EVOLUTION & DEVELOPMENT 16:1, 25–37 (2014)
DOI: 10.1111/ede.12060

Choanoflagellate and choanocyte collar-flagellar systems and the assumption of homology

Jasmine L. Mah, Karen K. Christensen-Dalsgaard, and Sally P. Leys

Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

*Author for correspondence (e-mail: sleys@ualberta.ca)

SUMMARY The similarities between the choanoflagellates and the choanocytes of sponges have been discussed for more than a century yet few studies allow a direct comparison of the two. We reviewed current knowledge of the collar and flagellum and compared their structure and function in the choanoflagellate *Monosiga brevicollis* and the sponge *Spongilla lacustris*. Collar microvilli were of similar length and number, but the shape of the collar differed between the two cells. In *Monosiga*, collars were flared and microvilli were joined by a single band of glycocalyx mid-way along their length; in *Spongilla*, collars formed a tube and microvilli were joined by a mesh of glycocalyx. *Monosiga* flagella beat at least four times faster than those in *Spongilla*. Flagellar vanes were found in both cell types. In both cells, the flagella and so probably also the vanes maintained moving points of contact with the microvilli, which suggested that collars and flagella were integrated systems rather than independent units. There were fundamental differences in how the collar and flagella interacted, however. In *Spongilla*, the flagellum bent upon contact with the collar; the flagellar amplitude was fitted to the collar diameter. In *Monosiga*, the flagellar amplitude was unaffected by the collar; instead the collar diameter appeared fitted to the flagellum. These differences suggest that though choanocytes and choanoflagellates are similar, homology cannot be taken for granted. Similarities in collar-flagellum systems separated by 600 million years of evolution, whether maintained or convergent, suggest that these form important adaptations for optimizing fluid flow through micro-scale filters.

INTRODUCTION

Choanoflagellates and choanocytes, the feeding cell type of sponges, are both characterized by an apical flagellum surrounded by a collar of microvilli; the flagellum draws water towards the cell and the collar slows the flow, potentially blocking particles from passing (Pettitt et al. 2002; Leys et al. 2011). In spite of a century-long discussion on the possible homology between the two cell types (Hibberd 1975; Mehl and Reiswig 1991; Maldonado 2004), it is still poorly understood to what extent the collar-flagellum systems of choanocytes and choanoflagellates are structurally and functionally similar.

The ultrastructural characteristics of the flagellum and collar of both choanoflagellates and choanocytes have been studied extensively over the last century (e.g., Afzelius 1961; Fjerdingstad 1961; Hibberd 1975; Simpson 1984; Mehl and Reiswig 1991; Weissenfels 1992; Leadbeater 2006) but typically only as separate and unrelated traits within individual species. As many structures are delicate and often lost or altered as a result of treatment (Mehl and Reiswig 1991; Leadbeater 2006), this complicates comparisons between choanocytes and choanoflagellates across studies. Moreover, in these ultrastructural studies, the functional significance of the observed structures has not, or has only superficially been taken into account. Conversely, functional studies have examined flow around the choanocyte and choanoflagellate flagella and collars (Larsen and Riisgård 1994; Orme et al. 2001, 2003; Pettitt et al. 2002; Riisgård and Larsen 2010; Roper et al. 2013) but these approaches have largely ignored the importance of features such as the flagellar vane and the glycocalyx mesh of the collar.

Importantly, in all previous work, the flagellum has been assumed to move independently of the collar, which is then seen as nothing but a filter that captures particles and imposes a resistance on the flow. In reality, it is well known that the width of the flagellar vane in both choanoflagellates and choanocytes may approach the diameter of the collar (Mehl and Reiswig 1991; Weissenfels 1992; Leadbeater 2006), and it is possible that the flagellum when beating could come within close proximity of the collar microvilli (Hibberd 1975; Weissenfels 1992; Imsiecke 1993; Pettitt et al. 2002). If so, interactions between the collar and the flagella axis or the vane might be functionally important. Therefore, morphological differences in collar structure between the two cell types could be indicative of functional differences.

In this article, we briefly review existing knowledge on the collar and the flagellum of choanocytes and choanoflagellates and describe new data from a detailed direct comparison of the ultrastructure as well as the function of the choanoflagellate *Monosiga brevicollis* and choanocytes of the freshwater sponge *Spongilla lacustris*. We used high-speed video recordings to investigate wave propagation with respect to the collar in both species and to study the flow around the collar. We used scanning and transmission electron microscopy of both cell types using

similar staining and fixation techniques to make direct comparisons of the collar and flagellar structures. Our results highlight important functional similarities as well as differences in the collar-flagellum system of this choanoflagellate and sponge choanocyte.

PRIOR WORK

An evolutionary perspective

The morphological and functional similarities between unicellular choanoflagellates and the choanocytes of sponges have been discussed since Henry James-Clark first noted the striking resemblance between the two, in particular with respect to their apical flagella encircled by a collar of microvilli (James-Clark 1866). The subsequent discovery of a colonial choanoflagellate, *Proterospongia haeckeli*, led biologists to suggest that a colonial choanoflagellate gave rise to sponges (Tuzet 1963), hence explaining the similarities through direct descent.

Molecular studies later supported the close relationship between Choanoflagellida and Metazoa (Carr et al. 2008), but also confirmed that the two groups were monophyletic, and so neither was a descendant of the other. In spite of this, the assumption that choanoflagellates and choanocytes were homologous at the whole cell level remained widely accepted due to their superficial morphological similarities (Woollacott and Pinto 1995; Maldonado 2004; Nielsen 2008).

The hypothesis that the collar cell as an entity has remained wholly present at each step of the evolutionary transition (Nielsen 2008) is unlikely to be true because the present-day sponge choanocyte develops from two different cell types, both of which lack a collar. During development from a gemmule, choanocytes arise from archaeocytes (Funayama 2005) and during metamorphosis from the larval stage they arise from ciliated larval epithelial cells (Amano and Hori 1996; Leys and Degnan 2002). Aside from this, little data exist that can shed light on whether the apparent similarities between choanocytes and choanoflagellates are due to homology, or whether they could arise by convergent evolution instead.

The fact that collars can arise through convergent evolution is suggested by the presence of apparently non-homologous collars in taxonomically unrelated protozoa. These include *Pteridomonas danica*, which feeds using an hispid flagellum surrounded by a collar of pseudopodia (Christensen-Dalsgaard and Fenchel 2003, 2004); species of the genus *Actinomonas*, which draw water through two rings of very fine filipodia protruding from the cell body (Sleigh 1964); and soil flagellates of the genus *Phalansterium* which have a small collar-like structure, the purpose of which remains unknown, surrounding the base of the flagellum (Ekelund 2002). Collar cells, in which a collar of microvilli surrounds a (typically non-motile) cilium, have also been found throughout the animal kingdom. These differ greatly in function and structure and are likely to have evolved convergently, although it has also been suggested that they could be homologous (e.g., Norrevang and Wingstrand 1970). They include cells from the digestive tract of the echinoderm *Marthasterias glacialis* (Martinez et al. 1991), the ciliary bands of the brachiolaria larva of *Asterias rubens* (Norrevang and Wingstrand 1970), and the trunk epithelium of the enteropneust *Harrimania kupfferi* (Norrevang 1964).

General morphology

Choanocytes and choanoflagellates are morphologically similar. The two cell types overlap in size. Choanoflagellates range from 1.2 to 10 μm in length (the longest axis of the cell, measured apical-basal) and 1.2 to 7 μm in width (the shorter axis of the cell) (Hibberd 1975; Leadbeater 1983), and choanocytes vary from 2 to 10 μm in length and 2 to 8 μm in width (Rasmont et al. 1958; Fjerdingstad 1961; Boury-Esnault et al. 1984; Muricy et al. 1999). The collars of choanoflagellates widen distally to form a funnel whereas the sponge collar tends to form a cylinder. Glycocalyx is present on the surface of both cells. In choanoflagellates, it forms an uninterrupted fibrous layer on the cell surface (Leadbeater 2008). In sponge choanocytes, the glycocalyx forms a mesh that connects adjacent collar microvilli (Fjerdingstad 1961; Simpson 1984; Mehl and Reiswig 1991; Weissenfels 1992) and in some members of Calcarea and Demospongiae, a glycocalyx coat is on the cell surface around the flagellum (e.g., Brill 1973; Ledger 1976) and on the exopinacocytes (Leys and Eerkes-Medrano 2006). In some demosponges, specific fixation and staining protocols reveal an additional layer of glycocalyx connecting the tips of neighboring collars to form a gasket-like structure. This may function similarly to the secondary reticulum of hexactinellids and a similar layer of cells in some demosponges that seals the space between adjacent choanocyte collars, possibly forcing water to pass through the collars (Mackie and Singla 1983).

A symmetric wing-like vane spanning the proximal two-thirds of the flagellum has been found in sponges and choanoflagellates (Afzelius 1961; Fjerdingstad 1961; Simpson 1984; Mehl and Reiswig 1991; Weissenfels 1992; Leadbeater 2006). In both cells, the vane is made of horizontal fibers of glycocalyx that extend perpendicularly from the flagellar axis (Simpson 1984; Mehl and Reiswig 1991; Leadbeater 2006), with additional diagonal and longitudinal fibers present in some choanoflagellates (Hibberd 1975; Leadbeater 2006). However, the choanoflagellate vane is both wider (3–4 μm wide, Hibberd 1975; Leadbeater 2006) and more delicate than the sponge vane (0.4–2.4 μm wide, Afzelius 1961; Brill 1973; Mehl and Reiswig 1991).

Ultrastructure

At a gross ultrastructural-level choanoflagellates and sponge choanocytes share many features, such as the presence of an accessory centriole, a transverse microtubule system and a

fibrillar rootlet that extends longitudinally from the accessory centriole (e.g., Karpov and Leadbeater 1998; Gonobobleva and Maldonado 2009; Pozdnyakov and Karpov 2013). But at the fine ultrastructural-level numerous differences emerge. In all choanoflagellates described to date, two central microtubules transition into a single filament at the flagellar transition zone (Karpov and Leadbeater 1998). In contrast, the flagellar transition zone of sponge choanocytes is more variable: two central microtubules insert into a membrane-limited transitional cylinder in *Halisarca dujardani* (Gonobobleva and Maldonado 2009), electron-dense material in *Sycon* sp. (Pozdnyakov and Karpov 2013), or a spiral structure above a transverse plate in *Ephydatia fluviatilis* (Karpov and Efremova 1994 in Gonobobleva and Maldonado 2009). The accessory centriole is consistently oriented at 90° to the flagellar basal body in choanoflagellates (Karpov and Leadbeater 1998) but it may be found at 30–45°, or 90° in sponges (Boury-Esnault et al. 1984; Gonobobleva and Maldonado 2009; Maldonado 2009; Pozdnyakov and Karpov 2013). Finally, in choanoflagellates, the rootlet microtubules that anchor the flagellum and collar microvilli radiate from several points near the basal bodies (Hibberd 1975) or from the composite arc, an electron-dense ring surrounding the flagellar basal body (Karpov and Leadbeater 1998). These microtubules extend shallowly under the surface of the plasma membrane and interdigitate at right angles with the microfilament cores of collar microvilli (Karpov and Leadbeater 1998). In sponges, these rootlet microtubules may emanate from a globular basal foot, the basal body, or a system of electron dense "satellites" near the basal body (Gonobobleva and Maldonado 2009; Pozdnyakov and Karpov 2013). In *H. dujardani*, the rootlet microtubules pass deep into the cytoplasm, at times connecting to the disto-lateral cell membrane (Gonobobleva and Maldonado 2009), while in *Haliclona rosea* rootlet microtubules appear to enter the collar microvilli (Garrone 1969).

Collar formation and dynamics

Choanoflagellates can typically alternate between long-collared feeding stages and short-collared dispersal stages (Leadbeater 1983; Dayel et al. 2011) suggesting that the choanoflagellate collar is highly dynamic. In addition, the choanoflagellate collar appears sensitive to irritation and rapidly contracts in response to membrane solubilizing chemicals such as glycerol or detergent (Karpov and Leadbeater 1998). On the other hand, video microscopy has demonstrated that the length of choanocyte collars does not vary in live intact sponges (Weissenfels 1992); rather, sponge choanocyte microvilli change length only in response to damage of the surrounding tissue (Saedeleer 1929; Fjerdingstad 1961; Leys and Hill 2012), though they can be retracted completely when differentiating into, for example, spermatozoa (Gaino et al. 1984; Kaye and Reiswig 1991; Lanna and Klautau 2010).

Further, there may be differences in how the collar is formed. In the sponge *Tetilla serica*, the collar initially forms as a single cytoplasmic tube that later divides into individual microvilli (Watanabe 1978). A similar pattern is seen in *Haliclona* sp. aggregates where developing choanocyte collars first arise as a tube (Leys, personal observation). In choanoflagellates, the collar always appears to occur as individual microvilli irrespective of the collar length, even during cell division (Karpov and Leadbeater 1997).

The mechanism underlying growth and retraction of collar microvilli in both choanoflagellates and choanocytes remains poorly understood. In vertebrate kidney epithelial cells, microvilli form when polymerization extends bundles of microfilaments from discrete nucleation sites below the membrane (Gorelik et al. 2003). Treadmilling of actin filaments allows microvilli to maintain a constant length, while retraction occurs when rates of depolymerization at the minus end exceed rates of polymerization at the plus end of microfilaments (Gorelik et al. 2003). This mode of microvillus formation, however, is not universal, and cannot be assumed to be applicable to sponge and choanoflagellate microvilli. The microvilli of sea urchin eggs, for instance, form by a two-step process instead: individual actin filaments are assembled and orientated so that one end contacts the membrane, then actin filaments are bundled laterally to form the core of a microvillus (Tilney and Jaffe 1980).

Feeding

In both choanocytes and choanoflagellates, the propagation of the flagellar wave is thought to draw water in through the microvilli of the collar, which then slows or blocks food particles, allowing them to be phagocytosed (Pettitt et al. 2002; Leys et al. 2011). This flow through the collar has been studied directly in choanoflagellates (Boenigk and Arndt 2000; Pettitt et al. 2002), but has yet to be shown in choanocytes; it has only been possible to visualize the movement of particles approaching the choanocyte surface (Weissenfels 1992; Imsiecke 1993). Hence, the study of the fluid dynamics of the choanocyte collar has largely been restricted to theoretical considerations (Larsen and Riisgård 1994).

In choanoflagellates, eddies may recirculate water in the proximity of the collar (Orme et al. 2001). Though it has been suggested that choanoflagellates can transport entrained food particles down the collar towards the cell surface (Boenigk and Arndt 2000), most are probably phagocytosed by tongue-like pseudopodia that form from one or more collar microvilli and migrate from the base of the collar (Leadbeater and Morton 1974). Sponges may feed through a similar mechanism, as particles have been shown to be engulfed by pseudopodia extending from the cell surface or consisting of several fused microvilli in the calcareous demosponge *Sycon coactum* (Leys and Eerkes-Medrano 2006). While the filtering mechanism of

choanocytes is often considered functionally identical to that of the choanoflagellate collar (e.g., Fenchel 1984; Larsen and Riisgård 1994; Pearse et al. 1994) differences in the flow profile may exist due to differences in collar morphology (Leys and Eerkes-Medrano 2006). Feeding experiments performed in the sponge *S. coactum* found only 0.1 μm beads in regular contact with collar microvilli (Leys and Eerkes-Medrano 2006). All larger beads were more commonly phagocytosed on the cell surface of choanocytes, suggesting that the collar primarily acted to slow flow rather than filter particles.

MATERIALS AND METHODS

Rearing *M. brevicollis* and *S. lacustris*

Choanoflagellate wheatgrass medium (CWM) was prepared by adding 1.2 g of wheat grass powder to 1 L of natural sea water or Instant Ocean (United Pet Group, Cincinnati, OH) that was adjusted to a specific gravity of 1.020–1.024 at 20–22°C. The solution was autoclaved, filtered through Whatman No. 1 filter paper, autoclaved again, and filtered through a 0.22-μm syringe filter. *Monosiga brevicollis* were subcultured by inoculating 35–40 ml of CWM with 1 ml of mature culture and incubating at 25°C for 4–5 days.

Adult *S. lacustris* were collected from Frederick and Rousseau Lakes near the Bamfield Marine Sciences Centre in BC, Canada. Gemmules were extracted, sterilized, plated on glass coverslips, and maintained in M-medium (Rasmont 1961) following the procedure outlined in Elliott and Leys (2007).

Video microscopy of live cells

Video microscopy of live cells was used to measure the parameters of the flagellar waveform as well as collar characteristics and flow around the collar. We used an upright compound microscope as well as an inverted scope, allowing observations from the top as well as the bottom of the chamber. The upright scope was a Zeiss Axioskop 2 plus fitted with a 40× air objective and a 40× immersion lens, both with phase contrast optics (Ph) as well as differential interference contrast (DIC) optics. The inverted scope was a Zeiss Axiovert M200 fitted with a 40× oil immersion objective with both Ph and DIC, and a 63× oil immersion objective with DIC only. Recordings were made using a high-speed video camera (Canadian Photonics: MS85K). Initial recordings at 1000 Hz were used to measure the frequency of the flagellar beat; subsequent recordings were made at approximately 10× the flagellar beat frequency for that given cell type to ensure adequate resolution of the flagellar wave propagation, and so at 500–600 Hz for *M. brevicollis* and 150–200 Hz for *S. lacustris*.

The *M. brevicollis* culture was transferred to a cover slip chamber created by mounting a 22 × 22 mm cover slip on a 24 × 60 mm cover slip for observations with the inverted scope and on a microscope slide for observations in the upright scope. The cover slip was mounted using balls of plasticine fitted at the corners, creating a chamber the depth of which depended on the size of the plasticine balls, but was a minimum of 400 μm.

Attached cells of *M. brevicollis* were defined as cells in which the flagellum was beating, creating a large-scale flow pattern around the cell, but producing no forward motion. The cells were in contact with and so presumably attached to the lower coverslip, but it was not possible to determine the attachment mechanism. Motile cells were defined as cells in which there was forward motion. Most recordings of motile cells were made of cells swimming in parallel to the coverslips, giving longer recording times. Recordings were made of cells swimming at various distances from the cover slip or slide surface, but as no differences were found, all data were grouped.

The choanocytes of *S. lacustris* were observed both in intact and dissociated individuals. Intact sponges were studied from above using the upright scope and the 40× immersion objective lowered into the 50 mm diameter petri dish. When studied from below using the inverted scope the gemmules were allowed to hatch directly on a 24 × 60 mm coverslip, and while still submerged in water, a 22 × 22 mm coverslip was fitted over the live sponges using plasticine balls as described above, ensuring that the balls were large enough to create a chamber that was minimum 1 mm deep. When the 24 × 60 mm coverslip was lifted out of the solution, the chamber remained liquid-filled and the slide could be dried and mounted on the microscope. In both cases, the choanocyte chambers were visible through the wall of the sponge.

For dissociated sponges, individual sponges were transferred to a drop of M-medium placed in a petri dish and pulled apart repeatedly using No. 5 Dumont straight forceps under a dissecting microscope. A drop containing dissociated cells was transferred to a coverslip chamber prepared as described above.

Analysis of videos was carried out with the Canadian Photonics AVI player program and in ImageJ (NIH, Washington, USA, http://rsbweb.nih.gov/ij/). The frequency of the flagellar beat was measured using the AVI player, and the amplitude, wavelength, central flagellar length, collar characteristics, and velocity of flow around collars were measured using ImageJ image analysis software with the JTrack plugin. All comparisons in Table 1 were done by the Mann–Whitney rank sum test.

Scanning and transmission electron microscopy

To preserve choanoflagellates for electron microscopy, we used a small plastic chamber created from a DNA purification column. The column material was removed and replaced by a custom cut circle of Duropore 0.45 μm membrane filter. Choanoflagellates were pipetted onto the filter and allowed to settle for 30 min before fixation with 0.8% OsO_4 (Electron Microscopy Sciences, Hatfield, PA) for 30 min. Filters were rinsed twice in distilled water and gradually dehydrated to 100% ethanol. Sponges were

Table 1. Comparison of the dimensions, collar angle, and flagellar beat frequency of the collar-flagellum system in *Monosiga brevicollis* and *Spongilla lacustris*

	n	Collar width at the base (μm)	Collar angle (θ)	Collar length (μm)	Flagellar central length (μm)	Frequency (Hz)
Choanoflagellate swimming	17	1.9 ± 0.1	–	2.1 ± 0.2	11.2 ± 0.4	68.0 ± 2.7
Choanoflagellate sessile	16	1.8 ± 0.1	19.1 ± 1.8	7.3 ± 0.5*	11.0 ± 0.5	49.9 ± 2.4*
Choanocyte whole sponge	20	3.1 ± 0.1*	−5.0 ± 3.8*	8.2 ± 0.2	10.4 ± 0.3	11.0 ± 1.1*
Choanocyte dissociated	46	3.4 ± 0.10	−0.5 ± 1.8*	4.8 ± 0.3*	10.4 ± 0.2	7.5 ± 0.4*

Values are mean ± SE.
*Significance, $P < 0.001$, for the comparison between the starred value and the one immediately above it.

fixed on coverslips, desilicified in 4% hydrofluoric acid while in 70% ethanol, and freeze fractured in liquid nitrogen according to the procedure outlined in Elliott and Leys (2007). For some experiments, ruthenium red (10%) was used in the fixative. In other experiments, choanoflagellates were incubated in Dimethylsulfoxide (DMSO) (0.01%) prior to fixation. Both choanoflagellates and sponge tissue were critical point dried, and tissues or filters were mounted on aluminum stubs using plastic adhesive or nail-polish, coated with gold, and viewed in a JEOL 6301F field emission scanning electron microscope. Sponges were prepared for transmission electron microscopy (TEM) as described by Adams (2010).

Collar measurements

The straight line tool in ImageJ 1.46r was used to measure microvillus width, spacing between adjacent microvilli, distance of the glycocalyx from the collar base, and width of the collar opening from scanning electron micrographs of *M. brevicollis* and *S. lacustris*. Average values and standard deviation were calculated in Microsoft Excel and graphed in SigmaPlot 12.0.

RESULTS

Collar morphology

Monosiga brevicollis collars emerged from the apical portion of the cell body, which rose to sheath the base of the microvilli with a "skirt" (Dayel et al. 2011; Fig. 1A). The collar had 16–28 microvilli that were 0.11 ± 0.02 μm wide, with spacing between microvilli increasing from 0.08 ± 0.02 μm at their bases to 0.60 ± 0.45 μm at their tips in SEM specimens (Fig. 1, A–C). With DMSO treatment *M. brevicollis* microvilli appeared as rods which were circled mid-way up their length (2.65 ± 0.31 μm from the base) by a fine ring of glycocalyx that measured 0.11 ± 0.02 μm thick (Fig. 1D); the DMSO may have added some thickness.

In contrast, there was no skirt around the collar of *Spongilla lacustris* (Fig. 1, E and F). *S. lacustris* choanocytes had 24–36 microvilli that were 0.12 ± 0.02 μm wide (Fig. 1F). In SEM specimens spacing between microvilli was 0.055 ± 0.015 μm at the base of the collar (Fig. 1F), but microvilli were held tightly together by the glycocalyx mesh for the distal two-thirds of the collar (Fig. 1, E–G). This mesh began 2.10 ± 0.47 μm from the base of the collar and measured 0.024 ± 0.006 μm thick in SEM specimens (Fig. 1I). In *S. lacustris*, the glycocalyx mesh was best preserved by fixation with ruthenium red (Fig. 1J).

In live specimens, the base of the collar of *M. brevicollis* was significantly narrower than that of *S. lacustris*, but it increased in diameter towards the opening to a maximum of 6.5 ± 0.4 μm, whereas the opening of the collar of *S. lacustris* narrowed slightly from the base upwards to 2.5 ± 0.2 μm (Table 1). For both *M. brevicollis* and *S. lacustris*, light microscopy confirmed the validity of the structures observed by SEM, although finer details such as the glycocalyx and the bulge in the lower region of the collar in *S. lacustris* could not be resolved by light microscopy.

A swimming and a sessile stage was present in *M. brevicollis*; the characteristics of the collar depended on the current stage of the cell. While swimming, the collar was retracted and significantly shorter than that of sessile cells (Table 1). There was no significant difference in length between the collars of sessile *M. brevicollis* cells and *S. lacustris* choanocytes in whole sponges. The collar angle was highly variable in short-collared dispersal cells of *M. brevicollis*, defined as cells with a collar between 1.5 and 3.5 μm (Fig. 2). At an average of 15° ± 8°, it was significantly smaller than that of medium-collared cells (mean: 26° ± 7°), here defined as cells with a collar length (CL) of 3.5–6 μm (*t*-test, $P = 0.004$). For cells with a CL greater than 3.5 μm, there was a significant negative correlation between CL and collar angle (Fig. 2, $\Theta = 33.4–1.80$ CL; $P = 0.0001$); that is, shorter collars flared more than longer collars. In *S. lacustris*, the collars always appeared to be full length, except in the case of dissociated choanocytes (Table 1).

Flagellar vane

We found a flagellar vane (or "velum," Weissenfels 1992) to be present in both *M. brevicollis* and *S. lacustris* (Fig. 3). Robust preservation of the vane in *M. brevicollis* was achieved by using

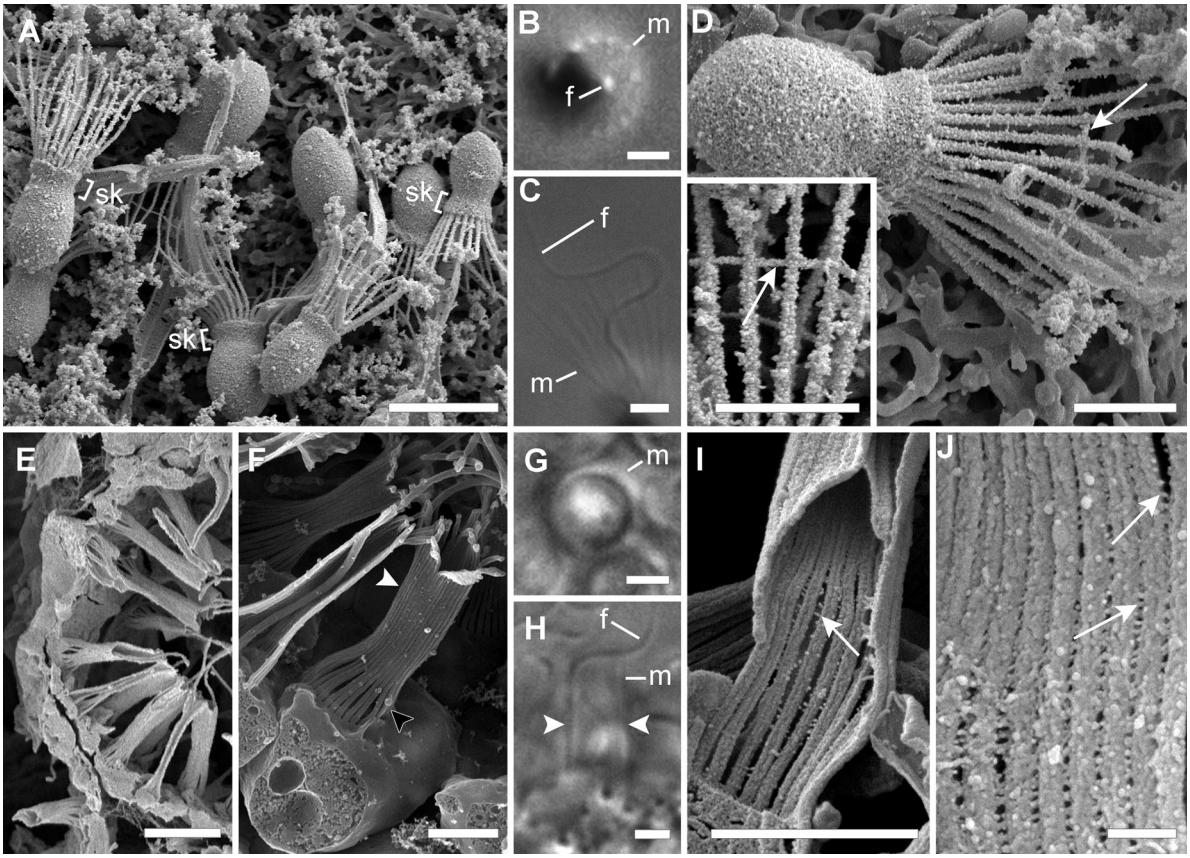

Fig. 1. Comparison of collar morphology in *Monosiga brevicollis* and *Spongilla lacustris*. A, D–F, I–J: Scanning electron microscopy; B, C, G, H: Light microscopy. (A) Several individuals of *M. brevicollis*. The collar emerges from a skirt (sk); grains are due to fixation in DMSO. (B) When viewed perpendicular to the flagellar axis the collar opening of *M. brevicollis* appears as a ring of dots, each formed by the tip of a microvillus, when viewed obliquely (C) it is flared. (D) In *M. brevicollis*, a glycocalyx fibril (arrow) joins collar microvilli. Inset: Enlarged view of the fibril (arrow) in a different individual. (E) Half of a spherical choanocyte chamber in *S. lacustris*; *S. lacustris* choanocytes lack a skirt. (F) An *S. lacustris* choanocyte. Spacing between microvilli is widest near the collar base, forming a bulge (black arrowhead) that narrows into a tight cylinder (white arrowhead). (G) The collar opening of a *S. lacustris* choanocyte appears as a continuous ring, but in profile (H) it is cylindrical (arrowheads indicate collar edges). (I) A cryo-fractured *S. lacustris* choanocyte collar showing the glycocalyx mesh fibrils (arrow). (J) In *S. lacustris*, at higher magnification the glycocalyx mesh appears as small fibers (arrows) closely linking adjacent microvilli. Scale bars: A, E, 5 μm; B–D, F–I, 2 μm; J, 300 nm. Abbreviations: m, microvilli; fl, flagellum.

DMSO in conjunction with OsO_4, although this also produced an insoluble precipitate that was deposited as a layer of small grains. In *M. brevicollis*, the vane formed a uniformly wide (1.6 ± 0.58 μm) sheet of glycocalyx along the length of the flagellum (Fig. 3, A and B). Although the width corresponded to the diameter of the collar base (Fig. 3B), as it was constant along the length of the flagellum it did not span the collar at the opening. It was best preserved for the proximal part of the flagellum closer to the cell body, and though present along the whole flagellum, it appeared damaged or frayed along the last third of the flagellum (Fig. 3C). Even in the best preserved vanes tears were apparent perpendicular to the axis of the flagellum (Fig. 3D).

In *S. lacustris*, DMSO had little effect on the quality of preservation of the flagellar vane. Instead the vane was best preserved with ruthenium red (Fig. 3E). The vane was 1.15 ± 0.27 μm wide, and stretched from the anterior-most tip where the flagellum extended out of the collar in the chamber towards the base of the flagellum, where it appeared to narrow or be absent (Fig. 3E). As in *M. brevicollis* vanes that were less well-preserved were torn (Fig. 3E). In contrast to *M. brevicollis*, the vane in *S. lacustris* appeared to span the full diameter of the collar with the edges of the two wings lying against and in most cases appearing to be attached to the inside of the collar (Fig. 3, F and G).

Flagellar waveform

In both *M. brevicollis* and *S. lacustris*, the flagellum beats in a perfect plane. When viewed parallel to the plane of beat, the flagellum appeared as a straight line (Fig. 4A, double-headed arrows). In both cells, the flagellum ran along the inside of the

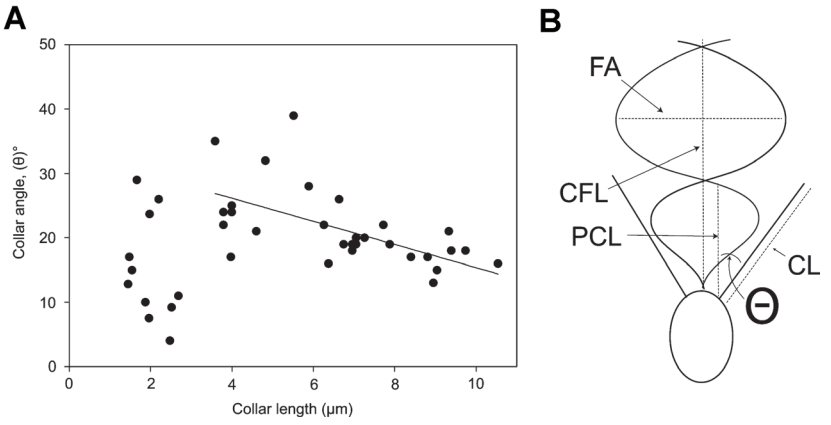

Fig. 2. The collar angle as a function of the total length of the collar microvilli of *Monosiga brevicollis*. (A) There was a significant negative correlation (shown as a solid line) between collar angle (Θ) and collar length for medium- to long-collared cells where the collar length (CL) was greater than 3.5 μm. (B) Illustration showing the parameters measured or calculated from measurements on light microscope images: Collar angle (Θ): the angle between the collar and the central axis, where Θ is measured positive in the direction away from the central axis. Collar length (CL): the length of the collar microvilli. Projected collar length (PCL): the length of the collar projected onto the central axis. Central flagellar length (CFL): the length of the flagellar waveform projected onto the central axis. Maximum flagellar amplitude (FA): the amplitude of the flagellar waveform furthest away from the cell.

fully grown collar when viewed perpendicular to the plane of beat (Fig. 4, Ai and Aii, Movies S1 and S2) and maintained moving points of contact with the collar at the maximum amplitude of the wave. However, one exception was seen in medium length collars of *M. brevicollis*, where the flagellum did not appear to make contact with the collar microvilli. Also, in *S. lacustris*, the flagellum made contact with the collar at a height of 2.2 ± 0.2 μm above the base (Fig. 4Aii). In *M. brevicollis*, the narrower base of the collar and flared collar shape meant that the flagellum approached the collar already at the base (Fig. 4Ai).

In *M. brevicollis*, the amplitude of the waveform increased distally concurrently with the increase in collar diameter. This increase in flagellar amplitude was unaltered by the relative length of the collar compared with the central length of the flagellum, and remained constant even when the collar was almost completely reduced. Hence, there was no correlation between maximum flagellar amplitude and the relative CL, projected CL/central flagellar length (PCL/CFL) (Fig. 4B, $A_{max} = 4.84 + 0.32$ (PCL/CFL); $r^2 = 0.02$; $P = 0.44$). In *S. lacustris*, the waveform within the approximately cylindrical collar approached that of a sinusoidal wave (Fig. 4Aii).

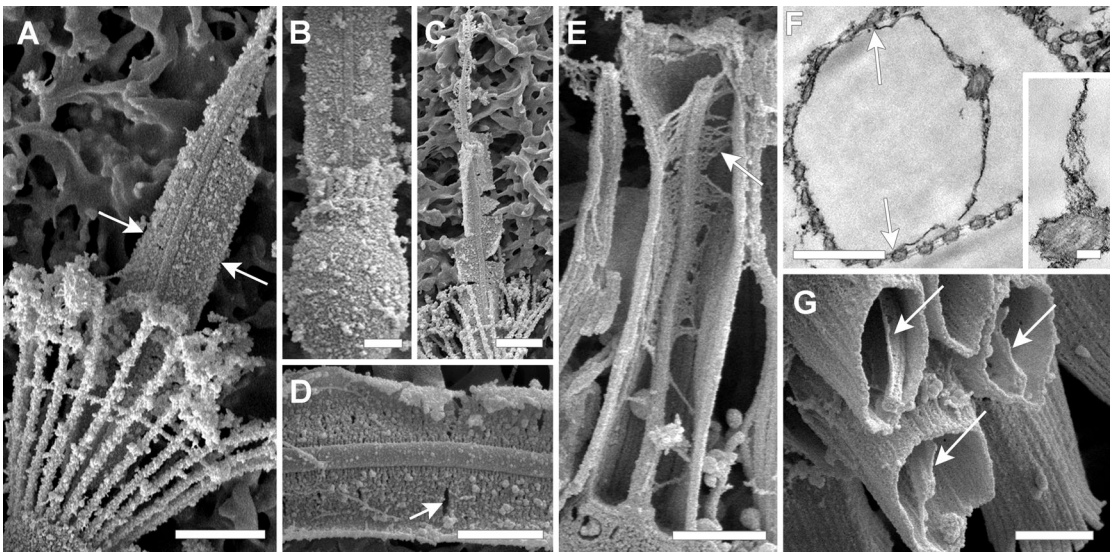

Fig. 3. Morphology of the flagellar vane in *Monosiga brevicollis* and *Spongilla lacustris*. A–E, G: Scanning electron microscopy; F: Transmission electron microscopy. (A) The flagellar vane of *M. brevicollis* consists of two symmetrical wing-like projections (arrows). (B) A short-collared *M. brevicollis* dispersal cell showing the vane spans the width of the base of the collar. (C) The vane was frequently torn along the last third of the flagellum. (D) *M. brevicollis* vane showing small tears (arrow) between fibrils. (E) A choanocyte from *S. lacustris* shows the flagellar vane spans the width of the collar. (F) Cross-section of a choanocyte fixed with ruthenium red showing the vane spans the diameter of the collar with its edges touching the collar wall (arrows). High magnification of the vane in *S. lacustris* (inset). (G) Fracture of collars in *S. lacustris* showing the edges of the vane (arrows) in contact with the collar. Scale bars: A, C, 2 μm; B, D–G, 1 μm; F (inset), 100 nm.

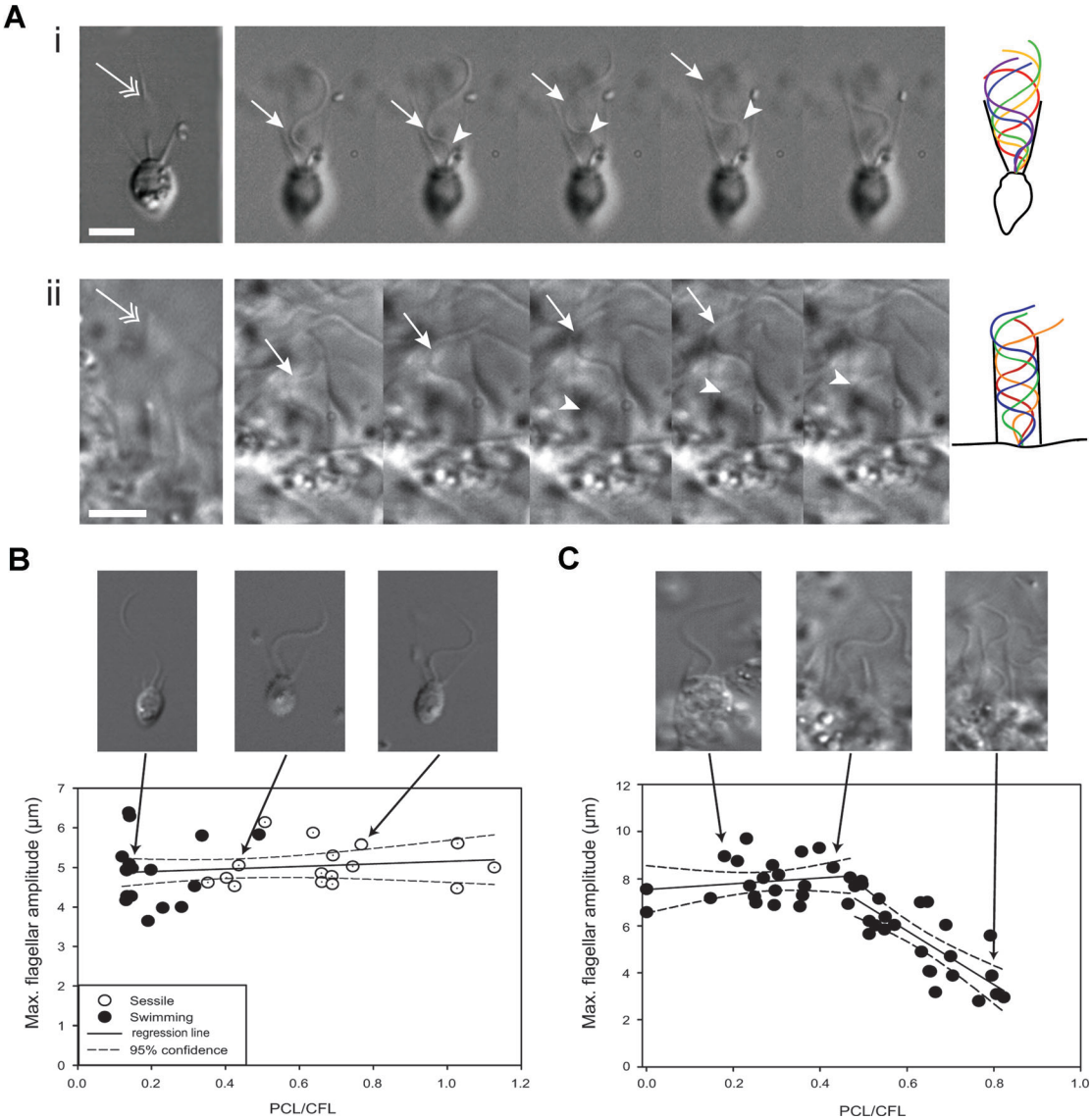

Fig. 4. Flagellar cycle of *Monosiga brevicollis* and *Spongilla lacustris*. (A) (i) *Monosiga brevicollis*, (ii) *Spongilla lacustris*. The flagellum beats in a single plane, as shown in the image to the left (double-headed arrows indicate the flagellar wave form). The drawings on the right show tracings of successive frames of the flagellar beat seen perpendicular to the plane of beat; single-headed arrows and arrowheads mark the progression of peaks in the flagellar waveform. (B, C) The maximum flagellar amplitude (FA) as a function of the fraction of the flagellar waveform that is contained within the collar (calculated as PCL/CFL, see also Fig. 2) in (B) *M. brevicollis* and (C) *S. lacustris*. Linear regressions are marked in solid lines, and the 95% confidence interval in dashed lines. No correlation was found between FA and relative collar length (PCL/CFL) in *M. brevicollis* and in short- to medium-collared cells in *S. lacustris*, but there was a significant negative correlation for medium- to long-collared cells of *S. lacustris* (defined as cells with a PCL/CFL > 0.47). Images above the graphs show short-, medium- and long-collared cells. Arrows indicate the data point obtained from the cell imaged. Scale bars, 5 µm.

However, upon reaching the opening of the collar, the amplitude of the waveform increased (Fig. 4C). For choanocytes with medium to long collars ($0.47 < \text{PCL/CFL} < 0.8$), the amplitude of the flagellar wave protruding from the collar varied linearly with the relative CL (Fig. 4C; $A_{max} = 12.66–11.46$ (PCL/CFL); $r^2 = 0.61$; $P < 0.0001$). For choanocytes with short to medium collars ($0 < \text{PCL/CFL} < 0.47$), the amplitude of this unconfined part of the flagellum was independent of the relative CL (Fig. 4C; $A_{max} = 7.55 + 1.24$ (PCL/CFL); $r^2 = 0.002$; $P = 0.45$).

Streamlines

The streamlines around sessile *M. brevicollis* cells generating feeding currents were not smooth; particles approached the

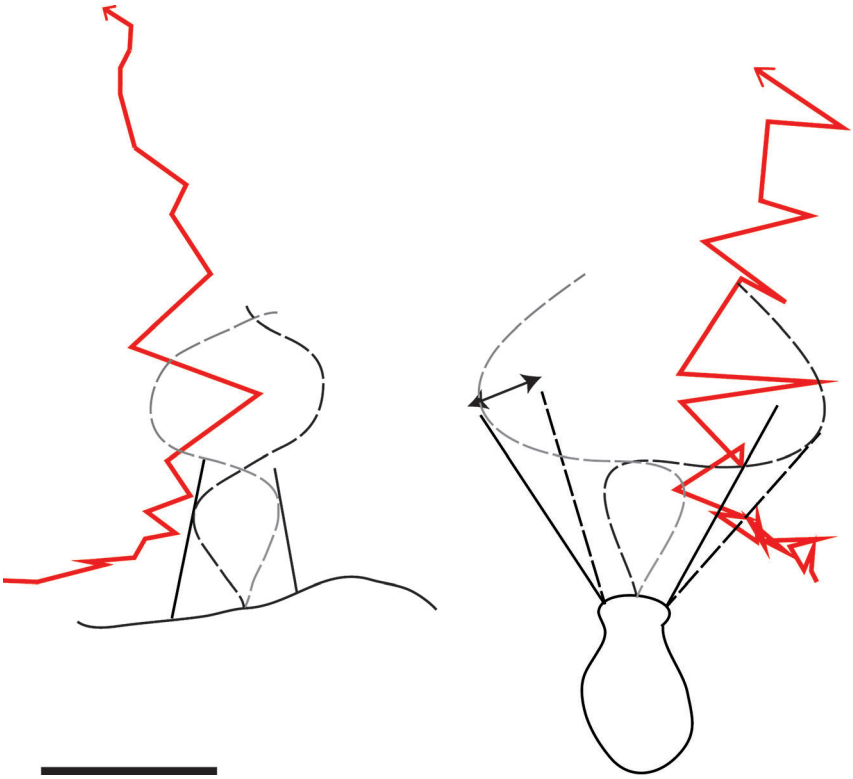

Fig. 5. Track of a particle passing past the collar of *Spongilla lacustris* and through the collar of *Monosiga brevicollis*. The track of a particle is shown by the bold solid line where changes in direction mark time between tracking points: 0.07 sec in *S. lacustris* (left) and 0.01 sec in *M. brevicollis* (right). The flagella at their two extremes are shown in dashed gray lines. The movement of the collar in *M. brevicollis* is shown with dashed black lines corresponding to the position of the darker flagellum. In *S. lacustris*, tracks of particles were only done on dissociated choanocytes with partially retracted collars. Scale bars, 5 μm.

collar surface in an undulating motion of alternating backwards and forwards movements that corresponded to the movements of the collar microvilli (Fig. 5). Particles pulled between the microvilli of the collar were moved from side to side both outside and within the collar by the flagellar movements, all the while maintaining a forward motion.

Due to the cellular density around the base of *S. lacustris* choanocytes, we could not see flow around the collars in intact choanocyte chambers. In the case of dissociated choanocytes, however, flow lines went up along the collar rather than through it (Fig. 5).

Flagellar beat frequency

The frequency of the flagellar beat was significantly lower in sessile as compared with free-swimming cells of *M. brevicollis*, but still approximately four times higher than that of *S. lacustris* choanocytes. The frequency of the flagellar beat of choanocytes decreased in dissociated cells and in some dissociated cells the flagellum stopped moving altogether (Table 1).

In *M. brevicollis*, the frequency of flagella in actively beating cells varied by less than a factor of two and ranged from 35.0 to 62.9 Hz, whereas in actively beating *S. lacustris* cells it varied by almost a factor of seven, ranging from 3.2 to 20.9 Hz; some choanocytes beat extremely slowly, while others beat quite fast. This appeared to be the case in all chambers we studied.

DISCUSSION

Function of the flagella-collar systems: an evolutionary perspective

In this study, we looked at only two species, and so differences observed between the two cannot be assumed to be indicative of differences between sponges and choanoflagellates as a whole. However, the study did highlight a number of fundamental differences as well as similarities between the choanoflagellate *M. brevicollis* and the choanocytes of *S. lacustris*. Hence, our results suggest that though homology between the two cell types is a possibility, it cannot be taken for granted.

We found similarities in the pumping mechanisms as well as the flagellum and collar of the two cells. In both cells, the flagellum maintained moving points of contact with the collar along most of the length of the microvilli. We found a flagellar vane in both cells and in both it appeared to be composed of horizontally oriented glycocalyx fibrils. Differences in integrity of the vanes have previously been used as an argument against homology between choanoflagellates and choanocytes (Mehl and Reiswig 1991), but our results suggest that integrity varies with preservation technique. We also found that with suitable fixing techniques, a ring joining the microvilli in *M. brevicollis* is present, and this appears similar to the many glycocalyx structures joining adjacent microvilli in sponge choanocytes.

On the other hand, the cells differed fundamentally in the response of the flagellum to the presence of the collar. In the flagellum of *S. lacustris*, the switch of the bend was determined through physical contact with the collar, whereas in *M. brevicollis* it did not appear to be affected by contact with the collar. Furthermore, though the microvilli in both were joined by glycocalyx structures, in *S. lacustris*, they were tightly joined together to form a tube throughout most of the length of the collar, and in *M. brevicollis*, they were much more loosely joined by a ring-like structure.

The differences between choanoflagellates and choanocytes can be interpreted in light of the different requirements of a single cell that alternates between a sessile and a dispersal stage compared with a cell that forms part of a multicellular organism. *Monosiga brevicollis* cells are independent organisms that must be able to transition effectively between free-swimming and sessile forms with variable CLs. Hence, they must be able to quickly and efficiently alter their CL without affecting the function of the flagellum. *Spongilla lacustris* choanocytes, on the other hand, form one cell type in a multicellular organism. Hence, they must, for example, have collars that can be packed and oriented within choanocyte chambers in a way that is optimal for the functioning of the organism as a whole. Optimization occurring at the level of the individual cell in *M. brevicollis* but on the level of the whole multicellular organism in *S. lacustris* might explain, for example, the much greater range in flagellar frequencies seen in *S. lacustris* than in *M. brevicollis*.

The collar-flagellum system

The fact that the flagellum maintained contact with the microvilli along most of the length of the collar both of *S. lacustris* and fully grown collars of *M. brevicollis* implies that the contact between the two is functionally important. Hence, the collar-flagellum system should be seen as a functionally integrated unit rather than two separate components found close to each other. In previous fluid-dynamical studies on choanoflagellates, the flagellum has been assumed to be smooth and to move independently of the collar, which then acted as nothing but a resistance imposed on the flow. This allowed the system to be modeled through slender body theory, by approximating the force exerted on the fluid by the flagellum through a line of stokeslets (Orme et al. 2001, 2003). But the presence of a vane and the contact between flagellum and collar would alter the fluid flow and an integrated collar-vane-flagellum system would require more complex modeling.

The flagellar vane has previously been reported only from freshwater species of choanoflagellates (Leadbeater 2006). We found it in *M. brevicollis*, a marine species, and so flagellar vanes may be more widespread among choanoflagellates than previously assumed. The width of the flagellar vanes corresponded to those reported from other species (Afzelius 1961; Simpson 1984; Weissenfels 1992; Imsiecke 1993; Leadbeater 2006) and were comparable in diameter to the collar base in both *M. brevicollis* and *S. lacustris*. Therefore, the vane must be rhythmically deformed into a concave shape, and be partially or completely in contact with the collar at the points of contact between the flagellum and microvilli. This would be the case to a greater extent in *S. lacustris* than in *M. brevicollis*, as the vane spanned the width of the collar along its whole length in *S. lacustris*, whereas the flared shape of the *M. brevicollis* collar resulted in an opening that was approximately three times the width of the vane. The deformation could occur either due to drag or through contact with the collar. We do not know the extent to which the vane is deformed due to drag as this would require an estimate of the rigidity of the vane.

The flow around feeding cells of *M. brevicollis* did not show the smooth streamlines predicted for flow generated by slender body theory (Pettitt et al. 2002; Orme et al. 2003; Roper et al. 2013); instead a back and forth fluid motion was imposed on the stokeslet flow as seen from the movement of particles approaching the collar. This corresponded to, and hence may have been caused by, the back and forth movements of the collar, which in turn were due to the movement of the flagellum against the microvilli. The flow pattern observed for dissociated choanocytes is unlikely to represent the flow in intact sponges. Not only are choanocytes in *S. lacustris* joined by a gasket that is thought to force the flow over the collar (Weissenfels 1992), but we also found the collar to be shorter in dissociated choanocytes. This would have changed the position of the center of the force with respect to the CL as well as potentially altering the resistance over the microvilli. Hence, finding a method for visualizing the flow over the collar in intact sponges is necessary for a direct comparison of the flow around choanoflagellates and choanocytes.

Contact between the flagellum and the collar suggests that structures could help maintain the position of the microvilli with respect to each other under the movement of the flagellum. We found that the *M. brevicollis* collar had a ring of glycocalyx joining the microvilli mid-way up the collar. As also shown in Weissenfels (1992) and Imsiecke (1993), the *S. lacustris* choanocytes had a dense mesh that joined the microvilli into a tube, with only a short region at the base where the microvilli "billowed" apart. The mesh began at a height above the cell surface that corresponded to the point of first contact between the flagellum and microvilli, as would be expected if the main purpose of the mesh was to provide support against the force exerted by the flagellum. The flagellar waveform was frequently flattened against the collar in *S. lacustris*. This could imply that the flagellum was pressed against the microvilli with greater force in this species providing a potential explanation for the need of a dense mesh.

The effect of collar width and length on flagellar amplitude

The fact that the flagellar waveform appeared to fit exactly to the width of the fully extended collar in spite of the variation seen for

collar dimensions suggests that some form of coordination occurred in both *S. lacustris* and *M. brevicollis*. However, there appeared to be fundamental differences in how the integration of the flagellum-collar system occurred in the two species. In *S. lacustris*, the flagellar waveform appeared modified to fit the width of the collar. In *M. brevicollis*, the collar appeared to be fitted to the waveform instead.

This difference was evident from the data showing that amplitude was modified in the presence of the collar. In short- to medium-collared cells of *S. lacustris*, the flagellar amplitude above the collar was constant and approximately three times as large as the collar diameter, suggesting that the flagella had inherent waveforms independent of the collar. When confined by the collar, however, the waveform fit the collar exactly or appeared slightly deformed against it, implying that the physical contact itself affected the switch of the bend. Cilia and flagella are known to have mechanosensory functions. This has been linked to the Ca^{2+} sensitive TRP V (Transient Receptor Potential - Vanilloid) channels in cilia of metazoans (Tobin et al. 2002; Kim et al. 2003) and similarly to TRP channels located proximally on the flagellum in the unicellular *Chlamydomonas* (Fujiu et al. 2011). However, according to the geometric clutch hypothesis for flagellar bend propagation, the switching of the bend occurs through internal changes in the total stress that acts across the axonemal scaffold (Lindemann and Lesich 2010). If true, it is possible that the imposed external forces caused by contact with the collar could alter the switch point directly.

In *M. brevicollis*, the flagellar amplitude was independent of the relative CL, and so the presence of the collar did not appear to alter the waveform. Instead, it is possible that the distal width of the collar could be modified to fit the flagellar waveform. This is supported by the change in collar angle as the length increased. In the dispersal stage, cells had a narrower collar, which would give less drag and potentially lower energy expenditure when swimming. During the transition to the sessile stage, however, the collar angle appeared to "overshoot" and become slightly wider than that of fully grown collars, only to narrow once approaching full length. This suggests that the collar microvilli of sessile choanoflagellates might be pulled in, perhaps by the glycocalyx ring, to fit the flagellar amplitude. More evidence is needed to determine whether this is the case.

Flagellar beat frequency

Our results suggest that a lower flagellar beat frequency might be advantageous for sessile as compared with free-swimming forms. In *M. brevicollis*, the beat frequency of sessile cells was significantly lower than that of free-swimming cells, and the permanently "sessile" choanocytes of *S. lacustris* beat at less than one-fourth the frequency of sessile *M. brevicollis* (Table 1). This could be related to the resistance imposed on the flow by the presence of the collar. Sessile choanoflagellates had a significantly longer collar than that of free-swimming forms (Table 1), as also shown for other species (Dayel et al. 2011). Also, the resistance of the *S. lacustris* choanocyte collar can be assumed greater than that of *M. brevicollis* due to the closer spacing of the microvilli and the fact that flow would be restricted to the short, flared basal area (Weissenfels 1992). Hence, it is possible that there is a correlation between collar resistance and optimal flagellar beat frequency.

CONCLUSIONS

We found notable similarities in the functional morphology of the collar-vane-flagellum pump system in two different organisms separated by at least 600 million years of evolution. This suggests that it is an important adaptation for optimizing fluid flow through micro-scale filters. But when studied as an integrated system fundamental differences in function emerged suggesting that homology cannot be assumed without question. Further studies of the functional significance of the flagellar vane and the contact between the microvilli and flagellum could shed new light on the physical requirements for life at low Reynolds numbers.

ACKNOWLEDGMENTS

We thank P. Holland and A. Smith for providing initial cultures of *M. brevicollis* and stimulating ideas for this work during a sabbatical by S. P. L. at Oxford University. G. Braybrook and N. Gerein provided assistance with SEM; E. D. M. Adams took the image in Fig. 3F. Portions of this work derive from J. M.'s honors thesis; we thank A. R. Palmer for early comments and advice on that work. This research was funded by an NSERC Discovery grant to S. P. L.

REFERENCES

Adams, E. D. M. 2010. Physiology and morphology of epithelia in the freshwater demosponge, *Spongilla lacustris*. M.Sc. Thesis, Biological Sciences, University of Alberta, Edmonton.

Afzelius, B. A. 1961. Flimmer-flagellum of sponge. *Nature* 191: 1318–1319.

Amano, S., and Hori, I. 1996. Transdifferentiation of larval flagellated cells to choanocytes in the metamorphosis of the demosponge *Haliclona permollis*. *Biol. Bull.* 190: 161–172.

Boenigk, J., and Arndt, H. 2000. Comparative studies on the feeding behavior of two heterotrophic nanoflagellates: the filter-feeding choanoflagellate *Monosiga ovata* and the raptorial-feeding kinetoplastid *Rhynchomonas nasuta*. *Aquatic Microb. Ecol.* 22: 243–249.

Boury-Esnault, N., De Vos, L., Donadey, C., and Vacelet, J. 1984. Comparative study of the choanosome of Porifera: 1. The Homoscleromorpha. *J. Morphol.* 180: 3–17.

Brill, B. 1973. Untersuchungen zur ultrastruktur der choanocyte von *Ephydatia fluviatilis* L. *Z. Zellforsch. Mikrosk. Anat.* 144: 231–245.

Carr, M., Leadbeater, B. S. C., Hassan, R., Nelson, M., and Baldauf, S. L. 2008. Molecular phylogeny of choanoflagellates, the sister group to Metazoa. *Proc. Natl. Acad. Sci. USA* 105: 16641–16646.

Christensen-Dalsgaard, K. K., and Fenchel, T. 2003. Increased filtration efficiency of attached compared to free-swimming flagellates. *Aquatic Microb. Ecol.* 33: 77–86.

Christensen-Dalsgaard, K. K., and Fenchel, T. 2004. Complex flagellar motions and swimming patterns of the flagellates *Paraphysomonas vestita* and *Pteridomonas danica*. *Protist* 155: 79–87.

Dayel, M. J., et al. 2011. Cell differentiation and morphogenesis in the colony-forming choanoflagellate *Salpingoeca rosetta*. *Dev. Biol.* 357: 73–82.

Ekelund, F. 2002. A study of the soil flagellate *Phalansterium solitarium* Sandon 1924 with preliminary data on its ultrastructure. *Protistology* 2: 152–158.

Elliott, G. R. D., and Leys, S. P. 2007. Coordinated contractions effectively expel water from the aquiferous system of a fresh water sponge. *J. Exp. Biol.* 210: 3736–3748.

Fenchel, T. 1984. Suspended marine bacteria as a food source. In M. J. R. Fasham (ed.). *Flows of energy and materials in marine ecosystems: theory and practice.* Plenum Press, New York.

Fjerdingstad, E. J. 1961. The ultrastructure of choanocyte collars in *Spongilla lacustris* (L). *Z. Zellforsch. Mikrosk. Anat.* 53: 645–657.

Fujiu, K., Nakayama, Y., Iida, H., Sokabe, M., and Yoshimura, K. 2011. Mechanoreception in motile flagella of *Chlamydomonas*. *Nat. Cell Biol.* 13: 630–632.

Funayama, N. 2005. Isolation of the choanocyte in the freshwater sponge, *Ephydatia fluviatilis* and its lineage marker, Ef annexin. *Dev. Growth Differ.* 47: 243–253.

Gaino, E., Burlando, B., Zunino, L., Pansini, M., and Buffa, P. 1984. Origin of male gametes from choanocytes in *Spongia officinalis* (Porifera, Demospongiae). *Int. J. Invert. Reprod. Dev.* 7: 83–93.

Garrone, R. M. 1969. Une formation d'ARN intranucléaire dans les choanocytes de l'Eponge *Haliclona rosea* O.S. (Démosponge, Haplosclériде). *C. R. Acad. Sci. Hebd. Seances. Acad. Sci. D* 269: 2219–2221.

Gonobobleva, E., and Maldonado, M. 2009. Choanocyte ultrastructure in *Halisarca dujardini* (Demospongiae, Halisarcida). *J. Morphol.* 270: 615–627.

Gorelik, J., et al. 2003. Dynamic assembly of surface structures in living cells. *Proc. Natl. Acad. Sci. USA* 100: 5819–5822.

Hibberd, D. J. 1975. Observations on ultrastructure of choanoflagellate *Codosiga botrytis* (Ehr) Saville-Kent with special reference to flagellar apparatus. *J. Cell Sci.* 17: 191–219.

Imsiecke, G. 1993. Ingestion, digestion and egestion in *Spongilla lacustris* (Porifera, Spongillidae) after pulse feeding with *Chlamydomonas reinhardtii* (Volvocales). *Zoomorphology* 113: 233–244.

James-Clark, H. 1866. Conclusive proofs of the animality of the ciliate sponges, and of their affinities with the *Infusoria flagellata*. *Am. J. Sci. Ser.* 2: 320–325.

Karpov, S. A., and Leadbeater, B. S. C. 1997. Cell and nuclear division in a freshwater choanoflagellate, *Monosiga ovata* Kent. *Eur. J. Protistol.* 33: 323–334.

Karpov, S. A., and Leadbeater, B. S. C. 1998. Cytoskeleton structure and composition in choanoflagellates. *J. Eukaryot. Microbiol.* 45: 361–367.

Kaye, H. R., and Reiswig, H. M. 1991. Sexual reproduction in four Caribbean commercial sponges. I. Reproductive cycles and spermatogenesis. *Invert. Reprod. Dev.* 19: 1–11.

Kim, J., et al. 2003. A TRPV family ion channel required for hearing in *Drosophila*. *Nature* 424: 81–84.

Lanna, E., and Klautau, M. 2010. Oogenesis and spermatogenesis in *Paraleucilla magna* (Porifera, Calcarea). *Zoomorphology* 129: 249–261.

Larsen, P. S., and Riisgård, H. U. 1994. The sponge pump. *J. Theor. Biol.* 168: 53–63.

Leadbeater, B. S. C. 1983. Life-history and ultrastructure of a new marine species of *Proterospongia* (Choanoflagellida). *J. Mar. Biol. Assoc. UK* 63: 135–160.

Leadbeater, B. S. C. 2006. The 'mystery' of the flagellar vane in choanoflagellates. *Nova Hedwigia*, *Beiheft* 130, p. 213–223.

Leadbeater, B. S. C. 2008. Choanoflagellate evolution: the morphological perspective. *Protistology* 5: 256–267.

Leadbeater, B. S. C., and Morton, C. 1974. Microscopical study of marine species of *Codosiga* James-Clark (Choanoflagellata) with special reference to ingestion of bacteria. *Biol. J. Linn. Soc.* 6: 337–347.

Ledger, P. W. 1976. Aspects of the secretion and structure of calcareous sponge spicules. Ph.D. Thesis, Zoology, University College of North Wales, Bangor.

Leys, S. P., and Degnan, B. M. 2002. Embryogenesis and metamorphosis in a haplosclerid demosponge: gastrulation and transdifferentiation of larval ciliated cells to choanocytes. *Invert. Biol.* 121: 171–189.

Leys, S. P., and Eerkes-Medrano, D. I. 2006. Feeding in a calcareous sponge: particle uptake by pseudopodia. *Biol. Bull.* 211: 157–171.

Leys, S. P., and Hill, A. 2012. The physiology and molecular biology of sponge tissues. *Adv. Mar. Biol.* 62: 1–56.

Leys, S. P., Yahel, G., Reidenbach, M. A., Tunnicliffe, V., Shavit, U., and Reiswig, H. M. 2011. The sponge pump: the role of current induced flow in the design of the sponge body plan. *PLoS ONE* 6: e27787.

Lindemann, C. B., and Lesich, K. A. 2010. Flagellar and ciliary beating: the proven and the possible. *J. Cell Sci.* 123: 519–528.

Mackie, G. O., and Singla, C. L. 1983. Studies on Hexactinellid sponges. I. Histology of *Rhabdocalyptus dawsoni* (Lambe, 1973). *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 301: 365–400.

Maldonado, M. 2004. Choanoflagellates, choanocytes, and animal multicellularity. *Invert. Biol.* 123: 1–22.

Maldonado, M. 2009. Embryonic development of verongid demosponges supports the independent acquisition of spongin skeletons as an alternative to the siliceous skeleton of sponges. *Biol. J. Linn. Soc.* 97: 427–447.

Martinez, A., Lopez, J., Villaro, A. C., and Sesma, P. 1991. Choanocyte-like cells in the digestive system of the starfish *Marthasterias glacialis* (Echinodermata). *J. Morphol.* 208: 215–225.

Mehl, D., and Reiswig, H. M. 1991. The presence of flagellar vanes in choanomeres of Porifera and their possible phylogenetic implications. *Z. Zool. Syst. Evol.* 29: 312–319.

Muricy, G., Bézac, C., Gallissian, M. F., and Boury-Esnault, N. 1999. Anatomy, cytology and symbiotic bacteria of four Mediterranean species of *Plakina* Schulze, 1880 (Demospongiae, Homosclerophorida). *J. Nat. Hist.* 33: 159–176.

Nielsen, C. 2008. Six major steps in animal evolution: are we derived sponge larvae? *Evol. Dev.* 10: 241–257.

Norrevang, A. 1964. Choanocytes in the skin of *Harrimania kupfferi* (Enteropneusta). *Nature* 204: 398–399.

Norrevang, A., and Wingstrand, K. G. 1970. On the occurrence and structure of choanocyte-like cells in some echinoderms. *Acta Zool.* 51: 249–270.

Orme, B. A. A., Otto, S. R., and Blake, J. R. 2001. Enhanced efficiency of feeding and mixing due to chaotic flow patterns around choanoflagellates. *IMA J. Math. Appl. Med. Biol.* 18: 293–325.

Orme, B. A. A., Blake, J. R., and Otto, S. R. 2003. Modelling the motion of particles around choanoflagellates. *J. Fluid Mechan.* 475: 333–355.

Pearse, V., Pearse, J., Buchsbaum, M., and Buchsbaum, R. 1994. Protozoans. In *Living invertebrates*. The Boxwood Press/Blackwell Scientific Publications, Inc., Pacific Grove.

Pettitt, M. E., Orme, B. A. A., Blake, J. R., and Leadbeater, B. S. C. 2002. The hydrodynamics of filter feeding in choanoflagellates. *Eur. J. Protistol.* 38: 313–332.

Pozdnyakov, I. R., and Karpov, S. A. 2013. Flagellar apparatus structure of choanocyte in *Sycon* sp. and its significance for phylogeny of Porifera. *Zoomorphology* 132(4): 351–357.

Rasmont, R. 1961. Une technique de culture des éponges d'eau douce en milieu controlé. *Ann. Soc. R. Zool. Belg.* 91: 147–156.

Rasmont, R., Bouillon, J., Castiaux, P., and Vandermeerssche, G. 1958. Ultra-structure of the choanocyte collar-cells in freshwater sponges. *Nature* 181: 58–59.

Riisgård, H. U., and Larsen, P. S. 2010. Particle capture mechanisms in suspension-feeding invertebrates. *Mar. Ecol. Prog. Ser.* 418: 255–293.

Roper, M., Dayel, M. J., Pepper, R. E., and Koehl, M. A. R. 2013. Cooperatively generated stresslet flows supply fresh fluid to multicellular choanoflagellate colonies. *Phys. Rev. Lett.* 110: 228104.

Saedeleer, H. 1929. Recherches sur les choanocytes; l'origine des Spongiaires (note préliminaire). *Ann. Soc. R. Zool. Belg.* 60: 16–21.

Simpson, T. L. 1984. *The cell biology of sponges*. Springer-Verlag, New York.

Sleigh, M. A. 1964. Flagellar movement of sessile flagellates *Actinomonas*, *Codonosiga*, *Monas* and *Poteriodendron*. *Q. J. Microsc. Sci.* 105: 405–414.

Tilney, L. G., and Jaffe, L. A. 1980. Actin, microvilli, and the fertilization cone of sea urchin eggs. *J. Cell Biol.* 87: 771–782.

Tobin, D. M., et al. 2002. Combinatorial expression of TRPV channel proteins defines their sensory functions and subcellular localization in *C. elegans* neurons. *Neuron* 35: 307–318.

Tuzet, O. 1963. The phylogeny of sponges according to embryological, histological, and serological data, and their affinities with the Protozoa and the Cnidaria. In E. C. Dougherty, Z. N. Brown, E. D. Hanson, and and W. D. Harman (eds.). *The lower Metazoa: comparative biology and phylogeny.* University of California Press, Berkeley.

Watanabe, Y. 1978. Structure and formation of collar in choanocytes of *Tetilla serica* (Lebwohl). *Demosponge Dev. Growth Differ.* 20: 79–91.

Weissenfels, N. 1992. The filtration apparatus for food collection in freshwater sponges (Porifera, Spongillidae). *Zoomorphology* 112: 51–55.
Woollacott, R. M., and Pinto, R. L. 1995. Flagellar basal apparatus and its utility in phylogenetic analyses of the Porifera. *J. Morphol.* 226: 247–265.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Movie S1. The flagellar wave cycle of *Monosiga brevicollis* slowed down to 1/31st (3.2%) of the real time flagellar beat frequency (57 Hz).

Movie S2. The flagellar wave cycles of five adjacent choanocytes within a choanocyte chamber in an intact *Spongilla lacustris*. The flagellar beat frequency is slowed down to 1/7th (14%) of the real time frequency (ranging from 7 to 20 Hz, depending on the choanocyte).