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Seeking Generalities in Salt Stress Effects on Herbivores: A Multi-Species Approach

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SEEKING GENERALITIES IN SALT STRESS EFFECTS ON HERBIVORES:
A MULTI-SPECIES APPROACH

by

Christy Marie Foust

A thesis submitted to the Department of Biology
in partial fulfillment of the requirements for the degree of

Master of Science in Biology

University of North Florida

College of Arts and Sciences

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CHAPTER 1

Seeking generalities in salt stress effects on herbivores: A multi-species approach

ABSTRACT

Stress has been identified as one of the primary factors influencing the structure of food webs, but few conclusions about the effects of stress on community dynamics have emerged. This study examined generalities that exist between salt stress and insect herbivore responses. Ambient salinity was artificially increased by adding 1.3 kg/m² of salt to six different plant species: *Avicennia germinans*, *Baccharis halimifolia*, *Batis maritima*, *Borrchia frutescens*, *Iva frutescens*, and *Spartina alterniflora*. By performing a multiple species examination, stress responses were examined to determine if consistencies within and between plant type, ambient salinity conditions, and host plant species (among other criteria) existed, allowing for generalizations to be made about stress and herbivore interactions. Herbivore responses were determined by direct visual counts and indirect visual evidence of insects (e.g. galls, stem boring, and leaf mining). A meta-analysis was then performed on the data to determine the factors that may result in consistent responses to salt stress. No differences were found between insect feeding guilds. Herbivory increased in the presence of that added salt in high ambient salinity conditions, increased on experimental *Spartina* plants, and

decreased on experimental *Iva* plants. These findings suggest that stress is generally a more important factor for plants in areas of already high ambient salinity. Results have been disparate in various studies, likely due to the difficulty in making comparisons between different field sites and experimental designs. This study addresses these issues, and ultimately finds that consistencies can be found among multiple species within communities.

INTRODUCTION

Stress is hypothesized to be one of the primary abiotic factors that influence the structuring of food webs (Sousa, 1960; Menge & Sutherland, 1987; Hacker & Gaines, 1997). To date, however, no consensus has emerged regarding the effects of salt-induced and other types of stress on trophic and community dynamics (White, 1978; Price, 1991; Bowdish & Stiling, 1998; Gonçalves-Alvim *et al.*, 2001; Moon & Stiling, 2002a). A number of researchers have suggested that differences in methodology and inherent abiotic variations in study sites may mask any generalities that might be present in past studies (Pennings & Moore, 2001; Goranson *et al.*, 2004; Moon & Stiling, 2004). Alternatively, each plant's osmoregulatory mechanisms could affect herbivory positively or negatively, and Price's (1991) "plant vigor" and White's (1978) "plant stress" hypotheses reflect the possibility for differences in effects based on plant species, plant quality, and herbivore type. These contrasting results further emphasize the myriad of variables affecting the suitability of each plant for herbivory, oviposition, and parasitism. They also underscore the need for a multi-species approach to the examination of stress effects on trophic interactions.

White (1978) and Price (1991) proposed two different hypotheses, based upon the plant's physiological and nutritional status, addressing the contradictory nature of these data; the plant stress hypothesis and the plant vigor hypothesis respectively. White's (1978) plant stress hypothesis predicts that herbivores will preferentially attack a physiologically stressed host both because stressed plants tend to mobilize nutritional amino acids in their tissues and produce fewer volatile, defensive

chemicals. Many studies have corroborated this hypothesis (White, 1970, 1976, 1984; Raffa & Berryman, 1982; Roades, 1983; Mattson & Haack, 1987; Fernandes & Price, 1988, 1991; DeBruyn, 1995; Cobb *et al.*, 1997). Conversely, Price's (1991) plant vigor hypothesis predicts that herbivores will choose vigorously growing plants or shoots, since young plants or plant parts may lack defensive chemicals and/or physical defenses yet contain high levels of available nitrogen. Numerous studies have also supported this hypothesis (Price *et al.*, 1987, 1990, 1997; Price, 1991; Hunter & Price, 1992; Feller, 1995; Preszler & Price, 1995; Feller & Mathis, 1997). Price (1991) recognized that the two hypotheses do not have to be mutually exclusive, since an herbivore could choose to eat the most vigorous portion of a stressed plant (Gonçalves-Alvim *et al.*, 2001). The diverse feeding mechanisms employed by the herbivores in the study system as well as the unique physiological and physical mechanisms employed by the plants in response to salt stress are likely to support instances of both the plant vigor and the plant stress hypotheses. These contrary findings result in little understanding of the overall effects of salt stress on these plant/insect systems. Differences in herbivore feeding modes and plant adaptations to salt stress, as well as the contradictory nature of data obtained from past studies, necessitate a multi-species approach when investigating the effects of stress on these insect/plant interactions.

Complex mechanisms of plant tolerance, avoidance, osmoregulation, and defense can influence the relationships between stressed plants and their herbivores in a variety of ways (Gonçalves-Alvim *et al.*, 2001; Moon *et al.*, 2000; Moon & Stiling,

2002a) and may further mask any generalities that may exist. Since each plant species has unique physiological methods for dealing with salt stress and exhibit different signs of salt stress, herbivory could be positively or negative affected (Bowdish & Stiling, 1998). For example, *Pissonotus quadripustulatus* (Homoptera: Delphacidae) planthoppers preferentially oviposit in the lower quality, woody stems of stressed *Borrchia* plants versus the higher quality, green stems (Moon & Stiling, 2006). Though *P. quadripustulatus* loses more eggs to plant-related mortality, this oviposition choice reduces parasitism by the wasp *Anagrus* sp. (Hymenoptera: Mymaridae), which have difficulty penetrating the woody epidermis of the plant with its ovipositor (Moon & Stiling, 2006). Therefore, *P. quadripustulatus* populations benefit from the stressed condition of *Borrchia*, while the parasitoids do not. Conversely, Denno *et al.* (1986) found that *Prokelisia marginata* (Homoptera: Delphacidae) prefer to feed on more vigorous members of the *Spartina* population to maximize growth, which suggests that high salinities would have a negative impact on *P. marginata* populations.

Phytophagous insects also influence these relationships since different herbivore feeding guilds have been shown to react to different plant stress conditions in different ways, thereby resulting in both positive and negative correlations (e.g. Denno *et al.*, 1986; Moon & Stiling, 2002b; Moon & Stiling, 2004). For example, Moon and Stiling (2002a) found that salt treatments decreased the number of *Asphondylia borrichiae* (Diptera: Cecidomyiidae) galls on *Borrchia*, which would support the plant vigor hypothesis. Contrary to this, Gonçalves-Alvim *et al.*

(2001) found an increase in *Cecidomyia avicenniae* (Diptera: Cecidomyiidae) galls with increased salinity on *Avicennia* located along a natural salinity gradient on a South American study site which supports the plant stress hypothesis. Similarly, several studies found that *Asphondylia borrichiae* (Diptera: Cecidomyiidae) females preferred to oviposit on *Borrichia* plants that received fertilizer, shade (Rossi & Stiling, 1998), or on plants that existed in higher ambient nitrogen conditions (Stiling & Rossi, 1996). Both plant species undergo morphological changes in high salt conditions (Gonçalves-Alvim *et al.*, 2001; Moon & Stilling, 2002a) which could affect the suitability of each plant to act as galling sites for each herbivore species. Similarly, Rossi and Stiling (1998) found that shade and fertilizer resulted in an increase of *Asphondylia borrichiae* (Diptera: Cecidomyiidae) galls and plant leaf nitrogen on *Borrichia*, with an even greater increase in galls when the treatments were combined.

Since stress is such an important factor influencing biotic communities, it is important that scientists fully understand how variable abiotic conditions associated with climate change affect these communities. It has been estimated that the world sea-level rise for the 20th century was 12-22 cm (Gilman *et al.*, 2008). Many climate models suggest that the rate of sea-level rise could accelerate in the coming decades (Church *et al.*, 2001, 2004; Cazemave & Nerem, 2004; Holgate & Woodworth, 2004; Thomas *et al.*, 2004; Church & White, 2006; Solomon *et al.*, 2007) and global sea-level projections through the end of the 21st century are 0.18-0.59 m (Solomon *et al.*, 2007), with the upper projections being more likely to occur (Church & White, 2006). Since increased salinities in coastal ecosystems, including salt marshes, can be a direct

result of relative sea-level rise by increasing salt water intrusion (Day & Templet, 1989; Ross et al., 1994), understanding the effects of salt stress on plants becomes an even more important question to address.

The primary focus of this study was to examine the effects of increased salinity on herbivore interactions with six native coastal plant species (hereafter referred to by genus name): *Avicennia germinans* (black mangrove), *Iva frutescens* (marsh elder), *Baccharis halimifolia* (salt bush), *Batis maritima* (saltwort), *Borrichia frutescens* (sea ox-eye daisy), and *Spartina alterniflora* (smooth cordgrass). Each of these plants is common to salt marshes in northeast Florida and represent dominant species at the research site (pers. obs.). As with many salt marsh plants, these species exist in distinct zones of vegetation based on the tide levels (Miller & Egler, 1950; Chapman, 1974; Nixon, 1982; Zedler, 1982) and salinity gradients (Oosting & Billings, 1942; Ehrenfeld, 1990; Stalter & Odum, 1993). *Iva* and *Baccharis* tend to grow in areas of lower salinity, with *Baccharis* being less salt-tolerant than *Iva* (Tolliver et al., 1997). Of the three herbaceous species examined in this study, *Borrichia* is the least salt tolerant and tends to be found in the back marsh near *Iva* and *Baccharis*. Moon and Stiling (2004) found *Borrichia* in areas with a salinity of approximately 25 ppt. while *Spartina* is located in the front marsh and is found in salinities similar to *Borrichia* (Pennings & Moore, 2001), although it should be noted that *Spartina* has not been found in back marsh areas due to competition with other plants (Pennings & Moore, 2001). Of the shrub species examined in this study, *Avicennia* is known to be extremely salt tolerant, existing in a broad range of habitats ranging from fresh

water to hypersaline conditions (Suárez *et al.*, 1998). *Batis* can be found in hypersaline conditions but is typically found in euryhaline salinities ranging from 10 to 50 ppt (Pennings & Richards, 1998).

An array of multivoltine herbivores utilize each of these plant species, each with different feeding (e.g. Denno *et al.*, 1986; Moon & Stiling, 2002b; Moon & Stiling, 2004) or ovipositing strategies (e.g. Bowdish & Stiling, 1998; Gonçalves-Alvim *et al.*, 2001; Moon & Stiling, 2002a; Moon & Stiling, 2006). These herbivores are categorized into various guilds based on the above characteristics; including, leaf chewers, leaf galls, phloem and xylem feeders, and leaf miners. A multi-species examination of these complex interactions will allow for a comprehensive assessment of guild responses to treatment versus non-treatment conditions. It is anticipated that herbivores of the same guild will react similarly to stress conditions, which allows for generalizations about the different guilds to be made.

Ultimately, data regarding plant growth, plant health, predation, and herbivore responses to these differences will be utilized to give an in-depth cross-species examination of a salt marsh system stressed by elevated salinity levels. It has been suggested that generalities can not exist within the realm of ecology (Lawton, 1999); however, just because they have not been consistently found in past studies does not mean that they do not exist. Meta-analysis can allow for the quantitative examination of emerging patterns due to combination of these variables including: treatment vs. non-treatment, herbaceous plants vs. shrubs, differences between herbivore feeding guilds, variability in interstitial salinity near each plot or plant, and

salinity tolerance of plants. Using this technique, this study has helped to elucidate which factors influence responses of herbivores to stressed host plants, and to what degree generalities exist in the effects of these factors.

MATERIALS AND METHODS

Twenty *Avicennia germinans*, *Baccharis halimifolia*, and *Iva frutescens* shrubs (respectively; each 1-2 m tall) were haphazardly flagged at the study site, located east and west of Hwy. A1A (approximately 29°40'41" N, 81°13'10" W) in the Guana-Tolomato-Matanzas National Estuarine Research Reserve (GTMNERR). In addition to these shrub species, twenty 1-m² monoculture plots of each herbaceous species were haphazardly established. These species included: *Borrchia frutescens*, *Batis maritima*, and *Spartina alterniflora*. Ten of the shrubs and plots were randomly chosen as control and experimental groups, respectively, and each shrub or plot was separated from other shrubs or plots by approximately 3m. Plants and plot sizes were chosen so that the biomass of all replicates of all species were approximately equivalent.

Salt stress on the plants was increased by distributing 1.3 kg/m² NaCl on the soil around all experimental plants or within experimental plots monthly according to the methods of Bowdish and Stiling (1998). This quantity of salt should increase the interstitial soil salinity around the plants by 20 – 30% (Moon & Stiling, 2000; 2002a), and actual soil salinities were found to increase by 25.3 – 31.4%.

Soil salinity was recorded prior to beginning the experiment, as well as one and two weeks after salt was administered, to ensure that the salt treatment was suitably increasing soil salinity levels. Soil sampling and salinity calculations were performed according to methods modified from Goranson *et al.* (2004). A soil core 15 cm deep was collected using an auger from 30 randomly chosen experimental plots and returned to the lab. The soil was homogenized, the mass of each sample was obtained, and the sample was oven dried. After drying, the mass was obtained again, excess DI water was added to the soil, and a salinity reading was obtained from the liquid with a refractometer. The original soil salinity was calculated with the following equation, adapted from Goranson *et al.* (2004):

$$(\text{diluted salinity} / \text{mL water added}) = (\text{original salinity} / \text{mL water lost}) \quad (1)$$

Herbivore responses to the salt treatment were also assessed monthly from March through October 2008. For the herbaceous species, 30 haphazardly chosen stems or culms per plot were visually inspected for the indirect presence of herbivores, including galled stems and leaves, bored stems, mined leaves, and rolled leaves in addition to the insects themselves. Insect herbivores on 30 haphazardly chosen shrub stem terminals (approximately 10 cm of leaf and stem) or 30 haphazardly chosen herbaceous stems (15 *Spartina* culms) were visually examined, and the stem or terminal counts were summed across all months and averaged to yield a single count per replicate. Predator densities, primarily spiders, were also monitored monthly; however, these densities did not vary significantly between treatments.

A Student's t-test was utilized to test the treatment effects on all herbivore response variables. SYSTAT 11 (Crane Software International, Bangladore, India) statistical software was utilized to carry out all of the statistical procedures. Only the most prevalent herbivores were included in the statistical and meta-analysis calculations.

In order to synthesize results across all plant species, a mixed effects model meta-analysis was performed on the herbivore response data using MetaWin 2.0 (Sinauer Associates, Sunderland, MA, USA) software. These data were assigned to a number of categories of interest; including, high or low ambient salinity, insect herbivore feeding guild, and plant genus. Soil salinities for the low ambient salinity plants ranged from 8.74 – 14.5 ppt, and high ambient salinity values ranged from 16.1 – 24.0 ppt. For each insect herbivore species on each plant species, an effect size was calculated (Hedge's *d*) as well as the sampling variance. Confidence intervals calculated via bootstrapping methods were utilized, as these have been found to be more conservative values than those derived from the more standard parametric or homogeneity statistics (Adams *et al.*, 1997). Effect sizes were determined to be significant if bootstrap confidence intervals did not overlap zero. A positive effect size indicated that insect herbivore densities were higher on experimental plants, while a negative value indicated higher insect densities on control plants. The meta-analysis allowed for the detection of patterns emerging within ambient salinity, plant type, and plant species, among other categories.

RESULTS

Herbivore responses to the salt treatment varied. Most insect herbivores did not respond to the salt treatment; however, the following insects did exhibit a response to this treatment. The number of leaf rollers on *Avicennia* increased marginally ($p = 0.099$) at higher concentrations of salt (Figure 2). There was also a trend for higher *Trirhabda bacharidis* densities on *Baccharis* in the presence of the salt treatment ($p = 0.094$; Figure 3). Stem borers on *Batis* increased significantly ($p < 0.001$) in the presence of the salt treatment (Figure 4). The salt treatment also had a positive, significant ($p = 0.044$) affect on *Pissonotus* densities on *Spartina* (Figure 5). Densities of all other insect species did not differ significantly between control and experimental plants (Figures 6 – 7).

The meta-analysis between high and low ambient salinities showed that there were significantly higher insect densities on the experimental plants within the high ambient salinity condition (Figure 8). Insects on plants in low salinity conditions (e.g. *Baccharis*, *Borrchia*, and *Iva*) did not show a significant response; however, there was a significant difference between plants in the high ambient salinity versus low ambient salinity ($p = 0.008$; Figure 8).

The meta-analysis showed that insect densities on *Iva* were significantly higher on the control plants (Figure 9). The opposite held true for *Spartina*: insect densities here were significantly higher on the experimental plants (Figure 10). All other plant species did not exhibit differences within control and experimental treatments; however, *Iva* and *Spartina* were significantly different from each other ($p = 0.004$).

Lastly, none of the insect herbivore feeding guilds exhibited significant differences between treatments (Figure 10).

DISCUSSION

This study's results did show some consistent trends. When the data were analyzed with a Student's t-test, all significant or near-significant ($p < 0.10$) findings showed an increase in insect densities within the experimental treatment (Figures 1, 3, 4, & 5) which would suggest that White's (1978) plant stress hypothesis is more prevalent than Price's plant vigor hypothesis (Price, 1991) within the plant species examined in this study. Meta-analysis supports this trend in most cases (Figures 8, 9, & 10); specifically the experimental group had higher insect densities in the high ambient salinity condition, within herbaceous species, and in particular within *Spartina*. *Iva* is the only species that showed contrary significant results, in that control plants had higher insect densities than experimental plants (Figure 10).

In the case of *Iva*, Price's (1991) plant vigor hypothesis was supported. Gallling mites were the predominant herbivores witnessed on *Iva* plants (pers. obs.). Currently, little is known about the physiology of *Iva* (Thursby & Abdelrhman, 2004), which makes it difficult to ascertain the mechanisms driving these interactions. However, Tooker *et al.* (2008) found that gall insects that utilize goldenrod (*Solidago altissima*) as a host were able to avoid the indirect defenses of the plant, or in some cases change the indirect defenses to their advantage. *Heliothis virescens*, a generalist caterpillar, elicited indirect plant defenses; however, the galling insects

examined did not. It was found that *Eurosta solidaginis* (Diptera: Tephritidae) was able to limit the release of volatile chemicals when *H. virescens* was present as well, and there was no increase of volatile chemicals when *E. solidaginis* existed in the absence of chewing herbivores. It is hypothesized that by manipulating the amount of secondary volatile chemicals, especially when other herbivores are present, that *E. solidaginis* were less susceptible to potential predators, since the plant is not releasing cues that it is being harmed by the galling insects (Tooker *et al.*, 2008).

Hacker and Bertness (1995) found that *Iva* plants that were indirectly salt stressed by the removal of black rush, *Juncus gerardi*, were morphologically and physiologically affected by the stress. Plants in high salinity had lower leaf and flower production, as well as stem growth (Hacker & Bertness, 1995). Physiologically, they exhibited lower photosynthetic and transpiration rates (Hacker & Bertness, 1995). These data suggest that increased salinity was a more powerful stressor to *Iva* than flooding and anoxic soil effects (Hacker & Bertness, 1995). These findings support the idea that *Iva* suffers from severe biological trade-offs in high stress conditions.

In the current study, galling insects or mites could be modifying their environment, indirectly decreasing predation (Tooker *et al.*, 2008) in the control plants. Also, since these galling insects can avoid indirect plant defenses the increased levels of defensive chemicals potentially present in less-stressed plants (White, 1978) may have no affect on insect herbivores. Since *Iva* does exhibit moderately extreme responses to stress (Hacker & Bertness, 1995), it is reasonable

that these responses may adversely affect insect herbivores, particularly ones that live inside plant tissues.

All other statistically significant herbivore responses supported White's (1978) plant stress hypothesis. There are a number of reasons why increased salinity could be more important to herbaceous coastal plant species in higher salinity areas. Many studies investigating community resilience to stressors have shown that community responses can vary based on the amplitude and the persistence of the stressor (Harrison, 1979). It appears that by increased salinity may push these smaller coastal plants over a stress threshold that causes them to respond physiologically or morphologically. These changes, in turn, benefit some insect herbivores.

Though salt stress results in increased insect densities in these examples, each plant species has different mechanisms for inducing these similar herbivore responses. Though few studies have examined herbivore responses in *Batis*, studies on its physiology have been performed (e.g. Kuramoto & Brest, 1979; Antlfinger & Dunn, 1983; Debez *et al.*, 2010). Antlfinger and Dunn (1983) found that *Batis* excludes some salt at the root, and Debez *et al.* (2010) found that *Batis* otherwise compartmentalizes salt in vacuoles. *Batis* seems to use Na^+ to control internal solute concentrations instead of amino acids (Debez *et al.*, 2010), so it is unlikely that herbivores are increasing in density due to this adaptation. Instead, the predominant herbivore found on *Batis* (i.e. a stem borer, pers. obs.) seems to be increasing in density due to morphological changes induced by plant stress. The "toughness" of *Batis* stems was not examined in this study; however, field observations suggest that

stressed *Batis* may exhibit the similar morphological changes in response to stress (pers. obs.) as *Borrchia* which could be beneficial to this stem boring herbivore. For instance, Moon and Stiling (2000) found that *Borrchia* plants that received fertilizer and/or shading exhibited a significant increase in the number of green, lush stems when compared to the control plants. Moon and Stiling (2006) found that *Pissonotus quadripustulatus* (Homoptera: Delphacidae) preferentially oviposited in *Borrchia* host plants that were of lower quality, because eggs were protected from parasitism by *Anagrus* sp. (Hymenoptera: Mymaridae).

Past studies of stress effects on *Borrchia* herbivores have also been variable. Albarracin and Stiling (2006) found no significant differences in herbivore densities or egg parasitism by *Anagrus* sp. (Hymenoptera: Mymaridae) due to increased ambient salt stress. However, when galls created by *Asphondylia borrichiae* (Diptera: Cecidomyiidae) were examined (Moon & Stiling, 2002a ; Stiling & Moon, 2005a), the galls were more prevalent on healthy, green stems located in ambient salinity, likely due to *A. borrichiae*'s ability to more easily penetrate the lush, green stems over the tougher, woody variety with their ovipositors (Moon & Stiling, 2002a; Moon & Stiling, 2005). Gall densities also decreased as salt levels were increased (Moon & Stiling, 2002a; Moon & Stiling, 2005). *Pissonotus quadripustulatus* densities as a whole were variable between the control, intermediate, and high salt treatments (Moon & Stiling, 2005), which suggests that though they prefer to oviposit on stressed plants (Moon & Stiling, 2000) that they reside and feed upon plants of varying stress levels equally.

Spartina herbivores have also exhibited mixed responses to stress in past studies (e.g. Denno et al., 1986; Bowdish & Stiling, 1998; Goranson et al., 2004). Goranson et al. (2004) found contrasting responses from two separate leaf chewing insects: *Orchelimum fidicinum* (Orthoptera: Tettigoniidae) and *Orphulella pelidna* (Orthoptera: Acrididae). *Orchelimum fidicinum* located on *Spartina* plants within high ambient salinity conditions significantly decreased when compared to low ambient salinity plants; whereas, *Orp. pelidna* densities on high ambient salinity plants increased significantly when compared to the plants in low ambient salinity (Goranson et al., 2004). Bowdish and Stiling (1998) found that artificially increased salt had no significant affect on *Prokelisia marginata* (Homoptera: Delphacidae) densities though they noticed decreased *P. marginata* densities within back-marsh areas which exhibit higher ambient salinity conditions. Denno et al. (1986) showed that *P. marginata* densities were lower on “inferior” host plants, and they exhibit smaller body size on less nutritious plants. This phloem-feeding insect inserts its beak-like mouthparts into the phloem of the plant and ingests free amino acids flowing through the vascular tissue (Brodbeck & Strong, 1987). Since many marsh plants accumulate amino acids to offset osmotic imbalances (Bowdish & Stiling, 1998), it is reasonable that phloem-feeder densities could be expected to increase in high stress conditions. The current study somewhat contradicts past research but ultimately supports White’s (1978) plant stress hypothesis in the cases where herbivores responded to the salt treatment.

When taken individually, each plant species mentioned reacts variably to increased stress conditions. Some exhibit morphological differences (e.g. Moon & Stiling, 2000), while others primarily exhibit physiological changes (e.g. Debez *et al.*, 2010; Hacker & Bertness, 1995). Alternatively, stress could also affect predator densities positively or negatively (Stiling & Moon, 2005a, 2005b), thereby masking herbivore responses to stress. Despite these differences, this study has shown that insects generally increase on plants that are stressed, thereby supporting White's (1978) plant stress hypothesis. These generalities are further strengthened since this study has shown the same trends within and between both traditional statistics (i.e. comparisons with a Student's t-test) as well as using meta-analysis as a tool. By performing large, multi-species community studies and utilizing meta-analysis when appropriate, thereby eliminating much of the variation caused by methodological logistics, ecological generalities may be found more easily than they have been in the past.

Appendix 1: Figures

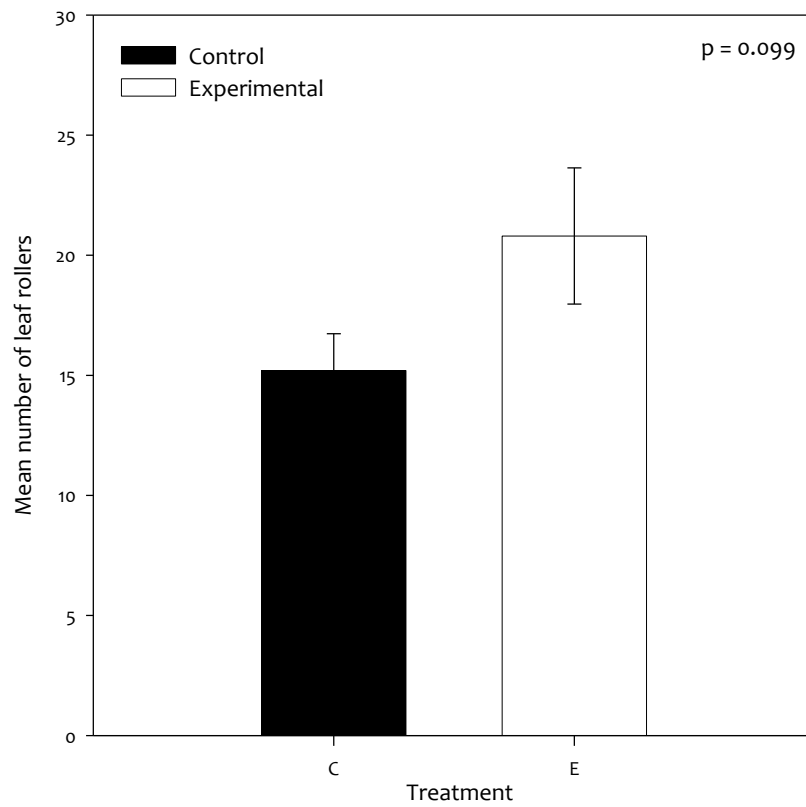


Figure 1.

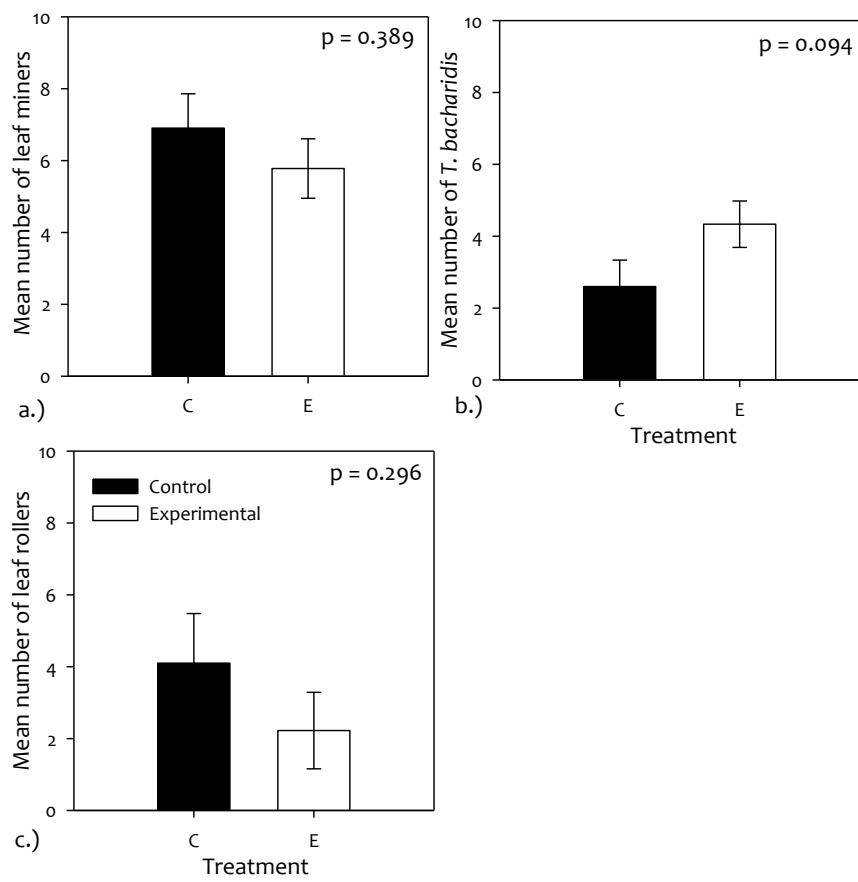


Figure 2.

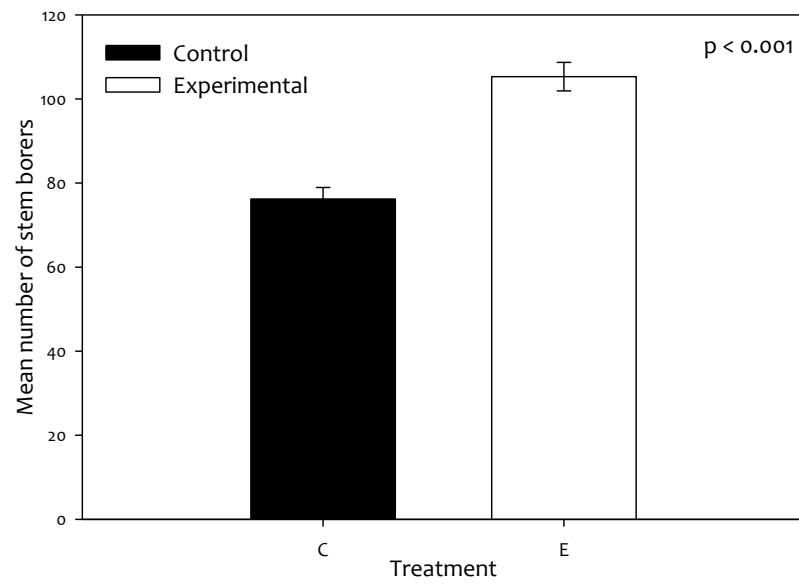


Figure 3.

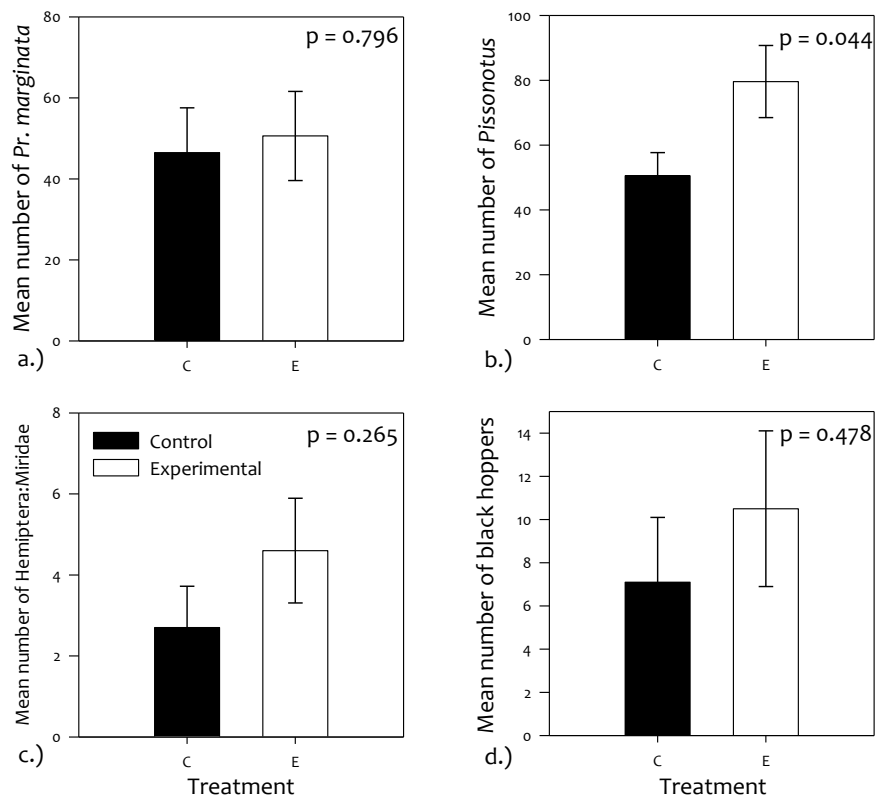


Figure 4.

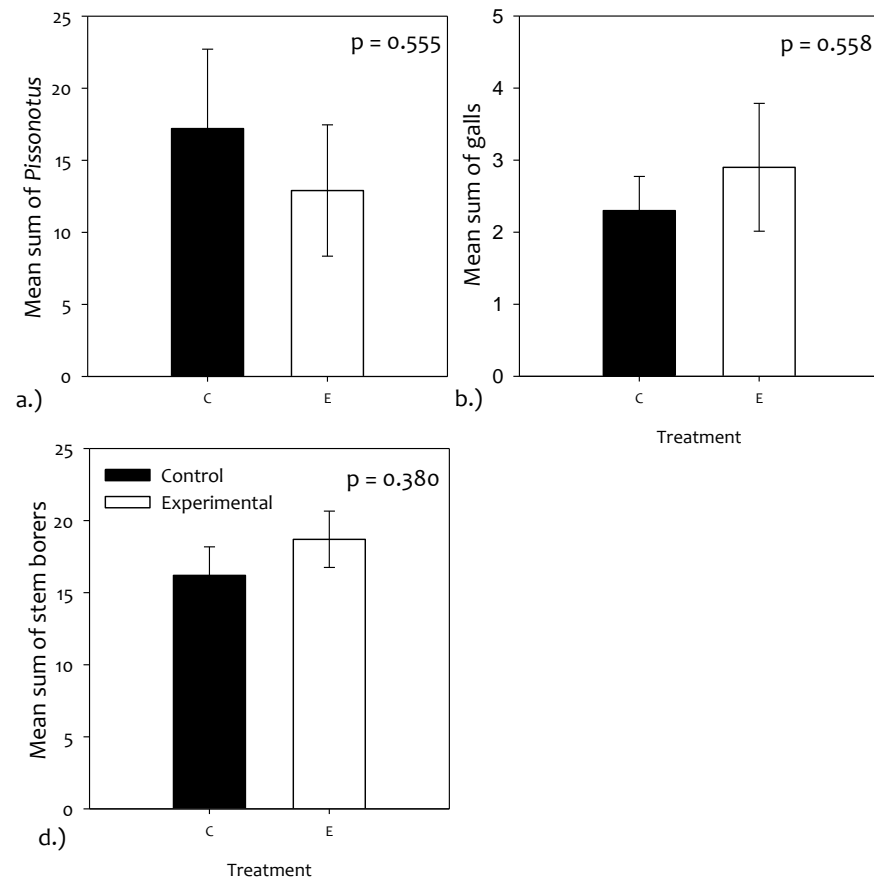


Figure 5.

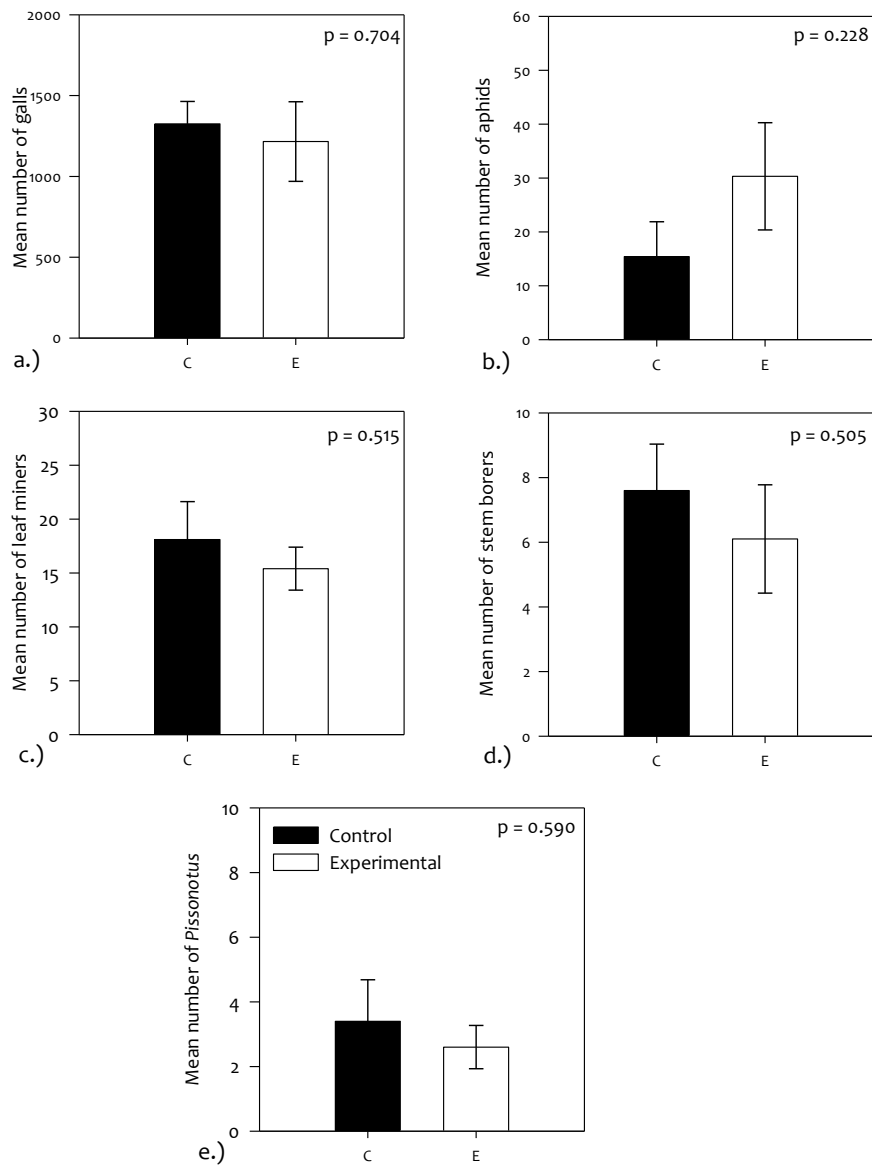


Figure 6.

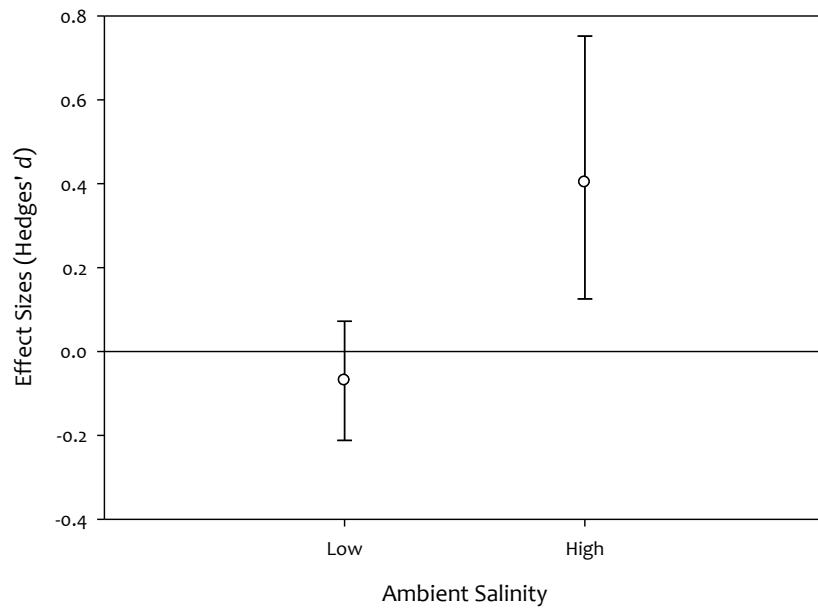


Figure 7.

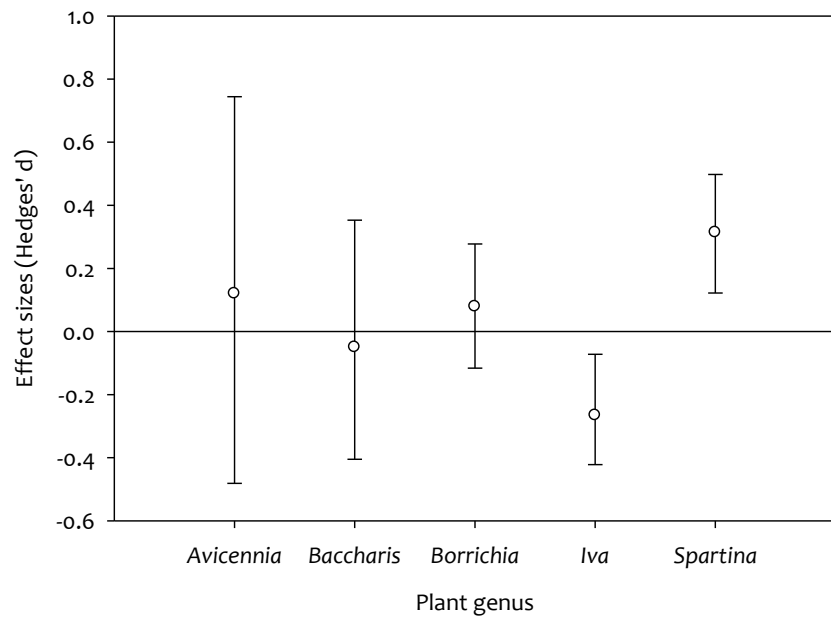


Figure 8.

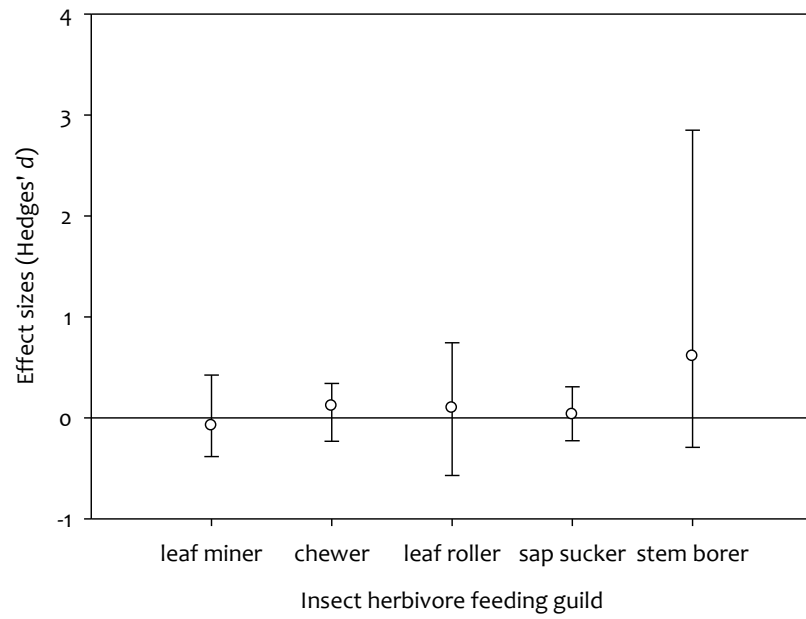


Figure 9.

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CHAPTER 2

Assessing salt stress effects at the organismal and molecular level in salt marsh plants

ABSTRACT

The primary objective of this study was to determine if assays of soluble protein content and catalase concentrations could be utilized to more accurately quantify stress effects in six coastal plant species (*Avicennia germinans*, *Baccharis halimifolia*, *Batis maritima*, *Borrchia frutescens*, *Iva frutescens*, and *Spartina alterniflora*). Stress has been identified as one of the primary factors influencing the structure of food webs. Even though stress has been identified as being highly important to community structures, a dichotomy exists in the methods used by field biologists and those used by physiological or molecular biologists. Field researchers primarily rely on measurements of morphological changes in the plant to attempt to quantify stress response, which is both time-intensive and variable. Parameters such as leaf and flower production, stem growth, and leaf area generally correlate with overall health of the plant; however, a more efficient and consistent method for assessing stress would be ideal. In this study, stem growth was significantly higher for the experimental plants for *Baccharis* and *Iva*, and significantly higher on control plants for *Batis*. Leaf production was significantly higher in the control plants for *Borrchia* and significantly higher in experimental *Iva* plants, while stressed *Borrchia*

stem densities were significantly lower than control plants. Measures of total soluble protein content in the leaves were significantly higher in stressed *Batis* plants, and catalase concentrations did not differ between treatments for any of the plant species examined. Lack of significant responses in catalase levels may have been due to plants maintaining elevated levels of the enzyme to combat consistent stressful conditions. More intense sampling efforts of both organismal and potential molecular markers should be examined in to determine their applicability in future studies.

INTRODUCTION

Stress is an important component structuring food webs (Sousa, 1979; Menge & Sutherland, 1987; Hacker & Gaines, 1997), and it is important to be able to detect if organisms are stressed and obtain accurate measures of these stress effects. Field researchers typically measure morphological changes in the plant structure as an attempt to quantify stress responses (Gonçalves-Alvim *et al.*, 2001; Pennings & Moore, 2001; Moon & Stiling, 2002, 2005). These methods can provide evidence that plants are living in high stress conditions; however, they tend to be time consuming and variable (pers. obs.). Fortunately, biotechnological advancements have allowed for the detection of reactive oxygen species (ROS) which are produced when plants (and other organisms) are stressed (Ferrat *et al.*, 2003). Biotic and abiotic stressors (Rijstenbill *et al.*, 1994) can trigger this release of ROS; including the metabolization of pollutants (Stegeman *et al.*, 1992; Cossu *et al.*, 1997).

Responses to counteract the presence of ROS in stressful conditions involve the up-regulation of antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT), among others (Ferrat *et al.*, 2003). These enzymes convert ROS into less reactive compounds (Ferrat *et al.*, 2003). In particular, SOD transforms the reactive superoxide ion (O_2^-) into a more stable hydrogen peroxide (H_2O_2) compound. CAT furthers the disassembly by catalyzing the reaction of hydrogen peroxide into water and oxygen (Ferrat *et al.*, 2003). The occurrence of these ROS detoxification pathways in cells proved to be highly beneficial to this study, because the enzyme

concentrations could be assessed, quantified, and hypothetically assist in determining whether a plant is stressed.

Plants have many other mechanisms, both biochemical and morphological, for coping with various stress conditions (Baisakh *et al.*, 2006; Parida *et al.*, 2004; Parida & Das, 2005); however, field biologists have tended to rely strongly on morphological changes in plant growth and/or structure, such as leaf area, plant growth, leaf and flower production, or stem morphology (Gonçalves-Alvim *et al.*, 2001; Moon & Stiling, 2000). Numerous studies have found that salinity significantly affects each of these morphological parameters in a variety of plants (Barbour, 1978; Lin & Sternberg, 1992; Smith & Snedaker, 1995; Moon & Stiling, 2000; Gonçalves-Alvim *et al.*, 2001; Richards *et al.*, 2005; Brown & Pezeshki, 2007). These methods can provide evidence that a plant is living in stressed conditions; however, data regarding the physiological mechanisms of stress can not be gauged via these procedures (Verslues *et al.*, 2006) and determining the cause of certain responses to stress is ambiguous without further data collection.

This portion of the study examined both the growth parameters as well as total soluble protein and CAT concentrations of six common coastal plants: *Avicennia germinans*, *Baccharis halimifolia*, *Batis maritima*, *Borrchia frutescens*, *Iva frutescens*, and *Spartina alterniflora*. Ultimately, these data regarding plant growth, plant health, and antioxidant enzyme production were utilized to give an in-depth cross-species examination of a salt marsh system stressed by elevated salinity levels. Determining plant stress conditions in the field has historically involved time consuming data

collection methods (Gonçalves-Alvim *et al.*, 2001; Pennings & Moore, 2001; Moon & Stiling, 2002, 2005), ideally CAT responses should mimic those found with the morphological properties of the plant and provide a good stress biomarker for future research.

MATERIALS AND METHODS

Twenty *Avicennia germinans*, *Baccharis halimifolia*, and *Iva frutescens* shrubs (respectively; each 1-2 m tall) were haphazardly flagged at the study site, located east and west of Hwy. A1A (approximately 29°40'41" N, 81°13'10" W) in the Guana-Tolomato-Matanzas National Estuarine Research Reserve (GTMNERR). In addition to these shrub species, twenty 1-m² monoculture plots of each herbaceous species were haphazardly established. These species included: *Borrchia frutescens*, *Batis maritima*, and *Spartina alterniflora*. Ten of the shrubs and plots were randomly chosen as control and experimental groups, respectively, and each shrub or plot was separated from other shrubs or plots by approximately 3m. Plants and plot sizes were chosen so that the biomass of all replicates of all species were approximately equivalent.

Salt stress on the plants was accomplished by broadcasting 1.3 kg/m² NaCl on the soil around all experimental plants or within experimental plots monthly according to the methods of Bowdish and Stiling (1998). This quantity of salt should increase the interstitial soil salinity around the plants by 20-30% (Moon & Stiling, 2000; 2002), and actual soil salinities were found to increase by 25.3 – 31.4%.

Plant responses to the addition of salt were monitored by assessing plant growth, leaf production, and flower production. For the shrubs, five branch terminals were flagged and monitored at the beginning, middle, and end of the experiment. For the herbaceous species, haphazardly chosen stems or culms were monitored on the same schedule. To assess plant growth of the shrubs, the length from the tip of the branch to the first node (approximately 10 cm) was determined on 5 branch terminals and these values were averaged for ten randomly selected plants of each species. Ten haphazardly chosen stems or blades from ten randomly selected plots of each herbaceous species were measured from soil to tip to assess the effects on plant growth, and these values were averaged to represent the changes in growth of each respective plot.

Leaf production was assessed by counting the number of leaves on each of the sampling units mentioned above (e.g. branch terminals for the shrubs and stems for the herbaceous species). In the case of *Spartina*, the number of blades per 10 culms was counted. In the appropriate flowering season for each plant, flowers on each of these 5 sampling units were counted, except in the case of *Spartina* where the flowers on 10 stems were counted. Flowers on *Batis* were not counted in this study. Finally, plant density was assessed in herbaceous plots by counting all stems present in a haphazardly selected quarter (25%) of the plot at the beginning, middle, and the end of the study to determine if plant densities changed. Since monocultures of *Batis* were difficult to come by, the quarter-plot densities of *Salicornia virginica* within *Batis* plots were also monitored.

Other stress indicators include SOD and CAT activity. These stress enzymes were monitored at the beginning, middle, and end of the study. Multiple undamaged, fully extended, haphazardly chosen leaves, stems, or blades were collected from 10 randomly chosen plants per species at the beginning, middle, and end of the experiment. The samples were kept on dry ice in the field to prevent enzyme denaturing. The samples were stored at -80°C until assays on these stress enzymes were processed.

Frozen plant samples (0.500 g wet mass, $n=5/\text{species/treatment}$) were thawed to room temperature. Enzymes were extracted in 500 μL of buffer (50 mM potassium phosphate buffer (pH 7.5) containing 1% (w/v) polyvinylpolypyrrolidone (PVP)-40, 0.25% Triton X-100, 0.04% protease inhibitor cocktail). Samples were homogenized with a Fast Prep-24 bead homogenizer with 10 zinc beads for 20s at 4.0 m/s. Protein, CAT, and SOD content was quantified from the resulting supernatant.

A Pierce[®] BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) was used to quantify total soluble protein (TSP) present in the samples. An Amplex[®] Red Catalase Assay Kit (Molecular Probes, Eugene, OR, USA) was used to quantify CAT activity in samples. A Superoxide Dismutase Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) was used to quantify SOD activity in samples; however, this data was not useable due to the leaf pigmentation of all leaves confounding the spectrophotometric readings associated with the kit. All kits were utilized within the constraints of the manufacturer's instructions. Enzyme activity was normalized to the protein concentration and units were expressed in U mg protein^{-1} .

RESULTS

The percent change in stem length for *Baccharis* was significantly higher ($p = 0.049$) on the experimental plants, *Batis* stems exhibited less regrowth in the experimental plots ($p = 0.041$), and *Iva* control stems were significantly shorter than experimental stems ($p = 0.037$); while all other plant species stem lengths did not significantly vary from the control plants (Figure 10). The percent change in leaf number for *Iva* was significantly higher ($p = 0.003$) in the experimental plants, and *Borrchia* exhibited lower leaf growth percentages ($p = 0.014$) in the experimental plots, while all other plant species did not vary from control values (Figure 11). The mean number of flowers on both shrub and herbaceous plants did not vary between treatments (Figure 12); however, *Borrchia* quarter-plot densities were significantly higher within the control plots ($p = 0.031$), though both decreased from initial densities (Figure 13). No other quarter-plot counts of herbaceous species varied between treatments (Figure 13).

For the assay data, the percent TSP by mass for *Batis* was significantly higher in both the July ($p < 0.0001$; Figure 14) and November sampling period ($p = 0.047$; Figure 15) within the experimental plants; however, all other protein values did not vary between treatments (Figures 14 & 15). None of the CAT concentrations varied significantly between treatments in either sampling period (Figures 16 & 17).

DISCUSSION

As mentioned in the previous chapter, each of the plants included in this study exist along a natural salinity gradient (Oosting & Billings, 1942; Ehrenfeld, 1990; Stalter & Odum, 1993). *Baccharis* is the least salt tolerant with *Iva*, *Borrichia*, *Spartina*, *Batis*, and *Avicennia* each increasing in salinity tolerance, respectively (Tolliver *et al.*, 1997; Pennings & Richards, 1998; Suárez *et al.*, 1998; Pennings & Moore, 2001; Moon & Stiling, 2004). Though each of these plants are able to tolerate salt, it should be noted that each species grows best at the lower end of its respective tolerance level (Tolliver *et al.*, 1997; Pennings & Richards, 1998; Suárez *et al.*, 1998; Pennings & Moore, 2001; Moon & Stiling, 2004). *Batis* is considered an obligate halophyte, growing best in approximately 200 mM NaCl (7 ppt) concentrations (Debez *et al.*, 2010), though it can grow in hypersaline conditions as well (Antlfinger & Dunn, 1979, 1983; Pennings & Richards, 1998). *Avicennia* tolerates a broad range of salinities as well, ranging from nearly fresh (Lugo & Snedaker, 1974) to hypersaline (Suárez *et al.*, 1998); however, stressed plants tend to exhibit stunted growth and sclerophyllic leaves (Gonçalves-Alvim *et al.*, 2001). *Borrichia* exhibits an increase in stressed “woody” stems when salt levels are artificially increased, while green “fleshy” stems are prevalent in plants in lower salinity (Moon & Stiling, 2000). *Spartina*’s salt tolerance is similar to *Borrichia*’s; however, it tends to exist in saltier conditions due to competition (Pennings & Moore, 2001). Stressed *Iva* plants also exhibit stunted growth compared to plants growing in areas of lower salinity (Hacker & Bertness,

1995). *Baccharis* tends to be the least salt tolerant of all of the plants examined in this study and has been seen in purely fresh water areas (pers. obs.).

All plant stress response variables examined in this study varied between treatments only rarely (Figures 10, 11, 13, & 14). Stressed *Iva* exhibited a significantly higher percent change in stem length ($p = 0.037$) and no significant differences in percent leaf changes and flower densities (Figure 10), which is contradictory to past studies. Hacker and Bertness (1995) found that *Iva* plants subjected to salt stress indirectly by removing surrounding *Juncus gerardi* (black rush) plants exhibited significantly lower growth, leaf area, and flower density. Thursby and Abdelrhman (2004) found that *Iva* living in areas subject to long and frequent flooding stress were shorter and exhibited less secondary growth in stems.

Batis exhibited less regrowth ($p = 0.041$) throughout the study in the experimental plots (Figure 10) and contained significantly higher TSP levels ($p < 0.0001$ & $p = 0.047$, respectively) in both post-treatment sampling periods (Figures 14 & 15). Debez *et al.* (2010) found that proline levels in *Batis* increased significantly in the shoots of the plant in the presence of salt. Though TSP is not a direct indication of free amino acid concentration, proteins have been found that accumulate in direct response to salt stress, as well as other forms of stress (Hurkman *et al.*, 1989; Pareek *et al.*, 1997; Ali *et al.*, 1999; Ashraf & Harris, 2004). It was hypothesized that the salt-stress proteins may provide a storage site for nitrogen during periods of high stress that could be utilized when stressful conditions diminish (Singh *et al.*, 1997; Ashraf & Harris, 2004), in addition to directly offsetting osmotic stress by increasing internal

solute concentrations (Ashraf & Harris, 2004). Pennings and Richards (1998) found that *Batis* stressed indirectly by the removal of wrack were significantly smaller and produced less biomass than less stressed plants, so it is logical that these physiological effects may be occurring as well.

Borrichia regrew significantly fewer leaves during the field season ($p = 0.014$; Figure 11) and had significantly lower ($p = 0.031$; Figure 13) stem densities within experimental plots. The stem density findings contradict Albarracin and Stiling's (2006) study that found higher *Borrichia* stem densities in sites with higher ambient salinity conditions, and Stiling and Moon (2005) found no significant difference in stem densities or leaf numbers between treatments. Though not directly examined in this study, Moon and Stiling (2000) found that stressed *Borrichia* stems exhibit "woody," thicker stems than plants that are not stressed. The vast majority of the stems at this study site were of the woody variety (pers. obs.). Albarracin and Stiling (2006) did not directly examine stem woodiness as a stress indicator of *Borrichia* at their sites, so this could account for the difference between their findings and those from this study.

Numerous studies have found that the morphological parameters examined are typically good indicators of salt stress (Brown & Pezeshki, 2007; Richards *et al.*, 2005; Gonçalves-Alvim *et al.*, 2001; Moon & Stiling, 2000; Smith & Snedaker, 1995; Lin & Sternberg, 1992; Barbour, 1978), and Moon and Stiling (2000; 2002) and Bowdish and Stiling (1998) found the amount of salt used in this study significantly stressed many of the plants used in this study.

Though this study found no significant differences in CAT concentrations between treatments (Figures 16 & 17), antioxidant enzymes have been used to quantify stress in organisms ranging from bacteria to animals (e.g. Storz *et al.*, 1990; Ferrat *et al.*, 2003; Jebara *et al.*, 2005; Parida & Das, 2005; Ross & Van Alstyne, 2007; Ross *et al.*, 2010). Since CAT is a ubiquitous enzyme in stressful conditions, the lack of significant results in this study could be due to individual differences in the responses of antioxidant enzymes in the study plants (Menezes-Benavente *et al.*, 2004; Sekmen *et al.*, 2007). Both SOD and CAT were found to be up-regulated in *Plantago maritima*, a salt-tolerant plant (Sekmen *et al.*, 2007) and *Oryza sativa*, an intermediately salt-sensitive rice species (Menezes-Benavente *et al.*, 2004), when subjected to certain salinity regimes for approximately one week. Enzyme activities did not vary significantly in *P. maritima* plants subjected to 100 mM NaCl, but they significantly increased at 200 mM NaCl concentrations (Sekmen *et al.*, 2007). Contrarily, SOD and CAT activities in salt-sensitive *P. media*, a salt-sensitive plant, decreased with increasing salinity (Sekmen *et al.*, 2007). Menezes-Benavente *et al.* (2004) showed that 6-week-old *O. sativa* plants exhibited diurnal variations in CAT-A and CAT-C concentrations, and that increased salt concentrations inhibited the accumulation of CAT-A. Takemura *et al.* (2000) showed that mangroves (*Bruguiera gymnorhiza*) grown from propagules in fresh water and transplanted to salt water experienced an immediate increase in both CAT and SOD activities. After 10 days, activities of these enzymes were five times greater in 250 mM NaCl and 8 times greater in 500 mM NaCl. The activity of SOD was maintained up to 1000 mM NaCl, while CAT activities

were maintained at 500 mM NaCl and inhibited at 1000 mM NaCl. These studies show that enzyme concentrations and activities can be variable even within the same plant species (e.g. *P. maritima* and *O. sativa*), though salt-tolerant plants all up-regulated CAT and/or SOD activities when salinity levels got high enough (Takemura *et al.*, 2000; Menezes-Benavente *et al.*, 2004; Sekmen *et al.*, 2007). In salt-sensitive species, antioxidant enzyme activity has been found to be down-regulated (Menezes-Benavente *et al.*, 2004), likely due to oxidative damage from ROS.

Since these studies are relatively short in duration, and the salt levels utilized in each study were held constant once administered (Takemura *et al.*, 2000; Menezes-Benavente *et al.*, 2004; Sekmen *et al.*, 2007), it is difficult to assess the enzyme activities in a system where abiotic factors are constantly changing (pers. obs.). Potentially, the plants utilized in this study could immediately combat ROS as stressful conditions arise if they accumulated and maintained antioxidant enzymes. If this does occur, responses enzyme activity responses in this study could have been masked since even control plants would have elevated enzyme activities.

In addition to general up-regulation of CAT and other antioxidant enzymes, each of the plant species examined have different physiological mechanisms for dealing with stress, many of which may not involve the parameters examined in this study. For example, *Avicennia* is known to possess salt glands that allow the plant to exude salt through the leaves, as well as excluding salt at the root level (S         *et al.*, 1998). Since salt is being directly removed from the plant by these mechanisms, *Avicennia* may not have to maintain high levels of antioxidant enzymes to combat salt

stress-related ROS levels. Similarly, *Batis* is known to compartmentalize salt into vacuoles (Flowers & Colmer, 2008), and *Spartina* exudes salt through its leaves (Denno *et al.*, 1986) thereby decreasing the damage caused by increased salt levels and the need for elevated antioxidant enzyme levels.

Plant pigmentation obscured results from the SOD assay kit, so another method would have to be used to analyze SOD concentrations in these plants. Other parameters that could be examined in addition to CAT and SOD include: free amino acid concentration, lipid peroxidation, protein carbonylation, heat shock protein concentration, and the presence and concentration of phenolic compounds (Ferrat *et al.*, 2003). Utilizing all of these parameters would make our understanding of coastal community physiology more robust, and coupling these data with herbivore response variables (Chapter 1) will allow for a more accurate comprehension of exactly how increased salinities from global sea-level rise (Day & Templet, 1989; Ross *et al.*, 1994) will affect these areas.

Appendix 2: Figures and Tables

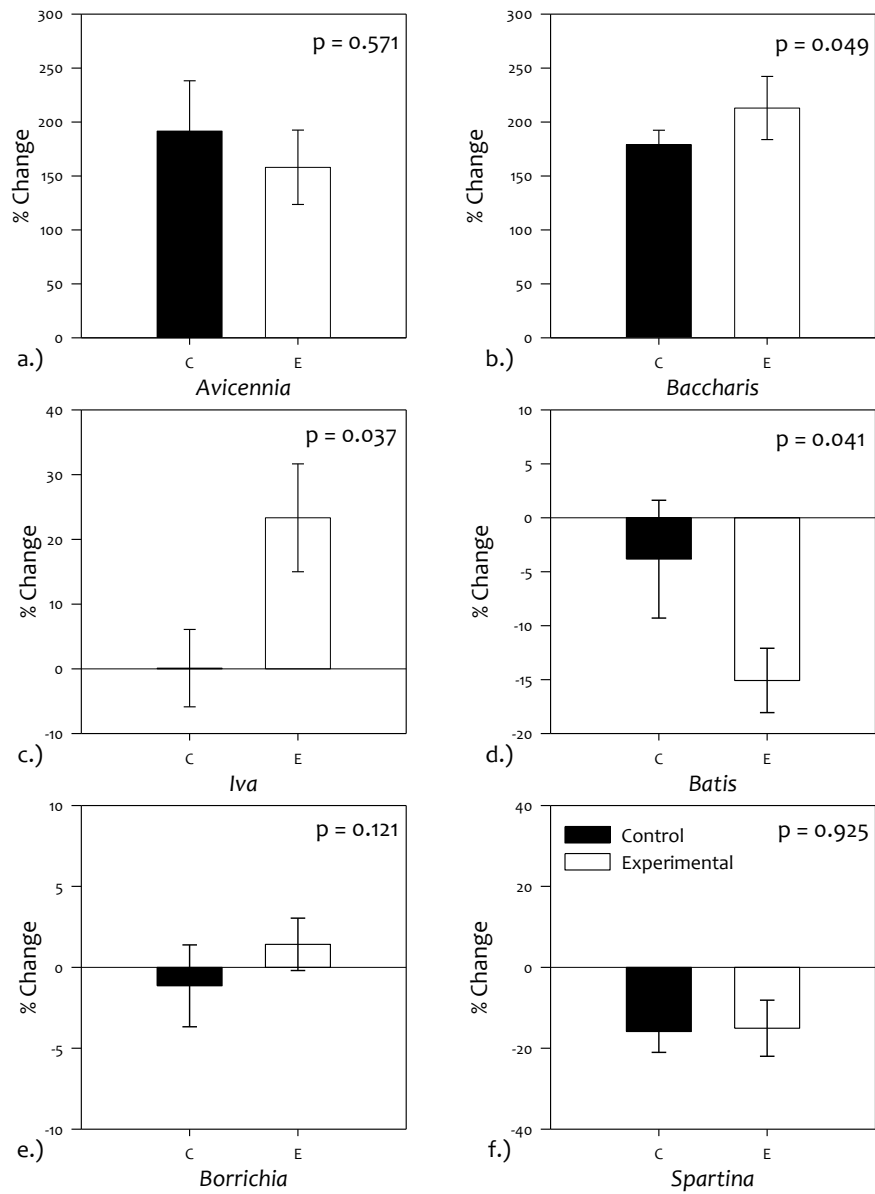


Figure 10.

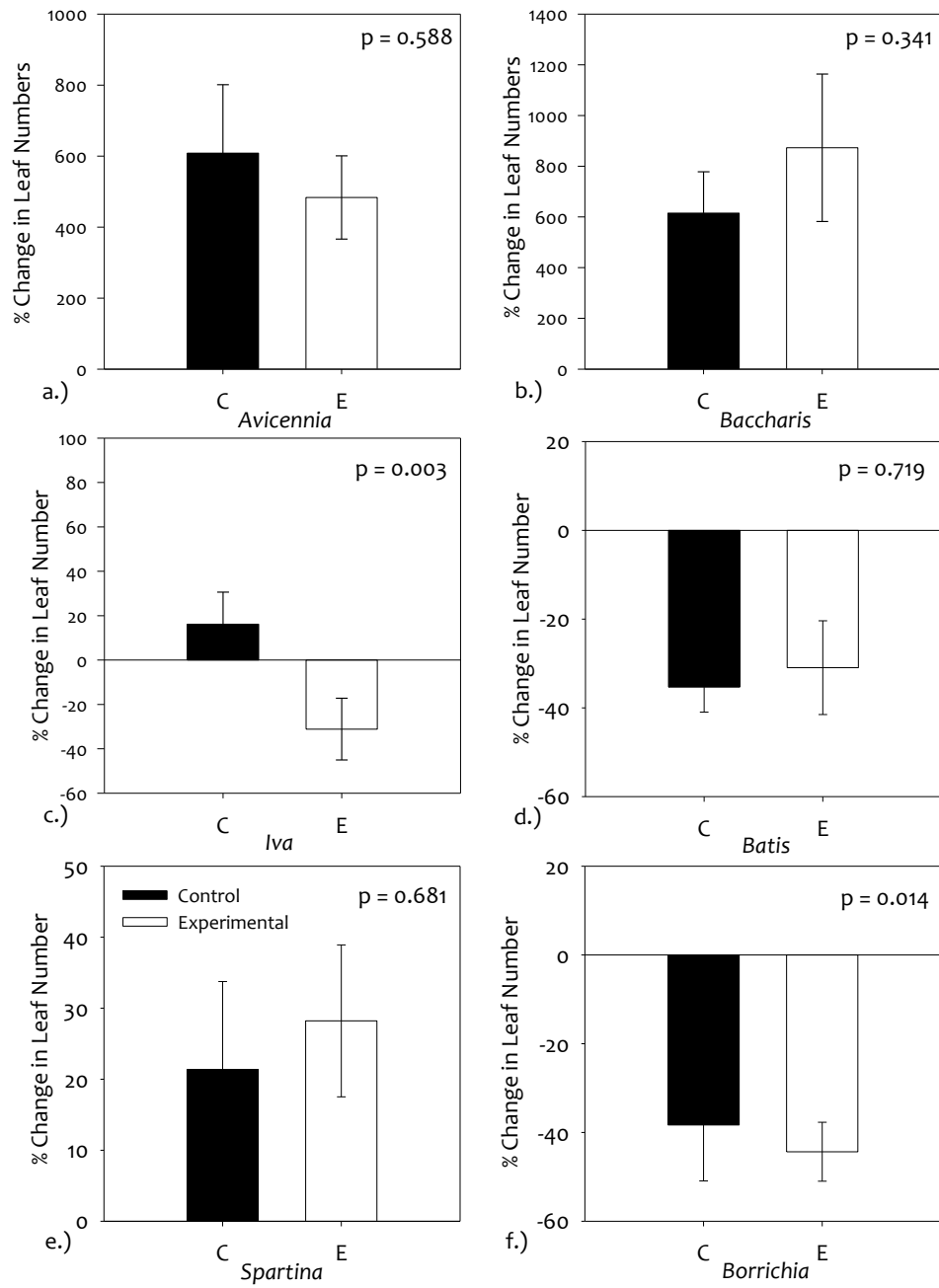


Figure 11.

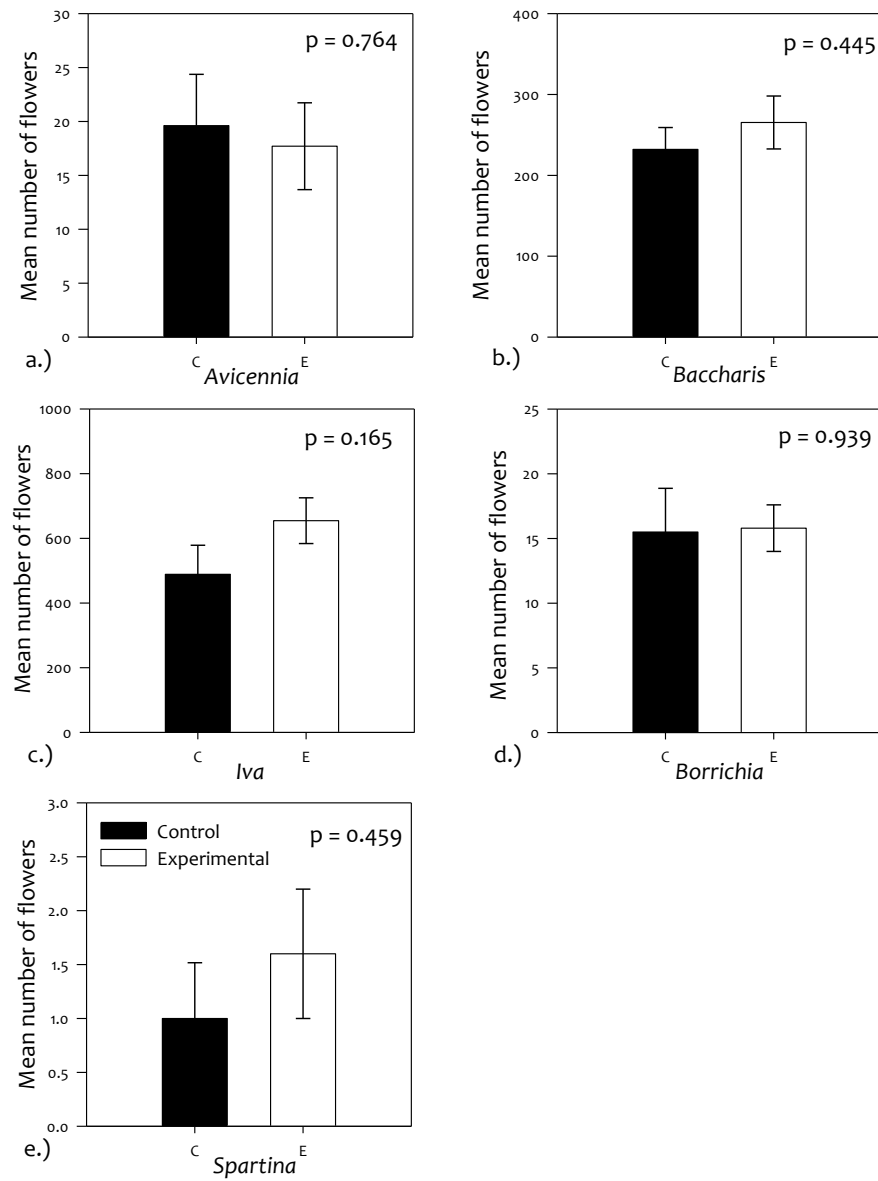


Figure 12.

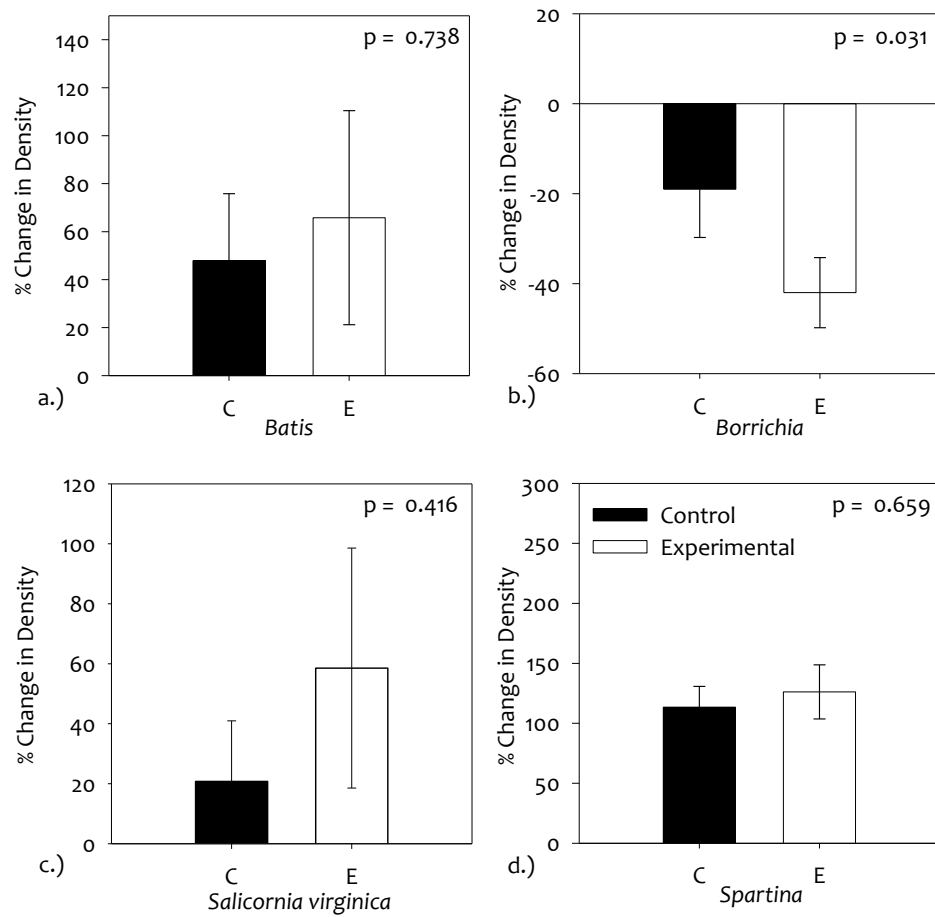


Figure 13.

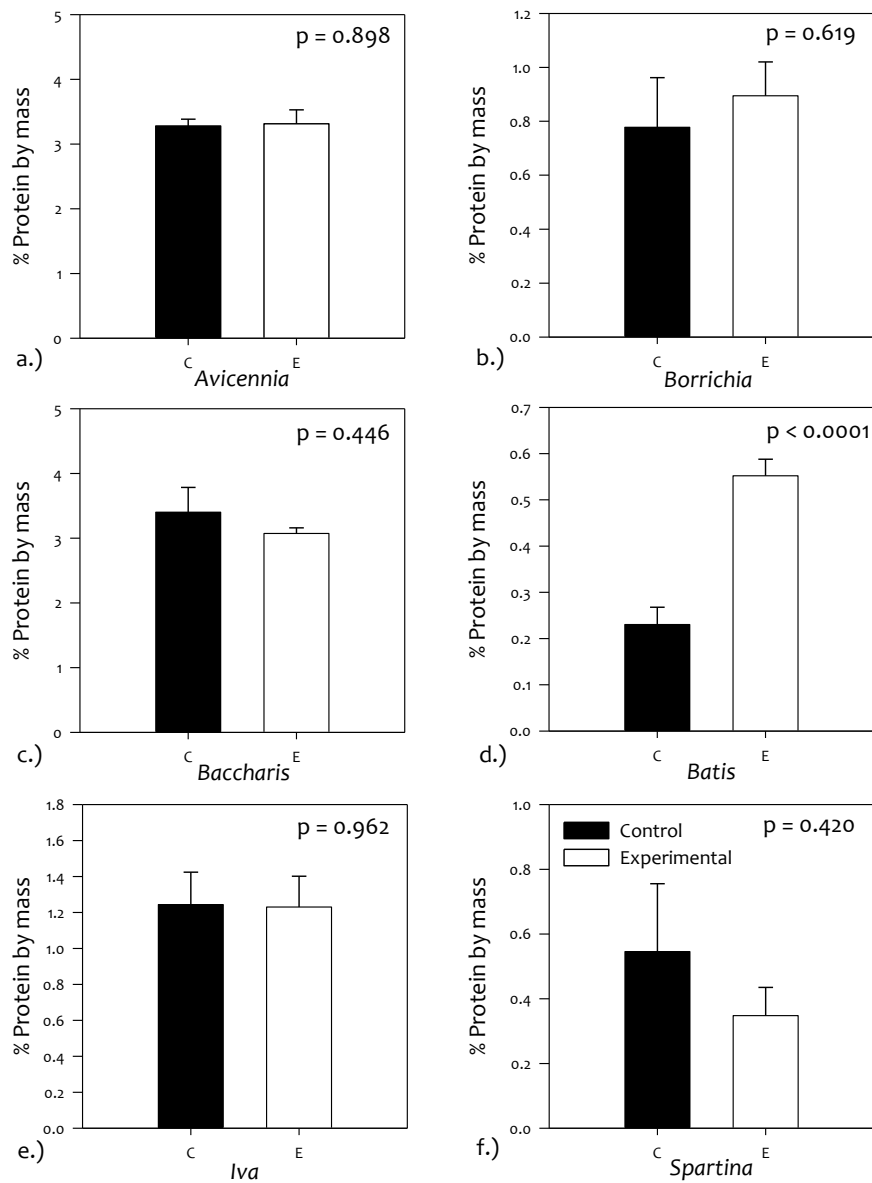


Figure 14.

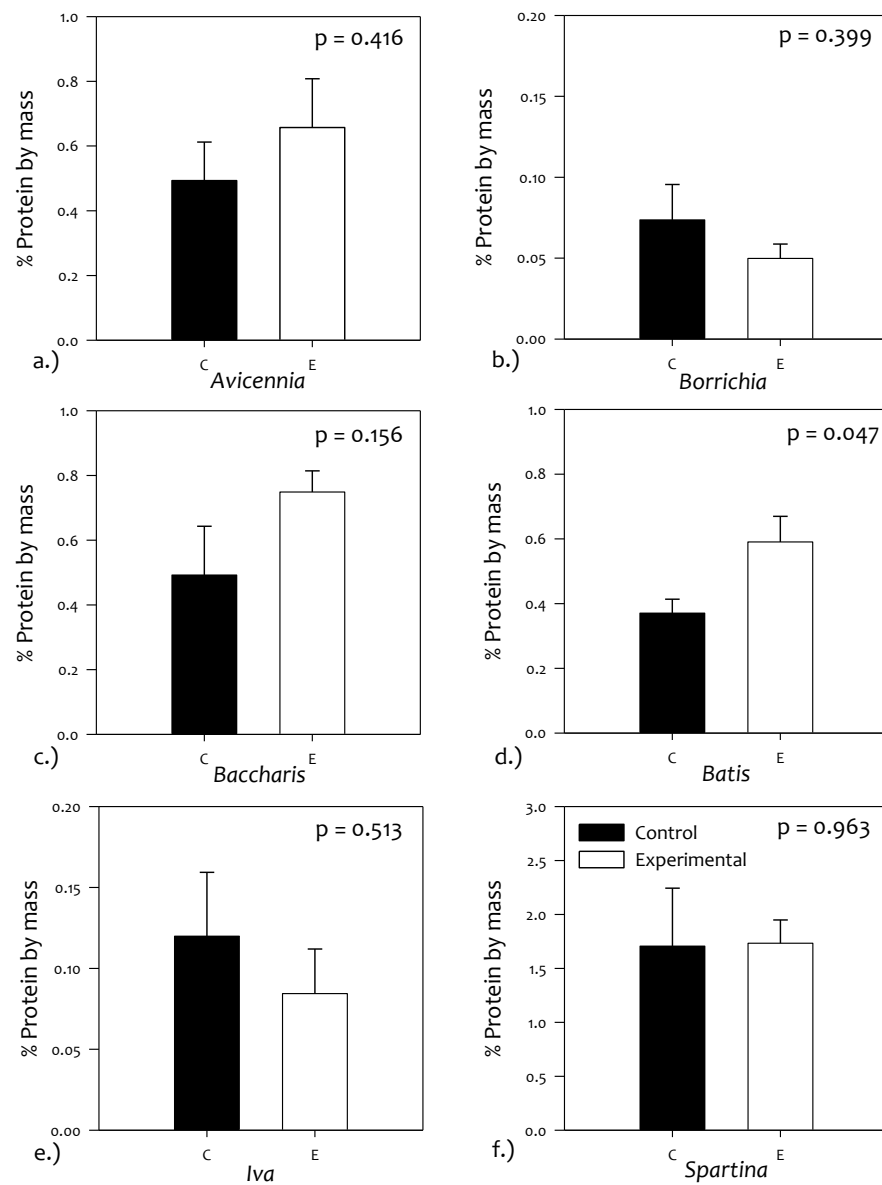


Figure 15.

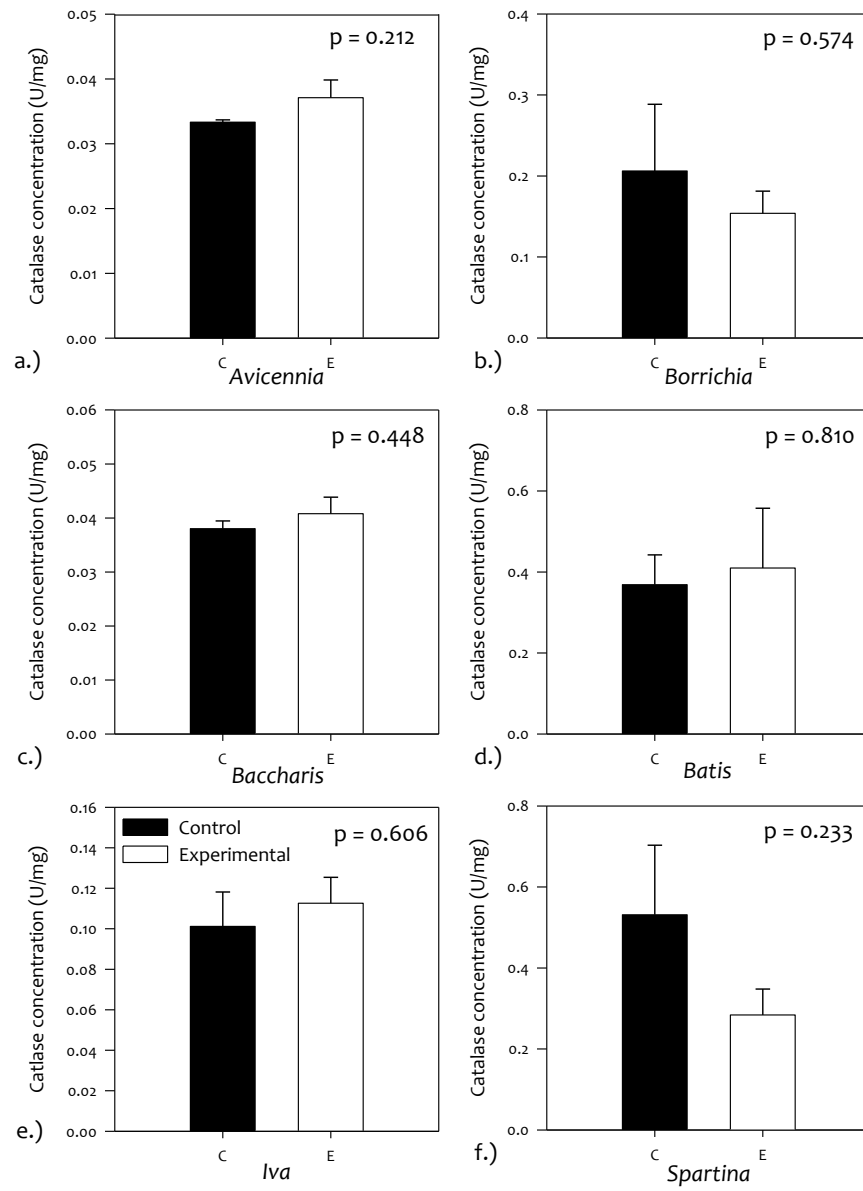


Figure 16.

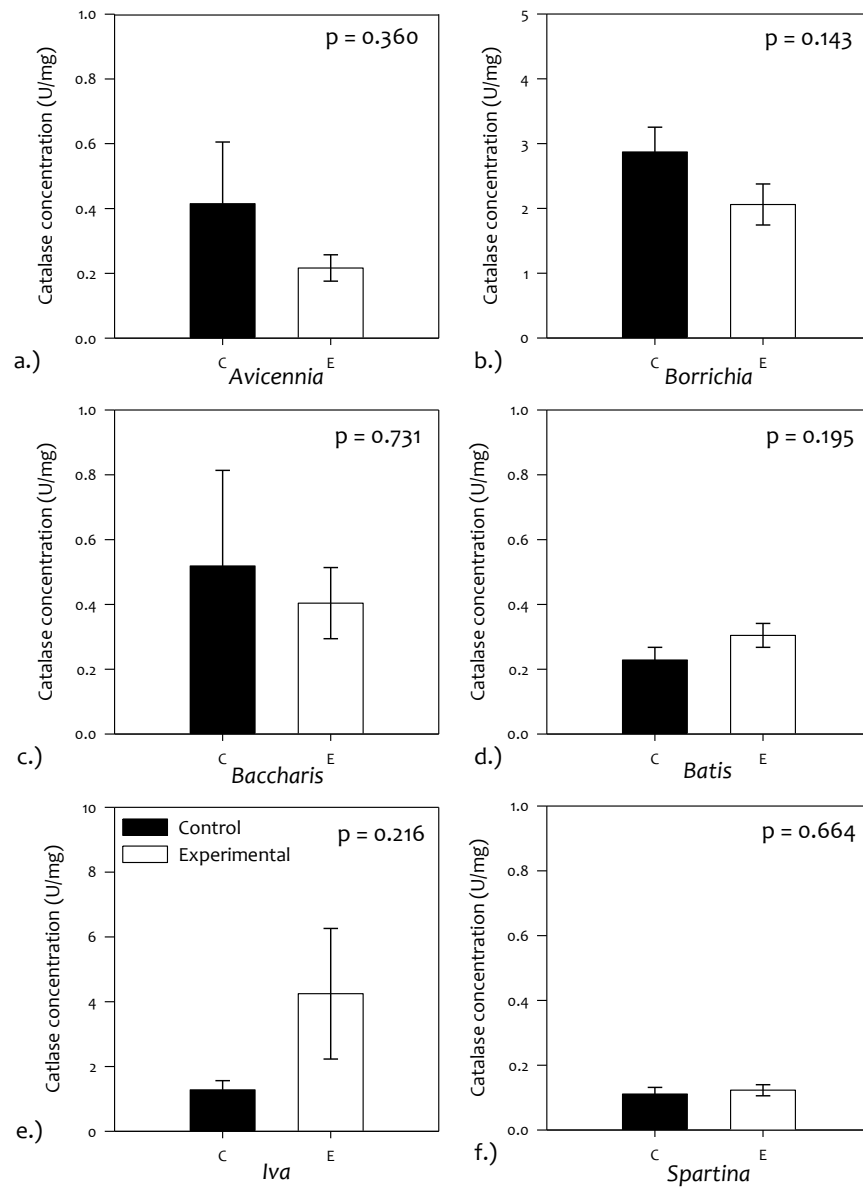


Figure 17.

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VITAE

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