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Direct Setting of Eastern Oyster (*Crassostrea virginica*) Larvae Confirmed with Calcein, a Fluorochrome Dye

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Abstract

Ongoing efforts to restore eastern oyster (*Crassostrea virginica*) populations in regions of low natural recruitment rely on stocking of juveniles (spat on shell) to rebuild populations. Often, remote setting is used, entailing releasing hatchery-produced larvae into recirculating tanks filled with oyster shells. Other restoration methods that rely on releasing *C. virginica* larvae directly onto the shell in situ, called "direct setting," have shown promise but were unable to definitively prove larval origin without the use of enclosures. The objective of this study was to determine if tagging *C. virginica* with calcein, a fluorochrome dye, could be a viable method for confirming larval origin in studies of direct setting in Chesapeake Bay. To do so, *C. virginica* larvae conditioned in water from adult *C. virginica* were marked with calcein and released by divers directly onto three 3.6-m^2 research sites constructed of oyster shell bags during July 2019 and September 2019 and recovered after 7 days. All shell bags were moved to flow-through tanks on land and spat on a subsample of valves were counted in each bag 8–12 days after deployment. Spat on the remaining valves were counted 42 to 46 days post deployment. A total of 119,020 spat were found on 84 shell bags from the two deployments during the initial settlement counts conducted just after shell bags were recovered. All recovered juveniles that were viewed under blue light excitation ($n = 84$) contained the calcein tag, indicating that these spat were derived from larvae released over the reefs. Initial settlement efficiencies on the sites ranged from 0.1 to 3.4% in July and September, respectively. The salinities experienced in July were below average and may have contributed to reduced larval survival compared to that in September. Shell bags contained zero to 90 spat per shell. Spat settlement was greatest closest to where the larvae were released (87% of spat were found in 12% of bags; the high-count bags were clustered around the larval release locations). Overall, 6 shell bags out of 190 deployed had spat per shell estimates similar to remote larval setting (hatchery) targets (10–20 spat per shell) and 6 had spat per shell values higher than hatchery targets. The presence of the calcein mark in recovered spat confirmed larval origin, and together with the observed setting efficiencies suggests there is promise for developing remote larval setting as a stock enhancement technique. However, more work is needed to understand the limitations of the technique, including its efficaciousness at a larger scale.

Keywords Oyster · Calcein · Fluorochrome dye · *Crassostrea virginica* · Marking · Mark and recapture · Direct setting

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Introduction

In coastal ecosystems, wild and farmed oysters function as keystone species providing vital habitat for fish and invertebrates (Rodney and Paynter 2006; Raj 2008; Shumway 2011). As suspension feeders, the eastern oyster (*Crassostrea virginica*) (Gmelin 1791) in Chesapeake Bay plays an important role in nutrient recycling, with intact *C. virginica* reefs shown to enhance denitrification rates, potentially reducing localized eutrophication effects (Newell 2004; Kellogg et al. 2014) and improving water quality (Grizzle et al. 2008). In this region, populations were once so abundant they were considered a significant navigational hazard, and wild harvest landings were the highest in the world (Kennedy and Breisch 1983; Baker and Mann 1992; Wharton 2010). Globally, native oyster populations have decreased roughly by 85% since the nineteenth century (Beck et al. 2011). In Chesapeake Bay, the deposition of sediment, diseases, overharvesting, and hypoxia and anoxia have contributed to reduced *C. virginica* populations (Newell 1988; Rothschild et al. 1994; Paynter 2007). One decade ago, *C. virginica* population abundances in Chesapeake Bay were estimated to be less than 1% of historic levels (Wilberg et al. 2011). In addition to the reduction in ecosystem services, the decrease in *C. virginica* populations in Chesapeake Bay has had negative economic impacts on the public oyster fishery (Kennedy and Breisch 1983). Most recently, *C. virginica* populations in 72% of the Maryland portion of Chesapeake Bay are increasing (2017–2020) and overfishing is occurring in 14% of the 36 oyster management areas in Maryland (MDNR 2020). Recently, the estimated abundance of market-sized *C. virginica* in Maryland is 452 million, the fifth highest estimate since 1999 (MDNR 2020). Despite the recent increase in population, oyster abundances in the Maryland portion of Chesapeake Bay are still estimated at less than half of what they were 40 years ago (Wilberg et al. 2011).

Due to the ecological and economic importance of *C. virginica* populations to coastal regions, oyster habitat in the USA is being restored in Chesapeake Bay and many other systems (e.g., Pamlico Sound, Long Island Sound, Gulf of Mexico) (USACE 2012; Puckett et al. 2014; McCann 2018; Carle et al. 2020). In these systems, the primary means of producing *C. virginica* for restoration, replenishing the public fishery, and supporting aquaculture in regions with low natural recruitment is spat-on-shell (SOS) production in remote setting facilities (Congrove et al. 2009). In this production method, suitable larval settlement substrate (called "cultch," usually oyster shell) is transported to facilities and placed in recirculating tanks while competent larvae and suitable food sources (algae) are added for a predetermined amount of time (e.g., 3 days) to allow the larvae to settle under controlled conditions (Congrove et al. 2009). This method can increase settlement by reducing predation, and controlling water temperature, food source, salinity, and dissolved oxygen (Supan 1990).

Although this production method has proven effective and is the primary means of SOS production across the East Coast oyster industry (Helm 2004; Kemp 2006; Congrove et al. 2009), it requires the acquisition, cleaning, transport, storing, loading, and planting of large quantities of settlement substrate. In Chesapeake Bay and in other regions, settlement substrate is costly and shell availability is decreasing (Mann et al. 2009; Kennedy et al. 2011; Theuerkauf et al. 2015). Hence, cost-saving alternatives to remote setting methods have been investigated (Coon and Fitt 1999; Theuerkauf et al. 2015; Steppe et al. 2016).

In Chesapeake Bay, and in other regions with different benthic invertebrate taxa (Arnold 2008), researchers have tested minimizing cultch material requirements by releasing larvae directly onto reefs in situ in a process called "direct setting" (Coon and Fitt 1999; Steppe et al. 2016). This method of reef seeding could reduce the need for settlement substrate and/or handling of the substrate. while attempting to increase reef seeding capacities. Direct setting techniques have been employed with corals and bay scallops *Argopecten irradians* in Australia and Florida, respectively (Heyward et al. 2002; Leverone et al. 2010) and on *C. virginica* in Chesapeake Bay (Coon and Fitt 1999; Theuerkauf et al. 2015; Steppe et al. 2016).

To date, research aimed at developing in situ direct setting methods for *C. virginica* have unequivocally shown the ability to produce juvenile oysters in numbers comparable to remote setting facilities, with the use of enclosures (Steppe et al. 2016). This direct setting technique proved efficient at producing desired spat densities on a natural oyster reef, but required the use of containment systems constructed of PVC barrier curtains to allow for water flow while retaining larvae, a method not readily deployable over large acreages, in rough seas, or in regions with vessel traffic. In addition, direct setting likely has produced spat without the aid of enclosures (Coon and Fitt 1999), although it was not possible to definitively prove that the spat were from hatchery stock and not from a natural set. Hence, despite promising results, the limitations related to validating larval origins and the challenging logistics of enclosures have precluded the adoption of direct settling techniques for *C. virginica*.

Recent advances in mark and recapture methodologies could provide tools for validating larval origin. Chemical mark and recapture techniques utilizing a fluorochrome dye (calcein) have been applied in field studies of *C. virginica* larval transport (Gancel et al. 2019), and recently have been shown to be effective for creating marks in *C. virginica* larval oysters that persist through metamorphosis and are readily identifiable for 4 weeks post settlement

(Spires et al. 2022). Calcein is a fluorochrome dye that binds with earth metals in suspension and is readily incorporated into the growing edge of multiple life stages of *C. virginica* via bath immersion techniques (Spires and North 2022; Spires et al. 2022). *C. virginica* is approved for calcein bath immersions and released into coastal waters on experimental levels in collaboration with the Investigational New Animal Drug (INAD) program of the U.S. Fish and Wildlife Service's Aquatic Animal Drug Approval Partnership (AADAP) program (U.S. Fish and Wildlife Service 2008, 2020).

Chemical tools also have been investigated to support restoring oyster reefs. *C. virginica* larvae have shown an increased settlement response to a number of dissolved chemical compounds (e.g., L-dopa, epinephrine, glycyl-glycyl-L-arginine, oyster-conditioned seawater) (Coon et al. 1985; Tamburri et al. 1992, 1996). Among these, oyster-conditioned seawater (OCW) is the only stimulus that would not require current Food and Drug Administration (FDA) testing and approval for use in systems where human consumption of oysters is possible. OCW contains concentrated waterborne substances released by *C. virginica* (e.g., Nh3, peptides) and the biofilm communities occupying their shells and is created by holding adult *C. virginica* in small static systems (Tamburri et al. 1992). Recently, OCW has been shown to increase setting efficiencies in field experiments (Hildebrandt 2021), with significantly higher spat per shell observed on trays of oyster shell direct set with larvae exposed to OCW than controls.

OCW is useful for cuing settlement because oyster larvae rely on natural stimulants released by adult oysters and their biofilms to cue settlement onto existing oyster communities (Bonar et al. 1986; Zimmer-Faust and Tamburri 1994; Tamburri et al. 1996; Tamburri et al. 2007), creating aggregations of conspecifics (Pawlik and Hadfield 1990). The gregarious settlement nature of reef-building organisms has community-level and individual benefits for reproduction, predator avoidance, survivorship, and feeding efficiencies (Hidu 1969; Tamburri et al. 1992, 2007; Whitman and Reidenbach 2012; Gercken and Schmidt 2014). By reacting to chemical cues, *C. virginica* larvae preferentially select habitats that should be beneficial for community preservation.

Although direct setting has shown promise, additional research is needed to determine the most beneficial OCW concentrations and larval exposure durations prior to release, and if direct setting could be a viable option to support small and large-scale oyster restoration, aquaculture, and commercial fisheries. Because direct setting exposes larvae to predation and could result in dispersal to unintended regions, it is not clear if direct setting has the potential to be effective over large spatial scales without enclosures. A tool for validating larvae origin would help address this question and advance research on the direct setting method.

Therefore, the objective of this study was to determine if tagging *C. virginica* larvae with calcein, a fluorochrome dye, could be a viable method for proving larval origin in studies of direct setting. The specific aims of this study were to use calcein-marked larvae to test the ability to directly seed oyster cultch material in situ without enclosures. We used OCW to enhance settlement likelihood, developed a diver-based method for direct setting of *C. virginica* larvae, and used calcein chemical tagging to verify larval origin.

Methods

Field trials for direct setting of *C. virginica* larvae took place in the Tred Avon River, a major tributary of the Choptank River, in Chesapeake Bay. The study site was in an oyster sanctuary adjacent to the Cooperative Oxford Laboratory (COL), in Oxford, Maryland. Oyster spawning, larval rearing, and marking of larvae were undertaken at the Maryland Department of Natural Resources Piney Point Aquaculture Center, in Piney Point, Maryland. Two direct setting trials occurred in the summer of 2019 (July and September) on research-scale oyster reefs (six total sites) constructed of oyster shell bags fixed to cargo nets in the Tred Avon River. The shell bag arrays were placed onto non-reef, hard mud river bottom within the designated oyster sanctuary. *C. virginica* larvae were released by divers directly onto shell bags and allowed to settle and grow for 7 days prior to being retrieved and moved to flow-through tanks at the COL where counts of juvenile *C. virginica* were conducted.

Broodstock Spawning and Larval Rearing

Adult *C. virginica* oysters were collected from oyster reefs adjacent to the Piney Point Aquaculture Center and used to produce the larvae for these experiments. The salinity in which the broodstock developed gametes (gametogenesis) was greater than 9.0 psu for both spawning events. *C. virginica* larvae were spawned and grown in tanks at the Piney Point Aquaculture Center following standard industry practices similar to those described in the FAO's Hatchery Culture of Bivalves (Helm 2004).

Calcein-Marking Procedures

Calcein-marking procedures follow those described in Spires et al. (2022) and are outlined here. These procedures were verified to mark larvae and retain the mark for 4 weeks after settlement (Spires et al. 2022). During this study, a 1000-L conical tank (Fig. 1) was filled with 600 L of filtered river water (salinity 10.1 on July 8 and 11.5 psu on September

Fig. 1 Conical tanks used for calcein bath immersions. Green liquid is calcein-stained water

4, 2019); then, 4900 mL of calcein was added and gently stirred. An additional 374 L of filtered river water was added to make a final calcein concentration of 50 mg/L. During filling, and for the duration of the marking procedure, the tank was continuously oxygenated with air stones. After mixing, temperature, salinity, and pH were analyzed using a YSI 6600 sonde. Sodium bicarbonate ($NaHCO_3$) was then added to raise the pH of the marking tank to a pH of 8.3 (July) and 8.4 (September) (Table 1), a necessary step due to acidic properties of calcein.

Oyster-marking methods were replicated in July and September of 2019. The only difference between the two procedures occurred when larvae were bundled for delivery to deployment sites and the number of deployment locations on each site. During the July deployment, one centralized larval release location was used at each of the three sites. During the September deployments, four larval release locations were used at each of the three sites. For both deployments (July and September), the same number of larvae was released at each site (1.6 million).

Table 1 Summary of water quality parameters during the larval marking period (July 8 and September 4, 2019) in the tanks at the Piney Point Aquaculture Center (a) and during the larval deployments and spat counting (b)

	July	September
a		
Dissolved oxygen (mg L^{-1})	7.4	8.0
Salinity (psu)	10.1	11.5
pH before calcein	8.3	8.1
pH after calcein	8.2	7.7
pH after buffering with ($NaHCO_3$)	8.4	8.3
Temperature (°C)	29.4	29.0
b		
Salinity (psu) during larval release	7.9	11.8
Salinity (psu) 2 weeks after release	8.9	12.0

To mark *C. virginica* larvae with calcein, 12 million (on July 8, 2019) and 10 million (on September 4, 2019) early pediveliger larvae (150–190 m in shell height) were added to the immersion tank. Early-stage pediveliger larvae were chosen to avoid larvae setting on the sides of immersion containers and because they have been shown to incorporate calcein into their growing larval shell (Spires et al. 2022). At that time, instant algae concentrate (Tetraselmis 3600 Premium 183 Fresh, Reed Mariculture, Inc.) was added at a stocking rate determined by hatchery staff.

Larvae were removed from the calcein bath after 24 h and reared to competency. Each tank with calcein was drained through a 125-micron sieve that was used to collect the marked larvae. A subsample of larvae was viewed on a stereo microscope at 50× using a Nightsea Bluestar® fluorescent detector microscopy kit to confirm that marks were visible and that larvae were alive and exhibiting normal behavior. All larvae collected on sieves were then rinsed into a 3000-L rearing tank for grow out to competency for settlement (i.e., when larvae have developed an eye spot and have a visible foot). Larval rearing tanks were set up according to standard Piney Point Aquaculture Center methods. Larvae were held in rearing tanks for up to 3 days, until deemed competent for settlement by Piney Point Aquaculture Center staff. During this time, larvae were checked by draining down larval tanks through a series of sieves (212 and 200 microns), twice daily by hatchery staff. Larvae that were caught on the 212-micron sieve were deemed suitable for settlement and were placed in a refrigerator while the remaining larvae were put back into the tanks to continue growing.

All larvae deemed suitable for settlement (captured on a 212-micron sieve) were divided by hatchery staff into three bundles of 1.6 million larvae (July) and twelve bundles of 400,000 larvae (September). Bundles were then refrigerated for 36 h until transport to the study site in the Tred Avon River.

Oyster-Conditioned Water

Oyster-conditioned water (OCW) was created following methods outlined in Tamburri et al. (1996). Twelve adult *C. virginica* were placed in a bucket containing 8 L of unfiltered river water with an air stone. The oysters were held in this system for 4 h, after which the adult *C. virginica* were removed and the OCW bucket was transported to the research vessel for use later that day.

Research-Scale Oyster Reefs

The location for the research-scale oyster reefs w selected based on substrate type (no oyster shell present), depths (5 m), and the presence of sediments firm enough to support shell bags without sinking into the bottom. Locations were preliminarily identified using multibeam side scan sonar data (Fig. 2) and ground-truthed with a sounding pole. Divers confirmed that the sediment was hard enough to support shell bags by dropping shell bags on the selected locations and observing whether or not the bags sunk into the sediment. Research sites were located within 150 m of each other in a line parallel to the shoreline, equidistant from the channel, at the same depth, and overlying visually similar sediment types to the scientific divers.

To construct the research-scale reefs (n = 6), bags of aged oyster shells (one shell is a single valve) were purchased from the Oyster Recovery Partnership. Oyster shell bags were made of polyethylene diamond mesh with a 20-mm opening. Each bag contained an average of 249 shells (Shannon Hood 2019, pers. comm).

Each site (the term used hereafter to describe each research-scale reef) was constructed with 31 or 32 oyster shell bags (3.6 m^2) that were individually numbered and secured to a cargo net using plastic cable ties (Fig. 3). On July 12, 2019, and again on September 9, 2019, three cargo nets with shell bags were deployed from the NOAA RV5502 at predetermined locations within the Cooperative Oxford Laboratory Oyster Sanctuary (Fig. 4) in the Tred Avon River. After the cargo nets were placed on the bottom by the research vessel, divers pulled each net flat on the bay bottom to ensure the shell bags were oriented correctly (as in Fig. 3). During the July deployments, a crab pot float was tied to the center bag of the research sites. These floats were used by scuba divers to find the location for deploying larvae in low-visibility conditions. During the September deployment, crab pot floats were attached to four shell bags to aid divers with releasing larvae at four locations on each site.

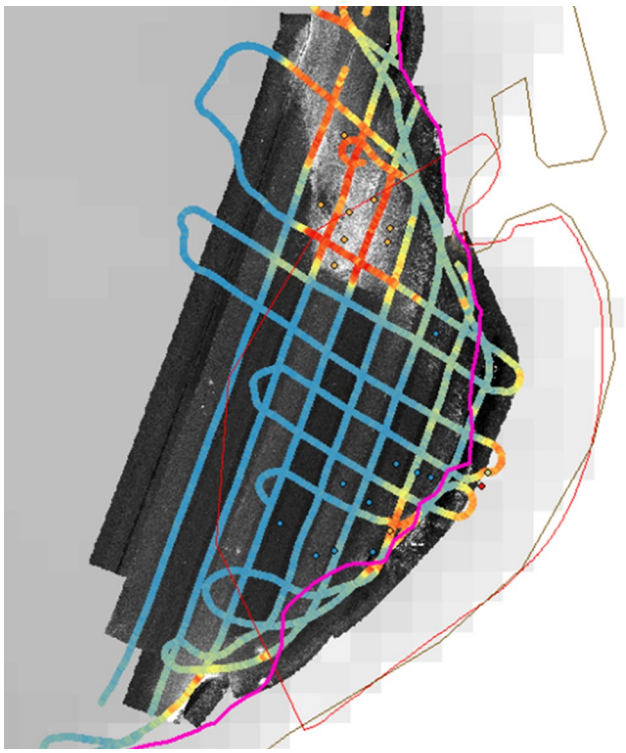

Fig. 2 Side scan sonar image of the Cooperative Oxford Laboratory Oyster Sanctuary. Colored lines indicate sonar vessel passes. Lighter colors in the black and white side scan image indicate hard substrate (600 kHz). Colors on track lines indicate roughness and hardness obtained by different frequencies (50 kHz). The Pink line is an unknown contour line (likely 3 m). The brown line is the shoreline and the orange polygon is the Cooperative Oxford Laboratory Oyster Sanctuary boundary. Image credit: NOAA Chesapeake Bay Office Habitat Assessment Team

Fig. 3 Photograph of one of six research scale shell bag sites that were deployed in the Cooperative Oxford Laboratory Oyster Sanctuary (Oxford, Maryland). Orange buoys were used by divers to identify where to release the larvae in low-visibility conditions

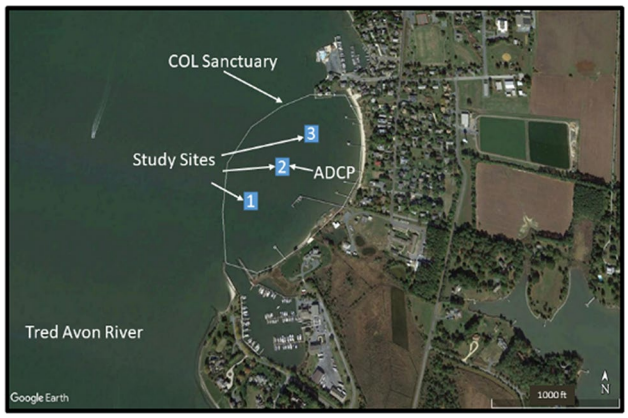

Fig. 4 Direct setting study site. The blue squares show the approximate deployment locations for the shell bag sites and ADCP within the Cooperative Oxford Laboratory Oyster Sanctuary (white polygon) in Oxford, Maryland. Numbers within blue squares are the study site numbers

Direct Setting

Using 2 L of OCW, larvae in each bundle were rinsed from the coffee filter and into a 2-L syringe (July) or screw-top plastic container (0.5 L) (September). The container was then filled with OCW and sealed for 15 min to allow chilled larvae to warm. After 15 min, larval movement was verified visually. On July 15, 2019, a 2-L syringe was used to deploy larvae into shell bags underwater. During the September deployments (9/10/2019), screw-top plastic containers were used instead because they were easier to control the operation of while releasing larvae underwater than the syringe. Divers released the larvae directly onto the shell bags marked with the crab pot floats by either depressing the plunger at one location on the site (July releases) or inverting the screw top container and then opening the lid and allowing the container to remain inverted for 10 s while maintaining direct contact with the shells in the bag at four locations on the site (September releases). The diver then returned to the surface and repeated this procedure on the remaining sites. Near-bottom water quality parameters (dissolved oxygen, temperature, salinity) were recorded at the time of deployment using a YSI 6600 Sonde.

Current Velocity Observations

Prior to larval releases, an Acoustic Doppler Current Profiler (ADCP, Nortek Aquadopp Profiler, 1 MHz) was deployed immediately adjacent to site #2 by divers (Fig. 4). The ADCP remained operational prior to and during the larval deployments until shell bags were retrieved from the bay bottom during both the July and September deployments. Current velocity data were analyzed to determine near-bottom current speed and direction while larvae were being released over the site and for 7 days afterward.

Recovery and Spat Counts

Seven days after larval releases, shell bags were recovered and moved to flow through tanks at the Cooperative Oxford Laboratory (Fig. 4). An initial estimate of settlers (initial settlement counts) was conducted by examining shells under a dissecting microscope. Each of 2016 shells was examined for newly settled spat using stereo microscopes by 20 biologists trained to identify *C. virginica* spat. Without looking at the shells, biologists reached into the oyster shell bag and haphazardly selected 7 shells from the top, middle, and bottom of each bag (stratified sampling), for a total of 21 sampled valves in each bag. The initial counts began one day after the shell bags were recovered and took 4 days to complete. The number of spat found within each bag and the location (top, middle, bottom) within each bag were recorded.

To determine if the natural recruitment of wild oyster larvae occurred, one shell from each bag containing spat ($n = 84$) was examined for the presence or absence of the spat with a calcein mark using an Olympus compound microscope with epifluorescence accessory for blue light excitation (490 nm). Note that inspecting shells for newly settled larvae with a compound microscope can be a time-consuming process All spat on each shell were examined for the presence of unmarked larvae, with numbers of spat ranging from 1 to dozens. Shells from each bag containing spat were then placed together in a new bag and put back into flow-through tanks. The remaining un-sampled shell bags were placed back into flow-through conditions where they remained for 35 more days.

Within 42 to 46 days after the completion of initial settlement counts, every valve remaining in each bag was visually inspected by 11 biologists without magnification for the presence of spat. During these counts (final counts) the total number of spat in each bag was recorded. The final counts took 4 days to complete.

Mortality Tracking

To monitor in-tank mortality between counts, valves with spat were haphazardly selected from among the valves that had been counted as part of the initial settlement counts. These were moved to polyethylene diamond mesh bags and kept in a flow-through tank. In July, 191 spat on 15 shells were monitored each week for mortality. In September, 93 spat on 11 valves were identified, moved to new shell bags, and monitored each week for mortality. The number of live individuals was identified using a dissecting microscope. Individuals were considered dead if their valves were gaping or missing and if the number of individuals counted in that

bag the previous week was greater. These subsampled spat were observed for a total of 35 days (time between initial counts and final counts).

In addition, mortality was estimated in each bag by subtracting the quotient of the number of live spat found during the final count divided by the number of spat found during the initial settlement count from 1. Because the final count does not include the 21 shells that were extracted from each bag during the initial settlement counts, the final counts were adjusted upward using the proportion of initial spat to total valves in each bag (249 valves per bag) and the final number of valves in each bag (228 shells).

Initial Settlement Spat per Bag Estimation

The abundance of spat on each site upon retrieval of shell bags was estimated using stratified random sampling techniques (Thompson 2012). The total number of spat on each site τ was estimated by summing the total spat in each bag. The number of spat in each bag was calculated as the sum of estimates of spat abundance in each stratum (top, middle, bottom). Hence, the total number of spat in each bag (τ) was estimated as:

$$\hat{\tau} = N_1\hat{\mu}_1 + N_2\hat{\mu}_2 + \ldots\ N_L\hat{\mu}_L = \sum_{i=1}^{L} N_i\hat{\mu}_i = \sum_{i=1}^{3} 21\hat{\mu}_i$$

where L is the number of strata, Ni is the number of sample units (shells) within stratum i, N is the number of sample units in the population (bags), and μ is the population mean.

The variance of the estimated total $\hat{\tau}$ is:

$$\widehat{var}(\hat{\tau}) = N^2\widehat{var}(\hat{u}) = \sum_{i=1}^{L} N_i^2\left(\frac{N_i - n_i}{N_i}\right)\left(\frac{S_i^2}{n_i}\right)$$

where S_i^2 is the estimate of the overall population variance from each strata i through L. The standard error of $\hat{\tau}$ is the square root of $\widehat{var}(\hat{\tau})$.

The total number of spat on each site was calculated as the sum of the estimated number of spat in each bag (τ) for all bags on the site.

Results

Calcein Marking

All shells with spat ($n \geq 84$ viewed under blue light excitation showed that spat on the shells contained the calcein tag encircling the umbo (Fig. 5). There was no evidence of natural recruitment (i.e., no untagged spat were observed). The visual appearance of each mark was similar to those observed by Spires et al. (2022) and distinct and new shell growth post-metamorphous was easily recognizable (Fig. 5).

Water Quality

Ambient salinity levels did not vary greatly during the larval releases, shell bag recoveries, and spat counting (Table 1b). Near-bottom (~20 cm off bottom) salinity was 7.9 psu at the time of the July larval release and 8.9 psu 2 weeks later. Salinity was 11.8 psu at the time of the September larval release and 12.0 psu 2 weeks later. During the larval deployments, near-bottom dissolved oxygen levels were 5.9 and 6.5 mg l^{-1}, respectively. There was negligible variability between any of the water quality parameters measured (salinity, temperature, dissolved oxygen) among the replicate research sites during larval deployment in either month. The maximum difference in salinity, temperature, and dissolved oxygen were 0.1 psu, 0.05 °C, and 0.4 mg/L, respectively.

Water quality parameters in the marking tanks during calcein immersion baths were similar during both immersion events (July and September) (Table 1a). A decrease in pH due to the addition of calcein was buffered by the addition of sodium bicarbonate ($NaHCO_3$).

Proximity of Spat to Release Site

During the July deployment, all larvae were released onto the center bag within each site. During this deployment, 23% of spat were found within the center release bags and 67% were found within one shell bag of the release site.

During the September deployments, the four shell bags with the highest number of settlers occurred at the locations of larval release (see green boxes in Fig. 6). During this deployment, 90% of all spat were found within the bags directly receiving larvae.

Settlement Estimations and Counts

The estimated number of initial settlers on each site was 13 times lower for the July deployment than the September deployment (Figs. 7a and 8) (Table 2). During the September deployment, the number of juveniles dropped by 87% between the 8–10-day estimates and the 42–46-day counts (Table 2). The counts conducted during the July deployment did not show the same magnitude of decrease as in the September deployment (Table 2).

Despite releasing the same number of larvae, the September deployment's initial settlement counts were 1,338% higher than the July deployment's initial settlement counts. The estimated setting efficiency (number of spat observed during initial settlement counts/number of larvae released) at the reef sites in July (0.1, 0.3, and 0.2% (Table 2)) was an order of magnitude lower than those in September (3.4, 2.0, and 2.4% (Table 2)). The initial spat count per m^2 (divide initial spat count per site by the footprint of the shell bags, 3.6 m^2) for the July deployment ranged between 276 and

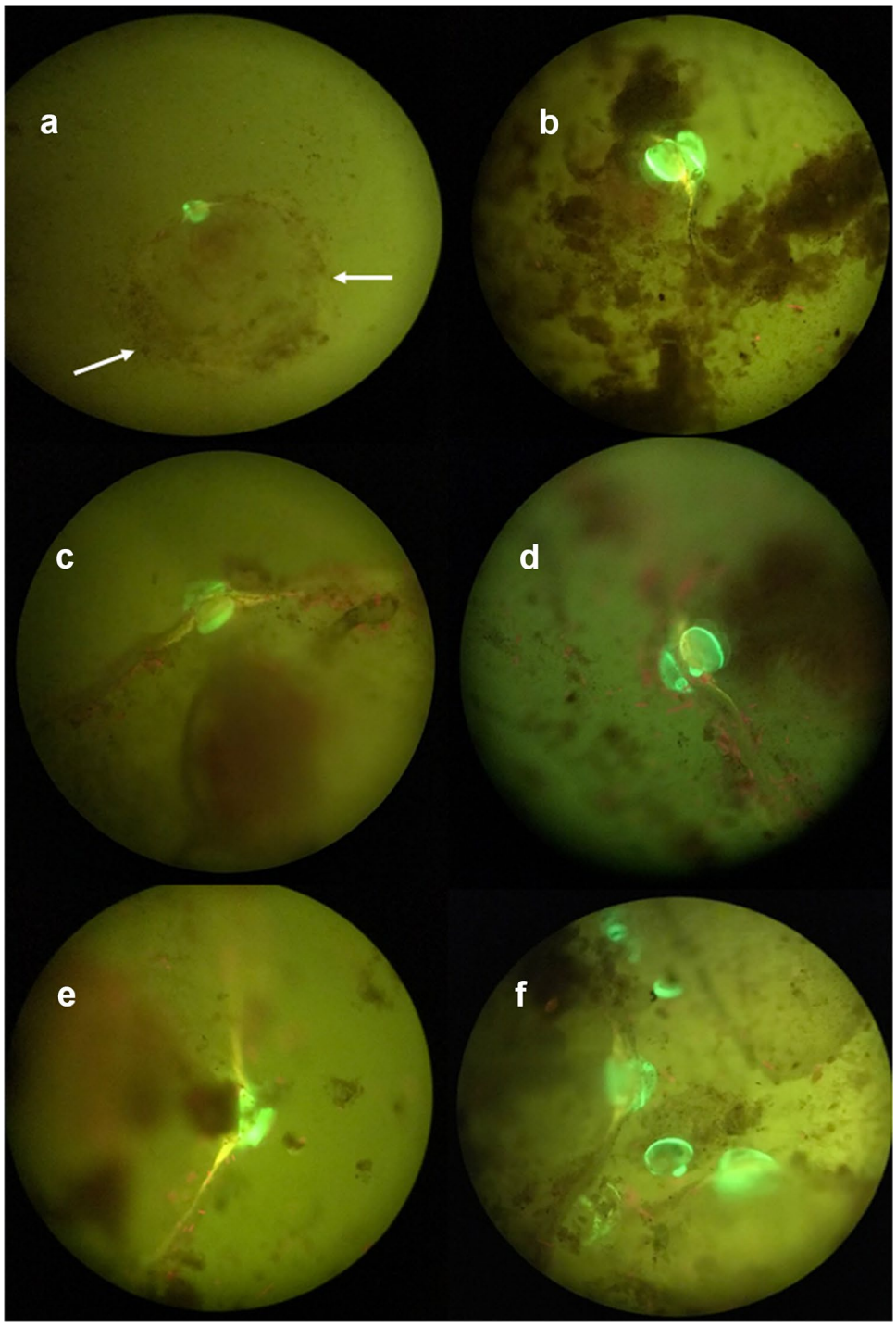

Fig. 5 Calcein-tagged spat from each site photographed under blue light excitation (490 nm) on July 26 (**a**, **c**, **e**), and September 20 (**b**, **d**, **f**). These photographs were taken 18 (**a**, **c**, **e**) and 16 (**b**, **d**, **f**) days after marking. Note that the larval shell, a small portion of the juvenile shell, fluoresces. The outline of the juvenile shell is most readily apparent in panel **a**, with white arrows pointing to the shell margin

1274 spat per m^2 and those in the September release ranged between 7911 and 13,349 spat per m^2 (Fig. 9).

The estimated number of spat per shell (number of spat observed during initial settlement counts /number of valves) in each bag for the July deployment was between 0.0 and 13.4 (Fig. 10). During the September deployment, the estimated number of spat per shell in each bag ranged between 0 and 92 (Fig. 10).

Stratified sampling of shell bags revealed that greater than 50% of the larvae settled in the top layer of the shell during both deployments. In July, 62%, 16%, and 22% (Table 3) of the spat were found in the top, middle, and bottom layers of the shell bags, respectively. Similar results were seen in from the September deployment where 56%, 26%, and 18% (Table 3) of the spat settled in the top, middle, and bottom layers of the shell bags respectively.

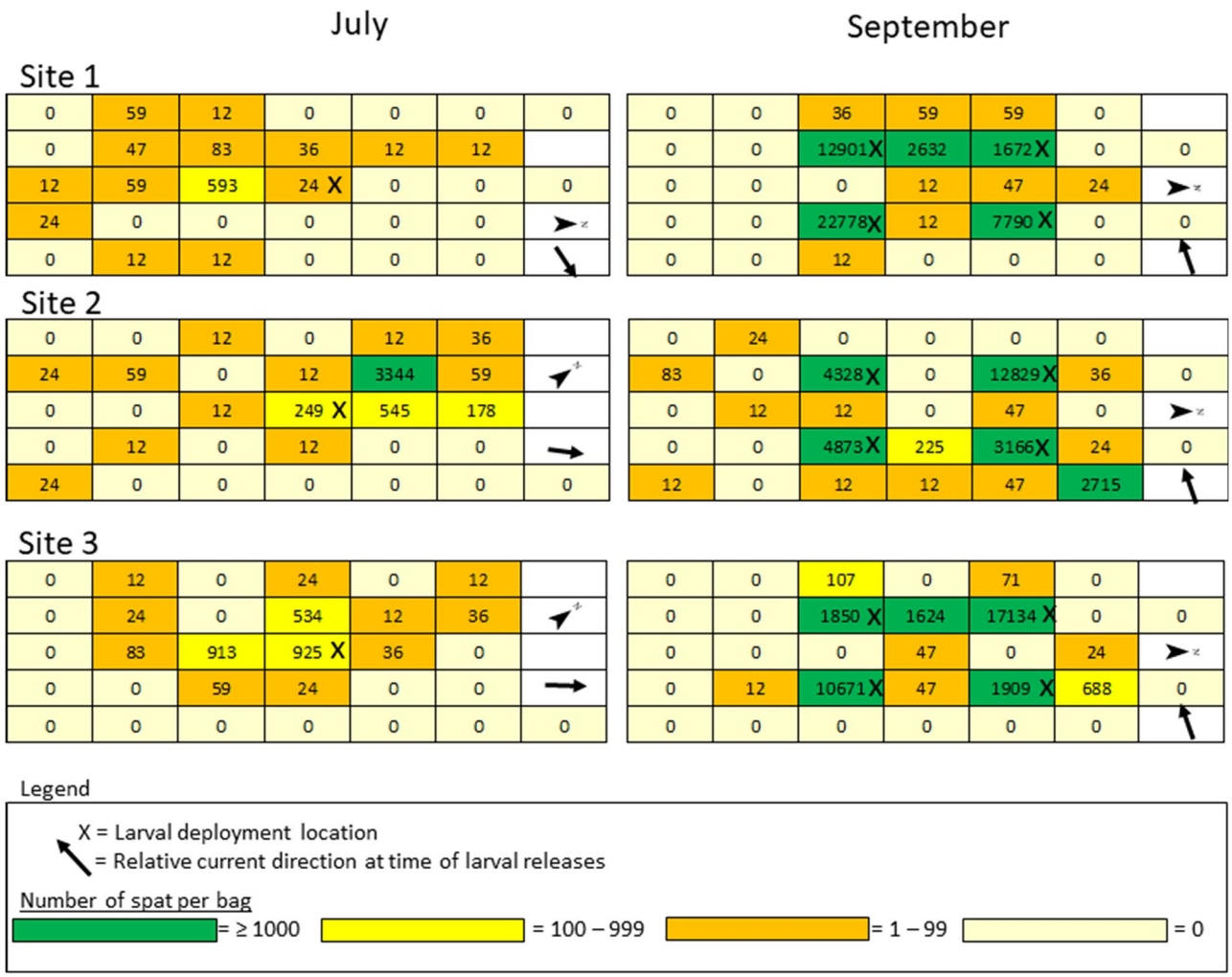

Fig. 6 Estimated total number of spat in each bag at each site after bags were retrieved from the river 8 to 12 days after larvae were released. Each grid cell represents the estimated number of initial settlers within a shell bag. An "x" within a grid cell represents the larval release location. 1.6 million marked larvae were released onto each site during each experiment (July 15 and September 10). The black arrow represents the direction of the current relative to each site averaged over the bottom boundary layer of the water column (0–0.75 m) at the approximate time of larval releases. During the July deployment, all 1.6 million larvae were released onto one location on each site (x). During the September deployments, the 1.6 million larvae were divided into 4 equal bundles (0.4 million) and released at 4 locations within each site (x). Blank cells indicate that no shell bags were in that location

In-Tank Mortality

For the individuals set aside and monitored between initial and final counts, the in-tank mortality varied between the July and September deployments. In July, 122 of the 191 spat monitored each week died (64% mortality). During September, 20 of the 93 spat monitored each week died (22% mortality).

The bag-specific mortality ranged from 12 to 100% between the initial and final spat counts (Fig. 11). All of the bags with at least 7790 ($n = 6$) estimated initial spat had greater than 93% mortality during this period.

Current Velocities

The observed bottom boundary (< 0.75 m) currents during the time of larval release were low for both deployments. During the deployment and for the 90 min immediately following the larval deployment, the current velocities averaged 5.7 ± 3.4 (mean ± SD) and 3.7 ± 1.3 cm s^{-1} in July and September, respectively. The minimum current velocity observed during the 7 days that the ADCP was deployed was 0.0 cm $s^{-1}$for both July and September, and the maximum velocity was 17.4 and 16.5 cm s^{-1} for July and September, respectively. There was no obvious relationship

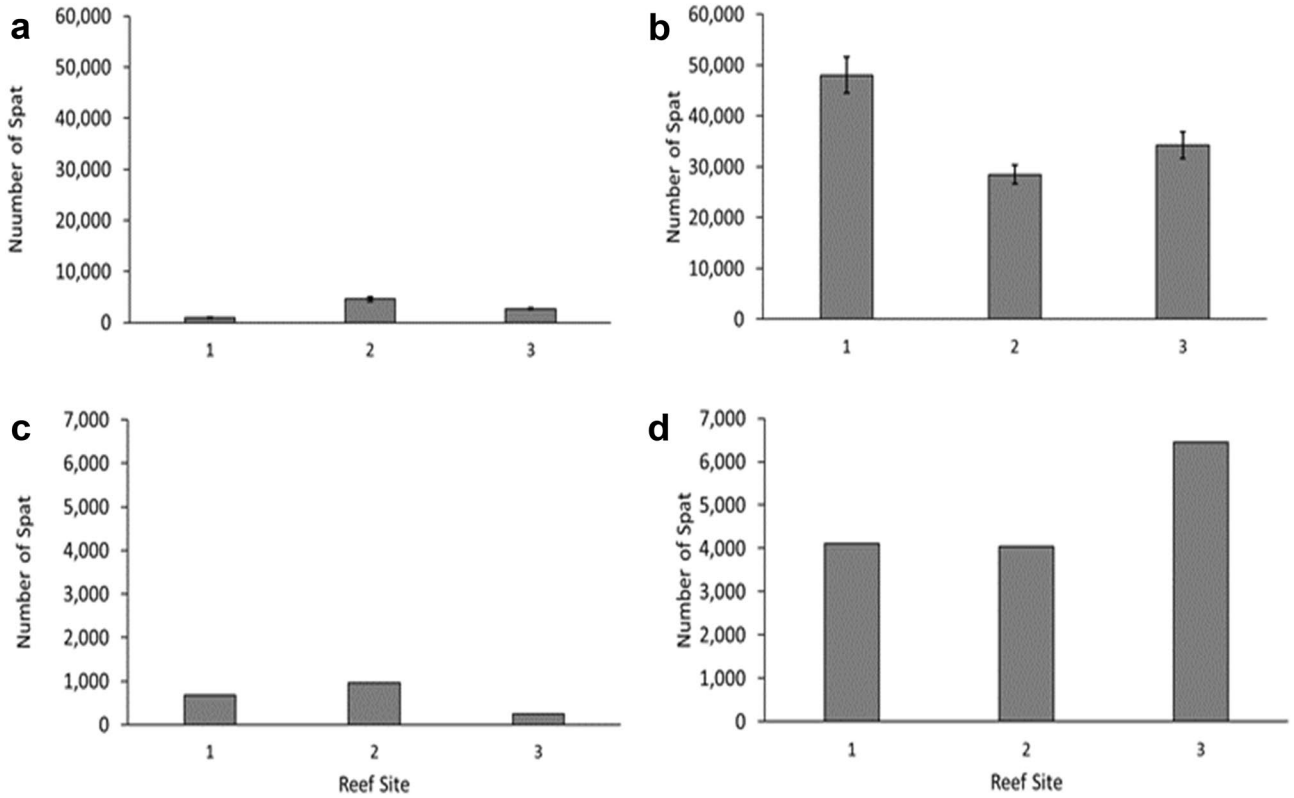

Fig. 7 Estimated number of spat on each site for July (**a**, **c**) and September (**b**, **d**) deployments. **a** and **b** represent the initial settlement counts (estimated number of settlers 8 to 12 days after larval release). **c** and **d** represent the final spat counts (total number of spat found on all shells 42 to 46 days after larval releases). Error bars represent one standard error of the total. Salinities at the time of larval releases were 7.9 and 11.8 psu during July and September, respectively. Salinities at the end of the grow out period in the tanks were 8.0 and 12.9 psu in July and September, respectively. Note that axes differ between upper and lower panels

between the settlement pattern of recovered spat and the direction of the current at the time of larval releases (see black arrows in Fig. 6).

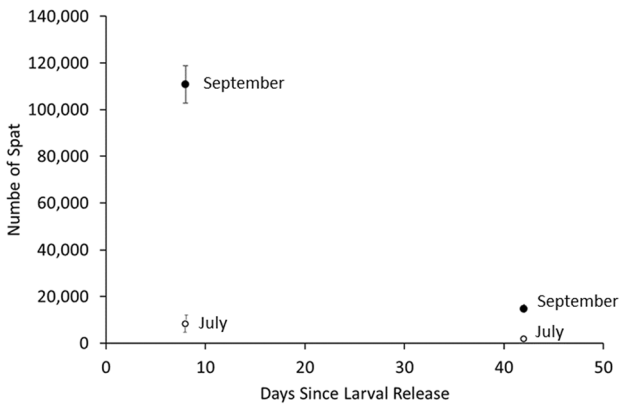

Fig. 8 Estimated number of initial settlers 8 to 12 days (initial settlements counts) after larval release and the number of spat counted 42 to 46 days (final spat counts) after larval releases for the July (open circles) and September (closed circles) deployments. Error bars represent one standard error of the total

Discussion

This study shows that calcein-marked *C. virginica* larvae cued with OCW and released directly onto suitable settlement material in situ without enclosures can settle and metamorphose into juveniles. In both July and September, spat were concentrated at larval release locations within each site and all spat that were viewed with a compound microscope under blue light excitation contained the calcein tag. While others provided evidence of the utility of direct setting without enclosures (Coon and Fitt 1999), this work is the first to definitely demonstrate successful direct setting of *C. virginica* with mark and recapture tools. Previous work has suggested the utility of direct setting without enclosures (Coon and Fitt 1999), and this work provides continued evidence of that idea.

Verification of Larval Origin

Results show that *C. virginica* pediveliger larvae tagged with calcein, released in situ, and recovered as spat do retain the calcein mark on the exterior shell, and that the

Table 2 Estimated settlement counts and efficiencies and observed number of spat for the July and September deployments

	Reef site 1	Reef site 2	Reef site 3
Initial settlement effiency (%)			
July	0.1	0.3	0.2
September	3.4	2.0	2.4
Estimated number of spat (8–12 days)			
July	996 ± 78	4588 ± 447	2691 ± 180
September	48,057 ± 3479	28,480 ± 1878	34,208 ± 2598
Number of spat (42–46 days)			
July	673	966	249
September	4117	4038	6445

Estimated setting efficiency (estimated number of spat/number of larvae released). The number of spat used as the numerator was the number estimated during the initial settlement counts (8–12 days after larval release). The denominator is the total larvae released at each deployment site (1,600,000 in both July and September). The number of spat (42–46 days) is the total number of spat counted when every valve was examined 42–46 days after larval release

mark can be confirmed without sacrificing individuals. Hence this study confirms that the laboratory results demonstrated in Spires et al. (2022) hold true in an applied setting. Tagging *C. virginica* larvae with calcein could be a viable method for proving larval origin in studies of direct setting. Tagging *C. virginica* larvae with calcein also has been used in an effort to understand planktonic transport in Mobile Bay, Alabama, with 2 veliger larvae of 22 million released larvae recovered after 8–10 days (Gancel et al. 2019).

Direct Setting Without Enclosures

This study demonstrates that larvae, cued with OCW, released directly on substrate, without enclosures, at slack tide, can settle, metamorphose, survive, and be quantified after seven days. The direct release and settlement of competent oyster

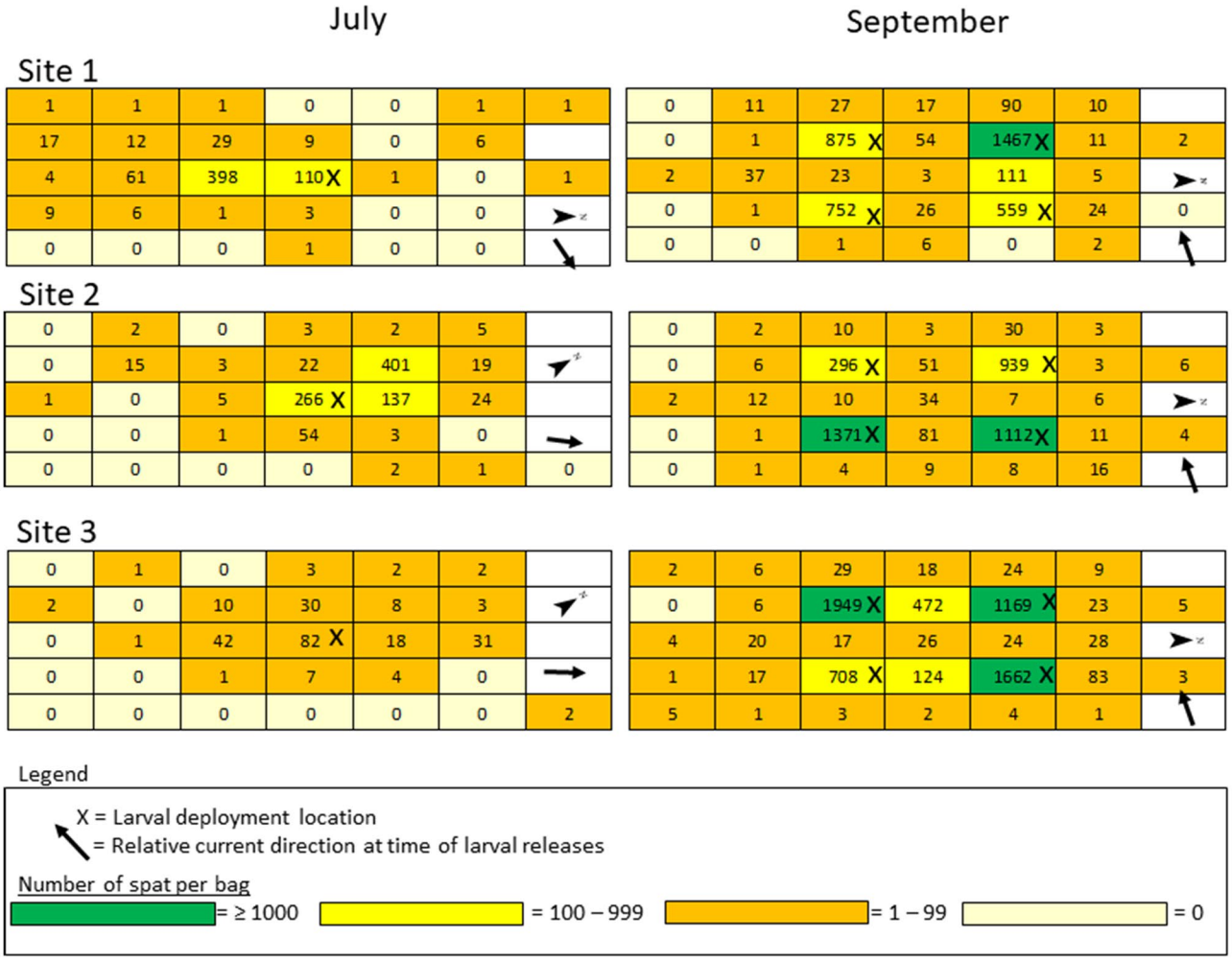

Fig. 9 Total number of spat found in each bag 42 to 46 days (final spat counts) after larval releases. An "x" within a grid cell represents the larval release location. Black arrows represent the direction of the current relative to each site averaged over the bottom boundary layer of the water column (0–0.75 m) at the approximate time of larval releases. Blank cells indicate that no shell bags were in that location. Counts reflect all shells in the bag minus the 21 used for initial counts

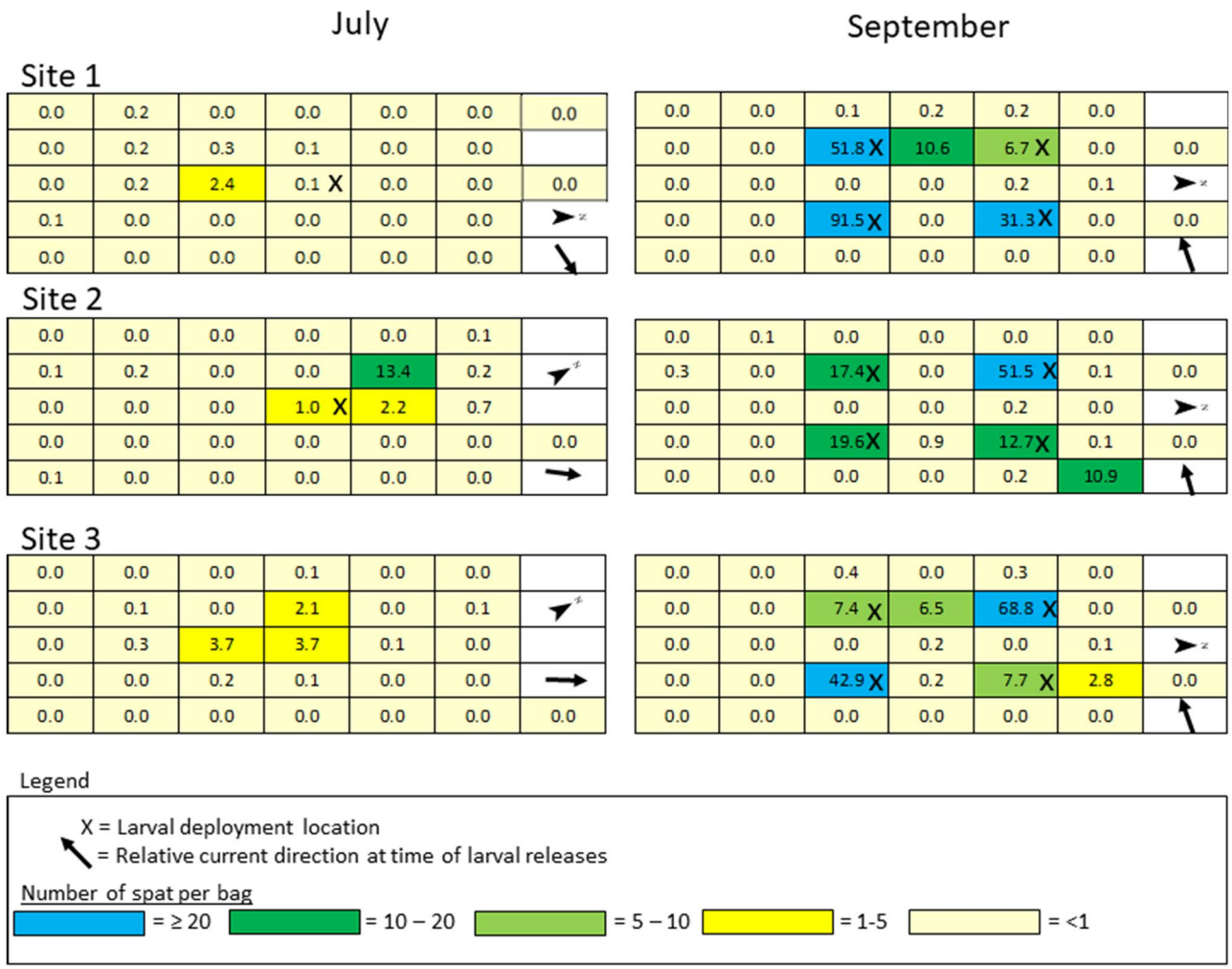

Fig. 10 Estimated number of spat per shell in each bag 8 to 12 days (initial settlement counts) after larvae were released at the three sites in July and September. Total number of estimated initial settlers per bag was divided by the total number of valves in each bag ($n = 249$). An "x" within a grid cell represents the larval release location. Black arrows represent the direction of the current relative to each site averaged over the bottom boundary layer of the water column (0–0.75 m) at the approximate time of larval releases. Blank cells indicate that no shell bags were in that location

larvae onto various substrates in situ has been demonstrated with *C. virginica* enclosures to minimize larval loss (Burke 2010; Rahall et al. 2011; Theuerkauf et al. 2015; Steppe et al. 2016). While successful, containment systems limit the scalability and increases the cost of direct setting.

Table 3 Estimated total number of spat from initial settlement counts, for all sites, both deployments combined

	July	September
Total initial settlement count	8275	110,745
% settlement top of bag	62	56
% settlement middle of bag	16	26
% settlement bottom of bag	22	18

The percent of spat that was found in each stratum of all of the bags used for the July and August deployments

Difference Between Deployments

The difference in spat settlement rates between the two deployments in 2019 may have been related to environmental conditions. *C. virginica* larvae for both deployments were reared in salinities above 10 psu (Table 1b). Salinity at the time of larval releases was 7.9 psu in July and 11.8 psu in September. At the same time, the total number of spat estimated on each site increased from a high of 8275 in July to 110,745 in September, and the average setting efficiencies for all three sites increased from 0.03 (July) to 3.4% (September). Pediveliger settlement rates have been shown to be reduced when they experience salinities below levels in which they underwent earlier larval developmental phases (Priester 2016), and salinities at the experiment site in July were lower

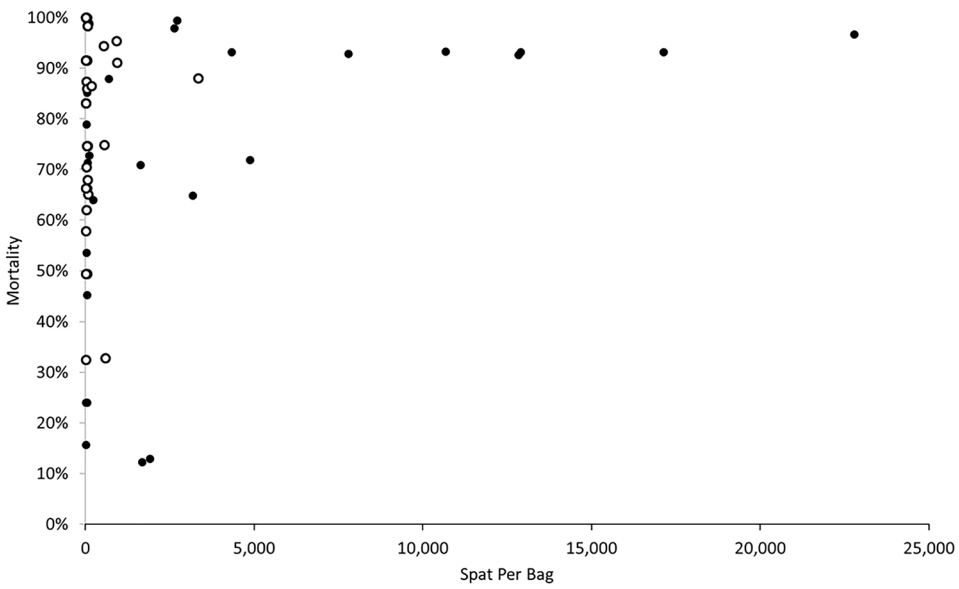

Fig. 11 Estimated mortality of spat in each bag for the July (open circle) and September (closed circle) experiments, plotted against the estimated number of spat per bag 8 to 12 days (initial settlement counts) after larvae were released. Morality was calculated with the number of spat per bag between the 8- to 12-day counts and the 42- to 46-day (final spat counts) counts

than those at the Piney Point Aquaculture Center hatchery (the larval source). In addition, the higher survival in September may be linked to the salinity in which the *C. virginica* broodstock underwent gametogenesis; *C. virginica* larvae survive at higher rates in conditions similar to those experienced by broodstock (Davis 1958; Scharping et al. 2019). The broodstock at the Piney Point Aquaculture Center (the larval source) underwent gametogenesis at salinities above 9 psu, hence larvae released into the river in July when salinity was 7.9 psu may have experienced suboptimal conditions.

Differences in observed spat settlement between July and September also may have been related to the release method. Spat settlement patterns for the September deployment (Figs. 6 and 9) show that 90% of the spat found were within the bag directly receiving larvae. During the July deployments, 90% of the spat were found in the shell bag directly receiving larvae or the adjacent bags. For the September deployment, divers removed the lid from a plastic screw-top container to release the larvae, whereas a large syringe was used during the July deployments. The difference in settlement locations relative to the deployment site may be related to the difference in deployment mechanisms, with the syringe pushing out larvae more forcefully and spreading them over a larger region compared with gentle emptying of the container.

Current velocity measurements and spatial patterns in settlement suggest that advection by currents was not a major factor influencing larvae when deployed at slack tide. Larval deployments were targeted for release on slack tides and ADCP data confirms that bottom (0 -0.75 m off the bottom) current velocities were minimal during and immediately after (90 min) the larval releases. In addition, there was no obvious influence of current direction on settlement patterns related to the release sites of the larvae (Fig. 6). Finally, very few spat were found more than two shell bags away from the release points, suggesting that currents did not transport larvae away from the release location.

Mortality Observations

The observations of in-tank mortality varied between the July and September deployments. During both experiments, a subset of valves was bundled and spat were tracked for survival for 35 days in the grow out tanks. Observed mortality differed markedly between the deployments, with 64 and 22% of the individuals dying in July and September, respectively. Mortality rates are variable from week to week in newly settled *C. virginica* populations (Roegner 1991) so the difference between deployments may not be unexpected.

The observed differences in spat mortality in the tanks between July and September may have been related to differences in salinity and to differences in the presence/abundance of predator communities in the grow out tanks at that time. The tanks used to grow out the spat did not contain filtration systems. Flatworms (*Stylochus ellipticus*), blue crabs (*Callinectes sapidus*) and mud crabs (*Panopeus herbstii* and *Eurypanopeus depressus* are all found in this region of Chesapeake Bay (Lippson and Lippson 1997) and are known to prey on juvenile *C. virginica* (Krantz and Chamberlin 1978; Abbe 1986; Bisker and Castagna 1987; Newell et al. 2000). Of the predators listed above, flatworms and mud crabs were observed in the tanks during the grow out period, but blue crabs were not. Published mortality rates for newly settled spat (Roegner 1991; Roegner and Mann 1995) are not directly comparable to

those in this study, because of the difference in the ages at which mortality rates were estimated and because the suite of predators was not the same (Newell et al. 2000).

Bag-specific mortality observations (Fig. 11) ranged from 0 to 100% with greater than 90% mortality occurring in bags containing as few as 12 initial settlers. In this study there did seem to be a threshold reached around 7,790 initial settlers: all bags containing more than this initial number of spat had greater than 93% mortality. It is likely that density-dependent mortality did occur due to competition for space and overcrowding in the majority of the bags containing significant numbers of *C. virginica* in this study, effects that have been shown to negatively influence the survival of *C. virginica* spat (Osman et al. 1989).

Comparison with Hatchery Targets

Setting efficiencies in this study were not directly comparable with current remote setting efficiencies. Local oyster hatcheries target initial spat on shell concentration of 10–15 spat per shell (Stephanie Alexander, personal communication). To achieve this spat density on shell bags, the Horn Point Oyster Hatchery staff typically adds 6–10 million larvae to a static tank containing ~31,000 l of water and 600 oyster shell bags (identical to the ones used in this experiment), then turns on the flow-through river water after 3 days. The initial estimates of spat per shell occur 48 h after larvae are released into the setting tanks (Stephanie Alexander, personal communication). In contrast, in our experiment 1.6 million larvae were released onto 31 shell bags in an open system, and spat set was quantified 8–12 days after larval release. The difference in timing of spat counts makes it difficult to compare spat on shell numbers.

It needs to be emphasized that this experiment produced setting concentrations on each site that were far too low for restoration purposes in some places and far too high in others (Fig. 10). Only 6 shell bags out of 190 deployed had an average of 10–20 spat per shell and 6 bags averaged 30–91 spat per shell (Fig. 10), a value considerably higher than the target 10–15 spat per shell ideal target the Horn Point Oyster Hatchery and the Piney Point Aquaculture Center strive to achieve.

Although most of the shell bags had spat sets that were below hatchery targets, there are several reasons why direct setting still may be useful for restoration and stock enhancement purposes. Hatchery operations incur many costs that are not part of direct setting (e.g., shell acquisition, shell cleaning, shell transport, shell storing, shell loading, shell planting, setting tank operation, large vessel/barge motoring to planting site, etc.). The potential may exist for direct setting to serve as an additional or alternative oyster restoration strategy in larvae-limited systems where suitable water quality and settlement substrate exists. The direct release of larvae via divers using the mechanisms employed in this study resulted in highly concentrated spat settlement on the specific shell bags where larvae were released. Notably, > 70% of the spat found in this study were on 6 of 190 shell bags, clustered on the larval release locations. To be most useful for restoration, aquaculture, or stock enhancement applications, additional studies should aim to better disperse larvae to achieve more even spat settlement.

Direct setting has been found to be successful in previous experiments. Direct setting using enclosures has been shown to produce setting efficiency rates comparable to remote setting operations (7–10%) (Bohn et al. 1995; Congrove 2008). Steppe et al. (2016) estimated an average setting efficiency of 15% after 3 days when pouring larval mixtures onto the water surface over planted oyster cultch contained within enclosures in 2.5 m of water. The range of setting efficiencies for this study (0.1–3.4%) are lower than the published average remote setting rates and those demonstrated by Steppe et al. (2016). Steppe et al. (2016) estimated settlement 3 d after release whereas this experiment did not attempt to quantify spat settlement until days 8–12. The difference in settlement efficiencies may be due to the shorter time between larval deployments and spat counts between this study and Steppe et al. (2016) that could reduce the amount of natural mortality on the settled larvae and the use of containment systems to reduce advective losses of larvae.

Direct setting without enclosures over larger areas has been attempted previously (Coon and Fitt 1999) and was thought to have achieved a detectable increase in juveniles over the treated site when compared to surrounding control reefs. Similar to our experiment, Coon and Fitt (1999) also used a chemical larval settlement inducer (L-3,4-dihydroxyphenylalanine) to encourage larvae to strike on suitable substrate. However, the authors of that study noted their inability to definitively prove larval origin because of a lack of a tagging mechanism and were unable to successfully replicate results. Unlike Coon and Fitt's (1999) work, this study observed similar results on three adjacent sites during both deployments (July and September).

Application and Next Steps

Methods to expand upon the lessons learned by this study and previous direct setting experiments (Coon and Fitt 1999; Steppe et al. 2016) should attempt to disperse larvae more evenly, on a variety of substrates, habitat types, (e.g., shell, stone, intact oyster reefs), and over areas larger than previously attempted. Currently, techniques to expand direct setting capabilities using vessels and towed mechanisms are being tested in the Tred Avon River Oyster Sanctuary and the Eastern Bay, a tributary of Chesapeake Bay. If efforts to release larvae over larger areas using towed apparatus produce oysters in quantities suitable for restoration and

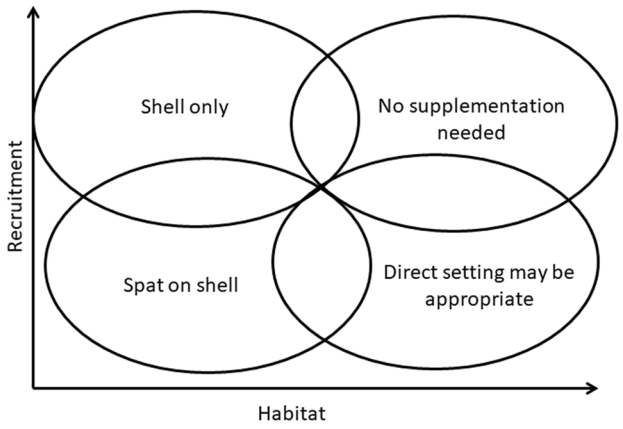

Fig. 12 A conceptualization of potential regions where direct setting and other supplementation methods may be useful stock enhancement tools

replenishment purposes, direct setting has the potential to contribute to oyster restoration, habitat management, public fishery replenishment efforts, and on-bottom aquaculture operations in regions that have intact oyster habitat but experience low recruitment (Fig. 12). In Chesapeake Bay, regions identified as having low recruitment and existing oyster habitat can be found in many tributaries throughout the estuary (Tarnowski 2019). Recent advances in rapid oyster habitat classification methods (Heggie and Ogburn 2021) coupled with recruitment monitoring like the efforts undertaken and described in the Maryland Oyster Status Reports (Tarnowski 2019) may help identify regions that have suitable amounts of settlement substrate but lack appreciable *C. virginica* recruitment; these could be candidate areas for direct setting.

This study has confirmed the ability to directly seed oyster cultch material in situ without enclosures through the aid of chemical tagging methods for *C. virginica* (Spires et al. 2022) and the aid of a larval settlement inducer (OCW) (Tamburri et al. 1996). Direct setting settlement efficiencies in this study were lower than typical remote setting operations (Bohn et al. 1995; Congrove 2008) and direct setting within enclosures (Steppe et al. 2016). However, our work is the first to demonstrate that direct setting is possible in 5 m of water using divers and no containment barriers. The potential cost savings incurred using a direct setting method that more evenly disperses larvae may encourage further refinement of these methods. Additional investigations to quantify the effectiveness of OCW to enhance settlement rates in situ and to test alternative OCW concentrations are being conducted at Hampton University (Sierra Hildebrandt 2021, pers. comm), and may validate the use of OCW as a beneficial additive to direct and remote setting approaches. The results of this study suggest that additional research direct setting of *C. virginica* larvae, including a cost-benefit economic analysis, is warranted to develop and test the technique as a tool for stock enhancement and restoration where suitable settlement material already is present.

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Data Availability The data that support the findings of this study are available from the corresponding author [JES].

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