SETTLEMENT PREFERENCE AND THE TIMING OF SETTLEMENT OF THE OLYMPIA OYSTER, *OSTREA LURIDA,* IN COOS BAY, OREGON

by

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THESIS ABSTRACT

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In the Pacific Northwest, populations of the Olympia oyster, *Ostrea lurida*, were once decimated by overharvesting and natural disasters. Their full recovery may now be limited by availability of hard substrata for larval settlement. I studied the timing of settlement and larval preferences for commonly available substrata, including conspecifics and the shells of Pacific oysters, *Crassostrea gigas*, which are often provided in restoration efforts. Settlement occurred from August-December with a peak in October. I found no significant settlement differences between live and dead oysters or between shells of Olympia or Pacific oysters. There was significantly higher settlement on bottoms of horizontal substrata than on tops. In the laboratory, larvae showed no clear preferences among various hard substrata. This lack of settlement preference has positive implications for restoration projects, since Pacific oyster shell is much easier to obtain and seems to be no less beneficial than the shells of conspecifics.

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TABLE OF CONTENTS

Chapter Page

LIST OF FIGURES

Figure Page

Figure Page

LIST OF TABLES

Table Page

Chapter II

Chapter III

Table Page

CHAPTER I

GENERAL INTRODUCTION

The Olympia oyster, *Ostria lurida* (Carpenter 1864), is the only native oyster on the west coast of the United States (Baker 1995). The population suffered decimation from severe overharvesting beginning in 1851 (Kirby 2004). Today only remnant populations remain in bays and estuaries throughout its range from central British Columbia to Baja California (Dall 1914, Gillespie 2009, Poulson 2009). Historically Olympia oysters played an important role in these ecosystems, providing water filtration as well as habitat for many fish and invertebrate species (Brumbaugh 2010). Recently much work has been done to restore Olympia oyster populations. However, for restoration efforts to be successful, the biology of *O. lurida* must be understood.

The Olympia oyster is a small oyster with an average size of 3.5 - 4.5 cm and a maximum size of 9 cm (Peter-Contesse and Peabody 2005, Gilespie 2009). It has a circular to elliptical thin grey shell with little sculpture (Baker 1995). *Ostrea lurida* is most commonly found in isolated bays and estuaries with salinities above 24, but is also capable of living in full seawater (Baker 1995, Peter-Contesse and Peabody 2005). Individuals of *Ostrea lurida* can reach maturity in five months and are protandrous hermaphrodites, alternating between male and female each reproductive season (Coe 1931). The females are viviparous and brood larvae for 10-14 days at which point they are released as veliger larvae. Once larvae are

competent to metamorphose, they will settle on available hard substrata (Hopkins 1937).

For most marine invertebrates that are sessile as adults, the mobile planktonic stage is the only dispersal stage and the selection of habitat by the larva will determine the success of the adult (Thorson 1950, Connell 1985). Since the discovery of larvae over 200 years ago, studies on the larval biology of marine invertebrates have been essential to understanding the ecology of populations and much of the work on early life history surrounds the process of settlement and the selection by larvae of a settlement site (Young 1990, Morgan 1995). Oyster larvae were the first larvae of marine invertebrates described (Young 1990) and many key concepts in larval biology and selective settlement were first observed in oysters including the discovery of gregarious settlement (Cole and Knight-Jones 1939). Over the years, numerous studies have examined the biology of oyster larvae, including studies on species within the genus *Ostrea*, which all brood their larvae (Andrews 1979). Most of these studies focused on the European flat oyster *Ostrea edulis* (Linnaeus 1758) (Prytherch 1929, 1931, 1934; Cole and Knight-Jones 1939, Loosanoff 1955, 1966; Cranfield 1973, 1974), but some have described the biology of other brooding members of the genus, including *O. chilensis* (Chaparro *et al*., 2006, 2008).

Early laboratory investigations of larvae of *Ostrea lurida* were conducted by Stafford (1914) in British Columbia, Hori (1933) with individuals transported to Japan, and Hopkins (1937) in Puget Sound. The first successful rearing of *O. lurida* larvae is attributed to Davis (1949), and Imai *et al.* (1954) conducted some of the

first experiments on larvae in tanks in Japan. From these early larval experiments, much is known about the biology of Olympia oyster larvae including brood size, larval life span, sizes of larvae, and temperatures that induce spawning. However, few experiments have directly examined choice of settlement location for *O. lurida,* which is important to understand for restoration efforts.

Restoration and preservation of oyster communities in the United States has been an important focus for over a hundred years, including restoring the native oyster on the east coast of the United States, *Crassostrea virginica* (Gmelin 1791). Since the early 1900s, there have been numerous efforts to restore oyster reefs, including projects in 15 states (Brumbaugh and Coen 2009). However, the Olympia oyster has been largely neglected by such projects until recent years. Large aggregations of Olympia oysters are valuable resources, serving an important ecosystem role by providing habitat for many animals, biofiltration of the West Coast estuaries, denitrification, and shoreline protection (Baker 1995, Brumbraugh 2010, Dinnel 2009, Groth and Rumrill 2009, McGraw 2009). In a community analysis of *Ostrea lurida* aggregations in California, 47 different species were found to be utilizing the benthic oyster habitat (Kimbro and Grosholz 2006). Despite the important role they serve, oyster reef habitats worldwide have been reduced by about 85%, more than coral reefs, mangroves and marshes, making them the most imperiled marine habitats on Earth (Brumbaugh 2010). In Puget Sound, remaining populations are only 4% of what they were prior to the commencement of the fishery in 1851 (Blake 2010).

Only in the past few years have *Ostrea lurida* populations been targeted for restoration. Earlier restoration attempts were initiated (i.e. by the State of Washington's establishment of oyster reserves in 1897) but attention eventually shifted to cultivation of a new imported oyster and the Olympia oyster was all but forgotten (Trimble *et al*. 2009). The Japanese or Pacific oyster *Crassostrea gigas* (Thunberg 1793) was imported from Japan as early as 1899 but not in large numbers until 1925 (White *et al.* 2009a). The Pacific oyster proved a beneficial substitution, as it is capable of growing faster and larger than *O. lurida* and is less susceptible to pollution (White *et al*. 2009a). However, restoration efforts have recently been reinitiated for Olympia oysters. Agencies, tribes, oyster growers and non-profit organizations have now spent more than 1 million dollars on re-introduction and restoration efforts along the west coast of the United States (Dinnel *et al*. 2009, McGraw 2009, White *et al*. 2009a). Current restoration efforts on the West Coast are underway in multiple locations including: San Francisco Bay and Tomales Bay in California; Siletz, Netarts and Coos Bays in Oregon; and Puget Sound, Willapa Bay and Fidalgo Bay in Washington (White *et al*. 2009b, Groth and Rumrill 2009, Dinnel *et al*. 2009, Buhle and Ruesink 2009).

Ostrea lurida has a different history in Coos Bay, Oregon than in other estuaries along the coast. When European settlers arrived in the area in the 1850's, there were no live *O. lurida* populations. Massive deposits of fossilized shells along the Coos Bay shoreline indicate that there was indeed a historic population and that it was a food source for local Native American populations (Baker 1995). Radiocarbon dating of existing shell middens places them around 400 years old ($\pm$60 yrs) (Groth

and Rumrill 2009). The population was most likely wiped out either by a large-scale Cascadia subduction zone earthquake and tsunami in 1700, or by a large fire event that occurred in the area in 1846 and may have deposited vast quantities of sediment in the estuary, suffocating the oysters with silt and sediment (Rumrill 2007, Groth and Rumrill 2009).

In Coos Bay, an inadvertent re-establishment of the species has occurred in the past few decades. It is likely that *Ostrea lurida* were re-introduced as "hitch-hikers" on the shells of *Crassostrea gigas* that were transported for aquaculture into Coos Bay from Willapa Bay, WA in the 1950's (Baker *et al.* 2000). In 1986, some individuals were found near commercial *C. gigas* plots and small Olympia oysters were also documented in Isthmus Slough in 1988. This inadvertent re-establishment eventually yielded self-sustaining populations in the east arm of Coos Bay (Baker 1995). Recent modifications of the habitat in Coos Bay laid the framework for successful *O. lurida* establishment (Rumrill 2007, Groth & Rumrill 2009). In the past 30 years, the water quality and benthic conditions have improved dramatically in the Coos Bay area. Additionally, it is likely that a change in salinity, brought about by the deepening of the channel, made it possible for larvae to recruit successfully (Baker 1995, Groth & Rumrill 2009).

Successive surveys of *Ostrea lurida* populations in Coos Bay (Groth and Rumrill 2009) document range expansion and increased densities of adults (average maximum density in 2006: 19.1 $\pm$ 8.6 per 0.25 m^2). Data from 2006 show a significantly broader range of small sizes than were present in 1997 suggesting that substantial recruitment has occurred over the previous years. Progress is apparent

but recovery is still slow. Current efforts are underway to restore populations of *O. lurida* in the South Slough estuary with a goal of outplanting 15,000 to 22,000 oysters and monitoring survival, growth and reproduction until 2015.

One of the reasons the Olympia oyster is an interesting subject is its failure to recover from previous stresses; although the stresses of the fishery were removed over 80 years ago, the population has failed to recover (Trimble *et al*. 2009, White *et al*. 2009b). Today, remnant aggregations are still all that remain throughout its range (Dinnel *et al*. 2009). This lack of recovery and its causes can be used as a model system for other struggling populations (Trimble *et al*. 2009). The many potential factors inhibiting the recovery of Olympia oyster populations include: reproductive limits, inadequate substratum availability, poor post-recruitment survival, predation, and competition (Groth and Rumrill 2009, White *et al.* 2009a). Predation by invasive oyster drills in Willapa Bay was found to have a low impact on Olympia oyster populations compared to other factors (Buhle and Ruesink 2009). In Coos Bay, as in Puget Sound and Willapa Bay in Washington, there is evidence that adequate larval supply and recruitment along with post-recruitment survival are the main factors preventing the successful recovery of Olympia oyster populations (Groth and Rumrill 2009, Trimble *et al*. 2009, White *et al*. 2009b). The importance of these factors varies with location, but in Coos Bay, the availability of substratum is a key limiting factor and will be the focus of this study.

The paucity of available hard substrata for oyster larvae to settle on is one the greatest challenges for oyster restoration. Indeed, besides overharvesting, the lack of sufficient substrata was one of the major causes of the decline of the *Ostrea*

lurida fishery along the West Coast, along with pollution, sedimentation and urbanization (McGraw 2009). The practice of shipping live oysters was also detrimental since it removed potential substrata (Baker 1995). The lack of available hard substrata at lower tidal elevations was found to be the limiting factor for recruitment in studies in Oregon and Washington (Trimble *et al.* 2009, Groth and Rumrill 2009, White *et al*. 2009b). One of the solutions is to increase the available substrata by outplanting shell to develop heterogeneous habitat and additional substrata for natural recruitment. Initially, *O. lurida* shell from the fishery was added for this purpose but once the aquaculture industry shifted to *Crassostrea gigas*, shells from the latter species are now added (Hopkins 1937, Cohen and Zabin 2009; Brumbaugh and Cohen 2009; McGraw 2009). Shell of *C. gigas* is readily available and useful in creating additional habitat, however, it is possible that it is not as effective as conspecific shell from *O. lurida* would be. Here, I will investigate larval settlement in the field on four different oyster shell substrata and compare these results to similar studies conducted with *O. lurida* larvae in the laboratory. I will also examine the timing of larval settlement in Coos Bay to determine the temporal pattern of attachment to substrata.

CHAPTER II

SETTLEMENT PREFERENCES AND TIMING OF SETTLEMENT OF *OSTREA LURIDA* IN THE FIELD

Introduction

Before European settlers arrived on the west coast of the United States in the mid 1800s, the Olympia oyster (*Ostrea lurida*) was abundant and formed populations that supported Native American fisheries and provided habitat for fish and invertebrates (Baker 1995). Extreme overfishing and live transport of oysters to the growing metropolitan areas along the coast led to decimation of the populations (White *et al.* 2009a). Although harvesting pressures were eliminated over 80 years ago, the remnant populations have failed to recover. Many projects are underway to re-introduce or restore these struggling populations in British Colombia, Washington, California and Oregon (Dinnel *et al*. 2009, White *et al.* 2009b).

For restoration efforts to succeed, the factors influencing the recovery of Olympia oysters, or lack thereof, must be identified. For Olympia oyster populations, low recruitment is the most probable cause of failure to re-establish thriving populations in many locations (Groth and Rumrill 2009, Trimble *et al.* 2009). Recruitment of post-larvae into a population depends on sufficient larval production, successful settlement of larvae on appropriate substrata, and survival of

early juveniles (Mann 1988). For Olympia oyster populations, a paucity of hard substrata for settlement has been identified as the primary limiting factor at many restoration sites (Groth and Rumrill 2009, Trimble *et al.* 2009).

Improved understanding of the settlement preferences of larvae of *Ostrea lurida* could help enhance recruitment for recovering populations. A common practice to increase larval settlement in naturally spawning populations is the addition of hard substrata, most often in the form of Pacific oyster shell (Breese and Malouf 1975). However, little is known about the settlement preferences of *Ostrea lurida* larvae. For example, it is not known if greater success occurs with the use of live oysters or conspecific shells. If *O. lurida* larvae do not have a strong settlement preference for conspecific shell or live oysters, it will validate continued deployment of *Crassostrea gigas* shell for restoration efforts. Knowledge of the exact timing of reproduction of *O. lurida* would also be helpful for planning when to deploy additional hard substrata into the bays or estuaries.

There is a lack of information on settlement of *Ostrea lurida* in Oregon estuaries. Recent studies in Washington and California have investigated: (1) the timing of reproduction (Seale and Zacherl 2009); (2) settlement and survival at various tidal levels (Trimble *et al*. 2009); (3) settlement in various habitats (White *et al*. 2009b); (4) genetics (Camara and Veldopalas 2009, Polson *et al*. 2009, Wight *et al.* 2009, Zacherl *et al.* 2009); and (5) predation (Buhle and Ruesink 2009). The timing of reproduction has been reported for *Ostrea lurida* in estuaries in Washington and California but no published data exist for Oregon estuaries. In California, at the southern extent of the range, two settlement peaks have been

noted: a large peak occurs in April followed by a smaller peak in October or November (Coe 1932, Seale and Zacherl 2009). In Fidalgo Bay, Washington, White *et al*. (2009b) report one small recruitment peak in late July while in Puget Sound, spawning occurs in May and early June with two spawning peaks 6-8 weeks apart (Hopkins 1937, Peter-Contesse and Peabody 2005). In British Columbia, peaks in spawning occur later in the year from July through September (Gillespie 2009). Hopkins (1937) linked the initiation of spawning to a minimum temperature of 12.5-13°C and Coe (1932) reported that spawning does not begin until water temperatures reach 16°C in the estuary, but recent studies in Southern California showed no correlation between temperature and the onset of spawning (Seale and Zacherl 2009).

Although *Ostrea lurida* is not particularly well studied, numerous detailed accounts of oyster reproduction around the world exist, including for *Ostrea edulis* (Linneas 1758) the European counterpart of *O. lurida* (Cole and Knight-Jones 1939, Andrews 1951, Walne 1958). Many species of oyster settle gregariously by responding to cues from other live oysters. Settlement of larvae can be induced by using chemical cues derived from conspecific adults (Bayne 1969, Veitch and Hidu 1971, Cranfield 1973, Coon and Bonar 1985, Turner *et al*. 1994, Zimmer Faust and Tamburri 1994, Tamburri *et al.* 2008). Working in a hatchery, Pascual and Zampatti (1995) have shown higher settlement of *Ostrea puelchana* (D'Orbigny 1842) larvae on living oysters than on dead shell. Additionally, Crisp (1967) reported that larvae of *Crassostrea virginica* (Gmelin 1791) responded to cues in the live organic material of conspecifics and in the insoluble organic layer of the periostracum of the oyster

shell. These chemical cues that facilitate gregarious settlement are useful in reef formation (Tamburri *et al.* 2008). However, many filter feeding animals, including oysters, feed on their own larvae (Thorson 1964, Young 1988, Tamburri *et al*. 2007) so gregarious tendencies at settlement may actually result in loss of larvae and lower recruitment. Tamburri *et al.* (2007) claim that adult oysters produce only weak ciliary currents that are rarely capable of capturing larvae but unpublished experiments by Young (*personal communication*) demonstrated significant predation of larvae by *C. virginica* under laboratory conditions. Neither predation on *Ostrea lurida* larvae nor the response of larvae to chemical cues has been tested.

Olympia oysters reach reproductive maturity as young as five months (Coe 1931). They are protandrous hermaphrodites, acting first as functional males and then alternating gender between male and female in successive spawning cycles (Coe 1932). The females brood 250,000-300,000 larvae in their mantle cavities for an average of 10 days after which they are released as veliger larvae (Hopkins 1937). Because they brood their larvae, the released veligers have a short larval life. Culture experiments by Imai (1954) in Japan document settlement of larvae at 10-16 days after release, although Hopkins recorded settlement after 30 days (1937). The competent pediveliger larva swims with the velum pointed upwards and the shell below (Andrews 1979). While searching for suitable substrata, this swimming position is suggested to increase the chance of encounter and settlement on the bottom of horizontal substrata (Hopkins 1937, Imai 1954). Top/bottom differences are among the most common ecological patterns in subtidal marine communities, and wide assortments of sessile animals prefer undersides of horizontal surfaces

(Witman and Dayton 2001). Other species of oyster are known to settle preferentially on the underside of horizontal surfaces, possibly because such undersides are free of silt and algal growth (Cole and Knight Jones 1939, Crisp 1967, Michener and Kenny 1991).

In Coos Bay, Groth and Rumrill (2009) compared size classes of Olympia oysters from surveys conducted in 1997 and 2006. The data show increased numbers of smaller size classes in 2006, indicating successive recruitment events over the preceding years. This suggests that there is a sufficient larval supply in Coos Bay, and that recruitment (most likely larval settlement or early survival) is the probable cause for the lack of large-scale recovery of the population.

In this study, I focused on issues surrounding the settlement of competent veligers on available hard substrata. My specific aims were to: (1) determine the timing of settlement in Coos Bay over a full annual cycle and to correlate settlement with seasonal temperature cycles; (2) determine if larvae of *Ostrea lurida* prefer to settle on conspecifics or introduced oysters; (3) determine whether larvae have a preference for live oysters or dead oyster shell; and (4) compare settlement on the top sides and bottom sides of horizontally oriented substrata.

Materials and Methods

The study site was a floating dock in downtown Coos Bay adjacent to one of the largest populations of adult *Ostrea lurida* in the Coos Bay region (Figure 1) (Baker 1995, Groth and Rumrill 2009). The dock belongs to Sause Bros. Ocean Towing Co. and is located at 155 Market Ave in downtown Coos Bay. They are a

private shipping company, and the docks cannot be accessed without consent from headquarters and thus receive little traffic compared to public docks in Coos Bay. The dock is also safe from vandalism. This site was used as a location to hang bags of *Crassostrea gigas* shell cultch for collection of *Ostrea lurida* spat that will be used for transplantation to other locations in Coos Bay and South Slough (Rumrill *personal communication*). *Ostrea lurida* larvae are known to consistently settle in significant numbers at this location. Previously, collection bags were suspended in the water for an entire year or more, so the timing of settlement peaks in Coos Bay remains unknown.

All juvenile oysters recorded in this study were assumed to be *Ostrea lurida*. Although Pacific oysters are grown near the study site for aquaculture, Clausen Oysters- the largest oyster producer in the state of Oregon- states on their webpage that "due to cold water conditions, oysters in Coos Bay do not spawn." For *Crassostrea gigas*, spawning does not typically occur unless water temperatures regularly exceed 19°C (Magoon and Vining 1981). Although this does occur in some protected bays in Washington, in Coos Bay temperatures did not reach 19°C in 2010 (see Figure 14). However, isolated adult individuals of *Crassostrea gigas* are occasionally observed along the shoreline of Coos Bay (Rumrill *personal communication*), but the abundance of these isolated individuals is very low and provides evidence that reproduction in Coos Bay of *C. gigas* is very rare. Additionally, juveniles of *C. gigas* were obtained from a local oyster grower and compared to *O. lurida* juveniles of a similar size collected throughout the field experiment (Figure 2). The former are much darker in color and contain obvious

ridges and pigment stripes that give them a different appearance than that of juveniles of *O. lurida*. Newly settled larvae of the two species also appear significantly different. Newly settled larvae of *C. gigas* exhibit an asymmetry and darker color of the shell and body which is quite distinct from the nearly transparent and symmetrical newly settled *O. lurida* larvae (Loosanoff *et al.* 1966, Trimble *et al.* 2009).

Four different oyster shell substrata were used for collection of *Ostrea lurida* juveniles. Live *O. lurida* were collected from the population adjacent to the study site, which is concentrated on the scattered hard substrata under the railroad bridge at the entrance to Isthmus Slough (see Figure 1). Dead shell of *O. lurida* was taken from a collection of shell from dredge spoils from Coos Bay. Live *Crassostrea gigas* were donated from Clausen Oysters in Coos Bay. The smallest possible individuals were selectively chosen to attempt to match the small size of live Olympia oysters. Dead shell of *C. gigas* was collected with permission of the Coos Bay Oyster Co. from the large piles of oyster shells next to the Charleston Bridge. Dead shell of *C. gigas* was smashed into pieces similar in size to live and dead shell of *O. lurida*. All shell substrata were soaked for at least 72 hours in seawater before initial placement into the field and then stored in running seawater tanks in order to establish a biofilm, which has been proven important for larval settlement (Gribben *et al.* 2009).

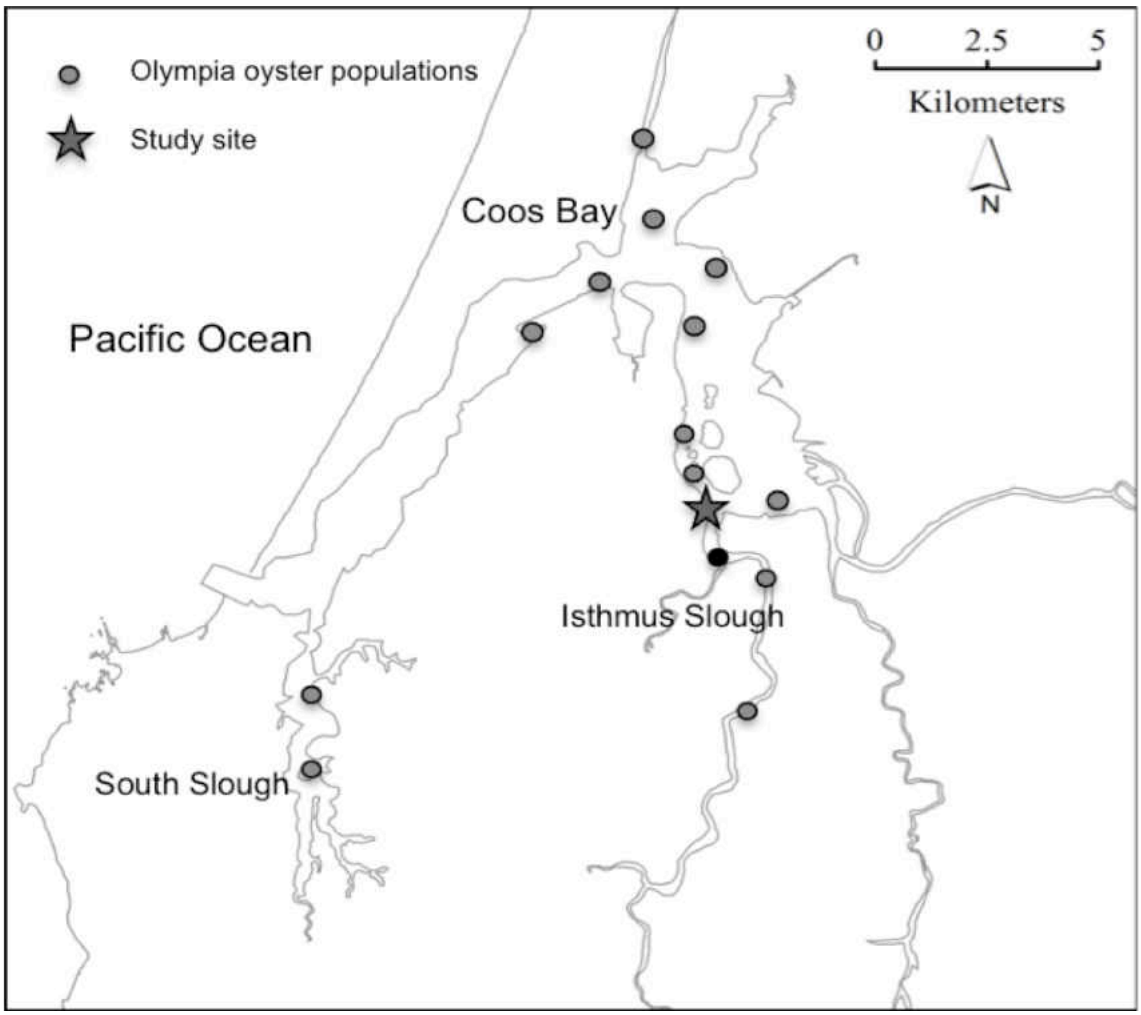

Figure 1. Map of the Coos Bay region indicating the location of the study site in Downtown Coos Bay (star) as well as the locations of Olympia oyster populations in Coos Bay and in South Slough. The large population of Olympia oysters concentrated under the railroad bridge is indicated by the black marker located just south of the study site at the mouth of Isthmus Slough.

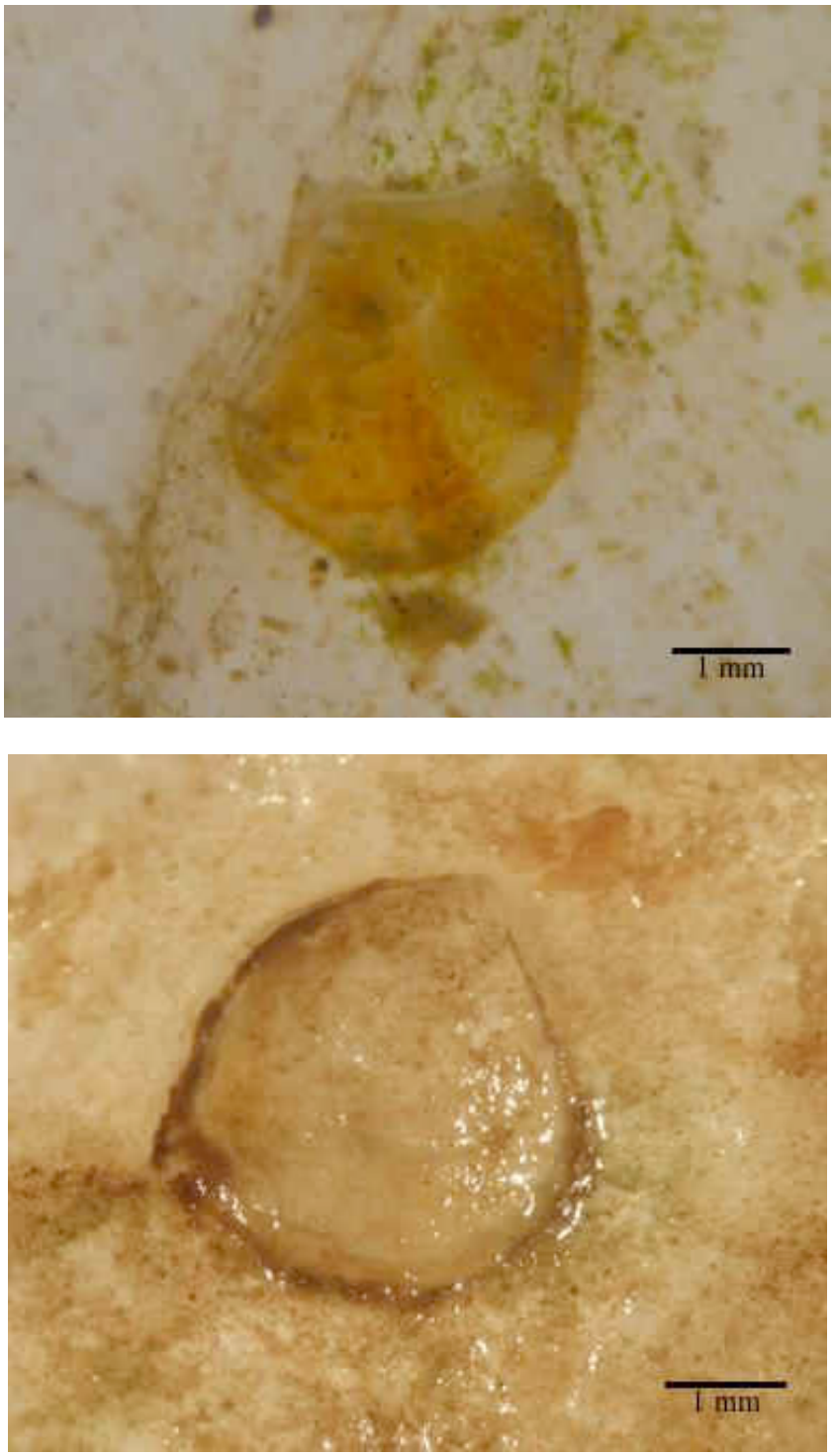

Figure 2. Juvenile *Crassostrea gigas* (Top; L=3.1 mm W=3.4 mm) compared to a juvenile *Ostrea lurida* (Bottom; L=3.8 mm W= 3.2mm). Both are settled on shell of *C. gigas.*

Sixty shell substrata were placed into in 20 mesh bags that were suspended along a 2.5 m section of PVC pipe. Each bag was attached with a cable tie to a loop of string on the PVC pipe spaced about 15 cm apart (Figure 3). Each mesh bag contained three horizontally oriented shells of the same type in a vertical stack, with a knot tied between each to separate them. A small rock was collected from the study site and tied at the bottom of each bag to act as a weight and to ensure that the bag hung straight down and maintained the substrata in a horizontal position (Figure 3). However, during the tidal exchange in Coos Bay water velocities near the study site are such that the small rocks were likely not enough weight to maintain the horizontal orientation of the shells or the mesh bags, but all bags were subjected to the same conditions. Of the 20 total bags, there were five replicate bags of each of the four substratum types. Each replicate bag contained three pseudoreplicates of the same substratum type and for statistical analyses the total settlement in each bag (replicate) was compared.

The three shell substrata hanging in each bag were spaced approximately 10 cm apart and oriented horizontally so each had two surfaces: a top (facing upwards) and a bottom (facing downwards) (see Figure 3). All dead shells were oriented with the surface of the shell that was the inside of the living oyster facing upwards. All live oysters were placed in a natural orientation so that the flat right valve was facing upwards (top) and the cupped left valve was facing downwards (bottom). This orientation was chosen because oysters are known to settle preferentially on the bottom of substrata in field experiments (Cole and Knight-Jones, 1939, Crisp 1967) and in this way, the rugose outer surface of the dead shell was oriented

downwards. Comparisons of *Ostrea lurida* settlement between the inner and outer surfaces of dead Pacific oyster shells (n=148) showed no significant difference between settlement on the what was the smooth inside surface of the living oyster versus the rough outside surface (one-way ANOVA F=0.15 p=0.70). Of the 148 shells sampled, each was randomly assigned to either the top or bottom category and the means were compared (n=74) ensuring that each shell corresponded to one independent data point.

Figure 3. Experimental set up shown before deployment: 20 mesh bags hung 15 cm apart from a 2.5 m long PVC pipe. Each mesh bag has three shells of the same substratum type with knots between each to maintain spacing. A rock is tied at the bottom of each bag for weight

Two separate sets of 20 mesh bags were constructed and suspended from the floating dock. Due to available space at the Sause Bros. dock, these two sites were positioned about 15 m apart. The bags were suspended just below the surface

consistent with findings by Hopkins (1937) that *O. lurida* settled mostly in the top 25 to 75 cm of the surface. The first set of 20 bags was placed in the field on July 14, 2010 and the second set was deployed on July 19, 2010. Each set was collected after it had been in the water for approximately two weeks. The collection dates were staggered so that one of the two sets was collected each week. Assuming there was no significant variation in settlement between the locations of the two sets, this produced data each week but allowed for sample intervals of two-weeks of settlement. A two-week sampling interval allows for a complete tidal cycle in case settlement varies between spring and neap tides in Coos Bay. This two-week interval was also employed in similar seasonal settlement studies in Southern California and Washington (Seale and Zacherl 2009, White *et al*. 2009b).

Data were collected approximately every week continuously from July 28 until Dec 13, 2010. Each week one set of 20 mesh bags was collected from the floating dock by cutting the cable ties and immediately replaced with another prepared set of bags. The collected bags were then brought into the lab at the Oregon Institute of Marine Biology and checked for *Ostrea lurida* juveniles under a dissecting microscope. Each shell was removed from the mesh bag and both sides inspected. When a juvenile was found, the substratum type and the side of the shell (top or bottom) was recorded. The mesh bags containing unexamined shells were stored in a flow through sea water table until they were all analyzed (within 48 hours). Once settlers were recorded, they were removed from the substrata. All substrata were cleaned of dirt and additional epiphytes with a wire brush and stored in a flow through sea table. These clean shells were then chosen haphazardly

and placed into the next set of 20 mesh bags constructed for the subsequent sampling interval.

The experiment continued for one full year with the intent of detecting a possible second earlier peak in settlement in the late spring or early summer. For three months during the winter (Dec 13, 2010 through March 15, 2011) the number of mesh bags deployed in the field was reduced. The water temperatures in the bay are not conducive to spawning at this time, so the number of replicate substrata was reduced in order to expedite the inspection process. Five bags of shell of *Crassostrea gigas* were hung off the floating dock and checked for settlement every month. In the early spring (March 15, 2011) the original experiment was resumed but with only one set of 20 mesh bags instead of two sets (Table 1). The same quantity of substrata was used over the same two-week collection period, but instead of weekly data collection, settlement data were collected every two weeks. Using the same experimental set up allowed comparisons of data between the summers of 2010 and 2011.

Table 1. Description of sample sizes, collection frequency, and data point intervals for the extent of the field experiment (July 2010-July 2011).

Sampling Structure	July – Dec 2010	Dec 2010 –Mar 2011	Mar –July 2011
Sample Size	2 sets of 20 Bags	5 Bags of *C. gigas* shell	1 set of 20 Bags
Collection Frequency	Every 2 weeks	Every month	Every 2 weeks
Data Point Interval	Every week	Every month	Every 2 weeks

To monitor the reproductive status of the adult population, adults were collected occasionally from the adjacent population to see if spawning could be induced. Fifteen individual *Ostrea lurida* from the Coos Bay population were collected on June 6, 2011 and two weeks later on June 22. Five oysters were placed in each of three containers and left out of the water overnight. Warm seawater (22°C) was then added to the containers to induce spawning. This is the same method that was used successfully to spawn ripe adults in August and September of 2010.

Adjustment of Raw Data to Densities

The Pacific oyster is a much larger species than the Olympia oyster with an average size of 8-15 cm for *Crassostrea gigas* and only 4-7 cm for *Ostrea lurida*. Although small live *C. gigas* were preferentially used for this experiment, it was still very difficult to acquire live individuals that were of comparable size to the much smaller live *O. lurida* (Figure 4). In most cases, the shell of *C. gigas* was broken with a hammer to produce shell fragments of equal size to live *O. lurida* but this process was obviously impractical for live oysters. In order to accurately compare the number of juveniles counted per substratum type, the raw counts were adjusted to densities (number of settlers per cm^2).

To calculate density, all of the shell substrata used throughout the duration of the experiment were collected and photographed (top and bottom). Then, using Image J software, the area of each shell was measured and a mean area ($cm^2 \pm SE$) for each substratum type was calculated (Table 2). The total number of settlers was

then divided by the mean area for the corresponding shell type to get the number of juveniles per cm^2.

Figure 4. Examples of the four substrata used in the experiment. From left to right: live *C. gigas*, dead shell of *C. gigas*, live *O. lurida*, and dead shell of *O. lurida*. Notice the size difference between the live *C. gigas* and the other substrata.

Table 2. Mean area and standard error of all shells of each substratum type used in the experiment.

Substratum type	Mean area (cm^2)	Std Err (+/-)
Dead shell *C. gigas*	20.75	0.74
Live *C. gigas*	35.27	0.68
Dead shell *O. lurida*	13.79	0.35
Live *O. lurida*	11.86	0.28

Statistical Methods for Settlement Choices (Substratum, Side, Date)

To compare differences in settlement among the four different substrata, a two-way ANOVA was used with substratum type and date as factors. For this analysis, the density of settlers (#/cm^2) on each shell was used. On each of the 20 collection dates, 60 shells were analyzed (20 mesh bags with 3 shells each). The three pseudoreplicate shells in each bag were summed to get a replicate total for each bag, resulting in five replicate bags of each treatment on each collection date (n=100 for each of the four treatments; 20 dates x 5 bags).

Because of the high prevalence of zeros, the data do not meet the ANOVA assumption of equality of variance (Levene's test: $F=4.61$ $p<0.0001$). However the F-value is small, indicating the high significance of the p-value is mostly due to the high number of replicates (n=400). According to Gamst (2008), in order to address the issue of unequal variances, a more stringent alpha level (p=.025) should be employed in interpreting the results.

In order to analyze differences in settlement on the top side of the shell versus the bottom side, a chi-square table was used. Each oyster shell consists of a top surface connected to a bottom surface, and this inherent link between the two surfaces causes them to not be independent of each other, so an ANOVA test could not be used (Zar 1996).

Settlement on the eight possible shell surfaces was compared (top and bottom of each of the four substrata). For the chi-square chart, the raw number of settlers on each substratum were used. The observed value is the total number of settlers counted on that surface for all dates. The expected value is predicted by the

mean area of each substratum type (see Table 2). From the grand total of the mean area for all four shell types, the percentage that each substratum represented was calculated. Since each substratum has two sides (top and bottom), half of that percentage is the actual percentage of the total available substrata represented by each side. The expected value is then the percentage of the total area per side multiplied by the total observed (all substrata) number of settlers recorded (Table 3). These expected values were used for the top and bottom of each indicated substratum.

If the chi-square analysis yielded a significant p-value (based on the df), then the test was followed up with an analysis of residuals to isolate the source of the deviation from independence (Agresti 2002). This cell-by-cell comparison shows the nature of the deviation from expected values by calculating a Z-score for each shell surface following the formula:

$$Z = (\text{Observed} - \text{Expected} / \sqrt{(\text{Expected})}) / \text{Standard Deviation}$$

The resulting value indicates how many standard deviations an observed value deviates above or below the expected value. If the absolute value of the Z-score is greater than 1.96, there is a significant variation from the expected value at a 95% confidence interval (Zar 1996).

The four substrata were subdivided into categories: dead shell was compared to live oysters and *Crassostrea gigas* substrata were compared to *Ostrea lurida* substrata. The low settlement recorded at the beginning and end of the field collection period contributed to a high prevalence of zero's in the data set. By using

only settlement data from the dates with the highest settlement (September 13, 2010 - November 18, 2010), the data set meets the equal variance assumptions.

Table 3. Calculations for the expected value of *Ostrea lurida* settlers per substratum type. The mean areas are calculated values from Table 2.

	Mean Area (cm^2)	% of Total Area	% of Total Area per side	Total Settlers Observed	Expected Value
Dead Shell *O. lurida*	13.79	16.9%	8.44%	1351	114.05
Live *O. lurida*	11.86	14.5%	7.26%	1351	98.10
Dead Shell *C. gigas*	20.75	25.4%	12.70%	1351	171.63
Live *C. gigas*	35.27	43.2%	21.59%	1351	291.72
Total area (cm^2)	81.67	100.00%			

Frequencies of Multiple Juveniles per Shell Substratum

A histogram of the number of settlers per shell for all shells collected (n=1200) was compared to a Poisson distribution. Poisson probabilities were calculated based on the average rate of settlers per shell (r=1.125) using the following equation for the probability of finding k settlers:

$$p(k) = r*k / (k!)(e*r)$$

A chi-square analysis was then used to compare the observed frequencies to the expected: p(k)*1200 (total shells). If the chi-square analysis resulted in a significant deviation in observed from expected frequencies, Z-scores were calculated for each

number of settlers found per shell. The Z-score value tells the degree and direction of the variation of each frequency from the expected.

Statistical Methods for Timing of Settlement

An ANOVA test was used to compare total settlement at each of the 20 collection dates over the five-month period. A StowAway Tidbit Underwater Temperature Data Logger was placed across the bay from the study site at the Eastside public docks (see Figure 1). This data logger recorded the water temperature every 15 minutes and data were then downloaded directly onto a computer. The average daily temperatures for the duration of the experiment were calculated. In order to compare temperature data to settlement data from the same time period, the average temperature over the exact period that the shell substrata were in the water and settlement was occurring was calculated.

Results

Due to limited space availability at the dock, two adjacent areas of the floating dock were used for hanging the two biweekly sampled sets of 20 mesh bags. These two sites were only 15 m apart on the same side of the dock and the spatial difference was assumed to be negligible. However, in order to be sure of this and allow for pooling of data from both sets of mesh bags, the number of settlers on all shells at both dock sites were compared to determine if settlement between sites varied significantly. The averages of all replicate totals from Site 1 and Site 2 (n=200 each) were not significantly different from each other when compared using

ANOVA (F= 0.12, p = 0.73; Figure 5). Since the two sites were not significantly different, the data from both sites were pooled to create a weekly sampled data set for all of the following analyses.

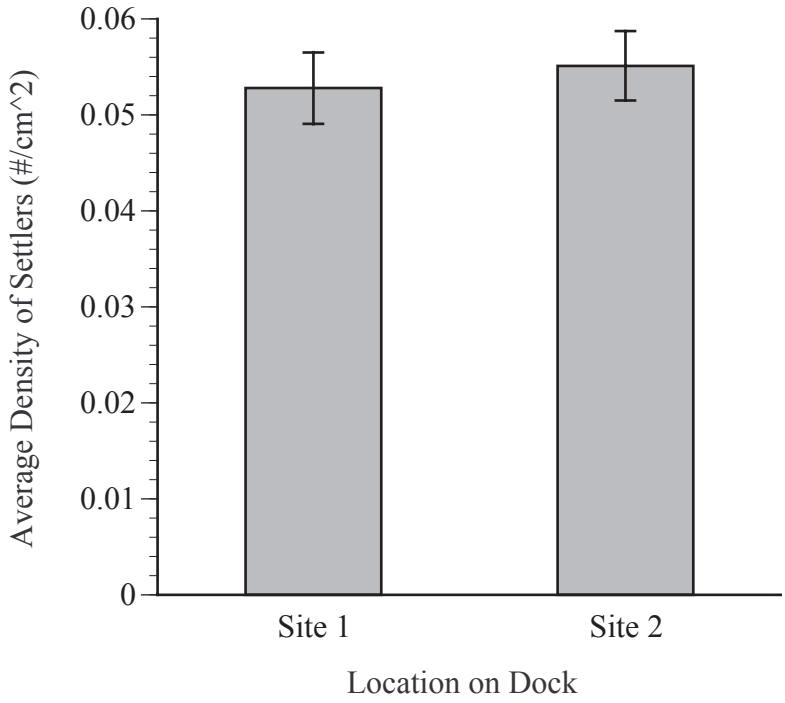

Figure 5. A comparison of the average settlement densities at Site 1 and Site 2 on the floating dock. There is no significant difference between the two sites (F=0.12 p=0.73). Error bars represent 1 SE (n=200).

Field Comparisons: Substratum Type

Table 4 shows the total number of settlers collected on each substratum type (total number of settlers from the 5 replicate bags) at each collection date throughout the experiment. Zero settlers were recorded after December 15, 2010 up until the last collection date of the experiment on June 22, 2011. The highest total number of settled juveniles recorded was on the October 5, 2010 collection date (see totals in bold in Table 4).

In order to compare settlement among the four substrata, raw numbers of settlers were converted to densities (#/cm^2; Table 5). Lower settlement was seen at the beginning and end of the experiment and higher settlement at the collection dates in the middle (Sept 13-Nov 18; bolded section of Table 5).

For the two-way ANOVA test, the total density of juveniles in each bag (sum of three pseudoreplicate shells) were used. Results from the two-way ANOVA with substratum type and date as factors are shown in Table 6. Because of the high prevalence of zero's in this data set, the ANOVA assumption of equal variance was not met, and the p-value was adjusted to 0.025 (Gamst 2008). There was a highly significant difference in *Ostrea lurida* settlement among the 20 collection dates ($F= 24.16$ $p<.0001$) but no significant difference in settlement among the four substratum types ($F=1.37$ $p=0.25$).

Although the two-way ANOVA (Table 6) shows no significant difference between settlement on the four different types of substrata (Figure 6), subdividing the four substrata into two subcategories of live shell (*Crassostrea gigas* and *Ostrea lurida*) versus dead shell and *C. gigas* shell (live and dead) versus *O. lurida* shell may reveal significant differences in settlement. For these comparisons, only the mid-dates of the experiment were compared (September 13 – November 18, 2010; see the bolded section of Table 5). Using only the mid-dates allowed for the reduction of zeros in the data set.

Table 4. The total number of juveniles counted on each substratum type on each of the 20 collection dates. Totals of all juveniles counted on all substrata are shown in the far right column. The highest numbers of juveniles recorded for each substratum type are in bold. The maximum total number of *Ostrea lurida* settlers was on the October 5, 2010 collection date.

Collection Date	Live *C. gigas*	Dead Shell *C. gigas*	Live *O. lurida*	Dead Shell *O. lurida*	Total
28-Jul	0	0	0	0	0
2-Aug	0	1	1	0	2
16-Aug	2	2	2	0	6
19-Aug	10	3	7	2	22
30-Aug	1	4	5	5	15
3-Sep	11	6	3	6	26
13-Sep	42	25	20	22	109
21-Sep	27	29	24	8	88
28-Sep	26	27	17	22	92
5-Oct	**107**	49	**31**	**32**	**219**
13-Oct	89	**61**	29	26	205
20-Oct	34	28	12	13	87
27-Oct	61	23	10	14	108
2-Nov	49	36	3	12	100
8-Nov	62	11	10	7	90
18-Nov	55	27	13	15	110
24-Nov	20	6	5	3	34
1-Dec	12	5	2	0	19
8-Dec	9	1	2	3	15
13-Dec	0	1	4	0	5

Table 5. The average density of settlers by substratum type (n=5 replicates per substratum). The bold numbers indicate dates when the average settlement was greater than 1.00 per cm^2 for most substratum types.

Collection Date	Live *C. gigas*	Dead Shell *C. gigas*	Live *O. lurida*	Dead Shell *O. lurida*
28-Jul	0.00	0.00	0.00	0.00
2-Aug	0.00	0.01	0.02	0.00
16-Aug	0.06	0.05	0.08	0.00
19-Aug	0.28	0.15	0.59	0.15
30-Aug	0.03	0.19	0.42	0.36
3-Sep	0.31	0.29	0.25	0.44
13-Sep	**1.19**	**1.21**	**1.69**	**1.60**
21-Sep	**0.82**	**1.59**	**2.19**	**0.80**
28-Sep	**0.68**	**1.11**	**1.27**	**1.38**
5-Oct	**3.03**	**2.36**	**2.61**	**2.32**
13-Oct	**2.52**	**2.94**	**2.45**	**1.89**
20-Oct	**0.96**	**1.35**	**1.01**	**0.94**
27-Oct	**1.73**	**1.11**	**0.84**	**1.02**
2-Nov	**1.39**	**1.74**	**0.25**	**0.87**
8-Nov	**1.76**	**0.53**	**0.84**	**0.51**
18-Nov	**1.56**	**1.30**	**1.10**	**1.09**
24-Nov	0.57	0.29	0.42	0.22
1-Dec	0.34	0.24	0.17	0.00
8-Dec	0.26	0.05	0.17	0.22
13-Dec	0.00	0.05	0.34	0.00
Average	0.87	0.83	0.84	0.69

Table 6. Results for a two-way ANOVA with substratum type and date as factors (n=400). Settlement varied significantly among dates but not among the four substratum types. There was no significant interaction effect.

Source of Variation	SS	df	F-value	p
Date	8.97	19	24.16	<.0001
Substratum type	0.08	3	1.37	0.25
Date*Substratum Type	1.16	57	1.04	0.40

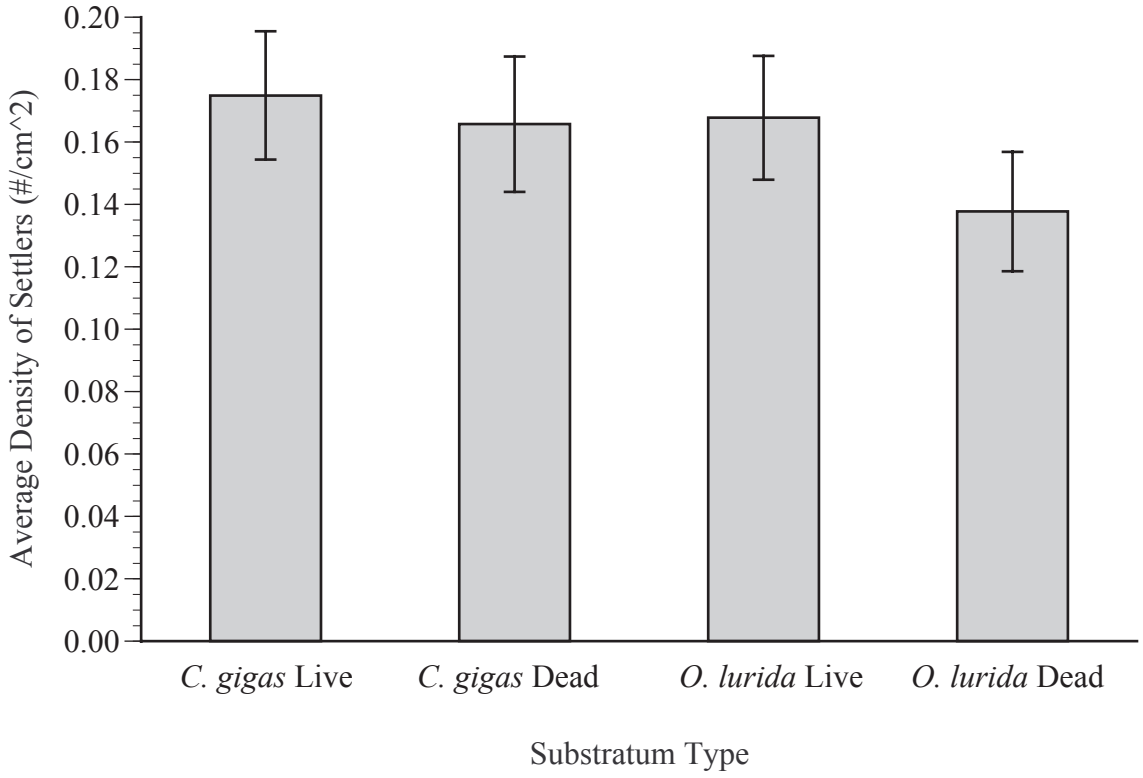

Figure 6. The average density of settlers for each of the four substrata. Error bars represent 1 SE (n=100). There is no significant difference in settlement among substrata (F=1.04 p=0.38).

The comparison between live oysters and dead oyster shell (Figure 7) shows no significant difference in settlement (one-way ANOVA F=0.73 p=0.40). The

comparison between settlement on *Crassostrea gigas* and *Ostrea lurida* substrata (Figure 8) also indicated no significant difference in settlement (ANOVA F=2.60 p=0.11).

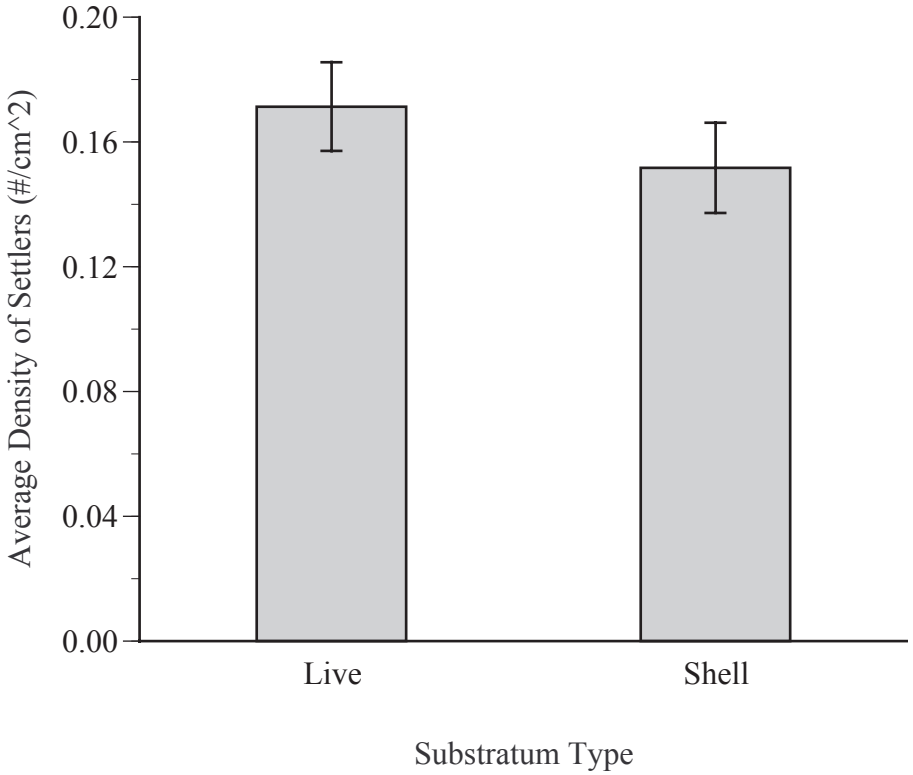

Figure 7. The average density of settlers (#/cm^2) on live oysters versus dead oyster shell. There was no significant difference in settlement between treatments (one-way ANOVA F=0.73 p=0.40). Error bars represent 1 SE (n=200).

Field Comparisons: Top vs. Bottom

Higher settlement occurred on the bottom surface of the horizontal shell substrata than the top surfaces (Figure 9).

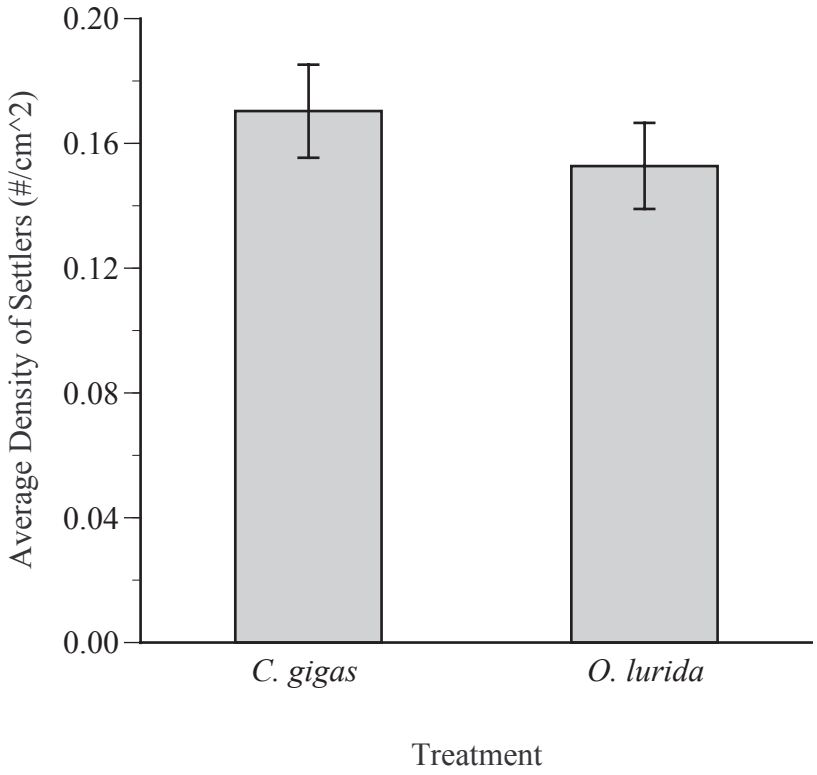

Figure 8. The average density of settlers (#/cm^2) on *Crassostrea gigas* shell (live and dead) and *Ostrea lurida* shell (live and dead). Error bars represent 1 SE (n=200). There is no significant difference in settlement (one-way ANOVA F=2.60 p=0.11).

The overall X^2 value (X^2 =563.78) indicates significant variation from the expected settlement (df=7 p<.0001). Z-scores for each surface type indicate the significance and direction of variation from the expected (see Table 3) number of settlers (Table 7). In all cases, there was significantly less settlement than expected on the top surfaces of all substratum types and significantly more settlement than expected on the bottoms (95% confidence = 1.96). All Z-scores are clearly significant (Table 7 and Figure 10).

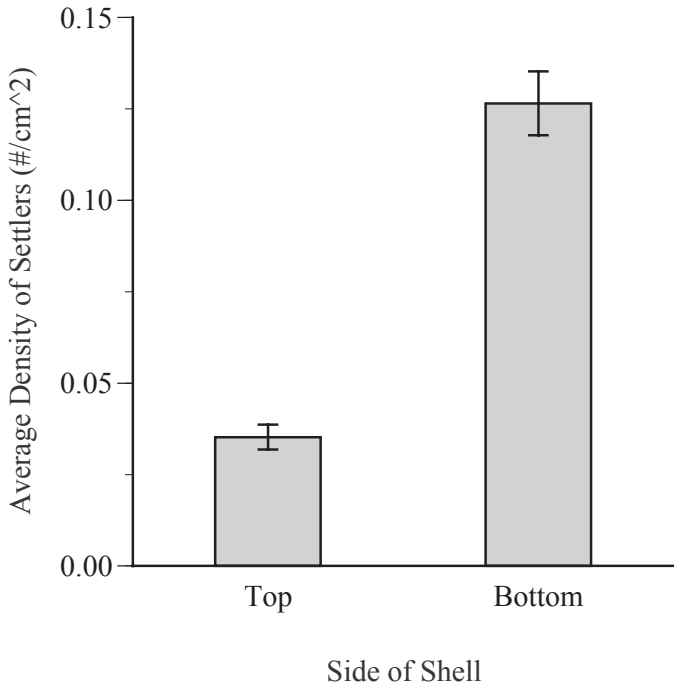

Figure 9. Average density of settlers on the top and the bottom of the shell substrata. Higher settlement occurred on the bottom of the shell substrata than on the top. Error bars represent 1 SE (n=200).

Table 7. Chi-square table for both sides of each substratum type. The Z-scores values indicate how many standard deviations the observed value is from the expected and in which direction. Z-scores with an absolute value greater than 1.96 are significant at a 95% confidence level.

Surface	Observed	Expected	X^2	Z-score
Dead Shell *O. lurida* top	36	114.06	53.42	-10.64
Dead Shell *O. lurida* bottom	154	114.06	13.99	5.57
Live *O. lurida* top	78	98.10	4.12	-2.97
Live *O. lurida* bottom	122	98.10	5.83	3.56
Dead Shell *C. gigas* top	39	171.63	102.49	-14.91
Dead Shell *C. gigas* bottom	305	171.63	103.65	15.86
Live *C. gigas* top	107	291.72	116.97	-16.57
Live *C. gigas* bottom	510	291.72	163.33	21.55
Totals	1351	1351.00	563.78	

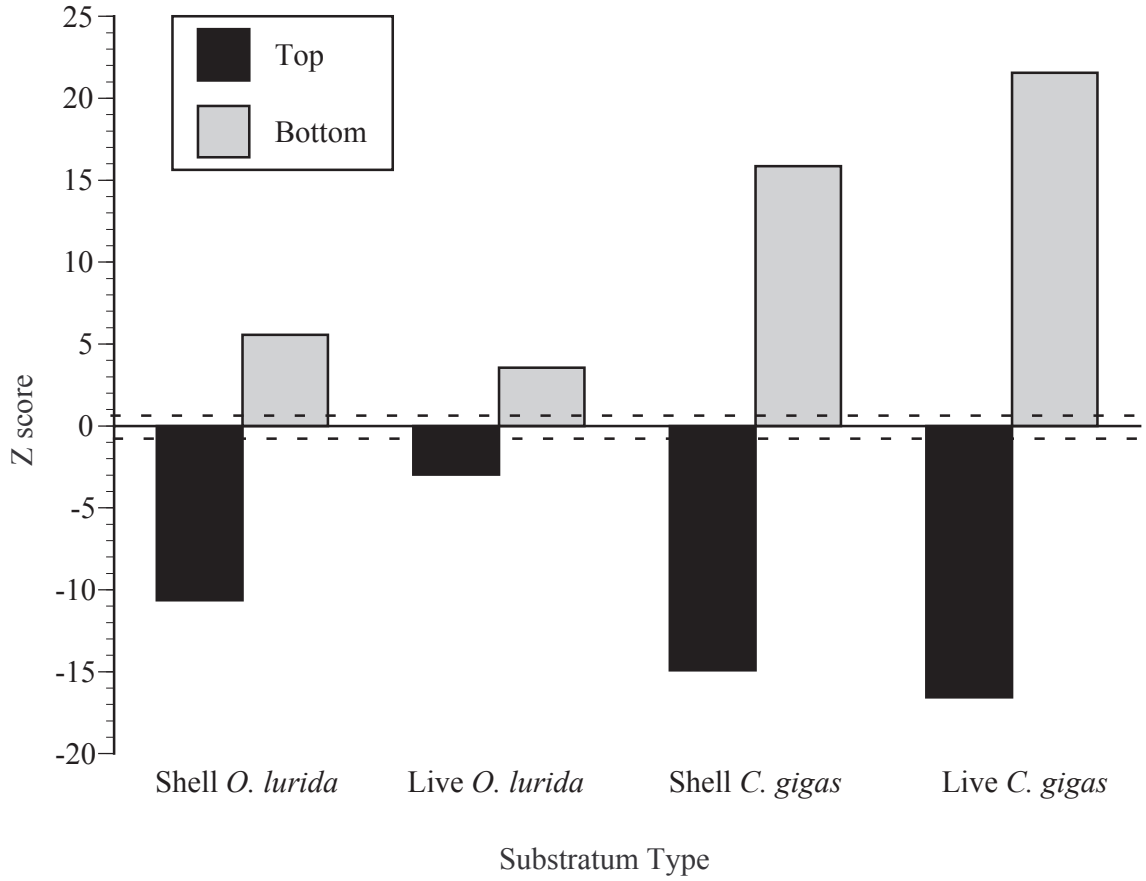

Figure 10. The Z-scores for both sides of each substratum. Significantly higher than expected settlement occurred on the bottom surface of all substrata while significantly lower than expected settlement occurred on the top of all substrata. The dashed lines represent the 95% confidence interval (1.96).

Frequencies of Multiple Juveniles per Shell Substratum

For each two-week period throughout the experiment, 0 to 16 *Ostrea lurida* individuals were counted per shell. A histogram of the settlement frequencies illustrates the number of settlers per shell (n=1200) over the collection period compared to the Poisson distribution of the expected values for each shell (Figure 11). The greatest frequency of observed number of settlers per shell was zero,

followed by shells with only one juvenile present, and a decreasing frequency of shells with higher densities of settlers. There were many shells with high numbers of settlers (20 shells with 9 or more juveniles settled). Table 8 shows that Z-scores for all shells with more than 9 settlers varied significantly from the expected values at those densities (0 in all cases), which led to the extremely high chi-square value (X^2=27,674,000). Z-score values showed no significant variation from expected values for frequencies of shells with less than 9 settlers (Table 8).

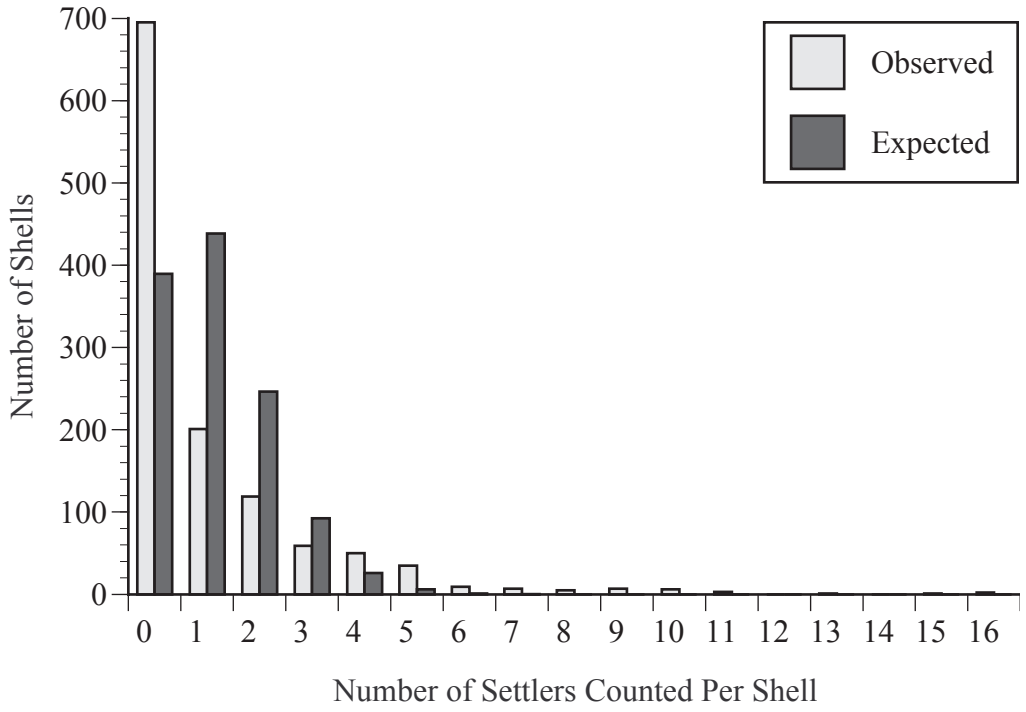

Figure 11. Number of settlers per shell compared to the calculated Poisson distribution. Average number of settlers = 1.125 (n=1200). Chi-square analysis showed significant variation of observed frequencies from expected (X^2=27,674,000 df=15 p<.0001).

Table 8. Observed and expected frequencies of number of settlers per shell. Observed frequencies varied significantly from expected (X^2=27674230 df=15 p<.0001). Significant Z-score values are in bold (95% confidence interval = 1.96).

Number of settlers per shell	Observed	Poisson Probability/ Expected	X^2	Z-scores
0	695	389.6	0.200	0.853
1	201	438.3	0.107	-0.540
2	119	246.5	0.055	-0.360
3	59	92.4	0.010	-0.147
4	50	26.0	0.018	0.195
5	35	5.9	0.121	0.496
6	9	1.1	0.047	0.309
7	7	0.2	0.220	0.664
8	5	0.0	0.832	1.291
9	7	0.0	13.1	**5.139**
10	6	0.0	86.0	**13.134**
11	3	0.0	210	**20.523**
12	0	0.0	0.000	0.000
13	1	0.0	2880	**75.921**
14	0	0.0	0.000	0.000
15	1	0.0	478002	**977.959**
16	2	0.0	27193035	**7377.767**
Total	1200	1200	27674230	

Timing of Settlement in Coos Bay

Table 4 and Figure 12 show that the highest numbers of settlers were counted on October 5, 2010 for almost all substratum types. Settlement varied significantly among the 20 collection dates with increased settlement from September- November and a distinct settlement peak in October.

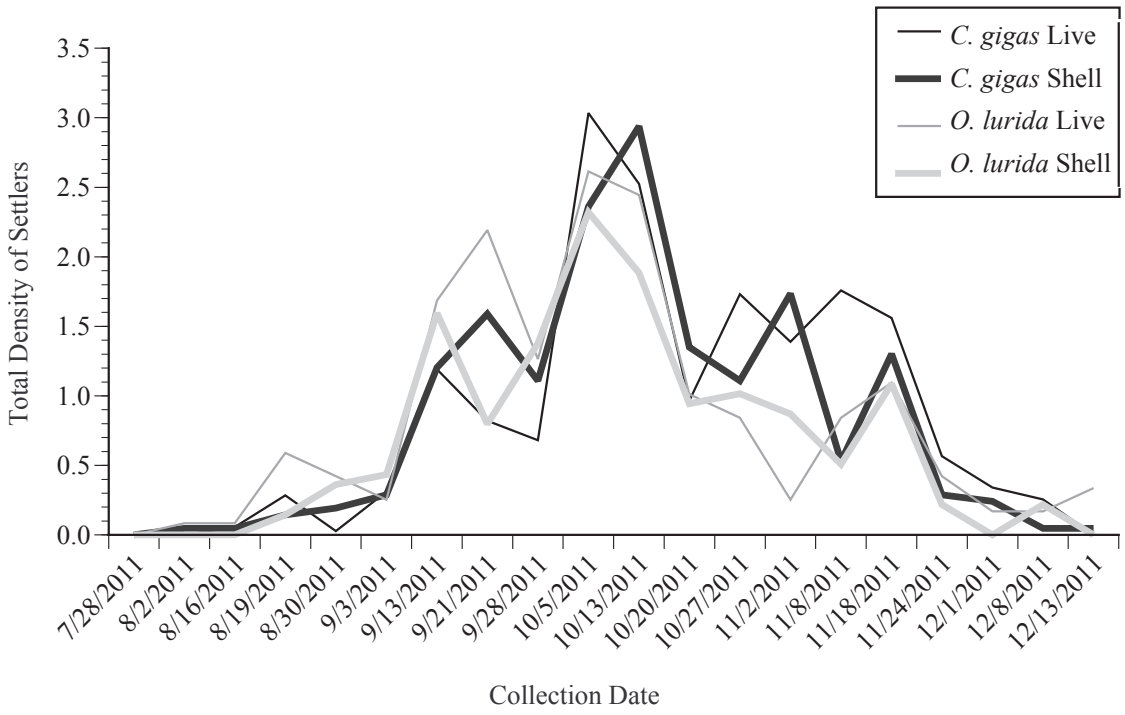

Figure 12. Total densities of settlement (#/cm^2) on each substratum throughout the season. Settlement on all four substratum types follows the same temporal pattern.

Temperature and Timing of Settlement

In order to investigate both settlement and temperature, the average water temperature was calculated for the time period the settlement substrata were suspended in the water column. Each temperature data point reflects a two-week period prior to the collection date and corresponds with the same two-week period during which actual settlement took place (Figure 13). There does not seem to be a

direct correlation between temperature and the onset of settlement of *Ostrea lurida* in Coos Bay. A decrease in temperature does coincide with the time at which settlement began to decrease, but no clear pattern is apparent.

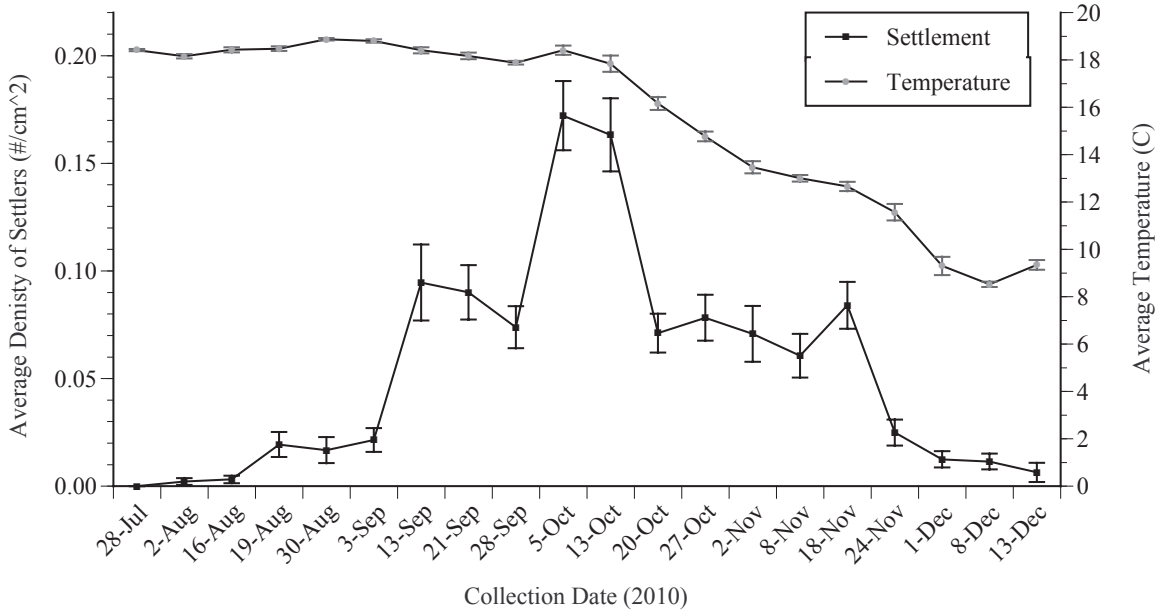

Figure 13. The average density of settlement on all substrata from July 2010 until December 2010 and the average temperature during the corresponding two-week settlement period. Error bars represent 1 SE.

Discussion

Settlement of larvae of *Ostrea lurida* in Coos Bay on four different substrata was monitored from July 2010 until July 2011. Settlement on the four substratum types did not vary significantly, although for all substrata there was a trend of higher settlement on the bottom surface than on the top surface of the horizontally oriented substrata.

On all four substrata there was lower than expected settlement on the top of the horizontal surface and higher than expected settlement on the bottom. This is

consistent with the reported preference of oyster larvae to settle on the undersides of substrata, perhaps to avoid siltation or due to their swimming orientation (Cole and Knight Jones 1939, Crisp 1967, Michener and Kenny 1991). For restoration projects, a mechanism for elevating shell off the bottom could enhance settlement on added substrata by allowing access to the preferred undersides of substrata.

Comparisons between live oysters and dead oyster shell and between *Crassostrea gigas* and *Ostrea lurida* substrata show no significant difference in densities of settlement. If *O. lurida* larvae had a strong response to cues for gregarious settlement, a preference for conspecific adults and thus increased settlement on live *O. lurida* would have been expected. Unlike other reef forming oyster species, *O. lurida* tends to settle on hard substrata such as cobble and form loose aggregations (Kimbro and Grosholz 2006). It is possible that larval settlement cues and selectiveness are less or differently expressed in this species, although clumps or clusters of *Ostrea lurida* are often found where multiple oysters have settled on top of each other. The lack of significant differences in settlement among the four substrata employed suggests a lack of selectivity in *O. lurida* larvae.

Juvenile *Ostrea lurida* were found from August 2 to December 13, 2010 with the greatest settlement from mid-September through mid-November. There was one peak in settlement in early October. This peak occurred later than those reported both north and south of Oregon and correlates most closely with the temporal patterns reported from British Colombia. Similar to recent timing of settlement experiments in Washington and California, no universal temperature can be linked directly to the initiation of reproduction and the first juveniles collected.

The timing of settlement in Coos Bay is consistent with data collected over the same time period of quantities of *Ostrea lurida* larvae in the water column near the study site. Plankton tows were taken every week in the bay across from the study site by OIMB post-doctoral investigator Laura Garcia-Petiero. She found larvae in the water column from late July until the end of sampling at the end of September. Peak larval abundance in the water column was on her sampling date of September 22 (100,000 larvae/m^3) which corresponds with the highest recorded settlement on the October 5th and October 13th collection dates, 14 and 22 days later respectively.

After December 13, 2010, deployment of substrata in the field continued until June 22, 2011, but no settlement was recorded. There may be no settlement early in the season and only one peak of settlement in Coos Bay. Attempts to spawn *Ostrea lurida* adults in June of 2011 corroborate this finding. Adults were collected from the Coos Bay population adjacent to the study site on June 6 and two weeks later on June 19, 2011. Both attempts to induce spawning were unsuccessful indicating that gonads had not yet matured.

Larvae of *Ostrea lurida* in Coos Bay show a peak in settlement from September to November. No strong larval preferences were seen in settlement on live and dead shell of *O. lurida* and *Crassostrea gigas*. Non-selective settlement on oyster shell substrata has positive implications for current *Ostrea lurida* restoration projects. Shell of the Pacific oyster is abundant and easy to come by whereas shell of the Olympia oyster is rare. It is easier to facilitate restoration with Pacific oyster

shell, and according to this study, there should be a comparable settlement response by doing so.

Bridge

While conducting these field experiments over the summer of 2010, I also designed similar experiments in the laboratory to look at larval preference among various substratum types. By performing experiments in the lab, specific variables can be isolated and compared to field experiment results. Settlement can also be easily examined on a smaller time scale, collecting data on *Ostrea lurida* settlement at 24-hour increments as opposed to two-week intervals. Larval choice experiments were conducted with various substrata that can be found near the field experiment site including: *Ostrea lurida* shell, *Crassostrea gigas* shell, clamshell (*Protothaca staminea*), rock, and wood.

CHAPTER III

SETTLEMENT CHOICES OF *OSTREA LURIDA* LARVAE IN THE LABORATORY

Introduction

Olympia oysters (*Ostrea lurida*) once formed dense beds along the west coast of the United States and Canada providing habitat for many fish and invertebrates. These populations were overfished by European settlers and although the pressures of the fishery were eliminated over 50 years ago, oyster populations have failed to recover. The availability of appropriate hard substrata is one of the key components for the successful restoration of Olympia oyster populations. Recent investigations of the lack of recovery of *O. lurida* populations along the west coast of North America have identified substratum availability as an important limiting factor in restoration efforts (White *et al*. 2009, Groth and Rumrill 2009, Trimble *et al*. 2009, Dinnel *et al*. 2009). The addition of hard substrata to the habitat was a common practice in efforts to restore the east coast native oyster (*Crassostrea virginica*) and is becoming common practice in Olympia oyster restoration as well (Brumbaugh 2009). However, in order to choose appropriate substrata for additions, the ecology of the Olympia oyster must be understood, especially the process by which settlement sites are selected by the pediveliger larvae.

As in most sessile marine invertebrates, the process of settlement for oysters occurs as the transition between the mobile planktonic stage and the immobile adult stage and occurs when a competent larva attaches to the substratum and undergoes metamorphosis (Chia and Rice 1978). The selection of a suitable substratum at settlement can influence survival and success of individuals and of the population as a whole (Thorson 1950, Connell 1985, Eckman 1996, Blythe and Pineda 2009). The settlement process is governed by multiple factors acting simultaneously including the availability of substrata, the water motion or hydrodynamics near the substratum, and larval behavior and selection of substrata (Pineda *et al*. 2008).

For *Ostrea lurida* populations in Coos Bay, there is a dearth of available hard substratum. The bottom of the bay consists mainly of mud, with some scattered cobble, gravel, rip-rap and wood. However, shell of Pacific oysters, *Crassostrea gigas*, are very abundant in the mid intertidal zone in commercial mariculture plots located throughout the mesohaline region of Coos Bay. Oysters are known to settle on a variety of hard surfaces and the populations of adult Olympia oysters in Coos Bay are concentrated at sites with available hard substrata, including wood, when no other hard substratum is present (Hopkins 1935, Cole and Knight-Jones 1939, Groth and Rumrill 2009).

Previous experiments with oyster pediveligers have shown that larvae select settlement sites actively based on biotic and abiotic cues, including chemicals from conspecific adults (Crisp 1967, Bayne 1969, Cranfield 1973, Veitch 1971, Bonar *et al.* 1990, Tamburri *et al*. 1992, Fitt and Coon 1992, Turner *et al.* 1994, Zimmer-Faust and Tamburri 1994, Paschual and Zampatti 1994). Observations of the mechanisms

of settlement behavior of *Ostrea lurida* larvae have been made in the laboratory (Hopkins 1936 and 1937, Hori 1933, Imai *et al.* 1954) but larval choice when presented with multiple different substrata has not been investigated.

Water movements can have both positive and negative effects on larval encounter of substrata in nature based on the velocity and direction of water movement (Hunt and Shielbing 1996, Pernet *et al.* 2003). Water flow can increase the rate of contact with substrata, but high water flow may restrict the active investigation of substrata by larvae (Eckman and Duggins 1998, Crimaldi *et al.* 2002) or make attachment difficult (Crisp 1955).

Low salinity is a known stressor to adult Olympia oysters, with prolonged exposure to low salinities resulting in die offs (Grosholz 2008, Wasson 2010). However, larvae of estuarine organisms have been shown to have high tolerances to salinity fluctuations (Morgan 1995). Olympia oysters are most successful at salinities over 25 and in Coos Bay are most commonly found at salinities of 22-28 and (Korringa 1976, Couch 1989, Baker 2000). Fluctuations in salinity may put *Ostrea lurida* larvae under stress and alter larval selection of substrata for settlement.

In the present study, lab experiments were designed to isolate factors in the settlement process of *Ostrea lurida* larvae and address the following questions:

1) If appropriate preferred substrata are not available to *Ostrea lurida* larvae, will they settle on whatever is available?
2) If larvae of *Ostrea lurida* are presented with multiple substratum choices for settlement, will they consistently select certain substrata over others?

3) Does water movement affect the capacity of larvae to choose settlement sites by increasing the rate of encounter with available substrata?
4) Does stress affect the selectivity of larvae; specifically do larvae raised at different salinities exhibit differential responses to offered substrata?

Materials and Methods

Single Substratum Offered

This experiment was designed to test the time course of settlement when larvae were presented with only one substratum choice at a time. Larvae used in the experiment were from the Whiskey Creek Shellfish Hatchery in Netart's Bay, OR and were transported by truck and received at the Oregon Institute of Marine Biology on July 17, 2010. These larvae were already eyed pediveligers and competent to settle and larval experiments were begun straight away.

Six different substrata were used for this experiment. Shells of *Ostrea lurida* were collected from Coos Bay, near a large population of adults. The shells of *Crassostrea gigas* were collected from a large pile of shucked shells across from Chuck's Seafood in Charleston, OR. Clamshells (*Protothaca staminea*) were collected from a sea table at OIMB. Small rocks (basalt and mudstone) and wood pieces were collected from the OIMB grounds. The sixth treatment was with no added substratum. All substrata were brushed clean with a wire brush and then soaked in seawater for at least 24 hours to build a biofilm (Gribben *et al.* 2009). Substrata were broken into small fragments about 1 cm^2 and were of approximately equal areas.

The experiment began on July 20, 2010 and ran for four days. One of six different substrata was placed in each of 30 finger bowls: *Crassostrea gigas* shell, *Ostrea lurida* shell, clamshell (*Protothaca staminea*), wood, rock and no added substratum. There were five replicates of each substratum type and each row of dishes contained all six substrata arranged randomly. An average of 192 $\pm$ 13 (SE) larvae were added with 40 mL of filtered seawater to each bowl. These larval densities are similar to those used in other oyster settlement experiments (Bayne 1969, Tamburri *et al*. 2008). The total number of larvae in each bowl was counted on the first day of the experiment. Since the total number of number of larvae in each bowl varied, the percentages of larval settlement for each bowl were used for analyses. The bowls were placed in a dark drawer at room temperature (19°C). Previous studies indicate no significant effect of light on settlement, so a dark environment was used to eliminate variations in light regimes as a factor (Hopkins 1937, Beiras and Widdows 1995).

The water was changed daily by reverse filtration and replaced with fresh filtered seawater at 19°C. Every 24 hours all substrata were examined under a dissecting microscope and the cumulative numbers of settled *Ostrea lurida* on each substratum were recorded. Previous settlement experiments with *Ostrea edulis* larvae also used 24-hour time periods to assess larval settlement (Cole and Knight-Jones 1939, Bayne 1969).

The cumulative percent settlement per replicate (cumulative number settled/ initial total), after each 24-hour period was compared using a repeated

measures ANOVA to analyze the effect of time as well as the within-subjects factor of substratum type on settlement.

Four-Way Substratum Choice Experiment

To determine whether larvae prefer certain substratum types, I compared cumulative settlement of *Ostrea lurida* larvae that were presented with four substratum choices simultaneously. Larvae received from the Whiskey Creek Shellfish Hatchery on July 17, 2010 were used in this four-choice experiment beginning on July 19. An average of 144.6 $\pm$ 4.2 (SE) *Ostrea lurida* larvae were placed in 100mL of filtered seawater in each of 12 Petri dishes. Each dish was divided in four quarters by raised glass partitions extending halfway up the dish and one substratum was placed in each quarter with enough water to fill the Petri dish sufficiently so that larvae could swim freely over the barriers between quadrants. The four hard substrata used were *Ostrea lurida* shell, C*rassostrea gigas* shell, clamshell (*Protothaca staminea*), and rock. All were of comparable size. Substrata were collected from the same sources as in the previous design and soaked in running seawater for at least 48 hours.

The twelve replicate Petri dishes were set out in three rows of four (Figure 1) and left in a dark drawer at room temperature (19°C). Water was changed daily by reverse filtration throughout the four-day experiment. Each substratum was checked for settlement after 24 hours under a dissecting microscope and the cumulative number of settlers was recorded for each substratum. The initial number of larvae in each dish was counted at the beginning of the experiment and the percent settlement was calculated for each substratum.

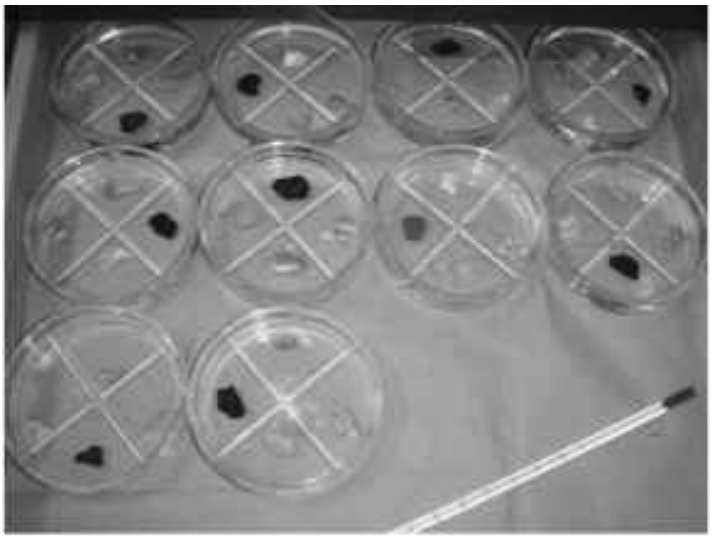

Figure 1. Experimental set up of Petri dishes showing 10 of the 12 Petri dishes, each with one of four substratum types in each quadrant.

Cumulative percent settlement was calculated for each replicate at every 24-hour period. A repeated measures ANOVA was used to analyze differential settlement over the four-day period with time as a within-subjects factor and substratum as a between-subjects factor.

Since the larval choice experiments were necessarily centered on choice between various substrata, the ANOVA assumption of independence was not met since the choice of a larva to settle on any one substratum excludes its settlement on the other substratum choices. Instead of using an ANOVA test to compare settlement between the various substrata, a contingency table and chi-square analysis were used to compare the observed settlement to the calculated expected settlement. Since settlement was cumulative, data from the last day of the experiments were used for the comparisons. Raw numbers of cumulative settlers on the last day of the experiment were totaled for all substrata and divided by the

number of substratum choices to obtain the expected settlement value. Using raw numbers of settlers versus percentages is advantageous because it eliminates zero's from the data set and allows for more accurate comparisons. If the chi-square test indicated a significant p-value, subsequent Z-scores were calculated for each substratum to isolate the source of the deviation from the expected values of settlement. Z-score values indicate how many standard deviations the observed value is from the expected value and Z-scores with an absolute value greater than 1.96 are significant at a 95% confidence interval.

Four-Way Substratum Choice Experiment with Water Motion

To address larval settlement choices between four substrata in moving water, experiments were performed on a Red Rotor Orbital Shaker set at low speed. The orbital shaker created water agitation with the intent of simulating conditions that are more realistic, thereby encouraging higher settlement rates. Such mechanisms have been employed to attain higher settlement in previous experiments with *Crassostrea gigas* larvae (Beiras and Widdows 1995). Larvae for the experiment were obtained by collecting and spawning adults from the Coos Bay population adjacent to the field study site. Fifteen adult *Ostrea lurida* were collected on August 11, 2010 and brought back to the laboratory at OIMB to induce spawning. All adults were brushed clean with a toothbrush and all epiphytes were removed. Five adults were placed in each of three tubs, left out of water overnight, and then moved into heated 22°C water baths to induce spawning. The larvae were cultured at 20°C with water exchanged by reverse filtration every other day and fed every

day (*Chaetoceros* sp. at 30,000 cells per mL) until they were eyed pediveligers at which point larval settlement experiments were initiated.

The four-way substratum choice experiment in quartered Petri dishes was repeated on the orbital shaker with the same four substratum choices. Twenty-five competent veliger larvae (25 days old) were placed in each of four Petri dishes along with 100 mL filtered seawater. The Petri dishes were placed on the orbital shaker in the dark, under a cardboard box. The number of settlers on each substratum was counted under a dissecting microscope after 24 hours and 48 hours.

A repeated measures ANOVA was used to compare cumulative settlement for each replicate on Day 1 and Day 2. To compare settlement between the four substratum choices, a chi-square analysis was used for data from the final day of the experiment.

Two-Way Substratum Choice Experiment with Water Motion

Larval settlement choice between two substrata (shell of *Crassostrea gigas* and of *Ostrea lurida*) in moving water was analyzed. On August 30, ten larvae from the spawned adults from the Coos Bay population were added to five finger bowls set on the orbital shaker. In this experiment, finger bowls were used instead of the quartered Petri dishes since less than four substratum choices were offered. Two substrata (*C. gigas* shell and *O. lurida* shell) of comparable size were placed in each bowl and the number of settlers was recorded after 48 hours. A chi-square analysis was used to compare observed settlement to the expected values.

Three-Way Substratum Choice Experiments with Water Motion I

Larvae from the Coos Bay larval culture were 27 days old when the experiment began on September 8, 2010. Twenty-five larvae were added to an array of 9 bowls with three different substrata (shell of *Crassostrea gigas*, shell of *Ostrea lurida* and rock) in each and left on the orbital shaker in the dark for two days. On Day 1 and Day 2, the cumulative numbers of settlers on each substratum was recorded.

A repeated measures ANOVA was used to compare settlement for each replicate on Day 1 and Day 2 as well as the two-way interaction between time and substratum type. To compare the three substratum choices, a chi-square analysis was employed.

Three-Way Substratum Choice Experiments with Water Motion II

The three-way choice experiment on the orbital shaker was repeated, but this time for 72 hours instead of 48 hours. Twenty-five larvae (29 days old) were placed into five finger bowls with the same three substrata in each (*Ostrea lurida* shell, *Crassostrea gigas* shell and rock). The number of larvae settled on each substratum was recorded after three days. For comparisons of settlement on the three substrata, a chi-square analysis was used.

Two-Way Substratum Choice Experiment at Five Salinities

Settlement was recorded for larvae raised at five different salinities to explore larval settlement choices under stress. On September 22, 2010 larvae were spawned using the same methods described above. Spawned larvae were divided into 30 beakers and raised in an incubator at 20°C under one of five salinity conditions: salinity 15, 20, 25, 29 or 33. They were fed *Chaetoceros* sp. (25,000-30,000 cells per mL) and the water was changed every other day in all beakers by reverse filtration. Each beaker started with a total of 58.4 $\pm$ 10.9 (SE) larvae in 200 mL of filtered seawater, but average of only 15.8 $\pm$ 1.2 (SE) larvae survived to the pediveliger stage when settlement experiment began (14 days).

Settlement experiments began on October 6, 2010 once the larvae were eyed pediveligers. The larvae raised at salinity 15 were not yet eyed pediveligers on this date and settlement experiments were not started until two days later on October 8. Larvae were placed into 6 replicate beakers at each salinity. In each beaker two oyster shell pieces of comparable size were added: one shell piece of *Ostrea lurida* and one shell piece of *Crassostrea gigas.* The substrata were checked for settlement every 2-3 days over a 13-day period at which point most larvae had died or settled (only one beaker had more than 4 larvae). After settlement was recorded, substrata were cleaned under the dissecting microscope and then added back into the beakers.

The cumulative percentage of larvae that settled in each beaker was calculated for both types of substrata (out of initial totals). A repeated measures ANOVA was used to compare the cumulative percent settlement on both substrata

at each salinity over the 13-day experiment. To create an equally sampled data set for the ANOVA test, zero settlement was assumed for Day 1 in all salinity 15 beakers since larvae were not yet competent. A complex mixed design ANOVAR was used for between subjects-factors of substratum (n=2) and salinity (n=5) analyzed over time (n=6). If two- or three-way interaction factors were found to be significant, post-hoc pairwise Bonferroni tests were performed to analyze the main effects.

Results

Single Substratum Offered

Settlement in this experiment was very low with a cumulative total percentage of 6.2% settlement (total number settled/total number of larvae). In addition, there was a very high prevalence of zero settlement even after the experiment ran four days (Figure 2). Results of the repeated measures ANOVA are shown in Table 1. Mauchly's W was significant ($W=0.24$ $p<0.0001$) so the assumption of sphericity was violated. Because the Huynh-Feldt epsilon value was greater than 0.7 ($e=0.735$) the Huynh-Feldt adjusted df were used for the within-subjects factor of time.

The cumulative percentage of settlement was significantly different among the four dates ($F=10.34$, $p<.0001$) and the six substrata ($F=3.13$, $p=0.015$). Stepwise Bonferroni comparisons indicated that settlement on *Ostrea lurida* shell is significantly higher than all other substrata on Day 3 and Day 4 but not on Day 1 or Day 2 consistent with the significant two-way interaction of time and substratum ($F=5.43$, $p<0.0001$).

Table 1. A repeated measures ANOVA with substrata as a between-subjects factor and time as a within-subjects factor. Both main effects are statistically significant was well as the two-way interaction.

Source	SS	df	F-value	p
Substrata	0.015	5.000	3.132	0.015
Date	0.000	2.207	10.343	<.0001
Date*Substrata	0.001	11.034	5.434	<.0001

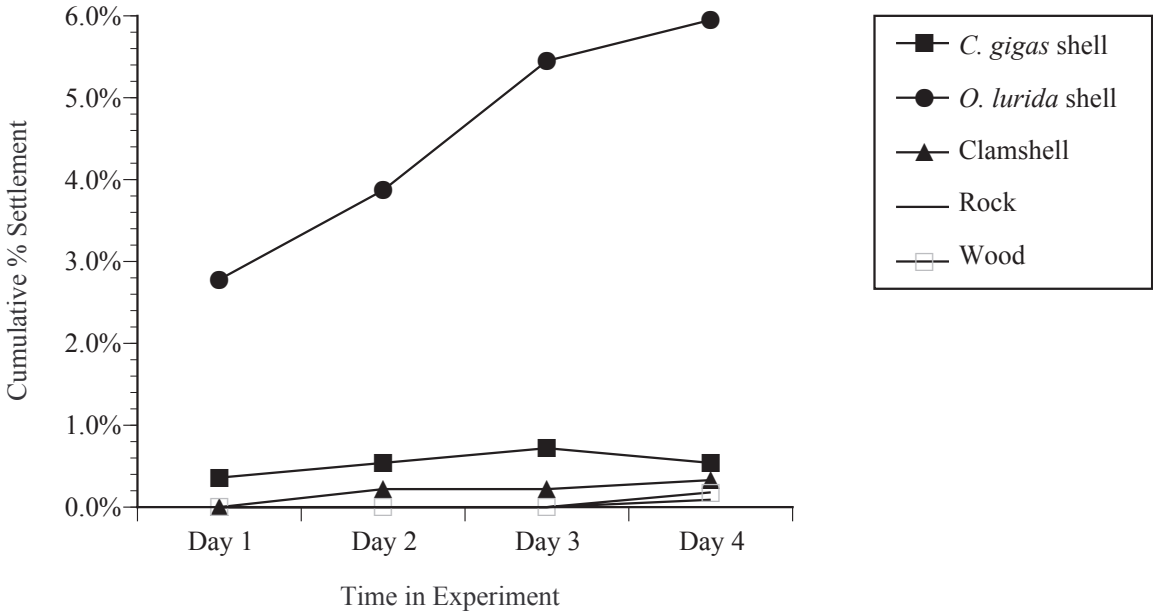

Figure 2. The cumulative percent settlement on each substratum over the four-day experiment. There was significantly higher settlement on the shell of *Ostrea lurida* than on all other substrata (p=0.015) and settlement was significantly different among days, with higher settlement on days 3 and 4 (p<.0001).

Four-Way Substratum Choice Experiment

Total cumulative settlement on the last day of the experiment (Day 4) was 19.39%. Results of the repeated measures ANOVA are shown in Table 2. The Mauchly's W value was significant (W=0.47 p<.0001) and the Huyhn-Feldt epsilon

was greater than 0.7 (e=0.84) so the Huynh-Feldt adjusted df were used for the within-subject factors. The two-way interaction effect was not significant (F=0.78 p=0.61) but settlement was significantly different over time in the four-day experiment (F=5.63 p=0.0023).

Table 2. Results of a repeated measures ANOVA with substrata as a between-subjects factor and time as a within-subjects factor. Both main effects are significant but there is no significant interaction effect.

Source	SS	df	F-value	p
Substrata	0.018	3.000	4.080	0.012
Date	0.005	2.523	5.629	0.002
Date*Substrata	0.002	7.568	0.783	0.612

The average percent settlement for each substratum over the four-day experiment is shown in Figure 3. The four substrata were compared using a chi-square analysis, which showed that settlement values were significantly different than expected (X^2= 13.75, df=3, p=0.003), consistent with the ANOVA results although the assumption of independence was violated. Calculations of the Z-score for each substratum showed significantly more settlement on *Ostrea lurida* and the clamshells than expected and significantly less than expected settlement on rocks (Figure 4).

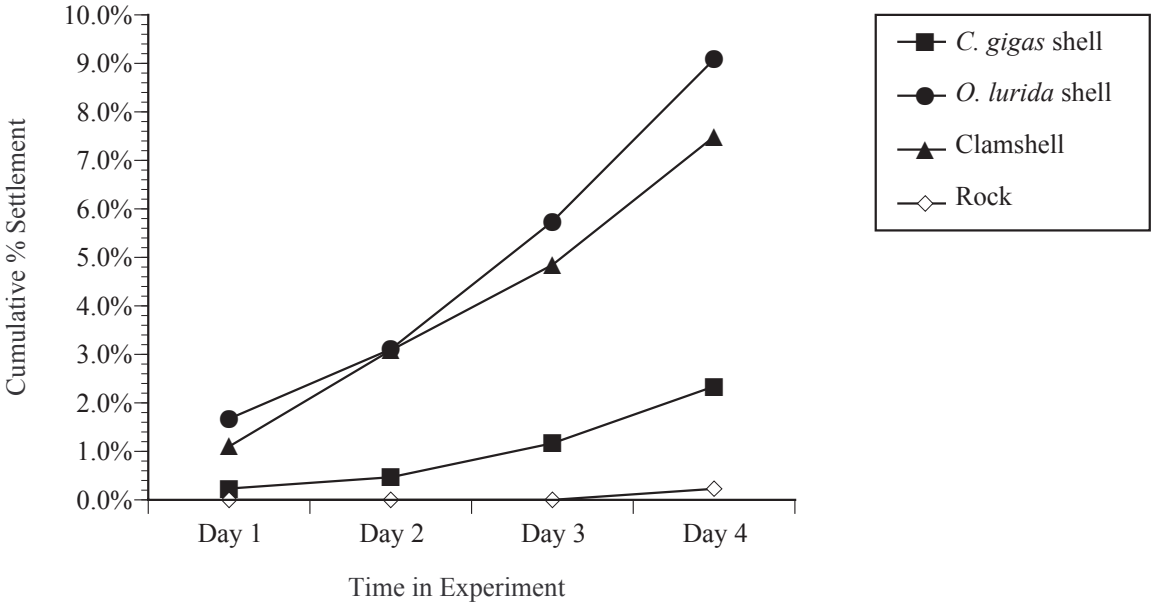

Figure 3. The cumulative percent settlement on each of the four substrata over the four-day experiment in Petri dishes (n=12).

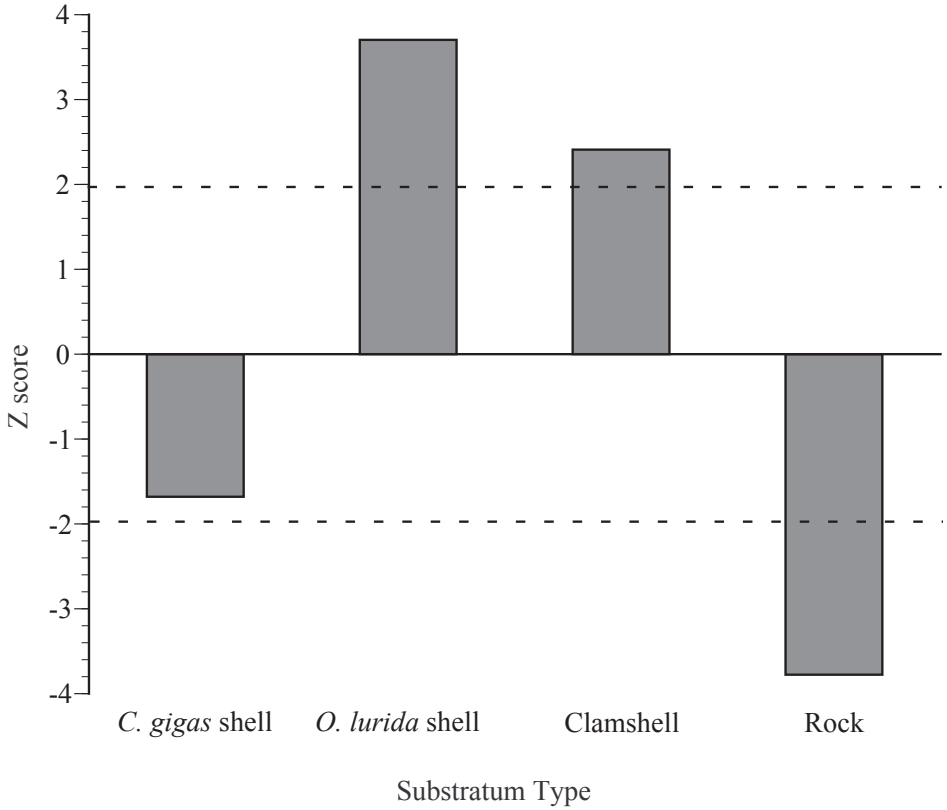

Figure 4. Graph of the Z-score values for all four substratum choices. Significantly more than expected settlement was observed on shell of both *O. lurida* and clamshell (*Protothaca staminea*). Less than expected settlement was seen on rock. Dashed lines indicate 95% confidence levels.

Four-Way Substratum Choice Experiment with Water Motion

Total settlement after 48 hours was 29%. A repeated measures ANOVA compared the number of larvae settled (out of the initial 25) at 24 and 48 hours. For Mauchly's Test for Sphericity, because there are only two dates there is only one correlation to compare and thus zero degrees of freedom and it must be assumed that the circularity assumption is met and the univariate ANOVAR results are read (Gamst 2008). The ANOVAR test (Table 3) showed no significant difference in settlement on the two dates or between the four substrata and no significant two-way interaction effect. The average cumulative settlement on Day 1 and Day 2 for each substratum is shown in Figure 5.

Table 3. Results of a repeated measures ANOVA with substrata as a between-subjects factor and time as a within-subjects factor. Neither main effect is significant, nor is there a significant interaction effect.

Source	SS	df	F-value	p
Substrata	7.094	3	0.374	0.773
Time	0.281	1	0.574	0.463
Time*Substrata	3.344	3	2.277	0.132

A contingency table and chi-square analysis were used to analyze the total number of settlers on each of the four substratum types at the end of the experiment (48 hours). There was no significant difference in settlement between substrata (X^2= 1.75, df=3, p=0.63). This is consistent with the lack of significant variations in settlement reported from the ANOVAR test.

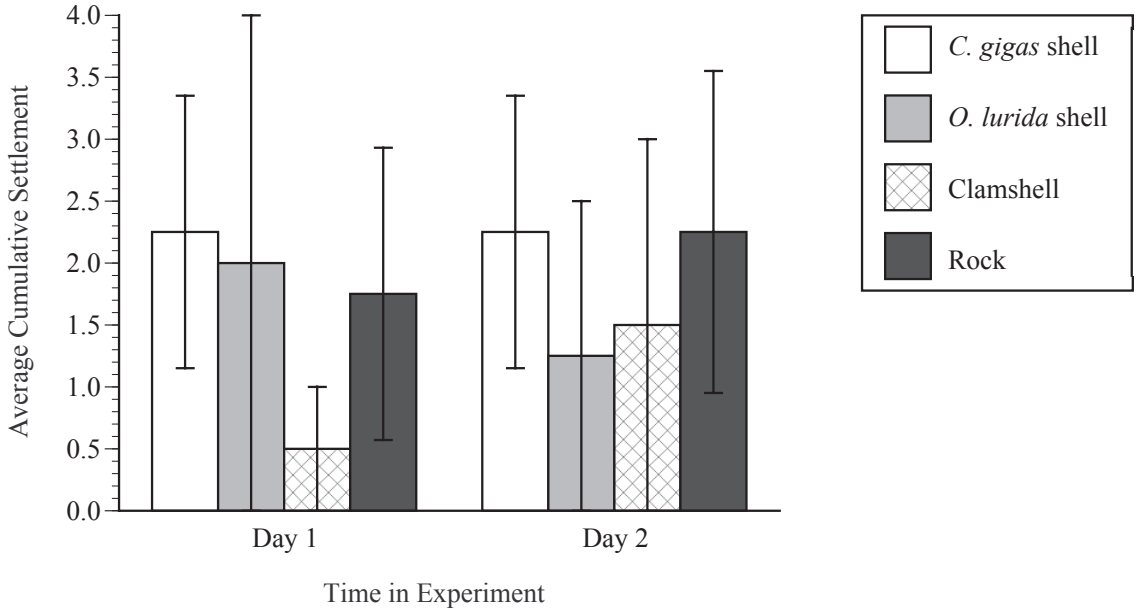

Figure 5. The average cumulative settlement (out of 25 total larvae) on Day 1 and Day 2 for each substratum type. Error bars represent 1 SE (n=4).

Table 4. Chi-square analysis for all four substrata. Observed settlement does not vary significantly from expected values (X^2= 1.75, df=3, p=0.63).

	Observed	Expected	X^2
C. gigas	9	7.25	0.422
O. lurida	5	7.25	0.698
Clamshell	6	7.25	0.216
Rock	9	7.25	0.422
TOTAL	29		1.759

Two-Way Substratum Choice Experiment with Water Motion

This two-way choice comparison of settlement on *Crassostrea gigas* shell to settlement on *Ostrea lurida* shell yielded a high total settlement of 78.38%. A chi-square analysis of the contingency table shows no significant difference in observed settlement from expected values (X^2=2.57, df=1, p=0.11).

Table 5. Chi-square analysis for settlement values on shell of *Ostrea lurida* and *Crassostrea gigas*. There is no significant variation in observed settlement from expected values (X^2=2.54, df=1, p=0.11).

	Observed	Expected	X^2
C. gigas	18	23.5	1.287
O. lurida	29	23.5	1.287
TOTAL	47		2.574

Three-Way Substratum Choice Experiment with Water Motion I

After 48 hours, total cumulative settlement was 65.33%. Results of the repeated measures ANOVA comparing settlement at 24 hours and at 48 hours are shown in Table 6. Because there are only two dates compared, again there is only one correlation and zero degrees of freedom. Thus, it is assumed that the Mauchly's Test for Sphericity is met and the univariate ANOVAR results are used. Significant difference was indicated in settlement on the two dates (F=15.21 p=0.0007), but the two-way interaction factor (date*substratum) was not significant (F=2.6 p=0.09; Figure 6).

Table 6. Results of a repeated measures ANOVA with substrata as a between-subjects factor and time as a within-subjects factor. Both main effects are significant, but there is not a significant interaction effect.

Source	SS	df	F-value	p
Substrata	70.037	2	3.366	0.051
Time	40.907	1	15.208	0.0006
Time*Substrata	14.037	2	2.609	0.094

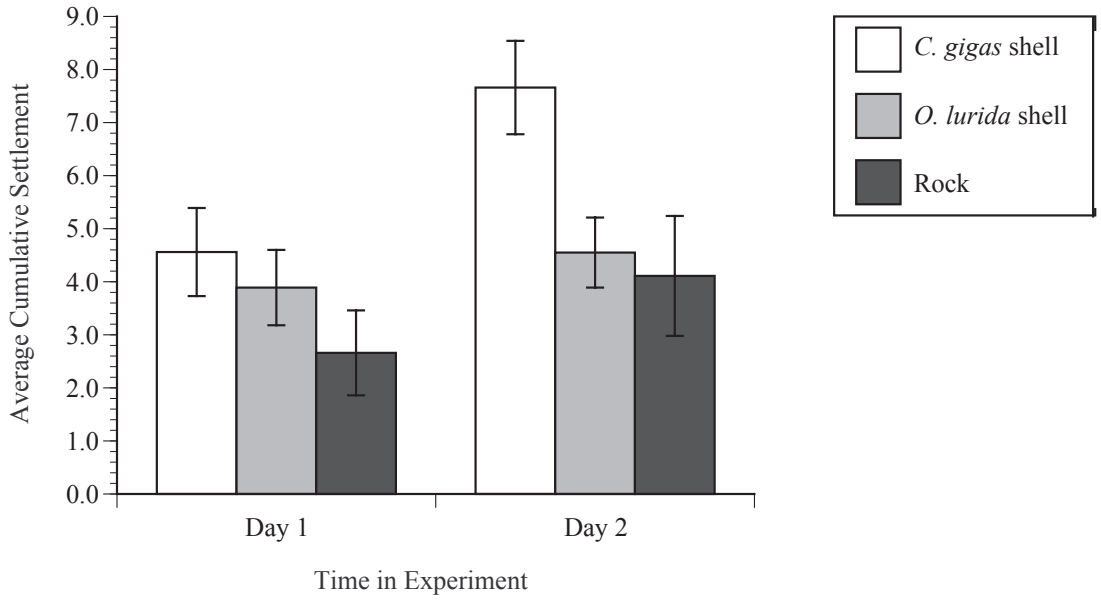

Figure 6. The average cumulative number of larvae settled (out of 25) for Day 1 and Day 2 on each substratum type. There is no significant difference in settlement on the two dates (F=15.21 p=0.0007). Error bars represent 1 SE (n=9).

A contingency table and chi-square analysis show significant variation from the expected settlement values (X^2=12.41, df=2, p=0.002). This clarifies and enhances the marginally significant (p=0.051) result from the ANOVA test. Individual Z-scores show higher than expected settlement observed on shell of *Crassostrea gigas* and lower than expected settlement on rock (Table 7, Figure 7).

Table 7. Chi-square table of the observed and expected values of settlement on the three different substrata. Observed values differed significantly from expected (X^2=12.41, df=2, p=0.002) and the Z-score was calculated for each substratum. Scores greater than 1.96 are significant at a 95% confidence level.

	Observed	Expected	X^2	Z-score
C. gigas	69	49	8.163	5.222
O. lurida	41	49	1.306	-1.940
Rock	37	49	2.938	-2.882
TOTALS	147	147	12.408	

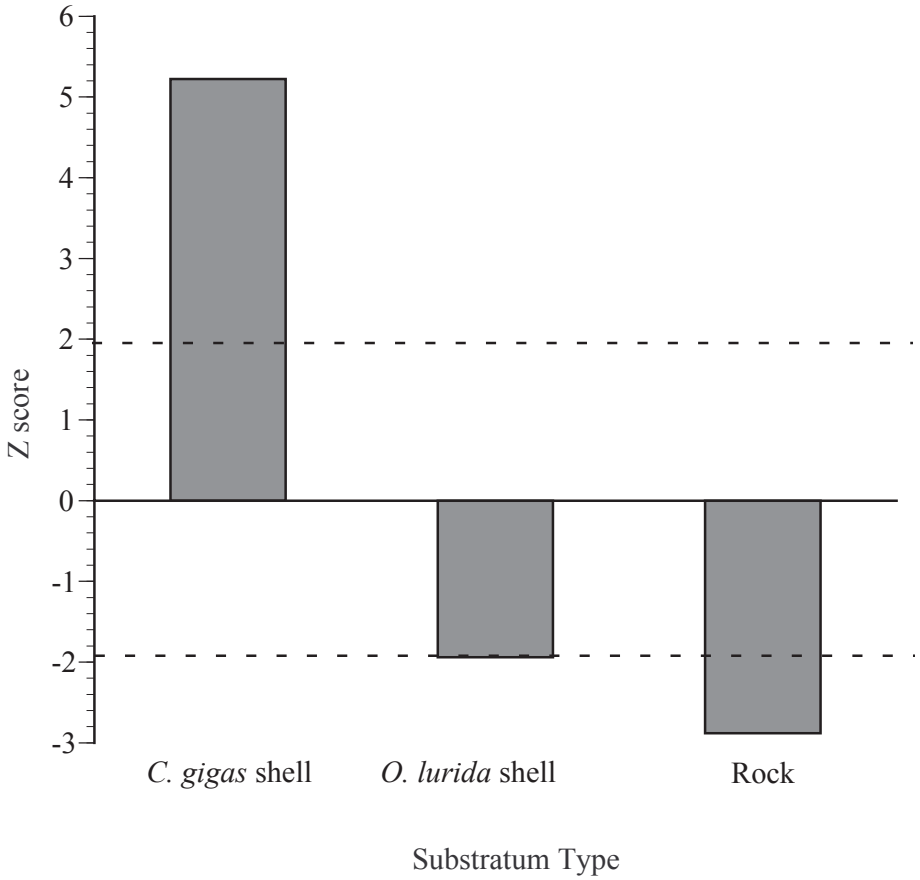

Figure 7. The Z-scores for each of the three substratum types. Higher than expected settled was seen on *C. gigas* shell and lower than expected settlement was seen on rock. Settlement on *O. lurida* shell was not significantly different than expected. Dashed lines represent the 95% confidence interval (1.96).

Three-Way Substratum Choice Experiment with Water Motion II

Total settlement at the end of the 72-hour experiment was 58.4%. A chi-square analysis of the contingency table (Table 8) for this three-way substratum choice experiment shows significant variation from the expected settlement trend (X^2=9.66, df=2, p=0.008). Individual Z-scores show more than expected settlement on rock and less than expected settlement on shell of *Ostrea lurida* and *Crassostrea gigas* (Figure 8).

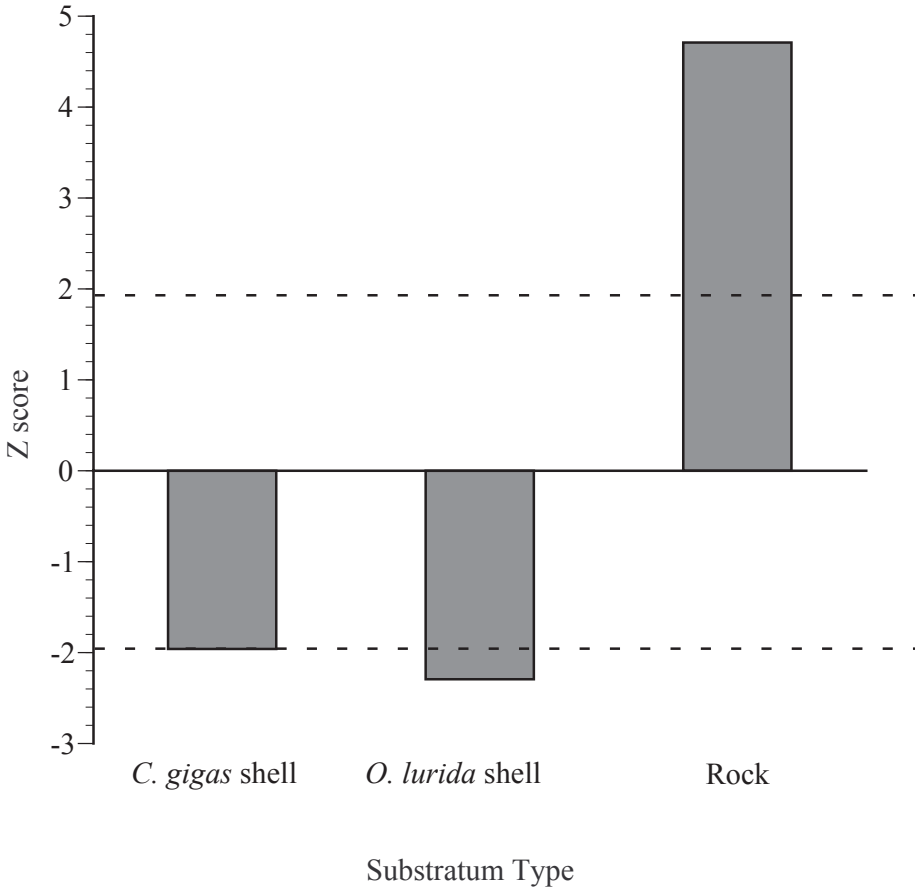

Figure 8. Z-scores for the three substratum choices. There was significantly higher than expected settlement on rock and significantly lower than expected settlement on shell of *Ostrea lurida* and *Crassostrea gigas*. The dashed lines represent 95% confidence intervals.

Table 8. Chi-square results for three-way choice experiment between *Crassostrea gigas, Ostrea lurida* and rock. The X^2 value indicates significant differences in settlement from the expected values (X^2=9.66, df=2, p=0.008). Z-scores greater than 1.96 indicate 95% confidence intervals.

	Observed	Expected	X^2	Z-score
C. gigas	18	23.667	1.357	-1.960
O. lurida	17	23.667	1.878	-2.294
Rock	36	23.667	6.427	4.708
TOTALS	71	71.001	9.662	

Two-Way Substratum Choice Experiment at Five Salinities

Results of the repeated measures ANOVA over the 13-day experiment are shown in Table 9. Mauchly's Test of Sphericity shows evidence of violation of the assumption of sphericity (W=0.006, p<.0001), so the Huynh-Felt epsilon value was examined. The epsilon value was less than 0.7 (e=0.51) but showed only a moderate degree of departure so the MANOVA results were used. All three main effects (time, substratum type, and salinity) were significant, but there was no significant three-way interaction. There was a significant two-way interaction between time and salinity (F=2.29, p=0.003) indicating that over time, differences in settlement were related to salinity but not to substratum type.

Table 9. Results of the repeated measures ANOVA with salinity and substrata as within-subjects factors and time and a between-subjects factor. The multivariate ANOVA results are reported for between-subjects factors. All three main effects were significant. There was also a significant two-way interaction between time and salinity.

Source	SS	df	F-value	p
Salinity	3.432	4.000	8.164	<.0001
Substrata	0.531	1.000	5.055	0.029
	Value	Hypothesis df		
Time	0.722	23.835	5.000	<.0001
Time*Salinity	0.700	2.078	2.078	0.006
Time*Substrata	0.071	0.707	0.707	0.621
Time*Salinity*Substrata	0.333	0.890	0.890	0.600

Figure 9 shows that over the course of the experiment, settlement at salinities of 25, 29 and 33 increased to above 60% by day 13 while average cumulative settlement at salinity 15 remained below 2%. Simple effects analyses (pairwise Bonferroni tests) of this two-way interaction showed that there were significant differences in settlement between settlement at salinity 15 and salinities 25, 29 and 33 (which did not vary significantly from each other) on Days 3-13, but not on Day 1 ($F=8.2$, $p<.0001$). Settlement at salinity 20 did not vary significantly from settlement at any of the other salinities.

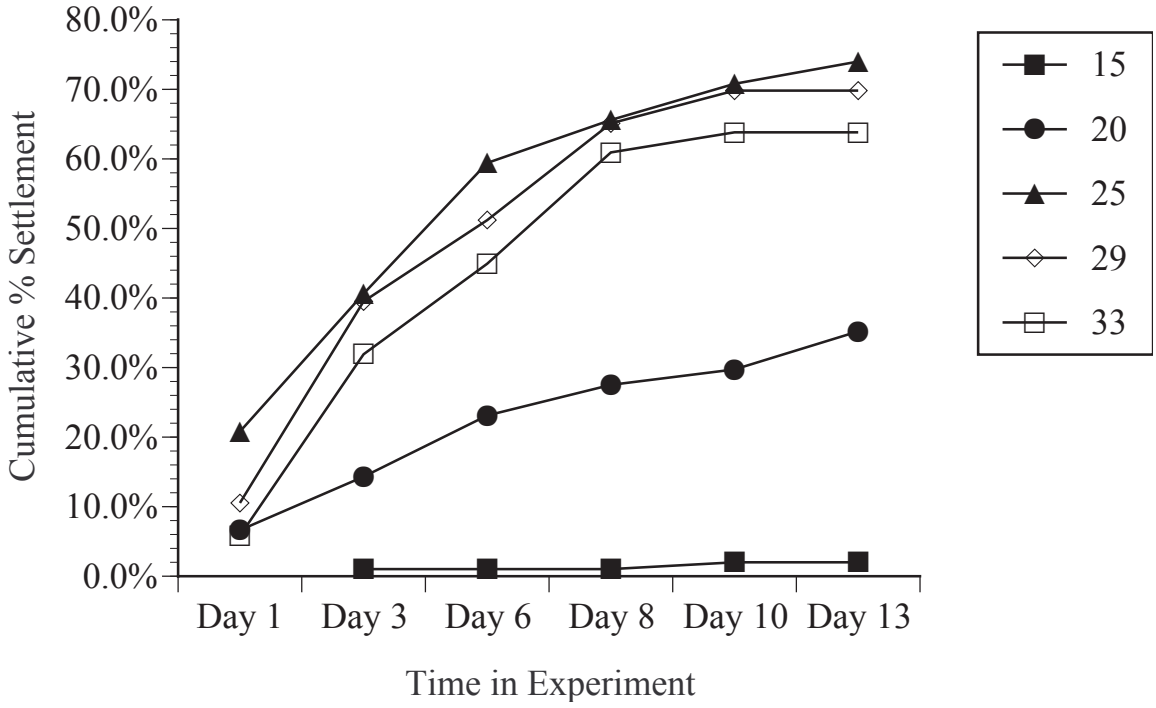

Figure 9. Cumulative percent settlement on all substrata at each salinity. Cumulative settlement increased over times except for on day 13 in which settlement is not significantly different than on day 10. Settlement at salinities 25, 29 and 33 was significantly higher than settlement at salinity 15 ($F=8.2$ $p<.0001$).

Cumulative settlement varied significantly over time, with increased cumulative percent settlement as the experiment progressed ($F=23.8$, $p<.0001$). Settlement on all dates was significantly different except on days 10 and 13

(Bonferroni tests). The between-subjects ANOVA shows significantly more settlement on shell fragments of *Crassostrea gigas* compared to the equally sized shell fragments of *Ostrea lurida* (F=5.05, p=0.03; Figure 10).

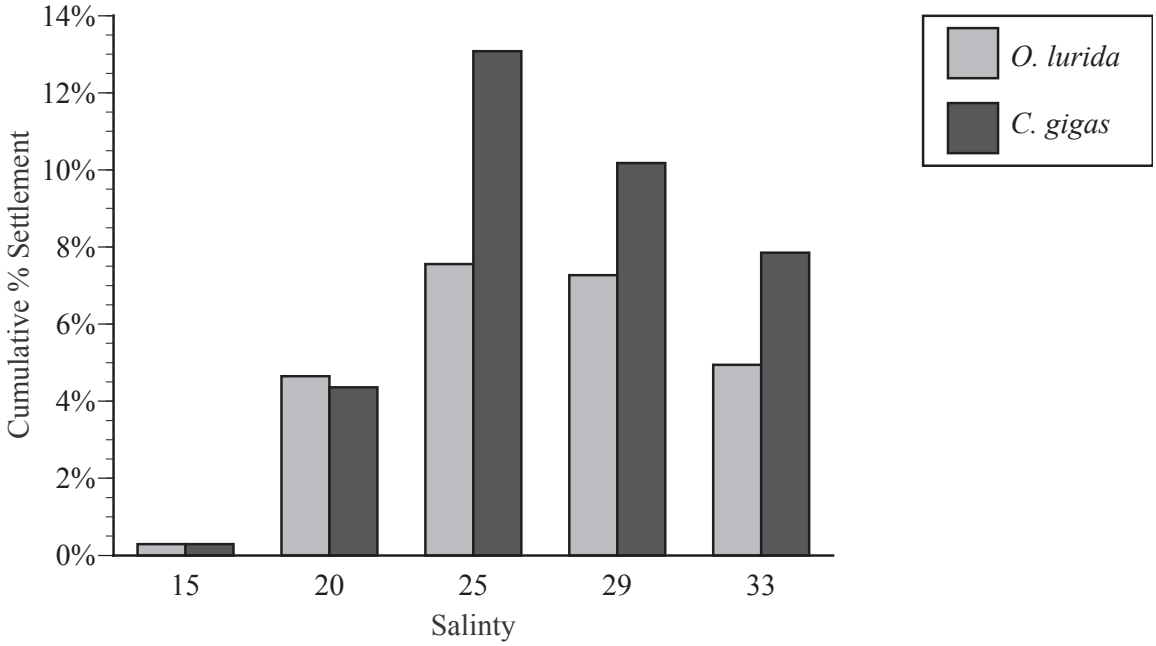

Figure 10. The cumulative percent settlement on *C. gigas* and *O. lurida* on the last day grouped by salinity. Settlement on shell of *C. gigas* was significantly higher than on shell of *O. lurida* (F=5.1 p=0.03).

Discussion

When *Ostrea lurida* larvae were presented with only one choice of substratum, they did not appear to settle indiscriminately. There was significantly more settlement on *O. lurida* shell than on all other substrata. In fact, cumulative settlement on all other substrata was under 1%, even on other shell substrata. Although there was significantly higher settlement on shell of *O. lurida* than on any other substratum, cumulative settlement was still quite low (less than 6%) even though larvae were given four days to settle. In other experiments, settlement was

high on these substrata but here it is not significantly different than settlement on wood or in bowls with no added substratum.

When presented with multiple substratum choices, larvae of *Ostrea lurida* did not exhibit consistency in selection of substrata for settlement. Various hard substrata were used for the 2-4 way choice experiments and it is possible that *Ostrea lurida* larvae do not discriminate between hard surfaces. If that were the case, however, settlement on all substrata should be comparable. Instead, cumulative settlement shows significant differences in settlement among substratum types in five out the seven experiments performed in the lab. However, it is clear there are no obvious trends in preference for any one substratum over another (Figures 4, 7, and 8). Although some lab experiments were replicated (four-way choice experiments and three-way choice experiments) attempts to pool the data were unsuccessful because in the trials differed significantly from each other (four-way choice: heterogeneous $G=12.18$, $df=3$, $p=.005$ and three-way choice: heterogeneous $G= 15.52$, $df=2$, $p=0.0004$). Significant deviation from expected settlement was seen in both directions for each substratum type with no uniformity between experiments. This lack of consistency suggests settlement preference may be based on other factors besides substratum type, such as gregarious settlement.

Gregarious settlement has been shown in other species of oyster (Crisp and Knight Jones 1953, Bayne 1969, Veitch and Hidu 1971, Michener and Kenny 1991, Tamburri *et al.* 2008). If larvae do indeed settle gregariously, after the first larva chose a substratum, additional larvae would be more likely to choose that same

substratum. Thus, after the initial larval choice, settlement would be based not on substratum type but on presence of conspecific larvae that had already settled.

Cumulative settlement was recorded for most experiments, making it hard to distinguish substratum choice from gregarious settlement. However, in the experiment at varying salinities shell substrata were cleared of settled larvae at each check date (every 48-72 hours). Comparing settlement over these six short time periods may clarify the possibility of gregarious settlement in *Ostrea lurida* larvae. From the data set of number of larvae counted at each check date, 8% (15/180) of the time more than three (3-14) larvae were recorded on the same shell piece. In 67% of these cases, there was zero settlement seen on the paired shell in that beaker. Consistent with results of significantly higher settlement on *Crassostrea gigas* shell, 80% of the time the shell with zero settlement was the *Ostrea lurida* shell. The frequency distribution of the number of settlers found on each shell in the field experiment in Chapter II showed significantly higher than expected frequencies of more than 9 settlers per shell. These tendencies of larvae to settle in larger than expected groups and predominately on only one of the available choices may indicate gregarious settlement but additional experiments designed with the specific aim of testing this should be performed.

Comparing the cumulative settlement percentages in static water experiments and experiments with water motion, the addition of water movement did result in higher settlement percentages (average cumulative settlement of 58%) than the total percent settlement in the initial experiments conducted in static water (average cumulative settlement of 13%). However, the two-way substratum choice

experiment at five salinities was also conducted in static water and resulted in high cumulative settlement of 61% at salinity 33. Thus, the addition of water motion alone does not account for differences in cumulative percent settlement. Perhaps the increased settlement percentages were more directly related to the larval culture sources than the factor of water movement. The experiments with moving water and the experiment at varying salinities both employed larvae spawned from Coos Bay adults rather than those transported from the Whiskey Creek Shellfish Hatchery. This correlation seems more probable but additional work would be needed to draw any definitive conclusions.

Settlement of larvae raised at the five different salinities showed significantly higher settlement at salinities 25, 29 and 33. At these three salinities, no significant difference in settlement was noted. This is consistent with the salinity range of naturally occurring adult *Ostrea lurida* at salinities above 25 (Korringa 1976, Couch 1989, Baker 2000). Significantly lower settlement was recorded at salinity 15 with under 2% total settlement over the 13 day experiment indicating decreased larval competency at low salinities.

Cumulative settlement increased over time in almost all experiments. For the salinity experiment, larvae were presented with substrata for settlement as soon as they were eyed pediveligers and cumulative settlement increased until Day 10. Cumulative settlement on Days 10 and 13 were not significantly different and almost all larvae had settled or perished by Day 13 (only 8% remained alive). One third of the larvae still alive at the end of the experiment were in the salinity 20

treatment, indicating decreased settlement at that salinity, but not enough stress to cause death of the larvae.

The lack of strong larval preference for settlement in the laboratory experiments matches findings of no significant preference between live and dead oyster shell substrata of *Crassostrea gigas* and *Ostrea lurida* from Chapter II. No significant difference in settlement was seen among *C. gigas* and *O. lurida* substrata in the field or in the lab. This lack of larval preference suggests that the addition of any hard substrata for restoration projects would be equally beneficial for settlement of *O. lurida* larvae.

CHAPTER IV

GENERAL DISCUSSION

The purpose of this study was to describe the timing of settlement of *Ostrea lurida* in Coos Bay, Oregon and to elucidate patterns of larval settlement on various surfaces with implications for restoration efforts. An annual collection of settlement data in Coos Bay showed one peak in settlement at the beginning of October with settlement occurring over 5 months from early August until December. Farther south, in California, spawning occurs over 7 months beginning in April and continuing until November (Coe 1932, Seale and Zacherl 2009). Coe (1932) suggested a temperature threshold of 16°C to initiate spawning which was not observed in this study nor in the 2009 study conducted by Seale and Zacherl, which set out to test that hypothesis in Southern California.

In Washington, spawning is reported to begin in May and early June, which is much earlier than seen in 2010 in Coos Bay (Hopkins 1937, Peter-Contesse and Peabody 2005, White *et al*. 2009b). In the protected bays and estuaries on the outer coast of British Columbia, spawning peaks are reported from July through September (Gillespie 2009). Timing of reproduction in British Columbia seems to most closely match the results observed in Coos Bay. From data collected in Coos Bay in the summer of 2011, settlement is not evident before July. Additionally,

attempts to spawn adults from the Coos Bay population were unsuccessful from mid May-July 2011.

In the field experiment, there was significantly higher than expected settlement on the bottom surface of the substrata compared to the top surface. This is consistent with previous studies on *Ostrea lurida* larvae and other oyster larvae indicating strong preferences of larvae to settle on the undersides of horizontal substrata (Hopkins 1937, Imai *et al.* 1954). Decreased settlement on the top of horizontal surfaces may prevent death by siltation and suffocation (Groth and Rumrill 2009, Wasson 2010, Smyth and Roberts 2010). For restoration efforts, attention should be paid to this trend and added substrata should be kept up off the bottom to allow for access to the undersides. This may enhance settlement by *Ostrea lurida* larvae by increasing access to this preferred location.

There was no significant difference in settlement on live oysters versus dead oyster shell. Previous lab experiments have demonstrated the ability of chemical cues derived from live conspecifics to induce settlement in oysters (Crisp 1967, Pascual and Zampatti 1995, Tamburri *et al.* 2008). Perhaps *Ostrea lurida* larvae respond differently than other oyster larvae to such cues. There may be no distinct preference for live oysters, or it was not detected in this experiment. This has positive implications for restoration efforts since it is much more difficult to provide for the addition of live oysters than of non-living oyster shell.

There was also no preference for shell of *Ostrea lurida* compared to shell of *Crassostea gigas* in the field. This result matches results from the laboratory portion showing no clear larval preference among hard substratum choices (*O. lurida* shell,

C. gigas shell, rock, and clamshell). Trends in settlement for lab experiments seem to fit with gregarious settlement reported in other oyster species (Cole and Knight-Jones 1939). Larval settlement may be based on the presence or absence of other settlers and not on the properties of the hard substrata itself. This was also seen in the field experiment histogram of the number of settlers per shell. All shells with 9 or more settlers varied significantly from the expected value. However, there was no significant preference for live conspecifics in the field experiment, which should have been the case if *O. lurida* larvae had a strong gregarious settlement response. When offered only one substratum choice, larvae did not settle indiscriminately. This indicates at least some level of larval selection. The addition of water movement did not yield results that were different from experiments performed in static water.

Olympia oyster larvae raised at five different salinities exhibited significant differences in settlement. Larvae from the salinity 15 treatment had significantly lower settlement than those raised at higher salinities as well as high mortality. Although settlement for larvae at salinity 20 did not differ significantly from any of the other salinities, one third of the surviving larvae at the end of the experiment were from the salinity 20 treatment, suggesting a decreased competency to settle. Cumulative settlement percentages at salinities 25, 29 and 33 did not differ significantly from each other, which corresponds to the habitat range of adult Olympia oysters. However, in Coos Bay adult *Ostrea lurida* are rarely found in the marine dominated region of the bay. It is possible there are other factors, such as competition, predation or larval flushing, limiting their distribution in higher

salinity areas. In lower salinity regions, adult oysters subjected to salinities of 15 showed 80% survival over 2 weeks (Baker 1995).

Although the lack of a strong preference for settlement on conspecific substrata has positive implications for the restoration effort, it may also have a negative effect. The only sources of *Crassostrea gigas* substrata in Coos Bay are live oysters raised for aquaculture. If *Ostrea lurida* larvae settle just as readily on *Crassostrea gigas* substrata, this could lead to their removal from the population with the harvest of mature Pacific oysters creating a "recruitment sink" (Trimble *et al.* 2009). However, due to the rapid maturation of Olympia oysters (5 months) they may be able to successfully reproduce before they are removed from the population and settlement in *C. gigas* aquaculture beds may still have a net positive impact on the population as a whole.

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