From physiology to salt marsh management challenges with sea level rise: the case of native *Spartina foliosa*, invasive *S. densiflora* and their hybrid

Blanca Gallego-Tévar¹,†, Procioipo Peinado-Torrubia¹,†, Rosario Álvarez¹, Brenda J. Grewell² and Jesús M. Castillo¹,*

¹Departamento de Biología Vegetal y Ecología, Universidad de Sevilla, Ap 1095, 41080 Sevilla, Spain
²USDA-ARS, Invasive Species and Pollinator Health Research Unit, Department of Plant Sciences MS-4, 1 Shields Avenue, University of California, Davis, CA 95616, USA

*Corresponding author: Departamento de Biología Vegetal y Ecología, Universidad de Sevilla, Ap. 1095, 41080, Sevilla, Spain. Email: manucas@us.es

Sea level rise (SLR) imposes increasing salinity and inundation stresses in salt marshes which simultaneously face invasions by exotic plant species. We aimed to improve and apply knowledge on the ecophysiological responses of halophytes to SLR to conservation management of salt marshes. In a mesocosm experiment, we measured and compared phosphoenolpyruvate carboxylase (PEPC) activity and related functional traits of the California-native *Spartina foliosa*, invasive *S. densiflora* and their hybrid *S. densiflora × foliosa* in response to increasing levels of salinity and inundation. *S. foliosa* was moderately sensitive to salinity, showing a 57% reduction in PEPC specific activity from freshwater to hypersalinity. This native species compensated for the reduction of PEPC activity with increased salinity through 80% higher enzyme activation by phosphorylation. PEPC functional trait responses of *S. foliosa* were mostly independent of inundation depth. In view of these results, managers should conserve undeveloped lands for accommodation space above current high tide lines to facilitate colonization of stress-tolerant *S. foliosa*. Our results on functional responses of PEPC traits recorded high sensitivity to salinity for *S. densiflora*. This was reflected by 65% lower PEPC specific activity together with increasing accumulation of free proline (+96%) and total proteins (+23%) with elevated salinity. These results suggest prioritized eradication of *S. densiflora* populations in brackish habitats. Measured PEPC responses support the high stress tolerance of the *S. densiflora × foliosa* hybrid. PEPC traits for the hybrid were mostly independent of salinity and inundation. The hybrid showed higher PEPC-specific activity than *S. foliosa* (+70%) and *S. densiflora* (+15%) in freshwater under intermediate inundation. Results suggest that eradication of the hybrid should be the highest management priority. Our study shows that the responses of key functional physiological traits to environmental stresses serve as biological indicators that can guide ecosystem management practices in a scenario of climate change.

**Keywords:** environmental stress, hybrid, inundation, PEPC, polyploid, salinity, transgressive traits

**Editor:** Kevin Hultine

Received 29 December 2019; Revised 12 May 2020; Editorial Decision 13 May 2020; Accepted 14 May 2020

**Cite as:** Gallego-Tévar B, Peinado-Torrubia P, Álvarez R, Grewell BJ, Castillo JM (2020) From physiology to salt marsh management challenges with sea level rise: the case of native *Spartina foliosa*, invasive *S. densiflora* and their hybrid. *Conserv Physiol* 8(1): coaa053; doi:10.1093/conphys/coaa053.
Introduction

Sea level rise (SLR) due to global warming is increasing salinity, and inundation depth and duration in the world’s salt marshes (IPCC, 2015). These environmental changes are highly significant since salinity and flooding are among the main abiotic stress factors determining the performance and distribution of halophytes in salt marshes (Engels and Jensen, 2010). In this context, halophytes may respond to SLR by migrating to less stressful habitats or survive in situ through genetic adaptation to changing conditions and phenotypic plasticity (Xue et al., 2018). In addition to SLR, salt marshes are being impacted by other human-mediated processes such as biological invasions. Global climate change and invasive species can also have interacting effects that compound uncertainty associated with each individual stress driver (Hellman et al., 2008). Exotic invasive species displace local biodiversity in salt marshes (Gedan et al., 2009). Sometimes, alien species hybridize with native species producing hybrids with high stress tolerance and competitive ability that also displace native biodiversity in salt marshes (Wong et al., 2018; Williams et al., 2019). This improved performance of hybrids may be related to transgressive traits due to non-additive gene expression (Favre and Karrenberg, 2011). Interacting environmental changes can decrease the effectiveness of invasive plant management, and it is important for conservation managers to identify which invasive species are likely to change (Hellman et al., 2008). Therefore, improved knowledge of functional and evolutionary traits supporting the invasiveness and relative impacts of exotic plant species is a priority (Drenovsky et al., 2012).

In the context of ongoing global environmental changes, preservation of salt marshes is essential since they carry out many important ecosystem services such as mitigating climate change by sequestering atmospheric carbon, providing food, regulating water and air quality, buffering the impacts of storms and tsunamis and offering natural spaces for recreational activities (Moomaw et al., 2018). Choosing and prioritizing salt marsh conservation goals is not an easy task, but knowledge of ecophysiological mechanisms underlying the survival and persistence of key halophyte species can inform decision-making to preserve ecosystem functions. In this context, improved knowledge of ecophysiological responses of both native and invasive species to a changing environment can provide scientifically based insight for design, prioritization and implementation of conservation management practices (French et al., 2017).

Spartina species (cordgrasses; Poaceae) are among the most widely distributed and abundant halophytes in salt marshes around the world. Many Spartina taxa behave as invasive alien species in their non-native geographical ranges where they have hybridized with resident native congeners forming transgressive hybrids (Strong and Ayres, 2013). However, some F1 Spartina hybrids are initially sterile, but research suggests they may become fertile and very competitive allopolyploid species (Aïnouche et al., 2004). Cordgrasses have a C4 photosynthetic metabolism, with phosphoenolpyruvate carboxylase (PEPC) playing a key role in CO₂ assimilation and other metabolic pathways. PEPC is regulated by allosteric positive (e.g. glucose-6-phosphate) and negative (e.g. L-malate) effectors (Jiao et al., 1991). In addition, PEPC enzymatic activity is regulated by reversible protein phosphorylation at its N-terminal domain and this process is controlled by a highly regulated Ca²⁺-independent protein-Ser/Thr kinase (Jiao et al., 1991). Light activates the kinase and consequently causes an increase in the activity and a decrease in the L-malate sensitivity of PEPC. The low sensibility to L-malate indicated a dephosphorylated PEPC at fresh-water conditions (Jiao and Chollet, 1992; Wang and Chollet, 1993). This regulatory phosphorylation depends not only on light but also on several abiotic factors such as salinity, carbon dioxide or inundation levels (Li and Chollet, 1994; Echevarría et al., 2001; Yordanova and Popova, 2007; Mateos-Narango et al., 2010). Thus, environmental changes in salt marshes due to SLR may alter PEPC activity in C4-halophytes and greatly influence changes in carbon fixation and ultimately change plant growth. In this sense, a recent study analyzed the effects of salinity on functional traits of PEPC in the European native Spartina maritima (Curtis) Fernald and the South American invasive Spartina densiflora Brongn. and their reciprocal hybrids and documented the development of some transgressive traits by the hybrids (Gallego-Tévar et al., 2019a). Studies of other Spartina hybrids (i.e. sterile S. x townsendii, a hybrid between Spartina alterniflora Loisel. and S. maritima in England; fertile S. alterniflora x foliosa hybrid in the San Francisco Estuary) have also revealed transgressive traits (Strong & Ayres, 2013). We reviewed 28 studies related to the response of S. foliosa, S. densiflora and their hybrids to salinity, 20 works related to inundation, and only one publication analyzing the combined effect of salinity and inundation on these taxa (Supplementary information: Literature review). To our knowledge, no previous studies have analyzed the combined effects of both salinity and inundation on PEPC performance and halophyte response.

Our study system included the California-native Spartina foliosa Trin. colonizing low to middle intertidal marshes, and invasive S. densiflora and their sterile F1 hybrid S. densiflora x foliosa growing mostly in middle/high marshes in the San Francisco Estuary (CA, USA) (Gallego-Tévar et al., 2020). Recent publications analyzing functional responses to salinity, inundation and their interaction have characterized S. foliosa as a stress-tolerant species, S. densiflora as a fast-growing species able to take advantage of low to moderate abiotic stress conditions and their invasive cross as a transgressive hybrid with high stress tolerance (Gallego-Tévar et al., 2019b, 2020). Our primary goals were to (i) improve present knowledge on the ecophysiological responses of these three halophytes to SLR and (ii) apply this new biological information to the management of salt marshes in the context of environmental change. We performed a mesocosm
experiment where, for the first time, PEPC activity and its regulation were recorded in response to the combined stress effects of salinity and inundation depth mimicking a SLR scenario. In addition, foliar free proline and malondialdehyde (MDA) concentrations were recorded as biological indicators of salt stress and oxidative damages, respectively (Mulholland & Otte, 2001; De Azevedo Neto et al. 2006). We hypothesized that native S. foliosa would have higher tolerances to salinity and flooding than invasive S. densiflora, whereas their hybrid would have greater tolerance than both parental species to both abiotic stresses due to influences of PEPC enzymatic levels. Our experimental results, in combination with the literature review (Supplementary information: Literature review), were then used to make conservation recommendations for native S. foliosa and to predict and prioritize counter measures in response to the invasion of S. densiflora and its hybrid.

**Material and methods**

**Studied taxa and plant material**

*Spartina foliosa* (2n = 62 chromosomes; Ayres et al., 2008) is the only native cordgrass in estuaries along the North American Pacific Coasts of California (USA) and Baja California (Mexico) (Mobberly 1956; Ayres et al., 2003). It plays a key role in primary succession of salt marshes and provides ecosystem services such as improved water quality, sediment accretion and habitat functions for fishes and wading birds (Ayres et al., 2003) (see Supplementary Fig. 1). In the San Francisco Estuary, *S. foliosa* has hybridized with invasive *S. alterniflora* and with invasive *S. densiflora* (Daehler and Strong, 1997; Ayres et al., 2008). For our experiment, we obtained *S. foliosa* individuals from middle to low marshes in the Carquinez Strait, in the northern reach of the San Francisco Estuary (38°57’57” N, 122°11’36” W).

*Spartina densiflora* (2n = 70) is a South American cordgrass that has invaded salt marshes in the Southwest Iberian Peninsula and along the Pacific Coast of North America (Bortolus, 2006) from the San Francisco Estuary north to British Columbia (Castillo et al., 2014). Invasive populations of *S. densiflora* show high phenotypic plasticity and low genetic diversity along the Pacific Coast of North America, where, in places, the species co-occurs with *S. foliosa* (Castillo et al., 2014, 2016, 2018; Grewell et al., 2016). The sterile hybrid *Spartina densiflora × foliosa* (diploid; 2n = 65 chromosomes) is primarily found in an overlapping range with *S. densiflora* within middle elevation salt marshes in the San Francisco Estuary (Ayres et al., 2008) (see Supplementary Fig. S1). This hybrid may have higher salinity tolerance than both parental species (Lee et al., 2016). *S. densiflora* and the hybrid *S. densiflora × foliosa* were collected from middle intertidal marshes in the Corte Madera Creek tributary to the San Francisco Estuary (37°56’27” N, 122°31’2” W).

**Experimental design**

*Spartina* rhizomes were cleaned and classified into weight classes to obtain similar-size experimental individuals according to the growth form of the different taxa (80–120 g for *S. foliosa* rhizomes; 230–280 g for *S. densiflora*; 20–50 g for the hybrid) at the Aquatic Weed Research Facility, University of California, Davis, in March 2017. Clean and standardized rhizomes were transplanted to 3.1 litre pots (pot size: 15 cm diameter × 17.5 cm height) containing sterile sand. Pots were sub-irrigated with freshwater for 2 months prior to arrangement in 16 500-l plastic mesocosms (1.3 m × 0.8 m × 0.6 m) (Rubbermaid, Atlanta, GA) for exposure to different salinity and inundation treatments. Treatments were randomly assigned within a randomized complete block design with treatments (4 salinity levels × 3 inundation depths × 3 taxa) nested within the 16 mesocosms. The split-plot, full-factorial experimental design included salinity as the whole plot factor, with each salinity level replicated 4 times. Inundation level was randomly assigned within salinity mesocosms as the subplot (within plot) factor, and the three focal taxa were nested within the subplots (n = 4 plants per taxon and treatment combinations). Salinity treatments ranged from freshwater to hypersalinity (0.5, 10, 20 and 40 ppt); treatments were prepared using a 20% Hoagland’s nutrient solution and Hoagland’s solution plus sea salts (Instant Ocean®, Aquarium Systems Inc., Mentor, OH) to achieve desired concentrations. EcoPond Clear biological product (Grow More Inc., Gardena, CA) was added to reduce algal proliferation. Salinity was gradually increased by 10 ppt per week to avoid osmotic shock in the higher salinity treatments. Three permanent inundation treatments were established: deep inundation (55.0 cm deep; pots placed on bottom of tank), intermediate inundation (35.5 cm deep; pots placed on concrete stands within tanks) and shallow inundation (4.5 cm deep; pots placed on stacked concrete stands within tanks). The experiment was carried out for 31 days (8–June 8 May 2017) in a glasshouse with controlled air temperature between 21–25°C. The natural photoperiod was extended to 12-h daily using high-intensity discharge lights (GE Lucalox LU1000/ECO HPS 1000 W, PARsource, Petaluma, CA). The photon flux density measured by a photometer (LI-COR LI-250A light meter; LI-COR Inc., Lincoln, NE) was 500 μmol m−2 s−1 at the canopy level and 100 μmol m−2 s−1 at the bottom of the mesocosms at midday. Plant material was collected at midday from the mid-section of randomly chosen flag leaves (first unfolded adult leaf from the apical leaf; ca. 2 g per plant) and lyophilized (n=3–4 per treatment combination).

**Abiotic stress indicators: proline and MDA quantification**

Free proline is a compatible organic solute that is a measure of plant response to salt stress, as reported previously for *Spartina* species (Mulholland & Otte, 2001). Foliar free proline concentrations were recorded as biological indicators of salt stress and oxidative damages, respectively (Mulholland & Otte, 2001; De Azevedo Neto et al. 2006). We hypothesized that native *S. foliosa* would have higher tolerances to salinity and flooding than invasive *S. densiflora*, whereas their hybrid would have greater tolerance than both parental species to both abiotic stresses due to influences of PEPC enzymatic levels. Our experimental results, in combination with the literature review (Supplementary information: Literature review), were then used to make conservation recommendations for native *S. foliosa* and to predict and prioritize counter measures in response to the invasion of *S. densiflora* and its hybrid.
proline content was determined following Bates et al. (1973). Lyophilized leaves (0.5 g) were homogenized in 10 ml of 3% sulfosalicylic acid and centrifuged at 15 000g for 5 min. Supernatant (2 ml) was combined with glacial acetic (2 ml) acid and acid-ninhydrin (2 ml) and boiled at 100°C for 1 h. Reaction was stopped in ice, and toluene (2 ml) was added. The upper toluene phase was analysed spectrophotometrically at 517 nm. Free proline concentration was calculated from a standard curve of L-proline.

Foliar MDA concentration was recorded to evaluate oxidative damage in relation to lipid peroxidation accumulation as a response to salinity and inundation stress (De Azevedo Neto et al. 2006; Zhu et al., 2019). MDA was assayed following Buege and Aust (1978). Lyophilized leaves (0.1 g) were homogenized in TCA–TBA reagent (2 ml; 20% (w/v) trichloroacetic acid (TCA) and 0.5% (w/v) 2-thiobarbituric acid (TBA)) and centrifuged at 13 000g for 2 min. The supernatant was boiled at 90°C for 30 min, then cooled and centrifuged at 15 000 g for 15 min. The absorbance of the supernatant was measured at 532 nm for MDA and at 600 nm for the non-specific absorption. MDA concentration was calculated using its molar extinction coefficient ($ε = 155 \text{mM}^{-1} \text{cm}^{-1}$).

**PEPC activity, L-malate test and soluble protein quantification**

Lyophilized leaf tissue (0.2 g) was ground with 1 ml of extraction buffer containing 0.1 M Tris–HCl pH 7.5, 20% (v/v) glycerol, 1 mM EDTA, 10 mM MgCl₂ and 14 mM mercaptoethanol. The homogenate was centrifuged at 17 000 g for 2 min, and the supernatant was used immediately as a clarified protein extract to determine the PEPC activity and sensitivity of PEPC to L-malate. PEPC activity was measured spectrophotometrically at optimal and suboptimal pH (8.0 and 7.3, respectively) using the NAD-malate dehydrogenase assay containing 2.5 mM phosphoenolpyruvate (PEP), 1 mM NaHCO₃, 5 mM MgCl₂, 5 units of NAD-malate dehydrogenase, 0.2 mM NADH and 100 mM Hepes/KOH (Echevarria et al., 1994). An enzyme unit (U) was defined as the amount of PEPC that catalyzes β-carboxylation of 1 μmol of PEP min⁻¹ at pH 8 and 30°C. Malate sensitivity was determined at suboptimal pH 7.3 in the presence of various concentrations of L-malate, where the malate inhibition of PEPC activity was expressed as IC₅₀ (50% inhibition of initial PEPC activity by L-malate). Previous studies validated the L-malate test as reflecting PEPC phosphorylation state (Feria et al., 2008). A high IC₅₀ is related to a high degree of PEPC phosphorylation (Echevarria et al., 1994). Protease and phosphatase inhibitors were not added to the extraction buffer to avoid PEPC activity lost since PEPC activity in leaf extracts was measured rapidly and very diluted (5 μl of crude extract in a final volume of 1000 μl); similar results have been obtained with and without adding inhibitors (Echevarria et al., 1990; Gallego-Tévar et al., 2019a). Extracts were not desalted as previous studies have shown similar results with and without desalting (Rodríguez-Penagos and Muñoz-Clares, 1999; Gandullo et al., 2019; Gallego-Tévar et al., 2019a). The total protein amount was determined following the colorimetric method of Bradford (1976), using bovine serum albumin as standard. PEPC activity was expressed as units per gram of protein (apparent specific activity).

**Statistical analysis**

Analyses were carried out using Sigma-Plot (Systat Software Inc., Point Richmond, CA; Windows version 12.0). Results were considered significant when $P ≤ 0.05$. Kolmogorov–Smirnov and Levene tests were used to verify the normality and homogeneity of variance of the data series. Data series for PEPC apparent specific activity and total protein amount were transformed using the function $\sqrt{x}$ to reach normality and homogeneity of variance. Each variable assayed was analysed using three-way analysis of variance (ANOVA) with taxa, salinity and inundation treatments as grouping factors. Tukey's honest significant different (Tukey–HSD) test was used as post hoc analysis. Relationships between PEPC traits, total protein content and proline and MDA concentrations among them and with inundation and salinity treatments were studied for every taxon using the Pearson correlation coefficient ($r$).

**Results**

**Abiotic stress indicators: proline and MDA accumulation**

Foliar free proline concentration showed significant differences among salinity and inundation treatments, and for taxa × salinity and taxa × inundation interactions (Table 1). Proline accumulation increased markedly with salinity for every taxon (Table 2). Proline concentration decreased with increasing total protein content for S. foliosa and the hybrid, showing the opposite response measured for S. densiflora. S. densiflora accumulated more proline than the other two taxa at hypersalinity under every inundation treatment. In contrast, S. foliosa and the hybrid increased their proline concentration at deeper inundations, especially at 20 and 40 ppt salinity (Fig. 1a; Table 2).

MDA exhibited significant differences among taxa, salinities and inundation depths, and taxa × salinity and salinity × inundation interactions (Table 1). MDA increased with proline concentration and salinity just for the hybrid, whereas both parental species accumulated less MDA at higher salinities. MDA was independent of inundation depth for every taxon (Fig. 1b; Table 2).

**PEPC traits**

PEPC apparent specific activity and IC₅₀ showed significant differences among taxa, salinities and taxa × salinity,
salinity × inundation, and salinity × inundation × taxa interactions (Table 1). *S. foliosa* tended to show lower PEPC apparent specific activity than *S. densiflora*, while their hybrid had intermediate specific activity values. In general, PEPC-specific activity tended to decrease at higher salinities for all taxa, but this relationship was only significant for the hybrid which showed the highest activity values in freshwater. PEPC-specific activity was independent of inundation depth for all taxa, except when under intermediate inundation in freshwater *S. foliosa* expressed its minimum activity and the hybrid its maximum activity values, with the hybrid having higher PEPC-specific activity of either parental species (Fig. 2a; Table 2).

IC$_{50}$ varied among taxa at 10 and 40 ppt salinity, with *S. densiflora* showing the highest values at 10 ppt salinity under lower inundation depths and at hypersalinity under deeper inundation. Thus, IC$_{50}$ increased together with total protein content and proline concentration in *S. densiflora*. IC$_{50}$ values increased with salinity for both parental species, and this relationship was much greater for *S. densiflora* than for *S. foliosa* (Pearson correlation coefficient, P < 0.001 and P < 0.01, respectively). IC$_{50}$ for the hybrid was independent of salinity. IC$_{50}$ was independent of inundation depth for *S. foliosa* and the hybrid, whereas it increased together with inundation at 20–40 ppt salinity for *S. densiflora* (Pearson correlation coefficient, P < 0.05) (Fig. 2b; Table 2).

The total amount of proteins changed significantly among taxa, salinity, and taxa × salinity and salinity × inundation × taxa interactions (Table 1). Total protein content decreased with increasing salinity for *S. foliosa* and its hybrid (especially at hypersalinity), whereas *S. densiflora* accumulated more proteins at higher salinities (Fig. 2c; Table 2).

**Discussion**

Native *S. foliosa* showed a moderate sensitivity to salinity in relation to PEPC activity and related functional traits. Thus, *S. foliosa* presented its maximum PEPC amounts in freshwater conditions, reflected in maximum PEPC apparent specific activity combined with minimum activation by phosphorylation (low IC$_{50}$ values). *S. foliosa* tried to compensate for the decrease in PEPC amount at higher salinity levels, with higher activation by phosphorylation as recorded previously for *Spartina maritima × densiflora* (Gallogo-Tévar et al., 2019a) and mutant *Amaranthus edulis* Spec. (Dever et al., 1997). Courtney et al. (2016) recorded that PEPC gene transcription was down-regulated at high salinities (> 12 ppt), which may explain our recorded decrease in PEPC amounts with increasing salinity. In contrast, the high marsh halophyte *Atriplex halimus* L. increased its foliar PEPC amount at high salinity (c. 30 ppt) (Alla et al., 2011). On the other hand, *S. foliosa* PEPC functional traits were independent of inundation depth, except that they had maximum PEPC-specific activity under shallow inundation in freshwater conditions. Thus, *S. foliosa* was moderately sensitive to salinity and to a minor degree, also somewhat sensitive to flooding in relation to its PEPC traits. These results improve the mechanistic understanding of findings from previous studies that characterized *S. foliosa* as moderately tolerant of salinity, given its capacity to colonize new sites by producing viable seeds even under high salinity, and due to its high tolerance to inundation (Gallogo-Tévar et al., 2020) (see Supplementary information: Literature review). Nevertheless, *S. foliosa* most often occurs in low marsh intertidal zones, which are expected to be highly impacted by SLR (Janousek et al., 2019). However, our results suggest that existing natural populations of *S. foliosa* should be able to tolerate a degree of increased salinity and inundation during
IC₅₀, PEPC phosphorylation state recorded as 50% inhibition of initial PEPC activity by L-malate. n = 34–48. Significant results (P < 0.05) are marked in bold.

SLR, as marshes transgress inland where possible and as the new habitat experiences increases in soil salinity and inundation (e.g. Fagherazzi et al. 2019). In this scenario, S. foliosa has the physiological tolerances to progressively replace upland vegetation as it colonizes the new salt marsh areas. Conservation managers should put highest priority on conservation of undeveloped lands for accommodation space above current high tide lines to facilitate natural migration and colonization of stress-tolerant S. foliosa.

Functional responses of PEPC characterized invasive S. densiflora as a halophyte highly sensitive to salinity. Maximum PEPC apparent specific activity was recorded in freshwater conditions, and activation by phosphorylation increased markedly at higher salinities as a compensatory mechanism (Gallego-Tévar et al., 2019a). The low sensitivity to L-malate (low IC₅₀ values) indicated a dephosphorylated PEPC at freshwater conditions (Jiao and Chollet, 1992; Wang et al., 1993). Additionally, the increase in activation
by phosphorylation with increasing salinity (+84%) co-occurred with high accumulation of free proline (+96%) and total proteins (+23%). Both proline and total protein (including antioxidant enzymes) accumulations are typical responses to salinity stress (Khalid et al., 2019), reflecting that the recorded sharp increase in PEPC activation by phosphorylation was also a stress response in S. densiflora. Changes in leaf anatomy that have been shown to drive PEPC activity in other Poaceae species in response to environmental factors such as light intensity (Ma et al., 2017) may also be operating in Spartina spp. responses to changes in salinity and flooded conditions. Following previous studies (Castillo et al., 2005, Gallego-Tévar et al., 2018a, 2020), the invasive halophyte S. densiflora was able to tolerate increasing salinity and flooding levels with markedly reduced fitness, but it was able to retain some capacity for seed production. Moreover, IC50 increased together with inundation at higher salinities for S. densiflora. In this sense, PEPC activity increased twofold after soil flooding in the C-4 plant Zea mays L. (Yordanova & Popova, 2007). In view of these results, and given limited resources for invasive species management, the management of S. densiflora-invaded marshes should prioritize eradication of new S. densiflora populations colonizing more benign (i.e. less saline soils at higher marsh elevations) habitats where growth rates and fruit set will be highest (Nieva et al., 2001). In this sense, S. densiflora and other Spartina taxa are being aggressively managed with the goal of estuary-wide eradication by mechanical and chemical methods in the San Francisco Estuary (Strong and Ayres, 2016). To a lesser degree, S. densiflora invasion control is also being carried out in the Humboldt Bay Estuary, northwestern California (Augyte and Pickart, 2014) and at the Odiel Marshes in the Southwest Iberian Peninsula (Castillo and Figueroa, 2009).

Our results on PEPC functional traits are in accordance with the previous characterization of S. densiflora as fast-growing species able to tolerate moderate stress levels (Gallego-Tévar et al., 2020; see Supplementary information: Literature review). The recorded increase in the phosphorylation state of PEPC at hypersalinity for S. densiflora from the San Francisco Estuary contrasted with the opposite response recorded for S. densiflora from the Odiel Estuary (Gallego-Tévar et al., 2019a). Álvarez et al. (2010) reported the existence of local adaptation in an ecotype of S. densiflora from high latitudes that was able to compensate low PEPC activation by phosphorylation with increasing amounts of the enzyme at freshwater conditions. Ecotypes with different PEPC activity levels have also been recorded for other wetland plants such as Phragmites australis (Cav.) Trin. ex Steud. (Zheng et al., 2000). Since S. densiflora at measured PEPC levels show higher performance in response to increasing salinity in the San Francisco Estuary than was recorded in the Odiel Estuary, managers should be especially aware of S. densiflora invading newly formed wetland restoration areas, and higher salinity salt marshes in recently flooded areas of the San Francisco Estuary.

Finally, Gallego-Tévar et al. (2020) described the hybrid S. densiflora × foliosa as a taxon relatively tolerant of both salinity and inundation. This is in accordance with our results in relation to its PEPC responses of the taxon, in which

Figure 1: Foliar free proline (a) and malondialdehyde (MDA) (b) concentration for native Spartina foliosa (yellow columns), invasive S. densiflora (blue columns) and their hybrid S. densiflora × foliosa (green columns) exposed to four salinity treatments (0.5, 10, 20 and 40 ppt) at three inundation depths (shallow inundation (SI), 4.5 cm deep; intermediate inundation (II), 35.5 cm deep; deep inundation (DI), 55.0 cm deep). Values are mean ± SEM (n = 4 for proline and 3 for MDA). Different letters indicate significant differences among taxa for the same inundation treatment at every salinity. Different numbers show significant differences among inundation treatments at the same salinity for each taxon (three-way ANOVA, Tukey–HSD, P < 0.05)
activity was mostly independent of both stress factors. However, in our experiment, the hybrid did express its maximum PEPC apparent specific activity in freshwater conditions. The sensitivity of the hybrid to salinity was reflected in oxidative stress, showing increasing MDA levels (+20%) at higher salinities. However, the hybrid tended to show mostly intermediate responses compared to responses of both parental species (additive inheritance). The hybrid had higher PEPC specific activity than S. foliosa (+70%) and S. densiflora (+15%) (best-parent heterosis) in freshwater under intermediate inundation depth. This heterotic response in PEPC enzymatic level coincided with the hybrid’s maximum net photosynthesis rates and maximum vegetative fitness (Gallego-Tévar et al., 2019c). Measured PEPC responses support the high stress tolerance of the S. densiflora × foliosa hybrid to increasing salinity, inundation and their interaction. Therefore, eradication of the hybrid should be the highest management priority. The hybrid should be eradicated before it potentially evolves to become a fertile, highly competitive and wide-spread allopolyploid species that will reduce local biodiversity, as has previously demonstrated in the case of the evolution of Spartina anglica C.E.Hubb. in European salt marshes (Aïnouche et al. 2004). In line with our suggestions on management of Spartina hybrid, the invasions by different hybrids between S. foliosa, S. alterniflora and S. densiflora are already under management as invasive species in the San Francisco Bay (Kerr et al., 2016). Our study shows that the responses of key functional physiological traits to increasing environmental stresses, such as specific enzymatic activities like PEPC in C4 species, interpreted together with other functional trait responses, serve as biological indicators that provide a mechanistic framework for improved ecosystem management practices in a scenario of biological invasions and climate change.

**Figure 2**: PEPC apparent specific activity (a), PEPC phosphorylation state (determined by L-malate assay, IC50) (b) and total proteins content (c) for native Spartina foliosa (yellow columns), invasive S. densiflora (blue columns) and their hybrid S. densiflora × foliosa (green columns) exposed to four salinity treatments (0.5, 10, 20 and 40 ppt) at three inundation depths (shallow inundation (SI), 4.5 cm deep; intermediate inundation (II), 35.5 cm deep; deep inundation (DI), 55.0 cm deep). Values are mean ± SEM (n = 3). Different letters indicate significant differences among taxa for the same inundation treatment at every salinity. Different numbers show significant differences among inundation treatments at the same salinity for each taxon (three-way ANOVA, Tukey–HSD, P < 0.05).

**Supplementary material**

Supplementary material is available at Conservation Physiology online.

**Funding**

This work was supported by Plan Propio de Investigación of Universidad de Sevilla by awarding a research contract to Blanca Gallego-Tévar and by a cooperative agreement.
between the United State Department of Agriculture, Agricultural Research Service (USDA-ARS) Invasive Species and Pollinator Health Research Unit, Davis, California, and the University of Seville [58-2030-6-043-F].

Acknowledgements

We thank the editors and anonymous reviewers for comments that improved the manuscript. Drew Kerr, San Francisco Estuary Invasive Spartina Project, and Christina Freeman, California State Parks, assisted with live plant collections for our experiment. We thank Dr Rebecca Drenovsky (John Carroll University) for input on experimental design and assistance with its harvest, and Dr Peter Baye (San Francisco State University, Romberg Tiburon Center) for insights on conservation priorities for preservation of salt marsh flora in California estuaries. We also thank Caryn J. Futrell and Meghan Skaer Thomason (USDA-ARS); Jessica Drost, Myra Huerta, Ann Le and Christopher McCort (University of California Davis); and Reina Nielsen and Rebecca A. Reichoff (John Carroll University) for maintenance and harvest of the experiment.

References

Abbas AM, Rubio-Casal AE, De Cires A, Figueroa ME, Lambert AM, Castillo JM (2012) Effects of flooding on germination and establishment of the invasive cordgrass Spartina densiflora. Weed Res 52: 269–276.

Aïnouche M, Baumel A, Salmon A (2004) Spartina anglica CE Hubbard: A natural model system for analyzing early evolution changes that affect allopolyploid genomes. Biol J Linnaean Soc 82: 475–484.

Alla MMN, Khedr AHA, Serag MM, Abu-Alnaga AZ, Nada RM (2011) Physiological aspects of tolerance in Atriplex halimus L. to NaCl and drought. Acta Physiol Plant 33: 547–557.

Álvarez R, Castillo JM, Mateos-Naranjo E, Gandullo J, Rubio-Casal AE, Moreno FJ, Figueroa ME (2010) Ecotypic variations in phosphoenolpyruvate carboxylase activity of the cordgrass Spartina densiflora throughout its latitudinal distribution range. Plant Biol 12: 154–160.

Augyte S, Pickart A (2014) Algal response to removal of the invasive cordgrass Spartina densiflora in a salt marsh at Humboldt Bay, California, USA. Nat Areas J 34: 325–331.

Ayres DR, Strong DR, Baye P (2003) Spartina foliosa (Poaceae)—a common species on the road to rarity? Madroño 50: 209–213.

Ayres DR, Grotkopp E, Zaremba K, Snoop CM, Blum MJ, Bailey JP, Anttila CK, Strong DR (2008) Hybridization between invasive Spartina densiflora (Poaceae) and native S. foliosa in San Francisco Bay, California, USA. Am J Bot 95: 713–719.

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39: 205–207.

Bortolus A (2006) The austral cordgrass Spartina densiflora Brong.: its taxonomy, biogeography and natural history. J Biogeogr 33: 158–168.

Bradford MM (1976) A rapid and sensitive method for the for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem 72: 248–254.

Brand LA, Smith LM, Takekawa JY, Athearn ND, Taylor K, Shellenbarger GG, Schoellhamer DH, Spenset R (2012) Trajectory of early tidal marsh restoration: elevation, sedimentation and colonization of breached salt ponds in the northern San Francisco Bay. Ecol Eng 42: 19–29.

Buege JA, Aust SD (1978) Microsom al lipid peroxidation. Methods Enzymol 52: 302–310.

Cain DJ, Harvey HT (1983) Evidence of salinity-induced ecophenotypic variation in cordgrass (Spartina foliosa Trin.). Madroño 30: 50–62.

Calado ML, Carvalho L, Pang KL, Barata M (2015) Diversity and ecological characterization of sporulating higher filamentous marine fungi associated with Spartina maritima (Curtis) Fernald in two Portuguese salt marshes. Microb Ecol 70: 612–633.

Canalejo A, Martínez-Domínguez D, Córdoba F, Torronteras R (2014) Salt tolerance is related to a specific antioxidant response in the halophyte cordgrass, Spartina densiflora. Estuar Coast Shelf Sci 146: 68–75.

Cantero JJ, Cisneros JM, Zobel M, Cantero A (1998) Environmental relationships of vegetation patterns in saltmarshes of central Argentina. Folia Geobot 33: 133.

Castillo JM, Rubio-Casal AE, Redondo S, Álvarez-López AA, Luque T, Luque C, Nieve FJ, Castellanos EM, Figueroa EM (2005) Short-term responses to salinity of an invasive cordgrass. Biol Invasions 7: 29–35.

Castillo JM, Figueroa E (2009) Restoring salt marshes using small cordgrass, Spartina maritima. Restor Ecol 17: 324–326.

Castillo JM, Brewell JG, Pickart A, Bortolus A, Peña C, Figueroa E, Sytma M (2014) Phenotypic plasticity of invasive Spartina densiflora (Poaceae) along a broad latitudinal gradient on the Pacific Coast of North America. Am J Bot 101: 448–458.

Castillo JM, Brewell BJ, Pickart AJ, Figueroa ME, Sytma M (2016) Variation in tussock architecture of the invasive cordgrass Spartina densiflora along the Pacific Coast of North America. Biol Invasions 18: 2159–2174.

Castillo JM et al. (2018) Low genetic diversity contrasts with high phenotypic variability in heptaploid Spartina densiflora populations invading the Pacific coast of North America. Ecol Evol 8: 4992–5007.

Cohen RA, Wilkerson FP, Parker AE, Carpenter EJ (2014) Ecosystem-scale rates of primary production within wetland habitats of the northern San Francisco Estuary. Wetlands 34: 759–774.

Courtney AJ, Xu J, Xyu Y (2016) Responses of growth, antioxidants and gene expression in smooth cordgrass (Spartina alterniflora) to various levels of salinity. Plant Physiol Bioch 99: 162–170.

Daehler CC, Strong DR (1997) Hybridization between introduced smooth cordgrass (Spartina alterniflora; Poaceae) and native Califor-
De León, M. E., García-Gómez, M. A., & de Freitas, G. (2019). Salt tolerance in native and exotic cordgrass species of San Francisco Bay, California, USA. *Am J Bot* 84: 607–611.

De León, M. E., García-Gómez, M. A., & de Freitas, G. (2019). Salt tolerance in native and exotic cordgrass species of San Francisco Bay, California, USA. *Am J Bot* 84: 607–611.

De León, M. E., García-Gómez, M. A., & de Freitas, G. (2019). Salt tolerance in native and exotic cordgrass species of San Francisco Bay, California, USA. *Am J Bot* 84: 607–611.

De León, M. E., García-Gómez, M. A., & de Freitas, G. (2019). Salt tolerance in native and exotic cordgrass species of San Francisco Bay, California, USA. *Am J Bot* 84: 607–611.

De León, M. E., García-Gómez, M. A., & de Freitas, G. (2019). Salt tolerance in native and exotic cordgrass species of San Francisco Bay, California, USA. *Am J Bot* 84: 607–611.

De León, M. E., García-Gómez, M. A., & de Freitas, G. (2019). Salt tolerance in native and exotic cordgrass species of San Francisco Bay, California, USA. *Am J Bot* 84: 607–611.

De León, M. E., García-Gómez, M. A., & de Freitas, G. (2019). Salt tolerance in native and exotic cordgrass species of San Francisco Bay, California, USA. *Am J Bot* 84: 607–611.

De León, M. E., García-Gómez, M. A., & de Freitas, G. (2019). Salt tolerance in native and exotic cordgrass species of San Francisco Bay, California, USA. *Am J Bot* 84: 607–611.

De León, M. E., García-Gómez, M. A., & de Freitas, G. (2019). Salt tolerance in native and exotic cordgrass species of San Francisco Bay, California, USA. *Am J Bot* 84: 607–611.

De León, M. E., García-Gómez, M. A., & de Freitas, G. (2019). Salt tolerance in native and exotic cordgrass species of San Francisco Bay, California, USA. *Am J Bot* 84: 607–611.
Conservation Physiology

Conservation Physiology

Kerr DW, Hogle IB, Ort BS, Thornton WJ (2016) A review of 15 years of
Jiao J, Chollet R (1992) Light activation of maize phosphoenolpyruvate
carboxylase protein-serine kinase activity is inhibited by mesophyll
and bundle sheath-directed photosynthesis inhibitors. Plant Physiol
98: 152–156.
Kerr DW, Hogle IB, Ort BS, Thornton WJ (2016) A review of 15 years of
Jiao JA, Vidal J, Echevarría C, Chollet R (1991) In vivo regulatory phos-
phorylation site in C4-leaf phosphoenolpyruvate carboxylase from
maize and sorghum. Plant Physiol 96: 297–301.
Jiao J, Chollet R (1992) Light activation of maize phosphoenolpyruvate
carboxylase protein-serine kinase activity is inhibited by mesophyll
and bundle sheath-directed photosynthesis inhibitors. Plant Physiol
98: 152–156.
Kerr DW, Hogle IB, Ort BS, Thornton WJ (2016) A review of 15 years of
Jiao JA, Vidal J, Echevarría C, Chollet R (1991) In vivo regulatory phos-
phorylation site in C4-leaf phosphoenolpyruvate carboxylase from
maize and sorghum. Plant Physiol 96: 297–301.
Jiao J, Chollet R (1992) Light activation of maize phosphoenolpyruvate
carboxylase protein-serine kinase activity is inhibited by mesophyll
and bundle sheath-directed photosynthesis inhibitors. Plant Physiol
98: 152–156.
Kerr DW, Hogle IB, Ort BS, Thornton WJ (2016) A review of 15 years of
Jiao JA, Vidal J, Echevarría C, Chollet R (1991) In vivo regulatory phos-
phorylation site in C4-leaf phosphoenolpyruvate carboxylase from
maize and sorghum. Plant Physiol 96: 297–301.
Jiao J, Chollet R (1992) Light activation of maize phosphoenolpyruvate
carboxylase protein-serine kinase activity is inhibited by mesophyll
and bundle sheath-directed photosynthesis inhibitors. Plant Physiol
98: 152–156.
Kerr DW, Hogle IB, Ort BS, Thornton WJ (2016) A review of 15 years of
Jiao JA, Vidal J, Echevarría C, Chollet R (1991) In vivo regulatory phos-
phorylation site in C4-leaf phosphoenolpyruvate carboxylase from
maize and sorghum. Plant Physiol 96: 297–301.
Smart RM, Barko JW (1978) Influence of sediment salinity and nutrients on the physiological ecology of selected salt marsh plants. *Estuar Coast Mar Sci* 7: 487–495.

Strong DR, Ayres DR (2013) Ecological and evolutionary misadventures of *Spartina*. *Annu Rev Ecol Evol Syst* 44: 389.

Strong DR, Ayres DA (2016) Control and consequences of *Spartina* spp. invasions with focus upon San Francisco Bay. *Biol Invasions* 18: 2237–2246.

Trilla GG, De Marco S, Marcovecchio J, Vicari R, Kandus P (2010) Net primary productivity of *Spartina densiflora* Brong in an SW Atlantic Coastal salt marsh. *Estuar Coast* 33: 953–962.

Trnka S, Zedler JB (2000) Site conditions, not parental phenotype, determine the height of *Spartina foliosa*. *Estuaries* 23: 572–582.

Ustin SL, Pearcy RW, Bayer DE (1982) Plant water relations in a San Francisco Bay salt marsh. *Bot Gaz* 143: 368–373.

Vicari RL, Fischer S, Madanes N, Bonaventura SM, Pancotto V (2002) Tiller population dynamics and production on *Spartina densiflora* (Brong) on the floodplain of the Parana River, Argentina. *Wetlands* 22: 347–354.

Wang YH, Chollet R (1993) Partial purification and characterization of phosphoenolpyruvate carboxilase protein-serine kinase from illuminated maize leaves. *Arch Biochem Biophys* 304: 496–502.

Watson EB, Byrne R (2009) Abundance and diversity of tidal marsh plants along the salinity gradient of the San Francisco Estuary: implications for global change ecology. *Plant Ecol* 205: 113.

Williams J, Lambert AM, Long R, Saltonstall K (2019) Does hybrid *Phragmites australis* differ from native and introduced lineages in reproductive, genetic, and morphological traits? *Am J Bot* 106: 29–41.

Wong JXW, Costantini F, Merloni N, Savelli L, Geelen D, Airoldi L (2018) The widespread and overlooked replacement of *Spartina maritima* by non-indigenous *S. anglica* and *S. townsendii* in north-western Adriatic saltmarshes. *Biol Invasions* 20: 1687–1702.

Xue L, Li X, Yan Z, Zhang Q, Ding W, Huang X, Tian B, Ge Z, Yin Q (2018) Native and non-native halophytes resiliency against sea-level rise and saltwater intrusion. *Hydrobiologia* 806: 47–65.

Yordanova RY, Popova LP (2007) Flooding-induced changes in photosynthesis and oxidative status in maize plants. *Acta Physiol Plant* 29: 535–541.

Zedler JB, Calaway JC, Desmond JS, Vivian-Smith G, Williams GD, Sullivan G, Brewster AE, Bradshaw BK (1999) Californian salt-marsh vegetation: an improved model of spatial pattern. *Ecosystems* 2: 19–35.

Zheng WJ, Zheng XP, Zhang CL (2000) A survey of photosynthetic carbon metabolism in 4 ecotypes of *Phragmites australis* in northwest China: Leaf anatomy, ultrastructure, and activities of ribulose 1,5-bisphosphate carboxylase, phosphoenolpyruvate carboxylase and glycollate oxidase. *Physiol Plant* 110: 201–208.

Zhu G, Chen Y, Ella ES, Ismail AM (2019) Mechanisms associated with tiller suppression under stagnant flooding in rice. *J Agron Crop Sci* 205: 235–247.