LOCALIZATION OF FLAVONOID COMPOUNDS IN *HALOPHILA JOHNSONII* EISEMAN IN RESPONSE TO LIGHT AND SALINITY VARIATION SUGGESTS ANTIOXIDANT FUNCTION

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CHAPTER ONE: LOCALIZATION AND ANTIOXIDANT CAPACITY OF FLAVONOIDS FROM INTERTIDAL AND SUBTIDAL HALOPHILA JOHNSONII AND HALOPHILA DECIPIENS
Abstract

The threatened seagrass *Halophila johnsonii* co-occurs subtidally with *Halophila decipiens*, but only *H. johnsonii* occurs intertidally. *H. johnsonii* contains 15 UV-absorbing flavonoids; the flavonoid content of *H. decipiens* is unknown. Intertidal *H. johnsonii* is exposed to much more variable and potentially stressful environmental conditions compared to subtidal populations. Abiotic stresses may cause overproduction of reactive oxygen species (ROS) in plants, which can lead to cell damage. Flavonoids have been demonstrated to protect plants against increased ROS. In this study, flavonoid localization, concentration and total antioxidant capacity in leaves of subtidal *H. decipiens* were compared to intertidal and subtidal *H. johnsonii*. *H. johnsonii* leaves had significantly higher flavonoid concentration and antioxidant capacity than *H. decipiens* leaves. Flavonoid concentration did not significantly differ between intertidal and subtidal *H. johnsonii* although antioxidant capacity was significantly higher in subtidal *H. johnsonii*. Confocal laser scanning microscopy of fresh leaf cross sections indicated that both species contained flavonoids in the outer cell wall and cuticle, but only *H. johnsonii* contained intracellular flavonoids. Intracellular flavonoids are better situated to perform antioxidant functions *in planta*. These results suggest that flavonoid compounds in *H. johnsonii* are capable of performing sunscreen and antioxidant functions while an antioxidant role for flavonoids within *H. decipiens* is not supported.

*Keywords*: Antioxidant; *Halophila*; Flavonoids; Confocal
1. Introduction

*Halophila johnsonii* Eiseman is a threatened seagrass that grows intertidally to depths of approximately 3-4 meters and has a distribution that is limited to a 200-km section of southeastern Florida (Kenworthy, 1993; Virnstein and Morris, 2007). *H. johnsonii* co-occurs with *H. decipiens* subtidally, but only *H. johnsonii* occurs intertidally (Virnstein and Morris, 2007). Compared with *H. decipiens*, *H. johnsonii* has greater tolerance to high irradiances, and to variations in temperature and salinity (Dawes et al., 1989). The presence of UV-absorbing pigments (UVPs) in *H. johnsonii* was suggested to provide protection from high irradiance and UV and allow this species to exploit the shallowest waters without competition from the closely-related, but UVP-lacking *H. decipiens* (Durako et al., 2003). The UVP compounds in *H. johnsonii* consist of 15 flavonoids (10 flavone glycosides and 5 flavones) that strongly absorb UVA radiation (absorption peak of 343-348) (Meng et al., 2008). However, experimental manipulations of photosynthetically active radiation (PAR) and UV in mesocosms did not elicit consistent changes in UVPs (Kunzelman et al., 2005) and results of subsequent mesocosm experiments suggested UVP concentrations responded more strongly to salinity variation than to variations in light quality (Kahn and Durako, 2008). A lack of consistent responses to UV or PAR treatments and a possible response to salinity variation raised questions regarding the principal physiological role of flavonoids in *H. johnsonii*.

Intertidal *H. johnsonii* is exposed to higher levels of light, temperature and desiccation than subtidal populations on daily and seasonal bases (Kenworthy et al., 2007). Any of these abiotic factors are capable of inducing oxidative stress alone, or in
combination, and can lead to an increase in production of reactive oxygen species (ROS) (Draper, 1997; Yamasaki et al., 1997; Alscher et al., 2002). Numerous studies have suggested that flavonoids commonly function as antioxidants and may protect plants against oxidative stress caused by suboptimal environmental conditions (Bohnert and Jensen, 1996; Rice-Evans et al., 1997; Tattini et al., 2004; Gould and Lister, 2006).

Antioxidant capacity of flavones is attributed to the high reactivity of the hydroxyl substituent, with the number of hydroxyl groups on the B-ring being most determinant of ROS scavenging capability (see Fig. 1 for flavone ring structure, Sekher et al., 2001; Burda, 2001; Heim et al., 2002). Eight of the fifteen flavone compounds in H. johnsonii possess a molecular structure indicative of high antioxidant activity (Fig. 2). Flavone compounds in H. johnsonii form two distinct groups, hydrophilic flavone glycosides and hydrophobic flavones (Fig. 2; Meng et al., 2008). Differences in solubility affect distribution and sub-cellular localization of flavonoids that may determine their ecological function (Hutzler et al., 1998).

Depending on tissue location, flavonoids could act directly in photoprotection against high solar irradiance by absorbing incident photons or indirectly as a result of their antioxidant activity. 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). As soluble sugars, flavone glycosides produced by H. johnsonii may also function as compatible solutes to help maintain osmotic pressure under extended alterations in salinity (Murphy et al., 2003). While seagrasses have been shown to change levels of flavonoids in response to abiotic stress,
Fig. 1. Molecular structure of flavone backbone. Illustration adapted from: Cao et al., 1997.
Fig. 2. Structure of flavone glycosides (1-10) and flavones (11-15) present in methanol extracts of *H. johnsonii* likely to possess high antioxidant activity due to 3'-4' ortho-di-hydroxyl configuration (3,7,8,11, 12) or 3'-4'-5' ortho-tri-hydroxyl configurations (1,2,15) on the B-ring (Sekher et al., 2001; Burda, 2001; Heim et al., 2002) Illustration adapted from: Meng et al., 2008.
the sub-cellular localization of these compounds is unknown (Trocine et al., 1981; Dawson and Dennison, 1996; Detres et al., 2001).

Due to the influence location has on flavonoid function \textit{in planta} the present study compared sub-cellular localization of flavonoid compounds between subtidal \textit{H. johnsonii} and \textit{H. decipiens}. Differences in total flavonoid concentration and antioxidant capacity were also compared among intertidal and subtidal \textit{H. johnsonii} and \textit{H. decipiens} to examine whether intertidal plants maintain higher flavonoid concentrations and antioxidant capacity. To the authors’ knowledge, this is the first study to visualize flavonoid localization within seagrasses.

2. Materials and methods

2.1. Sample Collection

All samples were collected August 17, 2009 at Munyon Island in Lake Worth Lagoon, Florida (26.82054° N, 80.04604° W) between 11:00 and 12:00 during a falling tide. This site was chosen because of the availability of intertidal \textit{H. johnsonii} and an intermixed subtidal bed of \textit{H. johnsonii} and \textit{H. decipiens}. A 10 x 10 cm sod plugger was used to extract rhizome segments of three to five leaf pairs from subtidal \textit{H. johnsonii} and \textit{H. decipiens}. Sod plugs were transplanted into 9 x 9 x 9 cm plastic planting pots pre-sterilized with bleach and placed in coolers filled with ambient seawater. Additional leaf material from subtidal \textit{H. johnsonii} (n=6) and \textit{H. decipiens} (n=6) and intertidal \textit{H. johnsonii} (n=6) plants was also collected. The second leaf pair back from an apical meristem was removed and placed in a polypropylene tube filled with 6ml HPLC grade methanol for pigment extraction and kept on ice in the dark. All plant materials were transported to the Center for Marine Science in Wilmington, NC within 24hr and potted
plants were placed into outdoor, seawater holding troughs (salinity 33.7) for one day before confocal images were recorded.

2.2. Confocal laser scanning microscopy (CLSM)

CLSM was used to visualize sub-cellular accumulation and localization of flavonoids in 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) of fresh *H. johnsonii* and *H. decipiens* leaves were obtained free hand using a razorblade. 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 minutes 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 ($\lambda_{exc} = 405$ nm) and 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 (Fig. 3).

2.3. **Leaf pigment extraction**

Leaf tissue samples collected in the field were placed between two glass microscopy slides adjacent to a ruler and photographed perpendicularly from above for determination of leaf surface 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 surface area was calculated from digital images using Image-Pro Plus © software. After being photographed, leaves were ground in the dark in a chilled mortar with 6 ml cold, HPLC grade methanol. Samples were allowed to extract overnight in refrigerated conditions and supernatant was collected after samples were centrifuged at 10,000 RPM for five minutes at 2 ºC using a Beckman Coulter™ Avanti ® J-25 high-performance centrifuge.

2.4. **Flavonoid concentration assay**

Total flavonoid concentration was determined according to the method of Ordonez et al. (2006). To 0.5 mL of methanol extract, 0.5 mL of 2% AlCl₃ ethanol solution was added. After one hour at room temperature, the absorbance was measured at
Fig. 3. Emission spectrum of blue-light excited ($\lambda_{\text{exc}} = 405$ nm) cross section of *H. johnsonii* before (solid black line) and after staining with NA (dashed line). Differences in emission spectra were used to select acquisition bandwidths for CLSM imaging where the blue channel ($\lambda_{\text{emission}} = 400$-$430$ nm) corresponded to autofluorescence from the cuticle, the ‘green’ channel ($\lambda_{\text{emission}} = 515$-$565$ nm) corresponded to NA-stained flavonoids and the ‘red’ channel ($\lambda_{\text{emission}} > 647$ nm) corresponded to chlorophyll fluorescence.
420 nm using an Ocean Optics© S2000 fiber optic spectrometer and a Mini-D2T© halogen/deuterium light source. Flavonoid content was calculated as quercetin equivalent (mg quercetin mm$^{-2}$ leaf area) based on a five point calibration curve prepared each day before measurements began. All determinations were performed in triplicate.

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). ABTS radical cation (ABTS$^+$) 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. 10µL of plant extract was added to 990 µL of ABTS$^+$ solution and absorbance was measured 4 minutes after initial mixing. Absorbances were read using a Milton Roy spectrophotometer (Spectronic 401) and a dose-response curve for trolox over the range of 0-20 µM (final concentration) was obtained. Results were corrected for dilution and expressed in µM trolox mm$^{-2}$ leaf area. All determinations were performed in triplicate.

2.6. Statistical analysis

Statistical comparisons between flavonoid concentration and antioxidant capacity were made between intertidal and subtidal *H. johnsonii* and between *H. johnsonii* and *H.
decipiens using one-way ANOVA. Variation between triplicate sub-samples was not significant and values were pooled for each replicate. Normality was tested using the Shapiro-Wilk test of normality and homogeneity of variance was tested using the Levene Median test (n=6). In cases where tests for normality failed, Kruskal-Wallis one-way ANOVA on ranks was applied. Linear regression analysis and Pearson product moment correlation were used to examine the relationship between total flavonoid content and antioxidant capacity and whether this differed among intertidal and subtidal H. johnsonii and subtidal H. decipiens. Regression diagnostics were performed (DFFITS and Cooks 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).

3. Results

Halophila johnsonii contained NA-stained flavonoid compounds in both abaxial and adaxial leaf tissues (Fig. 4a). Flavonoids were localized in epidermal cells of both leaf surfaces as well as the extracellular cuticular layer (Fig. 4b). Within epidermal cells, NA-stained flavonoids were absent from cell walls (Fig. 4c) and cellular organelles, including the central vacuole (Fig. 4c) and chloroplasts (Fig. 4d). Flavonoid fluorescence originated predominantly from soluble compounds within the cytosol that were released from cells damaged during sectioning (Fig. 4e).

CLSM images of Halophila decipiens showed NA-stained flavonoid compounds localized in the outer cell wall or cuticle of both the abaxial and adaxial surfaces but lacked flavonoid fluorescence within the cytosol, which contrasts with H. johnsonii
Fig. 4. CLSM images of chloroplast (red) and NA-stained flavonoid fluorescence (green) in *H. johnsonii*. (a) Z-stack of leaf mid-rib cross section. (b) Z-stack of leaf mid-rib, adaxial surface. Note flavonoids located in cytosol of intact epidermal cells (arrows) and within the cuticle (arrowhead). (c,d) Z-stacks of leaf epidermis collected below leaf cuticle. Note lack of flavonoids within central vacuole (arrowhead), cell wall (white arrow) (c), and chloroplast (white arrows) (d). (e) Leaf lamina. Note loss of NA-staining cells damaged during sectioning showed decreased NA-staining from release of soluble compounds (arrows).
Extracellular flavonoid fluorescence was clearly visible in the marginal teeth (Fig. 5a, b). Since the image is a combination of optical sections in the Z-direction, the intense green fluorescence above epidermal cells is attributed to fluorescence of flavonoid compounds localized in the cuticle above epidermal cells.

There was no statistically-significant difference between total flavonoid concentration of intertidal and subtidal *H. johnsonii*, but flavonoid concentration of *H. decipiens* was significantly lower than both intertidal and subtidal *H. johnsonii*. Median trolox equivalent antioxidant capacity (TEAC) of subtidal *H. johnsonii* was significantly higher than *H. decipiens* and intertidal *H. johnsonii* while TEAC of *H. decipiens* was significantly lower than both intertidal and subtidal *H. johnsonii* (Fig. 7). Subtidal and intertidal *H. johnsonii* showed significant positive correlations ($R = 0.959, p < 0.01$; $R = 0.882, p < 0.05$, respectively) between total flavonoid content and TEAC while *H. decipiens* did not ($R = 0.011, p = \text{ns}$, Fig. 8).

However, within subtidal *H. johnsonii* the correlation between total flavonoid content and TEAC was primarily driven by a single outlier (over two standard deviations from the median). When this point was removed from the regression the correlation was no longer significant ($R = 0.439, p = 0.459$). Similarly, when intertidal and subtidal *H. johnsonii* data were pooled there was a significant correlation between total flavonoid content and TEAC, but this relationship was not significant when the outlier was removed (Fig. 9). Due to the influence of this single point the relationship between flavonoid content and TEAC for subtidal *H. johnsonii* is unclear, but a significant, positive relationship between flavonoid content and TEAC was indicated for intertidal *H. johnsonii*. Intertidal *H. johnsonii* had smaller leaf surface area than subtidal *H. johnsonii*.
**Fig. 5.** Single channel fluorescent images ($\lambda_{\text{emission}} = 515\text{-}565\text{nm}$) of NA-stained leaf lamina cross sections of *H. johnsonii* (a) and *H. decipiens* (b). Note lack of intracellular flavonoids within epidermal *H. decipiens* tissues (bracket).
Fig. 6. Psuedo-colored CLSM fluorescence images of chloroplast (red) and NA-stained flavonoid fluorescence (green) in *H. decipiens*. Z-stack of NA-stained leaf margin shows flavonoids localized in cuticle and marginal teeth.
**Fig. 7.** (a.) Total flavonoid content (mg L$^{-1}$ quercetin mm$^{-2}$) and (b.) trolox equivalent antioxidant capacity (TEAC) (µM trolox mm$^{-2}$) for field collections of intertidal *H. johnsonii*, subtidal *H. johnsonii* and *H. decipiens*. Median = solid horizontal line, box = 25th and 75th percentile and error bars = 10th and 90th percentiles, n=6. Upper-case letter above box represents significant differences $p < 0.05$. 


**Fig. 8.** Correlations between total flavonoid content (mg L\(^{-1}\) quercetin mm\(^{-2}\)) and trolox equivalent antioxidant capacity (µM trolox mm\(^{-2}\)) for intertidal *H. johnsonii* (black circles), subtidal *H. johnsonii* (white circles), and *H. decipiens* (black triangles).
Fig. 9. Correlation between total flavonoid content (mg L\(^{-1}\) quercetin mm\(^{-2}\)) and trolox equivalent antioxidant capacity (µM trolox mm\(^{-2}\)) for intertidal and subtidal *H. johnsonii* with outlier (a) and with outlier removed (b).
although this difference was not significant. Leaf surface area of *H. decipiens* was significantly higher than intertidal and subtidal *H. johnsonii* (Fig. 10).

4. Discussion

*Halophila johnsonii* and *Halophila decipiens* significantly differ in antioxidant capacity, flavonoid localization and total flavonoid concentration, which may, in part, explain differences in their depth distributions. Due to its increased tolerance to high irradiances (Dawes et al., 1989), *H. johnsonii* was hypothesized to have higher antioxidant capacity and flavonoid concentration than *H. decipiens*. My data support this hypothesis, as both flavonoid concentration and trolox equivalent antioxidant capacity (TEAC) were significantly higher in *H. johnsonii* leaves. Higher concentrations of UV-absorbing flavonoid compounds and increased antioxidant capacity suggest *H. johnsonii* can better avoid damage from high-irradiance intertidal conditions and have greater protection against ROS, compared to *H. decipiens*. A positive correlation was present between flavonoid concentration and TEAC values for intertidal *H. johnsonii*, but not *H. decipiens*, which suggests flavonoids may perform antioxidant functions in leaf tissues of *H. johnsonii*, but not *H. decipiens*. This inference was supported by CLSM analysis of flavonoid localization. In addition to the cuticle, flavonoids in *H. johnsonii* were present in the cytosol of epidermal cells, surrounding sources of ROS production, while flavonoid compounds in *H. decipiens* were deposited exclusively in the cuticle of the leaf where they would be unlikely to perform antioxidant functions (Hutzler et al., 1998; Neil and Gould, 2003; Tattini et al., 2004). For *H. decipiens*, non-flavonoid pools of major antioxidants such as ascorbate, glutathione and alpha-tocopherol are likely responsible for the majority of the TEAC (Larson, 1988).
Fig. 10. Leaf surface area (mm$^2$) of leaves from field collections of intertidal *H. johnsonii*, subtidal *H. johnsonii*, and *H. decipiens*. Median = solid horizontal line and box = 25$^{th}$ and 75$^{th}$ percentile, n=6. Uppercase letter above box represents significant differences p < 0.01, n = 6.
There was no significant difference in flavonoid concentration between intertidal and subtidal *H. johnsonii* but TEAC was significantly higher for subtidal *H. johnsonii*. Intertidal *H. johnsonii* is exposed to higher levels of light, temperature and desiccation than subtidal populations on a seasonal and daily basis (Kenworthy et al., 2007). A similar concentration of flavonoids for intertidal and subtidal plants suggests flavonoids of *H. johnsonii* do not increase in response to the abiotic stress of the intertidal habitat, which supports previous observations that flavonoid concentrations of *H. johnsonii* leaves do not respond to changes in UV or PAR levels and may be produced constitutively (Kunzelman et al., 2005; Kahn and Durako, 2008). While total flavonoid concentration did not increase, it is possible that significant changes in concentration of individual flavonoids occurred. Markham et al., (1998) observed that raising incident UV-B light by 25% did not increase total flavonoid concentration but significantly elevated levels of luteolin relative to apigenin in the liverwort, *Marchantia polymorpha*. Luteolin is not thought to increase UV-B screening but B-ring ortho-dihydroxyflavones like luteolin are known to be significantly more effective antioxidants than B-ring monohydroxyflavones such as apigenin (Husain et al., 1987; Montesinos et al., 1995). Both of these flavonoids are produced by *H. johnsonii* and it is possible intertidal *H. johnsonii* may increase protection to UV-B without changing overall flavonoid concentration. A change in pigment ratios opposed to total concentration also occurs in the photoprotective mechanisms of carotenoids. For example, the carotenoid zeaxanthin up-regulates in several protection mechanisms of plants and accumulates when light intensity exceeds the rate of energy utilized by photosynthetic electron transport and carbon fixation (Demmig-Adams, 1996). However, zeaxanthin is synthesized from pre-existing
violaxanthin and therefore total carotenoid concentration may not increase despite increased photoprotection (Eskling, et al., 1997). Determination of flavonoid ratios in *H. johnsonii*, as opposed to total concentrations would be valuable and could be addressed through use of HPLC analysis to compare flavonoid contents between intertidal and subtidal populations.

Higher TEAC of subtidal *H. johnsonii* compared to intertidal plants may indicate antioxidant pools of intertidal *H. johnsonii* were more oxidized at the time of sample collection. Due to the inherent limitations of the TEAC test I was unable to judge whether reduced TEAC values for intertidal *H. johnsonii* were due to differences in light history at the time of sample collection or if intertidal *H. johnsonii* contained fewer antioxidant compounds and enzymes than subtidal plants. The TEAC assay relies upon a molecule’s capacity to scavenge the ABTS$^{\cdot-}$ radical and does not measure the concentrations of particular antioxidant compounds. Therefore, TEAC values reflect the product of potentially numerous interactions and turnover among antioxidant molecules, antioxidant enzymes and ROS, which may provide a more biologically relevant indicator of antioxidant status than obtained by measurements of individual antioxidant compounds and enzymes. However, if antioxidant compounds are oxidized prior to addition to the assay they will be unable to reduce the ABTS$^{\cdot-}$ radical (Re et al., 1999). All leaf samples were collected between 11:00 and 12:00 during a falling tide. The intertidal plants were exposed to direct sunlight at the time of collection and antioxidant pools may have been highly oxidized due to high irradiances and temperatures compared to subtidal plants, which were submerged at a depth of approximately 0.5 m during sampling. Therefore, even if intertidal *H. johnsonii* had higher concentrations of antioxidant compounds and
enzymes than subtidal plants, they could have lower TEAC values than subtidal *H. johnsonii* based upon differences in environmental conditions between collection sites. Future studies may answer this question by investigating the oxidative state of important antioxidant pools such as ascorbate and glutathione as well by sampling intertidal and subtidal plants before dawn to reduce the effect of different light intensities and history between sample sites.

In *H. johnsonii*, flavonoids were localized in the extracellular cuticular layer and within the cytosol of epidermal cells in both abaxial and adaxial leaf surfaces. Flavonoids isolated from *H. johnsonii* absorb UV light and a cuticular location may help shield underlying chloroplasts from excess high-energy quanta. In terrestrial plants adaxial leaf cells may receive higher amounts of PAR and UV than abaxial tissues which can be shaded by the cells above them. In response, flavonoids are often deposited in adaxial leaf surfaces (cuticle and cell-wall) or within the vacuole of adaxial epidermal cells to help shield the chloroplasts in mesophyll tissues below (Hutzler et al., 1998; Tattini et al., 2004). Seagrasses also rely on production of UV-absorbing pigments for protection against high irradiances and UV radiation, but unlike terrestrial leaves the leaf lamina of *H. johnsonii* is only two cells thick, thus, chloroplasts are epidermal (Trocine et al., 1981; Dawson and Dennison 1996; Hader et al., 1998). Although leaves of *H. johnsonii* possess clear abaxial and adaxial surfaces, when the leaf is supported by the water column they can be oriented in a nearly vertical position and wave oscillations or the direction of tidal flow may greatly influence which leaf surface receives higher irradiance. Thus, localization of sunscreen and antioxidant compounds preferentially in adaxial surfaces would be less advantageous in *H. johnsonii* compared to terrestrial leaves.
Some seagrass species possess sulphated flavonoids that can inhibit herbivory or have antibiotic or antifouling activity (Harborne, 1979; McMillan, 1986; Jensen et al., 1998). However, antifouling activity of these flavonoids in seagrasses is thought to be due to the incorporation of sulfate; flavonoids isolated from *H. johnsonii* are not sulfated (Harborne, 1977; Meng et al, 2008). *H. johnsonii* has high growth rates and *Halophila* species generally have high rates of leaf turnover (14.7 and 10.7 days for *H. hawaiiana* and *H. decipiens*, respectively) (Torquemada et al., 2005; Herbert, 1984; Kenworthy et al., 1993). Rapid leaf turnover reduces the time available for epiphytes to grow on leaf tissue. This may make antifouling compounds less advantageous than they would be for species with longer leaf retention times such as *Posidonia oceanica* (Linnaeus) Delile, which can retain leaves between 202 and 345 days (Hemminga et al., 1999).

Antiherbivoral or antifouling activity has not been examined for the flavonoids from *H. johnsonii*, but flavonoids that inhibit herbivory or have antifouling activity would be expected to be secreted from epidermal cells or be localized on adaxial and abaxial leaf surfaces.

Within epidermal cells of *H. johnsonii*, flavonoid fluorescence originated predominantly from soluble compounds within the cytosol that were released from cells damaged during sectioning. The chloroplast is responsible for a majority of ROS production in plant cells and chloroplasts in *H. johnsonii* are epidermal (Yamasaki et al., 1997; Asada and Yoshikawa, 1974). Flavonoids localized within the cytosol of epidermal cells are therefore well positioned to scavenge ROS. Oxidized flavonoids in the cytosol may be recycled to their parent state by cytosolic dehydroascorbic acid (DHA). DHA radicals can in turn be reduced enzymatically by monodehydroascorbic acid reductase (MDAR),
which completes the safe elimination of a flavonoid-scavenged ROS (Yamasaki et al., 1997). Alternatively, as soluble sugars, flavone glycosides produced by *H. johnsonii* may also function as compatible solutes to help maintain osmotic pressure under extended alterations in salinity (Murphy et al., 2003; Kahn and Durako, 2008). 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 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).

*H. decipiens* contained NA-stained flavonoids exclusively in the cuticle and they were clearly visible in marginal teeth. These compounds are unlikely to have sunscreen functions as *H. decipiens* is a deeper-water species and leaf extracts of *H. decipiens* show little UV absorbance (Durako et al., 2003). Isolation of the NA-staining compounds from *H. decipiens* is necessary to gain more insight to their molecular structure and function. It would be constructive to compare flavonoid localization between shallow and deep-water populations of *H. decipiens* to further our knowledge of possible functions for cuticular flavonoids in *H. decipiens*.

In summary, flavonoids in *H. johnsonii* were localized in the cuticle and within the cytosol of epidermal leaf cells in both adaxial and abaxial leaf tissues. Cuticular flavonoids are in locations favorable for UV screening while cytosolic flavonoids are ideally located to perform antioxidant or osmotic functions. *H. decipiens* contained extracellular flavonoids in adaxial and abaxial surfaces but lacked intracellular
flavonoids. The structure and function of these compounds in *H. decipiens* remains to be described. TEAC and flavonoid concentration assays suggest flavonoids in *H. johnsonii* may be multifunctional compounds produced at constitutive levels. The production of these compounds may provide photoprotection against high solar irradiance where cuticular flavonoids absorb incident UV radiation and cytosolic flavonoids may perform antioxidant functions. Flavonoids are a diverse group of compounds with a diverse array of known and proposed functions (reviewed in Gould and Lister, 2006). While knowledge of flavonoid localization has helped in our understanding of their potential physiological roles, questions remain as to their primary functions in marine plants. Measurements of changes in antioxidant capacity and flavonoid localization and concentration over diel cycles at different sites and seasons may provide greater insights into the role of these compounds in this unique group of vascular plants.

**References**


CHAPTER TWO: LOCALIZATION AND ANTIOXIDANT CAPACITY OF FLAVONOIDS IN HALOPHILA JOHNSONII IN RESPONSE TO EXPERIMENTAL LIGHT AND SALINITY VARIATION
Abstract

The threatened seagrass *Halophila johnsonii* contains 15 UV-absorbing flavonoids localized in the cuticle and epidermal cells of both abaxial and adaxial leaf surfaces, but previous studies indicate they 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 shading 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 trolox equivalent antioxidant capacity (TEAC), flavonoid concentration and localization and chlorophyll fluorescence parameters. Salinity had no significant effect on flavonoid concentration, but flavonoid concentrations were significantly higher in shade treatments at day 1. TEAC values were significantly higher for salinity 15 treatments at 14 and 21 days and TEAC responses to hyposalinity were not mitigated by a decrease in light as TEAC was similar between shaded and unshaded treatments. Shading and hyposalinity did not significantly affect photosynthetic characteristics of *H. johnsonii* as determined by rapid light curves (RLC), although shade-acclimated trends were observed in several RLC responses. Confocal laser scanning microscopy (CLSM) indicated distribution of flavonoids within leaf tissue was not altered by salinity or light treatments. These results do not support a sunscreen or compatible solute role for flavonoids in *H. johnsonii*, but suggest they are capable of antioxidant functions.

*Keywords:* Halophila; Hyposalinity; Antioxidant; Flavonoids; Confocal
1. Introduction

*Halophila johnsonii* Eiseman contains 15 flavonoids (10 flavone glycosides and 5 flavones) that strongly absorb UV radiation (absorption peak of 343-348) and are located in the cuticle and epidermal cells of both abaxial and adaxial leaf surfaces (Meng et al., 2008; Chapter one of this thesis). Experimental manipulations of photosynthetically active radiation (PAR) and UV levels did not elicit consistent changes in flavonoid concentration (Kunzelman et al., 2005) and results of subsequent mesocosm experiments suggested UVP concentrations responded more strongly to salinity variation than to variations in light quality (Kahn and Durako, 2008). However, no study has yet observed the cellular localization of flavonoids in *H. johnsonii* in response to such treatments.

Flavonoids vary in amount and accumulate in different plant tissues in response to environmental stimuli (Hutzler et al., 1998; Gould and Lister, 2006). In terrestrial plants, flavonoids are often deposited in adaxial leaf surfaces (cuticle and cell-wall) or within the vacuole of adaxial epidermal cells to help shield chloroplasts in mesophyll tissues below from excess light energy (Hutzler et al., 1998; Tattini et al., 2004). Excess light energy may also generate free radicals and reactive oxygen species (ROS), which if not reduced into less harmful substances may damage nuclear DNA, proteins and lipids (Yang et al., 2006). In addition, salinity-induced reduction in photosynthesis may lead to the production of free radicals and reactive oxygen species and many plants increase their ability to scavenge ROS during salinity stress by increasing their antioxidants (Qui et al., 2003). Flavonoids may also serve antioxidant functions, scavenging free radicals and ROS under both light- or salinity-stress conditions (Tattini et al., 2004; Bohnert and Jensen, 1996; Gould and Lister, 2006). *H. johnsonii* is significantly affected by salinity,
with maximum growth rates and survival observed at salinity 30, and significant reductions in both at salinities below and above 30 (Torquemada et al., 2005). Flavonoids in *H. johnsonii* may also function as compatible solutes as they are maintained in the cytosol and may help maintain osmotic pressure under extended alterations in salinity (Murphy et al., 2003).

Plants vary flavonoid concentration and localization in response to stress (Bohnert and Jensen, 1996; Rice-Evans et al., 1997; Tattini et al., 2004; Gould and Lister, 2006). In this study, the effects of controlled hyposaline and shading treatments on flavonoid concentration and localization in *H. johnsonii* were evaluated using mesocosm experiments. Hyposalinity was examined because of its hypothesized importance in limiting the distribution of this threatened species (Virnstein and Hall, 2009). The objective was to focus on the function of flavonoids as compatible solutes, sunscreens, and/or antioxidants in this species. The antioxidant capacity of *H. johnsonii* leaf extracts was also evaluated to determine whether flavonoids significantly contribute to overall antioxidant capacity in this species.

2. Materials and methods

2.1. Sample Collection

All samples were collected August 17, 2009 at Munyon Island in Lake Worth Lagoon, Florida (26.82054° N, -80.04604° W). This site was chosen because of the availability of intertidal *H. johnsonii* and an intermixed subtidal bed of *H. johnsonii* and *H. decipiens*. A 10 x 10 cm sod plugger was used to extract rhizome segments of three to five leaf pairs from subtidal *H. johnsonii* and *H. decipiens*. Sod plugs were transplanted
into 9 x 9 x 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 distilled 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 as 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 (36.7% 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 hrs prior to sample collection were used to determine recent PAR history for day 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, 1 min running average. Median temperatures 24 hrs prior to sample collection were used to determine recent temperature history for day 1, 7, 14 and 21. Treatment-aquaria were randomly assigned among four fiberglass mesocosm tanks flushed with medium seawater 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 surface 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 surface area was calculated from digital images using Image-Pro Plus © software. After being photographed, leaves were ground in the dark in a chilled mortar with 6 ml cold, HPLC grade methanol. Samples were allowed to extract overnight in refrigerated conditions and supernatant was collected after samples were centrifuged at 10,000 RPM for five minutes at 2 °C using a Beckman Coulter ™ Avanti ® J-25 high-performance centrifuge.
2.4. *Flavonoid content and antioxidant capacity assays*

Total flavonoid content and trolox equivalent antioxidant capacity (TEAC) of methanol leaf extracts were determined according to methods presented in chapter one.

2.5. *Confocal laser scanning microscopy*

CLSM analysis of flavonoid localization was performed according to methods described in chapter one.

2.6. *Chlorophyll fluorescence measurements*

At week three of the mesocosm experiments, rapid light curves (RLC) were obtained from plants in each treatment with a portable pulse amplitude modulated (mini-PAM) fluorometer (Walz, Germany). Ambient PAR was measured by a LICOR cosine-corrected (2π) quantum sensor. 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. Relative electron transport rate (rETR\textsubscript{max}, Beer et al. 2001) was estimated using the following equation: 
\[
\text{RETR} = \frac{([Fm' - F_s])}{Fm' \times \text{PPFD} \times 0.5}.
\]
Where Fm’ = light-acclimated maximal fluorescence, F = fluorescence yield for a given light 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
(Eₖ), and quantum efficiency of photosystem II (Fᵥ/Fₘ) were calculated for each treatment from RLC’s using a double exponential decay function as described by Ralph and Gademann (2005).

2.7. 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 concentration 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 while 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. Analysis was performed using SigmaPlot® for Windows 11.0 with significance determined at the 95% probability level (p < 0.05).

3. Results

Flavonoid localization was independent of salinity or light/shade treatments over short (one day) and long (three week) time periods. Flavonoids were consistently located in the cuticle and epidermal cells of both abaxial and adaxial leaf surfaces (Figs. 11, 12). This was consistent with localization described in chapter one of this thesis.
Fig. 11. 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.
Fig. 12. Psuedo-colored CLSM images of NA-stained flavonoid fluorescence ($\lambda_{\text{exc}} = 405$ 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).
Light/shade treatments had significant effects on mean values of flavonoid concentration, but salinity did not (Table 1). Among dates, flavonoid concentrations were significantly lower for week two than at other sample times, and other dates did not significantly differ from each other. Similarly, within salinity treatments, week two flavonoid concentrations were significantly lower among dates with the exception that day one, salinity 35 treatments did not significantly differ. Flavonoid concentrations were significantly lower for light treatments among dates and within day one and were consistently higher in shade treatments within salinity 15, but this difference was not significant. Among dates, flavonoid concentrations were highest at day one, decreased slightly at one week before a significant decrease from week one to two. Flavonoid concentrations then increased significantly from week two to week three and week three concentrations did not significantly differ from day one or week one (Fig. 13).

Salinity and date had statistically-significant effects on mean TEAC values while there were no significant effects of light or any treatment interactions (Table 2). Among dates, there were significant increases in TEAC values from day one to week one, followed by a slight, but statistically insignificant, decrease at week two. From week two to three there were significant decreases in TEAC values and week three TEAC values were lowest among all dates, but did not significantly differ from day one (Fig. 14). TEAC values did not significantly differ within day one or week one among salinity treatments. TEAC values for salinity 35 and salinity 25 declined from week one to week two while salinity 15 increased to a maximum among all salinity treatments. By week two, salinity 15 had significantly higher TEAC than salinity 25 while salinity 35 did not significantly vary from the other treatments. Similarly, within week three TEAC for
Table 1.

Results of three-way ANOVA on total flavonoid content (mg L\(^{-1}\) quercetin mm\(^{-2}\)) for *H. johnsonii* (factors: salinity, light and date). Levene’s test: Passed (p = 0.939)

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**Fig. 13.** Total flavonoid content (mg L\(^{-1}\) quercetin mm\(^{-2}\)) of *H. johnsonii* leaves from mesocosm treatments at one, seven, fourteen and twenty one days for salinity 35 (unfilled), 25 (right-slanted hash marks), 15 (grey) and light (unfilled) or shade (left-slanted hash marks) treatments. Upper-case letter above bars represents significant differences among dates \(p < 0.05\). Asterisk indicates significant difference between light/shade treatments within sample date.
Table 2.

Results of three-way ANOVA on trolox equivalent antioxidant capacity (µM trolox mm$^{-2}$) for *H. johnsonii* (factors: salinity, light and date). Levene’s test: Passed (p = 0.329)

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<tr>
<td>Total</td>
<td>95</td>
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Fig. 14. Trolox equivalent antioxidant capacity (µM trolox mm⁻²) for H. johnsonii leaves from mesocosms at one, seven, fourteen and twenty one days for salinity 35 (unfilled), 25 (right-slanted hash marks), 15 (grey) and light (unfilled) or shade (left-slanted hash marks) treatments. 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.
salinity 15 was significantly higher than salinity 35 and salinity 25, which were not significantly different from each other (Fig. 14). Flavonoid concentration and TEAC showed a significant, positive correlation when all data points were considered (Fig. 15). To determine if the relationship between flavonoid concentration and TEAC changed over time correlations among dates were compared. There were significant and positive correlations for all dates except week two (Fig. 16).

Leaf surface area was significantly lower in salinity 15 treatments within weeks two and three and leaves in this salinity treatment had become wrinkled along the margins and brittle (Fig. 17). There were no significant differences in leaf area between or among light or shade treatments. Leaf surface area for salinity 35 and salinity 25 treatments declined to minimum values from day one to week one. Within salinity 25 treatments week one leaf surface areas were significantly lower than at day one or week three. Leaf surface areas increased from week one through week three for salinity 35 and salinity 25 treatments, but increases were not significant. Two-way ANOVA indicated there were no significant treatment effects of salinity, light or their interaction on observed photosynthetic parameters. There was minimal separation among values of photosynthetic efficiency at sub-saturating irradiance (α) among treatments (Fig. 18a). This is in contrast to typical sun/shade photosynthetic patterns where α is higher for shaded plants compared to sun-exposed plants. In contrast, rETR_max reflected more typical shade/sun patterns as both salinity 15 and salinity 25 shade treatments were lower than those for the light treatments (Fig 18b). The irradiance at onset of saturation (E_K) was higher for light treatments than shade within all salinities, which also reflects the typical pattern for light/shade acclimation (Fig. 18c).
Fig. 15. Linear regression between total flavonoid content (mg L$^{-1}$ quercetin mm$^{-2}$) and trolox equivalent antioxidant capacity (µM trolox mm$^{-2}$) of *H. johnsonii* leaves, all mesocosm treatments pooled. n= 96.
Fig. 16. Linear regression with correlations between total flavonoid content (mg L⁻¹ quercetin mm⁻²) and trolox equivalent antioxidant capacity (µM trolox mm⁻²) for *H. johnsonii* leaves within salinity among date (n = 24).
Fig. 17. *Halophila johnsonii* leaf surface area (mm$^2$) for mesocosms at one, seven, fourteen and twenty one days for salinity 35 (unfilled), 25 (right-slanted hash marks), and 15 (grey) and light (unfilled) or shade (left-slanted hash marks) treatments. Asterisk above bar represents significant difference among salinities within date p < 0.05.
Fig. 18. *H. johnsonii* mean (±SD) rapid light curve coefficients for: (a) slope of rETR$_{\text{max}}$ versus photosynthetic photon flux density ($\alpha$; µmol electrons / µmol photons), (b) maximum relative electron transport rate (rETR$_{\text{max}}$; µmol electrons m$^{-2}$ s$^{-1}$), and (c) irradiance at the onset of saturation (E$_k$; µmol photons m$^{-2}$ s$^{-1}$).
Photochemical efficiency, (Fv/Fm) also did not significantly differ among salinity or light treatments but was lowest in the light treatment within salinity 15° (Fig. 19).

Median water temperature (°C) and mean PAR 24 hrs prior to sample collection for day 1, 7, 14 and 21 are shown in Fig. 20 and Fig. 21, respectively. Similar trends were observed as light and temperature were higher for day one and week one, declined at week two and remained lower through week three. Median temperatures for week two and week three were significantly lower than day one or week one. There were no significant differences between day one and week one or between week two and three.

Total PAR received by shaded plants was significantly lower (37.6% mean reduction of surface irradiance of PAR) compared to light treatments. However, due to high diurnal variability, there were not significant differences in mean PAR values between light and shade treatments in the 24 hrs prior to sample collection within or among dates (Fig. 21). Among dates the lowest mean PAR was at week two. This was significantly lower than day one or week one. Week three also had significantly lower PAR than day one, but did not significantly vary from week one or two.

4. Discussion

Flavonoid concentrations and sub-cellular localization within leaves of Halophila johnsonii did not significantly vary in response to the reduced salinity or light treatments used in this study. This suggests production of flavonoids may be constitutive in this species and 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
Fig. 19. *H. johnsonii* mean (±SD) maximum quantum yields (Fv/Fm) at week three of mesocosm experiments.
Fig. 20. Water temperature (C°) in treatment aquaria at day 1, 7, 14 and 21. Median = solid horizontal line and box = 25th and 75th percentile, n=49. Asterisk above line represents significant difference p < 0.05.
Fig. 21. Average (±stdev) PAR levels for shade (filled circles) and light (open circles) on each day of treatment, n=26. Uppercase letter above box represents significant differences p < 0.05.
plants compared to plants exposed to full sun (Lautenschlager-Fleury, 1955), but after three weeks of shading total flavonoid content did not significantly differ between light and shade treatments and shaded plants often had higher flavonoid concentration. This result supports findings of Kunzelman et al., (2005) and Kahn and Durako (2008) where changes in flavonoid concentration were not significantly affected by manipulations of PAR and UV. It is possible that the moderate decreases in PAR due to the shading levels used in this study were not great enough to lower flavonoid synthesis below constitutive levels. Correspondingly, the 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 concentrations of intertidal *H. johnsonii* collected in the field also were not significantly higher than those of subtidal plants and mean flavonoid content of intertidal *H. johnsonii* was within the range measured in mesocosm manipulations (see chapter one of this thesis). This indicates that even large changes in PAR or UV are unlikely to have a significant effect on flavonoid concentration or localization in this species.

If flavonoids in *H. johnsonii* were utilized as compatible solutes they should have decreased in concentration 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 concentration at short (day one) or long (week three) time periods. In addition, if cytosolic flavonoids consist of flavone glycosides, as suggested by their presence in an aqueous environment, it would be metabolically inefficient to utilize flavone glycosides as osmolytes when the sugar
moiety attached to the flavone 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 concentration occurred at week two in all salinity and light treatments. This decrease may reflect leaf turnover rather than any treatment effect. *H. johnsonii* produces new leaf pairs every 8.17 ± 4.12 days on average and leaf growth rate is not significantly affected by shading (70% and 40% reduction in ambient PAR) (Richmond et al., 2007). Week two leaf samples were therefore likely to have been the first leaves collected whose growth was 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 concentration in seagrasses is not known. The decrease in flavonoid concentration at week two also corresponded to a significant decrease in PAR, which began at day 12 and continued through week two sampling. When light limits photosynthesis all available carbohydrates can be shunted towards growth, which may reduce synthesis of carbon-based secondary metabolites (Bryant et al., 1988) and it is possible reduced carbon availability due to a combination of transplant stress and reduced light led to the significant reduction in flavonoid compounds observed at week two.

The lack of a significant effect between light treatments on TEAC indicated that the treatment light levels did not significantly influence antioxidant metabolism. Hyposalinity did have an effect as TEAC significantly increased at salinity 15 after three weeks. Many plants increase their ability to scavenge ROS during salinity stress by increasing their antioxidants (Qui et al., 2003) and it is probable *H. johnsonii* increased synthesis of antioxidant compounds in response to the physiological stress of
hyposalinity. There was a highly significant correlation between total flavonoid content and TEAC for all weeks except week two when flavonoid concentrations declined significantly, but TEAC did not. Furthermore, salinity 15 treatments had a highly significant effect on antioxidant capacity at week three but no effect on total flavonoid concentration. While it remains possible flavonoids localized within the cytosol of epidermal leaf cells function as antioxidants, the increase in TEAC for salinity 15 plants was unlikely the result of increased flavonoid synthesis.

Chlorophyll fluorescence has become a well-accepted technique for the assessment of plant stress on photosynthetic characteristics (Schreiber et al., 1997). Photosynthetic efficiencies (Fv/Fm), light-saturated photosynthesis (rETR_{max}) and the photosynthetic efficiency at sub-saturating light (α) did not significantly decrease in response to salinity or light/shade treatments, indicating hyposalinity and shading did not affect the ability *H. johnsonii* to utilize light energy for photosynthesis. While not statistically significant, a typical shading response was present as both rETR_{max} and E_{k} were reduced for shaded plants. This suggests *H. johnsonii* photo-acclimated to lower light conditions and was able to more efficiently capture and convert photons to chemical energy despite hyposaline conditions (salinity 15 and 25). Similarly, maximum quantum yield was lowest for the salinity 15, light treatment but this was not significantly lower than other treatments. A lack of separation in alpha values between 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 rates. Absence of significant effects of salinity or light on *H. johnsonii* photosynthetic parameters suggests *H. johnsonii* is tolerant to extended hyposalinity of salinity 15 and
that this tolerance is not affected by shading. Similarly, Dawes, et al., (1989) indicated photosynthetic parameters ($I_c$, $I_k$, and $P_{\text{max}}$ 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 (*ca.* 500 μmole-photons m$^{-2}$ s$^{-1}$). Ralph, (1998) also 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 the results from Torquemada et al. (2005), which indicated $\alpha$ and $P_{\text{max}}$ parameters decreased significantly with salinity among salinity treatments of 30, 20 and 10 in *H. johnsonii*. 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. Populations used in the present study were 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 (>5km from Palm Beach Inlet). Estuarine populations must tolerate large variations in salinity on a regular basis which may help *H. johnsonii* populations from Munyon Island to better tolerate hyposalinity treatments compared to Haulover Park populations. The lack of significant interactions between salinity and light on photosynthetic parameters mirrors results of total flavonoid content and antioxidant capacity analyses, which did not show a significant light effect and may have been due, in part, to an insufficient level of shading (36% reduction). Although
salinity and light had no significant effects on photosynthetic parameters, leaf surface area was significantly reduced by low salinity.

*H. johnsonii* is considered a rapid-growth plant with a reduced storage capacity (Dean and Durako, 2007). This implies that growth and photosynthetic rates should be closely coupled. A lack of light effect on leaf surface area in this study suggests that the light reduction of 36% in ambient PAR was not sufficient to significantly affect leaf size in *H. johnsonii*. This supports observations from Richmond, et al., (2009) where reductions of 40% and 70% in ambient PAR for nine days did not have a significant effect on leaf production or leaf size. However, in the present study, leaf surface area was significantly reduced at weeks two and three for salinity 15. Because photosynthetic rates were comparable among salinity treatments, reductions in leaf surface area may be a response to 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 increase ROS production and the higher TEAC for salinity 15 may reflect a response to this stress (Alscher, 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 salinity tolerance limits and a lower initial amount of HCO$_3^-$ available within treatment aquaria, which can occur when seawater is diluted with distilled water only (Dawes and McIntosh, 1981). This may explain why wrinkled, brittle leaves were observed in salinity 15 treatments but not salinity 25 or 35 and may also indicate carbon may have limited growth (leaf surface area) of plants at salinity 15. Whatever the cause,
at week three, salinity 15, treatments had the lowest mean quantum yield, highest mean flavonoid content, significantly higher TEAC and significantly smaller leaf surface area compared to the two higher salinity treatments. This combination of responses indicates *H. johnsonii* was likely stressed, but that it can tolerate a salinity of 15 for up to three weeks.

Flavonoid concentration and sub-cellular localization did not significantly vary in response to reduced salinity or light treatments used in this study. The lack of response to light reduction suggests production of flavonoids may be constitutive and flavonoids localized in the cuticle and cytoplasm of epidermal leaf cells are unlikely to primarily function as sunscreen pigments or compatible solutes, respectively. TEAC increased in response to hyposalinity suggesting there may have been an upregulation in antioxidant metabolism in response to hyposaline stress. The presence of flavonoid compounds in the cytosol, despite reduced salinity, suggests they may serve as antioxidants which could scavenge ROS produced by chloroplast and other cellular organelles. 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 is needed to determine their primary function(s) in *H. johnsonii*. This could be achieved through careful separation of cuticular and epidermal leaf tissues and the use of HPLC to identify differences in flavonoid chemistry within each tissue.

**References**


