



# Nitrogen Addition Increases Freeze Resistance in Black Mangrove (*Avicennia germinans*) Shrubs in a Temperate-Tropical Ecotone

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## ABSTRACT

Low temperature stress is the primary factor determining the latitudinal limits of tropical plants. As the climate warms, tropical species are migrating poleward, displacing native species and modifying ecosystem structure and function. Changes are particularly evident along latitudinal gradients with the highest velocity of change occurring in wetlands. In coastal wetlands, saltmarshes dominate at latitudes above 30°, whereas mangroves occur

mostly in the tropics because most species are intolerant of freezing temperatures, but others, like *Avicennia germinans* (black mangrove), do tolerate freezing temperatures. In response to a warmer climate and fewer killing freezes, mangroves are currently expanding into saltmarshes. However, the speed of the transition from saltmarsh to mangrove can also be modified by extreme events and nutrient subsidies. In a fertilization experiment along the Atlantic coast of North America, we found that nitrogen addition altered plant traits in *Avicennia*, which increased their resistance to freezing temperatures. This trait shift resulted in negligible freeze effects during a January 2018 extreme freeze event compared to unfertilized plants, which lost more than 80% of the leaves and more than 40% of the wood in their canopies. The freeze-killed litter from unfertilized plants provided a nutrient pulse that influenced recovery, growth and mangrove cover for three years following the freeze. Nutrient enrichment and recovery from the

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freeze effects led to increased growth and structural complexity of the mangrove canopy, which further enhanced freeze tolerance, shrub growth form and the ability of *Avicennia* to displace the saltmarsh in the temperate–tropical ecotone.

**Key words:** fertilization experiment; nitrogen limitation; plant traits; freeze resistance; microclimatic warming; extreme freeze events; saltmarshes; climate change; temperate–tropical ecotone.

## HIGHLIGHTS

- For the black mangrove (*Avicennia germinans*) growing near its range limit along the upper east coast of Florida, fertilization increased freeze resistance and reduced the negative effects caused by an extreme freeze event by more than 80%.
- Coastal eutrophication will accelerate the rate of regime shift from saltmarsh- to mangrove-dominated wetlands in this temperate–tropical ecotone.
- Recovery of freeze-affected black mangroves depends on nutrient availability and the severity of the effects.

## INTRODUCTION

Climate change is causing shifts in the distribution of species along latitudinal gradients, leading to changes in the structure and function of source and recipient ecosystems (Chuang and Peterson 2016). In the ecotone between temperate and tropical climates, global warming has resulted in a decreased frequency of extreme cold events over the past 30 years, allowing tropical and subtropical species to migrate poleward and establish populations beyond their historic range limits (Stevens and others 2016; Osland and others 2021). The highest velocity of climate-driven vegetation change is occurring in wetlands, affecting both saltmarshes and mangrove ecosystems (Loarie and others 2009). For coastal wetlands in the warm temperate zone (Lugo and others 1999), mangroves are expanding further poleward into temperate saltmarshes to form mixed ecotonal ecosystems (Perry and Mendelssohn 2009; Saintilan and others 2014; Cavanaugh and others 2015; Chen and others 2017).

Along the Atlantic coast of North America, this temperate–tropical ecotone spans more than two degrees of latitude, from 28.2°N to 30.5°N (Kennedy and others 2021). According to historical records, the northern and southern boundaries of mangrove distribution within this ecotone have expanded and contracted multiple times over the past 250 years in response to extreme freeze events caused by intense cold-air outbreaks (Roger and Rohli 1991; Cavanaugh and others 2019). Over this period, numerous freeze events had widespread effects on mangroves, including mortality, canopy loss, defoliation and stem breakage (Bidlingmayer and McCoy 1978). Between January 2021 and the extreme freeze of December 1989, which had a minimum temperature of -8.3 °C, this area also experienced seven freezes with temperatures between -4 °C and -7 °C (data obtained from the National Climatic Data Center, NCDC). These sublethal freezes had extensive effects on the canopy but caused low mortality of the mangroves (Osland and others 2020).

Even though mangrove area has declined globally (Valiela and others 2009), mangrove range has expanded latitudinally as climate has warmed (Cavanaugh and others 2015), and as tropical cyclones have continued to disperse mangrove propagules poleward of their current range limit (Kennedy and others 2020). Along Florida's NE coast, mangroves have doubled their area and progressively expanded their distribution northward over the past 30 years (Rodríguez and others 2016). But, unlike more tropical areas, mangrove forests in this ecotone are dominated by low-stature, multi-stemmed shrubs, a growth form typically associated with environmental disturbances and abiotic stress, including high salinity, low temperature and low soil fertility (Bellingham and Sparrow 2009). The black mangrove (*Avicennia germinans*), a prolific resprouter and the most freeze-tolerant mangrove in Florida, is the dominant species in these shrub mangrove stands. Expansion of shrub mangroves into herbaceous saltmarshes not only causes a change in community composition but also a dramatic transformation in the structure of coastal communities (Ross and others 2009). This shift is expected to have far-reaching consequences for ecological processes (Guo and others 2017) and associated ecosystem services (Kelleway and others 2017).

In addition to the effects of climate change on mangrove distribution, nutrient over-enrichment of coastal wetlands, a major threat worldwide (Malone and Newton 2020), can also hasten the expansion of mangroves into saltmarshes (Weaver

and Armitage 2018; Dangremond and others 2020). The detrimental effects of nutrient loading on mangroves and saltmarshes include eutrophication, hypoxia, establishment of non-native species and increased coastal erosion (Deegan 2012). When coupled with climate change, nutrient enrichment may also cause additional changes in coastal wetlands (Turner and others 2009).

Recent studies provide evidence of how mangroves and saltmarshes are affected by excess nutrients, which can influence their responses to global warming (Dangremond and others 2020). Experimental nutrient enrichment increases both above- and belowground mangrove biomass (Hayes and others 2017) and their capacity to store carbon (C) (Lovelock and others 2014), trap sediments, build soil and persist as mangrove habitats through time (McKee and others 2007). Nutrient enrichment also results in higher productivity, which is linked to a greater delivery and diversity of the numerous ecosystem services attributed to mangroves (Kelleway and others 2017). However, increased productivity has also been shown to make mangroves more vulnerable to extreme events, including droughts (Lovelock and others 2009) and tropical cyclones (Feller and others 2015). Increased mortality from extreme events in response to nutrient enrichment is expected to exert a negative feedback on C storage and surface elevation as mangroves replace saltmarsh in the temperate–tropical ecotone (Doughty and others 2016). In saltmarshes, some studies have also linked nutrient enrichment to increased erosion and loss of ecosystem services (Mo and others 2020). In contrast, other studies have found that nutrient enrichment resulted in increased belowground biomass and sediment capture, which stabilize wetlands (Morris and others 2013). However, the relationship between nutrient enrichment and vulnerability to freeze events remains unclear for both mangrove and saltmarsh ecosystems.

In this study, we investigated the effects of nutrient enrichment on mangrove responses to a freeze event in the temperate–tropical ecotone. Specifically, we investigated the plant traits associated with freeze effects and the recovery from a freeze in a mixed mangrove–saltmarsh ecosystem along Florida’s NE coast. Replicate plots containing an *Avicennia germinans* (hereafter *Avicennia*) shrub and an understory of *Avicennia* seedlings and herbaceous saltmarsh vegetation dominated by *Batis maritima* L. (hereafter *Batis*) and *Sarcocornia perennis* were fertilized annually since December 2012 with nitrogen (N) or phosphorus (P) or left unfertilized as the Control. Previous work showed

that plant growth in this experiment was N-limited; that is, N addition increased shoot elongation, leaf area index (LAI), percent cover, reproductive output, canopy height and volume of the *Avicennia* shrubs and it decreased cover and diversity of saltmarsh vegetation (Dangremond and others 2020). Nitrogen addition also altered plant traits often correlated with freeze tolerance (Cook-Patton and others 2015). In January 2018, a three-day sublethal freeze caused visible effects but no mortality in our fertilization experiment. This gave us an opportunity to study how the freezing event affected mangroves and saltmarsh plants, to determine how it varied among nutrient treatments and to track their recovery over the next three years. In this study, we tested the following three hypotheses (H1, H2 and H3):

- H1: Plants growing in nutrient-limited soil increase their C allocation to leaves, which is reflected in a suite of canopy- and leaf-level traits (for example, high leaf mass per area, slow growth, reduced canopy structure, low photosynthetic rates, more sclerophyllous leaves and efficient nutrient conservation strategies) that also make them more resistant to environmental stressors, including freezing temperatures (Ordóñez and others 2009). Although results vary by species, it is commonly assumed that fertilized plants are more tender and vulnerable to freezing (Charrier and others 2015), which predicts that the N-fertilized (hereafter + N) *Avicennia* plants in our experiment will experience more negative effects when exposed to a freeze event.
- H2: Increased complexity in the canopy structure of shrubs enhances microclimatic warming and buffers air temperatures beneath the canopy, which provides cold-intolerant plants with a mechanism to avoid negative effects during freeze events (Gratani and others 2003). This hypothesis predicts that our + N-fertilized *Avicennia* shrubs will experience fewer negative effects because of increased shrubbiness, cover and LAI. The lower stature and less complex structure of Control and P-fertilized (hereafter + P) plants are expected to have a smaller impact on microclimatic warming, which will result in more negative effects in these treatments.
- H3: In low-nutrient sites that are frequently exposed to sublethal freezing temperatures, basal resprouting is the primary mechanism for recovery from freeze damage in shrubs (Bellingham and Sparrow 2009). Resprouting from basal epicormic shoots allows freeze-damaged man-

groves to recover within one to three years (Osland and others 2020). However, recovery depends on plant size, nutrient resources and damage severity (Feller and others 2015).

We predict that differences in nutrient treatment will result in variable propagule production versus resprouting as mechanisms for recovery in freeze-affected *Avicennia* shrubs. Specifically, we expect that the + N plants in our experiment will reproduce sexually by producing propagules in contrast to Controls and + P plants, which will recover vegetatively by resprouting. We also predict that repetitive damage by sublethal freeze events and subsequent basal resprouting will increase the shrubbiness (stems/shrub) of *Avicennia* in this ecotone, which may increase their resistance to future freeze events (D'Odorico and others 2012).

To test these hypotheses, we quantified *Avicennia* freeze effects in our fertilization experiment following the January 2018 freeze. We examined relationships between freeze effects and morphological, physiological and structural traits at both leaf and canopy levels that have previously been correlated with increased vulnerability to cold stress (Hayes and others 2019). We also measured whole plant responses (for example, canopy traits, growth, resprouting) for three years post-freeze to track how these plants recovered from the effects of a sublethal freeze.

## MATERIAL AND METHODS

### Study site

Our fertilization experiment was set up in December 2012 within a mixed saltmarsh–mangrove wetland (140 ha) on Anastasia Island just north of Matanzas Inlet in the Guana Tolomato Matanzas National Estuarine Research Reserve (GTMNERR), St. John's County, FL (N29°43', W81°14'), USA, near the poleward range limit for mangroves along the Atlantic coast (Figure 1). Our initial objective was to determine whether increased nutrients affected the rate of mangrove expansion, as described in Dangremond and others (2020). To characterize the mangrove vegetation at this location, we used the point-centered quarter method (Cintrón and Schaeffer-Novelli 1984), with data obtained in June 2018 at 20 points at regular intervals across the widest part of the site, which was dominated by a dense stand ( $4.5 \text{ m}^{-2}$ ) of multi-stemmed (Mean  $\pm$  SE:  $2.6 \text{ stems} \pm 0.1 \text{ shrub}^{-1}$ ), low-stature *Avicennia* shrubs intermixed with an understory of saltmarsh species (*Spartina*, *Batis*, *Sarcocornia*).

The climate is transitional between subtropical and temperate zones, with a maximum air temperature of  $20.8^\circ\text{C} \pm 1.2$  during the winters (December–February) and  $31.4^\circ\text{C} \pm 1.3$  during the summers (June–September), and relatively constant during springs and falls ( $26.3^\circ\text{C} \pm 1.0$ ). From 2012 through 2017, and winter temperatures were relatively warm with no cold events lasting longer than one day (January 4, 2012,  $-1.1^\circ\text{C}$ ; February 2, 2012,  $-2.8^\circ\text{C}$ ; January 7, 2014,  $-3.3^\circ\text{C}$ ). On January 17, 18, 19, 2018, this area experienced a sublethal freeze with minimum temperatures of  $-1.1^\circ\text{C}$ ,  $-4.0^\circ\text{C}$ ,  $-2.8^\circ\text{C}$ , respectively, with freezing temperatures lasting for more than 10 hours each day (NCDC). No additional freeze events occurred at the experimental site during the three years following the January 2018 freeze.

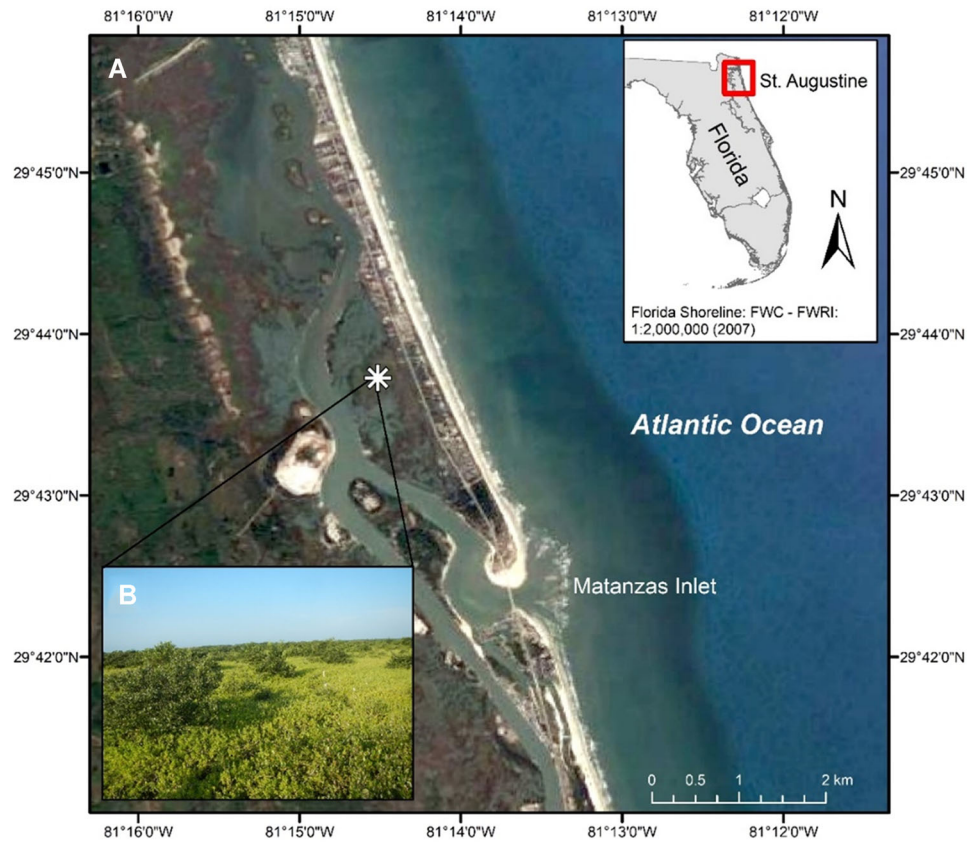
### Experimental design

*Avicennia* shrubs of similar stature, approximately 10 m apart, were selected within the mixed saltmarsh–mangrove wetland. Small plots ( $1 \text{ m} \times 1 \text{ m}$ ) were cordoned off around *Avicennia* shrubs, seedlings and saltmarsh to prevent trampling and to monitor fertilizer effects on the substrate and understory. The target shrubs were fertilized annually with one of three randomly assigned nutrient treatments (Control, + N, + P;  $N = 8$ ) with 300 g of N as  $\text{NH}_4$  (45:0:0) or P as  $\text{P}_2\text{O}_5$  (0:45:0), described in Feller (1995). Fertilizer (150 g) was sealed in dialysis tubing and placed in each of two holes (3 cm diameter 30 cm deep), cored into the substrate on opposing sides of an *Avicennia* shrub beneath the outermost margin (dripline) of the canopy and sealed.

### Freeze Effects

Initial symptoms of the January 2018 freeze included leaf browning and desiccation in the *Avicennia* canopy, which distinguished them from living (green) leaves (Osland and others 2020). The freeze-killed leaves quickly turned brown, but they remained attached to the plants for almost four months after the freeze event with most leaf fall during the following May.

We began measurement of freeze-killed leaves in February 2018, three weeks after the freeze. To quantify freeze effects to each fertilized plant, we counted all leaves including: live (green); senescent (yellow); dead non-senescent completely brown; and dead non-senescent mottled brown and measured leaf area and biomass on one stem/shrub. Because the mottled brown leaves were completely



**Figure 1.** (A) Location of a long-term fertilization experiment in a (B) mixed mangrove–saltmarsh wetland along Matanzas Inlet in NE Florida (USA) where an extreme freeze event occurred in January 2018.

brown when we revisited the site in April, the numbers of brown and mottled brown leaves were combined for a total of leaves killed by the freeze. Criteria for the stem selected were that it reached the top of the shrub canopy and was in a position that permitted access without damage to adjacent parts of the canopy. These values were multiplied by the number of stems/shrub to estimate leaf number, area and biomass for the whole canopy and were used to determine proportion of the leaves and leaf biomass killed by the freeze. We also measured the vertical distribution of freeze effect from ground level to the top of the canopy for each fertilized shrub. Subplots, 1.0 m × 0.5 m, adjacent to each fertilized shrub were used to quantify freeze effects on seedling and saltmarsh density, and size.

We monitored freeze effects in the fertilization experiment at frequent intervals (February, April, May, June and September 2018) to determine its impact on litter fall and stem death. By June 1, 2018, all freeze-killed leaves initially measured in February had fallen and provided an estimate of litter production by each fertilized *Avicennia*. We

also observed numerous defoliated stems and extensive top-kill in shrub canopies. To estimate the proportion of woody biomass killed by the freeze, we counted live and dead stems on each plant on June 1, 2018.

## Explanatory Variables

To assess the role of canopy structural and leaf traits that have been shown to influence the sensitivity of *Avicennia* to freeze effects, we assembled a database of explanatory variables, including canopy traits measured in February 2018 and leaf traits from Dangremond and others (2020) (Table 1). Canopy traits included height (m), crown area (m<sup>2</sup>), volume (m<sup>3</sup>), leaf area index (LAI, m<sup>2</sup> m<sup>-2</sup>), shrubbiness (stems/shrub), leaf density (leaves/shrub) and cover (%) of all canopy components. Leaf area index was measured with a Li-Cor 2250 Plant Canopy Analyzer (LI-COR, Lincoln, NE). Crown area was calculated as a regular ellipse based on the widest diameter and its orthogonal axis. Canopy volume was calculated using canopy height and crown area in a derived formula for shrub canopies (Thorne and others 2002). For ca-

**Table 1.** Responses of Canopy Structural Traits to Nutrient Enrichment Treatments at the Time of the January 2018 Freeze Event

| Trait                                               | Nutrient enrichment treatments |                          |                          | One-way ANOVA results |         |
|-----------------------------------------------------|--------------------------------|--------------------------|--------------------------|-----------------------|---------|
|                                                     | Control                        | Nitrogen                 | Phosphorus               | F                     | P       |
| <i>Avicennia germinans</i>                          |                                |                          |                          |                       |         |
| Plant height (m)                                    | 0.83 (0.02) <sup>a</sup>       | 1.2 (0.02) <sup>b</sup>  | 0.76 (0.01) <sup>a</sup> | 25.37                 | < 0.001 |
| Crown area (m <sup>2</sup> )                        | 2.03 (0.15) <sup>a</sup>       | 4.20 (0.26) <sup>b</sup> | 2.06 (0.11) <sup>a</sup> | 5.64                  | 0.011   |
| Leaf Area Index (m <sup>2</sup> · m <sup>-2</sup> ) | 2.47 (0.14) <sup>a</sup>       | 3.83 (0.24) <sup>b</sup> | 2.31 (0.22) <sup>a</sup> | 15.9                  | < 0.001 |
| Shrubiness (stems/shrub)                            | 4.5 (0.2)                      | 6.3 (0.3)                | 5.9 (0.3)                | Ns                    |         |
| <i>Avicennia</i> cover (%)                          | 51.6 (4.6) <sup>a</sup>        | 91.3 (3.8) <sup>b</sup>  | 58.9 (4.6) <sup>a</sup>  | 30.3                  | < 0.001 |
| Seedling density (m <sup>-2</sup> )                 | 48.5 (2.1)                     | 43.5 (3.0)               | 31.3 (2.2)               | Ns                    |         |
| Seedling height (cm)                                | 21.9 (0.1) <sup>a</sup>        | 25.3 (0.3) <sup>a</sup>  | 22.9 (0.1) <sup>a</sup>  | 9.63                  | 0.001   |
| <i>Batis maritima</i>                               |                                |                          |                          |                       |         |
| Saltmarsh height (cm)                               | 50.3 (0.6)                     | 52.1 (1.0)               | 46.2 (0.5)               | Ns                    |         |
| Saltmarsh cover (%)                                 | 29.1 (4.6) <sup>a</sup>        | 5.3 (3.2) <sup>b</sup>   | 24.3 (4.9) <sup>a</sup>  | 11.60                 | < 0.001 |

nopy cover, a 1 × 1 m nadir photograph of each shrub was analyzed with SamplePoint (Booth and others 2006).

We used analysis of covariance (ANCOVA) to evaluate the effects of nutrient treatment on freeze effects observed following the January 2018 freeze while controlling for differences between treatments, such as height. Only two traits, canopy volume and leaves/plant, had slopes that were significantly different between treatments. For canopy volume, ANCOVA results are reported; for all other traits, we proceeded with one-way analysis of variance (ANOVA) to assess the influence of nutrient enrichment treatments on the effect observed. When an ANOVA yielded significant effects, pairwise multiple comparison procedures (Holm–Sidak method) were applied. The proportion of leaves killed by the freeze was arcsine-square root transformed prior to analysis.

We evaluated the relationships between plant trait variables with freeze effects, using data and trait values for those variables that varied significantly in response to nutrient enrichment. We excluded variables from these analyses that were not significantly different among the three fertilizer treatments. Specifically, we assessed the relationships between freeze effects and leaf and canopy-level variables (that is, %C, %N, C:N, N:P, above-ground biomass allocation, chlorophyll content; see Table 2 for complete list). Leaf nutrient data were used to estimate the N and P content in the freeze-killed leaves. Because many of the explanatory variables were likely correlated, we used principal components analysis (PCA) to reduce the variables into fewer dimensions. Data were scaled and centered as part of the analysis.

**Table 2.** Contributions of 17 Leaf and Canopy Trait Variables to the First Two Principal Components, Expressed as Percentages

| Variable                                | PC1  | PC2  |
|-----------------------------------------|------|------|
| Total freeze damage (%)                 | 10.7 | 0.34 |
| Plant height (cm)                       | 10.1 | 0.01 |
| LAI                                     | 8.99 | 0.88 |
| Leaves (per shrub)                      | 8.95 | 0.97 |
| Canopy volume (m <sup>3</sup> )         | 8.90 | 0.03 |
| Canopy growth (cm y <sup>-1</sup> )     | 8.64 | 0.03 |
| Chlorophyll (mg m <sup>-2</sup> )       | 7.10 | 0.45 |
| Leaf size (cm <sup>2</sup> )            | 6.38 | 0.15 |
| Leaf N (%)                              | 6.36 | 7.67 |
| Leaf C (%)                              | 5.72 | 2.85 |
| Cover (%)                               | 5.28 | 0.80 |
| Crown area (m <sup>2</sup> )            | 5.18 | 10.4 |
| Leaf C:N <sub>at</sub>                  | 4.15 | 13.0 |
| Stems (per shrub)                       | 2.34 | 12.1 |
| Glycine betaine (μmol g <sup>-1</sup> ) | 0.94 | 15.5 |
| Leaf P (%)                              | 0.25 | 16.5 |
| Leaf C:P <sub>at</sub>                  | 0.01 | 18.3 |

As an additional leaf trait, we measured glycine betaine content of live, freeze-killed and senescent leaves from the fertilized shrubs because this compatible solute has been linked to freeze tolerance in *Avicennia* and many other species (Chen and Murata 2011). The protocol for analysis of glycine betaine accumulation was measured as described in Hayes and others (2019).

## Recovery

To track recovery of the *Avicennia* shrubs, we compared annual growth and allocation to above-ground biomass from the year before and the three

years after the January 2018 freeze. For annual growth, we collected terminal 0.5-m long stem sections in August 2020 from the top of the tallest stem of each experimental *Avicennia* shrub and located annual increments of growth of the main axis, which are delimited by a series of short internodes (winter growth) on either end separated by series of long internodes (summer growth), which together mark shoot elongation from the previous winter to the onset of the next winter (Duarte and others 1999). By locating these transitions in internode length proximal to the terminal bud, we determined the length of each annual increment within the harvested stem sections. Because the terminal buds were still intact, we used this method to reconstruct the history of annual growth rates for the year prior to the freeze even though some stems had been killed by the freeze. To compare differences in allocation to above-ground biomass among the fertilized *Avicennia* shrubs before and after the freeze, we also measured leaf mass fraction (LMF; leaf biomass (g): leaf + stem biomass (g)) and stem mass fraction (SMF; stem biomass (g): leaf + stem biomass (g)) for the annual increments harvested in October 2015 and in January 2021. In addition, we continued to measure LAI, percent cover and canopy volume at six-month intervals to track how nutrient enrichment affected recovery.

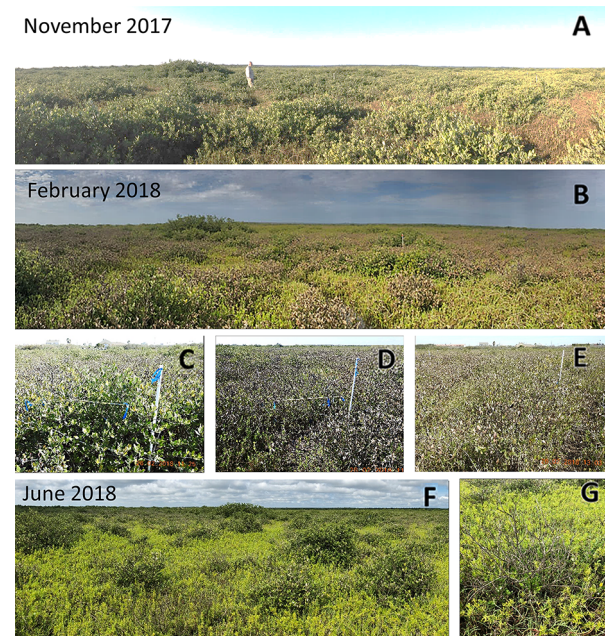
To determine whether the freeze altered the mode of regeneration by vegetative resprouting versus flowering, we also recorded the number of basal resprouts and the proportion of terminal shoots in flower in the canopy of each fertilized shrub. The proportion of terminal shoots with inflorescences was determined during dominant flowering season in June 2018, July 2019 and June 2021. Basal resprouts were initially counted in June 2018 and were recounted in January 2021.

During the recovery period, measurements of growth (shoot elongation), LAI, LMF, SMF and flowering were taken on the same individuals multiple times. To account for temporal pseudoreplication, we used linear mixed effects models with individual plant as a random effect and treatment and time as fixed effects, with AIC used for model selection. In the LAI analysis, the + N treatment was significantly different than the other two treatments, but Control and + P were not different from each other, so they were combined in the final model (Figure S1, Table S1). We analyzed epicormic shoot production per shrub in 2021 with a Poisson generalized linear model (GLM). Data analyses were performed in JMP 13.2 and R version 3.43 (R Core Team 2017).

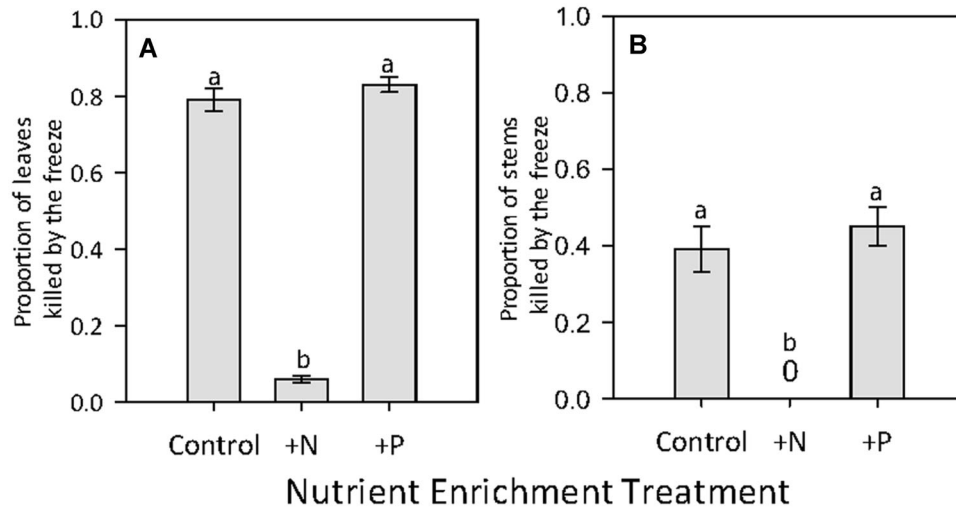
## RESULTS

### Freeze Effects

At the time of the freeze, multiple canopy structural traits of the fertilized *Avicennia* (for example, plant height, crown area, LAI, shrubbiness, cover), were significantly greater for + N plants than for Controls and + P plants (Table 1). In November 2017, no freeze effect was evident in the *Avicennia* shrubs at this site (Figure 2A). Two months later, immediately after a three-day freeze in January 2018, a layer of brown, non-senescent, freeze-killed leaves in the mangrove canopy was widespread across the entire site including our fertilization experiment (Figure 2B). There were significant differences among the nutrient enrichment treatments in the proportion of freeze-killed leaves (Figure 3A; one-way ANOVA,  $F_{2, 21} = 37.2$ ,  $p < 0.001$ ). Contrary to expectations from our H1, response to the freeze was negligible in + N compared to Control and + P plants, in which more than 80% of the leaves were killed by the freeze (Figure 2C-E). Although there was no difference between Control and + P treatments in the proportion of leaves killed by the freeze, within-canopy effects varied dramatically. From the ground

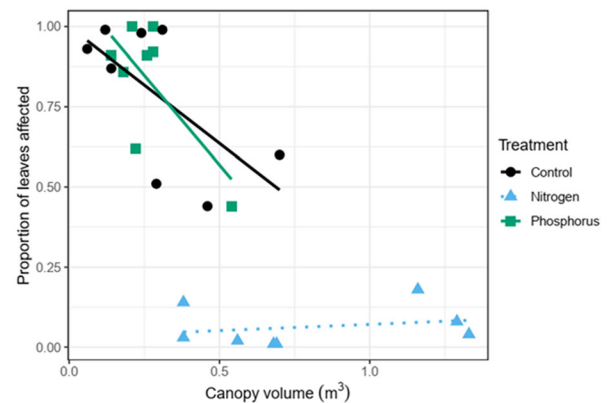


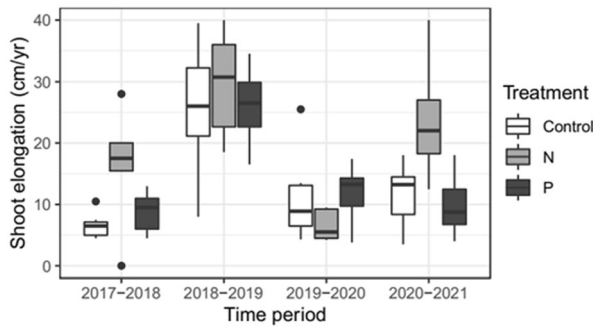
**Figure 2.** Fertilization experiment (A) eight weeks before and (B) three weeks after the 3-day freeze event in January 2018. (C) Freeze effect in N-fertilized plants was negligible. (D) Visible freeze effects were severely affected in the P-fertilized and (E) control plants with > 80% of their leaves killed. (F) By June 2018, leaf fall exposed (G) dead branches killed by the freeze.



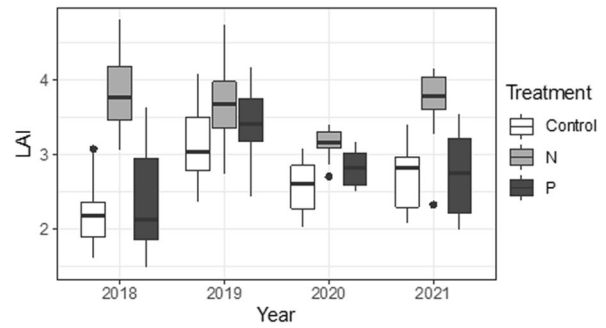
**Figure 3.** Proportion of (A) leaves and (B) stems in the canopies fertilized *Avicennia germinans* killed by a freeze in January 2018. Values are means  $\pm$  1 SE. Lowercase letters above bars show results of a one-way ANOVA for freeze-killed leaves and a Kruskal–Wallis one-way ANOVA on ranks for freeze-killed stems.

surface to a height coincident with the height of the surrounding saltmarsh (Table 1), *Avicennia* leaves below  $50.3 \text{ cm} \pm 1.7 \text{ cm}$  remained green with no visible freeze response. From that height to the top of the canopy, almost all the leaves in *Avicennia* canopies in the Control and + P treatments were completely brown and dead or mottled brown and dead because of freeze effect. In contrast, there were no freeze-killed leaves in any plants in the + N treatment, which suggested that increased N enhanced freeze resistance in *Avicennia* (Figure 2B). Other than small brown spots on a few green leaves at  $59.2 \text{ cm} \pm 1$  above the ground that accounted for less than 3% of the leaves, no freeze-killed leaves were observed in the + N *Avicennia*, which had larger canopy volumes and more leaves per plant than Control and + P plants. A larger canopy volume was associated with less freeze effect in Control and + P plants, but canopy volume did not affect the response to the freeze in + N plants (Figure 4; ANCOVA,  $F = 5.37$ ,  $p = 0.01$ ). Similarly, for a given number of leaves per plant, + N plants experienced less freeze effects than Control or + P plants (ANCOVA,  $F = 4.02$ ,  $p = 0.036$ ). The freeze-killed leaves fell into the leaf litter by June 1. Compared to + N plants, differences among treatments in the number of leaves killed per plant by the freeze also resulted in a three- to fourfold increase in brown, non-senescent litterfall biomass from the Control and + P plants. In addition, this litter originated from green leaves killed directly by the January 2018 freeze, which precluded nutrient resorption, resulting in twice as

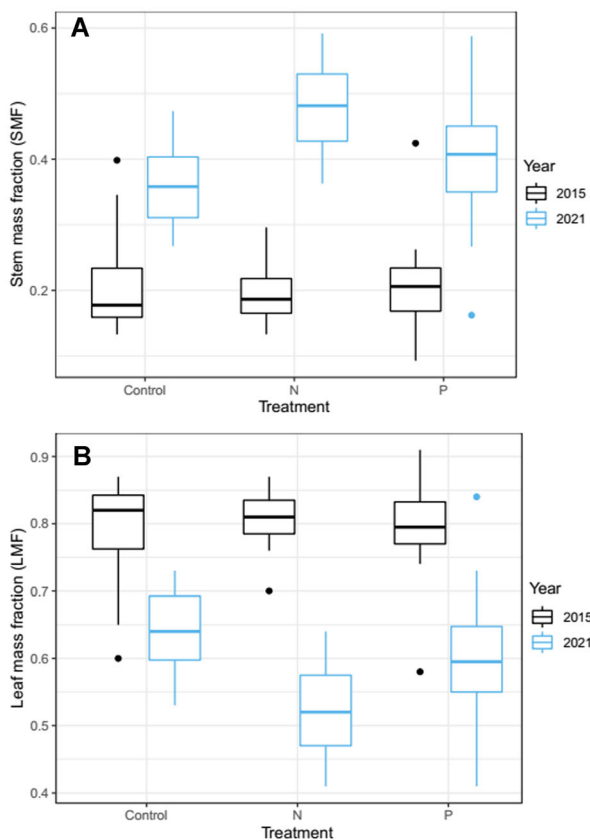




**Figure 6.** Effects of nutrient treatment on shoot elongation between 2017 and 2021.



**Figure 8.** Proportion of shoots flowering before and after the January 2018 freeze in each fertilizer treatment, 2016 to 2021.



**Figure 7.** Biomass allocation to (A) stems (stem mass fraction) and (B) leaves (leaf mass fraction) in 2015 and 2021.

much N in leaves entering the litter than from senescent leaves.

We also found differences among treatments in the proportion of stem tissue killed by the January 2018 freeze with multiple dead stems in the Control and + P plants and no effect in + N plants

(Figure 3B). After leaf fall of the freeze-killed leaves, we did not observe any dead stems in the + N plants. In contrast, widespread top-kill was evident with  $92\% \pm 8$  and  $100\% \pm 13$  of the stems in Control and + P plants, respectively, which were dead from the top of the *Avicennia* canopy to the top of the saltmarsh vegetation because of the freeze. In the lower portions of these stems beneath the adjacent saltmarsh canopy, the stems were still alive.

Unlike the *Avicennia* canopy, no freeze effect was observed to the saltmarsh understory throughout the site. Percent cover for the saltmarsh was significantly higher at Control and + P plants than at + N plants (Table 1). Each cordoned-off plot had a high density ( $41.1 \text{ m}^{-2} \pm 1.8$ ) of small *Avicennia* seedlings ( $23.4 \text{ cm} \pm 0.4$  tall) with no difference among treatments. There was no evidence of seedling mortality or freeze effect caused by the freeze in any of the plots.

## Explanatory Variables

The PCA summarized the 17 variables that we measured in response to nutrient treatments and identified four eigenvalues greater than 1 that accounted for 82% of the variation. The first two eigenvalues accounted for 64% of the variation (Figure 5). The explanatory variables contributing most to the first axis (Axis I) were canopy-level traits (that is, dead leaves and stems, plant height, canopy growth and LAI), whereas the explanatory variables that contributed most to the second axis (Axis II) were leaf-level traits: C:P, percentage P and glycine betaine (Table 2). Nitrogen-fertilized plants differed dramatically from Control and + P plants along Axis I, but they had more overlap along Axis II (Figure 5).

## Recovery

Prior to the freeze, annual growth rates, measured as shoot elongation from end of previous winter to beginning of following winter ( $\text{cm y}^{-1}$ ), varied significantly in response to nutrient enrichment with similar growth rates for Control and + P plants that were less than half that of + N plants. In the year following the freeze (2018–2019), growth rates dramatically increased in all treatments compared to the previous year, with annual growth rates in Control and + P plants similar to those of + N plants (Figure 6). In the following year (2019–2020), growth rates decreased to values similar to Control plants at the start of the time period. Shoot elongation of + N plants continued to increase compared to the other treatments between 2020 and 2021 (mixed effects model, Table S2). Leaf area index also increased dramatically for the Control and + P plants between 2018 and 2019, the year after the freeze. Leaf area index decreased in + N plants in 2020 but increased again the following year (Table S1).

The proportion of aboveground biomass allocated to leaves (LMF) versus stems (SMF) changed significantly before (October 2015) and after (January 2021) the January 2018 freeze (Figure 7). Although data from before the freeze showed that nutrient enrichment had little effect on LMF or SMF, the proportion of biomass allocated to leaves was significantly higher before the freeze than after, decreasing in all treatments (linear mixed effects model, Table S3), whereas allocation to stem biomass increased significantly after the freeze.

Following the freeze, nutrient enrichment also altered recovery through its effects on flowering and vegetative basal resprouting, which affected canopy shrubbiness. By June 2018,  $63\% \pm 7$  of the leaf-bearing shoots on + N plants had flowers compared to  $3\% \pm 1$  on Control and  $1\% \pm 1$  on + P plants. A linear mixed effects model showed that year, treatment and their interaction had significant effects on flowering (Table S4). Although + N plants overall had higher rates of flowering than Control and + P plants, all treatments experienced a decrease in 2018 compared to 2016 levels of flowering. By 2019, Control and + P plants had recovered flowering potential to pre-freeze levels, but + N plants had not (Figure 8).

In June 2018, none of the + N plants had formed any basal sprouts. However, 100% of the Control and + P shrubs were resprouting vigorously from their bases and had produced  $52.3 \pm 9.5$  and  $54.5 \pm 14.1$  epicormic sprouts, respectively. By 2021, epicormic shoot production was reduced in

the Control and + P shrubs to an average of  $2.5 \pm 0.5$  and  $4.5 \pm 0.7$  epicormic shoots per plant, respectively. The number of epicormic shoots produced by + P plants was significantly higher than Control plants (Poisson GLM,  $p = 0.04$ , Table S5). The + N plants had an average of 0.9 epicormic shoots per plant in 2021, which was significantly less than Control plants (Poisson GLM,  $p = 0.02$ , Table S5).

## DISCUSSION

Based on historical evidence, extreme but infrequent freeze events have set the poleward limits of mangrove distributions along the Florida coast (Lugo and Patterson-Zucca 1977; Kangas and Lugo 1990). Between 1835 and 1989, Florida's NE coast experienced 15 extreme freeze events that had minimum temperatures between  $-8^\circ\text{C}$  and  $-13^\circ\text{C}$  (Rogers and Rohli 1991; Attaway 1997), exceeding the lethal freeze threshold established for mangrove mortality along the Gulf of Mexico (Osland and others 2020). Based on NCDC weather data for 1977 and 2021, this area also experienced 11 sublethal freezes with minimum temperatures of  $-4^\circ\text{C}$  to  $-7^\circ\text{C}$  in addition to four extreme freeze events with minimum temperatures of  $-8.3^\circ\text{C}$  to  $-12.2^\circ\text{C}$ , all of which occurred in the 1980s. Although few studies have investigated the effects of past extreme freeze events on mangroves (Bidlingmayer and McCoy 1978; Lonard and Judd 1991), those effects can be inferred from proxy data on citrus and other crops (Attaway 1997). Despite limited firsthand data, diverse historical sources for Florida (for example, personal journals, plant and animal surveys, herbarium sheets) recorded details and observations for reconstructing changes in mangrove distribution and compelling evidence that widespread mangrove mortality coincided with extreme freeze events (Cavanaugh and others 2019). In addition, the sublethal freezes, which are cold enough to damage more than 50% of the leaves but not to kill the plants (Lugo and Patterson-Zucca 1977; Pickens and Hester 2011), are relatively common in this region during December and January. The duration of such sublethal freezes is also known to be equally or more important than minimum temperatures for citrus cultivation (Attaway 1997). Although few data are available from mangrove ecosystems, Coldren and Proffitt (2017) found that survival of *Avicennia* seedlings in a shade house experiment decreased with increased duration of exposure to a sublethal freeze.

In this study, we also found that a sublethal freeze did not cause mortality of mangroves, but it

did cause major damage to the mangrove canopy. In addition, our data showed that increased nutrient availability reduced freeze effect to the mangrove canopy and altered the mode of recovery following the freeze, which contrasts with other disturbances to mangroves where increased nutrient availability has been linked to greater defoliation and stem breakage in the canopy (Feller and others 2015).

Following a three-day, sublethal freeze in January 2018 with minimum temperatures reaching  $-4^{\circ}\text{C}$  on the second day, + N *Avicennia* shrubs in a long-term fertilization experiment had little to no visible freeze effect. In contrast, Control and + P plants were severely affected and had lost more than 80% of their leaf biomass. In addition, more than 40% of their stem biomass was pruned from the canopy. These data did not support our first hypothesis, which predicted that increased nutrient availability would increase freeze effect. Instead, these results suggest that coastal eutrophication will not only increase the growth and reproductive output of mangrove plants in this system, but it will also increase their cold tolerance and will further accelerate the regime shift from saltmarsh- to mangrove-dominated wetlands in this temperate-tropical ecotone.

Despite extensive defoliation and stem losses from the *Avicennia* shrubs in the Control and + P treatments, none of the plants at our study site were killed by the January 2018 freeze. Unlike the + N plants in the experiment, which had no basal resprouting, within four months Controls and + P plants initiated numerous vigorous vegetative basal sprouts that far outnumbered the stems killed by the freeze event. Within three years, several of the sprouts in each Control and + P plant survived to become part of the multi-stemmed *Avicennia* canopy. Our results suggest that sublethal freeze events coupled with an ability to regenerate vegetatively by vigorous basal resprouting after a disturbance have resulted in the multi-stemmed shrub growth form characteristic of *Avicennia* growing at its latitudinal limit (D'Ordico and others 2013). These findings support our second hypothesis that structural traits may contribute a protective mechanism that allows *Avicennia* shrubs to avoid exposure of their plant tissue to freezing temperatures, with little evidence of increased physiological freezing tolerance. They further suggest that frequent, repetitive sublethal freezes that occur in the temperate-tropical ecotone will cause increased canopy shrubbiness. This may enhance

the microclimatic warming provided by the *Avicennia* canopy and thereby create a positive feedback that leads to increased tolerance of future lethal and sublethal freeze events (Huang and others 2020). Similar results have been reported for other ecosystems where differences in vegetation can lead to changes in the local microclimate that temper the impact of freeze events (He and others 2015). Thus, by increasing growth, cover and complexity of the *Avicennia* shrub canopy, increased nutrient availability may create spatial heterogeneity that alters the microclimate to lessen the impacts of sublethal and extreme freeze events in the temperate-tropical ecotone. Our results suggest that the characteristic shrub architecture of *Avicennia* in this ecotone is an evolved trait that increases survival at its latitudinal range limit.

Initial results from our fertilization experiment showed that + N not only increased growth, but it also altered a range of leaf and canopy traits in *Avicennia* shrubs (Dangremond and others 2020, Table 6S). Similar results have been reported for other mangrove fertilization experiments in different environments (Reef and others 2010). It is broadly assumed that the vigorous new growth of leaves and stems that develops in response to nutrient enrichment is more sensitive to cold because it lacks the traits and structural rigidity typically associated with cold-tolerant plants (Niinemets 2016). However, our findings contrast with predictions that plants growing in high nutrient availability are more susceptible to cold stress than are nutrient-limited plants (Charrier and others 2015; Song and others 2020). Predictions from other experimental studies in temperate, boreal and Mediterranean climates are equivocal such that fertilization experiments had variable effects in diverse woody species, for example, cold tolerance increased (Taulavuori and others 2001), decreased (Heredia-Guerrero and others 2014) or did not change (Puertolas and others 2005), in response to nutrient enrichment.

Results from this study also show that the N-fertilized plants in our experiment were not visibly affected by the January 2018 freeze and that only the severely affected Control and + P plants regenerated with vigor. Energy to build and maintain the vigorous structure of the + N plants was provided by our annual additions of fertilizer. Yet, stress from the freeze event was still evident in the + N plants especially during their second year of recovery when they experienced a delayed and slower rate of growth similar to Controls, no

development of new stems from basal sprouting and a decrease in flowering.

For mangroves growing at their latitudinal limit, a suite of plant traits has been shown to make them more tolerant of cold temperatures (Stuart and others 2007; Cook-Patton and others 2015). However, fertilization experiments in various mangrove ecosystems have demonstrated similar structural and physiological adjustments in plant traits in response to differences in nutrient availability (Reef and others 2010). Based on a PCA of freeze effects with leaf and canopy traits, we found that + N plants were completely separated from Control and + P plants. These results show that variations in canopy structural traits (height, LAI, leaves per shrub, canopy volume) explain most of the differences that we observed in freeze effects (Table 2), which supports our second hypothesis. Thus, nutrient enrichment results in a more vigorous, complex and freeze-resistant canopy, which also increases microclimatic warming (Thompson and others 2017), contributes to mangrove dominance of coastal marshes in the temperate–tropical ecotone (Osland and others 2019) and facilitates poleward expansion of mangroves despite recurring freeze events.

Similar to other species of woody plants (Niinemets 2016), freeze resistance in mangroves is correlated with canopy traits that modify the surrounding microclimate and ameliorate air temperatures. Although few studies have quantified microclimatic warming in mixed mangrove–saltmarsh ecosystems, its importance for protecting mangrove seedlings in the understory from freeze damage is well recognized (Pickens and Hester 2011; D’Odorico and others 2013; Osland and others 2015, 2019). Both mangrove and saltmarsh vegetation have been shown to contribute to microclimatic warming in the understory that improves seedling survival during freeze events (Coldren and Proffitt 2017; Devaney and others 2017; Pickens and others 2019; Goldberg and Heine 2021). However, these studies found that air temperatures were consistently warmer under shrub mangrove canopies than under the co-occurring saltmarsh plants during freeze events. We suggest that survival of mangrove seedlings is facilitated by shrubby adults, thus creating a positive feedback that helps with mangrove infill and expansion.

Consistent with our third hypothesis, nutrient availability also interacted with the January 2018 freeze to alter reproduction and canopy regeneration in the *Avicennia* fertilization experiment. Even though the + N-fertilized plants were not visibly affected by the freeze event, their flowering was

significantly reduced for three years following the freeze. Only the severely affected Controls and + P plants were able to regenerate their canopies by vigorous basal sprouting as predicted. However, flowering by + N plants following the freeze was significantly lower than pre-freeze flowering, which only partially supports our hypothesis. Consistent with estimates from other mangrove ecosystems, recovery from the freeze event for both flowering and growth was approximately three years (Osland and others 2020).

Continued measurements of our fertilization experiment after the freeze showed that nutrient availability also had a significant effect on canopy recovery. In the year following the freeze, growth increased dramatically in the Control and + P plants as well as the + N plants. These data suggest that recycling of nutrients in the freeze-killed leaf litter following the freeze coincided with and may have stimulated the growth spurt that we observed. Rather than causing the predicted decline in productivity, the non-senescent leaves, which were killed by the freeze in mid-January 2018 and fell from the shrubs by June 2018, had more than 2% N compared to less than 1% N in litter composed of naturally senescent leaves (Dangremond and others 2020). However, these freeze-killed leaves were greenfall because they died while they were green. Consequently, nutrient resorption, an important physiological strategy for nutrient conservation in plants, was prevented. Recycling of this large pulse of higher quality litter may have provided approximately twice the concentration of N as in naturally senescent leaves. The growth spurt that we observed after the freeze may have been triggered by the greenfall of freeze-killed leaves that created a short-lived pulse of N in early June that was quickly recycled back into the mangrove. This pulse of N may have been the resource pulse that stimulated growth spurt of Controls and + P plants to the point that they were almost equivalent to our + N-fertilized *Avicennia*, which continued to grow vigorously but produced no freeze-killed litter. Similarly, Ellis and others (2006) reported results for white mangrove (*Laguncularia racemosa*) following a 2003 freeze event that also killed leaves while they were still green, which prevented nutrient resorption and likewise produced a pulse of enriched litter. Numerous studies in other forested ecosystems have reported analogous nutrient pulses following deposition and rapid decomposition of large amounts of greenfall caused by various types of disturbances where it provided N enrichment greater than background levels in litterfall and had positive effects on productivity

and recovery (for example, Yang and others 2008; Cantrell and others 2014). It seems less likely that the nutrient pulse that caused this short-lived growth could have been stimulated by nutrients from the nearby land or river. Thus, our results suggest sublethal freezes may also create a nutritive feedback response that increases resilience of the *Avicennia* ecosystems in the temperate–tropical ecotone, which potentially accelerates mangrove recovery and expansion and enhances resistance to future freeze events.

In conclusion, our results show that + N, the growth-limiting nutrient in coastal wetlands in the temperate–tropical ecotone, increases *Avicennia*'s tolerance of freezing temperatures. Freezes are relatively frequent during December and January along Florida's upper east coast, and this freeze tolerance contributes to the ability of mangroves to expand poleward. The increase in canopy structural complexity that we observed in response to the + N treatment correlated with an increase in freeze tolerance, which suggests that nutrient over-enrichment of the coastal zone may hasten the transition from saltmarsh to mangrove dominance in the ecotone. Similar to fertilization experiments, warming chamber experiments in mangroves and other ecosystems have found that warmer temperatures increase plant growth and shrubbiness and alter microclimatic warming, biogeochemistry and community structure (Chapman and others 2021). In contrast with the + N-fertilized *Avicennia*, freeze damage reduced the canopies of Control and + P plants by > 80% and prevented flowering and propagule production for more than a year. However, the canopies of these severely freeze-affected plants were able to begin recovery within four months via basal resprouting. Our data showed that both N enrichment and freeze effects stimulated increased complexity and shrubbiness of the *Avicennia* canopy, which we predict would result in increased microclimatic warming that may be an important mechanism to ameliorate the effect of episodic freeze events and facilitate the expansion of mangroves in the temperate–tropical ecotone.

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## DATA AVAILABILITY

All data available at Smithsonian Figshare <https://doi.org/10.25573/serc.21206261.v1>.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

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