

Coordinated contractions effectively expel water from the aquiferous system of a freshwater sponge

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Summary

In response to mechanical stimuli the freshwater sponge *Ephydatia muelleri* (Demospongiae, Haplosclerida, Spongillidae) carries out a series of peristaltic-like contractions that is effective in expelling clumps of waste material from the aquiferous system. Rates of contraction depend on the region of tissue they are propagating through: 0.3–1 $\mu\text{m s}^{-1}$ in the peripheral canals, 1–4 $\mu\text{m s}^{-1}$ in central canals, and 6–122 $\mu\text{m s}^{-1}$ in the osculum. Faster events include twitches of the entire sponge choanosome and contraction of the sheet-like apical pinacoderm that forms the outer surface of the animal. Contraction events are temporally and spatially coordinated. Constriction of the tip of the osculum leads to dilation of excurrent canals; fields of ostia in the apical pinacoderm close in unison just prior to contraction of the choanosome, apical pinacoderm and osculum. Relaxation returns the osculum, canals and the apical pinacoderm to their normal state, and three such coordinated ‘inflation–contraction’ responses typically follow a single stimulus. Cells in the mesohyl arrest crawling as a wave of contraction passes, suggesting an

extracellular signal may pass between cells. Bundles of actin filaments traverse endopinacocytes of the apical pinacoderm. Actin-dense plaques join actin bundles in adjacent pinacocytes to form continuous tracts spanning the whole sponge. The orchestrated and highly repeatable series of contractions illustrates that cellular sponges are capable of coordinated behavioural responses even in the absence of neurons and true muscle. Propagation of the events through the pinacocytes also illustrates the presence of a functional epithelium in cellular sponges. These results suggest that control over a hydrostatic skeleton evolved prior to the origin of nerves and true muscle.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/210/21/3736/DC1> [high quality copies of the videos are available from the authors on request (sleys@ualberta.ca)]

Key words: *Ephydatia muelleri*, peristalsis, evolution of conduction, Porifera, propagated contraction.

Introduction

Sponges (phylum Porifera) have a fossil record of over 600 million years (for example, see Conway-Morris, 1993). Despite their ancient origin, they possess a vast repertoire of genes that encode regulatory signalling molecules, many of which are homologous to those in higher animals (Morris, 1993; Müller, 2003; Adell et al., 2007). Recent studies also indicate that sponge embryogenesis is characterized by a spatio-temporal pattern of gene expression that structures different regions or layers of the developing larva (Larroux et al., 2006). Other research has shown that aspects of the immune response (Wiens et al., 2004), respiration, maintenance of homeostasis (Zocchi et al., 2001), and even control of body shape as a result of changes to the stiffness of the extracellular matrix (Wilkie et al., 2004), have many features in common with equivalent physiological processes in higher animals. These examples illustrate the sponge’s ability to regulate its developmental and physiological functions; however, coordinated movements of the whole animal in response to external stimuli – the quintessential feature of Eumetazoa – are not well known.

In contrast to its molecular and physiological complexity, the sponge is a structurally simple animal. The sponge body is composed of at least eight types of cells arranged around an extensive aquiferous canal system built for filter feeding (Simpson, 1984). It is often suggested that sponges lack conventional epithelia, with typical cell–cell junctions and a basement membrane, which would create sealed internal compartments (Tyler, 2003). However, sealing junctions, though not often dense or belt-form, are present in sponge epithelia (Woollacott and Pinto, 1995; Gonobobleva and Ereskovsky, 2004). Homoscleromorph sponges have a clear basement membrane containing type IV collagen, a diagnostic feature of basal laminae (Boute et al., 1996; Boury-Esnault et al., 2003), and complexes of extracellular matrix underlying the epithelium in other demosponges have recently been found to contain spongin short chain collagen that is functionally equivalent to Type-IV of basement membranes (Exposito et al., 1991; Aouacheria et al., 2006). Although sponges lack typical organs and nervous tissue, they do have contractile cells called myocytes [or actinocytes (Boury-Esnault and

Rutzler, 1997)] that structurally and by pharmacological manipulation resemble primitive smooth muscle cells, allowing certain contractile behaviour to occur (Parker, 1910; Prosser et al., 1962; Bagby, 1965; Prosser, 1967). The extent of coordination of this behaviour is the question addressed in the present study.

As a filter-feeder, it is likely that the main problem encountered by a sponge is intake of unwanted material into the aquiferous system. Like other filter feeders, sponges have developed mechanisms to control the feeding current – but these differ in the two physiologically distinct types of sponges. Glass sponges (Class Hexactinellida) form syncytial tissues during early embryogenesis, and this tissue allows them to arrest their feeding current by propagating action potentials (Lawn et al., 1981; Lawn, 1982; Mackie et al., 1983; Leys and Mackie, 1997; Leys et al., 1999). These animals apparently lack any contractile tissues. In contrast, cellular sponges (Classes Calcarea and Demospongiae) control their feeding current by contracting centralized sphincters or cells that line the aquiferous canal system (Leys and Meech, 2006). The slow rate of contractions recorded to date suggests there is no electrical coupling between cells as suggested by Mackie (Mackie, 1979) and reiterated by Nickel (Nickel, 2004). So far ultrastructural studies have not identified gap junctions in cellular sponges (Green and Bergquist, 1979; Garrone et al., 1980; Lethias et al., 1983), but since proteins immunoreactive to anti-connexin antibodies were found in penatulaceans and anemones (Anctil and Carette, 1994; Mire et al., 2000), innexin- or connexin-like molecules may yet surface from the current sponge genome project (Joint Genome Institute 2005-7). Nevertheless, in the presumed absence of such junctions cellular sponges must possess another mechanism for coordinating effective responses to stimuli.

Over a century of research has explored the intricacies of sponge responsiveness, but because each study has observed different structures (ostia, oscula or choanosome) in different animals, the events have been thought to be localized and decremental (not propagated); cellular sponges have not been considered capable of the coordinated behaviours of higher animals (Jones, 1962; Mackie, 1979; Pavans de Ceccatty, 1979). However, a fresh look at the activities of species in two demosponge genera, *Tethya* and *Ephydatia*, suggests cellular sponges are able to propagate contractions both endogenously and in response to external stimuli. *Tethya* is an opaque ball-shaped sponge that contracts rhythmically, shrinking to one third of its normal size in 21 min (Pavans de Ceccatty et al., 1960; Reiswig, 1971); similar contractions can be triggered by natural stimuli (e.g. touch of a crustacean) (Nickel, 2004) and by chemical stimuli (Parker, 1910; Emson, 1966; Ellwanger and Nickel, 2006). In juveniles of *Ephydatia muelleri*, a transparent encrusting sponge, waves of contraction travel through the canals and chambers, taking up to 1 h to entirely encompass the entire sponge (de Vos and Van de Vyver, 1981; Weissenfels, 1984; Weissenfels, 1990).

The objective of the present study was to determine whether responses to stimuli amount to a coordinated event; that is, do the various parts of the sponge – the pinacoderm, ostia, osculum and aquiferous canals – function together to propagate contractions in a directional manner throughout the sponge's

body. This study presents the first characterization of the inflation (dilation)–contraction behaviour of *E. muelleri*. Due to its small size and transparency, and the simplicity of its body design, *E. muelleri* offers a practical model for future physiological studies.

Materials and methods

Collecting and culturing of sponges

Gemmules (reduction bodies) and pieces of the freshwater sponge *Ephydatia muelleri* (Lieberkuhn 1955) were scraped from sunken trees or submerged rocks in Frederick Lake, BC, Canada (48°47'51.7559"; 125°2'58.5600") at a depth of 0–3 m and stored in unfiltered lake water at 4°C in the dark until use (Ricciardi and Reiswig, 1993). Bags with sponge pieces were aerated monthly, and gemmules stored in this way were viable for at least one year. The gemmules were removed from the spicule skeleton by gently rubbing sponge fragments between 2 pieces of wet corduroy. Loose gemmules were washed in cold distilled water (4°C) to remove debris, sterilized with a 1% hydrogen peroxide (H₂O₂) solution for 5 min, and rinsed with cold distilled water to remove excess H₂O₂.

Using sterile pipettes, gemmules were transferred to Petri dishes containing Strekal's growth medium (0.9 mmol l⁻¹ MgSO₄·7H₂O, 0.5 mmol l⁻¹ CaCO₃, 0.1 mmol l⁻¹ Na₂SiO₃·9H₂O, 0.1 mmol l⁻¹ KCl) (Strekal and McDiffett, 1974) or M-medium (0.5 mmol l⁻¹ MgSO₄·7H₂O, 1 mmol l⁻¹ CaCl₂·2H₂O, 0.5 mmol l⁻¹ NaHCO₃, 0.05 mmol l⁻¹ KCl, 0.25 mmol l⁻¹ Na₂SiO₃·9H₂O) (Funayama et al., 2005). For whole-mount preparations, single gemmules were placed on an ethanol-washed, flamed glass or plastic 22 mm² coverslip in Petri dishes. For sandwich preparations, one 18 mm² coverslip was mounted with dental wax (Hygenic Corporation, Arkon, OH, USA) at the corners on a cover slip-bottom culture dish (Willco Wells B. V., Amsterdam, The Netherlands) that had been sterilized in 30% H₂O₂ and rinsed with 100% ethanol prior to use. Two gemmules were placed at the edge of the raised coverslip, and dishes were left undisturbed at room temperature (21°C) in the dark. The growth medium was replaced every 48 h.

Digital video time-lapse microscopy and image analysis

Time-lapse imaging was carried out using either an inverted compound microscope (Zeiss Axioskop) or a stereomicroscope (Olympus SZX-12). Images were captured with digital cameras (QI-Cam monochrome with color filter, Retiga monochrome and Sony CCD), which were interchangeable on both microscopes. Image capture and analysis was carried out using Northern Eclipse version 7 (Empix Imaging Inc., Mississauga, ON, Canada) from both live video feed and digitally taped material. Stimulation of the juvenile sponges consisted of exposing sponges to water-soluble black calligraphy ink (Sumi black ink, Delta Art Supplies, Edmonton, AB, Canada) at a concentration of 1 drop (25 µl) of 100× diluted ink in 1 ml culture water (final dilution 4000×) or vigorous shaking (2–4 Hz) of the culture medium over the sponge in the Petri dish for 1 min [hereafter called agitation, as published elsewhere (de Vos and Van de Vyver, 1981)]. Images were captured by Northern Eclipse every 5, 10 or 20 s, as indicated for each

experiment. The use of water jets, pin-pricking or damage to sponge tissue did not solicit an inflation–contraction cycle; these stimuli only generated local contractions of tissue.

Changes in diameter of the canals for every first, fifth, tenth or 20th image of the aquiferous canals, ostia, osculum and apical pinacoderm were measured in triplicate using Northern Eclipse, and data were logged to MS Excel 2003. In whole preparations, measurements of the aquiferous canals were taken at the center (diameter $217.73 \pm 14.93 \mu\text{m}$), middle (diameter $107.38 \pm 3.75 \mu\text{m}$), and peripheral canals (diameter $40.16 \pm 1.39 \mu\text{m}$). In sandwich preparations, the inner diameter of the canals was measured at two locations (100 and 300 μm apart) along a single canal. For area measurements, images of ink-fed sponges were converted to greyscale with Adobe Photoshop, two regions of 1450 μm by 1350 μm (those occupied by canals) were thresholded from 0 to 130 and the black area (that occupied by canals) was calculated and expressed as a proxy for the contraction of canals.

Fixation for fluorescence and confocal microscopy

Juvenile sponges on glass coverslips (Fisher no. 1, Ottawa, ON, Canada) were placed directly into a mixture of 3.7% paraformaldehyde and 0.3% glutaraldehyde in phosphate-buffered saline (PBS; 100 mmol l^{-1}) for 24 h at 4°C. After fixation, preparations were washed in cold buffer and incubated in 1% sodium borohydride for 5 min to remove autofluorescent free aldehyde groups. Sponge tissues were permeabilized with 0.2% Triton-X100 in PBS for 2 min and washed in cold PBS. To label the actin cytoskeleton, coverslips were inverted onto a drop of solution containing Bodipy 591 Phalloidin, Alexa 594 Phalloidin or Bodipy 505 FL Phalloidin (Molecular Probes–Invitrogen, Carlsbad, CA, USA) in PBS with 10% bovine serum albumin (BSA). A 300 μl depression was made in a Parafilm™-covered Petri dish to prevent damage to the soft tissue by the gemmule. After 3 h at room temperature, preparations were rinsed three times in cold PBS. For mounting,

sponges were incubated in a 50:50 v/v glycerine:PBS solution, and mounted in 100% glycerine or in Mowiol with Dabco (antifade reagent; Polysciences, Warrington, PA, USA), and allowed to harden overnight. For best results slides were stored at 4°C. Preparations were viewed with a Zeiss Axioskop epifluorescence microscope or a Leica 2 photon confocal microscope.

Fixation for scanning electron microscopy

Juvenile sponges on plastic or glass coverslips were fixed in a cocktail consisting of 1% OsO_4 , 2% glutaraldehyde in 0.45 mol l^{-1} sodium acetate buffer (pH 6.4) with 10% sucrose for 24 h at 4°C (Harris and Shaw, 1984). The following day, preparations were washed with cold distilled water and dehydrated in cold 70% ethanol for 24 h at 4°C. Sponges on glass coverslips were desilicified in 4% HF in 70% ethanol for 2 h at 4°C. Once the sponge had lifted off the coverslip, it was placed into a new Petri dish with fresh 4% HF in 70% ethanol at 4°C until spicules were dissolved. After desilicification, the loose sponges were dehydrated to 100% ethanol and, while still in the vial of ethanol, fractured in liquid nitrogen. Sponges on plastic coverslips and fractured pieces of loose sponge were critical point dried, mounted on aluminium stubs with silver paste or nail polish, gold coated, and viewed in a field emission scanning electron microscope (SEM).

Results

Description of juvenile sponge

Gemmules of the freshwater sponge *Ephydatia muelleri* hatched in sterile culture dishes at room temperature (18–23°C) in the laboratory within 2–4 days of plating. Within 4 days of hatching, the apical pinacoderm (surface epithelium), choanocyte chambers, canal system and incipient osculum had begun to develop, and by 7–10 days, a filtering juvenile sponge was formed. 7–10-day-old sponges typically had a single osculum arising from two large excurrent canals that bifurcated around the gemmule and branched successively into finer canals

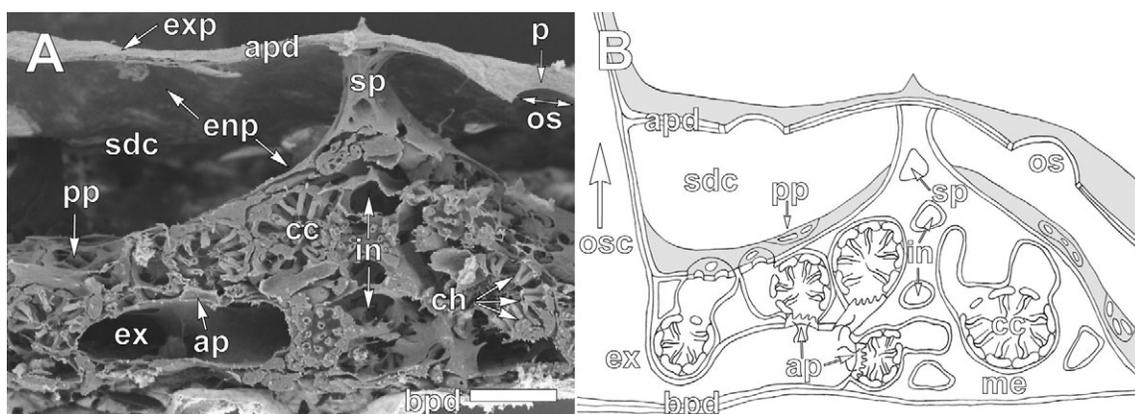


Fig. 1. Fracture (A) and schematic diagram (B) illustrating the principal features of *Ephydatia muelleri*: the apical pinacoderm (apd), sub-dermal cavity (sdc), choanocytes (ch), and basal pinacoderm (bpd). The apical pinacoderm consists of an inner layer of endopinacocytes (enp) and an outer layer of exopinacocytes (exp); porocytes (p), which form the ostia (os), are sandwiched between the two layers. The choanosome contains incurrent (in) and excurrent (ex) aquiferous canals, choanocyte chambers (cc) and spicule tracts (sp) that support the apical pinacoderm. A thin collagenous middle region (mesohyl, me) houses mobile cells. Prosopyles (pp), the entrance to chambers are formed by perforate ‘sieve’-like cells. Apopyles (ap) vent water from chambers. Scale bar, 20 μm .

lined by choanocyte chambers. In other specimens, the osculum was positioned directly over the gemmule and arose from a highly branched network of smaller excurrent canals (Fig. 1A,B).

Description of the inflation–contraction behaviour

The response triggered by stimulation of the sponge, either by adding ink to the water or by agitation of the dish, consisted of three phases (Fig. 2A–E; Movie 1 in supplementary material): an inflation phase, in which the major excurrent canals dilated; a plateau phase, involving dilation of smaller diameter canals (this phase was most pronounced in larger specimens); and a contraction phase, in which the excurrent canals constricted and there was a rapid contraction of the osculum. The sequence of events following either stimulus was similar, but changes in the morphology were more readily measured in the absence of ink.

Every response consisted of eight identifiable events: (1)

initial contraction of the osculum and apical pinacoderm (Fig. 2A); (2) Inflation phase: expansion of the sub-dermal cavity raising the apical pinacoderm and dilation of the excurrent canals travelling from the base of the osculum back along the aquiferous canal system; (3) dilation of the smallest peripheral canals; (4) contraction of porocytes (closure of ostia) in the apical pinacoderm; (5) Contraction phase: lowering of the apical pinacoderm forcing the water into the aquiferous canals; (6) a peristaltic-like wave of contraction that travelled along the excurrent canals from the distal edge of the sponge to the base of the osculum and caused the contraction of the choanocyte chambers (Fig. 2B); (7) a rapid contraction propagating from the base to the tip of the osculum (Fig. 2C); and (8) relaxation of the canals to their original diameter and extension of the osculum back to its original length (Fig. 2D). This sequence followed a predictable time course, and up to three such sequences occurred after a single stimulus, each separated by a recovery period.

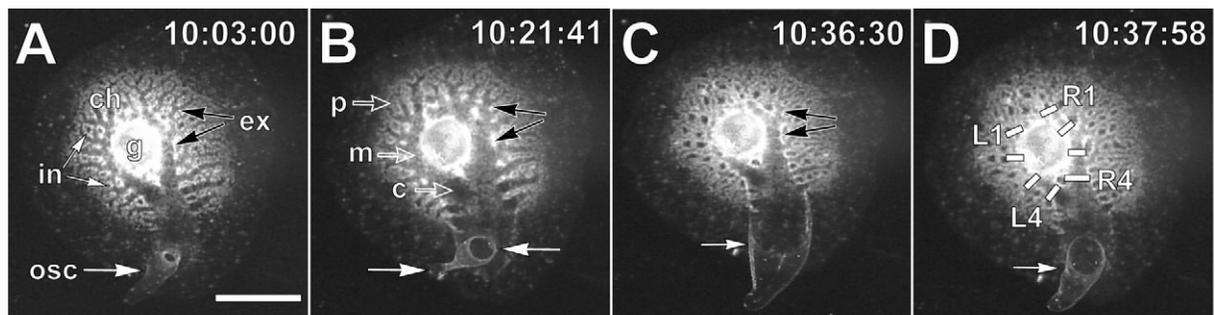


Fig. 2. The response of *E. muelleri* to mechanical agitation. (A–D) Light micrographs illustrating the changes to the excurrent aquiferous system (black arrows) during one inflation–contraction cycle. Choanosome (ch), excurrent canals (ex), gemmule (g), incurrent canals (in), and osculum (osc). Scale bar, 1 mm. (A) Initial contraction of the osculum: immediately after stimulation the base of the osculum contracts but the tip remains slightly open. (B) Inflation phase: excurrent canals dilate (black arrows); the base of the osculum begins to dilate, but the tip remains constricted (white arrows); hollow arrows indicate the locations of peripheral (p), middle (m) and central (c) canals. (C) Contraction phase: excurrent canals contract (black arrows) and the base of the osculum dilates (white arrow). (D) Contraction of the osculum (arrow) and return of canals to their original diameter. A–D correspond to phases *a–d*, respectively, in (E–G) below. (E–G) Changes in diameter of the largest excurrent canal and osculum (E) during the inflation–contraction cycle, and of all canals on the right (F) and left (G) sides of the sponge. R1–R4 and L1–L4 in D indicate locations of measurements plotted in F and G. (See Movie 1 in supplementary material.)

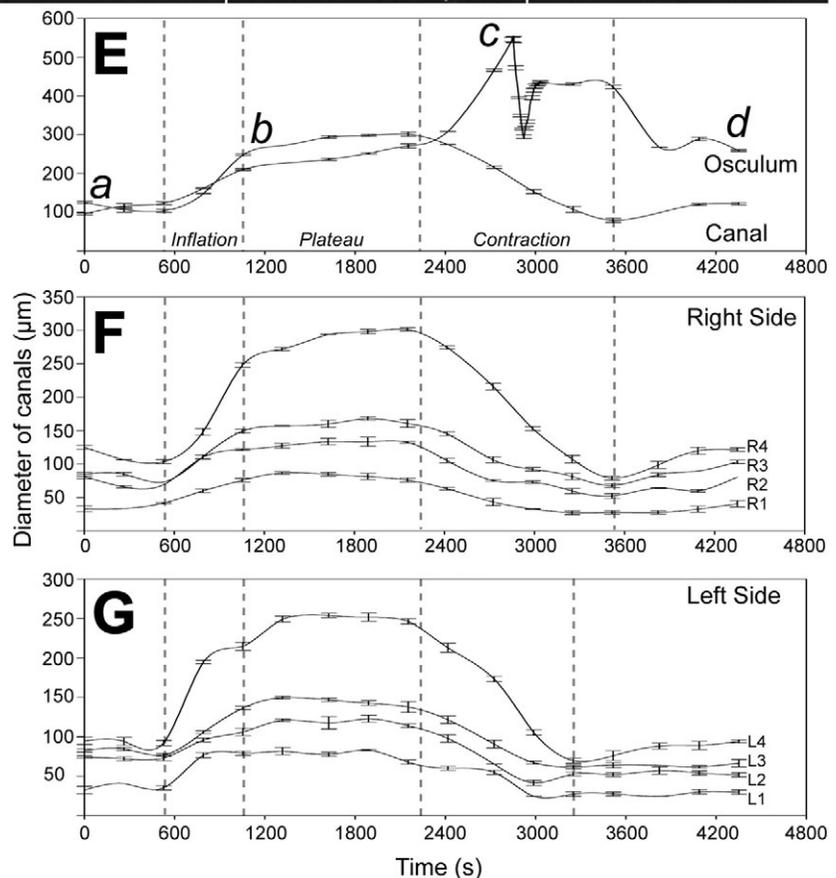


Table 1. Duration of the phases in the inflation–contraction cycle in response to different stimuli

Stimulus	Δt^I (min:s)	Δt^{Ch-C} (min:s)	Δt^{Os-C} (min:s)	Δt^{I-C} (min:s)
Ink 'fed'	11:30±4:43 (8)	15:55±3:19 (8)	1:43±0:24 (5)	30:45±2:01 (8)
Shaken	8:31±1:38 (12)	10:43±1:34 (12)	0:41±0:19 (3)	19:09±2:45 (12)
<i>P</i>	0.47	0.14	0.13	0.21

Values are means ± s.e.m.; *N* values are given in parentheses.

Δt^I Duration of inflation cycle; Δt^{Ch-C} , duration of choanosome contraction; Δt^{Os-C} , duration of oscular contraction.

Although the velocity of the propagated contraction varied as it progressed from the periphery of the sponge towards the osculum, both the left and right hand sides of the sponge inflated and contracted equally (Fig. 2F,G).

Response to the addition of inedible ink particles

The inflation–contraction cycle was first recorded in response to the addition of inedible ink to the culture dish. Ink taken into the sponge became lodged in the choanocyte chambers, making them black. Addition of the ink into the culture medium resulted in several events. The first was identical to the orchestrated series of responses triggered by agitation of the sponge as described above, but resulted in ejection of clumps of ink (supplementary material Fig. S1A–E; Movie 2). Additional responses followed minutes to hours later; up to three additional peristaltic-like waves traversed the entire sponge over a 48 h period.

The ink treatment also generated brief contractions that occurred simultaneously in different parts of the choanosome (like twitches), as well as short waves of contraction that propagated across portions of the choanosome in a linear direction: ripples. There were also local non-propagating inflations and contractions: local events. Addition of too little ink to the dish did not trigger the full 'inflation–contraction behaviour', but twitches and ripples still occurred. Similarly, too little agitation of the dish failed to trigger a full inflation–contraction cycle, but twitches, ripples and local events were common after any amount of agitation.

Attempts to trigger the full inflation–contraction cycles by focal tactile stimuli (pin pricks) and electrical stimuli have so far been unsuccessful.

The kinetics of the inflation–contraction cycle

Comparison of the duration of the entire cycle from start of inflation to end of contraction for ink 'fed' (30:45±2:1 min:s; *N*=8) and shaken sponges (19:9±2:45 min:s; *N*=12) suggests that addition of ink slows down the process (Table 1). Estimates of rates at which the events occur in different regions of the sponge (across the choanosome, along a canal, and up the osculum) depended upon the type of preparation (whole mount or sandwich) and type of stimulus applied. Contractions of the osculum were fastest, but contractions propagated across the choanosome more slowly during a full 'inflation–contraction' cycle (2–5 $\mu\text{m s}^{-1}$) than during ripples that occurred between cycles (4–11 $\mu\text{m s}^{-1}$) (Table 2). During an 'inflation–contraction' cycle, contractions usually propagated across all tissues from the periphery of the sponge to the base of the osculum; in some cases the waves travelled along the long axis of canals, but in others an entire canal expanded in unison as the wave propagated across it. All told, the rates appeared to be very dependent on the resulting effect of the contraction.

Canals

The full inflation–contraction behaviour had stereotypical 'inflation', 'plateau' and 'contraction' phases, but the duration of the entire event varied depending on the initial (resting) diameter of the aquiferous canals: sponges with larger resting canal diameter (e.g. 64.9, 76.9, 103.52, 154.13 and 213.35 μm) had a longer overall inflation–contraction phases (500, 899, 1399, 2052, 2988 s; Fig. 3 and supplementary material Fig. S2), extending the duration of the entire cycle from 15 to 40 min. Otherwise, the events occurred almost identically in sponges

Table 2. Rates of contraction in different regions of *E. muelleri* juveniles

Region (type of response; stimulus and preparation)	Contraction rate ($\mu\text{m s}^{-1}$)	Range ($\mu\text{m s}^{-1}$)
Choanosome		
Canals (full cycle; agitation; whole mount)		
Incurrent canal (inflation phase)	2.80±0.26 (5)	2.28–2.63
Excurrent canal (contraction phase)	3.30±0.45 (5)	2.31–4.99
Canals (full cycle; ink; sandwich preparation)		
Incurrent canal (inflation phase)	1.68±0.78 (6)	0.25–5.0
Excurrent canal (contraction phase)	0.49±0.13 (7)	0.32–1.0
Canals (ripple; agitation; whole mount)	7.09±0.95 (7)	4.5–11.72
Osculum (full cycle; agitation; whole mount)	71.85±32.4 (3)	11.59–122.80
(full cycle; ink; whole mount)	17.68±8.26 (5)	6.31–50.34

Values are means ± s.e.m.; *N* values are given in parentheses.

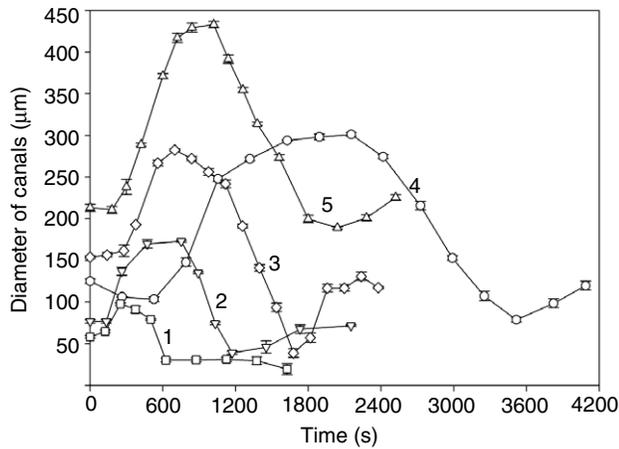


Fig. 3. The duration of the inflation–contraction cycle depends on the resting diameter of the largest excurrent canals. Responses to agitation were measured in five sponges with the same sized (diameter) choanosome, but with varying sizes (1–5) of excurrent canals: 64.9, 76.9, 103.52, 154.13 and 213.35 μm , respectively. Sponges with larger excurrent canal diameters have a longer inflation and contraction period (500, 899, 1399, 2988, 2052 s, respectively), but the rate of propagation of contractions along incurrent and excurrent canals was not significantly different (in: $2.80 \pm 0.26 \mu\text{m s}^{-1}$, $N=5$; ex: $3.30 \pm 0.45 \mu\text{m s}^{-1}$, $N=5$, $P=0.23$). Bars denote \pm s.e.m. of three measurements from one sponge (see supplementary material Fig. S2).

with quite different patterns of canals. The rates of dilation and contraction of the large excurrent canals were similar ($2.80 \pm 0.26 \mu\text{m s}^{-1}$, $N=5$ and $3.30 \pm 0.45 \mu\text{m s}^{-1}$, $N=5$, $P=0.23$, respectively; Table 2). Interestingly, the rate of the peristaltic-like contraction, measured from preparations in which specific points on the canals could be accurately tracked at high resolution, depended upon which region of the aquiferous

system it moved through. In the peripheral canals it traveled at $0.03\text{--}1 \mu\text{m s}^{-1}$, in the central canals at $1\text{--}4 \mu\text{m s}^{-1}$, and up the osculum at $6\text{--}122 \mu\text{m s}^{-1}$.

Sandwich preparations allowed clear observations of the waves of peristaltic-like contraction, and use of the ink stimulus provided a clear marker for incurrent and excurrent aquiferous canals. This preparation revealed that during the inflation phase, dilation of the excurrent canals was caused by a wave of contraction travelling along the incurrent canals. Ink entered incurrent canals rapidly filling choanocyte chambers

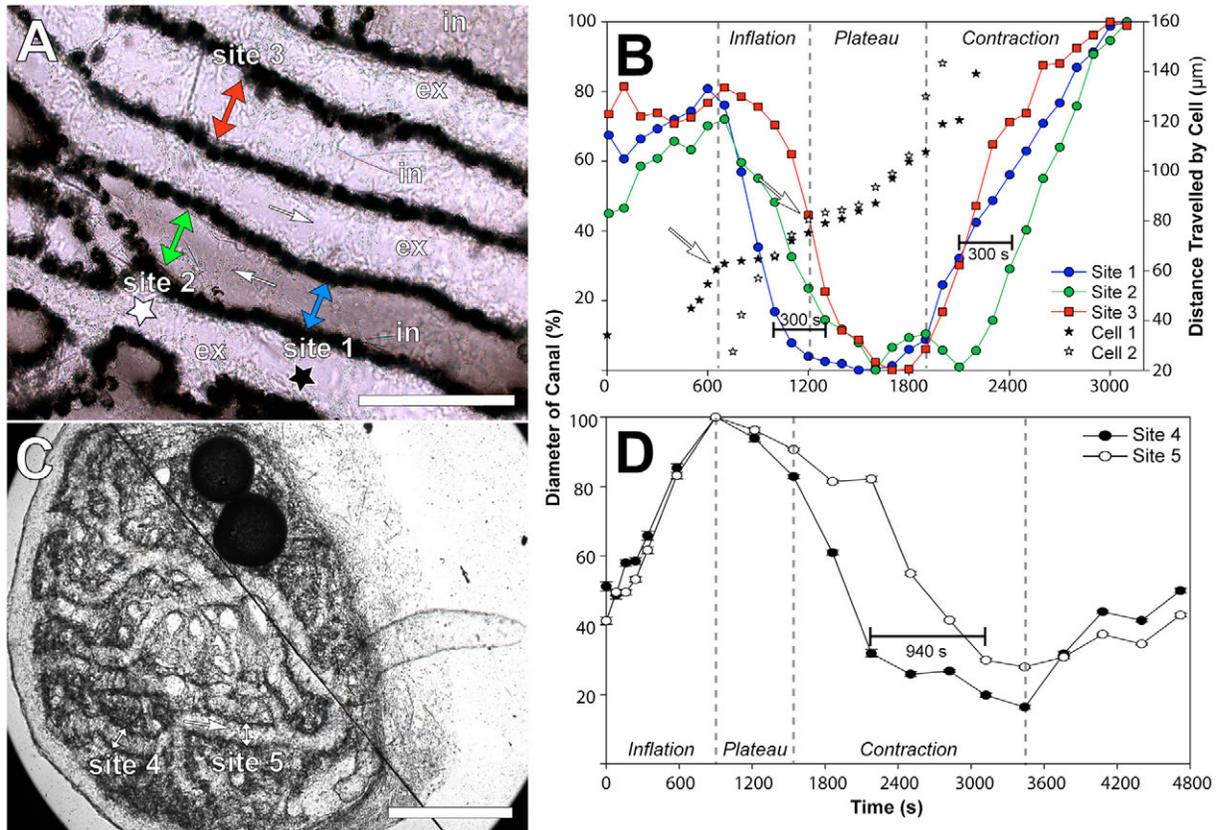


Fig. 4. Analysis of the kinetics of the contraction of incurrent (A,B) and excurrent (C,D) canals shows that waves of contraction propagate along and across canals. (A,C) Sponges grown as sandwich preparations were stimulated by addition of inedible ink. Measurements of incurrent (A) and excurrent (C) canal diameters over time are plotted in (B) and (D), respectively. (B,D) In B the wave of contraction propagated between sites 1 and 2 (100 μm apart) in 300 s, a rate of $0.33 \mu\text{m s}^{-1}$. The wave of contraction reached site 3 with a delay of 150 s. Cells crawling through the mesohyl (indicated by white and black stars on A and B) arrest movement for approximately 10 min (B, white arrows) while the wave of contraction passes. In D the wave of contraction propagated between sites 3 and 4 (300 μm apart) in 940 s, rate of $0.32 \mu\text{m s}^{-1}$. in, incurrent; ex, excurrent; arrows indicate direction of water flow in the canal. Scale bars, 100 μm (A); 300 μm (C). (Movie 3 in supplementary material.)

(supplementary material Fig. S3, Movie 3). Contraction of the incurrent canals condensed the ink in the chambers and incurrent canals and even forced some ink-filled water through the choanocyte chambers into the excurrent canals during the plateau phase. During the contraction phase, a wave of contraction propagated along the excurrent canals so as to cause the dilation of the incurrent canals. As the excurrent canals contracted, the flow of water (as seen by movement of ink) briefly reversed direction, and then remained stationary for up to 6 min. After one inflation–contraction cycle the sponge returned to a relaxed state. This type of preparation also illustrated that the wave of contraction propagated along two vectors, both along and across the incurrent and excurrent canals (Fig. 4A; supplementary material Movie 3). Contractions traveled across canals that were $310\ \mu\text{m}$ apart at a delay of 300 s (approximately $1\ \mu\text{m}\ \text{s}^{-1}$). Furthermore, cells crawling through the mesohyl arrested forward motion for about 10 min (approximately 600 s) as the wave of contraction passed by (Fig. 4B). The two cells tracked here were $1053\ \mu\text{m}$ apart, and they arrested with a delay of 600 s.

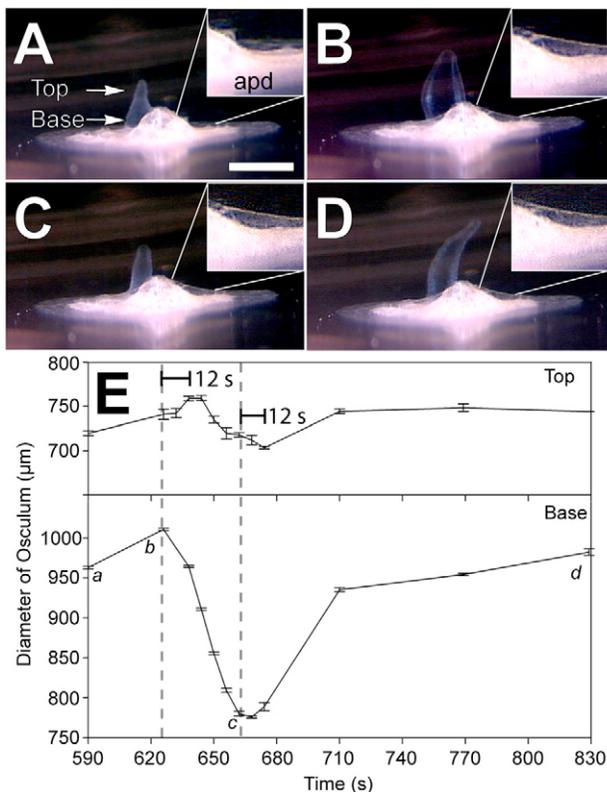


Fig. 5. Stereomicrographs (A–D) and changes in diameter (E) of the tip and base of the osculum (at positions indicated by arrows in A) during contraction of the osculum. (A) Immediately after stimulation by agitation, (B) during the contraction phase when its base is fully inflated, (C) fully contracted; and (D) when relaxed at the end of the cycle; insets show enlargements of the position of the apical pinacoderm. Scale bar, 1 mm. A–D correspond to phases a–d, respectively, in (E). Between A and D the canals inflate and the apical pinacoderm raises. The wave of contraction propagates from base to tip, a distance of $1473\ \mu\text{m}$, in 12 s, a rate of $122.8\ \mu\text{m}\ \text{s}^{-1}$. Bars denote \pm s.e.m. of an average of three measurements from one sponge. (Movie 4 in supplementary material.)

In sandwich preparations stimulated with ink, the contractions propagated along the incurrent canals slightly faster than along the excurrent canals (Table 2; Fig. 4C,D). Time-lapse images of these events in sandwich preparations suggest that cells in the mesohyl between two canals shorten, causing the choanocyte chambers to compress. These images also show that cells crawling through the mesohyl stop moving as the waves of contraction pass over them (supplementary material Movie 3).

Osculum

Immediately after agitation or addition of ink, the osculum contracted downwards. Then, as the aquiferous canals contracted, the base of the osculum dilated to become almost balloon-like. Only when the entire choanosome had completely contracted did a wave of contraction run from the base to the tip of the osculum (Fig. 5A–D). The final oscular contraction took $71.85 \pm 32.4\ \mu\text{m}\ \text{s}^{-1}$ ($N=3$) in agitated sponges and $17.68 \pm 8.7\ \mu\text{m}\ \text{s}^{-1}$ ($N=5$) in ink-fed sponges (range $6\text{--}122\ \mu\text{m}\ \text{s}^{-1}$), and was always followed by a slow extension (supplementary material Movie 4). Because precise changes in diameter of the osculum were difficult to track in ink-fed animals, measurements for those animals present a conservative estimate of the duration of the contraction event.

Apical pinacoderm

Upon agitation the apical pinacoderm contracted down towards the choanosome, lowering $50\text{--}200\ \mu\text{m}$ within 60 s. This contraction occurred after the initial response of the osculum, but before the inflation of the canals. The apical pinacoderm moved as a single unit, like a diaphragm, reducing the volume of the sub-dermal space. During the inflation phase, the apical pinacoderm moved back to its relaxed position (Fig. 5A,B), and just before the excurrent canals contracted, it lowered again. For sponges with a diameter of $3\text{--}5\ \text{mm}$, these waves of contraction traveled at $50\text{--}80\ \mu\text{m}\ \text{s}^{-1}$ propagating from the periphery of the sponge to the base of the osculum. In some instances a series of twitches occurred across the entire surface of the sponge just before the main wave of contraction that lowered the entire apical pinacoderm (supplementary material Movie 4).

Porocytes

In relaxed sponges, fields of porocytes – flat cells that formed the ostia, incurrent openings for water – littered the apical pinacoderm (Fig. 6). The margin of each cell was anchored in a collagenous extracellular matrix between the inner and outer epithelia of the apical pinacoderm. Each porocyte was surrounded by 3–4 plate-like exopinacocytes. In a relaxed sponge there were $10\text{--}12$ porocytes mm^{-2} of apical pinacoderm (Fig. 6A). After stimulus by agitation, fields of up to 35 ostia closed synchronously (Fig. 6A–C; supplementary material Movie 5). Individual ostia took approximately 40 s to close, and a field of ostia closed just before the contraction of excurrent canals in the choanosome. Ostia re-opened as canals relaxed (Fig. 6D).

In ink-fed sponges the ostia also closed just before canals contracted, and remained closed until the contractions had finished. Use of the ink as a stimulus revealed that in all cases

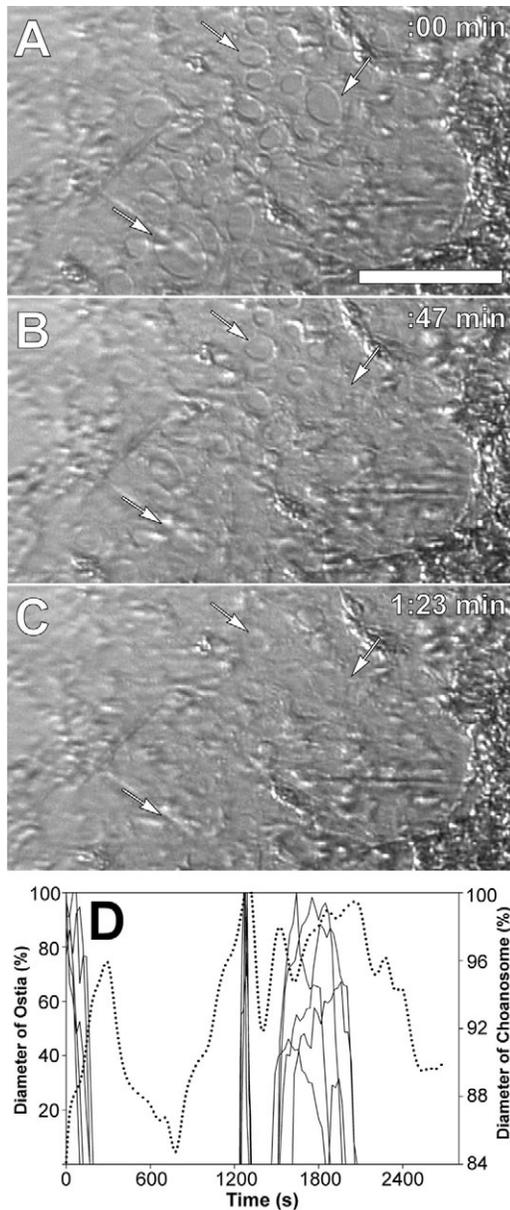


Fig. 6. Closure of a field of porocytes in the apical pinacoderm correlates with contraction of the choanosome after stimulus by agitation. (A–C) Stereo microscope images show that individual ostia (arrows) close within 50 s, and a field of 20 porocytes closes over a period of 83 s. Scale bar, 100 μm. (D) Constriction (closing) of eight ostia in the field shown in A is correlated with the contraction of the choanosome (broken line). Shortly after the sponge was stimulated the ostia closed and the choanosome contracted. A few ostia opened briefly at 1200 s during an expansion of the choanosome, but these closed again as the choanosome contracted. The field opened again with the expansion of the choanosome at $t=1500$ s (here approximately 22 min later), and again closed just prior to contraction of the choanosome at 2400 s.

a few ostia near the base of the osculum remained open, allowing a small amount of water to flush back out through the sub-dermal cavity (observed as puffs of ink in supplementary material Movie 6).

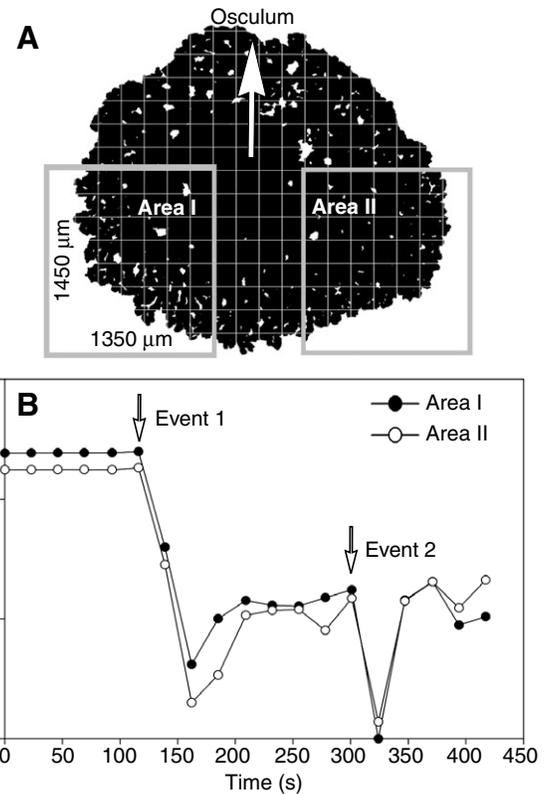


Fig. 7. Rapid contractions ('twitches') occur simultaneously in distinct regions of the choanosome after uptake of inedible ink. (A) Projected area (density of the tissue) was measured as a proxy for the extent of contraction of the tissue; a contraction was observed as a decrease in the area occupied by tissue (a decrease in blackness). (B) The change in projected area of each region shows that two contraction events occur within seconds of each other in Areas I and II of A, 700 μm apart. (Movie 2 in supplementary material.)

Kinetics of twitches, ripples and local contractile events

In between sequential inflation–contraction cycles, brief propagating and non-propagating contractions took place. In many experiments, waves of contraction rippled across portions of the sponge choanosome at a rate of $7.09 \pm 0.95 \mu\text{m s}^{-1}$ ($N=7$); these contractions did not travel towards the osculum and occurred without periodicity. Local contractile events also occurred in the interval between major inflation–contraction cycles. Here, a small region of the choanosome, usually no more than several hundred μm in diameter, inflated and contracted independently of any other activity of the sponge. In some experiments, the entire sponge choanosome contracted rapidly and apparently simultaneously like a twitch. These quick, global contractions of less than 20 s duration occurred nearly simultaneously (<5 s difference) in very different regions of the choanosome (Fig. 7A,B; supplementary material Movie 2).

Typically, an unstimulated sponge exhibited occasional ripples, twitches and local inflation–contraction events; however, only one full inflation–contraction cycle occurred during every 8 h of 48 h of observation.

The contractile apparatus of the sponge

Phalloidin-labelled sponges revealed dense tracts of actin in

endopinacocytes of the apical pinacoderm, canals and the osculum. In the apical pinacoderm, 2–3 bundles of filamentous actin traversed individual pinacocytes (Fig. 8A,B). Contacts between neighbouring cells labelled brightly, like adhesion plaques, and actin bundles in adjacent cells continued in the same direction so as to form tracts that were continuous for up

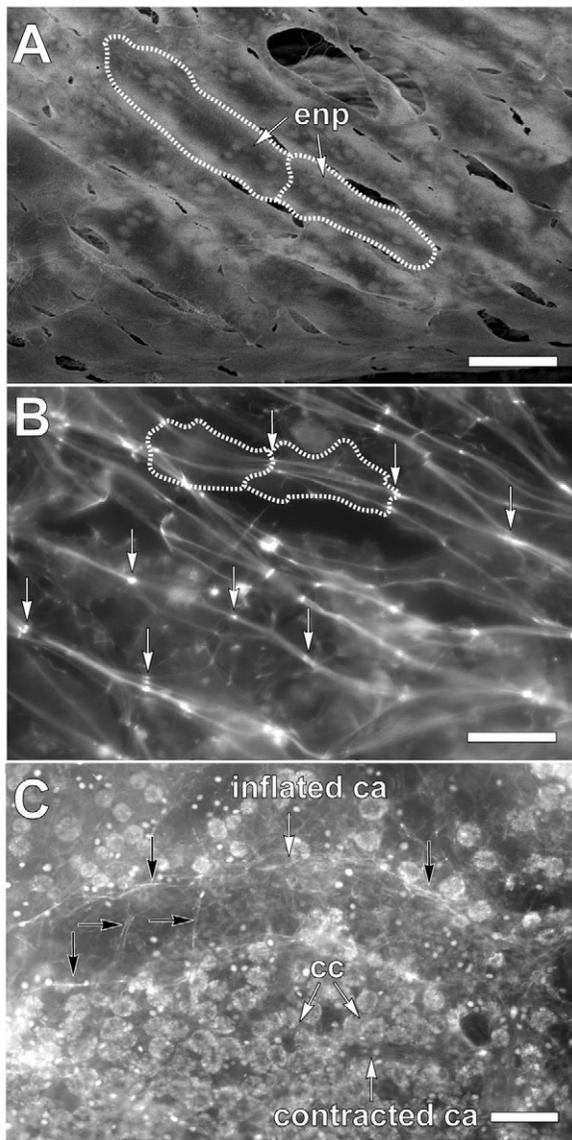


Fig. 8. Actin distribution and morphology of pinacocytes. (A) Scanning electron microscopy shows that endopinacocytes (enp) are elongated cells that form the underside of the apical pinacoderm. Dotted lines indicate cell boundaries. Scale bar, 10 μm . (B) Epifluorescence microscopy of Bodipy 505 FL Phalloidin-labelled tissue shows extensive tracts of actin in endopinacocytes of the apical pinacoderm, a region equivalent to that shown in A. Actin is brightly labeled in focal adhesion plaques between cells (arrows). Dotted lines indicate cell boundaries, demonstrating that the actin tracts continue in adjacent cells. Scale bar, 50 μm . (C) In cells lining excurrent canals of a sandwich preparation, actin tracts (black arrows) are much less brightly labelled. The preparation was fixed as a wave of contraction passed through the field of view. Dense packing of choanocyte chambers (cc) indicates that the lower canal was contracted. Scale bar, 100 μm .

to 3 mm (Fig. 8B). These tracts of actin stretched across the apical pinacoderm, around the perimeter of the sponge, and from the perimeter of the sponge to the top of the gemmule converging at the pinnacle of shafts of spicules that supported the overlying apical pinacoderm. Endopinacocytes lining the canals labelled much less intensely with phalloidin, and fine tracts of actin were visible only in sandwich preparations in which sponges grew in a 50 μm thick space between two coverslips (Fig. 8C). In unstimulated sponges, excurrent canals were lined by thin (1–3 μm) endopinacocytes, and choanocyte chambers were spherical (30 μm in diameter) (supplementary material Fig. S4A,B). In sponges fixed in a contracted state, endopinacocytes lining the excurrent canals were thicker (5–7 μm) and choanocyte chambers were compressed to the extent that their flagella projected out through the apopyle (supplementary material Fig. S4C,D).

Discussion

In response to a mechanical stimulus *Ephydatia muelleri* initiates a series of slow contractions (summarized in Fig. 9) that effectively expel water and wastes from the aquiferous system. The periodicity of contractions seen in unstimulated sponges, and reported in other species and genera, suggests this behaviour may also function to assist the sponge feeding and/or respiratory activity (Weissenfels, 1990; Nickel, 2004). There is growing evidence that sponges, like other metazoans, possess a broad repertoire of signaling molecules (e.g. Perovic et al., 1999; Nichols et al., 2006; Adell et al., 2007; Sakarya et al., 2007) and experiments have demonstrated that many of these substances trigger contractile responses in a variety of sponges (Emson, 1966; Prosser, 1967; Ellwanger and Nickel, 2006; Ellwanger et al., 2007). The exact nature of the contractile response has nevertheless been rather unclear, largely due to the difficulty of watching the animal at the cellular level. The small size and transparent tissues of *E. muelleri*, however, allow high magnification observations of specific regions of the sponge, which illustrate that it is not the speed of contraction but rather the temporal and spatial coordination of all the events that allows the sponge canal system to form an effective peristaltic-

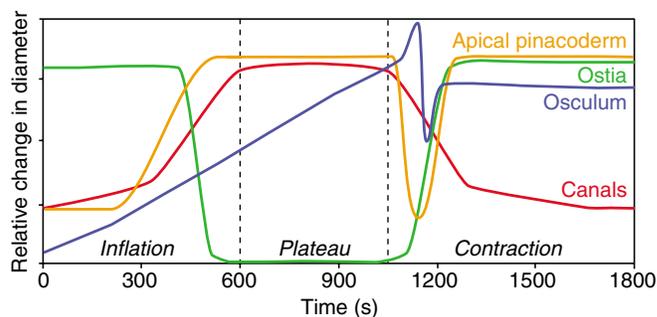


Fig. 9. Summary diagram illustrating the temporal coordination of contractions by the aquiferous canals, apical pinacoderm, ostia and osculum, during a single inflation–contraction event in *E. muelleri*. During the inflation phase, the apical pinacoderm, canals and osculum gradually dilate. The ostia contract for the duration of the inflation phase. The contraction of the apical pinacoderm and canals lead to the full inflation of the osculum and its rapid contraction. Ostia open only after all other components have relaxed.

like pump. Given the basal position of sponges within Metazoa, and the absence of nerves and true muscle in this group (Pavans de Ceccatty, 1989) (see references above), it can be inferred that what we see in sponges today represents a coordination system that predated the evolution of neuromuscular systems observed in higher Metazoa.

Rates of contraction

Rates of waves of contraction reported in cellular sponges are several orders of magnitude slower than electrically controlled contractile systems (for reviews, see Mackie 1979; Mackie et al., 1983). Contractions tend to be slightly slower in freshwater than marine sponges (presumably due to the lower calcium available), but rates of endogenous contractions reported in the literature largely depend on what region of a sponge was observed. For example, waves of endogenous contractions cross the surface (including the choanosome) of marine sponges (*Tethya wilhelma*, *Tethya lyncurium*, *Euspongia officinalis*) at 12–30.5 $\mu\text{m s}^{-1}$ (Pavans de Ceccatty, 1969; Pavans de Ceccatty, 1971; Nickel, 2004), and freshwater sponges (*Ephydatia fluviatilis*, *Eunapius fragilis*, *Spongilla lacustris*) at 8–11 $\mu\text{m s}^{-1}$ (de Vos and Van de Vyver, 1981; Weissenfels, 1990). Electrical stimuli applied to the base or tip of the osculum of *Ephydatia fluviatilis* triggered faster waves of contractions up and down (170 and 350 $\mu\text{m s}^{-1}$, respectively) the osculum (McNair, 1923); Prosser (Prosser et al., 1962) reports a similarly quick contraction of the oscula (1–5 s for 1 mm diameter oscula) in several marine species. Furthermore, precise measurements of rates are difficult to calculate from video recordings or a series of still images. For example, in *T. wilhelma*, periodic contractions have been documented by measuring the decrease in area of a projection of the sponge (Nickel, 2004). Subcontractions (equivalent to ripples) propagate at 12.5 $\mu\text{m s}^{-1}$ over the surface of the sponge, yet full contractions take 20–50 min to encompass the entire sponge; relaxation (inflation) takes somewhat longer. Although it is proposed that the contraction travels through the pinacoderm, because *Tethya* is an opaque sphere, the route that the contractile wave travels cannot be easily determined.

We encountered similar difficulty in determining precisely when contractions initiate at two points 100 μm apart along a canal. Cells in the mesohyl around the canal begin to change shape long before changes to the diameter of the canal are evident. Also, in some instances entire canals seemed to widen uniformly along their entire length, such that no ‘rate’ of propagation could be measured. In general, however, contractions propagated very slowly through the canals at the periphery of the sponge (0.3–1 $\mu\text{m s}^{-1}$), slightly faster through the large exhalent canals (1–4 $\mu\text{m s}^{-1}$), and even faster up the osculum (6–122 $\mu\text{m s}^{-1}$); these were part of the overall ‘inflation–contraction’ behaviour, while ripples and twitches occurred separately. Thus our study indicates that the actual speed of propagation of a contraction depends on the function of the contractile tissue (the effector). From this we infer that because each region comprises part of a hydrostatic skeleton whose function is to expel water from the aquiferous system, the rates observed indicate control of the body of water rather than the absolute ability to propagate a signal. The individual rates observed result from coordination of these regions.

Coordination of effectors

Coordination of the series of effectors is seen most acutely in the synchronous closure of fields of ostia independently of, and usually just before, the contraction of the apical pinacoderm. It has long been known that individual porocytes contract (Emson, 1966; Kilian and Wintermann-Kilian, 1979), but this is the first data showing that whole fields of porocytes contract and relax in unison. Synchronous closure of ostia is a remarkable event. The contraction of each porocyte sphincter takes some 60 s, but the fact that up to 50 ostia close over the same time frame, and just before the choanosome contracts, points either to some fairly rapid coordinating signal traversing the apical pinacoderm, or suggests that inflation of the entire sponge stretches the apical pinacoderm, triggering simultaneous closure of ostia (presumably by entry of calcium into each porocyte). It is interesting to note that in all experiments a few ostia remained open around the base of the osculum, allowing ink to be flushed back and out of the sub-dermal cavity. Reversal of flow by sponges has only been described by Storr (Storr, 1964) and likely refers to a similar back-flushing event during a periodic (cyclical) contraction event.

Contraction of inhalant and exhalant canals also demonstrates coordination of effectors. We initially thought that dilation of the exhalent canals occurred by passive inflation when the osculum closed in response to the initial stimulus. However, careful observation of videos shows that the osculum is never entirely closed – the tip constricts, but a fast stream of water continues to flow from it at all times (e.g. ink flows from the constricted osculum prior to explosion of ink from the choanosome, see supplementary material Movie 2). Sponges treated with cytochalasin B did not inflate the choanosome (dilate the incurrent or excurrent canals), even though the osculum did a small initial contraction when the dish was vigorously shaken; thus passive inflation of the choanosome is unlikely (data not shown). Because videos of sandwich cultures show that cells in the mesohyl bridging adjacent exhalent canals contract during the inflation period, we suggest that dilation of the exhalent canals seems to be at least partly due to the active contraction of inhalant canals. These observations explain why the rates of inflation and contraction are very similar regardless of the diameter of the canal (Table 1). What can also be seen is that water is absolutely stagnant for some part of the plateau phase (the ink front in the excurrent canal remains completely stationary for up to 6 min in one instance; supplementary material Movie 3), i.e. the sponge uses contractions to control the movement of water in its canals. This observation is the first precise visual demonstration that cellular sponges can stop their feeding current.

Evidence for effector tissue and signal propagation

Most studies suggest that endopinacocytes (the cells that line the inside of the sponge) are responsible for propagated contractions (de Vos and Van de Vyver, 1981; Pavans de Ceccatty, 1986; Nickel, 2004), but in sponges with a denser mesohyl, it is implied that either myocytes (cells in the mesohyl) or pinacocytes form sphincters that constrict flow through canals (Parker, 1910; Pavans de Ceccatty, 1960; Jones, 1962; Prosser et al., 1962; Bagby, 1965; Pavans de Ceccatty et al., 1970). The contractile apparatus has been difficult to pin down.

The actin cytoskeleton is only known from stationary basoendopinacocytes of freshwater sponges (Pavans de Ceccatty, 1986; Wachtmann and Stockem, 1992), and from myocytes in one marine sponge (*Microciona prolifera*). In basoendopinacocytes, the cytoskeleton is much like that of a fibroblast in which microfilaments form stress fibres across and around the cell. Actin filaments are slightly denser between neighboring cells, and between cells adhesion plaques reminiscent of early stage desmosomes in fish embryos (Lentz, 1966) can be seen in freeze–fracture electron micrographs (Pavans de Ceccatty, 1986). In contrast, myocytes in sphincters in the canals are well endowed with both thick and thin filaments (Bagby, 1965).

Our images show that a substantial actin network exists in the cells that form the lower portion of the apical pinacoderm, the endopinacocytes. Bundles of actin filaments form tracts traversing endopinacocytes, and each tract connects to another in neighboring cells through a dense plaque of actin; together these form the longest semi-continuous tracts known in sponges (1–3 mm). Continuity of the cytoskeleton in the apical pinacoderm is presumably necessary for the entire tent-like structure to lower in a single diaphragm-like movement in less than 60 s. Actin microfilaments appear as ‘rings’ around the circumference of the aquiferous canals; in both cases tracts connect to others in neighboring cells, as in the apical pinacoderm.

Earlier researchers favored mechanical tugging of one cell on another as the explanation of contractile waves (Parker, 1910; Pavans de Ceccatty et al., 1960; Emson, 1966; Pavans de Ceccatty, 1969). This hypothesis is difficult to test because damage to any portion of the sponge disrupts flow and interrupts contractions throughout the sponge. Furthermore, although mechanical ‘tugging’ might explain how waves of contraction propagate along canals, it does not readily explain how the waves propagate across canals or between completely distinct regions of the sponge as during twitches. It is possible that a change in pressure could result in signals being transmitted to a distant site, but how ink building up in the chambers could generate a pressure wave causing the osculum to constrict (the first event to occur) is unclear. Moreover, how pressure waves could orchestrate the spatio-temporal coordination of contractions in different regions is difficult to imagine.

Recent evidence that diffusible chemical messengers including amino acids (glutamate and GABA), biogenic amines and short-lived gases (e.g. nitric oxide) trigger or modulate contractions in *Tethya wilhelma* strongly suggest that signals travel through the mesohyl in a paracrine-like manner or through the aquiferous system (Ellwanger and Nickel, 2006; Leys and Meech, 2006; Ellwanger et al., 2007). Perhaps the most definitive evidence that a diffusible chemical messenger is involved in contractions in *Ephydatia* is that cells crawling through mesohyl stop moving as contractions pass by (Fig. 4), as also noted by other authors (de Vos and Van de Vyver, 1981). Since these cells are wandering through the mesohyl, not in contact with pinacocytes, it can be inferred that a signal passes through the mesohyl at least at $1.75 \mu\text{m s}^{-1}$ (a distance of $1053 \mu\text{m}$ in 600 s). It is quite possible that chemical and mechanical signalling function together to coordinate the propagation of contractions. Nevertheless, the rapid lowering of

the apical pinacoderm and rapid contraction of the osculum are faster events than can be explained by calcium signalling, which is generally up to $20 \mu\text{m s}^{-1}$ (Nedergaard, 1994).

Comparison with other contractile systems

We describe contractions in *E. muelleri* as ‘peristaltic-like’, but this is the first time the term peristalsis would be applied to an animal that lacks muscle. Peristalsis is usually considered to involve neurogenic modulation of myogenic contraction to propel a fluid through a tube (Randall et al., 2002). In the sponge the canals behave as a single motor complex, in which a period of dilation is followed by a propagated contraction that squeezes the water forward towards the osculum. Except that neuronal modulation is absent, the system does not appear much different from those composed of multi-unit smooth muscles (Randall et al., 2002).

Peristalsis seems to be a central feature of body plans in all animals. It is involved in moving fluid for nutrient transfer in the gastrovascular cavity (GVC) of the sea pansy *Renilla koellikeri* (Ancil, 1994), for burrowing by anemones, nemerteans, polychaetes and bivalves (Ansell and Trueman, 1968). Peristalsis is also involved in the contraction of the heart in tunicates and amphioxus (Holland et al., 2003). In each of these instances control is thought to be myogenic, although the role of nerves is not well understood. It is interesting to note that while contractions of the GVC of *Renilla* propagate at $1\text{--}1.3 \mu\text{m s}^{-1}$ (Ancil, 1994; Ancil et al., 2005), contractions of the body wall of the sessile anemone *Metridium senile* propagate at $\sim 500 \mu\text{m s}^{-1}$ (Batham and Pantin, 1950) and even slower in the tiny burrowing starlet anemone *Nematostella vectensis* (at $3\text{--}20 \mu\text{m s}^{-1}$) (S.P.L., unpublished observation). Cnidarians have the advantage of both muscle (epitheliomyocytes) and neurons, yet contractions are still slow. As previously suggested (Batham and Pantin, 1950), this is presumably due to the load the muscle acts against rather than intrinsic limitations, because when stimulated electrically, the same region of the body wall can contract much faster. Our observations suggest this is also true for sponges. In order to expel water, the tissues contract in a controlled and coordinated manner; but when water is not being pushed out of the aquiferous system faster contractions are possible, as when ripples run across portions of the sponge or the osculum contracts down in response to mechanical agitation. Evidently sponges have, without nerves or true muscle, evolved a way of coordinating contractions of cells to generate an effective mechanism of controlling water flow.

The next step is to determine what signal or mechanism controls each type of contraction. Because of its small size and transparency, the freshwater sponge promises to be an excellent model system for further study of the role of signalling molecules in inducing, controlling, and modulating behaviour in these ‘simple animals’.

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