Selection of vertical zones in low oxygen water columns by larval *Dendraster excentricus*

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Abstract

Decrease in dissolved oxygen levels is becoming more frequent in coastal marine environments. Causes may be of natural or anthropogenic origin, but human activities are having an impact on natural drivers. As organisms face many stressors in their environment, the need to understand how they respond to these increases. Most organisms have been studied at their adult life stage, while the embryo and larval stages are not considered. This study investigates the effect on marine invertebrate larvae of short-term exposure to hypoxia on selection of position in water column. It tests the hypothesis that larvae under oxygen-stress conditions will stay at the upper surfaces, where hypoxia is less likely to occur. An experimental setup was designed in which larvae could be placed in three columns, each filled with water a containing a different level of dissolved oxygen (DO) (three levels: 15 ± 1.71%, 36.7 ± 2.2% and 93.6 ± 0.97% DO). Larvae were then allowed to swim for 10 minutes, and their vertical positions in the experimental water columns were quantified. Three replicate swimming trials were conductued. . Larvae responded by choosing more the lower zones in hypoxic columns, while selection of upper zones was the most common in all treatments.

Introduction

Marine environments with dissolved oxygen (DO) levels lower than 2 mg l^{-1} may be characterized as hypoxic (Díaz & Rosenberg, 1995; Gray, Wu, & Ying, 2002). These conditions appear more frequently as decreases in DO in coastal marine environments become more frequent and spread globally (Diaz & Rosenberg, 2008; Doney et al., 2012; Hoegh-Guldberg et al., 2018; Rabalais et al., 2010; Vaquer-Sunyer & Duarte, 2008). Drivers of hypoxia can be either natural or anthropogenic (Levin et al., 2009), but because human activity is increasing the damaging impacts that naturally-caused hypoxic events might have on ecosystems overall impacts are predicted to increase (Gray et al., 2002; Wu, 2002).

As zones in the water column with lower DO levels become more common in coastal marine environments, the need to study effects of hypoxia on marine organisms increases. It is known that some species of fish and shrimp avoid waters with < 2 mg l^{-1} DO (Wannamaker & Rice, 2000), and that some benthic invertebrates react to a minimum threshold of DO (Levin et al., 2009). Gray et al. (2002) studied the effects of hypoxia on adult marine organisms in the water column. One experiment in Mexico studied the ecophysiological response of *Dendraster excentricus*, a common sand dollar, by assessing RNA/DNA ratio and *hsp*70 gene expression after individuals were exposed to a range of temperature and DO levels (Olivares-Bañuelos, Figueroa-Flores, & Carpizo-Ituarte, 2014). All of these studies suggest that hypoxia has strong negative effects on diverse adult marine invertebrates.

However, few studies have assessed effects of hypoxia on larval stages of invertebrates. This study uses echinoderm larval stages to test the hypothesis that, under hypoxic and other oxygen stress conditions, marine invertebrate larvae swim to and remain at the top layer of water where oxygen exchange might happen and where hypoxia is less likely to occur. This hypothesis

is motivated by the likelihood that hypoxia affects larval stages, and that the part of the water column likely to become hypoxic last and least is the atmosphere-exposed surface. To test this hypothesis, I quantified the position of larvae of the sand dollar, *Dendraster excentricus*, in laboratory water columns across a range of oxygen levels from normal to hypoxic.

Methods

Collection and spawning of sea urchins:

D. excentricus adults were collected during late July 2019, in Crescent Beach on Orcas Island, in the San Juan Archipelago (WA, USA). To induce spawning, procedures from Strathmann (1987) were followed. With a hypodermic needle, 2 mL of KCl 0.5 M were injected through the peristomial membrane. Then, the specimen was shaken and put on a beaker with overflowing seawater. The oral side was facing the bottom: if eggs were shed, the female would continue to shed them on the beaker; if sperm was spawned, the male would be separated to avoid sperm activation.

Fertilization and larval cultures:

Fertilization occurred on August 13, 2019. Gametes from one adult female and one adult male were obtained. Eggs were left to settle for 10 minutes on a beaker and water was decanted, conserving the eggs at the bottom. A small amount of sperm was put on 10 mL of filtered seawater (FSW) and mixed with the eggs. Fertilization envelope was observed in each sampled egg out of three replicates from a small amount taken with a pipette. Embryos were put on two separate 500 mL beakers and reared at ambient temperature.

After 48 hours, two armed plutei were observed. Larvae continued to be reared at ambient temperature and were fed daily a high concentration diet of *Dunalliela tertiolecta* (10^4

cells / mL), with water changes every day. In order to achieve these water changes, a 1 L tripour container was filled with 0.45 µm FSW and larvae were gently poured on a filter cup with a 15 µm mesh, submerged in the tripour. They were transferred to the culture flasks.

Dissolved oxygen manipulation:

In order to obtain the desired levels of DO, nitrogen was bubbled on 250 mL Erlenmeyer flasks filled with FSW. An air stone connected to a tank filled with nitrogen through a hose was used to inject nitrogen in the treatment FSW. To measure DO levels, a ProODO meter (YSI, OR, USA) was used to determine temperature, DO percentage and DO mg l^{-1}. Flasks were maintained at ambient temperature.

Experimental design:

4-armed, 4 day old echinoplutei were placed in experimental water columns containing three different levels of dissolved oxygen saturation: control (> 90% DO), low dissolved oxygen (between 30% and 40% DO) and hypoxic water (< 20% DO). Range of levels was determined according to Diaz & Rosenberg (1995). In order to analyze the selection of zones by larvae, approximately 10 individuals were put into three different cuvettes serving as columns, collocated within a cut rectangular flask that served as a water jacket to reduce fluctuations in temperature.

Dimension of cuvettes were approximately 44 mm x 12 x 12 mm. Total cuvette volume was 4.2 mL, as measured with a 10 mL graduated pipette. The cut flask surrounding the cuvettes was filled up to 150 ml of FSW. The experiment was carried on in a setup that consisted on five cuvettes attached to each other. The inner three were used for the experiment with different levels of DO. Outer cuvettes were filled with water to attenuate light, so that larvae were easier to detect and to aid in temperature regulation.

Cuvettes were filled and then capped with 2.4 mL of each treatment water. Capping reduced interactions between treatment water and air. Caps were designed in FreeCAD software (Riegel, Mayer & van Habre, 2001-2017) and printed on a MakerGear M3-ID 3D printer from (MakerGear, OH, USA). Larvae were taken from the culture flask, and 10 individuals were placed in a small, sectioned Petri dish filled with water for each treatment. Larvae were then injected with a pipette into the middle of the cuvettes, filling the remaining volume. Three trials served as replicates for the experiment. Placement of oxygen-manipulated FSW on columns was randomized to avoid confounding effect of columns. After placement of larvae, caps were again put on each of the three cuvettes to reduce surface interaction with air.

Data analysis:

Experiments were recorded for 15 to 20 minutes, having at least 10 minutes of footage after placement of larvae. Positions of larvae were determined at the end of these 10 minutes. Recordings were taken with a Logitech web camera modified to better capture close-up recording (Logitech, Lausanne, Switzerland). For image analysis, each column was divided into five zones: surface, upper, medium, lower and bottom. As each cuvette measured 44 mm on the recorded face, the upper, medium and lower zones were determined to measure 14 mm each, with the surface and bottom zones being part of the upper and lower layers. The software ImageJ was used to determine the location of larvae in these zones in the selected video frames (Rasband, 2018).

To determine whether zone, treatment and/or their interaction influenced selection of zone by larvae, a two-way analysis of variance (ANOVA) was applied to data. Fixed factors were treatment levels of DO (three levels of oxygen saturation) and selection zones (upper, medium and lower zones) in the water column. Instead of analyzing data under five selection

zones, “surface” and “bottom” were added to “upper” and “lower” zones to make three augmented zones.

Results

Experimental conditions of FSW:

Temperatures varied slightly between trials, but were nearly constant among treatments within each trial (Tables 1, 2 and 3). DO was set between a range and not an exact value, so variation was expected.

Table 1. Temperatures and dissolved oxygen levels obtained for normoxic trials. Averages with standard deviation (SD) included.

	Temperature	Dissolved oxygen (%)	Dissolved Oxygen (mg l^{-1})
Trial 1	19.9	94.7	7.19
Trial 2	18.9	92.8	7.17
Trial 3	18.9	93.4	7.22
Average ± SD	19.2 ± 0.57	93.6 ± 0.97	7.19 + 0.03

Table 2. Temperatures and dissolved oxygen levels obtained for oxygen stress trials. Averages with standard deviation (SD) included.

	Temperature	Dissolved oxygen (%)	Dissolved Oxygen (mg l^{-1})
Trial 1	19.7	34.2	2.61
Trial 2	19.2	38.1	2.93
Trial 3	18.9	37.9	2.13
Average ± SD	19.3 + 0.4	36.7 ± 2.2	2.56 ± 0.4

Table 3. Temperatures and dissolved oxygen levels obtained for hypoxic trials. Averages with standard deviation (SD) included.

	Temperature	Dissolved oxygen (%)	Dissolved Oxygen (mg l^{-1})
Trial 1	19.7	13.2	1
Trial 2	19.3	15.3	1.17
Trial 3	18.8	16.6	1.29
Average ± SD	19.3 ± 0.45	15 ± 1.71	1.15 ± 0.15

Zone selection in water column:

The medium and low layers were the least selected ones by larvae after ten minutes' exposure to treatments. Across all treatments and replicates, surface and upper zones were selected by most larvae (table 4).

Table 4. Total amount of larvae counted in each selection zone per treatment

	Surface			Upper			Medium			Lower			Bottom		
Hypoxic (n=16*, 10, 11*)	7	5	8	0	3	0	3	0	0	0	0	0	6	2	2
Low DO (n=10, 10, 10)	8	9	5	1	0	4	0	0	0	0	1	0	1	0	1
Control (n=10, 10, 10)	6	9	9	1	0	0	2	0	1	1	1	0	1	0	0

*These records were corrected to assume a sample of 10 instead, so that they could be comparable with the rest of the samples.

Larvae stayed more at the bottom in hypoxic treatments. Almost 26% of larvae in hypoxic water were found in this zone. More than 60% of larvae for each treatment selected to stay in upper and surface zones (Table 5).

Table 5. Average percentage of larvae per zone in treatment water column

	Surface	Upper	Medium	Lower	Bottom
Hypoxic	57.92	10.00	6.25	0.00	25.83
Low DO	73.33	16.67	0.00	3.33	6.67
Control	78.18	3.03	9.39	6.36	3.03

As seen in figure 1 and 2, selection for lower zone was higher on hypoxic treatment. More evident is that the highest concentration of larvae is encountered in the upper zone at the end of each trial. For the low DO treatments, no larvae chose to stay at the medium zone.

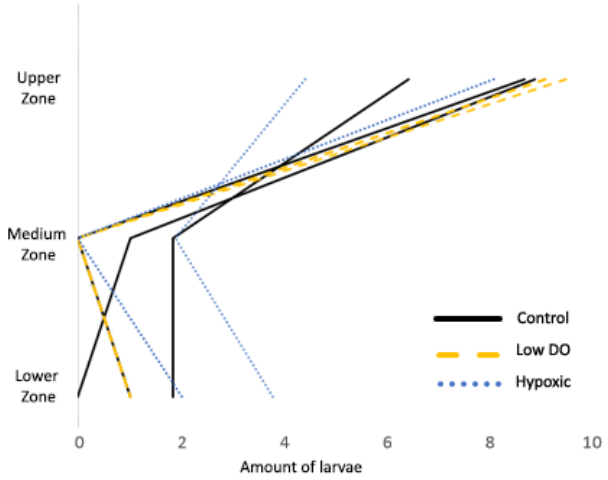

Figure 1. Number of larvae in each zone

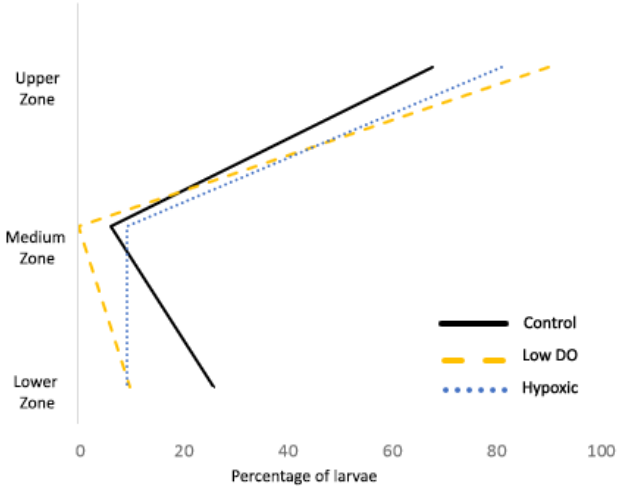

Figure 2. Percentage of average larvae in each zone at ends of trials.

Results of ANOVA demonstrated there was a significant difference of zone selection by larvae on treatments and on zones (table 6). The interaction between these factors was almost significant – including more replicates may possibly have shown a significant difference.

Table 6. Results of two-way analysis of variance of means in zone selection of larvae between treatments and zones.

Source	Nparm	DF	Sum of Squares	F ratio	Prob > F
Treatment	2	2	29.97654	4.8940	**0.0201**
Zone	2	2	249.011555	6536	**< 0.0001**
Treatment * Zone	4	4	33.58635	2.7417	0.0609

Values in bold indicate a significant difference.

Even though the result was not significant, there was a preference of larvae for staying at the lower and bottom zones in hypoxic treatments (figure 2). An "exploring" behavior of swimming along the vertical column was noticed during the 10 minutes of recording. This behavior was observed specially in larvae under hypoxia.

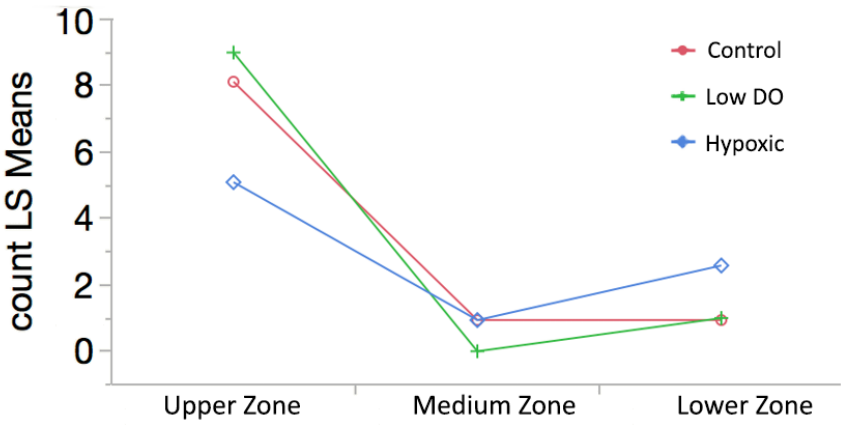

Figure 2. Means of larvae in treatments for each selection zone. 1: Upper zone; 3: Medium Zone; 5: Lower zone.

Discussion

Because hypoxic conditions are predicted to increase in frequency and severity in the next few decades, it is becoming more important to understanding how early life history stages respond to exposure to oxygen stresses (Dupont & Pörtner, 2013; Riebesell & Gattuso, 2015). Swimming behavior of larval invertebrates affects their exposure to hypoxia, and some larvae may alter swimming to reduce exposure. This study offers initial insights into behavioral responses by larval *D. excentricus* to short episodes of hypoxia or oxygen stress.

Echinoid larvae often present negative geotaxis and tend to swim upward (Mogami, Oobayashi, & Baba, 1988). In control and low DO treatments, more larvae were observed to be in the surface. Conversely, in hypoxic treatments, more individuals were observed near the bottom of experimental chambers. Furthermore, larvae that reached the bottom in hypoxic treatments did not swim up again (personal observation). A possible interpretation of this observation is that larvae, once they strayed into hypoxic water, lost the physical ability to swim upwards and hence were trapped. If so, selection of lower zones in hypoxic treatments may have been due to physiological malfunction, as opposed to behavioral choices.

Vertical swimming requires energy investment (Daigle & Metaxas, 2012) so larval respiration may be one factor that explains why more larvae were found at the bottom in hypoxic treatments: they may have ran out of oxygen to breath. All larvae were at the same developmental stage and of the same age, so although hypoxic effects may vary with age and stage, those variations did not contribute to the results in this study.

One noticeable aspect of this study is survival of *D. excentricus* larvae to hypoxic (average of 15 ± 1.71 % DO, 1.15 ± 0.15 mg l^{-1} DO) and, even more, their ability to swim up and down in the column. Physiological performance assessed as the swimming pattern observed under hypoxic columns may lead to further investigate what is the oxygen saturation threshold for echinoids.

This study was limited to quantify position of larvae in a water column at a determined time point after being placed in experimental conditions. Further analysis of hypoxia and low DO effects could be explored to quantify details of swimming behavior and speed, as done by Chan (2012) with *D. excentricus* larvae reared under ocean acidification conditions.

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