ABSTRACT

Title of Thesis:	EFFECTS OF SALINITY ON SETTLEMENT AND METAMORPHOSIS OF THE EASTERN OYSTER *(Crassostrea virginica)*
	Anna M. Priester, Master of Science, 2016
Thesis Directed By:	Donald W. Meritt, Principal Agent University of Maryland Center for Marine Estuarine Environmental Science

The eastern oyster *(Crassostrea virginica)* is a euryhaline species known for its historic populations, valuable fishery, and ecological importance. One of the most critical periods in the oyster's life cycle is its transition from a free-swimming pelagic larva into its sessile benthic form. Despite the importance of this transition, which includes attachment to a substrate (settlement) and metamorphosis into the juvenile, our understanding of salinity tolerance during these processes is limited. This study was designed to quantify the effects of salinity on settlement and metamorphosis, and to determine if those effects were influenced by the salinity in which the larvae were reared. Multiple cohorts of pediveliger larvae from hatcheries grown in Low (10), Medium (15-16.5) and High (22-27.5) salinities were allowed four days to settle in twelve salinity treatments ranging from 5 to 35. A set of additional experiments was

extended to 14 days to investigate if the settlers were also able to complete

metamorphosis and demonstrate juvenile growth within the same range of salinities.

Settlement consistently occurred all tested salinities (5-35), indicating that pediveliger

larvae can adapt to a broader salinity range than described in previous research.

Highest settlement rates were achieved in treatment salinities between 11 and 30 for

all three larval groups. Settlement performance outside that optimal range was highest

for the larvae group reared in salinities closest to those extremes. Settlers from the 14-

day experiments demonstrated metamorphosis and high post-settlement survivorship

in all salinity treatments, but juvenile growth rates were reduced in salinities less than

9 and above 30. This highly repeated study reveals the impressive capacity for

pediveliger larvae to tolerate a wide range of salinities, and has direct implications for

oyster aquaculture and our understanding of natural recruitment.

EFFECTS OF SALINITY ON SETTLEMENT AND METAMORPHOSIS
OF THE EASTERN OYSTER *(Crassostrea virginica)*

by

Anna Margaret Priester

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Advisory Committee:
Principal Agent Dr. Donald W. Meritt, Chair
Professor Dr. Standish K. Allen, Jr.
Associate Professor Dr. Kennedy T. Paynter
Assistant Professor Dr. Louis V. Plough

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Glossary

Competency- The capacity of larvae to settle or metamorphose following exposure to known inducers (Coon et al 1990). For this work, larvae were assumed to be competent if they had eye spots and actively searching feet.

Metamorphosis- The process of morphologically changing from a larva to a juvenile.

Pediveliger- The final larval developmental stage, indicated by the presence of an eye-spot and foot.

Remote setting- The practice of transferring and setting larvae in a different location than the hatchery.

Settlement- The behavioral process of larvae that leads up to, and includes, the permanent attachment to a substrate.

Settler- A larva that has attached to a substratum but has not completed metamorphosis (Baker and Mann 1994a; Roegner and Mann 1995).

Spat- Recently metamorphosed juvenile oysters.

Chapter 1: Laboratory studies on salinity tolerance during oyster larval settlement and metamorphosis

INTRODUCTION

The eastern, or American oyster, *Crassostrea virginica* (Gmelin 1791), is a bivalve mollusk native to estuaries and coasts of the western Atlantic, Gulf of Mexico, and Caribbean Islands (Carlton and Mann 1996), known for its historical abundances and valuable fishery (Rothschild et al. 1994). As an ecosystem engineer and a keystone species, the eastern oyster creates extensive reefs that provide substrate for colonization (Underwood and Denley 1984), refugia from predation, and foraging areas for many invertebrates and fishes (Beck et al. 2001, Soniat et al. 2004). Oyster reefs also augment coastline resilience by preventing erosion and serving as a break-wall for storm surges (Meyer et al. 1997). Oysters feed by filtering particles and algae from the water column, which in turn reduces eutrophication, (Officer et al. 1982; Newell 1988; Dame et al. 2002; Newell et al. 2007) increases water clarity, (Coen et al. 1999) and improves conditions for other habitats like seagrass beds (Newell and Koch 2004). Hence, the presence of oyster reefs improves ichthyofaunal diversity and the sustainability of other commercially important species (Breitburg 1999; Zimmerman et al. 1989; Meyer and Townsend 2000; Harding and Mann 2001).

Due to a combination of overharvest, diseases, reduced water quality, substrate limitation, and other contributing factors, the current yield from the wild-harvest fishery of the eastern oyster is a small fraction of the historical production (Jackson et al. 2001). For instance, the oyster population within the Chesapeake Bay, which formerly supported the largest oyster fishery in the world, is estimated to be

less than 1% of its historic abundance (Newell 1988; Wilberg et al. 2011). The decline of the oyster populations along the Atlantic coast and the Gulf of Mexico is well documented, as are the resulting economic and environmental ramifications of their absence (Newell 1988; Jackson et al. 2001; Kemp et al. 2005; Coen et al. 2007; Grabowski and Peterson 2007). Recently, oyster restoration efforts and aquaculture production have been greatly expanding, but are still far from filling the void created by the collapsed population (Coen et al. 2007; Mann and Powell 2007; Kennedy et al. 2011; Lipcius et al. 2015; Hudson and Murray 2016). As the culturing methods of the Eastern oyster evolve, gaps in our current knowledge of the basic physiological responses of the species are becoming apparent. Motivation for the current study stems from the greater activity in restoration and aquaculture in low salinity waters, conditions where oysters thrive, but information on some of the basic biology, such as larval behavior, is scant.

Eastern Oyster Ecology

The life cycle of the eastern oyster is similar to many benthic marine invertebrates, characterized by sedentary, spawning adults with pelagic larvae (e.g. Pawlik 1992, Rodriguez et al. 1993). They are commonly found in temperatures ranging from -2 to 36°C and salinities 5 to 40 (Butler 1954; Galtsoff 1964). Temperature and salinity are the two most important abiotic factors governing their biological processes, distribution of predators and diseases, and availability of food (as reviewed in Shumway 1996). Salinity, which can change dramatically on short temporal and spatial scales, has important effects on biological processes that can vary across the life cycle of an oyster and may depend upon their genetic makeup

(Davis 1958; Pierce 1992). Classic experimental work that dates back over half a century indicates that sensitivity to extreme salinity conditions decreases as larvae age (Davis 1958, Davis and Calabrese 1964, Loosanoff 1965). For example, the salinity range for successful egg fertilization is narrower than for the development of D-hinge larvae, and developing larvae can tolerate still a wider range of salinities as they mature. Davis (1958) and Davis and Calabrese (1964) also found that little to no fertilization can occur in salinities below 10, and larval growth rates are also minimal below salinities of 10. Older larvae can survive short periods of exposure to salinities as low as 5 (Loosanoff 1965).

Juvenile and adult oysters have a greater capacity to tolerate both a broader range and immediate change in salinity than larvae (Shumway 1996), but unlike larvae, the oyster's capacity to tolerate low salinities and higher temperatures decreases as it grows (Rybovich et al. 2016). The increased sensitivity to changes in salinity, temperature, and other environmental stressors in larger size classes has been reported in many studies of bivalves (Widdows 1978, Sukhotin et al. 2003, Peck et al. 2007, Yuan et al. 2010, Clark et al. 2013), and is likely due in-part to the proportionately increased metabolic demands of larger organisms (Bayne and Newell 1983). Oysters, like many osmoconformers, lack the ability to maintain osmotic homeostasis. Instead, they must conform at the cellular level, primarily by the regulation of ion channels and the synthesis or release of intracellular free amino acids (FAAs) (Pierce and Amende 1981; Zhao et al. 2012; Guo et al. 2015). The first line of defense for an oyster to a sudden change in intracellular sodium concentration is to close its valves and cease pumping, thereby creating a barrier between its tissues

and the surrounding water (Loosanoff 1953; Hand and Stickle 1977; Natochin et al. 1979). Because oysters cannot feed or respire without pumping, no gases are exchanged between the oyster and the surrounding medium when its valves are closed. The subsequent buildup of carbon dioxide (respiratory acidosis) is therefore one of the main reasons for summertime mortalities in oyster populations associated with unfavorable salinities and temperatures (Michaelidis et al. 2005; Lannig et al. 2008; Lombardi et al. 2013). Oysters that eventually open their valves due to respiratory stress must either osmoconform to the new salinity or die (Hoyaux et al. 1976; Hand and Stickle 1977).

Salinity tolerance in both larvae and adults varies because of prior exposure history, differences in genetics, or both. After conducting a few experiments using oysters from low salinity conditions in MD, Davis and Calabrese (1964) discovered that the salinity at which gametogenesis occurs affects the salinity range of successful larval development. For instance, when gametogenesis occurs in low salinities, the resulting larvae can tolerate lower salinities than if gametogenesis occurred in higher salinities. Pierce et al. (1992) also saw significant differences between adult populations when examining their intracellular response to osmotic stress. They proposed that adult oysters from the lower-salinity Chesapeake Bay were unable to adapt to high salinities without stepwise acclimation because of their smaller pools of FAAs. Recognizing these physiological differences, it is unlikely that a specific biological response to salinity will be the same across all populations of the eastern oyster.

Settlement and Metamorphosis

The persistence of self-sustaining oyster reefs depends on continued recruitment of new larvae attaching themselves to a substrate on the reef and then successfully metamorphosing into juveniles. Although settlement and metamorphosis are amongst the most critical periods in the life cycle of an oyster (Stafford 1913; Prytherch 1934; Cole and Knight-Jones 1949; Rodriguez et al. 1990), the process is difficult to study in the field because is it ephemeral. The development of larvae culture techniques has enabled study of many of the biological mechanisms, but still much of what we know about how they respond to their environment is based on loosely associative evidence riddled with exceptions. Even some of the effects from the most influential environmental parameters, like salinity, have yet to be adequately explored.

The process of settlement begins after the late-stage larva (called a pediveliger, characterized by the development of an eye-spot and foot), begins "settlement behavior" by swimming in a spiral pattern with the foot extended to search for a solid substrate (Prytherch 1934: Bonar et al. 1990). Similar to other settling invertebrates, if the larva encounters a surface that it deems unfavorable, it can resume swimming and search elsewhere (Bayne 1965; Scheltema 1974). Upon finding a suitable substrate, the larva will excrete a crystalline cement from its foot and permanently attach itself (Prytherch 1934). For this thesis, the process leading up to and including cementation will be henceforth referred to as "setting" or "settlement," and larvae that have successfully attached to a substrate will be called "settlers." Water-born chemical cues are responsible for initiating and enhancing

settlement, which originate from bacterial biofilms and conspecifics of recently set

and adult oysters (as reviewed in Pawlik 1992; Rodriguez et al. 1993; Kennedy

1996). Small increases in water temperature can also stimulate settlement behavior

(Lutz et al 1970). The mechanisms by which larvae choose to orient themselves on a

substrate are not fully understood, but they often prefer surfaces with less light

(negative phototaxis), the undersides of surfaces (geotaxis), and rougher surfaces

(rugotaxis) (Kennedy 1996; Baker 1997; Baker and Mann 1998; Saoud et al. 2000).

After cementation, the settler will begin metamorphosis into its juvenile form

(Galtsoff 1964; Baker and Mann 1994a). Metamorphosis is an energetically costly

process, during which the locomotive and feeding organ called the velum will be cast

off or resorbed and gills will fully develop (Galtsoff 1964; Bayne 1971; Baker and

Mann 1994a; Kennedy 1996). Additionally, the foot is resorbed, and the larval organs

revolve in an anterior-dorsal direction. A sharp transition in the mineralogy of the

shell deposition also occurs when the spat shifts from depositing aragonite to the

denser calcite (Carriker 1996). For this thesis, a fully-metamorphosed settler is

henceforth referred to as a "spat" or juvenile oyster.

The ability of larvae to settle and metamorphose is referred to as

"competency" (Coon et al. 1990a). Furthermore, Coon et al. (1990a) defined

metamorphic competence as the sum of behavioral competence (enabling them to

settle) and morphogenetic competence (enabling them to metamorphose).

Competency to settle can be independent of competency to metamorphose, as the

processes mark different stages of development and are triggered by different

chemical pathways (Coon et al. 1990a). True competency is tested by exposing larvae

to chemicals like L-3,4- dihydroxyphenylalanine (L-DOPA) and epinephrine that induce settlement and metamorphosis, respectively (Coon et al. 1990a, 1990b). For this work, however, all larvae used in experiments were assumed to be competent if they had an eye-spot and an actively searching foot.

Salinity is frequently implicated as the most important environmental factor determining the distribution and number of new juveniles recruited to an area. In general, higher salinities are associated with higher recruitment (Hopkins 1931; Chatry et al. 1983; Ulanowicz 1980; Kimmell and Newell 2007; Mann et al. 2009; Soniat et al. 2012; La Peyre et al. 2013). In Louisiana, setting intensity is highest between salinities 16-22 while virtually no setting takes place when mean summer salinity is less than 10 (Chatry et al. 1983). In Galveston Bay, TX, salinities above 20 yielded successful spat sets (Hopkins 1931), while successful settlement in the lower Laguna Madre, TX, was recorded at substantially higher salinities of 32 to 42 (Breuer 1962). In the Caloosahatchee estuary, FL, spat recruitment and growth rates are low in salinities 0-15 and more favorable between 15-25 (Barnes et al. 2007). Ulanowicz (1980) and Kimmell and Newell (2007) also found that the dominant factor increasing spat production in the upper Chesapeake Bay was sustained high salinity, although a range was not specified. Because recruitment is affected by the sum of many factors that affect various processes, including gametogenesis, larval development, settlement, and post-settlement mortality (Underwood and Denley 1984; Rumrill 1990), the degree to which each of these factors govern settlement on oyster reefs is hard to pin down (Chatry et al. 1983).

Recently, further clarification of the association between salinity and recruitment has been attributed to the role of river flow and hydrographic dynamics on larval transport. In systems like the Chesapeake Bay and Delaware Bay, the majority of larvae are transported downriver where salinity values are higher (North et al. 2008; North et al. 2010; Narvaez et al. 2012). Paradoxically, many of the largest and healthiest oyster populations in both systems are found in upriver, low salinity sites where disease and predation is low (Southworth and Mann 2004; North et al. 2010). The persistence of low-salinity reefs raises questions concerning our knowledge of the salinities in which settlement and metamorphosis is possible, particularly because it is a commonly held assumption that *C. virginica* larvae cannot successfully complete metamorphosis in salinities below 10.

To the best of my knowledge, only two laboratory studies have investigated the salinity tolerances of larvae during settlement and metamorphosis, and both of them involved larvae reared in salinities around 27 (Prytherch 1934; Davis 1958). In Prytherch's (1934) work concerning the role of copper in settlement and metamorphosis, he found that a small addition of copper can stimulate the setting behavior of pediveligers and used that method to test the salinities in which larval attachment is possible. The majority of his findings concerning copper have since been rejected, as the stimulatory effect he witnessed was likely due to larval avoidance of the sub-lethal addition of copper (Korringa 1952; Lund 1973; Pawlik 1992). Nonetheless, Prytherch (1934) provided evidence that larval attachment only occurs "with regularity" in salinities between 9 and 29, but observed that at least one larva settled in salinities as low as 5.6 and as high as 32.2. Years later, Davis (1958)

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investigated the effects of salinity on larval growth and extended one set of experimental larval cultures for a few days to get an indication of metamorphic success in different salinities. Results from this non-replicated study indicated that metamorphosis does not occur in salinities less than 10 (Davis 1958).

Recognizing that oyster reefs exist within waters regularly experiencing salinities below 10, there is a distinct need to re-examine how salinity affects oyster settlement and metamorphosis. Furthermore, the oyster hatchery located at the University of Maryland Center for Environmental Science (UMCES) Horn Point Laboratory (HPL) is currently the largest producer of *C. virginica* larvae in the world and operates in a salinity of approximately 10. The fact that this hatchery is successful within the lowest salinity deemed possible for larval development further emphasizes that conclusions from former studies using oysters from higher salinity areas may not be entirely appropriate. Additionally, HPL and other facilities in the Mid-Atlantic are increasingly engaging in the practice of "remote setting," whereby hatchery operators send bundles of competent larvae to be set in tanks at different locations. Therefore the need to examine the adaptive capabilities of larvae to a salinity change at the time of settlement and metamorphosis is of particular importance.

Project Objectives

The overall objectives of the present research were to 1) determine how salinity affects settlement and metamorphosis of larvae reared in low salinities, and 2) identify if larvae reared in higher salinities perform differently. To achieve this, settlement rates of HPL larvae placed in different salinity treatments were compared

to settlement rates of larvae reared in higher salinity hatcheries. A subset of these experiments was extended in order to investigate if settlement rates were also indicative of metamorphic rates. Results from this study will shed new light on how pediveliger larvae respond to a wide range of salinities, thereby furthering our understanding of the basic biological processes of *C. virginica* important to natural recruitment and the practices of oyster restoration and aquaculture.

MATERIALS AND METHODS

A series of experiments was conducted at the HPL Oyster Hatchery (HPL) in Cambridge, MD, from April through September of 2015 to quantify the effects of salinity on settlement and metamorphosis of *C. virginica* larvae. Preliminary experiments evaluated the feeding and larval size-selection protocols and established the range of treatment salinities to be tested. Afterwards, a total of 13 replicated experiments were conducted using different cohorts of pediveliger larvae originating from four hatcheries. These experiments tested the ability of larvae to settle when exposed to salinities ranging from 5 to 35 over a period of four days. Results from these settlement experiments motivated an additional subset of experiments in which settlers remained in settlement bags for an additional ten days to investigate their ability to complete metamorphosis and demonstrate juvenile growth.

Preliminary Experiments

It was deemed important to provide algae during the settlement experiments to guarantee that larvae from every hatchery did not go through a period of starvation before settlement (Laing 1995), and that they did not fail to settle simply because

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ambient food levels were too low (Baker 1994). It was first necessary to verify that algae cells remained intact and viable when exposed to different salinities, and therefore would not become a confounding factor within the subsequent settlement experiments. Two algae species, *Chaetoceros muelleri* (clone CHGRA, NMFS Milford laboratory, CT collection) and *Tetraselmis chui* (clone PLY 429, NMFS Milford laboratory, CT collection), were chosen for the experiments because they provide suitable nutrition for bivalve larvae undergoing metamorphosis, they support high growth rates in developing oyster spat (Enright et al. 1986; Utting et al. 1986; Jonsson et al. 1990; Wikfors et al. 1996), and consistent supplies are available at HPL. Aliquots of densely-cultured CHGRA and PLY 429 (grown in a salinity of 12) were placed in beakers of five treatment salinities; S-5, S-7, S-11.4 (ambient), S-25 and S-35. Samples from each salinity treatment were examined under a microscope every half hour for a period of three hours to monitor cell activity and cell lysis. At the end of the three-hour exposure, the only observed difference in the condition of the cells across all salinity treatments was an approximate 15% increase of lysed PLY 429 cells within the S-5 treatment. Despite this minor change, the two algae species demonstrated the ability to withstanding large changes in salinity and were deemed appropriate for their use within subsequent settlement experiments.

Another preliminary trial was conducted to determine the lower salinity limit that larvae from HPL could settle and to evaluate the practicality of including salinity treatments greater than 25 or 30 within future experiments. Approximately 400 eyed larvae were placed within duplicate Pyrex® beakers filled with 250mL of six low salinities (S-0, S-1, S-2, S-3, S-4, S-5), a control salinity of 10.5 (ambient river

water), and 4 higher salinities (S-25, S-27.5, S-30, and S-32.5). The protocols employed for salinity adjustment, aeration, temperature control, and feeding were the same as all subsequent settlement experiments described later in this section. Each beaker was examined under a dissecting scope once every 24 hours over a four-day period to observe larval activity and presence of settlers. Results from this preliminary experiment indicated that pediveliger larvae can adapt to a wider range of salinities than was expected. Larvae successfully set in salinities as low as 3 (S-3), many having exhibited metamorphosis and shell growth. (Two larvae were attached to the beaker in treatment S-2 on the second day of the experiment, but those settlers appeared to have died by Day 4.) Settlement increased as salinity increased up to the ambient treatment of S-10.5, at which point there were no distinguishable differences in settlement through S-30. Observed settlement success within these highest salinities proved that it was necessary to include a treatment salinity of 35 within the subsequent experiments.

The last preliminary experiment was designed to establish the larval size selection protocols to be used for all subsequent experiments. At HPL, late-stage larvae are graded by passing the larval cultures through a stack of metal sieves with descending screen sizes. Normally, larvae retained on both the 212μm and 224μm screen sizes display behavioral competence and are bundled for use in setting tanks. It was therefore necessary to decide which larval size (based on sieve size) would be most appropriate to use for the main settlement experiments. Using the methods of the main experiments described in detail below, larvae from one HPL cohort retained on the 212μm and 224μm screens were placed in a duplicate settlement array to

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compare their abilities to settle in salinities ranging from 5 to 30. Overall, larvae from the 224μm screen demonstrated higher settlement rates (Figure 1). Interestingly, however, settlement only differed substantially between the two larval sizes within treatments S-10 through S-30 but not within the lower salinity treatments. The larvae graded on the 224μm screen demonstrated a wider range of settlement responses to the treatment salinities, and therefore were chosen to be used in all subsequent experiments. This preliminary experiment also served as the first run of the main experiment, but only the settlement results from the 224μm group were included in later analyses.
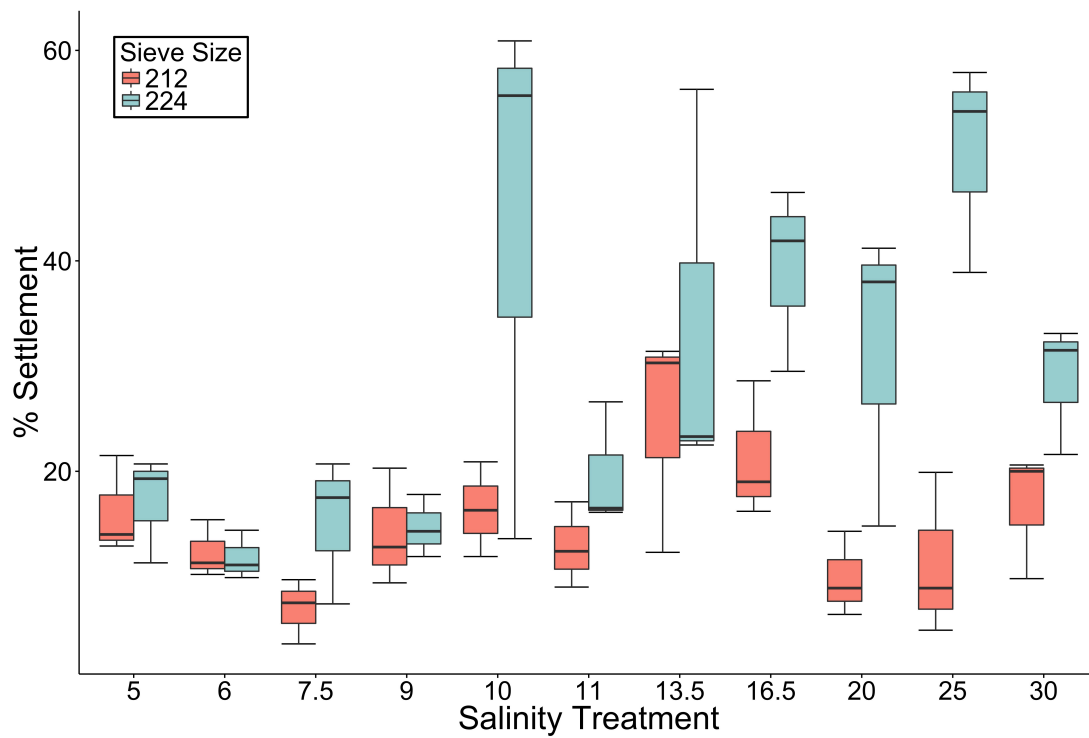
Figure 1. Settlement rates of HPL larvae graded on 212μm and 224μm sieve sizes. The median and the interquartile range (IQR) are displayed by the box, and maximum and minimum values are shown by the whiskers.

Main Experiments

Larval Sources and Selection

Multiple spawns, or cohorts, of pediveliger oyster larvae from four hatcheries were

obtained between May and September of 2015 to be tested in independent settlement

experiments listed in Table 1. The larvae were categorized into three groups (Low,

Medium, and High) according to the salinity in which they were spawned and grown

in their respective hatchery. The Low salinity larvae originated from the HPL oyster

hatchery and were reared within an average salinity of 10. The Medium salinity

larvae, reared at the Aquaculture Genetics and Breeding Technology Center (ABC) at

the Virginia Institute of Marine Science (VIMS) oyster hatchery in Gloucester, VA,

experienced salinities ranging from 15 to 16.5. The High salinity larvae originated

from both the Cherrystone oyster hatchery in Cape Charles, VA and the Rutgers

Aquaculture Innovation Center in Cape May, NJ. These two hatcheries reared their

larvae at salinities averaging 22 and 27.5, respectively. All cohorts were composed of

diploid larvae spawned from parents using broodstock sources normally used within

their respective hatcheries. In Table 1, the term "Wild" broodstock indicates that

oysters were obtained from naturally occurring oyster reefs within the upper

Chesapeake Bay. The broodstock lines "DEBY," "hANA," and "NEH[TM]" are

products of multiple generations of oysters selected for performance in the presence

of oyster diseases within the Mid-Atlantic, described in further detail in Proestou et

al. (2016). As a generalization across all hatcheries, larvae were obtained for these

experiments when deemed competent. Competency was indicated when the vast

majority of larvae had eyespots and feet and demonstrated searching behavior under a

compound microscope. Each cohort was used within only one experiment repetition (hereafter termed a "run"), but three of these cohorts were used in additional experiments that examined either the effect of larval size selection protocols or the ability for settlers to complete metamorphosis. The hatcheries provided between 100,000-500,000 larvae for each run. Prior to each run, competent larvae raised at HPL were bundled in moist cloths or paper towels and refrigerated. For other hatcheries, bundled larvae were shipped in coolers with gel packs and then refrigerated at HPL. All larvae were refrigerated for a total of 1-3 days before being introduced into the experimental array except for one Low salinity larval cohort that was refrigerated for only 4 hours.

Table 1. Hatchery sources and ambient salinity conditions of the 13 cohorts of diploid *C. virginica* larvae used in each experimental run. Larvae were classified as Low, Medium, or High according to their salinity of origin. Superscript letters represent the three larval cohorts used for multiple experiments that tested larval size selection protocols ([A]) or metamorphic completion ([B] and [C]).

Hatchery	Larval Rearing Salinity	Larval Salinity Group	Experiment Start Date	Experiment Duration	Broodstock
HPL	10	Low	5/1/15	4	Wild[A]
HPL	10	Low	5/1/15	4	Wild[A]
HPL	10	Low	5/27/15	4	Wild[B]
HPL	10	Low	5/27/15	14	Wild[B]
HPL	10	Low	6/11/15	4	Wild
HPL	10	Low	7/10/15	4	Wild
HPL	10	Low	7/20/15	4	Wild
HPL	10	Low	8/15/15	4	DEBY
HPL	10	Low	8/20/15	4	DEBY
VIMS	16.5	Medium	6/16/15	4	DEBY[C]
VIMS	16.5	Medium	6/16/15	14	DEBY[C]
VIMS	15	Medium	7/24/15	4	hANA
VIMS	15	Medium	7/28/16	4	hANA
Cherrystone	22	High	6/30/15	4	DEBY
Rutgers	27.5	High	6/22/15	4	NEH
Rutgers	27.5	High	7/23/15	4	NEH

Preparation and Enumeration of Competent larvae

Immediately prior to the start of every experimental run, each bundle of larvae was graded by size using on a 224μm stainless steel cloth sieve (W.S. Tyler) with cold filtered water (~3˚C, < 2μm) from the Choptank River. The larvae that did not pass through the sieve were placed in a 2-liter plastic pitcher filled with river water that had been adjusted to match the salinity of their originating hatchery. (See Salinity Adjustment section for details.) The pitcher was kept on ice to minimize larval activity, which prevented larvae from exuding mucus and clumping together or attaching to the beaker, ultimately enabling an evenly distributed larval suspension when mixed (Vlahovich 2009). A perforated plunger was used to continuously mix the solution while 1ml aliquots were pipetted onto to a Sedgewick-Rafter counting chamber to determine the larval density of the pitcher. Each aliquot was preserved using several drops of 10% formalin, and were counted using a compound microscope (Olympus BX40) at 40x magnification. These samples were repeated until the larval count from least five successive aliquots were within 10% of each other. The stocking density was determined by multiplying the average of the larval counts by the volume of water in the pitcher. Based on that estimate, a volume of water containing approximately 1,000 larvae was distributed into sterilized 15ml plastic vials. The vials were visually inspected to confirm that larvae were distributed uniformly, and were monitored for a few minutes to verify that larval activity increased as the water within the vials warmed to room temperature. Two additional vials were preserved with formalin and later counted to confirm that the number of

larvae distributed to the vials was approximately 1,000. The average of the two

verification counts from all runs were within 5% of 1,000.

An additional sample of approximately 2,000 larvae was removed from the

iced pitcher to determine the size distribution of each cohort. Larvae were placed in a

200mL vessel, suspended in a BCI ISOTONII electrolyte solution by a stir bar, and

were measured using a Beckman Coulter Counter Multisizer 4. Three successive

samples of 600 larvae were analyzed, resulting in three independent size distribution

logs.

Salinity Adjustment

Water used for all experiments originated from the Choptank River in the

mesohaline portion of the Chesapeake Bay and was filtered to a particle size $\leq 1\mu m$

by sand, charcoal, and string cartridge filters. The river water, which maintained a

salinity between 9.5 and 11 throughout the experimental period, was adjusted to one

of twelve salinity treatments ranging from 5 to 35 (Table 2).

Table 2. Salinity treatments tested within every experiment. An additional
salinity treatment (larval salinity), was adjusted to match the larval rearing
salinity of that cohort for each run..

5	6	7.5	9	11	13.5	16.5	20	25	30	35	Larval Salinity

Eleven consistent salinity treatments were accomplished for every

experimental run, while one salinity treatment was adjusted to match the salinity from

where the larvae originated (either 10, 15, 16.5, 22, or 27.5). The first nine salinity

values were chosen to maintain a consistent increase in the percentage of salt between

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treatments (~ 22.5%). These salinity increments enabled a higher resolution of treatment effects in lower salinities. Salinity treatments S-30 and S-35 were also included as a result of the preliminary experiments. Deionized water (DI) was added to decrease the salinity and Crystal Sea Marinemix® was added to increase the salinity. Crystal Sea was chosen because of its record of successful use within the HPL hatchery, and it is a recommended brand by the U.S Environmental Protection Agency for salinity adjustment within toxicity tests (U.S. Environmental Protection Agency 2002). The solutions for each salinity treatment were first mixed in a 30L bucket before being distributed into replicate treatments within the experimental array. Salinity was measured with a YSI Model 30 temperature and salinity probe that had been calibrated before each experimental run.

Water samples from 3 different runs were analyzed to observe how the addition of DI water and the Crystal Sea Marinemix® affected the carbonate chemistry of the river water. Water samples (30ml) from all 12 salinity solutions were retrieved from the 30L mixing bucket before the experiment started and preserved with 1µl of $HgCl_2$. A separate range of salinity solutions was made with only DI water and Crystal Sea and were sampled in the same manner. Total alkalinity, temperature, and pH (NBS scale) were measured using an SI Analytics TL 7750 automated titrator. Aragonite and calcite saturation coefficients (Ω_{ar} and Ω_{ca}) were calculated with the CO2SYS program (http://cdiac.ornl.gov/ftp/co2sys/) in Excel 2007 using the equilibrium constants K1 and K2 from Millero (2010) and KSO_4 from Dickson (1990).

Experimental Array

Larval settlement was tested in bags made from 0.254mm low-density

polyethylene tubing (25.4cm wide, VWR®) that was heat-sealed at the bottom to form

a square. Previous settlement experiments conducted at HPL using the same bag

material had demonstrated that 1) newly cemented larvae remain on the bags after a

drying process and can be counted at a later date, 2) the plastic is easy to manipulate

under a microscope to accurately count newly settled spat, and 3) the bags allow for

many concurrent replicates (Vlahovich 2009). Each settlement bag contained a

10.8cm^2 unglazed ceramic tile (Chesapeake Ceramics, Baltimore MD) that had been

thoroughly rinsed in tap water a day before the start of each run. Each bag was

initially filled with 2L of water and was aerated through a plastic tube (fitted with a

micropipette) connected to the HPL hatchery's low-pressure aeration system. For all

runs, each salinity was tested in triplicate (Figure 2). All bags within the array were

suspended in a water bath maintained at 27 ± 0.5 °C using a submersible water heater

(Innovative Heat Concepts, Homestead FL). Temperature uniformity within the water

bath was maintained by a 1.1AMP water pump that gently circulated the water.

Figure 2. Experimental array used for all settlement experiments. The settlement bags, filled with aerated, salinity-adjusted water, contained a ceramic tile, and were suspended in a temperature-controlled water bath.

Experimental Protocol: 4-Day Experiments

At the beginning of each "4-Day" run, 1,000 larvae were placed within each settlement bag filled with 2 liters of salinity-adjusted water. A mixture of algae was added to the bags once daily for 4 days. The first two algae additions consisted of 75,000 cells ml^{-1} of *Chaetoceros muelleri* (strain CHGRA, 70%) and *Tetraselmis chui* (strain PLY 429, 30%) given at the beginning of the experiment and again approximately 24 hours later. Salinity within all treatments was measured again on Day 2 (approximately 48 hours after beginning the experiment) and an additional 2 liters of water was added to each bag to maintain water quality and to adjust for any changes in salinity due to algae additions or evaporation. The approximate volume of algae added on Day 2 and 3 of the experiment remained the same as the prior two additions, but because the water volume had been doubled, the resulting density of algae was 37,500 cells ml^{-1} in the same proportions of CHGRA (70%) and PLY 429 (30%).

Upon completion of the 4-Day experiments (approximately 96 hours after larvae were added to the settlement bags), salinity was once again measured to confirm integrity of salinity in the trial. All runs remained within the salinity tolerances of the experiment. The tiles were removed from the settlement bags and placed on a rack to dry. Each bag was gently rinsed with water and hung upside down to dry. After at least one day on the drying rack, settlers were counted under 10x magnification using an Olympus S2X16 dissecting microscope. No distinction was attempted to identify recently attached settlers versus those that had settled and

metamorphosed. Settlement counts were summed from both the tile and bag to determine the setting rate for each replicate using the formula:

$$\frac{\text{Total Settlement}}{1,000\ \text{Larvae}} \times 100 = \text{Setting Rate (\%)}.$$

Experimental Protocol: 14-Day Experiments

Recognizing that no differentiation was made between recently attached settlers and fully metamorphosed juveniles within the 4-Day experiments, two longer-term experiments were conducted to observe if settlers were capable of undergoing metamorphosis across all salinity treatments. These "14-Day" experiments began simultaneously with two runs of the 4-Day experiments, using extra larvae provided with one Low salinity cohort and one Medium salinity cohort (Table 1). The 14-Day experiment was essentially an exact duplicate of the 4-Day experiment, starting at the same time and including the same triplicate salinity treatments. Immediately after the conclusion of the paired 4-Day experiment, water was emptied from the 14-Day settlement bags to remove any live or dead larvae. The bags were then refilled with 4 liters of water adjusted to the appropriate salinity treatments and the same ration of algae was provided daily (37.5 cells/ml of *Chaetoceros muelleri* (clone CHGRA, 70%) and *Tetraselmis chui* (clone PLY 429, 30%)). Complete water changes were conducted every 3-4 days. On Day 14, the experiment was concluded with the same rinsing and drying protocols employed for the 4-Day experiment. Unlike the newly attached settlers, however, some of the larger juveniles became dislodged from the bag during the rinsing process. Therefore the rinsing water was poured over a 110μm screen to capture any dislodged spat. The number of dislodged spat was added to the

bag and tile settlement tallies in order to calculate the total setting rate for each replicate.

After all tiles had been counted, the upper sides of the tiles were photographed to analyze spat growth within the different salinity treatments. The camera was mounted approximately 0.5m above the tile and a ruler was mounted next to the tile to calibrate the digital measurement tools within the image software Adobe Illustrator CC (version 14.2, 2013). Shell heights were measured as the distance between the umbo and the edge of the shell at the point of bisection. All shell heights (up to the maximum of 10) were recorded from each settlement tile. If the spat set on a particular tile was heavy, the tile was divided into 4 quadrants and the 2 spat closest to the center of the 4 quadrants and the center of the tile itself were measured.

Statistical Analysis

Effects of the 11 salinity treatments and the possible interaction with different larval salinity groups on settlement were examined using a linear mixed-effect model (lmer) with a weighted variance structure. The statistical model is:

$$Y_{ijkl} = \alpha_i + \beta_j + \alpha\beta_{ij} + \gamma_k + \varepsilon_{l(ijk)}$$

where α_i represents the 11 repeated salinity treatments, β_j represents the 3 larvae salinity groups, $\alpha\beta_{ij}$ is the salinity treatment by larval salinity interaction, γ_k is the "cohort effect," and $\varepsilon_{l(ijk)}$ is the error. The treatment salinities and larval salinity groups were modeled as fixed categorical factors. Because each larval cohort varies from one another even from the same hatchery (due to differences in gamete quality, algae cultures, water quality, competency, etc), there were differences in overall settlement performance for each repetition of the experiment. Therefore a random

factor representing the differences for each repetition, called "cohort effect," was also included within the statistical model. This enabled multiple experiments to be analyzed simultaneously with the *a priori* prediction that differences between larval batches would affect the overall settlement rates without changing the nature of differences between treatment levels. The best variance structure, as determined by comparing the Akaike Information Criterion (AIC) values of multiple models, was used to address the heterogeneity of variances by allowing different variances for each repetition (Zuur et al. 2009).

A two-way analysis of variance (ANOVA) was performed to test the null hypotheses that mean settlement rates across all salinities were equal, and to test if differences between the means were affected by the larval salinity group. Similarly, two-way ANOVAs were used to test the null hypothesis that there were no differences between the settlement rates of the different sized larvae of the first experimental cohort as well as differences between the 4-day and 14-day settlement rates across all salinity treatments.

Settlement results within each larval salinity group were individually examined using the same model described above, but excluded the larval salinity group or interaction terms. A one-way ANOVA was used to identify if there differences in settlement rates between salinity treatments. Afterwards, a Tukey's HSD (honest significant difference) test was performed to determine between which salinities the settlement rates were significantly different. Differences in spat lengths between salinity treatments from the 14-Day experiments were also analyzed in this

manner. All ANOVA calculations, tables, and figures were made using the statistical software R (2015).

Although traditional null-hypothesis testing is still the gold standard to identify treatment effects, recent scientific literature has emphasized the importance of describing the direction and magnitude of treatment effects even if there are no statistical differences between means (Nagawa and Cuthill 2007; Halsey et al. 2015). Effect size statistics are therefore increasingly being employed to assess differences between treatment results. For this study, effect sizes were calculated to illustrate how larvae performed in each salinity treatment relative to how they performed within their native salinity (acting as the "control"). The effect size for each salinity treatment within each cohort was calculated as a logarithmic response ratio (LnRR) using the following formula:

$$\left[R = \frac{\bar{X}_{\text{Treatment}}}{\bar{X}_{\text{Control}}} ; \ln(R) = L = Ln(\bar{X}_{\text{Treatment}}) - (\ln(\bar{X}_{\text{Control}})) \right].$$

A meta-analysis could then be performed by combining the effect sizes across cohorts within the same larval salinity group. Recognizing that effect size estimates within each experiment differ in precision (standard error), one can assume that the more precise estimate is closer to the true effect (Hedges et al. 1999). Therefore the estimated effect sizes across experiments could likely be improved by weighting them according to their variance, as detailed by Hedges et al. (1999). Effect size meta-analysis was particularly relevant for this data set because it provided a way to account for the variability between the overall performances of each cohort by standardizing the magnitude of the effect of the salinity treatments across all runs.

The statistical software Metawin (Rosenberg et al. 1996) was used to calculate the weighted effect sizes.

RESULTS

Salinities were maintained close to the designated treatment values throughout the experiment duration (Table 3), particularly within all salinity treatments below S-30. Salinities within treatments S-30 and S-35 varied the most, likely due to the daily additions of lower-salinity algae. Occasionally, bag seals were breached, which forced their removal from the experiment, therefore not all treatments contained three replicates. Overall settlement performance from each cohort varied substantially but appeared to have generally decreased as the larval season progressed (Figure 3). Larval size was not an indicator of overall settlement performance of each cohort, particularly from the more frequently tested Low salinity cohorts (Figure 4). The larval sizes obtained from coulter counter measurements are reported here as a spherical equivalent of volume, and therefore are not measurements of precise larval shell heights.

Evidence of gregarious settlement was repeatedly observed, as the majority of settlement was often concentrated on a small area of the bag or tile (Figure 5). Larvae preferred to set on the tiles as opposed to bags (57.83% and 42.17%, respectively), and set in substantially higher proportions on the undersides of tiles (59.29%) compared to the top (25.19%) or edges (15.52%).

As expected, settlement was highly variable between cohorts from the same hatchery, between similar salinities within a run, and among replicates. As an example, Figure 6 shows the individual settlement rates of each replicated treatment

from one run of a High salinity larval cohort. Generally speaking across all cohorts, as settlement averages increased, so did the variability. Despite this variability, clear settlement patterns across the range of salinities from each larval salinity group emerged.

Table 3. Maximum and minimum salinity values recorded for each salinity treatment throughout all measurements for across all repetitions. Salinities marked with (*) were only included in the experimental runs using larval cohorts reared within that salinity.

Salinity Treatment	Salinity Range
5	5.0-5.4
6	6.0-6.4
7.5	6.9-8.0
9	8.8-9.3
10*	9.7-10.4
11	10.7-11.6
13.5	13.0-14.0
15*	14.8-15.1
16.5	16.2-17.0
20	18.4-20.4
22*	21.4-22.3
25	24.1-25.2
27.5*	27.3-27.6
30	28.9-30.5
35	33.2-36.0
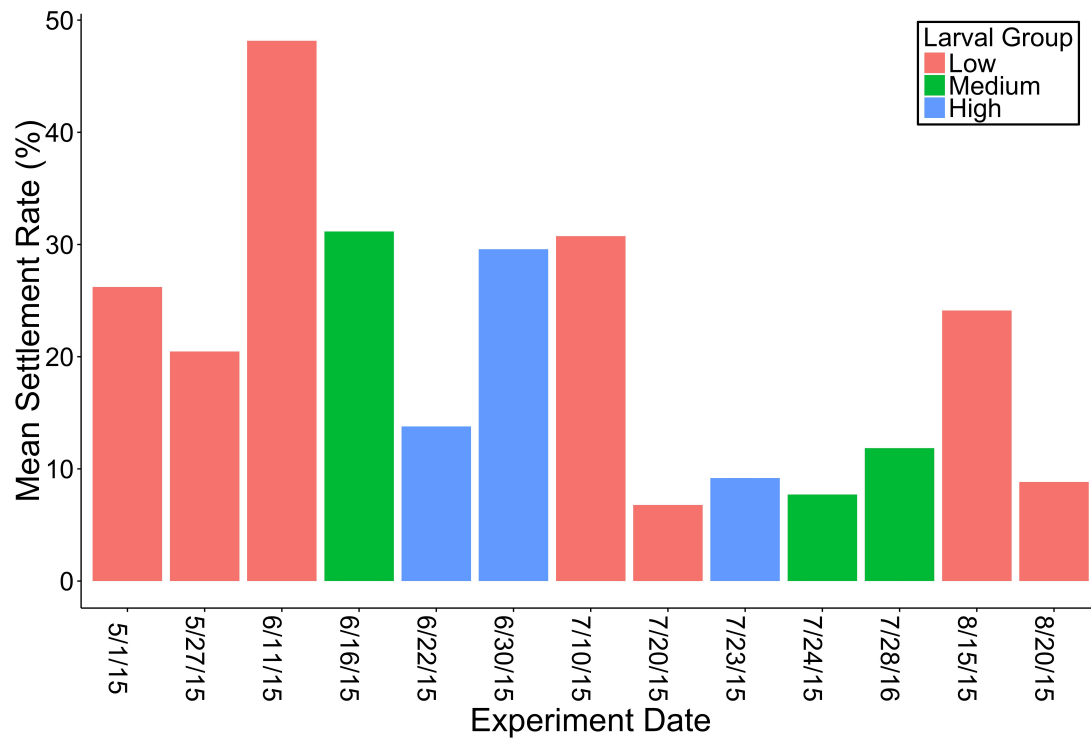
Figure 3. Mean settlement rate (across all salinity treatments) of each cohort from all three larval groups.
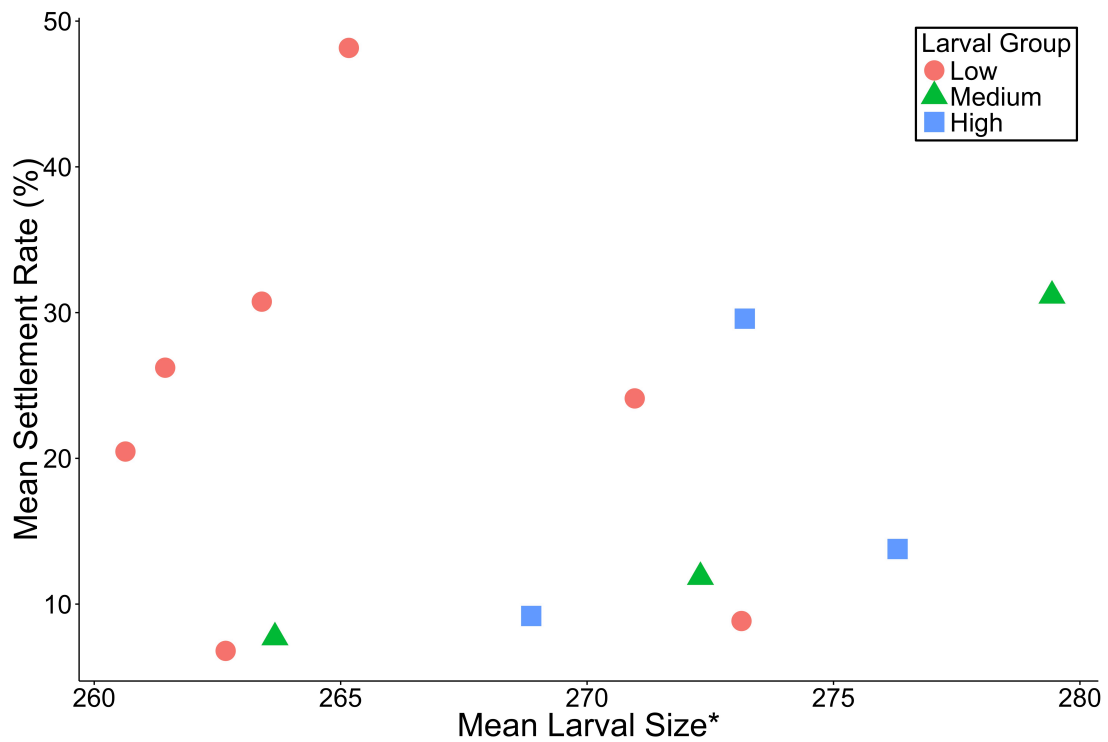
Figure 4. Mean larval sizes from each cohort, plotted against the average settlement across all salinity treatments. Mean larval sizes (*) are reported here as the spherical equivalents of volume produced by coulter counter measurements.
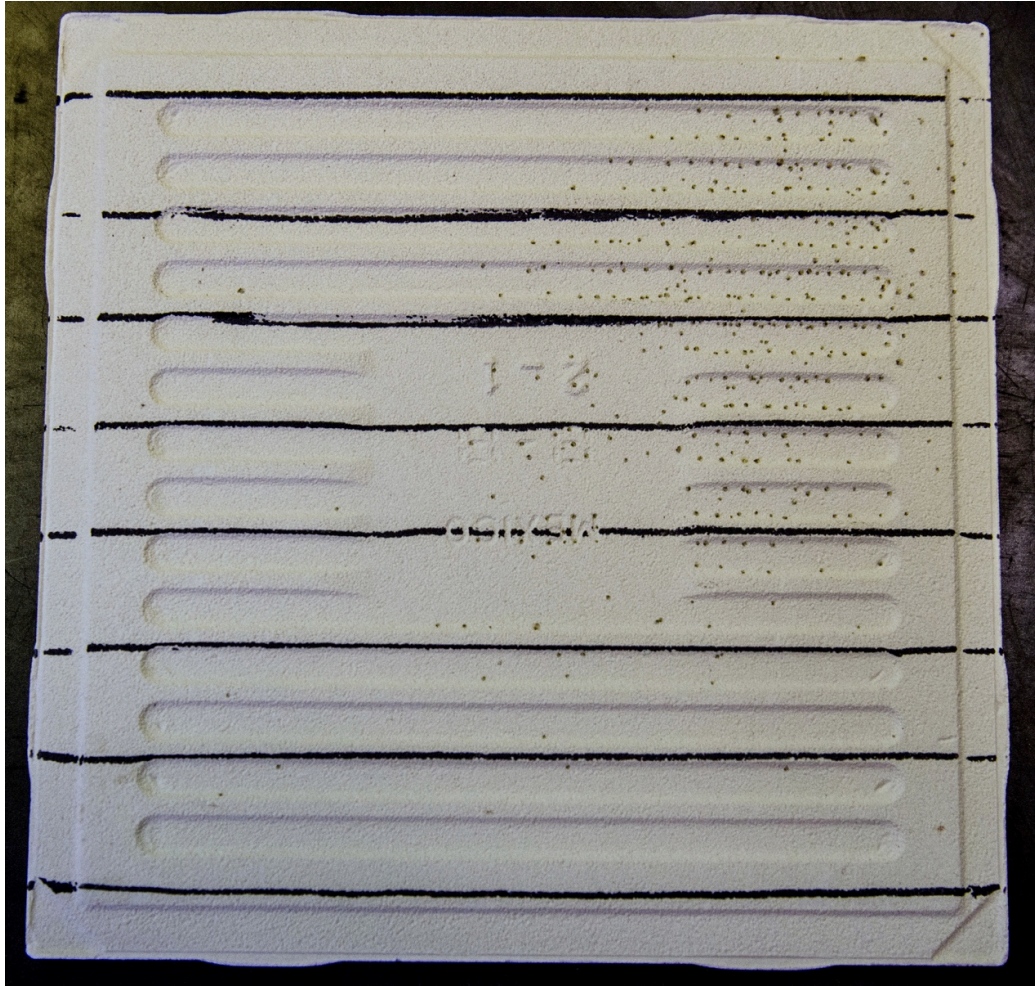
Figure 5. Example of gregarious settlement frequently observed on tiles. Black lines were drawn to aid in counting.
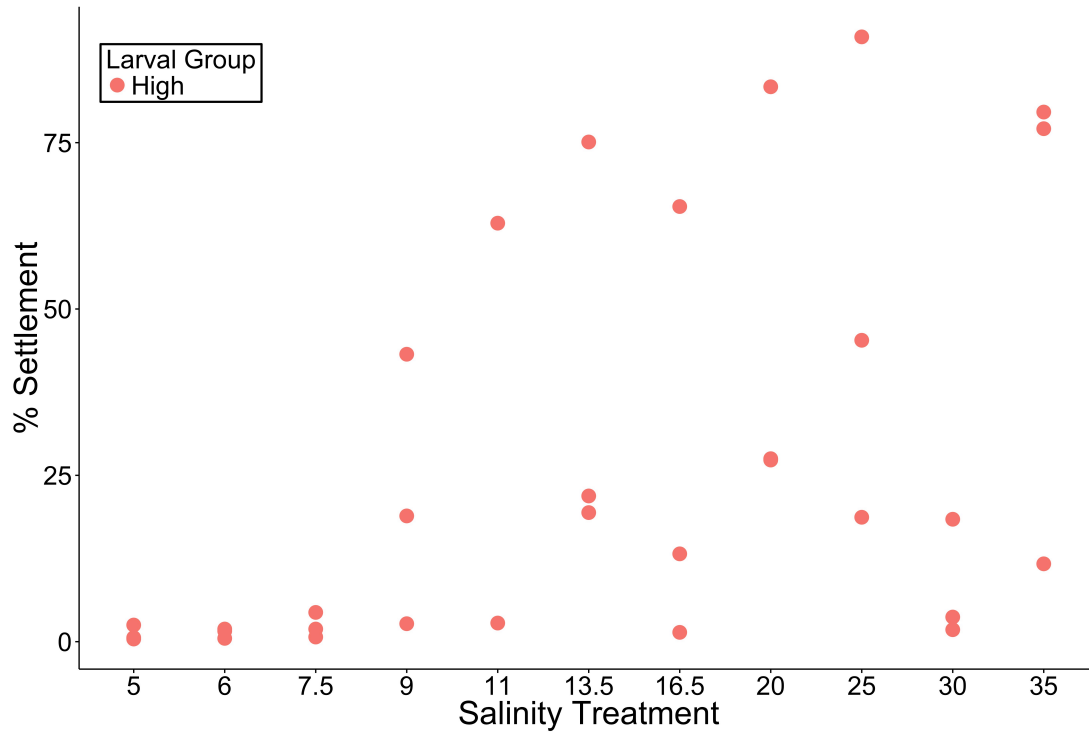
Figure 6. Example of settlement variability between replicates from one High salinity larval cohort.

4-Day Experiments

The effect of salinity treatments on settlement was highly significant (p<0.001, α<0.05, two-way ANOVA), indicating that settlement rates were affected by the salinity treatments (Table 4). Effects of larval rearing salinity on settlement were not significant (p=0.6148, α<0.05, two-way ANOVA). The interaction between the effects of salinity treatment and the larval rearing salinities approached the threshold of significance (p=0.0974, α<0.05, two-way ANOVA), suggesting that settlement in certain salinity treatments might be influenced by the salinity in which the larvae originated, but the effect was not strong enough to be significant under the nominal alpha threshold of .05.

The Tukey's HSD post-hoc analysis of each larval group revealed substantial similarities in settlement performance between the Low, Medium, and High larval salinity groups (Figure 6). Results from the Tukey's HSD also enabled a consistent method to define the salinity range of optimal settlement. This range was identified by selecting the salinity treatments that shared the same letter as the treatment that produced the highest average settlement from that salinity group. Interestingly, the salinity treatments S-11 through S-30 elicited peak settlement for all three larval groups. Additionally, the maximum settlement for all three larval groups occurred in S-25. Results from each larval salinity group are presented below in the following sections, where all average values are written as mean± standard error (SE) unless stated otherwise. The arcsine-transformed settlement means used for ANOVAs and Tukey's HSD were back transformed for all figures.

Table 4. Two-way ANOVA results of the effects of salinity treatment and hatchery salinity on larval settlement. Significant p-values (α=.05) are shown in bold.

Source	Df	F-value	P-value
Salinity Treatment	10	25.60602	**<.0001**
Larval Salinity Group	2	0.51099	0.6148
Salinity X Hatchery	30	1.44667	0.0974

Table 5. One-way ANOVA of settlement from Low salinity larval cohorts (rearing salinity of 9.8 to 10.6) across all salinity treatments. Significant p-values (α≤.05) are shown in bold.

Source	df	Den df	F-value	P-value
Salinity treatment	10	207	8.05196	**<.0001**

Table 6. One-way ANOVA of settlement from Medium salinity larval cohorts (rearing salinity of 15 and 16.5) across all salinity treatments. Significant p-values (α≤.05) are shown in bold.

Source	df	Den df	F-value	P-value
Salinity treatment	10	84	9.297656	**<.0001**

Table 7. One-way ANOVA of settlement from High salinity larval cohorts (rearing salinity of 22 and 27.5) across all salinity treatments. Significant p-values (α≤.05) are shown in bold.

Source	df	Den df	F-value	P-value
Salinity treatment	10	85	12.64338	**<.0001**
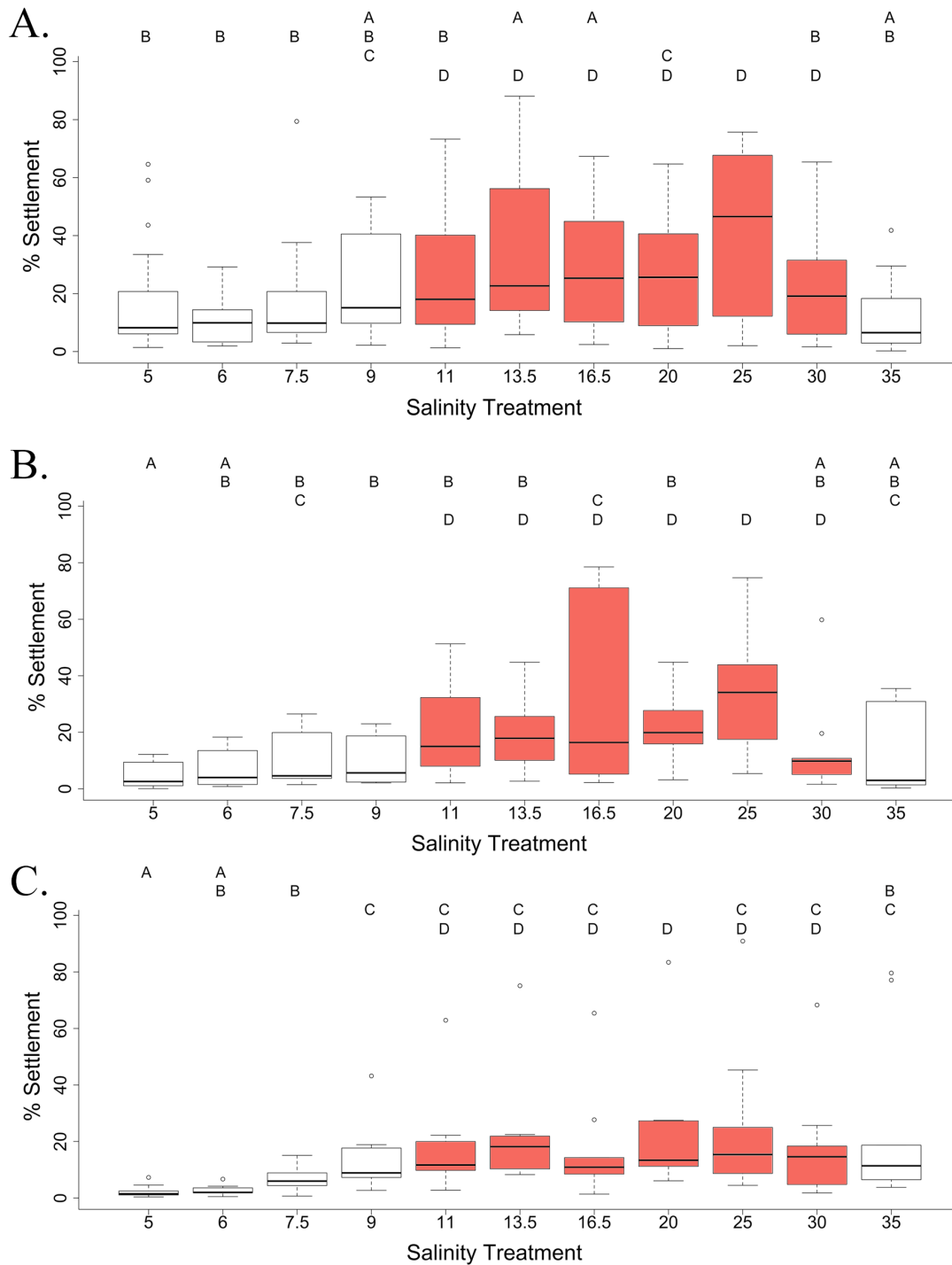
Figure 7. Settlement of Low (A), Medium (B) and High (C) salinity larval cohorts across the 11 consistent salinity treatments. Similar letters above the treatments indicate no significant differences in settlement, and the range of optimal settlement is highlighted in salmon. The median and the interquartile range (IQR) are displayed by the box, and maximum and minimum values are shown by the whiskers. Suspected outliers (defined as 1.5 x IQR) are displayed as open circles.

Low Salinity Larvae

A total of 7 larval cohorts were tested within the Low salinity group, originating from one hatchery that reared the larvae within salinities ranging from 9.8 to 11.2 during the experimental time frame. Because of this relatively small variation, the "control" treatment salinity tested to match the rearing salinity was always set at 10 (to be used for effect size calculations, described later in this section). Due to the high number of experiments and the variability among cohorts, the interquartile range across many of the salinities was large (Figure 6). For instance, the mean settlement across all salinities for one cohort was as low as 6.79%, while the cohort with the highest mean settlement was 48.16% (Figure 3). Overall, the mean settlement across all salinities for all runs was 23.51%. On average, the highest settlement occurred within S-25 (39.37±6.37%) and least in S-35 (11.32±2.52%). The mean settlement in the lowest salinity treatment (S-5) was 17.22±4.11%, which was higher than S-6 and S-7 (10.13±1.7% and 16.48±3.84%, respectively). Although the average settlement for S-5 was higher than for S-6 and S-7.5, the median settlement increased from 8.2% in S-5, to 9.9% and 9.8% in S-6 and S-7, indicating that a few high outlier settlement counts within S-5 are responsible for a higher mean within that treatment (Figure 6). The optimal salinity range was from S-11 (21.32±4.77%) to S-30 (23.16±4.51%).

Medium Salinity Larvae

The larvae within the Medium salinity group, tested across three replicate experiments, were obtained from one hatchery with salinity of 16.5 for one cohort and a salinity of 15 for another two. The average settlement rate across all salinities for all 3 replicates was 17.21%, ranging from the lowest average settlement of 7.3%

from one cohort to the highest of 31.85% from another. As seen with all salinity groups, on average the highest settlement occurred in S-25 (32.9±6.96%), and the salinity range of optimal settlement was from S-11 (18.59±6.65 %) to S-30 (17.85±6.81%) (). Settlement within S-25 was only slightly higher than settlement in the ambient salinity for the larvae of 16.5 (32.67±10.99%). The least amount of settlement occurred in the lowest salinity treatment, S-5 (4.54± 1.57%).

High Salinity Larvae

The three cohorts tested within the High salinity group originated from two hatcheries. One cohort was reared in a salinity of 22, and the other two cohorts were reared in salinity 27.5. The average settlement across all salinities for the High salinity larvae was 16.89%, ranging from the highest average of 28.34% from the cohort reared in a salinity of 22, to the lowest average of 9.19% with larvae reared in a salinity of 27.5. Although there was a relatively large difference in overall performance between cohorts from these two hatcheries, the patterns of settlement across salinity treatments closely mirrored each other. As with the Medium salinity larvae, the lowest settlement occurred in S-5 (2.39± .74%), and similar to the Low and Medium larval groups, the highest settlement occurred within S-25 (25.28 ± 9.18%) (Figure 6). Unlike the other two larval groups, however, settlement within S-35 was statistically higher than settlement within the lowest salinities S-5 and S-6. Differences in how the larval groups performed within the salinity extremes are illustrated more clearly with treatment effect sizes, described below.

Effect Size Analysis

Calculations of mean log response ratios (LnRR) revealed how each cohort performed within each salinity treatment relative to how they performed within their native, or "control," salinity during each repetition. Hence the LnRR, or effect size, for each larval group at their native salinity is equal to 0, and an increase or decrease in settlement performance is indicated by a positive or negative LnRR, respectively. The majority of the effect sizes were negative, indicating that the highest settlement rates occurred within the salinity treatment that matched the originating hatchery (Figure 7). The exception to that trend is seen only from the Low salinity larvae, which demonstrated higher settlement in S-13.5 through S-25 than within its native salinity of 10. However, the magnitude of the effect sizes between S-11 and S-30 for all 3 larval salinity groups were not profound, which is consistent with the results from Tukey's mean comparison tests (Figure 6).

The effect sizes outside of the optimal setting salinities (S-11 to S-30) became increasingly negative for all larval groups, but not in an equal manner for each larval group. The largest negative effects (which indicate the lowest settlement relative to their native salinity) occurred in salinity treatments S-5 and S-6 only for the Medium and High larval groups. In contrast, the negative effects on settlement within those salinity treatments were not as severe for the Low salinity larvae, indicating that the Low larval group could settle comparatively better within the lower salinities. The opposite is true within the highest salinity treatment, S-35, which produced the largest negative effect for Low salinity larvae, but did not induce a large decrease in settlement for the High salinity larvae.
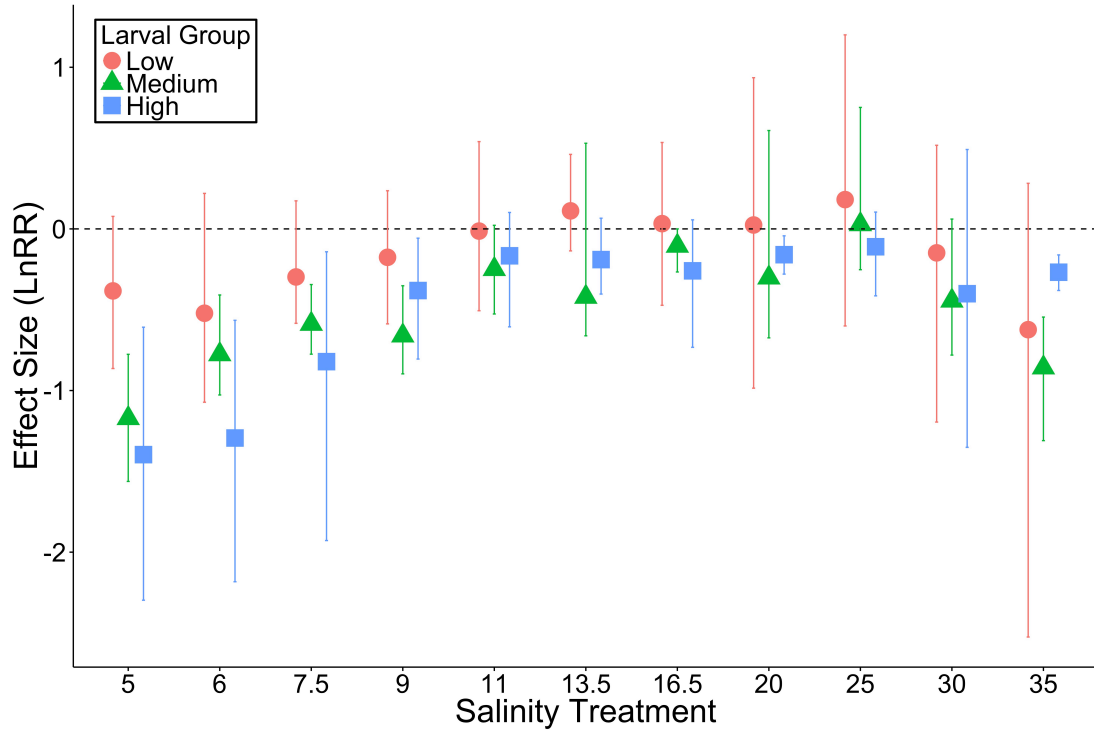
Figure 8. Mean Logarithmic Response Ratios (LnRRs), for each larval group across the 11 consistent salinity treatments. Symbols indicate the weighted mean, while the intersecting bars display the range of maximum and minimum LnRRs calculated for each cohort within the larval group. Negative LnRRs indicate reduced settlement compared to settlement within its native salinity.

14-Day Experiments

Metamorphosis and Post-settlement Survivorship

The 14-Day experiments examined the survivorship of settlers from two cohorts after the 4-Day settlement period and indicated that settlers within all salinity treatments were capable of metamorphic completion and subsequent juvenile growth. Differences in numbers of settlers between the 4-Day and 14-Day groups were analyzed statistically with two-way ANOVA testing (Tables 8, 9, and 10) and are illustrated by boxplots of settlement counts (Figures 8 and 9). As expected, settlement was significantly affected by salinity for both larval groups ($p<0.001$, $\alpha<0.05$; two-way ANOVA). Differences in settlement between the 4-day group and the 14-day group approached the significance threshold for the Low salinity larvae ($p=.0634$, $\alpha<0.05$; two-way ANOVA), but were non-significant for the Medium salinity larvae ($p=0.1273$, $p<0.05$, two-way ANOVA). Interactions between treatment salinities and experiment duration were non-significant for both the Low salinity larvae ($p=0.18515$, $\alpha<0.05$; two-way ANOVA) and the Medium salinity larvae ($p=0.9766$, $p<0.05$; two-way ANOVA). Figures 8 and 9 illustrate that with few exceptions, settlement rates within the 14-Day groups closely mirrored the settlement rates within the 4-Day groups for both larval cohorts. The only notable divergence between the 4-Day settlement rates and the 14-Day settlement and survivorship counts occurred with Low salinity larvae in the higher salinity treatments. Indeed, as Tables 8 and 9 demonstrate, a primary driver of the statistical differences is the divergence of settlement means in the treatment S-25. Although the means between the two settlement results at S-25 are quite different (62.95% and 25.17%), one replicate from

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the 14-day study contained a settlement and survivorship rate of 59.9%. Therefore, despite the differences in means, it was proven that settlers were physiologically capable of fully metamorphosing and growing in that salinity.

Table 8. Two-way ANOVA results of the effects of salinity treatment and experiment duration on settlement and survivorship of Low salinity larvae. Significant p-values (α=.05) are shown in bold.

Source	Df	F-value	P-value
Salinity Treatment	11	6.1478	**<.0001**
Experiment Duration	1	3.6080	0.06392
Salinity X Duration	11	1.4488	0.18515

Table 9. Two-way ANOVA results of the effects of salinity treatment and experiment duration on settlement and survivorship of Low salinity larvae, **excluding salinity treatment (S-25)**. Significant p-values (α=.05) are shown in bold.

Source	Df	F-value	P-value
Salinity Treatment	11	6.7110	**<.0001**
Experiment Duration	1	1.3448	0.2527
Salinity X Duration	11	0.7937	0.6349

Table 10. Two-way ANOVA results of the effects of salinity treatment and experiment duration on settlement and survivorship of Medium salinity larvae. Significant p-values (α=.05) are shown in bold.

Source	Df	F-value	P-value
Salinity Treatment	11	13.5677	**<.0001**
Experiment Duration	1	2.4102	0.1273
Salinity X Duration	11	0.3238	0.9766

Figure 9. Comparison of Low salinity larvae given 4 days to set (4-Days) with another group given 4 days to set and 10 days to grow (14-Days). The median and the interquartile range (IQR) are displayed by the box, and maximum and minimum values are shown by the whiskers.
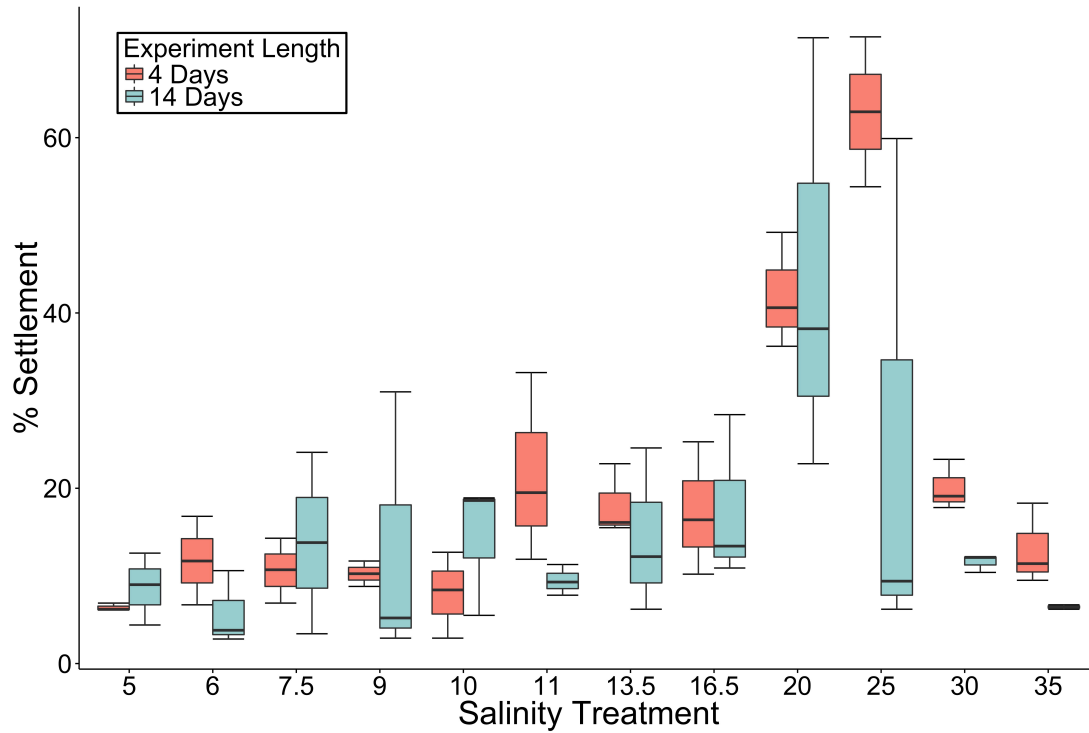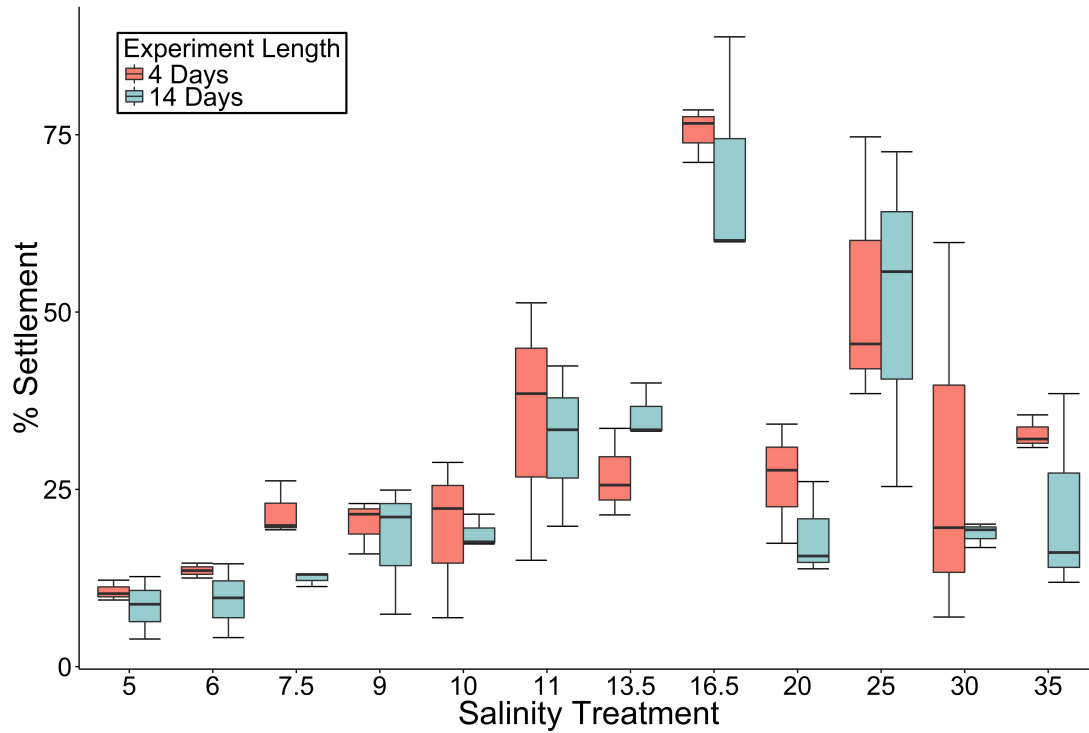
Figure 10. Comparison of Medium salinity larvae given 4 days to set (4-Days) with another group given 4 days to set and 10 days to grow (14-Days). The median and the interquartile range (IQR) are displayed by the box, and maximum and minimum values are shown by the whiskers.

Spat Growth

Settlers from both the Low and Medium salinity cohorts demonstrated appreciable juvenile growth in all salinity treatments. The effect of salinity on spat length was statistically significant ($p<0.001$, $\alpha<0.05$; one-way ANOVA) for both cohorts (Tables 8 and 9). Growth rates across the salinity treatments closely resembled the responses observed within the settlement experiments. Growth generally increased as salinity increased before a decline in S-35 (Figure 11). As with settlement, reduced growth in S-35 was more profound for the Low salinity cohort than the Medium salinity cohort. Additionally, as with settlement, both cohorts exhibited peak growth rates across the same salinity treatments as one another. Optimal salinities for growth can arguably be extended from S-11 through S-30 to include the S-9 treatment as well. Maximum shell heights also occurred in slightly lower salinities than the maximum settlement, which for both groups was within S-25. The largest average shell heights for the Low salinity larvae were measured in S-16.5, while the largest shell heights from the Medium salinity cohort were measured in S-20.

Table 11. One-way ANOVA of shell heights from a Low salinity cohort across all salinity treatments. Significant p-values ($\alpha \leq .05$) are shown in bold.

Source	df	Den df	F-value	P-value
Salinity treatment	11	287	13.946	**<.0001**

Table 12. One-way ANOVA of shell heights from a Medium salinity cohort across all salinity treatments. Significant p-values ($\alpha \leq .05$) are shown in bold.

Source	df	Den df	F-value	P-value
Salinity treatment	11	266	9.6907	**<.0001**
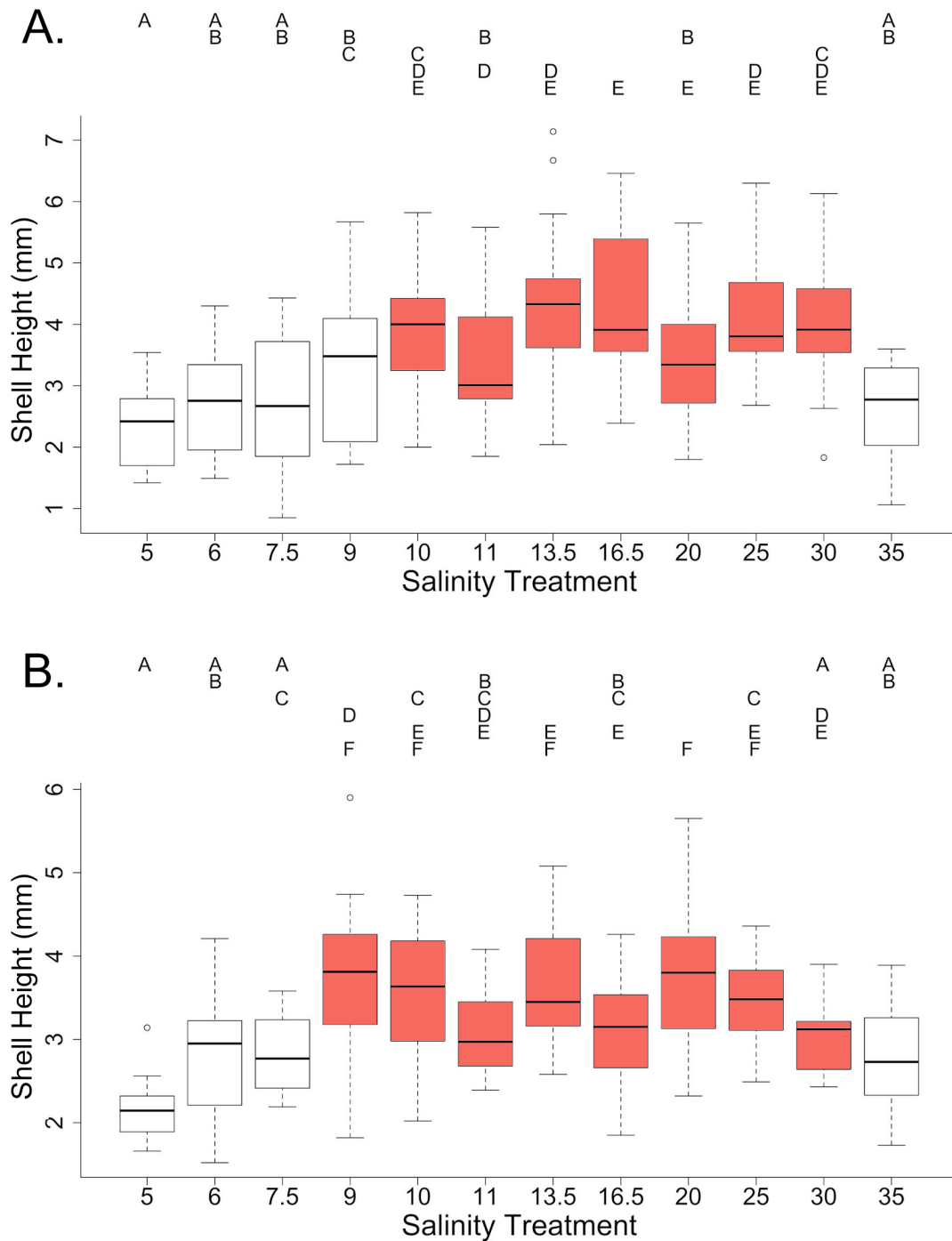
Figure 11. Juvenile Shell heights from Low salinity larvae (A) and Medium salinity larvae (B) after a 14-day period of settlement and growth. Similar letters above the boxplot indicate no significant differences in settlement among salinities. The median and the interquartile range (IQR) are displayed by the box, and maximum and minimum values are shown by the whiskers. Suspected outliers (defined as 1.5 x IQR) are displayed as open circles.

Carbonate Chemistry

Samples of the water used in the experimental treatments were analyzed to observe how the addition of DI water and Crystal Sea Marinemix® affected the carbonate chemistry of the filtered ambient river water. Figure 12 displays the aragonite saturation coefficients (Ω_{ar}) calculated by the program CO2SYS using the measured values of temperature, salinity, pH, and total alkalinity from 3 independent experimental runs. The addition of Crystal Sea Marinemix® appeared to cause a substantial increase in available calcium carbonate coinciding with increased salinity. The average ambient Ω_{ar} from filtered ambient Choptank water increased from 0.30±0.05 (mean±SD) to 7.05±1.304 within S-35. The addition of DI water also affected the carbonate chemistry, decreasing the ambient from Ω_{ar} 0.30±0.05 to 0.16±0.06.
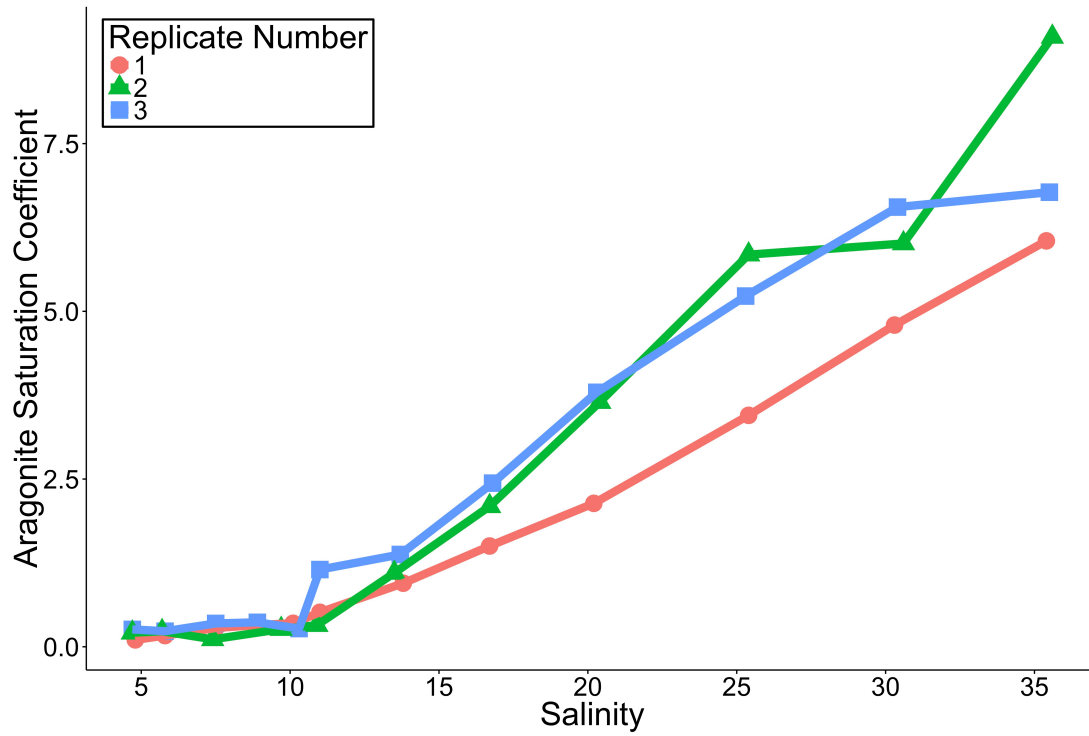
Figure 12. Aragonite saturation coefficients (Ω_{ar}) of all salinity treatments measured at the start of 3 experimental runs.

DISCUSSION

Salinity Adaptation During Settlement and Metamorphosis

Results from this study illuminate the impressive capabilities of *C. virginica*
to adapt to a wide range in salinity conditions during settlement and metamorphosis.
While the responses to salinity stress in bivalves have been shown to be less severe if
the salinity is changed gradually (Davis and Calabrese 1964; Hand and Stickle 1977;
Pierce et al. 1992; Yuan et al. 2010), this study shows that eyed larvae are capable of
tolerating changes in salinity of 20 or more without stepwise acclimation. In fact,
larvae were able to settle in greater proportions and in more extreme salinities than
described in prior research. Results from the preliminary experiments show that
settlement and metamorphosis can occur in salinity as low as 3, and larvae from all
cohorts consistently demonstrated settlement in all salinity treatments ranging from S-
5 to S-35. Furthermore, the experiments extended to 14 days confirmed that the
observed settlement rates after 4 days were also indicative of the number of larvae
capable of undergoing metamorphosis and demonstrating juvenile growth in the
tested salinities.

Although *C. virginica* is a species that normally lives in salinities from 5 to 40
(Shumway 1996), researchers have seen 100% larval and adult mortality following
exposure to smaller salinity changes than what the pediveligers experienced in this
study (Loosanoff 1953; Davis 1958; Davis and Calabrese 1964; Anderson and
Anderson 1974; Pierce et al. 1992). Therefore it is indeed surprising that larvae from
all cohorts could demonstrate such resilience to osmotic shock during this transitional
phase of their life. One might expect the added physiological stress of a salinity

change would severely impair their ability to complete the energetically expensive processes of settlement and metamorphosis. On the other hand, it is understandable that settling larvae have developed mechanisms enabling them to adapt to a variety of conditions on the benthos that may differ from the water column. Unfortunately, very little research has investigated the behavioral responses, cellular processes, or the genetic controls of osmoregulation during oyster settlement and metamorphosis. It is currently unknown if pediveligers have an increased capacity to regulate intercellular ionic concentration during settlement, or if and for how long they can buffer themselves from salinity shock by closing their valves. Although inhalent and exhalent currents have been observed through nearly all the stages of settlement and metamorphosis (Baker and Mann 1994b), pediveligers and newly-settled juveniles are capable of anaerobic metabolism in stressful conditions like hypoxia and anoxia (Baker and Mann 1992), and therefore could potentially remain closed for a period of time when initially placed in the settlement chamber. This study did not investigate whether the unsuccessful larvae within the salinity extremes died immediately due to salinity shock or if they were simply incapable of completing the behavioral process of searching for and cementing themselves to a substrate. Whether or not the larvae could maintain an osmotic barrier while they acclimated to the new salinity, it is clear that a surprisingly high percentage of larvae from all cohorts were eventually able to settle, osmoconform, and begin feeding and growing as juveniles. To my knowledge, the only observations of settlement in different salinities were made by Prytherch (1934), who noted that the process of cementation during settlement is substantially

slower in salinities less than 9 and above 29 due to reduced functioning of their byssal gland in salinity extremes.

A notable downfall of previous settlement experiments that have attempted to characterize the physiological tolerances and adaptive capabilities of larvae is the use of broodstock and larvae conditioned and reared in only one location. Research has shown that tolerance of *C. virginica* to extreme salinities differs among geographic regions (Davis 1958; Newkirk 1978; Pierce et al. 1992), therefore the use of only one larval source ignores the possibility that successful settlement in salinity extremes might be affected by the environment and genotype of its parents or by the environment during larval development. Davis (1958) saw evidence of maternal environment effects on early larval performance when he observed that optimal salinity for egg development was substantially higher when gametogenesis occurred in a salinity of 26 as opposed to 8.7. In a sense, the present study extends Davis's (1958) work to the next developmental stage, exploring if the salinity during gametogenesis and larval development influences salinity tolerance during settlement. The range of salinities for maximum settlement was the same for all larval groups (S-11 to S-30), implying that maternal effects do not affect salinity tolerances during settlement as much as they affect egg and larval development. These findings support the theory that the performance of older larvae in different salinities is less influenced from the environmental conditions during gametogenesis and spawning than younger larvae (Newkirk et al. 1977). The similar optimal salinity range between larval groups also suggests settlement preferences of the species as a whole falls within this range. Not surprisingly, most (if not all) research on salinity and *C. virginica* has found that

optimal salinity for all biological processes exists somewhere between 14 and 28 (Shumway 1996). Additionally, adult oysters have been shown to rapidly conform to salinities ranging from 10-30, and reach isosmotic conditions at the higher salinities much more quickly than in lower salinities (Anderson and Anderson 1975). Spat heights measured from both cohorts after the extended experiments further emphasizes that *C. virginica* functions best within this approximate salinity range (Figure 10).

Despite the consistent range of optimal setting salinities for all larval groups, the effect size analysis shows that the "home-field advantage" of settlement is most notable in the salinity extremes closest to their native salinity (Figure 7). For instance, larvae reared in the lowest salinity had the highest settlement in the lower treatment salinities (S-5 to S-9) relative to its native salinity than from all other larvae tested. Similarly, the High salinity larvae showed better performance in the highest salinity treatment (S-35). Previous studies that have explored the genotype-by-environment effects on larvae and adults have also indicated that survival and growth is optimized in the natal habitat over other environments (Newkirk et al., 1977; Newkirk 1978; Proestou et al. 2016). Recognizing that the current study was not designed to control for variations in broodstock lines and rearing conditions within each hatchery, differences in settlement between larval groups cannot be solely attributed to their rearing salinity. That being said, all selectively-bred oyster lines tested within these experiments (NEH, DEBY and hANA) have been shown to have high growth rates and low mortality within the Mid- Atlantic and are more successful in foreign environments than other lines developed in New England (Proestou et al. 2016).

Further research should be conducted to more specifically control for broodstock lineage when testing salinity tolerance during settlement.

As mentioned earlier, the observed adaptability of larvae to settle across a wide range of salinities was also reflective of their ability to complete metamorphosis and begin growing as juveniles. The lack of statistically significant differences between the 4-Day and 14-Day groups indicates that the results from all the 4-Day trials can reasonably represent the proportion of larvae that can successfully metamorphose in the tested salinities. The only curious exception to that was seen in S-30 and S-35 with the Low salinity larvae, having noticeably lower settlement means in the 14-Day Group. However, one 14-Day replicate within S-25 contained a comparably high settlement rate (60%) as the only two replicates in the 4-Day Group (71% and 54%), indicating that just as high of a proportion of larvae are technically capable of successful metamorphosis as they are of settlement. Additionally, the reduced number of replicates within those particular treatments (as a result of bag failures) prevents us from assuming that increased mortality in the higher salinity treatments was a trend.

Shell heights measured after the 14-Day experiments further emphasized that spat within the salinity extremes were capable of continued juvenile development. Growth across all salinity treatments for both the Low and Medium salinity cohorts was high, but as expected, differences between salinity treatments were statistically significant (Tables 8 and 9). Reduced spat growth observed in lower salinities is consistent with other literature indicating the same phenomenon with larvae, young juveniles, and adults (Loosanoff 1953; Chanley 1958, Rybovich et al. 2016). The

incremental decreases in shell heights that occurred in treatments below S-9 and above S-30 could be a result of; 1) later settlement (Collet et al. 1999), 2) slower progression through settlement and metamorphosis (Prytherch 1934), 3) reduced feeding rates (Loosanoff 1953), 4) decreased ability to assimilate nutrients, 5) differences in spat density (Roegner 1991), or by 6) higher energy costs associated with maintaining osmotic balance (Shumway 1996). That being said, the average juvenile shell heights observed in this study, ranging from 2.17 ± 0.44mm to 4.38 ± 1.16mm (mean $\pm$ SD), were approximately equal to or substantially larger than shell heights reported in other post-settlement growth studies of *C. virginica* regardless of the salinity treatment (Osman et al. 1989; Osman and Abbe 1994; Roegner and Mann 1995; Brownlee et al. 2005). Even though the experimental design did not control for settlement time or spat density, it is clear that juveniles in all salinity treatments were successfully consuming the algae provided and were likely not food limited.

It is possible that the adaptive capabilities of pediveligers to different salinities may depend on their level of competency at the time of introduction to the settlement apparatus. As previously noted, Prytherch (1934) found that competent larvae could settle in salinities as low as 5.6, while Davis (1958) found that larvae could only survive to metamorphosis if in salinities in 10 or higher. Castagna and Chanley (1973) hypothesized that the discrepancy between these two studies was due to possible differences in salinities during gametogenesis, differences in genetics or "physiologic races," or by differences in particulates from various methods of creating different salt concentrations. It seems, however, that publications have incorrectly described the Davis (1958) study by suggesting that the larvae used were

reared "almost to setting stage" before exposure to different salinities (Loosanoff and Davis 1963). In reality, Davis (1958) used larvae requiring an additional 8-11 days of growth within the different salinity treatments before they reached competency. Recognizing that older larvae can tolerate a wider range of salinities than younger larvae (Davis 1958; Davis and Calabrese 1964, Loosanoff 1965; Davis and Ansell 1962; Lough 1975), it seems possible that the differences between the studies by Prytherch's (1934) and the Davis (1958) could be the result of testing salinity tolerances at different points in larval development. Davis and Ansell (1962) addressed the issue of larval competency when testing the effect of salinity on growth and metamorphosis of the European flat oyster, *Ostrea edulis*. They concluded that if larvae were reared in normal conditions until just before settlement, the larvae which are truly ready for metamorphosis will do so and succeed in lower salinities than those in which the larvae could survive if they needed to develop further. They also noted that larvae within the low salinity treatments that failed to settle right away would eventually die. Conversely, the larvae that did not immediately settle within their preferred salinity range could continue to develop and eventually settle.

The question of larval competency and salinity tolerance was partially investigated by the preliminary experiment examining the settlement rates of two size classes from one cohort across all salinity treatments (Figure 1). Although the size of competent larvae of the same oyster species can vary from one hatchery to another (Figure 4) (Nosho and Chew 1991), and can vary by season or by brood within one hatchery (Figure 3) (Vlahovich 2009), larger larvae are generally regarded to be more competent than smaller larvae (Nosho and Chew 1991; Congrove 2008). Thus, even

though larvae from both size classes had eye-spots and actively searching feet, it was predicted that the larger sized larvae were more competent and would set in greater proportions. Interestingly, settlement rates in salinity treatments S-5 through S-9 were surprisingly similar between the two groups of larvae (Figure 1). In contrast, settlement differences between the two groups emerged in the optimal salinities of S-10 through S-30, where the larger larvae set in significantly higher proportions than the smaller larvae. Reasons for the divergence in settlement rates in only the optimal salinities were not investigated, but it is possible that differences in their abilities to respond to settlement cues could be a contributing factor. When testing the settlement response of eastern oysters to ammonia, Fitt and Coon (1992) found that newly competent larvae showed a weaker response to the stimulant than older larvae. If the larger larvae within this experiment were more capable of sensing and/or responding to the chemical cues that induce a gregarious settlement response, then either the chemical cues within the sub-optimal salinities weren't at high enough concentrations to elicit the gregarious response, or the ability to respond to chemical cues is reduced in sub-optimal salinities. Admittedly, further research is required to substantiate these possible explanations.

Although this study was not conducted in the exact manner as previous settlement studies, it appears that *C. virginica* has a wider range of optimal salinities for settlement than other commonly cultured oyster species. Lund (1972) found that the optimal salinity range for settlement of *C. gigas* pediveliger larvae was from 22-34, which was later corroborated by Henderson (1983) who found peak settlement occurring at a salinity of 30 and optimal range between 20 and 35. Davis and Ansell

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(1962) tested how eyed larvae of *O. edulis* reared in salinity of 26-27 performed in lower salinities and found the lower salinity limit for good growth and setting is about 22.5. Devakie and Ali (2000) tested *C. iredalei* settlement by methods similar to Henderson (1983) in salinities from 5 to 30 and reported highest settlement at 20 and proposed that the general optimum salinity range was between 14-24. Quite a few more experiments have involved the salinity tolerances of other oyster species during settlement, but like the Davis (1958) study, were designed to test larval growth and survivorship until metamorphosis rather than the effects of salinity change at the time of settlement. Langdon and Robinson (1996) reared D-stage *C. ariakensis* larvae at a salinity of 20, then transferred them to salinities 15, 20, 25, 30 and 35. They found that *C. ariakensis* larvae can grow and settle at salinities as low as 10, but grew and set best in 15 and 20, and no settlement occurred at 35. O'Connor et al. (2015) studied the effects of salinity on catecholamine–induced metamorphosis of *O. angasi* and found that settlement in salinities 30 and 35 was between 2 and 15 times higher than in salinities 25 or 20. Recognizing that investigators test different salinity treatments and employ different statistical methods to determine an "optimum" salinity range, one cannot definitely claim that *C. virginica* is the most tolerant to salinity changes. Nonetheless, it is clear that the eastern oyster is an excellent example of a euryhaline species, capable of living within tidal estuaries that receive variable freshwater inputs and is therefore tolerant of a wide range of salinities.

The extremely low carbonate availability within filtered ambient Choptank River water and the lower salinity treatments (as determined by the calculated aragonite saturation coefficient (Ω_{ar})), highlights our lack of understanding of the

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chemical interactions within low salinity estuaries and which chemical variables can impact the biological processes of the species. Recently, much attention has been focused on the influence of increasing pCO_2 and decreasing pH on the larval production of shellfish as a result of highly publicized hatchery failures of *C. gigas* in the Pacific Northwest (Feely et al. 2012; Waldbusser et al. 2016). Using measured pH, alkalinity, and temperature values, a calculated Ω_{ar} <1 indicates that the water is undersaturated in terms of carbonate ions, which can lead to shell malformations and subsequent mortality during early oyster larval development (Waldbusser et al. 2015). In contrast, research conducted with *C. gigas* and *M. mercenaria* larvae has indicated that Ω_{ar} >1 can lower the energetic cost of shell building, thus increasing the scope for growth (Waldbusser et al. 2016; Miller and Waldbusser 2016). During the present study, the calculated Ω_{ar} of the ambient river water used for the experiments was regularly below Ω_{ar} =0.25, which is consistent with summertime Ω_{ar} values measured at this facility (J. Alexander, UMCES, pers. comm.). The addition of DI water generally made the river water further undersaturated in terms of carbonate ions, while the addition of Crystal Sea Marinemix® greatly increased the saturation state (Figure 12). It is unknown whether the low Ω_{ar} values in the low salinity treatments were maintained through the duration of the experiment or if they negatively impacted settlement. Equally, no evidence exists to suggest that supersaturated conditions could negatively affect the biological processes of oysters. Research has indicated that *C. virginica* larvae appear to be substantially less sensitive to high CO_2 (and lower Ω_{ar}) than other US east coast bivalves such as hard clams (*M. mercenaria*) and bay scallops (*A. irradians*). However, the few experiments that have tested

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metamorphosis and juvenile growth of *C. virginica* have been conducted in treatments with substantially higher salinities and Ω_{ar} values than the present study (Talmage and Gobler 2009, 2010; Dickenson et al. 2012; Gobler and Talmage 2014; Waldbusser 2016). It is nonetheless apparent that the species can adapt to low Ω_{ar} values to some extent, as billions of *C. virginica* larvae have been successfully grown and set in the HPL facility where these conditions normally exist .

It is also important to note that the accuracy of Ω_{ar} calculations is reduced in low salinity waters. A possible source of error are pH measurements, as the technology to accurately calibrate pH measurements in salinities less than 20 has not yet emerged. The use of alkalinity measurements to estimate the total amount of carbonate may also be misleading, particularly if a high amount of organic acids and bases are present. Additionally, the program co2sys was developed for higher-salinity waters and the chosen K1 and K2 constants may inaccurately characterize the true carbonate availability within brackish waters (George Waldbusser, OSU, and A. Dickson, Scripps UCSD, pers. comm.). Even if the calculated Ω_{ar} values of the low-salinity treatments do not accurately reflect the true calcium carbonate availability, it is clear that additions of both DI water and salt mix substantially altered the alkalinity of the river water.

Alkalinity values measured from three sets of water samples from the experiment, as well as the alkalinities of salinity solutions made only with DI water and the salt mix, are displayed in Figure 13. Superimposed on the figure is the range of naturally occurring alkalinity and associated salinity values measured within the Chesapeake Bay (adapted from Waldbusser et al. 2013) and confirms that the

measured alkalinities of the filtered ambient Choptank River (approximate salinity of 10) were within the expected range. The addition of DI and the salt mix, however, substantially altered the natural salinity-alkalinity relationship found within the Bay. Alkalinity values from solutions made with only DI and the salt mix confirm that the DI water has reduced buffering capacity than natural river water. Accordingly, it makes sense that the added salt solution created such high alkalinity values, as the salts were developed for use in aquaria to counteract the low alkalinity of potable water. While further research is needed to determine the interactive effects of salinity, alkalinity, and aragonite saturation states on larval settlement and metamorphosis, these results serve as an important reminder that salinity is not independent of the many water quality variables that can influence the oyster throughout its life cycle.
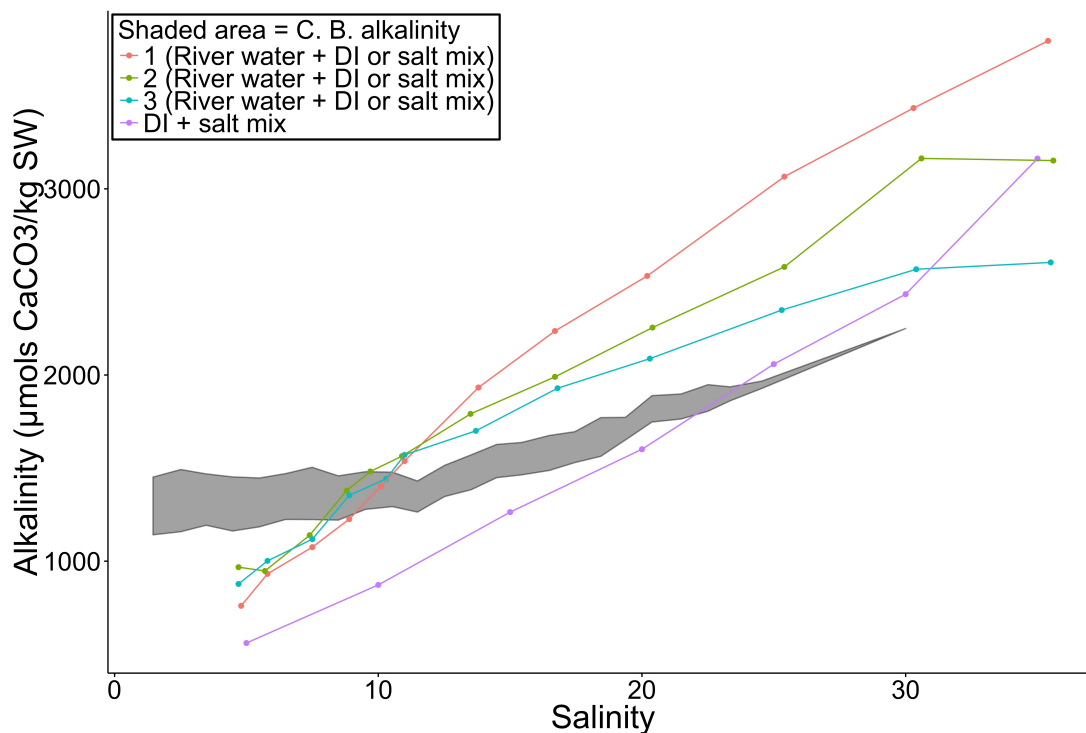
Figure 13. The effect of DI and salt mix on the relationship between salinity and alkalinity. Red, green, and blue lines indicate the alkalinity of twelve salinity treatments at the start of the experiment. For comparison, the purple line indicates the alkalinities of salinity solutions created only with DI water and salt mix. The shaded region represents field measurements of alkalinity vs. salinity within the Chesapeake Bay (adapted from Waldbusser et al. 2013)

Settlement variability

The uniquely large number of replicates in the present study enabled the detection of differences in settlement rates as a response to a range of salinities. However, the substantial variability in settlement that occurred within the experiment deserves further consideration. As noted by many researchers, high settlement variability between repeated experiments seems ubiquitous, even within carefully controlled conditions (Lund 1971; Davis and Ansell 1962; Laing 1995; Baker 1997; Brownlee et al 2005; Barnes 2008; Congrove 2008; Congrove et al. 2009; Steppe 2016). Additionally, individual oysters display a wide range of variability in response to salinity changes (Anderson and Anderson 1975). Variability within the present study existed in two forms: 1) variability in overall settlement rates between larval cohorts originating from the same hatchery, and 2) variability among replicates and/or between similar salinity treatments within the same run.

The opportunity to test seven different cohorts reared at HPL over the course of one production season provided clear evidence that the range of overall settlement performance can vary widely. (Figures 3 and 6). Despite the adherence to regimented protocols among hatchery staff for conditioning, spawning, feeding, water changes, and grading, the overall settlement means from each cohort ranged from 7.05% to 58.37%. Substantial differences in settlement rates were even seen between runs performed just 10 days after one another, from 50% to 13%, using larvae from broodstock obtained in the same location, refrigerated the same amount of time, and nearly identical in size (as determined from coulter counter spherical equivalent measurements). Particularly for this hatchery, there were no discernable correlations

between the size of larvae and settlement rates (Figure 4). However, there appeared to be a general trend of decreased settlement by cohorts raised later in the larval season (Figure 3). Although decreased success of egg fertilization, larval development, and settlement in later summer months is frequently described by hatchery operators (M. Congrove, S. Bennett, D Meritt and others, pers. comm.), the variability observed in this study cannot be explained by seasonal differences alone.

Any number of variables can influence larval growth and settlement performance, including minor differences in water quality, genetics, gamete quality, and algae (Helm et al., 1991; Berntsson et al. 1997; Jonsson et al. 1999). Variability in performance and survivorship within environments has also been correlated to genetic variability, where heterozygous individuals often show increased ability to tolerate stressors (Koehn and Shumway 1982; Rodhouse and Gaffney 1984; Galleger et al 1986; Borsa et al. 1992; Lourenço et al. 1997). Additionally, the nutritional value of algae strains can vary with differences in medium formulations, light, pH, temperature, and growth phase of algae at harvest (Webb and Chu 1983; Volkman et al. 1989; Wikfors et al. 1984) that can affect larval growth rates (Enright et al. 1986). As Berntesson et al. (1997) mentioned, the difficulty in maintaining a constant fatty acid composition of the diet will confound the interpretation of experimental factors in studies of condition and larval quality.

The variability in settlement observed between replicates within the same run was also striking. The example presented in Figure 6 illustrates the dramatic differences in settlement that occurred amongst replicates of each treatment. Generally speaking across all cohorts, as settlement averages increased, so did the

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variability. This variability could be caused by a variety of reasons, which unfortunately cannot be determined at this time. However, the repeated demonstration of gregarious settlement on the tiles could potentially be part of the explanation (Figure 5). We know that a substantial amount of genetic variation exists within a cohort of larvae (Taris 2009; Plough and Hedgecock 2012) and competency to settle is not uniform within a cohort, even for larvae of similar size (Vlahovich 2009). Additionally, it is well documented that osterid larvae respond positively to the water-soluble cues from settled conspecifics, evoking an increased settlement response (Cole and Knight-Jones 1949; Knight-Jones 1953; Crisp and Meadows 1962, 1963; Crisp 1967; Hidu 1969; Hidu 1971). As Hidu (1969) observed, the initial setting of *C. virginica* larvae appears to be "spontaneous," and as spontaneous setting proceeds, the presence of new spat on shells stimulates more larvae to set than would have otherwise. It is possible that minute differences in genetics and competency of the larvae affected the number of larvae that initially settled, which in turn could affect the number of larvae that set overall. This could explain why settlement within one bag could be an order of magnitude greater than another, even between replicates within the same salinity. Researchers have identified many of the chemicals responsible for eliciting a gregarious response (reviewed in Hadfield 2001), but the number of settlers needed to elicit a gregarious response within these experiments is unknown. Clearly, there are still many unanswered questions surrounding the unpredictable nature of settlement.

Implications of Research

Oyster Aquaculture and Restoration

Following the depletion of many wild stocks of the eastern oyster, the hatchery culture of larvae for aquaculture and restoration purposes has been increasing. Results from this study have direct implications for the production of oyster seed and are particularly relevant to facilities that set oyster larvae at a different location than where they were reared. This practice, called "remote setting," was developed in the Pacific Northwest in the 1970s and is currently the industry standard for the production of *C. gigas* oysters (Jones and Jones 1988; Nosho and Chew 1991). The adoption of remote setting practices by industries producing *C. virginica* are more recent, but are increasingly being employed within the Mid-Atlantic and Gulf of Mexico (Supan 1991; Hudson and Murray 2016; Stokes et al. 2014). Just as larvae was obtained for the current study, hatchery operators ship bundles of pediveliger larvae to an oyster farm or setting facility to be used for the production of single seed oysters (often set on shell fragments) or "spat-on-shell" (set on whole oyster shells). Remote setting offers the advantage of reduced shipping and transportation costs associated with the deployment of seed and spat-on-shell to locations far from an existing hatchery. Therefore remote setting also offers the opportunity to source larvae from hatcheries which experience water conditions that may be considerably different than the setting facility.

The current study addressed the question of how oyster larvae respond to a salinity change that they might experience during remote setting, and demonstrated that the four Mid-Atlantic oyster hatcheries participating in this study are producing

healthy larvae, capable of withstanding dramatic salinity changes without the need for stepwise acclimation. Although results indicate that hatchery-reared pediveliger larvae are more robust with regards to salinity changes than previously thought, a few rules of thumb emerged. First, the optimal salinity range for *C. virginica* settlement is approximately between 11 to 30, regardless of hatchery conditions or broodstock. Second, for salinities outside of their optimal range, larvae reared closest to the setting salinity perform better. Third, although larvae generally set best in salinities close their rearing conditions, larvae reared in the lowest salinities are tolerant to (and may prefer) salinities substantially higher than their own.

Aside from salinity tolerances, this study revealed a few other lessons pertinent to a setting operator. First, settlement performance between larval cohorts can vary substantially, even from the same hatchery. Second, larval size is not strictly indicative of the cohort's competency, but larger larvae show an increased capacity to set than smaller larvae of the same age. Finally, the observed variability among cohort performances (Figure 3) and among settlement responses among replicates (Figure 6) serves as a reminder that firm conclusions cannot be made from just one experience. Despite carefully controlling the experimental conditions within this study, it is evident that a myriad of variables besides salinity can influence settlement.

As a reminder, the conditions maintained in these experiments were not designed to achieve the highest amount of settlement possible, but rather, to provide the most consistent platform to measure the effect of salinity on settlement. Hence the settlement rates achieved in this study are relative across salinity treatments but are not absolute. Different results could be expected if changes were made to the

settlement chamber, water temperature, algae, or settlement substrate. The use of a ceramic tile (as opposed to oyster shell) in these experiments was to guarantee a consistent surface area, uniform composition, and to ensure there were no holes or crevices in which larvae could settle but not be seen under a microscope. However, as noted by Nosh and Chew (1991), setting and survival rates are probably not as high on ceramic tiles compared to oyster culch. It is also possible that the use of a more preferred substrate with a settlement-enhancing biofilm could increase the speed and number of settlers (Kirchman et al. 1982; Maki and Mitchell 1985; Weiner et al. 1985; Bonar et al. 1990; Fitt et al. 1990; Pawlik 1992; Tamburri et al. 2008; Hart 2009). However, the bacteria and other organisms that compose a biofilm would have likely changed over the course of the experimental period, which in turn would have added confounding variables between runs (Anderson 1996).

Although improvements in the setting process can lead to increased seed production from each cohort, it is also important to remember that settlement is just one phase of the multi-step process to produce oysters for profits and ecological benefits. For instance, setting operators might choose to accept slightly reduced settlement efficiencies if they have strategically located their operation in salinities below their settlement optima to avoid increased predation and disease that occurs in high salinities (Stokes et al. 2014). In summary, the degree to which a setting operator should manipulate their conditions to increase settlement success will be unique for each operation and need to be repeatedly tested at each location. Additionally, salinity is just one of the many variables that can be manipulated in order to affect settlement.

Natural Recruitment

Lessons from this study are also applicable to natural recruitment, as the results have altered our understanding of the physiological limitations of *C. virginica* larvae with regards to low salinities. Rumrill (1990) hypothesized that the four most important factors that limit natural recruitment success are low fertilization success from broadcast spawning, adverse hydrographic conditions, lack of adequate substrate, and predation. Researchers that have identified salinity as the most important environmental factor governing successful spatfall have not determined the exact mechanisms for the correlation, but note that larvae and spat are the most sensitive life stages to salinity extremes (Ulanowicz 1980; Kimmell and Newell 2007). This study isolated only a portion of these life stages, and by doing so, it was observed that competent larvae *can* settle, metamorphose, and grow in salinities as low as 5 (given the presence of competent larvae and adequate substrate). This fact confirms why oyster reefs can and do exist in low salinities, and supports the assumptions that salinity bears greater influence on earlier larval stages (Davis 1958, Davis and Calabrese 1964, Loosanoff 1965). Seeing that oyster predation and disease is generally reduced in lower salinities (Menzel et al. 1966; Cake 1983; Newell 1985; Paynter and Burreson 1991; La Peyre et al. 2003, 2010; Soniat 1996; Burreson and Ragone-Calvo 1996; Bushek et al. 2012), other variables in conjunction with salinity may also be at play.

Reasons why recruitment is reduced in low salinities may partially be explained by hydrodynamics. With recent advances in hydrographic instrumentation and computing ability, researchers have developed larval transport models that

predict the possible journey of a developing larva within an estuary. As a generalization, larval transport models of the Delaware Bay and tributaries of the Chesapeake Bay indicate that the majority of larvae will wind up further downriver (and in higher salinities) than from where they were spawned (North et al. 2008; North et al. 2010; Narvaez et al. 2012). Additionally, increases in precipitation likely increase the transport distance (Narvaez et al. 2012). Hence, precipitation is responsible for both creating the low salinity conditions and preventing the larvae from being present in lower salinities. Therefore the assimilation of information learned from the current research with what is known from hydrographic models leads to a logical hypothesis: reduced recruitment in low salinity regions may be more a function of larval availability than a function of the physiological abilities of larvae to settle there. Admittedly, salinity also exerts indirect control over the distribution of oyster populations by affecting the availability of food and presence of predators and diseases.

It is interesting to note that larvae from the Low salinity cohorts were both more tolerant of low salinity waters and were also tolerant to (and may even prefer) substantially higher salinities than their natal salinities. These results may be indicative of faster acclimation to higher salinities than lower salinities as observed in adult oysters (Anderson and Anderson 1975). However, the higher degree of plasticity exhibited by the Low salinity larvae is inconsistent with a few other studies that suggest oysters from lower salinity regions are less capable of adapting to salinity changes. Pierce et al. (1992) found that adult oysters taken from lower salinities of the Chesapeake Bay were unable to survive a direct transfer to a salinity of 30, which he

attributed to differences on osmolyte production and concentrations compared to the other Atlantic populations. Eirman and Hare (2013) also suggested that larvae from a high salinity oyster population showed higher plasticity across salinity treatments than larvae from parents originating from lower salinity populations. However, their conclusions should be viewed with caution. Replication was low within the Eirman and Hare (2013) study because many of the parental crosses failed to produce viable eggs, and none of their larval cultures could survive long enough for them to reach competency. In contrast, findings from the highly repeated experiments in this study support the assumption that larvae from low salinity regions are fully capable of adapting to and successfully completing settlement and metamorphosis in the higher salinities that they may encounter downriver.

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