NSF Project Description:

Colonization genetics of globally invasive Bryozoa (genus *Watersipora*): Is adaptation to temperature and copper prior/post-introduction important for determining spread?

I. Results from Prior NSF Research

REU SITE in Ecological & Evolutionary Research at Humboldt State University (NSF Award # DBI-0353673; $279,986; 2008-2010): Sean Craig (PI), Matt Johnson (Co-PI).

This continues a previous REU grant (2004-2006; Award # 0353673). Published products from the first REU Award are listed in the References Cited. There were 36 students over three 10-week research summers (12 students/year-despite funding level for only 10 students/year). Thirteen (36%) students were from underrepresented groups; 12 (92%) of the 13 are now working biologists or in graduate programs, 11 (85%) of the 13 presented research at meetings, and 7 (54%) published papers. Eighteen (50%) of the 36 REU participants presented talks, 20 (55%) presented posters at scientific meetings including 3 "best paper" awards and 1 "best poster" award (3 of the 4 award winners were mentored by Sean Craig, PI on this proposal). Several more publications are in preparation or in press. The current REU program is still in progress; while results have not yet been fully compiled (final report due March 2011), we anticipate similar success rates.

These statistics tell only part of the story, however. One Hispanic student (Vanessa Flores) from the most recent REU SITE grant in summer (2009) presented research on *Watersipora* copper tolerance (mentored by both Dr. Craig at HSU & Dr. Mackie at SJSU) which also won a "best talk" award at the West Coast Biological Sciences Undergraduate Research Conference (WCBSURC) at Santa Clara University in spring 2010. Vanessa also presented her work at the Western Society of Naturalists Meeting (WSN) in November, 2009 (Monterey, CA) and has was awarded a 2nd prestigious summer REU internship at the Univ. of Massachusetts at Boston this summer (2010) where she has gained further experience in population genetics and evolutionary biology of deep sea invertebrates in the laboratory of Dr. Ron J. Etter. We are currently working on a manuscript with her on *Watersipora* larval tolerance to copper, and fully expect Vanessa to go on to graduate school in ecology & evolutionary biology. More than 9 of our past REU students are in graduate programs at prestigious institutions including Michigan State University, Oregon State University, the University of Connecticut, and UC Davis (as well as our own M.S. program at HSU), and this number will be increase once all HSU-REU students are contacted this academic year.

II. Background: Invasions of Marine Bryozoa

The rate of new introductions and secondary spread of non-native species in shallow marine habitats is accelerating in connection with shipping traffic and other disturbances (Cohen & Carlton 1998, Hewitt et al. 2004). With high abundance in harbors, planktonic larvae may be entrained in ballast tanks of ships (Carlton & Geller 1993) or settle on ships' hulls (Carlton & Hodder 1995). Bryozoans in particular, a phylum of encrusting animals common in fouling communities the world over, are predisposed for human-mediated transport (Watts et al. 1998, Barnes 2002).

Species in the genus *Watersipora* Neviani, 1895 are likely among the most invasive of Bryozoans. Once released, populations of *Watersipora* grow explosively due to lateral growth of established colonies and co-settlement of short-lived larvae that may be retained near parents owing to short distance dispersal (larvae generally swim <24 h) (Lynch 1947, Wisely 1958). Multiple *Watersipora* species are relatively insensitive to antifouling paints (Allen 1953, Floerl et al. 2004, Piola et al. 2009).

Watersipora illustrate some key features of invasions, including the ability to disperse rapidly as a result of shipping movements. In line with the "tens rule" (Booth et al. 2003), sets of introductions almost definitely occur more frequently than is known; some introductions have probably occurred due to short-term influences such as El Niño patterns (Soule & Soule 1985b), and in other cases one *Watersipora* species has displaced another at a regional scale (Gordon & Mawatari 1992, Keough & Ross 1999). Interestingly, a number of different species have become prominent weedy species: namely, *W. arcuata*, *W. subtorquata*, *W. '*new sp', (a morphologically indistinguishable member of the *subtorquata*-like species complex), and *W. subovoidea.* This proclivity suggests unusually strong invasive ecological characteristics of the genus. In some areas, a *Watersipora* species is the most common infra-littoral zone bryozoan (Keough & Ross 1999, Rodriguez & Ibarra-Obando 2008). Accordingly, this relatively-little studied group may play a critical role in affecting the structure and diversity of shallow water ecosystems. Survival rates of *Watersipora*, and other widely dispersed fouling bryozoans, are strikingly high when compared to other species, such as mat-forming ascidians, in communities polluted with copper antifouling paint pollutants (Dafforn et al. 2008, Piola & Johnston 2009), or subject to simulated heat-wave conditions (Sorte et al. 2010). Such studies suggest shallow marine communities could be subject to 'bryozoanification', or at least substantial increases of a few bryozoans, as environmental conditions change.

Invasions are well recognized as dynamic processes, providing insight into evolution. In concert with introduction processes, adaptive changes may become manifested in a variety of ways, including via directional selection on additive genetic variation, selection on traits that assist in dispersal, and the generation of new allelic combinations by hybridization (Lee 2002). In plant populations it is apparent that genotypic diversity commonly plays a role in determining where, or under which environmental conditions, populations successful establish (Mack 1996, Williamson & Fitter 1996, Garant et al. 2007). The other general ecological aspect that is used to predict invasion success by a particular species is propagule pressure or introduction 'effort', whether intentional or non-intentional. Movements of more individuals from larger populations theoretically enhances the likelihood of successful establishment in overcoming the 'Allee' effect and in providing a pool of potentially successful genotypes (Leung et al. 2004, Verling et al. 2005, Johnston et al. 2008, Simberloff 2009). In marine ecological work generally, however, there are still few studies that synchronistically link genotype and colonization dynamics, and often "propagule pressure" arguments assume no importance of genetic variability among the invading propagules when modeling introduction processes (Verling et al. 2005, Simberloff 2009).

III. Goals of Proposed Research

Watersipora deserve detailed investigation for a number of reasons: they are dominant space occupiers in fouling communities with potential for rapid population growth (Rodriguez & Ibarra-Obando 2008), heavily impacting benthic communities; they facilitate other introduced species because they are resistant to antifouling paints and provide secondary substrata for other exotic species (Allen 1953, Floerl et al. 2004); and they are excellent model systems for studying evolution in marine habitats. The high introduction rate makes the genus a potentially useful candidate for examining underlying principles of marine introductions, including the role of adaptation to temperature and copper relative to the importance of transport vectors (e.g. ship hulls) in successful invasions. **The aim of this proposal is to provide genetic insight into the dynamics of introductions of a complex of taxonomically poorly-understood *Watersipora* species, focusing attention on the Western United States seaboard, and to provide an understanding of how genetic diversity shapes introduction success. We will do this by examining correlations between genotypes, inferred regions of origin, and geographic spread of long-isolated species.**

We will also experimentally examine whether populations defined by these units are affected in growth and survivorship under different temperature regimes and copper paint concentrations in order to determine if pre or post-introduction adaptation explains patterns of invasion in this Bryozoan genus.

IV. Invasions of Bryozoans in the *Watersipora* spp. complex

Introductions of *Watersipora* have been most extensively documented in southern Australia (Keough and Ross 1999) and New Zealand (Gordon and Mawatari 1992). California, like Australia and New Zealand, has experienced multiple watersiporid introductions. Introductions of *Watersipora* to the Pacific coast of the United States have been recorded since the 1950s (Osburn 1952, Banta 1969, Soule & Soule 1985a). All *Watersipora* populations on the Pacific US seaboard are thought to be non-native and effectively 'cryptogenic' (of unknown origin) because of taxonomic uncertainty (Gordon 1989, Cohen & Carlton 1995) and species boundaries are not well understood. *Watersipora subtorquata* (d'Orbigny), is characterized by slightly foliose colonies composed of relatively large (length ≈1 mm), flat, often orange or darkly pigmented zooids, with a subcircular (sinusoid) orifice. While *Watersipora 'subtorquata'* is recognized as a serious exotic pest, having become common on the Western US seaboard since the 1980s (Cohen & Carlton 1995, Boyd et al. 2002), the taxon is in fact a group of multiple species requiring formal separation. It has not been possible to document home ranges of *Watersipora* populations, due to strongly confused systematics. The problem of cryptic species in marine invertebrates in general makes accurate estimation of invasion rates difficult (Carlton 1996). One of the reasons why *Watersipora* has proven so taxonomically difficult is that all members of the genus lack spines, avicularia, and external ovicells – features which are highly available and useful in taxonomic diagnosis in most other bryozoans.

The following extended quotation from Cohen and Carlton (1995) explains distinctive morphological variations within the context of the state of knowledge of *Watersipora* invasions in California, as of 1995:

> "Since the 1960s two species of *Watersipora* have appeared in California where none were previously known. These species are distinguished from each other by the shape of the proximal border of the aperture, with the border curving into the aperture in *W. arcuata* (=*nigra*) and curving outward to form a sinus in *W. "subtorquata."* The identification of the latter species remains uncertain (the one or more species with a sinusoid aperture have been variously referred to as *W. subtorquata, subovoidea, cucullata, atrofusca, aterrima and edmundsoni* [*sic*.] due to variability in the characters used to distinguish sinusoid species and the unstable taxonomy of the genus (Gordon (1989), for example, referred to it as "a taxonomic 'can of worms'").

V. Preliminary Results: Population Genetic Structure of Watersipora spp. (complex)

Genetic analyses using the mitochondrial cytochrome c oxidase I (COI) gene have historically proven useful in verifying genetic divergence (typically showing nucleotide-divergences of 15% or more) across species, including putative sinusoid species which cannot be distinguishable morphologically using skeletal characters, including the shape of zooids and various microstructures on the operculum (Mackie et al. 2006, Ryland et al. 2009).

As shown in Figure 1, COI variation in California is stratified (Geller et al. 2008). There was a strong transition in the diversity of *Watersipora* lineages detected around ˚34-35 N, in the area of Santa Barbara and Point Conception, which is a widely recognized as a point of concentrated phylogenetic breaks in native biota (Dawson 2001, Kelley & Palumbi 2010).

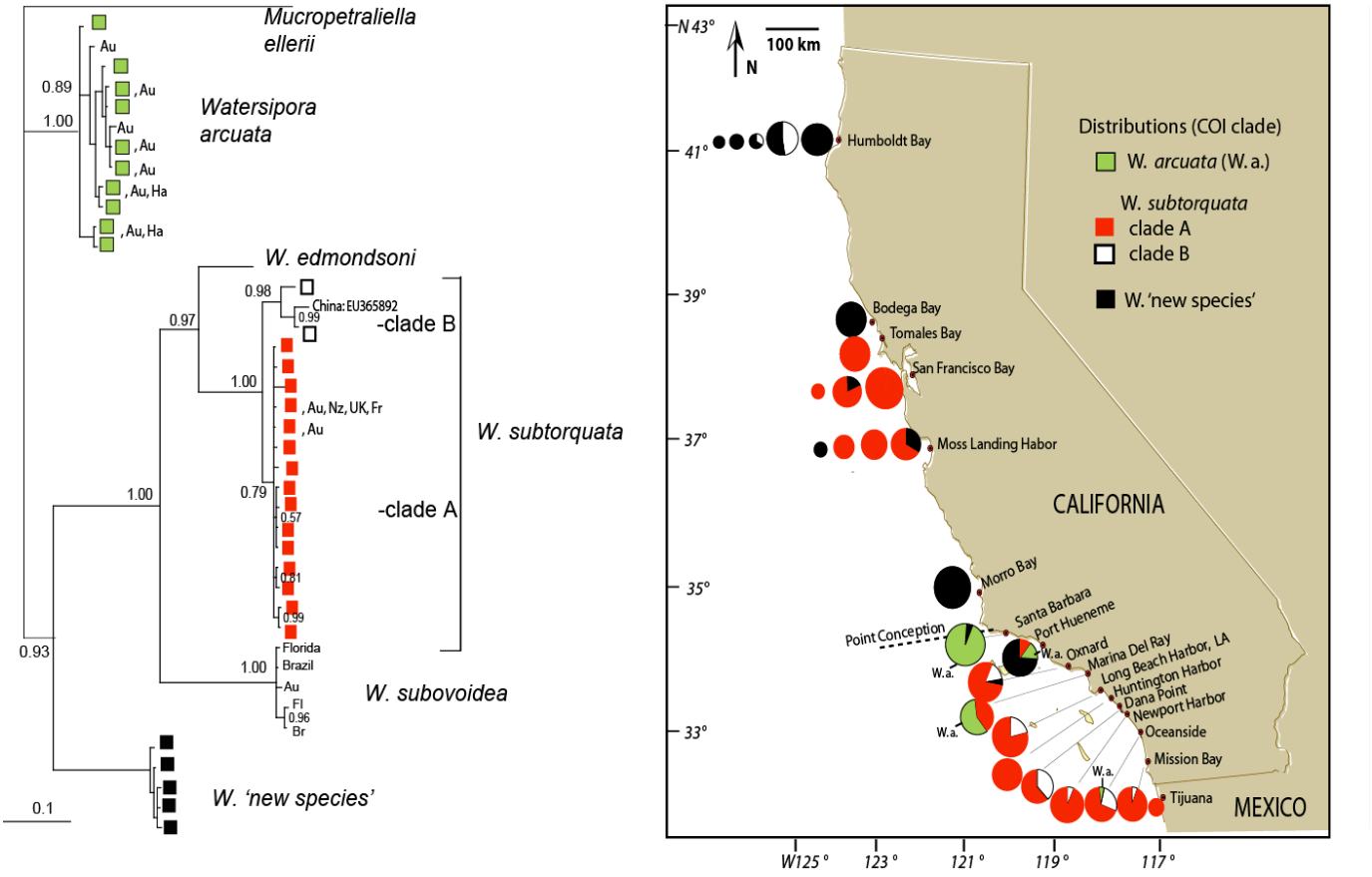


Figure 1. Left: Bayesian tree of COI (600-bp) haplotypes of the *Watersipora* complex developed by Mackie. Boxes indicate haplotypes found in California. Lineages sampled in other areas: Au, Australia; Ha, Oahu, Hawaii; NZ, Wellington, New Zealand; U.K. (southern England); and Fr, France. Posterior probabilities (>0.5) are shown at nodes. **Right:** Distribution of COI haplotype groupings at 26 sites in California; collections made between 2002-2010. Circles are sized in proportion to the number of colonies analyzed at each site, with separate circles depicting different localized sampling sites.

Genetic variation within the complex of invasive *Watersipora* species is more complex than may be inferred through morphological taxonomy (Geller et al. 2008). The presence of *W. subtorquata* A and B clades and *W. new sp* COI clade (Figure 1), suggests at least three separate introduction events to California, as opposed to the two suggested on the basis of the recognized morphological variations.

The distribution of major COI variants is as follows (delineating taxa that are considered important in bold). ***Watersipora arcuata***, a clearly differentiable, non-sinusoid species is common in the 33˚N-34˚N zone around Santa Barbara, often alongside (one or possibly more) sinusoid species (Figures 1 & 2). *Watersipora arcuata* and *W. subtorquata* also co-occur in Adelaide, Sydney and Perth, in Australia. Both regions have similar average sea-surface water temperatures (SST: see Figure 3). The *W.* **'new sp.'** clade, a sinusoid species found recently in introduced populations on natural reefs, has been collected from Bremerton, Washington in 2009, the northernmost known extent of *Watersipora* introduction (collector: G. Jenkins, University of Washington; Figure 2).

California remains the only known region of occurrence of the *W.* new sp. mitochondrial clade, however more sampling needs to be done. The **'*subtorquata*' type A** COI lineage, including the most common haplotype (WS1), has recently colonized the southern England coastline (Ryland et al. 2009). Some colonies collected at large-shipping docks in Humboldt Bay, in 2003, alongside *W.* new sp. (coll: L. McCann, Smithsonian Institution), had **'*subtorquata*' clade B** haplotype (Figure 1).

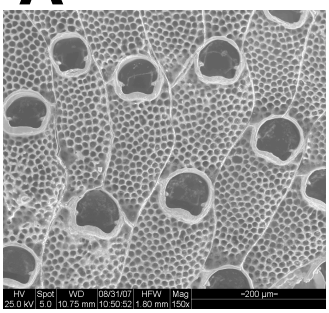
Morphologically we have found no evidence for separation of colonies of W. *subtorquata* and *'new sp.'* COI clades (Figure 2A, B), suggesting either a lack of fixed genetic differences (and perhaps mixing gene flow through hybridization), or, alternatively that these are truly morphologically invariant (cryptic) species. Intriguingly, colonies occurring in the North of California tended to have longer zooids, regardless of the COI-based mitotype than colonies in southern California (Figure 2C). Such a relationship has also been noted in inspection of different collections made in the genus *Watersipora* previously (Soule & Soule 1985a, Ryland et al. 2009). An inverse relationship between temperature and zooid size may indicate either plasticity in development, which has previously been found in Bryozoa (see for example, (O'Dea & Okamura 2000, Atkinson et al. 2006), or genetic variance in morphological variation responding to natural selection following introductions. This may occur in the time course following introduction; for example, introduced populations of *Drosophila subobscura* occurring along the West Coast of the US have heritable differences in wing-vein and wing surface area, with larger sizes occurring in the north of the distribution as well (Huey et al. 2000).

Watersipora subtorquata and *W.* 'new sp' mitotype lineages also show no apparent morphological differentiation in characters that have served to distinguish another sinusoid species, *Watersipora*'*subovoidea*' which was prior to COI analysis relegated to the '*subtorquata*' complex on practical grounds (Gordon & Mawatari 1992, Ryland et al. 2009). Thus far, there has been no test for hybridization among lineages defined by mitochondrial DNA using nuclear markers or breeding tests among these different lineages to distinguish some of these alternative explanations. However, the potential for inter-breeding and hybridization is high. Colonies with divergent mitotypes sometimes occur on the same harbor installations, and even share an alpha-bacterial symbiont (Anderson & Haygood 2007), with proximity close enough to facilitate inter-breeding.

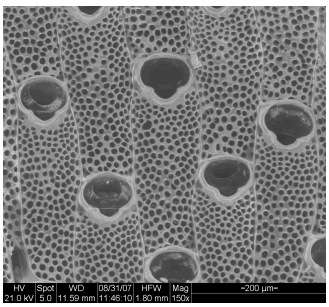
Looking across all sites that have been analyzed by COI sequence reveals come correspondence between phylogeny and mean environmental temperature in *Watersipora* (Figure 3). Similar COI lineages appear to occur in different parts of the globe in similar temperature zones. This suggests genetic pre-adaptation may mediate the occupation of different temperature niches, such that particular "cold-adapted" genotypes and/or species invade colder waters, and "warm-adapted" genotypes and/or species invade warmer waters.

The ability to track dispersal through the use of nuclear and organelle based genetic markers opens the window to studying the role of genetic (and phenotypic) polymorphism in successful establishment of *Watersipora* spp. In new sites. For example, the high similarity of sets of *W. arcuata* COI haplotypes, analyzed previously in Hawaii, Australia (Mackie et al. 2006), and California (Geller et al. 2008), suggests introductions may commonly arise from other invasive populations rather than their native range. Through analysis of multilocus data, the size of founder events in invasions can now be more clearly estimated (Estoup et al. 2001, Brown & Stepien 2009). In general, increasing understanding of population dynamic aspects of introductions—the number of independent introductions, the total genetic variation present in wide, established distributions of non-indigenous species, and the importance of genetic cohesion across the introduced range, in addition to phenotypic differences maintained in different areas—is considered important in predicting the scale at which mitigation of introduction effects should be enacted (Mack et al. 2000, Perrings 2005).

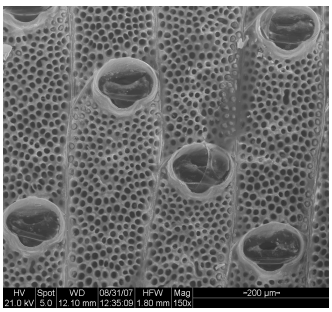
A



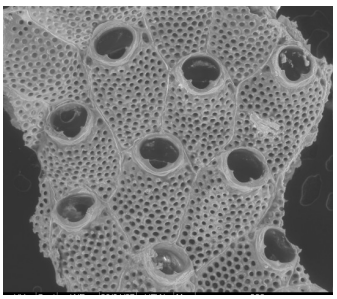
Watersipora arcuata
Collection locality:
Santa Barbara



W. subtorquata COI clade
Tomales Bay

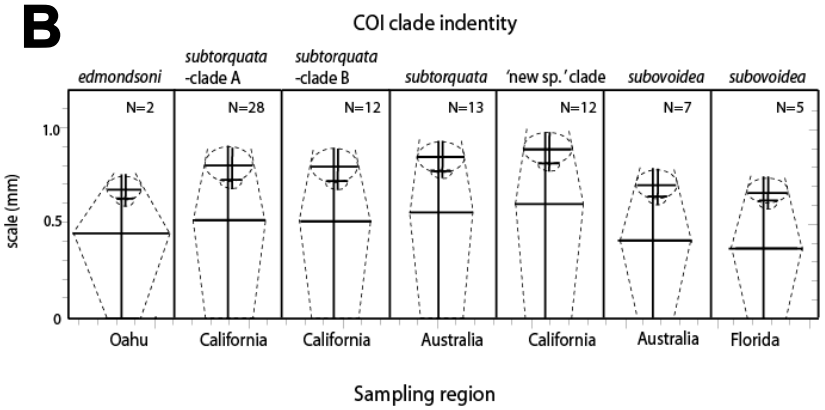


W. 'new species' COI clade
Bodega Bay



W. subovoidea
Florida (coll: Winston)

B



C

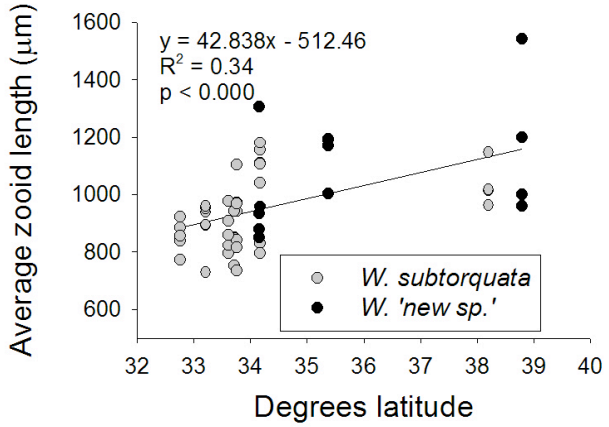


Figure 2A-C Descriptors of morphological variation in invasive sinusoid *Watersipora*. (**A)** Scanning electron micrographs of invasive *Watersipora* found on US coast lines. (**B)** Shape and size variation in sinusoid *Watersipora*, and corresponding COI gene clade (refers to Figure 1). Zooid motifs, are described by averages of five dimensions (indicated by dark lines) using measurements described in Ryland et al. (2009). The dark lines are means based on measurements made in multiple colonies (*N*, as shown). In each colony, a mean measurement was determined using ten zooids. (**C)** Variation in average zooid length of colonies of sinusoid *Watersipora* species of two divergent COI gene clades in California (Mackie, Darling and Geller in prep.).

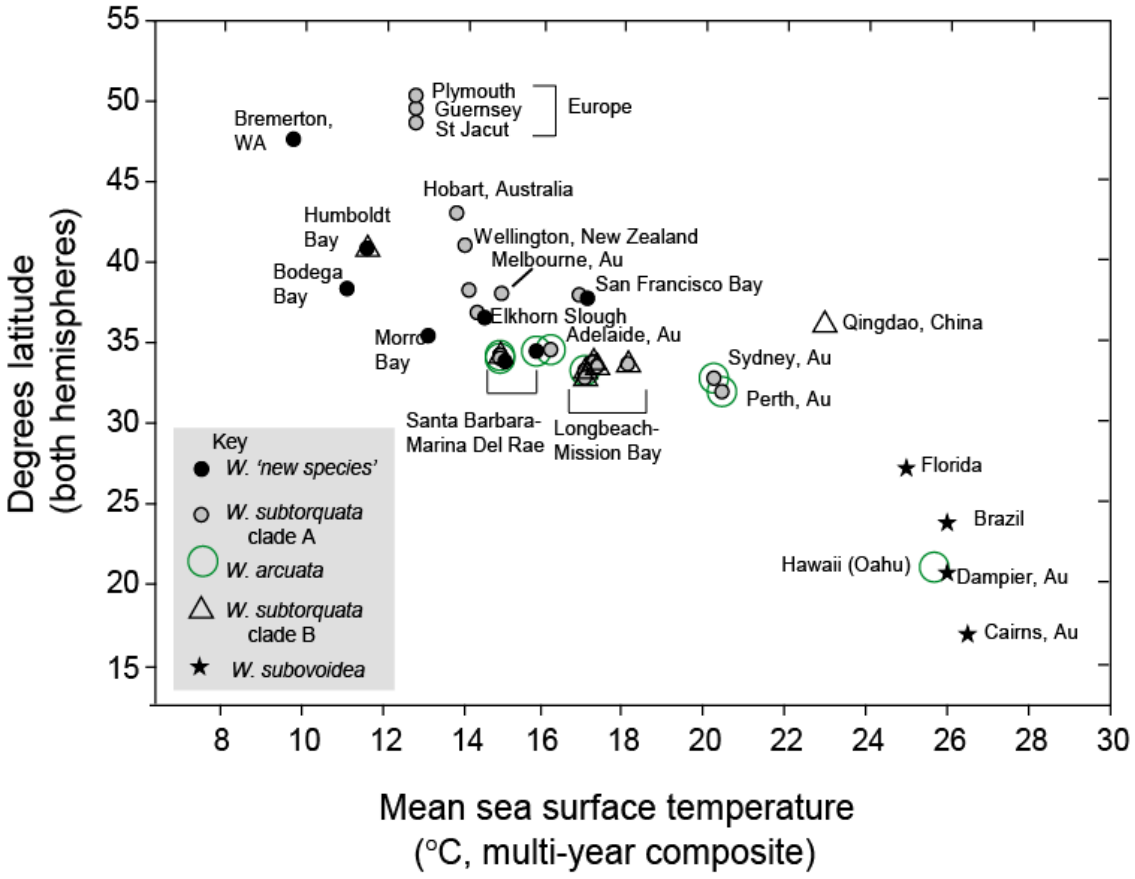


Figure 3 Distribution of widely invasive *Watersipora*-COI lineages in relation to latitude and average sea surface temperature (SST) of each collection region. Average SST is a regional rather than locale-level measurement. Temperature was determined to <1 ˚C approximately, by using monitoring data collected by local agencies, if available; or to within 1-2˚C, using NASA satellite time series measurements (Feldman & McClain 2010). Based on introduction records, all populations are non-native, with the possible exception of the Qingdao, China (Sun et al. 2009) and Florida populations, where further evidence of native versus non-native status is still needed. (Data from Mackie, Darling and Geller, in prep.)

It is possible that movement of fouling species on hulls, which are still commonly treated with copper-based paints, and historically copper (cuprous oxide) and or tin-based compounds including tributyltin (now largely discontinued), has affected genetic architecture of introduced fouling organisms. Metal-based marine paints exert an antifouling effect by killing or disabling larval settlers that enter a narrow zone of leached ions at the surface of the paint (Wisely 1962), or, as shown in some molluscs, by repelling larvae (Wisely 1963). Multiple invasive fouling species including *Watersipora* species have elevated copper tolerance, evidently in the larval and early growth phase, compared to similar native taxa in community level experiments (Mackie 2003, Piola & Johnston 2006, Dafforn et al. 2008). This suggests selective effects of the paints. Hypotheses addressing the evolution of antifouling paint tolerance, and whether it has occurred in the modern era, or is rather more accurately viewed as a pre-existing physiological response, have not been addressed. Currently genomic information is not available to determine the basis of physiological responses in bryozoans. An understanding of the genetic segregation of invasive lineages would provide a framework for

comparing tolerances of antifouling paint, and other potentially ecologically important responses, better allowing these to be tested as variables potentially determining population spread.

While heavy metal tolerance of a larva may not necessarily simply correlate with later colony performance, due to delayed ontogenetic impacts of exposure (Ng & Keough 2003), there is a basis to expect that is in part effectively adaptive-in allowing certain populations to colonize ship hulls, leading to widespread dispersal. A notable feature of a general *Watersipora* larval response to copper, is induction of settlement/ attachment of the larvae in the presence of low doses of copper (Wisely 1958, and Figure 4 below), which may enhance colonization of surfaces in the vicinity to antifouling paints. There have been few studies looking for the presence of intraspecific polymorphism in heavy metal tolerance, in fact only one that we know of, a study of *Bugula neritina*, showing differences in copper tolerance at a polluted and relatively non-polluted site by Piola & Johnston (2006). Among *Watersipora* populations, there are detectable differences in copper tolerance of larvae. We found in 2009 that larvae of a Humboldt Bay population have elevated copper tolerance compared to larvae collected at two other sites, Morro Bay and Moss Landing (Figure 4).

As with any trait, the variation in copper tolerance across a landscape is likely to reflect a wide range of factors, depending, on the genetic regulation and response to selection and drift, including whether there are associated fitness costs in certain environments (Mackie et al. 2010). Hypothetically, copper tolerance may be higher in areas in which the substrate includes painted vessel hulls, particularly if there are fitness costs in non-polluted environments. Alternatively, in the case of low-cost polymorphism, copper tolerance may reflect genetic divisions that have been spread over the landscape as a result of primary introductions, with little ecological patterning seen in comparing populations found in different habitats, for example kelp beds as opposed to marinas. The history of introductions, for example whether they occurred mostly on early wooden vessels, on shells of oysters, or on painted vessels hulls, like the number of generations occurring since introduction, could impinge on the distribution of copper tolerance variation; however, again, this has not been studied.

An aim of this proposal is to develop the use and interpretation-basis of a rapid, easily replicated LD-50 ('lethal dose' 50%) assay in comparing larval tolerance of dissolved copper. As shown successfully in studies revealing the selective pressure of ambient levels of polychlorinated biphenyls in populations of an estuarine fish (Nacci et al. 1999, Nacci et al. 2010), LD-50 values can provide a refined view of selection by correlation to environment. Ultimately we wish to compare LD-50 population values to phylogeographic variation assessed by multiple locus data in independent contrasts to determine if, where and when selection for increased copper tolerance in larvae of *Wateripora* has occured.

Understanding whether there is a link between genetically determined tolerance in *Watersipora*, a successful group of invaders, may be useful for regulating hull-cleaning practices, and planning for the use of a range of antifouling compounds, which include a increasing selection of non-metal based antifoulants. Tolerance possibly also interacts with other factors, such as placement of marinas and wharves in the environment in determining invasions.

reconnaissance

VI. General Aims

Focusing on populations in California, a coastline recently experiencing multiple *Watersipora* invasions, we have two major goals: (1) We will use microsatellite loci along with COI gene sequences to identify past/ present reproductive barriers in the complex of *Watersipora* species, both to better define the relevant units of natural selection (species), and to reconstruct the dynamics of introductions and spread of these species and genetic linkages across bays along the California coast. (2) Secondly, a major hypothesis that we wish to address through direct experiments is whether previous adaptation to temperature and/or copper paint strongly influences the overall pattern of

invasions within the *Watersipora* species complex. Using common environment experiments, we will compare developmental responses of different populations under different temperature regimes and copper concentrations (in antifouling paint), to assess whether there are fixed genetic characteristics in development that correlate with environmental temperature or copper conditions, and how this variation assorts relative to phylogeny.

VII. Basis for Collaboration

Both PIs involved in this project study marine invertebrate populations and have particular expertise in bryozoan species/genotype identification, using scanning electron microscopy and molecular-genetic techniques, including use of microsatellites, on adult and larval bryozoans. Craig (HSU) focuses on marine ecological questions and provides strength in techniques to grow and sub-clone bryozoans gained from NSF-NATO postdoctoral training at the University of North Wales (Bangor, UK) working in the laboratory of Dr. Roger N. Hughes (where the bryozoan *Celleporella hyalina* is raised in 2-liter soda bottles in enclosed cold rooms several miles from the sea). Craig will therefore direct both common-garden experiments, examining the role of adaptation to "cold" and "warm water" regimes, and copper tolerance experiments (in larval and adult *Watersipora*) at the Telonicher Marine Laboratory (TML) of Humboldt State University. Previous collaboration between Craig and Mackie (summer 2009) showed success in collecting and transporting these colonies from bays all along the California coast, after which they were maintained at TML to induce larval release, resulting in data which suggests differentiation among *Watersipora* populations in larval tolerance to copper (see Figure 4).

Mackie (SJSU) has a wealth of experience in bryozoan taxonomy and identification based on both morphological and molecular-genetic techniques, including COI gene sequencing (see Figure 1) and microsatellite markers. The latter, developed by Dr. John Darling (US-EPA) were recently tested with the help of an HSU REU-SITE student (Aki Larusen) shared with Craig in the summer of 2010. In addition to his experience in building molecular-genetic phylogenies, Mackie has postdoctoral experience gained in the laboratory of Dr. Jeffrey Levinton (SUNY-Stony Brook) which focused on rapid evolution of a freshwater oligochaete to heavy metals (Cadmium), and in with Dr Jonathan Geller (Moss Landing Marine Laboratories), focusing on *Watersipora*, and a separate project developing Quantitative realtime PCR and other methods for studying plankters. Mackie will therefore direct all work on further population genetic analysis of samples collected by both PI's in the first summer of this project (see timeline), and both PI's will involve CSU undergraduates in subsequent DNA extraction and PCR amplifications in year 1. Subsequent COI sequence analysis and microsatellite genotyping will be conducted in the Parr/Mackie Conservation Genetics Lab facility at SJSU, as well as the shared use facility run by Dr. Frank Cipriano (at San Francisco State University).

VIII. Analysis of invasive sinusoid Watersipora populations using microsatellites

A set of sixteen microsatellite markers has been isolated using genomic DNA from *Watersipora* by Dr John Darling at the U. S. EPA National Exposure Research Laboratory. SuperSNX linker-ligated fragments were enriched for microsatellites and cloned following procedures described in a similar study conducted previously by Darling (Schable et al. 2008). Genomic DNA used in microsatellite isolation and in extraction from ethanol-preserved *Watersipora* specimens for amplification trials was obtained using QIAGEN DNeasy ® kits, using the Tissue extraction protocol. In tests applying these markers to sinusoid (*subtorquata*/new sp. COI haplotype) *Watersipora* colonies collected from throughout California, twelve of the loci amplified successfully (success was defined as amplification in 70% or more of initial PCRs), and also demonstrated the presence of polymorphism (See Appendix

1 in supplemental materials, J. Darling unpubl. data). These twelve microsatellite loci provide a set of useful markers for in-depth population analyses.

Analyses will be carried out using both microsatellite markers and COI sequence haplotypes. Previous studies have amplified CO1 at a widely used 'barcoding' segment in invertebrate systematic studies, using primers LCO1490 and HCO2198 (Folmer et al. 1994) or nested bryozoan-specific primers, amplifying a segment internal to this region (Mackie et al. 2006). Previously, DNA has been extracted using Chelex ® 100 beads (Mackie et al. 2006) or the Qiagen ® kit (Geller et al. 2008). Ongoing work will be conducted using a CTAB DNA isolation protocol (Sambrook et al. 1989) which has been modified. This extraction is economical and yields high 'quality' DNA promoting good PCR-success. PCR and sequencing of COI will be conducted as reported previously. The microsatellite markers developed by Darling will be amplified using 55˚C-60˚C annealing steps in PCR, and other conventional PCR steps, using the QIAGEN Multiplex PCR Kit, which includes HotStarTaq DNA Polymerase, and proprietary additives (including Q-solution, which is beneficial in achieving consistent amplification, based on our initial PCRs).

Darling (unpublished data) used a touch-down protocol, carrying out amplicon PCR and binding of primers carrying a fluorophore (either 6-FAM, HEX, NED, PET, or VIC) to primers (Schuelke 2000). For ongoing work we will use standard flourescently labeled primers (supplied by Integrated DNA Technologies, Iowa). Prior-labeling more simply facilitates multiplexing of PCRs with similar optimized conditions. Products will be generated in the Conservation Genetics Laboratory in the Conservation Genetics Department at San Francisco State University, (a multi-user facility run by Dr Frank Cipriano: see supporting letter in supplemental documents). These products will be genotyped in reactions with up to four compatible (non-overlapping wavelength) fluorophores and sized against a ROX-400HD ladder on an ABI 3100 sequencer. Microsatellite profiles will be visualized using Genotyper ® software (Applied Biosystems).

Quality assurance of microsatellite scoring will include visual identification of loci showing stutter peaks resulting from unreliable PCR amplification, and the detection of loci with a high likelihood of allele 'drop out' by visual verification, both of which can effect downstream analysis based on deviation from Hardy−Weinberg Equilibrium or other applications such as Bayesian clustering (Dewoody et al. 2006). Micro-checker software (van Oosterhout et al. 2004) will be used to quantitatively check for scoring errors and potentially to adjust the allele and genotype frequencies based on the estimated null allele frequency, as necessary.

We aim to provide population genetic studies using a minimum of six microsatellite loci in individuals of a large 'sinsusoid' *Watersipora* collection from California. We will definitely include loci WS8, WS12, WS16, WS24 and WS30, as these microsatellites have already been used to successfully genotype *W. subtorquata* populations in the region of Sydney, Australia by Louise McKenzie (University of New South Wales, pers. comm. to Mackie). We will also determine whether the available markers can be used to genotype other species of *Watersipora,* namely *W. arcuata* and *W. subovoidea*, so that they might be included as outgroups to increase the scope for phylogenetic inference. Representative microsatellite sequence genotypes used in phylogenies will be submitted to GenBank for use by others.

IX. Population genetic goals

The microsatellites will be used to derive genotypes in order to understand the phylogenetic relationships of *Watersipora* spp. in California, and will also help to understand gene flow occurring via movement on ship hulls or other transport vectors.

We will sample at least four locales (20 colonies per locale) from within three regions of the Pacific Coast of the US, using sites selected based on COI phylogeny. We also have access to colonies that have been characterized previously using the COI gene which are stored in 85% ethanol

at SJSU. Fresh collections of colonies will also be made from locations in the Northern, Central and Southern regions of California to provide further insight into population genetic structure. Ideal collection locations, (with brief, ecologically relevant notes), are as follows (see table 1):

Table 1: Target sampling sites for microsatellite analyses of invasive 'sinusoid' *Watersipor*a in the NE Pacific:

(1) **Northern region**

- ●Bremeton, Harbor, Washington, site of recent population expansion (detected in 2009).
- ● Humboldt Bay, California ─ †Fields Landing Marina, Eureka, a large industrial dock
  ─ †Samoa Marina, Eureka, a large industrial dock
  ─ Eureka Marina, a marina used chiefly by small pleasure craft, (the marina was deployed in 2007)

(2) **Central California region**

- ● San Francisco Bay ─ †Richmond Marina, a large industrial dock
- ● Monterey Marina, Monterey Harbor, a population recently colonizing kelp beds in the vicinity of small boat slips
- ● Elkhorn Slough Harbor, a port used by fishing vessels and recreational vessels predominantly, lacking a history of inter-oceanic shipping, but with frequent boating traffic to San Francisco (Wasson et al. 2001)
- ● Bodega Bay
- ● Morro Bay ─ along with Bodega Bay, this harbor has shown only the presence of *W. '*new sp.' COI lineages, which provides an incentive to test for unique genotypic signatures

(3) **Southern California region**

- ● Long Beach Harbor, Los Angeles ─ part of an expansive, complex harbor system with a heavy volume of international shipping (1999 annual report, Port of Long Beach, cited in Lambert and Lambert 2003)
- ● Dana Point
- ● Mission Bay
- ● Oceanside ─ these three southern California localities, investigated previously, are relatively enclosed marinas (Lambert & Lambert 2003).

Three sites labeled † will include colonies sampled in 2002-2003 (L. McCann, Smithsonian). New collections will be made in these areas, and compared with earlier collections to assess whether genotypes have changed significantly over ten years.

The current study provides the ability to test for the occurrence of introgression in a bryozoan group. COI gene sequences in other bryozoans have served to validate allopatrically isolated species, highlighting the biological significance of fine-scale morphological variation (Mackie et al. 2002, Dick et al. 2003, Gomez et al. 2007). Particularly when it comes to measuring recent demographic processes, there is good reason to examine independent markers. Mitochondrial DNA, for example, can be misleading in inferring demographic processes because of the potential for genetic selection to produce sweeps that purge genetic variation (Barton & Etheridge 2004, Bazin et al. 2006).

We are proposing that introduction cannot be defined accurately as a process determined solely by the movement of the introduction vectors (e.g. ship's hulls), assessing these hypotheses by

genetic structural analyses. The major goal of microsatellite sampling is to determine whether genetic segregation occurs over a large scale in the sinusoid *Watersipora* species complex on the West Coast of the US, as suggested by mtDNA patterns.

Agreement or disagreement between mtDNA data (a haploid, uniparentally inherited marker) and diploid microsatellite allele variation provides a more comprehensive view of population processes than is available from any one type of marker (see for example, (Lu et al. 2001, Edwards et al. 2008, Zink 2008). We will assess whether population structure demonstrates reciprocally fixed differences, corresponding to the major mtDNA clade groups on the West Coast of the US. In each marker, we will determine whether there is a a clinal pattern, or regional 'blocking' of variation. We expect one of these spatial genetic patterns to be evident in both mtDNA and microsatellite variation if pre-existing genomic variation is correlated with the success of introduction in different areas.

Population genetic analyses

Examination of population diversity statistics in microsatellites will be conducted primarily using GenePop 4.0.10 Software (Rousset 2008). Population genetic structural measures (F_{ST} statistic comparisons among population pairs, and hierarchical Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) will be calculated using Arlequin 3.0 (Excoffier et al. 2005). We will use neighbor-joining based clustering to reconstruct relationships among populations via a dendrogram assessing branches for stability via bootstrapping (Felsenstein 1985). Phylogeographic dendrograms will be constructed using chord-distances (Cavalli-Sforza & Edwards 1967) or other distance measures, with appropriate assumptions for the data (Takezaki & Nei 1996). We will also use STRUCTURE 2.1 (Pritchard et al. 2000), which implements a Bayesian algorithm, to identify a number of populations (indicated by *k*, which is free to vary), that maximizes the probability of the data. Clustering processes will be used to find major genetic group divisions, and provide a basis for assessing mitochondrial marker and nuclear loci marker concordance. Within major genetic groupings, calculation of f-statistics, including linearized F_{ST}, a distance metric assuming stepwise mutation among alleles, developed for microsatellites (Slatkin 1995) ,will be used to estimate gene flow by conversion to *nM*.

The proposed sampling of colonies used to generate the core of the combined-marker analyses (shown above) should be appropriate for hierarchical assessment of population structure. The AMOVA framework provides a starting point for assessing genetic continuity across habitat types, where habitats reflect levels of shipping activity. Namely, we will test whether discrete, local introductions are likely to have resulted in the founding of separate introductions along the coastline, versus evidence of strong coast-wide dispersal of haplotypes.

A key hypothesis is that ancestral lineages assort within the invaded range based on pre-existing temperature-correlated fitnesses, as opposed to differences selected after introduction. We also propose that copper-tolerance, a factor which is elevated in fouling *Watersipora,* may play an historic role in determine patterns of introduction. Broad scale sampling (as described above) will thus be the focus of investigation in year one of this proposal. Locales that represent discrete sets of genotypes (defined by mtDNA and microsatellite genotypes) will then be used to sample colonies to test the validity of genetic adaptation to temperature or elevation of copper tolerance in some genetically defined propagules.

X. Is pre-existing (genotype specific) temperature tolerance important to coast wide invasions?

In many organisms, life history traits evolve predictably in response to the normal temperatures occurring during growth and reproductive phases. Accordingly, optimal growth rates of populations

experiencing different temperatures through ancestry may differ predictably in different temperature conditions (Vasi et al. 1994). Evidence for or against the influence of temperature on predicted growth efficiencies of different populations throughout California will be assessed using a common environment experiment. *Watersipora* colonies will be collected from different sites (and different biogeographic regimes with different SST's) and will be grown in the laboratory under controlled conditions (at Humboldt State University's Telonicher Marine lab).

Colonies from different places along the Californian coast will be collected live from the field for common-environment experiments. There are three putative collection areas, chosen to represent a wide range of sinusoid *Watersipora* genetic variation. Colonies will be chosen after the first year of research, during which populations will be genotyped at microsatellite loci to further determine which species (and genotypes) are restricted to "cold water" or "warm water" regimes. These colonies will be collected from the following 3 regions:

1) Southern California (Oceanside-near San Diego)
2) Central California (Elkhorn Slough, Moss Landing; and San Francisco Harbor)
3) Northern California (Humboldt Bay)

Colonies will be collected from at least two sites in each of these three regions. Individual colonies will be held in closed-circulating aquaria (to prevent further spread of invasive species) in the newly renovated wet-lab at the Telonicher Marine Laboratory (TML) in Trinidad, CA. Colonies will be maintained by feeding them a mixture of phytoplankton (*Tetraselmis* sp., *Isochrysis* sp., and *Rhinomonas reticulata*). Individual colonies will be mitotyped using COI sequences and genotyped at several microsatellite loci following several months of growth in the lab. At least four colonies from each site will be collected, brought back to the Telonicher Marine lab (HSU), and induced to release their larvae using standard techniques (light induction). We have already successfully used the light-induction technique to induce larval release at the Telonicher Marine Lab to examine larval resistance to copper in colonies collected from Morro Bay, Moss Landing and Humboldt Bay, and hence we are confident this technique will work again for this experiment. Larvae will be allowed to settle on thin acetate sheets placed in holding tanks, and grown to a large enough size to facilitate cutting and sub-cloning each colony into three or four clonal replicates. When colonies have obtained a size of between 4 and 8 zooids, these acetate sheets will be cut into separate colonies, removing different groups of colonies to allow them to be placed in common experimental tanks at either a low temperature (LT) 12˚C or a high temperature (HT) 21˚C. We will also grow colonies that are not clonally replicated to control for the possible effects of cutting and fragmenting colonies into multiple pieces.

In our main experiment, colonies (separated into ramets) will be grown at 12˚C, a typical "cold water" open coast sea surface temperature for August in Humboldt Bay, the northern extent of sampling, and 21˚C, a typical "warm water" August SST of southern populations such as those near San Diego bay.

Over the course of three weeks, we will compare several growth rate measures across genotypes (and within genets) within and across "warm" and "cold" temperature regimes. Measures of growth recorded will include the average number of zooids added weekly, the rate of change of total colony area, the average zooid length and width, the frontal surface area of individual modules/zooids, and the number of lophophores extended out to feed.

We will also monitor the number of larvae produced at different temperatures and their relationship with population source and genotype. Finally, the average larval volume (a sensitive indicator of the effects of temperature on development in larval *C. hyalina*, Atkinson et al. 2006) will be measured in digital images.

Data will be analyzed by repeated-measures MANCOVA, allowing us to test for the influence of (1) Region (3 regions: Northern vs Central vs Southern), (2) Source population (including sites such as Oceanside, Moss Landing, Elkhorn Slough, San Francisco Bay and Humboldt Bay), and (2) applicable genetic lineages (as defined by COI mitotype and microsatellite genetic signatures), on growth-related and other performance measures.

XI. Does larval tolerance to copper antifouling paints explain the spread of genotypes and/or species along the California coast?

The spread of species and/or genotypes of Watersipora spp. (complex), however, could alternatively (or additionally) be explained by the tolerance of larvae to different levels of copper (in antifouling paints on ship hulls), emphasizing the "hitch-hiking" nature of their dispersal into new bays and harbors around the world as the primary means by which these species have become such a globally successful invaders. To test this hypothesis, we will develop lab experiments at the Telonicher Marine Lab (TML) at Humboldt State University to test larvae (released within different colonies held in separate aquaria at ambient seawater temperatures representative of their source waters). These experiments will be structured in the same manner as those described above (in section X) for temperature adaptation: Larvae will be released from colonies collected from 3 regions (Northern vs Central vs Southern), each of which will contain colonies collected from at least 2 different sites (including Oceanside, Moss Landing, Elkhorn Slough, San Francisco Bay and Humboldt Bay) and these larvae will be genotyped (using COI sequences and microsatellite markers) to determine genetic identity.

From pilot data derived from experiments conducted in the summer of 2009 with HSU-REU students, we infer a range of copper concentrations (a dosage window of 0-20 uM of Cu^{2+}) as needed in 4-hr LC50 type trials to assess tolerance of larvae to dissolved copper in dishes of artificial seawater and dissolved copper. Both larval survival and larval settlement over a 4 hour period, will be assessed in replicate dishes (N=2-5) of 12 larvae released from each colony maintained in the laboratory. Induction of settlement in previous larval trials occurred above a threshold (c. 5 uM) of dissolved copper. This settlement occurred despite the fact that settlement dishes were vibrated through a common platform by a vortex (as suggested by (Wendt 1996)). Settled larvae also appear to have a higher survival function than larvae that remain swimming (Craig and Mackie, unpubl. data). As a result of larval settlement, which effectively 'censors' some individuals in any experiment, lowering their mortality risk, LD-50 measurements of copper-dosed *Watersipora* may not be simple, yielding for example, a typical s-shaped dose response curve. Nevertheless, broad differences in survival were evident among the three assayed populations using this method (see figure 4), and the approach warrants refinement and quantitative analysis. The tolerance differences (Humboldt Bay population larval tolerance being greater than the other two populations) were seen in separate, slightly different, experimental trials using larvae spawned from colonies on different occasions (Mackie and Craig, unpubl. data).

In addition, we plan to add a second experiment to examine the long-term effects of copper on larvae in the field, done by settling larvae released in the lab (from colonies collected from the 3 regions of the California coast as described above) onto ABS plastic panels coated with antifouling paint with 4 different levels of copper (Pettit® paints with 70%, 65%, 55% and 25% copper load). Ten (10) replicates of each panel (with 10 un-painted panels serving as controls), with at least 15 larvae settled on each panel in the laboratory (at TML) will be outplanted into the filed in Trinidad

Harbor, a site with very little shipping activity and few moored vessels. Results from this experiment will help to determine the relative ability of different *Watersipora* spp. (and genotypes) to settle on hulls with these commonly used antifouling paints in relatively pristine (low ambient copper concentrations in the water column, to be verified by testing) field conditions. These experiments will be conducted in year 2 of this project, and repeated in year 3 in a more "typical" harbor site (Humboldt Bay) where vessels of various sizes (painted with various antifouling paints) are more common.

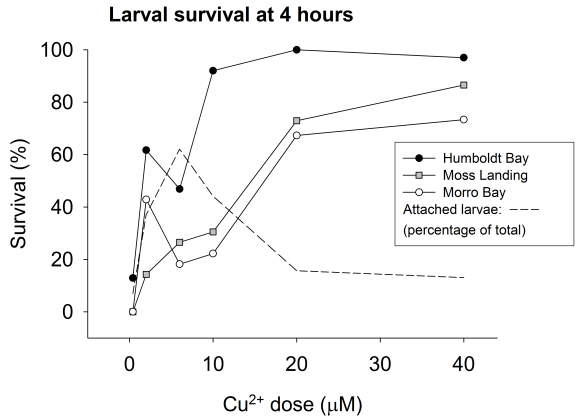


Figure 4 Comparison of survival rates of larvae from colonies of sinusoid *Watersipora* collected from three locales (Morro Bay, Moss Landing and Humboldt Bay) in dissolved Cu^{2+}. Colonies were collected in July 2009, and held in flow-through seawater for 14 d prior to the induction of larval spawning and exposure of the larvae to copper ions (as $CuSO_4$) in reconstituted seawater. Each exposure level was examined using two replicate dishes, each consisting of 15 exposed larvae (results averaged across dishes). Survivors included swimming larvae (showing actively beating cilia) and some attached individuals that were in pre-metamorphic stage; (the latter are indicated by the broken line, representing the total proportion attached from all populations). As shown, intermediate copper doses induced larval settlement/attachment.

XII. Expected Milestones:

Year 1, 2011 **June−Aug:** Travel and collect samples along entire California coast, from San Diego to Humboldt Bay for genetic analysis. Beginning in September, 2011 samples will be analyzed using COI & Microsatellite markers in the SJSU Conservation Genetics Lab.
Sept-Dec: Continue genotyping and genetic analysis

Year 2, 2012 **Jan-May:** Continue genotyping and genetic analysis
June-July: Collection of colonies along CA, establishment of cultures at HSU.
July−Aug: Commencement of experiments using cultured bryozoan stocks.
Sept-Oct: Finish common garden experiments at Telonicher Marine Lab (TML)
Dec: Completion of a manuscript describing microsatellite variation.

Year 3, 2013 **Jan−Aug:** Completion of lab-based experiments at (TML).
Aug-Oct: Completion of manuscript describing common-garden seawater temperature experiments, start manuscript describing copper antifouling paint experiments.