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Feeding in a Calcareous Sponge: Particle Uptake by Pseudopodia

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Abstract Sponges are considered to be filter feeders like their nearest protistan relatives, the choanoflagellates. Specialized "sieve" cells (choanocytes) have an apical collar of tightly spaced, rodlike microvilli that surround a long flagellum. The beat of the flagellum is believed to draw water through this collar, but how particles caught on the collar are brought to the cell surface is unknown. We have studied the interactions that occur between choanocytes and introduced particles in the large feeding chambers of a syconoid calcareous sponge. Of all particles, only 0.1-μm latex microspheres adhered to the collar microvilli in large numbers, but these were even more numerous on the choanocyte surface. Few large particles (0.5- and 1.0-μm beads and bacteria) contacted the collar microvilli; most were phagocytosed by lamellipodia at the lateral or apical cell surface, and clumps of particles were engulfed by pseudopodial extensions several micrometers from the cell surface. Although extensions of the choanocyte apical surface up to 16 μm long were found, most were 4 μm long, twice the height of the collar microvilli. These observations offer a different view of particle uptake in sponges, and suggest that, at least in syconoid sponges, uptake of particles is less dependent on the strictly sieving function of the collar microvilli.

Introduction

Sponges are some of the predominant benthic suspension feeders globally (Gili and Coma, 1998). Detailed *in situ* and *in vitro* studies have revealed feeding efficiencies in the range of 75%–99% on plankton 0.1–70 μm (Reiswig, 1974, 1975; Pile *et al.,* 1996, 1997; Ribes *et al.,* 1999). Sponges are capable of ingesting a great variety of types and sizes of plankton, but generally with higher grazing efficiencies on plankton smaller than 10 μm (Pile *et al.,* 1996; Turon *et al.,* 1997; Witte *et al.,* 1997; Ribes *et al.,* 1999). While concentration and particle size do affect retention ability (Huysecom *et al.,* 1988; Duckworth *et al.,* 2003), studies indicate that sponges can select nutritionally favorable cells (Frost, 1980). Two hypotheses have been proposed to explain particle selectivity: differential uptake, in which preferred particles are phagocytosed more "efficiently," and differential retention during digestion and egestion. From measurements of clearance rates of synthetic and natural particles, Francis and Poirrier (1986) concluded that since all particles were removed from the water equally, differential retention during digestion and egestion must explain selectivity. However, whether this can also explain the ability of sponge cells to differentiate between symbiotic bacteria and those in the ambient water, as demonstrated by Wilkinson *et al.* (1984), is not so clear. Few studies have addressed exactly how sponges take up food at the cellular level.

The sponge choanocyte is considered one of the few examples of a true sieve filter in metazoans (Riisgård and Larsen, 2001). A similar collar sieve is well known from choanoflagellates—colonial protozoans that strongly resemble the sponge choanocyte—which are now confirmed to be the sister group to sponges (Snell *et al.,* 2001; Cavalier-Smith and Chao, 2003; King *et al.,* 2003). The sponge choanocyte is said to be functionally identical to the choanoflagellate cell (Riisgård and Larsen, 2000): the beat of the flagellum draws water toward and through a collar of microvilli (Van Trigt, 1919; Kilian, 1952; Riisgård and Larsen, 2001; Pettitt *et al.,* 2002). In choanoflagellates, suspended particles are retained on the microvilli when water is drawn through the collar (Fenchel, 1982, 1986; Andersen, 1989), and particles can be seen to be phagocytosed by pseudopodia arising from the base of the collar

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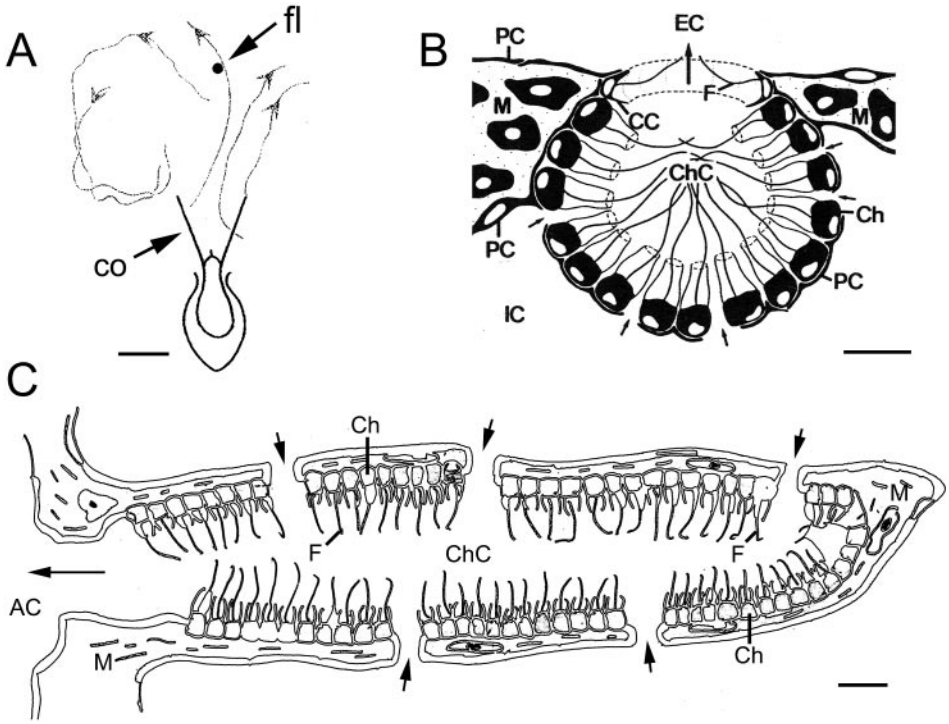

Figure 1. Diagrams illustrating the route of water filtered by (**A**) the choanoflagellate *Salpingoeca amphoridium**, (**B**) an ovoid choanocyte chamber in the demosponge *Haliclona elegans*†, (**C**) a finger-like choanocyte chamber in *Sycon coactum.* (A) The tip of the choanoflagellate flagellum (fl) is indicated by the black dot; dashed lines indicate the movement of particles traced from video recordings. Collar microvilli (co) are more widely spaced apically than basally. Scale bar: 5 μm. (B, C) Choanocyte chambers (ChC) of sponges are lined by many choanocytes (Ch) that have long narrow collars and long flagella (F). Pinacocytes (PC) form the outer epithelium and line canals. The choanoderm and pinacoderm sandwich a cellular middle layer, the mesohyl (M). Water enters the choanocyte chamber from the inhalant canal (IC, short arrows) and exits into the excurrent canal (EC) in demosponges (B), and into the atrial cavity (AC) in calcareous sponges (C). B, C Scale bars: 10 μm.

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†Reprinted from *Zoomorphology,* Vol. 106: 205–211, 1986, Body structure of marine sponges IV. Aquiferous system and choanocyte chambers in *Haliclona elegans* (Porifera, Demospongiae), P. F. Langenbruch and L. Scalera-Liaci, Fig. 9, Springer-Verlag, with kind permission of Springer Science and Business Media.

(Leadbeater and Morton, 1974). Sponge choanocytes are more difficult to observe *in vivo,* but studies that have examined particle uptake suggest that although choanocytes ingest India ink, carmine, bacteria, and latex microspheres, the collar microvilli do not stain with the dyes, nor are particles shown to be retained in great numbers on the collar microvilli (Van Trigt, 1919; Pourbaix, 1933; Schmidt, 1970; Willenz, 1980; Imsiecke, 1993).

Differences between the structure of sponge choanocytes and the choanoflagellate cell, and the fact that many choanocytes are arranged within single chambers in a sponge (Fig. 1), suggest that mechanisms of particle capture may differ. Choanoflagellates tend to have a short flagellum (2–3 times the height of the collar) and short, wide, flaring collars whose microvilli are more widely spaced apically than basally (0.7–0.1 μm) (Fig. 1A) (Fjerdingstad, 1961b; Leadbeater, 1983; Fenchel, 1986; Orme *et al.,* 2003), allowing relatively fast flow through the sieve (14–30 μm s^{-1}) (Fenchel, 1986; Larsen and Riisgård, 1994). In many choanoflagellates, microvilli contain a pair of microtubules that penetrate deep into the cell body (Leadbeader and Morton, 1974; Fenchel, 1982). In contrast, demosponges have long flagella (up to 6 times the height of the collar) and long narrow collars (Fjerdingstad, 1961a; Simpson, 1984; Langenbruch and Scalera-Liaci, 1986; Larsen and Riisgård 1994); collar microvilli are only 0.1 μm or less apart, and water is estimated to flow through the sieve at 2–3 μm s^{-1} (Reiswig, 1975; Riisgård *et al.,* 1993). Larsen and Riisgård (1994) argue that the long flagellum of sponges is adapted to provide a more powerful pump than the choanoflagellate cell, and that 80–100 such pumps in a single chamber are needed to create the pressure to overcome the resistance of

the extensive sponge canal system. The narrow collar is deemed appropriate to fit all the cells into a chamber, and though the flow field around the collar is thought to differ from that around free-living choanoflagellates, it is presumed that the alignment of collars and their possible touching at the tips, or the presence of a mesh "strainer" linking the tips (see Weissenfels, 1992) ensures that flow passes through the collar slits (Larsen and Riisgård, 1994).

The above studies, measurements, and models concern flow within the chamber of demosponges, all of which have a leuconoid body plan with incurrent canals leading to numerous ovoid chambers, as illustrated in Figure 1B. A small number of sponges in the Calcarea have vastly different chambers. Calcareous sponges make up only 5% of all Porifera and are the only group with ascon (choanocyte-lined tubes) and sycon (long choanocyte-lined chambers branching off a central cavity) as well as leucon body plans. In the calcareous sponge *Sycon coactum,* water enters through pores 10–20 μm apart into long chambers lined by several thousand pumping choanocytes (Fig. 1C). Furthermore, unlike those in demosponge collars, microvilli in *Sycon* are rarely neat filaments. As described in early studies (Haeckel, 1872; Schulze, 1875; Duboscq and Tuzet, 1939), the collar microvilli in *Sycon* frequently form short, lumpy projections that are often partially fused together. Although periods of anoxia can cause abnormal shapes of the choanocyte and its collar, our examination concurs with the earlier studies which suggest that collars are inherently variable in morphology regardless of fixative or handling. Early researchers suggested that the morphology of the collar microvilli reflect the state of feeding of the cell (Bidder, 1892; Duboscq and Tuzet, 1939). No recent work has examined this question, and it remains unclear how these chambers and the choanocytes that line them function in filter feeding.

To determine how choanocyte collars in large syconoid chambers filter, we fed the sponges bacteria and three sizes of latex microspheres. Sponges were fed both *in situ,* by divers using scuba, and *in vitro.* The sponges were preserved at intervals after feeding, by instant immersion in a large volume of fixative, and processed for examination by scanning electron microscopy.

Materials and Methods

Specimens of the calcareous sponge *Sycon coactum* were fed latex beads (Molecular Probes, OR) and heat-killed *Escherichia coli* bacteria both *in situ* and *in vitro* at the Bamfield Marine Sciences Centre, Bamfield, Canada. Solutions of beads in three sizes (1.0, 0.5, and 1.0 μm) were diluted to 1×10^9 in seawater collected from 20-m depth and mixed with 10% bovine serum albumin to prevent clumping, according to the manufacturer's instructions. For *in situ* feeding, sponges attached to dock piles at 10-m depth were fed by scuba divers. The sponges were first enclosed in 4×8 cm resealable zipper-type plastic bags, and 2 ml of the solution of 1-μm latex beads was injected into the bag, for a final concentration of 4×10^7 ml^{-1}. Bags were removed from the sponges after 10 min; after 20 min the sponges, together with some wood substrate, were carefully cut off the piles, slipped into plastic collection bags, and taken to the surface. Two sponges were fixed immediately (*i.e.,* 30 min post-feeding) in a cocktail fixative consisting of 1% OsO_4, 2% glutaraldehyde in 0.45 mol l^{-1} sodium acetate buffer pH 6.4, with 10% sucrose in the final mixture; the vial with fixative was kept cold until it was added to the sponges, and fixation was carried out on ice. The remaining sponges were suspended from the dock in mesh cages at 10-m depth and fixed at 2 and 6 h post-feeding as above. Sponges fed *in vitro* were slid, without removal from seawater, into 23-ml tubes of water from 10-m depth, and solutions of the three sizes of latex beads were added, to a final concentration of 1×10^8 ml^{-1}. Stock solutions of bacteria were more dilute; the final working concentration was 1×10^4 ml^{-1} in seawater. Two sponges from each treatment were fixed at 5, 10 and 20 minutes post-feeding. Sponges were not removed from seawater at any time before or during fixation.

Although *in situ* experiments were designed to cause the least disturbance to the sponge during particle uptake, the additional time involved with doing this experiment by scuba meant that the first fixation was considerably delayed after feeding. *In vitro* feeding allowed more control during addition of the "food" solutions, and sponges could be fixed soon after being exposed to particles. Sponges were maintained in cold seawater collected from 10-m depth, and particles were added within 10 min of collecting animals. Nevertheless, careful and minimal handling of all specimens during collecting, feeding, and fixation meant that the first animals were fixed some 5–10 min after particles were added. Controls were carried out to assess the effect of handling alone. In these experiments, after collection, sponges were knocked repeatedly for 5 min (while still submerged in seawater) prior to fixation, and others were fixed without the handling involved in adding beads or bacteria. To assess the possibility that the method of fixation affected cell morphology, five fixation protocols were used, and two types of anesthetics were applied in separate experiments (Eerkes-Medrano and Leys, 2006). To assess the effects of anoxia on sponge morphology, sponges were fixed after 30 min of sitting in cold still seawater.

After 4 h in fixative, pieces of sponge were rinsed twice in distilled water and decalcified in 5% ethylene diamine tetra-acetic acid (EDTA) disodium salt overnight. Decalcified pieces were dehydrated through a graded ethanol series and fractured in liquid nitrogen while in a vial of 100% ethanol. Fractured pieces were critical-point dried, mounted with nail polish on aluminum stubs, coated with gold, and

viewed in a field emission scanning electron microscope at 5 kV.

Pieces of fractured choanocyte chambers were surveyed for interactions between particles and choanocytes. The number and type of interactions of particles with collars and the cell surfaces at each time point (5, 10, 20, 30 min, 1, 2, and 6 h post-feeding) were counted on 312 images.

Results

Choanocyte morphology

The structure of choanocyte chambers and morphology of cells and particles were identical in sponges fed *in situ* and *in vitro.* Briefly, feeding chambers are finger-shaped cavities that radiate from a central atrial tube (Fig. 2A, B). Each chamber is carpeted by a single layer of cuboidal cells called choanocytes (Fig. 2C). About 10,000 choanocytes 3.5 μm in diameter were estimated to line an average-sized chamber (450 μm long, 100 μm in diameter) (Fig. 2B, C). Ostia (incurrent pores) were 10–20 μm apart in all specimens, with about 20 cells per ostia; however, few ostia were ever visible from the inside of the chamber (Fig. 2C, D). All the water vents out of the chamber through a single excurrent opening (the apopyle) into the atrial cavity (Fig. 2B).

At the apical surface each choanocyte has a 15-μm-long flagellum that is surrounded by a ring of microvilli (Fig. 2E, F). The shape of collar microvilli was highly variable in all sponges. Some collar microvilli were long and thin (2–3 μm long, 0.1 μm in diameter) and individual microvilli were linked by fine filaments, leaving a space between microvilli $\leq$ 0.1 μm (Fig. 2E), but many collars were short and their microvilli were thick ($\leq$0.5 μm long, up to 0.15 μm thick). In other collars, microvilli were fused to one another (Fig. 2F). Experimentation with a variety of fixatives and techniques has shown that fixation procedure, excess handling, and periods of anoxia can greatly affect the shape of the choanocyte cell body, but it was not clear that collar structure was equally sensitive. In our feeding experiments we took great care to reduce excess handling and processing time, but we still found that the morphology of collars was inconsistent, even among neighboring choanocytes in the same chamber. However, all choanocytes in this experiment, regardless of collar shape, were readily capable of particle uptake.

Full details of the fine structure of *Sycon coactum* are presented elsewhere (Eerkes-Medrano and Leys, 2006).

Time of particle uptake

Choanocytes of all sponges fixed 5–10 min after being fed were already filled with phagosomes containing either beads or bacteria, suggesting that uptake occurs within only a few minutes of particles entering the animal. Yet, in specimens fixed up to 1 h after feeding, chambers still contained beads or bacteria. Many instances of phagocytosis were found in sponges fixed 2 h after feeding, but no beads or bacteria were found in sponges fixed 6 h after feeding. While the time of uptake of particles was independent of particle size or type, the location and method of uptake varied considerably with both particle size and type.

Location of particle uptake

Most particles were found in association with the cell surface, lodged between neighboring choanocytes, rather than on the collar microvilli (Fig. 3, Fig. 4A, C, D). Few large particles (l-μm and 0.5-μm beads and bacteria) were found adjacent to collars, except where particles were very dense. For example, in an image of 180 cells, only 1–2 beads were found adjacent to a collar (Fig. 4A, B). In regions where beads or bacteria were dense, the ratio of particles to collar was 1:4 (4 particles on the collars of 16 cells; *e.g.,* Fig. 4C, D). Only 0.1-μm latex beads were found in large numbers on collar microvilli, but these beads adhered even more often to the cell surface (Fig. 5A–C). A comparison of collar interactions with 1-μm beads over time is shown in Figure 6. Those beads adherent on collars were found on the outside of the collar, and in a few instances one or several adjacent microvilli were wrapped around particles.

Mechanisms of particle uptake

Most 1.0- and 0.5-μm beads and some bacteria were engulfed by broad lamellipodia at the cell surface (Fig. 7, 8, 9). More particles were engulfed at the side of the cell than at its apical surface, but the number of interactions of particles with the apical surface increased with time (Fig. 8C).

Many cells also produced extensions of the cell surface that reached several micrometers out from the lateral or apical surface to contact beads or bacteria (Figs. 8, 10). The average length of extensions was twice that of the collar microvilli (membrane extension: 2.7 $\pm$ 1.6 μm, range 0.5–16 μm; collar length: 1.2 $\pm$ 0.5 μm, 0.4–3.5 μm, n = 44) (Fig. 8B). The longest extensions were found in contact with aggregates of a mucoid material, but in pieces fixed 30 min after being fed *in situ,* several 7-μm-long extensions were found wrapped around clumps of 1-μm latex beads (Fig. 10A–E). A total of 30 lateral and 12 apical extensions were counted in 44 images; 9 extensions were directed into the chamber, while 33 contacted particles that were between choanocytes. In two cases the extensions were clearly formed by fusion of collar microvilli (*e.g.,* Fig. 10C). Extensions for 0.5-μm beads typically involved more than one bead (Fig. 10F), and often two or more cells were in contact with the same particle (Fig. 10F, G). Although extensions did contact natural bacteria (Fig. 9A), fewer extensions touched heat-killed bacteria than latex beads or natural

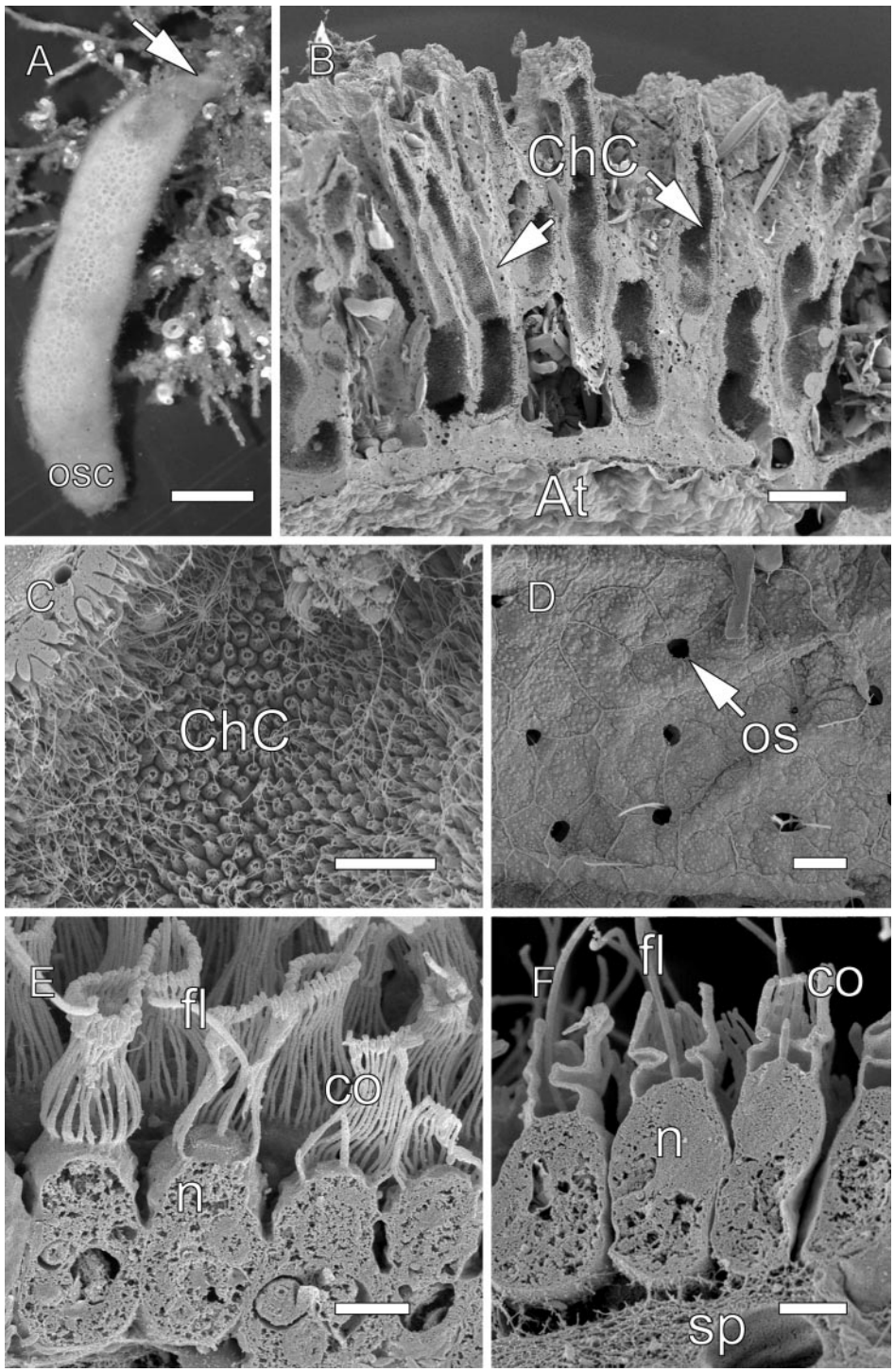

Figure 2. The general structure of *Sycon coactum.* (**A**) A digital photograph of the sponge attached to a bryozoan (arrow). Water is drawn in along the length of the animal and flows out of the single osculum (osc). Scale bar: 0.5 cm. (**B–F**) Scanning electron micrographs. (**B**) A fracture across the body wall showing the structure of the choanocyte chambers (ChC) that project from the atrial (At) cavity. Scale bar: 100 μm. (**C**) A portion of a choanocyte chamber (ChC) showing that the interior is lined by thousands of choanocytes. Scale bar: 20 μm. (**D**) The outer layer of the sponge, the pinacoderm, is formed of plate-like cells and ostia (os) formed by pore cells, through which water enters the chamber. Scale bar: 10 μm. (**E, F**) Collar microvilli (co) that surround the flagellum (fl) can be long and thin (**E**) or short, thick, and even fused (arrows) (**F**). The nuclei (n) of the choanocytes can be seen in the fracture, as can the space left by decalcification of a spicule (sp). E, F, scale bars: 2 μm.

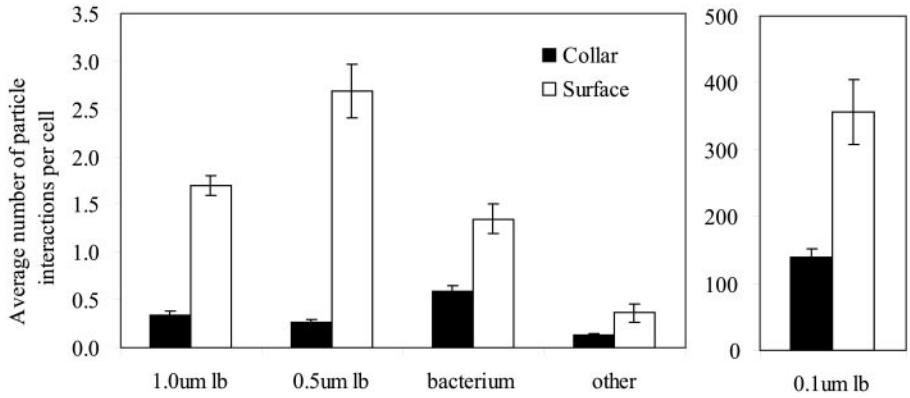

Figure 3. The average number of interactions per cell with either choanocyte collars (black bars) or choanocyte cell surface (white bars) for 1.0-, 0.5-, and 0.1-μm latex beads (lb), bacteria, and natural particles (other). Bars indicate standard error.

particles. Some heat-killed *E. coli* were engulfed with a lamellipodium (Fig. 9B), but in most instances the bacteria appeared to simply sink into the cell (Fig. 9C).

Only large particles (large beads and bacteria) were found in contact with flagella (24 instances observed). No pseudopodial extensions were found with 0.1-μm latex beads.

Uptake of natural food

The validity of the varied mechanisms of particle uptake we introduced experimentally is substantiated by examples of natural uptake of intact or broken diatoms (2–3 μm) by five separate choanocytes (Fig. 9D, E). Diatoms were always ingested at the apical surface of the cell, as were aggregates of mucous-bound material (2–3 μm in diameter) (Fig. 9F).

Discussion

Sycon coactum began to phagocytose particles immediately after they were introduced and uptake continued for at least 2 h after the initial feeding. All sizes and shapes of particles were ingested, and all choanocytes took up particles regardless of size or shape of collar microvilli. However, the mechanism of uptake varied with the size and type of particle. Only the finest particles (0.1-μm beads) adhered to collar microvilli in great numbers. Most particles contacted the cell surface, and larger particles and clumps of particles were attached to extensions of the cell surface. The longest extensions were produced 30–60 min after particles were introduced to the sponges. The results suggest that although the collar of syconoid sponges may entrain flow around the surface of the choanocyte, uptake of particles greater than 0.1 μm is independent of the collar.

The collar as a filter

Sponges are generally thought to filter with a true collar sieve (Riisgård and Larsen, 2000, 2001), yet in reality we know very little about the means by which particles are brought to, and interact with the collar microvilli, or how they are engulfed by the cell. To the best of our knowledge there are only two examples of sponge filters in which water is forced by physical barriers to pass through the collar microvilli. In the glass sponges *Oopsacas minuta* and *Rhabdocalyptus dawsoni,* particles are thought to be trapped at the collar microvilli by a thin layer of the syncytial trabecular reticulum that surrounds the distal region of each collar (Perez, 1996; Wyeth, 1999). The freshwater demosponge *Spongilla lacustris* has a similar feature made of a glycocalyx mesh that lies between the tips of adjacent collars and is thought to function like a strainer, forcing water through the collar microvilli (Weissenfels, 1992). In other sponges, no such barrier seems to exist, unless fixation techniques do not preserve it, and chambers could be interpreted as being leaky. In *Sycon* there is no membrane or protein mesh that links the tips of adjacent collar microvilli, yet all choanocytes were still capable of taking up particles. However, most particles were phagocytosed at the base of the collar; only 0.1-μm beads were found adhering to collars in substantial numbers, but these were even more numerous below the collars on the cell surface. Interestingly, those 0.1-μm beads that were attached to the collar were not retained by the fine mesh of the filter pores as would be expected for a true sieve filter (Rubenstein and Koehl, 1977); instead, they adhered to the outside of individual microvilli. Adhesion to the filter surface is not considered to occur in a true sieve (LaBarbera, 1984), but particles are also said to adhere to collars in choanoflagellates, which allows them to be transported down toward the surface of the cell (Lapage, 1925). The large numbers of 0.1-μm beads found at the base of collars 20 mins after feeding suggests that membrane transport of adherent particles may also occur down microvilli in *Sycon.*

It is generally imagined that particles such as bacteria that are caught on the collar microvilli, if not transported to the

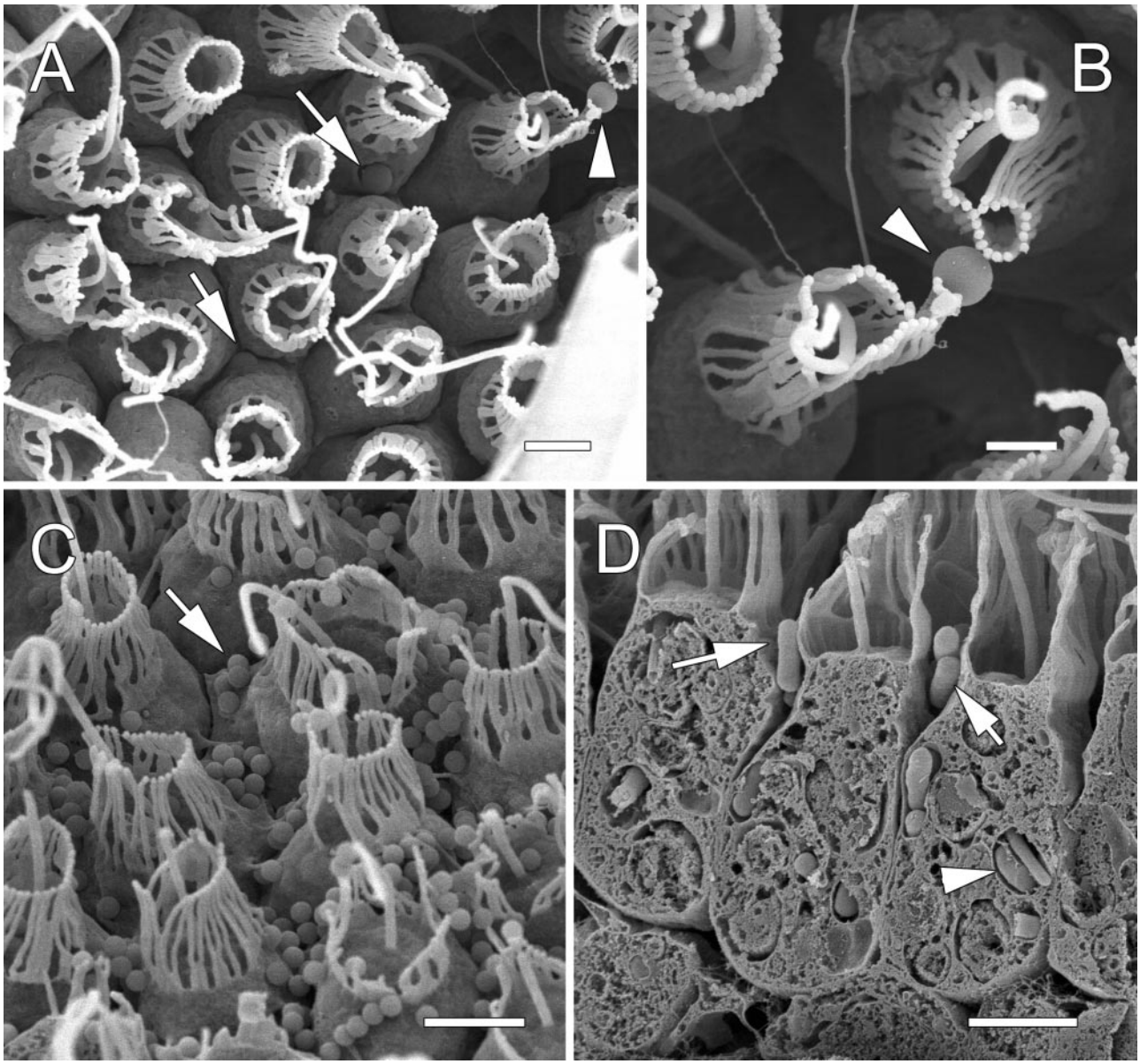

Figure 4. Scanning electron micrographs of interactions of large particles with cells. (**A**) For 1.0-μm latex beads, interactions with the surface (arrows) were more numerous than interactions with collars (arrowhead). Scale bar: 2 μm. (**B**) Higher magnification of the bead shown on the collar in (A). Scale bar: 1 μm. (**C**) A region where many 0.5-μm beads were captured. Most of the beads are lodged between neighboring choanoctyes (arrow). Scale bar: 2 μm. (**D**) Heat-killed *E. coli* bacteria between neighboring choanocytes (arrows) and in phagosomes (arrowhead). Scale bar: 2 μm.

base of the collar by membrane transport, are phagocytosed by pseudopodial extensions that grasp them off the collar. However, in 312 images of more than 50 specimens, we did not find one example of this. Pesudopodia were involved in feeding, but they did not phagocytose particles attached to the collar. We found that most large particles (bacteria and 0.5–1.0-μm beads) were engulfed either directly by the cell surface or with pseudopodial extensions, rather than the collar.

Phagocytosis by pseudopodial extensions

Phagocytosis of larger particles by pseudopodia has been shown to occur in many demosponges and is not in itself unusual (Schmidt, 1970; Willenz, 1980; Imsiecke, 1993). Pinacocytes can phagocytose particles as large as 5.7 μm on the surface of the sponge (Willenz and Van de Vyver, 1982), and it is generally assumed that the pinacocyte epithelium that lines canals can take up particles as large as 50

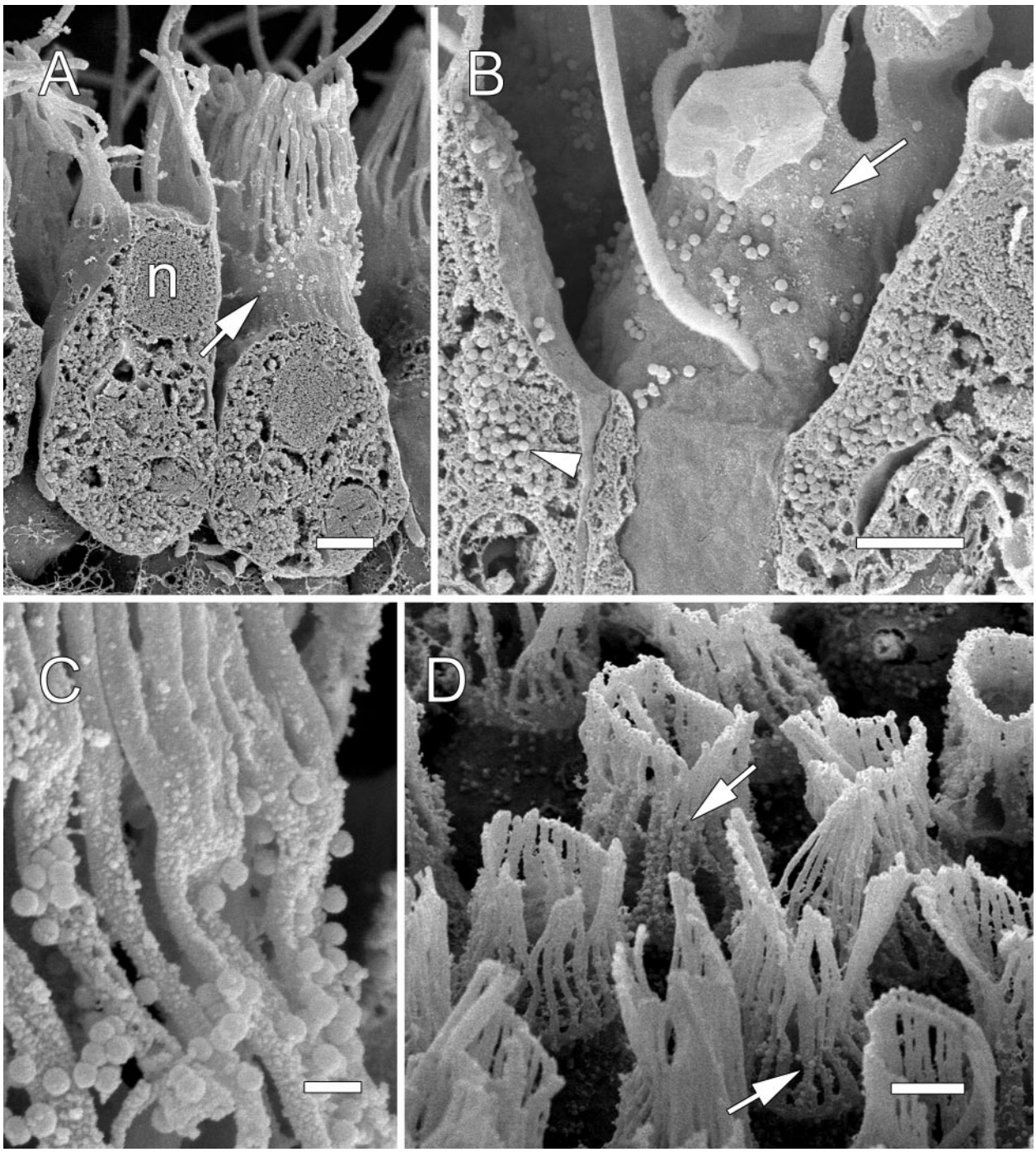

Figure 5. Scanning electron micrographs of 0.1-μm latex beads on collars and the choanocyte surface. (**A**) Beads on the cell surface (arrow) and the collar microvilli. Nucleus (n). Scale bar: 1 μm. (**B**) Beads (arrow) on the cell surface and in phagosomes (arrowhead). Scale bar: 1.0 μm. (**C, D**) Beads adhered to the collar microvilli. Scale bars: C, 200 nm; D, 1 μm.

μm (Weissenfels, 1992). Phagocytosis of particles at the apical surface of choanocytes is less common, since demosponge collars do not appear to allow large items within the collar microvilli. An exception may be the periflagellar sleeve of *Suberites massa,* a permanent sheath-like extension of the choanocyte surface (Connes *et al.,* 1971) that is thought to be involved in feeding (Simpson, 1984).

What is unusual and intriguing, however, is the manner in which the choanocyte surface in *Sycon coactum* extends laterally and apically to phagocytose the latex microspheres

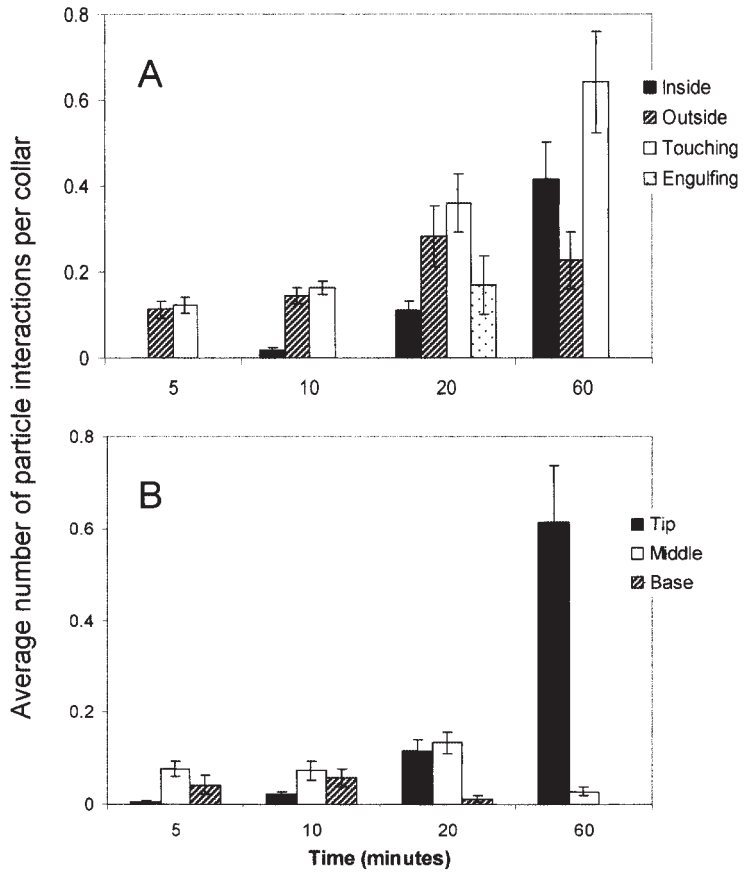

Figure 6. Precise method of interaction of 1.0-μm latex beads with the collar microvilli over time. (**A**) The average number of particles counted on the inside and the outside of the collar, and the type of contact with the collar, whether the microvilli are touching or engulfing the particles. (**B**) Location of the beads (tip, middle, or base) on the collar over time. Bars show standard error.

or bacteria, and the observation that often several cells extend pseudopodia apparently for the same particle (*e.g.,* Fig. 10G). Some pseudopodia are up to twice the height of the collar microvilli and contact a group of latex beads or piece of mucoid material well above the collar microvilli. The images capture a remarkable feat of the cell membranes. Not only can large amounts of membrane be generated over a relatively short time (lateral extensions 1–2 μm long were present in sponges fixed 5–10 min after being fed), but in some manner the cells must sense the particles even through they are several micrometers away from the cell surface; how this is achieved is unknown. The presence of membrane extensions 5 min after feeding indicates that the sponges are relatively quick to detect food particles. However, the increase in both collar and surface interactions for 1.0-μm beads over time shows that it takes time before the entire population of choanocytes in the chamber becomes involved in phagocytosis of particles.

Given the high volumetric pumping rates recorded from demosponges (Reiswig, 1971; Vogel, 1974), it is unknown how particles remain stationary long enough in the choanocyte chamber of *Sycon* to be phagocytosed by pseudopodial extensions. Several possibilities must be considered. First, could sponges have arrested their feeding current or become clogged shortly after the first beads entered the chambers? Only glass sponges (class Hexactinellida) are known to be able to arrest flagella in response to mechanical stimuli (see review by Leys and Meech, 2006). Although in *Sycon* the ostia close in response to excessive handling (as was seen in the controls used to assess the effects of handling), in sponges fixed during feeding experiments no ostia were closed or constricted or blocked by excess particles; thus it is unlikely that flow ceased at any point during feeding. Second, was the concentration of particles abnormally high, causing an aberrant mechanism of uptake? In fact, the concentration of bacteria fed to the sponges was 2-fold lower than in the sponge's natural environment (in July 2004, 1×10^6 ml^{-1} bacteria were counted in the Bamfield Marine Science Centre seawater system in water drawn from 25 m; Yahel *et al.,* 2006). We used a concentration of latex beads similar to that used in previous *in vitro* feeding studies of sponges (Willenz, 1980; Willenz and Van de

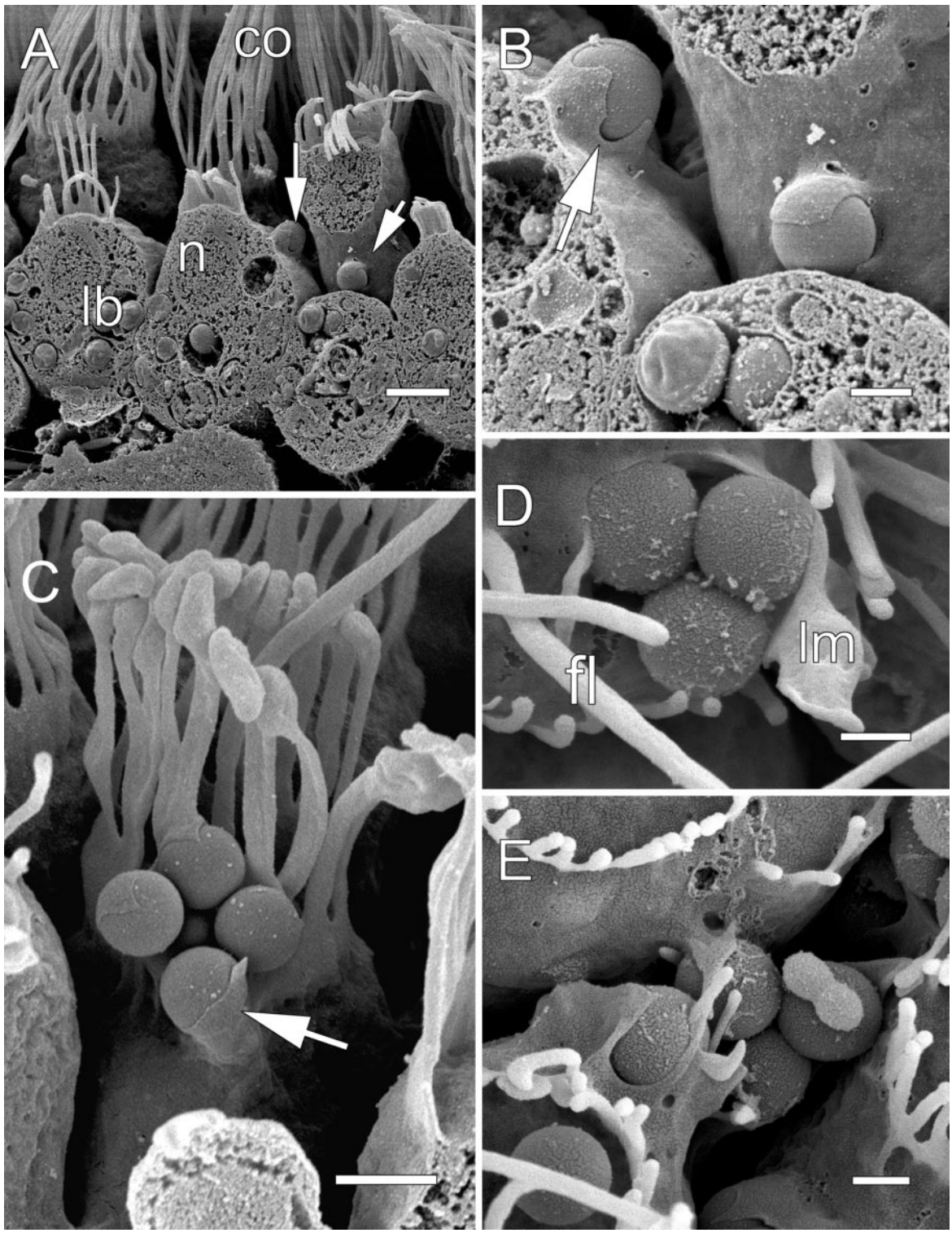

Figure 7. Scanning electron micrographs showing phagocytosis of 1.0-μm beads by lamellipodia. (**A**) Beads (lb) in cells and being engulfed (arrows) at the side of the cells (co, collar; n, nucleus). Scale bar: 2 μm. (**B**) Detail of the beads being engulfed by lamellipodia in (A). The cell membrane forms a neat grip (arrow) on the bead. Scale bar: 1 μm. (**C**) Lamellipodia engulf a group of beads at the base of the collar of a single cell. Scale bar: 1 μm. (**D**) A cluster of beads being engulfed by a lamellipodial (lm) extension at the apical surface between the flagellum (fl) and collar. Scale bar: 0.5 μm. (**E**) Three choanocytes use broad lamellipodia to engulf beads lodged between the cells. Scale bar: 0.5 μm.

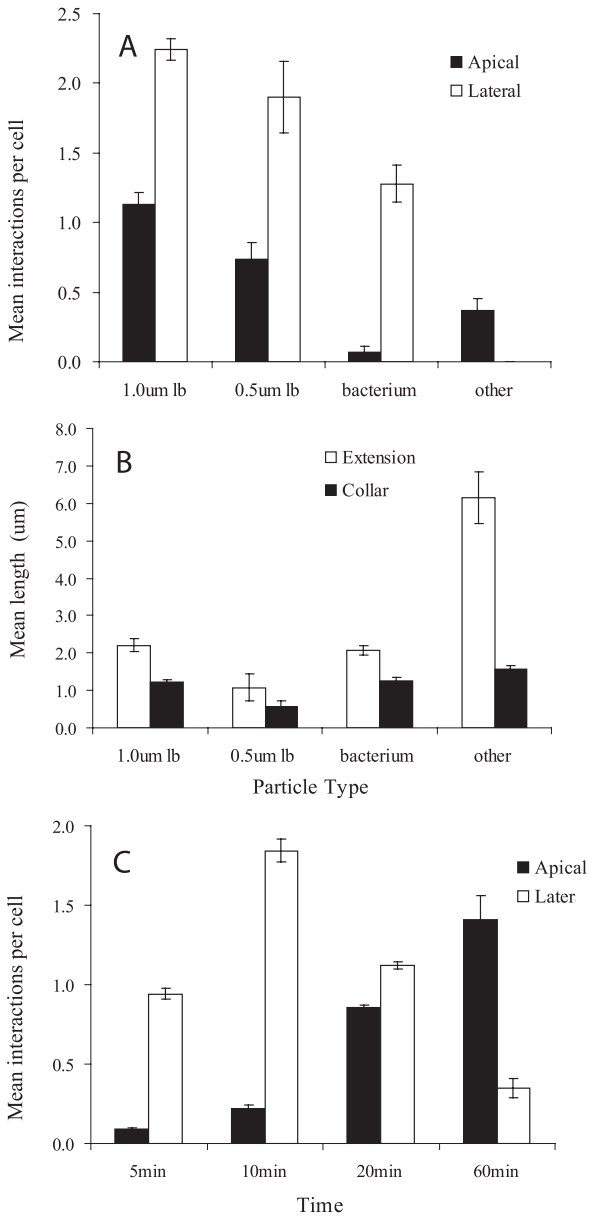

Figure 8. Interactions of beads (lb) and bacteria with the cell surface. (**A**) The mean number of apical (black bars) or lateral (white bars) interactions for each particle type. (**B**) The length of pseudopodial extensions (white bars) and collars (black bars) for each particle type contacted. (**C**) The mean number of apical (black bars) or lateral (white bars) particle interactions over time. Bars show standard error.

Vyver, 1982; Willenz *et al.,* 1986), but despite attempts to disperse the particles evenly, the distribution of both beads and bacteria was very patchy in any one sponge and even within a single chamber. While one region of a chamber was filled with particles, an adjacent region had only a few particles. Although we cannot rule out the possibility that exposure to high concentrations of particles could cause abnormal feeding behavior, choanocytes took up particles in a similar manner whether particles were sparse or dense. Third, could the particles (beads and bacteria) have fallen to their present locations after flow stopped in the sponge at the moment of fixation? Although this would be difficult to disprove, the presence of lamellipodia wrapped around portions of the beads (in the same way that the lamellipodia appeared to engulf diatoms, *e.g.,* Fig. 9D) suggests the contact was not one of chance. Furthermore, most 0.1-μm beads did not drop off the collar microvilli at fixation, nor did some 1-μm beads that were found on collars (*e.g.,* Fig. 4A,B).

The possibility that pseudopodia are used to reject or eject unwanted particles has also been considered. However, we found clear examples of egestion in samples fixed 6 h after being fed. During egestion, choanocytes lose normal morphology, become ameboid-shaped, and move up into the chamber with waste beads and broken diatom pieces. Exactly how this debris is ejected from the cell once in the chamber is not known.

The inference of living processes from static images is definitely problematic, and these difficulties will remain until a mechanism is developed to view live chambers in the process of feeding. However, consistent results with a variety of fixatives (see Eerkes-Medrano and Leys, 2006), and controls for handling suggest that phagocytosis by pseudopodia is not an artifact. Furthermore, excellent images of phagocytosis by pseudopodia in the choanoflagellate *Codosiga* (Leadbeater and Morton, 1974) show that choanflagellates can generate equally long membrane extensions; similar pseudopodial extensions were described by de Saedeleer (1929), who also first suggested that calcareous sponges fed much like choanoflagellates. In *Codosiga* it is suggested that the pseudopodia use the collar microvilli as a guide to locate the bacteria caught on the collar. In *Sycon* very few particles seemed to be caught on the collar, and some pseudopodia extended up from the apical surface of the cell, so it is not clear exactly how the collar is used in particle capture and uptake. One possibility is that the collar, rather than strictly sieving, may entrain the flow, drawing particles into an area of sluggish water at its base. Nevertheless, if the images are a correct representation of events frozen in time, then the fact that the largest extensions were observed in contact with natural food items suggests that the sponge may use pseudopodia to select the larger particles. If these particles (such as diatoms) are more nutritious, this mechanism could explain the particle selectivity found by Frost (1980).

Although feeding by long pseudopodia may be more common in demosponges than is generally thought, the images presented here that show uptake of whole diatoms and mucoid-coated material by choanocytes indicate that in syconoid chambers the technique has been perfected. This type of sponge may rely more on active capture of large food items than on passive filtering using the collar, to select nutritionally favorable items when food is abundant (*e.g.,*

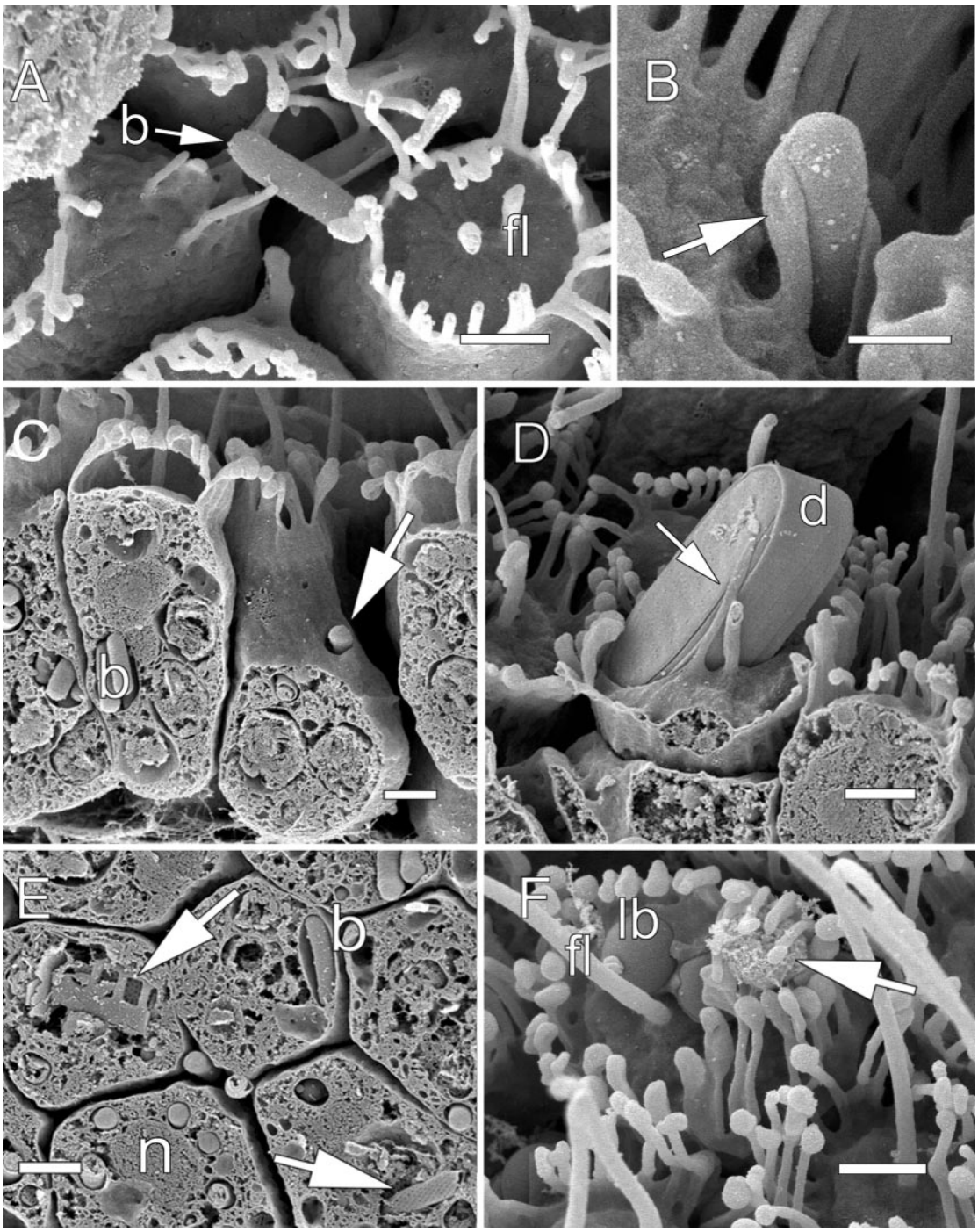

Figure 9. Uptake of natural particles and heat-killed *E. coli* bacteria (scanning electron microscopy). (**A**) Lateral extensions from more than two cells extended towards a natural bacterium (b) found in a bead-fed sponge experiment (fl, flagellum). Scale bar: 1 μm. (**B**) An *E. coli* bacterium being engulfed by a lamellipodium (arrow) at the side of a choanocyte. Scale bar: 0.5 μm. (**C**) *E. coli* bacteria (b, and arrow) phagocytosed by a choanocyte. Cells package several bacteria into single phagosomes. Scale bar: 1 μm. (**D**) A diatom (d) being engulfed at the apical surface of the choanocyte. A lamellipodium (arrow) lies along the length of the diatom. Scale bar: 1 μm. (**E**) A fracture through several choanocytes shows phagosomes containing broken diatoms (arrows) and bacteria. Nucleus (n). Scale bar: 1 μm. (**F**) A choanocyte ingesting 1.0-μm latex beads (lb) as well as mucous-bound material (arrow) at its apical surface. Collar microvilli are curled around the mucoid ball. Flagellum (fl). Scale bar: 1 μm.

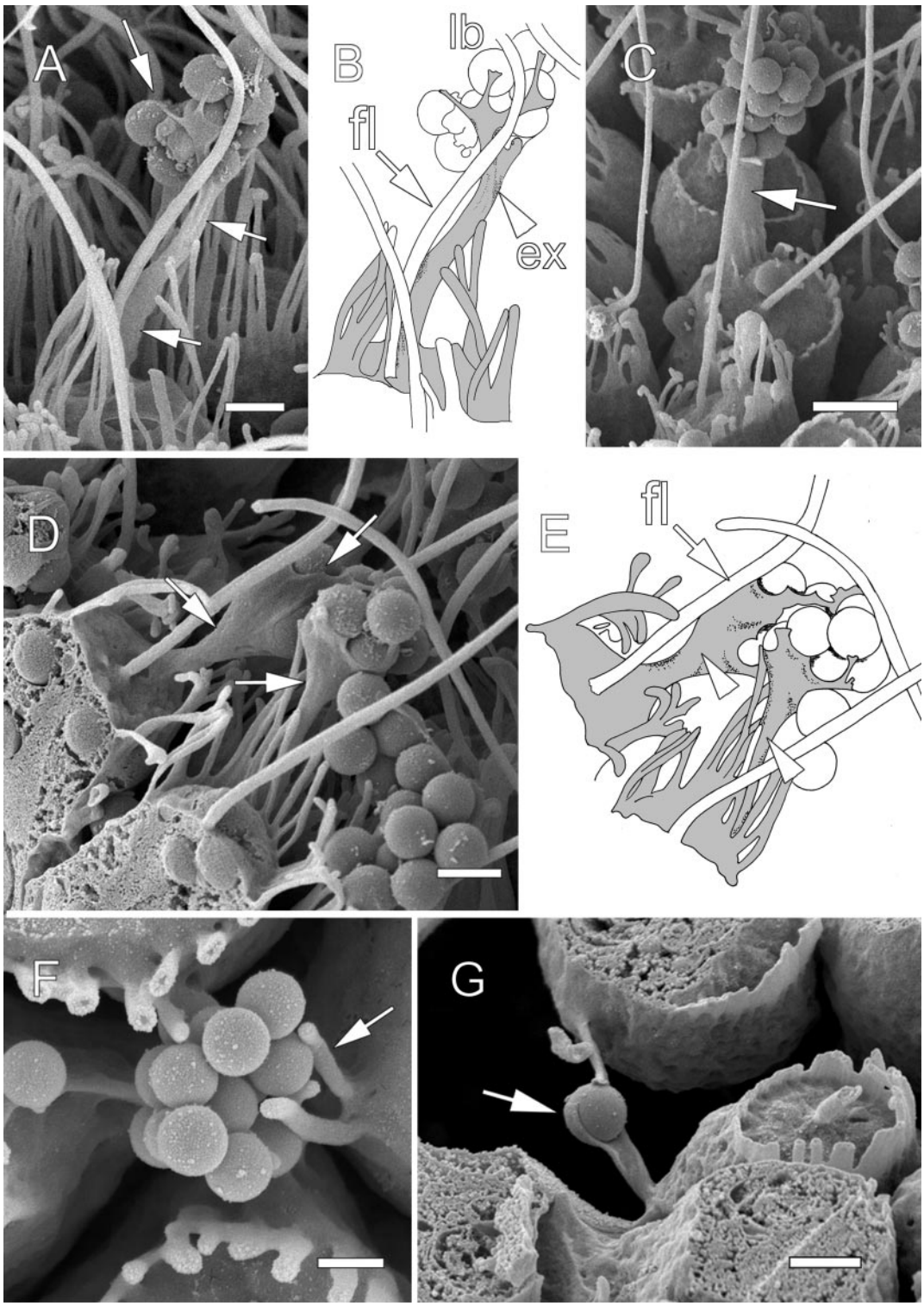

Figure 10. Uptake of 1.0-μm and 0.5-μm latex beads by pseudopodial extensions (see arrows). (**A**) Scanning electron micrograph (SEM) and (**B**) tracing of the micrograph, of a 7-μm-long extension (ex) around a cluster of 1.0-μm latex beads (lb; fl, flagellum). Scale bar: 1 μm. (**C**) SEM showing an extension formed by the fusion of collar microvilli enveloping a cluster of 1.0-μm beads. Scale bar: 2 μm. (**D**) SEM and (**E**) tracing of the micrograph showing two extensions (arrowheads) in contact with clusters of 1.0-μm beads (fl, flagellum). Scale bar: 1.0 μm. (**F**) SEM of 4 cells extending pseudopodia toward a cluster of 0.5-μm beads. Scale bar: 0.5 μm. (**G**) SEM of pseudopodial extensions from two cells in contact with the same particle. Scale bar: 1 μm.

during a summer diatom bloom). This mechanism would not be effective when food is scarce in winter, which may explain the ephemeral summer existence of this sponge.

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