Unraveling the mystery of leaf reddening in seagrasses

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Unraveling the mystery of leaf reddening in seagrasses

Abstract
Seagrass meadows around the world are declining due to natural and anthropogenic stressors, including global climate change. Recently, more attention has been given to identifying responses that offer resistance to stressors so that researchers can better manage seagrasses for resilience to environmental change. Leaf reddening, the expression of red coloration in leaves, is a well-documented response in terrestrial plants that has been shown to increase resilience to stress, but has been poorly understood in seagrasses. To increase our understanding of the prevalence, causes, and function of leaf in seagrasses, surveys were conducted in the world’s six seagrass bioregions and a series of experiments were performed with green- and red-leaved Thalassia testudinum shoots in the lower Florida Keys. Results show that leaf reddening is prevalent in seagrasses, occurring in numerous species growing in shallow waters with high light intensities around the world. In addition, experiments with T. testudinum demonstrate that the expression of red coloration is caused by the accumulation of anthocyanins, acts as a sunscreen during periods of high UV and visible light intensities, can be an indicator of UV-B exposure, and may be either transiently or permanently expressed in leaves. The findings of this study imply that leaf reddening could increase seagrass resilience to changes in atmospheric UV levels caused by global climate change by acting as a sunscreen and protecting photosynthetic mechanisms from damage.

Keywords
Biology, Ecology

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UNRAVELING THE MYSTERY OF LEAF REDDENING IN SEAGRASSES

BY

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DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Natural Resources and Earth System Sciences: Oceanography

September, 2011
This dissertation has been examined and approved.

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July 28th, 2011
Date
DEDICATION

This dissertation is dedicated to PEH, who initiated my interest in science and taught me to never take the path of least resistance.
ACKNOWLEDGEMENTS

I would like to acknowledge the following individuals and organizations for their support and assistance with my dissertation.

I thank my major professor, Dr. Fred Short, for his continual guidance and support through all aspects of my dissertation. I appreciate that he has always given me the freedom to pursue an independent project, has always shown faith in my research, and has been a strong advocate of my work. In reviewing my writing, Fred and his wife, Cathy, have provided invaluable comments while respecting my voice. I will greatly miss being a member of his lab.

I also extend my gratitude to my dissertation committee members: Drs. Clinton J. Dawes, Leland S. Jahnke, Thomas D. Lee, and J. Ru Morrison. I appreciate the useful suggestions and comments they provided that improved the overall quality of my project. They also developed my background in ecology and physiology.

I thank all my friends at Jackson Estuarine Laboratory and the technicians at Mote Marine Laboratory for their willingness to lend a hand. I would especially like to thank Nikki Sarette, Paul Sokoloff, Jon Felch, and Holly Bayley for their friendship and the many useful discussions we had on logistics, experimental design and implementation. In addition, I would like to thank Pamela Fraunruber and Lauren Gaskell for helping me in the field and lab during the summer of 2007.

Funding for this dissertation was provided by a number of institutions and organizations. I am indebted to NOAA’s Nancy Foster Scholarship Program for providing four years of financial support; without their generous contribution I would not
have been able to conduct this project. I would also like to acknowledge SeagrassNet, Ocean Park Conservation Foundation, the David and Lucile Packard Foundation, the Oak Foundation, the New Hampshire Charitable Foundation & Tom Haas, as well as the University of New Hampshire’s Natural Resources Department and Marine Program for covering equipment and travel. Additional travel awards were also provided by the Graduate School and the Farrington Fund at the University of New Hampshire.

Finally, I would like to extend my thanks to my family for their unwavering support. I thank my parents for teaching me early in life to love learning and for making my education their top priority. I also thank them for making numerous trips down to the Florida Keys to lend an extra hand in the field and lab, as well as to babysit. I am especially indebted to my husband, Chris, for being patient and for helping at all stages of this dissertation. I also appreciate him giving up his vacation time to search for “red” seagrasses. And last, but not least, I thank my newest field assistant, my daughter, Abby, as well as our little dog, Rudy, for patiently waiting each day for me to have time to take her for a walk.
# TABLE OF CONTENTS

DEDICATION.........................................................................................................................iv  
ACKNOWLEDGEMENTS........................................................................................................v  
LIST OF TABLES..................................................................................................................xiii  
LIST OF FIGURES.................................................................................................................xv  
ABSTRACT.............................................................................................................................xix

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Definition and origin of seagrasses</td>
<td>2</td>
</tr>
<tr>
<td>Morphological, anatomical and physiological adaptations of seagrasses</td>
<td>2</td>
</tr>
<tr>
<td>Leaves</td>
<td>3</td>
</tr>
<tr>
<td>Extensive root/rhizome system</td>
<td>3</td>
</tr>
<tr>
<td>Reproductive structures</td>
<td>4</td>
</tr>
<tr>
<td>Distribution of seagrasses</td>
<td>5</td>
</tr>
<tr>
<td>Importance of seagrass ecosystems</td>
<td>6</td>
</tr>
<tr>
<td>Impact of environmental stressors on seagrasses</td>
<td>6</td>
</tr>
<tr>
<td>Leaf reddening</td>
<td>8</td>
</tr>
<tr>
<td>Objectives</td>
<td>10</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>13</td>
</tr>
</tbody>
</table>
## II. LEAF REDDENING IN SEAGRASSES

| Abstract | 25 |
| Introduction | 25 |
| Methods | 27 |
| Results and Discussion | 27 |
| Acknowledgments | 29 |
| Literature Cited | 30 |

## III. LEAF REDDENING IN THE SEAGRASS *THALASSIA TESTUDINUM* IN RELATION TO ANTHOCYANINS, SEAGRASS PHYSIOLOGY AND MORPHOLOGY, AND PLANT PROTECTION

| Abstract | 39 |
| Introduction | 40 |
| Material and Methods | 43 |
| Site description and experimental design | 43 |
| Distribution of red pigments in cells | 44 |
| Quantification of pigments | 44 |
| Anthocyanin identification | 45 |
| Fluorescence measurements | 46 |
| Seagrass morphology and structure | 47 |
| Growth and plant constituents | 47 |
| Light and temperature | 48 |
| Statistics | 49 |
| Results | 49 |
IV. UV-B INDUCES LEAF REDDENING AND CONTRIBUTES TO THE MAINTENANCE OF PHOTOSYNTHESIS IN THE SEAGRASS *THALASSIA TESTUDINUM*
Experiment 1: Light induced leaf reddening in green-leafed

*T. testudinum* shoots ................................................................. 85

Experiment 2: Effect of light on red-leafed *T. testudinum*

shoots ............................................................................................. 85

Pigment Analyses ........................................................................... 86

Fluorescence measurements ................................................... 87

Statistics ......................................................................................... 88

Results ............................................................................................. 89

Experiment 1: Light induced leaf reddening in green-leafed

*T. testudinum* shoots ................................................................. 89

Color measurements ................................................................. 89

Quantification of anthocyanins .................................................. 89

Experiment 2: Effect of light on red-leafed *T. testudinum*

shoots ............................................................................................. 90

Color measurements ................................................................. 90

Pigment analyses ................................................................. 90

Fluorescence measurements .................................................. 90

Discussion ...................................................................................... 91

Acknowledgements ......................................................................... 94

Literature Cited ............................................................................ 95

V. TRANSIENT AND PERMANENT LEAF REDDENING IN THE SEAGRASS

*THALASSIA TESTUDINUM* ......................................................... 109
LIST OF TABLES

CHAPTER I

Table 1.1 A list of the 72 seagrass species of the world .............................................. 20

CHAPTER II

Table 2.1 Documented leaf reddening in seagrasses by species and location ................................................................. 34

CHAPTER III

Table 3.1 UV-absorbing compound and photosynthetic pigment content in green- and red-leafed shoots of *T. testudinum* ............................................. 68

Table 3.2. Morphological information for green and red-leafed shoots of *T. testudinum* ................................................................. 69

Table 3.3. Structural, growth, and nutrient content information for green and red-leafed shoots of *T. testudinum* ................................................................. 70

CHAPTER IV

Table 4.1 Experiment 2: Mean anthocyanin and UV-absorbing compound content in red-leafed shoots of *T. testudinum* after seven weeks for each treatment ................................................................. 100
Table 4.2 Experiment 2: Regression results for time (weeks) versus chlorophyll $a$, chlorophyll $b$, total chlorophyll, chlorophyll $a:b$ and carotenoid content in leaves of *in situ* red-leafed *T. testudinum* shoots grown in different light treatments over seven weeks ................................................................. 101

Table 4.3 Experiment 2: ANOVA results for each week showing differences among treatments in values of $\Delta F/ F_m$ for leaves of *in situ* red-leafed shoots .................................................................................................................. 102

CHAPTER V

Table 5.1. ANOVA results from within site comparisons of anthocyanin and UV absorbing (UV-B and UV-A) pigment content for green-and red-leafed shoots collected at 10 weeks post-transplantation .................................................................................................................. 124

Table 5.2. ANOVA results from within site comparisons of anthocyanin and UV absorbing (UV-B and UV-A) pigment content for each shoot color collected at 10 weeks in green compared to red patches .................................................................................................................. 125
LIST OF FIGURES

CHAPTER I

Figure 1.1 Global seagrass geographic bioregions ........................................ 23
Figure 1.2 Basic chemical structure of an anthocyanin molecule ................. 24

CHAPTER II

Figure 2.1 Survey site locations for seagrasses with red coloration in leaves .................................................................................................................. 37
Figure 2.2 Seagrasses exhibiting different patterns of redness ..................... 38

CHAPTER III

Figure 3.1 Map of the lower Florida Keys, USA, including study sites with patches of T. testudinum with entirely red-leafed shoots ....... 71
Figure 3.2 Red pigmented cells in leaf tissue of T. testudinum producing red-leafed shoots, with anthocyanins occurring in epidermal (E) and mesophyll (M) cells (A denotes location of arenchyma) ........................................................................ 72
Figure 3.3 Mean UV and visible absorption spectra of methanol extracts of leaves from green and red-leafed shoots of T. testudinum ....... 73
Figure 3.4 Mean anthocyanin content for leaves of green and red-leafed shoots of T. testudinum .................................................................................. 74
Figure 3.5 Regression between anthocyanin content and chlorophyll $a$
chlorophyll $b$, total chlorophyll, and carotenoid content for
leaves of green and red-leafed shoots of $T. testudinum$.............75

Figure 3.6 $\Delta F/ F_m$ (effective quantum yield) values for green and red-
leafed shoots of $T. testudinum$ at each site.................................76

Figure 3.7 Incident PAR at the leaf surface of green and red-leafed
shoots of $T. testudinum$ at each site, measured in July of 2007
on clear days near midday..............................................................77

Figure 3.8 Regressions between the average percentage of UV and
PAR bottom irradiance and leaf anthocyanin content for
patches of $T. testudinum$ with green and red-leafed shoots........78

CHAPTER IV

Figure 4.1 Map of the lower Florida Keys, USA with the location of
leaf reddening study sites (red dots)..............................................103

Figure 4.2 Section of a leaf from a green-leafed $T. testudinum$ shoot
showing reddening beginning at the base of the blade and
progressing up the central vein towards the tip.........................104

Figure 4.3 Experiment 1: Anthocyanin concentrations in leaves from
transplanted green-leafed $T. testudinum$ shoots grown under
different light treatments after one week ...............................105

Figure 4.4 Experiment 2: The relationship between time and chlorophyll
$a$, chlorophyll $b$, total chlorophyll, and carotenoid content in
leaves of *in situ* red-leafed *T. testudinum* shoots grown in different light treatments over seven weeks.

Figure 4.5 Experiment 2: Average weekly ΔF/ F m values from *in situ* red-leafed *T. testudinum* shoots from different light treatments at each site.

Figure 4.6 Experiment 2: Average weekly rETR values from *in situ* red-leafed *T. testudinum* shoots growing in different light treatments.

CHAPTER V

Figure 5.1 Location of study sites (red dots) in the Florida Keys, USA.

Figure 5.2 Transplanted green-leafed shoots growing in the red patch at Big Pine Key at week 4 post-transplantation.

Figure 5.3 Site and patch-level information on the number of transplanted green-leafed shoots with all green leaves and with new red leaf tissue at week 4 post-transplantation.

Figure 5.4 Mean anthocyanin content in transplanted green- and red-leafed shoots in the green and red patch at each site at 10 weeks post-transplantation.

Figure 5.5 Mean UV-B absorbing compound content in transplanted green- and red-leafed shoots in the green and red patch at each site at 10 weeks post-transplantation.
Figure 5.6  Mean UV-A absorbing compound content in transplanted
green- and red-leafed shoots in the green and red patch at each
site at 10 weeks post-transplantation..................................131
ABSTRACT

UNRAVELING THE MYSTERY OF SEAGRASSES WITH RED LEAVES

by

Alyssa B. Novak

University of New Hampshire, September, 2011

Seagrass meadows around the world are declining due to natural and anthropogenic stressors, including global climate change. Recently, more attention has been given to identifying responses that offer resistance to stressors so that researchers can better manage seagrasses for resilience to environmental change. Leaf reddening, the expression of red coloration in leaves, is a well-documented response in terrestrial plants that has been shown to increase resilience to stress, but has been poorly understood in seagrasses. To increase our understanding of the prevalence, causes, and function of leaf in seagrasses, surveys were conducted in the world’s six seagrass bioregions and a series of experiments were performed with green- and red-leafed *Thalassia testudinum* shoots in the lower Florida Keys. Results show that leaf reddening is prevalent in seagrasses, occurring in numerous species growing in shallow waters with high light intensities around the world. In addition, experiments with *T. testudinum* demonstrate that the expression of red coloration is caused by the accumulation of anthocyanins, acts as a sunscreen during periods of high UV and visible light intensities, can be an indicator of UV-B exposure, and may be either transiently or permanently expressed in leaves.
The findings of this study imply that leaf reddening could increase seagrass resilience to changes in atmospheric UV levels caused by global climate change by acting as a sunscreen and protecting photosynthetic mechanisms from damage.
CHAPTER 1

INTRODUCTION

Abstract

Seagrasses are a functional group of 72 species of marine angiosperms adapted to coastal environments throughout the world. They form extensive underwater meadows in estuaries, back reefs, and shallow marine waters in both temperate and tropical regions. They also provide a variety of ecological and economic services and are considered a vital component of coastal ecosystems. In recent decades, more attention has been given to understanding and predicting the responses of seagrasses to various environmental stressors since seagrass meadows are declining worldwide. Leaf reddening, a response commonly induced by abiotic or biotic stressors in terrestrial plants, has been reported in seagrass leaves, but research on the phenomenon in seagrasses is lacking. The objective of my research is to increase our understanding of: 1) the distribution and prevalence of seagrasses expressing red coloration in leaves; 2) the molecules responsible for red coloration; 3) the physiological and morphological characteristics associated with seagrasses expressing red coloration; 4) the potential function(s) of red coloration in leaves; 5) the factor(s) responsible for the induction of red coloration in leaves; and 6) the plasticity of red coloration in leaves. To accomplish the above objectives, I conducted the majority of my research in the lower Florida Keys with the seagrass *Thalassia testudinum*, the dominant species found in the tropical waters of the Atlantic and Caribbean.
Definition and origin of seagrasses

Seagrasses are an ecological group of angiosperms that live in estuarine or shallow marine environments. They are called seagrasses because most species superficially resemble terrestrial grasses of the Family Poaceae even though they are more closely related to terrestrial lilies and gingers. Researchers believe seagrasses evolved 70 million to 100 million years ago from a single lineage of terrestrial monocotyledons into three independent lineages of seagrass (Cymodoceaceae complex, Hydrocharitaceae, and Zosteraceae (Waycott et al., 2006). Today, there are approximately 72 seagrass species belonging to 6 families and 13 genera (Kuo and den Hartog, 2001; Moore and Short, 2006; Short et al., 2011) with each species classified according to ecological, reproductive, and vegetative characteristics including: blade width, blade tips, vein numbers, fiber distributions, epidermal cells, and roots and rhizomes (Kuo and den Hartog, 2001; Short et al., 2011). Five genera are placed in the family Cymodoceaceae (Amphibolis, Cymodocea, Halodule, Syringodium, and Thalassodendron), three in Hydrocharitaceae (Enhalus, Halophila, and Thalassia), one in Posidoniaceae (Posidonia), one in Ruppiaceae (Ruppia), one in Zannichelliaceae (Lepilaena) and two in Zosteraceae (Phyllospadix and Zostera; Kuo and den Hartog, 2001; Moore and Short, 2007; Short et al., 2011; Table 1.1).

Morphological, anatomical and physiological adaptations of seagrasses

Seagrasses have evolved a number of morphological, anatomical, and physiological adaptations that allow them to grow and reproduce in marine environments (Dawes, 1998; den Hartog, 1970):
Leaves

Seagrass leaves are well adapted for photosynthesis, absorption of nutrients, diffusion of gases, and buoyancy. Leaves consist of a basal sheath and a distal leaf blade, with blades differing greatly in morphology between species. Some species have long strap-like blades while others have cylindrical, ovate, or ovate-linear blades (den Hartog, 1970, Phillips and Meñez, 1988; Kuo and den Hartog, 2001). In comparison to terrestrial plants: 1) the epidermis serves as the primary site of photosynthesis; 2) epidermal cells have thick walls, as well as lack stomata and associated guard cells; and 3) companion cells are not distinct and vessel elements are absent. In addition, seagrasses have large thin-walled aerenchyma cells for facilitating gas and solute diffusion, as well as an extensive lacunal system with septae that protect the leaf from flooding (den Hartog, 1970; Phillips and Meñez, 1988; Dawes, 1998; Kuo and den Hartog, 2006).

Extensive root/rhizome system

All seagrasses have an indeterminate horizontal rhizome that produces roots, as well as shoots with leaves and flowers (den Hartog, 1970; Kuo and McComb, 1989). The rhizome is cylindrical or oval and found below ground in species with larger morphologies and just below the sediment surface in species that are more delicate. The rhizomes of most seagrass species have bundles of sclerenchyma fibers in the inner and outer cortex that make the below ground system rigid. The extensive root and rhizome system anchors plants into the substratum, thereby protecting seagrasses from waves and tidal action. The root and rhizome system also serves an important role in vegetative propagation, absorption of nutrients for growth, transport of oxygen, and storage of carbohydrates (den Hartog, 1970).
Roots of seagrasses are adventitious and grow on the lower surface of rhizomes at each node. Roots consist of a root cap, which protects meristematic cells and, depending on the species, may produce root hairs from epidermal cells. The cortex, which usually consists of parenchyma, also contains aerenchyma and lacunae. In addition to their anchoring function, seagrass roots assist in nutrient uptake from the substratum (den Hartog, 1970; Dawes, 1998; Kuo and den Hartog, 2006). For example, seagrass roots secrete oxygen into the sediment, creating an oxic zone around the seagrass roots that allows the conversion of ammonium to nitrate in the sediment and the nitrate is then taken up by the root (Phillips and Meñez, 1988).

Reproductive structures

Seagrasses are adapted for hydrophilous pollination and are either monoecious or dioecious. Flowering parts (petals, sepals, stamens, and pistils) are found on stems of reproductive shoots. In most genera, flowers are small and are produced underwater at the base of leaf clusters. The stamens and pistils extend above the petals to facilitate pollen release and pollination (Hemminga and Duarte, 2000). During sexual reproduction, pollen grains are transferred to female flowers and fertilization occurs to produce seeds. Seeds are poorly adapted for dispersal and are released just above or below the sediment surface. Some species produce long lived seeds that can remain in the sediment for 1-2 months, resulting in large seed banks. Asexual (vegetative) propagation may occur through vegetative expansion and/or via fragmentation of the rhizome, with vegetative fragments potentially providing an additional mechanism for dispersal (Cambridge et al., 1983; Ewanchuk and Williams, 1996; Ackerman, 2006).
Distribution of seagrasses

Seagrasses meadows are found in coastal waters along every continent except Antarctica, with their geographic and depth distribution controlled by a number of abiotic factors including light, water depth and clarity, temperature, salinity, current and wave patterns, nutrients, and substrate (Day et al., 1989; Short, Coles, and Pergent-Martini, 2001). According to Short et al. (2007), the distribution of seagrasses species can be divided into six geographic bioregions, based on assemblages of taxonomic groups in temperate and tropical areas and the physical separation of oceans. Within each bioregion, seagrass species may be further distributed according to physical habitat and/or different successional roles. The model suggests four Temperate bioregions and two Tropical bioregions: 1) The Temperate North Atlantic is a low diversity region with approximately 5 species occurring in estuaries, lagoons, and shallow coastal areas up to 12 meters deep; 2) The Mediterranean region has moderate diversity, with a temperate and tropical mix of 9 species occurring in coastal lagoons, shallow coastal areas, and deeper coastal waters up to 50 meters deep; 3) The Temperate North Pacific Region supports high species diversity with 15 species that occur in lagoons, estuaries, coastal surf zones, and deep coastal waters up to 20 meters deep; 4) The Temperate Southern Oceans region has low-to-high diversity with 18 species that often grow under extreme condition in lagoons, estuaries, shallow coastal areas, and deep coastal areas up to 50 meters deep; 5) The Tropical Atlantic is a high diversity region with 10 species that occur in lagoons; shallow coastal areas, back reefs, and deep coastal water up to 50 meters deep; and 6) The Tropical Indo-Pacific is the largest and highest diversity bioregion with 24 species that are located predominately on reef flats, but are also found in deep coastal areas up to 50
meters deep and in estuaries (For a listing of species in each bioregion refer to Short et al., 2007; Figure 1.1).

**Importance of seagrass ecosystems**

Seagrass meadows play an important ecological and economic role in coastal marine ecosystems. They are responsible for 15% of the carbon storage in the ocean (Duarte and Chiscano, 1999) and on average export 24% of their net production \((0.6 \times 10^{15} \text{ g C yr}^{-1})\) to adjacent ecosystems (Duarte and Cebrian, 1996). In addition to their high primary productivity, seagrass meadows filter sediments and nutrients and improve water quality through the direct trapping of suspended particles and the retention of organic matter (Heck et al., 1995; Short and Wyllie-Echeverria, 1996; Terrados and Duarte, 2000). They also provide food and habitat to a variety of organisms including microbes, invertebrates, and vertebrates that are often endangered, such as dugongs, or commercially important, such as fish and shrimp (Fry and Parker, 1979; Duarte, 2002). Finally, seagrasses are often viewed as indicators of coastal conditions because they are vulnerable to various forms of anthropogenic stressors including cultural eutrophication, oil spills, and commercial fishing (Orth, et al., 2006).

**Impact of environmental stressors on seagrasses**

In recent decades, there has been a tenfold increase in reports of seagrass declines (Orth et al., 2006). Waycott et al. (2009) estimated that a minimum of 29% of the known global extent of seagrass meadows has been lost since 1879 and that a greater area of loss is probable since many seagrass habitats (i.e., turbid, deep, and remote areas) have yet to
be mapped. The cause for declines has been attributed to both anthropogenic and/or natural stressors. The greatest threats to seagrasses worldwide have been eutrophication and sedimentation from urban and agricultural runoff, as well from fishery and aquaculture practices (Short and Wyllie-Eicheverria, 1996; Duarte, 2002; Short et al., 2007). Other anthropogenic stressors have included filling, land reclamation, dock and jetty construction. Natural stressors have included overgrazing (e.g., dugongs, urchins, sea turtles) bioturbation, and disease (e.g., wasting disease), as well as extreme climatic events (i.e., hurricanes, floods, and tsunamis; Duarte, 2002; Dawes, 2004; Orth et al., 2006; Short et al., 2007).

While the human factors associated with seagrass loss have been local or regional in scale, researchers believe that climate changes, including stratospheric ozone depletion and global warming, are further impacting seagrass distributions world-wide (Short and Neckles, 1999; Duarte, 2002; Orth et al., 2006; Björk et al., 2008). Stratospheric ozone depletion refers to the thinning of the stratospheric ozone layer (18-50 km) by ozone-depleting substances (e.g., CFCs, Halon, HBFCs, HCFCs, methyl bromide), which causes enhanced ultraviolet-B levels (280-320 nm) in many regions of the world (WMO, 2010; Mckenzie et al., 2011). Most seagrasses are sensitive to enhanced levels of ultraviolet radiation (UV; 100-400 nm), with researchers reporting declines in photosynthetic efficiency (Trocine et al., 1981; Larkum and Wood, 1993; Dawson and Dennison, 1996; Figueroa et al., 2002) and capacity (Dawson and Dennison, 1996; Dtres et al., 2001), photomorphogenic effects such as increased leaf thickness (Dawson and Dennison, 1996), and changes in secondary metabolism (Trocine et al., 1981; Larkum and Wood, 1993; Abal et al., 1994; Dawson and Dennison, 1996; Dtres et al., 2001; Kunzelman et
Because tolerance to UV radiation can vary between seagrass species (Dawson and Dennison, 1995), researchers have suggested species composition and distribution will shift over time in regions experiencing higher UV-levels (Björk et al., 2008).

“Global warming” refers to the warming of the atmosphere from increasing atmospheric concentrations of greenhouse gases (e.g., CO₂, NO, CFC, CH₄, N₂O, CFCs, SF₆, HFCs, and PFCs) caused by human activities, primarily the burning of fossil fuels and changes in land use and land cover. The increase in atmospheric concentrations of greenhouse gases alters radiative balances and warms the troposphere (0-18km). The large-scale changes associated with global warming include changes in the temperature of the ocean, sea-level rise, and increasing CO₂ concentrations. Researchers have also suggested that greenhouse gases trapped in the troposphere are causing unexpected increases in UV levels in the tropics and at high southern latitudes (Hegglin and Sheperd, 2009). The vulnerability of seagrasses to the large-scale changes associated with global warming will depend on the individual species’ tolerance to such changes. Researchers expect differential responses between seagrass species to global warming, leading to shifts in species composition and distribution (see reviews by Short and Neckles, 1999; Björk et al., 2008).

**Leaf reddening**

The expression of red coloration in leaves is well documented in terrestrial plants and has been shown to be due to the accumulation of anthocyanins, water-soluble flavanoid pigments (Figure 1.2; Gould and Lee, 2002). Anthocyanins occur in all major plant
groups and are synthesized regularly in the cytosol and subsequently transported into the vacuoles of palisade and spongy parenchyma and/or the lower or upper epidermal layers of leaves (Neill and Gould, 1999; Gould et al., 2000). Enhanced production of anthocyanins, and the reddening of otherwise green leaves, occurs in juvenile, senescing, or leaves exposed to environmental stressors, leading many researchers to believe that anthocyanins serve a functional role (Gould et al., 2002). The key hypotheses regarding anthocyanin function in terrestrial leaves include: (1) protection of chloroplasts from the adverse affects of excess visible light; (2) attenuation of UV-B radiation; and (3) antioxidant activity. While there are a large number of studies that support the sunscreen/antioxidant hypotheses in terrestrial plants, there are also a number of experiments that reject these hypotheses (Burger and Edwards, 1996; Lee et al., 2003; Kyparissis et al., 2007). Other research supports the role of anthocyanins in desiccation tolerance, cold-hardiness, or defense/camouflage from herbivores (Chalker-Scott, 1999; Gould et al., 2002; Gould, 2004; Karageorgou and Manetas, 2006; Manetas 2006; Archetti et al., 2009). Hence, there is no unified theory on the functional significance of anthocyanins in terrestrial plants.

Despite the attention leaf reddening has received in terrestrial plants, researchers have rarely reported the phenomenon in seagrasses. The first cases noted occurred in Australian species growing in intertidal and shallow subtidal waters (McMillan, 1983; Abal et al., 1994; Fyfe 2003, 2004), with two authors documenting high concentrations of anthocyanins in leaves expressing red coloration (McMillan, 1983; Fyfe 2003, 2004). While no formal studies were conducted to determine the factor(s) responsible for the red
coloration in leaves, the authors suggested that it was a response to high levels of UV or visible radiation (Abal et al., 1994; Fyfe 2003, 2004).

During a trip to Summerland Key in the lower Florida Keys, I observed *Thalassia testudinum* shoots with entirely red leaves (red-leafed shoots) growing in shallow subtidal waters (<0.5 m). Preliminary work showed that red coloration in leaves was caused by high concentrations of anthocyanins. In addition, red-leafed shoots were found to be morphologically and physiologically different than shoots with entirely green leaves (green-leafed shoots) growing at the same depth. Because red-leafed shoots appeared to be limited to waters exposed to a number of physical stressors (i.e., high temperatures, high UV and visible radiation, nutrient limitation), I proposed that the expression of red coloration in *T. testudinum* leaves was a stress response induced by plants to enhance survival.

**Objectives**

The objective of my dissertation is to increase our understanding of leaf reddening in seagrasses by determining: 1) the distribution and prevalence of seagrasses expressing red coloration in leaves; 2) the molecules responsible for red coloration; 3) the physiological and morphological characteristics associated with seagrasses expressing red coloration; 4) the potential function(s) of red coloration in leaves; 5) the factor(s) responsible for the induction of red coloration in leaves; and 6) the plasticity of red coloration in leaves. To accomplish these objectives, the majority of my research (Chapter 3-5) was conducted in the lower Florida Keys with the seagrass *Thalassia testudinum*, the dominant species found in the tropical waters of the Atlantic and Caribbean. I chose to work with *T. testudinum* in the lower Florida Keys because patches
with entirely red-leafed shoots growing adjacent to patches with entirely green-leafed shoots were found at multiple sites, providing me with the unique opportunity to conduct comparative and manipulative studies.

In Chapter 2, I use the literature, as well as information from SeagrassNet and four other locations to determine the prevalence of seagrasses with red leaves within the world’s six seagrass bioregions. The chapter was prompted by an evaluation of herbaria specimens and photographs from SeagrassNet, as well as discussions with my advisor that led us to believe that red coloration in seagrass leaves was more common than reflected in the literature.

In Chapter 3, I perform a comparative study of green- and red-leafed *T. testudinum* shoots to determine whether (a) red coloration in leaves is caused by the accumulation of one or more anthocyanin molecules, (b) under high light, physiological and morphological characteristics are different between green- and red-leafed shoots, and (c) red coloration in leaves serves a protective function by acting as a sunscreen during periods of high light intensity. I also explore the role of temperature, UV and visible radiation, as well as nutrient limitation as factors responsible for the induction of leaf reddening in this species.

In Chapter 4, I conduct two field experiments with *T. testudinum* using different light treatments to determine whether a) various components of the solar spectrum induce anthocyanin accumulation and red coloration in leaves of green-leafed shoots, and b) anthocyanin levels, red-coloration, and/or other physiological characteristics of leaves on red-leafed shoots are affected by reductions in light levels. The first experiment was prompted by results from Chapter 3, which showed that anthocyanin content in leaves of
green-leafed shoots was positively related to the percentage of surface light (i.e., UV and PAR). The second experiment was conducted to investigate if reducing light-levels caused red-leafed shoots to reduce anthocyanin content and/or turn green.

In Chapter 5, I perform reciprocal transplants of green- and red-leafed *T. testudinum* shoots using a common garden approach to test whether variations in light conditions affect anthocyanin concentrations and the persistence of red coloration in leaves. The shoots were monitored for three-years and information on anthocyanin content and coloration were collected for green- and red-leafed shoots. The study was conducted after results from Chapter 4 showed that reductions in light-levels did not immediately reverse anthocyanin content or red coloration in leaves of red-leafed shoots, leading me to believe that red-leafed shoots are a variant that are adapted to high light areas in the lower Florida Keys.

In Chapter 6, I provide a synthesis of my results, discuss the implications of research under current climate change scenarios, as well as provide recommendations for future studies.
Literature Cited


Commission, Fish and Wildlife Research Institute and the Tampa Bay Estuary Program, St. Petersburg, FL.


Karageorgou P. and Y. Manetas. 2006. The importance of being red when young: anthocyanins and the protection of young leaves of *Quercus coccifera* from insect herbivory and excess light. Tree Phys 26: 613-621.


Orth and CM Duarte (eds) Seagrasses: Biology, ecology and conservation.
Dordrecht, Springer.


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Figure 1.2. Basic chemical structure of an anthocyanin molecule. R’s denote locations where substitutions can occur.
CHAPTER II

LEAF REDDENING IN SEAGRASSES

Abstract

Red coloration in leaves is well documented in terrestrial angiosperms, but has rarely been reported in seagrasses. In a survey of the world’s six seagrass bioregions we documented leaf reddening in 12 seagrass species from intertidal and shallow subtidal waters at 25 locations in the Tropical Atlantic and Tropical Indo-Pacific. Including additional observations of seagrasses with red leaves from Australia, the phenomenon is now documented in 15 seagrass species at 29 locations worldwide. Similar to terrestrial angiosperms, leaf reddening in seagrass leaves may relate to enhanced production of anthocyanins after exposure to one or more stressors.

Introduction

The expression of red coloration in leaves is well documented in terrestrial plants and has been shown to be caused by the accumulation of anthocyanins, water-soluble flavonoid pigments (Lee and Gould 2002). In terrestrial plants, red coloration may be permanent or may transiently occur in juvenile, senescing, or leaves exposed to environmental stressors (Gould et al., 2002). Stressors that have been shown to induce leaf reddening in terrestrial plants include: exposure to increased visible or ultraviolet (UV) radiation (290-400 nm; Lindo and Caldwell 1978, Mancinelli 1995, Oren-Shamir and Levi-Nissim 1997,
Mendez et al. 1999); cold temperatures (Christie et al. 1994, Chalker-Scott 1999); nutrient limitation (Atkinson 1973, Hodges and Nozzolillo 1996, Kumar and Sharma 1999); pathogen attack (Hipskind et al. 1996); and wounding (Costa-Arbulu et al. 2001, Stone et al. 2001). There is currently no unified explanation for the functional role of anthocyanins in leaves; researchers suggest that anthocyanins are multifunctional, serving roles in photoprotection, osmoregulation, antioxidant activity, and/or defense against herbivory (Coley and Barone 1996, Gould et al. 2000, Gould et al. 2002).

Despite the attention that has been given to the occurrence of red coloration in leaves of terrestrial angiosperms, researchers have only alluded to the phenomenon in seagrasses. McMillan (1983) wrote of “small, purplish or reddish-brown leaves” in Halodule uninervis (Forsskål) Ascherson and Halophila ovalis (R. Brown) Hooker f. from intertidal areas in Shark Bay, Western Australia. A chromatographic comparison indicated that one unidentified anthocyanin was present in H. uninervis and two unidentified anthocyanins were present in H. ovalis (McMillan 1983). Abal et al. (1994) reported “pink coloration” in H. ovalis and Zostera capricorni Ascherson (conspecific with Zostera muelleri Irmisch ex Aschers, Short et al. 2007) in Moreton Bay, Queensland, Australia and suggested the color was due to the presence of anthocyanins. In 1996, purple leaves in Amphibolis antarctica (Labillardiere) Sonder et Ascherson, Heterozostera tasmanica (Martens ex Ascherson) den Hartog and Z. muelleri were seen in intertidal and shallow subtidal areas in Spencer Gulf, South Australia (Short pers. obs.). Most recently, Fyfe (2003, 2004) documented “red immature leaves and dark bronze adult leaves” of Z. capricorni having high concentrations of unidentified anthocyanins in shallow subtidal areas in Sussex Inlet, New South Wales, Australia.
Methods
We conducted wading and/or swimming surveys at low water at 42 SeagrassNet (http://www.SeagrassNet.org) and 4 other locations between 2003 and 2008 (Figure 2.1) to investigate the prevalence of red coloration in seagrasses leaves. Where red coloration in seagrass leaves was observed, the following information was collected: 1) GPS coordinates; 2) seagrass species composition, average water depth, tidal stage and pattern of reddening in each species; and 3) a photograph of each seagrass species present and of reddened seagrass species.

Results and Discussion
We found 12 seagrass species expressing red coloration in leaves out of the 23 species assessed (Table 2.1). Red coloration was most commonly seen in leaves of Cymodocea serrulata (R. Brown) Ascherson, Thalassodendron ciliatum (Forsskål) den Hartog, Halophila ovalis, and Cymodocea rotundata Ehrenberg & Hemprich ex Ascherson. Including previous reports from Australia, red coloration in leaves has been observed in a total of 15 seagrass species of eight genera and three families (Table 2.1). Red coloration was not seen in leaves of Halophila decipiens Ostenfeld, Halophila spinulosa (R. Brown) Ascherson, Ruppia maritima L., Syringodium filiforme Kützing, Syringodium isoetifolium (Ascherson) Dandy, Zostera caespitosa Miki, Zostera japonica Ascherson & Graebner, or Zostera marina L., despite the presence of these species at many survey locations, nor in Posidonia australis Hooker f. (Fyfe 2004).

We observed red coloration in seagrass leaves at 25 of the 46 locations we assessed. Including previous reports from Australia, the phenomenon is now documented
at 29 locations in the shallow subtidal or intertidal waters (< 0.5 m MLW) of the Tropical Atlantic, Tropical Indo-Pacific, and Temperate Southern Oceans bioregions (McMillan 1983, Abal et al. 1994, Short pers. obs. 1996, Fyfe 2003, 2004, Short et al. 2007, Figure 2.1; Table 2.1). Stressors to seagrasses in the intertidal and shallow waters of these bioregions may include enhanced visible and/or UV light exposure, water temperature extremes, and/or exposure to air at low tide. Of the above stressors, UV alone or a combination of cold temperatures with UV-B (Oren-Shamir and Levi-Nissim 1997) and/or high-intensity visible light (Leyva et al. 1995, Janda et al. 1996) have been shown to trigger leaf reddening in terrestrial angiosperms. Although the cause of red coloration in seagrass leaves is unknown, a link between enhanced UV radiation and reddening of seagrass leaves was suggested by Trocine et al. (1981), who observed reddish methanol-water fractions after exposing *Halophila engelmanni* Aschers to increased levels of UV-B in the laboratory.

Patterns of red coloration in seagrass leaves at the survey locations varied widely, from scattered shoots to small patches (1 m²) to large portions of meadows (e.g., 18 ha at Buda Island, Myanmar). In addition, the extent of red pigmentation varied between individuals of a species (Figure 2.2) and between leaves on a shoot, from small red spots on a leaf to shoots that were entirely red. Two species (*Cymodocea serrulata*, *Thalassodendron ciliatum*) exhibited consistent patterns of red coloration; *C. serrulata* often had red cross-stripes and *T. ciliatum* had red cross-stripes and margins, as well as red flowering parts. In *C. serrulata*, red cross-stripes along the leaves seem related to plant growth, with daily growth increments marked by each stripe. Even though red coloration in leaves was not mentioned in earlier descriptions of *C. serrulata* and *T.*
*ciliatum* (den Hartog 1970, Phillips and Meñez 1988, Kuo and den Hartog 2001), it is so common that these species are now illustrated with red cross-stripes in a recent field guide for the Indo-West Pacific (Waycott et al. 2004).

Our observations indicate that leaf reddening in seagrasses is not isolated to Australian seagrasses in the Temperate Southern Oceans bioregion, but is also found in numerous seagrass species growing in shallow subtidal and intertidal areas of the Tropical Atlantic and Tropical Indo-Pacific bioregions. Although it is evident that seagrasses with reddened leaves are widespread, we have not determined whether red seagrasses in these bioregions are recent or if researchers have previously overlooked the occurrence of this phenomenon. Additional studies are needed to increase our understanding of the occurrence and distribution of leaf reddening in seagrass leaves, as well as to determine its causes, costs, and protective functions.

**Acknowledgements**

We thank Cathy Short for technical editing. Research support was provided by: the National Oceanic and Atmospheric Administration’s Nancy Foster Scholarship, SeagrassNet, the Ocean Park Conservation Foundation, the David and Lucile Packard Foundation, the Oak Foundation, and the New Hampshire Charitable Foundation & Tom Haas. Jackson Estuarine Laboratory contribution series number 487.


Table 2.1 Documented leaf reddening in seagrass by species and location, including our observations (SeagrassNet sites and four other locations), two observations by Short 1996, and four observations reported in the literature (McMillan 1983, Abal 1994, Fyfe 2003, 2004).

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<th>Species</th>
<th>Location</th>
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<tr>
<td><em>Amphibolis antarctica</em> (Labillariere) Sonder et Ascherson</td>
<td>Spencer Gulf, South Australia (Short 1996).</td>
<td>Intertidal: leaves uniformly purple.</td>
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<td><em>Cymodocea rotundata</em> Ehrenberg &amp; Hemprich ex Ascherson</td>
<td>Xincun Bay, Hainan, China; Inhaca, Mozambique; Pulau Bada, Myanmar; Haad Chao Mai Marine Park, Trang, Thailand; Panwa Bay, Phuket, Thailand.</td>
<td>Intertidal: leaves uniformly red in parts of the meadow.</td>
</tr>
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<td><em>Cymodocea serrulata</em> (R. Brown) Ascherson</td>
<td>Green Is., Queensland, Australia; Manado, Indonesia; Ifaty, Madagascar; Pulau Gaya, Sabah, Malaysia; Inhaca, Mozambique.</td>
<td>Intertidal and subtidal: purple cross stripes (a typical characteristic of the species)</td>
</tr>
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<td><em>Halodule pinifolia</em> (Miki) den Hartog</td>
<td>Xincun Bay, Hainan, China.</td>
<td>High intertidal: red leaves except where covered by algae.</td>
</tr>
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<td><em>Halodule uninervis</em> (Forsskål) Ascherson</td>
<td>Shark Bay, Australia (McMillan 1983); Xincun Bay, Hainan, China; Inhaca, Mozambique; Haad Chao Mai Marine Park, Trang, Thailand.</td>
<td>Intertidal: uniformly red/purple leaves in parts of the meadow.</td>
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<td><em>Halodule wrightii</em> Ascherson</td>
<td>Lower Keys, Florida, U.S.A.</td>
<td>High intertidal and shallow subtidal: leaves uniformly purple in parts of the meadow.</td>
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<td><em>Thalassodendron ciliatum</em> (Forsskål) den Hartog</td>
<td>Wadi Gemal, Egypt; Komodo, Indonesia; Andavadoaka and Ifaty, Madagascar; Nyali Beach, Mombassa, Kenya; Inhaca Island, Mozambique; Chwaka and Chumbe, Zanzibar, Tanzania.</td>
<td>Reef zone and intertidal: partially red and/or red cross-stripes (a typical characteristic of the species).</td>
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1 Note: *C. serrulata* without red coloration was found in the Andaman Sea, Thailand.
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<td>Xincun Bay, Hainan, China.</td>
<td>Intertidal: reddish streaks along the leaf axis of some leaves.</td>
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<td><em>Halophila beccarii</em> Ascherson</td>
<td>Beimu Salt Fields, Bei Hai, China; Po Bay, Phuket, Thailand.</td>
<td>Intertidal: uniformly red or with red spots in the meadow.</td>
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<td><em>Halophila ovalis</em> <em>(R. Brown)</em> Hooker f.</td>
<td>Green Is. Queensland, Australia Moreton Bay, Australia (Abal et al. 1994); Shark Bay, Australia (McMillan 1983); Zhulin, Bei Hai, and Xincun Bay, Hainan, China; Wadi Gemal, Egypt; Andavadoaka and Ifaty, Madagascar; Inhaca Island, Mozambique; Pulau Bada, Myanmar, Ngchesar, Babelthraup, Palau; Bantangas and Guimaras, Philippines; Haad Chao Mai Marine Park, Trang, Thailand; Panwa Bay, Phuket, Thailand; Ha Long Bay, Vietnam.</td>
<td>Intertidal sand and mud flat: uniformly purple leaves, purplish spots, purple between cross veins, striations, purple petiole, or central vein pigmentation in parts of the meadow.</td>
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<tr>
<td><em>Thalassia hemprichii</em> <em>(Ehrenberg)</em> Ascherson</td>
<td>Inhaca Island, Mozambique. Green Is., Queensland, Australia.</td>
<td>Intertidal and sand flat: purple longitudinal stripes or purple spots in parts of the meadow.</td>
</tr>
<tr>
<td><em>Thalassia testudinum</em> Banks ex König</td>
<td>South Water Caye, Glover’s Atoll, Belize; Neguanje Bay, Colombia; Lower Florida Keys, U.S.A.</td>
<td>Intertidal and shallow subtidal: uniformly purple, purple stripes, or purple spots in parts of the meadow.</td>
</tr>
<tr>
<td><em>Zostera tasmanica</em> <em>(Martens ex Ascherson)</em> den Hartog</td>
<td>reported as <em>Heterozostera tasmanica</em> in Spencer Gulf, Australia (Short 1996).</td>
<td>Intertidal and shallow subtidal: uniformly purple.</td>
</tr>
<tr>
<td>Species</td>
<td>Location</td>
<td>Comments</td>
</tr>
<tr>
<td>-------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td><em>Zostera muelleri</em> Irmisch ex Ascherson</td>
<td>Moreton Bay, Australia (Abal et al. 1994) and Wegit Point, Australia (Fyfe 2003, 2004), reported as <em>Zostera capricorni</em>; Spencer Gulf, Australia (Short 1996).</td>
<td>Intertidal: pinkish, reddish, or entirely purple.</td>
</tr>
</tbody>
</table>
Figure 2.1 Surveys for leaf reddening were conducted at 42 SeagrassNet locations (Short et al., 2006; http://www.SeagrassNet.org for coordinates) and 4 other locations. Green triangles denote locations where no leaf reddening was found; red circles denote locations where leaf reddening was observed in species other than *Thalassodendron ciliatum* or *Cymodocea serrulata*; circles that are colored half red, half green with a thin black-stripe across the center denote locations where *T. ciliatum* and/or *C. serrulata* were the only red seagrass species found. One symbol is used for two or more locations when they are in close proximity (often the case for multiple SeagrassNet sites). Our map also includes four observations from the literature (McMillan 1993, Abal et al. 1994, Fyfe 2003, 2004).


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1 Leaf reddening was observed at the following survey locations not affiliated with SeagrassNet: Lower Florida Keys, USA at Big Pine (N 24° 39 22', W 81° 22 21'), Summerland (N 24° 39 65', W 81° 27 65'), Cudjoe (N 24° 39 87', W 81° 29 66'), and Sugarloaf (N 24° 39 33', W 81° 32 19'). Buda Island, Myanmar (N 10° 30 64', E 98° 14 30'); Phuket, Thailand at Po Bay (N 8° 3 60', E 98° 25 96') and Panwa Bay (N 7° 48 27', E 98 24 7'), and Xincun Bay, Haman, China (N 20° 3 35', E 100° 18 16').
Figure 2.2 (A) Individuals of *Halophila ovalis* (collected from the intertidal waters of Panwa Bay, Thailand; Wadi Gemal, Egypt; Babelthraup, Palau; and Bantangas, Philippines) showing different patterns of leaf reddening. (B) Green and red patches of *Cymodocea rotundata* observed at Pulau Bada, Myanmar. (C) *Thalassia testudinum* collected from subtidal waters of Summerland Key, Florida. The right shoot exhibits leaf reddening, while the left shoot is green. (D) Reddened *Cymodocea serrulata* consistently has red cross-stripes (blade width ca. 12mm).
CHAPTER III

LEAF REDDENING IN THE SEAGRASS *THALASSIA TESTUDINUM* IN RELATION TO ANTHOCYANINS, SEAGRASS PHYSIOLOGY AND MORPHOLOGY, AND PLANT PROTECTION

**Abstract**

Numerous seagrass species growing in high light environments produce leaves with red coloration, yet the ecophysiology of leaf reddening in seagrasses is poorly understood. To increase our understanding of the process of leaf reddening in *Thalassia testudinum* found in the lower Florida Keys (USA), we identified the molecules responsible for red coloration in leaves and compared physiological, morphological, and growth attributes of entirely red-leafed shoots to entirely green-leafed shoots. We determined that four anthocyanin molecules are responsible for red coloration in leaves. In addition, we found that red leaves had higher concentrations of photo-protective pigments (anthocyanins and UV-absorbing compounds), higher effective quantum yields (ΔF/ Fm) at midday, and were shorter, narrower, and weighed less than green leaves. No significant difference in growth rates was observed between red and green-leafed shoots, but patches of red-leafed shoots had shorter canopy heights and smaller LAI compared to patches of green-leafed shoots. Our results demonstrate that leaf reddening in *T. testudinum* is caused by high concentrations of anthocyanins, is associated with physiological and morphological
attributes, and acts as a sunscreen since red leaves were able to maintain high effective quantum yields at high light intensities.

**Introduction**

Leaf reddening, the expression of red coloration in leaves, is well documented in terrestrial plants. The phenomenon can occur during leaf growth, senescence, or in response to environmental or biotic stresses (Gould et al. 2002) and is often caused by the accumulation of anthocyanins (Lee 2002). Anthocyanins are water-soluble flavonoid pigments synthesized regularly in the cytosol of cells and sequestered in cell vacuoles (Gould et al. 2002). More than 400 anthocyanin molecules have been reported in nature, with each molecule consisting of an anthocyanidin (the aglycone chromophore) bonded to one or more glycosides (Harborne and Grayer 1988). The chromophore has a C6-C3-C6 configuration consisting of two aromatic rings, connected by a heterocyclic ring. The high degree of modification in the molecular structure of anthocyanins contributes to the unique ability of these molecules to absorb both ultraviolet (peak~280 nm) and visible radiation (green-yellow peak between 500-550 nm; Harborne 1967; Shirley 1996), leading researchers to propose that anthocyanins function as sunscreens/antioxidants against photoinhibition in high light environments (see reviews Chalker-Scott 1999; Gould et al. 2002; Gould 2004). While there are a large number of studies that support this hypothesis there are also a number of experiments that reject it (Burger and Edwards 1996; Lee et al. 2003; Kyparissis et al. 2007; Esteban et al. 2008). Moreover, researchers have suggested the role of anthocyanins in desiccation tolerance, cold-
hardiness, camouflage, or defense from herbivores (Chalker-Scott 1999; Gould et al. 2002; Gould 2004; Karageorgou and Manetas 2006; Manetas 2006; Archetti et al. 2009).

Seagrasses with reddened leaves were first reported in Australia (McMillan 1983; Abal et al. 1994; Short pers. obs. 1996; Fyfe 2003, 2004). Although the individual molecules responsible for red coloration in seagrasses were not identified, anthocyanins were reported in three species of seagrass (McMillan 1983; Fyfe 2004). In addition, two potential functional roles of reddening in seagrasses have been proposed: Abal (1994) suggested that pink coloration (due to the presence of anthocyanin-like pigmentation) in intertidal leaves of the seagrasses *Zostera capricorni* and *Halophila ovalis* was an adaptation to high ultraviolet (UV) levels while Fyfe (2004) suggested that red-bronze coloration produced by anthocyanins in *Z. capricorni* protected leaves from excess visible radiation.

In a recent survey, we extended the documented range of leaf reddening in seagrasses and concluded that the phenomenon is widespread, occurring in fifteen species from the intertidal and shallow subtidal waters of the Tropical Atlantic, Tropical Indo-Pacific, and Temperate Southern Oceans bioregions (Novak and Short 2010). We also noted that reddening occurs in areas where seagrasses are exposed to stressors known to induce reddening in terrestrial plants, including exposure to enhanced solar UV and/or visible radiation (Lindo and Caldwell 1978, Gould et al. 2002).

Seagrasses present varying physiological and morphological characteristics according to the environmental conditions in which they develop (Kuo and Hartog 2006). In high light environments where leaf reddening is prevalent (Novak and Short 2010) seagrasses have high concentrations of UV absorbing compounds (Abal et al. 1994;
Durako et al. 2003), with fifteen recently identified flavonoids in *Halophila johnsonii* (Meng et al. 2008). In addition, some seagrasses growing in high light environments have lower chlorophyll content (Abal et al. 1994; Dawson and Dennison 1996; Detres 2001), lower carotenoid content (Dawson and Dennison 1996; Detres 2001), lower tissue nitrogen (Abal et al. 1994; Grice et al. 1996) or lower photosynthetic efficiencies (Ralph et al. 1998) compared to seagrasses growing in lower light conditions. The low photosynthetic efficiencies at high irradiance levels are the result of photoinhibition (damage to photosystem II reaction centers) or the down-regulation of photosynthesis to prevent damage by non-photochemical quenching via the xanthophyll cycle (conversion of excess light energy to heat; Ralph et al. 1998; Silva and Santos 2003; Belshe, et al. 2007). Differences have been observed between green seagrasses in shallower and deeper water at the meadow scale, with shallow water plants exhibiting higher leaf area index (LAI, m² m⁻²) and shoot density (Ralph et al. 2007).

The present study was designed to identify the molecules responsible for red coloration in *Thalassia testudinum*, as well as to determine if physiological and morphological differences exist between entirely green-leafed shoots and entirely red-leafed shoots by comparing various plant parameters. We investigated whether (1) red coloration in *T. testudinum* leaves is caused by the accumulation of one or more anthocyanin molecules, (2) under high light, physiological and morphological characteristics are different between green and red-leafed shoots and, (3) reddening serves a protective function in *T. testudinum* by acting as a sunscreen during periods of high light stress. Leaf reddening in seagrasses is of interest because global climate change is causing increased levels of UV radiation in regions (Hegglin and Shepard...
2009) where seagrasses with red leaves are prevalent (Novak and Short 2010) and these plants could function as an indicator of UV exposure.

**Materials and Methods**

**Site description and experimental design**

The lower Florida Keys consist of thirty islands composed of carbonate sediments and rock that separate the Atlantic on the east from the Gulf of Mexico on the west (Schomer and Drew 1982). Nearshore waters are generally shallow and seagrass meadows, dominated by *T. testudinum*, are the primary benthic vegetation (Zieman et al. 1989; Fourquarean et al. 2001). Leaf reddening in *T. testudinum* occurs in subtidal waters <0.5 m depth on both the Atlantic and Gulf of Mexico sides of the lower Keys (Novak and Short 2010). Reddening may occur on one or more leaves on a shoot, with pigmentation varying from vertical or cross striations to uniformly red leaves.

We surveyed the subtidal waters around eight islands in the lower Florida Keys (Sugarloaf Key, Cudjoe Key, Summerland Key, Ramrod Key, Big Torch Key, Middle Torch Key, Lower Torch Key, and Big Pine Key) for patches of *T. testudinum* with entirely red-leaved shoots. Six sites were identified, each site containing one or more patches of entirely red-leaved shoots (red patch), as well as patches of entirely green-leaved shoots (green patch). For our study, four sites on the Atlantic side were selected for sampling based on their accessibility: Sugarloaf Key (N 24° 39.332, W 81° 32.194), Cudjoe Key (N 24° 39.868, W 81° 29.659), Summerland Key (N 24° 39.653, W 81° 27.647), and Big Pine Key (N 24° 39.219, W 81° 22.214; Figure 3.1). At each site, we selected one green patch and one red patch for physiological and morphological
measurements conducted during the week of July 1, 2007. All green and red patches selected were 2.8-3.5 m in diameter and located 10-25 m offshore. Green and red patches at each site were located at the same depth, although sites varied in depth (MLW): Sugarloaf Key 0.2 m, Cudjoe Key 0.5 m, Summerland Key 0.4 m, and Big Pine Key 0.3 m. Sample sizes for our pigment quantifications, fluorescence measurements, and plant morphological and structural measurements were determined from statistical power analyses conducted on data collected during the previous summer. Measurements were distributed evenly between green and red patches at each site and among sites.

**Distribution of red pigment in cells**

Fresh material was taken from three regions of the mid-section of the second youngest leaf of six red-leafed shoots from each site. Cross-sections were mounted on a cover-slide and the histological location of red pigment was noted under bright field microscopy with a BX-60 Olympus microscope. Photographs were taken with a Nikon Coolpix digital camera.

**Quantification of pigments**

Anthocyanins, UV-absorbing compounds, and photosynthetic pigments in fresh leaves were quantified in twenty-two shoots (eleven green and eleven red) of *T. testudinum* haphazardly collected at each site. Two 1 cm diameter discs taken from above the sheath of the second youngest leaf of each shoot were excised and weighed. The first disc was used for chlorophyll/carotenoid measurements and extracted in acetone/water (9:1, vol). The second disc was used for anthocyanin measurements and extracted in cold methanol/HCl/water (90:1:1, vol). The extracts were placed in the dark for 20 minutes and centrifuged before the absorption spectra were measured in 3 cm quartz cuvettes with
an Agilent Model 8453 Diode Array (Agilent, CA, USA). Chlorophyll (Chl \(a\), Chl \(b\), total) content (Porra 2002) and carotenoid content (Lichtenthaler 1987) were calculated. Total anthocyanin content was calculated using the Beer-Lambert equation, assuming a corrected absorbance of \(A_{529} - 0.288 A_{650}\) to compensate for the small overlap in absorbance at 529 nm by degraded chlorophylls (Sims and Gamon 2002) and a molar absorbance coefficient for anthocyanins at 529 nm of 30,000 \(\text{mol}^{-1} \text{cm}^{-1}\) (Murray and Hackett 1991). Concentrations of total UV-absorbing compounds (Day 1993) were estimated from 10-fold dilutions of the methanolic extracts as \(A_{300}\) (UV-B) and \(A_{350}\) (UV-A).

**Anthocyanin identification**

Approximately twenty red-leafed shoots were haphazardly collected from each site for identification of individual anthocyanin molecules using an HPLC coupled with a diode array spectrophotometer and ion trap mass spectrometer (LC/DAD/MS). To prepare samples for analyses, 3.64 g of leaf was ground (samples were combined from each site and we assumed that all red shoots produced leaves with the same combination of anthocyanin molecules), placed in 7.5 mL of ascorbic acid/HCl/methanol solution (dissolve 0.25 g ascorbic acid, 2.8 mL 37% HCl in 1000 mL methanol), extracted by sonication for 30 minutes, and then passed through a preconditioned C-18 Sep-Pak cartridge (Waters Associates, MA, USA). The adsorbed pigments were then washed with 5 mL of water, eluted by 2 mL of methanol, and stored at \(-20^\circ\text{C}\) until LC/DAD/MS analyses were performed by Brunswick Laboratories (Norton, MA) using the methods described by Wang et al. (2003).
**Fluorescence measurements**

Pulse amplitude modulated (PAM) chlorophyll fluorescence was measured *in situ* on green and red-leafed shoots with a Diving-PAM (Walz, Germany). The universal sample holder (DIVING-USH) was used to hold the fiber optics probe 10 mm from, and perpendicular to, the middle of the second youngest leaf on each shoot. Measurements were performed using the default instrument settings (measuring light intensity, 8; saturating pulse intensity, 8; saturating pulse width, 0.8; and gain, 2) at all sites.

Maximum quantum yield, a common indicator of photosynthetic stress, was estimated by the saturating-light method on leaves that were dark acclimated for ten minutes (Beer et al. 2001). Measurements were performed on eighteen shoots (nine green and nine red) at each site between 1100 and 1300 hrs (i.e., period of day when light intensity is greatest). Order was randomized between green and red-leafed shoots and leaves were held in their natural configuration for measurements. The equation for maximum quantum yield is expressed as \( \frac{F_{m} - F_{o}}{F_{m}} = \frac{F_{v}}{F_{m}} \) where \( F_{o} \) is the minimal fluorescence of a dark-acclimated leaf in which all photosystem II (PSII) reaction centers are open, \( F_{m} \) is the corresponding maximum fluorescence measured with all PSII reaction centers closed following a saturating light period, and \( F_{v} \) is the variable fluorescence determined from \( F_{m} - F_{o} \) (van Kooten and Snel 1990; Beer et al. 2001).

Effective quantum yield, an estimate of the photosynthetic efficiency of PSII when plants are light acclimated, was measured by the saturating-light method on leaves under ambient conditions. Measurements were performed on eighteen shoots (nine green and nine red) at each site between 1100 and 1300 hrs. Order was randomized between green and red-leafed shoots and leaves were held parallel to the surface to maximize
exposure to light. Incident underwater light reaching the leaf surface (i.e., PAR) was recorded in unison with fluorescence measurements by the Diving-PAM quantum sensor, which was fixed in the universal sample holder (DIVING-USH) next to the fiber optics probe. The equation for effective quantum yield is expressed as $(F_{m}'-F)/F_{m}' = \Delta F/F_{m}'$, where $F$ is the fluorescence of a leaf under ambient conditions, $F_{m}'$ is the corresponding fluorescence measured following a saturating light period, and $\Delta F$ is $F_{m}' - F$ (Genty et al. 1989; Beer et al. 2001).

Seagrass morphology and structure

At each site, 0.0625 m$^2$ quadrats were haphazardly tossed eleven times into the green colored patch of $T$. testudinum and eleven times into the red colored patch of $T$. testudinum and information was gathered on percent cover, canopy height and shoot density within each quadrat. In addition, one representative shoot consisting of both above and belowground material was collected on each toss and the number of leaves per shoot, as well as the length, width, and weight of the second youngest leaf of the collected shoot were measured. Distance between nodes on the rhizome (internode length) was also measured on each shoot. Leaf area index (LAI, m$^2$ m$^{-2}$) in each patch was calculated from shoot density, number of leaves per shoot, leaf width, and leaf length.

Growth and plant constituents

Growth of individual leaves was determined using the leaf-marking technique described by Short (1987). Twenty shoots (ten green and ten red) were haphazardly selected at each site and marked by making a pinhole with a syringe through the leaf sheath. Seven days after initial marking, the shoots were harvested and the distance between the pinhole
on each leaf and the residual scar on the sheath was measured along with leaf width. If a young leaf did not have a pinhole, it was considered new growth. The total area of new tissue added per shoot was divided by the number of days (Short and Duarte, 2001) and a linear relationship ($R^2 = 0.98$) between leaf area and g dry weight was used to estimate dry weight from leaf area. Shoot growth rate is expressed as mg dry weight day$^{-1}$.

Growth measurements were repeated during the week of June 25, 2010 on twenty shoots (ten green and ten red) at each site since leaves were broken and/or missing on a number of shoots collected during 2007. Growth data from 2010 was used for statistical analyses.

Carbon (C) and nitrogen (N) content in leaves was measured in sixteen shoots (eight green and eight red) randomly collected at each site. A sample of dried, ground material from the second youngest leaf of each shoot was weighed and combusted in a PerkinElmer® Series II CHNS/O Analyzer 2400.

Light and temperature

Total photon flux of UV was measured once a week at midday in green and red patches for 4 weeks (June 18-July 16, 2007) while visible (PAR) light was measured once a week at midday in green and red patches for 7 weeks (June 18-August 8, 2007). UV measurements were made using a UV dosimeter (Apogee Instruments, NV, USA) and PAR measurements were made using the quantum sensor on the Diving-PAM, which was calibrated underwater using a Li-190 light meter (LiCor, NE, USA). The UV and PAR sensors were leveled and measurements were taken directly above the surface of the water, directly below the surface of the water, and just above the substratum surface (bottom) in a location within the patches that was not influenced by shading of overlying
leaves. Light measurements were taken every minute for ten minutes and an average for the time interval was recorded. Temperature was recorded at 30-min intervals for a one-week period using iButton temperature loggers (Maxim Corporation, CA, USA), encased in silicon caulking and secured on the bottom at the center of each patch.

Statistics

Within-site comparisons for each color type were made using a one-way analysis of variance model (ANOVA) on the anthocyanin, UV-absorbing compound, photosynthetic pigment, fluorescence, morphological, growth, nutrient, and light data. Among site comparisons for each color type were also assessed on all datasets using a one-way ANOVA. The anthocyanins dataset was natural log transformed since it did not meet the assumptions of the Kolmogorov–Smirnov test of normality. All datasets met the assumptions of equal variance according to the Brown-Forsythe test. Tukey’s multiple comparisons tests were performed to identify which treatments were significantly different. Linear regression analyses were used to assess relationships between anthocyanin concentrations and chlorophyll \(a\), chlorophyll \(b\), total chlorophyll, carotenoids and light for both red and green patches. Analyses were performed using JMP (Version 6.0, SAS Institute Inc.) with significance determined at the 95% probability level (P<0.05). Values are reported as means and standard errors.

Results

Distribution of red pigment in cells

Red coloration was observed on both surfaces of the leaf in red-leafed shoots with the intensity of red coloration appearing similar. Leaf cross-sections revealed red coloration
in the majority of the epidermal and mesophyll cells near the surface of the leaf (Figure 3.2).

Quantification of pigments

The UV-visible light absorption spectra of leaf extracts of green and red-leafed shoots showed a characteristic peak absorbance of anthocyanins in the visible region at 530 nm in red leaves, which was not observed in green leaves. Red leaves also exhibited a higher peak absorbance than green leaves in the UV-B region at 300 nm and the UV-A region at 330 nm. Green leaves exhibited a small peak absorbance at 270 nm, which was not observed in red leaves (Figure 3.3).

Quantification of leaf extracts indicated that red leafed shoots had higher concentrations of anthocyanins (Figure 3.4, ANOVA for anthocyanins: Sugarloaf, $F_{1,20}=96.37$, $P<0.0001$; Big Pine, $F_{1,18}=121.15$, $P<0.0001$; Summerland, $F_{1,20}=127.44$, $P<0.0001$; Cudjoe, $F_{1,21}=717.18$, $P<0.0001$), as well as UV-B and UV-A absorbing compounds compared to green-leafed shoots (Table 3.1). Leaf anthocyanin content in green-leafed shoots decreased with depth and varied among some sites for red-leafed shoots (Figure 3.4, ANOVA: green, $F_{3,39}=49.51$, $P<0.0001$; red, $F_{3,40}=6.99$, $P=0.0007$). Both UV-B and UV-A absorbing compound content was lowest in green-leafed shoots at Cudjoe, the deepest site, while no difference among sites was observed for red-leafed shoots (Table 3.1).

At Cudjoe, red leaf-shoots had higher concentrations of chlorophyll $a$ and total chlorophyll in leaves than green-leafed shoots. Chlorophyll $b$ and carotenoid content was higher in red compared to green-leafed shoots at Summerland and Cudjoe while chlorophyll $a:b$ was higher in green compared to red-leafed shoots at those same sites.
Chlorophyll \(a\), chlorophyll \(b\), total chlorophyll, chlorophyll \(a:b\), and carotenoid content in leaves varied among some sites for green-leafed shoots while chlorophyll \(b\) and chlorophyll \(a:b\) varied among some sites for red-leafed shoots (Table 3.1).

A significant positive relationship between anthocyanin content and chlorophyll \(a\), chlorophyll \(b\), total chlorophyll, and carotenoid content was observed for leaves of green-leafed shoots for all sites combined (Figure 3.5, Linear regression: chl \(a\), \(R^2 = 0.482\), \(F_{1,43} = 40.03\), \(P < 0.0001\); chl \(b\), \(R^2 = 0.441\), \(F_{1,43} = 33.88\), \(P < 0.0001\); total chl, \(R^2 = 0.411\), \(F_{1,43} = 30.05\), \(P < 0.0001\); carotenoids, \(R^2 = 0.291\), \(F_{1,43} = 17.65\), \(P < 0.0001\)). No significant relationship was observed between anthocyanin content and these photosynthetic pigments for leaves of red-leafed shoots for all sites combined (Figure 3.5, Linear regression: chl \(a\), \(R^2 = 0.0003\), \(F_{1,40} = 0.01\), \(P = 0.9162\); chl \(b\), \(R^2 = 0.090\), \(F_{1,40} = 3.96\), \(P = 0.0535\); total chl, \(R^2 = 0.003\), \(F_{1,40} = 0.13\), \(P = 0.7232\); carotenoids, \(R^2 = 0.008\), \(F_{1,40} = 0.32\), \(P = 0.5744\)).

**Anthocyanin identification**

Four anthocyanin molecules were detected in leaves of red-leafed shoots and three were identified. The anthocyanin molecules identified include cyanidin 3-(malonoyl) glucoside, cyanidin 3-glucoside, and pelargonidin 3-(malonoyl) glucoside, which comprised 70.5%, 22.1%, and 3.7% of the area in the HPLC-UV spectrum, respectively. The anthocyanin molecules in leaves of green-leafed shoots occurred in low concentrations and were not investigated.

**Fluorescence measurements**

Maximum quantum yield (\(F_v/F_m\)) for green and red-leaf shoots ranged from 0.750 to 0.790. Values of \(F_v/F_m\) were not significantly different between green and red-leafed shoots.
shoots at each site (ANOVA: Sugarloaf, green, 0.76 ± 0.01, red, 0.77 ± 0.01, F_{1,15} = 0.12, P = 0.7367; Big Pine, green, 0.75 ± 0.01, red, 0.76 ± 0.01, F_{1,15} = 2.07, P = 0.1705; Summerland, green, 0.76 ± 0.01, red, 0.76 ± 0.01, F_{1,12} = 0.46, P = 0.5068; Cudjoe, green, 0.79 ± 0.01, red, 0.77 ± 0.01, F_{1,17} = 3.014, P = 0.1006). Values of F_v/F_m varied among sites for green-leafed shoots (i.e., shoots at Cudjoe had significantly higher values than shoots at Big Pine) while no significant difference in values of F_v/F_m was observed among sites for red-leafed shoots (ANOVA: green, F_{3,29} = 5.89, P = 0.0029; red, F_{3,30} = 1.19, P = 0.3282).

Effective quantum yield (ΔF/F_m) ranged from 0.234 to 0.596 for green-leafed shoots and 0.386 to 0.716 for red-leafed shoots. Red-leafed shoots had significantly higher ΔF/F_m values than green-leafed shoots at each site (Figure 3.6, ANOVA: Sugarloaf, F_{1,15} = 17.15, P = 0.0009; Big Pine, F_{1,15} = 6.60, P = 0.0213; Summerland, F_{1,16} = 7.11, P = 0.0169; Cudjoe, F_{1,16} = 11.12, P = 0.0042). Effective quantum yield values for green and red-leafed shoots varied significantly among sites (ANOVA: green, F_{3,34} = 28.72, P < 0.0001, red, F_{3,28} = 22.41, P < 0.0001), declining with depth (Figure 3.6).

Values for incident PAR at the leaf surface were not significantly different between green and red-leafed shoots at each site (Figure 3.7, ANOVA: Sugarloaf, F_{1,15} = 1.11, P = 0.3088; Big Pine, F_{1,15} = 1.41, P = 0.3011; Summerland, F_{1,14} = 4.40, P = 0.0545; Cudjoe, F_{1,16} = 2.55, P = 0.1297). No significant difference among sites in values for incident PAR at the leaf surface was observed for green or red-leafed shoots (Figure 3.7, ANOVA: green, F_{3,32} = 1.26, P = 0.3020; red, F_{3,32} = 1.77, P = 0.1724).
Seagrass morphology and structure

At each site, red-leafed shoots had shorter, narrower leaves that weighed less than leaves from green-leafed shoots. Leaf length varied among some sites for red-leafed shoots while leaf width and leaf weight varied among some sites for each color. Internode length was not different between colors at each site or among sites for each color (Table 3.2).

Red patches had significantly shorter canopy height and smaller LAI than green patches at all sites. Red patches had significantly lower percent cover and shoot density than green patches at Sugarloaf and Summerland. Canopy height and LAI were not different among sites for each color while percent cover varied among some sites for red patches and shoot density varied among some sites for green patches (Table 3.3).

Growth and plant constituents

Growth rates and percent leaf nitrogen content were not significantly different between green and red-leafed shoots at each site while the ratio of carbon to nitrogen (C:N) in leaves was higher in green compared to red-leafed shoots at Cudjoe. Growth rates of red-leafed shoots varied among some sites; percent nitrogen and C:N in green and red-leafed shoots varied among some sites (Table 3.3).

Light and temperature

The percent of UV and PAR surface irradiance reaching the bottom of patches was not different between green and red patches at each site (Figure 3.7, ANOVA: UV, Sugarloaf, $F_{1,4}=0.2868$, $P=0.6207$; Big Pine, $F_{1,6}=0.27$, $P=0.6197$; Summerland, $F_{1,8}=0.1155$, $P=0.7427$; Cudjoe, $F_{1,6}=2.90$, $P=0.1393$; PAR, Sugarloaf, $F_{1,12}=0.0017$, $P=0.9683$; Big Pine, $F_{1,9}=1.27$, $P=0.2888$; Summerland, $F_{1,12}=1.44$, $P=0.2538$; Cudjoe,
Green and red patches at Sugarloaf received a greater percentage of UV surface irradiance than green and red patches at Cudjoe (ANOVA: green, $F_{3,12} = 4.73$, $P = 0.0522$). Green and red patches at Sugarloaf received a greater percentage of UV surface irradiance than green and red patches at Cudjoe (ANOVA: green, $F_{3,12} = 4.2$, $P = 0.0290$, red, $F_{3,12} = 6.33$, $P = 0.0081$) while percent PAR surface irradiance reaching the bottom of green patches was greater at Sugarloaf and Big Pine compared to Cudjoe (ANOVA: green, $F_{3,23} = 4.00$, $P = 0.0198$, red, $F_{3,21} = 0.52$, $P = 0.6758$).

The average temperature ($N = 2329$) was similar within all sites and among patches (Sugarloaf, green, $33.55 \pm 0.05^\circ C$, max $38.05^\circ C$, red, $33.80 \pm 0.05^\circ C$, max $39.84^\circ C$; Big Pine, green, $33.61 \pm 0.05^\circ C$, max $38.37^\circ C$, red, $33.35 \pm 0.05^\circ C$, max $38.38^\circ C$; Summerland, green, $33.28 \pm 0.03^\circ C$, max $40.41^\circ C$, red, $33.23 \pm 0.04^\circ C$, max $37.82^\circ C$; Cudjoe, green, $33.35 \pm 0.05^\circ C$, max $38.49^\circ C$, red, $33.51 \pm 0.04^\circ C$, max $39.16^\circ C$).

**Light versus anthocyanin content**

A significant positive relationship was observed between the average percent of UV and PAR bottom irradiance and leaf anthocyanin content in patches of green-leafed shoots (Figure 3.8, Linear Regression: UV, $R^2 = 0.64$, $F_{1,43} = 76.50$, $P < 0.0001$; PAR, $R^2 = 0.71$, $F_{1,43} = 103.44$, $P < 0.0001$). No significant relationship was observed between the average percent UV and PAR bottom irradiance and leaf anthocyanin content in patches of red-leafed shoots (Figure 3.8, Linear Regression: UV, $R^2 = 0.02$, $F_{1,42} = 0.31$, $P = 0.5771$; PAR, $R^2 = 0.01$, $F_{1,42} = 0.52$, $P = 0.4763$).

**Discussion**

*Thalassia testudinum* shoots with red leaves have been found growing in high light areas in the lower Florida Keys (Novak and Short, 2010). In the present study, we compared
various plant parameters of *T. testudinum* shoots with entirely red leaves (red-leafed shoots) growing adjacent to *T. testudinum* with entirely green leaves (green-leafed shoots) and found morphological and physiological differences. In addition to having higher concentrations of anthocyanins that caused red coloration (Figure 3.4), leaves of red-leafed shoots had higher concentrations of other photo-protective pigments (UV-absorbing compounds), and were shorter, narrower and weighed less than leaves of green-leafed shoots (Tables 3.1, 3.2). Differences were also observed at the patch level, with patches of red-leafed shoots exhibiting shorter canopy heights and lower LAI compared to green patches (Table 3.3). Our study is the first to document physiological and morphological differences between green and red seagrasses other than leaf size (McMillan 1983).

Four anthocyanin molecules caused red coloration in *T. testudinum* leaves from our study sites. The dominant anthocyanin molecule identified in red leaves was cyanidin 3-(malonoyl) glucoside, followed by cyanidin 3-glucoside, the most common anthocyanin found in terrestrial plant leaves (Harborne 1967). Pelargonidin 3-malonoyl glucoside was also identified in leaves of red-leafed shoots, but in small quantities. In terrestrial leaves, cyanidin imparts a red-to-violet color while pelargonidin is typically orange (Harborne 1967). Cross-sections of red leaves indicated that anthocyanin molecules accumulate in the epidermis and outer mesophyll cells (Figure 3.2). In terrestrial leaves, anthocyanins occur within the lower or upper epidermal layers in some species; however, they are commonly found in the vacuoles of palisade and spongy parenchyma (Lee 2002). We are the first to identify specific anthocyanin molecules, as well as identify the location of anthocyanins, in seagrass leaves.
In our study, ΔF/ F_m (effective quantum yield) values were greater at deeper sites for both color types and were higher in red compared to green-leafed shoots at each site (Figure 3.6). The increase in effective quantum yield with depth should be due to a reduction in the amount of light available for photochemistry (Beer et al. 2001) even though we did not observe a difference in absolute PAR (μmol photon m^{-2} s^{-1}) among sites during effective quantum yield measurements (Figure 3.7). The higher effective quantum yield values in red compared to green-leafed shoots at each site (Figure 3.6) indicates that anthocyanins are acting as a sunscreen in leaves and reducing the amount of light reaching chloroplasts. Despite the anthocyanic screen in red-leafed shoots, F_v/F_m (maximum quantum yield) was high in both red and green-leafed shoots and no difference was observed between the two color types, indicating that neither was photoinhibited (i.e., damage to photosystem II) and anthocyanins were not protecting red leaves from photoinhibition during the time of our measurements. Because there was no evidence at our sites for another function of anthocyanins (e.g., desiccation tolerance, cold-hardiness, defense or camouflage from herbivores) we considered whether high anthocyanin content in leaves was compensating for the intrinsic physiological inferiority of red-leafed shoots in other aspects of their photoprotective machinery by preventing photoinhibition, as demonstrated in some terrestrial plants (Hughes and Smith 2007; Kytridis et al. 2008). Red-leafed shoots in our study, however, were not inferior to green-leafed shoots; red-leafed shoots had higher UV-absorbing compound concentrations in leaves than green-leafed shoots, leaf chlorophyll content was the same in green and red-leafed shoots (Table 3.1), and green and red-leafed shoots had similar internode lengths and growth rates suggesting similar photosynthetic capabilities for the
metabolic needs of growth and production (Table 3.2, 3.3). Thus, we suggest other mechanisms should be considered for the protective function of anthocyanins in leaves of *T. testudinum* growing under high irradiance, such as mitigating DNA damage from excess UV-B/visible radiation (Takahashi et al. 1991; Gould 2004) and/or serving as an antioxidant under high water temperatures with high irradiance since average water temperatures (33.23°C-33.80°C) at our sites exceeded the optimum temperatures for growth and photosynthesis (29.1 ± 0.3°C; Lee et al., 2007) and maximum water temperatures (37.82°C-40.41°C) at our sites were within the range (35-40°C) known to inhibit *T. testudinum* leaf survival (van Tussenbroek et al. 2006).

In terrestrial plants, the light-filtering effect of anthocyanins can cause leaves to develop the morphological and physiological attributes of shade leaves (Manetas et al. 2003; Kyparissis et al. 2007). We found that chlorophyll content in leaves increased with anthocyanin content (Figure 3.5) in green leaf shoots, suggesting that as leaves redden the light-filtering effect of anthocyanins causes leaves to increase photosynthetic capacity to enhance light capture. In red-leafed shoots, this relationship was not observed (Figure 3.5) and, except for high effective quantum yields at midday (Figure 3.6), red-leafed shoots did not develop characteristics associated with shade acclimation such as increased leaf surface area, lower chlorophyll a/b ratios in leaves, higher chlorophyll content in leaves, and/or reduced growth rates relative to green-leafed shoots (Dennison & Alberte, 1985; Duarte, 1991; Abal et al., 1994; Durako et al., 2003; Beer et al., 2006). In contrast, red-leafed shoots exhibited some characteristics associated with seagrasses growing under higher light intensities such as higher UV absorbing compound content and smaller (narrower and shorter) leaves than green-leafed shoots (Tables 3.1 and 3.2).
In addition, red-leafed shoots maintained the same growth rates as green-leafed shoots (Table 3.3).

The environmental factor(s) responsible for the induction of leaf reddening in seagrasses have yet to be identified. In terrestrial plants numerous stressors have been shown to induce reddening, including low temperatures and/or enhanced UV/visible radiation, as well as nutrient limitation (Chalker-Scott 1999). Water temperatures in green and red patches at our study sites were exceptionally warm (Mote Marine Laboratory data) indicating that cold temperatures were not responsible for the induction of anthocyanins in *T. testudinum* leaves. Our results show that anthocyanin content in leaves of green-leafed shoots increased with both visible light and UV-B (Figure 3.8), although the regression was driven by one site. Trocine et al. (1981) found that leaf extracts from laboratory grown seagrasses had a reddish hue after being exposed to increased levels of UV-B. Red-leafed *T. testudinum* had higher anthocyanin concentrations than green, but showed no change with either increasing visible light or UV-B (Figure 3.8).

Anthocyanin accumulation in *T. testudinum* was not caused by nitrogen limitation. Nitrogen limitation in seagrasses is usually defined as low leaf tissue nitrogen (<1.8%) and high C:N ratios (>20:1; Duarte 1990). Mean leaf nitrogen content was 2.2% ± 0.05% for green and red shoots, with mean concentrations falling within the range typically reported for *T. testudinum* (0.88% and 3.96% DW, Fourqurean et al. 1992; Jensen et al. 1998); Leaf C:N ratios in shoots at our sites were below 20:1 and not different between red and green shoots (Table 3.2). Our findings concur with Fourqurean
and Zieman (2002) who suggested *T. testudinum* growing in nearshore waters on the Atlantic side of the Florida Keys is not nitrogen limited.

Our study shows that anthocyanins cause leaf reddening in *Thalassia testudinum*, which serves as a sunscreen and allows plants to maintain high effective quantum yields at high light intensities. Despite the light-filtering effect of anthocyanins, we did not find that red leaves were less photo-inhibited than green leaves nor do our results indicate that the light-filtering effect of anthocyanins causes red-leafed shoots to develop characteristics associated with shade acclimation. Rather, red-leafed shoots in our study exhibited some physiological and morphological characteristics that are common in seagrasses growing in high light environments including high UV absorbing compounds, small leaf surface areas that reduce absorption of damaging wavelengths, and high shoot growth rates.

**Conclusions**

Our work demonstrates that leaf reddening in *Thalassia testudinum* is caused by anthocyanin molecules in high concentrations in epidermal and mesophyll cells, is associated with specific physiological and morphological attributes, and acts as sunscreen since red leaves were able to maintain high effective quantum yields during periods of high light stress. Although the factors that induce leaf reddening in *T. testudinum* have yet to be identified, our results show that high light (UV and/or PAR) is responsible. We are now exploring the functional roles of leaf reddening in seagrasses and factors responsible for enhanced anthocyanin production.
Acknowledgements

We thank Cathy Short for technical editing and two anonymous reviewers for their comments. Additional valuable assistance from Richard Novak, Christopher Mercer, Pamelia Fraungruber, Lauren Gaskell, Paul Sokoloff, Nicole Sarrette, Jon Felch, and Holly Bayley. Research support from: the National Oceanic and Atmospheric Administration’s Nancy Foster Scholarship, the Department of Natural Resources and Environment at the University of New Hampshire, SeagrassNet, and Mote Marine Laboratory. All research for this project was conducted under National Marine Sanctuary Permit FKNMS-2007-042. Jackson Estuarine Laboratory contribution number 502.
Literature Cited


Kytridis VP, Karageorgou P, Levizou E, Manetas Y (2008) Intra-species variation in transient accumulation of leaf anthocyanins in *Cistus creticus* during winter:


differential anthocyanin accumulation in juvenile and mature phase Hedera helix


equations for the accurate determination of chlorophylls a and b. Photosyn Res
73: 149–156.


Ralph PJ, Gademann R, Dennison WC (1998) In situ seagrass photosynthesis measured

Florida Bay and the Florida Keys, U.S. Fish and Wildlife Service Publ,
FWS/OBS-82/58.


Short FT (1987) Effects of sediment nutrients on seagrasses: Literature review and


Table 3.1 UV-absorbing compound and photosynthetic pigment content in green and red-leafed shoots of *T. testudinum*. Red leaves had significantly higher concentrations of UV-B and UV-A absorbing compounds compared to green leaves at all sites (means ± SE; “within site” comparisons: * denotes significant differences between colors within a site at *P* < 0.05 with the higher value marked). Significant differences in concentrations of some pigments were observed among some sites for each color (means ± SE; “among site” comparisons: different letters (green: a-c; red: A-C) in superscript denote Tukeys test results for significant differences among sites at *P* < 0.05.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Color</th>
<th>Site</th>
<th>Color</th>
<th>Site</th>
<th>Color</th>
<th>Site</th>
<th>among site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sugarloaf</td>
<td>Big Pnc</td>
<td>Summerland</td>
<td>Cudpe</td>
<td>Sugarloaf</td>
<td>Big Pnc</td>
</tr>
<tr>
<td>UV-B (AU g⁻¹ fresh wt)</td>
<td>Green</td>
<td>2.64 ± 0.21*</td>
<td>3.13 ± 0.36*</td>
<td>3.12 ± 0.48*</td>
<td>1.89 ± 0.26*</td>
<td>F₃, 4₁ = 3.03, <em>P</em> = 0.0401</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>4.46 ± 0.36**</td>
<td>5.75 ± 0.40**</td>
<td>5.68 ± 0.46**</td>
<td>4.85 ± 0.44**</td>
<td>F₃, 4₀ = 2.26, <em>P</em> = 0.0963</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F₁, 3₀ = 18.66, <em>P</em> = 0.0003</td>
<td>F₁, 3₀ = 23.15, <em>P</em> = 0.0001</td>
<td>F₁, 3₀ = 14.50, <em>P</em> = 0.0011</td>
<td>F₁, 3₀ = 34.65, <em>P</em> ≤ 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>UV-B (AU g⁻¹ fresh wt)</td>
<td>Green</td>
<td>4.57 ± 0.27*</td>
<td>4.07 ± 0.36*</td>
<td>4.48 ± 0.35*</td>
<td>2.92 ± 0.18*</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>5.6 ± 0.45**</td>
<td>6.32 ± 0.38**</td>
<td>6.68 ± 0.39**</td>
<td>5.65 ± 0.24**</td>
<td>F₃, 4₀ = 1.50, <em>P</em> = 0.2282</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F₁, 3₀ = 14.64, <em>P</em> = 0.0011</td>
<td>F₁, 3₀ = 18.10, <em>P</em> = 0.0004</td>
<td>F₁, 3₀ = 17.77, <em>P</em> = 0.0004</td>
<td>F₁, 3₀ = 85.97, <em>P</em> ≤ 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl a (mg g⁻¹ fresh wt)</td>
<td>Green</td>
<td>0.58 ± 0.04*</td>
<td>0.46 ± 0.03b</td>
<td>0.43 ± 0.04b</td>
<td>0.28 ± 0.02c</td>
<td>F₃, 4₂ = 16.12, <em>P</em> &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>0.49 ± 0.02A</td>
<td>0.46 ± 0.04A</td>
<td>0.49 ± 0.04A</td>
<td>0.56 ± 0.03**</td>
<td>F₃, 4₁ = 1.83, <em>P</em> = 0.1591</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>F₁, 3₀ = 4.64, <em>P</em> = 0.0437</td>
<td>F₁, 3₀ = 0.0445, <em>P</em> = 0.9470</td>
<td>F₁, 3₀ = 0.40, <em>P</em> = 0.5326</td>
<td>F₁, 3₀ = 56.68, <em>P</em> ≤ 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl b (mg g⁻¹ fresh wt)</td>
<td>Green</td>
<td>0.22 ± 0.01*</td>
<td>0.16 ± 0.01b</td>
<td>0.17 ± 0.02ab</td>
<td>0.09 ± 0.01c</td>
<td>F₃, 4₀ = 14.51, <em>P</em> = 0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>0.18 ± 0.01BC</td>
<td>0.14 ± 0.01c</td>
<td>0.27 ± 0.01AB</td>
<td>0.22 ± 0.01Bo</td>
<td>F₃, 4₂ = 18.27, <em>P</em> ≤ 0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F₁, 3₀ = 4.94, <em>P</em> = 0.0379</td>
<td>F₁, 3₀ = 1.37, <em>P</em> = 0.2582</td>
<td>F₁, 3₀ = 4.99, <em>P</em> = 0.0014</td>
<td>F₁, 3₀ = 73.49, <em>P</em> ≤ 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Chl (mg g⁻¹ fresh wt)</td>
<td>Green</td>
<td>0.81 ± 0.05*</td>
<td>0.62 ± 0.05b</td>
<td>0.57 ± 0.04b</td>
<td>0.38 ± 0.03c</td>
<td>F₃, 4₁ = 16.65, <em>P</em> = 0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>0.67 ± 0.03A</td>
<td>0.62 ± 0.06A</td>
<td>0.64 ± 0.10A</td>
<td>0.78 ± 0.04**</td>
<td>F₃, 4₂ = 1.92, <em>P</em> = 0.143</td>
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</tr>
<tr>
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<td></td>
<td>F₁, 3₀ = 4.80, <em>P</em> = 0.0424</td>
<td>F₁, 3₀ = 0.0001, <em>P</em> = 0.9927</td>
<td>F₁, 3₀ = 0.82, <em>P</em> = 0.3738</td>
<td>F₁, 3₀ = 63.76, <em>P</em> ≤ 0.0001</td>
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<td></td>
</tr>
<tr>
<td>chl a b</td>
<td>Green</td>
<td>2.67 ± 0.04ab</td>
<td>2.94 ± 0.09*</td>
<td>2.49 ± 0.13**</td>
<td>2.78 ± 0.04**</td>
<td>F₃, 4₀ = 3.47, <em>P</em> = 0.0247</td>
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<tr>
<td></td>
<td>Red</td>
<td>2.79 ± 0.07AB</td>
<td>3.00 ± 0.11A</td>
<td>1.89 ± 0.15C</td>
<td>2.59 ± 0.06B</td>
<td>F₃, 4₂ = 23.28, <em>P</em> ≤ 0.0001</td>
<td></td>
</tr>
<tr>
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<td>F₁, 3₀ = 2.75, <em>P</em> = 0.1126</td>
<td>F₁, 3₀ = 0.01, <em>P</em> = 0.9066</td>
<td>F₁, 3₀ = 6.45, <em>P</em> = 0.0212</td>
<td>F₁, 3₀ = 8.20, <em>P</em> = 0.0096</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotenoids (mg g⁻¹ fresh wt)</td>
<td>Green</td>
<td>0.16 ± 0.01*</td>
<td>0.14 ± 0.01*</td>
<td>0.13 ± 0.01ab</td>
<td>0.10 ± 0.01B</td>
<td>F₃, 4₂ = 7.11, <em>P</em> = 0.0006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>0.16 ± 0.01A</td>
<td>0.15 ± 0.01A</td>
<td>0.21 ± 0.04**</td>
<td>0.20 ± 0.01FS</td>
<td>F₃, 4₀ = 2.61, <em>P</em> = 0.0673</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>F₁, 3₀ = 0.04, <em>P</em> = 0.8488</td>
<td>F₁, 3₀ = 0.22, <em>P</em> = 0.6424</td>
<td>F₁, 3₀ = 8.92, <em>P</em> = 0.0087</td>
<td>F₁, 3₀ = 83.42, <em>P</em> ≤ 0.0001</td>
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</tr>
</tbody>
</table>
Table 3.2 Morphological information for green and red-leafed shoots of *T. testudinum*. Leaf length, width, and weight were less for red compared to green-leafed shoots at each site (means ± SE; “*within site*” comparisons: * denotes significant differences between colors within a site at P< 0.05 with the higher value marked). Significant differences in morphological characteristics were also observed among sites for one/both colors (means ± SE; “*among site*” comparisons: different letters (green: a-c; red: A-B) in superscript denote Tukey’s test results for significant differences among sites at P< 0.05).

<table>
<thead>
<tr>
<th>Plant Parameter</th>
<th>Color</th>
<th>Site</th>
<th>Length (cm)</th>
<th>Width (cm)</th>
<th>Weight (g leaf⁻¹)</th>
<th>Internode length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sugarloaf</td>
<td>13 9 ± 1 5*</td>
<td>0 50 ± 0 02*</td>
<td>0 17 ± 0 02*</td>
<td>F₁₃₀ = 0 34, P = 0 7994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Big Pine</td>
<td>14 5 ± 1 7*</td>
<td>0 43 ± 0 01b</td>
<td>0 28 ± 0 05a*</td>
<td>F₁₃₀ = 5 65, P = 0 0025</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Summerland</td>
<td>15 8 ± 1 3*</td>
<td>0 48 ± 0 03ab</td>
<td>0 31 ± 0 03ab*</td>
<td>F₁₃₀ = 3 51, P = 0 0239</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cubero</td>
<td>14 6 ± 1 1*</td>
<td>0 55 ± 0 02a</td>
<td>0 35 ± 0 05a*</td>
<td>F₁₃₀ = 0 70, P = 0 5593</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>within site</strong></td>
<td>F₁₃₀ = 6 28,</td>
<td>F₁₃₀ = 11 12,</td>
<td>F₁₃₀ = 10 51,</td>
<td>F₁₃₀ = 3 27, P = 0 0981</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P = 0 0209</td>
<td>P = 0 0035</td>
<td>P = 0 0003</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sugarloaf</td>
<td>10 5 ± 0 6A</td>
<td>0 09 ± 0 01b</td>
<td>0 12 ± 0 01ab</td>
<td>F₁₃₀ = 0 70, P = 0 5593</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Big Pine</td>
<td>8 5 ± 0 6A</td>
<td>0 53 ± 0 03A</td>
<td>0 50 ± 0 04A</td>
<td>F₁₃₀ = 0 20, P = 0 4855</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Summerland</td>
<td>11 4 ± 1 1A</td>
<td>0 50 ± 0 02A</td>
<td>0 48 ± 0 04A</td>
<td>F₁₃₀ = 0 7746</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cubero</td>
<td>8 2 ± 0 7b</td>
<td>0 41 ± 0 03A</td>
<td>0 48 ± 0 04A</td>
<td>F₁₃₀ = 0 7748</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>within site</strong></td>
<td>F₁₃₀ = 9 31,</td>
<td>F₁₃₀ = 10 37,</td>
<td>F₁₃₀ = 10 84,</td>
<td>F₁₃₀ = 0 20, P = 0 4855</td>
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<td></td>
<td>P = 0 0065</td>
<td>P = 0 0043</td>
<td>P = 0 0003</td>
<td></td>
</tr>
</tbody>
</table>

* = significant differences between colors within a site at P< 0.05 with the higher value marked.

Fᵢ = F value, P = significance level.
Table 3.3 Structural, growth, and nutrient content information for green and red-leafed shoots of *T. testudinum*. Canopy height and LAI were less for red compared to green-leafed shoots at each site (means ± SE; "within site" comparisons: * denotes significant differences between colors within a site at P < 0.05 with the higher value marked). Significant differences in structural, growth, and nutrient characteristics were also observed among sites for one/both colors (means ± SE; "among site" comparisons: different letters (green: a-b; red: A-C) in superscript denote Tukeys test results for significant differences among sites at P < 0.05).

<table>
<thead>
<tr>
<th>Plant Parameter</th>
<th>Color</th>
<th>Sugarloaf</th>
<th>Big Pine</th>
<th>Summerland</th>
<th>Cudjoe</th>
<th>among site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canopy height (cm)</td>
<td>Green</td>
<td>15 0 ± 1.2 ^a</td>
<td>15 5 ± 1.6 ^a</td>
<td>16 6 ± 1.3 ^a</td>
<td>14 1 ± 0.9 ^a</td>
<td>F3 40 = 0.67, P = 0.6670</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>11 1 ± 0.5 ^a</td>
<td>11 0 ± 1.1 ^a</td>
<td>10 5 ± 0.8 ^a</td>
<td>9 9 ± 0.4 ^a</td>
<td>F3 36 = 0.59, P = 0.6267</td>
</tr>
<tr>
<td></td>
<td>within site</td>
<td>F1 20 = 8.96, F1 18 = 4.77,</td>
<td>F1 20 = 14.58,</td>
<td>F1 17 = 19.22,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.0072</td>
<td>P = 0.0425</td>
<td>P = 0.0011</td>
<td>P = 0.0004</td>
<td></td>
</tr>
<tr>
<td>LAI</td>
<td>Green</td>
<td>1.2 ± 0.26 ^a</td>
<td>0.85 ± 0.09 ^a</td>
<td>1.1 ± 0.20 ^a</td>
<td>0.89 ± 0.18 ^a</td>
<td>F3 40 = 0.89, P = 0.4509</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>0.47 ± 0.06 ^a</td>
<td>0.51 ± 0.08 ^a</td>
<td>0.39 ± 0.06 ^a</td>
<td>0.38 ± 0.05 ^a</td>
<td>F3 38 = 0.77, P = 0.5140</td>
</tr>
<tr>
<td></td>
<td>within site</td>
<td>F1 20 = 8.06,</td>
<td>F1 18 = 6.53,</td>
<td>F1 20 = 10.73,</td>
<td>F1 20 = 6.52,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.0101</td>
<td>P = 0.0199</td>
<td>P = 0.0038</td>
<td>P = 0.0187</td>
<td></td>
</tr>
<tr>
<td>Percent Cover (%)</td>
<td>Green</td>
<td>40 ± 3 ^a</td>
<td>42 ± 6 ^a</td>
<td>52 ± 7 ^a</td>
<td>41 ± 6 ^a</td>
<td>F3 40 = 0.95, P = 0.4273</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>25 ± 2 ^BC</td>
<td>36 ± 3 ^a</td>
<td>18 ± 2 ^C</td>
<td>30 ± 4 ^AB</td>
<td>F3 39 = 6.12, P = 0.0017</td>
</tr>
<tr>
<td></td>
<td>within site</td>
<td>F1 20 = 22.42,</td>
<td>F1 18 = 0.48,</td>
<td>F1 20 = 21.58,</td>
<td>F1 20 = 1.64,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.0001</td>
<td>P = 0.4945</td>
<td>P = 0.0008</td>
<td>P = 0.2154</td>
<td></td>
</tr>
<tr>
<td>Shoot Density (shoots m ^2)</td>
<td>Green</td>
<td>31 ± 4 ^a</td>
<td>17 ± 2 ^b</td>
<td>15 ± 1 ^a</td>
<td>11 ± 2 ^b</td>
<td>F3 40 = 14.09, P &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>17 ± 2 ^a</td>
<td>18 ± 2 ^A</td>
<td>12 ± 2 ^A</td>
<td>15 ± 2 ^B</td>
<td>F3 38 = 2.28, P = 0.1066</td>
</tr>
<tr>
<td></td>
<td>within site</td>
<td>F1 20 = 11.64,</td>
<td>F1 18 = 0.30,</td>
<td>F1 20 = 5.03,</td>
<td>F1 20 = 2.80,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.0082</td>
<td>P = 0.5905</td>
<td>P = 0.0364</td>
<td>P = 0.1094</td>
<td></td>
</tr>
<tr>
<td>Growth (mg shoot ^1 day ^1)</td>
<td>Green</td>
<td>2.62 ± 0.49 ^a</td>
<td>2.92 ± 0.49 ^a</td>
<td>2.24 ± 0.18 ^a</td>
<td>1.58 ± 0.49 ^a</td>
<td>F3 25 = 1.75, P = 0.1806</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>1.57 ± 0.17 ^b</td>
<td>3.10 ± 0.31 ^A</td>
<td>2.54 ± 0.36 ^A</td>
<td>2.03 ± 0.30 ^AB</td>
<td>F3 34 = 3.50, P = 0.0257</td>
</tr>
<tr>
<td></td>
<td>within site</td>
<td>F1 16 = 4.05,</td>
<td>F1 19 = 0.03,</td>
<td>F1 15 = 0.42,</td>
<td>F1 15 = 0.69,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.0717</td>
<td>P = 0.8712</td>
<td>P = 0.5273</td>
<td>P = 0.4167</td>
<td></td>
</tr>
<tr>
<td>Leaf Nitrogen (%)</td>
<td>Green</td>
<td>2.2 ± 0.09 ^a</td>
<td>2.3 ± 0.09 ^a</td>
<td>2.3 ± 0.04 ^a</td>
<td>1.8 ± 0.13 ^b</td>
<td>F3 28 = 6.55, P = 0.0017</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>2.3 ± 0.10 ^AB</td>
<td>2.4 ± 0.06 ^a</td>
<td>2.1 ± 0.08 ^AB</td>
<td>2.0 ± 0.07 ^b</td>
<td>F3 27 = 4.49, P = 0.0111</td>
</tr>
<tr>
<td></td>
<td>within site</td>
<td>F1 14 = 6.91,</td>
<td>F1 14 = 1.54,</td>
<td>F1 14 = 2.86,</td>
<td>F1 15 = 2.33,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.0196</td>
<td>P = 0.2340</td>
<td>P = 0.1126,</td>
<td>P = 0.1506</td>
<td></td>
</tr>
<tr>
<td>Leaf C:N</td>
<td>Green</td>
<td>15.35 ± 0.45 ^a</td>
<td>14.95 ± 0.56 ^a</td>
<td>14.7 ± 0.33 ^b</td>
<td>19.66 ± 0.45 ^a</td>
<td>F3 28 = 20.1, P &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>15.3 ± 0.44 ^AB</td>
<td>14.98 ± 0.45 ^a</td>
<td>16.4 ± 0.73 ^AB</td>
<td>17.27 ± 0.58 ^b</td>
<td>F3 32 = 3.34, P = 0.0339</td>
</tr>
<tr>
<td></td>
<td>within site</td>
<td>F1 14 = 0.007,</td>
<td>F1 16 = 0.002,</td>
<td>F1 14 = 4.50,</td>
<td>F1 15 = 6.97,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.09453</td>
<td>P = 0.9683</td>
<td>P = 0.0522</td>
<td>P = 0.0206</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1 Map of the lower Florida Keys, USA. Patches of *T. testudinum* with entirely red-leafed shoots were found at Sugarloaf, Cudjoe, Summerland, Middle Torch and Big Pine Keys (red and yellow dots). Red dots represent the locations of study sites.
Figure 3.2 Red pigmented cells in leaf tissue of *T. testudinum* producing red-leafed shoots, with anthocyanins occurring in epidermal (E) and mesophyll (M) cells (A denotes location of aerenchyma): a) Surface of leaf at 10X magnification; b) Cross-section of leaf at 10X magnification; and c) Cross-section of leaf at 40X magnification.
Figure 3.3 Mean UV and visible absorption spectra for methanol extracts of green and red-leafed shoots of *T. testudinum* (N=8 for green leaves, N=7 for red leaves) collected at Big Pine Key. Peak absorbances are noted at 270 and 300 nm (UV-B wavelength), 330 nm (UV-A wavelength), and 530 nm (green wavelength; characteristic peak absorbance for anthocyanins, Markhum 1982; Harborne 1967; Durst and Wrolstad 2001). Similar spectra were observed at all sites.
Figure 3.4 Mean anthocyanin content for leaves of green and red-leafed shoots of *T. testudinum*. Significant differences (P< 0.05) in anthocyanin concentrations were observed between green and red leaves (horizontal grey lines) at each site. Significant differences among sites were also observed for each color (P< 0.05; means ± SE), with Tukey’s results denoted by different letters (green: a-c; red: A-B). Sites are ordered according to depth. Anthocyanin data was natural log transformed for analyses.
Figure 3.5 Regression between anthocyanin content (natural log) and chlorophyll \( a \), chlorophyll \( b \), total chlorophyll, and carotenoid content for leaves of green (squares) and red-leafed (triangles) shoots of \textit{T. testudinum}.
Figure 3.6 ΔF/ Fₘₑ (effective quantum yield) values for green and red-leafed shoots of *T.* *testudinum* at each site. Significant differences (P< 0.05) in ΔF/ Fₘₑ were observed between green and red leaves at each site (horizontal grey lines). Different letters (green: a-c; red: A-C) represent Tukey’s results for significant differences among sites for each color (P < 0.05; means ± SE). Sites are ordered according to depth.
Figure 3.7 Incident PAR at the leaf surface of green (squares) and red-leafed (triangles) shoots of *T. testudinum* at each site, measured in July of 2007 on clear days near midday. Sites are ordered according to depth.
Figure 3.8 Regressions between the average percentage of UV and PAR bottom irradiance (mean ± SE) and leaf anthocyanin content (natural log) for patches of *T. testudinum* with green (squares) and red-leafed (triangles) shoots.
CHAPTER IV

UV-B INDUCES LEAF REDDENING AND CONTRIBUTES TO THE MAINTENANCE OF PHOTOSYNTHESIS IN THE SEAGRASS *THALASSIA TESTUDINUM*

Abstract

Numerous seagrass species growing in intertidal and shallow subtidal areas around the world produce red leaves, but the factors responsible for the induction of leaf reddening in seagrasses are poorly understood. We investigated the responses of transplanted green-leafed and *in situ* red-leafed *Thalassia testudinum* shoots growing in high light areas in the lower Florida Keys, USA, to four light treatments: 1) full solar radiation with UV excluded (PAR); 2) full solar radiation with UV-B excluded (PAR + UV-A); 3) full solar radiation reduced by 50% (50% Ambient); and 4) full solar radiation (Ambient). In our first experiment, green-leafed shoots were transplanted from a 1 m depth (MLW) to the four light treatments in 0.2 m depth (MLW). In our second experiment, *in situ* red-leafed shoots growing at depths between 0.2 m and 0.5 m were exposed to the four light treatments. Within one week, new leaf tissue from green-leafed shoots transplanted into shallow water accumulated anthocyanins and began to turn red in treatments receiving full spectrum solar radiation (Ambient; 50% Ambient) while transplanted green-leafed shoots in the two treatments that excluded UV-B (PAR and PAR + UV-A) had low anthocyanin
content and remained green. Although we quickly induced red coloration in leaves of
green-leafed shoots, reducing light levels (including UV-B) for seven weeks did not
cause leaves of in situ red leafed shoots to decrease anthocyanin content or turn
green. Instead, red leaves increased photosynthetic pigments in all treatments except
Ambient. In addition, we observed lower effective quantum yields and relative
electron transport rates at midday in the PAR + UV-A treatment compared to the
PAR and 50% Ambient treatments. We conclude that exposure to UV-B induces
anthocyanin accumulation and red coloration in green-leafed shoots and contributes
to the maintenance of high levels of photosynthesis in red-leafed shoots of T.
testudinum. We also propose that T. testudinum in the clear, shallow waters of the
lower Florida Keys produces a red-leafed variant with permanently red leaves since
anthocyanin accumulation and red coloration in leaves of red-leafed shoots was not
reversible in this and a longer-term study.

Introduction
Numerous seagrass species with red leaves have been found growing in intertidal and
clear shallow subtidal waters of the Tropical Atlantic, Tropical Indo-Pacific, and
Temperate Southern Oceans bioregions (Short et al., 2007; Novak and Short, 2010).
Similar to terrestrial plants, red coloration in seagrass leaves is caused by the
accumulation of anthocyanins, water-soluble pigments produced via the flavonoid
In a previous study, we showed that anthocyanins can act as a sunscreen in
seagrasses, enabling red leaves to maintain higher effective quantum yields at midday
compared to green leaves (Novak and Short, 2011). Research with terrestrial plants has demonstrated that anthocyanins can serve as a sunscreen and antioxidant in leaves during periods of high light stress by absorbing both ultraviolet (280-400nm) and visible (400-750nm; also referred to as PAR) regions of the solar spectrum (see review Gould et al., 2002).

In terrestrial plants, leaves may be red throughout a plant’s life or they may turn red while growing, during senescence, or in response to environmental stress. Stressors shown to induce leaf reddening in terrestrial plants include enhanced ultraviolet (UV)/visible radiation, cold temperatures, nutrient limitation, herbivory and pathogen attack (Chalker-Scott, 1999; Gould et al., 2002). The permanent and/or transient nature of red coloration in seagrass leaves is not fully understood although there is evidence that reddening is photoinduced in some seagrasses. Trocine (1981) observed reddish methanol extracts after exposing the seagrass *Halophila engelmannii* to enhanced ultraviolet-B (UV-B; 280-320nm) radiation. More recently, we found that anthocyanin content in green-leaved *T. testudinum* shoots was positively related to UV and visible irradiance although no relationship was observed between anthocyanin content in red-leaved *T. testudinum* shoots and those same parameters (Novak and Short, 2011).

Ultraviolet irradiance reaching the Earth’s surface has increased over the last thirty years (Herman, 2010; McKenzie, 2011) and climate models predict global warming will cause further increases in the tropics and high southern latitudes even as the stratosphere recovers from ozone depletion (Hegglin and Shepard, 2009). While UV radiation can be beneficial to photosynthesis in some seagrasses growing in high
light environments (Figueroa et al., 2002; Hanelt et al. 2006, 2009) excess UV radiation has been shown to negatively affect photosynthetic capacity (Dawson and Dennison, 1996; Detres et al., 2001) and photosynthetic efficiency (Trocine et al., 1981; Larkum and Wood, 1995; Ralph and Burchett, 1995; Dawson and Dennison, 1996; Figueroa et al., 2002), with factors such as morphology, secondary metabolite production, and leaf epiphytes influencing the magnitude of the seagrass response (Trocine et al., 1981; Abal et al., 1994; Larkum and Wood, 1995; Dawson and Dennison, 1996; Detres et al., 2001; Brandt and Koch, 2003; Kunzelman et al., 2005).

The present field study was conducted in the shallow subtidal waters of the lower Florida Keys to determine whether 1) various components of the light spectrum induce anthocyanin accumulation and reddening in green-leafed *T. testudinum* shoots; and 2) reduction of various components of the light spectrum affects anthocyanin levels, redness, and/or other physiological characteristics of red-leafed *T. testudinum* shoots. Our work is part of an ongoing effort to develop a comprehensive understanding of the cause and adaptive significance of the expression of red coloration in seagrass leaves (Novak and Short, 2010; Novak and Short, 2011).

**Methods**

**Site Description and Experimental Design**

The lower Florida Keys comprise 30 carbonate islands that separate the Atlantic on the east from the Gulf of Mexico on the west (Schomer and Drew, 1982). Nearshore waters are generally shallow and seagrass meadows, dominated by *T. testudinum*, are the primary benthic vegetation (Zieman et al. 1989; Fourquarean et al. 2001).
*Thalassia testudinum* shoots with one or more leaves expressing red coloration have been observed in shallow subtidal waters on both the Atlantic and Gulf of Mexico sides of the lower Florida Keys (Novak and Short 2010). Red pigmentation in leaves varies from cross or vertical striations to leaves that are entirely red. Patches of *T. testudinum* consisting of shoots with entirely red leaves (red-leafed shoots) have been observed at a number of locations growing adjacent to patches of *T. testudinum* with entirely green leaves (green-leafed shoots). Red-leafed shoots have higher concentrations of photo-protective pigments (anthocyanins and UV-absorbing compounds), higher effective quantum yields (ΔF/Fm') at high ambient irradiance, as well as shorter, narrower, and lighter-weight leaves than leaves from green-leafed shoots (Novak and Short, 2011).

Two field experiments were performed in the lower Florida Keys between June 1 and August 17, 2007 each using four light treatments which included: 1) full solar radiation with UV excluded (PAR); 2) full solar radiation with UV-B excluded (PAR + UV-A); 3) solar radiation reduced by 50% (50% Ambient); and 4) full solar radiation (Ambient). The exclusion of UV was achieved using Acrylite OP3 polycarbonate sheets, which are opaque to wavelengths below 400 nm, but allow full transmittance underwater in the PAR region. The exclusion of UV-B was achieved using Mylar 92D sheets, which are opaque to wavelengths below 320 nm, but allow full transmittance underwater in the PAR region. To reduce ambient light by 50% we used two sheets of neutral density screen. Transmittance in the UV and visible region was verified with a UV dosimeter (Apogee, UT, USA) and a LI-COR meter (LI-COR, NE, USA). To ensure stability of the light filters, a PVC frame was placed
around the Acrylite, Mylar, and neutral density screens. Each 50 cm X 50 cm apparatus was placed 15 cm above the tips of the seagrass shoots and anchored into the sediment with stainless steel threaded rods in each corner. All filters remained submerged throughout the experimental period. Filters were cleaned daily to prevent fouling and transmittance of light through filters was checked weekly to ensure that the filters maintained their spectral properties. Water temperature was recorded under each light treatment at 30-min intervals using iButton temperature loggers (Maxim Corporation, MA, USA) to determine if light filters were affecting the temperature of the water column. The temperature loggers were encased in silicon and attached to stakes at the center of each light treatment. No difference in temperature was observed among the treatments in both experiments.

**Color Measurements**

Color hue of each leaf on all seagrass shoots was assessed in both experiments using the Royal Horticultural Society’s (RHS) color chart (Royal Horticulture Society, 2007). The RHS system consists of 884 numerically coded colors. Leaf color is determined by matching samples to color coded paint-chips.

**Quantification of Anthocyanins**

Anthocyanin content in both experiments was measured on the second youngest leaf of each shoot. One 1 cm disc from the base of the leaf (above the sheath) was excised, weighed, and extracted in cold methanol/HCl/water (90:1:1, vol). The extracts were placed in the dark for 20 min and centrifuged at 18,000 x G for 10 min before being assayed spectrophotometrically with an Agilent Model 8453 Diode Array Spectrophotometer (Agilent, CA, USA). Total anthocyanin content was calculated
using the Beer-Lambert equation, assuming a corrected absorbance of $A_{529} - 0.288 A_{650}$ to compensate for the small overlap in absorbance at 529 nm by degraded chlorophyll (Sims and Gamon, 2002) and a molar absorbance coefficient for anthocyanin at 529 nm of 30,000$^{\text{mol}^{-1} \text{cm}^{-1}}$, where $\ell$ is the length of the light path (Murray & Hackett, 1991).

**Experiment 1: Light induced leaf reddening in green-leafed *T. testudinum* shoots**

The first experiment was conducted on transplanted green-leafed shoots at Sugarloaf Key (N 24° 39.332, W 81° 32.194) to determine whether anthocyanin accumulation and red pigmentation in leaves is photoinduced in *T. testudinum* by high light intensities. For the experiment, we harvested 160 green-leaved shoots from 1 m depth. Ten shoots, each with 1 leaf bundle and 8 cm of rhizome, were transplanted into each of four replicates of the four light treatments located at a 0.2 m depth (i.e., depth at which red-leafed *T. testudinum* shoots were found at Sugarloaf Key).

**Experiment 2: Effects of light on red-leafed *T. testudinum* shoots**

The second experiment was conducted on *in situ* red-leafed shoots of *T. testudinum* at Sugarloaf Key (N 24° 39.332, W 81° 32.194), Cudjoe Key (N 24° 39.868, W 81° 29.659), Summerland Key (N 24° 39.653, W 81° 27.647), and Big Pine Key (N 24° 39.219, W 81° 22.214; Figure 4.1) to determine whether various components of light affect anthocyanin levels, redness in leaves, or other physiological characteristics of red-leafed shoots. For the experiment, a single patch of *in situ* red-leafed shoots was selected at each site, shoots within the patch were evenly divided among the same
light treatments as Experiment 1, and filters were erected over the shoots. Red
patches were 2.8 - 3.5 m in diameter, located 10 - 25 m offshore, and uniformly
colored. Sites varied in depth (MLW; Sugarloaf Key 0.2 m, Cudjoe Key 0.5 m,
Summerland Key 0.4 m, and Big Pine Key 0.3 m), with a tidal range of 0.3 m at all
sites except Sugarloaf (0.1 m). Color and pigment content (anthocyanins, UV-
absorbing compounds, and photosynthetic pigments) were assessed each week for
seven weeks on leaves from four shoots haphazardly collected from each light
treatment at each site. In addition, in situ fluorescence measurements were made on
leaves from eight shoots growing in each light treatment at each site.

**Pigment Analyses**

Concentrations of total UV-absorbing compounds were estimated from 10-fold
dilutions of the anthocyanin extracts (Day, 1993). The extracts were placed in the
dark for 20 min and centrifuged at 18,000 x G for 10 min before being assayed
spectrophotometrically. Absorbances for UV absorbing compounds were measured
at A300 (UV-B) and A350 (UV-A).

Chlorophyll and carotenoid content of red-leafed shoots was measured using
the second youngest leaf of each shoot. One 1 cm disc from the base of the leaf was
excised, weighed and extracted in acetone/water (9:1, vol). The extracts were placed
in the dark and centrifuged using the methods described above before being assayed
spectrophotometrically. Chlorophyll (chlorophyll a, chlorophyll b, total chlorophyll)
content was calculated using the equations of Porra (2002). Carotenoid content was
calculated using the Lichtenthaler and Wellburn (1983) equations.
Fluorescence Measurements

Pulse amplitude modulated (PAM) chlorophyll fluorescence was measured on in situ red-leafed shoots in all treatments with a Diving-PAM (Walz, Germany); its universal sample holder (DIVING-USH) was used to hold the fiber optics probe 5 mm from, and perpendicular to the second youngest leaf. Measurements were performed using the default instrument settings (measuring light intensity, 8; saturating pulse intensity, 8; saturating pulse width, 0.8; and gain, 2) at all sites.

Effective quantum yield, an estimate of the photosynthetic efficiency of PSII when plants are light acclimated, was measured by the saturating-light method on red-leafed shoots growing under each light treatment at each site. Fluorescence measurements were performed on forty shoots (8 per light treatment) at each site each week between 1100 and 1300 hrs, with order randomized among light treatments and with leaves held parallel to the surface to maximize exposure to light. Incident underwater light on the leaf surface (i.e., PAR) was recorded in unison with fluorescence measurements by the Diving-PAM quantum sensor, which was fixed in the DIVING-USH next to the fiber optics probe. The equation for effective quantum yield is \( \frac{F_m - F}{F_m} = \frac{\Delta F}{F_m} \), where \( F \) is the fluorescence of a leaf under ambient light and \( F_m \) is the corresponding fluorescence measured following a saturating light period (Genty et al. 1989; Beer et al. 2001).

Relative electron transport rates (rETR) in PSII were estimated on red-leafed shoots growing under each light treatment at each site for weeks 3 – 7. To estimate rETR we used the following equation: \( \text{rETR} = Y \times \text{PAR} \times 0.5 \times \Delta F \), where \( Y \) is the effective quantum yield in ambient light, PAR is the amount of photosynthetically
active radiation (400–700 nm) measured next to the leaf blade by the quantum sensor at the time of effective quantum yield measurements, 0.5 assumes half of the photons are absorbed by PSII for photosynthesis, and AF is the fraction of PAR absorbed by the leaf and used in photosynthesis (Genty et al., 1989; Beer et al., 2001). AF was assumed to be 0.81, the recommended AF value for *T. testudinum* with green leaves (Durako 2007), since we were unable to determine the amount of PAR that was absorbed by anthocyanins and no longer available to chloroplasts for photosynthesis in leaves of red-leafed shoots.

**Statistics**

For Experiment 1, anthocyanin data were compared among light treatments using a one-way analysis of variance model (ANOVA). The anthocyanin dataset met the assumptions of the Kolmogorov–Smirnov test of normality and the Brown-Forsythe test of equal variance. Tukey’s multiple comparisons tests were performed to identify which light treatments were significantly different.

For Experiment 2, a one way ANOVA was used to assess the effect of each light treatment on anthocyanins, UV absorbing compounds, and relative electron transport rates (rETRs) for each week. Because effective quantum yield (ΔF/Fₘ) is dependent upon ambient light conditions and our sites differed in depth, we present the effect of light treatment and week on this variable by site. All datasets met the assumptions of the Kolmogorov–Smirnov test of normality and the Brown-Forsythe test of equal variance. Tukey’s multiple comparisons tests were performed to identify which light treatments were significantly different. Linear regression analyses were
used to assess relationships between time and chlorophyll a, chlorophyll b, total chlorophyll, chlorophyll a:b, and carotenoids for each of the light treatments.

Analyses for both experiments were performed using JMP (Version 6.0, SAS Institute Inc.) with significance determined at the 95% probability level (p<0.05). Values are reported as means and standard errors.

**Results**

**Experiment 1: Light induced leaf reddening in green-leafed *T. testudinum* shoots**

**Color Measurements**

Leaves of transplanted green-leafed shoots remained green, RHS 146A, in the two light treatments that excluded UV-B (PAR and PAR + UV-A). All transplanted green-leafed shoots receiving full solar radiation (Ambient) had one or more leaves with red pigmentation, RHS N77A. Some transplanted shoots receiving 50% Ambient light had one or more leaves with red pigmentation, RHS 59B or N77A. Leaf reddening in the Ambient and the 50% Ambient treatments occurred in new leaf tissue on the youngest leaves and progressed from the base of the blade towards the tip (Figure 4.2).

**Quantification of Anthocyanins**

Concentrations of anthocyanins in the second youngest leaf of transplanted green-leafed shoots were low in the two treatments that excluded UV-B (PAR and PAR + UV-A), intermediate in the 50% Ambient treatments, and high under full solar radiation (Ambient; Figure 4.3).
Experiment 2: Effects of light reductions on red-leafed *T. testudinum* shoots

**Color Measurements**

All leaves of *in situ* red-leafed shoots were dark red, RHS N77A, and the color did not change throughout the experiment. In addition, *in situ* red-leafed shoots continued to produce new leaves of the color RHS N77A (Table 4.1).

**Pigment Analyses**

Anthocyanin and UV absorbing compound content of *in situ* red-leafed shoots was not significantly different among treatments after seven weeks (Table 4.1). Chlorophyll *a* and chlorophyll *b* content significantly increased over time in the second youngest leaf of red-leafed shoots growing in treatments where UV was excluded or reduced (PAR and 50% Ambient; Table 4.2; Figure 4.4). Total chlorophyll content and carotenoid content significantly increased in the second youngest leaf of red-leafed shoots growing in treatments where UV-B was excluded or reduced (PAR, PAR + UV-A, 50% Ambient; Table 4.2; Figure 4.4). No change in the ratio of chlorophyll *a* to chlorophyll *b* was observed in any treatment (Table 4.2).

**Fluorescence Measurements**

Red-leafed shoots in the 50% Ambient treatments had the highest effective quantum yields (ΔF/Fₘ) at midday for the majority of the experiment at all sites except Cudjoe, the deepest site; red-leafed shoots in the 50% Ambient treatment at Cudjoe had the highest ΔF/Fₘ values at midday in weeks 4, 6, and 7. By week five and for the rest of the experiment, red-leafed shoots with only UV-B excluded (PAR + UV-A) had the lowest ΔF/Fₘ values at midday of any treatment at each site while red-
leafed shoots in the PAR and Ambient treatments had the second highest \( \Delta F/ F_{n'} \)
values at most sites (Table 4.3; Figure 4.5).

Relative electron transport rates (rETRs) at midday were lowest in red-leafed
shoots in the treatment where UV-B was excluded (PAR + UV-A) and in the 50%
Ambient treatment for weeks 4, 5, 6, and 7. Red-leafed shoots in the PAR and
Ambient treatments had the highest rETRs for weeks 4, 5, 6, and 7 (Figure 4.6,
ANOVA: week 3, \( F_{3,12} = 1.95, P = 0.1762 \); week 4, \( F_{3,12} = 9.94, P = 0.0014 \); week 5,
\( F_{3,12} = 5.07, P = 0.0174 \); week 6, \( F_{3,12} = 5.87, P = 0.0105 \); week 7, \( F_{3,12} = 6.53, P =
0.0072 \)).

**Discussion**

We transplanted green-leafed *T. testudinum* shoots into shallow waters, with light
intensities higher than their natural environment, and exposed them to four light
treatments to determine whether the expression of red coloration in leaves can be
photo-induced in seagrasses. Experiment 1 shows that the expression of red
coloration in otherwise green leaves of *T. testudinum* is induced by exposure to UV-B
and is a response to enhanced UV-B levels (Figure 4.3). We show that new leaf
tissue in transplanted green-leafed shoots accumulated anthocyanins and turned red in
treatments receiving full spectrum solar radiation (Ambient; 50% Ambient) while
transplanted green-leafed shoots in the two treatments that excluded UV-B (PAR and
PAR + UV-A) did not accumulate anthocyanins and remained green (Figures 4.2 and
4.3). Our finding that UV-B exposure induces anthocyanin accumulation in seagrass
leaves is supported by Trocine (1981) who described reddish extracts after exposing
the seagrass *Halophila engelmanni* to high levels of UV-B. We estimate that transplanted green-leafed shoots in Ambient treatments were exposed to UV-B levels of 1300 w m\(^{-2}\) d\(^{-1}\) (Mote Marine Lab-U.S. EPA Data) when we observed anthocyanin accumulation and the expression of red coloration in leaves, which may be 60% more UV-B than they receive at 1 m depth (estimated from Barron et al., 2009).

Our study also demonstrates that red-leafed *T. testudinum* growing in high light environments uses UV-B to maintain high levels of photosynthesis. We show that effective quantum yield (\(\Delta F/ F_m\)) values and relative electron transport rates (rETRs) in red-leafed shoots decreased after four weeks when only UV-B was excluded (PAR + UV-A; Table 4.3; Figures 4.5 and 4.6). Because \(\Delta F/ F_m\) and rETRs did not change when UV-B and UV-A were excluded (PAR), we propose that photosynthesis in red-leafed shoots is impaired by UV-A alone or the combination of UV-A and PAR, as shown in some seagrasses with green leaves (Trocine, 1982). Our work also supports the suggestions of both Figueroa et al. (2002) and Hanelt et al. (2006) that seagrasses use UV-B as a photoreceptor in the recovery process of photosynthesis, as well as the suggestion of Hanelt et al. (2009) that the ameliorating effect of UV-B on photosynthesis is specific to seagrasses acclimated to high light environments. Our results and the studies discussed above are in contrast to most aquatic studies conducted at high light intensities because we demonstrate that high levels of photosynthesis in plants can be maintained, rather than impaired, by UV-B (Hader, 1991).

*In situ* red-leafed shoots exposed to reduced light levels in Experiment 2 increased photosynthetic capacity to enhance light capture (Figure 4.4), but did not
reduce anthocyanin content or turn green (Table 4.2; Figure 4.3). We show that after seven weeks all leaves on red-leafed shoots in all treatments remained dark red (RHS N77A) and all shoots continued to produce new dark-red leaves (RHS N77A), with high concentrations of anthocyanins and other UV-absorbing compounds (Table 4.2; Figure 4.3 and 4.4). Our results show that anthocyanin content and red coloration in leaves are not immediately reversed, and therefore, may be permanent in red-leafed *T. testudinum* shoots. A separate three-year study we conducted provides additional support for this hypothesis since red-leafed shoots transplanted to deeper depths continuously produced red leaves at reduced light intensities for the entire experimental period (Novak and Short, unpublished). Based on our findings, we propose that *T. testudinum* growing in high light environments in the lower Florida Keys produces a red-leafed variant, a genetically differentiated form with permanently red leaves while green-leafed shoots produce red leaves only during periods of exceptionally high light intensities (e.g., summer solstice, pers. obs.). Additional field studies are needed to understand the permanent versus transient nature of red coloration in seagrasses.

Ultraviolet-B radiation serves an important role in plant protection in *T. testudinum* growing at high light intensities in the clear waters of the lower Florida Keys. Our study shows that exposure to UV-B induces anthocyanin accumulation and red coloration in leaves of green-leafed shoots, as well as contributes to the maintenance of high levels of photosynthesis in red-leafed shoots. Although we demonstrate that leaf reddening can be used as an indicator of UV-B exposure in green-leafed shoots we also show that anthocyanins and red coloration in leaves of
red-leafed shoots are unaffected by light levels, leading us to believe that red-leafed shoots are a variant in this system. The selective advantage of producing red coloration in leaves only during periods of enhanced UV-B levels versus permanently maintaining red coloration in leaves should be investigated since seagrasses with red leaves are prevalent in regions exposed to increased ultraviolet radiation due to global climate change.

Acknowledgements

We thank Cathy Short for technical editing and two anonymous reviewers for their comments. Additional valuable assistance from Richard Novak, Pamela Fraungruber, Lauren Gaskell, Jonathon Felch, and Holly Bayley. Research support from: the National Oceanic and Atmospheric Administration’s Nancy Foster Scholarship, the Department of Natural Resources and Environment at the University of New Hampshire, SeagrassNet, and Mote Marine Laboratory. All research for this project was conducted under National Marine Sanctuary Permit FKNMS-2007-042. Jackson Estuarine Laboratory contribution number XXXX.
Literature Cited


Table 4.1 Experiment 2: Color values, as well as anthocyanin and UV absorbing compound content of *in situ* red-leafed *T. testudinum* after seven weeks. All leaves on *in situ* red-leafed *T. testudinum* shoots remained dark red, RHS N77A, in all treatments. No significant difference in anthocyanin content or UV-absorbing compounds were observed among treatments (means ± SE; ANOVA: anthocyanins, $F_{3,12} = 1.03$, $P= 0.4123$, UV-B absorbing, $F_{3,12} = 0.13$, $P= 0.9373$, UV-A absorbing, $F_{3,12}= 0.221$, $P= 0.8792$).

<table>
<thead>
<tr>
<th>Pigments</th>
<th>Light Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAR</td>
</tr>
<tr>
<td><strong>RHS value</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Anthocyanins</strong></td>
<td></td>
</tr>
<tr>
<td>(mg g$^{-1}$ fresh wt)</td>
<td>1.20 ± 0.19</td>
</tr>
<tr>
<td><strong>UV-B absorbing</strong></td>
<td>6.66 ± 0.51</td>
</tr>
<tr>
<td>(AU g$^{-1}$ fresh wt)</td>
<td></td>
</tr>
<tr>
<td><strong>UV-A absorbing</strong></td>
<td>7.83 ± 0.57</td>
</tr>
<tr>
<td>(AU g$^{-1}$ fresh wt)</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2  Experiment 2: Regression results for time (weeks) versus chlorophyll a, chlorophyll b, total chlorophyll, chlorophyll a:b and carotenoid content in leaves of *in situ* red-leafed *T. testudinum* shoots grown in different light treatments over seven weeks.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Light Treatment</th>
<th>Equation</th>
<th>$R^2$ value</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td>Chl a</td>
<td>PAR</td>
<td>$y = 0.0372x + 0.4552$</td>
<td>= 0.27</td>
<td>= 0.0043</td>
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<td>PAR + UV-A</td>
<td>$y = 0.0217x + 0.5381$</td>
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<td>= 0.0746</td>
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<td>$y = 0.0606x + 0.3909$</td>
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<td>Ambient</td>
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<tr>
<td>Chl b</td>
<td>PAR</td>
<td>$y = 0.0125x + 0.2036$</td>
<td>= 0.17</td>
<td>= 0.0290</td>
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<tr>
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<td>PAR + UV-A</td>
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<td>= 0.1082</td>
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<td>$y = 0.0241x + 0.1455$</td>
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<tr>
<td></td>
<td>Ambient</td>
<td>$y = 0.0039x + 0.1831$</td>
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<td>P = 0.5162</td>
</tr>
<tr>
<td>Total Chl</td>
<td>PAR</td>
<td>$y = 0.0579x + 0.6258$</td>
<td>= 0.30</td>
<td>= 0.0028</td>
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<td>$y = 0.0445x + 0.7150$</td>
<td>= 0.20</td>
<td>= 0.0182</td>
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<tr>
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<td>50% Ambient</td>
<td>$y = 0.0920x + 0.5263$</td>
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<td>&lt; 0.0001</td>
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<td>$y = 0.0206x + 0.6631$</td>
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<td>= 0.2955</td>
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<tr>
<td>Chl a:b</td>
<td>PAR</td>
<td>$y = 0.0435x + 2.422$</td>
<td>= 0.1</td>
<td>= 0.1000</td>
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<td>PAR + UV-A</td>
<td>$y = 0.0241x + 2.522$</td>
<td>= 0.02</td>
<td>= 0.4969</td>
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<td>50% Ambient</td>
<td>$y = -0.0044x + 2.677$</td>
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<td></td>
<td>Ambient</td>
<td>$y = -0.0175x + 2.629$</td>
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<td>= 0.6329</td>
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<td>Carotenoids</td>
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<td>$y = 0.0244x + 0.1388$</td>
<td>= 0.37</td>
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<td>$y = 0.0073x + 0.1720$</td>
<td>= 0.09</td>
<td>= 0.1271</td>
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Table 4.3 Experiment 2: ANOVA results for each week showing differences among treatments in ΔF/ F<sub>0</sub> values for <i>in situ</i> red-leaved <i>T. testudinum</i>.

<table>
<thead>
<tr>
<th>Site</th>
<th>Week</th>
<th>F statistics</th>
<th>P value</th>
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<td>Sugarloaf</td>
<td>1</td>
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<td></td>
<td>2</td>
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<tr>
<td></td>
<td>3</td>
<td>F&lt;sub&gt;3,36&lt;/sub&gt; = 8.40</td>
<td>&lt; 0.0002</td>
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<tr>
<td></td>
<td>4</td>
<td>F&lt;sub&gt;3,37&lt;/sub&gt; = 8.39</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>F&lt;sub&gt;3,36&lt;/sub&gt; = 12.16</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>F&lt;sub&gt;3,36&lt;/sub&gt; = 16.60</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>F&lt;sub&gt;3,36&lt;/sub&gt; = 28.78</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Big Pine</td>
<td>1</td>
<td>F&lt;sub&gt;3,30&lt;/sub&gt; = 8.31</td>
<td>&lt; 0.0004</td>
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<td>&lt; 0.0035</td>
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<td>Cudjoe</td>
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<tr>
<td></td>
<td>7</td>
<td>F&lt;sub&gt;3,34&lt;/sub&gt; = 17.76</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>
Figure 4.1 Map of the lower Florida Keys, USA with the location of study sites (red dots).
Figure 4.2 Section of a leaf from a green-leafed *T. testudinum* shoot showing reddening beginning at the base of the blade and progressing up the central vein towards the tip. Red coloration on the leaf is RHS 59B while green coloration is RHS 146A.
Figure 4.3 Experiment 1: Anthocyanin concentrations in leaves from transplanted green-leafed *T. testudinum* shoots grown under different light treatments after one week. Significant differences were observed among treatments at $P < 0.05$ (means ± SE; ANOVA: $F_{3,12} = 4.52, P = 0.0241$), with Tukey’s results denoted by different letters (A-B). RHS color values of the youngest leaves are denoted in parentheses (i.e. RHS 146A, RHS 59B).
Figure 4.4 Experiment 2: The relationship between time and chlorophyll $a$, chlorophyll $b$, total chlorophyll, and carotenoid content in leaves of $in situ$ red-leafed $T. testudinum$ shoots grown in different light treatments over seven weeks. Significant trends at $P<0.05$ are denoted by an asterisk. Legend symbols and regression lines are represented as follows: $\square$, $\Pi$, PAR; $\Diamond$, $\Pi \Pi$, PAR + UV-A; $\Delta$, $\Pi \Pi$, 50% Ambient; $\bullet$, $\Pi$, Ambient.
Figure 4.5 Experiment 2: Average weekly ΔF/ F₀ values from in situ red-leafed T. testudinum shoots from different light treatments at each site (A, Sugarloaf Key; B, Big Pine Key; C, Summerland Key; D, Cudjoe Key). Significant differences were observed among treatments at P< 0.05 (means ± SE), with Tukey’s results denoted by different letters (A-C).
Figure 4.6 Experiment 2: Average weekly rETR values from *in situ* red-leaved *T. testudinum* shoots growing in different light treatments. Significant differences were observed among treatments at $P<0.05$ (means ± SE), with Tukey’s results denoted by different letters (A-C).
CHAPTER V

TRANSIENT AND PERMANENT LEAF REDDENING IN THE SEAGRASS

*THALASSIA TESTUDINUM*

**Abstract**

Seagrasses with red leaves have been observed at numerous locations around the world growing in areas with high light intensities. To test whether variations in light conditions affect anthocyanin accumulation and red coloration in leaves, we performed reciprocal transplants of green- and red-leafed *T. testudinum* shoots among patches with high and low self-shading located along a depth related gradient of light availability in the lower Florida Keys, USA. We collected 40 green-leafed shoots with long leaves from a green patch (high self-shading) and 40 red-leafed shoots with short leaves from a red patch (low self-shading) at four sites that varied in depth (0.2 – 0.5 m) by harvesting sections of rhizomes with 2 to 5 shoots. Five shoots per collection site of each color were then transplanted into the green and red patch at each of the four sites and monitored for three years. Transplanted green-leafed shoots transiently turned red during periods of high solar UV and visible light intensity, with the reddening process influenced by self-shading and depth. We also found that red-leafed shoots continuously produced red leaves with high concentrations of anthocyanins regardless of self-shading or depth. We conclude that anthocyanin accumulation and the expression of red coloration can be
temporarily photo-induced in *T. testudinum* and this species produces a genetic variant with permanently red leaves in the shallow waters of the lower Florida Keys.

**Introduction**

Seagrasses with red leaves have been found growing in shallow waters with high light intensities at numerous locations around the world (Novak and Short, 2010). Research has shown that red coloration in leaves is caused by the accumulation of anthocyanins (McMillan, 1983; Fyfe, 2003, 2004; Novak and Short, 2011), which act as a sunscreen and enable leaves to maintain high effective quantum yields during periods of high light stress (Novak and Short, 2011). In terrestrial plants, leaves may be red throughout a plant’s life or they may transiently turn red while growing, during senescence, or in response to environmental stress. In a previous study, we exposed green- and red-leafed *Thalassia testudinum* shoots in the lower Florida Keys to different light treatments and showed that anthocyanin accumulation and red coloration in green-leafed shoots can be environmentally induced within one week by exposing shoots to high intensities of ultraviolet-B radiation (UV-B). We also showed that reductions in light levels, including UV-B, for seven weeks did not cause red-leafed shoots to reduce anthocyanin concentrations or turn green, leading us to believe that the environmental induction of red coloration in leaves is not reversible or that possibly *T. testudinum* produces a variant with permanently red leaves (Novak and Short, in press). Research on the seagrass species *Halodule wrightii* and *Halophila ovalis* has also suggested the occurrence of variants with permanently red leaves since red coloration detected among shoots in the field was maintained under the reduced light of laboratory conditions (McMillan, 1978; 1983).
Reciprocal transplant experiments with seagrasses are often performed to test whether differences in populations are caused by environmental or genetic factors (Calumpong and Fonseca, 2001). Phillips (1976) was one of the first seagrass researchers to use reciprocal transplants across an environmental gradient to demonstrate that some populations show phenotypic plasticity in morphology and adapt to new environmental conditions while other populations show little change, suggesting that they are genotypically differentiated. Genetic and genotypic variation are critical factors for maintaining seagrass ecosystem functioning and resilience to environmental change because they provide response diversity (Hughes and Stachowicz, 2004; Procaccini et al., 2007; Ehlers et al., 2008). *Thalassia testudinum* is one species that shows low genetic structure and high homogeneity within its distributional range (Waycott et al., 2006; Van Dijk et al., 2007) although genetically distinguishable clones have been reported in the lower Florida Keys at <0.25 m (Davis et al., 1999; Waycott et al., 2006).

In the present study, we performed reciprocal transplant experiments in the shallow waters of the lower Florida Keys with green- and red-leafed *T. testudinum* shoots to test whether variations in light conditions affect anthocyanin concentrations and the persistence of red coloration in leaves. Our study is part of an on-going effort to increase our understanding of the causes and adaptive significance of red coloration in seagrass leaves so that we can predict whether this phenomenon will enhance seagrass resilience to global climate change (Novak and Short, 2010; Novak and Short 2011; Novak and Short, in press).
Methods

Site Description and Experimental Design

The lower Florida Keys comprise 30 carbonate islands that separate the Atlantic on the east from the Gulf of Mexico on the west (Schomer and Drew, 1982). Nearshore waters are generally shallow and seagrass meadows, dominated by *T. testudinum*, are the primary benthic vegetation (Zieman et al. 1989; Fourqurean et al. 2001). Patches of *T. testudinum* consisting of shoots with entirely red leaves (red-leafed shoots) have been observed growing adjacent to patches of *T. testudinum* with entirely green leaves (green-leafed shoots) at a number of locations on both the Atlantic and Gulf sides. Patches of red-leafed shoots (red patches) have lower canopy heights and leaf area index compared to patches of green-leafed shoots (green patches; Novak and Short, 2011). Additionally, *T. testudinum* shoots have been observed in a transitional phase with one or more leaves expressing red coloration at Sugarloaf and Big Pine Key.

Two patches (1 green and 1 red) were selected at each of four sites for a reciprocal transplant experiment using a common garden approach in June 2007: Sugarloaf (N 24° 39.332, W 81° 32.194), Big Pine (N 24° 39.219, W 81° 22.214), Summerland (N 24° 39.653, W 81° 27.647), and Cudjoe (N 24° 39.868, W 81° 29.659; Figure 5.1). Water depth was similar within each site, but varied among sites (MLW; Sugarloaf, 0.2 m; Big Pine, 0.3 m; Summerland, 0.4 m; Cudjoe Key, 0.5 m) and tidal range was 0.3 m at all sites except Sugarloaf (0.1 m). Patches were 2.8 - 3.5 m in diameter and located 10 - 25 m offshore. Leaf color of green-leafed shoots in green patches was Royal Horticultural Society (RHS) color 146B while leaf color of red-leafed
shoots in red patches was RHS color N77A. Canopy height and LAI was higher in green compared to red patches (Novak and Short, 2011).

Reciprocal Transplants

Eighty shoots (40 green-leafed and 40 red-leafed) were collected from the green and red patches, respectively, at each site for the reciprocal transplant experiment by harvesting sections of rhizomes with 2 to 5 shoots. Rhizomes and an area 2 cm above the rhizome near the base of the sheath were marked and coded according to leaf color and collection site using different colored flagging tape. Rhizome sections with multiple shoots were then transplanted among green and red patches, including the donor patches, as follows: 20 green-leafed shoots (5 shoots/collection site) were transplanted into the green patch at each of the four sites; 20 green-leafed shoots (5 shoots/collection site) were transplanted into the red patch at each site; 20 red-leafed shoots (5 shoots/collection site) were transplanted into the green patch at each site; and 20 red-leafed shoots (5 shoots/collection site) were transplanted into the red patch at each site. Transplants were placed within the center of patches and evenly spaced (5 cm). We monitored transplants for three years and information on leaf color and pigment content of leaves was collected at periods of different solar light intensities: summer solstice (4 and 156 weeks post-transplantation), at the end of the summer during a spring tide and before the autumnal equinox (10 weeks post-transplantation), and around the winter solstice (26 weeks post-transplantation).

Color Measurements

We assessed the color hue of each leaf on transplanted green- and red-leafed shoots at weeks 4, 10, 26 and 156 post-transplantation. For the second youngest leaf on shoots, the
color hue was determined by clipping leaves and visually assessing them in the lab using the Royal Horticultural Society’s (RHS) color chart, which consists of 884 numerically coded colors (Royal Horticulture Society, 2007). Leaf color was determined by matching samples to color coded RHS paint chips in ambient light at a north-facing window.

In the field, color hue of the youngest leaf and leaves older than the second youngest leaf on each shoot was assessed by visually comparing leaves to the second youngest leaf. If a leaf on a shoot appeared to be a different color from the second youngest leaf, it was clipped, brought back to the laboratory, and assessed using the RHS color chart.

Pigment Analyses

Anthocyanin and UV-absorbing compound content was assessed on the second youngest leaf of transplanted green- and red-leafed shoots at each site at 10 weeks post-transplantation. One 1 cm disc from the bottom of the second youngest leaf was excised, weighed, and extracted in cold methanol/HCl/water (90:1:1, vol). Extracts were placed in the dark for 20 minutes and centrifuged at 18,000 X G before being assayed spectrophotometrically with an Agilent Model 8453 Diode Array Spectrophotometer. Total anthocyanin content was calculated using the Beer-Lambert equation, assuming a corrected absorbance of $A_{529} - 0.288 A_{650}$ to compensate for the small overlap in absorbance by degraded chlorophylls at 529 nm (Sims and Gamon, 2002) and a molar absorbance coefficient for anthocyanin at 529 nm of 30,000 \text{ mol}^{-1} \text{ cm}^{-1}, where $\ell$ is light path length (Murray & Hackett, 1991).

Total UV-absorbing compounds were estimated from 10-fold dilutions of the anthocyanin extracts. The extracts were placed in the dark, centrifuged, and assayed
spectrophotometrically using the methods described above. Absorbances for UV absorbing compounds were measured at $A_{300}$ (UVB) and $A_{350}$ (UVA; Day, 1993).

**Statistics**

Descriptive statistics for leaf color data are reported for weeks 4, 10, 26, and 156 post-transplantation. Within-site comparisons were performed using a Pearson’s Chi-Square Test to assess whether there was a difference in the frequency of green-leafed shoots producing new leaf tissue with red coloration in green compared to red patches at week 4 post-transplantation.

Within-patch comparisons were made on pigment data from week 10 post-transplantation using an ANOVA. We assessed differences in anthocyanin and UV (UV-B and UV-A) absorbing compound content between green- and red-leafed shoots, as well as between patches (green versus red) for green- and red leafed shoots. All datasets met the assumptions of equal variance according to the Brown-Forsythe tests.

Statistical analyses were performed using JMP (Version 6.0, SAS Institute Inc.) with significance determined at the 95% probability level ($p < 0.05$). Values are reported as means and standard errors.

**Results**

**Color Measurements**

**Green-leafed shoots**

Most green-leafed shoots transplanted in green and red patches were producing new leaf tissue with red coloration following the summer solstice at 4 weeks post-transplantation. We observed new leaf tissue that was red (RHS color Red-Purple Group, N77A) on 76%
of all transplanted green-leafed shoots while all older leaves on all transplanted green-leafed shoots remained green (RHS color Yellow-Green Group, 146A; Figure 5.2). We also found that at all sites except Sugarloaf more green-leafed shoots transplanted in red patches, compared to green-leafed shoots transplanted in green patches, produced leaves with new leaf tissue that was red. At Sugarloaf, the shallowest site, there was no difference between red and green patches in the number of transplanted green-leafed shoots producing leaves with red coloration (Figure 5.3, Sugarloaf, $\chi^2 (1, N = 54) = 0.43$, $p = 0.5137$; Big Pine, $\chi^2 (1, N = 41) = 7.78$, $p = 0.0053$; Summerland, $\chi^2 (1, N = 39) = 6.21$, $p = 0.0127$; Cudjoe, $\chi^2 (1, N = 49) = 14.78$, $p < 0.001$).

The reddening of green-leafed shoots at 4 weeks post-transplantation was temporary. At the end of the summer (10 weeks post-transplantation) and around the winter solstice (26 weeks post-transplantation) green-leafed shoots in green and red patches had all green leaves (RHS Yellow-Green Group, 146A). Variations in leaf color were not measured for the next two years; however, directly before the summer solstice at 156 weeks post transplantation, we observed new leaf tissue with red coloration (RHS color Red-Purple Group, N77A) on 10% of the green-leafed shoots in green and red patches. We did not observe any green-leafed shoots with brown or yellow-green leaves during the study period.

**Red-leafed shoots**

Most transplanted red-leafed shoots had red leaves (Red-Purple Group, N77A) and were producing new leaves that were red (Red-Purple Group, N77A) at 4, 10, 26, and 156 weeks post-transplantation. At 4 and 10 weeks post-transplantation, some red-leafed shoots (1% and 21%, respectively) appeared unhealthy and had one or more leaves with
brown (Grey-Brown Group, N199B) or green-yellow coloration (Yellow-Green Group, 146A). In addition, at 10 and 26 weeks post-transplantation, some red-leafed shoots (10% and 7%, respectively) in green and red patches were producing new leaves that were a different color red than the rest of the leaves on the shoot (Greyed-Orange Group, 166A; Greyed-Purple Group, 187A or Red-Purple Group, 59A compared to Red-Purple Group, N77A).

**Pigment Analyses**

Red-leafed shoots had significantly higher anthocyanin concentrations than green-leafed shoots at 10 weeks post-transplantation (Table 5.1; Figure 5.4). Red-leafed shoots transplanted into red patches at Big Pine Key had higher concentrations of anthocyanins than red-leafed shoots transplanted into green patches at that same site. There were no significant differences in anthocyanin content of green-leafed shoots transplanted into green compared to red patches at each site (Table 5.2; Figure 5.4).

At 10 weeks post-transplantation, red-leafed shoots transplanted into green patches at all sites except Summerland had significantly higher concentrations of UV-B absorbing compounds than green-leafed shoots transplanted into green patches at the same sites (Table 5.1; Figure 5.5). Green-leafed shoots transplanted into the red patch at Big Pine Key had higher concentrations of UV-B absorbing compounds than green-leafed shoots in the green patch at that same site. There was no significant difference in UV-B absorbing compound content of red-leafed shoots transplanted into green compared to red patches at each site (Table 5.2; Figure 5.5).

We observed no trends in UV-A absorbing compound content of either green- or red-leafed shoots. Green- and red-leafed shoots transplanted into the red patch at Big
Pine Key had higher UV-A absorbing compound content than green- and red-leafed shoots transplanted into the green patch at the same site (Table 5.2; Figure 5.6).

**Discussion**

We performed reciprocal transplants of green- and red-leafed *T. testudinum* shoots among green patches with high self-shading and red patches with low self-shading located along a depth related gradient of light availability to test whether variations in light conditions affect anthocyanin accumulation and red coloration in leaves. We showed that transplanted green-leafed shoots produce new leaf tissue with red coloration during periods of high solar UV and visible light intensity (summer solstice; Figures 5.2, 5.3), but at other times, produce green leaves with low concentrations of anthocyanins (Table 5.2; Figure 5.4). We further demonstrated that shading and depth can influence the process since more green-leafed shoots in red patches with low compared to high self-shading turned red at all sites except our most shallow site, Sugarloaf, where we found an equal number of transplanted green-leafed shoots with red leaves in green and red patches (Figure 5.3). Our study is the first to prove that seagrasses can transiently produce red leaves in response to light conditions. Moreover, our results support our previous hypothesis that anthocyanin accumulation and red coloration in seagrass leaves serves a photo-protective role against UV-B since green-leafed shoots only produced red leaves during periods when light intensities, including UV-B, were higher than normal (Novak and Short, 2010).

Researchers have shown that seagrasses can produce other UV-absorbing compounds besides anthocyanins for protection against high light intensities and UV
radiation (Trocine et al. 1981; Dawson and Dennison 1996; Meng, 2008). At ten weeks post-transplantation, UV-B and UV-A absorbing compound content in green-leafed shoots in our study was higher than previously documented (4.5 AU g\(^{-1}\) fresh wt versus 2.1 AU g\(^{-1}\) fresh wt; Figures 5.5, 5.6; Novak and Short 2011). In addition, we observed no difference between green- and red-leafed shoots in red patches in UV-B absorbing compound content or green- and red-leafed shoots in green and red patches in UV-A absorbing compound content (Table 5.2; Figures 5.5, 5.6). Our results are in contrast to our previous study in which we showed that green-leafed shoots growing adjacent to red-leafed shoots produce lower concentrations of UV-B and UV-A absorbing compounds (Novak and Short, 2011). The results of the present study demonstrate that green-leafed T. testudinum shoots in high light environments can increase their photo-protective capacity by increasing anthocyanin content, as well as increasing UV-absorbing compound content.

Our study further demonstrates that T. testudinum in this system produces shoots with leaves that are permanently red. We showed that red-leafed shoots in green and red patches continuously produced uniformly red leaves for three years regardless of light conditions, confirming our previous suggestion that red-leafed shoots are a variant, a genetically differentiated form, of T. testudinum in this system (Novak and Short, in press). The occurrence of a red-leafed variant is important given that levels of ultraviolet radiation in tropical areas are increasing (Hegglin and Shepard, 2009) and a permanent sunscreen in leaves allows seagrasses to minimize the risk of photo-damage while remaining in shallow waters. Moreover, a permanent sunscreen in leaves could enhance reproduction and survival, a hypothesis supported by our observation of red-leafed shoots
surviving during a dieback of green-leafed shoots following a month of cloudless days with high light intensities in the summer of 2007 (Novak, pers. obs.). The role of genetics in the maintenance of red coloration in seagrass leaves, as well as its effects on plant fitness deserves further attention.

*Thalassia testudinum* growing at high light intensities in the clear waters of the lower Florida Keys produce green-leafed shoots that have the ability to transiently produce red coloration in leaves, as well as permanently red-leafed shoots. While our reciprocal transplant experiments indicate a genetic basis for the permanent expression of red coloration of leaves, further research is needed. Moreover, genetic investigation of permanently versus transiently red plants would yield insight into the resiliency of seagrass populations to global climate changes.
Literature Cited


Table 5.1. ANOVA results from within site comparisons of anthocyanin and UV absorbing (UV-B and UV-A) pigment content for green- and red- leafed shoots collected at 10 weeks post-transplantation. Shoot color affected anthocyanin and UV-B absorbing content: 1) red- leafed shoots had significantly higher concentrations of anthocyanins than green-leafed shoots in all patches and sites; 2) red-leafed shoots compared to green-leafed shoots in green patches at all sites except Summerland had significantly higher UV-B absorbing compounds. Significant values at P< 0.05 are in bold.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Site</th>
<th>Patch Color</th>
<th>F statistics</th>
<th>P value</th>
</tr>
</thead>
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<td>Green</td>
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<tr>
<td></td>
<td></td>
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<td>Big Pine</td>
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<td>Red</td>
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Table 5.2. ANOVA results from patch color comparison of anthocyanin and UV absorbing (UV-B and UV-A) pigment content for each shoot color collected at 10 weeks at each site. At Big Pine Key, patch color affected anthocyanin and UV-absorbing compound content in shoots. Significant values at $P<0.05$ are in bold.

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<tr>
<th>Pigment</th>
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<th>F statistics</th>
<th>P value</th>
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Figure 5.1. Location of study sites (red dots) in the Florida Keys, USA
Figure 5.2. Transplanted green-leafed shoots growing in the red patch at Big Pine Key at week 4 post-transplantation. Most green-leafed shoots were producing new leaf tissue that was red (RHS, Red Purple Group, N77A) while older leaves and leaf tissue remained green (Yellow-Green Group 146A).
Figure 5.3: Site and patch-level information on the number of transplanted green-leafed shoots with all green leaves and with new red leaf tissue at week 4 post-transplantation. Number in parentheses after site name is MLW depth (m). At Sugarloaf, the shallowest site, almost all green-leafed shoots in red and green patches produced new leaf tissue with red coloration while at the remaining sites more green-leafed shoots in red patches compared to green patches produced new leaf tissue with red coloration. Labels on columns indicate the total number of shoots found in each patch for a given category. Grey horizontal bars indicate significant differences (P<0.05) between red and green patches at a site.
Figure 5.4. Mean anthocyanin content in transplanted green- and red-leafed shoots in the green and red patch at each site at 10 weeks post-transplantation. Horizontal grey bar indicates significant differences in anthocyanin content between transplanted green- and red-leafed shoots within each transplant patch (P < 0.05; Mean ± SE). Water depth at MLW of each site is in parentheses.
Figure 5.5. Mean UV-B absorbing compound content in transplanted green- and red-leafed shoots in the green and red patch at each site at 10 weeks post-transplantation. Horizontal grey bars denote significant differences in UV-B absorbing compound content between transplanted green- and red-leafed shoots within each transplant patch (P< 0.05; Mean ± SE). Depth of each site is in parentheses.
Figure 5.6. Mean UV-A absorbing compound content in transplanted green- and red-leafed shoots in the green and red patch at each site at 10 weeks post-transplantation. Horizontal grey bar denotes significant differences in UV-A absorbing compound content between transplanted green- and red-leafed shoots within each transplant patch (P< 0.05; Mean ± SE). Depth of each site is in parentheses.
CHAPTER VI

SYNTHESIS

Seagrass meadows around the world are declining due to natural and anthropogenic stressors, including global climate change (Waycott et al. 2009), with fourteen percent of species at risk for extinction (Short et al. 2011). Recently, more attention has been given to identifying responses that offer resistance to stressors so that researchers can better manage seagrasses for resilience to environmental change (Björk et al. 2008). Leaf reddening, the expression of red coloration in leaves, is a well-documented response in terrestrial plants that has been shown to increase resilience to stress (Gould et al. 2002, 2004, 2008), but has been poorly understood in seagrasses. My dissertation is the first comprehensive study on the prevalence, causes, and function of leaf reddening in seagrasses.

Prevalence of leaf reddening in seagrasses

Leaf reddening, the expression of red coloration in leaves, is well documented in terrestrial plants. The phenomenon is often caused by the accumulation of anthocyanins, flavonoid pigments, which have been shown to function in photoprotection, osmoregulation, antioxidant activity, and/or defense against herbivory (see reviews, Chalker-Scott 1999; Gould et al. 2000, Gould et al. 2002). In terrestrial plants, leaves may be red throughout a plant’s life or they may transiently turn during growth, senescence, or in response to environmental stress. In 2006, I observed shoots of the
seagrass *Thalassia testudinum* with entirely red leaves growing in the clear, shallow waters (<0.5 m) of the lower Florida Keys. After a review of the literature, as well as an evaluation of herbaria specimens and photographs from SeagrassNet, a global monitoring program, I was led to believe that seagrass leaf reddening was more common than reflected in the literature. The few reports that existed on seagrasses with red coloration in leaves were from Australia (McMillan 1983; Abal, 1994; F.T. Short personal observation, 1996; Fyfe 2003, 2004), with anthocyanins reported in three species (McMillan 1983; Fyfe 2004). Furthermore, two potential functional roles of red coloration in seagrasses had been proposed: Abal (1994) suggested that pink coloration (due to the presence of anthocyanin-like pigmentation) in intertidal leaves of the seagrasses *Zostera capricorni* and *Halophila ovalis* was an adaptation to high ultraviolet (UV) levels while Fyfe (2004) suggested that red-bronze coloration produced by anthocyanins in *Z. capricorni* protected leaves from excess visible radiation.

In Chapter II (Novak and Short 2010), I use information from the literature, as well as surveys from many locations around the world to determine the prevalence of leaf reddening in seagrasses within the world’s six seagrass bioregions (Short et al 2007; bioregions). I show that red coloration in leaves occurs in 15 seagrass species from intertidal and shallow subtidal waters at 29 locations in the Tropical Atlantic, Tropical Indo-Pacific, and Temperate Southern Oceans bioregions. I also show that patterns of red pigmentation vary, ranging from small red spots on a leaf to leaves that are entirely red. The findings of this chapter are significant because they demonstrate that red coloration in leaves is common in seagrasses growing in clear, shallow waters with high light intensities, providing support for the theory that leaf reddening may serve a role in
photoprotection and justifying further research on this phenomenon. The chapter also raises the question of whether leaf reddening in seagrasses is a recent product of our changing environment or has been previously overlooked by researchers.

**Leaf reddening and its relation to anthocyanins and plant protection**

In Chapter III (Novak and Short 2011), I conduct a comparative study with green- and red-leafed *T. testudinum* in the lower Florida Keys to determine if (a) red coloration in leaves is caused by the accumulation of one or more anthocyanin molecules, (b) under high light, physiological and morphological characteristics are different between green- and red-leafed shoots, and (c) red coloration in leaves serves a protective function by acting as a sunscreen during periods of high light intensity. I chose to work in the lower Florida Keys because the occurrence of patches of green-leafed *T. testudinum* shoots growing adjacent to patches of red-leafed *T. testudinum* shoots at multiple sites provided me the unique opportunity to conduct comparative and manipulative studies with this species. The results of this chapter show that four anthocyanin molecules are responsible for red coloration in *T. testudinum* leaves and demonstrate that red leaves have higher concentrations of photo-protective pigments (anthocyanins and UV-absorbing compounds), higher effective quantum yields (ΔF/Fₘ) at midday, and are shorter, narrower, and weigh less than green leaves. In addition, I show that anthocyanin content in green-leafed *T. testudinum* shoots is positively related to ultraviolet (UV) and visible irradiance. The findings of this chapter are significant because they show that red coloration in *T. testudinum* is caused by high concentrations of anthocyanins, is associated with physiological and morphological attributes, and acts as a sunscreen since red leaves are able to maintain high effective quantum yields at high light intensities.
Moreover, the positive relationship between anthocyanin content in green-leaves and UV and visible irradiance provides the first evidence that red coloration in leaves is photo-induced in this species.

**UV-B induction of leaf reddening**

In Chapter IV (Novak and Short in press), I further investigate the role of UV and visible light in the induction of red coloration in *T. testudinum* leaves by assessing the responses of transplanted green-leafed and *in situ* red-leafed *T. testudinum* shoots to four light treatments. I show that exposure to high levels of ultraviolet-B (UV-B) induces anthocyanin accumulation and red coloration in leaves of green-leafed shoots, as well as contributes to the maintenance of high levels of photosynthesis in red-leafed shoots by potentially protecting plants from damage caused by ultraviolet-A (UV-A) or by the combination of UV-A and visible light. The findings from this study are the first to demonstrate the environmental induction of red coloration in seagrass leaves and show that red coloration in leaves can be used as indicator of UV-B exposure in seagrasses. In addition, I provide preliminary evidence that *T. testudinum* produces a variant with permanently red leaves, as evidenced by anthocyanins and red coloration in red-leafed shoots being unaffected by light levels. The potential of red-leafed seagrass variants is significant given that seagrasses expressing red coloration in leaves are prevalent in regions affected by global changes in UV levels (Hegglin and Shepard, 2009) and a permanent sunscreen in leaves allows seagrasses to minimize the risk of photo-damage while growing in shallow waters.
Transient and permanent leaf reddening

In Chapter V, I perform reciprocal transplants of green- and red-leafed *T. testudinum* shoots among patches with high and low self-shading located along a depth-related gradient of light availability and monitor them for three years to determine the plasticity of red coloration in green- and red-leafed shoots. The results of this chapter confirm the findings of Chapter IV that anthocyanin accumulation and the expression of red coloration can be temporarily photo-induced in green leaves during periods of high light intensities, as well as support my hypothesis that *T. testudinum* also produces a variant in this system with leaves that are permanently red. My study is the first to demonstrate that seagrasses are similar to terrestrial plants because they can both transiently and permanently express red coloration in leaves. The extent to which other seagrass populations transiently and/or permanently express red coloration in leaves is unknown although it has been suggested that some *Halophila ovalis* populations in Thailand produce a red-leafed variant (A. Prathnep, pers. comm). Additional studies are needed to elucidate the transient versus permanent nature of reddening in seagrasses and determine if reddening affects fitness.

Conclusion

The findings from this dissertation provide the first in-depth analysis on the prevalence and eco-physiology of the expression of red coloration in seagrass leaves. I show that the expression of red coloration in *T. testudinum* leaves is caused by the accumulation of anthocyanins, acts as a sunscreen during periods of high UV and visible light intensities, can be an indicator of UV-B exposure, and is permanent in some plants. Based on my results, I propose that the prevalence of seagrasses with red leaves in clear, shallow
waters with high light intensities may be due to enhanced UV-B levels and may increase seagrass resilience to changes in atmospheric UV-B levels by acting as a sunscreen and protecting photosynthetic mechanisms from damage. Additional studies are needed to identify the mechanisms by which leaf reddening protects plants, determine whether leaf reddening affects fitness, and elucidate how long this phenomenon has been occurring in seagrasses.
**Literature Cited**


BIBLIOGRAPHY


150
Takahashi, A., Takeda K. and T. Ohnishi. 1991. Light-induced anthocyanin reduces the extent of damage to DNA in UV-irradiated 

Terrados, J. and C.M. Duarte. 2000. Experimental evidence of reduced particle Exper


