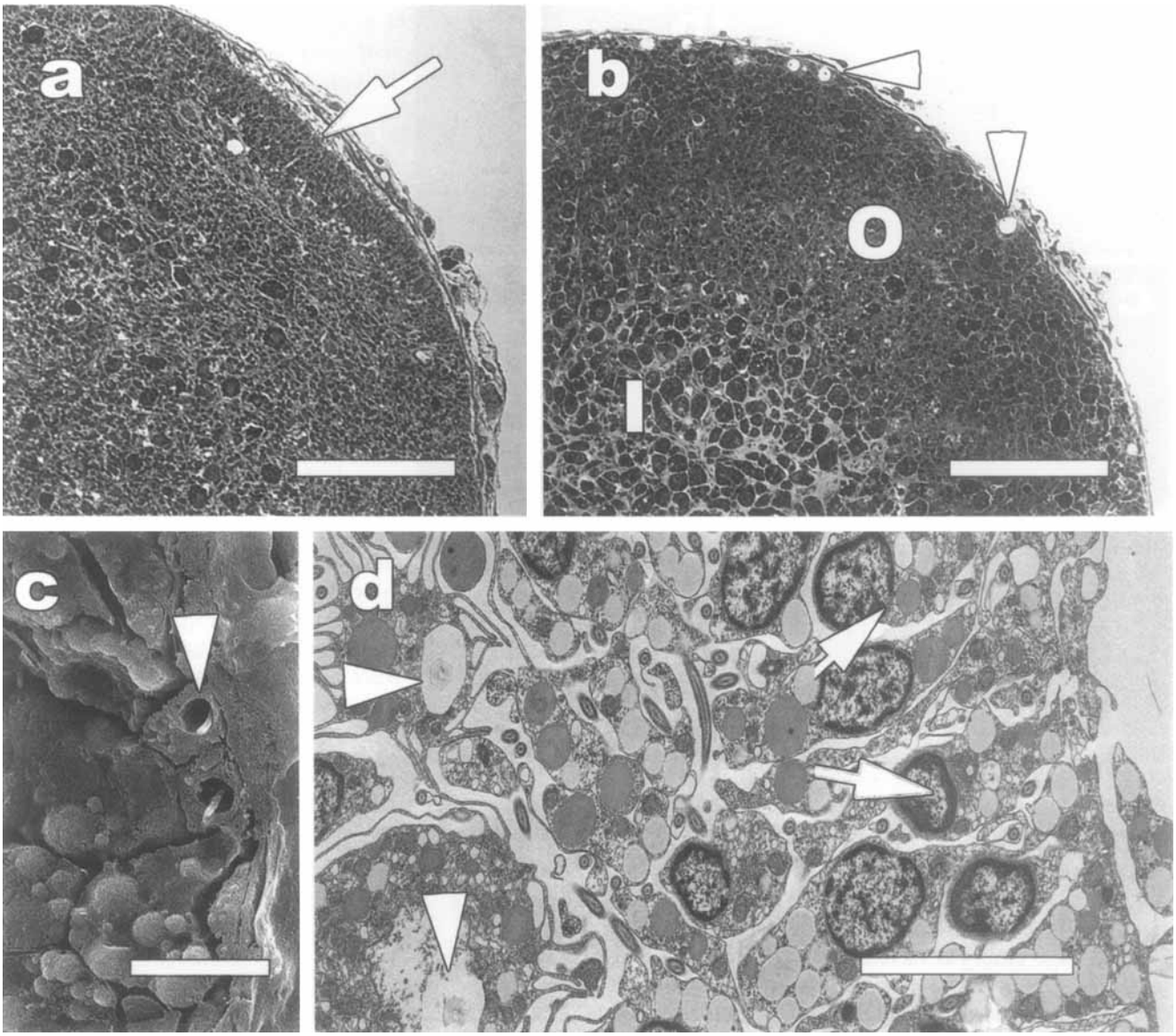

Fig. 4. Gastrulation in embryos of *Reniera* sp. (**a**) Late blastula with micromeres (arrow) at one side. LM, epoxy section. Scale bar, 50 μm. (**b**) Late gastrula: the inner region (I) has large osmiophilic cells and collagen whereas the outer region (O) has small cells. Arrowheads show holes left by the dissolution of spicules in cells at the periphery of the embryo. LM. Scale bar, 50 μm. (**c**) Edge of an embryo showing sclerocytes (arrowhead) identified by the proteinaceous core of the spicule that remains after desilicification of the fixed embryo. SEM. Scale bar, 4 μm. (**d**) Edge of a gastrula. Monociliated micromeres (arrows) are positioned at the periphery of the embryo forming the future epithelial layer. Sclerocytes (identified by the proteinaceous core in a vacuole within the cells; arrowheads) with numerous pseudopodia lie just beneath the emerging epithelial layer. TEM. Scale bar, 10 μm.

from the parent. The larvae sink to the bottom of containers and begin rotating on the anterior pole before attaching and beginning metamorphosis. Metamorphosis is complete with the formation of the osculum in the juvenile sponge 5–7 days after settlement (Fig. 6a–d). Rotation is effected by the short cilia of the ciliated epithelial layer, which continue to beat until attachment is complete. The long posterior cilia that control the direction of swimming cease beating when the larva is rotating on its anterior pole, but remain responsive to changes in light intensity until they are internalized or discharged during metamorphosis.

As the larva rotates on its anterior pole, the anterior hemisphere becomes flattened (Fig. 7a–c). When the larva ceases to rotate and begins to settle, the anterior pole becomes slightly indented (Fig. 7d).

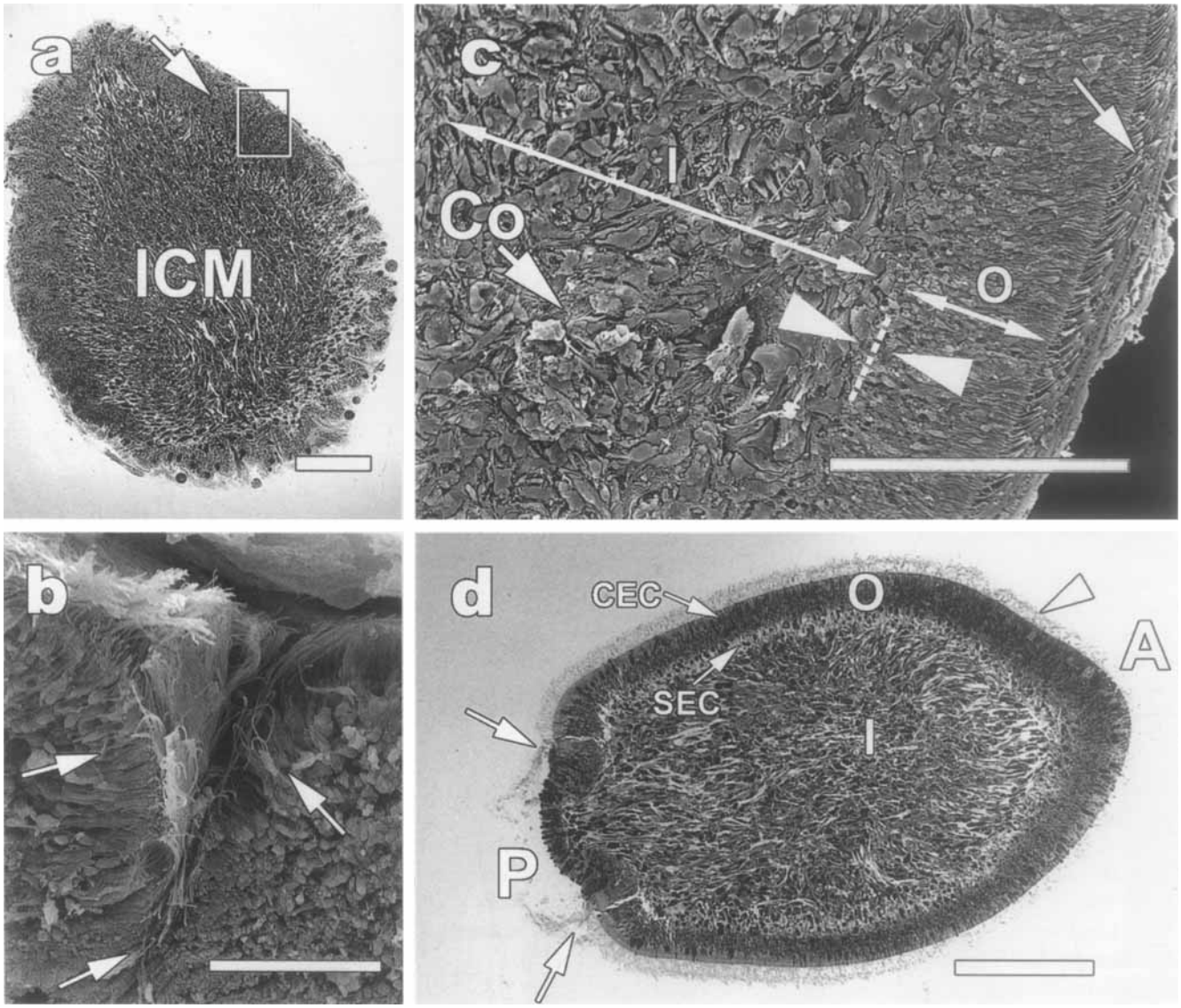

Fig. 5. Late embryos. (**a**) Late-stage diploblastic embryo. The embryo has a dense inner cell mass (ICM) and an outer monociliated epithelial layer (arrow). The columnar epithelial layer is initially folded. Epoxy section. Scale bar, 100 μm. (**b**) Detail of the box in (a). Arrows indicate the base of the columnar cells in the outer epithelial layer. SEM. Scale bar, 25 μm. (**c**) Embryo fractured in liquid nitrogen. Prior to release from the brood chamber the embryo elongates such that the epithelial layer is no longer folded. The outer layer (O) of monociliated epithelial cells is separated from an inner region (I) containing various amoeboid cells surrounded by collagen (Co). At the boundary of inner and outer cell layers is another, quite thin, layer of cells (arrowheads and dotted line), which lie perpendicular to the columnar monociliated epithelial cells and form a circumferential sheath around the larva. The cilia (upper right arrow) are bent under the embryonic follicle of maternal tissue. SEM. Scale bar, 50 μm. (**d**) Epoxy section of the embryo showing that it has an inner (I) region of at least 4 cell types, and an outer (O) region consisting of a columnar epithelial cells (CEC) and a layer of subepithelial cells (SEC). Short cilia (arrowhead) extend from the columnar monociliated epithelial layer. A small 15–20 μm region at the anterior pole (A) is non-ciliated. The posterior (P) pole has a larger (50–80 μm diameter) region which is non-ciliated and is bordered by pigmented cells that give rise to cilia 120–150 μm long (arrows). Scale bar, 100 μm.

During settlement, the anterior half becomes compressed (Fig. 8a,b). Scanning electron microscopy of postlarvae at various stages of metamorphosis shows that the short cilia first disappear from the anterior pole of the postlarva (Fig. 8c), and as metamorphosis progresses, gradually all short cilia are resorbed (Fig. 8d–f). The long posterior cilia remain on postlarvae that are otherwise covered in a smooth sheet of cells (Fig. 8d,f); it is unclear whether the long posterior cilia are finally resorbed or discharged. The

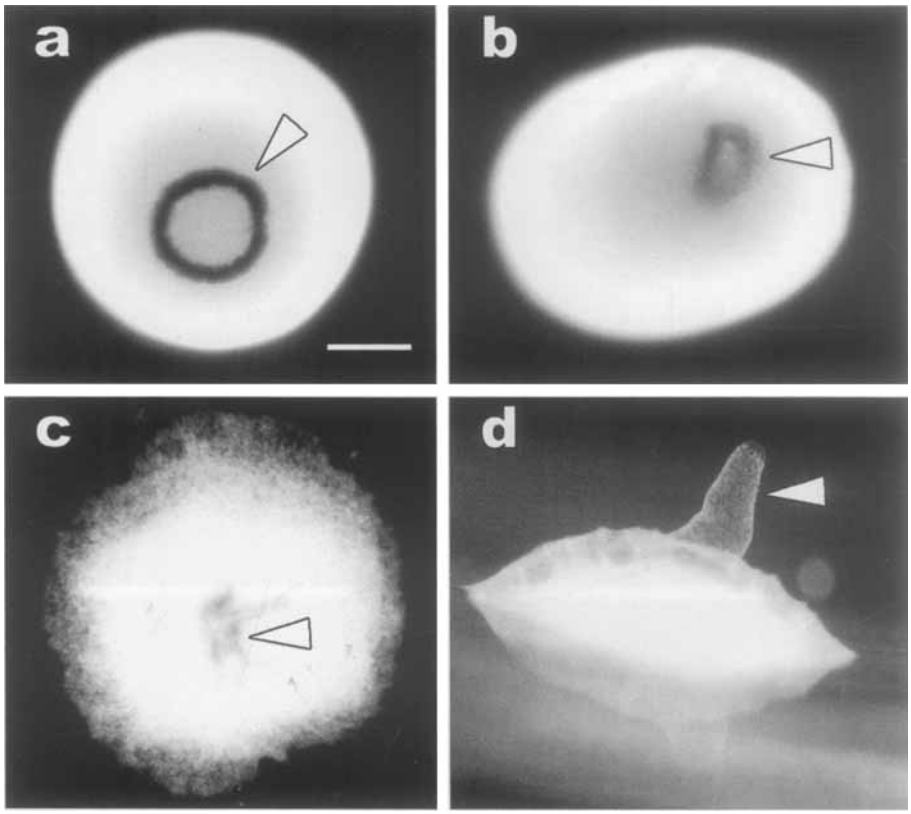

Fig. 6. Settlement and metamorphosis. LM. (**a**) The pigment ring (arrowhead) at the posterior pole is clearly visible in a larva that is rotating on its anterior pole prior to attachment. (**b, c**) During settlement and metamorphosis of the larva, the pigment ring (arrowheads) disappears. (**d**) The osculum (arrowhead) of the juvenile sponge develops at the former location of the pigment ring. Scale bar, 50 μm.

short lateral cilia of the metamorphosing postlarva are internalized (Fig. 8e).

Two-day-old metamorphosing sponges have a smooth covering of flat cells whose origin is unclear (Fig. 9a). Together with spherulous cells, these flat cells form the peripheral epithelial layer of the postlarval sponge. Each formerly ciliated epithelial cell, having internalized its axoneme, migrates towards the center of the metamorphosing larva (Fig. 9b). The axoneme remains intact during the initial migration of the cells into the interior of the juvenile sponge (Fig. 9c), but at later stages the axoneme is disassembled into its tubulin components within the cell (Fig. 9d and inset). Three days after settlement begins, the water canal system and flagellated chambers have all the attributes of a functioning juvenile sponge (Fig. 9e).

Because it was difficult to track the migration of the lateral ciliated cells after they had completely resorbed the cilium, the vital dye Cell Tracker CMFDA was used to follow the cells during metamorphosis of the larva. Larvae that were immersed for 30 min in the reagent showed fluorescence only in the outer epithelial layer, indicating that the dye did not move beyond this layer during labeling (Fig. 10a). Control larvae incubated in DMSO in seawater showed only autofluorescence that was evenly distributed throughout the larva (Fig. 10b).

Whereas control juveniles of any age showed no fluorescence at all (Fig. 10d), both 4- and 6-day-old metamorphosing sponges showed strong fluorescence, specifically in the choanocytes that form the flagellated chambers (Fig. 10c,e), and in an elongate cell type that was scattered throughout the juvenile sponge (Fig. 10f,g).

Discussion

This paper provides the first demonstration using a fluorescent cell-lineage tracer that the outer epithelial cells of the parenchymella sponge larva transform into the flagellated choanocytes and one type of elongate cell in the juvenile sponge.

The origin of sponge choanocytes during metamorphosis has long been in question. From studies of calcareous sponge development, Delage (1892) proposed that upon metamorphosis the outer "ectodermal" layer of the larva migrated in to become the flagellated "gastrodermal" layer of the adult sponge, thereby inverting the primary germ layers of sponges relative to all other metazoans. He proposed that sponges be placed in the phylum Enantiozoa ("inside out" animals) to reflect this inversion of layers. Both histological (Dubosq & Tuzet 1937; Lévi 1956; Borojevic & Lévi 1965; Borojevic 1966) and ultrastructural (Ama-

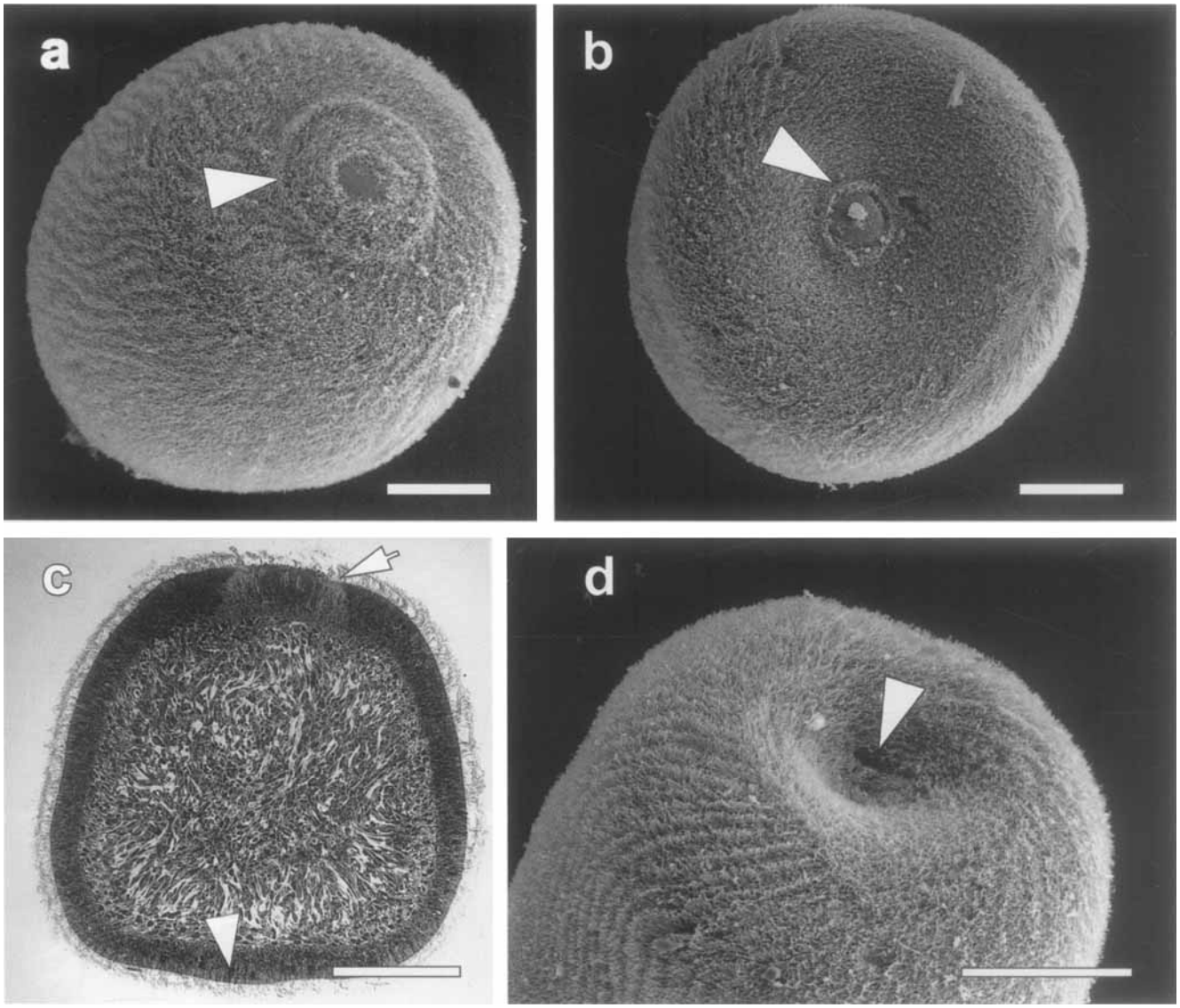

Fig. 7. Settlement. (**a**) The short cilia are flattened at the former anterior pole (arrowhead) in this specimen, which was fixed while rotating on its anterior pole prior to attachment. SEM. (**b, c**) The anterior pole (arrowhead) flattens as the larva settles. The pigment-containing cells with long cilia (arrow) are still visible at the posterior pole. b, SEM; c, LM. (**d**) At settlement the anterior pole forms an indentation (arrowhead). SEM. Scale bars, 100 μm.

no & Hori 1996) studies over the years suggest that inversion of layers at metamorphosis is quite common among sponges.

However, it has also been shown that some sponge larvae shed or phagocytize the outer ciliated epithelium at settlement and metamorphosis (Meewis 1939; Bergquist & Green 1977). Other sponge larvae precociously form the flagellated chambers in the larva before settlement (e.g., freshwater sponges: Brien & Meewis 1938; Wielspütz & Saller 1990; hexactinellids: Boury-Esnault et al. 1999). Furthermore, the one previous experimental approach that used a radioactive label to track cells during metamorphosis concluded that the outer epithelial cells of the larva become archaeocytes, but do not continue to form the choanocytes of the juvenile sponge (Misevic et al. 1990). Nonetheless, these results have generally been interpreted as exceptions to the normal mode of development in sponges.

The consensus of opinion over the course of the 20th century has been that the immigration of the larva's outer or epidermal layer to become the inner or gastrodermal layer at metamorphosis is actually gastrulation in sponges (Lévi 1963; Fell 1974; Simpson 1984; Ruppert & Barnes 1994). But interpretations of what represents gastrulation in the Porifera are highly

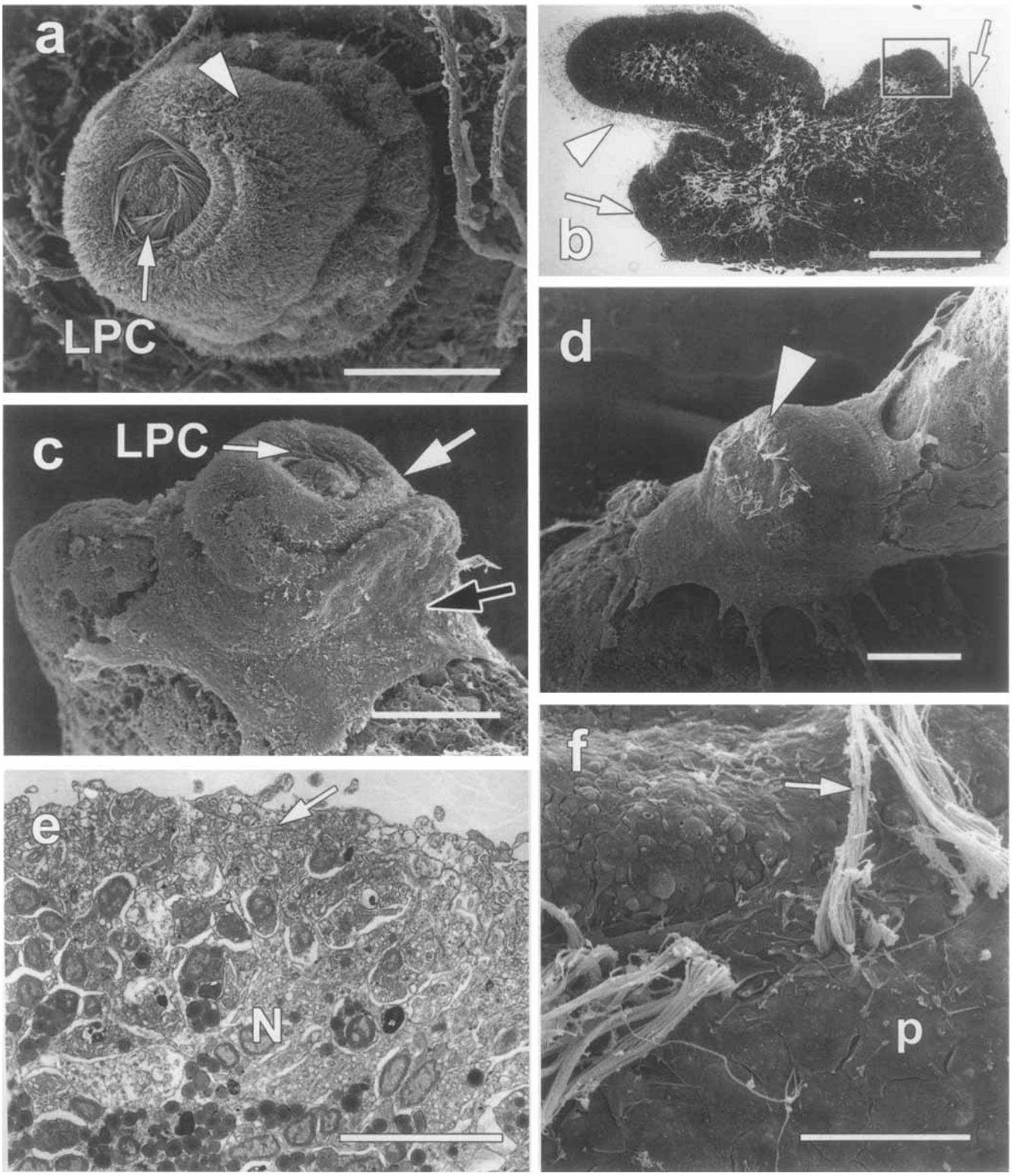

Fig. 8. Metamorphosis of postlarvae. (a,b) show postlarvae that have attached to algae (*Turbinaria ornata*). (**a**) The long posterior cilia (LPC) are recessed as the anterior of the postlarva is compressed on the alga. Short cilia (arrowhead) are still present on the postlarva. SEM. Scale bar, 200 μm. (**b**) The short cilia (arrowhead) are still present on the posterior half of the postlarva, but have been internalized on the anterior half (arrows). Cells at the anterior pole are migrating into the center of the postlarva. The boxed area is shown in (e). Epoxy cross section. Scale bar, 200 μm. (**c**) Postlarva metamorphosing on a sand grain. The cilia on the former anterior pole of the larva (black arrow) have been internalized, whereas

controversial. In other metazoans, gastrulation is the establishment of the primary germ layers, an event that typically occurs after the primary cleavages have finished forming a hollow or solid blastula (Brusca et al. 1997). Segregation of cell layers at gastrulation may occur by invagination, involution, delamination, epiboly, or uni- or multipolar ingression. Around this stage the first differentiated cell types appear (Brusca et al. 1997; Davidson 2001).

Our results show that cellular differentiation in embryos of *Reniera* sp. first becomes evident with the development of cilia on some of the blastomeres in the stereoblastula, a solid blastula, and is shortly followed by the appearance of cells of a distinct phenotype throughout the embryo (e.g., the red-staining spherulous cells). The cell migrations which occur after the final cleavages have been completed, and which establish the two cell layers of the larva, may be readily equated to gastrulation in the Cnidaria and in some triploblastic bilaterian metazoans. Furthermore, these cell migrations occur simultaneously with the appearance of pigmentation in the embryo. Polarity of the embryo is clearly observed at this stage, with the migration of the pigmented cells to the posterior pole of the swimming larva, suggesting that the anteroposterior axis is established earlier in development.

Although developmental modes among sponges are varied, the pattern of embryogenesis seen in *Reniera* sp. is nonetheless fairly standard for many parenchymella larvae. Nurse cells, or trophocytes, play a large role in the growth of the oocyte in parenchymellae (Fell 1969; Fell & Jacob 1979; Saller & Weissenfels 1985; Saller 1988), and to a lesser extent in the development of amphiblastula larvae (Gaino et al. 1987). The first cleavages are not always clear, because of the large number of trophocytes that are incorporated into the cytoplasm of the oocyte (Brien & Meewis 1938; Meewis 1939; Fell 1969). Where cleavage patterns are visible, they are reported to be either equal or unequal, both resulting in the formation of a solid blastula. Cellular differentiation leads to the formation of micro- and macromeres. The former migrate to the periphery in all cases documented, and in some instances it has been noted that these cells also move unequally to one end of the embryo (Meewis 1941).

Most parenchymella larvae remain solid until settlement and metamorphosis, but in the freshwater spongillids, precocious development of flagellated chambers and an inner cavity occurs in the free-swimming larva (Brien & Meewis 1938; Saller & Weissenfels 1985; Saller 1988), and in the dendroceratid sponge *Halisarca,* an inner ciliated chamber develops by an unusual invagination of the ciliated epithelium (Ereskovsky & Gonobleva 2000).

Other sponge larvae have notably different developmental patterns. Larvae of *Chondrosia reniformis* and *Polymastia robusta* (both oviparous demosponges in the subclass Tetractinomorpha; Hooper 2000) develop by equal cleavage of the oocyte to form a benthic coeloblastula larva. Most familiar from textbook descriptions is the semi-ciliated amphiblastula larva of the Calcaronea, a subclass of the Calcarea. The amphiblastula develops from a polarized oocyte into two distinct cell populations that lie adjacent to one another, and which literally turn inside out, as in the Volvocales, to produce a hollow larva with cilia on the anterior pole (Dubosq & Tuzet 1937; Tuzet 1973). In the trichimella larva of hexactinellid sponges, total and equal spiral cleavage gives rise to a coeloblastula that undergoes gastrulation by delamination to form a 2-layered embryo with macromeres on the inside and micromeres on the outside. The micromeres differentiate into an outer syncytial epithelium and multiciliated locomotory "cells" (these are joined to one another and to the multinucleated tissue by plugged cytoplasmic bridges); the macromeres form multinucleated sclerocytes and presumably also the archaeocytes (Okada 1928; Boury-Esnault et al. 1999).

What this study shows is that in the development of poriferan larvae, gastrulation as defined by the segregation of the primary germ layers clearly occurs during early embryogenesis. The modes of development of primary germ layers in the Cnidaria are equally as varied. Uni- or multipolar ingression is the most common means of gastrulation in the main cnidarian classes (Lowe 1926; Spaulding 1974; Martin et al. 1983; Martin & Archer 1986; Martin 1987; Thomas et al. 1987; Martin et al. 1997). Fossilized cnidarian embryos from the early Cambrian document a long history of gastrulation by unipolar ingression (Zhao & Bengston 1999). Gastrulation by delamination and epiboly have been reported in the Anthozoa and Hydrozoa, and

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those on the posterior half are still present (white arrow). The long posterior cilia (LPC) are recessed. SEM. Scale bar, 200 μm. (**d**) At a later stage during metamorphosis, all the short cilia have been internalized but the long cilia remain at the former posterior pole of the larva (arrowhead). SEM. Scale bar, 200 μm. (**e**) TEM of the boxed area in (b). Cilia (arrow) are internalized by the epithelial cells. Nucleus (n). Scale bar, 10 μm. (**f**) Long posterior cilia (arrow) and pinacocytes (p) of the new epithelial layer of the postlarval sponge. High-magnification SEM. Scale bar, 5 μm.

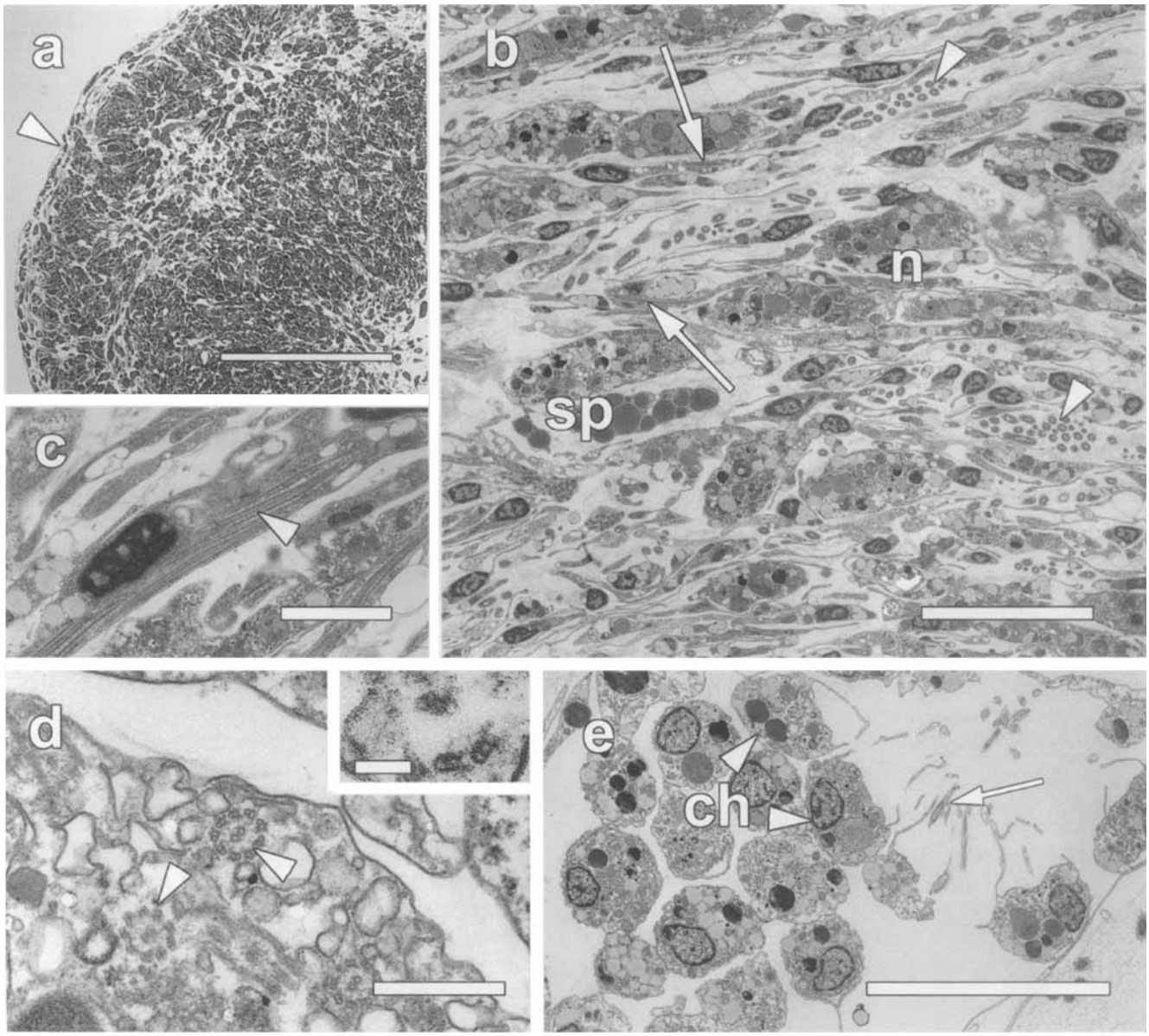

Fig. 9. Stages in the metamorphosis of postlarvae. (**a**) A 2-day-old metamorphosing postlarva such as that shown in Fig. 8d. The outer epithelium is smooth and lacks cilia (arrowhead), whereas the inner region is densely packed with cells in a collagenous extracellular matrix. Cross section (epoxy thick section). Scale bar, 100 μm. (**b**) Central region of a 2-day-old metamorphosing postlarva. Many cells have the axonemes of former cilia inside (arrows). The extracellular space is filled with collagen and numerous rod-shaped bacteria (arrowheads). Nucleus (n); spherulous cell (sp). TEM. Scale bar, 10 μm. (**c**) Former monociliated epithelial cell that has internalized its axoneme (arrowhead). TEM. Scale bar, 2 μm. (**d**) Cross section of axonemes (arrowheads) in a cell in a 2-day-old metamorphosing postlarva. The cilia have lost their membrane, and eventually even the microtubules of the cilium are broken down into their tubulin components (inset). TEM. Scale bar, 0.5 μm; inset, 0.1 μm. (**e**) Choanocyte chamber in a 4-day-old postlarval sponge. Choanocytes (ch); flagellum (arrow). TEM. Scale bar, 10 μm.

gastrulation by invagination is known from the Anthozoa and Scyphozoa (Spaulding 1974; Siebert 1974; Hirose et al. 2000), with one report in the Hydrozoa for *Halocordyle disticha* (Martin 1987).

Parallels can also be seen in the development of polarity in the embryos of parenchymella and planula larvae. Polarity of cnidarian embryos that undergo gastrulation by unipolar ingression is defined by the appearance of an indentation at the first site of ingression, which indicates the position of the future blastopore and oral opening (Martin & Thomas 1983; Martin 1987; Thomas et al. 1987; Zhao & Bengston 1999).

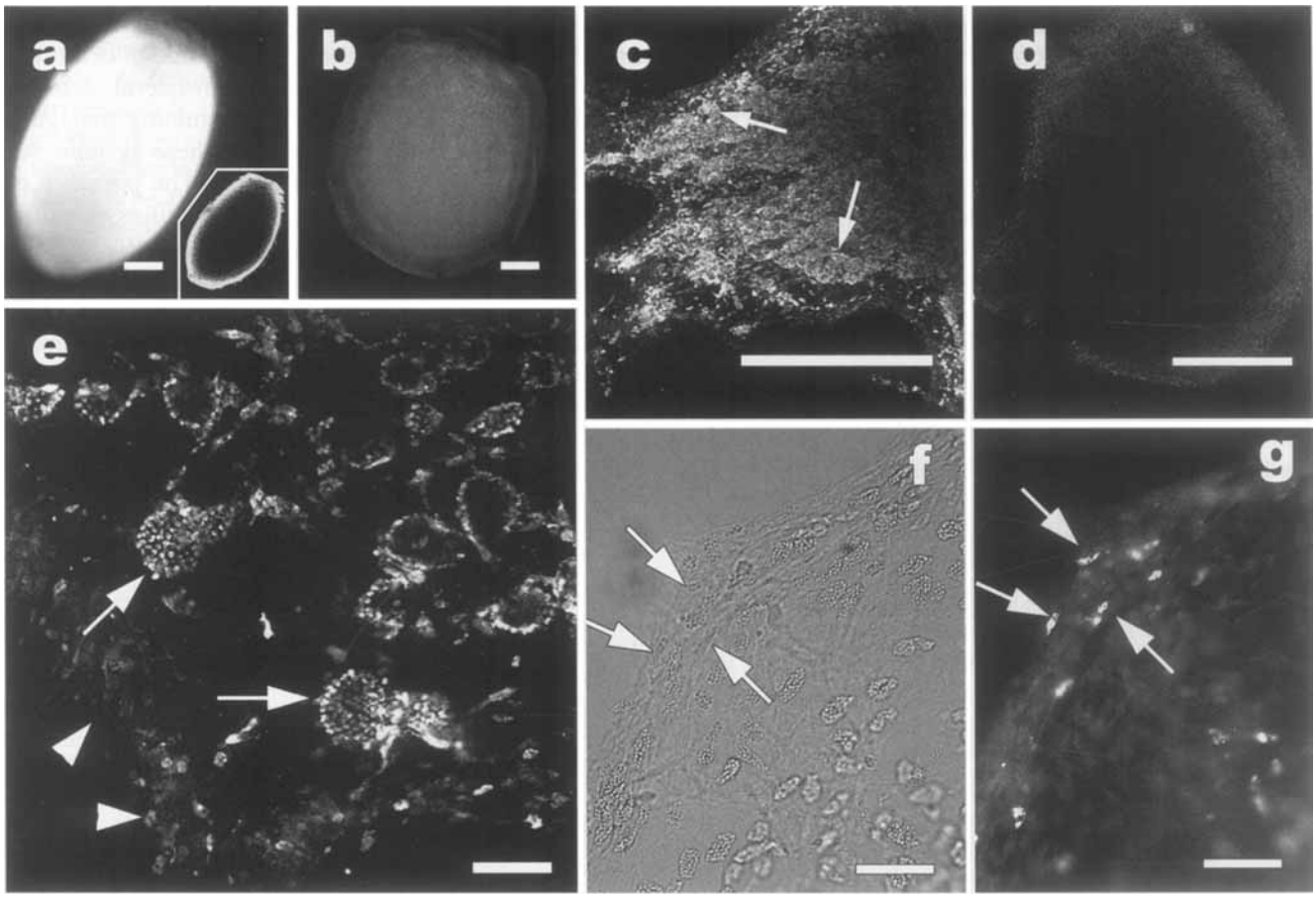

Fig. 10. Fluorescent labeling of the monociliated epithelial layer of larvae during metamorphosis. (**a**) Epifluorescence and (inset) confocal micrographs of larvae labeled with CMFDA for 30 min. The larvae fluoresce very brightly, indicating that the label has been taken up by the outer layer. (**b**) A control larva incubated in DMSO for 30 min shows only slight autofluorescence. Confocal microscopy. Scale bars for a,b, 100 μm. (**c**) 4-day-old and (**e**) 6-day-old postlarval sponges labeled with CMFDA prior to settlement and metamorphosis. Fluorescence is primarily in the choanocytes of the choanocyte chambers (arrows) and in particular amoeboid cells (arrowheads) around the edge of the juvenile sponge. Scale bars for c, 100 μm, e, 25 μm. (**d**) 4-day-old DMSO control juvenile sponge. Scale bar, 100 μm. (**f**) Bright field and (**g**) fluorescence microscopy of the elongate cells at the periphery of a 6-day-old juvenile sponge that was labeled with CMFDA prior to metamorphosis. Scale bars for f,g, 50 μm.

Polarity in poriferan embryos that gastrulate by uni- or multipolar ingression becomes evident with the accumulation of micromeres at one pole (Meewis 1941), or with the migration of pigmented micromeres to one pole, the future posterior pole of the larva (this paper). At settlement and metamorphosis, the former posterior pole gives rise to the osculum of the juvenile sponge. The indentation that occurs at the anterior pole of the larva during settlement does not reflect the inward migration of cells as seen during embryogenesis, but appears to indicate the initiation of the inward migration of the peripheral ciliated layer of the metamorphosing larva. Use of a fluorescent cell tracker to trace the fate of the larval locomotory cells in this study confirms work by Boury-Esnault (1976) which showed that the ciliated cells give rise to two cell types, the choanocytes and an elongate cell type, in the juvenile sponge. Although the role of the elongate cell type in the juvenile is unknown, it is clear that these cell migrations cannot be simply equated with an inversion of layers.

Transdifferentiation, the change of a fully differentiated cell into a different cell type involving a change in gene commitment and expression, once thought to be restricted to regeneration (Okada 1991), is known from cnidarians (Schmid 1992) and mammals (Patapoutian et al. 1995). The transdifferentiation of sponge larval ciliated cells may simply be an efficient means of recycling tubulin. Resorption of the cilium by epithelial cells during metamorphosis occurs in the Cnidaria (Martin et al. 1983) and Echinodermata (Chia & Burke 1978). Although the fate of the ciliated epithelial cells in other ciliated invertebrate larvae is not well

known, it is possible that they too are resorbed and recycled during metamorphosis. Clearly the cellular reorganization that occurs at metamorphosis is a complex process that requires further investigation in many invertebrate larvae as suggested by Nielsen (2000). We agree with the analysis by Borojevic (1970), who concluded that transdifferentiation of ciliated epithelial cells at metamorphosis need not be confused with the highly integrated cell movements that occur during gastrulation in the Porifera.

Cherdantsev & Krauss (1996) suggested that epithelialization lies at the heart of gastrulation: the resulting two primary germ layers differ in their degree of specialization with the inner cells remaining less specialized than the outer cells. This seems like a good way to understand gastrulation in the Porifera. In sponges the primary germ layers are established during embryogenesis of the larva when the ciliated cells migrate to the periphery (or are established at the anterior in amphiblastulae) and the pluripotent amoeboid cells remain in the center of the embryo. The sponge larva itself is a complex differentiated organism capable of sensing and responding to environmental stimuli (Simpson 1984; Wapstra & van Soest 1987; Woollacott 1990, 1993; Woollacott & Hadfield 1996; Maldonado & Young 1996; Leys & Degnan 2001). Metamorphosis of the larva into a juvenile sponge involves the reorganization of those cells that are as yet undifferentiated, and the de-differentiation of cells that have already been functioning in the differentiated larva; there is no evidence of extensive autolysis at metamorphosis.

The view that sponge development includes recognizable hallmarks of metazoan embryogenesis—formation of a blastula by cleavage and a 2-layered larva by gastrulation—provides a means to relate the ontogeny of the sponge body plan to that of other animals. Whether the sponge larval epithelium and inner cell mass can be equated to true ectodermal and endodermal layers awaits further investigation of cell junction specializations and of the architecture of basement membrane components known to exist in this group (e.g., Garrone 1995; Boute et al. 1996). Furthermore, the presence of a third cell layer, the subepithelial layer, and its differentiation from cells in the outer epithelial layer, is similar to the third cell layer formed from epithelia in many cnidarians (Hyman, 1940 p. 264). The development of the third cell layer in all the so-called diploblastic phyla deserves further attention.

Despite the uncertainty regarding the homology of the cell layers in sponges to those of other metazoans, it does appear that sponges use some of the mechanistic repertoire that creates these layers in animals with more complex body plans. The extensive cell movements occurring at gastrulation, which result in the regionalization of multiple differentiated cell types along the anteroposterior and centrolateral axes of sponge larvae, require basic cell signaling and gene regulatory systems (for a review of these systems see Carroll et al. 2001; Davidson 2001). The presence of a number of metazoan-specific gene families encoding transcription factors (e.g., Degnan et al. 1993, 1995; Manuel & Le Parco 2000) and components of signaling pathways (e.g., Brower et al. 1997; Wimmer et al. 1999) in sponge genomes suggests that these basal metazoans use the same molecular mechanisms to establish domains of differentiated cell types. Understanding the role of these genes during sponge development will allow us to gain insight into the ancestor that gave rise to sponges and the rest of the animal kingdom.

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