RUPPIA MARITIMA SEED AND *THALASSIA TESTUDINUM* SEEDLING RESPONSES TO FLUCTUATIONS IN SALINITY AND AMMONIUM

Amanda E. Kahn

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Approved by

Advisory Committee

Courtney Hackney

Larry Cahoon

Michael Durako
Chair

Accepted by

_____
Dean, Graduate School

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ABSTRACT

Six species of seagrasses are present in Florida Bay ranging from stenohaline to euryhaline species. The dominant species, *Thalassia testudinum* Banks ex. König, has optimum growth around marine salinity. The second species examined in this study was *Ruppia maritima* L, which tends to grow near fresh water outflows. Although many studies have examined the responses of mature plants to environmental stresses, this study examined the impacts on seeds and seedlings. As the focus behind the study was altered water flow into Florida Bay, it examined hypo- and hyper-salinity, as well as depleted and elevated ammonium concentrations. This study was conducted in mesocosms on material collected in Florida Bay. *R. maritima* seed germination and *T. testudinum* seedling survival, growth, photosynthesis and osmolality were investigated over a two-year study. Year one focused on direct introductions into hyper- and hypo-saline conditions and year two examined the impact of gradual increase/decrease in salinity as well as the addition of ammonium. *R. maritima* seeds did not germinate above 28 PSU and the highest percent germination occurred between 0 and 10 PSU. Elevated levels of ammonium decreased germination. Hypo-saline conditions were, however, detrimental to the fitness of *T. testudinum* seedlings, as were hyper-saline conditions. Plants at 0 and 70 PSU showed 100% mortality and decreased survival in the 60, 50 and 10 PSU treatments. Increased levels of ammonium decreased growth in the lower salinity treatments. Plants grown around 30-40 PSU showed the greatest growth (i.e. most productivity). Measurements of quantum yields as well as relative electron transport rate using PAM fluorometry showed a

decrease in photosynthetic performance on either side of this 30-40 PSU optimum. Tissue osmolality increased significantly with increased salinity and tissue remained distinctly and consistently hyperosmotic to the media. Results suggest that a change in fresh water flow as well as possible increase in ammonium may negatively impact the ability of *T. testudinum* seedlings to establish and possibly cause a shift in the floral composition of Florida Bay, favoring euryhaline species such as *Ruppia maritima*.

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CHAPTER 1: INTRODUCTION

The importance of seagrass beds in estuarine and shallow coastal marine environments has been intensively researched and documented over the last couple of decades. Seagrass bed die-off episodes around the world prompted scientists to examine the ecological role and function of submerged aquatic vegetation (SAV) habitats. In Florida Bay, commercially and recreationally important fishes, shrimp and other decapod crustaceans utilize seagrass beds as nursery habitat (Zieman et al., 1989; Chester and Thayer, 1990; Dennison et al., 1993; Erhardt and Legault, 1999; Thayer et al., 2000). Seagrass beds are also vital in substrate stabilization, both by preventing sediment resuspension (Gacia, 1999) and promoting deposition (Koch, 1999) and bio-filtration, their decline being linked with decreased water quality (EPA, 1990). Hence, a decline in fitness of the seagrass beds would lead to numerous ecological repercussions. Many stresses and disturbances impact the fitness of seagrasses both seasonally and annually, whether by human influence on habitat and water quality or naturally- occurring variations in the environment (Gunderson, 2001). Since Florida Bay seagrass beds are the dominant biological community and serve as the “through- route” from Everglades fresh water inflow to the Florida Keys marine ecosystems, changes in seagrass habitat characteristics in Florida Bay will conceivably lead to habitat changes in the adjoining ecosystems.

Florida Bay has been subjected to many hydrological changes over the last century. In the late 19th century, the first major plan to reroute water from Lake Okeechobee to the west, east and south via canals was put into action as a

means to drain the Everglades (Steinman et al., 2002). Not realizing the major sources of water to the Everglades were groundwater and precipitation, this plan did not fulfill its purpose and only served to increase the need for extended redirection of the water via the building of more canals, levees and pumps over the years to come. Increased drainage for agriculture, such as sugar plantations, in the early 20th century drove the change in hydrology even further. By 1915, construction of the Tamiami Trail and four major canals that cut directly through the Everglades prevented southward flow of water from the Everglades into Northern Florida Bay. In the mid-to-late 1900's, efforts to restore the Everglades were proposed and levees and pumps were constructed and rivers channelized, restoring flow from the Everglades to the southern Gulf of Mexico, Atlantic Ocean and Florida Bay (Sklar et al., 2002). Today, a new plan proposes to redirect water flow (diverting it from Biscayne Bay to the West) in an effort to increase freshwater input to the Everglades and Florida Bay via a new system of canals and pumps (Irlandi et al., 2002).

The increase in water flow brings with it a change in water quality for Florida Bay. Altering the volume and path of the fresh water entering the Bay will directly affect salinity, total nitrogen, phosphorous, sulfide and other chemical and biological parameters (Brand, 2001). It was changes to some of these parameters that were thought to have contributed to the mass mortality of *Thalassia testudinum* Banks ex König in Florida Bay, in 1987 (Robblee et al., 1991). The pattern of changes in water chemistry upon altering the water flow is difficult to predict due to the complex hydrology of the Bay. Florida Bay is not one large

basin, but an intricate series of small, shallow basins surrounded by mud banks and mangrove islands with an average depth of 2m (Lee et al., 2002). Water from Florida Bay flows through cuts in the Florida Keys possibly affecting coral reef ecosystems. Thus, seagrass beds in Florida Bay serve as a buffering zone between the fresh and sea water systems (Durako et al., 2002). The tolerance levels of seagrass species in this habitat need to be understood to determine what effects the proposed changes in hydrology in the Bay may have on the fitness and survival of these organisms.

The seagrass species in Florida Bay tolerate many stresses, but it is unclear as to how rapidly they adapt to changes in environmental conditions or what are their tolerance limits, especially at vulnerable germination and seedling stages. There are six species found in the Bay: *Ruppia maritima* L., *Halophila decipiens* Ostenfeld, *Halophila engelmanni* Ascherson, *Syringodium filiforme* Kuetzing, *Halodule wrightii* Ascherson and the predominant species, *Thalassia testudinum*. Their abundance varies throughout the Bay, based on environmental conditions (Fourqurean et al., 2002). For example, *H. wrightii* is generally euryhaline, whereas *T. testudinum* and *S. filiforme* prefer intermediate salinity ranges with a narrow range of tolerance between 20-40 PSU (Montague and Ley, 1993). In contrast, *R. maritima* generally thrives in areas of lower salinity near fresh water inputs (Fourqurean et al., 2002). *T. testudinum* was chosen for this study due to its present dominance in Florida Bay and marine-salinity optimum (Zieman, 1975). *T. testudinum* displays the growth strategy characteristics of a phalanx species; slow to establish, but not disturbed by short-term variations in the environment. *R.*

maritima was chosen in order to examine a plant at the other end of the salinity-optimum spectrum that prefers more hyposaline conditions. *R. maritima* also displays the characteristics of a more ephemeral species.

Thalassia testudinum Physiology

Thalassia testudinum exhibits physiological and anatomical adaptations to live in a submerged marine environment, many of which play a critical role in osmoregulation (Jagels,1973). The plasmalemma of the epidermal cells is greatly invaginated, forming pockets of cytoplasm in which most of the epidermal cell mitochondria are located. Both epidermal and mesophyll mitochondria contain cristae, which are convolutions of the mitochondrial envelope inner membrane that protrude into the interior mitochondrial matrix. The difference in size and shape of the cristae between the mesophyll and epidermal cells, however, suggests they undergo different levels of activity. The concentrated presence of cristae in mitochondria in the invaginated plasmalemma of epidermal cells may be evidence of transmembrane ion movement (osmoregulation) requiring ATP (Jagels, 1973).

T. testudinum lacks separate organs specialized for salt secretion so osmoregulation is not mediated through vesicles or microvacuoles. The sum of ultrastructural evidence supports the presumption that leaf epidermal cells are proficient in osmoregulation. There are no plasmodesmata connecting the mesophyll cells to epidermal cells or interconnecting the epidermal cells. Hence, symplastic transport may not be occurring between mature cells. This suggests considerable apoplastic transport, directly through the plasmalemma, which may

occur across young, thinner cell wall membranes and also possibly in the older, less membrane-permeable cell walls by the continual production of new and shedding of old cell layers. The plasmalemma membrane of *T. testudinum* is symmetrical suggesting two-way transport of ions. This may apply not only to salt (NaCl), but possibly other cations and anions, such as those involved in ion exchange during bicarbonate uptake. It is likely that plasmalemma-controlled ion transport allows for salt to be secreted as well as secluded at the plasmalemma boundary (Jagels, 1983). Symplastic backflow of brine solution does not occur due to the lack of plasmodesmata (Jagels, 1973).

Ruppia maritima Physiology

The adaptations in *Ruppia maritima* leaves differ from those in *T. testudinum*. Most likely, this is due to *R. maritima* frequently occurring in environments of lower salinity. *R. maritima* has been the topic of many debates as to whether or not it qualifies as a true seagrass. Husband and Hickman (1985) examined *R. maritima* growth rates in various environments and found an increase in growth rate in saline vs. freshwater conditions. Lazar and Dawes (1991) also showed increased photosynthetic productivity at sites with higher salinities than at a freshwater site. *R. maritima* is found in Florida Bay primarily near freshwater inputs and therefore may be the most greatly affected by fluctuations in salinity. It will be treated as a seagrass for the purpose of this study. However, the fact that *R. maritima* is most common in the lower-salinity portion of the Bay is most probably due to its distinctive physiological and anatomical adaptations.

The mitochondria of *R. maritima* epidermal cells are not located in cytoplasmic pockets due to diminished invagination of the plasmalemma. As the plasmalemma is directly responsible for NaCl ion transport, the degree of development of the mitochondrial plasmalemma transport system is the result of the salinity of the environment. The low-salinity environments in which *R. maritima* is frequently found require a lesser degree of development of the transport system than in *T. testudinum*, which prefers a more stenohaline marine environment. For *R. maritima,* osmoregulation requires significant metabolic changes. Hydrogen ions and sugar accumulate, protein is synthesized and structural adaptations occur as well. Increased salinity causes an increase in plasmalemma area and number of chloroplasts, thicker cell walls and a decrease in central vacuole size (Jagels and Barnabas, 1989). Andrea Wimmers (1998, unpublished) showed that extreme fluctuations in salinity with an amplitude of 30 PSU are harmful to *R. maritima* and growth is negatively impacted with exposure to high salinity. Combined with the effect salinity has on seed germination, extreme fluctuations in salinity in Florida Bay will likely be detrimental to *R. maritima* fitness.

Importance to Florida Bay

It is vital to understand that altering water flow patterns in Florida Bay implies alterations in magnitude and timing of fresh water input, which results in fluctuations in salinity and nutrient concentrations. It is important to investigate possible responses that dominant organisms might display that are indicative of exposure to fluctuations in salinity and nutrients, such as reduced seed

germination or seedling survival. Also, it is important to examine which extreme environmental changes or combination of changes the young seagrasses can tolerate. Hopefully this information may prevent anthropogenic alterations in environmental conditions that may push conditions beyond seagrass tolerances so that the seagrasses in Florida Bay will not be destroyed by hydrologic restoration.

This study examined response characteristics of immature seagrasses collected from Florida Bay (Fig. 1) when exposed to altered environmental conditions. Specifically, *R. maritima* seed germination and *T. testudinum* seedling growth and survival were examined under experimental conditions subjecting them to varying stressors and levels of stress. The effect of salinity as well as varying salinity with increased nitrogen levels was the focus of this research. Growth, respiration, photosynthetic rates and tissue osmolality and were measured in response to experimental manipulations in salinity and nitrogen levels. This study investigated the possible impacts a change in water chemistry in Florida Bay may have on the recruitment of seedlings of a marine (*Thalassia testudinum*) and estuarine (*Ruppia maritima*) seagrass species. The results were quantified in order to be applied to a physiologically-based unit model for seagrass seedlings in Florida Bay.

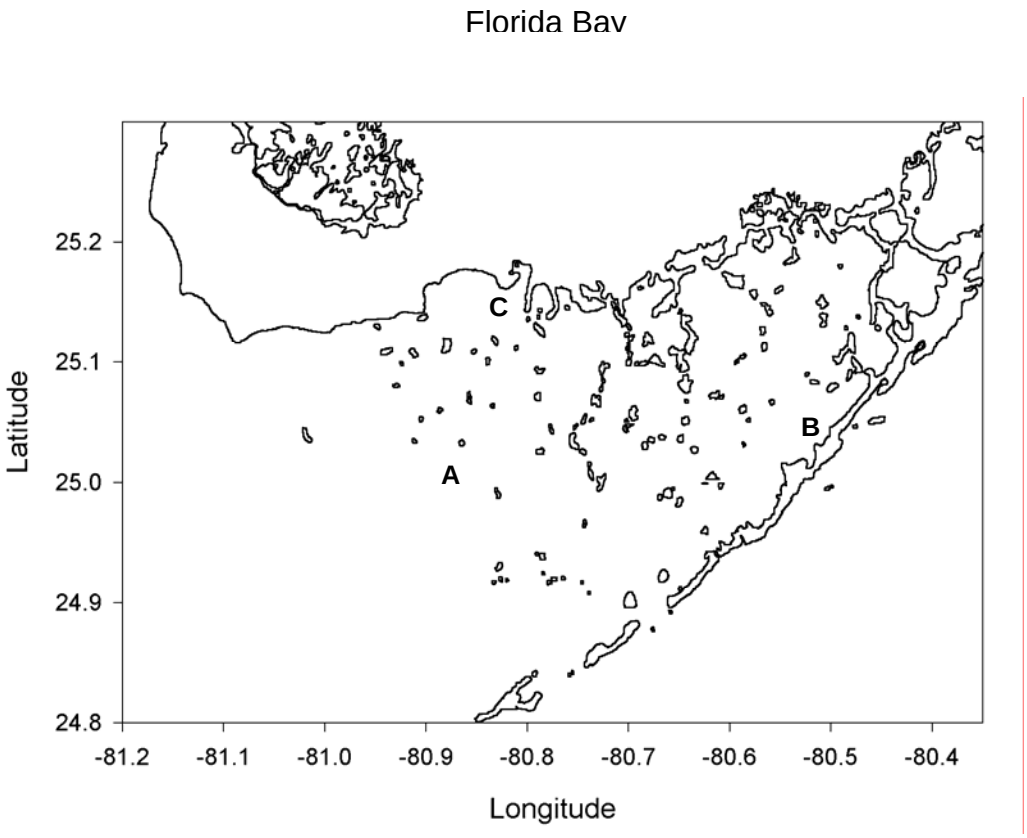

Fig. 1. Map of Florida Bay with collection sites of *Thalassia testudinum* seedlings: (A) Little Rabbit Key 24° 59.2'N, 80° 49.5' W and (B) Harry Harris Park, Tavernier Key and *Ruppia maritima* seeds(C) Garfield Bight 25° 09.8' N, 80° 48.5' W

CHAPTER 2: THE EFFECT OF SALINITY AND AMMONIUM ON SEED GERMINATION IN *RUPPIA MARITIMA* L. FROM FLORIDA BAY

1. Introduction

Ruppia maritima L. (Widgeon grass) is one of six species of submerged angiosperms found in Florida Bay, Florida, USA. Although *R. maritima* has the widest salinity tolerance of any submerged angiosperm (Kantrud, 1991), within Florida Bay this species is most common in euryhaline environments of the northern basins near fresh-water inflows from the Everglades (Fourqurean et al., 2002). However, populations of *R. maritima* in these areas are ephemeral and generally display seasonal die-backs (Montague and Ley, 1993). Recovery from seasonal die-backs is thought to require recruitment via seed germination (Kantrud, 1991).

Germination in *Ruppia spp.* is environmentally regulated (Brock, 1982a; Koch and Seeliger, 1988). Koch and Dawes (1991) found that salinity, rather than temperature, is the prime factor in ecotypic variation in *R. maritima* seed germination between northern and southern populations on the eastern coast of the United States and that seeds from Florida had higher germination at lower salinities. Using a statistical model based on water quality and seagrass distribution data, Fourqurean et al. (2003) recently predicted that an increase in seasonal freshwater delivery to Florida Bay, such as that proposed in the Comprehensive Everglades Restoration Program (CERP), would result in an expansion of seagrass beds dominated by euryhaline species such as *R. maritima* and *Halodule wrightii* Ascherson. However, there is some concern that the

proposed increases in freshwater inflow, may, in addition to lowering salinities, also result in an increase in the total nitrogen pool for central and eastern Florida Bay (Rudnick et al., 1999) and have negative consequences for the submerged angiosperms along the northern euryhaline margins of Florida Bay (Lapointe et al., 2002; Lapointe and Barile, 2004). In this study we examined germination of stratified and unstratified *R. maritima* seeds from Florida Bay under two types of salinity manipulations: 1) direct exposure and 2) exposure after incremental increases. The latter salinity experiment also examined the effect of increased nitrogen, in the form of ammonium. Ammonium levels chosen were based on nutrient concentrations found in northern Florida Bay (Lapointe et al., 2002).

2. Methods

2.1 Seed collection

Ruppia maritima seeds were collected in Garfield Bight in north-central Florida Bay (25° 09.8' N, 80° 48.5' W) in early August 2002 (year 1) and 2003 (year 2). The location of the seed collections was based on accessibility and the assumption that the population in Garfield Bight was representative of other *R. maritima* populations across the northern Bay. In 2002, seeds were collected by sieving (1 and 2 mm sieves) surface sediments or by direct collection from reproductive shoots. In 2003, seeds were only collected from reproductive shoots. Seeds were kept in ambient seawater and transported up to the Center for Marine Science, Wilmington, North Carolina. Within two days of collection, seeds were rinsed with DI water and placed in Magenta GA-7 flasks with 200 ml of autoclaved

DI water, 15 seeds were haphazardly selected for each flask. The flasks were stored in a dark growth chamber at 10°C (year one) or 6°C (year 2) for 2 weeks to undergo stratification, otherwise known as vernalization (Seeliger et al., 1984; Koch and Dawes, 1991).

2.2 Direct salinity treatment (Year 1)

After stratification, individual seeds were randomly placed into screw-cap test tubes containing 100 ml autoclaved Instant Ocean-based media at one of eight treatment salinities (0-70 PSU, increments of 10 PSU). Twenty replicate tubes were used for each salinity treatment, 10 were placed in a growth chamber with 12:12 light:dark (L:D) cycle (average PPFD of 385 μmole cm^{-2} s^{-1}), the remaining 10 placed in a growth chamber with 24 h darkness, and both growth chambers were kept at 24°C. Germination was monitored weekly.

A second set of 144 *R. maritima* seeds were rinsed with DI water then individually planted (no stratification) into aragonite shell hash from Florida in plastic nursery six-pack containers (5 cm x 5 cm x 8 cm). Three 30-l aquarium tanks were set at each salinity treatment (0-70 PSU, increments of 10 PSU) using Instant-Ocean and one six-pack was placed in each tank (n=18). The aquaria were located in a temperature-controlled (23 – 29°C) greenhouse with light-supplemented conditions (eight 500 W metal-halide lamps, 14:10 L:D, yielding an average mid-day PPFD of 1267 μmole cm^{-2} s^{-1}). The additional 2 hours of day length in the greenhouse, compared with the chambers, coincided with ambient dawn and dusk, at the start of the experiments. The higher light levels in the

greenhouse partially compensated for the fact that seeds were buried and in a 30-l tank. Germination was monitored weekly.

2.3 Salinity adjustment and ammonium (Year 2)

Following stratification, individual seeds were put into screw-cap test tubes containing 100 ml of DI water (0 PSU) in a growth chamber at 24°C, 12:12 L:D (average PPFD of 385 μmole cm^{-2} s^{-1}). After three days at 0 PSU, the media in all but 10 of the tubes was replaced with water of salinity 2 PSU. Ten seeds remained at 0 PSU for the rest of the experimental period. Every three days, the salinity in the tubes was raised by 2 PSU and at every salinity increment of 10 PSU, ten seedlings were maintained. This increment was chosen to emulate field conditions (Baskin and Baskin, 1998). Upon completion of the salinity-increase adjustments, ten seedlings were at each of the following salinities: 0, 10, 20, 30, 40, 50, 60 and 70 PSU. This salinity-adjustment regime was repeated for each of three ammonium levels: 0μM, 10μM and 20μM (n=10 for each salinity at each ammonium treatment). Ammonium was added in the form of ammonium chloride and levels were monitored when each media-change occurred. Salinity media were made with DI water and Instant-Ocean salts and medium in each tube was changed every three days, whether a change in salinity occurred or not. The pH was monitored before and after media changes and remained between 8.3 and 8.5. Germination was monitored weekly.

3. Results

3.1. Direct salinity treatment (Year 1)

Overall germination of *R. maritima* seeds was low in the test-tube scale experiment. In the light:dark (L:D) chamber, germination occurred in the 0, 10 and 20 PSU treatments (Fig. 2). On each of days 15, 20, 31 and 34, a single seed in the 0 PSU L:D treatment had germinated (40% total germination for that salinity treatment). By day 24, one seed in the 20 PSU L:D treatment had germinated and by day 38, one seed in the 10 PSU L:D treatment had germinated. Only one seed germinated in the 10 PSU 24h-dark treatment, after 143 days. No other seeds germinated in the dark treatment over the course of the monitoring period (5 months), nor did the non-stratified *R. maritima* seeds planted directly into sediments in the aquaria.

3.2 Salinity adjustment and ammonium (Year 2)

After 121 days post-stratification, 6.25 % out of the total 240 seeds had germinated (Fig. 2). In the 0µM ammonium treatment, 11.25% of the 80 seeds germinated. Only 3.75% germination occurred in both the 10 and 20µM ammonium treatments. The number of days post-stratification to germination ranged from 9 to 121 (Table 1). Some germination occurred during the up-ramping process at non-treatment salinities. Thus, the salinity of the media at the time at which the seed germinated was recorded. Germination occurred at salinities ranging from 0 to 28 PSU, with almost 70% of the observed germination (10 of the

15 seeds) occurring between salinities of 0 and 10 PSU. No seeds germinated above 28 PSU.

4. Discussion

Germination of *Ruppia maritima* seeds was greatest at the lowest salinities (0 and 10 PSU) in both years' experiments. However, germination was observed at higher salinities in the second year's experiments (up to 28 PSU) when seeds were exposed to incremental increases in salinity that were more indicative of field conditions (Baskin and Baskin, 1998). These results provide a mechanistic explanation for the prediction that a reduction in salinity in Florida Bay could result in an expansion of *R. maritima* distribution in northern Florida Bay (Fourqurean et al., 2003). Although overall germination was low in both sets of experiments, percent germination was comparable to previous shorter-term (< one year) germination results (Seeliger et al., 1984). Germination did not occur without stratification and occurred almost exclusively in L:D conditions. The former observation suggests an extended-dormancy requirement, beyond a year, for germination, while the latter observation suggests a signal involving a transition from darkness to light, such as movement to the surface following sediment disturbance, may also be involved in germination.

The lower overall germination observed in year one may have been due to the relatively high, but more ecologically relevant (Baskin and Baskin, 1998), stratification temperature of 10°C. This relatively high temperature was chosen because it has previously been suggested that the best germination of *R.*

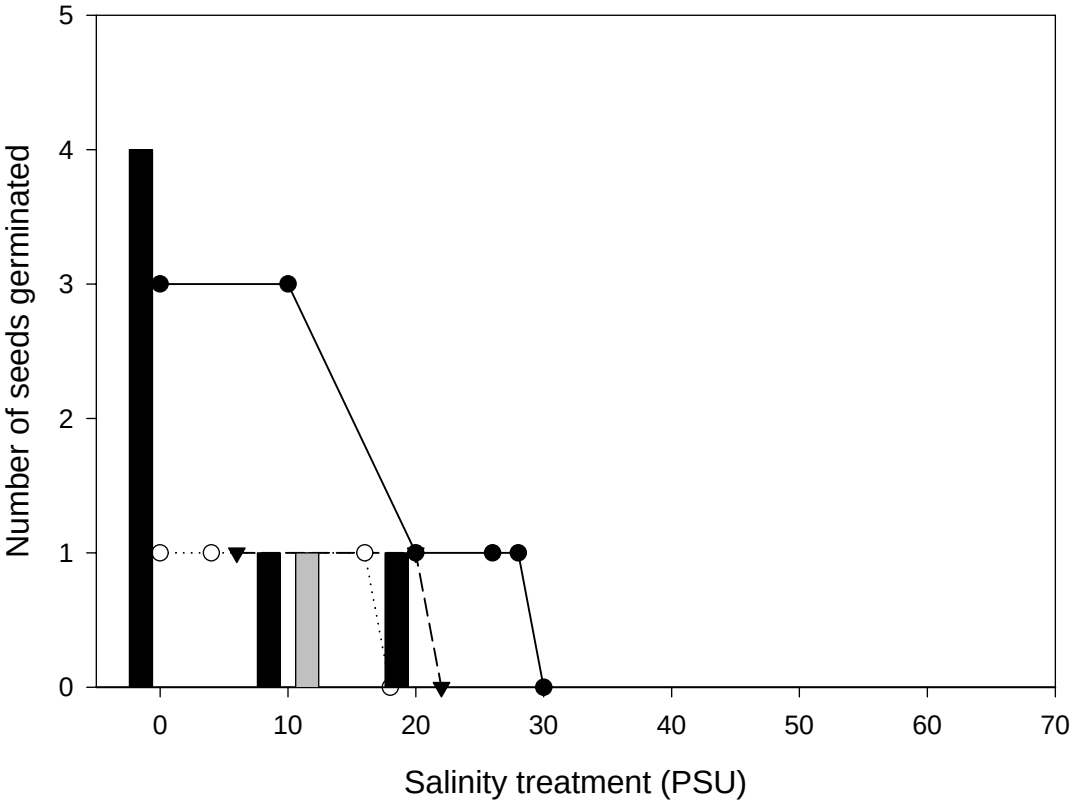

Fig 2. Salinity at which *Ruppia maritima* seeds germinated during the course of the experiment for year one in 12:12 L:D conditions (black bars) and 24hr dark (grey bars) and year two at the three ammonium treatments: 0μM (black circle), 10μM (white circle), and 20μM (black triangle).

Table 1. Number of *Ruppia maritima* seeds that germinated, out of a total n=10, at each salinity and ammonium treatment level (number of days post-stratification when germination occurred).

Salinity	0 µM NH_4^+	10µM NH_4^+	20µM NH_4^+
0	3 (9, 33, 91)	1 (100)	0
4	0	1 (6)	0
6	0	0	1 (9)
10	3 (18, 24, 84)	0	1 (15)
16	0	1 (27)	0
20	1 (121)	0	1 (30)
26	1 (40)	0	0
28	1 (44)	0	0

maritima seeds occurs at temperatures corresponding to the lowest winter temperatures at the site of collection (Seeliger et al., 1984). However, using a stratification temperature of 6°C, similar to that previously used by Seeliger et al. (1984) and Koch and Dawes (1991), but lower than winter minimum temperatures in Florida Bay, resulted in increased, albeit still low, germination rates. Other environmental or water-quality factors, such as dissolved-oxygen or nutrient levels (Moore et al., 1993; Brenchley and Probert, 1998), might also affect germination in *R. maritima* from this region. In this regard, we observed that germination decreased with increases in ammonium. Other possible synergistic effects between low salinity and changes in additional water chemistry parameters may be important for resource managers to consider.

Previous studies of *R. maritima* germination (Koch and Dawes, 1991; Koch and Seeliger, 1988) reported higher percent germination for seeds collected from sediment cores. It is unknown how long seeds from cores have been in the sediment, but their presence in sediment cores suggests the existence of a seed bank as in other submerged angiosperm populations (Brock, 1982b; Inglis, 2000). Seeds from Florida Bay, like other southern populations (Seliger et al., 1984; Koch and Seeliger, 1988), may require a period of dormancy in a seed bank to ensure greater germination in addition to increasing the ability to re-establish a population after disturbance events (Orth et al., 2000). For an ephemeral species like *R. maritima*, in a variable environment such as Florida Bay, the presence of a seed bank might help to explain the dramatic temporal population fluctuations observed in this system (Montague and Ley, 1993).

CHAPTER 3: FLORIDA BAY *THALASSIA TESTUDINUM* BANKS EX KÖNIG SEEDLING RESPONSES TO CHANGES IN SALINITY AND NITROGEN LEVELS

1. Introduction

Although seagrasses are clonal organisms, sexual reproduction plays a key role in increased genetic variation (Laushman, 1993; Schleuter and Guttman, 1998; Williams and Orth, 1998). Seedlings also increase the rate of re-establishment and recovery from stochastic disturbances (Whitfield et al., 2004). *Thalassia testudinum* displays the growth strategy characteristics of a phalanx species; slow to establish, but not disturbed by short-term variations in the environment. *T. testudinum* was previously thought to have been solely clonal. However, sexual reproduction and the release and survival of fruits and seeds are important for the expansion and persistence of seagrass beds (Kaldy and Dunton, 1999). Changes in the environment can greatly affect sexual reproduction in seagrasses (Orth and Moore, 1983; Koch and Dawes, 1991). The amplitude and duration of salinity change may affect seed viability and the vitality of young plants based on whether their physiological and anatomical adaptations are developed enough to acclimate to these changes.

This study considered the possible impacts a change in water chemistry in Florida Bay may have on the recruitment of seedlings of the dominant marine seagrass species. *T. testudinum* was chosen for this study due to its present dominance in Florida Bay and marine-salinity optimum (Zieman, 1975). Specifically, *T. testudinum* seedling growth and survival were evaluated under

increased and decreased levels of salinity and ammonium. Ammonium was chosen as the manipulated nutrient due to its expected fluctuations in the water column in response to changing freshwater flow (Rudnick et al., 1999). Fruit and seedlings of *T. testudinum* draw their nutrients from the water column before establishing themselves in the sediment. As submerged macrophytes, seagrasses are more susceptible to changes in water ammonium levels as nitrogen uptake cannot be regulated through the leaves. The response of seedlings to variations in salinity and ammonium will be quantified for application in a physiologically-based unit model for seagrass seedlings in Florida Bay.

The following null hypotheses were addressed in this study:

1) Direct introduction into hypo- and hyper-saline conditions does not affect *T. testudinum* seedling survival, growth, respiration, photosynthetic rates, or tissue osmolality.
2) Gradual introduction of seedlings into hyper- and hypo-saline conditions does not affect the fitness of *T. testudinum* seedlings
3) Physiological responses of *T. testudinum* seedlings to varying levels of salinity are not affected by increased or decreased levels of nitrogen.

2. Methods

2.1. Collection

2.1.1. Year 1

Thalassia testudinum seedlings were collected on August 13th, 2002 from the mangrove fringe on Little Rabbit Key in south- central Florida Bay. The seedlings

were kept in ambient seawater and transported to the Center for Marine Science, Wilmington, North Carolina on August 14th.

2.1.2. Year 2

Thalassia testudinum seedlings were collected in Florida on August 3rd, 2003. The seedlings were collected from fruits found floating in rack lines along Harry Harris Park, Tavernier Key. Upon returning to UNCW Center for Marine Science they were placed in a fiberglass mesocosm in the greenhouse for one week at salinity 30 PSU to allow seedlings to acclimatize to light and temperature conditions.

2.2. Year 1 salinity: Flask-scale experimental design

Thalassia testudinum seedlings were acclimated to 30°C, 29 PSU medium salt water for 24 h. Following acclimation, 80 seedlings were transferred to Magenta GA-7© flasks containing 200 ml of autoclaved Instant Ocean©-based medium, one seedling per flask, at salinities of 0, 10, 20, 30, 40, 50, 60 and 70 PSU. Ten seedlings were chosen at random for each of the salinity treatments. Flasks were incubated at 24°C in a growth chamber under 12:12 L:D photoperiod (average PPFD of 385 μmole cm^{-2} s^{-1}) for three months.

2.2.1. Morphometric measurements

Morphometric measurements were made weekly for the duration of the experiment. Individual roots and shoots were measured and the total lengths calculated. Average blade width was measured and multiplied by total blade length to calculate shoot leaf area. For the plants that survived the three-month period, remaining roots and shoots were dried and weighed upon experiment termination.

2.2.2 Fluorescence

Apparent and maximum quantum yields of photosystem II of the seedlings were measured once a month for the three-month duration of the experiment. A Walz Mini-PAM© (pulse amplitude modulated) fluorometer was used with the small, dark-adapting leaf clips (DLC-8). An apparent yield measurement (ΔF/Fm' = difference between initial and saturation pulse fluorescence/ fluorescence at saturation pulse for light acclimated leaves) was taken following a saturating light pulse and the clip shutter closed to allow the shoot to dark-acclimate. After five minutes, the shutter was opened and a maximum quantum yield measurement was taken (Fv/Fm = difference between maximal and initial fluorescence/ maximal fluorescence for dark acclimated leaves).

2.2.3. Osmolality

At the end of the experiment, osmolality of the leaf tissue was measured using a Wescor VAPRO Vapor Pressure Osmometer Model 5520©. The osmometer measured the total concentration of dissolved particles (osmolality) via a

measurement of vapor-point depression. When osmolality of solid samples such as leaf tissue is measured, a time delay must be determined to allow tissue to reach equilibrium. To determine the appropriate time-delay, fresh tissue samples from seedlings of the same age grown in ambient seawater were used and osmolality measured every two minutes for 30 minutes. After ten minutes, there were no significant differences between measurements (data not shown).

Seedling leaf tissue was cut underwater with a ¼ inch diameter hole-punch. When the tissue width was too small to cover the osmometer sample holder, two half-circle pieces were used. To determine the comparative osmolality of leaf tissue relative to the treatment seawater media, osmolality measurements were made on 10µl of media for each salinity treatment.

2.3. Year 1 salinity: Aquarium-scale experimental design

Following acclimation, an additional 96 *Thalassia testudinum* seedlings were planted in aragonite shell hash in plastic nursery six-pack containers and placed in 30-liter aquarium tanks, two tanks per salinity treatment. The aquaria were located in a temperature-controlled (23-29°C) greenhouse with light-supplemented conditions (eight 500 w metal-halide lamps, 14:10 L:D, yielding an average mid-day PPFD of 1267 μmole cm^{-2} s^{-1}). Blade number, length and width were measured weekly and total leaf area calculated. Remaining plant material was collected at the end of the experiment period and separated into seed, root and leaf material, dried for two days at 60°C, and weighed.

2.4. Year 2 salinity and ammonium: Aquarium-scale experimental design

Following transport to UNCW, initial measurements of root length and blade length and width were made of all seedlings before they were planted in aragonite shell hash in individual 2x2x4 inch plastic pots. Planted seedlings were placed in 30-liter aquarium tanks in the greenhouse with overhead lights providing ~10% increased daily PPFD (conditions same as in year one). Seedlings were maintained at a salinity of 30 PSU for one week to allow acclimatization to new conditions. After seven days, ten seedlings remained in the 30 PSU treatment and the remaining tank salinities were changed by 2 PSU every three days. Instant Ocean© salts were used to increase salinity and DI water was added to decrease the salinity. The up- and down-ramping was performed until ten seedlings were at each of the following salinity treatments: 0, 10, 20, 30, 40, 50, 60 and 70 PSU. For each of these salinity treatments, there were three ammonium treatments: 0, 10 and 20 μM NH_4^+. Ammonium was added in the form of NH_4Cl and levels monitored twice a week using the Koroleff reagent wet chemistry technique (Koroleff, 1969).

2.4.1. Morphometric measurements

Morphometric characteristics were measured weekly for each seedling. These included blade lengths and width from which total leaf area was calculated and a measurement of the length of the leaf that was brown. Certain anomalous characteristics were also noted such as red-colored blade tips and blade twisting.

2.4.2. Fluorescence

Photosynthetic rates were measured using PAM fluorometry when all seedlings had reached treatment salinities as well as after one month in the treatment salinities. Rapid light curves were used to calculate relative electron transport rate (RETR= Yield * PPFD * 0.5), and quantum yields of each seedling. Preliminary experiments on *T. testudinum* leaves were used to determine the PAM measurement protocol. RETR vs. irradiance curves were generated from which alpha and $RETR_{max}$ ($\mu mol\ e^{-}\ m^{-2}\ s^{-1}$) were calculated. To eliminate diel variation within plants, all measurements were taken between 2 and 4 p.m. and the plants were chosen arbitrarily.

2.4.3. Respiration

At the termination of the experiment, (one month after all seedlings had reached treatment salinity) seedling leaf respiration was measured with a Hansatech oxygen electrode. After the final morphometric measurements were made, a 1 cm^2 piece of *Thalassia* seedling blade (cut underwater) was placed within a Hansatech DW1 chamber, filled with 2 ml saltwater media of treatment salinity level at 20°C. The chamber was kept in the dark for 20 minutes and the level of oxygen in the chamber recorded every five minutes. Respiration rate ($\mu mole\ O_2\ cm^{-2}\ hr^{-1}$) was calculated as the slope of oxygen versus time for the linear portion of the incubation.

2.4.4. Osmolality

Leaf-tissue osmolality was also measured at the termination of the experiment with a Wescor vapor-pressure osmometer using the protocol determined in previous Year 1. Leaf, root, and seed tissues were then separated and dried for two days at 60°C for dry-weight determination.

2.5. Statistical analyses

Data were statistically analyzed using Jandel Scientific SigmaStat 2.0 and SAS statistical software. The effect of salinity on seedling growth and photosynthesis was assessed by one- way ANOVA's. The Tukey post hoc multiple pair-wise comparison test was applied to determine wherein the differences lay when alpha < 0.050. For the cases where normality failed, transformations were performed. Were the transformations unsuccessful, a Kruskal-Wallis one- way ANOVA on ranks was run followed by Dunn's multiple pair-wise comparisons. For repeated-measures analysis, linear repeated-measures analysis of covariance (SAS PROC-mixed) was used.

3. Results

3.1. Year 1: Flask-scale experiment

3.1.1. Survival and growth

For *Thalassia testudinum* in the flask-scale experiment, the extreme low- and high-salinity treatments had a negative impact on seedling survival after three weeks (Table 2). All seedlings in 0 PSU media were dead after 25 days. A

dramatic decrease in survival by day 30 was also observed in the seedlings grown at salinities 50, 60, and 70 PSU. The seedlings grown at 20, 30 and 40 PSU had a 100% survival over the experimental period. One seedling died in the 10 PSU treatment the final week of the experimental period and only one seedling at the 50 PSU treatment survived the 94-day experimental period.

Only values from surviving seedlings were used for comparison of morphometric characteristics (Fig. 3 a,b,c,d). There was a general trend of increasing total root length over time. Seedlings in the 20 PSU treatment had significantly greater root lengths than all other salinity treatments over the course of the experimental period in the treatment ($F_{7, 733}$=13.327 P<0.001). By the termination of the experiment, total leaf area was significantly less ($F_{7, 733}$=18.729 P<0.001) in the 50 PSU treatment than all other surviving treatments (10-40 PSU). This decrease in leaf area was due to a considerable decrease in blade width, which occurred around day 60. Blade width was slightly greater in the 10 PSU treatment plants, but blade length was not. Blade length was greater, though not significantly, in the 40 PSU treatment plants than in the other surviving treatments, but blade width was slightly less than the plants in the 10, 20, and 30 PSU salinity treatments. Prior to mortality, seedlings in the 0, 60, and 70 PSU salinity treatments showed very sharp decreases in total leaf area, which was an artifact of decrease in total blade length due to increased loss of blades.

Table 2. Year one: Average survival in days of *Thalassia testudinum* seedlings in flasks at eight treatment salinities over the 94-day experimental period (± stdev).

Salinity	0	10	20	30	40	50	60	70
Days till death	26 (±2)	94 (±1)	94 (±0)	94 (±0)	94 (±0)	46 (±20)	44 (±5)	42 (±8)

Table 3.Year one: Average survival in days of *Thalassia testudinum* seedlings in aquarium tanks at eight treatment salinities over the 80-day experimental period (± stdev).

Salinity	0	10	20	30	40	50	60	70
Days till death	16 (±0.82)	36 (±16)	72 (±12)	80 (±0)	80 (±0)	54 (±12)	19 (±0)	19 (±0)

Table 4. Year two: Average survival in days of *Thalassia testudinum* seedlings in aquarium tanks at three levels of ammonium and eight treatment salinities over the 116-day experimental period (± stdev).

Salinity	0	10	20	30	40	50	60	70
0µM NH_4^+	85 (±28)	100 (±33)	116 (±0)	116 (±0)	116 (±0)	116 (±0)	91 (±19)	74 (±6)
10µM NH_4^+	83 (±30)	114 (±5)	116 (±0)	116 (±0)	106 (±27)	93 (±37)	65 (±27)	76 (±10)
20µM NH_4^+	76 (±13)	85 (±45)	116 (±0)	115 (±4)	116 (±0)	84 (±31)	97 (±15)	74 (±12)

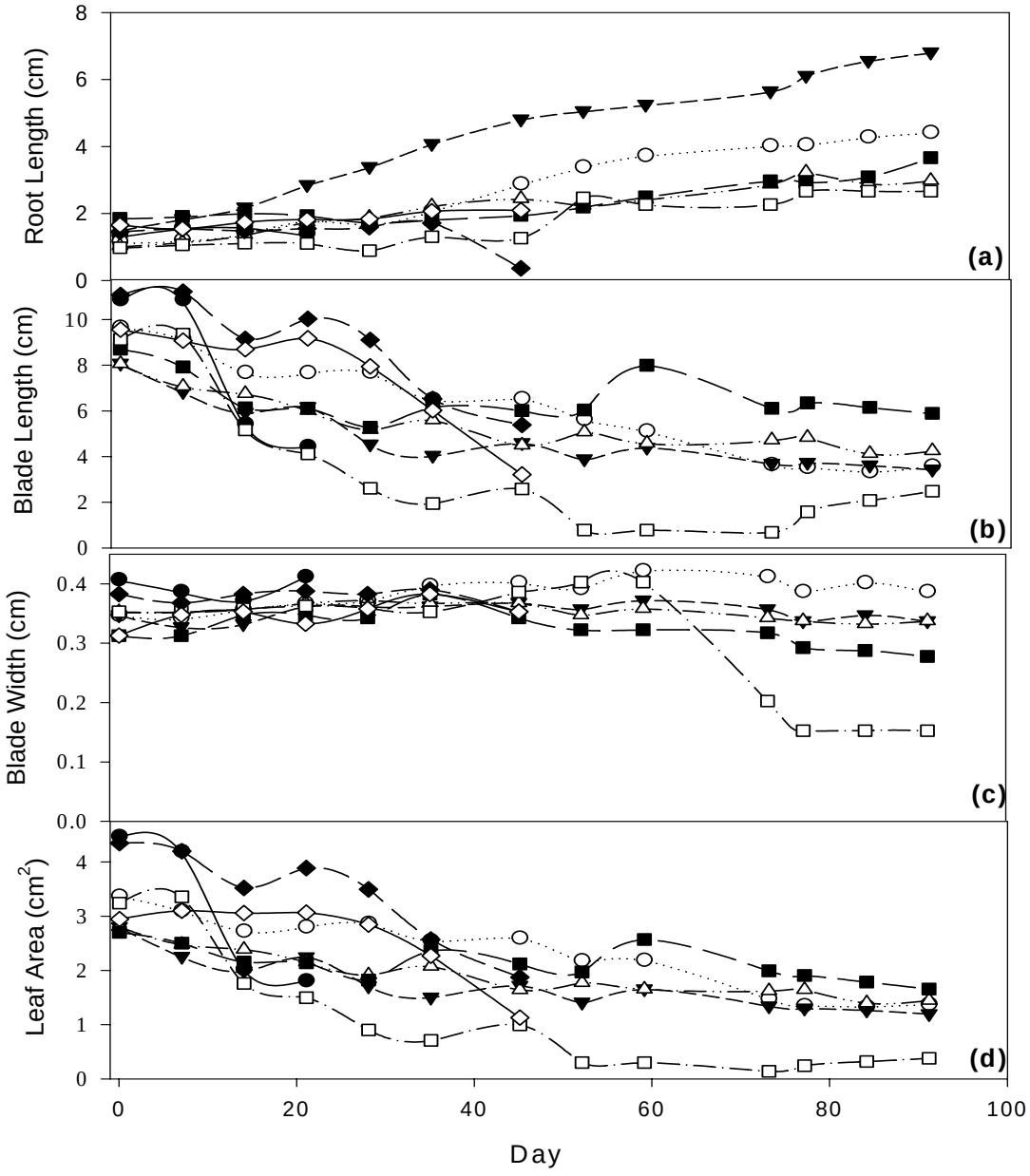

Fig. 3. Year One: Morphometric charactreristics of *Thalassia* seedlings grown in flasks a) total root length (cm), b) total blade length (cm), c) average blade width (cm), d) total leaf area (cm^2) for eight salinity tretaments: 0 (black circle), 10 (white circle), 20 (black triangle), 30 (white triangle), 40 (black square), 50 (white square), 60 (black diamond) and 70 PSU (white diamond).

3.1.2. Fluorescence

Quantum yield measurements, both effective and maximum, after one month at treatment salinity were significantly different ($P<0.001$ for both) among treatments at mid-salinity ranges, 20, 30 and 40 PSU, compared to those of extreme salinities of 0, 60 and 70 PSU (data not shown). After two months at treatment salinity, only plants at salinities 10-50 PSU remained for measurement and after three months at treatment salinities only plants in 10-40 PSU could be measured (Figs. 4 and 5). The one remaining seedling at the 50 PSU treatment was too small for the fluorescence measurement. At months two and three, effective yields for plants at 40 PSU were significantly greater than at 10 PSU ($F_{4, 49}=5.297$ $P=0.001$ and $F_{3,33}=5.022$ $P=0.006$, respectively). Effective yields decreased significantly from month one to month three for plants at 20 PSU ($F_{2, 27}=4.403$ $P=0.023$).

3.1.3. Osmolality

Osmolality showed a significant trend ($F_{4, 46}=253.112$ $P<0.001$) of increased tissue osmolality with increased salinity among all treatments (Fig. 6). Leaf tissue was distinctly hyperosmotic to media osmolality and maintained similar values of hyperosmolality among treatments with a mean del (difference between tissue osmolality and media osmolality) of 646 mmol/kg (±108.22). A regression of del of osmolality vs. salinity was not significant ($F_{1, 4}=2.340$ $P=0.224$).

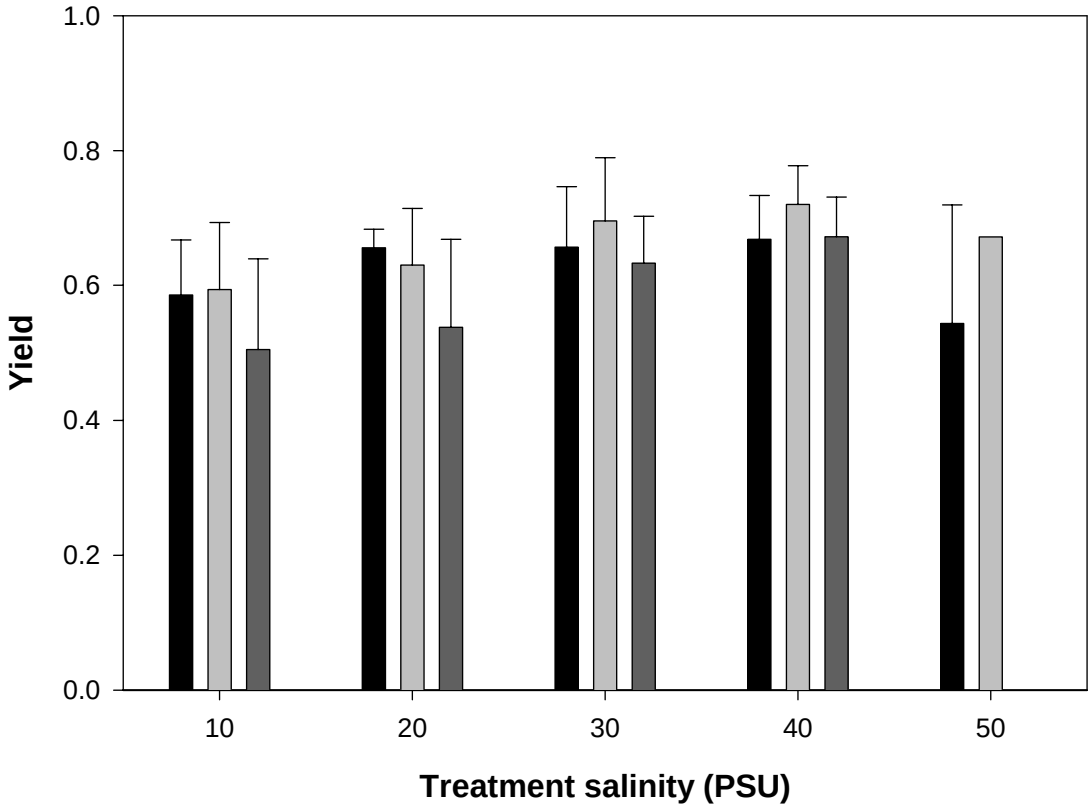

Fig. 4. Year one: Apparent quantum yields for *Thalassia testudinum* seedlings one (black), two (light grey) and three(dark grey) months at each treatment salinity (+/- stdev).

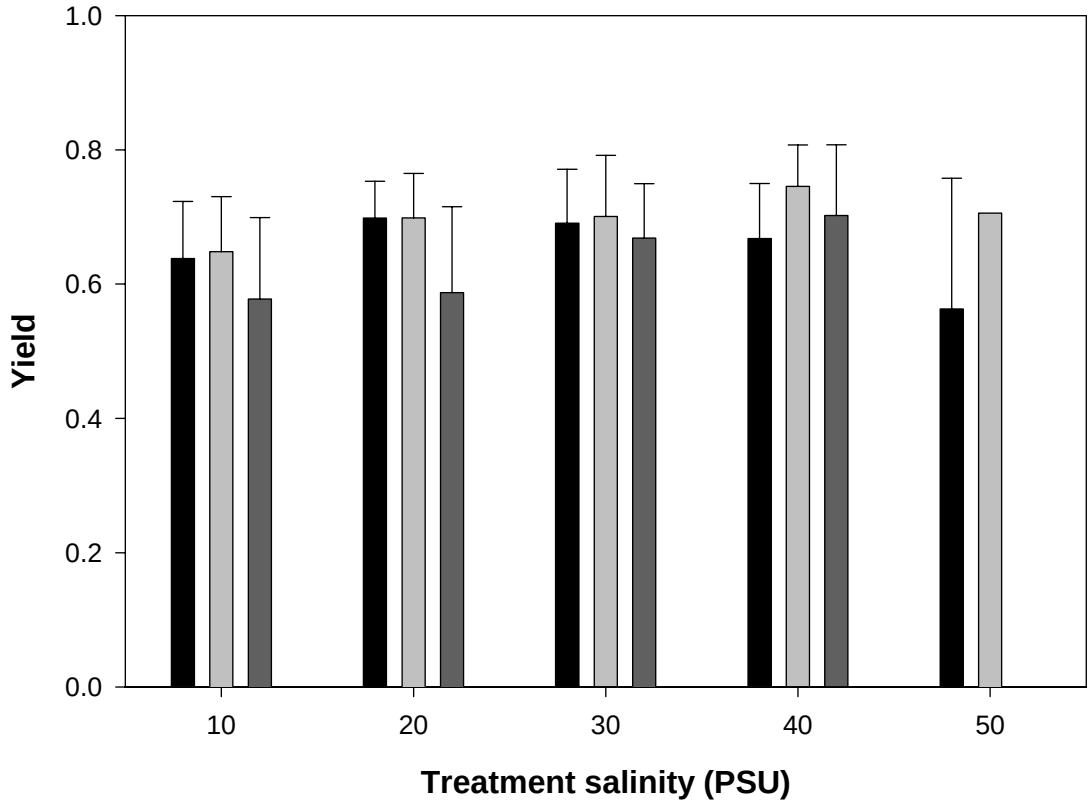

Fig. 5. Year one: Maximum quantum yields of *Thalassia testudinum* seedlings one (black), two (light grey) and three (dark grey) months at each treatment salinity (+/- stdev).

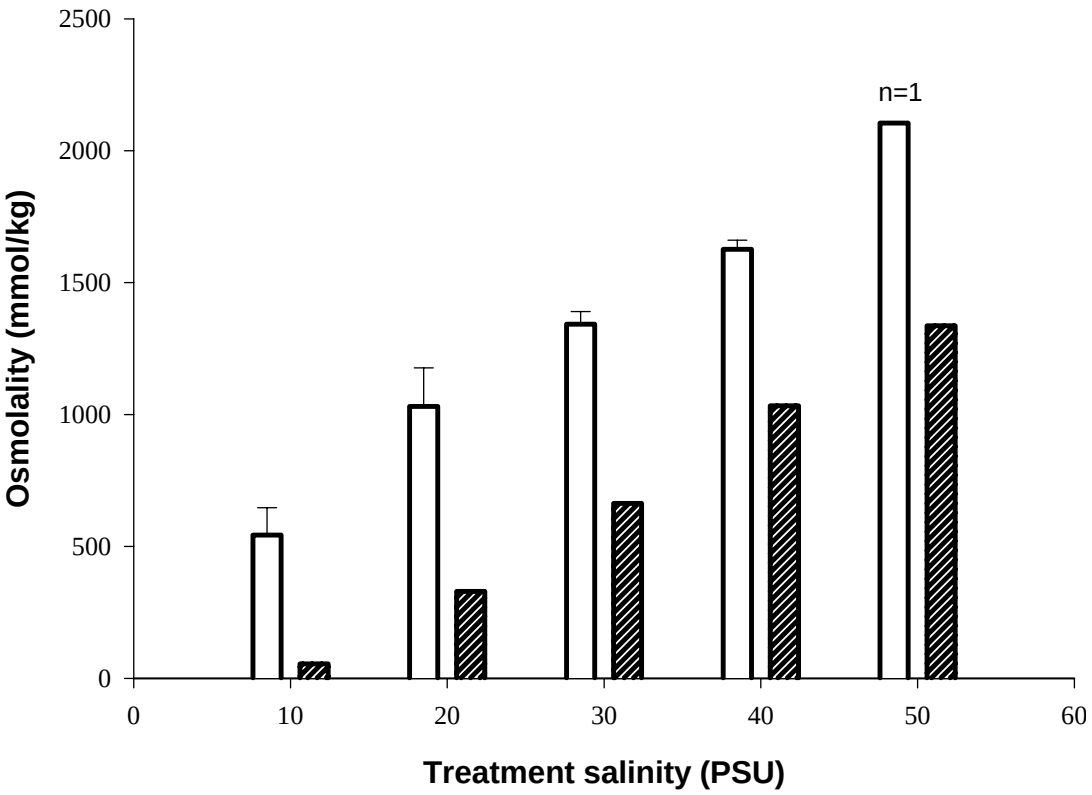

Fig 6. Year one: Intercellular osmolality of *Thalassia testudinum* seedling tissue (white) and treatment media (grey) at the eight salinity treatments (+/– stdev).

3.2. Year 1: Aquarium-scale experiment

3.2.1. Survival and growth

Seedlings in salinities of 0, 60, and 70 PSU had 100% mortality by day 19. Some seedlings at 10 PSU survived until day 45 and less than half of the seedlings at 50 PSU survived through the 80-day experimental period (Table 3). Of the surviving plants, those at salinity 50 PSU had a greater percent of total leaf length that was brown (Fig. 7b).

Plants at extreme salinities 0, 10, 60 and 70 PSU had significantly fewer blades than those at mid-range salinities, 20-40 PSU ($F_{7, 721}$=231.227 P<0.001), but were not significantly different in number of blades from one another over the course of the experimental period.

Blade width showed no significant differences among treatments over the course of the experimental period, but declined in the 50 PSU treatment in the last week of the experiment (Fig. 7c).

Total blade length (Fig. 7a) and leaf area (Fig. 7d) was greatest in the 30 and 40 PSU treatments. Leaf length and area for seedlings at 20 and 50 PSU were second greatest. Although those at 20 PSU had an overall greater average over the course of the experimental period, measurements were quite similar to those of the 50 PSU seedling by the termination of the experiment. By week 10, plants at 30 and 40 PSU had significantly greater total blade lengths than the surviving plants at 50 and 20 PSU ($F_{3, 39}$=3.774 P=0.019).

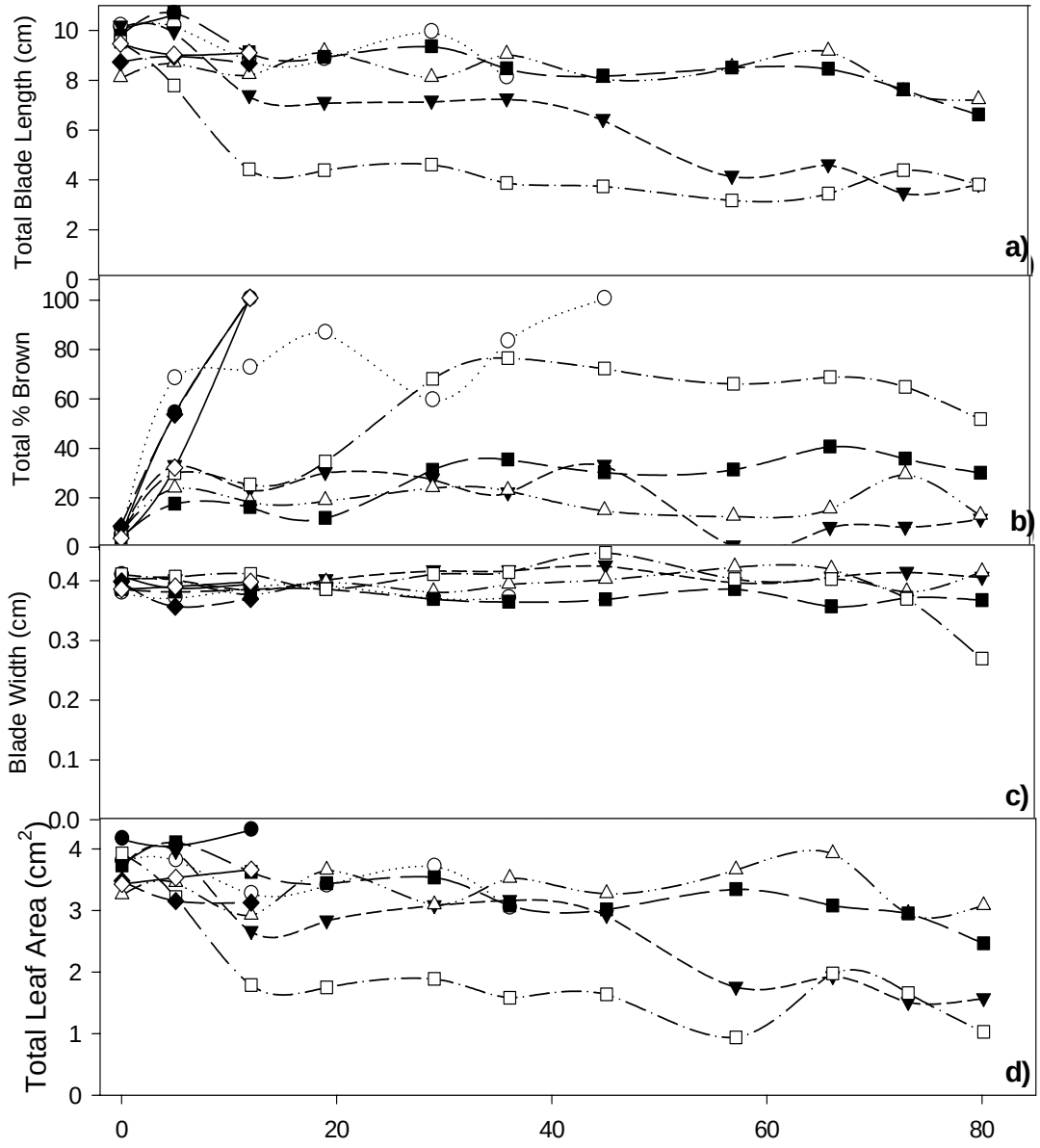

Fig. 7. Year One: Morphometric charactreristics of *Thalassia testudinum* seedlings grown in tanks a) total blade length (cm), b) % brown, c) average blade width (cm), d) total leaf area (cm^2) for eight salinity tretaments: 0 (black circle), 10 (white circle), 20 (black triangle), 30 (white triangle), 40 (black square), 50 (white square), 60 (black diamond) and 70 PSU (white diamond).

3.3. Year Two

3.3.1. Survival and Growth

Survival was greatest in the 10, 30, and 40 PSU treatments followed by the 50 PSU treatments at all ammonium levels (Table 4). Mortality was high in the hypo- and hyper-saline treatments (0, 60 and 70 PSU), but not as high as in year one.

For statistical analysis of morphological characteristics, plants were grouped into three salinity categories. “Low” was 0-10 PSU, “mid” 20-40 PSU, and “high” 50-70 PSU (refer to Tables 5- 8 for statistical data). Note that for weeks 9-15 all plants were at fixed treatment salinity, therefore decreasing the week*salinity interaction. If a plant died, its measurement was recorded as 0cm for graphical representation. At all ammonium levels, mid-salinity treatment plants had significantly greater total leaf area than the low or high salinity plants (Table 5, Fig. 8 a, b, c). This was due to the combination of significantly greater total blade length (Table 6, Fig. 9 a, b, c) as well as increased blade widths (Table 7, Fig. 10 a, b, c). At mid salinities, blade widths showed no significant differences among the three ammonium levels. There was, however, a decrease in total leaf area in the 0µM ammonium, mid-salinity treatments due to significant decrease in blade length. At low salinities, blade widths and in turn total leaf areas were significantly less in the 20µM ammonium treatment than the 10µM or 0µM treatments. Blade widths were significantly narrower in the high salinity treatments at all ammonium levels and the low salinity treatments showed a decrease in blade width at all ammonium treatments as well. Overall, the high ammonium treatment had a

negative impact on plants at low salinities and the low ammonium treatment had a negative impact on the plants at mid-range salinities. The effect of hypersalinity was greater than the impact of the ammonium treatment for seedling growth with much greater F values present for salinity effects (Tables 5-8).

3.3.2. Fluorescence

Overall alpha values were significantly greater ($F_{1, 169}$=16.083 P<0.001) upon reaching treatment salinity (initial) than one month later (Figs. 11 and 13). Initial alpha values were significantly greater ($F_{5, 118}$=9.117 P<0.001) in the 40 and 30 PSU treatment compared to the 0 and 10 PSU treatments. Initial values for $RETR_{max}$ across salinities were significantly greater ($F_{2, 118}$=3.081 P=0.05) in the 0µM ammonium than 20µM ammonium treatment (Fig 12). Values for initial $RETR_{max}$ across ammonium treatments for the 30 and 20 PSU treatments were significantly greater than the 0, 10 and 50 PSU treatments ($F_{5, 118}$=11.220 P<0.001). Neither $RETR_{max}$, nor alpha values were found to be significantly different among treatments after one month at treatment salinity (Figs. 13 and14).

3.3.3. Respiration

Plants at the 10µM ammonium treatment had significantly greater respiration rates than those at the 0 or 20µM ammonium treatments ($F_{2, 68}$=11.535 P<0.001) when pooled across salinities. Across all ammonium treatments, when examining

Table 5. Year two: The effects of salinity, ammonium and time on *Thalassia testudinum* total leaf area as determined by repeated measures analysis of covariance (** and * denote significance at p=0.01 and p=0.05, respectively).

Week	Effect	Model df	Total df	F Value	Pr>F
1-3	Salinity	2	231	1.32	0.2685
	Ammonium	2	231	2.85	0.0599
	Sal *Amm	4	231	0.64	0.6368
	Week	1	715	17.41	<.0001**
	Week*Sal	2	715	19.70	<.0001**
	Week*Amm	2	715	0.94	0.3901
4-8	Salinity	2	231	5.33	0.0054**
	Ammonium	2	231	2.13	0.1214
	Sal *Amm	4	231	2.38	0.0525
	Week	1	715	3.94	0.0474*
	Week*Sal	2	715	3.43	<.0001**
	Week*Amm	2	715	0.44	0.6440
9-15	Salinity	2	231	26.66	<.0001**
	Ammonium	2	231	4.14	0.0171*
	Sal *Amm	4	231	1.46	0.2159
	Week	1	715	73.83	<.0001**
	Week*Sal	2	715	0.54	0.5819
	Week*Amm	2	715	3.34	0.0360*

Table 6. Year Two: The effects of salinity, ammonium, and time on *Thalassia testudinum* total blade length as determined by repeated measures analysis of covariance (** and * denote significance at p=0.01 and p=0.05, respectively).

Week	Effect	Model df	Total df	F Value	Pr>F
1-3	Salinity	2	231	2.79	0.0633
	Ammonium	2	231	2.98	0.0530
	Sal *Amm	4	231	1.05	0.3805
	Week	1	715	44.58	<.0001**
	Week*Sal	2	715	11.89	<.0001**
	Week*Amm	2	715	0.79	0.4534
4-8	Salinity	2	231	3.58	0.0294*
	Ammonium	2	231	1.89	0.1532
	Sal *Amm	4	231	3.72	0.0059**
	Week	1	715	1.36	0.2433
	Week*Sal	2	715	20.87	<.0001**
	Week*Amm	2	715	0.14	0.8685
9-15	Salinity	2	231	21.28	<.0001**
	Ammonium	2	231	6.67	0.0015**
	Sal *Amm	4	231	1.57	0.1839
	Week	1	715	145.63	<.0001**
	Week*Sal	2	715	0.29	0.7479
	Week*Amm	2	715	5.40	0.0047**

Table 7. Year two: Effects of salinity, ammonium and time on *Thalassia testudinum* blade widths as determined by repeated measures analysis of covariance (** and * denote significance at p=0.01 and p=0.05, respectively).

Week	Effect	Model df	Total df	F Value	Pr>F
1-3	Salinity	2	231	.67	0.5141
	Ammonium	2	231	.46	0.6317
	Sal *Amm	4	231	1.44	0.221
	Week	1	715	5.03	0.0252*
	Week*Sal	2	715	18.30	<.0001**
	Week*Amm	2	715	1.34	0.2623
4-8	Salinity	2	231	10.76	<.0001**
	Ammonium	2	231	0.27	0.7666
	Sal *Amm	4	231	3.86	0.0047**
	Week	1	715	5.44	0.0199*
	Week*Sal	2	715	30.25	<.0001**
	Week*Amm	2	715	0.55	0.5763
9-15	Salinity	2	231	3.09	0.0473*
	Ammonium	2	231	0.23	0.7944
	Sal *Amm	4	231	3.78	0.0054**
	Week	1	715	65.10	<.0001**
	Week*Sal	2	715	24.48	<.0001**
	Week*Amm	2	715	1.72	0.1806

Table 8. Year two: The effects of salinity, ammonium and time on *Thalassia testudinum* number of blades as determined by repeated measures analysis of covariance (** and * denote significance at p=0.01 and p=0.05, respectively).

Week	Effect	Model df	Total df	F Value	Pr>F
1-3	Salinity	2	231	9.93	<.0001**
	Ammonium	2	231	5.99	0.0029**
	Sal *Amm	4	231	1.31	0.2688
	Week	1	715	78.63	<.0001**
	Week*Sal	2	715	8.78	0.0002**
	Week*Amm	2	715	1.58	0.2070
4-8	Salinity	2	231	0.52	0.5966
	Ammonium	2	231	0.61	0.5419
	Sal *Amm	4	231	2.10	0.0812
	Week	1	715	13.74	0.0002**
	Week*Sal	2	715	6.96	0.0010**
	Week*Amm	2	715	0.23	0.7949
9-15	Salinity	2	231	4.98	0.0076**
	Ammonium	2	231	2.27	0.1057
	Sal *Amm	4	231	1.76	0.1387
	Week	1	715	192.78	<.0001**
	Week*Sal	2	715	2.67	0.0702
	Week*Amm	2	715	1.80	0.1664

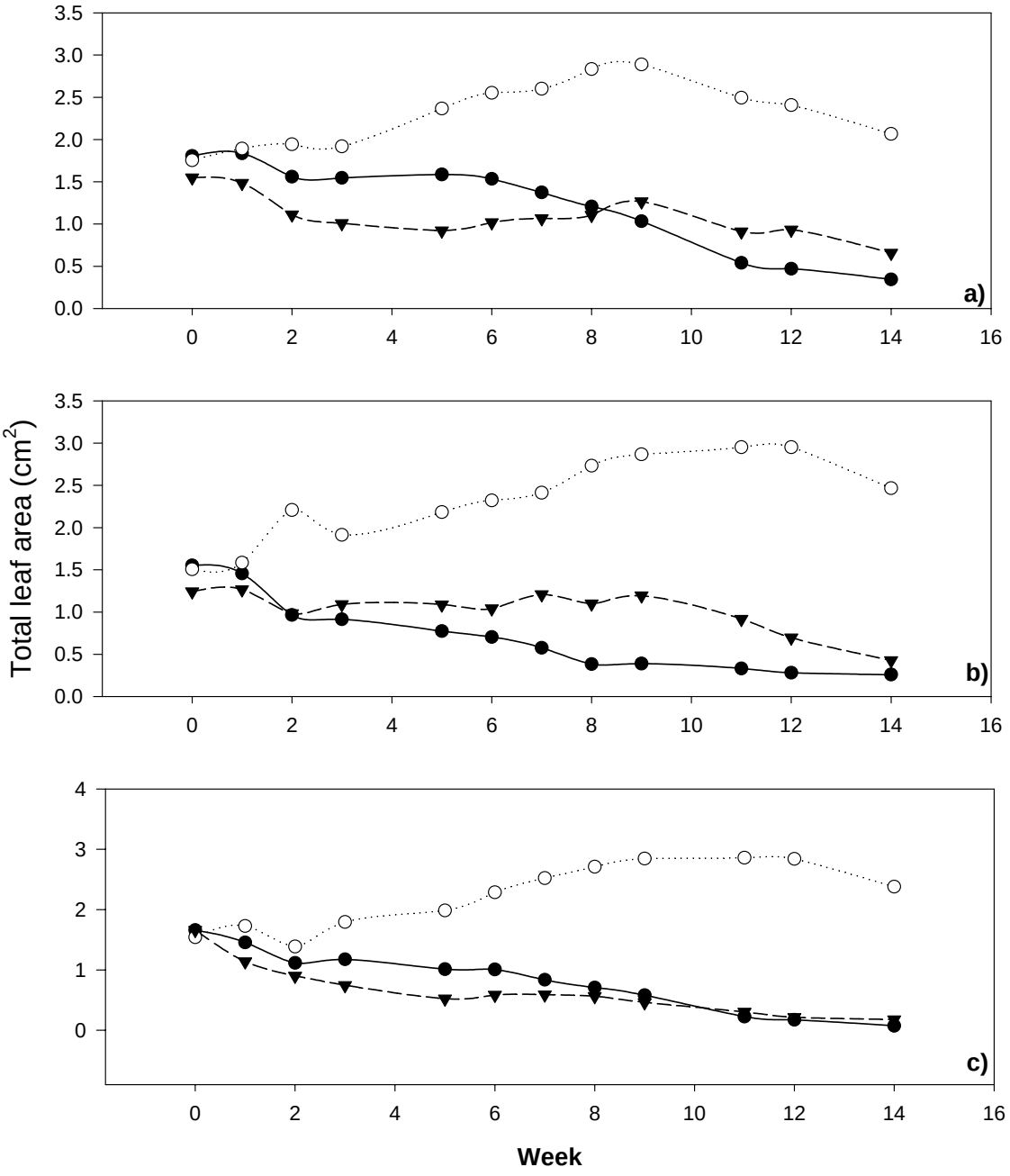

Fig. 8. Average total leaf area (cm^2) of *Thalassia testudinum* seedling over experimental period for the three salinity ranges: high (black circle), mid (white circle) and low (black triangle) for each ammonium treatment: a) 0μM, b) 10μM, and c) 20μM

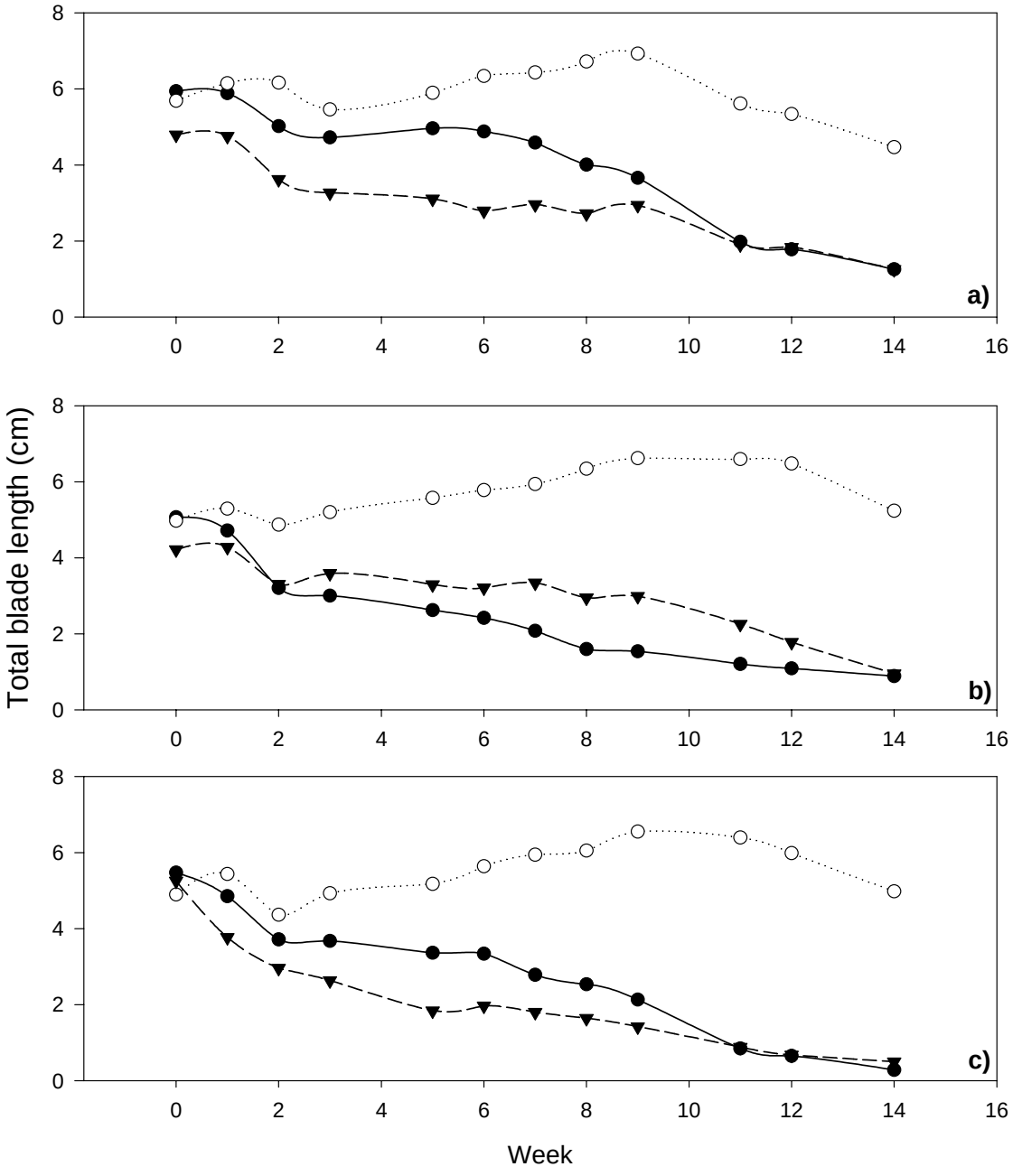

Fig 9. Average total blade length (cm) of *Thalassia testudinum* seedlings over experimental period for the three salinity ranges: high (black circle), mid (white circle), and low (black triangle) for each ammonium treatment: a) 0μM, b) 10μM and c) 20μM.

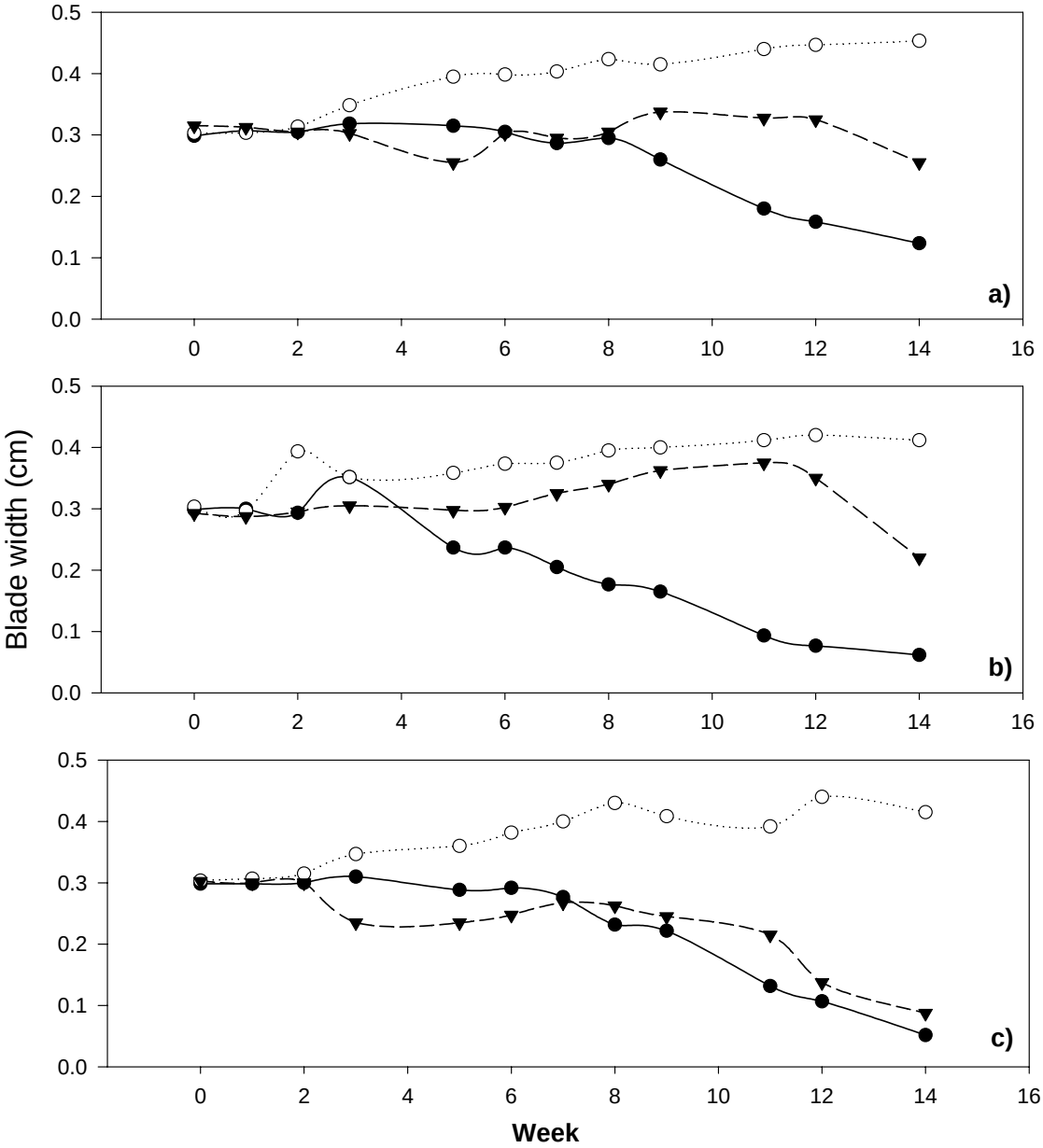

Fig 10. Average blade width (cm) of *Thalassia testudinum* seedlings over experimental period for the three salinity ranges: high (black circle), mid (white circle) and low (black triangle) for each ammonium treatment: a) 0μM, b) 10μM, and c) 20μM.

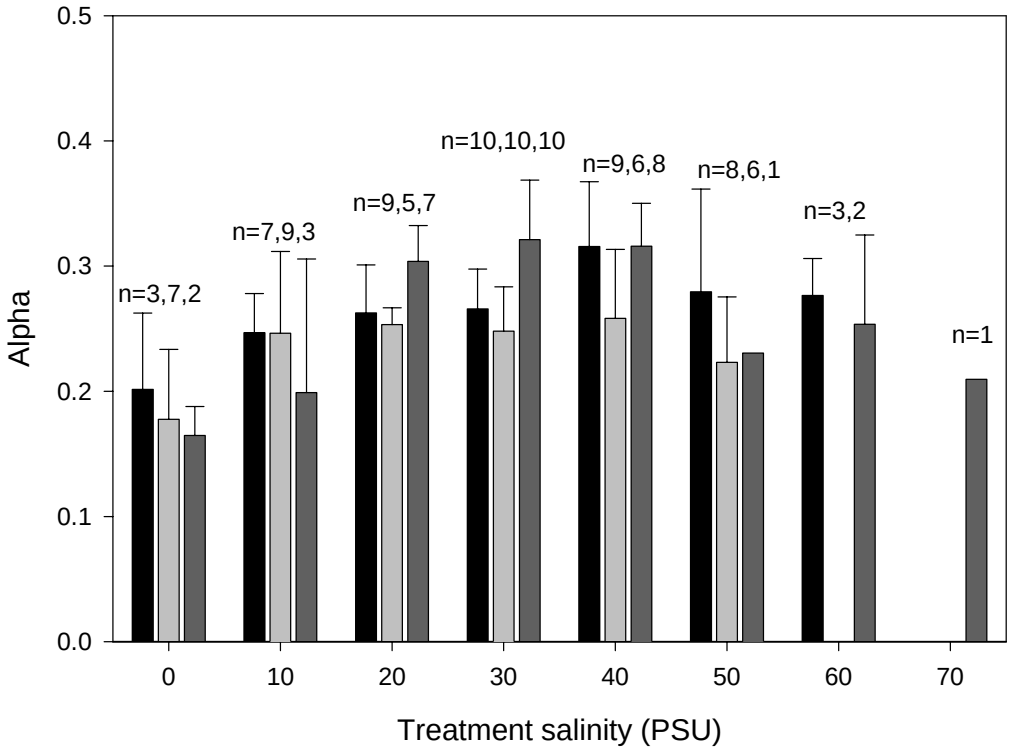

Fig. 11. Alpha values for *Thalassia testudinum* seedlings upon reaching treatment salinity for each ammonium treatment: 0μM (black), 10μM (light grey), and 20μM (dark grey) (+/-stdev). Number above bar represents number of replicates.

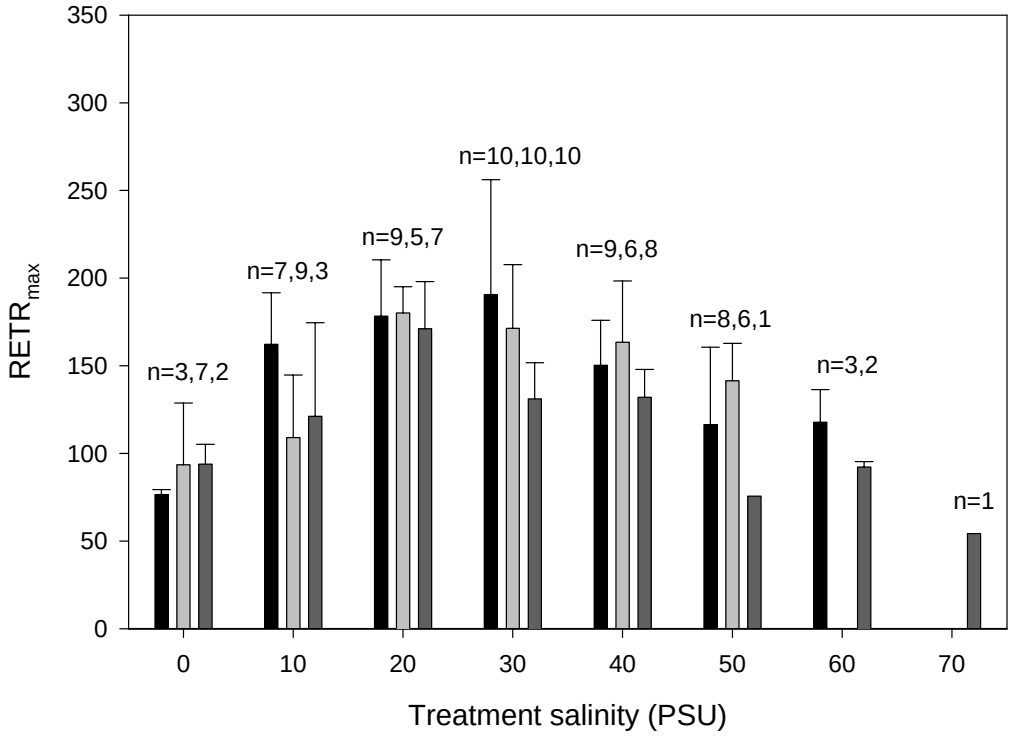

Fig. 12. RETR$_{max}$ for *Thalassia testudinum* seedlings upon reaching salinity treatment for each ammonium treatment: 0μM (black) 10μM (light grey) and 20μM (dark grey) (+/-stdev). Number above bar represents number of replicates.

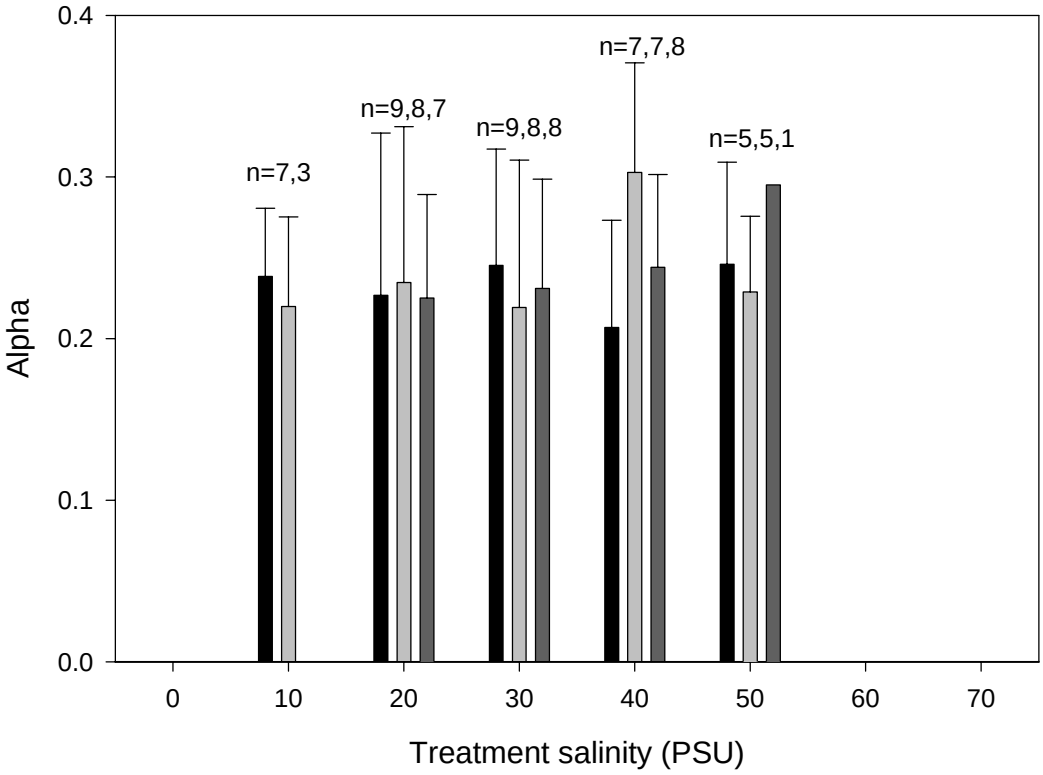

Fig. 13. Alpha for *Thalassia testudinum* seedlings after one month at treatment salinity for each of the three ammonium levels: 0μM (black), 10μM (light grey), 20μM (dark grey) (+/-stdev). Number above bar represents number of replicates.

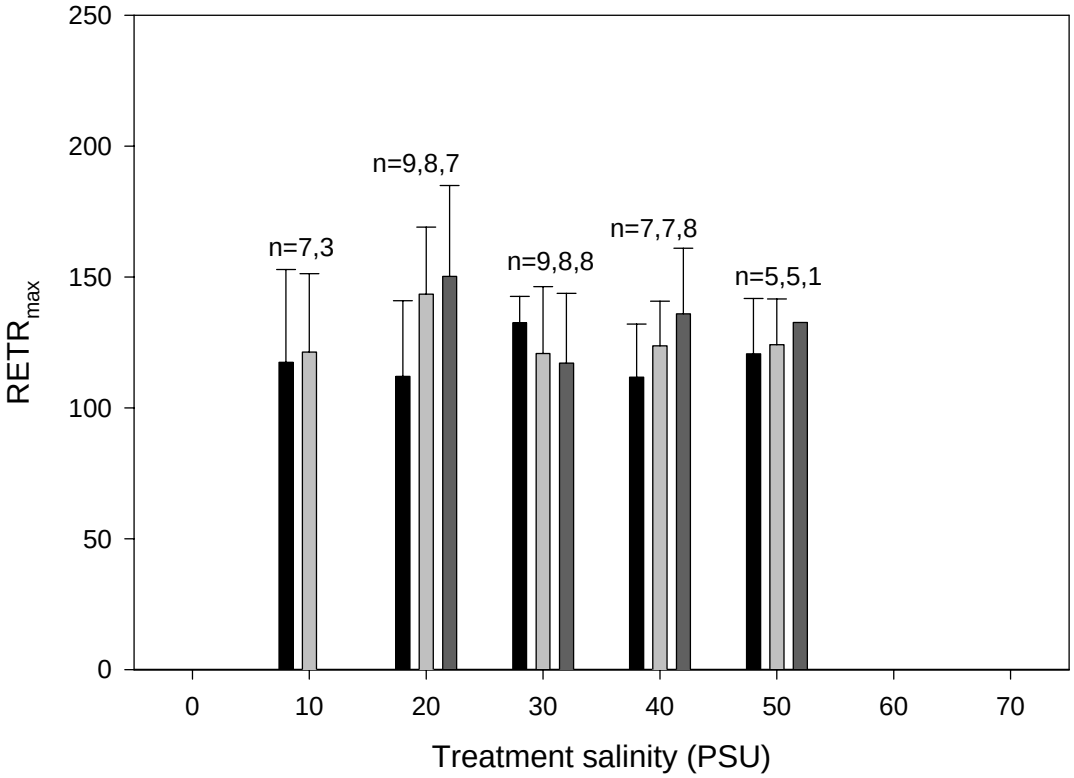

Fig. 14. RETR$_{max}$ for *Thalassia testudinum* seedlings after one month at treatment salinity for each ammonium treatment: 0μM (black), 10μM (light grey), and 20μM (dark grey) (+/-stdev). Number above bar represents number of replicates.

only the effect of salinity, plants at 20 PSU had significantly greater respiration rates than those at 10 PSU ($F_{4, 68}$=3.149 P=0.021). Interactions between salinity and ammonium were also significant ($F_{8, 68}$=4.433 P<0.001). Within the 10µM ammonium treatments, rates were greater at 20 PSU than 10, 30, and 40 PSU and greater at 50 PSU than 10 PSU. Within 40 PSU, respiration rates were greater in plants at ammonium levels of 20 than 0µM. Plants at both 20 and 50PSU had significantly greater respiration rates at 10µM than at 0 or 20µM ammonium (Fig. 15).

3.3.4. Osmolality

Salinity as a whole, with ammonium treatments pooled, had a significant impact ($F_{4, 66}$=242.025 P<0.001) on osmolality at each treatment salinity level (Fig. 16). Ammonium, with salinity treatments pooled, had little impact on osmolality, but it did have a significant interactive effect with salinity. At the lower ammonium levels, osmolality in the ramped tissue was more similar among the 30, 40 and 50 PSU treatment plants than among the same treatments at elevated ammonium levels. At ammonium of 0µM, there was a significant increase in osmolality ($F_{4, 24}$=105.459 P<0.001) with increasing salinity except between 30 and 40 PSU. For the 10µM ammonium treatment there was a significant increase in osmolality ($F_{5, 24}$=426.378 P<0.001) with increased salinity except between 40 and 50 PSU. For plants in the 20µM ammonium treatment there was a significant difference among some treatments ($F_{4, 18}$ =35.255 P<0.001), though not as many as in the other two ammonium treatments. Osmolalities of tissues from the 40 PSU treatment were not significantly different

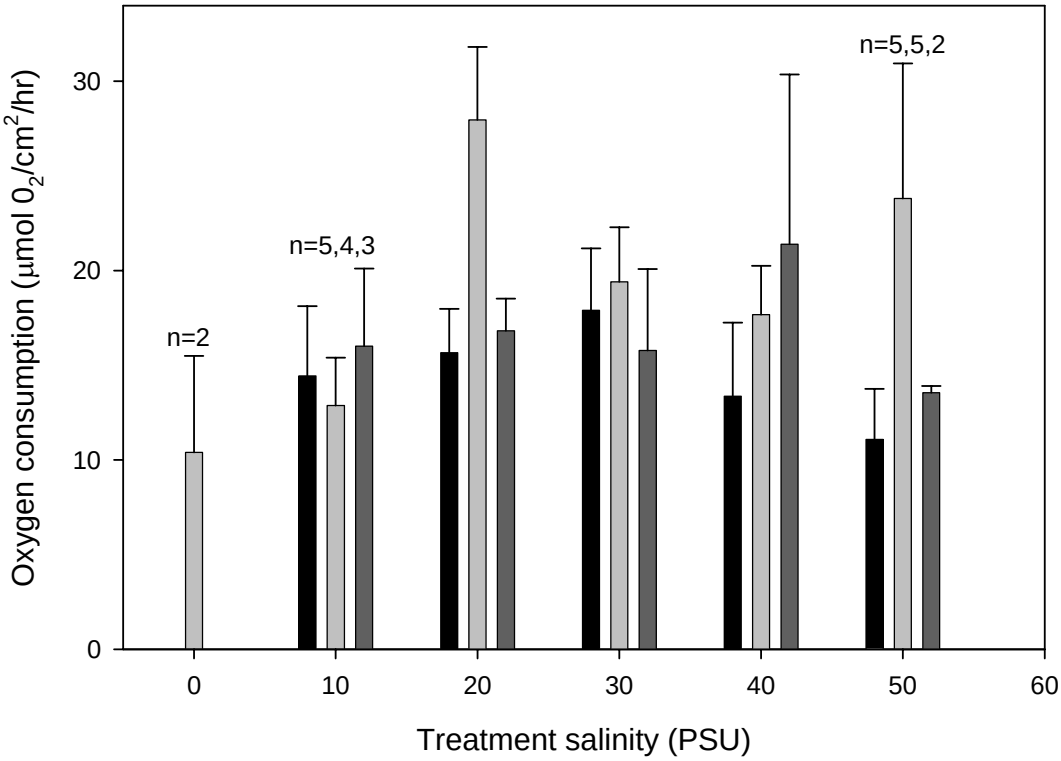

Fig. 15. Respiration of *Thalassia testudinum* seedlings as a function of oxygen consumption at each treatment salinity for each of the three ammoniumtreatments: 0μM (black), 10μM (light grey), 20μM (dark grey) (+/- stdev) n=5 unless otherwise noted.

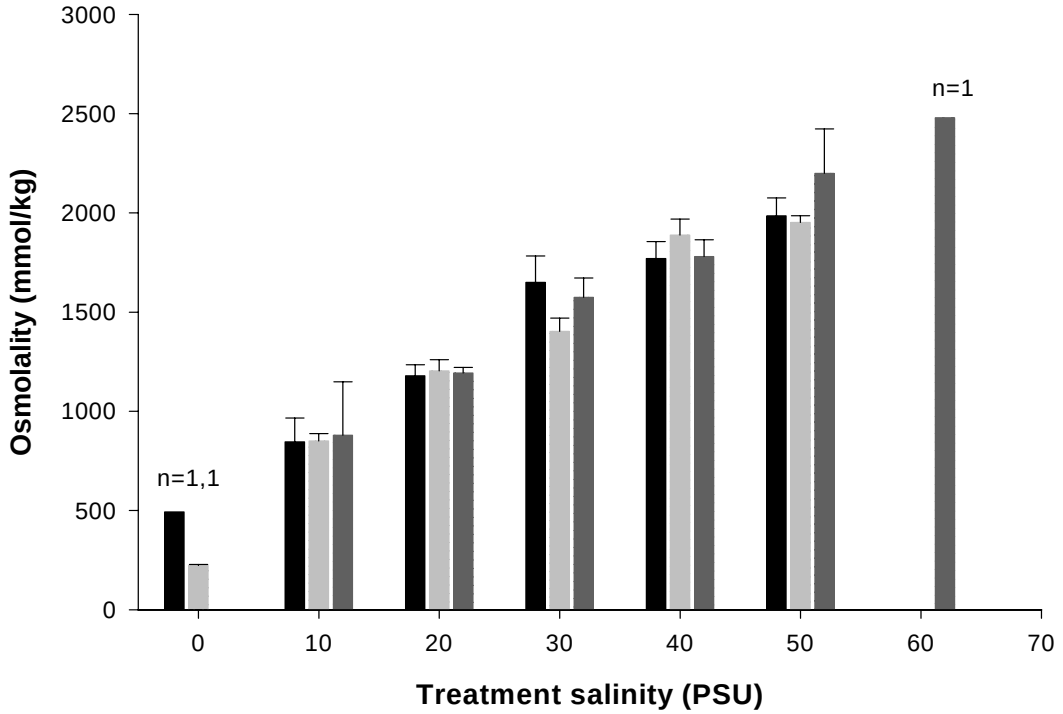

Fig. 16. Year two: Osmolality of *Thalassia testudinum* seedling tissue at each salinity treatment for each of the three ammonium treatments: 0μM (black), 10μM (light grey), and 20μM (dark grey) (+/- stdev).

than at 50 PSU or 30 PSU. Osmolality of plants at 50 PSU was not significantly different than in plants at 60 PSU and osmolality of the 20 PSU treatment plants was not significantly greater than that of 10 PSU treatment plants. Within salinity treatments, osmolality was significantly different at 30 PSU between the 0 and 10 μMNH_4^+ treatments ($P<0.001$).

4. Discussion

4.1. Survival and Growth

Thalassia testudinum seedlings are not adapted for extreme changes in salinity to ranges outside of what has been found to be their salinity optimum (20-40 PSU) This optimum has been supported by previous studies, both lab manipulations as well as field observations involving mature *T. testudinum* plants (Zieman, 1975; Doering and Chamberlain, 2000). Results of hypersalinity studies showed an arrest in growth after a salinity of 60 PSU (McMillan and Moseley, 1967) and decreased leaf elongation rates of *T. testudinum* at 56-60 PSU (Koch and Erskine, 2001). Salinity minimum studies showed a significant decrease in growth at salinities down to 6 PSU (Doering and Chamberlain, 2000). Field studies also support the negative impact of freshwater on blade width, shoot production and biomass per m^2 (Irlandi et al., 2002).

Both the McMillan and Moseley (1967) and Doering and Chamberlin (2000) studies involved incremental increase and decrease of salinity of 0.75 PSU per day and 1.5 PSU per day, respectively. Therefore, the results from year two with the incremental increase in salinity follows the growth and survival trends of

previous studies. Even with incremental increase/ decrease, it was clear that for the seedlings, survival and growth rates are less than for mature *T. testudinum* plants. Koch and Durako (2004) found that for mature *T. testudinum* plants grown in mesocosms, leaf growth and net number of shoots showed no significant change across salinity treatments of 38- 60 PSU for 55 days when grown between 28 and 32ºC, conditions similar to this study. These results for mature *T. testudinum* plants suggest that the mature plants are better adapted to high salinity regimes than are seedlings.

Ammonium did have a negative effect on salinity tolerance. Seagrasses take up their ammonium and other nutrients mostly from sediment pore water and the water column. Ammonium has been found to be toxic to other aquatic grasses, even at relatively low levels (Smolders et al., 1996; Katwijk et al., 1997), with a greater affinity for NH_4^+ uptake through the leaves of *T. testudinum* than the roots (Touchette and Burkholder, 2000). This may play a role in the magnitude of impact that elevated ammonium levels would have on seedlings that are floating in the water column and have no established root systems.

4.2. Fluorescence and respiration

Year-one measurements of effective quantum yields showed that treatment salinities below 30 PSU had a negative effect on yields. Yields at salinities 10 and 20 PSU decreased the most over the experimental period, indicating a decrease in ability to utilize light energy for photosynthesis. A study on *Halophila ovalis* (R.Br.) Hook. f. (Ralph, 1998) showed a similar quantum yield response to hyposalinity

stress. The decrease in quantum yield was attributed to damage of photosystem II via an ionic imbalance and seepage due to the difference in water potential between the less saline media and more saline leaf tissue. Under hypersaline conditions, *H. ovalis* was found to decrease maximum fluorescence, showing a greater initial tolerance to hypersaline conditions until photosynthetic inhibition occurred gradually at the point where the plant could no longer tolerate the extreme osmotic stress (Ralph, 1998). Studies on *Ruppia maritima* have also found the lowest quantum yields under extreme hypo- and hyper-saline conditions (Murphy at al., 2003). Maximum quantum yield and quantum efficiency were also negatively impacted by increased salinity in the green alga, *Ulva lactuca* (Xia et al., 2004). This study suggested that reaction centers of photosystem II were the target of the salinity stress and that salinity stress caused a reduction in the number of reaction centers as well as the decrease in evolution of oxygen. In some terrestrial plants that accumulate chloride and sodium within their leaves, after maximum fluorescence, photosynthetic quenching was found to be accelerated in conditions of increased salinity (Downton and Millhouse, 1985; Lee et al., 2004).

Year-two rapid light curve analyses show that initially $RETR_{max}$ and alpha exhibited a bell-shape curve trend when examining salinities from 0-60 PSU, with the lesser values at the two end members and greatest values at 30 PSU. By one month in treatment, only plants from 10-50 PSU survived for measurement. None of the end-members survived and in the remaining hyper- and hypo-saline treatments, only a portion of the plants survived. This indicates that although

plants can survive and grow in salinities surrounding the optimum range, actual establishment and colonization of the population may not be successful because of decreased numbers.

The results of respiration measurements suggest that in this case, an increased rate of respiration was due to an increase in metabolism rather than an increase in stress on the plant. Previous studies on *T. testudinum* under salinity stress also found no effect on respiration under hypo- and hyper-saline conditions (Burns, 2003). Respiration responses of *Zostera sp.* have also been studied in the past with no consistent correlation between salinity and respiration (Biebl and McRoy, 1971; Kerr and Strother, 1985). Although the patterns varied among ammonium treatments between salinities, the highest rates of respiration were found in plants, which according to growth data were the most productive. However, the lack of pattern in respiration rates at extremes may be reflective of the small sample size.

4.2. Osmolality

Tissue remains hyperosmotic to the media regardless of salinity or ammonium levels. Plants that were ramped up/ down showed intercellular tissue osmolality to be more hyperosmotic to the media than did those that were directly introduced into the treatment salinities. For the plants that remained at 30 PSU in both experiments, average osmolality at ambient ammonium levels was ca 1400 mmol/kg. This is supported by studies done on mature *T. testudinum* plants as well. Average osmolality for mature plants at salinities 40-60 PSU was found to be quite similar to those found in this study of seedling tissue osmolality (Koch and

Durako, 2004). Another study of mature *T. testudinum* showed an increase in osmolality with increased salinity and decrease in osmolality with decreased salinity at both the intra- and inter-cellular level (Berns, unpublished). Results from these studies provide evidence that regardless of environmental parameters (i.e. salinity or other stressors) or age, the internal osmolality of *T. testudinum* tissue is maintained within a certain ratio to the external environment. However, this osmotic adjustment to maintain the water potential may be taxing on the photosynthetic capacity of the plant (Xia et al., 2004; Ralph, 1998). Tyerman (1982) found that osmotic oscillations affect the physiology of seagrass by not only changing the concentration and balance of ions internally, but also by affecting the turgor pressure by a change in water potential at the cellular level. To deal with these physiological changes, the productivity of the plant may decrease by both a decrease in the energy allocated for photosynthesis as well as possible damage occurring to the photosystems.

4.3. Conclusions

In conclusion, *Thalassia testudinum* seedlings will tolerate salinity changes in their environment to around 10 PSU outside their optimum 20-40 PSU range. However, they are much less adaptable than mature individuals at surviving hypersaline conditions. Growth and survival were the best indicators of tolerance. Ammonium concentration influence varied among the various levels of salinity. At optimal salinity conditions (20-40 PSU), ammonium was not an influential factor in growth response. However, at lower salinities, increasing the level of ammonium

negatively impacted the growth response. This suggests that were the input of fresh water, containing higher levels of nitrogen, to increase, this would negatively impact *T. testudinum* seedling growth. This is important to consider when modeling the impact of a water-flow change on Florida Bay. The impact of fresh water in combination with increased ammonium and possibly other nutrients will likely alter the distribution of *T. testudinum* in Florida Bay and decrease the ability of new generations of genetically variant seedlings to successfully establish.

The fact that tissue remains hyperosmotic to the media even at salinities greater than seawater is interesting and should be further examined. The accumulation of ions may damage the photosystems as well as influence cellular water potential, therefore it would be interesting to examine the extent to which the inner cellular structures can tolerate these changes. Another aspect to consider is other possible biochemical processes that may be affected by an ionic imbalance and how the plant adjusts physiologically. Understanding these aspects may lead to a better understanding and possible prediction of salinity tolerance in these plants.

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