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How do compounds released by the green tide alga *Ulvaria obscura* affect development on invertebrate larvae?

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**HOW DO COMPOUNDS RELEASED BY THE GREEN TIDE ALGA ULVARIA
OBSCURA AFFECT DEVELOPMENT OF INVERTEBRATE LARVAE?**

By

Yolimar Rivera Vázquez

Accepted in Partial Completion
Of the Requirements for the Degree
Master of Science

Kathleen L. Kitto, Dean of the Graduate School

ADVISORY COMMITTEE

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MASTER'S THESIS

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Yolimar Rivera Vázquez
July 1 2014

**HOW DO COMPOUNDS RELEASED BY THE GREEN TIDE ALGA ULVARIA
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A Thesis

Presented to

The Faculty of

Western Washington University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

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Abstract

Green tides are vast accumulations of green macroalgae that, in the last decade, have become a common nuisance worldwide. Due to compounds the algae release, the blooms may negatively affect other organisms. *Ulvaria obscura*, a dominant contributor to green tides along the Pacific coast of the United States, produces, among other compounds, dopamine, a catecholamine and neurotransmitter known to affect settlement and metamorphosis of marine invertebrates. We tested the effects of *U. obscura* exudates and commercially purchased dopamine on fertilization, early development, and larval survival and morphology of the sand dollar *Dendraster excentricus* and Pacific oyster *Crassostrea gigas*. The exudate and dopamine treatments did not strongly affect fertilization success of *D. excentricus* or *C. gigas*, but they did affect early development and larval morphology of *D. excentricus* and *C. gigas*. We found significant differences in archenteron length of *D. excentricus* gastrulae and shell morphology of *C. gigas* veligers exposed to the exudates or dopamine. Morphology of *D. excentricus* plutei also varied significantly among the exudate treatments with larval arm lengths being affected. Our data indicate that compounds released by *U. obscura* can impact development and, presumably, survival of embryos and larvae, but that the effects differ between species. The impacts could affect development rates, larval dispersal, recruitment and population dynamics of invertebrate species.

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Introduction

Green tides are vast accumulations of green macroalgae that occur in dense mats in shallow coastal marine environments. Although green tide blooms are natural events, the magnitude and gravity of their occurrence has increased globally over the last three decades (Ye et al., 2011). Green tides, which appear to be related to eutrophication (Taylor, 1999; Ye et al., 2011), are common in the northern temperate zones. Their greatest effects have been seen in North American, European and Asia-Pacific areas, but they have also occurred in South America, Africa and Australia (Ye et al., 2011).

Green tides can have negative effects on tourism and local economies. When algal blooms wash on shore, they physically spoil beaches, interfering with recreational activities and producing noxious odors (Taylor, 1999). Green tides can also have important ecological effects, impacting other organisms in the environments in which they occur (Raffaelli et al., 1998). For example, adult barnacles (*Balanus balanoides*) showed high mortality in simulated tidepools where *Ulva lactuca* was present, suggesting that the algae produce some substance that concentrated in the simulated tidepools, killing the barnacles (Magre, 1974). Zoea stages of five species of estuarine crabs experienced 100% mortality 22 days after exposures to *U. lactuca* exudates (Johnson and Welsh, 1985). Fresh and decomposing cultures of *Ulva prolifera* killed juvenile abalone *Haliotis discus hannai* (Wang et al., 2011).

Many green ulvoid macroalgae (Phylum Chlorophyta, Order Ulvales) produce toxic or deterrent chemicals that protect the algae from grazers (Van Alstyne et al., 2001; Van Alstyne et al., 2006). Those chemicals include dimethylsulfoniopropionate (DMSP) and dopamine. These are both present in the tissues of *Ulvaria obscura*, a dominant contributor to green tides on the northwestern Pacific coast of North America (Nelson et al., 2003a). Van Alstyne et al. (2006) found that urchins avoided feeding on *U. obscura* due to the high concentrations of dopamine in the algal tissues (approximately 5% of the *Ulvaria* dry mass). Dopamine and its precursor, L-3,4-dihydroxyphenylalanine (L-DOPA), however, may have other effects. For example, both chemicals are known to influence development, settlement and metamorphosis of some marine invertebrate larvae (Burke, 1983; Bonar et al., 1990; Pires et al., 1997; Dobretsov and Qian, 2003; Adams et al., 2011).

The breakdown of dopamine produces other potentially damaging compounds. Nelson et al. (2003b) observed that exudates from *U. obscura* change water from a tawny-orange to a reddish-brown color and finally a dark brown or black color after the algae are exposed to desiccation. Tocher and Craigie (1966) reported that the darkening of the thallus of *U. obscura* indicated dopamine oxidase activity and Van Alstyne et al. (2011) saw that the oxidation of dopamine released from *U. obscura* changed water color consistent with the observations of Nelson et al. (2003b). Once in solution, dopamine is oxidized into reactive oxygen species (ROS) and quinones that produce the changes in water color; complete oxidation leads to insoluble dark brown or black melanins (Palmer,

1963; Stokes et al., 1999). Presumably, *U. obscura* release dopamine to interfere with competitors for space, nutrients and light (Nelson et al., 2003b).

Van Alstyne et al. (2014) studied the effects of dopamine on crabs and found that the survival of zoea stages of *Metacarcinus magister* was affected at concentrations above 168 μM dopamine and that the time to first molt of juveniles of *Cancer oregonensis* increases about 1.5 days when exposed to 738 μM dopamine. Adams et al. (2011) showed that 20 and 50 μM dopamine prevented the elongation of post-oral arms in 4-arm plutei of the sea urchin *Strongylocentrotus purpuratus*. These results suggest that a broad range of marine invertebrates might be affected by *U. obscura* exudates, and that the effects may be related to dopamine and its oxidation products.

If a green tide contains a large amount of *U. obscura*, the surrounding water could contain high concentrations of dopamine (mean concentrations of 3–563 μM according to laboratory experiments by Van Alstyne et al., 2011) and its various oxidation products like ROS and quinones, as well as any other chemicals released by the alga. It is unknown what impact such a “cloud” of chemicals traveling with the seawater mass could have on organisms, including eggs, embryos and larvae in the impacted area, but macroalgae produce allelochemicals (Hay and Fenical, 1988) that clearly affect growth and photosynthesis of phytoplankton and other macroalgae (Wium-Andersen et al., 1982; Gao et al., 2014).

The biological and ecological effects of such an accumulation have not yet been studied in detail, but could be significant. Invertebrate larvae provide new individuals to

adult populations, allow colonization of new areas through dispersal and promote genetic variability. Adult invertebrates might be affected by green tide chemicals (Magre, 1974; Fletcher, 1996), but early larval stages tend to be particularly sensitive to chemicals (Mohammed, 2013) and thus, the green tides might have particularly important impacts on larvae. Larvae can respond strongly to chemical stimuli and some chemicals serve as cues that naturally induce settlement and metamorphosis (Pawlik, 1992; Hadfield and Paul, 2001). Others can inhibit fertilization, produce developmental abnormalities, reduce settlement, interfere with metamorphosis, or kill the larvae outright. Hydrocarbons, metals and pesticides, can affect sperm motility, fertilization success, embryonic development, and larval survival (Johnson, 1988; Dinnel et al., 1989).

Gleason and Hofmann (2011) suggest that macroalgae have the potential to negatively affect larvae by releasing chemicals that induce larval mortality, delay larval development or prevent settlement. The brown crustose alga *Ralfsia verrucosa*, for example, produces tannins that affect the survival of nereid polychaetes and bivalve trochophores and veligers (Conover and Sieburth, 1966). Nelson et al. (2003b) and Nelson and Gregg (2013) showed that waterborne chemicals released from *U. obscura* and *Ulva fenestrata* cause larval mortality and abnormal development of *Crassostrea gigas*. Brock et al. (2007) showed that settlement of the barnacle *Balanus improvisus* is inhibited by seawater conditioned with desiccated *Fucus vesiculosus* and suggested that the inhibition was due to the release of phlorotannins from the algae. Therefore, it seems

likely that exudates from green tide algal blooms have allelopathic effects on the development of invertebrate larvae.

The duration of the planktonic period of invertebrate larvae is highly variable among species. Although many larvae can naturally extend their larval lives prior to metamorphosis (Pechenik, 1990), external forces in the environment could delay metamorphic competence and settlement. Independent of any direct effects of allelochemicals on larval survival, indirect effects through, for example, delayed development, could also have major implications for the larvae. A delay can also decrease post-metamorphic fitness of an individual, such as reducing the juvenile growth rate (Pechenik, 1985).

The aim of our study was to test the effects of *U. obscura* exudates on fertilization, larval development and larval survival of two kinds of marine invertebrate larvae. Because dopamine is a known allelochemical of *U. obscura*, we also tested its effects. To determine which life stages are most vulnerable to allelochemical impacts, we tested multiple developmental stages to address the following research questions: 1) Are eggs affected by *U. obscura* exudates, thus decreasing fertilization success? 2) If fertilized eggs are exposed to *U. obscura* exudates, do they then develop normally? 3) Do larvae survive after exposure to *U. obscura* exudates and if so, does their morphology change in any way? The sand dollar *Dendraster excentricus* (Phylum Echinodermata, Class Echinoidea) and the Pacific oyster *C. gigas* (Phylum Mollusca, Class Bivalvia) were used to answer these questions.

D. excentricus live in sand and mud flats in the low intertidal and shallow subtidal zones (Merill and Hobson, 1970). Adult sand dollars release eggs or sperm into the water column where fertilization and development occur. Their planktonic larvae spend several weeks in the plankton (Strathmann, 1987). In the Salish Sea, *D. excentricus* spawn from late March to late summer (Strathmann, 1987). *C. gigas* usually live in hard bottom habitats but can also be found in subtidal sand and mud flats. The adults reproduce by spawning and embryos develop into a planktonic veliger that spends 10 days to 1 month in the plankton (Strathmann, 1987). In the Strait of Georgia and the San Juan Archipelago, *C. gigas* spawns from late June to September (Strathmann, 1987).

Echinoderm larvae are used extensively as model organisms in embryology and toxicological testing (e.g., Dinnel et al., 1988; Chapman et al., 1995) and are thus a logical choice for this testing. Oysters, like *C. gigas*, are important aquaculture species and also provide an opportunity to test impacts on a very different kind of larva. Moreover, these species are ecologically relevant since *U. obscura* blooms occur in the habitats where these organisms reside.

Materials and Methods

Algal and chemical collection

Ulvaria obscura was collected from Ship Harbor, Anacortes, Washington, USA (48°30.53'N, 122°41.12'W) on 27 June and 1 August 2013 respectively for sand dollar (*D. excentricus*) and oyster (*C. gigas*) experiments. Because *U. obscura* occurs in the low intertidal and subtidal zones, the algae were collected by trawling from depths of 2–4 m, and immediately transported in buckets filled with seawater to the Shannon Point Marine Center (SPMC) in Anacortes, Washington, USA. The *U. obscura*, which has blades that are 1-cell thick, were distinguished from the similar looking alga *U. lactuca*, which are 2-cells thick, by examining thin sections under a compound microscope. The *U. obscura* were held overnight in flow-through seawater tables and used for experiments the following morning.

Chemicals in the blades of *U. obscura* were collected following the methods of Van Alstyne et al. (2011). The algae were gently blotted dry and 6 g portions were weighed and placed in each of 27, 8 cm diameter glass bowls. For the *D. excentricus* experiments, the bowls with algae were placed outdoors for 1 h to simulate a low tide and allow to desiccate. Overcast skies and rainy weather prevented us from desiccating the algae the same way for the *C. gigas* experiments. Those algae were instead placed in a 26.7 °C incubator with two full-spectrum light bulbs ($\approx 1.06 \pm 0.27$ quanta \cdot sec $^{-1}\cdot$ cm $^{-2}$) for 2 h.

Following the simulation of a low tide exposure, the bowls with the algae were filled to the top with ~140 mL of 0.5 μm filtered seawater (FSW) at 10.7 °C (*D. excentricus* experiments) or 12.7 °C (*C. gigas* experiments) to simulate an incoming tide. After 5 min, the algae were removed from the bowls and the water from all bowls was combined to create a 100% *U. obscura* exudate stock solution. We created 10% and 1% exudate treatments by diluting the 100% *U. obscura* stock solution with 0.5 μm FSW at 10.7 °C (*D. excentricus* experiments) or 12.7 °C (*C. gigas* experiments). This produced three *U. obscura* treatments at concentrations of 1%, 10% and 100%. For comparison to the exudate treatments, commercially purchased dopamine hydrochloride was used to create additional treatments of 10 μM and 100 μM dopamine in FSW.

Animal collection, spawning and larval rearing

Adult *D. excentricus* were collected during a -0.2 m MLLW tide on 25 April 2013 near Semiahmoo Spit in Blaine, Washington, USA (48°58.87'N, 122°47.36'W). The sand dollars were transported to SPMC where they were placed in a flow-through seawater table. Sand dollar spawning was induced by injecting 1–2 mL of 0.5 M KCl into their coelomic cavity (Strathmann, 1987) and eggs were collected in beakers filled with 5 μm FSW at 10.7 °C while sperm was collected without dilution in chilled petri dishes. An unfertilized egg stock was created by pooling unfertilized eggs from three females and a sperm stock was created by combining a single drop of undiluted sperm from each of three males in 50 mL of 5 μm FWS at 10.7 °C. The eggs were then fertilized by adding diluted sperm. After fertilization occurred, the culture was rinsed and placed in a 12 °C

incubator where the embryos developed into larvae that were later used in the larval experiment. A second spawning was conducted a week later and new stocks of unfertilized eggs and sperm were made following the methods described above. A portion of the new stock of unfertilized eggs was set aside to be used immediately for fertilization experiments. The other egg portion was fertilized with diluted sperm and after fertilization occurred, the culture was rinsed and used immediately for development experiments. As a result of these processes, on the day of the actual experiment we had unfertilized eggs, newly fertilized embryos and swimming pluteus larvae of *D. excentricus*.

Ripe *C. gigas* adults were collected by employees of Taylor Shellfish Inc. on 23 July 2013 from Dabob Bay, Washington, USA, and transported to flow-through seawater tables at SPMC. The oysters were dissected and an unfertilized egg stock was created by carefully macerating the gonads of four females through a piece of screen mesh into a beaker of 5 μ m FSW at 12.7 °C. This unfertilized egg stock was filtered twice to remove extra gonad tissue. A sperm stock was created by macerating gonads from two males in a petri dish, then mixing the undiluted sperm in 50 mL of 5 μ m FWS at 12.7 °C. As with *D. excentricus*, the *C. gigas* eggs were fertilized with diluted sperm and, after fertilization occurred, the culture was rinsed and placed in an incubator at 22 °C for later use in the larval experiment. A second oyster dissection was conducted a week later and new stocks of unfertilized eggs and sperm were made as described above. A portion of the new unfertilized egg stock was placed in another container for immediate use in fertilization

experiments. The other portion of the egg stock was fertilized with diluted sperm, rinsed and used immediately in development experiments. The result was that, on the day of the experiment we had freshly collected unfertilized eggs, newly fertilized embryos and swimming veliger larvae of *C. gigas*.

Fertilization experiment

To determine the effects of *U. obscura* exudates and dopamine solutions on fertilization of *D. excentricus* and *C. gigas*, unfertilized eggs were exposed to the freshly mixed solutions of 0% (FSW control), 1%, 10% or 100% *U. obscura* exudate or 10 μ M or 100 μ M dopamine. Our dopamine treatments were based on dopamine concentrations that are likely to be released from *U. obscura* (Van Alstyne et al., 2011). To start the experiment, 3–5 mL of unfertilized eggs from the *D. excentricus* or *C. gigas* unfertilized egg stocks were placed in each of 30, 250 mL plastic cups. Five replicate cups with the eggs were filled to the top with one of the *U. obscura* exudates (1%, 10% or 100%), the dopamine solutions (10 μ M or 100 μ M) or with the 5 μ m FSW control at 10.7 °C to yield a final concentration of 14–24 eggs \cdot mL⁻¹. The eggs were left for 15 or 25 min for *D. excentricus* or *C. gigas* respectively, then were filtered and placed in clean 250 mL plastic cups filled with 5 μ m FSW at 10.7 °C (*D. excentricus* experiments) or 12.7 °C (*C. gigas* experiments). Since sperm is diluted quickly once in contact with seawater, the window for an egg to be fertilized is relatively short, so the exposure to the treatments was intentionally kept short in order to simulate what might happen to eggs that encounter a “cloud” of exudates from *U. obscura*.

After the eggs were transferred to FSW, a drop of *D. excentricus* or *C. gigas* sperm was diluted in 50 mL of 5 μ m FSW and 1 mL of this solution was added to each cup. After 15 or 100 min, for *D. excentricus* or *C. gigas* respectively, the solutions were filtered, the eggs were placed in labeled 20 mL scintillation vials, and the samples were fixed with 10% buffered formalin. The longer period before fixation for the *C. gigas* samples was necessary to ensure that fertilized embryos could be identified since their fertilization envelope is harder to distinguish. The preserved samples were later examined under a compound microscope. At least 100 eggs were counted and categorized as fertilized or unfertilized. Fertilization was confirmed by the presence of a fertilization envelope in *D. excentricus* and by cell cleavage or a polar body in *C. gigas*.

Development experiment

To determine the effects of *U. obscura* exudates and dopamine solutions on early embryonic development, newly fertilized embryos were exposed to the exudate, dopamine or FSW treatments, then left to develop for two days, after which development success and morphology were measured. To accomplish this, samples of the fertilized egg stocks of *D. excentricus* or *C. gigas* with >90% fertilization success (determined by microscopic examination), were transferred to 30, 250 mL plastic cups. Five replicate plastic cups were then filled to the top with one of the *U. obscura* exudates, the dopamine solutions or the 5 μ m FSW control to yield a final concentration of 14–24 embryos·mL⁻¹. In an attempt to simulate what might happen in the field as a group of planktonic larvae encounter a bloom-impacted area, the exposure was for only 1 hr.

After 1 hr in the treatments, the embryos were filtered and transferred to clean 250 mL plastic cups filled with 5 μ m FSW. *D. excentricus* embryos develop to the gastrula stage 32–35 hr post fertilization (hpf) (Strathmann, 1987). Gastrulae can be identified by the presence of the archenteron (primitive gut), which extends from the vegetal pole towards the animal pole. *C. gigas* embryos develop to the straight-hinged veliger stage 48 hpf (Strathmann, 1987). Straight-hinged veligers can be identified by the morphology of their D-shaped shells.

Two days after the exposure to the treatments, the embryos (gastrulae for *D. excentricus* and straight-hinge veligers for *C. gigas*) were placed in labeled 20 mL scintillation vials and fixed with 10% buffered formalin. The embryos were later examined under a compound microscope to determine the percent that had developed normally to the gastrula or straight-hinged veliger stage, counting at least 100 larvae in each replicate. Ten haphazardly selected gastrulae or veliger larvae per replicate were then photographed under 10X or 40X magnification, respectively. Photos were analyzed with ImageJ software and length of the archenteron (*D. excentricus*) or shell length and width (*C. gigas*) were measured as an index of embryonic development (Figure 1).

Larval experiment

To determine the effects of *U. obscura* exudates and dopamine solutions on growth and development of individuals exposed as larvae, early 6–arm pluteus or straight-hinged veliger larvae of *D. excentricus* or *C. gigas* respectively were exposed to the treatments, then left to develop for 1 week, after which larval morphology was

measured. To accomplish this, early 6–arm pluteus and straight-hinged veliger larvae raised from fertilized egg stocks held in 12 °C (*D. excentricus*) or 22 °C (*C. gigas*) incubators were transferred to 30, 250 mL plastic cups. Five replicate plastic cups were then filled to the top with one of the *U. obscura* exudates, dopamine solutions or 5 µm FSW treatments to give a final density of 14–24 larvae·mL⁻¹. After soaking in the treatments for 1.5 or 2.0 hr for *C. gigas* and *D. excentricus*, respectively, the larvae were transferred to clean 250 mL plastic cups filled with 5 µm FSW.

After the larvae were change to FSW, the *D. excentricus* larvae were placed in a 12 °C incubator and were fed daily with a 1:1 mixture of the algae *Dunalliella tertiolecta* and *Rhodomonas lens* at a concentration of ~24,000 cells·mL⁻¹. *C. gigas* larvae were kept in a 22 °C incubator and were fed daily a 1:1 mixture of the algae *Isochrysis galbana* and *Thalassiosira pseudonana* at a concentration of ~24,000 cells·mL⁻¹. A higher temperature was used for *C. gigas* as recommended by Strathmann (1987). The water in all cultures was changed every other day and, after 1 week, the larvae were placed in labeled 20 mL scintillation vials and fixed with 10% buffered formalin. Ten haphazardly selected pluteus or veliger larvae per replicate were later photographed under 10X magnification and measured with ImageJ software. For *D. excentricus* larvae, measurements of arm length and body size were made following the procedures of Milonas et al. (2010) and measurements of shell length and width for *C. gigas* larvae were determined as shown in Figure 1.

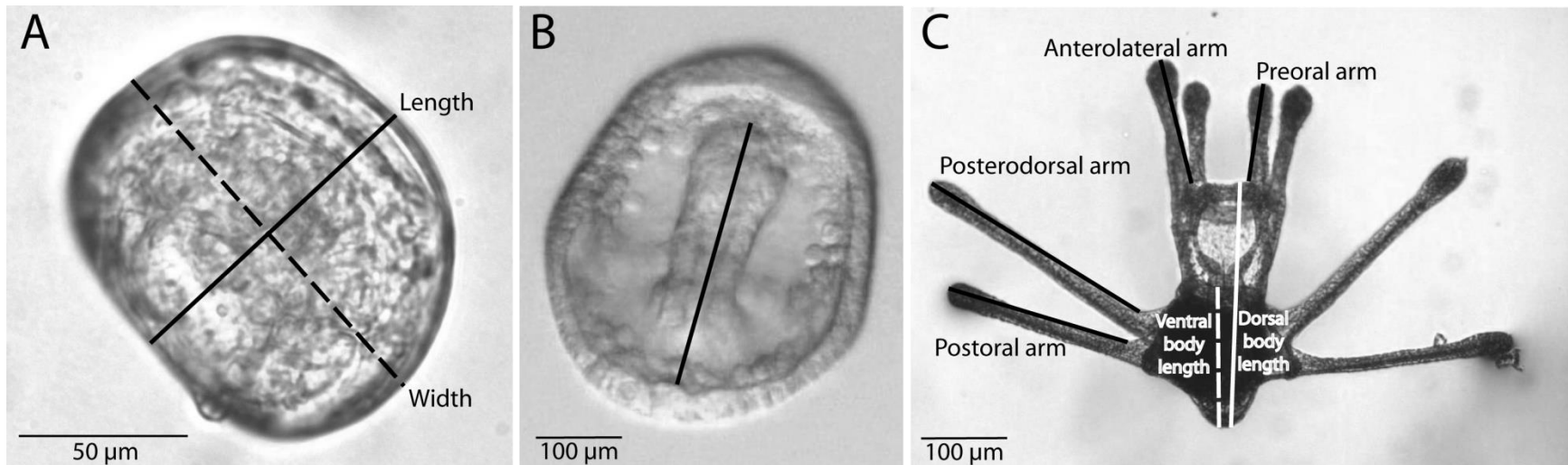


Figure 1. Larval measurements. **A)** Length and width of *C. gigas* straight-hinge veliger larva. **B)** Archenteron length of *D. excentricus* gastrula. **C)** Arm and body lengths of *D. excentricus* pluteus larva.

Statistical analyses

Percent fertilization and percent development to the gastrula (*D. excentricus*) or straight-hinged veliger (*C. gigas*) stage were all analyzed with 1-way ANOVA with the experimental treatments as the fixed factors. Archenteron length of *D. excentricus* and shell measurements of *C. gigas* larvae were analyzed with a 2-way ANOVA with the experimental treatments as the fixed factors and beaker nested in the treatments. Because we found no significant beaker-to-beaker variability at $\alpha = 0.25$ for archenteron length of *D. excentricus* and shell measurements of *C. gigas*, we pooled the beakers within each treatment and reanalyzed those data with a 1-ANOVA with experimental treatments as the fixed factor. Where significance was found, a Dunnett's test was performed to determine which treatments differed from the FSW control. A Levene's test was used to verify variance homogeneity prior to all analyses and statistical significance was evaluated at $\alpha = 0.05$. Data for percent development of *D. excentricus* gastrulae and *C. gigas* veligers, as well as archenteron length of *D. excentricus*, violated the assumption of equal variance and transforming the data did not resolve the problem so α was adjusted to 0.025 as recommended by Keppel and Wickens (2004).

Because the various larval measurements of *D. excentricus* plutei were correlated, the data dimensions were reduced using principal component analysis (PCA) following the procedures of Chan et al. (2011). PCA was performed on the correlation matrix of the larval dimensions with varimax rotation using Kaiser normalization to identify key components (Harris, 1975). The number of components retained was based on the eigenvalues and the shape of the scree plot. We performed nested ANOVAs for weighted

scores from on the two retained principal components with experimental treatment as the fixed factor and beaker nested in treatments. Where significance was found, a Dunnett's test was performed to determine which treatments were different from the FSW control. All analyses were performed with SPSS 20.

Results

Dendraster excentricus

Fertilization success of *D. excentricus* eggs exposed to the treatments was high (92.9– 98.8%) in all treatments, but significant treatment differences were found ($F_{5,24} = 2.88$, $P = 0.04$). Dunnett's test showed that only the 1% *U. obscura* exudate treatment was significantly different from the control (Figure 2). The *U. obscura* exudates and dopamine had no significant effect on percent development to the gastrula stage when newly fertilized eggs were exposed to the treatments ($F_{5,24} = 1.57$, $P = 0.21$). Development success was near 100% in all treatments (Figure 3A). Because we found no significant beaker-to-beaker variability in archenteron length ($F_{24,270} = 1.07$, $P = 0.38$), we pooled the beaker data and found a significant difference between the treatments ($F_{5,294} = 36.66$, $P < 0.001$; Figure 3B). Dunnett's test for the measurements showed that archenteron lengths were significantly smaller in the 1% and 100% *U. obscura* exudates and both dopamine solutions.

For *D. excentricus plutei*, PCA axis 1 included all the larval arm lengths with anterolateral and posterodorsal arms having the highest component scores, while PCA axis 2 included both body length dimensions (Table 1). A 2-way ANOVA for PC1 showed significant beaker-to-beaker variability ($F_{24,270} = 1.76$, $P = 0.02$), so no pooling was done. Despite this variability, there was still a significant difference between the treatments ($F_{5,24} = 9.04$, $P < 0.001$). Dunnett's test on PC1 showed that the 100% *U. obscura* exudate and both dopamine solutions were significantly different from the control (Figure 4A). For PC2, we again found beaker-to-beaker variability ($F_{24,270} = 1.86$,

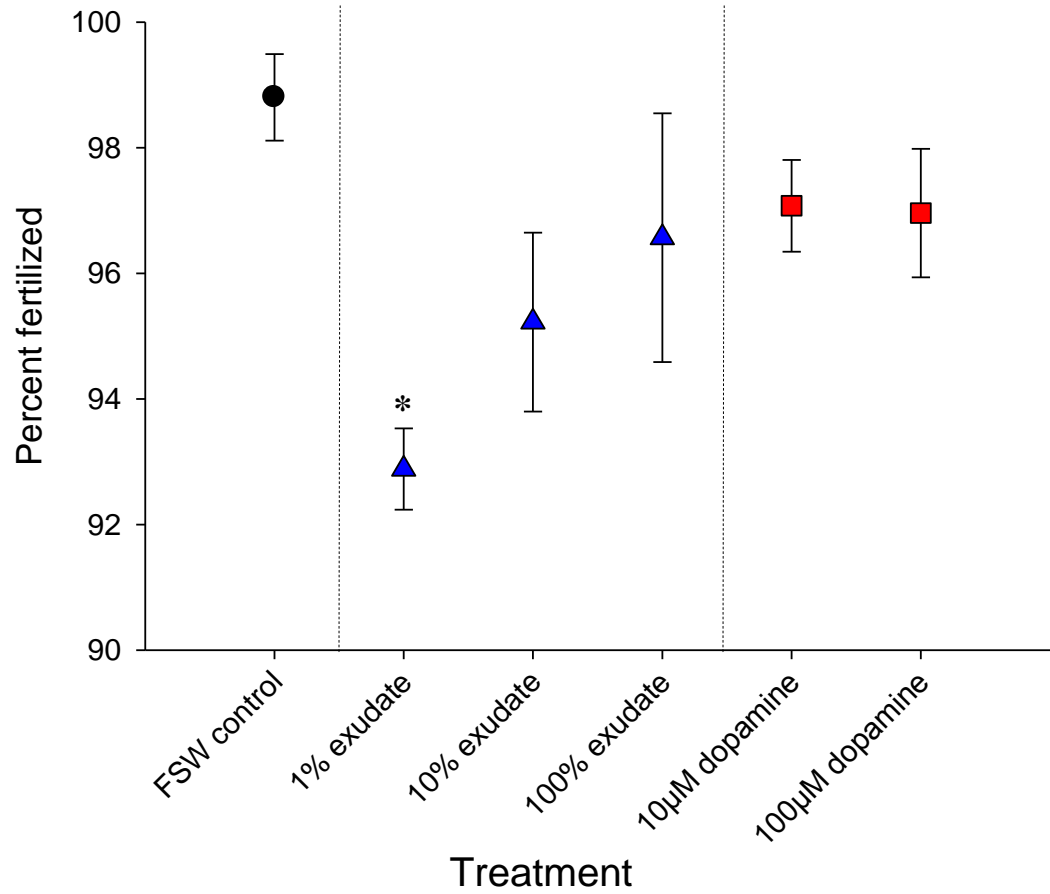


Figure 2. *D. excentricus* fertilization success in *U. obscura* exudates and dopamine solutions (n = 5). FSW = filtered seawater. Means and standard errors are shown. Asterisk indicates significant difference from the control (Dunnett's test).

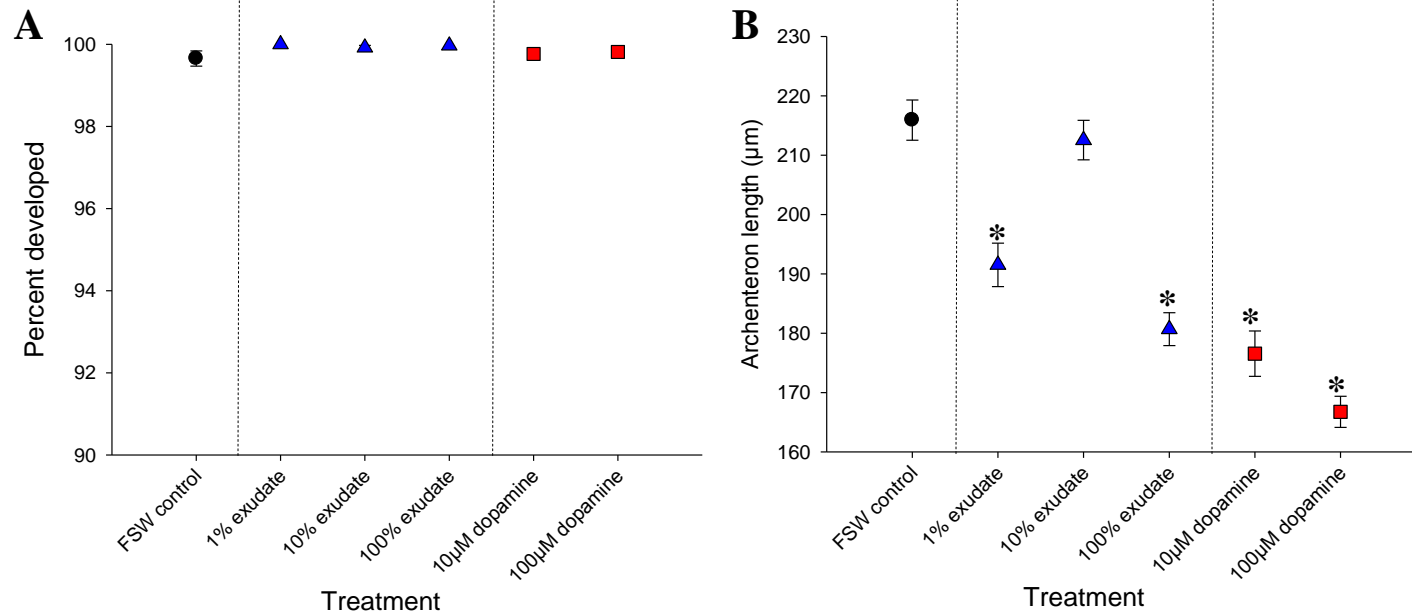


Figure 3. **A.** *D. excentricus* percent of embryos developed to the gastrula stage in *U. obscura* exudates and dopamine solutions (n = 5). **B.** *D. excentricus* archenteron length in *U. obscura* exudates and dopamine solutions (n = 5). FSW= filtered seawater. Mean and standard errors are shown. Asterisk indicates significant difference from the control (Dunnett's test).

Table 1. Principal component analysis (PCA) summary for *D. excentricus* pluteus exposed to *U. obscura* and dopamine solutions.

	PC1	PC2
Component scores	(0.86) Anterolateral arm	(0.87) Ventral body length
(loading factors)	(0.65) Posterodorsal arm	(0.80) Dorsal body length
	(0.56) Postoral arm	
	(0.50) Preoral arm	
Eigenvalue	2.26	1.16
% of variance	37.6	19.4

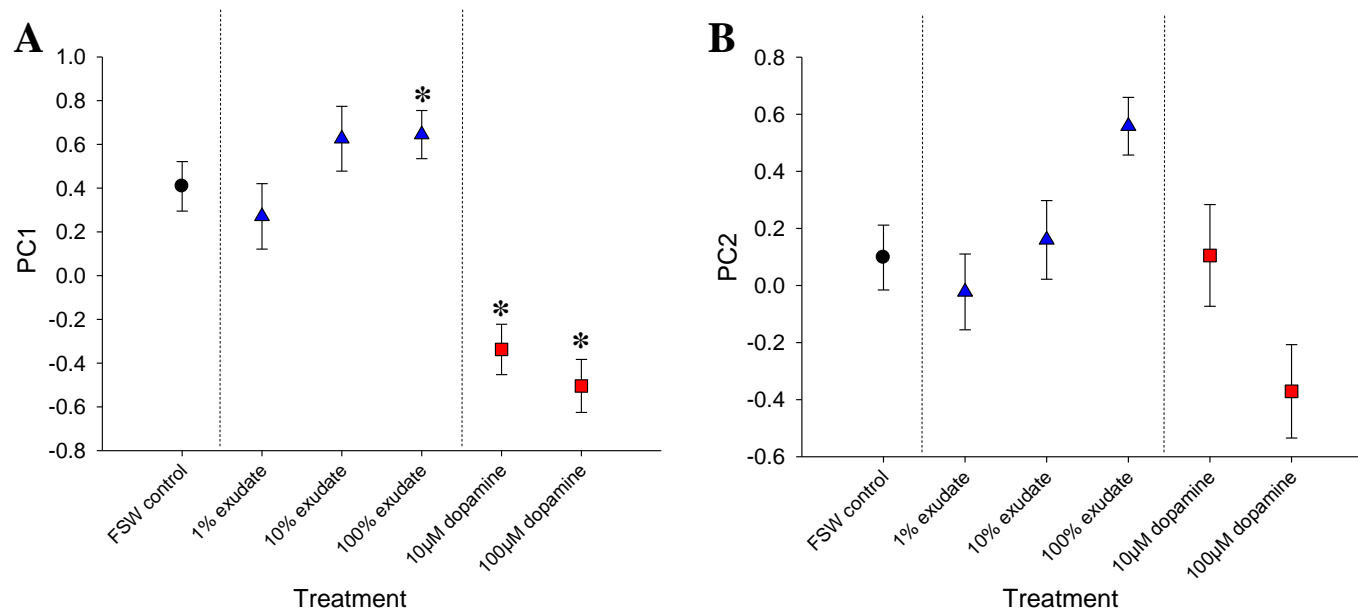


Figure 4. **A.** Principal component one (PC1) scores for *D. excentricus* larval arm lengths in *U. obscura* exudates and dopamine solutions (n = 5). **B.** Principal component two (PC2) scores for *D. excentricus* larval body lengths in *U. obscura* exudates and dopamine solutions (n = 5). FSW = filtered seawater. Means and standard errors are shown. Asterisk indicates significant difference from the control (Dunnett's test)

$P = 0.01$), but no significant difference between the treatments ($F_{5,24} = 1.08$, $P = 0.40$), indicating no significant difference in body lengths of plutei in the various treatments (Figure 4B).

Crassostrea gigas

Success of *C. gigas* fertilization after eggs were exposed to the treatments was high (92.5–96.0%) and no significant differences were found between the treatments ($F_{5,24} = 0.87$, $P = 0.52$; Figure 5). There was an effect of the treatments on percent of *C. gigas* embryos that reached the straight-hinged veliger stage ($F_{5,24} = 3.46$, $P = 0.02$; Figure 6A), but a Dunnett's test failed to detect significant differences between the controls and any of the treatments. The general trend, however, was for greater development success in the 100% exudate and 10 μ M dopamine treatments. Significant beaker-to-beaker variability was found in the shell length ($F_{24,270} = 2.30$, $P = 0.001$) and width ($F_{24,270} = 1.74$, $P = 0.02$) of the embryos that reached the straight-hinged veliger stage. There were also significant differences in shell length ($F_{5,24} = 11.58$, $P < 0.001$) and width ($F_{5,24} = 7.52$, $P < 0.001$) between the treatments (Figure 6B and 6C). Dunnett's test for the shell length measurements showed that the 10% and 100% *U. obscura* exudates and both dopamine solutions were significantly different from the control. The Dunnett's test for the shell width measurements showed that the 100% *U. obscura* exudates and the both dopamine solutions were significantly different from the control.

For larvae exposed to the treatments at the straight-hinged veliger stage, there was no significant beaker-to-beaker variability in either shell length ($F_{24,270} = 1.51$, $P = 0.06$)

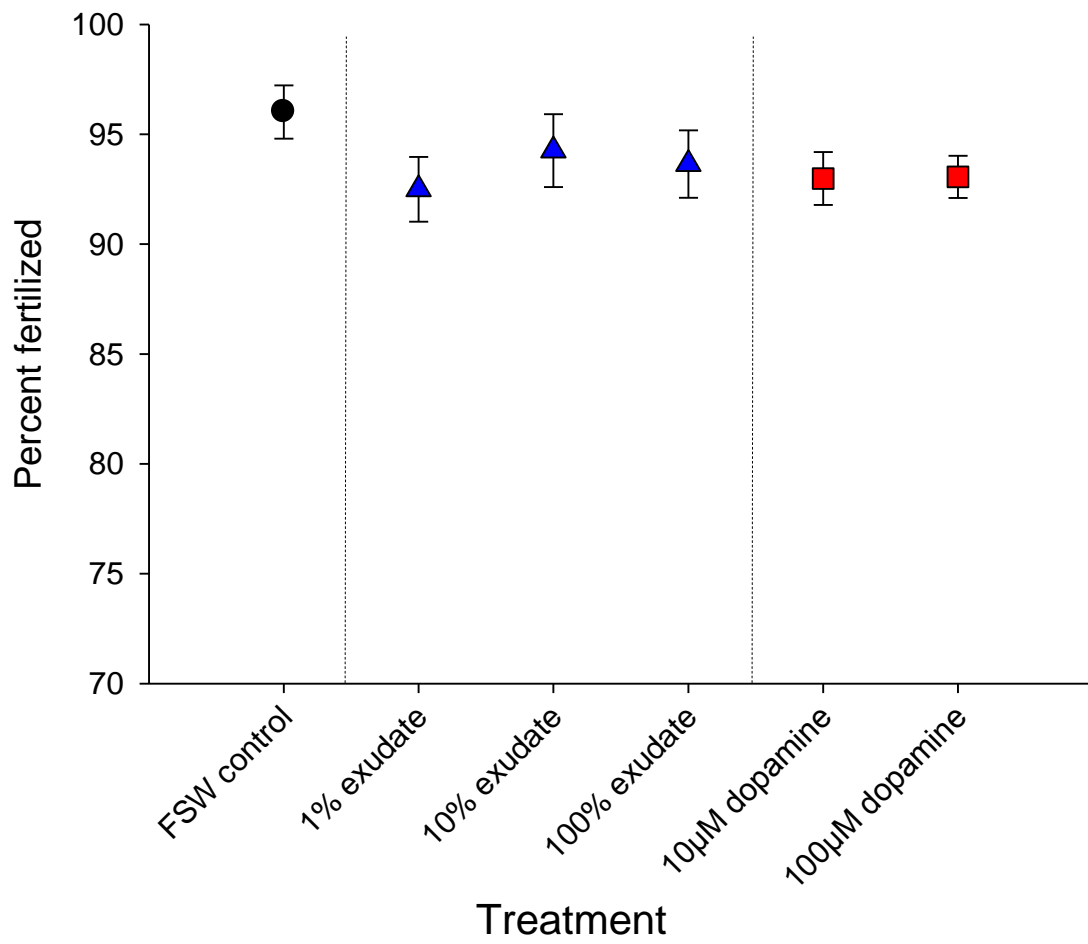


Figure 5. *C. gigas* fertilization success in *U. obscura* exudates and dopamine solutions (n = 5). FSW = filtered seawater. Mean and standard errors are shown.

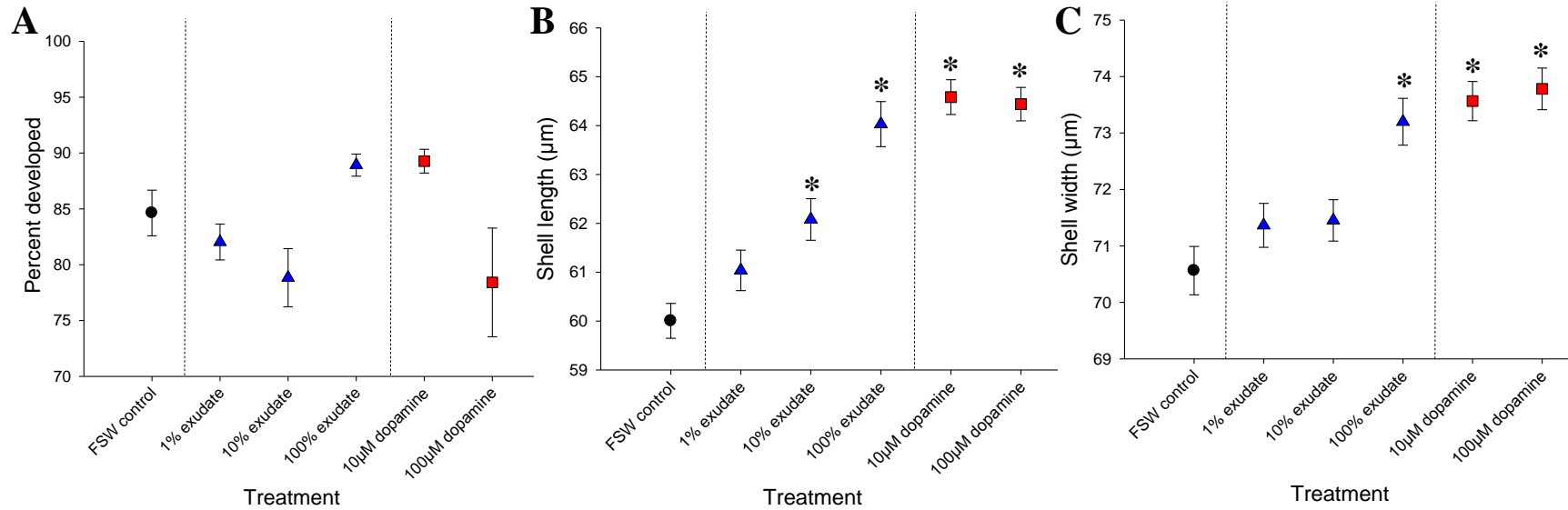


Figure 6. **A.** *C. gigas* percent of embryos developed to the straight-hinged veliger stage in *U. obscura* exudates and dopamine solutions (n = 5). **B** and **C.** *C. gigas* shell length and width of embryos developed to the straight-hinged veliger stage in *U. obscura* exudates and dopamine solutions from the development experiment (n = 5). FSW = filtered seawater. Mean and standard errors are shown. Asterisk indicates significant difference from the control (Dunnett's test).

or width ($F_{24,270} = 1.48$, $P = 0.07$), so beakers were pooled. The subsequent analysis showed significant differences in both shell length ($F_{5,294} = 10.69$, $P < 0.001$) and width ($F_{5,294} = 7.57$, $P < 0.001$) between the treatments (Figure 7). Dunnett's test showed that shell lengths in all *U. obscura* exudates and dopamine solutions were significantly different from the control. The Dunnett's test for the shell width measurements showed that all *U. obscura* exudates and the 100 μM dopamine solution were significantly different from the control.

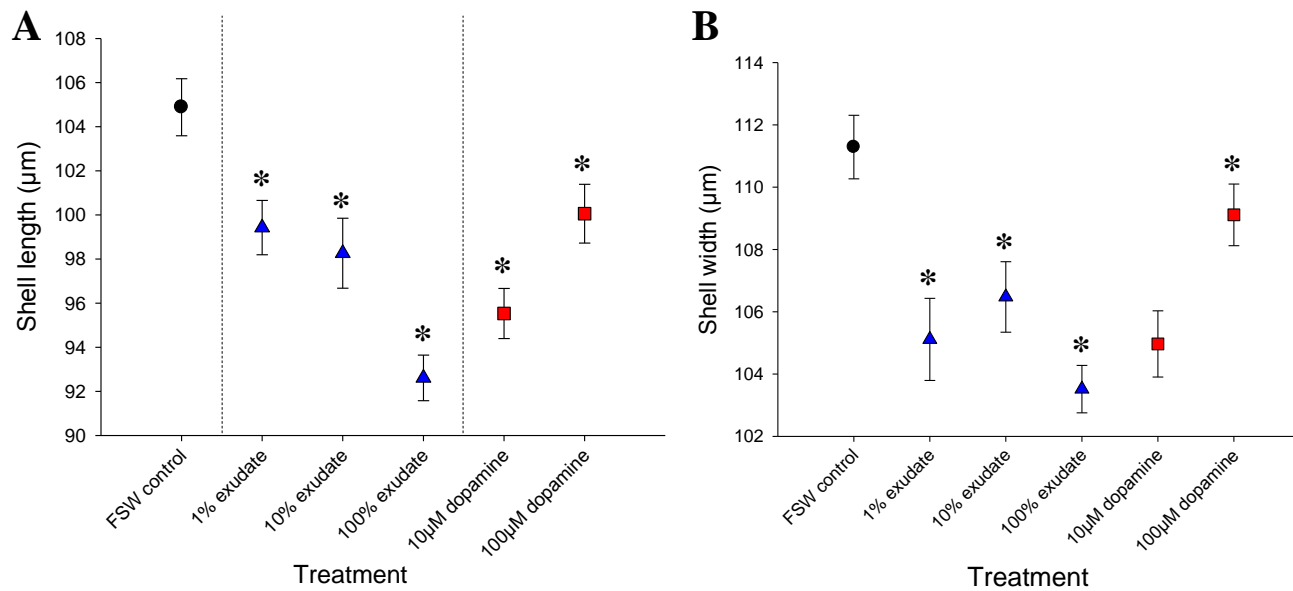


Figure 7. *C. gigas* shell length (A) and width (B) of veligers in *U. obscura* exudates and dopamine solutions from the larval experiment (n = 5). FSW = filtered seawater. Mean and standard errors are shown. Asterisk indicates significant difference from the control (Dunnett's test).

Discussion

Our results demonstrate that the exudates from *U. obscura* affect the development of *D. excentricus* and *C. gigas*, but that the effects varied with life history stage. *U. obscura* exudates and dopamine did not strongly affect fertilization success of either species, but did induce changes in larval morphology. This suggests that different larval stages have different sensitivities to *U. obscura* exudates and dopamine. The morphological changes, however, varied between treatment concentrations and the larval stages of *D. excentricus* and *C. gigas*.

U. obscura exudates and dopamine treatments had opposite effects on the early stages of *D. excentricus* and *C. gigas*. Embryos of *D. excentricus* exposed to *U. obscura* exudates and dopamine continued to develop normally to the gastrula stage, but had shorter archenterons. Although archenteron length was reduced overall (except in the 10% *U. obscura* treatment), the archenteron length was 0.5-1.5X shorter in the dopamine treatments than in the highest exudate concentration. The reduced size of the archenteron probably resulted from slower embryonic development in the exudate and dopamine treatments. Though these larvae appeared normal, slower development can have critical implications for larvae. Individuals that spend more time in the water column probably suffer higher mortality (Thorson, 1950; Strathmann, 1977). Pechenik (2006) reviewed several studies where the larval development of marine invertebrates was delayed and reported that even short delays can significantly reduce fitness. This is particularly obvious in reduced rates of growth or development and reduced survival of post-metamorphic juveniles and even in adults. In any case, our results suggest that *D.*

excentricus embryos that encounter *U. obscura* exudates could suffer slowed development and potentially lower survival.

In contrast, embryos of *C. gigas* exposed to the *U. obscura* exudates and dopamine continued to develop normally, successfully reached the straight-hinged veliger stage, and actually produced larvae that were 2–5X larger than the FSW control larvae. The dopamine treatments produced even larger larvae than the exudates. This increase in larval size could indicate faster development, a reduced planktonic period, reduced mortality and larger post-settlement juveniles, an event that has clear benefits (Stathmann, 1977). However, this does not consider the possibility of reduced tissue biomass or thinner larval shells.

Nelson et al. (2003b) exposed early embryos of *C. gigas* to *U. obscura* exudates at concentrations of 26–100% and saw no normal development. The larvae failed to even reach the straight-hinge veliger stage. We did not specifically monitor survival of *C. gigas* larvae through complete development, but we did end up with hundreds of viable larvae in our samples 2 days after their exposure to any of the exudates and saw no evidence of mortality. The difference in our outcomes likely results from differences in the duration of exudate exposure. Simply testing the effects of the chemicals themselves, Nelson et al. (2003b) exposed embryos to exudates for 24 hr whereas our exposure was for only 1 hr. Additionally, the time elapsed from collection of exudates to exposure (approximately 1.5–2 hr), likely affected the chemistry of the exudates. Therefore, the chemicals that affected our embryos and larvae were different in concentration and

presumably composition from those experienced by larvae in the Nelson et al. (2003b) study.

Nelson and Gregg (2013) similarly reported that early embryos of *C. gigas* develop abnormally after a 24 hr exposure to *U. obscura* exudates. In that study, they extracted *U. obscura* exudates at a ratio of 14 g·L⁻¹ of fresh algae and saw strong effects (an EC₅₀ of abnormal development to the straight-hinged veliger stage of 0.21 g·L⁻¹). Our extraction biomass was 3X as high, and we expected much higher concentrations of the chemical exudates than those used by Nelson et al. (2003b) and Nelson and Gregg (2013). It was a surprise, therefore, no developmental abnormalities and no evident mortality. However, our outcome differences, again, may have been due to differences in the exudates composition as explained above. Moreover, concentrations of chemicals in *U. obscura* can vary seasonally and spatially (Nelson et al., 2003a; Van Alstyne et al., 2011).

The *U. obscura* exudates and dopamine treatments also produced opposite effects on later stages of *D. excentricus* and *C. gigas*. Pluteus larvae of *D. excentricus* exposed to *U. obscura* exudates were affected, but only at the highest concentration. The effect was seen as an overall increase in larval arm length. Dopamine treatments, however, reduced arm lengths dramatically. Adams et al. (2011) found that the body rod (skeletal length of the larval body) of the sea urchin *S. purpuratus* did not change when larvae were exposed to dopamine suppressors, so the lack of an effect on body length in our study is not surprising.

Echinoderm larvae are well known for their morphological plasticity, which can be adaptive in a changing environment (Ebert, 1996). For example, under low food conditions, plutei can elongate the arms and ciliated bands that capture food particles, increasing the area for food capture. Alternatively, when food is abundant, they may save energy by developing shorter arms and reducing the length of the ciliated bands (Hart and Strathmann, 1994; Adams et al., 2011). Longer arms, like we saw in the highest *U. obscura* exudate treatment, could be a positive effect that increases feeding ability. However, morphological changes like those induced by exposure to dopamine are likely to affect the larvae negatively. Shorter arms may reduce feeding ability and could have serious consequences for larval survival.

In contrast with *D. excentricus* plutei and contrary to the *C. gigas* embryos that developed to straight-hinged veligers with larger shells, veliger larvae of *C. gigas* we exposed to the *U. obscura* exudates and dopamine treatments ended up producing 2–6X smaller larvae after 1 week of post-exposure development. However, shell size increased with increasing dopamine concentration, whereas shell size decreased with increasing exudate concentration, suggesting that the treatments interacted with the larvae in different ways. Reduced shells could indicate slower development, increased planktonic period and increased chances of mortality. Larval size differences can persist after metamorphosis (Marshall et al., 2003; Gehman and Bingham, 2010; Hettinger et al., 2012). Therefore, veligers affected by *U. obscura* exudates could produce less fit juveniles.

Undoubtedly, the exudates from the *U. obscura* used in our experiments did not contain only dopamine, but also the oxidation products of dopamine (including quinones, ROS and insoluble melanins; Palmer, 1963; Stokes et al., 1999), and any other chemicals present in the tissues of the algae. These substances were most likely at different concentrations in our *D. excentricus* and *C. gigas* experiments than in the studies done by Nelson et al. (2003b) and Nelson and Gregg (2013). Prior to conducting our experiments, we had assumed that dopamine would be the chemical most likely to affect development of embryos and larvae. However, the results from our study suggest that dopamine was not the primary cause of the effects. Although dopamine treatments produced some effects, they were different from those observed in the exudate treatments.

ROS in the exudates could have produced some of the effects. In a laboratory experiment, *U. obscura* released ROS into the surrounding water after being exposed to desiccation and then rehydrated with seawater (van Hees and Van Alstyne, 2013). In plants, ROS can damage lipids and proteins and can affect gene expression (Halliwell and Gutteridge, 1989; Apel and Hirt, 2004). In marine invertebrates, hydrogen peroxide and dopamine stop metamorphosis of the nudibranch *Phestilla sibogae* (Pires and Hadfield, 1991). ROS production, enhanced by ultraviolet light affects fertilization success of the sea urchin *Anthocardis crassispina* (Lu and Wu, 2005). Therefore, the effects produced by the *U. obscura* exudates might have been due to the ROS produced by dopamine breakdown, as much as by the dopamine itself.

Differences in responses of the species and stages tested in our experiments could be related to the method of exudate collection. *U. obscura* exudates are generally

collected by exposing the algae to desiccating conditions in direct sunlight (Nelson et al., 2003b; Van Alstyne et al., 2011; Nelson and Gregg, 2013). In our *D. excentricus* experiments, we used direct sunlight, but because of poor weather conditions, we created the exudates for the *C. gigas* experiments by desiccating the algae in an incubator. We were unable to measure the dopamine concentrations in the exudates, but expect they were lower in the *C. gigas* experiments because Van Alstyne et al. (2011) found that dopamine release was significantly correlated with solar intensity. However, we knew dopamine's breakdown products were present in the *D. excentricus* and *C. gigas* experiments because we observed the seawater color change associated with oxidation.

Our findings suggest that marine invertebrate larvae that are exposed to areas where they contact a green tide bloom may experience changes in embryonic or larval development that can affect survival and recruitment. Those changes will be dependent on the amount of algae in the bloom and the quantity of chemicals present, which depends on the severity of desiccation (Nelson, 2000; Van Alstyne et al., 2013). The impacts are likely to be greatest in nearshore areas or tidepools where flushing rates are lower and chemicals can accumulate. This suggests that larvae in open ocean areas might not be affected, but larvae retained near spawning areas might not be as fortunate. On the other hand, early embryos and competent larvae trying to find settlement substrata will be more prone to the effects from a green tide algal bloom, since they will be closer to the shore. This will all be affected by the timing and duration exposure. In any case, if green tides continue to expand in severity and extent, there could be important implications for larvae inhabiting the same waters.

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