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SETTLEMENT OF *CRASSOSTREA ARIAKENSIS* LARVAE: EFFECTS OF SUBSTRATE, BIOFILMS, SEDIMENT AND ADULT CHEMICAL CUES

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ABSTRACT The Suminoe oyster (*Crassostrea ariakensis*) is being considered for introduction into the Chesapeake Bay. However, our current understanding of the biology and ecology of *C. ariakensis* is insufficient to predict whether an introduction will be successful, provide desired benefits, or have adverse impacts. Behavior of native Eastern oyster (*C. virginica*) pediveligers has been studied for many years and it is well established that they use a variety of habitat characteristics when selecting a site for colonization. Perhaps the most important of these are chemical cues emitted by adult conspecifics, which can lead to gregarious larval settlement and dense, persistent reef communities. Conversely, almost nothing is known about the mechanisms that regulate larval settlement and metamorphosis for *C. ariakensis* or how pediveligers might respond to conditions found in Chesapeake Bay. In a comparative study with *C. virginica*, we examined how environmental factors such as substrate type, natural biofilms, sediment and waterborne chemical cues influence larval settlement for two *C. ariakensis* strains (''south China'' and ''west coast''). Our results demonstrate many similarities but also potentially important differences. Both species and strains of larvae greatly prefer natural substrates (e.g., shell) covered with biofilms for colonization but the west coast strain of *C. ariakensis* exhibited greater attachment onto manmade substrates (e.g., fiberglass) than *C. virginica*. Waterborne chemical cues emitted by adult oysters were also found to enhance substrate attachment for all larval forms but cues do not appear to be species specific. These results provide critical insight to the ability of *C. ariakensis* larvae to identify and colonize suitable substrates in the Chesapeake Bay, which will have a large impact on recruitment success and their ability to establish self-sustaining populations.

KEY WORDS: larval behavior, settlement, substrate preference, chemical cues, Suminoe oyster, *Crassostrea ariakensis*

INTRODUCTION

The Eastern oyster, *Crassostrea virginica* (Gmelin 1791), has historically supported a culturally and economically important fishery along the Atlantic and Gulf of Mexico coasts of the United States (Kent 1988, Mackenzie & Burrell 1997) and served as an important component of many estuarine ecosystems. Reefs built by oysters provide structure and habitat for a diverse assemblage of organisms, while consuming phytoplankton and reducing the accumulation of organic matter in the water column (Newell 1988, Lenihan & Grabowski 1998, Coen & Luckenbach 2000). *Crassostrea virginica* populations in the Chesapeake Bay are, however, at historic low levels as a result of several factors such as heavy harvesting pressure, habitat degradation, and high mortality caused by diseases (e.g., Homer et al. 1996, Ford & Ashton-Alcox 1998, Kirby & Miller 2005).

In response to this decline in native Eastern oysters, the states of Maryland and Virginia are considering introducing the Asian Suminoe oyster (*C. ariakensis* [Fujita 1913]) into the tidal waters of the Chesapeake Bay with the goal of establishing a naturalized, reproducing, and self-sustaining population of this nonnative species (see review by National Research Council 2004). However, neither the potential risks nor the potential benefits of introducing *C. ariakensis* to this region are adequately known. The current understanding of the biology and ecology of *C. ariakensis* is insufficient to predict whether an introduction will provide desired benefits or have substantial adverse impacts within the Chesapeake Bay or other Atlantic Coast estuaries over the short- or long-term.

To address this lack of knowledge, priority areas for research have been identified in the National Research Council (NRC) report on Nonnative Oysters in the Chesapeake Bay (NRC 2004) and the Scientific and Technical Advisory Committee (STAC) of the Chesapeake Bay program report on identifying and prioritizing research required to evaluate ecological risks and benefits of introducing diploid *Crassostrea ariakensis* to restore oysters to Chesapeake Bay (STAC 2004). Many of the recommendations and priorities focus on the larval biology and ecology of *C. ariakensis*. For example, the ability to answer the basic question of whether *C. ariakensis* can survive and reproduce in Chesapeake Bay is dependent in large part on understanding their larval behavior and settlement in response to environmental conditions found in the mainstem Bay and other tidal waters of Maryland and Virginia.

Many benthic organisms produce planktonic larvae whose dispersal and recruitment to benthic sites affects population dynamics and community structure (Olafsson et al. 1994, Palmer et al. 1996). The first step in larval recruitment is settlement (movement onto and attachment of a larva to the substratum). Although settlement and postsettlement events affect recruitment, there are many cases in which temporal and spatial patterns in recruitment are caused by larval settlement (review: Roughgarden et al. 1991, Rothlisberg & Church 1994). Habitat selection during settlement for sessile benthic animals, such as oysters, is of particular significance because there is essentially no chance of relocation after metamorphosis onto a substrate.

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It has been well established that *C. virginica* pediveligers can use a variety of habitat characteristics when selecting a site for colonization. For example, bacterial films and other organic factors that characterize a suitable substrate are involved in triggering behavioral changes at settlement (Veitch & Hidu 1971, Fitt et al. 1989). Perhaps the most important of such chemical cues are emitted by adults of the same species. It has been shown that for *C. virginica* and *C. gigas*, chemical signals exuded by adult conspecifics induce larval settlement from the water column (Tamburri et al. 1992, Turner et al. 1994, Zimmer-Faust & Tamburri 1994, Tamburri et al. 2007).

Larval behavioral responses to chemical signals released by adults of the same species often lead to dense, persistent aggregations of conspecifics (Hidu 1969, Pawlik 1992), such as oyster reefs (Bayne 1969, Tamburri et al. 1996, Tamburri et al. 2007). This gregariousness presumably increases postmetamorphic survival (Buss 1981, Highsmith 1982, Sebens 1983), and/or mating success (Pennington 1985, Denny & Shibata 1989, Levitan 1991). For oysters that are sessile broadcast spawners, neighboring conspecifics are obviously required to assure successful reproduction.

It is also well established that *C. virginica* requires sediment-free, hard substrate for metamorphosis. However, high sediment loads have become a common problem in the Chesapeake Bay and excessive siltation levels on reefs has been shown to impair habitat quantity and quality for settling *C. virginica* larvae and newly attached juveniles (Bahr 1976, Seliger & Boggs 1988). The preferred substrate for *C. virginica* is oyster shell, but pediveliger larvae begin the complex process of cementing to the substrate in response to other hard surfaces, such as rocks, pilings, etc. as well (Kennedy 1996).

It is unknown, however, what benthic habitats are most suitable for *C. ariakensis* and which substrate types/characteristics will trigger settlement and metamorphosis. There are also uncertainties about the reef-building characteristics of *C. ariakensis* in different regions where it naturally occurs (NRC 2004). Whereas known as a "reef-builder," there are observations of this species living as single individuals in areas where soft sediment predominates (Luckenbach, personal observations).

This study was designed to determine how the substrate preferences of *C. ariakensis* at the time of settlement differ from or resembles those of *C. virginica* and ultimately how these traits will impact recruitment, the likelihood of successful introduction into the Chesapeake Bay, and the potential for becoming a fouling nuisance species. Specifically, laboratory experiments were conducted to examine the substrate preferences of *C. virginica* and *C. ariakensis* larvae and their response to waterborne chemical cues. For this study, "settlement" is defined as contact with and attachment to a surface, including complete metamorphosis into spat.

MATERIALS AND METHODS

Larval Culture

All spawning, larval rearing and experiments were carried out in a quarantined laboratory at the Virginia Institute of Marine Science's Eastern Shore Laboratory in Wachapreague, VA. Experiments were conducted using larvae spawned from wild *C. virginica* collected in the vicinity of the laboratory and two strains of *C. ariakensis*: one strain, termed "west coast *C. ariakensis*" (and henceforth termed "WCA"), is derived from stocks imported into the United States west coast from Japan in the 1970s (Breese & Malouf 1977); the other strain, termed "south China *C. ariakensis*" (henceforth "SCA"), is an F3 generation from stocks imported from southern China. All effluent water from the laboratory was chlorinated (0.5 mL 5% Na hypochlorite L^{-1} of effluent) and dechlorinated (0.167 g Na thiosulfate mL^{-1} of Na hypochlorite) prior to discharge to ensure that no escape of gametes or larvae occurred.

Cohorts were produced from the eggs of single females and pooled sperm from several males and reared separately to the pediveliger stage. Larvae from individual cohorts were held in 10 L buckets maintained at 25°C to 26°C in a water bath and reared on a mixed diet of cultured *Isochrysis galbana*, *Chaetoceros neogracile*, *Pavlova pinguis*, and *Tetraselmis striata*. All brood stock conditioning, larval rearing, and experiments were conducted in 20 psu seawater made by diluting near full strength sea water from Wachapreague Channel with filtered freshwater. Experiments were conducted using individual cohorts as replicates; however, the logistics of conditioning, spawning, and larval rearing precluded simultaneously running the experiments with more than one species or strain. Thus, the experiments were conducted sequentially the spring and summer of 2004–2006. Care was taken to conduct all experiments and larval rearing under identical conditions of diet, temperature, and salinity.

Response to Substrate Type and Biofilms

The effects of six substrates (three natural and three man-made), each with and without natural biofilms, on settlement were examined for each of the three oyster species/strains (Table 1). The natural substrates included *C. virginica* shell, *C. ariakensis* shell and granite. The oyster shell substrates were

TABLE 1.

Substrate selection experimental design, indicating the numbers of replicates trials using each species/stock of oyster larvae. Cv = *Crassostrea virginica*, WCA = west coast *Crassostrea ariakensis*, SCA = south China *Crassostrea ariakensis*.

Substrate	No Biofilm	Biofilm
C. virginica shell	Cv: 14 reps	Cv: 14 reps
	WCA: 17 reps	WCA: 17 reps
	SCA: 18 reps	SCA: 18 reps
C. ariakensis shell	Cv: 8 reps	Cv: 8 reps
	WCA: 12 reps	WCA: 12 reps
	SCA: 12 reps	SCA: 12 reps
Granite	Cv: 8 reps	Cv: 8 reps
	WCA: 12 reps	WCA: 12 reps
	SCA: 12 reps	SCA: 12 reps
Fiberglass	Cv: 14 reps	Cv: 14 reps
	WCA: 17 reps	WCA: 17 reps
	SCA: 18 reps	SCA: 18 reps
PVC	Cv: 8 reps	Cv: 8 reps
	WCA: 12 reps	WCA: 12 reps
	SCA: 12 reps	SCA: 12 reps
Stainless steel	Cv: 8 reps	Cv: 8 reps
	WCA: 12 reps	WCA: 12 reps
	SCA: 12 reps	SCA: 12 reps

individual disarticulated valves of small *C. virginica*, WCA, or SCA. Shells were selected to have approximately the same surface area as the artificial substrates (see below). In trials with *C. virginica* larvae, conspecific shells, and those of WCA were used. In trials with larvae of either of the two *C. ariakensis* strains shells were used from the same strain as the larvae used in the treatment. All shells were cleaned of any tissue, thoroughly scrubbed, and air-dried prior to use. Granite pebbles with an upper surface area of approximately 5 cm^2 were cleaned and air dried. The man-made substrates included stainless steel, polyvinyl chloride (PVC), and fiberglass. We used stainless washers with an upper surface area = 3 cm^2; PVC and fiberglass were cut to the same dimensions as the washers. All substrate materials were thoroughly cleaned with freshwater and air dried before being used without biofilms or being subjected to biofilm development.

Natural biofilms were allowed to develop on one half of the substrates by soaking them for a minimum of 72 h in unfiltered flow-through seawater. The other half of the substrate replicates were maintained dry during this period and used as the *No Biofilm* treatment.

Individual pieces of a selected substrate type were added to small wells (measuring 4.2 cm × 6 cm × 4 cm deep) in a plastic (low density polyethylene) box such that each box contained one full set of test substrates at each biofouling level. Oyster shells were placed in the wells with the inside of the valve facing down. Because the curvature of the shells provided space for oyster larvae to have access to the undersides of the shells, we bent the stainless steel, PVC, and fiberglass rings slightly and placed them with the curved side down in the wells, thus allowing larvae access to both sides of these substrates. Fifty milliliters of filtered 20 psu seawater and approximately 50 oyster larvae were added with a pipette to each well, with each box receiving larvae from a single cohort. Boxes were placed in a water bath at 25°C to 26°C under ambient light and larvae were given 48 h to settle. At termination of the assays, the substrates and the walls of the wells were examined under a microscope and all oysters attached to the substrate or metamorphosed were counted as settled. For larvae settling onto the test substrates their position on the upper or lower surface of the substrate was recorded. Wells were rinsed with seawater and all unattached larvae, live and dead, were counted. Total larvae in each well was computed as the sum of settled (on the substrate and the box) and unattached (live and dead) larvae. The observed total numbers of larvae in each well (X = 53.2, SD = 12.9) were close to our nominal stocking density of 50, but in calculating the proportion of larvae settling on the substrate, we used the observed total for each well.

Our initial experiments included all six substrates × two biofouling levels and eight replicate cohorts for *C. virginica* and 12 each of WCA and SCA. Because the data suggested a greater settlement by WCA than *C. virginica* onto fiberglass, we conducted additional trials using *C. virginica* shells and fiberglass at both biofilm levels with 2 *C. virginica* cohorts, 5 WCA cohorts, and 6 SCA cohorts to increase statistical power for this comparison (see Table 1 for total replicate numbers at each treatment).

Response to Sediment

Similar assays as those described above were used to examine the impact of sedimentation on settlement. In this experiment three substrate treatments were used: (1) *C. virginica* shell with biofilm (as described above) and no sediment: *Shell only* treatment, (2) *C. virginica* shell with biofilm and sediment: *Shell + Sediment* treatment, and (3) *Sediment only* treatment. The sediment used in these experiments was obtained from an intertidal mudflat adjacent to the laboratory, sieved through a 100 μm-mesh screen and air dried. Treatment #2 was constructed by placing a *C. virginica* shell with biofilm in one of the plastic wells with 50 mL of filtered 20 psu seawater, as described above, and gently pipetting a slurry with ~1 g dry weight of sediment onto the upper surface of the shell. This amount was sufficient to cover the shell with a fine layer of sediment. The *Sediment only* treatment was constructed by covering the bottom of the well with ~1 mm depth of sediment. As in the previous experiment, approximately 50 larvae from either *C. virginica*, WCA, or SCA were added to each well in the plastic box, boxes were placed in a water bath and larvae given 48 h to settle. Experiments were conducted using larvae from single female cohorts for each species ($n = 5$ for *C. virginica* and WCA; $n = 11$ for SCA), and each treatment was replicated four times within each cohort. At termination the numbers of larvae attached to the substrate, attached to the walls of the wells and unattached were determined as described earlier.

Response to Waterborne Chemical Cues

To test for the effects of adult chemical signals that may induce settlement, and their specificity, bathwater solutions were prepared as described in detail by Tamburri et al. (1992). For these investigations, four types of solutions were made by bathing either 10 live: WCA (shell heights: 43–73 mm), SCA (SH: 31–110 mm), *C. virginica* (SH: 66–140 mm), or hard clams *Mercenaria. mercenaria* (L.) (SH: 50–96), separately in individual 10 L containers of 1 μm-filtered, 20 psu seawater for eight hours, to allow for the natural release of metabolites. *C. ariakensis* bathwater was made using adults from the same stock (WCA or SCA) as the larvae in the trials and using WCA for the *C. virginica* trials. Bivalves were then removed, the water passed through a 1-μm filter, and aliquots frozen until use. A control solution of 1 μm-filtered 20 psu seawater was frozen prior to use. Samples of each bathwater and control solution were analyzed for ammonium and total dissolved organic carbon concentrations (Table 2). Although the precise chemical cue(s) are not known, it has been found that signal production covaries with the release of these compounds by oysters (Tamburri et al. 1992, Zimmer-Faust & Tamburri 1994).

Settlement responses to the bathwater and control solutions were examined using assays similar to those described earlier.

TABLE 2.

Ammonium and dissolved organic carbon (DOC) concentrations in bathwater and control solutions.

Solution	NH_4 (mg/L)	DOC (mg/L)
C. virginica Bathwater	0.45	24.03
West Coast *C. ariakensis* Bathwater	0.30	58.59
South China *C. ariakensis* Bathwater	0.31	34.05
M. mercenaria Bathwater	0.41	22.10
Control (filtered bay water)	0.18	5.00

Pieces of either *C. virginica* shells with a biofilm or PVC without a biofilm (the most preferred and least preferred substrates, see Results) were placed in separate wells in replicate plastic boxes filled with one of the test solutions (Table 3). Approximately 50 larvae were then added to each box and the proportion of larvae that settled after 48 h determined. As before, larvae from separate single-female cohorts were used in replicate trials.

Statistical Analyses

Effects of oyster *Species* (=species or strain), *Biofilm* and *Substrate* on larval settlement were tested using the mixed models procedure in SAS (version 9). Mixed models analyses were selected because variances were heterogeneous even after applying standard transformations. Cohorts with <20% settlement on fouled *C. virginica* shell were assumed to have low competency and omitted from the analyses. Data for the remaining cohorts were normalized as the proportion settled on substrate X divided by the proportion settled on fouled *C. virginica* shell to obtain a relative settlement index (RSI). This normalization was used to reduce the effect of variation among oyster types in absolute settlement rates on analyses of differences in the response of the three oyster types to substrates and other factors. Substrates with RSI <1 were less preferred than fouled native oyster; substrates with RSI >1 were more highly preferred. Because the RSI for fouled *C. virginica* shell had a variance of zero, *C. virginica* shell was not included as a substrate choice in these analyses, but the data are shown graphically for comparison.

The effects of oyster species, biofouling, and substrate type on RSI were analyzed as a repeated measures design where cohort was the primary experimental unit and the substrates within cohort were the subunits. Inspection of AIC results indicated that an unstructured covariance structure yielded a better model fit than variance groupings based on substrate type or the interactions between substrate and fouling. Preselected *a posteriori* comparisons were conducted using sequential Bonferroni criteria for unequal variance *t*-tests of differences between least squares means. We present the results of main effects of fouling on substrate, as well as the interaction between substrate type and fouling. The most critical comparison, however, is the test for differences in settlement onto each substrate type between each of the *C. ariakensis* strains and *C. virginica*.

Because no larvae of either species settled in the *Sediment only* treatment, we analyzed the effects of *Substrate* (*Shell only* and *Shell + sediment*) and *Species* (*C. virginica*, SCA, WCA) on arcsin-square root transformed proportion of larvae settling on the substrate. We used a repeated measures mixed models analysis as described earlier. Inspection of AIC results and variances indicated that the homogeneous variance model best described the data once the *Sediment only* treatment was dropped.

The effects of waterborne chemical cues from adults on larval settlement were also analyzed using a repeated measures mixed model analysis and the arcsin-square root transformed proportion of larvae settling as the response variable. Inspection of AIC results for various covariance structures indicated that a model using variances grouped by the bathwater × substrate interaction without nonsignificant ($P > 0.25$) interaction terms provided the best fit. *A posteriori* comparisons of means were conducted using sequential Bonferonni criteria as mentioned earlier.

TABLE 3.

Adult chemical cue experimental design, indicating the number of replicate trials using each species/stock of oyster. Cv = *Crassostrea virginica*, WCA = west coast *Crassostrea ariakensis*, SCA = south China *Crassostrea ariakensis*.

Bathwater	Cv-fouled	PVC-clean
C. virginica	Cv: 8 reps	Cv: 8 reps
	WCA: 18 reps	WCA: 18 reps
	SCA: 14 reps	SCA: 14 reps
C. ariakensis	Cv: 8 reps	Cv: 8 reps
	WCA: 18 reps	WCA: 18 reps
	SCA: 14 reps	SCA: 14 reps
M. mercenaria	Cv: 8 reps	Cv: 8 reps
	WCA: 18 reps	WCA: 18 reps
	SCA: 14 reps	SCA: 14 reps
Control	Cv: 8 reps	Cv: 8 reps
	WCA: 18 reps	WCA: 18 reps
	SCA: 14 reps	SCA: 14 reps

RESULTS

Response to Substrate Type

Fifteen cohorts (2 *Cv*, 5 SCA and 8 WCA) out of a total of 49 were rejected from the analyses because <20% settlement was observed on the biofouled *C. virginica* shell treatment. Larval survival was high during the experiment and few dead larvae were observed. Settlement on the walls of the boxes was less than 1% (X = 0.61%, SD = 0.87%). Percent settlement on the treatment substrates ranged from 45.4% (SD = 28.0) for *C. virginica* larvae settling onto biofouled conspecific shells to 0% for *C. virginica* on the manmade substrates (Table 4).

The relative settlement index (RSI) was affected by *Substrate*, *Biofilm*, the interaction between the two, and the interaction between *Species* and *Substrate* (Fig. 1, Table 5). Because the significant 3-way interaction, required that we compare differences among species in relative settlement rates on the various substrates separately for fouled and unfouled substrates, we considered the *Species* × *Substrate* interaction only for fouled surfaces—the comparisons most relevant to field conditions.

Settlement was greatest on oyster shell, from both species, and next highest on granite. Little settlement was observed on the artificial substrates, though *C. ariakensis* exhibited a greater propensity to settle on fiberglass than did *C. virginica* (Fig. 1). Natural biofilms enhanced settlement response in both oyster species.

A posteriori least squares means comparisons indicated that RSI onto *C. ariakensis* shell was similar to that onto granite ($P = 0.04$, but nonsignificant because of the number of *a posteriori* comparisons), but significantly higher than that on fiberglass, PVC, or steel (all $P < 0.0009$); settlement onto PVC and steel did not significantly differ (all $P = 0.76$). Settlement was also higher on fouled than on clean substrates (Table 5). The effect of fouling varied among substrates, however. Fouling significantly increased settlement onto *C. ariakensis* shells and granite ($P = 0.0012$ and $P < 0001$, respectively), had a marginal, but nonsignificant effect on settlement onto fiberglass ($P = 0.021$), and did not affect settlement onto PVC ($P = 0.41$) or steel ($P = 0.27$).

TABLE 4.

Mean (SD) percent settlement for each oyster species on each substrate by biofouling level. Percent settlement calculated using the observed total number of larvae in each well (see text for explanation) and the number of oysters settled on both upper and lower surfaces of the substrate. See text for explanation of species abbreviations.

		% Settled on Substrate	
Larval Species	Substrate	Clean	Biofouled
SCA	CV-shell	3.7 (8.3)	19.3 (18.4)
SCA	CA-shell	4.0 (5.0)	16.7 (17.0)
SCA	Granite	1.4 (3.0)	7.1 (11.0)
SCA	PVC	0.1 (0.4)	0.6 (1.6)
SCA	Fiberglass	0.2 (4.6)	4.4 (6.1)
SCA	Steel	0.1 (0.5)	0.4 (0.9)
WCA	CV-shell	20.7 (25.3)	33.0 (22.2)
WCA	CA-shell	13.2 (22.8)	34.8 (19.0)
WCA	Granite	2.6 (6.3)	14.3 (19.1)
WCA	PVC	0.6 (0.9)	1.9 (3.13)
WCA	Fiberglass	3.0 (4.1)	9.2 (15.9)
WCA	Steel	0.3 (0.8)	0.2 (0.6)
CV	CV-shell	5.8 (13.5)	45.4 (28.0)
CV	CA-shell	10.5 (16.8)	41.3 (28.3)
CV	Granite	1.4 (3.1)	35.5 (23.7)
CV	PVC	0 (0)	0 (0)
CV	Fiberglass	0 (0)	0 (0)
CV	Steel	0 (0)	0 (0)

There were no significant differences among the three oyster types tested in their relative settlement on the various substrates tested ($P > 0.05$) with two exceptions. Both SCA ($P = 0.0049$) and WCA larvae ($P < 0.0001$) had a greater tendency to settle on fouled fiberglass than did *C. virginica* larvae. In addition, there was a nonsignificant trend toward higher RSI on fouled *C. ariakensis* shell by SCA than by WCA ($P = 0.035$).

Response to Sediment

No oysters of either species were observed to attach directly to the sediment in the *Sediment only* treatment (Fig. 2). Mean percent settlement did not differ between the remaining substrates (*Shell only* and *Shell + Sediment*), or the interaction between species and substrate (Table 6). Most oysters were observed to settle onto the underside of shells, which likely contributed to the lack of observed difference between the *Shell only* and *Shell + Sediment* treatments. In the few instances in which oysters were observed to settle on the upper surface of the *Shell + Sediment* treatment, close visual inspection of the shells revealed that these oysters had settled on areas of the shell that were locally devoid of sediment. The significant effects of species on settlement reflect slightly higher overall settlement by WCA than by SCA.

Response to Waterborne Chemical Cues

Proportion of larvae settling within chambers ranged from 0.60 for settlement in the presence of biofouled *C. virginica* shell in *C. ariakensis* bathwater to 0.01 for settlement onto clean PVC in *M. mercenaria* and control bathwaters for all species combined (Fig. 3). The mixed model analysis revealed significant main effects for *Substrate* and *Bathwater*, but no differences between *Species* (Table 7). The *Substrate* effect is a reflection of overall low settlement onto clean PVC. Our results for settlement onto substrates also indicate a significant *Species* × *Substrate* interaction reflecting a significantly stronger affinity for settlement on fouled shell by *C. virginica* larvae than by SCA or WCA larvae (*a posteriori* ls means $P < 0.005$ for both comparisons). *A posteriori* comparisons considering total settlement into chambers, not only settlement onto substrates, indicate significantly higher settlement in the *Crassostrea* bathwater treatments than in the *Control* and *Mercenaria* treatments (CV = CA > Control = MM).

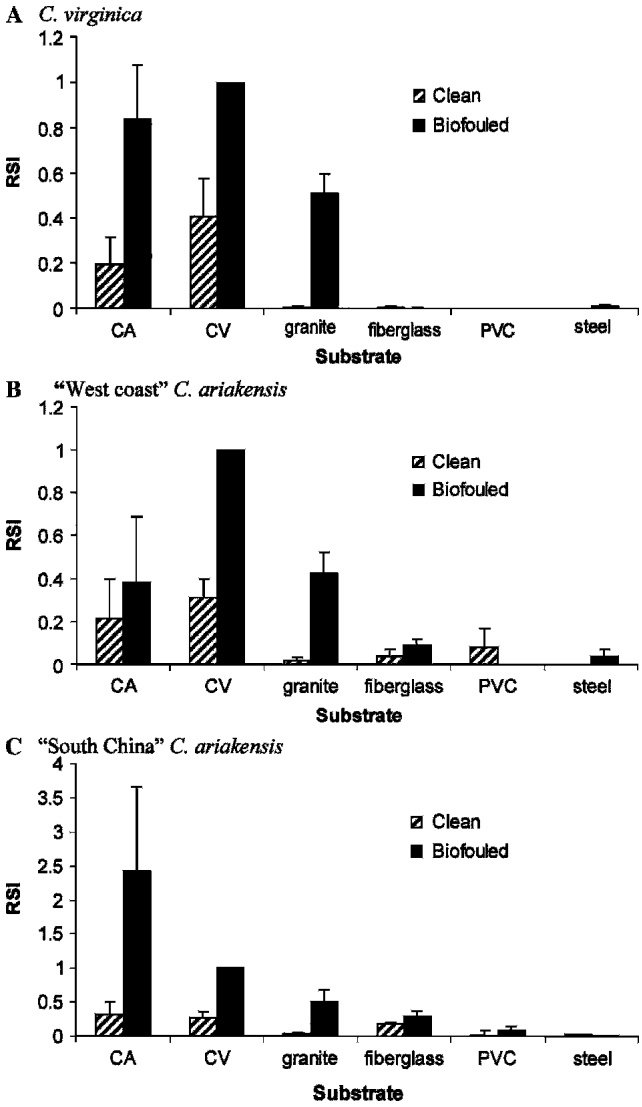

Figure 1. Mean (+SE) Relative Settlement Index (RSI) for each substrate and biofouling level for (A) *Crassostrea virginica*, (B) "West Coast" *Crassostrea ariakensis* and (C) "South China" *C. ariakensis*. Bars are one standard error above the means.

DISCUSSION

To determine if the introduction of Asian oyster, *C. ariakensis*, into the Chesapeake Bay can result in self-sustaining populations that replace a productive fishery and important

TABLE 5.

Mixed Models analysis of the effects of *Species*, *Substrate* and *Biofilm* on Relative Settlement Index (RSI).

Source	df	F	*P*
Species	2/19.6	3.44	0.0524
Substrate	4/19.8	19.30	<0.0001
Biofilm	1/28	63.10	<0.0001
Substrate x Biofilms	4/24	12.53	<0.0001
Species x Substrate	8/20.8	3.31	0.0134
Species x Biofilms	2/27.3	0.25	0.7783
Species x Substrate x Biofilms	8/20.8	2.53	0.0315

TABLE 6.

Mixed models analysis for the effects of *Species* and *Substrate* (Shell only and Shell + sediment) on mean percent settlement.

Source	df	F	*P*
Species	2/162	3.13	0.046
Substrate	1/162	1.98	0.161
Species x Substrate	2/162	1.50	0.225

ecosystem services previously provided by *C. virginica*, a basic understanding of similarities and differences in larval settlement responses between the two species is required. Our results demonstrate that there are many similarities in the larval settlement between *C. virginica* and the south China and west coast strains of *C. ariakensis* but also perhaps important differences.

Overall settlement rates in our experiments were lower for *C. ariakensis* than for *C. virginica*. However, this likely reflects a reduced ability on our part to determine competency in *C. ariakensis* larvae, because it has a less distinctive eyespot than competent *C. virginica* larvae. Thus, we advise caution in interpreting absolute settlement rates as reported in Table 4.

All species and strains tested preferred shell as a settlement substrate, and exhibited greatest settlement on solid, natural substrates covered with biofilms. However, our results indicate a potentially important difference between *C. virginica* and *C. ariakensis*. Both strains of *C. ariakensis* had significantly higher preferences for settlement onto fiberglass than did *C. virginica*. Whereas in nature, *C. virginica* has been found on several manmade substrates (Kennedy 1996; personal observations) and would appear to eventually colonize fiberglass to some extent as selectivity decreases with larval age, our direct comparison suggests that *C. ariakensis* may be more likely to attach to fiberglass in the wild. *Crassostrea ariakensis* might therefore pose a greater risk of becoming a fouling nuisance species.

The enhancement of larval settlement by natural biofilms growing on hard substrates has been demonstrated for many invertebrate species (Pawlik 1992), including *C. virginica* and *C. gigas* (e.g., Veitch & Hidu 1971, Fitt et al. 1989). Although we did not quantify or taxonomically characterize the biofilms present in our experimental treatments, the conditions in all assays were essentially identical, and the results demonstrate clearly that the presence of some biofilm growth greatly increase larval settlement of the south China and west coast strains of *C. ariakensis* when compared with clean substrates. Therefore, like *C. virginica*, a species introduction plan that utilizes oyster shell material with natural biofilms as initial bed or reef material for *C. ariakensis* will likely enhance larval recruitment.

Assays examining the impacts of sediment on settlement demonstrated that, like *C. virginica* (Bahr 1976, Seliger & Boggs 1988), the larvae of both strains of *C. ariakensis* require exposed clean substrates for colonization. All larvae tested either avoided or failed to settle directly on soft sediment and on sediment-covered shells, attaching only to small clean areas on the upper surface or to the clean undersurfaces of shells. Therefore, the problems associated with high sediment loads and excessive siltation in the Chesapeake Bay that impairs habitat quantity and quality for *C. virginica* larval settlement will also be a limiting factor in successful recruitment for both strains *C. ariakensis*.

Chemical cues have also long been known to mediate larval settlement for a variety of aquatic invertebrates (Pawlik 1992). In fact, the larvae of *C. virginica* and *C. gigas* have been shown to alter their behavior in the water column in response chemical cues emitted from adult conspecifics (Tamburri et al. 1992, Turner et al. 1994, Zimmer-Faust & Tamburri 1994, Tamburri et al. 2007). However, whereas this previous work with oyster larvae demonstrated significant changes in larval behavior prior to attachment, enhanced metamorphosis in response to dissolved cues was never found. Surprisingly, we found significant increases in the proportion of larvae that attached to surfaces when compounds emitted by oysters are present in the water,

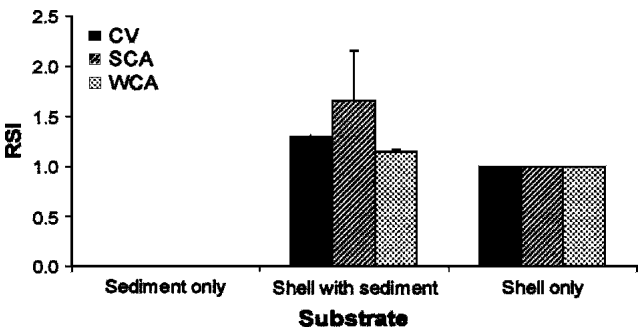

Figure 2. Mean (+SE) Relative Settlement Index (RSI) by oyster species onto the sediment treatments.

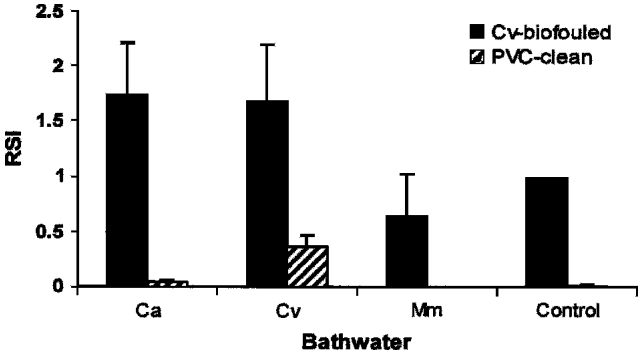

Figure 3. Mean (+SE) Relative Settlement Index (RSI) for all oyster species combined in each bathwater treatment by substrate type.

TABLE 7.

Mixed models analysis for the effects of *Species*, *Substrate* and *Bathwater* on the proportion of larvae settling onto test substrates. A significant bathwater effect ($P < 0.001$) was also found for total settlement in experimental chambers, which is a more relevant test for the influence of bathwater. Results reflect model runs without interaction terms with $P > 0.25$ in the full model. The three-way interaction, species × bathwater and bathwater × substrate terms were dropped for both response variables considered (settlement onto substrates and total settlement into chamber).

Source	df	F	*P*
Species	2/74.2	7.79	0.0008
Bathwater	3/33.3	5.92	0.0024
Substrate	1/85.7	206.51	<0.0001
Species x Substrate	2 /74.2	6.62	0.0023

especially if all settled larvae are considered including those attached to the walls of the plastic settlement chamber.

Our results also indicate that the waterborne chemical cues mediating oyster larval settlement are not species-specific. We found that dissolved compounds emitted from either oyster species/strain (congeneric) were as powerful as chemical signals from adults of the same species (i.e., *C. virginica* and *C. ariakensis* larvae settled similarly in response conspecific and congeneric cues). A comparable lack of chemical cue specificity has been demonstrated for *C. virginica* and *C. gigas* (Tamburri et al. 1992, Turner et al. 1994, Zimmer-Faust & Tamburri 1994, Tamburri et al. 2007). Because there are no obvious species-specific responses, gregarious settlement onto multigenerational and multispecies reefs may eventually occur if *C. ariakensis* is introduced into the Chesapeake Bay. Such a scenario raises important questions regarding the impacts of oyster cohabitation and interspecific competition.

Overall, our results demonstrate that like *C. virginica*, both strains of *C. ariakensis* use a variety of physical and chemical cues when selecting a site for colonization and that many of their responses mirror those of the native oyster species. This is not surprising, given similarities in larval behavior and settlement found previous between *C. virginica* and *C. gigas*. However, the question still remains about how these similarities and subtle differences will ultimately translate into the probability of a successful introduction and risks to native oysters. Whereas our results provide some evidence that *C. ariakensis* may be able to establish naturalized, reproducing, and self-sustaining populations in the Chesapeake Bay, much more information is required on other aspects of the biology, ecology, and physiology of this species to predict the ecosystem-level consequences of the proposed introduction.

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