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Localization and antioxidant capacity of flavonoids in *Halophila johnsonii* in response to experimental light and salinity variation

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ABSTRACT

The threatened seagrass *Halophila johnsonii* contains UV-absorbing flavonoids localized in the cuticle and cytosol of epidermal cells in both abaxial and adaxial leaf surfaces. Previous studies indicate that these compounds exhibit variations in response to salinity stress and may have physiological roles other than UV protection. In this study, controlled light and salinity mesocosm experiments were performed to investigate effects of salinity variation (35, 25 and 15) with or without light reduction in order to elucidate possible physiological roles of flavonoids in *H. johnsonii.* Response variables were measured over short (one day) and extended (three week) time periods and included flavonoid content and localization, Trolox equivalent antioxidant capacity (TEAC), and chlorophyll fluorescence parameters. Confocal laser scanning microscopy (CLSM) indicated that distribution of flavonoids in the cuticle and cytosol of abaxial and adaxial leaf epidermal cells did not change in response to the salinity or light treatments. Likewise, salinity and light did not have a significant effect on total flavonoid content overall, although within day 1, flavonoid content was significantly higher among shade treatments. Among salinities, TEAC values were significantly higher for salinity 15 at 14 and 21 days; TEAC values were similar between shaded and unshaded treatments. Neither salinity nor light had significant treatment effects on individual photosynthetic parameters, but a reduction of salinity and light did have a significant interactive effect as rapid light curves (RLCs) for salinity 15 and 25 shade treatments were significantly lower than all other treatments. Flavonoids were significantly correlated with TEAC values, suggesting a possible antioxidant function for cytosolic flavonoids in *H. johnsonii*. However, a sunscreen role for cuticular flavonoids in this species was not supported as total flavonoid content tended to be higher in shaded plants and intracellular flavonoid distribution was unaffected by light conditions.

1. Introduction

Halophila johnsonii Eiseman is a threatened seagrass that grows intertidally to depths of approximately 3–4 m and has a distribution that is limited to a 200 km section of southeastern Florida (Kenworthy, 1993; Virnstein and Morris, 2007). *H. johnsonii* contains 15 flavonoids (10 flavone glycosides and 5 flavones) that strongly absorb UV radiation (absorption peak of 343–348 nm, Meng et al., 2008). Experimental manipulations of photosynthetically active radiation (PAR) and UV levels did not elicit consistent changes in flavonoid quantity (Kunzelman et al., 2005) and results of subsequent mesocosm experiments suggested that flavonoid quantity responded more strongly to salinity variation than to variations in light quality (Kahn and Durako, 2008). However, no study has yet observed whether the cellular localization of flavonoids in *H. johnsonii* varies in response to such treatments.

Plants vary flavonoid content and localization in response to environmental stimuli (Gould and Lister, 2006; Hutzler et al., 1998). Hyposalinity-induced reduction in photosynthesis may lead to the production of free radicals and reactive oxygen species (ROS) and plants may increase their ability to scavenge ROS during hyposalinity stress by increasing their antioxidants (Lu et al., 2006). In addition, excess light energy may also generate free radicals and ROS, which if not reduced into less harmful substances may damage nuclear DNA, proteins and lipids (Yang et al., 2006). Depending on their tissue location, flavonoids could act in photoprotection against high solar irradiance by directly absorbing incident photons (i.e., act as sunscreens), or indirectly, as a result of their antioxidant activity (Bohnert and Jensen, 1996; Gould and Lister, 2006; Rice–Evans et al., 1997; Tattini et al., 2004). Cytosolic flavonoids may serve primarily as effective antioxidants while cuticular, vacuolar and cell-wall bound flavonoids can be more important in shielding chloroplasts from excess high-energy quanta (Hutzler et al., 1998; Neil and Gould, 2003; Tattini et al., 2004). In addition, as soluble sugars, flavone glycosides produced by *H. johnsonii* may function as compatible solutes (Manetas, 2006). Halophytic plants control vacuolar osmolality using ions, while the osmolality of the cytosol is affected by both ions and compatible solutes. Ion accumulation is beneficial for initial osmotic adjustments but may be insufficient in prolonged episodes of salinity stress (Murphy et al., 2003). To survive

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prolonged periods of salinity stress, halophytic plants maintain or restore turgor pressure through the accumulation of compatible solutes in cell cytoplasm (Wyn Jones and Gorham, 1983).

Flavonoids in subtidal and intertidal *H. johnsonii* are located in the cuticle and cytosol of both abaxial and adaxial leaf epidermal cells (Gavin and Durako, 2011). This distribution pattern would allow them to perform both sunscreen and antioxidant functions. In contrast, flavonoids only occur in the cuticle of the subtidal congeneric *Halophila decipiens*, suggesting that they do not perform an antioxidant function in this species. Due to the influence intracellular location has on flavonoid function *in planta* the present study compared the effects of controlled hyposaline and light-reduction treatments on flavonoid content and localization in *H. johnsonii* using mesocosm experiments. Hyposalinity was examined because of its hypothesized importance in limiting the distribution of this threatened species (Virnstein and Hall, 2009). Antioxidant capacities of *H. johnsonii* leaf extracts were also evaluated to determine the relationship between total flavonoid content and antioxidant capacity in response to treatments. The objective was to assess the possible functions of flavonoids as compatible solutes, sunscreens or antioxidants in this species.

2. Materials and methods

2.1. Sample collection

All plants were collected on August 17, 2009 at Munyon Island in Lake Worth Lagoon, Florida (26.82054° N, 80.04604° W). A 10×10 cm sod plugger was used to extract rhizome segments of three to five leaf pairs from subtidal *H. johnsonii*. Sod plugs were transplanted into 9×9×9 cm plastic planting pots pre-sterilized with bleach and placed in coolers filled with ambient seawater. All plant materials were transported to the Center for Marine Science in Wilmington, NC within 24 h.

2.2. Mesocosm salinity and light manipulations

Experimental mesocosm treatments consisted of three salinity (15, 25 and 35) and two light (shade and light) treatments. Salinity treatments were established in twelve 38 L glass aquaria using deionized (DI) water and Instant Ocean© synthetic sea salts; Von Stosch's enrichment media was also added to provide essential nutrients. Control-treatment salinity was 35, which is within the optimal range of *H. johnsonii* for photosynthesis (Dawes et al., 1989; Torquemada et al., 2005). Salinity was not decreased below 15 because previous studies have shown 100% mortality of *H. johnsonii* at salinity 10 after 10 days (Kahn and Durako, 2008). Shade treatments were created by covering half of each aquarium with a neutral density filter (37.6% mean reduction in PAR). A LICOR scalar (4π) quantum sensor was placed within a light and shade treatment aquaria at leaf canopy height and photosynthetically active radiation (PAR) was recorded at 30 minute intervals with a 5 min running average. Average daytime PAR values 24 h prior to sample collection were used to determine recent PAR history for days 1, 7, 14 and 21. Water temperature (°C) in treatment aquaria was monitored with HOBO® water temp pro data loggers and temperature was recorded at 30 min intervals, with a 1 min running average. Median temperatures 24 h prior to sample collection were used to determine recent temperature history for days 1, 7, 14 and 21. Treatment-aquaria were randomly assigned among four fiberglass mesocosm tanks, which were flushed with seawater from adjacent Masonboro Sound to regulate temperature. Mesocosms were placed on an outdoor platform arranged in an east–west orientation to reduce shading by mesocosm walls. Within 24 h post collection, four planting pots were placed into each salinity and light treatment for a total of 96 individual pots of *H. johnsonii*. Salinity was monitored and adjusted when necessary every two days using DI water and Instant Ocean© synthetic sea salts. After one day, and then one, two and three weeks under experimental conditions, the second leaf pair behind the rhizome apical meristem was collected from a single pot from each treatment aquaria for a total of four replicate leaf pairs for each treatment. One leaf was randomly selected for analysis of antioxidant capacity and flavonoid content; the second leaf was examined using confocal laser scanning microscopy (CSLM) to identify sub-cellular distribution of flavonoids.

2.3. Leaf area measurement and pigment extraction

Leaf tissue samples were placed between two glass microscopy slides adjacent to a ruler and photographed perpendicularly from above for determination of leaf area. The camera was placed on a tripod at a fixed distance (0.3 m) above the sample and a bubble level was placed on the camera to maintain a consistent photographic angle and distance. Leaf area was calculated from digital images using Image-Pro Plus © software (mean leaf area = 30.42 ± 2.07 mm^2). After being photographed, leaves selected for analysis of antioxidant capacity and flavonoid content were ground in the dark in a chilled mortar with 6 mL cold, HPLC grade methanol. Samples were allowed to extract in the dark overnight in refrigerated conditions and supernatant was collected after samples were centrifuged at 12,000 *g* for 5 min at 2 °C using a Beckman Coulter™ Avanti ® J-25 high-performance centrifuge.

2.4. Flavonoid content assay

Total flavonoid content was determined according to the method of Ordoñez et al. (2006). To 0.5 mL of methanol extract, 0.5 mL of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm using an Ocean Optics© USB4000 fiber optic spectrometer and a Mini-D2T© halogen/deuterium light source. Flavonoid content was calculated as nmol quercetin equivalent (molecular weight = 338.3 g mol^{-1}) based on a five point calibration curve prepared each day before measurements began and normalized to leaf area (mm^2).

2.5. Antioxidant capacity assay

The Trolox equivalent antioxidant capacity (TEAC) of methanol leaf extracts was determined using the improved ABTS (2, 2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay method described by Re et al. (1999). This spectrophotometric technique measures the relative ability of antioxidants to scavenge the long-lived ABTS radical cation chromophore ($ABTS^{\cdot+}$) in relation to that of Trolox, a water-soluble derivative of vitamin E (molecular weight = 250.29 g mol^{-1}). $ABTS^{\cdot+}$ was produced by reacting equal quantities of 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The solution was then diluted with methanol until an absorbance of 0.70 ± 0.02 units at 734 nm was obtained. A reagent blank was also taken. 10 μL of plant extract was added to 990 μL of $ABTS^{\cdot+}$ solution and absorbance at 734 nm was measured 4 min after initial mixing. Absorbances were read using a spectrophotometer (Milton Roy Spectronic 401) and a dose–response curve for Trolox over the range of 0–20 nmol was obtained. Results were corrected for dilution and expressed in nmol Trolox mm^{-2} leaf.

2.6. Confocal laser scanning microscopy (CLSM)

CLSM was used to visualize sub-cellular accumulation and localization of flavonoids in fresh plant tissues using an Olympus FV 1000 confocal microscope system with the Olympus Fluoview FV1000 version 1.6a software. CLSM gives the ability to analyze images from selected depths in the z direction, which can be viewed individually or combined to create a three-dimensional reconstruction of the sample (Z-

stack). Non-colored and non-fluorescent flavonoids were visualized through fluorescence induced by staining with diphenylboric acid 2-aminoethyl ester (Naturstoffreagenz A or NA). NA is a flavonoid-specific fluorescent stain that induces secondary fluorescence of flavonoid pigments and allows their sub-cellular distribution to be visualized by comparing the epifluorescence between unstained and stained specimens (Hutzler et al., 1998). Cross sections (approx. 15–30 μm thick and 4–5 cells wide) of fresh *H. johnsonii* leaves were obtained free hand using a razorblade. While cells along the edge of leaf sections were perforated during sample preparation, cells within the section remained intact. To avoid artifacts due to cell damage, optical sections (0.75–1.25 μm thick) and Z-stack images of both perforated and intact cells were compared to determine flavonoid localization. Sections were incubated on microscopic slides in a droplet (100 μL) of filtered seawater under a coverslip, and autofluorescence of the sample was recorded by CLSM. Leaf sections were then stained by adding a 100 μL drop of 0.1% (w/v) Naturstoffreagenz (NA) in filtered seawater under the coverslip using filter paper. The section was placed in the dark for 15 min and then excess stain was removed using filter paper to draw filtered seawater under the coverslip. Fluorescence-emission spectra were again collected using CLSM. An argon–ion laser was used for excitation ($\lambda_{exe} = 405$ nm). Specific band-pass filters (BP) were used to select three pseudo–colored emission channels to distinguish between major peaks in emission spectra where 'blue' (BP 400–430 nm) corresponded to autofluorescence from the cuticle; 'green' (BP 515–565 nm) corresponded to NA-stained flavonoid compounds and 'red' (Longpass filter > 647 nm) corresponded to chlorophyll fluorescence.

2.7. Chlorophyll fluorescence measurements

At week three of the mesocosm experiments, maximum quantum efficiencies and rapid light curves (RLC) were obtained from plants in each treatment using a portable pulse amplitude modulated fluorometer (Mini-PAM, Walz, Germany). Leaf pairs from the second node back from a primary apical bud were sampled to minimize age-related differences in photosystem development. Blades were gently wiped to reduce epiphyte and detritus cover. A dark leaf clip held the instrument's fiber optic 5 mm from the adaxial leaf surface. These clips standardize the geometry of the leaf surface illuminated and exclude ambient light during fluorescence measurements. Maximum quantum efficiency measurements were obtained pre-dawn (05:30–06:00 h). Minimum fluorescence was first measured (F_0). The leaf tissue was then subjected to a pulse of saturating light during which a second fluorescence reading was taken (F_m). Maximum (*i.e.*, dark-acclimated) quantum efficiencies, which measure maximum photochemical efficiency of PSII, were then calculated $((F_m - F_0)/F_m) = F_v/F_m$. RLCs were measured later that morning (10:00–11:00 h). For the RLCs, nine consecutive light levels of 0, 20, 49, 82, 136, 194, 298, 418 and 654 μmol photons $m^{-2}\ s^{-1}$ were applied at 10 s intervals. Relative electron transport rates (rETR, Beer et al., 2001) were estimated using the following equation: $rETR = ([F_m' - F_s])/F_m' * PPFD * 0.5$; where F_m' = light-acclimated maximal fluorescence, F_s = steady-state chlorophyll fluorescence yield in the light adapted state, PPFD = intensity of PAR at the corresponding RLC irradiance step and 0.5 assumes half of the photons are absorbed by photosystem II. Mean values of photosynthetic efficiency at subsaturating irradiance (α), irradiance at onset of saturation (E_K), and maximal relative electron transport rate ($rETR_{max}$) were calculated for each treatment from RLC's using a double exponential decay function as described by Ralph and Gademann (2005).

2.8. Statistical analysis

Three-way ANOVAs were used to examine the significance of treatment effects (date, salinity and light) and their interactions (date * salinity, date * light, salinity * light and date * salinity * light) on flavonoid content and antioxidant capacity. Normality was tested using the Shapiro–Wilk test of normality and homogeneity of variance was tested using the Levene Median test. When tests for normality failed, Kruskal–Wallis one-way ANOVA on ranks was applied. When a statistically-significant difference was identified, an all pairwise multiple comparisons test was used to isolate where those differences occurred. The Holm–Sidak method for multiple comparison procedures was used to test parametric data; Dunn's multiple comparisons test was used for nonparametric data. Linear regression analysis and Pearson product moment correlation was used to examine the relationship between total flavonoid content and antioxidant capacity in response to main treatment effects and their interactions. Regression diagnostics were performed (Cook's Distance) on each regression to identify significant outliers. Analyses were performed using SigmaPlot® for Windows 11.0 with significance determined at the 95% probability level ($p < 0.05$). Global curve fitting and the extra sum-of-squares F-test was performed using GraphPad Prism version 5.04 for Windows to compare rapid light curves between and among treatments where H_o = one curve for all data sets and H_a = different curves for each data set ($p = 0.05$). All data are reported as means ± standard error.

3. Results

Flavonoid localization did not change in response to salinity or light/shade treatments over short (one day) and long (three week) time periods. Flavonoids were consistently localized in both the cytoplasm and cuticle of leaf tissue of abaxial and adaxial leaf surfaces, regardless of salinity or light treatment (Figs. 1, 2).

Three-way ANOVA indicated that date had a significant effect on flavonoid content (Table 1). Among dates, flavonoid contents were similar from day 1 to 7 and decreased significantly from day 7 to 14. Flavonoid content then increased significantly from day 14 to 21 and flavonoid content at day 21 was significantly higher than at any other date (Fig. 3). Salinity and light treatments did not have a significant effect on total flavonoid content when all sample dates where included (Table 1).

TEAC values varied significantly among salinities and dates, but not in response to light (Table 2). Among dates, there were no significant changes in TEAC values from day 1 through day 14, but TEAC values decreased significantly from day 14 to 21 (Fig. 4). TEAC values did not significantly differ within day 1 or day 7, however, at day 14, salinity 15 treatments were significantly higher than salinity 25, and at day 21 TEAC values for salinity 15 treatments were significantly higher than salinity 25 or 35 treatments. While light treatments tended to have higher TEAC values than shade treatments among dates, the difference was not significant (Fig. 4).

Flavonoid content and TEAC were positively correlated when all treatments and dates were considered and although this correlation was weak, it was statistically significant (Fig. 5). However, when compared by sample date, the relationship between flavonoid content and TEAC is more highly correlated, except at day 14, when flavonoid content declined significantly but TEAC values did not (Fig. 6).

There were no significant differences in leaf area between light or shade treatments. Among salinity treatments, leaf area was significantly lower for salinity 15 treatments within days 14 and 21; by day 14 leaves at this salinity had become wrinkled along the margins and brittle. In contrast, leaves in salinity 35 and salinity 25 treatments did not become wrinkled or brittle and their areas increased from day 7 through 21 (Fig. 7).

Two-way ANOVA indicated no significant treatment effects of salinity, light or their interaction on individual photosynthetic parameters (Fig. 8). There was minimal separation among values of photosynthetic efficiency at sub-saturating irradiance (α) among treatments, but $rETR_{max}$ and E_K within salinities 15 and 25 reflected a typical pattern for light/shade acclimation as shade treatments had lower mean values than light treatments (Fig. 8). Maximum quantum efficiencies (Fv/Fm)

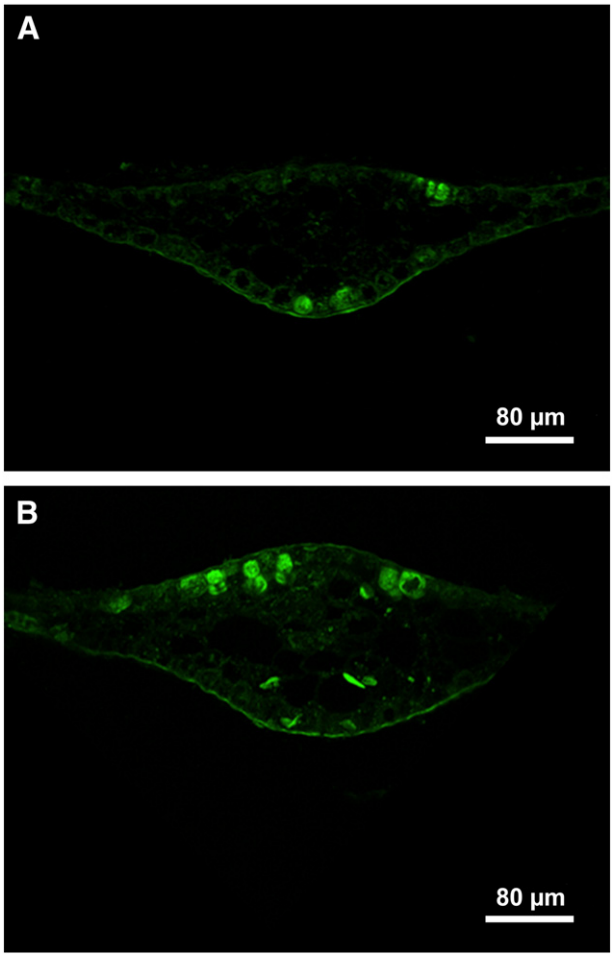

Fig. 1. Psuedo-colored CLSM images of NA-stained flavonoid fluorescence ($\lambda_{exe} = 405$ nm) in *H. johnsonii* leaf cross sections from day one, salinity 35, light treatment (A) and week three, salinity 15, shade treatment (B). Note flavonoid localization in cuticle and epidermal cells of adaxial and abaxial leaf surfaces. Images represent extremes in mesocosm treatments and are representative of the consistency in flavonoid localization throughout the experiment regardless of salinity or light treatments.

did not significantly differ among salinity or light treatments, but were lowest in salinity 15 light treatments (Fig. 9).

Global curve fitting of RLCs indicated there was no significant difference among light treatment RLCs regardless of salinity ($p = 0.66$; $F = 0.69$, $Dfn = 6$, $Dfd = 99$) and there was no significant difference between light and shade treatment RLCs within salinity 35 ($p = 0.64$; $F = 0.56$, $Dfn = 3$, $Dfd = 66$). In contrast, RLCs for salinities 15 and 25 shade treatments were significantly lower than light treatments ($p < 0.001$; $F = 8.66$, $Dfn = 3$, $Dfd = 138$ and $p < 0.001$; $F = 12.04$, $Dfn = 3$, $Dfd = 138$ respectively; Fig. 10).

Total PAR received by shaded plants was significantly lower (37.6% mean reduction of surface irradiance of PAR) compared to light treatments ($p < 0.05$). However, due to high diurnal variability, there were not significant differences in mean PAR values between light and shade treatments in the 24 h prior to sample collection within or among sample dates. Median water temperature and mean PAR 24 h prior to sample collection showed similar temporal trends, as maximum values for both occurred at day 1 (28.82 ± 0.49 °C, 707.36 ± 258.76 μmol photons m^{-2} s^{-1} respectively, Fig. 11a, b). Temperature and PAR values did not significantly vary between day 1 and day 7, but there was a significant decline in both parameters at day 14 ($p < 0.05$). Temperature and PAR then did not significantly vary from day 14 to 21 (Fig. 11a, b).

4. Discussion

Flavonoid content and sub-cellular localization within leaves of *H. johnsonii* did not significantly vary in response to the reduced salinity or light treatments used in this study. This suggests that flavonoids localized in the cuticle and cytoplasm of epidermal leaf cells are unlikely to primarily function as sunscreen pigments or compatible solutes, respectively. Flavonoids that perform sunscreen functions would be expected to be reduced in shaded plants compared to plants exposed to full sun (Agati et al., 2010). After three weeks of shading, total flavonoid content in *H. johnsonii* did not significantly differ between light and shade treatments. These results support findings of Kunzelman et al. (2005) and Kahn and Durako (2008) where flavonoid quantities were not significantly affected by manipulations of PAR and UV. It remains possible that the moderate decreases in PAR employed in this study were not great enough to produce an effect on flavonoid synthesis. Correspondingly, maximum irradiance in the light treatments may have been too low to induce an increase in flavonoid synthesis in this high-light adapted species. However, flavonoid content of intertidal *H. johnsonii* leaves collected in the field also were not significantly higher than those of subtidal plants and mean flavonoid content of the field-collected *H. johnsonii* was within the range measured in our mesocosm manipulations (Gavin and Durako, 2011). This suggests that even prolonged differences in PAR or UV do not have a significant effect on total flavonoid content.

If flavonoids in *H. johnsonii* were utilized as compatible solutes they should have decreased in quantity with reductions in salinity, as fewer osmolytes are required to maintain turgor pressure under hyposaline conditions (Wyn Jones and Gorham, 1983). However, there was no effect of salinity on flavonoid content at short (day one) or long (week three) time periods. In addition, if cytosolic flavonoids consist of mainly flavone glycosides, which comprise 10 of the 15 flavonoids identified in this species (Meng et al., 2008) and is suggested by their presence in the aqueous environment of the leaf epidermal cells (Fig. 1), it would seem metabolically inefficient to utilize flavone glycosides as osmolytes. The sugar moiety of a flavone glycoside is itself a compatible solute that could perform the same osmotic function with fewer enzymatic steps and lower carbon allocation.

A significant decline in flavonoid content occurred at day 14 across all salinity and light treatments. This decrease may reflect an artifact due to leaf turnover rather than any treatment effect. *H. johnsonii* produces new leaf pairs every 4–12 days and leaf growth rate is not significantly affected by shading (70% and 40% reduction in ambient PAR, Richmond et al., 2007). Day 14 leaf samples were therefore likely to have been the first leaves sampled whose growth was totally initiated after transplanting from the field to the mesocosm. Transplant stress can cause declines in chlorophyll concentration (Carter and Knapp, 2001), but its affect on flavonoid content in seagrasses is not known. Alternatively, the decrease in flavonoid content at day 14 also corresponded to a significant decrease in PAR, which began at day 12 and continued through day 14 sampling. When light limits photosynthesis all available carbohydrates may be shunted towards growth, which can reduce synthesis of carbon-based secondary metabolites (Bryant et al., 1988). It is possible that reduced carbon availability due to a combination of transplant stress and reduced light led to the significant reduction in flavonoid compounds observed at day 14.

Shading did not have a significant effect on TEAC values which suggests that antioxidant metabolism of *H. johnsonii* was not significantly affected by the differences in light utilized in this experiment. In contrast, hyposalinity did have a significant effect as TEAC values were significantly higher at the lowest treatment salinity (15) after three weeks. Plants can increase their ability to scavenge ROS produced during hyposalinity stress by increasing their antioxidants (Lu et al., 2006), and it is possible that *H. johnsonii* increased synthesis of antioxidant compounds in response to the physiological stress of

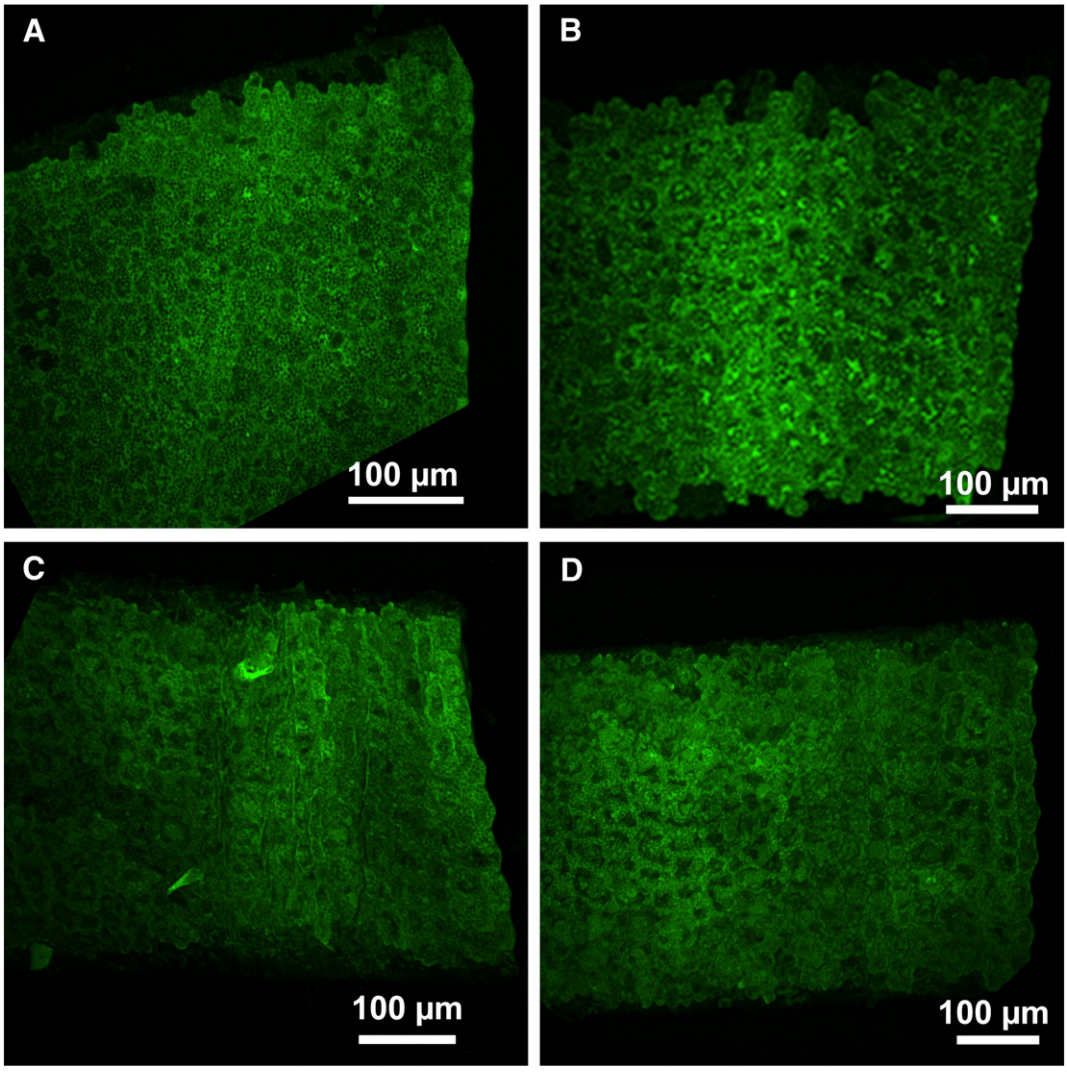

Fig. 2. Psuedo-colored CLSM images of NA-stained flavonoid fluorescence ($\lambda_{exe} = 405$ nm) in adaxial (A, C) and abaxial (B, D) leaf surfaces of *H. johnsonii* after 21 days in control treatment (salinity 35, light) (A, B) and experimental treatment (salinity 15, shade) (C, D).

hyposalinity. There was a highly significant correlation between total flavonoid content and TEAC for all sample times except day 14, where flavonoid content declined significantly but TEAC did not. Furthermore, salinity 15 treatments had a significant effect on antioxidant capacity at day 21 but no effect on total flavonoid content. This pattern could result from changes in flavonoid ratios that increase antioxidant capacity without an increase in total flavonoid quantity. Markham et al. (1998) observed that the liverwort, *Marchantia polymorpha* can significantly elevate levels of luteolin relative to apigenin

Table 1
Results of three-way ANOVA on total flavonoid content (nmol quercetin mm^{-2} leaf) for *H. johnsonii* (factors: salinity, light and date). Levene's test: passed ($p = 0.93$).

Source of variation	DF	SS	MS	F	P
Date	3	27.195	9.065	28.471	<0.001
Salinity	2	0.453	0.226	0.711	0.494
Light	1	0.913	0.913	2.869	0.095
Date × salinity	6	2.556	0.426	1.338	0.252
Date × light	3	1.364	0.455	0.428	0.242
Salinity × light	2	0.449	0.224	0.704	0.498
Date × salinity × light	6	1.211	0.202	0.634	0.703
Residual	72	22.925	0.318		
Total	95	57.066	0.601		

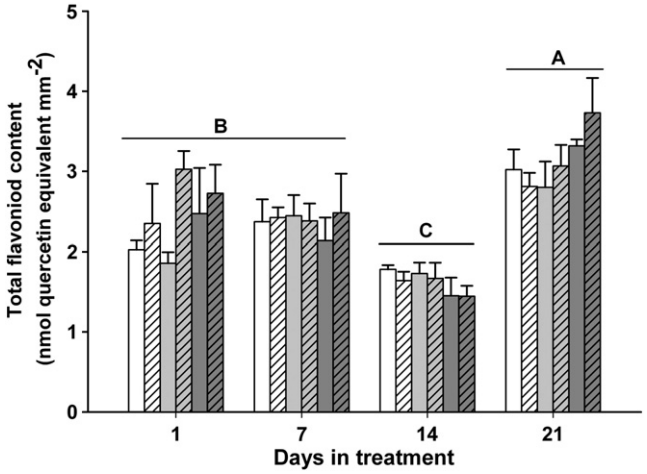

Fig. 3. Total flavonoid content (nmol quercetin mm^{-2} leaf) of *H. johnsonii* leaves from mesocosm treatments at one, seven, fourteen and twenty-one days (mean ± 1 SE). Treatments are salinity 35 (unfilled), 25 (light grey) and 15 (dark grey) and light (solid) or shaded (hatched). Upper-case letter above bars represents significant differences among dates $p < 0.05$.

Table 2
Results of three-way ANOVA on Trolox equivalent antioxidant capacity (nmol Trolox mm^{-2} leaf) for *H. johnsonii* (factors: salinity, light and date). Levene's test: passed ($p = 0.65$).

Source of variation	DF	SS	MS	F	P
Date	3	10556.550	3518.850	4.910	0.004
Salinity	2	11167.972	5583.986	7.791	<0.001
Light	1	1122.896	1122.896	1.567	0.215
Date × salinity	6	9746.627	1624.438	2.267	0.046
Date × light	3	1402.623	467.541	0.652	0.584
Salinity × light	2	1476.714	738.357	1.030	0.362
Date × salinity × light	6	458.771	76.462	0.107	0.995
Residual	72	51602.711	716.704		
Total	95	87534.865	921.420		

in response to increased UV-B light without an increase in total flavonoid concentration. Although luteolin does not increase UV-B screening, B-ring *ortho*-dihydroxyflavones like luteolin are significantly more effective antioxidants than B-ring mono-hydroxyflavones such as apigenin (Husain et al., 1987; Montesinos et al., 1995). *H. johnsonii* produces numerous B-ring ortho-dihydroxyflavones and B-ring mono-hydroxyflavones including luteolin and apigenin (Meng et al., 2008) and it is possible increased TEAC values after day 14 for salinity 15 treatments reflect an increase in the ratio of B-ring ortho-dihydroxyflavones to B-ring mono-hydroxyflavones which would not be reflected by measurement of total flavonoid content. While it remains plausible that flavonoids localized within the cytosol of epidermal leaf cells function as antioxidants, the increased TEAC for salinity 15 after day 14 was unlikely the sole result of flavonoids. The increase in antioxidant activity may instead have reflected changes in pools of antioxidant compounds not measured in this study, such as ascorbate and glutathione or changes in antioxidant enzymes such as catalase or superoxide dismutase, which would result in more reduced pools of antioxidants and higher TEAC values.

Chlorophyll fluorescence has become a well-accepted technique for the assessment of plant stress on photosynthetic characteristics (Schreiber et al., 1997). A lack of significant treatment effects for either light or salinity on individually-derived photosynthetic parameters suggests that *H. johnsonii* was able to efficiently capture and convert photons to chemical energy despite hyposaline (salinity 15 and 25) and shaded conditions. A lack of separation in alpha values among treatments may reflect the low number irradiance steps (3 to 4) recorded before the irradiance at onset of saturation (E_k) was reached, which can obscure differences in photosynthetic efficiency.

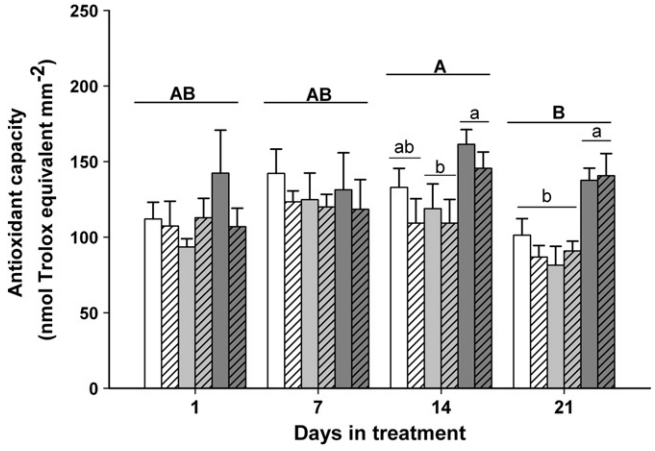

Fig. 4. Trolox equivalent antioxidant capacity (nmol Trolox mm^{-2} leaf) for *H. johnsonii* leaves from mesocosms at one, seven, fourteen and twenty one days (mean ± 1 SE). Treatments are salinity 35 (unfilled), 25 (light grey) and 15 (dark grey) and light (solid) or shaded (hatched). Upper-case letter above bar represents significant differences among dates, while lower-case letter above bar represents significant differences among salinities, within date ($p < 0.05$, $n = 4$).

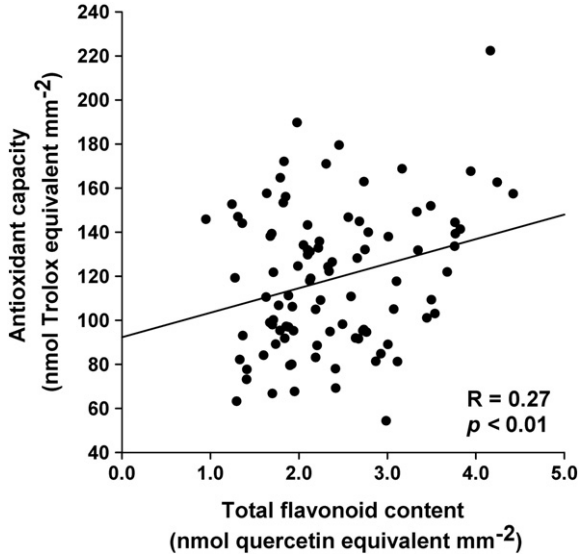

Fig. 5. Linear regression with correlation between total flavonoid content (nmol quercetin mm^{-2} leaf) and Trolox equivalent antioxidant capacity (nmol Trolox mm^{-2} leaf) of *H. johnsonii* leaves, all mesocosm treatments pooled ($n = 96$).

While shading or reduction in salinity did not have any significant effects on individual photosynthetic parameters, results of global curve fitting of RLCs indicated the ability of *H. johnsonii* to utilize light energy for photosynthesis was significantly reduced when shading and hyposalinity treatments were combined. This suggests that *H. johnsonii* may be able to photo-acclimate and maintain photosynthetic rates when light or salinity alone are reduced, but a combination of reduced light and salinity may significantly decrease the capacity of *H. johnsonii* to utilize light energy for photosynthesis. The tolerance of the *H. johnsonii*'s photosynthetic apparatus to reduced salinity is supported by Dawes et al. (1989) where photosynthetic parameters (I_c, I_k, and P_{max} levels) of estuarine, intertidal populations of *H. johnsonii* were tolerant to a wide range of salinities (15, 25, and 35) and were not photoinhibited by high PAR (~500 μmol photons m^{-2} s^{-1}). Similarly, Ralph (1998) showed the Fv/Fm ratio for the related species, *Halophila ovalis* (R. Br.) Hook. *f.* did not significantly decrease for 50% or 25% seawater treatments over a 5-day exposure period. These observations are in contrast to results from Torquemada et al. (2005), which indicated α and P_{max} parameters in *H. johnsonii* decreased significantly with salinity among salinity treatments of 30, 20 and 10. However, differences in photosynthetic response of *H. johnsonii* to hyposaline treatments between studies may be due to acclimation of *H. johnsonii* populations to different salinity conditions prior to experimental manipulation. Plants of *H. johnsonii* used in the present study were collected from Munyon Island, Fl while those utilized by Torquemada et al. (2005) were collected from Haulover Park, in northern Biscayne Bay, FL (USA, 25° 55′N; 80° 07′W). Haulover Park is within 1 km of a marine inlet (Haulover Inlet), which may result in higher and less variable salinity conditions than at Munyon Island (>5 km from Palm Beach Inlet). Estuarine populations must tolerate large variations in salinity on a regular basis, which may explain why *H. johnsonii* populations from Munyon Island exhibited better tolerance to hyposalinity treatments compared to Haulover Park populations. A lack of a significant light effect on photosynthetic parameters mirrors the results of total flavonoid content and antioxidant capacity analyses, which also were not significantly affected by light and may have been due, in part, to an insufficient level of shading. In addition, light treatments had no significant effect on leaf area, but leaf area was significantly affected by hyposalinity.

H. johnsonii is considered a rapid-growth clonal plant with a reduced storage capacity (Dean and Durako, 2007). This implies that growth and photosynthetic rates should be closely coupled. A lack

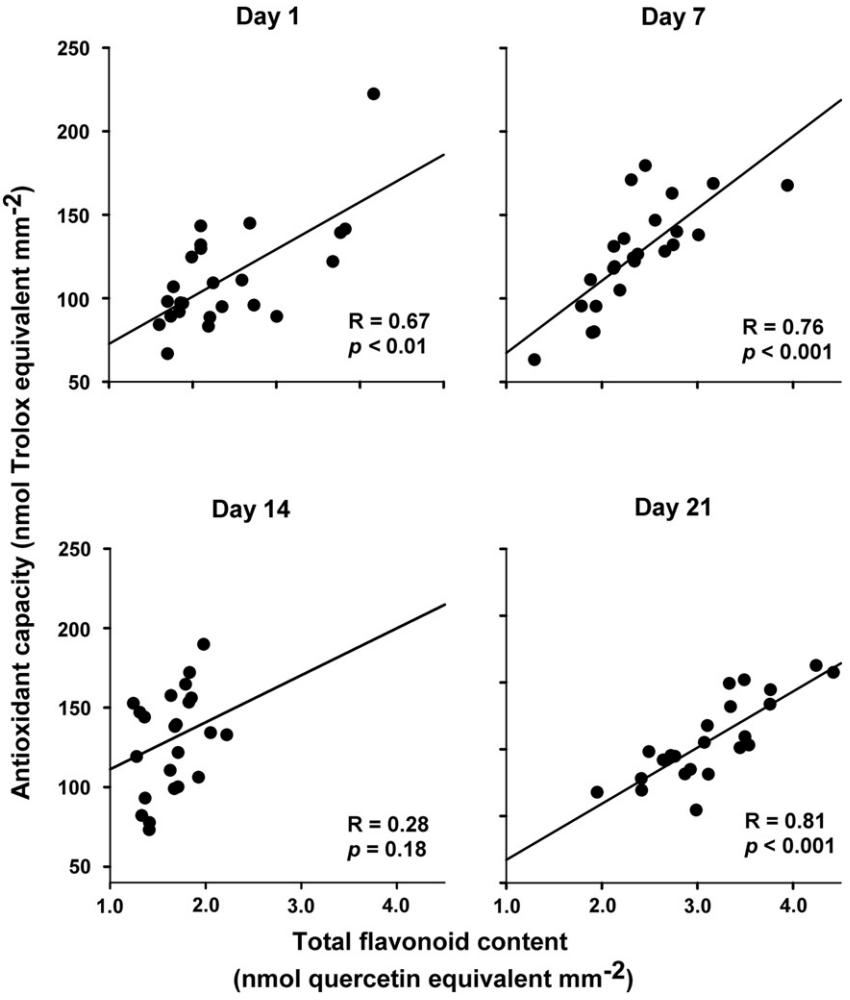

Fig. 6. Linear regression with correlations between total flavonoid content (nmol quercetin mm^{-2} leaf) and Trolox equivalent antioxidant capacity (nmol Trolox mm^{-2} leaf) for *H. johnsonii* leaves within salinity, among dates ($n = 24$).

of light effect on leaf area in this study suggests that the light reduction of 37.6% in ambient PAR for shaded treatments was not sufficient to significantly affect leaf size in *H. johnsonii* over the 21 day experimental period. This supports observations from Richmond et al. (2007) where reductions of 40% and 70% in ambient PAR for nine

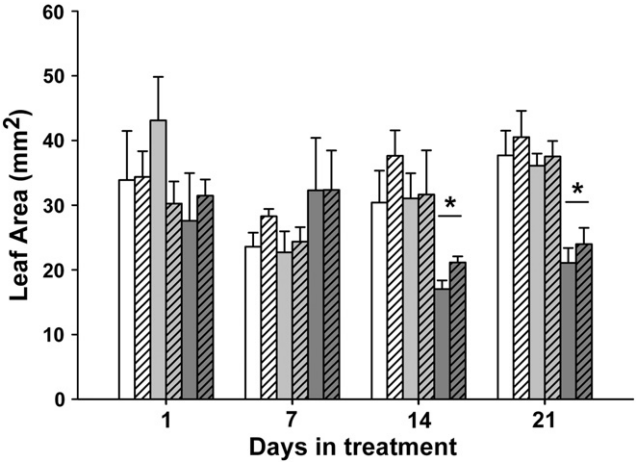

Fig. 7. *H. johnsonii* leaf surface area (mm^2) for mesocosms at days 1, 7, 14 and 21 (mean ± 1 SE). Treatments are salinity 35 (unfilled), 25 (light grey) and 15 (dark grey) and light (solid) or shaded (hatched). Asterisk above bar represents significant difference among salinities, within date ($p < 0.05$, $n = 4$).

days did not have a significant effect on leaf production or leaf size. Although shading did not have a significant effect on leaf size, in the present study, leaf area was significantly reduced at weeks two and three for salinity 15. Reductions in leaf area at salinity 15, regardless of light treatment, may reflect increased energy demands of growth in hyposaline conditions due to elevated metabolic costs associated with maintaining internal ionic balance (Sibly and Calow, 1989). Osmotic stress can also increase ROS production and the higher TEAC for salinity 15 treatments may reflect a response to this oxidative stress (Alscher et al., 2002; Yamasaki et al., 1997). Benjamin et al. (1999) reported wrinkled, brittle leaves and a reduction in leaf size in *H. ovalis* exposed to hyposaline treatments (salinity of 10) for four weeks. Leaf deformation was suggested to be a result of carbon limitation, due to increased energy demands of growth close to this species' salinity tolerance limits and a lower initial amount of HCO_3^- available within treatment aquaria, which can occur when seawater is diluted with distilled or deionized water (Dawes and McIntosh, 1981). This may explain why wrinkled, brittle leaves were observed in the present study at salinity 15 but not salinity 25 or 35 treatments and may also indicate carbon may have limited growth (as measured by leaf area) of plants at salinity 15. Alternatively, when a constant osmotic stress is applied to a cell wall, deformation occurs (Tyerman, 1982). Thus, the curling of *H. johnsonii* leaves observed at low salinity may be a result of cell wall deformation. Whatever the cause, at week three, plants in salinity 15 treatments had the lowest mean quantum yield, highest mean flavonoid content, significantly higher TEAC and significantly smaller leaf area compared to the two

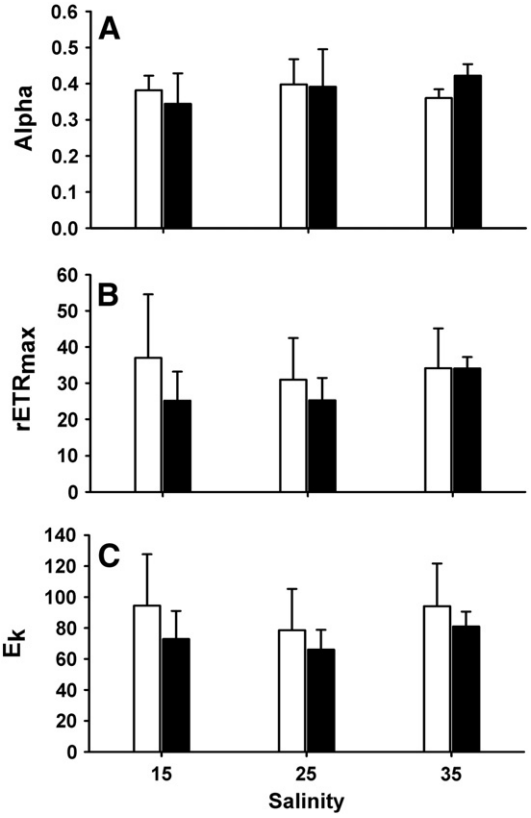

Fig. 8. *H. johnsonii* mean (±SE) rapid light curve coefficients of light (unfilled bars) and shade (filled bars) treatments for: (A) slope of $rETR_{max}$ versus photosynthetic photon flux density at sub-saturating light (α; μmol electrons/μmol photons), (B) maximum relative electron transport rate ($rETR_{max}$; μmol electrons m^{-2} s^{-1}), and (C) irradiance at the onset of saturation (E_k; μmol photons m^{-2} s^{-1}) at week three of mesocosm experiments ($n=4$).

higher salinity treatments. This combination of responses indicates that *H. johnsonii* was stressed, but that it is able to tolerate a salinity of 15 for up to three weeks.

Flavonoid content and sub-cellular localization did not significantly vary in response to reduced salinity or light treatments used in this study. Flavonoids localized in the cuticle may represent a constitutive component capable of a sunscreen function, while the presence of flavonoid compounds in the cytosol, despite reduced salinity, suggests they are not utilized as compatible solutes but rather may instead serve as antioxidants, which could scavenge ROS produced by chloroplast and other cellular organelles. TEAC increased

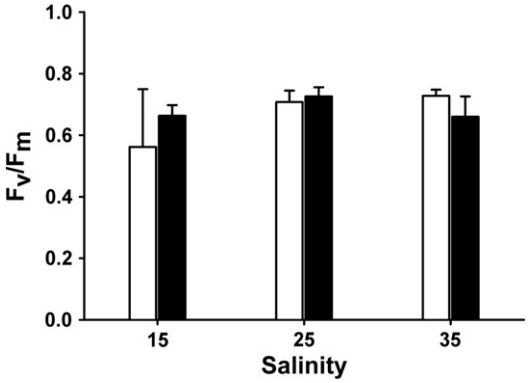

Fig. 9. *H. johnsonii* mean (±SE) maximum photochemical efficiency of photosystem II (Fv/Fm) at week three of mesocosm experiments for light (unfilled bars) and shade (filled bars) treatments ($n=4$).

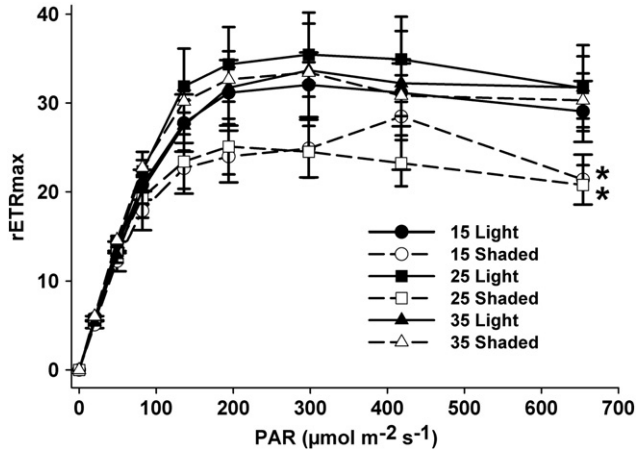

Fig. 10. *H. johnsonii* mean (±SE) rapid light curves (PAR; μmol photons m^{-2} s^{-1} vs. rETRmax; μmol electrons m^{-2} s^{-1}). Asterisks represent significant difference between shade treatments among salinity ($p<0.001$, $n=36$).

in response to hyposalinity, which suggests there was an upregulation in antioxidant metabolism in response to hyposaline stress. There are numerous other physiological functions suggested for flavonoid compounds including as antiherbivoral and antifouling compounds or growth regulators (reviewed in Gould and Lister, 2006). While knowledge of flavonoid localization in response to controlled treatments has helped in our understanding of their potential physiological roles, further information regarding the molecular structure of specific flavonoids within each subcellular location in response to environmental variation is needed to help determine their primary function(s) in *H. johnsonii*. This could be achieved through careful separation of cuticular and cytosolic components of leaf epidermal tissues and identification of the different flavonoids within each component.

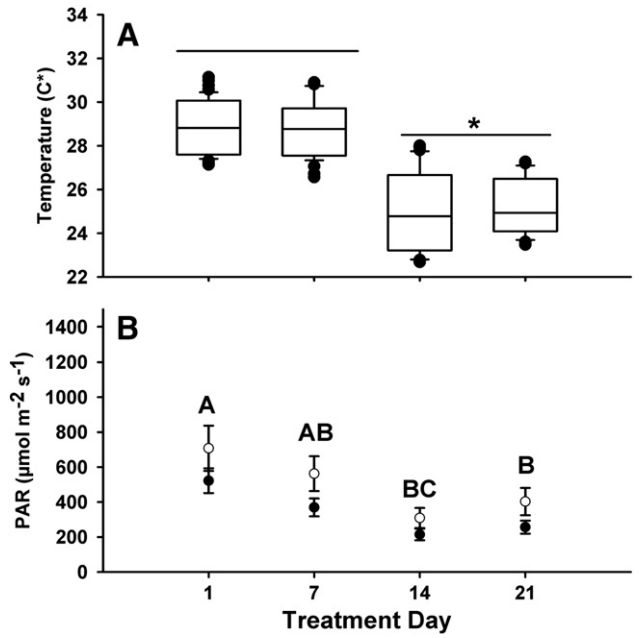

Fig. 11. Physical parameters for mesocosm at days 1, 7, 14 and 21. (A) Water temperature (°C) in treatment aquaria. Median = solid horizontal line and box = 25th and 75th percentile, $n=49$. Asterisk above line represents significant difference $p<0.05$. (B) Mean (±SE) PAR levels for shade (filled circles) and light (open circles) on each day of treatment ($n=26$). Uppercase letter above error bar represents significant differences among treatment days $p<0.05$.

Acknowledgments

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