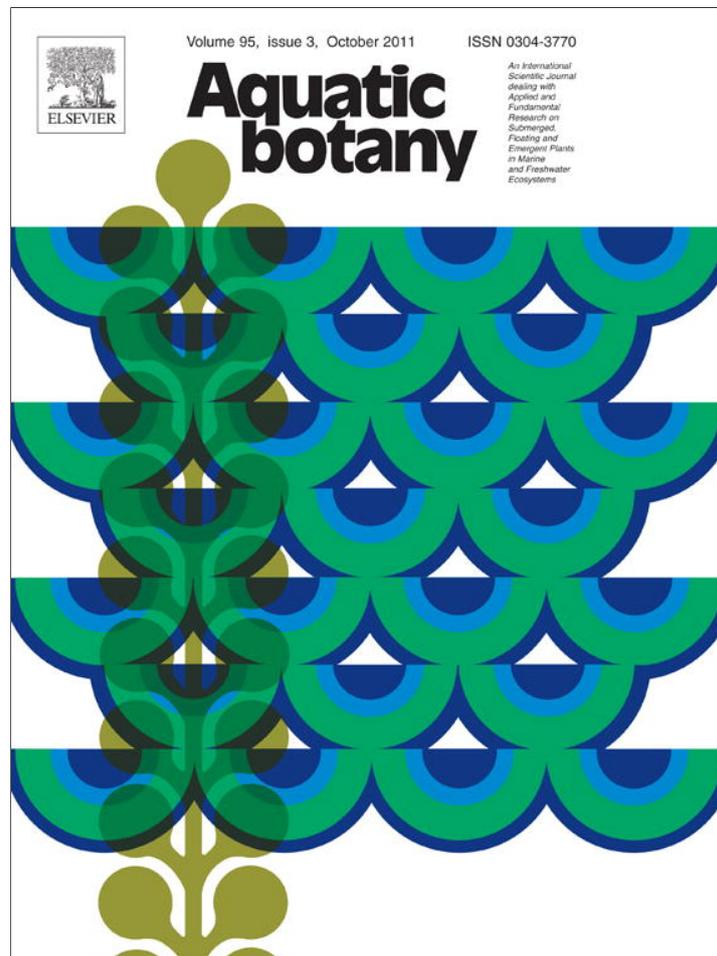


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Localization and antioxidant capacity of flavonoids from intertidal and subtidal *Halophila johnsonii* and *Halophila decipiens*

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ABSTRACT

In this study, flavonoid localization, content and total antioxidant capacity in leaves of subtidal *Halophila decipiens* were compared to intertidal and subtidal *Halophila johnsonii*. *H. johnsonii* leaves had significantly higher flavonoid content (3.5 and 3.8 nmol quercetin equivalent mm^{-2} leaf for intertidal and subtidal *H. johnsonii*, respectively) and antioxidant capacity (101.7 and 224.2 nmol Trolox equivalent mm^{-2} leaf for intertidal and subtidal *H. johnsonii*, respectively) than *H. decipiens* leaves (1.4 nmol quercetin equivalent mm^{-2} leaf and 21.0 nmol Trolox equivalent mm^{-2} leaf). Flavonoid content did not significantly differ between intertidal and subtidal *H. johnsonii*, however, antioxidant capacity was significantly higher in subtidal plants. Confocal laser scanning microscopy of fresh leaf cross sections indicated that both species contained flavonoids in the 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 sunscreen and antioxidant functions while an antioxidant role for flavonoids within *H. decipiens* is not supported.

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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* co-occurs with *Halophila decipiens* Ostenfeld 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 in *H. johnsonii* was suggested to provide protection from high irradiances and UV radiation and allow this species to exploit the shallowest waters without competition from the closely related *H. decipiens*, which lacks UV-absorbing pigments (Durako et al., 2003). The UV-absorbing pigments in *H. johnsonii* consist of 15 flavonoids (10 flavone glycosides and 5 flavones) that strongly absorb UVA radiation (absorption peak of 343–348 nm) (Meng et al., 2008). However, experimental manipulations of photosynthetically active radiation (PAR) and UV in mesocosms did not elicit consistent changes in UV-absorbing pigments (Kunzelman et al., 2005) and results of subsequent mesocosm experiments suggested UV-absorbing pigment

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 raises 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, 1997). 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; Alschner 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). The 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 correlated with ROS scavenging capability (Sekher et al., 2001; Burda and Oleszek, 2001; Heim et al., 2002). Eight of the fifteen flavone compounds identified in *H. johnsonii* possess molecular structures indicative of high antioxidant activity due to 3'-4'-ortho-di-hydroxyl or 3'-4'-5'-ortho-tri-hydroxyl configurations on the B-ring (Meng et al., 2008). Flavone compounds in *H. johnsonii* form two distinct groups, in terms of solubility: hydrophilic flavone glycosides and hydrophobic flavones (Meng et al., 2008). Differences in solubility affect

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distribution and sub-cellular localization of flavonoids, which may determine their physiological or ecological functions (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). While seagrasses have been shown to change levels of flavonoids in response to abiotic stress, 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 *in planta* the present study compared sub-cellular localization of flavonoid compounds between subtidal *H. johnsonii* and *H. decipiens*. Total flavonoid concentration and antioxidant capacity were also compared among intertidal and subtidal *H. johnsonii* and *H. decipiens* to examine whether intertidal plants maintain higher flavonoid concentrations and antioxidant capacity.

2. Materials and methods

2.1. Sample collection and physical measurements

All samples were collected August 17, 2009 at Munyon Island in Lake Worth Lagoon, FL (26.82054°N, 80.04604°W) between 11:00 and 12:00 h during a falling tide when intertidal plants were exposed and subtidal plants were immersed in 55 cm of water. This site was chosen because of the availability of intertidal *H. johnsonii* and an intermixed subtidal bed of *H. johnsonii* and *H. decipiens*. A LICOR scalar (4π) quantum sensor was used to measure photosynthetically active radiation (PAR) at the level of the leaf canopy immediately before plant collections, at intertidal ($998 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and subtidal depths ($829 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). Temperature and salinity were also measured at intertidal (30.9°C, salinity: N/A) and subtidal (30.5°C, salinity: 31.4) locations using an YSI Model 650 multiparameter display system. A 10 cm × 10 cm sod plugger was used to extract sediments containing rhizome segments with three to five leaf pairs of *H. johnsonii* and *H. decipiens*. Sod plugs were transplanted into 9 cm × 9 cm × 9 cm plastic planting pots pre-sterilized with bleach and placed in coolers filled with ambient seawater. Leaves from these plants were used for confocal laser scanning microscopy. Additional leaf material from subtidal *H. johnsonii* ($n=6$) and *H. decipiens* ($n=6$) and intertidal *H. johnsonii* ($n=6$) plants was also collected. The second leaf pair back from an apical meristem was removed; one leaf was placed in a polypropylene tube filled with 6 mL HPLC grade methanol for pigment extraction and kept on ice in the dark. The second leaf was fixed in 6 mL of 2.5% glutaraldehyde (w/v seawater) with 0.5% caffeine (w/v seawater). This leaf was used to determine leaf area. All plant materials were transported to the Center for Marine Science in Wilmington, NC, within 24 h and potted plants were placed into outdoor, flow-through seawater holding troughs (salinity: 33.7).

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 as individual optical sections or combined to create a three-dimensional recon-

struction 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* and *H. decipiens* 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. Sections were 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 ($\lambda_{\text{exc}} = 405 \text{ nm}$) 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. Plant material was not fixed at the time of collection because glutaraldehyde and formaldehyde caused strong autofluorescence of leaf tissue with emission wavelengths that overlapped fluorescence from the flavonoid-specific probe ($\lambda_{\text{emission}} = 515\text{--}565 \text{ nm}$) when viewed under CLSM.

2.3. Leaf area and pigment extraction

Fixed leaf tissue samples collected in the field were placed between two glass microscopy slides adjacent to a ruler and photographed for determination of leaf area. A digital camera was placed on a tripod at 0.3 m above the sample and a bubble level was used to maintain a perpendicular photographic angle. Leaf area was calculated from digital images using Image-Pro Plus[®] software. Leaves in the 6 mL of cold HPLC grade methanol were ground in the dark in a chilled mortar. Samples were allowed to extract overnight in refrigerated conditions and supernatant was collected after samples were centrifuged at $12,000 \times g$ for five minutes 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 Ordonez 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}) and normalized to leaf area (mm^{-2}) based on a five point calibration curve prepared each day before measurements began.

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 μ L of plant extract was added to 990 μ L of $ABTS^{\cdot+}$ solution and absorbance at 734 nm was measured 4 min 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 nmol was obtained. Results were corrected for dilution and expressed as nmol Trolox equivalent mm⁻² leaf.

2.6. Statistical analysis

Statistical comparisons between flavonoid concentration and antioxidant capacity were made between intertidal and subtidal *H. johnsonii* and between subtidal *H. johnsonii* and *H. decipiens* using one-way ANOVA. 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 (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 ($p < 0.05$). All data are reported as means \pm standard deviations.

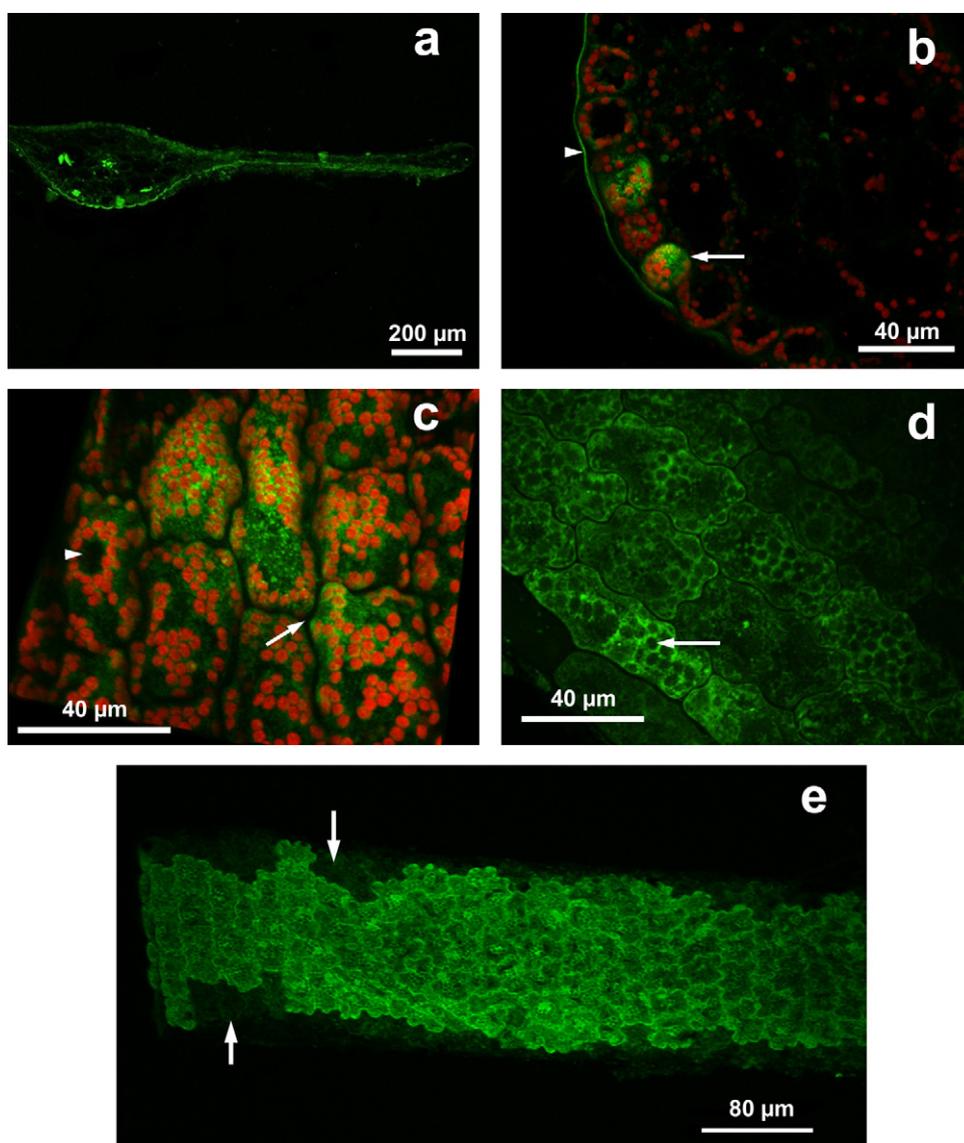


Fig. 1. CLSM images of chloroplast (red) and NA-stained flavonoid fluorescence (green) in *H. johnsonii*. (a) Z-stack of leaf mid-rib cross section. (b) Z-stack of leaf mid-rib, adaxial surface. Note flavonoids located in cytosol of intact epidermal cells (arrow) and within the cuticle (arrowhead). (c and d) Z-stacks of leaf epidermis collected below leaf cuticle. Note the lack of flavonoids within central vacuole (arrowhead), cell wall (white arrow) (c), and chloroplast (white arrow, d). (e) Leaf lamina. Note cells damaged during sectioning showed decreased NA-staining from release of soluble compounds (arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

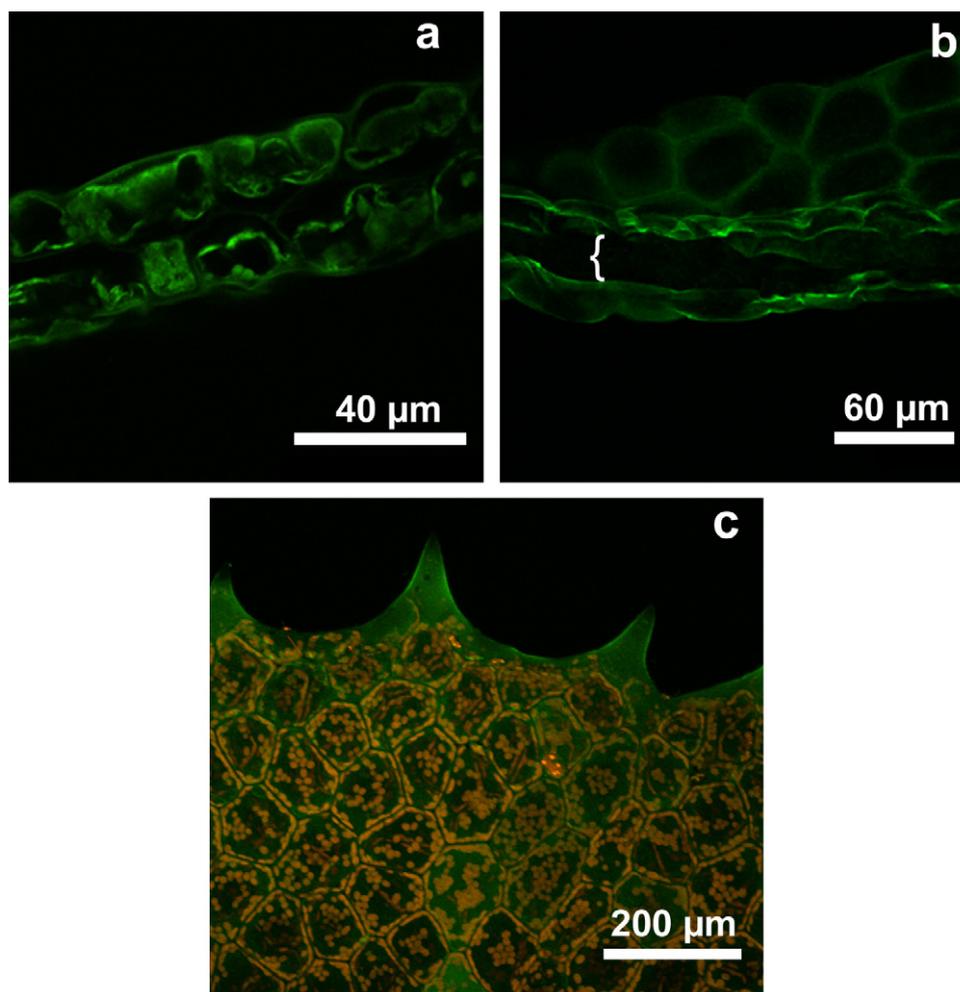


Fig. 2. Single channel CLSM fluorescent images ($\lambda_{\text{emission}} = 515\text{--}565\text{ nm}$) of NA-stained leaf lamina cross sections of *H. johnsonii* (a) and *H. decipiens* (b). Note lack of intracellular flavonoids within epidermal tissues of *H. decipiens* (b; white bracket). (c) Pseudo-colored 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results

H. johnsonii contained NA-stained flavonoid compounds in both abaxial and adaxial leaf tissues (Fig. 1a). Flavonoids were localized in epidermal cells of both leaf surfaces as well as the extracellular cuticular layer (Fig. 1b). Within epidermal cells, NA-stained flavonoids were absent from cell walls (Fig. 1c) and cellular organelles, including the central vacuole (Fig. 1c) and chloroplasts (Fig. 1d). Flavonoid fluorescence originated predominantly from soluble compounds within the cytosol. The soluble compounds were released from cells that were damaged during sectioning (Fig. 1e).

CLSM images of *H. decipiens* showed NA-stained flavonoid compounds localized in the cuticle of both the abaxial and adaxial surfaces, but lacked flavonoid fluorescence within the cytosol, which contrasts with *H. johnsonii* (Fig. 2a and b). Extracellular flavonoid fluorescence was clearly visible in the marginal teeth (Fig. 2c). Because Fig. 2c is a combination of optical sections in the z-direction, the intense green fluorescence that appears above several of the epidermal cells is due to fluorescence of flavonoid compounds localized in the cuticle above epidermal cells.

There was no statistically significant difference between total flavonoid content (nmol quercetin equivalent mm^{-2} leaf) of intertidal (3.5 ± 1.0) and subtidal (3.8 ± 1.2) *H. johnsonii* (Kruskal–Wallis ANOVA on Ranks: $H=0.92$, 1 d.f., $p=0.39$). Flavonoid content of

H. decipiens (1.4 ± 0.2) was significantly lower than both intertidal (ANOVA, $F_{1,10} = 28.39$, $p < 0.001$) and subtidal (Kruskal–Wallis ANOVA on Ranks, $H = 8.31$, 1 d.f., $p = 0.002$) *H. johnsonii*. Mean Trolox equivalent antioxidant capacity (TEAC, nmol Trolox equivalent mm^{-2} leaf) of subtidal (224.2 ± 100.9) *H. johnsonii* was significantly higher than intertidal (101.7 ± 16.6) plants (Kruskal–Wallis ANOVA on Ranks: $H = 8.31$, $p = 0.002$), while TEAC of *H. decipiens* (21.0 ± 7.3) was significantly lower than both intertidal (ANOVA, $F_{1,9} = 82.73$, $p < 0.001$) and subtidal (Kruskal–Wallis ANOVA on Ranks, $H = 7.5$, 1 d.f., $p = 0.004$) *H. johnsonii*. Both intertidal and subtidal *H. johnsonii* showed significant positive correlations between total flavonoid content and TEAC ($R = 0.90$, $p = 0.02$, $R = 0.96$, $p = 0.003$, respectively) while *H. decipiens* did not ($R = 0.04$, $p = 0.95$, Fig. 3a). However, for subtidal *H. johnsonii*, the relationship between total flavonoid content and TEAC was primarily driven by a single outlier (Cook's distance = 9.13). When this point was removed from the regression the correlation was no longer significant ($R = 0.44$, $p = 0.46$). Similarly, when intertidal and subtidal *H. johnsonii* data were pooled, the correlation between total flavonoid content and TEAC was significant ($R = 0.68$, $p = 0.02$), but the same point remained a significant outlier (Cook's distance = 3.63) which, upon removal, caused the correlation to lose significance ($R = 0.14$, $p = 0.68$, Fig. 3b). Due to the influence of this single point in the subtidal *H. johnsonii* data, the relationship between flavonoid content and TEAC is unclear for the subtidal plants, but a significant, positive rela-

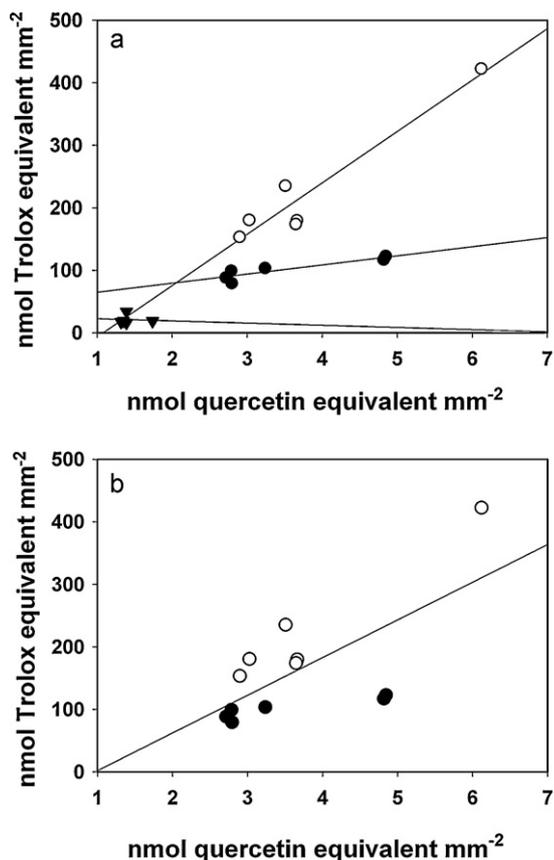


Fig. 3. (a) Correlations between total flavonoid content (nmol quercetin equivalent mm^{-2} leaf) and Trolox equivalent antioxidant capacity (nmol Trolox equivalent mm^{-2} leaf) for intertidal *H. johnsonii* (Hj I, $R = 0.90$, $p = 0.015$), subtidal *H. johnsonii* (Hj S, $R = 0.96$, $p < 0.01$), *H. decipiens* (Hd, $R = 0.04$, $p = 0.95$) and (b) intertidal and subtidal *H. johnsonii* pooled ($R = 0.74$, $p < 0.01$).

relationship between flavonoid content and TEAC was supported for intertidal *H. johnsonii*.

4. Discussion

H. johnsonii and *H. decipiens* significantly differ in antioxidant capacity, flavonoid localization and total flavonoid content, 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 content than *H. decipiens*. Our data support this hypothesis, as both flavonoid content and TEAC were significantly higher in *H. johnsonii* leaves. Higher contents 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 content and TEAC values for intertidal *H. johnsonii*, which suggests flavonoids may contribute to antioxidant capacity of leaf tissues in this species. In contrast, the lack of correlation between flavonoid content and TEAC values in *H. decipiens*, suggests flavonoids do not perform antioxidant functions in this species. 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 potential 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).

There was no significant difference in flavonoid content between intertidal and subtidal *H. johnsonii* but TEAC was significantly higher for subtidal plants. Intertidal *H. johnsonii* is exposed to higher levels of light, temperature and desiccation than subtidal populations on a seasonal and daily basis (Kenworthy, 1997). A similar content 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 levels 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).

Higher TEAC of subtidal *H. johnsonii* compared to intertidal plants was unexpected. However, antioxidant content does not always reflect stress tolerance associated with an intertidal distribution (Collén and Davison, 1999). 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 $\text{ABTS}^{\cdot+}$ radical (Re et al., 1999). Therefore, TEAC measurements of antioxidant capacity have inherent limitations and we were unable to judge whether reduced TEAC values for intertidal *H. johnsonii* were due to fewer antioxidant compounds or enzymes than subtidal plants or from differences in light history between intertidal and subtidal plants. Intertidal plants were exposed to direct sunlight at the time of collection and antioxidant pools may have been more highly oxidized due to higher irradiances and temperatures compared to subtidal plants. It should also be noted that these conclusions are drawn from a comparison of a single pair of sites, and comparison among multiple pairs of sites will be needed to determine whether these data represent a general pattern. 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 at multiple sites before dawn to reduce the effect of different light intensities and light histories 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 could 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; Häder 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 sulfated 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 (Harborne, 1997).

Antiherbivoral or antifouling activity has not been examined for the flavonoids from *H. johnsonii*, but flavonoids isolated from *H. johnsonii* are not sulfated (Meng et al., 2008).

Within epidermal cells of *H. johnsonii*, flavonoid fluorescence originated predominantly from soluble compounds within the cytosol. These compounds were absent from cells that were damaged during sectioning. Chloroplasts in *H. johnsonii* are epidermal and chloroplasts are responsible for a majority of ROS production in plant cells (Yamasaki et al., 1997). 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). In contrast to *H. johnsonii*, *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 pigment extracts of *H. decipiens* show little UV absorbance (Durako et al., 2003). Isolation and characterization 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 this species.

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 functions. *H. decipiens* contained extracellular flavonoids in adaxial and abaxial cuticular 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 levels 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.

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References

Alscher, R.G., Erturk, N., Heath, L.S., 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J. Exp. Bot.* 53, 1331–1341.

- Bohnert, H.J., Jensen, R.G., 1996. Strategies for engineering water stress tolerance in plants. *Trends Biotechnol.* 14, 89–97.
- Burda, S., Oleszek, W., 2001. Antioxidant and antiradical activities of flavonoids. *J. Agric. Food Chem.* 49, 2774–2779.
- Collén, J., Davison, I.R., 1999. Reactive oxygen production and damage in intertidal *Fucus* spp. (Phaeophyceae). *J. Phycol.* 32, 54–61.
- Dawes, C.J., Lobban, C.S., Tomasko, D.A., 1989. A comparison of the physiological ecology of the seagrass *Halophila decipiens* Ostenfeld and *H. johnsonii* Eiseman from Florida. *Aquat. Bot.* 33, 149–154.
- Dawson, S.P., Dennison, W.C., 1996. Effects of ultraviolet and photosynthetically active radiation on five seagrasses. *Mar. Biol.* 125, 629–638.
- Detres, Y., Armstrong, R.A., Connelly, X.M., 2001. Ultraviolet-induced responses in two species of climax tropical marine macrophytes. *J. Photochem. Photobiol. B: Biol.* 62, 55–66.
- Draper, J., 1997. Salicylate, superoxide synthesis and cell suicide in plant defense. *Trends Plant Sci.* 2, 162–165.
- Durako, M.J., Kunzelman, J.I., Kenworthy, W.J., Hammerstrom, K.K., 2003. Depth-related variability in the photobiology of *Halophila johnsonii* and *Halophila decipiens*. *Mar. Biol.* 142, 1219–1228.
- Gould, K.S., Lister, C., 2006. Flavonoid functions in plants. In: Andersen, O.M., Markham, K.R. (Eds.), *Flavonoids: Chemistry, Biochemistry and Applications*. CRC Press LLC, London, pp. 397–440.
- Häder, D.P., Kumar, H.D., Smith, R.C., Worrest, R.C., 1998. Effects on aquatic ecosystems. *J. Photochem. Photobiol. B: Biol.* 46, 53–68.
- Harborne, J.B., 1997. *Introduction to Ecological Biochemistry*, 4th ed. Academic Press, New York, p. 318.
- Harborne, J.B., 1979. Flavonoid pigments. In: Rosenthal, G.A., Janzen, D.H. (Eds.), *Herbivores: Their Interaction with Secondary Plant Metabolites*. Academic Press, New York, p. 718.
- Heim, K.E., Tagliaferro, A.R., Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships. *J. Nutr. Biochem.* 10, 572–584.
- Hutzler, P., Fischbach, R., Werner Heller, W., Jungblut, T., Reuber, S., Schmitz, R., Veit, M., Weissenbo, G., Schnitzler, J., 1998. Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. *J. Exp. Bot.* 49, 953–965.
- Jensen, P.R., Jenkins, K.M., Porter, D., Fenical, W., 1998. Evidence that a new antibiotic flavone glycoside chemically defends the sea grass *Thalassia testudinum* against zoospore fungi. *Appl. Environ. Microbiol.* 64 (4), 1490–1496.
- Kahn, A.E., Durako, M.J., 2008. Photophysiological responses of *Halophila johnsonii* to experimental hyposaline and hyper-CDOM conditions. *J. Exp. Mar. Biol. Ecol.* 367, 230–235.
- Kenworthy, W.J., 1993. The distribution, abundance and ecology of *Halophila johnsonii* Eiseman in the lower Indian River, Florida. Final Report to the Office of Protected Resources. National Marine Fisheries Services, Silver Spring, MD, p. 80.
- Kenworthy, W.J., 1997. An Updated Biological Status Review for *Halophila johnsonii* Eiseman and Summary of the Proceedings of a Workshop to Review the Biological Status of the Seagrass, *Halophila johnsonii* Eiseman. National Marine Fisheries Services, Office of Protected Resources, Silver Spring, MD, p. 24.
- Kunzelman, J.I., Durako, M.J., Kenworthy, W.J., Stapleton, A., Wright, J.C.L., 2005. Irradiance-induced changes in the photobiology of *Halophila johnsonii* Eiseman. *Mar. Biol.* 148, 241–250.
- McMillan, C., 1986. Sulphated flavonoids and leaf morphology in the *Halophila ovalis*–*H. minor* complex (Hydrocharitaceae) of the Indo-Pacific Ocean. *Aquat. Bot.* 25, 63–72.
- Meng, Y., Krzysiak, A.J., Durako, M.J., Kunzelman, J.I., Jeffrey, L.C., Wright, J.C.L., 2008. Flavones and flavone glycosides from sea grass *Halophila johnsonii*. *Phytochemistry* 69, 2603–2608.
- Neil, S.O., Gould, K.S., 2003. Anthocyanins in leaves: light attenuators or antioxidants? *Funct. Plant Biol.* 30, 865–873.
- Ordóñez, A.A.L., Gomez, J.D., Vattuone, M.A., Isla, M.I., 2006. Antioxidant activities of *Sechium edule* (Jacq.) Swart extracts. *Food Chem.* 97, 452–458.
- Re, R., Pellegrini, M., Prottogente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 26 (9/10), 1231–1237.
- Rice-Evans, C.A., Miller, N.J., Papanga, G., 1997. Antioxidant properties of phenolic compounds. *Trends Plant Sci.* 2, 152–159.
- Sekher, A., Pannala-Chan, T.S., O'Brien, P.J., Rice-Evans, C.A., 2001. Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. *Biochem. Biophys. Res. Commun.* 282, 1161–1168.
- Tattini, M., Galardi, C., Pinelli, P., Massai, R., Remorini, D., Agati, G., 2004. Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. *New Phytol.* 163, 547–561.
- Trocine, R.P., Rice, J.D., Wells, G.N., 1981. Inhibition of seagrass photosynthesis by ultraviolet-B radiation. *Plant Physiol.* 68, 74–81.
- Virnstien, R.W., Morris, L.J., 2007. Distribution and abundance of *Halophila johnsonii* in the Indian River Lagoon: an update. Technical Memorandum #51. St. Johns River Water Management District, Palatka, FL, p. 16.
- Yamasaki, H., Sakihama, Y., Ikehara, N., 1997. Flavonoid–peroxidase reaction as a detoxification mechanism of plant cells against H₂O₂. *Plant Physiol.* 115, 1405.