Reproduction and Genetic Structure in a Reef-Forming Glass Sponge, *Aphrocallistes vastus*

by

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Abstract

Reef-forming glass sponges are ecosystem engineers that provide habitat for a diverse assemblage of benthic marine species. Sixteen glass sponge reefs have recently been discovered at 100-200 m depth off the coast of British Columbia, Canada and are of conservation interest. Nothing is known of the genetic diversity or connectivity of these glass sponge populations or the extent of clonality, details that would better inform the design of protected areas. Previous work on the primary reef-forming species in the Strait of Georgia (SoG), *Aphrocallistes vastus*, has faced challenges in developing non-duplicated (diploid) markers. Here I develop a panel of single-copy, informative single nucleotide polymorphism (SNP) markers using a novel technique involving next generation sequencing (NGS). I examine the genetic structure of *A. vastus* at both reef and non-reef sites at multiple scales: 1) across individuals sampled within and between clumps in reefs, 2) between reefs, and 3) between sites within and outside the SoG. I show that the reefs are formed through sexual reproduction. Within a reef, and even within the SoG basin, genetic distance between individuals does not vary according to geographic distance, suggesting the presence of larvae that disperse throughout the SoG. Importantly, populations within the SoG are genetically distinct from populations in Barkley Sound, west of Vancouver Island. These results highlight the effectiveness of a new NGS methodology for overcoming problems posed by genomic duplication in some invertebrates, emphasize genetic mixing across reefs, and provide a baseline of connectivity that can provide insight into the management requirements of marine protected areas currently under discussion.

Preface

Chapter 2 of this thesis has been published as Brown, R.R., C.S. Davis, and S.P. Leys. 2014. SNP discovery in a reef-forming glass sponge, *Aphrocallistes vastus*, using the Ion Torrent next generation sequencing platform. Conservation Genetics Resources 6:49–51. I contributed to the development of and implemented the methodology, and was responsible for data collection, analysis, and manuscript composition. C.S. Davis provided concept formation and the design of the methodology. S.P. Leys was the supervisory author. Both coauthors contributed substantially to manuscript edits.

Chapter 3 (and Appendix A) of this thesis is a manuscript in preparation for submission and has also been coauthored by C.S. Davis and S.P. Leys. I was responsible for data collection, analysis and manuscript composition. C.S. Davis assisted with data collection and methodology. S.P. Leys conceived and designed the experiments. Both coauthors contributed to manuscript composition and substantial editing.

Appendix B has been published as Kahn, A.S., L.J. Vehring, R.R. Brown, and S.P. Leys. 2015. Dynamic change, recruitment and resilience in reef-forming glass sponges. Journal of the Marine Biological Association of the United Kingdom:in press. I collected data on the spermatocysts and provided guidance and assistance to L.J. Vehring in the collection of data on juveniles, and also contributed to manuscript edits.

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Many people have helped me to produce this thesis, either through direct assistance or emotional/other support.

First, I would like to thank my supervisor Sally Leys for driving me to be my best in everything I do, and for genuinely caring so deeply for all of her students. Her ingenuity and flexibility have brought me to where I am. I thank Corey Davis who has patiently taught me everything I know about molecular techniques. He has not only guided my project, but has been a constant support. I thank Warren Gallin for sticking with this project, asking me the tough questions, and always being a voice of reason.

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The Molecular Biology Service Unit (University of Alberta) provided assistance and use of facilities, and I'm grateful for the expertise and patience of Troy Locke, Cheryl Nargang, and particularly Sophie Dang. Though also largely dropped from my thesis, my microscopy

work would not have been possible without a number of people. I'm grateful to Arlene Oatway in the Microscopy unit, and to George Braybrook and Nathan Gerein in the Earth Sciences SEM lab, all of whom are incredibly good at what they do, making the rest of us look good in the process. I also thank Nhu Trieu for taking on some of the difficulty that is producing quality sections of *Aphrocallistes vastus*. Laura Vehring took on the challenge of identifying, counting, and measuring juveniles from reef footage, and brought cheer and enthusiasm to all interactions as both student and colleague.

Past and present Leys lab members have truly made this time enjoyable, and I thank them for their friendship and support. Amanda Kahn, especially, has shared her considerable Leys lab wisdom throughout my program, never getting annoyed by my endless questions. Pam Reid, Nathan Farrar, and Jasmine Mah, for their companionship and commiseration. I thank Danielle Pendlebury for being an awesome office-mate and travel companion, and for literally sheltering me from the storm along with her genial husband Brian Pendlebury. I thank Christianne McDonald for getting me into campus every day from said shelter, and for being a true friend. Kristen Kruper and Eugueni Matveev have been cheerful and welcome additions to the lab. I thank my many students from Bio 108 and Zoo 250 for helping me keep a fresh perspective and reminding me to be in awe of the little things. Many people in the 'entomology grad student area' have provided stimulating discussions and group brainstorming efforts, and especially Brian Brunet, Julian Dupuis, Boyd Mori have tirelessly offered troubleshooting tips.

Many of my close friends and family have offered support and encouragement from afar. I'm grateful to my nefarious siblings Jobi, Sterbie, Sessa, and John who have more than once funded a trip home for the holidays, and continue to patiently await my return. My parents for being so proud and encouraging, despite not entirely understanding what I do. And most of all, Davor Koračić whose endless patience and devotion have kept me going. I thank him for being here through it all, and for always finding ways to make my life easier, be it feeding me, providing technical support when I'd venture yet again into uncharted territory, or stepping in to help with image processing when I was desperately trying to prepare for a talk. For being great at being interested in biology, and for reminding me on occasion that I enjoy what I do.

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Chapter 1

Introduction

1.1 General Introduction

Sponges (Phylum Porifera; Figure 1.1) are a diverse phylum of non-motile animals considered to be 'ecosystem engineers' because their large structures form three dimensional habitat for other animals in the benthic community in both marine and freshwater ecosystems (Weinberg *et al.*, 2004; Du Preez & Tunnicliffe, 2011; reviewed in Leys *et al.*, 2007; Buhl-Mortensen *et al.*, 2010). Sponges range in size from centimeters to meters; larger sponges harbor commensal megafauna in their oscula, or even within their canals (Beazley *et al.*, 2013), and form vertical relief for larger animals (Miller *et al.*, 2012b). Dense aggregations of sponges form the base of vibrant, diverse communities known as sponge gardens, which significantly increase the abundance of associated animals (Du Preez & Tunnicliffe, 2011; Marliave *et al.*, 2009).

Glass sponges (Class Hexactinellida) are an ancient group (Figure 1.1) of enigmatic deep water sponges (generally >100 m but usually >500 m), unique among sponges for their six-rayed glass spicules and syncytium that forms from the fusion of early embryonic cells and allows transport and the propagation of electrical signals across the body (Leys *et al.*, 2007). Glass sponges are especially good at generating habitat because they are large (e.g. *Farrea occa* in Hecate Strait is up to 2 m high and wide) and their skeletons, which are composed of fused (dictyonine) or loose (lysaccine) spicules, remain in the environment as

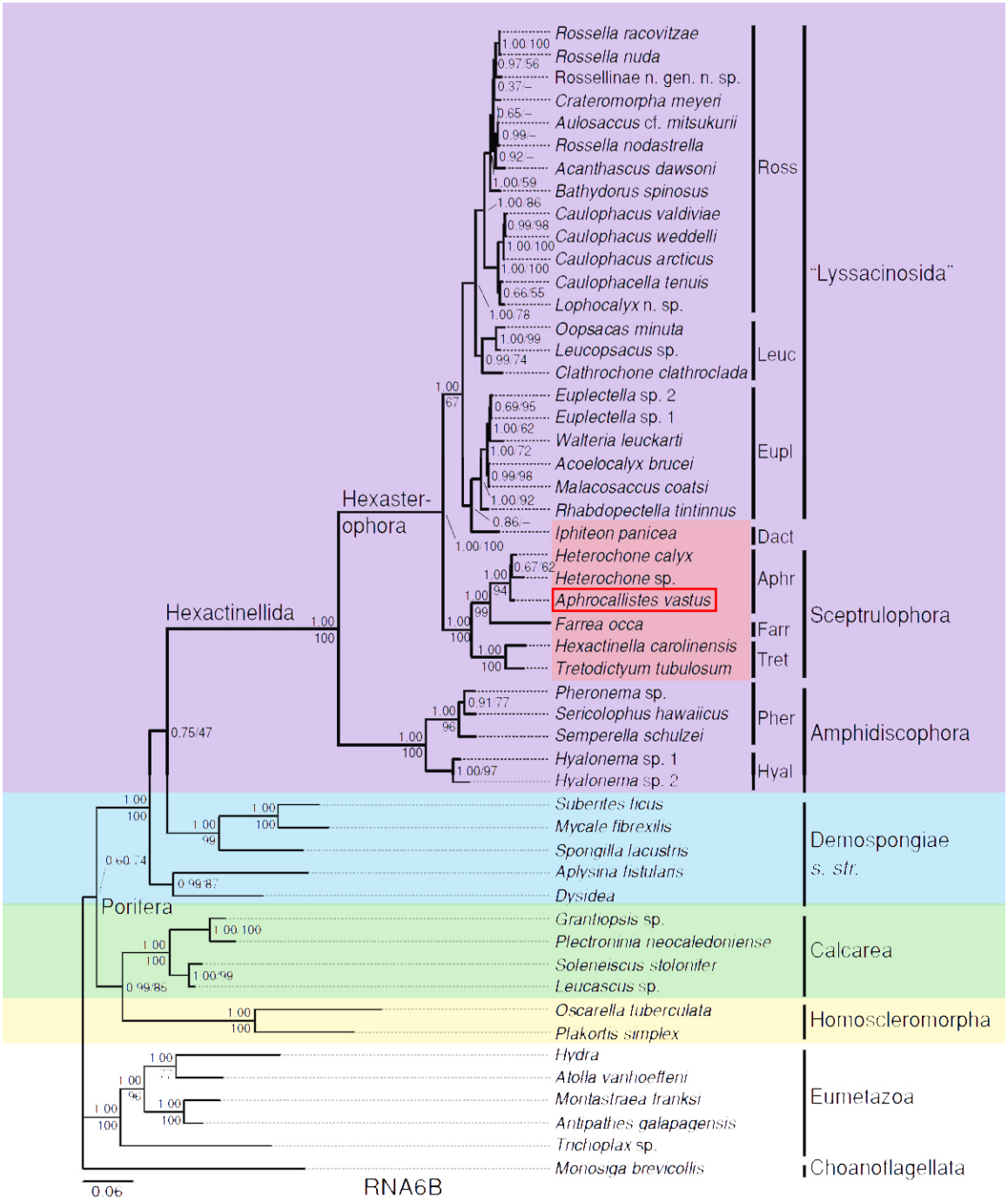

FIGURE 1.1: Sponge phylogeny with focus on Hexactinellida: Bayesian consensus tree of 18S, 28S, and 16S rDNA from sponges with cnidarians, a placozoan (*Trichoplax*), and a choanoflagellate. Glass sponges (purple) with dictyonal (rigid) skeletons (inset red) belong to Sceptrulophora and Dactylocalycidae (Dact). The red box emphasizes the placement of the study specimen, *Aphrocallistes vastus*. The remaining sponge classes are highlighted in blue, green and yellow for demosponges, calcareous sponges, and homoscleromorphs respectively. Scale bar = expected nucleotide substitutions per site. Modified from Dohrmann *et al.* (2008).

solid substrata long after the sponge dies. Lysaccine sponge spicules are woven into mats that remain intact through time and form a solid substrate on which other organisms settle. Mounds of lysaccine spicule mats significantly increase the abundance of benthic animals by an order of magnitude in the Porcupine Seabight southwest of Ireland (Bett & Rice, 1992) and the biomass and species diversity in Antarctica (Barthel, 1992). Sponges of the genus *Hyalonema* inhabit soft substrata in bathyal and abyssal depths by anchoring in sediments with stalks of woven glass spicules up to a meter long; these stalks over time become densely colonized by a diverse community of habitat-limited suspension feeders (Beaulieu, 2001). In bathyal basins off Southern California, glass sponge fragments have been associated with an increase in density of macrofauna (e.g. annelids, crustaceans, molluscs) (Jumars, 1976). Glass sponge gardens are common in Antarctica (Barthel & Gutt, 1992), the mid-Atlantic ridge (Felley *et al.*, 2008), the North Atlantic (Bett & Rice, 1992), and the Northeast Pacific where glass sponges also form large reefs (Leys *et al.*, 2004).

Sponge reefs differ from sponge gardens, however. Ancient sponge reefs were prevalent in the Tethys Sea from the late Triassic through the Cretaceous period, after which they disappear from the fossil record (Krautter *et al.*, 2001). At their peak in the late Jurassic, sponge reefs formed a discontinuous belt 7000 km in length along the northern Tethys Sea (what is now Newfoundland, Spain, Poland, and Romania), the largest biogenic structure ever formed. Modern sponge reefs formed by dictyonine glass sponges were discovered in Hecate Strait, BC in 1987-1988 (Conway *et al.*, 1991). Continued exploration uncovered a vast complex of reefs: four massive reefs in the Hecate Strait covering 425 km^2 at 165-240 m depth (Krautter *et al.*, 2001), up to 12 smaller reefs scattered across the Strait of Georgia at 90-210 m depth (Conway *et al.*, 2007; Figure 1.2), and Alaskan reefs on sills of fjords near the border of British Columbia (3 at 53-107 m depth) and in particularly shallow water near Juneau (22-56 m; Stone *et al.*, 2013). Reefs in Hecate Strait and Alaska are formed by three species of the Order Hexactinosida, *Farrea occa* (Farreidae), *Aphrocallistes vastus* (Aphrocallistidae; hereafter *Aphrocallistes*), and *Heterochone calyx* (Aphrocallistidae; hereafter *Heterochone*), though *Farrea occa* is absent from the reefs found in the Strait of Georgia.

Modern reefs began forming 9000 years ago (Krautter *et al.*, 2001). As glaciers retreated,

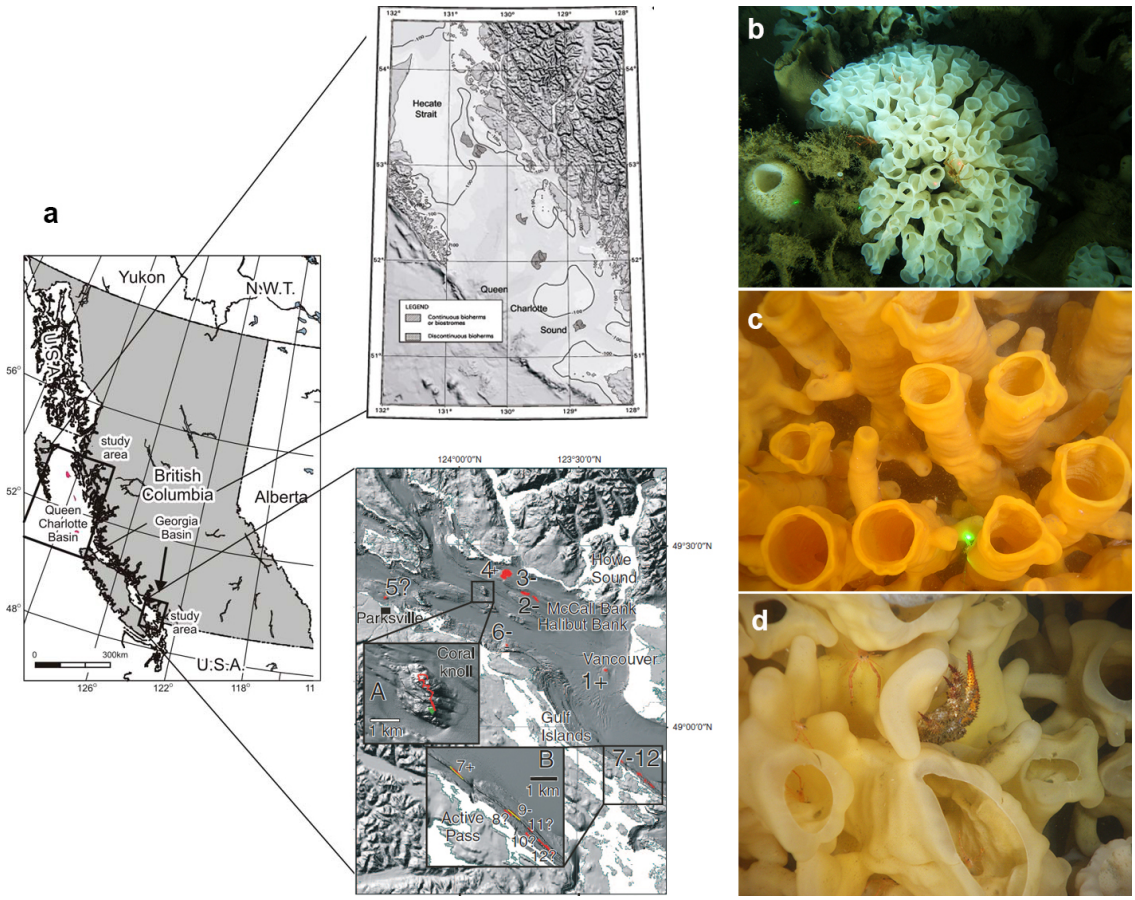

FIGURE 1.2: Glass sponge reefs: a) Maps of the known glass sponge reefs in British Columbia, modified from Conway *et al.* (2005b, 2007) and Krautter *et al.* (2006). b-d) Extant reef forming glass sponges: b) *Farrea occa*, c) *Heterochone calyx*, d) *Aphrocallistes vastus*. Images b-d by Sally Leys.

high sedimentation and isostatic rebound caused the sea floor to rise, creating a drop in sea level of 150 m below present day values. During this time, extensive iceburg furrowing left a network of scars and sills at depths now 250 m below sea level, providing the hard substrate that the first reef sponges settled on. Modern reefs, like some ancient reefs (Noe-Nygaard & Surlyk, 1985), are formed by new generations of glass sponges growing on intact skeletons of previous generations cemented together by sedimentation that occurs due to the baffling of bottom currents by the sponges (Krautter *et al.*, 2006). This process builds bioherms vertically from the point of original settlement, with the tallest modern reefs reaching 21 m above the bedrock (Conway *et al.*, 2005b).

Modern glass sponge reefs, as globally unique habitats and 'living fossils', have garnered

considerable interest since their discovery. They form nurseries for commercially important fish species (Cook *et al.*, 2008) in addition to providing habitat and substrate for a diverse array of other benthic animals (Chu & Leys, 2010). They are also important grazers, filtering as much as 165 vertical metres of water per square metre of reef (Kahn *et al.*, 2015a). However, the reefs are threatened by bottom trawl fishing, which is detrimental to sponges and corals and their associated communities (Freese *et al.*, 1999; Heifetz *et al.*, 2009; Du Preez & Tunnicliffe, 2011). This type of fishing creates a large disturbance from which glass sponges are slow to recover (Kahn *et al.*, 2015b). Evidence of trawl fishing on the reefs exists in the form of broken sponges (Conway *et al.*, 2007), piles of sponge rubble (Conway *et al.*, 2001), and scour marks made by trawl 'doors' (Conway *et al.*, 2001; Krautter *et al.*, 2001; Cook *et al.*, 2008). Therefore, Marine protected areas (MPAs) are currently being planned for some reefs, expanding on current fishing closures in Hecate Strait (Jamieson & Chew, 2002). Design of these closures would benefit from knowledge of the dispersal potential and population connectivity of reef-forming sponges, but we currently know little about either.

MPAs and conservation areas are generally created to protect biodiversity, and sometimes cultural, archaeological, or historic features, in areas threatened by anthropogenic impacts. These are typically intended to preserve ecosystems while simultaneously improving fishing yield in adjacent areas (Gaines *et al.*, 2010). There are many types of marine protected and conservation areas: while most types allow varying levels or types of commercial or non-commercial harvest or exploration, only 'no-take' marine reserves ban every type of fishing activity (Day *et al.*, 2012). Though marine reserves have been associated with an increase in size, density and diversity of species (Lester *et al.*, 2009), they represent a small percentage of MPAs (Spalding *et al.*, 2008). In all cases, a single conserved area has limited effect on the surrounding environment. To improve this, networks of MPAs are often created, with measurable effects on the surrounding areas (Gaines *et al.*, 2010). For a MPA network to produce greater ecological benefits than the combined benefits provided by each of its constituents, the constituents must be connected – that is, propagules of the species within the protected areas must connect to each other (Shanks *et al.*, 2003) and supply surrounding areas, and careful evaluation and testing should be done to ensure that appropriate connectivity is achieved (Grorud-Colvert *et al.*, 2014). Connectivity between populations provides

an exchange of individuals to replenish depleted numbers, introduce genetic material, and maintain diversity which help make a population more resilient to disturbance. This can be measured through biophysical modeling where knowledge of dispersal characteristics and oceanographic features exist, or through population genetics.

1.2 Reproduction in Sponges

Most sponges are viviparous hermaphrodites, though some are oviparious and gonochoristic, and nearly all produce free swimming dispersive larvae through sexual reproduction (Leys & Ereskovsky, 2006). Some sponges like *Tetilla japonica* and *T. serica* have direct development so dispersal only occurs with the gametes (Watanabe, 1978).

Asexual reproduction is also common in sponges and typically thought of as gemmulation, budding, or fission. Fission tends to be seen in encrusting sponges and can create highly clonal populations such as seen in *Crambe crambe* (Calderón *et al.*, 2007), where 76 of 177 sponges examined were clones from 24 lineages. Some sponges, such as *Tethya citrina* and *T. aurantium*, bud off fully functional juvenile forms (Gaino *et al.*, 2006). Some forms of asexual reproduction are less obvious. 'Dripping' of individual sponges onto lower substrates has been observed in *Chondrilla nucula* (Zilberberg *et al.*, 2006) and *Chondrosia reniformis* (Bonasoro *et al.*, 2001). Reattachment and growth of fragmented sponges could be thought of as simply a product of stellar regenerative abilities, however, the morphology of some sponges, such as the coral reef species *Iotrochota birotulata*, *Haliclona rubens*, and *Aplysina fulva*, makes them particularly prone to fragmentation so that as much as 30% of a population may consist of a single genet (Wulff, 1986), thus highlighting fragmentation as effective asexual reproduction.

Fusion to form chimeras has also been reported in some sponges. In *Scopalina lophyropoda* molecular data has shown that adult sponges can fuse: 13 sponges sampled four times each revealed 36 multilocus genotypes (Blanquer & Uriz, 2011). Two studies show fusion of larvae occurs. Ilan & Loya (1990) report that both kin and unrelated larvae and juveniles of *Chalinula* sp. fuse, in some instances with five larvae forming a single chimera, although adults discriminate between self and non-self, rejecting allogeneic tissue grafts. McGhee

(2006) found the same ontogenetic shift in propensity to fuse within *Haliclona* sp. Both studies suggested that larvae readily fuse in natural conditions, though Maldonado (1998) argues against the hypothesis of a natural tendency to form chimeras, showing that *Tedania ignis* larvae only fuse when placed forcibly in contact, with no apparent advantage for chimeras.

Sponge larvae are non-feeding (lecithotrophic), and thus have limited dispersal ability. Larvae are typically planktonic until they become competent to settle, switching to a demersal phase characterized by habitat exploration prior to settlement (Maldonado, 2006). The dispersal ability of lecithotrophic marine larvae depends on their yolk supply (length of planktonic phase), behaviour, and regional currents (Levin, 2006; Mariani *et al.*, 2006). There are eight sponge larval types described by Maldonado & Bergquist (2002) (amphiblastula, calciblastula, trichimella, cinctoblastula, clavablastula, parenchymella, dispherula, and hoplitomella; Figure 1.3), though most are morphologically similar, differing primarily by abundance and location of cilia, presence or absence of a body cavity, and size (50 μm - 5 mm). Exceptional larvae include hoplitomella, which are not ciliated and instead have radiating spicules (Vacelet, 1999), and the crawling, benthic larvae of hadromerid and halichondriid demosponges (Maldonado & Bergquist, 2002).

Descriptions of larval behaviour have been largely restricted to laboratory studies of shallow-dwelling demosponges (primarily parenchymella). Most larvae are short-lived, settling within minutes or days (<2 weeks) (Maldonado, 2006). After 72 hours in the plankton, mortality increases as energy reserves decline (Maldonado & Young, 1999; Maldonado *et al.*, 1997). Hoplitomella larvae, exceptionally, are thought to remain planktonic for months (Vacelet, 1999), and there have been reports of parenchymella larvae consuming dissolved organic carbon (Jaeckle, 1995) and bacteria (Ivanova, 1999). In the field, larvae are observed mostly spinning on their axis in the current with their anterior ends pointing upward, swimming horizontally if they encounter turbulence, strong light, or the boundary layer (Maldonado *et al.*, 2003). The behavioural repertoire of larvae observed in the lab is stunning for an organism with no nerves and includes fine-scale habitat selection (Whalan *et al.*, 2008a, 2012; Abdul Wahab *et al.*, 2011; Whalan & Webster, 2014). Swimming speed and direction is affected by light (Leys & Degnan, 2001; Leys *et al.*, 2002; Maldonado *et al.*,

2003; Elliott *et al.*, 2004), temperature (Maldonado & Young, 1996), gravity (Warburton, 1966), and flow direction (Maldonado & Young, 1999), while abrupt acceleration is thought to be achieved by changes in shape (e.g. becoming rounder)(Maldonado & Young, 1996; Mariani, 2005; Maldonado, 2006). Geotaxis, phototaxis and changes in buoyancy (Maldonado *et al.*, 1997; Maldonado, 2006) have all been suggested as means by which larvae move into or out of the boundary layer. Taxis and swimming ability affect realized dispersal and vary between species of sponges, with some "efficient-swimmers" (i.e. tufted parenchymella) possibly able to counteract currents through vertical migration to maintain their position near the parent population (Mariani *et al.*, 2006).

The above descriptions largely centre on demosponges. Much less is known of glass sponge reproduction and development because of the relative inaccessibility of deep-sea sponges. Sampling on ships with remote operated vehicles (ROVs) is expensive and deep-sea sponges are difficult to maintain in aquaria (sponges need a large volume of water, and deep sea species need cold water from depth). Therefore, development has been described in only two glass sponge species, *Farrea sollasii* (Okada, 1928) and *Oopsacas minuta* (Boury-Esnault *et al.*, 1999; Leys *et al.*, 2006), though larvae were described in *Vitrollula fertilis* (Ijima, 1904). Both *Farrea sollasii*, collected from the Sagami Sea, and *Oopsacas minuta*, collected from Mediterranean caves, reproduce year round (Okada, 1928; Boury-Esnault *et al.*, 1999; Leys *et al.*, 2006). Because glass sponges are syncytial, they undergo a unique process in their development. Embryos are cellular in early stages and form two cell types, an outer incomplete layer of smaller multiciliated cells, micromeres, and an inner layer of large cells, macromeres (Leys *et al.*, 2006). Macromeres extend projections which connect and fuse to each other before engulfing the micromeres with additional projections, thus forming the syncytial tissue of the larvae (Leys *et al.*, 2006). Cilia therefore project through the epithelial layer from the inner layer of cells, which also connect to the syncytium through bridges. Fully developed larvae have a pointed, yolk-packed posterior end and a rounded, lipid-packed anterior end, with a ciliated girdle (Leys *et al.*, 2006; Okada, 1928, as well although the orientation was inferred to be opposite). *Oopsacas* larvae swim slowly upwards in a rotating manner, with most larvae settling within 1-2 days of release (Leys *et al.*, 2007). Nothing is known of their dispersal ability in nature, nor that of any other glass sponge species, although we know from their distribution that both *Oopsacas* and *Aphrocallistes*

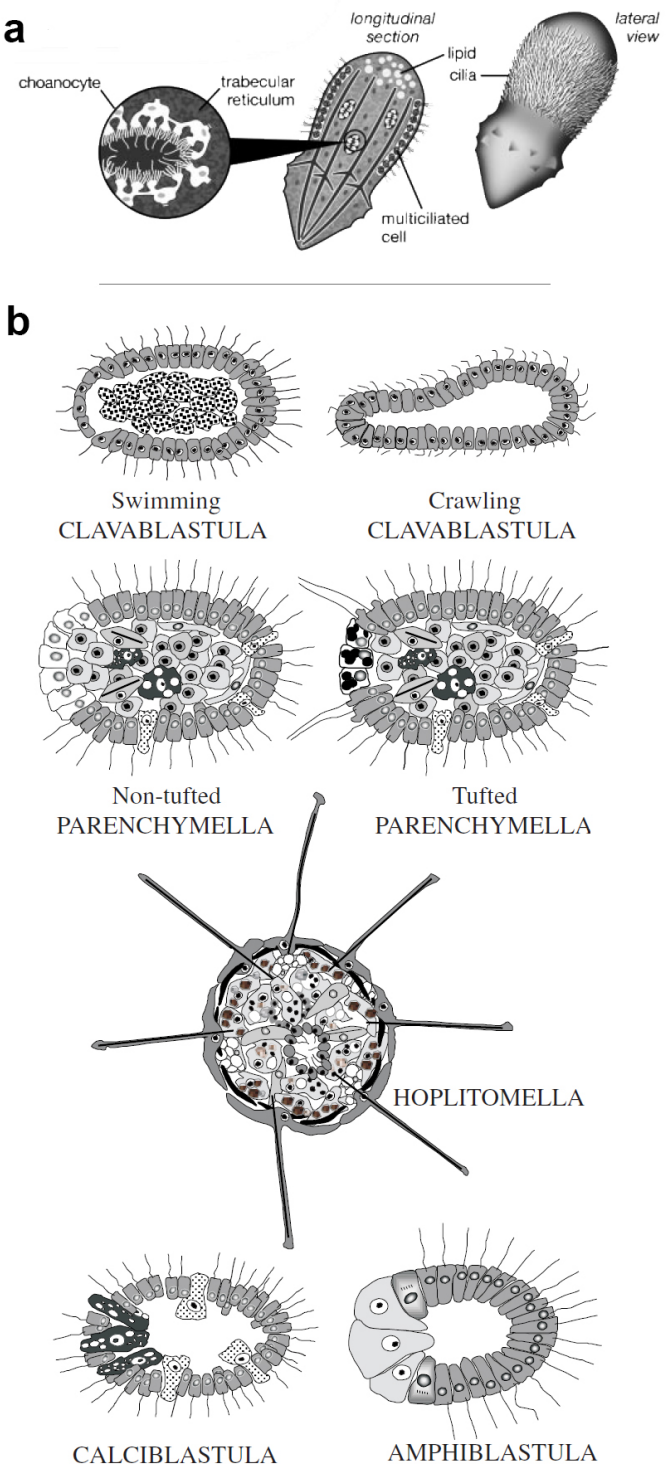

FIGURE 1.3: Morphology of six of the eight sponge larval types described by Maldonado & Bergquist (2002). a) Trichimella larvae of glass sponges, modified from Leys *et al.* (2006). b) Clavablastula, parenchymella, hoplitomella, calciblastula, and amphiblastula, modified from Maldonado (2006).

can disperse vertically well enough to settle at around 20 m depth (Vacelet, 1996; Leys *et al.*, 2004). To understand the dispersal of a species for which tagging or direct observations are impractical, population genetics is employed to study gene flow.

1.3 Population Genetics

The popularity of genetic marker types shifts over time with changing technology. Microsatellite markers (microsats), and mitochondrial DNA (mtDNA) markers have been popular choices in population genetics since the 1990s (Morin *et al.*, 2004; Seeb *et al.*, 2011) because microsats are highly variable multiallelic markers with a rapid mutation rate, while mtDNA shares an elevated mutation rate and provides phylogenetically informative sequence data in most animals. Recent criticisms point to limited resolution in mtDNA due to maternal inheritance (Morin *et al.*, 2004), and microsats suffer from complex mutation mechanisms, sensitivity to ascertainment bias, high rates of homoplasy, high genotyping error rates, difficulties replicating results between labs and low density throughout the genome (Väli *et al.*, 2008; Baric & Monschein, 2008; Ljungqvist *et al.*, 2010). Single Nucleotide Polymorphisms (SNPs) are bi-allelic point mutations found throughout the genome (i.e. genic and non-genic). SNPs have a simple mutation mechanism and high density (avg 1 per 300 bp of gDNA), low genotyping error rates, and are easily replicated between research groups (Morin *et al.*, 2009; Nussberger *et al.*, 2013). For any analysis more SNPs than microsats are required to resolve relationships (e.g. Hess *et al.*, 2011), but by using next generation sequencing (NGS) techniques thousands of markers can quickly be identified and genotyped (Helyar *et al.*, 2011). Thus SNPs, particularly when combined with NGS techniques and chip technology, have gained considerable popularity (reviewed in Seeb *et al.*, 2011; Grover & Sharma, 2014).

1.3.1 Population markers in sponges

Different genetic marker types have been used in sponges with varying degrees of success. Most population studies in sponges to date have used allozyme markers. These have successfully identified cryptic speciation (Lazoski *et al.*, 2001) and population structure; for

example, allozymes revealed a well-mixed, sexually produced population at reef level (up to a few hundred meters across) in *Haliclona* sp. from south-western Australia, with genetic distinction between reefs (several hundred meters) (Whalan *et al.*, 2005). Similarly, allozymes were used to determine that *Chondrilla nucula* has a genetically structured population (F_{ST} of 0.21) within a 2700 km range off Brazil (Klautau *et al.*, 1999). Studies using allozymes have also reported a lack of structure over large distances: Whalan *et al.* (2008b) found genetic homogeneity in the Great Barrier Reef sponge *Rhopaloeides odorabile* over a scale of tens of kilometers, and *Chondrosia reniformis* from Brazil and Bermuda lacked structure across 8,000 km (Lazoski *et al.*, 2001). COI was first used as a population marker in sponges by Duran *et al.* (2004a), with very little population differentiation found in *Crambe crambe*, although a follow-up study using microsatellite markers (microsats) revealed a highly structured population (Duran *et al.*, 2004b). Low resolution is consistent with a hypothesized low mitochondrial mutation rate in basal branching metazoans (Watkins & Beckenbach, 1999; Shearer *et al.*, 2002; Wörheide, 2006). In turn, microsats have become increasingly popular in sponge population genetics over the last decade (Calderón *et al.*, 2007; Blanquer *et al.*, 2009; Blanquer & Uriz, 2010; Dailianis *et al.*, 2011; Guardiola *et al.*, 2011; Giles *et al.*, 2013; Padua *et al.*, 2013).

The population structure of *Aphrocallistes* was initially investigated using mtDNA and microsats (Jensen, 2011; Figure 1.4). As suggested by previous work (Watkins & Beckenbach, 1999; Shearer *et al.*, 2002), COI, COII and ATP6 gene sequences were poor population level markers, displaying low nucleotide variability within *Aphrocallistes* and *Heterochone,* though they were able to resolve species level differentiation (Jensen, 2011). Five microsats were discovered and genotyped, but more than two alleles were amplified in samples at all five loci, indicating duplication of portions of the genome. Microsat discovery was performed a second time (by this author) using different probes, though only three microsats were attained and of those, only two were suitable for testing (data not shown). These challenges are not unique – other labs have described similar difficulties in assembling microsat libraries. Song *et al.* (2004) describes difficulties with genotyping microsats in soybean due to duplicated regions in the genome. Baums *et al.* (2005), building from previous attempts by three labs using five different protocols (Marquez *et al.*, 2000), located only five variable microsats in coral after identifying issues due to duplicated regions and a paucity of most

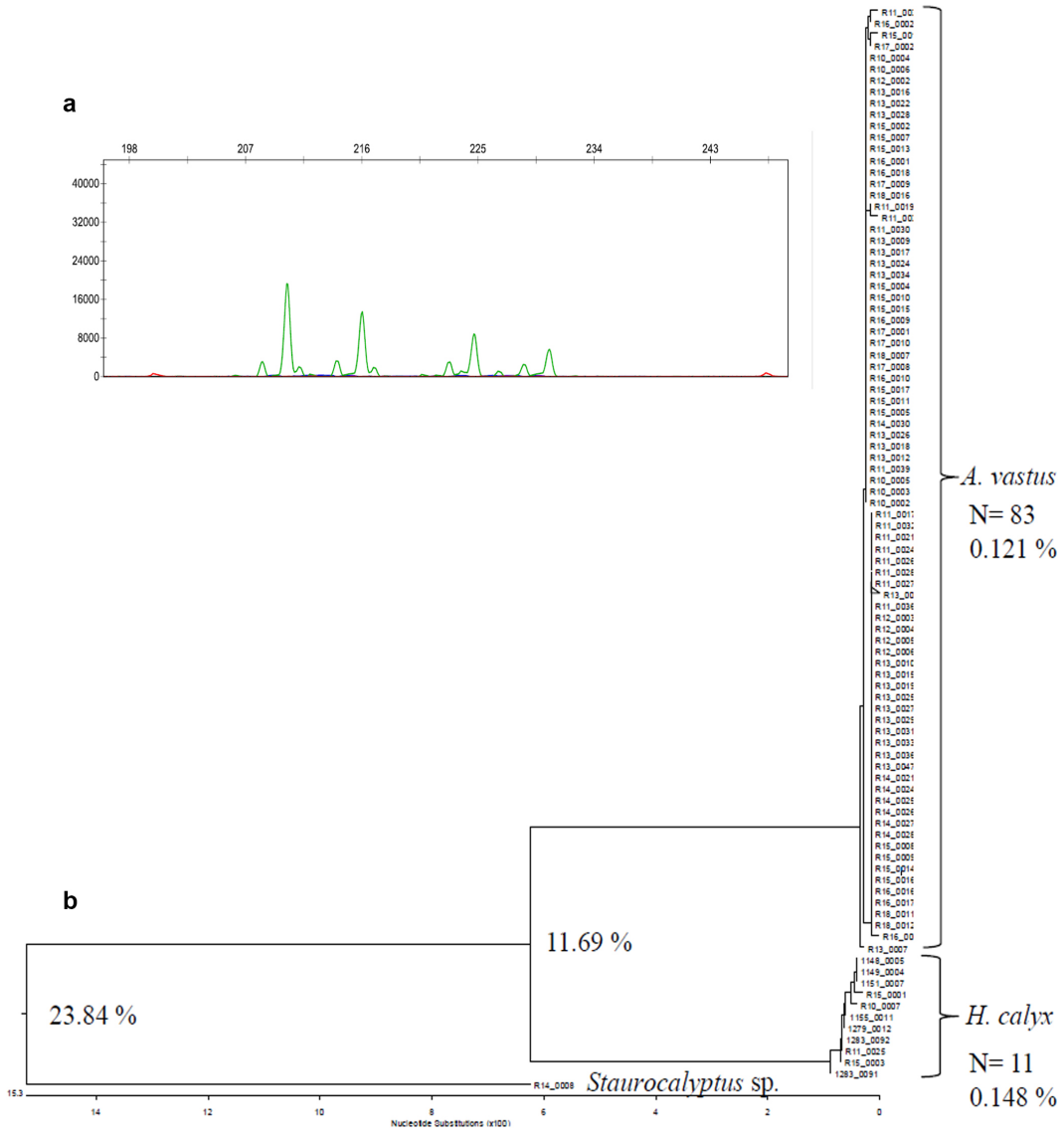

FIGURE 1.4: Microsatellite and COI markers in *Aphrocallistes vastus*: a) A microsatellite in a single individual, duplication indicated by four peaks corresponding to four different alleles. b) Distance based tree of COI in *Aphrocallistes vastus* and *Heterochone calyx*, showing low intraspecific variation. Modified from Jensen (2011).

repeat types using a southern blot. Similar difficulties appear common to Lepidopterans: as reviewed by Ji & Zhang (2004), in 20 published studies using microsats in Lepidopterans, 80% obtained libraries of five or fewer variable markers. Specifically, problems arise with low frequencies or attainment efficiency of microsats combined with a large percentage of duplicated loci, attributed to retrotransposons (Zhang, 2004).

1.4 Thesis Objectives and Approach

In this thesis, I investigate the genetic structure of the primary reef-building sponge in the Strait of Georgia, *Aphrocallistes*. I accomplish this by developing SNP markers for *Aphrocallistes* using a new technique that addresses the issue of duplication in the genome by allowing for extensive filtering of loci to identify single-copy markers. These markers are then applied to samples collected inside the Strait of Georgia and from Barkley Sound on the west coast of Vancouver Island, British Columbia for individual and population level analyses to imply the mode of reproduction (sexual or asexual) and to determine the level of genetic admixture between individuals in a clump, between individuals on a reef, and between sampling locations (including both reef and non-reef sites) within the Strait of Georgia, and between locations in the Strait of Georgia and Barkley Sound.

In Chapter 2, I develop informative single-copy genetic markers for *Aphrocallistes*. In order to work around the low attainment efficiency and duplication of microsat loci, I chose SNPs in combination with next generation sequencing because of the advantage provided by having a large number of markers from which to choose and the ability to avoid duplicated markers. I adapt reduced complexity genome sequencing using two restriction enzymes (RE) from Poland *et al.* (2012) to marker discovery, using the Ion Torrent Personal Genome Machine (Life Technologies) sequencing platform because of low in-house sequencing cost and long (400 bp) read lengths.

Reduced representation libraries (RRLs) allow for reduced genome sequencing by randomly sampling a small portion (ideally around 5%) of the genome that is sequenced consistently across multiple individuals (Peterson *et al.*, 2012; Slate *et al.*, 2009). Restriction-site associated DNA (RAD) tag sequencing is a particular brand of RRL that ligates specific barcoded adapters to digested DNA at RE cut sites before randomly shearing fragments and ligating a Y-shaped adapter, which allows selective amplification of sequences within 100 bp of those RE cut sites (Baird *et al.*, 2008). The promise of this type of sequencing approach for marker development in marine invertebrates has recently been highlighted in anthozoans (Reitzel *et al.*, 2013), and has proven popular, spawning many 'flavours' of RAD-type sequencing, all with small changes to the protocol. Examples include double-digest RAD

(ddRAD), which incorporates the use of a second RE in place of the shearing step and size selection to improve intersample consistency (Peterson *et al.*, 2012), and ezRAD, which applies the use of a kit (Toonen *et al.*, 2013). Considerable redundancy in the literature has ensued by the virtue of researchers publishing nearly identical methodologies under different labels, for example ddRAD and the two-enzyme genotyping-by-sequencing approach described by Poland *et al.* (2012). Though the approach to marker discovery used here is very similar to the Peterson and Poland methodologies, I avoid the growing problem of literature splitting in favor of calling it what it is: reduced complexity genome sequencing.

In Chapter 3, I apply the markers discovered in Chapter 2 to the question of reproduction and connectivity in *Aphrocallistes*. First, I explore the genetic population structure of *Aphrocallistes,* using a combination of traditional statistics (F_{ST}), Bayesian clustering analysis, and Mantel genetic and geographic distance correlations. I identify two distinct populations: one consisting of samples collected within the Strait of Georgia and the other consisting of samples collected from Barkley Sound. This clustering is supported by high pairwise F_{ST} values between locations within and outside of the Strait of Georgia, and low pairwise F_{ST} values between locations within the Strait of Georgia. I also show that there is little to no correlation between genetic distance and geographic distance between pairwise sample comparisons within the Strait of Georgia, suggesting a well-mixed population. Second, I show that there is no evidence of asexual reproduction in *Aphrocallistes* using pairwise multilocus genotype comparisons between individuals at all sites sampled.

In Chapter 4, I discuss my findings and their implications, and explore future directions particularly around larval and oceanographic work.

Chapter 2

SNP discovery in a reef-forming glass sponge, *Aphrocallistes vastus*, using the Ion Torrent next generation sequencing platform

Authors: Rachel R. Brown, Corey S. Davis and Sally P. Leys[1]

Abstract

Using next generation sequencing (NGS), we located 1121 single nucleotide polymorphism (SNP) markers in a reef-forming glass sponge, *Aphrocallistes vastus*. 16 SNPs were validated using Sanger sequencing. SNPs developed here are available for use in describing the genetic structure of glass sponge reefs, and demonstrate that NGS methods overcome problems posed by genomic DNA in some invertebrates.

[1]This Chapter has been reformatted from its original published version: Brown, R. R. *et al.* 2014. SNP discovery in a reef-forming glass sponge, *Aphrocallistes vastus*, using the Ion Torrent next generation sequencing platform. *Conserv. Genet. Resour.*, **6**: 49–51, see Preface for details.

2.1 Introduction

Glass sponge reefs form a globally unique habitat in the Northeast Pacific (Conway *et al.*, 2001). Marine Protected Areas (MPAs) have been proposed to protect reefs from fishing activity, and choice of which to include will rely in part on knowledge of their genetic diversity. Microsatellites are commonly used population markers, but where genomic duplication exists finding sufficient unduplicated markers *de novo* is difficult (e.g Ji & Zhang, 2004; Baums *et al.*, 2005). Here we adapt a method of selectively sampling identical genomic regions across several individuals (Poland *et al.*, 2012) to the discovery of SNPs, a high-density marker, on the Ion Torrent sequencing platform. This protocol allows duplicated loci to be removed from thousands of identified markers while retaining a high number of reliable SNPs.

2.2 Methods

2.2.1 DNA extraction

We collected tissue samples from *Aphrocallistes vastus* (Porifera: Hexactinellida), the dominant reef-building sponge in the Strait of Georgia, at eight discrete locations within four broad sample regions covering 900km of the Northeast Pacific from 2007-2011. We extracted DNA from ten samples (taken from all sample regions) with Qiagen DNeasy spin-columns following the manufacturer's instructions with these modifications: more tissue (approximately 5 mm^3) was digested in twice the reagent volumes (proteinase K, buffers ATL and AL, EtOH, and RNase A); tissue was incubated in lysis buffer overnight at 56°C and spin-columns were loaded in two steps. DNA was quantified using fluorometry.

2.2.2 Library development

A reduced representation library was developed using two restriction enzymes with paired linkers (Figure 2.1). Samples were digested with *PstI* (New England Biolabs (NEB)) in 10 separate 50 μl reactions (300 ng DNA, 5U *PstI*, 1X BSA, 1X Buffer 3) for 3 hours at

37°C. Modified Ion Xpress^{TM} Barcode Adapters (Life Technologies) were ligated to 200 ng of *PstI* digested DNA in separate 50 μl reactions (0.25 pmol adapter, 100 U T4 DNA ligase (NEB), 10pmol rATP). Barcoded samples were pooled, purified with the MinElute PCR Purification kit (Qiagen), and digested with *MspI* (NEB) in a single 50 μl reaction (5U *MspI,* 1X Buffer 4) for 3 hours at 37°C. Following a second column purification, a reverse Y-adapter (P1Y) including the Ion Torrent P1 primer sequence was ligated to the pooled DNA library in a 50 μl reaction (0.25 pmol adapter, 100 U T4 DNA ligase, 1X ligase buffer) at room temperature for 30 min. E-Gel SizeSelect Gels (Invitrogen) were used to extract bands at 200, 250, 300, and 350 bp both before and after PCR. Fragments were amplified in a 100 μl reaction using 2U Phusion High Fidelity DNA polymerase (NEB), 200 μM of each dNTP, 1X Phusion HF buffer, 0.5 μM of forward and reverse primers, and 3% DMSO, under the following conditions: 96°C for five minutes, 30 cycles of 96°C (45s), 60°C (60s), 72°C (60s), and a final extension at 72°C for five minutes (Figure 2.1b). Sequencing was performed on the Ion Torrent PGM (Life Technologies) using a 316 chip, resulting in 3,286,118 read fragments (416 Mbp, 126 bp mean read length).

2.2.3 Assembly and genotyping

In the absence of a reference genome, short contigs were assembled in CLC Genomics Workbench v6.0 (CLCbio) from 3,203,077 unsorted sequences after trimming to remove adapters, low quality sequences and short (<25 bp) reads, using a minimum contig length of 100 bp, word size of 21 and bubble size of 50. 1,899,488 reads were matched into 42,066 contigs averaging 149 bp long (minimum 18 bp, maximum 478) for a total assembled length of 6,257,020 bp.

1,885,052 demultiplexed reads (mean read length 125.93 bp) were aligned to the reference contigs with stringent similarity fractions (0.95) after trimming adapters and low-quality regions. Potential SNPs were identified with quality-based variant calls and filtered for variability and locus duplication. Symptoms of duplication included contigs with 100% heterozygosity and more than two alleles per locus. Variable loci required a minimum of one heterozygote and one homozygote genotype. 1121 filtered SNPs were obtained from 7393 variants called by CLC .

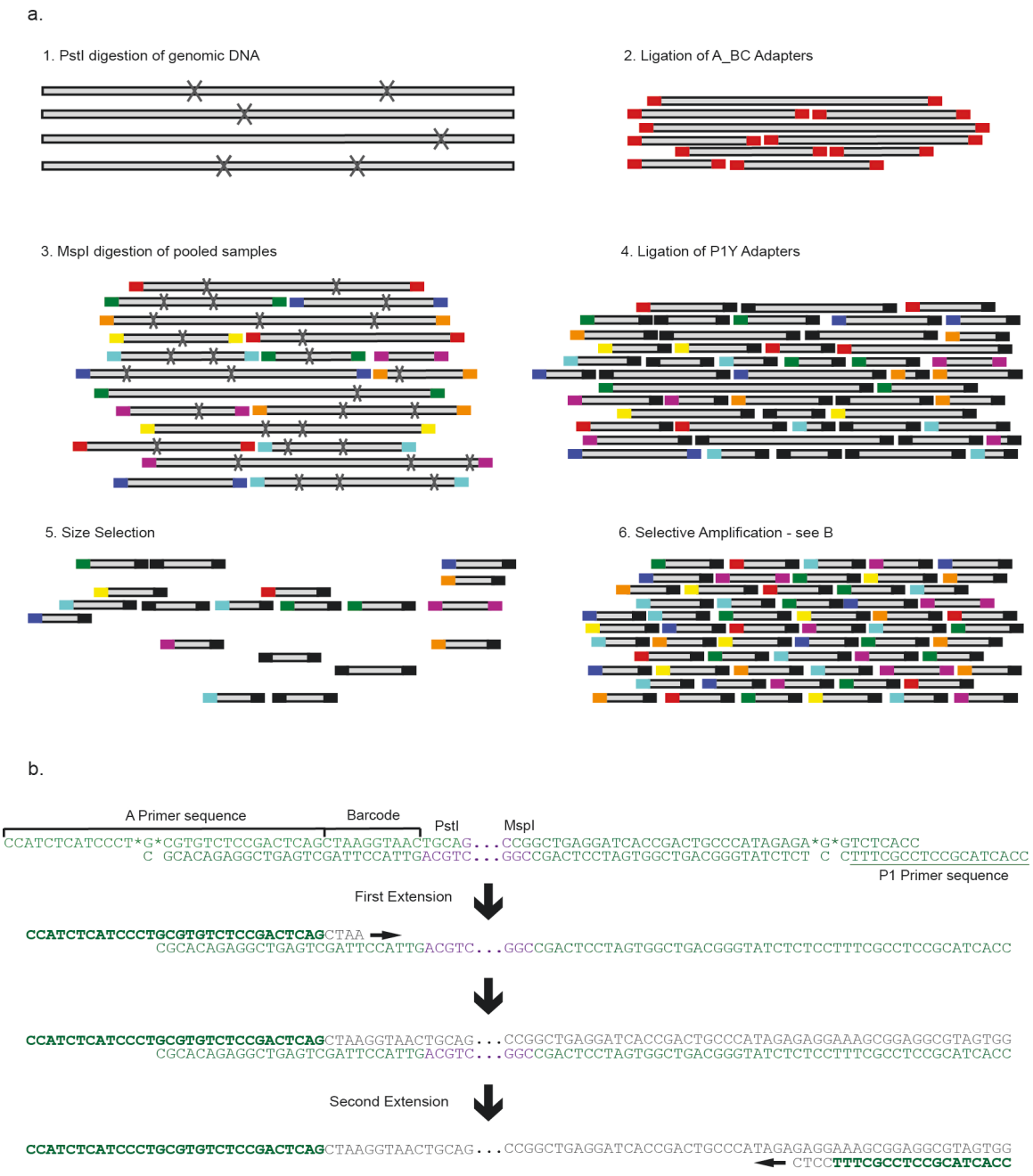

FIGURE 2.1: a) Reduced Representation Library construction (1) uncut genomic DNA is digested with a rare (6 bp) cutter RE, followed by (2) ligation of barcoded adapters; all barcoded samples are pooled, (3) digested with a common (4 bp) cutter, and (4) ligated to a Y shaped adapter; (5) Size selection is performed prior to (6) selective amplification of fragments with an A_BC adapter. b) Illustration of the first two PCR cycles (Primers are bold): P1 primers do not have a binding site until a complimentary strand is created using the A primer, resulting in amplification of only the fragments which have both A_BC and P1Y adapters, or 2 A_BC adapters (rare - eliminated in the sequencing). 2B adapted from Poland *et al.* (2012).

2.2.4 Validation

Ion Torrent genotype data was validated for 16 loci using Sanger sequencing on the same 10 samples. Paired forward and reverse primers were designed using Primer3 (Rozen & Skaletsky, 2000) with product lengths ranging from 60-120 bp. Samples were amplified using touchdown PCR conditions: 16 cycles of decreasing annealing temperature (56°-48°C in 0.5°steps) followed by 14 cycles at 48°C in 10 μl reactions (0.03 U colourless GoTaq Flexi DNA polymerase (Promega Corp.), 1X GoTaq buffer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 20 ng DNA template and 0.5 μM of each forward and reverse primer). Products were sequenced with Big Dye Terminate v1.1 chemistry on a 3730 DNA Analyzer (Applied Biosystems). Resulting sequences matched 93% of the Ion Torrent genotypes.

2.3 Conclusion

Using the methods described here, thousands of molecular markers can be developed in-house for species with unknown, complex, and duplicated genomes. The SNP markers developed in this study will be used to describe the population connectivity and diversity of the glass sponge reefs.

Acknowledgements

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Chapter 3

Genetic structure of the glass sponge *Aphrocallistes vastus* in British Columbia, Canada suggests broad dispersal among sponge reefs

3.1 Abstract

Reef-forming glass sponges are ecosystem engineers that provide habitat for many other marine species. Sixteen glass sponge reefs exist in deep waters off the coast of British Columbia, Canada and are of conservation interest. Due to their deepwater habitat, little is known about the mode of reproduction of glass sponges and the genetic connectivity between reefs. The genetic structure of populations of *Aphrocallistes vastus*, the primary reef-builder in the Strait of Georgia (SoG), British Columbia, was examined using single nucleotide polymorphisms (SNPs) at both reef and non-reef sites. Structure was examined at multiple scales: 1) across individuals sampled within and between clumps in reefs, 2) between reefs, and 3) between sites within and outside the SoG. Pairwise comparisons of multilocus genotypes were used to determine whether asexual reproduction is common in *A.*

vastus. Sixty-seven SNPs were genotyped across 79 samples from areas in and around the SoG, including four sponge reefs and individual sponges at nearby sites. Our results show that the reefs are formed through sexual reproduction. Within a reef, and even within the SoG basin, genetic distance between individuals does not vary according to geographic distance (R^2=0.0156). Importantly, populations within the SoG are genetically distinct from populations in Barkley Sound, west of Vancouver Island. High population structure was seen across all sample sites (global F_{ST} 0.247), with much higher differentiation between inside and outside SoG locations (average pairwise F_{ST} 0.2512) than within the SoG (average pairwise F_{ST} 0.0221). These results suggest genetic mixing across the reefs through well-dispersive larvae, and provide a baseline of connectivity that will provide insight into the management requirements of marine protected areas currently under discussion.

3.2 Introduction

Connectivity, the exchange of genetic material via dispersal of individuals or gametes, directly affects the resilience of populations (Cowen & Sponaugle, 2009). As such, habitat management efforts focus heavily on understanding connectivity in areas of interest for conservation (e.g. Robinson *et al.*, 2005; Bors *et al.*, 2012; Underwood *et al.*, 2013). Exploitation (e.g. mining; reviewed in Ramirez-Llodra *et al.*, 2011) has necessitated a better understanding of population connectivity in the deep sea so as to generate a baseline of data for management (Hilário *et al.*, 2015).

In marine systems, dispersal of offspring occurs via microscopic planktonic larvae that are nearly impossible to track, so population connectivity is often assessed indirectly using population genetics and/or biophysical modeling. However, biophysical modeling relies on prior knowledge of biological factors such as larval duration, behaviour, buoyancy, food, predation, and mortality, and these are unknown for many deep sea species (>200 m depth). Therefore deep sea connectivity work relies on population genetics or models of maximum dispersal based on estimated larval duration (Hilário *et al.*, 2015).

Sponges and corals are of particular interest in conservation efforts because they contribute to the high biodiversity seen in many deep-sea habitats by increasing habitat heterogeneity (Jumars, 1976; Buhl-Mortensen *et al.*, 2010). Sponges and corals both reproduce sexually and have free-swimming non-feeding larvae, but they may also rely to varying extents on asexual reproduction. Feeding larvae are capable of spending extended periods in the water column, such as veliger larvae from the genera *Cymatium*, *Charonia* and *Tonna* (Mollusca: Gastropoda) that remain pelagic for over 8 months (Scheltema, 1971). Non-feeding (lecithotrophic) larvae are limited by their energy reserves, and therefore have more strict dispersal boundaries, but also exhibit a large range of durations across taxa: many spend only hours or days in the water column while some persist for weeks. Remarkably, Graham *et al.* (2008) report maximum larval durations of more than six months in five sceleractinian coral genera that lack zooxanthellate symbionts – symbiont-carrying larvae can persist for periods comparable to most feeding larvae. Studies of larval duration in sponges suggest most settle within 1-3 days in laboratory conditions (Maldonado & Young, 1999; Leys & Degnan, 2001; Maldonado & Bergquist, 2002); larval settlement competency beyond that period and larval longevity *in situ* are unknown.

Glass sponges (Class Hexactinellida) are deep water sponges reaching SCUBA depths (approximately 30 m) in only two regions world-wide: the Northeast Pacific and submarine caves in the northern Mediterranean. They are particularly abundant in continental shelf waters of Antarctica and the Pacific coast of North America. Reproductive periods are often elusive (Kahn *et al.*, 2015b) so development has been described in only two species, *Farrea sollasii* and *Oopsacas minuta* (Ijima, 1904; Okada, 1928; Boury-Esnault *et al.*, 1999; Leys *et al.*, 2006). The larvae from *Oopsacas* and *Farrea*, known as trichimella, are 100 μm and 250 μm long respectively and rich in lipid and yolk (Okada, 1928; Leys *et al.*, 2006). *Oopsacas* larvae have a ciliated belt and rotate slowly upwards in laboratory settings (Leys *et al.*, 2006), generally settling within 1-2 days but can last 7 days (Leys *et al.*, 2007). *Oopsacas* provides the only clues to glass sponge larval behaviour thus far, and details of the potential or realized dispersal of the larvae are still unknown.

One Order of glass sponges, Hexactinosida, is able to form a three dimensional skeleton of fused silica which resists erosion after the sponge is dead, forming a scaffold for settlement

and growth of new individuals (Krautter *et al.*, 2006). On the Canadian and Alaskan coasts, three species of hexactinosidan glass sponges form massive reefs up to 21m high and tens of kilometers long that provide habitat for many other species (Conway *et al.*, 2005a; Cook *et al.*, 2008; Chu & Leys, 2010). The rigid scaffold of sponge skeletons is cemented together by sediment so that only the uppermost living layer is exposed (Conway *et al.*, 1991). The modern reef, therefore, has a patchy distribution with areas of dense live sponge cover consisting of tight clumps of individuals (2-10 m in diameter) separated by dead sponges or areas of bare mud (Chu & Leys, 2010). Sporadic sampling has shown that spermatocysts occur in winter (Appendix B) but larvae have yet to be found. Sexual reproduction with release of larvae is expected to be common, however, because juveniles less than 5 cm diameter are abundant among the reefs (Kahn *et al.*, 2015b; Appendix B). It is also expected that, as in other sponges, dispersal is limited by the nature of lecithotrophic larvae.

A consistently short larval duration across sponge species would lead to highly structured populations, and many sponge populations are known to be highly structured (e.g. Duran *et al.*, 2004b; Blanquer *et al.*, 2009; Guardiola *et al.*, 2011). In contrast, Whalan *et al.* (2008b) found genetic homogeneity in the Great Barrier Reef sponge *Rhopaloeides odorabile* over a scale of tens of kilometers, with a similar pattern described across 8,000 km in *Chondrosia reniformis* (Lazoski *et al.*, 2001). However, both studies used allozymes as markers, which may have less power to detect population structure relative to microsatellite markers, now commonly used for studying both population structure and mating systems. Single nucleotide polymorphisms (SNPs) are abundant throughout the genome (avg 1 per 300 bp), yet SNPs have yet to be used to analyze sponge population genetics. SNPs may provide an additional advantage in some species. Single-copy microsatellite loci have proven difficult to ascertain in some invertebrate species of barnacles (Barazandeh & Davis, 2011), coral (Marquez *et al.*, 2000), and lepidopterans (Zhang, 2004), as well as the glass sponge species studied here (Brown *et al.*, 2014), possibly from duplication in portions of the genome. Although it is expected that SNPs would also be duplicated in these genomes, it is easier to determine which loci are duplicated because of their biallelic nature. Their even spread throughout the genome may also increase the probability that single-copy loci are obtained.

We[1] examined the genetic structure of a reef-forming glass sponge in southern waters of British Columbia, Canada using SNP markers. Specifically, we examined population structure across individuals sampled within and between clumps in reefs, between reefs, and between sites (both reef and non-reef) within and outside the SoG. We also looked for evidence of asexual reproduction by comparing multilocus genotypes. In doing so, we aim to uncover the mode of reproduction in reef-forming sponges and highlight the role of dispersal in creating sponge reefs. Sponge reefs have a patchy distribution which could be a product of asexual reproduction, such as budding, or local recruitment of larvae (Chu & Leys, 2010; Figure 3.1a). An individual sponge begins with one osculum, the vent for filtered water (exhalant flow) and unit of organization in sponges, but can develop more as it grows larger. Each sponge can have 5-10 oscula although the bases of sponges may be dead, are not all visible in a clump, and are often covered by sediment (Figure 3.1b). Because of this, it is unclear from imagery whether individuals in clumps are discrete or physically connected, but sponges generally appear as distinct individuals due to slight differences in morphological aspects and colour. The extent to which the sponges in a clump are related, either as clones or as kin, remains unknown. We are able to address these questions by combining the molecular methods with the high-tech capabilities of the Remotely Operated Platform for Ocean Sciences (ROPOS) to determine relatedness of sponges within a clump using precise GPS coordinates along with video and still images recorded of each sample collected. As glass sponge reefs have been designated areas of interest for future status as a Marine Protected Area (MPA) (Jamieson & Chew, 2002), understanding the level of connectivity between the reefs is important for conservation efforts.

3.3 Methods

3.3.1 Study region and species

The Strait of Georgia (SoG) is a semi-enclosed basin 200 km by 30 km that lies between the British Columbia mainland and Vancouver Island. The basin is 420 m deep in the central

[1]This chapter is written for publication and will be submitted to Conservation Genetics. See Preface for details.

Strait, but the average depth is 155 m. To the south the SoG connects to the Pacific Ocean through the Strait of Juan de Fuca and to the north it connects via Johnstone Strait and other very small channels to Queen Charlotte Sound (Figure 3.1).

Sponge reefs in the SoG are formed by two species of rigid (dictyonine), reef-forming glass sponge, *Aphrocallistes vastus* and *Heterochone calyx* (family Aphrocallistidae; hereafter referred to as *Aphrocallistes* and *Heterochone*). Both species are also found in non-reef habitats in the SoG including fjords and inlets throughout the BC coast (Leys *et al.*, 2004) where they colonize hard surfaces (rock walls, outcrops, and even sunken ships) as shallow as 18 m but usually below 25 m depth. In some places, they reach densities of up to 240 individuals/10 m^2 (Leys *et al.*, 2004), but the highest density of glass sponges known occurs in sponge reefs (463 individuals/10 m^2; Chu & Leys, 2010).

Heterochone has a hydroid symbiont that often gives it a characteristic orange colour (Schuchert & Reiswig, 2006). *Aphrocallistes* is thought to be symbiont-free, making it a better choice for molecular work. Though they can appear very similar *in situ*, the two species can be differentiated by their spicule skeleton complement. We targeted *Aphrocallistes* for this study by avoiding orange coloured sponges and confirmed species identity by microscopy.

3.3.2 Sampling methods

Using the remote operated vehicle (ROV) ROPOS, pieces of sponge tissue approximately 5 cm^2 were collected from both reef and non-reef sites by manipulator arm or suction sampler on 31 dives between 2007 and 2011. We sampled four reefs in the SoG: Howe Sound Reef, Galiano Ridge Reef, Fraser Ridge Reef, and McCall Bank Reef (hereafter referred to as Howe, Galiano, Fraser, and McCall Reefs). Non-reef sites included two adjacent sites at San Jose Islands and Josie Islets in Barkley Sound (hereafter grouped together as Barkley Sound) on the west coast of Vancouver Island and three sites within the SoG: Sabine Channel on Texada Island (hereafter Texada), Coral Knoll, and McCurdy Point in Saanich Inlet, Vancouver Island (collected by SCUBA). Coordinates for sample locations are listed in Table A.1 and mapped in Figure 3.1c. Samples from Learmonth Bank (north of Haida Gwaii

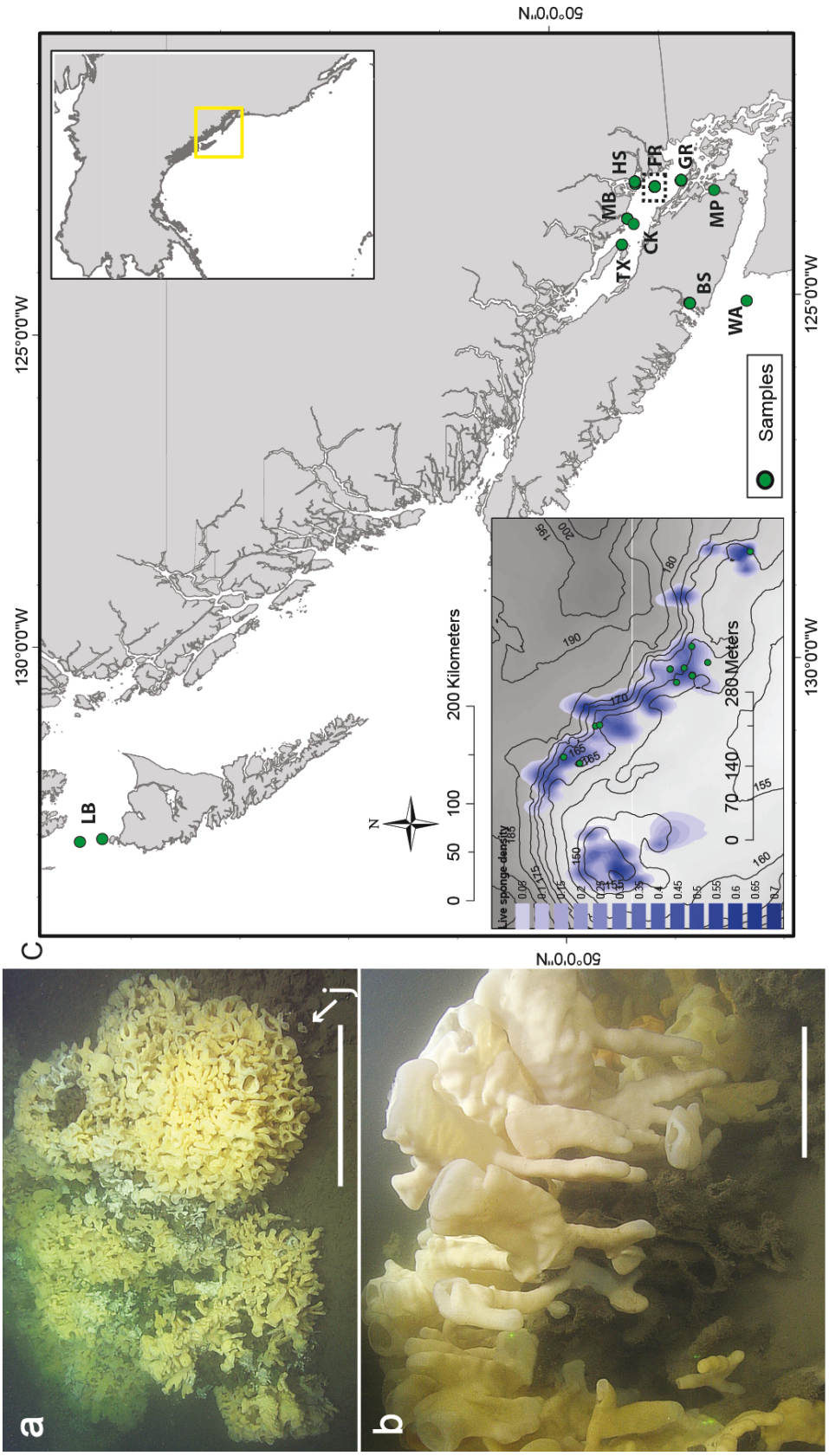

FIGURE 3.1: a) A clump of reef sponges *Aphrocallistes vastus* and *Heterochone calyx*, surrounded by sediment anchoring skeletons of past generations. A juvenile (j) on skeleton is visible close to the live sponges. Scale bar = 1 m. b) Individuals within a clump are often dead at the base, which may be obscured by sediment. Scale bar = 20 cm. c) Map of sample sites, including those collected for SNP discovery in Washington and Learmonth Bank: Sabine Channel at Texada Island (TX), McCall Bank Reef (MB), Howe Sound Reef (HS), Coral Knoll (CK), Fraser Reef (FR), Galiano Reef (GR), Barkley Sound (BS), Olympic Coast National Marine Sanctuary in Washington (WA), and Learmonth Bank (LB). Inset corresponds to the dashed box and shows detail of sampling in Fraser Reef as an example of reef sampling, with density of live sponge cover in blue (provided by Chu & Leys (2010)) shown for context.

B.C.) and from the Olympic Coast National Marine Sanctuary in Washington (courtesy of Jim Boutillier, Pacific Biological Station) were used for marker discovery (Brown *et al.*, 2014; Chapter 2), to assess SNP quality and strength, and to examine relatedness between individuals.

At each reef in the SoG, pieces were taken from up to 5 sponges in a clump (defined as a group of sponges 1-5 m in diameter around which the ROV could settle without damaging adjacent sponges) and from clumps across the reef (ranging from 5 m to 2.2 km apart) (Figure 3.1c). Self-self controls were taken by sampling the same individual twice. Precise spatial positioning recorded by the ROV provided GPS coordinates for all samples, which were placed into separate, closed collection boxes (shown in Figure A.4b). High definition video and still images accompanied all sample collections. On the ship, using a new razor blade for each collection, a 2 cm^2 piece was cut from each sample and stored in 95% ethanol at -20°C for transport to the University of Alberta. Species identity was confirmed by microscopy. To determine that specimens were not reproductive at the time of collection and therefore contained no larvae with additional parental genotypes, a second piece from each collection was fixed in Bouin's for 1-2 days, rinsed twice in water, dehydrated through a graded ethanol series and embedded in paraffin wax. Sections 30 μm thick were stained with hematoxylin and eosin and viewed with a Zeiss Axioskop microscope.

3.3.3 SNP discovery

Our first efforts isolated microsatellite markers which were duplicated (Jensen, 2011). We therefore chose to isolate SNP markers using a reduced genomic complexity next generation sequencing approach. This approach provides a large number of typically bi-allelic markers sampled randomly through the genome, providing a greater chance of sampling non-duplicated genomic regions and also allowing for identification of duplicated markers. Marker discovery is described in Brown *et al.* (2014) (Chapter 2) and summarized here. Genomic DNA was extracted with Qiagen DNeasy spin-columns using the manufacturer's instructions with some modification (see Chapter 2). A subset of ten samples selected from all sample regions were used to construct reduced representation libraries that were pooled and sequenced on an Ion Torrent Personal Genome Machine (PGM; Life Technologies). Variant

detection in the CLC Genomics Workbench v6.0 (CLCbio) identified 7393 variants *de novo.* Variants were manually filtered to remove uninformative or apparently duplicated markers, based on 100% heterozygosity in all individuals or greater than 3 alleles at a locus across all individuals. If one or more locus on a contig showed signs of genomic duplication, all loci on that contig were discarded. Marker validation was performed using Sanger sequencing technology on 16 SNPs, after which additional filtering to remove duplicated sequences (identified by divergent flanking sequences), low coverage SNPs (more than eight individuals had less than 30 reads each), and SNPs with inadequate flanking sequences for primer design was carried out manually in CLC. Eighty-one contigs containing 93 SNPs passed the filters and were retained for genotyping. Forward and reverse primers were designed in Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Product size ranges of 50-125, 100-200, and 200-250 bp were targeted. Optimal primer size was set to 20 bp (min 16 bp - max 24 bp), Tm was set to 60°C (min 55°C - max 65°C; max difference of 1°C within primer pair), and GC% was set to 50% (min 20% - max 80%). Sequence specific primers were designed with *"Glenn"* (forward) and *trPI* (reverse; Ion Torrent, Life Technologies Inc.) primer tails (see Table A.3 for tail sequences) to serve as primer binding locations in a second barcoding PCR.

3.3.4 Amplification and sequencing

SNP containing amplicons for 96 samples including a negative (no DNA template) control were amplified in four multiplexed reactions for each sample (Qiagen multiplex PCR kit). Multiplexes were generated by sorting the 81 primer pairs into four sets (3x 20 and 1x 21; Table A.3) based on the design length of the amplicon, clustering similar lengths while avoiding duplicate lengths to limit amplification bias by PCR and facilitate visualization of PCR products for optimization. Multiplex PCR reactions in a final volume of 10 μl included 1X Type-It PCR master mix (Qiagen), 2 μM each primer, and 20 ng of template DNA. Primer pools 1 and 2 (Table A.3) were amplified using an annealing temperature of 51°C (15 minutes at 95°C, 30x [30 sec at 94°C, 2 min at 51°C, 60 sec at 72°C], and 30 min final extension at 60°C). Primer pools 3 and 4 were run using touchdown PCR conditions which used the same settings except for the annealing stage: 16 cycles of decreasing annealing

temperature (56°C - 48°C in 0.5°C steps) followed by 14 cycles at 48°C. The four multiplex reactions were pooled for each individual and diluted 1:500. Ion Xpress^{TM} Barcode Adaptors (Life Technologies) modified to contain *Glenn* (forward primer) tails were amplified with *trP1* (reverse primer) into fragments using 3 μl of the pooled, diluted PCR product as template in a second PCR in 10 μl reaction volumes (0.3 U Phusion High Fidelity DNA polymerase (New England Biolabs), 160 μM dNTPs, 3% DMSO, 1X Phusion HF buffer, 1.5 mM $MgCl_2$, 1.6 μM each of *trPI* and barcode primers; 5 min at 94°C, 35x (30 sec at 94°C, 30 sec at 55°C, 60 sec at 72°C), and 10 min final extension at 72°C). The amplification workflow is summarized in Figure A.1. Small fragments (e.g. primer dimers) were excluded by sequential PCR purification (Qiagen Qiaquick PCR purification column) and gel purification (Qiagen Qiaquick gel extraction with 1% agarose gel) prior to sequencing.

Samples were run on the Ion Torrent PGM using a 316 chip and a 200 bp read kit according to the manufacturer's protocol. Controls consisted of three samples from the SNP discovery process (across-run controls), one sample extracted twice (in-run control), three self-self controls, one different species (*Heterochone*) sample, and one negative control (no DNA template).

3.3.5 Genotyping and analysis

Assembly and SNP genotyping were completed in SeqMan NGen, SeqMan Pro and Arraystar of Lasergene Suite 12 (DNASTAR, Inc). The minimum read depth required for minimize false homozygote calls (probability set at 0.01) was calculated for each locus separately based on observed heterozygosity and average read depth as described by Chenuil (2012). Genotypes not meeting the minimum read depth requirement were discarded. Individuals and loci with greater than 10% missing data (genotypes either missing from sequence data or with insufficient read depth) were removed from analysis. Marker characteristics including allele frequencies, observed and expected heterozygosities, and probability of identity were calculated using GenAlEx 6.5 (Peakall & Smouse, 2006, 2012). Deviations from Hardy-Weinberg equilibrium and pairwise linkage disequilibrium were tested using Genepop 4.2 (Raymond & Rousset, 1995; Rousset, 2008). Genetic distance between populations was assessed using F statistics, and within and between populations using Mantel

tests in GenAlEx. Mantel tests used pairwise linear codominant genetic distance calculated in GenAlEx by weighting genotypic differences from 0 to 4 (0 being identical genotypes, 1 being a homozygote and heterozygote combination, and 4 being opposing homozogous genotypes) and pairwise linear geographical distance calculated in km from decimal latitude and longitude coordinates, recorded by the ROV.

Samples were assigned to genetically homogenous populations (K) inferred using a Bayesian clustering algorithm based on allele frequencies and Hardy Weinberg equilibrium assumptions without prior geographical information using STRUCTURE v2.3.4 (Pritchard *et al.*, 2000). An admixture model was used with correlated allele frequencies and 1,000,000 MCMC iterations (burn-in of 50,000), repeated 20 times for each value of K from 1 to 8. The most likely value of K was determined using Evanno's *ad hoc* ΔK statistic, which describes the second order rate of change for the probability of the data (Evanno *et al.*, 2005), calculated and plotted by Structure Harvester web v0.6.94 (Earl & VonHoldt, 2012). The 20 replicates of optimal K were aligned using the *FullSearch* algorithm in the software package CLUMPP v1.1.2 (Jakobsson & Rosenberg, 2007), then graphed using DISTRUCT v1.1 (Rosenberg, 2004). Individuals that assigned with greater than 80% ancestry to the largest cluster (i.e. SoG below) were isolated and run again through the same analysis to test for additional levels of substructure.

To examine relatedness between individuals, multilocus genotypes were compared in GenAlEx using the 'multilocus matches' function, which outputs a list of pairwise comparisons and the number of differing locus genotypes. Missing data was ignored. Genotypes at each locus were scored as either a 0 (perfect match) or 1 (different), totaled for each pairwise comparison, and graphed as a percent of total loci. A generalized extreme studentized deviate (ESD) test for outliers was performed on the experimental sample set (without controls) using the Real Statistics Resource Pack software (Release 3.8: Copyright (2013 – 2015) Charles Zaiontz. www.real-statistics.com).

3.4 Results

3.4.1 Description of the reefs

The sponge reefs appear as fields of orange and white pillars 1-2m high, each with many mitten-shaped extensions (Figure 3.1a). There are at least 12 reef complexes in the Strait of Georgia (Conway *et al.*, 2005a, 2007), each having a signature identified by multibeam mapping and side-scan sonar (Conway *et al.*, 2005a). The four reefs we sampled in the SoG are between 21 km and 68.5 km apart (Fraser and Howe are closest to each other, while McCall and Galiano are furthest apart). Reefs are separated by expanses of mud which dictyonine sponges are not known to colonize.

3.4.2 Genotypic variation

Of 106 samples sequenced (including 10 samples from the marker discovery run), genotypes of 92 samples including the single *Heterochone* control sample were retained (excluding therefore the negative control and 13 samples with high rates of missing data (>10%)). Of 93 loci, two did not produce informative reads, ten had high rates of missing data (>10%), and three were of low quality (the sequence was unresolved or adjacent to homopolymer region). Two loci were monomorphic and nine were removed because of physical linkage. In total, 67 loci were used for analyses. Though all loci used in analyses were in Hardy-Weinberg equilibrium and linkage equilibrium when samples were grouped by site after carrying out Bonferroni correction (Table 3.1), disequilibrium was observed at global and regional scales, indicating that random mating occurs within sample sites but that there is population structure at larger scales. Probability of identity for all loci in each sample site ranged from $4.19\text{x}10^{-19}$ - $6.35\text{x}10^{-15}$ (Table 3.2). All loci were amplified and sequenced in *Heterochone* and were homozygous.

TABLE 3.1: Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) values for three hierarchical sampling levels (Global: all samples; Regional: inside or outside Strait of Georgia; Population: sample site; Exp: expected type I error; Obs: number of observed loci in disequilibrium at that significance level). No Result is due to monomorphism in HWE tests and one column or row in contingency table for LD tests.

	Global HWE		**Regional HWE**		**Population HWE**	
Significance	**Exp**	**Obs**	**Exp**	**Obs**	**Exp**	**Obs**
0.05	3.35	22	6.7	20	20.1	30
0.01	0.67	12	1.34	7	4.02	11
Bonferroni	[0.00075]	7	[0.00037]	2	[0.00012]	0
No Result		0		4		51
	Global LD		**Regional LD**		**Population LD**	
Significance	**Exp**	**Obs**	**Exp**	**Obs**	**Exp**	**Obs**
0.05	110.55	328	221.1	136	663.3	234
0.01	22.11	178	44.22	43	132.66	38
Bonferroni	[2.26E^{-5}]	41	[1.13E^{-5}]	1	[3.77E^{-6}]	0
No Result		2		1182		3153

TABLE 3.2: Sample size (N), mean observed (Ho) and expected (He) heterozygosities, and probability of identity (PID) by sample site (Texada (TX), Fraser Reef (FR), Galiano Ridge (GR), Howe Sound (HS), McCall Bank (MB), Barkley Sound (BS)).

Site	**N**	**Ho**	**He**	**PID**
TX	5	0.304	0.307	1.20465E^{-18}
FR	17	0.369	0.319	1.8249E^{-19}
GR	14	0.420	0.320	1.82084E^{-19}
HS	20	0.342	0.323	1.03331E^{-19}
MB	11	0.350	0.312	4.19481E^{-19}
BS	11	0.267	0.240	6.35248E^{-15}

3.4.3 Genetic distance between and within populations

All population level analyses, including Hardy-Weinberg and linkage disequilibrium tests, probability of identity, FST, and STRUCTURE, were performed on 78 samples after removing all *Aphrocallistes* controls (7), the single *Heterochone* control, and locations with fewer than five samples (Learmonth Bank-2, Saanich Inlet-1, Washington-2, and Coral Knoll-1). Global F_{ST} across all sites was 0.247 (p=0.001), indicating a high degree of population differentiation. Pairwise F_{ST} values ranged from 0 to 0.057 between sites inside the SoG and from 0.191 to 0.274 between Barkley Sound and SoG sites (Table 3.3). A model for two genetically homogenous clusters (K) had the highest likelihood (Figure A.2a) determined by Bayesian clustering analysis (STRUCTURE; Figure 3.2a). Barkley Sound and Texada

were distinguished from SoG reefs by cluster assignment. No further population structure was found after running the analysis on samples assigned with greater than 80% ancestry to the largest cluster, which dominated the SoG (n=64; Figure A.2b). No correlation between geographic and genetic distance was found between populations within the SoG (Mantel test, R^2=0.0156, p=0.030; Figure 3.2b) or at the reef level within each reef (Figure 3.2c-f).

TABLE 3.3: Pairwise F_{ST} values between six sample sites (Texada (TX), Fraser Reef (FR), Galiano Ridge (GR), Howe Sound (HS), McCall Bank (MB), Barkley Sound (BS)). P-values are above the diagonal (bolded values are significant after Bonferroni correction).

	TX	**FR**	**GR**	**HS**	**MB**	**BS**	**N**
TX	-	*0.040*	*0.088*	*0.166*	*0.256*	***0.001***	5
FR	0.056	-	*0.324*	*0.095*	*0.053*	***0.001***	17
GR	0.043	0.000	-	*0.129*	*0.044*	***0.001***	14
HS	0.015	0.015	0.013	-	*0.169*	***0.001***	20
MB	0.010	0.030	0.032	0.009	-	***0.001***	11
BS	0.191	0.274	0.270	0.252	0.269	-	11

3.4.4 Relatedness between individuals

Genotypic variation, measured by the percent difference between multilocus genotypes in pairwise comparisons between *Aphrocallistes* samples (n=91, 4095 pairwise comparisons), showed no identical multilocus genotypes outside of the control groups (Figure 3.3), that is, there was no evidence of asexual reproduction. The DNA in-run control pair differed at 0% of loci, while the three across-run control pairs (from the marker discovery sequences) displayed 2.6%, 6.5%, and 9.1% genotypic difference. The three sponge self-self control pairs (individuals that were sampled twice) differed at 1.3% (two pairs) or 9.1% (one pair) of loci. Two exceptional cases among comparisons between "experimental pairs" (i.e. pairs sampled from distinct oscula) were found in two reefs (Figure A.4). At Howe Reef, two sponges with 5.2% genotypic difference were less than 0.5 m apart and had dead bases that appear to be connected by a mostly buried ridge of dead skeleton. At Galiano Reef, three closely packed oscula had similar multilocus genotypes: two differed from each other at 10.4% of loci, and the third differed from both of the others at 16.9% of loci. The two pairwise comparisons with 5.2% and 10.4% difference were the only statistically significant outliers within the experimental sample set ($p<0.05$; ESD test for up to seven outliers). The

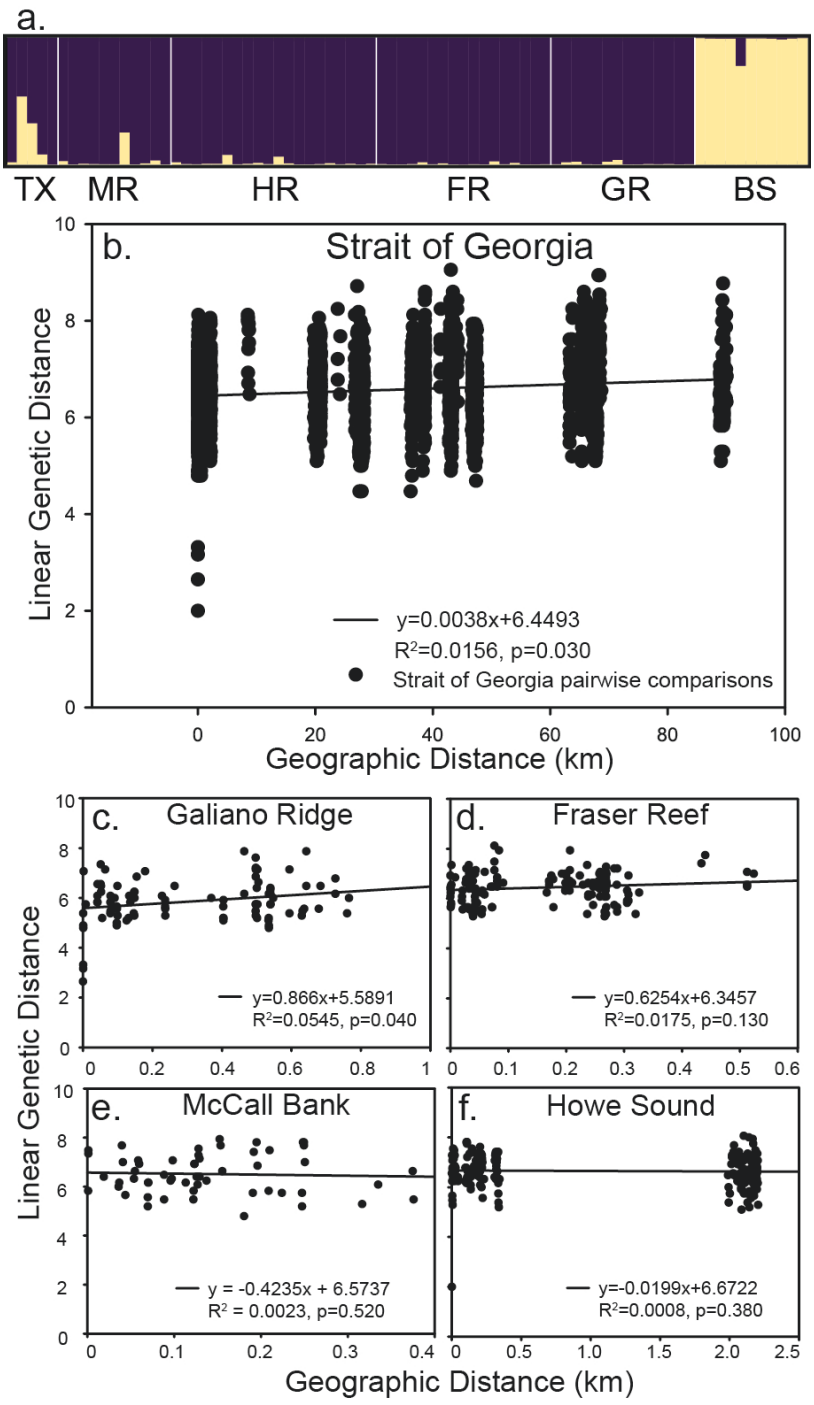

FIGURE 3.2: Genetic structure. a) STRUCTURE Bayesian analysis for two clusters (K) determined using the Δ K method (Evanno *et al.*, 2005). Each column represents a sample assigned proportionally to each genetic cluster. b) Mantel test comparing individuals of *Aphrocallistes vastus* in the Strait of Georgia populations, including Texada, Galiano Ridge, Fraser Reef, Howe Sound, and McCall Bank sites (n=68). c-f) Mantel tests comparing individuals of *Aphrocallistes vastus* within each of the four reefs.

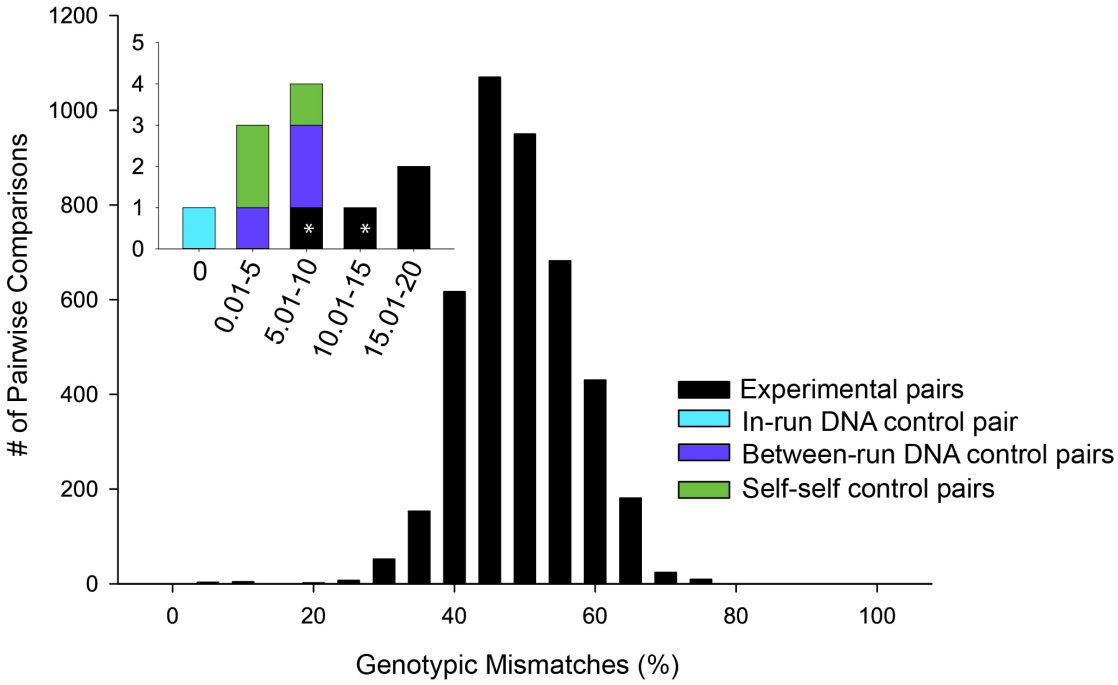

FIGURE 3.3: Relatedness between individuals of *Aphrocallistes vastus* as shown by the percent of genotypic differences between pairwise comparisons of individuals (N=91). Genotypes between individuals are compared at each locus (of 77), where mismatches are totaled for the pair and presented as a percent. Missing genotypes are ignored. Starred experimental pairs are statistically significant outliers ($p<0.05$; ESD test). Control pairs were created by extracting DNA twice from the same sample and sequencing in the same run (In-run), sequencing the same samples on different sequencing runs (Between-run), and sampling the same *Aphrocallistes* individual twice (Self-self), and are presented as a comparison to the sample population. For more details on controls see Figure A.3.

5.2% data point lies clearly within the 0-9.1% difference range seen in the control pairs and the 10.4% data point falls outside of the controls by a single allele, so neither are considered sufficiently different from the controls to describe distinct individuals. Genotypic differences for the sample (experimental) population averaged 46.5% and ranged from 5.2% to 74.0% (without the two exceptions, minimum difference is 20.8%). Overall, adjacent individuals had distinct multilocus genotypes with no greater relatedness than random sample pairs.

Methodology workflows for both the marker discovery and genotyping phases are summarized in Figure A.3.

3.5 Discussion

The glass sponge reefs in the Strait of Georgia provide a unique opportunity to study dispersal in a deep-sea species within a semi-enclosed marginal sea. Though samples are difficult and expensive to collect, they are more readily accessible in the SoG than from deep populations off the continental shelf.

Our results show high regional genetic structure in the reef-building glass sponge *Aphrocallistes vastus*, distinguishing populations in the SoG from those on the west coast of Vancouver Island; however, reef and non-reef sites in the SoG show little genetic differentiation, suggesting that larvae (and/or broadcast sperm) disperse extensively within this region. Within reefs the genetic distinctness of adjacent sponges and the general absence of identical multilocus genotypes both imply that even 1-5 m diameter clumps of sponges in a reef are the result of sexual reproduction and that larvae rarely settle and recruit within the parent clump.

3.5.1 Genetic distance between regions

Sites on the west coast of Vancouver Island (Barkley Sound) are genetically distinct from the sites within the SoG. The distinction between the SoG and Barkley Sound populations could be caused by geography alone, because the distance from Barkley Sound to the closest sample site (Galiano Ridge) around the southern tip of Vancouver Island is 275 km. Samples within the SoG appear to belong to the same population, which may be self-recruiting or sourced from deeper waters in the Pacific, presumably arriving to the SoG via its southern entrance at the Strait of Juan de Fuca. Samples collected from Texada Island in Sabine Channel, which connects to the northern SoG, showed mixed ancestry according to STRUCTURE analysis. This relationship, although weak, suggests that individuals on the edges of the SoG may be more genetically similar to outside populations, and that the area around Texada Island represents an admixture zone. The widely-distributed deep-water coral *Lophelia pertusa* shows a similar pattern in structure between open and fjord populations, attributed to differences in geological and hydrological characteristics, or divergence in populations since the formation of the fjords (Goff-Vitry *et al.*, 2004).

3.5.2 Between reef and within-reef homogeneity and genetic distinction of individuals

Within the SoG, little population structure was found. There is little genetic differentiation between individuals in clumps relative to between clumps or between sites (reefs or non-reef) in the SoG, indicating larvae must disperse widely throughout the SoG basin.

Individuals within reefs were equally genetically distinct at all spatial scales (within clumps and between clumps), so the clustered pattern of sponges within a reef may be best understood as a response to the high flow required to sustain considerable grazing rates (Kahn *et al.*, 2015a) or substrate availability. Identical multi-locus genotypes (MLGs) between individuals generally provide identification of clones (asexual reproduction) in a population. Only one shared MLG was observed between two adjacent samples presumed to be different individuals, which appeared to be connected by skeleton. Thus it is likely that these two sponges represent reduced living portions of a large, old individual sponge, rather than an example of discrete asexual reproduction. This absence of clonality in the population is surprising because examples of asexual reproduction are common in benthic invertebrates, and range in degree from species like *Lophelia pertusa* that are predominantly sexually reproducing but use asexual reproduction when larval recruitment is low (Goff-Vitry *et al.*, 2004), to species like *Pocillopora damecornis*, *Tubastrea diaphana,* and *T. coccinea*, which produce larvae asexually (Ayre & Resing, 1986; Yeoh & Dai, 2010). *Aphrocallistes* can apparently form 'drips' of tissue that Austin (2003) proposed may be a form of asexual reproduction; however, we have never observed "dripping" in reef populations, and unique MLGs observed between adjacent individuals indicate that reefs arise by sexual reproduction. In a population study of *Crambe crambe*, an intertidal encrusting demosponge from the Mediterranean, 76 of 177 sponges examined were clones from 24 lineages, with an average distance between clone mates of 20 cm (Calderón *et al.*, 2007), a pattern which could be similarly formed through growth and recession as seen in this exceptional case of two identical but physically separated individuals. The three adjacent oscula with a genetic difference of 10.4-16.9% at Galiano Reef present another exception. Whereas samples taken within clumps were generally collected from apparently distinct individuals that were clustered within approximately 5 m of each other, this trio was collected from three adjacent

oscula that appeared to be the same individual, though the base could not be seen. Because the pairwise comparison of two oscula with 10.4% difference represents a statistical outlier and is virtually indistinguishable from an error rate of nearly 10%, those oscula are likely two branches of the same individual as would be expected. The third osculum differs from the previous two by 16.9%, outside of the error range, and likely comprises a related individual. Although the general trend points to wide dispersal of larvae, this could represent an additional layer of complexity in dispersal as an instance of local recruitment within a clump. Further sampling within tightly clustered oscula presumed to be from the same individual could determine whether this is a unique occurrence or a pattern.

Individual heterozygosity was normally distributed across the sample set with no outliers (data not shown), which, combined with the fact that individuals sampled twice had the same MLG, suggests that separate individuals do not fuse to form chimeras as seen in some other sponge species (e.g. Blanquer & Uriz, 2011). Genetic distinctness between individuals of *Aphrocallistes* is further supported by an inability of tissue pieces from different individuals to fuse, whereas the syncytial tissue from self-self controls are able to fuse (Table A.2). Fusion in a glass sponge, which is entirely syncytial, means joining cytoplasm and nuclei under a single cell membrane.

3.5.3 Accuracy of the SNP sequencing approach

It was originally envisioned that microsatellite markers would be used to conduct this study, however during microsatellite development and testing we encountered what appears to be duplication of portions of the genome (Jensen, 2011). The ineffectiveness of COI as a population marker in sponges, consistant with low mitochondrial mutation rates in basal branching metazoans (Watkins & Beckenbach, 1999; Shearer *et al.*, 2002), was also confirmed in *Aphrocallistes* and *Heterochone* through a paucity of intraspecific variation. SNPs were therefore chosen in combination with next generation sequencing because they offered the advantage of having a large number of markers from which to choose and the ability to identify duplicated markers. However, there were high rates of non concordance (<9.1%) between multilocus genotypes in pairs of control samples. Differences in accuracy between methodologies are highlighted in four DNA control pairs. The three across-run

control pairs, which consisted of DNA extractions that were sequenced twice using two differing methodologies (reduced representation library and amplicon sequencing), differed at 2.6% - 9.1% of loci. In contrast, no difference was found between the in-run control pair consisting of DNA extracted twice from the same sample and sequenced with the same methodology (amplicon sequencing). Amplicon sequencing is more likely to provide accurate results than the reduced genome sequencing used for marker discovery through more selective amplification offering greater read depths. But there were also high rates of non concordance (1.3% - 9.1%) in the three self-self control pairs, sponge oscula sampled twice but sequenced in the same amplicon-sequencing run. These error rates, comparable to the across-run DNA controls, suggest that high error rates may also arise from error inherent in PCR and sequencing, as well as genotyping calls. However, this may also be caused by genomic duplication which, despite stringent marker filters, could cause an increase in false genotyping calls due to skewed read percentages and sampling error. To compensate for the high error rates, we relaxed the range allowed for genotypic difference of identical MLGs, but this did not affect the inference made about population structure.

The species control provided by *Heterochone* further supports the accuracy of the results and highlights an interesting relationship between species. In *Heterochone*, the 100% call rate paired with 100% homozygosity for *Aphrocallistes* markers (all genotypes matched one of the two alleles identified in *Aphrocallistes*) would be expected in comparing two closely related species with a divergence time of approximately 2-3 my (Miller *et al.*, 2012a). The phylogeny of the family Aphrocallistidae is uncertain; recent molecular analyses have placed *Heterochone* and *Aphrocallistes* as sister species with a recent divergence (Dohrmann *et al.*, 2011) consistent with the above date.

3.5.4 Implications for larval dispersal in the Strait of Georgia

The results described here suggest larvae from glass sponge reefs are widely distributed in the SoG presumably by currents. In the SoG, there is a strong estuarine flow out to the Pacific Ocean fed primarily by the Fraser River, with a return flow at 50 to 200 m depth (Pawlowicz *et al.*, 2007). Strong semidiurnal tidal currents reach 1.3 m s^{-1} through the Straits (Davenne & Masson, 2001), though these apparently diffuse in the central SoG basin

to an average of 0.5 m s^{-1}. Two eddies in the central SoG promote east-west transport across the Strait (Stacey *et al.*, 1987; Snauffer, 2013), which may cause a degree of isolation at the northern Texada site. Currents accelerate over the ridges formed by the reefs by up to 0.5 m s^{-1} higher than surrounding flow (Bedard, 2011), though flow at the level of the sponge oscula is much slower, from 0-15 cm s^{-1} (Leys, in prep). High currents over the reefs may facilitate larval dispersal, while the extensive boundary layer may make settlement easier at the reefs. In a hypothetical situation of maintained currents that directly connected the reefs sampled, particle transport from one location to another could be accomplished in 11.7 - 38 hrs, the approximate larval duration time recorded in other sponges. Given the dominance of tidal currents, which add to the complexity of flow along with local features such as eddies, it is expected that larval transport between reefs would take longer. A high resolution circulation model for the SoG (Soontiens et al. in prep), will afford a more realistic idea of transport time and potentially elucidate source/sink relationships between sites. If larvae remain elusive, this may provide further hints of the larval behaviour and duration in this deep-sea species.

Distribution patterns may also be influenced by historical biogeography, as glass sponges are relatively old and slow-growing animals - estimated to live up to 400 years (Leys & Lauzon, 1998; Fallon *et al.*, 2010), while the reefs are less than 9,000 yo (Conway *et al.*, 1991).

3.5.5 Implications for conservation

As slow-growing, sessile animals, glass sponges are vulnerable to damage by fishing activity (Freese *et al.*, 1999; Heifetz *et al.*, 2009), and slow to recover from large scale damage (Kahn *et al.*, 2015b). Marine protected areas have been proposed to limit damage to the sponge reefs by fishing and other anthropogenic disruptions, but would management decisions, such as the selection of which geographic regions to protect, benefit from knowledge of genetic structure of the reefs? The genetic similarity of *Aphrocallistes* in adjacent reefs suggests a healthy dispersal ability and the existence of a potentially self-sustaining population within the SoG. The fact that our results show two distinct populations of glass sponges – inside and outside the SoG – implies that it is important to protect sponge communities (reef

and non reef) both outside and within the SoG basin to maintain genetic diversity. Our results, however, did not resolve directionality to gene flow (sources and sinks), probably because of insufficient sampling. Until this can be resolved it is safest to assume that all reefs should are equally important. Management decisions would benefit from further study of directional dispersal, which could be clarified through modeling efforts, or information on larval development and behaviour. Other considerations are also important to keep in mind: based on the *in situ* mapping of live and dead regions of reefs (Chu & Leys, 2010) some reefs are clearly healthier than others (more live cover relative to that found underlying the surface by multibeam mapping), so environmental factors such as flow, sedimentation, and oxygen levels, should also be considered in conservation management decisions.

3.5.6 Summary

Although sponges at sites outside of the SoG were found to be genetically distinct from those inside the SoG, no evidence of clonality and little within-region population structure were found, indicating that larvae are sexually produced and are widely dispersed by local currents. Neighboring individuals are genetically distinct, and genetic distances between sponges do not vary over a range of geographical scales, giving the appearance of a single population in the SoG that may be self-sustaining or arise from larvae sourced by outside populations.

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Chapter 4

Conclusions

4.1 General Conclusions

This thesis accomplishes two major objectives: 1) development of a method of marker discovery for population studies that works well with difficult, non-model organisms, and 2) description of the genetic population structure of a reef-building glass sponge in and around the Strait of Georgia. In accomplishing the first objective, the *Aphrocallistes* genome was surveyed using reduced representation libraries to find informative markers, removing those with signs of duplication. Over 7000 markers were discovered, allowing for very stringent filter settings to obtain reliable markers. Without a reference genome, it is impossible to know that the SNPs used in the analysis were single-copy, but the extensive filtering that I performed raises my confidence that they were. Loci were removed if they were 100% heterozygous across discovery samples, if there were more than two allelic variants within an individual or across the ten discovery individuals, and if the trimmed flanking sequences of the contig differed. If variants on a contig appeared duplicated, then all variants on that contig were discarded. In the analysis, 67 SNPs were used, an extremely low number compared to most studies using NGS sequencing methodologies but comparable to 6 - 10 microsatellites, an average to good number of markers for population analysis. Importantly, this technique made analysis of an organism possible for which 6 - 10 acceptable microsatellite markers would have been prohibitively expensive, if not impossible, to discover.

The results of this thesis provide important new data about the reproduction of a reef-forming glass sponge. First, *Aphrocallistes* does not appear to reproduce asexually by budding, nor is there evidence of oscula breaking off and reattaching in a new location. This is interesting considering that, like many other sponges, *Aphrocallistes* is capable of attaching to new substrates – live pieces of sponge attached to tanks when kept in seawater trays at the Bamfield Marine Sciences Centre (pers. obs.). Conditions at the reefs are calm compared to shallower environments so natural breakage is unlikely to occur. Fragmented sponges are observed following human activity around the reefs, so either this does not occur frequently enough to contribute to population dynamics, or sponges have a difficult time reattaching in the reefs, where their skeletons provide the only available hard substrate. Speculation aside, it is clear that the reef sponges reproduce sexually.

Overall, I found that *Aphrocallistes* individuals on and off the reefs are genetically distinct, and within a clump individuals are no more genetically similar than across or between sites. But there were also two unusual cases in which adjacent 'individuals' were much more closely related. In one case, it appeared from the ROV video of the collection that the two sponges might have been living remnants of a larger bush. Oddly, however, this was not observed often in our results, given the prevalence of dead bases and known growth pattern. In the second case, two adjacent oscula were from the same individual (% difference similar to error rate) with a closely related third adjacent osculum possibly from a sibling or parent/offspring relationship, either of which could result from the larvae settling close to their release point. This was the only time samples were collected from three oscula that appeared to be from the same individual, so while this could reflect added complexity in dispersal, sampling focused on 'individuals' would be required to examine this further.

The lack of structure in the SoG reef and non-reef locations suggests the larvae are sexually produced and highly dispersive. Dispersal could also presumably be achieved through spermcasting, although where it has been studied, sperm are not known to last long in the water column (Lévi & Lévi, 1976). The lack of differentiation between SoG sites could be explained by recent population expansion – which has not been tested – however, low heterozygosity might be expected. No evidence of inbreeding was detected, and sites within the SoG had higher (0.304-0.420) average heterozygosity than the Barkley Sound

population on the other side of Vancouver Island (0.267). This doesn't account for the possibility that Barkley Sound also experienced recent population expansion, though, and further sampling and testing would be needed to a rule out a genetic bottleneck.

If the sponge reefs and non-reef locations are connected by larval dispersal, then it is likely that the larvae last longer than two days in the water column. Planktonic duration in a lecithotrophic larva depends on its energy reserves and thus is inherently limited. In a study of larval duration in the demosponge *Sigmadocia caerulea*, Maldonado & Young (1999) report higher juvenile mortality for larvae that settled after 72 hours, which they suggest is due to energy depletion. This is difficult to extrapolate to other species and situations, as yolk reserves and energy expenditure vary by species, and larvae may behave differently in lab settings. Larvae in moving water are reported to primarily maintain a vertical "resting" phase in which they passively spin on their axis (Maldonado *et al.*, 2003) with only sporadic bursts of horizontal swimming (Uriz *et al.*, 2008). The relative amount of time spent in this restive spinning phase varies by species as well (Uriz *et al.*, 2008). Active directional swimming is likely to use up more energy reserves and tends to be the dominant phase for larvae in still water, particularly when they are regularly 'jostled', as is the case in most laboratory behaviour studies. Because of this, behaviour studies may emphasize sharper declines in energy reserves than would be seen *in situ*. Larval duration could also potentially be extended through the consumption of dissolved organic carbon (Jaeckle, 1995) or bacteria (Ivanova, 1999).

A genetic difference between reef and non-reef individuals is not evident from the data, supporting the idea that the reefs are concentrations of individuals that occur where conditions support it. Dictyonine sponges have massive siliceous skeletons – to support a dense community such as a reef, dissolved silicate levels need to be high (Chu *et al.*, 2011). Glass sponges are sensitive to temperature, requiring cold water for electrical impulse conduction through their tissues (Leys & Meech, 2006). Sponge reefs also require sedimentation rates that are high enough to allow for the construction of mounds (Whitney *et al.*, 2005), and a flow rate high enough to sustain considerable grazing rates (Kahn *et al.*, 2015a). These conditions are met on the coasts of British Columbia and Alaska, where coastal

upwelling supplies cold, nutrient-rich deep water, fluvial outflow supplies high sedimentation rates (Whitney *et al.*, 2005), and ridges increase tidal current velocities (Bedard, 2011). This raises the questions of whether other dictyonine sponges could form reefs in the proper conditions and if there is potential for creating reefs artificially. There are reports of *Aphrocallistes* settling on manmade structures such as ship wrecks (Austin, 2003) and lines (Levings & McDaniel, 1974), but the larvae do seem particular about settlement substrates. Despite finding many juveniles in Galiano and Fraser reefs, no larvae settled on substrates provided in 12 recruitment tubes deployed within the two reefs from 2011-2013/14, although there was one instance of recruitment to a small mesh square set on the surface of the sediment (Kahn *et al.*, 2015b). If artificial substrates were to be used to create more reefs, considerable planning would need to go into deciding what is used to seed reefs and where.

In general, planktonic larval dispersal is greatly influenced by prevailing oceanographic conditions and features which may hinder or facilitate dispersal. For example, the larvae of organisms inhabiting hydrothermal vents can be entrained in the rising vent plume, enabling horizontal dispersal of 1000s of meters at depths 100s of meters above the vents (Mullineaux *et al.*, 1995). In contrast, strong cross-currents (Won *et al.*, 2003) or geographic disruptions such as breaks in ridges (Vrijenhoek, 2010) can create barriers to dispersal in vent species. In coastal environments larvae may be whisked toward or away from adjoining sites by eddies (Watson *et al.*, 2010). Additionally, a suite of biological, ecological and physical variables create annual differences in dispersal and recruitment, making population connectivity stochastic for many marine organisms (Siegel *et al.*, 2008). For longer lived species, examining the population genetics can provide an approximation of the net gene flow.

4.2 Future Directions

To fully understand dispersal in *Aphrocallistes*, additional complementary research should be undertaken. Future studies should examine oceanographic currents in the Strait of Georgia to determine flow between sites of interest and the changes over time. If possible,

larval and development work would also greatly contribute to the current knowledge of glass sponges.

4.2.1 Oceanographic modeling / particle tracking

Modeling larval dispersal of marine animals on ocean currents has been gaining considerable traction in the last decade as a tool to describe the patterns observed in population connectivity and recruitment (Gallego *et al.*, 2007). Examples of its applications include directing conservation efforts (Robinson *et al.*, 2005; Treml *et al.*, 2007), providing framework for broad multi-species comparisons of marine dispersal (Kinlan *et al.*, 2005), providing context for interpretation of population genetics results (Torda *et al.*, 2013), quantifying coastal connectivity of a regional environment (Mitarai *et al.*, 2009), and discovering unknown populations (Yearsley & Sigwart, 2011).

Specific features of flow in the SoG are well studied, including tidal, residual, and ocean currents (Davenne & Masson, 2001), flow directly over the Fraser Reef (Bedard, 2011) gyres (Stacey *et al.*, 1987), and estuarine flow (Pawlowicz *et al.*, 2007). Remaining questions regarding the deep flow for much of the Strait are being addressed. The Victoria Experimental Network Under the Sea (VENUS) hosts three stationary observation nodes in the SoG which have monitored current vectors among a suite of oceanographic variables since deployment in 2008. A new high resolution Nucleus for European Modelling of the Ocean (NEMO) model has been developed for the Salish Sea (Soontiens et al. in prep). This model will provide an excellent opportunity to model larval dispersal across the SoG through lagrangian particle tracking (Siegel *et al.*, 2003). This may easily be accomplished using offline particle tracking software such as Ariane (http://stockage.univ-brest.fr/~grima/Ariane/), which can be run forward or backward in time to track both destinations and sources of particles. Ariane is a post-processing software package that calculates 3D trajectories from the output velocity fields of OGCMs, and is commonly used with NEMO. These and similar methods have been used in a variety of ocean environments to project expected larval dispersal ranges (Pfeiffer-Herbert *et al.*, 2007; Mitarai *et al.*, 2009; Watson *et al.*, 2010; Yearsley & Sigwart, 2011). Potential connectivity is determined statistically by the probability densities gathered from several runs consisting of thousands of particle tracks, and source and sink

potential is quantified using potential connectivity matrices (Watson *et al.*, 2010). These reflect the probability of larval dispersal from a specific source site to any set of destination sites (and vice versa) given currents and larval duration.

4.2.2 Larval development and behaviour

Descriptions of larval development and behaviour would be immensely useful in understanding the dispersal ability of *Aphrocallistes*. Though larvae have remained elusive in our sampling, the reproductive season seems to be over the winter months (Appendix B). Sampling with an ROV is not possible for much of that season largely because it is difficult to obtain ship time (via NSERC and the Department of Fisheries and Oceans) in winter, but *Aphrocallistes* could be collected in November/December and maintained in the Bamfield Marine Sciences Centre aquaria on the west coast of Vancouver Island. Bamfield is unique among marine stations in that their open water system draws water from 30m depth and so is chemically ideal for maintaining glass sponges. *Aphrocallistes* has been held there for months successfully, and in 2011 samples housed at Bamfield developed spermatocysts, a good indication that they will reproduce in the aquaria. If larvae could be obtained, they would provide valuable developmental data, particularly changes over time to larval composition with regards to spicule development and relative levels of yolk and lipids (which affect larval buoyancy and orientation). Various aspects of larval behaviour could be studied such as swimming ability, tolerances for environmental factors, phototaxis, geotaxis, and settlement cues through a series of observations and manipulative experiments modeled after those done with other larval types (Warburton, 1966; Elliott *et al.*, 2004; Whalan *et al.*, 2008a; Abdul Wahab *et al.*, 2011). These might include measurements of distance traveled over time in gridded Petri-dishes, selection of light/dark environments, maintenance of vertical position, and settlement/metamorphosis rates with and without the presence of biofilms and glass sponge skeleton. Perhaps most importantly, experiments on larval duration that incorporate moving water could be done to more accurately to reflect natural conditions.

4.3 Conservation

The glass sponge reefs are known to be nursery habitat for commercially important species of rockfish (*Sebastes* spp; Du Preez & Tunnicliffe, 2011; Conway *et al.*, 2007; Cook *et al.*, 2008); removal of *Aphrocallistes* and *Heterochone* by fishing is correlated with a decrease in rockfish abundance (Du Preez & Tunnicliffe, 2011; Cook *et al.*, 2008). This creates interest in the reefs for the fishing industry as well – if properly managed, fishing yield in nearby areas should increase (Gaines *et al.*, 2010), affording sustainable fishing. Because the reefs are slow to recover from large-scale damage (Kahn *et al.*, 2015b), potentially even from the level of damage caused by fishing lines (Austin *et al.*, 2007), reef sponges would benefit from 'no-take' marine reserve designation. Sources and sinks have not yet been established, so all reefs should be considered equally important, although the connectivity of the reefs in the SoG shown here make them prime candidates for a network of MPAs. The varying 'health' of the reefs, as inferred by percent live cover relative to that found underlying the surface by multibeam mapping (Chu & Leys, 2010), means that continued exchange of individuals between sites in the SoG may be critical to maintaining numbers and diversity within the basin.

A multitude of other factors will affect conservation efforts of the reefs and should also be considered in planning. Glass sponge reefs represent complex communities consisting of many other species; genetic distinction of these other animals between reefs should be assessed. Temperature may increasingly become a concern as the upper layers of the SoG warm (Masson, 2007). Impacts from other community interactions such as competition by *Desmacella austini* (Lehnert *et al.*, 2005; Kahn *et al.*, 2015b), and even predation (Chu & Leys, 2012), could also be examined further.

Appendix A

Supplementary Data for Chapter 3

A.1 Figures

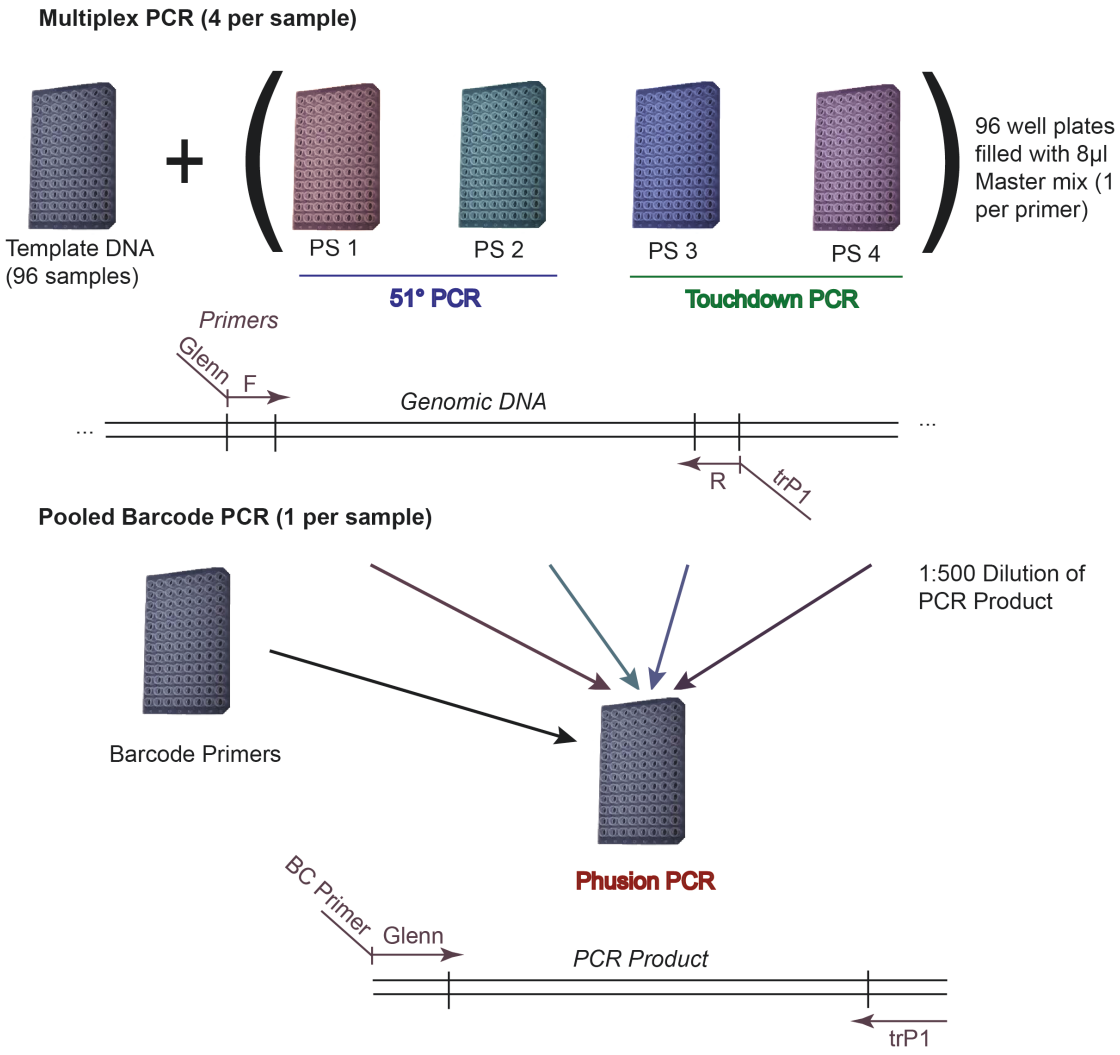

FIGURE A.1: Four multiplex PCRs, each with twenty primer pairs, were performed with DNA template from 96 samples. Sequence-specific forward primers were designed with Glenn primer tails and reverse primers were designed with trP1 primer tails. The first two primer sets (PS 1 and PS 2) were amplified using a 51°C annealing temp, and the other two primer sets (PS 3 and PS 4) were amplified using touchdown settings. Products were diluted and pooled by individual before Ion Torrent A barcode primers were attached to fragments in a second PCR.

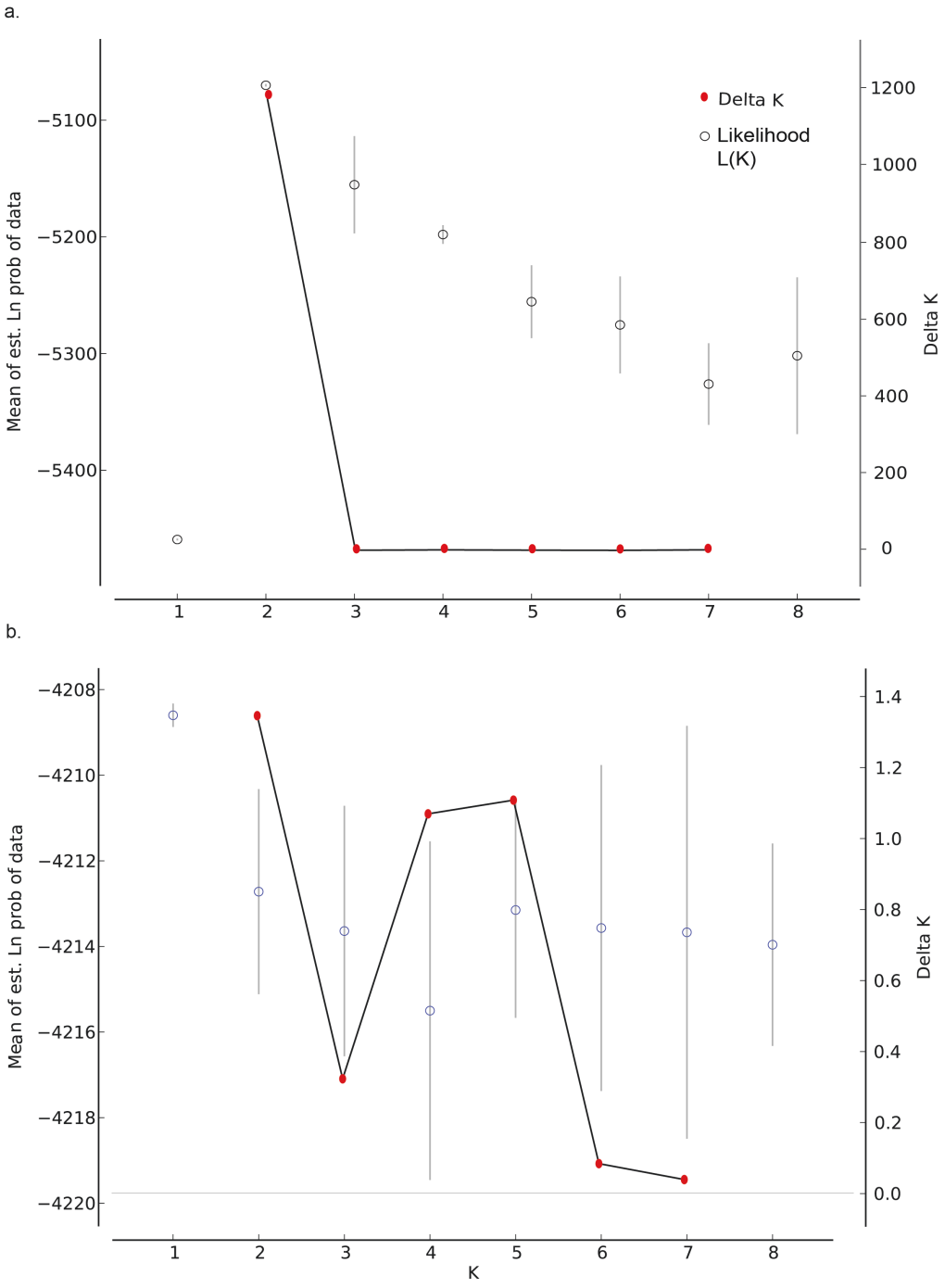

FIGURE A.2: STRUCTURE likelihood plots for most likely number of genetically homogenous clusters (K): mean of estimated Ln likelihood with standard deviation bars for K=1-8, and corresponding ΔK (Evanno *et al.*, 2005) calculated with Structure Harvester. a) All population level *Aphrocallistes vastus* samples (excluding controls and samples from sites with n<5; n=78). b) Samples from a) that assigned with greater than 80% ancestry to the largest cluster.

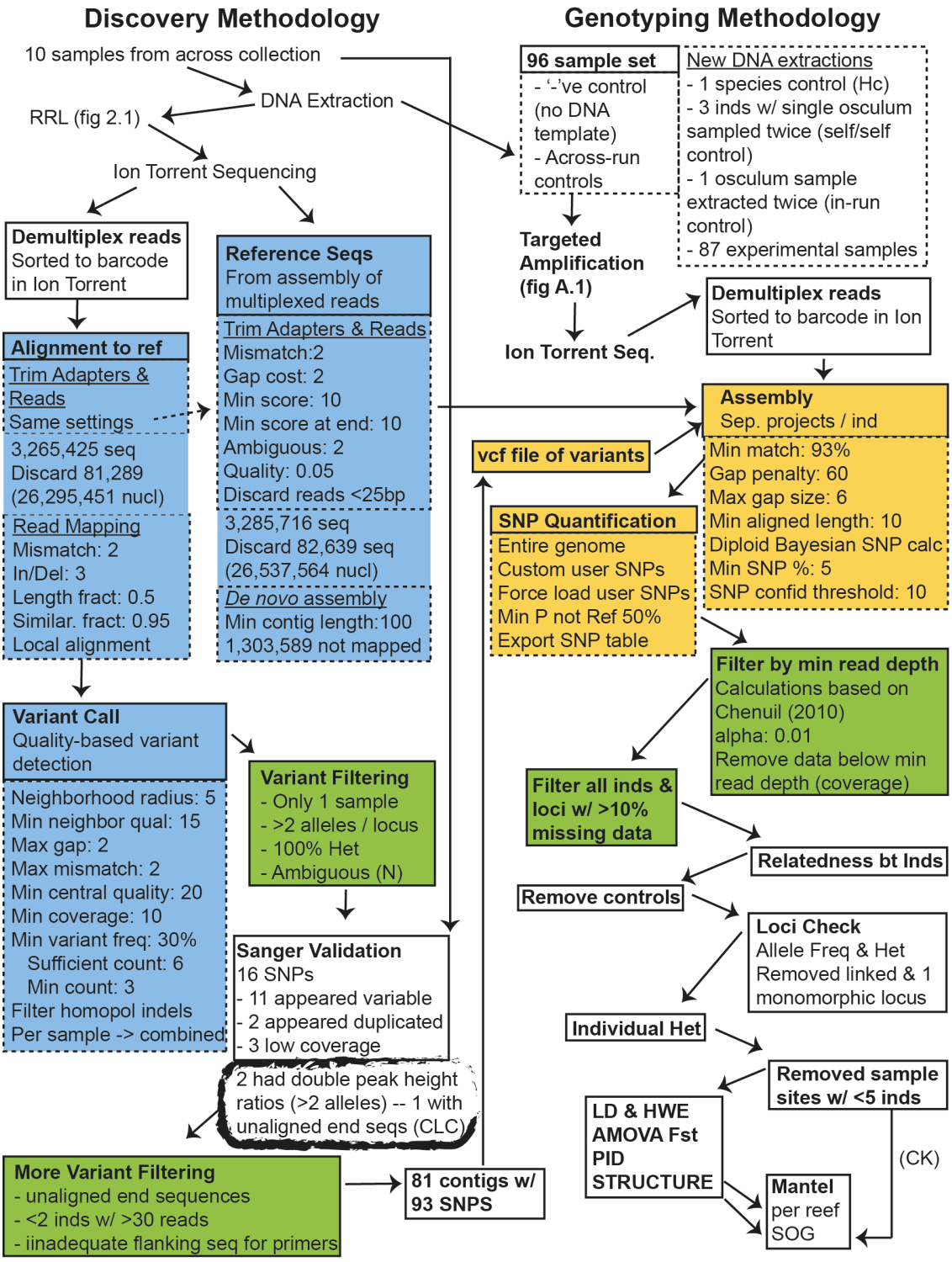
Discovery Methodology
Genotyping Methodology
10 samples from across collection
DNA Extraction
RRL (fig 2.1)
Ion Torrent Sequencing
Demultiplex reads
Sorted to barcode in Ion Torrent
Alignment to ref
Trim Adapters & Reads
Same settings
3,265,425 seq
Discard 81,289
(26,295,451 nucl)
Read Mapping
Mismatch: 2
In/Del: 3
Length fract: 0.5
Similar. fract: 0.95
Local alignment
Reference Seqs
From assembly of multiplexed reads
Trim Adapters & Reads
Mismatch:2
Gap cost: 2
Min score: 10
Min score at end: 10
Ambiguous: 2
Quality: 0.05
Discard reads <25bp
3,285,716 seq
Discard 82,639 seq
(26,537,564 nucl)
De novo assembly
Min contig length:100
1,303,589 not mapped
Variant Call
Quality-based variant detection
Neighborhood radius: 5
Min neighbor qual: 15
Max gap: 2
Max mismatch: 2
Min central quality: 20
Min coverage: 10
Min variant freq: 30%
Sufficient count: 6
Min count: 3
Filter homopol indels
Per sample -> combined
Variant Filtering
- Only 1 sample
- >2 alleles / locus
- 100% Het
- Ambiguous (N)
Sanger Validation
16 SNPs
- 11 appeared variable
- 2 appeared duplicated
- 3 low coverage
2 had double peak height ratios (>2 alleles) -- 1 with unaligned end seqs (CLC)
More Variant Filtering
- unaligned end sequences
- <2 inds w/ >30 reads
- iinadequate flanking seq for primers
81 contigs w/ 93 SNPS
96 sample set
- '-'ve control (no DNA template)
- Across-run controls
New DNA extractions
- 1 species control (Hc)
- 3 inds w/ single osculum sampled twice (self/self control)
- 1 osculum sample extracted twice (in-run control)
- 87 experimental samples
Targeted Amplification (fig A.1)
Ion Torrent Seq.
Demultiplex reads
Sorted to barcode in Ion Torrent
Assembly
Sep. projects / ind
Min match: 93%
Gap penalty: 60
Max gap size: 6
Min aligned length: 10
Diploid Bayesian SNP calc
Min SNP %: 5
SNP confid threshold: 10
vcf file of variants
SNP Quantification
Entire genome
Custom user SNPs
Force load user SNPs
Min P not Ref 50%
Export SNP table
Filter by min read depth
Calculations based on Chenuil (2010)
alpha: 0.01
Remove data below min read depth (coverage)
Filter all inds & loci w/ >10% missing data
Relatedness bt Inds
Remove controls
Loci Check
Allele Freq & Het
Removed linked & 1 monomorphic locus
Individual Het
Removed sample sites w/ <5 inds
LD & HWE
AMOVA Fst
PID
STRUCTURE
(CK)
Mantel
per reef
SOG

FIGURE A.3: Summary and workflow of marker discovery and genotyping methodologies. Tasks carried out in CLC Genomics are highlighted in blue, tasks completed in Microsoft Excel are highlighted in green, and work completed in DNAStar Lasergene Suite is highlighted in yellow. Multiple controls were run in the genotyping phase: a negative control without template DNA was run to check for contamination in the sequencing run; a *Heterochone* sample was run to look for identifiable differences between species and as a second check that no *Heterochone* were sequenced; across run controls resequenced DNA extracted for the marker discovery to assess differences in genotypes between RRL and targeted amplicon methodologies; an in-run control of a tissue sample that was extracted twice was used to check for errors within targeted amplicon sequencing pipeline beginning with extraction; oscula were sampled twice for self/self controls in order to check for errors in the sampling level of methodology. Error within the discovery phase (RRL pipeline) was assessed through the Sanger validation of 16 SNPs based on differing genotypes of the same samples sequenced on the two platforms (7.7%). Error within the genotyping phase (targeted amplicon pipeline) was assessed using combined control data – different genotypes between across-run pairs, in-run pair, and self/self pairs (0-9.1%).

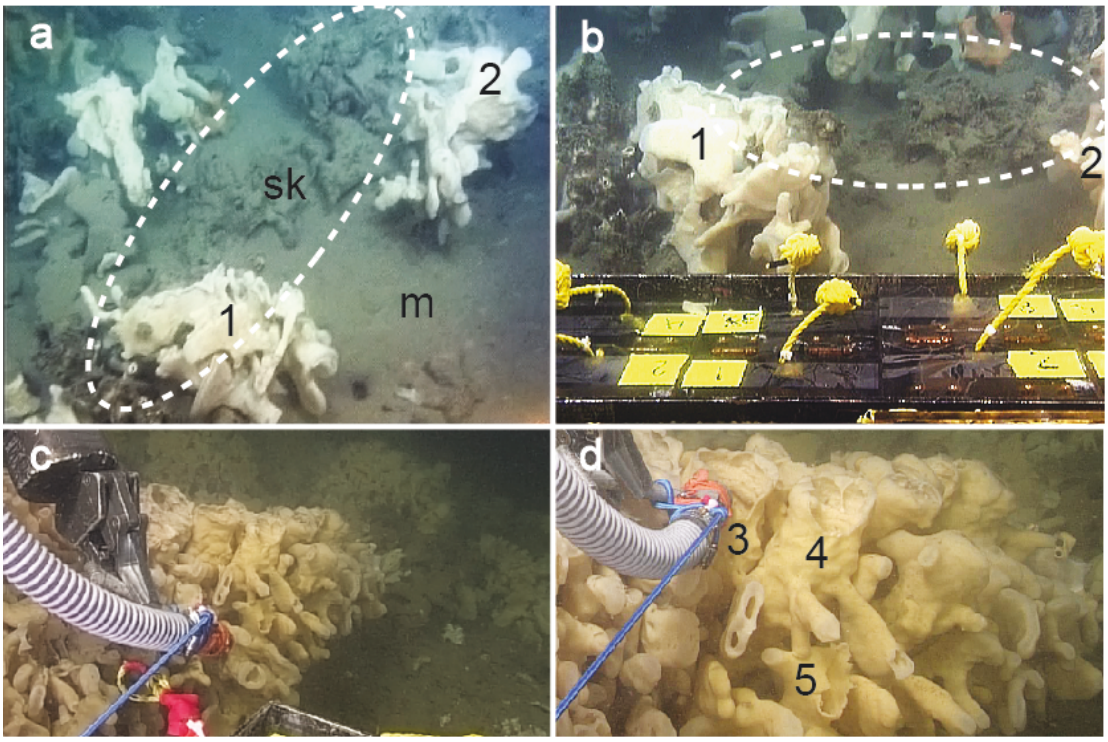

FIGURE A.4: Individual relatedness exceptional cases. a-b) 'Twins' - two samples (1 and 2) that differ at 5.2% of loci, within the range of paired controls, separated by sediment (m) and partially buried skeleton (sk, dashed circle). The extent of the two live portions are shown in a) with a closer angle showing dead/live tissue connections and the sampling boxes in which each sample is stored separately after being collected by the manipulator arm of the ROV. c-d) 'Triplets' - three samples (3, 4 and 5) that are closely grouped and have 10.4%-16.9% genotypic differences, outside the paired control range but closely related. A large portion of the clump is shown in c) for context, with a close-up of the sampled trio in d). Samples in c and d were collected by suction into separate tubes.

A.2 Tables

Table A.1: Geographic coordinates of sample locations.

Sample location	**Latitude**	**Longitude**
Howe Sound Reef	49°20' 2.5" N	123°17' 46.2" W
Galiano Ridge Reef	48°54' 51.5" N	123°19'27.7" W
Fraser Ridge Reef	49°9' 15.7" N	123°23' 3.7" W
McCall Bank Ridge Reef	49°25' 43.1" N	123°48' 25.6" W
San Jose Islands	48°53' 55.0" N	125°3' 2.1" W
Josie Islets	48°54' 15.8" N	125°2' 37.1" W
Sabine Channel, Texada Island	49°29' 42.2" N	124°10' 3.2" W
Coral Knoll	49°22' 27.8" N	123°53' 30.2" W
McCurdy Point, Saanich Inlet	48°33' 39.2" N	123°31' 15.2" W

TABLE A.2: Self/non-self recognition experiments on *Aphrocallistes vastus* specimens from Fraser Reef and Howe Sound carried out in 2009. Within Fraser Reef, samples were collected from three distinct clumps of individuals. Three experiments were carried out: pairwise couplings of samples (indicated by ■) within Clump 1 (n=5, 10 cross-sample pairs and 5 controls), within Clump 3 (n=5, 10 cross-sample pairs and 5 controls) and across reefs (n=6, 9 cross-sample pairs and 3 controls). Samples that appeared to be fused were fixed for SEM to determine whether tissues were fused or spicules were simply ensnared. Six control pairs (two pieces of tissue from the same individual placed next to each other) were the only samples to fuse (indicated by ●).

	Fraser Reef											**Howe Reef**		
	Clump 1					**Clump 2**	**Clump 3**							
Samples	**05**	**06**	**07**	**08**	**09**	**12**	**15**	**16**	**17**	**18**	**19**	**45**	**50**	**59**
05	■	■	■	■	■	-	-	-	-	-	-	■	■	■
06		■	■	■	■	-	-	-	-	-	-	-	-	-
07			■	■	■	-	-	-	-	-	-	-	-	-
08				■	■	-	-	-	-	-	-	-	-	-
09					■	-	-	-	-	-	-	-	-	-
12						-	-	-	-	-	-	■	■	■
15							■	■	■	■	■	-	-	-
16								■	■	■	■	-	-	-
17									●	■	■	-	-	-
18										●	■	■	■	■
19											●	-	-	-
45												●	-	-
50													●	-
59														●

TABLE A.3: Characteristics of 93 SNPs isolated from 84 individuals of *Aphrocallistes.* Greyed loci were removed for analysis: LC - low coverage, HP - homopolymer sequence, PL - physically linked loci, MM - monomorphic, MS - missing sequence, LQ - low quality. (Where more than one SNP are described are amplified by the same primers, only the row containing the discarded SNP is grey.)

Contig	Amplification Primers (5'-3')[3]*	Tm[4]§	Primer set	Length	Locus	H_0	H_E	p-value[5]†	Fixation Index	
12	F: GTCCCTTGCATGTCCACT	56.8	2	66	64	0.167	0.278	**0.000**	0.400	
	R: TTCTGAAAATGTTGCACAATAA	56.1								
13	F: CATGTTCCGCCCAGTCA	60.2	1	67	56	0.457	0.378	0.061	-0.208	
	R: ATCGAGGAATTCGCCAAA	59.2								
21	F: GTTAGGTGACTGTCGCACCA	59.8	4	96	85	0.064	0.491	0.000	0.869	**LC**
	R: AGGCTCCCCTTGATCAGC	60.3								
35	F: CGGGAGGTGTGGAATATCACT	59.2	1	120	94	0.083	0.101	0.102	0.178	
	R: GCAGATCACCTACCTCGCTG	60.2								
80	F: CGATAGAGGGTCGACGAAAG	59.8	2	94	108					**HP**
	R: CAGGTATTGTTTCCAAATTAGCC	59								
129	F: AGAGCTCCGCAGACAGAATC	59.7	1	74	58	0.131	0.330	**0.000**	0.603	
	R: TCGCTCACTTCCAACTTGTG	60								
134	F: GGGACCTAAGCAGGGAGTTA	58.3	3	108	69	0.262	0.363	0.011	0.278	
	R: CAGGAACTCTCCTGCAACAA	59								
154	F: TCTGAACTCGGAAGGAAACG	60.4	4	109	107	0.024	0.046	**0.000**	0.488	

Contig	Amplification Primers (5'-3')[3]*	Tm[4]§	Primer set	Length	Locus	H_0	H_E	p-value[5]†	Fixation Index	
	R: ACAAACAGCGAGCAGATTCA	59.6								
159	F: TAGCGCGGTCCATTTAGTCT	59.9	3	146	110	0.524	0.427	0.039	-0.225	
	R: GGAGATGATGAGCAGAGCAA	59.1								
235	F: AACGTCGTACTGGTCTTCCA	58.2	4	121	155	0.205	0.288	0.008	0.290	
	R: CAAACCTAATGCCACTCACG	59.2								
240	F: TCTCGGCAGAGTTCCGTAGT	60	3	119	75	0.042	0.086	0.000	0.511	**LC**
	R: GACACAGCATCAGTGGCATC	60.3			114	0.152	0.180	0.042	0.158	**LC**
258	F: TCTCACCCATCTCCCAAAAG	60	3	100	90	0.286	0.444	0.001	0.357	
	R: AGACCTGGAGACGAAGCAGA	60.1								
266	F: GCGAGAGTCCGTCACTTCTT	59.6	3	103	102					**LQ**
	R: TACGTGAACGGACAGCTGAG	60.1								
274	F: TCTTAGGGTAGGATGGCGGT	59.7	1	101	67	0.590	0.435	0.001	-0.358	
	R: ACCAGAGATGATCGCAAACCA	59.7								
347	F: CGGATCCGTACGCGAAATCT	60.3	2	97	48	0.024	0.024	0.912	-0.012	
	R: CTCTTGCCTCGGCCTGG	59.8								
481	F: AGCACTGCCAGCTATCAGC	60.2	1	92	61	0.536	0.440	0.047	-0.216	
	R: TGCAAGCATTTGAGATTTCCA	56.7								
487	F: GATGCTGTGGTCCGAGTGG	60.4	4	190	139	0.500	0.497	0.963	-0.005	
	R: TTTGGTGACTGGAGATGCCG	60.3								

Contig	Amplification Primers (5'-3')[3]*	Tm[4]§	Primer set	Length	Locus	H_0	H_E	p-value[5]†	Fixation Index	
558	F: TGACTAATTTGAGCGGAAATGA	56.1	1	99	41	0.548	0.499	0.370	-0.098	
	R: GCAGGCATCAAGTGTGTAGTG	59.5								
655	F: CTGGATATAGACGACTTCTTATCA	55.1	2	101	31	0.099	0.124	0.008	0.197	**PL**
	R: AGTTGCTGTGCTATCCCGTC	60.1			39	0.429	0.398	0.476	-0.078	
668	F: CGGACACTTACATAACACTCAAGG	59.4	4	142	113	0.238	0.262	0.410	0.090	
	R: ATTGATTGTGTCGTGTGGGT	57.7								
677	F: ACGATGTCGGTAGCAACGAA	59.8	1	95	89					**MM**
	R: GCACCTTACGTGAATGGAGC	59.3								
692	F: TGGTCAGGCCAGTCAGGT	60.1	4	104	41	0.145	0.255	0.000	0.430	**LC**
	R: TGGTCAGGTCCATTGCTTCG	60.3			83	0.129	0.461	0.000	0.720	**LC**
697	F: CTGCTGACCTCTGACGTACC	59.8	1	96	36	0.476	0.427	0.296	-0.114	
	R: GCTCTTTCGATGTTCTCCGC	59.4								
744	F: TGCTAGCTCAATACCGCGAG	60	2	99	54	0.119	0.191	**0.001**	0.378	
	R: ACACACACGAGTTGTACTCTGA	59.3								
774	F: CAGGAGTGATCTTGACAGCGA	59.8	4	136	89	0.405	0.444	0.413	0.089	
	R: ACAGTCTTGATTGTGTGTCCGT	60.2			132	0.480	0.463	0.604	-0.039	**PL**
805	F: CAAGGAGCTCAACCCTTGGT	59.9	3	139	73	0.500	0.495	0.933	-0.009	
	R: TGTATCTGCCGTTCTCCTGG	59.2								
835	F: ACTTGTCCCAGGGAGCTGG	61.2	1	90	33	0.108	0.164	0.002	0.340	

Contig	Amplification Primers (5'-3')[3]*	Tm[4]§	Primer set	Length	Locus	H_0	H_E	p-value[5]†	Fixation Index	
	R: GCACGAATCCCTGACTCAG	57.9								
990	F: CCATCGCGCAGATCGACTT	60.6	2	96	71	0.238	0.387	**0.000**	0.384	
	R: CTGAGAGAGATCCCAGCTCG	59								
992	F: CCGGTTGTAATTGCCATGTGT	59.5	3	152	42	0.205	0.203	0.925	-0.010	
	R: GCCATTTCTCACAATCTAAACACA	58			95	0.522	0.469	0.131	-0.112	**PL**
1014	F: TAGCTAGCGGGACTCCTGG	60.2	2	119	115	0.405	0.350	0.152	-0.156	
	R: CTCACTCGCAGCTGGGAAC	60.7								
1026	F: GCAGCATTCTCGTTAATTGCG	58.9	3	96	76	0.429	0.444	0.743	0.036	
	R: ACGCAAGGAAGTGAATACCCT	59.4								
1169	F: GACTGTCCGACGGCTTTTCA	60.6	1	81	28	0.048	0.046	0.823	-0.024	
	R: TCACCGCACTGCTTGATGTA	59.7			42	0.039	0.039	0.789	-0.020	**PL**
1227	F: TGTCAACGCAGAGAAGGCAT	60	2	111	54	0.518	0.443	0.123	-0.169	
	R: GACCTCTGCGCCCATCTCTT	62			55	0.484	0.417	0.463	-0.159	**PL**
1283	F: GGACTAGCACCATGGATCCG	60	4	119	41	0.000	0.000	0.000		**MM**
	R: GCAGCATATCCGCATTCCTC	59.2			103					**LQ**
1321	F: CGACGAACCCAACGAGCTTA	60.4	2	125	115	0.393	0.488	0.074	0.195	
	R: GCGGATGCACAACTTTCTGT	59.4								
1386	F: CAGGTCTCCTCCTCCCTCC	60.1	3	123	80	0.524	0.495	0.600	-0.057	
	R: CGACATATCGGATGACCCCA	59								

Contig	Amplification Primers (5'-3')[3]*	Tm[4]§	Primer set	Length	Locus	H_0	H_E	p-value[5]†	Fixation Index	
1424	F: AGTGAACGTTAACAAGGAAATGCT	59.4	4	117	112	0.500	0.495	0.933	-0.009	
	R: GTGCCGCCAGTATCAGT	56.5								
1438	F: CCGCACTTAGAACTTGGGTCA	60.3	2	108	99	0.060	0.080	0.020	0.255	
	R: TCAGCTGATAATGCCTCCCC	59.2			121	0.082	0.089	0.315	0.074	**PL**
1441	F: GGAATAGTCCACTGATGCTC	55.3	1	57	67	0.373	0.406	0.467	0.080	
	R: GGGCAGGGGGAGAACAATTA	59.4								
1448	F: CTTGGCAGACTCTCTTCCCG	60.1	2	120	29	0.179	0.201	0.314	0.110	
	R: GCTAACGATGGTCGAAACGC	60								
1522	F: CTTGGCAGACTCTCTTCCCG	60.1	4	201	48					**MS**
	R: GGAAGTGGGTGTGACGTCAT	60								
1546	F: AGAGAGGAGAGACAGCTGGG	60	2	79	47	0.438	0.425	0.791	-0.030	
	R: GGGAGCTGCGACACGAG	60.2								
1598	F: GGACAGCTCCGTCTCCAT	58.4	2	142	134	0.095	0.112	0.171	0.149	
	R: GCAGTGGGACGAGATGAACA	60								
1608	F: TTCACTCCCTTAGCAGAGCT	58.1	1	85	67	0.512	0.498	0.801	-0.027	
	R: CTGGTGGCGGGAATTTTTGG	60								
1737	F: AGACTACATCGCTCAAGCAT	56.7	2	68	66	0.337	0.468	0.011	0.279	
	R: CCGTCCTTGAACAAGTTAGTCC	58.9								
1789	F: TGGATATTCCTATTAACACTGCGT	57.8	2	85	93	0.810	0.500	**0.000**	-0.620	

Contig	Amplification Primers (5'-3')[3]*	Tm[4]§	Primer set	Length	Locus	H_0	H_E	p-value[5]†	Fixation Index	
	R: TGGAGATATCCATCTAACTCAGCT	58.4								
1800	F: GGGACCGAATCGTCCGTATC	60	2	103	62					**MS**
	R: ATGCGGATGAGCGCGAG	60.3								
1819	F: AGAGTTGCTGATGAGGTCTGA	58.5	4	112	88	0.410	0.369	0.317	-0.113	
	R: GCAGCTGCTAGCCATTCATC	59.4								
1882	F: CATCTTGGTCAGGAGGTCGT	59.1	4	128	226	0.583	0.456	0.010	-0.280	
	R: AGAGGGTGTAGACAACTCGGA	59.9								
2056	F: CGGGTTAATTATCGAATCTACGAG	57.1	4	106	63	0.488	0.440	0.321	-0.108	
	R: ACCAATTGTAGTAACTCTCCGCT	59.5								
2089	F: GCAGCAGCTTCCCTCTATGA	59.5	3	97	71	0.070	0.198	0.000	0.645	**LC**
	R: GCAGTTCAATTGATTCGAAAGT	55.9								
2169	F: GCATTACCGTTAATGAAAGAGACA	57.6	1	53	60	0.223	0.474	0.000	0.528	**LC**
	R: TCTCGTTTCAACAGGAATTTCCA	58.5								
2186	F: AGTTATCGAAATCCAATCTGGGT	57.6	1	65	88	0.363	0.434	0.139	0.165	
	R: GTGACAGGTTGAAGTATCCCCA	59.7								
2248	F: ATGGAATGATGCAAGACAGAA	55.6	4	188	132	0.250	0.270	0.502	0.073	
	R: CGGAATCTGATAGTGGCGTAGA	59.4								
2256	F: ACTTTGCCCTGCGTCTCTAC	60	3	111	81	0.298	0.286	0.701	-0.042	
	R: TTCGAGCCTCTGTCTGATGC	59.8								

Contig	Amplification Primers (5'-3')[3]*	Tm[4]§	Primer set	Length	Locus	H_0	H_E	p-value[5]†	Fixation Index	
2268	F: TCCACAGCTAACAGGGTCAAC	59.9	1	118	68	0.512	0.491	0.703	-0.042	
	R: AGGTGAAGTGCCATTCTCCC	59.7								
2290	F: CGGGAGCCAGCTGTGAAATT	61	3	160	140	0.470	0.435	0.461	-0.081	
	R: GCAGCATAGAGTACATCCCA	56.8			148	0.566	0.499	0.072	-0.134	**PL**
2418	F: CGACCCCGATGACTCCATTT	59.8	3	183	156	0.120	0.134	0.354	0.102	
	R: TCGCAGAGAATTCCCTTAGTGG	59.8								
2467	F: GGACTACGACCTCTGCCAG	59.2	1	109	35	0.024	0.218	0.000	0.891	**LC**
	R: CATCTCGCGGGAGTACGAG	59.7								
2526	F: TCGCATTCAGAGTGCACTGT	60	4	160	78	0.494	0.500	0.910	0.013	
	R: CCAGTTTCTGCTTCCCCGAT	60								
2543	F: GGACTCCCGTTTCTTCATTTTGG	60.1	4	101	81	0.440	0.392	0.259	-0.123	
	R: CCAACGAGTCGGACCTCAC	60.1								
2545	F: TGCTAACCAGTGCATTGGAGA	59.6	4	97	82	0.524	0.472	0.311	-0.111	
	R: ACATCTCTCTGTAACAACACTTCC	58.5								
2551	F: GTTCTCCACGTTCGCCTTTC	59.5	2	160	89	0.385	0.444	0.234	0.135	
	R: GGATATCCTGGGATTAAGGGCG	60.1								
2553	F: GCAGGTCGAAAGGAACAAATC	57.8	1	63	38	0.262	0.490	**0.000**	0.465	
	R: TCAATTCCAGGTCGAACACA	57.1								
2576	F: GGAACTGGTCCATGAATGCC	58.9	3	135	86	0.429	0.387	0.320	-0.109	

Contig	Amplification Primers (5'-3')[3]*	Tm[4]§	Primer set	Length	Locus	H_0	H_E	p-value[5]†	Fixation Index	
	R: GTTTCTCCTGCCTGGACCAA	59.9								
2667	F: CCCCAATCCTGTGCGTCTAT	59.5	3	131	111	0.536	0.440	0.047	-0.216	
	R: GCAGTTTAATCGAGTTCGTAATCA	57.7								
2685	F: CGTCGCACACTCTAACAGGA	59.8	1	83	103	0.482	0.484	0.974	0.004	
	R: TCAGGTGGTGTGTGCCAAG	60.2								
2712	F: TCCTTACAGGACCGCATGC	59.8	2	83	48	0.136	0.311	0.000	0.562	**LC**
	R: GTGTCTGATGTGAGGGTCGG	60.1								
2834	F: GCTGGAGGAGAGACAGAAGC	59.8	1	78	32	0.434	0.439	0.914	0.012	
	R: TTATTCAGGACGGCCGCATC	60.5								
2899	F: TGGCCGTGATGGTGAGTAAG	59.8	3	86	49	0.357	0.436	0.097	0.181	
	R: GCAATGGAAACTTGACTTTATTCT	56.2								
3139	F: CCGGCAGTGATCCAAATTCC	59.3	4	132	41	0.122	0.176	0.005	0.307	
	R: GCAGTCAGAAAACAACTCTGCT	59.4								
3290	F: AACAGGTATTCTTCATTTTCAGAT	55.4	3	157	154	0.610	0.495	0.036	-0.231	
	R: TGTCAATAGCTTGAGACACCA	56.9								
3378	F: AATGGCGCGTTGGAAACAC	60	4	140	133	0.190	0.172	0.335	-0.105	
	R: TGTACTCGTTCGCACTCCAC	60								
3454	F: ATGGAAATGGTGCAGTGCTA	59.2	3	126	59	0.060	0.080	0.020	0.255	
	R: CCACCAGTCAACACTCTAATGC	59.7								

Contig	Amplification Primers (5'-3')[3]*	Tm[4]§	Primer set	Length	Locus	H_0	H_E	p-value[5]†	Fixation Index	
3818	F: AGAACAGCATCACGTCTCCG	60.1	2	81	69	0.346	0.387	0.346	0.107	
	R: CGAGACAGACGGACGAGTTC	60.2								
3845	F: AAGTCGTTCTGCACCACCTC	60.2	2	132	62	0.331	0.356	0.347	0.070	**PL**
	R: GTACATCACAGACAACCGCG	59.3			64	0.274	0.301	0.409	0.090	
					83	0.354	0.405	0.089	0.126	**PL**
3864	F: CGGTCACCTCCTGAGTGAAG	59.8	4	100	39	0.274	0.357	0.034	0.232	
	R: GCCCCTAGCGATTTCAATTTCG	60.3								
5726	F: ACCGATCTGGGACTGAAGGA	60	3	99	30	0.083	0.101	0.102	0.178	
	R: CTTCCCACTCAGCTCCCTCT	60.6								
8023	F: CGGATCTAACAATTCTAAGCACT	56.4	3	141	65	0.381	0.350	0.419	-0.088	
	R: GTCGCTGCAAATTCACAAGGA	59.7								
13023	F: TTCTGATGTACTCGCGTGCT	59.5	1	103	37	0.175	0.266	0.000	0.340	**LC**
	R: CCGCTCCTGCCCTCCA	60.8								
13533	F: GTCATGCCAGTGCTAATGGC	59.6	1	70	61	0.463	0.499	0.521	0.071	
	R: ACTCATCAATTTCTGTACGAGGA	57.3								

[3]*All forward primers have CAGTCGGGCGTCATCA (Glenn) tails; all reverse primers have CCTCTCTATGGGCAGTCGGTGAT (*trPI*) tails.
[4]§Tm excludes tails
[5]†Bolded P-values signify significant deviation (after bonferroni correction) from Hardy Weinburg equilibrium

Appendix B

Dynamic change, recruitment, and resilience in reef-forming glass sponges

Authors: Amanda S. Kahn, Laura J. Vehring, Rachel R. Brown, and Sally P. Leys[1]

B.1 Abstract

Glass sponge reefs on the continental shelf of western Canada and southeast Alaska are considered stable deep-sea habitats that do not change significantly over time. Research cruises using a remotely operated vehicle equipped with accurate GPS positioning have allowed us to observe the same sponges at two reefs in the Strait of Georgia, British Columbia to document recruitment, growth, and response to damage over time. Spermatocysts and putative embryos found in winter suggest annual, asynchronous reproduction. Juvenile sponges (2-10 cm in osculum diameter) in densities up to 1 m^{-2} were more concentrated near live sponges and sponge skeletons than away (Spearman rank correlations, $p<0.0001$

[1]This Appendix has been reformatted from its original published version: Kahn, A. S. *et al.* 2015b. Dynamic change, recruitment and resilience in reef-forming glass sponges. *J. Mar. Biol. Assoc. United Kingdom*, in press. doi:10.1017/S0025315415000466, see Preface for details.

for live cover and for skeletons), suggesting that recruitment occurs in particular regions using sponge skeletons as substrate. Most sponges showed no change in shape or size over 2-3 years, but some had died while others showed growth of 1-9 cm yr^{-1}. Deposition rates of reef-cementing sediments were 97 mm yr^{-1} at Galiano Reef and 137 mm yr^{-1} at Fraser Reef, but sediments eroded so that there was no net gain or loss over time. Sponges recovered within one year from small-scale damage that mimicked bites by fish or nudibranchs; however sponges did not recover from crushing of a large area (1.5 x 2 m^2) even three years later. These observations and experiments show that while recruitment and growth of sponge reefs is more dynamic than previously thought, the reefs are not resilient in the face of larger-scale disturbances such as might be inflicted by trawling.

B.2 Introduction

Glass sponges (Class Hexactinellida) are conspicuous members of the deep-sea fauna and are thought to be adapted to the long-term constancy of deep water. Glass sponges are estimated to live up to 400 years (Leys & Lauzon, 1998; Fallon *et al.*, 2010), yet yearly monitoring has also revealed rapid responses to changing conditions and seasonal or annual spurts of growth in both shallow and deep-water populations (Leys & Lauzon, 1998; Kahn *et al.*, 2012; Dayton *et al.*, 2013; Fillinger *et al.*, 2013).

Glass sponges are typical members of deep-sea fauna, but on the Pacific coast of North America they are highly abundant in fjord habitats and also form reefs covering hundreds of kilometers of seafloor on the continental shelf. Glass sponge reefs are thought to have formed between 6,000 and 9,000 years ago after glaciers retreated from the continental shelf of western Canada and southeast Alaska (Conway *et al.*, 1991). Since their first discovery during seafloor mapping of the shelf waters in the late 1980s, it is known that 4 large reefs exist in Hecate Strait and Queen Charlotte Sound, British Columbia and over 12 more are known in southern waters of the Strait of Georgia.

Whereas in northern reefs three glass sponge species make up the reef structure - *Aphrocallistes vastus, Heterochone calyx* and *Farrea occa* - in the Strait of Georgia *F. occa* is

absent. These three species differ from most glass sponges in having secondary silica deposition that fuses their spicules into a three-dimensional scaffold. When the sponge dies the scaffold resists decay and is eventually buried by sediment. Before burial however, larvae settle and grow up to form the next generation (Krautter *et al.*, 2006). In the Strait of Georgia *A. vastus* and *H. calyx* settle and grow upon previous generations forming mounds up to 21m high cemented together by sediment (Conway *et al.*, 2005a).

Glass sponge reefs and sponge gardens serve important ecosystem functions: they are important nursery habitats for commercially important species (Cook, 2005; Marliave *et al.*, 2009; Chu & Leys, 2010; Miller *et al.*, 2012b), they contribute to local silica cycling (Chu *et al.*, 2011), and they are major grazers of plankton in deep water (Kahn *et al.*, 2015a). Glass sponges throughout the northeast Pacific are easily damaged by trawl and other fishing activity (Freese *et al.*, 1999; Heifetz *et al.*, 2009). In western Canadian waters glass sponges have been recorded as bycatch with between 0.086 kg^{-1} and 6.041 kg min^{-1} catch per unit effort in trawls through reefs (Jamieson & Chew, 2002); there are also many anecdotal records of damage to sponges by recreational prawn fisheries. What is not known is how resilient glass sponges are to damage, nor generally how dynamic growth and regeneration of individuals and populations are.

We used a remotely operated vehicle with highly accurate GPS navigation underwater to monitor change over time at two sponge reefs in the Strait of Georgia. Our aim was to observe and document reproduction and recruitment events, measure normal growth rates, and to determine whether dense glass sponge communities are resilient to disturbance.

B.3 Materials and Methods

Fraser and Galiano sponge Reefs were visited in 2005, 2007, 2009, 2011, 2013, and 2014 during cruises on the *CCGS Vector* and *CCGS Tully* (Fraser Reef: 49°9' 15.7" N, 123°23' 3.7" W; Galiano Reef: 48°54' 51.5" N, 123°19'27.7" W). Work was carried out using the remotely operated vehicle (ROV) ROPOS (http://ropos.com) which uses an ultra-short baseline navigation (USBL) Global Acoustic Positioning System (GAPS) with a LOKI Kalman filter that allows positioning within 1 m.

B.3.1 Recruitment and growth

Both Fraser and Galiano sponge reefs were mapped extensively using grids of non-overlapping photos taken 1 m above the seafloor in 2005 and 2007 (Galiano Reef: 214 photos covering 594 m^2; Fraser Reef: 109 photos covering 69 m^2) (Chu & Leys, 2010). From the same set of ROV photos juvenile sponges - those with maximum osculum width of <10 cm and minimal branching - were counted and osculum diameters measured using ImageJ software. No attempt was made to differentiate between the two species because *Heterochone calyx* and *Aphrocallistes vastus* cannot be distinguished from photos alone. The density of juvenile sponges was calculated from the total photo area for each survey grid point, plotted on maps of the reefs using ArcGIS (ArcInfo version 10.2, ESRI), and compared to the distribution of adult sponges documented by Chu & Leys (2010) using Spearman rank correlation (SYSTAT 12).

To look for evidence of reproduction (whether as spermatocysts, eggs, or embryos in tissue), pieces of *Aphrocallistes vastus* collected by SCUBA and by ROV were preserved in a cocktail fixative of 1% OsO_4, 2% glutaraldehyde, and 0.45 M sodium acetate buffer with 10% sucrose (Harris & Shaw, 1984). The fixative was replaced after 30 minutes and specimens were left at 4°C overnight. Specimens were dehydrated through a graded ethanol series to 70% ethanol, and then desilicified in 4% HF in 70% ethanol. Following desilicification, samples were rinsed twice in 70% ethanol, dehydrated to 100% ethanol and fractured in liquid nitrogen. Fractured pieces were critical point dried (Bal-Tec CPD 030), mounted on aluminum stubs, sputter-coated with gold (Xenosput XE200), and viewed in either a Hitachi S-3500N or a field emission scanning electron microscope at 5 keV (JEOL 6301F).

To monitor growth (changes in size of branches) or death (loss of live tissue) over time, the ROV was positioned at the exact same heading and same GPS position to match the camera's view to photos from previous years. Lasers 10 cm apart fixed to the camera provided a scale.

B.3.2 Sediment accumulation

To determine how much sediment accumulates at the reefs, sediment traps and erosion markers were placed at Fraser and Galiano Reefs between 2007 and 2009. Sediment traps were PVC tubes 0.5 m long with 45-cm^2 opening attached to stakes that were pushed into the sediment until the bottom of the tube was at the sediment surface. Each tube trap was pre-filled with hypersaline water and capped with a lacrosse ball that was removed by an elastic cord after the trap was positioned. Traps and makers were placed adjacent to and about 2-3 m away from clumps of sponges in 2007. In 2009 sediment traps were re-sealed with lacrosse balls to prevent loss of contents, recovered using the ROV, and frozen at -20°C for transport to the University of Alberta. The sediment was pushed out of the traps while still frozen and the total height of accumulated sediment measured. Sediments were dried in an oven at 60°C until less than 3% change in mass was achieved for two days in a row.

Erosion markers were PVC poles with 2.5-cm wide black and white markings. Not all fourteen erosion poles were found each cruise but for those revisited the number of markings above- and below-ground were counted for each pole.

B.3.3 Recovery after disturbance

Disturbance experiments were carried out in 2011, 2013, and 2014. First a clump of sponges was selected for large-scale disturbance at Galiano Reef using an asymmetrical BACI design, with one treatment patch and two control patches to minimize damage to the reef (Underwood, 1994). Two PVC markers, described above, were placed on either side of the clump. Overlapping still images of the entire clump including the marker poles were taken 1 meter above the seafloor as the ROV moved around and across the clump. Then the ROV was lowered onto the sponges to crush an approximately 1.5 x 2 m large area. A second image series was captured after damaging the sponges. Two control sites were surveyed using the same methods. All three sites were revisited in 2013 and 2014 and an identical image survey carried out. Seventy photos were selected from each survey based on clarity, image quality, and coverage of the site and adjusted for optimal contrast using Photoshop. Three-dimensional structure-from-motion reconstructions of each BACI site were created

using 123d Catch (Autodesk, www.123dapp.com/catch) to visualize changes to the sites from all angles.

Second, in 2013 four sponges adjacent to PVC erosion markers were selected for small-scale damage experiments at Galiano reef. Images were captured of 'mitten'-like extensions and then the ROV manipulator arm was used to remove 3-10 cm portions from the tips of the sponges. The same sponges were revisited in 2014 using heading and GPS coordinates to match the view from images from 2013. Images were captured of the same view.

B.4 Results

B.4.1 Recruitment of new sponges

The smallest sponges visible on the reef using HD video from the ROV were less than 5 cm in overall height and width with oscula as small as 2 cm in diameter. All juvenile sponges observed were attached to dead skeleton, had centrally placed oscula, and had broad ridges indicating the first growth of mitten-like projections.

The density of juvenile sponges (0.4 ± 0.9 juveniles m^{-2}; mean ± standard deviation, SD) was greater in areas where adult sponge density was highest (Figure B.1a; Spearman rank correlation, ρ=0.299, p<0.0001) and where dead sponge cover (skeletons) was highest (Spearman rank correlation, ρ=0.272, p<0.0001). Live and dead sponge cover were strongly correlated (Spearman rank correlation, ρ=0.666, p<0.00001). While density did not differ between reefs (Mann-Whitney U-test, U=10,905, df=1, p=0.218), juveniles found at Fraser Reef were significantly smaller (1.6 ± 0.8 cm maximum osculum width, mean ± SD) than juveniles at Galiano (2.6 ± 1.7 cm; Mann-Whitney U-test, U=1,376.5, df=1, p=0.004) and had a narrower size distribution (Figure B.1b-d).

Adult tissues from one ROV collection in November 2011 had spermatocysts by December of the same year, and tissue collected by SCUBA in a nearby fjord (Saanich Inlet) in December 2012 and February 2013 also contained spermatocysts (Figure B.1e). Developing

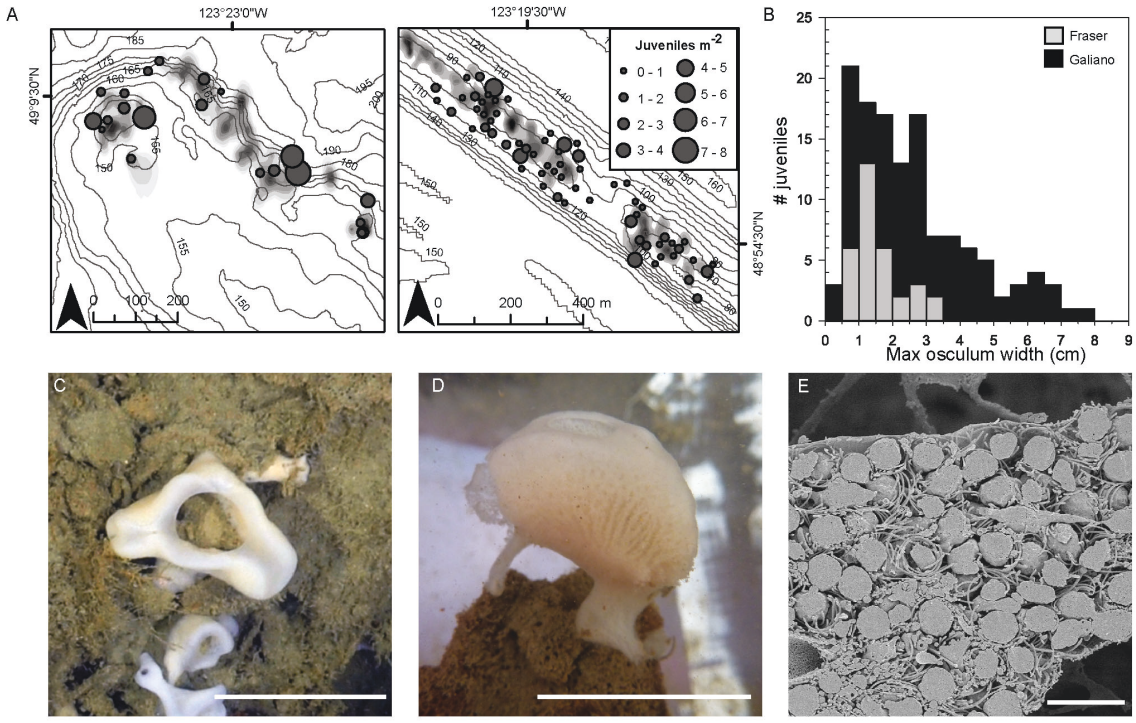

FIGURE B.1: Recruitment in glass sponge reefs. A. Map of the density of juvenile sponges at Fraser Reef and Galiano Reef. Juvenile density is correlated with adult sponge density (shaded gray). B. Size distributions of juveniles found at Fraser (gray) and Galiano reefs (black). C-D. Juvenile sponges have a maximum osculum width less than 10 cm. E. Spermatocysts from a specimen of *Aphrocallistes vastus* collected in November, kept alive in seawater tables at Bamfield Marine Sciences Centre, and fixed in December. Scale bars: C: 5 cm, D: 2 cm, E: 10 μ m.

embryos were found in a single specimen of *Aphrocallistes vastus* collected by scuba by one of us (Leys) in November 1995 (Figure B.2).

B.4.2 Growth

Because *Aphrocallistes vastus* and *Heterochone calyx* grow in three dimensions and extend mitten-like projections to increase surface area, it is difficult to find an accurate growth parameter to measure. We compared images captured at the same angle (ROV heading) each year to measure changes in shape and change in either height of an osculum or length of a projection. Changes included one sponge found growing around a pole in 2013 that had been absent in 2011, and was dying in 2014 (Figure B.3a). Individual oscula grew in diameter in a multicolored clump of sponges in 2011, 2013, and 2014 but a portion of

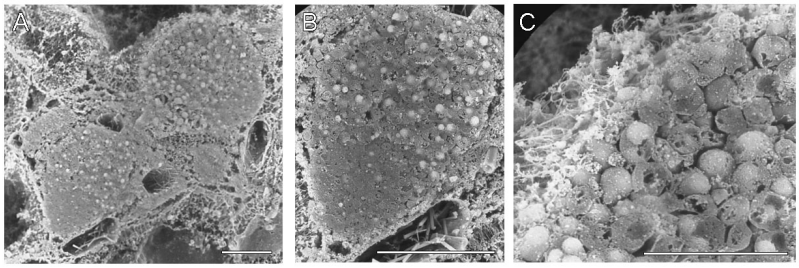

FIGURE B.2: Supplemental: Two putative embryos found in tissue of a single specimen of *Aphrocallistes vastus* collected in November 1995. A. Two putative embryos amongst feeding chambers of the sponge. B. Closer view of one of the possible embryos, showing a pointed anterior end and a yolk-filled posterior end. C. Cilia lining the outside of the embryos. Scale bars: A: 10 cm, B: 10 cm, C: 5 μ m.

that clump died back between 2013 and 2014 (Figure B.3b). A juvenile grew an estimated 3 cm yr^{-1} (Figure B.3c). Three large sponges grew between 1 and 3 cm yr^{-1}taller while projections from oscula grew faster, between 7 and 9 cm yr^{-1} (Figure B.3d).

B.4.3 Sediment accumulation

Sediment traps showed sedimentation accumulation of 97 mm y^{-1} at Galiano and 137 mm y^{-1} at Fraser reef over 2 years (2007-2009); however, the PVC poles showed both accumulation and erosion with no net overall change over the two years (Figure B.4). On some poles at Galiano reef the markings at sediment level were erased by scouring caused by high currents.

B.4.4 Recovery after disturbance

Large-scale damage: The site crushed by the ROV showed no recovery after 3 years (Figure B.5). Large clumps of sponges immediately adjacent to the damaged site survived through 2014 but did not spread into the space vacated by the dead sponges, nor were new juvenile sponges seen anywhere on the damaged skeletons (Figure B.6). The two undisturbed control sites showed both new growth and patches of sponges that had died. New growth occurred as overgrowth of skeletons by both glass sponges and by the demosponge *Desmacella austini.*

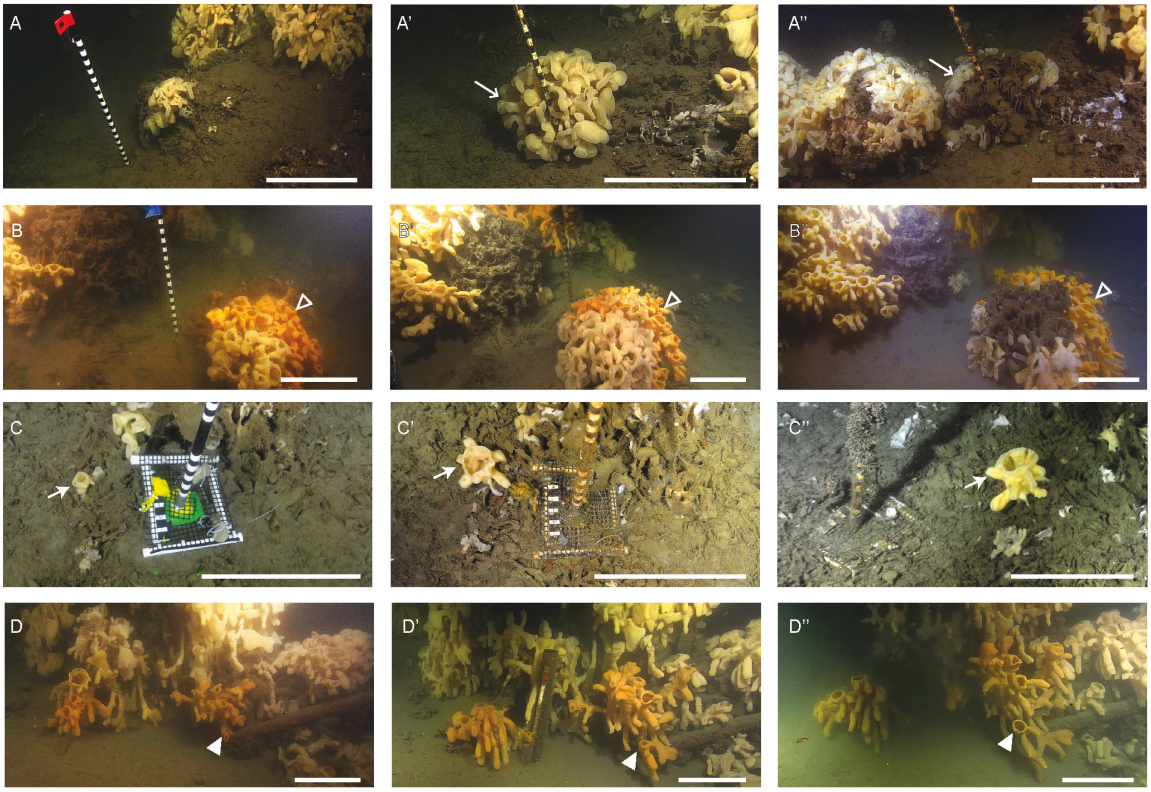

FIGURE B.3: Growth and change in glass sponge reefs. A. A marker pole planted in open sediments in 2011 (A) had a 20-cm tall sponge (arrow) surrounding it in 2013 (A'), probably dislodged by the ROV. In 2014 (A"), the sponge had died but other sponges had grown up nearby. The photo from 2011 was rotated to provide the same view as in 2013 and 2014. B. A clump of sponges (arrowhead) adjacent to a sediment marker persisted from 2011 (B) to 2013 (B'), but was dead in 2014 (B"). C. Growth of a small sponge (filled arrow) beside a marker. The marker was moved after photographing in 2013, so it now is to the left of the sponge and a juvenile appeared on the settlement plate in 2014 (C: 2011, C':2013, C": 2014). D. Several sponges (filled arrowheads) overgrew a discarded cable we encountered at Fraser Reef. The projections on these sponges grew 7-9 cm yr^{-1} (D: 2011, D': 2013, D": 2014). All scale bars: 50 cm.

Small-scale damage: All four sponge projections that were damaged had repaired completely within one year. In each instance, the sponge had regenerated a soft growing edge to close the damaged area. On one sponge, the damaged projection had grown into what appeared to be an osculum while an undamaged projection of the same sponge had grown 9 cm in length (Figure B.6).

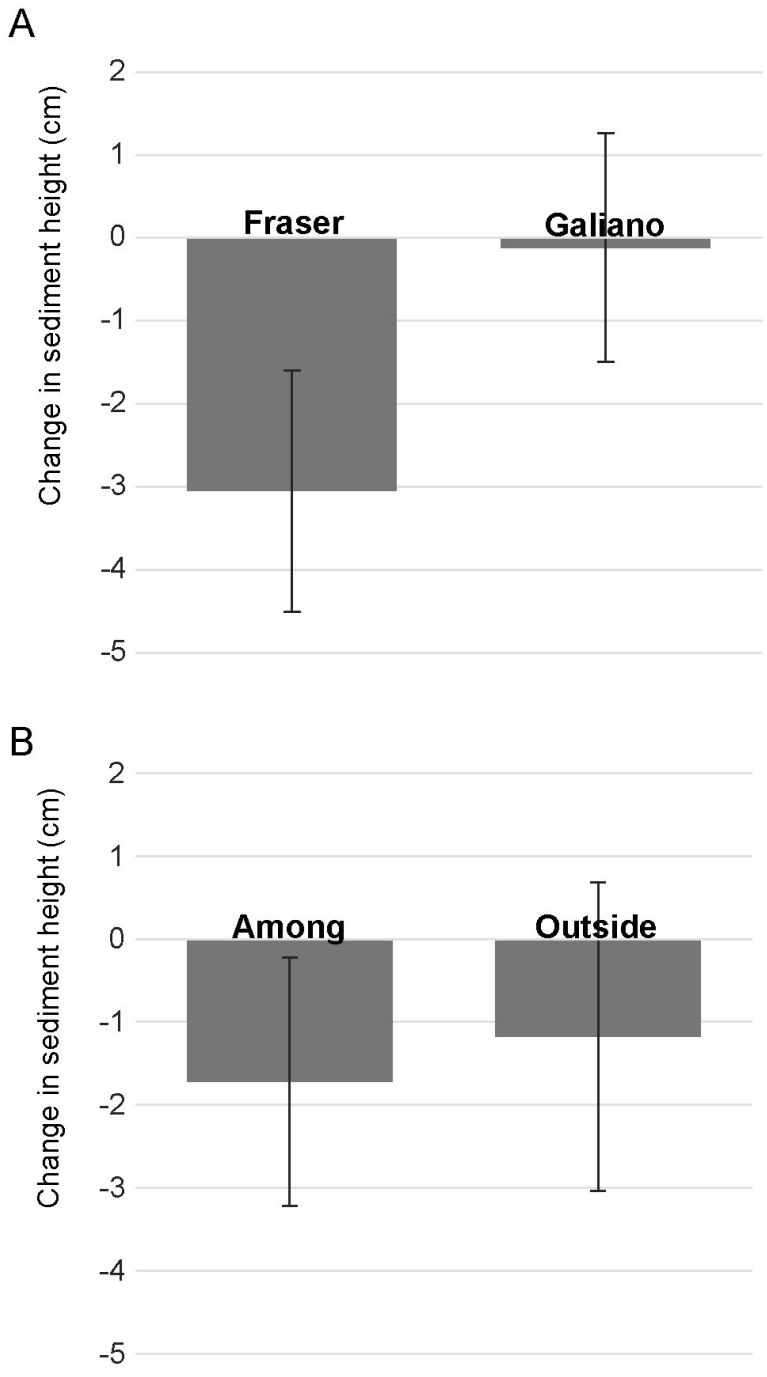

FIGURE B.4: Supplemental: Mean (± SE) changes in sediment height were measured using sediment poles surveyed between 2005 and 2013. Not all poles could be visited during each research cruise. Changes in sediment height were compared between poles deployed at Fraser or Galiano reefs (A), and between poles deployed among or outside of patches of reef (B).

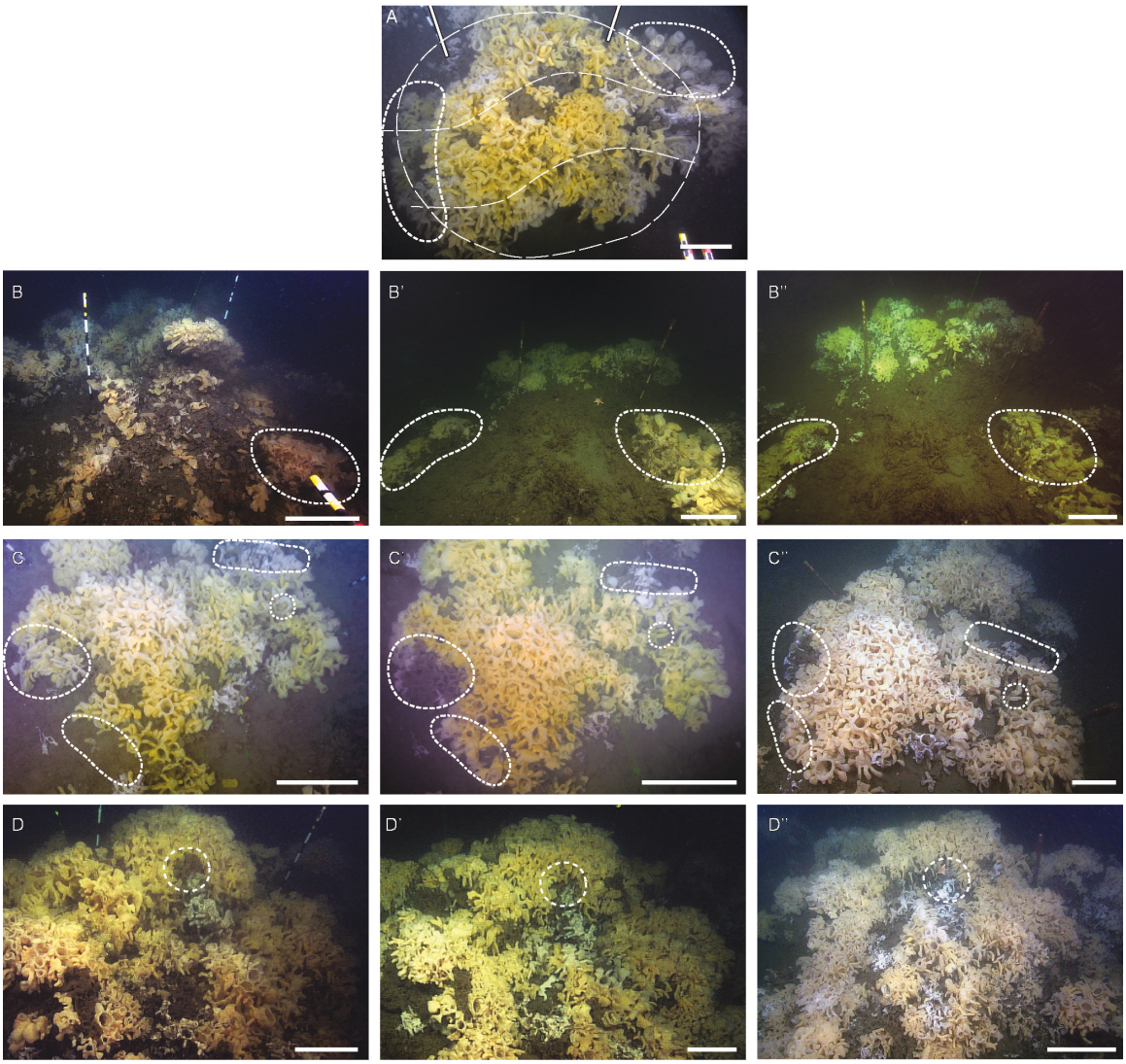

FIGURE B.5: Disturbances to the sponge reefs using an asymmetrical BACI design. A. Images of 'impacted' site in 2011 before physical disturbance with paths for ROV surveys shown by dashed lines. (B, B', B") Image surveys following disturbance in 2011, 2013, and 2014 show little recovery, though adjacent sponges appeared unaffected. Control sites 1 (C: 2011, C': 2013, C": 2014) and 2 (D: 2011, D': 2013, D": 2014) showed overgrowth of sponge tissue, death of other tissue, and colonization by *Desmacella*. Dotted circles indicate regions of growth or death between years. All scale bars: 50 cm.

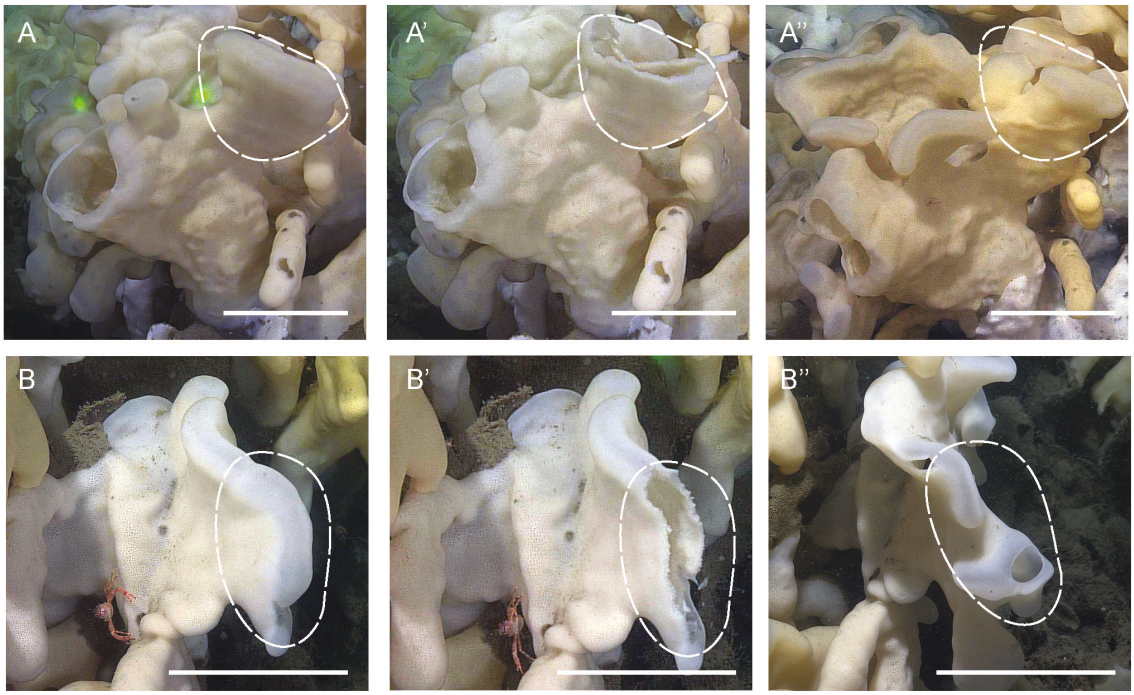

FIGURE B.6: Recovery of reef sponges from small-scale damage. All panels show regions with projections of sponges that were damaged in 2013. A, B: 2013 Still image captured from HD video before damage occurred. A', B': 2013 Still image from HD video taken after the projections had been damaged. A", B": 2014 Still image from HD video taken one year later. All scale bars: 10 cm.

B.5 Discussion

We took advantage of repeat visits to the glass sponge reefs in the Strait of Georgia British Columbia using a ROV to observe recruitment and changes to individual sponges over time. We found that the changes were similar to those that occur to sponges in shallow-water habitats including growth, death, and resilience to disturbance.

B.5.1 Recruitment of new sponges

The many juvenile sponges observed on the reefs with one distinct size class of osculum diameter (1-3 cm) are indicative of one or more reproductive events per year. We have looked for signs of reproduction in reef sponge tissues during every visit by SCUBA or ROV since the late 1990s. Although putative embryos have only been found in one specimen collected in November 1995 and not in tissues collected in July 2005, October 2007, 2009,

2011 or November 2011, 2013, 2014, nevertheless we found spermatocysts in tissue collected in December 2012 and February 2013. From these three observations of spermatocysts and embryos we conclude that gametes develop asynchronously within a single individual of *Aphrocallistes vastus* and that reproduction is asynchronous among individuals, with spawning of sperm and development of embryos by brooding occurring over winter months.

Finding so many very small sponges gives new insight into reproduction and recruitment at the reefs. Given a growth rate of 1-3 cm yr^{-1} , juveniles (2-10 cm) seen on the reefs in November 2013 and 2014 may have settled the previous year. Because larvae recruit to dead sponge skeleton we wondered whether high sedimentation might impede settlement and recruitment. Our two methods of measuring sediment accumulation suggest this is not the case. First, the density of juveniles was independent of sedimentation rates: Fraser reef had double the sediment fallout of Galiano reef, but density of juvenile sponges at both reefs did not differ. Second, the erosion markers showed very little accumulation of sediment even over several years, which explains why reef skeletons remain exposed as a good substrate for recruitment.

The fact that juveniles were found near both adult sponges and dead skeleton suggests that either larvae settle close to their site of release as found by Uriz and colleagues for a demosponge (Uriz *et al.*, 1998), or that larvae settle gregariously based on chemical cues from the material growing on dead skeletons (Ettinger-Epstein *et al.*, 2008). A third possibility is that those skeletons and live sponges provide both good substrate and good growing conditions for sponges. These hypotheses cannot be tested with our data since the density of live sponges and dead skeletons were strongly correlated. In all visits to the reefs, there was no evidence that reef species reproduce asexually by forming tissue drips as suggested by Austin *et al.* (2007).

B.5.2 Growth

Because we could carry out repeated visits to the same sites on the reefs we could see changes to individual sponges over time. Rates we calculate of 1-3 cm yr^{-1} for very young reef sponges are slower than those estimated for overall growth for the hexactinellid

Rhabdocalyptus dawsoni, which can grow in length by up to 12% per year in small specimens and by 6% per year in larger specimens (Leys & Lauzon, 1998). Projections of reef sponges grew more rapidly, at 7-9 cm yr^{-1}, which is in agreement with rates measured by Austin and colleagues (Austin *et al.*, 2007). Generally it seems then that smaller sponges grow relatively quickly or episodically (Leys & Lauzon, 1998; Dayton *et al.*, 2013; Fillinger *et al.*, 2013). Appearance and growth of new sponges, and death of patches of sponge, indicate that the population is continually renewing and replacing across annual timescales as described for other glass sponge populations (Kahn *et al.*, 2012). In 2013 we re-visited an erosion pole planted in 2011. A large sponge now lay against it (Figure B.3a), which suggests that this sponge was dislodged from elsewhere and came to rest against the pole. If the sponge was dislodged in 2011 and was still alive two years later then glass sponges can reattach following breakage. There is little chance that this sponge settled and grew from a larva to some 30 cm in diameter in 2 years, although that possibility should not be ruled out. This was a single observation however, and further observations are needed to determine whether this sort of reattachment could happen more often in sponge reefs due to disturbance by sharks or high currents.

B.5.3 Recovery after disturbance

Glass sponges are easily broken with trawls and prawn traps (Freese *et al.*, 1999; Wassenberg *et al.*, 2002; Ardron & Jamieson, 2006; Heifetz *et al.*, 2009), but the ability of reefs to recover from damage is unknown. We therefore took advantage of the ability to make repeat visits to identical sites to determine the ability of reef forming sponges to regenerate.

Having expected that deep sponge communities change slowly, we were surprised to find that small parts of sponges intentionally damaged by the ROV could recover completely in one year. Similar regeneration rates were found previously in shallower populations of glass sponges wounded experimentally (0.05cm^2 d^{-1} or 18 cm^2 yr^{-1}; Leys & Lauzon, 1998). Naturally broken fragments of sponges and damage to oscula and projections were frequently seen on our ROV dives. This damage was thought to be caused by both fish bites and knocks by fish tails (e.g. dogfish, ratfish) because in 2014 we watched as both

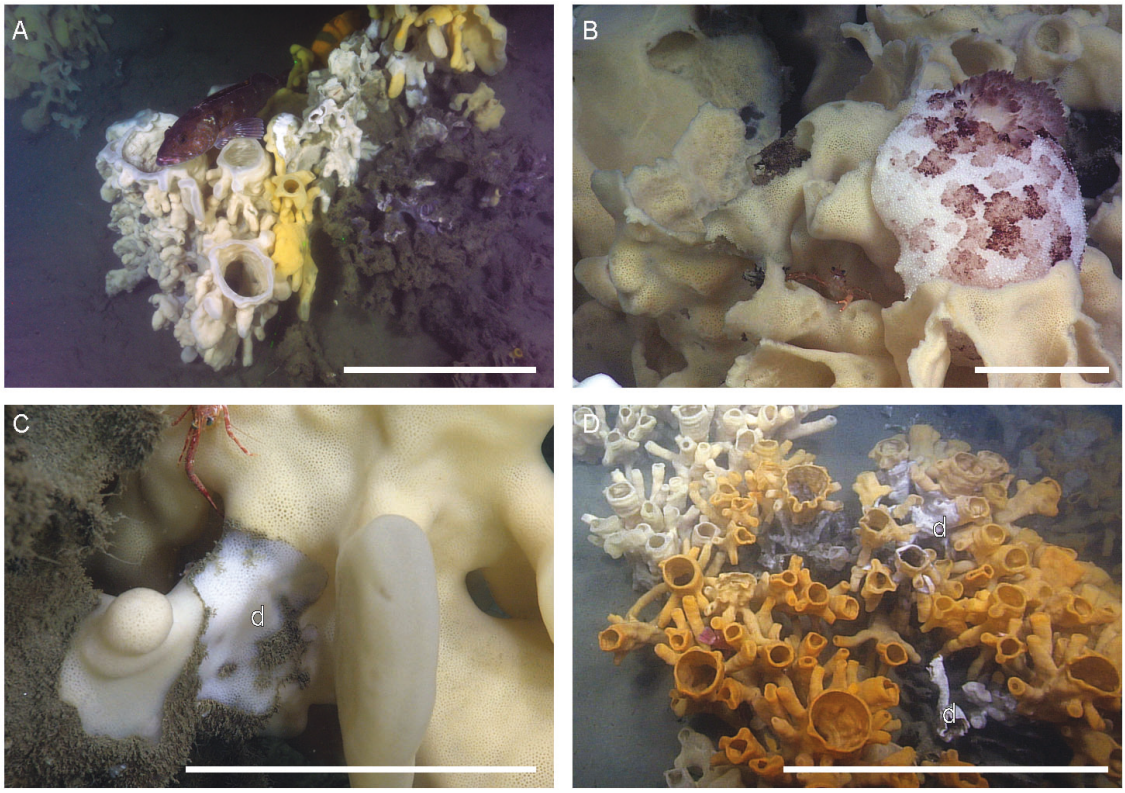

FIGURE B.7: Other ecological players in sponge reefs. A. A lingcod (*Ophiodon elongatus*), one of many fish that frequent the reefs and can cause disturbance from bites and knocks, perches on the sponges. B. The nudibranch *Peltodoris lentiginosa* on a sponge; adjacent oscula showing damage from grazing. C. *Desmacella austini* (d), a demosponge that occupies the skeleton, here at the base of *Aphrocallistes vastus*. D. *Desmacella* was commonly seen at the base of glass sponges in the reefs. Scale bars: A & D: 50 cm, B & C: 5 cm. All scale bars are approximations.

the Pacific spiny dogfish (*Squalus acanthius*) and the lingcod (*Ophiodon elongatus*) caused this sort of damage when they caught crustacean prey from among the sponges (Figure B.7a). Grazing by the nudibranch *Peltodoris lentiginosa* also causes extensive damage to the uppermost projections of sponges (Figure B.7b).

We were even more surprised to learn that, in contrast to the quick repair of small parts of the sponges, no new sponges grew into or colonized the 'impacted' BACI site even after three years. Because sponges at the edge of the damaged site grew into the area but no new sponges recruited, we suspect that it was the extensive damage to the skeletons, used as substrate by reef sponges, that prevented new recruitment. Trawling has similar effects

in removing rugosity and creating flat terrain (Puig *et al.*, 2012), so damage caused by trawling in regions with sponge reefs and gardens is expected to be long lasting.

In addition to anthropogenic disturbance, reef sponges face predation as described above and also by Chu and Leys (Chu & Leys, 2012) as well as competition. The demosponge *Desmacella austini* grows on and takes over the skeletons of reef sponges. Three different colour morphs - possibly different species - of *Desmacella* were seen at both reefs. *Desmacella* was most common on dead glass sponge skeleton (Figure B.7a), but careful study of the high definition video showed that *Desmacella* occupied the base of many individuals of glass sponges (Figure B.7b). Whether *Desmacella* colonizes live glass sponges or takes advantage of areas of skeleton left exposed by regressing tissue as the sponges grow upward is unknown. Its growth on dead skeletons however, may prevent larvae from settling.

B.6 Conclusions

Three years of observations of deep sponge reefs showed that many aspects of reef ecology - recruitment, growth, and resilience to disturbance - were comparable to those of shallower sponges. Recruitment occurs annually with growth rates for the smallest sponges of 1-3 cm yr $^{-1}$ and projections of the larger sponges at up to 7 cm yr^{-1}. New sponges continually grow and replace sponges that have died. Glass sponge reefs are resilient to minor natural disturbance, but show no signs of recovery from large-scale physical breakage within the timescale of this study.

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