Knowledge advances by steps, not by leaps.

Thomas Babington Macaulay

**University of Alberta**

# Physiology and morphology of epithelia in the freshwater demosponge, *Spongilla lacustris*.

by

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# Abstract

Epithelia form protective barriers and regulate molecule transport between the mesenchyme and environment. Amongst all metazoans, only sponges are said to lack 'true' epithelia however the physiology of sponge cell layers are rarely studied empirically. Aggregates and gemmules of a freshwater demosponge, *Spongilla lacustris*, were used to grow confluent tissue over permeable culture wells which are required for transepithelial recordings. The transepithelial potential (TEP) of *S. lacustris* was slightly negative (-3mV), indicating possible control of ion transport. Transepithelial resistance (TER) was recorded between 1-2 k $\Omega cm^2$, the same order of magnitude as many vertebrate epithelia. Cultures with high resistance blocked the passage of the small tracer molecules $^{14}$C-PEG, $^{3}$H-Inulin and ruthenium red. Pinacocytes were spatially stable over time and epithelial layers were morphologically similar in freshwater and marine species. These results suggest that sponge cell layers are able to control solute and ion transport, the physiological attributes of functional epithelia.

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# Chapter One

## A general introduction to epithelial physiology in sponges and other animals.

### 1.1 Evolutionary concepts in biology

Many questions in evolutionary biology concern the appearance of particular forms in animals.

- How did multicellularity evolve? Which proteins were required?
- When did the first coordination and tissue systems develop?
- What is the physiological function of certain cellular structures?

These types of questions are often explored in model animals using a variety of physiological, histological and genetic techniques. However the study of model organisms alone cannot elucidate all questions, particularly when they concern the origin of animal physiology. Phyla which evolved early during evolution can offer insight into the overall patterns of animal evolution.

The first evidence of sponges in the paleontological record is of demosponge specific sterols in rocks dating from 635 million years ago, at a time period before the Cambrian explosion and subsequent diversification of animal life (Love et al., 2006).

Of all known phyla, sponges are most often considered the earliest diverging multicellular animals (Fig. 1.1) (Phillipe et al., 2009). The Porifera (sponges) are defined as complex, sessile filter-feeding metazoans (Bergquist, 1978). These features are also present in ancient sponges found in Ediacaran age rocks which appear to share a similar morphology with modern species (Li et al., 1998). Because they are thought to have remained relatively unchanged since they first appeared in the geological record, sponges represent a unique window into the evolutionary past. Among the first steps in animal evolution must have been the integration of previously independent cells into cooperative units which led to a division of labour and allowed cells to specialize for particular functional roles. These specializations eventually resulted in the development of complex tissue and organ systems which facilitated a range of novel evolutionary behaviours and allowed adaptations within new ecological niches. Characterization of sponge cell physiology will clarify the functional ability of Poriferan tissues and inform key questions in evolutionary biology concerning the origin of animal forms.

## 1.2 Introduction to epithelia

### *1.2.1 Morphology and protein composition of epithelia*

The evolution of proteins associated with cell-to-cell connectivity, communication and sealing must have been an important factor leading to the diversification of tissue types. Modifications in cell adhesion and permeability determine the function of epithelial tissue, for example to seal organs completely, as in a urinary bladder, or remain somewhat permeable, like in intestines (Claude and

Goodenough, 1973). Since epithelia are a fundamental component of multicellular animals and are present throughout the body, dysfunctions in epithelia are associated with diverse pathophysiologies affecting many organ systems (Miyoshi and Takai, 2005, González-Mariscal et al., 2007). Understanding the physiology of epithelia is thus important in the study of numerous diseases and their treatments.

Epithelia tissues are classified according to the shape and organization of their component cells. These tissues vary in complexity from simple, squamous, pentagonal cells (e.g. endothelium) to ornamented, stratified layers (e.g. epidermis) depending on specific functional requirements (Furuse et al., 2002). Epithelia are tissue structures which encase a whole body, organs or body cavities and they separate an internal environment from the external environment. Their major function is compartmentalization which controls secretion or absorption of water, chemicals, proteins, and ions between disparate body regions. Epithelia are also important for structural stability and protection from the external environment, both of which are particularly important on land.

Epithelia require a specialized set of cell-cell junctions for attachment, molecule regulation, and communication. Tissue integrity depends on stable connections between adjacent cells via desmosome spot contacts (*macular adherens*) and belt-form *adherens junctions* (AJs - *zonula adherens*) (Knust and Bossinger, 2002). Stability also depends on connections to underlying connective tissue layers via focal adhesions (Petit and Theiry, 2000). Belt-form AJs are composed of a complex of proteins which are integrated with the actin cytoskeleton of a cell (Itoh et al., 1997). Cadherins, a large family of transmembrane proteins, all have an

extracellular domain which extends into the intercellular space and adheres to cadherins expressed on adjacent cells. This adhesion is dependent on calcium which controls the conformation of cadherin extracellular domains. The intracellular cadherin domain binds a complex of catenin proteins. The most well-studied complex associated with adhesion junctions includes E-cadherin, α & β catenin, and p120 catenin which are all expressed near the apical cell membrane (reviewed in Cereijido et al., 2004, Miyoshi and Takai, 2005, Magie and Martindale, 2008). In addition to cell layer stability, AJs are also involved with cell signalling and coordination of cell behaviour (Lewis-Tuffin & Anastasiadis, 2008). Protein-protein binding between adjacent cells also occurs in punctate desmosome structures. Desmosomes can be identified from other cell junctions by the expression of the cytoplasmic proteins desmoplakin and desmoglein (Schwarz et al., 1990). Focal adhesion plaques are required for cell stability, signalling and mobility. Integrins, the major constituent proteins of focal adhesions, are transmembrane proteins which are formed by glycosylated heterodimers made up of α and β subunits with large extracellular domains. They anchor the actin cell cytoskeleton to an extracellular matrix composed of fibronectin, collagen, laminin and vitronectin (Petit and Thiery, 2000). This process involves several cytoplasmic proteins including talin, vinculin, paxillin and α-actinin. Integrin based focal adhesions play an important role in 'outside-in' cell signalling via associations with kinases, such as FAK (focal adhesion kinase), which are activated in response to external mechanical stress on the paracellular membrane (Banno and Ginsberg, 2008). Signalling from within the cell can change the activation state of integrins during 'inside-out' signaling via talin proteins (Banno and Ginsberg, 2008). This suite of adhesion junctions, both cell-to-cell and cell-to-

substrate, are thus responsible for the mechanical strength and stability of the entire epithelial layer.

Another major set of junctions is responsible for controlling the permeability of the epithelial cell layer. The tight junction (TJ- *zonula occludens*) transmembrane proteins, claudin and occludin, selectively control molecule passage through the paracellular space (Anderson and Van Itallie, 2009). Unlike occludins, claudins are able to form paracellular barriers in the absence of calcium but are thought to seal only after stable cellular attachment with adhesion junction proteins. Both claudins and occludins are considered to be restricted to vertebrates TJs in a complex at the apex of the cell-cell interaction surface. They form minute pores which selectively control the size and charge of molecules passing across the epithelial sheet. Septate junctions are often understood to perform the occluding junction role in invertebrates (Benerjee et al., 2006). However, they are morphologically distinct structures compared to vertebrate tight junctions and are positioned below adherens junctions rather than above - like TJs. Additionally, some invertebrates express both TJs and septate junctions which further implies that they are not homologous structures (Hudspeth and Revel, 1971). However, it is also known that some proteins are shared between the two regions (Hortsch and Margolis, 2003). This discovery has prompted some investigators to regard the junctions as having a common origin but the true nature of metazoan occluding junctions remains unresolved.

Gap junctions, consisting of innexin proteins in invertebrates allow communication between cells. Vertebrate gap junctions permit the exchange of signalling molecules and ions between closely apposed cells and are composed

predominately of connexins (Shestopalova and Panchin, 2008). These effectively form full or semi channels in the plasma membrane through which ions pass into the cytoplasm of an adjacent cell.

The preceding set of junctions are arranged in a characteristic manner which has been named a "terminal bar". Vertebrate tight junctions are present apically at the lateral cell surface. Adherens junction lie below the more external occluding structures with desmosomes located at intervals below this region. Although there have been some reports of tight junction structures in invertebrates, septate junctions, located basally to AJs, are typically thought to perform the occluding role outside of vertebrates (see Magie and Martindale, 2008).

### *1.2.2 General epithelial physiology*

The physiology of animal epithelia has been investigated for over 100 years. One of the preeminent philosophers in animal biology, Claude Bernard introduced and explored concepts which later developed into modern physiology (Bernard, 1949). In 1885, Bernard was the first to realize that maintaining an internal milieu was of particular importance for independent life. This work was followed by that of W.B. Cannon who coined the term "homeostasis" referring to the steady-state which is controlled by overlapping systems within living beings (Cannon, 1939). Understanding the processes which govern how cells interact to control an internal environment has long been a focus for biological research. H. Ussing and his contemporaries developed methods to study the electrical physiology of skin

(Ussing, 1960). A major development from this period was so-called "Ussing chambers", a closed system in which electrodes record the ion current passing across an epithelial bridge separating two liquid filled chambers. This method measures ionic gradients maintained on either side of the epithelia. Since cell membranes are relatively impervious to the passage of molecules, the ability to maintain an ionic gradient is determined primarily by mechanisms for controlling diffusion between adjacent cells (paracellular transport route) and by the active pumping of ions against osmotic pressures (reviewed in Teukita et al., 2001).

Epithelia vary in their ability to seal the paracellular space and this has led to classifications of 'leaky' or 'tight' tissues (Claude and Goodenough, 1973). In vertebrates, epithelial tight junctions have been shown to be the major limiting structures responsible for controlling the passive diffusion of molecules between cells. They are sometimes considered analogous to resistors in a simple electrical circuit. Thus Ohm's law (Resistance=Voltage/Current) has been used in epithelial physiology to determine transepithelial resistance after a voltage pulse. Molecule flux and epithelial conductance methods have been used extensively in the study of diseases related to deficient paracellular permeability and are related to fields like the study of Crohn's disease (Hyun et al., 1995). Additionally, transepithelial resistance is relevant to the study of drug delivery across the blood brain barrier.

### *1.2.3 The cell biology of sponge epithelia*

The current scientific paradigm describes sponges as lacking characteristic Eumetazoan traits including bilateral symmetry, body axis polarity, true tissues,

organs, nerves, and a mesoderm (the third germ layer). Specifically, they are said to lack cellular polarity, a basal lamina, and cell-cell junctions; traits required to constitute a so-called 'true' epithelium (Green and Bergquist, 1982, Tyler, 2003, Magie and Martindale, 2008). The major functional unit of a sponge body consists of a layer of juxtaposed cells termed pinacocytes due to their flat, thin pavement-like appearance. These layers bound external and internal regions within the sponge body. Choanocytes represent a second functional unit and are responsible for feeding. These cells possess several specialized elaborations, principally a long flagellum surrounded by a ring of microvilli and a round base as the site of particle uptake and digestion. An additional 13 or so cell types have been described in sponges, most of which are not found in surface layers, but exist as migratory cells within the sponge mesohyl - middle ground material (Bergquest, 1978, Simpson, 1984). Some mesohyl cell types include sclerocytes for spicule secretion, collencytes/lophocytes for laying down the collagen mat, spongocytes for spongin secretion, and cells with inclusions such as spherulous, gray, globoferous and rhabdiferous cells (Simpson, 1984). Although this suite of cells has been previously identified in some sponge species, further work describing sponge cell biology is required to understand physiological systems operating throughout these animals.

Spongeologists have used the term 'epithelium' to describe a sponge pinacoderm layer however, they have been classed 'epithelioid' by other researchers when compared to higher animals (Woollacott and Pinto, 1995). The argument that sponges lack epithelia was primarily based on the absence of evidence showing stable belt-form junctions and a basal lamina in sponges (Tyler, 2003, Cereijido et al., 2004). However, the degree of connectivity between sponge cells has not been

previously determined by physiological methods. Much confusion resulted from the reliance on images that were produced using fixation techniques, which inherently caused damage. Authors thus observed that intercellular distances were large and lacked permanent cell junctions structures like those in other animal tissues, while other studies have clearly shown a variety of junctions throughout the Porifera (reviewed by Leys et al., 2009). These junctions have been sporadically described as transient or 'leaky', suggesting that cells are able to slide in and out of epithelia (Ledger, 1975, Green and Bergquist, 1982). Morphologically, the most compelling case for the existence of sponge cell junctions are desmosomes in *Ephydatia fluviatalis* (Feige, 1969, Pavans de Ceccacaty, 1974) which are associated with 5-10nm cytoplasmic microfilaments and septate junctions between sclerocytes in calcareous sponges (Ledger, 1975). The recently published genome of *Amphimedon queenslandica*, a marine demosponge, contained cadherins, α & β catenin, p120 catenin, α & β integrins, and γ laminin proteins (Srivastava et al., 2010). It has also been found that sponges contain MAGI (membrane associated gyuanlate kinase linker protein with inverted arrangement), a molecule that is associated with tight junctions in vertebrates (Adell et al., 2004). Although no evidence of occludin or claudins exists within the Porifera, a member of the tetraspanin family of proteins is expressed in pinacoderm regions (Adell et al., 2004, Srivastava et al., 2010). Sponges are also known to produce a network of collagen below pinacoderm layers in an arrangement similar to the basement membrane. In homoscleromorphs this region contains type IV collagen while in demosponges it contains spongin short chain collagen - a homolog to type IV (Boute et al., 1996, Aouacheria et al., 2006 ). Evidence from recent molecular work revealed that sponges contain many post-

synaptic protein coding genes although they do not contain any nerves (Sakarya et al., 2007). This indicates a potential disconnect between functional physiology - since propagating electrical signals have not yet been found in cellular sponge and contractions are slow, genetics - since post synaptic proteins are present, and morphology - since no nerve structures have been found. This type of disconnect could form the basis of a hypothesis postulating that the collagenous structures present in sponges could adequately serve as a functional basement membrane in the absence of type IV collagen. The presence of junction related genes and some corresponding ultrastructural features suggests that the designation of sponge pinacoderms as epithelioid may be misleading.

## 1.3 The evolution and phylogeny of basal metazoans

The resolution of phylogenetic patterns during early metazoan evolution has been a topic of considerable research with contradictory conclusions. Traditional character-based phylogenies place sponges as the most basal multicellular animal taxon. This is due to morphological similarities between the choanocytes of sponges and choanoflagellate protists (James-Clark, 1868) as well as the lack of nerves, radial symmetry, gut and other bilaterian features. They have been considered metazoans since the 19th century based on spermatogenesis (Schulze, 1885). Sponges were long considered evolutionary oddities which branched early in animal history and reached a dead-end. However molecular phylogenies of basal metazoans have generated conflicting theories which could indicate that all animals had a sponge-like last common ancestor (LCA).

Sponges can be grouped into three major classes Demospongiae (Sollas 1885), Hexactinellida (Schmidt 1870) and Calcarea (Browerbank 1864) based predominately on the structure and composition of their spicules. Two of these classes are siliceous sponges; demosponges, are cellular with mono or tetra-axial spicules and glass sponges (hexactinellids), which are syncytial with tri-axial (six-rayed) spicules (Bergquist, 1978). Cellular calcareous sponges produce extracellular calcium carbonate spicules typically in a three-ray arrangement. By far the most species rich group is Demospongiae which make up 75%- 95% of all sponges (Bergquist, 1978, Hooper & van Soest, 2002). An additional subclass of sponges, the homoscleromorphs, are emerging as an important phylogenetic group. Although they have long been considered basal demosponges because of their siliceous skeletons, more recent morphological and genetic studies have suggested that they may be a phylogenetically distinct class and more recently derived. A paraphyletic Porifera concept was presented in a theory advanced a decade ago. The theory suggests that sponges are a paraphyletic grade (Zrzavý et al., 1998, Collins, 1998, Medina et al., 2001, Erpenbeck and Worheide, 2007). This scheme typically shows Demospongiae and Hexactinellida appearing first during evolution followed by Calcarea and Homoscleromorpha as the sister group to Eumatazoa (Kruse et al., 1998, Borchilinni et al., 2001, Sperling and Peterson, 2007) (Fig. 1.2). The validity of the paraphyletic hypothesis has profound importance for evolutionary theories related to the development of animal synapomorphies. Paraphyly strongly suggests that all animals descended from a sponge-like last common ancestor (Halanych, 2004, Nielsen 2008) because parsimony would dictate that multiple sponge branches would share an ancestor with sponge features. In this scenario, the evolution of complex traits that

were present in all Poriferans could directly inform the study of similar features in higher order animals. However, the base of the animal tree of life has continued to be restructured in recent years and the identity of the last common animal ancestor remains controversial. The branching order of early metazoan taxa differs depending on the quantity and type of genes selected, as well as the number of different species sampled and the analysis technique used. In particular, long-branch length attraction may have greatly impacted the structure of phylogenies. Other recent hypotheses have stated that either ctenophores (Dunn et al., 2008) or placozoans (Schierwater et al., 2009) are the most basal multicellular animals. Although neither theory is definitively confirmed, the fact that such differing opinions exist on this sort of fundamental question highlights a particular difficulty with evolutionary biology. The validity of these phylogenetic relationships determines whether hypotheses generated from early metazoan research are interpreted correctly. The most recent examination of this issue (Pick et al., 2010) has reanalysed the data from Dunn et al. (2008) and concluded that sponges are monophyletic and are the first branch of multicellular animals. This scenario was also resolved from data gathered from protein coding genes (Phillipe et al., 2009). It should be explicitly noted that sponge monophyly does not exclude the possibility of a last common metazoan ancestor having many sponge features. Only with more rigorous testing using a large number of genes and with particular use of a larger assortment of early branching taxa, including many sponge representatives, can a more accurate tree be resolved and accepted.

## 1.4 Freshwater demosponges

### *1.4.1 Sponge ecology*

Sponges are found throughout the world from deep marine habitats, to coastal waters, estuaries and lakes. They grow over a variety of surfaces including rocks, branches, mud, gastropod shells and other sponges. There are over 8340 described species with the majority belonging to the Demospongiae class (Worheide et al., 2005, van Soest et al., 2008). Sponges reproduce both sexually with free swimming larvae and asexually by budding, reaggregation and gemmulation (Simpson and Gilbert, 1974). Gemmulation tends to occur during the winter in sponges living at high latitudes at a time when adult tissue regresses (Fig 1.3). Gemmulation involves the grouping of pluripotent cells into round masses which are encased in collagen sheaths containing embedded siliceous spicules (Simpson and Fell, 1974). In the spring, cells from the gemmules migrate out and recolonize the 'parent' spicule framework left after regression the previous winter (Simpson and Gilbert, 1973). Freshwater demosponges can have a global distribution and constitute more than 30 species growing in diverse forms (Harrison and Warner, 1986). Although the origin of freshwater sponges is not known for certain, it is believed that they diverged multiple times from marine haplosclerids during the Tertiary about 50 million years ago (Muller, 1982). It has been postulated that the advent of gemmulation in some marine haploscerid demosponge ancestors promoted a greater dispersal ability in their freshwater descendents (Manconi and Prozanto, 2007). Gemmules could populate new lakes while circumventing desiccation. The fact that cosmopolitan freshwater

sponges tend to have gemmules while those without reduction bodies tend to have limited distributions seems to support this hypothesis.

Although many sponges are thought to have few natural predators due to spicule defensive strategies and repulsive chemical toxins, freshwater sponges are known to be a significant nutrient source for larval caddisflies and "spongillaflies" (Resh, 1976, Manconi and Pronzato, 2007). Sponge species often encrust on rocks or other solid substrates. For this study, *Spongilla lacustris* and *Ephydatia muelleri* were found growing in patches on submerged logs and rocks in lakes. They tend to live in shaded zones in a low flow environment with minimal turbidity (Harrison and Warner, 1986). As with other Poriferans, freshwater sponges feed on small particulates including flagellates, bacteria and detritus and in particular have associations with zoochlorella, a green alga which is responsible for their green colouring (Frost and Williamson, 1980). Whether these symbionts have beneficial properties for their sponge hosts has not been conclusively determined but based on growth patterns which show sponges with symbionts grow at a faster rate, it seems likely that the algae are beneficial (Frost and Williamson, 1980).

### *1.4.2 Sponges as laboratory models*

Working with a non-traditional model like sponges presents inherent challenges. Basic anatomy and physiology are not completely understood, although genetic data for sponges is a growing field, the genetics of *E. muelleri* and *S. lacustris* are not yet fully annotated and published, polyclonal antibodies raised against non-sponge proteins do not typically bind sponge epitopes so are required to be made de

novo at significant expense and time, and sponge culturing is not always successful. While many such challenges do exist, sponges allow study based on a whole animal multi-methodology approach that is often impossible in higher animals.

Freshwater sponges are also promising laboratory model animals due to their ephemeral life-history where they form over-wintering gemmules (cysts of pluripotent cells encased in a collagenous sheath). Once collected, gemmules can be easily stored in a laboratory for up to a year at 4$^{o}$C and potentially longer if frozen at -80$^{o}$C. Gemmules are cultured on cover slips in artificial freshwater M-Medium and form juvenile sponges after only a week on the bench top (Ricciardi and Reiswig, 1993). Furthermore sponge tissue, and *Spongilla lacustris* in particular, has the ability to reaggregate (Fig 1.3) into a functional sponge after being disassociated into a pool of single cells. This behaviour has long fascinated researchers because it demonstrates the high degree of plasticity and wound repair possible in the sponge bauplan (Henry and Hart, 2005). After extensive research, the mechanism for cell aggregation is now known to be a large proteoglycan molecule which has been called an aggregation factor (Schutze et al., 2001, Harwood and Coates, 2004). This molecule not only promotes cellular aggregation but it is also responsible for cell sorting. The process results in cells selectively migrating together according to species, individual and cell type. The ability of sponges to regenerate from aggregates and gemmules made it possible to grow sponge tissue over permeable membrane supports which then permitted electrophysiological recordings.

## 1.5 Thesis objectives and hypotheses

The objective of this thesis was to determine if freshwater sponges have functional epithelia with the physiological characteristics of sealing and ion transport. It asked if freshwater sponges have the ability to regulate an internal milieu and what morphological features may be involved and shared between species. I aimed to (a) empirically test the physiology of freshwater sponge tissues for sealing of paracellular ion and molecule transport and to (b) describe a suite of morphological and behavioural characteristics of sponge epithelia in different species. I asked the following questions:

1. Do sponges have the ability to occlude ion and molecule passage through the epithelial sheets?

2. What morphological and molecular features are present in sponge epithelia throughout the phylum? Do cells in the pinacoderm layer behave as a cohesive unit?

### *1.5.1 Aims of Chapter Two*

In Chapter Two, I describe the physiological sealing capacity of sponge tissues. *S. lacustris* was shown to have a relatively high transepithelial resistance that is comparable to other transporting epithelia in higher order metazoans. As with other epithelia, sponge pinacoderms were shown to occlude molecules such as inulin and ruthenium red in an inverse relationship to resistance. These results suggest that the

first multicellular animals, sponges, had already evolved mechanisms for cell-cell occlusion and coordination.

### *1.5.2 Aims of Chapter Three*

In chapter three, I show the stability of the pinacoderm layers compared to other mesohyl regions in sponge tissues. I present a description of morphological similarities between different sponge species using transmission and scanning electron microscopy, fluorescence and light microscopy. In detail I show junction structures between adjacent freshwater sponge cells, focusing on similarities between sponge cell layers and discuss different body forms in marine species. Morphological comparisons were made to begin to address the question of whether epithelial characteristics recorded in freshwater sponges were independently derived or could be found throughout the phyla and homologous to features in other animals.

### *1.5.3 General Discussion*

In chapter four, I discuss how these findings may impact evolutionary theories about epithelia. I also suggest further experiments which may add more information to these fields.

**Fig 1-1 Phylogeny of metazoan relationships:** Phylogenetic tree representing protein coding gene and morphological data adapted from Philippe et al., 2009 and Borchiellini et al., 2001. Sponges are here considered the most basal metazoans and are monophyletic. Definitive relationships between homoscleromorph and calcareous sponges and other animals remain uncertain. A - Bilateria, B- Eumetazoa, C - Metazoa.

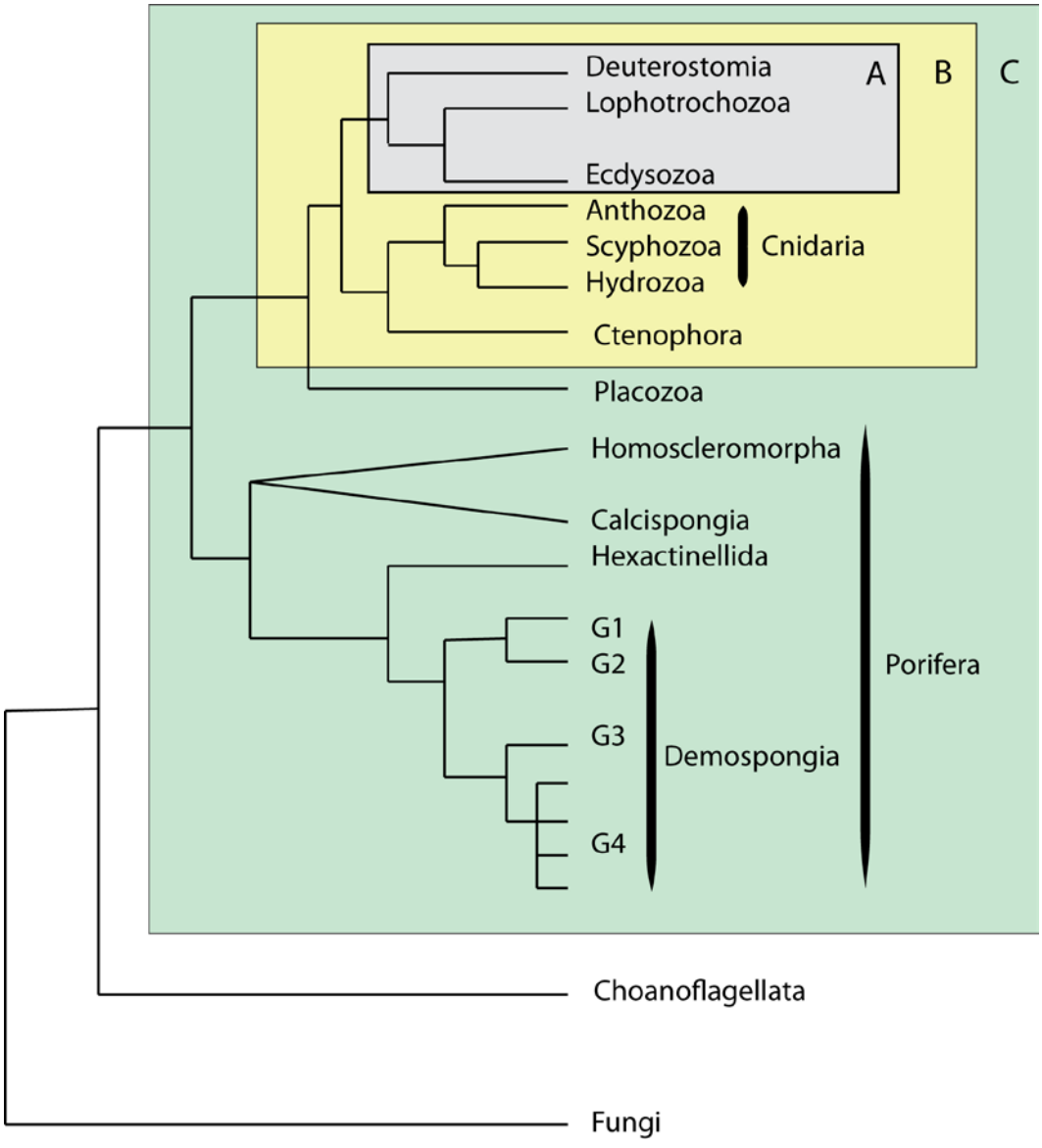
A
B
C
Deuterostomia
Lophotrochozoa
Ecdysozoa
Anthozoa
Scyphozoa
Hydrozoa
Cnidaria
Ctenophora
Placozoa
Homoscleromorpha
Calcispongia
Hexactinellida
G1
G2
G3
G4
Demospongia
Porifera
Choanoflagellata
Fungi

**Figure 1-1**

**Figure 1-2 Sponge monophyly versus paraphyly.** Two alternate hypothesis suggest that sponges may be a monophyletic basal clade (**A**) or a paraphyletic grade (**B**). Hm - Homoscleromorphs, Hx - Hexatinellids, De - Demosponges, Ca - Calcareous sponges.

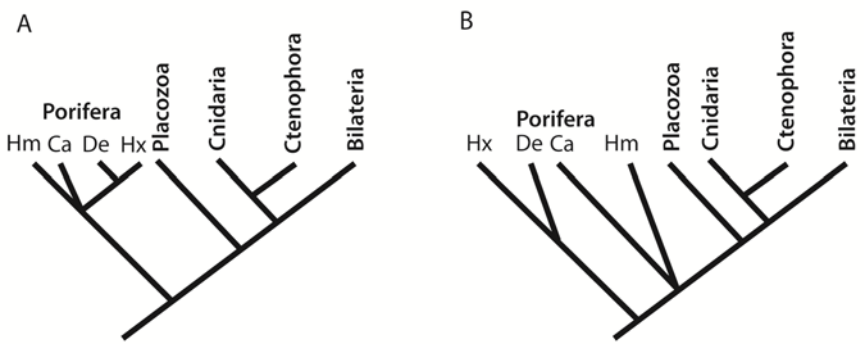
A
Porifera
Hm Ca De Hx
Placozoa
Cnidaria
Ctenophora
Bilateria
B
Hx
Porifera
De Ca
Hm
Placozoa
Cnidaria
Ctenophora
Bilateria

**Figure 1-2**

**Fig 1-3 Aggregate and gemmule cultures of *Spongilla lacustris* and *Ephydatia muelleri*.** (A) *S. lacustris* gemmules. (B) *E. muelleri* juvenile grown from a gemmule on a cover slip. (C) *S. lacustris* juvenile grown on a cover slip from aggregates. (D) One-week old S. *lacustris* aggregates. (E) Adult *S. lacustris* grown on submerged branches in Rousseau Lake [Photo. S. Leys]. (F) *S. lacustris* tissue grown from aggregates on permeable membrane culture wells.

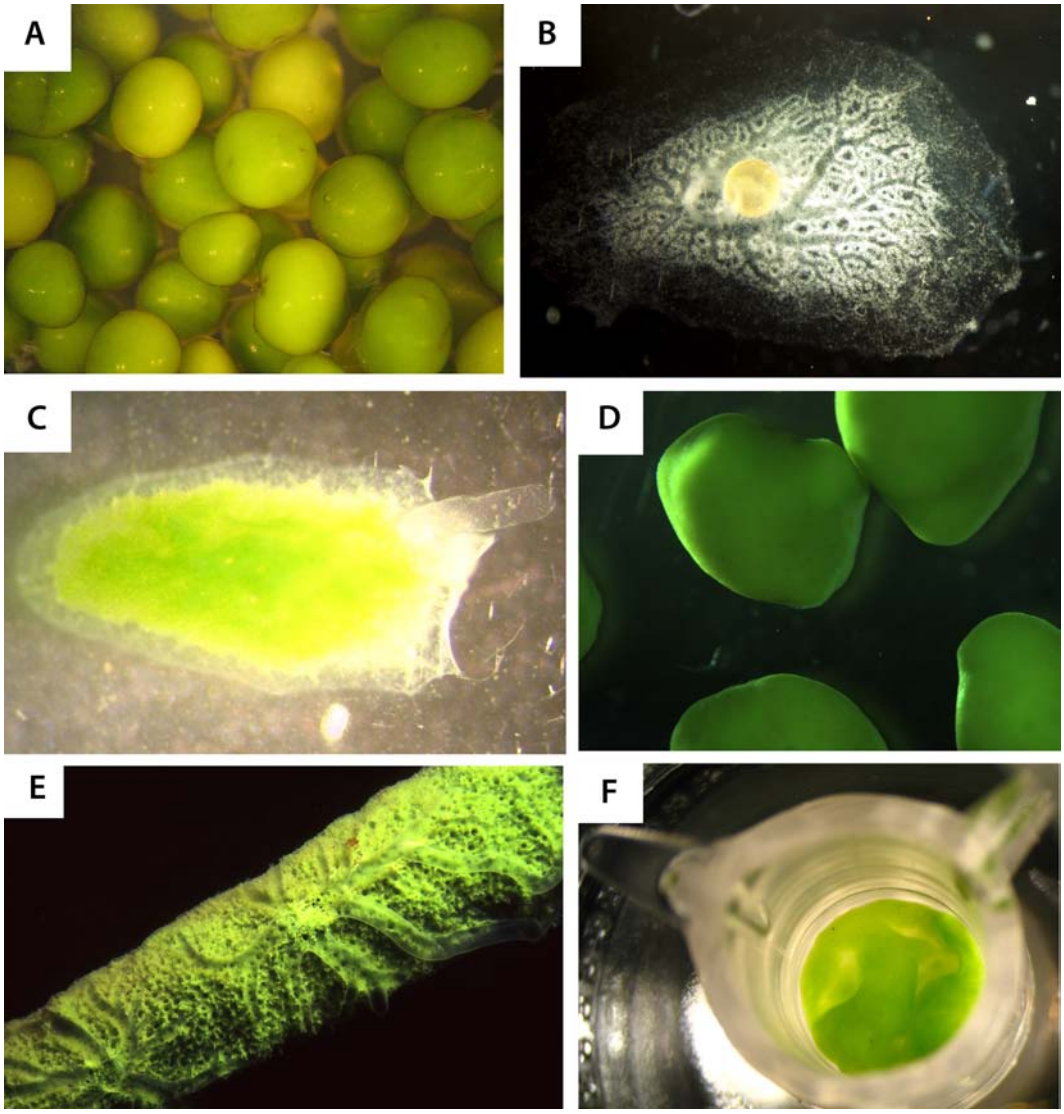


**Figure 1-3**

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# Chapter Two

## Freshwater sponges have sealed and ion transporting epithelia.

### 2.1 Introduction

Epithelial tissue — the sealed and polarized layer of cells that regulates transport of ions and solutes between the environment and the internal milieu — is a defining characteristic of the Eumetazoa. A sealed epithelium is necessary to control the internal milieu of animals but it is particularly important in fresh water where osmotic stress and loss of ions can disrupt cell physiology (Shaw, 1958). Epithelia are considered to have first evolved in ancestors of the Cnidaria (Philippe et al., 2009) so sponges (Porifera), which diverged up to 200 million years prior to other metazoans (Love et al., 2009), are thus the only multicellular animals thought to lack true epithelial tissues (Mackie, 1984, Tyler, 2003, Cereijido et al., 2004, Srivastava et al., 2010). Although most phylogenomic analyses show sponges as the most basal animals (Phillipe et al., 2009) other authors have found evidence that animals with occluding epithelia, such as ctenophores or placozoans (Dunn et al, 2008, Schierwater et al., 2008), are more basal than sponges. If these scenarios were correct, it suggests that sponges must have lost functional epithelia. However, freshwater demosponges

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must have experienced the same evolutionary pressures to regulate an internal environment as eumetazoans, and therefore they provide a unique model to study the emergence of key features of functional epithelia.

Epithelia are composed of simple or stratified layers of polarized cells that are connected by intercellular junctions. In most animals, desmosomes and adherens junctions (AJs) control whole tissue integrity (Cereijido et al., 2004), while semi-permeable occluding junctions such as tight junctions (TJs) or septate junctions (SJs) selectively control molecule and ion passage between cells (Anderson et al., 2009). Epithelia are also usually supported by an extracellular basement membrane (the basal lamina), which prevents dedifferentiation and migration of cells into the mesenchyme (Timpl, 1996). Although epithelial characteristics are often rigidly defined, epithelia are actually dynamic structures with variable integrity and tightness depending on physiological requirements throughout the body or during ontogeny (Leys et al., 2009). Moreover, the recent discovery of new types of cell junctions in vertebrate tissues (Franke et al., 2009) demonstrates that the characterization of cell-cell interactions is still a developing field. There must be a wide diversity and redundancy of junction proteins since the presence of known junctional molecules does not always match with junction morphology. Sealing and adhesion may therefore occur in the absence of classical occluding and adherens molecules or junctions.

Although early morphologists classified sponge surfaces as syncytial tissues, the advent of electron microscopy revealed clear cell boundaries. The external surface of sponges is formed by a single layer of thin, pentagonal cells called pinacocytes

(pavement cells) that enclose a collagenous mesohyl (middle layer) containing mobile amoeboid cells. Sponge pinacoderms are polarized by the unilateral secretion of proteins and express the polarity genes Frizzled, discs large, Par, and Crumbs (Adell et al., 2003,Srivastava et al., 2010). The membranes of adjacent pinacocytes are typically closely apposed, separated by a small 15 nm intercellular gap (Green & Bergquist, 1982 ). Desmosome- and septate-like junctions have been found between cells in all sponges (Leys et al., 2009), even though only a few of the genes associated with SJs appear to be present (e.g., discs large (Srivastava et al., 2010) and neurexin (Nichols et al., 2006)). Regions of membrane fusion (Revel, 1966) and sealing (Eerkes-Medrano, 2006) also occur in sponges and some tight junction-related genes (e.g., members of the tetraspanin and MAGI family of proteins) are expressed in the pinacoderm (Adell et al., 2004). Although some junction related genes have been found in sponges along with evidence of junction structures in microscopy, no evidence of physiological function has been determined.

A distinct basement membrane with immunoreactivity to type IV collagen has been found in several homoscleromorph sponges (Boute et al., 1996). Although other sponges appear to lack an obvious basement membrane, a structurally similar network containing fibronectin and a homolog of type IV collagen underlies pinacoderms (Labat-Robert et al., 1981, Aouacheria et al., 2006, Leys et al., 2009) and integrins are localized at the basal cell surface (Brower et al., 1997, Srivastava et al., 2010). Thus sponges possess the components for a functional extracellular matrix capable of supporting an epithelium. Recent work demonstrated that freshwater sponge pinacocytes are static over time (Leys et al., 2009 - Chapter Three) and that glutaminergic signalling controls a coordinated inflation and contraction behaviour,

in effect, a sponge ‘sneeze’ (Elliott and Leys, 2010). These behavioural traits are evidence that pinacocytes must establish stable attachments for communication and coordination. But the ultimate demonstration of a true epithelium is the ability to selectively regulate the passage of solutes, that is, its ability to seal and create a transepithelial potential.

## 2.2 Materials & Methods

### *2.2.1 Collection, culturing and fixation of tissues.*

During the summers of 2008 and 2009, tissue of the freshwater demosponge *Spongilla lacustris* (Linnaeus 1759) were collected off submerged branches by snorkelling in Sarita and Rosseau Lakes, Vancouver Island, B.C., Canada. Sponges were stored in unfiltered 18°C lake water for approximately one week at the Bamfield Marine Sciences Centre, B.C., Canada with daily water changes. Gemmules from *S. lacustris* and another freshwater demosponge, *Ephydatia muelleri* (Lieberkühn 1955) were collected during the winter months from Rosseau and Frederick Lake, B.C and stored in lake water at 4$^{o}$C for up to one year at the University of Alberta, Edmonton, Canada.

For microscopy, gemmules were gently excised from their adult spicule framework, cleaned with 1% $H_2O_2$, and plated on ethanol flamed cover slips in 20 mL Petri dishes. Gemmules hatched after two days and developed into juvenile sponges within a week of plating. Sponges were fixed for electron microscopy with 1% $OsO_4$, 2% glutaraldehyde, 0.45M sodium acetate buffer pH 6.4, and 10% sucrose

at 4° C for six hours (Elliot and Leys 2007). Samples were rinsed in water, dehydrated through a graded ethanol series (70%, 95%, 100% EtOH) and either embedded in Spurs resin (Electron Microscopy Services, Hatfield, PA, USA) for Transmission Electron Microscopy (TEM) on a Philips/FEI Morgagni microscope or critical point dried, mounted and sputter coated with gold for Scanning Electron Microscopy (SEM) using a JEOL 6301F field emission microscope. For fluorescence microscopy, sponges were fixed in 3.7% paraformaldehyde, 0.3% glutaraldehyde in 10 mM phosphate buffered saline (PBS) at 4° C overnight. Sponges were rinsed in PBS, permeabilized with PBTX-100 for two minutes and rinsed again for 30 minutes in PBS. Samples were labelled with Bodipy Fluorescein Phallacidin (Molecular Probes, OR) for three hours and counterstained with 1 mM Hoechst. Cover slips with attached sponges were inverted and mounted on slides in Mowiol with Dabco (antifade reagent: Polysciences, Warrington, PA, USA), for viewing on a Zeiss Axioskop 2 Plus microscope.

### *2.2.2 Tissue culture*

For tissue culture, pieces of *S. lacustris* approximately 10 $cm^2$ were manually disassociated (by pressing) through either 25 μm or 100 μm Nitex mesh into Petri dishes with artificial freshwater (AFW) (M-Medium: 0.5 mM $MgSO_4 \bullet 7H_2O$, 1 mM $CaCl_2 \bullet 2H_2O$, 0.5 mM $NaHCO_3$, 0.05 mM KCl, 0.25 mM $Na_2SiO_3$) (Rasmont, 1966). Cell suspensions were kept at 12-15° C with gentle rotation and the medium was changed daily. At one week post disassociation (1 wpd), approximately three 5 $mm^3$ round aggregates were seeded into each Millicell hanging cell culture insert; culturing

inserts were made of polyethylene terephthalate with 0.4 µm pore sizes and a 0.33 $cm^2$ filter area (Millipore™, Billerica, MA, USA). Inserts were suspended in a basin with approximately two litres AFW with aeration to generate water circulation. This set-up was key to the growth of healthy sponge tissue. Without additional aeration or when cultures were grown in a small volume of liquid, sponge tissue quickly died. Under optimum conditions, cultures formed confluent sheets three or more days after seeding. Tissue cultures were alternatively prepared by hatching *S. lacustris* gemmules directly onto culture inserts (25 cysts per dish).

### *2.2.3 Transepithelial Resistance*

Inserts which appeared to contain fully confluent sponge cultures were placed in a 24 well Falcon culture dish with symmetrical apical (0.25 ml) and basal (1.5 ml) AFW solutions. Transepithelial electrical resistance (TER) was measured with an electron voltohmeter (EVOM; W.P.I., Sarasota, FL). Because AFW had insufficient ions to record current, salts were added to AFW medium to reach 0.06-0.099% w/v NaCl [1.5 mM $MgSO_4 \bullet 7H_2O$, 1.4 mM $CaCl_2 \bullet 2H_2O$, 0.6 mM $NaHCO_3$, 0.45 mM KCl, 0.25 mM $Na_2SiO_3$, 11 mM-17 mM NaCl, 0.6-0.9 mM $MgCl_2 \bullet 6H_2O$]. Resistance (R) values were corrected by subtraction of the R of an empty Millicell insert bathed in each medium from each recorded R value. To recording medium without $CaCl_2$, 0.5mM EGTA was added for calcium chelating and 1mM NMDG-Cl was added to maintain osmotic balance by replacing the missing calcium ions. In recording medium without $MgSO_4$, $CaCl_2$, and $MgCl_2$, calcium and magnesium were removed by the addition of 0.5mM EDTA and those ions in recording medium were

replaced with 2mM NMDG-Cl and 1.5mM NMDG-$SO_4$. Chloride-free TER medium contained 12.7 mM Na-gluconate, 1.95 mM $MgSO_4 \bullet 7H_2O$, 1.1 mM Ca-gluconate, 0.56 mM $NaHCO_3$, 0.32 mM K-gluconate, and 0.25 mM $Na_2SiO_3$; sodium-free TER medium contained 1.5 mM $MgSO_4$, 1.4 mM $CaCl_2$, 0.45 mM $KHCO_3$, 11 mM NMDG-Cl, and 0.6 mM $MgCl_2$. Chemicals were obtained from Sigma (Oakville, ON.) unless otherwise noted.

### *2.2.4 Paracellular molecule flux.*

To measure paracellular molecule flux, 1 μCi mL-1 of [$^3$H] inulin or [$^{14}$C] PEG was added to the apical side of *Spongilla* cultured on Millicell inserts. At time zero, 10 μl baseline samples were taken from the apical side to determine initial value; thereafter 100 μl was sampled from the basolateral side every 30, 60, 90, 120 and 180 minutes. Samples were stored at 4°C, mixed with 4 ml of ACS Fluor scintillation counting fluid (Fisher, Ottawa, ON) and radioactivity was measured with a Beckman LS 6000 scintillation counter.

Epithelial permeably was also assessed in TEM by examining the passage of a ruthenium red (RR) tracer. Tissues were fixed for one hour in 2% glutaraldehyde with 1mg $mL^{-1}$ RR in 0.02 M cacodylate buffer; rinsed three times with 0.02 M cacodylate buffer and post-fixed in 1% $OsO_4$ with 1mg $ml^{-1}$ RR in 0.02M cacodylate buffer. Following dehydration in ethanol the tissue was embedded in Spurs (Electron Microscopy Services, Hatfield, PA, USA) and thin sections (60-70nm) were cut on a Leica Ultracut T ultramicrotome. Sections were mounted on 200nm copper grids or

formvar coated grids, stained with uranyl acetate for one hour and lead citrate for eight minutes and then viewed in a Morgani Field Emission I. Transmission Electron Microscope (TEM).

## 2.3 Results & Discussion

### *2.3.1 Culturing and TER*

To determine if freshwater sponge epithelia can seal and generate an electrochemical potential I took advantage of the ability of sponge cells to form aggregates from dissociated cells (Wilson, 1907) and hatch from gemmules, cysts of undifferentiated cells, to grow cell cultures on permeable membranes. *Spongilla lacustris* cells formed confluent layers 3-5 days after seeding with either aggregates or gemmules. I measured the transepithelial electrical resistance on either side of the sponge tissue using chopstick electrodes inserted into the upper (apical) and lower (basolateral) chambers (Fig. 2.1 A). The electrode in the apical chamber was set as zero while the electrode in the basal chamber recorded the resistance or voltage. Scanning electron microscopy (Fig. 2.1 B) showed that the cultures had all the attributes of a typical sponge with closely apposed exopinacocytes in the dermal tissue or surface of the sponge (Fig. 2.2 A, B), canals lined by endopinacocytes, choanocyte chambers and an osculum or chimney-like vent. The sponge basopinacoderm (which directly contacts the culture membrane) is formed by exopinacocytes which secrete proteins that form an attachment to surfaces. Since the canal system is contiguous with the external environment, resistance to ion passage occurred through two simple cell layers, the endopinacoderm and basopinacoderm

(Fig. 2.1 A inset). The basopinacocytes completely covered the membrane by forming a mosaic of cells that resembled vertebrate epithelial cell cultures (Fig. 2.2 C). Punctate regions between exopinacocytes were associated with 10 nm diameter actin fibres that extended into the cytoplasm (Fig. 2.2 B). These regions corresponded to actin-dense plaques found in the exopinacocyte layers of both *S. lacustris* and *Ephydatia muelleri* (Fig. 2.2 D, E).

Cultures that were confluent across culturing membranes had resistances (R) above 300 $\Omega$ cm$^2$ and up to 5500 $\Omega$ cm$^2$, comparable to the resistance of vertebrate epithelia (Fig. 2.3). Cultures formed from gemmules had a higher resistance (mean 1932.5 $\Omega$ cm$^2$ $\pm$ 253.5 s.e., n= 22) than those formed from aggregates (mean 1098.5 $\Omega$ cm$^2$ $\pm$ 671.5 s.e., n= 33), possibly because the cultures made from gemmules contained more cells (Fig. 2.4). Initial resistances of the sponge epithelium were even higher, but dropped when I began recording because it was necessary to add salt (<0.1% w/v NaCl) to the medium so that the instrument could measure a current. The resistance stabilized at 30% of their initial value after 10 minutes; and it was after two minutes in the recording medium that resistances were reported.

Epithelial resistance varies by orders of magnitude between different tissue types and animals studied. Freshwater fish gill epithelia have amongst the highest recorded resistance (up to 40,000 $\Omega$ cm$^2$) (Table 2.1). However tissues considered to be true epithelia, including proximal kidney tubule, can also have very low resistances when they are associated with nutrient or waste exchange (Claude and Goodenough, 1973). Sponges represent a mid range of resistance, and typically a resistance of 500 – 1500 $\Omega$ cm$^2$ is considered a tight epithelium (Claude et al., 1971).

### *2.3.2 Paracellular solute flux*

Sponge cultures with a high electrical resistance excluded the flux of a 5 kD 1.5 nm diameter $^{3}$H-inulin tracer (Fig. 2.5 A). For cultures with the highest resistance, only 0.8% of the tracer was able to pass through after three hours (Fig. 2.5 B). A higher molecular weight tracer ($^{14}$C PEG MW = 7000) was also excluded from passage through sponge cultures that had a high resistance (Figure 2.6). The electron dense tracer ruthenium red (MW = 859) was excluded from the paracellular space and was not able to enter the sponge mesohyl (Fig. 2.5 C, D). A potential site for this occlusion would be regions where the membranes of adjacent cells appeared fused (Fig. 2.5 E).

To better understand the characteristics of ion occlusion by sponge epithelia cultures were treated with the divalent cation chelators EGTA and EDTA. In the absence of calcium, it was expected that — as in vertebrate models — disruption of calcium-dependant adhesion would result in lower resistance. However, neither chelator reduced the resistance of sponge epithelia more than control cultures in recording medium alone (Fig. 2.7 A). Both controls and EGTA-treated cultures recovered high resistance after two hours in culture medium. These results suggested that the mechanism of occlusion in freshwater sponges may not be calcium dependent. Alternatively, sponge occlusion may occur apical to adhesion rather than in a typical terminal bar arrangement. Although not definitive, ruthenium red was excluded to the apical side of sponge tissues in the presence of cation chelators. This finding suggests that the tracer occlusion occurs apically in the intercellular space with potential calcium dependent adhesion occurring below. Studies in vertebrate

systems have highlighted the interaction between tight and adherens junctions and it appears that disruption of one junction type may damage the other. In sponges this system may be more uncoupled. However, these results were confounded by a drop in TER that always occurred when the sponges were placed in normal recording medium. If this issue were solved with future work, it could be possible to untangle a potential drop in TER due to cation chelation from the drop in saline medium.

In symmetrical recording medium (the same solution in both compartments) the sustained transepithelial electrical potential across sponge cultures was slightly negative (approximately -3 mV, Fig. 2.7 B) indicating active transport occurs between the sponge and the water. To begin to understand the source of the net negative potential, symmetrical chloride-free or sodium-free media were used. In $Cl^-$ free media, the voltage was positive (Fig. 2.7 B), suggesting a net loss of chloride into the apical compartment (Fig 2.7 C). In the absence of sodium, voltage was slightly less negative than in the control recording medium (Fig. 2.7 B) and sodium was likely lost into the apical compartment (Fig 2.7 D). Although the voltage may arise from net loss of ions, it is unlikely that in normal freshwater medium sodium would be lost from tissues. Therefore the negative potential in the normal recording medium is most readily explained by active net uptake of sodium or loss of chloride across the basal pinacoderm (Fig. 2.7 E). In the future, a more accurate measurement of ion movement would require isolation of a single cell layer which would allow recording between a 'mesohyl' and 'environment' compartment. This advance could be used to record asymmetrical properties between the mesohyl-facing and water-facing membrane. The current results indicate that under normal ionic conditions the apical sponge tissues (endopinacoderm and dermal tissues) are tight, as supported by

the lack of tracer flux. Most importantly, the results suggest that sponge epithelia, like epithelia of other animals, maintain an electric potential.

In this study, epithelia in *Spongilla lacustris* was shown to have relatively high electrical resistances, impede the passage of small molecules, and control their membrane potential by transport of ions. These results provide the first demonstration of high resistance, occlusion of small tracers, and ion transport in freshwater sponge tissues, three features that are key indices of epithelial function in vertebrate *in vivo* and *in vitro* systems. Occlusion could be localized at regions where adjacent cell membranes are in tight contact (Fig. 2.5 E). Alternatively, proto-occluding junctions could form a diffuse network along the intercellular cleft as occurs in the epithelia of developing vertebrates (Franke, 2009). If so, the resistance of sponge epithelia to ion transport and ability to occlude small molecules may be related to the "membrane spacing factor" defined by Green and Bergquist (Green and Bergquist, 1982) and would also control the regular cell spacing between pinacocytes in the absence of septae or other electron dense proteins. Although septate junctions are clearly present between sclerocytes in calcareous sponges, clear septae have not been shown between pinacocytes. The exact protein responsible for occlusion in freshwater sponges warrant more investigation.

## 2.4 Conclusions

These data show that freshwater sponges have functional epithelia with high transepithelial electrical resistance (TER), a transepithelial potential (TEP), and low

permeability to small-molecule diffusion. These results are intriguing since the *Amphimedon* sponge genome lacks many classical occluding genes like occludins and claudins (Srivastava et al., 2010) that regulate sealing and control ion transport. Depending on which phylogenetic relationship is ultimately correct, different evolutionary hypotheses could explain this new data. One theory, not excluded by these data, is that the mechanism for occlusion in *S. lacustris* is an independently derived feature only found in freshwater sponges. Future work on marine species will address this possibility. Not all sponges are as easy to culture, yet the similarity in morphology of sponge epithelia in general suggests that the epithelia of marine sponges may also seal. It would be informative to culture homoscleromorph sponges to measure resistance and exclusion of ruthenium red. This could reveal physiological differences due to the presence of type IV collagen in the only known sponge group with a true basement membrane. Freshwater sponges have arisen from marine haplosclerid sponges (Meixner et al., 2007), which are common in shallow and intertidal habitats worldwide. Given the daily changes in salinity and humidity shallow marine haplosclerids must experience, it may be expected their tissues also regulate the passage of solutes.

The molecular components underlying freshwater sponge physiology may have also been responsible for ionic regulation in evolutionarily ancient organisms (e.g., the last common ancestor of animals) and these may have been retained throughout the metazoa. If they are not the most basal animals, sponges must have secondarily lost genes for occlusion but retained functionality. Further study on basal node metazoans and comparisons to known vertebrate systems may reveal overlooked or hidden components of epithelial ion regulation and pathophysiologies

in ‘higher’ animals. The results imply that functional epithelia evolved prior to the origin of Cnidaria and therefore forces a re-examination of the classical definition of true epithelia and the Eumetazoa.

The fact that sponges have polarized epithelia that seal and control the passage of solutes implies that at least freshwater sponges have a tissue rather than cellular level of organization. These results suggest that sponge have an internal milieu and are more than just transiently sealed. However, further testing would be required to determine if the ionic concentration of the internal milieu differs markedly from that of the environment. Eumetazoa have been defined by the presence of a mouth and gut and tissue level organization (Hyman, 1940). If sponges are understood to have functional epithelial tissues, then the presence of tissues can no longer be used as a purely eumetazoan character. Moreover, if true epithelia exist in sponges, then this implies that epithelia either arose within demosponges or evolved earlier and was a defining feature of the first multicellular animals.

**Figure 2-1 Structure of the sponge epithelium**. (A) Diagram of the experimental TER chamber. (B) Scanning electron microscopy (SEM) of sponge tissue showing: the bilayered dermal tissues (dt), suspended over the subdermal space (ss) by shafts of spicules (spi); the choanosome (ch) with the choanocyte pumps; excurrent canals (ec); and an osculum (o). For transepithelial recordings, apical and basolateral compartments were effectively separated by two confluent cell layers - the basopinacoderm (bp) and endopinacoderm (enp) - surrounding a thin mesohyl (m) (inset). Scale bar: 100μm.

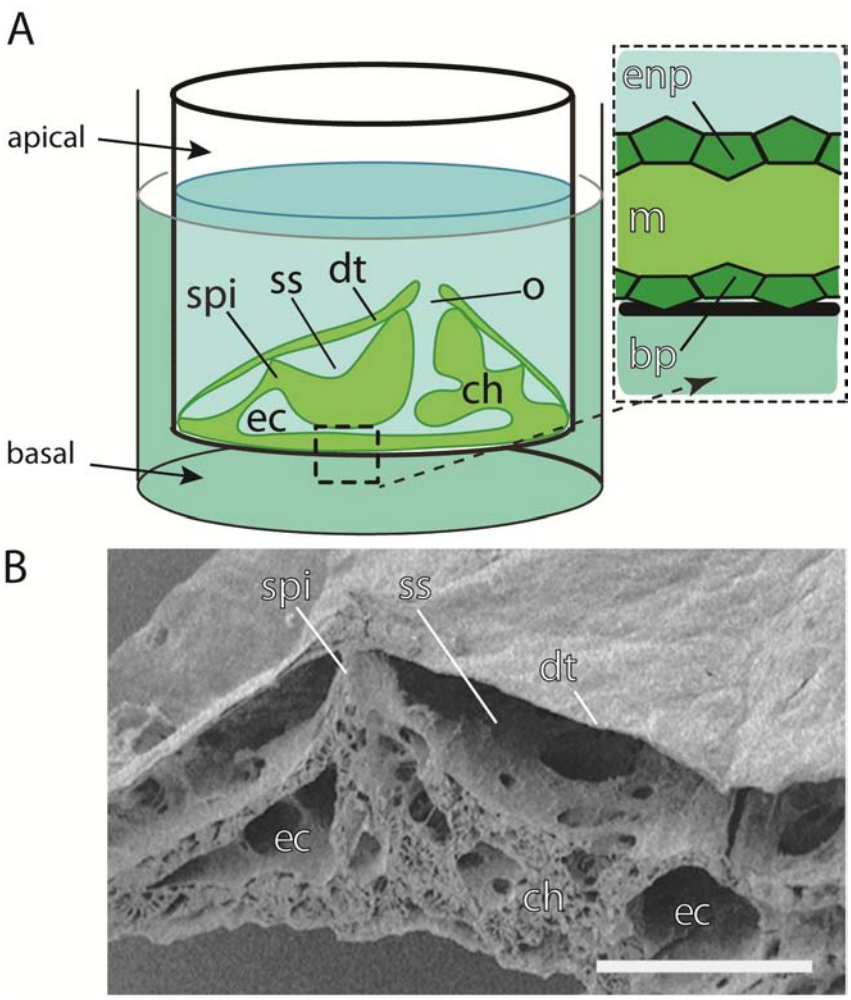


**Figure 2-1**

**Figure 2-2 Exopinacocyte attachment** (A) SEM of the dermal tissue showed exopinacocytes are very close to one-another (opposing arrows show cell-cell spacing); scale 1μm. (B) Transmission electron microscopy of exopinacocytes showed tight membrane apposition between adjacent cells (arrow). These areas were associated with 10 nm diameter cytoskeletal fibres (arrowheads); scale 200nm. (C) Fluorescent labelling of the basopinacoderm with the steryl dye FM 1-43 highlighted the borders of cells and showed that a confluent layer of cells covered culture membranes; scale 10μm. (D & E) Fluorescent labelling using phallacidin revealed dense plaques of actin (arrowhead) between cells over the entire surface of a juvenile sponge. These plaques occur near regions of tight membrane apposition and are likely associated with cell-cell adhesion. Nuclei in blue as stained by Hoechst. Scales: D = 5μm, E = 100μm.

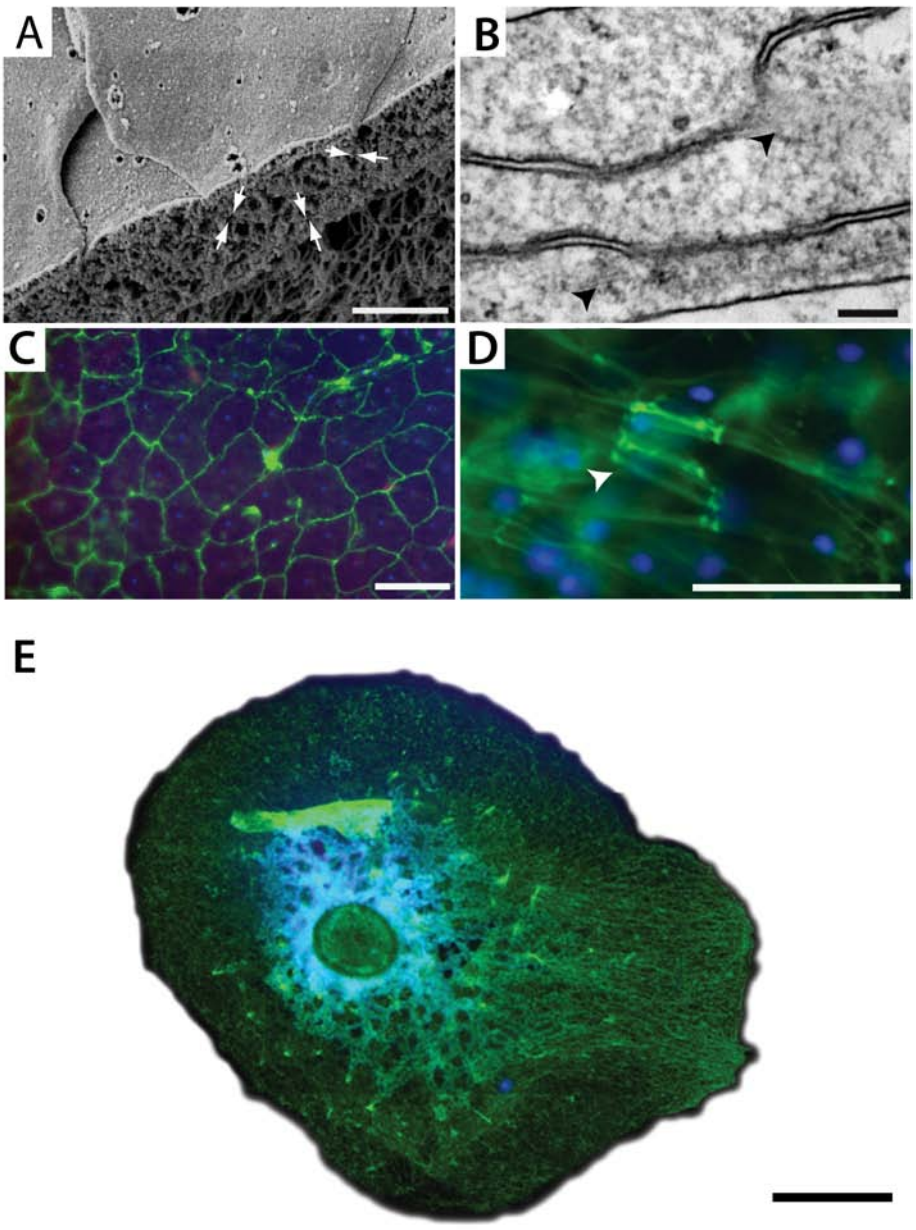


**Figure 2-2**

**Figure 2-3 Transepithelial resistance of *S. lacustris* gemmule and aggregate cultures compared to cultures of vertebrate tissues:** The resistance to ion passage of *Spongilla* epithelia derived from aggregates (Ag) (1098.5 Ω $cm^2$ ± 671.5 s.e. n=33) and gemmules (Gm) (1932.5 Ω $cm^2$ ± 253.5 s.e. n=22) was comparable to the resistance of epithelia from vertebrate tissues (MPT= mouse proximal tubule, MGB= mouse gall bladder, SPT= salamander proximal tubule, RC= rabbit colon, MDT = mouse kidney distal tubule, FTS= frog skin, FWG= freshwater fish gill, TUB= toad urinary bladder). Data from sources in Table 2.1.

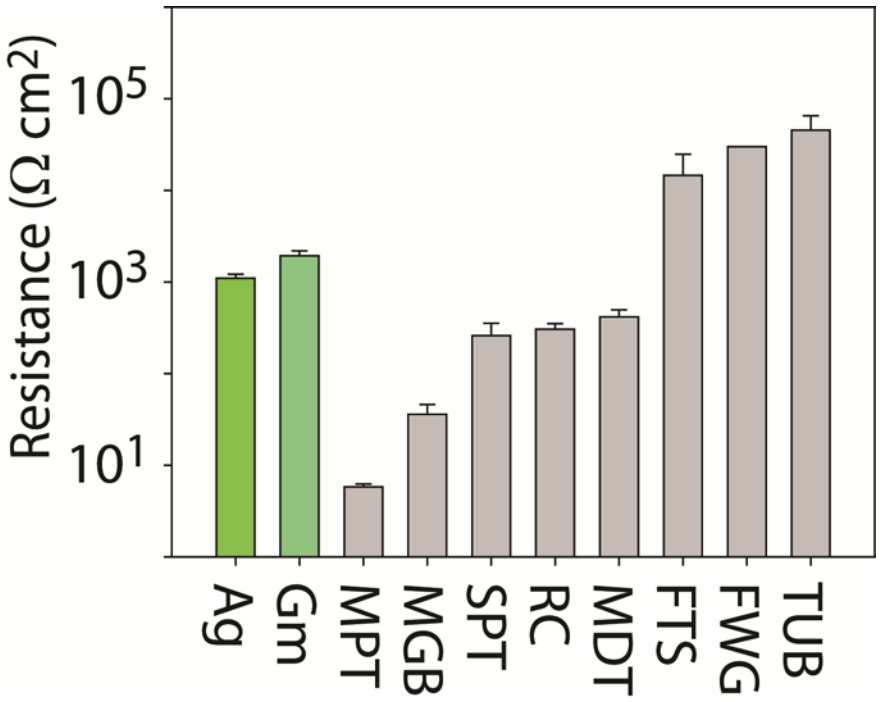
Resistance (Ω cm²)
10⁵
10³
10¹
Ag
Gm
MPT
MGB
SPT
RC
MDT
FTS
FWG
TUB

**Figure 2-3**

**Figure 2-4 Resistance of freshwater sponge cultures**: Box plots showing the resistance of tissue cultures from *S. lacustris* gemmules (mean 1932.5 Ω $cm^2$, n=22, s.e. 253.5) and aggregates (mean 1098.5 Ω $cm^2$, n=33, s.e. 116.9). Mean= dashed line, Median = solid line, all outliers are shown.

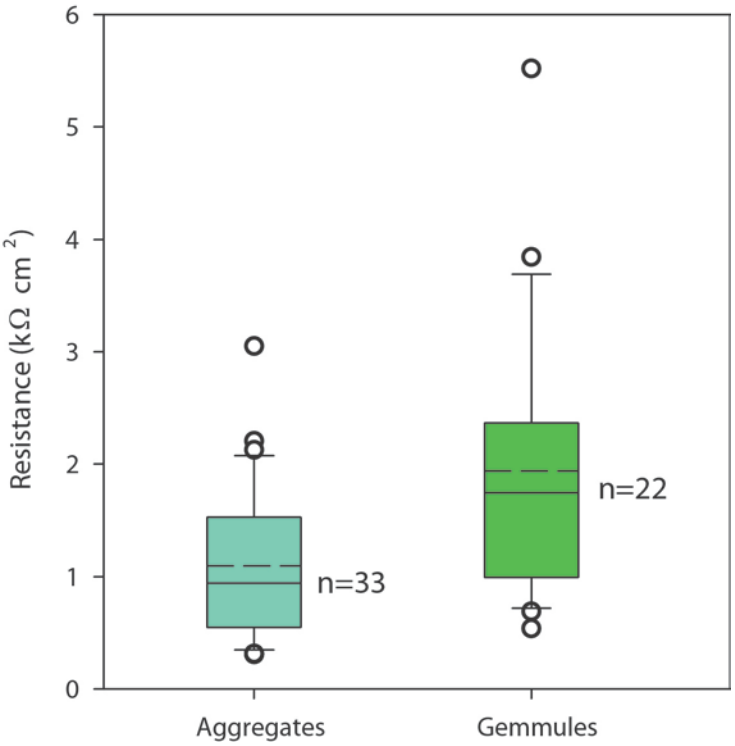


**Figure 2-4**

**Table 2-1** Data sources for meta analysis of epithelial cultures (Adapted from Boulpaep & Seely, 1971 , Claude & Goodenough 1973 and Powell, 1981).

| Tissue Type | Resistance Value | Reference |
|---|---|---|
| **Dog Proximal Tubule** | 6.96 | Lutz, M. D. et al,. (1973) *Am. J. Physiol*. **225,** 729-773 |
| **Dog Proximal Tubule** | 5.6 | Boulpaep, E. L. & Seely, J.F. (1971) *Am. J. Physiol.* **221,** 1084-1096 |
| **Rat Proximal Tubule** | 4.9-5.7 | Hegel, U., Frömter, E. & Wick. T. (1967) *Arch. Ges. Physiol.* **294,** 274-290 As Cited in: Boulpaep, E. L. & Seely, J.F. (1971) *Am. J. Physiol.* **221,** 1084-1096 |
| **Necturus Proximal Tubule** | 260 | Guggino, W. B. (1982) J. Membr. Biol. **67,** 143-154 |
| **Necturus Proximal Tubule** | 70 | Boulpaep, E. L. (1969) As Cited in: Boulpaep, E. L. & Seely, J.F. (1971) *Am. J. Physiol.* **221,** 1084-1096 |
| **Necturus Proximal Tubule** | 450 | Anagnostopoulos, T. et al,. (1980) *J. Gen. Physiol.* **75,** 553-587 |
| **Human Gallbladder** | 58 | Rose et al,. (1973) *Am. J. Physiol.* **224,** 1320-1326 |
| **Rabbit Gallbladder** | 20 | Henin et al., (1977) *J. Membr. Biol.* **34**, 73-91 |
| **Rabbit Gall bladder** | 30 | Frömter, E. & Diamond, J. (1972) *Nat. New Biol.* **235,** 9. |
| **Frog skin** | 34400 | Erlij, D. (1976) *Pflügers Archiv*. **363,** 91-93 |
| **Frog skin** | 8700 | Lewis et al., (1975) *Physiologist*, **8**, 291 As Cited in Powell (1981) *Am. J. Physiol*. **241**, G275-288. |
| **Toad skin** | 763 | Bruus et al., (1976) *Acta. Physiol. Scand.* **97**, 31-47 As Cited in Powell (1981) *Am. J. Physiol*. **241**, G275-288 |

| | | |
|---|---|---|
| **Dog distal tubule** | 600 | Boulpaep, E. L. & Seely, J.F. (1971) *Am. J. Physiol.* **221,** 1084-1096. |
| **Rat distal tubule** | 300 | Giebisch, G. & Malnic, G. (1968) Cited in: Boulpaep, E. L. & Seely, J.F. (1971) *Am. J. Physiol.* **221,** 1084-1096. |
| **Rat distal tubule** | 350 | Malnic, G. & Giebisch, G. (1972) *Am. J. Physiol*. **223**, 797-808 |
| **Rabbit colon** | 385 | Anderson and Van Itallie (2009) *Cold Spring Harb Perspect Biol*, **1**:a002584, 1-16. |
| **Rabbit colon** | 330 | Powell, D. W. (1981) *Am. J. Physiol.* **241,** G275-G288 |
| **Rabbit colon** | 200 | Ghandehari, H. et al., (1997) *J. Pharm. Exp. Thera.* **280**, 747-753 |
| **Freshwater Fish Gill** | 30000 | Tsui et al., ( 2009) *J. Exp. Biol*. **212**, 878-892. |
| **Urinary bladder toad** | 3800 | Reuss and Finn (1974) *J. Gen. Physiol.* **64,** 1-25 |
| **Urinary bladder toad** | 49400 | Erlij, D. (1976) *Pflügers Archiv*. **363,** 91-93 |
| **Urinary bladder toad** | 83000 | Erlij, D. (1976) *Pflügers Archiv*. **363,** 91-93 |

**Figure 2-5 Permeability of *S. lacustris* epithelia:** (A) Permeability to $^{3}H$ inulin decreased with increasing resistance of sponge cultures. (B) $^{3}H$ inulin gradually accumulated on the basolateral side of cultures with low resistance (solid circles, <500 Ω $cm^2$), but was excluded by high resistance (open circles, >800 Ω $cm^2$) epithelia. (C) Ruthenium red (rr, the dark precipitate) was excluded by both exopinacocytes (exp) and endopinacocytes (enp) so it was not able to enter the internal collagenous (col) mesohyl containing mesohyl cells (m). The tracer was flushed into the subdermal space with the buffering medium though ostia in the dermal tissue. It therefore coated the external underside of the endopinacoderm lined 'roof' of the subdermal space (ss); scale, 500nm. (D) A close up revealed that ruthenium red was excluded from paracellular clefts (black arrow); scale, 200 nm. (E) Tracer occlusion could have occurred due to membrane fusions. As seen by transmission electron microscopy (TEM), these fusion point were sometimes present near the most apical point of cell contact between exopinacocytes; scale 100nm.

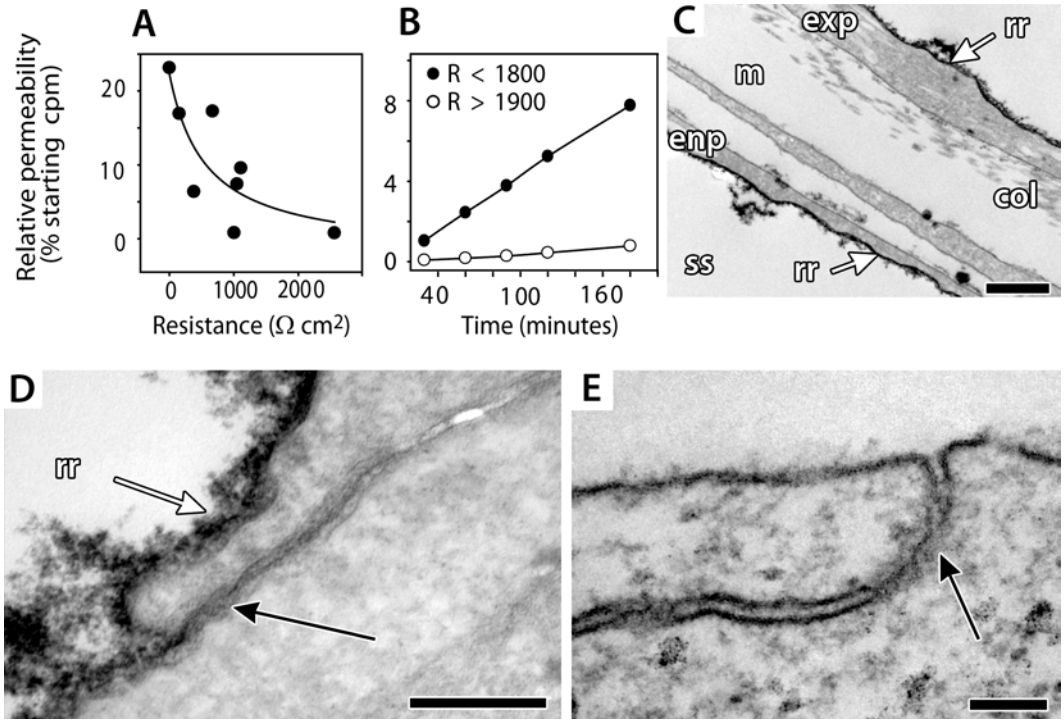
A
B
C
D
E
Relative permeability
(% starting cpm)
20
10
0
0
1000
2000
Resistance (Ω cm2)
R < 1800
R > 1900
8
4
0
40
100
160
Time (minutes)
exp
rr
m
enp
col
ss
rr
rr

**Figure 2-5**

**Figure 2-6 $^{14}$C-PEG permeability through cultures made from aggregates**. Preparations with a higher resistance (977 Ω cm$^2$, 908 Ω cm$^2$) excluded more radioactive tracer than cultures with a low resistance (231 Ω cm$^2$). In all cases the amount of tracer that could pass through the sponge tissue did not increase over time.

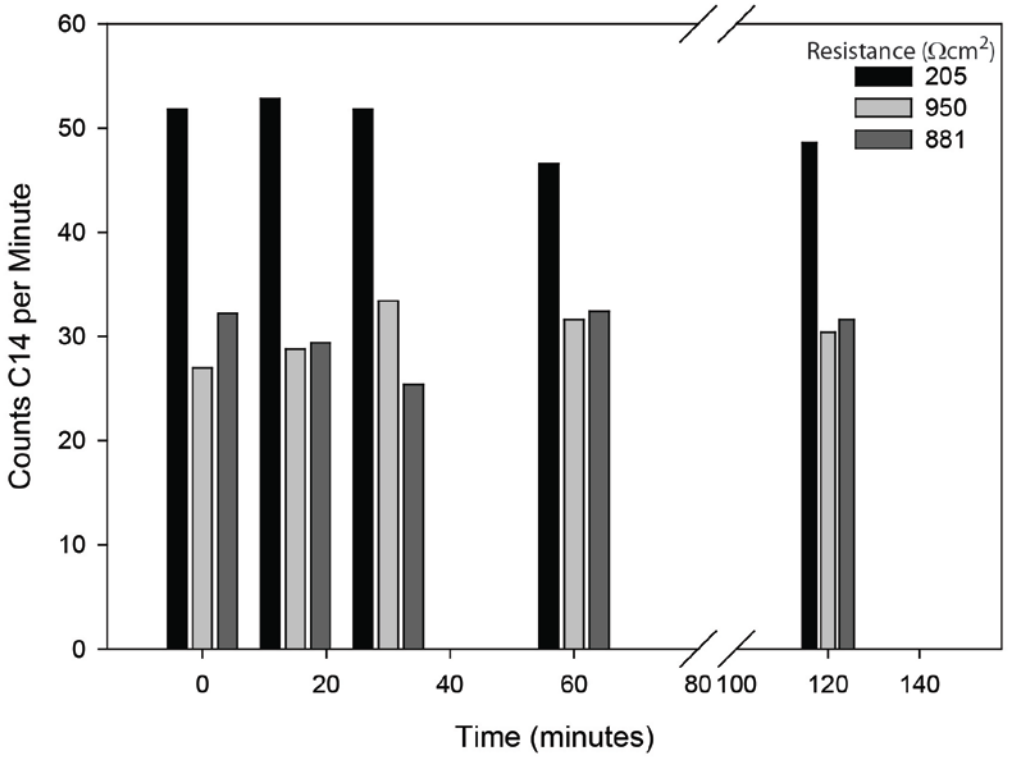
Resistance (Ωcm2)
205
950
881
Counts C14 per Minute
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
Time (minutes)

**Figure 2-6**

**Figure 2-7 Cation chelation, membrane potential and ion transport in sponge epithelia**. (A) Effect of EGTA or EDTA treatment on transepithelial resistance of sponge cultures. All cultures lost resistance in the recording medium and recovered high resistance after 2 hours in the culture-medium. (B) Voltage across the epithelium was negative in the normal culture medium and in sodium-free medium and positive in chloride-free medium. (C) The positive voltage recorded in $Cl^-$ free media suggests that more chloride ions may have accumulated in the apical compartment. (D) Negative voltage in $Na^+$ free media suggests that sodium ions may have preferentially accumulated in the apical compartment. These two hypotheses theorize that passive flux of anions and cations down artificial concentration gradients created by the removal of ions in culturing media may preferentially occur from the mesohyl and across apical pinacoderms rather than across the basal pinacoderm. (E) A schematic showing the theorized uptake of sodium in normal recording medium to generate a negative potential. This model assumes that in slightly saline media, sodium ions would likely enter the sponge mesohyl rather than flow out into the apical compartment against a concentration gradient. It is theorized that active transport processes occur across the basal pinacoderm.

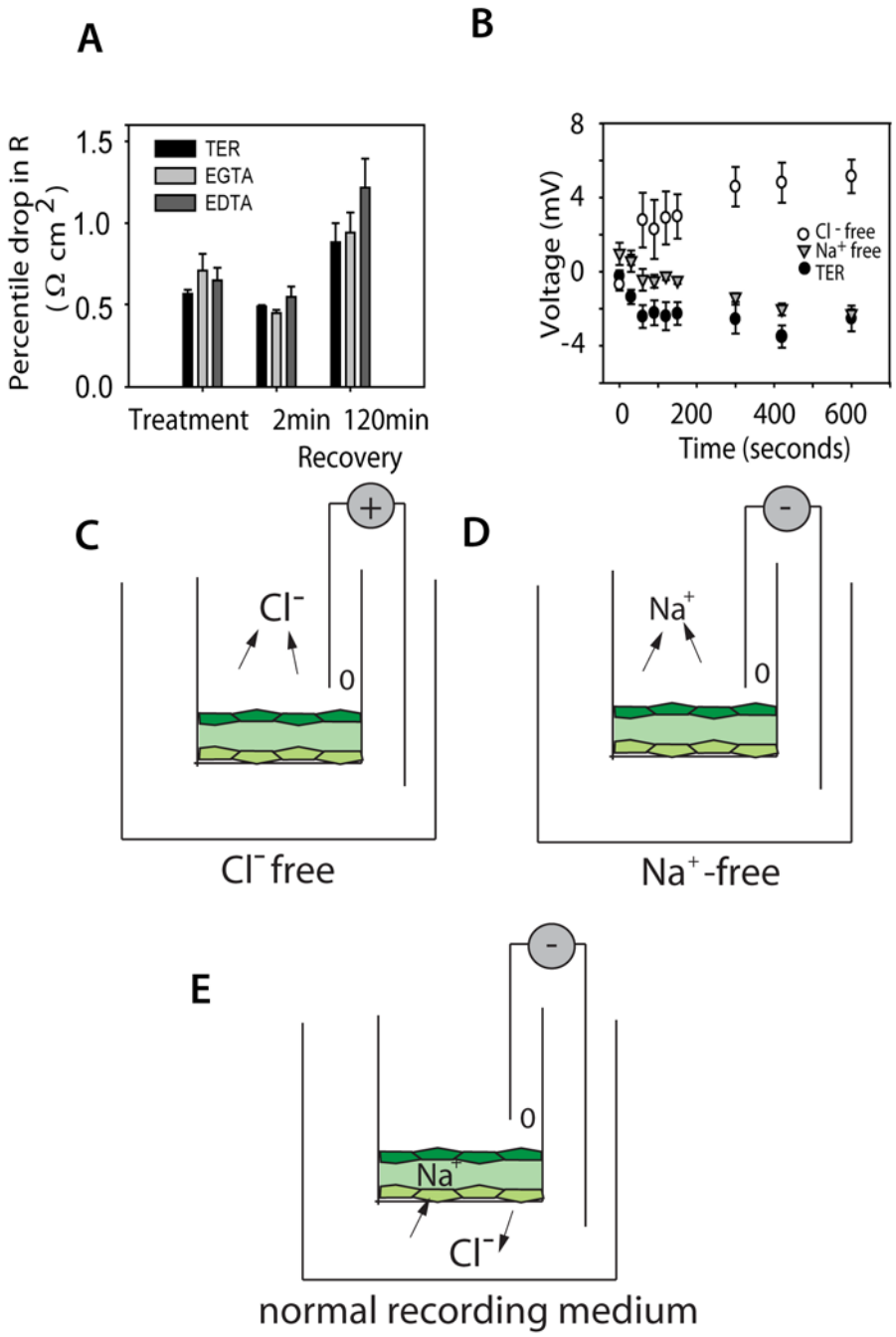
A
B
Percentile drop in R
( Ω cm 2 )
1.5
1.0
0.5
0.0
TER
EGTA
EDTA
Treatment
2min
120min
Recovery
Voltage (mV)
8
4
0
-4
0
200
400
600
Time (seconds)
Cl - free
Na+ free
TER
C
D
Cl-
Na+
0
+
-
Cl- free
Na+ -free
E
Na+
Cl-
normal recording medium

**Figure 2-7**

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# Chapter Three

## Comparative epithelial morphology, cell behaviour, and function in freshwater and marine sponges.

### 3.1 Introduction

Sponges, the earliest diverging metazoans, can offer insight into the evolution of animal multicellularity. A proxy for this important evolutionary transition is the origin of epithelia, which could be considered the most basic multicellular tissue structure. Historically, sponges were said to lack 'true' epithelia (Tyler, 2001) and 'true' tissues (Muller, 1982), but these contentions were made in the absence of physiological data which empirically tested the sealing ability of sponge tissues (Adams et al., 2010 - Chapter 2). The view of sponges as epithelioid animals was based on a perceived absence of cell polarity, junctions and a basement membrane in sponges, the defining features of epithelial tissues. Confusingly, the term epithelia has been used, albeit inconsistently, to describe cell layers in sponges (e.g. Levi, 1970) however, when the topic of epithelial evolution has been addressed directly, sponges have been said to have a cellular rather than tissue based organization (Tyler, 2003). Descriptions of cellular morphology in sponges have been misleading, partially due to difficulty in achieving proper tissue fixation as well as a lack of knowledge concerning functional cell ability. Cell biology of the Porifera has been

**Figure 3- 10 presented in this chapter was published in: Leys, S. P., Nichols, S. A. & Adams, E. D. M. (2009) Epithelia and integration in sponges. *Integr. Comp. Biol.* 49: 167-177.**

oversimplified by comparisons to vertebrates. Although a significant amount of work has focused on epithelial physiology, the molecular mechanisms for proper sealing function are not fully resolved even in mammals (Franke, 2009). As described in chapter two, the discovery that freshwater sponge tissues have high transepithelial resistance and the ability to block solute transport suggests that freshwater sponge epithelia effectively compartmentalize internal extracellular spaces from the external environment. The re-examination of cell morphology in sponges in this thesis aims to identify the structural basis behind the physiology of sponge epithelia. In this chapter I describe the anatomy of cell monolayers within the freshwater sponge body plan, compare the behaviour of cells in epithelial layers to other cell types and offer a morphological comparison to select marine sponges.

The history of morphological studies on sponge cell biology has been marked by periods of uncertainty and changing opinion. For the early part of the 20th century, sponges were understood to have an epithelium of multinucleated syncytial tissue which lacked defined cells (Wilson, 1907, Parker, 1919). This viewpoint has been attributed to the limitation in resolution at a light microscope level. The small size of sponge cells meant that cell boundaries were not discerned by early researchers. However, the advent of electron microscopy allowed investigators to view sponge morphology at a more detailed scale and this technological advance has been used to prove that most sponges, including Demospongiae and Calcarea, are made up of mononucleated distinct cells (Simpson, 1984). The two types of differentiated cells present in all sponges are pinacocytes, forming surface layers and choanocytes, which are feeding structures (Levi, 1970). Only hexactinellid glass

sponges have been confirmed by TEM to have synctial tissues (Mackie and Singla, 1983, Reiswig and Mehl, 1991).

The functional roles of pinacoderm layers have been investigated by researchers in the past. In particular, reports on feeding have suggested that exo- and endo-pinacocytes can phagocytose particles larger than 5 um in size which cannot enter choanocyte chambers (Francis and Poirrier, 1986). This behaviour has been particularly noted in the endopinacoderm of incurrent canals. However, many studies used non-natural food particles such as latex beads or non-native bacteria in addition to carrying out experiments in non-natural and extremely low-flow environments (Willenz and Van De Vyver, 1982, Turon et al., 1997, Duckworth and Pomponi, 2005). These issues cast doubts on the biological relevance of feeding studies since it seems likely that natural water flow around a sponge would restrict the number of bacteria able to colonize the surface and be available for phagocytosis. In addition, methods which purport feeding behaviour based only on observations of food particles in the mesohyl may have overlooked fixation artifacts. A moderate view would suggest that the phagocytotic ability of exopinacocytes is primarily for cleaning the cell surface rather than as a primary place for food digestion (Harrison, 1974). Histochemical data supports this supposition. Harrison (1974) reported that apical pinacocytes of the freshwater sponge *C. carolinensis* contain low amounts of carbohydrate, lipid, and protein inclusions as well as low amounts of acid phosphatase activity. He theorized that these conditions meant pinacocytes do not have a major role in food storage or digestion (Harrison, 1974). A high level of acid phosphatase was present in choanocytes, indicating that active intracellular digestion takes place in these known feeding structures (Harrison, 1974). Past workers have

shown that the basal pinacoderm has several functional roles including phagocytosis, digestion, and osmoregulation via contractile vacuoles (Harrison, 1972). Differences in cell activity between individual pinacoderm regions could support the hypothesis generated from Chapter Two (Adams et al. 2010). The first recordings of voltage across sponge tissues suggested that the basal pinacoderm may be a site for ion exchange while apical surfaces could be regions of more passive ion movements. This theory was based on the assumption that sodium ions were entering the mesohyl from the basolateral compartment in recording medium while they were passively lost across the apical membrane down their concentration gradients in sodium-free media. It is therefore possible that apical surfaces are primarily for protection and to act as a barrier between connective tissue regions and the external environment.

Physiological data which suggest that sponge epithelia are tight alongside the absence of characteristic genes involved in sealing indicates that this group may possess a type of ancestral cell connection not identified by our current classification of cell junctions. Studying the morphology of cell connections within sponges may therefore illuminate features presently overlooked within vertebrate epithelia.

## 3.2 Materials & Methods

### *3.2.1 Sponge collection*

The marine sponges *Haliclona* cf. *permollis*, *Suberites sp.*, *Tethya leysae*, *Neopetrosia* cf. *vanilla* and *Haliclona* cf. *mollis* were collected from Bamfield, B.C., Canada by diving or by hand collection from the intertidal near Bamfield Marine

Science Centre, Vancouver Island, Canada during the summers of 2008 and 2009. The freshwater sponges *Ephydatia muelleri* and *Spongilla lacustris* were collected by snorkelling in lakes near Bamfield, B.C.. During the winters of 2008 and 2009 gemmules of *E. muelleri* and *S. lacustris* were collected and stored at the University of Alberta for up to a year at 4$^{o}$C. The homoscleromorph sponges, *Plakortis sp*. and *Plakinistrella sp*, were collected in 2007 at the STRI in Bocas, Panama by Dr. S. Leys.

### *3.2.2 Electron microscopy*

Sponges were fixed for electron microscopy in a cocktail of 1% $OsO_4$, 2% glutaraldehyde, 0.45M sodium acetate buffer pH 6.4, and 10% sucrose at 4°C for six hours (Elliott and Leys, 2007). To observe molecule occlusion through freshwater sponge tissues in transmission electron microscopy (TEM), ruthenium red was added to the cocktail fix at 0.01mg/ml. Alternately, ruthenium red was put into a pre-fix containing glutaraldehyde and 0.2M cacodylate buffer as well as post-fix with osmium and 0.2M cacodylate buffer (Luft, 1971). Sponges were left in pre-fix for 1 hour, rinsed with cacodylate buffer and then post-fixed. Processing consisted of one brief rinse in $dH_2O$, followed by an ethanol dehydration series (50, 70, 95 to 100% EtOH with three 10 minutes rinses at each dehydration level). For SEM, while immersed in absolute ethanol, sponges were fractured by freezing in liquid nitrogen. Sponge fragments were critical point dried using a Bal-Tec CPD 030 before mounting on metal stubs with nail polish, sputter coating with gold on a Nanotek SEMprep 2 and viewing on a JEOL 6301F field emission microscope.

For TEM, sponges were dehydrated to 100% EtOH after fixation as above but were then slowly infiltrated with Spurs (Electron Microscopy Services, Hatfield, PA, USA) going from 25, 50, 75, to 100 % resin over the course of several days. The resin was set in an oven at 60°C and then embedded sponges were mounted on metal stubs. Thin 60 nm sections of the blocks were cut for TEM on a Leica Ultracut T ultramicrotome and mounted on 200 nm copper grids. Sections were stained for 1 hour with uranyl acetate and then for 8 minutes in lead citrate to enhance contrast. Images were taken on a Philips/FEI Morgagni transmission electron microscope. Tissue sections fixed with ruthenium red were not double stained so as to allow proper identification of the tracer.

### *3.2.3 Time-lapse*

Gemmules trapped within a spicule framework were extracted by rubbing between two pieces of corduroy. Intact cysts were cleaned with 2% hydrogen peroxide and stored at 4°C in $dH_2O$. *E. muelleri* and *S. lacustris* gemmules hatched within two days on ethanol flamed cover slips in M-Medium (Rasmont, 1966) and formed functional sponges after a week. For microscopy, pinacoderm outlines were stained with the lipophilic- fluorescent dye FM 1-43 (Invitrogen, Burlington, On). Images were captured every minute for one hour on a Zeiss Axioskop 2 Plus microscope using Northern Eclipse software (Empix, Mississauga, On). Phase contrast microscopy was used to observe mesohyl cell movements.

### *3.2.4 Image Analysis - Cell dimensions*

Cell dimensions and speed were measured using Image J software (Rasband, 1997). Photoshop CS5 and Illustrator CS5 (Adobe, San Jose, Ca) were used for image and diagram design.

## 3.3 Results & Discussion

### *3.3.1 Functional anatomy of freshwater sponges*

Textbooks categorize sponges based on the complexity of the canal branching structure from simple (asconoid) to more complex (synconoid and leuconoid) (Rupert and Barnes, 1994). However, these terms are now understood to have no phylogenetic basis since all three patterns can be found within a single sponge class. Although the phylogeny of the Porifera is not yet clear, recent genetic data has indicated that sponges can be split into four main groups (Fig 3.1). Freshwater sponges all belong to a suborder Spongillina and are considered Haplosclerid demosponges but the phylogenetic relationship between freshwater sponges and marine species is not yet fully understood. The morphology of sponges can be most readily understood by following the path of water though an animal and considering cell layers in terms of functional units.

Sponges are known to feed on bacteria, unicellular protists, and small eukaryotes (Reiswig 1971, Imsieke, 1993). Water is transported though the body of sponges and suspended nutrient particles are captured in choanocyte chambers

(Langenbruch and Jones, 1990). Choanocytes generate this flow of water by continuous movement of long flagella. Water velocity is thought to be controlled primarily by the morphometrics of the water canal system, and specifically canal diameter although cellular sponges are also able to control the rate of flagella beating (Elliott and Leys 2010, Leys and Meech, 1999) Within *E. muelleri* and *S. lacustris* water enters through ostia, openings in the external tissues formed by specialized pinacocytes called porocytes which possess a contractile hole. Water is drawn into a subdermal space, down incurrent canals, through choanocyte chambers, out excurrent canals and then exits via the chimney-like osculum (Figure 3.2). Throughout the canal system the water is thus channelled past two types of epithelia – pinacoderm and choanoderm.

### *3.3.2 Exopinacoderm and endopinacoderm in freshwater sponges*

The outer surface of *S. lacustris* and *E. muelleri* is formed by a cellular tissue which appears as a tent suspended between vertical siliceous spicules and act like tent-poles. This feature has long been called a dermal membrane (Feige, 1969, Bagby, 1970, Simpson, 1984, Teragawa, 1986) and recently the apical pinacoderm (Elliott and Leys, 2007 ); however both terms are problematic. Dermal membrane was a term derived when all sponge tissue was considered syncytial. Thus the surface layer was thought to be a continuous membrane not formed of multiple cells. Although thin, the 'tent' is composed of two layers of pentagonal, squamous pinacocytes (pavement cells) surrounding a narrow mesohyl (middle space) containing collagen and a population of crawling amoeboid cells (Bergquist, 1978,

Simpson, 1984). Thus the singular term -- dermal membrane -- is a misnomer for this multilayered structure composed of different cell types. Likewise the apical pinacoderm refers only to the outermost exopinacoderm layer and not the entire tent. Hereafter I will refer to this tent structure as the "dermal tissues" and the components as mesohyl, exo- and endo-pinacoderms respectively.

Apical exopinacocytes in *E. muelleri* (Fig. 3.3 A) and *S. lacustris* (Fig. 3.3 B) are arranged in a tile-like pattern with overlapping edges (Fig. 3.3 C). They are very thin 0.25 um (0.030 s.e.; n=20). This is similar to marine demosponges with 0.2 μm thick exopinacocytes (Johnson and Hildemann, 1982). Cells in the sheet are tightly held together and so well integrated that it is sometimes difficult to observe cell boundaries even in SEM. It is unsurprising therefore that early researchers believed that demosponges were syncytial. These cells sit over a mat of fibrillar collagen which is approximately 1.72 um thick (0.53 s.e.; n=20). The collagen fibers are 16nm in diameter (1.2nm s.e.; n=10). Below the collengenous mesohyl the thin endopinacoderm (0.30 um think (0.071 s.e.; n=20) is a confluent cell layer like the exopinacoderm (Fig. 3.3 D, E). TEM revealed that adjacent exopinacocytes are consistently held apart at a distance of 15-20 nm (Figs. 3.4 A, B) and this figure agrees with published literature (Green and Bergquist, 1982). In several sections the outline of these cells had complex morphologies that were perfectly mirrored by a neighbouring cell (Fig. 3.4 D). This construction suggests that sponge cells are connected by a physiological mechanism which has been called a 'membrane spacing factor' (Green and Bergquist, 1982).

Classifying sponge junctions as only simple parallel regions underestimates their potential functional significance. The ability of sponge cell layers to generate an electrical potential and resistance suggests that these sponges possess junctions that can occlude molecules (Adams et al. 2010 - Chapter Two). Although definitive septate, tight or adhesion junctions were not observed, regions of apparent cell-cell membrane contact were noted (Fig. 3.4 C). These regions appeared to resemble a single "kissing" point, a hallmark of tight junctions morphology. Furthermore, these regions often had filamentous projections leading into the cytosol indicating cytosketal integration and thus possibly an anchoring or stabilizing role similar to desmosomes. Similar structures have been shown in *Ephydatia mulleri* (Pavans de Ceccatty, 1986). Faint septae were noted in some places between adjacent cell membranes (e.g. Fig. 3.6 E) however these septae were not unambiguous like those observed in calcareous sponges (Ledger, 1975)

As shown in chapter two, dermal tissues exclude the passage of ruthenium red into the mesohyl of *S. lacustris* (Figs. 3.5 A, B) and *E. muelleri* (Fig 3.5 C). Here the tracer was only able to pass through approximately 450nm of the paracellular space in *S. lacustris* (Fig. 3.5 D). Further, the endopinacoderm located apical to the subdermal space had large vacuoles which did not contain any tracer (Fig. 3.5 E). Detailed views at cell contact zones in the endopinacoderm show that this layer also hindered the passage of tracer through the intracellular space (Fig. 3.5 F). The occlusion of tracer (Fig. 3.5 G) is highly reminiscent of studies showing lack of tracer penetration past insect septate junctions (Hori, 1986).

In freshwater sponges the tissue forming the 'floor' of the subdermal space is also composed of a thin endopinacoderm with overlapping cells (Figs. 3.6 A, B, C). Like the ostia through dermal tissues, openings in this layer permit the passage of water into the choanosome. These openings lead to incurrent canals and have the ability to constrict during a whole body contraction (Elliott and Leys, 2009). Cells lining the base of the subdermal space act as a barrier which contains the choanosome. The ruthenium red tracer was also shown to be excluded by cells in this layer (Figs. 3.6 D, E).

The canals in freshwater sponges were lined with flat endopinacocytes that overlapped in the same manner as cells in the exopinacoderm (Figs. 3.7 A, B, C). Unlike the exopinacoderm, a mat of collagen was not evident under endopinacoderm layers. In some regions, choanocyte or mesohyl cells directly contacted the canal wall (Figs. 3.7 D, E). Freshwater sponge canals appeared wider than those observed in marine species and the mesohyl of these species was less dense than other sponges (e.g. Figs. 3.13 C, D).

The basal surface of freshwater sponges is responsible for stable attachment to substrates. As such, this area is a challenge to observe with microscopy because it is typically damaged during collection. The basopinacoderm can be better visualised in sponges cultured from gemmules on glass cover slips. Hydrofluoric acid (HF) treatment dissolves siliceous spicules and etches the cover slip which permits the sponge to be lifted gently from its substrate (Fig. 3.8 A). Because HF treatment can only occur post-fixation, staining with ruthenium red tracer to determine permeability of basal cell layers has not been successful. Since sponges attach tightly to the

substrate, the tracer did not contact the basal surface during the first fixation stage. Cells in basopinacoderms are pentagonal, tightly connected to each other and arranged in a similar pattern to those in other pinacoderm layers (0.23um thick (0.016 s.e.; n=20)) (Figs. 3.8 B, C, D). Unlike other regions, the basopinacoderm lacks any openings so it is a continuous cell surface. Basopinacocytes appeared slightly rounded up rather than forming a smooth, flat surface like the apical pinacoderm (Fig. 3.8 E). The shingle-like appearance is likely due to the contractile nature of the basal pinacoderm. A layer of secreted material (49 nm thick (7nm s.e. ; n=5)) below the basopinacoderm is thought to help attach the sponge to the substrate (Fig. 3.8 C). In TEM this appeared as a thin acellular coat punctuated by dark round inclusions 71nm in diameter (8.4 nm s.e.; n=5). It is unlikely that these inclusions are the same type as the larger salt crystals which are present in SEM coating the basopinacoderm surface (1605 nm diameter; 173nm s.e. n=10) (Fig. 3.8 E). In adult fixed tissues a stiff layer of collagen underlies the basal tissue. The cross banding periodicity of these fibres was 45nm (0.56 s.e.; n=5) and they had a diameter of 90nm (5.7nm s.e.; n=10). It is likely that these structures were bundles since single microfibrils were measured to be 10nm (Garrone and Mazzorana, 1985). Although genetic surveys have only reported Type IV collagen in homoscleromorphs (Boute et al. 1996) a homologous protein, spongin short chain collagen has been found in demosponges (Aouacheria et al., 2006).

The cells which make up the surface of the osculum are continuous with the apical exopinacoderm (Fig. 3.9 A, B). They are thin pentagonal pinacocytes and are tightly arranged together to form a smooth sheet (Fig. 3.9 C) . However, like the basopinacoderm, cells in the osculum can appear slightly rounded and there are no

ostia openings on the surface of the structure. The inside of the osculum is composed of an endopinacodem arising from and continuous with the major excurrent canals. Adjacent pinacocytes are held together with a spacing of 20nm (Figs. 3.9 D, E). This region is notable for the presence of small primary cilia on every cell. Recent evidence suggests that these organelles are involved with flow sensing (Ludeman and Leys, pers com.).

### *3.3.3 Cell Behaviour*

Cells in sponges are often indiscriminately considered highly mobile with emphasis on their potential to leave the pinacoderm and migrate into the mesohyl (Simpson, 1984). However, a functional epithelium acting as a boundary must be composed of a fairly stable population of cells. Time-lapse imaging was used to track cell movement in the different regions of the dermal tissue. This illustrated that exopinacocytes, even at the periphery of a sponge where damage and cell movements are presumably at a maximum, do not rapidly exit or enter the epithelial layer (Fig. 3.10). Exopinacocytes retained their connections to neighbouring cells for the duration of filming (1 hour). Furthermore the adhesion between adjacent cells was evident during a period of whole tissue contraction. During this event the cells acted as a coherent sheet rather than a collection of independent cells. By contrast, mesohyl cells were highly mobile. Cells with large vacuoles migrated slowly at approximately 1 um/min and seemed to rotate rather than travel in a linear direction. These cells also underwent few shape changes with the large vacuole remaining essentially unchanged over time. Other amoebocytes moved more rapidly at approximately 9um/min and

underwent conformational changes. They were noted to have leading and trailing edges with ruffled lamelopodia. Cells in this layer migrated in multiple directions, often changing orientation quickly and interacted with other cells only intermittently.

These cell migration results agree with the findings of stable pinacoderm layers in another freshwater sponge *Ephydatia fluviatilis* which was grown between cover slips in a sandwich preparation (Efremova, 1967). *E. fluviatilis* pinacocytes were said to have "hardly moved" in three hours (Efremova, 1967). The results presented in this thesis with *E. muelleri* used a lipophilic dye to highlight cell membranes which made it possible to track and document the connections between many cells for a longer time period of one hour. Furthermore, the recording of these movements in a non-sandwich culturing preparation may better represent cell movements in native conditions.

### *3.3.4 Proposal for a marine model sponge*

Although this work has focused mainly on freshwater species to compliment the physiological data presented in chapter two (Adams et al., 2010), it was also necessary to examine the morphology of marine species to formulate a hypothesis about how ubiquitous functional epithelia are within the Porifera. In particular, analysis of the physiology in *Haliclona* sp. could provide a useful comparison to freshwater sponges. Haliclonids are marine haplosclerids, a closely related group of demosponges to the freshwater Spongillina (Fig 3.1). Additionally *Haliclona* sp. live in intertidal regions where environmental pressures favour the evolution of protective cell barriers much like conditions in freshwater. The dermal tissues of *Haliclona* cf.

*mollis* were found to be similar to freshwater varieties. A tent-like structure was present over a large subdermal space (Fig. 3.11 A). This structure was bi -layered (Fig. 3.11 B) and punctuated by porocytes. The exopinacoderm was composed of thin, overlapping cells overlying a thick collagen mat (Figs. 3.11 B, C). These features are also present in fully marine species such as *Neopetrosia* cf. *vanilla* (Fig. 3.11 D). The similarities noted between freshwater and marine species indicate that further studies on the permeability and physiology of *Haliclona* tissues may help expand the scope of this work beyond the freshwater habitat to encompass the entire sponge phylum. They suggest that the features observed in freshwater sponges were not derived only in that environment and that marine sponges may also have physiological sealed epithelia.

### *3.3.5 Sponge choanoderm*

The choanoderm of freshwater sponges have already been described in great detail (e.g. Weissenfels, 1992). However it is worthwhile to briefly illustrate the nature of this second epithelial structure in sponges. In *Haliclona* cf. *permollis* the choanosome region was particularly well preserved. Fractures through the sponge body revealed a network of endopinacocyte lined canals which fully enclosed a densely packed mesohyl interspersed with choanocyte chambers (Fig. 3.12 A). As water passes through the sponge it is channelled into the choanocyte chambers and between microvilli where a pressure drop and subsequent decrease in water velocity allows particles to be phagocytosed at the base of choanocytes. A glycocalyx protein mesh has been shown within the choanocyte chambers of freshwater sponges

(Weissenfels, 1992). This structure is thought to have a role in controlling water flow and is similar to the secondary reticulum present in hexactinellids. The secondary reticulum is also believed to stop backward flow of water against the polarity of the canal system (Reiswig, 1979). Although these structures are often not preserved during fixation, in *Haliclona* cf. *mollis* a complex, cellular reticulum was stretched between and around a ring of microvilli (Figs. 3.12 B, C). Water entering the chambers near the base of choanocytes is drawn between adjacent microvilli (Fig. 3.12 D) and travels up the flagellum and out an apopyle opening into an excurrent canal. As water passes between the collar microvilli a pressure drop occurs. The complex arrangement of cells throughout the canal system controls the size of water channels and thus the velocity of water flow. This control allows particle capture to occur via phagocytosis at the base of choanocytes at the site of the pressure drop.

### *3.3.6 Comparison of epithelial layers in marine and freshwater sponges.*

Homoscleromorphs possess a cilia (6.87 μm (0.69 um s.e.; n=10)) extending into the middle of the canal on each cell (Fig. 3.13 A, B). Similar cilia are present in demosponges, but these are not thought to be distributed on every cell of the canal (Leys pers. com.). Cilia are known to occur on every cell on the internal surface of the osculum in *E. muelleri* (Leys unpublished data). Although the subdermal space is found in marine sponges including *Haliclona* sp. and *Neopetrosia* sp., this feature is not present in every lineage (Fig. 3.14). *Tethya leysae*. does not contain a continuous subdermal space but cavities called lacunai are evident below a thicker dermis especially when the sponge is contracted (Nickel, 2006) (Fig. 3.14 A). The surfaces

of some sponges such as *Suberites* sp. and *Plakinistrella* sp. are only bounded by an exopinacoderm directly overlying the mesohyl and feeding choanosome (Fig. 3.14 B). The exopinacoderm in all sponges observed in this study consisted of thin overlapping pinacocytes lying on a collagen sheet. Differences were mainly noted in the thickness of the mesohyl and the size of the subdermal space.

## 3.4 Conclusions

Sponges have epithelia composed of either pinacocytes or choanocytes which form the continuous linings of the aquiferous system. These layers are typically squamous with the exception of the pseudostratified columnar epithelium in sponge larva and the columnar epithelium involved in secreting a gemmule coat. External layers lack many junction structures typical of vertebrate models although regions similar in appearance to desmosomes are evident in many sponges (Leys et al. 2009). Septate junctions are found internally and are thought to be involved in $CaCO_3$ spicule production in calcareous sponges (Ledger, 1975). Cells in pinacoderm layers typically sit on an ECM made up of collagen fibers forming, in many cases, a mat but lacking type IV collagen like most basement membranes. In homoscleromorphs this layer is considered equivalent to a basal lamina and does include type IV collagen (Boute et al., 1996). Whether such layers in other sponges act as functional basal lamina requires further functional study. Morphological descriptions of pinacodem layers alongside evidence of spatial stability, ion transport and solute occlusion suggest that sponges have an epithelial system which is functionally similar to higher metazoans. It also indicates that these mechanisms could have been inherited by other

eumetazoans and have gone unrecognized due to lack of obvious junctions in ultrastructure.

**Figure 3-1 Phylogeny of sponges.** Homoscleromorphs are considered as a separate class which is more closely related to Eumetazoans (Philippe et al. 2009) or as a subclass of demosponges designated as G0 (Wang and Lavrov, 2008). Although the freshwater Spongillidae has long been thought to be closely related to marine haplosclerids they typically fall into the G4 clade (★), *Haliclona* sp. and *Neopetrosia* sp. belong to G3, *Tethya leysae* and *Suberites* sp. are G4.

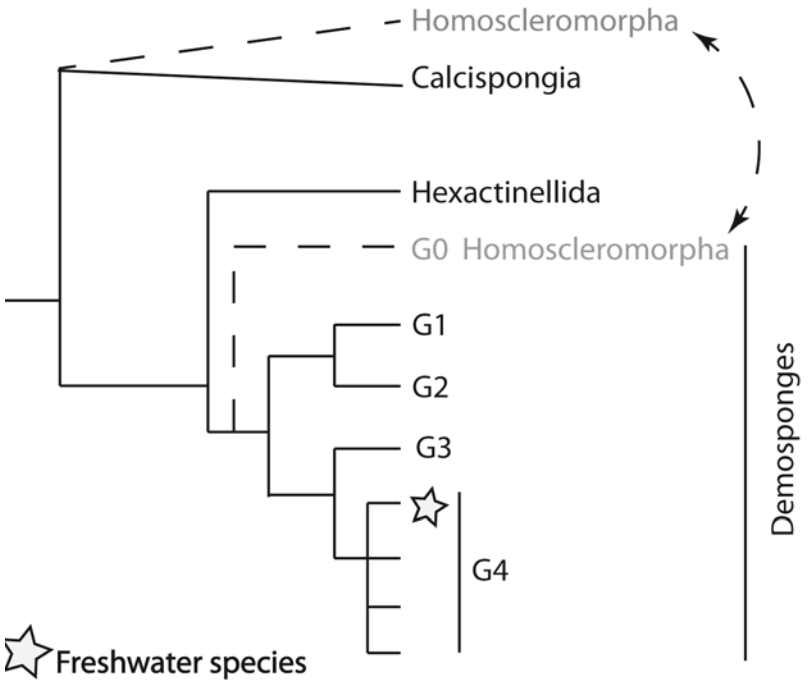
Homoscleromorpha
Calcispongia
Hexactinellida
G0 Homoscleromorpha
G1
G2
G3
G4
Demosponges
Freshwater species

**Figure 3-1**

**Figure 3-2 Diagram and SEM depicting *S. lacustris* morphology.** The body plan of a freshwater sponge grown from a gemmule (A) can be understood by focusing on the water vascular system which is observable in SEM (B) (modified from Elliott and Leys, 2007). Water is drawn into the sponge via ostia in the dermal tissue (dt) layer, composed of exopinacoderm (exp), mesohyl (m) and endopinacoderm (enp), which drapes between spicule struts (spi) creating a subdermal space (ss). Water passes down through contractile seive cells (sc) within incurrent canals (ic) and is drawn into choanocyte chambers (cc) through prosopyle (prp) openings. Food particles suspended in the water are phagocytosed by choanocytes before the water passes out apopyles (ap) into excurrent canals (ec) and then exits via a chimney-like osculum (o).

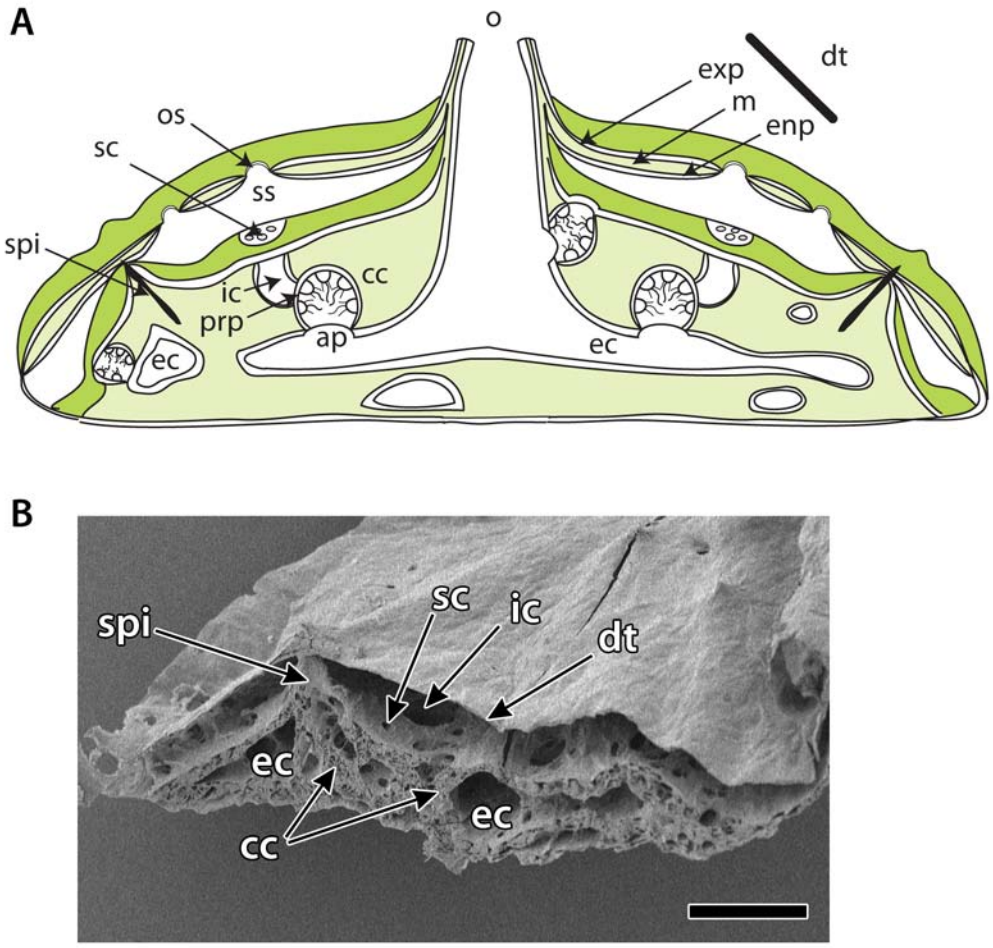


**Figure 3-2**

**Figure 3-3 Scanning electron microscopy of dermal tissues from freshwater sponges**: In *E. muelleri* (A) and *S. lacustris* (B & C) exopinacocytes (outlined - exp) are thin and pentagonal with overlapping boundaries and interdigitations (C). Cross sections in SEM of *E. muelleri* (D) and *S. lacustris* (E) show exopinacocyte (exp) and endopinacocyte (enp) layers separate a mesohyl (m) space containing collagen strands (col) and amoboid mesohyl cells (mc) from the external environment. Scales: A=2μm, B=10μm, C,D,E=1μm.

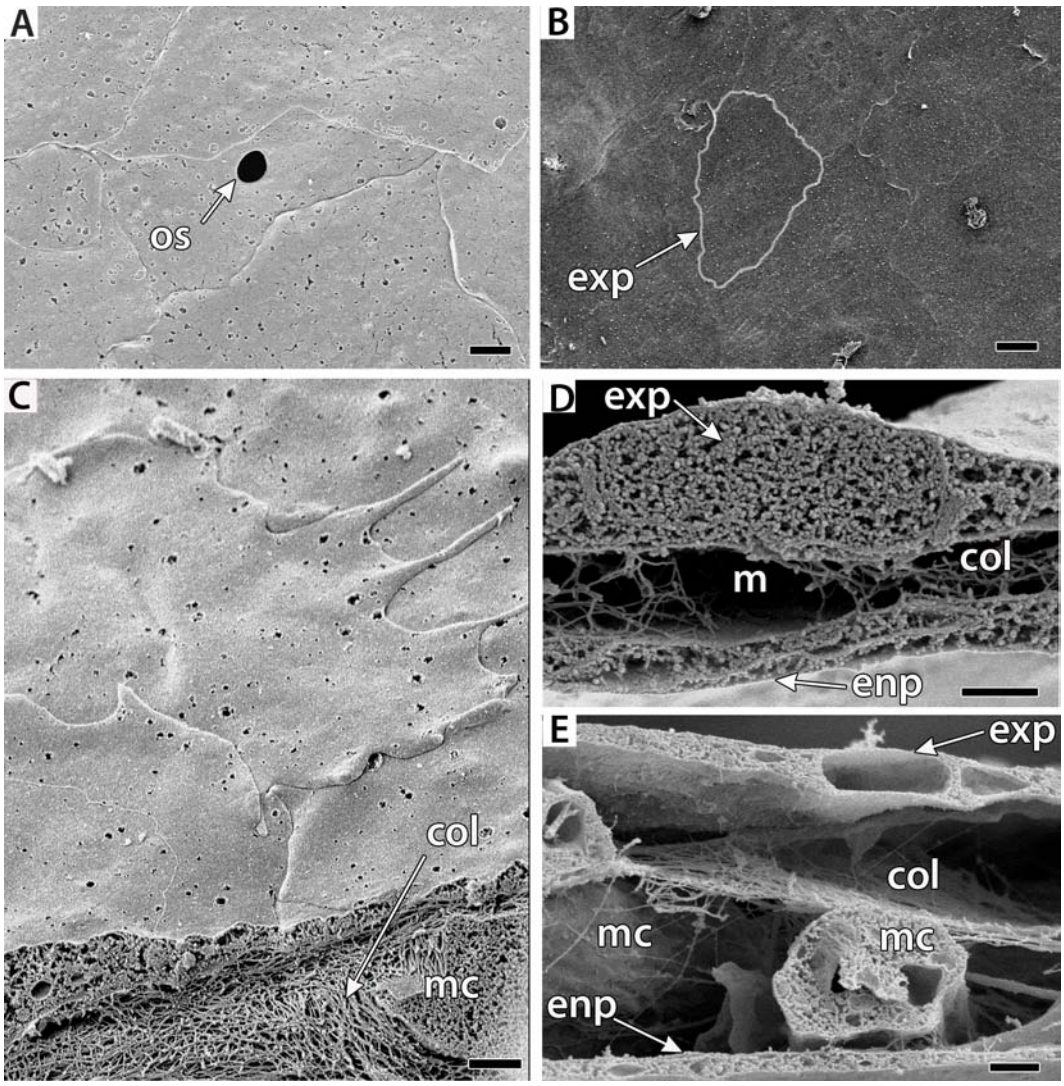


**Figure 3-3**

**Figure 3-4 Transmission electron microscopy of dermal tissues from freshwater sponges**: (A) Adjacent cells in the exopinacoderm of *S. lacustris* were held closely together. Exopinacocytes (exp) membranes maintained a paracellular gap of 15-20 nm. [mesohyl cell = mc, nucleus = n]. (B) Cell layers appeared confluent. Pinacocytes are likely replaced by cells originating in underlying mesohyl regions (black arrow). (C) The paracellular gap was absent in places (black arrow) where adjacent cell membranes appeared to fuse in a punctate pattern, reminiscent of tight junctions. (D) Contact zones were sometimes complex with paracellular spacing remaining constant at between 15-20nm. Contact points similar to desmosomes (black arrows) were observed. These regions appeared to be associated with cytoskeletal filaments (white arrows). Three presumptive junction structures were noted: simple parallel, tight junction-like, and desmosome-like. These structures may act for stability or be a possible morphological basis for solute occlusion. Scales: A, D=500nm, B, C=200nm.

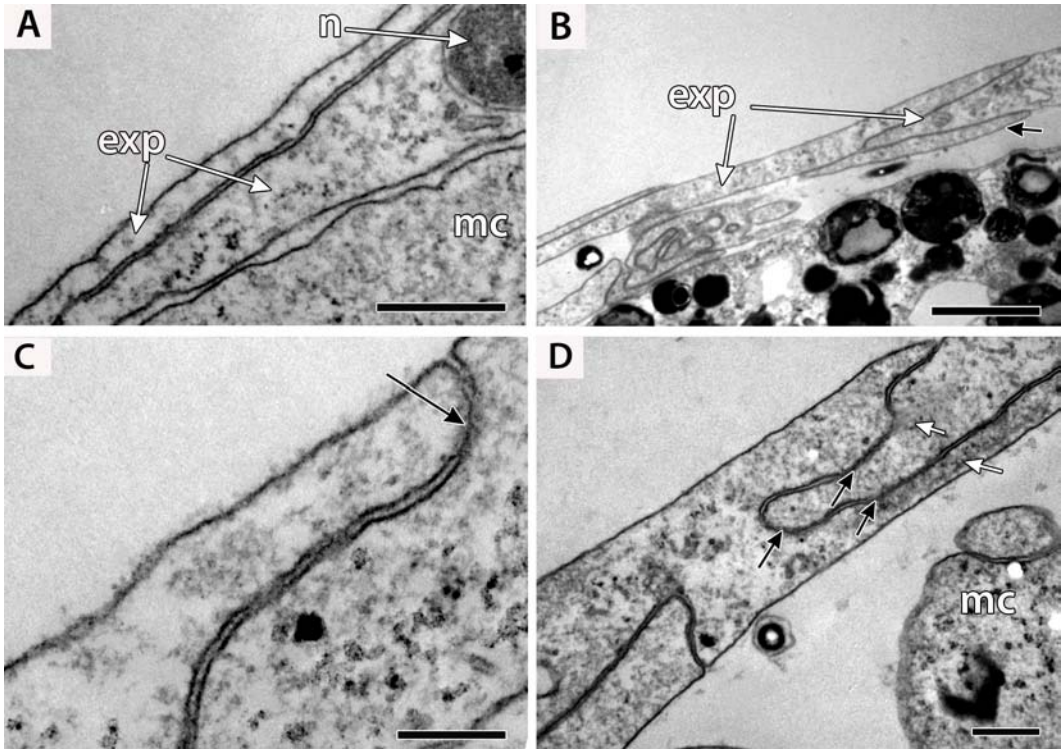


**Figure 3-4**

**Figure 3-5 Occlusion of ruthenium red in *S. lacustris* and *E. muelleri* dermal layers**: As described briefly in Chapter 2, ruthenium red (rr), a tracer which should penetrate paracellular spaces in the absence of cell junctions, was occluded to the external sides of dermal tissues (A & B). Ruthenium red adhered and stained proteoglycans on the external surfaces of exopinacocytes (exp) and endopinaocodytes (enp). No tracer was observed inside the mesohyl (m) space, staining the surface of mesohyl cells (mc), collagen strands (col) or in vacuoles (v). Occlusion of rr was observed in both *E. muelleri* (C) and *S. lacustris* (D) where ruthenium red was excluded to the outside of this tissue layer and at points of cell-cell contact was only able to penetrate a limited distance (arrow) between adjacent exopinacocytes. In addition, ruthenium red was also occluded by the apical endopinacoderm layer which lines the top of the subdermal space (E,F G). Some cell layers were notable by the presence of large vacuoles (E). These are thought to have an osmoregulatory role. No ruthenium red was present in any such spaces. Scales: A=1μm, B, E=300nm, C=200nm, D=150nm, F=800nm, G=80nm.

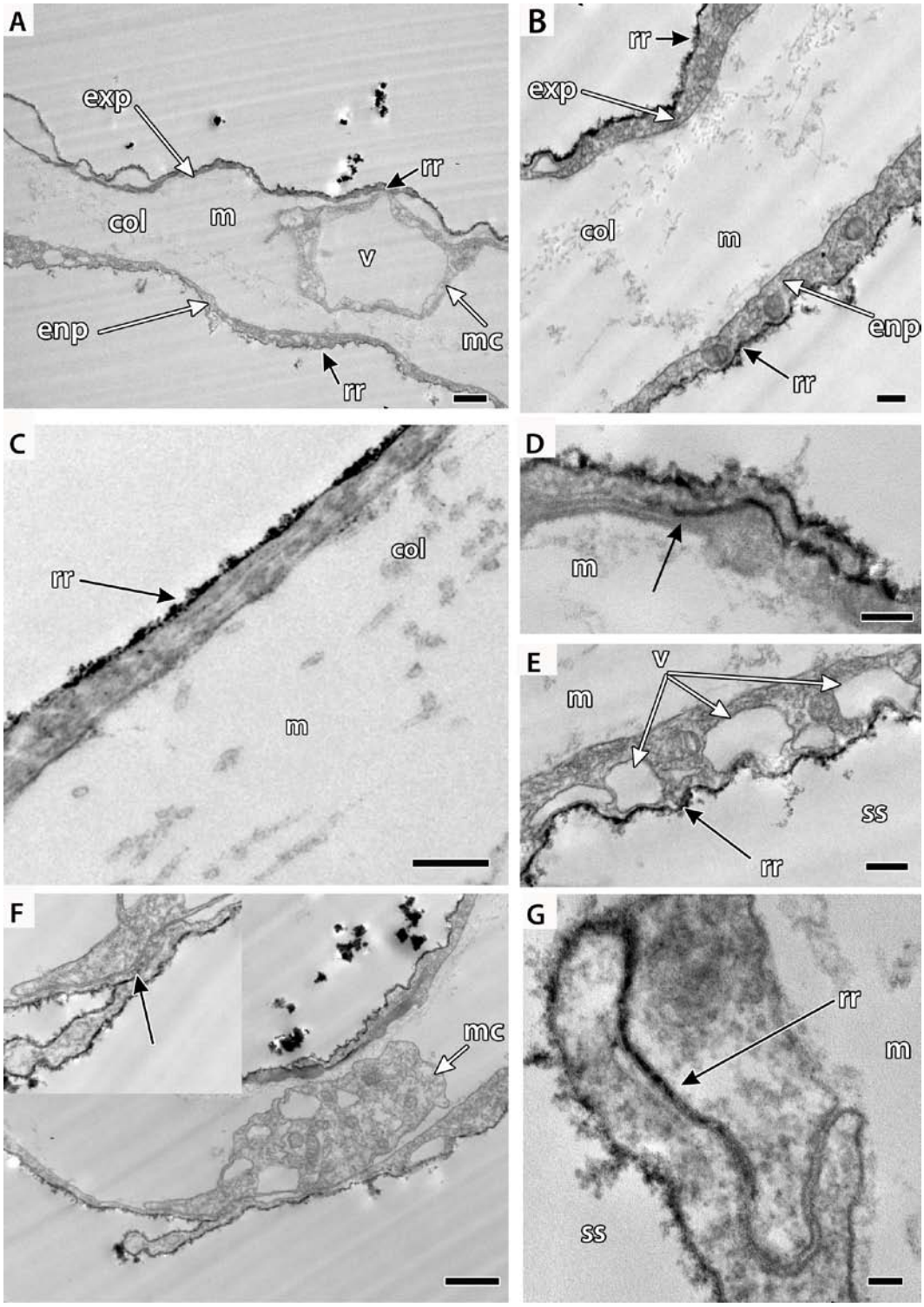
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**Figure 3-5**

**Figure 3-6 SEM of the endopinacoderm below the subdermal space and TEM of ruthenium red penetration.** (A & B) Below the dermal tissues (dt), an endopinacoderm (enp) layer lines the 'floor' of the subdermal space (ss). The endopinacoderm encloses the choanosome (cs), which contains choanocyte chambers (cc), incurrent and excurrent canals (ec), and other migratory cells. [m= mesohyl, bp=basopinacoderm, ap=apopyle] (C) Pinacocytes in the endopinacoderm resembled exopinacocytes. They were thin and tightly overlapping. (D) As in the apical layers, ruthenium red (rr) did not penetrate the entire interpinacocyte distance (black arrow). (E) Intercellular spacing was constant and some regions appeared to have faint septate (white arrows). Scales: A=100μm, B,C = 10μm, D= 400nm, E=150nm.

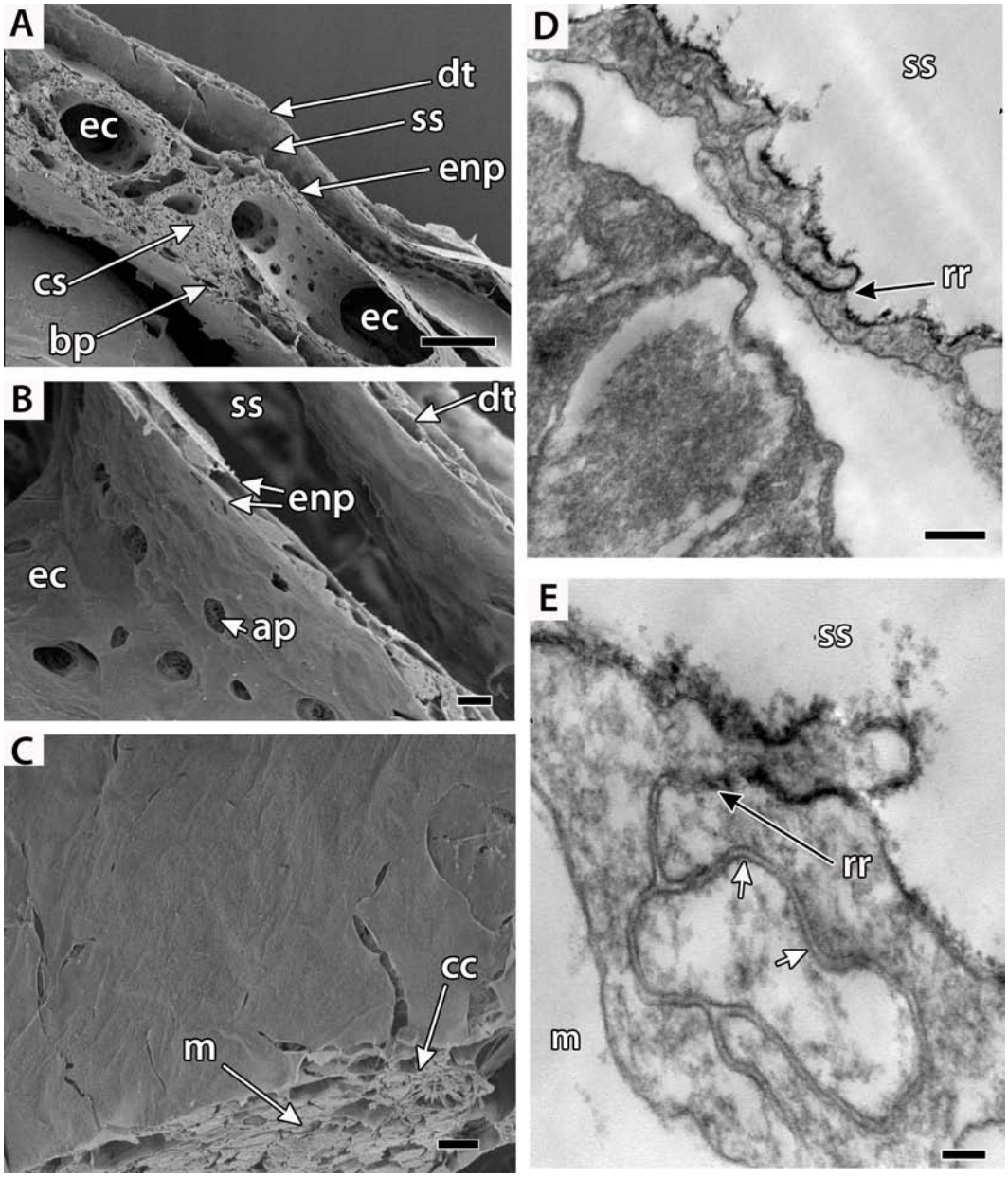


**Figure 3-6**

**Figure 3-7 The canal epithelium in freshwater sponges**: (A) The canal epithelium of *E. muelleri* was made of a single thin layer of squamous endopinacocytes (enp) held tightly together like the cells in apical layers. The canals are large and extensive in freshwater sponges and appear as a network of branching pipelines. Water enters the choanosome through vertical incurrent canals (ic). Large irregularly shaped sieve cells (sc) extend to either side of the canal surface and are thought to control canal diameter. The beating of flagella in choanocyte chambers (cc) generates water current. Water exits choanocyte chambers via apopyle (ap) openings (B- *E. muelleri*, C- *S. lacustris*) that lead into excurrent canals. [bp=basopinacocytes]. Endopinacodems in freshwater sponges are very thin with minimal associations with collagen. (D- *E. muelleri*, F- *S. lacustris*). Scales: A=100µm, B,C = 10µm, D= 2µm, E= 20 µm.

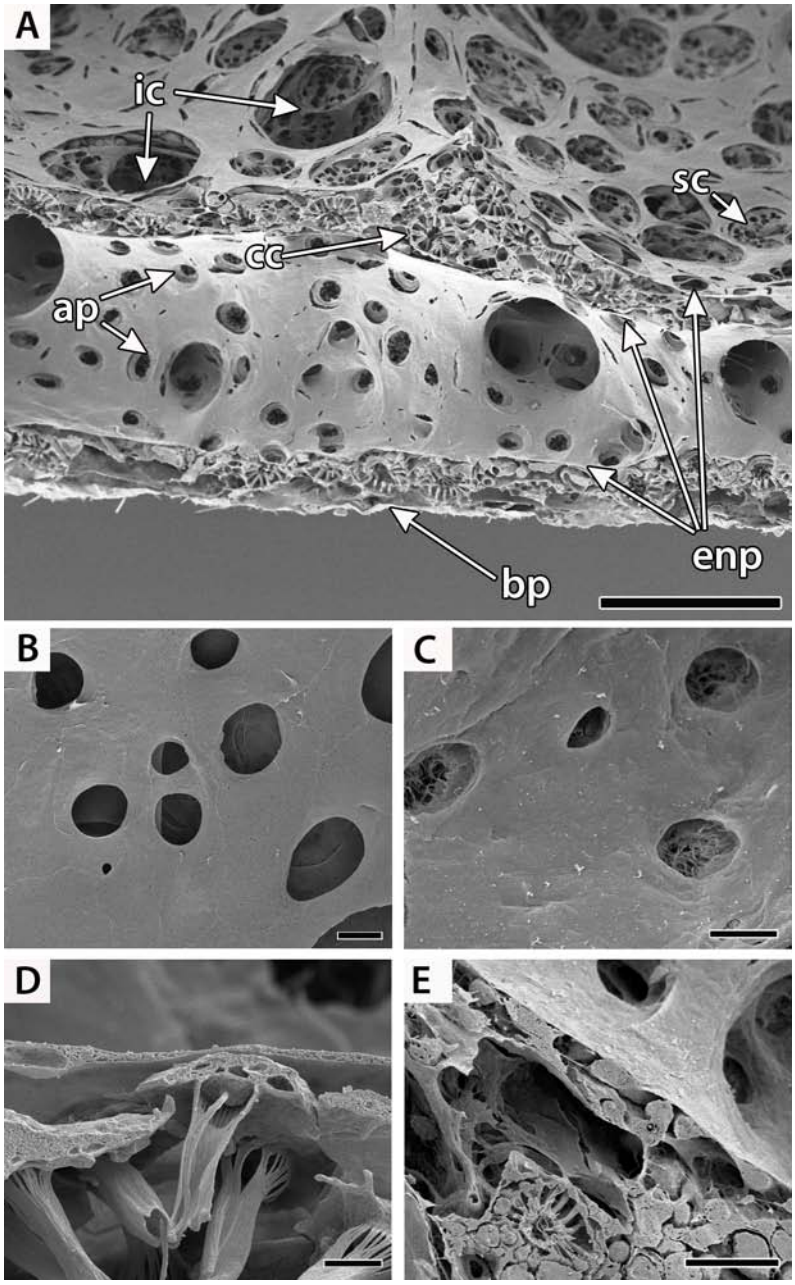


**Figure 3-7**

**Figure 3-8 Basopinacoderm in *S. lacustris*:** (A) The basopinacoderm (bp) in freshwater sponges can be observed, undamaged, if sponges are cultured on glass cover slips and then removed with hydrofluoric acid. This process gently lifts the sponge off the glass substrate leaving the sponge intact. [dt=dermal tissues, gem= gemmule] (B) Cells in the basal layer are thin and have a similar morphology and spatial arrangement as pinacocytes on the apical surface. [cc= choanocyte chambers, m=mesohyl] There are no porocytes with ostia openings in the basopinacoderm layer. (C) It is evident in TEM that the basopinacoderm is a single confluent layer of thin pinacocytes. Below the layer is an acellular substance which is likely a mucus coat (muc) containing secreted collagen with dark inclusions. (D) In SEM, crystals (cr) of potassium are found coating the basal surface. (E) Basopinacocytes are held tightly together and overlap with a shingle-like appearance. Scales: A=100 μm, B, D, E= 10μm, C= 150nm.

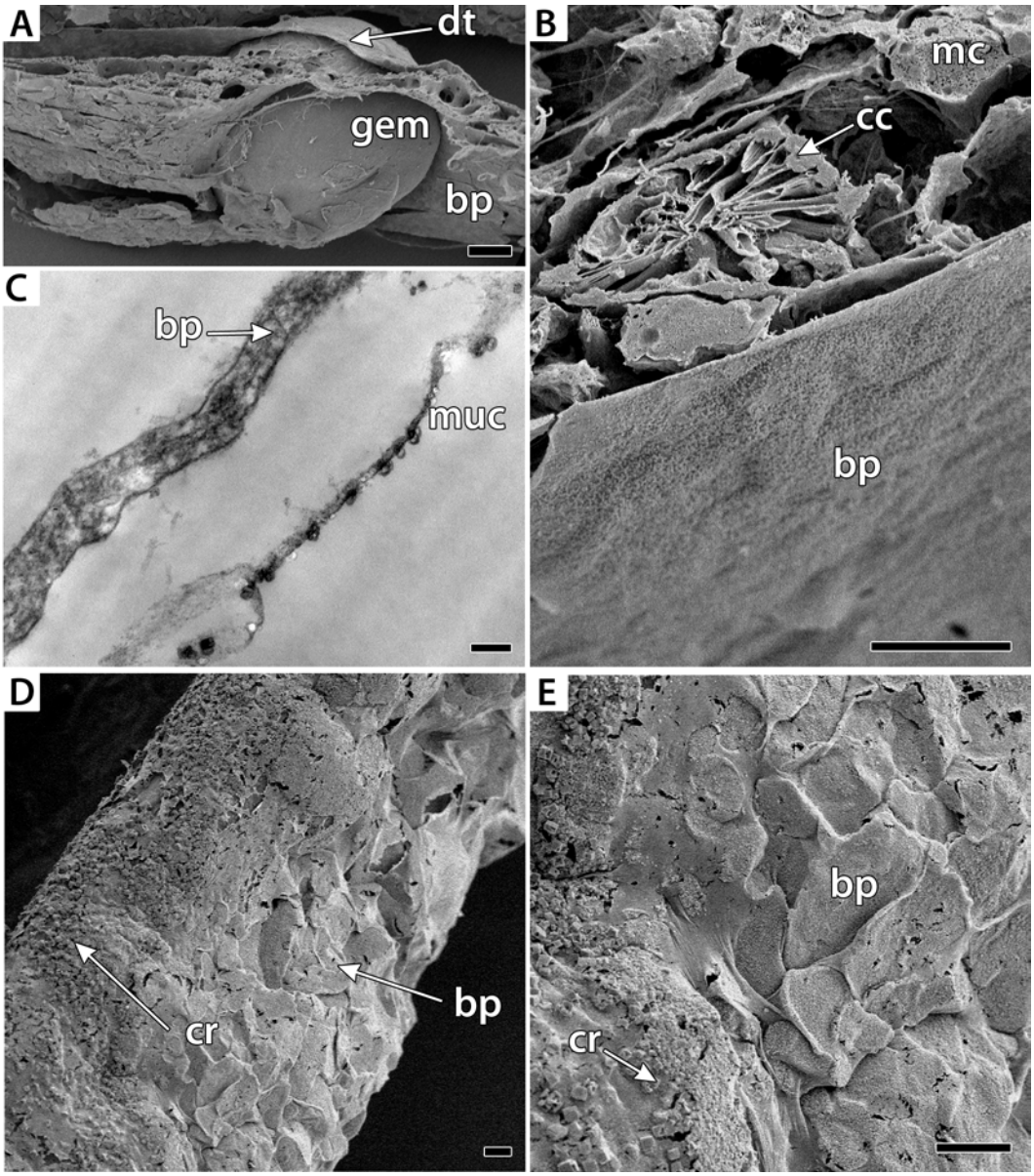


**Figure 3-8**

**Figure 3-9 Osculum in freshwater sponges**. (A) An osculum -- the exit of the aquiferous system -- is the most noticeable structure on the apical surface of freshwater sponges. Juvenile sponges grown from gemmules on cover slips typically have a single osculum (o) arising near the gemmule husk (gem). The osculum is composed of two cell layers consisting of exopinacoderm continuous with the apical surface and endopinacoderm continuous with the excurrent canals. (B & C) Exopinacocytes in *S. lacustris* are held tightly together to form a continuous sheet of cells with no evident ostia. (D & E) TEM of *E. muelleri*. Cells had constant intracellular spacing like that of the exopinacoderm and no specialised junctions were noted. Scales: A=1mm, B=100μm, C= 10μm, D= 6 μm, E=2μm.

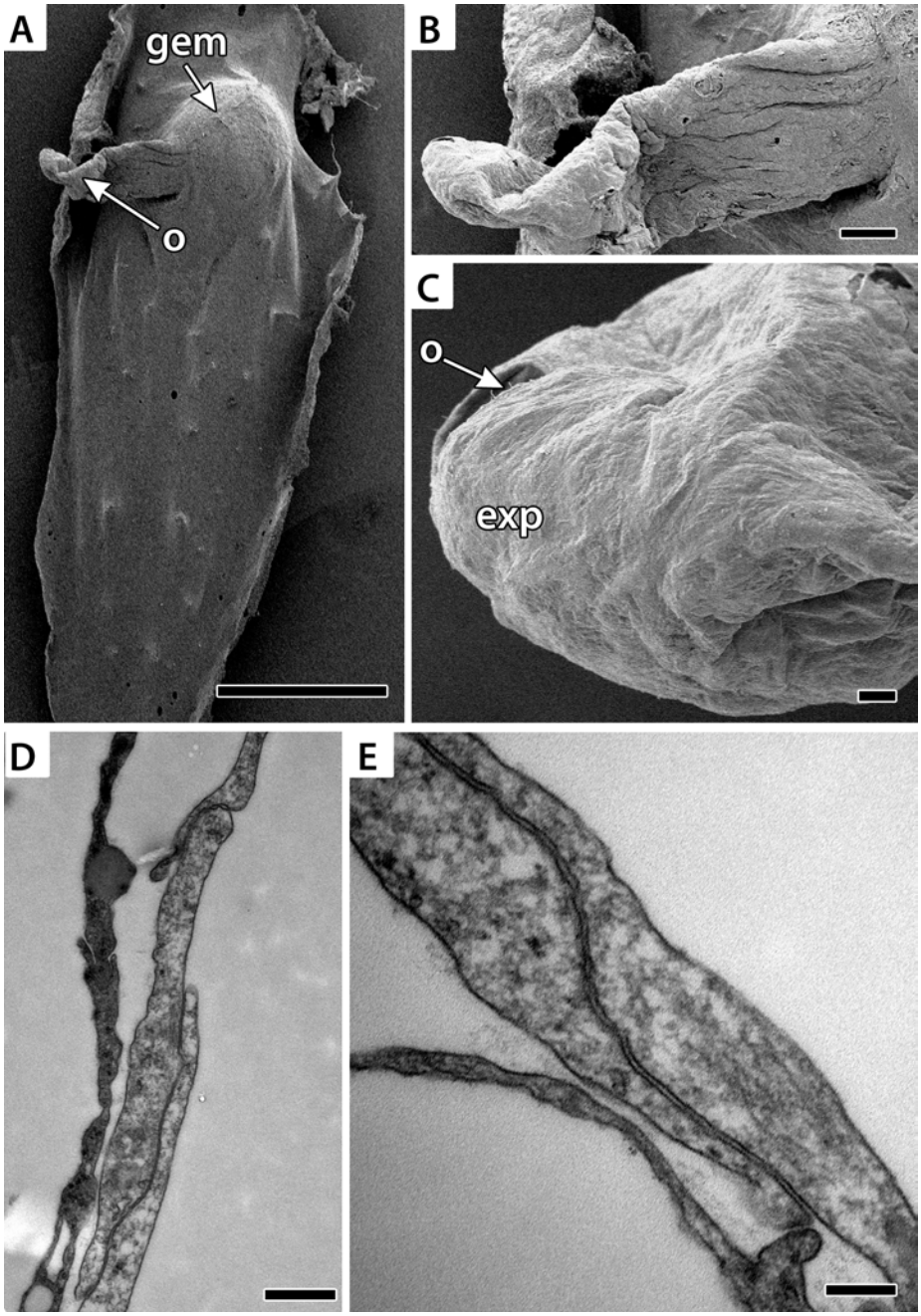


**Figure 3-9**

**Figure 3-10 Tracing cell movement in the pinacoderm and mesohyl layers.** Sponge pinacoderm cells are spatially stable compared to cells in the mesohyl. (A) Staining with FM 1-43 revealed that the exopinacoderm of *Ephydatia mulleri* is composed of pentagonal cells arranged like tiles. (B) A series of images of the pinacoderm were taken every minute for one hour. Every tenth frame/minute the outlines of these cells were traced and the tracings were then overlapped. In one hour the cells in the pinacoderm did not migrate away from their neighbouring cells and remained stable in the pinacoderm layer. (C) Cells in the mesohyl are shown with brightfield microscopy after exposure to FM 1-43. Cells in this layer are morphologically more varied than the overlying pinacocytes. Some cells contain round vacuoles, others have extended projections, and others contain granules. (D) An image of the mesohyl was taken every minute for one hour. Cells in this layer were very active – they changed shape, direction and speed of movement over time. The outline of two cells in this layer were traced every fifth frame/minute and overlapped to show both direction of migration (arrows) and shape change over time (darkening colour). The green vacuolated cell resembling a cystencyte moved slowly at 1 µm/min while the blue cell was migrated faster at a maximum of 9 µm/min. Scale = 30 µm

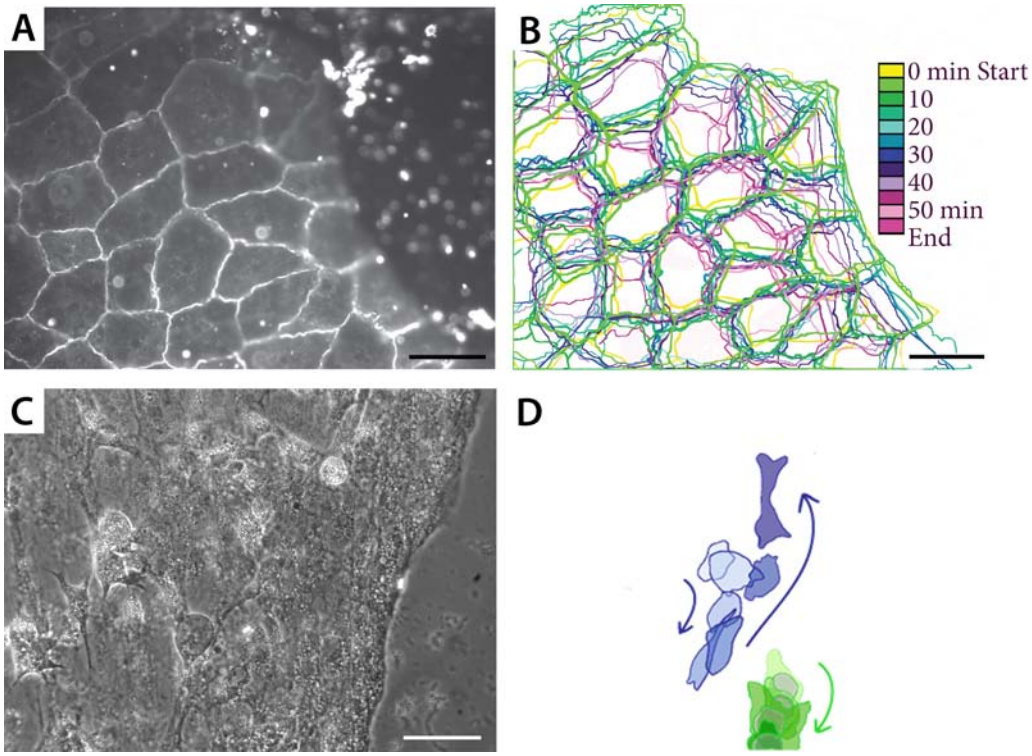


**Figure 3-10**

**Figure 3-11 Dermal tissues of marine sponges:** (A) Like freshwater sponges, *Haliclona* cf. *permollis* has a subdermal space (ss) below the thin dermal tissue (dt) layers containing prominent spicule shafts (spi). Dermal tissues formed a tent-like structure which was stiffer than the more delicate tissues of freshwater sponges. (B) The dermal tissue was formed of two cell layers: exopinacoderm (exp) and endopinacoderm (enp) with a thin mesohyl containing amoboid cells (mc) and small mat of collagen (col). Like freshwater sponges, *H. permollis* dermal tissues contained porocytes (po) with a contractile ostia (os). (C) Exopinacocytes were thin and sat directly over a mat of collagen (D). The exopinacoderm of *Neopetrosia* cf. *vanilla* contained similar structures to Haliclonids. Scales: 20μm, B, D= 2μm, C= 1μm.

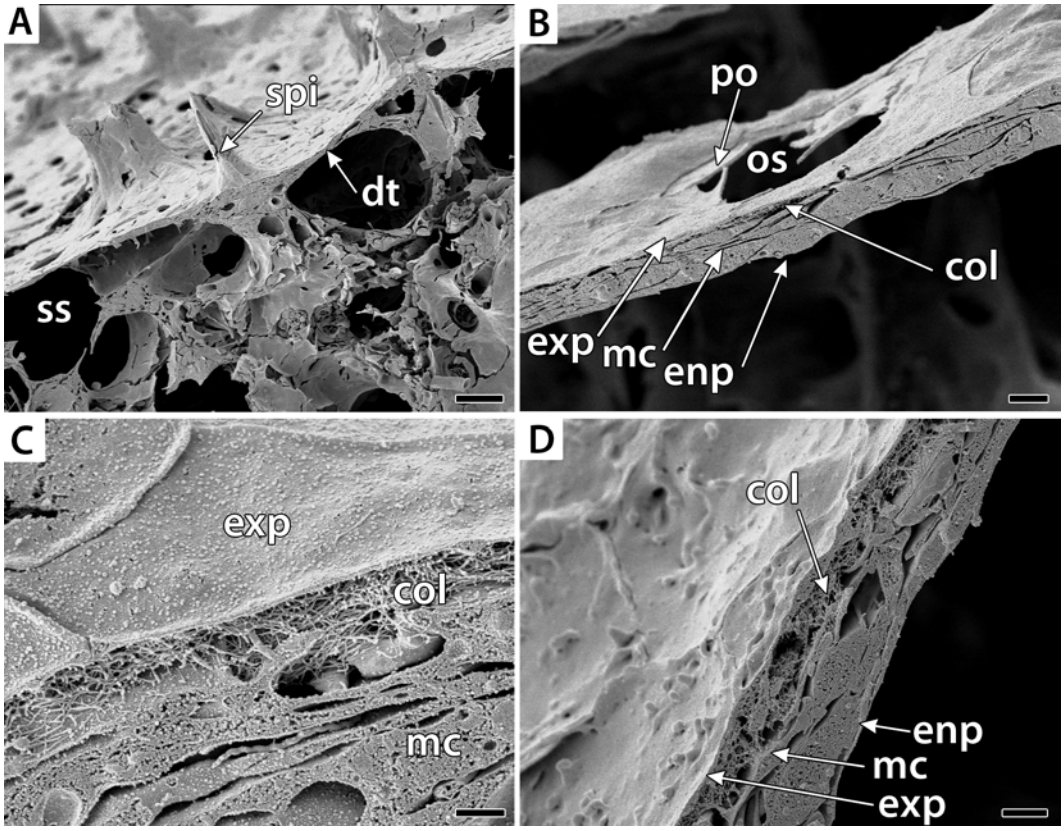


**Figure 3-11**

**Figure 3-12 Choanoderm of *Haliclona* spp. in SEM**. (A) In *Haliclona* cf *permollis* choanocyte chambers (cc) are packed within a dense meshyl surrounded by a smooth endopinacoderm (enp). (B). In some species, such as in *Haliclona* cf. *mollis* a substantial secondary reticulum (sr) lies within each choanocyte chamber (cc). (C) This structure appeared to be cellular (arrows). (D) Water flows into choanocyte chamber through prosopyle openings, passes between microvilli (mv) near the base of choanocytes (coc), travels up flagella (fg) and out through the ring. Scales: A, B=10μm, C, D= 2μm.

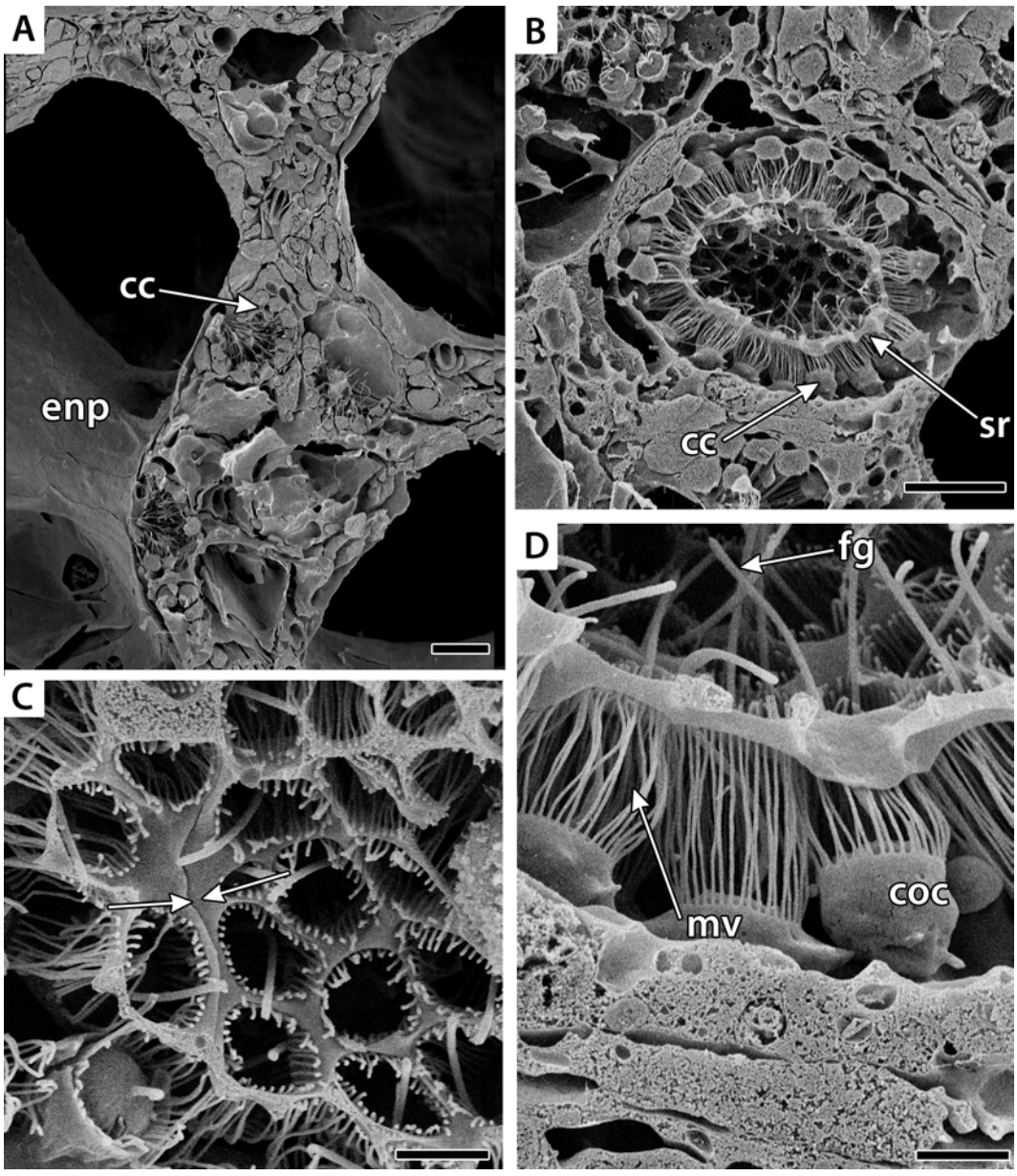


**Figure 3-12**

**Figure 3-13 Canal epithelium in homoscleromorph sponges**. (A) Every cell making up the canal endopinacoderm of *Plakinstrella* sp had a 10 μm long cilia which extended into the center of the canal (arrow). (B) A ciliated canal epithelium was also evident in the homoscleromorph *Plakortis* sp. (C) The choanosome of homoscleromorph sponges was more densely packed with cells than freshwater sponges. (D) Close examination revealed that the mesohyl of this sponge was filled with marine bacteria (black arrows). Collagen (col) layers in Homoscleromorphs were thicker and more dense than in those freshwater sponges but fibres were similarly arranged in a matted net of material. A, B, C =10μm, A inset= 2μm, D= 2μm.

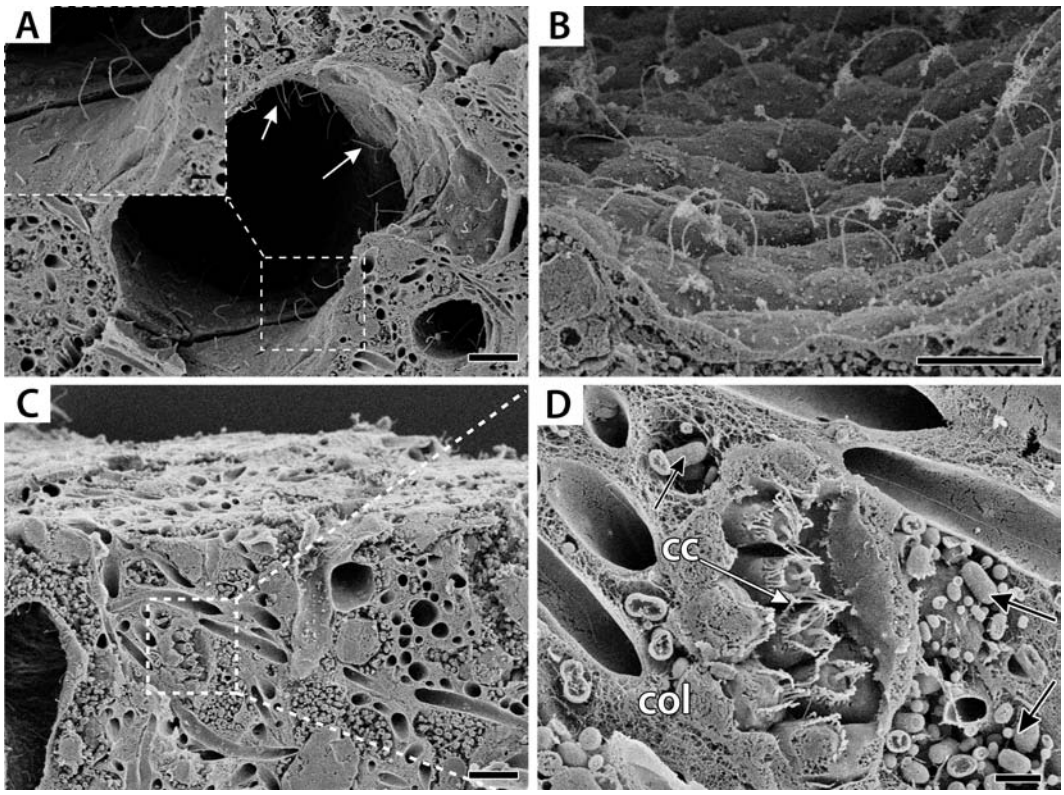


**Figure 3-13**

**Figure 3-14 Dermal tissue organization in thick walled marine sponge species**. (A) *Tethya leysae,* unlike freshwater sponges has a thin pinacoderm (exp) that did not overlie a subdermal cavity. Large cavities called lacunai (la) were present below the surface. The exopinacoderm sat on a collagen mat above a dense cortex region (cor) with the chanosome below. (B) *Suberites* sp. also lacked a subdermal space. This species has a globular, dense body form with an exopinacoderm (exp) lying over a mesohyl (m) with many parallel spicule (spi) tracks present perpendicular to the sponge surface. Scales: A=150µm, B= 100µm.

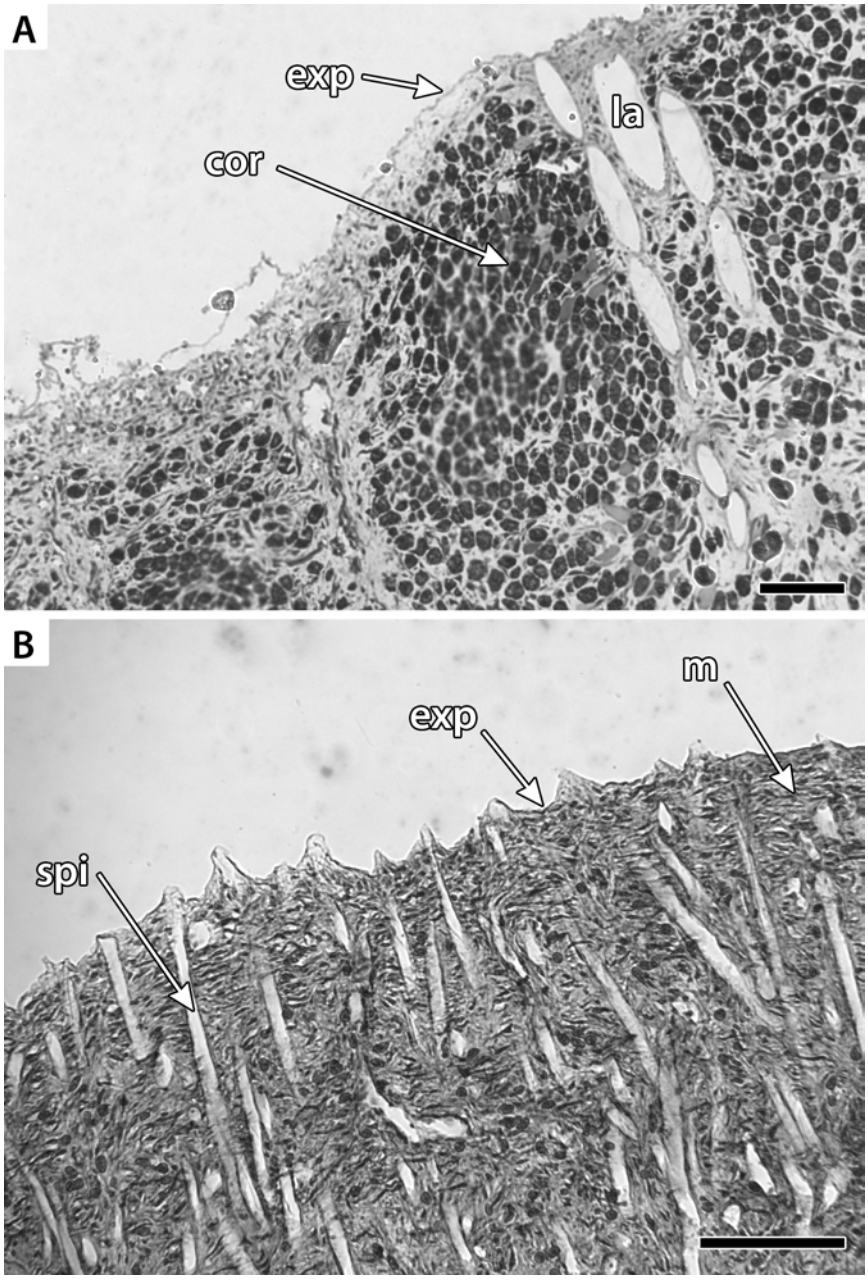


**Figure 3-14**

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# Chapter Four

# A general discussion of the implications of functional epithelia in sponges.

## 4.1 Introduction and broad research goal

Multicellularity appeared independently in at least 16 eukaryotic lineages but it is thought to have evolved only once in animals (King, 2004). Phylogenetic reconstructions place the unicellular protists choanoflagellates as the closest extant outgroup to all metazoan lineages (Phillipe et al., 2009, Pick et al., 2010, Srivastava et al., 2010). The transition from a unicellular to multicellular existence is hypothesized to include a colonial unicellular stage (King et al., 2004). This scenario is similar to some choanoflagellates that group together while individual cells remain autonomous. Following the development of molecular mechanisms for permanent adhesion and interaction, signalling systems developed for growth, cell fate and patterning. Once cells became specialized for particular tasks, they were eventually restricted to a single fate and were no longer capable of independent life. Metazoan organisms developed complex forms, different modes of development and various life histories.

The evolution of metazoan organisms remains somewhat mysterious. The first animals, sponges, are thought to have originated prior to the Cambrian explosion

at a time of rapid evolutionary change (Sperling and Peterson, 2007, Love et al., 2009, Sperling et al., 2010, Van Roy et al., 2010). Although the known paleontological record does not include spicules in pre-Cambrian sediment, demosponge specific chemicals have been identified in 635 million year old formations (Love et al., 2009). Conflicting metazoan phylogenies produced by different authors within the last ten years have cast doubt on the identity of the first metazoan (Dunn et al., 2008, Schierwater et al., 2009, Pick et al., 2010). Most models suggest that sponges evolved after choanoflagellates and are the first multicellular metazoans (Zrzavý et al., 2003, Borchiellini et al., 2004, Phillipe et al., 2009, Pick et al., 2010). Whether a sponge-like body plan evolved once or multiple times is also unclear. Competing theories have argued that the Porifera is a paraphyletic grade where Calcarea and Homoscleromorphs are more closely related to higher metazoans than other sponges (Borchiellini et al., 2003, Zrzavý et al., 2003). Alternately, a more traditional monophletic Porifera emerged from genetic analyses on protein coding genes (Philippe et al., 2009, Pick et al., 2010). In any case, genetic data have shown that sponges possess many genes associated with higher animal physiology. These data refute the concept that sponges are evolutionary oddities and a dead-end belonging to a separate subkingdom (Hanson, 1958). It should be noted that no published phylogenetic analysis is without flaws. Low taxon sampling, biased gene selection, and choice of analytical approach introduce uncertainly as to the validity of research findings. Only strategies which employ a wide variety of taxa and many genes should be considered sufficient to address the problem of basal metazoan relationships. Determining early metazoan phylogeny is not a trivial debate. This

knowledge is required to accurately form a framework on which to base grand evolutionary concepts including those related to epithelial evolution.

The evolution of transporting epithelia is also unclear. The complex assortment of junction proteins, ion channels and signalling molecules present in epithelia likely evolved in stages (Cereijido et al., 2004). The data presented in this thesis suggests that sponges have the ability to control their extracellular environments. A homeostatic balance between the external environment and internal body regions is thought to be critical for normal cell behaviour in all other metazoans (Evans et al., 1999). Control of ion, solute and signalling molecule transport between various body regions is required for feeding, reproduction and cell signalling. The presence of functional epithelia in sponges indicates a level of organization beyond the cell-level.

The main research goal of this thesis was to examine if sponges have functional epithelial layers. This chapter will include a discussion of the main research findings which will be followed by a brief commentary relating the relevance of this work to larger debates concerning multicellular evolution and physiological complexity in Poriferans. Future studies are also suggested which may expand on the findings to date. These studies could be broken into three broad categories: physiology, genetics and morphology.

## 4.2 Summary of research findings

It is convenient in evolutionary biology to emphasize those features that early branching organisms lack as a means of simplifying concepts about increasing biological complexity. Many molecular components in higher order animals are now known to be present throughout the Porifera although their biological function in sponges is often unknown (Srivastava et al., 2010). Since definitive TJ, SJ, and AJ structures and genes associated with epithelial tissue in higher animals (particularly type IV collagen) are missing in most sponges it has been suggested that Poriferans had not evolved true epithelia (Pavans de Ceccatty, 1974, Magie and Martindale, 2008). The designation as 'true' has been used without consideration for the physiology of pinacoderm layers in sponges.

Research presented within this thesis included the first recordings of transepithelial resistance (TER) across sponge tissues and it was concluded that sponges have epithelial layers which are functionally similar to those of Eumetazoans (Adams et al. 2010 - Chapter 2). Sheets of sponge pinacocytes resisted the passage of small solute tracers and maintained a membrane potential of -3mV which is small compared to frog skin (90mV) but similar to proximal tubules (2mV) (Ussing et al., 1974). This suggests that differential ion transport occurs across sponge cell layers. The morphological bases for these physiological properties has not yet been resolved. Many junctions present in invertebrate and vertebrate systems were not present in sponges when epithelial morphology was examined in detail (Chapter Three). No unambiguous septate or tight junctions were observed although many regions of apparent cell membrane contact were noted near the apex of the lateral cell-cell contact zone. Additionally, faint septae could be discerned between some adjacent pinacocytes. The constant intracellular distance of less than 20nm between adjacent

pinacocytes was similar to that reported by other authors (Green and Bergquist, 1982). This feature had previously been named a 'simple parallel junction', hypothesized to contain a secreted membrane spacing factor. Although the membrane spacing factor was first suggested as a mechanism controlling cell-to-cell positioning, the physiological data presented here suggests that the factor could also share an occluding role .

## 4.3 Importance of this work

The culturing techniques developed during this thesis could be applied in future studies on sponge physiology or for epithelial physiology in general. Expression of cell adhesion, occlusion and communication molecules is complex thus making study of protein function difficult. Individual components of an epithelial phenotype must be isolated to identify the function of a specific protein. In model animals, this can be achieved with gene knockout studies. Sponges represent an animal model with few of the genes thought to be necessary for occlusion in vertebrates (Srivastava et al., 2010) and yet they are physiologically sealed. Sponges could thus represent a kind of natural gene knockdown model. Identifying the molecular mechanisms for epithelial function in sponges may reveal either sponge-specific proteins that are capable of functional sealing or proteins with a sealing function that underlie the epithelial physiology in other animals which have gone unnoticed.

The presence of functional epithelia in the Porifera has the potential to alter the definition of Eumetazoa. The current definition requires all so-called "true animals" to possess epithelial tissues as well as a gut and nervous system. I have shown that functional epithelia are present in a metazoan animal. The criteria for both Eumetazoan classification and 'true' epithelia may thus have to be re-examined. The absence of classical basal lamina in demosponges evidently does not inhibit epithelial function. It seems likely that collagens present in sponges are able to perform the same role as Type IV collagen in higher animals. Many other basal lamina proteins such as integrins and fibronectin are known to be present in sponges (Brower et al., 1997, Labat-Robert et al., 1981). Although sponges do not possess a nervous system, their genome encodes post-synaptic scaffolding genes (Sakaraya et al., 2007). The line between Eumetazoa and Metazoa is beginning to be unclear (Fig. 4.1).

Errors in the molecular mechanisms associated with the advent of multicellularity are tightly linked to epithelial pathophysiologies. An epithelial to mesenchymal transition (EMT) occurs when cells in layers delaminate, become fibroblastic and migrate through the basal lamina to other regions of the body (reviewed in Hay, 2005). This process is associated with tumorigenesis in higher animals (Guarino et al., 2007). The tropical marine sponge *Amphimedon queenslandica* contains genes involved in the transition to metazoan multicellularity including those related to cell adhesion , signalling, and apoptosis (Srivastava et al 2010). Errors in genes associated with these fundamental multicellular processes are considered to be linked to cancer. It has been reported that epithelial cells in sponges do not undergo mitosis (Harrison, 1972), inferring that they are replaced from a population of mesohyl cells. Although sponges appear to have sealed boundaries,

this type of strategy for homeostasis suggests that movement of cells into epithelial regions can occur. It is possible that the genetic basis underlying these cell replacement events is also associated with changes in cell adhesion which could lead to pathophysiologies such as cancer in the event of disruptions.

## 4.4 Future directions: Studying sponge epithelia

### *4.4.1 Further characterization of ion transport physiology*

The data presented in this manuscript suggest that cell layers in sponges have some control over the ion composition of the environment on either side of pinacoderms. Ion transport includes both passive and active processes. Passive flux tends to occur through the paracellular route and is controlled by occluding junctions while active transport occurs via ion channels through a transcellular path across cell membranes (Frömter, 1972, Reuss and Finn, 1974). A wide diversity of ion channels including voltage gated, antiporters, and exchangers control ion transport, particularly of sodium and chloride, across epithelia (Decoursey, 2003). The first freshwater sponges likely experienced similar environmental pressures to control their internal extracellular and intracellular ionic concentrations as freshwater fish. It has been shown that gill epithelia of freshwater fish take up sodium ions in an energetically costly direction against an osmotic gradient. Understanding the mechanisms for this process is an ongoing focus for research (Parks et al., 2008). The physiology of ion transport in sponges by contrast to other animals is only in its infancy. To understand ion flux in freshwater sponges, the uptake of radioactive labelled sodium into sponge tissues must be determined. Such an experiment is expected to show unidirectional

transport of sodium in the inward direction. Sponge tissues would be first incubated with the radioisotope $^{22}Na$ before being rinsed and lysed. The concentration of radiolabelled tracer present in mesohyl regions would then be measured on a scintillation counter. A similar experiment could use $^{36}Cl$ to measure chloride flux, although it is not expected that chloride would be transported into freshwater tissues. These data could be used to clarify unidirectional flux of ions across sponge tissues.

Freshwater sponge tissues were shown to resist paracellular solute flux and seems to able to transport ions across their epithelia. The availability of the *Amphimedon queenslandica* genome as well as 454 sequencing for *Ephydatia muelleri* increase the likelihood that genes encoding sponge cell sealing and ion transport could be identified if they are homologs of known proteins. Although genetic knockdown of presumptive ion transport genes in sponges would require additional work, the use of specific pharmacological inhibitors could provide more immediate insight into the molecular mechanisms underlying sponge physiology. In particular, amiloride has been shown to disrupt epithelial sodium channels (ENaC) in freshwater fish (Goss et al., 2001) which reduces sodium uptake. If this drug causes a knockdown of sodium transport in *S. lacustris* it suggests that sponges may have a functional Na+/H+ exchanger responsible for sodium regulation. Other treatments could include bafilomycin, an inhibitor of vacular $H^+$-ATPase, phenamil, an inhibitor of sodium channels (Lin et al., 2005, Tsui et al., 2009) and acetazolamide which has been shown to disrupt chloride exchange (Evans et al., 1999). It must be noted that the use of these types of inhibitors can be complicated by differential drug response by cells in different animals (Clauss, 2001, Sobczak, 2006) but they do offer the

chance to identify molecular components of sponge epithelia without requiring genetic data.

### *4.4.2 The genetics of sponge cell junctions*

Recent genetic studies have found cell junction genes in sponges and choanoflagellates. A suite of MAGUK (membrane associated guanylate kinase) proteins are now known to have evolved prior to the advent of multicellularity (de Mendoza et al., 2010). These scaffolding proteins are known to be associated with tight junctions, synapses and cell polarity. Known sponge MAGUK proteins include Discs Large (DLG), ZO-1, and MAGI. Expression studies have shown that MAGI and DLG are only expressed in pinaocderms (Adell et al., 2004, Sakarya et al., 2007). MAGUK proteins may form complexes at regions like tight junctions in sponges (de Mendoza et al., 2010). Further work should focus on genes involved with ion transport, collagen and junction formation in sponges including MAGI, occludin, tetraspannin, disc large and beta-catenin using data generated from *Ephydatia* 454 sequencing. Comparing the compliment of genes to the known *Amphimedon* genome as well as genome of higher order animals could reveal the likely evolutionary histories of multicellular animals and determine if these genes are homologous.

### *4.4.3 Comparative physiology of freshwater and marine sponges*

Marine sponge species are more common than freshwater groups and they branched earlier during evolution (Simpson, 1984). It is therefore important to

determine if transepithelial solute occlusion was present in the last common ancestor of all sponges, or if this phenotype evolved independently as an adaption to freshwater. Comparative study of a suite of marine and freshwater sponge species can potentially clarify this issue. Since the membrane potential reported for freshwater sponges was low in open circuit Evom conditions, it would be more accurate to record under voltage clamp conditions in a modified Ussing chamber (Karnaky, 1992). A likely hypothesis would expect marine sponges to have a lower TER than freshwater species but have functional epithelia which could block transcellular solute passage.

Of all sponges, freshwater species have likely had the strongest pressures to evolve tightly sealed epithelia and mechanisms to regulate the ionic composition of their mesohyl space. Chapter Three demonstrated that pinacoderm morphology of freshwater sponges was not substantially different from marine species however, this is not definitive evidence of occlusion and ion transport. To empirically test if marine sponge tissue can seal, the TER of several marine species should be recorded. Of particular interest should be *Haliclona permollis*, an intertidal marine haplosclerid demosponge which is easily accessible from Bamfield, Vancouver Island, Canada. Freshwater sponges are thought to be closely related to marine haplosclerids (Meixner et al., 2007) so haliclonids are well placed to research when an ion transporting epithelial arose in sponges. *H. permollis* is easily disassociated and undergoes reaggregation and spreading within a similar timeframe as *S. lacustris*. Preliminary work using this species was hampered by a marine ciliate, originating either from the seawater or the sponge itself, which infested and killed the tissues (Adams, personal observation). Optimization of culturing conditions may overcome

this problem with additional seawater filtrations, sterilizations, antibiotic treatments, or dissociation of sponge cells into low salinity water. Determining a protocol that encourages healthy cell culture is not trivial. Marine invertebrates are notoriously problematic sources for successful cell culturing. Sponges in particular pose challenges to culturing because their symbiotic bacteria could be killed by antibiotic treatments. However these difficulties would be worth overcoming. As an intertidal sponge, *Haliclona* sp. must withstand extreme daily changes in environmental conditions including changes in sunlight, temperature, and water flow. In addition, during times of heavy rain, the resulting drop in salinity would put this species under similar pressures to avoid ion losses as freshwater genera. At low tide, some individuals are completely exposed to air and thus it is probable that *H. permollis* would have sealed epithelia to avoid desiccation and prevent internal cell damage.

TER recording from *H. permollis* could be compared to those attained from the gills of diadromous fish which must tolerate both salt and freshwater conditions. The ancestors of these two organisms were adapted for a marine environment so methods for ion regulation in freshwater may differ between these animals and be an example of convergence. Asymmetrical TER recordings where fresh and salt-water were applied to either side of the sponge tissue culture at one time could reveal information about the environmental and physiological tolerances of this species. The epithelial physiology of a fully marine sponge could also be compared to freshwater and intertidal species in order to determine if all sponges are capable of sealing. The most promising model is likely the use of gemmules from a marine haplosclerid. These could be grown in culture wells and TER could be recorded in a salt water media. It is important to determine if sponge epithelial physiology is more similar in

closely related groups or in species living in similar habitats. This could reveal if the physiology of freshwater sponges represent an evolutionary relevant transition which then evolved in other animals or a convergent character state only found in freshwater sponges. Finally, recording TER in a homoscleromorph sponge could determine if the presence of a basal lamina in this lineage (Boute et al., 1996) is associated with higher resistance or other physiological differences.

### *4.4.4 Morphology: Protein localization and comparative cell biology*

Thin-sectioning of *S. lacustris* tissues revealed the presence of punctate regions of apparent cell membrane fusion. The occlusion of ruthenium red suggested that sponge cells are able to block the paracellular route of molecule transport. Putative junctions identified in Chapter Three must be identified as having either adhesion, occlusion or hybrid functions. This could be achieved by the localization of proteins associated with these roles in other organisms within sponge cells by electron immunogold labelling. Comparison of cell junction genes in sponges and other animals would first identify similar antibody target sequences. If protein epitopes were conserved, commercial antibodies from animal models could be used to reveal protein expression in sponge cells. Differential localization of actin, integrins, discs large, MAGI and tetraspanin throughout the architecture of sponge cells could act as markers of functional identity. Integrins have a role in cell signalling and could be involved in a sponge epithelial-muscular system which controls pinacoderm contraction (Brower et al. 1997). A known tetraspannin protein was shown to be expressed in the pinacoderm of some demosponges (Adell et al.

2004) but the function and cellular localization of this protein has not been determined. The TER of freshwater sponge cells suggests that this or another protein are present in sponge occluding junctions. Discs large is known from septate junctions of Drosophila and its possible function as a membrane spacer and occlusion molecule in sponges requires further verification (Banerjee et al. 2006). Thus the localization of either tetraspanin, discs large or integrins at regions of apparent cell membrane fusions or regions resembling desmosomes could determine if these structures are for cell sealing or adhesion.

## 4.5 Concluding statements

The electrical recordings across sponge tissues suggests that *S. lacustris* possess functional sealed and transporting epithelia. This is the first evidence for resistant epithelia in the Porifera and it may indicate that a sealed epithelium was a prerequisite for the onset of multicellularity.

**Figure 4-1 Simplified metazoan phylogeny:** modified from Srivastava et al 2010. Evidence that sponges have functional epithelia alongside behavioural studies on sponge coordination in response to stimuli (Elliott and Leys, 2007) and future work on sponge development may support the inclusion of the Porifera with other 'true' animals - Eumetazoa.

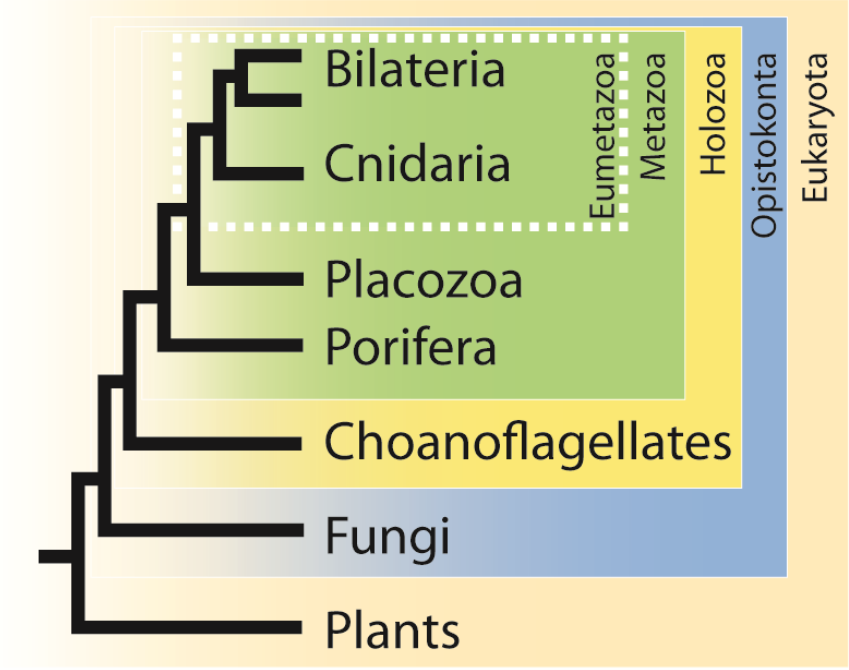
Bilateria
Cnidaria
Placozoa
Porifera
Choanoflagellates
Fungi
Plants
Eumetazoa
Metazoa
Holozoa
Opistokonta
Eukaryota

**Figure 4-1**

## 4.6 References


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