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MARKING THE SHELLS OF PEDIVELIGER EASTERN OYSTERS *CRASSOSTREA VIRGINICA*, WITH A CALCEIN FLUOROCHROME DYE

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ABSTRACT The continued development of effective mark and recapture tools for marine invertebrates is required to better understand fundamental planktonic processes such as larval transport and mortality. Mass chemical marking methods provide researchers with the ability to mark large quantities of individuals at one time with reduced handling stress and costs. Although 3-day-old larvae of *Crassostrea virginica* (eastern oyster) have been marked with calcein (a fluorochrome dye) and recaptured, pediveligers have not been marked nor has mark retention through metamorphosis been verified. To track mark retention through metamorphosis, 12,000 pediveligers were marked for 24 and 48 h in calcein of 25 and 50 mg L^{-1} concentrations, and mark retention was tracked every 7 days for 4 wk. Results indicated that calcein did not affect initial settlement rates, that the distinct bands on larval shells were visible after metamorphosis, that individuals marked as larvae were positively identified up to 4 wk postsettlement without sacrificing individuals, and that lower calcein concentrations than previously documented were effective. This study demonstrates that calcein is a reliable method for marking *C. virginica* pediveligers and suggests that relatively low concentrations of calcein can produce marks and could reduce costs and potential harmful effects.

KEY WORDS: oyster, larvae, calcein, fluorochrome, *Crassostrea virginica*, marking, mark and recapture

INTRODUCTION

Understanding planktonic dispersal and population connectivity in marine ecosystems remains a fundamental obstacle in the management of many marine organisms (Cowen et al. 2007, Krueck et al. 2017). Discerning patterns of larval movement have been attempted with numerical models (Paris et al. 2005, Cowen et al. 2006, North et al. 2008, Narváez et al. 2012, Puckett et al. 2014, OysterFutures Stakeholder Workgroup 2018, Gancel et al. 2019), genetics (Gilg & Hilbish 2003, Taylor & Hellberg 2003, Pujolar et al. 2013), trace elements (DiBacco & Levin 2000, Becker et al. 2007), and recently with chemical markers (Gancel et al. 2019). Confirmation of chemical mark retention from larval stages through metamorphosis would provide a method to deduce origin of juveniles and further validate larval transport model predictions of population connectivity.

Understanding population connectivity of shellfish such as the eastern oyster (*Crassostrea virginica*) could help site restoration and population enhancement areas. Decreasing *C. virginica* populations in Chesapeake Bay and along the Atlantic USA seaboard (Beck et al. 2011, Wilberg et al. 2011) have spurred the construction and designation of numerous oyster restoration and replenishment projects (Wesson et al. 1995, Southworth & Mann 1998, USACE 2012, Bersoza Hernández et al. 2018). Oysters and their reef communities provide vital ecosystem services (Newell 2004), serve as a keystone species by providing three-dimensional structure amid vast mud flats (Coen et al. 1995, Raj 2008), and support local and regional wild and farmed fisheries (Supan 2002, Wieland 2008, Maryland Department of Natural Resources 2018). The ability to mark larval *C. virginica* and reidentify them as juveniles would provide a new tool to inform oyster restoration efforts, such as direct setting during which larvae are released into the water column over suitable settlement habitat (Coon & Pitt 1995, Steppe et al. 2016). Past attempts to directly seed oyster larvae have succeeded in producing juveniles, but were hampered by the restriction of a containment barrier system (Fredriksson et al. 2016, Steppe et al. 2016) or the inability to definitively identify larval origins of spat (Coon & Pitt 1995).

Chemical mark and recapture techniques may provide a method for identifying larval origin. These techniques allow large quantities of individuals to be marked at one time (Warren-Myers et al. 2018), and can be time and cost-effective when compared with mechanical means (Beckman & Schulz 1996, Hammer & Lee Blankenship 2001, Lü et al. 2020). In addition, chemical marks can have little or no negative effect on the organism's survivorship or growth (Mohler 1997, Kaehler & McQuaid 1999, Moran & Marko 2005, Mahé et al. 2010, Sutphin & Morinaka 2010, Spires & North 2021) and may produce markings that do not increase the likelihood of predation (Leips et al. 2001, Mohler et al. 2002). Chemical marking techniques have been used with success in larval fish (Wilson et al. 1987, Hendricks et al. 1991, Secor et al. 1991, Beckman & Schulz 1996, Leips et al. 2001, Crook et al. 2009, Caraguel et al. 2015) and bivalves (Castell & Mann 1994), and have facilitated evaluation of larval fish-stocking programs (Hendricks et al. 1991).

One chemical used for chemical marking of marine invertebrate larvae is the fluorescent dye calcein ($C_{30}H_{26}N_2O_{13}$). Calcein is a fluorochrome dye compound that binds with earth metals in suspension and is incorporated into calcium carbonate structures, whereas organisms grow, resulting in an increase in fluorescence. It has been confirmed to provide 100% marking success in early life stages of several marine molluscs, including *Perna canaliculus* (New Zealand green-lipped mussel) (Fitzpatrick et al. 2013), *Argopecten irradians* (bay scallop)

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and *Mytilus trossulus* (bay mussel) (Moran & Marko 2005), *Nucella ostrina* (sea snail) (Moran 2000), and *Crassostrea virginica* (Gancel et al. 2019). In addition, calcein mark retention through metamorphosis was visible on juvenile *A. irradians* after settlement. Toxicity from immersion in calcein has been observed in some species, with 10-day larval *P. canaliculus* suffering significant mortality when held in calcein concentrations of 50, 100, or 200 mg L^{-1} for 24 h (Fitzpatrick et al. 2013).

Calcein has also been used to study early life history of *Crassostrea virginic*a larvae in Mobile Bay, AL (Gancel et al. 2019). Although Gancel et al. (2019) and other studies of bivalves (Moran & Marko 2005) have found calcein to be an effective marker, the ability of the marker to be maintained through metamorphosis and the effect of different concentrations of calcein on survival and growth of pediveligers and spat have not yet been assessed. This study tests the effectiveness of different concentrations and immersion times for marking *C. virginica* pediveliger larvae with calcein, the duration of mark retention through metamorphosis, and the effect of calcein immersion on initial settlement rates and postsettlement growth. A broad goal was to identify a cost-efficient and nontoxic concentration of calcein for marking of *C. virginica* pediveliger larvae.

METHODS

Marking experiments took place at the Cooperative Oxford Laboratory in Oxford, MD, during the summer of 2018. Pediveliger larvae (150–180 μm) were treated with a combination of two immersion concentrations: low (25 mg L^{-1}) and high (50 mg L^{-1}), and two immersion exposure times: short (24 h) and long (48 h). Each of the four treatments included three pseudoreplicates and three control groups for each exposure time (Table 1). The control groups underwent the same manipulations as the treatment groups, except exposure to calcein. In total, there were 18 different treatment containers for marking larvae (Fig. 1A) and 18 bags with ceramic tiles for observing settlement (Fig. 1B). The influence of calcein treatments on settlement rates and postsettlement growth was tracked for 34 days, as was the presence/absence of calcein marks on the larval and juvenile shells. Oyster pediveligers were marked using Calcein (SE-Mark), a nontoxic liquid that contains 1% calcein.

Pretreatment Care and Handling of Oyster Larvae

Pediveligers of *Crassostrea virginica* (150–180 μm) were obtained from the University of Maryland Center for Environmental Science Horn Point Oyster Hatchery. The broodstock used to produce the larvae were sourced from oyster reefs in the Choptank River, MD. Oyster larvae were acquired in a bundle in a moist coffee filter inside a plastic container. The container with larvae was held in a cooler with ice during the 35-min transport to Cooperative Oxford Laboratory. Onsite, the larval container, was refrigerated at 4°C for 1 day prior to initiation of experiments.

Larval Concentration Estimates

Before experiments started, the larval bundle was placed into a 2-L plastic container filled with 5 μm-filtered estuarine water from the Tred Avon River, Oxford, MD. During enumeration, the larval container was held on ice to minimize movement of larvae (Vlahovich 2009, Priester 2016). The salinity of the Tred Avon River water used was 10.0 psu and the temperature was 27.1°C. Using a perforated plunger to continually mix the suspension, 0.5-mL aliquots were pipetted onto a Sedgewick-Rafter counting cell to determine larval concentrations. Larval concentration estimates were calculated as follows:

$$C = (D \times 2) \times V$$

where D is the larval abundance per 0.5-mL aliquot and V is the volume of water in milliliters in the plastic container.

Pretrial Assessment of Calcein Concentrations

A preliminary trial was conducted to test the calcein concentration of 100 mg L^{-1} that was previously shown to successfully mark larvae of *Crassostrea virginica*, *Argopecten irradians*, and *Mytilus trossulus* (Moran & Marko 2005, Gancel et al. 2019) and a lower untested concentration (50 mg L^{-1}). Based on the larval concentration estimates derived above, 1,000 larvae were transferred to five sterilized 1-L glass beakers containing 0.5 L of filtered (5 μm) river water during July 2018. The protocols for feeding, aeration, larval transfer, temperature control, and settling chambers were the same as the subsequent experiment

TABLE 1.

Treatments for calcein marking of oyster pediveliger larvae.

Treatment combinations	Number of pseudo replicates	Number in each pseudo replicate	Mean percent settlement	Mean initial shell height (mm)	Mean final shell height (mm)
Low—short	3	1,000	12 ±5	1.1 ± 0.1 (30)	3.9 ± 0.3 (11)
Low—long	3	1,000	18 ± 6	1.2 ± 0.1 (30)	3.1 ± 0.2 (27)
High—short	3	1,000	24 ± 11	1.1 ± 0.1 (30)	3.3 ± 0.1 (22)
High—long	3	1,000	13 ± 2	1.2 ± 0.1 (30)	3.5 ± 0.2 (24)
Control—short	3	1,000	14 ± 4	1.1 ± 0.1 (30)	2.8 ± 0.2 (21)
Control—long	3	1,000	14 ± 7	1.2 ± 0.1 (30)	2.9 ± 0.2 (20)

Total individuals in treatment and control groups for each combination of marking durations (short, long) and concentrations (low, high) were 3,000. Treatment concentrations were high (50 mg L^{-1} calcein), low (25 mg L^{-1} calcein), and control (no calcein). Treatment durations were short (24 h) and long (48 h). Initial shell heights were measured 7 days after larvae were immersed in calcein. Final shell heights were measured 35 days after larvae were immersed in calcein. Mean percent settlement and mean initial and final shell heights were calculated by averaging across pseudoreplicates. Numbers in parentheses show sample sizes. Error estimates (±) are SEM.

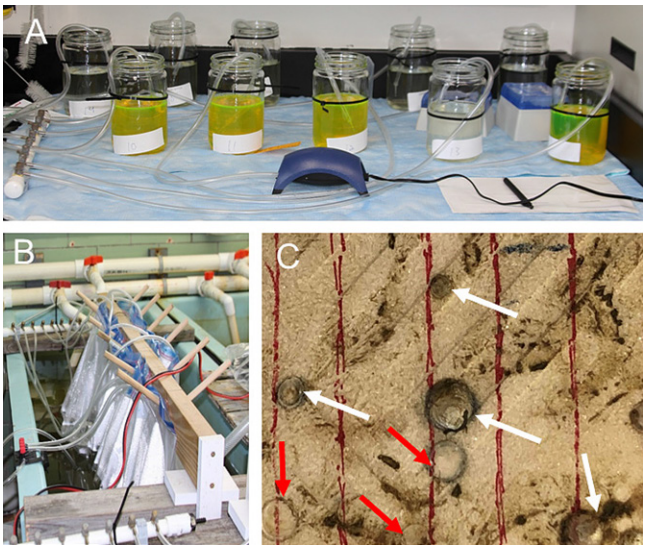

Figure 1. Experimental tools and events. (A) Aerated immersion vessels during larvae labeling with calcein (fluorescent green) or controls (clear). (B) Aerated HDPE settlement chamber bags immersed in flowing, ambient estuarine waters. (C) Unglazed ceramic settlement plate with spat attached. White arrows indicate live spat and red arrows indicate residual valves of dead spat (spat scars).

described below. Four beakers were selected haphazardly to receive calcein: two of these beakers received 5.0 mL of calcein (high treatment of 100 mg L^{-1}) and two received 2.5 mL of calcein (low treatment of 50 mg L^{-1}). The remaining beaker did not receive any calcein and was designated as control. Results of this trial showed zero individuals surviving to settlement in the 100 mg L^{-1} group and successful marking of larvae and settlement in the 50 mg L^{-1} group. Based on these results, the 100 mg L^{-1} concentration was removed from further experiments and a lower concentration (25 mg L^{-1}) was added.

Calcein Immersion Bath

Using the same protocol for estimating larval concentrations described above, 1,000 larvae were transferred to 18 sterilized 1-L glass beakers (18,000 total) (Fig. 1A) containing 0.5 L of filtered (5 µm) estuarine river water on August 8, 2018. Each beaker was aerated with a plastic tube fitted with a micropipette tip and connected to low-pressure aeration. Beakers were observed for several minutes to confirm that larvae were active within each chamber. Ambient salinity at the time of larval transfer was 9.3 psu and the water temperature was held at 27.0°C using the room's thermostat. Twelve beakers were selected haphazardly to receive calcein, where six beakers received 2.5 ml of calcein (high treatment of 50 mg L^{-1}) and six received 1.25 mL of calcein (low treatment of 25 mg L^{-1}). The remaining six beakers did not receive any calcein, and were designated as controls. All beakers were then gently stirred with a small plastic spatula (one for each beaker).

Larval Transfer

After 24 h (short treatments) or 48 h (long treatments), the larvae in each marking chamber (glass beaker) were poured through stacked stainless steel sieves (170 µm, 240 µm) and rinsed with filtered (5 µm) estuarine river water. Larvae were rinsed multiple times with filtered river water until effluent calcein was no longer visually present in rinse water. All rinsing was performed over plastic buckets to capture calcein waste. Larvae on all sieves for each beaker were then transferred to predetermined settlement chambers (polyethylene bags). One or two larvae from each settlement chamber were collected with a micropipette and observed under a microscope to confirm motility and normal development.

Settlement Chambers

Larval settlement chambers were constructed from low-density polyethylene bags (7.6 L), a material proven to be suitable in previous larval setting experiments (Vlahovich 2009, Priester 2016). During construction, each bag was heat sealed at the bottom corners to create square bottom areas. Within each chamber, one prerinsed ceramic unglazed tile (103 cm^2) was placed at the bottom of each square (Fig. 1C). Two liters of filtered (5 µm) river water were placed into each chamber. Aeration was provided by plastic tubes fitted with micropipette tips connected to a low-pressure aeration system (Fig. 1B). Settlement chambers were suspended in a flowthrough system to maintain water temperature similar to ambient river water (28°C) (Fig. 1B).

Settlement Experiment

Larvae were held in their respective settlement chambers for 5–6 d, and were removed when they were the same age, after 6 days postmarking for the short treatment and 5 days for the long treatment. After 48 h, 0.5 L of filtered (0.5 µm) river water was added to each chamber to maintain water quality.

During the experiment, live cultures of the algae *Chaetoceros muelleri* and *Tetraselmis chui* were obtained from Horn Point Oyster Hatchery. During and after marking, larvae were fed algal mixtures consisting of 75,000 cells mL^{-1} of *C. muelleri* (70%) and *T. chui* (30%) at the beginning of the experiment and daily during the experiment. These phytoplankton species and feeding concentrations were used successfully to support late-stage *Crassostrea virginica* larval growth, settlement, and metamorphosis by Priester (2016).

At the end of the settlement experiment, all settlement tiles were removed from their chambers. Immediately after removal from settlement chambers, all attached settlers on each tile were counted using a stereo microscope. Water was removed from the settlement chamber bags and the bags were then inverted and hung to dry. After air-drying for 24 h, the internal surface of each settlement chamber bag was inspected, and settlers were counted. No attempt was made to differentiate between settlers and individuals that had both settled and metamorphosed (i.e., settlement was defined as attachment to substratum, independent of metamorphosis). The total number of settlers per treatment was derived by adding the number of settlers observed on each tile with the number of settlers on the sides of each settlement chamber bag.

After enumeration of settlers on each tile, tiles were moved to a flowthrough system inside the laboratory for growth observations. At this time, feeding with cultured algae ceased, and spat relied on phytoplankton from the Tred Avon River that were delivered with ambient water by the laboratory's flowthrough

system. One day after the settlement period concluded (1 DPS), confirmation of calcein marks was obtained by viewing all spat on each settlement tile with an Olympus BHS compound microscope with epifluorescence accessory for blue light excitation (490 nm) and UV excitation (340 nm), and equipped with an Olympus DP72 digital camera and Olympus cellSens 1.15 imaging software.

Six days after tiles were removed from settlement chambers (6 DPS), shell height measurements from 10 spat on each tile were obtained using the Olympus BHS compound microscope and imaging software described earlier (total n = 10 spat × 18 pseudoreplicates = 180). Shell heights were measured as the distance between the dorsal umbo and the ventral edge of the shell. Individuals were selected for shell height measurements by dividing the tile into five sections and measuring the two spat closest to the centers of the five sections (Fig. 1C), similar to methods used by Priester (2016). Shell heights were recorded weekly for three additional weeks. Some mortality occurred during this time period. If fewer than 10 individuals were on the settlement tile, then all individuals were measured.

Calcein marks were monitored each week by viewing oyster spat on the settlement plates by epifluorescence microscopy under blue light excitation. Autofluorescent elements were assessed by alternating the epifluorescence excitation of the specimen from blue light (490 nm) to UV light (340 nm). Although calcein is only fluorescent under blue light excitation, autofluorescent objects also routinely fluoresce under UV excitation. This step was taken to distinguish between oyster shells intentionally labeled with calcein and autofluorescent fouling organisms and oyster structures.

Analysis

Mean shell height observations were compared across groups to determine whether there were significant differences at each time point, using a pairwise t-test with a Bonferroni adjustment ($\alpha = 0.05$) for multiple comparisons. The mean number of initial settlers within each treatment group was tested for significant differences using an ANOVA F-test ($\alpha = 0.05$). Statistical analyses were conducted using R software version 3.5.3.

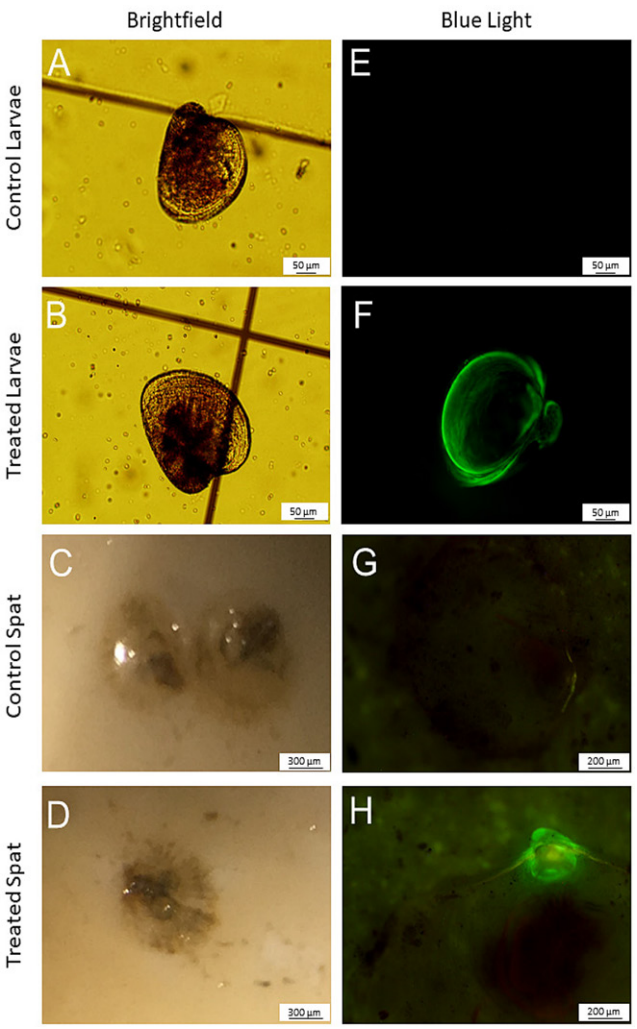

Figure 2. ***Crassostrea virginica*** **larvae and spat imaged under blue light excitation (490 nm) or brightfield illumination. All panels are different individuals except for A and E. Panels (A)–(D) show labeled and control** ***C. virginica*** **larvae and spat under brightfield illumination, panels (E)–(H) show labeled and control** ***C. virginica*** **larvae and spat under blue light excitation (490 nm). Only calcein-labeled larvae show fluorescent valve margins (2F), which remain prominent in the umbo region of a week-old juvenile oyster spat (2H).**

RESULTS

Marking

Calcein concentrations of 25 and 50 mg L^{-1} were both effective for marking valves of *Crassostrea virginica* pediveligers. All of the larvae examined from each treatment that had been immersed in calcein showed the fluorescent signature directly after being removed from the calcein immersion bath (Fig. 2F). In addition, all spat that had been treated with either amount of calcein concentration showed the fluorescent mark when viewed under blue light excitation on August 16, 2018, when settlers were first counted (Fig. 2H). Finally, all spat on the setting tiles from both calcein treatments that were examined once per week were found to retain the calcein mark during the 34-day grow-out phase after settlement. The visual appearance of the mark was similar across treatments. In contrast, none of the control larvae or spat showed fluorescent valve marks (Fig. 2E, G).

After 3-wk postsettlement, potential false-positive markings became visible on spat in all treatment groups (Fig. 3). These conflicting markings in the umbo region were identified as the tensilia elements of growing hinge ligaments, which autofluoresce under multiple excitation wavelengths. This distinction was confirmed by the use of alternating excitation of blue light (490 nm) (Fig. 3A) and UV light (340 nm) (Fig. 3B) on the same specimens, to differentiate calcein fluorescence under 490-nm excitation only, from noncalcein autofluorescence under both 490- and 340-nm excitations.

Although mark retention was not quantified after 34 days, individuals who had been treated with calcein and maintained in flowthrough conditions were examined weekly for mark retention for the next 3 mo. By 9-wk postmarking, most of the marked individuals showed little or no visible external sign of the mark (Fig. 4C). During this time period, the autofluorescent hinge ligament became larger and more apparent (Fig. 4C).

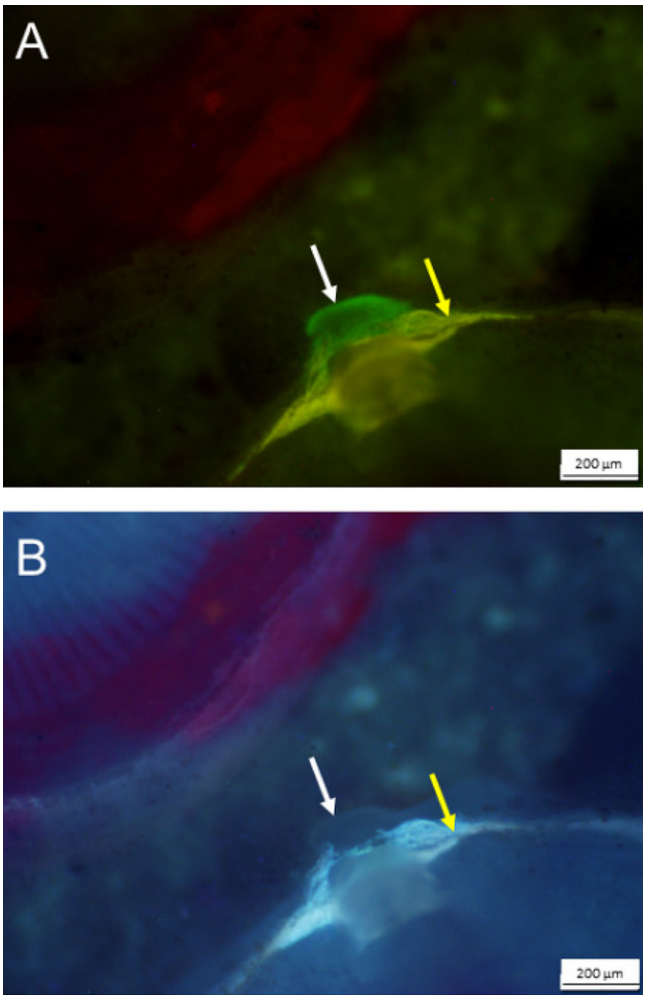

Figure 3. Umbo region of a calcein-labeled *Crassostrea virginica* spat at 3 wk postsettlement, under (A) blue light excitation (490 nm) and (B) UV excitation (340 nm). White arrows indicate locations of the calcein label in the valve umbo of a spat that was imprinted during larval immersion with calcein. Yellow arrows indicate paired lateral hinge ligament tensilia that autofluorescence prominently under both blue and UV light excitation. Panels (A) and (B) are the same view of the same oyster spat.

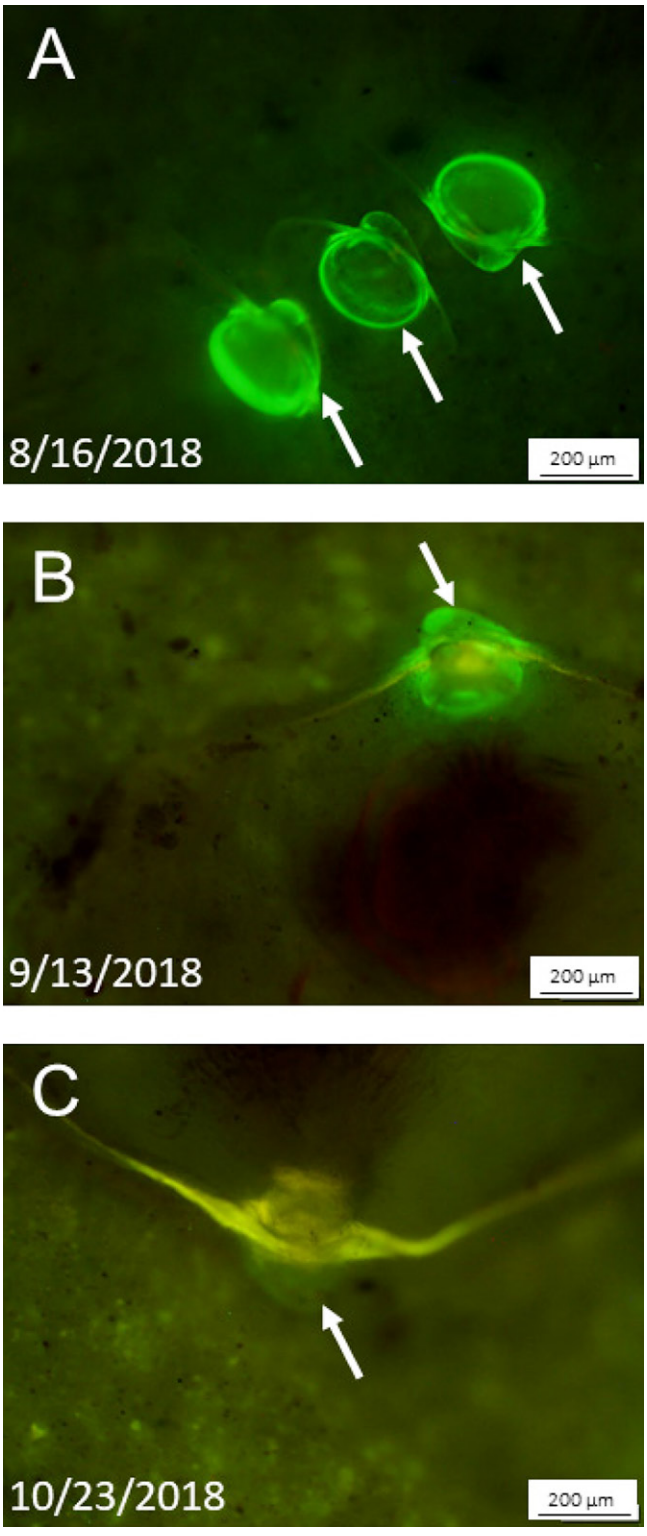

Figure 4. *Crassostrea virginica* spat imaged under blue light excitation (490 nm). Images correspond to (A) 1 day after marking; (B) 28 days after marking; and (C) 66 days after marking. White arrows in panels indicate calcein imprints in the umbo regions of oyster spat valves. Panel (A) contains three different individuals. Labeling in panel (C) is barely detectable. All panels contain images of different individuals.

Growth

From the beginning to the end of the 34-day growth observation period, dimensions of treated individuals were either significantly larger or no different than those of controls. The low, high, and control individuals in the 24-h immersion group grew on average 2.8 ± 1.0, 2.2 ± 1.1, and 1.7 ± 0.8 mm, respectively (Table 1). During the same time span, the low, high and control individuals in the 48-h immersion group grew on average 1.9 ± 1.3, 2.3 ± 0.9, and 2.3 ± 0.8 mm, respectively (Table 1). Mean shell heights of individuals in treatment groups during the 4-wk grow-out period were either not different or were significantly higher ($P < 0.05$) than those of control groups, indicating no significant negative effects of marking on growth (Fig. 5). Within the 24-h immersion group, spat subjected to low concentrations of calcein (25 mg L^{-1}) were significantly ($P < 0.05$) larger than those of control treatment groups on weeks 3 and 4, and spat in

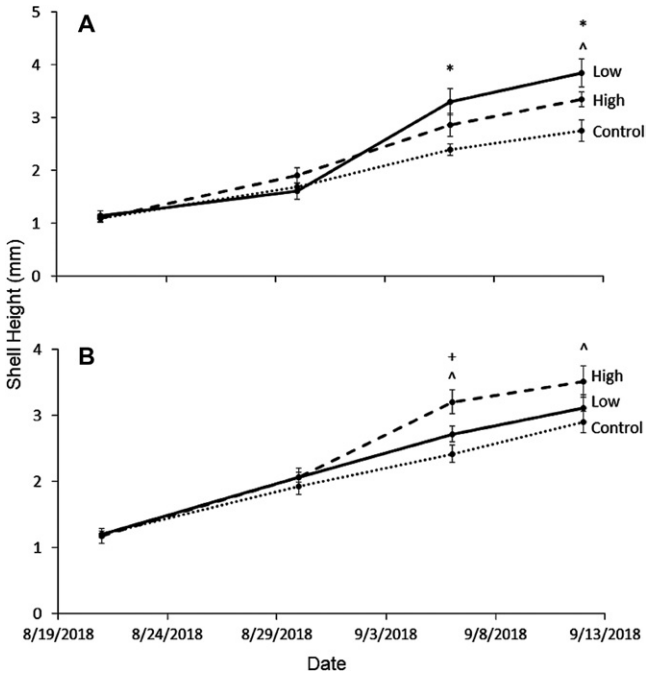

Figure 5. Mean shell height (mm) (±SEM) of *Crassostrea virginica* spat over time among individuals subjected to the (A) short (24 h) and (B) long (48 h) immersion treatment schedules in control (dotted line), low (25 mg L^{-1}) immersion concentration (solid lines), and high (50 mg L^{-1}) immersion concentration (dashed lines) treatments. Initial shell heights were measured 1 wk after removal from settlement chambers (August 21, 2018). Appended labels indicate the following statistical differences by pairwise t-test with Bonferroni adjustment ($P < 0.05$). * A significant difference in shell height between low and control treatments. ^ A significant difference in shell height between high and control treatments. + A significant difference in shell height between low and high treatments.

the high concentration (50 mg L^{-1}) group were significantly ($P < 0.05$) larger than those of the controls on week 4. In the 48-h (long) immersion group, spat subjected to low (25 mg L^{-1}) concentrations of calcein were significantly ($P < 0.05$) larger than the controls on week 3, but not on week 4. In addition, spat subjected to the high concentrations (50 mg L^{-1}) of calcein were significantly ($P < 0.05$) larger than controls on weeks 3 and 4.

Settlement

The mean initial numbers of settlers were similar between all treatment groups: no significant differences were detected. In the 24-h immersion group, average settlement ranged from 11% to 24% across all treatments and controls (Table 1). In the 48-h immersion group, on average 13%–18% of the larvae settled successfully across the low, high, and control groups (Table 1). Variability between pseudoreplicates was high (Fig. 6), with the high concentration and 48-h immersion treatment and the low concentration and 48-h immersion treatment (Fig. 6B) having the narrowest and widest data ranges, respectively.

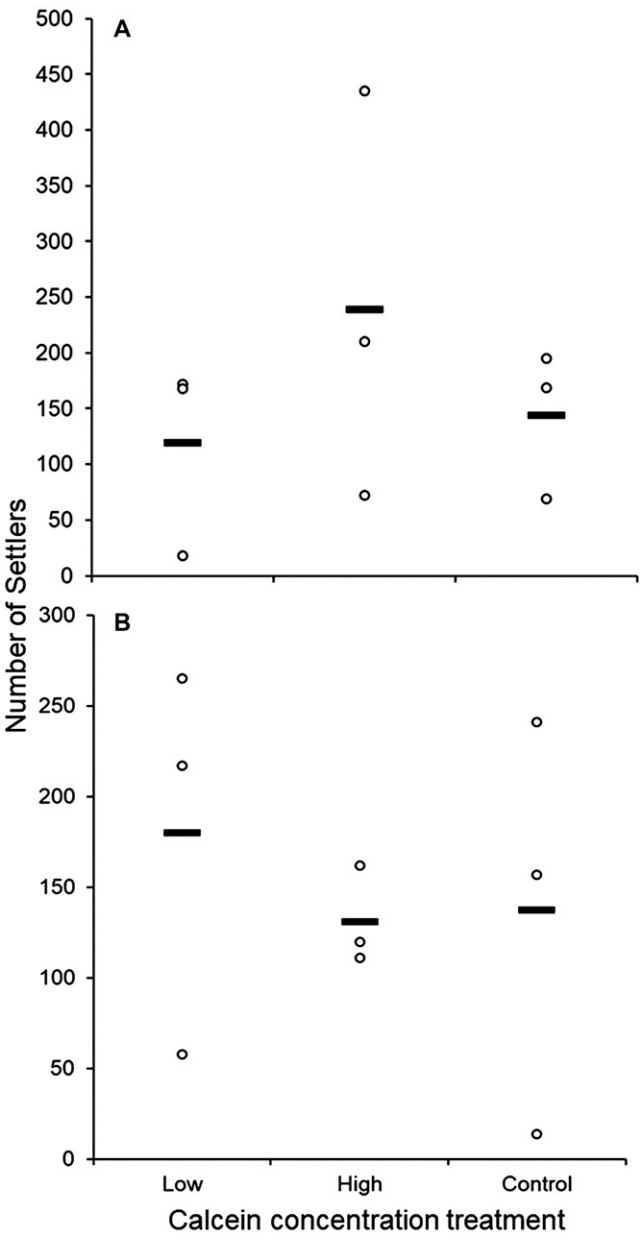

Figure 6. Number of settles (larva that has attached to a substratum) for the (A) short (24 h) and (B) long (48 h) immersion treatments. Pseudoreplicates ($n = 3$) consisted of 1,000 individuals each. Circles indicate individual pseudoreplicates from each treatment, and black bars represent the means of the pseudoreplicates for each treatment. No significant difference between group means was detected (ANOVA, *F*-test, $P < 0.05$).

DISCUSSION

Marking Success

Calcein concentrations of 25 and 50 mg L^{-1} and immersion times of 24 and 48 h appear to be effective in creating marks in late-stage *Crassostrea virginica* larvae. Marks were visible in all examined larvae after immersion, marks carried through metamorphosis, and the marks were visible for at least 34 days in all juveniles. No obvious differences in mark clarity were detected between calcein labeling concentrations, and the visual appearance of the mark was similar across all treatments. The mark on juveniles encircled the valve umbo region (Fig. 4), which corresponds to the location of the prodissoconch or larval shell in the juvenile oyster (Kennedy et al. 1996). Similar to the results of calcein marking experiments in other marine bivalves *Argopecten irradians* and *Mytilus trossulus* (Moran & Marko

2005), results of this experiment indicate that calcein immersion appears to be a simple and effective method for creating marks that are retained through metamorphosis in *C. virginica*.

After metamorphosis, the quality and retention of the mark in *Crassostrea virginica* was high through 34 days, a result similar to that observed for *Argopecten irradians* and *Mytilus trossulus*, two species that had readily visible marks up to 47 days after metamorphosis (Moran & Marko 2005). The degradation of the mark after metamorphosis in *C. virginica* may be because of ongoing shell dissolution and degenerative processes related to pH (Waldbusser et al. 2011). The valves of pediveliger oyster larvae are typically 4- to 6 μm thick, and are composed primarily of aragonite (Kennedy et al. 1996) during the stage when larvae were marked in this experiment. After metamorphosis, complex biochemical changes occur at the cellular level as the juvenile oyster transitions from constructing its shell from aragonite to constructing it from calcite, a less soluble form of calcium carbonate (Doney et al. 2009). The aragonite larval shell will dissolve more rapidly than the predominantly calcite juvenile/adult shell (Miller et al. 2009). Hence, the disappearance of the calcein mark may have been because of the dissolution of the aragonite shell, and not necessarily to the degradation of the calcein label itself. In future work, cross sectioning the shell of juveniles (Spires & North 2021) could be used to determine whether the mark degrades as the shell dissolves, or is retained internally.

Changes in morphology of the rapidly growing juveniles may have led to false-positive marks as the hinge ligament grew and autofluoresced under the same excitation light source as calcein (Fig. 3). The false-positive mark in the *Crassostrea virginica* hinge ligament was identified by alternating the light source on the same specimen between blue light (490 nm) and UV light (340 nm) (Fig. 3). Autofluorescence in bivalves also has been noted in larvae of the giant clam (*Tridacna noae*) (Braley et al. 2018) and in juveniles of the winged pearl oysters stained with calcein (*Pteria sterna*) (Cáceres-Puig et al. 2011). The autofluorescence observed in *P. sterna* juveniles is similar to that observed in our study of *C. virginica*; autofluorescence was detected in the umbo region and appeared as a thin band radiating out along the growing margin. Green autofluorescence, such as that of the hinge ligament in *C. virginica*, could confound studies using calcein. Researchers working with green fluorescent labels (e.g., calcein) should be aware of the potential for development of false-positive marks in organisms of interest.

Calcein marking of larval *Crassostrea virginica* using the methods described in this manuscript will provide researchers a tag that can be readily identified on the exterior of juveniles for at least 4 wk postmetamorphosis. The testing of higher calcein concentrations on larval *C. virginica* may lead to longer mark retention periods and could be investigated further. The pretrial experiment using a calcein concentration of 100 mg L^{-1} (after Gancel et al. 2019) ended in 100% mortality after 48 h, but could be repeated with different broodstock and environmental conditions.

Growth

No deleterious effects on postmetamorphic growth were detected in any treatment group, similar to the results observed in marking experiments on *Argopecten irradians* larvae through metamorphosis (Moran & Marko 2005). For *Crassostrea virginica*, the effects of calcein on growth were positive in all treatments, because the mean shell heights of all individuals exposed to calcein were significantly larger than those of controls by the end of the observation period. Enhanced growth of marked spat compared with controls also was observed in juvenile *C. virginica* spat exposed to calcein (Spires & North 2021). Oyster growth rates are inherently variable (Singh & Zouros 1978), and can be influenced by numerous factors, including water quality, genetics, gamete quality, and available phytoplankton prey (Helm et al. 1991, Berntsson et al. 1997, Jonsson et al. 1999, Priester 2016). Interestingly, this study is the third study to demonstrate that bivalves immersed in calcein experienced higher growth (Spires & North 2021) or survival rates increased (Moran & Marko 2005) when compared with controls. Understanding the role of calcein on growth would be important for growth studies of, for example, hatchery-produced shellfish compared with wild shellfish.

Settlement

The mean number of settlers was similar between treatments in both the short (24 h) and long (48 h) treatment groups. Variability within pseudoreplicates was observed (Fig. 6) but is not unexpected, as competency to settle even within the same cohort is not uniform (Vlahovich 2009). The lack of significant variability in the initial number of settlers between treatments suggests that the methods used in this investigation did not alter the ability for competent *Crassostrea virginica* pediveligers to settle under suitable conditions of salinity and food availability.

CONCLUSION

Results presented here indicate that calcein is an effective marker of pediveliger *Crassostrea virginica* at concentrations of 25 and 50 mg L^{-1}, with the ability to confirm marked individuals in 100% of juveniles immediately after metamorphosis, for up to 39 days postimmersion, and for 34 days postsettlement. No negative effects on initial settlement or postsettlement growth were observed, suggesting that calcein is an effective marker for *C. virginica* larvae at lower salinities (approximately 10 psu) and lower calcein concentrations than previously tested (approximately 15 psu, 100 mg L^{-1}) (Gancel et al. 2019), and can be used to facilitate mark and recapture investigations in salinity regimes similar to the middle of Chesapeake Bay (10–12 psu).

Based on this assessment, creating marks with calcein could have multiple uses for resource managers, fisheries scientists, and private oyster production facilities. The ability to mark pediveliger *Crassostrea virginica* and to reidentify them as juveniles would facilitate testing and validating tests of *in situ* setting methods, such as direct setting (Coon & Pitt 1995, Steppe et al. 2016), which could be enhanced with the ability to definitively identify larval origin (Coon & Pitt 1995). Oyster restoration managers charged with restoring oyster populations in coastal regions, where larval transport helps to maintain population stability through source and sink mechanisms, could enhance siting decisions by using larval transport models (North et al. 2008, Puckett et al. 2014) that have been validated with short-term (e.g., 3–4 days) field deployments of tagged larvae. Such validation studies face significant challenges because of advective losses; however, similar efforts have recently been undertaken within Mobile Bay, AL, and were successful in recapturing two individuals out 22 million released (Gancel et al. 2019), indicating proof of concept and that further development of field methods could increase recaptures. For private oyster growers, calcein-tagged larvae

could be used to determine the contribution of hatchery versus wild larvae to recruitments at on-bottom oyster leases.

Recommendation

Future work to investigate rapid high-throughput detection methods of marked larvae or spat would support field programs that are currently limited by time-consuming manual or semiautomated confirmation methods (Gancel et al. 2019). Researchers wishing to mark multiple batches of larvae with calcein for release into the same system could investigate the potential to produce multiple marks, as is possible on juvenile *Crassostrea virginica* (Spires & North 2021), which may allow multiple cohorts to be distinguished.

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