



0001-7272(95)00020-8

Use of Sandwich Cultures for the Study of Feeding in the Hexactinellid Sponge *Rhabdocalyptus dawsoni* (Lambe, 1892)

Russell C. Wyeth, Sally P. Leys and George O. Mackie

Department of Biology, University of Victoria, Victoria, British Columbia, Canada V8W 2Y2

(Accepted for publication 18 July 1995)

Abstract

Wyeth, R. C., Leys, S. P. & Mackie, G. O. 1996. Use of sandwich cultures for the study of feeding in the hexactinellid sponge *Rhabdocalyptus dawsoni* (Lambe, 1892).—*Acta Zoologica* (Stockholm) 77: 227–232.

Fragments of sponge tissue were cultured between glass slides and coverslips, permitting direct observation of cytoplasmic movements and tissue organization *in vitro*. The cut surfaces healed and the cultures lived for periods of several weeks. Cytoplasmic organization appeared similar to that described from study of sectioned material. Uptake of food particles (*Escherichia coli*, *Isochrysis galbana*) and latex beads took place primarily in the region of the flagellated chambers. Cytoplasmic streams were seen throughout the preparation and may serve for distribution of nutrients in these syncytial animals. It is proposed that the sandwich cultures are valid models of the intact sponge. Copyright © 1996 The Royal Swedish Academy of Sciences. Published by Elsevier Science Ltd.

Russell C. Wyeth, Department of Biology, University of Victoria, Victoria, British Columbia, Canada V8W 2Y2.

Introduction

The Hexactinellida are a major sponge group now treated as a subphylum distinct from the other three classes of the Porifera (Reiswig & Mackie 1983). They differ most obviously from other sponges in having an open trabecular network instead of canals, and in their extensive syncytiality (Mackie & Singla 1983). They inhabit relatively deep waters, which makes them inaccessible in most parts of the world. As a result, there have been few physiological studies on members of the group and, in particular, there is little information regarding their feeding (Mackie & Singla, 1983; Reiswig 1992).

Almost all sponges are filter feeders. Water is pumped through dermal ostia into incurrent canals or spaces which lead through prosopyles to flagellated chambers. The water exits from the chambers through apopyles, eventually leaving the sponge's body via the osculum. As the water flows through the sponge, particulates, colloidal material, and/or dissolved organic matter are filtered out (Rasmont 1968). Particle uptake is by phagocytosis.

In demosponges (the group where feeding has been most thoroughly investigated) most surfaces are used for particle uptake, including the layer of exopinacocytes of the dermal surface (Willenz & Van de Vyver 1982; Schmidt 1970), the endopinacocytes of the incurrent canals (Reiswig 1971; Diaz 1979), and the choanocyte collars in the flagellated chambers (Schmidt 1970; Reiswig 1971; Imsiecke 1993). A sealed mucus strainer system, which creates a complete filter between incurrent and excurrent flows inside the flagellated chamber, is seen in one species of demosponge and may be more widespread within this class (Weissenfels, 1992). The

trabecular net of hexactinellids is a much more open structure than the canal system of demosponges, there are more entrances into the flagellated chambers in hexactinellids, and there are marked structural differences between flagellated chambers of the two groups. Given these differences it would not be surprising to find that internal water flow patterns, filtration mechanisms, and uptake of food materials also differed in the two groups, but information is generally lacking.

Very little is known regarding the natural food of hexactinellids. Mackie & Singla (1983) found ultrastructural evidence of phagocytosis of bacteria. *In situ* studies by Reiswig (1990) suggested that *Aphrocallistes vastus* filters both colloidal particles and bacteria, while *Rhabdocalyptus dawsoni* retains only dissolved organic carbon. However, *Rhabdocalyptus* in its natural habitat has a surface coating containing many epibiotic organisms (Boyd 1981), making it difficult to study the sponge's feeding capabilities in isolation.

In demosponges, phagocytosed materials are transferred to amoebocytes, or the phagocytic cells become amoebocytes (Diaz 1979; Imsiecke 1993); the amoebocytes then migrate through the spacious mesohyl, distributing nutrients. It is doubtful if amoebocytes play such a role in hexactinellids, as the mesohyl is relatively poorly developed, and the only cells which might function as amoebocytes, the archaeocytes, do not appear to have the long pseudopodial processes expected of motile cells. They occur in clusters, closely linked by plugged junctions (Mackie & Singla 1983). The vigorous cytoplasmic streaming seen in regenerating hexactinellid tissues and in syncytial aggregates formed from dissociated tissues suggests that hexactinellids may use such streams for nutrient translocation, making amoebocytic transport redundant (Leys & Mackie 1994).

Observation of the intact sponge's tissues is difficult because it is very hard to obtain by dissection along the thin, flat pieces of healthy tissue, free of spicule fragments and other debris, needed for optical clarity. Plated aggregates made from dissociated *Rhabdocalyptus* tissue offer excellent visibility but differ from normal sponge tissue in lacking flagellated chambers (Leys & Mackie 1994). The present study was undertaken in an attempt to develop a preparation in which the tissues retain their normal structural and functional relationships but which provides clear visibility. We report here that explanted hexactinellid tissues maintained as sandwich cultures heal to form functioning model sponges, in which cytoplasmic streaming is observed, and in which uptake of food particles can be monitored directly.

Materials and methods

Specimens of *Rhabdocalyptus dawsoni* were collected in plastic bags by SCUBA at depths of <30 meters in Saanich Inlet and Barkley Sound, British Columbia. They were transferred to a darkened sea water tank kept at 10°C at the University of Victoria, where they were maintained through the course of the experiments.

Sandwich cultures

Two types of sandwich culture were used. Coverslip sandwich cultures were constructed from two coverslips separated by spacers cut from plastic coverslips and sealed with Vaseline (Fig. 1A). Slide sandwich cultures used a coverslip and slide separated by the spacers (Fig. 1B).

Tissue was prepared by dissecting out a piece of the animal and clipping off the outer spicule mass. The tissues and spicules were teased apart into pieces 1 or 2 spicule layers thick using forceps and microscissors. Approximately ten teased pieces were placed inside each sandwich. The distance between the two glass surfaces was approximately 0.25 mm. Thicker sandwiches were not used because they were too thick for good visibility.

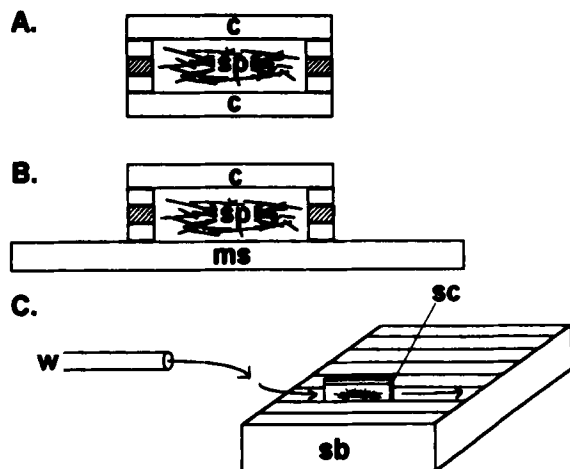


Fig. 1. Sandwich culture preparation. A. Schematic diagram of the coverslip sandwich culture construction. The spicule preparations (sp) were held inside two coverslips (c) separated by two spacers (shaded) and sealed with Vaseline (clear). B. Schematic diagram of the slide sandwich culture construction, as in (A) except that a microscope slide (ms) was used instead of the bottom coverslip. C. The cultures were held in a plastic microscope slide box (sb) with strips of plastic siliconed into the slide slots. The sandwich cultures (sc) were inserted into the gaps between the plastic strips with their open ends facing the sides of the holder such that a water current (w) flowing across the entire holder resulted in a small proportion flowing through the sandwich culture as shown by the arrows.

The cultures were kept in a holder with water slowly flowing across them (Fig. 1C) in the same darkened sea water tank as the whole sponges. Water flow was monitored using food colouring dyes. For long term observations under the microscope, cultures were kept in a perfusion chamber, irrigated with a steady flow of filtered sea water.

Feeding

Escherichia coli (ca. 1.0 μm) labelled with sulforhodamine 101 (Sigma Chemical Co.), *Isochrysis galbana* (ca. 4.0 μm), and fluorescently labeled 1 μm latex beads (Molecular Probes, Inc.) were fed to the sponges. The *I. galbana* did not require a label as they were strongly autofluorescent. Fluorescence was observed using a rhodamine filter block (Leitz N2.1, excitation wavelength 515–560 nm). The sponge tissues were only slightly autofluorescent in the rhodamine emission range. Although the particles were selected for fast and easy identification under fluorescence microscopy, both the algae and latex beads were readily visible with differential interference contrast microscopy (DIC). Due to the damaging effects of UV light, fluorescence was chiefly used for quick identification of the food particles, while routine observations were done with DIC.

- (1) *Whole piece feeding test.* Whole pieces of sponge body wall (1.0 cm^3) were dissected into a watch glass containing a suspension of either *E. coli* or *I. galbana*. After 30 min the food suspension was replaced with fresh sea water for 15 min. Sandwich cultures were prepared from the fed pieces 0.5–22 hours after feeding.
- (2) *Slide sandwich feeding test.* Slide sandwich cultures were prepared and allowed to regenerate for at least 24 hours. Approximately 0.5 ml of *E. coli* or *I. galbana* suspension was perfused slowly through the sandwich. The fed cultures were then placed in their underwater holders and observed 0.5–24 hours later.
- (3) *Coverslip sandwich feeding test.* Coverslip sandwich cultures were prepared and allowed to regenerate for at least 24 hours. They were then placed within a perfusion chamber, and maintained at 8°C while *E. coli* or latex bead suspension was added. Observations were made over a maximum period of 6 hours. A control test was also performed by feeding latex beads to a heat-killed sandwich culture.

Light microscopy

For short term DIC or rhodamine fluorescence viewing, a Leitz Aristoplan microscope with a 50 \times water immersion objective was used. For long term DIC viewing of sandwich cultures in the perfusion chamber, a Zeiss 2033 microscope was used with the 50 \times water immersion lens. This microscope was equipped with thermo-electric cooling stage set to maintain the temperature in the perfusion chamber at 8°C.

Electron microscopy

Coverslip sandwich cultures were fed latex beads by perfusion and fixed 2 hours after feeding in a mixture of 1% OsO_4 , 2% glutaraldehyde, and 10% sucrose, in 0.45 M sodium acetate buffer at pH 6.4, for 2 hours on ice. Preparations were dehydrated through a graded ethanol series, pausing to desilicify the specimens in 4% hydrofluoric acid in 80% ethanol overnight. Preparations were stained in 0.5% uranyl acetate while in 80% ethanol and subsequently embedded in Epon. Silver sections were cut with glass knives on a Reichert ultramicrotome, stained with lead citrate, and viewed in a Joel, JEM 1200 Ex electron microscope.

Results

Sandwich cultures

The sponge tissues held inside the sandwich cultures consisted of an irregular mass of spicules spanned by flat sheets of tissue perforated by pores (Fig. 2A) and elsewhere by irregular strands representing the trabecular system of the intact sponge (Fig. 2B). Flagellated chambers were sus-

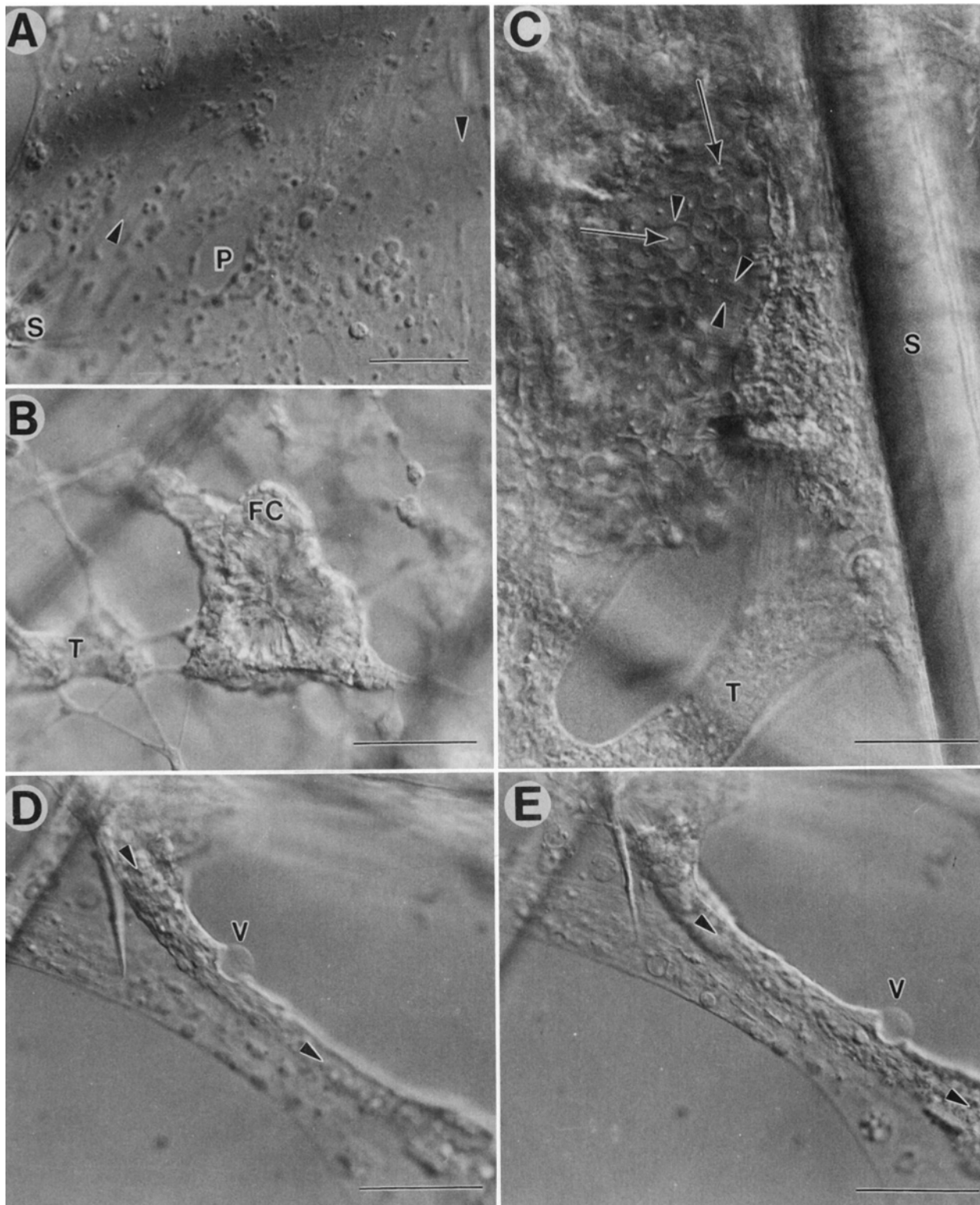


Fig. 2. Histology of cultured tissues. **A.** External membrane with several cytoplasmic streams. Direction of flow is shown with *arrowheads*. Spicules (S) and pores (P) are both found in this tissue. This thin, sheet-like morphology was typical of much of the trabecular tissue, and was not confined to the outside of the spicule preparation. Scale bar 30 μm . **B.** A flagellated chamber (FC) within the trabecular network (T). Scale bar 30 μm . **C.** A portion of a flagellated chamber with flagella (*arrows*) and collars (*arrowheads*), spicules (S), and a trabecular strand (T). This kind of chamber occurred in thicker areas of the preparation and it was possible to focus through several planes to see the whole depth of the chamber. Scale bar 20 μm . **D, E.** A cytoplasmic stream inside a trabecular strand flowing to the right (*arrowheads*). A large vesicle (V) is being transported towards the right. Photographs are 20 seconds apart. Scale bars 30 μm .

pendent within this trabecular net (Fig. 2B, C). The perforated sheets and strands appeared to be parts of a continuous system, with cytoplasmic streams flowing between them. The trabecular structure was quite variable, with strands of

many lengths and widths. In addition to spanning the gaps between spicules, the trabecular syncytium was observed to cover some or all of the spicules themselves. This general morphology was preserved throughout the life of the prep-

aration. When maintained with adequate flow, sandwich cultures could be kept alive for at least two weeks. One preparation still had beating flagella after 38 days.

Although considerable damage was incurred during dissection, resulting in broken spicules and ragged or torn trabeculae, such signs of damage decreased progressively over time. Further healing was apparent with the development within two days of an external membrane consisting of flat, perforated sheets of tissue around the whole preparation, spanning the gaps between many of the outer spicules (Fig. 2A). Flagellated chambers damaged during dissection did not appear to be repaired or removed, as clusters of flagella representing chamber remnants were still present in the oldest preparations observed.

Cytoplasmic streaming was observed in all preparations (Fig. 2A, D, E). Individual organelles and vesicles flowed singly or in masses of varying sizes. The entire contents of some trabecular strands consisted of a single stream. Streams flowed through all tissues except those associated with flagella and collar bodies. Immediately following dissection, and for several days afterwards, there were large numbers of cytoplasmic streams. The number and size of streams declined in older preparations.

The shape of the tissue was constantly changing, especially in young preparations. The cytoplasmic streams, particularly the wider ones, appeared to be an integral part of these changes. Spreading and retraction of tissue over spicules took place in association with streaming. Over the course of several days, the external membrane enveloping the preparation was observed to increase in size, presumably by influx of streaming cytoplasm. Sometimes a portion of a stream would become blocked, and the cytoplasm arriving from upstream would cause a swelling on the proximal side of the block. If the blockage was released, streaming would be seen again on the distal side and the swelling would gradually diminish in size. In one case, a blocked stream was observed to swell, and then spread out, eventually forming a whole new layer of tissue, itself complete with streams.

Beating flagella were observed in whole and broken chambers in preparations of all ages. Many immobile flagella were also observed. Collars were usually visible around the flagella in intact flagellated chambers (Fig. 2C) but they could not always be seen, and it appeared from the erratic and unconstrained beating of some flagella that their collars were incomplete or missing.

After approximately two weeks, signs of regression were usually seen. The trabecular tissues started to withdraw from some of the spicules. In very old preparations, the trabeculae tended to retract completely, forming isolated masses of tissue.

Feeding

Feeding of *I. galbana* to whole pieces of sponge imparted a brownish tinge to the white tissues of the sponge. Only the thin dermal and atrial layers, and the areas next to cut edges retained their white colouration. Observations following the feeding tests on whole pieces and on sandwich cultures demonstrated that *I. galbana* and *E. coli* were both taken into the cytoplasm. Transfer from the extra- to the intracellular domain took place gradually over several

minutes, and was presumably accomplished by phagocytosis, though the level of microscopic resolution was not sufficient to verify this by direct observation. Internalized *I. galbana*, visualized by DIC (Fig. 3A) and fluorescence microscopy (Fig. 3B), were seen lying in the cytoplasm near flagellated chambers. Similarly, latex beads were abundant in the cytoplasm in the region of the flagellated chambers after feeding (Fig. 3C, D). Electron microscopy of thin sections of fed preparations showed beads lying within phagosomes in the cytoplasm of the trabecular syncytium, near flagellated chambers (Fig. 3E).

The great majority of internalized food particles observed were found around the flagellated chambers. When uptake was observed during the perfusion feeding tests, it was seen only in the vicinity of the flagellated chambers. In all feeding tests, many parts of the trabecular net were observed to have food particles around, or even resting against their outer surfaces, but only in a very few cases could food particles be seen inside the cytoplasm. Once or twice during hundreds of observations, latex beads and *E. coli* were seen moving within trabecular cytoplasmic streams far from any flagellated chambers. The control test on the heat-killed sandwich culture showed no tendency for particles to concentrate in the vicinity of flagellated chambers, indicating that their concentration in the regions in the living preparation is due to active processes.

Sandwich cultures of *Rhabdocalyptus dawsoni* have many characteristics which suggest that they can serve as valid models of intact sponges. The preparations showed flagellated chambers with beating flagella, cytoplasmic streaming, and the ability to feed and to heal their cut surfaces. Despite some variability between preparations, the structure of the trabecular network and flagellated chambers, whether in preparations half an hour or thirty days old, always conformed to classical descriptions of hexactinellid histology (Ijima 1904; Reiswig 1979). The regenerated external membrane was morphologically similar to the dermal membrane of whole sponges. Where differences from normal histology were observed, they could usually be attributed to dissection damage or to regression. Thus, because the preparations showed no signs of changing from their original form, and because they bore a strong resemblance to intact sponge tissues, there is much in favour of the view that they were structurally and functionally equivalent to whole sponges. Although the sandwich cultures failed to grow and eventually underwent regression, fragments of *Rhabdocalyptus* do have the potential to regenerate into whole sponges: a few have been observed to form minute sponges, complete with an osculum, when kept in running sea water (Leys, unpublished observations).

Cytoplasmic streaming observed in these preparations was similar to that described in the substrate-attached aggregates made from dissociated hexactinellid sponge tissues (Leys & Mackie 1994), providing further evidence that cytoplasmic streaming is a basic physiological process made possible by the lack of membrane barriers in these syncytial organisms. Streaming appears to play a vital role in the creation of new tissue, and the extensive streaming seen in recently damaged preparations suggests that it is also important in healing processes. Streams are probably significant in nutrient transport, but further experiments will be needed to verify this.

Our experiments on sandwich cultures showed that 1.0

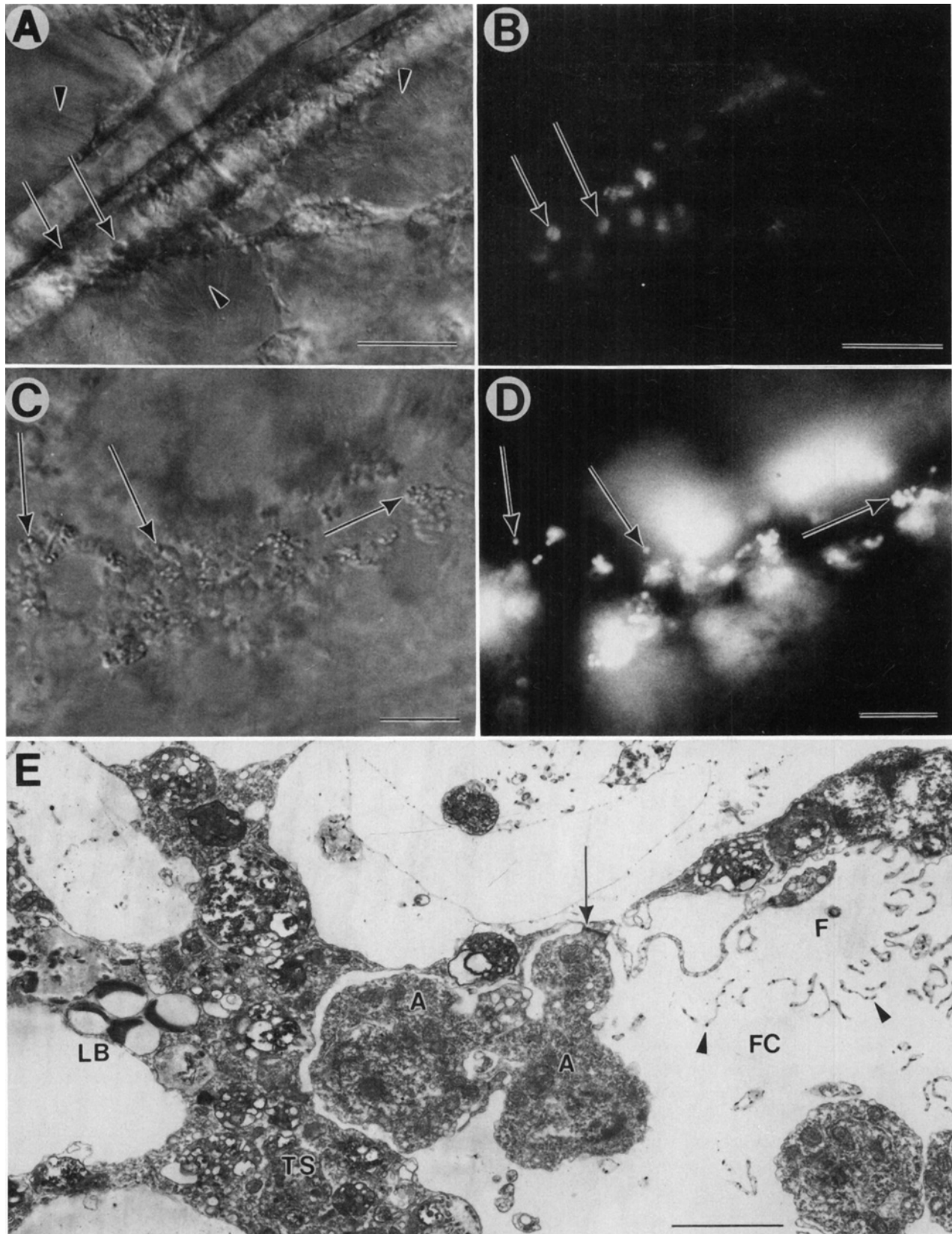


Fig. 3. Uptake of particles by sandwich cultures of *R. dawsoni*. A,B. The internalized golden-brown algae, *I. galbana*, (arrows) can be seen near flagellated chambers. Algae are seen by DIC (A) and by fluorescence microscopy (B). Flagella and collars serve to locate flagellated chambers (arrowheads). Scale bars 20 μm . C,D. 1 μm latex beads (arrows) are incorporated into tissues near flagellated chambers, observed by DIC (C) and fluorescence microscopy (D). Scale bars 20 μm . E. Electron micrograph of a preparation fixed 2 hours after being fed with latex beads. Four latex beads (LB) are visible inside the trabecular syncytium (TS) near a flagellated chamber (FC). A plugged junction (arrow) connects archaeocytes (A) to the trabecular syncytium. Flagellum (F); Collar microvilli (arrowheads). Scale bar 2 μm .

μm bacteria, $4.0 \mu\text{m}$ unicellular algae and $1.0 \mu\text{m}$ inorganic particles could all be internalized. We have not investigated uptake of colloidal material or dissolved organic matter, but the ability of the culture tissues to take up particles suggests that *R. dawsoni* in nature feeds at least in part on particulates, contrary to an earlier report (Reiswig, 1990).

The majority of the internalized food particles seen in sandwich cultures were located close to flagellated chambers, having presumably been phagocytized by collar bodies in the chambers as in other sponges, or by the trabecular syncytium associated with the chambers, but the details of uptake have not been verified by direct observation. Observations on the sandwich cultures showed that while free food particles were present close to trabecular strands in many regions there was little evidence of uptake outside the flagellated chambers. In the feeding tests on whole pieces of intact sponge tissue, internalized food particles were seen almost exclusively in the immediate vicinity of the flagellated chambers, indicating that their concentration here in sandwich cultures was not an artefact. Furthermore, the control test on killed tissue in sandwich cultures showed no concentration of food particles in or around the flagellated chambers, showing that their concentration here in living preparations was not due to passive flow anomalies caused by sandwiching the tissue between glass sheets. Rather, these results suggest that uptake occurs by an active process and is primarily a function of the flagellated chambers. This does not preclude the possibility that some particle uptake can occur in the trabecular system. Indeed, phagocytosis of latex beads has been observed in plated aggregates, which lack flagellated chambers (Leys, unpublished).

The development of a tissue preparation which adequately models whole hexactinellids opens a number of avenues for further investigation. For example, video microscopy could now be used to record the long term details of feeding, regeneration, morphogenesis, regression and the involvement of cytoplasmic streaming in these processes. Specific points of interest are whether nutrients are transported in the streams, how they are transferred into the streams from the flagellated chambers, whether digestion continues during translocation or only in the choanoderm, how much uptake (if any) occurs in the trabecular network and rates of digestion. It would be desirable to improve culture conditions so as to allow the cultures to survive for longer periods, to grow in size and possibly to develop new flagellated chambers etc., so that morphogenetic processes could be directly observed. Finally, ultrastructural studies using pulse feeding and fixation methods could be used to examine the finer

details of phagocytosis, particle and nutrient transport, digestion and egestion.

Acknowledgements

We thank the Natural Sciences and Engineering Research Council of Canada for an undergraduate student research award (RCW), postgraduate scholarship (SPL), and for operating grant No. OGP001427 (GOM). We are grateful to A. R. Fontaine for loan of equipment, and to L. A. Hobson, L. R. Page, and N. M. Sherwood for assistance in the algal and bacterial cultures.

References

- Boyd, I. 1981. The spicule jungle of *Rhabdocalyptus dawsoni*: a unique microhabitat. B.Sc. Honours Thesis, University of Victoria.
- Diaz, J. P. 1979. Variations, différenciation et fonctions des catégories cellulaires de la demosponge d'eaux saumâtres, *Suberites massa* Nardo, au cours du cycle biologique annuel et dans des conditions expérimentales. Ph.D. Thesis, Université des Sciences et Techniques du Langue-doc.
- Ijima, I. 1904. Studies on the Hexactinellida. Contribution IV. (Rosellidae).—*Journal of the College of Science, Imperial University, Tokyo, Japan* 28: 5–307.
- Imsiecke, G. 1993. Ingestion, digestion, and egestion in *Spongilla lacustris* (Porifera, Spongillidae) after pulsed feeding with *Chlamydomonas reinhardtii* (Volvocales).—*Zoomorphology* 113: 233–244.
- Leys, S. P. & Mackie, G. O. 1994. Cytoplasmic streaming in the hexactinellid sponge *Rhabdocalyptus dawsoni* (Lambe, (1873)). In van Soest, van Kempen and Braekman (eds): *Sponges in Time and Space*, pp. 417–423. Balkema, Rotterdam.
- Mackie, G. O. & Singla, C. L. 1983. Studies on hexactinellid sponges. I. Histology of *Rhabdocalyptus dawsoni* (Lambe, (1873)).—*Philosophical Transactions of the Royal Society of London B* 301: 365–400.
- Rasmont, R. 1968. Nutrition and digestion. In M. Florkin & B. T. Scheer (eds): *Chemical Zoology*, vol. 2, pp. 43–50. Academic Press, New York.
- Reiswig, H. M. 1971. Particle feeding in the natural populations of three marine demosponges.—*Biological Bulletin* 141: 568–591.
- Reiswig, H. M. 1979. Histology of Hexactinellida (Porifera). In C. Levi & N. Boury-Esnault (eds): *Colloques internationaux du C.N.R.S. No. 291—Biologie des spongiaires*, pp. 173–180. Edition du Centre National de la Recherche Scientifique, Paris.
- Reiswig, H. M. 1990. *In situ* feeding in two shallow water hexactinellid sponges. In K. Rützler (ed): *New perspectives in sponge biology*, pp. 504–510. Smithsonian Institution Press, Washington, D.C.
- Reiswig, H. M. & Mackie, G. O. 1983. Studies on hexactinellid sponges. III. The taxonomic status of Hexactinellida within the Porifera.—*Philosophical Transactions of the Royal Society of London B* 301: 419–428.
- Schmidt, I. 1970. Phagocytose et pinocytose chez les Spongillidae. Etude *in vivo* de l'ingestion de bactéries et de protéines marquées à l'aide d'un colorant fluorescent en lumière ultra-violette.—*Zeitschrift für vergleichende Physiologie* 66: 398–420.
- Weissenfels, N. 1992. The filtration apparatus for food collection in freshwater sponges (Porifera Spongillidae).—*Zoomorphology* 112: 51–55.
- Willenz, Ph. & Van De Vyver, G. 1982. Endocytosis of latex beads by the exopinacoderm in the fresh water sponge *Ephydatia fluviatilis*: An *in vitro* and *in situ* study in SEM and TEM.—*Journal of Ultrastructure Research* 79: 294–306.