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Article

Ecotypes of Aquatic Plant *Vallisneria americana* Tolerate Different Salinity Concentrations

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Abstract: Increased salinity caused by saltwater intrusion or runoff from de-icing salts can severely affect freshwater vegetation and deteriorate aquatic ecosystems. These habitats can be restored with freshwater ecotypes (locally adapted populations) that tolerate above-normal salinity. *Vallisneria americana* is a prominent species in many freshwater ecosystems that responds differently to abiotic conditions such as substrate composition and fertility, so in this study we evaluated the effects of salt stress on 24 ecotypes of *V. americana*. Instant Ocean aquarium salt was used to create saline solutions [0.2 to 20.0 parts per thousand (ppt)], then plants were abruptly exposed to these solutions and maintained in these concentrations for 5 weeks before being visually assessed for quality and destructively harvested. Analysis of variance and non-linear regression were used to calculate LC_{50} values – the lethal concentration of salt that reduced plant biomass and quality by 50% compared to control treatment. Growth rate and visual quality varied significantly among ecotypes, and ecotypes that were most and least sensitive to salt had 50% biomass reductions at 0.47 and 9.10 ppt, respectively. All ecotypes survived 10.0 ppt salinity concentration but none survived at 20.0 ppt, which suggests the maximum salinity concentration tolerated by these ecotypes is between 15.0 and 20.0 ppt.

Keywords: aquatic macrophytes; freshwater systems; salinity tolerance; intraspecific variation; lethal concentration; genotypic variability; ecotype; salt stress; effective concentration; growth rate

1. Introduction

Local adaptation is a well-established phenomenon whereby habitat-mediated natural selection drives the differentiation of populations [1]. By definition, a distinct form of a plant species that occupies a particular ecosystem or habitat is called an ecotype. Intraspecific variation or ecotypic variability in salt tolerance has been investigated in several plant species [2–5]. For example, different ecotypes of *Spartina patens* from the Gulf Coast of the United States reportedly tolerate different salinity concentrations [3]. Such differences are the result of local adaptations and originate from genotypic traits as opposed to non-heritable acclimation to adverse conditions. Selection of ecotypes that are capable of tolerating extreme salinity conditions is important and useful in developing strategies for stabilization and revegetation of deteriorating marshes and wetlands that are subject to saltwater intrusion [6,7].

Vallisneria americana is a key species in many aquatic ecosystems [8]. This perennial submersed macrophyte provides food and habitat for fish, mammals, and invertebrates and affects nutrient cycling, sediment stability, and water clarity in lakes and estuaries [9]. Gettys and Haller [10] reported that *V. americana* ecotypes differ in their substrate and nutrient requirements, so variability in salt tolerance reported for this plant might also be due to ecotypic differences. The species is found in fresh and brackish water, but sporadic high salinity events could induce salt stress in this plant and affect its growth and establishment. Sporadic salinity can occur due to elevation difference from natural saline systems such as the ocean, and such prolonged flooding can have a direct influence on plant survival [11]. Various studies have shown that *V. americana* can tolerate 5.0 to 15.0 parts per thousand (ppt) salinity [8]. Salt-tolerant ecotypes of *V. americana* could be useful for restoration of wetlands that are at risk of saltwater intrusion and estuaries that are threatened by sea level rise. Also, the ability to assess salt sensitivity among ecotypes can be used to increase our understanding of the physiological and biochemical mechanisms underlying salt tolerance.

In this study, we used *V. americana* ecotypes that were collected from various locations within Florida, USA. It is recommended that field-collected plants being used for ecotype assessments should be maintained in culture to eliminate field acclimations and subsequent (cultured) generations should be used for experimentation [5,12]. Therefore, all ecotypes of *V. americana* used in these studies were vegetatively propagated and maintained as isolated cultures in the greenhouse for a minimum of 5 years prior to these experiments to remove environmental influences and acclimations of collection sites. The exact provenance of some of these ecotypes is unknown; however, phenotypic differences were evident and ecotypes varied somewhat in leaf size (i.e., width and length), leaf color (light green to reddish brown), and sex (male or female).

In this study, we aimed to answer the following questions:

1. How is *V. americana* impacted by increased salinity?
2. Is there variability in salt tolerance among *V. americana* ecotypes?

This study will provide information regarding what salinity levels are lethal to *V. americana* and will elucidate the relationship between ecotype and salt tolerance, which could yield valuable information to facilitate plant selection for better management of lakes, restoration of estuarine systems, and revegetation of littoral zones endangered by saltwater intrusion.

2. Materials and Methods

A total of 24 different *V. americana* ecotypes were gathered from various regions in Florida (Figure 1) and maintained in culture at the University of Florida IFAS Ft. Lauderdale Research and Education Center in Davie, FL, USA. Plastic 0.4 L (14 oz) containers were filled with coarse silica sand (Banaszak Concrete Corporation, Davie, FL, USA), and amended via incorporation of 2.0 g per container of controlled-release fertilizer (Osmocote Plus $15N{:}9P_2O_5{:}12K_2O$ formulated for 220-day release; ICL Specialty Fertilizers, Dublin, OH, USA). Filled containers were planted with a single 12 to 15 cm long plant of *V. americana*, and 24 containers were prepared for each ecotype to provide four replications per salinity level. Planted containers were maintained in six separate 1700 L HDPE tanks filled with pond water (salinity 0.2 ppt) for four weeks to allow establishment of plants.

Figure 1. Twenty four ecotypes of *Vallisneria americana* were collected from various locations within the state of Florida. Each circle (●) represents the approximate collection site.

After four weeks, Instant Ocean aquarium mix (Spectrum Brands Company, Cincinnati, OH, USA), was used to mimic natural seawater salinity [13] and was added to each tank to reach target salinity levels of 2.0, 4.0, 10.0, 15.0, and 20.0 ppt. An untreated control tank was not treated with salt but instead retained the natural pond water salinity of 0.2 ppt. Additional pond water was added to all tanks as needed to compensate for evaporation and to maintain salinity levels within ±0.7 ppt of the target level. Data loggers (HOBO Water Temperature Pro v2 Data Logger-U22-001, Onset HOBO Data Loggers, Bourne, MA, USA) were placed in four randomly selected tanks to record water temperature for the duration of the experiment. Salinity and pH were monitored weekly using a portable TDS/conductivity meter (Oakton Con 110, Oakton Instruments, Vernon Hills, IL, USA) and a handheld pH/mV/thermometer (IQ 150, Spectrum Technologies, Inc., Plainfield, IL, USA), respectively.

After five weeks of salinity exposure, all plants were individually evaluated by three trained individuals and assigned a visual quality score on a 0 (complete plant death) to 10 (no visible damage) scale. All live aboveground biomass was then destructively harvested; plant material was rinsed to remove algae and other debris and placed in a forced-air oven at 65 °C for two weeks before weighing to obtain dry weights. Mean daily growth rate was evaluated by the method adapted from Hunt [14]:

$$RGR = (\ln DW2 - \ln DW1)/(T2 - T1), \quad (1)$$

in which DW1 refers to total dry weight of sample at the beginning of the experiment (T1=0), and DW2 after the final harvest (T2=35). For measuring the initial biomass (DW1) four extra pots of each ecotype were harvested at the start of the experiment before increasing salinity levels.

Lethal concentration (LC_{50}) is the salinity concentration that reduces plant biomass and visual quality by half compared to the salinity concentration where plants had the best performance (in these experiments, 2.0 ppt). A nonlinear regression was used to fit visual quality and dry weight of each ecotype along the salinity gradient [15] and LC_{50} estimates for visual rating (LC_v) and dry weight (LC_d) data sets was calculated using the method described by Gettys and Haller [16]. Statistical analysis was performed using JMP® Pro 14.0.1 (SAS Institute Inc., Cary, NC). Dry weight, visual rating, and growth rate data sets were analyzed using standard least square analysis, and Tukey-Kramer was performed where significant differences were detected ($P < 0.05$). To provide an overall ranking of the relative performance of ecotypes under the salinity treatments, ecotypes were numerically ranked from "best" to "worst" based on visual rating, growth rate, dry weights, LC_v and LC_d. The mean of these five ranking values was then calculated for each ecotype. Ecotypes with tied mean ranks were given the same overall ranking.

3. Results

3.1. Environmental conditions

pH remained consistent throughout the experiment and ranged from 7.8 to 9.2 with no differences among treatments. Temperature was similar in all mesocosms and mean temperature ranged from 28.2 to 29.3 °C.

3.2. Impact of increased salinity on V. americana

Increased salinity significantly affected visual rating of *V. americana* (P < 0.0001; Table 1). For example, at 0.2 ppt visual rating averaged 6.7 among all ecotypes but at 2.0 ppt visual rating increased to 8.0 on average (Figure 2). Visual rating significantly decreased at 10.0 ppt (2.9) and at 15.0 ppt most ecotypes were obviously stressed, with an average visual rating of 0.8. All *V. americana* ecotypes were eliminated at 20.0 ppt (0.0). Increased salinity also impacted the growth rate of *V. americana* (P < 0.0001; Table 1). All ecotypes had an average growth rate of 14 mg day^{-1} at 0.2 ppt, but at 2.0 ppt growth rates were increased and averaged 22 mg day^{-1} among ecotypes (Figure 3). Growth rates decreased to 13 and 10 mg day^{-1} at 4.0 and 10.0 ppt, respectively, and at 15.0 ppt, plants lost biomass, with a growth rate of -3 mg day^{-1}, on average.

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Table 1. Two-way analysis of variance showing the effect of salinity concentration (2.0, 4.0, 10.0, 15.0 and 20.0 ppt), ecotype (24 different ecotypes) and their interaction on visual rating and growth rate of *Vallisneria americana*.

Parameter	Source	DF	F Ratio	Prob > F
Visual rating	Ecotype	25	11.02	<.0001
r^2=0.87	Salinity	4	508.43	<.0001
	Ecotype*Salinity	100	2.95	<.0001
Growth rate	Ecotype	25	9.64	<.0001
r^2=0.71	Salinity	4	88.71	<.0001
	Ecotype*Salinity	100	3.33	<.0001

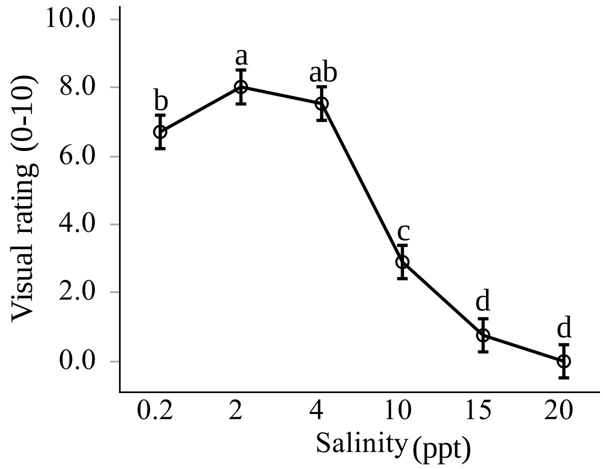

Figure 2. Visual rating of *Vallisneria americana* ecotypes. Each circle and error bar represent 96 observations. Letter differences on top of the bars denote significant (*P* < *0.05*) differences among ecotypes.

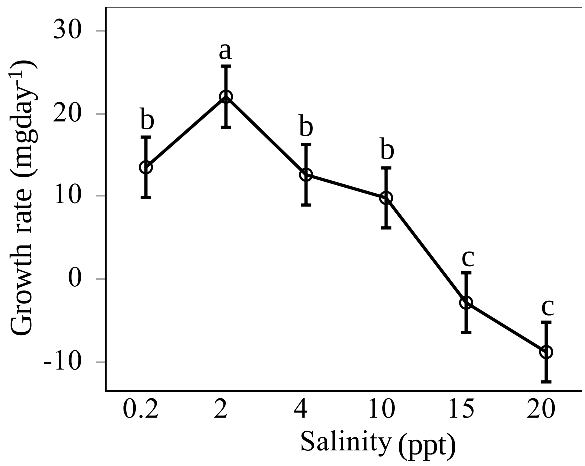

Figure 3. Growth rates (mg day^{-1}) of *Vallisneria americana*. Each circle and error bar represent 96 observations. Letter differences on top of the bars denote significant (*P* < *0.05*) differences among ecotypes.

3.2. Variability among V. americana ecotypes

Ecotypes of *V. americana* responded differently to increased salinity (P < 0.0001; Table 1). For example, Bird, Kennedy and Toho ecotypes had the highest visual rating among ecotypes and averaged 6.8, 4.9 and 4.9, respectively (Table 2). Trafford, Weekie and Harris had the lowest visual ratings, which averaged 3.3, 3.2 and 2.8, respectively. Bird, George and Mann had the highest growth

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rates among ecotypes and averaged 40, 16 and 10 mg day-1, respectively, across the salinity gradient (Table 2). Ecotypes with the lowest growth rates were Snarrow, Fairview and Caloosa, which averaged -1, -5 and -5 mg day-1, respectively. Few ecotypes were unaffected by the salinity treatments. For example, visual rating and growth rate of Caloosa, Rainbow, and Snarrow ecotypes did not differ across the salinity gradient (P > 0.05; Table A1 and A2). Also, the growth rates of STA and Suwanee ecotypes were not affected by increased salinity, but their visual ratings differed among salinity levels.

Table 2. Visual rating (0-10) and growth rate (mg day^{-1}) of each *Vallisneria americana* ecotype is compared across different salinity levels. Plants were assigned a visual quality score on a numerical scale of 0 through 10, where 0 = dead; 5 = fair quality, somewhat attractive form and color, little to no chlorosis or necrosis; and 10 = excellent quality, perfect condition, healthy and robust. Growth rates were calculated using initial and final dry weights for the duration of the study.

Ecotype	Visual rating**		Growth rate**	
Bird	6.8	A	40	A
George	4.6	BCD	16	BC
Mann	4.7	BC	10	BCD
Toho	4.9	AB	9	BCDE
Monroe	4.5	BCD	9	BCDE
Ballen	4.8	B	9	BCDE
Okeech	4.2	BCD	8	BCDE
Trafford	3.3	BCDE	7	BCDE
Kennedy	4.9	AB	7	BCDE
Wekiva	3.5	BCDE	4	CDE
Wakulla	3.8	BCD	4	CDE
Rockstar	4.1	BCD	4	CDE
Pierce	3.4	BCDE	4	CDE
Harris	2.8	DEFG	3	CDE
STA	3.7	BCDE	3	CDE
Suwanee*	2.8	CDEFG	2	CDE
Weekie	3.2	BCDEF	2	CDE
Harney	3.5	BCDE	1	CDE
Rainbow*	1.7	EFG	1	CDE
Feather	3.6	BCDE	0	DE
Biven	4.5	BCD	0	DE
Snarrow*	1.0	G	-1	DE
Fairview	4.3	BCD	-5	E
Caloosa*	1.3	FG	-5	E

* Ecotype did not show a significant response to salinity gradient.

** Means in a column with the same letter are not significantly different (*P* > *0.05*).

Calculated LC_{50} values were developed using regression components from visual rating and dry weight data. The r^2 values revealed that the visual rating was more directly related to increased salinity than dry weight and hence had higher r^2 values (Table 3). Based on LC_{50} values, ecotypes were considered different if their 95% confidence intervals (CI) did not overlap. For example, Bird's visual rating was reduced by 50% (LC_v) at a salinity of 9.00 ppt (lower and upper 95% CI 6.58 and 14.24 ppt, respectively), which was higher than LC_v values for Feather, George, Harris, Pierce, Toho and Wekiva. Harris was the most salt-sensitive ecotype and had the lowest LC_v value (1.13 ppt; lower and upper 95% CI 0.86 and 1.62 ppt, respectively). Based on the LC_d values, Bird had 50% reduction in biomass at 9.1 ppt (lower and upper 95% CI 5.55 and >20.00 ppt, respectively), which was higher

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than LC_d values for Wakulla and Wekiva (Table 2). Feather also had a higher LC_d value (and this was more salt-tolerant) than Wakulla and Wekiva.

Table 3. Lethal concentration (LC_{50}) of salt expected to cause a 50% reduction in visual rating (LC_v) and dry weight (LC_d) of *Vallisneria americana* compared with plants grown at 2.0 ppt saline solution (salinity level with the best plant performance). LC_{50}, upper and lower 95% confidence interval are calculated based on nonlinear regressions fitted for visual rating and dry weight data. Ecotypes are considered different if their upper and lower confidence intervals do not overlap.

Ecotype	LC_v	Lower	Upper	r^2	LC_d	Lower	Upper	r^2
Bird	9.00	6.58	14.24	0.76	9.10	5.55	>20.00	0.51
Biven	5.94	4.00	11.53	0.70	7.42	4.30	>20.00	0.45
Mann	5.48	4.12	8.16	0.86	6.65	4.03	18.95	0.57
Rockstar	5.42	3.53	11.65	0.68	9.85	4.59	>20.00	0.24
Fairview	5.37	3.75	9.45	0.76	4.83	2.79	18.28	0.55
Ballen	5.35	3.98	8.17	0.84	5.34	3.76	9.19	0.76
Kennedy	5.28	4.35	6.73	0.93	3.87	2.46	9.01	0.70
Monroe	5.06	3.56	8.77	0.78	7.22	3.82	>20.00	0.39
Okeech	4.78	3.37	8.20	0.80	4.78	2.83	15.28	0.57
Toho	4.64	3.71	6.19	0.92	5.72	3.68	12.77	0.65
George	4.47	3.73	5.59	0.95	4.87	2.94	14.33	0.60
Trafford	4.36	2.61	13.29	0.62	5.92	3.02	>20.00	0.33
STA	4.30	2.99	7.63	0.80	NA	NA	NA	NA
Harney	4.29	2.88	8.45	0.76	7.33	3.60	>20.00	0.31
Feather	4.14	3.16	6.00	0.89	12.74	5.16	>20.00	0.16
Waqulla	3.81	2.46	8.50	0.69	4.68	2.25	>20.00	0.31
Pierce	3.76	2.84	5.58	0.89	2.62	1.16	10.39	0.39
Suwanee	3.76	2.14	15.68	0.59	NA	NA	NA	NA
Weekie	3.45	2.20	7.94	0.74	3.11	1.83	10.44	0.66
Wekiva	2.98	1.99	5.95	0.81	2.09	1.00	>20.00	0.49
Harris	1.13	0.86	1.62	0.93	0.47	0.24	10.54	0.87
Rainbow	NA	NA	NA	NA	NA	NA	NA	NA
Snarrow	NA	NA	NA	NA	NA	NA	NA	NA
Caloosa	NA	NA	NA	NA	NA	NA	NA	NA

NA Ecotype did not show a significant response to salinity gradient.

4. Discussion

Some researchers have categorized salinity stress in plants into phase one (salt shock or osmotic stress) and phase two (ionic stress) [17,18]. Phase one is caused by short-term exposure to high sodium concentrations, which affects plants through imbalanced osmotic pressure and causes wilting. Phase two of salt stress elicits long-term physiological responses such as reduced growth rate and production of osmo-protectant compounds such as sugars, amino acids, and proteins. In our study, we did not intend to study plant response to the short-term salinity stress (salt shock or phase one) and hence plants were exposed to a five-week period of elevated salinity, long enough to assess long-term plant response such as growth rate.

In this experiment, visual rating and growth rate of most ecotypes were affected by salinity treatments; however, a few ecotypes (Suwanee, Rainbow, Snarrow and Caloosa) did not respond to increased salinity concentrations (Table A1). These ecotypes had very low visual ratings and growth rates regardless of salinity levels; therefore, statistical analysis did not detect significant difference among salinity levels. Results from the visual rating evaluations showed that all ecotypes perished at 20.0 ppt, which suggests that this level was higher than tolerable salinity for *V. americana* (Figure 2). Most ecotypes survived five weeks of exposure to 15.0 ppt salinity, but Caloosa, Snarrow, Feather, and STA did not (Table A2). These four ecotypes had very low growth rates across all salinity levels and at 15.0 ppt they lost shoots more quickly than they were able to replace via normal growth. When the rate of shoot loss increased, they failed to maintain enough photosynthesizing tissue and were decimated (Table A2). This is supported by research conducted by Munns [19], who reported that salt-stressed plants tend to accumulate salts in their older tissues, and when salt concentration in old leaves reaches a toxic level, plants drop their "old" leaves and rely on new growth for photosynthesis. Prolonged salinity exposure could lead to a complete loss of photosynthesizing tissue and ultimately kill the plant. At 15.0 ppt, several other ecotypes such as Toho and Mann lost shoots at very high rates (-27 to -15 mg day^{-1}, respectively), but they accumulated enough photosynthesizing tissue to survive five weeks of 15.0 ppt salinity exposure (Table A2). Bird and Trafford ecotypes had positive growth rates (5 to 25 mg day^{-1}) at 15.0 ppt, which means that their biomass accumulation surpassed their leaf deterioration and shoot loss. Although this experiment ran for five weeks, one could expect that at a given salinity, ecotypes with positive growth rate could tolerate longer salinity exposure (more than five weeks). However, the ability to endure longer salinity exposure does not necessarily make a species or ecotype salt tolerant.

It is suggested that salt tolerant species exhibit stimulated growth under increased salinity until salinity concentration reaches toxic level [20]. In our experiment, we observed that most ecotypes had higher growth and visual rating at 2.0 ppt compared to 0.2 ppt (no salt added) (Figure 2 and 3). We could argue that an increase in growth at such a low salinity concentration (2.0 ppt) could be a hormetic response and not an indication of salt tolerance [12]. Hormesis is defined as the stimulation of growth by low levels of toxic compounds [21]. In addition, the aquarium salt mix used in this study for increasing salinity concentration has a complex elemental composition similar to natural seawater and contains macro and micronutrients [13]. At 2.0 ppt concentration, these nutrients could enhance plant growth, provided the concentration of harmful compounds (e.g., sodium and chlorine) remain below toxic levels. Nevertheless, 2.0 ppt salinity could have indirectly increased growth of *V. americana* by limiting growth of other competing organisms such as algae.

In our experiment, Bird had the highest growth rate among ecotypes and performed best under 15.0 ppt, for instance it had visual rating of 5.3 and growth rate of 25 mg day^{-1} at 15.0 ppt which were only decreased by 53% and 32% compared to 2.0 ppt treatment. Reduced growth under salt stress is a common observation in salt-sensitive plants, yet the question remains whether high growth rate per se could impart salt tolerance. Lee et al. [22] used growth curves to study salt tolerance among *Paspalum vaginatum* ecotypes and suggested that under salinity condition ecotypes with higher growth rates could be considered salt tolerant. Conflicting results were reported by Marcum and Murdoch [23], who found that salt tolerant ecotypes of *P. vaginatum* had lower growth rates than salt-sensitive ecotypes. In another example, salt-tolerant ecotypes of *Arabidopsis* used slow growth as a mechanism to better partition sodium into shoots and hence ecotypes with lower growth rates had greater ability for tolerating salt [24]. Rawson et al. [25] conducted an experiment on three species of barley, wheat and triticale, and argued that greater growth under salinity conditions does not infer greater salt tolerance. They suggested that measuring high growth under the absence of salt is a better indicator of salt tolerance than growth rate under increased salinity. In our experiment, STA and Feather ecotypes were ranked 15 and 20 for growth rate and died at 15.0 ppt, while ecotypes with lower growth rates such as Biven and Fairview (ranked 21 and 23 for growth rate, respectively) survived at 15.0 ppt. Consequently, lower growth rate does not translate to lower salt tolerance and high growth rate does not necessarily mean that a plant is salt-tolerant, but adequate growth may allow for potential recovery from salt injury [22].

In this study, visual ratings allowed us to accurately evaluate the health and survival of the plants, and dry weight data were used to calculate growth rate of each ecotype. Both data sets were utilized for the nonlinear regression analysis and calculating LC_{50} values. Nonlinear regression can be legitimately used for estimating LC_{50} values if the experimental design includes adequate coverage of the response range for treatments (i.e., different salinity levels) and having more than five

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treatments that include lethal and sublethal concentrations increases the likelihood of having an accurate regression [15,26]. In this study, we had six salinity levels which would provide a good response range for the regression; however, as discussed in the previous section, at 2.0 ppt ecotypes had better visual quality and produced larger biomass than 0.2 ppt. Inclusion of 0.2 ppt in the analysis would cause overestimation of LC_{50} estimates. Therefore, 0.2 ppt was removed from the analysis and 2.0 ppt was considered the control treatment for LC_{50} analyses, so our treatments were decreased to 5 levels instead of having 6 salinity treatments (0.2, 2.0, 4.0, 10.0, 15.0 and 20.0 ppt).

The ecotype that performed best based on overall rankings was Bird, with highest dry weight, growth rate, and visual quality (Table 4). LC_{50} values indicated that Bird has an exceptional ability to grow under high-salinity conditions and loses half of its biomass only when salinity concentration is 9.10 ppt. High growth rate is critically important for restoration and revegetation project to succeed, because introduced plants and transplants need to quickly establish at the target site to survive herbivory and competition with existing vegetation. Based on our results, Bird ecotype could be a good candidate for restoration purposes because it had the highest growth rate among ecotypes (40 mg day^{-1}, on average) (Table 2 and 4).

Table 4. *Vallisneria americana* ecotypes are ranked based on visual rating, growth rate, dry weight and lethal concentration (LC_{50}) estimated using visual rating (LC_v) and dry weight (LC_d) models. In each column ecotypes are ranked from best (1) to worst (24).

Ecotype	Visual rating	Growth rate	Dry weight	LC_v	LC_d	Mean rank	Overall ranking	
Bird	1	1	1	1	4	1.6	1	A
Mann	5	3	5	3	9	5	2	AB
Toho	3	4	3	10	11	6.2	3.5	AB
Ballen	4	6	4	6	12	6.4	3.5	AB
George	6	2	2	11	14	7	5	ABC
Monroe	8	5	10	8	8	7.8	6.5	ABCD
Rockstar	11	12	11	4	2	8	6.5	ABCD
Biven	7	21	7	2	6	8.6	8	ABCDE
Kennedy	2	9	12	7	19	9.8	9	ABCDEF
Trafford	18	8	6	12	10	10.8	10.5	ABCDEF
Okeech	10	7	13	9	17	11.2	10.5	ABCDEF
Fairview	9	23	8	5	16	12.2	12.5	ABCDEF
STA*	13	15	16	14	3	12.2	12.5	ABCDEF
Feather	14	20	14	16	1	13	14.5	BCDEFG
Wakulla	12	11	9	17	18	13.4	14.5	BCDEFG
Harney	15	18	21	15	7	15.2	16	BCDEFG
Suwanee*	20	16	19	19	5	15.8	17	BCDEFG
Wekiva	16	10	15	22	22	17	18	DEFG
Pierce	17	13	20	18	21	17.8	19	CDEFG
Weekie	19	17	18	21	20	19	20.5	FG
Snarrow*	24	22	24	13	13	19.2	20.5	EFG
Rainbow*	22	19	22	20	15	19.6	22.5	FG
Harris	21	14	17	24	24	20	22.5	FG
Caloosa*	23	24	23	23	23	23.2	24	G

* Ecotype did not show a significant response to salinity gradient.

In this study, all environmental conditions such as sunlight, water depth, temperature, and salinity levels were equal among ecotypes, yet there were drastic differences in response to salinity treatments. For instance, Harris, with an overall ranking of 22.5, had a growth rate of 3 mg day^{-1}, on

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average, which was 13x and 5x less than Bird's and George's growth rate, and Bird's growth rate was 2.5x higher than George (Table 2). Harris lost half its biomass at 0.80 ppt (LC_d), which is 11-fold and 6-fold lower than the LC_d values calculated for Bird and George ecotypes (Table 3). These drastic differences could be derived from differences in growth traits and biomass allocation, for example, production of stolons, roots, and other traits such as leaf elongation and leaf area [22,27]. Measuring these traits could provide valuable information for the comparison of ecotypes; however, it was not feasible for the scale of our experiment.

Various experiments have reported that *V. americana* species can tolerate salinity concentrations between 5.0 to 15.0 ppt [8,28,29]. Although research methodologies vary and ecotypes used in experiments are different, we can confirm that all *V. americana* ecotypes used in this study survived exposure to 10.0 ppt salinity. Ecotypes may lose as much as 50% of their biomass upon exposure to 5.15 ppt salinity concentration (on average), and 20 out of 24 ecotypes tolerated five weeks of exposure to 15.0 ppt. Salt tolerance thresholds reported in different experiments are highly dependent on the method of salinity induction/initiation (i.e., abrupt vs. gradual salinity increase), elemental composition of salt used (i.e., seawater vs. artificial salts), period/length of exposure [13,30], and the ecotype used in the experiment. Nevertheless, salt tolerance is a natural phenomenon with a complex mechanism. Sea level rise and saltwater intrusion impose pressure on plant populations to gradually evolve specialized population that can tolerate higher salinity conditions [7]. It is possible that ecotypes of *V. americana* with higher tolerance or sensitivity to salt could exist but were not included in our limited ecotype selection.

5. Conclusions

Our results revealed that *V. amerciana* is significantly affected by increased salinity. Growth rate and visual quality ratings along the salinity gradient varied among ecotypes, which suggests that ecotypes respond differently to salt stress. Final dry weight measurement was not as good as visual quality rating for assessment of plants under salt stress because stressed plants had discoloration or altered leaf shape, size and/or width, and dry weight measurement is not able to detect such symptoms in plants. Also, dead plants retain biomass, which may not correlate well with the level of stress that plants have experienced. Therefore, visual quality rating is a better indicator of plant health if performed properly. Ecotypes with lower growth rates did not die at lower salinity concentration; hence, growth rate per se is not the main determinant of salt tolerance, although the ecotype with the highest growth rate performed better than others.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Effect of salinity on visual rating and growth rate of *Vallisneria americana* ecotypes.

Ecotype	Parameter	N	Df	F ratio	prob>F
Ballen	Visual rating	5	5	38.06	<0.0001
Ballen	Growth rate	5	5	6.92	0.0009
Bird	Visual rating	5	5	36.89	<0.0001
Bird	Growth rate	5	5	4.49	0.0079
Biven	Visual rating	5	5	5.99	0.0020
Biven	Growth rate	5	5	3.59	0.0198
Caloosa*	Visual rating	5	5	2.05	0.1262
Caloosa*	Growth rate	5	5	1.25	0.3314
Fairview	Visual rating	5	5	12.30	<0.0001
Fairview	Growth rate	5	5	5.11	0.0048
Feather	Visual rating	5	5	59.27	<0.0001
Feather	Growth rate	5	5	14.27	<0.0001
George	Visual rating	5	5	125.10	<0.0001
George	Growth rate	5	5	5.70	0.0029
Harney	Visual rating	5	5	17.14	<0.0001
Harney	Growth rate	5	5	3.85	0.0163
Harris	Visual rating	5	5	17.55	<0.0001
Harris	Growth rate	5	5	9.87	0.0001
Kennedy	Visual rating	5	5	151.93	<0.0001
Kennedy	Growth rate	5	5	5.47	0.0031
Mann	Visual rating	5	5	13.11	<0.0001
Mann	Growth rate	5	5	9.88	0.0001
Monroe	Visual rating	5	5	27.42	<0.0001
Monroe	Growth rate	5	5	7.61	0.0005
Okeech	Visual rating	5	5	74.65	<0.0001
Okeech	Growth rate	5	5	10.68	<0.0001
Pierce	Visual rating	5	5	26.40	<0.0001
Pierce	Growth rate	5	5	3.04	0.0387
Rainbow*	Visual rating	5	5	2.56	0.0670
Rainbow*	Growth rate	5	5	1.57	0.2225
Rockstar	Visual rating	5	5	10.78	<0.0001
Rockstar	Growth rate	5	5	3.28	0.0281
Snarrow*	Visual rating	5	5	2.41	0.0797
Snarrow*	Growth rate	5	5	1.74	0.1802
STA	Visual rating	5	5	14.32	<0.0001
STA*	Growth rate	5	5	1.97	0.1349
Suwanee	Visual rating	5	5	3.82	0.0155
Suwanee*	Growth rate	5	5	1.57	0.2199
Toho	Visual rating	5	5	154.74	<0.0001
Toho	Growth rate	5	5	14.59	<0.0001
Trafford	Visual rating	5	5	7.26	0.0007
Trafford	Growth rate	5	5	3.00	0.0383
Wakulla	Visual rating	5	5	8.37	0.0004
Wakulla	Growth rate	5	5	3.28	0.0297
Weekie	Visual rating	5	5	6.96	0.0009

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Weekie	Growth rate	5	5	6.59	0.0012
Wekiva	Visual rating	5	5	13.99	<0.0001
Wekiva	Growth rate	5	5	3.24	0.0292

* Ecotype did not show a significant response to salinity gradient based on standard least square analysis ($P < 0.05$).

Table A2. Effect of salinity on visual rating and growth rate of *Vallisneria americana* ecotypes. Plants were assigned a visual quality score on a numerical scale of 0 (complete plant death) through 10 (no visible damage). Growth rate was calculated using Hunt [14] method.

Ecotype	Salinity	Visual rating (0-10)		Growth rate (mg day^{-1})	
Ballen	0.2	9.00	A	30	AB
Ballen	2.0	9.00	A	42	A
Ballen	4.0	10.00	A	26	AB
Ballen	10.0	4.25	B	4	ABC
Ballen	15.0	0.75	C	-8	BC
Ballen	20.0	0.00	C	-21	C
Bird	0.2	9.00	A	56	A
Bird	2.0	10.00	A	77	A
Bird	4.0	10.00	A	44	AB
Bird	10.0	8.50	A	63	A
Bird	15.0	5.25	B	25	AB
Bird	20.0	0.00	C	-12	B
Biven	0.2	5.75	AB	-6	AB
Biven	2.0	8.50	A	15	A
Biven	4.0	8.00	A	5	AB
Biven	10.0	5.00	AB	4	AB
Biven	15.0	1.00	B	-8	AB
Biven	20.0	0.00	B	-17	B
Caloosa	0.2	1.33	NS**	-4	NS**
Caloosa	2.0	4.00	NS	-4	NS
Caloosa	4.0	1.50	NS	-5	NS
Caloosa	10.0	1.00	NS	-5	NS
Caloosa	15.0	0.00	NS	-7	NS
Caloosa	20.0	0.00	NS	-7	NS
Fairview	0.2	4.75	ABC	-11	AB
Fairview	2.0	7.67	AB	9	AB
Fairview	4.0	9.25	A	13	A
Fairview	10.0	4.00	BCD	-7	AB
Fairview	15.0	0.50	CD	-20	B
Fairview	20.0	0.00	D	-20	B
Feather	0.2	6.67	A	6	AB
Feather	2.0	8.00	A	1	BC
Feather	4.0	7.75	A	3	B
Feather	10.0	2.25	B	16	A
Feather	15.0	0.00	C	-9	C
Feather	20.0	0.00	C	-9	C
George	0.2	10.00	A	45	AB

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George	2.0	10.00	A	62	A
George	4.0	8.75	A	26	ABC
George	10.0	3.33	B	12	ABC
George	15.0	1.00	C	-1	BC
George	20.0	0.00	C	-21	C
Harney	0.2	5.25	A	1	AB
Harney	2.0	7.00	A	2	AB
Harney	4.0	9.00	A	6	A
Harney	10.0	1.25	B	1	AB
Harney	15.0	0.25	B	-2	B
Harney	20.0	0.00	B	-3	B
Harris	0.2	6.75	AB	10	AB
Harris	2.0	9.75	A	20	A
Harris	4.0	2.75	BC	-1	B
Harris	10.0	1.00	C	1	B
Harris	15.0	0.25	C	-2	B
Harris	20.0	0.00	C	-2	B
Kennedy	0.2	9.25	A	18	AB
Kennedy	2.0	9.75	A	11	A
Kennedy	4.0	9.25	A	32	AB
Kennedy	10.0	4.50	B	10	AB
Kennedy	15.0	1.00	C	-2	B
Kennedy	20.0	0.00	C	-5	B
Mann	0.2	7.50	A	36	AB
Mann	2.0	9.00	A	21	A
Mann	4.0	8.75	A	13	AB
Mann	10.0	5.25	AB	8	A
Mann	15.0	0.50	BC	0	B
Mann	20.0	0.00	C	-2	B
Monroe	0.2	8.75	A	8	AB
Monroe	2.0	8.50	A	9	ABC
Monroe	4.0	10.00	A	7	A
Monroe	10.0	3.50	B	4	ABC
Monroe	15.0	0.50	B	1	BC
Monroe	20.0	0.00	B	-1	C
Okeech	0.2	10.00	A	0	A
Okeech	2.0	8.00	A	4	AB
Okeech	4.0	10.00	A	0	BC
Okeech	10.0	2.75	B	0	BC
Okeech	15.0	0.25	C	0	C
Okeech	20.0	0.00	C	-1	C
Pierce	0.2	6.75	A	8	A
Pierce	2.0	7.67	A	5	A
Pierce	4.0	7.25	A	13	A
Pierce	10.0	1.50	B	12	A
Pierce	15.0	0.50	B	-4	A
Pierce	20.0	0.00	B	-6	A

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Rainbow	0.2	2.75	NS	-1	NS
Rainbow	2.0	5.00	NS	-2	NS
Rainbow	4.0	2.00	NS	1	NS
Rainbow	10.0	0.75	NS	-2	NS
Rainbow	15.0	0.75	NS	-2	NS
Rainbow	20.0	0.00	NS	-2	NS
Rockstar	0.2	7.50	AB	9	A
Rockstar	2.0	7.00	AB	4	A
Rockstar	4.0	9.75	A	8	A
Rockstar	10.0	3.25	BC	7	A
Rockstar	15.0	0.50	C	0	A
Rockstar	20.0	0.00	C	-4	A
Snarrow	0.2	1.50	NS	2	NS
Snarrow	2.0	0.33	NS	3	NS
Snarrow	4.0	4.25	NS	9	NS
Snarrow	10.0	0.25	NS	0	NS
Snarrow	15.0	0.00	NS	2	NS
Snarrow	20.0	0.00	NS	-3	NS
STA	0.2	5.25	AB	18	NS
STA	2.0	7.75	A	54	NS
STA	4.0	8.67	A	23	NS
STA	10.0	2.00	BC	23	NS
STA	15.0	0.00	C	-27	NS
STA	20.0	0.00	C	-27	NS
Suwanee	0.2	3.50	NS	-11	NS
Suwanee	2.0	6.50	NS	28	NS
Suwanee	4.0	5.75	NS	15	NS
Suwanee	10.0	0.75	NS	0	NS
Suwanee	15.0	1.00	NS	5	NS
Suwanee	20.0	0.00	NS	-11	NS
Toho	0.2	9.25	A	7	A
Toho	2.0	10.00	A	32	A
Toho	4.0	10.00	A	-4	A
Toho	10.0	3.75	B	8	A
Toho	15.0	0.50	C	-4	B
Toho	20.0	0.00	C	-11	B
Trafford	0.2	7.25	AB	7	A
Trafford	2.0	6.75	AB	11	A
Trafford	4.0	7.75	A	5	A
Trafford	10.0	1.50	BC	0	A
Trafford	15.0	0.50	C	-3	A
Trafford	20.0	0.00	C	-3	A
Wakulla	0.2	6.50	AB	10	AB
Wakulla	2.0	9.67	A	20	A
Wakulla	4.0	5.00	ABC	7	AB
Wakulla	10.0	2.25	BC	-1	AB
Wakulla	15.0	2.00	BC	-1	AB

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Wakulla	20.0	0.00	C	-4	B
Weekie	0.2	6.00	AB	30	AB
Weekie	2.0	8.00	A	42	A
Weekie	4.0	6.25	AB	26	ABC
Weekie	10.0	1.25	BC	4	BC
Weekie	15.0	0.50	BC	-8	BC
Weekie	20.0	0.00	C	-21	C
Wekiva	0.2	7.25	A	56	AB
Wekiva	2.0	9.25	A	77	A
Wekiva	4.0	6.25	A	44	AB
Wekiva	10.0	1.00	B	63	AB
Wekiva	15.0	0.75	B	25	AB
Wekiva	20.0	0.00	B	-12	B

Means in a column with the same letter are not significantly different ($P < 0.05$).

NS** Ecotype did not show a significant response ($P > 0.05$) to salinity gradient based on standard least square analysis.

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