ABSTRACT

Title of Document: CHEMICAL INDUCTION OF SETTLEMENT IN LARVAE OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA* (GMELIN)

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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, I treated competent *C. virginica* larvae with select chemicals to identify those which are capable of inducing settlement behavior at an average salinity of 9.6 ($\pm$0.1). The compounds γ-aminobutyric acid and acetylcholine chloride, both at 10^{-4}M, did not significantly increase the percentage of larvae exhibiting settlement behavior. As compared with the control, a significant increase in settlement behavior was induced by treatment with 3-isobutyl-1-methylxanthine, 5-hydroxytryptamine, and L-3, 4-dihydroxyphenylalanine all at 10^{-4}M, as well as ammonia as a solution of 7.9mM NH_4Cl (pH=8.0). These findings differ somewhat from the results of similar studies involving other species in the *Crassostrea* genus and may be of value to hatchery personnel or researchers interested in the chemical induction of settlement behavior in the eastern oyster.

CHEMICAL INDUCTION OF SETTLEMENT IN LARVAE OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA* (GMELIN)

By

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Thesis submitted to the Faculty of the Graduate School of the
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Dedication

I dedicate this work to my father, Gregory James North, who not only introduced me to the beauty and wonder of the natural world, but who gave me the tools necessary to explore it; and to my children, Annabelle and Willem, the next generation of explorers.

Acknowledgements

First and foremost, I would like to thank my husband, Adam, for his unwavering support. Not only has he been an amazing cheerleader who is gifted at putting everything in perspective, but he also has done more than his fair share of housework while I sat hunched over a pile of journal articles, fingers typing furiously at the keyboard. My children, while they are too young to know it, are a constant source of inspiration and amusement and I thank them for being so understanding when Mama has had to work.

To Emily Vlahovich I give enormous thanks for listening when I needed an ear and for all of her moral support, help, and advice over the years. I am so grateful that we were in this process together, but I am even luckier to have her as a dear friend. I am deeply indebted to my advisor Mutt for having the flexibility and patience to mentor a student who has two young children. His support made my dream of pursing this degree a reality. I thank committee members Dave Kimmel, for helping me through some challenging statistics and kindly answering my many e-mails, and Stan Allen, for his valuable insight on my thesis drafts. For tolerating my invasion of the Dry Lab and MLT Room, and for all of their assistance during my project, I extend a big "thank you" to the Hatchery Crew, particularly Stephanie Alexander, Melissa Radcliffe, Courtney Hargens, and Brittany Burtner. I also thank Jeff Alexander, who volunteered his time to help me brush up on my chemistry skills and who provided welcome advice throughout this project. In addition, I would like to acknowledge Dr. Michael Masser of the Southern Regional Aquaculture Center and Dr. Steven Coon for granting me permission to use figures from their

publications. Special thanks are extended to Dr. Coon for reviewing my description of his settlement model.

Finally, I am profoundly grateful for my parents, who made many sacrifices to ensure that my sister and I had the best schooling possible and in doing so taught us the value of a good education.

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Chapter 1: Introduction

1.1 Life History of Oyster Larvae

Crassostrea virginica (Gmelin 1791), also known as the American or eastern oyster, is a bivalve mollusk native to estuaries and coasts of the western Atlantic and the Gulf of Mexico (Newball and Carriker 1983; Andrews 1991). Adult *C. virginica* are sedentary, reef-forming organisms which reproduce by releasing gametes into the water column in salinities above 5 (Kennedy 1996). Here fertilization takes place and non-feeding trochophore larvae develop (Figure 1-1). After approximately 24-48 hours, these free-swimming larvae progress into the veliger stage and begin grazing on phytoplankton, bacteria, and detritus (Kennedy 1996). They remain in this phase for about 2 weeks (Prytherch 1924), a period of time which varies depending upon water temperature and the availability of food (Kennedy 1996). Larval survival is influenced by a number of factors, possibly including the quality and quantity of food available to spawning females (and thus the amount of lipid reserve in trochophore larvae) and the quality of the food supply available to veligers (Kennedy 1996). If any of these variables are insufficient, the pelagic phase may be extended, thereby increasing exposure to diseases and predation (Underwood and Fairweather 1989).

Eventually pelagic larvae will enter into the benthic stage and metamorphose into their adult form (Figure 1-1). They begin this process by searching for a suitable substrate on which to set; during this phase called settlement, pediveliger (late-stage) larvae swim through the water in a spiral manner until they make contact with a solid

object, whereupon they crawl on the substrate surface, apparently sensing cues with an organ known as the foot (Prytherch 1934). Settlement is formally defined as a behavioral activity that occurs before permanent attachment (Burke 1983, Tamburri et al. 1992, Kennedy 1996). It is reversible in that if the larvae deem the surface unsuitable, they can resume swimming and search elsewhere (Scheltema 1974). The term "settlement behavior" is also used to describe the larval action of evaluating the surface with the foot (Coon et al. 1986, Bonar et al. 1990, Coon et al. 1990a, Coon et al. 1990b, Fitt and Coon 1992, Walch et al. 1999). When the appropriate substrate (called "cultch") has been located, a larva will excrete crystalline cement from its foot and permanently attach its left valve to the substrate (Harper 1992). After cementation, the newly attached larva, now called a "spat", will commence metamorphosis whence they lose their larval feeding organ (velum), develop gills, resorb the foot, and excrete adult shell (Kennedy 1996).

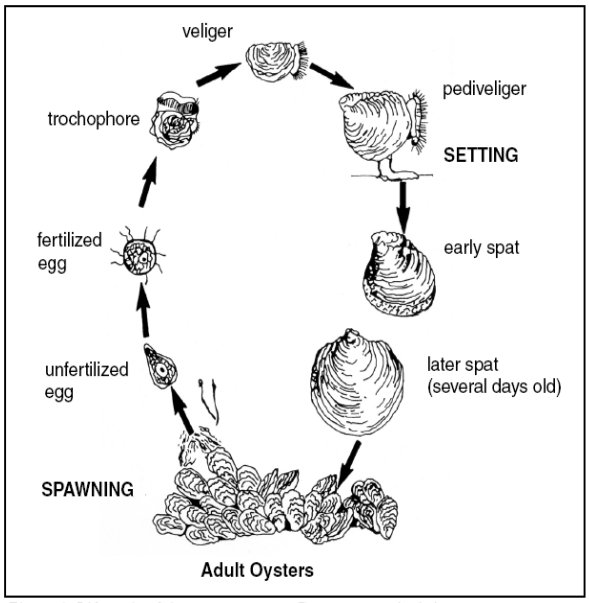

Figure 1-1. Life cycle of the eastern oyster *Crassostrea virginica* (Wallace 2008).

1.2 Hatchery Culture of Spat on Shell

Chesapeake Bay, located in Maryland and Virginia, is an estuary that once sustained a great abundance of fish and wildlife, including the eastern oyster (Kennedy and Breisch 1983). In addition to supporting a large shellfish industry, *C. virginica* has served a significant role ecologically. Not only have oyster reefs provided valuable habitat for aquatic organisms (Breitburg 1995, Zimmerman et al. 1989), but oysters themselves have also served as filters for the Bay's water (Newell 1988). Through their filter-feeding action, oysters remove light-blocking particles and algae from the water column. Unfortunately over-harvesting, degradation of water quality, habitat destruction, and the extensive presence of oyster disease have resulted in the decline of the native oyster and its filtering potential (Newell 1988).

In an effort to restore the population of *C. virginica* in Chesapeake Bay and to support oyster research, the Horn Point Oyster Culture Facility at University of Maryland's Center for Environmental Sciences operates a large-scale spat production operation. This requires intimate knowledge of the eastern oyster's life cycle. Using increases in water temperature or gonadal material isolated from reproductively competent adults, hatchery personnel trigger oysters to spawn in a controlled environment. The progeny of these oysters are raised on a multi-species algal diet in large fiberglass tanks. As they approach two weeks old, these larvae are monitored closely for the presence of an eye spot, an indication that they are nearing metamorphic competence. If the larvae also possess an active foot and searching behavior (Figure 1-2), they are sorted by size class through a series of sieves. Each size class is again assessed for competence (presence of an eye spot and actively

searching foot) and those that are deemed ready to "set," or rather attach to a substrate, are distributed in setting tanks which contain clean oyster shell. After two days, a random sampling of shells from the tanks is assessed and a setting efficiency value, or quantitative measure of the set, is calculated before the spat-on-shell are deployed. The setting efficiency value is defined as $\frac{\#SpatInTank}{\#LarvaePlacedInTank} x100$, where the number of spat in the tank is an estimate derived from examining random shell samples. More larvae may be added to the tank if the set is particularly poor.

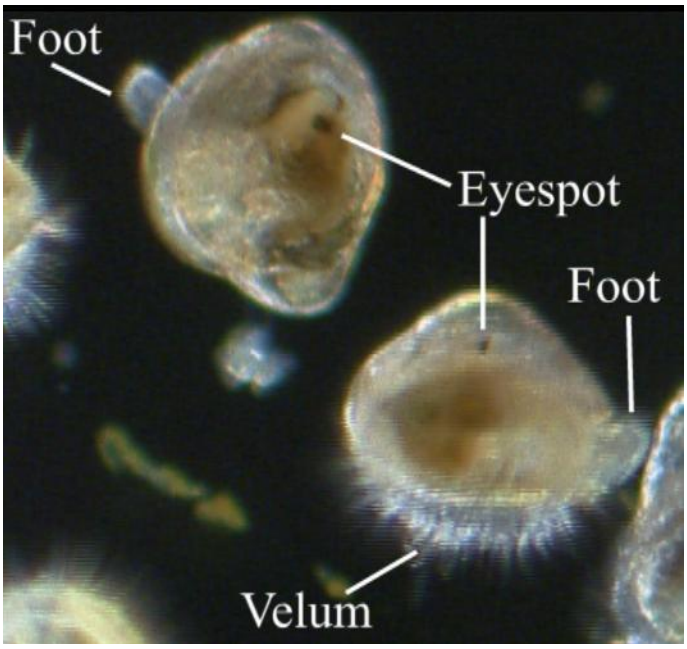

Figure 1-2. Fifteen day-old *C. Virginica* larvae exhibiting competence.

While approximately 137 million spat-on-shell were produced at Horn Point Oyster Culture Facility in 2007, setting efficiencies averaged 15.6% (S. Alexander, pers. comm.). This figure is consistent with results at other hatcheries (Henderson 1983, Jones and Jones 1983, Nosho and Chew 1991, Green and Grizzle 2005), but indicates that the majority of larvae did not set on shell. Because a higher setting

efficiency could result in more oysters being deployed for restoration purposes, the process of settlement and metamorphosis in hatchery larvae was the focus of my research. The project findings presented here provide valuable information that may aid in increasing hatchery setting efficiencies.

Chapter 2: Chemical Induction of Settlement in Eastern Oyster Larvae

2.1 Introduction

The study of settlement in oyster larvae began in the early 1930s. Prior to this time, it was known that oysters undergo a period of settlement, however the mechanisms driving this process were not yet clear (Prytherch 1934, Cole and Knight-Jones 1939). Then, after observing an increased spat set at low tide, Prytherch (1931) investigated the abiotic factors that could be potentially responsible for triggering eastern oyster settlement. He subsequently determined copper to be an element capable of producing a "positive setting reaction". This was one of the first studies to investigate external factors capable of inducing settlement in oyster larvae.

An indication that chemical cues may play a role in larval settlement came mid-century when researchers began reporting gregarious settlement in several marine sessile invertebrates (Cole and Knight-Jones 1949, Knight-Jones and Stephenson 1950, Knight-Jones 1952a, Knight-Jones 1952b, Knight-Jones and Crisp 1953, Knight-Jones 1953). These findings inspired researchers to determine how larvae recognize the presence of other conspecifics. Crisp and Meadows (1962, 1963) found that cypris larvae of *Balanus balanoides* recognize an insoluble protein from other barnacles. It was then determined that conspecific shell proteins promote settlement in both barnacles (Crisp 1965) and oysters (Crisp 1967). Hidu (1969) later discovered that spat confined to a mesh bag stimulated settlement outside of the bags in *Crassostrea virginica.* He therefore concluded that a pheromone released from nearby spat must be the stimulatory agent. In another experiment, researchers

examined the induction ability of oyster shell liquor (OSL), the liquid contained within the shells of closed oysters that consists of seawater and materials secreted or excreted by the oyster (Hidu et al. 1969, Veitch and Hidu 1971). They reported that OSL and water in which adult oysters had filtered both induced settlement. Attempts made to extract a setting factor from the oyster shell liquor suggested that it is a thyroprotein (Veitch and Hidu 1971).

While these researchers were investigating the stimulus behind gregarious settlement, others were examining the role that bacteria may be playing in settlement induction. In 1955, Wilson discovered that small amounts of acid-cleaned substrate which had been soaked in seawater attracted more polychaetous worm larvae to settle than acid-cleaned substrate which not been soaked. He also found that cleaning and sterilizing these soaked sands removes their attractiveness to the larvae. Wilson therefore concluded that the presence of microorganisms, namely bacteria, could be a setting factor. Similarly, Scheltema (1961) found that destroying the biological component of substratum (e.g. bacteria and algae) by washing, boiling and then incinerating it reduces settlement in *Nassarius obsoletus*. In a 1960 letter to the journal *Nature*, Crisp and Ryland discussed the theory that surface bacterial films may indeed be a prerequisite for settlement. After offering a number of surface textures that were either clean or filmed to larvae of *Spirorbis borealis* and *Bugula flabellate*, they found that preferences to a filmed surface vary by species. In a similar investigation by Young and Mitchell (1973), Plexiglas plates were deployed to serve as a settlement surface for *C. virginica*. These researchers reported a positive correlation between the number of bacteria composing a surface film on the plates

and the number of eastern oysters that set on that surface. Later, research progressed to include the isolation of bacteria that are associated with oysters and oyster habitats (Weiner et al. 1985). After the melanin precursor, L-β-3, 4-dihydroxyphenylaline (L-DOPA) was detected in marine surface films, Weiner et al. (1985) isolated the bacterium responsible for producing it and found that it did indeed attract *C. virginica* larvae. This bacterium was subsequently classified as *Alteromonas colwelliana* (Weiner 1988) and later reclassified as *Shewanella colwelliana* (Coyne et al. 1989). Thereafter, Tamburri et al. (1992) confirmed that exposure to metabolites (ammonium and dissolved organic carbon) released by biofilms and by adult oysters induced eastern oyster larvae to initiate settlement behavior.

Aware of findings from several studies that reported the inductive effect of neuroactive compounds and their structural analogs on other molluscan species, Coon et al. (1985) launched an investigation to evaluate the effects of compounds derived from L-DOPA on the settlement of the Pacific oyster *Crassostrea gigas*. Of the chemicals these researchers studied, L-DOPA, epinephrine, and norepinephrine consistently produced an inductive effect. However, larvae treated with the latter two chemicals metamorphosed without settlement, while larvae treated with L-DOPA exhibited settlement behavior and many cemented themselves to the substratum before metamorphosing (Coon et al. 1985). Coon et al. (1986) later published methods for producing cultchless *C. gigas* and *C. virginica* oyster spat using epinephrine and norepinephrine. In 1999, Walch et al. confirmed that L-DOPA does indeed induce settlement behavior and improve hatchery sets of *C. virginica* at a salinity of 15; treatment with 10^{-4}M L-DOPA for 20 minutes induced behavior in

nearly 100% of the larvae and treating them with the same concentration for 10 minutes before presenting them with bagged cultch increased the set by 30% over untreated controls .

2.1.1 A Model of Oyster Settlement and Metamorphosis

The responses of the larvae to neuroactive compounds led scientists to create a model of oyster settlement and metamorphosis. On the basis of their findings, Coon et al. (1985) suggested that a larva extends its foot in response to waterborne L-DOPA-mimetic molecules possibly released from bacterial films and/or other juvenile or adult oysters. If the larva senses that the environment is appropriate, it will cement itself to the substratum. Coon et al. (1985) then hypothesized that the release of endogenous epinephrine- or norepinephrine-mimetic molecules induces metamorphosis.

Researchers later expanded on this explanation and created a model for the control of oyster metamorphosis (Bonar et al. 1990, Coon et al. 1990a) (Figure 2-1). They postulated the presence of two serial pathways: 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-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).

In their natural environment, the first step in the settlement response of oysters 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). 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). Bonar et al. (1990) postulate that, at this point, the endogenous catecholamine norepinephrine is released and 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. This would explain why treating larvae with norepinephrine or epinephrine results in the production of cultchless spat: doing so bypasses the behavioral search phase of settlement and triggers solely the morphogenetic phase of metamorphosis.

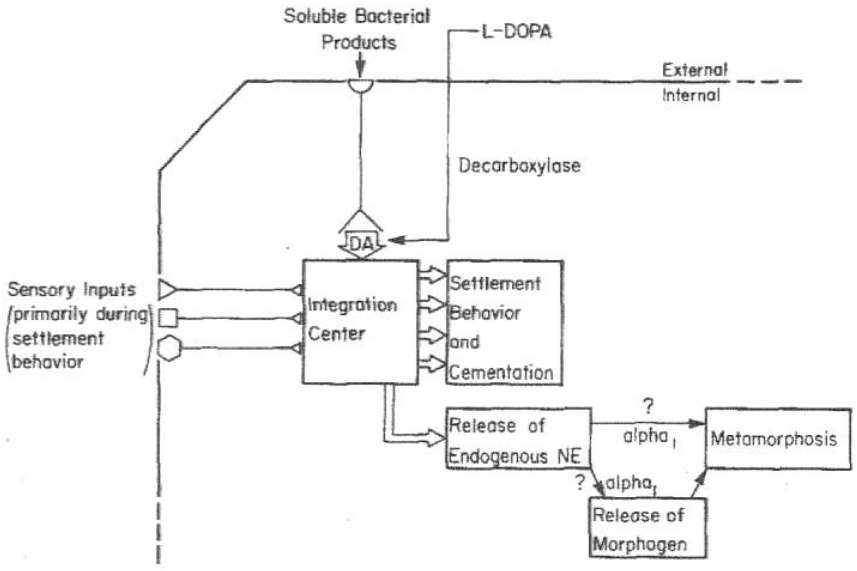

Figure 2-1. Model of oyster settlement and metamorphosis (Coon et al. 1990a).

2.1.2 Species Differences in Response to Chemical Exposure

There exist some inconsistencies in the literature regarding the response of oysters within the *Crassostrea* genus to the chemical induction of settlement. As stated previously, in their 1985 article on *C. gigas*, Coon et al. report that L-DOPA, epinephrine, and norepinephrine are the only chemicals of the twenty they studied to consistently and significantly induce metamorphosis. They cite these results in a later article, and use them to draw conclusions about the ability of oysters in the entire *Crassostrea* genus to respond to neuroactive agents (Bonar et al. 1990). However, data from two other studies contradicts these conclusions. Beiras and Widdows (1995) tested the inductive ability of several of the same compounds. In contrast to statements made by Coon et al. (1985) and Bonar et al. (1990), acetylcholine was

found to effectively induce settlement and metamorphosis in *C. gigas* at 10^{-4}M 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) at 10^{-5}M, while other researchers report that this chemical is ineffective in producing settlement and metamorphosis in *C. gigas* at the same concentration (Coon et al. 1985, Bonar et al. 1990, Beiras and Widdows, 1995). 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).

These findings bring into question whether species of the same genus do indeed have the same response to attempts at chemical induction. 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 (Beiras and Widdows 1995, Dobretsov and Qian 2003, Zhao et al.2003, Urrutia 2004), γ-aminobutyric acid (Tan and Wong 1995, Doroudi and Southgate 2002, Zhao et al.2003), 3-isobutyl-1-methylxanthine (Dobretsov and Qian 2003, Zhao et al. 2003) , and serotonin (Beiras and Widdows 1995, Zhao et al. 2003, Urrutia 2004) were shown to induce settlement in other marine bivalves, but have not been tested on the eastern oyster *C. virginica*. Only one compound, L-DOPA (Walch et al. 1999), has been tested at the mesohaline salinities (5 to 18, MDNR) typical of the mid-Chesapeake Bay region. Such knowledge could greatly benefit the hatchery production of spat-on-shell. Here, I present a study which aimed to answer the question: will competent *C. virginica* larvae demonstrate

settlement behavior at low salinities in response to treatment with chemicals that have been proven to induce such behavior in other oyster species? I hypothesized that acetylcholine chloride (ACh), γ-aminobutyric acid (GABA), 3-isobutyl-1-methylxanthine (IBMX), 5-hydroxytryptamine (serotonin, or 5-HT), and L-3, 4-dihydroxyphenylalanine (L-DOPA) all at 10^{-4}M and ammonia (NH_3) as a solution of .0079M NH_4Cl (ph = 8.0) would induce a significantly greater percentage of *C. virginica* larvae to exhibit settlement behavior than those in the control treatments.

2.2 Methods

Conducting my research at the Horn Point Oyster Culture Facility provided me with a unique opportunity to raise oyster larvae for this project. I was able to control the larval diet, as well as the salinity and temperature of the water in which the larvae were raised. In addition, I was able to observe the general health of the larvae as they matured and personally determine when the larvae were nearing metamorphic competence and thus were eligible for the study.

2.2.1 Larval Culture

Reproductively competent adult *Crassostrea virginica* (Gmelin) were spawned in a controlled setting at Horn Point Oyster Culture Facility, Cambridge, Maryland. For the collection of gametes and to prevent polyspermy, males and females were separated upon the commencement of spawning. After controlled fertilization, I placed 4 million eggs in each 600L fiberglass cone (Gemini Fiberglass

Products Inc., Golden CO) with 1 μm filtered seawater (FSW) from the Choptank River at a concentration of 10 larvae/ml. 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 an average temperature of 25.9°C (± 0.3°C) with an average salinity of 9.6 (± 0.1). After day 8 the larvae concentration was reduced to 2 larvae/ml. Larvae were fed a mixed algal diet of *Isochrysis galbana* (C-Iso), *Thalassiosira pseudonana* (3H), and *Tetraselmis chui* (Ply-429) daily (Appendix I). Occasionally, the quality or availability of algae declined and thus I made slight amendments to the ratio of algal species offered.

2.2.2 Larval Preparation

For each replicate of the experiment, I used larvae obtained from different spawns. (Hereafter, I use the term "broods" to identify these groups.) On average, 19 adult males and 30 adult females produced the larvae in these broods. When spat began to appear on a test shell suspended in the culture cone, the cone was drained and the larvae were caught on a 100μm sieve. The larvae were then graded through a series of sieves (200μm, 212μm, 224μm; manufactured by W.S. Tyler). All experiments were conducted with larvae collected on a 224 μm sieve. I determined whether the larvae were competent to respond to chemical induction by observing through a compound microscope (Olympus BX51) the presence of a well developed eyespot and actively searching foot. As is standard practice in the oyster hatchery at Horn Point, 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). Using a Sony DXC-390 camera and StreamPix3 software, I recorded microscopy video footage of larval samples from each brood and later calculated that on average 16.9% of the larvae among all broods exhibited settlement behavior prior to the experiment (Table 2-1). The average length of the larvae among all broods was 318.9 μm (±14.3μm; Table 2-1). The average shell length did not differ significantly between the broods (Table 2-2).

Upon collection, I placed the larvae in clean plastic vessels containing 0.2μm filtered, autoclaved seawater with 10 μl ml^{-1} of penicillin-G, streptomycin sulfate, and neomycin sulfate at an ambient salinity of approximately 9.5-10 (= antibiotic seawater ABS). Antibiotics were added to reduce the buildup of pathogenic bacteria and to eliminate the potential for settlement cues from replicating bacteria. This is a common practice in studies of this nature (Hadfield 1984, Bonar et al. 1990, Coon et al. 1990a, Beiras and Widdows 1995). I did note that the addition of antibiotics reduced settlement behavior (searching with the foot extended) to <1.2% prior to chemical induction.

Table 2-1. Size and competence of *C. virginica* larvae prior to behavioral assays.

Brood	Age (days)	Shell length† (μm) (mean ± SD)	Percent Searching‡ (mean ± SD)
F	15	318.6 (± 12.7)	15.5 (± 22.2)
G	14	319.4 (± 16.3)	17.0 (± 15.7)
H	14	318.8 (± 13.8)	18.1 (± 15.7)
All*	14.3	318.9 (± 14.3)	16.9 (±18.2)

* F, G, and, H combined. Data from one brood replicate (E; 19-July-07) are absent due to a hard drive failure.

†Photos of larval samples were taken using an Olympus D70 camera affixed to an Olympus BX51 compound microscope. Larvae were later measured manually using Image Pro 6.0 software.

‡ Assessment of metamorphic competence in larvae occurred during a 1.25 minute period recorded immediately pre-treatment (no chemicals, no ABS).

Table 2-2. Analysis of variance for larval shell length of *C. virginica* broods used in chemical induction experiments.

Source	df	Sum of squares	Mean square	F-ratio	p
Brood*	2	9.44†	4.72	0.02	0.98
Error	83	17266.22	208.03		

*Analysis conducted with broods F, G, and H only.

†Type III

2.2.3 Experimental Protocol

Stock solutions of chemicals were prepared fresh daily in distilled water (DI) approximately 1 hour prior to the initiation of the experiment. Chemicals were obtained from Sigma-Aldrich. I tested potential chemical inducers of larval settlement at concentrations and exposure times determined to induce a response in other species (Coon & Bonar 1985, Bonar et al. 1990, Coon et al. 1990a, Coon et al. 1990b, Tamburri et al. 1992, Beiras & Widdows 1995, Tan & Wong 1995, Walch et

al. 1999, Doroudi & Southgate 2002, Urrutia et al. 2004, Zhao et al. 2003). The time required to carefully assess each treatment during the experiment limited the number of treatments I was able to include in my project. The information from the aforementioned studies allowed me to narrow the concentrations I chose to investigate and preliminary experiments aided me in determining the concentrations most likely to induce a response without mortality (Table 2-3). These informal assessments were conducted in the same manner as is described below for the formal bioassays but lacked replication. I chose to assess the inductive ability of the following six chemicals plus two controls. The chemical compounds acetylcholine chloride (ACh), γ-aminobutyric acid (GABA), 3-isobutyl-1-methylxanthine (IBMX), 5-hydroxytryptamine (serotonin, or 5-HT), and L-3, 4-dihydroxyphenylalanine (L-DOPA) were tested at 10^{-4}M. I also tested the compound ammonia (NH_3) as a solution of .0079M NH_4Cl (ph = 8.0). This corresponds to an NH_3 concentration of 310 μM (Coon et al. 1990b). Hereafter, the chemicals will be denoted as follows: ACh, GABA, IBMX, 5-HT, L-DOPA, and NH_3, respectively.

Table 2-3. Observations from informal experiments conducted to determine chemical concentrations

Chemical Treatment	Concentration	Observations of *C. virginica* larvae	Conclusion*
IBMX	10^{-3}M	• Very few searching • Majority have closed shells and are not moving	10^{-4}M will be used in accordance with Zhao et al. (2003) and because 10^{-3}M appeared to have toxic effects here
	10^{-4}M	• Several searching, others swimming	
GABA	10^{-3}M	• Behaved very much like control	10^{-4}M will be used in accordance with Tan and Wong (1995) and because 10^{-3}M did not appear to induce more settlement behavior here
	10^{-4}M	• One searching • Overall seems very much like control group	
Acetylcholine	10^{-4}M	• Majority have closed shells • One has tip of foot extended	10^{-4}M will be used in accordance with Beiras and Widdows (1995) and because there were no larvae searching in my 10^{-5}M treatment
	10^{-5}M	• Slightly more movement than in 10^{-3}M AC, but none searching	
Serotonin	10^{-4}M	• Many searching, others swimming or spinning	10^{-4}M will be used in accordance with Urrutia (2004) and because fewer larvae were searching in my 10^{-5}M treatment
	10^{-5}M	• Fewer searching than in 10^{-4}M group • Does not seem less toxic than 10^{-4}M	
L-DOPA	10^{-4}M	• Many searching, others swimming	10^{-4}M will be used in accordance with Beiras and Widdows (1995), Coon et al. (1990a), and Walch et al. (1999) and because fewer larvae were searching in my 10^{-5}M treatment
	10^{-5}M	• A few searching, others swimming • Does not seem to be less toxic than 10^{-4}M	
NH_3	400µm	• Several searching • Slightly less movement compared to 310µM; more with shells closed than 310µM	310µM will be used in accordance with Coon et al. (1990b) and because the higher concentration used here appears to show the beginnings of toxic effects
	310µm	• Several searching, others swimming	
Control	n/a	• Nearly all swimming • Only one searching	n/a

*Based on personal observations and results from studies investigating chemical induction in other marine bivalves.

I conducted bioassays of settlement behavior using methods similar to those described by other researchers (Coon et al. 1985, Coon et al. 1990a, Coon et al. 1990b, Beiras and Widdows 1995, Tan and Wong 1995, Walch et al. 1999). As previously described, settlement in oyster larvae consists of a number of behaviors, but that which is most easily quantified is the extension of the foot, which the larvae use to inspect the surface prior to setting.

The experiments were conducted in Costar 24-well tissue culture plates containing 0.35ml ABS (100 µl ml^{-1} each of penicillin-G, streptomycin sulfate, and neomycin sulfate) and 0.15ml stock chemical (Figure 2-2). To determine the number

of larvae to be placed in each well, I conducted a preliminary experiment (Table 2-4). My objective was to determine the larval density that would allow me to clearly view as many larvae as possible and evaluate whether such a density induced a behaviorally-mediated settlement response. During this informal assessment, I observed larvae from one brood in 3 treatment replicates for each of 4 larval densities. The larvae were placed in microwells with 1μm FSW and were not treated with ABS or chemicals. After allowing the larvae to acclimate to the treatment wells for 30 minutes, I recorded the number searching in each replicate during a 30 second period. As a result of this informal investigation, I determined that 50-70 larvae could be placed in each well without invoking density-dependent settlement effects and without hindering my ability to clearly see and assess them. Coon et al. (1985) also noted that experimental results were not significantly affected by larval densities in the microwells over a range of 0.5-100 larvae per ml.

Table 2-4. Observations from an informal experiment conducted to assess effects of *C. virginica* larval density on settlement behavior in microwells. For each density, three samples of larvae from one brood were placed in separate microwells without antibiotics or chemical treatment. The number of larvae exhibiting foot extension in each well was recorded for 30 seconds to assess settlement behavior.

Larval Density Level	Larvae/ml in each Microwell	Mean Number of Larvae/Frame (± SD)	Mean Number of Larvae Searching/Frame (± SD)	Mean Percentage of Larvae Searching (± SD)	Level Mean Percentage of Larvae Searching (± SD)
I	25	8.3 (± 1.2)	0.3 (± 0.6)	4.8 (± 8.2)	3.2 (± 6.3)
	30	10.3 (± 1.2)	0.0 (± 0)	0.0 (± 0)	
	23	8.7 (± 3.8)	0.3 (± 0.6)	4.8 (± 8.2)	
II	52	13.3 (± 2.1)	0.7 (± 0.6)	5.4 (± 4.8)	6.0 (± 3.9)
	73	21.7 (± 3.5)	1.3 (± 0.6)	6.0 (± 1.8)	
	60	20.7 (± 1.5)	1.3 (± 1.2)	6.5 (± 5.7)	
III	152	39.3 (± 1.2)	1.0 (± 0)	2.5 (± 0.1)	3.0 (± 2.2)
	131	30.0 (± 5.3)	1.7 (± 0.6)	5.4 (± 1.1)	
	132	30.7 (± 1.5)	0.3 (± 0.6)	1.0 (± 1.8)	
IV	203	43.0 (± 7.5)	3.0 (± 1.0)	7.0 (± 2.2)	8.6 (± 2.8)
	184	46.3 (± 6.0)	5.0 (± 1.0)	11.0 (± 3.5)	
	275	64.0 (± 5.2)	5.0 (± 0)	7.8 (± 0.6)	

Each treatment well received the following components:

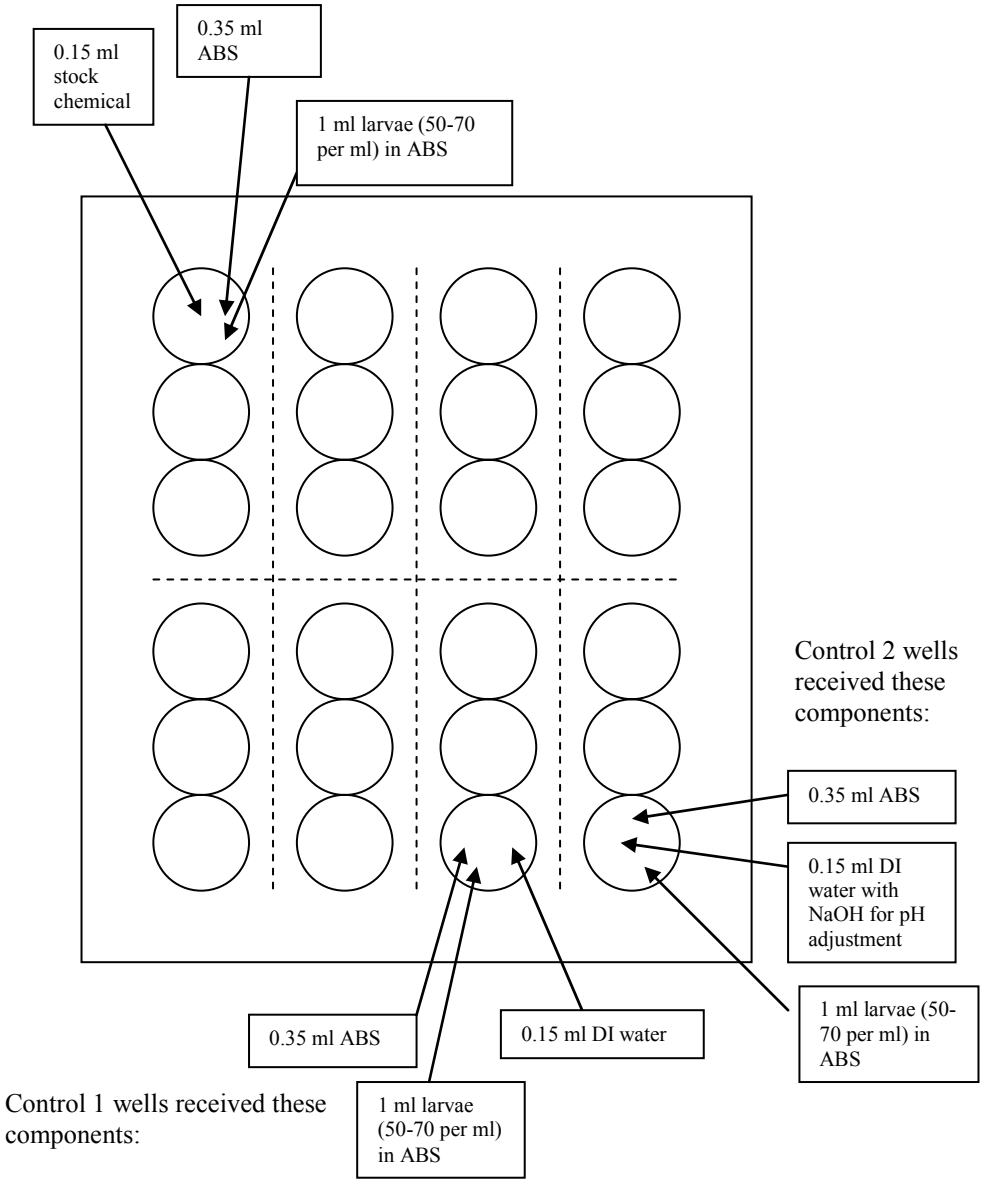

Figure 2-2. Microwell setup for bioassays of chemically induced settlement behavior.

At the initiation of the official experiment, I extracted 50-70 larvae from their holding container by gently but thoroughly mixing the larval milieu with a perforated plunger while withdrawing 1ml aliquots with a pipette. Each prepared treatment well then received one of these aliquots, bringing the final chemical concentration in the

microwell to 10^{-4}M for ACh, GABA, IBMX, 5-HT, and L-DOPA and 310μM for NH_3. Previous studies have revealed that a <10% reduction in salinity has no effect on experimental results (Coon et al. 1985, Beiras and Widdows 1995). The salinity reduction here was 11.1% and thus a control with an equivalent reduction in salinity was also run for comparison (Control 1). To account for any possible effects due to the pH adjustment for the ammonia treatment, I included a control with adjusted pH (Control 2). The location of the chemical treatments in the microwell plate was randomly varied and a new tissue culture plate was used for each treatment replicate.

Each experiment was recorded by StreamPix3 video recording software using a Sony DXC-390 camera affixed to an Olympus SZX16 dissecting microscope (Figure 2-3). This allowed me to look at individual video frames and quantify the number of larvae swimming or crawling with the foot extended in each frame. For each treatment well, I evaluated 10 frames in a 30-s period at 5 to 10-min intervals for 1hr, the timing of which is similar to procedures conducted by Walch et al. (1999). I executed the experiment using 6 broods of larvae, running 3 replicates for each treatment to gauge treatment variability. The ability to record these experiments and analyze individual frames allowed me to collect 110 data points for every time interval of each treatment (Appendix III). Unfortunately, data loss due to a hard drive failure resulted in only 4 brood replicates of useable data; however only 2 of the 3 treatment replicates remained for one of these brood replicates. The exception here was the data set from the ammonia treatments, which after data loss had only 2 brood replicates.

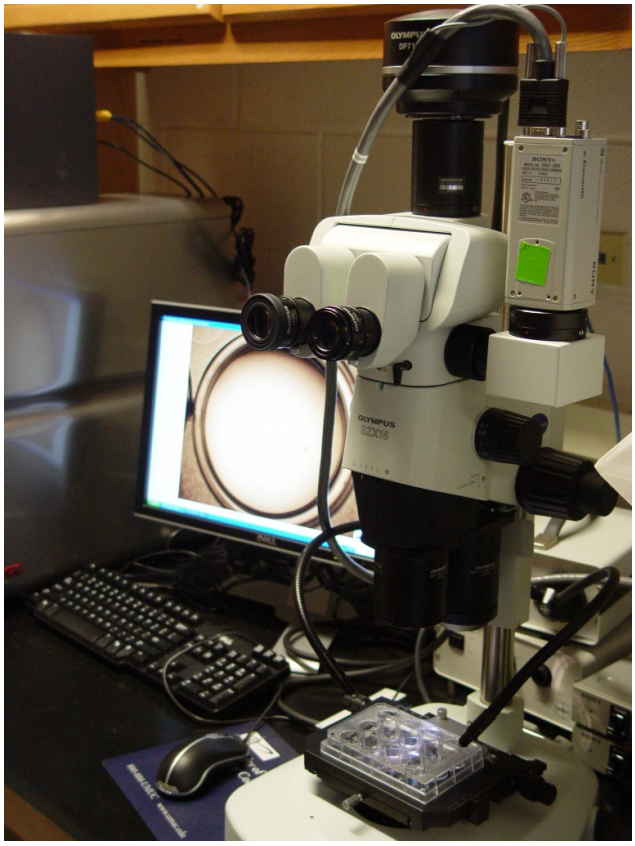

Figure 2-3. Set-up for microscopy video evaluation of *C. virginica* settlement behavior.

2.2.4 Data Analysis

To statistically separate the chemical treatments that were effective settlement inducers, I first conducted a Wilcoxon analysis of the proportion of larvae *not* displaying settlement behavior in each treatment. My null hypothesis was that there would be no difference between the proportions of larvae not searching in each chemical treatment and that of its control. Those that differed significantly from their controls were then compared using the arcsine-transformed percentage of larvae that *were* exhibiting settlement behavior for a one-way analysis of variance and Tukey test with a 95% significance level. This allowed me to determine how the chemicals compared during each time interval. For comparison to the ammonia group, which

had fewer replicates, the ANOVA was performed using the same sample size (n=60) for every treatment. (To insure that reducing the number of data points did not affect the overall results for the remaining treatments, I first ran the analysis using their full data sets. Reducing the sample size did not significantly affect the results.) I also executed a t-test to compare the proportion of larvae not searching in the treatments that induced similar levels of response as indicated by Figure 2-4. This allowed me to determine if these chemicals differed significantly from one another over the entire treatment period. Finally, I used a Kruskal-Wallis test to determine if there were significant differences in the brood responses for each effective chemical treatment, and a repeated measures univariate ANOVA to identify the effect of time.

2.3 Results

2.3.1 Visual Observations

In response to an active chemical inducer [ammonia (NH_3), 3-isobutyl-1-methylxanthine (IBMX), 5-hydroxytryptamine (5-HT), and L-3, 4-dihydroxyphenylalanine (L-DOPA)], larvae decreased their swimming speed and many began crawling with the foot extended. Those in the control treatments and γ-aminobutyric acid (GABA) treatment continued to swim rapidly without foot extension. In the acetylcholine (ACh) treatment, all larvae stopped swimming, sank to the bottom with veli withdrawn, and closed their shells. They remained this way for the duration of the experiment, with only a few occasionally extending the tip of the foot. Of the active inducers, many larvae in the 5-HT treatments swam in a constant circular, rotating fashion with the foot extended, while those in the remaining chemical treatments (NH_3, L-DOPA, IBMX) exhibited normal swimming and searching behavior.

2.3.2 Identification of Chemical Inducers and Effects of Exposure Time on Settlement Behavior

The raw data are presented in Appendix II. The results of the Wilcoxon analysis comparing the proportion of larvae not searching in each chemical treatment to that of its control are displayed in Table 2-5. The NH_3 ($p<.0002$), IBMX ($p<.0006$), L-DOPA ($p<.0003$), and 5-HT ($p<.0001$) treatments differed significantly from their controls. Hereafter, I refer to these chemicals as "effective," as they induced settlement behavior in larvae. Table 2-7 gives the results from the Tukey

test, which I ran in conjunction with an ANOVA in order to elucidate whether the percentage of larvae exhibiting settlement behavior in the effective chemical treatments differed significantly from one another during each time interval. The means from this analysis enabled me to rank the chemical treatments by the strength of their inductive ability for each time interval. This analysis indicated that L-DOPA and 5-HT often shared the same rank, as did NH_3 and IBMX (Table 2-7). Figure 2-4 also demonstrates the similarities in the levels of response between L-DOPA and 5-HT and between NH_3 and IBMX. While the percentage of larvae searching in the chemical treatments varied with time, overall there was no difference in the proportion of larvae not searching between the IBMX and NH_3 treatments (Table 2-6). The same was true for the larvae in the L-DOPA and 5-HT treatments.

Table 2-5. Summary of results from t-tests comparing the proportion of *C. virginica* larvae not searching in each chemical treatment to that of its control.

Chemical	Wilcoxon p-value	Mean Proportion Searching
Acetylcholine	0.1324	0.04
Ammonia	0.0002	0.46
GABA	0.2837	0.05
IBMX	0.0006	0.54
L-DOPA	0.0003	0.89
Serotonin	0.0001	0.96

Table 2-6. Summary of results from t-tests comparing larval response* in "effective" chemical treatments of *C. virginica* larvae.

Comparison	Wilcoxon p-value
IBMX v. Ammonia	0.2656
L-DOPA v. Serotonin	0.0718
IBMX v. L-DOPA	0.0019
L-DOPA v. Ammonia	0.0041
Serotonin v. IBMX	0.0005
Ammonia v. Serotonin	0.0007

* Proportion not exhibiting settlement behavior

Table 2-7. Summary of results from Tukey analysis comparing percentage of *C. virginica* larvae searching in each "effective" chemical treatment*

Chemical	Time Interval† 1	2	3	4	5	6	7	8	9	19	11	12
Ammonia (A)	Mean=3.7. Differs from ALL: B (p=0.0027), C (p=0.0080), D (p=0.0008)	Mean=17.1. Differs from D (p<.0001) only. Same as B (p=0.1246) and C (p=0.8571).	Mean=18.3. Differs from C and D (p<.0001). Same as B (p=0.1601).	Mean=17.0. Differs from all. (p=0.0154 for B; p<.0001 for C and D).	Mean=9.5. Same as B (p=0.999). Differs from C and D (p<.0001)	Mean=5.0 Same as B (p=0.2683). Differs from C and D (p<.0001)	Mean=7.6. Same as B (p=0.2775). Differs from C and D (p<.0001)	Mean=4.8. Same as B (p=0.2253). Differs from C and D (p<.0001)	Mean=3.8. Same as B (p=0.0489). Differs from C and D (p<.0001)	Mean=2.5. Differs from all (p=.0022 for B, p<.0001 for C and D)	Mean=4.1. Same as B (p=0.9356). Differs from C and D (p<.0001)	Mean=3.3. Same as B (p=0.3585). Differs from C and D (p<.0001)
IBMX (B)	Mean = 0.0 Differs from A (p=0.0027) and D (p<.0001). Same as C (p=0.9889).	Mean=13.4. Differs from D (p<.0001) only. Same as A (p=0.1246) and C (p=0.4932).	Means=14.7. Differs from C and D (p<.0001). Same as A (p=0.1601).	Mean=12.6. Differs from all. (p=0.0154 for A; p<.0001 for C and D).	Mean=9.6. Same as A (p=0.999). Differs from C and D (p<.0001)	Mean=7.7. Same as A (p=0.2683). Differs from C and D (p<.0001)	Mean=10.3. Same as A (p=0.2775). Differs from C and D (p<.0001)	Mean=7.5. Same as A (p=0.2253). Differs from C and D (p<.0001)	Mean=7.7. Same as A (p=0.0489). Differs from C and D (p<.0001)	Mean=7.6. Differs from all (p=.0022 for A, p<.0001 for C and D)	Mean=5.0. Same as A (p=0.9356). Differs from C and D (p<.0001)	Mean=5.8. Same as A (p=0.3585). Differs from C and D (p<.0001)
L-DOPA (C)	Mean=0.3 Differs from A (p=0.0027) and D (p<.0001). Same as B (p=0.9889).	Mean=15.8. Differs from D (p<.0001) only. Same as A (p=0.8571) and B (p=0.4932).	Means=29.3. Differs from A and B (p<.0001). Same as D (p=0.4572)	Mean=33.8. Differs from A and B (p<.0001). Same as D (p=1.0).	Mean=38.7. Same as D (p=0.0630). Differs from A and B (p<.0001).	Mean=38.2. Differs from all (p=0.0044 for D; p<.0001 for A and B)	Mean=37.7. Same as D (p=0.8318). Differs from A and B (p<.0001)	Mean=36.3. Same as D (p=0.4199). Differs from A and B (p<.0001)	Mean=35.1. Same as D (p=0.5661). Differs from A and B (p<.0001)	Mean=31.3. Same as D (p=0.7260). Differs from A and B (p<.0001)	Mean=29.6. Differs from all (p<.0001 for A and B, p=.0063 for D)	Mean=29.7. Differs from all (p<.0001)
Serotonin (D)	Mean=7.8 Differs from ALL: A (p=0.0008), B (p<.0001), C (p<.0001)	Mean=32.2. Differs from all (p<.0001).	Mean=31.7. Differs from A and B (p<.0001). Same as C (p=0.4572)	Mean=33.8. Differs from A and B (p<.0001). Same as C (p=1.0).	Mean=34.9. Same as C (p=0.0630). Differs from A and B (p<.0001).	Means=33.1. Differs from all (p=0.0044 for C; p<.0001 for A and B)	Mean=36.5. Same as C (p=0.8318). Differs from A and B (p<.0001)	Mean=34.1. Same as C (p=0.4199). Differs from A and B (p<.0001)	Mean=33.2. Same as C (p=0.5661). Differs from A and B (p<.0001)	Mean=32.7. Same as C (p=0.7260). Differs from A and B (p<.0001)	Mean=34.7. Differs from all (p<.0001 for A and B, p=.0063 for C)	Mean=36.6. Differs from all (p<.0001)
Rank by effectiveness	1. Serotonin 2. Ammonia 3. L-DOPA and IBMX	1. Serotonin 2. Ammonia, IBMX, & L-DOPA	1. L-DOPA and serotonin 2. Ammonia and IBMX	1. L-DOPA and serotonin 2. Ammonia 3. IBMX	1. L-DOPA and serotonin 2. Ammonia and IBMX	1. L-DOPA 2. Serotonin 3. Ammonia and IBMX	1. L-DOPA and serotonin 2. Ammonia and IBMX	1. L-DOPA and serotonin 2. Ammonia and IBMX	1. L-DOPA and serotonin 2. Ammonia and IBMX	1. L-DOPA and serotonin 2. Ammonia 3. IBMX	1. Serotonin 2. L-DOPA 3. Ammonia and IBMX	1. Serotonin 2. L-DOPA 3. Ammonia and IBMX

*Analysis was conducted using arcsine-transformed percentages. The mean percentages listed here have been calculated from transformed data.

†Assessments occurred during a 30 second period during each time interval. Each time interval is equivalent to 5 minutes, with the first interval being 0-5 minutes, the second 5-10 minutes, and so forth.

As shown in Table 2-8, the results from the repeated measures univariate analysis of variance show that exposure time had a significant effect on the percentage of larvae exhibiting settlement behavior ($p<.0001$). Furthermore, these results reveal a significant interaction effect between time and the chemical ($p<.0001$). Figure 2-4 shows the response of the larvae to the chemicals throughout the exposure period. The larvae responded most rapidly to 5-HT and NH_3; however the 5-HT treatment induced more larvae to exhibit settlement behavior as compared to the NH_3 treatment. The peak settlement response of the larvae in both the NH_3 and IBMX treatments occurred approximately 10 minutes after the initiation of the experiment. Of all chemical treatments, L-DOPA and 5-HT induced the greatest percentage of larvae to commence settlement behavior. The peak settlement response in the L-DOPA and 5-HT treatments was obtained more slowly than in the NH_3 and IBMX treatments however. In the L-DOPA treatment, the peak response occurred approximately 25 minutes after initial exposure, while the peak response in the serotonin treatment is indicated as occurring at 55 minutes, but may have been achieved with further exposure. The response in the L-DOPA and 5-HT treatments also seemed to be maintained longer throughout the exposure period than in the NH_3 and IBMX treatments.

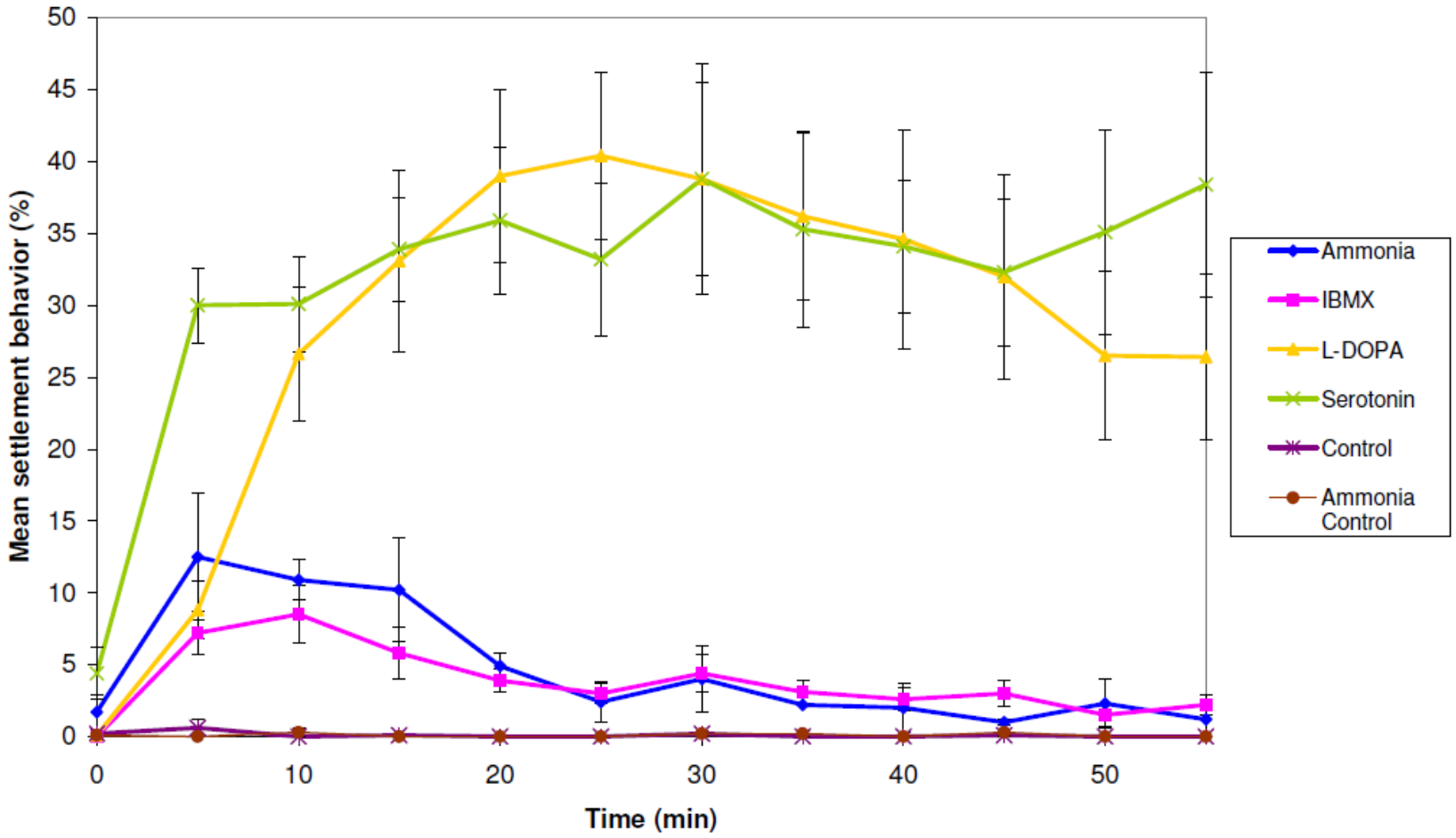

Figure 2-4. Average percentage of *C. virginica* larvae induced to exhibit settlement behavior as a function of length of exposure to test compounds. Prior to placement in ABS and treatment with test compounds, an average of 16.9% of larvae exhibited settlement behavior. Data have not been transformed. Bars represent standard error between brood

Table 2-8. Results from the repeated measures univariate analysis of variance for the effect of time on the settlement response* of chemically induced *C. virginica* larvae.

Source	df	Sum of squares	Mean square	F-ratio	p
Time	11	82665.96	7515.09	202.29	<.0001
Time*Chemical	33	72743.62	2204.35	59.33	<.0001
Error (Time)	2596	96443.90	37.15		

*Percentage of larvae exhibiting settlement behavior

2.3.3 Comparison of Brood Responses to Chemical Induction

Results from the Kruskal-Wallis analysis indicate that there are significant differences between some of the brood replicates in each of the chemical treatments (Table 2-9). These results are outlined in detail for each of the effective chemical treatments in Tables 2-10, 2-11, 2-12, and 2-13, and graphically in Figures 2-5, 2-6, 2-7, and 2-8.

Table 2-9. Presence or absence of a significant difference between the average responses of *C. virginica* brood replicates to chemical induction of settlement behavior*

Treatment	Time Interval†											
	1	2	3	4	5	6	7	8	9	10	11	12
L-DOPA	yes (p=0.0031)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)
Serotonin	yes (p=0.0002)	yes (p=0.0011)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)
Ammonia	yes (p=0.0003)	yes (p=0.010)	no (p=0.55)	no (p=0.063)	no (p=0.25)	yes (p=0.010)	yes (p=0.0008)	no (p=0.30)	yes (p=0.0013)	no (p=0.10)	yes (p=0.030)	yes (p<.0001)
IBMX	no (p=1.0)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p=0.0019)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p<.0001)	yes (p=0.0031)	yes (p=0.0053)	yes (p=0.0008)

*As indicated by the Kruskal-Wallis Test.

†Assessments occurred during a 30 second period during each time interval. Each time interval is equivalent to 5 minutes, with the first interval being 0-5 minutes, the second 5-10 minutes, and so forth.

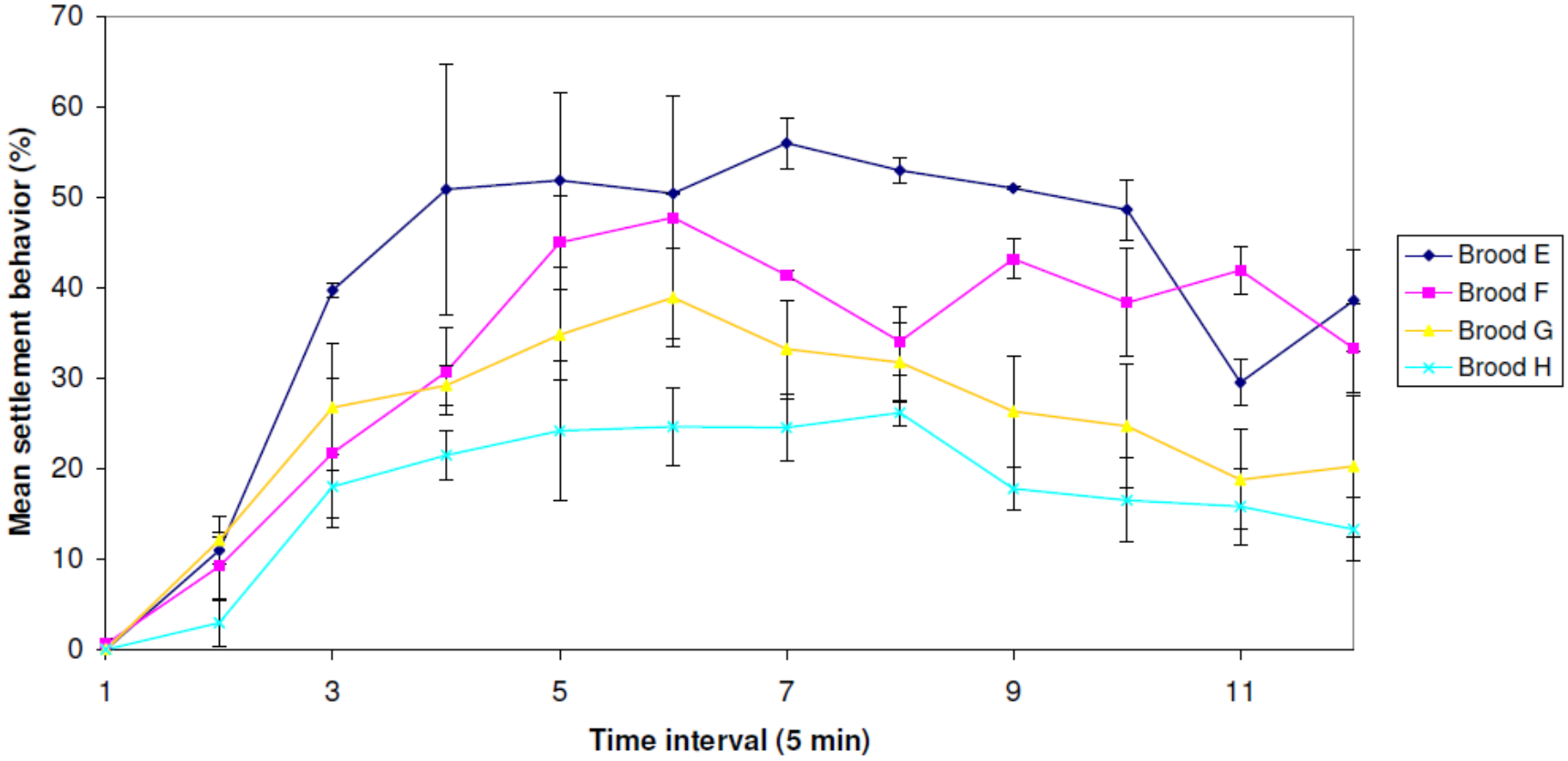

Figure 2-5. Comparison of *C. virginica* larval brood responses (mean percent exhibiting settlement behavior) to L-DOPA treatment as a function of length of exposure to chemical. Data have not been transformed. Bars represent standard error within each replicate.

Table 2-10. Summary of results from Tukey analysis comparing the average percentage of *C. virginica* larvae exhibiting settlement behavior in each brood replicate exposed to 10^{-4}M L-DOPA*

	Time Interval†											
Brood	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
E	Mean = 0. Same as all: F (p=0.14), G (p=1.0), H (p=1.0)	Mean = 19.4. Same as F (p=0.57) and G (p=1.0), differs from H (p<.0001).	Mean = 39.4. Differs from all: F (p=0.0003), G (p=0.035), (p<.0001).	Mean = 37.4. Same as F (p=0.17) and G (p=0.083), differs from H (p<.0001).	Mean = 51.9. Differs from all: F (p=0.0041), G (p<.0001), H (p<.0001).	Mean = 45.3 Same as F (p=0.94) and G (p=0.16), differs from H (p<.0001).	Mean = 50.1. Differs from all (p<.0001).	Mean = 45.9. Differs from all (p<.0001).	Mean = 45.5. Same as F (p=0.32), differs from G (p<.0001) and H (p<.0001).	Mean = 44.2. Same as F (p=0.11), differs from G (p<.0001) and H (p<.0001).	Mean = 32.7. Differs from all: F (p=0.026), G(p<0.012), H (p=0.0016).	Mean = 34.8. Same as F (p=1.0), differs from G (p=0.0097) and H (p<.0001).
F	Mean = 1.8. Same as E (p=0.14), differs from G (p=0.016) and H (p=0.016).	Mean = 16.0. Same as E (p=0.57) and G (p=0.19), differs from H (p<.0001).	Mean = 25.8. Same as G (p=0.14) and H (p=0.97), differs from E (p=0.0003).	Mean = 33.3. Same as E (p=0.17) and G (p=0.96), differs from H (p=0.0005).	Mean = 42.1. Differs from all: E (p=0.0041), G (p=0.013), H (p<.0001).	Mean = 43.5. Same as E (p=0.94) and G (p=0.14), differs from H (p<.0001).	Mean = 40.0. Differs from all: E (p<.0001), G (p=0.0066), H (p<.0001).	Mean = 35.4. Same as G (p=0.77), differs from E (p<.0001) and H (p=0.0074).	Mean = 41.1. Same as E (p=0.32), differs from G (p<.0001) and H (p<.0001).	Mean = 38.1. Same as E (p=0.11), differs from G (p<.0001) and H (p<.0001).	Mean = 40.3. Differs from all: E (p=0.026), G (p<.0001), H (p<.0001).	Mean = 35.0. Same as E (p=1.0), differs from G (p<.0001) and H (p<.0001).
G	Mean = 0. Same as E (p=1.0) and H (p=1.0), differs from F (p=0.016).	Mean = 19.8. Same as E (p=1.0) and F (p=0.19), differs from H (p<.0001).	Mean = 30.6. Same as F(p=0.14), differs from E (p=0.035) and H (p=0.049).	Mean = 32.6. Same as E (p=0.083) and F (p=0.96), differs from H (p=0.0026).	Mean = 35.9. Differs from all: E (p<.0001), F (p=0.013), H (p=0.0024).	Mean = 38.5. Same as E (p=0.16) and F (p=0.14), differs from H (p=0.001).	Mean = 34.9. Differs from all: E (p<.0001), F (p=0.0066), H (p=0.0032).	Mean = 34.0. Same as F (p=0.77) and H (p=0.097), differs from E (p<.0001).	Mean = 29.8. Differs from all: E (p<.0001), F (p<.0001), H (p=0.019).	Mean = 29.0. Differs from all: E (p<.0001), F (p<.0001), H (p=0.017).	Mean = 24.4. Same as H (p=0.82), differs from E (p=0.012) and F (p<.0001).	Mean = 24.9. Same as H (p=0.17), differs from E (p=0.0097) and F (p<.0001).
H	Mean = 0. Same as E (p=1.0) and G (p=1.0), differs from F (p=0.016).	Mean = 6.2. Differs from all (p<.0001).	Mean = 24.7. Same as F(p=0.97), differs from E (p<.0001) and G (p=0.049).	Mean = 27.4. Differs from all: E (p<.0001), F (p=0.0005), G (p=0.0026).	Mean = 28.6. Differs from all: E (p<.0001), F (p<.0001), G (p=0.0024).	Mean = 29.4 Differs from all: E (p<.0001), F (p<.0001), G (p=0.0001).	Mean = 29.5. Differs from all: E (p<.0001), F (p<.0001), G (p=0.0032).	Mean = 30.5. Same as G (p=0.097), differs from E (p<.0001) and F (p=0.0074).	Mean = 24.4. Differs from all: E (p<.0001), F (p<.0001), G (p=0.019).	Mean = 23.2. Differs from all: E (p<.0001), F (p<.0001), G (p=0.017).	Mean = 22.8. Same as G (p=0.82), differs from E (p=0.0016) and F (p<.0001).	Mean = 20.4. Same as G (p=0.17), differs from E (p<.0001) and F (p<.0001).

*Analysis was conducted using arcsine-transformed percentages. The mean percentages listed here have been calculated from transformed data.

†Assessments occurred during a 30 second period during each time interval. Each time interval is equivalent to 5 minutes, with the first interval being 0-5 minutes, the second 5-10 minutes, and so forth.

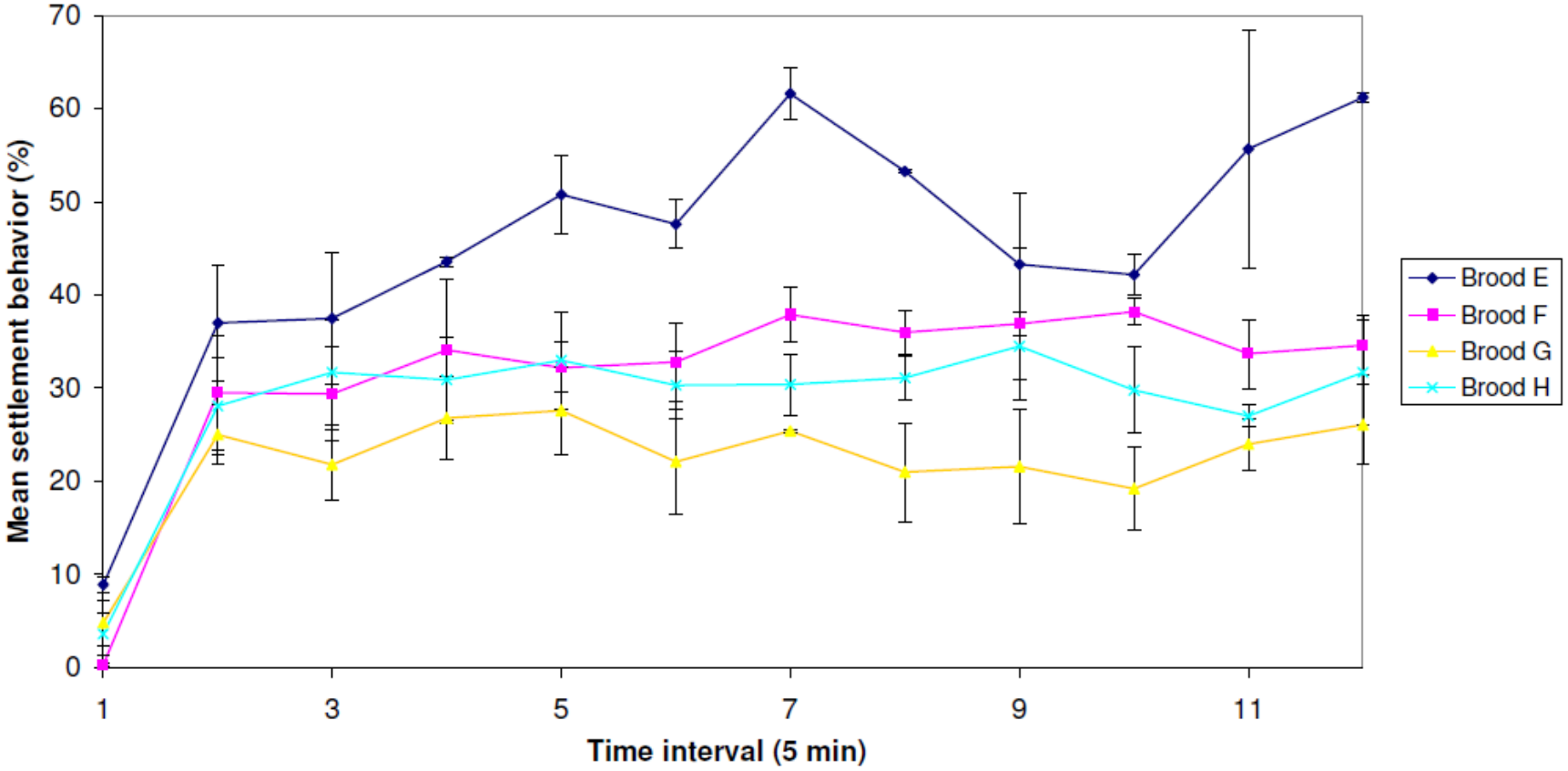

Figure 2-6. Comparison of *C. virginica* larval brood responses (mean percent exhibiting settlement behavior) to serotonin treatment as a function of length of exposure to chemical. Data have not been transformed. Bars represent standard error within each replicate.

Table 2-11. Summary of results from Tukey analysis comparing the average percentage of *C. virginica* larvae exhibiting settlement behavior in each brood replicate exposed to 10^{-4}M serotonin*

Brood	Time Interval† 1	2	3	4	5	6	7	8	9	10	11	12
E	Mean = 12.8. Same as G (p=0.74) and H (p=0.40), differs from F (p=0.0006).	Mean = 42.4. Differs from all (p<.0001).	Mean = 41.9. Differs from all: F (p<.0001), G (p<.0001), H (p=0.0009).	Mean = 41.5. Differs from all: F (p=0.035), G (p<.0001), H (p=0.0033).	Mean = 48.0. Differs from all (p<.0001).	Mean = 42.1. Differs from all: F (p=0.0035), G (p<.0001), H (p=0.0002).	Mean = 53.4. Differs from all (p<.0001).	Mean = 46.9. Differs from all (p<.0001).	Mean = 45.5. Differs from all: F (p=0.0030), G (p=<.0001), H (p=0.0005).	Mean = 40.5. Same as F (p=0.54), differs from G (p<.0001) and H (p=0.0002).	Mean = 55.9. Differs from all (p<.0001).	Mean = 51.8. Differs from all (p<.0001).
F	Mean = 1.7. Differs from all: E (p=0.0006), G (p=0.0003), H (p=0.0043).	Mean = 32.6. Same as G (p=0.14) and H (p=0.92), differs from E (p<.0001).	Mean = 32.5. Same as H (p=0.65), differs from E (p<.0001) and G (p=0.0041).	Mean = 35.4. Same as H (p=0.67), differs from E (p=0.035) and G (p=0.023).	Mean = 34.4. Same as G (p=0.15) and H (p=0.99), differs from E (p<.0001).	Mean = 34.8. Same as H (p=0.70), differs from E (p=0.0035) and G (p<.0001).	Mean = 38.0. Differs from all (p<.0001).	Mean = 36.8. Same as H (p=0.084), differs from E (p<.0001) and G (p<.0001).	Mean = 37.1. Same as H (p=0.87), differs from E (p=0.0030) and G (p<.0001).	Mean = 38.1. Same as E (p=0.54), differs from G (p<.0001) and H (p<.0003).	Mean = 35.4. Differs from all: E (p<.0001), G (p<.0001), H (p=0.0011).	Mean = 35.9. Same as H (p=0.44), differs from E (p<.0001) and G (p=0.0002).
G	Mean = 10.0. Same as E (p=0.74) and H (p=0.85), differs from F (p=0.0003).	Mean = 29.8. Same as F (p=0.14) and H (p=0.43), differs from E (p<.0001).	Mean = 27.6. Differs from all: E (p<.0001), F (p=0.0041), H (p<.0001).	Mean = 30.9. Same as H (p=0.29), differs from E (p<.0001) and F (p=0.023).	Mean = 31.5. Same as F (p=0.15) and H (p=0.08), differs from E (p<.0001).	Mean = 27.4. Differs from all: E (p<.0001), F (p<.0001), H (p=0.0010).	Mean = 30.2. Differs from all: E (p<.0001), F (p<.0001), H (p=0.010).	Mean = 26.8. Differs from all (p<.0001).	Mean = 27.1. Differs from all (p<.0001).	Mean = 25.6. Differs from all (p<.0001).	Mean = 29.2. Same as H (p=0.23), differs from E (p<.0001) and F (p<.0001).	Mean = 30.5. Differs from all: E (p<.0001), F (p=0.0002), H (p=0.025).
H	Mean = 8.4. Same as E (p=0.40) and G (p=0.85), differs from F (p=0.0043).	Mean = 31.8. Same as F (p=0.92) and G (p=0.43), differs from E (p<.0001).	Mean = 34.1. Same as F (p=0.65), differs from E (p=0.0009) and G (p<.0001).	Mean = 33.7. Same as F (p=0.67) and G (p=0.29), differs from E (p=0.0033).	Mean = 34.9. Same as F (p=0.99) and G (p=0.075), differs from E (p<.0001).	Mean = 33.2. Same as F (p=0.70), differs from E (p=0.0002) and G (p=0.0010).	Mean = 33.3. Differs from all: E (p<.0001), F (p<.0001), G (p=0.010).	Mean = 33.8. Same as F (p=0.084), differs from E (p<.0001) and G (p<.0001).	Mean = 35.8. Same as F (p=0.87), differs from E (p=0.0005) and G (p<.0001).	Mean = 32.8. Differs from all: E (p=0.0002), F (p=0.0003), G (p<.0001).	Mean = 31.2. Same as G (p=0.23), differs from E (p<.0001) and F (p=0.0011).	Mean = 34.1. Same as F (p=0.44), differs from E (p<.0001) and G (p=0.025).

*Analysis was conducted using arcsine-transformed percentages. The mean percentages listed here have been calculated from transformed data.

†Assessments occurred during a 30 second period during each time interval. Each time interval is equivalent to 5 minutes, with the first interval being 0-5 minutes, the second 5-10 minutes, and so forth.

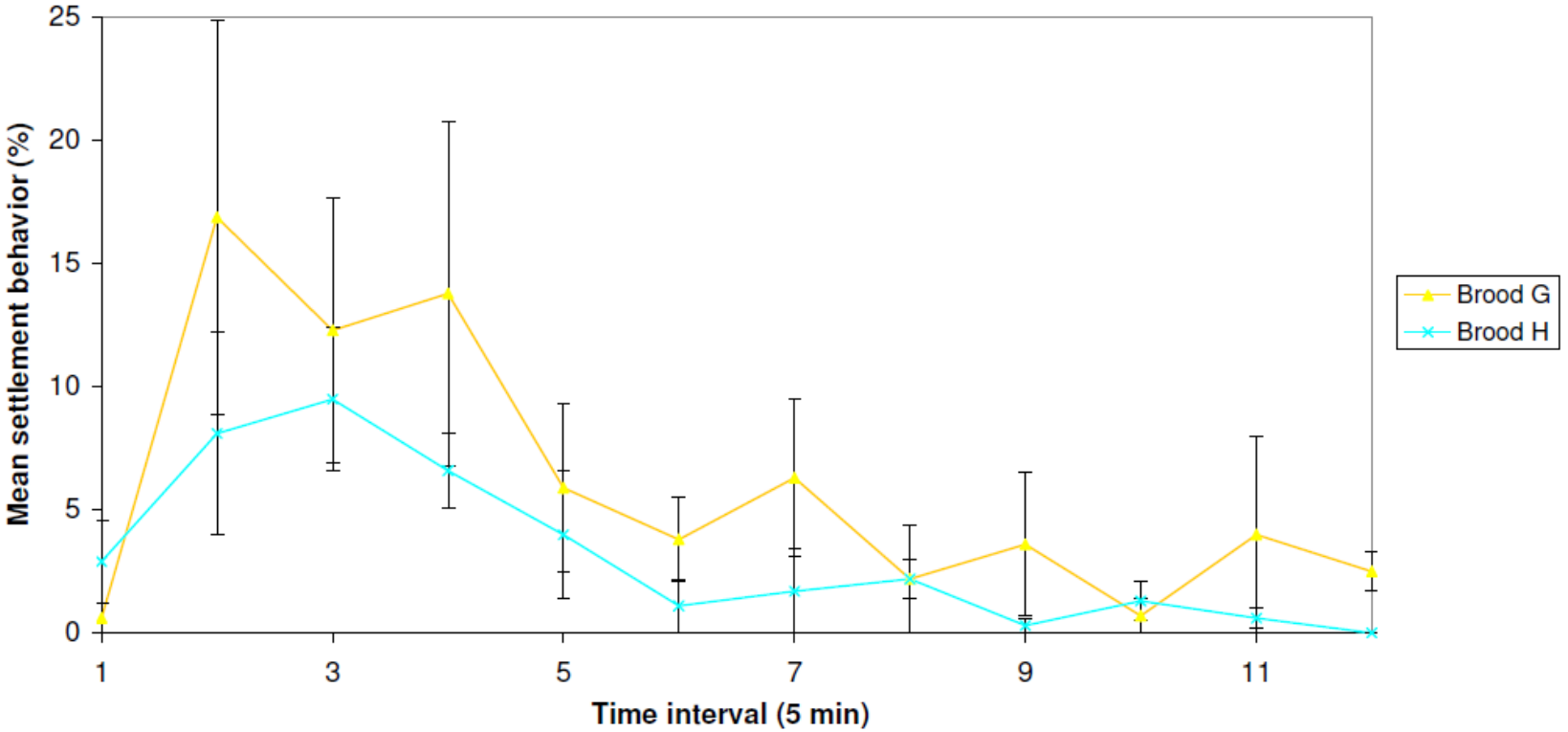

Figure 2-7. Comparison of *C. virginica* larval brood responses (mean percent exhibiting settlement behavior) to ammonia treatment as a function of length of exposure to chemical. Data have not been transformed. Bars represent standard error within each replicate. Data for broods E and F are absent due to a hard drive failure.

Table 2-12. Summary of results comparing the response of each brood of *C. virginica* larvae (G and H) exposed to 310μM ammonia

Time Interval	Mean settlement behavior (%)*	Presence of significant difference between broods†
1	G: 0.6 H: 2.9	yes (p=0.0003)
2	G: 16.9 H: 8.1	yes (p=0.0101)
3	G: 12.3 H: 9.5	no (p=0.5490)
4	G: 13.8 H: 6.6	no (p=0.0613)
5	G: 5.9 H: 4.0	no (p=0.2523)
6	G: 3.8 H: 1.1	yes (p=0.0104)
7	G: 6.3 H: 1.7	yes (p=0.0008)
8	G: 2.2 H: 2.2	no (p=0.3024)
9	G: 3.6 H: 0.27	yes (p=0.0013)
10	G: 0.75 H: 1.3	no (p=0.0966)
11	G: 4.0 H: 0.62	yes (p=0.0297)
12	G: 2.5 H: 0	yes (p<.0001)

*Analysis was conducted using arcsine-transformed percentages. The mean percentages listed here have been calculated from transformed data.

†As indicated by the Kruskal-Wallis Test

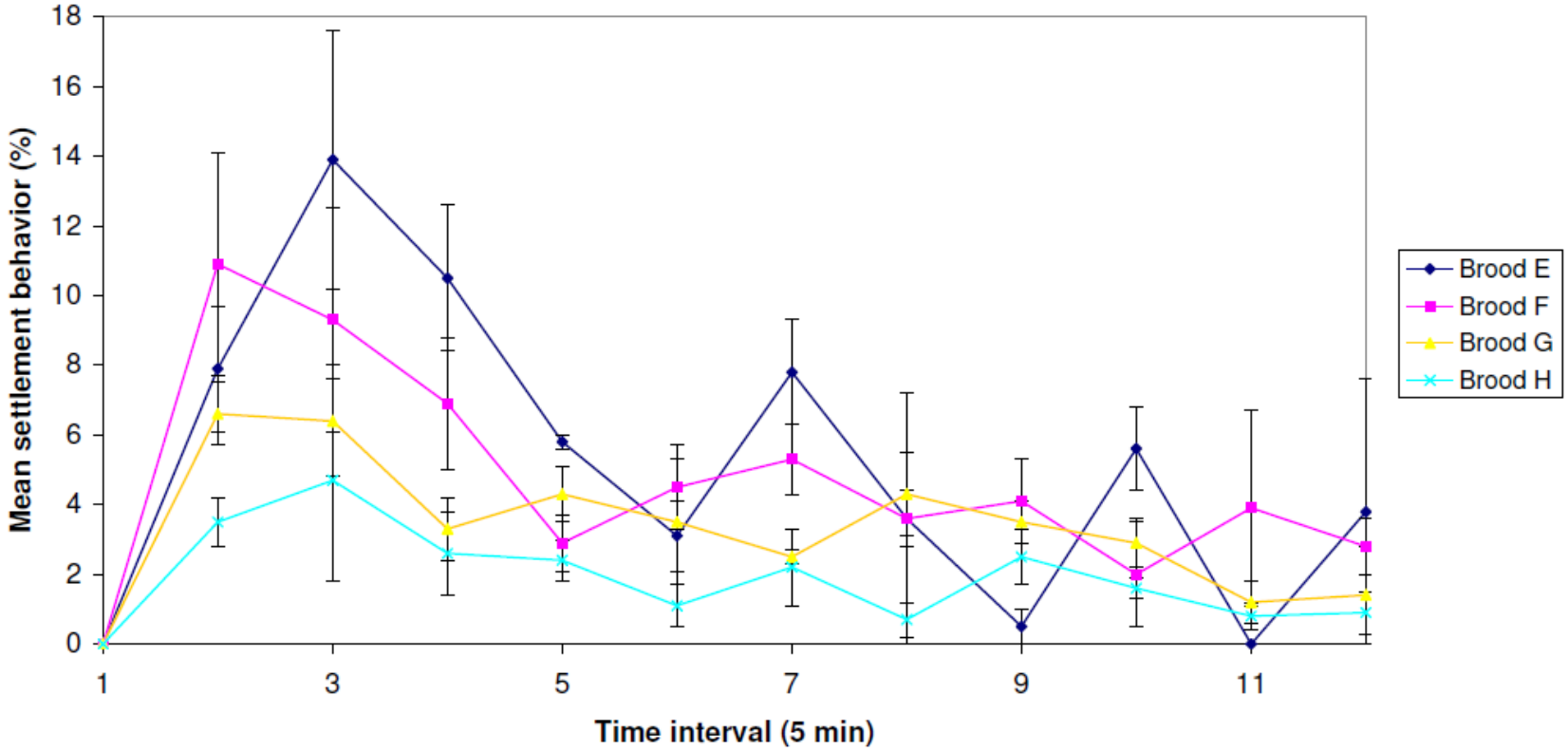

Figure 2-8. Comparison of *C. virginica* larval brood responses (mean percent exhibiting settlement behavior) to IBMX treatment as a function of length of exposure to chemical. Data have not been transformed. Bars represent standard error within each replicate.

Table 2-13. Summary of results from Tukey analysis comparing the average percentage of *C. virginica* larvae exhibiting settlement behavior in each brood replicate exposed to 10^{-4}M IBMX*

Brood	Time Interval† 1	2	3	4	5	6	7	8	9	10	11	12
E	Mean = 0. Differs from all (p<.0001).	Mean = 11.0. Same as G (p=0.54) and H (p=0.77), differs from F (p=0.0046).	Mean = 23.4. Same as F (p=0.079), differs from G (p=0.0005) and H (p<.0001).	Mean = 20.4. Differs from all: F (p=0.016), G (p<.0001), H (p<.0001).	Mean = 11.8. Same as all: F (p=0.30), G (p=1.0), H (p=0.056)	Mean = 3.8. Same as G (p=0.18) and H (p=1.0), differs from F (p=0.0038).	Mean = 10.1. Same as all: F (p=0.67), G (p=0.69), H (p=0.32).	Mean = 0. Same as H (p=0.65), differs from F (p<.0001) and G (p<.0001).	Mean = 0. Differs from all: F (p<.0001), G (p<.0001), H (p=0.0021).	Mean = 12.1. Same as G (p=0.26), differs from F (p=0.0095) and H (p=0.0081).	Mean = 0. Same as G (p=0.35) and H (p=0.68), differs from F (p=0.0051).	Mean = 0. Same as G (p=0.14), and H (p=0.55), differs from F (p<.0001).
F	Mean = 0. Differs from all (p<.0001).	Mean = 18.7. Differs from all: E (p=0.0046), G (p=0.019), H (p<.0001).	Mean = 17.1. Same as E (p=0.079) and G (p=0.092), differs from H (p=0.0012).	Mean = 14.8. Differs from all: E (p=0.016), G (p<.0001), H (p<.0001).	Mean = 8.3. Same as all: E (p=0.30), G (p=0.10), H (p=0.65).	Mean = 11.4. Same as G (p=0.16), differs from E (p=0.0038) and H (p<.0001).	Mean = 12.6. Same as E (p=0.67), differs from G (p=0.011) and H (p=0.0006).	Mean = 9.6. Same as G (p=0.84), differs from E (p<.0001) and H (p<.0001).	Mean = 10.8. Same as G (p=0.95), differs from E (p<.0001) and H (p=0.0049).	Mean = 5.1. Same as G (p=0.22) and H (p=1.0), differs from E (p=0.0095).	Mean = 7.6. Same as G (p=0.070), differs from E (p=0.0051) and H (p=0.0087).	Mean = 8.3. Differs from all: E (p<.0001), G (p=0.0062), H (p<.0001).
G	Mean = 0. Differs from all (p<.0001).	Mean = 14.0. Same as E (p=0.54), differs from F (p=0.019) and H (p=0.0087).	Mean = 12.7. Same as H (p=0.45) and F (p=0.092), differs from E (p=0.0005).	Mean = 8.9. Same as H (p=0.81), differs from E (p<.0001) and F (p<.0001).	Mean = 11.6. Same as E (p=1.0) and F (p=0.10), differs from H (p=0.0042).	Mean = 8.2. Same as E (p=0.18) and F (p=0.16), differs from H (p=0.015).	Mean = 7.7. Same as E (p=0.69) and H (p=0.82), differs from F (p=0.011).	Mean = 10.7. Same as F (p=0.84), differs from E (p<.0001) and H (p<.0001).	Mean = 10.1. Same as F (p=0.95), differs from E (p<.0001) and H (p=0.025).	Mean = 8.1. Same as all: E (p=0.26), F (p=0.22), H (p=0.19).	Mean = 3.7. Same as all: E (p=0.35), F (p=0.070), H (p=0.87).	Mean = 4.0. Same as E (p=0.14) and H (p=0.63), differs from F (p=0.0062).
H	Mean = 0. Differs from all (p<.0001).	Mean = 8.9. Same as E (p=0.77), differs from F (p<.0001) and G (p=0.0087).	Mean = 10.0. Same as G (p=0.45), differs from E (p<.0001) and F (p=0.0012).	Mean = 7.7. Same as G (p=0.81), differs from E (p<.0001) and F (p<.0001).	Mean = 6.7. Same as E (p=0.056) and F (p=0.65), differs from G (p=0.0042).	Mean = 3.5. Same as E (p=1.0), differs from F (p<.0001) and G (p=0.015).	Mean = 6.4. Same as E (p=0.32) and G (p=0.82), differs from F (p=0.0006).	Mean = 2.1. Same as E (p=0.65), differs from F (p<.0001) and G (p<.0001).	Mean = 6.5. Differs from all: E (p=0.0021), F (p=0.0049), G (p=0.025).	Mean = 5.0. Same as F (p=1.0) and G (p=0.19), differs from E (p=0.0081).	Mean = 2.5. Same as E (p=0.68) and G (p=0.87), differs from F (p=0.0087).	Mean = 2.4. Same as E (p=0.55) and G (p=0.63), differs from F (p<.0001).

*Analysis was conducted using arcsine-transformed percentages. The mean percentages listed here have been calculated from transformed data.

†Assessments occurred during a 30 second period during each time interval. Each time interval is equivalent to 5 minutes, with the first interval being 0-5 minutes, the second 5-10 minutes, and so forth.

2.4 Discussion

2.4.1 Chemical Induction of Settlement

Studies have shown that a number of neuroactive compounds are capable of inducing settlement in oyster larvae at salinities ranging from 15-30 (Coon & Bonar 1985, Bonar et al. 1990, Coon et al. 1990a, Coon et al. 1990b, Fitt and Coon 1992, Tamburri et al. 1992, Beiras & Widdows 1995, Tan & Wong 1995, Walch et al. 1999, Doroudi & Southgate 2002, Zhao et al. 2003). However, of these studies only two used *Crassostrea virginica* as a test subject and the inductive abilities of only two compounds, L-DOPA and ammonia, were evaluated with this species (Fitt and Coon 1992, Walch et al. 1999). Thus until now it was unknown whether *C. virginica,* at the low salinities (9-10) often encountered at mid-Chesapeake oyster hatcheries, would exhibit a response similar to the other species that were examined.

Results of my research show that ammonia (NH_3), 3-isobutyl-1-methylxanthine (IBMX), 5-hydroxytryptamine (5-HT), and L-3, 4-dihydroxyphenylalanine (L-DOPA) consistently and repeatedly induce settlement behavior in *C. virginica* at low salinities when exposed for 55 minutes, while acetylcholine chloride (ACh) and γ-aminobutyric acid (GABA) do not. The effective chemicals induced varied levels of response in the larvae. While their responses varied with exposure time, the reaction of the larvae to L-DOPA over the entire assessment period is not significantly different from that of the 5-HT treatment. Similarly, there is no difference in the proportion of larvae not searching in the IBMX and NH_3 treatments over the entire assessment period.

As noted, after using the standard hatchery procedure of visual inspection to determine competence, I calculated that approximately 16.9% of the larvae used in this study were exhibiting settlement behavior prior to placement in antibiotic seawater (ABS) and treatment with neuroactive compounds. However, placement in ABS reduced searching behavior to <1.2% while the larvae seemed otherwise unaffected by their environment. A number of other researchers used ABS in studies similar to this one (Hadfield 1984, Bonar et al. 1990, Coon et al. 1990a, Fitt and Coon 1992, Beiras and Widdows 1995). One of these studies mentioned that although competent larvae were used, none metamorphosed in the control treatment and several of the chemical treatments which they later deemed ineffective (Beiras and Widdows 1995). The authors did not discuss this further. As I added antibiotics to reduce the accumulation of pathogenic bacteria and to eliminate the potential for settlement cues from replicating bacteria, one can only surmise that doing so may have removed a bacterial cue from the water and thus reduced settlement behavior in the larvae. It is also possible that the addition of antibiotics acted on the larvae at a molecular level, perhaps "turning off" the settlement mechanism that was later reactivated by the effective compounds. It is unknown whether the results from chemical induction in this study would have been higher without the use of ABS.

2.4.2 Comparison of Brood Responses to Chemical Induction

The differences in the response of each brood replicate to chemical treatment are representative of the variability inherent to larval oyster culture. During normal hatchery operations, there are variations in the degree of settlement behavior both between broods and within a brood of larvae (Meritt, pers. comm.). Attempts were

made during this project to control variables such as water quality, larval size, and diet, the latter of which was altered slightly on only 1 day for broods E and F (in this case, the quantity of C-Iso was doubled to replace 3H). The broods did not differ significantly in size (Tables 2-1, 2-2; size data for brood E was unavailable). Broods E and F were 15 days old at the time of the experiment, while broods G and H were 14 days old. However, the responses of broods E and F often differed significantly from one another, as did broods G and H (Tables 2-10, 2-11, 2-12, 2-13). Determining the source of the variability between the broods was not within the scope of this study.

2.4.3 Comparisons to Other Species

Several of my results differ from those pertaining to other species in the literature. Acetylcholine chloride (ACh) at 10^{-4}M, for example, was found to be capable of inducing *Crassostrea gigas* to exhibit settlement behavior (Beiras and Widdows 1995), while serotonin (5-HT) at 10^{-4}M was deemed ineffective (Coon et al. 1985, Beiras and Widdows 1995). The discrepancy with the acetylcholine results could lie in the amount of time larvae were exposed to the compound. Beiras and Widdows (1995) found maximum settlement and metamorphosis in the ACh treatment to occur after 48 hours. The formal assessment of the larvae in my study occurred within 1 hour. However, an informal assessment of the larvae in my ACh treatment at 48 hours revealed little to no metamorphosis. Some of the larvae had resumed swimming and a few were exhibiting settlement behavior, but the percentage of larvae searching did not appear to differ from the controls. Interestingly, Coon et

al. (1985) found acetylcholine chloride to be ineffective at inducing settlement and metamorphosis in *C. gigas* after a 24-48 hour exposure time. It should also be noted that the larvae in my study (and in that of Coon et al.) were exposed to an experimental environment very similar to those in the study performed by Beiras and Widdows (1995) (with the exception of salinity which was much lower in my investigation). Further study of this chemical may clarify this issue.

Another compound which seems to produce a varying response amongst the *Crassostrea* genus is γ-aminobutyric acid (GABA). As my results demonstrate, this chemical was found to be ineffective at inducing settlement behavior in *C. virginica* at the concentrations and exposure times tested. Similarly, Coon et al. (1985) reported that *C. gigas,* when exposed for 1 hour or 24 hours, did not initiate settlement behavior and metamorphosis in response to GABA at 10^{-6}-10^{-4}M. In contrast, when GABA was evaluated under similar conditions for *C. belcheri,* it was found to be an effective inducer of settlement and metamorphosis (Tan and Wong 1995). Treating competent larvae with 10^{-6}-10^{-4}M GABA for 1 hour yielded a setting percentage of 70-71% in this study (Tan and Wong 1995).

2.4.4 Mechanisms of Chemical Induction of Settlement

Although the purpose of this study was not to determine the method by which effective chemicals induce settlement, this topic does warrant some discussion. As previously described, competent pediveliger oyster larvae typically undergo settlement (whence they crawl upon the substrate actively searching for an appropriate place to set) before metamorphosis. If the location is deemed suitable,

they will commence metamorphosis by initially cementing to the substrate then changing into their adult form. Coon et al. (1985) proposed a model of two serial control pathways which orchestrate this process: a dopaminergic pathway that controls settlement behavior, and an adrenergic pathway controlling metamorphosis (Figure 2-1).

These researchers later found that *C. gigas* larvae convert L-3, 4-dihydroxyphenylalanine (L-DOPA) to dopamine which then interacts with dopaminergic receptors (Coon et al. 1987). This indicates that exogenous L-DOPA acts through an internal transduction event, rather than as an environmental cue. Typically, appropriate environmental cues trigger the release of dopamine, thus initiating settlement. The results from this study and those of several others support the finding that L-DOPA is capable of producing settlement behavior by triggering the dopaminergic pathway (Coon et al. 1985, Coon et al. 1990a, Beiras and Widdows 1995, Walch et al. 1999).

As presented here, ammonia (NH_3) is also capable of triggering the settlement neuropathway in *C. virginica*. This is consistent with results seen from studies on *C. gigas* and *C. virginica* (Coon et al. 1990b, Fitt and Coon 1992). Unlike L-DOPA, however, it likely functions as a natural settlement cue (Fitt and Coon 1992). Coon et al. (1990b) argue that the presence of high NH_3 levels in the oyster reef environment, the rapid induction of settlement behavior by NH_3, and the quick reversibility of inductive effects of NH_3 all suggest that it is a natural environmental cue. They further postulate that it acts on the dopaminergic pathway by increasing pH, although the exact mechanism has not been confirmed.

Serotonin (5-HT) is a derivative of tryptophan, a known neurotransmitter and modulator in both vertebrate and invertebrate nervous systems (as cited by Zhao et al. 2003). Its function in bivalves has not been fully described; however it has been reported to be the most effective chemical inducer of settlement and metamorphosis in *Pinctada maxima,* the silver- or goldlip pearl oyster (Zhao et al. 2003). It has also been found to induce metamorphosis in *Ruditapes philippinarum,* the Japanese short-neck clam (Urrutia et al. 2004). Interestingly, Cann-Moisan et al. (2002) found a marked increase in serotonin and dopamine in king scallop *Pecten maximus* larvae as they approached metamorphosis followed by a sharp decrease during post-larval life. While 5-HT did induce *C. virginica* to exhibit settlement behavior in my study, the behavior differed somewhat from that induced by other test compounds. In the 5-HT treatments, the larvae would extend their feet, but most often would be motionless or spinning in circles, rather than crawling. When I informally assessed these larvae after 48 hours of exposure to 5-HT, I noticed that the vela on many larvae had become quite enlarged and a number of vela were observed detached from the larvae. In addition, those larvae that were still moving were spinning in circles, as were the detached vela. Such behavior was not observed to this extent in other treatments. This raises questions as to how serotonin is influencing the settlement process. Results from laboratory studies conducted by Kuang and Goldberg (2001) suggest that serotonin regulates ciliary activity in another mollusk, the pond snail *Helisoma trivolvis,* and the rotational behavior it exhibits as an embryo. Considering that the velum in the eastern oyster is a ciliated organ (Galstoff 1964), my observations lead me to infer that serotonin is probably not acting as an external settlement cue, but as

an endogenous neurotransmitter, controlling the velum and its release after settlement; it also seems to be triggering the portion of the dopaminergic pathway involving foot extension. Whether or not *C. virginica* larvae are able to complete metamorphosis after exposure to 5-HT has yet to be determined.

The mechanism of induction is also not known for 3-isobutyl-1-methylxanthine (IBMX). As reported by Zhao et al. (2003), this chemical "is a xanthine derivative, which can elevate intracellular cAMP levels by inhibiting the phosphodiesterase responsible for the degradation of cAMP (Pawlik, 1990) and affects calcium transport (Holm et al.1998)". In the report of their findings, Zhao et al. (2003) stated that IBMX induced larval settlement of *Pinctada maxima.* According to Morse et al. (1984), induced changes in cAMP and calcium concentrations participate in the transduction of the settlement-inducing signal in *Haliotis* (gastropod) larvae. From this, it seems logical to deduce that IBMX may be acting at membrane receptors to elevate cAMP levels in eastern oyster larvae, thus participating in the transfer of the settlement-inducing signal.

2.4.5 Use of Chemical Inducers in the Hatchery Setting

Given the results presented here, it appears that L-3, 4-dihydroxyphenylalanine, ammonia, 5-hydroxytryptamine, and 3-isobutyl-1-methylxanthine could serve as useful inducers of larval settlement in hatcheries producing *C. virginica* spat. These compounds 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.

In an oyster culture facility, it is assumed that larvae which reach a certain size and exhibit settlement behavior are competent to set and progress through metamorphosis (Meritt, pers. comm.). However, according to Coon et al. (1990a), *C. gigas* larvae can exhibit settlement behavior prior to becoming morphogenetically competent. Perhaps then, the practical application of these chemical inducers would involve challenging a larval sample with epinephrine (which, as previously mentioned, produces cultchless spat) prior to settlement induction to ensure their ability to metamorphose. If a certain percentage of the challenged larvae metamorphosed in response to epinephrine, their cohort would then be treated with one of the settlement inducers described in this study to cue them to set on the offered substrate. During this procedure, the length of exposure to these chemical inducers would be of important consideration; the results from my study indicate that the length of exposure to the effective test chemicals had a significant effect on the settlement response of the larvae ($p<.0001$; Table 2-8). Further research is needed to determine if the results seen in my small scale study could be duplicated in a large scale setting, but this concept is particularly promising considering that results from chemical induction in the laboratory have proven to be more variable than those from induction in the hatchery setting (Walch et al. 1999).

It would also be insightful to determine if chemical induction could boost production and/or produce more consistent results under less than ideal circumstances, such as those which occurred in the summer of 2007 when this study was conducted. This was a particularly challenging season for the Horn Point Oyster Culture Facility: production of spat-on-shell dropped from 335.5 million in 2006 to

140.3 million in 2007, with great variability in setting efficiencies in 2007 from 1.2 – 44.9% (S. Alexander, pers. comm.). Metamorphosis was not evaluated in this experiment; however the range of larvae exhibiting settlement behavior in the L-DOPA treatment after 25 minutes, for example, was 12.5-81.3%. It is assumed by hatchery personnel that an increase in the percentage of larvae exhibiting settlement behavior is followed by an increase in the percentage of larvae that set and metamorphosis (Meritt, pers. comm.). It is also possible that, but not known if, chemical treatment could induce an even greater percentage of competent larvae to exhibit settlement behavior and metamorphose under favorable conditions, such as those which were present in 2008 when setting efficiencies averaged 27.5% and 527.6 million spat-on-shell were produced (S. Alexander, pers. comm.).

Metamorphic success is determined not only by the number of larvae that set on shell in the hatchery, but also by the ability of these spat to survive and grow post-metamorphosis. With the exception of ammonia, a number of studies indicate that various bivalve larvae treated with these compounds progress from settlement to metamorphosis (Coon et al. 1985, Coon et al. 1990a, Beiras and Widdows 1995, Tan and Wong 1995, Zhao et al. 2003); however, for *C. virginca,* this has only been confirmed for L-DOPA (Walch et al. 1999). Walch et al. (1999) also noted that 4 week old *C. virginica* and *C. gigas* spat appeared normal after being induced by L-DOPA exposure. Preliminary studies to evaluate metamorphosis in chemically induced *C. virginica* larvae have already been completed at Horn Point Laboratory. Work evaluating large scale settlement induction with L-DOPA, ammonia, IBMX, and serotonin, as well as post-induction survival and growth, are planned.

Appendices

Appendix I. Algal diet for *Crassostrea virginica* larvae raised for behavioral assays.

Larval Age (days)	Larval Density (larvae/ml)	C-Iso (cells/ml)	3H (cells/ml)	Ply-429 (cells/ml)
0	10	0	0	0
1	10	27,000	0	0
2	10	27,000	0	0
3	10	27,000	0	0
4	10	27,000	0	0
5	10	13,500	13,500	0
6	10	31,500	31,500	0
7	10	21,000	21,000	2,100
8	2	10,000	10,000	1,000
9	2	10,000	10,000	1,000
10	2	10,000	10,000	1,000
11	2	13,200	13,200	1,320
12	2	20,000	20,000	2,000
13	2	26,666	26,666	2,666
14	2	26,666	26,666	2,666
15	2	30,000	30,000	3,000

Appendix II. Results from assays of settlement behavior in *C. virginica* larvae.

Larval Treatment	Brood Replicate	Percentage of Larvae Searching* (Mean ± SE†) Time Interval											
		1	2	3	4	5	6	7	8	9	10	11	12
Control 1	E	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	F	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	G	0.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	H	0.0 ± 0.0	2.4 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
	All	0.2 ± 0.1	0.6 ± 0.6	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
L-DOPA	E	0.0 ± 0.0	11.0± 1.8	39.7 ± 3.1	50.9 ± 4.0	51.9 ± 2.7	50.4 ± 2.8	56.0 ± 1.6	53.0 ± 1.9	51.0 ± 2.0	48.6 ± 3.3	29.5 ± 2.5	38.6 ± 2.1
	F	0.6 ± 0.3	9.2 ± 1.2	21.8 ± 2.7	30.7 ± 2.0	45.0 ± 1.8	47.8 ± 3.8	41.4 ± 1.7	34.1 ± 1.8	43.2 ± 1.2	38.4 ± 1.9	41.9 ± 1.7	33.3 ± 1.9
	G	0.0 ± 0.0	12.1 ± 1.3	26.8 ± 2.3	29.2 ± 1.3	34.8 ± 2.5	38.9 ± 1.9	33.2 ± 2.2	31.7 ± 1.8	26.3 ± 2.5	24.7 ± 2.5	18.8 ± 2.2	20.3 ± 2.5
	H	0.0 ± 0.0	3.0 ± 0.7	18.0 ± 1.2	21.5 ± 1.2	24.2 ± 2.4	24.6 ± 1.7	24.5 ± 1.3	26.2 ± 1.3	17.8 ± 1.4	16.5 ± 1.5	15.8 ± 1.4	13.3 ± 1.6
	All	0.1 ± 0.1	8.8 ± 2.0	26.6 ± 4.7	33.1 ± 6.3	39.0 ± 6.0	40.4 ± 5.8	38.8 ± 6.7	36.2 ± 5.8	34.6 ± 7.6	32.0 ± 7.1	26.5 ± 5.9	26.4 ± 5.8
Serotonin	E	8.9 ± 2.4	37.0 ± 2.9	37.5± 4.3	43.6 ± 2.2	50.8 ± 2.8	47.6 ± 1.6	61.6 ± 1.5	53.3 ± 1.9	43.3 ± 2.0	42.2 ± 2.1	55.7 ± 3.2	61.2 ± 1.1
	F	0.3 ± 0.1	29.5 ± 1.7	29.4 ± 1.8	34.1 ± 2.2	32.2 ± 1.2	32.8 ± 1.6	37.9 ± 1.1	36.0 ± 1.2	37.0 ± 2.3	38.2 ± 1.1	33.7 ± 1.6	34.6 ± 1.1
	G	4.8 ± 0.9	25.0 ± 1.1	21.8 ± 1.2	26.8 ± 1.8	27.6 ± 1.4	22.1 ± 1.8	25.4 ± 0.6	21.0 ± 1.5	21.5 ± 1.7	19.2 ± 1.3	24.0 ± 1.1	26.1 ± 1.4
	H	3.6 ± 0.7	28.1± 1.5	31.7 ± 1.6	30.9 ± 1.3	33.0 ± 1.7	30.3 ± 1.5	30.4 ± 1.5	31.1 ± 1.2	34.5 ± 1.5	29.8 ± 1.5	27.0 ± 0.8	31.7 ± 1.8
	All	4.4 ± 1.8	30.0 ± 2.6	30.1 ± 3.3	33.9 ± 3.6	35.9 ± 5.1	33.2 ± 5.3	38.8 ± 8.0	35.3 ± 6.8	34.1 ± 4.6	32.3 ± 5.1	35.1 ± 7.1	38.4 ± 7.8
IBMX	E	0.0 ± 0.0	7.9 ± 1.3	13.9 ± 2.1	10.5 ± 1.1	5.8 ± 0.9	3.1 ± 0.9	7.8 ± 1.4	3.6 ± 1.2	0.5 ± 0.4	5.6 ± 1.2	0.0 ± 0.0	3.8 ± 0.9
	F	0.0 ± 0.0	10.9 ± 1.1	9.3 ± 1.0	6.9 ± 0.6	2.9 ± 0.4	4.5 ± 0.4	5.3 ± 0.5	3.6 ± 0.5	4.1 ± 0.4	2.0 ± 0.5	3.9 ± 1.0	2.8 ± 0.3
	G	0.0 ± 0.0	6.6 ± 0.7	6.4 ± 0.9	3.3 ± 0.5	4.3 ± 0.4	3.5 ± 0.6	2.5 ± 0.3	4.3 ± 0.6	3.5 ± 0.3	2.9 ± 0.4	1.2 ± 0.3	1.4 ± 0.4
	H	0.0 ± 0.0	3.5 ± 0.5	4.7 ± 0.9	2.6 ± 0.4	2.4 ± 0.5	1.1 ± 0.3	2.2 ± 0.7	0.7 ± 0.3	2.5 ± 0.5	1.6 ± 0.3	0.8 ± 0.3	0.9 ± 0.3
	All	0.0 ± 0.0	7.2 ± 1.5	8.5 ± 2.0	5.8 ± 1.8	3.9 ± 0.8	3.0 ± 0.7	4.4 ± 1.3	3.1 ± 0.8	2.6 ± 0.8	3.0 ± 0.9	1.5 ± 0.8	2.2 ± 0.7

*Calculated from raw percentages before dropping any data points for analysis.

†Standard error represents between brood variability.

Appendix II (continued). Results from assays of settlement behavior in *C. virginica* larvae.

Larval Treatment	Brood Replicate	Percentage of Larvae Searching* (Mean ± SE) Time Interval 1	2	3	4	5	6	7	8	9	10	11	12
GABA	E	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	F	0.0 ± 0.0	0.0 ± 0.0	2.1 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	G	1.0 ± 0.3	0.0 ± 0.0	1.2 ± 0.4	2.1 ± 0.5	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	H	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	2.7 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.2
	All	0.2 ± 0.2	0.0 ± 0.0	0.8 ± 0.5	0.5 ± 0.5	0.8 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
Acetylcholine	E	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.4	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	1.8 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	F	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.1 ± 0.6	3.4 ± 0.6	0.0 ± 0.0	1.0 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	G	0.0 ± 0.0	1.4 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	H	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	All	0.0 ± 0.0	0.3 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.5 ± 0.5	1.0 ± 0.9	0.5 ± 0.5	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Control 2	E	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	F	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
	G	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	H	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.3	0.7 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	All	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.2 ± 0.2	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
Ammonia	E†	-	-	-	-	-	-	-	-	-	-	-	-
	F†	-	-	-	-	-	-	-	-	-	-	-	-
	G	0.6 ± 0.6	16.9 ± 2.7	12.3 ± 1.6	13.8 ± 2.2	5.9 ± 1.1	3.8 ± 0.9	6.3 ± 1.1	2.2 ± 0.5	3.6 ± 0.9	0.7 ± 0.4	4.0 ± 1.3	2.5 ± 0.5
	H	3.0 ± 0.6	8.1 ± 1.2	9.5 ± 1.0	6.6 ± 0.7	4.0 ± 0.9	1.1 ± 0.5	1.7 ± 0.6	2.2 ± 0.8	0.3 ± 0.2	1.3 ± 0.4	0.6 ± 0.3	0.0 ± 0.0
	All	1.7 ± 1.2	12.5 ± 4.4	10.9 ± 1.4	10.2 ± 3.6	4.9 ± 0.9	2.4 ± 1.4	4.0 ± 2.3	2.2 ± 0.0	2.0 ± 1.7	1.0 ± 0.2	2.3 ± 1.7	1.2 ± 1.2

*Calculated from raw percentages before dropping any data points for analysis.

†Data absent due to hard drive failure.

Appendix III. Sample table of raw data collected during behavioral assays of *C. virginica* larvae in the serotonin treatment.

Replicate*	Frame†	Time Interval	Total # Larvae in Frame	# Larvae Searching in Frame	Percent Searching	Transformed Percent Searching‡
E1	15;57;06	7	14	9	64.3	53.3
	15;58;10	7	11	7	63.6	52.9
	15;59;01	7	12	8	66.7	54.7
	16;01;16	7	7	4	57.1	49.1
	16;02;28	7	12	8	66.7	54.7
	16;04;10	7	13	9	69.2	56.3
	16;05;26	7	14	9	64.3	53.3
	16;07;03	7	15	10	66.7	54.7
	16;09;01	7	18	11	61.1	51.4
	16;10;17	7	17	11	64.7	53.6
E2	31;05;27	7	7	3	42.9	40.9
	31;08;27	7	6	3	50.0	45.0
	31;12;18	7	11	7	63.6	52.9
	31;15;02	7	11	7	63.6	52.9
	31;18;21	7	13	9	69.2	56.3
	31;21;14	7	12	8	66.7	54.7
	31;24;19	7	12	7	58.3	49.8
	31;27;22	7	13	8	61.5	51.7
	31;31;04	7	12	6	50.0	45.0
	31;34;21	7	13	8	61.5	51.7
F1	37;43;16	7	20	8	40.0	39.2
	37;46;28	7	21	9	42.9	40.9
	37;50;04	7	19	9	47.4	43.5
	37;56;15	7	15	7	46.7	43.1
	37;58;13	7	19	8	42.1	40.5
	38;00;20	7	17	7	41.2	39.9
	38;03;00	7	15	7	46.7	43.1
	38;05;10	7	17	8	47.1	43.3
	38;09;04	7	23	9	39.1	38.7
	38;13;29	7	22	10	45.5	42.4
F2	36;53;16	7	23	9	39.1	38.7
	36;56;28	7	26	10	38.5	38.3
	37;00;02	7	27	10	37.0	37.5
	37;03;15	7	28	10	35.7	36.7
	37;06;29	7	30	12	40.0	39.2
	37;10;01	7	29	11	37.9	38.0
	37;13;14	7	26	9	34.6	36.0
	37;15;27	7	24	7	29.2	32.7
	37;19;00	7	22	6	27.3	31.5
	37;23;22	7	25	7	28.0	32.0

*Replicates shown are those that remained after data loss due to a hard drive failure. Letter denotes brood replicate. Number denotes treatment replicate.

†Frame identification does not necessarily correspond with the actual experiment time.

‡Arcsine transformation.

Appendix III (continued). Sample table of raw data collected during behavioral assays of *C. virginica* larvae in the serotonin treatment.

Replicate	Frame	Time Interval	Total # Larvae in Frame	# Larvae Searching in Frame	Percent Searching	Transformed Percent Searching
F3	27;49;09	7	14	5	35.7	36.7
	27;52;15	7	14	5	35.7	36.7
	27;55;27	7	16	7	43.8	41.4
	27;57;16	7	17	7	41.2	39.9
	27;59;00	7	14	5	35.7	36.7
	28;02;16	7	13	4	30.8	33.7
	28;05;28	7	15	4	26.7	31.1
	28;09;01	7	15	5	33.3	35.3
	28;12;17	7	15	5	33.3	35.3
	28;14;17	7	14	5	35.7	36.7
G1	34;33;14	7	16	4	25.0	30.0
	34;36;27	7	15	4	26.7	31.1
	34;40;00	7	16	4	25.0	30.0
	34;43;15	7	17	5	29.4	32.8
	34;46;28	7	20	4	20.0	26.6
	34;50;01	7	20	5	25.0	30.0
	34;53;16	7	20	5	25.0	30.0
	34;56;29	7	20	5	25.0	30.0
	35;00;02	7	21	5	23.8	29.2
	35;03;17	7	19	5	26.3	30.9
G2	32;45;08	7	19	3	15.8	23.4
	32;48;17	7	39	11	28.2	32.1
	32;51;29	7	33	11	33.3	35.3
	32;55;02	7	32	9	28.1	32.0
	32;58;16	7	32	9	28.1	32.0
	33;01;28	7	35	8	22.9	28.6
	33;04;01	7	35	8	22.9	28.6
	33;07;15	7	38	10	26.3	30.9
	33;10;27	7	35	9	25.7	30.5
	33;15;00	7	33	9	27.3	31.5
G3	29;02;17	7	18	6	33.3	35.3
	29;05;26	7	23	6	26.1	30.7
	29;09;00	7	23	5	21.7	27.8
	29;12;15	7	23	6	26.1	30.7
	29;15;27	7	25	6	24.0	29.3
	29;17;10	7	25	6	24.0	29.3
	29;19;01	7	23	6	26.1	30.7
	29;22;16	7	25	6	24.0	29.3
	29;25;28	7	25	6	24.0	29.3
	29;29;02	7	21	5	23.8	29.2

Appendix III (continued). Sample table of raw data collected during behavioral assays of *C. virginica* larvae in the serotonin treatment.

Replicate	Frame	Time Interval	Total # Larvae in Frame	# Larvae Searching in Frame	Percent Searching	Transformed Percent Searching
H1	34;37;00	7	32	9	28.1	32.0
	34;40;15	7	35	8	22.9	28.6
	34;43;27	7	34	9	26.5	31.0
	34;47;01	7	33	8	24.2	29.5
	34;50;16	7	34	8	23.5	29.0
	34;53;28	7	32	9	28.1	32.0
	34;57;02	7	31	6	19.4	26.1
	35;00;17	7	33	8	24.2	29.5
	35;03;29	7	33	8	24.2	29.5
	35;07;03	7	32	8	25.0	30.0
H2	33;10;00	7	27	5	18.5	25.5
	33;13;15	7	29	9	31.0	33.9
	33;15;27	7	25	7	28.0	32.0
	33;19;01	7	28	8	28.6	32.3
	33;22;16	7	27	8	29.6	33.0
	33;25;28	7	26	8	30.8	33.7
	33;29;02	7	27	11	40.7	39.7
	33;32;17	7	28	14	50.0	45.0
	33;35;29	7	28	15	53.6	47.1
	33;36;04	7	28	14	50.0	45.0
H3	33;29;15	7	11	4	36.4	37.1
	33;32;27	7	25	7	28.0	32.0
	33;36;01	7	24	6	25.0	30.0
	33;39;16	7	25	8	32.0	34.5
	33;42;28	7	28	8	28.6	32.3
	33;46;02	7	26	8	30.8	33.7
	33;49;17	7	27	8	29.6	33.0
	33;52;29	7	26	8	30.8	33.7
	33;56;03	7	25	8	32.0	34.5
	33;59;18	7	26	8	30.8	33.7

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