Gene expression and sensory structures in sponges: Explorations of sensory-neural origins in a non-bilaterian context

by

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Abstract

The nervous system is present in all but two animal phyla – one of them being Porifera, sponges. Sponges have no neurons and yet have organized behavior and finely tuned sensation. Furthermore, sponges have genes involved in the nervous system of other animals (informally called 'neural' genes). Do these genes impart a sensory capacity in sponges and does their presence suggest that the sponge sensory system is homologous to the nervous system? There are few manipulative genetic techniques for sponges; instead approaches include looking for the expression of 'neural' genes in sponge structures. I reviewed the literature of sponge gene expression studies and found little correlation between the expression of 'neural' genes and sponge sensory structures. Instead non-sensory cells in sponges expressed just as many, if not more, 'neural' genes. I carried out an RNA-seq study to determine whether candidate 'neural' genes might be differentially upregulated in the osculum, a demonstrated sensory structure that is the excurrent vent of the sponge filtration system. Four candidate 'neural' genes – mGluR, GABAR, K_{ir} and Bsh – were significantly upregulated in sponges with oscula compared to those in which oscula were still developing or in sponge body tissues. While glutamate (L-Glu) and GABA have been shown to trigger and arrest (respectively) sponge contraction behavior, glutamate and GABA receptors themselves may have roles in normal metabolic processes and therefore their upregulation in tissues may reflect differential activity of other activities that occur in the osculum. Taken together, the data presented in this thesis suggest that genes involved in the nervous system of bilaterians are ineffective markers for sensory/coordinating systems in sponges. Instead, studying 'neural' genes without the assumption that they hold sensory or coordinating functions may provide a less biased way of investigating sensory-neural origins.

Preface

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Chapter Three is an RNA-seq experiment conceived by JLM and SPL. SPL collected sponge specimens. JLM and SPL plated and harvested the sponge tissue. JLM performed all RNA extractions. Library preparation was performed by Delta Genomics and sequencing occurred through the University of Alberta Molecular Biology Facility (MBSU). JLM performed the data analysis and wrote the chapter.

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List of Symbols, Nomenclature, and Abbreviations

AMPA GluR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate glutamate receptor
ANTP	antennapedia
AP3	DL-2-amino-3-phosphonopropionic acid
Aqu	*Amphimedon queenslandica*
Ava	*Aphrocallistes vastus*
Bcat	ß-catenin
bHLH	basic helix-loop-helix
BLAST	Basic Local Alignment Search Tool
Bra	brachyury
Bsh	brain-specific homeobox
BUSCO	Benchmarking Universal Single-Copy Orthologs
CAMKII	calcium/calmodulin-dependent protein kinase II
CAP	cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins
CASK	calcium/calmodulin-dependent serine protein kinase
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
ColS1	short-chain collagen
CRIPT	cysteine-rich interactor of PDZ three
CRISPR	clustered regularly interspaced short palindromic repeat
Cry2	cryptochrome 2
DLG	discs-large
Dsh	dishevelled
Dvl	dishevelled
E-value	Expect value
Elav	embryonic lethal abnormal visual system
EphR	ephrin receptor
ErbB	erythroblastic leukemia viral oncogene homolog receptor
Eya	eyes absent
FC	fold change
FDR	false discovery rate
FPKM	fragments per kilobase of transcript per million mapped reads

Fzd	frizzled
GABA	gamma-aminobutyric acid
GABABR	gamma-aminobutyric acid type B receptor
GABAR	gamma-aminobutyric acid receptor
GKAP	guanylate kinase-associated protein
Gli	glioma-associated oncogene
GLIPR1-like	glioma pathogenesis related-1-like
GO	gene ontology
GRIP	glutamate receptor-interacting protein
Gro	groucho
Hand	heart, autonomic nervous system, neural crest derivatives
Hmx	H6-like-homeobox
IP3R	inositol triphosphate receptor
K_{ir}	inward rectifier potassium channel
L-Glu	L-glutamate
Lhx	LIM homeobox
LIM	LIN-11, Isl1, MEC-3
LimK	LIM-kinase
LIN-7	lineage abnormal 7
Lrp5/6	LDL-receptor-related protein 5/6
M-medium	mineral medium
MAGI (S-SCAM)	membrane-associated guanylate kinase with inverted orientation (synaptic scaffolding molecule)
mGluR	metabotropic glutamate receptor
Msi	musashi
Msx	muscle segment homeobox
Myc	myelocytomatosis virus oncogene homolog
MyoRb	myogenic repressor b
NCBI	National Center for Biotechnology Information
NF-Kb	nuclear factor kappa B
NM myhc	non-muscle myosin type II heavy chain
NMDA GluR	N-methyl-D-aspartate glutamate receptor
NO	nitric oxide
NOS	nitric oxide synthase

NSCL	neurological SCL
ORF	open reading frame
Pax	paired box
PC	principal component
PICK1	protein interacting with C-kinase 1
Piwi	P-element-induced wimpy testis-interacting
PKC-a	protein kinase C alpha
PLOD	procollagen lysyl hydroxylase
PMCA	plasma-membrane Ca^{2+} ATPase
PON	pre-oscular node
Ras	rat sarcoma
RIN	RNA integrity number
RNA-seq	RNA sequencing
RNAi	RNA interference
ROPOS	Remotely Operated Platform for Ocean Sciences
RRMcp	RNA recognition motif-containing protein
RSEM	RNA-seq by Expectation Maximization
SCL	stem cell leukemia
SFRP	secreted frizzled related protein
Six	sine oculus homeobox
Sla	*Spongilla lacustris*
Smad	small mothers against decapentaplegic
SNM	sensory-neural marker
Sox	Sry-related HMG box
SPAR	spine-associated RapGAP
ST myhc	striated muscle myosin type II heavy chain
SynGap	synaptic GTPase activating protein
Tcf	T cell transcription factor
TGF-ß	transforming growth factor beta
TIR1	transport inhibitor response 1
TMM	trimmed mean of M-values
TRP	transient receptor potential channel
Wnt	wingless/int-1

Chapter 1. The cryptic biology of sponges

1.1. Extant sponge biology

Thoughts, sensations, and behavior emerge from the nervous system, a distinctly animal innovation. Where did the nervous system come from? Core elements of the nervous system likely arose early in animal evolution. Sponges, one of the earliest diverging group of animals (Figure 1-1), have sensation and behavior, but lack neurons. However, they do possess genes that are homologous to those with key functions in the nervous system of other animals. Studying the sponge sensory and coordinating system may yield deep insight into the origin of the nervous system.

The defining feature of the poriferan body plan is the aquiferous system, a network of canals essential for filter feeding (Simpson 1984). Water enters the sponge through pores called ostia and travels through a series of incurrent canals to chambers lined with choanocytes. Choanocytes, a cell type characterized by microvilli encircling a beating flagellum, drive water through the sponge and capture food particles entrained in the flow. A pressure drop occurs as water is forced through the minute holes of the glycocalyx mesh of the choanocyte microvilli (Leys et al. 2011). Food particles are thought to be baffled by the microvilli and drawn towards the base of the cell, where they are phagocytized (Leys and Eerkes-Medrano 2006). Upon passing through the microvilli, water is shunted through excurrent canals ultimately exiting through a single excurrent chimney called the osculum. Filter feeding is highly efficient. A single square meter of a glass sponge reef can clear the equivalent of 165 m of water above it (Kahn et al. 2015) and water exiting the osculum has been measured to be near-sterile (Reiswig 1971b). In juvenile freshwater demosponges the location of the osculum and configuration of the canals is constantly dynamic (pers. obs.).

Sponges possess cell types that are highly dissimilar to those of other animal phyla. Porocytes form ostia, the incurrent openings on the surface of the sponge. Pinacocytes, plate-like epithelial cells, form the outer layer of the sponge and line the inner surface of canals. Between these two layers of pinacocytes is a collagenous mesohyl layer with a diversity of motile cells. Amongst these are sclerocytes, which secrete the sponge skeleton (spicules), and pluripotent stem cells called archaeocytes. Archaeocytes give rise to choanocytes, pinacocytes, oocytes, sperm and possibly other cell types (Simpson 1984, Boury-Esnault et al. 1999). For instance, subsets express cell-specific markers for sclerocytes suggesting that these archaeocytes

are precursors to sclerocytes (Mohri et al. 2008). Sperm, in contrast, can also arise from transdifferentiation of choanocytes. There is no doubt that sponges harbor cryptic subtypes of cells remaining to be found. For example, Kahn and Leys (2017) observed mesohyl cells towing immature choanocyte chambers through the mesohyl. It is currently unknown whether these are archaeocytes or a new cell type.

As with all animals, sponges shape and are shaped by a rich world of ecological interactions. Sponges are prey to fish and other organisms and have evolved various defense mechanisms such as the production of toxins (Green 1977, Gillor et al. 2000). Alternatively, sponges can be predators. Carnivorous sponges possess mobile digestive cells that travel to envelope prey ensnared upon spicules (Vacelet and Boury-Esnault 1995, Vacelet and Duport 2004). Sponges structure their surrounding environment by providing habitat (Maldonado et al. 2016), participating in benthic-pelagic coupling (Gili and Coma 1998), exerting spatial competition (Jackson and Buss 1975), and removing live bacteria from the water column (Kahn et al. 2015). In return, they must react to fluctuating environmental conditions, including turbidity (Reiswig 1971a), seasonal temperature changes (Maldonado and Young 1996) and sediment plumes (Fabricius et al. 2007). Larvae navigate the environment to find an optimal settling habitat, using cues from light, gravity, or currents to guide them (Warburton 1966, Maldonado and Young 1996). The complex interactions sponges encounter demand efficient and integrated responses.

1.2. Sponges as dynamic animals

1.2.1. Sponge contractile behavior

Sponges are largely sessile but far from inanimate. Aristotle was first to observe that adult sponges can undergo contractions (Aristotle, edited by Gaza, 1498). Contractions involve the whole body including the osculum, ostia, pinacoderm, and choanocyte chambers (Nickel 2004, Elliott and Leys 2007, Meech 2008). One type of contraction, the inflation-contraction behavior, has been further explored in demosponges in a laboratory setting revealing that sponge contractions can consist of a complex, multi-step, stereotyped behavior involving multiple tissue types (Elliott and Leys 2007). In some sponges pinacocytes are thought to be the contractile cells (Nickel et al. 2011) and contractions are likely coordinated by paracrine signaling (Elliott and Leys 2007, Elliott and Leys 2010). One possibility is that control of this behavior centers around the osculum: it may be a sensory and coordinating hub. In two freshwater sponge species, contractions can originate at the osculum (Elliott and Leys 2007) and

removal of the osculum extinguishes contractions (Ludeman et al. 2014). First Elliott (2009) and later Ludeman et al. (2014) described cilia that line the inner surface of the osculum. These are non-motile and were determined to be primary cilia. When the cilia were removed, using chloral hydrate, contractions ceased (Ludeman et al. 2014). Thus, as seen in other animals, primary cilia may hold a sensory function in the osculum. Contractions have been observed to occur when particles, such as ink, are added to the water suggesting that contracting may be a response to prevent clogging, analogous to a sneeze (Elliott and Leys 2007, Leys 2015). Filter-feeding sponges presumably share a need to flush irritants from the aquiferous system, and contractions have been witnessed in all classes of sponge (Nickel 2010). However, contractions can also be triggered by electrical stimulation and have even been correlated to seasonal changes in temperature (McNair 1923, Jones 1957, Pavans de Ceccatty et al. 1960, Reiswig 1971a), so they may serve additional, as yet unknown, functions.

Neuroactive molecules have been implicated in the sponge contractile behavior. Elliott and Leys (2010) found pools of amino acid neurotransmitters in *E. muelleri*. Of these the excitatory neurotransmitter glutamate triggered an inflation-contraction behavior in a dose-dependent manner while the inhibitory neurotransmitter GABA prevented this (Elliott and Leys 2010). Likewise, chemical inhibitors of the glutamate receptor blocked the behavior. Nitric oxide synthase localized to the osculum and pinacoderm and a cGMP assay suggested that a NO-like signaling pathway is active in *E. muelleri*. Many other neuroactive molecules, such as caffeine, glycine, adrenaline, nicotine and serotonin have been observed to modulate the rhythm or profile of contractions, and change pumping activity (Emson 1966, Ellwanger and Nickel 2006, reviewed in Nickel 2010). It is difficult to determine whether the sponges in these studies possess the full inflation-contraction behavior but it is apparent that many sponges possess some degree of reactivity to these chemicals.

Compared to freshwater demosponges glass sponges are not known to contract. Instead, upon sensing mechanical or electrical stimuli glass sponges arrest pumping (Leys and Mackie 1997, Leys et al. 1999, Tompkins-MacDonald and Leys 2008). While contractions in demosponges occur slowly, arrests in glass sponges occur immediately (Leys et al. 1999). Studies in tanks using sediment as a stimulus showed that the profile of an arrest varies with the concentration and duration of sediment exposure (Tompkins-MacDonald and Leys 2008). Furthermore, it was found that *Aphrocallistes vastus* and *Rhabdocalyptus dawsoni* become less sensitive to gradual increases in sediment concentration and that the arrest profile of these two

species, which live in habitats with different sediment loads, varies (Tompkins-MacDonald and Leys 2008). Accordingly, the fine-tuned arrest response may serve a similar function to the sneeze-like contractions seen in other sponges. Inspection by scanning electron microscopy after the short term addition of sediment showed that the feeding collars were not covered in sediment, though bacteria were present (Tompkins-MacDonald and Leys 2008). In the glass sponge *Aphrocallistes vastus* pumping was estimated to account for up to 28% of the total energy expenditure, leading Leys et al. (2011) to hypothesize that the arrest behavior may help balance the energetic equation of living in the deep sea environment. Glass sponge sensitivity walks a fine balance. Though mechanical stimuli trigger feeding current arrests, many benthic animals can be seen sheltering within glass sponge oscula. In addition, sediment may play a role in gluing together glass sponge reefs (Conway et al. 1991, Whitney et al. 2005). Thus, Tompkins-MacDonald and Leys (2008) suggest that the arrest response may represent a tradeoff between clogging and the necessity of living in a sediment-filled environment.

1.2.2. Larval behavior

Another well-documented behavior is larval phototaxis, which has been observed in a range of larva including the calcarean amphiblastula and demosponge parenchymella (Leys and Degnan 2001, Elliott et al. 2004, Maldonado 2006). Phototaxis is thought to guide larvae to swim out of the osculum or to settle in dark habitats or at lower depths on benthic substrates (Maldonado 2006). The parenchymella larvae of *Amphimedon queenslandica* possess a posterior crown of pigmented cells among which strikingly long cilia protrude. Lining the inner perimeter of this crown is a ring of pigmented epithelial cells, while a cap of non-ciliated cells occupy the posterior pole itself. Short beating cilia covering the rest of the larva propels it in lazy spirals through the fluid environment (Leys and Degnan 2001). Increase in light exposure causes the long cilia of the posterior cells to snap straight while decreasing light causes these cilia to fold over the pole. Each pigmented ciliated cell acts independently, and as the larva moves through a gradient of light this ciliary action occurs in a wave across the cells. This higher-level behavior is suggested to be a shadow response (Leys and Degnan, 2001). The pigmented cells opposite to the incoming light shadow the base of each ciliated cell, where the photoreceptor presumably resides. Thus, the ciliated cells opposite to the light fold their cilia, while those directly in the face of the light straighten, guiding the larva like a rudder to turn away from the light (Leys and Degnan 2001). In this way, the posterior ciliated cells act as both sensor and effector.

1.2.3. Hypothesized sensory cells

Other larval cell types have been hypothesized to be sensory. The *Amphimedon queenslandica* larva has globular cells, which are concentrated at the bald patch of the posterior pole, and flask cells, that increase in density towards the anterior third of the larva (Leys and Degnan 2001). Though phototactic, no photoreceptive cell has been confirmed in calcarean amphiblastula larvae (Elliott et al. 2004). However, the amphiblastula larva possesses anterior-posterior hemispheres of distinct cell types that are bisected by four cross cells spaced evenly around the equator. Tuzet (1973) hypothesized that these "cellules en croix" may be photoreceptive, though no functional experiments have confirmed this. Choanocytes have been compared to mechanosensory cells based on gross morphology and it has been suggested that they possess mechanosensory and chemosensory functions that may somehow be involved in capturing food particles (Jacobs et al. 2007).

Thus, although the extant sponge body plan appears alien and minimalist it is the culmination of intimate ecological and environmental interactions. Contrary to being passive animals, sponges have evolved a suite of tailored behaviors that allow them to engage and navigate a fluctuating environment.

1.3. What is biological complexity?

1.3.1. Sponges as 'almost-animals': a review of traditional descriptions

The first scientific description of sponges was presented by Aristotle (Johnston 1842, reviewed in Hooper and Van Soest 2002), who also precociously noted that they possessed behavior. However, later scientists did not follow in his footsteps. Linnaeus, noting the absence of many characters found in other animals, initially classified sponges as "cryptogamous algae" (Linnaeus 1759). These absences also guided Haeckel to place sponges at the very base of his Genealogical Tree of Humanity (Haeckel 1879). Sollas (1885) then relegated sponges to an exclusive clade called the Parazoa – or "beside the animals". Even though his classification has long been abandoned, the absence of complex characters is still used to maintain this sidelined status. Other animals are characterized according to tangible characters- such as the nervous system, or sensory organs. But in non-bilaterian metazoans, 'absence' is a character in and of itself.

Yet 'absence' is relative. In sponges, for instance, it depends on what life stage one observes. Symmetry does not seem to be conserved in many adult sponge body plans, yet sponge

larvae are clearly highly symmetric (Leys and Degnan 2001). Additionally, it has been argued that some adult sponges do in fact have radial, axial or semi-spherical symmetry (Manuel 2009). It also depends on how one defines the character. Can the sponge osculum be considered an organ? Indeed, it is a sensory and coordinating entity whose tissues and cell types work together to sense water flow and initiate an inflation-contraction behavior, though it may not look like a typical sensory organ (Hyman 1940).

1.3.2. Defining the absence of 'higher' characters in sponges

By necessity we must define characters to study them. But how these definitions are drawn also determines their absence in sponges, and whether this is useful in the study of the origin and evolution of characters is debatable. An ultrastructural definition of tissue precludes sponges since they lack clear belt junctions or in some cases, a basal lamina (Leys et al. 2009). While many epithelia are 'leaky' to a certain degree, this has led some to suggest that the sponge pinacoderm is particularly permeable (Tyler 2003). Paleontological papers have followed this misconception, purporting to have found sponge fossils that lack epithelial connections altogether (Yin et al. 2014). At the physiological level, however, the sponge pinacoderm functions as an epithelium: it has a transepithelial potential, undergoes selective uptake of ions and has sealing junctions capable of blocking ion entry (Adams et al. 2010). Indeed, sponges have several types of junctions and in homoscleromorphs the pinacoderm is underlain with a basal lamina, to which type IV collagen localizes (Ledger 1975, Boute et al. 1996).

Oddly, the strict definition of an epithelium does not apply to other organisms. The basement membrane is wholly absent in acoels (Pedersen 1991), but it is still maintained that they have epithelia. Presumably, this is because absence is attributed to loss. But given that sponges are monophyletic, if the homoscleromorph basement membrane is not convergent, then it has been lost in the other sponge lineages. Loss is often not considered when examining sponges. Instead, absence is assumed ancestral. Thus, epithelia in sponges are absent according to strict criteria defined in other clades featuring characters that are not present by default. A more flexible definition, built around poriferan physiology and function, suggests otherwise. Sponges possess orthologues of essential cell polarity, cell junction, and basal lamina genes (Fahey and Degnan 2010, Srivastava et al. 2010b, Riesgo et al. 2014), presenting an avenue to test the heritage of the sponge epithelium.

1.3.3. Sponges lack a bilaterian-like neuron

The hunt for a poriferan neuron followed similar themes to studies of the sponge epithelium. Since the late nineteenth century scientists have searched for a neuron-like cell among the unconventional cell types of sponges (Sollas 1888, Lendenfeld 1889). Pavans de Ceccatty (1955) reported the presence of classical and aberrant neurons, and sensory, neuromuscular, vesiculous and arachnoid cells, all presumably components of a sponge nervous system (Pavans de Ceccatty 1955, 1959). His description of a classical sponge neuron was that of a fusiform or triangular cell with dendritic fibres extending from one side and a single fibre at the other pole – in other words, the archetype of a traditional bilaterian neuron. The search continued with Lentz (1966)'s histochemical experiments. Fibril-displaying cell types around the collar of the osculum stained for various neurotransmitters or enzymes involved in neurotransmitter production. Even so, no synapse has ever been found in sponges. Pavans de Ceccatty (1955) hypothesized that his classical sponge neuron possessed an "intracellular so-called synapse", where neuronal fibres connected inside the cell as a syncytium. The closest resemblance of a synapse is Lethias et al. (1983)'s observation of the exchange of vesicles between cells (as reviewed in Leys 2015). Jones (1962) noted that the characters of putative sponge neurons could easily be preservation artefacts, cells preserved in midst of contractions, the remains of spicules, or non-specific staining. Ultimately, consensus converged on the conclusion that there is no neuron in Porifera, or at least a cell type that matches the classical bilaterian cytology and histochemistry of a neuron (Jones 1962). Perhaps, however, the expectation of finding a neuron in sponges is not useful for the study of the origin of the nervous system.

1.3.4. Summary

Absence does not need to be a defining character of sponges. By default, bounding characters according to a bilaterian standard makes it unlikely that they or their equivalent will be found in sponges – these features arose in later diverging clades. This leads one to question, perhaps, why bilaterian characters are not defined according to non-bilaterian biology. The dominating concept of absence encourages one to think of sponges as a 'first step', from single-celled simplicity to eumetazoan complexity. Much derided, Aristotle's Scala Naturae nevertheless persists. Instead, we must let the animal guide us.

1.4. Sponge complexity in the molecular age

The study of evolution in sponges has entered the molecular age. For now, manipulative genetic techniques are not available for sponges. Thus molecular studies have largely taken a

candidate gene approach. Which genes are selected as candidates reflects existing approaches to sponge biology and ultimately strongly influences our interpretation of eumetazoan character evolution.

The first sponge genome was sequenced from *Amphimedon queenslandica*. Srivastava et al. (2010b) pursued candidate genes underlying the most basic tenets of multicellularity: cell growth and cycling, cell death, cell differentiation, cell-cell and cell-matrix adhesion, gene regulation and developmental signaling, and self-recognition and innate immunity. Notably, it was rare that *A. queenslandica* possessed all members of a bilaterian pathway and proteins often lacked eumetazoan domains (Srivastava et al. 2010b). Srivastava et al. (2010b) commented that pathways increase in complexity as one progresses towards Bilateria – yet if these pathways are characterized by bilaterian pathways, it is not surprising they are 'incomplete'. Furthermore, expansion of gene families is not unheard of in sponges, or indeed in other non-bilaterians (Liebeskind et al. 2015, Francis et al. 2017). Srivastava et al. (2010b) also identified sets of genes proposed to underlie the emergence of gross morphological complexity in animals. Complexity was defined as the number of described cell types in model organisms of each complexity 'grade' (non-bilaterians, invertebrates, vertebrates). Yet the cell biology of non-bilaterians (e.g. *Trichoplax*, see Smith et al. (2014)) is vastly less understood than that of bilaterian model organisms. Furthermore, it is difficult to understand what the biological significance is of the number of different cell types and organismal complexity. However, it is true that the definition of complexity in biology remains difficult to formulate (Koonin 2004). Rather than signaling complexity, perhaps the Srivastava et al. (2010b) genes simply indicate difference from Bilateria.

As molecular studies have progressed, an alternate perspective has arisen – that of the "surprising genetic complexity" of sponges (Riesgo et al. 2014, Borisenko et al. 2016). Sponges possess a well-represented repertoire of signaling, neural, epithelial, adhesion, innate immunity, and reproductive genes (Figure 1-2) (Nichols et al. 2006, Riesgo et al. 2014, Francis et al. 2017). Riesgo et al. (2014) found that 93% of genes in the pathways investigated were present across eight sponge transcriptomes. The fact that sponges possess genes that function in complex bilaterian systems while apparently lacking such features is often remarked upon (Nichols et al. 2006, Degnan et al. 2009, Riesgo et al. 2014). Intuitively, one thinks they should correspond, as they do in many other animals. A similar approach exists with gene expression studies. For instance, the expression of several neural-related genes in the globular cell of the *A.*

queenslandica larva led Richards et al. (2008) to suggest the presence of a proto-neuron, as further discussed in Chapter Two. "Almost-pathways" are used to homologize "almost-characters" to bilaterian features.

1.4.1. Summary

Perhaps it can be argued that molecular data, much like morphological data, is still interpreted through a lens of 'primitiveness'. The candidate gene approach is open to interpretation. When genes that fit our idea of basic-ness are searched for and found it agrees with the archetype of a 'simple animal', even if these genes by definition are present in most animals. In contrast, when bilaterian genes are found we are surprised. Perhaps it's reasonable to expect these genes to directly correlate with 'complex' bilaterian characters and looking for these characters are an obvious next step. Yet we do not even know if these genes interact in a similar fashion, and if not, must approach their study without the assumption that bilaterian function is conserved in an earlier diverging animal. Molecular studies often feature a figure which shows a bilaterian pathway, and elements coloured according to whether they are present in sponges – the image of an unfinished animal (Figure 1-2) (eg. Sakarya et al. 2007, Alie and Manuel 2010). Indeed, the *A. queenslandica* genome has been described as "unicellular-multicellular", in a paper that looked for "bilaterian-like" promoters (Fernandez-Valverde and Degnan 2016). Perhaps one should re-define our approach to sponge genes. Arguably, all genes that can be traced from Bilateria to sponges may perhaps be better thought of as non-bilaterian genes. Understanding 'bilaterian' genes in light of non-bilaterian biology may prove insightful.

1.5. Hidden biology

We have a narrow window of insight into non-bilaterian biology that is limited by the hypotheses we ask and the technology we use to test them. Sponges are "obscure in character and possessed of less interest than attaches every other" (Johnston 1842), and even today this dearth persists. This compelled Dunn et al. (2015) to ask: to what degree are we blind to the non-bilaterian context surrounding the evolution of bilaterian characters (Figure 1-3A)?

The universe of proteins accessible to study has grown in step with technical advances. We can now examine molecular underpinnings of non-bilaterian biology (Babonis et al. 2016); yet sponge studies remain largely limited to bilaterian genes (Fernandez-Valverde and Degnan 2016, Pena et al. 2016). Unannotated genes are quantified in genome and transcriptome studies, but none have been further characterized (Riesgo et al. 2014, Guzman and Conaco 2016). Even species-specific biology can be a source of insight into broader evolutionary principles, as

demonstrated by studies of the evolution of directional symmetry and the emergence of higher levels of biological organization (eg. Dunn 2005). The study of sponge-specific characters may allow us to explore the evolution of filter feeding in a fluid, bacteria-filled environment – the context within which the first metazoan may have arisen.

One group of proteins remains largely absent in the study of nervous system evolution: those that arose at the dawn of Metazoa, but were lost before the emergence of Bilateria. A dual-branching phylogeny tends to lend itself to picturing a stepwise assembly of bilaterian machinery. But perhaps the evolution of a character can be better imaged as a continuous series of nested gains and losses. By examining only bilaterian genes in non-bilaterians, we see only the genes that were gained and subsequently conserved in Bilateria. Thus, we miss genes that were lost – genes that once provided a rich context of interactions to those same bilaterian genes we are interested in. These hidden genes may have helped shape the interactions from which the extant nervous system arises.

It is highly likely that candidate genes experience divergent interactions in non-bilaterians. For instance, sponge genes often possess different domains, as exemplified by Hedgling (Figure 1-3B) (Adamska et al. 2007b). Unique to cnidarians and sponges, Hedgling possesses the Hedge, but not the Hog domain, which together characterize the bilaterian signaling gene Hedgehog (Adamska et al. 2007b). Additionally, important bilaterian molecular pathways are present only in a piecemeal fashion in sponges, implying that either non-bilaterian proteins populate the pathway or that the members that are present act alone in distinctly different ways (Srivastava et al. 2010b, Riesgo et al. 2014). The glass sponge *Aphrocallistes vastus* provides a compelling example. Although it has most members of the canonical Wnt pathway, including the Wnt Inhibitory Factor (WIF), so far all attempts to find Wnt itself has failed (Riesgo et al. 2014).

1.6. Thesis objectives and outline

Broadly, this thesis presents an attempt to escape a bilaterian view by critically evaluating how functional inferences are made from *in situ* hybridization data and by characterizing the sponge osculum from a non-bilaterian view.

Chapter Two is a review of the literature of *in situ* hybridization studies. I ask: Are the current data sufficient to conclude that sponge 'neural' genes are involved in sensory functioning in sponges and thus may indicate homology of sponge sensory systems to the bilaterian nervous

system? Manipulative genetic techniques are not accessible for sponges yet, and so inferences rely on correlating gene expression and sensory structures. I explore whether it is effective to draw correlations from gene function to sponge biology. I conclude that the neural genetic signal is either not present or divergent to the degree that it is not currently recognizable in sponges.

In Chapter Three I present the results of an RNA-seq experiment to study differences in genes expressed during oscular development in the freshwater demosponge *Spongilla lacustris* and in the osculum and body of the glass sponge *Aphrocallistes vastus*. To do so, samples of the pre-oscular sponge (before an osculum has formed) and the juvenile (with osculum) in *S. lacustris* were compared, and samples of the osculum and body in *A. vastus* were contrasted. I searched for significantly upregulated candidate 'neural' genes and performed gene ontology enrichment. Finally, I examined whether any orthologues are shared among the set of upregulated genes in the *A. vastus* osculum and the top region of the calcareous sponge *Sycon ciliatum*.

In Chapter Four I reflect on the challenges, limitations, and future directions suggested by the above chapters.

Figure 1-1| Metazoan phylogeny.

The four non-bilaterian phyla are Porifera, Ctenophora, Placozoa, and Cnidaria. Porifera possesses four classes: Demospongiae, Hexactinellida, Calcarea and Homoscleromorpha. It is currently unknown whether Porifera or Ctenophora is the earliest diverging animal lineage.

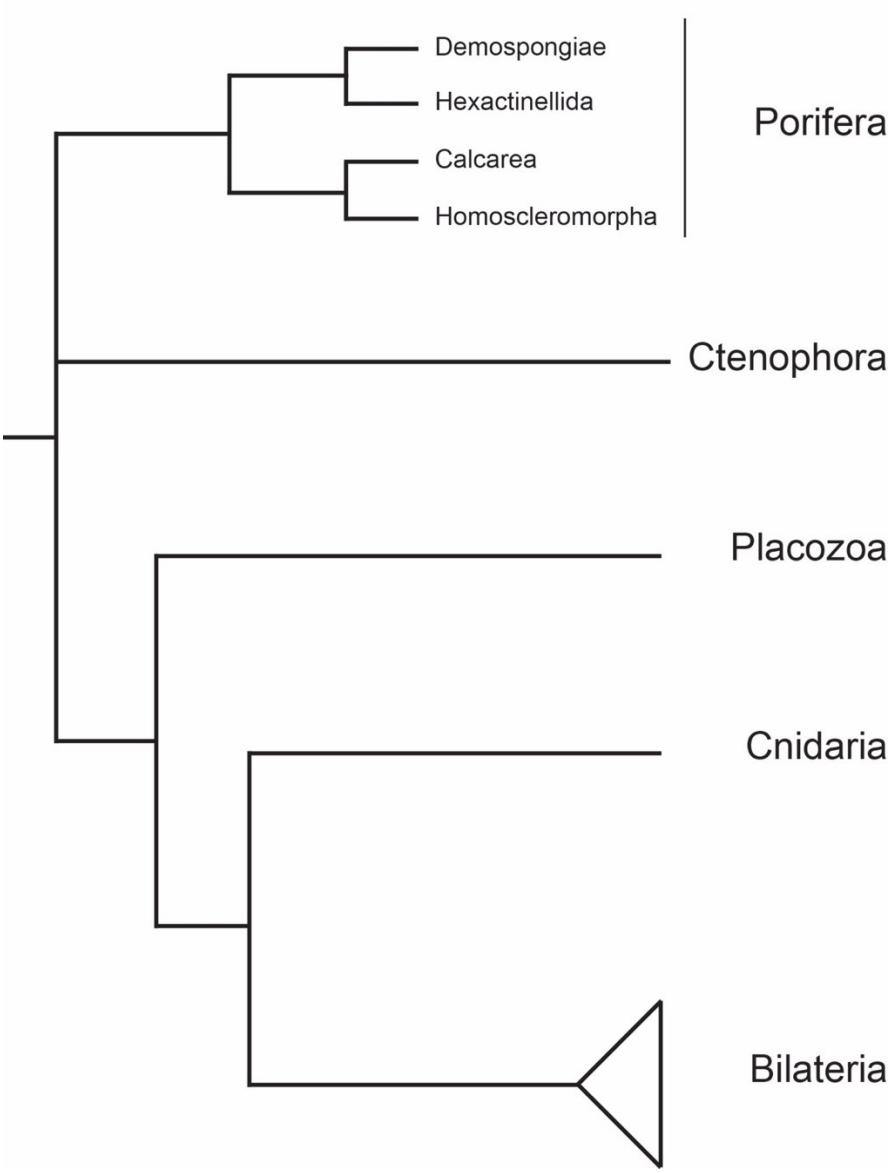

Demospongiae
Hexactinellida
Calcarea
Homoscleromorpha
Porifera
Ctenophora
Placozoa
Cnidaria
Bilateria

Figure 1-2| An example of a portrayal of an 'almost-synapse'.

Vertebrate postsynaptic density proteins are shown according to the interactions they undergo in a bilaterian postsynapse, as adapted from Sakarya et al. (2007). Colours indicate when each protein originated during metazoan evolution.

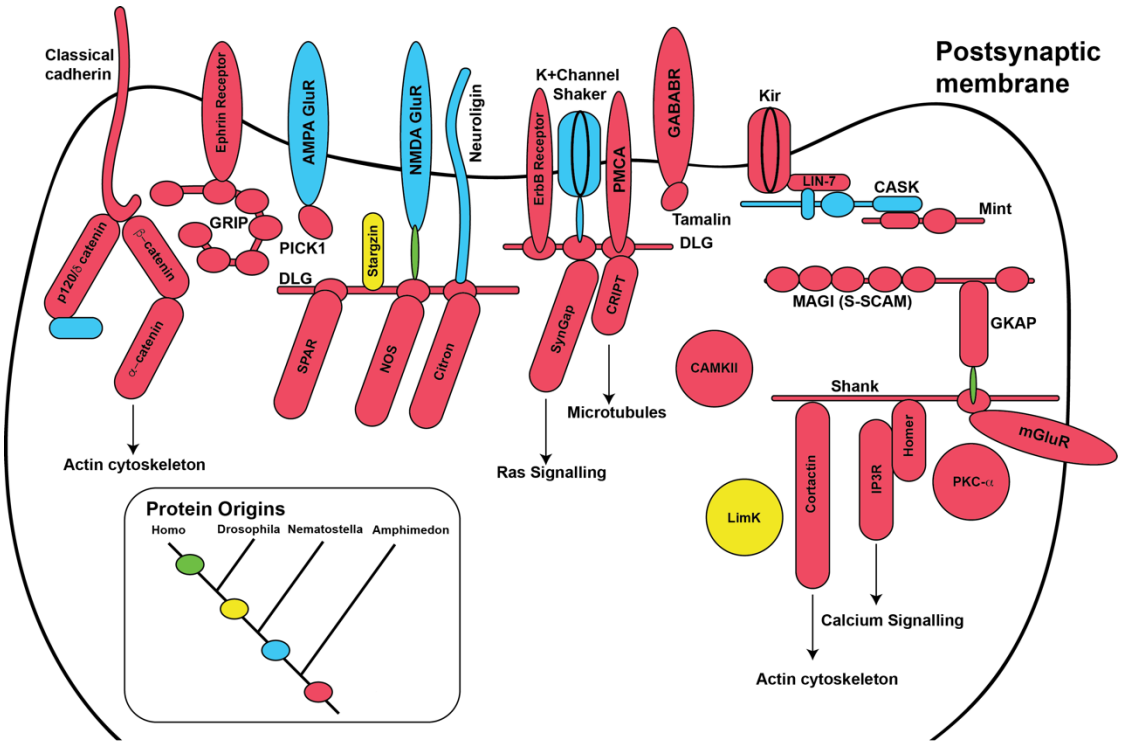
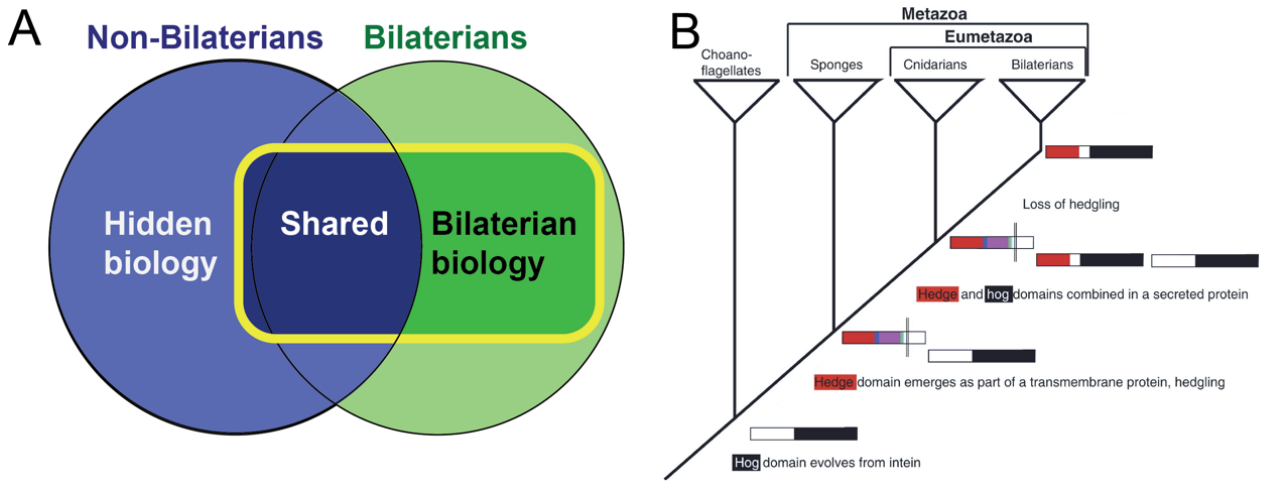

Postsynaptic membrane
Classical cadherin
Ephrin Receptor
AMPA GluR
NMDA GluR
Neuroligin
K+Channel Shaker
ErbB Receptor
PMCA
GABABR
Kir
LIN-7
CASK
Mint
Tamalin
DLG
GRIP
PICK1
Stargzin
p120/δ catenin
β-catenin
α-catenin
SPAR
NOS
Citron
SynGap
CRIPT
MAGI (S-SCAM)
GKAP
CAMKII
Shank
mGluR
Homer
IP3R
PKC-α
LimK
Cortactin
Actin cytoskeleton
Microtubules
Ras Signalling
Calcium Signalling
Actin cytoskeleton
Protein Origins
Homo
Drosophila
Nematostella
Amphimedon

Figure 1-3| Non-bilaterian biology is underexplored.

(A) Our current knowledge (yellow box) largely consists of an understanding of bilaterian biology (green) and that non-bilaterian biology (blue) that is shared with Bilateria. (B) Sponges possess non-bilaterian-specific proteins that have been lost in Bilateria. Rectangles represent proteins with the Hog domain (black) and/or the Hedge domain (red). The domains of Hedgling, which include the Hedge domain, is indicated in red, blue and purple. The Hedgehog protein (red and black rectangle) arose from the combination of the Hedge and Hog domains. (A, adapted from Dunn et al. (2015), B, modified from Adamska et al. (2007b) and reprinted with permission from Cell Press).


A
Non-Bilaterians
Bilaterians
Hidden biology
Shared
Bilaterian biology
B
Metazoa
Eumetazoa
Choano-flagellates
Sponges
Cnidarians
Bilaterians
Loss of hedgling
Hedge and hog domains combined in a secreted protein
Hedge domain emerges as part of a transmembrane protein, hedgling
Hog domain evolves from intein

Chapter 2. Think like a sponge: the genetic signal of sensory cells in sponges[1]

2.1. Abstract

A complex genetic repertoire underlies the apparently simple body plan of sponges. Among the genes present in poriferans are those fundamental to the sensory and nervous systems of other animals. Sponges are dynamic and sensitive animals and it is intuitive to link these genes to behaviour. The proposal that ctenophores are the earliest diverging metazoan has led to the question of whether sponges possess a 'pre-nervous' system or have undergone nervous system loss. Both lines of thought generally assume that the last common ancestor of sponges and eumetazoans possessed the genetic modules that underlie sensory abilities. By corollary extant sponges may possess a sensory cell homologous to one present in the last common ancestor, a hypothesis that has been studied by gene expression. I have performed a meta-analysis of all gene expression studies published to date to explore whether gene expression is indicative of a feature's sensory function. In sponges I find that eumetazoan sensory-neural markers are not particularly expressed in structures with known sensory functions. Instead it is common for these genes to be expressed in cells with no known or uncharacterized sensory function. Indeed, many sensory-neural markers so far studied are expressed during development, perhaps because many are transcription factors. This suggests that the genetic signal of a sponge sensory cell is dissimilar enough to be unrecognizable when compared to a bilaterian sensory or neural cell. It is possible that sensory-neural markers have as yet unknown functions in sponge cells, such as assembling an immunological synapse in the larval globular cell. Furthermore, the expression of sensory-neural markers in non-sensory cells, such as adult and larval epithelial cells, suggest that these cells may have uncharacterized sensory functions. While this does not rule out the co-option of ancestral sensory modules in later evolving groups, a distinct genetic foundation may underlie the sponge sensory system.

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2.2. Introduction

Sponges lack muscles, a gut and a nervous system, and consequently have emerged as a model to study complex character evolution in Metazoa. But sequencing has revealed substantial genetic complexity despite an apparently simple body plan (Srivastava et al. 2010b, Riesgo et al. 2014). The presence of so-called 'neural' genes and evident sensory behaviour in sponges intuitively suggests that the latter emerges from the former. The proposal that ctenophores are basal to all other metazoans (Dunn et al. 2008, Ryan et al. 2013, Moroz et al. 2014), has led to discussion about whether these genes are characters of a 'pre-nervous' system or the remnants of a lost nervous system (Richards et al. 2008, Nickel 2010, Ryan and Chiodin 2015). However it is not yet possible to distinguish between these two hypotheses; our paper examines the data from the perspective that nervous system loss has not occurred. Additionally, there is no evidence for the presence of an extant poriferan neuron and thus we do not search for one. Instead, we ask if the available data are sufficient to test whether the last common ancestor of sponges and eumetazoans possessed the genetic modules underlying the nervous system's sensory and coordinating phenotype. By inference this suggests that these genetic modules led to a specialized sensory cell type in the last common ancestor. Alternatively, if these modules arose after the divergence of sponges the co-opted genes may have held non-sensory functions in the last common ancestor. Thus it is problematic to refer to these genes as 'neural', especially given the fact that gene function may diverge in an extant animal. Instead, we use the term 'sensory-neural markers' (SNM) to indicate genes involved in the sensory and neural systems of neuralians.

Sponges, a quintessential non-model organism, have so far resisted the development of direct tests of gene function. The vast majority of data on SNMs has arisen from gene expression studies which have localized these genes to specific structures and cell types in sponges. With few exceptions there is very little access to higher-level genetic data, such as gene network interactions (eg. Arendt et al. 2016) and more nuanced approaches to homology are currently not possible. Instead, the presence/absence data of *in situ* hybridization is often interpreted as a kind of genetic signal, in which the collection of genes expressed in a structure is inferred to suggest heritage to another cell type. In this way, the genetic signal approximates a molecular fingerprint. However these are two distinct concepts with subtle differences. Arendt (2008) defines a molecular fingerprint as the unique set of both the transcription factors and the effector genes they govern that give rise to a specific cell type. While effectors are indicative of a cell's phenotype, regulatory genes may be either used in neural or non-neural functions.

In this framework investigating the possibility of a neural heritage within sponges faces several distinct difficulties. First, the majority of genes expressed in a neuron are not neural-specific (Bucher and Anderson 2015). Second, it is not readily apparent what to compare this genetic signal to. The genetic repertoire of neurons is vastly diverse – no gene is universally and uniquely expressed in all neurons (Bucher and Anderson 2015) – though non-exclusive "pan-neuronal" genes have been studied (Stefanakis et al. 2015, Arendt et al. 2016). Indeed, the definition of a neuron as drawn at the genetic, morphological, or functional level is not wholly agreed upon (Bucher and Anderson 2015). Thus, the question arises as to what degree SNMs allow us to trace neural heritage and infer a sensory function of a sponge sensory structure.

Current research aims to infer homology of a sponge structure to the nervous system by attempting to determine whether SNMs are expressed in that structure and hold a sensory function. The idea that the sensory and nervous systems are intimately related stems from Mackie (1970) who theorized that a cell type specialized for signaling may have emerged through separation of functions in an ancestral neuro-sensory precursor cell. A step-wise evolution of the complete sensory cell system was envisioned, starting with a single sensory cell acquiring an effector property, and then separation of sensor and effector with the addition of a nerve cell (Mackie 1970). The genes underlying the sponge sensory system may be distinct from those of the nervous system.

Animal sensory cells are generally associated with nerves. The sensory cell functions as a 'receptor' and transmits information to an 'effector' via the neuron. However, without nerves, what does a sponge sensory cell do? Examples in which a sensory cell directly activates separate effector cells include kidney epithelia, in which primary cilia detect changes in flow and provide feedback to other cells, which change their secretion/absorption of solutes (reviewed in Berbari et al. 2009). Cnidarian nematocytes show a graded receptor and effector interaction. Some cells can be triggered to fire the nematocyst capsule by activation directly via a sensory neuron, or as independent effectors via cilia on the nematocyte itself that receive a mechanical or chemical trigger (Holstein 2012). At least one example of a sensor-effector cell in sponges is seen in the ciliated pigmented cells of the *Amphimedon queenslandica* larval photoreceptive organ, which acts to both detect light and steer the larva in response (Leys and Degnan 2001).

In light of these considerations, context is paramount when interpreting a genetic signal. Alone, a genetic signal presents a limited hypothesis. It does not consider the sponge's unique biology, which has been shaped over evolutionary time by highly specific selective forces. Yet morphological, functional and genetic pieces of the puzzle are often missing in the study of the sponge sensory system. Here I aim to provide some context by providing a broad, objective view of gene expression in both sensory and non-sensory features, and providing an analysis of how this genetic data may be informative about the above hypotheses.

2.3. Sensory cells in sponges

Several types of evidence are used to identify sensory regions and cells in sponges, including phototaxis or geotaxis of larvae, contractions of the osculum or ostia of whole sponges, and more recently, expression of marker genes. Original workers saw the osculum retract in response to mechanical or chemical stimuli (Parker 1910, McNair 1923, Prosser et al. 1962, reviewed in Leys 2015). Pore cells are also responsive (Elliott and Leys 2007), as are cells or sphincters around canals that vent into the atrial cavity, although these are often hidden within the osculum and so difficult to see (Reiswig 1971a). All these cells may share a developmental origin as 'sieve cells', 'pore cells' and contractile sphincters, because at least in one freshwater sponge, a small (20 μm diameter) ostium was found to develop into the osculum (Weissenfels 1980). In general sponges contract (see examples in Nickel 2010) and it is usually considered that canal epithelia are responsible (Nickel et al. 2011) but the best-known examples of sponge sensation come from studies of larval behaviour.

Phototaxis in parenchymella larvae results from rapid bending or straightening of long cilia in cells at the posterior pole of the larva (Leys and Degnan 2001, Leys et al. 2002, Maldonado et al. 2003, Collin et al. 2010). These cells are elongate, with a basal nucleus, and they lie just adjacent to cells containing many inclusions with pigment granules, as well as cells with large globular inclusions at the posterior pole (Figure 2-1A,C). Calcareous amphiblastula larvae are also phototactic (Elliott et al. 2004) and although cross cells were long ago suggested to be responsible (Tuzet 1973), the mechanism by which they would cause changes in ciliary beat is not understood. Cross cells and the ciliary cells of the *Sycon* larva are fairly transparent, but ultrastructure shows unusual striated inclusions in the cross cells, and the ciliated cells contain dense inclusions (Figure 2-1B,D).

These are the main receptors for which functional evidence has been demonstrated, but other sensory cells/regions are inferred from gene expression patterns. One hypothesis holds

that choanocytes, in light of their flagellum (Jacobs et al. 2007) and the expression of NK genes (Renard et al. 2009), might be sensory. Another is that globular/mucous cells and flask-shaped cells (Leys and Degnan 2001) of the ciliated epithelia in the *Amphimedon* larva might be sensory due to varied gene expression (Richards et al. 2008). Functional data for these hypotheses are needed and will resolve whether certain genes act as a genetic signal for function.

2.4. A genetic context for sensory cells in sponges

SNM expression in specific cell types or structures has provoked hypotheses that sensory mechanisms in sponges are homologous to eumetazoan sensory systems and therefore a shared proto-neuron existed (Richards et al. 2008). The expanding array of *in situ* hybridization data allows us to frame this hypothesis within a more complete genetic context.

Until a direct test of gene function is available function is inferred from gene expression pattern. Inferences predominantly stem from two types of information: (a) the function of the gene as characterized in other animals, and (b) the function of the feature in which the gene is expressed. The vast majority of genes have been solely or most thoroughly characterized in Bilateria. Thus, underlying this premise is the assumption that function is conserved in clades that arose after the divergence of Porifera. Inversely the second approach extends conclusions from feature to gene. This is a top-down method which suggests that sensory function, a highly emergent character, can be used to extend inferences down to the genetic level. These two approaches provide different views. The first focuses a bilaterian 'lens', coined by Dunn et al. (2015), on gene function, while the second provides a more immediate poriferan-centred approach.

There are caveats to both approaches. Without additional context it is precarious to use either method of inference alone, a fact that becomes especially evident when their conclusions contradict each other. For instance when a gene has been characterized as non-sensory or non-neural in other animals it is rarely suggested to be sensory in sponges, even if it is expressed in a sponge sensory feature (Larroux et al. 2006, Gauthier and Degnan 2008). Similarly when SNM expression occurs in a non-sensory feature that gene is generally not proposed to be sensory (Okamoto et al. 2012, Fortunato et al. 2014b, Nakayama et al. 2015). Yet until the function of that gene is directly tested both hypotheses remain viable. Furthermore, the presence of a greater number of SNMs expressed within the same structure is interpreted as stronger evidence for a sensory function, especially given the fact that the *in situ* hybridization technique involves

whole sample exposure. But *in situ* hybridization studies also face the challenge of being limited by the number of genes that can be examined. Sampling bias and a smaller sample size can distort the catalogue of gene expression observed for a feature. Thus a comprehensive view of both SNM and putatively non-sensory genes is required.

2.4.1. Gene expression studies: A survey

An encompassing review of gene expression data may give a broader, objective platform from which to base inferences. My co-author and I have performed a meta-analysis of all the currently available *in situ* hybridization data (Figure 2-2, Figure 2-3; Supplemental Figure S2-1). SNMs were defined as those possessing a conserved role in sensory or neural systems across Bilateria. These include developmental regulatory as well as effector genes, those genes responsible for sensory functions themselves. Features in which gene expression has been predominantly reported are included for the three sponges (*Amphimedon queenslandica*, *Sycon ciliatum*, *Ephydatia fluviatilis/muelleri*) in which sensory structures or cell types have been hypothesized or investigated (Supplemental Figure S2-2, S2-3). *A. queenslandica* and *S. ciliatum* have gained prominence in gene expression studies due to the availability of a genome, while *E. fluviatilis* and *E. muelleri* have benefitted from their ability to be grown and maintained on the lab bench. For our meta-analysis, we used gene expressions that were clearly corroborated by images shown in published work. In some instances we were unable to confirm that gene expression occurred in a particular cell type because images showed regional expression. However, these patterns were still included (Supplemental Figure S2-3).

Non-model organisms offer little from which to infer gene function. However, there is a rich database of functional gene data as characterized in other animals, and the dominant approach has been to use this data to test functional hypotheses in non-model organisms. Indeed, as demonstrated in cnidarian and ctenophore neurodevelopment, the genes of non-bilaterian metazoans do display functional conservation (Marlow et al. 2009, Simmons et al. 2012). Being aware of the fact that *in situ* hybridization data can be open to interpretation and methodological difficulties, analysis of gene expression data nevertheless reveals distinct insights into sponge biology.

SNMs are indeed expressed in sponge sensory structures, supporting the hypothesis that they play a sensory role in sponges. By corollary this also suggests homology to the sensory structures of other animals, though the story is complex. One example is the expression of SNMs in the *A. queenslandica* photoreceptive organ (Figure 2-2). The presence of a 440 nm peak on

the action spectrum prompted Leys et al. (2002) to first hypothesize that a flavin or carotenoid may be the photo-pigment underlying the photoreceptive organ. No peak was seen at 500 nm, the characteristic absorption peak of opsin (Leys et al. 2002), and searches of the *A. queenslandica* and *Oscarella carmela* genomes have not yet found this protein (Plachetzki et al. 2007, Feuda et al. 2012). Instead, Rivera et al. (2012) found that the photoreceptive organ of the *Amphimedon* larva expresses a cryptochrome, rather than an opsin. Opsin expression unites morphologically distinct photoreceptors across non-poriferan animals (Arendt 2008), but opsins have yet to be found in sponge genomes. Thus, while colloquially called an 'eye' the photoreceptive organ in the sponge larva is most likely a convergent structure. However, patterning of the underlying sensory domain may yet be homologous to other sensory regions, as evidenced by the expression of NK5/6/7B, Lhx3/4 and Lhx1/5 (Figure 2-2).

The osculum expresses comparatively fewer SNMs than the photoreceptive organ (Figure 2-2), but as oscular expression data is available for only two species of sponges (*Sycon ciliatum* and *Halisarca dujardini*) low sample size may play a factor (Fortunato et al. 2012, Fortunato et al. 2014a, Leininger et al. 2014, Borisenko et al. 2016). SNMs in *H. dujardini* have not yet been explored but in *S. ciliatum* H6-like-homeobox (Hmx) and muscle segment homeobox (Msx) show oscular expression patterns (Fortunato et al. 2014a). Hmx is involved in CNS development in bilaterians, but little non-bilaterian data is currently available (Wang and Lufkin 2005). Among its many roles, Msx is involved in muscle development in both bilaterians (Lord et al. 1995, Houzelstein et al. 1999) and non-bilaterians (Galle et al. 2005), in addition to neural functions (Wang et al. 1996, Miljkovic-Licina et al. 2004, Ramos and Robert 2005). The difficulty of drawing conclusions from genes with multiple functions is not limited to Msx. Oscular expression patterns may yet occur in other sponges, such as *Ephydatia*, but difficulties in viewing the osculum obscure this.

But the correlation between SNMs and sensory structures is not exclusive; non-sensory cells also express SNMs (Figure 2-2). Choanocytes, pinacocytes, archaeocytes, and skeletogenic elements do not appear to have a sensory function, yet all express SNMs. But if we are to maintain the view that SNM gene function is conserved in sponges, new hypotheses emerge: putatively non-sensory cell types may hold cryptic sensory functions. In *Ephydatia muelleri* PaxB may regulate Six1/2 (Rivera et al. 2013) and both are expressed in the pinacocytes lining the canals. Canal growth is a dynamic process, likely in response to flow. One possibility may be that these cells provide sensory feedback contributing to canal maintenance (Rivera et al. 2013).

The presence of SNM expression in functionally uncharacterized cell types has also prompted novel hypotheses. The *A. queenslandica* larva possesses globular/mucous cells and flask cells and the larva of *S. ciliatum* possesses cross cells (Duboscq and Tuzet 1938, 1941, Leys and Degnan 2001, Richards and Degnan 2012). Both larvae are phototactic (Leys and Degnan 2001, Elliott et al. 2004), and the *A. queenslandica* larva has also been shown to settle in response to various cues (Jackson et al. 2002). In the face of these behaviors the expression of SNMs in these specialized cell types has led to suggestions that they are sensory (Richards et al. 2008, Fortunato et al. 2014b, Nakanishi et al. 2015b, Ueda et al. 2016). However, no direct functional tests have been performed on these cell types.

The hypotheses that globular cells and flask cells are sensory are based on different classes of evidence. The expression of an atonal-like gene (Richards et al. 2008), several postsynaptic density genes (Sakarya et al. 2007), and NOS (Ueda et al. 2016) in globular cells suggest a sensory function, although the morphology of globular cells is not similar to that of other animal sensory cells. They lack a cilium, and though the apex of globular cells extends beyond the surface of the larva the cell also possesses large inclusions indicative of mucous. Thus follows the first functional interpretation of this cell, which is that it serves to secrete mucous externally (Leys and Degnan 2001). In contrast, flask cells do possess a recessed cilium suggestive of a possible sensory functionality (Leys and Degnan 2001) yet they do not appear to express any of the SNMs censused to date (Figure 2-2). However, given the diversity and proximity of cell types in the sponge epithelium one cannot rule out the possibility that some of these expression patterns may have been associated with the wrong cell type. Thus in these two examples the traditional measure of morphology and the untested evidence of molecular data are in dispute. It is relevant to note that although globular cells, flask cells, and the photoreceptive organ are all hypothesized to be sensory, no SNM expression is shared amongst them (Figure 2-2).

If functional hypotheses are to be built upon gene expression, the entire repertoire of expressed genes must be considered. Globular cells express the innate immunity genes TIR1, pellino, and NF-kb, while cross cells also express the germline genes vasa and nanos (Figure 2-3). The sensory potential of these cells is most often emphasized, yet these expression patterns suggest a multi-modal functionality or even the possibility that a sensory function is secondary. For example, perhaps expression of both synaptic and innate immunity genes in globular cells

and the apposition of ciliated flask cells hints at the ancestry of the immunological synapse (Angus and Griffiths 2013, Le Borgne and Shaw 2013). It is much easier to infer gene function if expression occurs in a cell type that is recognizably comparable to that of other animals. But sponge cells allow few parallels and the relation of these specialized, but functionally uncharacterized, cell types remains elusive.

2.4.2. Challenges of tracing homology within Porifera

Both the pre-nervous system and nervous system loss hypotheses predict that a sensorineural-like cell was present at the stem of Porifera, implying that a homologous representative could be present in extant sponges. Thus, cell type comparisons between sponge species are invaluable. However, sponges offer several challenges. Poriferan lineages are characterized by long branches allowing ample time for divergence (Philippe et al. 2009). This evolutionary distance is embodied by the presence of unique, class-specific cell types including the globular, flask, and cross cells. All three are hypothesized to possess neural links (Richards et al. 2008, Fortunato et al. 2014b, Fortunato et al. 2015, Nakanishi et al. 2015b) but none can be compared to the other. Attempts at cross-species comparisons have been made but the criteria used is broad by necessity. For instance, morphological similarities have been drawn between various larval cells and globular cells: they are bottle or oval-shaped larval cells with or without cilia possessing numerous small vesicles (Renard et al. 2009). Even differentiating between cell types within the same species can be challenging. For example, there was early confusion in the identification of globular vs. flask cells (Sakarya et al. 2007, Richards et al. 2008, Renard et al. 2009). Furthermore, undescribed cell types may exist. Globular cells are often treated as a single cell type (Richards et al. 2008, Gauthier et al. 2010) but Sakarya et al. (2007) noted that subsets of globular cells expressed different complements of postsynaptic density genes . In fact a closer look at the ultrastructure shows that there are a number of different cell types in the larval epithelium that have not been described (Figure 2-1), so perhaps selective gene expression is not to be unexpected. Thus, a deeper understanding of basic sponge cytology is required if new hypotheses on cell function are to be made.

In situ hybridization studies follow the candidate gene approach, and what we learn is strongly dependent on what is tested. Most genes selected for study, including many SNMs, are transcription factors. Perhaps unsurprisingly they are expressed extensively during development. As more *in situ* hybridization studies have been published an increasing number of gene expressions have been replicated across species. However of the SNMs studied so far only a handful have been investigated in more than one species (Figure 2-2). Interestingly, of all

genes examined so far, only one gene - Gata - is expressed in the same structure or cell type in different species (Figure 2-2). This may be due to the limited number of structures available for study in each species. The most well-studied life stage in *Amphimedon queenslandica* is the larva while the juvenile sponge is the most common subject of study in *Ephydatia*. In contrast, studies featuring *Sycon ciliatum* examine both the juvenile and larval stages as the adult is small and tends to contain all the developmental stages. Work is done on whole small sponges, or portions of these sponges, containing embryos and larvae. Finally, sponge genes themselves offer additional challenges. A common scenario is for sponge genes to be most closely related to other sponge genes, clustering amongst themselves rather than within better characterized bilaterian clades (Tompkins-MacDonald et al. 2009, Fortunato et al. 2014b).

2.5. Higher-level approaches

Several SNM studies have introduced higher-level multi-dimensional data beyond that of expression patterns. Most often protein function, as deduced from domain structure, is not tested despite the fact that protein interactions in sponges may very well not match that of bilaterians. Richards et al. (2008) demonstrated through heterologous expression that an atonal-related bHLH from *Amphimedon queenslandica* has proneural properties in *Xenopus laevis* and *Drosophila*. Similarly the *A. queenslandica* cryptochromes, which lie in a clade sister to both photolyase and cryptochromes, were tested for bona fide photoreceptive abilities through in vitro assays (Rivera et al. 2012). Other studies have examined potential gene interactions. Rivera et al. (2013) found that PaxB may regulate Six1/2 in *E. muelleri*, and indeed these two genes localize to the same cell type. In contrast, Conaco et al. (2012a) found that many postsynaptic density genes are not co-expressed and thus may not assemble into a unified scaffold. Globular cells however, do express five postsynaptic density genes (Sakarya et al. 2007) and Conaco et al. (2012a) note that small modules of interactions may persist. Evidence for gene interactions may also derive from the temporal and spatial information *in situ* hybridizations provide. Richards et al. (2008) hypothesized that the order of expression of notch, delta, and bHLH in globular cells and putative globular cell precursors suggests the presence of a genetic circuit.

Ultimately, the underlying goal is to link SNMs to organismal behavior and sensation. Ludeman et al. (2014) found that fluorescent molecules that function as calcium channel blockers label both primary cilia in the osculum and inhibit the inflation-contraction behaviour, leading to the hypothesis that TRP channels may localize to the cilia and function in detection of water flow. Ueda et al. (2016) demonstrated that nitric oxide triggers larval metamorphosis in *A.*

queenslandica, suggesting a link between nitric oxide synthase in the globular cells and detection of nitric oxide. Studies characterizing SNMs at a higher-level represent invaluable progress, but still face challenges until direct tests of gene function are available. Those that investigate protein function or gene network interactions struggle to link to higher-level organismal behaviours and studies that do examine organismal behaviour must draw links to genes by association.

2.6. Emerging molecular approaches and future directions

Despite challenges, bottom-up molecular approaches may provide a path forward. RNA-seq offers unbiased access to the entire genetic complement of a sensory feature, providing greater genetic context from which to base homology inferences. In particular single cell RNA-seq may allow targeted access of sponge sensory cells, which are often sparsely distributed within tissues. Importantly, these sequencing techniques allow access to uncharacterized proteins. Testing uncharacterized orthologues shared among non-bilaterian metazoan sensory structures may be key to understanding sensory function origins. In this vein, interactome studies may be invaluable. Given that many genes fundamental to neurons are not neural-specific understanding the emergence of the molecular interactions underlying the neural phenotype will be insightful. Interactions conserved in basal metazoans, but since lost in Bilateria, may provide the molecular context within which neurons evolved. RNAi has so far been tested in *Ephydatia muelleri* and *Tethya wilhelma* (Rivera et al. 2011, Rivera et al. 2013) and further development of this technique in other sponge species will prove promising. CRISPR is not yet accessible due to lack of access to early embryos and difficulties in delivering the molecules into cells, but techniques are continually being refined. Finally, while molecular techniques are powerful this data cannot be interpreted without an understanding of an organism's basic biology. Morphological, functional, and physiological characterizations provide a foundation for discovery. Molecular data often supersedes morphological data, but larvae clearly have many undescribed cell types (Figure 2-1). Studies that closely describe ultrastructure and cell interactions coupled with gene expression will lead to a better understanding of function (Richards et al. 2008, Nakayama et al. 2015, Kahn and Leys 2016).

The genetic resources available for sponges have advanced in step with the increasing efficiency and affordability of molecular technology. As of the writing of this thesis four genomes (*Amphimedon queenslandica* (Srivastava et al. 2010b), *Sycon ciliatum* (Fortunato et al. 2014a, Leininger et al. 2014), *Tethya wilhelma* (Francis et al. 2017), and *Oscarella carmela* (Nichols et al. 2012)) are available. Other genomes are in progress. In addition, assembled transcriptomes

have been published or made available for at least 24 species of sponges (Supplemental Table S2-2). These include the transcriptomes of closely related species, which may offer insight into how divergence affects genetic interpretations (eg. *Sycon ciliatum* vs *Sycon coactum* (Leininger et al. 2014, Riesgo et al. 2014), *Haliclona tubifera* vs *Haliclona amboinensis* (Guzman and Conaco 2016) and *Ephydatia fluviatilis* vs *Ephydatia muelleri* (Alie et al. 2015, Pena et al. 2016)).

2.7. Think like a sponge

As noted by Dunn et al. (2015) most organismal knowledge has been gathered in Bilateria, but non-bilaterian biology extends beyond this perimeter. Functional data from other animals affords a limited window of insight into sponge genes. Another source of information, the function of the sponge feature gene expression occurs in, presents alternate hypotheses. Might SNMs contribute to non-sensory functions in sponges? As noted previously, SNM expression occurs in non-sensory cells. Musashi, which is involved in neural stem cell maintenance in Bilateria (Richter et al. 1990, Nakamura et al. 1994), is expressed in the archaeocytes of *Ephydatia fluviatilis* (Okamoto et al. 2012) (Figure 2-2). Archaeocytes act as stem cells in sponges presenting the possibility that musashi may be involved in the broader function of general stem cell maintenance (Okamoto et al. 2012). Another striking example is that SoxB1 acts a marker for spicule transporting cells in *E. fluviatilis* (Nakayama et al. 2015) (Figure 2-2). So far no stem cell function has been uncovered for these spicule transporters and SoxB1 is not expressed in archaeocytes (Nakayama et al. 2015), leaving the function of SoxB1 uncertain. Of course, sponges are not the only non-bilaterian that displays this phenomenon. Pang and Martindale (2008), for example, found it surprising that brain-specific homeobox is expressed in the tentacle apparatus rather than the sensory apical organ of the *Mnemiopsis leidyi* larva.

A sister hypothesis is that predominantly non-neural genes hold sensory functions in sponge sensory structures. For instance, Wnt has widespread developmental roles (McMahon and Moon 1989) and is expressed in two polarized sponge structures: the larval photoreceptive organ of *A. queenslandica* and the osculum (Figure 2-3). However, Wnt is also involved in neurodevelopment in Bilateria (Thomas and Capecchi 1990). Thus Wnt may also hold sensory patterning roles in these sponge structures, though it may not be possible nor meaningful to delineate between these two possibilities.

Thus a conundrum is presented when gene function, as characterized in other animals, conflicts with the function of the structure it is expressed in. But should we expect gene function to be conserved in sponges if these functions were characterized in later diverging clades? Instead, alternate hypotheses directly drawn from poriferan characters may offer insight that is not constrained within a bilaterian framework.

2.8. Conclusion

The study of nervous system evolution seeks to understand at what node the genetic modules underlying sensorineural functions originated. Most published work suggests that SNMs are associated with sensory abilities in the last common ancestor to sponges and eumetazoans. But our analysis suggests that the correlation is weak. While sponge sensory structures do express some SNMs, many putatively non-sensory cell types do too (Figure 2-2). When forming hypotheses about the function of uncharacterized cell types based on gene expression we must be exceedingly cautious. Indeed, conceiving hypotheses in general is a difficult task as insight is narrowed by a lack of broader genetic context. The fact that some SNMs are expressed alongside genes suggestive of alternate functions raises the question of whether other hypotheses exist beyond the small window of candidate genes selected for study. Furthermore, drawing correlations between gene expression in sponges and gene function as characterized in other animals may be misleading when working with a non-bilaterian non-model organism. This top-down approach lends a bilaterian 'lens' (Dunn et al. 2015) when interpreting genetic data in sponges when equally viable hypotheses emerge from a more poriferan-centred approach. Currently, we lack sufficient data to conclude that the sponge sensory and eumetazoan nervous systems are homologous. Our analysis suggests that the null hypothesis, that SNMs may hold non-sensory functions, is equally possible, if not more likely. But this is a nascent and exciting field, and further advances may yet transform the enticing insights genetic data has delivered so far.

Acknowledgements

We thank Jim Kaufman (University of Cambridge) for his insights on the immunological synapse. Funding from NSERC Discovery grants to SPL is gratefully acknowledged.

2.9. Definitions box

Pinacocytes: Plate-like cells that form the sponge epithelium. Pinacocytes form the outer surface of the sponge as well as line the canals of the canal system

Choanocytes: A specialized feeding cell. Choanocytes are arrayed in chambers connected to the sponge canal system. Choanocytes possess an apical crown of microvilli encircling a beating flagellum, which drives water through the canals. Food particles entrained in the current are filtered out and subsequently phagocytized by the sponge.

Archaeocytes: Motile stem cells found within the middle layer of the sponge. Archaeocytes give rise to several sponge cell types, including choanocytes and pinacocytes.

Spicules: Spicules are structural elements which act as a lattice to support sponge tissues. They are made of silica or calcite.

Skeletogenic elements: All cell types involved in the creation and organization of spicules. These include sclerocytes, which directly secrete and shape spicules, as well as cells that are involved in transporting spicules throughout the sponge body.

Ostia: Incurrent openings on the outer surface of the sponge through which water enters.

Atrial cavity: A space to which all excurrent canals converge. Water is deposited into the atrium before exiting through the osculum.

Osculum: The single excurrent vent through which water exits the sponge.

Pore cells: Cells or groups of cells that form the pores through which water enters the sponge.

Sieve cells: A sieve-like contractile cell which may serve to regulate water flow through the canal system (Steinmetz et al. 2012).

Amphiblastula: A calcareous free-swimming larva possessing an anterior hemisphere of ciliated columnar cells and a posterior hemisphere of large cells.

Cross cells: Four cells of unknown function spaced equidistantly around the equator of the calcareon amphiblastula

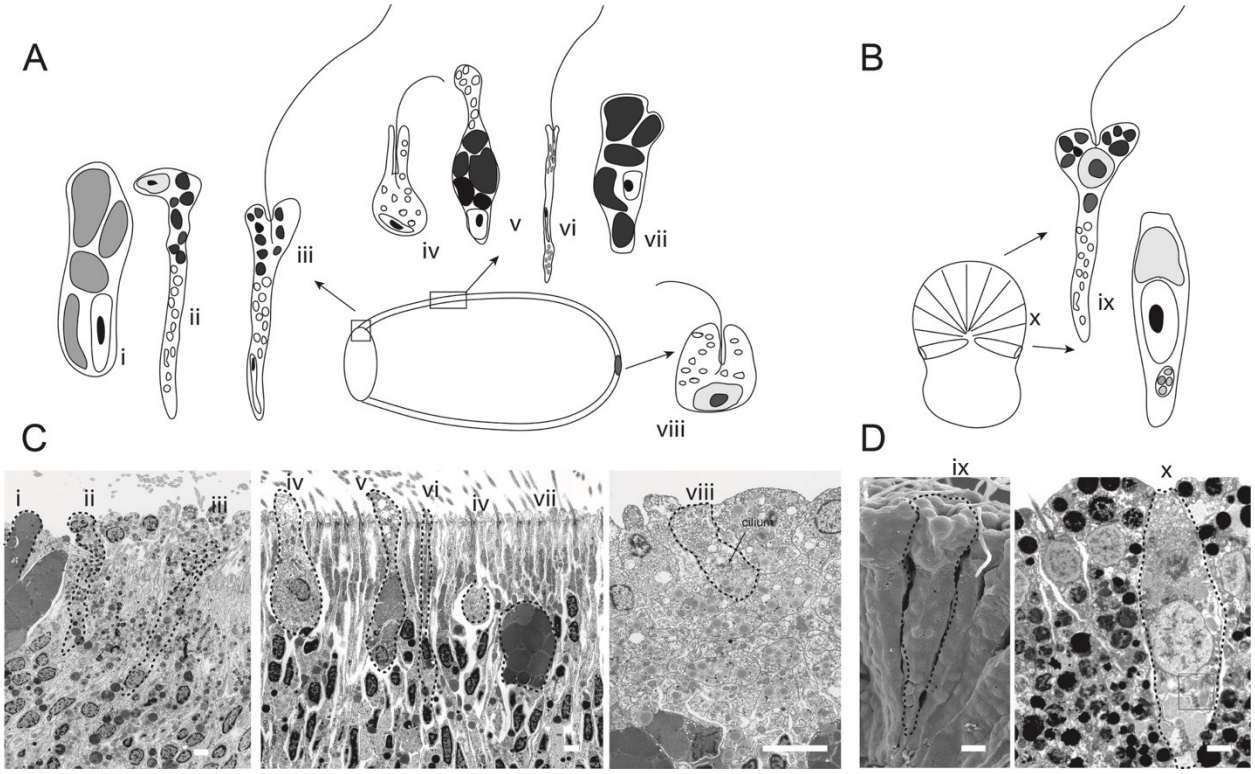
Parenchymella: An ovoid free-swimming larva found in Demospongiae.

Globular or mucous cells: Large cells with electron-dense inclusions found embedded within the epithelium of the parenchymella larva *Amphimedon queenslandica* (Leys and Degnan 2001).

Flask cells: Bottle-shaped cells embedded within the epithelium of the *A. queenslandica* parenchymella larvae. Flask cells have clear cytoplasmic vesicles and a single sunken epithelium extending from the apical surface (Leys and Degnan 2001).

Figure 2-1| The diversity of known and putative sensory cells in poriferan larvae.

A,C*, Amphimedon queenslandica* parenchymella larva. Illustrations (A) and electron micrographs (C) of sensory cells at the posterior (i-iii), middle (iv-vii) and anterior (viii) poles. The posterior pole contains cells with mucous inclusions (i), cells with pigment (ii), and cells with long posterior cilia (iii). The mid-section of the larva has flask cells (iv) and several types of globular cells (v, vii) in addition to the ciliated columnar epithelial cell (vi). The anterior pole has cuboidal cells with small, clear inclusions and recessed cilia (viii). B,D, *Sycon coactum* amphiblastula. Illustrations (B) and electron micrographs (D) of ciliated cells (ix) and cross cells (x). Box in D indicates inclusions with laminated structures. Previously unpublished images. For methods of preservation see Leys and Degnan (2001) and Eerkes-Medrano and Leys (2006). Scale bars are 2 μm.


A
i
ii
iii
iv
v
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vii
viii
B
x
ix
C
i
ii
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vi
iv
vii
viii
cilium
D
ix
x

Figure 2-2| A meta-analysis of structural and cell-specific localization of sensory-neural markers and putative non-sensory gene expression.

Orthologous or paralogous genes were collapsed under a single heading, indicated in bold. All genes can be viewed in Supplemental Figure S2-1. Unstable classifications are indicated with * (see Fortunato et al. (2012), Schnitzler et al. (2014)). A blank indicates absence of data or that expression was not detected. Blue triangle: *Amphimedon queenslandica*, green circle: *Ephydatia muelleri* or *Ephydatia fluviatilis*, red square: *Sycon ciliatum*. References are listed in Supplemental Table S2-1.

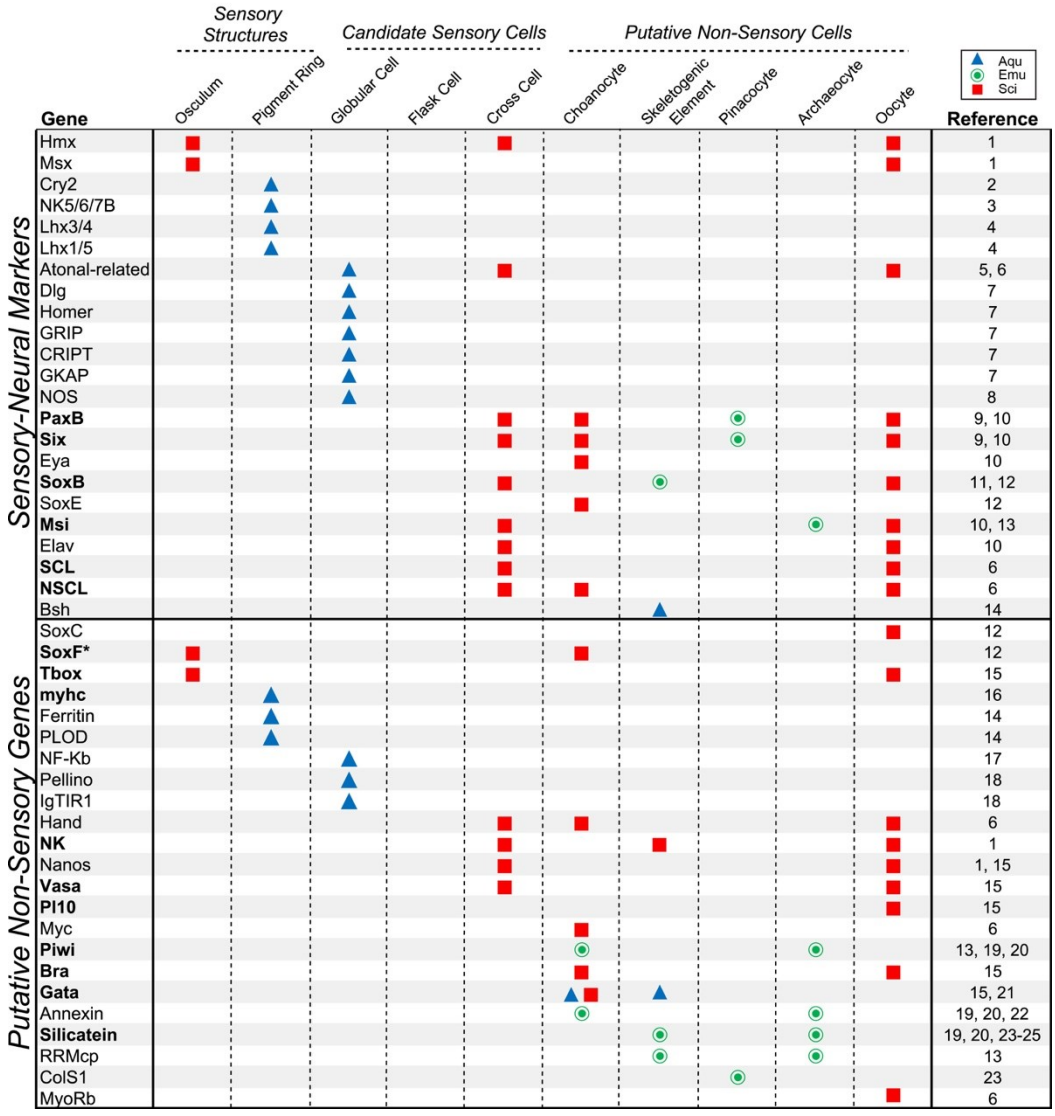
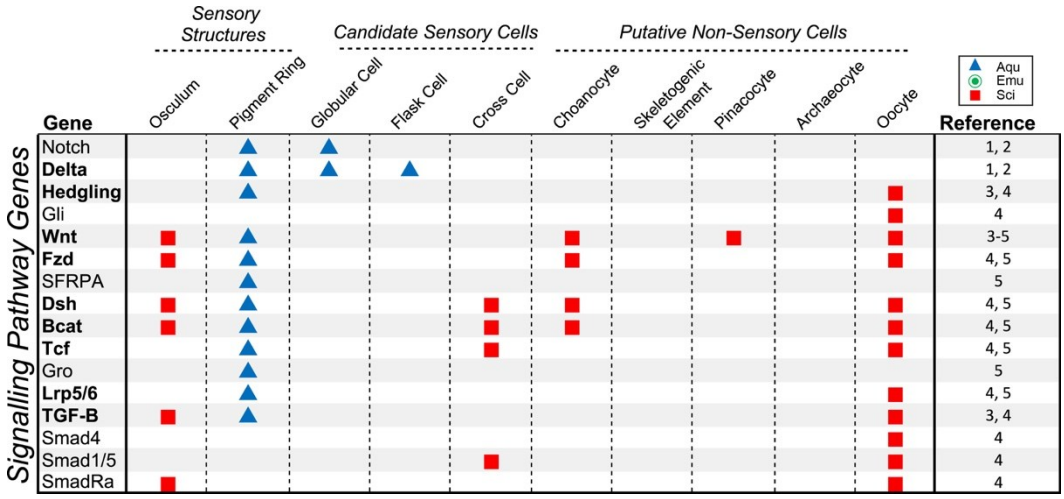

Sensory Structures
Candidate Sensory Cells
Putative Non-Sensory Cells
Aqu
Emu
Sci
Gene
Osculum
Pigment Ring
Globular Cell
Flask Cell
Cross Cell
Choanocyte
Skeletogenic Element
Pinacocyte
Archaeocyte
Oocyte
Reference
Sensory-Neural Markers
Hmx 1
Msx 1
Cry2 2
NK5/6/7B 3
Lhx3/4 4
Lhx1/5 4
Atonal-related 5, 6
Dlg 7
Homer 7
GRIP 7
CRIPT 7
GKAP 7
NOS 8
PaxB 9, 10
Six 9, 10
Eya 10
SoxB 11, 12
SoxE 12
Msi 10, 13
Elav 10
SCL 6
NSCL 6
Bsh 14
Putative Non-Sensory Genes
SoxC 12
SoxF* 12
Tbox 15
myhc 16
Ferritin 14
PLOD 14
NF-Kb 17
Pellino 18
IgTIR1 18
Hand 6
NK 1
Nanos 1, 15
Vasa 15
PI10 15
Myc 6
Piwi 13, 19, 20
Bra 15
Gata 15, 21
Annexin 19, 20, 22
Silicatein 19, 20, 23-25
RRMcp 13
ColS1 23
MyoRb 6

Figure 2-3| A meta-analysis of structural and cell-specific localization of signaling pathway gene expression.

Orthologous or paralogous genes were collapsed under a single heading, indicated in bold. A blank indicates absence of data or that expression was not detected. All genes can be viewed in Supplemental Figure S2-1. Blue triangle: *Amphimedon queenslandica*, green circle: *Ephydatia muelleri* or *Ephydatia fluviatilis*, red square: *Sycon ciliatum*. References are listed in Supplemental Table S2-1.


Sensory Structures
Candidate Sensory Cells
Putative Non-Sensory Cells
Osculum
Pigment Ring
Globular Cell
Flask Cell
Cross Cell
Choanocyte
Skeletogenic Element
Pinacocyte
Archaeocyte
Oocyte
Aqu
Emu
Sci
Gene
Reference
Signalling Pathway Genes
Notch 1, 2
Delta 1, 2
Hedgling 3, 4
Gli 4
Wnt 3-5
Fzd 4, 5
SFRPA 5
Dsh 4, 5
Bcat 4, 5
Tcf 4, 5
Gro 5
Lrp5/6 4, 5
TGF-B 3, 4
Smad4 4
Smad1/5 4
SmadRa 4

Chapter 3. The genetic signal of the osculum: RNA-seq of a sponge sensory structure

3.1. Introduction

Sponges lack neurons, yet have organized behavior and finely tuned sensation – two characters closely tied to the nervous system in other animals (Elliott and Leys 2007, Ludeman et al. 2014). Whether they use neural-like mechanisms to achieve this has long underlain studies of sensation and coordination in sponges.

The sponge body plan centres around a complex of canals called the aquiferous system (Figure 3-1). As filter feeders, they pump water through the canals and filter food particles from the flow using choanocytes, which possess a beating flagellum surrounded by a ring of microvilli. Ultimately, all water exits through a single excurrent vent – the osculum.

Various nervous system-like characters have been searched for in sponges. Early researchers used histology and histochemistry to look for a poriferan neuron, but found none (Lendenfeld 1889, Pavans de Ceccatty 1955, 1959, Jones 1962, Lentz 1966). Since then, several sensory cells have been hypothesized. The ultrastructure of the *Amphimedon queenslandica* larval flask cell has been compared to sensory neurons, and the gross morphology of choanocytes has been suggested to resemble mechanosensory cells (Jacobs et al. 2007, Nakanishi et al. 2015b). In contrast, while lacking a typical 'neuron-like' morphology, the *A. queenslandica* globular cell and *Sycon ciliatum* cross cell both express several neural-related genes (Richards et al. 2008, Fortunato et al. 2012, Fortunato et al. 2014a, Fortunato et al. 2014b). However, so far a sensory function has not been found for any of these specific cell types. One of the few characterized sensory cells, the pigmented ciliated cells of parenchymella larvae, is distinct from that of many other animal sensory cells in being both a sensor and effector (Leys and Degnan 2001, Maldonado 2006). These cells, which form a ring at the posterior pole of the larva, detect light and respond by straightening their cilia, steering the larva away from the light (Leys and Degnan 2001).

Another approach has been to investigate the sponge sensory/coordinating system at a tissue level. Though lacking neurons, sponges do possess excitable tissue. Mechanical or electrical stimuli lead glass sponges to propagate an action potential through their syncytial tissues, ultimately leading to flagellar arrest (Leys and Mackie 1997). This action potential is

slow and involves calcium (Leys et al. 1999). In contrast to the immediate arrest of the feeding current triggered by electrical signalling in glass sponges, other sponges propagate slow contractions (e.g. 0.3-122 μm/s) through their tissues (Nickel 2004, Elliott and Leys 2007, Hamer et al. 2007) that are considered to be used to clear the aquiferous system of irritants. These contractions can be triggered by neuroactive molecules, but the contractions are too slow to be coordinated through electrical signals (Emson 1966, Ellwanger et al. 2004, Ellwanger and Nickel 2006, Ellwanger et al. 2007, Elliott and Leys 2010, reviewed in Nickel 2010). In the demosponge *Ephydatia muelleri* Elliott and Leys (2010) found that the excitatory neurotransmitter glutamate can trigger a multi-step inflation-contraction behavior in a dose dependent manner. Likewise, the inhibitory neurotransmitter GABA can inhibit contractions, even in the face of repeated addition of glutamate (Elliott and Leys 2010).

The osculum may be a sensory and coordinating hub for this behavior. Elliott (2009) observed primary cilia lining the inner surface of *E. muelleri*. Primary cilia play a multitude of sensory roles in other animals and removal of the osculum or inhibition of ciliary receptors abolished the inflation-contraction behavior, suggesting that these cilia may also be sensory in *E. muelleri* (Ludeman et al. 2014). This sensory mechanism might be present in many sponges. The osculum is a near-universal feature and primary cilia have been found in the oscula of at least nine species of sponges, including the glass sponge *Aphrocallistes vastus* (Nickel 2006, Nickel 2010, Ludeman et al. 2014).

Despite lacking neurons, sponges do possess genes that in other animals are considered to have a neural function (Sakarya et al. 2007, Srivastava et al. 2010b, Riesgo et al. 2014). Given this absence of neurons in sponges and the fact that these genes are not restricted to neural or sensory functions (Woods et al. 1996, Carmeliet and Tessier-Lavigne 2005, Julio-Pieper et al. 2011, Bucher and Anderson 2015), calling them 'neural' is problematic. I will refer to these genes as sensory-neural markers (SNM, Mah and Leys 2017, Chapter Two). In general it is difficult to compare sponge biology to that of other animals as there is little similarity. But SNMs present an opportunity to examine the homology of the sponge sensory system. Molecular technology is constantly improving, but for now we cannot test gene function directly in sponges. Instead, we rely on finding the presence of gene expression in an organ of interest – a kind of genetic signal – to deduce whether these genes may contribute to an organ's sensory character and whether this organ may be homologous to other organs (Mah and Leys 2017).

Naturally it is intuitive to link SNMs to sponge behavior and they suggest the potential for neural-like mechanisms to underlie sponge sensation. But currently the identity of the earliest diverging metazoan is not clear. It can no longer be assumed that sponges occupy this position; instead it is possible that ctenophores do (Dunn et al. 2008, Ryan et al. 2013, Moroz et al. 2014). If ctenophores are basal to sponges, nerves may have arisen convergently once in ctenophores and once in cnidarians and bilaterians. A kind of "proto-nervous" system may be conserved in extant sponges. Alternatively, a nervous system may have arisen at the origin of Metazoa, with nervous system loss occurring in sponges and placozoans. Other genetic evidence, such as genome density, suggests that sponges may be sister to Eumetazoa (Fernandez-Valverde and Degnan 2016). Even so, emerging molecular work suggests that several neural gene families may have arisen more than once during animal evolution and so nervous system complexity may have arisen independently several times (Liebeskind et al. 2015, Francis et al. 2017). Thus the study of the sponge sensory/coordinating system must occur within this nuanced context.

RNA-seq has emerged as a powerful tool to examine the molecular underpinnings of non-bilaterian biology. This technique has been used to examine the upregulation of genes implicated in vision and sensory organ development in the cnidarian rhopalium (Nakanishi et al. 2015a, Ames et al. 2016) and the expression of potential neuropeptide receptors, gap junction proteins, and other nervous system-related proteins in the aboral organ of *Pleurbrachia bachei* (Moroz et al. 2014). In sponges, RNA-seq of the developmental stages of *A. queenslandica* showed that global co-upregulation of postsynaptic density proteins does not occur (Conaco et al. 2012a). RNA-seq has been performed on one hypothesized sensory cell type, choanocytes, but SNMs were not examined (Pena et al. 2016). While other sponge RNA-seq studies examining differential expression have been performed, none have specifically targeted a potential sensory organ (e.g. Conaco et al. 2012b, Alie et al. 2015, Pena et al. 2016).

The osculum is a well-studied sensory structure (Parker 1910, McNair 1923, Elliott and Leys 2007). It is accessible and amenable to physical manipulation, such as dissection. As detailed above, there is also independent, physiological evidence that there may be neural-like mechanisms underlying the osculum's possible sensory and coordinating abilities (Elliott and Leys 2010). Given its near-universal presence in sponges, the oscular system may have originated at the stem of Porifera. Thus, the osculum presents a good candidate for a structure that potentially expresses SNMs. Even so, the osculum is poorly described at the molecular level

– so far, only three gene expression studies found that the osculum expressed particular genes (Fortunato et al. 2014a, Leininger et al. 2014, Borisenko et al. 2016).

I used RNA-seq to test whether SNMs are significantly upregulated in sponges with oscula compared to sponges in which oscula had not yet developed. I also used RNA-seq to compare the osculum to body tissues. I focus on two divergent sponge groups, the freshwater demosponge *Spongilla lacustris* and the glass sponge *Aphrocallistes vastus* (Figure 3-1 B-D). *S. lacustris* undergoes the inflation-contraction behavior when mechanically stimulated and possesses non-motile cilia in the osculum (Ludeman et al. 2014). The osculum has been found to play an essential role in this contraction behaviour (Ludeman 2010). Like other freshwater sponges, *S. lacustris* forms overwintering cysts called gemmules which can be hatched in the lab and monitored during development (Simpson and Gilbert 1973). When canals begin to develop, a pre-oscular stage can be isolated and studied (Figure 3-1B). The sponge subsequently progresses to the juvenile stage, which possesses a full osculum (Figure 3-1C). Thus to examine oscular development, I compared the pre-oscular and juvenile stages of gemmule hatching. The osculum of *S. lacustris* was not used for RNA-seq because, despite harvesting at least two hundred oscula, there is so little tissue in that structure that it was not possible to obtain enough RNA for library preparation. Oscula from *S. lacustris* juvenile sponges are very small (roughly 1 mm long and less than 0.25 mm across) and consist of only two layers of thin pinacocytes with some mesohyl in between. Instead, to study the osculum itself I compared the oscular and body tissue of *A. vastus* (Figure 3-1D), which I anticipate having a similar sensory mechanism as *E. muelleri*.

3.2. Materials and methods

3.2.1. Tissue collection

Spongilla lacustris gemmules were collected by SPL from Rousseau, British Columbia, Canada and stored in the dark at 4˚C in lake water at the University of Alberta. Gemmules were cleaned from the adult tissue following Elliott and Leys (2007) and then plated in M-medium (Rasmont 1961) in 5cm Petri dishes. Generally, within 2-4 days most gemmules hatched and immediately progressed to the pre-oscular stage (no osculum present) and within 5 days most sponges had developed an osculum (juvenile stage) (Figure 3-1B,C). Flame-sterilized forceps or sterile cell scrapers were used to harvest sponge tissue from the Petri dishes. Tissue was spun down in Eppendorf tubes, excess M-medium was removed, and the tissue was immediately flash

frozen in liquid nitrogen and stored at -80˚C. Corresponding pre-oscular and juvenile tissue for three separate individuals of *S. lacustris* was obtained.

Specimens of *Aphrocallistes vastus* were collected at Fraser Ridge, Vancouver Island, Canada using ROPOS, a remotely operated vehicle (Figure 3-1 D). Upon being brought to the surface, the thinner, flexible oscular tissue was manually dissected from the sponge, flash frozen in liquid nitrogen and stored at -80˚C for transport to the University of Alberta. Oscular tissue was obtained from three separate individuals. The same procedure was followed for body tissue. While body tissue was also collected for three separate individuals only one sample corresponded to an oscular tissue sample from the same individual.

3.2.2. RNA extraction and sequencing

Sponge tissue was homogenized using a mini-pestle attached to a drill. Total RNA was extracted using the Single Cell RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada) and eluted into 20 µl nuclease-free water. RNA was then stored in a solution containing 1/10th the volume of 3M sodium acetate (pH 5.5) (Ambion/ThermoFisher Scientific, Waltham, MA, USA) and 3 times the volume of 100% ethanol. RNA purity and concentration was measured using a Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, DE, USA) and RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the Eukaryote Total RNA Nano assay (Supplemental Table S3-1). Only samples with a RIN greater than or equal to 9 progressed to sequencing (Supplemental Table S3-1).

cDNA libraries were made from 1 µg of RNA (20 ng/ul) with the TruSeq RNA Library Prep Kit v2 (Illumina, Inc., San Diego, CA, USA) by Delta Genomics (Edmonton, AB, Canada). All six *S. lacustris* samples (3 replicates each of pre-oscular and juvenile sponges) and six *A. vastus* samples (3 replicates of body and oscular tissue) were sequenced in a single lane on an Illumina NextSeq 500 using the NextSeq Series High-Output Kit (Illumina, Inc., San Diego, CA, USA) to obtain 2x150 bp reads at an average depth of 124x (Supplemental Table S3-1). Reads sequenced previously by LC Sciences (Houston, TX, USA) were also incorporated into the transcriptome assembly. These reads were sequenced from adult specimens of *S. lacustris* and *A. vastus* that were collected at a separate time. Libraries had been created by LC Sciences from the TruSeq RNA Sample Preparation Kit (Illumina, Inc.) and sequenced on the HiSeq2000 to produce 2x100 bp reads (Riesgo et al. 2014).

3.2.3. Bioinformatics

FastQC v.0.11.3 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, last accessed July 22, 2017) was used to assess read quality before and after trimming. Reads were trimmed of adapters and bases with quality scores below Q20 using Trimmomatic-0.33 (Bolger et al. 2014). Only reads at least 50 bp long were retained. Reads previously sequenced by LC Sciences and those from the current experiment were used to assemble a reference transcriptome for each species using Trinity v.2.0.6 (Grabherr et al. 2011). Descriptive transcriptome statistics were determined using the TrinityStats.pl script from the Trinity package and transcriptome completeness was assessed with BUSCO v.1.2 (Simao et al. 2015) using the Metazoa dataset on the transcriptome setting (Table 3-1, Supplemental Table S3-2). Transcripts were filtered for potential bacterial, viral, human, and plant contaminants using BLAST (script available in Appendix 2).

Reads were aligned to the full, unfiltered transcriptome to allow contaminant reads to align to contaminant contigs. Transcript quantification was performed with RSEM v.1.2.19 (Li and Dewey 2011) using the Trinity wrapper script 'align_and_estimate_abundance.pl' and 'abundance_estimates_to_matrix.pl'. Counts from contaminant contigs were discarded and all following analyses were performed on the remaining 'clean' counts. The Trinity wrapper script 'run_DE_analysis.pl' was used to perform differential expression analysis with DESeq2 v.1.14.1 (Love et al. 2014) and heatmaps of all differentially expressed genes were made using the Trinity script 'analyze_diff_expr.pl' using the cutoff $p_{adj} < 0.05$ and $\log_2FC > 1$. All differential expression analyses were performed at the gene level, which considers the sum of expression values of a gene's isoforms. A candidate gene was considered significantly differentially expressed if it had a Benjamini-Hochberg adjusted p-value < 0.05, a false discovery rate (FDR) < 0.05, and at least a two-fold difference in expression ($\log_2FC > 1$). Heatmaps of candidate genes were made using R v.3.3.2 (R Development Core Team, 2016) and edited using Adobe Illustrator (Adobe Systems, San Jose, CA, USA). A principal component analysis was computed using the Trinity wrapper script 'PtR'.

Transdecoder v.2.1.0 (https://transdecoder.github.io/, last accessed July 22, 2017) was used to find the coding and peptide sequences of the reference transcriptomes. For sequence annotation, peptide sequences were blasted against the UniProtKB/Swiss-Prot database (downloaded November 15, 2016) using BLASTP (BLAST+ v. 2.2.29+) (Altschul et al. 1997). Gene ontology (GO) terms were retrieved from the UniProtKB/Swiss-Prot BLAST results. Gene

ontology enrichment using GOSeq v.1.26.0 (Young et al. 2010) was performed through Trinotate v3.0.1 (https://trinotate.github.io/, last accessed July 22, 2017) and the Trinity script 'analyze_diff_expr.pl' at default settings. A GO term was classified as 'neural' if its name included reference to neurons, neurotransmitters, neuronal cell parts (e.g. synapse, dendrite), neural organs (e.g. brain) or neurodevelopmental processes.

Candidate genes were found by using TBLASTN (BLAST+ v.2.2.31+) to search the *S. lacustris* and *A. vastus* reference transcriptomes. The candidate SNMs used were those that have been previously published in past sponge papers. The *S. lacustris* and *A. vastus* transcripts that were found were blasted with BLASTX against the NCBI protein database using the web interface with default settings (last accessed July 6, 2017). Then a reciprocal TBLASTN back to the *S. lacustris* or *A. vastus* transcriptome using the top NCBI hit was performed.

Where default settings were not used, full commands for the scripts can be found in Appendix 2.

3.2.4. OrthoMCL

Leininger et al. (2014) published a set of 2x100 bp reads sequenced from the top, upper middle, lower middle, and bottom regions of the calcareous sponge *Sycon ciliatum*. Using the same protocol outlined above, these reads were trimmed and aligned to the *S. ciliatum* transcriptome, which was also made available by Leininger et al. (2014). As the top region of *S. ciliatum* contains the osculum, differential expression analyses were performed to find the upregulated genes in the top region as compared to the upper middle region. TransDecoder was then used to translate these upregulated genes to protein. All settings for each program are identical to that outlined above, except for trimming (Appendix 2). OrthoMCL v2.0.9 (Li et al. 2003) was used with default settings to find shared orthologues between the set of significantly upregulated proteins in the *A. vastus* osculum and the top region of *S. ciliatum*. These orthologues were then identified by blasting against the NCBI protein database with BLASTX. Where default settings were not used, full commands for the scripts can be found in Appendix 2.

3.3. Results

The *Spongilla lacustris* transcriptome had 188,554 transcripts with an N50 of 1,748 bp (Table 3-1). A search of the transcriptome found 81% of the BUSCO genes from the Metazoa data set (Table 3-1). The *Aphrocallistes vastus* transcriptome produced 83,052 transcripts with an N50 of 2,324 bp (Table 3-1). In the *A. vastus* transcriptome 70% of the BUSCO genes were

found. For *S. lacustris*, 87,175 transcripts possessed at least one open reading frame and of these 63.0% returned a hit in the UniProtKB/Swiss-Prot database. The *A. vastus* transcriptome had 41,565 transcripts with at least one open reading frame, of which 55.3% returned a hit from UniProtKB/Swiss-Prot. These assembly statistics are comparable to that seen in the de novo transcriptomes of other non-bilaterian animals (e.g. Conaco et al. 2012b, Babonis et al. 2016). BUSCO scores fell well within the range seen in other sponge assemblies (Supplemental Table S3-2).

A distinct set of genes was differentially expressed during *S. lacustris* oscular development (Figure 3-2 A) and in the *A. vastus* osculum (Figure 3-2 B). Of these, 2,312 genes in *S. lacustris* were significantly differentially expressed with at least a two-fold difference ($\log_2$FC > 1) and an adjusted p-value less than 0.05. 1,151 genes were differentially expressed at those levels in *A. vastus*. A principle component analysis showed that biological replicates from the same sample type grouped together (Figure 3-3).

Several candidate SNMs were significantly differentially expressed in *S. lacustris* and *A. vastus*. Three genes were significantly upregulated in the juvenile stage of *S. lacustris*: the metabotropic glutamate receptor (mGluR), the gamma-aminobutyric acid receptor (GABAR), and the inward rectifier potassium channel (K_{ir}) (Figure 3-4 A). In *A. vastus* GABAR was significantly upregulated in the body tissue while brain-specific homeobox (Bsh) was significantly upregulated in the osculum (Figure 3-4 B). While other candidate genes were expressed, none were differentially expressed at a significant level (Figure 3-4). Some candidate genes found in a previous assembly of *S. lacustris* and *A. vastus* (Riesgo et al. 2014) were not found here; these include citron, CRIPT, EphR, ErbB-R, GRIP and SPAR for *S. lacustris* and citron, CRIPT, GKAP, NOS, PMCA, SPAR, and Lin-7 for *A. vastus*. This may be due to differences in temporal expression or transcriptome assembly protocol. Of the ten most upregulated genes in each sample type at least one, plexin-A4, is known to have a neural function in bilaterians (Table 3-2) (Suto et al. 2005).

Alignment of *Sla*mGluR to mGluR1 sequences showed that *Sla*mGluR possesses some of the residues required for binding glutamate (Supplemental Figure S3-1). Similarly, alignments of *Sla*GABAR and *Ava*GABAR to GABABR1 show that these sequences also possess some, but not all, of the GABA binding residues (Supplemental Figure S3-2). The two transmembrane domains and the pore domain appear to be conserved in *Sla*K_{ir} (Figure 3-5). However, the pore

domain sequence 'TXGYG' is present as 'TIGFG' in *Sla*K_{ir} and position 171, which determines the strength of inward rectification, is occupied by a threonine ('T') (Figure 3-5).

Hill et al. (2004) found a sequence in *Halichondria bowerbanki* that had homeodomain residues characteristic of both Bsh and Bar, a closely related ANTP homeobox gene. Given this, and the fact that *Ava*Bsh blasted to *H. bowerbanki* BarX/Bsh with a moderately high E-value (Supplemental Table S3-3), I performed an alignment of the homeodomains of Bsh and Bar sequences. *Ava*Bsh possesses residues characteristic of both Bar and Bsh, as seen in Hill et al. (2004) (Figure 3-6). In particular, *Ava*Bsh possesses a string that differs by two residues from 'LSETQVKTWFQNR' (positions 149-164) and an arginine ('R') at position 110, both of which Hill et al. (2004) propose act as a diagnostic residue for Bsh. Likewise the Bar-specific residues lysine at position 24 and tryptophan at position 56 are present in *Ava*Bsh. Interestingly, the *Mnemiopsis leidyi* Bsh sequence possesses the same Bsh and Bar-specific residues as *Ava*Bsh and lacks the same two residues in the 'LSETQVKTWFQNR' string, though it has replaced them with different residues from *Ava*Bsh.

Significantly enriched neural-related GO terms were found in all sample types (Figure 3-7). In general, with the exception of the GO terms for the *A. vastus* body region, non-neural GO terms were more significantly enriched than neural GO terms (Figure 3-7). No significantly enriched neural-related GO terms were associated with *Ava*Bsh, *Ava*GABAR, or *Sla*K_{ir} (Supplemental Table S3-4 to 6). However, *Sla*GABAR was associated with significantly enriched GO terms describing the regulation of neurotransmitter secretion and *Sla*mGluR had a GO term associated with synaptic plasticity (Supplemental Table S3-7, 8).

Given that the osculum is a near-universal poriferan feature, I searched for orthologues shared between the set of significantly upregulated genes in the *A. vastus* osculum and the *Sycon ciliatum* top region, where the osculum resides. Of the 248,955,540 paired reads from *S. ciliatum*, 204,526,894 survived trimming. Upon alignment to the *S. ciliatum* transcriptome (Leininger et al. 2014), differential expression analysis revealed that 68 genes were significantly upregulated in the top region (Figure 3-8A). Four orthologues were shared between the set of genes significantly upregulated in the *A. vastus* osculum and *S. lacustris* top region. These transcripts blasted with moderate to high support to genes involved in cell adhesion (thyroid receptor-interacting protein 6/paxillin-like), ion pumping (multidrug resistance-associated protein 4) and members of the CAP superfamily (peptidase inhibitor 15 and 16, GLIPR1-like

protein), a family of secreted proteins involved in mammalian cancers, among other varied functions (Figure 3-8B) (Wang and Gilmore 2003, Gibbs et al. 2008, Russel et al. 2008, Lin and Lin 2011).

3.4. Discussion

3.4.1. Sensory-neural marker expression

Recent studies on homology of the sponge sensory/coordinating system and the nervous system have examined sensory-neural markers, genes typically expressed in the nervous tissues of bilaterians (e.g. Sakarya et al. 2007, Richards et al. 2008, Fortunato et al. 2014b). Co-expression of SNMs is often interpreted as a genetic signal, suggestive of the presence of a sensory function and thus perhaps also homology. I performed an RNA-seq experiment to determine whether a similar genetic signal may be present in sponges undergoing oscular development or in the osculum, a sponge structure with strong evidence of sensory abilities.

The distinct set of genes upregulated in the osculum and during oscular development suggests that at the molecular level the osculum is a specialized structure (Figure 3-2, Figure 3-3). Localized gene expression at the osculum has also been seen in *in situ* hybridization studies, both as a distinct ring at the lip of the osculum and as a gradient of expression where the gene is expressed most strongly towards the tip of the osculum (Fortunato et al. 2012, Fortunato et al. 2014a, Leininger et al. 2014, Borisenko et al. 2016). Among the set of differentially expressed genes, I found four that were significantly upregulated in the juvenile sponge or in the osculum (Figure 3-4).

GABAR was significantly upregulated in both the *Spongilla lacustris* juvenile and in the *Aphrocallistes vastus* body tissue. mGluR was significantly upregulated in the *S. lacustris* juvenile. Both GABA and glutamate induce contractions in several species and change the kinetics of contraction in *Tethya wilhelma* (Ellwanger et al. 2004, Ellwanger et al. 2007, Elliott and Leys 2010, Nickel 2010). In particular, glutamate can trigger the multi-step inflation-contraction behavior in *Ephydatia muelleri*, while GABA inhibits it – functions perhaps reminiscent of their roles as excitatory and inhibitory neurotransmitters in the bilaterian nervous system (McCormick 1989, Meldrum 2000). Furthermore, the ability of an allosteric inhibitor of mGluR to eliminate glutamate-triggered contractions suggests the involvement of a receptor with structural and functional similarity to mGluR (Elliott and Leys 2010). These findings are consistent with the presence of a mGluR-GABAR module in *E. muelleri* which, at

the behavioral level, possesses similar functional roles as that seen in the bilaterian nervous system.

However, these experiments are unable to identify the genes underlying the behavioral effects of GABA and glutamate. Past experiments have used chemicals that target conventional mGluRs and GABARs, but it is possible the sponge gene is neither - Perovic et al. (1999) identified a sequence with characteristics of both mGluR and GABAR. *Ava*GABAR is expressed in body tissue and not the osculum. In *S. lacustris*, *Sla*mGluR and *Sla*GABAR are expressed in the juvenile which, as a whole sponge, may have many other significantly upregulated non-sensory/coordinating activities. It is possible several mGluR and GABAR subtypes exist. Francis et al. (2017) found that both genes have undergone an expansion in demosponges and Elliott and Leys (2010) suggest that in *E. muelleri* there may be sublocalization of mGluR subtypes based on the selective activity of AP3, an mGluR antagonist. In bilaterians neither mGluR nor GABAR are exclusively expressed in neural tissues (He et al. 2001, Pacheco et al. 2006, Julio-Pieper et al. 2010). Thus, while upregulation of *Sla*mGluR, *Sl*aGABAR, and *Ava*GABAR suggests they play biologically important roles, these genes may not necessarily be involved in sensation and coordination.

Tompkins-MacDonald et al. (2009) found that the *Amphimedon queenslandica* K_{ir} delivers a strong inward rectifying K^+ current when heterologously expressed in *Xenopus laevis* oocytes. Thus, sponge K_{ir} channels possess the potential for conserved functional roles at the protein level, at least in a bilaterian cell. This drove me to compare *Aqu*K_{ir} to *Sla*K_{ir}. The highly conserved pore domain sequence, 'TXGYG' is present as 'TIGFG' in *Sla*K_{ir} (Figure 3-5). This unconventional pore domain sequence is also seen in the K_{ir} 6.x family, among other potassium channels (Figure 3-5) (Capener et al. 2000). *Aqu*K_{ir} A and *Aqu*K_{ir} B, in contrast, have 'TIGYG' (Tompkins-MacDonald et al. 2009). In their work Tompkins-MacDonald et al. (2009) found that *Aqu*K_{ir} is a strong inward rectifier, and consistent with this is the presence of aspartic acid ('D') at position 171, the residue that determines rectification strength (Wible et al. 1994). Interestingly, *Sla*K_{ir} has neither an asparagine (weak) nor aspartic acid (strong) residue at this position; instead it has threonine (Figure 3-5). These differences suggest that at the protein level there is a distinction between how *Sla*K_{ir} and *Aqu*K_{ir} function, though it is not possible to infer more until functional tests are performed. In the nervous system, K_{ir} channels are involved in regulating action potentials through controlling the excitability of neurons (Day et al. 2005, reviewed in Hibino et al. 2010). However, while there is a possibility (albeit remote) that action

potentials may occur in demosponges (Loewenstein 1967, Reiswig 1979, as noted by Leys and Farrar 2015), there is no electrical signaling in *S. lacustris*. K_{ir} channels are a large family of proteins that hold both neural and non-neural functions (Moulton and Attwood 2003). This, and the fact that K_{ir} channels may have undergone independent radiations in sponges and chordates (Tompkins-MacDonald et al. 2009), indicate that it is not yet possible to determine whether *Sla*K_{ir} holds a role in the *S. lacustris* sensory or coordinating system.

The fourth candidate gene of interest which showed a significant difference in expression was brain-specific homeobox (Bsh). Expression of Bsh has been studied in both ctenophores and sponges (Larroux et al. 2006, Pang and Martindale 2008). Both studies suggested that Bsh did not have a sensory or neurodevelopmental function in either non-bilaterian. Instead, Bsh was expressed in the tentacle apparatus of *Mnemiopsis leidyi* and the spicule-producing cells of *A. queenslandica* (Larroux et al. 2006, Pang and Martindale 2008). Like the BarX/Bsh sequence of *Halichondria bowerbanki*, *Ava*Bsh possesses residues characteristic of both Bsh and Bar (Figure 3-6). Given that there is a growth zone where spicule formation occurs in the *A. vastus* osculum (Kahn and Leys 2017), perhaps *Ava*Bsh is also involved in spicule formation. However, the presence/absence patterns of diagnostic Bar and Bsh residues more closely followed that of *M. leidyi* rather than that of the *Halichondria* sp. or *A. queenslandica* sequences (Figure 3-6). The similarity in binding residues with *M. leidyi* suggests that *Ava*Bsh may possess a similar, as yet unknown function as seen in the ctenophore.

Thus, although a limited number of SNMs was searched for I found several significantly differentially expressed in the *S. lacustris* juvenile and in the osculum of *A. vastus*. However, although these genes are expressed in what is known to be a sensory structure we cannot directly link these upregulated genes to a sensory or coordinating function. Modules of conserved neural-like function may indeed underlie the sponge sensory/coordinating system, especially with regards to *Sla*mGluR and *Sla*GABAR. However, based on our current knowledge of these genes' function and expression patterns in sponges it is equally possible these significantly upregulated SNMs hold cryptic, non-sensory, non-coordinating functions.

3.4.2. Gene ontology

Gene ontology (GO) enrichment analysis tests for the co-upregulation of sets of genes known to be involved in particular biological processes, and so perhaps can be interpreted as another, broader genetic signal. Like candidate genes, inferences based on GO enrichment analysis rely on the functional conservation of genes linked to each GO term. Of the four

significantly upregulated genes, only *Sla*mGluR and *Sla*GABAR were associated with neural GO terms that were significantly enriched for the sample these genes were upregulated in (ie. the *S. lacustris* juvenile, for these genes) (Supplemental Table S3-7, 8). The genes linked to the neural-related GO terms of *Sla*mGluR and *Sla*GABAR may be useful future targets for experiments exploring whether sponges use neural-like mechanisms. Should *Sla*K_{ir}, *Ava*Bsh, and *Ava*GABAR possess conserved neural-like interactions, the set of genes defining these interactions were either not sufficiently co-upregulated to be detectable by GO enrichment analysis or the GO database does not have terms encompassing the neural-related functions of K_{ir} and Bsh (Table S3-4 to 6). Alternatively, the molecular networks *Sla*K_{ir}, *Ava*Bsh and *Ava*GABAR participate in may not be related to neural processes. Finally, a third possibility is that GO terms may be poorly suited to describe the molecular processes *Sla*K_{ir}, *Ava*Bsh and *Ava*GABAR may participate in, should they be involved in sensory functions.

"Ear development" (GO:0043583) and "retina layer formation" (GO:0010842) are among the most significantly enriched GO terms, yet sponges possess neither ears nor retinas (Figure 3-7). GO terms, which are largely defined according to bilaterian biology, may not be sufficient to define sponge biology. GO enrichment analysis identified neural-related GO terms that were overrepresented in each sample type compared to the opposite sample type (Figure 3-7), but until genes can be functionally tested all GO terms must be considered with the same degree of uncertainty. Proteins responsible for non-bilaterian interactions, which may be just as important if not more for the emergent functions of *Sla*mGluR and *Sla*GABAR, will be missing from the list of genes linked to neural-related GO terms. Several of the most highly upregulated genes expressed in *S. lacustris* and *A. vastus* produced no hits against the NCBI protein database (Table 3-2). This suggests that uncharacterized proteins, and their interactions, may underlie important functions in these sponges.

3.4.3. Shared 'oscula' genes

The hypothesis that the sponge sensory/coordinating system is homologous to the bilaterian nervous system suggests the potential for this sensory/coordinating system to be shared across Porifera. However, I found only four orthologues shared between the set of significantly upregulated genes in the *A. vastus* osculum and the *S. ciliatum* top region (with osculum) – the molecular signal for an osculum itself is weak, at least as defined between these two species (Figure 3-8). Perhaps unsurprisingly, these four genes do not appear to be closely associated with neural functions but are instead involved in basic cellular functions (Figure 3-8B).

The presence of only four shared orthologues may signal extreme divergence. *A. vastus*, a glass sponge, is evolutionarily distant from the calcareous sponge *S. ciliatum* (Dohrmann et al. 2008). The functions occurring in these regions of these sponges are diverse: choanoblast and spicule formation occurs in the osculum of *A. vastus* while choanocyte chamber formation occurs at the top region of *S. ciliatum*. (Leininger et al. 2014, Kahn and Leys 2017).

Alternatively, this may be a case of convergence. Wnt signalling may play a role in the development of the osculum of the demosponge *Ephydatia muelleri* (Windsor and Leys 2010). Application of Wnt pathway agonists lead to the formation of multiple oscula (Windsor and Leys 2010). Wnt is also upregulated in the osculum of *S. ciliatum* (Leininger et al. 2014). Yet all searches of the *A. vastus* transcriptome, including my own (data not shown), have failed to find a Wnt orthologue (Riesgo et al. 2014). The formation of a glass sponge osculum has never been reported, mostly due to the inaccessibility of their habitat. Indeed, as mentioned above the functions of sponge oscula are highly diverse and alternatively may reflect different evolutionary heritages. Deeper knowledge of the genetic regulatory networks underlying these diverse oscula are needed.

It is possible that a neural genetic signal remains more recognizable in a different species of sponge, such as *S. lacustris*. However, the fact that few orthologues are shared between the osculum or top region of these two species suggests an alternative – that some sponge sensory/coordinating systems, even if 'neural-like', may be a taxon-specific innovation. A comparison of the osculum to other non-bilaterian sensory systems rests on the assumption that it is representative of a pan-poriferan sensory system. Yet these findings suggest that sponge oscula may strongly differ amongst themselves. Even within sponges, the molecular definition of an osculum is elusive.

3.4.4. Conclusion

Among the set of significantly upregulated genes that characterize the osculum and oscular development, several are sensory-neural markers. The expression of *Sla*mGluR, *Sla*GABAR, *Sla*K_{ir}, *Ava*GABAR and *Ava*Bsh can perhaps be interpreted as part of an SNM signal, adding to the oscular expression patterns of Msx and Hmx (Fortunato et al. 2014a). However, currently we cannot directly link the genes found in this study to sensation or coordination in *Aphrocallistes vastus* or *Spongilla lacustris*. Indeed, from what we know of the biology of these two sponges an equally viable hypothesis is that these SNMs are not involved in

sensory or coordinating functions. Neural-related GO terms are significantly enriched, but a bilaterian definition may not accurately reflect sponge sensory or coordinating biology. The fact that there are few orthologues shared between the *A. vastus* and *S. ciliatum* osculum suggests that the possibility of divergence or convergence must be considered before a genetic signal can be proposed as representative of sponge biology. Neural-like modules may yet contribute to the sensory and coordinating abilities of sponges and this study has found an SNM signal associated with the osculum. However, sponge biology is unlike that of bilaterian biology and the use of this signal to infer function, and perhaps homology, may not be rigorous.

Table 3-1| Descriptive statistics for de novo assemblies of *Spongilla lacustris* and *Aphrocallistes vastus*.

The number of transcripts with at least one blast hit was found by translating each transcript and blasting (BLASTP) each protein to the UniProt database. Only BLAST hits with an E-value of at least 10^{-5} was considered a valid hit. BUSCO scores refer to the percentage of complete BUSCO genes found in the transcriptome using the Metazoa data set. ORF, open reading frame, FPKM, fragments per kilobase of transcript per million mapped reads.

Statistic	*Spongilla lacustris*	*Aphrocallistes vastus*
No. raw paired reads	621,746,448	609,237,878
No. trimmed paired reads	582,086,944	594,575,482
No. transcripts	188,554	83,052
No. genes	90,193	46,049
Total assembled bases	197,584,629	98,043,873
Average transcript length	1,033.01	1,162.95
Median transcript length	582	537
N50	1,748	2,324
No. transcripts with at least one ORF	87,175	41,565
No. transcripts with at least one BLAST hit	54,924	22,973
No. transcripts expressed > 1 FPKM	146,243	69,233
% GC content	46.01%	41.64%
BUSCO Complete score	81%	70%

Table 3-2| BLAST hits of the most significantly upregulated transcripts in *Spongilla lacustris* and *Aphrocallistes vastus*.

BLASTX was used to query the ten most significantly upregulated sequences from each sample against the NCBI protein database. The first BLAST hit is given. Fold change in expression level relative to the other sample condition is given as $\log_2$ of the value. The p-value for differential expression has been adjusted using the Benjamini-Hochberg procedure for multiple testing.

Transcript	Log_2FC	DE p_{adj}	NCBI Accession	BLASTX Hit	E-value
S. lacustris PON					
TR2129|c0_g2_i1	12.00	1.22e-211	N/A	No hit	N/A
TR20962|c0_g1_i1	10.82	3.84e-173	BAK19071.1	PL-toxin II	> 0
TR14949|c0_g2_i1	10.66	4.30e-118	XP_020901074.1	Cell-number regulator 2-like	5e-35
TR26506|c0_g2_i3	9.76	7.05e-108	WP_051502233.1	Uncharacterized protein	1e-75
TR26506|c0_g4_i2	11.08	6.84e-98	N/A	No hits	N/A
TR26504|c3_g3_i2	11.14	1.65e-92	XP_014774744.1	Kielin/chordin-like protein	4e-14
TR16074|c0_g1_i1	9.95	4.27e-75	XP_011408345.1	TNF receptor-associated factor 5-like	2e-71
TR26506|c0_g1_i1	9.97	3.18e-74	N/A	No hits	N/A
TR6845|c0_g1_i2	10.17	2.04e-71	XP_001335438.1	Natterin-like protein	> 0
TR18939|c1_g1_i1	11.4	5.34e-71	XP_013388481.1	Natterin-like protein	4e-5
S. lacustris juvenile					
TR27931|c0_g1_i1	7.82	9.17e-54	XP_013403007.1	Uncharacterized protein	8e-36
TR6441|c0_g1_i1	6.07	2.07e-47	XP_019855032.1	Uncharacterized protein	2e-37
TR24384|c2_g1_i19	5.65	2.62e-30	N/A	No hits	N/A
TR17207|c4_g1_i1	3.69	4.43e-27	XP_019849626.1	Mucin-like protein	4e-47
TR18170|c0_g1_i2	3.57	9.32e-26	XP_011410422.1	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit B-like	2e-107
TR26481|c1_g2_i2	10.04	7.08e-25	P18503.1	Short-chain collagen C4	3e-70
TR16347|c0_g1_i6	3.67	8.10e-25	XP_021002771.1	Nephrin-like	> 0
TR30701|c0_g1_i1	3.64	1.25e-24	KOF81273.1	Uncharacterized protein	4e-22
TR24426|c0_g2_i9	6.86	2.70e-24	XP_019851059.1	X-ray repair cross-complementing protein 5-like	9e-109
TR15578|c1_g8_i4	9.14	9.93e-21	XP_004994984.1	Hypothetical protein	3e-8
A. vastus body					
TR5655|c0_g3_i1	3.95	1.37e-35	XP_003384066.1	Dual oxidase 1-like	0.0
TR8845|c0_g1_i1	2.49	2.70e-35	XP_005488521.1	Xanthine dehydrogenase/oxidase	0.0
TR3959|c2_g3_i12	8.09	1.24e-32	XP_013410897.1	Uncharacterized protein	8e-13
TR2672|c1_g2_i1	2.76	7.58e-32	XP_008076081.1	SH3-domain-containing protein	> 0
TR12926|c0_g1_i3	6.34	4.69e-30	KHJ97811.1	Ubiquitin-protein ligase	1e-67
TR17255|c1_g2_i16	11.29	6.00e-25	XP_006892261.1	PX domain-containing protein kinase-like protein isoform X3	2e-111
TR2672|c1_g3_i4	2.44	1.98e-21	N/A	No hits	N/A
TR16173|c3_g4_i1	4.10	3.08e-19	WP_007595573.1	Sugar ABC transporter substrate-binding protein	> 0
TR7072|c0_g1_i5	9.49	4.23e-19	XP_015777477.1	Ubiquitin carboxyl-terminal hydrolase isozyme L3-like isoform X1	7e-80
TR778|c0_g1_i14	9.29	2.99e-18	XP_020513209.1	Suppression of tumorigenicity 5 protein isoform X2	2e-107
A. vastus osculum					
TR9593|c0_g1_i1	4.81	9.86e-80	BAS21353.1	Glassin	8e-34

TR15760|c0_g1_i1	5.47	2.19e-72	XP_019863042.1	Uncharacterized	2e-13
TR9660|c0_g1_i2	5.79	8.42e-69	N/A	No hits	N/A
TR11731|c0_g1_i1	4.51	5.51e-55	KOF98557.1	Uncharacterized protein	3e-7
TR3033|c0_g5_i1	5.67	5.21e-52	XP_005388779.1	ADAMTS-like protein 1 isoform X2	9e-26
TR1938|c0_g1_i1	6.20	1.82e-46	XP_020611710.1	Aquaporin 7-like	5e-60
TR6359|c0_g2_i3	3.35	1.51e-45	BAV53121.1	Brachyury transcription factor	1e-93
TR5619|c3_g3_i6	2.85	5.19e-45	XP_019633459.1	Plexin-A4-like	2e-168
TR8472|c0_g1_i1	4.62	2.20e-42	XP_018579510.1	Polycomb protein eed-B	6e-110
TR14639|c0_g1_i5	2.57	2.34e-42	XP_019853696.1	Cartilage-associated protein-like	2e-59

Figure 3-1| Functioning and development of the sponge osculum.

(A) Cross-sectional diagram of a juvenile *Spongilla lacustris* individual. Water is driven through canals by choanocyte chambers, ultimately exiting through a single osculum. (B) At the pre-oscular stage (PON), *S. lacustris* undergoes canal development and lacks an osculum. (C) At the juvenile stage, *S. lacustris* possesses a fully functioning canal system with an osculum. (D) The osculum of *Aphrocallistes vastus* is distinguishable from the rest of the body tissue as a flexible lip. osc, osculum. Scale bars: 1 mm.

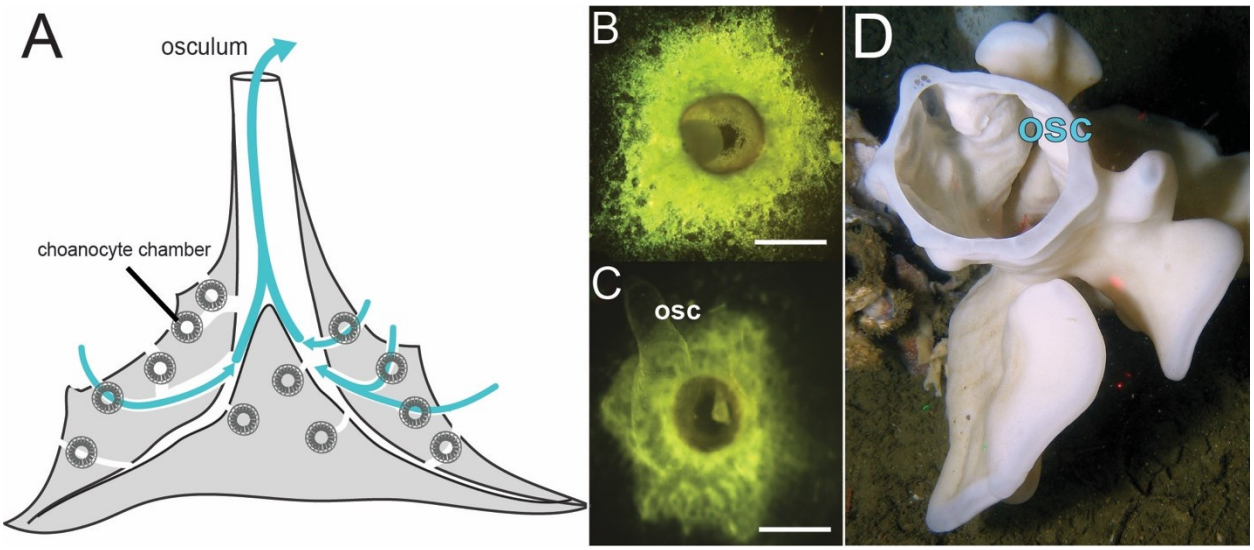

A
osculum
choanocyte chamber
B
C
osc
D
osc

Figure 3-2| Differential gene expression during oscular development and in the osculum.

Heatmaps of differentially expressed genes in (A) the pre-oscular (PON) and juvenile stages of *Spongilla lacustris* and (B) the body and oscular samples of *Aphrocallistes vastus*. Each row is a differentially expressed gene and each column is a biological replicate. Expression levels are indicated in the legend as median centred $\log_2$(TMM-normalized FPKM). Higher gene expression is indicated in yellow while lower gene expression is represented in purple.

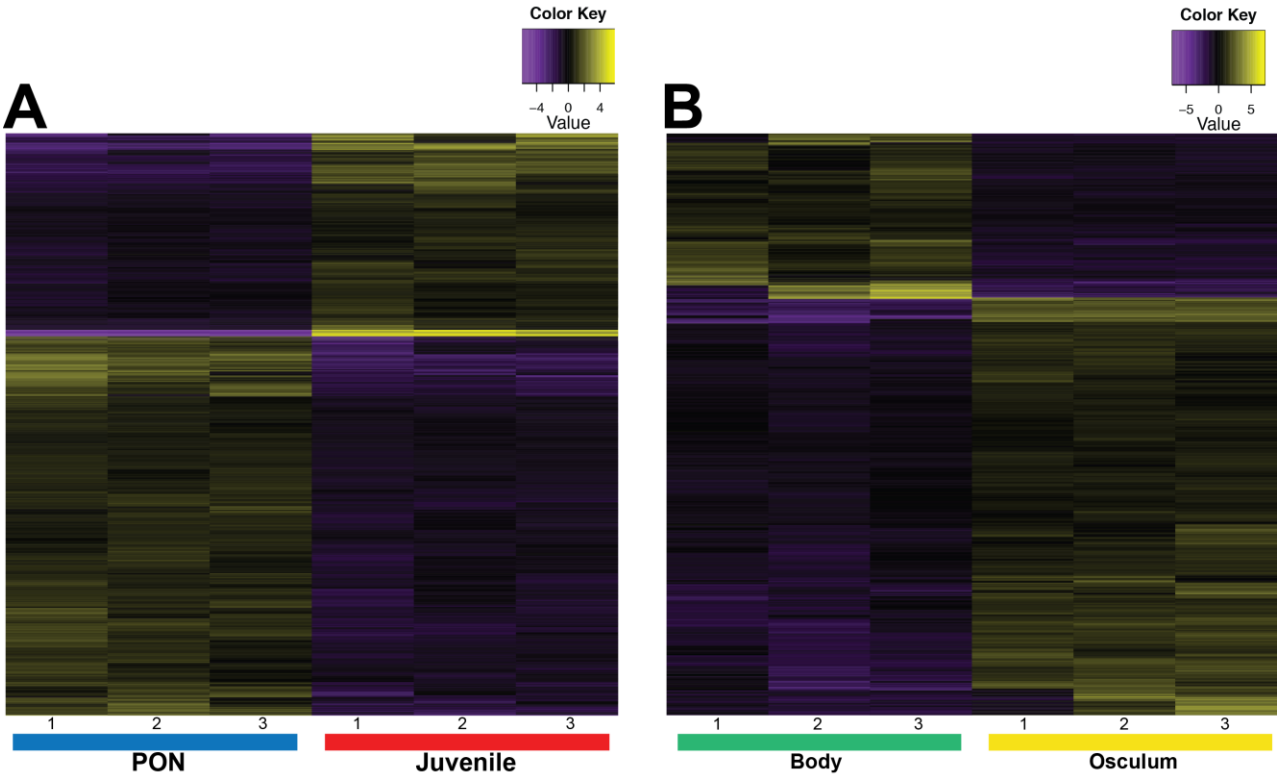

A
Color Key
−4 0 4
Value
1 2 3 1 2 3
PON
Juvenile
B
Color Key
−5 0 5
Value
1 2 3 1 2 3
Body
Osculum

Figure 3-3| Principal component analysis of gene expression levels in RNA-seq samples.

Principal component analysis of the expression profiles of (A) the *Spongilla lacustris* pre-oscular (PON) and juvenile stages, and (B) the *Aphrocallistes vastus* body and osculum tissue demonstrates the distinctness of each sample type. The first two principal components are given.

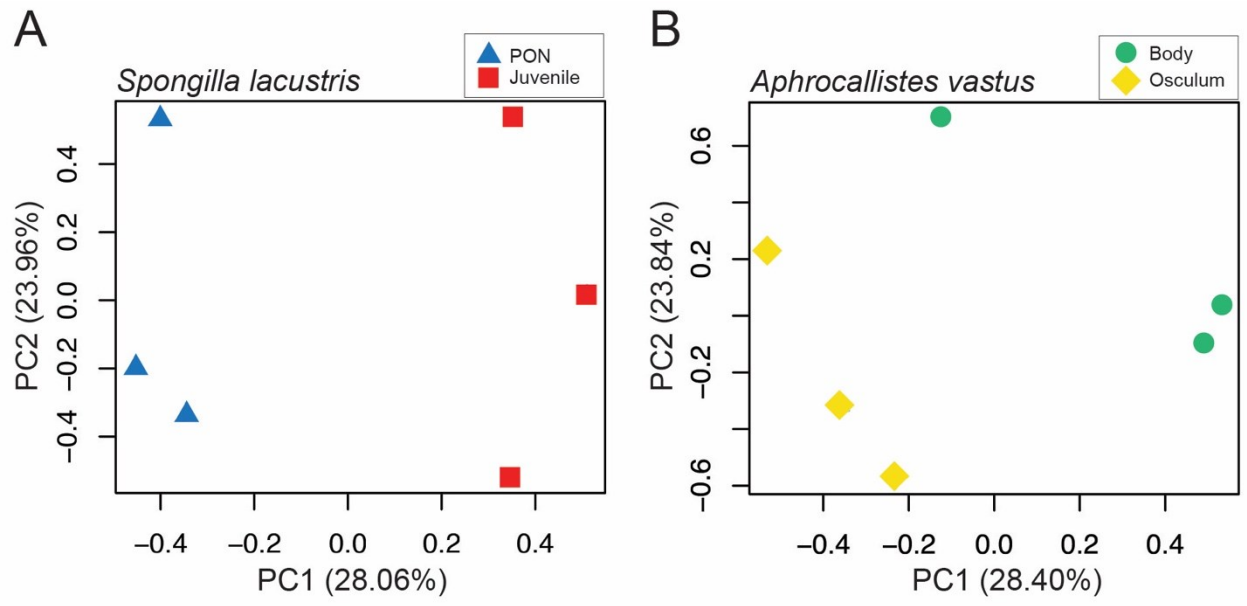

A
Spongilla lacustris
PON
Juvenile
PC2 (23.96%)
PC1 (28.06%)
B
Aphrocallistes vastus
Body
Osculum
PC2 (23.84%)
PC1 (28.40%)

Figure 3-4| Differential expression of sensory-neural markers in oscular tissues.

(A) Three genes (mGluR, GABAR and K_{ir}) were significantly upregulated in the *Spongilla lacustris* juvenile, which possesses an osculum. (B) In *Aphrocallistes vastus*, GABAR was significantly upregulated in the body tissue, while Bsh was significantly upregulated in the osculum. Candidate neural genes that are present but not significantly differentially expressed are portrayed in the upper heatmaps, while significantly differentially expressed genes are in a separate heatmap below this. The p-value for differential expression is given below each significantly differentially expressed gene, with $p< 0.05$ as the cutoff. The p-value for differential expression has been adjusted using the Benjamini-Hochberg procedure for multiple testing. The legend indicates expression levels as TMM-normalized FPKM. Red indicates higher expression while yellow represents lower expression.

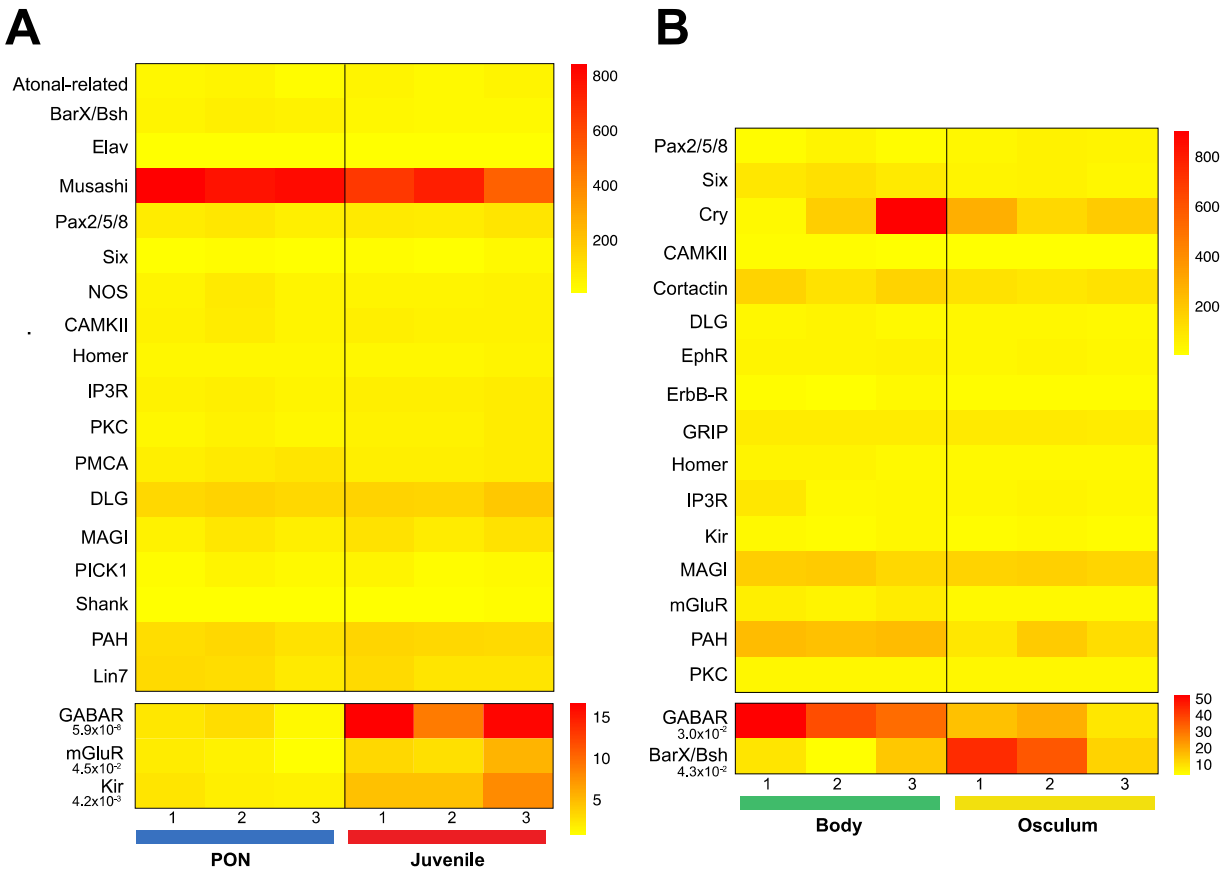

A
Atonal-related
BarX/Bsh
Elav
Musashi
Pax2/5/8
Six
NOS
CAMKII
Homer
IP3R
PKC
PMCA
DLG
MAGI
PICK1
Shank
PAH
Lin7
800
600
400
200
GABAR
5.9x10-8
mGluR
4.5x10-2
Kir
4.2x10-3
15
10
5
1 2 3 1 2 3
PON
Juvenile
B
Pax2/5/8
Six
Cry
CAMKII
Cortactin
DLG
EphR
ErbB-R
GRIP
Homer
IP3R
Kir
MAGI
mGluR
PAH
PKC
800
600
400
200
GABAR
3.0x10-2
BarX/Bsh
4.3x10-2
50
40
30
20
10
1 2 3 1 2 3
Body
Osculum

Figure 3-5| Alignment of the transmembrane and pore domains of K_{ir} sequences.

Solid black bars above the sequences mark transmembrane region 1 (M1) and 2 (M2), and the pore loop (P). The pore-domain motif is highlighted in red. Residue 171, which affects the strength of inward rectification, is marked in blue. Dots indicate that a portion of the alignment has been skipped. Number of amino acid positions follow Hsap_Kir1.1 (accession: NP_000211.1). Hsap, *Homo sapiens*, Sla, *Spongilla lacustris*, Aqu, *Amphimedon queenslandica*, Ifas, *Ircinia fasciculata,* Pfi, *Petrosia ficiformis,* Psu, *Pseudospongosorites suberitoides*, Ava, *Aphrocallistes vastus,* Sco, *Sycon coactum*, Cca, *Corticium candelabrum*.

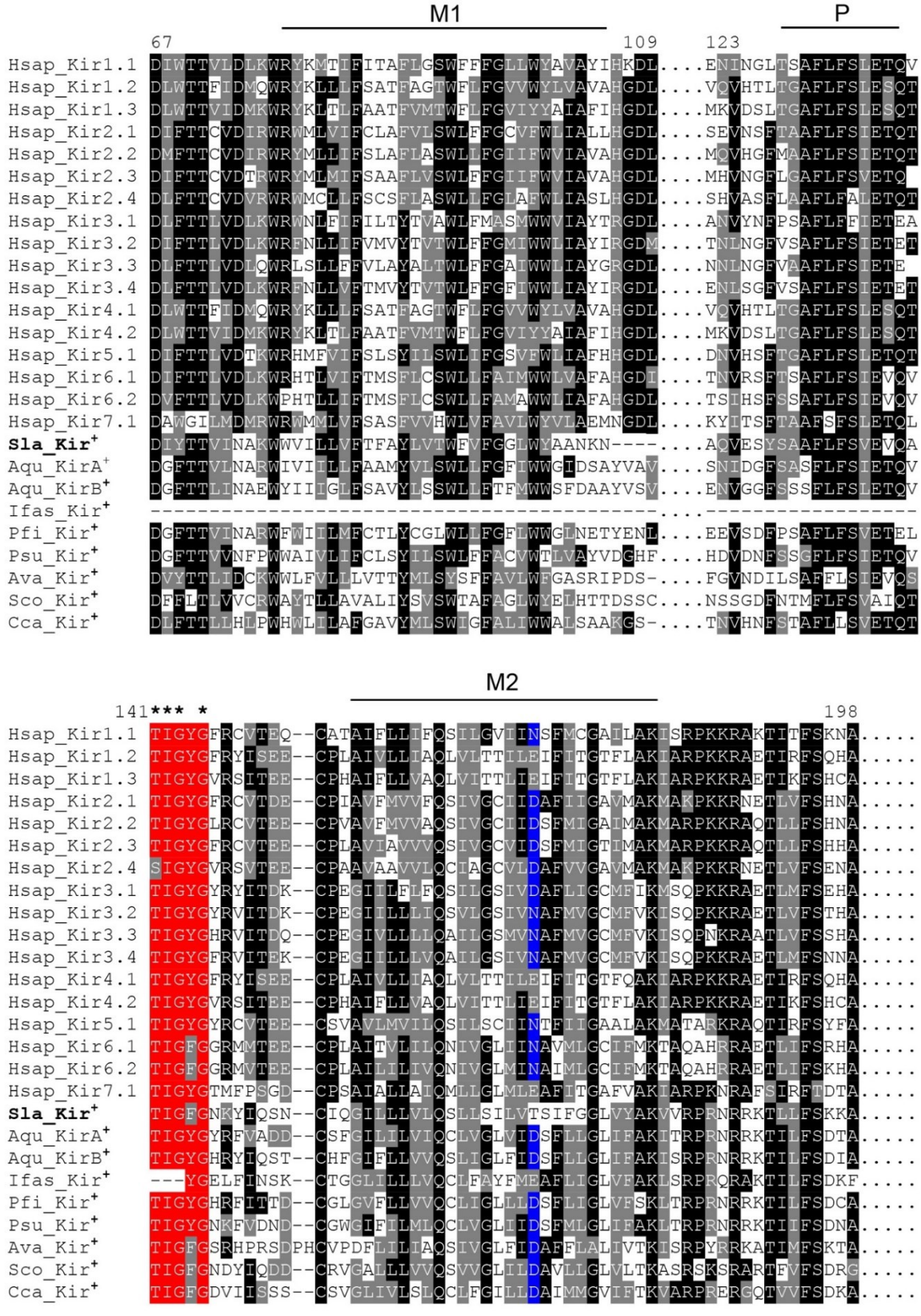

M1
P
67
109
123
Hsap_Kir1.1
Hsap_Kir1.2
Hsap_Kir1.3
Hsap_Kir2.1
Hsap_Kir2.2
Hsap_Kir2.3
Hsap_Kir2.4
Hsap_Kir3.1
Hsap_Kir3.2
Hsap_Kir3.3
Hsap_Kir3.4
Hsap_Kir4.1
Hsap_Kir4.2
Hsap_Kir5.1
Hsap_Kir6.1
Hsap_Kir6.2
Hsap_Kir7.1
Sla_Kir+
Aqu_KirA+
Aqu_KirB+
Ifas_Kir+
Pfi_Kir+
Psu_Kir+
Ava_Kir+
Sco_Kir+
Cca_Kir+
M2
141
198
Hsap_Kir1.1
Hsap_Kir1.2
Hsap_Kir1.3
Hsap_Kir2.1
Hsap_Kir2.2
Hsap_Kir2.3
Hsap_Kir2.4
Hsap_Kir3.1
Hsap_Kir3.2
Hsap_Kir3.3
Hsap_Kir3.4
Hsap_Kir4.1
Hsap_Kir4.2
Hsap_Kir5.1
Hsap_Kir6.1
Hsap_Kir6.2
Hsap_Kir7.1
Sla_Kir+
AquKirA+
AquKirB+
Ifas_Kir+
Pfi_Kir+
Psu_Kir+
Ava_Kir+
Sco_Kir+
Cca_Kir+

Figure 3-6| Alignment of the homeodomains of brain-specific homeobox (Bsh) and Bar.

Bsh-specific residues are highlighted in red while diagnostic Bar residues are in blue. The *Aphrocallistes vastus* Bsh sequence found in this study is highlighted in bold, and all sponge sequences are indicated with a '+'. Bsh or Bar-specific residues present in the *A. vastus* sequence is highlighted with a *. Hsap, *Homo sapiens,* Mmus, *Mus musculus*, Dme, *Drosophila melanogaster*, Adi, *Acropora digitifera*, Mle, *Mnemiopsis leidyi*, Ava, *Aphrocallistes vastus*, Hbo, *Halichondria bowerbanki*, Aqu, *Amphimedon queenslandica*.

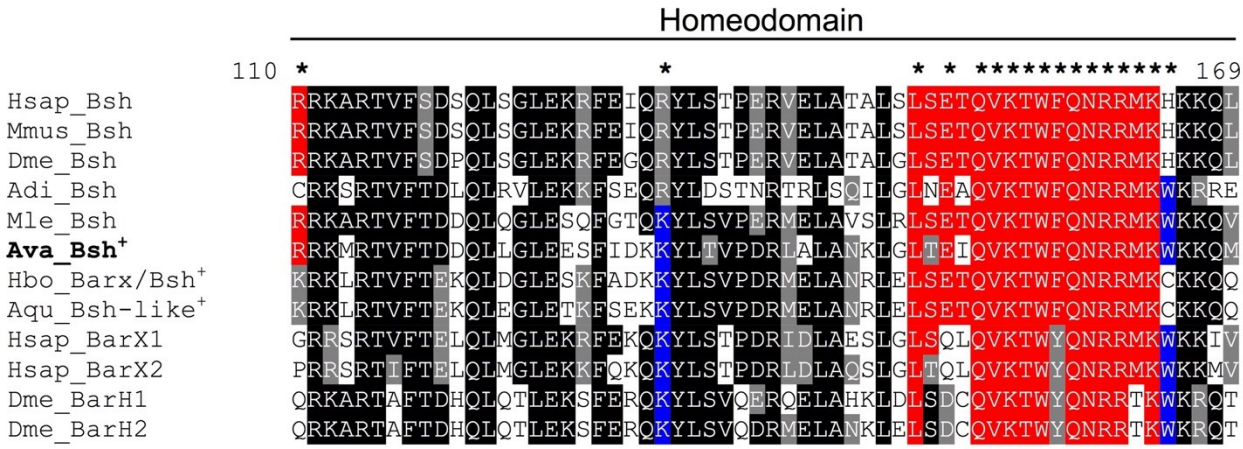

Homeodomain
110
169
Hsap_Bsh
Mmus_Bsh
Dme_Bsh
Adi_Bsh
Mle_Bsh
Ava_Bsh+
Hbo_Barx/Bsh+
Aqu_Bsh-like+
Hsap_BarX1
Hsap_BarX2
Dme_BarH1
Dme_BarH2

Figure 3-7| Significantly enriched gene ontology terms for *Spongilla lacustris* and *Aphrocallistes vastus*.

Biological process gene ontology (GO) terms for the *S. lacustris* (A) pre-oscular and (B) juvenile stages and the *A. vastus* (C) body and (D) oscular samples. The five most significantly enriched GO terms are indicated by the red bars. Below these, the five most significantly enriched neural-related GO terms are portrayed with the blue bars. Significance values are represented as $-\log_{10}$ of the p-value for significant over-representation. Non-neural GO terms associated with the *S. lacustris* pre-oscular and juvenile stages, and the *A. vastus* osculum were more significantly enriched than neural GO terms (A, B, D). However, in the *A. vastus* body sample several neural-related GO terms (indicated by *) were among the most significantly enriched terms overall (C).

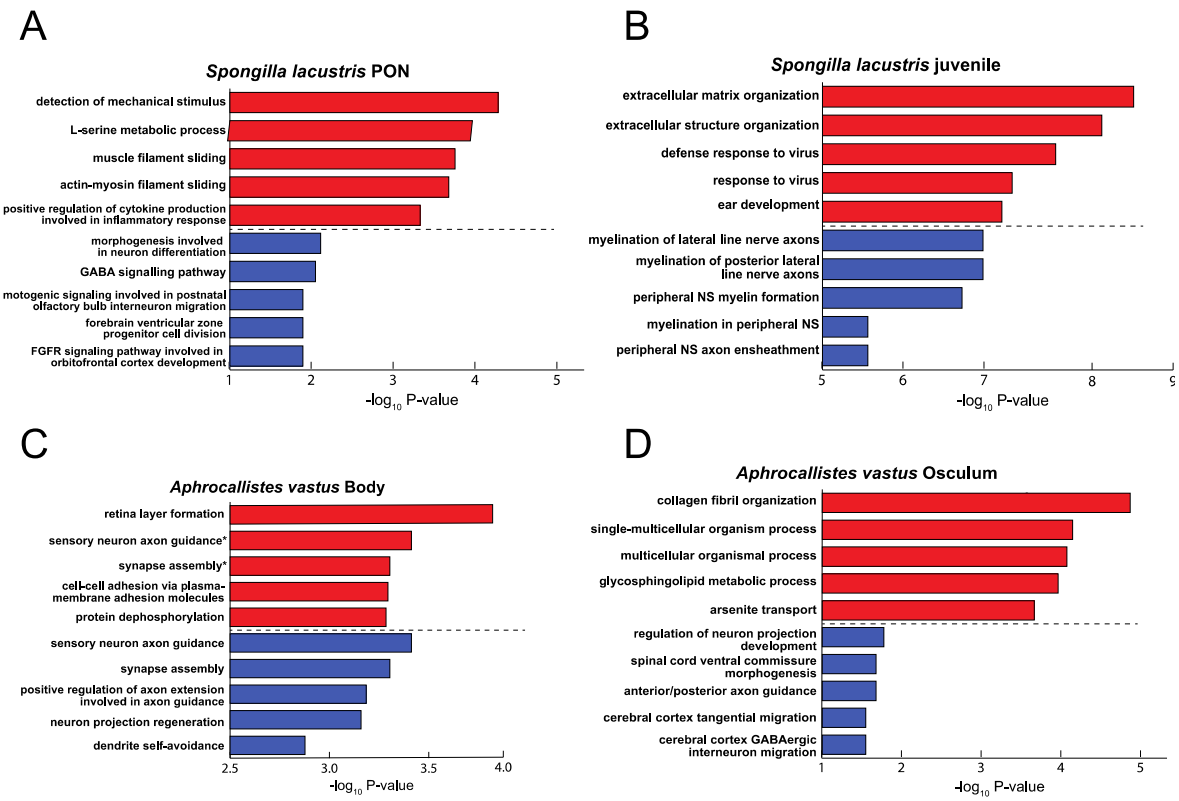
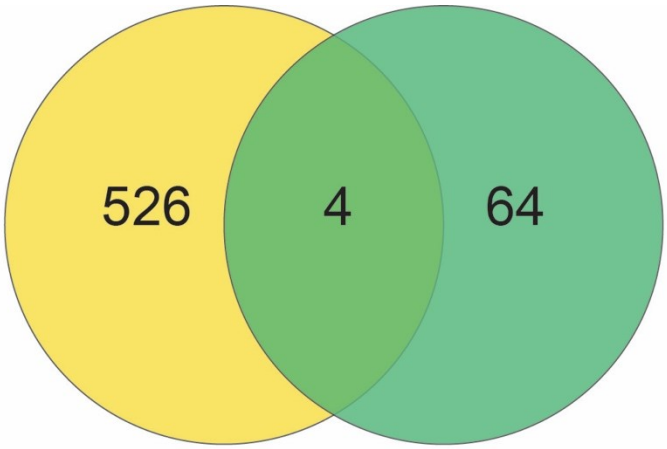

A
Spongilla lacustris PON
detection of mechanical stimulus
L-serine metabolic process
muscle filament sliding
actin-myosin filament sliding
positive regulation of cytokine production involved in inflammatory response
morphogenesis involved in neuron differentiation
GABA signalling pathway
motogenic signaling involved in postnatal olfactory bulb interneuron migration
forebrain ventricular zone progenitor cell division
FGFR signaling pathway involved in orbitofrontal cortex development
1
2
3
4
5
-log10 P-value
B
Spongilla lacustris juvenile
extracellular matrix organization
extracellular structure organization
defense response to virus
response to virus
ear development
myelination of lateral line nerve axons
myelination of posterior lateral line nerve axons
peripheral NS myelin formation
myelination in peripheral NS
peripheral NS axon ensheathment
5
6
7
8
-log10 P-value
C
Aphrocallistes vastus Body
retina layer formation
sensory neuron axon guidance*
synapse assembly*
cell-cell adhesion via plasma-membrane adhesion molecules
protein dephosphorylation
sensory neuron axon guidance
synapse assembly
positive regulation of axon extension involved in axon guidance
neuron projection regeneration
dendrite self-avoidance
2.5
3.0
3.5
4.0
-log10 P-value
D
Aphrocallistes vastus Osculum
collagen fibril organization
single-multicellular organism process
multicellular organismal process
glycosphingolipid metabolic process
arsenite transport
regulation of neuron projection development
spinal cord ventral commissure morphogenesis
anterior/posterior axon guidance
cerebral cortex tangential migration
cerebral cortex GABAergic interneuron migration
1
2
3
4
5
-log10 P-value

Figure 3-8| The *Aphrocallistes vastus* osculum and *Sycon ciliatum* top region share few significantly upregulated orthologues

(A) The number of significantly upregulated transcripts in the *A. vastus* osculum (left, yellow) and the *S. ciliatum* top region (right, green), which contains the osculum. Four orthologues were shared between them. (B) The top blast hit (BLASTP) for each of the four shared orthologues, each row being a separate orthologue. The hit for the *A. vastus* orthologue is given in the left column and the hit for the corresponding *S. ciliatum* orthologue in the right column. Given below the name of the top hit is the NCBI accession number and the E-value of the top hit.

A



B

	Aphrocallistes vastus	*Sycon ciliatum*
1	**thyroid receptor-interacting protein 6-like** XP_013794212.1, 3e-50	**paxillin-like** XP_013775220.1, 2e-127
2	**peptidase inhibitor 15-like** XP_011422872.1, 5e-41	**peptidase inhibitor 16** XP_014393960.1, 2e-31
3	**GLIPR1-like protein 1** XP_012623892.1, 2e-35	**peptidase inhibitor 16-like** XP_016403951.1, 1e-32
4	**multidrug resistance-associated protein 4** XP_007196906.1, 6e-147	**multidrug resistance-associated protein 4-like** XP_019646603.1, 0.0

Chapter 4. General discussion and directions for future research

The identity of the earliest diverging metazoan can no longer be taken for granted. Long held to be sponges, some hypothesize that ctenophores may hold this title (e.g. Dunn et al. 2008, Ryan et al. 2013, Moroz et al. 2014, Whelan et al. 2015). The potential of a ctenophore-first history of animal evolution has challenged the intuitive, stepwise thinking that quietly dominates the study of character evolution. Given that ctenophores have recognizable complex characters, such as striated muscles, a through-gut, and a nervous system (Hernandez-Nicaise 1973, Mackie et al. 1988, Presnell et al. 2016), in this scenario these characters must have arisen convergently or, unintuitively, were lost in sponges and placozoans. While a stepwise view of character evolution is widely acknowledged as misplaced, it nevertheless persists and often implicitly underlies studies on sponges (eg. Leininger et al. 2014, Fernandez-Valverde and Degnan 2016).

The current debate on whether sponges have a proto-nervous system or have undergone nervous system loss circles around the presence of what are often referred to as 'neural' genes in sponges (Richards et al. 2008, Srivastava et al. 2010b, Ryan and Chiodin 2015). To both sides of the nervous system debate, these genes signal the presence of a homologous sensory system in sponges. At this time there are no techniques to directly test gene function or interactions in sponges making it difficult to predict whether presumptive neural genes do in fact give rise to sponge sensation. Yet in evolutionary development a crucial test for homology is whether the compared structures or phenotypes emerge from the same genetic mechanism (Brigandt 2003).

This thesis investigates whether sponge sensory structures express a 'neural' genetic signal. Chapter Two examines whether 'neural' genes, termed sensory-neural markers, are more frequently expressed in sponge sensory structures compared to their expression in putatively non-sensory cell types. Chapter Three explores whether the osculum expresses a distinct genetic signal and whether sensory-neural markers are among the set of upregulated oscular genes. Here, in Chapter Four I discuss the challenges, limitations, and future directions that have emerged from this work.

4.1. Chapter Two: The challenge of defining 'sensory'

Chapter Two arose from a symposium on the development and evolution of sensory cells and organs at the 2016 EuroEvoDevo meeting, where SPL gave a presentation. Initially meant to

encompass only those studies examining sensory-neural marker expression in sponges, I came to realize that an unbiased interpretation could only occur in the context of all gene expression studies. Ultimately, given the fact that many putatively non-sensory structures also express sensory-neural markers, I concluded that the use of these markers to predict sensory functions in a sponge is not robust in the absence of other experimental work.

Our figures explicitly defined which genes are sensory and which are not. My approach to this consisted of determining whether a gene possessed any sensory or neural function in an invertebrate and vertebrate, according to the literature. Ultimately, however, these classifications were an interpretation. For example, I classified nanos as non-sensory given its prominent role in germ cell specification (Tsuda et al. 2003), though it does have neural function (Kanska and Franck 2013, Bhogal et al. 2016). This did not change the overall interpretation of the data, but must be considered nevertheless. Conceptually, the definition of 'sensory' is challenging. Most if not all cells are sensory to a certain degree. Not only might cells and organs hold multiple functions, genes may too. For instance, genes involved in axon guidance may also play a role in blood vessel formation (Woods et al. 1996, Carmeliet and Tessier-Lavigne 2005, Julio-Pieper et al. 2011). 'Sensory' is not often a discrete classification. The operational definition of 'sensory' used in Chapter Two is open to a degree of subjective interpretation.

4.2. Chapter Three: The technical challenges of non-bilaterian biology

In Chapter Three I sought to determine whether there was a defined set of genes expressed in the sponge osculum, and whether any of these genes were sensory-neural markers. I uncovered four significantly differentially expressed sensory-neural markers in *S. lacustris* and *A. vastus*. Previous experiments suggest that sponge orthologues of mGluR, GABAR and K_{ir} may hold similar functions to their counterparts in Bilateria (Ellwanger et al. 2007, Tompkins-MacDonald et al. 2009, Elliott and Leys 2010). However, it is important to note that only bilaterian functions have been tested for. Experiments involving the application of neuroactive molecules in sponges is not unlike the candidate gene approach (e.g. Ellwanger and Nickel 2006, reviewed in Nickel 2010). In the case of K_{ir},, functional tests were performed in a bilaterian cell (Tompkins-MacDonald et al. 2009). The mGluR and GABAR sequences from *S. lacustris* are promising targets for future experiments given the role of glutamate and GABA in the inflation-contraction behavior in *Ephydatia muelleri.* But mGluRs and GABARs have undergone an expansion in demosponges (Francis et al. 2017) so it is possible that these are not the same receptors targeted in Elliott and Leys (2010). Evidence is converging to suggest that in

these instances there may be conserved bilaterian-like genetic modules underlying the osculum, but synthesis of these ideas remains elusive for now.

Non-model organisms present many challenges. I encountered two particular difficulties during the design and execution of the RNA-seq study. The first was optimization of my transcriptome assemblies. I initially used a previously assembled transcriptome of the same species as a benchmark for transcriptome completeness and quality. However, given that the LC Sciences constructed transcriptome was made from RNA derived from a different specimen collected at a different time and sequenced on a different sequencer, it may not have been valid to expect the two to have identical transcriptome quality metrics. Furthermore, it is not possible to know a priori whether one assembly is more 'correct' than the other. Contaminant filtering also proved difficult. For reference-based filtering, a database of many, varied sequences of the study organism must be available. The NCBI protein database contains only ~35,000 sponge sequences – fewer than my transcriptomes – with ~69% originating from *Amphimedon queenslandica*. If I were to consider only sequences that blasted to sponge sequences, I would select only those that resembled sequences in one sponge. It is important to search more broadly, but it is unclear how far to go. Not all sequences may appear metazoan-like given that sponges are early diverging. Early in my degree I attempted to optimize a non-reference based method of contaminant filtering, which capitalized on the fact that GC content and codon usage were characteristic of an organism. However, this method did not provide enough resolution.

4.3. Future directions

Pursuing non-bilaterian biology at the molecular level, when the gene networks of many non-model bilaterians are equally unknown, will be extremely difficult. However, the molecular technology, and thus the chance to do so, exists.

It is challenging to take an integrated approach to studying gene function in sponges. While genes themselves can be sequenced, it is difficult to connect this to an emergent phenotype like sensation or behavior. Meanwhile, behavioral-level experiments cannot identify the genes underlying these behaviors. The first step may be to perform *in situ* hybridizations of the significantly upregulated genes I found in Chapter Three to confirm their localization. This can be followed by the approach taken by Perovic et al. (1999), Elliott and Leys (2010), and others with the application of agonists and antagonists to determine whether these genes play a role in behavior or sensation. As it is difficult to connect the results of these physiological experiments with the exact gene they manipulate, the results of an RNAi experiment targeting

the candidate genes can be compared to the phenotype that results from the physiological experiments. However these approaches target candidate genes with the assumption that they function similarly to bilaterian genes. I would then perform pull-downs to identify whether the candidate genes undergo uncharacterized, non-bilaterian interactions. If uncharacterized proteins are found to not only interact with the candidate gene but are also themselves highly upregulated in the osculum, they become the next targets for experiments.

Genes that have been lost may be equally important to the origin of the nervous system as genes that have been conserved to Bilateria. If I hypothesize that sensory organs, or genetic modules within them, are homologous across Metazoa, there may exist a core set of defining, conserved orthologues. With my assembly of the *A. vastus* osculum, there are now assembled transcriptomes of major sensory organs for all non-bilaterian phyla (excluding Placozoa, which has no identified sensory organ) (Moroz et al. 2014, Ames et al. 2016). Using OrthoMCL, it is possible to identify shared orthologues between these organs (Li et al. 2003). Following this, identifying when the shared set of genes arose or were lost can be achieved with EvolMap, a program that predicts the ancestral gene content at nodes (Sakarya et al. 2008). The set of genes that were gained at the dawn of Metazoa and subsequently lost in Bilateria would prove to be exciting targets for future work. Little manipulative genetic techniques exist for sponges, but better optimized systems are available for ctenophores and cnidarians. Finding conserved interactions shared between ctenophores and cnidarians would allow us to make inferences on the role these 'hidden' genes may have played in the origin of sensory organs in Metazoa.

4.4. Conclusion

This thesis examines the presence and interpretation of sensory-neural marker expression in sponges. I find that sensory-neural marker expression does not strongly correlate to sponge sensory structures. Sensory-neural marker genes are expressed in the *A. vastus* osculum and *S. lacustris* juvenile but it is not possible to determine the function of these upregulated genes in my study organisms. The presence of sensory-neural marker expression is sometimes interpreted to signal a sensory function and, perhaps, homology to the bilaterian nervous system. However my results suggest that in the context of sponge biology there is not sufficient data for this approach to be robust. Examining sensory-neural markers in a non-bilaterian context may yield deep insight into the origins of the nervous system.

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Appendix 1 Supplemental material for Chapter Two

Procedure for selecting expression patterns around sensory structures

Gene expression patterns that overlapped with the photoreceptive organ or were adjacent to its outer border during the ring to larval stages were reported. Expression domains that featured exclusively in the centre of the ring, but not overlapping with the ring, were not included.

Gene expression patterns that were more strongly expressed at the osculum, in a ring around the osculum, or in cells localized around the osculum, were considered oscular expression patterns.

Table S2-1| Corresponding references listed in Figure 2-2, Figure 2-3, and Supplemental Figure S2-1.

Reference Number	Citation
1	Fortunato et al. (2014a)
2	Rivera et al. (2012)
3	Fahey et al. (2008)
4	Srivastava et al. (2010a)
5	Richards et al. (2008)
6	Fortunato et al. (2016)
7	Sakarya et al. (2007)
8	Ueda et al. (2016)
9	Rivera et al. (2013)
10	Fortunato et al. (2014b)
11	Nakayama et al. (2015)
12	Fortunato et al. (2012)
13	Okamoto et al. (2012)
14	Larroux et al. (2006)
15	Leininger et al. (2014)
16	Steinmetz et al. (2012)
17	Gauthier and Degnan (2008)
18	Gauthier et al. (2010)
19	Alie et al. (2015)
20	Funayama et al. (2010)
21	Nakanishi et al. (2014)
22	Funayama et al. (2005a)
23	Funayama et al. (2005b)
24	Mohri et al. (2008)
25	Richards and Degnan (2012)
26	Adamska et al. (2007b)
27	Adamska et al. (2007a)
28	Adamska et al. (2010)

Table S2-2| Available assembled sponge transcriptomes.

Species	Class	Reference
Amphimedon queenslandica	Demospongiae	Srivastava et al. (2010b), Fernandez-Valverde et al. (2015)
Aphrocallistes vastus	Hexactinellida	Riesgo et al. (2014)
Chondrilla nucula	Demospongiae	Riesgo et al. (2014)
Corticium candelabrum	Homoscleromorpha	Riesgo et al. (2014)
Crambe crambe	Demospongiae	Versluis et al. (2015)
Crella elegans	Demospongiae	Perez-Porro et al. (2013)
Ephydatia fluviatilis	Demospongiae	Alie et al. (2015)
Ephydatia muelleri	Demospongiae	Pena et al. (2016)
Haliclona amboinensis	Demospongiae	Guzman and Conaco (2016)
Haliclona tubifera	Demospongiae	Guzman and Conaco (2016)
Halisarca dujardini	Demospongiae	Borisenko et al. (2016)
Ircinia fasciculata	Demospongiae	Riesgo et al. (2014)
Leucosolenia complicata	Calcarea	Fortunato et al. (2014a)
*Oscarella sp.**	Homoscleromorpha	Nichols et al. (2012)
Oscarella carmela	Homoscleromorpha	Nichols et al. (2012)
Petrosia ficiformis	Demospongiae	Riesgo et al. (2014)
Pseudospongosorites suberitoides	Demospongiae	Riesgo et al. (2014)
Scopalina sp.	Demospongiae	Francis et al. (2017)
Spongilla lacustris	Demospongiae	Riesgo et al. (2014)
Stylissa carteri	Demospongiae	Ryu et al. (2016)
Sycon ciliatum	Calcarea	Fortunato et al. (2014a)
Sycon coactum	Calcarea	Riesgo et al. (2014)
Tedania anhelens	Demospongiae	Francis et al. (2017)
Xestospongia testudinaria	Demospongiae	Ryu et al. (2016)

*A sister species to *O. carmela* lodged at Compagen

Figure S2-1| Meta-analysis of structural and cell-specific localization of all gene expression patterns surveyed.

Gene names are given as they were referred to in the cited text, except for procollagen lysyl hydroxylase, which was abbreviated to PLOD. Unstable classifications are indicated with * (see Fortunato et al. (2012), Schnitzler et al. (2014)). A blank indicates absence of data or that expression was not detected. Blue triangle: *Amphimedon queenslandica*, green circle: *Ephydatia muelleri* or *Ephydatia fluviatilis*, red square: *Sycon ciliatum*. References are listed in Supplemental Table S2-1.

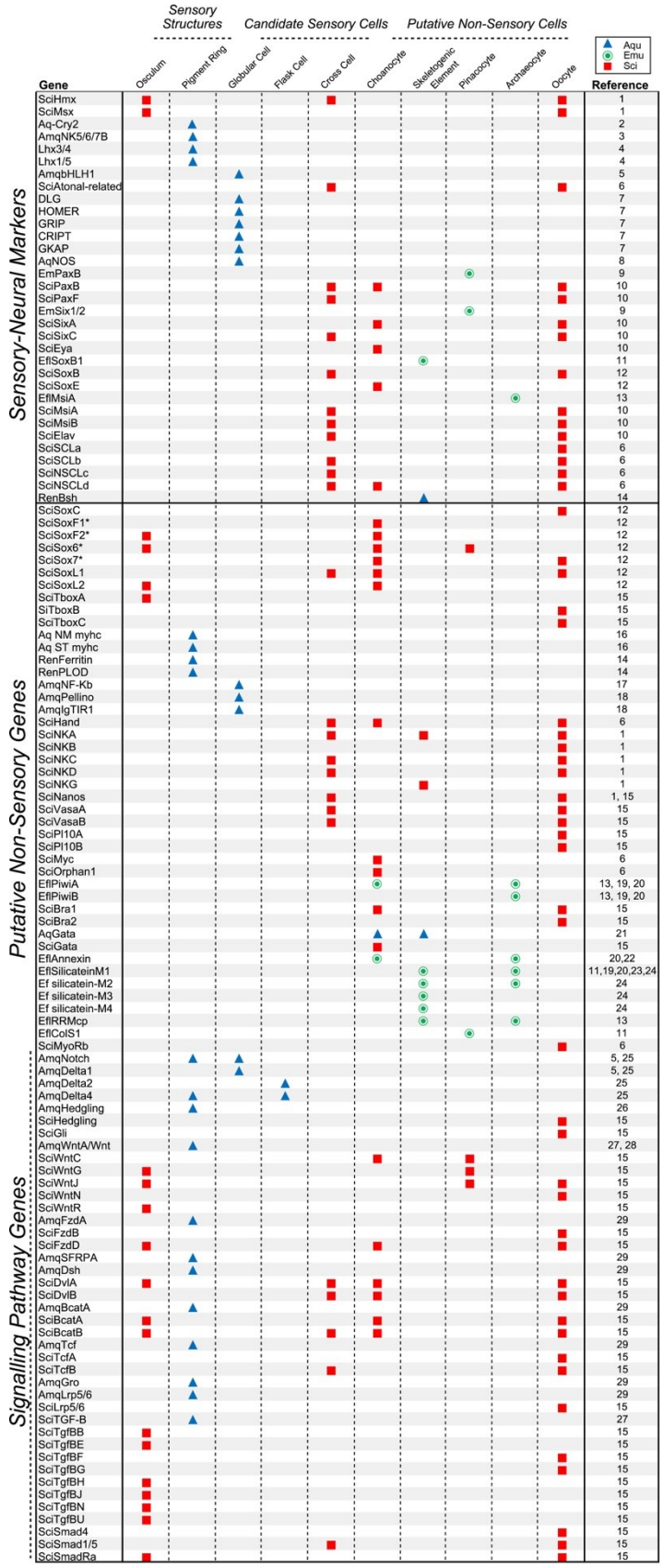

Sensory Structures
Candidate Sensory Cells
Putative Non-Sensory Cells
Aqu
Emu
Sci
Osculum
Pigment Ring
Globular Cell
Flask Cell
Cross Cell
Choanocyte
Skeletogenic Element
Pinacocyte
Archaeocyte
Oocyte
Gene
Reference
Sensory-Neural Markers
Putative Non-Sensory Genes
Signalling Pathway Genes
SciHmx 1
SciMsx 1
Aq-Cry2 2
AmqNK5/6/7B 3
Lhx3/4 4
Lhx1/5 4
AmqbHLH1 5
SciAtonal-related 6
DLG 7
HOMER 7
GRIP 7
CRIPT 7
GKAP 7
AqNOS 8
EmPaxB 9
SciPaxB 10
SciPaxF 10
EmSix1/2 9
SciSixA 10
SciSixC 10
SciEya 10
EflSoxB1 11
SciSoxB 12
SciSoxE 12
EflMsiA 13
SciMsiA 10
SciMsiB 10
SciElav 10
SciSCLa 6
SciSCLb 6
SciNSCLc 6
SciNSCLd 6
RenBsh 14
SciSoxC 12
SciSoxF1* 12
SciSoxF2* 12
SciSox6* 12
SciSox7* 12
SciSoxL1 12
SciSoxL2 12
SciTboxA 15
SiTboxB 15
SciTboxC 15
Aq NM myhc 16
Aq ST myhc 16
RenFerritin 14
RenPLOD 14
AmqNF-Kb 17
AmqPellino 18
AmqIgTIR1 18
SciHand 6
SciNKA 1
SciNKB 1
SciNKC 1
SciNKD 1
SciNKG 1
SciNanos 1, 15
SciVasaA 15
SciVasaB 15
SciPl10A 15
SciPl10B 15
SciMyc 6
SciOrphan1 6
EflPiwiA 13, 19, 20
EflPiwiB 13, 19, 20
SciBra1 15
SciBra2 15
AqGata 21
SciGata 15
EflAnnexin 20,22
EflSilicateinM1 11,19,20,23,24
Ef silicatein-M2 24
Ef silicatein-M3 24
Ef silicatein-M4 24
EflRRMcp 13
EflColS1 11
SciMyoRb 6
AmqNotch 5, 25
AmqDelta1 5, 25
AmqDelta2 25
AmqDelta4 25
AmqHedgling 26
SciHedgling 15
SciGli 15
AmqWntA/Wnt 27, 28
SciWntC 15
SciWntG 15
SciWntJ 15
SciWntN 15
SciWntR 15
AmqFzdA 29
SciFzdB 15
SciFzdD 15
AmqSFRPA 29
AmqDsh 29
SciDvlA 15
SciDvlB 15
AmqBcatA 29
SciBcatA 15
SciBcatB 15
AmqTcf 29
SciTcfA 15
SciTcfB 15
AmqGro 29
AmqLrp5/6 29
SciLrp5/6 15
SciTGF-B 27
SciTgfBB 15
SciTgfBE 15
SciTgfBF 15
SciTgfBG 15
SciTgfBH 15
SciTgfBJ 15
SciTgfBN 15
SciTgfBU 15
SciSmad4 15
SciSmad1/5 15
SciSmadRa 15

Figure S2-2| Examples of gene expression patterns in each cell type featured in Figures 2-3, 2-4 and Supplemental Figure S2-1.

Expression of musashi in archaeocytes (A), NOS in globular cells (B), delta in flask cells (C), Sox6 in choanocytes and pinacocytes (D), PaxB in oocytes (E), Smad1/5 in cross cells (F), and silicatein in sclerocytes (G). (C) is one of three images showing punctate expression, which was interpreted by Richards and Degnan (2012) as occurring in flask cells. Images are from (A) Okamoto et al. (2012), (B) Ueda et al. (2016), (C) Richards and Degnan (2012), (D) Fortunato et al. (2012), (E) Fortunato et al. (2014b), (F) Leininger et al. (2014), and (G) Nakayama et al. (2015). pp indicates posterior pole of *Amphimedon queenslandica* larva. pin and ch indicate the pinacocytes and choanocytes of the *Sycon ciliatum* larva. Scale bars in original publications.

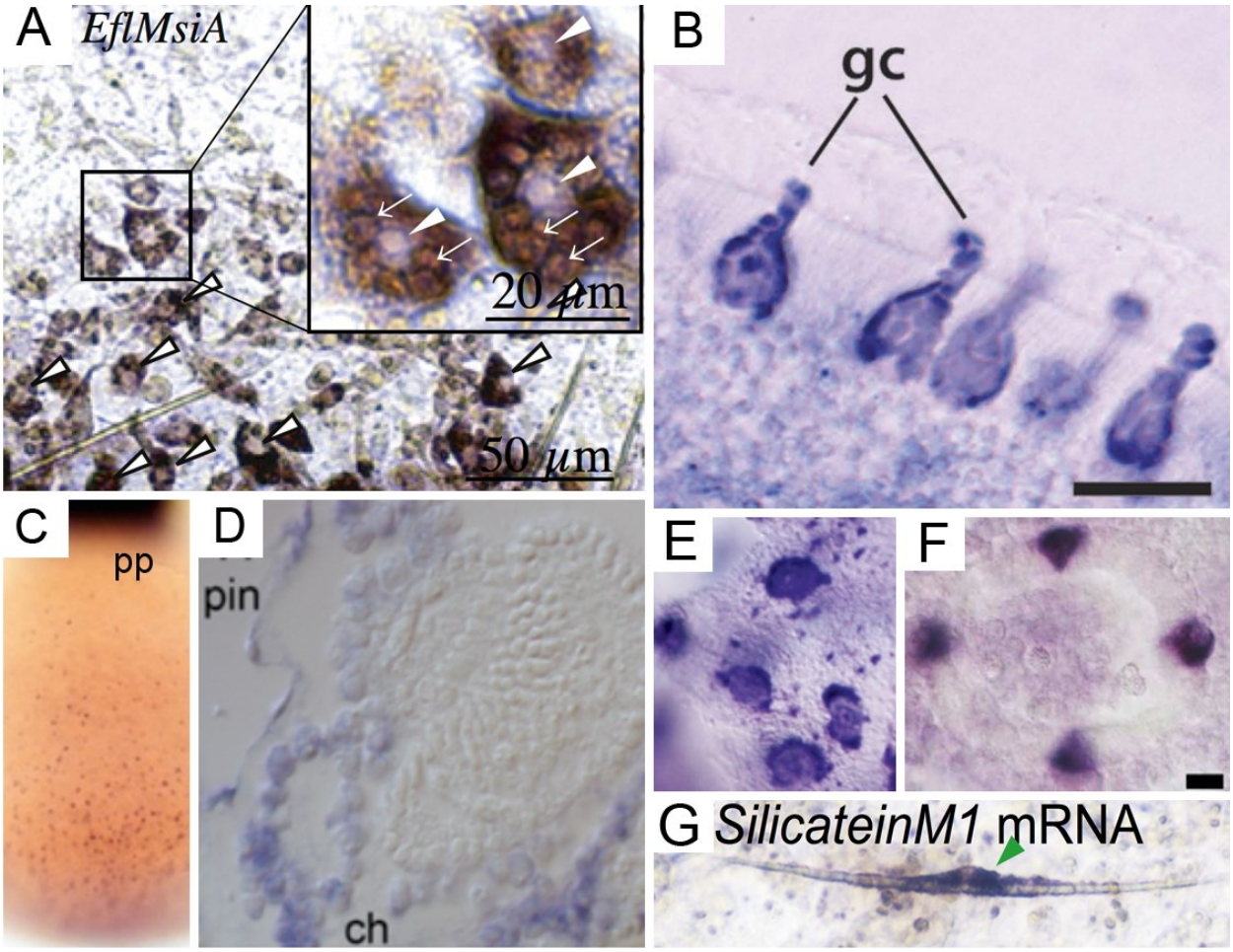
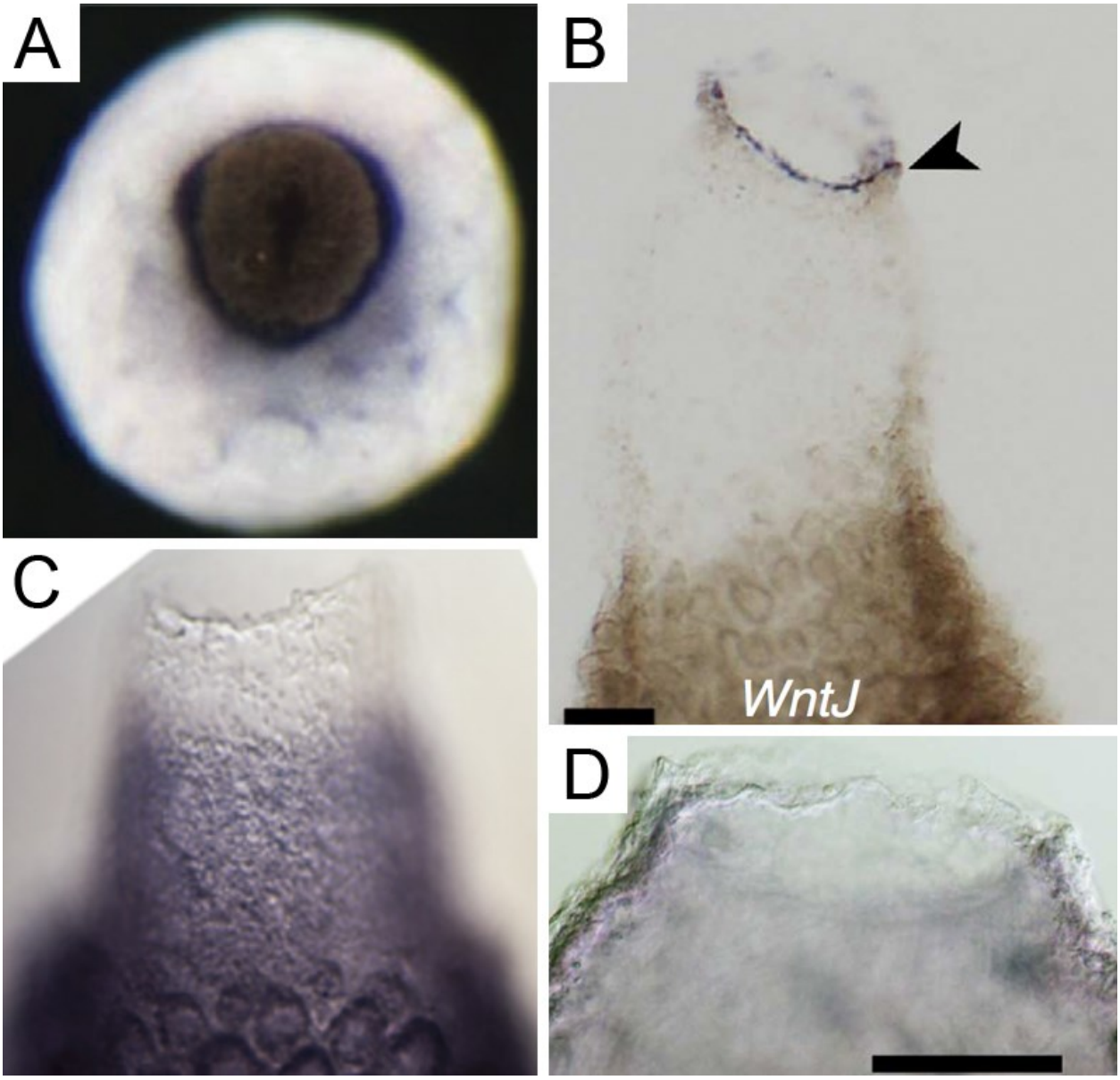

A EflMsiA
20 μm
50 μm
B
gc
C
pp
D
pin
ch
E
F
G SilicateinM1 mRNA

Figure S2-3| Examples of regional gene expression.

Expression of cryptochrome 2 around the forming ring of the *Amphimedon queenslandica* larva (A). Wnt J expression at the oscular tip of *Sycon ciliatum* (B). An example of regional expression meant to demonstrate expression in choanocytes. Sox7 is expressed. We took the authors' word for the interpretation of these expression patterns (C). An example of vague expression, also interpreted as occurring in choanocytes, that was not included in the study. (D). Images are from (A) Rivera et al. (2012), (B, D) Leininger et al. (2014) , and (C) Fortunato et al. (2012). Scale bars in original publications.


A
B
WntJ
C
D

Appendix 2 Supplemental material for Chapter Three

Bioinformatics: Full Commands

Read trimming - Trimmomatic

Spongilla lacustris and Aphrocallistes vastus:

trimmomatic-0.33.jar PE -threads 24 -phred33 –trimlog log.txt Sample1_R1.fastq.gz Sample1_R2.fastq.gz Sample1.1P.fq.gz Sample1.1U.fq.gz Sample1.2P.fq.gz Sample1.2U.fq.gz ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:1:true SLIDINGWINDOW:4:20 CROP:145 MINLEN:50

Sycon ciliatum:

trimmomatic-0.33.jar PE -threads 24 -phred33 –trimlog log.txt Sample1_R1.fastq.gz Sample1_R2.fastq.gz Sample1.1P.fq.gz Sample1.1U.fq.gz Sample1.2P.fq.gz Sample1.2U.fq.gz ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:1:true SLIDINGWINDOW:4:20 CROP:95 MINLEN:36

Transcriptome assembly - Trinity

Trinity --seqType fq --max_memory 200G --left Trimmed_Reads_R1.fq.gz --right Trimmed_Reads_R2.fq.gz --CPU 24 --normalize_reads --min_kmer_cov 10 --no_cleanup --verbose --output Trinity_output

Assessing transcriptome completeness - BUSCO v.1.2

BUSCO_v1.2.py -o Busco_output –in transcriptome.fasta -l metazoa -m trans -c 12

Contaminant filtering - BLASTX

blastx -query transcriptome.fasta -db nr –out blast_output.txt -evalue 1e-5 -num_threads 24 -max_target_seqs 5 -max_hsps 1 -outfmt "6 qseqid staxids sseqid sacc sgi pident evalue bitscore score sscinames sskingdoms"

Transcript quantification - Trinity wrapper scripts for RSEM

align_and_estimate_abundance.pl --transcripts Trinity.fasta --est_method RSEM --aln_method bowtie --trinity_mode --prep_reference --thread_count 24

align_and_estimate_abundance.pl --transcripts Trinity.fasta --seqType fq --left Trimmed_Reads_R1.fq.gz --right Trimmed_Reads_R2.fq.gz --est_method RSEM --aln_method bowtie --trinity_mode --output_prefix Sample_RSEM --output_dir RSEM_output --thread_count 24

abundance_estimates_to_matrix.pl --est_method RSEM Sample1.genes.results Sample2.genes.results Sample3.genes.results Sample4.genes.results Sample5.genes.results Sample6.genes.results

Differential expression analysis - Trinity wrapper scripts for DESeq2

run_DE_analysis.pl --matrix RSEM_genes.counts.matrix --method DESeq2 --samples_file Samples.txt

analyze_diff_expr.pl --matrix RSEM_genes.TMM.fpkm.matrix --samples Samples.txt --P 0.05 --C 1

Gene ontology enrichment and BLASTP for annotation against UniProt database

blastp -query protein_seq.fasta -db UniProt_DB.pep -num_threads 8 -max_target_seqs 1 -outfmt 6 > GO_blast_output.outfmt6

analyze_diff_expr.pl --matrix RSEM_genes.TMM.fpkm.matrix --samples Samples.txt --examine_GO_enrichment --GO_annots GO_file.txt --gene_lengths gene_lengths.txt

Candidate gene search – TBLASTN

tblastn -query query_pep_seqs.fasta –db reference_transcriptome_db.fasta –out tblastn_output.txt -num_threads 4 -max_hsps 1 -outfmt "6 qseqid sseqid pident evalue"

Script for identifying contaminants from BLAST results

```
#! /usr/bin/env python
import sys
import re
from ete3 import NCBITaxa
ncbi = NCBITaxa()

from sqlite3 import OperationalError

'''
Usage:  blast_decontamination.py formatted_blast_file.txt > contaminants_list.txt

Classify as contaminant if:
- if there is an E-value gap > 5 between two hits that are not of the same species, hit with lower E-value taken as classification
- all blast hits identical
- only 1 blast hit and E-value < 5
- if all hits are Bacteria, Archaea, Virus or human - classified contaminant
'''

#definitions
Column=[]
hit = []
hitnum=0
superkingdom_list=[]
species_list=[]
E_list=[]
algae_list=[]

#input: formatted blast output file
File = sys.argv[1]
File = open(File, 'rU')
for Line in File:
        Line = Line.strip('\n')
        Column = Line.split('\t')


        if Column[0] != "*":
                #hitnum: keep track of which hit this is
                hitnum += 1
                #save all blast data in hit[]
                hit.append([hitnum,Column[0:21]])

                #Is data for superkingdom, species missing?
                if Column[10]==False:
                        superkingdom_list.append("N/A")
                else:
                        superkingdom_list.append(Column[10])

                if Column[9]==False:
                        species_list.append("N/A")
                else:
                        species_list.append(Column[9])

                #store only the exponent of the E-value
                if Column[6][1] == "e":
                        E_list.append(int(Column[6][3:7]))
                #except when E-value = 0.0
                else:
                        #if E == 0, make "infinitely large"
                        if Column[6] == "0.0":
                                Column[6] = "1000"

                #Query NCBI taxdb to see if query sequence is an alga
                Tax = Column[1].split(';')
                Tax = Tax[0]
                try:
                        tax_rank = ncbi.get_rank(ncbi.get_lineage(Tax))
                        if tax_rank == "":
                                algae_list.append("N/A")
                #if ncbi.get_lineage(Tax) returns empty set, just continue
                except (ValueError,OperationalError,TypeError):
                        algae_list.append("N/A")
                        continue
                #33090 = Viridiplantae, 3041 = Chlorophyta
                if tax_rank.has_key(33090) or tax_rank.has_key(3041):
                        algae_list.append("algae")
                else:
                        algae_list.append("N/A")
```

```
		# "*" = end of list of hits for this query sequence
		if Column[0] == "*":

			#if there was only 1 hit
			if hitnum == 1:
				if E_list[0] > 4 or E_list[0] ==0:
					#check if contaminant
					if species_list[0] == "Homo sapiens" or superkingdom_list[0] in {"Bacteria","Archaea","Viruses"} or
algae_list[0]=="algae":
						print hit[0][1][0],"\t","contaminant (single hit)"

						#clear variables
						hit=[]
						hitnum=0
						superkingdom_list=[]
						species_list=[]
						E_list=[]
						algae_list=[]
						continue

			#if there is more than one hit in the list of hits
			if superkingdom_list.count(superkingdom_list[0]) == len(superkingdom_list) and superkingdom_list[0] in
{"Bacteria","Archaea","Viruses"}:
				print hit[0][1][0],"\t","contaminant (all bacteria, archaea, or viruses)"
				hit=[]
				hitnum=0
				superkingdom_list=[]
				species_list=[]
				E_list=[]
				algae_list=[]
				continue
			if algae_list.count(algae_list[0]) == len(algae_list) and algae_list[0] == "algae":
				print hit[0][1][0],"\t","contaminant (all plant/algae)"
				hit=[]
				hitnum=0
				superkingdom_list=[]
				species_list=[]
				E_list=[]
				algae_list=[]
				continue


			for n in range(hitnum):
				#are all of the hits the same contaminant?
				if n+1 == hitnum:
					if species_list.count(species_list[0]) == len(species_list):
						if species_list[0] == "Homo sapiens" or superkingdom_list[0] in
{"Bacteria","Archaea","Viruses"} or algae_list[0]=="algae":
							print hit[n][1][0],"\t","contaminant (all same contaminant)"
							break


				#is there a gap in E-values > 4 between contaminant hit and rest of hits?
				else:
					if species_list[n] != species_list[n+1] and species_list[n] != "N/A" and species_list[n+1] != "N/A":
						if E_list[n] - E_list[n+1] > 4:
							if species_list[n] == "Homo sapiens" or superkingdom_list[n] in
{"Bacteria","Archaea","Viruses"} or algae_list[n]=="algae":
								print hit[n][1][0],"\t","contaminant"
							break


#clear variables for the next query sequence
			hitnum=0
			hit=[]
			superkingdom_list=[]
			species_list=[]
			E_list=[]
			algae_list=[]
```

Table S3-1| RNA quality and read output for each RNA-seq sample.

RNA was extracted using the Norgen Single Cell Kit. The RNA integrity number (RIN) was measured using an Agilent 2100 Bioanalyzer and the A260/280 ratio by a Nanodrop ND-1000. RNA was sequenced on an Illumina NextSeq 500 to produce 2x150 bp reads at an average depth of 124x.

Sample	RIN	A260/280	No. raw paired reads
Spongilla lacustris PON			
PON 1	9.3	2.14	91,528,788
PON 2	9	2.52	110,290,718
PON 3	10	1.97	107,482,432
Spongilla lacustris Juvenile			
Juvenile 1	10	2.57	115,988,118
Juvenile 2	9.3	2.01	97,531,374
Juvenile 3	9.2	1.85	98,925,018
Aphrocallistes vastus Body			
Body 1	9.3	1.94	114,280,832
Body 2	9.2	1.91	104,425,048
Body 3	9.7	1.93	78,237,712
Aphrocallistes vastus Osculum			
Osculum 1	9.8	1.97	114,026,040
Osculum 2	9.3	1.88	89,141,906
Osculum 3	10	1.84	109,126,340

Table S3-2| BUSCO scores of all currently published, pre-assembled sponge transcriptomes.

BUSCO v1.2 on the transcriptome setting was used to search each transcriptome for 843 metazoan single copy orthologues. The percentage of full-length or near full-length orthologues found in each transcriptome is given. Statistics for the transcriptomes produced in Chapter Three are bolded.

Species	Class	BUSCO (%)	Reference
Amphimedon queenslandica	Demospongiae	83	Fernandez-Valverde et al. (2015)
Aphrocallistes vastus	**Hexactinellida**	**70**	**Chapter Three**
Aphrocallistes vastus	Hexactinellida	74	Riesgo et al. (2014)
Chondrilla nucula	Demospongiae	43	Riesgo et al. (2014)
Corticium candelabrum	Homoscleromorpha	31	Riesgo et al. (2014)
Crambe crambe	Demospongiae	33	Versluis et al. (2015)
Crella elegans	Demospongiae	N/A	Perez-Porro et al. (2013)
Ephydatia fluviatilis	Demospongiae	N/A	Alie et al. (2015)
Ephydatia fluviatilis	Demospongiae	67	S. Leys
Ephydatia muelleri	Demospongiae	83	Pena et al. (2016)
Ephydatia muelleri	Demospongiae	76	S. Leys
Haliclona amboinensis	Demospongiae	61	Guzman and Conaco (2016)
Haliclona tubifera	Demospongiae	64	Guzman and Conaco (2016)
Ircinia fasciculata	Demospongiae	26	Riesgo et al. (2014)
Leucosolenia complicata	Calcarea	76	Fortunato et al. (2014a)
Oscarella carmela	Homoscleromorpha	86	Nichols et al. (2012)
Petrosia ficiformis	Demospongiae	N/A	Riesgo et al. (2014)
Pseudospongosorites suberitoides	Demospongiae	15	Riesgo et al. (2014)
Scopalina sp.	Demospongiae	70	Francis et al. (2017)
Spongilla lacustris	**Demospongiae**	**81**	**Chapter Three**
Spongilla lacustris	Demospongiae	74	Riesgo et al. (2014)
Stylissa carteri	Demospongiae	29	Ryu et al. (2016)
Sycon ciliatum	Calcarea	82	Leininger et al. (2014)
Sycon coactum	Calcarea	60	Riesgo et al. (2014)

Sycon coactum	Calcarea	33	S. Leys
Tedania anhelens	Demospongiae	73	Francis et al. (2017)
Xestospongia testudinaria	Demospongiae	46	Ryu et al. (2016)

Table S3-3| BLAST hits and differential expression of significantly upregulated candidate genes.

Each transcript was identified by blasting (BLASTX) to the NCBI protein database. The fold change and the adjusted p-value for differential expression was measured at the gene-level. Log_2FC, Log_2 fold change, DE p_{adj}, adjusted p-value for differential expression.

	BLASTX Hit	BLASTX Hit Accession No.	E-value	Log_2FC	DE p_{adj}
***S. lacustris* Juvenile**					
***Sla*mGluR** TR10301|c0_g1_i1	metabotropic glutamate receptor-like	XP_003388055.1	3e-162	1.80	0.045
***Sla*GABAR** TR35706|c0_g2_i6	gamma-aminobutyric acid type B receptor subunit 1-like	XP_019856011.1	6e-140	192	5.9e-08
***Sla*K_{ir}** TR8389|c0_g1_i1	potassium inward rectifier channel A	NP_001266219.1	1e-79	1.45	4.2e-03
***A. vastus* Body**					
***Ava*GABAR** TR669|c0_g2_i2	gamma-aminobutyric acid type B receptor subunit 1	XP_017116958.1	9e-46	1.91	0.030
***A. vastus* Osculum**					
***Ava*Bsh** TR8705|c0_g1_i1	BarX/Bsh [Halichondria bowerbanki]	AAQ24371.1	2e-20	1.84	0.043

Table S3-4| Significantly enriched *Ava*Bsh gene ontology terms.

Gene ontology (GO) terms associated with *Ava*Bsh were cross-referenced with the most significantly enriched GO terms derived from the upregulated genes expressed in the *Aphrocallistes vastus* osculum.

GO ID	Over-represented p-value	GO term
GO:0044699	0.022	single-organism process
GO:0051216	0.026	cartilage development

Table S3-5| Significantly enriched *Ava*GABAR gene ontology terms.

Gene ontology (GO) terms associated with *Ava*GABAR were cross-referenced with the most significantly enriched GO terms derived from the upregulated genes expressed in the *Aphrocallistes vastus* body tissue.

GO ID	Over-represented p-value	GO term
GO:0045761	0.015	regulation of adenylate cyclase activity
GO:0031279	0.016	regulation of cyclase activity
GO:0051339	0.017	regulation of lyase activity
GO:0030817	0.021	regulation of cAMP metabolic process
GO:0030802	0.023	regulation of cyclic nucleotide biosynthetic process
GO:0030808	0.025	regulation of nucleotide biosynthetic process
GO:1900371	0.025	regulation of purine nucleotide biosynthetic process
GO:0030799	0.025	regulation of cyclic nucleotide metabolic process
GO:1900542	0.032	regulation of purine nucleotide metabolic process
GO:0006140	0.033	regulation of nucleotide metabolic process

Table S3-6| Significantly enriched *Sla*K_{ir} gene ontology terms.

Gene ontology (GO) terms associated with *Sla*K_{ir} were cross-referenced with the most significantly enriched GO terms derived from the upregulated genes expressed in the *Spongilla lacustris* juvenile.

GO ID	Over-represented p-value	GO term
GO:1902578	0.028	single-organism localization
GO:0044765	0.042	single-organism transport

Table S3-7| Significantly enriched *Sla*mGluR gene ontology terms.

Gene ontology (GO) terms associated with *Sla*mGluR were cross-referenced with the most significantly enriched GO terms derived from the upregulated genes expressed in the *Spongilla lacustris* juvenile. Neural-related terms are bolded.

GO ID	Over-represented p-value	GO term
GO:0007186	4.95e-05	G-protein coupled receptor signaling
GO:0044707	1.25e-04	single-multicellular organism process
GO:0032501	1.58e-04	multicellular organismal process
GO:0050896	3.39e-04	response to stimulus
GO:0019233	1.38e-03	sensory perception of pain
GO:0007166	3.01e-03	cell surface receptor signaling
GO:0007600	7.92e-03	sensory perception
GO:0051239	0.012	regulation of multicellular organismal process
GO:0007165	0.018	signal transduction
GO:0048169	**0.030**	**regulation of long-term neuronal synaptic plasticity**
GO:0010647	0.030	positive regulation of cell communication
GO:0043408	0.031	regulation of MAPK cascade
GO:2000026	0.032	regulation of multicellular organismal development
GO:0044057	0.032	regulation of system process
GO:0051480	0.036	regulation of cytosolic calcium ion concentration
GO:0023056	0.040	positive regulation of signaling
GO:0048584	0.044	positive regulation of response to stimulus

Table S3-8| Significantly enriched *Sla*GABAR gene ontology terms.

Gene ontology (GO) terms associated with *Sla*GABAR were cross-referenced with the most significantly enriched GO terms derived from the upregulated genes expressed in the *Spongilla lacustris* juvenile. Neural-related terms are bolded.

GO ID	Over-represented p-value	GO term
GO:0007186	4.95e-05	G-protein coupled receptor signaling
GO:1903531	1.51e-04	negative regulation of secretion by cell
GO:0051048	2.16e-04	negative regulation of secretion
GO:0050896	3.39e-04	response to stimulus
GO:0007166	3.01e-03	cell surface receptor signaling pathway
GO:0051051	3.75e-03	negative regulation of transport
GO:0030154	6.73e-03	cell differentiation
GO:0014052	**0.010**	**regulation of gamma-aminobutyric acid secretion**
GO:0014053	**0.010**	**negative regulation of gamma-aminobutyric acid secretion**
GO:0014060	**0.010**	**regulation of epinephrine secretion**
GO:0032811	**0.010**	**negative regulation of epinephrine secretion**
GO:0033602	**0.010**	**negative regulation of dopamine secretion**
GO:0033604	**0.010**	**negative regulation of catecholamine secretion**
GO:0014049	**0.011**	**positive regulation of glutamate secretion**
GO:0060124	0.012	positive regulation of growth hormone secretion
GO:0051957	0.012	positive regulation of amino acid transport
GO:0046888	0.013	negative regulation of hormone secretion
GO:0014048	0.014	regulation of glutamate secretion
GO:0051953	0.014	negative regulation of amine transport
GO:0051956	0.014	negative regulation of amino acid transport
GO:0030817	0.015	regulation of cAMP biosynthetic process
GO:0032891	0.015	negative regulation of organic acid transport
GO:0030814	0.016	regulation of cAMP metabolic process
GO:1903792	0.017	negative regulation of anion transport
GO:0051955	0.017	regulation of amino acid transport
GO:0014059	**0.018**	**regulation of dopamine secretion**

GO:0007165	0.018	signal transduction
GO:0030802	0.019	regulation of cyclic nucleotide biosynthetic process
GO:0060123	0.019	regulation of growth hormone secretion
GO:0030808	0.021	regulation of nucleotide biosynthetic process
GO:1900371	0.021	regulation of purine nucleotide biosynthetic process
GO:0030799	0.021	regulation of cyclic nucleotide metabolic process
GO:0051954	0.022	positive regulation of amine transport
GO:0050433	**0.022**	**regulation of catecholamine secretion**
GO:0035094	0.029	response to nicotine
GO:0090087	0.029	regulation of peptide transport
GO:0010647	0.030	positive regulation of cell communication
GO:1900542	0.030	regulation of purine nucleotide metabolic process
GO:0006140	0.032	regulation of nucleotide metabolic process
GO:0045761	0.034	regulation of adenylate cyclase activity
GO:0051952	0.036	regulation of amine transport
GO:0031279	0.039	regulation of cyclase activity
GO:0051339	0.039	regulation of lyase activity
GO:0043271	0.040	negative regulation of ion transport
GO:0023056	0.040	positive regulation of signaling

Figure S3-1| Alignment of the binding domain of the metabotropic glutamate receptor 1.

Glutamate binding residues as characterized in mGluR1 are indicated in red. Blue indicates residues found across all mGluR subtypes. Binding residues that are present in the *S. lacustris* sequence is indicated with *. The *S. lacustris* sequence is highlighted in bold, and all sponge sequences are indicated with +. Ligand binding (LB) domain 1 and 2 are indicated respectively with solid and dotted lines above the alignment. Dots indicate that a portion of the alignment has been skipped. Amino acid numbering follows the Hsap_mGluR1 sequence (accession: P23385.1). Hsap, *Homo sapiens*, Mmus, *Mus musculus*, Dre, *Danio rerio*, Sko*, Saccoglossus kowalevski*, Dme, *Drosophila melanogaster*, Cel, *Caenorhabditis elegans,* Cgi, *Crassostrea gigas*, Sla, *Spongilla lacustris*, Ifas, *Ircinia fasciculata*, Cnu, *Chondrilla nucula* , Pfi, *Petrosia ficiformis*, Psu, *Pseudospongosorites suberitoides*, Ava, *Aphrocallistes vastus*, Sycon, *Sycon coactum*, Cca, *Corticium candelabrum*.

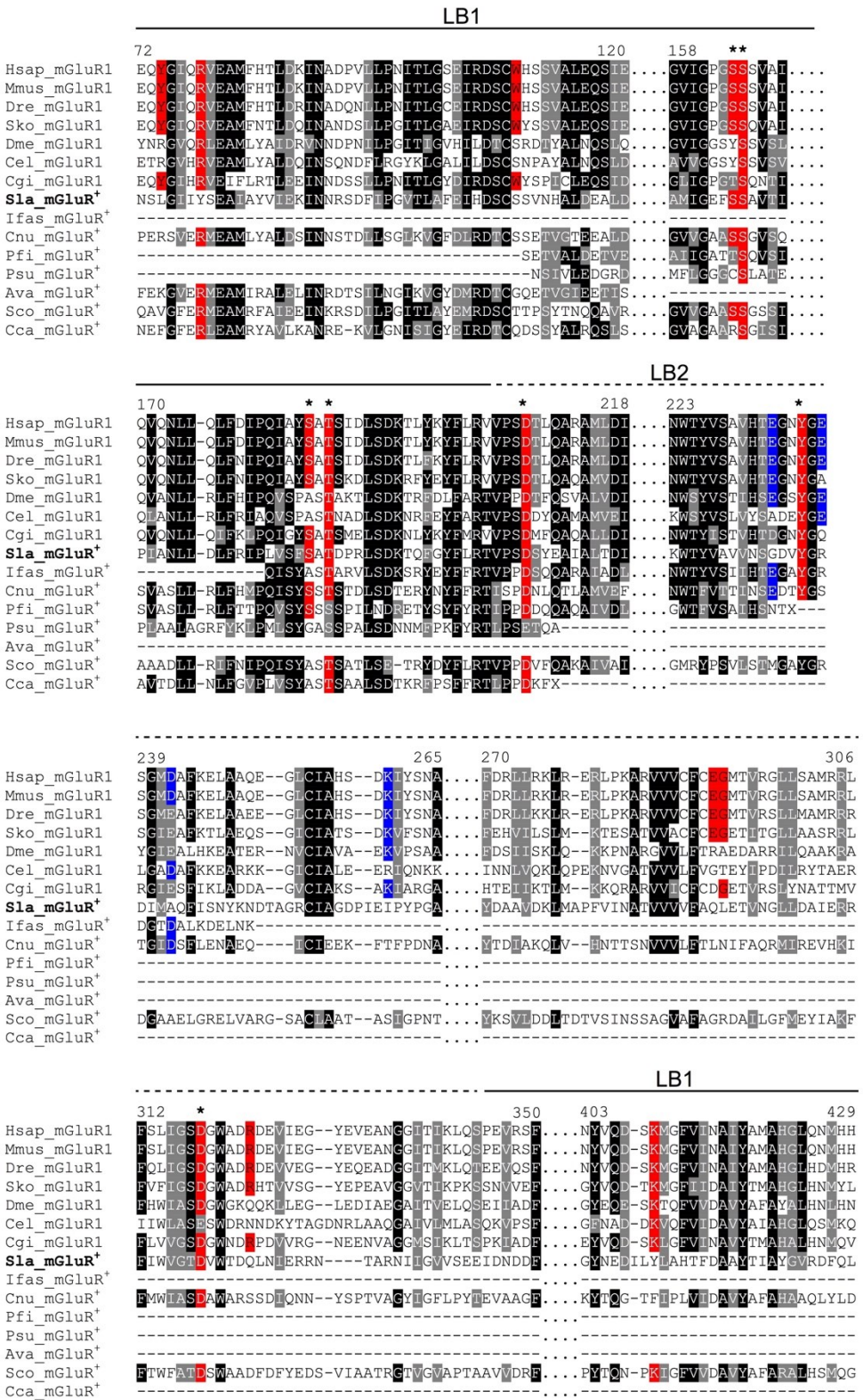
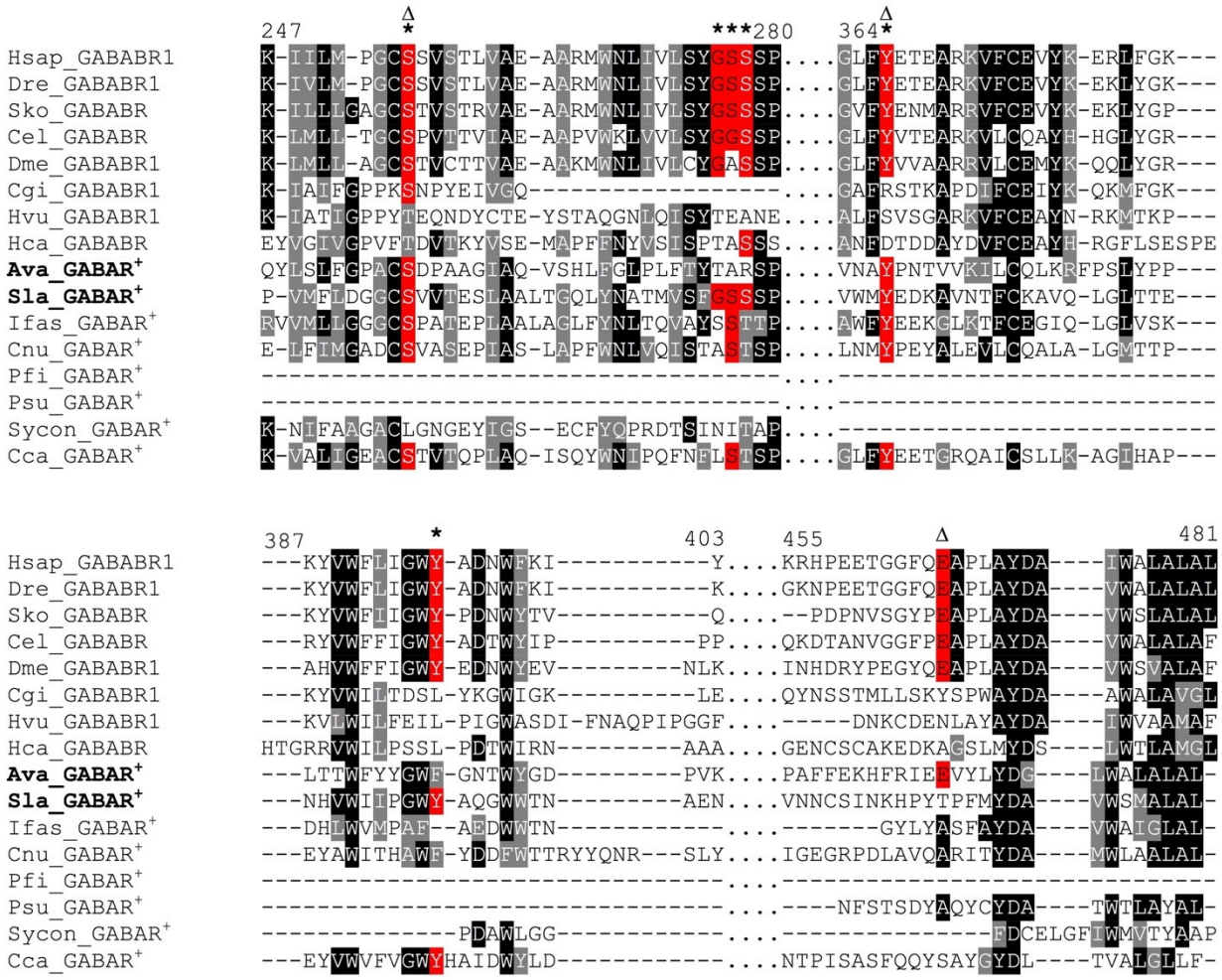

LB1
LB2
LB1
Hsap_mGluR1
Mmus_mGluR1
Dre_mGluR1
Sko_mGluR1
Dme_mGluR1
Cel_mGluR1
Cgi_mGluR1
Sla_mGluR+
Ifas_mGluR+
Cnu_mGluR+
Pfi_mGluR+
Psu_mGluR+
Ava_mGluR+
Sco_mGluR+
Cca_mGluR+

Figure S3-2| Alignment of the extracellular domain of the gamma-aminobutyric acid receptor.

GABABR1 residues involved in binding GABA are highlighted in red. The *Aphrocallistes vastus* and *Spongilla lacustris* sequences found in this study are highlighted in bold, while all sponge sequences are indicated with a +. Binding residues present in the *A. vastus* sequence are indicated with Δ and those present in the *S. lacustris* sequence is indicated with *. Dots indicate that a portion of the alignment has been skipped. Number of amino acid positions follow Hsap_mGluR1 (accession: Q9UBS5.1). Hsap, *Homo sapiens,* Dre, *Danio rerio*, Sko, *Saccoglossus kowalevski*, Cel, *Caenorhabditis elegans*, Dme, *Drosophila melanogaster*, Cgi, *Crassostrea gigas*, Hvu, *Hydra vulgaris*, Hca, *Hormiphora californensis*, Ava, *Aphrocallistes vastus,* Sla, *Spongilla lacustris*, Ifas, *Ircinia fasciculata*, Cnu, *Chondrilla nucula* , Pfi, *Petrosia ficiformis*, Psu, *Pseudospongosorites suberitoides*, Sycon, *Sycon coactum*, Cca, *Corticium candelabrum*.


247
280
364
Hsap_GABABR1
Dre_GABABR1
Sko_GABABR
Cel_GABABR
Dme_GABABR1
Cgi_GABABR1
Hvu_GABABR1
Hca_GABABR
Ava_GABAR+
Sla_GABAR+
Ifas_GABAR+
Cnu_GABAR+
Pfi_GABAR+
Psu_GABAR+
Sycon_GABAR+
Cca_GABAR+
387
403
455
481
Hsap_GABABR1
Dre_GABABR1
Sko_GABABR
Cel_GABABR
Dme_GABABR1
Cgi_GABABR1
Hvu_GABABR1
Hca_GABABR
Ava_GABAR+
Sla_GABAR+
Ifas_GABAR+
Cnu_GABAR+
Pfi_GABAR+
Psu_GABAR+
Sycon_GABAR+
Cca_GABAR+