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Chemical induction of settlement behavior in larvae of the eastern oyster *Crassostrea virginica* (Gmelin)

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ABSTRACT

Although numerous studies have been conducted to examine the effects of neuroactive compounds on bivalve larvae, few have identified chemicals capable of inducing settlement behavior in the eastern oyster *Crassostrea virginica*. In this study, we placed competent *C. virginica* larvae in the microwells of tissue culture plates and treated them with select chemicals to identify those that were capable of inducing larvae to exhibit settlement behavior. These behavioral assays were recorded using video microscopy and the extension of the foot was the characteristic searching behavior that was quantified. The compounds γ-aminobutyric acid (10^{-4} M) and acetylcholine chloride (10^{-4} M) did not significantly increase the percentage of larvae exhibiting settlement behavior at a salinity of approximately 8.5 ± 0.2. As compared with a control, a significant increase in settlement behavior was induced at this salinity by treatment with 3-isobutyl-1-methylxanthine (10^{-4} M), 5-hydroxytryptamine (10^{-4} M), and L-3,4-dihydroxyphenylalanine (10^{-4} M) as well as ammonia as a solution of 7.9 mM NH_4Cl (pH = 8.0). These new findings differ from the results of similar studies involving other species in the genus *Crassostrea* and have the potential to improve setting efficiencies on a large scale by allowing hatchery personnel to trigger a controlled, synchronized settlement event of these larvae.

1. Introduction

Adult *Crassostrea virginica* (Gmelin 1791) are sedentary, reef-forming oysters that reproduce by releasing gametes into the water column in salinities above 5 (Kennedy, 1996). Here fertilization takes place and pelagic larvae develop. The pediveliger is the final larval stage prior to settlement and metamorphosis. The first step in the settlement response of oysters in situ is likely initiated when soluble bacterial products, such as peptides, serve as appropriate environmental cues to trigger the release of dopamine (Bonar et al., 1990). At this point, settlement behavior is initiated, a period during which the larvae swim through the water in a spiral manner until they make contact with a solid object. They then crawl on the substrate surface, apparently sensing cues with an organ known as the foot (Kennedy, 1996; Prytherch, 1934). Further information from the environment (sensory inputs such as surface texture, water current, light intensity, etc.) may then advance settlement behavior through its subsequent phases with cementation being the culmination of the settlement process (Bonar et al., 1990; Coon et al., 1990a). After cementation, the newly attached larvae, now called "spat", will commence metamorphosis whence they lose their larval feeding organ (velum), develop gills, resorb the foot, and excrete adult shell (Kennedy, 1996).

Researchers have postulated the presence of two serial pathways that control this settlement process: a dopaminergic behavioral pathway and an adrenergic morphogenetic pathway. The results from pharmacological studies conducted by Bonar et al. (1990) suggest that externally applied L-3,4-dihydroxyphenylalanine (L-DOPA) enters the larva and is converted to dopamine (DA), triggering searching behavior by acting at dopaminergic receptors. Other weak amine bases (e.g. ammonia) excreted by a dense community of oysters, are also capable of triggering some degree of search behavior through a non-specific activation of this pathway, although the mechanism is suspected to differ from that of L-DOPA (Fitt and Coon, 1992); researchers found that the dopaminergic antagonist, sulpiride, blocked the ability of L-DOPA to induce settlement behavior, but did not do so to ammonia (Coon et al., 1990b). Bonar et al. (1990) postulate that the adrenergic phase begins when the endogenous catecholamine norepinephrine is released. This compound acts on alpha-1 adrenoreceptors, thereby controlling the morphogenetic phase of metamorphosis, either through direct interaction with target tissues or perhaps by triggering the release of a morphogenetic agent.

There exist some inconsistencies in the literature regarding the response of oysters within the *Crassostrea* genus to artificial chemical induction of settlement. In their 1985 article on *Crassostrea gigas*, Coon et al. reported that L-DOPA, epinephrine, and norepinephrine were the only chemicals of the twenty that they studied to consistently and significantly induce metamorphosis. They cited these results in a later article when discussing the ability of oysters in the entire *Crassostrea* genus to respond to neuroactive agents (Bonar et al., 1990). However, data from two other studies contradict their conclusions. Beiras and Widdows (1995) tested the settlement-inducting

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ability of several of the same compounds. In contrast to statements made by Coon et al. (1985) and Bonar et al. (1990), acetylcholine (ACh; 10^{-4} M) was found to effectively induce settlement and metamorphosis in *C. gigas* under similar test conditions. Similarly, Tan and Wong (1995) reported that *Crassostrea belcheri* larvae can be induced to set when exposed to γ-aminobutyric acid (GABA; 10^{-5} M), while other researchers reported that this chemical was ineffective in producing settlement and metamorphosis in *C. gigas* at the same concentration (Beiras and Widdows, 1995; Bonar et al., 1990; Coon et al., 1985). Interestingly, species differences in the degree of response to L-DOPA, epinephrine, and norepinephrine have also been noted for *C. virginica* and *C. gigas* (Coon et al., 1986; Walch et al., 1999). Furthermore, while not all of these studies evaluated mortality, those that did demonstrate that the results could be quite variable. For example, after 6 h of exposure to each chemical treatment at 10^{-4} M, survivorship of *Pinctada maxima* did not differ from the controls and was nearly 100% (Zhao et al., 2003). This concentration also induced a significant settlement response in the 3-isobutyl-1-methylxanthine, γ-aminobutyric acid, and serotonin treatments for this species (Zhao et al., 2003). In contrast, a high mortality rate, even after induced metamorphosis, was observed in *Ruditapes philippinarum* after treatment with serotonin and acetylcholine (Urrutia et al., 2004).

These findings bring into question whether species of the same genus do indeed have the same response to settlement-inducing chemicals. Most of the research evaluating the inductive ability of various chemical compounds has been conducted on the Pacific oyster *C. gigas* at coastal ocean salinities. Several chemicals, including acetylcholine (Alfaro et al., 2011; Beiras and Widdows, 1995; Dobretsov and Qian, 2003; Sánchez-Lazo and Martínez-Pita, 2012; Urrutia et al., 2004; Yu et al., 2008; Zhao et al., 2003), γ-aminobutyric acid (Alfaro et al., 2011; Doroudi and Southgate, 2002; Sánchez-Lazo and Martínez-Pita, 2012; Tan and Wong, 1995; Yu et al., 2008; Zhao et al., 2003), 3-isobutyl-1-methylxanthine (Dobretsov and Qian, 2003; Sánchez-Lazo and Martínez-Pita, 2012; Yu et al., 2008; Zhao et al., 2003), and serotonin (Alfaro et al., 2011; Beiras and Widdows, 1995; Urrutia et al., 2004; Yu et al., 2008; Zhao et al., 2003) were shown to induce settlement in other marine bivalves, but have not been tested on the eastern oyster *C. virginica*. Amongst all of these chemicals, only one compound, L-DOPA (Walch et al., 1999), has been tested at the mesohaline salinities (5 to 18) typical of the mid-Chesapeake Bay region. Given the variability in the results of studies of these chemicals, but considering that each was effective at inducing settlement behavior in at least one bivalve species, we chose to investigate whether acetylcholine chloride, γ-aminobutyric acid, 3-isobutyl-1-methylxanthine, 5-hydroxytryptamine (serotonin), L-3,4-dihydroxyphenylalanine (L-DOPA), all at 10^{-4} M, and ammonia (as a solution of .0079 M NH_4Cl; pH = 8.0) were capable of inducing settlement behavior in *C. virginica*.

Knowledge about the inductive potential of the aforementioned chemicals on *C. virginica* larvae could greatly benefit the hatchery production of eastern oyster spat. While over 800 million spat-on-shell were produced in 2012 at Horn Point Oyster Hatchery at University of Maryland Center for Environmental Sciences, setting efficiencies averaged 22.2% (defined as $\frac{\#SpatOnShellInTank}{\#LarvaePlacedInTank} \times 100$). This figure is consistent with results at other hatcheries (Greene and Grizzle, 2005; Henderson, 1983; Jones and Jones, 1983; Nosho and Chew, 1991), but indicates that the majority of larvae did not set on shell. The ability to synchronize setting events could potentially yield a higher setting efficiency and may result in the production of more oysters (Yu et al., 2008). By allowing hatchery personnel to trigger settlement of larvae, neuroactive compounds may shorten the time that the group would otherwise need to be offered cultch, as well as perhaps reduce the loss experienced when larvae set on the tank or do not set at all. This study sought to determine if competent *C. virginica* larvae would demonstrate settlement behavior at low salinities in response to treatment with chemicals that have been proven to induce such behavior in other oyster species.

2. Materials and methods

2.1. Larval culture

Reproductively competent adult *C. virginica* (Gmelin) were spawned in a controlled setting at Horn Point Oyster Hatchery, Cambridge, Maryland. For the collection of gametes and to prevent polyspermy, males and females were separated upon the commencement of spawning. After controlled fertilization with sperm collected from these males, we placed 4 million eggs in each 600 l fiberglass cone (Gemini Fiberglass Products Inc., Golden CO) with 1 μm filtered seawater (FSW) from the Choptank River to yield a final concentration of 10 larvae ml^{-1}. The water was gently aerated. Every 2 days, the cones were drained and cleaned with freshwater and a scrub brush. The water was replaced and maintained at a mean temperature of 25.9 °C (±0.3 °C) with a mean salinity of 9.6 (±0.1). After day 8, the larval concentration was reduced to 2 larvae ml^{-1}. Larvae were fed a fixed algal diet of *Isochrysis galbana* (C-Iso), *Thalassiosira pseudonana* (3H), and *Tetraselmis chui* (Ply-429) daily. This protocol was repeated for each of 6 spawns, each of which was treated as an experimental replicate.

2.2. Larval preparation

For each experiment, we used larvae obtained from different spawns. (Hereafter, the term "broods" will be used to identify these groups, each of which had different parents.) On average, 19 adult males and 30 adult females produced the larvae in these broods. When spat began to set on a clean oyster shell (test shell) suspended in the culture cone, the cone was drained and the larvae were caught on a 100 μm sieve. The larvae were then graded through a series of stainless steel sieves (200 μm, 212 μm, 224 μm; manufactured by W.S. Tyler). We determined whether the larvae were competent to respond to chemical induction by observing through a compound microscope (Olympus BX51) for the presence of a well-developed eyespot and actively searching foot (Fig. 1). As is standard practice in the oyster hatchery at Horn Point Laboratory, if larvae had set on the test shell and several larvae were observed searching in a sample, their size class was deemed competent to set (so as to avoid a substantial loss of larvae due to settlement in the larval tanks). All experiments were conducted with larvae retained on a 224 μm sieve.

Upon collection, we placed the larvae in clean plastic vessels containing 0.2 μm filtered, autoclaved seawater with 10 μl ml^{-1} of penicillin-G, streptomycin sulfate, and neomycin sulfate at an ambient salinity of approximately 9.6 ± 0.2 (= antibiotic seawater ABS).

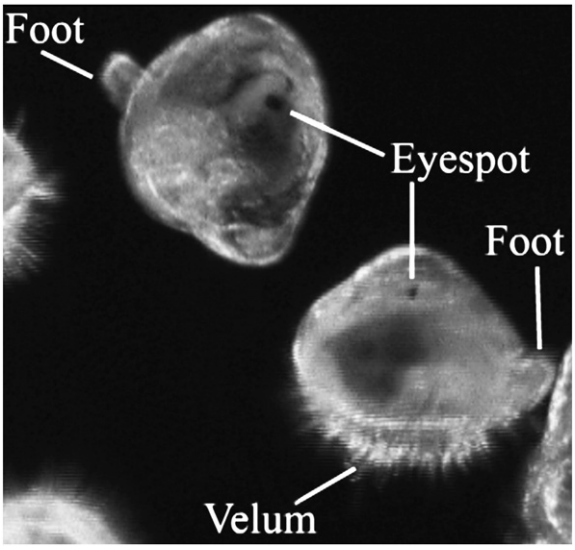

Fig. 1. Fifteen day-old *C. virginica* larvae exhibiting competence.