Journal of
Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2022) **88**: eyac004. https://doi.org/10.1093/mollus/eyac004
Published online 25 February 2022

Marking the shells of juvenile and adult eastern oysters, *Crassostrea virginica*, with the fluorochrome dye calcein and measuring growth and mortality after marking

Jason E. Spires[1,2] and Elizabeth W. North[1]

[1]*Horn Point Laboratory, University of Maryland Center for Environmental Science, 2020 Horns Point Road, Cambridge, MD 21613, USA; and*
[2]*NOAA Cooperative Oxford Laboratory, National Centers for Coastal Ocean Science, National Ocean Service, National Oceanic and Atmospheric Administration, 904 S. Morris Street, Oxford, MD 21654, USA*

Correspondence: J.E. Spires; e-mail: Jason.spires@noaa.gov

(Received 24 May 2021; editorial decision 21 September 2021)

ABSTRACT

Techniques for positive re-identification of finfish are well developed and are used to estimate vital rates, migration patterns and stock structure. Fluorochrome dyes, like nontoxic calcein, have been used with success to mark hard parts of fish and the shells of some molluscs. Yet, despite the commercial and ecological importance of the eastern oyster *Crassostrea virginica*, a systematic evaluation of the effectiveness of calcein for marking this species does not exist. In this study, methods that have been used for marking freshwater mussels with calcein were tested on 2-week- and 2-month-old juvenile *C. virginica* and on 3-year-old adult *C. virginica*. Individuals were immersed in 250 mg l^{-1} of calcein for 24 h either once (single treatment) or three times (triple treatment), and then observed to determine whether marks were visible, how long they remained visible and whether calcein influenced growth and mortality of the different age classes. Results indicated that all marked individuals were recognizable under blue light, that multiple distinct marks were possible on juveniles and that marks on juveniles could remain visible for 3.75 years. Growth of juveniles and adults treated with calcein was not significantly different from controls after 11 months. Survivorship did not differ between treated and control individuals for all treatments and ages, except the 2-month-old juvenile oysters that were immersed three times. Results indicate that calcein is an effective and safe marking technique for juvenile oysters from robust cohorts, and could be used to enhance studies of growth, mortality, larval transport and settlement *in situ*.

INTRODUCTION

Globally, oyster populations have declined to less than 85% of historic levels (Beck *et al.*, 2011), with restoration projects currently underway in numerous coastal systems (USACE, 2012; McCann, 2018). Targeted restoration efforts are supplementing wild populations by planting hatchery-spawned juveniles in protected regions to rebuild broodstock levels and restore ecosystem function (NOAA Restoration Center, 2007; USACE, 2012). Current restoration monitoring strategies in Chesapeake Bay focus on the commercially and ecologically important eastern oyster *Crassostrea virginica* (Gmelin, 1791) and include assessments of abundance of individuals in planted areas (NOAA, 2017). However, the long-term survival and growth of introduced individuals is difficult to assess in regions with natural recruitment. The testing and development of an approved method for tagging oysters would allow introduced individuals to be distinguished from wild stock and help monitor growth and survival of planted oysters.

Numerous physical and chemical methods have been used to tag and release fish and molluscs. Attempts to mark post-metamorphosed bivalves have been primarily focused on internal and external tagging methods requiring direct handling of each individual. Internally, acoustic passive integrated transponder tags have been placed in the mantle cavity of freshwater mussels (Kurth *et al.*, 2007). Externally, numerous methods have been employed with some success for small numbers of relatively large individuals, including etching (Mattice & Wright, 1986; McMahon & Williams, 1986), paints and dyes (Buttner & Heidinger, 1980), and glue-on and wire tags (Lim & Sakurai, 1999; Bayne, 2002; Evans, Hick & Whittington, 2016; Twist, Rayment & Hepburn, 2016).

Chemical tagging methods have proven effective in mass marking fish and some molluscs. Oxytetracycline, an antibacterial chemical (Stewart, 2011), has been used to mark otoliths, fin rays and scales in fish (Lorson & Mudrak, 1987; Secor, White & Dean, 1991; Isermann, Bettoli & Sammons, 1999). Calcein, a fluorescent (fluorochrome) chemical (U.S. Fish and Wildlife Service, 2008), has been used with success to mark fish otoliths (Wilson, Beckman & Dean, 1987), fin rays and scales (Alcobendas *et al.*, 1991; Gelsleichter *et al.*, 1997) and abalone

(Day, Williams & Hawkes, 1995; Riascos *et al.*, 2007). Calcein marking techniques are effective on many members within the class Bivalvia: marine mussels (Kaehler & McQuaid, 1999) and quahog (*Arctica islandica*; Beirne, Wanamaker & Feindel, 2012; Wanamaker & Gillikin, 2019); larval bay scallops and mussels (Moran & Marko, 2005); pearl oysters (Linard *et al.*, 2011; Joubert *et al.*, 2014); tropical scallops (Thébault *et al.*, 2006); burrowing bivalves (*Loripes lacteus*; van der Geest *et al.*, 2011); freshwater mussels (Eads & Layzer, 2002); hard clams (*Mercenaria mercenaria*; Goodwin *et al.*, 2021); and 3-d-old oyster larvae (*C. virginica*; Gancel *et al.*, 2019).

Calcein (SE-Mark®) is a nontoxic liquid that contains 1% calcein ($C_{30}H_{26}N_2O_{13}$). Calcein is a fluorochrome dye compound that binds with earth metals in suspension resulting in an increase in fluorescence. It is approved for bath marking treatments on finfish and select invertebrate organisms under the Investigational New Animal Drug (INAD) programme of the U.S. Fish and Wildlife Service's Aquatic Animal Drug Approval Partnership (AADAP) programme (U.S. Fish and Wildlife Service, 2008, 2020). INADs are drugs that are in the approval process under the Food and Drug Administration but are not yet fully approved. Prior to the initiation of this study, *C. virginica* were not included on the list of species that were approved to be treated with calcein and then placed *in situ*. The durable markings created by calcein staining have had minimal effects on the growth and survival of bivalves (Eads & Layzer, 2002; Moran & Marko, 2005; Lucas *et al.*, 2008). However, certain species have experienced deleterious effects on growth and/or survivorship from calcein exposure (Brooks, Heidinger & Kohler, 1994; Gelsleichter *et al.*, 1997; Fitzpatrick, Jeffs & Dunphy, 2013). This study was initiated to determine the viability of using calcein as a chemical marker on *C. virginica*. The objectives were to measure mark retention and to determine whether calcein could affect the growth and mortality of treated individuals.

MATERIAL AND METHODS

Oyster marking experiments took place at the Cooperative Oxford Laboratory (COL) in Oxford, MD, during the summer of 2016. Three age classes of *Crassostrea virginica* (2 weeks, 2 months and 3 years old) were tested with two types of exposures (one 24-h immersion and three 24-h immersions in 250 mg l^{-1} of calcein). Growth, mortality and tag retention were tracked for 330 d after immersion. Mark retention was tracked on a subset of individuals for 3.75 years. Statistical tests for differences in growth and mortality between treatments were conducted.

Oyster source and pre-immersion care

Cultured 3-d-old juvenile *C. virginica*, which had settled on oyster shell (spat-on-shell), were obtained from the University of Maryland Center for Environmental Science Horn Point Oyster Hatchery in Cambridge, MD, on 25 May and 6 July 2016; these individuals would become the 2-month- and 2-week-old cohorts at the time of marking, respectively. These two cohorts of juveniles were spawned from different broodstock, aligning with FAO's Hatchery Culture of Bivalves standard practices (Helm, 2004), with care taken to ensure that multiple male and female oysters contribute gametes to each cohort. Adults that were *c.* 3 years old were obtained from a commercial aquaculture company, Marinetics, Inc., in Cambridge, MD, on 13 May 2016. Age groups were chosen based on a study in which two freshwater mussels, *Lampsilis cardium* and *Actinonaias pectorosa*, were successfully marked with calcein (Kaehler & McQuaid, 1999).

Before immersion, oysters were held in cages connected to the COL pier on the Tred Avon River, Oxford, MD. The cages were lowered to near bottom (water depth *c.* 3.0 m) of the river, where the oysters experienced ambient river conditions. No attempt to supplement food was made. The 2-month-old cohort (obtained on 25 May 2016) experienced significant mortality (*c.* 80% loss) during the holding period compared to mortality in the 2-week-old and adult cohorts, which was negligible. Three days prior to immersion, oysters were moved to an outdoor, *c.* 3,750-l shaded cylindrical tank maintained on a flow-through system with ambient Tred Avon River water that had an initial salinity of 10.7 and temperature of 27.9 °C. Dissolved oxygen, temperature and pH were monitored using a calibrated YSI 6600 data sonde.

Shell heights of oysters were measured on day 1 of the single immersion treatment (25 July 2016). The total shell height of all adults was measured from the umbo to the leading ventral edge using callipers. For 2-week-old (obtained on 6 July 2016) and 2-month-old (obtained on 25 May 2016) juveniles, a random subsample ($n = 50$) from each group was measured with callipers. Throughout the experiment, care was taken not to break off the delicate growing edges of shells. The initial mean shell heights (± SE) of the 2-week and 2-month age classes were 3.8 ± 1.2 and 23.5 ± 5.8 mm, respectively. Adults had a mean initial shell height of 77.9 ± 7.9 mm.

Batch immersions of oysters

The single immersion treatment was conducted on 25–26 July 2016 and post-immersion monitoring began on 26 August 2016. For the triple immersion treatment, the 24-h immersions began on 2, 18 and 31 August 2016 and followed the same protocol as the single immersion treatment described below. In between the immersion dates, and after the 3-d observation period, the treated and control oysters were placed in cages and suspended from the COL pier for 11 d, making a total of 14 d in between treatments. For these oysters, post-immersion monitoring began on 7 October 2016.

For the single and triple immersion treatments, individuals were selected at random from a pool of similarly aged oysters and were placed into bags before the treatments began to help with transport and handling (Table 1). For both treatments combined, the 2-week-old group contained *c.* 1,200 individuals split into 12 bags of 100 oysters each, with 3 bags receiving one calcein immersion treatment, 3 bags receiving three calcein immersion treatments and 6 control bags (Table 1). The 2-month-old group contained 128 individuals, which were split into four bags of 32 individuals each, with one bag receiving one calcein immersion treatment, one bag receiving three calcein immersion treatments and two control bags. The 3-year-old group contained 400 individuals split into bags of 20 individuals each ($n = 20$ bags), with 5 bags receiving one calcein immersion treatment, 5 bags receiving three calcein immersion treatments and 10 control bags. A total of *c.* 864 individuals of all ages in 18 bags were exposed to calcein between the two immersion schedules (single and triple) and a total of 864 individuals in 18 bags were used as controls to facilitate side-by-side comparisons. All bags containing oysters were the same size and were made of polyethylene diamond mesh with a 20-mm opening. Bags were acquired from an aquaculture supplier and are commonly used throughout the Chesapeake Bay oyster aquaculture industry.

All treated individuals were immersed in a calcein solution for 24 h, either once (single treatment), or three times (triple treatment) separated by 2-week intervals. The 1% calcein solution was obtained from Western Chemical Incorporated. A single concentration of 250 mg l^{-1} of calcein (derived from the 1% calcein solution) and immersion time (24 h) was used for all groups. The concentration and immersion schedule was based on the findings and recommendations of previous investigations on marking freshwater and marine mussels (Kaehler & McQuaid, 1999; Eads & Layzer, 2002) and followed INAD immersion concentration protocols (U.S. Fish and Wildlife Service, 2008, 2020). For each age group,

Table 1. Number of individuals in treatment and control groups for each age group of oysters.

Oyster age groups	Mean shell height (mm) ±SD (date measured)		Single immersion treatment	Single immersion controls	Triple immersion treatment	Triple immersion controls	Total in study
2 weeks	3.8 ± 1.2 (25 June 2016)	Total number	300	300	300	300	1,200
		Replicates	3	3	3	3	
		Number in each replicate	100	100	100	100	
2 months	23.5 ± 5.8 (25 June 2016)	Total number	32	32	32	32	128
		Replicates	1	1	1	1	
		Number in each replicate	32	32	32	32	
3 years	77.9 ± 7.9 (1 June 2016)	Total number	100	100	100	100	400
		Replicates	5	5	5	5	
		Number in each replicate	20	20	20	20	

Mean shell heights (mm) before treatments began were calculated with oysters from each age group before they were separated into treatment and control groups. Sample sizes for shell height measurements were $n = 50$ for 2-week-old oysters, $n = 50$ for 2-month-old oysters and $n = 400$ for 3-year-old oysters.

Figure 1. Example of calcein immersion containers with adult *Crassostrea virginica* undergoing calcein immersion treatment (right) and controls (left). Treated water appears green because of the addition of calcein. Containers were covered during the 24-h marking treatment to prevent breakdown of calcein by exposure to light.

control individuals were treated identically to treatment individuals except that they were not exposed to calcein (Fig. 1).

During the treatments, each bag was placed into a separate 16-l plastic container filled with 8 l of filtered Tred Avon River water mixed to a concentration of 250 mg l^{-1} calcein (Fig. 1). Initial water quality conditions in the plastic containers were similar to the ambient conditions described above. All 16-l plastic immersion tanks were covered with opaque plastic lids to prevent breakdown of fluorochromes by light (Shapiro, 2005; Honeyfield *et al.*, 2008). Each container was continuously oxygenated using air stones. River water was filtered through a series of polyester felt bag filters, the smallest pore size of which was 1 μm. Immersion for each age group began at the same time. Oysters were fed with a suspension of instant alga concentrate (Tetraselmis 3600 Premium Fresh; Reed Mariculture, Inc.) at the time of immersion because marking success may depend on feeding (Day *et al.*, 1995; Eads & Layzer, 2002). Algal suspensions measured an initial concentration of 100,000 cells ml^{-1} (Kemp, 2006). Two additional feedings at 8 and 16 h post-immersion occurred over the course of the 24-h immersion period using the same concentration of algae.

After 24 h, all bags were removed from immersion tanks and rinsed with filtered river water. All rinse water from calcein-treated oysters was captured and stored for disposal. All bags of oysters were placed into separate cages for each age group and held in a shaded outdoor flow-through tank for 3 d for observation. After 3 d, all oysters were inspected for obvious signs of mortality and then oysters in each treatment and age group were placed in cages suspended in the Tred Avon River (NOAA, 2021) from the COL pier. The cages were lowered to near bottom (water depth *c.* 3.0 m). This depth simulates light intensities experienced on intact oyster reefs found in the Oxford Laboratory Oyster Sanctuary (MDNR, 2016); these reefs surround the pier and have oysters at depths ranging from 2 to 5 m, based on hand tong and diver observations by Jason E. Spires. Maintaining similar depths to natural oyster reefs was important because breakdown of fluorochrome compounds due to sunlight may impact mark retention (Shapiro, 2005; Honeyfield *et al.*, 2008).

Post-immersion mark detection

Fluorescent mark retention was evaluated immediately after each 24-h immersion treatment and then repeatedly over time to determine whether the marks degraded. Calcein exhibits a bright green fluorescence when exposed to blue light of *c.* 500 nm wavelength. The SE-Mark® fluorescent detector available from Western Chemical Co. and the Nightsea Bluestar® fluorescent detector were acquired for initial mark confirmation to ensure that marks were visible with both instruments. The Nightsea Bluestar® fluorescent detector was used for all subsequent photography and mark confirmation. This combines a blue-light handheld flashlight and filter glasses; the blue light excites the fluorescence from the labelled oysters and the filter glasses block the reflected excitation light so that the mark is visible for detection by eye (Fig. 2). Positive mark confirmation was obtained by viewing oysters through the Nightsea Bluestar® fluorescent detector in a dark room with no additional light sources.

A random subsample of oysters ($n = 30$) from each treatment group was selected and photographed during the initial 3-d observation period, 1 month post-immersion and 6 months post-immersion to determine mark retention and intensity. In addition, to track long-term mark retention, 34 individuals from the 2-week- and 2-month-old triple immersion treatment groups were moved to a separate bag in July 2017, suspended from the COL pier at *c.* 3.0 m depth, and their mark retention was assessed periodically through March 2020.

To examine the structure of the calcein marks within the shell, shells were sectioned following the methods used by Mahé *et al.* (2010). Five individuals from the 2-week-old age group, which were

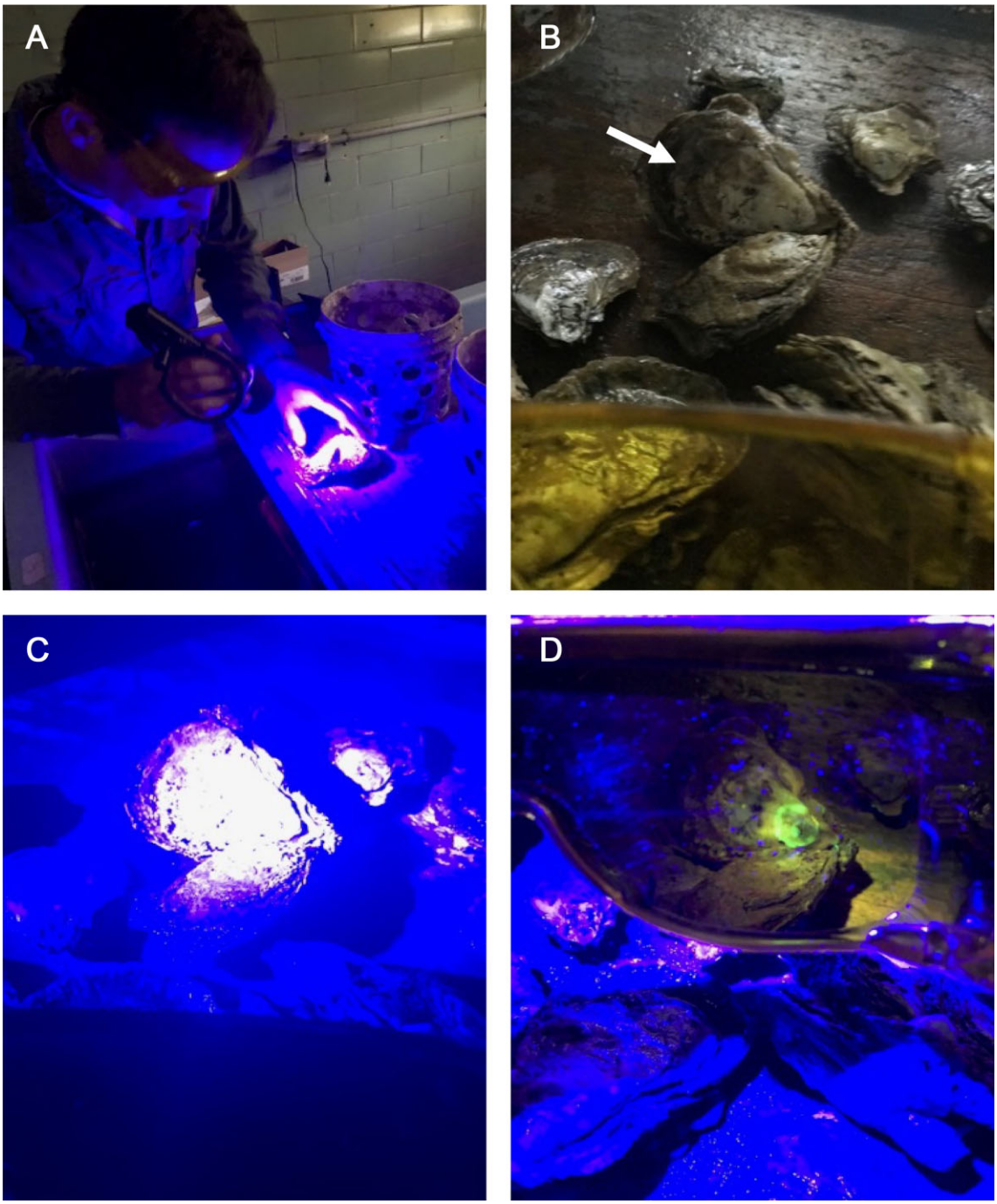

Figure 2. Photographs of *Crassostrea virginica* and the Nightsea Bluestar® fluorescent detector used for detecting calcein-induced fluorescent marks on oyster shells. **A.** Author Jason E. Spires using the Nightsea Bluestar® glasses and blue-light flashlight to check for a fluorescent mark on an oyster in a dimly lit room. **B.** A 2.75-year-old oyster that was marked with calcein when it was 2 months old and the Nightsea Bluestar® glasses in the foreground. **C.** The same oyster under the blue light of the Nightsea Bluestar® flashlight. **D.** The same oyster under the blue light when viewed through the detector glasses, with the green fluorescent mark clearly visible. The white arrow in panel (B) indicates the oyster of interest. The oyster was marked on 2 August 2016 and photographs were taken on 3 May 2019.

subjected to multiple immersions, were sacrificed when they were 7 months old and the valves were embedded in an epoxy mould. One thin section from each oyster was cut transversely along the growing axis from the umbo to the outermost posterior end. One section from each individual was examined under fluorescence microscopy using an Olympus BX41 microscope equipped with a 460–490-nm excitation filter.

Post-immersion observations of growth and mortality

After immersion and the 3-d observation period, mortality and growth were recorded monthly for 11 months. Each month, number of surviving individuals and number of dead individuals were recorded after inspecting all oysters in each age group. In addition, shell height observations were conducted on a random subsample

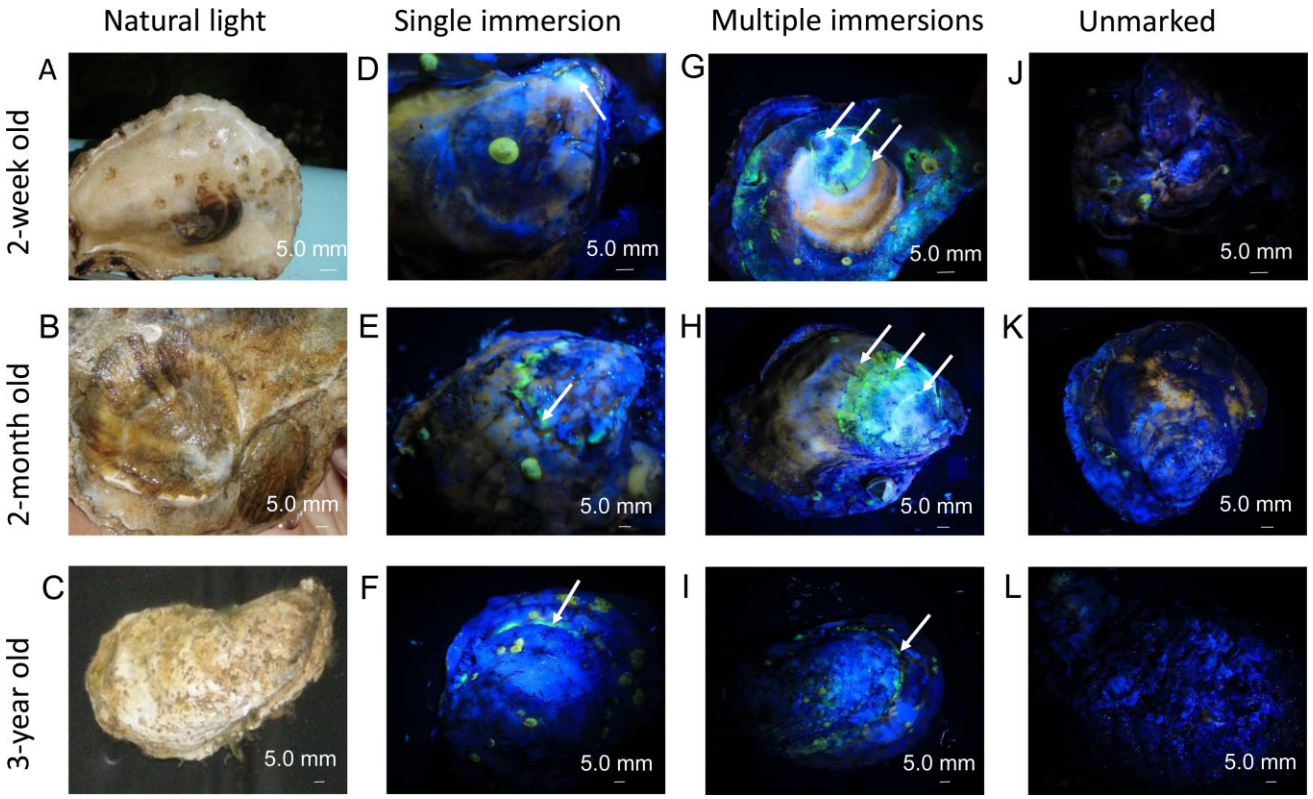

Figure 3. Labelled *Crassostrea virginica c.* 240 d after initial treatment in calcein, photographed under Nightsea Bluestar fluorescent light and filter. **A–C.** *Crassostrea virginica* under natural light. **D–F.** *Crassostrea virginica* that underwent a single calcein immersion for 24 h. **G–I.** *Crassostrea virginica* that underwent three calcein immersions, each for 24 h. **J–L.** Control *C. virginica* that were handled similarly but were not treated with calcein. Rows contain different ages of oysters, which were 2 weeks old (top row), 2 months old (middle row) and 3 years old (bottom row) at the time of marking. White arrows indicate the location of the calcein label in the oyster shell. Bright green circles are autofluorescing barnacles that were not marked and grew after treatment.

($n = 50$) of individuals from the 2-week- and 3-year-old age groups. All oysters in the 2-month age group were measured due to limited abundances. Dead oysters and remnant shells were discarded after each count.

Average shell heights, determined by shell height measurements, were used to indicate signs of stress. The percentage of individuals surviving at each sampling period (P) was calculated as

$$P = 100 \times \frac{N}{N_0}, \tag{1}$$

where N_0 is the number of oysters prior to immersions (e.g. number of oysters initially) and N_t is the number of oysters observed at time t. Cumulative percent mortality (M) was calculated as $1 - P$ at $t = 11$ months.

Statistical analyses

Using slower growth as an indication of stress, average shell heights were compared between treated and control individuals in the same age group. Welch two-sample t-tests ($\alpha = 0.05$) were used to test for difference between shell heights of treated and control individuals within each age group. Normality was tested with a Shapiro–Wilk test and homogeneity of variance was tested with Bartlett's and Levene's tests. Statistical analyses were conducted using R v. 3.5.3 (R Core Team, 2020).

To test whether the value of M differed significantly between treated and control individuals in the same group, the equality of the two proportions was tested against the alternative that they were not equal. In statistical notation, this test was

$$H_o : M_1 = M_2 \quad \text{versus} \quad H_A : M_1 \neq M_2,$$

where M_1 and M_2 are the cumulative percent mortality for the treated and control groups, respectively. The test statistic ($\hat{Z}$) for testing the difference in the two population proportions, that is, for testing the null hypothesis

$$H_o : M_1 - M_2 = 0,$$

was

$$\hat{Z} = \frac{(\hat{M}_1 - \hat{M}_2) - 0}{\sqrt{\hat{M}(1-\hat{M})\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}} \tag{2}$$

with

$$\hat{M} = \frac{Y_1 + Y_2}{n_1 + n_2} \tag{3}$$

where M is the proportion of 'mortality' in the two samples combined, Y is the number of individuals that died in each group and n is the total number of individuals in that group (Pennsylvania State University, 2018).

RESULTS

Marking

All individuals exposed to calcein showed signs of marking on the growing edge of the shell immediately after immersion and retained this mark throughout the 11-month monitoring period. After 240 d post-immersion, 2-week- and 2-month-old juveniles that were immersed in calcein multiple times showed distinct markings

Table 2. Mean initial and final shell height (mm) ± SE of oysters in the single and triple immersion treatments.

	2-week-old shell height (mm)		2-month-old shell height (mm)		3-year-old shell height (mm)	
	Control ($n = 50$)	Treated ($n = 50$)	Control ($n = 32$)	Treated ($n = 32$)	Control ($n = 50$)	Treated ($n = 50$)
Single immersion						
Initial	4.6 ± 0.1*	2.8 ± 0.1	20.5 ± 1.0*	24.1 ± 0.7	76.3 ± 0.8*	79.4 ± 0.8
Final	42.1 ± 1.2	44.8 ± 1.1	56.5 ± 2.2*	50.1 ± 2.0	94.6 ± 1.6	96.96 ± 1.4
Triple immersion						
Initial	4.0 ± 0.2	3.9 ± 0.1	24.1 ± 1.0	25.6 ± 0.7	76.4 ± 0.9	79.2 ± 0.6
Final	39.6 ± 1.2	41.7 ± 1.2	54.1 ± 2.1	57.2 ± 0.9	96.1 ± 1.6	95.9 ± 1.3

Initial shell heights were measured within 1 d of the initial immersion. Final shell heights were measured 329 d after the initial measurement. Asterisks indicate significant difference in shell height between treated and control individuals at that time point (Welch two-sample *t*-test, $P < 0.05$).

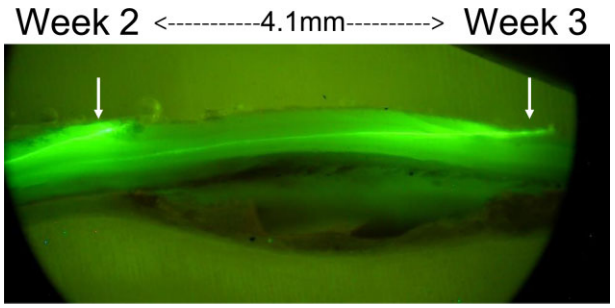

Figure 4. Cross-sectioned portion of right valve of juvenile *Crassostrea virginica* that was treated with three calcein immersions of 24-h duration spaced 2 weeks apart. The valve was sectioned along the axis of growth. Brilliant green lines at the upper surface of the shell (white arrows) indicate the location of the shell margin at the time of immersion for two of the three immersion treatments that this individual underwent. The distance between labelled microstructure growth marks (white arrows), as observed under 4× fluorescence microscopy (460–490 nm), was 4.1 mm, indicating that this oyster grew 4.1 mm in 2 weeks. Due to the distance between marks and the field of view of the microscopy equipment, capturing the marks from all three immersion events was not possible within one image.

(Fig. 3G, H). Individuals exposed to one immersion did show marks (Fig. 3D–F), but singular bandings created from one exposure to calcein were not as easily seen as the marks on individuals that had been exposed multiple times. Marked adults had mottled calcein labelling along the growing margin, which was not as readily identifiable as in the juveniles (Fig. 3F, I).

Of 34 juveniles that were suspended *c.* 3.0 m below the surface for long-term mark retention (marks detected using the Nightsea Bluestar® fluorescent detector; Fig. 2), 100% had detectable marks 2 years after immersion, 33 of the 34 individuals had detectable marks 2.9 years after immersion and 33 of the 34 individuals had detectable marks 3.75 years after immersion. On average, shell length was 86.06 ± 7.8 mm (SD) after 3.75 years.

To verify internal marking, a cross-section of the right valve of five individuals was viewed under fluorescence microscopy. The shell margin at time of immersion was readily apparent (e.g. Fig. 4). The distance between markings (e.g. 4.1 mm on a 28.2-mm-long valve; see Fig. 4) was measured using an ocular micrometer. The external distance between markings from immersion events, measured with callipers, was within 0.1 mm of the internal measured distance between microstructure marks on all five individuals.

Growth

Comparison of mean shell heights indicated that, overall, there were no systematic differences in growth between treated and control individuals for 1 year after immersion (Table 2, Figs 5 and 6). At most time points (59 out of 63), the shell heights of treated oysters were not significantly lower than controls for all age class and immersion treatments (Figs 5 and 6). In the cases where significant differences were detected, 7 of the 11 tests indicated that shell heights of treated individuals were larger than controls, suggesting that no systematic reduction in growth occurred in the calcein-treated oysters.

Survivorship

No gaping or missing valves in control or treated oysters were observed at the end of the 3-d observation window after each immersion treatment.

The proportion of treated individuals surviving at any time point was equal to or higher than control individuals in most age and treatment groups (five of six) (Fig. 7). No more than 19% of individuals in each group died from one time point to another throughout the year-long monitoring period, except for 2-month-old juveniles that were immersed three times. This group experienced a significantly greater mortality than controls in the first month after marking, losing 52% of the treated oysters compared to 0% of the controls in this time period. This 2-month-old group was the only group that had fewer treated individuals surviving than controls at the end of the monitoring period, with a cumulative mean percent mortality of 28 ± 16% (SE) for single immersion and 56 ± 18% for triple immersion groups (Fig. 8). Cumulative mortality of all other treatment and control groups was ≤34% over the 11-month observation period.

DISCUSSION

Marking success

Calcein appears to be a suitable method to mark the shells of juvenile *Crassostrea virginica*: multiple ages were successfully marked, multiple marks were created (Fig. 3) and the marks were detectable on some individuals for at least 3.75 years after marking. The clarity of the mark and the duration of detection varied depending on the age of the individuals exposed and the number of exposures, with best results for juvenile oysters. The presence of the fluorochrome mark was visible on the growing edge of the oyster shell just after marking, as has been observed in the mussels *Lampsilis cardium* and *Actinonaias pectorosa* (Eads & Layzer, 2002) and the abalone *Haliotis rubra* (Day *et al.*, 1995), and was detectable within the upper surface of the shell (Fig. 4).

All juveniles exposed to a single immersion of calcein at 2 weeks of age had a distinct labelling encircling the umbo (Fig. 3D) 8 months after marking. Over time, this singular mark may become harder to detect due to degradation of the fluorochrome compound (Shapiro, 2005; Honeyfield *et al.*, 2008), as well as due to the fact that oyster shells dissolve and weather (Waldbusser, Steenson & Green, 2011). Multiple discrete marks were produced by multiple immersions spaced 2 weeks apart (Fig. 3G, H) and marks on juvenile oysters were readily detectable after 3.75 years. Using

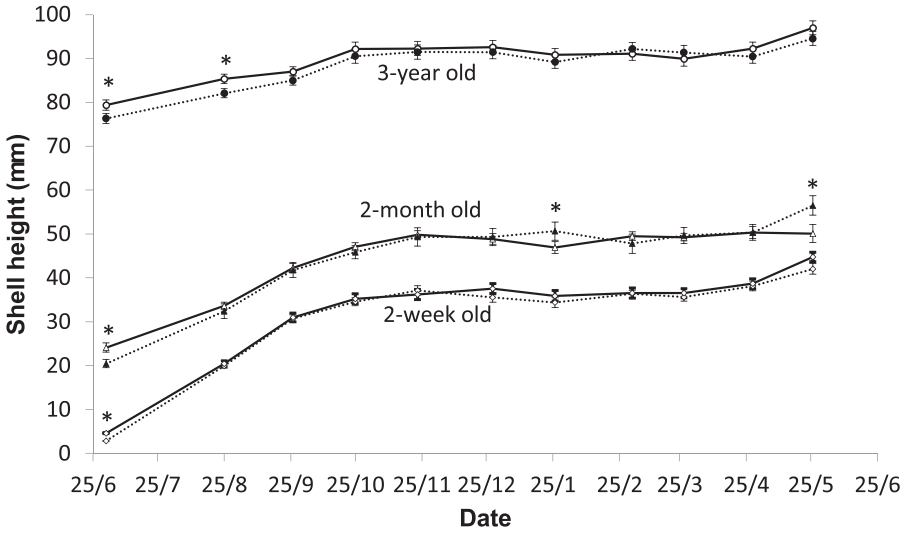

Figure 5. Mean shell height ± SE (mm) of *Crassostrea virginica* over time of control (dashed line) and single immersion treatment (solid lines) in the three age classes, 2 weeks old (bottom lines), 2 months old (middle lines) and 3 years old (top lines). Initial shell heights were measured on the day of treatment (25 July 2016). An asterisk indicates significant difference in shell height between treated and control individuals at that time point (Welch two-sample *t*-test; $P < 0.05$).

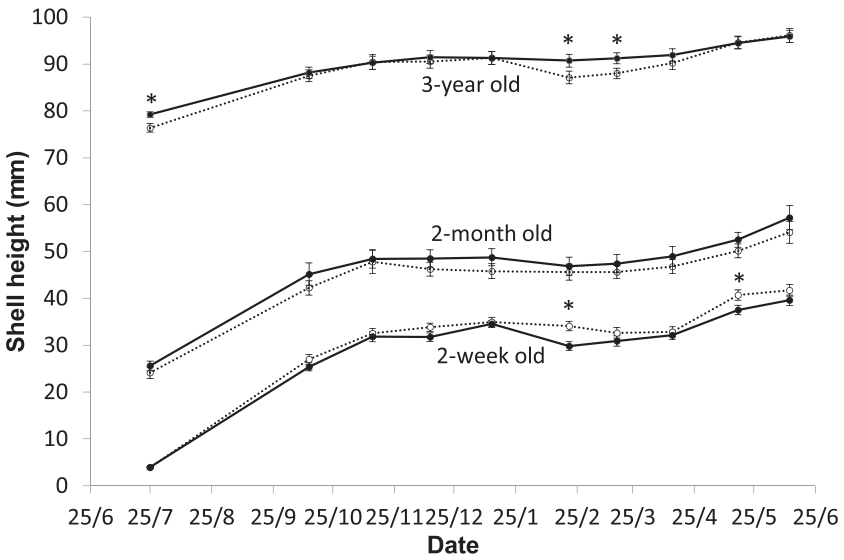

Figure 6. Mean shell height ± SE (mm) of *Crassostrea virginica* over time of control (dashed line) and triple immersion treatment (solid lines) in the three age classes, 2 weeks old (bottom lines), 2 months old (middle lines) and 3 years old (top lines). Initial shell heights were measured on the day of treatment (2 August 2016). An asterisk indicates significant difference in shell height between treated and control individuals at that time point (Welch two-sample *t*-test; $P < 0.05$).

multiple marks may prove desirable for researchers in regions of severe weathering or rapid shell dissolution, or in studies documenting growth rates.

Marking success was less clear on adult oysters (3-year-olds). Mottled labelling along the growing edge was variable and inconsistent (Fig. 3F, I). The difference between the success of labelling younger individuals and that of adults may be due to differences in growth rates. Older oysters grow at a slower rate than younger oysters (Vølstad *et al.*, 2007), thereby producing less labelled shell during immersions than their younger counterparts. Longer immersion times may be necessary for marking adults.

Labelling of internal microstructure growth marks was confirmed by cross-sectioning the right valves of labelled oysters (Fig. 4). The shell margin at the time of each immersion was readily apparent, allowing for the measurement of growth between immersion events internally using an ocular micrometer. Internal labelling of microstructure growth rings in oysters may be useful when using stable isotope ratios to understand how environmental conditions influence growth and could be used for oyster sclerochronology, the study of periodic increments in skeletal organisms (Kirby, Soniat & Spero, 1998).

Mortality and growth

Similar to studies on brown mussels (*Perna perna*; Kaehler & McQuaid, 1999), adverse effects of calcein (SE-Mark®) on

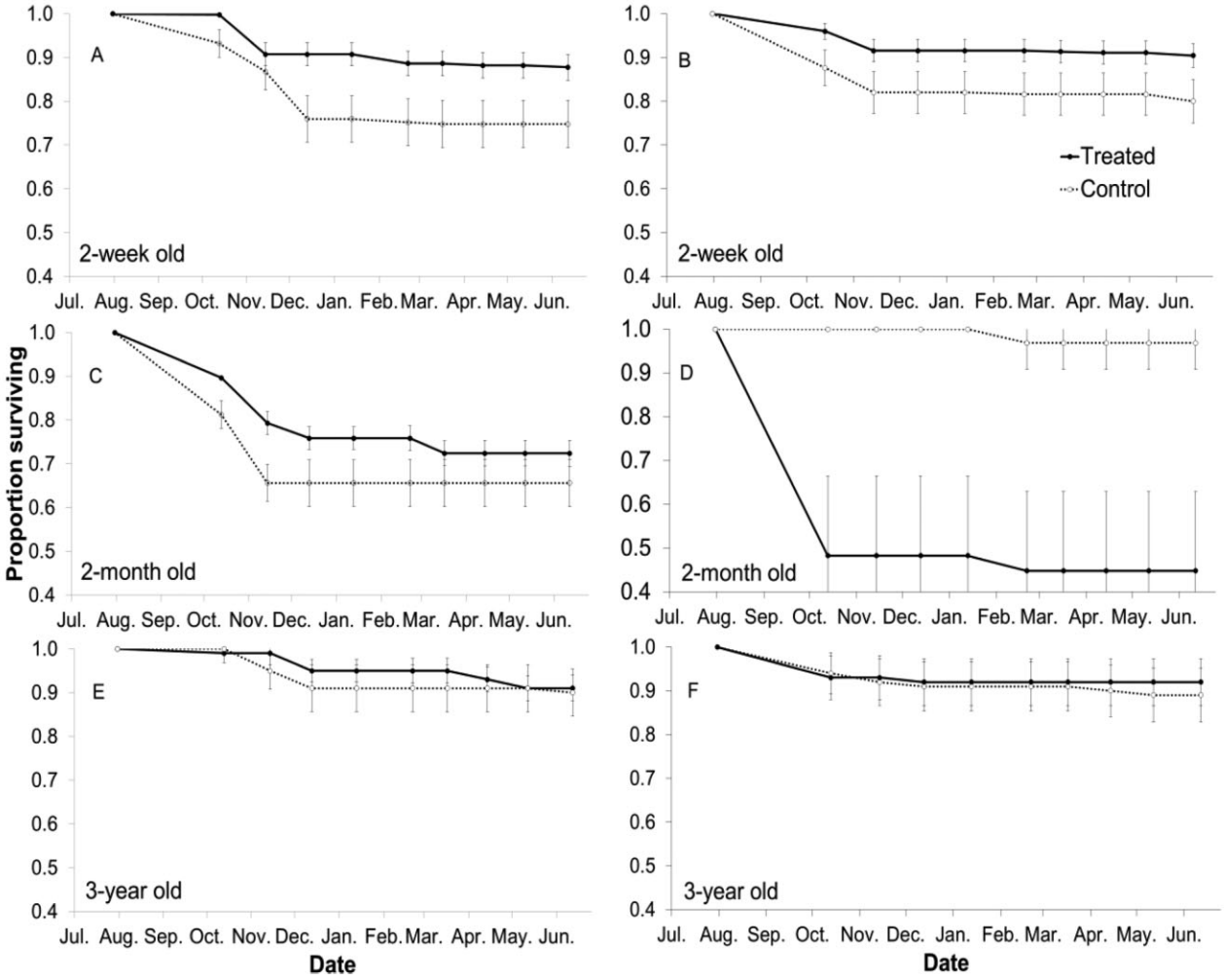

Figure 7. Proportion of surviving *Crassostrea virginica* over time for treated (solid line) and control (dashed line) animals in the single calcein immersion treatment (**A**, **C**, **E**) and the triple calcein immersion treatment (**B**, **D**, **F**). Rows contain different ages of oysters, which were 2 weeks old (**A**, **B**), 2 months old (**C**, **D**) and 3 years old (**E**, **F**) at the time of marking. The error bars represent the 95% confidence limits.

C. virginica were negligible for robust cohorts. Juvenile oysters as young as 2 weeks old were exposed to calcein with no deleterious effects on growth and survivorship in either the single or triple immersion treatments. In contrast, 2-month-old oysters survived well in the single immersion treatment but suffered 55% mortality in the triple immersion treatment. This cohort suffered significant pre-experiment mortality (80%) that was not observed in the 2-week-old cohort, both of which were suspended in cages near bottom in the Tred Avon River in close proximity (2 m apart). The higher pre-experiment mortality of the 2-month-old cohort may have been due to predation (Newell *et al.*, 2000) or due to a higher genetic mutation load, which can result in one cohort having higher mortality rates than others (Plough, Shin & Hedgecock, 2016). Survival of all other labelled oysters was similar to or better than controls, including the youngest 2-week-old group, which would be expected to be more sensitive than older juveniles. Hence, the difference in survivorship between the treated and controls in the 2-month-old group cannot completely rule out the possible negative effect of calcein on some cohorts of juvenile oysters that are predisposed to high mortality.

Mean shell height of labelled individuals was not significantly lower than controls at most time points for all age groups and immersion treatments. However, initial shell heights of four of the six treatment groups were significantly larger than the controls (Figs 5 and 6). Treated cohorts that started out larger than their control group counterparts were equal to or larger than controls at the first time point after calcein immersion, indicating that calcein did not appear to negatively affect growth. Over the whole study, the slight but statistically significant differences in shell heights that were observed may not be biologically meaningful because of the inherent variability in oyster growth rates (Singh & Zouros, 1978). Also, the slight and unavoidable differences in handling when retrieving cages could have chipped off the growing edge of some shells in an unequal fashion.

Previous studies using fluorochromes on other bivalves have shown mixed post-treatment mortality results, with cumulative mortality rates as high as 49%, with shorter exposure durations and lower concentrations (Day *et al.*, 1995; Kaehler & McQuaid, 1999; Eads & Layzer, 2002; Mahé *et al.*, 2010). The deleterious effects observed in this study were isolated to one of six treatment groups that had already experienced substantial mortality before the treatments began. Similar to Eads & Layzer (2002), who exposed freshwater mussels multiple times to calcein, there could be a negative effect on cohorts that are prone to high mortalities. Multiple immersions do not appear to have reduced the growth or survivorship of robust juveniles and adults.

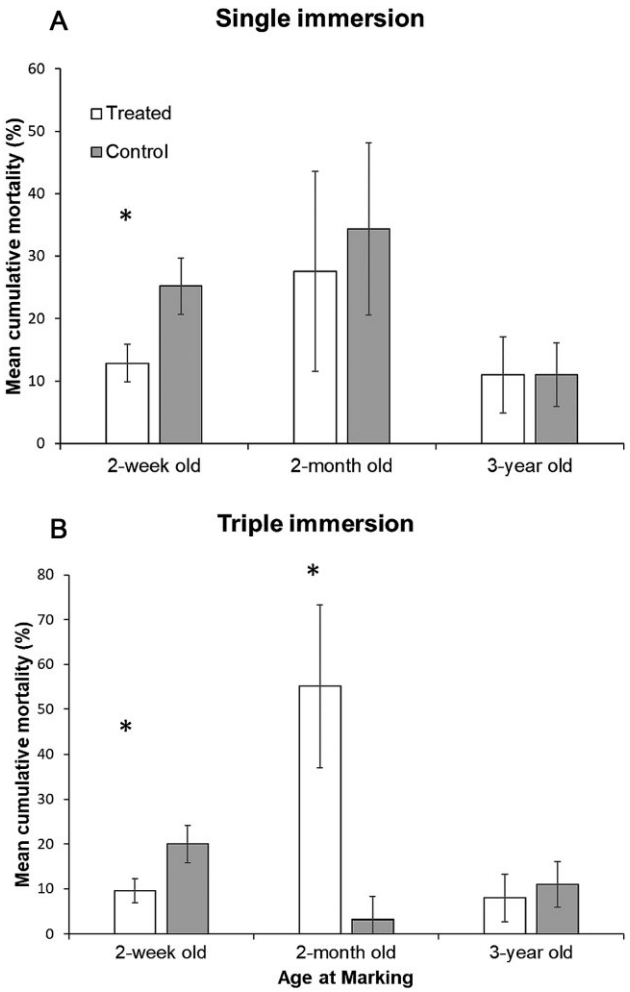

Figure 8. Mean cumulative percent mortality of all *Crassostrea virginica* oysters 11 months post-immersion in the single calcein immersion treatment (**A**) and triple calcein immersion treatment (**B**) by age class. Control oysters were treated identically to treated oysters, except that they did not receive a 24-h immersion in calcein. The error bars represent the 95% confidence limits. An asterisk indicates significant difference in shell height between treated and control individuals ($P < 0.05$).

Recommendations

Based on the results of this study, oysters that are 2 weeks and 2 months old can be readily marked by calcein using an immersion time of 24 h with a concentration of 250 mg l^{-1}. In addition, juveniles can be marked multiple times with the expectation of good growth and survivorship for robust cohorts. That being said, further testing with additional cohorts is warranted due to the high pre- and post-trial mortality experienced in one of the 2-month-old cohorts. Because shell deposition of bivalves is influenced by temperature and food availability (Joubert *et al.*, 2014), spatial-, temporal- and age-specific growth should be considered when developing marking methods (concentration, duration and time between immersions) for *C. virginica* in different regions or for other oyster species.

It is clear that calcein labelling offers a rapid noninvasive method for mass marking of juvenile oysters. In fact, based in part on the results of this study, oysters have been added to the list of approved species to be treated with the INAD calcein, under the U.S. Fish and Wildlife Service's AADAP programme, allowing labelled *C. virginica* to be marked and released when used within the established INAD guidelines (U.S. Fish and Wildlife Service, 2008, 2020).

Calcein marking may support growth studies, product health tracking, enforcement of no-take fishing zones and monitoring of intentionally introduced conspecifics among wild populations, the latter of which is a common practice in oyster restoration. This last application may be of particular interest in Chesapeake Bay and other regions where large numbers of oyster restoration projects are currently underway (Beck *et al.*, 2011; USACE, 2012; McCann, 2018) because this technique makes it possible to distinguish hatchery-reared individuals from wild stock, estimate their survival and determine growth rates.

Future studies to test progeny of additional broodstock and improve efficiency and effectiveness of methods for calcein marking are needed to ensure that this technique has wide applications across the range of *C. virginica* with minimal adverse effects. In addition, future research could focus on calcein as a mark for various stages of *C. virginica* larvae. If so documented, then calcein could be used to enhance siting of restoration projects within interconnected reef systems (USACE, 2012), and to calibrate and validate larval transport models (North *et al.*, 2008; Haase *et al.*, 2012; Narváez *et al.*, 2012; Arnold *et al.*, 2017).

ACKNOWLEDGEMENTS

This work was funded by the National Oceanic and Atmospheric Administration National Centers for Coastal Ocean Science (NCCOS Science Portfolio Project No. 449). The Cooperative Oxford Laboratory provided valuable facility and staff support. We thank Danny Furman, Ava Ellett, Shawn McLaughlin, Amy Freitag and Gretchen Messick for providing invaluable laboratory support. Howard Townsend, Eric Weissberger, Michael Wilberg, Chris Dungan, Frank Marenghi, John Jacobs, Stephanie Westby, Donald Merritt, Stephanie Alexander, Suzanne Skelley and A.K. Leight provided the valuable advice and comments. The Horn Point Oyster Hatchery provided oyster spat. This is UMCES-HPL publication no. CN 6081.

REFERENCES

ALCOBENDAS, M., LECOMTE, F., CASTANET, J., MEUNIER, F.J., MAIRE, P. & HOLL, M. 1991. Technique de marquage en masse de civelles (*Anguilla anguilla* L.) par balnéation rapide dans le fluorochrome. Application au marquage à la tétracycline de 500 kg de civelles. *Bulletin Français de la Pêche et de la Pisciculture*, **321**: 43–54.

ARNOLD, W.S., MEYERS, S.D., GEIGER, S.P., LUTHER, M.E., NARVÁEZ, D., FRISCHER, M.E. & HOFMANN, E. 2017. Applying a coupled biophysical model to predict larval dispersal and source/sink relationships in a depleted metapopulation of the eastern oyster *Crassostrea virginica*. *Journal of Shellfish Research*, **36**: 101–118.

BAYNE, B.L. 2002. A physiological comparison between Pacific oysters *Crassostrea gigas* and Sydney Rock oysters *Saccostrea glomerata*: food, feeding and growth in a shared estuarine habitat. *Marine Ecology Progress Series*, **232**: 163–178.

BECK, M.W., BRUMBAUGH, R.D., AIROLDI, L., CARRANZA, A., COEN, L.D., CRAWFORD, C., DEFEO, O., EDGAR, G.J., HANCOCK, B. & KAY, M.C. 2011. Oyster reefs at risk and recommendations for conservation, restoration, and management. *Bioscience*, **61**: 107–116.

BEIRNE, E.C., WANAMAKER, A.D. & FEINDEL, S.C. 2012. Experimental validation of environmental controls on the $\delta^{13}C$ of *Arctica islandica* (ocean quahog) shell carbonate. *Geochimica et Cosmochimica Acta*, **84**: 395–409.

BROOKS, R.C., HEIDINGER, R.C. & KOHLER, C.C. 1994. Mass-marking otoliths of larval and juvenile walleyes by immersion in oxytetracycline, calcein, or calcein blue. *North American Journal of Fisheries Management*, **14**: 143–150.

BUTTNER, J.K. & HEIDINGER, R.C. 1980. Seasonal variations in growth of the Asiatic clam, *Corbicula fluminea* (Bivalvia: Corbiculidae) in a southern Illinois fish pond. *Nautilus*, **94**: 8–10.

DAY, R.W., WILLIAMS, M.C. & HAWKES, G.P. 1995. A comparison of fluorochromes for marking abalone shells. *Marine and Freshwater Research*, **46**: 599–605.

EADS, C.B. & LAYZER, J.B. 2002. How to pick your mussels out of a crowd: using fluorescence to mark juvenile freshwater mussels. *Journal of the North American Benthological Society*, **21**: 476–486.

EVANS, O., HICK, P. & WHITTINGTON, R.J. 2016. Comparison of two external tagging methods used for the identification of individual adult Pacific oysters, *Crassostrea gigas*. *Journal of Shellfish Research*, **35**: 837–840.

FITZPATRICK, M.P., JEFFS, A.G. & DUNPHY, B.J. 2013. Efficacy of calcein as a chemical marker of green-lipped mussel (*Perna canaliculus*) larvae and its potential use for tracking larval dispersal. *Aquaculture Research*, **44**: 345–353.

GANCEL, H.N., CARMICHAEL, R.H., PARK, K., KRAUSE, J.W. & RIKARD, S. 2019. Field mark-recapture of calcein-stained larval oysters (*Crassostrea virginica*) in a freshwater-dominated estuary. *Estuaries and Coasts*, **42**: 1558–1569.

GELSLEICHTER, J., CORTÉS, E., MANIRE, C.A., HUETER, R.E. & MUSICK, J.A. 1997. Use of calcein as a fluorescent marker for elasmobranch vertebral cartilage. *Transactions of the American Fisheries Society*, **126**: 862–865.

GOODWIN, D.H., GILLIKIN, D.P., JORN, E.N., FRATIAN, M.C. & WANAMAKER, A.D. 2021. Comparing contemporary biogeochemical archives from *Mercenaria mercenaria* and *Crassostrea virginica*: insights on paleoenvironmental reconstructions. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **562**: 110110.

HAASE, A.T., EGGLESTON, D.B., LUETTICH, R.A., WEAVER, R.J. & PUCKETT, B.J. 2012. Estuarine circulation and predicted oyster larval dispersal among a network of reserves. *Estuarine, Coastal and Shelf Science*, **101**: 33–43.

HELM, M.M. 2004. *Hatchery culture of bivalves: a practical manual*. FAO, Rome.

HONEYFIELD, D.C., KEHLER, T., FLETCHER, J.W. & MOHLER, J.W. 2008. Effect of artificial sunlight on the retention of external calcein marks on lake trout. *North American Journal of Fisheries Management*, **28**: 1243–1248.

ISERMANN, D.A., BETTOLI, P.W. & SAMMONS, S.M. 1999. Efficacy of identifying stocked crappies in a Tennessee reservoir through oxytetracycline marking. *North American Journal of Fisheries Management*, **19**: 1122–1123.

JOUBERT, C., LINARD, C., Le MOULLAC, G., SOYEZ, C., SAULNIER, D., TEANINIURAITEMOANA, V., KY, C.L. & GUEGUEN, Y. 2014. Temperature and food influence shell growth and mantle gene expression of shell matrix proteins in the pearl oyster *Pinctada margaritifera*. *PLoS One*, **9**: e103944.

KAEHLER, S. & McQUAID, C.D. 1999. Use of the fluorochrome calcein as an *in situ* growth marker in the brown mussel *Perna perna*. *Marine Biology*, **133**: 455–460.

KEMP, P.S. 2006. *Oyster hatchery manual*. Carteret Community College, Morehead City, NC.

KIRBY, M.X., SONIAT, T.M. & SPERO, H.J. 1998. Stable isotope sclerochronology of Pleistocene and Recent oyster shells (*Crassostrea virginica*). *Palaios*, **13**: 560–569.

KURTH, J., LOFTIN, C., ZYDLEWSKI, J. & RHYMER, J. 2007. PIT tags increase effectiveness of freshwater mussel recaptures. *Journal of the North American Benthological Society*, **26**: 253–260.

LIM, B.K. & SAKURAI, N. 1999. Coded wire tagging of the short necked clam *Ruditapes philippinarum*. *Fisheries Science*, **65**: 163–164.

LINARD, C., GUEGUEN, Y., MORICEAU, J., SOYEZ, C., HUI, B., RAOUX, A., CUIF, J.P., COCHARD, J.-C., Le PENNEC, M. & Le MOULLAC, G. 2011. Calcein staining of calcified structures in pearl oyster *Pinctada margaritifera* and the effect of food resource level on shell growth. *Aquaculture*, **313**: 149–155.

LORSON, R.D. & MUDRAK, V.A. 1987. Use of tetracycline to mark otoliths of American shad fry. *North American Journal of Fisheries Management*, **7**: 453–455.

LUCAS, T., PALMER, P., WANG, S., SCOONES, R. & O'BRIEN, E. 2008. *Marking scallops for release and recapture*. Final report to the Fisheries Research and Development Corporation, FRDC Project 2005/016. Department of Primary Industries & Fisheries, Queensland, Australia.

MAHÉ, K., BELLAMY, E., LARTAUD, F. & de RAFÉLIS, M. 2010. Calcein and manganese experiments for marking the shell of the common cockle (*Cerastoderma edule*): tidal rhythm validation of increments formation. *Aquatic Living Resources*, **23**: 239–245.

MATTICE, J.S. & WRIGHT, L.L. 1986. Aspects of growth of *Corbicula fluminea*. *American Malacological Bulletin*, **2**: 167–178.

McCANN, M. 2018. *New York City oyster monitoring report: 2016–2017*. Nature Conservancy, New York.

McMAHON, R. & WILLIAMS, C.J. 1986. A reassessment of growth rate, life span, life cycles and population dynamics in a natural population and field caged individuals of *Corbicula fluminea* (Mueller) (Bivalvia: Corbiculacea). *American Malacological Bulletin*, **2**: 151–166.

MDNR. 2016. Appendix A: characterization of individual sanctuaries. In: *Oyster management review: 2010–2015*, pp. 285–289. Maryland Department of Natural Resources, Annapolis, MD.

MORAN, A.L. & MARKO, P.B. 2005. A simple technique for physical marking of larvae of marine bivalves. *Journal of Shellfish Research*, **24**: 567–571.

NARVÁEZ, D.A., KLINCK, J.M., POWELL, E.N., HOFMANN, E.E., WILKIN, J. & HAIDVOGEL, D.B. 2012. Modeling the dispersal of eastern oyster (*Crassostrea virginica*) larvae in Delaware Bay. *Journal of Marine Research*, **70**: 381–409.

NEWELL, R.I.E., ALSPACH, G.S., KENNEDY, V.S. & JACOBS, D. 2000. Mortality of newly metamorphosed eastern oysters (*Crassostrea virginica*) in mesohaline Chesapeake Bay. *Marine Biology*, **136**: 665–676.

NOAA. 2017. *2016 Oyster reef monitoring report*. Available at: https://chesapeakebay.noaa.gov/images/stories/pdf/2016oysterreefmonitoringreport.pdf. Accessed 1 March 2020.

NOAA. 2021. *Chart 12266*. U.S. Department of Commerce, National Oceanic and Atmospheric Administration, Washington, DC.

NOAA RESTORATION CENTER. 2007. *West Coast native oyster restoration: 2006 workshop proceedings*. U.S. Department of Commerce, NOAA Restoration Center, Silver Spring, MD.

NORTH, E.W., SCHLAG, Z., HOOD, R.R., LI, M., ZHONG, L., GROSS, T. & KENNEDY, V.S. 2008. Vertical swimming behavior influences the dispersal of simulated oyster larvae in a coupled particle-tracking and hydrodynamic model of Chesapeake Bay. *Marine Ecology Progress Series*, **359**: 99–115.

PENNSYLVANIA STATE UNIVERSITY 2018. *Introduction to mathematical statistics – 9.4 comparing two proportions*. Available at: https://online.stat.psu.edu/stat415/lesson/9/9.4. Accessed 1 April 2018.

PLOUGH, L., SHIN, G. & HEDGECOCK, D. 2016. Genetic inviability is a major driver of type III survivorship in experimental families of a highly fecund marine bivalve. *Molecular Ecology*, **25**: 895–910.

R CORE TEAM. 2020. *R: a language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.R-project.org/. Accessed 1 March 2020.

RIASCOS, J., GUZMAN, N., LAUDIEN, J., HEILMAYER, O. & OLIVA, M. 2007. Suitability of three stains to mark shells of *Concholepas concholepas* (Gastropoda) and *Mesodesma donacium* (Bivalvia). *Journal of Shellfish Research*, **26**: 43–49.

SECOR, D.H., WHITE, M.G. & DEAN, J.M. 1991. Immersion marking of larval and juvenile hatchery-produced striped bass with oxytetracycline. *Transactions of the American Fisheries Society*, **120**: 261–266.

SHAPIRO, H.M. 2005. *Practical flow cytometry*. John Wiley & Sons, New York.

SINGH, S.M. & ZOUROS, E. 1978. Genetic variation associated with growth rate in the American oyster (*Crassostrea virginica*). *Evolution: International Journal of Organic Evolution*, **1978**: 342–353.

STEWART, D.R. 2011. The efficacy of mass-marking channel catfish fingerlings by immersion in oxytetracycline. In: *Proceedings of the Oklahoma Academy of Science*, pp. 31–36.

THÉBAULT, J., CHAUVAUD, L., CLAVIER, J., FICHEZ, R. & MORIZE, E. 2006. Evidence of a 2-day periodicity of striae formation in the tropical scallop *Comptopallium radula* using calcein marking. *Marine Biology*, **149**: 257–267.

TWIST, B.A., RAYMENT, W.J. & HEPBURN, C.D. 2016. Movement patterns of adult scallops (*Pecten novaezealandiae*) within a customary fisheries reserve: implications for fine scale spatial management. *Fisheries Research*, **174**: 160–166.

USACE. 2012. *Chesapeake Bay oyster recovery: native oyster restoration master plan*. U.S. Army Corps of Engineers, Baltimore, MD/Norfolk, VA.

U.S. FISH AND WILDLIFE SERVICE. 2008. Investigational new animal drug (INAD) exemption for calcein. INAD #10-987.

U.S. FISH AND WILDLIFE SERVICE. 2020. Investigational new animal drugs. Available at: https://www.fws.gov/fisheries/aadap/inads/Calcein-Se-Mark-INAD-10-987.html. Accessed 1 April 2021.

VAN DER GEEST, M., VAN GILS, J.A., VAN DER MEER, J., OLFF, H. & PIERSMA, T. 2011. Suitability of calcein as an *in situ* growth marker in burrowing bivalves. *Journal of Experimental Marine Biology and Ecology*, **399**: 1–7.

VØLSTAD, J.H., CHRISTMAN, M.C., LEWIS, D., WEBER, E.D. & DEW, J. 2007. *A demographic model of oyster populations in the Chesapeake Bay to evaluate proposed oyster-restoration alternatives. Final programmatic environmental impact statement for oyster restoration in Chesapeake Bay including the use of a native and/or nonnative oyster*. Versar, Inc., Maryland.

WALDBUSSER, G.G., STEENSON, R.A. & GREEN, M.A. 2011. Oyster shell dissolution rates in estuarine waters: effects of pH and shell legacy. *Journal of Shellfish Research*, **30**: 659–669.

WANAMAKER, A.D. & GILLIKIN, D.P. 2019. Strontium, magnesium, and barium incorporation in aragonitic shells of juvenile *Arctica islandica*: insights from temperature controlled experiments. *Chemical Geology*, **526**: 117–129.

WILSON, C.A., BECKMAN, D.W. & DEAN, J.M. 1987. Calcein as a fluorescent marker of otoliths of larval and juvenile fish. *Transactions of the American Fisheries Society*, **116**: 668–670.