Spicule and flagellated chamber formation in a growth zone of *Aphrocallistes vastus* (Porifera, Hexactinellida)

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**Abstract.** Three species of glass sponges (Class Hexactinellida) form massive deep-water reefs by growing on the skeletons of past generations, with new growth largely vertical and away from sediment that buries the lower portions. Growth is therefore essential for reef health, but how glass sponges produce new skeleton or tissue is not known. We used fluorescence, light, and electron microscopy to study skeletal and tissue growth in the reef-forming glass sponge *Aphrocallistes vastus*. The sponge consists of a single large tube (the osculum), usually with several side branches, each of which can function as an effective excurrent vent. New tissue forms at the tips of each of these extensions, but how this occurs in a syncytial animal, and how the tubes expand laterally as the sponge gets larger, are both unknown. The fluorescent dye PDMPO labeled more spicule types in the tips of the sponge than elsewhere, indicating growth that was concentrated at the edge of the osculum. New tissue production was tracked using the thymidine analog EdU. EdU-labeled nuclei were found predominantly at the edge or lip of the osculum. In that region new flagellated chambers were formed from clusters of choanoblasts that spread out around the enlarging chamber. In cellular sponges clusters of choanocytes form flagellated chambers through several rounds of mitotic divisions, and also by immigration of mesohyl cells, to expand the chamber to full size. By contrast, chambers in glass sponges expand as choanoblasts produce enucleate collar bodies to fill them out. Growing chambers with enucleate structures may be an adaptation to life in the deep sea if chambers with cells, and therefore more nuclei, are costly to build.

**Additional key words:** Porifera, Hexactinellida, spicules, syncytia, EdU

Glass sponges (Phylum Porifera, Class Hexactinellida) are found throughout the deep sea where they serve important ecosystem functions as habitat for other animals and as a link between the water column and the seafloor through their filter feeding activity (Beaulieu 2001; McClintock et al. 2005; Kahn et al. 2015). Some of the greatest densities of glass sponges are found in the northeastern Pacific (Kahn et al. 2015), where three species—*Farrea occa* BOWERBANK 1862, *Heterochone calyx* (SCHULZE 1886), and *Aphrocallistes vastus* SCHULZE 1886—form reefs by new recruits settling and growing on the skeletons of previous generations (Fig. 1A) (Conway et al. 2001). In the northeastern Pacific, glass sponges colonized glacially carved ridges after the last glacial maximum, and reefs have grown almost 20 m vertically in the 9000 years since. Despite this impressively long time forming, only 1–2 m of glass sponge skeleton—both living and dead—projects out of the sediments (Krautter et al. 2001). Growing away from the sediments can be seen as a race against burial by sediment; in addition, growing upwards might allow reef sponges to reach stronger ambient currents and presumably would enhance feeding, as is known to be important for corals (Genin et al. 1986). How glass sponges grow is not known. Glass sponges are syncytial, and so exactly how they form new tissue and the skeletal scaffolding upon which it lies with multinucleate tissues and a shared cytoplasm is puzzling.

The tissue of glass sponges has been well described (Mackie & Singla 1983; Reiswig & Mehl 1991; Leys 1995, 1999; reviewed in Leys et al. 2007). It is almost entirely formed by a continuous, multinucleate syncytium called the trabecular reticulum, which covers a scaffolding of siliceous spicules that can be either fused together into a rigid framework or lie individually (Leys et al. 2007). The trabecular reticulum includes the dermal and atrial surfaces

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and runs continuously through the choanosome. At the flagellated chambers, branches of the trabecular reticulum called the primary and secondary reticula support the collar-flagella pump units (Fig. 1B–D) (Mackie & Singla 1983; Reiswig & Mehl 1991; Leys 1999). There is a very thin collagenous mesohyl and no mobile cells. Instead, transport of materials for feeding, growth, and repair occurs via cytoplasmic streaming along microtubule tracts through the trabecular reticulum (Leys 1995). Dense transport strands have previously been called “cord syncytia” (Reiswig & Mehl 1991). The sponge is a mixture of these multinucleated tissues and regions that function as cells but are tethered to the rest of the tissue by cytoplasmic bridges (Mackie 1981; Mackie & Singla 1983). At the flagellated chambers, groups of uninucleated cells called choanoblasts give rise via cytoplasmic extensions to collar–flagella units; each unit has a beating flagellum surrounded by a ring of microvilli, but these arise from an enucleate base (Mackie & Singla 1983; Leys 1999).

Spicules are conspicuous and well-studied structures in sponges. In glass sponges, spicules are secreted intrasyncytially but not in the trabecular reticulum. It was at first believed that distinctly separated cells called scleroblasts were needed because of the particular microenvironment needed for silica deposition (Mackie & Singla 1983), but it is now known that spicule-secreting regions are multinucleate, may be very large and, as with all the sponge’s tissues, are connected to all other tissues by cytoplasmic bridges (Leys 2003). Studies of

**Fig. 1.** Anatomy of mature chambers of *Aphrocallistes vastus*. A. The fused spicule skeleton of a dead sponge (brown) persists after the live tissue (yellow) has died. B. The trabecular syncytium makes up most of the tissue of the sponge, including the atrial and dermal surfaces. Dermal spicules project from the dermal membrane down to a large vestibule. Beneath the vestibule lie flagellated chambers. White boxes with black lettering outline regions that are magnified in C and D. C. Chambers are lined with two membranes: a primary reticulum perforated with prosopyles through which water enters the chamber, and a secondary reticulum. Enucleate collar bodies pump water through the flagellated chambers and canals. Collar bodies lie embedded in the primary reticulum with collars of microvilli projecting through holes in the secondary reticulum. D. Collar bodies are produced by mononucleate choanoblasts. Scale bars: A=10 cm, B=100 μm, C=10 μm, D=10 μm. 1R, primary reticulum; 2R, secondary reticulum; c, flagellated chamber; cb, collar body; chb, choanoblast; d, dermal surface; p, prosopyle; ts, trabecular syncytium; v, vestibule.
freshwater sponges have suggested that siliceous spicules grow deposited around an organic membrane—the silicalamella—that surrounds the entire spicule (Simpson & Vaccaro 1974). In the glass sponges Caulophacus sp. and Euplectella aspergillosum Owen 1841, it was inferred that spicules form as amorphous silica is deposited around a hydroxylated collagen substrate, and this later fuses into the spicule structure using a silica-calcite composite (Ehrlich et al. 2010, 2011).

To understand where spicules form in a growing sponge, and how new syncytial and cellular regions of tissue are made, we studied regions of new growth in the reef-forming, dictyonine glass sponge Aphrocallistes vastus (Order Hexactinosida, Family Aphrocallistidae). We used a ratiometric dye that labels newly deposited silica as well as fluorescence, scanning, and transmission electron microscopy to understand the morphology and processes involved in growth of new tissue and skeleton.

Methods

Specimen collection

Pieces of Aphrocallistes vastus (10×10 cm) were collected either by SCUBA from Saanich Inlet, British Columbia (40 m depth) or from the Galiano Ridge sponge reef by using the remotely operated vehicle ROPOS with the Canadian Coast Guard Ship Vector. Sponges were maintained in an incubator at 9°C or in flow-through seawater tanks at the Bamfield Marine Sciences Centre, Bamfield, BC.

Spicule growth

Three 2×8 cm strips were cut from the main body up to the osculum lip and incubated in 250 mL of 0.5 μmol L⁻¹ 2-(4-pyridyl)-5-((4-(2-dimethylaminoethylamino)carbamoyl)methoxy)phenyl)oxazole (PDMPO; RatioWorks®) in seawater for 2 d. Seawater was filtered (0.22 μm) to remove diatoms, which could take up the PDMPO. Sponges were fixed in Bouin’s fixative overnight, rinsed twice briefly in seawater and dehydrated to 70% ethanol for long-term storage. Thin strips of sponge tissues were mounted and viewed under epifluorescence on a Zeiss Axioskop2 Plus using a long-pass DAPI filter.

EdU labeling of cells

Tissue pieces 3×5 mm were cut from the main body of the sponge (where the skeleton was fused) and from the soft osculum lip region where the skeleton was not fused. Tissue pieces were allowed to regenerate cut membranes in seawater for 24 h in an incubator (at 9°C), prior to being immersed in 5 mL (1 tissue piece) or 20 mL (2 tissue pieces) of 100 μmol L⁻¹ 5-ethyl-2'-deoxyuridine (EdU) in 0.2-μm filtered seawater for 1–6 d. Seawater and EdU were refreshed daily. Using a pulse-chase protocol, tissue pieces were incubated in EdU for 3 d and transferred to filtered seawater without EdU for 1, 2, or 3 d before fixation. Sponges were fixed in 8% paraformaldehyde and 0.03% glutaraldehyde for up to 24 h. Following fixation, pieces were rinsed twice in phosphate buffered saline (PBS), dehydrated to 70% ethanol, and desilicified in 4% hydrofluoric acid in 70% ethanol for 1–2 d. Desilicified sponge tissue was dehydrated and embedded in paraaffin. Sections 7 μm thick were collected at least 30 μm medial to the cut face of the tissue to avoid regenerative or repairing tissue. To fluorescently tag the EdU, sections were dewaxed and labeled with Alexa Fluor 594-azide using click chemistry following the standard protocol for the Click-iT EdU whole animal imaging kit (Life Technologies). Nuclei in the same sections were labeled with 100 μM Hoechst 33342 (Molecular Probes®) for 30 min, then sections were rinsed three times with PBS and mounted in Mowiol.

Thick sections and electron microscopy

Sponge sections (2×2 cm) were prepared for electron microscopy in a cocktail fixative consisting of 1% OsO₄, 1% glutaraldehyde, and 0.45 mol L⁻¹ sodium acetate buffer with 10% sucrose (Leys 1995), except twice the concentration of osmium was used in the first 30 min to account for any dilution of the first mixture by seawater in the sponge tissues. The fixative was replaced after 30 min and samples were left overnight at 4°C. Specimens were rinsed once briefly in freshwater to remove salts, dehydrated through a graded series to 70% ethanol, and desilicified in 4% hydrofluoric acid in 70% ethanol for 1–2 d. Once desilicified, separate pieces were prepared for scanning and transmission electron microscopy (SEM, TEM).

Samples prepared for SEM were dehydrated to 100% ethanol, fractured in liquid nitrogen, and critical-point dried (Bal-Tec CPD 030). Dried specimens were mounted onto aluminum stubs, sputter-coated with gold (Xenosput XE200) and viewed in a JEOL 6301F field emission scanning electron microscope. Samples prepared for TEM were dehydrated to 100% ethanol and embedded in epoxy resin (TAAB 812, Electron Microscopy Sciences). Thick sections
(1 μm) were cut on a Leica Ultracut T and stained with Richardson’s stain (Richardson et al. 1960). Thin sections (60 nm) were mounted onto copper grids, stained with lead citrate and uranyl acetate, and viewed in a Philips/FEI (Morgagni) transmission electron microscope.

**Results**

**Spicule growth**

A survey of the tissue from all parts of the sponge showed newly forming spicules throughout the body, but different spicule types formed in the fused and unfused regions (Fig. 2A,B). In all cases, when spicules were labeled with PDMPO the whole spicule was fluorescent, not just a tip or segment (Fig. 2C).

The only new spicules labeled in the lower regions of the sponge where spicules were fused into a scaffold were microscleres. By contrast, all spicule types (both microscleres and megascleres) were labeled at the growing edge of the osculum (hereafter called the osculum lip) (Fig. 2D,D’). Furthermore, secondary silica deposition that locked the loose spicules into a rigid framework was also seen between

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**Fig. 2.** Spicule formation in the growing edge of a glass sponge. A. Image of the osculum showing both the opaque region where the spicule skeleton is fused and a newly formed projection where the spicules are not yet fused. A piece was excised from the projection as shown by the dashed line. B. Image of the excised piece showing the soft region where spicules are not fused and the thicker region where spicules are fused. C. Microscleres and megascleres labeled with PDMPO. Clockwise from top left: oxyhexaster (microsclere), pinular hexactin (megasclere), scopule (microsclere). D and D’. The unfused region of the skeleton, showing microscleres and megascleres labeled with the fluorescent dye PDMPO (D: PDMPO label. D’: PDMPO label overlaid onto a brightfield view of all spicules.) E and E’. PDMPO labeling of secondary deposition of silica on the dictyonine skeleton in the transitional area between the fused and unfused skeleton. (E: PDMPO label. E’: PDMPO label overlaid onto a brightfield view of all spicules.) Scale bars: A=5 cm; B=5 mm; C=250 μm; D,D’=1 mm; E,E’=500 μm. f, fused; u, unfused.
the fused and unfused parts of the skeleton (Fig. 2E,E’).

**Growth of flagellated chambers**

Nuclei found scattered throughout the trabecular syncytium never took up the EdU label. Cells adjacent to flagellated chambers did take up EdU, but only after 4 d incubation; whether these were archaeocytes or choanoblasts could not be determined.

More activity was visible in the osculum lip region. There, choanoblasts took up EdU after 24 h, initially labeling as tight clusters of typically five or more (Fig. 3A), but later becoming arranged in single-file rows of four or more (Fig. 4A). Clumps of microvilli were visible near the clusters of choanoblasts with collar bodies already produced but not inserted into a larger chamber structure (Fig. 3B–D). Images captured by all methods (SEM, thick sections, and TEM) showed that once choanoblasts were arranged in single-file rows the collar bodies had also moved apart to form a mature flagellated chamber with regularly spaced collar bodies surrounded by the primary and secondary reticula (Fig. 4E). Naturally, from static views, it could not be determined when the chambers became functional. Chambers were also found that were intermediate between the early clustered choanoblast stage and the more mature single-file choanoblast stage (Fig. 3E,F).

The transition from clusters of collar bodies to the regular spacing observed in mature chambers could not be tracked with EdU, but several observations...
from the thick sections and scanning and transmission electron micrographs suggest different stages. Beginning with the lowest degree of organization, microvilli and flagella of the collar bodies were sometimes found surrounded by tissue that extended up into the chamber (Fig. 5A–C). In other chambers, tissue surrounded each collar body but provided more space around each flagellum (Fig. 5D). In yet other chambers, collar bodies were regularly spaced and embedded in a primary reticulum with tissue near either collar microvilli or a flagellum (Fig. 5E). In these chambers, only the secondary reticulum surrounded the microvilli (Fig. 5F).

**Discussion**

Tissue and spicule production in the glass sponge *Aphrocallistes vastus* both occur at the uppermost edges—the tips of the oscula—of the mature sponge. Spicules are formed throughout the sponge, but megascleres only form and secondarily fuse near the growing tips of the sponge. Flagellated chambers appear to form by division of choanoblasts first into clusters, then by arrangement of those choanoblasts into a string, and then by expansion of the chamber and separation of the choanoblasts to lie around it through production of collar bodies and the primary and secondary reticula. Still unclear are how new syncytial tissues form elsewhere in the body and how the smaller nuclei in the syncytium form. It would appear that no chambers form as the sponge tube widens; however, this should be confirmed with additional studies of regions closer to the middle and base of the sponge.

**Spicule growth**

The silicification process is thought to be the same between mega- and microscleres (Uriz 2006), so both types were expected to take up PDMPO label.
if they were depositing new silica, and indeed new silica deposition was seen on megascleres and microscleres. Complete labeling of spicules by PDMPO during a short incubation period supports the prevailing model of silicification, that siliceous spicules grow in layers around an axial filament (reviewed by Uriz 2006; Schröder et al. 2007; Wang et al. 2011). This differs from calcareous sponges in which spicules grow from a seed nucleus either from the center to the tips or from discrete calcification sites (Ilan et al. 1996; Sethmann & Wörheide 2008).

That different regions of the sponge produce different spicule complements demonstrates regionalization across the body. Sponges with fused (dictyonine) skeletons cannot change their shape as other sponges can, so growth would be expected to occur only in regions without fused skeletons. It is therefore tempting to propose an interpretation of the stages of spicule production from early in the unfused tip of the sponge to later as one moves toward the fused skeleton. If this were the case, most spicule-forming activity would occur in tissue that lies at the unfused tips of the sponges, with both microscleres and megascleres being produced. Once they have grown for some time and reached terminal size, megascleres would then be fused into the rigid scaffolding (as previously described in Leys et al. 2007). In this case, it is expected that more mature regions of the sponge already have the megascleres needed for structure, so only microscleres would be produced. Not all microscleres were labeled, so in the future one may be able to quantify the number of spicules deposited, and surface area of spicule deposition, by incubating the sponge in PDMPO for different lengths of time.

**Growth of flagellated chambers**

Flagellated chambers were also found forming in the unfused tips of the sponge, where they formed a

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**Fig. 5.** Proposed steps of formation of the reticula around the microvilli and flagella. A and B. Tissue projecting down among the collars of microvilli and around the flagella. C. A top-down view of a similar area showing tissue surrounding the flagellum and the collars. D. Wider spaces around the flagella while collars of microvilli were still surrounded by tissue. E. All tissue surrounding the flagella is gone but the collars of microvilli are still surrounded by tissue. F. The secondary reticulum above the primary reticulum with only a thin tissue layer above the collars remaining. Scale bars: 1 μm. 1R, primary reticulum; 2R, secondary reticulum; cb, collar body; chb, choanoblasts; f, flagellum.
surprisingly discrete band of new growth. Directional growth that is confined to one area suggests ontogenetic processes which merit further study. In the growth zone, chambers began as clusters of choanoblasts that first replicated and then produced collar bodies to expand into a fully sized chamber. The organization and arrangement of collar bodies and the primary and secondary reticula may occur in the order described here; however, it is possible that the stages of reticulum formation are reversed, such that what we describe is in fact the degradation and loss of a chamber over time. In earlier work on the glass sponge Rhabdocalypthus dawsoni Lambe 1893, flagellated chambers were thought to be resorbed after individuals were kept in a tank for several days (Leys 1999); while that may be possible here too, all of our current observations came from the growth zone of the sponge and were never observed elsewhere in the tissue. Flagellated chambers may be lost over time, either because they are resorbed back into the body or because that region of the body has a different function, such as when archaeocyte congeries adjacent to the chambers produce spermatocysts (Boury-Esnault et al. 1999; reviewed by Leys et al. 2007).

The formation of flagellated chambers by expansion of enucleate collar bodies is unique to glass sponges. Other sponges produce chambers through transdifferentiation of larval ciliated larvae with a possible archaeocyte intermediate (Amano & Hori 1996; Leys & Degnan 2002), by immigration of stem cells from the mesohyl (Kahn & Leys 2016), by fusion of chambers, or by 4–5 mitotic divisions of a founding archaeocyte into smaller and smaller cells until all choanocytes are made (Tanaka & Watanabe 1984). The rosettes of choanoblasts that appear in early stages of chamber formation in glass sponges are remarkably similar to the small, new chambers described from cellular sponges. What is interesting is that while flagellated chambers of cellular sponges expand to full size through mitosis, transdifferentiation, or stem cell immigration, the flagellated chambers of glass sponges expand without producing any more choanoblasts at all, instead creating collar bodies. In comparison with choanocyte cells of other sponges, collar bodies are smaller, lack nuclei and some organelle types including polyribosomes and phagosomes (Mackie & Singla 1983; Boury-Esnault & Vacelet 1994), and are not thought to be involved in particle capture (Perez 1996; Wyeth 1999). Using a limited number of choanoblasts to produce a chamber with collar bodies as small repeating units may be an energy-efficient strategy of living in food-poor deep water.

Different growth forms can also confer energetic advantages depending on flow conditions. The hydrozoan Millepora alcicornis Linnaeus 1758, scleractinian coral Pocillopora damicornis (Linnaeus 1758), and the sponges Haliclona oculata (Linnaeus 1759) and Halichondria panicea (Pallas 1756) all have thin, branching morphologies in areas with low flow and are more compact in areas with higher flow (Palumbi 1984, 1986; Kaandorp 1999). Mathematical models of accretive growth in a sponge or stony coral show that a branching, upward growth form becomes a dominant morphology for suspension feeders in areas with high flow and high nutrients (Kaandorp & Sloot 2001). High flow and high nutrient conditions are what reef sponges are exposed to and so might explain the directional growth observed for both their tissue and their spicule skeletons.

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