The gill slits and pre-oral ciliary organ of *Protoglossus* (Hemichordata: Enteropneusta) are filter-feeding structures

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Observations on the hemichordate worm *Protoglossus graveolens* demonstrate that the gill slits, pre-oral ciliary organ (POCO), and the lining of the cylindrical pharynx are used in filter feeding. Pumping of water is generated by cilia that line the lateral gill bars and the POCO directs water from the dorsal surface of the proboscis to the mouth. Particles are trapped and concentrated on the primary and secondary gill bars and transported ventrally and posteriorly by cilia that line the pharynx. The oesophageal organ functions as a barrier to water flow and to squeeze excess water from the mucus-food cord. Particles that passed freely through the gill pores were a maximum of 1.28 μm in size. Diluted milk entered the mouth and was pumped through the pharynx at a rate up to 4.05 mm s^{-1}. The Reynolds number and propulsive efficiency of the filter-feeding structures are estimated using algebraic models. *Protoglossus* elegantly orchestrates the movements of food particles captured by simultaneous filter feeding and deposit feeding or may, from one moment to the next, switch back and forth between deposit and filter feeding. Structural and functional similarities with the cephalochordate pharynx suggest that a wheel organ/ POCO and a filter-feeding pharynx may have been present in the common ancestor to the deuterostomes. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, **98**, 898–906.

ADDITIONAL KEYWORDS: chordate origins – deuterostome evolution – Enteropneusta – pharynx.

INTRODUCTION

There exist two prevailing hypotheses on the filter-feeding structure that is ancestral to the deuterostomes. The first suggests that filter-feeding *tentacles* are ancestral because they are expressed, in almost identical form, in crinoids (arms and tube feet) and pterobranch hemichordates (arms and tentacles) (Romer, 1967; Jefferies, Brown & Daley, 1996) (Fig. 1). The second hypothesis submits that filter-feeding ciliary bands are ancestral because of their common occurrence on the surface of enteropneust hemichordate larvae (tornaria) and the larvae of many echinoderms (Garstang, 1928; Lacalli & West, 1993; Nielsen, 1999; Nielsen & Hay-Schmidt, 2007) (Fig. 1). A third hypothesis, namely that the ancestral

deuterostome may have filtered food by creating a unidirectional flow through a chordate-like pharynx, perforated with gill slits, has not been explored, despite the likeness of the enteropneust–hemichordate pharynx to that of cephalochordates (Ruppert, Cameron & Frick, 1999; Cameron, 2005). Although structurally similar, their homology has been dismissed for two reasons. First, the pterobranch hemichordates, which use tentacles to filter feed, are regarded as the primitive clade of hemichordates. Second, enteropneusts are assumed to be deposit, not filter feeders (Knight-Jones, 1953; Burdon-Jones, 1962; Thomas, 1972); thus, tentacles (or ciliary bands) are primitive and the pharynx is likely to be convergent in the enteropneusts and chordates. Molecular phylogenetic studies of hemichordates, however, indicate that the Pterobranchia may be the sister group to the Enteropneusta (Winchell *et al.*, 2002; Cameron, 2005; Delsuc *et al.*, 2008),

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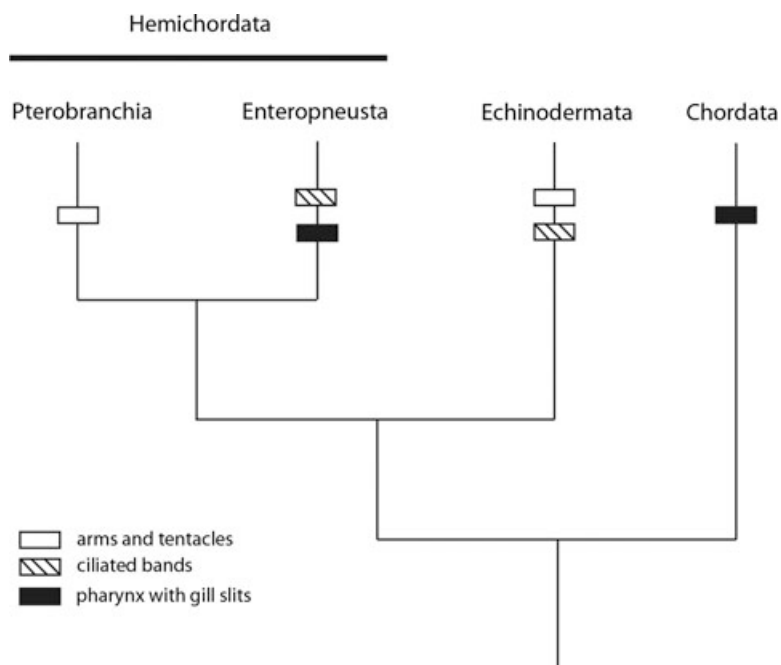
Figure 1. Prevailing hypotheses suggest that the ancestral deuterostome fed with either ciliated bands like those in enteropneust and echinoderm larvae or with arms and tentacles such as those in pterobranchs and echinoderms. The present study supports the hypothesis that a pharynx perforated with gill slits used in filter feeding is ancestral to the deuterostomes.

or derived within Enteropneusta (Halanych, 1995; Cameron, Garey & Swalla, 2000; Bourlat *et al.*, 2003). It is therefore reasonable to advance the hypothesis that a pharynx with gill slits, used in filter feeding is ancestral to deuterostomes. Support for this hypothesis, however, requires a demonstration of chordate-like filter feeding in enteropneusts.

Evidence of filter feeding in the Enteropneusta first came from Barrington (1940) who observed carmine particles passing into the mouth of *Glossobalanus minutus* Kowalevsky, 1866, and observations on the directions of flow and beating cilia along the proboscis, pre-oral ciliary organ (POCO), pharynx, and gill bars of *Saccoglossus horsti* Brambell, Rogers & Goodhart, 1941 (Knight-Jones, 1953). Burdon-Jones (1952) described the filter-feeding behaviour of juvenile *S. horsti*, and the sieving of material from the respiratory current in *Balanoglossus gigas* Müller, 1893 (Burdon-Jones, 1962). Cameron (2002b), described suspension feeding mechanisms by *Harrimania planktophilus* Cameron, 2002, and quantified particle velocity and size-selectivity. Despite these observations, the gill slits are regarded as respiratory (Knight-Jones, 1953), even though they are not specifically involved in gas exchanges in *G. minutus* (Pardos & Benito, 1988) and filter feeding is regarded as being of secondary (Knight-Jones, 1953; Burdon-Jones, 1962) or of little importance (Thomas, 1972) to deposit feeding.

The U-shaped POCO is situated on the posterioventral end of the enteropneust proboscis just anterior to the mouth. Suggestions that it may be sensory (Brambell & Cole, 1939) or an organ of taste (Knight-Jones, 1953) are reasonable given its position in front of the mouth and that it is underlain by a thickened nerve plexus (Welsch & Welsch, 1978). The POCO of *S. horsti* and *Saccoglossus cambrensis* Brambell, Rogers, & Goodhart, 1939, capture particles on a mucous cord during deposit or suspension feeding, further supporting the hypothesis that it may be a chemoreceptive organ (Burdon-Jones, 1962). Although ubiquitous among the enteropneusts, its functional role remains largely unknown.

The genus *Protoglossus* (family Harrimaniidae), as the name implies, is presumed basal among Enteropneusta because of its simple morphology (Burdon-Jones, 1956), and therefore may provide some insight to the evolution of the filter-feeding pharynx in the deuterostomes. The present study aimed: (1) to determine that *Protoglossus graveolens* Giray and King, 1996 filter feeds using its pharynx and gill slits; (2) to determine the particle size selectivity and rate of flow into the mouth; (3) to provide a simple fluid dynamic model to estimate the energy requirements of operating the pharyngeal pump; and (4) to determine the function of the POCO.

MATERIAL AND METHODS

Protoglossus graveolens was collected from Lowes Cove (43°56′N, 69°34′W), a tidal mudflat in the Damariscotta River estuary, Maine. Specimens were kept in their natural sediments in an aquarium under a flow of ambient seawater. One at a time, 20 worms were removed from their sediment and placed in a dish where they were cleaned of sediment and fed orange-coloured plastic particles (Dayglo Color Corp.) or diluted milk, in order to visualize and record the current entering the mouth and pharynx and exiting the gill pores. Particle velocity was measured for three individuals using still images taken at a regular interval and analysed with IMAGEJ (Abramoff, Magelhaes & Ram, 2004). Particle size was recorded from particles entering the mouth, and exiting the gills. Size selectivity was determined by comparing the size range of particles fed with those escaping the gill pores. Videos and photographs were taken with a QImaging Retiga 2000 camera mounted on an Olympus SZX16 microscope. Particle movement inside the pharynx was observed through the gill pores and through the body wall. Five specimens were relaxed for 10 min in 7% $MgCl_2$ and, after being fed, were pinned and dissected open with a longitudinal incision along the dorsal or the ventral midline. Observations on particle retention and transport in the pharynx were thus corroborated post-interception, so that dissection could not interfere with particle capture. Measurements of mouth diameter, pharynx length, and the number of gill pores were taken for each worm filmed and were used to calculate (i) the Reynolds number:

$$Re = lU/\nu$$

where l is the inside diameter of the pharynx (m), U is the flow velocity (m s^{-1}) at the entrance to the mouth, and ν is the kinematic viscosity of seawater ($= 1.047 \times 10^{-6}$ m s^{-1} at 20 °C) (Vogel, 1994).

(ii) the resistance to flow through the pharyngeal cylinder:

$$R = 8 \ l/\pi a^4$$

where l is the length of the pharynx (m), a is the inside radius of the pharynx (m), and μ is the dynamic viscosity of seawater ($= 1.072 \times 10^{-3}$ Pa s^{-1} at 20 °C) (Vogel, 1994).

(iii) the power consumption P (W) of the pharyngeal pump:

$$P = Q^2R$$

where Q is the volumetric flow rate (m^3 s^{-1}) and R is the resistance through the pharynx.

For scanning electron microscopy, the proboscis and dorsal pharynx were dissected and fixed in 4% glut-araldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 and postfixed in 1% osmium tetroxyde in 0.1 M sodium cacodylate buffer. They were then dehydrated through a graded series of ethanol solutions, followed by critical point drying. After sputter coating with an alloy of gold-palladium, the specimens were examined with a JEOL-JSM 35S scanning electron microscope. Cilia length were measured on the proboscis, POCO, pharynx and gill slits.

RESULTS

STRUCTURE OF THE POCO

The POCO of *P. graveolens* is a U-shaped structure situated on the posterio-ventral end of the proboscis, in a small depression encircling the ventro-lateral stalk (Fig. 2A). The POCO is delimited by a continuous elevated ridge covered by a dense boundary of long cilia (mean length = 25 ± 3 μm, $N = 10$). This ciliated band encircles a central ciliated field of shorter cilia (mean length = 11 ± 2 μm, $N = 10$) (Fig. 2B, C). On the ventral midline where the two lateral branches of the U converge, the POCO has an inverted heart shape (Fig. 2A). At this medial spot, the dorsal bands meet and their cilia face each other (Fig. 2D).

STRUCTURE OF THE GUT

Protoglossus worms are fragile and commonly break during collection but in the most complete animals, where the pharynx, oesophagus, and a large portion of intestine were present, the pharynx represented approximately 40% of the total length of the trunk. Gill slits and their associated external gill pores are numerous: 114.7 ± 21.0 ($N = 7$) and evenly spaced every 0.21 ± 0.04 mm ($N = 20$). Primary gill bars and secondary (tongue) bars are not interconnected by collagenous bridges (synapticles) (Fig. 2E). The gill bars form the dorso-lateral walls of the pharynx lumen and on their lateral sides are a thin band of long cilia (mean length = 14 ± 2 μm, $N = 10$) (Fig. 2F, inset). Shorter cilia (mean length = 6 ± 1 μm, $N = 10$) cover the frontal face of the primary and secondary gill bars (Fig. 2F).

OBSERVATIONS ON FILTER FEEDING AND PARTICLE TRANSPORT

When in the presence of sediment, *Protoglossus* can deposit feed and filter feed simultaneously. Deposit feeding is achieved by the muscular proboscis which traps particles on a secreted tube shaped mucous sheet. The mucus and particles are transported posteriorly in a conveyor belt fashion on a ciliated epidermis. As the mucous tube approaches the mouth and collar, it may enter the mouth or pass
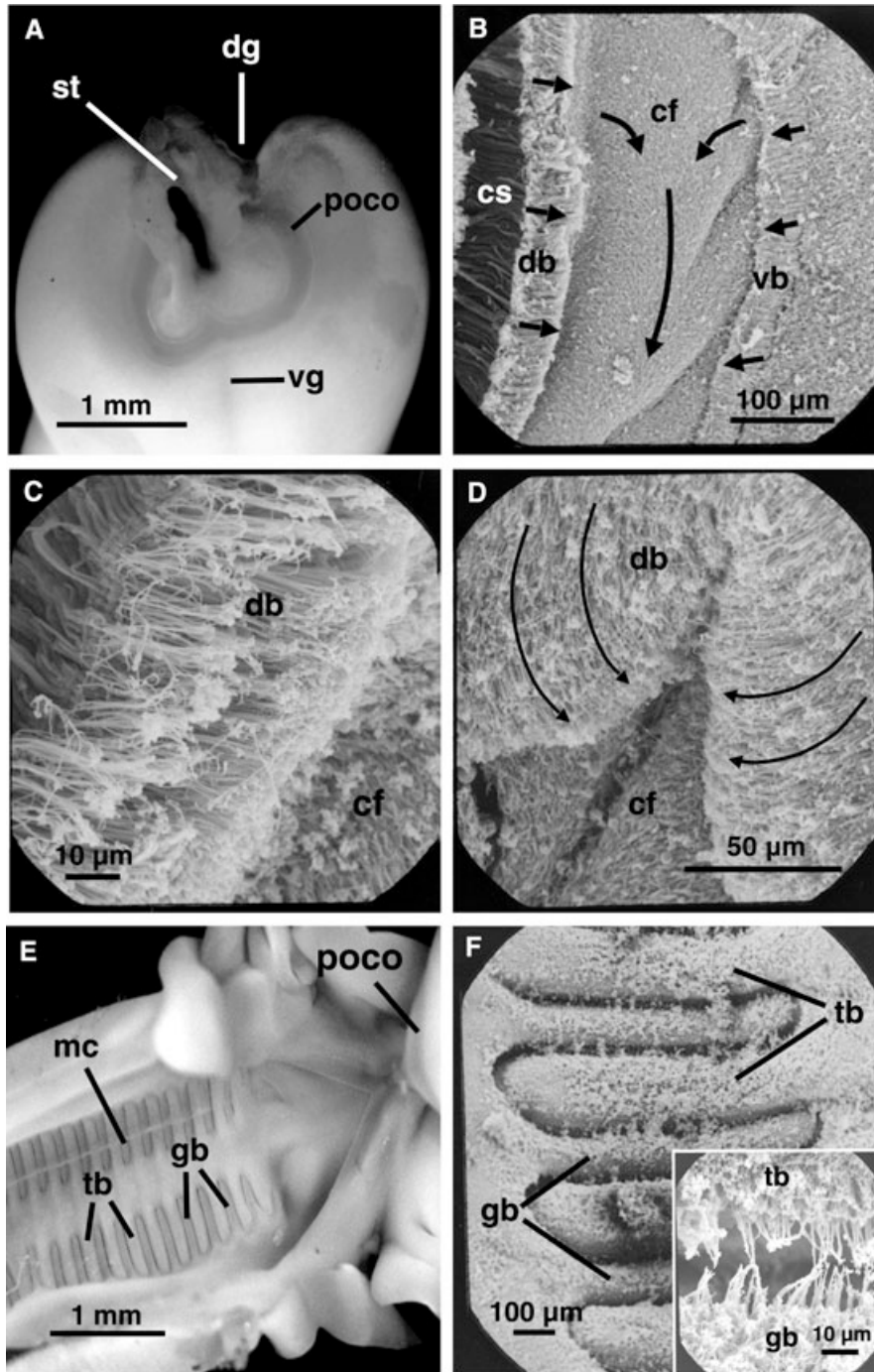
Figure 2. A, light microscopic photograph of the anterior proboscis and pre-oral ciliary organ (POCO) of *Protoglossus graveolens*. The proboscis stalk was cut, exposing the severed, black skeleton and the POCO. B, Scanning electron microscopy (SEM) photograph of the lateral right end of the POCO. The arrows show the beating direction of cilia. A tear in the preparation shows the columnar cells that form the ciliated bands. C, SEM image of cilia on the POCO dorsal band. D, the medio-ventral part of the dorsal POCO cilated band. Arrows show the beating direction of the cilia. E, light microscopic photograph of the dorsal pharynx was taken after a ventral midline dissection. Particles bound in a mucous cord are floating freely above the pharynx, unable to reach the dissected ventral pharynx. F, SEM photograph of the dorsal pharynx. (inset: high magnification view of the lateral cilia between adjacent primary and secondary gill bars). cf, ciliated field; cs, columnar cells; db, dorsal band; dg, dorsal groove; gb, primary gill bar; mc, mucous cord; poco, pre-oral ciliary organ; tb, secondary or tongue bar; st, proboscis stalk; vb, ventral band; vg, ventral groove.
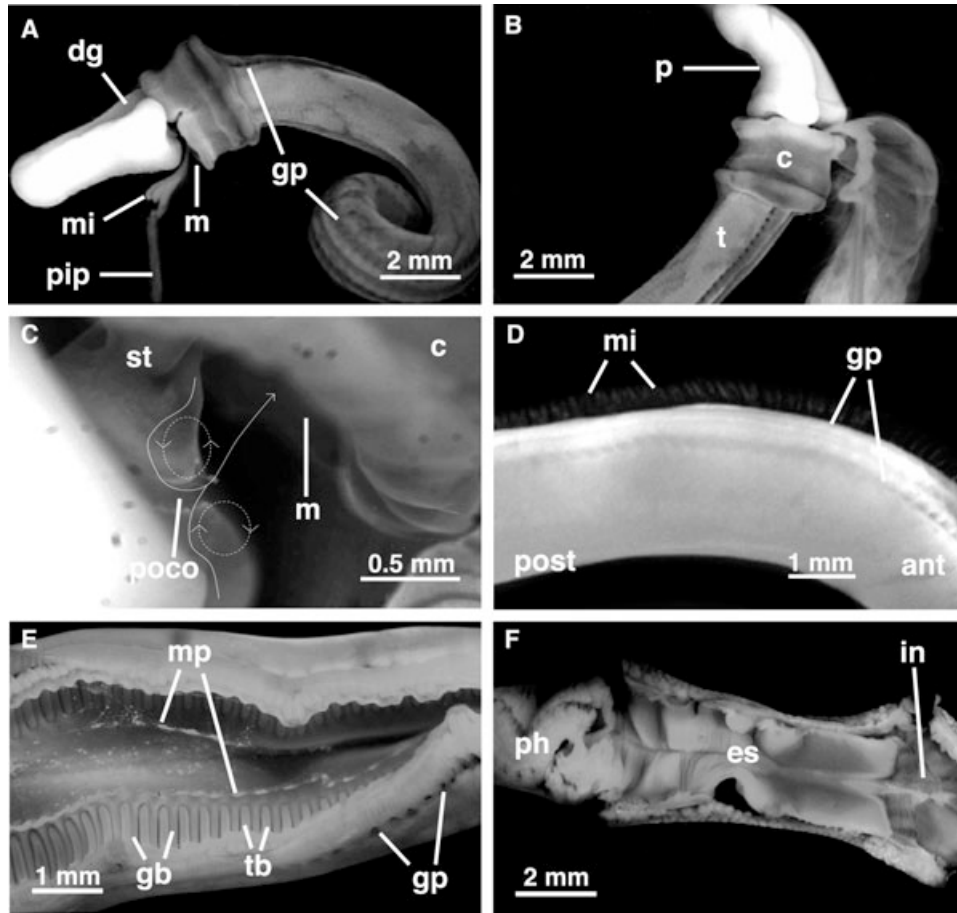
Figure 3. Light micrographs showing the pumping, filtering and digestive system of *Protoglossus graveolens* during filter feeding. A, B, flow of diluted milk entering the mouth. C, close-up of the buccal cavity. Dashed lines represent the vortices produced at the mid ventral region of the pre-oral ciliary organ. Solid lines represent the trajectory of particles into the centre of the mouth. D, diluted milk exiting the gill pores. E, mucus bound particles being transported to the ventral pharynx. F, dorsal dissection showing the muscular oesophagus. c, collar; dg, dorsal groove; es, oesophagus; gp, gill pores; in, intestine; m, mouth; mi, milk; mp, mucus bound particles; p, proboscis; ph, pharynx; poco, pre-oral ciliary organ; pip, pipette; st, proboscis stalk; t, trunk.

directly over the mouth onto the muscular collar. The posterior transport of the mucous tube stops at the junction with the trunk where it would otherwise block water from passing out the gill pores. To simultaneously filter feed, two well-defined grooves on the posterior proboscis, one dorsal and one ventral, create a temporary gap in the mucous tube through which suspended particles pass.

When only filter feeding, the mouth of *Protoglossus* functions like an inhalant siphon (Fig. 3A, B; Supporting Information S1, S2). Plastic particles (0.1–2.5 μm, average 0.78 ± 0.57 μm, $N = 100$) placed up to 4 mm away accelerate as they are sucked towards the mouth. Maximum velocities at the entrance to the mouth varied between individuals in the range 1.80–4.05 mm s^{-1} (mean $v_{max} = 3.19 \pm 1.21$ mm s^{-1}, $N = 3$). The Reynolds number in the pharynx is approximately 13, which means the flow is laminar and dominated by viscous forces. The average mouth area A_{buc} is 9.46 ± 1.15 mm^2 ($N = 3$). Average estimated pumping rates (volumetric flow rates) are $P = v_{max}A_{buc} = 0.031 \pm 0.013$ mL s^{-1} ($N = 3$). The resistance to flow through the pharynx is $R = 9.29.10^6 \pm 2.08.10^6$ Pa s^{-1} m^{-3} ($N = 3$). The power output of the water pumping system is $P = 0.009 \pm 0.006$ μW ($N = 3$). Dynamics of particles at the entrance to the mouth show a velocity profile, where the highest flow is at the center and decreases to zero at the wall of the buccal cavity. After removal of the proboscis, including the POCO, a weak pumping action is observed ($v_{max} = 0.34$ mm s^{-1}, $N = 1$) but the velocity profile is similar. This velocity profile suggests that cilia on the gill slits drive the flow and not cilia that line the walls of the pharynx.
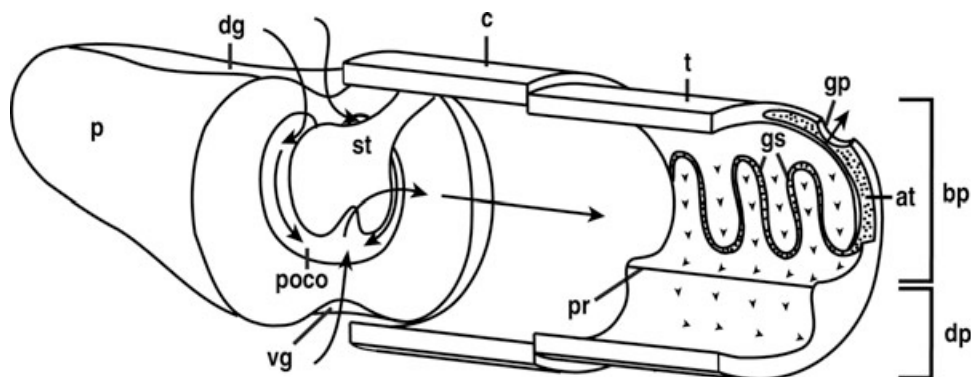
Figure 4. Organization of the filter-feeding morphology of *Protoglossus graveolens*. Water and particles (arrows) are pumped into the mouth by cilia of the pre-oral ciliary organ and pharyngeal gill bars then pass through the gill slits and atrial cavities and exit via the gill pores. Larger particles (arrowheads) are intercepted by cilia on the gill bars and are transported ventrally over the parabranchial ridge. Once in the digestive pharynx, the food laden mucous cord is transported posteriorly towards the oesophagus. at, atrial cavity; bp, branchial pharynx; c, collar; dg, dorsal groove; dp, digestive pharynx; gp, gill pore; gs, gill slits; p, proboscis; poco, pre-oral ciliary organ; pr, parabranchial ridge; st, stalk; t, trunk; vg, ventral groove.

The POCO functions to draw particles from the dorsal and lateral sides of the proboscis stalk to the ventral side and to eject these particles into the inhalant flow of the mouth (Fig. 4; Supporting Information S2). All cilia in the region of the POCO, including those on the posterior face of the proboscis and on the proboscis stalk (posterior to the POCO), beat in the direction of the POCO. This arrangement of cilia beating transports particles along the epidermis to the POCO where they are then ejected into the inhalant flow of the mouth. At the ventral midline, where cilia from the dorsal bands face each other, it creates vortices that also propel particles into the feeding current (Fig. 3C; Supporting Information S2).

Filtered water and small particles (minimum size: 0.1 μm, maximum size: 1.28 μm, average size: 0.78 ± 0.40 μm, $N = 100$) pass through the gill slits and atrial cavities and exit via the epithelial gill pores (Figs 3D, 4). We were unable to observe interception of suspended particles but presume that this is achieved by the dense lateral gill bar cilia. Observations through the gill pores and from dissected specimens clearly show that the frontal cilia are responsible for the ventral transport of trapped particles over the parabranchial ridges to the ventral digestive pharynx (Fig. 4). Mucus secreted from the pharynx wall traps the food particles and, in the ventral digestive pharynx, forms a mucus-food cord that is then transported posteriorly to the muscular oesophagus (Fig. 3E). Situated between the pharynx and intestine, the muscular oesophageal organ (Fig. 3F) functions as a plug to water flow into the intestine, to squeeze excess water from the food, and probably to macerate food particles before they enter the digestive intestine. In the most complete speci-men (pharynx length = 11.8 mm, oesophagus + intestine length = 18.3 mm), the passage time through the trunk was 3 h 25 min.

DISCUSSION

A facultative filter feeder, *Protoglossus* is able to deposit feed and filter feed simultaneously. It feeds on particulate matter in the same size range as many filter feeders and at relatively low energetic cost. It filters particles even less than 1.3 μm in size, which is the approximate minimal size filtered by the enteropneust *H. planktophilus* (Cameron, 2002b). The mucous net sieving of ascidians and lancelets enable particle capture down to 2–3 μm and 0.06 μm in size, respectively (Ruppert, Nash & Smith, 2000). Our mathematical model of the power demand of the *P. graveolens* pumping system suggests that it is much lower (0.009 μW) than that of the sponge *Haliclona urceolus* Rathke & Vahl, 1806 (0.677 μW), polychaetes (0.451 μW), bivalves (*Mytilus edulis* Linnaeus, 1758; 10 μW), and ascidians (*Styela clava* Herdman, 1881; 2.3 μW) (Riisgard & Larsen, 2000). This low calculation is the result of a low volumetric flow rate. Because no data exist on the respiratory physiology of enteropneusts, we are unable to estimate the total metabolic power expenditure (R_t) of *Protoglossus* to calculate the overall efficiency P/R_t of the pumping system for comparison with other filter feeders.

Similar to *G. minutus* (Pardos, 1988) and cephalochordates (Ruppert, Fox & Barnes, 2004) the pumping activity in the pharynx of *Protoglossus* is maintained by long cilia that line the lateral edges of the gill bars and beat orthogonally to their surface. Short cilia that line the frontal face of the gill bars

transport mucus and trapped food particles to the ventral (dorsal in cephalochordates) digestive pharynx. The velocity profile observed at the entrance of the mouth suggests that gill bar cilia pump the water: if the cilia covering the pharynx walls were responsible for creating the current, the highest velocity would be seen along the buccal walls, and not at the centre of the cylindrical pharynx. Observations of orange particles through the body wall and gill pores corroborate observations from dissected specimens: trapped particles are transported ventrally to the digestive gut. The food and sediment laden mucous cord is then transported posteriorly to the oesophageal organ and intestine. *Protoglossus* lacks the mucous net and endostyle that characterize the chordate pharynx (but see also Ruppert *et al.*, 1999), although recent phylogenetic studies that place Cephalochordata as basal chordates (Delsuc *et al.*, 2008; Putnam *et al.*, 2008) make further comparative studies with the enteropneusts germane.

The enteropneust POCO may be homologous to the wheel organ of cephalochordates and in part to the vertebrate adenohypophysis (Welsch & Welsch, 1978). Similar to the wheel organ, the POCO is ectodermal (Goodrich, 1917), and composed of columnar cells (Fig. 2B) (Sahlin & Olsson, 1986) that form a ciliated band and is positioned anterior to the buccal cavity. These organs function to transport suspended particles towards the mouth. In enteropneusts, the POCO is U-shaped and envelopes the proboscis stalk ventrally and laterally, whereas, in adult cephalochordates, the band is elongate and sinuous. A thickening in the nerve plexus under the POCO suggests a sensory function (Welsch & Welsch, 1978). The long peripheral cilia create a feeding current that transports particles from the dorsal and lateral proboscis to the ventral midline where they are then ejected into the incurrent flow to the mouth. Unlike the wheel organ, the POCO of *Protoglossus* does not transport particles from the ciliary field to the mouth on a mucous cord, although this method of particle transport has been described for the POCO of *S. cambrensis* (Knight-Jones, 1953) and *B. gigas* (Burdon-Jones, 1962). Further research using ultrastructure, protein, and gene expression are needed to support the potential homology of the wheel organ and the POCO.

Protoglossus uses the gill slits and pharynx to filter feed much in the same way as protochordates. Increasing evidence provided by comparative morphology, development, and gene expression data supports the hypothesis that the gill slits were present in the ancestor to hemichordates and chordates. The *pax1/9* orthologue is expressed in the gill endoderm of the adult enteropneust *Ptychodera flava* Eschscholtz, 1825 (Ogasawara *et al.*, 1999), juvenile *Saccoglossus kowalevskii* Agassiz, 1873 (Lowe *et al.*,

2003), and chordates (Neubuser, Koseki & Balling, 1995). The posterior expression of the *otx* domain is at the first pair of gill slits in chordates and *Saccoglossus* (Lowe *et al.*, 2003). Supporting the dorsal–ventral inversion hypothesis is the expression of the nk-2 class genes in dorsal pharyngeal endoderm of *Saccoglossus* (Lowe *et al.*, 2006) and the ventral pharynx of a cephalochordate (Holland *et al.*, 2003). Moreover, the gill bars of hemichordates and cephalochordates are composed of acellular invertebrate type II collagen (Cameron, 2005; Rychel *et al.*, 2006).

Recent progress in the molecular phylogenetic position of the deuterostomes suggest that *Xenoturbella*, an unusual marine worm lacking a through gut, coelomic cavities, and gill slits, is a possible stem-group ambulacrarian (Bourlat *et al.*, 2003). If this is true, then either the through gut and gill slits of chordates and hemichordates are homoplasious or have been lost in the branch leading to *Xenoturbella*. Three lines of evidence support the latter hypothesis. The first is the mounting molecular developmental evidence cited above for shared gene expression domains in enteropneusts and chordates (Lowe *et al.*, 2003, 2006); the second is that *Xenoturbella* arguably lost these structures because it is likely a consumer of mollusks (Bourlat *et al.*, 2003; Telford, 2008); and the third is the presence of gill slits, coelomic cavities, and complete gut in fossils of basal deuterostome vetulicolians (Shu *et al.*, 2001) and carpoid echinoderms (Jefferies, 1986; Dominguez, Jacobson & Jefferies, 2002).

Sufficient data have emerged to make some general observations on filter feeding in the Enteropneusta. By contrast to the report of Knight-Jones (1953), who detected a current entering the burrow of *S. horsti*, and Burdon-Jones (1952) who observed the filter-feeding behaviour in juveniles of the same species, we have not observed filter feeding in adult *Saccoglossus pusillus* Ritter, 1902, *Saccoglossus bromophenolosus* King, Giray & Kornfield, 1994, or *S. kowalevskii* suggesting that adult *Saccoglossus* generally do not filter feed. This genus, characterized by a long proboscis, is a specialized surface deposit feeder. The filter feeders *H. planktophilus* (Cameron, 2002a), *B. gigas* (Burdon-Jones, 1962) and *P. graveolens* have a short proboscis and do not appear to construct permanent burrows that open into the water column. Unlike most filter feeders, which are pelagic or epifaunal, these animals instead exploit interstitial pore water in the same respect as lamprey larvae and cephalochordates (Mallatt, 1982; Ruppert *et al.*, 2000). Nothing is known of the feeding biology from the enteropneust families Spengeliidae, Saxipendidae, and Torquaratoridae; thus, we are unable to determine the number of losses or gains of filter feeding in the Enteropneusta. The most current hemi-

chordate phylogenies are incongruent (Cameron *et al.*, 2000; Winchell *et al.*, 2002; Cameron, 2005) and grossly undersampled but, as filter feeding is known in the morphologically most complex genera (*Balanoglossus*: family Ptychoderidae) and the most simple (*Protoglossus*: family Harrimaniidae), it thus appears to be an ancient trait, perhaps as old as the protodeuterostome.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

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

Video S1. Video recording of plastic particles being pumped into the mouth of *Protoglossus graveolens*. c, collar; p, proboscis; st, stalk.

Video S2. Video recording of plastic particles being pumped into the mouth of *Protoglossus graveolens*. Close up of the buccal cavity showing the pre-oral ciliary organ. c, collar; p, proboscis; poco, pre-oral ciliary organ; st, stalk.

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