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Impacts of changing water temperatures on the life histories of two invasive ascidians in the Gulf of Maine: Botryllus schlosseri and Botrylloides violaceus

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IMPACTS OF CHANGING WATER TEMPERATURES ON

THE LIFE HISTORIES OF TWO INVASIVE ASCIDIANS IN

THE GULF OF MAINE: *BOTRYLLUS SCHLOSSERI* AND

BOTRYLLOIDES VIOLACEUS

BY

ERICA WESTERMAN
BS Yale University, 2003

THESIS

Submitted to the University of New Hampshire
In Partial Fulfillment of
the Requirements for the Degree of

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in
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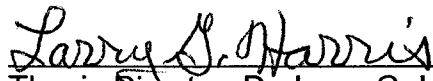
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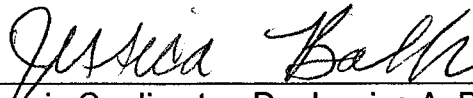
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_4/26/2007_
Date

DEDICATION

To my grandparents, Dr. Kenneth Westerman, Carol Westerman,

Merrill Whitcomb, and Marian Stearns, and my constant companions,

Samwise and Daisy Mae.

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ACKNOWLEDGEMENTS

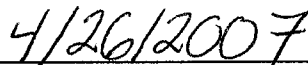Any research project that encompasses field sites in three states and daily laboratory work can only be conducted with a great deal of assistance. First and foremost I would like to thank my advisors, Dr. Larry Harris and Dr. Jessica Bolker, and my committee member Dr. Michael Lesser for their insight, advice, and direction over the last two and a half years. Dr. Phillip Yund also offered invaluable assistance in the how to of culturing *Botryllus schlosseri* and *Botrylloides violaceus*.

My brother, Michael Westerman, both came field sampling and offered me the hospitality of his home for numerous early morning sampling trips. Other members of the Harris laboratory, Jennifer Dijkstra, Sarah Teck, and Linda Auker, came sampling, helped with culturing, and engaged in hours of thoughtful insight and enlightenment. My roommate, Rebecca Story, not only helped my mental stability throughout the last two years, but also went field sampling and helped with late night zooid counts. Steven Fay, Morgan Eastman, Hillary Sherman, Andrew Collins, Cameron Brooks, Liam Kean, Kaitin Bonner, Travis Godkin, and Jason Goldstein all assisted in the culturing of my tunicates. And lastly, I'd like to thank my parents, for their undying support and encouragement, even when that meant sampling off a dock in the middle of winter.

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ABSTRACT

IMPACTS OF CHANGING WATER TEMPERATURES ON THE LIFE HISTORIES OF TWO INVASIVE ASCIDIANS IN THE GULF OF MAINE: *BOTRYLLUS SCHLOSSERI* AND *BOTRYLLOIDES VIOALCEUS*

by

Erica Westerman
University of New Hampshire, May, 2007

The impact of temperature on organism life cycles has received great attention due to heightened awareness of global warming. Relative growth and reproductive development were monitored in two dominant ascidians from the Gulf of Maine to compare effects of rising temperatures on established (*Botryllus schlosseri*) versus recently arrived (*Botrylloides violaceus*) species. Settlement panels were deployed at three sites with different temperature regimes (Damariscotta, ME, Newcastle, NH, and Salem, MA) during two growing seasons June 2005 to December 2006, and a recruitment study conducted May to December 2006. Both species had elongated breeding seasons in Salem, MA relative to the other sites. *Botryllus schlosseri* settled two weeks earlier than *Botrylloides violaceus* at all sites, however this delay in settlement can be attributed to the longer brooding period of *Botrylloides violaceus*. Timing of initial settlement may not be as indicative of temperature tolerances as timing of initial egg production in brooding animals.

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CHAPTER I

Introduction

<u>Climate Change and Ascidians</u>

Average global temperatures have risen 0.6°C over the past century
(Walther et al. 2002). During this time there has also been an increase in
species movement to new habitats, primarily assisted by humans (Carlton 1996,
Stachowitcz et al. 2002). These nonindigenous species have altered their new
ecosystems through competition for space, food, and other resources (Carlton
1996). As global temperatures continue to change, it is imperative to know how
shifting environmental conditions affect species diversity in general and
invasibility of communities in particular.

One way to study the impact global temperature change has on
communities is through examining the impact of temperature change on life
histories of individual species. Temperature effects on life cycle traits such as
increased growth, earlier maturation, higher resource use efficiency, and earlier
reproduction at higher temperatures are well documented in many species

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(Brunetti et al. 1980, Grosberg 1982, Fielder et al. 2000, Stachowitcz et al. 2002, Newlon et al. 2003, Rodriguez & Bustamente 2003, Durant et al. 2004). These positive, or negative, effects of temperature on life history traits are of particular interest when looking at interactions between invasive and local species in the context of ongoing climate change. A higher tolerance for seasonal temperature variability, coupled with a lack of predators, may increase the viability of an invasive species and enable it to out compete native species and disrupt the local ecosystem.

Ascidians appear to be a particularly successful group of invaders in a variety of marine environments (Berman et al. 1992, Stachowitcz et al. 2002, Lambert & Lambert 2003). Colonial ascidians are sessile, active suspension feeders that reproduce both sexually and asexually; they have grown in dominance on near shore sea bottom communities worldwide over the past forty years (Lambert & Lambert 2003). Part of this invasive success may be due to rapid growth and reproductive rates as well as highly flexible colony growth patterns. Colonies can grow over flat surfaces, around corners, over solitary tunicates, and around mussels. When space is limited they are also able to form long lobes and grow on themselves (Brunetti 1974, Sabbadin 1955). These flexible growth patterns and high growth rates have caused problems for shell fisheries, as ascidians are able to cover vast areas of the sea floor, over grow mussel lines, and produce biochemicals that make settlement and growth difficult for economically valuable mussels (Bryan et al. 2003, Bullard et al. 2007).

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Ascidian invasive success may also be driven by the fishing down of coastal ecosystems and the reduction of top predators such as Atlantic cod (Jackson et al. 2001). Simple communities with little diversity appear to be more susceptible to invasion than communities with high biodiversity (Case 1990, Dunne et al. 2002). As the Gulf of Maine has particularly low species diversity relative to other temperate coastal ecosystems, it may be more susceptible to invasion by highly competitive taxa such as ascidians.

The cause of increasing ascidian dominance is unknown and most likely multifactorial, however environmental change is one likely contributor. Previous studies have shown that colonial ascidian asexual growth and settlement are affected by temperature and salinity (Yamaguchi 1975, West & Lambert 1976, Brunetti et al. 1980, Grosberg 1988, Newlon et al. 2003). However, temperature related reaction norms for the reproduction of dominant colonial ascidians in the Gulf of Maine have not been studied. The increase in water temperatures over the last thirty years (Conversi et al. 2001, Greene et al. 2003, Dijkstra et al. 2007), combined with the increase in colonial ascidian populations (Stachowicz et al. 2002, Bullard et al. 2007) make the Gulf of Maine an ideal area for examining temperature related changes in life cycle traits. To clarify the relationship between colonial invasive ascidian success and temperature, I studied the effects of temperature on reproduction in two species of colonial ascidians in the Gulf of Maine.

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Study Organisms

This study focuses on the colonial ascidians *Botryllus schlosseri* (Pallas 1766) and *Botrylloides violaceus* (Oka 1927). *Botryllus schlosseri*, the star tunicate, has been a member of the Gulf of Maine subtidal community since the mid 1800s (Gould 1870). Transported to New England from the North Sea, this species was the historically dominant colonial tunicate in the Gulf of Maine until the late 1980s (Harris & Tyrrell 2001). *Botrylloides violaceus*, the orange sheath tunicate, is native to the west Pacific and arrived in the Gulf of Maine in the early 1980's, most likely from Japanese oysters brought into the Damariscotta River (Dijkstra et al. 2007). Although *Botrylloides violaceus* is considered a warm water species and *Botryllus schlosseri* is considered a cold-water species(Dijkstra et al. 2007), they currently share the same range in the Gulf of Maine, from Cape Cod MA to Eastport ME (Dijkstra et al. 2007).

Both *Botryllus schlosseri* and *Botrylloides violaceus* belong to the subfamily Botryllidae (Phylum Chordata, Subphylum Tunicata, Class Ascidiacea, Order Stolidobranchia, Family Styelidae). They are cyclical hermaphrodites, have internal fertilization, and brood their young. Colonies of both species are made up of genetically identical individuals, known as zooids, whose circulatory systems are connected to each other through blood vessels between the zooids and structures called ampullae. Ampullae are round, protruding structures at the ends of all interstitial blood vessels often found along the edges of the colony under the tunic; they may also be more centrally located among zooids in older

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colonies. Botryllidae is one of the few ascidian families containing animals with interconnecting blood vessels between zooids and ampullae around the edges of the colonies. These ampullae contain muscle tissue and contract on a regular basis to assist in pumping blood throughout the colony (Newberry 1965, Burighel & Brunetti 1971). Ampullae also help the colony adhere to surfaces, and assist in self/non-self recognition (Hirose et al. 1990, Rinkevich 2002, Litman 2006).

All ascidians are hermaphroditic, and each zooid within a colony is capable of producing both ovaries and testes. Ovaries in *Botryllus schlosseri* are located on the left side of the body above the stomach, while testes are located on both sides of the body: above the stomach on the left and slightly lower on the right (Berrill 1950). Gonads are located in a slightly different location in *Botrylloides violaceus*. A single ovary and testis are on each side at the base of the zooid in the bottom of the colony with testes located above the ovaries (Mukai 1977). Ovaries appear pink and testes white when observed through the tunic under a microscope.

Reproduction

All colonial ascidians undergo two different types of reproduction: asexual reproduction, through a budding process known as blastogenesis, and sexual reproduction through cross-fertilization and egg brooding. Asexual reproduction (often called colony growth) in the Botryllidae can occur through two different

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methods: palleal budding or vascular budding (Rinkevich 2002). In palleal budding, the most common method, a bud is formed out of the side of an existing zooid or bud. Zooids can have zero to four buds, and all buds in a colony reach adulthood at the same time (Sabbadin 1955). The replacement of old adult zooids by fully developed buds is called takeover, while the life span of one generation of adult zooids, from takeover to takeover, is referred to as a blastogenic cycle.

Three different generations of zooids at different stages of development are visible within a colony: adult zooids, which are heavily pigmented, large, and share an atrial siphon with two to fifteen other zooids; primary buds, which are buds off of the adult zooids and are generally positioned high on the left of the zooid and low on the right side of the zooid (a single primary bud is located on the left side); and secondary buds, which are small, mostly clear and relatively undefined buds attached to the left and right sides of the primary buds (Berrill 1941). Takeover occurs when the primary buds are mature and the adult zooids go through apoptosis and degenerate. Immediately following takeover, colonies comprise three generations of zooids: senescing zooids, adult zooids, and primary buds. Secondary buds form on the primary buds within a day (Berrill 1941).

The second form of asexual reproduction, vascular budding, occurs when a bud is formed from the wall of a blood vessel within the colony but is not attached to an adult zooid. This may occur when a piece of tissue without any zooids is removed from a colony and allowed to settle and grow on its own

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(Rinkevich 2002), or when a colony comes in contact with another colony (personal observation); however, vascular budding has rarely been observed in nature (Rinkevich 2002).

Sexual reproduction in a colonial ascidian occurs after the colony has gone through a series of blastogenic cycles and become sexually mature. *Botryllus schlosseri* colonies become mature after five to seven blastogenic cycles; it is unknown how many asexual cycles are required for *Botrylloides violaceus* colonies to become sexually mature (Brunetti 1974, Grosberg 1988). Once a colony reaches sexual maturity, each zooid within the colony is capable of producing eggs and sperm (Sabbadin 1955, Brunetti 1974). In *Botryllus schlosseri* colonies, initial testes production occurs at least one blastogenic cycle before initial egg production. Once eggs are being produced, however, both testes and eggs are produced in the same generation, though at different times during the life cycle of the zooid. This is one of the many ways that colonial ascidians prevent self fertilization (Milkman 1967).

Eggs are fertilized internally, then deposited in a brood pouch where incubation begins (Berrill 1950). During the brooding period in *Botrylloides violaceus*, which can last at least four weeks (Mukai et al. 1987), the adult zooid, which originally produced the egg, degenerates and is absorbed into the colony during takeover (Stewart-Savage et al. 2001a). Therefore, developing larvae are supported by a minimum of two different generations of zooids within the colony, only one of which produced the original oocytes. In *Botryllus schlosseri*, the brooding period is the duration of one blastogenic cycle, which is considerably

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shorter than that of *Botrylloides violaceus*, and larvae are released into the water column immediately before takeover is completed (Yund et al. 1997). However, if an egg is fertilized late in the life span of the adult zooid, the developing embryo will not develop in time, will not be released before takeover occurs, and will be reabsorbed back into the colony (Yund et al. 1997, Stewart-Savage et al. 2001a).

Botryllus schlosseri eggs are stored in a common brood pouch near the shared atrial opening between each system of zooids (Berrill 1950). Though housed within the tunic, embryos do not receive any nutrients from surrounding zooids during development. In contrast, *Botrylloides violaceus* embryos are stored in individual brood pouches near the shared atrial opening between each system of zooids, and these brood pouches have blood vessels running through them, bringing nutrients from the entire colony to the developing embryos (Millar 1971, Mukai et al. 1987).

Once released from the colony, larvae from both species normally spend less than 24 hours in the water column before settling on a hard substrate and metamorphosing (Sabbadin 1955, Millar 1971). Larvae can either attach to primary substrates such as rocks, wood, and cement piling, or to secondary substrates such as algae, scallop shells, and other tunicates (Berrill 1950). After settlement, larvae go through metamorphosis, absorbing their tail and opening atrial and oral siphons to become the original zooid of a new colony, known as the oozooid (Sabbadin 1955). This oozooid is identical in every way to future zooids in the colony (known as blastozooids) produced during the ensuing

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asexual budding, except that oozooid organs are formed from different tissues than those of blastozooids, and oozooids are not capable of becoming sexually reproductive (Brunetti & Burighel 1969).

Since each zooid within a mature colony can produce eggs and sperm, one can calculate the fecundity of a colony based on two measures: 1) number of eggs per zooid and 2) average size of the testes (testes size correlates directly with sperm count) (Yund 1998). Sexually mature colonies of both *Botryllus schlosseri* and *Botrylloides violaceus* continue to undergo periods of asexual reproduction while producing viable eggs and sperm, though at a diminished rate.

Past Studies on Abiotic Factors

Botryllus schlosseri has been a model system for the study of colonial ascidians for over fifty years, and a model system for the study of allorecognition for the last twenty years (Sabbadin 1955, Brunetti 1974, Brunetti et al. 1980, Grosberg 1988, Chadwick-Furman & Weissman 1995, Cohen et al. 1998). These studies have included in-depth descriptions of this species and how its life history is impacted by changes in temperature in both Venice, Italy and Massachusetts, USA. (Brunetti et al. 1980) found that *Botryllus schlosseri* colonies from Venice would grow in the laboratory at 10°C but not at 3°C, and that all colonies died by day 11 when cultured at 3°C in the laboratory. Brunetti

et al. (1980) also found that *Botryllus schlosseri* colonies from Venice would not reproduce when cultured in the laboratory at temperatures below 13°C. Grosberg (1982) performed an extensive study on the life history of *Botryllus schlosseri* in Eel Pond on Cape Cod. He found that the duration of blastogenic cycles correlated inversely with increases in temperature, from 35 days at 5°C to 7 days at 20°C. These growth rates are very similar to those of colonies from the Venetian Lagoon (Mukai 1977, Brunetti et al. 1980), suggesting that the duration of blastogenic cycle may be fixed relative to temperature. However, as the annual temperature regimes of Eel Pond and the Venetian Lagoon are very similar (Brunetti et al. 1980, Grosberg 1982) it is likely that *Botryllus schlosseri* populations of these two sites are also adapted to similar temperature ranges. Thus, the temperature tolerance similarities between these two populations may not be indicative of all *Botryllus schlosseri* populations, particularly those that may have adapted to different environmental conditions.

A transplant study comparing reproduction in *Botryllus schlosseri* colonies from Eel Pond, MA, with those from the Damariscotta River, ME found that colonies from Eel Pond had lower egg production than those of Damariscotta when grown at the Darling Marine Center (Stewart-Savage et al. 2001b). Stewart-Savage et al. (2001b) also found that genetically identical *Botryllus schlosseri* colonies grown upriver had significantly lower reproductive output than clones grown downriver in the Damariscotta River, ME. These data demonstrate the importance of treating each population as a unique entity and examining its life history traits in its new habitat before assuming it will behave as it has in other

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environmental conditions. Such considerations are especially critical in areas like the Gulf of Maine, where water temperatures are changing (Conversi et al. 2001, Greene et al. 2003) and an influx of invasive species has altered benthic communities during the last thirty years (Berman et al. 1992, Harris & Tyrrell 2001).

Botrylloides leachi, a sister species of *Botrylloides violaceus* found throughout Europe, has been extensively studied in Italy (Brunetti 1976 1967, Brunetti et al. 1980). Brunetti (1976) found that the breeding season of *Botrylloides leachi* in the Venetian Lagoon began when water temperatures reached 17-18°C and ended when they rose to 24-25°C. He also found evidence of a second reproductive period in early fall that occurred when water temperatures fell from 25°C to 16-17°C. *Botrylloides leachi* colonies would not grow at 11°C, but would grow at 16°C (Brunetti et al. 1980). *Botrylloides leachi* was found to hibernate through the winter months. Hibernation in *Botryllid* ascidians is when a colony has many ampullae but few to no functional zooids. The only evidence that the colony was still alive was blood flowing through common blood vessels throughout the colony. *Botrylloides leachi* entered hibernation when water temperatures went below 10°C (Brunetti 1976).

Botrylloides violaceus is less commonly used in laboratory studies than *Botryllus schlosseri*, but its recent dominance of the Gulf of Maine benthic community has resulted in a heightened interest in its environmental limitations. Stachowicz et al. (2002) found that colonies of *Botrylloides violaceus* from Woods Hole, MA increased number of zooids in response to temperature at a

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higher rate than *Botryllus schlosseri* during a one-week study. Their one week

study used water temperatures between 15 and 24°C, and recorded increase in

number of zooids instead of duration of blastogenic cycle or increase in colony

area. A second study by McCarthy et al. (2007) that examined increase in

colony area over one week found that neither *Botryllus schlosseri* nor

Botrylloides violaceus colonies from Groton, CT responded significantly to colder

or warmer water temperatures relative to ambient water temperatures. The

contrasting results from these two experiments and the brief duration of both

suggest that the relative response of New England colonies of *Botryllus*

schlosseri and *Botrylloides violaceus* to temperature merits further study.

These studies illustrate that the physical tolerances of different

Botrylloides populations differ from each other and also differ greatly from those

of *Botryllus schlosseri*. A particularly interesting and often overlooked study by

Mukai (1977) found that *Botrylloides violaceus* colonies collected and cultured in

Shizuoka, Japan had a blastogenic cycle that was eight days long at 12-13°C

while *Botryllus schlosseri* colonies collected and cultured in Venice, Italy had a

blastogenic cycle of seven days at 17°C. *Botrylloides violaceus* would also

reproduce at 13°C, the lowest recorded temperature for *Botryllus schlosseri*

reproduction. This suggests that while *Botrylloides violaceus* found in New

England waters may be considered a warm water species, it may be more cold

tolerant than *Botryllus schlosseri* in its native environment.

The apparent warm water preference of *Botrylloides violaceus* in Atlantic

coastal waters coupled with the increase in its abundance coinciding with

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increased water temperatures in the Gulf of Maine suggests that temperature may be one factor driving the success of this invasive species. It is therefore important to examine whether increased temperatures result in heightened growth and reproduction for *Botrylloides violaceus* populations relative to those of *Botryllus schlosseri* in the Gulf of Maine. As water temperatures throughout the Gulf of Maine are highly variable and dispersal distances are short, populations from different areas may not only differ from those of other species or from populations from other major geographical regions, but from other areas within the Gulf of Maine as well. Therefore, studying different populations within the Gulf of Maine is vital to increasing our understanding of the interactions between the life histories of these two species and changing environmental conditions such as temperature.

Objectives

The objectives of this study are to 1) establish temperature minima for gametogenesis in *Botryllus schlosseri* and *Botrylloides violaceus* in the Gulf of Maine, 2) define the reproductive seasons of *Botryllus schlosseri* and *Botrylloides violaceus* under different temperature regimes in the Gulf of Maine, 3) establish the impact of increased water temperature on asexual reproduction of *Botryllus schlosseri* and *Botrylloides violaceus*, and 4) determine temperature impacts on observed abundance patterns of *Botryllus schlosseri* and *Botrylloides violaceus*.

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I hypothesize that *Botrylloides violaceus* will tolerate a larger range of temperatures than *Botryllus schlosseri* as well as populations that acclimatize more readily to new temperature regimes because it is a more recent invader and has thus had fewer generations than *Botryllus schlosseri* to adapt to conditions in the Gulf of Maine. To test this hypothesis, I will look at how temperature affects a) gametogenesis, and b) duration of the reproductive cycle. In addition, I hypothesize that ascidian populations from different temperature regimes will have different temperature minima for sexual reproduction, correlated with their natural local climate. *Botryllus schlosseri* has been found throughout the Gulf of Maine for a longer period of time than *Botrylloides violaceus* (Stachowicz 2002, Berman et al. 1992). Therefore, I expect *Botryllus schlosseri* to exhibit greater variability between populations and less plasticity within populations in response to temperature than *Botrylloides violaceus*.

Determining time of ascidian gamete production and temperature minima for gametogenesis in different areas within the Gulf of Maine will clarify the temperature range at which sexual reproduction can occur in these ascidian species. Establishing the effect temperature has on the duration of the reproductive cycle for different populations of different species will enable me to compare the impact of temperature on reproductive output in these species, as well as whether there is a change in response to temperature based on the colony's climate of origin.

CHAPTER II

Materials and Methods

Field Sites

When looking at the impact of different local climates on the life history of a species, it is important to observe the behaviors of different populations exposed to differing environments in the field. Therefore three different study regions throughout the Gulf of Maine were used for field observations and experiments. .

The three study regions within the Gulf of Maine were 1) the Damariscotta Estuary, Maine; 2) Portsmouth Harbor, New Hampshire; and 3) Salem Harbor, Massachusetts (Figure 1). These three regions of the Gulf of Maine all differ in yearly temperature and average range (Figure 2). All sites contain high densities of *Botryllus schlosseri* and *Botrylloides violaceus* (Bullard et al. 2007).

The Damariscotta River (43° 56.0' N, 69° 34.8' W) is the northernmost site. The site has a yearly water temperature range of 0.73°C to 20.51°C with an average of 9.89°C. Panels were attached to a floating dock at the University of

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Maine Darling Marine Center (DMC), which is located in a protected area of the estuary, with a more constant water temperature than the other two sites.

Portsmouth Harbor (43° 04'18" N, 70° 42'37" W) is the middle site, with a yearly water temperature range of -0.61°C to 20.138°C, and an average of 10.66°C. Panels were attached to a floating dock at the University of New Hampshire Coastal Marine Laboratory (CML) in New Castle NH. The large fluctuations in water temperature at this site are due to tides and its proximity to the Piscataqua estuary.

The southern site, Salem Harbor (42° 31'17" N,70° 52'55" W), experiences a yearly water temperature range of 1.9°C to 24.15°C with an average of 12.17°C. Panels were attached to a float at the Hawthorne Cove Marina (HCM) in Salem MA, which has moderate temperature fluctuations and is the most affected by urban contaminants of the three sites, being near the Salem Harbor Station power plant.

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Figure 1: Map of Study sites. DMC has a temperature range of 0.73°C to 20.51°C, CML has a temperature range of -0.61°C to 20.138°C, and HCM has a temperature range of 1.9°C to 24.15°C.

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2005/2006 Average Biweekly Water Temperatures
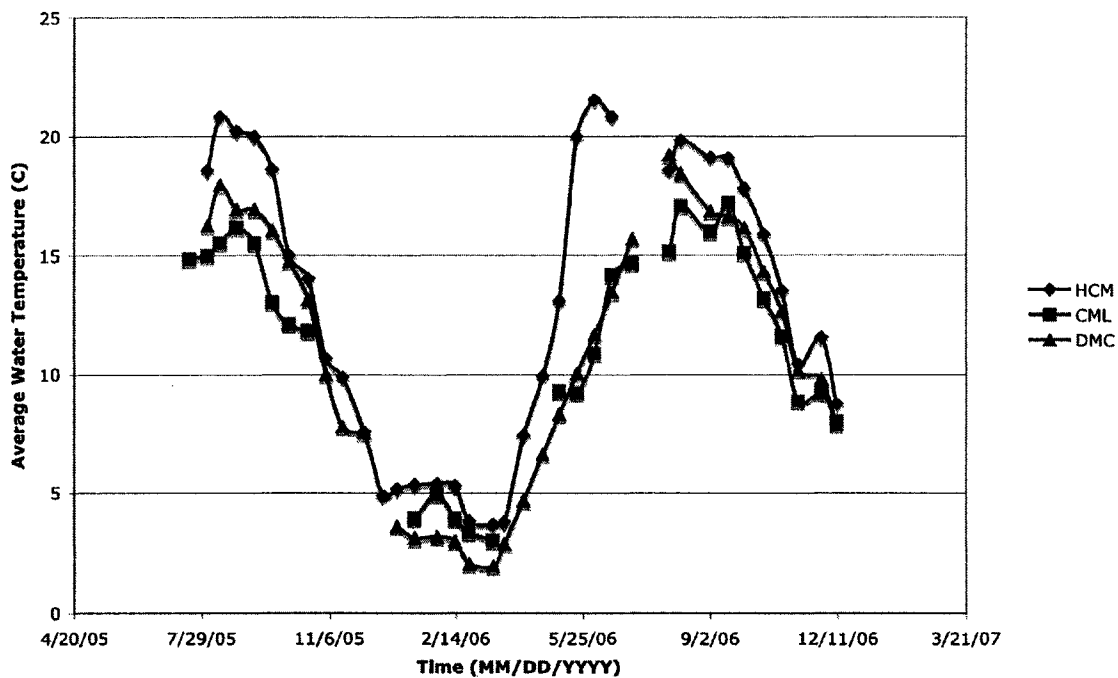
Figure 2: Average sea surface water temperatures 7/12/2005-12/11/2006. HCM data are marked by diamonds, CML data are marked by squares, and DMC data are marked by triangles.

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Data Collection

Physical Data

HOBO data loggers located between 0.5 and 1.5m below water surface at the three sites monitored temperature during the field study. We used two types of loggers, HOBO temperature loggers from July 2005 to July 2006 and HOBO temperature and light recorders from July 2006 to December 2006, with measurements taken every 30 minutes by both machines. Salinity and water temperatures were gathered from GoMoos (Gulf of Maine Ocean Observing System, http://www.gomoos.com) Buoy E (43° 42'47" N, 69° 21'20" W). GoMoos Buoy B (43° 10'51" N, 70° 25'40" W), and GoMoos Buoy A (42° 31'40" N, 70° 33'59" W), buoys up to nine miles off shore, November 2004-August 2006 (Figure 3). Salem State College has an aquaculture facility in Cat's Cove MA (42° 31'54"N, 70° 52'10" W), which is one cove north of the Hawthorne Cove Marina. Salem State students measured salinity and temperature data for Cat's Cove from 2001 to 2006. Further data were collected from a sub-tidal multiparameter YSI at the University of New Hampshire Coastal Marine Laboratory (43° 04'18" N, 70° 42'37" W).

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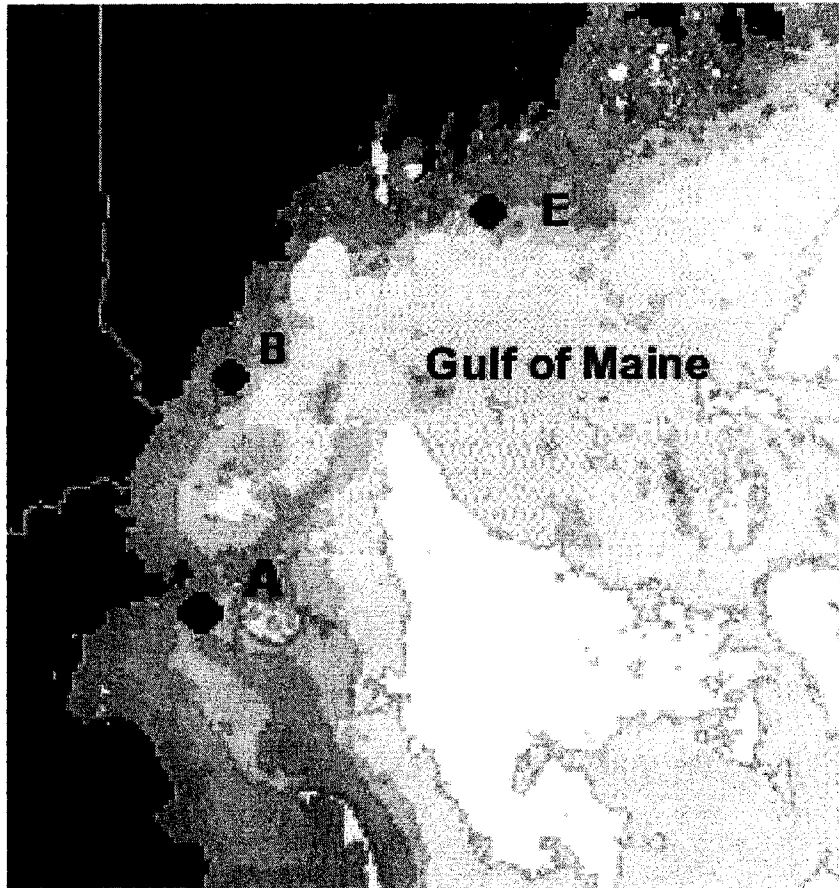
Figure 3: Map of Gulf of Maine showing the location of GoMoos Buoys A (near Salem, MA), B (near Portsmouth, NH) and E (near Walpole, ME).

2005 Spring Water Temperature Cross Calibration

In order to compare site 2004/2005 and 2005/2006 winter and spring water temperatures at DMC and HCM, 2005 data was cross-calibrated using GoMoos buoy 2005 and 2006 water temperatures and on site HOBO 2006 water temperatures. A linear regression between GoMoos and HOBO water temperatures was performed on data from January 1st, 2006 to August 31st 2006. Data from GoMoos buoy E were compared with those from DMC. Spring water temperatures at the two sites were highly correlated, with an R^2 of 0.8826 (Figure 4b). The equation from the linear regression of this relationship was used to calibrate spring water temperatures at DMC for 2005 (y=1.1352x-0.732). GoMoos buoy E water temperatures from January 1st 2005 to August 31st 2005 were then used to estimate spring water temperatures at DMC (Figure 4a). Data from GoMoos buoy A were compared with those at HCM. Spring water temperatures at the two sites were highly correlated, though less than those at GoMoos buoy E and DMC, with an R^2 of 0.71156. A polynomial fit was then performed on the same data, and was found to be a better fit, with an adjusted R^2 of 0.871, p value <0.001 and an N of 4, 605 (Figure 4b). The equation from the second degree polynomial fit of this relationship was used as a calibration for spring water temperatures at HCM for 2005 (y=-2.198+2.158x-0.187(x-7.971)^2). GoMoos buoy A water temperatures from January 1st 2005 to August 31st 2005 were then used to estimate spring water temperatures at HCM (Fig 4b).

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Estimated spring 2005 temperatures were compared with spring 2006 water temperatures at HCM and DMC.

Estimations of spring 2005 CML water temperatures were unnecessary, as the multiprameter YSI located at CML had been deployed prior to the study period. Therefore actual spring temperatures from 2005 could be compared to spring temperatures from 2006.

Correlations between physical conditions and biotic results of field studies were analyzed using a regression analysis and a correlation matrix in the JMP statistical package.

Field Studies

Deployment of Panels For All Field Studies Unless Otherwise Noted.

Six 100 cm^2 Plexiglas® panels were attached to bricks and deployed horizontally one meter below the water surface off of floating docks at DMC, CML and HCM. Every two weeks panels were photographed using a Nikon coolpix 995, Nikon coolpix 950, or a Canon rebel 2000, June 2005 to December 2006.

22
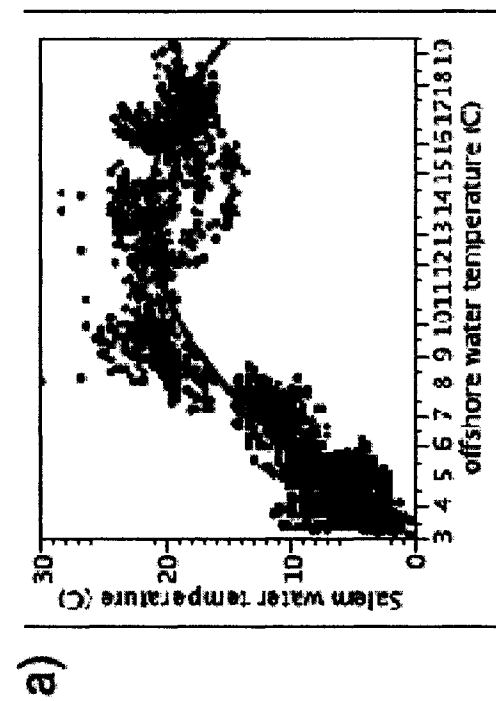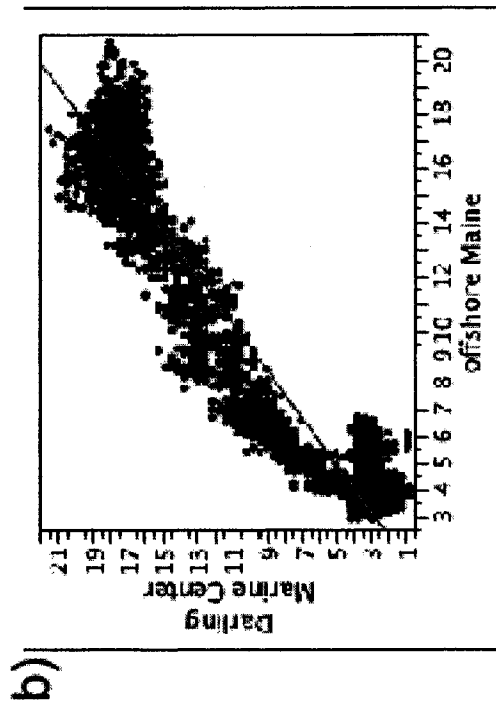
Figure 4: a) HCM correlation between on site 2006 water temperatures and offshore GoMoos Buoy A 2006 water temperatures. b) DMC correlation between on site 2006 water temperatures and offshore, GoMoos Buoy E 2006 water temperatures. y-1.1352x-0.732, R^2=0.892.

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Percent Cover by Species.

Three of the six settlement panels were experimentally selected for each species, either *Botryllus schlosseri* or *Botrylloides violaceus*. The allotted species was allowed to settle and grow on each panel while all other organisms were removed, resulting in a monoculture of one of the two species growing on each panel. This removal allowed for the observation of maximum growth under the different field conditions by ascidians unfettered by spatial interspecific competition. Panels were deployed twice, from late June 2005 to January 2006 and from February 2006 to December 2006.

Percent cover was calculated from images viewed in iPhoto® using a point count analysis. Time of initial recruitment of each species at each site was recorded and analyzed using a two way ANOVA. Percent cover data were arc-sin transformed and space occupancy patterns between species, sites and years were analyzed using repeated measures MANOVA. Interactions between percent cover and temperature were analyzed using a linear regression model. Abundance patterns between species and sites were analyzed using a two way ANOVA. Statistical analyses were performed using the JMP statistical analysis package.

Single Colony.

Six settlement panels were deployed at HCM and DMC from May 2006 to December 2006. Two colonies of either *Botrylloides violaceus* or *Botryllus schlosseri* were allowed to settle on each panel, with three replicates for each

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species. All other organisms were removed. Two recruits were allowed to settle to ensure survival of at least one adult colony, as survival rates of recently settled juvenile colonies are low (Keough & Downes 1982). Once the two colonies reached approximately 100 zooids, one was removed leaving a single colony on each panel. Panels were cleaned of all other organisms and were deployed at HCM and DMC from June 2006 to December 2006. CML was not used for this study due to the severe flooding that occurred in May 2006, which caused recruitment to be delayed relative to DMC and HCM.

Initial recruitment and time of larval brooding in the colonies were recorded and analyzed using a two way ANOVA. Colony growth was measured as percent cover of the Plexiglas® panel using a point count analysis. Data were arc-sin transformed and spatial coverage patterns between species and site were analyzed using repeated measures MANOVA. Correlations between percent cover and temperature were analyzed using linear regression. Statistical analyses were performed using the JMP statistical analysis package.

Recruitment.

Six 100 cm^2 Plexiglas® recruitment panels were deployed in May 2006. Panels were replaced and taken back to the laboratory every two weeks and examined under a Leica S6D dissecting microscope. Panels were deployed from May 2006 to December 2006. The presence, absence, and quantity of *Botrylloides violaceus* and *Botryllus schlosseri* were recorded. Recruitment was calculated

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instead of settlement because deceased colonies, unidentifiable to species level, were observed on the panels, suggesting that I was not observing complete settlement, but was instead recording two week recruitment. Therefore recruitment for this study is defined as those juveniles that have settled during the two week period and maintained space until panel removal. Data were analyzed using a two way ANOVA. Interactions between temperature and recruitment were analyzed using linear regression. All statistical analyses were performed using the JMP statistical package.

Tunicate Culturing.

Colonies of both *Botrylloides violaceus* and *Botryllus schlosseri* were collected at all three field sites and brought back into the laboratory where they were settled onto microscope glass slides by removing all the debris from the underside of the colonies and placing them on the microscope slides inside a humid settlement box for two to three hours. After settlement, the colonies were hung vertically in a tank with low flow until they attached to the slide. (see (Phillippi et al. 2004 for details) Colonies for all laboratory studies were maintained in either 5-gallon tanks or 4-gallon plastic tubs containing 8.7l to 11.4l of 32 psu seawater. Water was changed every two weeks in 2005 and every week in 2006. Animals were fed Coralife® Invertebrate Smorgasbord™ daily (five drops a day for the first five colonies, and an extra drop for every colony containing over fifty zooids in the tank or tub) and were in constant light. All

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colonies were monitored using a Leica S6D dissecting microscope, and periodically photographed using a Canon power shot S40 attached to the Leica dissecting microscope. Established colonies were then utilized in the different experiments as described in the following sections.

These studies were conducted during August-January 2005 and March-December 2006. There were two tubs containing 9.5l 32 psu seawater at every temperature in 2005 and three tubs/tanks containing 9.5-10.5l at every temperature in 2006. Growth and reproduction studies were initiated four times: August 2005, March 2006, and June 2006 and July 2006. Colonies were kept until death, and as colony lifespans were often many months, there was overlap between cohorts in the tanks. However, all colonies in the laboratory at the same time experienced the same water conditions, food quantities and environmental parameters at each specific temperature, and food, temperature, and environmental parameters remained constant during the entire two-year period colonies were kept in the laboratory. Replicates were variable for each species from each site at the different temperatures and cohorts, ranging from 4-24.

Calculating colony growth.

There are currently four different methods in the literature for calculating colony growth in colonial ascidians. These methods can be divided into two groups, those calculating an increase over time, and those calculating an increase based on the asexual reproductive cycle of the colony. The two

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methods that have been used based on time are percent increase in number of zooids over a one-week period (Stachowicz et al. 2002) and percent increase in area of colony over a set interval of time such as a week or month (McCarthy et al. 2007). The two methods of calculating growth that incorporate the biological process of colony growth are duration of blastogenic cycle (Mukai 1977) and percent increase in number of zooids after a blastogenic cycle (Brunetti et al.1980). Percent increase in number of zooids over a one week period, though calculated, was not used for this study because duration of blastogenic cycle was often greater than one week for temperatures under 17°C (Mukai 1977, Brunetti et al. 1980, Grosberg 1982) which resulted in highly variable growth rates when weeks were compared. Percent increase in colony area was not used for this study because colonies are able to increase their area by either increasing the number of zooids, increasing the amount of interstitial tissue, or increasing the number of ampullae in a colony (personal observation) and two of these methods of increasing area do not necessarily involve increasing numbers of bud or egg producing structures. The duration of blastogenic cycle, though calculated, was not used for this study because it has been found to be highly variable throughout the life span of a single colony, and colonies of *Botryllus schlosseri* cultured in the same container were found to cycle together (De Tomaso, personal communication).

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Growth Rate and Reproduction.

To test the effect of temperature on growth, colonies were placed in five different temperature controlled rooms (5°C, 10°C, 15°C, 20°C and 25°C). After a two week acclimatization period, colonies were monitored daily throughout their life span (two weeks to over six months) (Brunetti 1974). Number of zooids, percent increase in zooid number per week, time of take over, percent increase in zooids at take over, presence of gametes, number of eggs, and size of testes were recorded. Percent increase in zooid number at takeover and reproductive success were compared between species and across temperatures using a two way ANOVA in the JMP statistical package.

CHAPTER III

Results

__Physical Conditions for the Hawthorne Cove Marina, the University of New Hampshire Coastal Marine Laboratory, and the University of Maine Darling Marine Center January 2005-December 2006__

__Salinity__

Salinity levels dropped below 25 psu five times at GoMoos Buoy A, twice at GoMoos Buoy B, and never dropped below 25 psu at GoMoos Buoy E throughout the two-year study period (Figure 5). Salinity levels did not drop below 20 psu at any of the three buoy sites.

__May 2006 Flooding__

There was a 100 year flooding event in New England that occurred in May 2006. This resulted in lower salinities than normal and a broken panel set up at

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the Hawthorne Cove Marina. Rains were heavy throughout New England,

however there was less precipitation in the Maine midcoast region, where the

Darling Marine Center is located, than in coastal New Hampshire or Salem

Harbor (Figure 5).
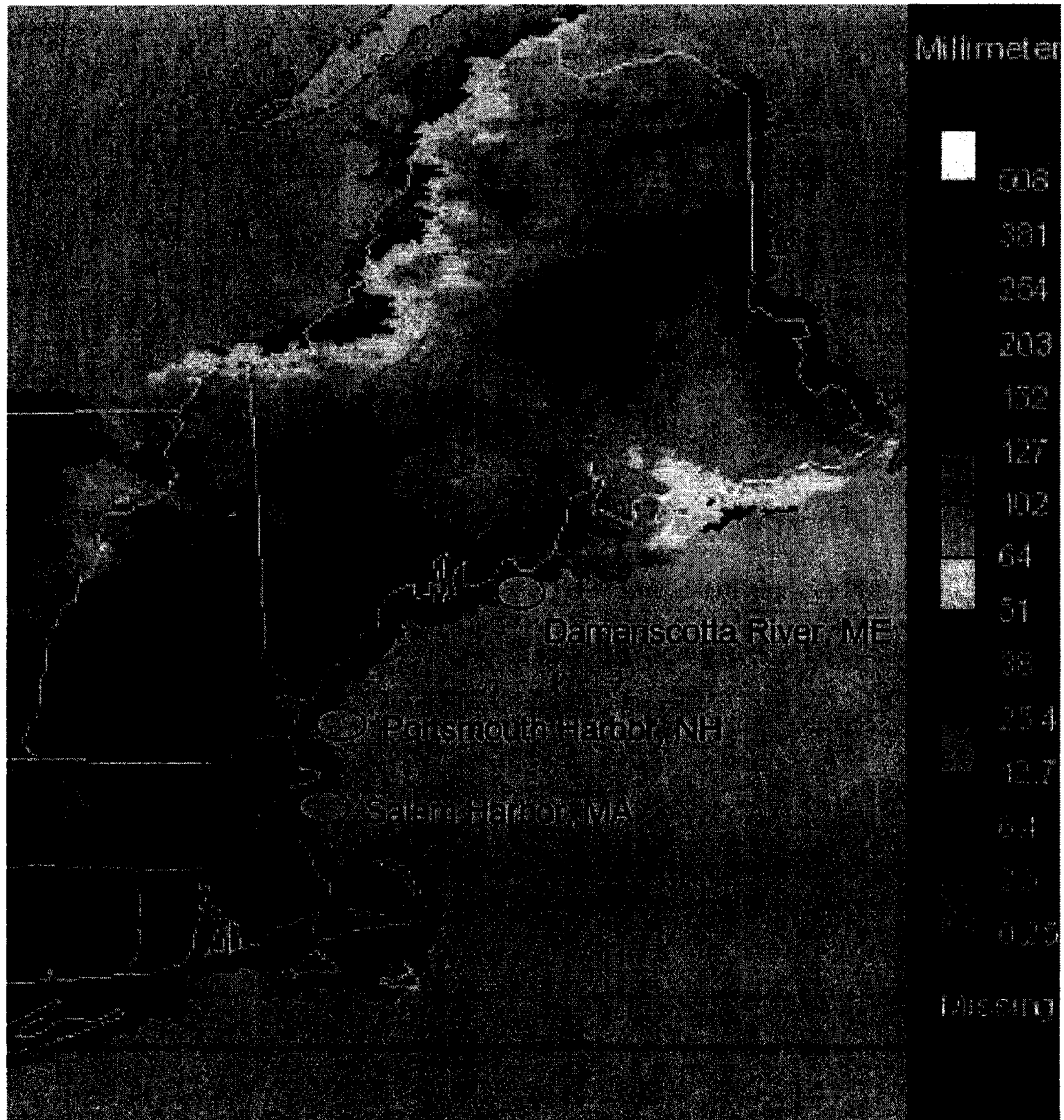
Figure 5: May 2006 Precipitation map for New England from NOAA's National
Weather Service. Most intense rainfall occurred in coastal New Hampshire and
the Cape Anne area, with less rainfall at the Damariscotta River.

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Additional Data for the Hawthorne Cove Marina

Data from Cat Cove were particularly helpful when looking at the effect of the spring 2006 flooding on the recruitment and growth patterns of *Botryllus schlosseri* and *Botrylloides violaceus* at HCM. While there was a 100-year flooding event in New Hampshire and parts of Massachusetts in May of 2006, salinity levels in Cat's Cove did not drop below 23 psu during the flooding in May 2006 (Table 1). These results differ from those from GoMoos Buoy A, and were considered a more accurate representation of salinities at HCM due to Cat's Cove's proximity to the study site.

Date	Temperature (C)	Salinity (psu)
6/20/04	17.9	31.4
7/1/04	18	31.7
8/22/05	23.3	24
9/6/05	19.7	25
10/14/05	14	30.5
10/27/05	12	26.3
11/3/05	9.8	
11/10/05	8.1	
5/22/06		27
6/12/06	16.6	30.8

Table 1: Abiotic data from Cat's Cove, MA. Periodic records of temperature and salinity from 2004 to 2006.

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Water Temperature

Water temperatures collected from the HOBO data loggers during the study period of June 2005 to December 2006 varied at the three different sites. Summer water temperatures were highest at HCM for both summers, while CML had the lowest (Figure 2). Winter temperatures were again highest at HCM, however they were lowest at DMC. Biweekly average water temperatures were above 10°C 72 more days at HCM than at DMC during 2005, but only 26 more days during 2006. Biweekly average water temperatures were above 13°C 26 more days at HCM than at DMC during 2005 and 54 more days in 2006 (Table 2). Biweekly average water temperatures were above 10°C 37 more days in 2006 than in 2005 at DMC and 9 fewer days in 2006 than in 2005 at HCM.

33
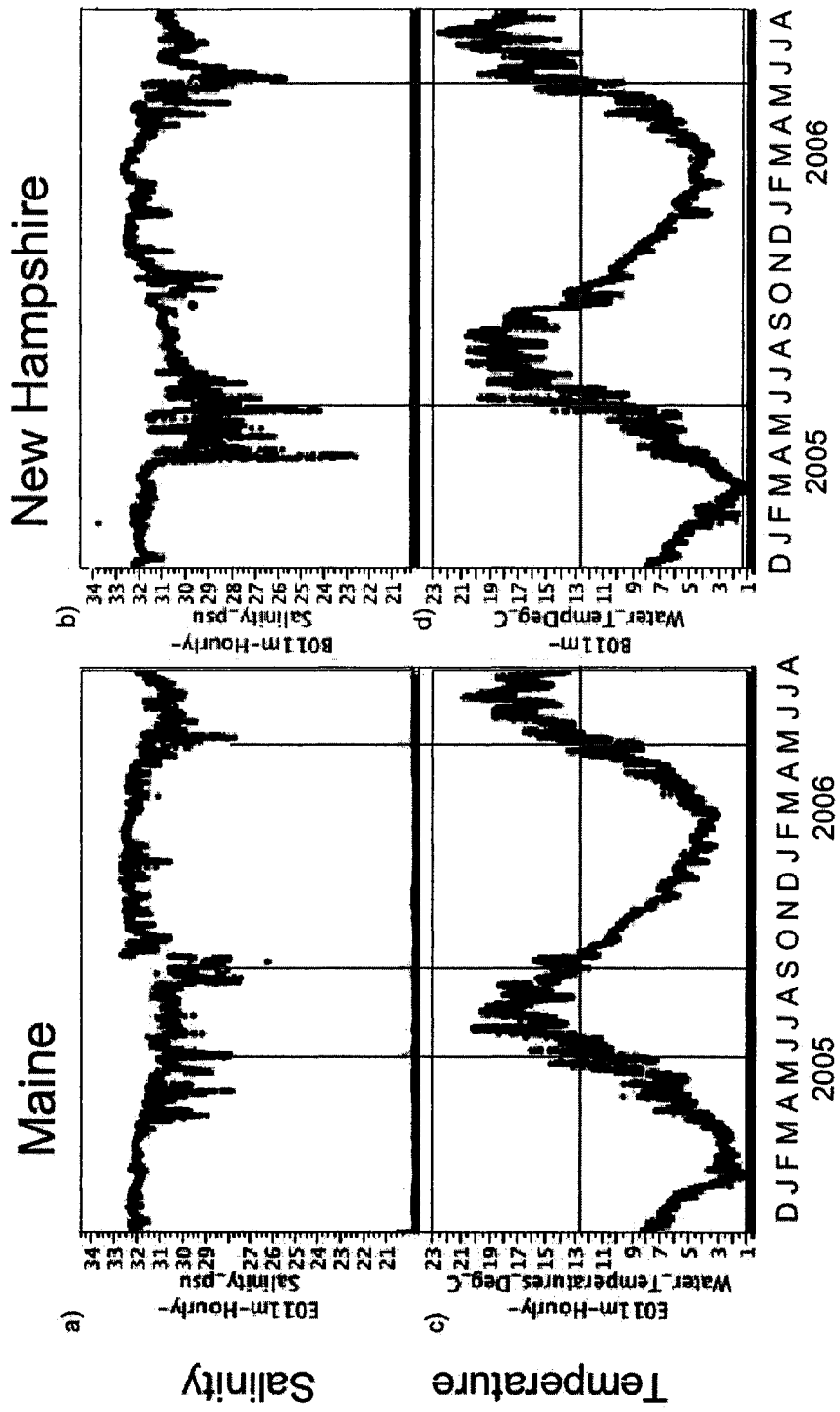
Figure 6: a) 2005 and 2006 salinity at GoMoos Buoy E. b) 2005 and 2006 salinity at GoMoos Buoy B. c) 2005 and 2006 water temperature at GoMoos Buoy E. d) 2005 and 2006 water temperature at GoMoos Buoy B. Timing of increasing salinities and initial temperatures above and below 13°C coincide at both sites.

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Site	Biweekly average temperature above 10C		Biweekly average temperature below 10C		
	Average temperature	Date	Average temperature	Date	Days above 10C
DMC		6/14/05	7.83	11/16/05	155
DMC	10.8	5/20/06	9.82	11/28/06	195
CML	10.9	6/3/06	8.89	11/16/06	166
HCM		4/18/05	9.9	11/16/05	227
HCM	13.11	5/6/06	8.76	12/10/06	218
	Biweekly average temperature above 13C		Biweekly average temperature below 13C		
	Average temperature	Date	Average temperature	Date	Days above 13C
DMC		6/14/05	10.01	11/3/05	143
DMC	13.49	6/17/06	12.72	10/28/06	134
CML	14.19	6/17/06	10.7	10/28/06	134
HCM		5/19/05	10.7	11/3/05	169
HCM	13.11	5/6/06	10.45	11/10/06	188

Table 2: Timing above 10°C and 13°C at HCM, CML, and DMC. These are the two different temperatures documented as the critical temperatures for reproduction in *Botryllus schlosseri*.

Biweekly average water temperatures were above 13°C 9 fewer days in 2006 than in 2005 at DMC, and 19 more days in 2006 than in 2005 at HCM. As both 10°C and 13°C are potential critical temperatures for *Botryllus schlosseri* reproduction (13°C is the lowest tested temperature that gamete production has been observed in the laboratory while 10°C is the highest temperature gamete production has not been observed in the laboratory), the opposite changes in number of days above these temperatures between the two years for these three sites may give us a better idea in which temperature is more important for Gulf of Maine *Botryllus schlosseri* populations

Biweekly average water temperatures did not differ significantly between years over the summer and fall months at any of the three sites (Figure 7). However, though these seasonal patterns did not vary between years at any of

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the sites, 2006 spring temperatures rose above 10°C sooner than the

relationship between offshore and inshore temperature predicted for spring 2005

water temperatures for both DMC and HCM (Figure 8c, d, Table 2). At CML the

difference between summer and winter temperatures was greater in 2006 than in

2005 (2006 slope=-5.8202, R^2=0.3973, p<0.000, 2005 slope= -4.6746, R^2=0.277,

p<0.000) suggesting that spring and early summer temperatures were higher in

2006 than in 2005 (Figure 9).

Spring water temperatures followed a similar pattern when compared

between sites as between years, with the sites all having similar patterns.

However, the southern most site (HCM) warmed earlier than the northern site

(DMC) and reached a higher maximum temperature. This pattern was found in

both the 2006 and estimated 2005 spring water temperatures (Figure 8).

36
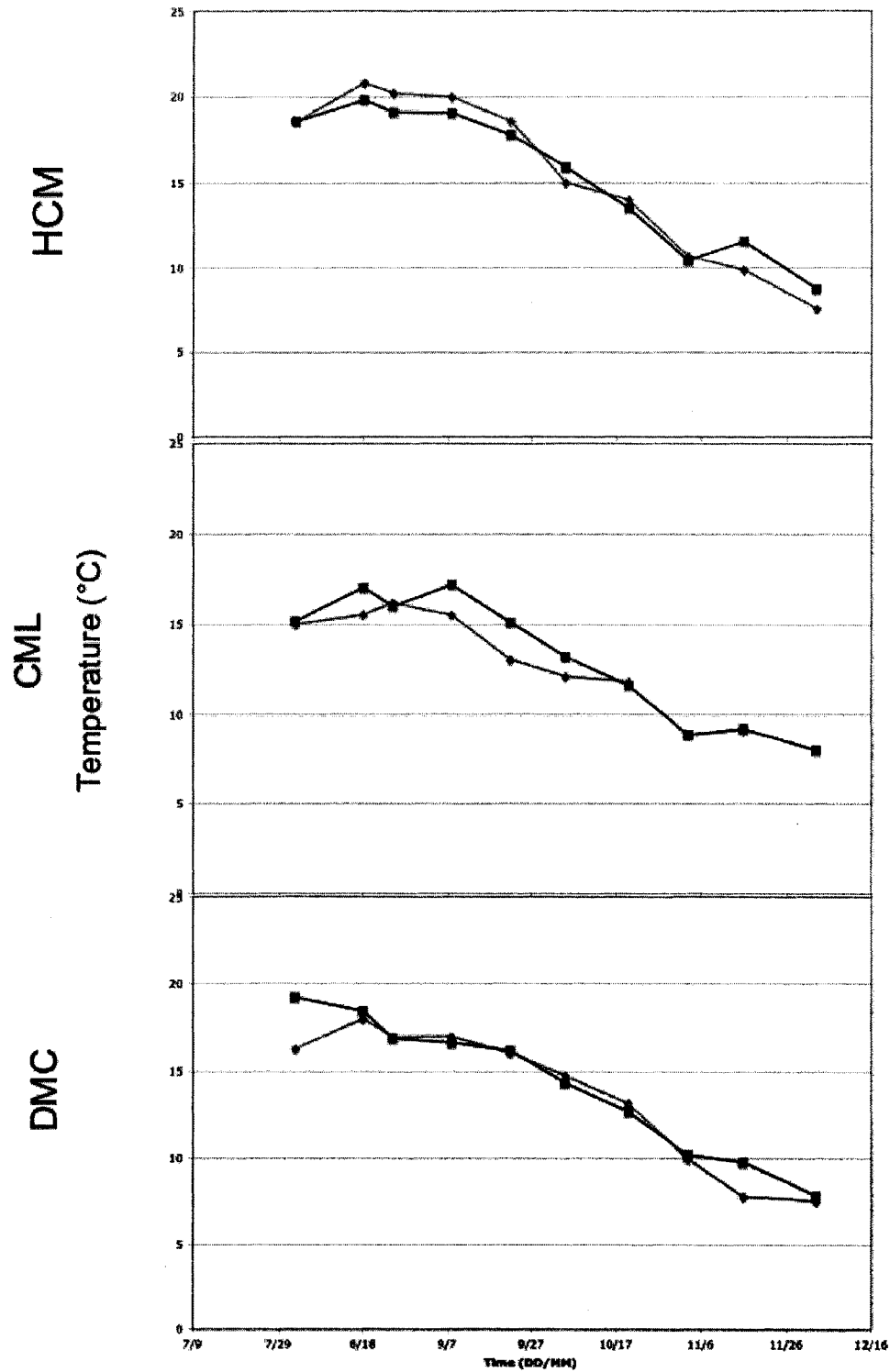
Figure 7: Summer and fall biweekly average water temperatures for 2005 (grey diamonds) and 2006 (black squares) a) HCM. There was no significant difference in water temperature. b) CML. No significant difference. c) DMC. No significant difference between years.

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HCM Water Temperatures

DMC Water Temperatures

a)

b)

c)

d)
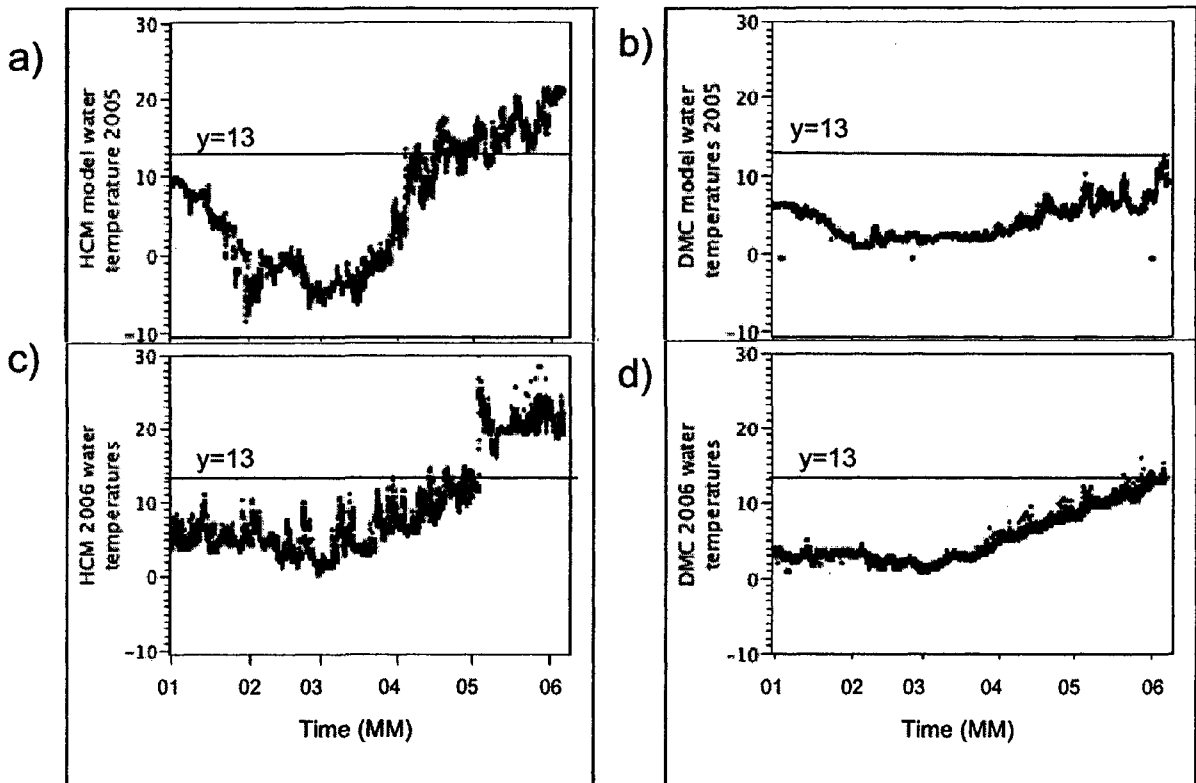
Figure 8: a) Model spring 2005 water temperatures at HCM. b) Model spring 2005 water temperatures at DMC. c) Spring 2006 water temperatures at HCM. d) Spring 2006 water temperatures at DMC.

38
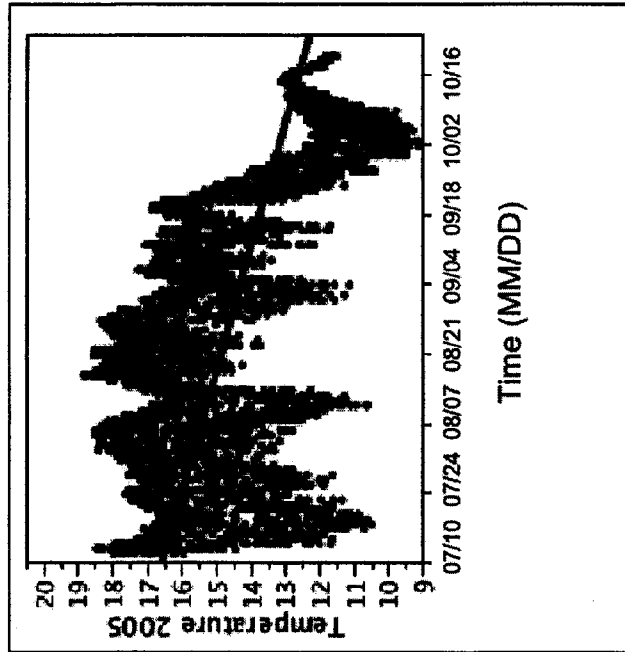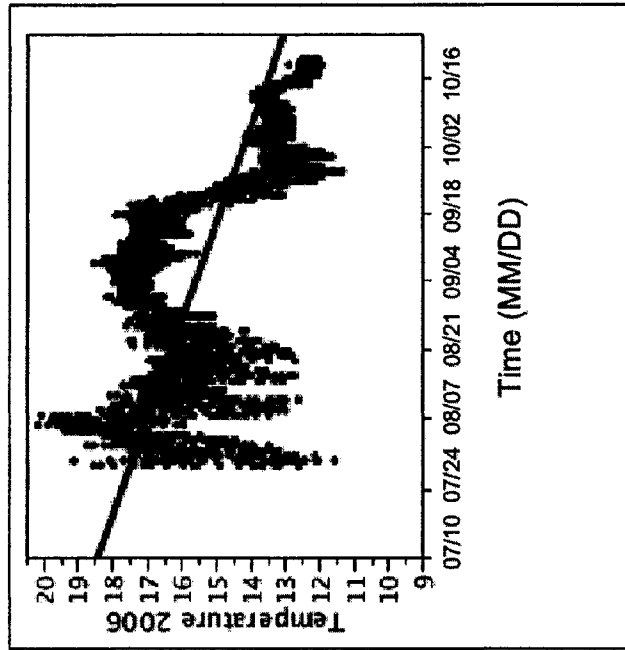
Figure 9: a) 2005 CML July –November water temperatures. Slope=-4.6746, R^2=0.277, p<0.000. b) 2006 CML July-November water temperatures. Slope=-5.8202, R^2=0.3973, p<0.000.

Abundance and Growth Patterns

Hawthorne Cove Marina

Description of Colony Abundance and Growth Patterns.

Botrylloides violaceus had two local maxima of percent cover during both 2005 and 2006 at HCM, with an overall maximum percent cover on August 13 in both 2005 and 2006 (Figure 10a). A local minimum in percent cover of *Botrylloides violaceus* occurred during both summer growing seasons, though earlier in 2005 (September 23) than in 2006 (October 7). *Botryllus schlosseri* also had two local maxima of percent cover during both 2005 and 2006 at HCM, with a maximum percent cover on August 13 in 2005 and almost a month earlier in 2006 (July 18) (Figure 10a). A local minimum in percent cover of *Botryllus schlosseri* occurred during both summer growing seasons, on September 9 during 2005 and on September 23 during 2006. The maximum percent cover for *Botrylloides violaceus* occurred at the same time as the maximum percent cover for *Botryllus schlosseri* in 2005 and one month after the maximum percent cover for *Botryllus schlosseri* in 2006. The minimum percent cover during the growing season for *Botrylloides violaceus* occurred two weeks after that of *Botryllus schlosseri* in 2005 and in 2006. The maximum number of colonies of *Botrylloides violaceus* found on a single panel was 47 during 2005, and 36 during 2006 (Figure 10b). The maximum number of colonies of *Botryllus schlosseri* found on a single panel was 9 during 2005 and 8 during 2006.

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HCM Percent Cover and Colony Number, 2005 and 2006
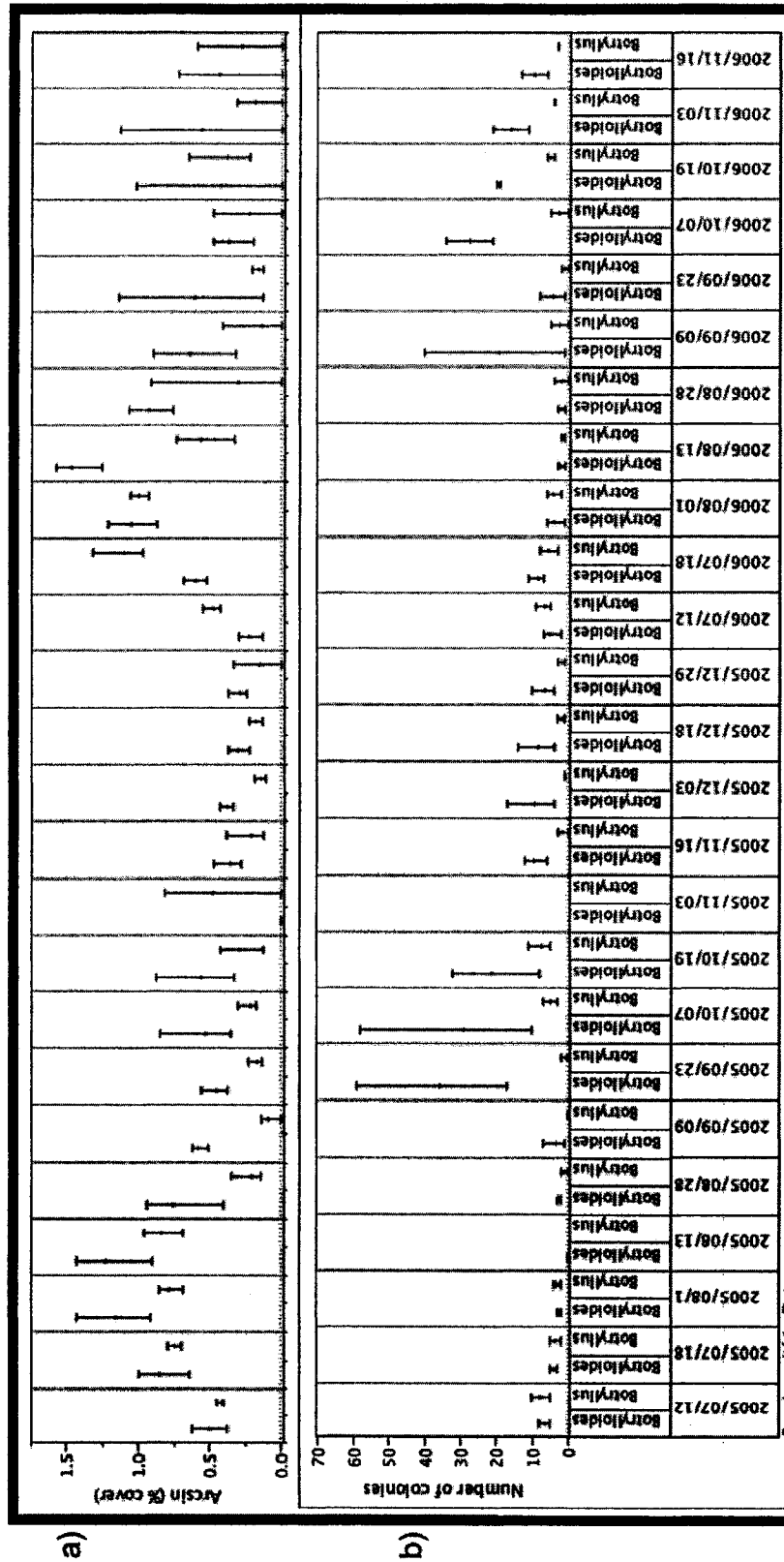
Figure 10: a) Percent cover of *Botryllus schlosseri* and *Botrylloides violaceus* at HCM. There are two peaks of percent cover for both species both years. b) Average number of colonies at HCM. There are large increases in number of colonies in late September and October for *Botrylloides violaceus* but not *Botryllus schlosseri*.

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CML Percent Cover and Colony Number, 2005 and 2006
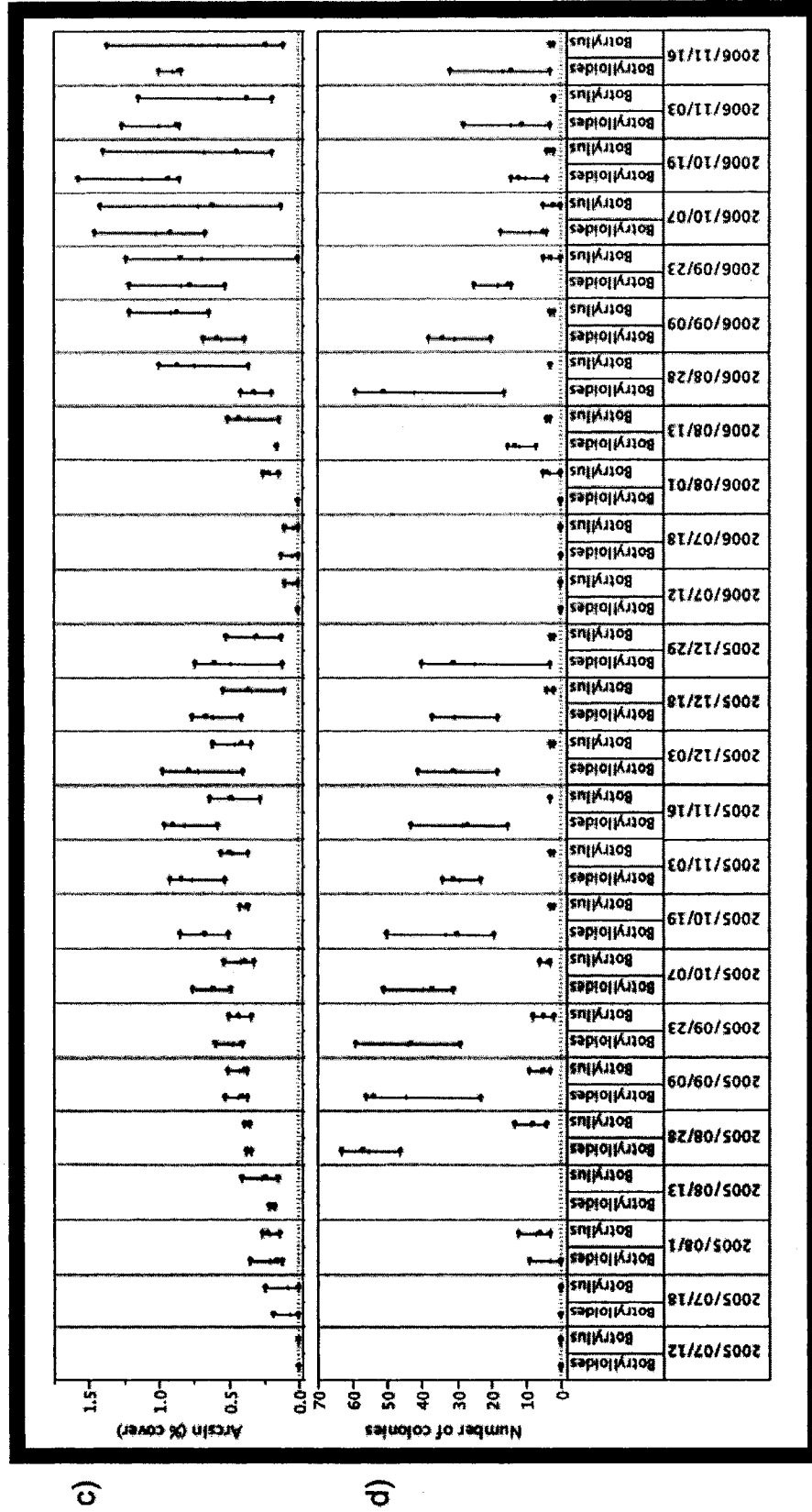
Figure 10: c) Percent cover of *Botryllus schlosseri* and *Botrylloides violaceus* at CML. There is one peak of percent cover for both species both years. d) Average number of colonies at CML.

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DMC Percent Cover and Colony Number, 2005 and 2006
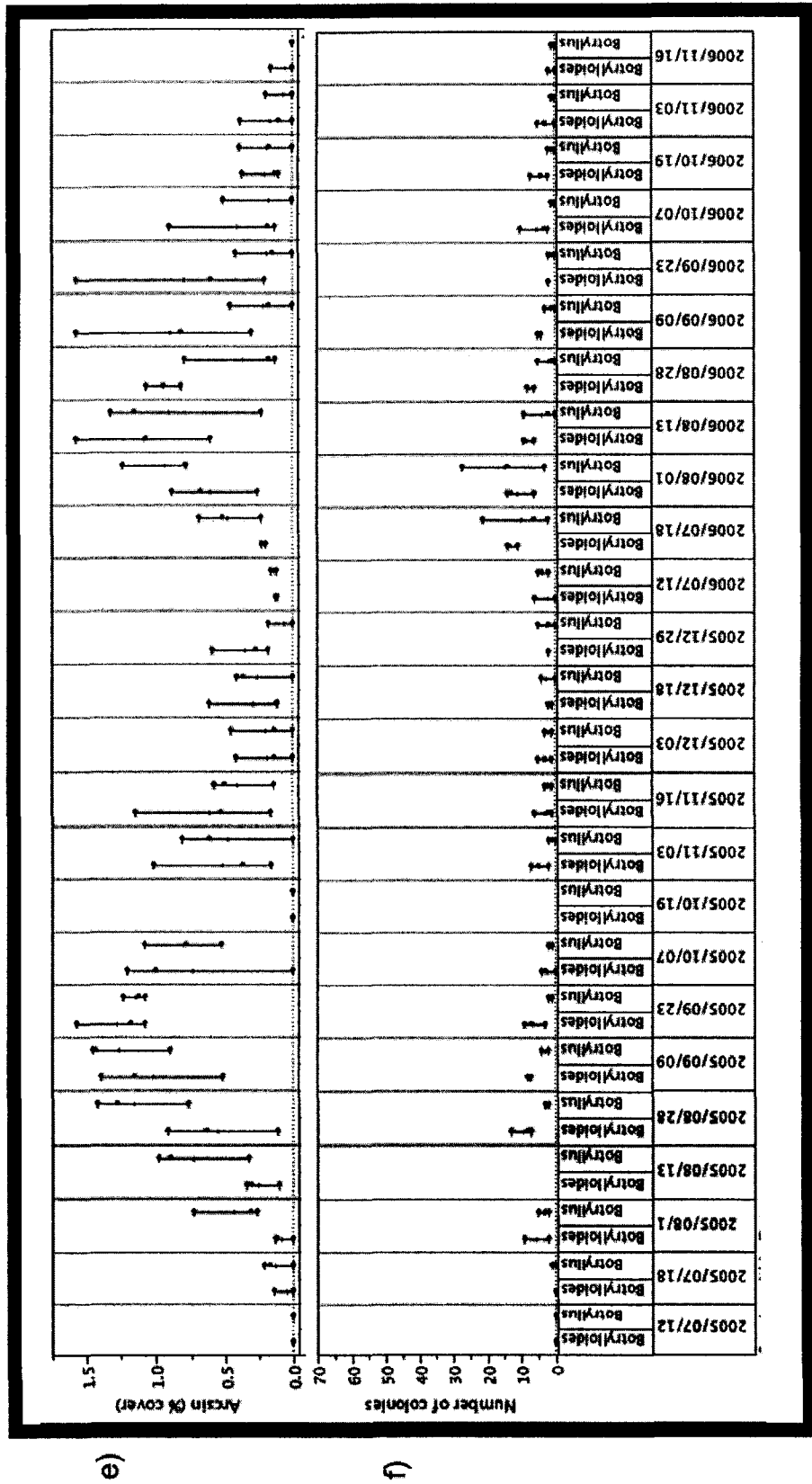
Figure 10: e) Percent cover of *Botryllus schlosseri* and *Botrylloides violaceus* at DMC. One peak percent cover.
f) Average number of colonies at DMC.

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<u>Spatial Coverage Patterns Between Species.</u>

During 2005, there was a significant difference in the spatial coverage patterns of *Botryllus schlosseri* and *Botrylloides violaceus* at HCM (repeated measures MANOVA, p=0.019), though this was primarily due to quantity of cover rather than timing of peak percent cover, as *Botrylloides violaceus* colonies covered a larger area of their panels than *Botryllus schlosseri* colonies did (Fig 11a). There was no difference in the timing of peak percent cover for the two species in 2005. The two species showed similar spatial coverage patterns at HCM during 2006 (Fig 11b). However, this may be due to the flooding in May 2006, which dropped the salinity of Salem Harbor to 23 psu and destroyed the panel set up at HCM (Table 1). This led to replacement of all panels on June 6, over a month after water temperatures had reached 10°C at HCM.

<u>Spatial Coverage Patterns Between Years.</u>

Botrylloides violaceus showed similar spatial coverage for the two years at HCM. However, there were significant differences in the spatial coverage patterns between the two years for *Botryllus schlosseri* at HCM (repeated measures MANOVA, p=0.008) with peak abundance occurring earlier in 2006 than in 2005 (Figure 12a).

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<u>Spatial Coverage and Temperature.</u>

Percent cover was positively correlated with average biweekly water temperature June through December 2005 for *Botrylloides violaceus* (slope=3.188, R^2=0. 414, p<0.0001) but not *Botryllus schlosseri* (Figure 13a, 13d). Percent cover was positively correlated with average biweekly water temperature May-December 2006 at HCM for *Botrylloides violaceus* (R^2=0.602, p=0.014) but not *Botryllus schlosseri* (Fig. 14a, 14d). However, percent cover for both *Botryllus schlosseri* and *Botrylloides violaceus* had a greater correlation with time than temperature from July to December 2006. Percent cover of *Botryllus schlosseri*, though not correlated with temperature, was negatively correlated with time. This correlation suggests a decline in one cohort of colonies that is not replaced by an increase of a second cohort. Percent cover of *Botrylloides violaceus* was correlated with time by a second-degree polynomial, suggesting that the growth in the cohort of *Botrylloides violaceus* colonies is delayed relative to that of *Botryllus schlosseri* (Figure 15a and 15d).

45
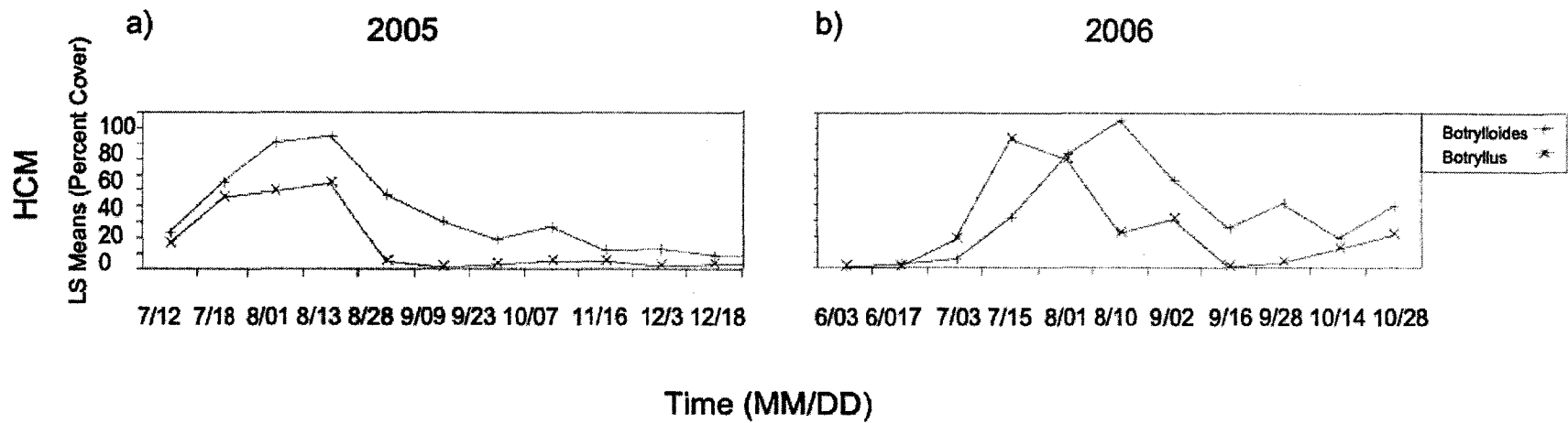
Figure 11: a) Percent cover of both *Botryllus schlosseri* (dark) and *Botrylloides violaceus* (grey) at HCM during 2005. There was a significant difference in the abundance patterns throughout the year, repeated measures MANOVA p=0.019. b) Percent cover of *Botryllus* and *Botrylloides* at HCM during 2006. There was no significant difference in the abundance patterns of the two species.
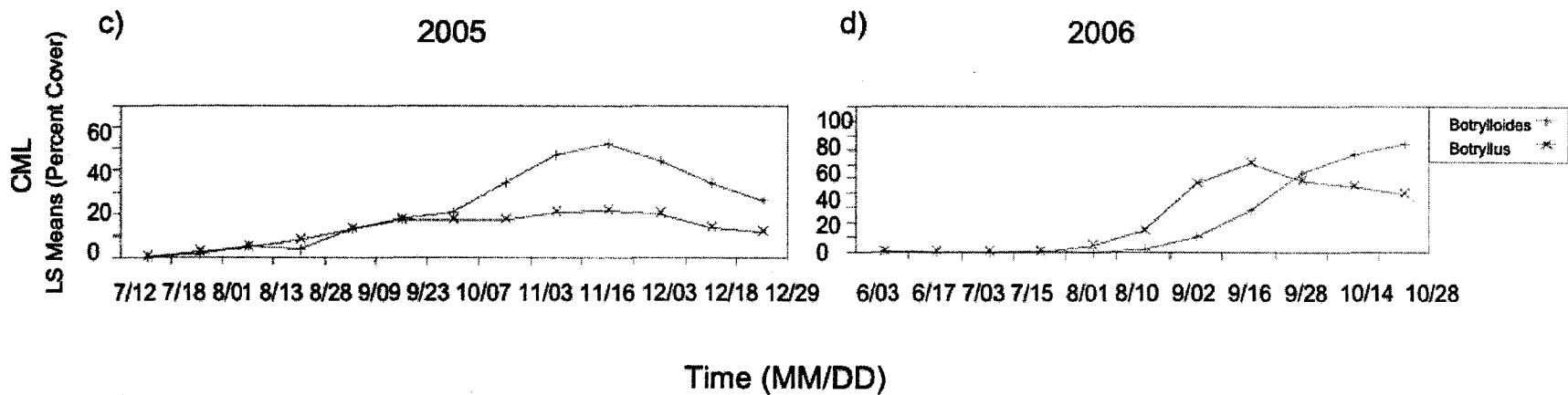
Figure 11: c) Percent cover of *B. schlosseri* and *B. violaceus* at CML during 2005. While there was a difference in the abundance during the fall, there was not a significant difference in the abundance patterns of the two species at this site. d) Percent cover of *Botryllus schlosseri* and *Botrylloides violaceus* at CML during 2006. No significant difference in abundance patterns.

e) 2005

f) 2006

Botrylloides

Botryllus

7/12 7/18 8/01 8/13 8/28 9/09 9/23 10/07 11/03 11/16 12/03 12/18 12/29

6/03 6/17 7/03 7/15 8/01 8/10 9/02 9/16 9/28 10/14 10/28

DMC LS Means (Percent Cover)

Time (MM/DD)
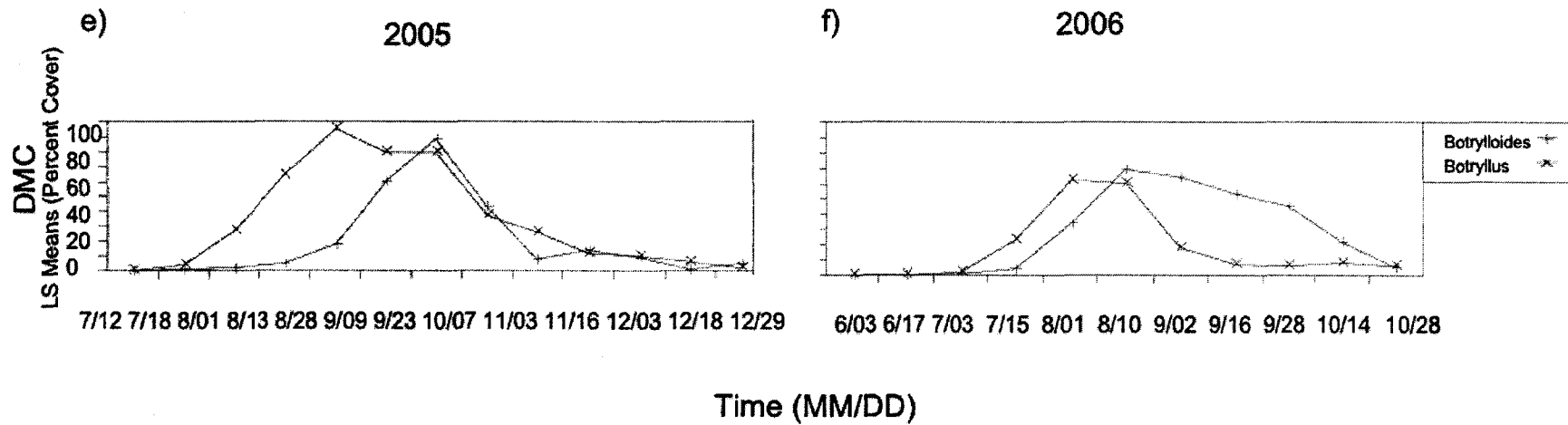
Figure 11: e) Percent cover of *B. schlosseri* and *B. violaceus* at DMC during 2005. There was an almost significant difference in the abundance patterns of these two species, repeated measures MANOVA p=0.057. f) Percent cover of *Botryllus schlosseri* and *Botrylloides violaceus* at DMC during 2006. No significant difference in abundance patterns of the two species.

48
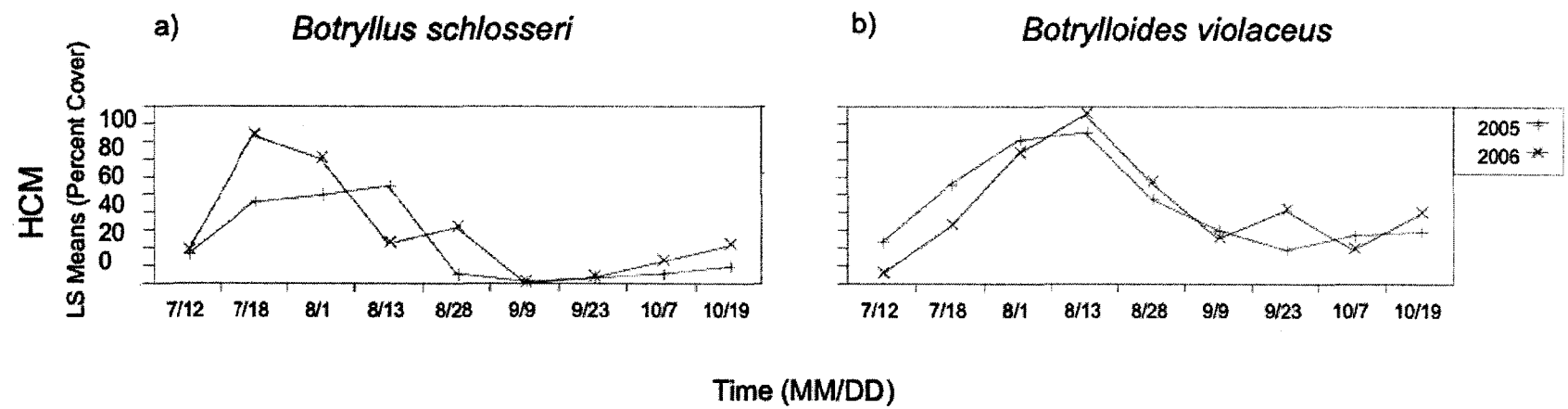
Figure 12: a) Percent cover of *Botryllus schlosseri* at HCM for 2005 (light) and 2006 (dark). There was a significant difference in the abundance patterns between the two years, repeated measures MANOVA, p=0.008. b) Percent cover of *Botrylloides violaceus* at HCM for 2005 and 2006. No significant difference between years.

50
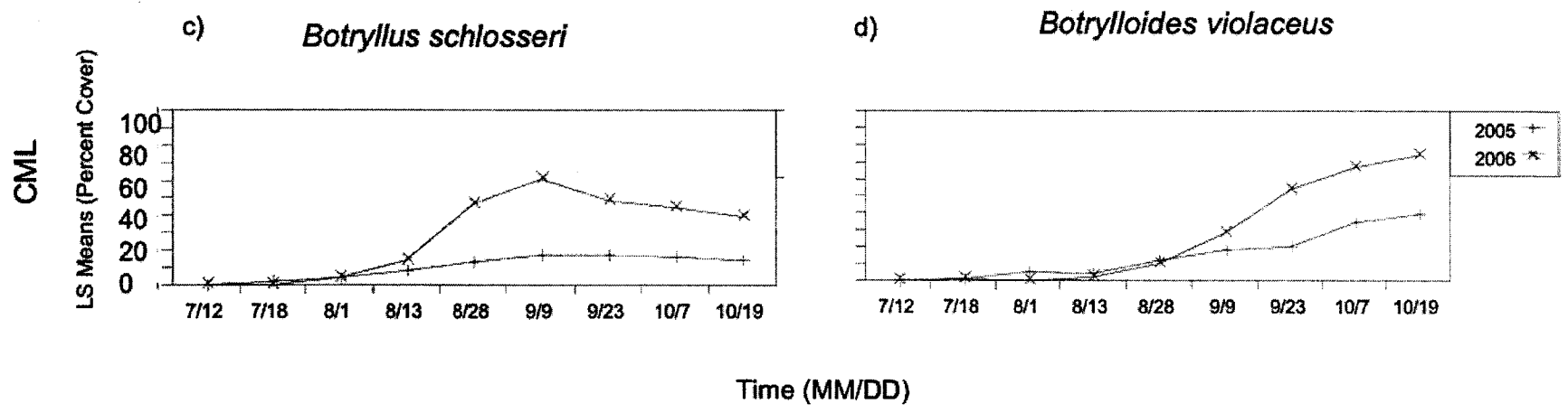
Figure 12: c) Percent cover of *Botryllus schlosseri* at CML for 2005(light) and 2006 (dark). No significant difference between years. d) Percent cover of *Botrylloides violaceus* at CML for 2005 and 2006. No significant difference between years.
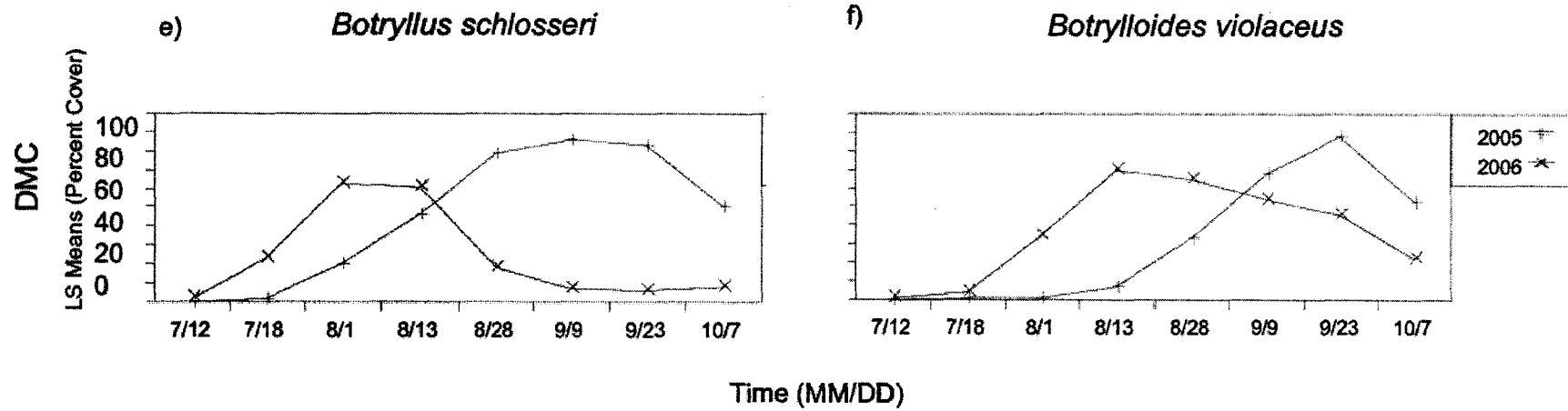
Figure 12: e) Percent cover of *Botryllus schlosseri* at DMC during 2005(light) and 2006 (dark). There was a significant difference in the abundance patterns between the two years, repeated measures MANOVA, p=0.008. f) Percent cover of *Botrylloides violaceus* at DMC during 2005 and 2006. While there was no significant difference in the abundance patterns, there was a trend towards an earlier time of initial presence and growth in 2006.

Figure 13: a) Linear regression between 2005 *Botryllus schlosseri* percent cover and average biweekly water temperature at HCM.$y=1.257x-3.173$, $R^2=0.130$, $p=0.0307$. b) Linear regression between 2005 *Botryllus schlosseri* percent cover and average biweekly water temperature at CML, $y=-1.545x+33.510$, $R^2=0.122$, $p=0.095$. c) Linear regression between 2005 *Botryllus schlosseri* percent cover and average biweekly water temperature at DMC, $y=4.86x-19.703$, $R^2=0.417$, $p=0.0002$. d) Linear regression between 2005 *Botrylloides violaceus* percent cover and average biweekly water temperature at HCM. $y=3.188x-12.061$, $R^2=0.414$, $p<0.0001$. e) Linear regression between 2005 *Botrylloides violaceus* percent cover and average biweekly water temperature at CML, $y=-6.956x+116.092$, $R^2=0.537$, $p<0.0001$. f) Linear regression between 2005 *Botrylloides violaceus* percent cover and average biweekly water temperature at DMC, $y=1.650x+13.247$, $R^2=0.046$, $p=0.251$.

Botryllus schlosseri

2005

a) HCM

b) CML

c) DMC

Botrylloides violaceus

2005

d) HCM

e) CML

f) DMC

54
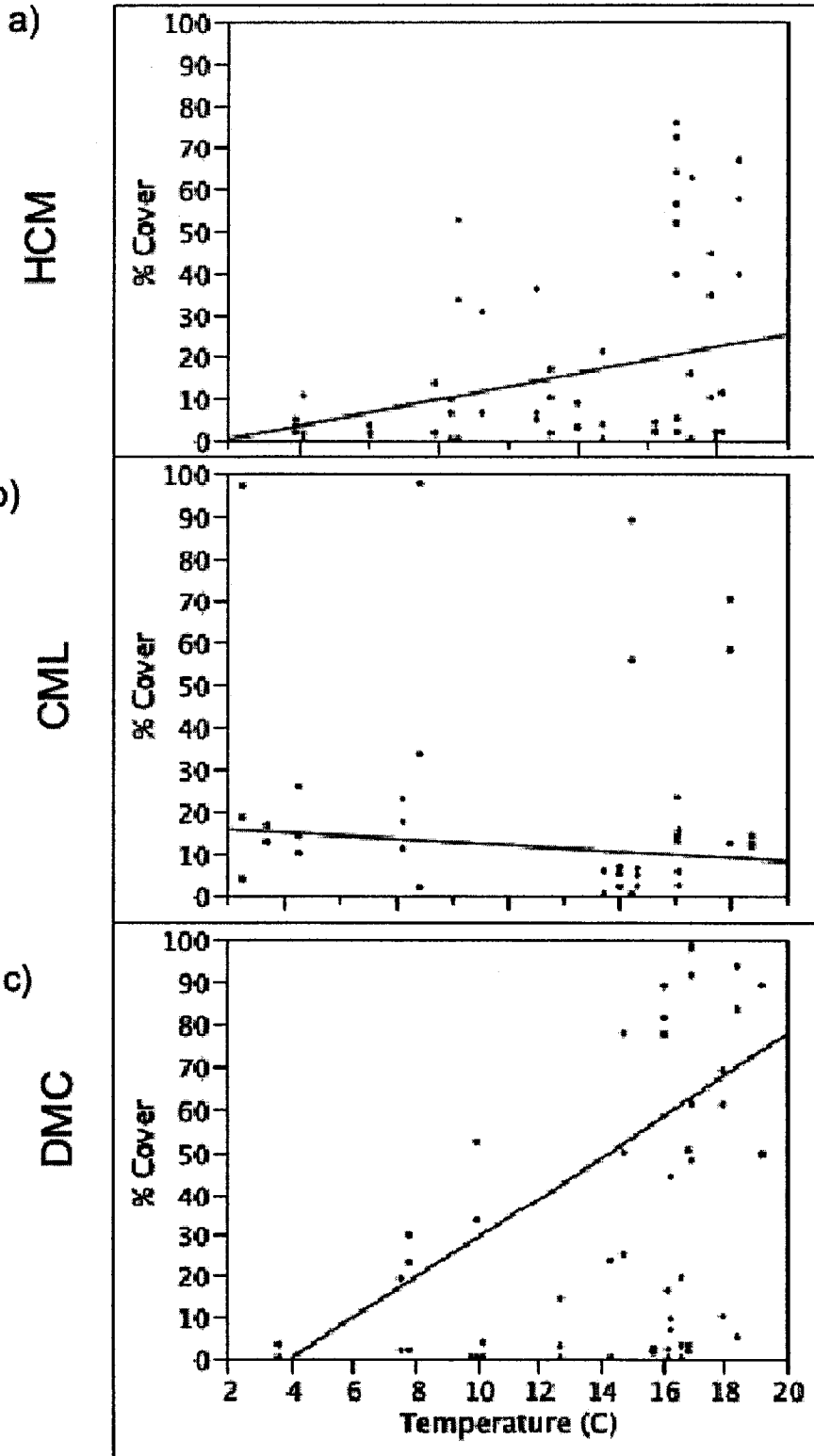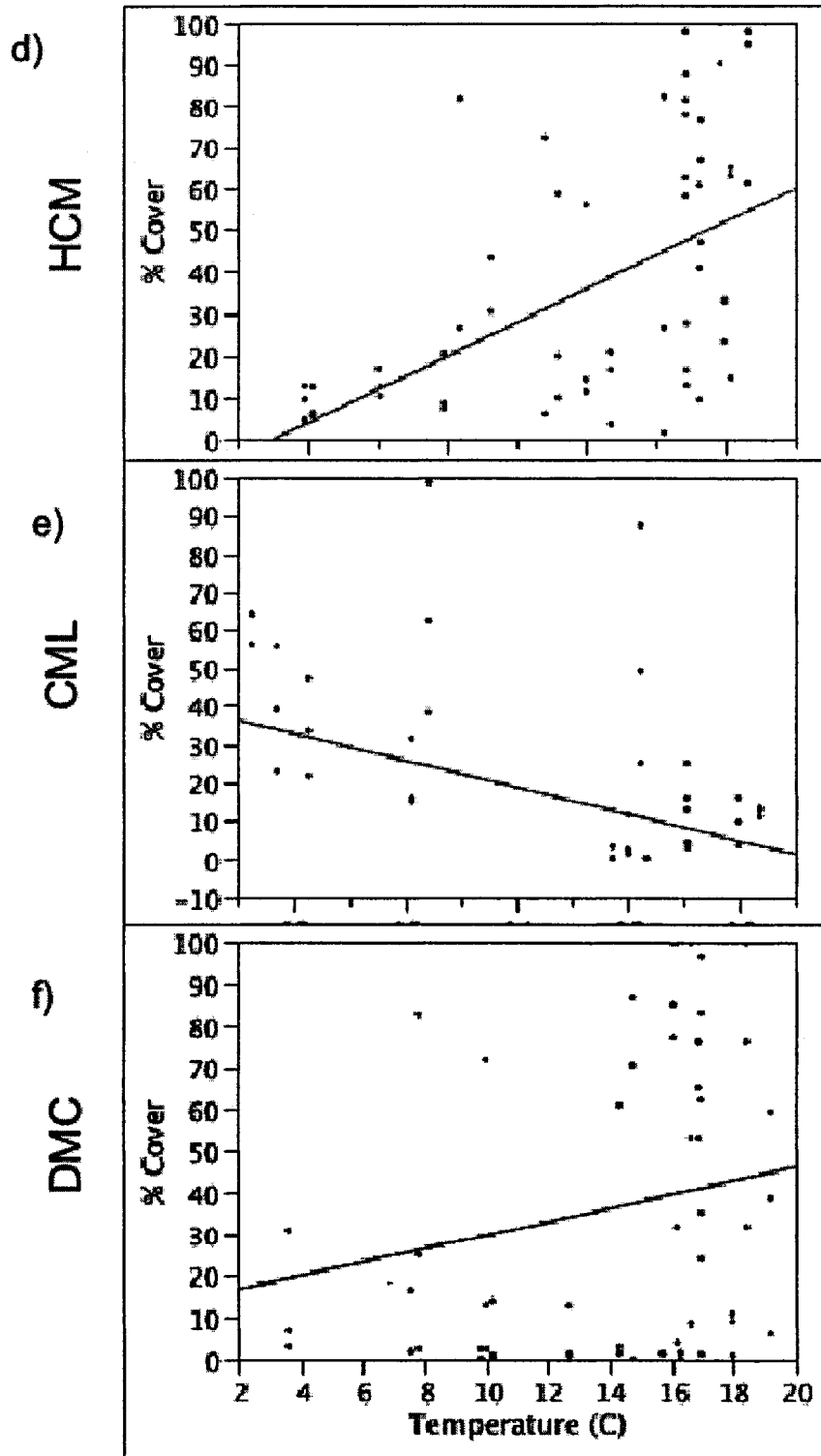
University of New Hampshire Coastal Marine Laboratory

Description of Colony Abundance and Growth Patterns.

Botrylloides violaceus had one period of maximum percent cover during

the growing seasons of both 2005 and 2006. Maximum percent cover occurred

November 16 in 2005 and almost a month earlier in 2006 (October 19) (Figure

10c). *Botryllus schlosseri* also had one period of maximum percent cover during

the growing seasons of both 2005 and 2006. Maximum percent cover occurred

November 16 in 2005 and two months earlier in 2006 (September 9). Maximum

percent cover for *Botrylloides violaceus* occurred at the same time as maximum

percent cover for *Botryllus schlosseri* in 2005, and six weeks later than maximum

percent cover for *Botryllus schlosseri* in 2006. The decline in percent cover at

the end of the growing season for *Botrylloides violaceus* occurred two weeks

after that of *Botryllus schlosseri* in 2005 and six weeks after that of *Botryllus*

schlosseri in 2006. The maximum number of colonies of *Botrylloides violaceus*

found on a single panel was 63 during 2005 and 56 during 2006 (Figure 10d).

The maximum number of colonies of *Botryllus schlosseri* found on a single panel

was 11 during 2005 and 7 during 2006.

Figure 14: a) Percent cover was not correlated with temperature for *Botryllus schlosseri* at HCM in 2006. (R^2=0.099, p value=0.124) b) Percent cover was correlated with temperature for *Botrylloides violaceus* at HCM. 3rd degree polynomial, y=0.344(x-15.727)^3+3.235(x-15.727)^2+1.289x-1.488, R^2=0.602, p value=0.014. c) Percent cover versus temperature for *Botryllus schlosseri* at CML was not correlated. d) Percent cover was negatively correlated with temperature for *Botrylloides violaceus* at CML, y= -8.366x+150.205, R^2=0.543, p value=0.0005. e) Percent cover was positively correlated with temperature for *Botryllus schlosseri* at DMC, y=5.396x-63.806, R^2=,0.388 p-value <0.002. f) Percent cover was positively correlated with temperature for *Botrylloides violaceus* at DMC, y=5.138x-52.988 R^2=0.355, p value<0.0004.

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Botryllus schlosseri
2006

a) HCM

b) CML

c) DMC

Temperature (C)

57

Botrylloides violaceus

2005

d) HCM

e) CML

f) DMC

58
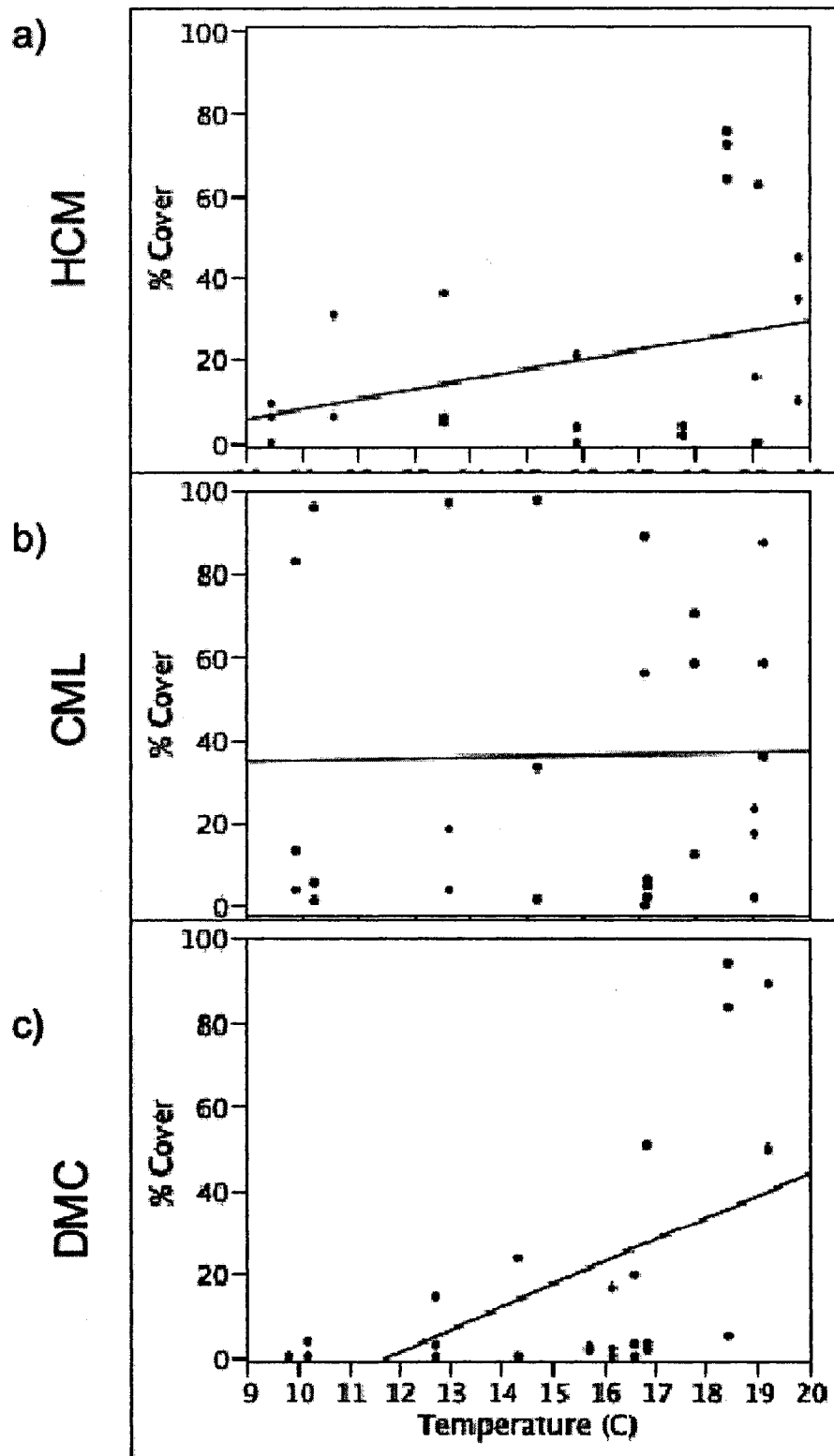
Figure 15: a)2006 *Botryllus schlosseri* percent cover was negatively correlated with time at HCM, y=15416.262-47035e-6x, R^2=0.3538, p=0.004. b) 2006 *Botrylloides violaceus* percent cover was correlated with time by a 3rd degree polynomial, y=44203.86-0.0000135x-1.963e-12(x-3.27e+9)^2+6.49e-16(x-3.27e+9)^3, R^2=0.466, p=0.0008 . c) 2006 *Botryllus schlosseri* percent cover was not correlated with time, R^2=0.144, p=0.0291. d) 2006 *Botrylloides violaceus* percent cover was positively correlated with time, y=7.8012e^{-6}x-25494.44, R^2=0.714, p<0.001. e) 2006 *Botryllus schlosseri* percent cover was negatively correlated with time at DMC, y=10642.881-3.247e-6x, R^2=0.182, p=0.0133. f) *Botrylloides violaceus* percent cover was correlated with time by a 2nd degree polynomial, y=4380.54-1.322e-6x-1.874e-12(x-3.27e+9)^2, R^2=0.406, p=0.0002.
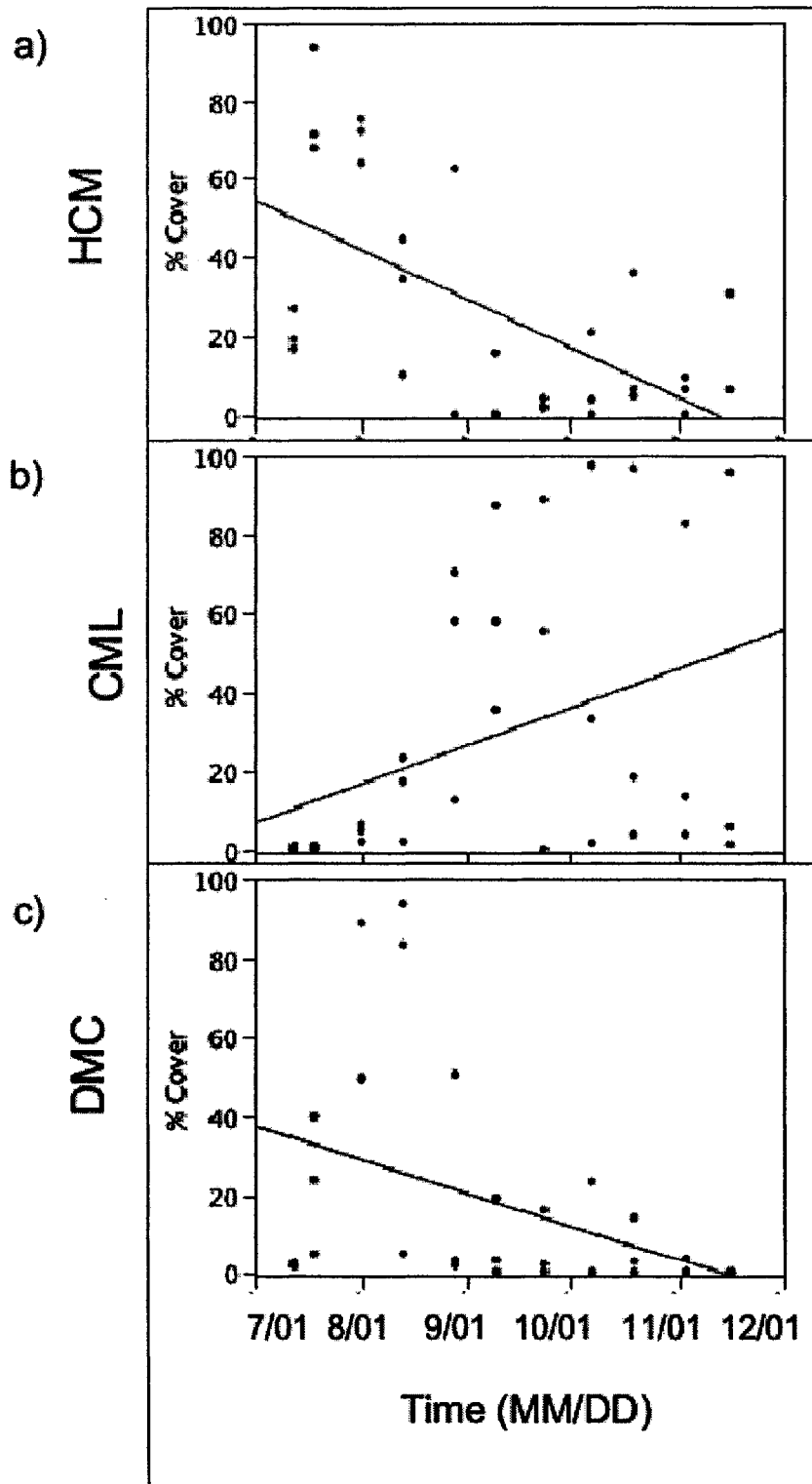
59

Botryllus schlosseri
2006

Botrylloides violaceus

2006
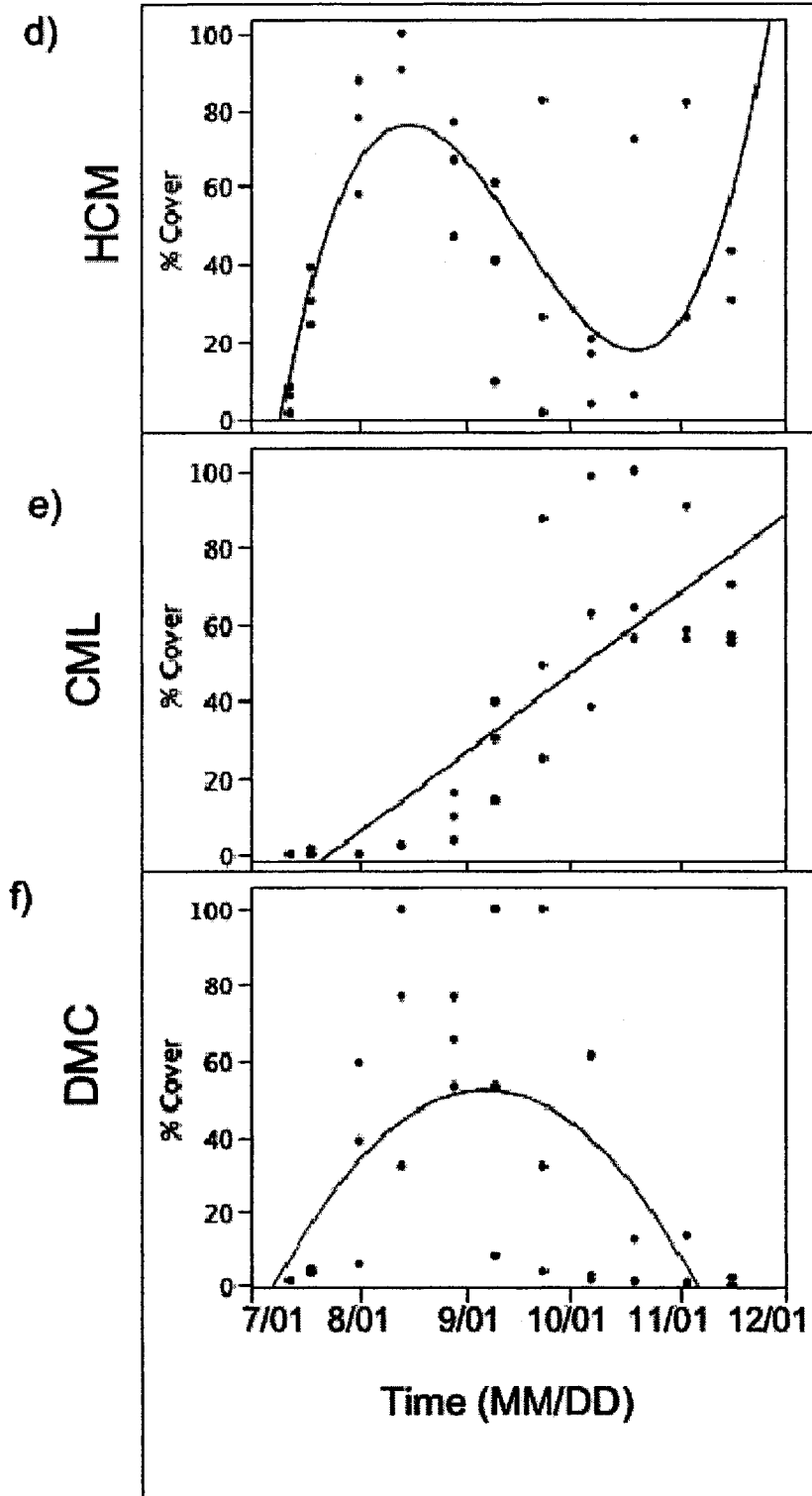
Botrylloides violaceus and Botryllus schlosseri showed similar abundance patterns at CML in both 2005 and 2006 (Figure 11c and 11d). They also showed similar abundance patterns between 2005 and 2006 at CML (Figure 12c and 12d). Botrylloides violaceus percent cover was negatively correlated with temperature at CML in 2005 (R^2=0.515, p<0.001, one way ANOVA) (Figure 13e). In 2006 Botrylloides violaceus percent cover was negatively correlated with temperature (y= -8.366x+150.205, R^2=0.543, p value=0.0005) and positively correlated with time at CML (Fig 14e and 15e). Botryllus schlosseri percent cover was correlated with neither temperature nor time at CML in 2005 or 2006.

University of Maine Darling Marine Center

Description of Abundance and Growth Patterns.

Botrylloides violaceus had one period of maximum percent cover during the growing seasons of 2005 and 2006. Maximum percent cover occurred September 23 in 2005 and a month earlier in 2006 (August 13) (Figure 10e). Botryllus schlosseri also had one period of maximum percent cover during the growing seasons of 2005 and 2006 at DMC. Maximum percent cover occurred September 9 in 2005 and two weeks earlier, on August 1, in 2006. The maximum percent cover for Botrylloides violaceus occurred two weeks after the maximum percent cover for Botryllus schlosseri in 2005 and 2006. The decline

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in percent cover at the end of the growing season for *Botrylloides violaceus* also occurred two weeks after that of *Botryllus schlosseri* in 2005 and 2006. The maximum number of colonies of *Botrylloides violaceus* found on a single panel was 12 during 2005 and 13 during 2006 (Figure 10f). The maximum number of colonies of *Botryllus schlosseri* found on a single panel was 4 during 2005 and 23 during 2006 (Figure 10f).

Spatial Coverage Patterns Between Species.

There was an almost significant difference in the spatial coverage patterns between species at DMC during 2005 (repeated measures MANOVA, $p=0.057$) (Figure 11e). There was a trend towards earlier growth of *Botryllus schlosseri* relative to that of *Botrylloides violaceus* at the DMC, an area that did not receive as much rainfall during the spring of 2006 as the other two sites, though this trend was not statistically significant (Figure 11f and Figure 5).

Spatial Coverage Patterns Between Years.

There was a trend towards earlier settlement and growth of *Botrylloides violaceus* at DMC in 2006 relative to that in 2005, however this relationship was not statistically significant (Fig 13f). There were significant differences in the spatial coverage patterns between the two years for *Botryllus schlosseri* at DMC, with peak percent cover occurring later, lasting longer, and being greater in 2005 (repeated measures MANOVA, $p=0.008$) (Fig 12e).

<u>Spatial Coverage Patterns and Temperature.</u>

Botryllus schlosseri percent cover was positively correlated with average biweekly water temperature in 2005 (y=4.86x-19.703, R^2=0.417, p= 0.002) (Figure 13c) while Botrylloides violaceus percent cover was not correlated with temperature. Percent cover was positively correlated with average biweekly water temperature May-December 2006 at DMC for both Botrylloides violaceus (y=5.138x-52.988 R^2=0.355, p value<0.004) and Botryllus schlosseri (y=5.396x-63.806, R^2=,0.388 p-value <0.002) (Figure 14c, 14f). Percent cover of Botryllus schlosseri was negatively correlated with time at DMC during 2006 suggesting that there was only one cohort of colonies on the panels. Percent cover of Botrylloides violaceus was parabolically correlated with time at DMC 2006, suggesting that Botrylloides violaceus has a similar pattern to that of Botryllus schlosseri (growth, reproduction, and death, with colonies sloughing off the panel) but that it's initiated two weeks later (Figure 15c, 15f).

Comparisons between field sites

<u>Abundance and growth patterns between sites.</u>

There was a significant difference in the seasonal timing of the growth patterns between the three sites in 2006 for both species, with initial growth bursts occurring first at HCM, then DMC, and finally at CML (Figure 16a and 16b). Botrylloides violaceus did not exhibit significantly different patterns of growth across the three sites for either 2005 or 2006. Botryllus schlosseri

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percent cover patterns were significantly different between the three sites during 2005, with initial growth first occurring at HCM and then at DMC (repeated measures MANOVA p=0.004) (Fig 16c). There was also a second peak in percent cover for both species at HCM that didn't occur at DMC, suggesting a longer growth season at HCM than DMC.

Botryllus schlosseri

Botrylloides violaceus
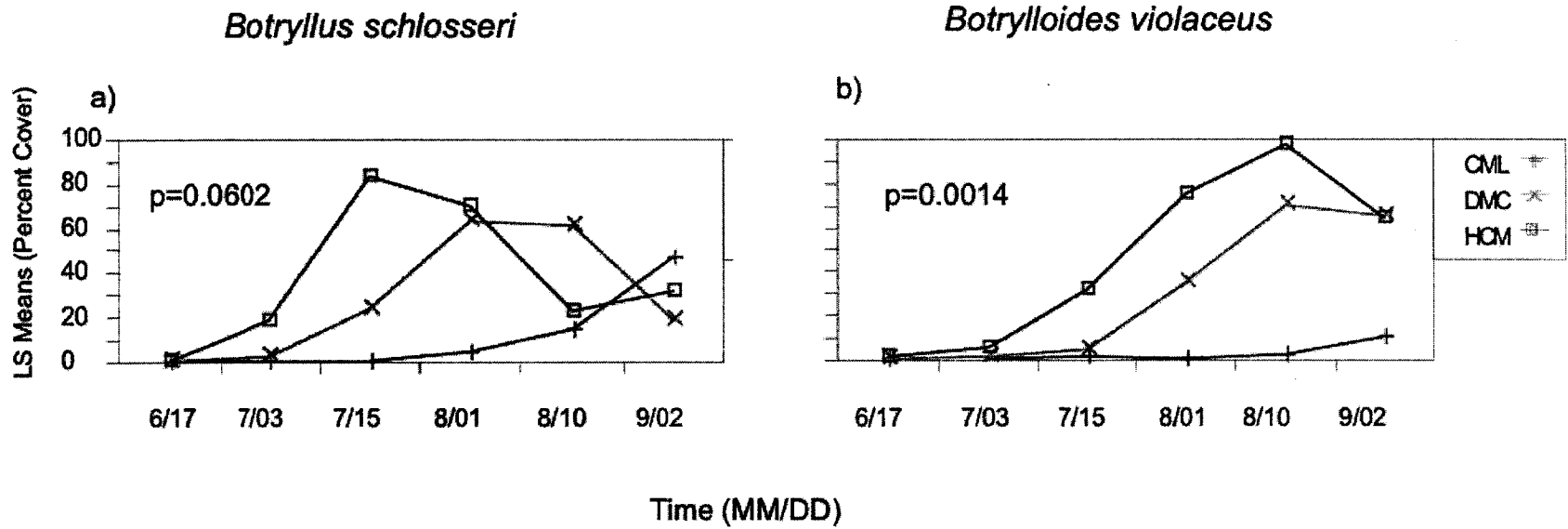
Figure 16: a) Percent cover of *Botryllus schlosseri* July-September 2006. There was a significant difference in abundance patterns across the three sites, repeated measures MANOVA, p=0.0602. b) Percent cover of *Botrylloides violaceus* July-September 2006. There was a significant difference in abundance patterns across the three sites, repeated measures MANOVA, p=0.0014.

c) *Botryllus schlosseri*

d) *Botrylloides violaceus*

p=0.0025

7/12 7/18 8/13 8/28 9/09 9/23 10/07 11/03 11/16 12/03 12/18 12/29

7/12 7/18 8/01 8/13 8/28 9/09 9/23 10/07 11/16 12/03 12/18 12/29

LS Means (Percent Cover)

CML
DMC
HCM

Time (MM/DD)
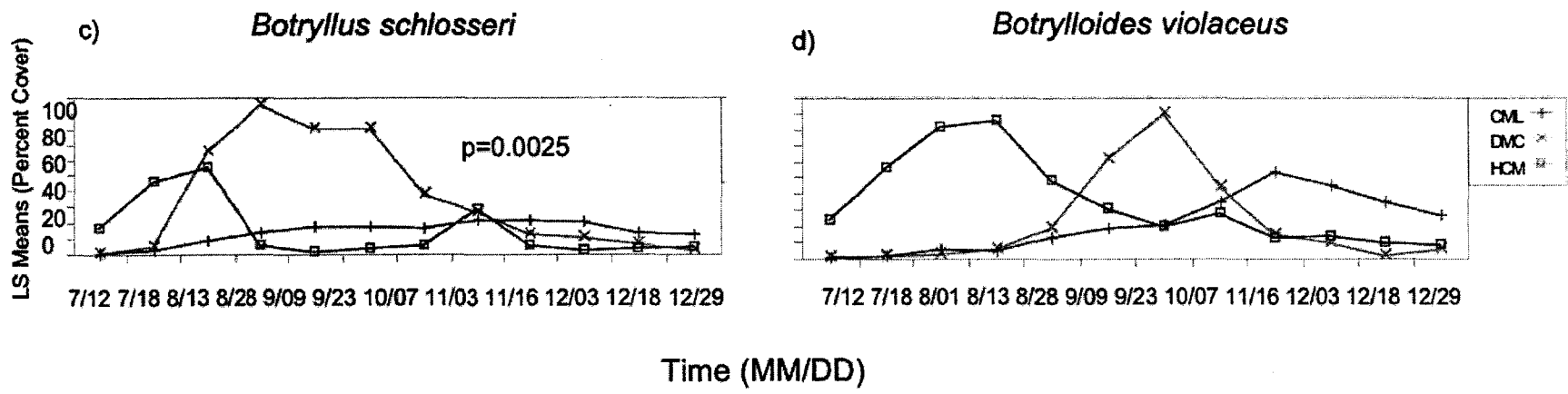
Figure 16: c) Percent cover of *Botryllus schlosseri* throughout 2005. A significant difference between the abundance patterns across the three sites was found using a repeated measures MANOVA, p=0.0025. d) Percent cover of *Botrylloides violaceus* throughout 2005. No significant differences in abundance patterns.

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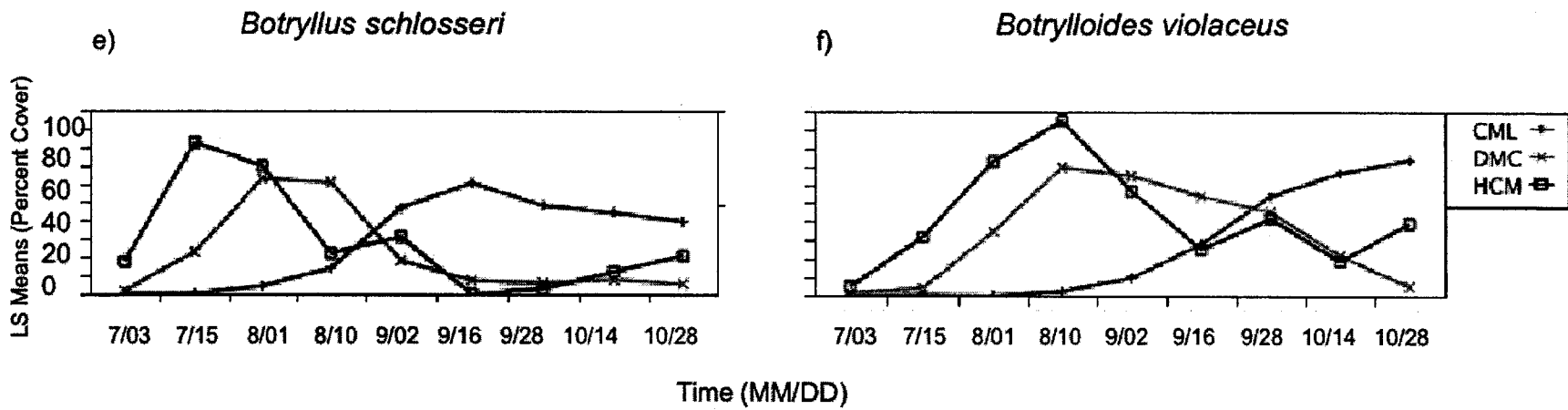
Figure 16: e) Percent cover of *Botryllus schlosseri* throughout 2006. There was no significant difference in abundance patterns. f) Percent cover of *Botrylloides violaceus* throughout 2006. There was no significant different in abundance patterns.

Hawthorne Cove Marina

Growth rates differed significantly between the two species at HCM, with

Botrylloides violaceus growing faster and having a higher maximum percent

cover than *Botryllus schlosseri* (repeated measures MANOVA, p=0.037) (Figure

17a). *Botrylloides violaceus* percent cover is negatively correlated with

temperature (y=-0.0932x+1.867, R^2=0.763, p=0.001) (Figure 18). Percent cover

of *Botrylloides violaceus* was parabolically correlated with that of *Botryllus*

schlosseri at HCM suggesting that the timing of the life cycles of these two

species at HCM are offset (y=-13.467(x-0.099)^2+3.418x+0.225, R^2=0.565,

p=0.0019) (Figure 19).
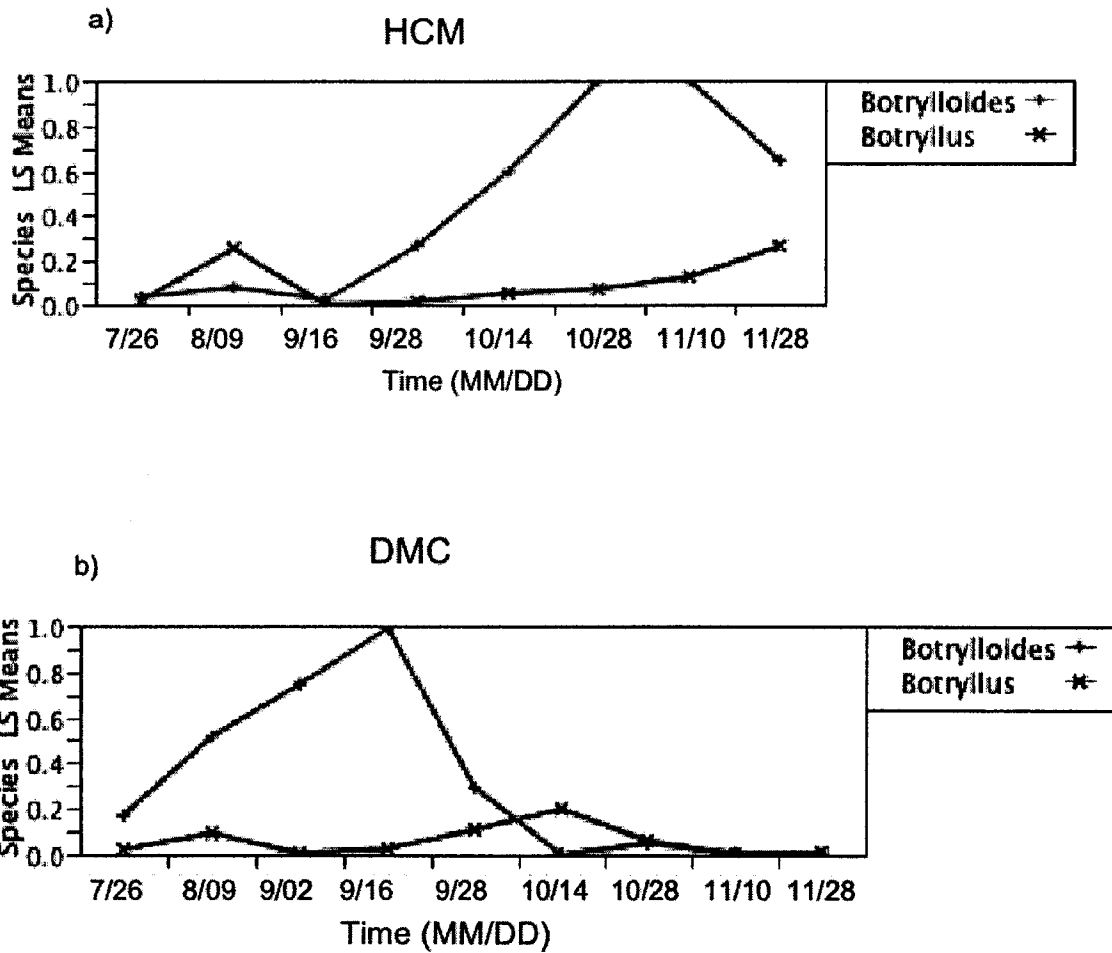
Figure 18: a) Single colony percent cover for *Botryllus schlosseri* and *Botrylloides violaceus* at HCM. Significant differences in abundance patterns, repeated measures MANOVA, p=0.037 b) Single colony percent cover for *Botryllus schlosseri* and *Botrylloides violaceus* at DMC. Almost significant differences in abundance patterns, repeated measures MANOVA, p=0.082.
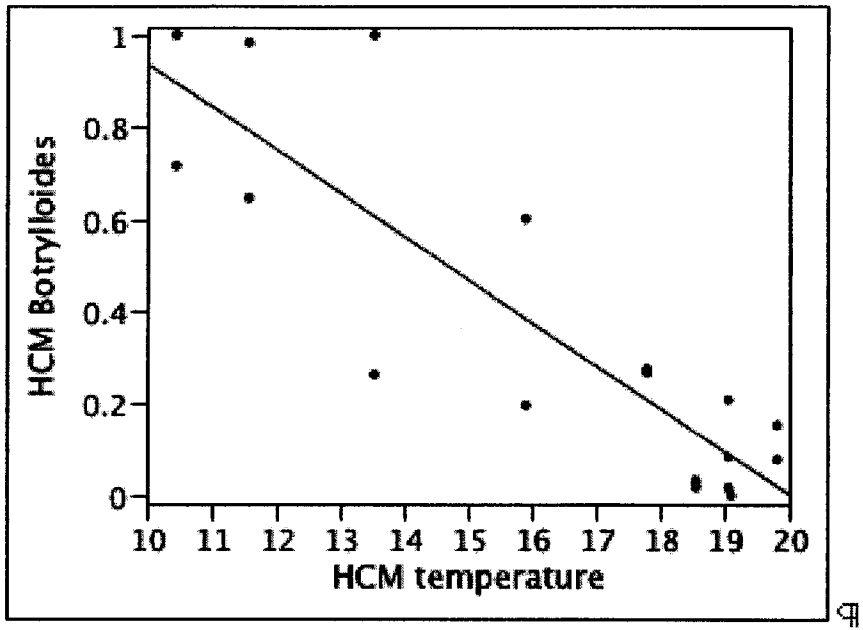
Figure 18: *Botrylloides violaceus* single colony percent cover at HCM is negatively correlated with temperature, y=-0.0932x+1.867, R^2=0.763, p=0.001.
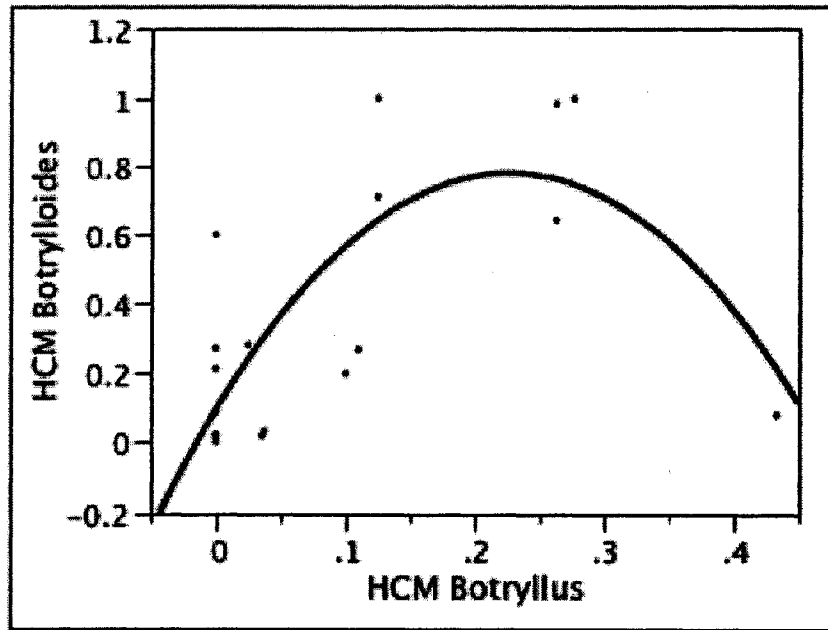
Figure 19: Correlation between *Botryllus schlosseri* percent cover and *Botrylloides violaceus* at HCM. ($y=-13.467(x-0.099)^2+3.418x+0.225$, $R^2=0.565$, $p=0.0019$).

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There was an almost significant difference in growth rates between the two species at DMC, with *Botrylloides violaceus* growing faster and achieving a higher percent cover than *Botryllus schlosseri* (repeated measures MANOVA, $p=0.082$) (Figure 17b). Percent cover of *Botrylloides violaceus* was not correlated with that of *Botryllus schlosseri*.

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Comparisons Between Sites

There was no significant difference in growth rates of either species between HCM and DMC (Figure 20). However, at HCM the pattern of percent cover exhibited by *Botrylloides violaceus* was almost identical to that found at DMC, but occurred two months later, suggesting that these growth patterns have little to do with temperature but that the initial start time of growing might, and that the species may have acclimated to their local environments (Fig 20b).

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a)

Botryllus schlosseri

b)

Botrylloides violaceus
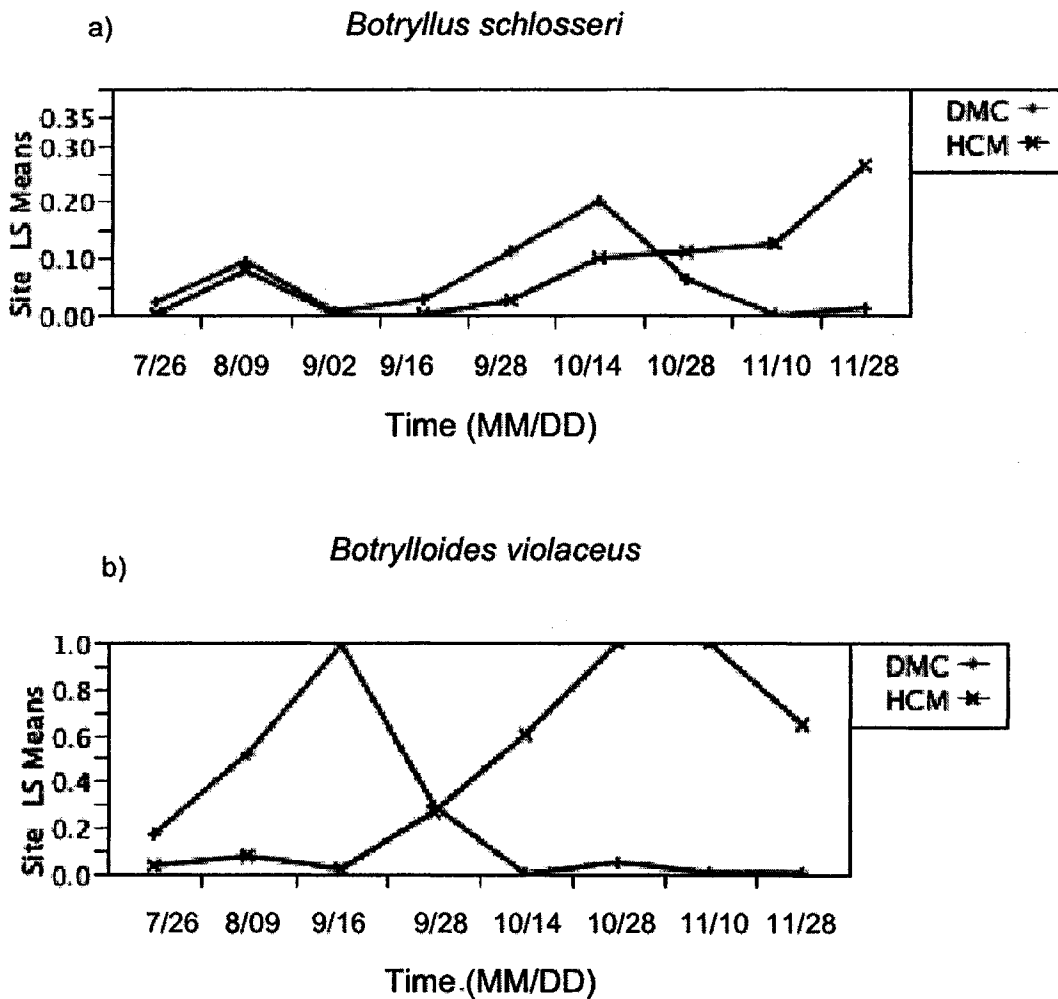
Figure 20: a) Single colony percent cover for *Botryllus schlosseri* at DMC and HCM. no significant difference in abundance patterns (repeated measures ANOVA, p-value=0.843). b) Single colony percent cover for *Botrylloides violaceus* at DMC and HCM, no significant difference in abundance patterns (repeated measures MANOVA, p-value=0.2063).

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Recruitment

Hawthorne Cove Marina

A substantial spring storm broke the panel set up at HCM in 2006,
resulting in a hole in the recruitment data set that may have coincided with peak
recruitment. Consequently, initial timing of *Botryllus schlosseri* recruitment
relative to that of *Botrylloides violaceus* is unknown. However, recruitment
occurred for both species throughout the growing season, and peak recruitment
for both *Botryllus schlosseri* and *Botrylloides violaceus* occurred during the two
weeks prior to September 9 (Figure 21a). There were also a significantly greater
number of *Botrylloides violaceus* recruits than *Botryllus schlosseri* recruits over
the entire recorded breeding season (Figure 21a). The recorded recruitment
peak occurred within a month of peak percent cover and coincided with a decline
in percent cover for both species. A second increase in percent cover also
occurred a month after peak recruitment (Fig 22a). There is a positive correlative
trend between recruitment and temperature for *Botryllus schlosseri* at HCM
(R^2=0.243, p-value=0.123) (Fig 23a). Recruitment was not correlated with
percent cover for either species at HCM.

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Coastal Marine Laboratory

Recruitment occurred throughout the growth season at CML, with peak recruitment for *Botryllus schlosseri* during the two weeks prior to July 18, and peak recruitment for *Botrylloides violaceus* during the two weeks prior to August 28 (Fig 21b). Outside of the month and a half from July 18 to September 9 number of recruits was comparable for both species. Recruitment was not correlated with temperature or percent cover for either species at CML. There was no direct correlation between high recruitment and high percent cover, though there was a two to three week lag in peak percent cover after peak recruitment (Figure 22b).

76
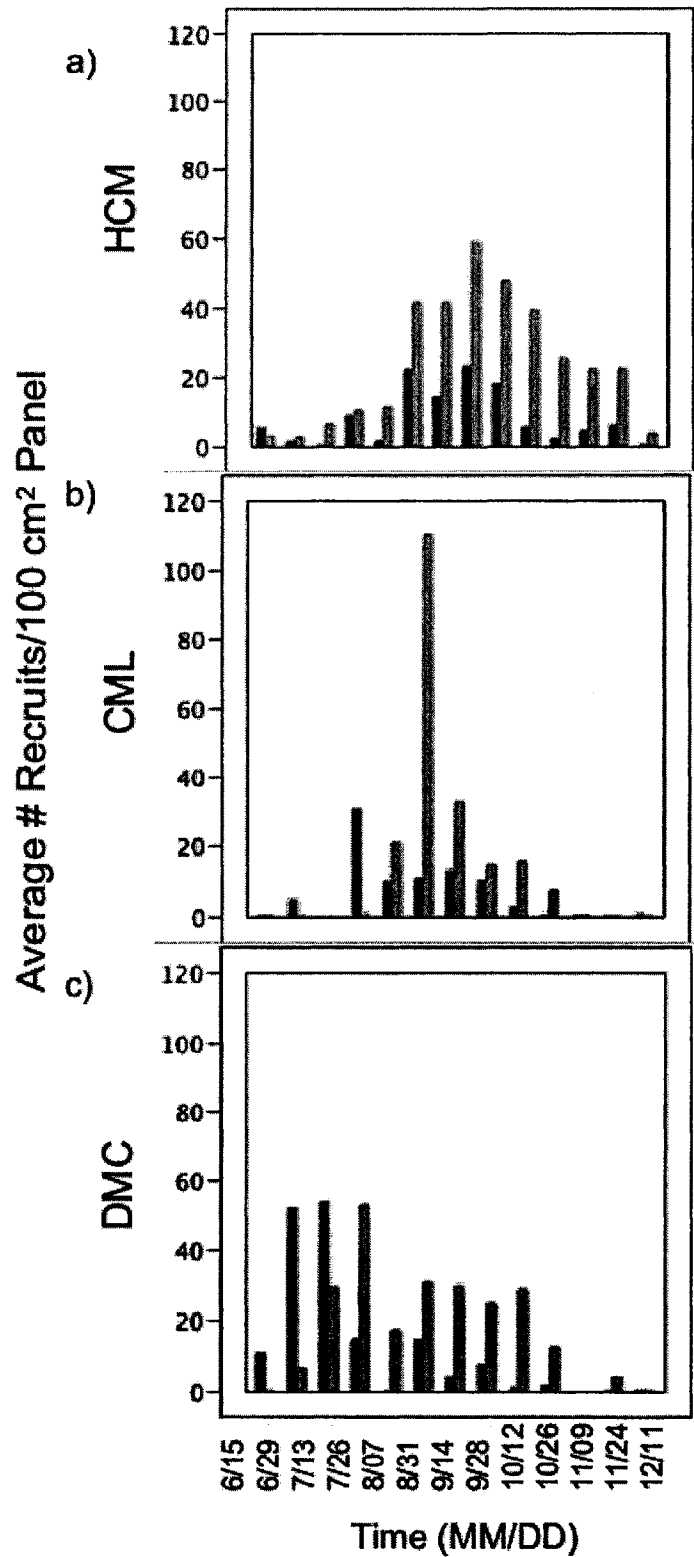
Figure 21: Recruitment of *Botryllus schlosseri* (black) and *Botrylloides violaceus* (grey) at HCM. b) Recruitment of *Botryllus schlosseri* and *Botrylloides violaceus* at CML. c) Recruitment of *Botryllus schlosseri* and *Botrylloides violaceus* at DMC.

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Figure 22: a) Recruitment and percent cover for *Botryllus schlosseri* and *Botrylloides violaceus* at HCM. b) Recruitment and percent cover for *Botryllus schlosseri* and *Botrylloides violaceus* at CML. c) Recruitment and percent cover for *Botryllus schlosseri* and *Botrylloides violaceus* at DMC.

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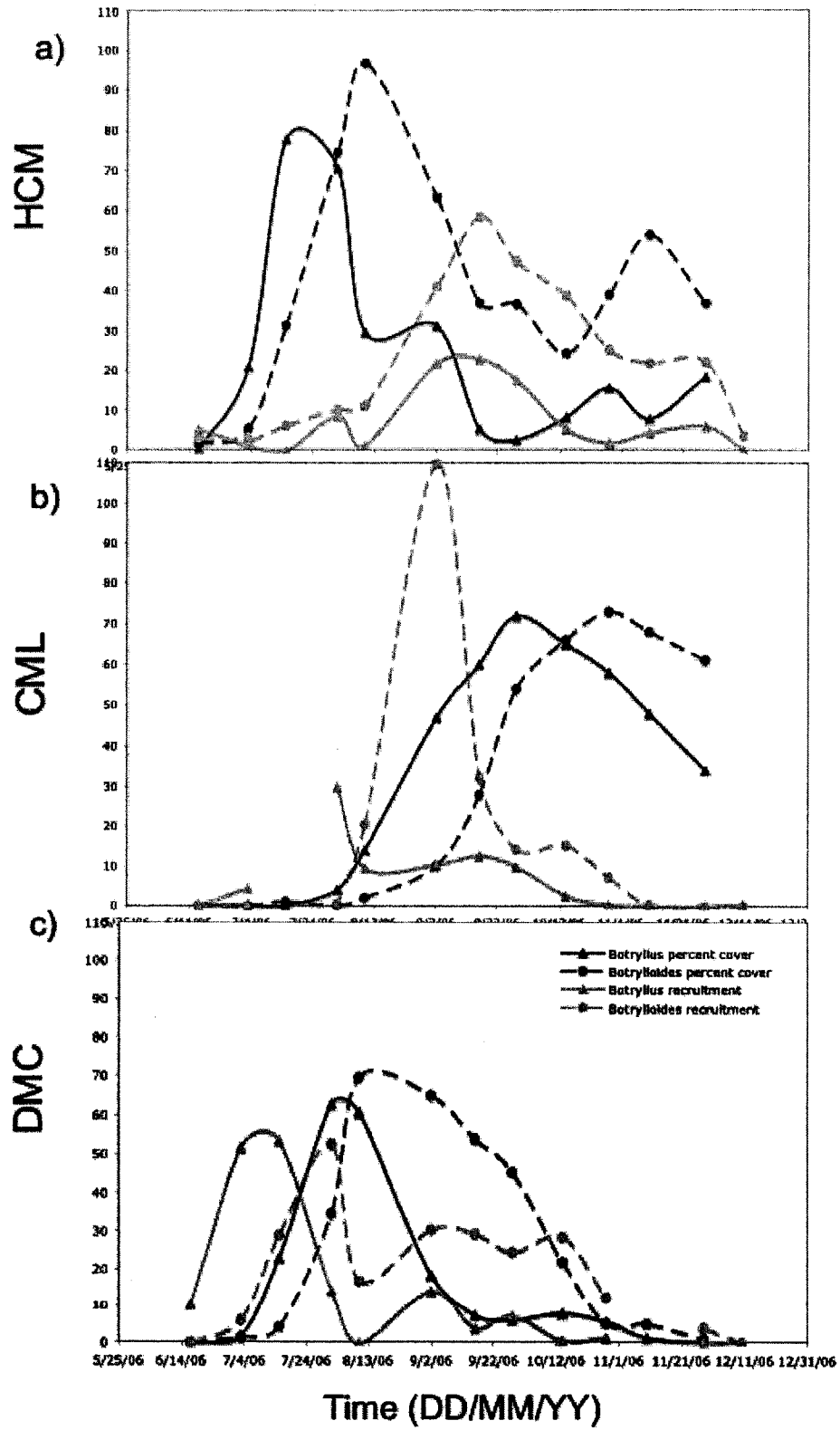
Figure 23: Recruitment is not significantly correlated with temperature for *Botryllus schlosseri* at HCM. R^2=0.243, p-value=0.123 b) Recruitment is not correlated with temperature for *Botrylloides violaceus* at HCM, R^2=0.053, p-value=0.498. c) Recruitment is not correlated with temperature for *Botryllus schlosseri* at DMC. R^2=0.085, p-value=0.38 d) Recruitment is positively correlated with temperature for *Botrylloides violaceus* at DMC,y=3.461x-32.0347, R^2=0.546, p-value=0.009.

Botryllus schlosseri

Botrylloides violaceus

a) HCM — HCM Botryllus recruitment vs HCM Temperature

b) HCM Botrylloides recruitment vs HCM Temperature

c) DMC — DMC Botryllus recruitment vs DMC Temperature

d) DMC Botrylloides recruitment vs DMC Temperature
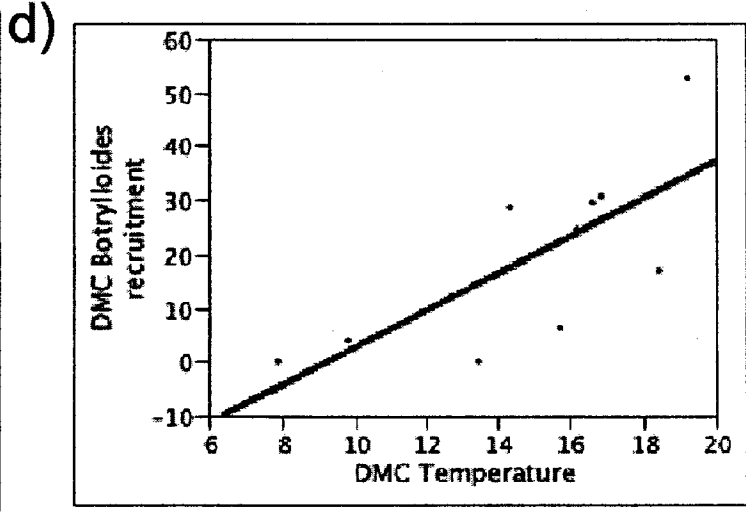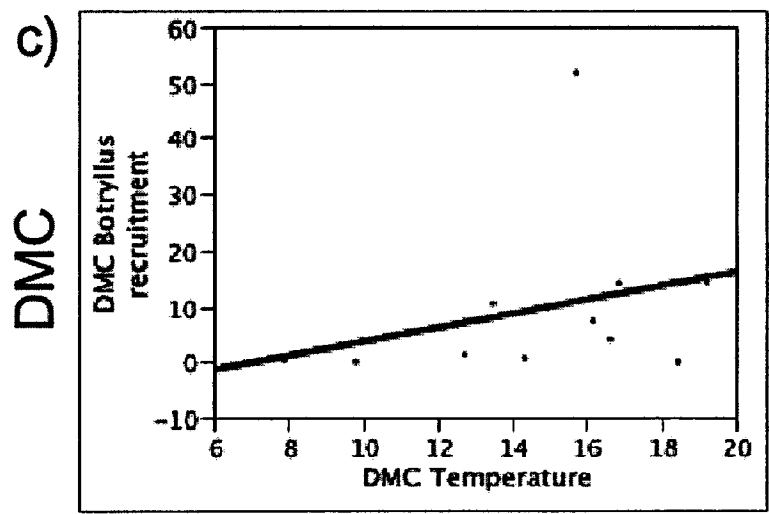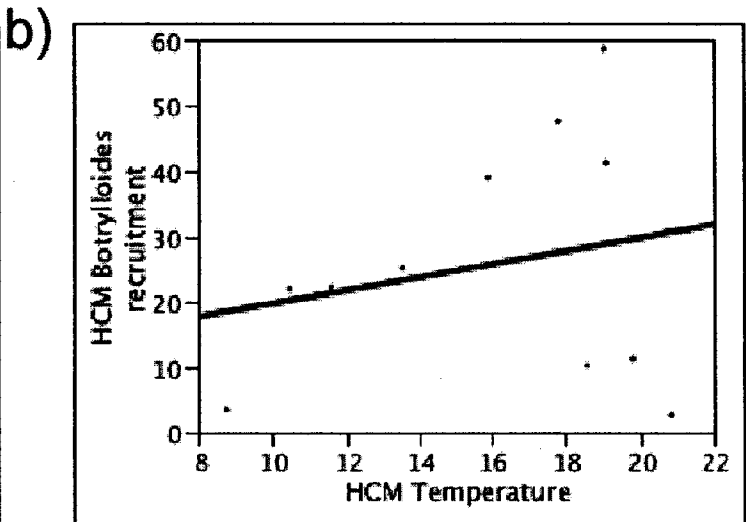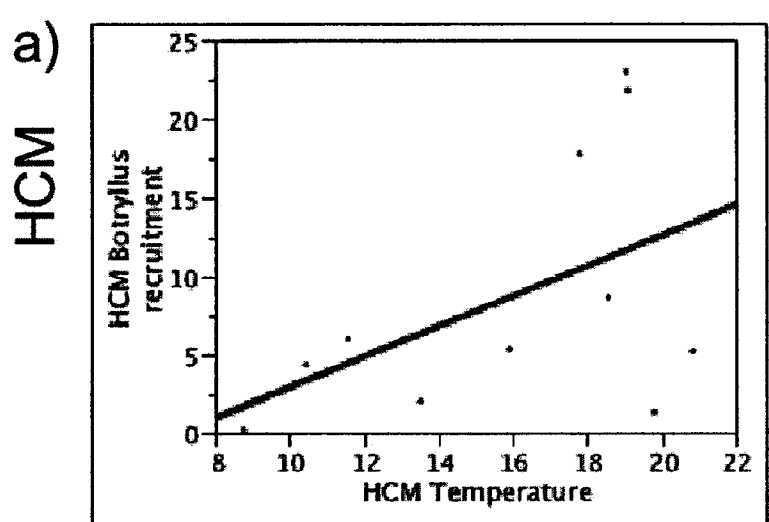
Darling Marine Center

       Recruitment occurred throughout the growing season at DMC, with peak recruitment for *Botryllus schlosseri* during the two weeks prior to July 12 and for *Botrylloides violaceus* during the two weeks prior to July 18. Initial recruitment of *Botryllus schlosseri* occurred two weeks prior to that of *Botrylloides violaceus* (Fig 21c). Peak recruitment of *Botryllus schlosseri* (68) was higher than that of *Botrylloides violaceus* (53), however overall recruitment of *Botrylloides violaceus* was higher than that of *Botryllus schlosseri*. Recruitment was positively correlated with temperature for *Botrylloides violaceus* at DMC (y=3.461x-32.0347, R^2=0.546, p-value=0.009) (Fig 23d). Recruitment was positively, almost significantly correlated with percent cover for *Botrylloides violaceus* at DMC (y=0.280x-13.670, R^2=0.249, p-value=0.118) (Fig 24). There was no direct correlation between high recruitment and high percent cover, however there was a two to three week lag in peak percent cover after peak recruitment for both species (Fig 22c).

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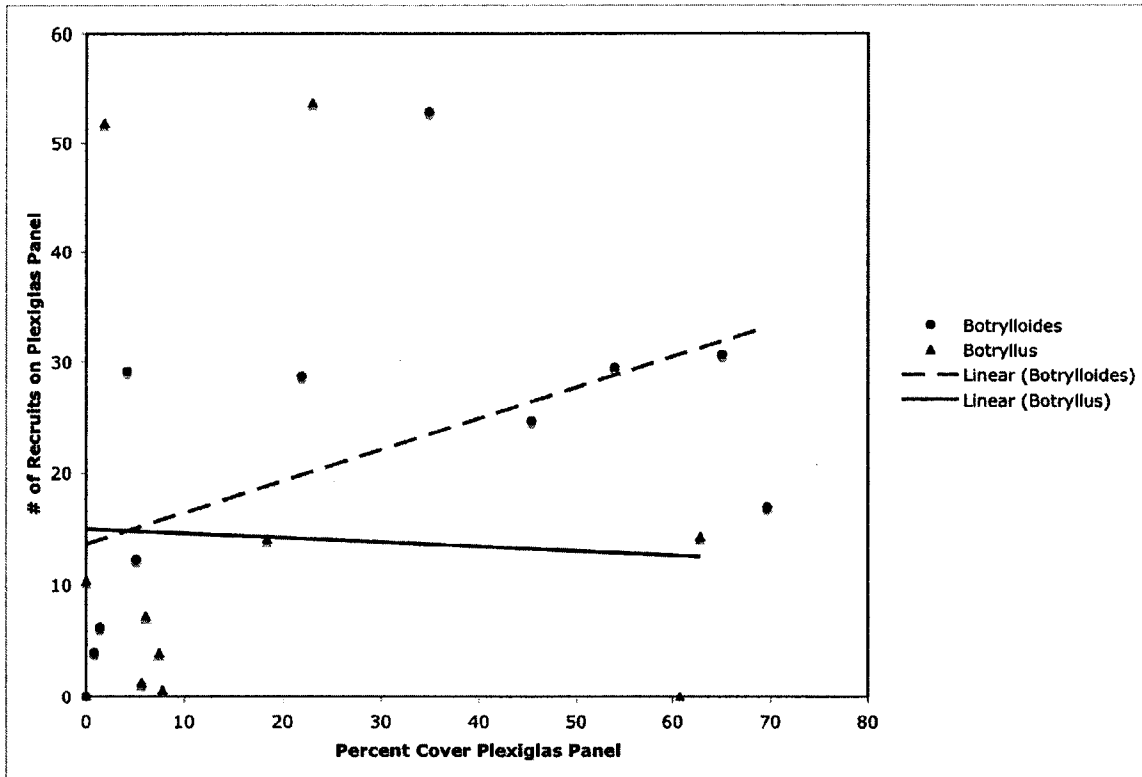
Figure 24: Recruitment is positively, but not significantly correlated with percent cover for *Botrylloides violaceus* at DMC, y=0.280x-13.670, R^2=0.249, p-value=0.118.

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Comparisons Between Sites

Initial recruitment for *Botryllus schlosseri* occurred at HCM a month earlier than at DMC and a month and a half earlier than at CML (Fig 25a). Initial recruitment for *Botrylloides violaceus* occurred at HCM a month earlier than at DMC and two months earlier than at CML (Fig 25b). *Botryllus schlosseri* recruitment continued a month longer at HCM than at DMC and CML. *Botrylloides violaceus* recruitment continued at HCM two weeks longer than at DMC and a month longer than at CML (Figure 25a). Initial recruitment for both species at CML occurred six weeks after initial recruitment at DMC; the end of the recruitment period at CML coincided with that of DMC (Figure 25).

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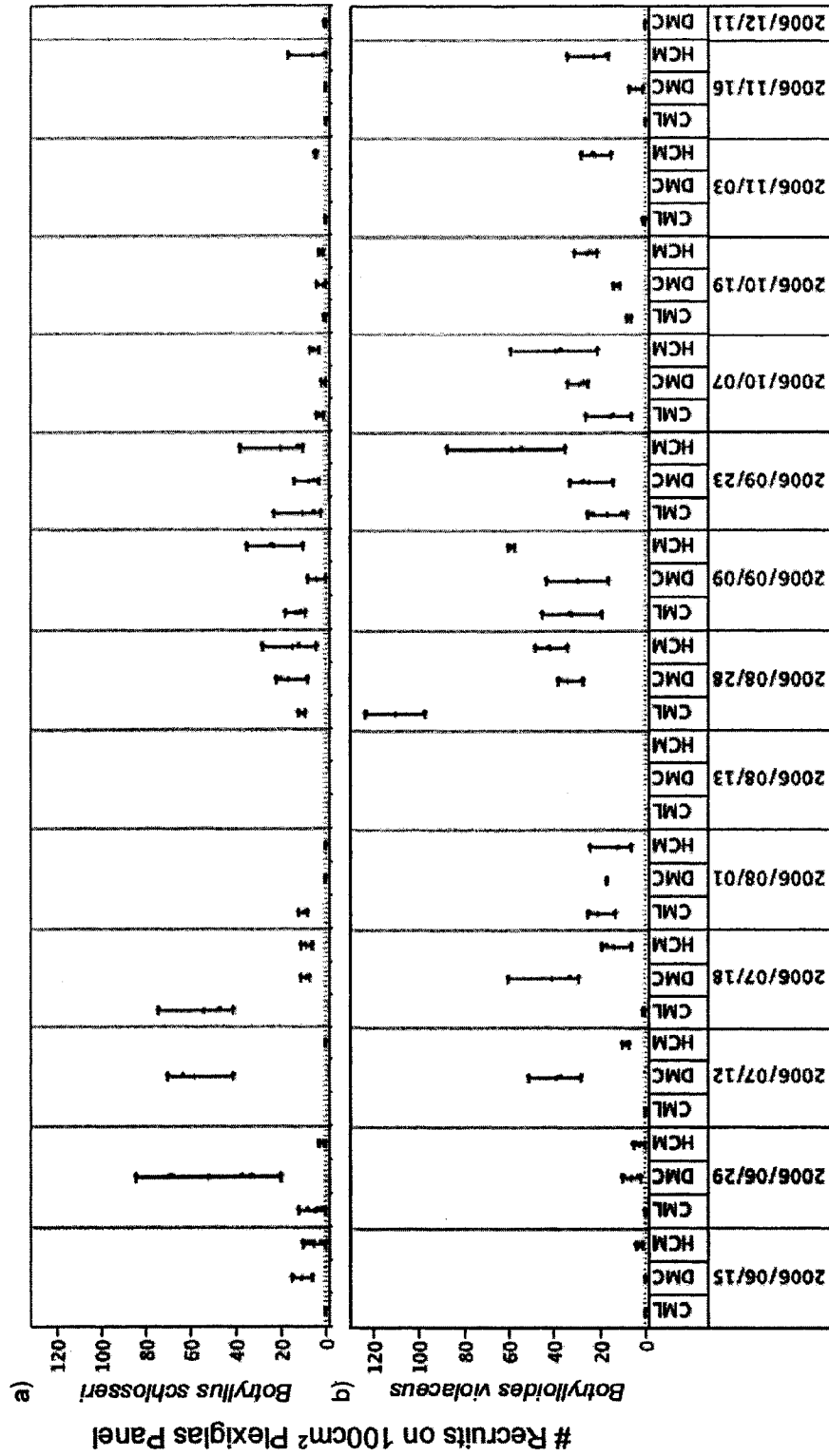
Figure 25: a) Mean recruitment for *Botryllus schlosseri* at HCM, CML, and DMC.
b) Mean recruitment for *Botrylloides violaceus* at HCM, CML, and DMC.

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Laboratory Studies

Colony Growth and Temperature

In order to reduce effects of the high amount of variability in growth both between colonies and within the life span of each colony, (Millar 1971) only the initial month of growth was used to examine the response of tunicates to temperature. During a one month period in the laboratory colonies went through zero to three asexual reproductive cycles depending on temperature, species, health, and reproductive stage of the colony.

Both *Botryllus schlosseri* and *Botrylloides violaceus* responded to increases in temperature by increasing the number of zooids after the first or second week of culturing and decreasing the duration of first and second blastogenic cycle (DBC) (N=135,y=-0.650x+25.741, R^2=0.073 p=0.0015) (Figure 26 and 27). However, temperature correlated more consistently with DBC than with weekly percent increase in number of zooids in both species (Tables 2-6). Also, when there was a positive correlation between DBC and percent increase in number of zooids in week 1 there was a negative correlation between DBC and percent increase in week 2, demonstrating that the asexual reproduction cycle took more than a week under these culturing conditions, often even at 20°C (Tables 2-6).

Both ascidian species showed similar patterns of shorter blastogenic

cycles at higher temperatures. However, when one examined the timing of

takeover in one colony relative to those of other colonies in the same tank, it

became apparent that all colonies in the tank were cycling together. Not only

were *Botryllus schlosseri* colonies cycling with other *Botryllus schlosseri* colonies

and *Botrylloides violaceus* colonies cycling with other
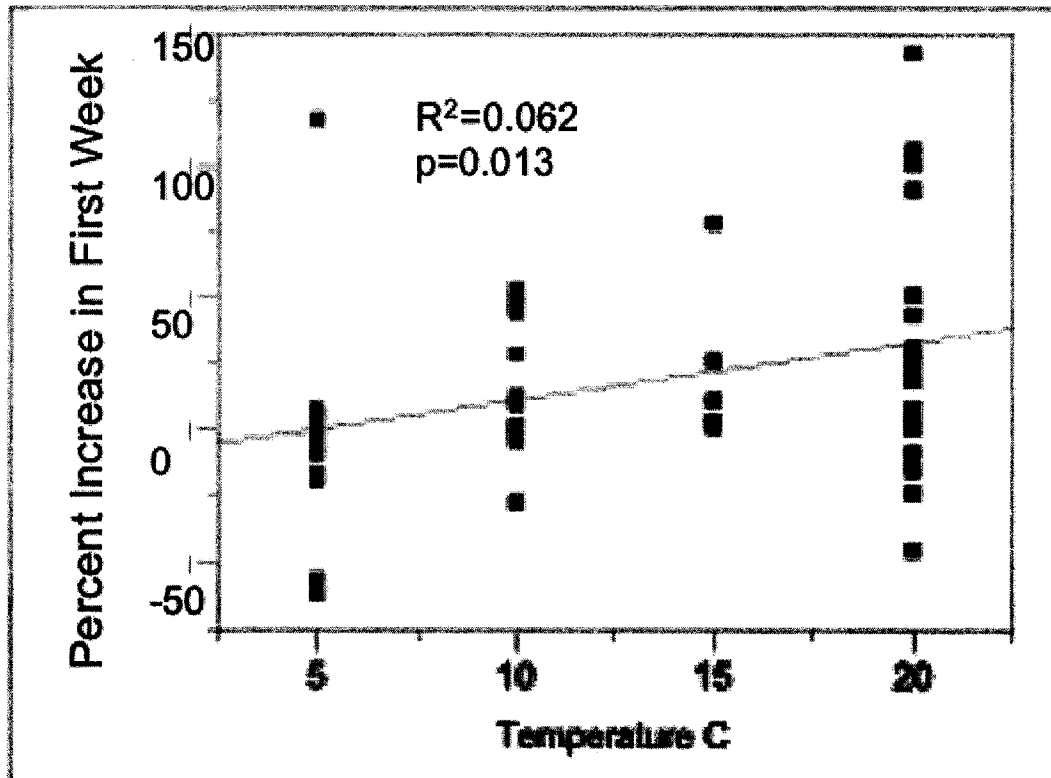
Figure 26: Percent increase in number of zooids at the end of week one for *Botryllus schlosseri* colonies from all sites combined. N=54, y=2.004x-6.857, R^2=0.062 p=0.0139.
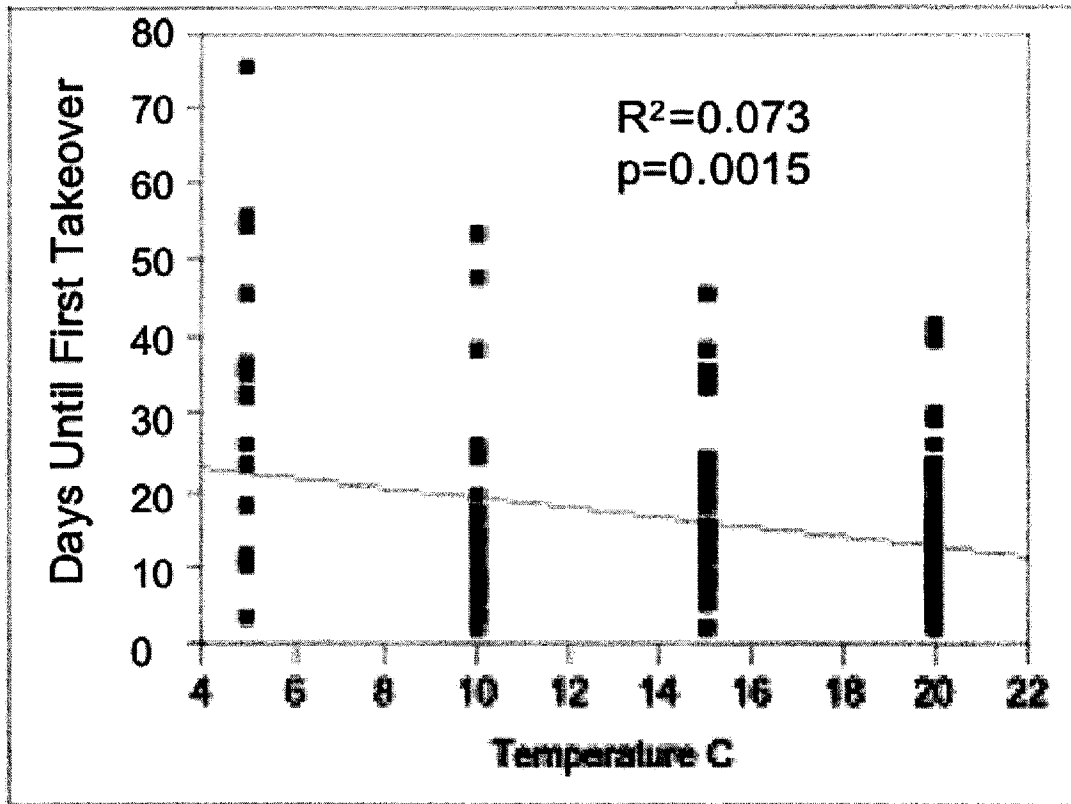
Figure 27: Bivariate fit of first DBC across temperature for laboratory reared colonies of both *Botryllus schlosseri* and *Botrylloides violaceus*. There is a significant decrease in time to take over with an increase in temperature, N=135,y=-0.650x+25.741, R^2=0.073 p=0.0015.

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	Temperature C	Percent Increase in first week	Percent increase in second week	Days until first take over	Life Span
Temperature C	1.0000	-0.1551	0.3155	-0.2576	0.2617
Percent Increase in first week	-0.1551	1.0000	-0.1721	-0.1547	-0.1999
Percent increase in second week	0.3155	-0.1721	1.0000	-0.2302	0.1111
Days until first take over	-0.2576	-0.1547	-0.2302	1.0000	-0.1142
Life Span	0.2617	-0.1999	0.1111	-0.1142	1.0000

Table 3: Correlations between temperature, varying measures of growth, and life span in *Botrylloides violaceus* colonies from the Darling Marine Center.

	Temperature C	Percent Increase in first week	Percent increase in second week	Days until first take over	Life Span
Temperature C	1.0000	0.2272	-0.2564	-0.1499	-0.1806
Percent Increase in first week	0.2272	1.0000	-0.0274	0.0278	0.5617
Percent increase in second week	-0.2564	-0.0274	1.0000	0.7559	0.1813
Days until first take over	-0.1499	0.0278	0.7559	1.0000	0.2776
Life Span	-0.1806	0.5617	0.1813	0.2776	1.0000

Table 4: Correlations between temperature, varying measures of colony growth, and life span in *Botrylloides violaceus* colonies from the Coastal Marine Laboratory.

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	Temperature C	Percent Increase in first week	Percent increase in second week	Days until first take over	Life Span
Temperature C	1.0000	0.3416	-0.3858	0.1919	-0.2898
Percent Increase in first week	0.3416	1.0000	-0.4475	0.1266	-0.0761
Percent increase in second week	-0.3858	-0.4475	1.0000	-0.1295	-0.0653
Days until first take over	0.1919	0.1266	-0.1295	1.0000	0.2500
Life Span	-0.2898	-0.0761	-0.0653	0.2500	1.0000

Table 5: Correlations between temperature, varying measures of colony growth, and life span in *Botrylloides violaceus* colonies from the Hawthorne Cove Marina.

	Temperature C	Percent Increase in first week	Percent increase in second week	Days until first take over	Life Span
Temperature C	1.0000	0.3214	0.0440	-0.2773	0.0007
Percent Increase in first week	0.3214	1.0000	-0.2024	-0.3831	-0.2133
Percent increase in second week	0.0440	-0.2024	1.0000	0.0385	0.2900
Days until first take over	-0.2773	-0.3831	0.0385	1.0000	-0.0877
Life Span	0.0007	-0.2133	0.2900	-0.0877	1.0000

Table 6: Correlations between temperature, varying measures of colony growth, and life span in *Botryllus schlosseri* colonies from the Darling Marine Center.

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	Temperature C	Percent Increase in first week	Percent increase in second week	Days until first take over	Life Span
Temperature C	1.0000	0.5995	0.1989	-0.9544	-0.0858
Percent Increase in first week	0.5995	1.0000	0.5706	-0.4554	-0.6505
Percent increase in second week	0.1989	0.5706	1.0000	0.0825	-0.2675
Days until first take over	-0.9544	-0.4554	0.0825	1.0000	0.0902
Life Span	-0.0858	-0.6505	-0.2675	0.0902	1.0000

Table 7: Correlations between temperature, varying measures of colony growth, and life span in *Botryllus schlosseri* colonies from the Hawthorne Cove Marina.

Botrylloides violaceus colonies, but *Botryllus schlosseri* colonies were cycling with *Botrylloides violaceus* colonies. Since both species were going through asexual cycles at the same time, comparing the difference in the change in duration of blastogenic cycle at different temperatures between species became a poor method of comparison. Therefore, only increase in number of zooids at takeover was used for comparisons between species.

Temperature and Asexual Cycling

Botryllus schlosseri and *Botrylloides violaceus* colonies maintained at 20°C and 15°C cycled together when cultured in the same tank. *Botrylloides violaceus* colonies stopped cycling with both other *B. violaceus* colonies and

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Botryllus schlosseri colonies at 10°C. *Botryllus schlosseri* colonies stopped cycling with other *B. schlosseri* colonies at 5°C.

Low Temperatures and Growth

Both *Botrylloides violaceus* and *Botryllus schlosseri* underwent what appeared to be normal colony growth and blastogenic cycling at all temperatures but 5°C. This temperature appeared to be low enough to alter their growth patterns, though in very different ways for the two species.

Botryllus schlosseri laboratory colonies reared at 5°C underwent very slow blastogenic cycles, but most of the time did not stop bud production. However, different individual colonies began growing in very different ways. One colony doubled the number of zooids in the colony after every blastogenic cycle but did not increase its surface area. The colony accomplished this by producing smaller zooids every cycle until they were too small to function, at which point the colony died. Other colonies reduced the amount of interstitial tissue between rosettes, slowly destroyed the blood vessels between them, and formed isolated rosettes that cycled together, were genetic clones, but were no longer connected by either blood vessels or interstitial tissue. Some colonies stopped cycling altogether and just maintained one generation.

Botrylloides violaceus colonies had a very different, but much less variable, response to the cold temperatures. They halted blastogenic cycles and slowly increased the number of ampullae on the surface of the colony. Zooids

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became full of pigmented cells and started to shrink as ampullae number

increased throughout the colony. Zooid number continued to decrease and

ampullae number increased until the colony under the tunic was an almost solid

mat of ampullae with a few very small zooids interspersed between the ampullae.

All outward appearances of the colony suggested it was dead, however when

one flipped the microscope slides holding these colonies over, one could see

blood flowing, at times quite quickly, through the colony. Colonies would

maintain this condition for a period of months, and then slowly come out of what

has become dubbed "hibernation" (Brunetti 1976). If kept at 5°C, the colony

would die a few days after it came out of hibernation.

Temperature and Growth in DMC Colonies

When looking at the data from DMC there is no correlation between

temperature and percent increase in number of zooids after the second

blastogenic cycle observed in the laboratory for *Botrylloides violaceus*, but a

positive correlative trend between temperature and percent increase in number

of zooids for *Botryllus schlosseri* (N=15, slope=4.66, R^2=0.263, p=0.0505)

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Percent increase in number of zooids, 2nd Takeover

Botryllus schlosseri   **Botrylloides violaceus**

HCM

a)

b)

DMC

c)
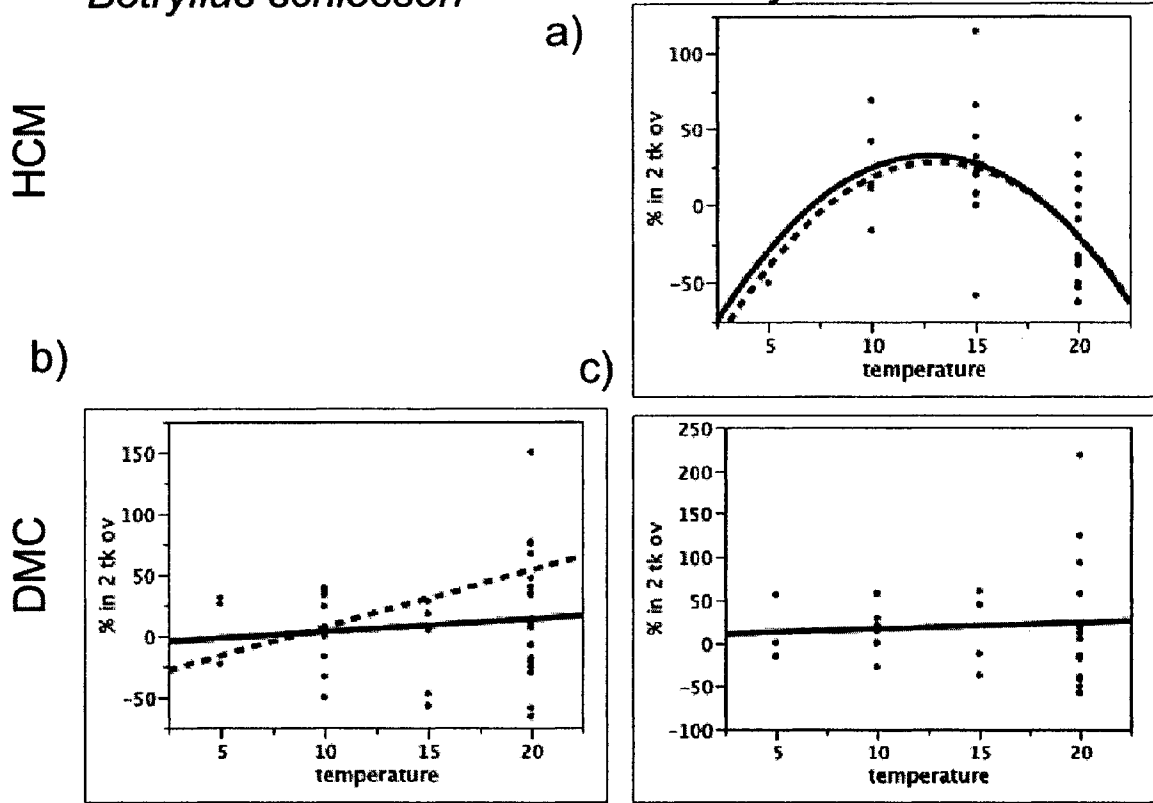
Figure 28: a) Bivariate fit of percent increase in second take over and temperature for HCM *Botrylloides violaceus*. $y=128.29-6.745x-1.023(x-16.47)^2$, $R^2=0.272$, p-0.0072. b) Bivariate fit of percent increase in second take over and temperature for *DMC Botryllus schlosseri*. Solid line is all trials combined, $y=-7.042+1.03x$, $R^2=0.014$, p=0.49. Dotted line is trial started on July 17, $y=-39.78+4.66x$, $R^2=0.263$, p=0.05. c) Bivariate fit of percent increase in second take over and temperature for DMC *Botrylloides violaceus*. No significant relationship, $R^2=0.005$, p>0.7.

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(Figure 28). However, there was an increase in variability of percent increase in number of zooids with an increase in temperature, as well as an increase in the maximum percent increase for both species. Also, the maximum percent increase in number of zooids for *Botrylloides violaceus* was 218% while that of *Botryllus schlosseri* was only 150%. There was no correlation in percent increase after first blastogenic cycle and percent increase after second blastogenic cycle when both species were combined, but there was a negative correlative trend between percent increase during second and first blastogenic cycles for *Botrylloides violaceus*.

Temperature and growth in HCM colonies

Data from HCM show a parabolic relationship between temperature and percent increase after second blastogenic cycle for *Botrylloides violaceus* (N=34, R^2=0.225, p=0.0072) with the greatest increase in number of zooids occurring at 15°C (Figure 28). There was not enough data (N=7) to observe a relationship between temperature and percent increase after second blastogenic cycle for *Botryllus schlosseri*. The greatest amount of variability in percent increase for *Botrylloides violaceus* was also at 15°C, as was the greatest percent increase, 115%. The greatest percent increase in number of zooids recorded for *Botryllus schlosseri*, 145%, also occurred at 15°C, but there weren't any colonies from HCM cultured at 20°C that survived, so it is unknown if *Botryllus schlosseri* would produce more buds per zooid at a higher temperature.

While there appear to be some differences in the responses of *Botrylloides violaceus* from DMC relative to those from HCM, such conclusions warrant caution because colonies from DMC were collected on July 17th while those from HCM were collected on June 15th. This disparity in collecting time occurred because the life cycles of these two species at these two sites are offset by approximately one month, so in order to have colonies in the laboratory that were approximately the same age I had to collect them at different times from the different sites. Age of colony was important for measuring time to reproduction and for comparing growth rates, as DBCs may be highly variable throughout the lifespan of the colony (Millar).

Lifespan was negatively correlated with temperature in colonies from HCM and CML (Tables 3,4,6). Life span was not correlated with temperature in colonies from DMC (Tables 2 & 5).

Reproduction and Temperature

Botryllus schlosseri colonies from all sites failed to develop gametes at 10°C or below. Both eggs and sperm were produced at 15°C and 20°C; however, colonies were more productive at 20°C than 15°C, as measured by number of eggs per colony, size of testes per zooid, and number of colonies with gonads (Table 7). *Botrylloides violaceus* testes were never observed in laboratory-reared cultures, though this may have been due to the location of the testes and not a lack of testes production. Unfertilized eggs were observed in colonies at 15

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and 20°C, though on a much less consistent rate than that found in *Botryllus*

schlosseri colonies. This suggests that culturing methods were not conducive to

reproductive development in *Botrylloides violaceus,* and that temperature limits of

reproduction for New England populations of *Botrylloides violaceus* could not be

established in this study. However, the complete lack of growth of these colonies

at 5°C suggests that they are not reproductively viable at this temperature

(Figure 28).

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Species	5C	10C	15C	20C
# of colonies with gonads				
Botryllus	0	0	4	5
Botrylloides	0	0	1	0
Average # of asexual cycles with eggs per colony				
Botryllus	0	0	1.75	1.6
Botrylloides	0	0	1	0
Average # of eggs per zooid				
Botryllus	0	0	0.66	0.44
Botrylloides	0	0	0.22	0
# of testes per zooid				
Botryllus	0	0	1	2
Botrylloides	0	0	0	0
Average size of testes per zooid				
Botryllus	0	0	small	large
Botrylloides	0	0		

Table 8: Laboratory cultured sexual reproduction of *Botryllus schlosseri* and *Botrylloides violaceus*. *Botryllus schlosseri* colonies had more than one clutch per colony, and were slightly more fertile at 15°C than at 20°C.

CHAPTER IV

Discussion

Abiotic Factors

Abiotic factors other than temperature varied between the three sites

during the study period. However, there was a striking difference in the number

of days above the reproductive critical temperature for both *Botryllus schlosseri*

and *Botrylloides violaceus* at HCM compared to both CML and DMC (Table 2).

This makes duration above critical temperature a potential cause of observed

growth pattern variation between the sites. Also, salinity, one of the abiotic

factors known to impact the growth and reproduction of these species in other

habitats, was similar at HCM and DMC during the study period, and is not below

27 psu offshore when water temperature are above 13°C for either DMC or CML

(Figure 6). As salinities lower than 15 psu are required to affect the heart rates of

Botryllus schlosseri and *Botrylloides violaceus* (Dijkstra et al, submitted), it is

unlikely that low salinities significantly affected the ability of these two species to

grow and reproduce once water temperatures had reached 13°C. Therefore, it is

highly likely that observed correlations between water temperature and initial

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recruitment, initial bursts of growth, initial periods of decline, or final recruitment are indicative of a functional relationship.

Since late summer and fall average water temperatures did not differ significantly between the two years for any of the sites (Figure 7), it is unlikely that variation observed in the abundance and life history patterns of *Botryllus schlosseri* and *Botrylloides violaceus* between 2005 and 2006 resulted from changes in ambient water temperature. However, since spring and late winter water temperatures did vary between years, (Figures 8 and 9) spring water temperatures are a potential contributor to observed changes in life history traits between years.

Abundance and Growth Patterns of *Botryllus schlosseri* and *Botrylloides violaceus*

Temperature Effects on Reproductive Timing

The two-week delay in start time for the growth pattern of *Botrylloides violaceus* colonies relative to *Botryllus schlosseri* colonies observed in a majority of the comparisons implies that *Botryllus schlosseri* is more tolerant of lower water temperatures than *Botrylloides violaceus* (Figures 10 and 11). This tolerance should allow *Botryllus schlosseri* to have a longer growing season than *Botrylloides violaceus*, and ultimately allow it to extend its range further north than that of *Botrylloides violaceus*. However, as these two species share the

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same northerly limit on the Atlantic coast (Dijkstra et al. 2007) and *Botrylloides violaceus* has a similar northern range limit to *Botryllus schlosseri* on the eastern Pacific coast (Stoner et al. 2002, Lambert 2003, deRivera et al. 2006), the assumption that *Botryllus schlosseri* is more cold tolerant than *Botrylloides violaceus* is questionable. Further support for similar temperature tolerances of the two species are similar durations of blastogenic cycles of the two species at specific temperatures, and similar lowest recorded temperature for gamete production (Muaki 1977, Brunetti 1980).

Greater tolerance of lower temperatures should permit a longer growing season for *Botryllus schlosseri* than for *Botrylloides violaceus*. However, this is not the case (Figures 10 and 11). *Botryllus schlosseri* colony growth may start two weeks earlier than *Botrylloides violaceus*, but it also peaks, declines, and levels off earlier, resulting in similarly lengthened, offset growing seasons for the two species, one starting roughly two weeks earlier than the other. Since the observed growing seasons for these two species are the same length, they have the same northern range limit on the western Atlantic coast and *Botrylloides violaceus* has the greater northern limit on the east Pacific coast, the one observation that supports the idea that *Botryllus schlosseri* is more cold water tolerant than *Botrylloides violaceus* is the earlier spring starting time. However, this delay in start time for *Botrylloides violaceus* may be an artifact of the amount of time required to grow a larva to hatching age rather than a result of differing temperature tolerances. *Botrylloides violaceus* larvae are brooded in an adult colony at least two weeks longer than those of *Botryllus schlosseri*, therefore

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Botrylloides violaceus eggs fertilized at the same time as *Botryllus schlosseri* eggs will hatch approximately two weeks later (Sabbadin 1955, Mukai et al. 1987). *Botrylloides violaceus* colonies would have to become fertile two weeks earlier than *Botryllus schlosseri* colonies if larvae were to appear on panels at the same time, and there would be a two week delay in recruits if the two species became reproductive at the same time. The two week delay actually observed in *Botrylloides violaceus* growth patterns strongly suggests that the two species become reproductive at the same time, and that they reproduce in similar temperature ranges.

Alternatively, it is possible that initial recruitment of these two species was missed and that the observed patterns are not the result of concurrent reproduction and delayed larval release. New *Botryllus schlosseri* recruits are much smaller than those of *Botrylloides violaceus* (Millar 1971), making them harder to see on settlement panels. Therefore, it is possible that recruits arrive on the panels earlier than they are first observed, or that *Botrylloides violaceus* recruits are observed at an earlier stage in their life cycle. *Botryllus schlosseri* colonies may start reproducing earlier than *Botrylloides violaceus* colonies, but this particular study was unable to detect them. Single zooid colonies of both species were observed, suggesting that the animals were first observed at similar ages; however, the possibility of undetected earlier *Botryllus schlosseri* recruits cannot be ruled out.

Abundance

Many more *Botrylloides violaceus* than *Botryllus schlosseri* colonies were found on the panels at HCM and CML (Figure 10b, 10d and 10f). While this did not always result in a higher percent cover, it does suggest that there are more *Botrylloides violaceus* colonies in the community. The quantitative imbalance between of *Botrylloides violaceus* and *Botryllus schlosseri* becomes even more pronounced when one considers that *Botrylloides violaceus* colonies are only able to produce one larva per zooid, and take a month to brood each larva, while *Botryllus schlosseri* colonies are able to produce up to four larvae per zooid and the larvae are ready to be released into the water column at the end of a single blastogenic cycle (six to ten days) (Muaki 1977). This means the same number of *Botryllus schlosseri* zooids can produce up to eight times as many larvae as *Botrylloides violaceus* zooids in the same time period. Therefore there must be many more *Botrylloides violaceus* zooids than *Botryllus schlosseri* zooids in the nearby benthic and/or fouling communities at CML and HCM. Interestingly, the numbers of *Botrylloides violaceus* colonies found on panels at DMC were much more similar to those of *Botryllus schlosseri* than those found at other sites (Figure 10f). This suggests that *Botryllus schlosseri* is a more successful competitor in the Damariscotta River than in either Portsmouth or Salem. *Botryllus schlosseri* success could be due to a variety of reasons: 1) there are large colonies of *Didemnum* sp. at DMC, which may be competing with *Botrylloides violaceus* populations; 2) water temperatures may be cold enough

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long enough to significantly slow down *Botrylloides violaceus* growth in the spring; 3) *Botryllus schlosseri* has been in the Damariscotta longer than *Botrylloides violaceus* (Dijkstra et al. 2007) and has become better adapted to the local environmental conditions.

The impact of *Didemnum* sp. on *Botrylloides violaceus* may be ascertained by conducting a panel study observing the effects of competition on the growth and reproductive output of these two species. Panels should be deployed and two colonies allowed to settle on each, either two *Botrylloides violaceus*, two *Didemnum* sp., or one *Botrylloides violaceus* and one *Didemnum* sp. As the growing seasons for these two species differ at DMC (personal observation) panels should be monitored for two years and the impact of *Didemnum* sp. on *Botrylloides violaceus* fecundity observed and recorded. Reduced fecundity on the interspecific panels would suggest that *Didemnum* sp. is negatively impacting *B. violaceus* and allowing *Botryllus schlosseri* to maintain a competitive advantage.

Differentiating between chilly spring water temperatures and acclimatization is difficult, because reduced acclimatization in *Botrylloides violaceus* may be the cause of observed low tolerance of cold spring waters. However, one can test whether chilly spring water temperatures are slowing *Botrylloides violaceus* growth by monitoring the growth of overwintering colonies in the spring, comparing between species, and correlating the growth with water temperature. Increased growth of *Botryllus schlosseri* at colder temperatures than that of *Botrylloides violaceus* would suggest that it stays colder long enough

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at DMC for *Botryllus schlosseri* to maintain it's competitive advantage, even without the help of *Didemnum* sp. Reduced *Botrylloides violaceus* growth resulting from low spring water temperatures should increase global warming concerns. *Botrylloides violaceus* is a highly successful competitor at both CML and HCM (Dijkstra et al. 2007, personal observation), and if water temperatures continue to warm, it may continue it's dominance march up the New England coast, impacting and changing benthic and fouling communities as it goes.

Abundance, Percent Cover and Chimaerism

The reduction in number of colonies with increased percent cover (Figure 10), most likely due to fusion, has broad implications for assessing the genetic diversity of colonial ascidian communities. This reduction suggests very high rates of fusion, and therefore high numbers of *Botryllus schlosseri* and *Botrylloides violaceus* chimeras in the benthic and fouling communities of northern New England. The high number of chimeras may result in the underestimation both of numbers of larvae recruiting into a community, and of the genetic diversity of the ascidian population. As chimeras contain multiple gene lines in their oocytes (Magor et al. 1999), chimaerism has the potential to increase the speed of natural selection. This chimaerism may help colonial ascidians acclimatize to new environments rapidly, and may be partially responsible for their global success as invasive species.

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Length of Growing Season

The number of colonies decreased as percent cover increased for both species both years at all three sites (Figure 10). This could have been caused by either competition between colonies or fusion between colonies. Competition should lead to portions of colonies that have overgrown earlier ones (Grosberg 1982). However, as this was not the case, and fusion events were observed on all panels, it is highly likely that the decrease in number of colonies that occurred at the same time as an increase in percent cover was a result of sibling or closely related colonies fusing together and becoming one large colony covering a high percentage of the panel. This hypothesis is also supported by evidence that recruits preferentially settle near related individuals (Rinkevich & Weissman 1987, Feldgarden & Yund 1992). Due to fusion, manual removal of all spatial competitors, and the ability of larvae to settle on and fuse into adult colonies (Laird et al. 2005), each panel contained one age cohort of healthy colonies. Data thus revealed the number of sequential cohorts possible in a single growing season at each of the three sites (Figure 10). Each decline in percent cover at a site reflected the death of a cohort. Therefore, the two local maxima of percent cover found at HCM for both *Botryllus schlosseri* and *Botrylloides violaceus* imply that the growing season is long enough for the animals to produce two cohorts of reproductive colonies before going into hibernation in the winter. This same pattern is observed further south at Woods Hole, MA and Groton, CT (Grosberg

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1988) as well as upriver from the DMC in the Damariscotta River (Yund & Stires 2002). The single peak in percent cover per growing season for both species at CML and DMC suggests that the growing season at these sites is only long enough to produce one cohort of reproductive colonies. While there are a variety of different possible causes for this shortened growth season at the northern sites, water temperatures are above 13°C for a much shorter time at CML and DMC than they are at HCM (Table 2). This may result in *Botryllus schlosseri* and *Botrylloides violaceus* running out of time to produce a second generation as is possible during the longer season at HCM.

Temperature

The relationship between percent cover and temperature was highly variable for *Botryllus schlosseri* and *Botrylloides violaceus* across the three sites, with *Botrylloides violaceus* percent cover being positively correlated with temperature at HCM, negatively correlated with temperature at CML, and uncorrelated with temperature at DMC, the only site at which *Botryllus schlosseri* was correlated with temperature (Figures 13 and 14). However, initial growth for each species occurred at very similar temperatures across all sites (Figure 29). This suggests that reaching a critical temperature may be key for initiating growth and reproduction in these animals, but that as long as temperatures stay above the minimum critical temperature, other factors such as food availability, competition, salinity, and turbidity, have greater impacts on their dominance. The

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growth pattern of *Botrylloides violaceus* at CML is a good example of this, as low salinities in the spring may delay reproduction and growth, which may therefore lead to high percent cover in the fall instead of the summer months, though the end of the growing season was delineated by a drop below critical temperatures even at CML.

Consequently, increased warming may result in a longer uninterrupted growing season for these colonial tunicates. However, Brunetti (1976) observed two periods of reproduction during the growing season of *Botrylloides leachi*, one of which stopped when water temperatures reached 24°C. If *Botryllus schlosseri* and *Botrylloides violaceus* both have critical maximum reproductive temperatures as well as critical minimum temperatures, we may start seeing breaks in reproduction during the growing season, and even two shorter, separate

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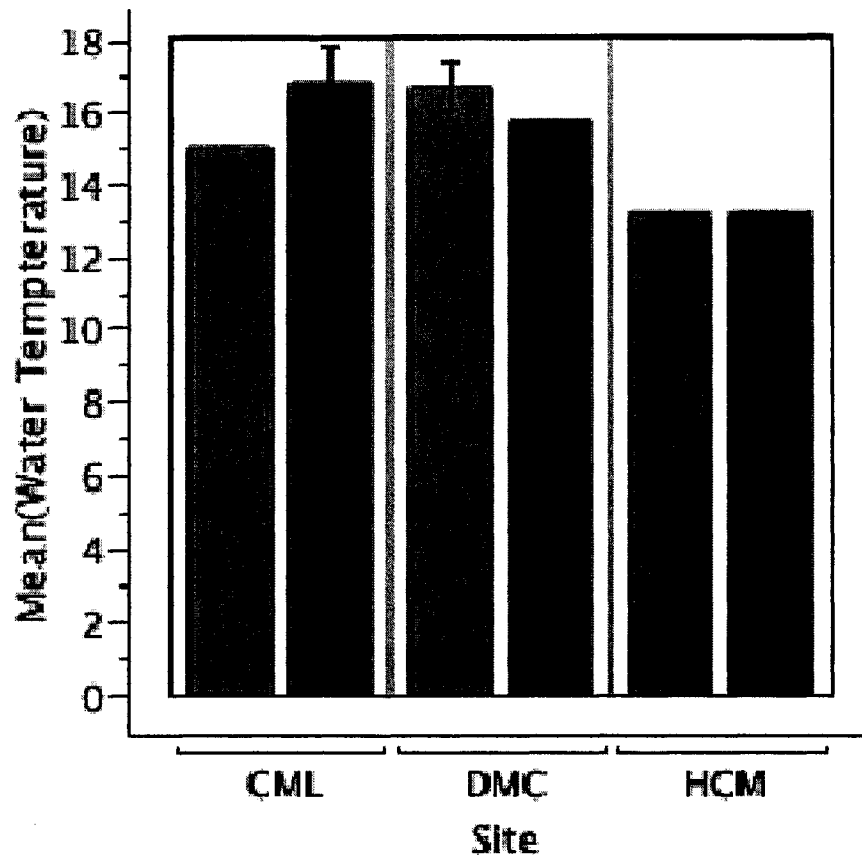
Figure 29: Temperature of initial starting growth. *Botryllus schlosseri* is in black and *Botrylloides violaceus* is in grey.

growing seasons, a shift that could reduce the impact of these two colonial ascidians on coastal temperate ecosystems.

The multi-colony study was initiated to address how these two different species respond to different environments. While results elucidated the spatial patterns and abundance of these two species in the absence of spatial competition, the variable numbers of colonies on each panel made it difficult to compare growth rates between sites or species (Figure 10). This led to a second study to examine single colony growth rates at DMC and HCM.

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Single Colony

Growth rates for *Botrylloides violaceus* and *Botryllus schlosseri* did not differ significantly between HCM and DMC (Figure 20). As the two sites had different temperature regimes (Figure 2, Table 2), different fouling communities (personal observation), and potentially different quantities of food, these similar growth patterns suggest that both species have been in these environments long enough to adapt to them; they are tolerant of a wide variety of environmental conditions (have high levels of phenotypic plasticity) or both. *Botrylloides violaceus* was first documented in the Gulf of Maine less than thirty years ago (Berman et al. 1992), and has a maximum of two generations a year (Figure 10), meaning that the species had adapted to its new environment in under sixty generations. This is a short number of generations for effecting such change; phenotypic plasticity seems a more likely explanation of similar growth patterns. However, the combination of three facts: the length of *Botrylloides violaceus* and *Botryllus schlosseri* breeding seasons being dependent on temperature (Millar 1971, Brunetti et al. 1980), populations are likely genetically isolated from each other (Yund 1995), and colony growth patterns are statistically the same regardless of environmental condition or seasonal timing (Figure 20), suggests that this response is due to adaptation rather than to levels of plasticity. Both *Botryllus schlosseri* and *Botrylloides violaceus* are highly successful invasive species in the Atlantic and Pacific Oceans (Millar 1971, Lambert & Lambert 2003, Dijkstra et al. 2007). Their ability to adapt quickly to new environments may be

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one of the reasons they are now so widespread. Conversely, there is strong selection for high plasticity in heterogeneous environments, and this perceived ability to adapt quickly may instead be reflective of high levels of plasticity. A transplant study determining whether larvae hatched at one site and transplanted to another responded to physical conditions at the second site in the same manner as native recruits would help determine whether adaptation or acclimatization occurred.

While growth rates for each species did not differ between sites, they did differ between species at each site (Figure 18). *Botrylloides violaceus* grew faster than *Botryllus schlosseri* did at both HCM and DMC. This means that *Botrylloides violaceus* zooids were producing a greater number of buds than *Botryllus schlosseri* zooids in the field. The ability to produce a greater number of buds and therefore larger colonies than *Botryllus schlosseri* may enable *Botrylloides violaceus* to produce comparable numbers of larvae as *Botryllus schlosseri* in similar amounts of time, as well as to occupy more space more quickly. However, a two week time period between observations did not allow me to determine whether *Botrylloides violaceus* colonies were actually producing more buds per zooid than *Botryllus schlosseri* colonies, or if they were simply undergoing blastogenic cycles at a faster rate.

My inability to answer this question of manner of growth due to the inadequate time scale of data collection underscores the impact duration between field sampling may have on research results. We are in a day and age when it is very difficult to observe aquatic communities and subtidal animals in

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the amount of detail currently used in terrestrial studies. However, fouling communities, and the propensity of sessile animals to attach to conveniently deployed settlement panels, give us a unique opportunity to study marine organisms in (semi) natural environments with a level of detail normally reserved for terrestrial systems. I cannot ascertain how *Botrylloides violaceus* grows faster than *Botryllus schlosseri* with my long-term biweekly data set, but a month long study of daily observations would not only solve this puzzle, but might add clarity to a variety of other mysteries surrounding the fusion capabilities and competitive advantages of these animals.

Many sessile invertebrates have relatively short lifespans (Millar 1971, Brunetti 1974, Yamaguchi 1975, Brunetti 1976, Harvell & Grosberg 1988, Satoh 1994, Krik 1997), and the current method of conducting long term studies with monthly, or even biweekly, snapshots of a system may result in inaccurate conclusions about the interactions and driving forces behind observed community changes. It is therefore very important to know the time scales relevant to the organisms being observed when designing and analyzing a study, both to insure one's questions can be answered and to prevent misinterpretation of a result due to an overlooked life history event.

Single to Multi Colony

Spatial coverage patterns for single colonies appeared to differ from those on the multi colony panels in two ways: 1) single colonies never reached as

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great a percent cover as the multi colony panels in all *Botryllus schlosseri* colonies and in all but one *Botrylloides violaceus* colony at each site (Figures 11 and 20); and 2) *Botrylloides violaceus* single colonies from HCM reached maximum growth at a later time than colonies from DMC, while the opposite was true for the multi colony panels (Figures 12 and 20). The slower increase in spatial coverage by single colonies compared to multi colony panels suggests that the high increases in percent coverage of both species are due to the combined growth of many colonies. The fact that none of the *Botryllus schlosseri* colonies and only one of the *Botrylloides violaceus* colonies (which may have been an unrecognized fused chimera) covered the entire panel before dying suggests that single colonies may not be capable of reaching extremely large sizes. Therefore most colonies observed to be more than 100 cm^2 were probably chimeras. This hypothesis is further supported by the fact that when attempts were made to follow single colony growth on the multi colony panels, every colony randomly selected for monitoring fused with another colony during the period of observation. High fusion rates may be responsible for both the greater-than-exponential growth rate and the large colonies observed in long-term field studies of fouling communities.

The reversal in timing of maximum growth between sites observed in the single and multi colony panels may be due to the delay in deployment of the single colony panels relative to initial increase in percent cover at HCM. Initial increase in percent cover occurred almost a month later at DMC than it did at HCM, but the single colony panels were deployed at the same time at both sites

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to eliminate any temporal or environmental effects caused by starting the experiment at the time of initial percent increase at each site instead of concurrently at both sites. Unfortunately, this delay in deployment until recruits had been observed at both sites resulted in HCM panel deployment during a period of low ascidian larvae settlement and in DMC panel deployment during high ascidian larvae settlement (Figure 25). Since colonies both grew and cycled together at the different sites, recruitment peaks were amplified, and obtaining new recruits during periods of low larvae release was very difficult. Ironically, therefore, these single colony growth rate results may still be confounded by time, though this is an artifact of variable ascidian life cycles and not timing of panel deployment.

Temperature

Botryllus schlosseri percent cover was not correlated with water temperature at either site, and *Botrylloides violaceus* percent cover was negatively correlated with water temperature at HCM (Figure 18). The lack of a significant positive interaction between colony growth and temperature in the field suggests that colony growth rate is not driven by temperature. Laboratory studies have demonstrated that colonies do grow faster at higher temperatures when all else is equal (Grosberg 1982, Brunetti et al. 1984, Stachowicz et al. 2002, McCarthy et al. 2007), so the lack of a significant relationship between temperature and growth in the field suggests that some other factor with a

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greater influence is masking the effect of temperature. Laboratory studies have also shown that colony growth rates change throughout their life cycles, that they slow down once becoming reproductive, and that some colonies die shortly after one reproductive cycle (Milkman 1967, Millar 1971, Brunetti & Copella 1978, Mukai et al. 1987, Grosberg 1988, Harvell & Grosberg 1988). As this study lasted the duration of each colony's life, it is possible that the changing growth rates masked the impact of temperature on percent cover. It is also possible that colonies reached reproductive maturity, released larvae, and died slightly before or a few weeks after the occurrence of maximum water temperature, which could result in either no correlation with water temperature or a negative correlation with water temperature (Figure 13 and 14). This is illustrated by the inclusion of two reproductive cohorts, each of which experiences a decline in percent cover after larvae release, in one temperature cycle at HCM, making the lack of correlation with temperature an expected result (Figure 10). As the predicted results from these conditions match the actual results from this study, it is very likely that something besides summer water temperature is driving the life cycles and reproduction of these animals. One potential driving factor is food availability. Animals often have peak growth and reproductive output at times of peak food availability (Van Schaik & Van Noordwijk 1985, Harvell & Grosberg 1988, Svensson & Nilsson 1995, Fielder et al. 2000, Ottersen et al. 2001, Rubenstein & Wikelski 2003, Durant et al. 2004), and as this may not always correlate with temperature for ascidians, food driven life cycles would not be correlated with temperature. Panels were not deployed when water

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temperatures were below 10°C so I was unable to determine the significance of a lower critical temperature on these colonies.

Recruitment

Interesting differences appear between recruitment patterns at CML and those at the other two sites. Unlike HCM and DMC, both *Botryllus schlosseri* and *Botrylloides violaceus* showed a pronounced burst in settlement followed by very low recruitment for the rest of the growing season (Figure 21). This suggests that either the vast majority of the colonies at CML are semelparous or that most colonies became reproductive at the same time at CML, but that timing of reproductive maturity was more variable at the other two sites. One strong possibility is that the flooding in May 2006 that strongly affected the fouling community in Portsmouth Harbor may have reset the colonies at CML so that they all became reproductive at the same time. However, one cannot rule out the possibility that this population is isolated from the other two populations and contains fewer iteroparous colonies that are releasing multiple sets of larvae into the water column per colony. In this case, one would not see a steady stream of recruits. Instead one would observe very sharp peaks of recruitment followed by months of very low recruitment from the stragglers, until the new cohort reached reproductive maturity.

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A simple colony dissection study could be conducted to ascertain whether *Botryllus schlosseri* and *Botrylloides violaceus* colonies at CML are all semelparous instead of simply having the same temporal onset of gametogenisis. Eggs of both species are brooded outside of the zooids and are visible to the naked eye (Mukai 1977, Boyd et al. 1986). Colonies monitored throughout the breeding season with fertile eggs for more than two consecutive weeks for *Botryllus schlosseri* and more than four consecutive weeks for *Botrylloides violaceus* (multiple sets of viable eggs) would be iteroparous, and those with only one set of viable eggs before colony death would be semelparous. The ratio of iteroparous to semelparous colonies could then be compared between sites to see if there was a higher dominance of semelparous colonies at CML relative to DMC and HCM. If not, the large burst observed in recruitment in 2006 was most likely a result of synchronous reproduction in the colonies at CML.

Initial recruitment occurred two weeks earlier for *Botryllus schlosseri* than for *Botrylloides violaceus*, and peak recruitment at both CML and DMC occurred earlier for *Botryllus schlosseri* than it did for *Botrylloides violaceus*, suggesting that *Botryllus schlosseri* may be more tolerant of cold than *Botrylloides violaceus* (Figure 21). However, the picture changes when one recalls that *Botrylloides violaceus* larvae take two weeks longer to brood than *Botryllus schlosseri* larvae (Milkman 1967, Mukai et al. 1987). Therefore, a two week lag in initial and peak recruitment for *Botrylloides violaceus* would mean that colonies of the two species became reproductive at the same time, and a co-occurrence of initial

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recruitment would mean that *Botrylloides violaceus* colonies become reproductive at least two weeks earlier than *Botryllus schlosseri* colonies. The similar timing of initial reproduction (Figure 21) supports the findings of the spatial coverage study that suggest the two species have similar critical temperatures for reproduction and therefore started reproducing and becoming active at the same time.

Recruitment abundance patterns mirrored colony abundance patterns observed for the two species at the two sites (Figures 10 and 21). There were more *Botrylloides violaceus* recruits relative to *Botryllus schlosseri* at HCM and CML, suggesting a greater number of *Botrylloides violaceus* zooids in the benthic and fouling communities at these sites, as well as higher spatial dominance of settled recruits. Recruitment data between the species were more similar at DMC, suggesting that *Botrylloides violaceus* currently has a smaller competitive advantage there than at either HCM or CML.

The offset of peak recruitment and peak percent cover suggests that colonies are growing, reaching maximum percent cover, reproducing, releasing larvae, and then dying (Figure 22). This life cycle is characteristic of the semelparous colonies Grosberg found in Eel Pond (Grosberg 1988). Due to the interaction between percent cover and recruitment, there did not seem to be any iteroparous colonies of either species on the panels (Figure 22). However, as there was recruitment throughout the entire growing season for both species at both sites (Figure 21), it is entirely possible that there were iteroparous colonies in the benthic and fouling communities that were releasing multiple sets of larvae.

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None was observed on the panels, however, and fertilized eggs can be seen by the naked eye in both species (personal observation), so missing reproductive cycles in the observed colonies would be unlikely.

The peak in recruitment observed prior to peak percent cover in both species at DMC and CML suggests that initial recruitment was observed in this study. The lack of both a secondary recruitment peak and secondary percent cover peak at these two sites demonstrates that only one cohort of colonies is able to complete the colonial ascidian life cycle in one growing season. However, the occurrence of peak recruitment after peak percent cover for both species at HCM not only suggests that initial and peak growing season were missed, but that two cohorts are able to reach reproductive maturity and complete their life cycle during the growing season at this site. A two-cohort growing season is also observed in Cape Cod MA, Groton CT, Venice Italy, and upriver Damariscotta ME (Brunetti 1976, Brunetti & Copella 1978, Grosberg 1982, Yund & Stires 2002).

Initial and ending recruitment occur at very similar temperatures at HCM, CML and DMC, further supporting the idea of a critical temperature range suggested by many laboratory studies (Milkman 1967, Mukai 1977, Grosberg 1982, Brunetti et al. 1984, Mukai et al. 1987) and by the spatial cover study conducted earlier (Figures 10 and 11). However, as the reproductive temperature limits of these populations of *Botryllus schlosseri* and *Botrylloides violaceus* have not been tested in the laboratory, it is possible that the species have changed since leaving their native habitats, and have acclimated to reproducing at lower

temperatures. It is therefore important to take colonies from the field site into the laboratory and culture colonies at one degree increments to find the actual critical temperature for reproduction in these two species. A meticulous field study consisting of daily observations and correlations with temperature to corroborate reproductive limits with laboratory results would further isolate the reproductive critical temperature for *Botryllus schlosseri* and *Botrylloides violaceus*.

Growth and Temperature

Grosberg (1988) stated that the duration of blastogenic cycles was fixed at set temperatures based on his and Milkman's (1967) work in Woods Hole and Brunetti et al. (1984) and Sabaddin's (1955) work in Venice. However, the temperature regimes of these locations differ from those at the three sites used in this experiment. Colony duration of blastogenic cycle in this study was longer than that found in other locations (Figure 27) (Sabaddin 1955, Milkman 1967, Brunetti et al, 1980, Grosberg 1988). This could be caused by a variety of factors. Colonies in this study were cultured in a closed system of seawater that was changed every week, while Grosberg (1988) and Milkman (1967) changed water daily and Brunetti (1984) and Sabaddin (1955) used flow through systems. This may have resulted in lower food intake by the zooids than in other studies due to either a slower flow of water or a reduction in amount of food in the water column (most studies using flow through systems found supplemental feeding unnecessary). However, if lower food intake can extend the blastogenic cycle

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then it is not fixed at a specific temperature and other factors may change it, contradictory to Grosberg's claim. Grosberg (1988) also stated that duration of blastogenic cycle was fixed at a set temperature unless the colonies were being starved. Perhaps a reduction in water flow resulted in reduced food intake and therefore starvation in this study, but this does not explain colonies continuing to increase in number of zooids as well as producing gonads, a very energetically expensive process. The continued production of both high numbers of buds and gonads implies that the colonies were not starved, and that the longer blastogenic cycles were a result of something besides food availability (Table 8, Figure 28). Thus duration of blastogenic cycle may not in fact be fixed for these species at specific temperatures.

Both species increased the maximum number of zooids produced with increases in temperature when from DMC, though there was no significant correlation between average percent increase in zooids and temperature (Figure 28). The widening of the range of possible zooid increases with increased temperature suggests that as temperatures rise other factors such as food availability, dips in salinity, competition, and space availability may play a greater roll in determining how many buds are produced. As this happened for both species, the pattern of temperature having a greater influence at lower temperatures and a smaller influence at higher temperatures may be constant throughout temperate Botryllid colonial ascidians.

Botrylloides violaceus and *Botryllus schlosseri* both increased the maximum percent increase with increased temperature, but they did not do so at

the same rate (Figure 28). The maximum percent increase of *Botrylloides violaceus* was 100% more than that of *Botryllus schlosseri*, meaning that in at least one colony, zooids were producing twice as many buds as in the most productive *Botryllus schlosseri* colony. If this observed trend also occurs in natural conditions, *Botrylloides violaceus* colonies would have a clear advantage over *Botryllus schlosseri* at warm temperatures. The single colony study demonstrated that *Botrylloides violaceus* colonies grew faster in the field than *Botryllus schlosseri* colonies (Figure 20). Laboratory results suggest that higher growth rates are due to greater potential for bud production in *Botrylloides violaceus.*

Botrylloides violaceus colonies from HCM exhibited a different pattern than those at DMC (Figure 28), which was interesting for a variety of reasons. The decline in percent increase in zooids from 15°C to 20°C suggests that *Botrylloides violaceus* colonies from HCM do not necessarily increase growth indefinitely with temperature. This response to temperature could be caused by an increase in reproductive output at higher temperatures or be because HCM *Botrylloides violaceus* colonies are less fit at 20°C. These two reasons seem unlikely, however, as water temperatures at HCM are higher than those at DMC, and colonies from DMC did best at 20°C. One possible explanation for the decreased growth at 20°C is that the colonies from HCM were collected a month earlier than the colonies from DMC. Perhaps these colonies were adapted to grow fastest at lower temperatures to maximize space occupancy at a fast pace before sea waters reached maximum temperatures. *Botrylloides violaceus'*

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suboptimal growth at maximum temperature should be further examined by monitoring growth rates at multiple temperatures of different cohorts from the different sites.

Lifespan

Colonies kept at cold temperatures generally lived longer than those at warmer temperatures. Physiologically this may be the result of a general slowing down of colony metabolism due to temperature, but there may be an evolutionary reason for this as well. Grosberg (1988) found colonies of *Botryllus schlosseri* to either live a short period of time and reproduce quickly in the summer, or to live a long period of time and wait to reproduce in the winter. Both *Botrylloides violaceus* and *Botryllus schlosseri* colonies are known to overwinter in the field (Brunetti 1974) but they appear to simply maintain their space occupancy, or to shrink slightly in the case of *Botrylloides violaceus* (Brunetti 1974, Dijkstra, personal communication). The longer life span coupled with slower blastogenic cycle and reduced bud production may be how these two species have evolved to handle temperate climate winter conditions, in which there is less food, higher turbulence, and colder water temperatures. If this is the case, then the two species appear to have evolved different methods of surviving the winter, with *Botrylloides violaceus* going into a morphologically unique state of hibernation while *Botryllus schlosseri* simply slows or stops growth.

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Botryllus schlosseri colonies were able to live longer than *Botrylloides violaceus* colonies at cold temperatures, suggesting it may be a more cold tolerant species (Tables 2-6). However, *Botryllus schlosseri* colonies cultured in the laboratory were able to live longer in general, and may simply be more tolerant of laboratory conditions than *Botrylloides violaceus*. The high durability of *Botryllus schlosseri* relative to other *Botrylloid* ascidians may be one of the reasons it has become a model organism for histocompatibility studies, and may skew the results of comparative studies between it and other, less durable species in the laboratory.

Reproduction

The minimum temperature for reproductively viable colonies of both *Botryllus schlosseri* and *Botrylloides violaceus* established in this study was similar to those previously recorded for both species (Sabbadin 1955, Brunetti et al. 1984, Mukai et al. 1987). This suggests that the reproductive temperature range for these highly cosmopolitain species may be fixed. Moreover, it is the same for both species, confirming the field results in which recruits occurred on panels at times that would imply comparable reproductive temperatures (Table 8, Figure 21). While both species would produce eggs, and sperm were observed in *Botryllus schlosseri*, there were no viable offspring from laboratory-cultured animals. This occurred because the animals within a tank cycled together. As

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animals are cyclically hermaphroditic, all the colonies were male at the same time, so when sperm were released in a tank there were no viable eggs to fertilize.

Concurrent Colony Cycling

The concurrent cycling of *Botryllus schlosseri* and *Botrylloides violaceus* in the laboratory is particularly interesting because it suggests that colonies of the two species communicate. Interspecies communication in terrestrial animals such as birds is fairly common, but is most often in the form of alarm call recognition, food item location, or guarding (Baptista & Gaunt 1994, Zuberbuhler 2000, Lewenza et al. 2002). Some animals might initiate their reproductive cycles when they obtain signals from food items, but interspecies communications controlling a behavior that is most likely used to prevent interbreeding is unusual. If colonies do have intraspecific selective settlement near relatives (Rinkevich & Weissman 1987), it would be logical for colonies to cycle together so that they are not fertilized by their siblings. However, the evolutionary advantage of cycling with a near neighbor of a different, but very similar species would be prevention of hybridization.

Colonial tunicates are highly selective of sperm and some species are able to store desirable sperm and reject those genetically similar to there own (Bishop & Ryland 1991, Bishop et al. 1996). This illustrates a complex sperm

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recognition process that should be able to identify and remove unsuitable sperm such as those from other species. However, *Botrylloides* and *Botryllus* are very similar genera, quite difficult to tell apart in both the field and the laboratory, and may even belong in one genus: *Botryllus*. As little experimental work has been conducted to see if different *Botryllus* species will interbreed, and even less on the reproductive compatibility of *Botryllus* and *Botrylloides* species, it is possible that *Botryllus schlosseri* and *Botrylloides violaceus* are similar enough that cross fertilization is a potential problem. *Botryllus schlosseri* has been found to be able to self fertilize (Mukai et al. 1987). This may be due to a lack of pre-zygotic barriers to selfing. If *Botryllus schlosseri* is unable to differentiate between highly related sperm and highly desirable sperm, it may also be unable to differentiate between conspecific sperm and closely related, non-conspecific sperm. If this were the case one would expect *Botryllus schlosseri* to have evolved a mechanism for preventing interactions with nearby non-conspecific sperm. One way to do this would be to release chemical signals that would cause all Botryllidae colonies that received the signals to cycle with the chemical releasing colony. Conversely, the *Botryllus schlosseri* or *Botrylloides violaceus* colonies may be releasing such strong and genera general cycling signals that all colonies of the genera in the area will cycle together regardless of a total lack of evolutionary advantage.

There are a few experiments that could be performed to test whether *Botryllus schlosseri* is sending out a general cycling signal, or is using cycling as a way to prevent hybridization. Reproductive *Botryllus schlosseri* and

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Botrylloides violaceus colonies that are out of sync (one male and one female) could be placed in the same tank to see if they fertilize each other. Hybridization would support the theory that strong cycling evolved to prevent both nearby inter and intra specific mating, while incompatible gonads would support a strong, genera general cycling signal.

Concurrent cycling of *Botrylloides violaceus* and *Botryllus schlosseri* stops as temperature drops below 10°C. *Botrylloides violaceus* colonies also stopped cycling with other *Botrylloides violaceus* colonies at 10°C, five degrees before the behavior was observed to stop in *Botryllus schlosseri*. This difference in minimum temperature at which the colonies will cycle with each other is the one clear piece of evidence from this study that suggests that *Botryllus schlosseri* is more cold tolerant that *Botrylloides violaceus*.

The breakdown of a communication system possibly used to prevent interbreeding implies that there are either not enough resources to support the behavior or that the system simply can no longer function in the present environmental conditions. A similar breakdown in species identification due to changes in temperature has been observed in rotifers (Suatoni, personal communication, personal observation). It is important to note that the breakdown of cycling occurs at temperatures below the known critical minimum reproduction temperature in both these species. Maintaining the ability to decrease the likelihood of inbreeding at these temperatures is not critical to these animals. However, this loss may be a cost of surviving at such low temperatures, and as it is exhibited at higher temperatures in *Botrylloides violaceus* than in *Botryllus*

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schlosseri; thus water temperatures below 10°C may indeed be less tolerable for *Botrylloides violaceus*. A higher cold water tolerance in *Botryllus schlosseri* is to be expected, as the species has been in the Gulf of Maine at least 100 years longer than *Botrylloides violaceus* (Gould 1870, Berman et al. 1992) and has therefore had a longer amount of time to adapt to local conditions such as long periods of low water temperatures.

It is important to note that preliminary data show these patterns of concurrent cycling to occur in the field as well as in the laboratory. While all colonies may not be cycling together in the field, concurrent cycling with near neighbors, if those neighbors were relatives, would be advantageous for preventing inbreeding. Further field studies with daily observations need to be conducted, along with genetic tests of near neighbor colonies, to establish how viable the prevention of interbreeding is as a evolutionary cause for concurrent cycling.

CHAPTER V

Conclusions

Results from all three field studies give strong evidence supporting the extension of the breeding season of both *Botryllus schlosseri* and *Botrylloides violaceus* due to increased temperature. These colonial ascidians (though not migrating like birds) exhibit a similar response to longer periods of warmer water temperatures. Many migrating passerines whose breeding seasons are defined by summer temperatures have been found to increase the amount of double clutching as periods of warm temperatures increase (Brown et al. 1999, Rodriguez & Bustamente 2003, Moller 2006). These responses, which are found across many taxa, appear to be driven by critical reproductive temperatures. There has been a great deal of interest in what aspects of global warming and changing temperature will impact species the most: extreme temperatures, average temperatures, temperature trends, or earlier critical temperatures. Shifting climates have resulted in range shifts towards the poles on a global scale, most likely driven by critical temperatures required for organism survival (Parmesan & Yohe 2003). As critical temperatures also seem to be driving the elongation of breeding seasons for both birds and ascidians, critical temperatures

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appear to be a very important factor in controlling the changing life cycle patterns observed in nature.

Botrylloides violaceus and *Botryllus schlosseri* both appear to be temperate species with almost identical reproductive temperature ranges and similar growth seasons. However, they have different temperature ranges for competitively dominant growth patterns, with *Botryllus schlosseri* growing faster at lower temperatures and *Botrylloides violaceus* growing faster at higher temperatures. Differences in relative responses to temperature for sexual and asexual reproduction in these two species underscore the importance of examining the impact of a factor such as temperature over the entire lifespan of an organism when attempting to understand how observed patterns are influenced by environmental elements.

These observed variations in response to temperature suggest that other factors such as food availability, competition, salinity, and turbidity may greatly influence the life cycles of *Botrylloides violaceus* and *Botryllus schlosseri*, making it very hard to gauge the relative importance of different factors. The large number of recruits, high abundance, and fast growth of *Botrylloides violaceus* demonstrate that it is clearly a more successful competitor than *Botryllus schlosseri* throughout the Gulf of Maine, and its success has coincided with an increase in water temperature over the past thirty years. However, data are inconclusive as to whether the increase in water temperature per se has facilitated this increase in *Botrylloides violaceus*: resolution of this question will require further studies of the impacts of other environmental factors on

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reproduction and growth. Future studies on the relative abilities of these two species should address: 1) whether increased food availability results in increased growth, earlier onset of gametogenesis, and higher fecundity; 2) whether these species compete for the same food resources, and whether one is better at metabolizing the food source; 3) whether short term changes in salinity impact growth rates differently for these two species; 4) whether increased turbidity reduces growth, delays onset of gametogenisis, and reduces fecundity. Only after conducting this series of studies will we be able to identify when specific abiotic variables are most important for predicting invasive ascidian growth and reproductive patterns.

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