Dissolved oxygen concentration affects δ¹⁵N values in oyster tissues: implications for stable isotope ecology

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Abstract. To investigate the potential effects of physiological stress on stable isotope (SI) ratios in oysters, we transplanted hatchery-reared oysters to reefs that experienced different concentrations of dissolved oxygen (DO) in Mobile Bay, Alabama, USA, a freshwater dominated estuary that experiences periodic hypoxia. We monitored physical, biological, and chemical parameters of the environment and biological responses in oysters, including growth, survival, and carbon and nitrogen SI ratios in tissues. Oysters at the low DO site (3.66 ± 0.05 mg/L) had ~1 % higher δ¹⁵N values in tissue than oysters at the higher DO site (6.00 ± 0.06 mg/L). Oysters grown at the low DO site also had lower survival (25%) and no growth compared to oysters at the higher DO site, which had 96% survival and growth rates up to 38 mm/d. Multivariate and regression statistics related DO concentration to changes in δ¹⁵N values in three different oyster tissues (adductor muscle, gut gland, and gill), regardless of similarity in suspended particulate matter and chlorophyll a concentrations (food quantity), C:N (food quality), and SI composition in available foods between sites. The increased δ¹⁵N values in oyster tissues under low DO conditions during this study were consistent with known starvation responses in which oysters stop feeding and catabolize tissues. These data indicate that environmental conditions related to physiological stress can affect SI ratios in oysters in a similar manner to changes in diet composition. Small changes in SI ratios, therefore, may be physiologically significant indicators of environmental stress. When analyzing SI ratios for food web dynamics and source tracing, potential for confounding effects of environmental stressors should be considered.

Key words: adductor muscle; Crassostrea virginica; gill; gut gland; metabolism; starvation; stress.

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INTRODUCTION

Stable isotope (SI) ratios in soft tissues of animals are primarily controlled by the composition of their food sources, with biotic and abiotic factors contributing to variation (Boecklen et al. 2011). Because more metabolically active tissues turnover faster than less active tissues (MacAvoy et al. 2006), some abiotic factors such as temperature have been linked to changes in metabolic or growth rates that alter SI fractionation values away from the standard 0–1‰ for carbon and 2–5‰ for nitrogen (De Niro and Epstein 1978, Minagawa and Wada 1984, Dattagupta et al. 2004, Barnes et al. 2007). Other environmental variables that cause physiological stress and affect metabolism, therefore, may also affect SI ratios in stressed biota, but the effects of most potential stressors on SI ratios have not been examined.

If environmental stressors such as temperature alter SI values, then environmental conditions and associated organismal responses could confound
food web reconstructions and source tracing efforts. Low dissolved oxygen (DO), for example, is known to create physiological stress on organisms (Diaz and Rosenberg 1995, Vaquer-Sunyer and Duarte 2008) by impairing growth, reproduction, and immune functions, and reducing available habitat. Accordingly, low DO has been associated with the loss and failed recovery of many oyster reefs (Cheney et al. 2001, Beck 2011). These effects of low DO stress on biota are of particular concern because low DO conditions are globally pervasive, and the number of affected water bodies is increasing (Diaz 2001, Diaz and Rosenberg 2008). Defining how dominant environmental stressors such low DO affect SI values could provide an important tool for detecting sublethal physiological stress, while also better informing traditional SI-based ecological studies.

Oysters (Crassostrea virginica) naturally occur in nearshore environments where natural and anthropogenic factors commonly cause low DO conditions that may pose a threat to their survival (Bishop et al. 2006). Oyster tolerance to low DO conditions combined with the often ephemeral and episodic nature of low DO conditions (Breitburg 1992, Johnson et al. 2009), however, have made it challenging to relate low DO to effects on growth and survival of oysters and other commercially important species (Hoback and Barnhart 1996, Carmichael et al. 2004, Keppler et al. 2005, 2006). Survival under different DO conditions is size, condition, temperature, and salinity dependent, but bivalve mollusks such as C. virginica can survive hypoxia (<1 mg/L) for 3 to >28 d (Stickle et al. 1989, Gray et al. 2002). Potential sublethal effects of low DO on oysters include changes in stress protein expression (Patterson et al. 2014) and reduced feeding, settlement, growth, and reproduction (Baker and Mann 1992, 1994a, b, Breitburg 2009, Johnson et al. 2009, Wallace et al. 2002). Detection of sublethal and cumulative effects of DO on oysters is necessary to enhance fundamental understanding of oyster physiology and will benefit site selection for restoration and management efforts ongoing worldwide (Beck 2009, Peterson et al. 2003, Schulte et al. 2009). Furthermore, understanding how low DO concentrations specifically affect SI ratios in oyster tissues will refine food web analyses and anthropogenic source tracing efforts that include or rely on these and similar species and could provide an early physiological indicator of stress to avoid mortality.

In this study, we tested the effects of low DO on SI ratios ($\delta^{15}N$ and $\delta^{13}C$) in tissues of juvenile and adult oysters transplanted to two reefs that differed in DO concentration in Mobile Bay, Alabama, USA, an embayment that experiences periodic sustained low DO conditions. To gauge variables that could affect SI ratios in transplanted oysters, we measured (1) environmental variables, including DO, salinity, temperature, turbidity, and pH; and (2) available food supply in terms of chlorophyll $a$ concentration, C:N, and C and N SI composition. We assessed oyster response to these environmental variables by measuring oyster growth and survival, and SI values in tissues. To further determine whether variation in SI ratios was due to tissue-specific effects on metabolic rates, we measured SI ratios in adductor muscle, gut gland, and gill, which are known to be metabolically different (Malet et al. 2007). We hypothesize that the oysters experiencing lower DO conditions will have reduced growth, higher mortality, and increased $\delta^{15}N$ (due to catabolism of tissues associated with stress). Tissues with higher turnover rates (such as gut gland) will detect the stress response sooner than tissues with lower turnover rates (adductor muscle). We used multivariate and regression analyses to determine the relative importance of different environmental attributes to SI values in oyster tissues.

**Methods**

**Mobile Bay description**

Mobile Bay is located in the northern Gulf of Mexico (Fig. 1). The bay is relatively wide (25–50 km), but shallow (average depth 3 m), with a deeper ship channel running the length of the Bay (Schroeder and Wiseman 1988, Park et al. 2007). The Mobile River system discharges an average of 1900 m$^3$/s into Mobile Bay yearly, making it the third largest freshwater discharge among major river systems with watersheds in the United States, with widespread annual salinity variation (Park et al. 2007, US Census Bureau 2012). Tides are diurnal, averaging 0.4 m, and wind is the primary forcing mechanism for circulation and mixing in the bay. Due to these depth, salinity, wind, and circulation patterns, DO fluctuates throughout the Bay, and strong stratification events result
in periodic and at times sustained low DO conditions (Cowan et al. 1996, Park et al. 2007). The reef sites used for this study are documented to represent historically more hypoxic (Denton reef) and typically normoxic (Sand reef) locations in the Bay (May 1973).

**Oyster transplants**

Oysters were transplanted at two sites, ~13 km apart, that historically differed in DO characteristics, Denton and Sand reefs (Fig. 1) in Mobile Bay, Alabama, USA. Because the relief at each reef (largely remnant) varied gradually up to a height of 1 m, across several meters, we opted to simulate natural relief by deploying caged oysters at fixed heights (0.1 and 1.0 m) above the bottom on two existing pilings at each reef. Oysters were deployed during summer 2008 and 2010. To capture ontogenetic variation of common oyster stocks at each site in response to low DO conditions, we deployed juvenile and adult hatchery-reared oysters obtained from the Auburn University Shellfish Laboratory, Dauphin Island, Alabama. In 2008, 100 juveniles (40.1 ± 0.3 mm) and 50 adults (52.6 ± 0.4 mm) were placed in cages measuring 30 cm wide × 40 cm long × 10 cm deep, with one cage of juveniles and two cages of adults (25 per cage) deployed at each site. In 2010, we deployed 50 juveniles per cage (40.1 ± 0.3 mm) and 45 adults per cage (62.8 ± 0.4 mm) in cages measuring 30 cm wide × 40 cm long × 10 cm deep, with three cages of each age class deployed at each site. Cages were used to protect oysters from predation and keep them at the fixed depth, with individual oysters considered experimental units within each cage. Given the known flow patterns in the area, open structure of the cages, densities that mimic typical oyster clusters on natural reefs, and consistent caging conditions between sites, caging effects were considered negligible. Additionally, these same cages have been used in multiple studies with a range of bivalve species on the US Atlantic and Gulf of Mexico coasts without evidence for caging effects (Carmichael et al. 2004, Biancani et al. 2011, Patterson et al. 2014, Darrow et al. 2017). Cages were cleaned and survival was recorded every two weeks, at which time up to 10 individuals from each age class were collected for growth measurements and SI analysis in 2008, and up to 3 adults and 15 juveniles were sampled from each cage in 2010. A higher number of oysters were sampled in 2010 due to sampling for a companion study on stress protein expression (described in Patterson et al. 2014). To capture oyster responses during multiple low DO events, which varied by year, oysters were transplanted for 112 and 138 d, respectively, in 2008 and 2010.

**Measuring site characteristics**

**Environmental attributes.**–Environmental data were collected using a YSI 6600v2 datasonde (YSI, Yellow Springs, Ohio, USA) attached to each piling at the two depths (0.1 and 1.0 m above the bottom) where oysters were transplanted at both reef sites. In 2008, sondes were equipped with probes to measure DO, conductivity, temperature, depth, and turbidity, and in 2010, pH was added. Sondes were programmed to record five-minute averages of each variable every 15 min. Data were downloaded and the sondes recalibrated approximately...
every two weeks. Data for DO, temperature, and salinity were corroborated using a handheld YSI 85 (YSI) during each calibration.

**Food supply.**—To quantify site-specific differences in food supply, we measured food quantity and quality every two weeks. Food quantity was measured as chlorophyll a (chl a) concentration of different particle size classes (5, 20, 200 μm), and food quality was measured in terms of C:N and SI values. Measurements were made by collecting one liter of filtered (200 μm) water at the depth (1.0 or 0.1 m above the bottom) of transplant cages at each site. Water was filtered through a pre-ashed 0.7-mm Whatman GF/F filter to collect suspended particulate matter (SPM) for chl a and SI analyses. In addition, to further determine potential differences in composition of different particle size classes at each site, an additional two liters of water was collected monthly in 2008 and biweekly in 2010 and filtered through 5- and 20-μm mesh. For chl a analysis, filters were stored at −20°C until extraction with DMSO/90% acetone and analysis by fluorometry (MacIntyre and Cullen 2005). For SI analyses, filters were dried at 60°C, packaged into tin capsules, and analyzed as described below for tissue samples. C:N was determined from C and N content measured during SI analyses.

**Growth and survival of transplants**

Oyster growth was measured in terms of change in shell height (longest dimension). Before transplanting, oysters were cleaned, dried, and marked with water-resistant black permanent ink to indicate the outer margin of shell. Shell height was then measured to the nearest 0.1 mm, using vernier calipers. Past studies have shown consistent allometric relationships among individual hatchery-reared oysters transplanted at sites in Mobile Bay, Alabama, making shell height a suitable indicator of shell growth (Daskin et al. 2008). Survival was noted on each sampling date, and dead oysters were removed from cages. Percent survival was calculated as the number of oysters alive through the end of the study, divided by the number initially deployed, multiplied by 100.

**Stable isotope analysis analyses**

To measure δ15N and δ13C values in oyster tissues, tissues were removed from the shell and separated into adductor muscle, gut gland, and gill tissue. Tissues were stored at −20°C until processing. All tissues were dried at 60°C and ground with a mortar and pestle, then weighed and packed in tin capsules (1.0 ± 0.2 mg). Lipid extraction was not performed because preliminary studies demonstrated that lipid extraction was not necessary for glycogen storing oyster tissues (Patterson and Carmichael 2016). Samples were sent to the University of California Davis Stable Isotope Facility (http://stableisotopefacility.ucdavis.edu/) for analysis by continuous flow isotope ratio mass spectrometry (IRMS; PDZ Europa, Sercon, UK) after combustion in an elemental analyzer (PDZ Europa Automatic Analyzer-Gas Solid Liquid).

**Statistical analysis**

All data were analyzed for sampling dates up to 87 d for which comparable data were available at both sites and in both years; all data were not available for comparison among all sampling dates due to environmental variation that prevented sampling and differences in mortality rates between sites and years. Minitab (version 16) was used for general linear model analyses of variance (ANOVA), followed by a Tukey’s post hoc test for multiple comparisons, when appropriate. ANOVAs were performed using depth, site, and day as fixed factors for environmental variables and included age class for biological comparisons. We ran a full factorial linear model for differences among factors and examined all first-order interactions. Data were log-transformed to meet normality assumptions when needed. Regression analyses and tests for homogeneity of slopes were performed in SigmaPlot (version 16, Microsoft, Redmond, Washington, USA). A general linear model was performed to compare differences among significant regression lines, using day as a covariate. An α value of 0.05 was used for all tests. Error is reported as standard error of the mean. Data for bottom (0.1 m above the bottom) and top (1.0 m above the bottom) sites or adult and juvenile oysters were combined for subsequent analyses when no significant differences were detected between data sets. For survival, data were pooled to yield a single value for each treatment in 2008, but collected separately from each cage in 2010 to allow calculating a standard error for survival. To determine the relative importance of environmental or food supply attributes to δ15N values in oyster tissues at each site, a distance-based
multivariate analysis for a linear model (DISTLM) was run in PRIMER 6 and PERMANOVA+ (PRIMER-E, UK) on all site data. DISTLM uses a resemblance matrix and permutations rather than fixed Euclidean distance and normality assumptions of standard linear models and selects the best model without having to use a cutoff value, such as an Akaike information criterion. Because environmental conditions varied during the study and different tissues may respond to environmental variation at different rates, we opted to use the two-week mean (we had previously tested 2-, 5-, and 14-d periods) of relevant environmental attributes (DO, salinity, temperature) and food supply parameters (chl a, C:N, δ15N SPM) in the DISTLM model for comparison of δ15N values measured in oyster tissues throughout the study. To specifically test for the effects of DO on final δ15N values in oyster tissues after SI values reached equilibrium in the field, we performed single attribute regression analyses, combining data for all treatments and comparing SI values to the five-day and two-week means for DO. Because there was a better relationship with the five-day mean, the DISTLM was performed again, replacing the two-week DO values with the five-day DO values. These time periods were chosen to bracket known estimates for water movement along the eastern side of Mobile Bay where our reef sites were located, which typically range from 4 to 9 d, depending on precipitation (Park et al. 2007).

RESULTS

Environmental attributes

Mean daily DO concentrations ranged from ~2 to 4 mg/L at Denton reef and 3–6 mg/L at Sand reef (Fig. 2, Table 1). Dissolved oxygen differed between sites and years (ANOVA, $F_7 = 61.24$, $P < 0.001$), with Denton reef having significantly lower DO than Sand reef in both years, and 2008 having lower DO than 2010 (Fig. 2, top panels; Table 1). Salinity also differed between sites and years (ANOVA, $F_7 = 60.55$, $P < 0.001$), with significantly lower salinity at Denton reef (ranging: 14–18) than Sand reef (ranging: 19–25), and lower in 2010 than 2008 (Fig. 2, middle panels; Table 1). Temperature did not differ between sites or years.

Overall chl a concentrations (200 μm pre-filtered) differed between sites with Denton reef having a chl a concentration nearly two times higher than Sand in 2008, but Sand reef having a higher chl a concentration than Denton reef in 2010 (ANOVA, $F_3 = 5.58$, $P = 0.002$; Table 2). When separated into 5- and 20-μm size classes, there was no difference between sites or years. In 2008, total SPM available as food to oysters at Sand reef had a higher C:N than at Denton reef, but in 2010, C:N was the same between sites (ANOVA, $F_3 = 100.30$, $P < 0.001$; Tukey’s post hoc $P < 0.05$, Table 2).

Oyster growth and survival

Oysters showed significant shell growth through time for some treatments, but not others. In 2008, juvenile oysters showed significant growth at all sites, except Denton reef bottom (0.1 m above the bottom). For adults, only oysters at Sand reef top sites (1.0 m above the bottom) showed significant growth. In 2010, juvenile oysters at both sites and adult oysters at Sand reef showed growth (Fig. 3). In both years, oysters at Sand reef top had higher growth than oysters at Sand reef bottom or at Denton reef (homogeneity of slopes, 2008, $F_3 = 24.15$, $P < 0.001$; 2010, $F_4 = 3.72$, $P = 0.02$; Tukey’s post hoc $P < 0.05$ for all significant comparisons, Fig. 3). Similarly, survival was higher at Sand reef in both years (Table 3), with survival ranging from lowest (25–50%) at Denton bottom to highest (89–98%) at Sand reef top sites. Overall, survival also was higher in 2010 compared to 2008 (Table 3).

Stable isotope ratios

δ15N and δ13C values in oyster tissues were different between sites in both years such that oysters from Denton reef had higher δ15N and lower δ13C values in tissues than oysters from Sand reef (ANOVA, adductor muscle: δ15N $F_3 = 29.85$, $P < 0.001$, δ13C $F_3 = 143.42$, $P < 0.001$; gut gland: δ15N $F_3 = 43.94$, $P < 0.001$, δ13C $F_3 = 135.96$, $P < 0.001$; gill: δ15N $F_3 = 44.42$, $P < 0.001$, δ13C $F_3 = 179.40$, $P < 0.001$; Tukey’s post hoc, $P < 0.05$; Fig. 4). While both years showed similar patterns in SI values between sites, δ13C values in oysters transplanted in 2010 were somewhat lower compared to 2008 (Fig. 4). Overall, the mean difference in SI values between Denton and Sand reefs was 0.82% at N and 1.4% at C in 2008 and 0.66% at N and 1.6% at C in 2010. While the overall SI values
differed, the overall difference between the two sites did not differ greatly.

SI values also differed among the different tissues in oysters. At Denton and Sand reefs in both years, δ15N values were highest in adductor muscle and lightest in gut gland (ANOVA; Denton 2008 $F_3 = 24.21$, $P < 0.001$, Denton 2010 $F_3 = 77.69$, $P < 0.001$, Sand 2008 $F_3 = 175.40$, $P < 0.001$, Sand 2010 $F_3 = 198.77$, $P < 0.001$, Tukey’s post hoc $P < 0.05$; Fig. 4). Similarly, δ13C differed among tissues, with gut gland having the lowest values and adductor muscle the highest (ANOVA; Denton 2008 $F_3 = 51.45$, $P < 0.001$, Denton 2010 $F_3 = 216.68$, $P < 0.001$, Sand 2008 $F_3 = 112.08$, $P < 0.001$, Sand 2010 $F_3 = 43.41$, $P < 0.001$, Tukey’s post hoc $P < 0.05$; Fig. 4). SI values in SPM (δ15N 6.53 ± 0.25‰, δ13C −25.16 ± 0.39‰) did not differ between sites or depths in 2008, allowing us to combine data for subsequent analyses. In 2010, SI values in SPM differed only by site (Denton δ15N 4.70 ± 0.14‰, δ13C −26.23 ± 0.14‰, Sand δ15N 5.11 ± 0.16‰, δ13C −24.47 ± 0.22 ‰).

When examining the mean fractionation between oysters and their available food (SI in oyster tissue; SI in SPM), for δ15N there was a difference in fractionation by site and year (Table 4;
ANOVA, adductor muscle \(F_3 = 250.93, P < 0.001\); gut gland \(F_3 = 47.72, P < 0.001\); gill \(F_3 = 192.20, P < 0.001\), with Denton reef typically having greater fractionation than Sand reef, and greater fractionation in 2010 compared to 2008 for all tissue types (Tukey’s post hoc, \(P < 0.05\)). For \(d^{13}C\), mean fractionation was greater in 2008 than in 2010 for all tissue types (Table 4, ANOVA, adductor muscle \(F_3 = 31.03, P < 0.001\); gut gland \(F_3 = 15.51, P < 0.001\); gill \(F_3 = 27.16, P < 0.001\), but the only site difference was found for fractionation from adductor muscle to SPM, which was greater at Denton reef in 2010.

Table 1. Mean (±standard error) dissolved oxygen (DO), salinity, and temperature measured at top (1.0 m) and bottom (0.1 m) depths at Denton and Sand reefs in 2008 and 2010.

<table>
<thead>
<tr>
<th></th>
<th>Site</th>
<th>DO (mg/L)</th>
<th>N</th>
<th>Group</th>
<th>Salinity</th>
<th>N</th>
<th>Group</th>
<th>Temp (°C)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>Denton</td>
<td>2.31 ± 0.19</td>
<td>88</td>
<td>EF</td>
<td>17.66 ± 0.42</td>
<td>88</td>
<td>C</td>
<td>29.01 ± 0.10</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Denton</td>
<td>1.75 ± 0.19</td>
<td>100</td>
<td>F</td>
<td>17.40 ± 0.44</td>
<td>100</td>
<td>C</td>
<td>28.92 ± 0.09</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Sand</td>
<td>3.60 ± 0.15</td>
<td>101</td>
<td>CD</td>
<td>21.68 ± 0.47</td>
<td>101</td>
<td>B</td>
<td>29.10 ± 0.10</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Sand</td>
<td>3.01 ± 0.23</td>
<td>42</td>
<td>DE</td>
<td>25.41 ± 0.60</td>
<td>42</td>
<td>A</td>
<td>28.65 ± 0.18</td>
<td>42</td>
</tr>
<tr>
<td>2010</td>
<td>Denton</td>
<td>4.35 ± 0.17</td>
<td>100</td>
<td>B</td>
<td>13.93 ± 0.43</td>
<td>97</td>
<td>D</td>
<td>29.79 ± 0.15</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Denton</td>
<td>3.59 ± 0.18</td>
<td>100</td>
<td>CD</td>
<td>14.05 ± 0.49</td>
<td>100</td>
<td>D</td>
<td>29.88 ± 0.13</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Sand</td>
<td>5.91 ± 0.09</td>
<td>99</td>
<td>A</td>
<td>18.52 ± 0.47</td>
<td>99</td>
<td>C</td>
<td>30.03 ± 0.13</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Sand</td>
<td>3.92 ± 0.15</td>
<td>100</td>
<td>BC</td>
<td>21.57 ± 0.41</td>
<td>100</td>
<td>B</td>
<td>29.65 ± 0.12</td>
<td>100</td>
</tr>
</tbody>
</table>

Note: Number of data points and similarity groupings are shown for each mean environmental attribute (group based on GLM followed by Tukey’s post hoc, \(P < 0.05\)).

Table 2. Mean (±standard error) chlorophyll \(a\) content (µg/L) for different size classes of suspended particulate matter and C:N measured at Denton and Sand reefs in 2008 and 2010.

<table>
<thead>
<tr>
<th>Variable</th>
<th>2008 Denton</th>
<th>2008 Sand</th>
<th>2010 Denton</th>
<th>2010 Sand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size class (µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.00 ± 1.17</td>
<td>3.62 ± 0.84</td>
<td>3.76 ± 0.41</td>
<td>3.04 ± 0.32</td>
</tr>
<tr>
<td>20</td>
<td>8.37 ± 1.12</td>
<td>5.90 ± 1.42</td>
<td>8.40 ± 0.54</td>
<td>9.12 ± 0.81</td>
</tr>
<tr>
<td>200</td>
<td>12.35 ± 1.69</td>
<td>6.37 ± 0.28</td>
<td>9.10 ± 0.49</td>
<td>14.13 ± 1.80</td>
</tr>
<tr>
<td>C:N</td>
<td>6.24 ± 0.24</td>
<td>7.18 ± 0.28</td>
<td>3.55 ± 0.14</td>
<td>4.07 ± 0.17</td>
</tr>
</tbody>
</table>

ANOVA, adductor muscle \(F_3 = 250.93, P < 0.001\); gut gland \(F_3 = 47.72, P < 0.001\); gill \(F_3 = 192.20, P < 0.001\), with Denton reef typically having greater fractionation than Sand reef, and greater fractionation in 2010 compared to 2008 for all tissue types (Tukey’s post hoc \(P < 0.05\)). For \(d^{13}C\), mean fractionation was greater in 2008 than in 2010 for all tissue types (Table 4, ANOVA, adductor muscle \(F_3 = 31.03, P < 0.001\); gut gland \(F_3 = 15.51, P < 0.001\); gill \(F_3 = 27.16, P < 0.001\), but the only site difference was found for fractionation from adductor muscle to SPM, which was greater at Denton reef in 2010.

Effects of environmental variables on stable isotope ratios in oysters

To quantify the effect of different environmental attributes on SI values in oyster tissues, we used a distance-based multivariate analysis (DISTLM) to compare data at different time periods during the study. Because different tissues may respond to environmental variation at different rates, we tested the two-week means of relevant environmental attributes. Overall, we found that food supply (chl \(a\), C:N) and DO were the dominant variables determining \(\delta^{15}N\) values in oyster tissues at two-week intervals (Table 5, Fig. 5). Adductor muscle was less responsive to environmental variation than gill and gut gland, showing no significant relationship with any of the tested environmental variables in 2008, but in 2010, pH and DO explained 34% of the variation in \(\delta^{15}N\) values. In contrast, gill and gut gland were more responsive to environmental variation, particularly during 2008 when DO was significantly lower at our field sites. In 2008, DO alone explained 49% and 63% of the variation in \(\delta^{15}N\) values in gut gland and gill, respectively. When chl \(a\) concentration was included, the cumulative percentage of variation in gut gland and gill increased to 57 and 70 (Table 5). During 2010, when DO concentrations were higher at field sites, DO alone explained only 9–18% of variation in \(\delta^{15}N\) among all tissues (Table 5). The combination of C:N and DO was the driving variable for \(\delta^{15}N\) gill and gut gland in 2010 (Fig. 5 middle, Table 5). Additionally, other
attributes of food supply (chl, δ\(^{15}\)N in SPM) were also important drivers of δ\(^{15}\)N in gill.

We further tested the relationship between two-week mean DO and final δ\(^{15}\)N in tissues after they reached equilibrium in the field. Results were similar to our DISTLM analyses. We again found that δ\(^{15}\)N values in gut gland and gill tissues were higher when DO
concentrations were lower in 2008, with the relationship to DO explaining >60% of the variation in $\delta^{15}$N values in both tissues (Fig. 6, left panels). In 2010, we found similar relationships for adductor muscle and gill (but not gut gland), with DO alone explaining up to ~30% of the variation in $\delta^{15}$N values (Fig. 6, right panels).

**Discussion**

Overall $\delta^{15}$N values were higher in tissues from oysters at low DO sites, despite comparable or better food resources at the low DO site. While food supply attributes were major determinants of $\delta^{15}$N values in oyster tissues (as expected), DO concentration and individual tissue metabolic characteristics had a significant effect on SI values among tissues and between sites and years. Despite the many possible environmental attributes affecting oysters at the study sites, multivariate analysis repeatedly found a relationship between $\delta^{15}$N and DO as well as food attributes in all tissues tested.

Tissue-specific differences in SI values are known to be related to differences in tissue turnover rate (Tieszen et al. 1984) and, in addition, for carbon, the amount of lipid in the tissue type (due to isotopic fractionation between pyruvate and acetyl coenzyme A; De Niro and Epstein 1977). The tissue-specific SI values we measured in adductor muscle (highest), gill (intermediate), and gut gland (lowest) were similar to relationships determined by Cabanellas-Reboredo et al. (2009) in the fan mussel and are consistent with known metabolic attributes of each tissue. Adductor muscle, for example, has slower tissue turnover rates than gut gland (Cabanellas-Reboredo et al. 2009). Importantly, DO was identified as a driving variable in the DISTLM, which considered the relationship between DO and $\delta^{15}$N through time while tissues were coming to equilibrium with food sources and environmental conditions, as well as in the regression analyses, which considered only the relationship between DO and $\delta^{15}$N after tissues had reached equilibrium. As far as we know, this is the first study to link variation in $\delta^{15}$N ratios to DO concentration.

We also found that available food resources could not fully account for other observed patterns in oyster condition. In 2008, oyster growth and survival were lower at Denton reef than at Sand reef despite higher food quantity (chl a content) at Denton, indicating that food supply was not the factor limiting growth. Similarly, oysters at Sand reef had lower growth in 2010 than in 2008 (when DO concentrations were lower), despite higher chl a content. On average, C:N in SPM was lower in 2010 than 2008, indicating a potential difference in food quality (Redfield et al. 1963, Table 2). This difference was not reflected in oyster tissues, however, which had similar C:N between years (data not shown). Increased Hsp70 (a stress protein biomarker) expression also has been documented in tissue of oysters in relation to low DO in Mobile Bay, corroborating this attribute as a stressor for oysters at our study sites during the study period.
(Patterson et al. 2014). The slightly higher oyster density in 2010 may also have contributed to lower growth at Sand reef and higher d15N values at both sites if food became limiting. Food resources and oyster survival, however, were higher in 2010 when oyster densities were higher and differences in growth, survival, and SI ratios were greater between sites than between years, with growth already so low at Denton reef that higher oyster density had no additional effect. Overall, growth and survival patterns among oysters were better related to patterns in DO concentration than differences in food supply (quantity or quality), with reduced growth and survival at low DO conditions.

Nutritional status, particularly starvation, has been implicated in changes in SI ratios leading to an enrichment of d15N values in tissues (Hobson et al. 1993, Doucett et al. 1999, Cherel et al. 2005). In response to declining oxygen tension, oysters can alter their oxygen consumption until oxygen levels are low enough that they switch to anaerobic metabolism (Shumway and Koehn 1982). Under anaerobic metabolism, oysters will catabolize available resource pools including carbohydrates, amino acids, and proteins. Under

Table 4. Mean fractionation (SI ratio in tissue—SI ratio in mean suspended particulate matter [SPM]) of adductor muscle (AM), gut gland (GG), and gill (Gill) after SI ratios in the tissues had reached equilibrium.

<table>
<thead>
<tr>
<th>Variable</th>
<th>AM</th>
<th>GG</th>
<th>Gill</th>
<th>AM</th>
<th>GG</th>
<th>Gill</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denton</td>
<td>3.98 ± 0.16</td>
<td>3.03 ± 0.13</td>
<td>2.32 ± 0.12</td>
<td>3.83 ± 0.11</td>
<td>2.24 ± 0.11</td>
<td>3.13 ± 0.11</td>
</tr>
<tr>
<td>Sand</td>
<td>2.85 ± 0.09</td>
<td>0.95 ± 0.28</td>
<td>4.74 ± 1.11</td>
<td>3.75 ± 0.11</td>
<td>2.21 ± 0.12</td>
<td>4.02 ± 1.14</td>
</tr>
<tr>
<td>2010</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denton</td>
<td>6.03 ± 0.05</td>
<td>3.13 ± 0.11</td>
<td>5.82 ± 0.07</td>
<td>3.29 ± 0.04</td>
<td>1.27 ± 0.09</td>
<td>2.35 ± 0.06</td>
</tr>
<tr>
<td>Sand</td>
<td>5.41 ± 0.06</td>
<td>3.27 ± 0.08</td>
<td>4.86 ± 0.09</td>
<td>2.68 ± 0.06</td>
<td>1.55 ± 0.11</td>
<td>2.27 ± 0.07</td>
</tr>
</tbody>
</table>

Note: Mean SPM is the average from the start of the experiment to the date preceding collection.

Table 5. PRIMER-E PERMANOVA distance-linear model (DISTLM) step-wise model outputs comparing relationships between environmental attributes and d15N in tissues, including adductor muscle (AM), gut gland (GG), and gill (Gill) from oysters grown in Mobile Bay, Alabama, USA, in 2008 and 2010.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Environmental attribute</th>
<th>AICc</th>
<th>Psuedo-F</th>
<th>P</th>
<th>Var (%)</th>
<th>Cum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>chl a</td>
<td>−56.56</td>
<td>2.02</td>
<td>0.18</td>
<td>7.76</td>
<td>7.76</td>
</tr>
<tr>
<td>GG</td>
<td>DO</td>
<td>−57.39</td>
<td>23.67</td>
<td>0.00</td>
<td>48.63</td>
<td>48.63</td>
</tr>
<tr>
<td></td>
<td>chl a</td>
<td>−59.45</td>
<td>4.47</td>
<td>0.05</td>
<td>8.05</td>
<td>56.69</td>
</tr>
<tr>
<td></td>
<td>Salinity</td>
<td>−60.52</td>
<td>3.51</td>
<td>0.08</td>
<td>5.74</td>
<td>62.43</td>
</tr>
<tr>
<td>Gill</td>
<td>DO</td>
<td>−61.97</td>
<td>40.46</td>
<td>0.00</td>
<td>62.77</td>
<td>62.77</td>
</tr>
<tr>
<td></td>
<td>chl a</td>
<td>−64.88</td>
<td>5.39</td>
<td>0.04</td>
<td>7.07</td>
<td>69.84</td>
</tr>
<tr>
<td>2010</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>pH</td>
<td>−74.98</td>
<td>7.21</td>
<td>0.01</td>
<td>22.39</td>
<td>22.39</td>
</tr>
<tr>
<td>GG</td>
<td>DO</td>
<td>−76.87</td>
<td>4.29</td>
<td>0.06</td>
<td>11.76</td>
<td>34.15</td>
</tr>
<tr>
<td></td>
<td>C:N</td>
<td>−34.91</td>
<td>5.39</td>
<td>0.04</td>
<td>21.24</td>
<td>21.24</td>
</tr>
<tr>
<td>Gill</td>
<td>DO</td>
<td>−35.03</td>
<td>2.60</td>
<td>0.14</td>
<td>9.47</td>
<td>30.71</td>
</tr>
<tr>
<td></td>
<td>chl a</td>
<td>−58.61</td>
<td>5.47</td>
<td>0.03</td>
<td>17.95</td>
<td>17.95</td>
</tr>
<tr>
<td></td>
<td>C:N</td>
<td>−62.45</td>
<td>4.76</td>
<td>0.06</td>
<td>12.10</td>
<td>41.54</td>
</tr>
<tr>
<td></td>
<td>15N SPM</td>
<td>−63.99</td>
<td>4.07</td>
<td>0.05</td>
<td>9.13</td>
<td>50.66</td>
</tr>
</tbody>
</table>

Notes: Var (%) = percent of d15N in tissue explained by each variable. Cum (%) = cumulative percent d15N explained by each variable in combination with the variables listed above it in the table for a given tissue. AICc, Akaike’s information criterion, corrected for sample sizes.
Fig. 5. Distance-based redundancy plots from the distance-linear model run in PRIMER PERMANOVA+,
prolong anoxia, resulting in increased starvation, carbohydrate reserves are reduced, protein catabolism increases, and nitrogen excretion increases (Bayne 1973, Riley 1980, De Zwaan 1983). Accordingly, we observed higher δ15N values coincidental with slower growth and reduced survival during both years at the chronically lower DO site (Denton reef; ~1.8–4.4 mg/L). In contrast, we did not see higher δ15N in tissues at Sand reef, and oysters showed significant growth at this site despite low DO comparable to Denton Reef (3.9 mg/L) at the bottom depth (0.1 m above the bottom) in 2010. Oysters at Sand reef, however, did show greater fractionation from measured food sources to tissues in 2010, which may indicate some relative enrichment of values in tissues compared to previous years, despite similar absolute values. It is not clear, therefore, whether oysters at Sand bottom reached the point of protein catabolism during the study period in 2010. These findings suggest that oysters likely must experience persistent stressed conditions, greater than the episodic low DO exposure typically found at Sand Reef, before

![Fig. 6. δ15N in adductor muscle, gut gland, and gill tissue of oysters (from all sites combined) compared to two-week average DO concentration (mg/L) at each site, after stable isotope ratios in tissues reached equilibrium.](image)
undernourishment and protein catabolism will lead to slower growth, increased SI values, and reduced survival (Hatch 2012).

Environmental variables, such as salinity, have been previously related to \( \delta^{13}C \) (Fry 2002), and our \( \delta^{13}C \) tissue values reflected the differences in food supply and salinity between sites and years (Figs. 1, 4, Table 1). Denton reef located further north in Mobile Bay had lower salinity and lower \( \delta^{13}C \) values than Sand reef, located near the mouth of Mobile Bay with higher salinity and higher \( \delta^{13}C \). It has previously been shown that salinity affects oyster growth and survival (Davis 1958, Galtsöff 1964). Although Denton reef had lower salinity, the range for optimum growth of oysters is between 15\(^{\text{‰}}\) and 27\(^{\text{‰}}\) (Davis 1958), and the mean and median of salinity at Denton was within this range. Hence, major salinity effects on growth or survival are highly unlikely. Like DO, salinity has the potential to affect SI ratios in tissues because it can affect oyster metabolism and growth. While we observed a relationship between salinity and \( \delta^{13}C \), we found little or no relationship between salinity and \( \delta^{15}N \). Temperature also has previously been reported to affect SI ratios (Bosley et al. 2002, Barnes et al. 2007). There was no difference in temperature through time or between our study sites and it was not implicated in the DISTLM, indicating that temperature was not responsible for the observed patterns in \( \delta^{15}N \) values. While other environmental attributes had potential to affect SI ratios in oysters by affecting food resources or oyster physiology, in this study, attributes besides DO were not likely major factors.

**Conclusion**

The idea that environmental variables (besides food supply) alter SI ratios in animal tissues is not new, but has rarely been tested or quantified to inform our understanding of organismal physiology or refine the use of SI analyses as an ecological tool. During this study, salinity, temperature, and food supply at the low DO site were comparable to or at ranges better suited to support growth and survival of oysters than at the higher DO site. Hence, DO was the dominant stressor detected in our system during this study and, in part, mediated the resulting metabolic responses of oysters and associated changes in \( \delta^{15}N \) values in oyster tissues. These findings suggest that any variable that causes stress on an organism and affects feeding (leading to nutritive stress) has potential to alter SI ratios in consumer tissues and cause changes in apparent fractionation that would not be expected based on composition of food supply alone. The results of this study have important implications for analyses commonly performed in ecology, including trophic assessments and organic matter source tracking. Given that a trophic shift is typically 2–4\(^{\text{‰}}\) for N, a difference of 1.2\(^{\text{‰}}\) for \( \delta^{15}N \) (such as found between oysters at Denton and Sand reefs in this study) means that low DO stress accounted for 25–50% of the value of a trophic shift. Our findings support the long-held notion that SI ratios are a promising tool for physiological ecologists (Griffiths 1991, Gannes et al. 1998) by demonstrating that small changes in SI ratios may be physiologically significant indicators of environmental stress. In addition, the timescale of SI turnover and response by different tissue types could be applied to answer questions on different timescales. Taking all of these points into consideration, our data suggest that (1) \( \delta^{15}N \) has potential to reflect the effects of DO concentration (and other mechanistically similar cumulative sublethal stressors) in organisms, and (2) physiological condition of biota must at least be considered when making any SI-based ecological assessments.

**Acknowledgments**

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**Literature Cited**


