

Three-dimensional fate mapping of larval tissues through metamorphosis in the glass sponge *Oopsacas minuta*

Sally P. Leys,^{1,a} Afyqah Kamarul Zaman,¹ and Nicole Boury-Esnault²

¹Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

²Aix Marseille Université, CNRS, IRD, Avignon Université, IMBE UMR 7263, Marseille 13397, France

Abstract. The tissue of glass sponges (Class Hexactinellida) is unique among metazoans in being largely syncytial, a state that arises during early embryogenesis when blastomeres fuse. In addition, hexactinellids are one of only two poriferan groups that already have clearly formed flagellated chambers as larvae. The fate of the larval chambers and of other tissues during metamorphosis is unknown. One species of hexactinellid, *Oopsacas minuta*, is found in submarine caves in the Mediterranean and is reproductive year round, which facilitates developmental studies; however, describing metamorphosis has been a challenge because the syncytial nature of the tissue makes it difficult to trace the fates using conventional cell tracking markers. We used three-dimensional models to map the fate of larval tissues of *O. minuta* through metamorphosis and provide the first detailed account of larval tissue reorganization at metamorphosis of a glass sponge larva. Larvae settle on their anterior swimming pole or on one side. The multiciliated cells that formed a belt around the larva are discarded during the first stage of metamorphosis. We found that larval flagellated chambers are retained throughout metamorphosis and become the kernels of the first pumping chambers of the juvenile sponge. As larvae of *O. minuta* settle, larval chambers are enlarged by syncytial tissues containing yolk inclusions. Lipid inclusions at the basal attachment site gradually became smaller during the six weeks of our study. In *O. minuta*, the flagellated chambers that differentiate in the larva become the post-metamorphic flagellated chambers, which corroborate the view that internalization of these chambers during embryogenesis is a process that resembles gastrulation processes in other animals.

Additional key words: Hexactinellida, 3-D, Porifera, cell fate, gastrulation, evolution and development

Glass sponges (Porifera, Hexactinellida) are deep water animals that can occur at shallower depths in fjords, caves, and shelf habitats where local conditions mimic the deep. Much about their biology is unusual and quite likely an adaptation to this specialized habitat. Their tissues are largely syncytial, formed by the fusion of cells during early development. Syncytial tissues allow these sponges, alone among Porifera, to propagate electrical signals that arrest the feeding current, which is presumed to be a protective response as seen in other filter feeders (e.g., Mackie et al. 1974; Mackie 1995) to prevent damage to the filter caused by resuspended sediments. Sponges are among the earliest metazoans to have evolved, and although it is unknown when

glass sponges diverged from cellular sponges, “cruciform” spicules like those of glass sponges from 535 Ma provide the oldest unequivocal fossil record of metazoans (Antcliffé et al. 2014). A better understanding of why and how syncytial tissues are formed and maintained might shed light on the conditions that gave rise to these unusual animals.

Because glass sponges are difficult to access, studies on their reproduction and development are scarce. Embryos and larval development are described from only three species (Ijima 1904; Okada 1928; Boury-Esnault & Vacelet 1994; Boury-Esnault et al. 1999; Leys et al. 2006). Glass sponge embryos form a hollow blastula until the 64-cell stage when macromeres fuse and envelop micromeres, a process that has been equated to epiboly (Boury-Esnault et al. 1999). The larva is called a trichimella. It has a single multinucleated syncytial

^aAuthor for correspondence.

E-mail: sleys@ualberta.ca

tissue that forms the dermal epithelium and whose thin strands penetrate and surround cells such as archaeocytes, choanoblasts, sclerocytes, and multiciliated cells. Cells—defined as a region of distinct cytoplasm with a single nucleus (Mackie & Singla 1983)—and multinucleated syncytia are connected by cytoplasmic bridges such that the plasma membrane is continuous throughout.

In cellular sponges the larval tissues reorganize at metamorphosis to form adult structures. This occurs by migration of cells from the ciliated epithelium inwards to redifferentiate into choanocytes and other cell types (Wielsputz & Saller 1990; Amano & Hori 1993; Leys & Degnan 2002; Gonobobleva & Ereskovsky 2004; Nakanishi et al. 2014). In glass sponge tissues, however, no cell is motile. Nutrient transport occurs intrasyncytially along multinucleated tissues termed “cord syncytia” (Reiswig 1979; Leys 1995); even archaeocytes are transported along with their cytoplasmic bridge as an intact tether (Leys 1995). In that case, one wonders how the syncytial tissues of the glass sponge larva are restructured to form the adult at metamorphosis while maintaining distinct control of regionality.

Syncytia are not uncommon in animals (reviewed in Mackie & Singla 1983; Leys 1996, 2003b) and are thought to allow coordination across large regions of cytoplasm. Most syncytia also have mechanisms to control regions of cytoplasmic separation, as in *Drosophila* embryos prior to cellularization (Anderson et al. 2013). In the glass sponge, this is done in part by an unusual protein plug formed by the Golgi apparatus (Mackie 1981). Plugs have a trilaminar structure with pores 6–8 nm in diameter that can contain membranous vesicles. That the plug acts as a selective filter is suggested by the distinct cytoplasm found on either side of the plug (Mackie & Singla 1983).

We have studied the fate of larval tissues of *Oopsacas minuta* TOPSENT 1927, a glass sponge that inhabits particular submarine caves in the northern Mediterranean and Adriatic where deep cold water is trapped. It is one of the smallest glass sponge species known (6–7 cm) (Vacelet et al. 1994; Bakran-Petricioli et al. 2007) but is reproductive year round. Typical methods used to map the fate of larval tissues include transmission electron microscopy (TEM) where cellular markers are clear (Boury-Esnault 1976; Amano & Hori 1996, 2001), fluorescent markers such as CMFDA that label cell types, or diI, a cell tracker which tags a tissue for a few days (Leys & Degnan 2002; Nakanishi et al. 2014). In some cases particular cells, such as sclerocytes

(Leys 2003a), can be identified and traced through metamorphosis, but fluorescent markers are not ideal for glass sponges because they spread through the syncytial tissues (Leys 1995). In this study, we therefore used a novel method to map the fate of larval tissues in *O. minuta*, by rendering three-dimensional (3-D) models reconstructed from larvae sectioned at different stages of metamorphosis. These 3-D models complement the perspective given by electron microscope images and allow us to follow regions of extremely thin tissues through metamorphosis into the juvenile sponge.

Methods

Light microscopy

Larvae were obtained from adult *Oopsacas minuta* (Leucopsacidae, Lyssacinosa, Hexasterophora, Hexactinellida) collected by SCUBA in November 2002 in a submarine cave near La Ciotat, France (Vacelet et al. 1994). Pieces of rock with sponges attached were chipped from the wall of the cave using a hammer and chisel, and collected into a glass canning jar for transport to the Station Marine d'Endoume. Sponges were maintained in cooled seawater to allow release of larvae, and although some larvae swam out autonomously, the sponges were dissected so that the tugging caused more to be released. *Trichimella* larvae were pipetted into 5-cm-diameter Petri dishes in which coverslips were placed on the bottom and floated on the surface of the water for settlement of larvae on either surface. To capture the change in larval to adult tissues, various stages of development were fixed for microscopy, from free-swimming larvae to 6-week post-settlement juveniles. For fixation, first the amount of water was reduced by pipetting out as much as possible without allowing the two coverslips to touch, and then a cocktail fixative of 2% glutaraldehyde, 1% osmium tetroxide in 0.45 M sodium acetate buffer of pH 6.4 was added. Larvae and metamorphosing juveniles were prepared for epoxy infiltration as previously reported (Leys et al. 2006). Semi-thin serial sections (0.5 μ m) were cut from each stage with a histo Jumbo diamond knife (Diatome), and ribbons were transferred to glass slides according to Blumer et al. (2002). Sections were stained with toluidine blue and viewed with a Zeiss Axioskop2 microscope in pure white light illumination. Colored images of serial sections were captured with a Zeiss AxioCam HR camera and Axiovision LE software. *Trichimella* larvae grow in diameter as they

metamorphose; therefore, depending on the age of each sponge, the number of serial sections obtained and images taken varied from 100 to 300 per specimen.

Fate mapping and 3-D reconstruction

To ensure that all images had uniform background color and were in correct orientation relative to one another, images were further processed in Adobe Photoshop 5.5 (Adobe System). Serial images of each sponge were either automatically aligned with AutoAligner software (Bitplane) or manually aligned with Reconstruct software (Fiala 2005). Image stacks were viewed with IMARIS software and tissues of interest were identified based on the description by Leys et al. (2006) and from TEM images taken in this study. In SURPASS mode, each tissue type was either drawn semiautomatically or manually depending on its conspicuousness. Specifically, the following tissues were traced manually in all image slices that they appeared in: the trabecular reticulum, multiciliated cells, spicule spaces, flagellated chambers (based on how collar bodies were positioned), choanoblasts, lipid inclusions, and yolk inclusions.

Transmission and scanning electron microscopy

Ultrathin sections of pre- and newly post-settled larvae were cut with a diamond knife and collected on copper grids. Sections were stained with uranyl acetate for 10 min, lead citrate for 8 min, rinsed with water and air-dried. Sections were examined with a Philips (FEI) Morgagni 268 transmission electron microscope and images were captured with a Gatan

Orius CCD Camera. For scanning electron microscopy (SEM) larvae were dehydrated to 100% ethanol and prepared as described by Leys et al. (2006).

Results

Description of larval behavior in *Oopsacas* and the process of metamorphosis

Glass sponge larvae are not easily encountered, and therefore this represents one of the few opportunities to describe their behavior, albeit in a laboratory setting. Adult sponges can be collected intact by chipping the rock substrate on which they are settled (Fig. 1A). Larvae are abundant in the sponge tissue (Fig. 1B) and readily swam out from the parent tissue in Petri dishes in the lab (Fig. 1B, inset). The larva is white, ~100 μm long, with a shiny opaque anterior swimming pole and translucent mid and posterior regions; the anterior is rounded mid and the posterior pointed (Fig. 1C). Larvae swam upwards in still water, in a slow left-hand rotation. No rapid turns were seen, nor did the larvae probe the substrates. They were weak swimmers (in comparison to larger demosponge larvae such as *Amphimedon queenslandica*), and like most sponge larvae in Petri dishes of still water, they became trapped in the surface tension unless they were pushed back down by a jet of water from a pipette. In initial work, some larvae did not settle for over a week, but we did not record how active they were, whether swimming or not, nor if or when they finally settled. In all subsequent experiments, larvae settled within 12–24 h after release from the adult sponge. Most larvae preferred grooves in plastic coverslips or the Petri

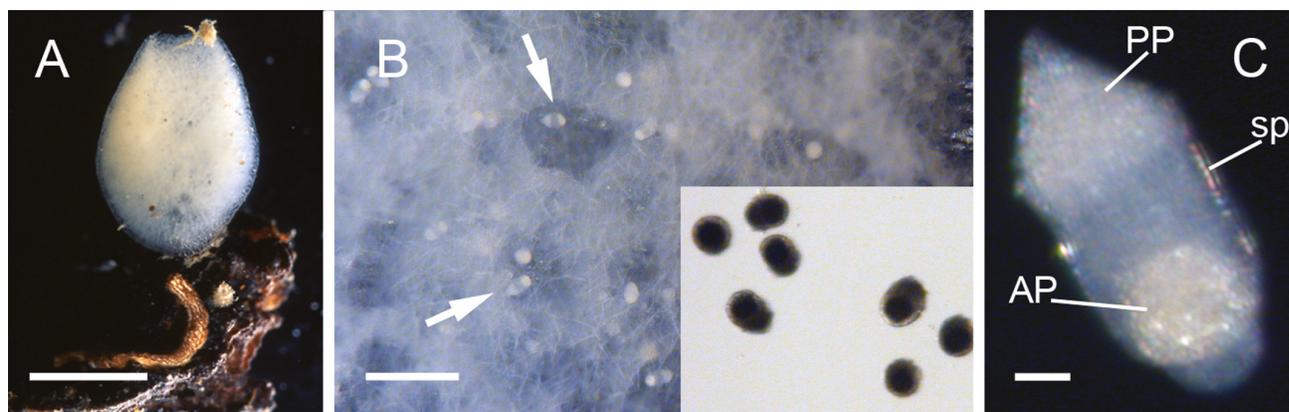


Fig. 1. The glass sponge *Oopsacas minuta*, adult and larvae. **A.** Whole sponge on a rock chip. **B.** Larvae in the adult tissue (arrows) and free swimming in a Petri dish (inset). **C.** Swimming larva with birefringent lipids in the anterior pole (AP), a dense conical posterior pole (PP), and a translucent mid-region bordered by spicules (sp). Scales: A, 0.5 cm; B, 500 μm ; C, 20 μm .

dish, and many settled on the surface coverslips and could be observed as they underwent the first stages of metamorphosis.

Morphology of the larva

The trichimella larva is highly polarized with a rounded anterior pole filled with large opaque, smooth appearing lipid inclusions and a pointed posterior pole filled with irregularly shaped inclusions of yolk (Fig. 2A,B). Yolk and lipid inclusions

are directly held in the trabecular reticulum, a single continuous membrane-bound tissue which forms the epithelium of the entire animal and links together all discrete uni-nucleated components (cells) by cytoplasmic bridges. Between the two ends, the larval body is elongated and cylindrical. Multiciliated cells girdle this region arranged side by side like a series of batteries (Fig. 2C,D). Each cell has up to 50 cilia that pierce the larval epithelium, and while each cell has a single nucleus and is rich with mitochondria, the cells are connected to each other and to other

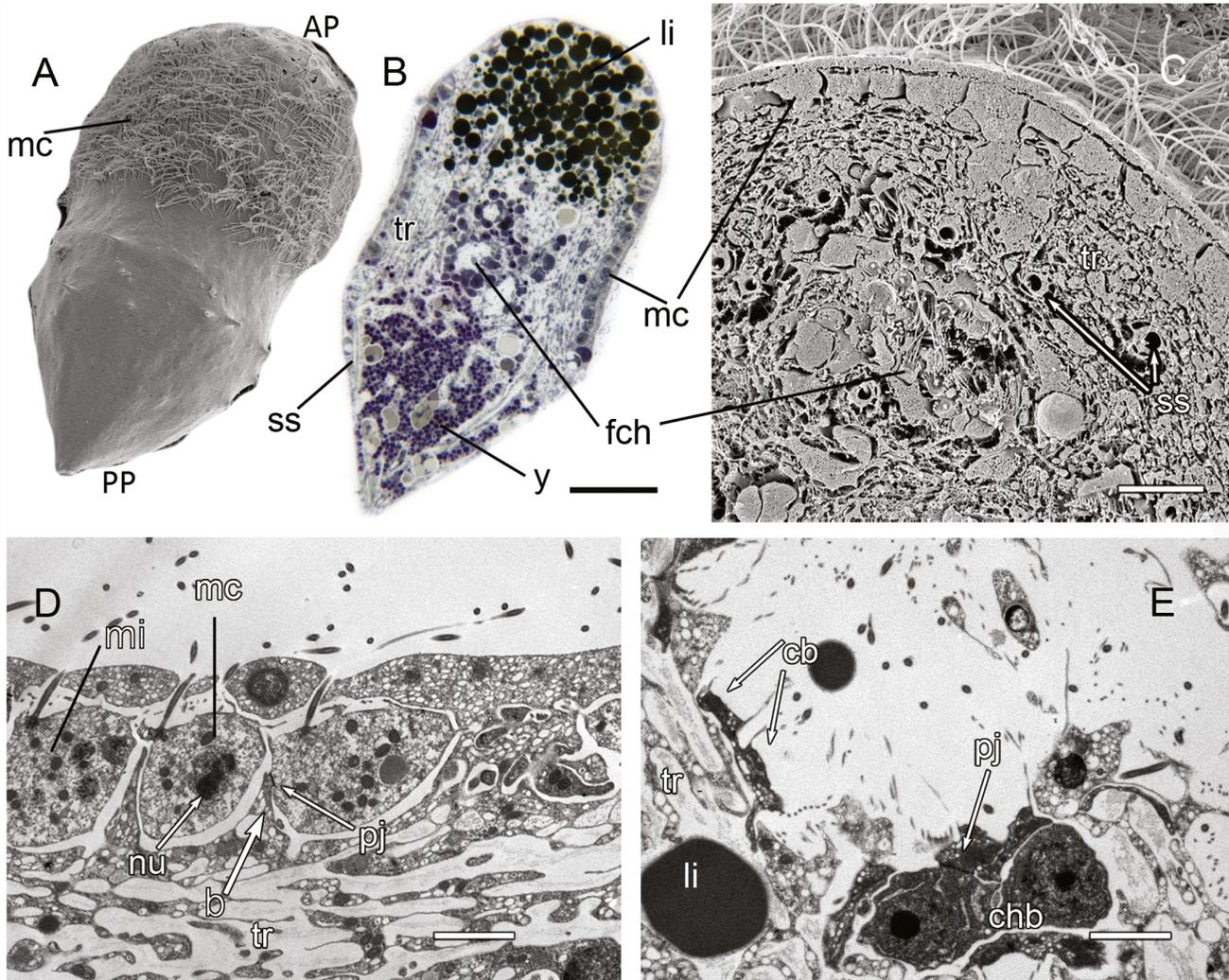


Fig. 2. Larval features of *Oopsacas minuta*. **A.** Whole larva showing the smooth syncytial covering and multiciliated cells. **B.** Longitudinal section. **C.** Cross section showing flagellated chambers and multiciliated cells. **D.** Section of the mid-region showing multiciliated cells with cilia piercing the syncytial epithelium. Multiciliated cells each have a single nucleus and are mitochondria-rich, but are connected to the multinucleated trabecular reticulum by cytoplasmic bridges with plugged junctions. **E.** A flagellated chamber in a larva showing developed choanoblasts connected by cytoplasmic bridges to collar bodies. Scanning electron (A,C), light (B) and transmission electron (D,E) microscopy. AP, anterior pole; b, cytoplasmic bridges; cb, collar bodies; chb, choanoblast; fch, flagellated chamber; li, lipid; mc, multiciliated cells; mi, mitochondrion; nu, nucleus; pj, plugged junction; PP, posterior pole; ss, spicule spaces; tr, syncytial trabecular reticulum; y, yolk. Scales: A,B, 20 µm; C, 5 µm; D,E, 2 µm.

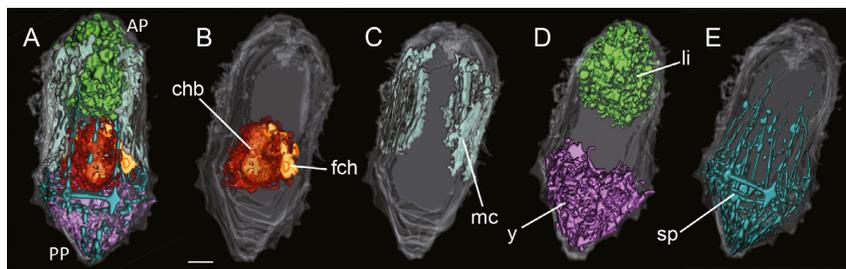


Fig. 3. Three-dimensional reconstructions of a larva of *Oopsacas minuta*. **A.** Composite. **B.** Flagellated chambers (pale orange) and choanoblasts (rust). **C.** Multiciliated cells (pale blue). **D.** Lipid (green) and yolk (purple). **E.** Larval spicules (steel blue). AP, anterior swimming pole of the larva; chb, choanoblast; fch, flagellated chamber; li, lipid; mc, multiciliated cells; PP, posterior swimming pole of the larva; sp, spicules; y, yolk. Scale 20 μm .

syncytial tissues by a continuous cytoplasmic bridge (Fig. 2D).

Bordering the yolk inclusions near the posterior pole are the larval flagellated chambers (Fig. 2C,E). Unlike other sponges, glass sponges do not have nucleated choanocytes. Instead the chambers are lined by “branched choanocytes” (Leys et al. 2007): a few nucleated choanoblasts have cytoplasmic connections to enucleate collar bodies, so the collar-flagella structure lies on a thin cytoplasmic base that has no nucleus of its own (Fig. 2E). Choanoblasts can be easily recognized by their prominent large nucleus and mitochondria (chb, Fig. 2E). Plugged junctions are found connecting one multiciliated cell to another, multiciliated cells to the syncytial trabecular reticulum, choanoblasts to each other, and choanoblasts to the syncytial trabecular reticulum (Fig. 2D,E).

Larval spicules are stauractins whose long ray is just shorter than the length of the larva. The lateral rays arise about 40 μm from the anterior pole of the larva and form a cage around the soft tissues of the larva (Leys 2003a). In sections, the space left by spicules is distinguished by the presence of a square proteinaceous axial filament and some collagenous material (Fig. 2C).

The most prominent aspects of the larva which could be readily followed through metamorphosis were the lipid and yolk inclusions, the flagellated chambers, choanoblasts, and spicules (Fig. 3A–E). These structures were traced at each stage, and their position relative to each other and the juvenile sponge structure was inferred from the resulting three-dimensional reconstruction (Supporting information Video S1).

Stages of metamorphosis in *Oopsacas minuta*

Larvae settled on their anterior pole or their side, but never on the posterior pole. The lipid inclusions

at the anterior pole reflected light, and during metamorphosis the region containing the lipid spheres spread out to form a darker plaque on the coverslip. As the post-larva changed shape, the stauractin spicules remained together at the conical end and spread apart where the larva attached to the substrate, forming an upside down basket that enveloped the rounded post-larva and looked like a pyramid (Fig. 4A,B). Metamorphosis was slow, and during the first week post-settlement there was a gradual change in tissue density as the settler became wider and rounder (to about 300 μm in diameter) and the tissue much thinner, with the posterior pole no longer conical but dome-shaped (Stage 2, Fig. 4C). The settler remained much the same, growing slightly and broadening out, until at 6 weeks post-settlement it formed a nearly spherical juvenile sponge, in which new types of spicules including discohexasters were evident in the surface tissues, and the sponge had clearly visible large flagellated chambers (Fig. 4D). However, neither an osculum nor ostia were evident. Only small sponges found on rocks chipped from the wall of the cave (Fig. 4E), or small individuals that had settled on a polypropylene rope that hung in the cave as a guide for divers, had oscula (Fig. 4F). It is assumed these sponges were older than 2 months.

At the final stage of metamorphosis, most larval tissues had disappeared or were reorganized, and only the yolk and lipid inclusions and the flagellated chambers with choanoblasts were carried through to the end of metamorphosis. The spicules in the young 6-week-old juvenile sponge were pentactins and discohexasters, which are adult spicules. There was no evidence of discarded stauractins.

Transitions of the flagellated chambers and choanoblasts

All stages of metamorphosing settlers had flagellated chambers and it did not appear that they were

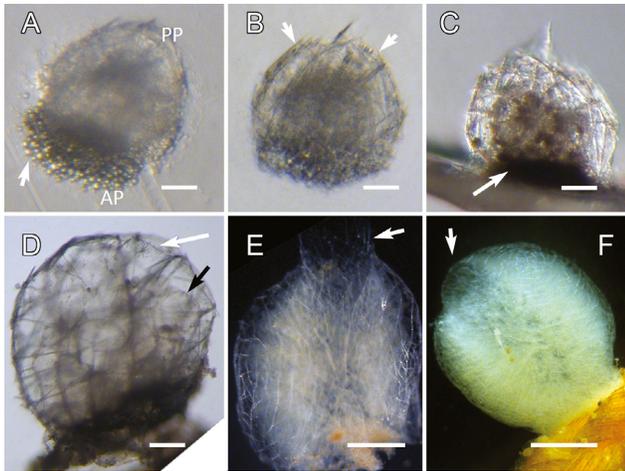


Fig. 4. Stages of metamorphosis and growth of the young glass sponge *Oopsacas minuta*. **A.** The first stage of attachment looks like a small pyramid extending up from the birefringent lipid granules (arrow). Former anterior swimming pole (AP) and posterior swimming pole (PP) are marked and are the same orientation for all images in this figure. **B.** Slightly later stage of metamorphosis during which the settler begins to broaden apically at the former larval posterior swimming pole (arrows). (A,B viewed through a plastic coverslip floated on the surface of the water.) **C.** Early sphere stage. The plastic of the dish was pried up at the base (arrow) to image the settler from the side. **D.** Sphere stage, pried off a rock chip, shows several flagellated chambers (black arrow) and discohaster spicules (white arrow). **E,F.** Juvenile glass sponges from the cave, both showing oscula (arrows) as a raised lip in E, and as a depression in a slightly older stage in F. Scales: A,B, 20 μm ; C, 50 μm ; D, 200 μm ; E, 500 μm ; F, 0.5 cm.

lost and reformed at any stage during metamorphosis (Fig. 5A–C). Instead, as metamorphosis progressed the flagellated chambers grew in size, doubling in diameter from approximately 45 μm in the larva, to 100 μm in the pyramid stage (Fig. 5A), and to 170 μm by the sphere stage (Fig. 5C).

In the newly settled larva, the choanoblasts formed most of the chamber surface and several were aggregated to one or the other side with collar bodies lying above them, whereas in early settlers, the choanoblasts were more dispersed across the chamber surface (Figs. 5A,C, 6A–I). The three-dimensional reconstructions showed that in early settlers choanoblasts were right against the flagellated chambers (Fig. 5A' arrow), but in 6-week-old sponges, choanoblasts had moved further from the chambers (Fig. 5C' arrow). The chambers presumably enlarged by expansion of the trabecular reticulum which extended out around the chamber with yolk inclusions. The collar bodies also became more distant from the choanoblasts so that the stolons connecting

the two expanded (Fig. 6). TEM images of new settlers show many choanoblasts with large plugged junctions, but no mitotic spindles were found (Fig. 6A–C). At this stage, choanoblasts often had more than one flagellum arising from cytoplasmic extensions. These observations suggest that collar bodies were being formed and plugs produced to separate the collar bodies from choanoblasts. In slightly later stage settlers, syncytial tissues containing yolk inclusions surrounded the choanoblasts and small chambers (Fig. 6D–F). By 6 weeks post-settlement, flagellated chambers had enlarged, much less yolk was present, and groups of choanoblasts were farther apart around the chambers (Fig. 6G–I).

Changes in amount and location of yolk and lipid

Throughout metamorphosis yolk inclusions appeared to be used up more quickly than the lipid inclusions. In the larva, densely packed lipid and yolk inclusions created a compact filling at both ends (Fig. 3D), but neither overlapped with the flagellated chambers (Fig. 3A). When the larva had settled, the lipid inclusions became spread across the flattened tissues at the base (Fig. 5A,A''), and the yolk inclusions were present throughout the central region of the larva. In the newly settled post-larva yolk inclusions occupied the same region as the flagellated chambers and were found adjacent to the choanoblasts (Figs. 5B,B'', 6D–F). At six weeks post-settlement, there were far fewer yolk inclusions, while lipid inclusions were now distributed flatly across the base of the juvenile sponge (Fig. 5C,C'').

Fate of the multiciliated cells

Even in the earliest settling larvae, the multiciliated cells were difficult to find. No cell in the post-larva had the same features as the larval multiciliated cells, and it was clear in several newly settling larvae at the pyramid stage that at least some, if not all, of the multiciliated cells were discarded (Fig. 7A–C). In these settlers, the former multiciliated cells were condensed into a small region at the basal edge, and sections showed cells with unusual shapes, some only attached by a thread of syncytial tissue to the settler (Fig. 7B,D). The cells and cilia were disorganized, but the large numbers of mitochondria as well as the many vesicles they contained suggested they were multiciliated cells from the larva (Fig. 7E). In several pyramid-stage settlers, these looked detached from the rest of the larva; where they remained attached they were not near flagellated chambers, but instead mixed with the lipid-

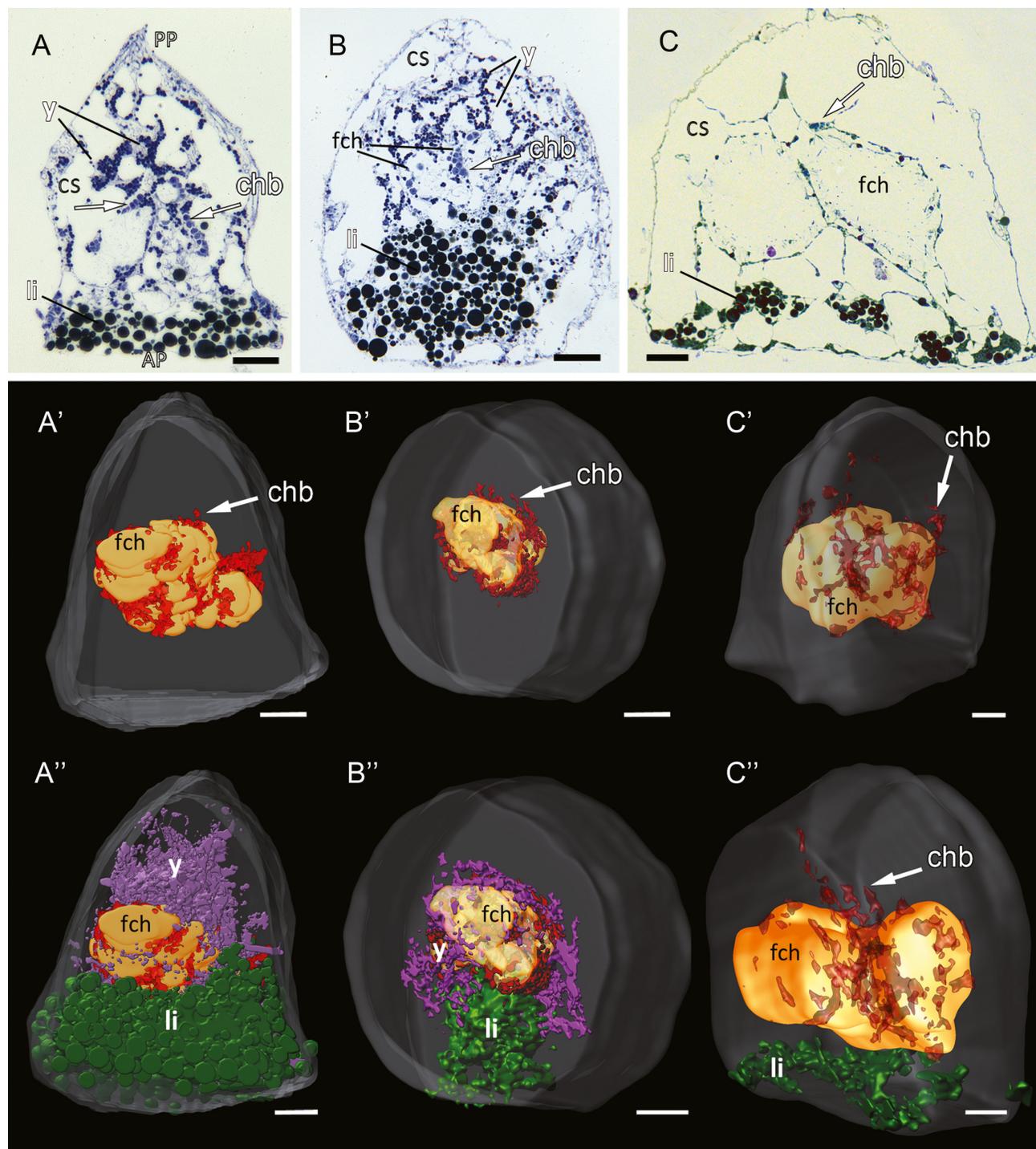


Fig. 5. Fate of the flagellated chambers, yolk, and lipid in settlers of *Oopsacas minuta*. **A.** Pyramid (newly settled larva). **B.** Early sphere (~2 weeks post-settlement). **C.** Late sphere (~6 weeks post-settlement). Epoxy sections (A–C), 3-D reconstructions (A'A''–C'C''); all images show the same orientation. Color scheme: Flagellated chambers (pale orange), choanoblasts (rust), lipid (green) and yolk (purple). Choanoblasts are associated with chambers in early stages (A,A', B,B' arrows), and by late stages (C,C') some are now located away from the chambers. Yolk is associated with the chambers in the pyramid stage (A,A'') and early sphere (B,B'') but is absent in the late sphere (C,C''). Lipid is basal in all stages, but there are fewer lipid granules in the later stage sphere compared to the pyramid stage (A–C, A''–C''). AP, former anterior swimming pole of the larva; chb, choanoblast; cs, canal space; fch, flagellated chamber; li, lipid; PP, former posterior swimming pole of the larva; y, yolk. All scales: 50 μ m.

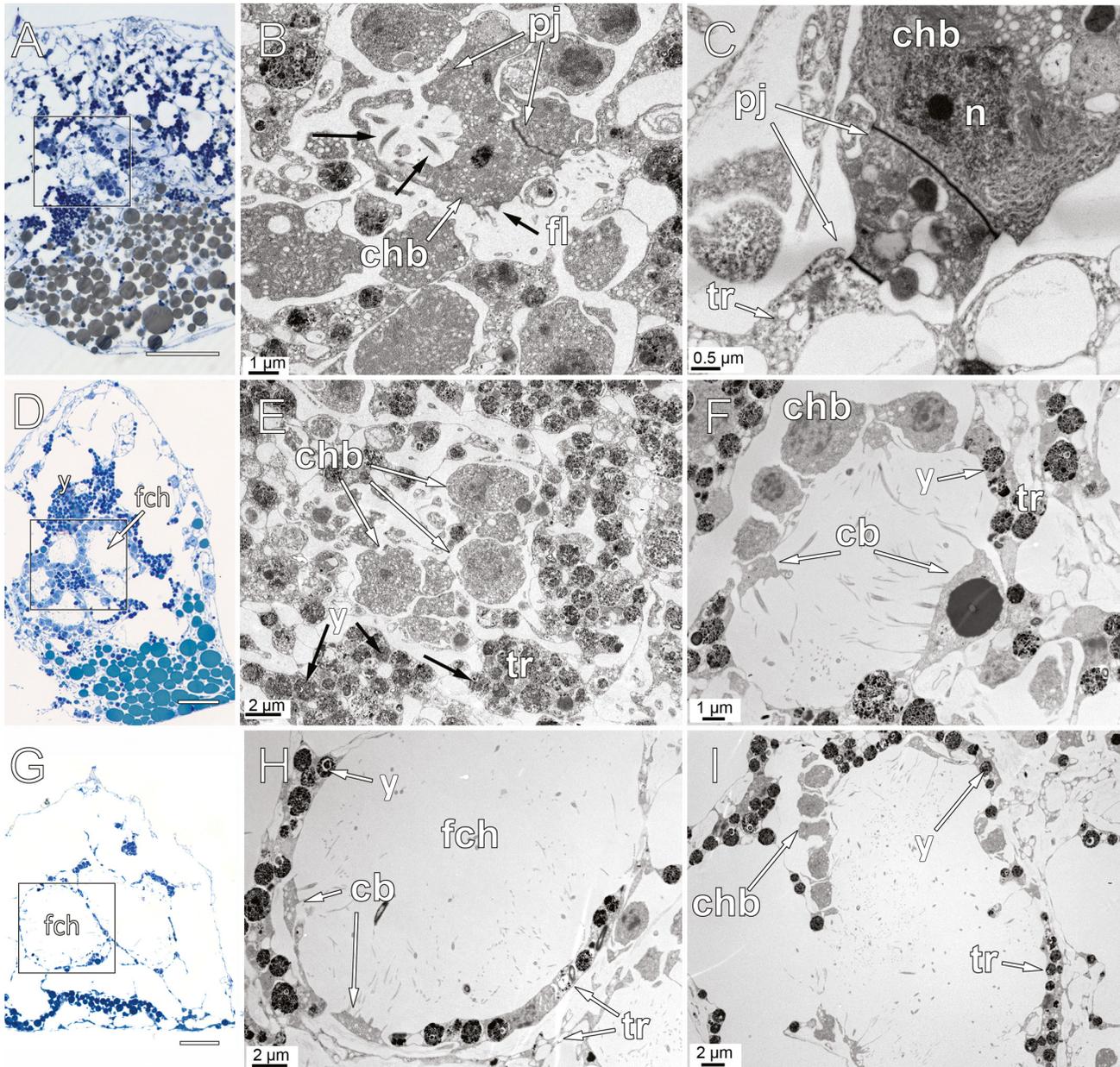


Fig. 6. Fate of the flagellated chambers. **A–C.** Late larva/early settler. **D–F.** Pyramid stage. **G–I.** Sphere stage. Panel **B** shows a choanoblast with a nucleus and a large plugged junction. From the choanoblast arise several flagella (black arrows) suggesting these may become collar bodies. Panel **C** shows a choanoblast with a nucleus adjacent to a flagellated chamber, in which two large plugged junctions separate regions of distinct cytoplasm and connect it to the trabecular reticulum. In **E**, yolk-filled (black arrows) trabecular tissue is aggregated around the nascent flagellated chamber with choanoblasts. Panel **F** is a section showing three choanoblasts connected to collar bodies as well as the yolk-filled syncytial tissue of the trabecular reticulum. **G–I.** Views of the flagellated chambers in the sphere stage when the trabecular reticulum, still with some yolk inclusions, is thinner and supports the collar bodies. Choanoblasts are only evident at one side of the chamber. Light (**A,D,G**) and transmission electron micrographs (TEM) (**B,C,E,F,H,I**). Boxes in **A,D,G** show regions illustrated by TEM in the panels to their right. cb, collar bodies; chb, choanoblast; fl, flagella; n, nucleus; pj, plugged junction; tr, trabecular reticulum; y, yolk. Scales: **A,D,G**, 50 μm ; **B,F**, 1 μm ; **E,H,I**, 2 μm ; **C**, 0.5 μm .

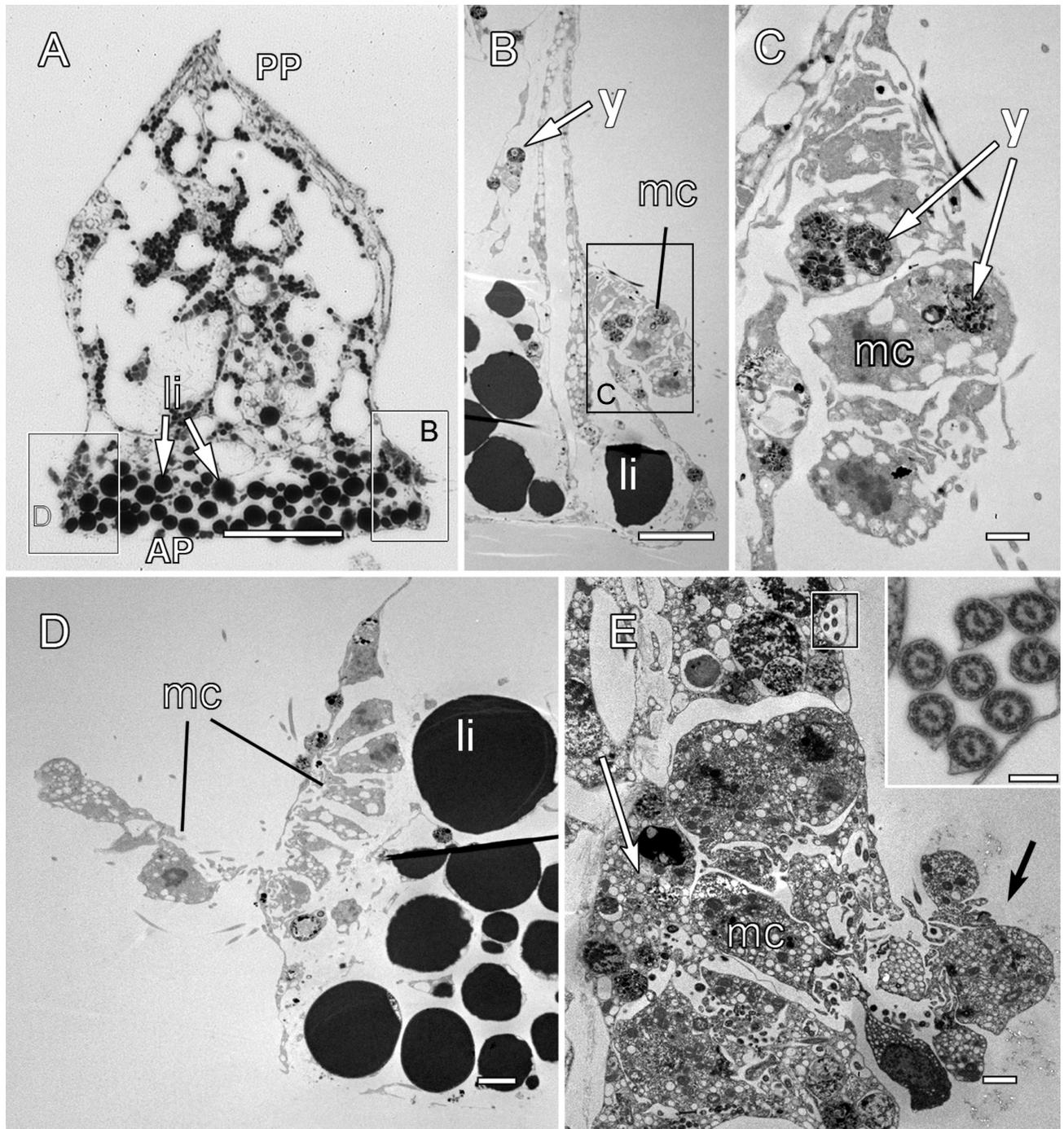


Fig. 7. Fate of the multiciliated cells of *Oopsacas minuta* at metamorphosis. **A.** Longitudinal section of a settler at the pyramid stage. **B.** Enlarged box from **A.** Former multiciliated cells are in a bundle at the lower edge of the settled larva. Many have unusual shapes and some contain yolk inclusions. **C.** An enlargement of the boxed region shown in **B.** **D.** Enlarged from **A.** Multiciliated cells at the lower left of the larva shown in **A.** are detached from the settler. **E.** Multiciliated cells from another settler showing cilia (box and inset) as well as some cells detached (black arrow) and others possibly in the process of being extruded or alternatively in the process of being internalized (white arrow). Orientation is the same for all images. AP, former larval anterior swimming pole; li, lipid; mc, multiciliated cells; PP, former larval posterior swimming pole; y, yolk. Scales: A, 20 μm ; B, 5 μm ; C, 1 μm ; D, 2 μm ; E, 1 μm ; E inset, 200 nm.

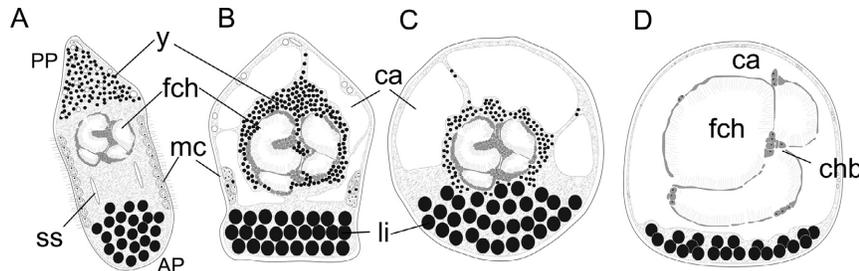


Fig. 8. Cartoons illustrating the stages of settlement and metamorphosis in *Oopsacas minuta*. **A.** Larva. **B.** Pyramid (newly settled larva). **C.** Early sphere (2-week-old settler). **D.** Late sphere (6-week-old sponge); attachment is on the anterior pole, so orientation is the same for all images. Changes involve the appearance of canal areas (B), thinning of the trabecular tissue (B,C), association of yolk with growing flagellated chambers (B,C), and enlargement of the chambers (D). AP, anterior swimming pole; ca, canal spaces; chb, choanoblast; fch, flagellated chamber; li, lipid; mc, multiciliated cells; PP, posterior swimming pole; ss, spicule spaces; y, yolk.

dense region. Some sections of newly settled larvae showed cells with groups of cilia at the edge of the settler, but it was not possible to determine whether these were being internalized by the tissues since they were still intact (Fig. 7E inset). The above processes are summarized in Fig. 8A–D.

Discussion

Glass sponges are an important group in which to seek clues as to the evolution of developmental processes because sponges are one of the oldest metazoan lineages (Dohrmann et al. 2008; Philippe et al. 2009; Pick et al. 2010; Nosenko et al. 2013) and have the oldest validated fossil record (Antcliff et al. 2014).

Glass sponges are also unusual in being syncytial. The embryos are cellular like other sponges, but after the 64-cell stage macromeres (large cells) fuse during early development and as they do so envelop smaller cells, which then form rudimentary flagellated chambers and multiciliated cells of the larva (Leys et al. 2006). Our study shows that at metamorphosis those chambers remain to become the first feeding chambers of the adult sponge. Therefore, the processes that occur during embryogenesis are those which establish cells in their positions to form the adult tissues, including the feeding epithelium.

Development of the flagellated chambers

Flagellated chambers of the larva enlarged in the settled sponge and it appeared that this happened in two ways. First the yolk-filled syncytial tissues from the larva aggregated around the chambers and strands of the syncytium containing the yolk started to form the primary and secondary reticula (the two

strands of the trabecular reticulum that support the flagellated chambers in adult glass sponges). In later stages (6 weeks after settlement), the chamber walls were much thinner and resembled those of the adult sponge. Therefore, it seems likely that the larval yolk was used to build and extend the chamber walls. Second, although we saw cells undergoing mitosis in larvae, no mitotic spindle was found in any choanoblast of the settling larvae or young sponges. It did not appear that choanoblasts divide to form the larger chambers. Instead they became spread out across the now much larger chambers. It appeared that there was extensive protein production in choanoblasts, production of very large plugged junctions, and differentiation of cytoplasm from archaeocytes or choanoblasts (cells adjacent to chambers which could not be distinguished as one or the other at that stage). It seems that these cells were in the process of producing more collar bodies, and so the walls of the flagellated chambers became populated by branches of the choanoblasts (the collar bodies) rather than by more choanoblasts.

Only one other study examined chamber formation in glass sponges. Okada (1928) thought flagellated chambers in *Farrea sollasii* SCHULZE 1886 developed from ameboid cells that had wandered into the center of the embryo. Although Okada considered the whole sponge embryo cellular, his drawings suggest syncytial strands exist and his description of a “hollow chamber” lined by “small spherical cells” is very similar to our observations of *Oopsacas minuta* at approximately the same stage. While Okada (1928) thought collars did not arise until the larva was released, he still called the cells choanocytes. In *O. minuta*, the larva already had collar-flagella units attached by stolons to the choanoblasts (“branched choanocytes” from Leys et al. 2007), but none were functional since an osculum

does not seem to open until many weeks after settlement.

Fate of the multiciliated cells

Our sections and reconstructions suggest that in *O. minuta* the multiciliated cells are discarded at metamorphosis. Wielsputz & Saller (1990) reported that larval ciliated cells of the freshwater sponge *Ephydatia fluviatilis* (LINNAEUS 1759) were discarded, and those larvae also already had flagellated chambers. We have found that ciliated cells can also be discarded from larvae of *Spongilla lacustris* (LINNAEUS 1759) (Windsor 2014). However we also found that in the same larva other ciliated cells are phagocytosed by cells directly below them in the larval epithelium, and it is unclear whether these later can become choanocytes and form chambers. In other demosponges, it is quite clear that some larval ciliated cells transdifferentiate into choanocytes, while others become ameoboid cells (Leys & Degnan 2002; Nakanishi et al. 2014), and so not all ciliated cells share the same fate. In *O. minuta*, although reuse of some of the multiciliated cells in the juvenile cannot be ruled out because our sections only show us a static view, transdifferentiation does not appear to be the typical case. Considering we have confirmed that there do not appear to be any ameoboid or migratory cells in *O. minuta* larvae and settlers, we suggest that it is unlikely the multiciliated cells give rise to new chambers.

How effective are methods of tracing cell identity?

Thin sections showed that the multiciliated cells lost their cuboidal morphology just prior to larval settlement. Those cells quickly disappeared after settlement, apparently being sloughed off, but it would have been better if that could be confirmed by using other methods. Finding adequate tracers to follow sponge cells is difficult because bath application of dye will not solely label the ciliated cells. For example, demosponge larvae are not typically ciliated around their entire circumference. Some cells protrude between the ciliated cells, others form a distinct anterior region, and yet others at the posterior pole are non-ciliated cells. Therefore, incubation of whole demosponge larvae in fluorescent markers will label anterior, posterior, and mucous cells, in addition to the larval ciliated cells in the outer epithelium. Injection of markers is possible, but given the very small area of each cell that is exposed at the larval surface, usually several cells are marked at once, and so again it is not possible to be precise

about marking specific cells and not others. So in the end, the fate of a suite of differentiated cells is followed without being able to distinguish between them or know their precise origin, and this is the case for previous studies that have used fluorescent cell tracers on larvae (e.g., Leys & Degnan 2002; Nakanishi et al. 2014). In some sponges markers may also be transferred to other cells via phagocytosis of the larval ciliated cells at metamorphosis (reviewed in Mukhina et al. 2006).

Finding a more reliable marker to trace cell fate in sponges is challenging because of the small cell size. Usually markers are inserted into blastomeres at an early stage in development by injection, but few sponges produce embryos that develop independently of the adult and none so far has been found with embryos that can be studied *in vitro* to use injectable cell trackers. However, for the glass sponge, the syncytial tissues of the larva will continue to be a problem even for those tracers since they will distribute largely around the syncytial tissues as they were shown to do in dissociated and reaggregated tissues *in vitro* (Leys 1995). Reconstructing the tissues from tracings of sections is an alternative approach that gives more depth of view for the thin tendrils of tissues found in the glass sponge. For future studies on *O. minuta*, it may be possible to improve on this method by taking advantage of the different refraction properties of the tissues using laser light sheet imaging of live larvae during metamorphosis.

Implications for gastrulation in sponges

How complex cellular events such as gastrulation came about during the evolution of early animals is intriguing. A range of morphogenetic movements exhibited by sponge cells during embryogenesis has been argued to constitute gastrulation-like events (reviewed in Leys 2004), but the topic is contentious largely because of the unclear fate of larval cells at metamorphosis (Ereskovsky & Dondua 2006; Dondua & Kostyuchenko 2013).

At the heart of the matter is whether sponge cells have a restricted developmental fate or not. Haeckel first described gastrulation in calcareous sponges (Haeckel 1872), suggesting that the formation of the opening of the osculum was the equivalent of the blastopore of other animals, essentially homologizing germ layers across animals. Work by Delage (1892) showed the story was not so simple, and that in forming the osculum/opening, the outer ciliated epithelial cells of the larva were internalized to become the feeding cells of the juvenile sponge; thus,

he called sponges “inside out animals,” or *Enantiozoa* (reviewed in Leys 2004). Over the last century studies have shown that what happens at metamorphosis is even more complicated, with some cell types retaining their embryological fate and others redifferentiating to take on a new cell fate (e.g., Meewis 1941; Borojevic 1970; Amano & Hori 1993, 1996; Fell 1997; Leys & Degnan 2002; Nakanishi et al. 2014). Thus, it seems that for many sponges cell fate is or can be labile.

Nonetheless, our findings show this is not the case for the glass sponge *O. minuta*. Here, because cells are not mobile, they do not dedifferentiate or transdifferentiate, and do not migrate to take on a new fate at metamorphosis. The cell fate is determined already in the embryo, and because the cells that will form the future feeding epithelium are internalized during early development, there appears no good reason not to equate these movements with gastrulation by epiboly, as suggested by Boury-Esnault et al. (1999).

Glass sponges are not the only group in which cell fate is determined early during embryogenesis. For example, *Tetilla japonica* LAMBE 1886 undergoes direct development, with the fertilized egg attaching to the substrate so that cleavage produces the tissues of the adult sponge (Watanabe 1978). But few direct developers are known, and most sponges do not form chambers in the larva. The freshwater sponges are the only group other than glass sponges in which all larvae have chambers. The larval chambers are used in the adult sponge, but new chambers can also form by transdifferentiation, or from totipotent stem cells called archaeocytes. In other species that do form what appear to be chambers in the larva (e.g., *Halisarca dujardini* JOHNSTON 1842 [Gonobobleva & Ereskovsky 2004] or *Halichondria melanodocia* DE LAUBENFELS 1956 [Woollacott 1990]), it is unclear whether these are used in the adult.

One might think that forming chambers during the larval stage might be an effective way of ensuring the ability to feed soon after metamorphosis. However, in *O. minuta* we found neither an osculum nor any ostia within 6 weeks of settlement, a period in which substantial morphogenetic events occurred, including formation of canal spaces and enlargement of the chambers. Therefore, having chambers already formed does not necessarily lead to readiness to filter soon after metamorphosis. By contrast, juveniles of demosponges and calcareous sponges that do not have flagellated chambers in the larval stage are able to form a fully functional aquiferous system within 1–3 d of settlement, if not sooner (Lévi 1956; Leys & Degnan 2002; Eerkes-Medrano

& Leys 2006); thus, a lack of chambers in the larva does not hinder feeding soon after settlement. Unfortunately sponge phylogeny does not shed light on the direction of evolutionary change for time of onset for chamber formation because we lack sufficient morphological data about the larvae of many sponge groups. However, as more data are gathered over time, this would be an interesting focus for future work.

Acknowledgments. We thank Jean Vacelet, Claude Jalong, and Nelson Lauzon for diving assistance and fruitful discussions on this topic, the director and staff at the Station Marine d’Endoume, Marseille, for use of facilities, and members of the Leys Lab group for feedback on a draft of this manuscript. The research was funded by an NSERC Discovery Grant to S.P.L. The authors declare no conflict of interest with respect to the work carried out and reported here.

References

- Amano S & Hori I 1993. Metamorphosis of calcareous sponges II. Cell rearrangement and differentiation in metamorphosis. *Invertebr. Reprod. Dev.* 24: 13–26.
- 1996. Transdifferentiation of larval flagellated cells to choanocytes in the metamorphosis of the demosponge *Haliclona permollis*. *Biol. Bull.* 190: 161–172.
- 2001. Metamorphosis of coeloblastula performed by multipotential larval flagellated cells in the calcareous sponge *Leucosolenia laxa*. *Biol. Bull.* 200: 20–32.
- Anderson CA, Eser U, Korndorf T, Borsuk ME, Skotheim JM, & Gladfelter AS 2013. Nuclear repulsion enables division autonomy in a single cytoplasm. *Curr. Biol.* 23: 1999–2010.
- Antcliff JB, Callow RHT, & Brasier MD 2014. Giving the early fossil record of sponges a squeeze. *Biol. Rev.* 89: 972–1004.
- Bakran-Petricioli T, Vacelet J, Zibrowius H, Petricioli D, Chevalloné P, & Rada T 2007. New data on the distribution of the ‘deep-sea’ sponges *Asbestopluma hypogea* and *Oopsacas minuta* in the Mediterranean Sea. *Mar. Ecol.* 28: 10–23.
- Blumer MJF, Gahleitner P, Narzt T, Handl C, & Ruthensteiner B 2002. Ribbons of semithin sections: an advanced method with a new type of diamond knife. *J. Neurosci. Methods* 120: 11–16.
- Borojevic R 1970. Différenciation cellulaire dans l’embryogenèse et la morphogenèse chez les spongiaires. *Zool. Soc. Lond.* 25: 467–490.
- Boury-Esnault N 1976. Ultrastructure de la larve parenchymella d’*Hamigera hamigera* (Schmidt) (Demosponge, Poecilosclerida). *Origine des cellules grises. Cah. Biol. Mar.* 17: 9–20.
- Boury-Esnault N & Vacelet J 1994. Preliminary studies on the organization and development of a hexactinellid

- sponge from a Mediterranean cave, *Oopsacas minuta*. In: Sponges in Time and Space. Proceedings of the Fourth International Porifera Congress. van Soest RWM, van Kempen TMG, & Braekman J, eds. pp. 407–416. AA Balkema, Rotterdam.
- Boury-Esnault N, Efremova S, Bézac C, & Vacelet J 1999. Reproduction of a hexactinellid sponge: first description of gastrulation by cellular delamination in the Porifera. *Invertebr. Reprod. Dev.* 35: 187–201.
- Delage Y 1892. Embryogénie des éponges. Développement post-larvaire. *Arch. Zool. Exp. Gén.* 10: 345–498.
- Dohrmann M, Janussen D, Reitner J, Collins AG, & Wörheide G 2008. Phylogeny and evolution of glass sponges (Porifera, Hexactinellida). *Syst. Biol.* 57: 388–405.
- Dondua AK & Kostyuchenko RP 2013. Concerning one obsolete tradition: does gastrulation in sponges exist? *Russ. J. Dev. Biol.* 44: 267–272.
- Eerkes-Medrano D & Leys SP 2006. Ultrastructure and embryonic development of a syconoid calcareous sponge. *Invertebr. Biol.* 125: 177–194.
- Ereskovsky AV & Dondua AK 2006. The problem of germ layers in sponges (Porifera) and some issues concerning early metazoan evolution. *Zool. Anz.* 245: 65–76.
- Fell PE 1997. Porifera, the Sponges. In: *Embryology: Constructing the Organism*. Gilbert SF & Raunio AM, eds., pp. 39–54. Sinauer, Sunderland, MA.
- Fiala JC 2005. Reconstruct: a free editor for serial section microscopy. *J. Microsc.* 218(1): 52–61.
- Gonobobleva EL & Ereskovsky AV 2004. Metamorphosis of the larva of *Halisarca dujardini* (Demospongiae, Halisarcida). *Bull. Inst. R. Sci. Nat. Belgique Biol.* 74: 101–115.
- Haeckel E 1872. Die Kalkschwämme. Eine Monographie Verlag von Georg Reimer, Berlin. 484 pp.
- Ijima I 1904. Studies on the Hexactinellida. Contribution IV. (Rossellidae). *J. Coll. Sci. Imp. Univ. Tokyo* 28: 13–307.
- Lévi C 1956. Étude des *Halisarca* de Roscoff. Embryologie et systématique des démosponges. *Arch. Zool. Exp. Gén.* 93: 1–184.
- Leys SP 1995. Cytoskeletal architecture and organelle transport in giant syncytia formed by fusion of hexactinellid sponge tissues. *Biol. Bull.* 188: 241–254.
- 1996. Cytoskeletal architecture, organelle transport, and impulse conduction in hexactinellid sponge syncytia. Doctoral dissertation, University of Victoria, Victoria. 234 pp.
- 2003a. Comparative study of spiculogenesis in demosponge and hexactinellid larvae. *Microsc. Res. Tech.* 62: 300–311.
- 2003b. The significance of syncytial tissues for the position of the Hexactinellida in the Metazoa. *Integr. Comp. Biol.* 43: 19–27.
- 2004. Gastrulation in sponges. In: *Gastrulation: From Cells to Embryo*. Stern C, ed., pp. 23–31. Cold Spring Harbor Press, New York.
- Leys SP & Degnan BM 2002. Embryogenesis and metamorphosis in a haplosclerid demosponge: gastrulation and transdifferentiation of larval ciliated cells to choanocytes. *Invertebr. Biol.* 121: 171–189.
- Leys SP, Cheung E, & Boury-Esnault N 2006. Embryogenesis in the glass sponge *Oopsacas minuta*: formation of syncytia by fusion of blastomeres. *Integr. Comp. Biol.* 46: 104–117.
- Leys SP, Mackie GO, & Reiswig H 2007. The biology of glass sponges. *Adv. Mar. Biol.* 52: 1–145.
- Mackie GO 1981. Plugged syncytial interconnections in hexactinellid sponges. *J. Cell Biol.* 91: 103a.
- 1995. Defensive strategies in planktonic coelenterates. *Mar. Freshw. Behav. Physiol.* 26: 119–129.
- Mackie GO & Singla CL 1983. Studies on hexactinellid sponges. I Histology of *Rhabdocalypptus dawsoni* (Lambe, 1873). *Philos. Trans. R. Soc. Lond., Ser. B* 301: 365–400.
- Mackie GO, Paul DH, Singla CM, Sleigh MA, & Williams DE 1974. Branchial innervation and ciliary control in the ascidian *Corella*. *Proc. R. Soc. Lond. B Biol.* 187: 1–35.
- Meewis H 1941. Contribution à l'étude de l'embryogénèse des éponges siliceuses. *Ann. Soc. R. Zool. Belg.* 72: 126–149.
- Mukhina Y, Kumeiko V, Podgornaya O, & Efremova S 2006. The fate of larval flagellated cells during metamorphosis of the sponge *Halisarca dujardini*. *Int. J. Dev. Biol.* 50: 533–41.
- Nakanishi N, Sogabe S, & Degnan BM 2014. Evolutionary origin of gastrulation: insights from sponge development. *BMC Biol.* 12: 1–9.
- Nosenko T, Schreiber F, Adamska M, Adamski M, Eitel M, Hammel J, Maldonado M, Müller WEG, Nickel M, Schierwater B, Vacelet J, Wiens M, & Wörheide G 2013. Deep metazoan phylogeny: when different genes tell different stories. *Mol. Phylogenet. Evol.* 67: 223–233.
- Okada Y 1928. On the development of a hexactinellid sponge, *Farrea sollasii*. *J. Fac. Sci. Imp. Univ. Tokyo* 4: 1–29.
- Philippe H, Derelle R, Lopez P, Pick K, Borchiellini C, Boury-Esnault N, Vacelet J, Renard E, Houliston E, Queinnec E, Da Silva C, Wincker P, Le Guyader H, Leys S, Jackson D, Schreiber F, Erpenbeck D, Morgenstern B, Wörheide G, & Manuel M 2009. Phylogenomics revives traditional views on deep animal relationships. *Curr. Biol.* 19: 706–12.
- Pick KS, Philippe H, Schreiber F, Erpenbeck D, Jackson DJ, Wrede P, Wiens M, Alié A, Morgenstern B, Manuel M, & Wörheide G 2010. Improved phylogenomic taxon sampling noticeably affects nonbilaterian relationships. *Mol. Biol. Evol.* 27: 1983–1987.
- Reiswig HM 1979. Histology of Hexactinellida (Porifera). *Coll. Intern. CNRS*. (Lévi C & Boury-Esnault N, eds.) 291:173–180.
- Vacelet J, Boury-Esnault N, & Harmelin J 1994. Hexactinellid Cave, a unique deep-sea habitat in the scuba zone. *Deep Sea Res. Part 2(41)*: 965–973.

- Watanabe Y 1978. The development of two species of *Tetilla* (Demosponge). Nat. Sci. Rep., Ochanomizu Univ. 29: 71–106.
- Wielsputz C & Saller U 1990. The metamorphosis of the parenchymula-larva of *Ephydatia fluviatilis* (Porifera, Spongillidae). Zoomorphology 109: 173–177.
- Windsor PJ 2014. Evolution of the sponge body plan: Wnt and the development of polarity in freshwater sponges. Doctoral dissertation, University of Alberta, Edmonton.
- Woollacott RM 1990. Structure and swimming behavior of the larva of *Halichondria melanadocia* (Porifera: Demospongiae). J. Morphol. 205: 135–145.

Supporting information

Additional Supporting information may be found in the online version of this article:

Video S1. Three-dimensional reconstruction of the larva of *Oopsacas minuta*. Spicules are shown first, then lipid in green at the larval anterior pole. Flagellated chambers (orange) and choanoblasts (rust) are shown in the center, followed by yolk in purple at the larval posterior pole. The last layer to be added shows the larval multiciliated cells in gray.