This thesis has been prepared in the style and format consistent with the journal Marine Biology.
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ABSTRACT

Prolonged hyposalinity has been identified as the most serious environmental stress to the threatened seagrass *Halophila johnsonii* Eiseman. In order to determine the minimum salinity tolerance of *H. johnsonii* and the impact of hyposaline conditions on its physiology, an outdoor mesocosm study was performed on field-collected transplants. Plants were exposed to either pulsed hyposalinity treatments of 30, 15, 10 and 8, or gradual salinity reductions of two every two days. When salinity was pulsed, the lowest salinity that plants were able to tolerate without exhibiting signs of stress was a salinity of 15. Survivorship remained high in the control and salinity of 15 treatments but declined in the salinity of 10 and 8 treatments. Similarly, maximum quantum yields of plants in the control and salinity of 15 did not decline over time while those in the salinity treatments of 10 and 8 did. Leaf osmolality declined with respect to salinity treatment but the difference between leaf and media osmolality remained constant. In contrast, when salinity was gradually reduced, the lowest salinity that plants were able to tolerate without showing signs of stress was a salinity of 6. Survivorship remained high from salinities of 30 to 4, and maximum quantum yields remained high from salinities of 30 to 6. Leaf osmolality declined linearly with respect to media osmolality and the difference between leaf and media osmolality remained constant from salinities of 30 to 2. Antioxidant activity declined over time in both salinity reduction experiments and in all pulsed salinity treatments. Overall, the results indicate that *H. johnsonii* is more tolerant of hyposalinity than has previously been reported and that gradually reducing salinity extended its tolerance threshold by around a salinity of 10.
ACKNOWLEDGMENTS

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INTRODUCTION

*Halophila johnsonii* is one of the smallest seagrass species with a canopy height of 2-5 cm and considerably less biomass than other seagrass genera (Kenworthy 1997; Dean and Durako 2007; Virnstein et al. 2009). It also has limited reproductive capabilities, as only female flowers have been observed (Eiseman and McMillan 1980; Kenworthy 1997). *Halophila johnsonii* has a restricted geographical distribution, occupying around 200 km of coastal estuaries and lagoons from Sebastian Inlet (27°51’N, 80° 27’W) in the northern Indian River Lagoon (IRL) to North Biscayne Bay (25°45’N, 80°07’W), Florida (Eiseman and McMillan 1980; Virnstein and Hall 2009). Within its range, *H. johnsonii* has a patchy and disjunct occurrence, often appearing and disappearing from specific locations from one season to the next in so called “pulsating patches” (Kenworthy 1997; Dean and Durako 2007; Virnstein et al. 2009). Because of its small carbon store, limited reproductive capabilities and restricted geographical distribution, *Halophila johnsonii* is particularly susceptible to environmental disturbances and is the only marine plant listed as threatened under the US Endangered Species Act (63 FR 49035, Kenworthy 1997).

*Halophila johnsonii* is sensitive to changes in water quality such as turbidity, chromophoric dissolved organic matter (CDOM), temperature, and salinity (Kahn and Durako 2008). However, recent studies indicate that prolonged hyposalinity may be the most serious threat to *H. johnsonii* (Torquemada et al. 2005; Kahn and Durako 2008). Optimal growth and survival of *H. johnsonii* have been observed at a salinity of 30, decreasing under both hyper- and hypo-salinities (Torquemada et al. 2005). Decreased survivorship and photosynthetic rates were observed when *H. johnsonii* was immediately exposed to salinities of 10 and 0 (Torquemada et al. 2005) and 100% mortality of *H. johnsonii* has been observed after 10 days in a salinity of 10 (Kahn and Durako 2008). However, *H. johnsonii* can survive for several weeks at salinities of
20 and 15 (Gavin 2010).

Because of the closed nature and restricted circulation of lagoons and estuaries, both natural and anthropogenic disturbances can drastically alter their salinities, placing submerged plants under osmotic stress. Severe storm events like hurricanes create pulsed fresh water inputs, which have severely impacted seagrass beds, including *Halophila* species (Ralph 1998; Steward et al. 2006). It’s predicted that with climate change there will be more frequent and severe rainfall events in tropical regions that will lead to hypo-osmotic conditions (Steward et al. 2006). In 2004, four major hurricanes (Charley, Frances, Ivan, and Jeanne) directly impacted the east coast of Florida and generated runoff conditions that decreased water quality over much of the IRL (Steward et al. 2006). The large input of freshwater decreased salinities to $<15$, which led to significant declines in seagrass densities and changes in species composition (Steward et al. 2006).

Freshwater inputs from tropical storms are a natural process. However, humans have greatly modified the watersheds in Florida through a series of canals, levees, and water control structures, diverting a larger volume of freshwater into the habitats where *H. johnsonii* resides (Hall et al. 2010; IRL CCMP). Freshwater inputs often occur in pulses associated with flood-control canal releases, rapidly decreasing the salinity in localized to lagoonal-scale regions. The decrease in seagrass densities following the 2004 hurricanes highlights the negative effects of pulsed and prolonged hyposalinity on seagrass survival (Steward et al. 2006). Because *H. johnsonii* is negatively affected by hyposalinity, it’s important to determine the impact of freshwater releases via watershed management on its physiology and survival. This will allow implementation of adaptive water management practices to ensure this species’ continued existence.
Previous experiments have placed *H. johnsonii* directly in the treatment salinity, in what is termed a “pulsed” event (Torquemada et al. 2005; Kahn and Durako 2008; Gavin 2010). However, when gradually exposed to a change in salinity, seagrasses are able to osmoregulate (Jagels 1983) and they have been shown to tolerate a salinity of 10 higher when incrementally exposed to hypersaline conditions versus exposure to a rapid pulsed event (Koch et al. 2007). In this study, *H. johnsonii* was subjected to hyposaline conditions under both pulsed and gradual salinity reductions to determine (1) its minimum salinity tolerance and (2) if its tolerance threshold could be further extended if salinity was gradually reduced rather than pulsed. It was hypothesized that by gradually decreasing salinity, *H. johnsonii* would have sufficient time to osmoregulate and therefore tolerate lower levels of salinity than if exposed to a pulsed salinity decrease.
METHODS

Collection Site

*Halophila johnsonii* Eiseman transplants were collected on May 18, 2010 from Munyon Island, Lake Worth, Florida (26°49’N, 80°02’W) using a 9 x 9 cm sod plugger. This sampling location is located near the middle of the geographic range of this species. Transplants consisted of rhizome segments with > 4 leaf pairs and associated sediments, which were placed in 9 x 9 x 8 cm plastic pots. Transplants were transported in seawater-filled coolers to the University of North Carolina Wilmington Center for Marine Science (UNCW/CMS). Upon arrival (within 24 h of collection), the plant pots were transferred to fiberglass vaults supplied with flow-through seawater, which was kept at near-ambient temperatures (23°-29°C) to act as water baths for temperature regulation. There were four fiberglass vaults and each contained four aquaria (16 aquaria total). Temperatures in the treatment aquaria during the experiment varied between 24.4°C and 38.8°C. Plants were allowed to acclimate to a control salinity of 30 for 10 days.

Experimental design: pulsed hyposalinity

After the 10-day acclimation, three aquaria were held at a salinity of 30 while the salinity of the other nine aquaria was immediately decreased to salinities of 15, 10, or 8. Replicate aquaria (N=3) were staggered in position to offset any spatial differences in the location of the fiberglass tank. Physiological measurements of maximum quantum yield (pre-dawn), leaf osmolality, and total antioxidant activity, along with salinity and plant survivorship were obtained daily for the first week, once every two days during the second week, and once per week for the next two weeks. Within treatments, each plant pot was assigned a number (1-15); plants were randomly sampled using a random numbers table in Excel©. Salinity was checked daily with a
conductivity meter (YSI Model 80) and adjusted as needed to maintain treatment level (±0.2). Instant Ocean© salts and de-ionized (DI) water were used for salinity adjustments.

Experimental design: gradual hyposalinity acclimation

After acclimation, the salinity of three 40-1 aquaria in the fourth fiberglass vault was decreased by two every two days. The fourth aquarium was held at a salinity of 30 as a control. This gradual acclimation regime was chosen because it mimics natural field conditions and allowed for achievement of target salinity levels in a reasonable amount of time. Previous studies have used similar adjustment rates to test the salinity tolerance of seagrasses (Kahn and Durako 2005; Koch et al. 2007). Maximum quantum yield, osmolality and total antioxidant capacity, along with salinity and plant survivorship were measured 24 hours after salinity adjustment. Random sampling was accomplished in the same way as the “pulsed” treatments. Salinity was adjusted by adding Instant Ocean© salts or DI water.

Physiological measurements of stress: Fv/Fm

Measurements of maximum quantum yield were made using a pulse amplitude modulated (PAM) fluorometer (Mini-PAM, Walz GmbH), which is a non-destructive and non-invasive method used to assess plant health (Ralph 1998; Durako et al. 2003; Biber 2008; Chartrand et al. 2009). Maximum quantum yield measures the amount of light absorbed by a plant that is directed for photosynthesis, specifically the efficiency of the photosystem II, and is a common measure of stress in seagrasses (Ralph 1999). The leaf is exposed to a pulse of light during which two fluorescence measurements are made: one before (minimum fluorescence, Fo) and
one during the burst of light (maximum fluorescence, Fm). Maximum quantum yields are calculated as Fv/Fm, where Fv= Fm-Fo. All measurements were performed on the second intact leaf pair back from the rhizome apical meristem. Measurements were made at pre-dawn on dark-acclimated leaves to represent the maximum potential of photosystem II as all reaction centers are open and primary electron acceptors able to be oxidized (Murphy et al. 2003). A leaf clip was used to ensure equal distance and geometry between the fiber optic tip and leaf tissue.

The fluorescence signal was obtained from approximately the middle of the adaxial side of the leaf. Maximum quantum yields nearing zero were indicative of mortality. Additionally, mortality was indicated by the absence of leaves.

Physiological measurements of stress: osmolality

Leaf osmolality was measured using a Wescor VAPRO Vapor Pressure Osmometer 5520© following the protocols described by Murphy et al. (2003), Kahn and Durako (2006), and Koch et al. (2007). The osmometer measures the total concentration of dissolved particles using a vapor-point depression. When measuring solid tissue samples, a time delay had to be used to allow for vapor pressure and temperature to equilibrate. To determine the appropriate time delay for *H. johnsonii* leaf tissue, initial time delay readings were made every 2 min for 30 minutes. After 10 minutes there was no significant change in readings ($F_{1,32}=0.86$, $P=0.58$, data not shown).

To measure leaf osmolality, one mature leaf from each replicate aquarium was cut under water and submerged in a 15 mL centrifuge tube. A 10 mm section of leaf tissue was cut from the leaf, blotted with a Kimwipe to remove media liquid, and placed on the osmometer sample holder. Readings were taken after 10 minutes. Osmolality was also measured on 10 µL samples
of the treatment media to allow for comparison of leaf tissue to treatment salinity.

Physiological measurements of stress: total antioxidant activity

Antioxidant activity of leaf cells was measured using the trolox equivalent antioxidant capacity (TEAC) assay (Sigma-Aldrich). The assay uses different concentrations of Trolox (1-10µM), a vitamin E analog, as a quantitative reference for antioxidant activity (Re et al. 1999). The percent inhibition of absorbance at 734 nm is calculated as a function of decolorization by the ABTS•+ radical:

\[
\% \text{ inhibition} = \frac{A_{\text{734 initial}} - A_{\text{734 final}}}{A_{\text{734 initial}}}
\]

*Halophila johnsonii* leaves were extracted in methanol for 24 h in the dark at 7.8°C. Absorbance of 7 mM ABTS (990 µL) at 734 nm was measured. 10 µL of the leaf methanol extract was added to the ABTS solution and absorbance was measured 4 min after initial mixing. Decolorization in leaf extracts was compared to a Trolox standard curve (0 to 10 µM) to determine the level of antioxidant activity (µM Trolox g Fresh weight\(^{-1}\)) in *H. johnsonii* leaf tissue.

Statistical analyses

Statistical analyses were performed using JMP7® and SigmaStat® software. Two-way fixed factor ANOVAs (factor one salinity, factor two time) were used to determine the strength of each factor and their combined interaction on each of the physiological measurements (maximum quantum yield, leaf osmolality, antioxidant activity) in the pulsed salinity treatments over time. Linear regressions and one-way fixed factor ANOVAs were used to determine if there were
significant changes (P<0.05) in each physiological measurement (maximum quantum yield, leaf osmolality, antioxidant activity) over time in the gradual salinity reduction. Tukey’s honestly significant difference (HSD) test was performed when a significant effect was detected. Normality of data was tested using the Shapiro Wilk Goodness of Fit test. When normality failed, log transformations were attempted, if transformation failed, non-parametric tests (Kruskal Wallis) were performed.
RESULTS

Pulsed Salinity Reduction

In the pulsed salinity treatments, there was high survivorship of *Halophila johnsonii* in the control (30) and salinity of 15 treatments over the duration of the mesocosm experiment. However, there was a relatively linear decline in survivorship in the 10 and 8 salinities over time. Survivorship was consistently lowest in the pulsed salinity treatment of 8 (Figure 1).

Both salinity and time had significant effects on maximum quantum yield (Fv/Fm) values of *H. johnsonii* (salinity: $F_{2,3}=9.12$, $P<0.0001$; time: $F_{2,12}=3.11$, $P=0.0008$) but there was no significant salinity*time interaction ($F_{2,36}=1.52$, $P=0.052$). There was no difference in maximum quantum yield between the control (30) and 15 salinity treatments; additionally, regression analyses indicated that their yields did not change over time (Figure 2, Table 1). However, the maximum quantum yields of individuals in the pulsed salinity treatments of 10 and 8 were significantly lower than the control and 15; additionally, regression analyses indicated that their yields declined significantly over time (Figure 2, Table 1).

Salinity had a significant effect on leaf tissue osmolality of *H. johnsonii* in the pulsed salinity treatments but time did not (salinity: $F_{2,3}=110.08$, $P<0.0001$; time: $F_{2,12}=0.81$, $P=0.81$) nor was there a salinity*time effect ($F_{2,36}=1.09$, $P=0.36$). There was a direct, linear relationship between leaf and media osmolality (Figure 3) as the slope of the line regressing leaf tissue against media osmolality does not differ significantly from one ($t_{177}=-0.113$, $P=0.91$).

The difference in osmolality between leaf tissue and media did not differ significantly among the four pulsed salinity treatments or over time ($F_{51,104}=0.88$, $P=0.69$). *Halophila johnsonii* plants in the four pulsed salinity treatments maintained an internal leaf tissue osmolality that was $675\pm177$ mmol kg$^{-1}$ greater than their media.
Figure 1. Survivorship (percent % alive) of *Halophila johnsonii* in each of the four pulsed salinity treatments (30, 15, 10, 8). Measurements of survivorship began on day 7 of mesocosm.
Figure 2. Maximum quantum yields (Fv/Fm) of *Halophila johnsonii* in the four pulsed salinity treatments: (a) 30, (b) 15, (c) 10, (d) 8. The mean ± standard deviation are represented (N=3).
Table 1. Linear regression analysis for maximum quantum yields (Fv/Fm) of *Halophila johnsonii* in the pulsed salinity treatments over the duration of the mesocosm. An asterisk (*) indicates significance (P < 0.05).

<table>
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<tr>
<th>Salinity</th>
<th>$r^2$</th>
<th>F-value</th>
<th>df</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>30</td>
<td>0.02</td>
<td>1.21</td>
<td>1.64</td>
<td>0.27</td>
</tr>
<tr>
<td>15</td>
<td>0.01</td>
<td>0.48</td>
<td>1.37</td>
<td>0.49</td>
</tr>
<tr>
<td>10</td>
<td>0.12</td>
<td>4.89</td>
<td>1.37</td>
<td>0.03*</td>
</tr>
<tr>
<td>8</td>
<td>0.12</td>
<td>4.99</td>
<td>1.37</td>
<td>0.03*</td>
</tr>
</tbody>
</table>
Figure 3. Osmolality of *Halophila johnsonii* leaf tissue compared to media for each of the four pulsed salinity treatments (30, 15, 10, 8). The slope of the regression line does not differ significantly from one (dotted line). The mean ± standard deviation are represented (control N=62, treatment N=39).
Antioxidant activity of *Halophila johnsonii* did not differ among pulsed salinity treatments however, it did decline significantly over time (salinity: $F_{2,3}=1.63$, $P=0.19$; time: $F_{2,7}=14.15$, $P<0.0001$). There was no salinity*time effect ($F_{2,21}=1.58$, $P=0.08$). Pooled treatment antioxidant activity of *H. johnsonii* declined significantly by day seven ($F_{1,98}=13.25$, $P<0.0001$; Figure 4).

When examining the antioxidant activity of plants in each salinity treatment over time, those individuals in the control had significantly higher antioxidant activity on day four than all other days ($F_{1,27}=10.23$, $P<0.0001$; Figure 5); plants in salinity of 15 exhibited no change in antioxidant activity over time ($F_{1,22}=1.95$, $P=0.13$), though their highest average values were on day one and lowest on day 13 (Figure 5). Plants in the salinity of 10 had the lowest average antioxidant activity on day 13 and the next lowest on day 27 (Figure 5); and antioxidant activity on days 2, 7, 13, 21, and 27 were significantly lower than day 1 ($F_{1,27}=11.89$, $P<0.0001$).

*Halophila johnsonii* plants in the treatment salinity of 8 had the lowest antioxidant activity on day 27, which was significantly lower than days 1, 3, and 4 ($F_{1,23}=5.04$, $P=0.004$; Figure 5).
Figure 4. Total antioxidant activity (μM Trolox g fwt⁻¹) of *Halophila johnsonii* over time using pooled data from four pulsed salinity treatments. Data were pooled because salinity did not have a significant treatment effect on leaf antioxidant activity. The mean ± standard deviation are represented (N=13).
Figure 5. Total antioxidant activity (μM Trolox g fwt⁻¹) of *Halophila johnsonii* plants in four pulsed salinity treatments (30, 15, 10, 8). The mean ± standard deviation are represented (N=3).
Gradual Salinity Reduction

When salinity was gradually reduced, there was 100% survivorship of *Halophila johnsonii* from salinities 30 to 22, and 80% from salinities 20 to 4 (Figure 6). Survivorship decreased to 60% when a salinity of 2 was reached and finally to 0% at salinity of zero (Figure 6). In the gradual salinity reduction treatment, maximum quantum yield values of *H. johnsonii* remained high and did not differ significantly until a salinity of 6 (day 23), after which they began to decline significantly (*F*$_{1,47}$=42.16, *P*<0.0001; Figure 7). Tukey’s HSD indicated that yields at salinities of 4 and 2 were significantly lower than salinity of 6 and salinity of 0 yields were significantly lower than all other salinities.

As salinity was gradually reduced, leaf tissue osmolality significantly differed (*F*$_{1,47}$=22.22, *P*<0.0001; Figure 8). There was a direct, linear relationship between leaf versus media osmolality as the slope of the line regressing leaf tissue against media osmolality does not differ significantly from one (*t*$_{46}$=1.12, *P*=0.27; Figure 8). From salinities 30 to 2, the difference in osmolality between leaf tissue and media did not differ significantly (*F*$_{1,44}$=1.55, *P*=0.15). From salinities 30 to 2, *H. johnsonii* maintained an internal osmolality in their leaf tissue that was 638±161 mmol kg$^{-1}$ greater than their aqueous medium. It was not until freshwater conditions that the difference in osmolality between leaf and media neared zero. Furthermore, the difference in osmolality between leaf tissue and media did not differ significantly between plants in the pulsed and gradual salinity reductions (*t*$_{222}$=1.23, *P*=0.22).

As salinity was gradually reduced, there was a significant decline in antioxidant activity over time (*r*²=0.65, *F*$_{1,21}$=36.49, *P*<0.0001). Antioxidant activity was significantly lower by salinity of 16 (day 13) after which it continued to decline but not significantly (*F*$_{1,21}$=12.85, *P*<0.0001; Figure 9).
Figure 6. Survivorship (percent % alive) of *Halophila johnsonii* in response to gradual salinity reduction.
Figure 7. Maximum quantum yields (Fv/Fm) of Halophila johnsonii in response to gradual salinity reduction. The mean ± standard deviation are represented (N=3).
Figure 8. Osmolality of *Halophila johnsonii* leaf tissue compared to media osmolality in response to gradual salinity reduction. The slope of the line does not differ significantly from one (dotted line). The mean ± standard deviation are represented (N=3).
Figure 9. Antioxidant activity (µM Trolox g fwt⁻¹) of Halophila johnsonii plants in response to gradual salinity reduction. The mean ± standard deviation are represented (N=3).
DISCUSSION

The results of this study indicate that *Halophila johnsonii* is tolerant of hyposalinity and can be considered a euryhaline species. Survivorship of plants in the control and salinity treatment of 15 remained high (~80%) by day 30 of the mesocosm experiment (Figure 1). Although survivorship declined over time, 40% of plants in the salinity treatment of 10 were still alive by day 30 (Figure 1), a value much higher than has previously been reported (Torquemada et al. 2005; Kahn and Durako 2008). Kahn and Durako (2008) reported 100% mortality of *H. johnsonii* plants after 10 days at a salinity of 10. The different responses of *H. johnsonii* to hyposalinity may be due to plant origin and mesocosm design. Individuals in this study were collected from Munyon Island, Florida where the salinity averages around 32, but can vary from 37 to 15, more typical of an estuarine environment (SFWMD.gov). *Halophila johnsonii* plants from Kahn and Durako (2008) were obtained from Jupiter Inlet, Florida, where salinity only varies from 32 to 30, more characteristic of a marine environment (CIRP 2011). A similar survivorship trend was observed with marine versus estuarine *Halophila ovalis* plants, where marine plants were intolerant to low salinities but estuarine plants thrived (Benjamin et al. 1999). The different salinity tolerances of *H. johnsonii* observed in this mesocosm and Kahn and Durako (2008) suggest that these two populations represent different ecophenes.

Another explanation for the high survivorship in the salinity treatments of 10 and 8 may be due to mesocosm design. Previous studies on *H. johnsonii* have directly placed plants in treatment salinities within 24 hours of collection and subsequently observed high mortality in hyper- and hypo-salinity treatments (Torquemada et al. 2005; Kahn and Durako 2008; Gavin 2010). Plants in this study were allowed to acclimate for ten days to control salinity conditions before the salinity reduction treatments were initiated. This acclimation period most likely
allowed enough time for recovery from transplant shock and for new growth under mesocosm conditions.

Maximum quantum yield values of plants in the pulsed salinity treatments of 10 and 8 were significantly lower than those in the treatment salinities of 30 and 15 (Figure 2), and values in these two lowest treatments declined over time (Table 1). Maximum quantum yield values for healthy non-stressed seagrasses range between 0.7-0.8 (Ralph 1999; Durako et al. 2003). Plants in the control and salinity treatment of 15 had average yields within this range (Figure 2). However, yields of plants in the pulsed salinity treatments of 10 and 8 varied over time and often fell below 0.7 (Figure 2), indicating that photosynthetic efficiencies were compromised and that plants were stressed at these two lowest salinities. Previous studies have also measured decreases in photosynthetic parameters of *H. johnsonii* when exposed to hyposaline conditions (Torquemada et al. 2005; Kahn and Durako 2008).

There was a direct, linear relationship between leaf osmolality and treatment salinity in the four pulsed salinity treatments (Figure 3). However, the difference in osmolality between leaf and media did not differ among salinity treatments or over time; *H. johnsonii* remained hyperosmotic to its environment by $675\pm 177$ mmol kg$^{-1}$. The seagrasses, *Thalassia testudinum, Halodule wrightii*, and *Ruppia maritima*, also maintain an internal osmolality that is hyperosmotic to the media and show a linear trend between leaf osmolality and media salinity (Murphy et al. 2003; Kahn and Durako 2006; Koch et al. 2007).

As halophytes, seagrasses undergo both physical and mechanical changes to deal with salt stress; this includes managing turgor pressure, synthesizing compatible solutes in the cytosol, and accumulating ions inside of vacuoles such that the plant is hyperosmotic to its environment and water will flux in (Touchette 2007). Under hypo-osmotic stress, plants will release ions
from their vacuoles and degrade or metabolize compatible solutes (Bisson and Kirst 1995). In this mesocosm experiment, cytoplasmic non-vacuolar osmolality was measured because osmolality readings were made on fresh *H. johnsonii* leaves (Murphy et al. 2003; Koch et al. 2007). This suggests that the concentration of compatible solutes in *H. johnsonii* leaf cells decreased under increased hyposalinity. This is consistent with data for *Ruppia maritima* in which the concentration of proline, a primary compatible solute, decreased under hyposaline conditions as well (Murphy et al. 2003).

Hyposalinity stress is thought to act in the same way as high temperature stress by destabilizing membranes (Los and Murata 2004). Besides helping to maintain an equal osmotic potential across a cell, compatible solutes also function to stabilize membranes (Bisson and Kirst 1995). The decreased concentration of solutes with increased hyposalinity observed in this study may explain the decrease in maximum quantum yields observed in both the pulsed and gradual salinity reductions as stability of thylakoid membranes was lost (Figures 2 and 7).

Compatible solute synthesis and ion sequestering are slow processes (hours to days) and function in longer-term salinity adjustments (Murphy et al. 2003; Touchette 2007). To initially respond to salinity change, seagrasses control water flux by the hydraulic conductivity of the plasma membrane and elastic properties of the cell wall (Tyerman 1982; Touchette 2007). When exposed to a hyposaline solution, there is an immediate influx of water to cells. The elastic properties of the cell wall will determine how much water fluxes in. Cells with flexible cell walls have a low elasticity and will swell when exposed to a high osmotic flux; cells with rigid cell walls have high cell wall elasticity and prevent water from entering (Touchette 2007).

*Halophila ovalis*, a euryhaline seagrass species able to tolerate salinities from 10 to 45, has a high cell wall elasticity but because of a rudimentary cuticle, can adjust to hypo-osmotic
conditions within 24 hours (Ralph 1998). A rigid cell wall may explain why many seagrasses can tolerate hyposalinity better than hypersalinity (Ralph 1998). The leaves of *H. johnsonii* are only two cells thick at the margins, which would allow for rapid exchange between cells and the environment, and subsequent adjustment to salinity change. Further studies measuring the cell wall elasticity or turgor pressure of *H. johnsonii* would provide insight into the mechanisms used to achieve short-term osmotic equilibrium during pulsed salinity changes.

During the pulsed salinity reduction, it was observed, beginning at around day 13, that leaves in the lowest pulsed salinity treatments (10 and 8) were often wrinkled along the margins. It has been noted in other seagrass species that extreme hyposalinity conditions can cause physical damage (Ralph 1998). Rapid leaf senescence and wrinkled leaf margins have been observed in *H. ovalis* under hypo-osmotic conditions (Ralph 1998; Benjamin et al. 1999). When a constant stress, such as fresh water influx from hyposalinity, is applied to a cell wall, deformation occurs (Jagels 1973; Tyerman 1982). Because *Halophila johnsonii* is only two cells thick at the margins, any cell deformation would be observed phenotypically. Wrinkling of *H. johnsonii* leaves at the margins observed in the low salinity aquaria, may be the phenotypic result of cell deformation under the constant stress of low salinity.

Total antioxidant activity did not differ with respect to salinity but it did differ with respect to time. Under stress, free radicals and reactive oxygen species (ROS: O$_2^-$, OH, H$_2$O$_2$) accumulate and damage plant cells (Yang et al. 2006; Touchette 2007). To counteract these harmful products, plants produce antioxidants (xanthophylls, peroxidases, and catalases) (Touchette 2007). Plants increase antioxidant concentrations in order to prevent damage from oxidative stress (Noctor and Foyer 1998). Therefore, it was expected that antioxidant activity would increase under hypo-osmotic stress, however a decrease in total antioxidant activity was
observed in all treatments, including the control (Figures 4 and 5). One explanation for this measurable decrease may be that under hypo-osmotic stress, antioxidants are consumed at a high rate, which may be faster than they are regenerated (Lu et al. 2006). The TEAC assay depends on the antioxidant’s ability to reduce the ABTS* radical (Re et al. 1999), which may be lowered if the antioxidants are being rapidly oxidized by ROS. Future studies examining the concentrations of specific antioxidants and enzymes could elucidate more detailed antioxidant responses. Another explanation for the observed decrease in total antioxidant activity may be that the mesocosm had a treatment effect on the plants, as antioxidant activity of plants in the control treatments decreased as well.

In the gradual salinity reduction, there was a tiered response to stress manifestation: total antioxidant activity declined significantly by salinity of 16 (Figure 9); from salinities 30 to 6, maximum quantum yields ranged between 0.7 and 0.8, suggesting that plants did not compromise their photosynthetic efficiencies until a salinity of 4 when yields fell below 0.7 (Figure 7); survivorship of plants remained high (80-100%) from salinities 30 to 4 and did not reach 0% until fresh water conditions (Figure 6); and lastly, the difference in osmolality between leaf and media did not differ from salinities 30 to 2, suggesting that plants were osmoregulating to maintain a relatively constant level of cell turgor until freshwater conditions (Figure 8). Since cell turgor is necessary in maintaining cell and plant form, and essential for normal cell functions (Bisson and Kirst 1995; Taiz and Zeiger 2006), it seems logical that this would be the last process to fail. Gradually reducing the salinity, compared to pulsed salinity reduction, extended the thresholds of all four of the parameters measured (Table 2).

Gradually introducing an osmotic stress allowed time for *H. johnsonii* to acclimate and therefore extend its survivorship. This increased tolerance pattern to hyposalinity is similar to
Table 2. The lowest salinity that plants were able to tolerate without showing signs of stress in the pulsed versus gradual salinity reductions. Time is represented for antioxidant activity in the pulsed reduction since salinity did not have a significant effect.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Pulsed</th>
<th>Gradual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fv/Fm</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>Leaf osmolality</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Antioxidant activity</td>
<td>Day 7</td>
<td>Day 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(salinity 16)</td>
</tr>
<tr>
<td>Survivorship</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>Overall</td>
<td>15</td>
<td>6</td>
</tr>
</tbody>
</table>
the increased tolerance to hypersalinity exhibited by *Thalassia testudinum*, *Halodule wrightii*, and *Ruppia maritima* when exposed to gradually increasing salinities versus pulsed salinity increase (Koch et al. 2007). Under hypo-osmotic stress, plants must transport inorganic ions out of their vacuoles and cells and degrade or metabolize organic solutes in order to maintain a relatively constant cell turgor (Bisson and Kirst 1995). However, since these processes are slow (hours to days) gradually reducing the salinity by two every two days most likely allowed enough time for these processes to occur and to counteract the influx of fresh water.

Conclusions

*Halophila johnsonii* exposed to a gradual salinity reduction was able to extend its minimum salinity tolerance by almost 10, based on the parameters measured in this study (survivorship, maximum quantum yields, osmolality, and antioxidant activity). When salinity was pulsed, the lowest salinity *H. johnsonii* could tolerate without showing signs of stress was 15. However, when salinity was gradually reduced plants did not begin to show signs of physiological stress or decreased survivorship until a salinity of 6 reached (Table 2). The results of this mesocosm study also indicate that there is a primacy given to osmoregulation and the maintenance of cell turgor, as *H. johnsonii* remained at a relatively constant hyperosmotic condition to its environment while other physiological processes, like photosynthesis and antioxidant activity, declined.

With climate change, it is anticipated that there will be more storm events that will increase the volume of freshwater naturally entering the estuaries and lagoons of south Florida (Steward et al. 2006). Coupled with human alteration of watersheds, which diverts additional
freshwater into these coastal systems, the resulting salinity changes may have significant implications for the species living within these systems. Understanding how reduced salinities affect submerged marine angiosperms, like *H. johnsonii*, will allow for implementation of adaptive management practices to ensure their survival. The results of this study suggest that *H. johnsonii* is more tolerant of hyposalinity than has previously been reported and that gradually reducing salinity further extends its tolerance threshold. Future investigations should focus on *H. johnsonii’s* resilience and ability to recover from low salinities at both the physical and cellular levels.
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