Sponges as sensitive animals: sensory systems and energetics of filtration in demosponges

by

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Abstract

Sponges (Porifera) are abundant in most marine and freshwater ecosystems, and as suspension feeders they play a crucial role in filtering the water column. Their active pumping enables them to filter up to 900 times their body volume of water per hour, recycling nutrients and coupling the benthic and pelagic communities. Despite the ecological importance of sponge filter feeding, little is known about how sponges control the water flow through their canal system or how much energy it costs to filter the water. Sponges lack conventional muscles and nervous tissue, yet respond to stimuli through coordinated behaviours. Here, I show the presence of non-motile cilia in the canal system of sponges and study their role as flow sensors. I demonstrate that molecules known to block cationic channels in sensory cilia in other organisms reduce or eliminate sponge behaviour. In addition, removal of the cilia using chloral hydrate eliminates sponge contractions, suggesting the cilia are flow sensors and involved in controlling water flow through the canal system. Sponges have long been considered textbook examples of animals that use current-induced flow. I show evidence that suggests some species of demosponge do not use current-induced flow; rather, they respond behaviourally to increased ambient currents by reducing their pumping volume. Using a morphometric model of the canal system, I also show that filter feeding may be more energetically costly than previously thought. Measurements of pumping volume and oxygen removal in five species of demosponges show that pumping rates are variable within and between species, with more oxygen consumed the greater the pumping volume. Together, these data suggest that sponges have a lot of control over the volume of water pumped, which may be an adaptation to reduce the energetic cost of filtration in times of high stress.

Preface

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Chapter One

A general introduction to behaviour and filter-feeding in sponges

1.1 Ecology of sponges

Sponges (Porifera) are an important structural and functional component of most benthic marine and freshwater environments. Their huge abundance, diversity, and biomass is such that they interact with most other organisms either by providing habitat or acting as competitors, symbionts, hosts of symbionts, consumers, or prey (Diaz and Rützler, 2001; Wulff, 2006). A wide diversity of animals inhabit the interior cavities and canals of sponges (Rützler, 1976; Villamizar and Laughlin, 1991; Ribeiro et al., 2003), with some sponges supporting whole communities of other organisms, such as the glass sponge reefs off the coast of British Columbia spanning hundreds of kilometers in size (Krautter et al., 2001). In addition to animals living in the canal system of sponges, many photosynthetic and chemosynthetic symbionts ranging from bacteria to algae are found in the tissue of sponges, occupying almost 40% of the tissue volume (Vacelet, 1975; Wilkinson, 1978). Sponges also play an important functional role by filtering out particles from the overlying water column, assimilating carbon, and linking the benthic and pelagic environments in a process termed benthic-pelagic coupling (reviewed in Gili and Coma, 1998). By filtering out food from the water column, sponges recycle nutrients and are known to influence levels of primary production (Corredor et al., 1988; Diaz and Ward, 1997; Southwell, 2007; Southwell et al., 2008) and contribute substantially to organic matter cycling in the water column (Reiswig, 1971; Reiswig, 1974; Reiswig, 1981; Pile et al., 1996; Pile et al., 1997; Turon et al., 1997; Yahel et al., 2003). This thesis focuses on the behaviour of pumping water through the sponge body to filter out food particles.

As suspension feeders, the sponge body plan is designed to filter as much water as needed for feeding and respiration (Reiswig, 1975). Their huge range in habitats and their long evolutionary history, however, have led to many adaptations in both body form and pumping rates. A variety of pumping rates have been documented both within individual sponges and between species, with differences in water temperature (Riisgård et al., 1993), suspended sediment concentration (Gerrodette and Flechsig, 1979), sponge body form (Reiswig, 1975), microbial content (Weisz et al., 2008), and tissue density (Turon et al., 1997) all known to influence the volume of water filtered by the sponge. Sponges are a common component of most benthic aquatic environments ranging from the tropics to the poles, including the abyssal deep sea to freshwater rivers and lakes. Substrate type can range from hard rocky bottom, soft sediment, to infaunal, and a wide variety of sponge shapes and sizes exist (Figure 1-1 a-g). Some sponges have even strayed from filter feeding and evolved a carnivorous feeding strategy (Figure 1-1 h), using their spicule skeleton to snag crustaceans in the surrounding water column (Vacelet and Boury-Esnault, 1995). It is unclear exactly what role habitat plays on the filtration and pumping activity of sponges, though sponges have likely adapted their pumping activity to coincide with local food and water dynamics.

Despite their huge abundance, diversity, and ecological importance in most marine and freshwater habitats, there is much about sponge physiology and ecology that is not fully understood (Bergquist, 1978; Wulff, 2006). Sponges lack many of the tissues and systems that define the Metazoa, with a body plan so distinct from other animals that it is often difficult for sponge biologists to communicate results to a broader audience. Historically, it has been challenging to maintain maximal pumping activity in whole sponges in the laboratory, which has led to difficulties in understanding sponge physiology and behaviour in relation to filter feeding (Bergquist, 1978; Hadas et al., 2008; Weisz et al., 2008). Recent advances in technology, however, have led to powerful sensors that can be used *in situ* to measure oxygen removal and pumping rates of sponges such as thermistor flow meters (Reiswig, 1971; Reiswig, 1974; Vogel, 1977), acoustic Doppler velocimeters (ADVs'; Leys et al, 2011), and oxygen optodes (Hadas et al., 2008). In addition, recent improvements in our understanding of water flow and oxygen requirements for sponges has led to the ability to study sponge filter feeding and behaviour in the laboratory

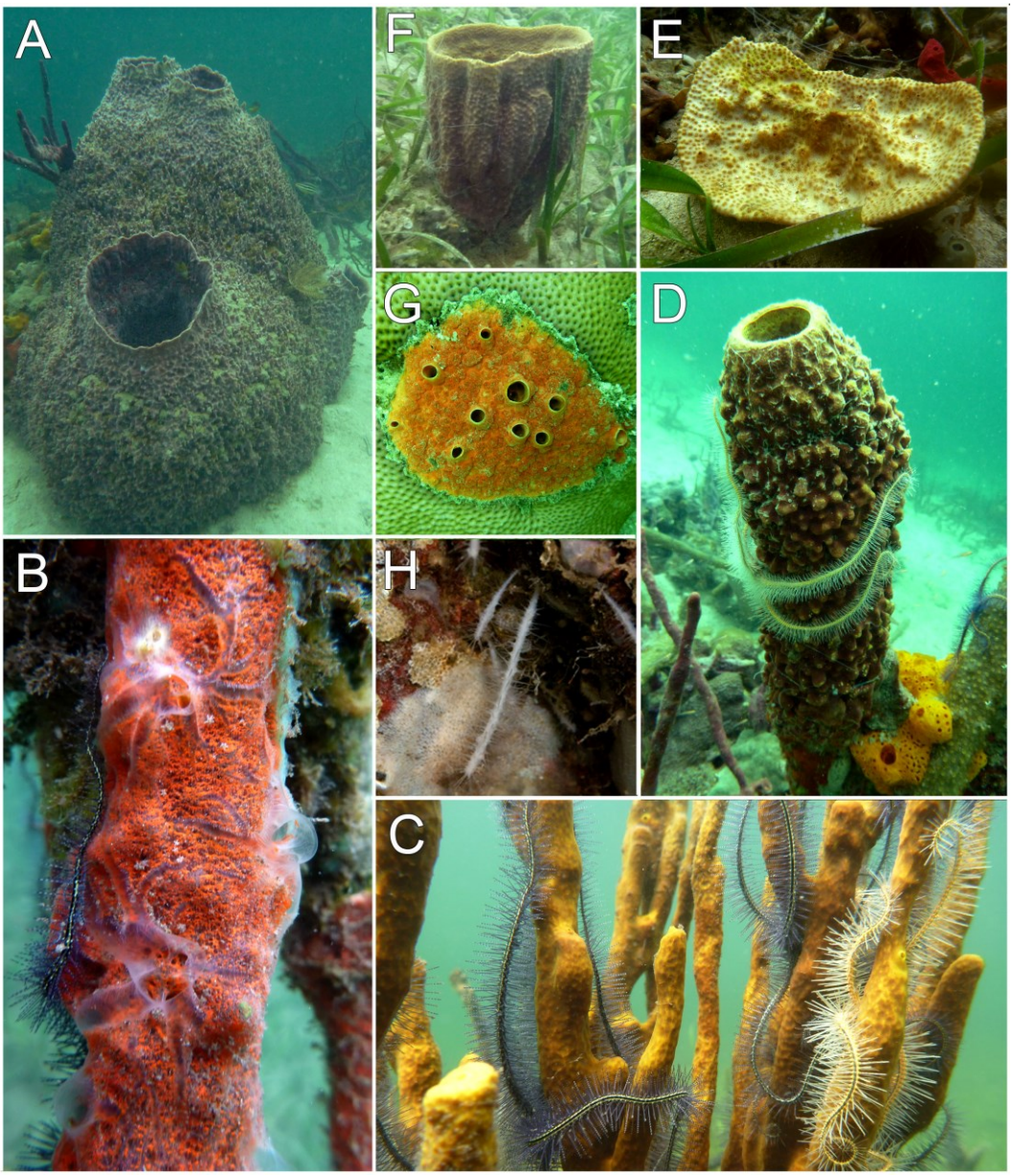

Figure 1-1| The diversity of sponge body forms found in Bocas del Toro, Panama

counter-clockwise from top left: (A) barrel sponges (*Xestospongia)* that can grow to a few metres in height, (B) the encrusting sponge *Mycale*s, shown here growing on mangrove roots, (C) rope sponges (*Aplysina)*, (D) columnar (*Aplysina)* and (E) plate-like sponges (*Ircinia)*, (F) vase shaped sponges (*Xestospongia)*, and (G) the boring sponge (*Cliona)* that dissolves through coral skeleton [Photos (A-G) taken by N. Lauzon] (H) In addition to suspension feeding sponges, some sponges evolved a carnivorous feeding strategy such as *Asbestopluma* found in British Columbia, Canada, that uses its spicule skeleton to snag small crustaceans in the water column [Photo (H) from Chu and Reiswig (2014)].

(Hadas et al., 2008; Leys et al., 2011). Furthermore, the more widespread use of freshwater sponges as model organisms has led to a greater understanding of some basic anatomy and physiology in relation to the water canal system of sponges (McNair, 1923; Wintermann, 1951; Elliott and Leys, 2007; Adams et al., 2010; Elliott and Leys, 2010). These advances have set the stage to answer some important questions about how sponges control water flow through their bodies and what the energetic cost is for a sponge to pump water. In Chapter Two I use freshwater sponges as model organisms to study how sponges sense their environment to maintain water flow through their canal system. In Chapter Three I describe use of fiber optic oxygen sensors and profiling ADVs to study sponge pumping and oxygen removal *in situ* and *in vitro* to study the energetic cost of filter feeding in sponges.

1.2 Anatomy of sponges

As suspension feeders, sponges pump large volumes of water through a branching canal system to obtain both food and oxygen as well as excrete wastes and release gametes. This water canal system, termed the aquiferous canal system, penetrates all regions of the sponge body (Figure 1-2) leading to an absence in regional specialization. Water flow is unidirectional through the sponge, entering the sponge often through many small holes on the outer surface, termed ostia, and exiting the sponge through one large opening termed the osculum (Figure 1-3). The ostia are formed by porocytes, sphincter-like cells that can control the inflow of water to the canal system. Once water has entered into the canal system it flows through branching incurrent canals that decrease in diameter until they reach the choanocyte chambers through an aperture termed the prosopyle. This branching canal system drastically increases cross-sectional area, decreasing the velocity of water as it enters the choanocyte chambers, just as blood capillaries do. Here, choanocytes act as both a pump and a filter to draw water through the canal system using their flagella and filter out particles using the microvillar collar. Once the water passes through the filter it exits the choanocyte chamber via the apopyle into the excurrent canals. The excurrent canals are similar to a mirror image of the incurrent canals, increasing in diameter and merging together resulting in a jet of filtered water and waste out of the osculum, the terminal opening located at the back-end of the canal system. The entire canal system is lined

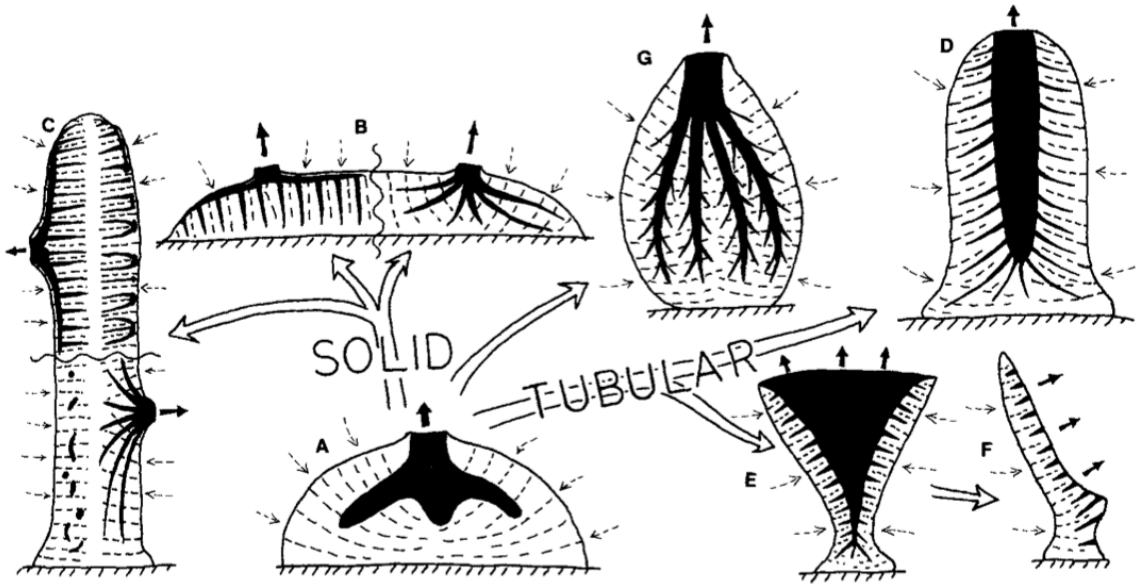

Figure 1-2| Major organizational types of Demosponges

Despite many different body forms of sponges, the aquiferous canal system (shown in black) penetrates all regions of the sponge body leading to a reduction in regionalization (taken from Reiswig, 1975).

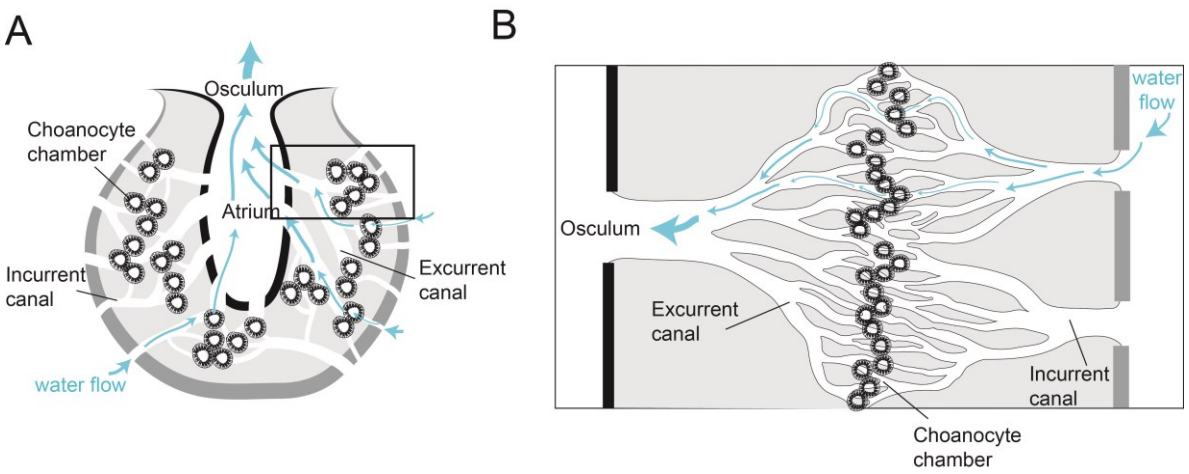

Figure 1-3| Aquiferous canal system in sponges

(A) Schematic drawing showing water flow through a sponge. Water enters through pores on the sponge surface, into incurrent canals to the choanocyte chambers where the water is filtered, then out through the excurrent canals to the osculum. The branching is such that water is only filtered once in a chamber. (B) Schematic representation showing water flow through a sponge highlighting the huge increase in cross-sectional area as the water enters the choanocyte chambers, which slows the water for filtration. The cross-sectional area then decreases as the water leaves the choanocyte chambers, jetting the water out through the osculum**.**

with a tight epithelial tissue (Adams et al., 2010) made out of contractile endopinacocytes providing the sponge with a lot of control over the water flow through each region (Nickel, 2004).

1.3 Sponges are sensitive to their environments

Although at first glance sponges may appear to be static animals spending their entire adult life stationary on the sea floor, larvae and adults in all sponge classes have been documented to display responsive behaviour (Leys and Meech, 2006), albeit slower than most other animals. In fact, many of the coordinated responses of sponges are too slow for human eyes to detect, so that we usually rely on time-lapse photography to capture sponge behaviour (Pavans de Ceccatty, 1974). Only then is it easy to see that sponges display coordinated responses to the mechanical touch of other organisms (Nickel, 2004) with some species also undergoing periodic endogenous contractions (Reiswig, 1971; Weissenfels, 1984; Nickel, 2004). Local cellular contractions in the canal system have also been documented and suggested to regulate the flow of water through contractions at myocytes (Bagby, 1966), porocytes (Simpson, 1984), and endopinacocytes lining the canals (Nickel, 2004). In addition, the osculum at the back-end of the canal system is highly contractile in some species (McNair, 1923; Prosser et al., 1962; Emson, 1966; Pavans de Ceccatty, 1969), sometimes taking on a variety of forms. These small adjustments to canal diameters probably occur constantly in sponges allowing them to maintain adequate flow through the aquiferous system.

Much of the early work on sponge behaviour has been limited to laboratory experiments observing how individual structures respond. These studies have demonstrated that sponges respond to mechanical (Parker, 1910; McNair, 1923; Prosser et al., 1962; Emson, 1966), chemical (Parker, 1910; Emson, 1966; Prosser, 1967), and electrical stimulation (Emson, 1966). More complex coordinated behaviours in response to external stimuli have also been demonstrated in a variety of sponges, beginning with the use of sandwich preparations by Wintermann (1951) that provided the first hint that local contractions may be part of a more ‘global’ behaviour. Since then, sponges have been shown to display endogenous rhythmic or diurnal patterns in behaviour (Pavans de Ceccatty et al., 1960; Reiswig, 1971; Weissenfels, 1984; Nickel, 2004) as well as whole

body contractions in response to mechanical or chemical stimuli (Nickel, 2004; Elliott and Leys, 2007; Ellwanger et al., 2007; Elliott and Leys, 2010), sometimes causing the whole animal to shrink to one third of its original size. Laboratory studies of this global contractile behaviour have provided insight into the role of ligand-based receptor systems in coordinating signals through the sponge body (Ellwanger et al., 2007; Elliott and Leys, 2010). Such coordinated contractions in sponges have been suggested to help clear debris or eject gametes from the sponge (Pavans de Ceccatty, 1969; Reiswig et al., 1976; Elliott and Leys, 2007), and usually result in periodic cessations or reductions in pumping activity (Reiswig, 1971; Elliott and Leys, 2007).

Although the link between sponge contractions and pumping activity is not always made, the contraction of canals will undoubtedly influence the volume of water that a sponge pumps. Sponges, as suspension feeders, rely on water flow through their bodies for both food and oxygen, and therefore should have a high degree of control over the water flow through their bodies. Sponges have been shown to respond to the flow regime around them and suspended sediment in the water column, altering their pumping rates accordingly (Reiswig, 1971; Gerrodette and Flechsig, 1979; Tompkins-MacDonald and Leys, 2008). Increased suspended sediment in the water column results in periodic cessations of pumping activity (Gerrodette and Flechsig, 1979; Tompkins-MacDonald and Leys, 2008). In addition, pumping volume and behaviour have been linked to wave strength in the sponge *Tethya crypta* when Reiswig (1971) measured excurrent velocity in 50 individuals of a population over many months while taking images of their oscula. He found that during storms, the increased wave strength and resulting sand scour caused rapid and total oscular closure of all individuals of the population (Figure 1-4), with their effective pumping rate reduced to 51% of their maximum. It is not clear, though, whether the reduced pumping rates were a result of the increased wave action, changes in pressure, or the increased sediment in the water during the storm. Because of the high risk of clogging from sediment, sponges have likely adapted to respond to many different environmental cues that would predict high sediment in the water column.

For an animal to respond to its surroundings requires not only an ability to sense changes in the environment but also a means to relay that information to cells or tissues that

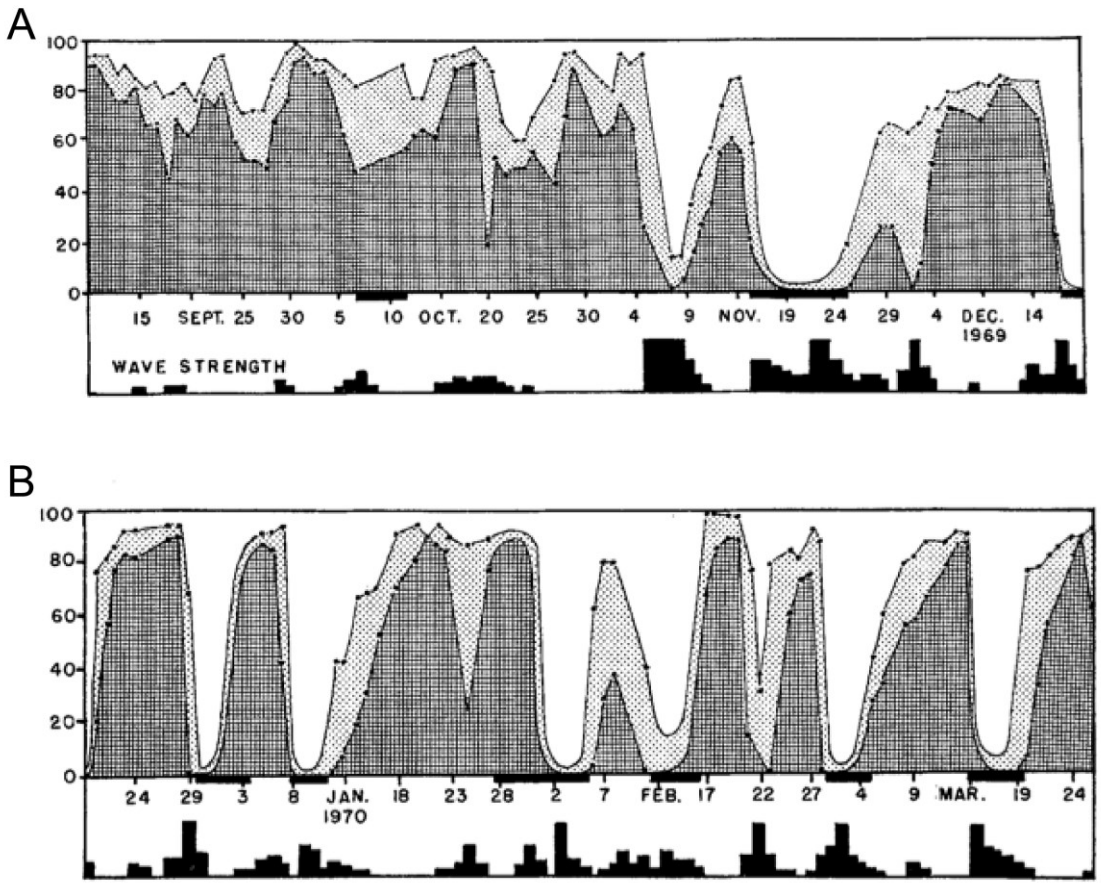

Figure 1-4| Oscular area of sponges from a population of *Tethya crypta* in relation to wave strength

The relative dilation of 50 oscula were measured and cumulated by Reiswig (1971) each day and plotted as either percent fully dilated (dark), percent fully closed (white), or percent partially dilated (grey). Relative wave strength is shown at the bottom of each graph. High wave strength corresponds to most of the population having fully closed oscula.

can effect a response. For most animals this involves both nerves and muscles; however, sponges lack conventional nerve and muscle tissue yet still respond to their surroundings. The contractile ability of some types of sponge cells is now fairly well known, and recent studies have begun to provide an understanding of some of the paracrine signaling mechanisms in sponges that are involved in coordinating such behaviours (Ellwanger et al., 2007; Elliott and Leys, 2010). It is still unknown, however, what cells are used to sense environmental changes to trigger a response. Recently, short non-motile cilia have been found in the excurrent canal system and osculum of freshwater sponges (Leys et al., 2009) and it has been suggested that these are sensory cilia that may play a role in controlling water flow through the sponge (Elliott, 2009).

1.4 Filter feeding and its energetic cost in sponges

Sponges, as suspension feeders, pump large volumes of water through their aquiferous canal system to remove bacteria and other small plankton from the water column. Although pumping rates can vary greatly both within and between individuals and species, a single sponge has been shown to pump up to 900 times its body volume in one hour (Reiswig, 1974). This high rate of filtration exerts a major impact on the ecosystems in which sponges reside, shaping planktonic communities and coupling energy between the benthos and plankton (reviewed in Maldonado et al., 2012). Scaling up, it has been estimated that a population of the sponge *Halichondria panicea* in the western Baltic Sea can filter 3.7 m^3 m^{-2} d^{-1}, which is equivalent to 37 % of the overlying water column each day (Riisgård et al., 1993). Another example is a single 20-hectare glass sponge reef off the coast of British Columbia, which has been estimated to filter 80,000 L of water a second (Chu and Leys, 2010) and consume up to 180,000 g of carbon a day (Kahn et al., 2015). This high rate of filtration could filter an Olympic sized swimming pool in just over 30 seconds. Sponge populations, therefore, can have a pronounced effect on nutrient cycling in the overlying water column.

Pumping large volumes of water to extract food is an active process, requiring energy to bring water through the canals to be filtered in chambers. A balance is needed between the amount of energy used to pump a volume of water per unit time and the

amount of food and oxygen gained. For all suspension feeders, energy is allocated to a variety of needs such as feeding, growth, reproduction, and basal metabolism. This resource allocation can shift depending on environmental factors such as temperature, food availability, photoperiod, and stress (Brown and Howard, 1985; Coma et al., 1998; Weber et al., 2006). For example, in temperate waters seasonality can result in more energy allocated to feeding and growth during times of high food availability (Coma et al., 1998).

On the other hand environmental stresses can increase an animal's energetic expenditure to deal with the stress, leaving less energy available for important processes such as growth and reproduction. During increased sediment exposure corals will produce mucus to help clear the sediment from the coral surface, an energetically costly process that can further lead to bleaching and necrosis (Weber et al., 2006). Sedimentation has also been shown to decrease sponge growth rates and reproduction (Roberts et al., 2006) and reduce their survival (Maldonado et al., 2008). Sessile suspension feeders obtain their energy by bulk feeding on minute prey, and cannot move to regions of high food abundance to increase the amount of food, and thus energy, they consume. If suspension feeding is a costly process, then environmental stresses that reduce the amount of energy sponges can allocate to feeding would further impact the amount of food energy they can consume and allocate to growth and reproduction. It is therefore important to understand the energetic cost of filter feeding in sponges to determine how sensitive they may be to environmental stresses.

In sponges, the energetic cost of filter feeding is directly proportional to the resistance of water flow through the various regions of the aquiferous canal system (Riisgård et al., 1993); the greater the resistance through the filter and canals, the more energetically expensive it is to pump water. Sponges also feed on bacteria that can be smaller than 1 μm in size (Yahel et al., 2007), requiring very small dimensions at the filter. The cost of filter feeding in sponges has previously been estimated by calculating the resistance, or head loss, across each region of the canal system (Riisgård et al., 1993; Leys et al., 2011). Riisgård and colleagues (1993; 1995) suggest that the cost of pumping is quite low at less than 1% of their total metabolism (Riisgård et al., 1993; Riisgård and Larsen, 1995), which is in line with the hypothesis of Jørgensen (1975) which suggests that filter feeders evolved a low cost of pumping to allow continuous feeding at low rates. However, sponges do not

pump continuously, and it has been suggested that the lower pumping rates at night in *Tethya crypta* are due to lower abundances of food availability (Reiswig, 1971). This would agree with the hypothesis by Taghon (1981) which suggests that for suspension feeders to maximize their energy intake, they must vary their ingestion rate (or pumping rate) as a function of food quantity and quality. In addition, recent experimental measurements have shown that 25% of oxygen consumed in the sponge *Negombata magnifica* is used for pumping water (Hadas et al., 2008), suggesting that filter feeding is more energetically expensive than previously thought.

1.5 Use of current-induced flow

If the cost of pumping is energetically expensive, then sponges could reduce the metabolic cost of pumping by supplementing active pumping with ambient water velocities in a process termed current-induced flow (Vogel, 1974; Vogel, 1977). There are three different mechanisms in which flow in a large pipe can be induced through a smaller one (Figure 1-5), and it is thought that sponges are designed to allow water to flow passively through them, thus reducing their metabolic cost of pumping (Vogel, 1974). It is not yet clear, though, exactly how flow could be induced through a sponge. Using thermistor flow meters, Vogel (1974, 1977) measured excurrent water velocities from multiple species of marine demosponges (class Demospongaie) both in the laboratory and *in situ,* while experimentally changing the velocity of water around the sponge. He found that the excurrent water velocity from the sponge osculum increased when ambient velocity increased, suggesting that sponges do take advantage of ambient currents to increase the amount of water they process. However, Vogel did not measure oxygen during these recordings and so it is not known if there was an increase in the amount of energy used when excurrent velocity increased. An alternative hypothesis has been posed suggesting that the increase in excurrent water velocity from sponges may be a result of indirect effects of the ambient currents, such as higher concentrations of food particles available (Harrison and Cowden, 1976). One difficulty, therefore, in interpreting Vogel's results is distinguishing between passive flow moving through the sponge versus increased active

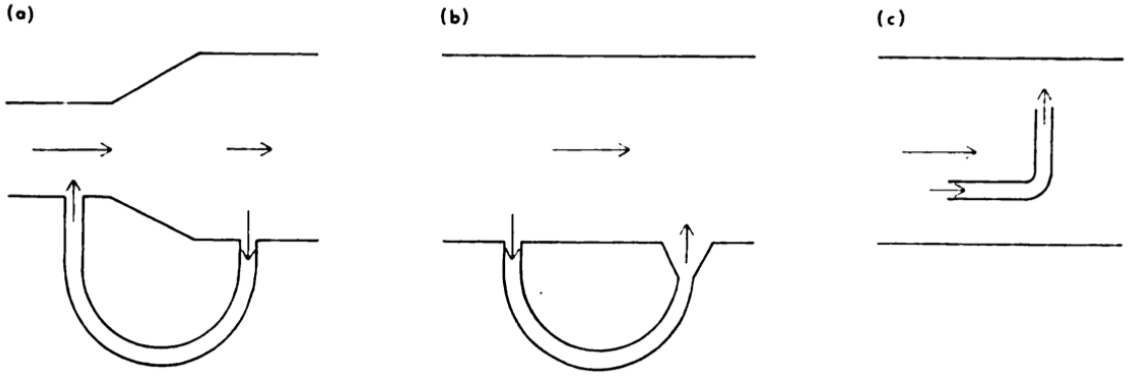

Figure 1-5| Current-induced flow through pipes

Three mechanisms in which Vogel proposes fluid can flow passively from a large pipe into a smaller pipe based on a) Bernouilli's Principle, b) viscous entrainment, and c) dynamic pressure (taken from Vogel, 1974).

pumping behaviour when there is higher food availability. A second difficulty with his results is his method used to increase ambient currents. Thermistor flow meters cannot distinguish the direction of water velocity. During experiments, Vogel increased ambient velocity using a scuba fin, which would have resulted in turbulent flow in multiple directions. Therefore, the thermistor flow meters inside the osculum of the sponge may have been reading the increased turbulence from the finning method rather than increased excurrent velocity. A further look at the use of current-induced flow in sponges is required.

Recently, Leys et al. (2011) looked at the use of current-induced flow in glass sponges (class Hexactinellida). Glass sponges are distinct from the demosponges that Vogel used in that they have wider canals and larger choanocyte chambers and oscula, and thus are considered good candidates in which to expect current-induced flow. Leys et al. (2011) found that at ambient velocities greater than 15cm s^{-1}, the excurrent velocity of *Aphrocallistes vastus* did increase. Interestingly, however, they found that *A. vastus* could still arrest pumping during high ambient velocities, suggesting they still have a lot of control over the water flow through their canal system. In addition, using a morphometric model, Leys et al. (2011) measured the resistance through the filter and canal system to predict the ability to induce flow through the glass sponge. When comparing glass sponges to demosponges, Leys et al. (2011) suggested that the resistance through the filter and canal system in demosponges may be too high to allow passive flow. The use of current-induced flow, therefore, may not be adaptive for all sponges.

There are many sponge species that inhabit relatively quiet water habitats and are thus unlikely to ever experience ambient flows that could result in passive flow (Reiswig, 1975). Rather, the ability to cease pumping under unfavourable conditions is a more likely adaptation for sponges inhabiting these quiet waters. Other sponges inhabit areas that are prone to storms and high currents that bring in high suspended sediment loads. Here, increased ambient flows would be indicative of high sediment in the water column, and passive flow would not be adaptive as it would likely lead to damage of the filter and canals. Reiswig (1971) found that high ambient currents during storms in Jamaica result in cessation of pumping in *Tethya crypta* during high wave strength (Figure 1-4). Savarese et al. (1997) also found that sponges in Lake Baikal exhibit negative correlations between

ambient current and excurrent flow. It therefore remains unclear what the contribution and importance of passive flow is in sponges.

1.6 Thesis objectives and outlines

Broadly, this thesis aims to understand the behavioural control and energetic cost of filter feeding in sponges. I aimed to determine the function of the non-motile cilia previously found in the aquiferous system of sponges and study their role as flow sensors. In addition, I aimed to investigate the energetic cost of filtration through the use of morphometric models, as well as assess the use of passive flow in sponges.

In Chapter Two, I show the presence of short, non-motile cilia lining the inner epithelium of the sponge osculum in seven species of demosponges. 'Primary' non-motile cilia are involved in sensation in animals ranging from invertebrates to humans. Here, I show that drugs known to inhibit primary cilia sensation in other organisms reduce or eliminate sponge contractions. In addition, both chemical removal of the cilia and physical removal of the whole osculum in sponges reversibly eliminate sponge contractile behaviour. These findings suggest the cilia in sponges are sensory and involved in the coordination of simple behaviour, and may represent the first step in the evolution of sensory systems.

In Chapter Three I studied the ability of sponges to control water flow through the aquiferous system indirectly by examining oxygen consumption and filtration rates, as well as by modelling resistance through the sponge aquiferous system. First I re-evaluated the cost of pumping in sponges and three other groups of filter feeders using a morphometric model. I show that new measurements for both filter dimensions and pumping rates increase the cost of pumping up to five times that of previous estimates, suggesting that filter size and pumping rates contribute most to the energetic cost of pumping and that this cost may be higher than previously thought. I then compare the design of the aquiferous canal system and the cost of pumping in five species of demosponges from both temperate and tropical waters using measured canal and filter dimensions, pumping rates, and oxygen consumption. Although each species has broad differences in its overall aquiferous canal system, the design of the choanocyte chamber, and specifically of the collar apparatus, is

strikingly similar among the five species. In all species but one, oxygen consumption increased with increased pumping volume. To assess whether the two tropical species use current-induced flow to reduce the cost of pumping I looked at excurrent flow rates and oxygen consumption under different ambient velocities. Interestingly, high ambient currents resulted in a behavioural response by the sponge to change excurrent velocity, suggesting demosponges respond to ambient currents to control the water flow through their aquiferous canal system.

In Chapter Four, I reflect on the above findings and their implications with our current understanding of sponge behaviour and ecology.

1.7 References

Adams, E. D., Goss, G. G. and Leys, S. P. (2010). Freshwater sponges have functional, sealing epithelia with high transepithelial resistance and negative transepithelial potential. *PloS one* **5**, e15040.

Bagby, R. M. (1966). The fine structure of myocytes in the sponges *Microciona prolifera* (Ellis and Solander) and *Tedania ignis* (Dechassaing and Michelotti). *Journal of Morphology* **118**, 167-182.

Bergquist, P. R. (1978). Sponges: Univ of California Press.

Brown, B. and Howard, L. (1985). Assessing the effects of "stress" on reef corals. *Advances in Marine Biology* **22**, 1-63.

Chu, J. W. F. and Leys, S. P. (2010). High resolution mapping of community structure in three glass sponge reefs (Porifera, Hexactinellida). *Marine Ecology Progress Series* **417**, 97-113.

Chu, J. W. F. and Reiswig, H. M. (2014). Mechanisms of propagule release in the carnivorous sponge *Asbestopluma occidentalis*. *Invertebrate Biology* **133**, 109-120.

Coma, R., Ribes, M., Gili, J.-M. and Zabala, M. (1998). An energetic approach to the study of life-history traits of two modular colonial benthic invertebrates. *Marine Ecology Progress Series* **162**, 89-103.

Corredor, J. E., Wilkinson, C. R., Vicente, V. P., Morell, J. M. and Otero, E. (1988). Nitrate release by Caribbean reef sponges. *Limnology and Oceanography* **33**, 114-120.

Diaz, M. C. and Rützler, K. (2001). Sponges: an essential component of Caribbean coral reefs. *Bulletin of Marine Science* **69**, 535-546.

Diaz, M. C. and Ward, B. B. (1997). Sponge-mediated nitrification in tropical benthic communities. *Marine Ecology Progress Series* **156**, 97-107.

Elliott, G. R. D. (2009). The cytology and physiology of coordinated behaviour in the freshwater sponge, *Ephydatia muelleri*. In *Department of Biological Sciences*, vol. Degree of Philosophy, pp. 252: University of Alberta.

Elliott, G. R. D. and Leys, S. P. (2007). Coordinated contractions effectively expel water from the aquiferous system of a freshwater sponge. *The Journal of Experimental Biology* **210**, 3736-48.

Elliott, G. R. D. and Leys, S. P. (2010). Evidence for glutamate, GABA and NO in coordinating behaviour in the sponge, *Ephydatia muelleri* (Demospongiae, Spongillidae). *The Journal of Experimental Biology* **213**, 2310-21.

Ellwanger, K., Eich, A. and Nickel, M. (2007). GABA and glutamate specifically induce contractions in the sponge *Tethya wilhelma*. *Journal of Comparative Physiology A* **193**, 1-11.

Emson, R. H. (1966). The reactions of the sponge *Cliona celata* to applied stimuli. *Comparative Biochemistry and Physiology* **18**, 805-827.

Gerrodette, T. and Flechsig, A. O. (1979). Sediment-induced reduction in the pumping rate of the tropical sponge *Verongia lacunosa*. *Marine Biology* **55**, 103-110.

Gili, J.-M. and Coma, R. (1998). Benthic suspension feeders: their paramount role in littoral marine food webs. *Trends in Ecology and Evolution* **13**, 316-321.

Hadas, E., Ilan, M. and Shpigel, M. (2008). Oxygen consumption by a coral reef sponge. *The Journal of Experimental Biology* **211**, 2185-90.

Harrison, F. and Cowden, R. (1976). External Currents and Sponge Feeding. In *Aspects of Sponge Biology*, pp. 46. USA: Academic Press Inc.

Jørgensen, C. B. (1975). Comparative physiology of suspension feeding. *Annual Reviews in Physiology* **37**, 57-79.

Kahn, A.S., Yahel, G., Chu, J.W.F., Tunicliffe, V., and Leys, S.P. (2015). Benthic grazing and carbon sequestration by deep-water glass sponge reefs. *Limnology and Oceanography* doi: 10.1002/lno.10002.

Krautter, M., Conway, K. W. and Barrie, J. V. (2001). Discovery of a "Living Dinosaur": Globally unique modern Hexactinellid sponge reefs off British Columbia, Canada. *Facies* **44**, 265-282.

Leys, S. P. and Meech, R. W. (2006). Physiology of coordination in sponges. *Canadian Journal of Zoology* **84**, 288-306.

Leys, S. P., Nichols, S. A. and Adams, E. D. (2009). Epithelia and integration in sponges. *Integrative and Comparative Biology* **49**, 167-77.

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.

Maldonado, M., Giraud, K. and Carmona, C. (2008). Effects of sediment on the survival of asexually produced sponge recruits. *Marine Biology* **154**, 631-641.

Maldonado, M., Ribes, M. and van Duyl, F. C. (2012). Nutrient fluxes through sponges: biology, budgets, and ecological implications. *Advances in Marine Biology* **62**, 113-82.

McNair, G. T. (1923). Motor reactions of the freshwater sponge *Ephydatia fluviatilis*. *Biological Bulletin* **64**, 153-166.

Nickel, M. (2004). Kinetics and rhythm of body contractions in the sponge *Tethya wilhelma* (Porifera: Demospongiae). *The Journal of Experimental Biology* **207**, 4515-24.

Parker, G. H. (1910). The reactions of sponges, with a consideration of the origin of the nervous system. *The Journal of Experimental Zoology* **8**, 1-41.

Pavans de Ceccatty, M. (1969). Les systemes des activites motrices, spontanées et provoquées des éponges. *Comptes Rendus de l'Académie des Sciences – Series III Sciences de la Vie* **269**, 596-599.

Pavans de Ceccatty, M. (1974). Coordination in sponges: the foundations of integration. *American Zoologist* **14**, 895-903.

Pavans de Ceccatty, M., Cargouil, M. and Coraboeuf, E. (1960). Les réactions motrices de l'éponge *Tethya lyncurium* (Lmk.) à quelques stimulations expérimentales (I). *Vie et Milieu* **11**, 594-600.

Pile, A. J., Patterson, M. R., Savarese, M., Chernykh, V. I. and Fialkov, V. A. (1997). Trophic effects of sponge feeding within Lake Baikal's littoral zone. 2. Sponge abundance, diet, feeding efficiency, and carbon flux. *Limnology and Oceanography* **42**, 178-184.

Pile, A. J., Patterson, M. R. and Witman, J. D. (1996). *In situ* grazing on plankton <10 μm by the boreal sponge *Mycale lingua*. *Marine Ecology Progress Series* **141**, 95-102.

Prosser, C. L. (1967). Ionic analyses and effects of ions on contractions of sponge tissues. *Zeitschrift für vergleichende Physiologie* **54**, 109-120.

Prosser, C. L., Nagai, T. and Nystrom, R. A. (1962). Oscular contractions in sponges. *Comparative Biochemistry and Physiology* **6**, 69-74.

Reiswig, H. M. (1971). *In situ* pumping activities of tropical Demospongiae. *Marine Biology* **9**, 38-50.

Reiswig, H. M. (1974). Water transport, respiration and energetics of three tropical marine sponges. *Journal of Experimental Marine Biology and Ecology* **14**, 231-249.

Reiswig, H. M. (1975). The aquiferous systems of three marine Demospongiae. *Journal of Morphology* **145**, 493-502.

Reiswig, H. M. (1981). Partial carbon and energy budgets of the bacteriosponge *Verongia fistularis* (Porifera: Demospongiae) in Barbados. *Marine Ecology* **2**, 273-293.

Reiswig, H. M., Harrison, F. and Cowden, R. (1976). Natural gamete release and oviparity in Caribbean Demospongiae. *Aspects of Sponge Biology*, 99-112.

Ribeiro, S. M., Omena, E. P. and Muricy, G. (2003). Macrofauna associated to *Mycale microsigmatosa* (Porifera, Demospongiae) in Rio de Janeiro State, SE Brazil. *Estuarine, Coastal and Shelf Science* **57**, 951-959.

Riisgård, H. U. and Larsen, P. S. (1995). Filter-feeding in marine macro-invertebrates: pump characteristics, modelling, and energy cost. *Biological Reviews* **70**, 67-106.

Riisgård, H. U., Thomassen, S., Jakobsen, H., Weeks, J. M. and Larsen, P. S. (1993). Suspension feeding in marine sponges *Halichondria panicea* and *Haliclona*

urceolus: effects of temperature on filtration rate and energy cost of pumping. *Marine Ecology Progress Series* **96**, 177-188.

Roberts, D. E., Cummin, S. P., Davis, A. R. and Chapman, M. G. (2006). Structure and dynamics of sponge-dominated assemblages on exposed and sheltered temperate reefs. *Marine Ecology Progress Series* **321**, 19-30.

Rützler, K. (1976). Ecology of Tunisian commercial sponges. *Tethys* **7**, 249-264.

Savarese, M., Patterson, M. R., Chernykh, V. I. and Fialkov, V. A. (1997). Trophic effects of sponge feeding within Lake Baikal's littoral zone. 1. In situ pumping rates. *Limnology and Oceanography* **42**, 171-178.

Simpson, T. L. (1984). The Cell Biology of Sponges. New York, New York: Springer-Verlag.

Southwell, M. W. (2007). Sponges impacts on coral reef nitrogen cycling, Key Largo, Florida: PhD thesis, pp 218 The University of North Carolina at Chapel Hill.

Southwell, M. W., Weisz, J. B., Martens, C. S. and Lindquist, N. (2008). In situ fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida. *Limnology and Oceanography* **53**, 986-996.

Taghon, G. L. (1981). Beyond selection: optimal ingestion rate as a function of food value. *The American Naturalist* **118**, 202-214.

Tompkins-MacDonald, G. J. and Leys, S. P. (2008). Glass sponges arrest pumping in response to sediment: implications for the physiology of the hexactinellid conduction system. *Marine Biology* **154**, 973-984.

Turon, X., Galera, J. and Uriz, M. J. (1997). Clearance rates and aquiferous systems in two sponges with contrasting life-history strategies. *The Journal of Experimental Zoology* **278**, 22-36.

Vacelet, J. (1975). Étude en microscopie électronique de l'association entre bactéries et spongiaires du genre *Verongia* (Dictyoceratida). *Journal de Microscopie et de Biologie Cellulaire* **23**, 271-288.

Vacelet, J. and Boury-Esnault, N. (1995). Carnivorous sponges. *Nature* **373**, 333-335.

Villamizar, E. and Laughlin, R. (1991). Fauna associated with the sponges *Aplysina archeri* and *Aplysina lacunosa* in a coral reef of the Archipielago de Los Roques, National Park, Venezuela. In *Fossil and Recent Sponges*, pp. 522-542: Springer.

Vogel, S. (1974). Current-induced flow through the sponge, *Halichondria*. *Biological Bulletin* **147**, 443-456.

Vogel, S. (1977). Current-induced flow through living sponges in nature. *Ecology* **74**, 2069-2071.

Weber, M., Lott, C. and Fabricius, K. E. (2006). Sedimentation stress in a scleractinian coral exposed to terrestrial and marine sediments with contrasting physical, organic and geochemical properties. *Journal of Experimental Marine Biology and Ecology* **336**, 18-32.

Weissenfels, N. (1984). Bau und Funktion des Süßwasserschwamms *Ephydatia fluviatilis* (Porifera). *Zoomorphology* **104**, 292-297.

Weisz, J. B., Lindquist, N. and Martens, C. S. (2008). Do associated microbial abundances impact marine demosponge pumping rates and tissue densities? *Oecologia* **155**, 367-76.

Wilkinson, C. R. (1978). Microbial associations in sponges. III. Ultrastructure of the *in situ* associations in coral reef sponges. *Marine Biology* **49**, 177-185.

Wintermann, G. (1951). Entwicklungsphysiologische Untersuchungen an Süsswasserschwammes, Zool. *Zoologische Jarhrbücher Abteilung für Anatomie und Ontogenie de Tiere* **71**, 427-486.

Wulff, J. L. (2006). Ecological interactions of marine sponges. *Canadian Journal of Zoology* **84**, 146-166.

Yahel, G., Sharp, J. H., Marie, D., Häse, C. and Genin, A. (2003). *In situ* feeding and element removal in the symbiont-bearing sponge *Theonella swinhoei:* Bulk DOC is the major source for carbon. *Limnology and Oceanography* **48**, 141-149.

Yahel, G. Whitney, F., Reiswig, H.M., Eerkes-Medrano, D.I., and Leys, S.P. (2007). *In situ* feeding and metabolism of glass sponges (Hexactenellida, Porifera) studied in a deep temperate fjord with a remotely operated submersible. *Limnology and Oceanography* **52,** 428-440.

Chapter Two

Evolutionary origins of sensation in metazoans: functional evidence for a new sensory organ in sponges

2.1 Introduction

Sensory systems use specialized cells or organelles to receive signals that are conducted through the body electrically or chemically (Ryan and Grant, 2009). Signal transduction in many unicellular eukaryotes occurs via cilia, which often have both motile and sensory roles (Dunlap, 1977; Singla and Reiter, 2006; Fujiu et al., 2011). The evolution of multicellularity necessarily involved the ability to transduce signals over longer distances, which in animals is now done by nerves (Meech, 2008) to allow rapid coordinated movements of the whole organism (Mackie, 1970). Although cilia play an important role in sensing the environment in both unicellular and multicellular animals, the evolutionary relationship of sensory cilia in unicellular eukaryotes, fungi and metazoans is unclear. Studies of sensory systems in the earliest evolving metazoans could shed light on shared common mechanisms of sensation.

Sponges lack a nervous system and while they are usually considered representatives of the first multicellular animals (Philippe et al., 2009; Sperling et al., 2009; Nosenko et al., 2013; Roure et al., 2013), some recent phylogenomic analyses place ctenophores more basally (Dunn et al., 2008; Nosenko et al., 2013) calling into question our understanding of the evolution of nerves and the ancestral metazoan state. Analysis of sponge genomes and transcriptomes has revealed a complex assortment of signaling molecules and proteins necessary for a post-synaptic scaffold (Sakarya et al., 2007; Conaco et al., 2012). Together with physiological evidence that glutamatergic signaling occurs in sponges (Elliott and Leys, 2007; Ellwanger et al., 2007; Elliott and Leys, 2010) this suggests that a signaling system similar to that seen in other metazoans may be used to

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coordinate sponge behavior. Whereas sensory organs are well-known from ctenophores, in sponges the mechanism for transducing sensory information from the environment has as yet remained unknown.

Here we provide experimental data which suggest that an array of non-motile cilia in the sponge osculum–the chimney-like structure through which water exits from the sponge–functions as a sensory system to detect changes in flow and control whole animal responses. We used an emergent model system, the freshwater sponge, to investigate the ultrastructure and physiology of the cilia. We also studied the molecular evolution of sensory channels of the Transient Receptor Potential (TRP) family in Porifera. Regardless of whether sponges as we know them today were or were not the earliest multicellular animals to evolve, it is intriguing to consider that an array of sensory cilia like this in sponge oscula could have given rise to more complex signalling cells, such as nerves and sensory sensilla, in the early evolution of animals.

2.2 Methods

2.2.1 Summary of experimental design

The presence of short, non-motile cilia lining the osculum of sponges led us to hypothesize that the cilia are flow sensors and involved in behaviours that maintain water flow in the sponge canal system. To test this, we conducted pharmacological experiments on freshwater sponges in the laboratory to determine whether the cilia play a role in sponge behaviour. The drugs used in experiments are known to either inhibit ciliary signalling through TRP channels in other animals or remove cilia completely. As a negative control, a calcium channel blocker was used which does not have an effect on ciliary signaling in other animals. We also looked in the transcriptomes of sponges for the presence of TRP channel sequences.

2.2.2 Collecting and culturing of sponges

Gemmules of the freshwater sponges *Ephydatia muelleri* and *Spongilla lacustris* were collected from Frederick Lake, BC and Rousseau Lake, BC, respectively, at a depth of 0-3 m and stored at 4°C in unfiltered lake water, aerated monthly, until use. These species were selected because of their transparent canal system and their ability to gemmulate over the winter, allowing us to culture them in the laboratory to conduct experiments. The spicule skeleton was removed from the gemmules by gently rubbing between two pieces of corduroy, and the gemmules were then sorted, sterilized (using 1% H_2O_2 for 5 min), and rinsed in cold distilled water. Single gemmules placed onto ethanol sterilized glass coverslips in Petri dishes containing M-medium (Rasmont, 1961), hatched in 2-3 days; culture medium was changed every 2d post hatching (dph). Only 5-10dph sponges with fully developed aquiferous canal systems were used in experiments.

2.2.3 Fixation for fluorescence microscopy

Sponges on coverslips were fixed in 3.7% paraformaldehyde and 0.3% glutaraldehyde in 100 mM phosphate-buffered saline (PBS) for 12-24 h at 4°C. Preparations were rinsed three times in cold PBS, permeabilized with PBS + 0.1% Triton X-100 (PBTX) for two minutes and rinsed in PBS. Either whole juvenile sponges or individual oscula (pulled off of the sponge by pinching the base of the osculum with fine forceps) were labeled with mouse anti-acetylated alpha tubulin (Sigma-Aldrich, Oakville, ON) in 10% goat serum (GS) and PBS at 1:1000 at RT overnight. Preparations were rinsed in PBS and incubated in goat anti-mouse 488 (Molecular Probes, Burlington, ON) at 1:100 in 10% GS and PBS overnight. Nuclei were counterstained with Hoescht 33342 (Sigma-Aldrich) 1:100 in PBS for 10 min. Some preparations were stained for actin using Alexa 594 phalloidin (Molecular Probes) in BSA-PBS. Labelled oscula were sliced open using a microscalpel and mounted on a slide in 100% glycerol, which was sealed with nail polish. Whole sponges on coverslips were inverted onto a slide in 100% glycerol and viewed with

a Zeiss Axioskop2 Plus. Confocal images were taken using a Zeiss LSM 710, and surface rendering was done using Imaris v7.2 (Bitplane, Zurich, Switzerland).

2.2.4 Fixation for scanning and transmission electron microscopy (SEM, TEM)

Hatched sponges were fixed and prepared for electron microscopy as described previously (Elliott and Leys, 2007). For SEM oscula were removed from the sponges, dehydrated to 100% ethanol and critical point dried. Dried oscula were mounted on aluminum stubs using adhesive tabs and gold-coated prior to viewing using a scanning electron microscope (JEOL 6301 F field emission or a Zeiss EVO MA 15). For TEM oscula were dehydrated through 100% ethanol and embedded in epoxy (TAAB 812). Ultrathin sections (60 nm) were stained with uranyl acetate and lead citrate and viewed in a Hitatchi H-7000 or Phillips Morgagni (FEI) TEM and images captured with an AMT or Gatan digital camera respectively.

2.2.5 Orientation analysis

To assess orientation of each cilia pair with respect to the direction of water flow along the osculum, a line was drawn between the base of the two cilia and the angle between that line and a line defining the long axis of the cell was calculated using ImageJ (v1.43r; NIH, Bethesda, MD). Circular statistics calculated with Oriana v. 3.13 (KCS, Wales, UK) gave the mean angle of the orientation of cilia pairs and a V-test was performed to determine difference from the long axis of the cell.

2.2.6 Assessment of the possible sensory role

Stock solutions of 10 mM neomycin sulfate (Fisher BioReagents, New Jersey), 1 g/L (178.5 M) FM 1-43FX (Fixable analog; Molecular probes, Invitrogen), 10 mM of $GdCl_3$ (Sigma-Aldrich), 20 mM Verapamil (Sigma-Aldrich), and 1 M Chloral hydrate (Sigma-Aldrich) were kept covered at 4°C and used at 300 µM, 35 µM, 5 µM, 10 µM, and

4 mM respectively. Neomycin sulfate and FM1-43FX were added to the Petri dish and the sponge was stimulated using agitation (vigorous shaking of the Petri dish for 30 s) 10 min later for *E. muelleri* or 2 hr later for *S. lacustris*. Gd^{3+} and Verapamil were added to the Petri dish 2 hr prior to stimulation with 75-90 μM L-Glutamate. Treatment in chloral hydrate was for 20 hr prior to stimulation with 75-90 μM L-Glutamate; during washout the M-medium was changed every 2d and the sponge was then stimulated with 75-90 μM L-Glutamate. Oscula were removed by pinching the base of the osculum with fine-tipped forceps, and the sponge was stimulated at 2 hr and then again at 24 hr using agitation. Care was taken to add each treatment to the side of the Petri dish away from the sponge, and the solution was mixed by pipetting gently 5-6 times. Images were captured every 10 s for 50 min, or until the sponge had completed an inflation/contraction cycle. Still images were captured in Northern Eclipse v.7 (Empix Imaging Inc., Mississauga, ON, Canada). Changes in canal diameter were measured every tenth image for the first 60 images, and then every 20^{th} image, using ImageJ (v1.43r; NIH). The neomycin and FM1-43 study had three treatment groups of neomycin, FM1-43, and control (n=8). Due to high variation in changes in canal diameters within a single sponge, three canals in each sponge were measured for the neomycin and FM test and a nested ANOVA was run in R (v.2.4.1). Variance between groups was expected to be greater than either the variance between canals or the variance within a treatment group. The variance between canals within a single individual did not account for any of the variance in the dataset, therefore only one canal was measured per sponge in the remaining experiments and tested via a one-way ANOVA in R (v.2.4.1). For the Gadolinium study there were three treatment groups of control, gadolinium, and gadolinium with 1d washout (n=3). For the verapamil study there were also three treatment groups of control, verapamil, and neomycin (n=5). For the choral hydrate study there were five treatment groups of control, chloral hydrate, 24hr washout, 72hr washout, and 120hr washout (n=5). All data were tested for normality using a Shapiro-Wilks test, with Gd^{3+} data log(x) transformed and chloral hydrate data square root transformed.

Cilia length of sponges treated with neomycin sulfate, FM1-43FX and Gd^{3+}, for one hour each, were measured from fluorescence images with ImageJ (v1.43r). Untreated sponges were used as controls. Reversibility of Gd^{3+} treatment was demonstrated by

washing out the blocker for 1 hr in culture medium prior to fixation. Cilia and flagella length of Gd^{3+}-treated sponges were measured from SEM images. The measurements were log(x) transformed and analyzed using a nested ANOVA in R (v.2.4.1), with the number of cilia nested in individual sponge nested in treatment group.

Texas-Red conjugated neomycin (TR-Neo) was made by shaking neomycin sulfate (50 mg/ml in K_2CO_3) and Texas Red (Molecular Probes, Invitrogen; 2 mg/ml in dimethylformamide) overnight (Steyger et al., 2003), and added to M-medium to a final concentration of 300 μM neomycin sulfate. *S. lacustris* was treated for 2 min in TR-Neo followed by three rinses in M-medium, 5 min in 1 μM YO-PRO1 (Invitrogen) (Santos et al., 2006; Ou et al., 2009), and three more rinses in M-medium prior to viewing live using a 40X Zeiss water immersion objective.

Both whole *S. lacustris* and oscula removed from the sponge were treated in 4 mM chloral hydrate for 20 hr or 70 hr (medium changed daily to maintain concentration), and fixed for fluorescence microscopy with anti-tubulin and for SEM as described above. Click-iT EdU imaging kit (Invitrogen) was used to label newly synthesized cells post osculum removal. *E. muelleri* was incubated in 50 μM EdU in M-medium for 8 hr or 24 hr after the osculum was removed, fixed for fluorescence, and labeled using the click copper-catalyzed covalent reaction. Sponges were labelled with acetylated alpha tubulin and Hoechst as described above.

2.2.7 BioInformatics

The transcriptomes of 8 sponge species (*Ephydatia muelleri, Spongilla lacustris, Petrosia ficiformis, Chondrilla nucula, Ircinia fasciculata, Corticium candelabrum, Sycon coactum, Aphrocallistes vastus)* were sequenced using Illumina and assembled *de novo* in either Trinity or CLCGenomics Workbench 5.1 (Riesgo et al., 2012). TRP sequences in these transcriptomes and also in the *Amphimedon queenslandica* genome (Srivastava et al., 2010) were detected using HMMer (Janelia.org) using HMM profiles formed with *pkd1* and *pkd2* sequences collected from NCBI or by blasting NCBI sequences against the transcriptome datasets using the tblastn suite in CLC Genomics Workbench. Sequence

identity and domain conservation was confirmed by BLAST and NCBI's conserved domain search as well as EMBL's InterPro Scan; domain illustrations were conceived using DOG2.0 and 3D models projected using Phyre2 (Kelley and Sternberg, 2009). TRP channel and PKD channel sequences from bilaterians were downloaded from SwissProt following the (vertebrates) taxon sampling for TRP and PKD domains in Pfam (Punta et al., 2012); SwissProts accession numbers are indicated in the sequence labels. *Chlamydomonas reinhardti* PKD2 ABR14113.1 was downloaded from NCBI. For phylogenetic analysis sequences were aligned in MAFFT (Katoh et al., 2002) using the E-INSI algorithm, and positions shared by 85% of the taxa were selected using MEGA5.1 (Tamura et al., 2011) for further phylogenetic analyses. Evolutionary relationships were inferred by ML using the evolutionary model LG [41] + GAMMA + Invariants as implemented in RAxML (Stamatakis, 2006). The statistical support of the branches was obtained by generating 1000 bootstrap pseudoreplicates. (The full alignment of 344aa and tree are shown in Additional file 1: Figures S2, S3.) The same dataset was analyzed under the Bayesian Inference framework /Phylobayes-MPI (Lartillot et al., 2013) under the CAT-GTR (Lartillot and Philippe, 2004) model (Additional file 1: Figure S4). The tree search was conducted during 7,500 cycles, and a burning of 1000 trees (sub-sampling every 10 trees) was used to discard the trees before the search reached the likelihood optima.

2.3 Results and Discussion

2.3.1 Sponge oscula are ciliated

Sponges are unusual in possessing both cilia and flagella (named for their differing beat patterns (Linck, 1973) on somatic cells. These include ciliated epithelial cells of sponge larvae which are involved in locomotion and also photoresponses (Leys and Degnan, 2002; Rivera et al., 2012), ciliated cells at the exit of the feeding choanocyte chambers (de Vos et al., 1990; Leys and Hill, 2012) and flagellated choanocytes involved in pumping water through the canal system (reviewed in Leys and Hill, 2012). In contrast, the epithelia of adult sponges are usually naked. We were therefore surprised to find cilia on all cells forming the epithelial lining of the osculum in the freshwater sponge *Ephydatia*

muelleri, a demosponge that can be cultured in the laboratory (Figure 2-1a). The osculum is the most prominent feature of a sponge, and is the final exit of water filtered through the sponge body for food and oxygen.

In *E. muelleri* a pair of cilia, each 4-6 microns long, emerges above the nucleus of every epithelial cell (Figure 2-1 b-f). A survey of 6 other demosponges showed that in each, the oscula are also lined by ciliated cells; in some species the cells have a single cilium, and others up to 4 cilia, all arising centrally above the cell nucleus (Appendix 1: A1.1). Even glass sponges (class Hexactinellida), which are syncytial, have cilia at the lip of their large oscula. There is no data available so far for the other two taxa, Calcarea and Homoscleromorpha, although the latter is known to have cilia throughout the canals, and therefore presumably also up to the oscula lip.

Serial sections through the base of the cilium in *E. muelleri* show basal bodies are simple, with no structures linking pairs of cilia in a cell (Figure 2-2a). In contrast to the flagella of choanocyte chambers, which have a central pair of microtubules, in cross section the oscula cilia have a 9 + 0 axonemal skeleton (Figure 2-2b), which is characteristic of sensory cilia in other organisms (Singla and Reiter, 2006). Both fluorescence and scanning electron microscopy show pairs of cilia in *E. muelleri* are oriented perpendicular to the water flow (Figure 2-2c), which may be important for sensing changes in flow. In live animals the cilia label with the vital dye FM 1-43, and high frequency time-lapse microscopy showed that they are non-motile and only vibrate in the flow that passes out of the osculum (Figure 2-2d, and Appendix 1: Movie A1.6).

2.3.2 Cationic channel blockers inhibit sponge behaviour

In the last decade it has been recognized that most cells in the vertebrate body, and many in invertebrates, possess specialized sensory structures called ‘primary’ cilia, which function as sensory organelles as in kidney epithelial cells, chondrocytes, odontoblasts, embryonic endocardial cells, and ‘Kupffer’s vesicle’ (Praetorius and Spring, 2005). Primary cilia, although similar to motile cilia in their basic structure, lack the radial spokes and dynein arms that enable motility. Instead they possess stretch-activated cationic channels that are part of the transient receptor potential (TRP) channel superfamily (Nauli

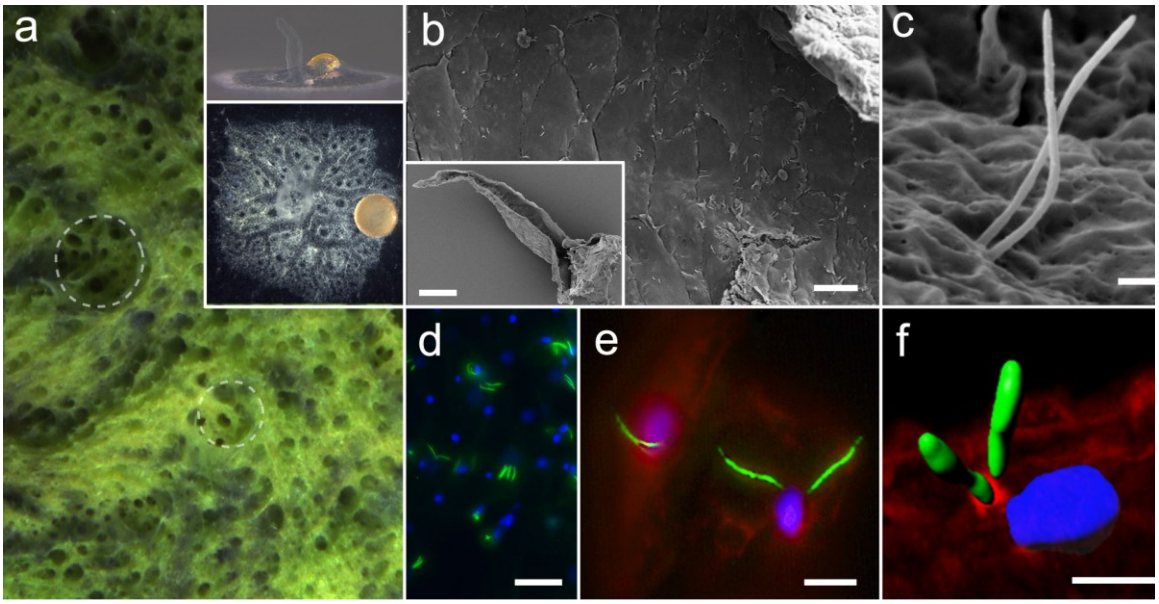


Figure 2- 1| Cilia are found on the epithelia lining the osculum

a. The sponge *Ephydatia muelleri* in the lake, and grown in the lab viewed from the side (upper inset) and from above (lower inset). The oscula (white dashed circles) extend upwards from the body. **b,c**, Scanning electron micrographs show cilia arise from the middle of each cell along the entire length of the inside of the osculum; **b** the lining of the osculum with cilia on each cell (inset shows an osculum removed from the sponge and sliced in half longitudinally); **c,** two cilia arise from each cell. **d,e**, Cilia in the oscula labeled with antibodies to acetylated α-tubulin (green), nuclei with Hoechst (blue, n), actin with phalloidin (red). **f,** A 3D surface rendering illustrates how the cilia arise just above the nucleus of the cell**.** Scale bars **a** 5 mm; inset 1 mm; **b** 20 µm; inset 100 µm **c**, 1 µm **d**, 20 µm **e,f** 5 µm.

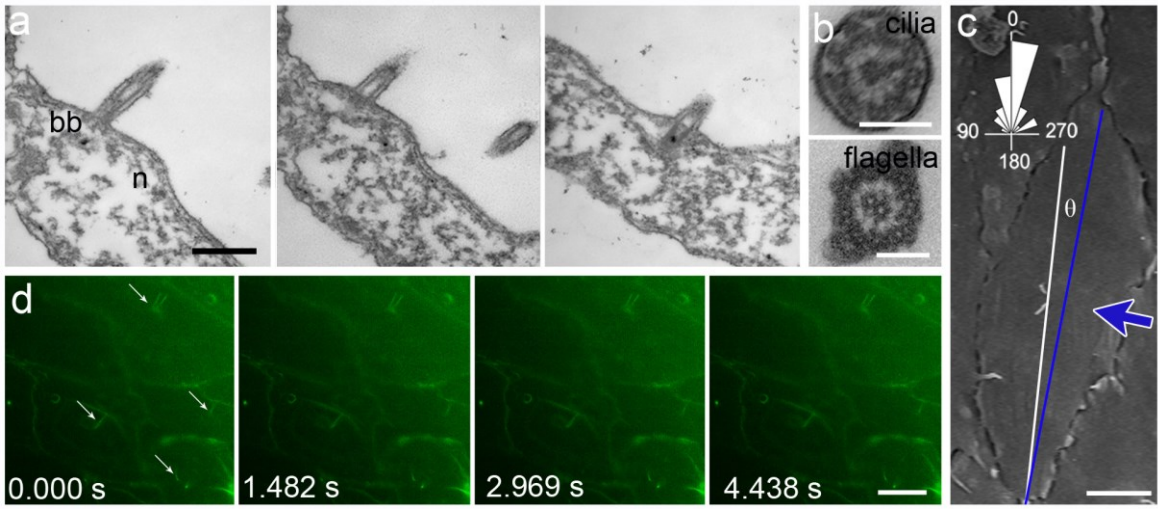


Figure 2- 2| Cilia are non-motile and are oriented perpendicular to the direction of water flow in the osculum

a. Serial longitudinal sections (86 nm apart) show each cilium arises just above the cell nucleus (n) from simple basal bodies (bb); no links between the bases of the ciliary pair were found. **b**. In cross-section the cilium lacks a central microtubule pair in contrast to the cross section of a flagellum from a choanocyte chamber. **c.** Cilia pairs are aligned parallel to the long axis of the cells in the osculum, and both the cilia pairs and the cells' long axes lie perpendicular to the direction of water flow (shown by the blue arrow) at 345.12 ± 4.72° (mean ± SE) (rose diagram: H_A:0°; V = 0.841; $p < 0.001$; n = 49). **d**. Still images from high-frequency time-lapse imaging of live cilia (arrows) labeled with FM1-43 (see Additional file 2: Movie S1). Scale bars: **a**, 500 nm **b**, 100 nm **c**, 10 μm **d**, 20 μm.

et al., 2003) including polycystin-1 (PC1) and polycystin-2 (PC2) (Nauli et al., 2003) or their homologs, which allow them to function as sensory organelles (Nauli et al., 2003; (Nauli et al., 2003; Praetorius and Spring, 2003; Praetorius and Spring, 2005; Singla and Reiter, 2006). Remarkably, TRP channels are responsible for almost all forms of sensation experienced by eukaryotic cells, including movement, taste, smell, temperature, vision and osmolarity.

The function of TRP channel sensation is difficult to assess directly, and is therefore usually done by behavioral assay; for example inhibition of an avoidance reaction by the unicellular alga *Chlamydomonas* using TRP channel blockers has shown that TRP11 is involved in mechanosensation (Fujiu et al., 2011). In multicellular organisms it is difficult to study the function of primary cilia in living tissues, except in cell culture. In contrast, freshwater sponges are small and transparent, and cilia can be viewed live. Furthermore, both of the freshwater sponges *E. muelleri* and *S. lacustris* can be triggered to inflate and then contract their whole body (a behaviour termed a 'sneeze' (Elliott and Leys, 2007; Elliott and Leys, 2010) in response to mechanical or chemical stimuli (Figure 2-3a). Because the osculum is the final channel through which water exits the sponge, we hypothesized that the cilia have a sensory role in controlling the canal diameter to optimize normal flow through the sponge filter, and in particular during the sneeze behaviour. Three commonly used chemicals–the antibiotic neomycin sulfate, styryl dye FM1-43, and cationic channel blocker Gadolinium (Gd^{3+})–have been shown to inhibit sensory ability of primary cilia in other organisms (Gale et al., 2001; Praetorius and Spring, 2001). These drugs are all thought to block TRPP2 (PC2) channels on the ciliary membrane. In sponges natural stimuli (sediment, vigorous mechanical agitation) as well as bath treatments of 75-90 μM L-glutamate trigger the inflation and contraction of the excurrent canals (Elliott and Leys, 2007; Elliott and Leys, 2010). Treatment of sponges with neomycin sulfate (300 μM) and FM 1-43 (35 μM) reduced the maximum amplitude of the inflation response by 60% (Figure 2-3b) in both cases, and treatment with Gd^{3+} (5 μM) eliminated the response; the effects were reversible (Figure 2-3b). After recovery, the Gd^{3+}-treated sponges showed an enhanced response to L-Glu (Figure 2-3b). This knock-down and knockout of the sponge behaviour by drugs that are known to affect channels on ciliary membranes implicates the

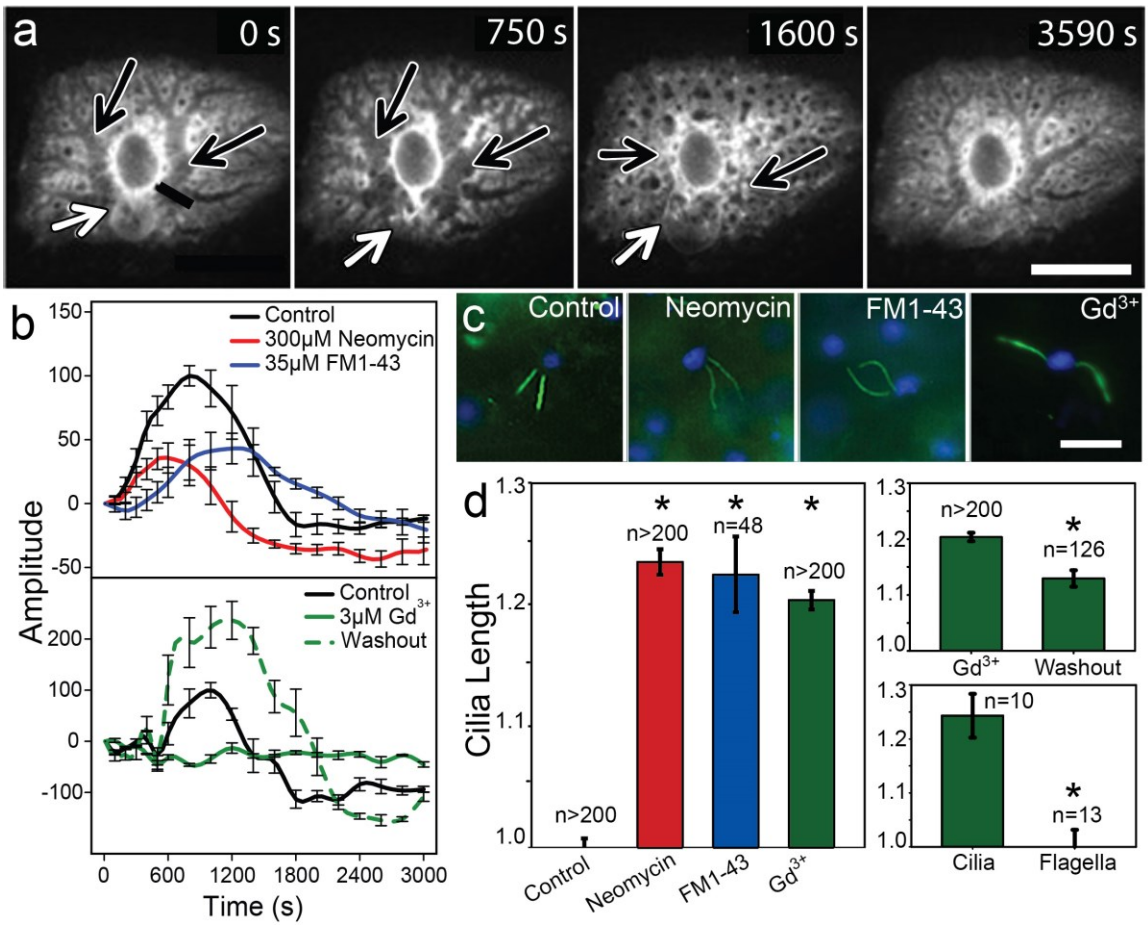


Figure 2- 3| Cationic channel blockers reduce the 'sneeze' response

a. The sponge 'sneeze' behaviour involves contraction of the osculum (white arrows), inflation, then contraction of canals (black arrows) and recovery (bar shows canal diameter). **b**. Neomycin sulfate (red) and FM1-43 (blue) reduce the peak amplitude of the behaviour in *Ephydatia muelleri* (n = 8; $p < 0.001$). Gd3+ (solid green) eliminated all response (n = 3; $p = 0.015$), but after recovery for 24 h the sponge response was even greater than before (dotted green). **c,d** All three compounds caused lengthening of cilia relative to controls (left), but had no effect on choanocyte flagella (bottom right) in *E. muelleri* (*significance at $p < 0.001$; error bars show ± SE). Scale bars: **a**, 1,000 µm **c**, 10 µm.

cilia in sensing stimuli and transducing them into behaviour. Further support for this idea comes from the direct effect the drugs had on ciliary length.

Lengthening of primary cilia in other organisms has been proposed to increase their sensitivity (Besschetnova et al., 2010; Miyoshi et al., 2011). Ciliary (and flagellar) length is determined by a dynamic process of intraflagellar transport (IFT) which continuously brings molecules, including tubulin, up and down the cilium (Rosenbaum and Witman, 2002). Chemical or mechanical stimuli that interfere with Ca^{2+} influx have been shown to alter IFT, thereby changing cilium length (Besschetnova et al., 2010; Miyoshi et al., 2011). In *E. muelleri* cilia length increased 1.2-fold after only one hour of treatment in all three drugs (Figure 2-3c,d), and Gd^{3+} treated sponges recovered partially after a one-hour washout. These data suggest that the drugs interfere with IFT in the oscula cilia. Unlike cilia, the flagella in choanocyte chambers of *E. muelleri* did not change length (Figure 2-3d), implying that the effects of the drugs reported here are only on ciliated cells.

Although pharmacology is almost universally used to study the sensory roles of cilia and flagella in other organisms (Gale et al., 2001; Praetorius and Spring, 2001; Harris et al., 2003; Besschetnova et al., 2010; Fujiu et al., 2011), neomycin sulfate, FM 1-43, and Gadolinium can also affect other calcium transport processes in tissues including smooth muscle contractility. We therefore tested whether another calcium channel blocker could equally affect the sponges. In contrast to neomycin sulfate which eliminates all response in the sponge, the L-type calcium channel blocker Verapamil (10 μM) had no effect on the amplitude of the sneeze reflex (Figure 2-4a). This finding is consistent with experiments on vertebrate primary cilia (Gale et al., 2001; Praetorius and Spring, 2001). We found that longer incubation in neomycin sulfate (2 hr in *S. lacustris* compared to 10 min for *E. muelleri*) repressed the sneeze reflex for longer. FM 1-43 is fluorescent and was clearly localized primarily to the cilia (Appendix 1: Movie A1-S1), but to determine where neomycin sulfate localized we incubated sponges in Texas Red-conjugates of neomycin sulfate. Cells in the sponge osculum labeled within 2 minutes of incubation in the dye, and the same cells co-labelled with YO-PRO1, which selectively labels hair cells in the lateral line of zebrafish (*Danio rerio*) (Santos et al., 2006; Ou et al., 2009) (Figure 2-4b). Taken together, the effect of these treatments suggests that stretch-activated, nonselective cation channels are involved in the sponge behaviour.

While we cannot rule out the possibility that any of these drugs have other effects on the sponge in addition to working on the cilia, in our experience very few molecules cause the sponge to relax–most trigger contractions (Ellwanger et al., 2007; Elliott and Leys, 2010). However, to confirm that the cilia in the osculum, and the osculum itself, are indeed required for the sponge sneeze reflex we used both chloral hydrate to deciliate the sponge and removed the osculum, and tested the responsiveness of the sponge in each instance. Chloral hydrate is known to remove cilia from cells, causing a loss of behaviour in both metazoans (Praetorius and Spring, 2003) and unicellular eukaryotes (Dunlap, 1977; Fujiu et al., 2011) after 20 hr exposure. It is thought to act by weakening the attachment of the cilium to the basal body, with full loss of cilia occurring after 68 hr in kidney epithelial cells (Praetorius and Spring, 2003). We found that 20 hr exposure to 4 mM chloral hydrate eliminated the sneeze reflex and it took 120 hr for recovery of sensitivity (Figure 2-4c-e). As in kidney cells (Praetorius and Spring, 2003), it took 70 hr to remove all cilia from the epithelium of the osculum (Figure 2-4f).

We have found that when removed, a new osculum forms after 8 hours. De-osculated sponges could not be triggered to sneeze (Figure 2-4g), and although the sponge continued to filter water at all times during repair of the osculum, it was only after the osculum had fully formed that the sneeze response returned. Together these results suggest that both the osculum and the cilia lining it are necessary for the sneeze reflex. To determine when ciliated cells first appear on newly formed oscula, we labeled sponges from which the osculum had been removed with the cell proliferation marker EdU and detected incorporation of uridine into new cells using Click-iT (Molecular Probes, Invitrogen). At 8 hr after the osculum was removed, cilia were found on cells in a few discrete places on the surface of the sponge (Figure 2-4h). Pinacocytes in the sponge surface are not usually ciliated, therefore we interpreted the differentiation of cilia on pinacocytes as an early marker of the location of a new osculum. Furthermore, although mesohyl cells were labeled within 6 hrs of incubation in EdU, cells of the new osculum were not labeled with EdU, and it was only 24 hr after the new osculum was formed that a few new ciliated cells labeled (Figure 2-4i). Although we were unable to trace the migration of cells in live animals, we interpret these data to suggest that cilia differentiate on cells in the surface of the sponge, thereby identifying the region as a potential osculum; then as the osculum grows to full

Figure 2-4| Cilia are specifically involved in the sponge behaviour. a. In contrast to Neomycin sulfate (solid red) which eliminates the 'sneeze' response (n = 3, p = 0.035), the calcium channel blocker Verapamil (dotted red) does not affect amplitude of the sneeze behaviour in *Spongilla lacustris* (n = 5, p = 0.573). **b**. Texas-Red Neomycin sulfate conjugate (red) and YO-PRO1 (green) selectively label cells in the osculum. **c**. A 20 hr treatment in chloral hydrate eliminates the sneeze behaviour in *S. lacustris* (solid green; n = 5, p = 0.004), which does not return until more than 3 days after recovery (dotted green; n = 5, 24 hr washout p = 0.003, 72 hr washout p = 0.018, 120 hr washout p = 0.864; error bars show ± SE). **d-f(SEM) d'-f'(fluorescence).** Cilia are removed by chloral hydrate treatment; *S. lacustris* 0 hr (**d,d'**), 20 hr (**e,e'**), and 70 hr (**f,f'**) treatment in chloral hydrate. **g**. The sneeze behaviour in *S. lacustris* cannot be triggered when the osculum is removed (solid blue; n = 3, p = 0.010) until it has fully regrown (dotted blue; n = 3, p = 0.275). **h**. Ciliated cells on the surface of *Ephydatia muelleri* 8 hr post osculum removal and (**i)** in the newly formed osculum 24 hr post osculum removal. Ciliated cells do not become labeled with EdU until after the osculum has regrown suggesting they arise by migration of newly formed mesohyl cells which differentiate into ciliated pinacocytes. Cilia are labeled with acetylated α-tubulin (red), nuclei with Hoechst (blue), and newly synthesized DNA with EdU (green). Scale bars: **b**, 50 μm inset 10 μm **d,e**, 5 μm **d',e',f,f',h,i** 10 μm

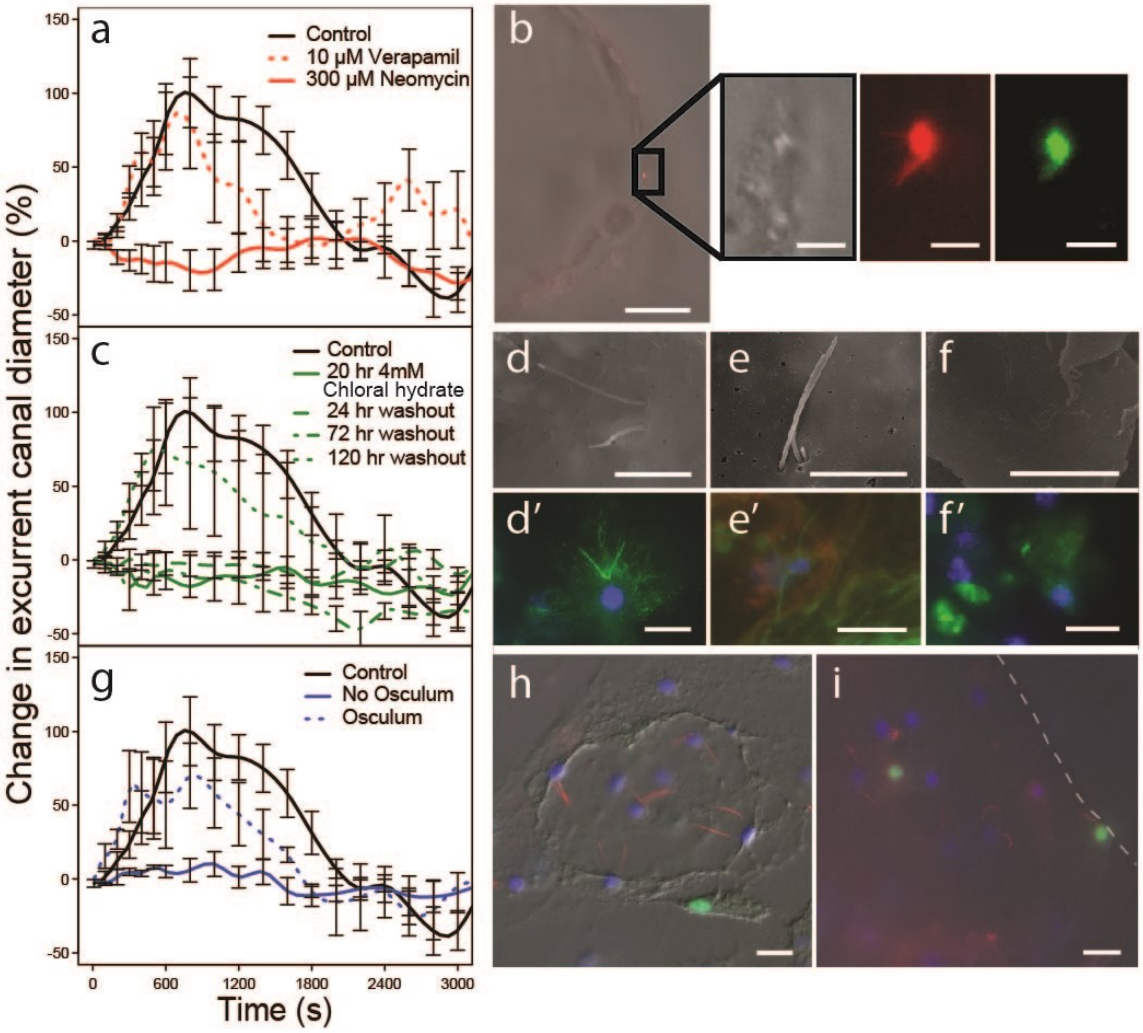


Figure 2- 4| Cilia are specifically involved in the sponge behaviour

height using cells already present in the sponge, new ciliated epithelial cells differentiate from newly formed mesohyl cells.

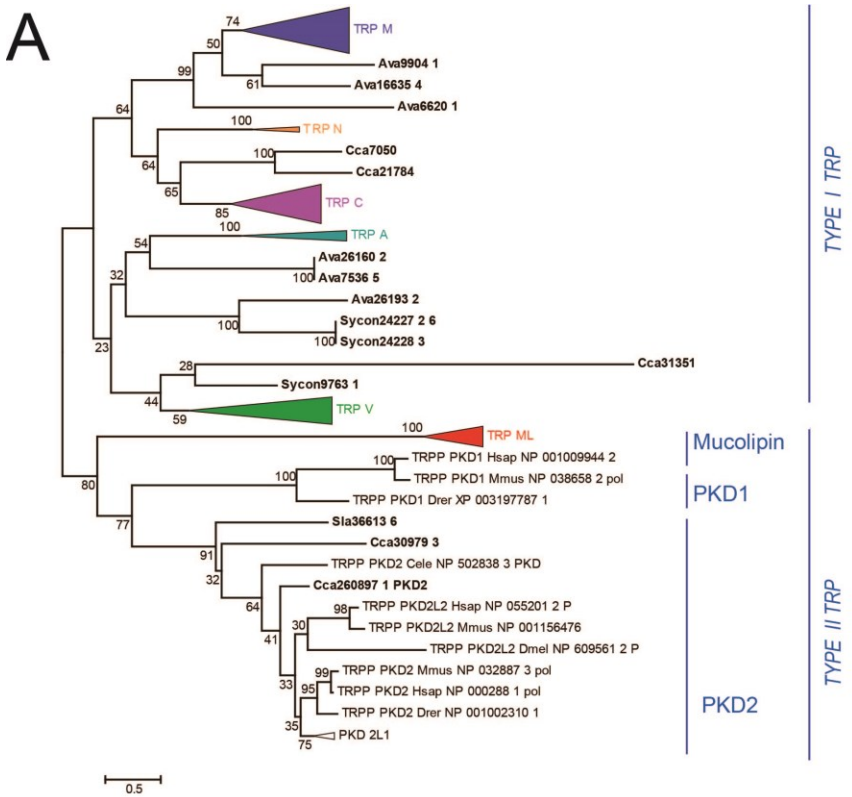
2.3.3 Sponges possess a repertoire of transient receptor potential channels

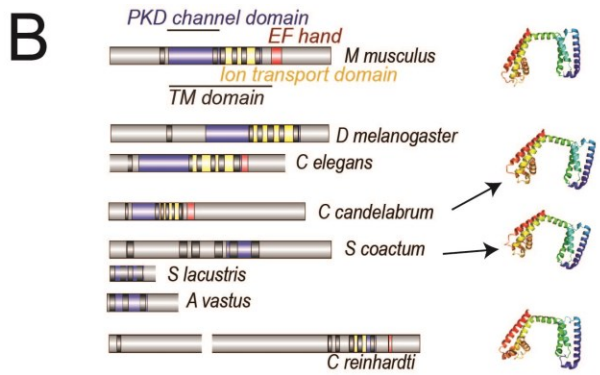
Considering the conserved role of TRP channels, and in particular PKD in sensory behaviour across eukaryotes (Fujiu et al., 2011), we searched the transcriptomes of 8 sponge species for homologs of both *pkd1* and *pkd2* and other TRP channels. A 700aa homolog of *pkd2* (Type II TRP) was identified in *Corticium candelabrum* (Homoscleromorpha) and a 178aa sequence of a *pkd2* (Type II TRP) gene was found in the freshwater *Spongilla lacustris* (Demospongiae) (Figure 2-5a, Appendix 1: A1.2, A1.3). We found a 978aa sequence of a Type II TRP (ML) in *Sycon coactum* (Calcarea), and several sequences with similarity to various Type I TRP channels were found in all 4 Porifera classes (Figure 2-5a-c, Appendix 1: A1.5). These candidates were included in an alignment containing more than 100 representatives for all the TRP families across bilaterians (Figure 2-5a; Appendix 1: A1.2 and A1.3). The ability to retrieve protein sequences depends on the quality of the transcriptome and the divergence of sequences in transcriptomes.

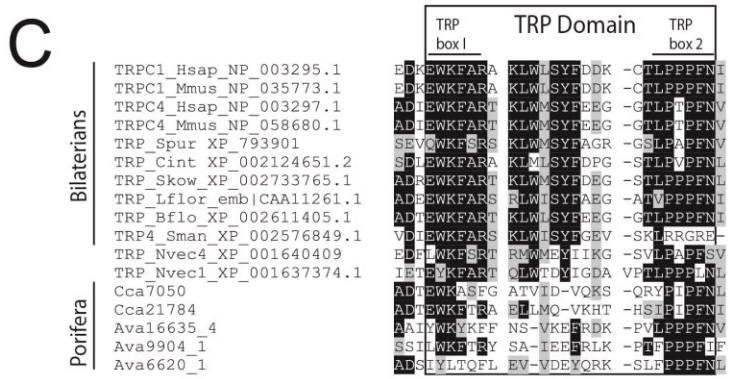
Negative results do not imply conclusive absence. Our phylogenetic analysis grouped sponge *pkd* sequences with Type II TRP and specifically *pkd2* channels genes from bilaterians with high support (91% bootstrap). Sponge *pkd* channel sequences showed similar domain architecture and proposed 3D protein folding to both mouse and *Chlamydomonas* sequences (Figure 2-5b), and other sponge sequences showed amino acids indicative of the TRP domain (Figure 2-5c; Appendix 1: A1.5). Although the pharmacology of the sponge cilia is similar to that of cilia known to have *pkd2* channels, several TRP channels from *Chlamydomonas* have also been found to transduce mechanical signals so we cannot rule out the possibility that other TRP channels are involved in flow sensing in sponges.

Figure 2- 5| Phylogenetic analysis of sponge TRP genes

a. Evolutionary relationships of sponge TRP Type I and II genes, values in the nodes indicate Boostrap Support and Posterior Probabilities (see methods); sponge sequences are in bold. **b**. Domain diagrams showing the PKD channel domain, transmembrane domain (TM), EF hand domain, and ion transport domains for the *pkd2* genes from mouse, *Mus musculus*; Cca, *Corticium candelabrum* (Homoscleromorpha); Cel, *Caenorhabditis elegans;* Sla, *Spongilla lacustris* (Demospongiae; Sco; *Sycon coactum* (Calcarea); Ava, *Aphrocallistes vastus* (Hexactinellida); Cre, *Chlamydomonas reinhardtii*, and 3D models of the proteins from mouse, *Corticium, Sycon,* and *Chlamydomonas*. **c**. Alignment of bilaterian, cnidarian and sponge TRP sequences showing the TRP domain and TRPbox (Hsap, *Homo sapiens;* Mmus, *Mus musculus;*Spur, *Strongylocentrotus purpuratus;* Cint, *Ciona intestinalis,* Sko, *Saccoglossus kowaleskii,* Lforb, *Loligo forbesi*, Bflo, *Branchiostoma floridae,* Sman, *Schistosoma mansoni,* Nvec, *Nematostella vectensis*). For the full tree and alignment see Appendix 1: A1.2 and A1.4.


A
TRP M
Ava9904 1
Ava16635 4
Ava6620 1
TRP N
Cca7050
Cca21784
TRP C
TRP A
Ava26160 2
Ava7536 5
Ava26193 2
Sycon24227 2 6
Sycon24228 3
Cca31351
Sycon9763 1
TRP V
TRP ML
TRPP PKD1 Hsap NP 001009944 2
TRPP PKD1 Mmus NP 038658 2 pol
TRPP PKD1 Drer XP 003197787 1
Sla36613 6
Cca30979 3
TRPP PKD2 Cele NP 502838 3 PKD
Cca260897 1 PKD2
TRPP PKD2L2 Hsap NP 055201 2 P
TRPP PKD2L2 Mmus NP 001156476
TRPP PKD2L2 Dmel NP 609561 2 P
TRPP PKD2 Mmus NP 032887 3 pol
TRPP PKD2 Hsap NP 000288 1 pol
TRPP PKD2 Drer NP 001002310 1
PKD 2L1
0.5
TYPE I TRP
TYPE II TRP
Mucolipin
PKD1
PKD2


B
PKD channel domain
EF hand
M musculus
Ion transport domain
TM domain
D melanogaster
C elegans
C candelabrum
S coactum
S lacustris
A vastus
C reinhardti


C
TRP Domain
TRP box I
TRP box 2
Bilaterians
Porifera
TRPC1_Hsap_NP_003295.1
TRPC1_Mmus_NP_035773.1
TRPC4_Hsap_NP_003297.1
TRPC4_Mmus_NP_058680.1
TRP_Spur XP_793901
TRP_Cint XP_002124651.2
TRP_Skow_XP_002733765.1
TRP_Lflor_emb|CAA11261.1
TRP_Bflo_XP_002611405.1
TRP4_Sman_XP_002576849.1
TRP_Nvec4_XP_001640409
TRP_Nvec1_XP_001637374.1
Cca7050
Cca21784
Ava16635_4
Ava9904_1
Ava6620_1

-

2.4 Conclusions

Obstruction of the canals by particulates in the feeding current would cause changes in pressure across the system; the osculum is the single exit of the entire system and is expected to be sensitive to this change, so it is plausible that the cilia detect changes in water flow or pressure. The absence of motility of the cilia, and their specific localization to the inner lining of the sponge osculum strongly suggest a sensory role for the osculum; the pharmacology and ablation experiments also support the hypothesis that the cilia have a sensory function. The primary cilium, which extends out from the cell and has a high surface-area to volume ratio, is an ideal organelle for both sensing and transducing signals (Singla and Reiter, 2006). These cilia in the sponge osculum have all the characteristics of primary cilia.

While the role of cilia in sensing information may have evolved many times within eukaryotes, the sponge sensory system described here is certainly very similar to signalling via primary cilia in other metazoans (Praetorius and Spring, 2005). The role of cilia in the sponge osculum suggests either a convergent role in sensing and transducing flow information into behaviour across all metazoa, or implies that primary cilia had an ancient evolutionary role in transducing sensory information, and in particular flow, in early multicellular animals. Given the unique position of Porifera as extant representatives of one of the first groups of multicellular animals (Roure et al., 2013), and in particular their lack of conventional nervous and coordination systems, the finding of such an organized array of sensory cells in sponges provides new insight into possible mechanisms of evolution of early sensory systems.

2.5 References

Besschetnova, T. Y., Kolpakova-Hart, E., Guan, Y., Zhou, J., Olsen, B. R. and Shah, J. V. (2010). Identification of signaling pathways regulating primary cilium length and flow-mediated adaptation. *Current Biology* **20**, 182-7.

Conaco, C., Bassett, D. S., Zhou, H., Arcila, M. L., Degnan, S. M., Degnan, B. M. and Kosik, K. S. (2012). Functionalization of a protosynaptic gene expression network. *Proceedings of the National Academy of Sciences of the United States of America* **109 Suppl**, 10612-8.

de Vos, L., Boury-Esnault, N. and Vacelet, J. (1990). The apopylar cells of sponges. In: New Perspectives in Sponge Biology. 153-158.

Dunlap, K. (1977). Localization of calcium channels in *Paramecium caudatum*. *Journal of Physiology* **271**, 119-133.

Dunn, C. W., Hejnol, A., Matus, D. Q., Pang, K., Browne, W. E., Smith, S. A., Seaver, E., Rouse, G. W., Obst, M., Edgecombe, G. D. et al. (2008). Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature* **452**, 745-749.

Elliott, G. R. D. and Leys, S. P. (2007). Coordinated contractions effectively expel water from the aquiferous system of a freshwater sponge. *The Journal of Experimental Biology* **210**, 3736-48.

Elliott, G. R. D. and Leys, S. P. (2010). Evidence for glutamate, GABA and NO in coordinating behaviour in the sponge, *Ephydatia muelleri* (Demospongiae, Spongillidae). *The Journal of Experimental Biology* **213**, 2310-21.

Ellwanger, K., Eich, A. and Nickel, M. (2007). GABA and glutamate specifically induce contractions in the sponge *Tethya wilhelma*. *Journal of Comparative Physiology A* **193**, 1-11.

Fujiu, K., Nakayama, Y., Iida, H., Sokabe, M. and Yoshimura, K. (2011). Mechanoreception in motile flagella of *Chlamydomonas*. *Nature Cell Biology* **13**, 630-2.

Gale, J. E., Marcotti, W., Kennedy, H. J., Kros, C. J. and Richardson, G. P. (2001). FM1-43 dye behaves as a permeant blocker of the hair-cell mechanotransducer channel. *The Journal of Neuroscience* **21**, 7013-25.

Harris, J. A., Cheng, A. G., Cunningham, L. L., MacDonald, G., Raible, D. W. and Rubel, E. W. (2003). Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (*Danio rerio*). *Journal of the Association for Research in Otolaryngology* **4**, 219-34.

Katoh, K., Misawa, K., Kuma, K.-i. and Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**, 3059-66.

Kelley, L. A. and Sternberg, M. J. E. (2009). Protein structure prediction on the Web: a case study using the Phyre server. *Nature Protocols* **4**, 363-71.

Lartillot, N. and Philippe, H. (2004). A Bayesian mixture model for across-site heterogeneities in the amino-acid replacement process. *Molecular Biology and Evolution* **21**, 1095-109.

Lartillot, N., Rodrigue, N., Stubbs, D. and Richer, J. (2013). PhyloBayes MPI : phylogenetic reconstruction with infinite mixtures of profiles in a parallel environment. *Systematic Biology*.

Leys, S. P. and Degnan, B. M. (2002). Embryogenesis and metamorphosis in a haplosclerid demosponge: gastrulation and transdifferentiation of larval ciliated cells to choanocytes. *Invertebrate Biology* **121**, 171-189.

Leys, S. P. and Hill, A. (2012). The physiology and molecular biology of sponge tissues. *Advances in Marine Biology* **62**, 1-56.

Linck, R. W. (1973). Chemical and structural differences between cilia and flagella from the lamellibranch mollusc, *Aequipecten irradians*. *Journal of Cell Science* **12**, 951-81.

Mackie, G. (1970). Neuroid conduction and the evolution of conducting tissues. *The Quarterly Review of Biology* **45**, 319-332.

Meech, R. W. (2008). Non-neural reflexes: sponges and the origins of behaviour. *Current Biology* **18**, R70-R72.

Miyoshi, K., Kasahara, K., Miyazaki, I. and Asanuma, M. (2011). Factors that influence primary cilium length. *Acta Medica Okayama* **65**, 279-85.

Nauli, S. M., Alenghat, F. J., Luo, Y., Williams, E., Vassilev, P., Li, X., Elia, A. E. H., Lu, W., Brown, E. M., Quinn, S. J. et al. (2003). Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nature Genetics* **33**, 129-37.

Nosenko, T., Schreiber, F., Adamska, M., Adamski, M., Eitel, M., Hammel, J., Maldonado, M., Müller, W. E. G., Nickel, M., Schierwater, B. et al. (2013). Deep metazoan phylogeny: when different genes tell different stories. *Molecular Phylogenetics and Evolution* **67**, 223-33.

Ou, H. C., Cunningham, L. L., Francis, S. P., Brandon, C. S., Simon, J. a., Raible, D. W. and Rubel, E. W. (2009). Identification of FDA-approved drugs and bioactives that protect hair cells in the zebrafish (*Danio rerio*) lateral line and mouse (*Mus musculus*) utricle. *Journal of the Association for Research in Otolaryngology* **10**, 191-203.

Philippe, H., Derelle, R., Lopez, P., Pick, K., Borchiellini, C., Boury-Esnault, N., Vacelet, J., Renard, E., Houliston, E., Quéinnec, E. et al. (2009). Phylogenomics revives traditional views on deep animal relationships. *Current Biology* **19**, 706-12.

Praetorius, H. A. and Spring, K. R. (2001). Bending the MDCK cell primary cilium increases intracellular calcium. *Journal of Membrane Biology* **184**, 71-79.

Praetorius, H. A. and Spring, K. R. (2003). Removal of the MDCK cell primary cilium abolishes flow sensing. *Journal of Membrane Biology* **191**, 69-76.

Praetorius, H. A. and Spring, K. R. (2005). A physiological view of the primary cilium. *Annual Review of Physiology* **67**, 515-529.

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J. et al. (2012). The Pfam protein families database. *Nucleic Acids Research* **40**, D290-301.

Rasmont, R. (1961). Une technique de culture des eponges d'eau douce en milieu controle. *Annales de la Société Royale Zoologique de la Belgique* **91**, 149-155.

Riesgo, A., Pérez-Porro, A. R., Carmona, S., Leys, S. P. and Giribet, G. (2012). Optimization of preservation and storage time of sponge tissues to obtain quality mRNA for next-generation sequencing. *Molecular Ecology Resources* **12**, 312-22.

Rivera, A. S., Ozturk, N., Fahey, B., Plachetzki, D. C., Degnan, B. M., Sancar, A. and Oakley, T. H. (2012). Blue-light-receptive cryptochrome is expressed in a sponge eye lacking neurons and opsin. *The Journal of Experimental Biology* **215**, 1278-86.

Rosenbaum, J. L. and Witman, G. B. (2002). Intraflagellar transport. *Nature Reviews Molecular Cell Biology* **3**, 813-25.

Roure, B., Baurain, D. and Philippe, H. (2013). Impact of missing data on phylogenies inferred from empirical phylogenomic data sets. *Molecular Biology and Evolution* **30**, 197-214.

Ryan, T. J. and Grant, S. G. N. (2009). The origin and evolution of synapses. *Nature Reviews Neuroscience* **10**, 701-12.

Sakarya, O., Armstrong, K. a., Adamska, M., Adamski, M., Wang, I.-F., Tidor, B., Degnan, B. M., Oakley, T. H. and Kosik, K. S. (2007). A post-synaptic scaffold at the origin of the animal kingdom. *PloS one* **2**, e506.

Santos, F., MacDonald, G., Rubel, E. W. and Raible, D. W. (2006). Lateral line hair cell maturation is a determinant of aminoglycoside susceptibility in zebrafish (*Danio rerio*). *Hearing Research* **213**, 25-33.

Singla, V. and Reiter, J. F. (2006). The primary cilium as the cell's antenna: signaling at a sensory organelle. *Science* **313**, 629-33.

Sperling, E. A., Peterson, K. J. and Pisani, D. (2009). Phylogenetic-signal dissection of nuclear housekeeping genes supports the paraphyly of sponges and the monophyly of Eumetazoa. *Molecular Biology and Evolution* **26**, 2261-74.

Srivastava, M., Simakov, O., Chapman, J., Fahey, B., Gauthier, M. E., Mitros, T., Richards, G. S., Conaco, C., Dacre, M., Hellsten, U. et al. (2010). The *Amphimedon queenslandica* genome and the evolution of animal complexity. *Nature* **466**, 720-6.

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688-90.

Steyger, P. S., Peters, S. L., Rehling, J., Hordichok, A. and Dai, C. F. (2003). Uptake of gentamicin by bullfrog saccular hair cells *in vitro*. *Journal of the Association for Research in Otolaryngology* **4**, 565-78.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731-9.

Chapter Three

It costs more to pump more: energetic cost of filtration and behavioural response to ambient currents in demosponges

3.1 Introduction

Benthic suspension feeders can have a major impact on marine ecosystems by ingesting suspended particulates and dissolved nutrients from the overlying water column and releasing them for use by other organisms (Gili and Coma, 1998). Recycling of nutrients in this way provides an important link between the benthic and pelagic communities, known as benthic-pelagic coupling (Gili and Coma, 1998). Water quality is declining in most coastal marine ecosystems by processes including increased suspended sediment from fishing trawls (Puig et al., 2012), increased eutrophication resulting in harmful algal blooms (Hallegraeff, 1993), oxygen dead zones (Diaz and Rosenberg, 2008), and increased pollutants especially near ports and coastal communities. Suspended sediment in particular is known to impact the efficiency and ability of filter feeders to process water, leading to reduced pumping activity and increased metabolic demand (Gerrodette and Flechsig, 1979; Ellis et al., 2002; Bannister et al., 2012). Knowing the energetic constraints that might affect suspension feeders is therefore important to understand the impacts these additional stresses may have on benthic communities, yet the energetic cost of this type of feeding in invertebrates is still debated.

Many suspension feeders are sessile and use ciliary or muscular pumps to draw water with food towards themselves. Invertebrates such as bivalves, ascidians, polychaetes, and sponges use filters to strain out particles from the water that are too small to be captured individually (Jørgensen, 1966). Where suspended particulates are dilute, this approach can be highly efficient if huge volumes of water are processed (Jørgensen, 1955). It has been suggested that filter feeders evolved a low energetic cost of filtration to allow

continuous feeding rates (Jørgensen, 1975). Yet, food availability varies hugely on a temporal basis, with fluctuations occurring seasonally, daily, and with the ebb and flow of the tide. It would therefore be adaptive to sense the variations in food availability and feed when concentrations are high. Although few studies have focused on this question, two examples suggest this hypothesis is correct. Bivalves respond to food availability by reducing filtration and respiration rates when food is scarce (Thompson and Bayne, 1972; Griffiths and King, 1979), and the demosponge *Tethya crypta* reduces pumping activity at night when ambient currents are lower (Reiswig, 1971) which would therefore bring reduced food availability (Newell and Branch, 1980). As all filter feeders demonstrate some fluctuations in pumping rates in response to various environmental cues, filtration may be more costly than previously thought and the animals may be finely adapted to habitats that support the energetic cost to obtain food.

Previous work modeling the filter and pump system for a number of invertebrates has suggested that filter feeding is inexpensive, at less than 4% of total metabolism (Riisgård and Larsen, 1995). In contrast, direct measurements of the uptake of oxygen going from non-feeding to feeding has shown that filter feeding in bivalves accounts for up to 50% of total metabolism (Thompson and Bayne, 1972; Newell and Branch, 1980) and in sponges 25% of total respiration (Hadas et al., 2008). In addition, the resistance through the filter of sponges may be much higher than previously thought due to a difficult to preserve fine glycocalyx (mucus) mesh on the collar (Leys et al., 2011).

In support of the idea that cost of filtration may be high, some animals seem to reduce energy expended to feed by using ambient currents in the water column to enhance flow by dynamic pressure, the Bernoulli effect or viscous entrainment. For example, in high current speeds barnacles will switch from active to passive feeding and orient their bodies toward the current (Trager et al., 1990). Cnidarians (Best, 1988), ascidians (Young and Braithwaite, 1980; Knott et al., 2004), and brachiopods (Labarbera, 1977) also orient their bodies with the current, while other invertebrates may take advantage of current-induced flow through tubes (Vogel, 1977; Murdock and Vogel, 1978; von Dassow, 2005; Shiino, 2009). Sponges are often considered ‘textbook’ examples of the use of current-induced flow in nature (Bidder, 1923; Vogel, 1974; Vogel, 1977) but experiments to confirm this have been equivocal (Leys et al. 2011).

Even if models are correct and cost of filtration is not great, a universal cost for all sponges is difficult to accept since structural differences in the sponge canal system in relation to body form (Reiswig, 1975a), microbial content (Weisz et al., 2008), and tissue density (Turon et al., 1997) can cause wide differences in pumping rates between species. Temperature and food availability vary across habitats causing differences in seawater viscosity and enzyme function and potentially leading to differences in the metabolic cost of filtering in different habitats. Estimates of the cost of pumping for a range of sponge species and habitats are therefore required to better understand sponge energy budgets.

Do differences in sponge shape, size, pumping volume, and habitat affect the energetic cost of pumping in sponges? I have studied energetics of filtration in five species of demosponge from tropical and temperate habitats using *in situ* measurements of oxygen consumption and pumping rate, experimental tests of pumping at different ambient flow rates, and by morphometric analysis of the canal and filter structures. To evaluate the importance of accurate measures of filter dimensions and volume of water processed, I first examined the effect of changes in these values on cost of filtration calculated in previous work. The results from that analysis focused our attention on these aspects in our own experimental analyses.

3.2 The importance of mesh size and volume flow rates

Animals allocate energy to a variety of processes including growth, reproduction, feeding, and digestion. For filter feeders, the energy allocated to feeding is generally considered to be low, at 0.1 – 4% of total metabolism based on theoretical models (Jørgensen, 1955; Jørgensen et al., 1986; Jørgensen et al., 1988; Riisgård, 1988; Riisgård, 1989; Riisgård et al., 1993). In these studies, the cost of filtration is assumed to be equivalent to the energy lost due to frictional resistance as water flows through the filter and canals. Models can be informative about which structures contribute most to energetic costs, however, their accuracy depends on having correct dimensions for each region of the filter and canal system as well as volume flow rates. This same approach used by Riisgård and colleagues was recently used for the filtration system of the glass sponge *Aphrocallistes vastus* (Leys et al., 2011) where the cost of pumping was found to be 28% of

the total metabolism. Here, the resistance through the filter was found to be much higher than previous estimates due to the small spaces of the glycocalyx mesh (Leys et al., 2011), a structure that is often not preserved with common fixation techniques and was not included by Riisgård et al (1993). In addition, the volume flow rate in *A. vastus* was quite high compared to the 'standard sponge' studied by Riisgård and colleagues (1993) where volume flow rate was obtained indirectly using clearance rates of particles (flagellate cells) during incubation in a closed vessel. Closed vessels have been shown to cause reduced pumping behaviour of sponges (Yahel et al., 2005; Hadas et al., 2008), and the sponge may re-filter the water if sampling times are not well adjusted to pumping rates (Yahel et al., 2005).

I carried out a meta-analysis using data from the literature to determine the cost of pumping in four filter feeding invertebrate group used by Riisgård and colleagues (summarized in Riisgård and Larsen, 1995). I calculated dimensions from electron micrographs of mucus filters (Figure 3-1), where available, and volume flow rates that were obtained using 'direct' methods. By changing filter size and volume flow rate, the estimate for the cost of filtration increased to more than 5 times previous values (Table 3 -1). This suggests that both filter dimensions and volume flow rates contribute substantially to the cost of pumping in filter feeding invertebrates. It also suggests that accurate measurements of filter dimensions and volume flow rates are important when modeling the cost of filtration and forced my attention on these for my own study.

3.3 Methods

3.3.1 Overview

I conducted *in situ* and *in vitro* studies to measure excurrent flow rates and oxygen removal from five species of demosponge, *Neopetrosia problematica, Haliclona mollis, Tethya californiana, Callyspongia vaginalis,* and *Cliona delitrix* (hereafter referred to by genus). These species were selected due to their abundance at each of the study locations as well as the large size of their oscula. Paired flow and oxygen recordings were done on a mimimum of six oscula per species. During paired recordings for *Cliona* and *Callyspongia,*

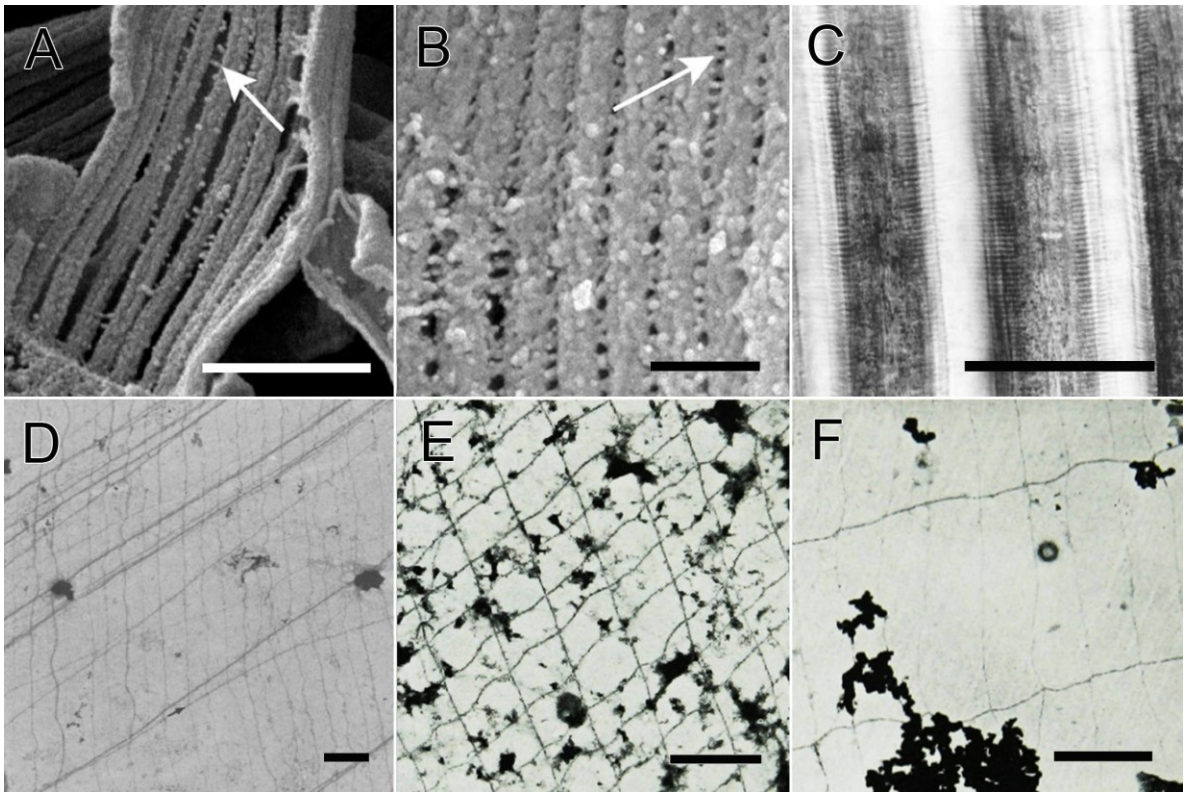


Figure 3- 1| Feeding filters in four groups of invertebrates used to re-estimate the cost of filtration

(A,B) Scanning electron micrograph (SEM) of the collar of *Spongilla lacustris,* showing the glycocalyx mesh fibrils (arrows) linking adjacent microvilli (Mah et al., 2014) (C) Gill filament of *Mytilus edulis* showing the latero-frontal cirri. (Jones et al., 1992) (D-F) Transmission electron micrographs of the mucus nets in *Chaetopterus variopedatus* (Flood and Fiala-Medioni, 1982) (D), *Ciona intestinales* (Flood and Fiala-Medioni, 1981) (E), and *Styella plicata* (Flood and Fiala-Medioni, 1981) (F) Scale bars (A, D-F) 1 μm (B) 300 nm (C) 100 μm.

Table 3- 1| Estimated cost of pumping (%) for four different groups of filter feeders

Using new measurements from the literature for filter dimensions and volume flow rates (blue). In the absence of volume flow rates for *Spongilla lacustris,* estimates for *Haliclona permollis* (blue) were used instead. Estimates for the cost of pumping (% of metabolism) are based on the morphometric model summarized by Riisgård and Larsen (1995) and outlined in equations 3-8. Original estimates for the cost of pumping are in black and the new estimates are shown in red.

Species	Filter dimensions	Filter Dimension Reference	Volume Flow rate	Volume Flow rate Reference	Estimate of the cost of pumping (%)	
Sponges						
Haliclona urceolus	d=0.14um, b=0.25um	Riisgard et al (1993)	6 mL/min	Riisgard et al (1993)	**0.850**	
	d=0.14um, b=0.25um	Riisgard et al (1993)	6 mL/min	Riisgard et al (1993)	**1.021**	*
Haliclona permollis	d=0.14um, b=0.25um	Riisgard et al (1993)	18.84 mL/min	Reiswig (1975)	**3.206**	
Spongilla lacustris	h1=0.048um, h2=0.041um, d=0.04um	Mah et al (2014)	6 mL/min	Riisgard et al (1993)	**1.594**	
	h1=0.048um, h2=0.041um, d=0.04um	Mah et al (2014)	18.84 mL/min	Reiswig (1975)	**5.004**	
Bivalves						
Mytilus edulis	L=200um, l=40um	Jorgensen et al 1986a, 1988	60 mL/min	Jorgensen et al 1986a, 1988	**1.562**	
	L=200um, l=40um	Jorgensen et al 1986a, 1988	67.8 mL/min	Riisgard et al 2011	**1.765**	
	L=200um, l=16um	Jones et al (1992)	60 mL/min	Jorgensen et al 1986a, 1988	**4.131**	
	L=200um, l=16um	Jones et al (1992)	67.8 mL/min	Riisgard et al 2011	**4.668**	
Polychaetes						
Chaetopterus variopedatus	h1=2.3, h2=1.4, d=0.02	Riisgard (1989)	18 mL/min	Riisgard (1989)	**4.032**	
	h1=2.3, h2=1.4, d=0.02	Riisgard (1989)	30 mL/min	Grove et al (2000)	**6.719**	
	h1=0.76 h2=0.46, d=0.02um	Flood and Fiala-Medioni (1982)	18 mL/min	Riisgard (1989)	**10.903**	
	h1=0.76 h2=0.46, d=0.02um	Flood and Fiala-Medioni (1982)	30 mL/min	Grove et al (2000)	**18.172**	
Ascidians						
Styella clava	h1=0.35um, h2=1.35um, d=0.020um	Riisgard and Larsen (1995)	45.6 mL/min	Riisgard and Larsen (1995)	**0.191**	
	h1=0.35um, h2=1.35um, d=0.020um	Riisgard and Larsen (1995)	45.6 mL/min	Riisgard and Larsen (1995)	**0.724**	**
Mean of three species	h1=0.35um, h2=1.35um, d=0.020um	Riisgard and Larsen (1995)	57.5 mL/min	Fiali-Medioni (1978)	**0.913**	
Mean of six species	h1=1.002, h2=0.366, d = 0.025	Flood and Fiala Medioni (1981)	45.6 mL/min	Riisgard and Larsen (1995)	**0.815**	
Styella plicata	h1=1.959, h2=0.5055, d=0.020	Flood and Fiala Medioni (1981)	83.1 mL/min	Fiali-Medioni (1978)	**0.885**	
Ciona intestinales	h1=0.640, h2=0.405, d=0.020	Flood and Fiala Medioni (1981)	21.5 mL/min	Fiali-Medioni (1978)	**0.376**	

* Cost of pumping re-estimated to use consistent temperatures for kinematic viscosity

** Cost of pumping re-estimated using corrected head loss at the filter

ambient velocity was increased using an underwater pump for one individual to determine use of current-induced flow. Following experiments, pieces of sponge were fixed for both scanning electron microscopy (SEM) and histology to estimate dimensions of the aquiferous canal system. These dimensions along with excurrent flow rates and respiration were used to estimate the resistance through the sponge and cost of filtration for each species, using the models by Riisgård and colleagues (1995) and Leys and colleagues (2011).

3.3.2 Field and lab studies

Work was carried out at two research laboratories: the Bamfield Marine Sciences Centre (BMSC) in Bamfield, British Columbia, Canada, and the Smithsonian Tropical Research Station (STRI) on Isla Colon in Bocas del Toro, Panama (Figure 3-2).

At BMSC the three temperate species of demosponge *Neopetrosia, Haliclona,* and *Tethya* were collected via SCUBA by Amanda Kahn and Sally Leys from Wizard Island and kept in seawater tables with high water flow (up to 3000 L/min) supplied from deep water (30m depth) in Bamfield Inlet. Experiments with acoustic Doppler velocimeters (ADVs) were conducted in a large circular seawater tank approximately 1m in diameter. Samples were collected both in September 2012 and December 2013. Pumping rates, respiration, and passive flow experiments were carried out in December 2013.

At STRI, in July 2013, experiments on *Cliona* and *Callyspongia* were conducted *in situ* by snorkeling at STRI point, GPS coordinates 9°21.169'N 82°15.528'W (Diaz, 2005) at approximately 2 m depth. An aluminum frame made from 80/20 (80/20 Inc., Columbia City, IN) was placed over the sponge and instruments were attached using loc-line (Lockwood Products, OR) and clamps (Figure 3-2). The instruments were tethered via cables to a laptop computer on a boat anchored near the study site to monitor the data collection in real time.

At both STRI and BMSC, photos were taken using a GoPro Hero 2 camera with underwater housing as well as a Panasonic Lumix DMC-TS4 underwater camera.

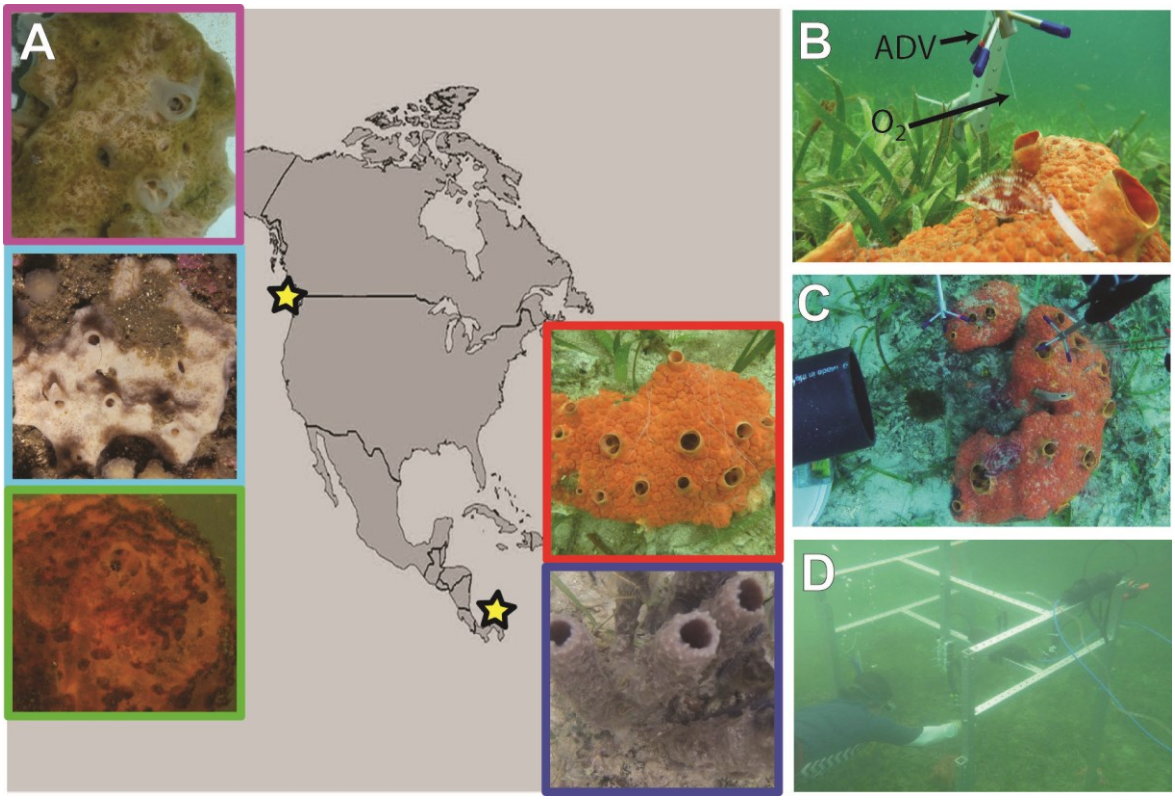


Figure 3- 2| Experimental set-up and species used.

(A) *Haliclona mollis* (purple), *Neopetrosia problematica* (cyan), and *Tethya californiana* (green) were collected and studied *in vitro* in Bamfield, British Columbia, Canada (top star on the map). *Callyspongia vaginalis* (blue) and *Cliona delitrix* (red) were studied *in situ* in Bocas del Toro, Panama (bottom star). **(B)** Excurrent velocity was measured out of the osculum using a Nortek Vectrino II acoustic Doppler velocity (ADV) and removal of oxygen was measured using a FirestingO_2 bare fiber sensor (O_2) positioned inside of the sponge osculum. **(C)** Ambient velocity was increased using an underwater pump attached to a PVC tube (shown) positioned at the sponge. **(D)** Instruments were mounted *in situ* on a frame positioned over the sponge using snorkel.

3.3.2.1 Measurements of excurrent velocity

Maximum excurrent velocity from each sponge species was measured using a Vectrino II profiling acoustic Doppler velocimeter (ADV; Nortek). The profiling ADV is a non-intrusive instrument that measures the velocity of water by sending acoustic waves that reflect off particles moving in three dimensions (X, Y and Z) within a small cylindrical "sampling volume" 45-75 mm from the probe head. The sampling volume is 6 mm in diameter, 30 mm long, and divided into 1 mm intervals or profiles. Because the sampling volume is some distance from the probe head, and because the excurrent flow from a sponge osculum may be small, and may not travel very far from the osculum lip, positioning it over the sponge osculum is challenging. The profiling capabilities of the Vectrino II help because they provide measurements over the full 30 mm profile, and as a result the lip of the sponge osculum itself disrupts the profile, showing exactly where above the sponge the sampling volume is. Using this method it is possible to be sure that the velocity being recorded is just above or inside the sponge osculum, and this becomes important in differentiating between ambient and excurrent water velocities. Fluorescein dye diluted in filtered seawater was used to visualize the excurrent flow, and a plastic cable tie was used to indicate the specific position of the sampling volume by blocking the signal and confirm that the readings were just above or inside the sponge osculum.

The ADV sensor was attached to loc-line to allow fine positioning above the sponge osculum. Movement of the sensor above the sponge while watching the velocity recordings on a computer allowed us to determine the position that gave the maximum excurrent velocity from the sponge osculum. Once the sensor was oriented correctly, excurrent velocity was recorded for 5 minutes at 25 Hz on low power (it was found that high power could push the excurrent flow down, a process termed streaming). Only the Z (vertical – or in line with the excurrent flow and ADV) direction of velocity was used in analysis to differentiate from the ambient velocities. All of the flow exiting the sponge osculum may not be in the exact same direction, and some flow may drift or shear out of the sampling volume; therefore, the Z direction is a conservative estimate of the excurrent velocity. Data were binned using a 5 second median filter in MATLAB (vR2013b). Images of each sponge osculum were taken using a GoPro Hero2 during recording and their diameters

measured in ImageJ (v. 1.43r; NIH) to calculate sponge pumping volume, assuming plug flow in which velocities are equal across the osculum plane.

The ratio of sponge volume to dry weight was calculated by drying three pieces of each species at 100°C to constant weight. This ratio was then used to standardize volume flow rate to dry weight.

3.3.2.2 Measurements of oxygen consumption

Ambient and excurrent oxygen were measured using two 2-channel FireStingO_2 optical oxygen meter (Pyro Science, Germany) with 250 μm diameter bare fiber minisensor probes. Although these sensors are extremely stable, minor differences due to construction of fiber optics meant probes were calibrated separately and can therefore deviate minimally when in the same water volume. Therefore, prior to positioning the excurrent sensor into the sponge osculum, both probes were left in ambient water for at least 5 minutes to obtain the difference in readings between the two sensors (here termed the offset value), and this difference was subtracted from the difference between ambient and excurrent oxygen for all analyses. Oxygen readings were calibrated using an external temperature probe. In addition, the ambient and excurrent sensors were positioned at the same height in the water column, such that any temperature fluctuations were accounted for when offsetting between probes. Data were collected every 1 s and binned using a 5 s median filter using MATLAB (vR2013b). Oxygen removal per hour was calculated using the volume of water filtered per hour, and standardized to per gram dry weight.

3.3.2.3 Test of passive flow

To assess the effect of changes in ambient current velocities on sponge excurrent flow, experiments were conducted on *Cliona* and *Callyspongia* at STRI by manipulating ambient flow with an underwater aquarium pump (Eheim compact + 3000). The pump was anchored near the sponges with a weight and the outflow directed through a 50 cm long, 10 cm diameter PVC pipe at and over the sponges, after Genin and Karp (1994). The aquarium pump had variable speeds that could generate flow at 5 to 40 cm/s through the PVC pipe

when positioned 30 cm from the sponge, as measured in a flow flume. Three flow speeds were used in experiments by setting the pump to low, medium, and high speeds. The ambient velocity was recorded using the Vectrino I point ADV (Nortek) with a sampling volume of 6 mm diameter by 7 mm length, positioned about 10 cm from the sponge perpendicular to the pump outflow (Figure 3-2). The ADV could not be positioned right next to the sponge due to interference between the ADVs. Data were measured with a transmit length of 1.8 mm at 25 Hz on high power and binned using a 5 s median filter using MATLAB (vR2013b).

Paired excurrent velocities and oxygen removal were measured during experiments as described above (sections 3.3.1.1 and 3.3.1.2). The profiling ADV (Nortek) was first positioned to ensure maximum excurrent velocity recordings from the osculum. Then the oxygen sensor was positioned inside the osculum, ensuring that it did not interfere with the ADV sampling volume as determined by a probe check analysis. Paired recordings were measured for 5 minutes at zero flow, 5 minutes with the pump on the 'low' setting, 5 minutes with the pump on the 'medium' setting, and 5 minutes with the pump on the 'high' setting, repeated three times through.

A GoPro Hero2 with underwater dive housing was positioned on the frame above both *Cliona* and *Callyspongia* to record osculum size during the experiment; images were captured every 30 s and a ruler was positioned in one of the images for calibration. Changes in osculum area were measured using a script developed for MATLAB (vR2013b) (Appendix 3). Volume filtered was calculated using excurrent velocity and area of the osculum. The ratio of sponge volume to dry weight was used to standardize volume flow rate and oxygen removal to per gram dry weight.

3.3.2.4 Statistical analyses

If the cost of filtration in sponges was low, as predicted by Jørgensen (1975) and Riisgård and colleagues (1993), then there would be no relationship between the volume of water a sponge pumps and the amount of oxygen it removes. In addition, if all species of sponge used passive flow as predicted by Vogel (1974; 1977), then there would be a positive relationship between ambient velocity and excurrent velocity. All statistical

analyses were done using SigmaStat in SigmaPlot v12.5. Data were tested for normality and linearity and subsequently variables were tested for association using a Spearman's rank order correlation test to allow for non-linearity.

3.3.3 Morphometric analysis of sponges

3.3.3.1 Scanning electron microscopy

For scanning electron microscopy (SEM), sponges were cut into 1 mm^3 pieces and fixed in a cocktail consisting of 1% OsO_4, 2% gluteraldehyde in 0.45 mol L^{-1} sodium acetate buffer with 10% sucrose at 4°C for 6-12 h (Harris and Shaw, 1984). In some preparations 10% ruthenium red was added to the fixative to preserve the fine structure of the glycocalyx mesh on the collar filter. We found that applying 4% OsO_4 directly to the sponge tissue prior to cutting and placing into the cocktail fixative helped to minimize contraction of canals. After 6-12 h, preparations were washed with distilled water and dehydrated to 70% ethanol. Sponges were desilicified in 4% Hydrofluoric Acid (HF) in 70% ethanol at room temperature (RT) for 24-72 h or until the spicules were dissolved. After desilicification, the sponge pieces were dehydrated to 100% ethanol and fractured while still in ethanol, in liquid nitrogen. Fractured pieces were critical point dried and mounted on aluminum stubs with clear nail polish, gold coated, and viewed in a field emission scanning electron microscope (JEOL 6301 F). For some pieces of *Cliona* and *Callyspongia,* the pieces were embedded in paraffin wax, sectioned at 12 or 30 μm and mounted on aluminum stubs. Prior to embedding, *Cliona* was placed in 5% EDTA for 24 hr to remove the coral skeleton. After sectioning the wax was removed by placing the stubs in toluene for 15 minutes. The stubs were then gold coated and viewed in a field emission scanning electron microscope.

3.3.3.2 Histology

For wax embedding, sponges were cut into 1cm^3 pieces and fixed in 4% paraformaldehyde in filtered seawater for 24 h. Pieces were rinsed in phosphate buffered

saline (PBS), dehydrated to 70% ethanol and transported back to the laboratory in Edmonton, Alberta where they were processed by Nhu Trieu in the Department of Biological Sciences Microscopy Unit. Preparations were desilicified in 4% HF in 70% ethanol at RT for 24-72 h until the spicules were dissolved. *Cliona* was further placed in Cal-Ex Decalcifier (Fisher Scientific) for 24 h to remove the coral skeleton. Sponges were embedded in paraffin wax and sectioned at 5 μm for *Tethya,* 12 μm for *Haliclona* and *Cliona*, and 30 μm for *Neopetrosia* and *Callyspongia,* with section width dependent on the density of the tissue. Wax was removed with toluene and slides were hydrated and stained using Masson's trichrome stain in Hematoxylin for 1 min 20 sec, Ponceau acid fuchsin for 2 min, and Aniline Blue for 3 min. The slides were then dehydrated to 100% ethanol and cleared in Toluene prior to mounting with Permount. Slides were viewed using a Zeiss Axioskop2 Plus and captured with a QiCam using Northern Eclipse v.7 software (Empix Imaging Inc., Mississauga, ON, Canada).

3.3.3.3 Measurements of the canal system

The approach here was to replicate the methods used by Reiswig (1975a) to compute the dimensions through each portion of the aquiferous system to determine resistance. Dimensions of the aquiferous canal system were measured from both SEM and histological images using ImageJ (v. 1.43r; NIH). Care was taken to select regions of the canal system that were not contracted by looking at the tissue surrounding the canal system. Because pieces imaged by SEM were smaller than one millimeter, SEM images showed the smaller canals but did not capture larger canals; therefore dimensions and path lengths of larger canals were obtained from histological sections. It was not always possible to identify incurrent vs. excurrent canals; in these instances it was assumed that the dimensions and path length were the same between excurrent and incurrent canals following Riisgård et al. (1993). Choanocyte density was also calculated from histological sections. The cross-sectional area of each region of the aquiferous system was calculated for a 100 mm^3 (100 μl) piece after Reiswig (1975a) and Leys et al. (2011). Due to differences in the shape of the sponge body, the dimensions for this 100 μl piece differed for each species. For *Neopetrosia, Haliclona, Tethya* and *Cliona* inhalant surface was 4.5 x

4.5 mm^2 and the wall 5 mm thick. The body wall of *Callyspongia,* however, is only 3 mm thick and therefore a larger inhalant surface was used (5.77 x 5.77 mm^2) to generate the same 100 µl volume for the piece.

Sponge volumes and surface area for each of the species were calculated by measuring the dimensions of the sponge from images taken of whole animals *in situ*, and using ImageJ (v. 1.43r; NIH). Most sponges have irregular shapes, therefore volumes and surface areas were estimated by selecting a more regular shape that the sponge resembles: *Callyspongia* most resembles a cylinder, *Cliona* and *Haliclona* an ellipsoid, and *Tethya* a sphere. When there was more than one osculum per sponge, sponge volume was calculated and divided by the number of oscula to get sponge volume/osculum. The ratio of sponge to coral skeleton in *Cliona* was estimated by dissolving the coral skeleton using 5% EDTA for a small piece (~ 2 cm^3, or 4.5 g in weight) and scaling up to the whole specimen using the relative immersed volumes. *Neopetrosia* is highly irregular, therefore a combination of triangles was used to estimate volume and surface area.

3.3.3.4 Estimating resistance through the canal system

The velocity of water through each region of the aquiferous canal system, u_i, was calculated using the estimated cross-sectional areas for each part of the sponge and known excurrent velocity from the osculum (Reiswig, 1975a):

$$u_i = \frac{u_{ex} A_{osc}}{A_i} \quad (1)$$

where A_i is the cross sectional area of the region (see section 3.3.3.3), A_{osc} is the cross sectional area of the osculum, and u_{ex} is the measured excurrent velocity from the osculum (see section 3.3.2.1).

Two separate approaches were used to estimate the resistance through the canal system of sponges. The first uses a different equation to model each region of the canal system based on the characteristics of the region (an approach summarized by Riisgård and Larsen, 1995). The different equations in this first approach reflect the estimated different architectures of different regions of the sponge. The second approach uses only one equation for the whole canal system (Leys et al, 2011). This approach assumes that

different equations do not capture the accurate differences between regions and therefore one equation is more straightforward and just as accurate.

Following the approach by Riisgård and colleagues (Jørgensen et al., 1986; 1988; Riisgård, 1988; 1989; Riisgård et al., 1993; Riisgård and Larsen, 1995), the hydraulic head loss through each region of the canal system H_i may be due to apertures, frictional resistance in canals, or pressure drop across lattice nets. Most flows in biological systems occur at low Reynolds numbers $R_e = ud/v$, where d = diameter of tube or aperture, u = is mean velocity, and v = is kinematic viscosity. As water flows from a large diameter canal into a smaller one, the flow becomes fully developed after a length of about $L_d \approx 0.1dR_e$. Flow in the sponge canals is at low R_e and is fully developed. Head loss for ostia, prosopyles, and apopyles was calculated using equation 15 from Riisgård and Larsen (1995) for creeping flow through a circular aperture with diameter *d* (equation originally from Happel and Brenner, 1983):

$$\Delta H_i = 6\pi v u_{i-l}/gd \tag{2}$$

where v = kinematic viscosity; u_{i-l} = mean velocity of the flow upstream of the structure; g = the acceleration of gravity and d is the diameter of the circular aperture. Head loss at the canals was calculated using a rendition of the Hagen-Poiseuille equation for fully developed laminar flow in a circular tube of length *L* and radius *r,* which is equation 19 in Riisgård and Larsen (1995) (originally in Fox et al., 1998):

$$\Delta H_i = 8v u_{i-l} L/gr^2 \tag{3}$$

where *L* is the length of the canal, and r is the radius of the canal. For the subdermal space, which is a region below the dermal membrane, head loss was calculated using equation 21 in Riisgård and Larsen (1995) for flow between parallel plates spaced *l* distance apart (originally in Walshaw and Jobson, 1962):

$$\Delta H_i = 12v u_{i-l} L/gl^2 \tag{4}$$

where L is the length of the subdermal space, and l is the width of the subdermal space. Head loss across the rectangular lattice mesh, equation 17 in Riisgård and Larsen (1995) and originally described by Silvester (1983), is calculated as:

$$\Delta H = K\nu u_{i-l}/gh \tag{5}$$

where $K = 8\pi/(1 - 2\ln\left(\frac{\pi d}{h_0}\right) + \left(\frac{\pi d}{h_0}\right)^2/6)$; $h_0 = h_1 h_2/\sqrt{(h_1^2 + h_2^2)}$; d = diameter of the cylindrical fiber and h are the dimensions of the mesh where: $h = h_1 h_2/(h_1 + h_2)$; h_1 = width of the mesh; and h_2 = length of the mesh. The contribution of head loss from the velocity of water leaving the sponge osculum can be estimated from the kinetic head loss, equation 22 in Riisgård and Larsen (1995), as:

$$\Delta H_i = u_{ex}^2/2g \tag{6}$$

where u_{ex} is the velocity of water leaving the osculum. The total head loss through the system is equal to the sum of the head losses at each region of the canal system.

As a comparison to the model by Riisgård and Larsen (1995), we also used the simplified model developed by Leys et al. (2011) which assumes head loss through each region of the aquiferous canal system can be calculated based on the Hagen-Poiseuille equation for fully developed laminar flow in a tube (Equation 3).

As the values used for both the density of seawater, ρ, and kinematic viscosity, ν, depend on the temperature of seawater, a temperature of 12°C was used for the temperate demosponges and a temperature of 30°C was used for the tropical demosponges. To estimate the overall pump efficiency (η), and therefore the cost of pumping, we used equation 25 from Riisgård and Larsen (1995):

$$\eta = \frac{P_p}{R_{tot}}, \tag{7}$$

Where R_{tot} is the total metabolic power expenditure (total measured respiration) and P_p is the pumping power expressed by the linear energy equation for steady, incompressible flow through a controlled volume, equation 24 in Riisgård and Larsen (1995):

$$\rho g \Delta H Q = P_p. \quad (8)$$

Here ρ is the density of seawater, g the acceleration of gravity, Q the volume flow rate through the system, and ΔH the total pressure drop, or head loss, through the system.

3.4 Results

3.4.1 Experimental work

3.4.1.1 Volume flow rates and oxygen removal

Mean excurrent velocity, volume flow rate, and oxygen removal for each species are provided in Table 3-2. *Cliona* had the fastest excurrent velocity; however, *Callyspongia* had the highest volume flow rate and oxygen removal due to its large osculum size. *Tethya* filtered the least volume of water per unit time and removed the least oxygen of all five species.

All sponges, irrespective of temperature, habitat or species, removed more oxygen when more water was filtered. (Figure 3-3; and see Appendix 2: Figure A2.1). Mean oxygen removal for one osculum from each species was positively correlated with the amount of water filtered (Spearman r = 0.843, $p < 0.0001$; Figure 3-3 A). For one individual of each species measured over a five minute period, oxygen removal also increased with volume filtered for all species except *Cliona* (*Neopetrosia* Spearman r = 0.813, $p<0.0001$, *Haliclona* Spearman r = 0.869, $p<0.0001$; *Tethya* Spearman r = 0.905, $p<0.0001$; *Cliona* Spearman r = -0.180, p = 0.169; and *Callyspongia* Spearman r = 0.734, $p<0.0001$; Figure 3-3B).

Table 3- 2| Mean excurrent velocity, volume flow rate, and oxygen removal from five species of demosponges. Both volume flow rate and oxygen removal are standardised by sponge volume (per mL sponge) and sponge weight (per gram dry weight, gDW).

Species	n	Excurrent Velocity (cm s^{-1})	Volume Flow Rate (L h^{-1})	Volume Flow Rate (L h^{-1} mL^{-1} sponge)	Volume Flow Rate (L h^{-1} gDW^{-1} sponge)	Oxygen removal (µmol L^{-1})	Oxygen removal (µmol h^{-1} mL^{-1} sponge)	Oxygen removal (µmol h^{-1} gDW^{-1} sponge)
Cliona delitrix	8	11.04 ± 0.54	175.04 ± 38.83	0.39 ± 0.02	4.33 ± 0.21	2.20 ± 1.04	0.83 ± 0.38	9.32 ± 4.29
Callyspongia vaginalis	10	5.93 ± 0.67	44.49 ± 7.29	1.13 ± 0.18	18.02 ± 2.95	2.63 ±0.53	3.12 ± 0.99	49.72 ± 15.87
Tethya californiana	9	1.95 ± 0.30	5.16 ± 0.73	0.09 ± 0.007	0.28 ± 0.02	2.71 ± 0.60	0.23 ± 0.04	0.71 ± 0.12
Haliclona mollis	10	3.04 ± 0.30	2.92 ± 0.47	0.13 ± 0.01	1.08 ± 0.11	2.32 ± 0.53	0.31 ± 0.08	2.53 ± 0.63
Neopetrosia problematica	6	1.37 ± 0.25	0.53 ± 0.11	0.28 ± 0.05	2.26 ± 0.41	1.35 ± 0.14	0.38 ± 0.07	3.08 ± 0.63

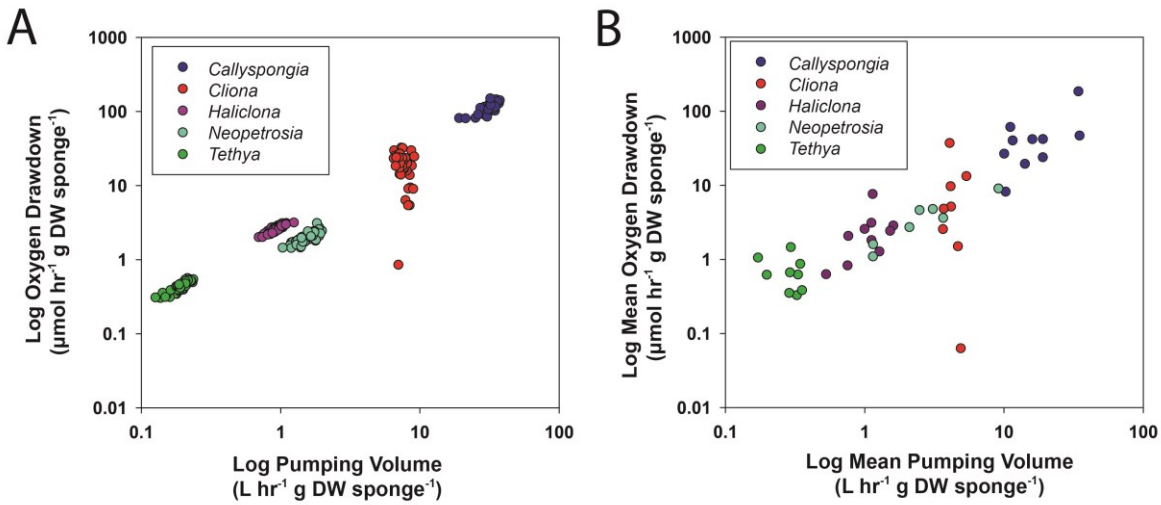


Figure 3- 3| Volume flow rates and oxygen removal

a) Sponge pumping volume (L hr^{-1} g DW $sponge^{-1}$) and oxygen removal (µmol hr^{-1} g DW $sponge^{-1}$) were recorded over a five-minute period and plotted for one individual of each of five species of demosponges. Oxygen removal increased as the pumping volume increased both within an individual and between species, with the exception of *Cliona* (red). This same trend can be seen in b) when the mean oxygen removal and pumping volume over a 5 minute period is plotted for multiple individuals of each species (*Callyspongia* n=11; *Cliona* n=8; *Haliclona* n=10; *Neopetrosia* n=7; *Tethya* n=8).

3.4.1.2 Effect of ambient flow on pumping rates

Tests to determine the effect of increased ambient flow showed that the excurrent velocity of *Callyspongia* did increase slightly with increasing ambient flow (Spearman $r = 0.141$, $p<0.001$, Figure 3-4 a,b), although over the course of the experiment the excurrent velocity decreased from about 9 cm/s to below 8 cm/s (Figure 3-4 a). When the ambient velocity went above 20 cm/s, the excurrent velocity decreased in the first and third run, but not in the second, and when the pump was turned off, the excurrent velocity slowly increased. Images of the osculum showed the diameter did not change during the experiment, therefore pumping volume would show the same relationship. Interestingly, oxygen removal ($\mu mol\ hr^{-1}$) was negatively correlated with ambient flow (Spearman $r = -0.221$, $p<0.0001$, Figure 3-4 a,c); however, oxygen removal at the beginning and end of the experiment was the same despite a decreased excurrent velocity.

The excurrent velocity of *Cliona* was positively correlated with ambient flow (Spearman $r = 0.485$, $p<0.0001$, Figure 3-5). However, the osculum constricted during the experiment with the area reducing from 4 cm^3 to less than 2 cm^3 with increasing ambient flow (Figure 3-5). As such, less water was filtered; therefore, while excurrent velocity increased with increasing ambient flow, the total volume filtered was less (Spearman $r = -0.407$, $p<0.0001$). Oxygen removal ($\mu mol\ hr^{-1}$) also decreased with increasing ambient flow (Spearman $r = -0.456$, $p<0.0001$).

3.4.2 Estimating the cost of filtration

Dimensions of each region of the aquiferous canal system for the five species of demosponges studied are given in Table 3-3. The path of water is illustrated in Figure 3-6. Briefly, water flows in through minute holes (ostia) in the dermal membrane (a three layered tissue) into a large subdermal space in four of the five species (except possibly for *Cliona*). From there, water enters into the largest incurrent canals which branch into smaller and smaller canals leading to the choanocyte chambers (Figure 3-7). *Callyspongia* is distinct from the other species in having water flow from the smallest incurrent canals into a lacunar space that holds all of the choanocyte chambers (Johnston and Hildemann, 1982).

Figure 3-4| Effect of ambient currents on *Callyspongia vaginalis.* (A) Top graph: Ambient (black) and excurrent (blue) velocity (cm/s) over time. Ambient velocity was increased every 300 s (5 min) using an underwater aquarium pump. Excurrent velocity remains at around 9 cm/s until about 1500 s when it decreases to below 8 cm/s as the ambient velocity increases above 20 cm/s. Bottom graph: Oxygen removal (μmol/L) over time. Oxygen is stable at about 4 μmol/L for the first 300s until the pump is turned on, when it decreases to around 2 μmol/L. **(B)** There is a slight positive correlation between ambient and excurrent velocity (Spearman $r = 0.141$, $p < 0.001$) **(C)** and a negative correlation between ambient velocity and oxygen removal (Spearman $r = -0.221$, $p < 0.0001$).

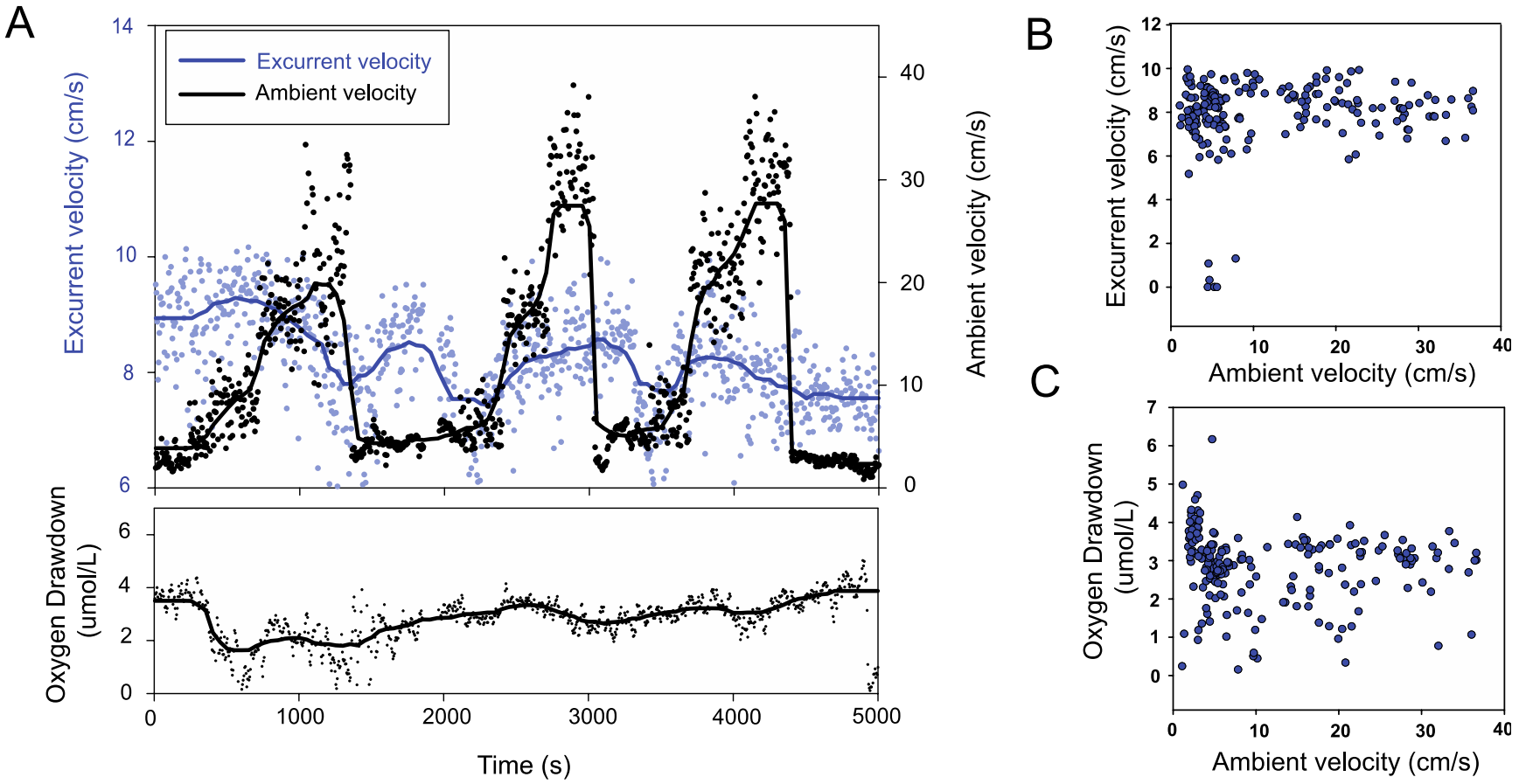


Figure 3- 4| Effect of ambient currents on *Callyspongia vaginalis*

Figure 3-5| Effect of ambient currents on *Cliona delitrix*. (A) Top graph: Ambient (black) and excurrent (grey dashed) velocity (cm/s) over time. Ambient velocity was increased every 300 s using an underwater aquarium pump. Excurrent velocity increases with increasing ambient velocity, although pumping volume (L/hr; red) calculated using osculum area (top images) does not increase. Middle graph: Oxygen removal (μmol/L) over time. Oxygen is highly variable when the pump is off, and then slowly decreases once the pump is turned on. Bottom graph: osculum area over time until the camera battery died after about one hour. **(B)** There is a positive correlation between ambient and excurrent velocity (Spearman $r = 0.485$, $p<0.0001$), **(C)** although a negative correlation between ambient velocity and pumping volume (Spearman $r = -0.407$, $p<0.0001$). **(D)** as well as ambient velocity and oxygen removal (Spearman $r = -456$, $p<0.0001$).

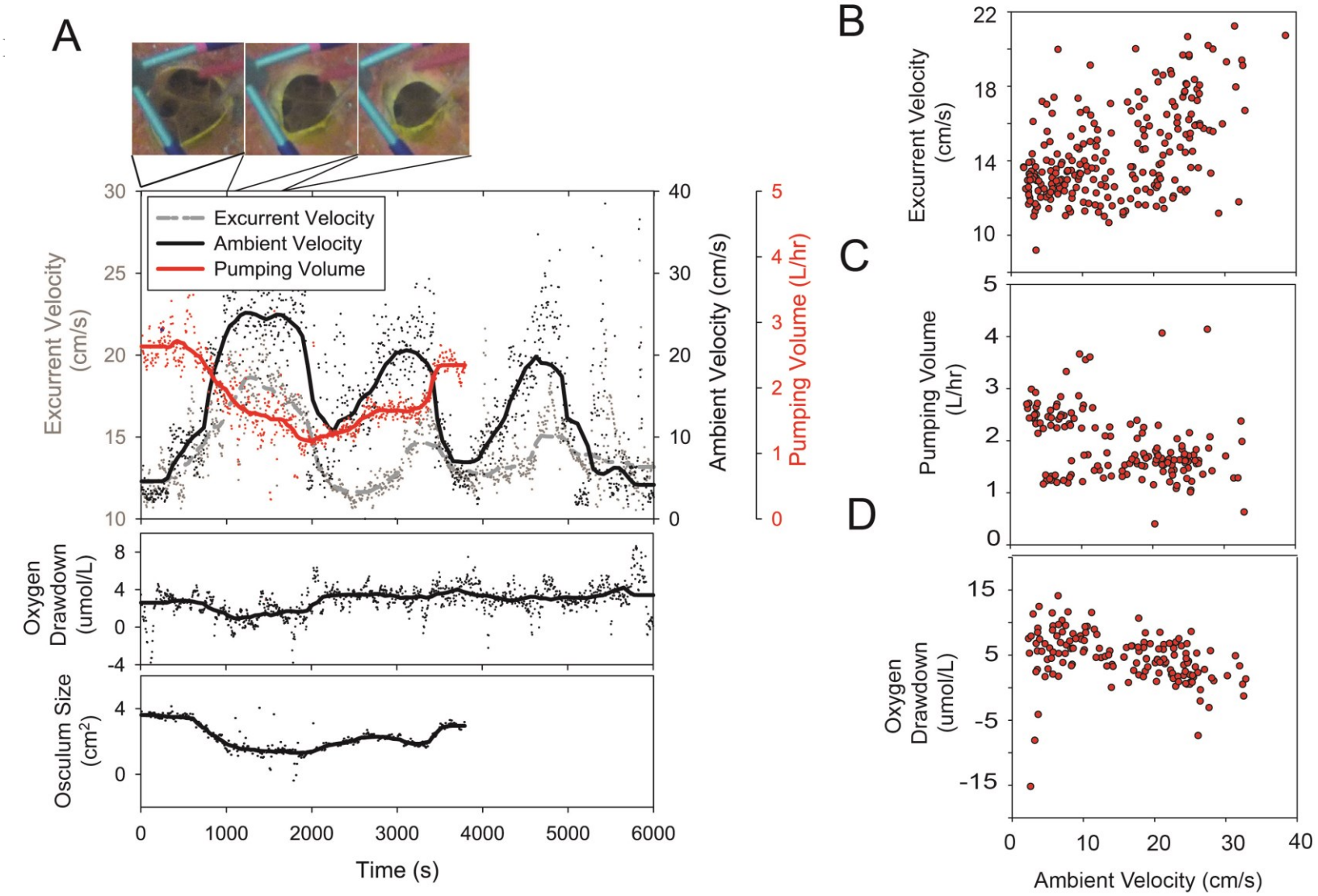

A
B
C
D
Excurrent Velocity
Ambient Velocity
Pumping Volume
Excurrent Velocity (cm/s)
Ambient Velocity (cm/s)
Pumping Volume (L/hr)
Oxygen Drawdown (umol/L)
Osculum Size (cm²)
Time (s)
Ambient Velocity (cm/s)

Table 3- 3| Dimensions of the aquiferous canal system in sponges

Numbers represent means of 3-75 measurements taken from 1-6 images from either scanning electron microscopy (SEM) or histology and light micrographs. Dimensions of collar slit, including the glycocalyx mesh on the collar, are in bold representing the filtration apparatus.

	Haliclona permollis*		***Aphrocallistes vastus****		***Neopetrosia problematica***		***Haliclona mollis***		***Tethya californiana***		***Callyspongia vaginalis***		***Cliona delitrix***	
Region of the aquiferous canal system	Diameter (µm)	Path length (µm)	Diameter (µm)	Path length (µm)	Diameter (µm)	Path length (µm)	Diameter (µm)	Path length (µm)	Diameter (µm)	Path length (µm)	Diameter (µm)	Path length (µm)	Diameter (µm)	Path length (µm)
Ostia	20.6		3.89	0.50	24.5	0.5	14.3	0.5	40.6	0.5	31.2	0.5	37.3	0.5
Subdermal space			90	82	242	86.1	95.0	50.6	177	105	168	131		
Large incurrent canal	50-340	3000	366	2000	383	2944	333	930	678	1118	407	923	294	1130
Medium incurrent canal				529	156	648	140	834	170	1118	195	725	144	969
Small incurrent canal				237	33	250	51.3	151.9	34.7	68.9	43.8	1	60.1	251
Prosopyles	1 to 5		2.15		3.60	0.5	2.37	0.5	4.52	0.5	1.61	0.5	2.59	0.5
Pre-collar space			2	2	1.3	2.6	5.7	3.6	1.6	2.2	0.5	2.6	0.69	2.19
Glycocalyx mesh			**0.045**	**0.010**	**0.095**		**0.166**		**0.059**		**0.052**		**0.118**	
Collar slit	**0.120**	**0.140**	**0.119**	**0.070**	**0.074**	**0.118**	**0.110**	**0.100**	**0.066**	**0.086**	**0.069**	**0.109**	**0.070**	**0.099**
Glycocalyx mesh			**0.045**	**0.010**										
Post-collar space			2	2	2.6	2.1	3.3	3.3	2.2	3.4	2.6	1.6	2.2	2.65
Chamber	30		56	56	23.3	23.3	28.5	28.5	21.1	21.1	19.7	19.7	16.0	16.0
Apopyle	14	1	26.4	2	16.0	0.5	14.1	0.5	0.90	0.5	5.97	0.5	4.23	0.5
Small excurrent canal				118	45.2	173	51.9	189	34.9	74	52.9	0.5	60.1	251
Medium excurrent canal					130	648	155	546	170	994	179	1096	144	969
Large excurrent canal	102-235	3000	405	2840	282	2944	411	930	678	994	339	1342	294	1130
Osculum	2300		44734	279000	3464	6676	5527	7420	8666	2184	16209	198410	22666	9983
Chambers per mm^3	12,000		1,876		9,792		2,684		14,403		14,358		35,175	
Collars per chamber	95		260		80		139		99		93		50	
Microvilli per collar	28		38		40		40		39		33		33	

* *Haliclona permollis* measurements taken from Reiswig (1975), with path lengths from Riisgard et al. (1993)
** *Aphrocallistes vastus* measurements taken from Leys et al. (2011)

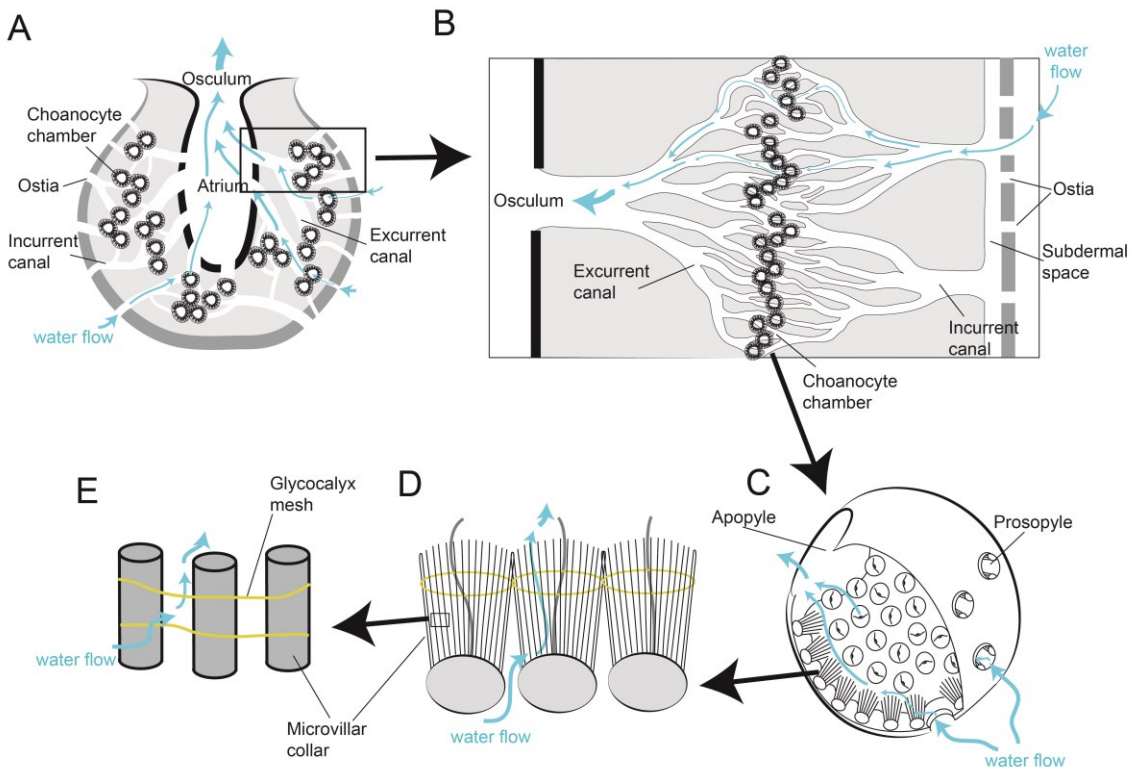


Figure 3- 6| Water flow through the aquiferous canal system of sponges.

Schematic drawings showing water flow through the aquiferous canal system of sponges. (A) Water enters through pores (ostia) on the sponge surface, into incurrent canals to the choanocyte chambers where the water is filtered, then out through the excurrent canals to the osculum. (B) There is a huge increase in cross-sectional area of the aquiferous system as the water enters the choanocyte chambers, which slows the water for filtration. The cross-sectional area then decreases as the water leaves the choanocyte chambers, jetting the water out through the osculum. (C) Water enters the choanocyte chamber through prosopyles and exits via the apopyle. (D) Glycocalyx (yellow) forms a gasket that connects all of the collars of choanocyte cells together, forcing the water through the microvilli of the choanocyte chambers. (E) Each microvillus is connected by a glycocalyx mesh (yellow), forcing the water through narrow rectangular openings.

In *Callyspongia,* water flows freely through the lacunar space before entering the choanocyte chambers through openings between choanocytes (Figure 3-8a). As there are no prosopyles in *Callyspongia*, openings between the choanocytes were considered to be prosopyles when estimating resistance through the canal system (Figure 3-8b). In the other four species, water enters the choanocyte chambers from the smallest incurrent canal through one or more prosopyles. At choanocyte chambers water moves through the collar microvilli. In *Haliclona* a set of cells forms a flat layer that, like a gasket, connects all collars in the chamber (Figure 3-8 c). In both *Neopetrosia* and *Callyspongia* a mucus glycocalyx mesh connected to each of the collars also serves as a gasket (Figure 3-8 b,d). Although a gasket has not been found yet in *Tethya* and *Cliona* (Figure 3-8 e,f), this sort of structure may be more common in demosponges than has previously been appreciated since those made from mucus glycocalyx are difficult to preserve. A glycocalyx mesh was found between the microvilli of the choanocyte cells in each species studied (Figure 3-8 b), but in the case of *Tethya* and *Cliona* it was only found in a few well-preserved choanocytes within a chamber. After passing through the glycocalyx mesh filter on the collar, the water flows into the chamber and from there, out of the apopyle (exit of the sponge choanocyte chamber). In *Tethya,* the apopyle consists of a sieve-plate (Figure 3-8 e); in others it is a circular aperture. From the apopyle the water enters small excurrent canals that merge into increasingly larger canals before flowing out of the osculum.

Cross-sectional area, velocity of water flow, and head loss for each region of the canal system based on Riisgård and colleagues (1995) model are given in Table 3-3. In each species, the cross-sectional area increases as the water enters the choanocyte chambers (Figure 3-9). Velocity through each region (u_i) was calculated from total cross-sectional area of each region (i), A_i, and excurrent (ex) velocity out of the sponge osculum, u_{ex}, using equation (1). The effective velocity u_i through the collar slit of the two warm-water species, *Callyspongia* and *Cliona*, was 0.011 mm/s and 0.010 mm/s respectively, which is 2-10 times higher than the effective velocity in the temperate species (*Neopetrosia* = 0.005 mm/s, *Haliclona* = 0.004 mm/s, and *Tethya* = 0.0011 mm/s), a difference resulting from the higher excurrent pumping rates of those species. The total head loss through the canal system – the sum of head loss through each region calculated using equations (1-6) – is also 5-38 times higher for the tropical species (Table 3-4).

Figure 3-7| Histological sections in five species of demosponges. Incurrent (ic) and excurrent (ec) canals in **(A)** *Neopetrosia* **(B)** *Haliclona* **(C)** *Tethya* **(D)** *Cliona* **(E)** and *Callyspongia.* Insets show choanocyte chambers. Scale bars: 1 mm; insets: 100 μm.

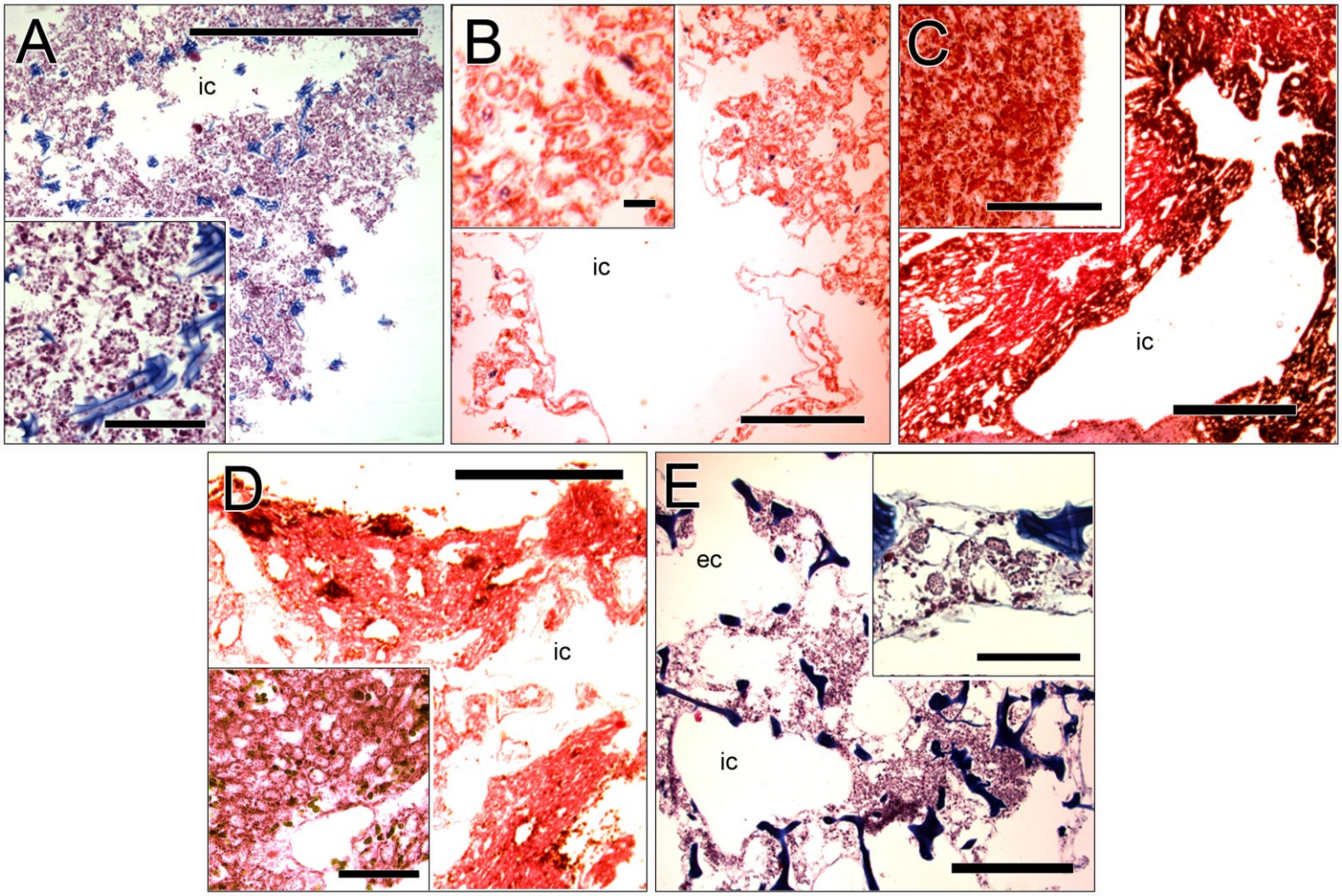


Figure 3- 7| Histological sections in five species of demosponges

Figure 3-8| Scanning electron micrographs of choanocyte chambers in five species of demosponges. (A,B) Choanocyte chambers (cc) of *Callyspongia* within the lacunar spaces (ls)**.** Each choanocyte cell (cho) has a collar of microvilli (mv) that are connected by glycocalyx mesh (gly). **(C)** Large choanocyte chamber in *Haliclona* showing the cellular gasket (g) that connects each choanocyte cell. Water exits the chamber via the large apopyle (apo). **(D)** A glycocalyx gasket (gly) connects the collars in *Neopetrosia.* **(E)** The apopyle in *Tethya* consists of a sieve plate (sp). **(F)** Smaller choanocyte chamber in *Cliona* with the flagella protruding from the apopyle.Scale bars **A,C,E** 10 μm **B,** 2 μm inset, 1 μm **D,** 1 μm **F,** 5 μm.

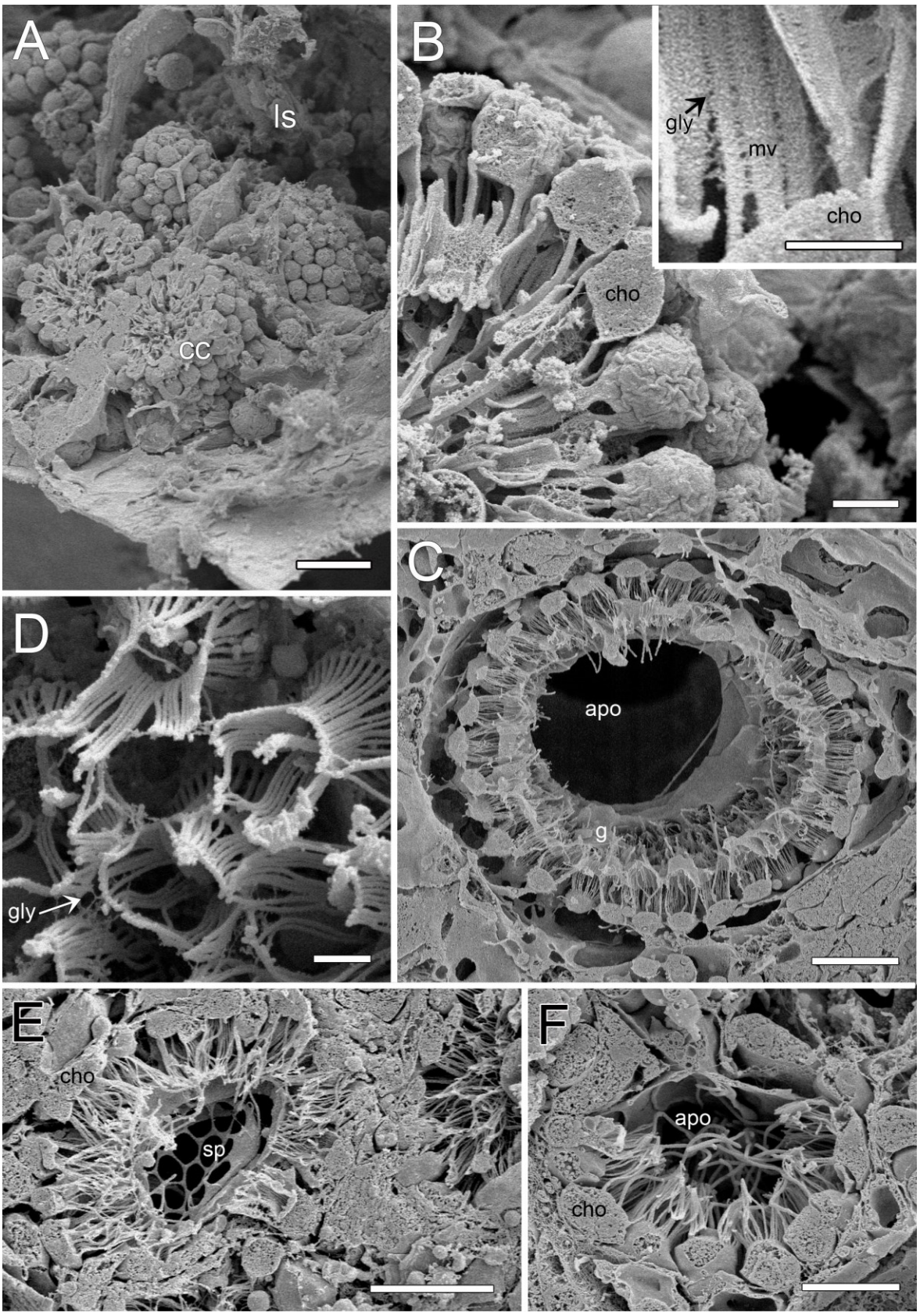


Figure 3- 8| Scanning electron micrographs of choanocyte chambers in five species of demosponges

Figure 3-9| Morphometric model of five species of demosponges. (A) Total cross-sectional area of the aquiferous canal systems from the inhalant surface to the osculum for five species of demosponges: *Haliclona mollis* (purple), *Neopetrosia problematica* (cyan), *Tethya californiana* (green), *Callyspongia vaginalis* (blue) and *Cliona delitrix* (red). **(B)** Estimated water velocity (solid lines) and relative head loss (dotted lines) through the aquiferous canal system as water travels from the inhalant surface to the osculum for the same five species of demosponges.

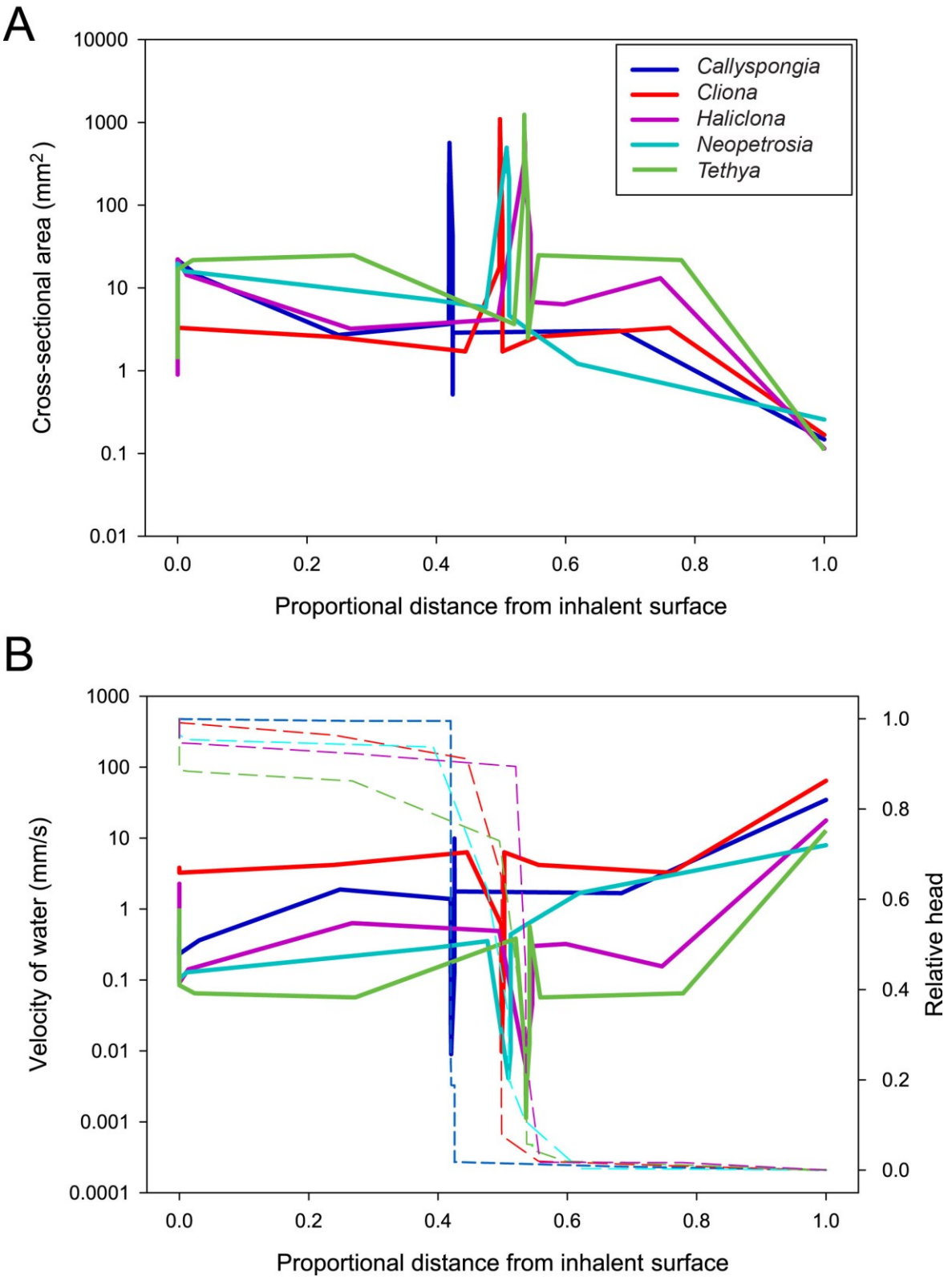


Figure 3- 9| Morphometric model of five species of demosponges

To determine the cost of filtration, the power required to pump water across the sponge, $P_{p,}$ was calculated using the total head loss, ΔH, and volume flow rate, $Q,$ and divided by the total measured oxygen removal R_{tot} using the conversion 1 μL O_2 hr^{-1} = 5.333 μW and the mean oxygen removal and volume flow rates reported in Table 3-2. Using the model by Riisgård and Larsen (1995) the estimates of the cost of filtration were quite variable, ranging from 0.97% for *Tethya* and 40.27% for *Cliona.* The simplified model developed by Leys et al (2011) gives the same relationship for cost of filtration between the five species of demosponge, although the range is slightly lower at 0.89% for *Tethya* and 31.9% for *Cliona.* It also estimates a higher cost of pumping for *Callyspongia* at 16.97% total respiration. Both of the warm-water species *Callyspongia* and *Cliona* had the highest cost of filtration regardless of the model used (Table 3-4).

3.5 Discussion

Our study aimed to assess the energetic cost of filtration in demosponges and to determine whether sponges reduce their cost of filtration by taking advantage of ambient currents. Using a suite of different approaches we have shown that the cost of pumping in demosponges is quite variable and depends on the volume of water pumped. Of the five species of demosponges studied, the tropical species *Cliona* and *Callyspongia* filtered the most water and extracted the most oxygen per gram of tissue, leading to higher estimates for the energetic cost of filtration than the three temperate demosponges *Neopetrosia, Haliclona,* and *Tethya*. This suggests that the cost of filtration for demosponges is higher than previously estimated and that reducing the volume of water filtered would reduce the cost of filtration in times of low food availability. Interestingly, we found that demosponges can control the water flow through their bodies by responding behaviourally to changes in ambient flow. Both *Cliona* and *Callyspongia* reduce the amount of water filtered at very high ambient velocities, which may be a mechanism to protect themselves from damage during storms.

Table 3-4| Morphometric model of the aquiferous system in five species of demosponges. Estimated total cross-sectional area for each region was measured from the dimensions listed in Table 4-3 as well the density of the structures as found in SEM and histological images. The velocity of water flow through each area u_∞ was calculated from cross-sectional area A_i and measured excurrent velocity u_{ex} out of the osculum using equation (4). Head loss H in each region was calculated using equations (3-6) from dimensions and velocity u_∞ of each region. Riisgård and Larson's (1995) model used a different equation of head loss for each region of the aquiferous canal system, whereas Leys et al's (2011) model used only equation (3). The sum of the head loss ΔH and measured volume flow rate are used to calculated the pumping power P_p using equation (2). The cost of pumping η (%) is then estimated using equation (1) from the pumping power P_p and the measured respiration rate R_{tot}. The collar slit is in bold, representing the filtration apparatus.

Table 3- 4| Morphometric model of the aquiferous canal system in five species of demosponges

	Neopetrosia problematica				***Haliclona mollis***				***Tethya californiana***				***Callyspongia vaginalis***				***Cliona delitrix***			
			Head loss, H (µm H_20)				Head loss, H (µm H_20)				Head loss, H (µm H_20)				Head loss, H (µm H_20)				Head loss, H (µm H_20)	
Region of the aquiferous canal system	Cross-sectional area, A_i (mm^2)	Velocity, u_i (mm/s)	Riisgard and Larson (1995)	Leys *et al* (2011)	Cross-sectional area, A_i (mm^2)	Velocity, u_i (mm/s)	Riisgard and Larson (1995)	Leys *et al* (2011)	Cross-sectional area, A_i (mm^2)	Velocity, u_i (mm/s)	Riisgard and Larson (1995)	Leys *et al* (2011)	Cross-sectional area, A_i (mm^2)	Velocity, u_i (mm/s)	Riisgard and Larson (1995)	Leys *et al* (2011)	Cross-sectional area, A_i (mm^2)	Velocity, u_i (mm/s)	Riisgard and Larson (1995)	Leys *et al* (2011)
Ostia	3.37	1.04	111	4	0.90	3.90	709	42	1.38	1.76	113	2	12.8	0.68	51	1	2.82	6.57	409	9
Subdermal space	19.7	0.18	56	7	22.2	0.16	239	96	16.7	0.14	46	26	21.8	0.40	10	12				
Large incurrent canal	15.9	0.22	16	16	14.4	0.24	6	6	21.7	0.11	2	2	14.1	0.62	9	9	3.31	5.60	338	338
Medium incurrent canal	7.21	0.49	26	26	3.21	1.09	45	45	24.8	0.10	19	19	2.70	3.25	47	47	2.57	7.22	1026	1026
Small incurrent canal	5.79	0.61	491	491	4.16	0.84	278	278	3.66	0.66	25	25	3.67	2.39	3	3	1.71	10.84	1969	1969
Prosopyles	494	0.007	438	103	346	0.010	922	330	172	0.014	380	71	55.2	0.159	3434	1806	17.7	1.04	9714	3186
Pre-collar space	255	0.014	49	49	775	0.005	5	5	504	0.005	55	55	170	0.051	6768	6768	264	0.070	19158	19158
Collar slit	**376**	**0.009**	**288**	**797**	**546**	**0.006**	**471**	**73**	**1237**	**0.0020**	**147**	**521**	**492**	**0.018**	**668**	**8202**	**1095**	**0.017**	**2300**	**1969**
Post-collar space	412	0.009	13	13	405	0.009	9	9	1077	0.0022	6	6	566	0.015	17	17	1019	0.018	37	37
Chamber	408	0.009	2	2	171	0.020	1	1	488	0.005	0	0	406	0.022	3	3	674	0.027	4	4
Apopyle	208	0.02	1	0	44.6	0.08	4	0	110	0.02	14	13	40.3	0.22	8	1	52.1	0.36	15	3
Small excurrent canal	4.66	0.75	6	6	6.79	0.52	24	24	2.47	0.98	6	6	0.52	16.9	0	0	1.71	10.84	97	97
Medium excurrent canal	3.47	1.01	128	128	6.33	0.55	52	52	24.8	0.10	149	149	2.87	3.05	2288	2288	2.57	7.22	1985	1985
Large excurrent canal	1.21	2.90	165	165	13.1	0.27	13	13	21.7	0.11	1	1	3.04	2.88	140	140	3.31	5.60	371	371
Osculum	0.26	13.66	10	33	0.12	30.44	47	33	0.11	21.95	25	3	0.15	59.33	179	176	0.17	110.41	622	8

Volume flow rate, Q (mL/min)	**9.0**		**48.6**		**82.1**		**742**		**2668**	
Respiration, R_{tot} (µW)	**87**		**790**		**1396**		**14218**		**42102**	
Head loss, ΔH (µm H_20)	**1799**	**1840**	**2825**	**1008**	**988**	**900**	**13626**	**19474**	**38043**	**30161**
Pumping Power, P_p (µW)	**3**	**3**	**23**	**8**	**14**	**12**	**1688**	**2412**	**16955**	**13442**
Cost of pumping, η (%)	**3.12**	**3.19**	**2.91**	**1.04**	**0.97**	**0.89**	**11.87**	**16.97**	**40.27**	**31.93**

3.5.1 The cost of pumping

Cost of filtration was estimated by determining the energy required to overcome the resistance through the aquiferous system. Although there are differences in gross morphology among the five species, we found similar morphology at the filter for each of the species studied. The cost of pumping was variable ranging from 0.97% for *Tethya* to 40.27% for *Cliona* of the total oxygen consumed (assuming oxygen consumed is used for all metabolism including filtration). Previous estimates on the energetic cost of pumping in demosponges also show a large range, from 0.4% for *Haliclona urceolus* (Riisgård et al., 1993) to 25% for *Negombata magnifica* (Hadas et al., 2008). Although this variability may indicate inconsistencies between the theoretical models used by Riisgård et al. (1993) and direct measurements done by Hadas et al. (2008), our results suggest that the variability in 'cost' may simply reflect differences in the volume of water pumped. Although our findings in Section 3.2 suggest that filter dimensions contribute a large amount to the cost of filtration (Table 3-1), the dimensions of the glycocalyx mesh are smaller in *Tethya* than *Cliona* and therefore do not account for the difference in costs. Rather, volume flow rates contribute mostly to differences in the energetic cost of filtration, with the two species found in the tropics having the largest volume flow rates resulting in the largest cost of filtration (Table 3-4). In addition, when individuals of each species filtered more water they also consumed more oxygen (Figure 3-3), suggesting that the energetic cost of pumping is variable within an individual depending on how much water is being pumped at any one time. This implies that sponges could save energy in times of low food availability by lowering the volume of water pumped.

That pumping more water would cost more energy in sponges is intuitive. How a sponge changes its pumping rate and volume filtered, however, is not known. One mechanism, in demosponges, may be to dilate the canals while maintaining excurrent velocity, increasing the volume of the canal system and therefore the volume filtered. The more water flowing through the canal system at any one time would increase the resistance and therefore also the energetic cost of pumping. Another mechanism could be to increase the rate of flagellar beating in the choanocyte chambers. This would increase the velocity of

water through the canal system and therefore increase the volume of water filtered as well as the energetic cost of pumping. Sponges likely control the rate of flagellar beating to save energy when the sponge contracts. We did not look at the energy spent on the flagellar beat nor the drag on the flagella when estimating the cost of pumping, which is a limitation in the model and probably makes our estimates more conservative in our cost of filtration.

It is important to consider why the two tropical species of demosponge had higher volume flow rates and therefore higher estimates for the cost of filtration in this study. Differences in pumping rates between species can be caused by structural differences in the sponge canal system (Reiswig, 1975a), microbial content (Weisz et al., 2008), and tissue density (Turon et al., 1997). In addition, Riisgård et al. (1993) found that volume flow rate in *Haliclona permollis* increased up to ten times with a change in temperature from 6°C to 15°C. The higher volume flow rate per gram weight in the two tropical species in this study therefore may be a result of the higher temperature of water and lower viscosity. However, due to the high energetic cost to pump more water it would only be adaptive to have such high volume flow rates if there is enough food in the water to support this.

Sponges are found in almost every marine and freshwater habitat and, with the exception of carnivorous sponges, all feed on ultraplankton and dissolved organic carbon. Increasing cross-sectional area through the canal system slows the velocity of water, enabling food capture either in the small incurrent canals or at the filter. Slight differences in filtration ability and size of plankton captured between different species of sponges are known (Reiswig, 1975b; Turon et al., 1997; Yahel et al., 2007). There is little information, however, on the relationship between filtration ability, diet, and microarchitecture of the aquiferous canal system. Among the five species studied here, slight variations in the architecture of the aquiferous canal system were found such as the lacunar space in *Callyspongia* and the sieve-plate apopyle of *Tethya.* We also found wide differences in the velocities of water measured out of the osculum, although similar velocities of water at the filter (Table 3-4). This suggests that despite wide differences in volume flow rates and adaptations to a variety of habitats and ecological niches, the canal system of sponges is designed to slow the velocity of water down to a certain speed that enables food capture at the filter. The small differences that were found in velocities at the filter may reflect small differences in preferred plankton size or filtration ability. The model predicted that both

tropical species *Callyspongia* and *Cliona* had faster velocities of water at the collar filter. In addition, *Callyspongia* lacks the small incurrent canals leading into the choanocyte chambers, suggesting that it does not rely on pinacocyte capture of plankton in the canals. The diet and filtration ability of these two species is unknown, although it would be interesting to determine the relationship between microarchitecture, velocity of water at the filter, and diet of the sponges.

3.5.2 Response to ambient currents

One way to reduce the cost of pumping in sponges would be to take advantage of ambient currents through passive flow. In both *Cliona* and *Callyspongia* excurrent volume flow rates were not correlated to the ambient currents, suggesting they do not use passive flow. Rather, both species decreased their volume flow rate when ambient velocities reached a certain level, which may be to reduce damage caused by high currents or sediments during storms. High velocities through the canal system may cause damage to some of the smaller spaces, including the fine glycocalyx mesh at the filter. In addition, storms can stir up sediment into the water column which has been shown to have a negative impact on sponge filtration and respiration (Gerrodette and Flechsig, 1979; Tompkins-MacDonald and Leys, 2008; Bannister et al., 2012).

Sponges have long been considered textbook examples of animals that can take advantage of current-induced flow (Bidder, 1923; Vogel, 1974; Vogel, 1977). In the 1970's Vogel used thermistor flow meters to record excurrent flow while increasing ambient currents, and found a strong correlation between excurrent and ambient velocities. His method, however, did not take into account changes in dimensions of the osculum. Moreover, Vogel only plotted points in time rather than the full time series. Therefore, the increased excurrent velocity recorded by Vogel (1977) in the species *Amphimedon viridis* (referred to as *Haliclona viridis*)*, Ircinia variabilis* (referred to as *Ircinia fasciculata*)*,* and *Aplysina fistularis* (referred to as *Verongia fistularis*), could also have been behavioural responses, as shown here in *Cliona delitrix.* In this study, although excurrent velocity in *Cliona* did increase with increasing ambient currents, this was due to the sponge osculum contracting resulting in a decrease in volume flow rate as ambient currents increased.

In addition to positive correlations between ambient velocity and oscular flow, there are reports in the literature of a negative correlation. When studying *in situ* pumping rates for the globose freshwater sponge *Baikalospongia bacillifera* in Lake Baikal, Savarese et al. (1997) noted that two individuals monitored over a diel cycle showed negative correlations between ambient and oscular velocities. In addition, they noted periodic cessation of pumping on the order of minutes to hours that did not correlate with ambient flow. Reiswig (1971) also found periodic oscular closures and therefore cessations of pumping with *Tethya crypta* that were negatively correlated to wave action (Figure 1-4). Here, we found both *Callyspongia* and *Cliona* reduced the volume of water filtered when ambient currents reached a certain threshold. Some demosponges therefore show a large amount of control of their excurrent volume flow rate despite fluctuations in the ambient water velocity.

3.5.3 General conclusions

The over-arching finding of this work is that demosponges control the water flow through their aquiferous systems. The architecture and design of the aquiferous canal system is such that the velocity of water slows at the filter to enable food capture, although each species accomplishes this in slightly different ways. Despite the broad similarity in the canal system architecture among demosponge species, the amount of water filtered by each (volume flow rate) varies considerably, resulting in different costs of pumping. For the three temperate demosponges, our estimates for the cost of pumping are comparable to that found by Riisgård et al. (1993) for what he termed the 'standard sponge'. However, the two tropical demosponges we studied had much higher volume flow rates with much higher cost of pumping; the cost of pumping for *Cliona* was more comparable to the cost of pumping found by both Leys et al. (2011) and Hadas et al. (2008). It is likely that habitat and ecological niche of sponges has led to adaptations in body form and physiology over time and together these play a large role in differences in cost of pumping.

We also found that demosponges respond to ambient currents, reducing the excurrent velocity and volume filtered as ambient flows increase. Previously, we have shown that demosponges have sensory cilia in their osculum, which allow them to sense and respond to changes in their environment (Chapter Two). Here, we show that

demosponges also respond to changes in ambient currents, possibly to reduce their cost of filtration or to reduce damage caused by high currents or sediment.

3.5 References

Bannister, R. J., Battershill, C. N. and de Nys, R. (2012). Suspended sediment grain size and mineralogy across the continental shelf of the Great Barrier Reef: impacts on the physiology of a coral reef sponge. *Continental Shelf Research* **32**, 86-95.

Best, B. A. (1988). Passive suspension feeding in a Sea Pen: Effects of ambient flow on volume flow rate and filtering efficiency. *Biological Bulletin* **175**, 332-342.

Bidder, G. P. (1923). The relation of the form of a sponge to its currents. *Quaterly Journal of Microscopical Sciences* **67**, 293-323.

Diaz, M. C. (2005). Common sponges from shallow marine habitats from Bocas del Toro Region, Panama. *Caribbean Journal of Science* **41**, 465-475.

Diaz, R. J. and Rosenberg, R. (2008). Spreading dead zones and consequences for marine ecosystems. *Science* **321**, 926-9.

Ellis, J., Cummings, V., Hewitt, J., Thrush, S. and Norkko, A. (2002). Determining effects of suspended sediment on condition of a suspension feeding bivalve (*Atrina zelandica*): results of a survey, a laboratory experiment and a field transplant experiment. *Journal of Experimental Marine Biology and Ecology* **267**, 147-174.

Fiala-Medioni, A. (1978). Filter-feeding ethology of benthic invertebrates (Ascidians). IV. Pumping rate, filtration rate, filtration efficiency. *Marine Biology* **48**, 243-249.

Flood, P. R. and Fiala-Medioni, A. (1981). Ultrastructure and histochemistry of the food trapping mucous film in benthic filter-feeders (Ascidians). *Acta Zoologica* **62**, 53-65.

Flood, P. R. and Fiala-Medioni, A. (1982). Structure of the mucous feeding filter of *Chaetopterus variopedatus*. *Marine Biology* **72**, 27-33.

Fox, R. W., McDonald, A. T. and Pritchard, P. J. (1998). Introduction to fluid mechanics, vol. 5, pp. 360: John Wiley & Sons New York.

Genin, A. and Karp, L. (1994). Effects of flow on competitive superiority in scleractinian corals. *Limnology and Oceanography* **39**, 913-924.

Gerrodette, T. and Flechsig, A. O. (1979). Sediment-induced reduction in the pumping rate of the tropical sponge *Verongia lacunosa*. *Marine Biology* **55**, 103-110.

Gili, J.-M. and Coma, R. (1998). Benthic suspension feeders: their paramount role in littoral marine food webs. *Trends in Ecology and Evolution* **13**, 316-321.

Griffiths, C. L. and King, J. A. (1979). Some relationships between size, food availability and energy balance in the ribbed mussel *Aulacomya ater*. *Marine Biology* **51**, 141-149.

Grove, M. W., Finelli, C. M., Wethey, D. S. and Woodin, S. A. (2000). The effects of symbiotic crabs on the pumping activity and growth rates of *Chaetopterus variopedatus*. *Journal of Experimental Marine Biology and Ecology* **246**, 31-52.

Hadas, E., Ilan, M. and Shpigel, M. (2008). Oxygen consumption by a coral reef sponge. *The Journal of Experimental Biology* **211**, 2185-90.

Hallegraeff, G. M. (1993). A review of harmful algal blooms and their apparent global increase. *Phycologia* **32**, 79-99.

Happel, J. and Brenner, H. (1983). Low Reynolds number hydrodynamics: with special applications to particulate media, The Hague, Netherlands. Springer.

Harris, P. and Shaw, G. (1984). Intermediate filaments, microtubules and microfilaments in epidermis of sea urchin tube foot. *Cell Tissue Research* **236**, 27-33.

Johnston, I. S. and Hildemann, W. H. (1982). Cellular organization in the marine demosponge *Callyspongia diffusa*. *Marine Biology* **67**, 1-7.

Jones, H. D., Richards, O. G. and Southern, T. A. (1992). Gill dimensions, water pumping rate and body size in the mussel *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology* **155**, 213-237.

Jørgensen, C. B. (1955). Quantitative aspects of filter feeding in invertebrates. *Biological Reviews* **30**, 391-453.

Jørgensen, C. B. (1975). Comparative physiology of suspension feeding. *Annual Reviews in Physiology* **37**, 57-79.

Jørgensen, C. B., Famme, O., Kristensen, H. S., Larsen, P. S., Møhlenberg, F. and Riisgård, H. U. (1986). The bivalve pump. *Marine Ecology Progress Series* **34**, 69-77.

Jørgensen, C. B., Larsen, P. S., Møhlenberg, F. and Riisgård, H. U. (1988). The mussel pump: properties and modelling. *Marine Ecology Progress Series* **45**, 205-218.

Knott, N. A., Davis, A. R. and Buttermer, W. A. (2004). Passive flow through an unstalked intertidal ascidian: Orientation and morphology enhance suspension feeding in *Pyura stolonifera*. *Biological Bulletin* **207**, 217-224.

Labarbera, M. (1977). Brachiopod orientation to water movement. 1. Theory, laboratory behaviour, and field orientations. *Paleobiology* **3**, 270-287.

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.

Mah, J. L., Christensen-Dalsgaard, K. K. and Leys, S. P. (2014). Choanoflagellate and choanocyte collar-flagellar systems and the assumption of homology. *Evolution & Development* **16**, 25-37.

Murdock, G., R. and Vogel, S. (1978). Hydrodynamic induction of water flow through a keyhole limpet (Gastropoda, Fissurellidae). *Comparative Biochemistry and Physiology. Part A: Comparative Physiology* **61A**, 227-231.

Newell, R. and Branch, G. (1980). The influence of temperature on the maintenance of metabolic energy balance in marine invertebrates. *Advances in Marine Biology* **17**, 329-396.

Puig, P., Canals, M., Company, J. B., Martin, J., Amblas, D., Lastras, G. and Palanques, A. (2012). Ploughing the deep sea floor. *Nature* **489**, 286-9.

Reiswig, H. M. (1971). *In situ* pumping activities of tropical Demospongiae. *Marine Biology* **9**, 38-50.

Reiswig, H. M. (1975a). The aquiferous systems of three marine Demospongiae. *Journal of Morphology* **145**, 493-502.

Reiswig, H. M. (1975b). Bacteria as food for temperate-water marine sponges. *Canadian Journal of Zoology* **53**, 582-589.

Riisgård, H. U. (1988). The ascidian pump: properties and energy cost. *Marine Ecology Progress Series* **47**, 129-134.

Riisgård, H. U. (1989). Properties and energy cost of the muscular piston pump in the suspension feeding polychate *Chaetopterus variopedatus*. *Marine Ecology Progress Series* **56**, 157-168.

Riisgård, H. U., Egede, P. P. and Barreiro Saavedra, I. (2011). Feeding behaviour of the mussel, *Mytilus edulis*: new observations, with a minireview of current knowledge. *Journal of Marine Biology* **2011**, 1-13.

Riisgård, H. U. and Larsen, P. S. (1995). Filter-feeding in marine macro-invertebrates: pump characteristics, modelling, and energy cost. *Biological Reviews* **70**, 67-106.

Riisgård, H. U., Thomassen, S., Jakobsen, H., Weeks, J. M. and Larsen, P. S. (1993). Suspension feeding in marine sponges *Halichondria panicea* and *Haliclona urceolus:* effects of temperature on filtration rate and energy cost of pumping. *Marine Ecology Progress Series* **96**, 177-188.

Savarese, M., Patterson, M. R., Chernykh, V. I. and Fialkov, V. A. (1997). Trophic effects of sponge feeding within Lake Baikal's littoral zone. 1. *In situ* pumping rates. *Limnology and Oceanography* **42**, 171-178.

Shiino, Y. (2009). Passive feeding in spiriferide brachiopods: an experimental approach using models of Devonian *Paraspirifer* and *Cyrtospirifer*. *Lethaia* **43**, 223-231.

Silvester, N. R. (1983). Some hydrodynamic aspects of filter feeding with rectangular-mesh nets. *Journal of Theoretical Biology* **103**, 265-186.

Thompson, R. J. and Bayne, B. L. (1972). Active metabolism associated with feeding in the mussel *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology* **9**, 111-124.

Tompkins-MacDonald, G. J. and Leys, S. P. (2008). Glass sponges arrest pumping in response to sediment: implications for the physiology of the hexactinellid conduction system. *Marine Biology* **154**, 973-984.

Trager, G. C., Hwang, J.-S. and Strickler, J. R. (1990). Barnacle suspension-feeding in variable flow. *Marine Biology* **105**, 117-127.

Turon, X., Galera, J. and Uriz, M. J. (1997). Clearance rates and aquiferous systems in two sponges with contrasting life-history strategies. *The Journal of Experimental Zoology* **278**, 22-36.

Vogel, S. (1974). Current-induced flow through the sponge, *Halichondria*. *Biological Bulletin* **147**, 443-456.

Vogel, S. (1977). Current-induced flow through living sponges in nature. *Ecology* **74**, 2069-2071.

von Dassow, M. (2005). Flow and conduit formation in the external fluid-transport system of a suspension feeder. *The Journal of Experimental Biology* **208**, 2931-8.

Walshaw, A. C. and Jobson, D. A. (1962). Mechanics of fluids, pp. 183: Longmans.

Weisz, J. B., Lindquist, N. and Martens, C. S. (2008). Do associated microbial abundances impact marine demosponge pumping rates and tissue densities? *Oecologia* **155**, 367-76.

Yahel, G., Marie, D. and Genin, A. (2005). InEx - a direct *in situ* method to measure filtration rates, nutrition, and metabolism of active suspension feeders. *Limnology and Oceanography: Methods* **3**, 46-58.

Yahel, G., Whitney, F., Reiswig, H. M., Eerkes-Medrano, D. I. and Leys, S. P. (2007). *In situ* feeding and metabolism of glass sponges (Hexactinellida, Porifera), studied in a deep temperate fjord with a remotely operated submersible. *Limnology and Oceanography* **52**, 428.

Young, C. M. and Braithwaite, L. F. (1980). Orientation and current-induced flow in the stalked ascidian *Styela montereyensis*. *Biological Bulletin* **159**, 428-440.

Chapter Four

A general discussion on filter feeding in sponges

4.1 Overview

Sponges (Porifera) are sessile suspension feeders that actively pump water through their canal system bringing in food and oxygen and excreting metabolic wastes. This suspension feeding has a major impact on ecosystems, shaping planktonic communities as well as coupling nutrients between the water column and the benthos (reviewed in Gili and Coma, 1998). Recently, sponges have been rapidly disappearing in some marine habitats (Wulff, 2006). The reason for this decline is still not known, although episodic changes in salinity, temperature, or sedimentation are a possible cause. Increasingly, sedimentation from resource exploitation such as oil exploration or fishing trawls is having severe impacts on benthic suspension feeders including sponges, which are sensitive to materials that can clog their filtration system (Ellis et al., 2002; Bannister et al., 2012; Puig et al., 2012). Before we can understand the impacts of additional stresses in the environment, however, we must first answer some important questions on sponge physiology and behaviour.

Some demosponges have been shown to deal with short pulses of high sediment by carrying out slow rhythmic contractions that help to clear out debris (Nickel, 2004; Elliott and Leys, 2007; Ellwanger et al., 2007). It is still not understood, however, how sponges detect the increased sediment and coordinate a response without the use of nerves. High suspended sediment loads have also been linked to reduced pumping activity (Gerrodette and Flechsig, 1979; Tompkins-MacDonald and Leys, 2008) and increased metabolic demand (Bannister et al., 2012), which is thought to be a result of clogging of the filtration apparatus. Yet, the amount of energy required for a sponge to filter water under normal conditions is still not known. Thus, to further understand the impacts of additional stresses such as sediment, we must first understand the energetic constraints that might affect sponges. In addition, sessile filter feeders depend on water currents to bring in food and

carry wastes away, yet increased water currents during storms usually bring in increased suspended sediment loads. The same stimulus – water velocity – can therefore be either beneficial or damaging to a sponge, and it is not yet understood how sponges detect and respond to a range of ambient current speeds.

This thesis examines how sponges control water flow during filter feeding. Chapter Two reveals the presence of sensory cilia in the water canal system and suggests that the cilia are involved in responding to stimuli and coordinating behaviour in the sponge. This thesis also assesses the amount of energy that it takes the sponge to filter water for food. Chapter Three examines the energetic cost of filter feeding and suggests that the cost of pumping is quite variable both within and between species, depending on the volume of water pumped. In addition, Chapter Three shows that demosponges control the amount of water flow through their canal system by responding behaviourally to increased ambient currents. Here, in Chapter Four, I discuss several new questions and areas for future research on filter feeding and behaviour in demosponges.

4.2 Sponges respond to their environment using sensory cilia

Sponges typically respond to stimuli within minutes to hours, compared to the milliseconds that our nerves and muscles effect a response. Though it has long been known that sponges do not possess nerve cells or nervous tissue (Jones, 1962; Pavans de Ceccatty, 1974), until now it has largely been unknown how sponges detect and respond to their external surroundings. In Chapter Two I showed the presence of short, non-motile sensory cilia in the excurrent canal system of six species of demosponge and one species of glass sponge. These cilia closely resemble the primary cilia that function as sensory organelles found in most cells in the vertebrate body and in many cells in invertebrates (Praetorius and Spring, 2005). I have suggested that these cilia detect changes in water flow or pressure in the aquiferous canal system of the sponge and coordinate a response in the form of whole body contractions.

These sensory cilia have eluded detection by scientists until now, mostly because of the difficulty in preserving them during fixation. In addition, they are very small (4-6 μm in length) and found in localized regions of the sponge, therefore one must be looking

specifically at the endopinacocyte cells of the osculum or regions of the excurrent canal system to observe them. Now that these cilia have been observed, it would be important for other researchers to look for the cilia in a range of species to determine just how widespread the cilia are among sponges. It would also be important to understand exactly where the cilia are located in each species of sponge to further understand the role they may play in detecting environmental signals and controlling behaviours.

In Chapter Two I used chemicals commonly used to block channels on the ciliary membrane and which inhibit sensory function in other organisms (Gale et al., 2001; Praetorius and Spring, 2001). These drugs are thought to block TRPP2 (PC2) channels on the ciliary membrane, with TRP channels responsible for almost all forms of sensation in eukaryotic cells (Nauli et al., 2003; Praetorius and Spring, 2003; Praetorius and Spring, 2005; Singla and Reiter, 2006; Fujiu et al., 2011). TRP channels have not previously been known in sponges; here, we showed the presence of *pkd2* sequence in transcriptomes of sponges. Developing a TRP channel antibody for the sponge would be a powerful tool to determine where sensory cells are located in the sponge body, although this would not be an easy task. It has previously been done in *Chlamydomonas* to determine the location of a variety of TRP channels along the length of the flagella (Fujiu et al., 2011), although a good sequence is necessary to develop a proper antibody. Another important area for further research in the sponge would be to link Ca^{2+} signalling, L-glutamate, and channels on the ciliary membrane in order to further understand the role of these cilia in triggering responses in the sponge. Previously, influential work on Ca^{2+} signalling in kidney cell primary cilia has been done using fluorescent markers for calcium (Praetorius and Spring, 2001), and a similar study in sponges would be very informative.

A common misconception about sponges is their lack of responsiveness, despite many studies showing that sponges have whole body behaviours and respond to stimuli (for example see Reiswig, 1971; Weissenfels, 1984; Nickel, 2004; Elliott and Leys, 2007). While carrying out experiments in both Chapters Two and Three of this thesis I was surprised at how sensitive sponges are – sometimes just the vibrations of the computer would be enough to trigger the sneeze behaviour (described in Ch 2). More research is required to explore this sensitivity and the range of behaviours that sponges exhibit. Underwater time-lapse photography is a powerful tool that can help us to visualize and

study these behaviours. Technological advances have allowed underwater cameras to be set up year-round, paired with instruments that measure abiotic factors such as the North East Pacific Time-series Underwater Networked Experiment (NEPTUNE), part of Ocean Networks Canada. This tool enables researchers to study long-term behaviours and phenomena that would previously be missed with SCUBA, snorkelling, or traditional ship-based exploration alone. It also enables sponge behaviours to be observed *in situ*, and by pairing the responses with abiotic factors it will help to understand how factors such as temperature, salinity, pressure, suspended sediment load, or current velocity may play a role in behaviour. Since contractions of the canal system are linked to changes in volume of water pumped (Reiswig, 1971), understanding the factors that may influence behaviour may help to understand the stresses that impact filter feeding in sponges.

4.3 Demosponges control water flow through their bodies

It has long been recognized that sponges show considerable morphological plasticity (Bidder, 1923; Warburton, 1960; Palumbi, 1986) and behaviour (Reiswig, 1971; Vogel, 1974; Vogel, 1977) to different flow conditions. However, it is still not fully understood how different flow conditions affect pumping volume in the sponge. The results in Chapter Three suggest that higher ambient flow rates actually reduce the volume of water pumped, which is contrary to previous studies suggesting sponges use current-induced flow (Vogel, 1974; Vogel, 1977; Leys et al., 2011). Other studies have also found that higher ambient flow can be correlated with reduced pumping activity in the demosponges *Tethya crypta* (Reiswig, 1971) and *Baikalospongia bacilifera* (Savarese et al., 1997). This suggests that sponges are not just passive conduits for water and have a lot more control over the water flow through their bodies than previously thought. The ambient current speeds we used in Chapter Three were higher than the current speeds the sponges would naturally experience, except perhaps during storms. In addition, due to logistics and time, we were only able to study one individual for each species properly. More replicates are therefore required to determine the relationship between ambient velocity and pumping volume.

Sponges do rely on at least some ambient water flow to bring food in and carry wastes away. Yet our results showed that high ambient currents caused sponges to reduce their pumping volume in at least some species, suggesting intermediate ambient flow conditions would result in the maximum amount of water that sponges process. This is in line with studies for other suspension feeders that have found rates of particle capture and growth are highest at an intermediate ambient flow speed (Best, 1988; Shimeta and Jumars, 1991; Eckman and Duggins, 1993). During our *in situ* experiments on *Cliona,* it was evident that the sponge responded favourably to a small increase in ambient flow. At the beginning of one experiment, we arrived at the study location to find that the sponge oscula had been eaten by fish and the sponge was fully contracted. We decided to direct flow over the sponge from a pump we had brought while setting up the instruments, and found that over the course of about one hour the sponge relaxed and resumed pumping, though not as strongly as the previous day. We then continued to use this technique on contracted sponges prior to beginning our experiments. While maintaining sponges in the laboratory in Bamfield it was also determined that to obtain maximal pumping volume the inflow to the tanks needed to be on high flow with the hoses directed at the sponges. Not all sponges, however, seem to respond to ambient flow conditions. Reiswig (1971) found that the species *Mycale sp.* did not alter its pumping volume despite changes in wave action, and predicted that species with thin walls and low pumping velocities would show little to no changes in pumping activity over time. Some flow is therefore required to obtain maximal pumping in many demosponges, though there is still much to learn about the effect of ambient currents on sponge pumping volume.

The amount of water that a sponge processes is directly proportional to the amount of food that it obtains. So why do some sponges reduce their pumping volume in high ambient flow conditions? In suspension feeders that use external filters to capture food particles, high ambient flows can cause deformation and damage to the filter (Best, 1988; Shimeta and Jumars, 1991). For sponges, however, the filter is inside the sponge body and thus does not come in direct contact with ambient flow. The morphometric model used in Chapter Three however suggests that increasing the velocity of water out of the osculum without changing the canal dimensions would also increase the velocity at the filter. This suggests that if the sponge increases pumping volume with increasing ambient flow rates to

take advantage of higher food concentrations, the velocity at the filter will also increase and may reach a threshold in which damage to the filter might occur. Therefore, to reduce damage, sponges may control the velocity of water at the filter by contracting their canals. More studies are required to understand the mechanisms used to alter pumping volumes under a range of ambient flow conditions.

4.4 It is energetically expensive to filter water

4.4.1 Optimal foraging with respect to habitat

Sessile filter feeders are unable to move to search out food and instead rely on water currents to bring food in as well as carry wastes away. To optimize their net rate of energy gain, sessile suspension feeders may alter their ingestion rates to feed more when food is more abundant (Taghon, 1981). This also suggests that suspension feeders will have evolved optimal levels of filtration that are fine-tuned to their habitat, and that they will alter their filtration rate based on food availability, seasonality, wave action, suspended sediment loads, temperature, and predation. Chapter Three reports on experiments which suggest that for five species of demosponge in both temperate and tropical habitats, pumping volumes do differ within an individual, between individuals of the same species, and between each of the five species. Pumping rates among sponges are therefore quite variable, likely as a mechanism to increase net energy gain.

Why do sponges not continuously pump high volumes of water to obtain more food? Jørgensen (1966) suggested that filter feeding is inexpensive to allow for continuous rates of feeding. However, my results in Chapter Three suggest that there is a trade-off to pumping more water because it costs more energy to do so. There would therefore be an optimal level of filtration that is dependent on the sponge's environment. Although sponges do selectively feed on certain particles within the water column (for example see Yahel et al, 2007), they cannot move to search out more or better food, suggesting that during low food conditions sponges could reduce their pumping rates to save energy.

4.4.2 Amount of oxygen consumed by sponges

The results of this thesis suggest that it is energetically expensive for sponges to pump large volumes of water and that sponges that pump more water also consume more oxygen. But how do the rates of oxygen consumption found in this thesis compare to those reported for other sponges? Previously, oxygen consumption for sponges has been found to range from 7.42 μmol O_2 h^{-1} g_{DW}^{-1} for *Tethya crypta* to 46.58 μmol O_2 h^{-1} g_{DW}^{-1} for *Mycale sp* (Reiswig, 1974). In Chapter Three, I reported oxygen consumption values that ranged from 0.71 μmol O_2 h^{-1} g_{DW}^{-1} for *Tethya californiana* to 49.72 μmol O_2 h^{-1} g_{DW}^{-1} for *Callyspongia vaginalis.* In fact, each of the three temperate demosponges *Tethya californiana, Haliclona mollis,* and *Neopetrosia problematica* had oxygen consumption values less than 3 μmol O_2 h^{-1} g_{DW}^{-1}, which is much lower than the known range of oxygen consumption for other sponges. Although this low oxygen consumption can be attributed to the low pumping volumes recorded for each of the species, the reason for the low pumping volumes is not certain. One reason may be the method of measurement. The small osculum sizes of the temperate species may have led to conservative estimates in excurrent velocity when using an acoustic Doppler velocimeter (ADV). The sampling volume of the ADV used in our experiments measures the velocity of water in a 6mm diameter, which is larger than some of the oscula measured. Another reason for the lower pumping volumes may be seasonality since the measurements were done during the winter months. Further studies need to be done to determine what role seasonality plays in both pumping volumes and oxygen consumption in temperate sponges.

A recent study has suggested that sponges do not require much oxygen, and even goes so far as to say that sponges may have evolved prior to the oxygenation of the oceans (Mills et al., 2014). Mills et al. (2014) reported that *H. panacea* could both respire and feed at oxygen levels 0.5% – 4% present atmospheric levels, and even reported some sponge 'growth' at these levels. This reported 'growth' was the elongation of several of the sponge protrusions, but because the weight of the sponge was not measured it is unknown if this elongation resulted in an increase in tissue. Sponges have been known to relocate under poor environmental conditions by extending fibrils and 'crawling' along the substrate (Maldonado and Uriz, 1999). This elongation of protrusions, therefore, may have been an

escape mechanism to try to find more suitable habitat. In addition, the clearance rates reported by Mills et al. (2014) were 40% clearance rates previously reported for *H. panacea* (Riisgård et al., 1993). Based on the results from this thesis, the reduced clearance rates reported by Mills et al. (2014) may be an indication of reduced pumping rates to save energy under unfavourable conditions. Increased filtration to obtain food comes with increased energy and thus oxygen uptake. Therefore, although some sponges are able to withstand short periods of low oxygen it is unlikely that they would be able to thrive under these conditions.

4.5 Concluding statements

This thesis presents the first evidence of non-motile sensory cilia in the osculum of sponges, which may represent the first steps in the evolution of sensory and coordination systems in metazoans. I show evidence suggesting these sensory cilia provide the sponge with the ability to sense changes in the environment and control the amount of water flow through the aquiferous canal system. Sponges rely on this water flow to filter out particles for food. However, a repeatedly overlooked component of filter feeding is the amount of energy required to pump the large volumes of water needed to meet food requirements. My results suggest that this filter feeding is energetically costly, and that in times of low food availability or other additional stresses sponges may be able to reduce the volume of water filtered to lower the cost of filtration.

4.6 References

Bannister, R. J., Battershill, C. N. and de Nys, R. (2012). Suspended sediment grain size and mineralogy across the continental shelf of the Great Barrier Reef: Impacts on the physiology of a coral reef sponge. *Continental Shelf Research* **32**, 86-95.

Best, B. A. (1988). Passive suspension feeding in a Sea Pen: Effects of ambient flow on volume flow rate and filtering efficiency. *Biological Bulletin* **175**, 332-342.

Bidder, G. P. (1923). The relation of the form of a sponge to its currents. *Quaterly Journal of Microscopical Sciences* **67**, 293-323.

Eckman, J. E. and Duggins, D. O. (1993). Effects of flow speed on growth of benthic suspension feeders. *Biological Bulletin* **185**, 28-41.

Elliott, G. R. D. and Leys, S. P. (2007). Coordinated contractions effectively expel water from the aquiferous system of a freshwater sponge. *The Journal of Experimental Biology* **210**, 3736-48.

Ellis, J., Cummings, V., Hewitt, J., Thrush, S. and Norkko, A. (2002). Determining effects of suspended sediment on condition of a suspension feeding bivalve (*Atrina zelandica*): results of a survey, a laboratory experiment and a field transplant experiment. *Journal of Experimental Marine Biology and Ecology* **267**, 147-174.

Ellwanger, K., Eich, A. and Nickel, M. (2007). GABA and glutamate specifically induce contractions in the sponge *Tethya wilhelma*. *Journal of Comparative Physiology A* **193**, 1-11.

Fujiu, K., Nakayama, Y., Iida, H., Sokabe, M. and Yoshimura, K. (2011). Mechanoreception in motile flagella of *Chlamydomonas*. *Nature Cell Biology* **13**, 630-2.

Gale, J. E., Marcotti, W., Kennedy, H. J., Kros, C. J. and Richardson, G. P. (2001). FM1-43 dye behaves as a permeant blocker of the hair-cell mechanotransducer channel. *The Journal of Neuroscience* **21**, 7013-25.

Gerrodette, T. and Flechsig, A. O. (1979). Sediment-induced reduction in the pumping rate of the tropical sponge *Verongia lacunosa*. *Marine Biology* **55**, 103-110.

Gili, J.-M. and Coma, R. (1998). Benthic suspension feeders: their paramount role in littoral marine food webs. *Trends in Ecology and Evolution* **13**, 316-321.

Jones, W. C. (1962). Is there a nervous system in sponges? *Biological Reviews* **37**, 1-50.

Jørgensen, C. B. (1966). Biology of Suspension Feeding. Oxford: Pergamon Press.

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.

Maldonado, M. and Uriz, M. J. (1999). An experimental approach to the ecological significance of microhabitat-scale movement in an encrusting sponge. *Marine Ecology Progress Series* **185**, 239-255.

Mills, D. B., Ward, L. M., Jones, C., Sweeten, B., Forth, M., Treusch, A. H. and Canfield, D. E. (2014). Oxygen requirements of the earliest animals. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 4168-4172.

Nauli, S. M., Alenghat, F. J., Luo, Y., Williams, E., Vassilev, P., Li, X., Elia, A. E. H., Lu, W., Brown, E. M., Quinn, S. J. et al. (2003). Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nature Genetics* **33**, 129-37.

Nickel, M. (2004). Kinetics and rhythm of body contractions in the sponge *Tethya wilhelma* (Porifera: Demospongiae). *The Journal of experimental biology* **207**, 4515-24.

Palumbi, S. R. (1986). How body plans limit acclimation: responses of a demosponge to wave force. *Ecology* **67**, 208-214.

Pavans de Ceccatty, M. (1974). Coordination in sponges: the foundations of integration. *American Zoologist* **14**, 895-903.

Praetorius, H. A. and Spring, K. R. (2001). Bending the MDCK cell primary cilium increases intracellular calcium. *Journal of Membrane Biology* **184**, 71-79.

Praetorius, H. A. and Spring, K. R. (2003). Removal of the MDCK cell primary cilium abolishes flow sensing. *Journal of Membrane Biology* **191**, 69-76.

Praetorius, H. A. and Spring, K. R. (2005). A physiological view of the primary cilium. *Annual Review of Physiology* **67**, 515-529.

Puig, P., Canals, M., Company, J. B., Martin, J., Amblas, D., Lastras, G. and Palanques, A. (2012). Ploughing the deep sea floor. *Nature* **489**, 286-9.

Reiswig, H. M. (1971). *In situ* pumping activities of tropical Demospongiae. *Marine Biology* **9**, 38-50.

Reiswig, H. M. (1974). Water transport, respiration and energetics of three tropical marine sponges. *Journal of Experimental Marine Biology and Ecology* **14**, 231-249.

Riisgård, H. U., Thomassen, S., Jakobsen, H., Weeks, J. M. and Larsen, P. S. (1993). Suspension feeding in marine sponges *Halichondria panicea* and *Haliclona urceolus:* effects of temperature on filtration rate and energy cost of pumping. *Marine Ecology Progress Series* **96**, 177-188.

Savarese, M., Patterson, M. R., Chernykh, V. I. and Fialkov, V. A. (1997). Trophic effects of sponge feeding within Lake Baikal's littoral zone. 1. In situ pumping rates. *Limnology and Oceanography* **42**, 171-178.

Shimeta, J. and Jumars, P. A. (1991). Physical mechanisms and rates of particle capture by suspension-feeders. *Oceanography and Marine Biology Annual Review* **29**, 191-257.

Singla, V. and Reiter, J. F. (2006). The primary cilium as the cell's antenna: signaling at a sensory organelle. *Science* **313**, 629-33.

Taghon, G. L. (1981). Beyond selection: optimal ingestion rate as a function of food value. *The American Naturalist* **118**, 202-214.

Tompkins-MacDonald, G. J. and Leys, S. P. (2008). Glass sponges arrest pumping in response to sediment: implications for the physiology of the hexactinellid conduction system. *Marine Biology* **154**, 973-984.

Vogel, S. (1974). Current-induced flow through the sponge, *Halichondria*. *Biological Bulletin* **147**, 443-456.

Vogel, S. (1977). Current-induced flow through living sponges in nature. *Ecology* **74**, 2069-2071.

Warburton, F. E. (1960). Influence of currents on form of sponges. *Science* **132**, 89.

Weissenfels, N. (1984). Bau und Funktion des Süßwasserschwamms *Ephydatia fluviatilis* (Porifera). *Zoomorphology* **104**, 292-297.

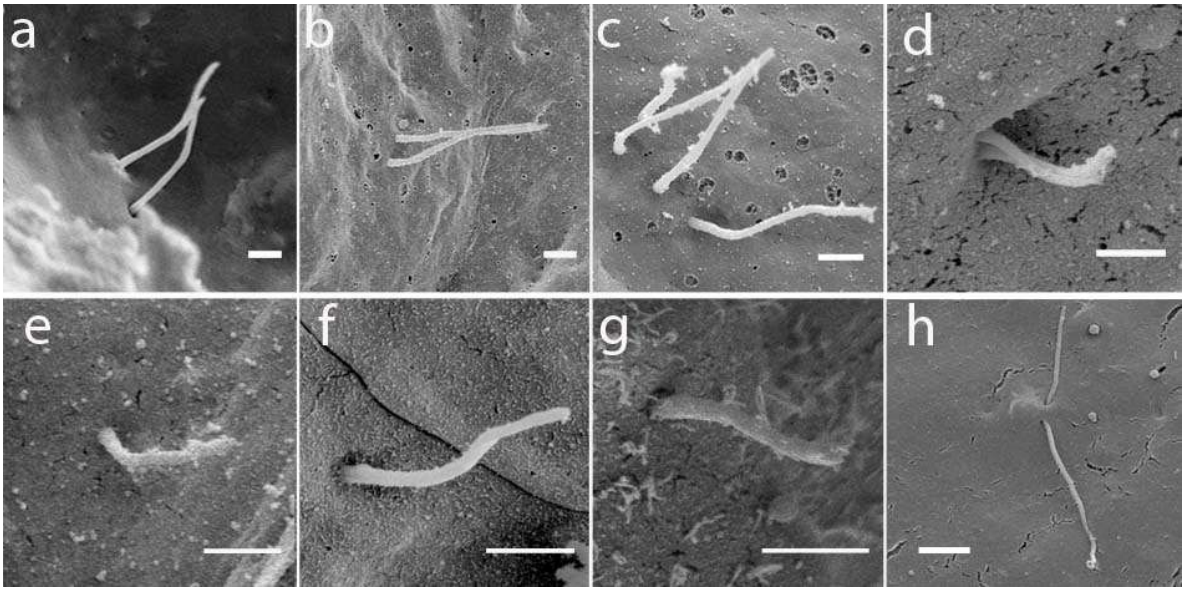
Wulff, J. L. (2006). Rapid diversity and abundance decline in a Caribbean coral reef sponge community. *Biological Conservation* **127**, 167-176.

Yahel, G. Whitney, F., Reiswig, H.M., Eerkes-Medrano, D.I., and Leys, S.P. (2007). *In situ* feeding and metabolism of glass sponges (Hexactenellida, Porifera) studied in a deep temperate fjord with a remotely operated submersible. *Limnology and Oceanography* **52,** 428-440.

Appendix One

Supplemental material for Chapter Two

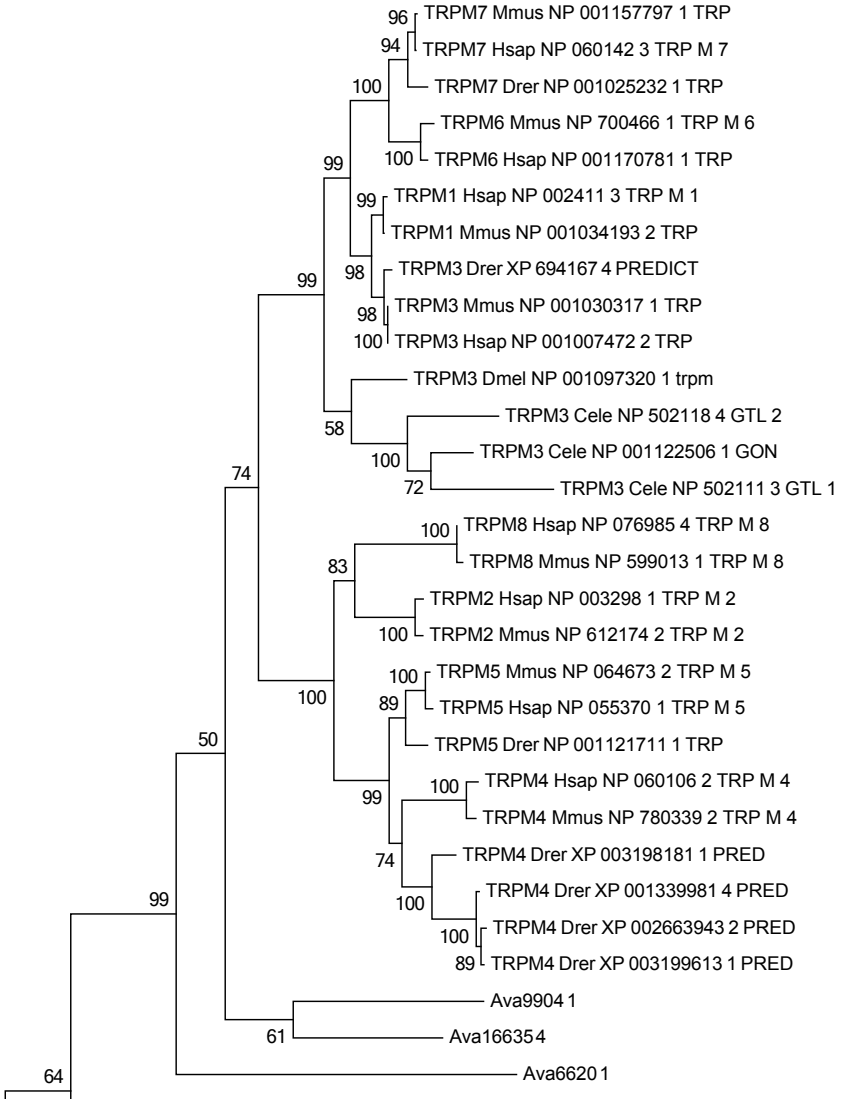
A1.1 Cilia in the oscula of various sponges

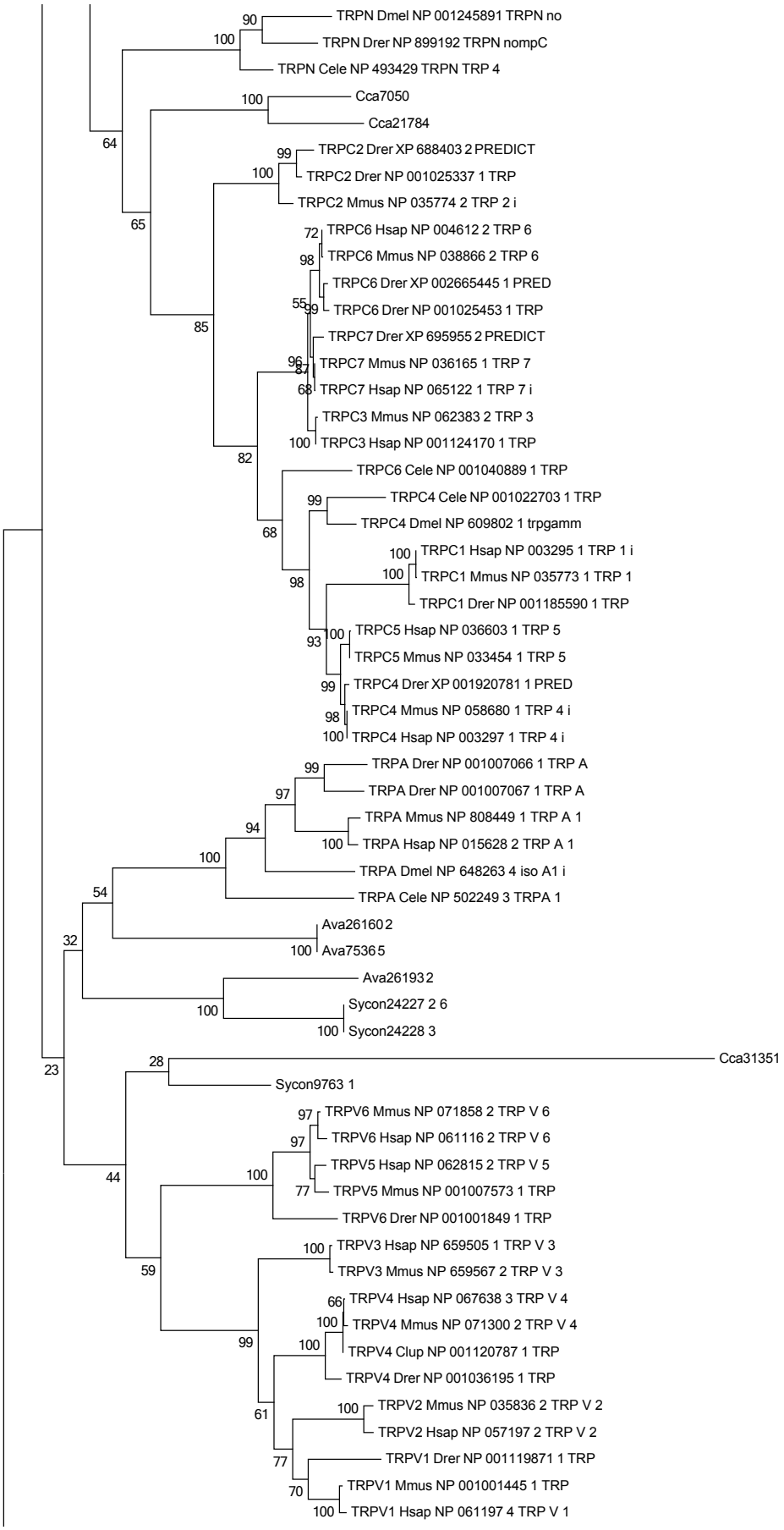


a. *Ephydatia muelleri*, **b, c.** *Spongilla lacustris*, **d**. *Neopetrosia vanilla*, **e.** *Haliclona mollis*, **f**. *Haliclona sp*,. **g.** *Neopetrosia problematica*, **h**. *Aphrocallistes vastus*. Scale bars 1 μm.

A1.2 Uncompressed tree showing the evolutionary relationships of sponge TRPType I and II genes.

Values at nodes indicate Bootstrap support. (Tree has been split into three pages for viewing. For the full scale complete tree see http://www.biomedcentral.com/1471-2148/14/3/additional)

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Cca7050
Cca21784
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TRPC2 Mmus NP 035774 2 TRP 2 i
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TRPA Cele NP 502249 3 TRPA 1
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Ava75365
Ava261932
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Sycon24228 3
Cca31351
Sycon9763 1
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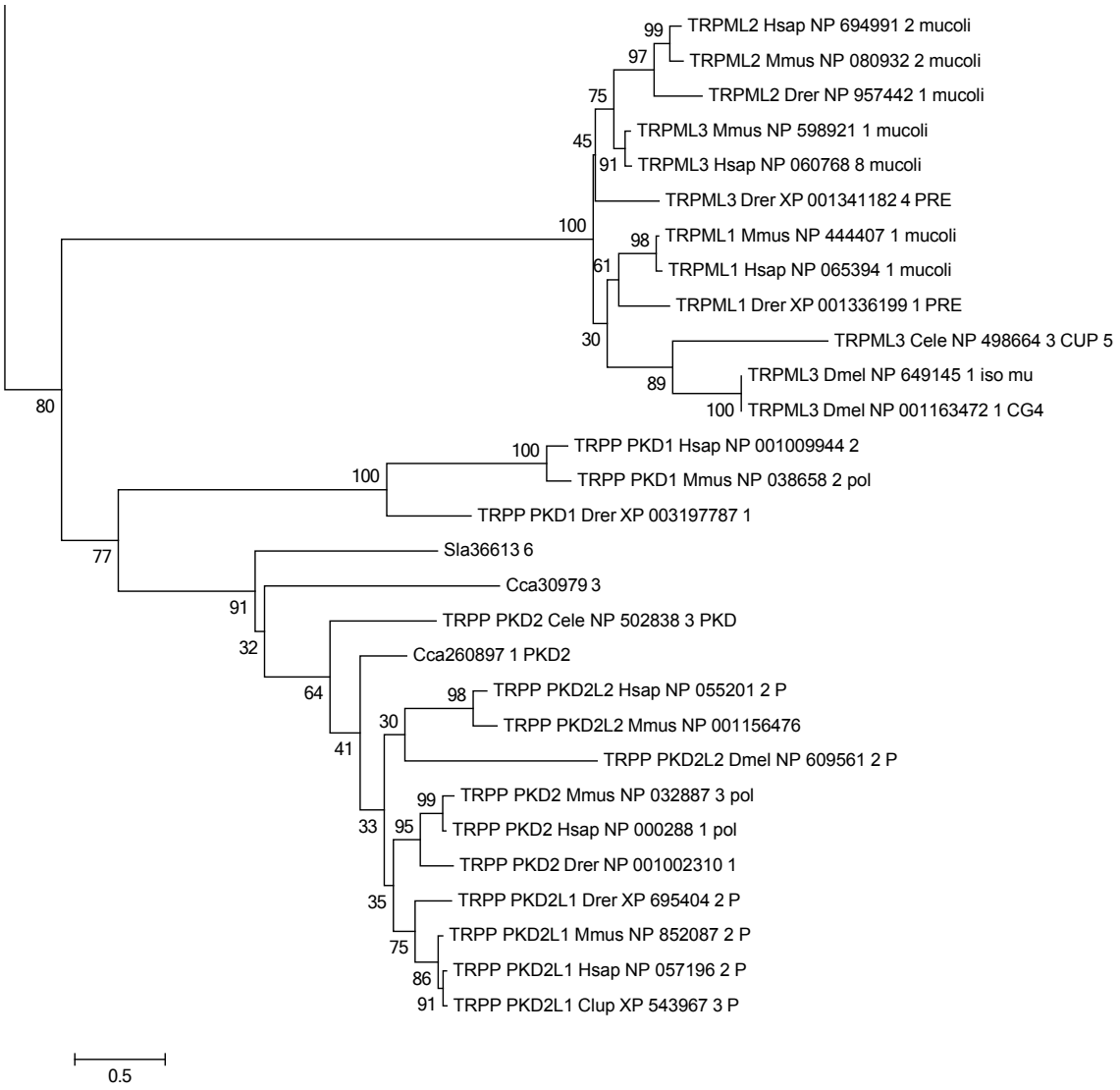
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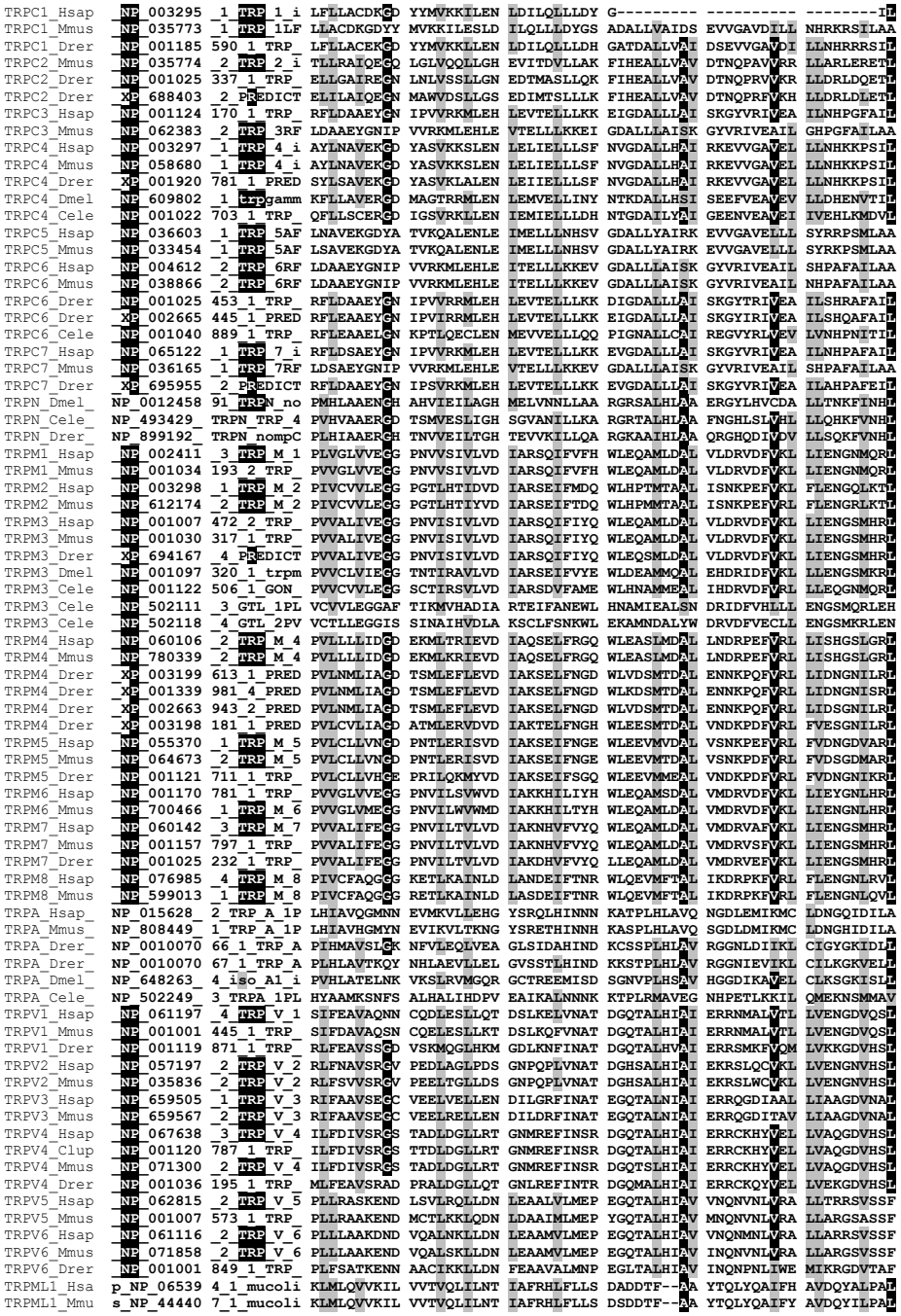
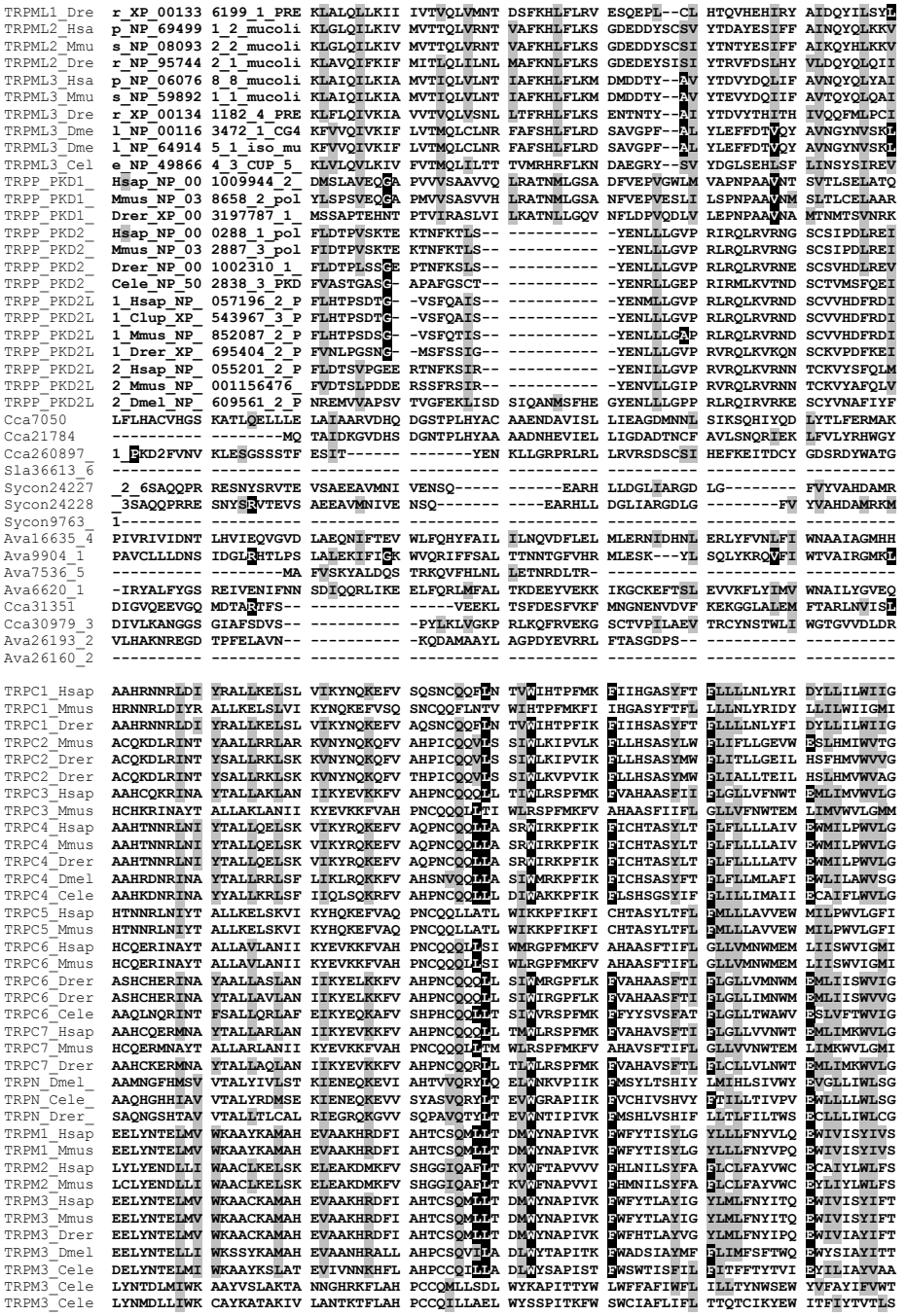
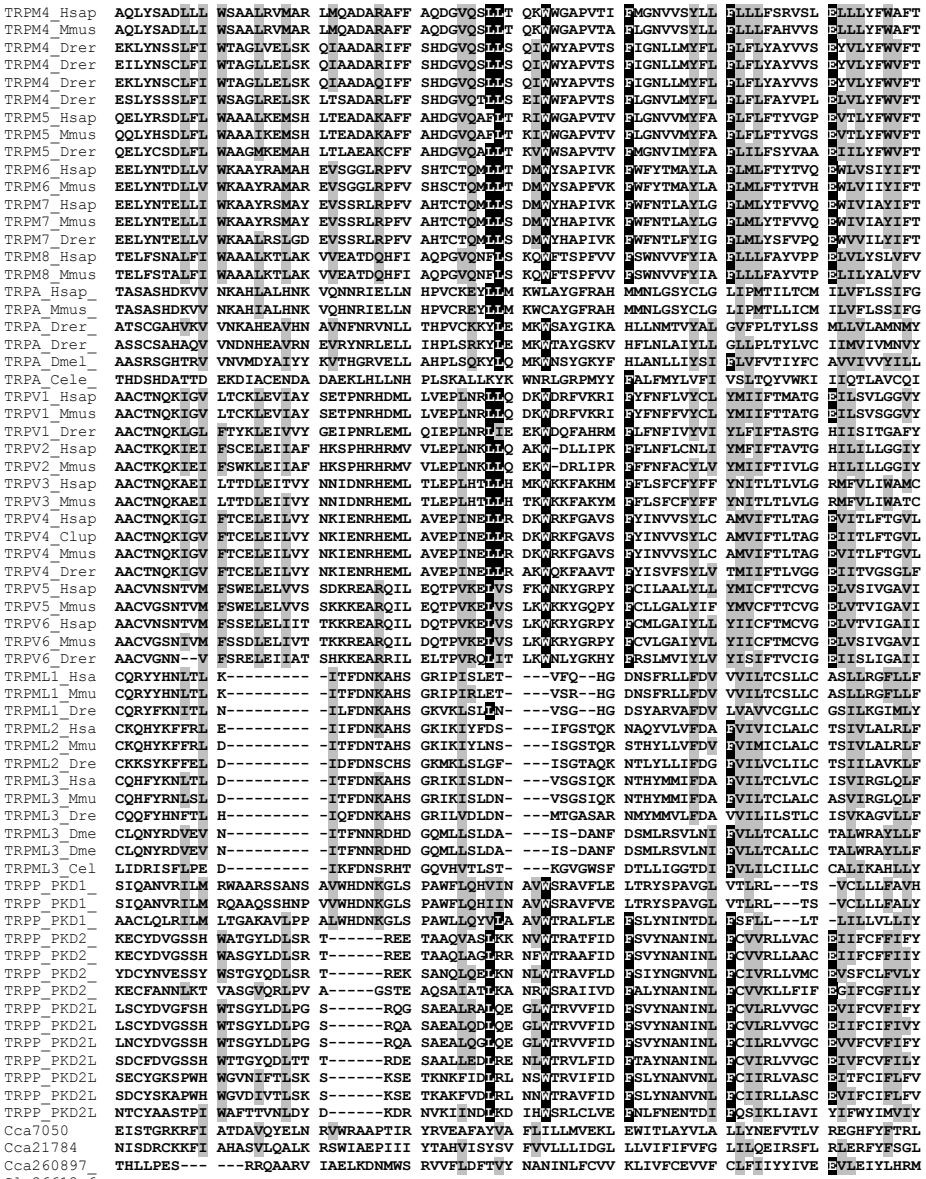
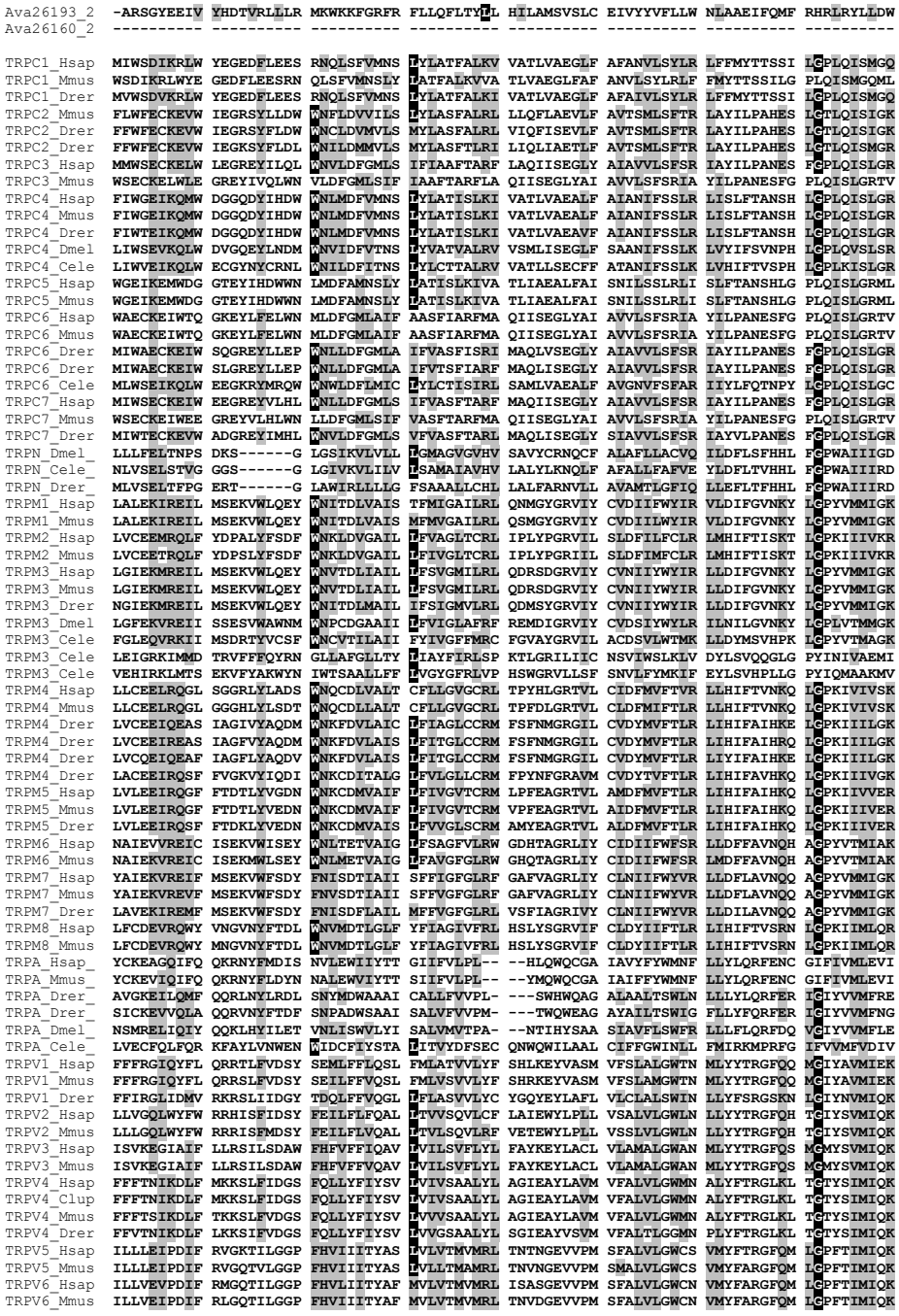
A1.3 Full alignment of TRP sequences for uncompressed tree in Fig 2-5a









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Ava26160_2 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

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TRPC1_Mmus WSDIKRLWYE GEDFLEESRN QLSFVMNSLY TATFALKVVA TLVAEGLFAF ANVLSYLRLF FMYTTSSILG PLQISMGQML
TRPC1_Drer MVWSDVKRLW YEGEDFLEES RNQLSFVMNS TYLATFALKI VATLVAEGLF AFAIVLSYLR LFFMYTTSSI LCPLQISMGQ
TRPC2_Mmus FLWFECKEVW IEGRSYLLDW WNFLDVVILS LYLASFALRL LLQFLAEVLF AVTSMLSFTR LAYILPAHES LCTLQISIGK
TRPC2_Drer FFWFECKEVW IEGRSYFLDW WNCLDVMVLS MYLASFALRL VIQFISEVLF AVTSMLSFTR LAYILPAHES LCTLQISIGK
TRPC2_Drer FFWFECKEVW IEGKSYFLDL WNILDMMVLS MYLASFTLRI LIQLIAETLF AVTSMLSFTR LAYILPAHES LCTLQISMGR
TRPC3_Hsap MMWSECKELW LEGREYILQL WNVLDFGMLS IFIAAFTARF LAQIISEGLY AIAVVLSFSR IAYILPANES FCPLQISLGR
TRPC3_Mmus WSECKELWLE GREYIVQLWN VLDFGMLSIF IAAFTARFLA QIISEGLYAI AVVLSFSRIA YILPANESFG PLQISLGRTV
TRPC4_Hsap FIWGEIKQMW DGGQDYIHDW WNLMDFVMNS TYLATISLKI VATLVAEALF AIANIFSSLR LISLFTANSH LCPLQISLGR
TRPC4_Mmus FIWGEIKQMW DGGQDYIHDW WNLMDFVMNS TYLATISLKI VATLVAEALF AIANIFSSLR LISLFTANSH LCPLQISLGR
TRPC4_Drer FIWTEIKQMW DGGQDYIHDW WNLMDFVMNS TYLATISLKI VATLVAEAVF AIANIFSSLR LISLFTANSH LCPLQISLGR
TRPC4_Dmel LIWSEVKQLW DVGQEYLNDM WNVIDFVTNS TYVATVALRV VSMLISEGLF SAANIFSSLK LVYIFSVNPH LCPLQVSLSR
TRPC4_Cele LIWVEIKQLW ECGYNYCRNL WNILDFITNS TYLCTTALRV VATLLSECFF ATANIFSSLK LVHIFTVSPH LCPLKISLGR
TRPC5_Hsap WGEIKEMWDG GTEYIHDWWN LMDFAMNSLY TATISLKIVA TLIAEALFAI SNILSSLRLI SLFTANSHLG PLQISLGRML
TRPC5_Mmus WGEIKEMWDG GTEYIHDWWN LMDFAMNSLY TATISLKIVA TLIAEALFAI SNILSSLRLI SLFTANSHLG PLQISLGRML
TRPC6_Hsap WAECKEIWTQ GKEYLFELWN MLDFGMLAIF AASFIARFMA QIISEGLYAI AVVLSFSRIA YILPANESFG PLQISLGRTV
TRPC6_Mmus WAECKEIWTQ GKEYLFELWN MLDFGMLAIF AASFIARFMA QIISEGLYAI AVVLSFSRIA YILPANESFG PLQISLGRTV
TRPC6_Drer MIWAECKEIW SQGREYLLEP WNLLDFGMLA IFVASFISRI MAQLVSEGLY AIAVVLSFSR IAYILPANES FCPLQISLGR
TRPC6_Drer MIWAECKEIW SLGREYLLEP WNLLDFGMLA IFVTSFIARF MAQLISEGLY AIAVVLSFSR IAYILPANES FCPLQISLGR
TRPC6_Cele MLWSEIKQLW EEGKRYMRQW WNWLDFLMIC TYLCTISIRL SAMLVAEALF AVGNVFSFAR IIYLFQTNPY LCPLQISLGC
TRPC7_Hsap MIWSECKEIW EEGREYVLHL WNLLDFGMLS IFVASFTARF MAQIISEGLY AIAVVLSFSR IAYILPANES FCPLQISLGR
TRPC7_Mmus WSECKEIWEE GREYVLHLWN LLDFGMLSIF VASFTARFMA QIISEGLYAI AVVLSFSRIA YILPANESFG PLQISLGRTV
TRPC7_Drer MIWTECKEVW ADGREYIMHL WNVLDFGMLS VFVASFTARL MAQLISEGLY SIAVVLSFSR IAYVLPANES FCPLQISLGR
TRPN_Dmel_ LLLFELTNPS DKS------G LGSIKVLVLL TGMAGVGVHV SAVYCRNQCF ALAFLLACVQ ILDFLSFHHL FCPWAIIIGD
TRPN_Cele_ NLVSELSTVG GGS------G LGIVKVLILV LSAMAIAVHV LALYLKNQLF AFALLFAFVE YLDFLTVHHL FCPWAIIIRD
TRPN_Drer_ MLVSELTFPG ERT------G LAWIRLLLLG FSAAALLCHL LALFARNVLL AVAMTLGFIQ LLEFLTFHHL FCPWAIIIRD
TRPM1_Hsap LALEKIREIL MSEKVWLQEY WNITDLVAIS TFMIGAILRL QNMGYGRVIY CVDIIFWYIR VLDIFGVNKY LCPYVMMIGK
TRPM1_Mmus LALEKIREIL MSEKVWLQEY WNITDLVAIS MFMVGAILRL QSMGYGRVIY CVDIILWYIR VLDIFGVNKY LCPYVMMIGK
TRPM2_Hsap LVCEEMRQLF YDPALYFSDF WNKLDVGAIL TFVAGLTCRL IPLYPGRVIL SLDFILFCLR LMHIFTISKT LCPKIIIVKR
TRPM2_Mmus LVCEETRQLF YDPSLYFSDF WNKLDVGAIL TFIVGLTCRL IPLYPGRIIL SLDFIMFCLR LMHIFTISKT LCPKIIIVKR
TRPM3_Hsap LGIEKMREIL MSEKVWLQEY WNVTDLIAIL TFSVGMILRL QDRSDGRVIY CVNIIYWYIR LLDIFGVNKY LCPYVMMIGK
TRPM3_Mmus LGIEKMREIL MSEKVWLQEY WNVTDLIAIL TFSVGMILRL QDRSDGRVIY CVNIIYWYIR LLDIFGVNKY LCPYVMMIGK
TRPM3_Drer NGIEKMREIL MSEKVWLQEY WNITDLMAIL IFSIGMVLRL QDMSYGRVIY CVNIIYWYIR LLDIFGVNKY LCPYVMMIGK
TRPM3_Dmel LGFEKVREII SSESVWAWNM WNPCDGAAII TFVIGLAFRF REMDIGRVIY CVDSIYWYLR ILNILGVNKY LCPLVTMMGK
TRPM3_Cele FGLEQVRKII MSDRTYVCSF WNCVTILAII FYIVGFFMRC FGVAYGRVIL ACDSVLWTMK LLDYMSVHPK LCPYVTMAGK
TRPM3_Cele LEIGRKIMMD TRVFFFQYRN GLLAFGLLTY TIAYFIRLSP KTLGRILIIC NSVIWSLKLV DYLSVQQGLG PYINIVAEMI
TRPM3_Cele VEHIRKLMTS EKVFYAKWYN IWTSAALLFF LVGYGFRLVP HSWGRVLLSF SNVLFYMKIF EYLSVHPLLG PYIQMAAKMV
TRPM4_Hsap LLCEELRQGL SGGRLYLADS WNQCDLVALT CFLLGVGCRL TPYHLGRTVL CIDFMVFTVR LLHIFTVNKQ LCPKIVIVSK
TRPM4_Mmus LLCEELRQGL GGGHLYLSDT WNQCDLLALT CFLLGVGCRL TPFDLGRTVL CLDFMIFTLR LLHIFTVNKQ LCPKIVIVSK
TRPM4_Drer LVCEEIQEAS IAGIVYAQDM WNKFDVLAIC TFIAGLCCRM FSFNMGRGIL CVDYMVFTLR LIHIFAIHKE LCPKIIILGK
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TRPM4_Drer LVCQEIQEAF IAGFLYAQDV WNKFDVLAIS TFITGLCCRM FSFNMGRGIL CVDYMVFTLR LIYIFAIHKE LCPKIIILGK
TRPM4_Drer LACEEIRQSF FVGKVYIQDI WNKCDITALG TFVLGLLCRM FPYNFGRAVM CVDYTVFTLR LIHIFAVHKQ LCPKIIIVGK
TRPM5_Hsap LVLEEIRQGF FTDTLYVGDN WNKCDMVAIF TFIVGVTCRM LPFEAGRTVL AMDFMVFTLR LIHIFAIHKQ LCPKIIVVER
TRPM5_Mmus LVLEEIRQGF FTDTLYVEDN WNKCDMVAIF TFIVGVTCRM VPFEAGRTVL AIDFMVFTLR LIHIFAIHKQ LCPKIIIVER
TRPM5_Drer LVLEEIRQSF FTDKLYVEDN WNKCDMVAIS TFVVGLSCRM AMYEAGRTVL ALDFMVFTLR LIHIFAIHKQ LCPKIIIVER
TRPM6_Hsap NAIEVVREIC ISEKVWISEY WNLTETVAIG TFSAGFVLRW GDHTAGRLIY CIDIIFWFSR LLDFFAVNQH ACPYVTMIAK
TRPM6_Mmus NAIEKVREIC ISEKMWLSEY WNLMETVAIG TFAVGFGLRW GHQTAGRLIY CIDIIFWFSR LMDFFAVNQH ACPYVTMIAK
TRPM7_Hsap YAIEKVREIF MSEKVWFSDY FNISDTIAII SFFIGFGLRF GAFVAGRLIY CLNIIFWYVR LLDFLAVNQQ ACPYVMMIGK
TRPM7_Mmus YAIEKVREVF MSEKVWFSDY FNVSDTIAII SFFVGFGLRF GAFVAGRLIY CLNIIFWYVR LLDFLAVNQQ ACPYVMMIGK
TRPM7_Drer LAVEKIREMF MSEKVWFSDY FNISDFLAIL MFFVGFGLRL VSFIAGRIVY CLNIIFWYVR LLDILAVNQQ ACPYVMMIGK
TRPM8_Hsap LFCDEVRQWY VNGVNYFTDL WNVMDTLGLF YFIAGIVFRL HSLYSGRVIF CLDYIIFTLR LIHIFTVSRN LCPKIIMLQR
TRPM8_Mmus LFCDEVRQWY MNGVNYFTDL WNVMDTLGLF YFIAGIVFRL HSLYSGRVIF CLDYIIFTLR LIHIFTVSRN LCPKIIMLQR
TRPA_Hsap_ YCKEAGQIFQ QKRNYFMDIS NVLEWIIYTT GIIFVLPL-- --HLQWQCGA IAVYFYWMNF LLYLQRFENC GIFIVMLEVI
TRPA_Mmus_ YCKEVIQIFQ QKRNYFLDYN NALEWVIYTT SIIFVLPL-- --YMQWQCGA IAIFFYWMNF LLYLQRFENC GIFIVMLEVI
TRPA_Drer_ AVGKEILQMF QQRLNYLRDL SNYMDWAAAI CALLFVVPL- ---SWHWQAG ALAALTSWLN LLLYLQRFER ICIYVVMFRE
TRPA_Drer_ SICKEVVQLA QQRVNYFTDF SNPADWSAAI SALVFVVPM- ---TWQWEAG AYAILTSWIG FLLYFQRFER ICIYVVMFNG
TRPA_Dmel_ NSMRELIQIY QQKLHYILET VNLISWVLYI SALVMVTPA- --NTIHYSAA SIAVFLSWFR LLLFLQRFDQ VGIYVVMFLE
TRPA_Cele_ LVECFQLFQR KFAYLVNWEN WIDCFIYSTA TITVYDFSEC QNWQWILAAL CIFFGWINLL FMIRKMPRFG IFVVMFVDIV
TRPV1_Hsap FFFRGIQYFL QRRTLFVDSY SEMLFFLQSL FMLATVVLYF SHLKEYVASM VFSLALGWTN MLYYTRGFQQ MGIYAVMIEK
TRPV1_Mmus FFFRGIQYFL QRRSLFVDSY SEILFFVQSL FMLVSVVLYF SHRKEYVASM VFSLAMGWTN MLYYTRGFQQ MGIYAVMIEK
TRPV1_Drer FFIRGLIDMV RKRSLIIDGY TDQLFFVQGL TFLASVVLYC YGQYEYLAFL VLCLALSWIN LLYFSRGSKN LGIYNVMIQK
TRPV2_Hsap LLVGQLWYFW RRHISFIDSY FEILFLFQAL TTVVSQVLCF LAIEWYLPLL VSALVLGWLN LLYYTRGFQH TGIYSVMIQK
TRPV2_Mmus LLLGQLWYFW RRRISFMDSY FEILFLVQAL TTVLSQVLRF VETEWYLPLL VSSLVLGWLN LLYYTRGFQH TGIYSVMIQK
TRPV3_Hsap ISVKEGIAIF LLRSILSDAW FHFVFFIQAV TVILSVFLYL FAYKEYLACL VLAMALGWAN MLYYTRGFQS MGMYSVMIQK
TRPV3_Mmus ISVKEGIAIF LLRSILSDAW FHFVFFVQAV TVILSVFLYL FAYKEYLACL VLAMALGWAN MLYYTRGFQS MGMYSVMIQK
TRPV4_Hsap FFFTNIKDLF MKKSLFIDGS FQLLYFIYSV TVIVSAALYL AGIEAYLAVM VFALVLGWMN ALYFTRGLKL TGTYSIMIQK
TRPV4_Clup FFFTNIKDLF MKKSLFIDGS FQLLYFIYSV TVIVSAALYL AGIEAYLAVM VFALVLGWMN ALYFTRGLKL TGTYSIMIQK
TRPV4_Mmus FFFTSIKDLF TKKSLFVDGS FQLLYFIYSV TVVVSAALYL AGIEAYLAVM VFALVLGWMN ALYFTRGLKL TGTYSIMIQK
TRPV4_Drer FFVTNIKDLF LKKSIFVDGS FQLLYFIYSV TVVGSAALYL SGIEAYVSVM VFALTLGGMN PLYFTRGLKL TGTYSIMIQK
TRPV5_Hsap ILLLEIPDIF RVGKTILGGP FHVIIITYAS TVLVTMVMRL TNTNGEVVPM SFALVLGWCS VMYFTRGFQM LGPFTIMIQK
TRPV5_Mmus ILLLEIPDIF RVGQTVLGGP FHVIIITYAS TVLLTMAMRL TNVNGEVVPM SMALVLGWCS VMYFARGFQM LGPFTIMIQK
TRPV6_Hsap ILLVEVPDIF RMGQTILGGP FHVLIITYAF MVLVTMVMRL ISASGEVVPM SFALVLGWCN VMYFARGFQM LGPFTIMIQK
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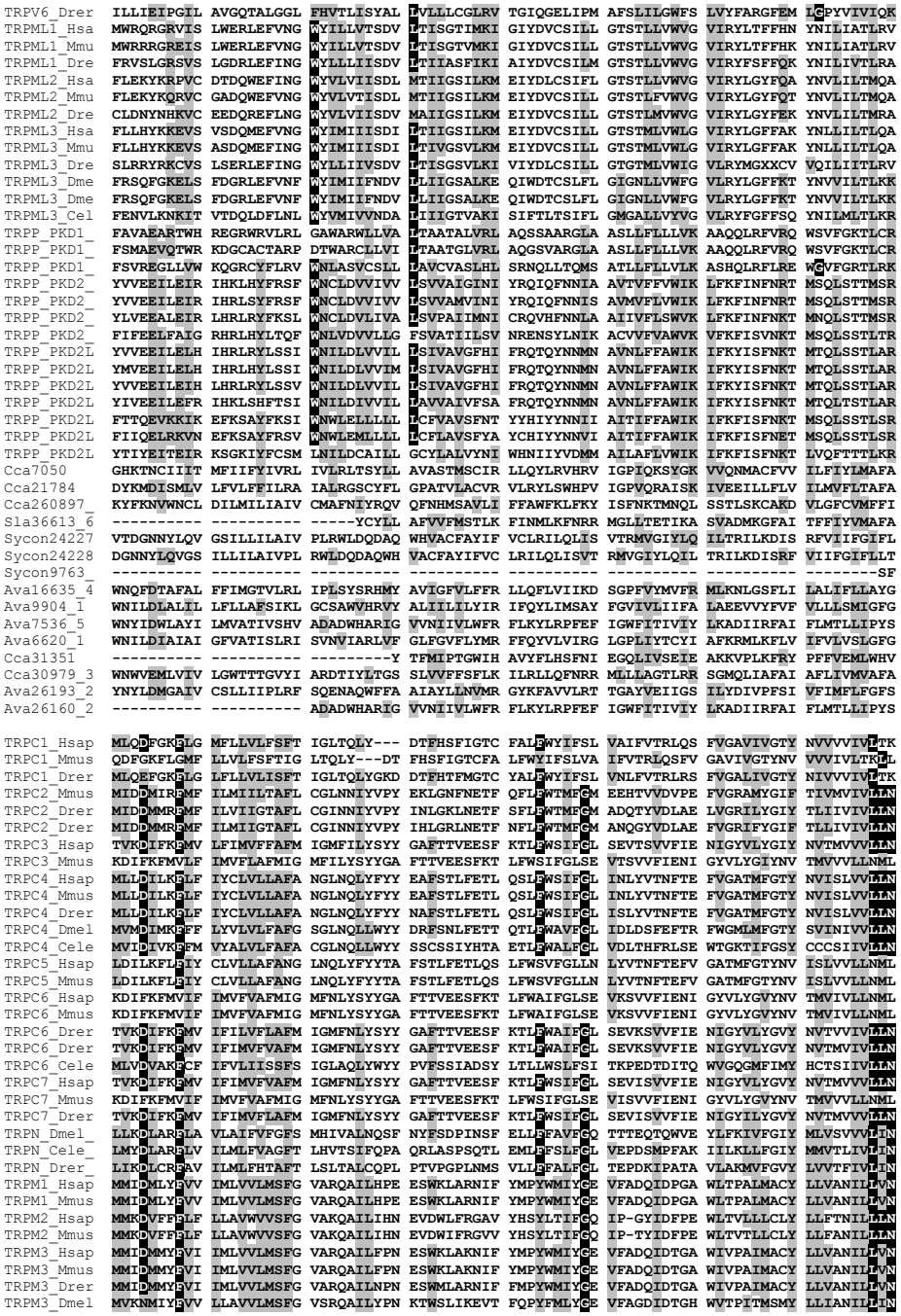
TRPV6_Drer ILLIEIPGIL AVGQTALGGL FHVTLISYAL FVLLLCGLRV TGIQGELIPM AFSLILGWFS LVYFARGFEM LCPYVIVIQK
TRPML1_Hsa MWRQRGRVIS LWERLEFVNG WYILLVTSDV FTISGTIMKI GIYDVCSILL GTSTLLVWVG VIRYLTFFHN YNILIATLRV
TRPML1_Mmu MWRRRGREIS LWERLEFVNG WYILLVTSDV FTISGTVMKI GIYDVCSILL GTSTLLVWVG VIRYLTFFHK YNILIATLRV
TRPML1_Dre FRVSLGRSVS LGDRLEFING WYLLLIISDV FTIIASFIKI AIYDVCSILM GTSTLLVWVG VIRYFSFFQK YNILIVTLRA
TRPML2_Hsa FLEKYKRPVC DTDQWEFING WYVLVIISDL MTIIGSILKM EIYDLCSIFL GTSTLLVWVG VIRYLGYFQA YNVLILTMQA
TRPML2_Mmu FLEKYKQRVC GADQWEFVNG WYVLVTISDL MTIIGSILKM EIYDVCSILL GTSTLFVWVG VIRYLGYFQT YNVLILTMQA
TRPML2_Dre CLDNYNHKVC EEDQREFLNG WYVLVIISDV MAIIGSILKM EIYDVCSILL GTSTLMVWVG VIRYLGYFEK YNVLILTMRA
TRPML3_Hsa FLLHYKKEVS VSDQMEFVNG WYIMIIISDI FTIIGSILKM EIYDVCSILL GTSTMLVWLG VIRYLGFFAK YNLLIILTLQA
TRPML3_Mmu FLLHYKKEVS ASDQMEFING WYIMIIISDI FTIVGSVLKM EIYDVCSILL GTSTMLVWLG VIRYLGFFAK YNLLIILTLQA
TRPML3_Dre SLRRYRKCVS LSERLEFING WYLLIIVSDV FTISGSVLKI VIYDLCSILL GTGTMLVWIG VLRYMGXXCV VQILIITLRV
TRPML3_Dme FRSQFGKELS FDGRLEFVNF WYIMIIFNDV FLIIGSALKE QIWDTCSLFL GIGNLLVWFG VLRYLGFFKT YNVVILTLKK
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TRPML3_Cel FENVLKNKIT VTDQLDFLNL WYVMIVVNDA FIIIGTVAKI SIFTLTSIFL GMGALLVYVG VLRYFGFFSQ YNILMLTLKR
TRPP_PKD1_ FAVAEARTWH REGRWRVLRL GAWARWLLVA FTAATALVRL AQSSAARGLA ASLLFLLLVK AAQQLRFVRQ WSVFGKTLCR
TRPP_PKD1_ FSMAEVQTWR KDGCACTARP DTWARCLLVI FTAATGLVRL AQGSVARGLA ASLLFLLLVK AAQQLRFVRQ WSVFGKTLCR
TRPP_PKD1_ FSVREGLLVW KQGRCYFLRV WNLASVCSLL LAVCVASLHL SRNQLLTQMS ATLLFLLVLK ASHQLRFLRE WCVFGRTLRK
TRPP_PKD2_ YVVEEILEIR IHKLHYFRSF WNCLDVVIVV LSVVAIGINI YRQIQFNNIA AVTVFFVWIK LFKFININFNRT MSQLSTTMSR
TRPP_PKD2_ YVVEEILEIR IHRLSYFRSF WNCLDVVIVV LSVVAMVINI YRQIQFNNIS AVMVFLVWIK LFKFININFNRT MSQLSTTMSR
TRPP_PKD2_ YLVEEALEIR LHRLRYFKSL WNCLDVLIVA LSVPAIIMNI CRQVHFNNLA AIIVFLSWVK LFKFININFNKT MNQLSTTMSR
TRPP_PKD2_ FIFEELFAIG RHRLHYLTQF WNLVDVVLLG FSVATIILSV NRENSYLNIK ACVVFVAWVK VFKFISVNKT MSQLSSTLTR
TRPP_PKD2L YVVEEILELH IHRLRVLSSI WNILDLVVIL LSIVAVGFHI FRQTQYNNMN AVNLFFAWIK IFKYISFNKT MTQLSSTLAR
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TRPP_PKD2L YIVEEILEFR IHKLSHFTSI WNILDIVVIL LAVVAIVFSA FRQTQYNNMN AVNLFFAWIK IFKYISFNKT MTQLTSTLAR
TRPP_PKD2L FTTQEVKKIK EFKSAYFKSI WNWLELLLLL FCFVAVSFNT YYHIYYNNII AITIFFAWIK IFKFISFNKT MSQLSSTLSR
TRPP_PKD2L FIIQELRKVN EFKSAYFRSV WNWLEMLLLL FCFLAVSFYA YCHIYYNNVI AITIFFAWIK IFKFISFNET MSQLSSTLSR
TRPP_PKD2L YTIYEITEIR KSGKIYFCSM LNILDCAILL GCYLALVYNI WHNIIYVDMM AILAFLVWIK IFKFISFNKT LVQFTTTLKR
Cca7050 GHKTNCIIIT MFIIFYIVRL IVLRLTSYLL AVASTMSCIR LLQYLRVHRV IGPIQKSYGK VVQNMACFVV ILFIYLMAFA
Cca21784 DYKMDISMLV LFVLFFILRA IALRGSCYFL GPATVLACVR VLRYLSWHPV IGPVQRAISK IVEEILLFLV ILMVFLTAFA
Cca260897_ KYFKNVWNCL DILMILIAIV CMAFNIYRQV QFNHMSAVLI FFAWFKLFKY ISFNKTMNQL SSTLSKCAKD VLGFCVMFFI
Sla36613_6 ---------- ---------- -----YCYLL AFVVFMSTLK FINMLKFNRR MGLLTETIKA SVADMKGFAI TFFIYVMAFA
Sycon24227 VTDGNNYLQV GSILLILAIV PLRWLDQDAQ WHVACFAYIF VCLRILQLIS VTRMVGIYLQ ILTRILKDIS RFVIIFGIFL
Sycon24228 DGNNYLQVGS ILLILAIVPL RWLDQDAQWH VACFAYIFVC LRILQLISVT RMVGIYLQIL TRILKDISRF VIIFGIFLLT
Sycon9763_ ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------SF
Ava16635_4 WNQFDTAFAL FFIMGTVLRL IPLSYSRHMY AVIGFVLFFR LLQFLVIIKD SGPFVYMVFR MLKNLGSFLI LALIFLLAYG
Ava9904_1 WNILDLALIL LFLLAFSIKL GCSAWVHRVY ALIILILYIR IFQYLIMSAY FGVIVLIIFA LAEEVVYFVF VLLLSMIGFG
Ava7536_5 WNYIDWLAYI LMVATIVSHV ADADWHARIG VVNIIVLWFR FLKYLRPFEF IGWFITIVIY LKADIIRFAI FLMTLLIPYS
Ava6620_1 WNILDIAIAI GFVATISLRI SVNVIARLVF GLFGVFLYMR FFQYVLVIRG LGPLIYTCYI AFKRMLKFLV IFVLVSLGFG
Cca31351 ---------- ---------- ---------Y TFMIPTGWIH AVYFLHSFNI EGQLIVSEIE AKKVPLKFRY PFFVEMLWHV
Cca30979_3 WNWVEMLVIV LGWTTTGVYI ARDTIYLTGS SLVVFFSFLK ILRLLQFNRR MLLLAGTLRR SGMQLIAFAI AFLIVMVAFA
Ava26193_2 YNYLDMGAIV CSLLIIPLRF SQENAQWFFA AIAYLLNVMR GYKFAVVLRT TGAYVEIIGS ILYDIVPFSI VFIMFLFGFS
Ava26160_2 ---------- ---------- ADADWHARIG VVNIIVLWFR FLKYLRPFEF IGWFITIVIY LKADIIRFAI FLMTLLIPYS
TRPC1_Hsap MLQDFGKGLG MFLLVLFSFT IGLTQLY--- DTFHSFIGTC FALFWYIFSL VAIFVTRLQS FVGAVIVGTY NVVVVIVLTK
TRPC1_Mmus QDFGKFLGMF LLVLFSFTIG LTQLY---DT FHSFIGTCFA LFWYIFSLVA IFVTRLQSFV GAVIVGTYNV VVVIVLTKLL
TRPC1_Drer MLQEFGKGLG LFLLVLISFT IGLTQLYGKD DTFHTFMGTC YALFWYIFSL VNLFVTRLRS FVGALIVGTY NIVVVIVLTK
TRPC2_Mmus MIDDMIRGMF ILMIILTAFL CGLNNIYVPY EKLGNFNETF QFLFWTMFGM EEHTVVDVPE FVGRAMYGIF TIVMVIVLLN
TRPC2_Drer MIDDMMRGMF ILVIIGTAFL CGINNIYVPY INLGKLNETF SFLFWTMFGM ADQTYVDLAE LVGRILYGIY TLIIVIVLLN
TRPC2_Drer MIDDMMRGMF ILMIIGTAFL CGINNIYVPY IHLGRLNETF NFLFWTMFGM ANQGYVDLAE FVGRIFYGIF TLLIVIVLLN
TRPC3_Hsap TVKDIFKGMV LFIMVFFAFM IGMFILYSYY GAFTTVEESF KTLFWSIFGL SEVTSVVFIE NIGYVLYGIY NVTMVVVLLN
TRPC3_Mmus KDIFKFMVLF IMVFLAFMIG MFILYSYYGA FTTVEESFKT LFWSIFGLSE VTSVVFIENI GYVLYGIYNV TMVVVLLNML
TRPC4_Hsap MLLDILKGLF IYCLVLLAFA NGLNQLYFYY EAFSTLFETL QSLFWSIFGL INLYVTNFTE FVGATMFGTY NVISLVVLLN
TRPC4_Mmus MLLDILKGLF IYCLVLLAFA NGLNQLYFYY EAFSTLFETL QSLFWSIFGL INLYVTNFTE FVGATMFGTY NVISLVVLLN
TRPC4_Drer MLLDILKGLF IYCLVLLAFA NGLNQLYFYY NAFSTLFETL QSLFWSIFGL ISLYVTNFTE FVGATMFGTY NVISLVVLLN
TRPC4_Dmel MVMDIMKGFF LYVLVLFAFG SGLNQLLWYY DRFSNLFETT QTLFWAVFGL IDLDSFEFTR FWGMLMFGTY SVINIVVLLN
TRPC4_Cele MVIDIVKGFM VYALVLFAFA CGLNQLLWYY SSCSSIYHTA ETLFWALFGL VDLTHFRLSE WTGKTIFGSY CCCSIIVLLN
TRPC5_Hsap LDILKFLGIY CLVLLAFANG LNQLYFYYTA FSTLFETLQS LFWSVFGLLN LYVTNFTEFV GATMFGTYNV ISLVVLLNML
TRPC5_Mmus LDILKFLGIY CLVLLAFANG LNQLYFYYTA FSTLFETLQS LFWSVFGLLN LYVTNFTEFV GATMFGTYNV ISLVVLLNML
TRPC6_Hsap KDIFKFMVIF IMVFVAFMIG MFNLYSYYGA FTTVEESFKT LFWAIFGLSE VKSVVFIENI GYVLYGVYNV TMVIVLLNML
TRPC6_Mmus KDIFKFMVIF IMVFVAFMIG MFNLYSYYGA FTTVEESFKT LFWAIFGLSE VKSVVFIENI GYVLYGVYNV TMVIVLLNML
TRPC6_Drer TVKDIFKGMV IFILVFLAFM IGMFNLYSYY GAFTTVEESF KTLFWAIFGL SEVKSVVFIE NIGYVLYGVY NVTVVIVLLN
TRPC6_Drer TVKDIFKGMV IFIMVFVAFM IGMFNLYSYY GAFTTVEESF KTLFWAIFGL SEVKSVVFIE NIGYVLYGVY NVTMVIVLLN
TRPC6_Cele MLVDVAKGCF IFVLIISSFS IGLAQLYWYY PVFSSIADSY LTLLWSLFSI TKPEDTDITQ WVGQGMFIMY HCTSIIVLLN
TRPC7_Hsap TVKDIFKGMV IFIMVFVAFM IGMFNLYSYY GAFTTVEESF KTLFWSIFGL SEVISVVFIE NIGYVLYGVY NVTMVVVLLN
TRPC7_Mmus KDIFKFMVIF IMVFVAFMIG MFNLYSYYGA FTTVEESFKT LFWSIFGLSE VISVVFIENI GYVLYGVYNV TMVVVLLNML
TRPC7_Drer TVKDIFKGMV IFIMVFLAFM IGMFNLYSYY GAFTTVEESF KTLFWSIFGL SEVISVVFIE NIGYILYGVY NVTMVVVLLN
TRPN_Dmel_ LLKDLARGLA VLAIFVFGFS MHIVALNQSF NYFSDPINSF ELLFFAVFGQ TTTEQTQWVE YLFKIVFGIY MLVSVVVLIN
TRPN_Cele_ LMYDLARGLV ILMLFVAGFT LHVTSIFQPA QRLASPSQTL EMLFFSLFGL VEPDSMPFAK IILKLLFGIY MMVTLIVLIN
TRPN_Drer_ LIKDLCRGAV ILMLFHTAFT LSLTALCQPL PTVPGPLNMS VLLFFALFGL TEPDKIPATA VLAKMVFGVY LVVTFIVLIN
TRPM1_Hsap MMIDMLYGVV IMLVVLMSFG VARQAILHPE ESWKLARNIF YMPYWMIYGE VFADQIDPGA WLTPALMACY LLVANILLVN
TRPM1_Mmus MMIDMLYGVV IMLVVLMSFG VARQAILHPE ESWKLARNIF YMPYWMIYGE VFADQIDPGA WLTPALMACY LLVANILLVN
TRPM2_Hsap MMKDVFFGLF LLAVWVVSFG VAKQAILIHN EVDWLFRGAV YHSYLTIFGQ IP-GYIDFPE WLTVLLLCLY LLFTNILLLN
TRPM2_Mmus MMKDVFFGLF LLAVWVVSFG VAKQAILIHN EVDWIFRGVV YHSYLTIFGQ IP-TYIDFPE WLTVTLLCLY LLFANILLLN
TRPM3_Hsap MMIDMMYGVI IMLVVLMSFG VARQAILFPN ESWKLAKNIF YMPYWMIYGE VFADQIDTGA WIVPAIMACY LLVANILLVN
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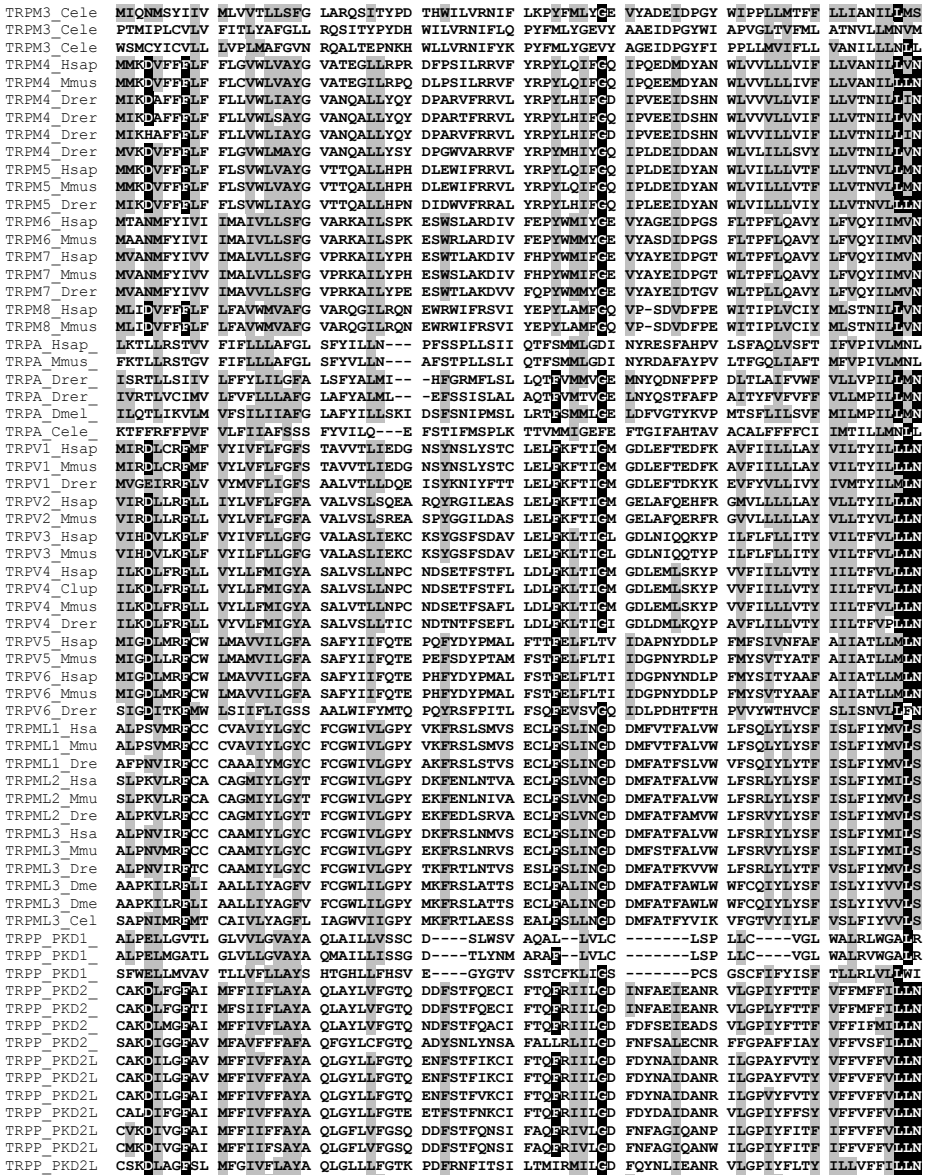
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TRPM4_Mmus MMKDVFFFLF FLCVWLVAYG VATEGILRPQ DLPSILRRVF YRPYLQIFGQ IPQEEMDYAN WLVVLLLIVF LLVANILLLN
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TRPM4_Drer MIKHAFFFLF FLLVWLIAYG VANQALLYQY DPARVFRRVL YRPYLHIFGD IPVEEIDSHN WLVVILLVIF LLVTNILLIN
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TRPM5_Mmus MMKDVFFFLF FLSVWLVAYG VTTQALLHPH DLEWIFRRVL YRPYLQIFGQ IPLDEIDYAN WLVILLLVTF LLVTNVLLMN
TRPM5_Drer MIKDVFFFLF FLSVWLIAYG VTTQALLHPN DIDWVFRRAL YRPYLHIFGQ IPLEEIDYAN WLVILLLVIY LLVTNVLLLN
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TRPA_Drer_ ISRTLLSIIV LFFYLILGFA LSFYALMI-- -HFGRMFLSL LQTFVMMVGE MNYQDNFPFP DLTLAIFVWF VLLVPILLMN
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TRPA_Cele_ KTFFRFFPVF VLFIIAFSSS FYVILQ---E FSTIFMSPLK TTVMMIGEFE FTGIFAHTAV ACALFFFCI IMTILLMNLL
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Sla36613_6 MFAFLLFGTQ AAFGSFGSTI TSLFLFVLGT YDFESMQAQP FLGPVFFFLF IFIVFFGLQA MFLTIIIEAF ETVRTDVKQK
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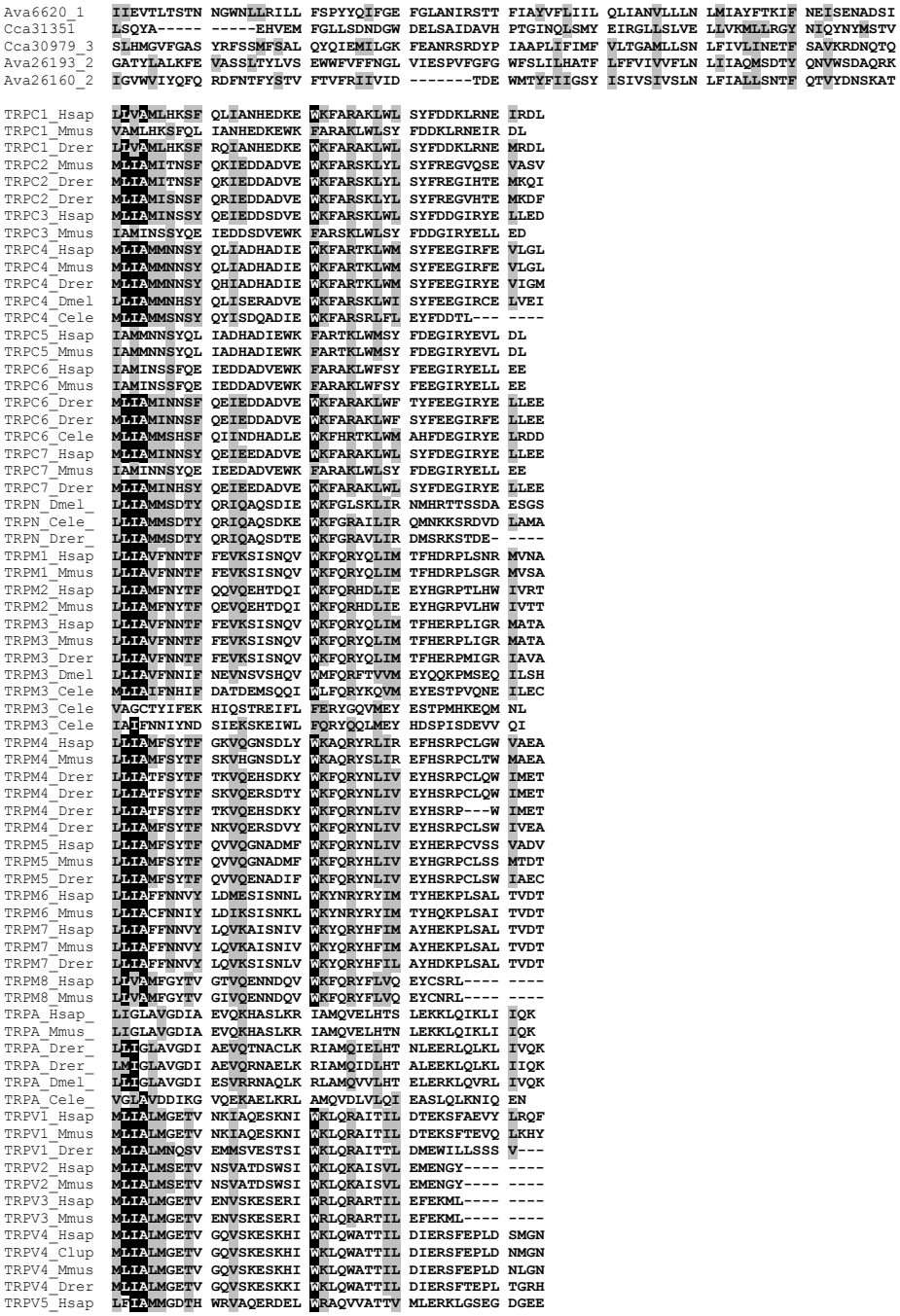
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Ava26193_2 GATYLALKFE VASSLTYLVS EWWFVFFNGL VIESPVFGFG WFSLILHATF LFFVIVVFLN LIIAQMSDTY QNVWSDAQRK
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TRPC1_Drer LIVAMLHKSF RQIANHEDKE WKFARAKLWL SYFDDKLRNE MRDL
TRPC2_Mmus MLIAMITNSF QKIEDDADVE WKFARSKLYL SYFREGVQSE VASV
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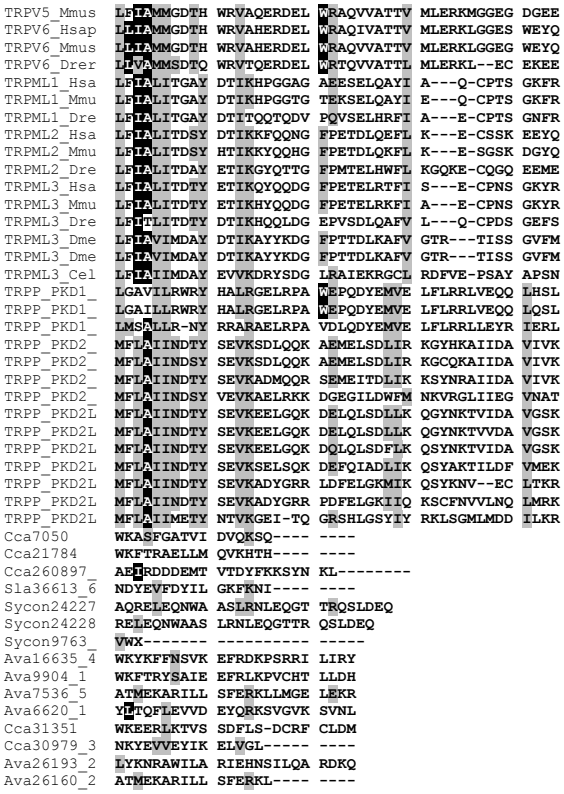
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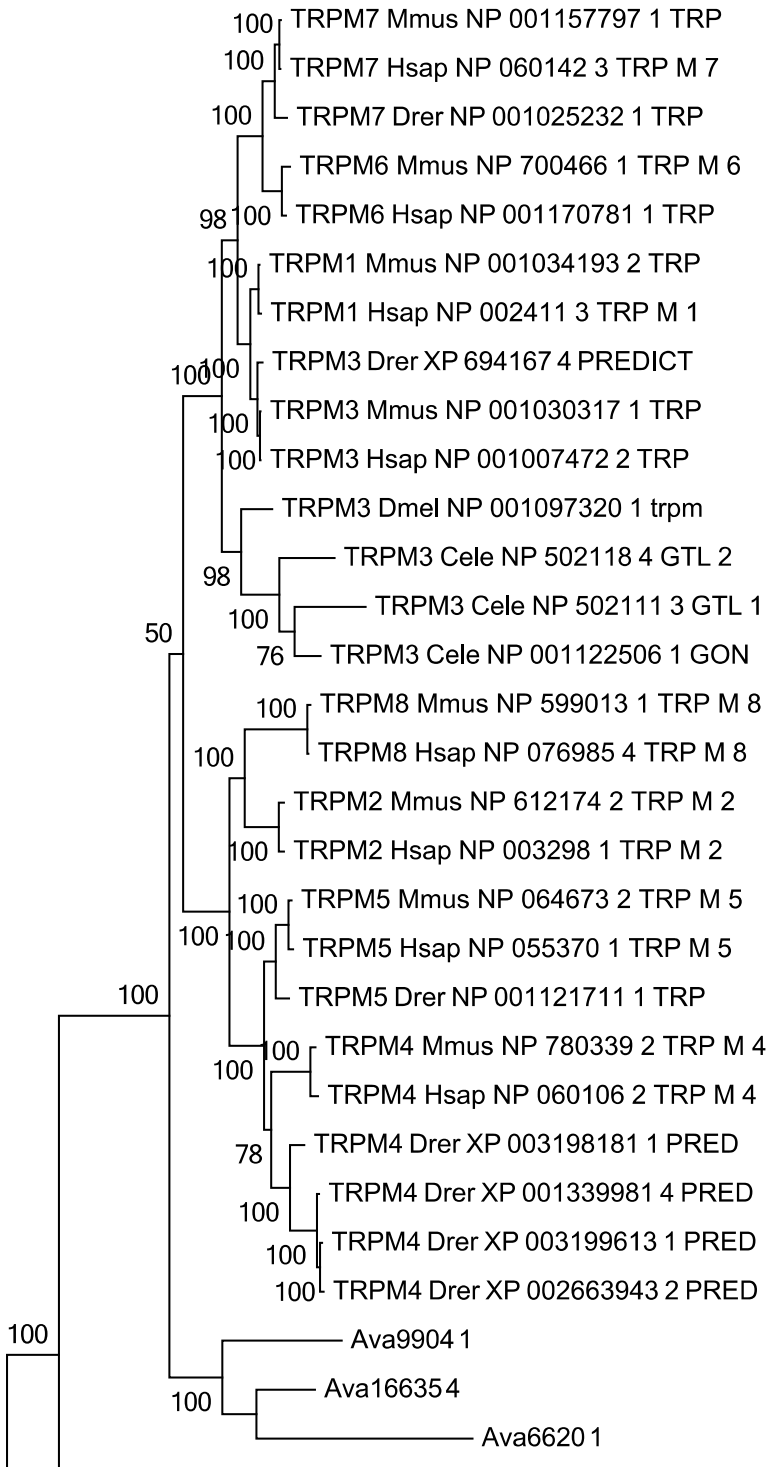
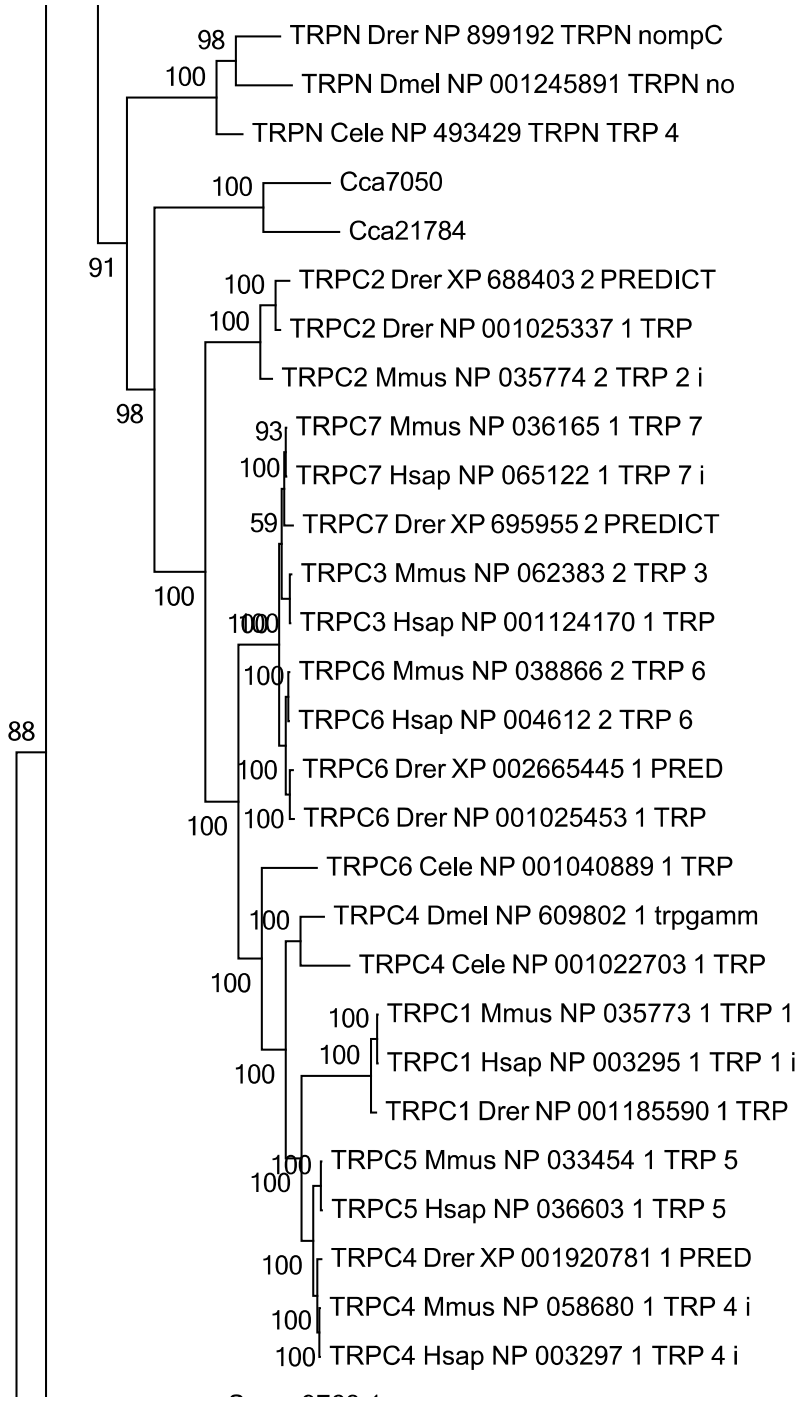
TRPP_PKD1_ LGAILLRWRY HALRGELRPA WEPQDYEMVE LFLRRLVEQQ LQSL
TRPP_PKD1_ LMSALLR-NY RRARAELRPA VDLQDYEMVE LFLRRLLEYR IERL
TRPP_PKD2_ MFLAIINDTY SEVKSDLQQK AEMELSDLIR KGYHKAIIDA VIVK
TRPP_PKD2_ MFLAIINDSY SEVKSDLQQK AEMELSDLIR KGCQKAIIDA VIVK
TRPP_PKD2_ MFLAIINDTY SEVKADMQQR SEMEITDLIK KSYNRAIIDA VIVK
TRPP_PKD2_ MFLAIINDSY VEVKAELRKK DGEGILDWFM NKVRGLIIEG VNAT
TRPP_PKD2L MFLAIINDTY SEVKEELGQK DELQLSDLLK QGYNKTVIDA VGSK
TRPP_PKD2L MFLAIINDTY SEVKEELGQK DELQLSDLLK QGYNKTVVDA VGSK
TRPP_PKD2L MFLAIINDTY SEVKEELGQK DQLQLSDFLK QSYNKTVIDA VGSK
TRPP_PKD2L MFLAIINDTY SEVKSELSQK DEFQIADLIK QSYAKTILDF VMEK
TRPP_PKD2L MFLAIINDTY SEVKADYGRR LDFELGKMIK QSYKNV--EC LTKR
TRPP_PKD2L MFLAIINDTY SEVKADYGRR PDFELGKIIQ KSCFNVVLNQ LMRK
TRPP_PKD2L MFLAIIMETY NTVKGEI-TQ GRSHLGSYIY RKLSGMLMDD ILKR
Cca7050 WKASFGATVI DVQKSQ---- ----
Cca21784 WKFTRAELLM QVKHTH---- ----
Cca260897_ AEIRDDDEMT VTDYFKKSYN KL--------
Sla36613_6 NDYEVFDYIL GKFKNI---- ----
Sycon24227 AQRELEQNWA ASLRNLEQGT TRQSLDEQ
Sycon24228 RELEQNWAAS LRNLEQGTTR QSLDEQ
Sycon9763_ VWX------- ---------- -----
Ava16635_4 WKYKFFNSVK EFRDKPSRRI LIRY
Ava9904_1 WKFTRYSAIE EFRLKPVCHT LLDH
Ava7536_5 ATMEKARILL SFERKLLMGE LEKR
Ava6620_1 YLTQFLEVVD EYQRKSVGVK SVNL
Cca31351 WKEERLKTVS SDFLS-DCRF CLDM
Cca30979_3 NKYEVVEYIK ELVGL----- ----
Ava26193_2 LYKNRAWILA RIEHNSILQA RDKQ
Ava26160_2 ATMEKARILL SFERKL---- ----

A1.4 Phylobayes alignment of data in 2-5c.

(Tree has been split into four pages for viewing. For the full scale complete tree see http://www.biomedcentral.com/1471-2148/14/3/additional)

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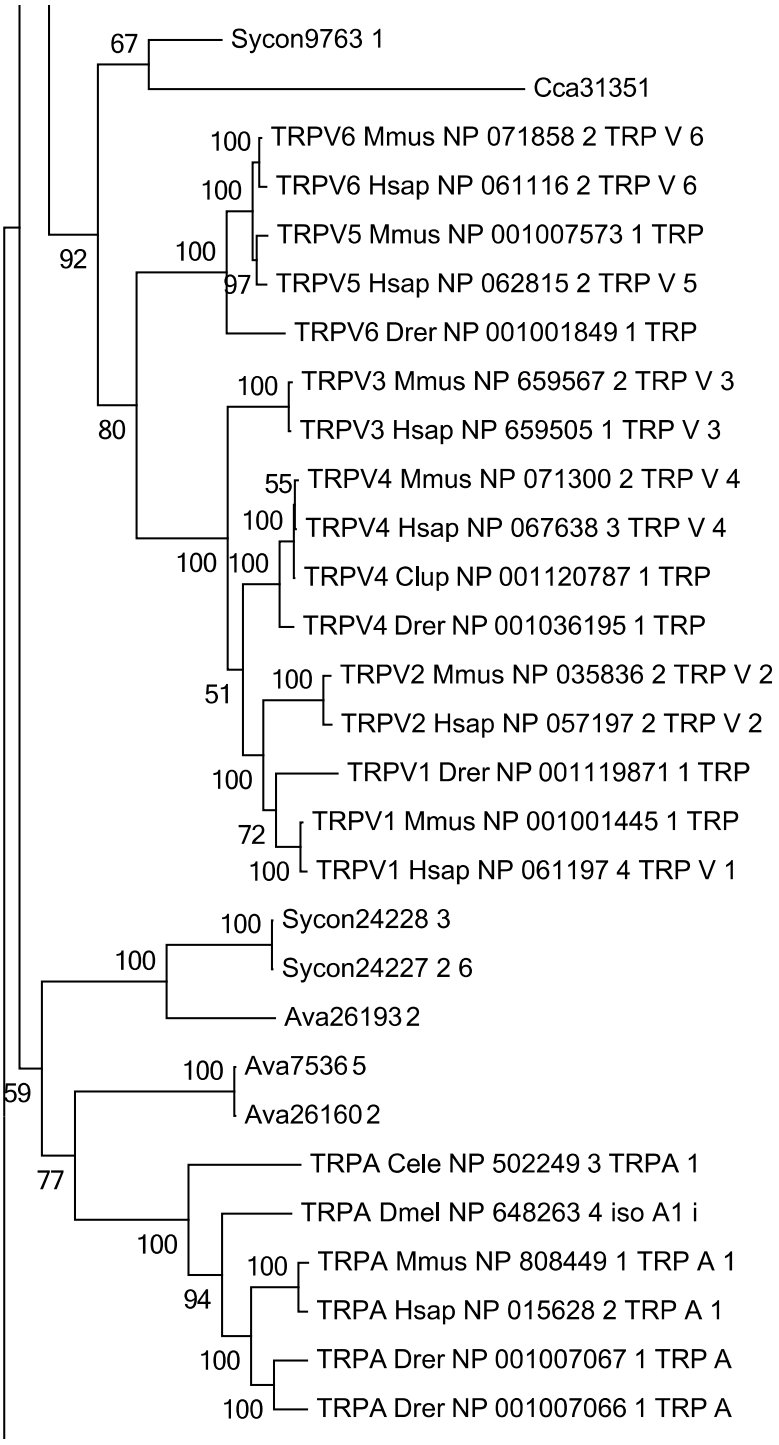
TRPN Dmel NP 001245891 TRPN no
TRPN Cele NP 493429 TRPN TRP 4
Cca7050
Cca21784
TRPC2 Drer XP 688403 2 PREDICT
TRPC2 Drer NP 001025337 1 TRP
TRPC2 Mmus NP 035774 2 TRP 2 i
TRPC7 Mmus NP 036165 1 TRP 7
TRPC7 Hsap NP 065122 1 TRP 7 i
TRPC7 Drer XP 695955 2 PREDICT
TRPC3 Mmus NP 062383 2 TRP 3
TRPC3 Hsap NP 001124170 1 TRP
TRPC6 Mmus NP 038866 2 TRP 6
TRPC6 Hsap NP 004612 2 TRP 6
TRPC6 Drer XP 002665445 1 PRED
TRPC6 Drer NP 001025453 1 TRP
TRPC6 Cele NP 001040889 1 TRP
TRPC4 Dmel NP 609802 1 trpgamm
TRPC4 Cele NP 001022703 1 TRP
TRPC1 Mmus NP 035773 1 TRP 1
TRPC1 Hsap NP 003295 1 TRP 1 i
TRPC1 Drer NP 001185590 1 TRP
TRPC5 Mmus NP 033454 1 TRP 5
TRPC5 Hsap NP 036603 1 TRP 5
TRPC4 Drer XP 001920781 1 PRED
TRPC4 Mmus NP 058680 1 TRP 4 i
TRPC4 Hsap NP 003297 1 TRP 4 i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Cca31351
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TRPV6 Hsap NP 061116 2 TRP V 6
TRPV5 Mmus NP 001007573 1 TRP
TRPV5 Hsap NP 062815 2 TRP V 5
TRPV6 Drer NP 001001849 1 TRP
TRPV3 Mmus NP 659567 2 TRP V 3
TRPV3 Hsap NP 659505 1 TRP V 3
TRPV4 Mmus NP 071300 2 TRP V 4
TRPV4 Hsap NP 067638 3 TRP V 4
TRPV4 Clup NP 001120787 1 TRP
TRPV4 Drer NP 001036195 1 TRP
TRPV2 Mmus NP 035836 2 TRP V 2
TRPV2 Hsap NP 057197 2 TRP V 2
TRPV1 Drer NP 001119871 1 TRP
TRPV1 Mmus NP 001001445 1 TRP
TRPV1 Hsap NP 061197 4 TRP V 1
Sycon24228 3
Sycon24227 2 6
Ava261932
Ava753655
Ava261602
TRPA Cele NP 502249 3 TRPA 1
TRPA Dmel NP 648263 4 iso A1 i
TRPA Mmus NP 808449 1 TRP A 1
TRPA Hsap NP 015628 2 TRP A 1
TRPA Drer NP 001007067 1 TRP A
TRPA Drer NP 001007066 1 TRP A
67
92
100
100
100
97
80
100
55
100
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100
51
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72
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100
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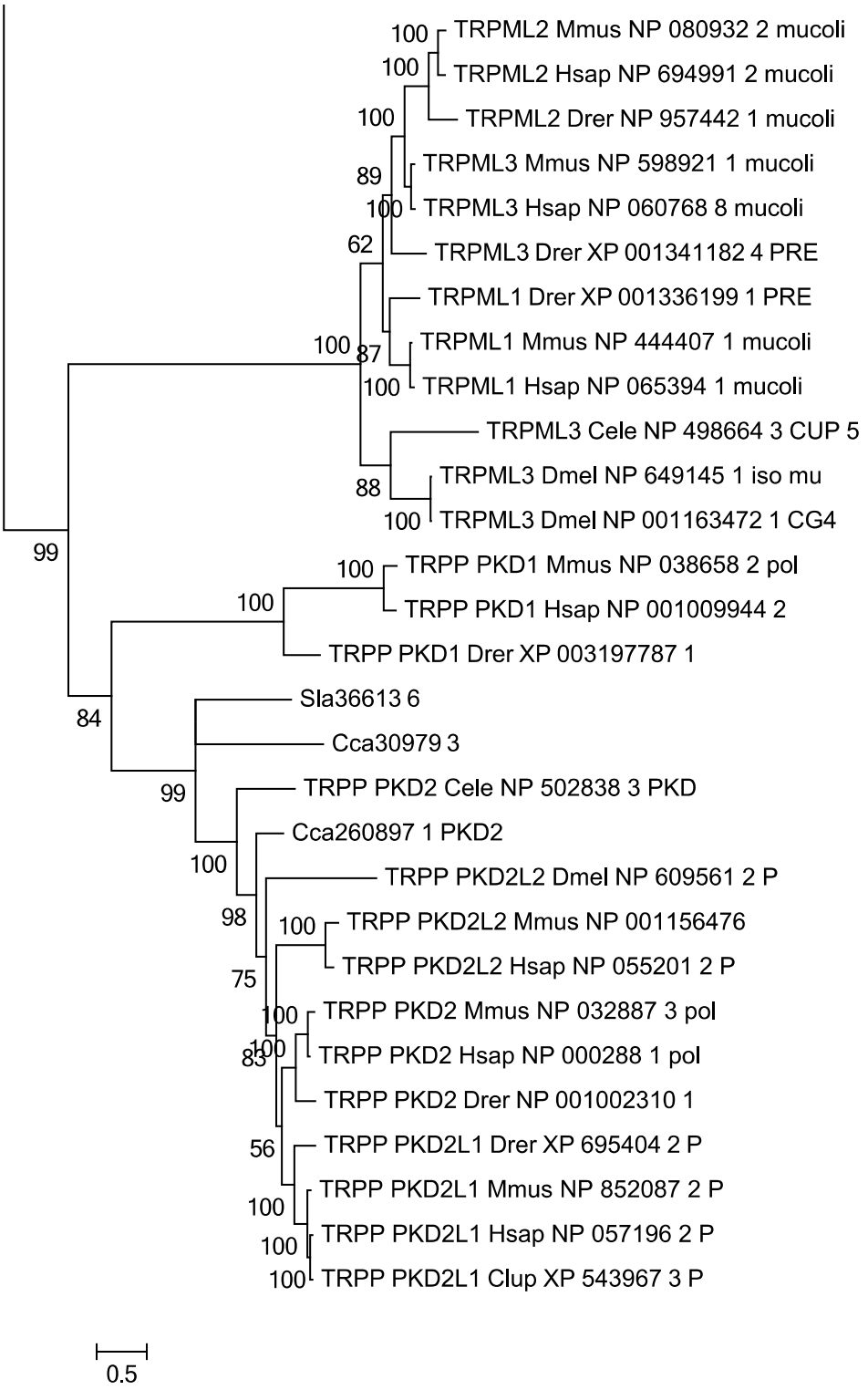
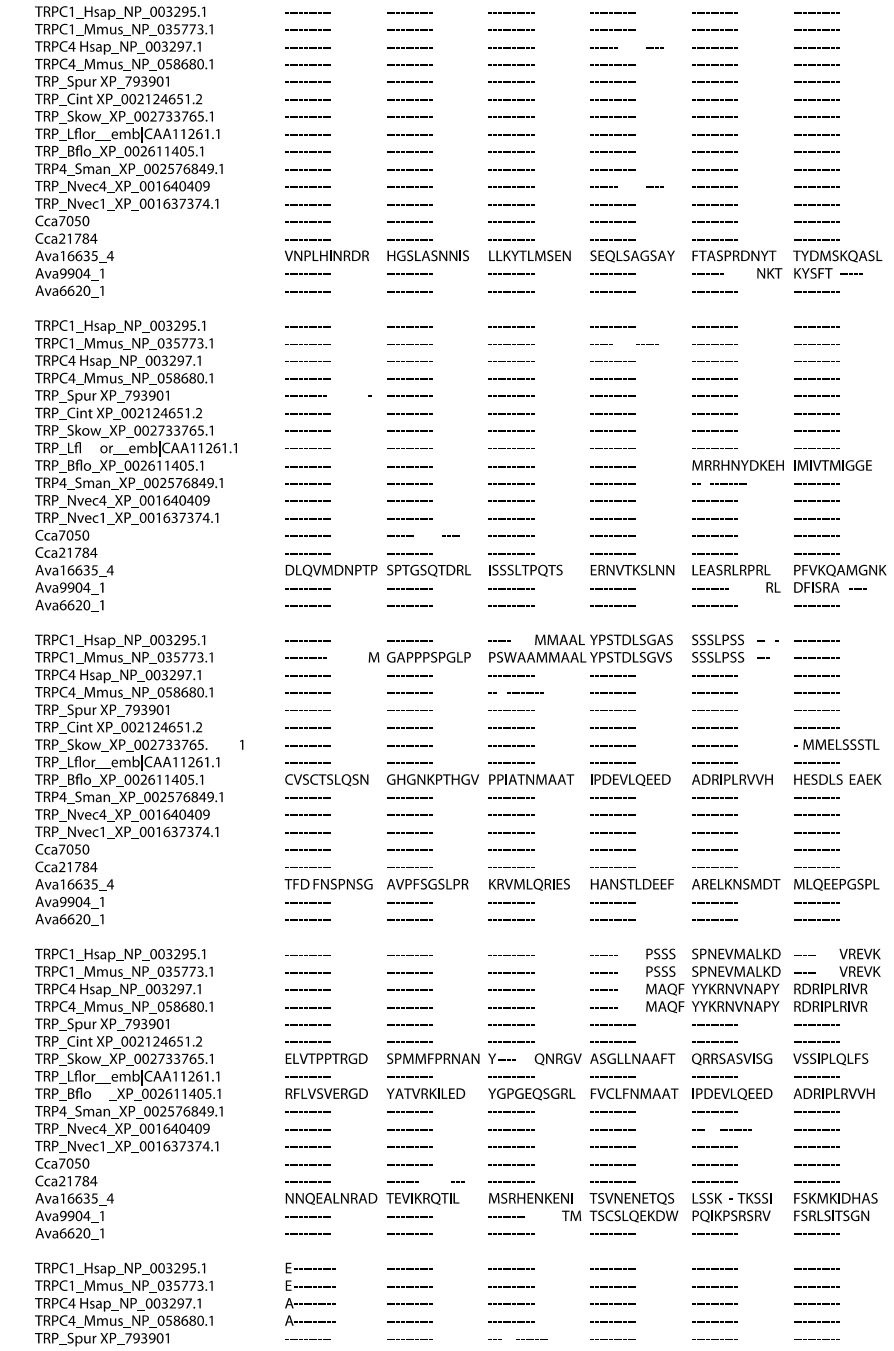
59
100
77
100
100
94
100
100


TRPML2 Mmus NP 080932 2 mucoli
TRPML2 Hsap NP 694991 2 mucoli
TRPML2 Drer NP 957442 1 mucoli
TRPML3 Mmus NP 598921 1 mucoli
TRPML3 Hsap NP 060768 8 mucoli
TRPML3 Drer XP 001341182 4 PRE
TRPML1 Drer XP 001336199 1 PRE
TRPML1 Mmus NP 444407 1 mucoli
TRPML1 Hsap NP 065394 1 mucoli
TRPML3 Cele NP 498664 3 CUP 5
TRPML3 Dmel NP 649145 1 iso mu
TRPML3 Dmel NP 001163472 1 CG4
TRPP PKD1 Mmus NP 038658 2 pol
TRPP PKD1 Hsap NP 001009944 2
TRPP PKD1 Drer XP 003197787 1
Sla36613 6
Cca30979 3
TRPP PKD2 Cele NP 502838 3 PKD
Cca260897 1 PKD2
TRPP PKD2L2 Dmel NP 609561 2 P
TRPP PKD2L2 Mmus NP 001156476
TRPP PKD2L2 Hsap NP 055201 2 P
TRPP PKD2 Mmus NP 032887 3 pol
TRPP PKD2 Hsap NP 000288 1 pol
TRPP PKD2 Drer NP 001002310 1
TRPP PKD2L1 Drer XP 695404 2 P
TRPP PKD2L1 Mmus NP 852087 2 P
TRPP PKD2L1 Hsap NP 057196 2 P
TRPP PKD2L1 Clup XP 543967 3 P
0.5

A1.5 Full alignment of sequences in Figure 2-5c and list of Sponge TRP Fastas

Supplementary Figure S4



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TRP_Cint_XP_002124651.2    ---------- ---------- ---------- ---------- ---------- ----------
TRP_Skow_XP_002733765.1    E--------- ---------- ---------- ---------- ---------- ----------
TRP_Lflor__emb|CAA11261.1  ---------- ---------- ---------- ---------- ---------- ----------
TRP_Bflo_XP_002611405.1    H--------- ---------- ---------- ---------- ---------- ----------
TRP4_Sman_XP_002576849.1   ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec4_XP_001640409     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1   ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                    ---------- ---MSTDCLD VDMLVMLCQS DVDLLLSDEN NITAMHHMVE KHRPTDAHLV
Cca21784                   ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                 PGDHVEMNKL FTVPD-SISN TEITPLLDQH TGNVENEIEK TVDGHIHFPT RHGRKDANYL
Ava9904_1                  EKAEFLEEQL QNVLSVTYQG SDISEIPCVH SKALKKSDEI EIDTDFEYSC GNVKFEGAYE
Ava6620_1                  ---------- ---------- ---------- ---------- ---------- ----------

TRPC1_Hsap_NP_003295.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC1_Mmus_NP_035773.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC4_Hsap_NP_003297.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC4_Mmus_NP_058680.1     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Spur_XP_793901         ---------- ---------- ---------- ---------- ---------- ----------
TRP_Cint_XP_002124651.2    ---------- ---------- ---------- ---------- ---------- ----------
TRP_Skow_XP_002733765.1    ---------- ---------- ---------- ---------- ---------- ----------
TRP_Lflor__emb|CAA11261.1  ---------- ---------- ---------- ---------- ---------- ----------
TRP_Bflo_XP_002611405.1    ---------- ---------- ---------- ---------- ---------- ----------
TRP4_Sman_XP_002576849.1   ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec4_XP_001640409     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1   ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                    HTLASERP-- ---------- ---------- ---------- ---------- ----------
Cca21784                   ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                 IIPPQASPS- ---------D LLDVLSKTWK ---------- ----LPSPKI IISLPGTSQ-
Ava9904_1                  VADPAKKPKI VSITPKCEVN QLPKIHKYFK YDATHREYFV HHFRLCKPNL TLILPSTTDH
Ava6620_1                  ---------- ---------- ---------- ---------- ---------- ----------

TRPC1_Hsap_NP_003295.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC1_Mmus_NP_035773.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC4_Hsap_NP_003297.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC4_Mmus_NP_058680.1     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Spur_XP_793901         ---------- ---------- ---------- ---------- ---------- ----------
TRP_Cint_XP_002124651.2    ---------- ---------- ---------- ---------- ---------- ----------
TRP_Skow_XP_002733765.1    ---------- ---------- ---------- ---------- ---------- ----------
TRP_Lflor__emb|CAA11261.1  ---------- ---------- ---------- ---------- ---------- ----------
TRP_Bflo_XP_002611405.1    ---------- ---------- ---------- ---------- ---------- ----------
TRP4_Sman_XP_002576849.1   ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec4_XP_001640409     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1   ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                    ---------- ---------- ---------- ---------- ---------- ----------
Cca21784                   ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                 ------LSQG SRFRQMLKEN LIRIAATTKA WFLTEGVNKG ISAFVGSCLQ SHAYKRFAKK
Ava9904_1                  CPENAIRNRD PNYMKNFLSD MRQIIRNTDT WVLTNGLNWG LTRIIGENIA TRVLSPFTAE
Ava6620_1                  ---------- ---------- ---------- ---------- ---------- ----------

TRPC1_Hsap_NP_003295.1     ---------- ---------- ---------- ---------- ---------- -------ENT
TRPC1_Mmus_NP_035773.1     ---------- ---------- ---------- ---------- ---------- -------ENT
TRPC4_Hsap_NP_003297.1     ---------- ---------- ---------- ---------- ---------- -------ESE
TRPC4_Mmus_NP_058680.1     ---------- ---------- ---------- ---------- ---------- -------ESE
TRP_Spur_XP_793901         ---------- ---------- ---------- ---------- ---------- -------MPG
TRP_Cint_XP_002124651.2    ---------- ---------- ---------- ---------- ---------- ----------
TRP_Skow_XP_002733765.1    ---------- ---------- ---------- ---------- ---------- -------GNR
TRP_Lflor__emb|CAA11261.1  ---------- ---------- ---------- ---------- ---------- MSKKPPRKRD
TRP_Bflo_XP_002611405.1    ---------- ---------- ---------- ---------- ---------- -------ESD
TRP4_Sman_XP_002576849.1   ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec4_XP_001640409     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1   ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                    ---------- ---------- ---------- ---------- ---------- -------YDF
Cca21784                   ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                 EQNQRDWFIG TGFGDYDAPI PIIGMVNPEN IYKGTLFQNP TDSITYGKDN SSLCFPDRNL
Ava9904_1                  T--------- ---------I ELIGIQKFEK L--PATVRNC ISRLSMKAQL EEDTIP-RGC
Ava6620_1                  ---------- ---------- ----FVKKDS HTVMARNEEL IPLREREEIQ EVLPIRHSDN

TRPC1_Hsap_NP_003295.1     LN--EKLELL ACDKGDYYMV KK--------- ---------- ----------
TRPC1_Mmus_NP_035773.1     LN--EKLELL ACDKGDYYMV KK--------- ---------- ----------
TRPC4_Hsap_NP_003297.1     LSPSEKAYLN AVEKGDYASV KK--------- ---------- ----------
TRPC4_Mmus_NP_058680.1     LSPSEKAYLN AVEKGDYASV KK--------- ---------- ----------
TRP_Spur_XP_793901         SNHKEFGELL ARSPMTETER PR--------- ---------- ----------
TRP_Cint_XP_002124651.2    ---------- ---------- MV--------- ---------- ----------
TRP_Skow_XP_002733765.1    LTAKERMEME AAEKGDNATI QK--------- ---------- ----------
TRP_Lflor__emb|CAA11261.1  LNNEERQELG AVARGDVGSV KQ--------- ---------- ----------
TRP_Bflo_XP_002611405.1    LSEAEKFELV SVERGDYATV RK--------- ---------- ----------
TRP4_Sman_XP_002576849.1   ---------- ---------- ---------- ---------- ----------
```

```
TRP_Nvec4_XP_001640409     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1   ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                    NTIRDSLGLH ACVHGSKATL QE-------- ---------- ---------- ----------
Cca21784                   ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                 LDANHSHGIV LSKSISGNSL DQ-WSC---- ---------- ---------- --------IE
Ava9904_1                  LEENHSLGLS LKDNLDRDEI ETFWDT---- ---------- ---------- --------IE
Ava6620_1                  LTMKHWEYVE AARLDNREQQ PNFWKIPFAF GHSLVDDSKD DRLVTHYKHV PQKSIHQETK

TRPC1_Hsap_NP_003295.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC1_Mmus_NP_035773.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC4_Hsap_NP_003297.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC4_Mmus_NP_058680.1     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Spur_XP_793901         ---------- ---------- ---------- ---------- ---------- ----------
TRP_Cint_XP_002124651.2    ---------- ---------- ---------- ---------- ---------- ----------
TRP_Skow_XP_002733765.1    ---------- ---------- ---------- ---------- ---------- ----------
TRP_Lflor__emb|CAA11261.1  ---------- ---------- ---------- ---------- ---------- ----------
TRP_Bflo_XP_002611405.1    ---------- ---------- ---------- ---------- ---------- ----------
TRP4_Sman_XP_002576849.1   ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec4_XP_001640409     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1   ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                    ---------- ---------- ---------- ---------- ---------- ----------
Cca21784                   ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                 ET-------- ---------- ---------- ---------- ---------- ----------
Ava9904_1                  HTQIIKRIED IKAATCRESI ILDLEQKWDK NREAPNTPKT KTSLVELSTK RNSEVGEPKP
Ava6620_1                  DEGDLVKKPS TKFYDIVEIP ILDDPSNIDF LKYAKSLKQI LEVGKDLAYD RFNYRSKIDF

TRPC1_Hsap_NP_003295.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC1_Mmus_NP_035773.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC4_Hsap_NP_003297.1     ---------- ---------- ---------- ---------- ---------- ----------
TRPC4_Mmus_NP_058680.1     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Spur_XP_793901         ---------- ---------- ---------- ---------- ---------- ----------
TRP_Cint_XP_002124651.2    ---------- ---------- ---------- ---------- ---------- ----------
TRP_Skow_XP_002733765.1    ---------- ---------- ---------- ---------- ---------- ----------
TRP_Lflor__emb|CAA11261.1  ---------- ---------- ---------- ---------- ---------- ----------
TRP_Bflo_XP_002611405.1    ---------- ---------- ---------- ---------- ---------- ----------
TRP4_Sman_XP_002576849.1   ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec4_XP_001640409     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1   ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                    ---------- ---------- ---------- ---------- ---------- ----------
Cca21784                   ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                 ---------- -VSNHLKIGK EPL------- ---------- ---------- -----PIVRI
Ava9904_1                  DGDLIEMGKL EKANFVIFED EPTRVGRTID SGPRIPSEFQ NLWFKQIEFK DPDINPAVCL
Ava6620_1                  FNMIIYIPEI TKTGAGLLAV PNTAVAILGK VITDLLTKNK ALIILDARAK GPITNLVLES

TRPC1_Hsap_NP_003295.1     ---------- ---------- ----ILEENS SG-DLNINCV DVLG------ --------RN
TRPC1_Mmus_NP_035773.1     ---------- ---------- ----ILEENS SG-DLNINCV DVLG------ --------RN
TRPC4_Hsap_NP_003297.1     ---------- ---------- ----SLEEAE IYFKINININCI DPLG------ --------RT
TRPC4_Mmus_NP_058680.1     ---------- ---------- ----SLEEAE IYFKINININCI DPLG------ --------RT
TRP_Spur_XP_793901         ---------- ---------- ---------- ---------- ---------- ----------
TRP_Cint_XP_002124651.2    ---------- ---------- ----NLLRAF TNRNIGLGVI GFNG------ --------NV
TRP_Skow_XP_002733765.1    ---------- ---------- ----CLKHPD P---VNVNVT NILG------ --------RS
TRP_Lflor__emb|CAA11261.1  ---------- ---------- ----ALAEAE ERF-IDISCK DSLG------ --------RS
TRP_Bflo_XP_002611405.1    ---------- ---------- ----ILEDYG P--EMDINCT DFLS------ --------RT
TRP4_Sman_XP_002576849.1   ---------- ---------- ---------- ---------- --MG------ ----------
TRP_Nvec4_XP_001640409     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1   ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                    ---------- ---------- ---------- ---------- ---------- ----------
Cca21784                   ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                 VIDNTLHVIE QVGQSMNKNV ATV-VISSNE LMRKIHSYIF TLTV------ --------RD
Ava9904_1                  LLDNSIDGLR HTLHAIRNRV PTILVIGSGI AADMIHQIM- -ILS------ --------RD
Ava6620_1                  LERAKHDGVK QITERIQNRL NFL-GFYDKP LKQDIDPEAP NALGNKRYLL STDGCRELKS

TRPC1_Hsap_NP_003295.1     AVTITIENEN LDILQL---- ---------- ---------- --LLD-Y--- ----------
TRPC1_Mmus_NP_035773.1     AVTITIENES LDILQL---- ---------- ---------- --LLD-Y--- ----------
TRPC4_Hsap_NP_003297.1     ALLIAIENEN LELIEL---- ---------- ---------- --LLS-F--- ----------
TRPC4_Mmus_NP_058680.1     ALLIAIENEN LELIEL---- ---------- ---------- --LLS-F--- ----------
TRP_Spur_XP_793901         ------TRGG IDIIVV---- ---------- ---------- --LLE-H--- ----------
TRP_Cint_XP_002124651.2    AAGIAVELKD DLLIKL---- ---------- ---------- --IVVEH--- ----------
TRP_Skow_XP_002733765.1    ALQISVDNEN IEIVEL---- ---------- ---------- --LLAQP--- ----------
TRP_Lflor__emb|CAA11261.1  SLVIAIENEN DDLVSL---- ---------- ---------- --LLN-Y--- ----------
TRP_Bflo_XP_002611405.1    ALLIAIENEN LELVGL---- ---------- ---------- --LVS-Y--- ----------
TRP4_Sman_XP_002576849.1   ---------- ---------- ---------- ---------- --LLGRF--- ----------
TRP_Nvec4_XP_001640409     ---------- IEMIRL---- ---------- ---------- --LLK-N--- ----------
TRP_Nvec1_XP_001637374.1   ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                    ---------- ---------- ---------- ---------- --LLQ----- ----------
Cca21784                   ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                 QKMASTK--- QDFLKLCCGF LKNEAQSEEI LTNIGFILDK PNLFTMF--- ----------
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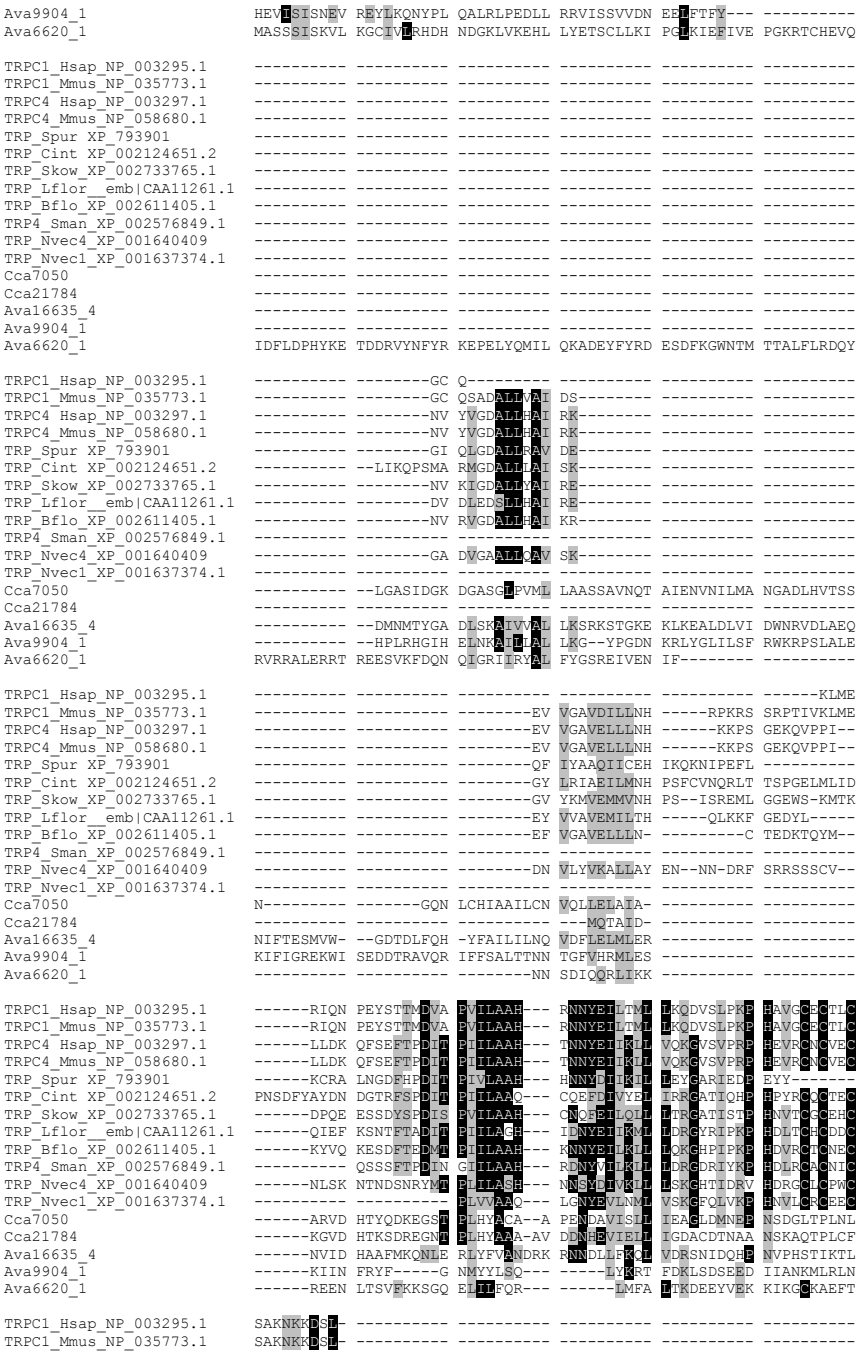

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TRPC4 Hsap_NP_003297.1       VSSSDVDSL- ---------- ---------- ---------- ---------- ----------
TRPC4_Mmus_NP_058680.1       VSSSDVDSL- ---------- ---------- ---------- ---------- ----------
TRP_Spur XP_793901           AFSTETNTL- ---------- ---------- ---------- ---------- ----------
TRP_Cint XP_002124651.2      TAMRRDDAL- ---------- ---------- ---------- ---------- ----------
TRP_Skow_XP_002733765.1      TEKQHEDSL- ---------- ---------- ---------- ---------- ----------
TRP_Lflor__emb|CAA11261.1    LRGSLVDVL- ---------- ---------- ---------- ---------- ----------
TRP_Bflo_XP_002611405.1      AEGYRTDAL- ---------- ---------- ---------- ---------- ----------
TRP4_Sman_XP_002576849.1     TKARRENGL- ---------- ---------- ---------- ---------- ----------
TRP_Nvec4_XP_001640409       QSLGR---I- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1     ----QADNF- ---------- ---------- ---------- ---------- ----------
Cca7050                      SIKSQHVNNV IPLIRAMVDI TKC------- ---------- ---------- ----------
Cca21784                     AVLSNQVTNI VPLIRSSADI RKA------- ---------- ---------- ----------
Ava16635_4                   KPQNKNSNLP NQT------- ---------- --NGQIPEDQ QKIPIAE--- ----------
Ava9904_1                    FSESKHRKDP RQILYEIGKL IKELMGNDYV CFYNHLPISR KKREYEEENI KKNVNSCYNF
Ava6620_1                    ASLE------ ---------- ---------- ---------- ---------- ----------

TRPC1_Hsap_NP_003295.1       ---------- ---------- ---------- ---------- RHSRFRLDIY RCLASP---A
TRPC1_Mmus_NP_035773.1       ---------- ---------- ---------- ---------- RHSRFRLDIY RCLASP---A
TRPC4 Hsap_NP_003297.1       ---------- ---------- ---------- ---------- RHSRSRLNIY KALASP---S
TRPC4_Mmus_NP_058680.1       ---------- ---------- ---------- ---------- RHSRSRLNIY KALASP---S
TRP_Spur XP_793901           ---------- ---------- ---------- ---------- QHSLGMLNIY RALASQ---A
TRP_Cint XP_002124651.2      ---------- ---------- ---------- ---------- NFCLSRLNAY KGLASP---V
TRP_Skow_XP_002733765.1      ---------- ---------- ---------- ---------- RHSLHRINTY KALASP---A
TRP_Lflor__emb|CAA11261.1    ---------- ---------- ---------- ---------- GHSRARINAY RALASP---S
TRP_Bflo_XP_002611405.1      ---------- ---------- ---------- ---------- RHSRQRLNVY RALASP---S
TRP4_Sman_XP_002576849.1     ---------- ---------- ---------- ---------- QHSTLRINTY RALTSP---S
TRP_Nvec4_XP_001640409       ---------- ---------- ---------- ---------- GTSLHRLYTY RALASPVYMS
TRP_Nvec1_XP_001637374.1     ---------- ---------- ---------- ---------- RESQRRLDIY RGLANP---V
Cca7050                      ---------- ---------- ---------- ---------- GLQDCIYQDL QILAQP---E
Cca21784                     ---------- ---------- ---------- ---------- GLQKLRIEKL QLLARP---E
Ava16635_4                   ---IGKLLKY VLK------- --YSFKDVYV ENEGMHDIIE NHPIDNLFIW AVLTQRWRMA
Ava9904_1                    LRHIATLMKQ CLKRFRVRKL TAQTKKLKLV RAEKAQRLFE QFPEEQVFIW CLLFHRWEMA
Ava6620_1                    ---VVKFLQL AIEKLMGDGF LWNIPDVQRH DMQLNLTHYE VYFAEYIMVW GALSQRYEVA

TRPC1_Hsap_NP_003295.1       LIMLT----- ---------- ---------- -----EEDPI LRAFELSADL K---------
TRPC1_Mmus_NP_035773.1       LIMLT----- ---------- ---------- -----EEDPI LRAFELSADL K---------
TRPC4 Hsap_NP_003297.1       LIALS----- ---------- ---------- -----SEDPF LTAFQLSWEL Q---------
TRPC4_Mmus_NP_058680.1       LIALS----- ---------- ---------- -----SEDPF LTAFQLSWEL Q---------
TRP_Spur XP_793901           YISLT----- ---------- ---------- -----SLDPI NTGFERCVKL R---------
TRP_Cint XP_002124651.2      YMSIV----- ---------- ---------- -----TRDPV RHALKLCHEL S---------
TRP_Skow_XP_002733765.1      WISLT----- ---------- ---------- -----SSDPI LTAFKLSWEL E---------
TRP_Lflor__emb|CAA11261.1    LIALS----- ---------- ---------- -----SKDPI LTAFELSWEL R---------
TRP_Bflo_XP_002611405.1      LIMLS----- ---------- ---------- -----SDDPI LTAFQLSWEL RGSVTTRLFN
TRP4_Sman_XP_002576849.1     FIILT----- ---------- ---------- -----SNDPI LTAFELSWEL K---------
TRP_Nvec4_XP_001640409       LTFLS----- ---------- ---------- -----CKDPV RQAFELNKEL V---------
TRP_Nvec1_XP_001637374.1     FISMT----- ---------- ---------- -----SNDPF LTAFELSSEL E---------
Cca7050                      IIEA------ ---------- ---------- -----SPKPL EYTLMLSSFF E---------
Cca21784                     MIQA------ ---------- ---------- -----SAEPL KFVLELSYLY R---------
Ava16635_4                   EVLLSNTNSV I--------- ---------- FNAV-AAKAL IAGMHHHLKD D---------
Ava9904_1                    ILILKYCNKT C--------- ---------- LTTVLSAARL IRGMKLKYQS R---------
Ava6620_1                    ELFLEYSGGH LQELLTRDRE FYRTQKGVYQ IANALAISRL LYGVEQELRF D---------

TRPC1_Hsap_NP_003295.1       ---------- ---ELSLVEV EFRNDYEELA RQCKMFAKDL LAQ---ARNS RELEV--ILN
TRPC1_Mmus_NP_035773.1       ---------- ---ELSLVEV EFRNDYEELA RQCKMFAKDL LAQ---ARNS RELEV--ILN
TRPC4 Hsap_NP_003297.1       ---------- ---ELSKVEN EFKSEYEELS RQCKQFAKDL LDQ---TRSS RELEI--ILN
TRPC4_Mmus_NP_058680.1       ---------- ---ELSKVEN EFKSEYEELS RQCKQFAKDL LDQ---TRSS RELEI--ILN
TRP_Spur XP_793901           ---------- ---KLCQINP EFSVEFNTLA GQCEQFAADI LGH---IRNQ AEQTC--VLY
TRP_Cint XP_002124651.2      ---------- ---KMATMEK EFKKDYLKLV HQLYDYLEKL LDI---CRNS EEVNA--MLS
TRP_Skow_XP_002733765.1      ---------- ---HLAMREN EFKDVYQQLS HQSKTYAVDL LEQ---CRSS EEVIA--VLN
TRP_Lflor__emb|CAA11261.1    ---------- ---NLSEMEN EFRAEYEELG ITCQNFAVDL LEQ---IRGS KELEV--ILN
TRP_Bflo_XP_002611405.1      SFHHQFLAKT FLLTLSTLEN EFKSEYEQLS KQCKKFASDL LSH---TRGT RELQV--ILN
TRP4_Sman_XP_002576849.1     ---------- ---KLGELEN EFRTEYEELE AKCQKFATEM LAQ---TRSS SELSI--VLN
TRP_Nvec4_XP_001640409       ---------- ---ELADIEY EFRSDYLQLS QQCEEFAVAL LDQ---CRDM SEIEQ--MMS
TRP_Nvec1_XP_001637374.1     ---------- ---VIARRED EYEKDYLQLS QQCSQFTIGL LDE---CRTS REQRC--ILN
Cca7050                      ---------- ---RMAKERM RLKESYMALS VQLEDAAIDL IEG---LHSS QLQGL--VLH
Cca21784                     ---------- ---HWGYNHP LQKARYEALS MEMEELAVSM VDS---APPV LLLKV--LTN
Ava16635_4                   ---------- -----QIVSE NDLGLLVEVA DKFEMTAVGM MEE-AYKRDK EQAHL--ILQ
Ava9904_1                    ---------- -----TDVDE LARDRLENKA NDFENLAITL LGEFSYSAHS DLKLK--LLL
Ava6620_1                    ---------- -----TAVST DDRETLIQKA AYFEELAVGL LDN---AIGK NEFTLNELFN

TRPC1_Hsap_NP_003295.1       ---------- ------HTSS -D-------- --EPLDKRGL LEE------- ----------
TRPC1_Mmus_NP_035773.1       ---------- ------HTSS -D-------- --EPLDKRGL LEE------- ----------
TRPC4 Hsap_NP_003297.1       ---------- ------YRDD ---------- --NSLIEE-- -QS------- ----------
TRPC4_Mmus_NP_058680.1       ---------- ------YRDD ---------- --NSLIEE-- -QS------- ----------
TRP_Spur XP_793901           ---------- ------HDPC ---------- -FYSIGES-- ---------- ----------
TRP_Cint XP_002124651.2      GNYSDSWKGL ARKQSGRTSS NEITNNHLMK SFSNVADE-- HID------- ----------
TRP_Skow_XP_002733765.1      ---------- ------RDND SE-------- --DGFAEEDV MGG------- ----------
```

```
TRP_Lflor__emb|CAA11261.1  ---------- ------HDCR ---------- -SPDQDEE-- -KE------- ----------
TRP_Bflo_XP_002611405.1    ---------- ------HSDD ---------- ----MVES-- ATS------- ----------
TRP4_Sman_XP_002576849.1   ---------- ------HYTG ADVSGCHVSG INQSLSED-- ISE------- ----------
TRP_Nvec4_XP_001640409     ---------- ------VPDM -D-------- --GALTKN-- --G------- ----------
TRP_Nvec1_XP_001637374.1   ---------- ------YPGE ---------- -NGSVEDYA- ---------- ----------
Cca7050                    ---------- ---------- ---------- ---------- ---------- ----------
Cca21784                   ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                 ---------- ------QNNR L--------- ---------- ---------- ----------
Ava9904_1                  ---------- ------KNRR VECSELIGLE VAACQDDKPP VEGDNLLMRE QDDSTTYIWP
Ava6620_1                  ---------- ---------- ---------- ---------- ---------- -----TKIPF

TRPC1_Hsap_NP_003295.1     RMNLSRLKLA IKYNQKEFVS QSNCQQFLNT VW-------- -FGQMSGYRR KPTCKKIMTV
TRPC1_Mmus_NP_035773.1     RMNLSRLKLA IKYNQKEFVS QSNCQQFLNT VW-------- -FGQMSGYRR KPTCKKIMTV
TRPC4_Hsap_NP_003297.1     GNDLARLKLA IKYRQKEFVA QPNCQQLLAS RW-------- -YDEFPGWRR RHWAVKMVTC
TRPC4_Mmus_NP_058680.1     GNDLARLKLA IKYRQKEFVA QPNCQQLLAS RW-------- -YDEFPGWRR RHWAVKMVTC
TRP_Spur_XP_793901         GIGPYKVKIA IHHEQRKFVA HPHCQQHLTQ LW-------- -YEGLPSWYM TNWISSTLST
TRP_Cint_XP_002124651.2    HPRLYQLKLA IKYGVKGFVA NPNCQLQLIA IW-------- -NRDLSWLRQ LSIGYKFLLA
TRP_Skow_XP_002733765.1    KITLIRLKLA LKFEQKRFVA HAHTQQLLTS IW-------- -YEGLPGWRK HNAFVKLLIC
TRP_Lflor__emb|CAA11261.1  LKKLSRLKLA IKYKQKKFVA HPNCQQLLAA KW-------- -YEGLGNFRR KPILKQLTVA
TRP_Bflo_XP_002611405.1    SNSLARLKLA IKYHQKEFVS QPNCQQLLSS MW-------- -YEGFPSWRR KHWAVKVLIC
TRP4_Sman_XP_002576849.1   RMQLSRLKLA IRYGQKQFVA HPHCQQLLAA IW-------- -YEGLPGFRQ KPVFAQLTTI
TRP_Nvec4_XP_001640409     SKSLGVLDYA INNKNGRFVA HPYCQMMLNS VI-------- -YGDFHTWEK MGVTLKILFA
TRP_Nvec1_XP_001637374.1   ENSLGLVNSA ISYNQKEFVA HPFCQHLVMQ HI-------- -FGDITGWRT NHFLYRIAYV
Cca7050                    ---GNVLEMA ISTGRKRFIA TDAVQYELNR VW-------- -HE------W QEPSYKALLK
Cca21784                   ----EIVEMA ISDRCKKFIA HASVLQALKR SW-------- -AG------E NSLPLRLILS
Ava16635_4                 YDDNNCIEMA VTGRCMTFLS HPCVQSLLDE QW-------- -KKPL---YH QNKLWKKLLS
Ava9904_1                  WHENNAFDMA IKGDCKHFIA HPLIQDYANQ KW-------- -YSKI---RR LNKEWKLIMC
Ava6620_1                  WNHSTAIELA STAGKQLFFT HEGVQGHVRN VWNGIAKLQE QYEKTNSDQN INIGVQLKNS

TRPC1_Hsap_NP_003295.1     LTVGIFWPVL S---LCYLIA PKS------- ---------- ---------- --------QF
TRPC1_Mmus_NP_035773.1     LTVGIFWPVL S---LCYLIA PKS------- ---------- ---------- --------QF
TRPC4_Hsap_NP_003297.1     FIIGLLFPVF S---VCYLIA PKS------- ---------- ---------- --------PL
TRPC4_Mmus_NP_058680.1     FIIGLLFPVF S---VCYLIA PKS------- ---------- ---------- --------PL
TRP_Spur_XP_793901         SAIGFGFPIL C---LLYIIY PWG------- ---------- ---------- --------NI
TRP_Cint_XP_002124651.2    VGMSLFMPVL S---IAYFVA PRS------- ---------- ---------- --------KV
TRP_Skow_XP_002733765.1    LAVILLLPLM A---LCYLLF PHT------- ---------- ---------- --------KI
TRP_Lflor__emb|CAA11261.1  FGILFGFPIL S---GIYMIA PHS------- ---------- ---------- --------KF
TRP_Bflo_XP_002611405.1    ASIALAFPLL G---ICYLCA PKS------- ---------- ---------- --------SL
TRP4_Sman_XP_002576849.1   GTLCSLFPLL A---TFYMLA PHS------- ---------- ---------- --------KL
TRP_Nvec4_XP_001640409     LLFTIFIPVW A---IVYFFT PTS------- ---------- ---------- --------CL
TRP_Nvec1_XP_001637374.1   LTQVIIFPVL A---VIYFFM PFL------- ---------- ---------- --------EV
Cca7050                    STIIFLLQAI ARPFVILVVW PLI------- ---------- -----VLNIQ VFGYTFSWNI
Cca21784                   LTFVFILKAM VRPFVFFCIW PFL------- ---------- -------LVR DSCCCITWDS
Ava16635_4                 LIFPFLIYFL I---SFSDPQ PSLP------ ---------- ---------- -------LGY
Ava9904_1                  FFIPFLIPFV L---ELHSRR KRLEHHMNPQ KDPVTKETRF RQKSFCHFFL FLFFDFFRGI
Ava6620_1                  LRHPFSKKSL N---FFY--- ---------- ---------- ---------- ----------

TRPC1_Hsap_NP_003295.1     GRIIHTPFMK FIIHGASYFT FLLLLNLYSL V--------- ---------- ----------
TRPC1_Mmus_NP_035773.1     GRIIHTPFMK FIIHGASYFT FLLLLNLYSL V--------- ---------- ----------
TRPC4_Hsap_NP_003297.1     GLFIRKPFIK FICHTASYLT FLFLLLLASQ H--------- ---------- ----------
TRPC4_Mmus_NP_058680.1     GLFIRKPFIK FICHTASYLT FLFLLLLASQ H--------- ---------- ----------
TRP_Spur_XP_793901         GRLMRVPHVQ FVCHISSMLV FLLLLGLQSS ---------- ---------- ------FTPK
TRP_Cint_XP_002124651.2    VRTMKSPFCK FATQGMSYAQ FLMLLILNCM DRVEGFEFSS T--------- ------FTFL
TRP_Skow_XP_002733765.1    GQLLRTPFMK FMNHSISFGF FLLLLVLAST VRFEGTRPAS PSDTWVD--- ----------
TRP_Lflor__emb|CAA11261.1  GKLCRKPFIK FLLHSASYLS FLVLLILVSQ R--------- ---------I ENPFFFLFPM
TRP_Bflo_XP_002611405.1    GKLIRKPFLK FICHTTSYLF FLALLYLASN PSLVGDEADD RPDQQGPVPN TVEWMIFVWV
TRP4_Sman_XP_002576849.1   GSMMRKPFIK FLCHSSSYLS FLALLTLAAT R--------- ---------- ---VEFLLTN
TRP_Nvec4_XP_001640409     SQKLATPILK FIMHTGAFGL FLLLLILSSI QRFISSNT-- ---------- ----------
TRP_Nvec1_XP_001637374.1   GRKIKRPFVK FINHTSSFVV FLILLAVSSH HQFE------ ---------- ----------
Cca7050                    LRPRAAPTIR YRVEAFAYVA FLILLMVEAA DH-------- ---------- ----------
Cca21784                   LRPIAEPIII YTAHVISYSV FVVLLLIDAL KL-------- ---------- ----------
Ava16635_4                 LKFYTSPSIK FITYVLAFIL YLTLYSFVVL FQWHPSPN-- ---------- ----------
Ava9904_1                  YSFYNAPKIK YWLHFLSHLV YTILFTIVGV TPYRPIRNRT T--------- ----------
Ava6620_1                  -TYYISPSTT FYIHSLVYFI FLVLYTVHVL FTWEPSHKNG ---------- ----------

TRPC1_Hsap_NP_003295.1     YNEDKK-NTM GPALERIDYL LILWIIGMI- ---------- ---------- ----------
TRPC1_Mmus_NP_035773.1     YNEDKK-NTM GPALERIDYL LILWIIGMI- ---------- ---------- ----------
TRPC4_Hsap_NP_003297.1     IDRSDL-NRQ GPPPTIVEWM ILPWVLGFI- ---------- ---------- ----------
TRPC4_Mmus_NP_058680.1     IDRSDL-NRQ GPPPTIVEWM ILPWVLGFI- ---------- ---------- ----------
TRP_Spur_XP_793901         YDDDPDVDQR AEVPKPTEWL ILHGMAMELM EIPPTTMLYS TMEIPTAFTD DEFTTSGETP
TRP_Cint_XP_002124651.2    LHQTSR-DGR HTQFNWCEVF IMLYIAGLL- ---------- ---------- ----------
TRP_Skow_XP_002733765.1    -------SPR GREPNFIEVL IALWVFGFI- ---------- ---------- ----------
TRP_Lflor__emb|CAA11261.1  KEEKKISELR GSPATIVELM ILGYVIGLI- ---------- ---------- ----------
TRP_Bflo_XP_002611405.1    LERRQV-RPA EPVPNTVEWM IFVWVLGMI- ---------- ---------- ----------
TRP4_Sman_XP_002576849.1   SLDQRM-HDR GPTPSLTESA LVIYVI---- ---------- ---------- ----------
TRP_Nvec4_XP_001640409     -------FLL KFLFNFSDIL ITIWIFGLI- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1   -------IRF RKMPSGLEWL IFSWILGVA- ---------- ---------- ----------
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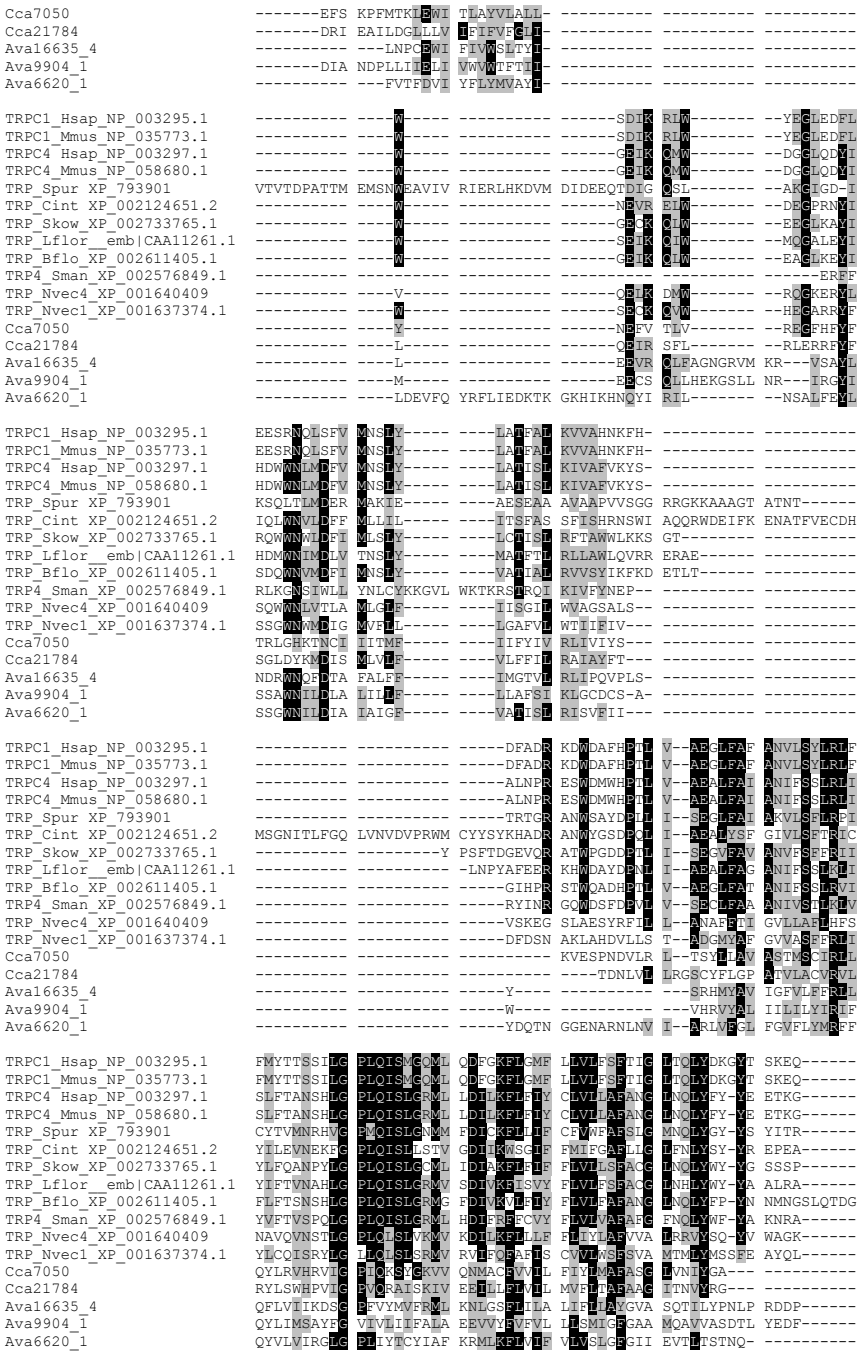

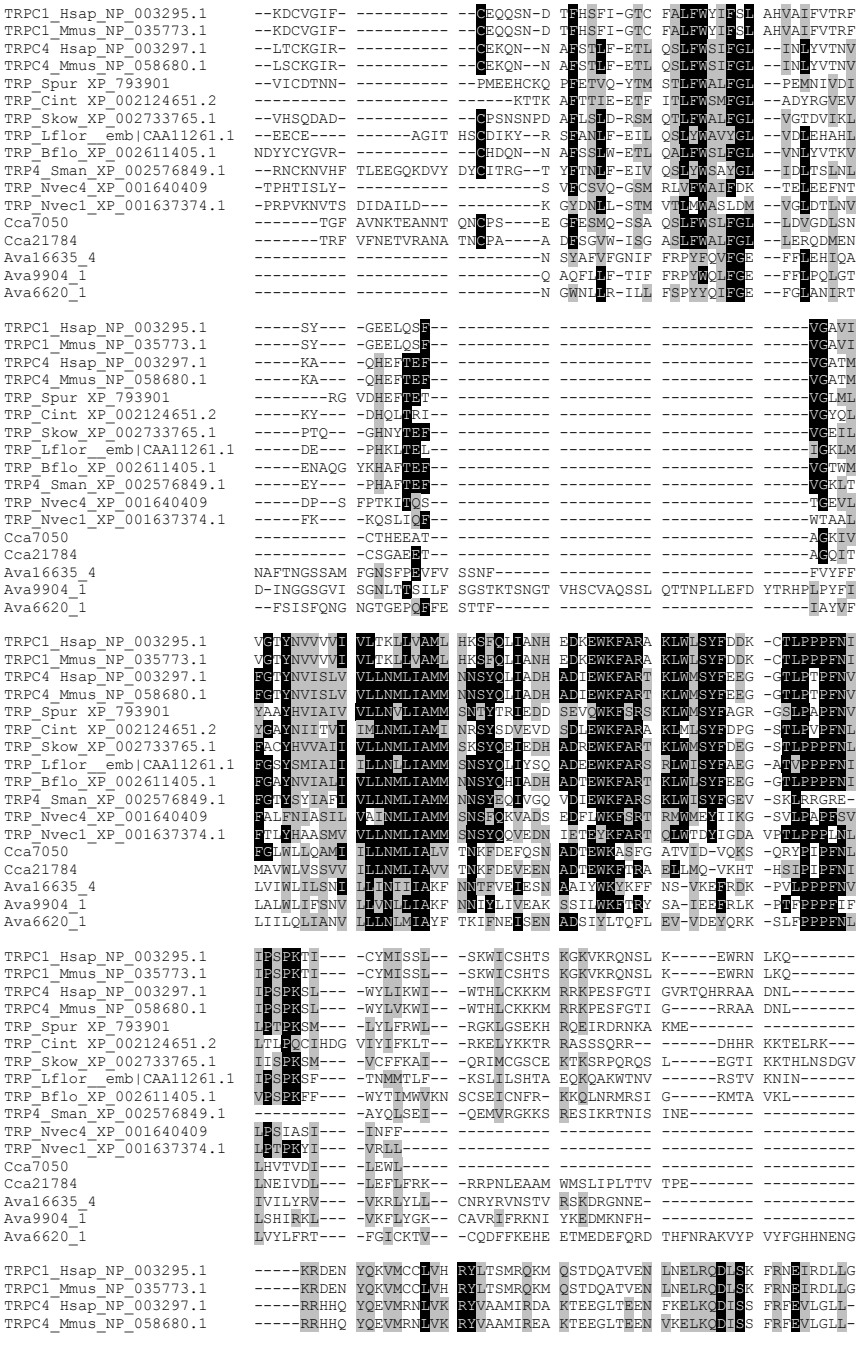
TRPC1_Hsap_NP_003295.1
TRPC1_Mmus_NP_035773.1
TRPC4_Hsap_NP_003297.1
TRPC4_Mmus_NP_058680.1
TRP_Spur_XP_793901
TRP_Cint_XP_002124651.2
TRP_Skow_XP_002733765.1
TRP_Lflor__emb|CAA11261.1
TRP_Bflo_XP_002611405.1
TRP4_Sman_XP_002576849.1
TRP_Nvec4_XP_001640409
TRP_Nvec1_XP_001637374.1
Cca7050
Cca21784
Ava16635_4
Ava9904_1
Ava6620_1

```
TRP_Spur XP_793901          -----EKEQQ YREIMGKLVK RYIFDAKRDE --DENNQEQW VNRLKQDISG FKYEMFEALT
TRP_Cint XP_002124651.2     -----RMSMH HINVMTRIVK RYVLRAQVEE AGGSNTSEAD LQEIKHDISS LRYEVIGGLT
TRP_Skow_XP_002733765.1     IGPPLQENIK YQDVMKRLVS RYIHQQKAEQ K-QDGVNEDD LNEIKQDISS LRYELRADR-
TRP_Lflor__emb|CAA11261.1   -----KREIR YQYVIRNLIS RYIMNKQRPQ K-DEMVSQDD INELKQDIST FREELLTILS
TRP_Bflo_XP_002611405.1     -----RKDMA YKDVIQNLVR RYIANEIRDR ESSEGLCEDD INELKQDISS FRYEMLALL-
TRP4_Sman_XP_002576849.1    -----NKSTQ SKPLFSDIKQ ----PVHRKI TTSTGINKSD FNKTLLPSST SIGGIIGPV-
TRP_Nvec4_XP_001640409      ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1    ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                     ---------- ---------- ---------- ---------- ---------- ----------
Cca21784                    -----RRFEA QKELLQTLKR K-----EERE GMESPSTKSD MRKVSEQLHE LK--------
Ava16635_4                  -----HKEKE KEKILFDFEK RCVLACNFNL EQTNEL---- --NLDKRITE VDNRLKNT--
Ava9904_1                   -----RKNAE RWKWTKDIQQ QGLNRWESKR LSEQEE---- --SVRNLLSN VETKLEDVYR
Ava6620_1                   FADEYWRNRE KIRIKGNLQ- --LKKRQKQV EYLTQKSQEQ SLHRKYTVKY FYFNFTGVRR

TRPC1_Hsap_NP_003295.1      FRTSKYAMFY --PRN----- ---------- ---------- ---------- ----------
TRPC1_Mmus_NP_035773.1      FRTSKYAMFY --PRN----- ---------- ---------- ---------- ----------
TRPC4_Hsap_NP_003297.1      -RGSKLSTIQ --SANASKES SNSADSDEKS DSEGNS---- --------KD KKKNFSLFDL
TRPC4_Mmus_NP_058680.1      -RGSKLSTIQ --SANA---- ASSADSDEKS QSEGNG---- --------KD KRKNLSLFDL
TRP_Spur XP_793901          GMDKKMSEME QRIEDGGVKE PGTQMFHKME DVVKRPLYQP D-------SM QSVISGCSDL
TRP_Cint XP_002124651.2     RWDSKIEELF NSLEKQKTEE GQIQISTAVA QIERKS---- --------KN ERLAPPVLDA
TRP_Skow_XP_002733765.1     -KKEQMRGTS QIENVKHEII GELRGISGAP ENESTTIVGA PRRSLSTVNP PSFISQSFDK
TRP_Lflor__emb|CAA11261.1   DNGFETPTVH Q-AKTSSRLD RMWKNLSAAT EGQTETLMEE AGLDEE---- ----------
TRP_Bflo_XP_002611405.1     -KSDGGEGGP --DSLQDERP SRRKRRTGKY SLEALS---- --------QP RSRSMNNLDR
TRP4_Sman_XP_002576849.1    -DNPAFENEE --VIVNKTSI SSSVDLDGKK QGKSTTVLDQ KTSVSDVTSQ QLNPNFIPCT
TRP_Nvec4_XP_001640409      ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1    ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                     ---------- ---------- ---------- ---------- ---------- ----------
Cca21784                    ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                  ---------- --QHLIVTLT NKVDSLVRST GSF--E---- --------SE QDFFSLQGDT
Ava9904_1                   NCHTLLDHMG SDEPLLTMMT SEIREVRRQL ADFGVH---- --------SF QNYLLILHLV
Ava6620_1                   KGVKSVNLFF CAYPFNLX-- ---------- ---------- ---------- ----------

TRPC1_Hsap_NP_003295.1      ---------- ---------- ---------- ---------- ---------- ----------
TRPC1_Mmus_NP_035773.1      ---------- ---------- ---------- ---------- ---------- ----------
TRPC4_Hsap_NP_003297.1      TTLIHPRSAA IASERHNISN --GSALVVQE PPREKQRKVN FVT------- ----------
TRPC4_Mmus_NP_058680.1      TTLIHPRSAA IASERHNLSN --GSALVVQE PPREKQRKVN FVA------- ----------
TRP_Spur XP_793901          MTAASKDLMN SKGSNNADNS PILDDGLSDP STKNRRQPKS TSMPCNIAGY LYPPDWEKNE
TRP_Cint XP_002124651.2     TEINK----- ---------- ---------- ---------- ---------- ----------
TRP_Skow_XP_002733765.1     SELESLKDDL IHTIRSEIKE ELQESLRR-- ---------- ---------- ----------
TRP_Lflor__emb|CAA11261.1   ---------- ---------- ---------- ---------- ---------- ----------
TRP_Bflo_XP_002611405.1     LEE-HPAVDE TDSAKACLSR PLAAAAVRSV FPVLEQRQRN -VM------- ----------
TRP4_Sman_XP_002576849.1    SAVVCEETIT NNTDQNTVEK QLQQSKLKQP PSQLSIETKT PSM------- ----------
TRP_Nvec4_XP_001640409      ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1    ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                     ---------- ---------- ---------- ---------- ---------- ----------
Cca21784                    ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                  MGSMHGRRIL IRYFNNVSIL QLFISPLSYT TSPVLYQERL NIF------- ------FILC
Ava9904_1                   I--KHITRST HFKITGLLLL PLYIYTVYSC TSPLCYFPKT FIFVVTIQYF PINICLFLFC
Ava6620_1                   ---------- ---------- ---------- ---------- ---------- ----------

TRPC1_Hsap_NP_003295.1      ---------- ---------- ---------- ---------- ---------- ----------
TRPC1_Mmus_NP_035773.1      ---------- ---------- ---------- ---------- ---------- ----------
TRPC4_Hsap_NP_003297.1      ---------- --DIKNFGLF HRRSKQNAAE QNANQIFSVS EEVARQQAAG PLERNIQLES
TRPC4_Mmus_NP_058680.1      ---------- --DIKNFGLF HRRSKQNAAE QNANQIFSVS EEITRQQAAG ALERNIELES
TRP_Spur XP_793901          PILFMDEDPD VPILGGPPRK ESKKRRFSAN HMMNHIGSRH LKRKKSHTKN TTRV------
TRP_Cint XP_002124651.2     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Skow_XP_002733765.1     ---------- ---------- --ALNSAAQE SSVNGLLPTS SDLYHTHLYT QL--------
TRP_Lflor__emb|CAA11261.1   ---------- ---------- ---------- ---------- ---------- ----------
TRP_Bflo_XP_002611405.1     ---------N GQVAGAADRT WSRGNPGGGL QDGNPLLLQT ERVS---SMG RISRTFVTLA
TRP4_Sman_XP_002576849.1    ---NIIKNDK MPNLKNSTFT NETRSQTVKQ DHADKIESVE NEATSQLFSD RKSTDHKTIE
TRP_Nvec4_XP_001640409      ---------- ---------- ---------- ---------- ---------- ----------
TRP_Nvec1_XP_001637374.1    ---------- ---------- ---------- ---------- ---------- ----------
Cca7050                     ---------- ---------- ---------- ---------- ---------- ----------
Cca21784                    ---------- ---------- ---------- ---------- ---------- ----------
Ava16635_4                  MKVSEDLVVL SR-------- ---------- ---------- ---------- ----------
Ava9904_1                   ---SSRMIYD SMLCCV---- ---------- ---------- ---------- ----------
Ava6620_1                   ---------- ---------- ---------- ---------- ---------- ----------

TRPC1_Hsap_NP_003295.1      ---------- ---------- ---------- ---------- ---------- ----------
TRPC1_Mmus_NP_035773.1      ---------- ---------- ---------- ---------- ---------- ----------
TRPC4_Hsap_NP_003297.1      RGLASRGDLS IPGLSEQCVL VDHRERNTDT LGLQVGKRVC P-FKSEKVVV EDTVPIIPKE
TRPC4_Mmus_NP_058680.1      KGLASRGDRS IPGLNEQCVL VDHRERNTDT LGLQVGKRVC STFKSEKVVV EDTVPIIPKE
TRP_Spur XP_793901          ---------- ---------- ---------- ---------- ---------- ----------
TRP_Cint XP_002124651.2     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Skow_XP_002733765.1     ---------- ---------- ---------- ---------- ---------- ----------
TRP_Lflor__emb|CAA11261.1   ---------- ---------- ---------- ---------- ---------- ----------
TRP_Bflo_XP_002611405.1     SPVLGRRRRQ QRKGLDE--- -----EEDTE LGAMSRTNRN FANENDMGSV FTCAPITEID
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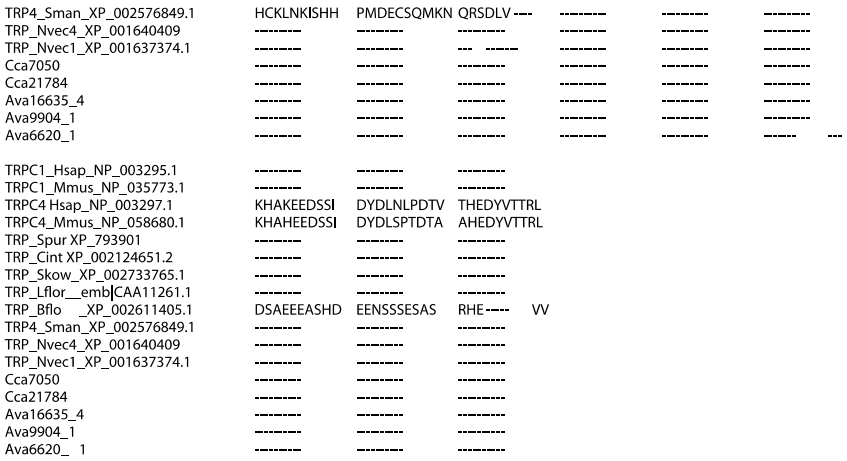


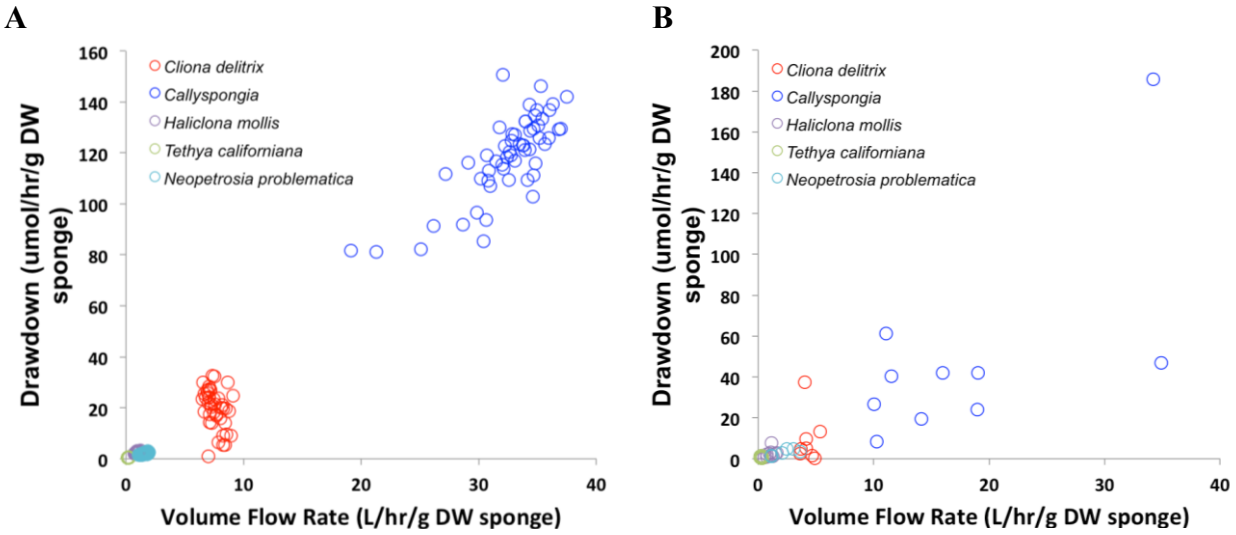
Supplementary Figure S4| Full alignment of sequences in Figure 5c.

A1.6 Movie: Cilia in the osculum of a live sponge, *Ephydatia muelleri*, labeled using FM1-43.

High-frequency time-lapse microscopy (images taken at 50 millisecond intervals with exposure of 50 milliseconds) indicates that the cilia are non-motile and only vibrate in the flow that passes out the osculum. (to view the movie visit http://www.biomedcentral.com/1471-2148/14/3/additional)

Appendix Two

Supplementary material for Chapter Three



A2.1 Volume flow rates and oxygen removal in five species of demosponges.
To better understand the relationship between volume flow rate and oxygen removal, Figure 3-3 was re-plotted without the axes log transformed. a) Sponge pumping volume (L hr^{-1} g DW $sponge^{-1}$) and oxygen removal (µmol hr^{-1} g DW $sponge^{-1}$) were recorded over a five-minute period and plotted for one individual of each of five species of demosponges. Oxygen removal increased as the pumping volume increased both within an individual and between species, with the exception of *Cliona delitrix* (red). This same trend can be seen in b) when the mean oxygen removal and pumping volume over a 5 minute period is plotted for multiple individuals of each species (*Callyspongia* n=11; *Cliona* n=8; *Haliclona* n=10; *Neopetrosia* n=7; *Tethya* n=8).

Appendix Three

Automated image analysis for quantifying behaviour in the sponge

A3.1 Introduction

Advances in optical underwater imaging techniques over the past 50 years have allowed biologists to gain new insights into seafloor processes and ecosystem dynamics (Solan et al., 2003), but the voluminous data collected are often difficult to analyze. SCUBA depth limitations, boat access, costs of equipment, and limitations on the time spent underwater, all make underwater photography a useful, non-destructive tool for observing processes that occur on the seafloor. Images can be stored indefinitely and can be re-analysed at any time for new questions. Underwater time-lapse or video data can also provide information on otherwise cryptic behaviour, such as animals that behave differently when humans are around or behaviours too slow for humans to observe in real-time.

Here, I use examples of behaviour carried out *in situ* and *in vitro* by sponges (Porifera) to describe an efficient and simple method for quantifying behaviour and detecting events in time-lapse videos using the mathematical software MATLAB (v.R2013b; Mathworks). Sponges are good examples of sessile animals that are difficult to understand in real time, but which react to stimuli in the environment by changing their shape. I use this image analysis method to describe a range of behaviours carried out by sponges over minutes to hours, imaged *in vitro* and *in situ*. The primary aim is to demonstrate the utility of automated analysis of images in describing the variety of behaviours of animals such as sponges. Use of these automated approaches however, has highlighted the interesting differences in behaviour of sponges, and allows us to better understand the different stimuli that sponges respond to.

A3.2 Materials and Methods

MATLAB (v.R2013b; Mathworks) is a cross-platform (Windows, Mac, Linux) technical language software that is used across a wide range of disciplines for numerical computation, data analysis and visualization, and programming. Using the Image Processing Toolkit, it can read a diverse set of image and video formats and is capable of a high-level of image processing and analysis including a variety of image segmentation techniques (Gonzales et al., 2009). Although the use of MATLAB requires scripted input, there are many built-in tools as well as tools available for download online that use a Graphical User Interface (GUI), making it more user-friendly than traditional programming languages. Matlab is especially useful if the images to be analyzed come together with large amounts of data from other instruments, which also require Matlab analysis.

The simplest method to divide a digital image into its pixel components for analysis (called segmentation) uses a tool called thresholding (Arifin and Asano, 2006). The purpose of thresholding is to separate objects from their background by creating a binary image in which the subject is one colour of pixels (usually white, greyscale value = 255) and the background another colour (usually black, greyscale value = 0). The size of the objects can then easily be assessed based on the sum of white vs. black pixels in the binary image. Thresholding can therefore be used in any image set in which contrast exists between the object and the background. The optimal threshold value chosen to differentiate foreground and background can either be done manually by the user or automatically using a set of algorithms. Here, we used the well-known Otsu's method of automatic thresholding (Otsu, 1979) that selects an optimal value based on discriminant analysis. Thresholding can be sensitive to noise in the image, potentially introducing artefacts during analysis due to changes in illumination, focus, or movement of an object over the course of the experiment. Therefore, any images associated with outliers on the graph should be assessed for changes in illumination, focus, or object movement.

A3.3 MATLAB Script

A script for analysing time-lapse videos in MATLAB is provided here to allow researchers the ability to use the powerful image analysis software in MATLAB without having to learn programming language. This script is then applied to three different scenarios in sponges: sponge behaviour *in vitro,* osculum contractions *in situ* in response to ambient currents, and contractions of sponges in response to sediment in tanks.

```
%% Using thresholding to quantify animal behaviour - Batch
analysis of multiple images
% This script will run through multiple images in a folder and calculate the
% percent tissue in each, storing the results into an excel file

%% Load images
clear all;close all;clc;
%Get names of files
display('Select ALL of the files that you want to analyze in the folder')
[filenames,pathname] = uigetfile('*.tif','Select File(s)','multiselect',
'on');

%% Create Regions of Interest (ROI), select threshold value, and analyze
percent tissue
PctTissue = zeros(size(filenames)); %creates a matrix of zeros that is the
%same size as the number of images to analyze
%For loop that will go through every image and:
% 1) Crop out the region of interest
% 2) Convert the image to a binary B&W image according to a threshold level
%  selected
% 3) Analyze the binary image so that the percent tissue (white) is recorded
for ii=1:length(filenames)
  fn=fullfile(pathname,filenames{ii});
  if ii==1 % For the first image only:
    temp=imread(fn); %Load the image
    display('Using the cursor, draw a rectangle to select an ROI')
    display('then double-click in the centre of the ROI selected')
    [crop, RECT]=imcrop(temp); % Opens the image to select
                  % a Region of Interest (ROI)
%Selection of an ROI is by clicking and dragging using the cursor.
%The size of the rectangle can then be re-sized or positioned as needed.
%Once the ROI is adequately placed, double-click in the centre to select
%The coordinates of the ROI is saved as RECT
    figure, imshow(crop); %Show the cropped image
    imwrite(crop,'select_image_name.tif'); % Save the cropped image
    threshval(ii) = graythresh(crop(:,:,1));
    channelMask = im2bw(crop, threshval(ii));
    %%The # of White Pixels is the sum of all pixels (white pixel=1)
    numberOfWhitePixels=sum(channelMask(:));
    %%The # of Black Pixels are all remaining pixels (black pixels=0)
    numberOfBlackPixels=numel(channelMask)-numberOfWhitePixels;
    %%To calculate the percent of white pixels in the image:
    a=numberOfWhitePixels+numberOfBlackPixels;
    b=numberOfWhitePixels/a;
    PctTissue(ii) = b*100;
else %The same sequence will now run for every other image, using the
    %coordinates of the cropped image i=1 and the threshold value
```

```
    %selected for i=1
    temp=imread(fn); % Loads the image
         crop=imcrop(temp, RECT); % Crops the image using the coordinates
         saved from image i=1
    threshval(ii) = graythresh(crop(:,:,1));
    channelMask = im2bw(crop, threshval(ii));
         numberOfWhitePixels=sum(channelMask(:));
         numberOfBlackPixels=numel(channelMask)-numberOfWhitePixels;
         a=numberOfWhitePixels+numberOfBlackPixels;
    b=numberOfWhitePixels/a;
    PctTissue(ii) = b*100;
     end
end

%% Look at some of the thresholded images
% to ensure that the ROI and thresholded value chosen is measuring what you
% want it to
int=50;% spacing between thresholded images to save
t=10;% time interval between each photo of the time series
for j=1:int:size(filenames,2);
  fn=fullfile(pathname,filenames{j});
  temp=imread(fn); % Loads the image
  crop=imcrop(temp, RECT); % Crops the image using saved coordinates
  channelMask = im2bw(crop,threshval(j));
  figure, imshow(channelMask,[]);
  imwrite(channelMask,['select image name',num2str((j*t)-t),'.tif']);
         %Comment to not save the thresholded images
end

%% Save the results
%to combine data into one matrix, each column must be the same length.
%Repmat is used for all data that is only one value, to create columns
%that are all the same length
xcoord = repmat(RECT(:,1), size(PctTissue));
ycoord = repmat(RECT(:,2), size(PctTissue));
height = repmat(RECT(:,3), size(PctTissue));
length = repmat(RECT(:,4), size(PctTissue));
% To add a column of the time for each photo:
[t1 ,t2] =size(filenames); % t2 is the number of photos
time=(0:10:((t2*t)-t)); %Creates a time series starting at time '0'
     %and increasing by t (time between photos chosen on line 79)

% Store the results
result= ...
  [{'FILENAME','Time','Pct Tissue','Threshold Level','X Coordinate', 'Y
Coordinate', 'Height', 'Length','Date Analyzed'}; ...
  filenames', ...
  num2cell(time')...
  num2cell(PctTissue'), ...
  num2cell(threshval'),...
  num2cell(xcoord'),...
  num2cell(ycoord'),...
  num2cell(height'),...
  num2cell(length'),...
  repmat({datestr(now)},size(PctTissue'))];
%%Save a .mat file to call on for later analysis or graphs
'variable name' = result;
%Use this line for the first time that the .mat file is created
save('filename.mat','variable name');
%To add a variable to an existing .mat file, uncomment the line below
%save('filename.mat','variable name, '-append');
```

```
%%Write to Excel
xlswrite('Filename.xls',result, 'Sheetname);
%For mac and linux users, xlswrite does not work properly and will default
%to write a .csv file. Try downloading the xlwrite function


%% Create a graph of the results
x=getcolumn(cell2mat(result(2:end,2:3)),1);
y=100-(getcolumn(cell2mat(result(2:end,2:3)),2));
yy=smooth(y,20);
roi=num2str(RECT);
name=filenames{1,1};
% Scatterplot with points
plot(x,y,'.')
hold on
set(findobj('type','axes'),'fontsize',16, 'fontname', 'Arial', 'fontweight',
'b')
xlabel('Time (s)');
ylabel('Canal space (%)');
plot(x,yy,'-');
title(char(name,'ROI coordinates',roi));
hold off
%%
```

For an RGB image, use the following script prior to running the batch analysis to select which color channel to use. Then replace the “1” in the graythresh tool with the color channel number (“1” for red, “2” for green, and “3” for blue).

```
%% First, open and explore representative image
% Read a single image
[filenames,pathname] = uigetfile('Select File');
imgName = fullfile(pathname,filenames);
rgbImg = imread(imgName);

%% Explore color channels with Explore RGB
exploreRGB(rgbImg); % On line 176 of exploreRGB.m script, comment it
out so that it does not provide an error message
% Extract out each color channel
fR=rgbImg(:,:,1); %Red
fG=rgbImg(:,:,2); %Green
fB=rgbImg(:,:,3); %Blue
histR=imhist(fR);
histG=imhist(fG);
histB=imhist(fB);

% Graph the histograms of each color channel
figure, plot(histR,'r')
hold on
set(gca, 'xtick', [0:50:255]);
plot(histG, 'g');
plot(histB, 'b');
legend('Red Channel', 'Green Channel', 'Blue Channel');
hold off
```

A3.4 Assessment

A3.4.1 Sponge behaviour in vitro

Freshwater sponges can be triggered to inflate and contract their whole body in response to mechanical or chemical stimuli, in a process termed a 'sneeze' (Elliott and Leys, 2007; Elliott and Leys, 2010; Ludeman et al., 2014). This behaviour is a useful tool for understanding coordination systems in sponges, but it is quite slow (15-60 minutes long), and is most easily viewed using time-lapse imaging. As spaces in the sponge expand and then contract, dimensions of these spaces can be measured to quantify the behaviour. Not only are manual measurements time-consuming, but they reflect observer bias. The custom computer image analysis tool-set I have developed converts the time-lapse videos into quantitative data. This approach may not always provide equivalent results, as shown previously by Elliott and Leys (2010, fig 1). Therefore, to determine how faithfully this new tool-set could reproduce results obtained manually, I compared the results with those obtained previously for a series of experiments looking at the pharmacological effects on the 'sneeze' behaviour (Figure A3-1;(Ludeman et al., 2014).

Still images were captured in Northern Eclipse v.7 (Empix Imaging Inc., Mississauga, ON, Canada) using an Olympus SZX Stereoscope every 10 s for 50 min, or until the sponge had completed an inflation/contraction cycle. Images were collected in greyscale to minimize storage space. To determine whether this computer image analysis workflow could differentiate changes in the behaviour in response to treatments, we looked at the sneeze response when the sponges were treated with the non-selective calcium channel blocker Gadolinium chloride. 5 μM Gadolinium chloride was added to a Petri dish with the freshwater sponge *Ephydatia muelleri,* and the sneeze response was triggered 2 hours later using 90 μM of the neurotransmitter L-glutamate (n=3). The drug was then washed out with culture medium over 24 hours and the sneeze response triggered again (n=3).

During the 'sneeze' response, the excurrent canals stereotypically inflate and then contract. To measure the change in the excurrent canals over time, a region of interest (ROI) that contained many branching excurrent canals and surrounding tissue was selected

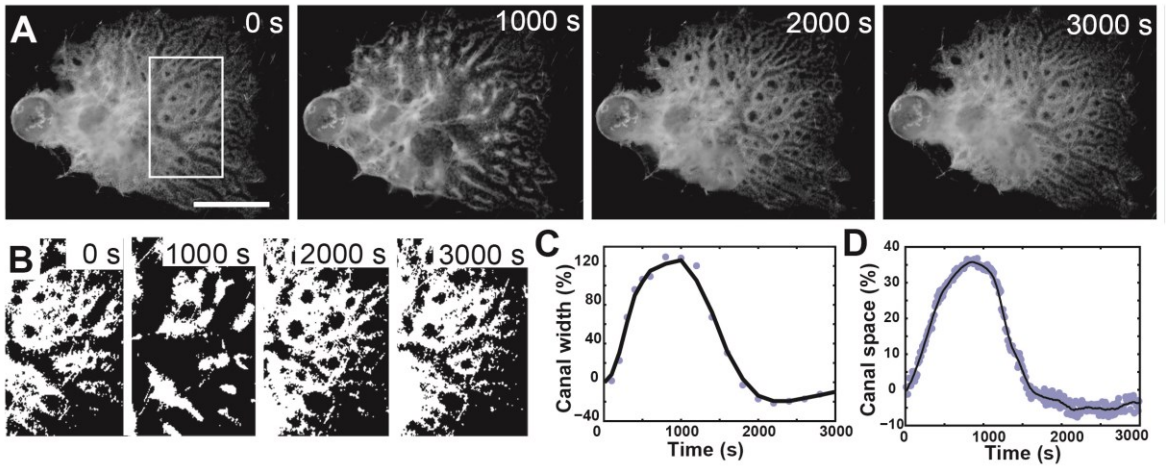


Figure A3-1| A comparison of manual and automated measurement of the sneeze response in *Ephydatia muelleri.* (A) The sneeze response in *Ephydatia muelleri* was captured by time-lapse photography and **(B)** using the computer image analysis technique developed in MATLAB a region of interest was selected and the images were then thresholded**. (C)** Manual measurements, as described in Chapter Two, were then compared to **(D)** the automated results calculated using the percent canal space (black pixels) in the thresholded images.

(Figure A3-1 a). As many of the images had unimodal histograms, we used Otsu's method for selecting the optimal threshold value to differentiate the canal from the sponge tissue (Figure A3-1 b), designated by the function 'graythresh' in MATLAB (Otsu, 1979). After segmentation the tissue surrounding the canals was classified as white pixels and the canals classified as black pixels. We graphed the percent of black pixels or area fraction over the series of images to look for a change in canal size over time.

Previously the canal diameters had been measured manually using the line tool in ImageJ (v1.43r; NIH) (Ludeman et al., 2014); but to reduce time taken, only every tenth image for the first 60 images, and then every 20th image were used. We used the data collected from those experiments for comparison with digital image analysis tools here.

Neither manual nor computer image analysis detected a 'sneeze' response in sponges exposed to Gadolinium (Figure A3-2 a,b). Once the drug had been washed out over 24hr, each method was able to detect an increase and then a decrease in canal space (Figure A3-2 a,b), which is highly correlated ($r^2 = 0.899$; Figure A3-2 c).

A3.4.2 Osculum contractions in situ *in response to ambient currents*

Despite a wide understanding of the importance of water flow for sponges, very few studies have assessed behavioural responses in sponges to increased ambient currents. In 1971 Reiswig documented the correlation between wave strength and oscular openings in *Tethya crypta* by using time-lapse photography over many days along with velocity measurements. Concurrent measurements of ambient velocity, excurrent velocity, and osculum area, as done by Reiswig (1971), can provide powerful data to understand how sponges respond to water flow.

A GoPro Hero2 camera in underwater housing was mounted over the sponge *in situ* using an aluminium frame (see Chapter Three) off of STRI point in Bocas del Toro, Panama. The camera was positioned to capture changes in osculum sizes of the sponge *Cliona delitrix* during an experiment in which the ambient current speed was manipulated using an underwater aquarium pump. Images were captured in colour (RGB) every 30 sec at 5 MP resolution for approximately one hour. Acoustic Doppler velocimeters were set up

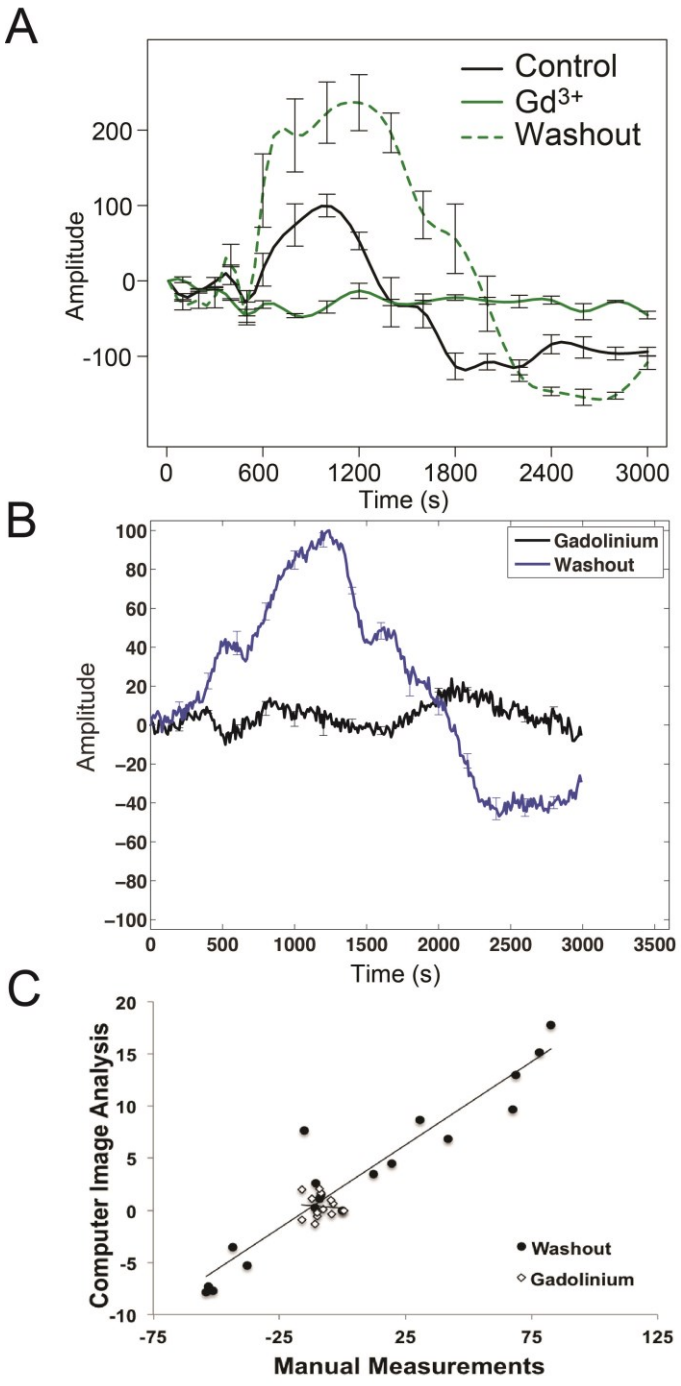


Figure A3-2| Comparison of results between manual and computerized image analysis of the sneeze response during pharmacological treatment with Gadolinium. **(A)** Manual measurements using canal diameter**. (B)** Automated measurements using percent canal space. **(C)** Scatterplot showing the positive correlation between computer and manual measurements ($r^2 = 0.899$).

to record both ambient water velocity beside the sponge and excurrent water velocity exiting the sponge. The underwater pump was directed at the sponge and run through four settings (off, low, medium, and high) for 5 minutes each, three times through. The camera battery did not last the entire experiment, however, therefore only two runs were analyzed here. Refer to Chapter Three for a more detailed description of these methods.

To detect changes in osculum area over the course of the experiment, a region of interest and some surrounding tissue was selected in *Cliona delitrix* (Figure A3-3 a) The red colour level was selected for each of the images and thresholded using 'graythresh' in MATLAB. A ruler was placed next to the osculum in one of the images to convert the number of pixels to osculum area and used to calculate pumping volume from the excurrent velocity (Figure A3-3 b,c).

A3.4.3 Contractions of sponges in response to sediment in tanks

Sponges, as suspension feeders, are sensitive to sediment and other materials in the water column that can clog their filtration system. It has been shown that some sponges respond to sediment by carrying out slow, rhythmic contractions to help clear out debris (Nickel, 2004; Elliott and Leys, 2007; Ellwanger et al., 2007) People have also long noted that sponges contract their excurrent opening, or osculum, in response to various stimuli (Parker, 1910; McNair, 1923; Emson, 1966; Prosser, 1967). Changes in both osculum diameter and sponge volume can be readily captured using time-lapse imaging, and it is important to be able to quantify the extent of contraction.

A GoPro Hero2 camera was mounted over the sediment chamber and positioned to capture changes in the volume and changes in osculum sizes of the sponge *Suberites* sp. during the experiment. Images were captured in colour (RGB) every 30 sec at 5 MP resolution for approximately eight hours. A low concentration of sediment (~12mg/L) was added to the chamber for the first hour.

For the analysis of sponge volume, the green colour level was selected and two threshold levels were chosen using the 'multithresh' function in MATLAB. The pixel intensities between the two threshold levels were then converted to white pixels during

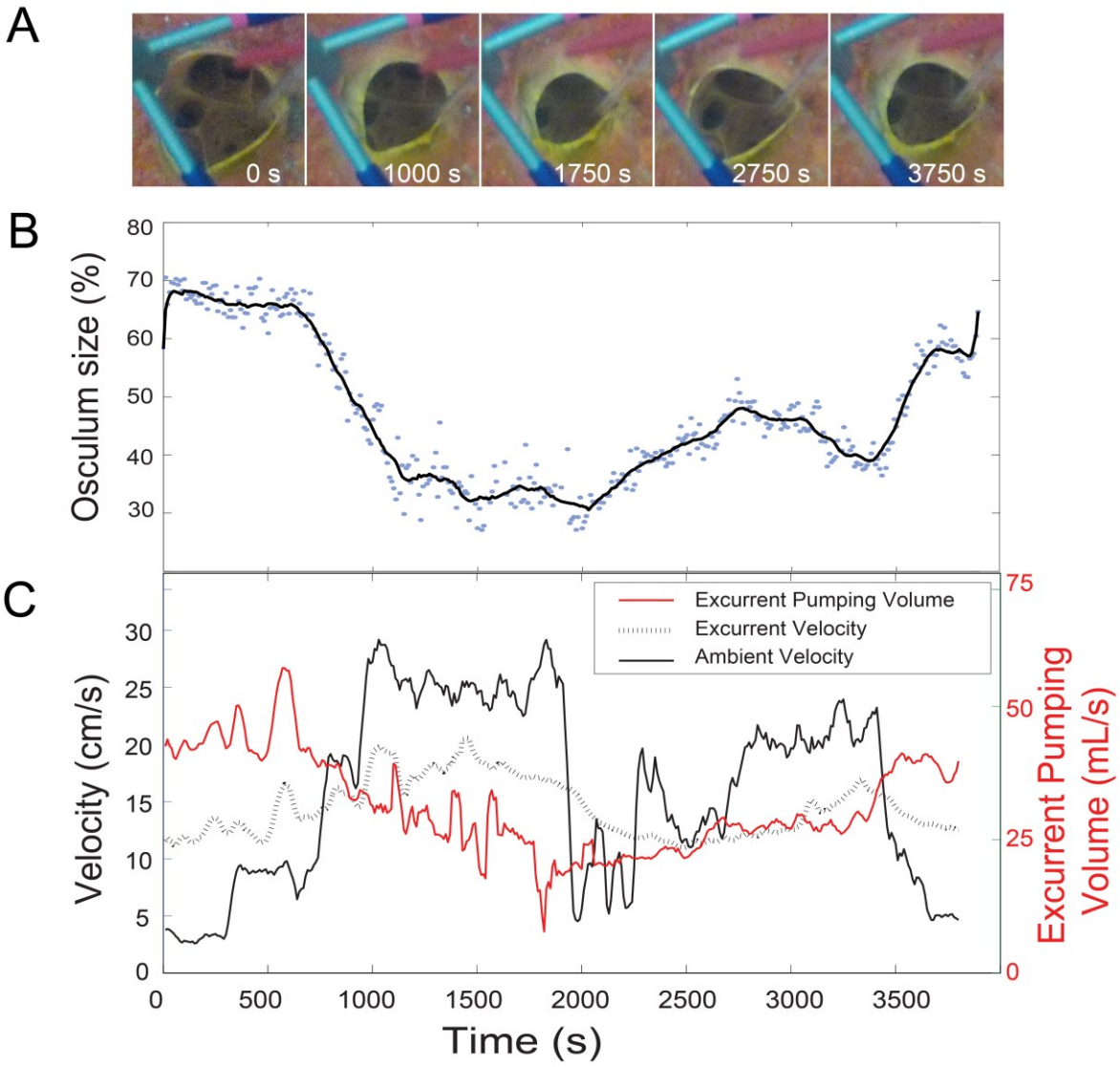


Figure A3-3| The sponge *Cliona delitrix* contracts its osculum in response to increasing ambient flow rates. (A) Images of the sponge osculum were taken using time-lapse photography and **(B)** osculum size was determined using thresholding**. (C)** The osculum contracts with increasing ambient flow rates, resulting in a decrease in pumping volume and an increased in excurrent velocity.

segmentation to differentiate the sponge from the dark background and the light colored rocks (Figure A3-4 a,b). A region of interest of the osculum and some surrounding tissue area was selected to detect movement of the osculum during the course of imaging. The red colour level was chosen as it provided the best distinction between sponge and osculum. Only one threshold value was selected, and the sponge tissue was converted to white pixels during segmentation. The change in the number of black pixels was then analysed over time (Figure A3-4 c).

A3.5 Discussion

Here we provide a tool for the analysis of behaviour of aquatic organisms using time-lapse photography and computer image analysis. This tool is appropriate for cryptic animal behaviour, for studying growth of sessile benthic animals, and to capture events in video imagery. The rapid analysis of video data allows more time to be spent on interpreting the data. The use of computer image analysis may also reveal events or changes that were otherwise undetected by observers, either due to cryptic changes or from the ability to analyse many more images than would have been measured manually. The accuracy of measurements obtained using this computer image analysis will depend on the quality of image as well as the regions of interest and thresholding levels chosen. Comparison with manual measurements for the sponge 'sneeze' response, however, suggests that the results should be accurate for most applications and can differentiate between treatments. Computer image analysis will never be able to replace the critical observer of an informed experimenter, and therefore careful visual checks throughout the analysis workflow will ensure accuracy and prevent artefacts from occurring during the measurements. Importantly, this computer image analysis workflow minimizes or prevents human observer bias when analysing behaviour in time-lapse videos.

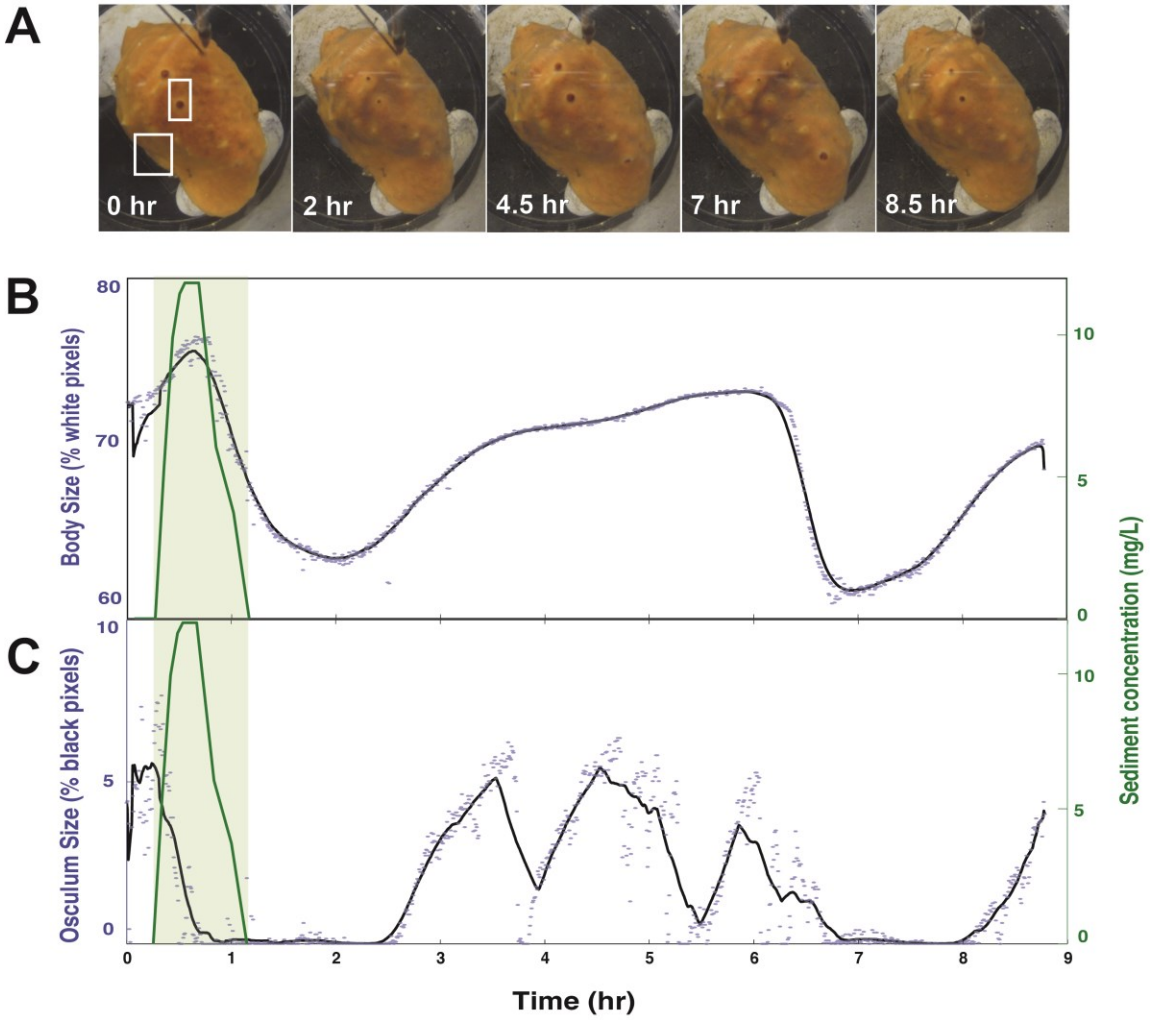


Figure A3-4| The sponge *Suberites sp.* responds to increased suspended sediment in a tank. (A) Time-lapse photography was used to image the sponge during one hour of sediment addition to a tank followed by eight hours of recovery**. (B)** A region of interest along the body wall was thresholded and analyzed, and shows an increase in body volume during sediment addition (shaded green) followed by a contraction event. During recovery the sponge underwent a second inflation and contraction response. **(C)** A region of interest around an osculum and surrounding tissue was thresholded and demonstrates an immediate contraction of the osculum with an increase in sediment, followed by multiple osculum contractions during the recovery stage.

A3.6 References

Arifin, A. Z. and Asano, A. (2006). Image segmentation by histogram thresholding using hierarchical cluster analysis. *Pattern Recognition Letters* **27**, 1515-1521.

Elliott, G. R. D. and Leys, S. P. (2007). Coordinated contractions effectively expel water from the aquiferous system of a freshwater sponge. *The Journal of Experimental Biology* **210**, 3736-48.

Elliott, G. R. D. and Leys, S. P. (2010). Evidence for glutamate, GABA and NO in coordinating behaviour in the sponge, *Ephydatia muelleri* (Demospongiae, Spongillidae). *The Journal of Experimental Biology* **213**, 2310-21.

Ellwanger, K., Eich, A. and Nickel, M. (2007). GABA and glutamate specifically induce contractions in the sponge *Tethya wilhelma*. *Journal of Comparative Physiology A* **193**, 1-11.

Emson, R. H. (1966). The reactions of the sponge *Cliona celata* to applied stimuli. *Comparative Biochemistry and Physiology* **18**, 805-827.

Gonzales, R. C., Woods, R. E. and Eddins, S. L. (2009). Digital Image Processing using MATLAB. USA: Gatesmark Publishing.

Ludeman, D. A., Farrar, N., Riesgo, A., Paps, J. and Leys, S. P. (2014). Evolutionary origins of sensation in metazoans: functional evidence for a new sensory organ in sponges. *BMC Evolutionary Biology* **14**.

McNair, G. T. (1923). Motor reactions of the freshwater sponge *Ephydatia fluviatilis*. *Biological Bulletin* **64**, 153-166.

Nickel, M. (2004). Kinetics and rhythm of body contractions in the sponge *Tethya wilhelma* (Porifera: Demospongiae). *The Journal of Experimental Biology* **207**, 4515-24.

Otsu, N. (1979). A threshold selection method from gray-level histograms. *IEEE Transactions on Systems, Man, and Cybernetics* **9**, 62-66.

Parker, G. H. (1910). The reactions of sponges, with a consideration of the origin of the nervous system. *The Journal of Experimental Zoology* **8**, 1-41.

Prosser, C. L. (1967). Ionic analyses and effects of ions on contractions of sponge tissues. *Zeitschrift für vergleichende Physiologie* **54**, 109-120.

Reiswig, H. M. (1971). *In situ* pumping activities of tropical Demospongiae. *Marine Biology* **9**, 38-50.

Solan, M., Germano, J. D., Rhoads, D. C., Smith, C., Michaud, E., Parry, D., Wenzhöfer, F., Kennedy, B., Henriques, C., Battle, E. et al. (2003). Towards a greater understanding of pattern, scale and process in marine benthic systems: a picture is worth a thousand worms. *Journal of Experimental Marine Biology and Ecology* **285-286**, 318-338.

References

Full list of references

Adams, E. D., Goss, G. G. and Leys, S. P. (2010). Freshwater sponges have functional, sealing epithelia with high transepithelial resistance and negative transepithelial potential. *PloS one* **5**, e15040.

Arifin, A. Z. and Asano, A. (2006). Image segmentation by histogram thresholding using hierarchical cluster analysis. *Pattern Recognition Letters* **27**, 1515-1521.

Bagby, R. M. (1966). The fine structure of myocytes in the sponges *Microciona prolifera* (Ellis and Solander) and *Tedania ignis* (Dechassaing and Michelotti). *Journal of Morphology* **118**, 167-182.

Bannister, R. J., Battershill, C. N. and de Nys, R. (2012). Suspended sediment grain size and mineralogy across the continental shelf of the Great Barrier Reef: impacts on the physiology of a coral reef sponge. *Continental Shelf Research* **32**, 86-95.

Bergquist, P. R. (1978). Sponges: Univ of California Press.

Besschetnova, T. Y., Kolpakova-Hart, E., Guan, Y., Zhou, J., Olsen, B. R. and Shah, J. V. (2010). Identification of signaling pathways regulating primary cilium length and flow-mediated adaptation. *Current Biology* **20**, 182-7.

Best, B. A. (1988). Passive suspension feeding in a Sea Pen: Effects of ambient flow on volume flow rate and filtering efficiency. *Biological Bulletin* **175**, 332-342.

Bidder, G. P. (1923). The relation of the form of a sponge to its currents. *Quaterly Journal of Microscopical Sciences* **67**, 293-323.

Brown, B. and Howard, L. (1985). Assessing the effects of "stress" on reef corals. *Advances in Marine Biology* **22**, 1-63.

Chu, J. W. F. and Leys, S. P. (2010). High resolution mapping of community structure in three glass sponge reefs (Porifera, Hexactinellida). *Marine Ecology Progress Series* **417**, 97-113.

Chu, J. W. F. and Reiswig, H. M. (2014). Mechanisms of propagule release in the carnivorous sponge *Asbestopluma occidentalis*. *Invertebrate Biology* **133**, 109-120.

Coma, R., Ribes, M., Gili, J.-M. and Zabala, M. (1998). An energetic approach to the study of life-history traits of two modular colonial benthic invertebrates. *Marine Ecology Progress Series* **162**, 89-103.

Conaco, C., Bassett, D. S., Zhou, H., Arcila, M. L., Degnan, S. M., Degnan, B. M. and Kosik, K. S. (2012). Functionalization of a protosynaptic gene expression network. *Proceedings of the National Academy of Sciences of the United States of America* **109 Suppl**, 10612-8.

Corredor, J. E., Wilkinson, C. R., Vicente, V. P., Morell, J. M. and Otero, E. (1988). Nitrate release by Caribbean reef sponges. *Limnology and Oceanography* **33**, 114-120.

de Vos, L., Boury-Esnault, N. and Vacelet, J. (1990). The apopylar cells of sponges. In: New Perspectives in Sponge Biology. 153-158.

Diaz, M. C. (2005). Common sponges from shallow marine habitats from Bocas del Toro Region, Panama. *Caribbean Journal of Science* **41**, 465-475.

Diaz, M. C. and Rützler, K. (2001). Sponges: an essential component of Caribbean coral reefs. *Bulletin of Marine Science* **69**, 535-546.

Diaz, M. C. and Ward, B. B. (1997). Sponge-mediated nitrification in tropical benthic communities. *Marine Ecology Progress Series* **156**, 97-107.

Diaz, R. J. and Rosenberg, R. (2008). Spreading dead zones and consequences for marine ecosystems. *Science* **321**, 926-9.

Dunlap, K. (1977). Localization of calcium channels in *Paramecium caudatum*. *Journal of Physiology* **271**, 119-133.

Dunn, C. W., Hejnol, A., Matus, D. Q., Pang, K., Browne, W. E., Smith, S. A., Seaver, E., Rouse, G. W., Obst, M., Edgecombe, G. D. et al. (2008). Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature* **452**, 745-749.

Eckman, J. E. and Duggins, D. O. (1993). Effects of flow speed on growth of benthic suspension feeders. *Biological Bulletin* **185**, 28-41.

Elliott, G. R. D. (2009). The cytology and physiology of coordinated behaviour in the freshwater sponge, *Ephydatia muelleri*. In *Department of Biological Sciences*, vol. Degree of Philosophy, pp. 252: University of Alberta.

Elliott, G. R. D. and Leys, S. P. (2007). Coordinated contractions effectively expel water from the aquiferous system of a freshwater sponge. *The Journal of Experimental Biology* **210**, 3736-48.

Elliott, G. R. D. and Leys, S. P. (2010). Evidence for glutamate, GABA and NO in coordinating behaviour in the sponge, *Ephydatia muelleri* (Demospongiae, Spongillidae). *The Journal of Experimental Biology* **213**, 2310-21.

Ellis, J., Cummings, V., Hewitt, J., Thrush, S. and Norkko, A. (2002). Determining effects of suspended sediment on condition of a suspension feeding bivalve (*Atrina zelandica*): results of a survey, a laboratory experiment and a field transplant experiment. *Journal of Experimental Marine Biology and Ecology* **267**, 147-174.

Ellwanger, K., Eich, A. and Nickel, M. (2007). GABA and glutamate specifically induce contractions in the sponge *Tethya wilhelma*. *Journal of Comparative Physiology A* **193**, 1-11.

Emson, R. H. (1966). The reactions of the sponge *Cliona celata* to applied stimuli. *Comparative Biochemistry and Physiology* **18**, 805-827.

Fiala-Medioni, A. (1978). Filter-feeding ethology of benthic invertebrates (Ascidians). IV. Pumping rate, filtration rate, filtration efficiency. *Marine Biology* **48**, 243-249.

Flood, P. R. and Fiala-Medioni, A. (1981). Ultrastructure and histochemistry of the food trapping mucous film in benthic filter-feeders (Ascidians). *Acta Zoologica* **62**, 53-65.

Flood, P. R. and Fiala-Medioni, A. (1982). Structure of the mucous feeding filter of *Chaetopterus variopedatus*. *Marine Biology* **72**, 27-33.

Fox, R. W., McDonald, A. T. and Pritchard, P. J. (1998). Introduction to fluid mechanics, vol. 5, pp. 360: John Wiley & Sons New York.

Fujiu, K., Nakayama, Y., Iida, H., Sokabe, M. and Yoshimura, K. (2011). Mechanoreception in motile flagella of *Chlamydomonas*. *Nature Cell Biology* **13**, 630-2.

Gale, J. E., Marcotti, W., Kennedy, H. J., Kros, C. J. and Richardson, G. P. (2001). FM1-43 dye behaves as a permeant blocker of the hair-cell mechanotransducer channel. *The Journal of Neuroscience* **21**, 7013-25.

Genin, A. and Karp, L. (1994). Effects of flow on competitive superiority in scleractinian corals. *Limnology and Oceanography* **39**, 913-924.

Gerrodette, T. and Flechsig, A. O. (1979). Sediment-induced reduction in the pumping rate of the tropical sponge *Verongia lacunosa*. *Marine Biology* **55**, 103-110.

Gili, J.-M. and Coma, R. (1998). Benthic suspension feeders: their paramount role in littoral marine food webs. *Trends in Ecology and Evolution* **13**, 316-321.

Gonzales, R. C., Woods, R. E. and Eddins, S. L. (2009). Digital Image Processing using MATLAB. USA: Gatesmark Publishing.

Griffiths, C. L. and King, J. A. (1979). Some relationships between size, food availability and energy balance in the ribbed mussel *Aulacomya ater*. *Marine Biology* **51**, 141-149.

Grove, M. W., Finelli, C. M., Wethey, D. S. and Woodin, S. A. (2000). The effects of symbiotic crabs on the pumping activity and growth rates of *Chaetopterus variopedatus*. *Journal of Experimental Marine Biology and Ecology* **246**, 31-52.

Hadas, E., Ilan, M. and Shpigel, M. (2008). Oxygen consumption by a coral reef sponge. *The Journal of Experimental Biology* **211**, 2185-90.

Hallegraeff, G. M. (1993). A review of harmful algal blooms and their apparent global increase. *Phycologia* **32**, 79-99.

Happel, J. and Brenner, H. (1983). Low Reynolds number hydrodynamics: with special applications to particulate media, The Hague, Netherlands. Springer.

Harris, J. A., Cheng, A. G., Cunningham, L. L., MacDonald, G., Raible, D. W. and Rubel, E. W. (2003). Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (*Danio rerio*). *Journal of the Association for Research in Otolaryngology* **4**, 219-34.

Harris, P. and Shaw, G. (1984). Intermediate filaments, microtubules and microfilaments in epidermis of sea urchin tube foot. *Cell Tissue Research* **236**, 27-33.

Harrison, F. and Cowden, R. (1976). External Currents and Sponge Feeding. In *Aspects of Sponge Biology*, pp. 46. USA: Academic Press Inc.

Johnston, I. S. and Hildemann, W. H. (1982). Cellular organization in the marine demosponge *Callyspongia diffusa*. *Marine Biology* **67**, 1-7.

Jones, H. D., Richards, O. G. and Southern, T. A. (1992). Gill dimensions, water pumping rate and body size in the mussel *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology* **155**, 213-237.

Jones, W. C. (1962). Is there a nervous system in sponges? *Biological Reviews* **37**, 1-50.

Jørgensen, C. B. (1955). Quantitative aspects of filter feeding in invertebrates. *Biological Reviews* **30**, 391-453.

Jørgensen, C. B. (1966). Biology of Suspension Feeding. Oxford: Pergamon Press.

Jørgensen, C. B. (1975). Comparative physiology of suspension feeding. *Annual Reviews in Physiology* **37**, 57-79.

Jørgensen, C. B., Famme, O., Kristensen, H. S., Larsen, P. S., Møhlenberg, F. and Riisgård, H. U. (1986). The bivalve pump. *Marine Ecology Progress Series* **34**, 69-77.

Jørgensen, C. B., Larsen, P. S., Møhlenberg, F. and Riisgård, H. U. (1988). The mussel pump: properties and modelling. *Marine Ecology Progress Series* **45**, 205-218.

Kahn, A.S., Yahel, G., Chu, J.W.F., Tunicliffe, V., and Leys, S.P. (2015). Benthic grazing and carbon sequestration by deep-water glass sponge reefs. *Limnology and Oceanography* doi: 10.1002/lno.10002.

Katoh, K., Misawa, K., Kuma, K.-i. and Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**, 3059-66.

Kelley, L. A. and Sternberg, M. J. E. (2009). Protein structure prediction on the Web: a case study using the Phyre server. *Nature Protocols* **4**, 363-71.

Knott, N. A., Davis, A. R. and Buttermer, W. A. (2004). Passive flow through an unstalked intertidal ascidian: Orientation and morphology enhance suspension feeding in *Pyura stolonifera*. *Biological Bulletin* **207**, 217-224.

Krautter, M., Conway, K. W. and Barrie, J. V. (2001). Discovery of a "Living Dinosaur": Globally unique modern Hexactinellid sponge reefs off British Columbia, Canada. *Facies* **44**, 265-282.

Labarbera, M. (1977). Brachiopod orientation to water movement. 1. Theory, laboratory behaviour, and field orientations. *Paleobiology* **3**, 270-287.

Lartillot, N. and Philippe, H. (2004). A Bayesian mixture model for across-site heterogeneities in the amino-acid replacement process. *Molecular Biology and Evolution* **21**, 1095-109.

Lartillot, N., Rodrigue, N., Stubbs, D. and Richer, J. (2013). PhyloBayes MPI : phylogenetic reconstruction with infinite mixtures of profiles in a parallel environment. *Systematic Biology*.

Leys, S. P. and Degnan, B. M. (2002). Embryogenesis and metamorphosis in a haplosclerid demosponge: gastrulation and transdifferentiation of larval ciliated cells to choanocytes. *Invertebrate Biology* **121**, 171-189.

Leys, S. P. and Hill, A. (2012). The physiology and molecular biology of sponge tissues. *Advances in Marine Biology* **62**, 1-56.

Leys, S. P. and Meech, R. W. (2006). Physiology of coordination in sponges. *Canadian Journal of Zoology* **84**, 288-306.

Leys, S. P., Nichols, S. A. and Adams, E. D. (2009). Epithelia and integration in sponges. *Integrative and Comparative Biology* **49**, 167-77.

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.

Linck, R. W. (1973). Chemical and structural differences between cilia and flagella from the lamellibranch mollusc, *Aequipecten irradians*. *Journal of Cell Science* **12**, 951-81.

Ludeman, D. A., Farrar, N., Riesgo, A., Paps, J. and Leys, S. P. (2014). Evolutionary origins of sensation in metazoans: functional evidence for a new sensory organ in sponges. *BMC Evolutionary Biology* **14**.

Mackie, G. (1970). Neuroid conduction and the evolution of conducting tissues. *The Quarterly Review of Biology* **45**, 319-332.

Mah, J. L., Christensen-Dalsgaard, K. K. and Leys, S. P. (2014). Choanoflagellate and choanocyte collar-flagellar systems and the assumption of homology. *Evolution & Development* **16**, 25-37.

Maldonado, M. and Uriz, M. J. (1999). An experimental approach to the ecological significance of microhabitat-scale movement in an encrusting sponge. *Marine Ecology Progress Series* **185**, 239-255.

Maldonado, M., Giraud, K. and Carmona, C. (2008). Effects of sediment on the survival of asexually produced sponge recruits. *Marine Biology* **154**, 631-641.

Maldonado, M., Ribes, M. and van Duyl, F. C. (2012). Nutrient fluxes through sponges: biology, budgets, and ecological implications. *Advances in Marine Biology* **62**, 113-82.

McNair, G. T. (1923). Motor reactions of the freshwater sponge *Ephydatia fluviatilis*. *Biological Bulletin* **64**, 153-166.

Meech, R. W. (2008). Non-neural reflexes: sponges and the origins of behaviour. *Current Biology* **18**, R70-R72.

Mills, D. B., Ward, L. M., Jones, C., Sweeten, B., Forth, M., Treusch, A. H. and Canfield, D. E. (2014). Oxygen requirements of the earliest animals. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 4168-4172.

Miyoshi, K., Kasahara, K., Miyazaki, I. and Asanuma, M. (2011). Factors that influence primary cilium length. *Acta Medica Okayama* **65**, 279-85.

Murdock, G., R. and Vogel, S. (1978). Hydrodynamic induction of water flow through a keyhole limpet (Gastropoda, Fissurellidae). *Comparative Biochemistry and Physiology. Part A: Comparative Physiology* **61A**, 227-231.

Nauli, S. M., Alenghat, F. J., Luo, Y., Williams, E., Vassilev, P., Li, X., Elia, A. E. H., Lu, W., Brown, E. M., Quinn, S. J. et al. (2003). Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nature Genetics* **33**, 129-37.

Newell, R. and Branch, G. (1980). The influence of temperature on the maintenance of metabolic energy balance in marine invertebrates. *Advances in Marine Biology* **17**, 329-396.

Nickel, M. (2004). Kinetics and rhythm of body contractions in the sponge *Tethya wilhelma* (Porifera: Demospongiae). *The Journal of Experimental Biology* **207**, 4515-24.

Nosenko, T., Schreiber, F., Adamska, M., Adamski, M., Eitel, M., Hammel, J., Maldonado, M., Müller, W. E. G., Nickel, M., Schierwater, B. et al. (2013). Deep metazoan phylogeny: when different genes tell different stories. *Molecular Phylogenetics and Evolution* **67**, 223-33.

Otsu, N. (1979). A threshold selection method from gray-level histograms. *IEEE Transactions on Systems, Man, and Cybernetics* **9**, 62-66.

Ou, H. C., Cunningham, L. L., Francis, S. P., Brandon, C. S., Simon, J. a., Raible, D. W. and Rubel, E. W. (2009). Identification of FDA-approved drugs and bioactives that protect hair cells in the zebrafish (*Danio rerio*) lateral line and mouse (*Mus musculus*) utricle. *Journal of the Association for Research in Otolaryngology* **10**, 191-203.

Palumbi, S. R. (1986). How body plans limit acclimation: responses of a demosponge to wave force. *Ecology* **67**, 208-214.

Parker, G. H. (1910). The reactions of sponges, with a consideration of the origin of the nervous system. *The Journal of Experimental Zoology* **8**, 1-41.

Pavans de Ceccatty, M. (1969). Les systemes des activites motrices, spontanées et provoquées des éponges. *Comptes Rendus de l'Académie des Sciences – Series III Sciences de la Vie* **269**, 596-599.

Pavans de Ceccatty, M. (1974). Coordination in sponges: the foundations of integration. *American Zoologist* **14**, 895-903.

Pavans de Ceccatty, M., Cargouil, M. and Coraboeuf, E. (1960). Les réactions motrices de l'éponge *Tethya lyncurium* (Lmk.) à quelques stimulations expérimentales (I). *Vie et Milieu* **11**, 594-600.

Philippe, H., Derelle, R., Lopez, P., Pick, K., Borchiellini, C., Boury-Esnault, N., Vacelet, J., Renard, E., Houliston, E., Quéinnec, E. et al. (2009). Phylogenomics revives traditional views on deep animal relationships. *Current Biology* **19**, 706-12.

Pile, A. J., Patterson, M. R. and Witman, J. D. (1996). *In situ* grazing on plankton <10 μm by the boreal sponge *Mycale lingua*. *Marine Ecology Progress Series* **141**, 95-102.

Pile, A. J., Patterson, M. R., Savarese, M., Chernykh, V. I. and Fialkov, V. A. (1997). Trophic effects of sponge feeding within Lake Baikal's littoral zone. 2. Sponge

abundance, diet, feeding efficiency, and carbon flux. *Limnology and Oceanography* **42**, 178-184.

Praetorius, H. A. and Spring, K. R. (2001). Bending the MDCK cell primary cilium increases intracellular calcium. *Journal of Membrane Biology* **184**, 71-79.

Praetorius, H. A. and Spring, K. R. (2003). Removal of the MDCK cell primary cilium abolishes flow sensing. *Journal of Membrane Biology* **191**, 69-76.

Praetorius, H. A. and Spring, K. R. (2005). A physiological view of the primary cilium. *Annual Review of Physiology* **67**, 515-529.

Prosser, C. L. (1967). Ionic analyses and effects of ions on contractions of sponge tissues. *Zeitschrift für vergleichende Physiologie* **54**, 109-120.

Prosser, C. L., Nagai, T. and Nystrom, R. A. (1962). Oscular contractions in sponges. *Comparative Biochemistry and Physiology* **6**, 69-74.

Puig, P., Canals, M., Company, J. B., Martin, J., Amblas, D., Lastras, G. and Palanques, A. (2012). Ploughing the deep sea floor. *Nature* **489**, 286-9.

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J. et al. (2012). The Pfam protein families database. *Nucleic Acids Research* **40**, D290-301.

Rasmont, R. (1961). Une technique de culture des eponges d'eau douce en milieu controle. *Annales de la Société Royale Zoologique de la Belgique* **91**, 149-155.

Reiswig, H. M. (1971). *In situ* pumping activities of tropical Demospongiae. *Marine Biology* **9**, 38-50.

Reiswig, H. M. (1974). Water transport, respiration and energetics of three tropical marine sponges. *Journal of Experimental Marine Biology and Ecology* **14**, 231-249.

Reiswig, H. M. (1975a). The aquiferous systems of three marine Demospongiae. *Journal of Morphology* **145**, 493-502.

Reiswig, H. M. (1975b). Bacteria as food for temperate-water marine sponges. *Canadian Journal of Zoology* **53**, 582-589.

Reiswig, H. M. (1981). Partial carbon and energy budgets of the bacteriosponge *Verongia fistularis* (Porifera: Demospongiae) in Barbados. *Marine Ecology* **2**, 273-293.

Reiswig, H. M., Harrison, F. and Cowden, R. (1976). Natural gamete release and oviparity in Caribbean Demospongiae. *Aspects of Sponge Biology*, 99-112.

Ribeiro, S. M., Omena, E. P. and Muricy, G. (2003). Macrofauna associated to *Mycale microsigmatosa* (Porifera, Demospongiae) in Rio de Janeiro State, SE Brazil. *Estuarine, Coastal and Shelf Science* **57**, 951-959.

Riesgo, A., Pérez-Porro, A. R., Carmona, S., Leys, S. P. and Giribet, G. (2012). Optimization of preservation and storage time of sponge tissues to obtain quality mRNA for next-generation sequencing. *Molecular Ecology Resources* **12**, 312-22.

Riisgård, H. U. (1988). The ascidian pump: properties and energy cost. *Marine Ecology Progress Series* **47**, 129-134.

Riisgård, H. U. (1989). Properties and energy cost of the muscular piston pump in the suspension feeding polychate *Chaetopterus variopedatus*. *Marine Ecology Progress Series* **56**, 157-168.

Riisgård, H. U. and Larsen, P. S. (1995). Filter-feeding in marine macro-invertebrates: pump characteristics, modelling, and energy cost. *Biological Reviews* **70**, 67-106.

Riisgård, H. U., Egede, P. P. and Barreiro Saavedra, I. (2011). Feeding behaviour of the mussel, *Mytilus edulis*: new observations, with a minireview of current knowledge. *Journal of Marine Biology* **2011**, 1-13.

Riisgård, H. U., Thomassen, S., Jakobsen, H., Weeks, J. M. and Larsen, P. S. (1993). Suspension feeding in marine sponges *Halichondria panicea* and *Haliclona urceolus:* effects of temperature on filtration rate and energy cost of pumping. *Marine Ecology Progress Series* **96**, 177-188.

Rivera, A. S., Ozturk, N., Fahey, B., Plachetzki, D. C., Degnan, B. M., Sancar, A. and Oakley, T. H. (2012). Blue-light-receptive cryptochrome is expressed in a sponge eye lacking neurons and opsin. *The Journal of Experimental Biology* **215**, 1278-86.

Roberts, D. E., Cummin, S. P., Davis, A. R. and Chapman, M. G. (2006). Structure and dynamics of sponge-dominated assemblages on exposed and sheltered temperate reefs. *Marine Ecology Progress Series* **321**, 19-30.

Rosenbaum, J. L. and Witman, G. B. (2002). Intraflagellar transport. *Nature Reviews Molecular Cell Biology* **3**, 813-25.

Roure, B., Baurain, D. and Philippe, H. (2013). Impact of missing data on phylogenies inferred from empirical phylogenomic data sets. *Molecular Biology and Evolution* **30**, 197-214.

Rützler, K. (1976). Ecology of Tunisian commercial sponges. *Tethys* **7**, 249-264.

Ryan, T. J. and Grant, S. G. N. (2009). The origin and evolution of synapses. *Nature Reviews Neuroscience* **10**, 701-12.

Sakarya, O., Armstrong, K. a., Adamska, M., Adamski, M., Wang, I.-F., Tidor, B., Degnan, B. M., Oakley, T. H. and Kosik, K. S. (2007). A post-synaptic scaffold at the origin of the animal kingdom. *PloS one* **2**, e506.

Santos, F., MacDonald, G., Rubel, E. W. and Raible, D. W. (2006). Lateral line hair cell maturation is a determinant of aminoglycoside susceptibility in zebrafish (*Danio rerio*). *Hearing Research* **213**, 25-33.

Savarese, M., Patterson, M. R., Chernykh, V. I. and Fialkov, V. A. (1997). Trophic effects of sponge feeding within Lake Baikal's littoral zone. 1. In situ pumping rates. *Limnology and Oceanography* **42**, 171-178.

Shiino, Y. (2009). Passive feeding in spiriferide brachiopods: an experimental approach using models of Devonian *Paraspirifer* and *Cyrtospirifer*. *Lethaia* **43**, 223-231.

Shimeta, J. and Jumars, P. A. (1991). Physical mechanisms and rates of particle capture by suspension-feeders. *Oceanography and Marine Biology Annual Review* **29**, 191-257.

Silvester, N. R. (1983). Some hydrodynamic aspects of filter feeding with rectangular-mesh nets. *Journal of Theoretical Biology* **103**, 265-186.

Simpson, T. L. (1984). The Cell Biology of Sponges. New York, New York: Springer-Verlag.

Singla, V. and Reiter, J. F. (2006). The primary cilium as the cell's antenna: signaling at a sensory organelle. *Science* **313**, 629-33.

Solan, M., Germano, J. D., Rhoads, D. C., Smith, C., Michaud, E., Parry, D., Wenzhöfer, F., Kennedy, B., Henriques, C., Battle, E. et al. (2003). Towards a greater understanding of pattern, scale and process in marine benthic systems: a picture is worth a thousand worms. *Journal of Experimental Marine Biology and Ecology* **285-286**, 318-338.

Southwell, M. W. (2007). Sponges impacts on coral reef nitrogen cycling, Key Largo, Florida: PhD thesis, pp 218 The University of North Carolina at Chapel Hill.

Southwell, M. W., Weisz, J. B., Martens, C. S. and Lindquist, N. (2008). In situ fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida. *Limnology and Oceanography* **53**, 986-996.

Sperling, E. A., Peterson, K. J. and Pisani, D. (2009). Phylogenetic-signal dissection of nuclear housekeeping genes supports the paraphyly of sponges and the monophyly of Eumetazoa. *Molecular Biology and Evolution* **26**, 2261-74.

Srivastava, M., Simakov, O., Chapman, J., Fahey, B., Gauthier, M. E., Mitros, T., Richards, G. S., Conaco, C., Dacre, M., Hellsten, U. et al. (2010). The *Amphimedon queenslandica* genome and the evolution of animal complexity. *Nature* **466**, 720-6.

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688-90.

Steyger, P. S., Peters, S. L., Rehling, J., Hordichok, A. and Dai, C. F. (2003). Uptake of gentamicin by bullfrog saccular hair cells *in vitro*. *Journal of the Association for Research in Otolaryngology* **4**, 565-78.

Taghon, G. L. (1981). Beyond selection: optimal ingestion rate as a function of food value. *The American Naturalist* **118**, 202-214.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731-9.

Thompson, R. J. and Bayne, B. L. (1972). Active metabolism associated with feeding in the mussel *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology* **9**, 111-124.

Tompkins-MacDonald, G. J. and Leys, S. P. (2008). Glass sponges arrest pumping in response to sediment: implications for the physiology of the hexactinellid conduction system. *Marine Biology* **154**, 973-984.

Trager, G. C., Hwang, J.-S. and Strickler, J. R. (1990). Barnacle suspension-feeding in variable flow. *Marine Biology* **105**, 117-127.

Turon, X., Galera, J. and Uriz, M. J. (1997). Clearance rates and aquiferous systems in two sponges with contrasting life-history strategies. *The Journal of Experimental Zoology* **278**, 22-36.

Vacelet, J. (1975). Étude en microscopie électronique de l'association entre bactéries et spongiaires du genre *Verongia* (Dictyoceratida). *Journal de microscopie et de biologie cellulaire* **23**, 271-288.

Vacelet, J. and Boury-Esnault, N. (1995). Carnivorous sponges. *Nature* **373**, 333-335.

Villamizar, E. and Laughlin, R. (1991). Fauna associated with the sponges *Aplysina archeri* and *Aplysina lacunosa* in a coral reef of the Archipielago de Los Roques, National Park, Venezuela. In *Fossil and recent sponges*, pp. 522-542: Springer.

Vogel, S. (1974). Current-induced flow through the sponge, *Halichondria*. *Biological Bulletin* **147**, 443-456.

Vogel, S. (1977). Current-induced flow through living sponges in nature. *Ecology* **74**, 2069-2071.

von Dassow, M. (2005). Flow and conduit formation in the external fluid-transport system of a suspension feeder. *The Journal of Experimental Biology* **208**, 2931-8.

Walshaw, A. C. and Jobson, D. A. (1962). Mechanics of fluids, pp. 183: Longmans.

Warburton, F. E. (1960). Influence of currents on form of sponges. *Science* **132**, 89.

Weber, M., Lott, C. and Fabricius, K. E. (2006). Sedimentation stress in a scleractinian coral exposed to terrestrial and marine sediments with contrasting physical, organic and geochemical properties. *Journal of Experimental Marine Biology and Ecology* **336**, 18-32.

Weissenfels, N. (1984). Bau und Funktion des Süßwasserschwamms *Ephydatia fluviatilis* (Porifera). *Zoomorphology* **104**, 292-297.

Weisz, J. B., Lindquist, N. and Martens, C. S. (2008). Do associated microbial abundances impact marine demosponge pumping rates and tissue densities? *Oecologia* **155**, 367-76.

Wilkinson, C. R. (1978). Microbial associations in sponges. III. Ultrastructure of the *in situ* associations in coral reef sponges. *Marine Biology* **49**, 177-185.

Wintermann, G. (1951). Entwicklungsphysiologische Untersuchungen an Süsswasserschwammes, Zool. *Zoologische Jarhrbücher Abteilung für Anatomie und Ontogenie de Tiere* **71**, 427-486.

Wulff, J. L. (2006). Ecological interactions of marine sponges. *Canadian Journal of Zoology* **84**, 146-166.

Yahel, G., Marie, D. and Genin, A. (2005). InEx - a direct *in situ* method to measure filtration rates, nutrition, and metabolism of active suspension feeders. *Limnology and Oceanography: Methods* **3**, 46-58.

Yahel, G., Sharp, J. H., Marie, D., Häse, C. and Genin, A. (2003). *In situ* feeding and element removal in the symbiont-bearing sponge *Theonella swinhoei:* Bulk DOC is the major source for carbon. *Limnology and Oceanography* **48**, 141-149.

Yahel, G., Whitney, F., Reiswig, H. M., Eerkes-Medrano, D. I. and Leys, S. P. (2007). *In situ* feeding and metabolism of glass sponges (Hexactinellida, Porifera), studied in a deep temperate fjord with a remotely operated submersible. *Limnology and Oceanography* **52**, 428.

Young, C. M. and Braithwaite, L. F. (1980). Orientation and current-induced flow in the stalked ascidian *Styela montereyensis*. *Biological Bulletin* **159**, 428-440.