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On particle filtration by amphioxus (*Branchiostoma lanceolatum*)

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We show in this paper that amphioxus (*Branchiostoma lanceolatum*) is capable of collecting sub-micron particles on its mucous filter. This is made by an endostyle in some respects simpler than those of tunicates, and that unlike the filters in tunicates, the strands of the amphioxus filter are sticky. It therefore does not act simply as a sieve.

INTRODUCTION

Filter feeding in amphioxus was first carefully described in this journal just over 90 years ago by Orton (1913). Orton showed that the ciliated gill bars were not respiratory in function, as previously supposed, but a filter-feeding apparatus. He pointed out that small particles were captured on mucus secreted by the endostyle, and sometimes formed strings, which passed vertically upwards over the inclined gill bars to reach a dorsal groove, from which they were carried backwards to the oesophagus. To trace the pathways followed by the particles trapped in mucus, Orton used carmine particles, or water containing diatom particles and methylene blue. Although it was evident that amphioxus could collect small particles, he did not consider what minimum particle size could be collected.

Subsequently, Gosselck et al. (1978) examined the gut contents of *Branchiostoma senegalense* after feeding in the wild, and after a mixed diet in the laboratory which included sub-micron activated carbon particles. They concluded that the upper limit for ingestion was 300 μm, and that bacteria (approximately the same size as the carbon particles), could be ingested. More recently, Riisgård & Svane (1999) determined (by feeding amphioxus with a mixture of different-sized flagellates) that 4 μm particles were collected with 100% efficiency. Lastly, Ruppert et al. (2000) fed the Florida amphioxus (*B. floridae*) on particles of different sizes, and by examining the faeces showed that particles down to 0.06 μm were captured. However, this approach gave no indication of the efficiency of the process, nor (as for Gosselck et al.'s (1978) observations) whether the particles had been aggregated when collected.

The different ascidian species that have been examined have mucus-net filters capable of retaining small particles, for example *Ciona* traps 1 μm carbon particles efficiently (Jorgensen & Goldberg, 1953). Our own experiments using sepia ink show that *Ciona* can capture 0.3 μm particles, but capture efficiency was not examined. These remarkable mucus filter nets have very regular sub-micron meshes (Flood & Fiala-Medioni, 1981), so too do those of salps (Bone et al., 1991, 2000). In appendicularian tunicates, the filter is less regular (Diebel & Lee, 1987), but this may be artefactual, due to the great difficulty of preserving the filter in these small animals. At all events, they can capture sub-micron particles (Deibel & Lee, 1992; Flood et al., 1992).

In amphioxus, Flood (1981) figured a portion of a regular mucous net filter, similar to those of ascidians, but accurate measurements of filter mesh (such as those made on the ascidian filters) were not possible. This paper in part extends the feeding experiments of Riisgård & Svane (1999) to determine the efficiency of feeding on sub-micron particles, and also examines how the mucus produced by the endostyle actually captures particles.

MATERIALS AND METHODS

Adult amphioxus (*Branchiostoma lanceolatum* Pallas) were trawled from the Eddystone shell gravel off Plymouth (Smith, 1932) and maintained in the laboratory circulation until required. To visualize the feeding current and particle capture, individual animals were observed in Petri dishes and 'fed' either with carmine particles or with small polystyrene beads labelled with FITC. The former were examined on a binocular with transmitted light, the latter with a similar instrument using epifluorescence and appropriate filters. With both methods, it was easy to see (as Orton (1913) did) particles passing into the pharynx via the velar aperture and either rapidly running the length of the pharynx to exit via the atriopore, or, becoming attached to the gill bars and endostyle. For particle retention experiments, 10–12 animals were placed in crystallizing dishes with 200 ml of circulation seawater to which various sizes of microspheres had been added. No attempts were made to measure flow rates. Two ml samples were taken at 15 min intervals for periods of 1 h and examined in a Becton Dickinson Facsort flow cytometer. The samples were analysed with the flow cytometer programme Win MDI v. 2.8.

For scanning microscopy of the gill bars and endostyle, animals were fixed in various ways prior to dehydration and critical point drying. The most successful approach was to place the endostyle and the bottom half of the gill bars dissected from the animal, in small dishes to which algal

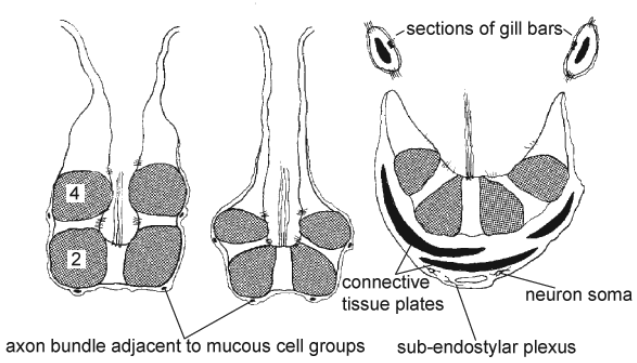

Figure 1. Cross-sections of endostyles of tunicates and amphioxus (not to scale). The cell groups 2 and 4 (shaded in each) secrete the strands of the mucous filter. Other cell groups apparently do not secrete mucous filter strands. Left, an ascidian (*Ciona*), partly after Holley (1986); middle, a salp, drawn from semi-thin resin sections; right, amphioxus (*Branchiostoma*), drawn from serial sections of mid-region of body.

suspensions had been added. The endostyle continues to secrete mucus under these conditions, and the gill bar cilia continue to beat, hence algal cells are captured and pass to the tops of the bars as they do in the intact animal. In tunicates (personal observations) and so too in amphioxus, when anaesthetized, the gill bar cilia beat continuously, but no mucus is secreted by the endostyle, hence algal cells are not captured. Fixation with 1–2% osmium tetroxide was followed by dehydration in 70% ethyl alcohol and critical point drying from acetone or absolute ethanol. Other portions of gill bars were frozen in liquid nitrogen and thawed in 2% OsO_4 before similar dehydration. Scanning electron microscopy studies of the gill bars used a JEOL 35C and a JEOL JSM-5600LV.

RESULTS

Structure of the endostyle

The filtering system of amphioxus is basically similar to that of ascidian tunicates, with similar ciliary tracts on the gill bars (Orton, 1913). In ascidians and salps, the endostyle opens into a more or less deep groove, and the filter net is elaborated within this groove (Bone et al., 2000). In amphioxus, however, the endostyle is an open U in transverse section (Figure 1), so that the mucous filter is deployed more or less directly into the pharynx from the secretory cells of the endostyle, over the bases of the gill bars. Significantly, this means that the filter has to be built within a much shorter distance from the secretory cells than in these tunicates, closer to the endostyle margin. In our preparations, the central very long cilia of the endostyle are collapsed upon themselves (Figure 2) or partially covered with a mucous sheet (Figure 3). There is no evidence for the special filament-spacing rows of cilia seen in the ascidian and salp endostyles (Holley, 1986; Bone et al., 2000).

Under the endostyle there are coelomic and blood spaces and a rich nerve plexus, the whole being finally covered by a thin layer of atrial epithelium, as in Figure 1. The nerve plexus contains an abundance of multipolar neuron somata, as does that on the gut (Bone, 1961).

The endostylar filter and its operation

Observation of the living animal under a binocular microscope entirely confirms Orton's careful account of the way in which the filter entraps particles, though of course the filter itself is not visible. Often, discontinuous horizontal lines of mucus and trapped particles travel upwards along the gill bars, (Figure 4) remaining more or less horizontal until they reach the top of the bars to become incorporated in the mucus passing to the gut along the dorsal ciliated groove. That is, they do not pass upwards along the long axis of the bars, but at an angle to it. This is because the frontal cilia of the gill bars are aligned at an oblique angle to the long axis of the bar (Figure 5), producing (as Orton, 1913 observed) a current passing directly dorsally. Our views of the pharyngeal side of the gill bars after freezing in liquid nitrogen show the frontal cilia set obliquely along the bars at some 30–35° to their long axes (Figure 5). Holley (1984), using a water immersion objective and thus at much higher magnification than the binocular microscope, observed the passage of a 'mucus sheet' across the pharyngeal surface of the bars, at 38–48° to their long axes.

On the atrial side of the frontal cilia (i.e. away from the pharynx) longer lateral cilia (Figures 4, 7&8) act as (Orton described) to draw water from the pharynx to the atrium and thence to the atriopore. All are not always active, and some on portions of gill bars are still, even when the animal is feeding actively, and then, disposed more or less at right angles to the gill bar surface, they are so long that the tips of the cilia from adjacent bars touch, as seen in Figure 8.

Evidently amphioxus can inhibit the lateral cilia. Entry of distasteful water into the pharynx causes rapid inhibition of the lateral cilia. In our excised gill preparations and even in small, transparent intact animals actively feeding, the cilia on some gill bars and some cilia on many bars, do not beat. If anaesthetized, all lateral cilia beat continuously, and mucus is no longer secreted by the endostyle.

In operation, as Knight Jones (1954) showed, the direction of metachronal beat on apposed cilia is from the border of the endostyle, algal cells are rapidly ejected from the endostylar surface onto the surface of the gill bars to join those passing obliquely upwards from the base of the gill bars. In fixed material examined with the scanning microscope, similar lines of aggregated strings of mucus can be seen (Figure 4) overlying the frontal cilia of the gill bars. After feeding algal cells, such as *Isochrysis galbana*, (kindly provided from the MBA Plymouth algal culture collection), they are entrapped in a dense web of mucous filaments some 44–65 nm in diameter that overlie the frontal cilia of the gill bars, or amongst compound mucus strands over them (Figures 4, 6–8). The mean of measurements of these filaments from material frozen with liquid N_2 before fixation was 51.5 nm (N=9). These fibres also span the gaps between the gill bars at intervals, above the lateral cilia (as seen from the pharyngeal surface. On occasion (Figure 6), close to the endostyle, the filter fibres overlay each other to form an irregular kind of network, even occasionally a suggestion of a rectangular net (Figure 6 insert).

We have examined many series of gill bars fixed in different ways whilst the animal was feeding. Unlike the continuous salp or ascidian filter, we have been unable to demonstrate

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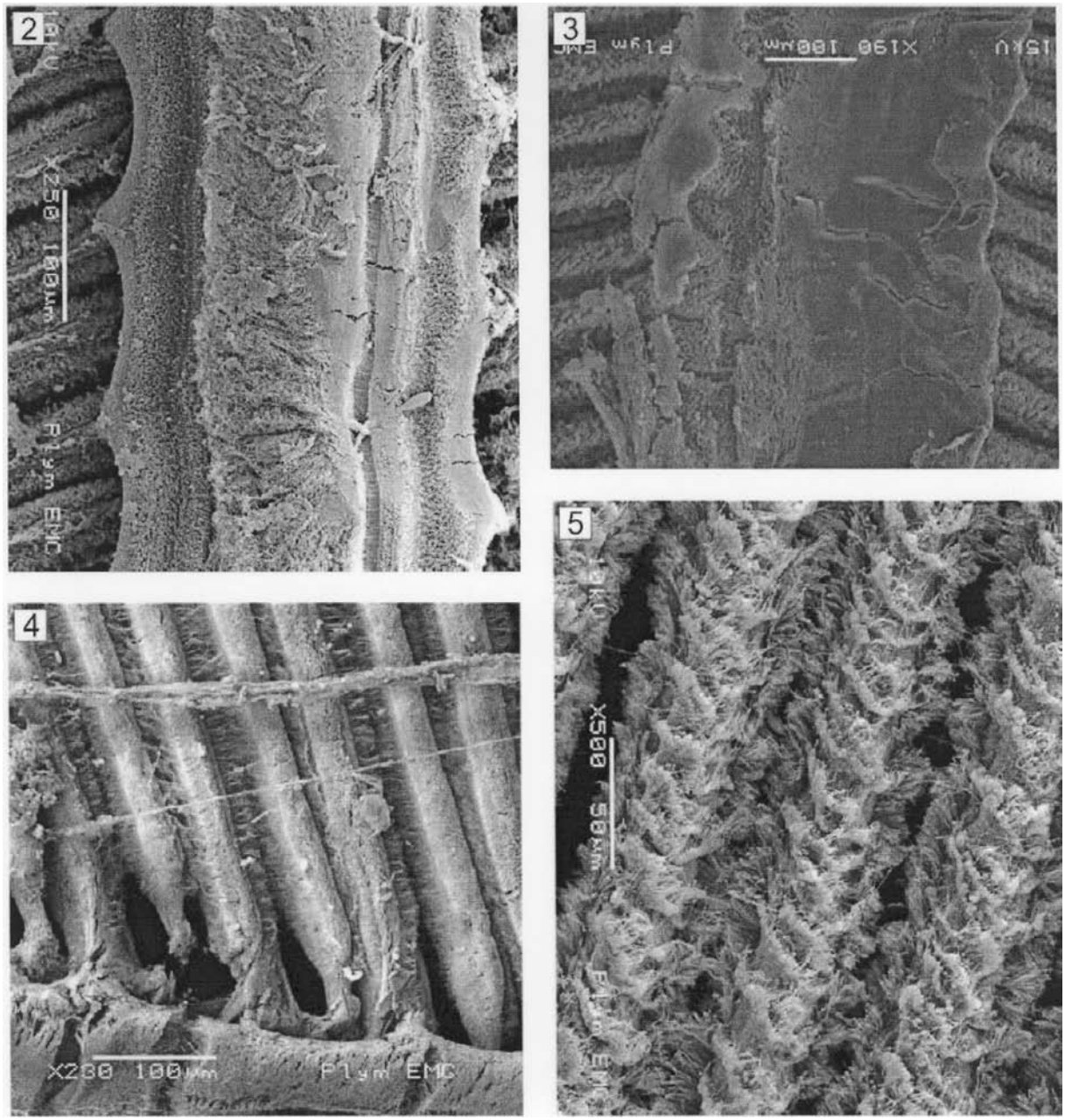

Figure 2. Endostyle from pharyngeal aspect, anterior upper right. Central long cilia of zone 1 partly entangled in mucus. No obvious lines of regularly spaced cilia on lateral regions of the endostyle. Leading to bases of gill bars. Scale bar: 100 µm.
Figure 3. Similar view, anterior to right. The left side of the endostyle is covered with a mucuous sheet, from which the tips of the cilia from zone 1 emerge mid right. The bases of the gill bars on right are partially covered with fragments of the mucuous sheet. Scale bar: 100 µm.
Figure 4. Aggregated more or less horizontal strings of mucus on gill bars fixed whilst animal was feeding. Scale bar: 100 µm.
Figure 5. Pharyngeal aspect of gill bars, dorsal at top. Note metachronal pattern of lateral cilia, and less organized beat of central frontal cilia. Scale bar: 50 µm.

a filter of regular mesh in any of our preparations. Perhaps the filter may emerge from the endostyle as a more regular net, but very soon after it passes onto the gill bars it seems to become disrupted and irregular. This is not, naturally, to say that such a regular net filter may not occasionally or even regularly be produced: if so, we have been unable to demonstrate it. Significantly, there is also another difference between amphioxus and the filters of tunicates, which is that the strands of the amphioxus filter are invariably coated with small particles suggesting that they must be sticky.

In addition to the fine mucous filaments on the gill bars, on several occasions we have observed sheets of mucus covering parts of the bars (Figure 7), or more often, overlying the endostyle. As already noted, the endostyle is associated with a plexus of nerve fibres containing neuron somata, and at least some of these fibres may be secreto-motor to the mucus glands of the endostyle, since mucus is not secreted when amphioxus is anaethetized. In our gill bar preparations, the descending rami of the atrial nerves, linking the cns with the gills and endostyle, have been cut.

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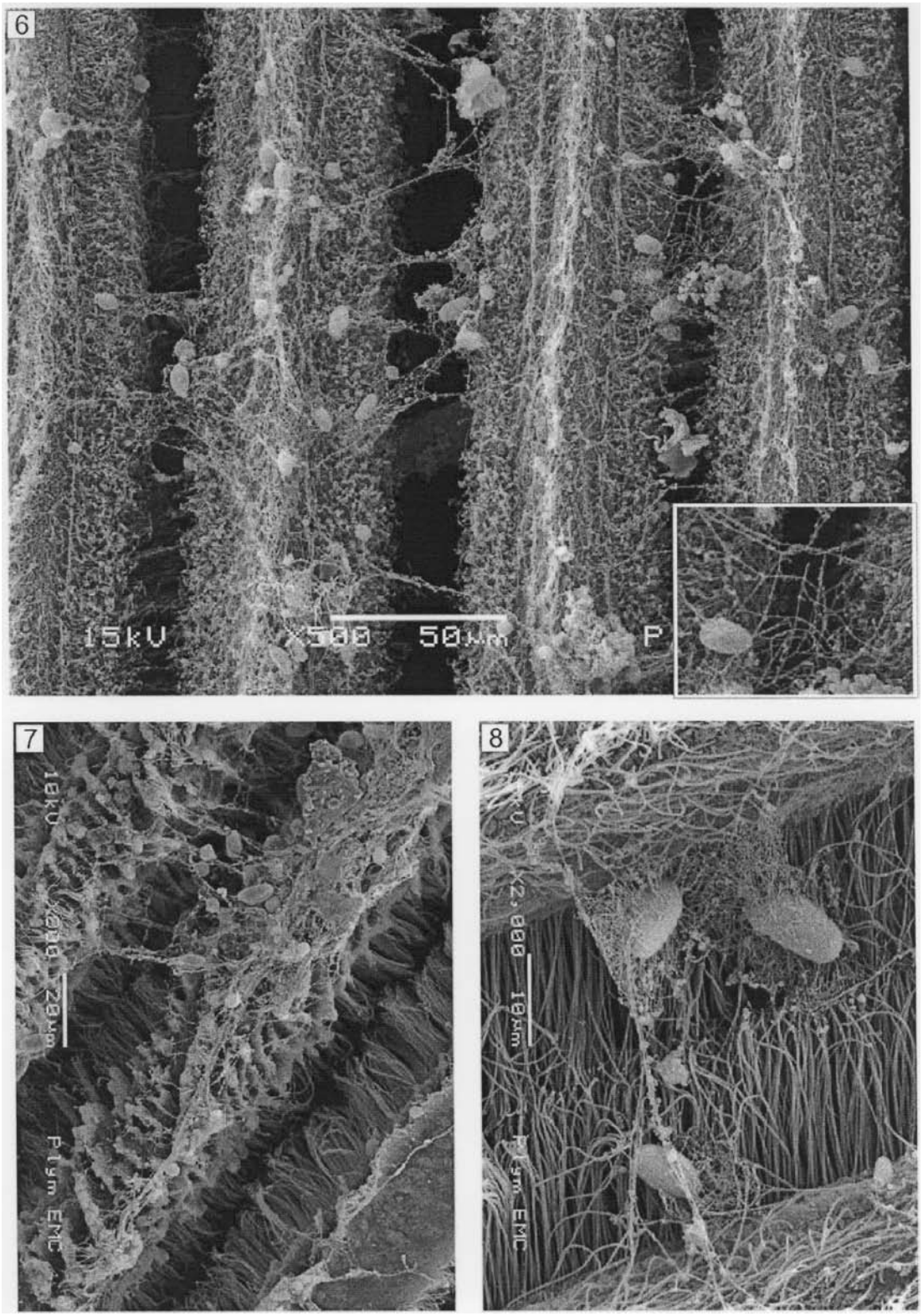

Figure 6. Pharyngeal face of gill bars fixed whilst feeding. *Isochrysis* cells haved been captured by the filter, some strands crossing between bars. Scale bar: 50 µm. Inset: portion outlined at higher magnification, probably showing remnant of an original rectangular portion of the filter.
Figure 7. Similar view, at higher magnification, showing *Isochrysis* cells caught on filter strands overlying frontal cilia. At bottom right, a mucous sheet covers the gill bar. Scale bar: 20 µm.
Figures 8. *Isochrysis* cells trapped in filaments of the filter net. The lateral cilia are apparently inactive in this view. Scale bar: 10 µm.

Particle feeding experiments

Our experiments have shown that amphioxus is capable of capturing particles down to 0.2 µm with reasonable efficiency. Figure 9 shows the simultaneous measurement of clearances from a mixture of different sizes of polystyrene beads (0.2, 0.5, 1.0 and 2.0 µm diameter). The sedimentation rates of the beads were also measured in controls without animals (not shown) and did not exceed 5% of the percentage change in

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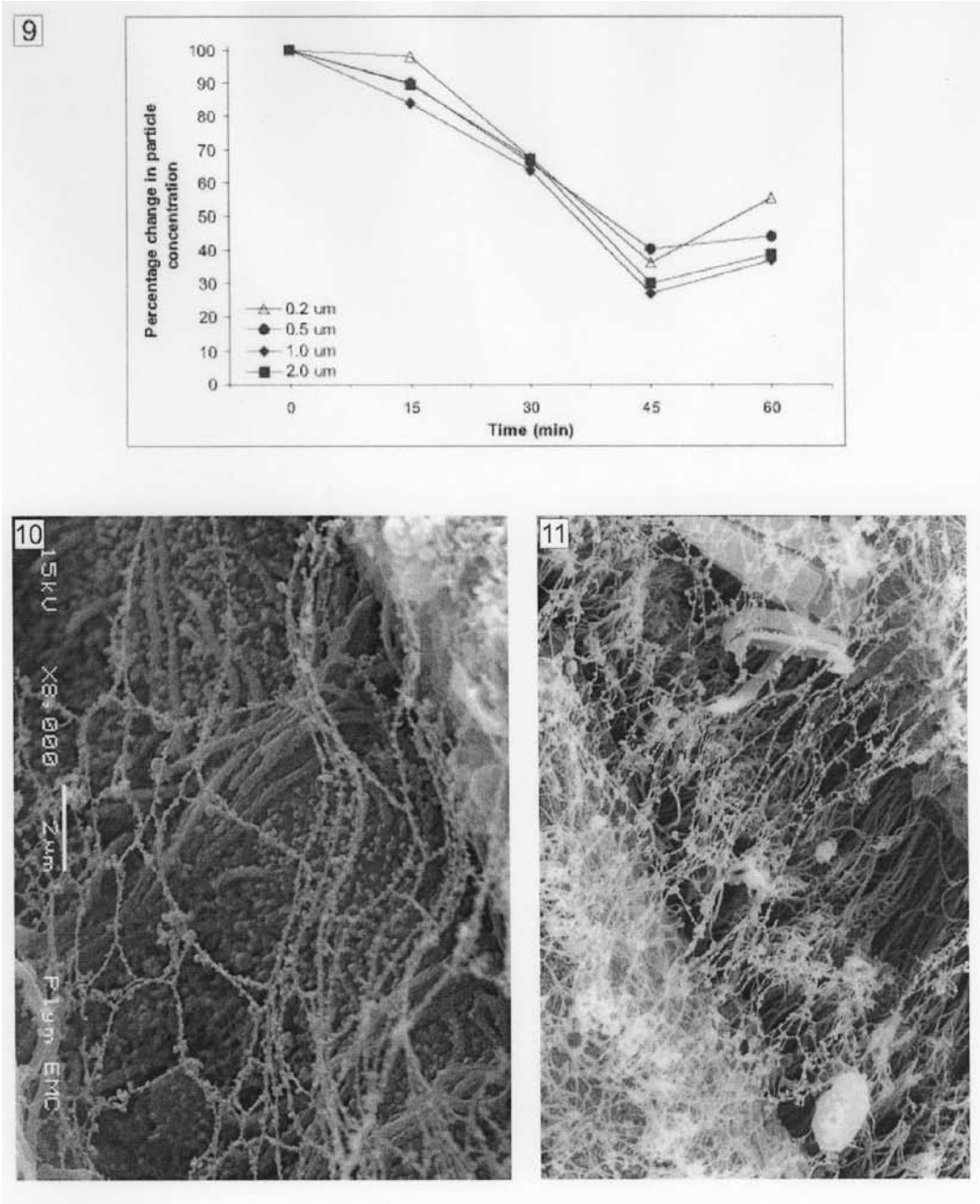

Figure 9. The clearance rates of various sized particles by the amphioxus filter during 1 hour.
Figures 10 & 11. Two views of the filter, showing the amphioxus characteristic of particles attached to filter strands. Scale bars: 2 μm and 20 μm.

particle concentration. In our experiments, the percentage change in particle concentration between 0 and 45 min was similar for all four bead sizes, with retention efficiency (RE) between 60 and 70% (Figure 9). For the 2 μm beads, RE was ~44%, for the 1 μm beads 56%, whilst for the 5 μm and 2 μm beads RE was ~60%. After 45 min, as seen in Figure 9, there is a slight decrease in RE, perhaps a consquence of exceeding the mean residence time for particles in the gut. In *Branchiostoma floridae*, Ruppert et al. (2000) noted that the mean residence time for particles in the gut was 80 min.

DISCUSSION

In this brief note we have shown that amphioxus is capable of collecting sub-micron particles on its mucus filter and although we have not been able to view a regular-mesh filter of the ascidian or salp type, we have been able to see that the filter strands are some 51.5 nm thick in fixed preparations, and apparently sticky. This compares with the thinner strands (10–40 nm) of different ascidian filters, the 30–200 nm strands of different salp filters and the 200 nm strands of a large appendicularian filter, none of which show apparent stickiness. Mesh sizes of the ascidian filter nets vary from 640–0.5 μm (Flood & Fiala-Medioni, 1981). They are constructed from fine mucous filaments arranged in two series at right angles to each other, so forming a filter net of square or rectangular mesh. In salps, the filter is equally regular, but the filaments are thicker (30–200 nm), and mesh size ranges from 0.3–5.4 μm (see Bone et al., 2003).

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There have been three suggestions about the way in which these delicate mucous filter nets of ascidians and salps are constructed. Flood (1981) figured a scanning micrograph of the amphioxus filter net (his figure 1b) that was less regular than the accompanying figures of several ascidian species, and the polychaete *Chaetopterus*. He suggested that the net-structure was a fundamental intrinsic property of mucus, and that the basic two- or three-dimensional structure of mucus simply meant that when extruded by glandular cells in the endostyle a regular mucus filter was inevitably formed in all of these animals.

Holley (1986) suggested a different mechanism, based on his deductions (from transmission micrographs) of the direction of beat in the endostylar cilia of the ascidian *Ciona*. His view was that the sheet of mucus secreted by the gland cells was 'combed' by regularly arrayed sets of cilia, as the mucus passed upwards along the inner faces of the endostyle from the gland cells to the upper pharyngeal opening of the endostyle. The spacing of the filter mesh was thus dictated by the spacing of the cilia, and not by any intrinsic property of mucus.

Most recently Bone et al. (2000) examined filter production in the pelagic tunicates *Salpa* and *Pegea*, mainly by scanning microscopy. They concluded, like Holley (1984), that the endostylar cilia were involved in spacing the filter mesh, and were able to see a series of filter strands passing from the tips of a regular row of cilia to form one side of the filter mesh, to which another series then became attached at right angles. Thus, despite some uncertainties, and as yet lack of success in seeing a similar process in ascidian tunicates, (though there is reason to suppose that this exists in *Pyrosoma*), at least in salps the formation of the filter on a more or less vertical flat endostylar surface is fairly clear.

As Holley (1986) pointed out, the endostyle of amphioxus is differently organized to that of ascidians and salps, for instead of multiciliated cells differing in structure across the endostyle, those in amphioxus are monociliated and similar in structure. Furthermore, there is no approximately vertical flat inner surface of the endostyle like those in tunicates on which the filter is constructed. Lastly, the amphioxus sub-endostylar plexus apparently differs from those of tunicates for it contains many multipolar neuron somata: none have been found in ascidians (Burighel et al., 2003) or in salps (personal observations).

The central connections of the endostylar plexus pass down from the top of the gill bars, thus these were interrupted in our preparations, and if, as in ascidians (Arkett, 1987), gill ciliary activity is regulated by central cells, this might have disrupted normal activity. However, striking observations by Courtney & Newell (1965) strongly suggest that the activity of the gill bar lateral cilia is (at least in part) controlled by the sub-endostylar plexus. This plexus also controls mucus filter production, blocked by anaesthesia. Possibly the unusual appearance of sheets of mucus on the endostyle and over parts of the gill frontal cilia may be related to loss of the normal link between the sub-endostylar plexus and the central nervous system. The role of the presumably sensory cells on the gill bars themselves (Bone et al., 1966), is unclear.

It is certainly striking that the amphioxus endostyle seems much less complex than that of ascidians or salps, and Holley (1986) suggested that perhaps the filter net was less complex than that of tunicates. Nevertheless even if this may be so, the animal is capable of capturing sub-micron particles with considerable efficiency. Our feeding experiments, during which it was clear that 0.2 μm particles were easily captured, unfortunately do not throw any light upon the mesh size of the filter itself. This is because the filter is unlikely to act as a simple sieve. Perhaps ascidian tunicate mucus filters may act in this way, for measured mesh sizes are in fair agreement with the retention efficiency of various sizes of particles (see Bone et al., 2003). As Silvester (1983) recognized in his influential analysis of filter feeding with nets, smaller particles than the mesh size can be trapped if the strands of the filter are sticky (see also Riisgård & Larsen, 2001). The strands of the amphioxus filter seen in scanning micrographs are certainly sticky for they invariably have particles stuck to them.

We suggest therefore that amphioxus collects particles on a less geometrically regular filter than those of ascidians and salps. The amphioxus filter has sticky strands and so can easily trap particles much smaller than if its irregular mesh simply acted as a simple sieve. Like the irregular filters of oikopleurid tunicates, it traps sub-micron particles by virtue of the stickiness of its strands, which are much thicker than those of ascidians, and more similar to those in the filters of salps and appendicularian tunicates.

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