ABSTRACT

Title of Document: RELATIONSHIPS BETWEEN LARVAL
                                      MORPHOMETRICS AND SETTING
                                      EFFICIENCY IN THE EASTERN OYSTER,
                                      CRASSOSTREA VIRGINICA

                                      Emily Ann Vlahovich, Master of Science, 2009

Directed By: Dr. Donald W. Meritt, Senior Agent, University
                                      of Maryland Center for Environmental Science

In oyster hatcheries, the decision to move larvae from rearing tanks to setting tanks is

based on physical and behavioral characteristics. These criteria can suggest

conflicting action and a more reliable method may result in higher spat production. I

observed hatchery reared *Crassostrea virginica* larvae, beginning with larvae retained

on a 200 µm sieve. Aliquots of larvae were measured or placed in a setting vessel,

and the remaining were returned to the culture cone daily. Each day had an

associated setting efficiency, loss, and set of larval morphometrics, including shell

height and length and eyespot diameter. Day was most strongly correlated with

setting efficiency. Eyespot diameter was moderately correlated with setting

efficiency, and shell morphometrics were weakly correlated with setting efficiency. I

estimated daily spat production, which peaked on day 2. These results suggest spat

production may be increased by altering current hatchery methods to consider eyespot

diameter or days past retention on a 200 µm sieve when deciding to place larvae in

setting tanks.

RELATIONSHIPS BETWEEN LARVAL MORPHOMETRICS AND SETTING
EFFICIENCY IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

By

Emily Ann Vlahovich

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
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Advisory Committee:
Dr. Donald W. Meritt, Chair
Dr. David G. Kimmel
Dr. Roger I. E. Newell

Dedication

I dedicate this work to my dear friends Rocky Taco and Lotus Bean, who were with me when I started this journey, but are not here to witness the completion; also to my sister, Laura Rose Vlahovich, who persists in the face of incredible challenges, and has kept me laughing the entire time.

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I am fortunate to have been surrounded by wonderful people throughout this process. I offer many thanks to my husband, Patrick, who has supported me in countless ways, and has made this process a little less daunting. He has sacrificed much of his free time to pick up my slack and has endured more than his fair share of stress-induced crabbiness. I will never fully be able to express my extreme gratitude to my fellow student, Melissa Grant, who has been with me every step of the way, offering assistance, advice, and friendship. I cannot imagine being stuck with a more enjoyable person.

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Lastly, thanks to my family, including Sucia. I have missed far too many gatherings and celebrations due to my school obligations, and I look forward to mud pits and stump dances. Special thanks to my mother, who taught me the joys and wonders of the animal kingdom at a very young age; and to my father for our amazing Alaskan summers. I am so fortunate to have been exposed to the natural world, and hold these experiences very near to my heart.

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Eastern Oyster Distribution

Crassostrea virginica (Gmelin 1791), the eastern oyster, is distributed from the Gulf of St. Lawrence to the Gulf of Mexico, the Caribbean, and the Brazilian and Argentinean coasts. It is primarily an estuarine organism generally inhabiting areas with salinity levels of 5 to 30, however can be found in full strength seawater (Galtsoff, 1964). In the Chesapeake Bay, they are exposed to seasonal fluctuations in salinity of about 10 (Andrews, 1991) and throughout their distribution, they are exposed to temperatures ranging from approximately 0°C to 36°C (Kennedy and Breisch, 1981). *Crassostrea virginica* forms reefs on hard and semi-hard surfaces (Galtsoff, 1964) and occurs in the intertidal and subtidal (Carriker and Gaffney, 1996). Adults can grow up to 36 cm in shell height (Galtsoff, 1964) and the shape of the shell can vary greatly due to growing conditions such as type of substrate, density of oysters, and salinity (Andrews, 1991).

Larval Life History

Crassostrea virginica are broadcast spawners (Galtsoff, 1964). Eggs and sperm are released into the water column where fertilization occurs. Five to nine hours after fertilization, the trochophore larva develops. The rate of development is affected by environmental conditions such as salinity, temperature, and oxygen levels, and the condition of the egg (Galtsoff, 1964). The non-feeding trochophore is

able to swim and this stage lasts for one to two days in the laboratory (Galtsoff, 1964; Kennedy, 1996). The next stage, the veliger, is marked by the presence of the ciliated velum that allows for stronger swimming (Stafford, 1913) and aids in feeding (Galtsoff, 1964). The veliger grows for approximately two weeks (Prytherch, 1924) before the next stage, the pediveliger, develops. By this time, the larval shell may have grown approximately five times its original shell length[1] (Stafford, 1913; Figure 1) and Galtsoff (1964) observed *C. virginica* larvae with heights[2] (Figure 1) over 300 µm during the pediveliger stage. This stage is distinct due to the presence of a foot, which is a strong, well-developed organ used to crawl on a substrate (Stafford, 1913). The foot extends from between the two valves during swimming or crawling, or can be retracted into the shell (Galtsoff, 1964; Kennedy, 1996). When swimming, the foot points in different directions, and may play a role in 'steering' the larva (Galtsoff, 1964).

Another characteristic of pediveligers are eyespots. Each larva has two eyespots, which are pigmented spots that are visible on either side of the larva (Thompson et al., 1996). The function of eyespots is not fully understood. Nelson (1926) described them as photosensitive (reviewed by Thompson et al., 1996) however; Prytherch (1934) found no evidence of photosensitivity. The presence of eyespots and foot activity is an indication that the larvae have become competent to metamorphose (Prytherch, 1934; Galtsoff, 1964; Dupuy et al., 1977; Jones and Jones, 1988).

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[1] Length is defined as 'maximal anterior-posterior dimension of the shell parallel to the hinge line; (Carriker, 1996).

[2] Height is defined as 'maximal dorsoventral dimension perpendicular to the hinge' (Carriker, 1996).
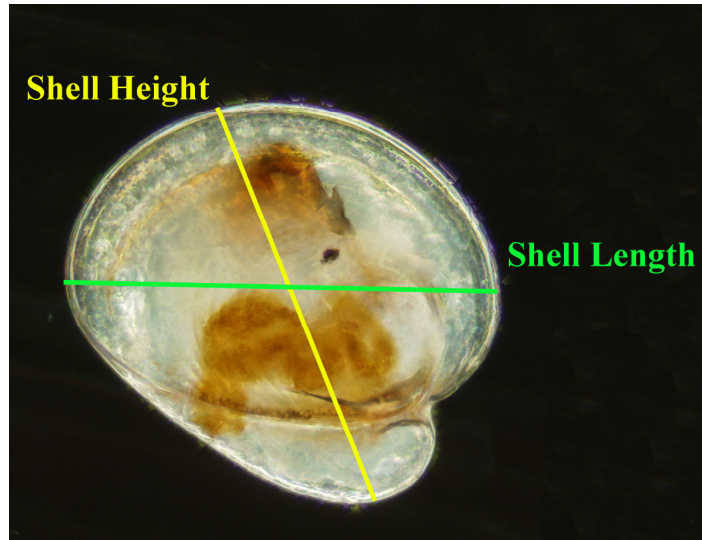
Figure 1. *Crassostrea virginica*. **This is a micrograph of an eyed larva, right valve up. The left valve umbo is visible. Shell height and length are labeled.**

Metamorphosis is the process whereby the oyster larva permanently attaches itself to a hard substrate, exchanging its previous pelagic existence for a benthic life (Galtsoff, 1964). Prior to metamorphosis, settlement occurs, which Burke (1983) defines as a behavior that an individual may repeat (Scheltema, 1974). This behavior begins with the larva swimming in the water column, in a spiral pattern, with its foot outstretched and waving, searching for a solid substrate. When the substrate is located, the larva crawls on the surface with its foot extended, exploring the suitability of the substrate (Nelson, 1924; Prytherch, 1934; Galtsoff, 1964). If it is not sufficiently attractive, the larva resumes swimming in search of a more appropriate substrate (Galtsoff, 1964; Kennedy, 1996). When the larva finds an attractive substrate on which to attach, it secretes cement from the byssus gland in its foot and permanently cements its left valve to the substrate (Stafford, 1913; Nelson, 1924; Prytherch, 1934; Galtsoff, 1964). Immediately after cementation (often referred to as "setting"), the larva is termed a "spat", and the process of metamorphosis begins

(Galtsoff, 1964). During metamorphosis, the larva undergoes permanent morphological changes such as loss of the velum, resorption of the foot, and the development of gills (Galtsoff, 1964; Baker and Mann, 1994; Kennedy, 1996).

When larvae are in the presence of known inducers, and they do not respond with settlement or metamorphosis, the larvae are considered pre-competent (Coon et al., 1990). The capacity of larvae to metamorphose following exposure to known inducers is termed competency (Coon et al., 1990). Competency in oyster larvae has two components, known as behavioral and morphogenetic competency. Behavioral competency refers to the ability of larvae to respond to stimuli with characteristic behavior, such as extension of the foot beyond the ventral margin of the shell during swimming or crawling, and can be tested in the laboratory by exposure to L-3,4-dihydroxyphenylalanine (L-DOPA) (Coon et al., 1990). Morphogenetic competency is characterized by the ability to metamorphose after exposure to endogenous stimuli, which can be tested in the laboratory by exposure to epinephrine (Coon et al., 1990; Fitt et al., 1990). In *C. gigas* larvae, behavioral competency is evident at approximately the same time that the larvae begin to develop eyespots, however behavioral competency does not depend on the presence of fully developed eyespots (Coon et al., 1990). Behavioral competency can be reached 2-4 days prior to attaining morphogenetic competency in *C. gigas* larvae (Coon et al., 1990).

History of Oyster Culture

There is evidence of oyster culture since ancient times (Stafford, 1913; Heral and Deslous-Paoli, 1991). Techniques practiced by ancient Romans are known from

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paintings on pottery, and those techniques are still used in the same geographical area today (Günther, 1897). Early techniques involved collecting oysters and placing them in accessible areas for ease of harvest. Romans also used spat collectors in these areas (Heral and Deslous-Paoli, 1991).

Oyster culturing techniques have changed from a rather passive approach in ancient times to a highly intensive approach in the last half-century (Loosanoff, 1971). Before this change occurred, researchers were required to expand their understanding of the life cycle of oysters, the survival requirements of larvae, and how to support their growth in the laboratory. These advances took decades to complete (Loosanoff, 1954, 1971).

In 1882, F. Winslow collaborated with W. K. Brooks and J. A. Ryder to raise large numbers of *C. virginica* larvae to spat and to establish conditions for survival and growth (Winslow, 1884). These were ambitious goals, as no researcher had successfully reared any species of oyster larvae to spat in the laboratory. Handling and raising larvae in the laboratory was difficult. Sieves to retain small larvae had not yet been developed, so the researchers were unable to perform water changes on larval cultures. They attributed earlier experimental failures to the lack of water changes. In addition, they suspected the larvae required food, but the diet of larvae was unknown (Winslow, 1884). The researchers were not successful in raising any larvae to spat during this project; however, they did develop basic techniques that aided in the progress of oyster culture.

As was standard protocol at the time, Winslow and his colleagues strip-spawned adult oysters to obtain gametes. This process involved cutting the gonads

and collecting the gametes in the water. The time taken to remove gametes, place them in water, and the quality of gametes proved to be of great importance when raising larvae (Winslow, 1884). Winslow developed a protocol for removing gametes from adults, fertilizing eggs, and transferring eggs between containers, while leaving unfertilized eggs and excess spermatozoa behind (Winslow, 1884). This assisted in starting larval cultures as cleanly as possible which was significant since water changes were not performed. Winslow found that larval concentration is critical in keeping cultures healthy, and cautioned against overcrowding eggs or larvae (Winslow, 1884).

Brooks and Winslow attempted to feed their laboratory raised oyster larvae by collecting water and mud near an established oyster reef and adding small amounts to the cultures. They observed larvae digesting some material, but the larvae did not survive to metamorphosis. They attributed this failure to lack of sufficient food (Winslow, 1884).

At the close of Winslow, Brooks, and Ryder's ambitious, but disappointing season, Winslow made a prediction. "…I am convinced that it will require a series of pains-taking experiments, extending over considerable time and conducted under many dissimilar conditions, before the artificial production and culture of the oyster is made a matter of practical importance"(Winslow, 1884).

Around this same time, Ryder experimented with pond culture in Maryland (Ryder, 1883). Pond culture involved releasing artificially fertilized eggs into a mostly enclosed area with spat collectors. Spat collectors provided a hard surface on which larvae may attach and metamorphose into spat. Pond culture was practiced in

other countries, but had not yet been attempted in the United States. Ryder found that larvae did attach to spat collectors and the spat grew in the pond at the same rate as spat in the nearby open bay (Ryder, 1883). This was the first reported success of raising spat from strip spawned eastern oysters.

Nearly 40 years after Winslow, Brooks, and Ryder set out to raise larvae to spat in the laboratory, W. F. Wells was successful (*The New York Times*, 1920). At the time, his accomplishment did not receive the recognition it deserved (Loosanoff, 1971). I could not obtain a copy of Wells' 1920 article, but later researchers described his findings (Loosanoff, 1971). Wells devised a successful method to change the larval culture water. Like Winslow (1884), he removed unused spermatozoa and unfertilized eggs. Additionally, every two days he used a milk separator to remove larvae from the water, which he would replace with fresh seawater (Loosanoff, 1971). At the same time, he divided the larvae into two different culture vessels, thus decreasing larval concentration. Wells did not supply the larvae with food outside of what was present in the culture water, so dividing the larvae into different vessels supplied more food per larva at each division. His success is attributed to this practice (Loosanoff, 1971).

In 1923, H. F. Prytherch continued the advancement of oyster culture by using temperature to stimulate the spawning of oysters, instead of strip spawning. Prytherch expressed strong feelings regarding strip spawning, calling it "unreliable, crude, wasteful, unnatural, and in most cases unnecessary, and undoubtedly accounts for many of the failures in the various attempts to artificially propagate oysters" (Prytherch, 1924). Prytherch induced spawning by placing adult *C. virginica* in a

tank warmed by sunlight. This method worked well, resulting in large numbers of

fertilized eggs and no sacrificed broodstock (Prytherch, 1924).

In 1936, H. A. Cole conducted a series of experiments with the European flat

oyster, *Ostrea edulis*. *Ostrea edulis* is a brooding species, and Cole conducted the

experiment with larvae already liberated from the female oysters. Cole hoped to

devise methods that would allow oyster culturists with no scientific background or

access to laboratory equipment feed their larval cultures. He ground fresh flesh of the

local brachyuran crab *Carcinus sp.* with fine sand and the mixture was diluted and

distributed throughout the culture tanks daily (Cole, 1937). This controlled organic

enrichment method was used successfully by others (Hughes, 1940). In a separate

study, Cole fed larvae a pure culture of flagellates, and found that these larvae

exhibited significantly more growth than unfed larvae (Cole, 1937). This was the

first reported incident where a pure algal culture was correlated with larval growth in

the laboratory.

While Cole was conducting the aforementioned studies, J.R. Bruce, M.

Knight, and M.W. Parke were also experimenting with *Ostrea edulis* larvae and pure

cultures of flagellates (Bruce et al., 1939). They observed different setting

efficiencies between larval cultures fed different flagellates, demonstrating that

flagellates have different food value to larvae (Bruce et al, 1939).

V. L. Loosanoff made a vital discovery in the advancement of oyster research

and culture in 1945. He found adult *C. virginica* can be induced to develop gametes

outside of the natural spawning season (Loosanoff, 1945). Loosanoff took oysters

from near freezing water and warmed them to room temperature within 24 hours,

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where they stayed for an additional 48-72 hours. They were then divided into three treatments and kept at 20°C, 25°C, or 30°C for 30 days. Most oysters in the 20°C treatment developed eggs or active spermatozoa. The oysters in the warmer treatments had well developed gonads, some equal in quantity to gonad developed under natural conditions (Loosanoff, 1945). The conclusions from this study, coupled with those of a later study (Loosanoff, 1954), gave culturists access to ripe oysters outside of the natural spawning season. Loosanoff discovered that summer spawning may be delayed by keeping the adults in colder temperatures which allows slower development of the gonad and reduces spawning (Loosanoff, 1954). These adults can then be spawned in the fall, while other broodstock are being conditioned in warmer water to be spawned in the winter. The ability to induce ripening and delay spawning is helpful because it greatly extends the research and culturing season. This method is widely used in hatcheries today (Utting and Spencer, 1991; Gibbons et al., 1992; Helm and Bourne, 2004; D. Meritt, Horn Point Laboratory, UMCES, personal communication).

Advances in oyster culturing techniques have continued over the years, but by the 1950s, the basic techniques existed. In general, researchers understood the larval stage and morphology, larval development, raising larvae in the laboratory to setting stage, feeding larvae, inducing gonadal development, and controlling the timing of spawning. With a foundation established, researchers were able to study other topics such as genetics and disease while culturists could focus on problems specific to their own geographical areas and hatcheries (Loosanoff, 1954; Hidu et al., 1969).

Oyster Hatcheries in Chesapeake Bay, Maryland

The vast majority of advancements discussed above occurred in the North Eastern United States. The concepts may be applied to other areas; however, the exact methods may not be successful throughout the range of *C. virginica*. Loosanoff and C. A. Nomejko (1951) collected adult oysters from Massachusetts, New Jersey, New York, Virginia, North Carolina, and Florida. Their study revealed oysters from northern states spawned during a Connecticut summer while the majority of the southern oysters did not spawn when held in Connecticut. They believe this indicated oysters from different geographical areas have different temperature requirements for spawning. This supported the idea of different physiological races among *C. virginica* populations (Loosanoff and Nomejko, 1951; Loosanoff, 1971).

In 1958, *Haplosporidian nelsoni*, a protozoan that causes MSX disease in oysters, was found in Delaware Bay (Haskin et al., 1966). A year later, the parasite was discovered in Chesapeake Bay, and already the Delaware oyster industry had been devastated by the mortality caused by MSX (Andrews and Wood, 1967). The concerned states of New Jersey, Delaware, Maryland, and Virginia joined efforts to attempt a restoration of the oyster industry (Hidu et al., 1969). However, based on Loosanoff and Nomejko's study (1951) and similar studies (reviewed by Stauber, 1950), it was first necessary to assess the feasibility of culturing oysters in southern areas (Hidu et al., 1969).

Hidu et al. (1969) conducted and reviewed experiments throughout the Chesapeake Bay area, in high and low salinities. They found Chesapeake Bay oysters do not act as northern oysters in several respects. Southern oysters can be

conditioned for spawning out of season, however they require more conditioning time than northern oysters. An increase in the temperature of running water stimulates southern oysters to spawn, especially with the addition of gonad from a sacrificed oyster, as opposed to standing water that has been successful with northern oysters. There had also been success with delaying spawning to save ripe oysters for colder seasons (Hidu et al., 1969). Although some techniques needed adjustments for southern oysters, the authors believed that oyster hatcheries in Maryland and Virginia could be successful (Hidu et al., 1969).

Horn Point Laboratory Oyster Hatchery

The University of Maryland established Horn Point Oyster Hatchery in the early 1970s in Cambridge, MD in an effort to overcome damage to shellfish stocks caused by Tropical Storm Agnes in 1972. Agnes deposited record setting amounts of rainfall over the entire Chesapeake Bay watershed, resulting in low salinity, huge sediment deposition and very high run off (Lynch, 2005). The already struggling oyster population was devastated by this event (Haven et al., 1976). The goal of Horn Point Oyster Hatchery was to produce low salinity tolerant spat to replenish the reefs destroyed by Agnes (D. Meritt, Horn Point Laboratory, UMCES, personal communication).

Over the years, the facility has grown in size and in production, although the goal to restore Maryland's oyster population remains. In 2009, the hatchery produced 4.1 billion eyed larvae and 750 million spat on shell (S. Alexander, Horn Point Laboratory, UMCES, personal communication). Until recently, despite large spat

production numbers, the hatchery has struggled with low and inconsistent setting

efficiencies. Setting efficiency, the percent of larvae that metamorphose in the setting

tanks, is the measure of how well larvae set. In 2004, Horn Point Laboratory opened

a new oyster hatchery facility, which allowed for greater control over larval and algal

culturing conditions. The great increase in setting efficiencies over the last several

seasons is attributed to the new facility, particularly the vast improvement in our

ability to consistently produce high quality algae. Although the general trend for

setting efficiencies has increased since 2005, we still observe inconsistent setting

efficiencies, as demonstrated by the large standard deviations shown in figure 2.
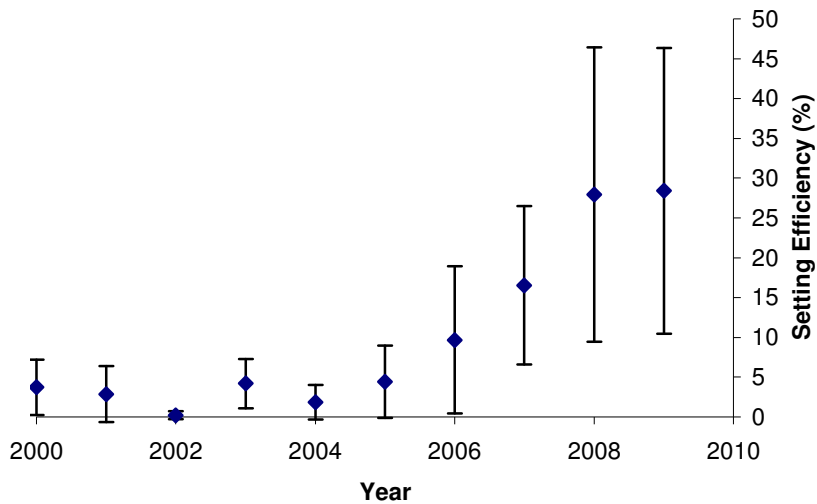
Figure 2. *Crassostrea virginica*. Mean setting efficiency ($\pm$ SD) observed
at Horn Point Laboratory oyster hatchery. Note that rearing and setting
efficiency estimate methods have changed over time.

Chapter 2: The relationships between larval morphometrics and setting efficiency in hatchery reared eastern oyster larvae

Introduction

At the Horn Point Laboratory oyster hatchery, larvae are monitored more intensely when individuals in the brood reach shell lengths of approximately 180 µm. When this occurs, a dead acid-washed *C. virginica* adult shell attached to a string, known as the test shell, is placed in the larval tank. The test shell is acid-washed after each use to remove spat that have set on the shell. This allows an accurate assessment of newly set spat when that test shell is reused. When larvae in the brood begin developing eyespots, the test shell is inspected with the naked eye for spat. Additionally, larvae are collected on 200 and 212 µm sieves, by running the sieves through the larval culture water. Larvae retained from each sieve are examined under a compound microscope at 40x magnification for an estimate of percentage of eyed larvae and for the presence of searching behavior (larvae crawling with the foot extended). The decision to return the larvae to the rearing tanks or to introduce them into the setting tanks is based on the observations from the test shell and the larvae from the sieves; however, these criteria do not always point in one direction. For instance, larvae from the sieves may show little or no searching behavior, but the test shell may have many spat on it. This may imply a relatively small percentage of larvae were competent to metamorphose, and they did so on the test shell. Additionally, it may indicate that the majority of the larvae may be competent to set

soon, however it is unknown if that will occur in hours or days. Alternatively, it may suggest many larvae metamorphosed in the culture tank, and the larvae still swimming represent those not ready to metamorphose. This scenario underscores the subjectivity in this decision making process and the need for more reliable quantitative methods to assess when larvae have reached the developmental stage where they are ready to metamorphose.

The timing of the removal of larvae from the rearing tanks is critical. If the larvae are left in a rearing tank too long, they will set on the sides of the tank. Spat attached to a tank are useless to the hatchery because they must be scraped off, resulting in death. Removing larvae from the larval tank prior to competency and placing them in a setting tank may result in too few spat. It is unknown if larvae undergo additional development in the setting tanks, so larvae that are not quite ready to metamorphose may not mature enough to reach competency and successfully attach. The setting tanks are less hospitable than larval tanks because they are filled with unfiltered (raw) river water. The setting tanks are static; there is no water flow until the ambient water is turned on at least three days after the larvae are first introduced. Cultured algae are not added to the setting tanks, so the only food available to the larvae are food which comes in with the raw water. Zooplankton, such as copepods and rotifers, are also present in the water and may compete with oyster larvae for food. Additionally, the setting tanks are generally filled a day prior to the introduction of larvae. This gives zooplankton in the tank time to consume phytoplankton before the introduction of oyster larvae, further decreasing available phytoplankton that may be consumed by the larvae.

There are chemical and physical factors that stimulate settlement and metamorphosis in *C. virginica* larvae. Larvae are gregarious, and prefer to settle near spat and adult oysters (Crisp, 1967; Hidu 1969). Crisp (1967) demonstrated that larvae are attracted to organic compounds found on oyster shells, and to cultch that has been steeped in extracts of *C. virginica* bodies. Larval settlement is also enhanced by cultch with bacterial film (Young and Mitchell, 1973; Kingsley-Smith and Luckenbach, 2008; Tamburri et al., 2008). Nelson (1908) stated that shells serving as cultch should be placed in the water 2-3 days prior to the anticipated setting event, to allow time for a sufficient film to develop. Weiner et al. (1985) isolated a bacterium from holding tanks housing *C. virginica* spat, and found the bacterium increased settlement in larvae. Tamburri et al. (1992) cleared up any confusion between the exact sources of inducers that were increasing settlement. They found both bacterial biofilms and conspecific adults and juveniles to be sources of inducers.

A physical factor that affects larval preference for location of metamorphosis is the orientation of the cultch. In laboratory and field experiments, larvae generally prefer to settle on the underside of cultch (Crisp, 1967; Baker, 1997), however a preference for upper surfaces has been observed in field studies (Kennedy, 1980), but has been explained by high turbidity and low light penetration. Larvae have displayed negative phototaxis in the laboratory and preferentially settled on shaded surfaces (Ritchie and Menzel, 1969). Baker (1997) suggested *C. virginica* larvae chose lower surfaces for settlement through geotaxis, as the settling pattern holds in experiments conducted in the dark.

Morphological Characteristics

Age is generally not a meaningful way to describe larval development, as many factors may affect their growth rate (Davis and Calabrese, 1964). Loosanoff (1959) reported that clam larvae (*Venus mercenaria*) grown at higher temperatures metamorphose faster than those grown at lower temperatures and summarized that the same species of lamellibranch larvae metamorphose at the same size, regardless of the temperature at which they were cultured. Dupuy (1975) reported slower growth rates of *C. virginica* larvae when fed presumably less nutritious algae from older algal cultures. Similarly, Nascimento (1980) found differences in growth rate in *C. gigas* larvae when fed different diets. Dupuy (1975) also found a difference in growth rate between larvae grown in different sized culture containers. Larvae grown in smaller vessels had a mean size that was 73 µm less than those grown in larger vessels. Conversely, Walne and Spencer (1968; as reported by Helm and Spencer, 1972) observed the opposite pattern in relation to vessel size and larval growth. Helm and Spencer (1972) found significantly more eyed larvae in cultures grown with higher aeration rates versus lower aeration. However, they also found that larvae in lower aeration developed eyespots at a smaller shell size.

Instead of age, physical characteristics (most commonly shell length and eyespot presence) are used to describe larvae. *Crassostrea virginica* larvae are asymmetrical, with a larger, more convex left valve and umbo, which are increasingly noticeable as the larva grows (Strafford, 1912; Prytherch, 1934; Galtsoff, 1964). Hu et al. (1993) described *C. virginica* larvae as dorsal-ventrally flattened when compared to other *Crassostrea* species, and with a tapered anterior end. Nelson

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(1917; as reported by Carriker, 1951) described the maximum size of *C. virginica* larvae to range from 320-400 µm, and Chanley and Andrews (1971) reported the maximum to be 350 µm. Prytherch (1934) stated that *C. virginica* larvae are fully grown when the greatest diameter is 330 µm and the width is 220 µm. Loosanoff et al. (1966) reported lengths in excess of 300 µm, and Dupuy et al. (1977) considered *C. virginica* larvae to be fully developed at 290 µm. The Virginia Institute of Marine Science hatchery at Gloucester Point reported the mean length of eyed larvae (wild and hatchery reared) to range from 280-357 µm during the 2009 season (K. Hudson, VIMS, personal communication). Loosanoff et al. (1966) reported that there were no significant differences in maximum size in larvae from differing geographical areas. No significant difference was found in maximum size between laboratory cultured larvae and wild larvae (Carriker, 1996).

There are several published length and height measurements for larger *C. virginica* larvae (Table 1). Loosanoff et al. (1966) reported the growth of *C. virginica* larvae under laboratory conditions throughout its entire pelagic life. Salinity was not reported, but typical salinity at Milford Laboratory is 27 (Loosanoff and Davis, 1963). They found length to be greater than height until both length and height reached approximately 95-100 µm. From this point, height normally increased faster than length, resulting in a height of approximately 10-15 µm greater than length (Loosanoff and Davis, 1963; Loosanoff et al., 1966; Carriker, 1996; Chanley and Andrews, 1971). Conversely, Hu et al. (1993) reported heights generally larger than lengths until approximately 240 µm in length, when the ratio shifted and length equaled or exceeded height. Then, shell lengths between 280-320 µm were larger

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than height. Carriker (1950) observed length to exceed height when length surpassed approximately 270 µm. Forbes (1967) also reported lengths greater than heights, as did Stafford (1909 and 1912); however, others disagreed with Stafford's measurements (Loosanoff and Davis, 1963; Loosanoff et al., 1966).

Table 1. Published length and height shell measurements (µm) for *Crassostrea virginica* larvae. * denotes mean values

Length	Height	Location	Source
340	310	Prince Edward Island	Stafford, 1909
276	262	Canada	Stafford, 1912
345	297	Canada	Stafford, 1912
262	262	New Jersey	Carriker, 1951
260	266	New Jersey	Carriker, 1951
266	270	New Jersey	Carriker, 1951
273	266	New Jersey	Carriker, 1951
270	252	New Jersey	Carriker, 1951
280	273	New Jersey	Carriker, 1951
284	273	New Jersey	Carriker, 1951
277	283	Connecticut	Loosanoff et al., 1966
303	308	Connecticut	Loosanoff et al., 1966
301*	268*	Florida	Forbes, 1967

Eyespot presence is often reported to characterize the stage of larval development. There are variations between studies in the reported shell lengths at the initiation of eyespot development. Loosanoff and Davis (1963) observed *C. virginica* larvae develop eyespots at shell lengths of 270 µm and Loosanoff et al. (1966) reported *C. virginica* metamorphosed at lengths of 275-315 µm, yet some larvae measuring 355 µm had not yet metamorphosed and were still swimming in the culture. Chanley and Andrews (1971) reported that larvae metamorphosed between

shell lengths of 310-350 µm. Dupuy et al. (1977) observed that eyespots developed when larvae measured 280 µm (presumably in length), however the eyespots were irregular and they state larvae introduced into setting tanks at this stage resulted in few or no spat.

The size at which oyster larvae develop eyespots and metamorphose is quite variable between studies and is also variable within larval cultures. Loosanoff and Davis (1963) reported that *C. virginica* larvae raised together under identical conditions metamorphosed at different ages, with larvae metamorphosing over a span of 27 days. Davis and Calabrese (1964) also observed a wide range of days over which larvae set. They reported the following ranges that represent the beginning of setting to the end of setting for *C. virginica* larvae (with temperature treatment): 10-20 days (raised at 30.0-32.5°C), 14-30 days (27.5°C), 24-40 days (25.0° C), 28-46 days (22.5°C), and 36-38 days (20.0°C).

The relationships between size and metamorphosis have been found to be weak, variable, or non-existent. Holiday et al. (1991) investigated *Saccostrea commercialis* and *C. gigas* and found no significant relationship between shell size and eyespot diameter. In the field, Carriker (1951) observed variation in *C. virginica* larval size in relation to eyespot development. However, Coon et al. (1990) summarized that shell length and eyespot development were partially correlated with setting in *C. gigas*. The lack of significant relationship between shell size and eyespot development, the variation between shell size and eyespot development, and the only partial correlation between shell length and setting and eyespot development and setting may be counterintuitive for oyster culturists, who heavily rely on larval

size and eyespot presence to determine when to remove larvae from rearing tanks and place them in setting tanks.

Metamorphosis

Setting efficiencies can vary a great deal between species and location. Henderson (1983) (as reported by Devakie and Ali, 2000) stated that commercial hatchery setting efficiencies for *C. gigas* ranged from 20-28%. Holiday et al. (1991) observed 85% and 68% setting efficiencies for *S. commercialis* and *C. gigas*, respectively, in their Australian laboratory study. Dupuy et al. (1977) reported setting efficiencies between 50% and 70% for *C. virginica* in Virginia. Ranges of 4-13%, 1-13%, and up to 38% were reported in Baker's review (1994) for *C. virginica*. In his own laboratory study in Virginia, Baker's observed setting efficiencies for *C. virginica* larvae ranged from 7-32% for hatchery reared larvae and 56-81% for wild larvae. Hidu et al. (1969) quoted Mercer (1963) regarding the Bluepoint *C. virginica* hatchery in New York: "The stage to examine closely is that of the eyed larvae through setting and two days past setting. Here is the greatest loss in hatchery work. We estimated that there is no more than a 3-8% recovery of larvae in setting tanks."

Problem Statement

At the Horn Point Laboratory oyster hatchery, we are focused on increasing the setting efficiencies of *C. virginica* larvae in our facility, as well as decreasing the variation observed in the efficiencies. Hatcheries commonly rely on larval size and presence and size of eyespots to determine when larvae are competent to set (Dupuy

et al., 1977; Jones and Jones, 1988; Gibbons et al., 1992; Coon et al., 1990). However, the few studies that relate to this topic do not support this practice. As mentioned previously, a large variation in shell length at time of metamorphosis for *C. virginica* has been reported, ranging from lengths of 275-350 µm (Loosanoff et al., 1966; Chanley and Andrews, 1971). This implies a variable or non-existent relationship between metamorphosis and shell size, which has also been reported by Carriker (1951). He noted a variable relationship between larval size and metamorphosis of *C. virginica* from field observations. Studies on other oyster species have yielded similar results, with no significant relationship observed between shell size and eyespot diameter in *S. commercialis* or *C. gigas* (Holiday et al. 1991) and only a partial correlation observed between shell length and metamorphosis and eyespot development and metamorphosis in *C. gigas* (Coon et al., 1990). Taking into consideration the lack of formal studies conducted on *C. virginica* larvae in reference to the relationships between shell and eyespot size and metamorphosis, and the widespread use of these factors in oyster hatcheries to determine competency, I investigated the relationship between larval morphometrics and setting efficiency. I hypothesized that larval morphometrics, particularly eyespot diameter, would strongly correlate to setting efficiency. Additionally, I expected right valve and left valve morphometrics to correlate with each other, and anticipated observing a significant difference between the mean eyespot diameters of those measured from the right versus the left valve. Furthermore, the study will numerically describe the morphometric changes that occur at the end of the larval stage.

Methods

Standard Hatchery Practices

All oyster larvae were spawned and raised at the University of Maryland Center for Environmental Science's Horn Point Laboratory (HPL) oyster hatchery in Cambridge, MD between April and June, 2008. The study took place between May and July 2008.

Prior to inclusion in the study, the larvae were raised using the following typical HPL oyster hatchery practices. Wild stocks of adult *C. virginica* were collected from Chesapeake Bay, MD. The exact origin of all adults was unknown; however, the majority of oysters came from the Choptank and Chester rivers. Adult *C. virginica* were spawned in the hatchery by subjecting gravid individuals to a temperature rise of 10°C by altering the water temperature in the overlying running water. When such temperature treatment did not result in spawning, gametes were dissected from other oysters and these gametes were suspended in the overlying water to stimulate spawning. As individuals began to spawn, females and males were separated from each other to allow sperm and eggs to be collected separately into large containers. When spawning ceased, sperm were added to the eggs and the resulting fertilized eggs were suspended in 1 µm filtered Choptank River water in approximately 38,000 liter larval tanks for rearing. When the salinity of the incoming Choptank River water was below nine, it was increased to at least nine using sea salts (Crystal Sea Marinemix). Unless noted otherwise, this water treatment was used

throughout the study. The mean ($\pm$ SD) salinity and temperature of the larval cultures was 9.1 ($\pm$ 0.2) and 26.7°C ($\pm$ 0.6).

Larvae were fed a daily standard hatchery diet of the following species of monocultured microalgae: *Isochrysis sp.*, *Thalassiosira pseudonana*, *Chaetoceros muelleri*, and *Tetraselmis chui* (Table 2). At least twice weekly, the larval tanks were drained through mesh sieves constructed from Nitex mesh to collected larvae, and the tanks were cleaned and refilled before larvae were returned to them. When larval shell lengths reached approximately 180 µm, a test shell was placed in each larval tank and this shell was checked daily for spat. When spat were present or when the larvae showed signs of nearing metamorphosis (i.e., when the vast majority of larvae had large eyespots and there was some foot activity, such as crawling with the foot extended, as observed under a compound microscope at 40x magnification), the brood was graded by size. The grading process used 224, 212, 200, and 100 µm stainless steel cloth sieves (W.S. Tyler). Larvae from each of the three larger size groups were assessed for eyespot presence and foot activity as described above. Larvae determined competent to set were introduced into setting tanks and non-competent larvae were returned to the larval tanks. Those larvae were checked again for eyespot presence and foot activity every one to two days.

Table 2. *Crassostrea virginica.* The standard HPL hatchery diet. The larvae are fed different amounts of algae depending on when the larval tank is drained. This is because there are usually leftover algae in the larval tank from the previous day, so the larvae are fed a lesser amount of algae on days that the larval tank is not drained. Cells fed are based on the following equivalencies: 1 cell *Isochrysis sp.* = 1 cell *Thalassiosira pseudonana* = 0.5 cell *Chaetoceros muelleri* = 0.1 cell *Tetraselmis chui*. Larvae were fed *Isochrysis sp.* at any age, *Thalassiosira pseudonana* beginning on day 2, *Chaetoceros muelleri* beginning at day 6-8, and *Tetraselmis chui* beginning atday 8-10. Larvae were fed as many algal species each day as were available. Day does not have the same meaning as it does in the study. Here, day refers to the age of the larvae.

Larval age (days)	Cells^{-ml} fed to larvae (drained)	Cells^{-ml} fed to larvae (not drained)
1	20,000	20,000
2	20,000	20,000
3	30,000	20,000
4	40,000	30,000
5	50,000	40,000
6	60,000	50,000
7	70,000	60,000
8	80,000	70,000
9	90,000	80,000
10+	100,000-120,000	100,000

Selection of Larvae

Once larvae from a hatchery brood, regardless of sieve size, were determined competent to set (as described above), the larvae from the 200 µm grading sieve from that brood were eligible to be entered into the study (day 0). I chose this sieve size because it was the smallest size sieve from which the hatchery takes larvae to introduce into the setting tanks. After I obtained larvae for my study, with the exception of two replicates (broods 1 and 5), the remaining larvae from the 200 µm sieve were deemed competent to be introduced into the setting tanks by the hatchery personnel. All 11 replicates in the study were from different broods or mixes. Mixes were composed of at least two broods, and were made when a single brood of larvae

had inadequate numbers of larvae to fill a larval tank. The mean ($\pm$ SD) number of females and males which contributed eggs or sperm to each brood or mix was 59 ($\pm$ 32) and 49 ($\pm$ 26) individuals, respectively. Larvae ranged from 13 to 30 days old, with a mean ($\pm$ SD) age of 19.8 ($\pm$ 4.4) days on day 0.

Experimental Protocol

On day 0, I took the larvae directly from the grading station and placed them in a beaker with 3 l of 2 µm filtered cold river water. The beaker was kept on ice to decrease larval activity. Under cold conditions, larvae discontinued swimming, closed their shells and sank to the bottom of the container. This lack of activity decreased the likelihood of larvae attaching to the surface of the beaker. I used a perforated plunger to distribute the larvae uniformly in the water column and I did this every time a subsample was taken. I took an aliquot of larvae and placed it in a scintillation vial on ice. Later, usually within 1 hour, I used a pipette to remove as much water as possible, and preserved the larvae with 2% glutaraldehyde in 0.1M piperazine-N,N′-bis[2-ethane-sulfonic acid] buffer (pH of 7.6) (Coon et al., 1990). The preserved larvae in the scintillation vial were stored in the refrigerator at 4°C.

I took a 1 ml subsample from the larvae remaining in the beaker and placed it on a Sedgewick-Rafter slide. The subsample of larvae was preserved using several drops of 10% formalin. Depending on the similarity of counts, I counted three to six subsamples of larvae on a compound microscope (Olympus BX40) at 40x magnification. The mean number of subsamples counted daily for a brood was 3.8. To estimate the number of larvae in the beaker, I multiplied the mean of the counts by the volume of water in the beaker.

Based on the estimate of larval numbers, I used a pipette to remove 2000 larvae and deposited them in the setting bag. The setting bags were made from 0.6 mm low-density polyethylene tubing (25.4 cm wide), heat sealed at the bottom to form a "V" shaped base. The bag contained one ceramic tile (semi-gloss, glazed on one side, 10.8 cm by 10.8 cm) to provide a setting surface (Figure 3). The bag was chosen as a vessel for the tile because previous studies have demonstrated that larvae tend not to settle on the clean plastic surface as readily as they set on the hard substrate of the ceramic tile (Newell, Horn Point Laboratory, UMCES, personal communication). The bags were also easily manipulated and allowed sufficient replicates to be maintained concurrently. I conditioned each tile for two days in 1 μm filtered Choptank River water, to encourage the growth of bacterial film. These bags were then filled with 4 l of water, which produced a round bag with a conical bottom (Figure 3) and each bag was suspended in a tank serving as a water bath. Oxygen levels in the bags were maintained with a slow bubbling airstone. A submersible heater (Clepco Smart Heater QSUBMM15-W Cleveland Process Corp.) was used to maintain a temperature of 26.5°C ($\pm$ 0.3) in the water bath. The mean salinity and temperature of the water in the bags was 9.1 ($\pm$ 0.3) and 26.5°C ($\pm$ 0.2), respectively.

After 2000 larvae were introduced into the setting bag, I put the remaining larvae into a fiberglass cone (Gemini Fiberglass Products Inc., Golden, Colorado) filled with filtered Choptank River water and aerated with an air stone. Cones were filled with water the day before larvae were added to allow the water temperature to acclimate to ambient air temperature. When the water was needed, so as not to place the larvae into a 'conditioned' cone, the water was pumped into a clean cone. The

larval concentration was maintained at 2 larvae ml^{-1}, and the mean ($\pm$ SD) salinity and temperature of the water in the cones was 9.1 ($\pm$ 0.3) and 26.7°C ($\pm$ 1.5), respectively. The same water was also used to fill the setting bag, so all larvae from one brood were in water from the same source.

Each cone and bag received the equivalent of 37,500 cells larva^{-1} of *Isochrysis sp.* day^{-1} respectively. Equivalencies were based on the following relationship: 1 cell *Isochrysis sp.* = 1 cell *Thalassiosira pseudonana* = 0.5 *Chaetoceros muelleri* = 0.1 *Tetraselmis chui* (Helm, et al., 2004). The daily diet was composed of as many algal species as were available from the HPL algal culture facility on that particular day. Throughout the study, on a given day, the larvae were fed three algal species 47.9% of the time, two algal species 45.8% of the time, and one algal species 6.3% of the time. Every effort was made to control for diet, but not all algal species were available on each day.

On day 1, I drained the larval cone through a 100 µm Nitex sieve. I collected the larvae from the sieve, placed them into a beaker, and counted them as previously described. Using the known number of larvae placed into the cone on day 0, I calculated the number of larvae lost by day 1, and calculated the percent of larvae lost between days 0 and 1 (number of larvae collected from cone on day 1 subtracted from number of larvae put into cone on day 0, multiplied by 100 and divided by number of larvae put into cone on day 0). Throughout the study, percent lost was calculated using the previous day's numbers and was associated with the previous day's morphometrics (percent loss on day 3 was calculated using numbers from days 2 and 3, and was associated with morphometric data from day 2). Using the method

described previously for sampling larvae, I then took a sample of larvae for morphometric analysis and 2000 larvae to introduce into the setting bags. The remaining larvae were placed in a new larval cone.
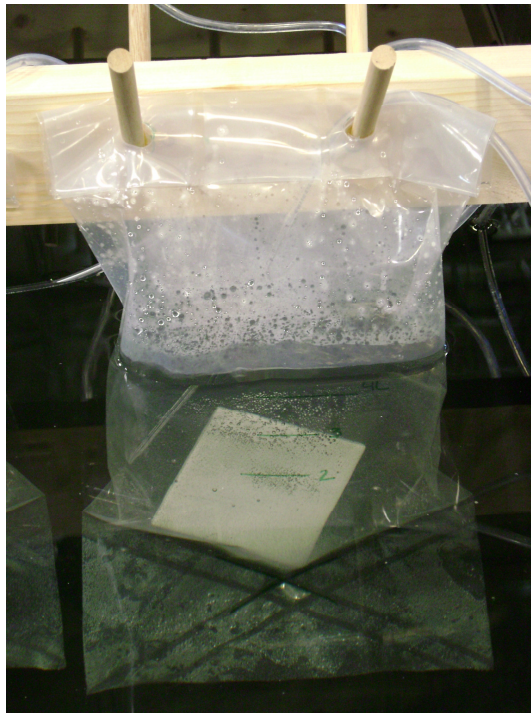
Figure 3. *Crassostrea virginica.* The aerated setting bag containing the conditioned tile which served as the setting surface for larvae. The bag is suspended in the water bath to maintain a controlled temperature. A known amount of larvae were placed in the bag. The tile was removed 24 hours later, and any spat on the tile were counted. This number was used to calculate a setting efficiency.

On day 1, I removed the setting tile from the setting bag from day 0, 24 hours after the larvae were introduced. Tiles were examined under a dissecting microscope (Olympus SZX12), and I counted any spat that had set on each tile (Figure 4 is an example of a tile after it had been removed from the setting bag). I calculated a setting efficiency (number of spat divided by 2000, multiplied by 100) and it was

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associated with morphometric data taken on day 0. The study continued daily in the

manner described until less than 10% of the original number of larvae remained in the

larval cone.

I used a compound microscope (Olympus BX51), digital camera (Olympus

DP 70), and Image Pro Plus 6.0 software for morphometric analysis. Image Pro Plus

software can measured many variables quickly. With the proper settings, the program

can identify the objects of interest and automatically measure them. The

measurements focused on the shell and eyespots. The following settings were used

for both analyses: acquisition resolution = 4080 x 3072 (Pixel Shift 9), preview

resolution = normal (bin 1x1), and capture depth = 48 bit depth (color). For shell and

eyespot analysis, exposure time for preview and acquisition = 00.250.000 and

00.003.000, respectively.

For shell analysis, I took digital micrographs of larvae at 40x magnification,

using phase contrast (Figure 5 is an example of a typical micrograph from this study).

I placed a small number of larvae on a Sedgewick-Rafter counting slide, and

manipulated them to reduce overlap. I used the count/size tool to select the larvae on

the micrographs and Image Pro Plus measured the following: area, diameter max,

diameter min, perimeter, roundness, size width, feret min, feret max, feret mean, and

size length (descriptions of the measurements are presented in Table 3). I noted

which valve was measured for each larva, so a right or left orientation was associated

with all morphometric data (Figure 6). Larvae that were tilted were not included in

analyses (see Figure 6 for an example).

For eyespot analysis, I used extended depth of focus imaging. I took multiple digital micrographs at different planes of focus and compiled them into one image to allow for better focus over a greater depth of field than a single micrograph can provide (Figure 7 is an example of a typical extended depth of focus image from this study). To complete this process, I took micrographs of larvae at 100x magnification with bright field. I made extended depth of focus micrographs for analysis, with an average of four micrographs in each composite image. Each image contained multiple larvae, so the number of micrographs in each composite image depended on the number of images were necessary to capture all eyespots in focus. I used the rectangular area of interest (AOI) tool and then the count/size tool with automatic dark object setting to select the eyespot for measuring. Image Pro Plus measured the following: area, diameter mean, perimeter, and roundness (Table 3). As with shell measurements, I noted the orientation of each larva.
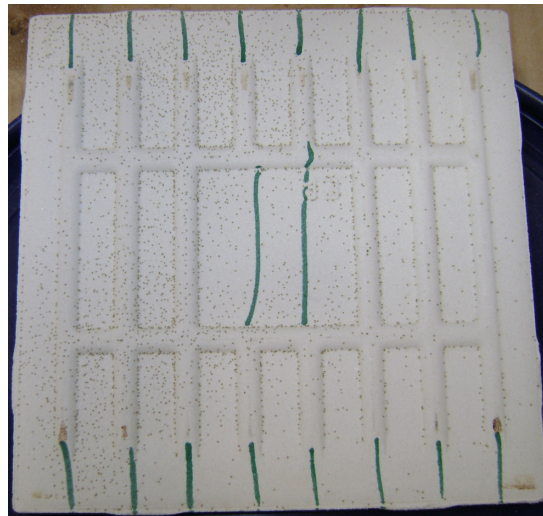
Figure 4. A tile with *Crassostrea virginica* spat set to it. The tile served as a setting surface in the setting bags. Larvae were placed in the bags for 24 hours and a setting efficiency was calculated based on the number of spat on the tile. This tile was heavily set upon. The green lines are to aid in counting.
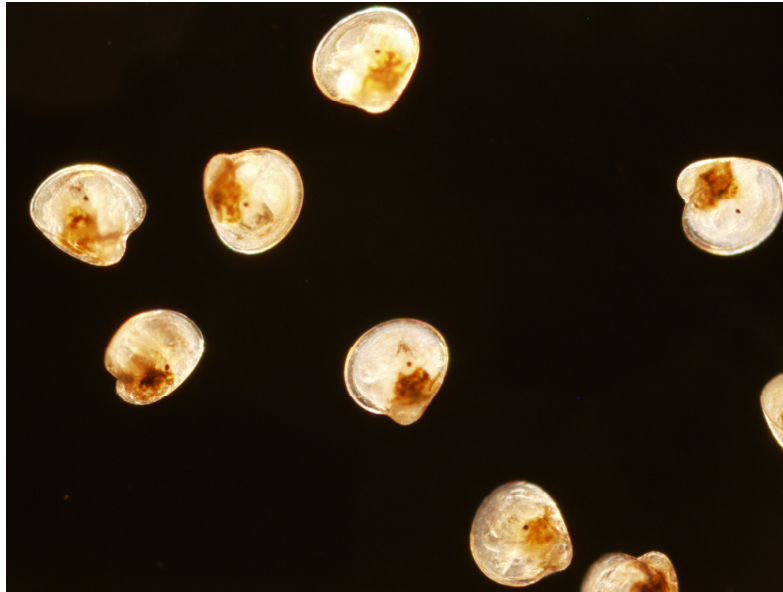
Figure 5. *Crassostrea virginica*. A typical micrograph from this study. The larvae were fixed, placed under a compound microscope at 40x magnification using phase contrast, and the micrograph was captured via a digital camera that was attached to the microscope. Larval shell morphometrics were generated using Image Pro Plus software, which automatically measured shell dimensions from the micrograph.

Table 3. The measurements and definitions used for morphometric analysis of *Crassostrea virginic*a larvae. Definitions are taken from Image Pro Plus 6.0 start-up guide for Windows, 2006.

Measurement	Image Pro Plus Definition
Area	Reports the area of each object.
Diameter max	Reports the length of the longest line joining two outline points and passing through the centroid.
Diameter mean	Reports the average length of the diameters measured at two degree intervals joining two outline points and passing through the centroid.
Diameter min	Reports the length of the shortest line joining two outline points and passing through the centroid.
Feret max	Reports the longest caliper (feret) length.
Feret mean	Reports the average caliper (feret) length.
Feret min	Reports the shortest caliper (feret) length.
Perimeter	Measurement to report the length of the outline of each object using a polygonal outline. The perimeter of interior holes are not included in this measurement.
Roundness	Reports the roundness of each object, as determined by the following formula: $(perimeter^2)/(4*pi*area)$. Circular objects will have a roundness = 1; other shapes will have a roundness < 1.
Size length	Reports the feret diameter (caliper length) along a major axis of the object.
Size width	Reports the feret diameter (caliper length) along a minor axis of the object.
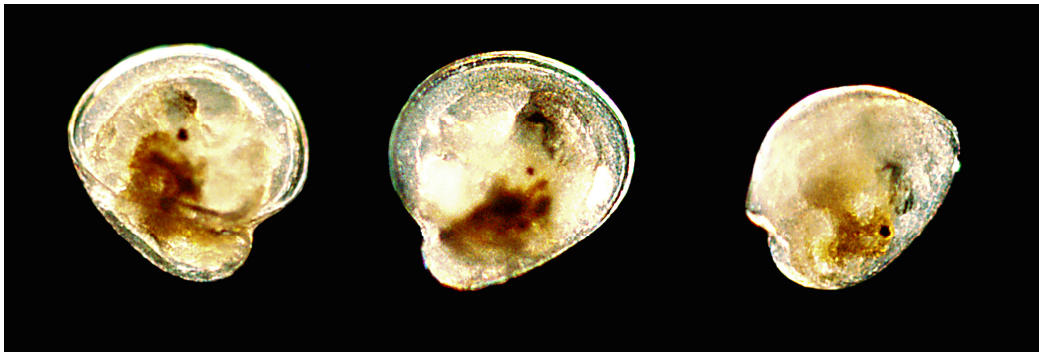
Figure 6. *Crassostrea virginica.* These micrographs show different orientations of larvae that were often encountered during the study. From left to right: right valve up and two larvae with left valves up. The first two larvae are in ideal positions for measuring, while the larva at the far right is tilted to such a degree that measurements would be inaccurate. Larvae in such positions were not included in analyses. The valve from which measurements were taken was recorded. These micrographs were taken under a compound microscope at 40x magnification.
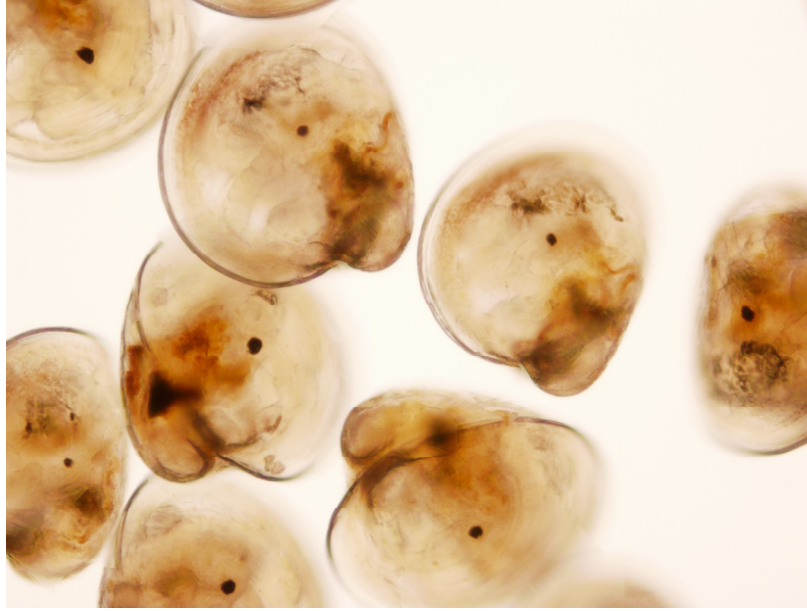
Figure 7. *Crassostrea virginica*. A typical extended depth of focus image from this study. The larvae were fixed, placed under a compound microscope at 100x magnification using bright field, and a series of micrographs were captured at different planes of focus of the same field of view. The micrographs were captured via a digital camera that was attached to the microscope. Image Pro Plus software was used to generate the extended depth of focus images, and the software also automatically measured eyespot dimensions from the composite images.

Statistical Analysis

Percent setting efficiencies and percent of loss were arcsine transformed prior to statistical analyses. Normality of the data were determined using the Shapiro-Wilke test. I used analysis of variance to determine the presence of a brood and side-of-larvae-measured effect (valve effect), and Tukey-Kramer analysis for multiple means comparisons. I hypothesized there would be a valve effect for eyespot diameter and eyespot roundness. A p-value of 0.05 was used to determine significance in all analyses. All analyses were run with SAS 9.1 software.

Correlations

Correlations were run by brood, and a mean Spearman correlation coefficient (rho) and standard deviation were calculated based on correlations of the 11 replicate broods. Due to the manner in which I performed the correlations, it was not possible to calculate a p-value for each mean correlation. Instead, I summed the number of significant and non-significant correlations for each relationship. I categorized correlations comprised of eight or more significant individual correlations as a significant correlation. All other correlations were considered non-significant. I used the following definitions to categorize the strength of the correlations: strong correlations had rho values of 0.75-1.0, moderate correlations had rho values of 0.5-0.74, weak correlations had rho values of 0.49-0.25, and rho values less than 0.25 were considered to have negligible or no correlation.

Results

All of the variables measured were statistically non-normal (Shapiro-Wilke, $p < 0.05$), however I did not transform the data. The distributions of the data were approximately normal based on histograms and plots; additionally, I used non-parametric statistical tests to accommodate the non-normality of the data. Based on the practicality of measuring and grading in a hatchery setting, I chose to focus on the following variables: shell feret mean (hereafter termed 'shell height'), shell diameter max (hereafter termed 'shell length'), eyespot diameter mean, eyespot roundness, setting efficiency, loss, and day. Additional measurements were made, and are

presented in the appendix (Appendix 1-6). Shell height was reported only for the

right valve due to the nature in which Image Pro Plus obtains measurements. Image

Pro Plus can automatically generate the minimum, maximum, and mean

measurements of an image. Shell height does not represent a minimum or maximum

measurement, so there was no way to instruct Image Pro Plus to automatically take

that measurement. However, a previous study found that feret mean does represent

shell height (D. Yarmchuck, Horn Point Laboratory, UMCES, personal

communication). To confirm this, I compared feret mean measurements to manual

height measurements, and found there was no significant difference between those

measurements taken on right valves (t-test, $p < 0.05$). There was a significant

difference between manual left valve measurements and feret mean, so it may not be

used as an accurate measurement of left valve height.

Larval shell measurements were significantly affected by brood (replicate)

and valve measured (ANOVA, $p < 0.05$; Table 4). Tukey-Kramer multiple mean

comparison showed a significant difference between mean shell length based on

valve ($p < 0.005$), so further analyses were run by valve. The significant differences

between means of different replicates (brood effect), are shown in Tables 5-7.

Eyespot diameter mean (hereafter termed 'eyespot diameter') was not significantly

different based on side of larvae (ANOVA, $p = 0.2305$; Table 4), however eyespot

roundness was affected by the side measured (ANOVA, $p = 0.011$; Table 4). There

was a brood effect detected for eyespot diameter (ANOVA, $p < 0.0001$; Table 4), and

the difference in means between broods are listed in Table 8. Right and left eyespot

roundness showed no significant brood effect (ANOVA, $p = 0.09$; Table 4).

Table 4. *Crassostrea virginica* . Analysis of variance for measurements by side and brood. Shell height was not tested for side, because only right valves were measured. Side refers to the valve which was measured. *Type III

Measurement	Source	DF	Sum of Squares	Mean Square	F-ratio	P > F
R shell height	Model	10	11028.61	1102.86	8.87	<0.0001
	Error	999	124253.61	124.38		
	Brood	10	11028.60968*	1102.86	8.87	<0.0001
Shell length	Model	11	35617.59	3237.96	25.40	<0.0001
	Error	1601	204125.65	127.50		
	Side	1	13046.41*	13046.41	102.33	<0.0001
	Brood	10	22528.61*	2252.86	17.67	<0.0001
Eyespot diameter	Model	11	635.85	57.80	3.75	<0.0001
	Error	957	14733.89	15.40		
	Side	1	22.17*	22.17	1.44	0.2305
	Brood	10	610.79*	61.08	3.97	<0.0001
Eyespot roundness	Model	11	0.22	0.02	2.23	0.0116
	Error	957	8.71	0.01		
	Side	1	0.06*	0.06	6.49	0.011
	Brood	10	0.15*	0.01	1.64	0.09

Table 5. *Crassostrea virginica* . Tukey-Kramer multiple mean comparison analysis for brood effect on right valve height. Brood effect examines significant differences in right valve height means between broods (replicates).   + denotes a significant p-value (<0.05), - denotes a non-significant p-value (>0.05).

Brood	1	2	3	4	5	6	7	8	9	10	11
1		+	+	+	-	+	+	+	-	-	-
2	+		-	-	+	-	-	-	+	+	+
3	+	-		-	+	-	-	-	-	-	-
4	+	-	-		+	-	-	-	+	-	+
5	-	+	+	+		-	+	+	-	-	-
6	+	-	-	-	-		-	-	-	-	-
7	+	-	-	-	+	-		-	-	-	-
8	+	-	-	-	+	-	-		-	-	-
9	-	+	-	+	-	-	-	-		-	-
10	-	+	-	-	-	-	-	-	-		-
11	-	+	-	+	-	-	-	-	-	-	

Table 6. *Crassostrea virginica* . Tukey-Kramer multiple mean comparison analysis for brood effect on right valve length. Brood effect examines significant differences in left valve length means between broods (replicates). + denotes a significant p-value (<0.05), - denotes a non-significant p-value (>0.05).

Brood	1	2	3	4	5	6	7	8	9	10	11
1		+	+	+	-	+	+	+	+	+	-
2	+		-	-	+	-	-	-	-	-	+
3	+	-		-	+	-	-	-	-	-	+
4	+	-	-		+	-	-	-	-	-	+
5	-	+	+	+		+	+	+	-	-	-
6	+	-	-	-	+		-	-	-	-	-
7	+	-	-	-	+	-		-	+	+	+
8	+	-	-	-	+	-	-		-	-	-
9	+	-	-	-	-	-	+	-		-	-
10	+	-	-	-	-	-	+	-	-		-
11	-	+	+	+	-	-	+	+	-	-	

Table 7. *Crassostrea virginica* . Tukey-Kramer multiple mean comparison analysis for brood effect on left valve length. Brood effect examines significant differences in left valve length means between broods (replicates). + denotes a significant p-value (<0.05), - denotes a non-significant p-value (>0.05).

Brood	1	2	3	4	5	6	7	8	9	10	11
1		+	+	-	-	-	-	-	-	-	-
2	+		-	-	+	-	-	-	-	-	-
3	+	-		-	+	-	-	-	-	-	-
4	-	-	-		+	-	-	-	-	-	-
5	-	+	+	+		+	+	-	-	-	-
6	-	-	-	-	+		-	-	-	-	-
7	-	-	-	-	+	-		-	-	-	-
8	-	-	-	-	-	-	-		-	-	-
9	-	-	-	-	-	-	-	-		-	-
10	-	-	-	-	-	-	-	-	-		-
11	-	-	-	-	-	-	-	-	-	-	

Table 8. *Crassostrea virginica*. Tukey-Kramer multiple mean comparison analysis for brood effect on eyespot diameter. Brood effect examines significant differences in eyespot diameter means between broods (replicates). + denotes a significant p-value (<0.05), - denotes a non-significant p-value (>0.05).

Brood	1	2	3	4	5	6	7	8	9	10	11
1		-	-	-	-	-	-	-	-	-	-
2	-		-	-	-	-	-	+	-	-	-
3	-	-		-	-	-	-	-	-	-	-
4	-	-	-		-	-	-	-	-	-	-
5	-	-	-	-		-	-	+	-	-	-
6	-	-	-	-	-		-	-	-	-	-
7	-	-	-	-	-	-		-	+	+	-
8	-	+	-	-	+	-	-		+	+	-
9	-	-	-	-	-	-	+	+		-	-
10	-	-	-	-	-	-	+	+	-		-
11	-	-	-	-	-	-	-	-	-	-	

Correlations

The correlations between the variables are reported in Tables 9 and 10. Additional correlations between all variables measured are reported in Appendix 1-6. Eyespot roundness was not significant correlated with any variables (Tables 9 and 10). The correlations between shell morphometrics and setting efficiency were weak. Right valve morphometrics were weakly correlated with eyespot dimensions (Table 9). Left valve length was not significantly correlated with any larval morphometrics, but was weakly correlated with setting efficiency, loss and day (Tables 9 and 10). Setting efficiency was most strongly correlated with day (Table 10).

Morphometrics Over Time

Kruskal-Wallis analyses indicate that all variables had some significant difference between means over time (p<0.0001), with the exception of right and left eyespot roundness (p>0.05). These relationships are presented in Figures 8-15, which also include the results from Tukey-Kramer multiple means comparison analyses. The exact means and standard deviation for each measurement is reported in Table11, and the means and standard deviations for measurements for each replicate are reported by day in Appendix 7-17.

Table 9. *Crassostrea virginica*. Spearman correlation matrix examining shell measurements. Mean rho ($\pm$ SD) values are reported. Mean rho is calculated from rho for each of the individual correlations of the 11 replicates. The fraction reported below rho refers to the number of significant correlations (p<0.05) over the number of non-significant correlations (p>0.05) of the 11 replicates. R and L refer to measurements taken on the right or left larval valve. Significant mean relationships are defined as those comprised of at least 8 individual significant relationships. Gray shading denotes significant correlations.

	R valve height	R valve length	L valve length
R valve height	1 ± 0	0.898 ± 0.045 11/0	0.302 ± 0.128 4/7
R valve length	0.898 ± 0.045 11/0	1 ± 0	0.205 ± 0.132 4/7
L valve length	0.302 ± 0.128 4/7	0.205 ± 0.132 3/8	1 ± 0
Eyespot diameter	0.421 ± 0.154 10/1	0.333 ± 0.174 8/3	0.378 ± 0.179 7/4
R eyespot roundness	0.029 ± 0.186 1/10	$-3.89E-4 \pm 0.192$ 2/9	0.023 ± 0.164 1/10
L eyespot roundness	0.074 ± 0.148 0/11	0.066 ± 0.139 1/10	-0.005 ± 0.204 0/11
Setting efficiency	0.462 ± 0.246 11/0	0.367 ± 0.187 9/2	0.414 ± 0.200 8/3
Loss	0.488 ± 0.238 10/1	0.365 ± 0.216 8/3	0.414 ± 0.157 8/3
Day	0.585 ± 0.173 11/0	0.455 ± 0.162 11/0	0.494 ± 0.147 10/1

Table 10. *Crassostrea virginica*. Spearman correlation matrix examining eyespot, setting efficiency, and loss measurements. Mean rho (± SD) values are reported. Mean rho is calculated from rho for each of the individual correlations of the 11 replicates. The fraction reported below rho refers to the number of significant correlations (p<0.05) over the number of non-significant correlations (p>0.05) of the 11 replicates. R and L refer to measurements taken on the right or left larval valve. Significant mean relationships are defined as those comprised of at least 8 individual significant relationships. Gray shading denotes significant correlations.

	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency	Loss	Day
R valve height	**0.421 ± 0.154** **11/0**	0.029 ± 0.186 1/10	0.074 ± 0.148 0/11	**0.462 ± 0.246** **10/1**	**0.488 ± 0.238** **9/2**	**0.585 ± 0.173** **11/0**
R valve length	**0.333 ± 0.174** **8/3**	-3.89E-4 ± 0.192 2/9	0.066 ± 0.139 1/10	**0.367 ± 0.187** **9/2**	**0.365 ± 0.216** **8/3**	**0.455 ± 0.162** **11/0**
L valve length	0.378 ± 0.179 7/4	0.023 ± 0.164 1/10	-0.005 ± 0.204 0/11	**0.414 ± 0.200** **8/3**	**0.414 ± 0.157** **8/3**	**0.494 ± 0.147** **10/1**
Eyespot diameter	1 ± 0	0.111 ± 0.171 2/9	-0.045 ± 0.168 1/10	**0.603 ± 0.227** **10/1**	**0.583 ± 0.164** **11/0**	**0.683 ± 0.135** **11/0**
R eyespot roundness	0.111 ± 0.171 2/9	1 ± 0	-0.070 ± 0.164 1/10	0.002 ± 0.174 1/10	0.026 ± 0.099 0/11	0.019 ± 0.161 1/10
L eyespot roundness	-0.045 ± 0.168 1/10	-0.070 ± 0.164 1/10	1 ± 0	0.016 ± 0.169 1/10	0.021 ± 0.130 1/10	0.005 ± 0.170 1/10
Setting efficiency	**0.603 ± 0.227** **10/1**	0.002 ± 0.174 1/10	0.016 ± 0.169 1/10	1 ± 0	**0.720 ± 0.285** **10/1**	**0.796 ± 0.260** **11/0**
Loss	**0.583 ± 0.164** **11/0**	0.026 ± 0.099 0/11	0.021 ± 0.130 1/10	**0.720 ± 0.285** **10/1**	1 ± 0	**0.916 ± 0.100** **11/0**
Day	**0.683 ± 0.135** **11/0**	0.019 ± 0.161 1/10	0.005 ± 0.170 1/10	**0.796 ± 0.260** **11/0**	**0.916 ± 0.100** **11/0**	1 ± 0

Table 11. *Crassostrea virginica* . All broods. Mean values of broods are reported ($\pm$ SEM) for variables. R and L refer to measurements taken on the right or left larval valve. Individual broods lasted for different lengths of time. Days 0, 1, 2: N=11, Day 3: N=10, Day 4: N=4, Day 5: N=1. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting Efficiency (%)	Loss (%)
0	277.02 $\pm$ 0.89	298.70 $\pm$ 1.15	292.35 $\pm$ 1.05	9.04 $\pm$ 0.47	1.11 $\pm$ 0.01	1.10 $\pm$ 0.01	4.445 $\pm$ 1.23	10.31 $\pm$ 1.98
1	287.44 $\pm$ 0.93	306.67 $\pm$ 1.21	299.18 $\pm$ 1.21	12.17 $\pm$ 0.64	1.09 $\pm$ 0.01	1.13 $\pm$ 0.02	29.38 $\pm$ 6.515	15.25 $\pm$ 4.15
2	293.09 $\pm$ 0.76	311.01 $\pm$ 1.09	305.33 $\pm$ 1.22	13.99 $\pm$ 0.72	1.09 $\pm$ 0.01	1.11 $\pm$ 0.01	39.86 $\pm$ 6.94	41.76 $\pm$ 2.0
3	295.27 $\pm$ 1.74	313.58 $\pm$ 1.63	307.73 $\pm$ 1.32	16.13 $\pm$ 0.37	1.10 $\pm$ 0.01	1.11 $\pm$ 0.01	57.04 $\pm$ 3.67	60.79 $\pm$ 7.51
4	295.30 $\pm$ 3.71	314.08 $\pm$ 3.38	309.01 $\pm$ 3.99	15.50 $\pm$ 0.99	1.10 $\pm$ 0.02	1.13 $\pm$ 0.02	55.96 $\pm$ 17.43	63.74 $\pm$ 10.52
5	293.02	311.99	317.87	16.49	1.14	1.06	67	59.20

Right Valve Measurements

The means of right valve height and length followed a similar pattern over time (Figures 8 and 9). Both measurements increased until day 4 and then decreased; however, there were no significant differences between the means of days 3, 4, and 5. Both height and length had the same Tukey-Kramer multiple mean comparison results. The mean from day 0 significantly differed from all days, day 1 differed from days 0, 2, 3, and 4, day 2 differed from days 0, 1, and 3, day 3 differed from days 0, 1, 2, day 4 differed from days 0 and 1, and day 5 differed from day 0 ($p<0.05$). Height measurements ranged from 249-321 µm and length measurements ranged from 270-350 µm.

Left Valve Measurements

Left valve length mean increased by approximately 25 µm during the study period. The mean increased daily, however the means on days 2, 3, and 4 were not significantly different from each other (Figure 10). Days 0, 1, and 5 significantly differed from all others. Length measurements ranged from approximately 275-339 µm.
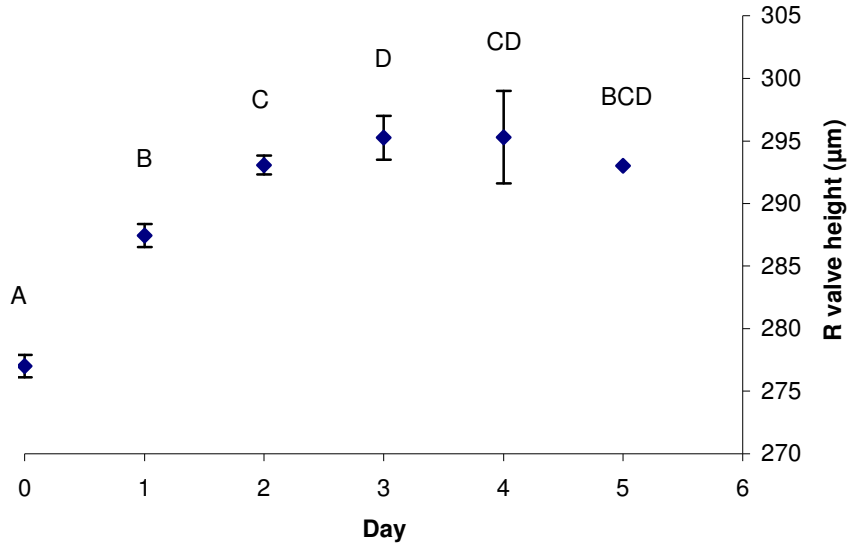
Figure 8. *Crassostrea virginica*. Mean (± SEM) right valve height over time. Means were calculated from all broods in the study. Individual broods lasted for different lengths of time. Days 0, 1, 2: N=11, Day 3: N=10, Day 4: N=4, Day 5: N=1. Different letters denote significant differences (Tukey-Kramer multiple mean comparison, p<0.05).
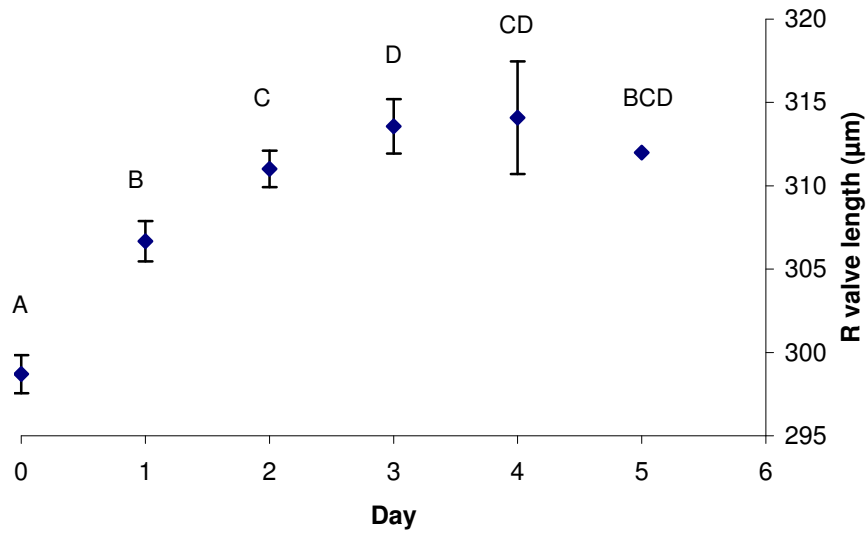
Figure 9. *Crassostrea virginica*. Mean (± SEM) right valve length over time. Means were calculated from all broods in the study. Individual broods lasted for different lengths of time. Days 0, 1, 2: N=11, Day 3: N=10, Day 4: N=4, Day 5: N=1. Different letters denote significant differences (Tukey-Kramer multiple mean comparison, p<0.05).
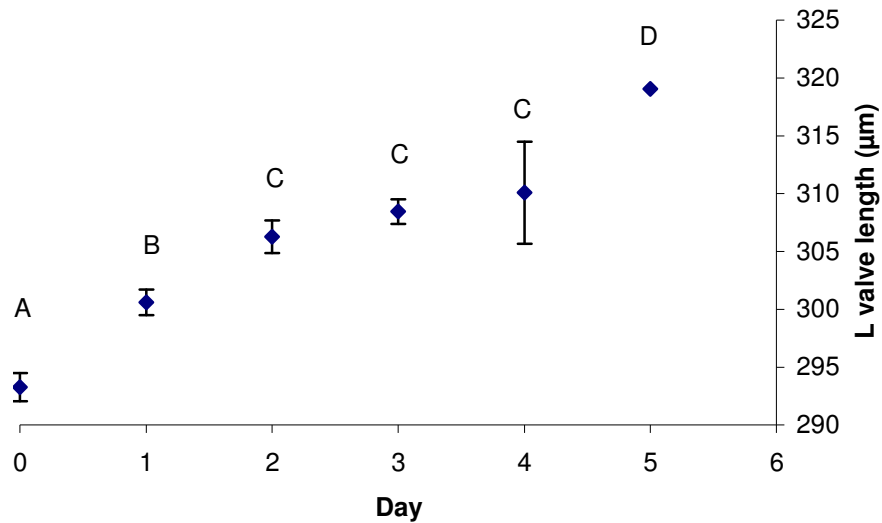
Figure 10. *Crassostrea virginica*. Mean ($\pm$ SEM) left valve length over time. Means were calculated from all broods in the study. Individual broods lasted for different lengths of time. Days 0, 1, 2: N=11, Day 3: N=10, Day 4: N=4, Day 5: N=1. Different letters denote significant differences (Tukey-Kramer multiple mean comparison, p<0.05).

Eyespot Measurements

Eyespot diameter peaked on day 5, but did not increase steadily, as a decline occurred on day 4 (Figure 11). Days 0 and 1 were significantly different from all days, day 2 differed days 0, 1, 3, and 4, and days 3, 4, and 5 did not differ from each other. Eyespot mean diameter measurements ranged from 3-28 µm. The mean of right eyespot roundness (Figure 12) was lowest on day 1, and peaked on day 5, contrary to the mean of left eyespot roundness (Figure 13) which peaked on day 1 and dipped on day 5. Both measurements had large standard SEM and there were no significant differences between days (Tukey-Kramer multiple mean comparison, p>0.05).

Figure 11. *Crassostrea virginica.* Mean (± SEM) eyespot diameter over time. Means were calculated from all broods in the study. Individual broods lasted for different lengths of time. Days 0, 1, 2: N=11, Day 3: N=10, Day 4: N=4, Day 5: N=1. Different letters denote significant differences (Tukey-Kramer multiple mean comparison, p<0.05).

Figure 12. *Crassostrea virginica.* Mean (± SEM) right eyespot roundness over time. Means were calculated from all broods in the study. Individual broods lasted for different lengths of time. Days 0, 1, 2: N=11, Day 3: N=10, Day 4: N=4, Day 5: N=1. A value of 1 represents a perfect circle. Tukey-Kramer multiple means comparison found no significant difference between the means (p>0.05).
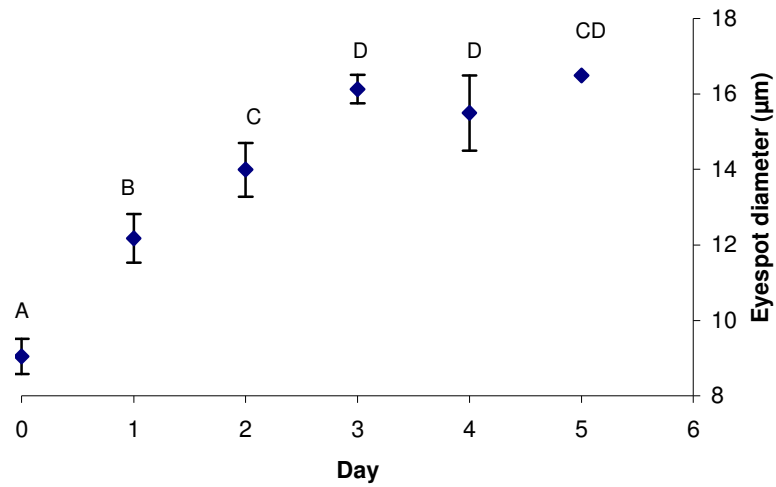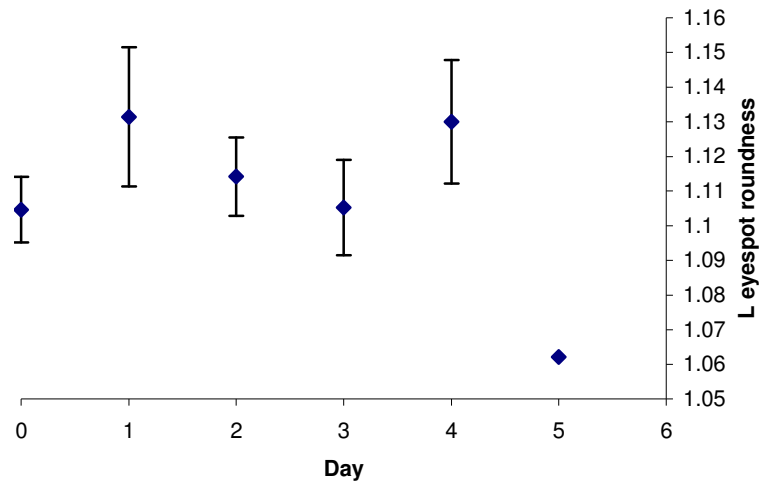
Figure 13. *Crassostrea virginica*. Mean ($\pm$ SEM) left eyespot roundness over time. Means were calculated from all broods in the study. Individual broods lasted for different lengths of time. Days 0, 1, 2: N=11, Day 3: N=10, Day 4: N=4, Day 5: N=1. A value of 1 represents a perfect circle. Tukey-Kramer multiple means comparison found no significant difference between the means ($p>0.05$).

Setting Efficiency and Loss

Mean setting efficiency increased through day 3, decreased on day 4, and peaked on day 5 (Figure 14). Mean loss increased through day 4 and decreased on day 5 (Figure 15). Tukey-Kramer results were identical for setting efficiency and loss. Days 0, 1, and 2 differed from all other days and days 3, 4, and 5 differed from days 0, 1, and 2. Setting efficiency ranged from 0.2-85.7% and loss ranged from 1.0-92.0%.
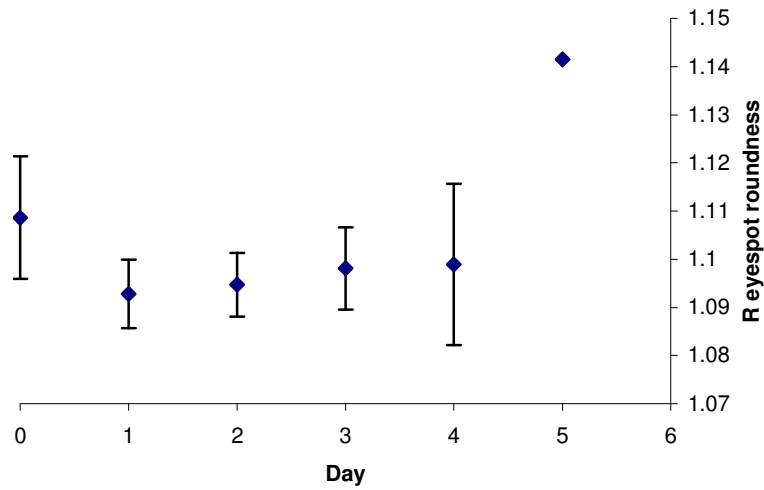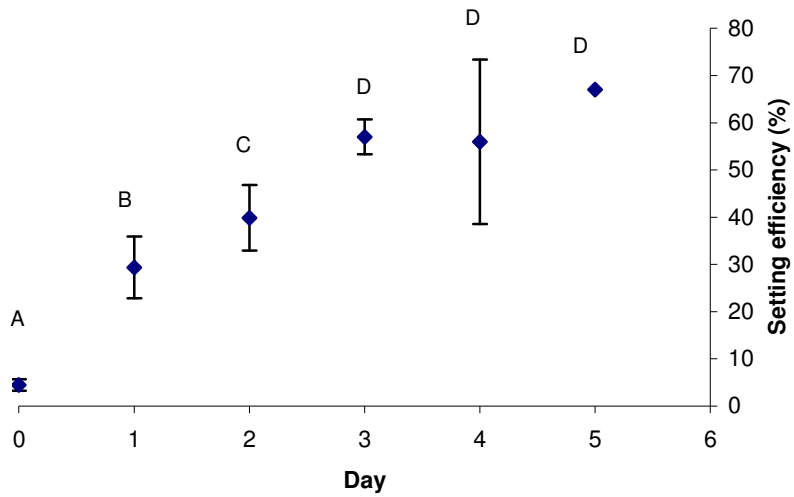
Figure 14. *Crassostrea virginica.* Mean (± SEM) setting efficiency over time. Means were calculated from all broods in the study. Individual broods lasted for different lengths of time. Days 0, 1, 2: N=11, Day 3: N=10, Day 4: N=4, Day 5: N=1. Different letters denote significant differences (p<0.05).
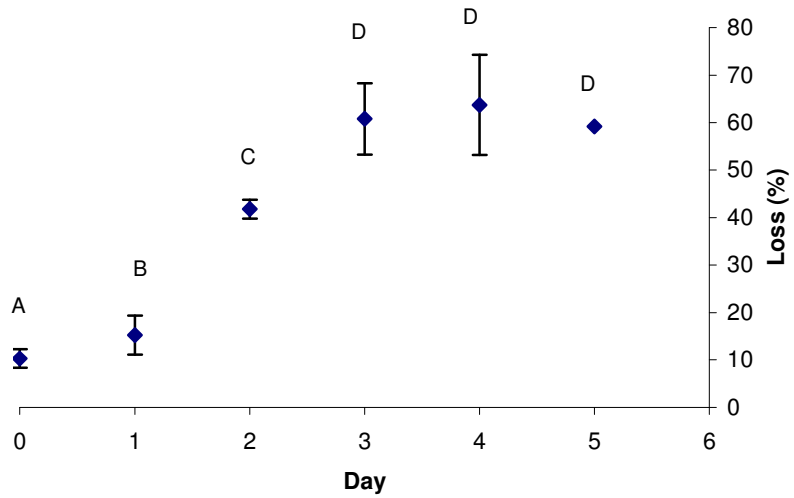
Figure 15. *Crassostrea virginica.* Mean (± SEM) loss over time. Means were calculated from all broods in the study. Individual broods lasted for different lengths of time. Days 0, 1, 2: N=11, Day 3: N=10, Day 4: N=4, Day 5: N=1. Different letters denote significant differences (p<0.05).

Discussion

Setting Efficiency Correlations

Day was most strongly correlated with setting efficiency (Table 10), and may be the most practical and convenient parameter to use when determining larval competence. The lack of strong correlations between setting efficiency and any shell or eyespot morphometric (Tables 9 and 10) highlights the fact that competency to set cannot solely be judged upon physical characteristics. Behavioral competency can be present 2-4 days prior to morphogenetic competency in *C. gigas* (Coon et al., 1990; Fitt et al., 1990). A temporal difference such as this in *C. virginica* would explain the lack of strong correlations between morphometrics and setting efficiency in my study. I did not quantify foot activity, however nine of eleven larval broods in my study were deemed competent to set by hatchery personnel on day 0. Therefore, I can assume at least nine of the broods exhibited some behavioral competency on day 0, yet the mean ($\pm$ SEM) setting efficiency of 4.45% ($\pm$ 1.23) (Table 11) implies that the vast majority of larvae were not morphogenetically competent.

Setting Efficiency and Loss

I observed the highest setting efficiency on day 5 (Figure 14), however only one replicate lasted until day 5. In this case, maximizing setting efficiency was not associated with maximizing spat production. This is because only a small fraction of larvae remained in the larval cone by day 5 (the other larvae had died or had set in the larval cone), resulting in low spat production. This demonstrates the importance of focusing on maximizing spat production, not on achieving the highest setting

efficiency, as I will illustrate here. In table 12, I use setting efficiency and loss to estimate a hypothetical amount of larvae that may metamorphose each day. In this theoretical situation, I start with 1 million larvae similar to the larvae I used in the study. One million larvae are multiplied by the loss for day 0. The product is larvae lost. Larvae lost from day 0 are subtracted from remaining larvae from day 0, and result is remaining larvae for day 1. Spat for day 0 is calculated by multiplying day 0 setting efficiency by day 0 remaining larvae. All days are calculated in this manner.

Table 12. *Crassostrea virginica*. Hypothetical outcome when using setting efficiency and loss to determine spat production on any given day. In this theoretical scenario, we begin with 1,000,000 larvae. One million larvae are multiplied by 'loss' for Day 0 and the product is 'larvae lost'. 'Larvae lost' from Day 0 are subtracted from 'remaining larvae' of Day 0, and results in 'remaining larvae' of Day 1. Day 0 'setting efficiency' is multiplied by Day 0 'remaining larvae' to result in Day 0 'spat'. Calculations continue in this manner to complete the table.

Day	Setting Efficiency	Loss	Remaing Larvae	Larvae Lost	Spat Produced
0	0.0445	0.1031	1000000	103100	44500
1	0.2938	0.1525	897000	136793	263539
2	0.3986	0.4176	760208	317463	303019
3	0.5704	0.6079	442745	269145	252542
4	0.5596	0.6374	173600	110653	97147
5	0.67	0.592	62947	37265	42175

Table 12 demonstrates the number of larvae (remaining) in the culture daily if the larvae are never removed to be placed in a setting vessel. The number decreases due to mortality and larvae metamorphosing in the culture vessel. It also shows the number of spat which would result if all the larvae in the culture were removed and placed in a setting vessel on a given day. It is evident that the greatest number of spat produced would occur on day 2, but days 1 and 3 would also produce many spat. If larvae were removed from the rearing tanks to be set on only one day, day 2 would

result in the most spat. However, if larvae were removed on days 1, 2, and 3, 819,000 spat would result, which represents approximately 80% of the original number of larvae. Removing larvae on multiple days means larvae placed in the setting vessel must be separated from larvae which will be placed back in the culture tank.

The easiest method to separate larvae is through grading. Table 13 reports mean shell lengths and heights by valve, caught on different size grading sieves used in the HPL oyster hatchery. Right valve lengths for days 1-3 range from approximately 307-313 µm. Table 13 shows larvae of these sizes can be retained primarily by a 200 µm sieve, which retains a mean ($\pm$ SEM) size of 305.55 ($\pm$ 2.01) µm. This appears promising, as if it is possible to separate the size group of interest successfully, however, the mean ($\pm$ SEM) right valve length of day 0 from the study equals 298.7 ($\pm$ 1.15) µm (Table 11). These means should be approximately equal to each other, because they both are describing larvae retained on a 200 µm sieve. The difference in the means shows the inability of sieves to separate larvae on a precise scale, therefore grading in this way is not a dependable tool to separate groups of larvae that have small size differences from each other.

Mean eyespot diameter for days 1-3 range from approximately 12-16 µm (Table 11 and Figure 11) and this measurement shows the largest percent of increase between days. The increase makes eyespot diameter a better candidate to distinguish between larvae to place in a setting vessel and larvae to remain in the culture tank. However, it is not possible to physically separate large amounts of larvae based on eyespot diameter. Instead, larvae from a 200 or 212 µm sieve may be analyzed for

mean eyespot diameter. Broods with mean eyespots less than 12 µm (for example)

may be returned to a culture tank instead of being placed in the setting vessel.

Table 13. *Crassostrea virginica*. Mean sizes (±SEM) of larvae
retained on the grading sieves used by the Horn Point Laboratory
oyster hatchery. There were 3 replicates for the 200 µm sieve, and
4 replicates for the 212 and 224 µm sieves. At least 100 larvae
were measured for each replicate, with a total of approximately
1200 larvae measured. All measurements are reported in µm.

Size	Valve	Shell Length	Shell Height
200	L	297.05 ± 2.97	ND
212	L	309.16 ± 4.34	ND
224	L	318.68 ± 3.32	ND
200	R	305.55 ± 2.01	280.04 ± 2.27
212	R	317.66 ± 2.69	292.52 ± 3.20
224	R	324.87 ± 3.12	299.63 ± 3.89

Brood Effects

Brood effects, or the significant difference of means between replicates, were

common in measurements (Tables 4-8). This may be explained by genetic

differences between the broods. Another possible explanation is the difference in diet

during the study. Larvae were fed equivalent diets (based on the number of cells fed

larvae^{-1} day^{-1}), but not identical diets. Although it would have been ideal to raise

larvae under completely controlled conditions, and feed them an identical diet, this

proved to be impractical. I did attempt this during a previous season, and was unable

to raise larvae from fertilized eggs in sufficient numbers to use in the study.

However, I believe these are minor inconsistencies and are of little concern, based on

the small SEM observed during the study (Figures 8-15). Although there were

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statistically significant differences of means between some replicates, the SEMs

imply that these differences were not practically significant.

Morphometrics Through Time

The mean maximum right and left valve lengths of 314 and 318 µm (Table

11) that I observed fall within the range of other reported maximum lengths for *C.*

virginica larvae (Carriker, 1951; Prytherch, 1934; Loosanoff et al., 1966); K. Hudson,

VIMS hatchery, personal communication). Mean maximum right valve height of 295

µm (Table 11) is generally larger than reported values (Stafford, 1909; Stafford,

1912; Carriker, 1951; Loosanoff, 1961; Forbes, 1967); however, those values are not

necessarily considered the maximum height.

Like Stafford (1909, 1912), Carriker (1950) and Forbes (1967), I found length

to be greater than height (Table 11), however the difference between length and

height was not as large as Stafford and Forbes reported. On average, the right length

measurement from my study was 19.3 µm greater than height (Table 11), while

Stafford and Forbes reported maximum differences of 48 and 33 µm, respectively.

The length to height relationship of *C. virginica* measured in this study differs from

that observed by Loosanoff and Davis (1963) and Loosanoff et al. (1966).

To my knowledge, there are no reported data on eyespot growth in any species

of oyster larvae. In this study, the mean maximum eyespot diameter of

approximately 16 µm was reached when the right shell length was approximately 313

µm, or on days 3-5 (Table 11). This is in contrast to Dupuy et al.(1977), who state

that eyespot development begins at approximately 280 µm in length, and will be fully developed about 24 hours later, when the shell length is approximately 290 µm.

When comparing my measurements to others', it must be noted that rarely are studies conducted at the low salinity that I used. Salinity affects growth of larvae, but not to the same extent as parameters, such as temperature (Davis and Calabrese, 1964). *Crassostrea virginica* larvae can tolerate and grow in a wide range of salinities; however, the salinity of this study (9.1) is lower than most studies. In other low salinity studies, the larvae often came from broodstock who developed their gametes while in high salinity water (Davis, 1958), which adds an additional factor that does not apply to my work.

Right and Left Valve Measurements

Studies involving oyster larvae often report mean valve length, and only occasionally do researchers specify whether the left or right valve was measured. Stafford (1912) reports several measurements taken on left valves. Loosanoff and Davis (1966) mention measurements were taken on larvae in the same orientation, and their photos, although not labeled, imply the right valves were measured. Forbes (1967) reported measurements from only left disarticulated valves. These mentions of valve side appear to be the exception and presumably, most researchers measure the side that happens to land facing up on the microscope slide. Observations from my study show that there are significant differences between the means of shell measurements taken on the right versus the left valve (Table 4). This difference is likely due to the tilt of the larvae on the slide. The umbos and curvature of both valves result in a tilting of the larva (Figure 6), thus changing the outline of the shell

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(Loosanoff et al., 1966) and affecting the measurements. Note that the left valve is larger, however right valve measurements are generally greater because a portion of the left valve umbo is visible and is included in the right height measurement (Hu et al., 1993; Figure 1).

Eyespots develop on the right side of *C. gigas* larvae prior to developing on the left side; consequently, there is a difference in eyespot size between eyespots visible from the left and right valves (Coon et al., 1990). Asymmetrical development of eyespots has not been reported for *C. virginica*, and to my knowledge, has not been reported for any other oyster species. I did not observe asymmetrical development of eyespots however, that does not mean it does not occur in *C. virginica*. I worked primarily with eyed larvae, so although I observed growth of the eyespots, I did not observe the beginning of the development. Coon and his colleagues discriminated between early-eyed and eyed larvae based on the size of eyespot on the right side and the presence of eyespots on both sides of the larvae, respectively. Larvae with right side eyespots of less than half the diameter of a fully developed eyespot were considered early-eyed larvae. A fully developed eyespot was not defined. I observed a variation in maximum eyespot diameter (Appendix 7-17), similarly to maximum valve size (Appendix 7-17), and this may be related to environmental conditions or the presence or absence of appropriate stimuli to induce metamorphosis.

Correlations Between Morphometrics

The strong correlation within right valve measurements (Table 9) was expected based on the nearly linear relationship between height and length reported by Loosanoff et al. (1966). The lack of significant correlations between right shell

measurements and left shell measurements (Table 9), although somewhat surprising, may be explained by the differences in shape of the right and left valves. The more convex left valve may be growing in a more three dimensional manner than the right valve, and my methods did not permit analysis in this way.

The weak or non-significant correlations observed between shell measurements and eyespot diameter (Table 9) is surprising from a hatchery perspective, as culturists rely on shell size and presence of eyespot to indicate the readiness of larvae to be introduced into setting tanks. Despite the findings in the literature that suggest partial or no correlation between shell length and eyespot presence, hatcheries continue to use shell length as an important factor when choosing larvae to place in setting tanks. This does not emphasize culturists' ignorance; rather it points to the limitations of working with small organisms on a large scale.

Comments on Image Pro Plus Software

Image Pro Plus 6.0 software allowed me to measure many variables automatically, and to measure variables that I would be unable to measure with a micrometer, such as roundness. This was helpful for a study such as this, which examined a variety of measurements in an attempt to find significant relationships to setting efficiency. Many of those measurements were not discussed, but can be found in the Appendix (1-6). Image Pro Plus takes measurements of micrographs, and so it requires additional equipment and time to capture the micrographs. For those interested in measuring dimensions that are easy to identify, such as shell height and length, I recommend doing so with a standard micrometer.

Application and Future Work

The possible application of using setting efficiency, loss, and shell measurement data to assist in determining the optimal time to place larvae in setting tanks is not likely realistic for a production hatchery. As explained above, the sieves catch overlapping sizes of larvae (Table 13), and the larvae retained on a sieve may depend on the quantity of larvae present on the sieve, the previous sieve, and the orientation in which the larvae pass through the sieve openings. This makes sieves an ineffective tool for separating larvae on such a scale. Mean eyespot diameter of larvae from a given sieve may have more practical potential in determining when larvae should stay in a culture tank or be placed in a setting tank.

Setting efficiency is most strongly correlated with day. This is a convenient result for culturists, because after larvae are retained on a 200 µm sieve, it eliminates the need to examine larvae under the microscope for shell and eyespot size. Instead, culturists may return the larvae from the 200 µm sieve to a larval tank for a predetermined amount of time (based on the scenario presented in Table 12). It is unlikely culturists will forego examining larvae under the microscope as they near metamorphosis, but the relationship between setting efficiency and days past retention on a 200 µm sieve may assist culturists when they are indecisive regarding the course of action to take with a particular batch of larvae. However, any application beyond the scale of this study would require additional research to determine if larvae behave similarly when reared in larger tanks or placed in larger setting tanks with an unfiltered water source, oyster shells as cultch, and without the addition of cultured algae.

An additional option to increase setting efficiency may be the use of chemical induction. As previously mentioned, a possible explanation for the lack of strong correlation between setting efficiency and larval characteristics may be related to competency. The larvae, although behaviorally competent on day 0 (demonstrated by crawling behavior when examined by hatchery personnel), were likely not morphogenetically competent (supported by the low setting efficiency observed, Figure 14). A study conducted at the HPL hatchery has demonstrated that settlement behavior in *C. virginica* can be induced with L-DOPA, serotonin, 3-isobutyl-1-methylxanthine (IBMX) and ammonia (Grant, 2009). L-DOPA-induced settlement behavior in *C. virginica* does result in increased rates of metamorphosis (Walch et al., 1999). A practical approach to using these inducers in a hatchery involves challenging a sample of the larvae with a metamorphosis-inducing chemical, as suggested by Coon et al. (1990) for *C. gigas*. An aliquot of larvae may be treated with epinephrine. If an adequate percentage respond by metamorphosing, the larvae can be considered morphogenetically competent. Synchronous setting could then be triggered with an inducer prior to or upon the introduction of larvae into the setting tank. If the larvae fail to respond to epinephrine in sufficient numbers, they may be returned to a larval tank for continued rearing. However, additional research investigating morphometric induction in a low salinity setting is needed (Grant, 2009).

Another possible way to increase setting efficiencies is to adjust the conditions in the setting tanks. If larvae in setting tanks continue to mature as they do in rearing tanks, selection of larvae to place in setting tanks would not be as critical;

however if larvae in setting tanks do not undergo any further development, then timing of placement into the setting tanks is important. Currently, it is standard practice at HPL oyster hatchery to begin ambient water flow into the setting tank approximately 72 hours after the introduction of the larvae into the setting tank. However, if the setting efficiency estimate indicates a poor set, additional larvae are introduced into the setting tank. When this occurs, ambient water flow is turned on 96 hours after the first introduction of larvae into the setting tank. Based on this study, the majority of larvae can develop from behaviorally competent to morphogenetically competent within that time period. Changing methods to create a more supportive setting environment may result in larvae reaching morphogenetic competence while in the setting tanks, and ultimately, in higher setting efficiencies. Possible methods to support larval maturation in setting tanks may include the use of filtered water to decrease possible competitors for algae, and supplementing the water with cultured algae. The addition of cultured algae would likely support larval maturation more than other possible method changes, however it is also the most difficult to implement. This is because algae are generally a limiting factor in larval rearing, even without factoring in the use of algae in setting tanks. However, if studies show that feeding larvae in setting tanks results in a greatly increased setting efficiency, hatcheries could use fewer larvae to produce equal or greater numbers of spat. This would make algae that was generally fed to larvae in larval tanks available to be used in setting tanks.

Appendices

Appendix 1. *Crassostrea virginica.* Spearman correlation matrix examining right and left valve measurements. Mean rho (± SD) values are reported. Mean rho is calculated from rho for each of the individual correlations of the 11 replicates. The fraction reported below rho refers to the number of significant correlations (p<0.05) over the number of non-significant correlations (p>0.05) of the 11 replicates. R and L refer to measurements taken on the right or left larval valve. Significant mean relationships are defined as those comprised of at least 8 individual significant relationships. There are no significant correlations in this matrix.

	R valve area	R valve perimeter	R valve roundness	R valve diameter min	R valve length	R valve feret min	R valve feret max	R valve height	R valve size width	R valve size length
L valve area	0.291 ± 0.127	0.253 ± 0.119	-0.133 ± 0.172	0.291 ± 0.152	0.200 ± 0.122	0.288 ± 0.149	0.205 ± 0.115	0.278 ± 0.121	0.253 ± 0.142	0.163 ± 0.101
	4/7	3/8	1/10	6/5	2/9	5/6	2/9	4/7	5/6	0/11
L valve perimeter	0.305 ± 0.132	0.272 ± 0.128	-0.139 ± 0.185	0.307 ± 0.153	0.208 ± 0.133	0.307 ± 0.153	0.214 ± 0.130	0.293 ± 0.125	0.270 ± 0.149	0.166 ± 0.117
	6/5	5/6	3/8	7/4	3/8	7/4	3/8	6/5	4/7	1/10
L valve roundness	-0.022 ± 0.182	-0.009 ± 0.165	0.054 ± 0.175	-0.020 ± 0.231	-0.011 ± 0.145	-0.006 ± 0.210	-0.014 ± 0.151	-0.017 ± 0.182	0.005 ± 0.202	-0.012 ± 0.138
	1/10	1/10	2/9	3/8	1/10	3/8	1/10	0/11	1/10	1/10
L valve diameter min	0.247 ± 0.156	0.211 ± 0.132	-0.122 ± 0.161	0.250 ± 0.183	0.166 ± 0.140	0.249 ± 0.178	0.169 ± 0.138	0.235 ± 0.147	0.220 ± 0.167	0.136 ± 0.127
	3/8	3/8	1/10	5/6	3/8	5/6	3/8	3/8	4/7	2/9
L valve length	0.317 ± 0.132	0.290 ± 0.140	-0.141 ± 0.202	0.332 ± 0.161	0.205 ± 0.132	0.324 ± 0.161	0.212 ± 0.126	0.302 ± 0.128	0.282 ± 0.166	0.150 ± 0.120
	5/6	4/7	2/9	6/5	3/8	7/4	3/8	4/7	5/6	2/9
L valve feret min	0.250 ± 0.154	0.213 ± 0.138	-0.118 ± 0.160	0.251 ± 0.180	0.168 ± 0.142	0.251 ± 0.177	0.171 ± 0.138	0.239 ± 0.146	0.222 ± 0.165	0.137 ± 0.128
	3/8	3/8	1/10	5/6	3/8	5/6	2/9	3/8	4/7	1/10
L valve feret max	0.312 ± 0.136	0.288 ± 0.145	-0.133 ± 0.187	0.327 ± 0.168	0.206 ± 0.135	0.321 ± 0.167	0.214 ± 0.130	0.298 ± 0.132	0.282 ± 0.172	0.153 ± 0.125
	5/6	5/6	1/10	7/4	3/8	7/4	3/8	5/6	5/6	1/10
L valve size width	0.248 ± 0.157	0.213 ± 0.139	-0.102 ± 0.159	0.248 ± 0.180	0.170 ± 0.151	0.247 ± 0.178	0.174 ± 0.149	0.238 ± 0.15	0.217 ± 0.165	0.139 ± 0.139
	3/8	3/8	1/10	5/6	2/9	4/7	3/8	3/8	4/7	1/10
L valve size length	0.289 ± 0.140	0.283 ± 0.140	-0.123 ± 0.205	0.282 ± 0.167	0.266 ± 0.264	0.272 ± 0.166	0.182 ± 0.143	0.254 ± 0.139	0.238 ± 0.171	0.128 ± 0.147
	4/7	4/7	2/9	5/6	4/7	5/6	4/7	3/7	4/7	1/10

Appendix 2. *Crassostrea virginica*. Spearman correlation matrix examining right valve measurements. Mean rho (± SD) values are reported. Mean rho (± SD) values are reported. Mean rho is calculated from rho for each of the individual correlations of the 11 replicates. The fraction reported below rho refers to the number of significant correlations (p<0.05) over the number of non-significant correlations (p>0.05) of the 11 replicates. R and L refer to measurements taken on the right or left larval valve. Significant mean relationships are defined as those comprised of at least 8 individual significant relationships. Gray shading denotes significant correlations.

	R valve area	R valve perimeter	R valve roundness	R valve diameter min	R valve length	R valve feret min	R valve feret max	R valve height	R valve size width	R valve size length
R valve area	1 ± 0	0.917 ± 0.79	-0.226 ± 0.144	0.946 ± 0.019	0.879 ± 0.053	0.947 ± 0.018	0.884 ± 0.050	0.995 ± 0.006	0.927 ± 0.023	0.0784 ± 0.081
		11/0	7/4	11/0	11/0	11/0	11/0	11/0	11/0	11/0
R valve perimeter	0.917 ± 0.079	1 ± 0	0.0425 ± 0.216	0.857 ± 0.090	0.840 ± 0.069	0.868 ± 0.075	0.847 ± 0.070	0.929 ± 0.065	0.858 ± 0.065	0.759 ± 0.084
	11/0		5/6	11/0	11/0	11/0	11/0	11/0	11/0	11/0
R valve roundness	-0.2256 ± 0.144	0.043 ± 0.216	1 ± 0	-0.287 ± 0.148	-0.093 ± 0.157	-0.267 ± 0.160	-0.081 ± 0.150	-0.188 ± 0.148	-0.243 ± 0.175	-0.044 ± 0.135
	7/4	5/6		8/3	3/8	7/4	3/8	7/4	7/4	0/11
R valve diameter min	0.946 ± 0.019	0.857 ± 0.090	-0.287 ± 0.148	1 ± 0	0.741 ± 0.107	0.984 ± 0.011	0.745 ± 0.106	0.929 ± 0.028	0.952 ± 0.022	0.650 ± 0.136
	11/0	11/0	8/3		11/0	11/0	11/0	11/0	11/0	11/0
R valve length	0.879 ± 0.053	0.840 ± 0.069	-0.093 ± 0.157	0.741 ± 0.107	1 ± 0	0.752 ± 0.098	0.994 ± 0.003	0.898 ± 0.045	0.791 ± 0.082	0.938 ± 0.019
	11/0	11/0	3/8	11/0		11/0	11/0	11/0	11/0	11/0
R valve feret min	0.947 ± 0.018	0.868 ± 0.075	-0.267 ± 0.16	0.984 ± 0.011	0.752 ± 0.098	1 ± 0	0.755 ± 0.098	0.933 ± 0.023	0.972 ± 0.014	0.667 ± 0.123
	11/0	11/0	7/4	11/0	11/0		11/0	11/0	11/0	11/0
R valve feret max	0.884 ± 0.050	0.847 ± 0.070	-0.081 ± 0.150	0.745 ± 0.106	0.994 ± 0.003	0.755 ± 0.098	1 ± 0	0.904 ± 0.043	0.789 ± 0.084	0.941 ± 0.018
	11/0	11/0	3/8	11/0	11/0	11/0		11/0	11/0	11/0
R valve height	0.995 ± 0.006	0.929 ± 0.065	-0.188 ± 0.148	0.929 ± 0.028	0.898 ± 0.045	0.933 ± 0.023	0.904 ± 0.043	1 ± 0	0.917 ± 0.025	0.803 ± 0.077
	11/0	11/0	7/4	11/0	11/0	11/0	11/0		11/0	11/0
R valve size width	0.9267 ± 0.023	0.858 ± 0.065	-0.243 ± 0.175	0.952 ± 0.022	0.791 ± 0.082	0.970 ± 0.011	0.789 ± 0.084	0.917 ± 0.025	1 ± 0	0.729 ± 0.101
	11/0	11/0	7/4	11/0	11/0	11/0	11/0	11/0		11/0
R valve size length	0.0784 ± 0.081	0.759 ± 0.084	-0.044 ± 0.135	0.650 ± 0.136	0.938 ± 0.019	0.667 ± 0.123	0.941 ± 0.018	0.803 ± 0.077	0.729 ± 0.101	1 ± 0
	11/0	11/0	0/11	11/0	11/0	11/0	11/0	11/0	11/0	

Appendix 3. *Crassostrea virginica*. Spearman correlation matrix examining right valve and eyespot measurements. Mean rho (± SD) values are reported. Mean rho is calculated from rho for each of the individual correlations of the 11 replicates. The fraction reported below rho refers to the number of significant correlations (p<0.05) over the number of non-significant correlations (p>0.05) of the 11 replicates. R and L refer to measurements taken on the right or left larval valve. Significant mean relationships are defined as those comprised of at least 8 individual significant relationships. Gray shading denotes significant correlations.

	R valve area	R valve perimeter	R valve roundness	R valve diameter min	R valve length	R valve feret min	R valve feret max	R valve height	R valve size width	R valve size length
Eyespot area	0.435 ± 0.151	0.396 ± 0.149	-0.204 ± 0.17	0.464 ± 0.156	0.332 ± 0.173	0.456 ± 0.156	0.329 ± 0.168	0.421 ± 0.153	0.428 ± 0.148	0.282 ± 0.136
	10/1	10/1	5/6	10/1	8/3	10/1	8/3	10/1	10/1	7/4
Eyespot perimeter	0.445 ± 0.148	0.403 ± 0.147	-0.275 ± 0.223	0.472 ± 0.154	0.339 ± 0.170	0.466 ± 0.154	0.334 ± 0.166	0.430 ± 0.15	0.437 ± 0.144	0.287 ± 0.147
	10/1	10/1	5/6	10/1	8/3	10/1	8/3	10/1	10/1	6/5
R eyespot roundness	0.035 + 0.176	0.021 ± 0.187	-0.086 ± 0.119	0.051 ± 0.168	3.89E-4 ± 0.192	0.059 ± 0.161	-0.009 ± 0.203	0.029 ± 0.186	0.046 ± 0.181	0.006 ± 0.167
	1/10	1/10	0/11	1/10	2/9	1/10	2/9	1/10	1/10	1/10
L eyespot roundness	0.068 + 0.141	0.081 ± 0.138	0.079 ± 0.157	0.046 ± 0.157	0.066 ± 0.139	0.026 ± 0.137	0.077 ± 0.143	0.074 ± 0.148	0.034 ± 0.137	0.073 ± 0.123
	0/11	0/11	2/9	0/11	1/10	0/11	1/10	0/11	0/11	0/11
Eyespot diameter	0.435 + 0.153	0.397 ± 0.150	-0.201 ± 0.171	0.464 ± 0.156	0.333 ± 0.174	0.455 ± 0.157	0.330 ± 0.170	0.421 ± 0.154	0.428 ± 0.150	0.284 ± 0.137
	10/1	10/1	5/6	10/1	8/3	10/1	8/3	10/1	10/1	7/4
Setting efficiency	0.475 ± 0.246	0.436 ± 0.227	-0.193 ± 0.213	0.485 ± 0.272	0.367 ± 0.187	0.483 ± 0.270	0.366 ± 0.185	0.462 ± 0.246	0.450 ± 0.245	0.304 + 0.161
	10/1	10/1	6/5	9/2	9/2	9/2	9/2	10/1	9/2	7/4
Loss	0.502 + 0.231	0.445 ± 0.230	-0.224 ± 0.194	0.527 ± 0.233	0.365 ± 0.216	0.523 ± 0.24	0.362 ± 0.217	0.488 ± 0.238	0.483 ± 0.241	0.303 ± 0.208
	9/2	9/2	5/6	9/2	8/3	9/2	8/3	9/2	9/2	6/5
Day	0.599 + 0.168	0.545 ± 0.170	-0.245 ± 0.175	0.615 ± 0.172	0.455 ± 0.162	0.612 ± 0.181	0.454 ± 0.163	0.585 ± 0.173	0.572 ± 0.171	0.380 ± 0.157
	11/0	11/0	6/5	11/0	11/0	10/1	11/0	11/0	11/0	8/3

Appendix 4. *Crassostrea virginica*. Spearman correlation matrix examining left valve measurements. Mean rho ($\pm$ SD) values are reported. Mean rho is calculated from rho for each of the individual correlations of the 11 replicates. The fraction reported below rho refers to the number of significant correlations ($p<0.05$) over the number of non-significant correlations ($p>0.05$) of the 11 replicates. R and L refer to measurements taken on the right or left larval valve. Significant mean relationships are defined as those comprised of at least 8 individual significant relationships. Gray shading denotes significant correlations.

	L valve area	L valve perimeter	L valve roundness	L valve diameter min	L valve length	L valve feret min	L valve feret max	L valve size width	L valve size length
L valve area	1 ± 0	0.962 ± 0.025	-0.418 ± 0.194	0.940 ± 0.024	0.761 ± 0.074	0.946 ± 0.019	0.737 ± 0.080	0.938 ± 0.025	0.575 ± 0.105
		11/0	7/4	11/0	11/0	11/0	11/0	11/0	11/0
L valve perimeter	0.962 ± 0.025	1 ± 0	-0.237 ± 0.254	0.879 ± 0.050	0.776 ± 0.064	0.894 ± 0.037	0.758 ± 0.068	0.888 ± 0.036	0.600 ± 0.095
	11/0		6/5	11/0	11/0	11/0	11/0	11/0	11/0
L valve roundness	-0.418 ± 0.194	-0.235 ± 0.254	1 ± 0	-0.528 ± 0.177	-0.084 ± 0.182	-0.500 ± 0.193	-0.051 ± 0.174	-0.490 ± 0.207	0.027 ± 0.185
	7/4	6/5		11/0	1/10	10/1	2/9	8/3	1/10
L valve diameter min	0.940 ± 0.024	0.879 ± 0.050	-0.528 ± 0.177	1 ± 0	0.586 ± 0.097	0.991 ± 0.005	0.562 ± 0.103	0.974 ± 0.020	0.398 ± 0.118
	11/0	11/0	11/0		11/0	11/0	11/0	11/0	9/2
L valve length	0.761 ± 0.074	0.776 ± 0.064	-0.084 ± 0.182	0.586 ± 0.097	1 ± 0	0.587 ± 0.103	0.989 ± 0.005	0.589 ± 0.106	0.930 ± 0.021
	11/0	11/0	1/10	11/0		11/0	11/0	11/0	11/0
L valve feret min	0.946 ± 0.019	0.894 ± 0.037	-0.500 ± 0.193	0.991 ± 0.005	0.587 ± 0.103	1 ± 0	0.561 ± 0.110	0.987 ± 0.01	0.400 ± 0.126
	11/0	11/0	10/1	11/0	11/0		10/1	11/0	9/2
L valve feret max	0.737 ± 0.080	0.758 ± 0.068	-0.051 ± 0.174	0.562 ± 0.103	0.989 ± 0.005	0.561 ± 0.110	1 ± 0	0.559 ± 0.144	0.940 ± 0.016
	11/0	11/0	2/9	11/0	11/0	10/1		11/0	11/0
L valve size width	0.938 ± 0.025	0.888 ± 0.036	-0.490 ± 0.207	0.974 ± 0.020	0.589 ± 0.106	0.987 ± 0.010	0.559 ± 0.114	1 ± 0	0.410 ± 0.132
	11/0	11/0	8/3	11/0	11/0	11/0	11/0		9/2
L valve size length	0.575 ± 0.105	0.600 ± 0.095	0.027 ± 0.185	0.398 ± 0.118	0.930 ± 0.021	0.400 ± 0.126	0.940 ± 0.016	0.410 ± 0.132	1 ± 0
	11/0	11/0	1/10	9/2	11/0	9/2	11/0	9/2	

Appendix 5. *Crassostrea virginica.* Spearman correlation matrix examining left valve and eyespot measurements. Mean rho ($\pm$ SD) values are reported. Mean rho is calculated from rho for each of the individual correlations of the 11 replicates. The fraction reported below rho refers to the number of significant correlations (p<0.05) over the number of non-significant correlations (p>0.05) of the 11 replicates. R and L refer to measurements taken on the right or left larval valve. Significant mean relationships are defined as those comprised of at least 8 individual significant relationships. Gray shading denotes significant correlations.

	L valve area	L valve perimeter	L valve roundness	L valve diameter min	L valve length	L valve feret min	L valve feret max	L valve size width	L valve size length
Eyespot area	0.385 ± 0.177	0.377 ± 0.175	-0.145 ± 0.219	0.358 + 0.188	0.378 ± 0.178	0.350 ± 0.186	0.367 ± 0.177	0.351 ± 0.194	0.317 ± 0.170
	9/2	9/2	4/7	8/3	8/3	8/3	7/4	8/3	7/4
Eyespot perimeter	0.382 ± 0.180	0.376 ± 0.178	-0.143 ± 0.219	0.355 + 0.190	0.372 ± 0.180	0.356 ± 0.187	0.361 ± 0.180	0.346 ± 0.196	0.309 ± 0.180
	8/3	9/2	4/7	4/7	7/4	7/4	7/4	6/5	7/4
R eyespot roundness	0.032 ± 0.185	0.045 ± 0.168	72E-04 ± 0.133	0.044 + 0.166	0.023 ± 0.164	0.040 ± 0.0175	0.027 ± 0.163	0.035 ± 0.166	-0.010 ± 0.158
	0/11	0/11	0/11	1/10	1/10	1/10	0/11	1/10	0/11
L eyespot roundness	0.016 ± 0.149	0.012 ± 0.141	-0.041 ± 0.142	0.021 + 0.150	-0.005 ± 0.204	0.020 ± 0.135	)64E-04 ± 0.197	0.008 ± 0.139	-0.020 ± 0.190
	0/11	0/11	0/11	0/11	0/11	0/11	0/11	0/11	0/11
Eyespot diameter	0.385 ± 0.177	0.377 ± 0.176	-0.145 ± 0.218	0.358 + 0.188	0.378 ± 0.179	0.359 ± 0.185	0.367 ± 0.178	0.350 ± 0.193	0.317 ± 0.170
	10/1	9/2	4/7	8/3	7/4	8/3	7/4	8/3	7/4
Setting efficiency	0.397 ± 0.226	0.400 ± 0.220	-0.093 ± 0.216	0.367 + 0.233	0.414 ± 0.200	0.371 ± 0.231	0.403 ± 0.193	0.361 ± 0.245	0.358 + 0.181
	8/3	8/3	2/9	7/4	8/3	7/4	8/3	7/4	8/3
Loss	0.408 ± 0.189	0.407 ± 0.192	-0.107 ± 0.211	0.362 + 0.218	0.414 ± 0.157	0.370 ± 0.223	0.390 ± 0.167	0.365 ± 0.232	0.333 ± 0.155
	7/4	8/3	3/8	7/4	8/3	7/4	8/3	7/4	7/4
Day	0.474 ± 0.171	0.482 ± 0.160	-0.097 ± 0.217	0.426 + 0.204	0.494 ± 0.147	0.432 ± 0.201	0.480 ± 0.150	0.424 ± 0.211	0.424 ± 0.145
	10/1	10/1	3/8	8/3	10/1	8/3	10/1	8/3	9/2

Appendix 6. *Crassostrea virginica*. Spearman correlation matrix examining eyespot measurements. Mean rho (± SD) values are reported. Mean rho is calculated from rho for each of the individual correlations of the 11 replicates. The fraction reported below rho refers to the number of significant correlations (p<0.05) over the number of non-significant correlations (p>0.05) of the 11 replicates. R and L refer to measurements taken on the right or left larval valve. Significant mean relationships are defined as those comprised of at least 8 individual significant relationships. Gray shading denotes significant correlations.

	Eyespot area	Eyespot perimeter	R eyespot roundness	L eyespot roundness	Eyespot diameter	Setting efficiency	Loss	Day
Eyespot area	1 ± 0	0.978 ± 0.040	0.115 ± 0.169	-0.044 ± 0.167	1.0 ± 2.373E-04	0.602 ± 0.228	0.583 ± 0.163	0.683 ± 0.134
		11/0	2/9	1/10	11/0	10/1	11/0	11/0
Eyespot perimeter	0.978 ± 0.040	1 ± 0	0.205 ± 0.167	-0.026 ± 0.152	0.977 ± 0.040	0.590 ± 0.227	0.574 ± 0.161	0.673 ± 0.130
	11/0		4/7	0/11	11/0	10/1	11/0	11/0
R eyespot roundness	0.115 ± 0.169	0.205 ± 0.167	1 ± 0	-0.070 ± 0.164	0.111 ± 0.171	0.002 ± 0.174	0.026 ± 0.099	0.019 ± 0.161
	2/9	4/7		1/10	2/9	1/10	0/11	1/10
L eyespot roundness	-0.044 ± 0.167	-0.026 ± 0.152	-0.070 ± 0.164	1 ± 0	-0.045 ± 0.168	0.016 ± 0.169	0.021 ± 0.130	0.005 ± 0.170
	1/10	0/11	1/10		1/10	1/10	1/10	1/10
Eyespot diameter	1.0 ± 2.373E-04	0.977 ± 0.040	0.111 ± 0.171	-0.045 ± 0.168	1 ± 0	0.603 ± 0.227	0.583 ± 0.164	0.683 ± 0.135
	11/0	11/0	2/9	1/10		10/1	11/0	11/0
Setting efficiency	0.602 ± 0.228	0.590 ± 0.227	0.002 ± 0.174	0.016 ± 0.169	0.603 ± 0.227	1 ± 0	0.720 ± 0.285	0.796 ± 0.260
	10/1	10/1	1/10	1/10	10/1		10/1	11/0
Loss	0.583 ± 0.163	0.574 ± 0.161	0.026 ± 0.099	0.021 ± 0.130	0.583 ± 0.164	0.720 ± 0.285	1 ± 0	0.916 ± 0.100
	11/0	11/0	0/11	1/10	11/0	10/1		11/0
Day	0.683 ± 0.134	0.673 ± 0.130	0.019 ± 0.161	0.005 ± 0.170	0.683 ± 0.135	0.796 ± 0.260	0.916 ± 0.100	1 ± 0
	11/0	11/0	1/10	1/10	11/0	11/0	11/0	

Appendix 7. *Crassostrea virginica*. Brood 1. Mean values ($\pm$ SD, where applicable) are reported for variables. R and L refer to measurements taken on the right or left larval valve. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency (%)	Loss (%)
0	280.57 $\pm$ 7.81	303.86 $\pm$ 9.73	297.77 $\pm$ 9.15	7.78 $\pm$ 1.86	1.12 $\pm$ 0.12	1.08 $\pm$ 0.07	11.3	12.06
1	291.69 $\pm$ 9.36	311.70 $\pm$ 10.57	304.35 $\pm$ 9.61	11.44 $\pm$ 2.00	1.06 $\pm$ 0.03	1.13 $\pm$ 0.05	10.3	8.54
2	296.44 $\pm$ 9.70	315.62 $\pm$ 10.74	312.51 $\pm$ 9.98	13.83 $\pm$ 1.91	1.07 $\pm$ 0.07	1.07 $\pm$ 0.06	34	21.51
3	299.92 $\pm$ 8.79	319.72 $\pm$ 11.62	311.45 $\pm$ 9.19	16.56 $\pm$ 4.36	1.10 $\pm$ 0.07	1.09 $\pm$ 0.06	59.75	43.76
4	301.32 $\pm$ 7.56	320.68 $\pm$ 10.27	314.11 $\pm$ 10.23	15.27 $\pm$ 2.80	1.08 $\pm$ 0.04	1.11 $\pm$ 0.06	76.95	42.61

Appendix 8. *Crassostrea virginica*. Brood 2. Mean values ($\pm$ SD, where applicable) are reported for variables. R and L refer to measurements taken on the right or left larval valve. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency (%)	Loss (%)
0	275.27 $\pm$ 9.22	297.55 $\pm$ 9.37	290.61 $\pm$ 8.58	9.28 $\pm$ 3.19	1.09 $\pm$ 0.03	1.09 $\pm$ 0.04	0.2	13.43
1	282.89 $\pm$ 7.75	303.01 $\pm$ 9.99	297.47 $\pm$ 6.37	13.71 $\pm$ 2.52	1.08 $\pm$ 0.03	1.08 $\pm$ 0.02	30.1	12.31
2	291.42 $\pm$ 5.94	309.10 $\pm$ 7.19	298.32 $\pm$ 10.52	15.13 $\pm$ 2.29	1.08 $\pm$ 0.03	1.06 $\pm$ 0.03	61.5	35.12
3	289.09 $\pm$ 11.78	305.31 $\pm$ 13.17	301.91 $\pm$ 9.05	16.24 $\pm$ 2.00	1.07 $\pm$ 0.01	1.06 $\pm$ 0.2	64.15	68.86

Appendix 9. *Crassostrea virginica*. Brood 3. Mean values ($\pm$ SD, where applicable) are reported for variables. R and L refer to measurements taken on the right or left larval valve. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency (%)	Loss (%)
0	278.57 $\pm$ 8.35	301.22 $\pm$ 9.97	291.61 $\pm$ 9.35	9.52 $\pm$ 2.86	1.11 $\pm$ 0.07	1.11 $\pm$ 0.08	10.05	20.38
1	288.91 $\pm$ 6.98	304.75 $\pm$ 8.05	303.29 $\pm$ 7.01	14.07 $\pm$ 2.12	1.11 $\pm$ 0.06	1.16 $\pm$ 0.10	18.5	47.43
2	292.55 $\pm$ 8.09	310.66 $\pm$ 9.98	298.34 $\pm$ 8.82	14.68 $\pm$ 1.95	1.11 $\pm$ 0.07	1.20 $\pm$ 0.24	61.9	80.66

Appendix 10. *Crassostrea virginica*. Brood 4. Mean values ($\pm$ SD, where applicable) are reported for variables. R and L refer to measurements taken on the right or left larval valve. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency (%)	Loss (%)
0	279.21 $\pm$ 6.13	300.43 $\pm$ 6.39	294.56 $\pm$ 8.21	10.62 $\pm$ 3.11	1.10 $\pm$ 0.07	1.09 $\pm$ 0.07	5.7	13.22
1	285.42 $\pm$ 5.31	305.06 $\pm$ 5.16	298.30 $\pm$ 6.41	11.40 $\pm$ 2.49	1.13 $\pm$ 0.16	1.08 $\pm$ 0.01	24.7	11.28
2	291.01 $\pm$ 8.77	309.52 $\pm$ 8.70	305.47 $\pm$ 9.97	13.50 $\pm$ 3.17	1.09 $\pm$ 0.03	1.10 $\pm$ 0.08	4.6	23.96
3	283.38 $\pm$ 7.74	305.92 $\pm$ 9.31	303.68 $\pm$ 8.99	13.68 $\pm$ 2.54	1.08 $\pm$ 0.03	1.07 $\pm$ 0.04	47.75	37.08
4	286.18 $\pm$ 11.33	305.65 $\pm$ 12.63	302.10 $\pm$ 11.73	13.11 $\pm$ 2.45	1.09 $\pm$ 0.06	1.16 $\pm$ 0.23	6.0	54.79

Appendix 11. *Crassostrea virginica*. Brood 5. Mean values (± SD, where applicable) are reported for variables. R and L refer to measurements taken on the right or left larval valve. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency (%)	Loss (%)
0	277.30 ± 8.06	301.37 + 9.56	293.10 ± 11.99	9.23 ± 1.76	1.18 ± 0.09	1.11 ± 0.05		5.23
1	292.75 ± 5.41	313.89 ± 10.89	304.62 ± 12.97	14.38 ± 3.24	1.06 ± 0.07	1.11 ± 0.09		0.96
2	297.36 ± 9.16	316.68 ± 9.35	307.07 ± 8.72	12.91 ± 1.75	1.11 ± 0.07	1.08 ± 0.05	16.55	2.89
3	298.73 ± 9.12	317.13 ± 11.01	307.81 ± 14.67				50.4	15.28
4	292.32 ± 11.19	311.76 ± 13.61	317.52 ± 10.25	15.65 ± 2.55	1.08 ± 0.04	1.09 ± 0.04	58.45	65.56
5	293.02 ± 6.38	311.99 ± 9.91	317.87 ± 11.23	16.49 ± 1.74	1.14 ± 0.18	1.06 ± 0.02	67	59.20

Appendix 12. *Crassostrea virginica*. Brood 6. Mean values (± SD, where applicable) are reported for variables. R and L refer to measurements taken on the right or left larval valve. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency (%)	Loss (%)
0	273.50 ± 6.20	294.67 ± 7.17	289.01 ± 6.59	9.50 ± 2.44	1.15 ± 0.18	1.06 ± 0.03	2.5	4.62
1	286.24 ± 6.74	306.26 ± 7.08	296.05 ± 9.09	12.92 ± 2.10	1.07 ± 0.06	1.11 ± 0.08	55.55	7.55
2	290.04 ± 8.83	306.62 ± 9.02	306.88 ± 7.27	7.62 ± 1.25	1.10 ± 0.06	1.09 ± 0.05	57.35	46.43
3	293.70 ± 8.48	311.51 ± 10.76	311.46 ± 11.53	16.31 ± 1.53	1.12 ± 0.12	1.08 ± 0.05	59.95	52.14
4	301.38 ± 11.67	318.23 ± 13.38	302.29 ± 8.81	17.97 ± 1.93	1.15 ± 0.21	1.16 ± 0.10	82.45	91.98

Appendix 13. *Crassostrea virginica* . Brood 7. Mean values ($\pm$ SD, where applicable) are reported for variables. R and L refer to measurements taken on the right or left larval valve. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency (%)	Loss (%)
0	273.95 $\pm$ 7.51	291.68 $\pm$ 8.09	289.51 $\pm$ 10.78	10.23 $\pm$ 3.10	1.09 $\pm$ 0.07	1.13 $\pm$ 0.07	5.45	8.72
1	284.34 $\pm$ 8.36	299.67 $\pm$ 9.01	293.74 $\pm$ 7.54	6.72 $\pm$ 1.30	1.11 $\pm$ 0.10	1.12 $\pm$ 0.10	2.35	20.71
2	291.09 $\pm$ 11.82	307.85 $\pm$ 13.38	304.19 $\pm$ 10.46	14.60 $\pm$ 2.29	1.08 $\pm$ 0.05	1.15 $\pm$ 0.08		45.24
3	298.36 $\pm$ 5.94	314.72 $\pm$ 7.09	303.69 $\pm$ 9.52	16.03 $\pm$ 2.73	1.10 $\pm$ 0.09	1.07 $\pm$ 0.03	85.7	81.52

Appendix 14. *Crassostrea virginica* . Brood 8. Mean values ($\pm$ SD, where applicable) are reported for variables. R and L refer to measurements taken on the right or left larval valve. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency (%)	Loss (%)
0	275.96 $\pm$ 8.07	295.28 $\pm$ 7.24	292.42 $\pm$ 5.39	5.25 $\pm$ 1.65	1.04 $\pm$ 0.04	1.09 $\pm$ 0.04	0.4	
1	285.31 $\pm$ 6.16	306.97 $\pm$ 7.65	292.84 $\pm$ 0.45	11.23 $\pm$ 3.45	1.11 $\pm$ 0.09	1.07 $\pm$ 0.04	35.05	10.09
2	293.14 $\pm$ 7.99	311.84 $\pm$ 9.93	305.64 $\pm$ 9.15	13.92 $\pm$ 2.62	1.06 $\pm$ 0.05	1.13 $\pm$ 0.09	47.75	34.81
3	292.57 $\pm$ 7.02	310.18 $\pm$ 8.71	304.52 $\pm$ 10.63	15.93 $\pm$ 2.31	1.09 $\pm$ 0.07	1.10 $\pm$ 0.07	51.25	58.72

Appendix 15. *Crassostrea virginica*. Brood 9. Mean values (± SD, where applicable) are reported for variables. R and L refer to measurements taken on the right or left larval valve. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency (%)	Loss (%)
0	282.16 ± 8.17	302.68 ± 8.49	297.96 ± 8.52	10.64 ± 3.44	1.13 ± 0.11	1.11 ± 0.10	4.4	5.44
1	286.52 ± 4.52	304.30 ± 6.62	298.88 ± 6.81	13.25 ± 2.55	1.10 ± 0.06	1.14 ± 0.06	58.85	6.23
2	290.94 ± 8.90	306.82 ± 9.14	305.92 ± 9.66	16.18 ± 1.79	1.14 ± 0.12	1.10 ± 0.05	16.6	44.48
3	298.58 ± 9.13	316.25 ± 10.25	306.87 ± 10.12	16.99 ± 2.05	1.15 ± 0.16	1.12 ± 0.05	45.8	79.60

Appendix 16. *Crassostrea virginica*. Brood 10. Mean values (± SD, where applicable) are reported for variables. R and L refer to measurements taken on the right or left larval valve. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency (%)	Loss (%)
0	273.29 ± 6.69	296.60 ± 7.10	290.21 ± 9.40	7.89 ± 2.73	1.11 ± 0.03	1.09 ± 0.02	0.4	1.44
1	289.55 ± 8.24	309.00 ± 9.17	300.04 ± 5.83	12.97 ± 2.35	1.08 ± 0.01	1.06 ± 0.01	51.55	33.83
2	294.01 ± 6.44	310.53 ± 8.43	306.75 ± 9.5	16.48 ± 2.28	1.08 ± 0.02	1.07 ± 0.02	33.75	54.88
3	299.20 ± 8.94	315.65 ± 9.96	315.36 ± 7.30	17.82 ± 2.09	1.08 ± 0.02	1.07 ± 0.02	51.95	83.96

Appendix 17. *Crassostrea virginica* . Brood 11. Mean values (± SD, where applicable) are reported for variables. R and L refer to measurements taken on the right or left larval valve. Measurements are reported in µm, unless stated otherwise.

Day	R height	R length	L length	Eyespot diameter	R eyespot roundness	L eyespot roundness	Setting efficiency (%)	Loss (%)
0	277.40 ± 10.27	300.32 ± 10.90	288.38 ± 7.92	9.54 ± 2.01	1.14 ± 0.08	1.16 ± 0.10	4.05	18.60
1	288.18 ± 7.03	308.78 ± 6.92	301.39 ± 9.77	11.82 ± 2.14	1.10 ± 0.08	1.09 ± 0.03	6.85	8.86
2	295.98 ± 6.53	315.90 ± 7.87	307.54 ± 8.63	15.07 ± 2.53	1.08 ± 0.03	1.09 ± 0.04	64.6	69.37
3	299.15 ± 12.20	319.38 ± 13.69	310.54 ± 9.53	15.63 ± 1.80	1.09 ± 0.04	1.20 ± 0.24	53.65	87.00

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