THE HYBRIDS OF CHENOPODIACEAE AND POACEAE HALOPHITIC PLANTS:

their role in structuring and functioning of salt marshes

PhD Thesis

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LOS HÍBRIDOS DE QUENOPODIÁCEAS Y GRAMÍNEAS HALÓFITAS: SU PAPEL EN LA ESTRUCTURACIÓN Y EL FUNCIONAMIENTO DE LOS ECOSISTEMAS DE MARISMAS

Tesis presentada por Blanca Gallego Tévar
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THE HYBRIDS OF CHENOPODIACEAE AND POACEAE HALOPHITIC PLANTS: THEIR ROLE IN STRUCTURING AND FUNCTIONING OF SALT MARSHES

Thesis submitted by Blanca Gallego Tévar to apply for the degree of PhD

DEPARTAMENTO DE BIOLOGÍA VEGETAL
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CHAPTER I

General introduction

CHAPTER I: GENERAL INTRODUCTION

The process of hybridization

Interspecific hybridization is the process of mating between different species, leading to the production of viable hybrids. Hybridization is characterized by the genetic exchange between divergent taxa that results in genetic, epigenetic and phenotypic variations (Salmon et al. 2005, Cara et al. 2013). Molecular alterations occurring during hybridization comprise chromosomal rearrangements, genome enlargement, gene silencing, histone modifications, small RNA regulation and/or DNA methylation (Baack and Rieseberg 2007, Groszmann et al. 2013, Jackson 2017). These molecular changes determine the phenotype of the hybrid offspring through differences in gene expression (Comai 2000, Chen 2007). Thus, the expected value for a certain phenotypic trait in a hybrid is the intermediate value between both parents (mid-parent value), that occurs when gene expression is additive. However, hybrids can also exhibit certain traits equal to one or both parents if a dominant expression takes place, as well as higher or lower values (transgressive) than both parents when gene expression is overdominant (Bassene et al. 2010, Fridman 2015).

Depending on the different phenotypic inheritance patterns, hybrids fitness may therefore be higher, lower or the same as the parental species (Arnold and Hodges 1995). The process of hybridization that results in lower fitness hybrids is called ‘hybrid breakdown’ (Johansen-Morris and Latta 2006), while heterosis or ‘hybrid vigor’ is the production of F1 hybrids fitter than the parental species (Lippman and Zamir 2007). Heterosis can be fixed in the following generations of hybrids if the duplication of the genetic material occurs, leading to the production of a new allopolyploid taxon (Chen 2010).

Despite of allopolyploidization (Ma and Gustafson 2005), polyploidy can also be achieved by genome duplication without hybridization (autopolyploidy) (Parisod et al. 2010). The high success of polyploids in nature has been related

to the formation of new phenotypes with adaptive significance that occupy new ecological niches (Osborn et al. 2003, Leitch and Leitch 2008), especially in the case of polyploids with two different origins or allopolyploids (Soltis and Soltis 2000). Among these adaptive changes are the increase in genomic and phenotypic plasticity related to higher heterozygosity in allopolyploids, contributing to an increase in their tolerance to high contrasting habitats (Hahn et al. 2012, Jackson 2017). It has also been described that phenotypic plasticity can increase through homoploid hybridization and increasing heterozygosity (Weber and D'Antonio 1999, Lukhtanov et al. 2015).

Hybridization and evolution

Hybridization has been recognized as an evolutionary force (e.g Arnold et al. 2012; Arnold, 2006; Rieseberg et al. 2000; Yakimowski and Rieseberg, 2014) especially for its role in speciation (Abbott et al. 2013, Vallejo-Marín and Hiscock 2016). Hybridization gives rise to adaptive changes more rapidly than through mutation (Long 1991, Minder et al. 2007), being speciation by hybridization related to the establishment of hybrid zones in which these changes lead to adaptive divergence of the new taxon and, sometimes, to the reproductive isolation with its parental species (Baack and Rieseberg 2007, Abbott et al. 2013, Lukhtanov et al. 2015). Barton (2001) observed that if the fitness of hybrids is lower or equal to the parents, reproductive isolation is promoted and hybrid zones remain stable over time promoting speciation. By contrast, if hybrids are fitter, they could adapt better than the parents to new environments, escaping to the hybrid zone that would become unstable. However, Arnold et al. (2012) argued that genotype x environment interaction can make fitter hybrids adapt to certain ecological conditions also favoring their reproductive isolation. Moreover, reproductive isolation of hybrids populations is not only provided by ecological differentiation (pre-zygotic barriers) but it

can also occur through post-zygotic barriers such as suppressing recombination or developing sterility (Faria and Navarro 2010).

On the other hand, the evolutionary role of hybridization is also associated to the extinction of the parental lineages through crossing with their hybrids (introgression) (Rhymer and Simberloff 1996) and the consequent dilution of the parental genome (Huxel 1999). Extinction by introgression is conditioned by factors such as the initial proportion of genetic material of each parent, the fitness difference between hybrids and the parental taxa and the frequency of crossing (Epifanio and Philipp 2000).

Native plant hybrids

Hybridization is an abundant process both between animal and between plant species from a given region, being more frequent in the latter group (Yakimowski and Rieseberg 2014). In addition, certain plant phylogenetic groups are more prone for hybridization than others (Whitney et al. 2010).

Two genera of plants in which numerous studies have been conducted on native plant hybrids, especially from the evolutionary point of view, are the annual sunflowers *Helianthus* and the Lousiana *Irises*, both from North America (Arnold and Martin 2010). Precisely, the sunflower hybrid *Helianthus paradoxus* Heiser between *Helianthus annuus* L. and *Helianthus petiolaris* Nutt. (Rieseberg 1991) is an example of speciation by ecological differentiation in relation to its parental species. *H. paradoxus* colonizes saline environments while its parents are both glycophytes (Welch and Rieseberg 2002b). The adaptation of this hybrid to high salinities has been related to the development of transgressive traits (Lexer et al. 2003b, Karrenberg et al. 2006, Edelist et al. 2009), being a process in which anthropic influence does not seem to intervene (Welch and Rieseberg 2002a). On the other hand, introgressive hybrids between *Iris fulva* Ker Gawl. and *Iris brevicaulis* Raf. exhibit fitness dependent on genotype x environment interactions (Arnold et al. 2012). Thus, fitness-related

traits of these hybrids can be superior, equal or inferior to the parents, not showing a clear tendency to transgressivity (Arnold and Hodges 1995, Johnston et al. 2004, Taylor et al. 2009) and tending to be similar to that of the parents by introgression (Cruzan and Arnold 1993, Tang et al. 2010).

The genus *Sarcocornia* of the family Chenopodiaceae is another taxon in which hybridization is a frequent process that has taken place between different species in South Africa and Europe (Castroviejo and Lago 1992, Figueroa et al. 2003, Steffen et al. 2015). These are polyploid species with high morphological variability of overlapping traits that make their taxonomic differentiation challenging (de la Fuente et al. 2013, Guilló et al. 2014). This, together with the description of backcrossing with the parents in other genera of the family, suggests the existence of hybrid swarms as a result of introgression, but this phenomenon has not been studied yet (Shepherd et al. 2004).

Exotic/invasive plant hybrids

Biological invasions that occur after the introduction of exotic species in new environments have been pointed out as a relevant conservation issue strongly associated with human activity and global environmental change (Pejchar and Mooney 2009, Vitousek et al. 2017). When an exotic species arrives at a certain region, one of the forms of invasion that may take place is the genomic invasion by hybridization with native species (Mallet 2005). This invasion at the genetic level can be enhanced if the attenuation of the native parental genetic material through introgression takes place (Abbott 1992, Petit 2004).

Ellstrand and Schierenbeck (2000) described that invasiveness in hybrids increase with respect to ancestral invasive lineages, related to enhanced adaptive performance for the creation of novel genotypes, increase of genetic diversity and development of transgressive traits that confer them hybrid vigor. Examples (25) of increased invasiveness in hybrids and allopolyploids of the families Rosaceae, Poaceae, Chenopodiaceae Brassicaceae, Onagraceae,

Polygonaceae, Asteraceae, Laminaceae, Aizoaceae, Lythraceae, Ericaceae and Violaceae in North America, Europe and Australia were included by the authors. After this study, several authors have tested the occurrence of enhanced invasiveness in different hybrid taxa. For instance, the hybrid *Typha x glauca* Godron between the native *Typha latifolia* L. and the invasive European *Typha angustifolia* L. in the Great Lakes Region of North America has been considered the most invasive cattail species of this region (Travis et al 2010). Numerous field and common garden studies have shown the higher competitive ability of *T. x glauca* with respect to the parental species and other native coexisting species (e.g. Larkin et al. 2012a 2012b, Freeland et al. 2013, Pieper et al. 2018) and greater tolerance to environmental factors (e.g. Waters and Shay 1990, Freyman 2008, Bunbury-Blanchette et al. 2015). High levels of heterosis (Zapfe and Freeland 2015) and genetic diversity (Bastien Lavergne and Molofsky 2007) has been associated to increased invasiveness in this species.

Another example of invasive hybrid widely studied is *Fallopia x bohemica* Chrtek and Chrtkova in Europe between the native *Fallopia sachalinensis* (C.F.Schmidt) Ronse Decr. and the invasive Asian *Fallopia japonica* (Houtt.) Ronse Decr. (e.g. Bailey et al. 1995, Gammon et al. 2007, Siemens and Blossey 2007, Rouifed et al. 2011, Parepa et al. 2014). It has been found that the high phenotypic plasticity displayed by *F. x bohemica* is a key factor in its improved invasiveness (Richards et al. 2008).

Additionally, different hybrids between native and invasive species have been described in the genus of the Poaceae family *Spartina* (cordgrasses) in North America and Europe, showing greater fitness than their parents (Castillo et al. 2010a, Strong and Ayres 2013, Lee et al. 2016). In Europe, the chromosomal duplication of *Spartina x townsendii*, the hybrid between the European small cordgrass *S. maritima* (Curtis) Fernald and the invasive from the East Coast of North America *S. alterniflora* Loisel, has led to the formation of the allopolyploid *Spartina anglica* C.E.Hubb. that is a highly invasive species dominating the marshes where it is present (Thompson 1991, Hacker et al. 2001).

Salt marshes as model ecosystems

For all the above, the presence of native and invasive hybrid plant taxa can have a relevant role in ecosystems. In this sense, coastal salt marshes represent model systems for the study of the functioning and structuring of ecosystems since they harbor relatively low number of species that are subjected to marked environmental gradients related to the tidal influence and determining their configuration and performance (Adam 1990, Pennings et al. 2005, Silvestri et al. 2005). In addition, they are ecosystems with high ecological, socio-economic and cultural value that provide a wide range of provisioning, regulating and cultural services (Barbier et al. 2011). However, salt marshes are currently threatened in many parts of the world as a consequence, for example, of their occupation for different human activities, the increase of invasive species and/or sea level rise associated with climate change (Kennish 2001, Gedan et al. 2009, Crosby et al. 2016). A solid scientific basis on the knowledge of the functioning, structuring and composition of these wetlands is necessary to properly elaborate conservation strategies and management plans (Christensen et al. 1996).

1.1. Objectives

Despite the study of hybridization as an evolutionary force and promoter of invasiveness, there is a lack of comparative studies about the role of native and invasive hybrids on the functioning and structuring of ecosystems. With this main objective, we studied the ecological performance of native hybrids of the genus *Sarcocornia* (Chenopodiaceae) and exotic hybrids of the genus *Spartina* (Poaceae) in coastal salt marshes.

The specific goals were:

1. To study the genetic structure of hybrid populations between native *Spartina maritima* and invasive *S. densiflora* in an invaded range in the salt marshes of the Gulf of Cadiz (Southwest Iberian Peninsula) (Chapter III).

2. To examine the response of reciprocal hybrids between *S. maritima* and *S. densiflora* from the Gulf of Cadiz to different environmental factors such as salinity (Chapter IV and V).

3. To study the tolerance of the invasive hybrid *Spartina densiflora x foliosa* from San Francisco Bay (California) to the combination of salinity and inundation (Chapter VI and VII).

4. To analyze the role of native *Sarcocornia* hybrids and invasive *Spartina* hybrids in native zonation pattern of the salt marshes in the Gulf of Cadiz (Chapter VIII).

5. To analyze the role of native *Sarcocornia* hybrids and invasive *Spartina* hybrids in the ecological succession of salt marshes in the Gulf of Cadiz (Chapter IX).

6. To investigate the effect of climate change on the hybridization process between native *S. maritima* and invasive *S. densiflora* (Chapter X).

CHAPTER II

Study sites

The sites where our work was conducted is described in this character.

2.1 Gulf of Cadiz

Most of the studies of this doctoral thesis were carried out in marshes of three estuaries of the Gulf of Cadiz: Guadiana River (37º10'–37º16'N, 7º16'–7º28'W), Piedras River (37º12'–37º18'N, 7º06'–7º12'W), and Tinto-Odiel Rivers (37º08'–37º20'N, 6º45'–7º02'W) Estuaries. This area is located on the Atlantic Coast of the Southwest Iberian Peninsula and it is under a Mediterranean climate with Atlantic influence. Mean temperatures are 17-24 ºC with an amplitude of 9-13ºC, an annual precipitation of 250-850 mm with 75-85 days of rain per year and 4-5 months of dried period around June and September (AEMET, 2018; CLIMA, 2018) when evapotranspiration leads to hypersalinity on the highest parts of the marshes (Castellanos et al. 1994). The yearly average significant wave height is 1 m comprised of both sea and swell, generating a predominant longshore current towards the E and SE (Benavente et al. 2000). Storms events are generally frequent over the autumn and winter months with significant wave height values reaching up to 7 m (Plomaritis et al. 2015). The semidiurnal tides have a mean range of 2.10 m and a mean spring tidal range of 2.97 m, representing 0.40–3.37 m above Spanish Hydrographic Zero (SHZ). Mean sea level is +1.85 m relative to SHZ. Three different zones are distinguished in these salt marshes based on the tidal influence and their elevation: Low marshes are defined between Mean High Water Neap (MHWN; +2.44 m SHZ) and Mean High Water (MHW; +2.91 m SHZ), middle marshes go from MHW to Mean High Water Spring (MHWS; +3.37 m SHZ), and high marshes from MHWS to Highest Astronomical Tide (HAT; +3.71 m SHZ) (Long and Mason 1983).

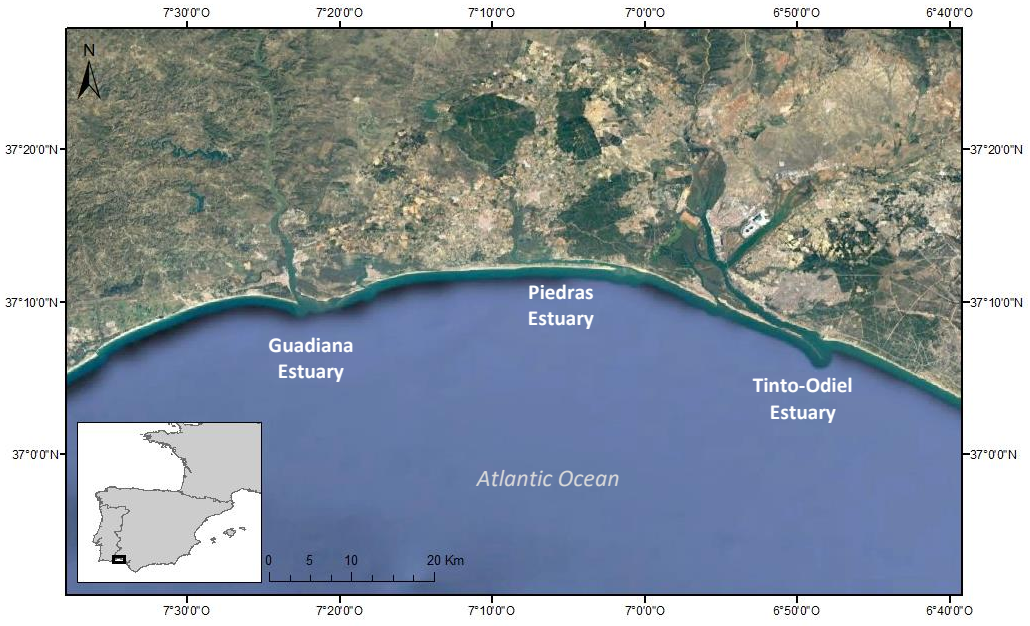

Fig. 2.1. Map of the Gulf of Cadiz (Southwest Iberian Peninsula). Source: Google Earth 2018 [Accessed 1 December 2018].

The typical plant zonation in these marshes is depicted in Appendix 8.B. The vegetation is characterized by its diversity and heterogeneity. Lower marsh areas tend to be dominated by *Spartina maritima* (Curtis) Fernald, but they can also be colonized by the South American invasive species *Spartina densiflora* Brongn, *Salicornia europaea* agg. and *Sarcocornia perennis* (Mill.) A. J. Scott. Higher on the marsh the vegetation tends to be dominated by long-lived, shrubby species *Atriplex portulacoides* L. and *S. densiflora* become dominants in some marshes at intermediate elevations. On the highest parts of the marsh, *Sarcocornia fruticosa* (L.) A.J.Scott, *Arthrocnemum macrostachyum* (Moric.) Moris, *Limoniastrum monopetalum* (L.) Boiss, *Suaeda vera* Forssk. ex J.F. Gmel., *Limonium ferulaceum* (L.) Chaz. and *L. diffusum* (Pourr.) Kuntze and are prominent. The banks of creeks and channels in mature marsh typically support stands of *S. densiflora*, *A. portulacoides* and *Inula crithmoides* L. After disturbance, other species can occur where sand has blown on to the surface of the marsh and where the influence of tides is very small (Castellanos et al. 1994, Castillo et al. 2008b, Curado et al. 2014). Moreover, two different hybridization

events have been described between salt marsh halophytic species in the Gulf of Cadiz. One between natives *S. perennis* and *S. fruticosa* (family: Chenopodiaceae) (Figueroa et al. 2003) and other between native *S. maritima* and invasive *S. densiflora* (family: Poaceae) (Castillo et al. 2010a). The native *Sarcocornia* hybrids are fertile (Castroviejo and Lago 1992, Figueroa et al. 2003), while the exotic *Spartina* hybrids are sterile and they are the consequence of different hybridization processes where both parental species act as seed parent, resulting in the hybrid *S. maritima x densiflora* in low elevation marshes and *S. densiflora x maritima* in middle marshes.

2.2 San Francisco Bay

San Francisco Bay (37º24'–38º12'N, 122º31'–121º41'W) is located on the Pacific Coast of North America (Fig. 2.2), in the confluence of the Sacramento and San Joaquin Rivers. This area is subject to Mediterranean climate, with mean daily temperature varying between 10° and 18°C, according to the climatic series (1971–2000) (Castillo et al. 2014). Average annual precipitation is 600 mm with 68 days of rain per year and a dried period in summer (NCDC, 2018). The tides are mixed diurnal and semidiurnal with a maximum amplitude of 2 m and generate tidal currents that, together with the abundant winds in summer and in winter storms, determine the hydric and sedimentary dynamics of the Estuary. The Bay is divided in North, Central and South Bay, being the northern area functioning more dependent on river inflow and the southern area more influenced by tidal fluctuation (Conomos et al. 1985).

As other estuaries on the Pacific coast, salt marshes of the San Francisco Estuary are relatively recent (some of them just few hundred years), which has been linked to their low species richness (Macdonald and Barbour 1997, Atwater et al. 1979). In low elevation salt marshes, the native cordgrass *Spartina foliosa* Trin. forms almost monospecific stands up to the ecotone with the other native abundant species *Salicornia virginica* L., located at higher elevations in

the tidal gradient (Mahall and Park 1976). In these salt marshes, a total of 5 different cordgrass species has been recorded: *S. foliosa, S. patens* (Aiton) Muhl, *S. alterniflora* Loisel., *S. densiflora* and *S. anglica* C. E. Hubb. (Daehler and Strong, 1996), only being *S. foliosa* a native species (Mobberley, 1956; Spicher and Josselyn, 1985). Both invasive *S. alterniflora* and *S. densiflora* have hybridized with *S. foliosa* in San Francisco Bay (summarized in Strong and Ayres 2013). While the former has given rise to a hybrid swarm product of backcrossing with the parental species (Daehler and Strong 1997), the reciprocal hybrids between *S. foliosa* and *S. densiflora* are sterile and restricted to areas close to the parental species, both in the case of *S. densiflora x foliosa* and *S. foliosa x densiflora* (Ayres et al. 2008).

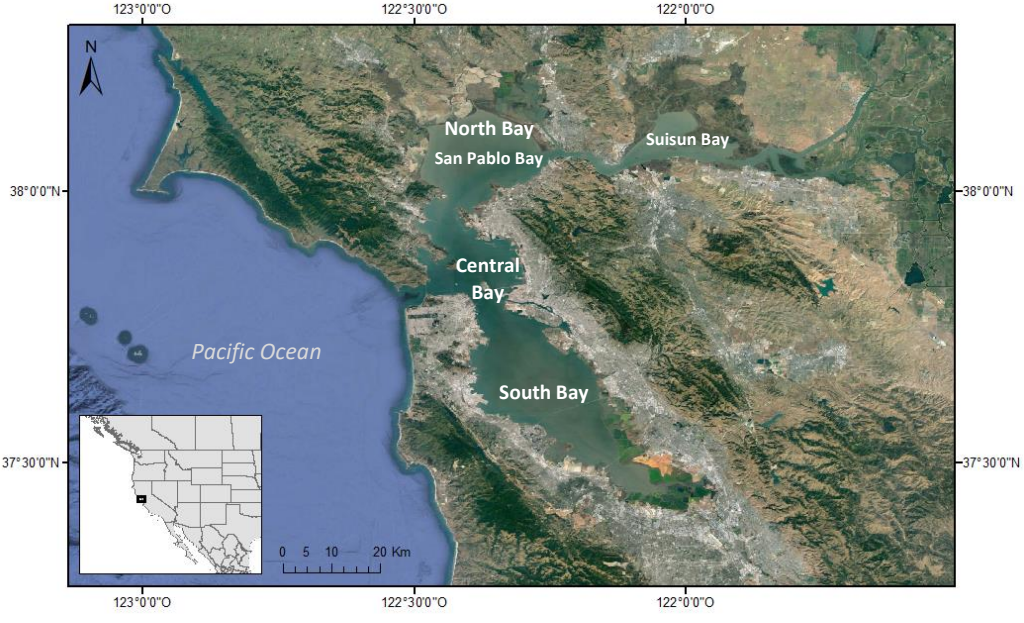

Fig. 2.2. Map of the San Francisco Bay (Pacific Coast of North America). Source: Google Earth 2018 [Accessed 1 December 2018].

CHAPTER III

Genetic structure of *Spartina* hybrids between native *Spartina maritima* and invasive *Spartina densiflora* in Southwest Europe

CAPÍTULO 3: La estructura genética de los híbridos entre la nativa Spartina maritima y la invasora Spartina densiflora en el Suroeste de Europa

Resumen

La hibridación interespecífica es un proceso evolutivo que resulta en la formación de nuevos genotipos. Los cambios genómicos que ocurren como resultado de la hibridación afecta tanto a la estructura del genoma como a la expresión génico y, por tanto, determina el fenotipo y la ecología de los híbridos. Este estudio aporta nuevos datos de la dinámica de invasión de híbridos exóticos, integrando los efectos de la genética, el fenotipo, la situación geográfica y el medio ambiente en la hibridación tras la invasión de una especie exótica vegetal en una comunidad de halófitas. Para ello, analizamos la estructura genética espacial de las poblaciones de híbridos F1 estériles de *Spartina* establecidas en el Golfo de Cádiz (Suroeste de la Península Ibéria) y de sus especies parentales, la natica *S. maritima* y la invasora *S. densiflora*, usando secuencias de ADN nuclear (microsatélites) y ADN cloroplástico. También analizamos las relaciones entre la estructura genética espacial de los híbridos, su variabilidad fenotípica y el medio abiótico. Las poblaciones estudiadas de híbridos de *Spartina* se establecieron en zonas con una estructura genética heredada de ambos parentales. Los híbridos fueron genéticamente más similares a la especie nativa que a la invasora. Las poblaciones con mayor diferenciación genética fueron aquellas que estuvieron más separadas espacialmente entre ellas y que presentaron ambientes sedimentarios más distintos, revelando respectivos procesos de aislamiento por distancia y por ambiente. Los híbridos del estuario del río Guadiana fueron los más genéticamente diferenciados y que presentaron un comportamiento más transgresivo en lo que se refiere a la altura de tallos.

CHAPTER 3: Genetic structure of Spartina hybrids between native Spartina maritima and invasive Spartina densiflora in Southwest Europe

Abstract

Interspecific hybridization represents an evolutionary force resulting in novel genotypes. The genomic changes that occur as a result of hybridization affect both genome structure and gene expression and consequently determine hybrid phenotypes and ecology. This study provides new data on the dynamics of exotic hybrid invasions, integrating effects of the genetic, phenotypic, geographical and environmental scenario with hybridization following invasion of a halophyte community by an exotic plant species. We analyzed the spatial genetic structure of sterile *Spartina* F1 hybrid populations established at the Gulf of Cadiz (Southwest Iberian Peninsula) and that of their parental species native *S. maritima* and invasive *S. densiflora* using nuclear DNA (Simple Sequence Repeats) and chloroplast DNA sequences. We also analyzed the relationships between the spatial genetic structure of the hybrids, their phenotypic variability and their marsh environment. The studied populations of Spartina hybrids were establishing hybrid zones with a spatial genetic structure inherited from both parental species. The hybrids were genetically more similar to the native than to the invasive species. The hybrid populations with greater genetic differentiation were those more spatially separated from each other and that were present in more contrasted sedimentary environments, revealing respective isolation processes by distance and by environment. The hybrids in the Guadiana Estuary were the most genetically differentiated and with the highest transgressive behavior in terms of tiller height.

3.1 Introduction

Interspecific hybridization represents an evolutionary force (Yakimowski and Rieseberg 2014) that promotes rapid genetic, epigenetic and phenotypic changes (Salmon et al. 2005, Chen and Ni 2006, Cara et al. 2013). By resulting in novel genotypes, hybridization can lead to more rapid adaptive changes than through mutation (Long 1991, Minder et al. 2007). To identify the role of hybrids in the structuring of plant communities, it is necessary to analyze their underlying population genetics background, their dispersion mechanisms and the intensity of selection (Sloop et al. 2011). Hybrid zones are considered important tools for the study of evolutionary processes between divergent populations and genetic structure at the community level (Whitham et al. 1999, Minder et al. 2007, Sloop et al. 2011).

The regional-scale genetic structure that we observe today is determined by the differences generated between ancestral colonizers (Slatkin 1987, Sloop et al. 2011). Thus, according to the classical population genetic approach, genetic similarity among populations decreases with geographic distance, explained by the isolation by distance (Wright 1943, Malécot 1948) and the stepping-stone models (Kimura and Weiss, 1964). This has been directly related to the reduction of gene flow between more distant populations leading to reproductive isolation promoting genetic drift and selection (Slatkin 1987, Ellstrand 2014). In the particular case of introduced species, genetic drift may be emphasized as a consequence of bottlenecks leading to a general reduction of genetic variability (founder effect) in the introduced population in relation to the genetic variability of the species in its native range (Dlugosch and Parker 2008, Brzyski et al. 2014, Castillo et al. 2018). In addition, environmental (biotic and abiotic) heterogeneity may lead to genetic differentiation between populations, mainly by selective pressure that favors local adaptation (Linhart and Grant 1996, Hübner et al. 2009) as a consequence of isolation by environment (Wang and Bradburd 2014). The effect that hybridization may

have on the established spatial genetic structure will depend on different factors such as the genetic variability of the parental species, the dispersion capacity of the hybrids, their fertility and backcrossing frequency with the parental species (introgression) and seedling recruitment (Abbott 1992, Graham et al. 1995, Ellstrand and Schierenbeck 2000, Rieseberg et al. 2003, Arnold 2006). Thus, populations of hybrids with a marked genetic structure related to reproductive isolation and local adaptation can be found (Sloop et al. 2011), as well as others without genetic differences between populations in different geographic locations when introgression and sexual reproduction homogenize populations (Yatabe et al. 2009).

The genomic changes that occur as a result of hybridization affect both genome structure and gene expression and consequently determine hybrid phenotypes (Baack and Rieseberg 2007). In hybrid taxa, the phenotype is the combination of different types of gene expression that can be additive (equal to the mid-parent value), dominant (equal to the value of one parent) or overdominant (higher or lower than the values of both parents) (Bassene et al. 2009). Depending on these differences in gene expression, hybridization may affect the evolutionary process in different ways, being negative if low fitness hybrids with limited ecological range are produced that can lead to reproductive isolation, or having positive effects when the new genotypes hybrids with greater fitness and large ecological amplitudes (Barton 2001). The latter are the result of the predominance of transgressive traits (exceeding trait values of both parents) related to heterosis or hybrid vigor (Lippman and Zamir 2007, Chen 2010). In the last decades, there have been important advances in the knowledge of molecular mechanisms underlying gene expression of hybrids, although there is still lack of understanding of the genetic regulation of the phenotypes of hybrids (Tirosh et al. 2009, Yoo et al. 2014, Bird et al. 2018). In hybrids deriving from at least one invasive species (crossed with a native or non-native species), it has been reported that heterosis frequently increases the invasiveness of the offspring (Ellstrand and Schierenbeck 2000, Ellstrand 2009, Hovick and

Whitney 2014) such as the hybrids between the native *Spartina foliosa* Trin. and the invasive *S. alterniflora* Loisel in San Francisco Bay (Ayres et al. 2004). Also, hybridization involving an invasive species usually counteracts the decrease in population genetic variability as a consequence of bottlenecks in punctual invasions (Ellstrand and Schierenbeck 2000), increasing the success of the invasion in the offspring.

In the genus of polyploid grasses *Spartina* (Poaceae), there are several examples of hybrids and allopolyploids between invasive and native species which exhibit transgressive traits that confer hybrid vigor (Ayres et al. 2004, Ainouche et al. 2009, Castillo et al. 2010a, Pakenham-Walsh et al. 2010, Lee et al. 2016). Along the Atlantic Coast of the Gulf of Cadiz (Southwest Iberian Peninsula), populations of hybrids between the native *Spartina maritima* (Curtis) Fernald. (2n = 6x = 60) and the invasive from the west coast of South America *Spartina densiflora* Brongn. (2n = 7x = 70) have been described in at least three different estuaries: the joint estuary of Tinto and Odiel Rivers, the estuary of Piedras River and the estuary of Guadiana River. Two different hybrids have been found: *S. maritima x densiflora* (2n = 9.5x = 95), product of the fecundation of unreduced ovules of *S. maritima* fertilized by *S. densiflora* pollen in low marshes, and *S. densiflora x maritima* (2n = 6.5x = 65) obtained by the fecundation of regular ovules of *S. densiflora* by *S. maritima* pollen in middle marshes (Castillo et al. 2010a). These reciprocal hybrids are transgressive in their tiller height, lateral expansion of their tussocks, and survival along the intertidal gradient (Castillo et al. 2010a).

In this study, the spatial genetic structure of the *Spartina* hybrid populations established at the Gulf of Cadiz and that of their parental species *S. maritima* and *S. densiflora* was explored using nuclear DNA (Simple Sequence Repeats, SSRs). These co-dominant markers are potentially polymorphic and allow for genetic discrimination among populations (Baisakh et al. 2009, Gedye et al. 2012, Vieira et al. 2016) and are useful in detecting hybrids combining different parental allelic combinations (Ayres et al. 2008, Sloop et al. 2011,

Hogle and Zaremba 2014). Additionally, chloroplast (cp) DNA sequences were used to assess the maternal origin of hybrid taxa (Ferris et al. 1997, Baumel et al. 2003). We also analyzed the relationships between the spatial genetic structure of the hybrids, their phenotypic variability and their marsh environment. Hybrids and parental populations were phenotypically examined by measuring twelve vegetative morphological traits, and their sedimentary abiotic environment was characterized. This enabled us to study the genetic structure of populations of hybrids between the native *S. maritima* and the invasive *S. densiflora* in relation to their parental species. More specifically, we analyzed the relationship between genetic distances among populations and (1) their geographic distance to assess the relative contribution of gene flow and drift in the population structure and consequent isolation by distance, (2) their abiotic environmental distances to identify the influence of processes of local adaptation and isolation by environment, and (3) their phenotypic differentiation by using morphological markers to evaluate the relationship between genetic and phenotypic distances. Considering the sterility of the studied *Spartina* hybrids, we hypothesized that the genetic structure of these hybrids would be determined by the genetic structure of the parental populations, whose genetic differentiation would increase together with the geographical distance between estuaries and with the environmental distance due to the contrasted habitat of both parental species.

3.2 Material and Methods

Study sites

This study was carried out in coastal salt marshes of three estuaries along the Atlantic Coast of Gulf of Cadiz (Southwest Iberian Peninsula): Tinto-Odiel, Piedras and Guadiana Estuaries (Fig. 3.1). Location, climate, physical environment and marsh vegetation of these estuaries are described in *Chapter 2*.

Plant collection

Before collecting the plant material for this study, we conducted field trips to locate tussocks of *Spartina* hybrids between *S. maritima* and *S. densiflora* following the first description by Castillo et al. (2010a). The hybrids were identified using the intermediate character of their adaxial crests (deeper than *S. maritima* and less than *S. densiflora*) and leaves (longer than *S. maritima* and shorter than *S. densiflora*) and the transgressive character of the tussock height (Castillo et al. 2010a). During these field trips, we recorded that the presence of hybrids was abundant in the Guadiana Estuary, with some hundreds of hybrids in just one marsh location known locally as 'San Bruno', whereas only five tussocks of hybrids were located in the Piedras Estuary and eleven were documented in the Tinto-Odiel Estuary. The collection of plant material was carried out in June-July 2016 from all known hybrid tussocks in the joint estuary of Tinto and Odiel Rivers (5 hybrids from a middle marsh close to 'La Rábida' monastery (37° 13' 42" N, 6° 54' 39" W), 3 hybrids from a middle marsh close to the sandspit known locally 'La Cascajera' (37° 11' 2" N, 6° 57' 21" W), 2 hybrids from a middle marsh at 'Don Claudio II' marsh (37° 11' 10" N, 6° 56' 22" W), and 1 hybrid from a low marsh at 'Estero del Colmenar' (37° 14' 17" N, 6° 59' 21" W)). Collections also included the only five known hybrid tussocks in the estuary of Piedras River including 1 from a low marsh area are close to the road from 'El Terrón' seaport to 'La Antilla' beach (37° 13' 2" N, 7° 10' 51" W) and 4 from two different middle marsh areas (37° 13' 4" N, 7° 10' 14" W). Collections were made of 10 hybrid tussocks from 'San Bruno' marsh in the estuary of Guadiana River, including 5 from a low marsh area (37° 11' 41" N, 7° 24' 22" W) and 5 from a middle marsh area (37° 11' 46" N, 7° 24' 10" W) (Fig. 3.1). The plant material of the parental species was sampled from the closest tussocks of *S. maritima* and *S. densiflora* to the sampled hybrids (N = 18 for *S. maritima*; N = 22 for *S. densiflora*). In total, 66 individuals were collected and analyzed in this study.

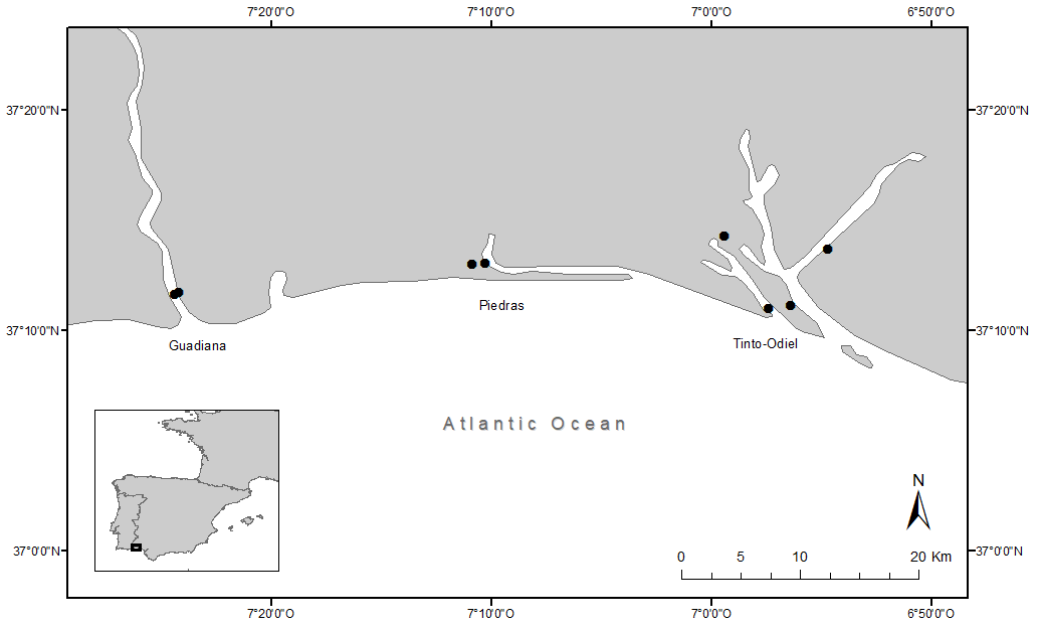

Fig. 3.1. Map of the Gulf of Cadiz (Southwest Iberian Peninsula) with the three estuaries sampled for hybrids between S*partina maritima* and *S. densiflora*: Tinto-Odiel, Piedras and Guadiana Estuaries. Symbols mark sampling points.

Chloroplast DNA sequencing

DNA was extracted from the collected dried leaves of *S. maritima, S. densiflora* and their hybrids by employing a NucleoSpin Plant Extraction Kit (Macherey-Nagel GmbH & Co, Düren, Germany). The *trnT-trnF* chloroplast DNA (Cp-DNA) region was amplified using the primer pair *a* and *b* to recover the *trnT-trnL* segment, and primer pair *c* and *f* to recover the *trnL-trnF* segment (Taberlet et al. 1991). The Polymerase Chain Reaction (PCR) was performed in a Mastercycler thermal cycler (Eppendorf AG, Hamburg, Germany) and underwent denaturation for 2 min at 94 ºC, followed by 35 cycles at 94 ºC for 30 s, 54 ºC for 30 s, 72 ºC for 1 min 30 s, followed by an extension phase at 72 ºC for 7 min. PCR products were purified using a NucleoSpin Gel and PCR purification kit (Macherey-Nagel GmbH & Co), and sequenced directly (on both directions) by Sanger at Macrogen Europe sequencer (Amsterdam, The Netherlands).

Simple Sequence Repeats (SSRs)

Genetic diversity was analyzed at eight microsatellite (SSR) loci, using eight primer pairs (MS2, MS7, MS13, MS14, MS15, MS16, MS17 and MS18) (Table 3.1) designed on genomic assemblies from *Spartina maritima* and successfully employed in *S. densiflora* (Castillo et al. 2018). The protocol used for microsatellite locus amplification followed that of Baumel et al. (2016) and Castillo et al. (2018). DNA extracts were diluted to a concentration of 10 ng μL^{-1} and amplified using the 8 microsatellite primer pairs selected. We used FAM labelled reverse primers for PCR and amplification products were diluted to 1/30 before subsequent analysis. An aliquot of 2 µL of these diluted PCR products were mixed with 10 µL of formamide solution (975 µL formamide + 25 µL of Liz-500 size marker) and separated by electrophoresis in an ABI PRISM 16-capillary 3130xl Genetic Analyzer (Applied Biosystems Inc., Waltham, USA). Alleles were identified and binned using GeneMapper 4.1 software (Applied Biosystems Inc., Waltham, USA).

Table 3.1. Nucleotide sequences, of the selected 8 microsatellite primer pairs and their corresponding repeated motifs (as detected in *S. maritima* genomic assemblies) analyzed in three populations of *S. maritima, S. densiflora* and their hybrids in Southwest Iberian Peninsula.

Locus	Forward primer sequence	Reverse primer sequence	SSR
MS2	ATATTCCGATCCCCTCCTTG	TTCGATCGGTCATGTTTTGA	$(AAAG)_n$
MS7	CAGAATCACCATCATCAGCG	TTCCATTTTTCAGGGTGAGC	$(TGGCAG)_n$
MS13	CTTGACCGCAACCAGTATGA	CCCAGGGCAATGGTTATACA	$(TTCT)_n$
MS14	TGAGTTTGAGTTCACGGTTCA	ATGTGATGCCATTTCCACAA	$(AAAG)_n$
MS15	TGCATTGCAGCAAGAGAATC	CGCTAGCTGATCCTGGAAAC	$(GATG)_n$
MS16	GGGACACGGGATAGGAAAGT	CCGCCGTGCAATTATTTATC	$(GTGGA)_n$
MS17	TTTGTTCAGCTTCAGCATGG	TTCTTGCAGTCGTTCTGTGC	$(GAAA)_n$
MS18	TCTTATGGACCCCTTGCAGT	CATCCGATTGGCGTAAGATT	$(TGATA)_n$

Sedimentary variables and phenotypic plant traits

The sedimentary environment variables and phenotypic plant traits for the studied tussocks of *S. maritima*, *S. densiflora* and their hybrids were recorded at low tide during June and July 2016. Every sedimentary variable was measured at a depth of 0-5 cm in monospecific plots (ca. 50 x 50 cm) where discrete tussocks were located. Sediment redox potential (Eh) and elevation above SHZ were measured *in situ* in the field, being each measurement the mean of 3 sub-replicates. We used a Leica NA 820 theodolite (Singapore) with a resolution of 2 cm for recording elevation. Measurements were referred to m above SHZ by using tidal extremes as reference points (Ranwell et al. 1964). Sediment Eh was obtained by using a portable meter and an electrode system (Crison pH/mV p-506). On the other hand, pH of interstitial water, electrical conductivity (mS cm^{-1}) and sediment water content (%) were determined in the laboratory after collecting sediment samples in 200 ml sealed recipients. Distilled water was added to the sediment (1:1, v/v) for the determination of pH (pH/redox Crison with the electrode M-506) and double water volume was used (1:2, v/v) for the measurement of electrical conductivity (conductivity meter, Crison-522) (Curado et al. 2014). Sediment water content was measured as the difference between fresh weight (FW) and dry weight (DW) after drying ca. 100 g of sediment in a stove at 80 °C for 48 h.

The following vegetative plant traits were recorded at the same time than the collection of plant material for genetic analyses and the characterization of the sedimentary environment: number of live and dead leaves, length, base width and weight of the flag leaf (first adult leaf completely unfolded on a tiller), foliar area, the Specific Leaf Area (SLA), diameter and length of tillers and the number of leaves per tiller length, tiller density, and the Leaf Area Index (LAI). The number of live and dead leaves, and the length, base width and weight of the flag leaf were measured in 5 tillers randomly selected from each tussock. Foliar area was calculated as a triangle using leaves width at the base and length.

Then, foliar areas were used to calculate SLA (m^{-2} g^{-1}) (Garnier et al. 2001) dividing by the DW of the same leaves. The difference in leaf width between unfolded (manually) and folded (in the field) leave area was expressed in percentage to calculate leaf adaxial rolling (Premachandra et al. 1993). In addition, diameter and length of tillers and the number of leaves per tiller length (leave cm^{-1}) were recorded for the same 5 tillers per tussock. Also, the number of tillers in 10 x 10 cm plots (n = 5 plots) was counted for the determination of tillers density (tiller cm^{-2}). LAI (m^2 $m\text{-}^{2}$) (Jonckheere et al. 2004) was estimated as the product between the mean foliar area, the mean number of leaves per tiller and the mean tiller density of a given tussock divided by the soil occupied area by that tussock at the base of its tillers or by its aerial above-ground structures, including the area occupied by its tilted tillers.

Data analyses

Chloroplast DNA sequences of the hybrids were compared to those from *S. maritima* and *S. densiflora* collected in the three estuaries, in order to assess the maternal origin of the hybrids. These sequences are deposited in Genbank under accession numbers (*in progress*). They were compared to those previously published for *S. maritima* (GB accessions KP176438, Rousseau-Gueutin et al. 2015, AF 275669, Baumel et al. 2001) and *S. densiflora* (GB accession AF372629, Baumel et al. 2002). Sequences were aligned using Geneious 9.1.2 software (Kearse et al. 2012) and a phylogenetic (Maximum Parsimony) analysis was performed using MEGA X software (Kumar et al. 2018)

SSR alleles were determined by comparison with the standard marker size (GeneScan-500 LIZ Size Standard), and the different “genotypes” (harbouring different allele combinations) were scored. Since the studied *Spartina* taxa are all polyploid, allelic dosage per individual cannot be ascertained. SSRs were then treated as "dominant markers", a convenient procedure for genetic analyses in polyploids (Obbard et al. 2006). Alleles were

recorded as either present or absent using the GenAlex 6.502 software (Peakall and Smouse 2006, 2012). Up to 10 different alleles could be expected per locus since the taxa with the highest level of ploidy was *S. maritima x densiflora* (2n = 9.5x = 95 chromosomes) (Castillo et al. 2010a). Genetic distances (GD) between individuals and populations of *S. maritima, S. densiflora* and their hybrids were calculated following Huff et al. (1993) and Nei (1987), respectively. The intra-population genetic diversity parameters, effective number of alleles and Shannon Information Diversity Index (Brown and Weir 1983) were obtained for each studied population of the *Spartina* hybrids at the estuary level. In addition, Analysis of Molecular Variance (AMOVA) was used to determine the proportion of genetic differentiation within and among different hybrid populations at the estuary level. The fixation index (Φ_{PT}), analogous to Wright's (F_{ST}), was obtained following the standardization of Meirmans (2006) as an indicator of population genetic differentiation for dominant data.

Two-way analysis of variance (ANOVA) were performed to compare mean sedimentary variables and vegetative plant traits (dependent variables) for the *Spartina* hybrids and their parental species among estuaries, using taxon and estuary as grouping factors. In case of significant ANOVA, Tukey's honestly significant difference (HSD) was used as post hoc analysis. Before using the parametric tests, dependent variables were assessed for homoscedasticity using Levene's test and for normality using the Shapiro-Wilk test. When these requirements for parametric analysis were not met, transformations of type inverse (for LAI) and square root (for number of dead leaves and tiller density) were carried out. These analyses were conducted using SigmaPlot (Systat Software, San Jose, CA) version 14 for Windows. Deviations of all data were calculated as standard error of the mean (SE) and a significance level (α) of 0.05 was applied for every analysis.

Phenotypic (PD) and sedimentary (SD) distances between plants in the field were calculated as Gower's similarity index ranging between 0 and 1

(Gower 1971) using the package *vegan* (Oksanen et al. 2018) of R-software to obtain a pairwise dissimilarity matrix. In order to compare genetic, phenotypic, sedimentary and geographic distance matrices, different Mantel's test for Matrix Correspondence (Mantel 1967) were conducted following the method of Smouse et al. (1986) and Smouse and Long (1992) using GenAlEx 6.502 software. Principal Coordinate Analysis (PCoA) with the SSR-based matrices of phenotypic and genetic distances, with the algorithm of Orlóci (1975), was also carried out using GenAlEx 6.502 software.

3.3 Results

Genetic diversity

Chloroplast DNA sequences enabled the differentiation of *S. maritima* and *S. densiflora* plastomes and the determination of the maternal genome donor in hybrids. The *trn*L-*trn*T region exhibits diagnostic nucleotide substitutions and indels between *S. maritima* and *S. densiflora*, with a notable 384 bp deletion in *S. densiflora* (Fig. 3.2). There is almost no sequence variation among accessions from the same species. One *S. maritima* individual from Piedras Estuary displayed a unique 29 bp deletion, corresponding to a repeated region. This individual was not included in subsequent genetic comparisons, as it was not involved in the parentage of the detected hybrids (see below). Six hybrids from Tinto-Odiel, 1 from Guadiana (from low marshes) and 4 from Piedras Estuaries had *S. maritima* as maternal genome donor, whereas 5 hybrids from Tinto-Odiel, 1 from Guadiana (from middle marshes) and 1 from Piedras Estuaries had *S. densiflora* as maternal parent (Fig. 3.2). The analyses of one hybrid individual from low marsh and another one from middle marsh in Guadiana Estuary corroborated the maternal origin of both populations previously analysed by Castillo et al. (2010a).

The eight SSR primer pairs amplified 1-7 different alleles per locus with an average of 5 alleles per locus and a total sum of 41 alleles. On average, the number of different alleles found per locus and individual for the parental species was 2 (1- 4) and 2 (1-6) for *S. densiflora* and *S. maritima*, respectively. At the 8 analyzed loci, 31 different alleles were found for *S. maritima* and 24 for *S. densiflora* (Table 3.2). According to Nei's index, the genetic distance (GD) among populations of each parent was greater between Guadiana and Tinto-Odiel (*S. densiflora*: 0.045, *S. maritima:* 0.144) and Guadiana and Piedras Estuaries (*S. densiflora*: 0.063, *S. maritima*: 0.144) than between Tinto-Odiel and Piedras Estuaries (*S. densiflora*: 0.023, *S. maritima*: 0.005). The GD between populations of both parental species in the same estuary was higher in the Guadiana Estuary (0.684) than in Tinto-Odiel (0.449) and Piedras (0.428) Estuaries.

In hybrid plants, an average of 3 (0-6) different alleles per locus and individual was obtained, with a total of 36 different alleles for the 8 surveyed loci. All the alleles observed in the parental species were also present in different proportions in the hybrids, except one absent allele of *S. densiflora* (MS18-3) and three of *S. maritima* (MS14-4, MS15-7, MS18-7). Thirteen alleles of the hybrids were unique in *S. maritima*, 8 alleles were exclusive of *S. densiflora* and 15 alleles of the hybrids were found in both parental species. This high number of *S. maritima* alleles compared to S. *densiflora* was maintained in both *S. maritima x densiflora* (13 unique alleles of *S. maritima* and 7 of *S. densiflora*) and *S. densiflora x maritima* (11 of *S. maritima* and 8 of *S. densiflora*) hybrids, although the maternal proportion increased for *S. maritima x densiflora* (Table 3.2). As for the parental species, the greatest GD for the hybrids according to the Nei's index, was found between the Guadiana and Tinto-Odiel (0.167) and between Guadiana and Piedras (0.125) Estuaries; while the GD between Tinto-Odiel and Piedras Estuaries was lower (0.028).

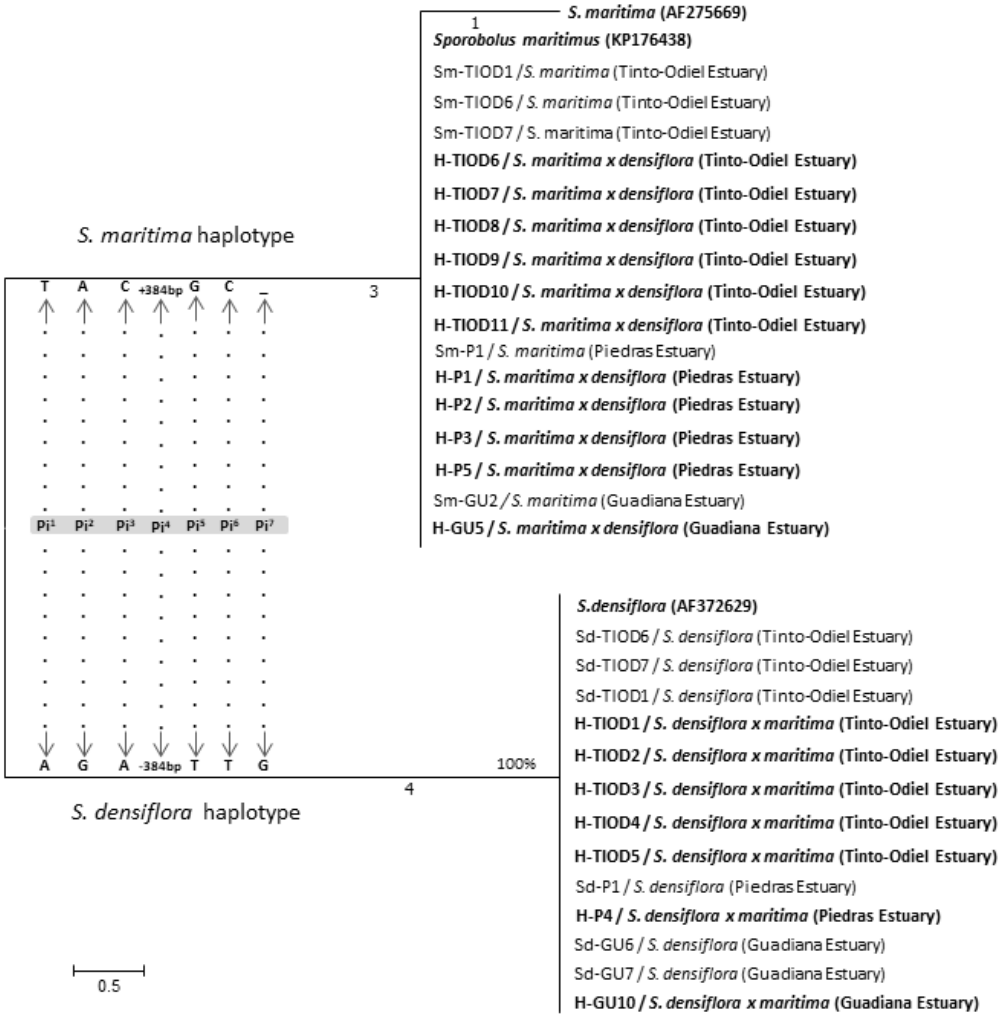

Fig. 3.2. Phylogenetic analysis (Maximum Parsimony) of the intergenic trnT-trnL sequences from *S. densiflora*, *S. maritima* and their hybrids (745 aligned nucleotide sites). The following accessions from Genbank were included in the analysis for comparison: KP176438 and AF275669 for *S. maritima* and AF372629 for *S. densiflora*. All positions containing gaps and missing data were eliminated. Two informative indels have been coded (as present/absent additional character states): one deletion of 384 pb characteristic of the *S. densiflora* haplotype and its corresponding insertion characteristic of the *S. maritima* haplotype; and the other indel at one nucleotide site where the *S. densiflora* haplotype has an additional nucleotide (G) that is absent (gap) from the *S. maritima* haplotype. Differential character states of eight parsimony informative characters (Pi 1 to 7) which distinguish the two reference haplotypes are indicated on the respective branches. Five are represented by substitutions and two by indels of 384 bp (Pi^4) and 1 bp (Pi^7). One out of 10 most parsimonious trees (length = 8) is shown. The consistency index is (1.0), the retention index is (1.0). Bootstrap % values (500 replicates) are indicated.

Table 3.2. Allele occurrence at 8 microsatellite loci in three populations of *Spartina maritima*, *S. densiflora* and their hybrids in three different estuaries of the Gulf of Cadiz (Southwest Iberian Peninsula), TIOD, Tinto and Odiel Estuary; PI, Piedras Estuary; GU, Guadiana Estuary.

		MS 2			MS7				MS13							MS14			
		1	*2*	*3*	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*1*	*2*	*3*	*4*
TIOD	*S. densiflora*	1	0	0	1	0.3	0	0	0.1	0.1	0	1	0.3	0	0	0	0.8	1	0
	S. maritima	0.1	0.1	1	0	0	1	0.1	0.4	0.1	0.1	1	0.1	0.4	1	0	0	1	0
	Hybrid	0.6	0.1	1	1	0	1	0	0	0	0	1	0	0	1	0	0.5	1	0
PI	*S. densiflora*	1	0	0.4	1	0.2	0	0	0.4	0	0	1	0	0	0	0	1	1	0
	S. maritima	0	0	1	0	0	1	0.2	0.6	0	0	1	0	0.6	1	0	0	1	0.2
	Hybrid	1	0	1	1	0.4	1	0.2	0.4	0	0	1	0	0	1	0	0.8	1	0
GU	*S. densiflora*	1	0	0	1	0.2	0	0	0	0.9	0	1	0	0	0	0	0.9	1	0
	S. maritima	0	0	1	0	0	1	0.2	0	1	0.8	1	1	1	0.8	0.8	0	1	0
	Hybrid	1	0	1	1	0	1	0	0	1	0.8	0.9	1	0.9	0.9	0.6	0.9	1	0

		MS15							MS16				MS17				MS18						
		1	*2*	*3*	*4*	*5*	*6*	*7*	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*4*	*1*	*2*	*3*	*4*	*5*	*6*	*7*
TIOD	*S. densiflora*	0	1	0.4	1	0.8	0	0	0	1	0.1	1	0.3	0.1	1	0	1	0	0	0.3	0.9	0	0
	S. maritima	1	1	0.8	0	0	1	0	1	0	0	1	0	1	1	0.1	0	0	0	0	0	1	0.1
	Hybrid	0.8	1	0.5	0.4	0.6	0.6	0	0.9	0.8	0.5	0.9	0	1	0.8	0	0	0	0	0	0	1	0
PI	*S. densiflora*	0	1	0.8	0.8	0.8	0	0	0	1	0.4	1	0.2	0	1	0	1	0	0	0	0	0	0
	S. maritima	1	1	1	0	0	1	0	1	0	0	1	0	1	1	0	0	0	0	0	0.2	1	0
	Hybrid	1	1	0.8	0.4	0.4	1	0	1	1	0.2	1	0	1	1	0	0	0	0	0	0	1	0
GU	*S. densiflora*	0	1	0.9	0.7	0.8	0	0	0	1	0.3	1	1	0	1	0	1	0	0.1	0.4	1	0	0
	S. maritima	1	1	1	0.4	0	1	0.2	1	0	0.2	1	0	1	1	1	0	1	0	0	0	1	0
	Hybrid	1	1	1	0.2	0.8	1	0	1	0.9	0	1	0.8	1	1	0.8	0.9	0.9	0	0.1	0.5	1	0

Five unique alleles were found in Guadiana Estuary (MS14-1 and MS18-2 of *S. maritima* and the hybrids, MS15-7 of *S. maritima* and MS18-3 and MS18-4 of *S. densiflora*), 2 unique alleles were recorded in Tinto-Odiel Estuary (MS2-2 of *S. maritima* and the hybrids and MS18-7 of *S. maritima*), and 1 unique allele was found in Piedras Estuary (MS14-4 of *S. maritima*) (Table 3.2). Genetic differentiation of *Spartina* hybrids at the estuary level (Φ_{PT} = 0.547) was higher among populations (55%) than within populations (45%) (Table 3.3).

These genetic differences are represented in the PCoA of GD that allowed distinguishing the parental species along the PC-2 axis and positioning the hybrids in the center, closer to *S. maritima* than to *S. densiflora*. The PC-1 axis separated individuals from different estuaries, with *Spartina* populations from the Guadiana Estuary showing negative values and those of the Tinto-Odiel and Piedras Estuaries with positive values (Fig. 3.3A).

As expected, the hybrids showed higher genetic diversity (I) than their parental species at all estuaries, except at Piedras Estuary where it was similar for the hybrids and *S. densiflora*. In the Guadiana Estuary, the hybrids displayed a genetic diversity 2.00 times higher than *S. densiflora* and 2.50 times than *S. maritima*, while in both Tinto-Odiel and Piedras Estuaries it was 1.25 and 1.50 higher, respectively. The intra-population genetic diversity of the hybrids in the Guadiana Estuary was twice than that of Tinto-Odiel and Piedras Estuaries. Despite having higher total number of alleles, *S. maritima* exhibited lower inter-individual genetic diversity than *S. densiflora* (Table 3.4).

Ecology and phenotypic diversity of the hybrids

The Guadiana Estuary had lower sediment salinity (17.1 ± 1.3 mS cm^{-1}) and elevation (2.62 ±0.04 m) for every taxon than interstitial sediment salinity and elevation measured in Tinto-Odiel (24.8 ± 1.3 mS cm^{-1} and 2.96 ± 0.05 m, respectively) and Piedras Estuaries (22.9 ± 1.6 mS cm^{-1} and 3.08 ± 0.05 m, respectively). Moreover, sediments from the Tinto-Odiel Estuary exhibited lower redox potential (-9 ± 16 mV) than the other estuaries (Guadiana: 100 ± 16 mV, Piedras: 79 ± 19 mV). The Tinto-Odiel Estuary also had higher sediment pH (6.9 ± 0.1) than the Guadiana (6.7 ± 0.1) and Piedras (6.4 ± 0.1) sites. On the other hand, the recorded sedimentary variables did not change significantly between *Spartina* taxa within estuaries, except for sediment characteristics in the lower elevation occupied by *S. maritima* in the Tinto-Odiel Estuary as compared to areas occupied by the other studied taxa (Table 3.5; see Appendix 3.A for statistical tests).

The vegetative phenotypes of the *Spartina* hybrids were characterized by some traits similar to one or to both parental species, others that were intermediate between the parental species, and some

Table 3.4. Number of different alleles (Na), number of effective alleles (Ne), Shannon's Information Index (I) for populations of *Spartina maritima*, *S. densiflora* and their hybrids from three different estuaries of the Gulf of Cadiz (Southwest Iberian Peninsula). Values are mean ± SE.

	Tinto-Odiel Estuary			Piedras Estuary			Guadiana Estuary		
	S. maritima	*S. densiflora*	Hybrids	*S. maritima*	*S. densiflora*	Hybrids	*S. maritima*	*S. densiflora*	Hybrids
Na	0.854 ± 0.129	0.780 ± 0.133	0.780 ± 0.128	0.585 ± 0.110	0.634 ± 0.125	0.780 ± 0.118	0.780 ± 0.113	0.707 ± 0.127	1.146 ± 0.124
Ne	1.075 ± 0.029	1.117 ± 0.041	1.174 ± 0.053	1.059 ± 0.031	1.122 ± 0.044	1.111 ± 0.040	1.102 ± 0.042	1.141 ± 0.047	1.296 ± 0.063
I	0.089 ± 0.026	0.114 ± 0.033	0.142 ± 0.041	0.057 ± 0.026	0.106 ± 0.035	0.102 ± 0.034	0.088 ± 0.032	0.120 ± 0.037	0.234 ± 0.048
N	8	8	11	5	5	5	5	9	10

transgressive traits that were superior to both parents. Thus, SLA (except for hybrid individuals from the Piedras Estuary), the number of leaves and the leaf width for the hybrids were similar to those of *S. maritima* and greater than the values recorded for *S. densiflora*. In contrast, the tiller diameter of hybrids only for the Guadiana Estuary, and the number of dead leaves and the foliar area for all measured hybrids were similar to those of *S. densiflora* and greater than those of *S. maritima*. In addition, the number of leaves per tiller length for the hybrids was similar to this metric for *S. densiflora* and 2.5 times lower than data recorded for *S. maritima*. In contrast, leaf length, leaf rolling and tiller density for the hybrids were intermediate between the lower values of *S. maritima* and the higher values of *S. densiflora*. Tiller length for the hybrids at Guadiana Estuary (49.8 ± 2.6 cm) and almost LAI for every estuary (*P*-value = 0.072) exceeded those of both parental species (*S. maritima*: 13.5 ± 3.7 cm, *S. densiflora*: 29.6 ± 2.6 cm) (see Appendix 3.A for statistical tests).

In general, *Spartina* plants of parents and hybrids from the Guadiana Estuary had 16% shorter leaves than leaves measured in the other estuaries. Plants from the Guadiana Estuary for every taxon showed also 71% higher tiller densities, 17% lower numbers of leaves and 16% smaller leaf areas than those of Piedras Estuary. Plants from the Piedras Estuary exhibited 34% lower LAI than Tinto-Odiel Estuary and 16% greater leaf width than the other two estuaries. *Spartina maritima* growing at Tinto-Odiel Estuary showed greater SLA than at Piedras, wider tillers than at Guadiana Estuary, and taller tillers and fewer leaves per tiller length than both estuaries (Table 3.5; see Appendix 3.A for statistical tests).

Fig. 3.3. Principal Coordinates Analysis (PCoA) of of *Spartina maritima* (Sm, black symbols), *S. densiflora* (Sd, white symbols) individuals and their hybrids (H, gray symbols) based on (A) Huff genetic distance using 8 SSRs loci, and (B) on Gower dissimilarity index using 12 phenotypic plant traits for 66 clumps from three different estuaries on the Gulf of Cadiz (Southwest Iberian Peninsula): TIOD (squares), Tinto and Odiel Estuary; PI (triangles), Piedras Estuary; GU (circles), Guadiana Estuary.

Table 3.5. Phenotypic traits and sedimentary variables for three populations of *Spartina maritima, S. densiflora* and their hybrids in three estuaries of the Gulf of Cadiz (Southwest Iberian Peninsula), TIOD, Tinto and Odiel Estuary; PI, Piedras Estuary; GU, Guadiana Estuary (values are mean ± SE).

		Spartina maritima			*Spartina densiflora*			*Spartina* hybrids		
		TIOD	PI	GU	TIOD	PI	GU	TIOD	PI	GU
Phenotypic traits	Number of leaves	5 ± 0.3	6 ± 0.4	4 ± 0.4	3 ± 0.3	4 ± 0.4	3 ± 0.3	4 ± 0.3	5 ± 0.4	4 ± 0.3
	Number of dead leaves	0.3 ± 0.4	1 ± 0.5	0.3 ± 0.5	3 ± 0.4	2 ± 0.5	2 ± 0.4	2 ± 0.4	3 ± 0.5	4 ± 0.4
	Leaves per tiller (cm^{-1})	0.17 ± 0.02	0.30 ± 0.02	0.31 ±0.02	0.08 ± 0.02	0.12 ± 0.02	0.11 ± 0.01	0.11 ± 0.02	0.11 ± 0.02	0.09 ± 0.01
	Leaf width (cm)	0.6 ± 0.03	0.8 ± 0.04	0.6 ± 0.04	0.5 ± 0.03	0.6 ± 0.04	0.5 ± 0.03	0.6 ± 0.03	0.7 ± 0.04	0.7 ± 0.03
	Leaf length (cm)	17.4 ± 2.3	19.3 ± 2.7	13.66 ± 2.7	39.1 ± 2.3	38.8 ± 2.7	29.7 ± 1.9	27.7 ± 2.3	27.8 ± 2.7	28.8 ± 1.9
	Leaf area (cm^2)	11.5 ± 1.9	15.4 ± 2.2	7.9 ± 2.2	20.5 ± 1.9	22.2 ± 2.2	16.3 ± 1.6	17.8 ± 1.9	20.5 ± 2.2	19.9 ± 1.6
	SLA ($m^2 \cdot g^{-1}$)	0.013 ± 0.001	0.009 ± 0.001	0.011 ± 0.001	0.007 ± 0.001	0.009 ± 0.001	0.008 ± 0.001	0.012 ± 0.001	0.010 ± 0.001	0.010 ± 0.001
	Leaf Rolling (%)	4 ± 5	0 ± 0	7 ± 5	38 ± 5	31 ± 6	40 ± 4	14 ± 5	11 ± 6	25 ± 4
	Tiller diameter (mm)	3.5 ± 0.2	3.8 ± 0.2	3.0 ± 0.2	3.5 ± 0.2	3.2 ± 0.2	3.2 ± 0.1	3.3 ± 0.2	3.7 ± 0.2	3.6 ± 0.1
	Tiller length (cm)	31.4 ± 3.1	19.6 ± 3.7	13.5 ± 3.7	37.7 ± 3.1	33.9 ± 3.7	29.6 ± 2.6	42.2 ± 3.1	43.5 ± 3.7	49.8 ± 2.6
	Tillers density (cm^{-2})	0.15 ± 0.07	0.06 ± 0.08	0.19 ± 0.08	0.49 ± 0.07	0.37 ± 0.08	0.54 ± 0.06	0.27 ± 0.07	0.23 ± 0.08	0.38 ± 0.06
	LAI	4.72 ± 1.58	1.84 ± 1.58	3.04 ± 1.58	6.11 ± 1.34	5.26 ± 1.58	2.29 ± 1.12	7.71 ± 1.33	5.22 ± 1.58	6.29 ± 1.12
Sedimentary variables	pH	7.0 ± 0.1	6.5 ± 0.2	6.6 ± 0.1	7.0 ± .1	6.4 ± 0.2	6.7 ± 0.2	7.0 ± 0.1	6.4 ± 0.2	6.7 ± 0.1
	Conductivity (mS cm^{-1})	23.0 ± 2.3	19.5 ± 2.7	13.8 ± 2.7	27.2 ± 2.3	22.9 ± 2.3	19.2 ± 2.0	24.2 ± 3.1	26.3 ± 2.4	18.2 ± 1.9
	Water content (%)	58 ± 5	54 ± 7	48 ± 7	52 ± 5	48 ± 7	52 ± 5	44 ± 5	62 ± 6	48 ± 5
	Redox potential (mV)	- 65 ± 28	109 ± 33	85 ± 33	16 ± 33	59 ± 33	143 ± 25	21 ± 28	69 ± 33	73 ± 23
	Elevation (m above SHZ)	2.41 ± 0.08	3.02 ± 0.09	2.52 ± 0.09	3.22 ± 0.08	3.20 ± 0.09	2.67 ± 0.07	3.23 ± 0.03	3.04 ± 0.08	2.68 ± 0.03

The phenotypic difference between both parental species and their hybrids is shown in the graphic representation of the PD that separated *S. maritima* from the other taxa along the PC-2 axis. *Spartina densiflora* and the hybrids appeared mixed, with a slight tendency to separate *S. densiflora* in the opposite direction to *S. maritima* along the PC-2 axis. Instead of the difference reported above, the combination of the recorded phenotypic plant traits did not distinguish the populations from the three estuaries for any taxon (Fig. 3.3B).
The Mantel tests showed that the PD of the *Spartina* hybrids was independent of their sedimentary, genetic and geographic distances. PD between hybrids tended to increase with their GD, however this relationship was not significant (*P*-value = 0.060) (Fig. 3.4A). On the other hand, GD between hybrids increased with their sedimentary and geographic distances (Fig. 3.4B, C).

3.4 Discussion

Our results show that the genetic structure of sterile *Spartina* F1 hybrids between the native *S. maritima* and invasive *S. densiflora* along the coast of the Gulf of Cadiz (Southwest Iberian Peninsula) was mainly determined by the genetic structure of the parents, the exotic hybrids being genetically more similar to the native than to the invasive species.

Although *S. maritima* individuals exhibited more different alleles (31) than *S. densiflora* (24) at the 8 investigated loci, their genetic diversity among tussocks from the same estuary was the lowest of the studied taxa. This low inter-individual variability of *S. maritima* is consistent with its low sexual reproduction (Castellanos et al. 1994, 1998) and previous molecular investigations in Europe (Yannic et al. 2004). It also agrees with its high levels of local adaptation to stressful and stable low marshes (Castellanos et al. 1994, Castillo et al. 2008b, Contreras-Cruzado et al. 2017). *S. densiflora*, with hybrid origin (Fortune et al. 2008), has a higher ploidy level (7x) than *S. maritima* (6x), which most likely increases genetic diversity (Petit et al. 1999, Soltis and Soltis 2000).

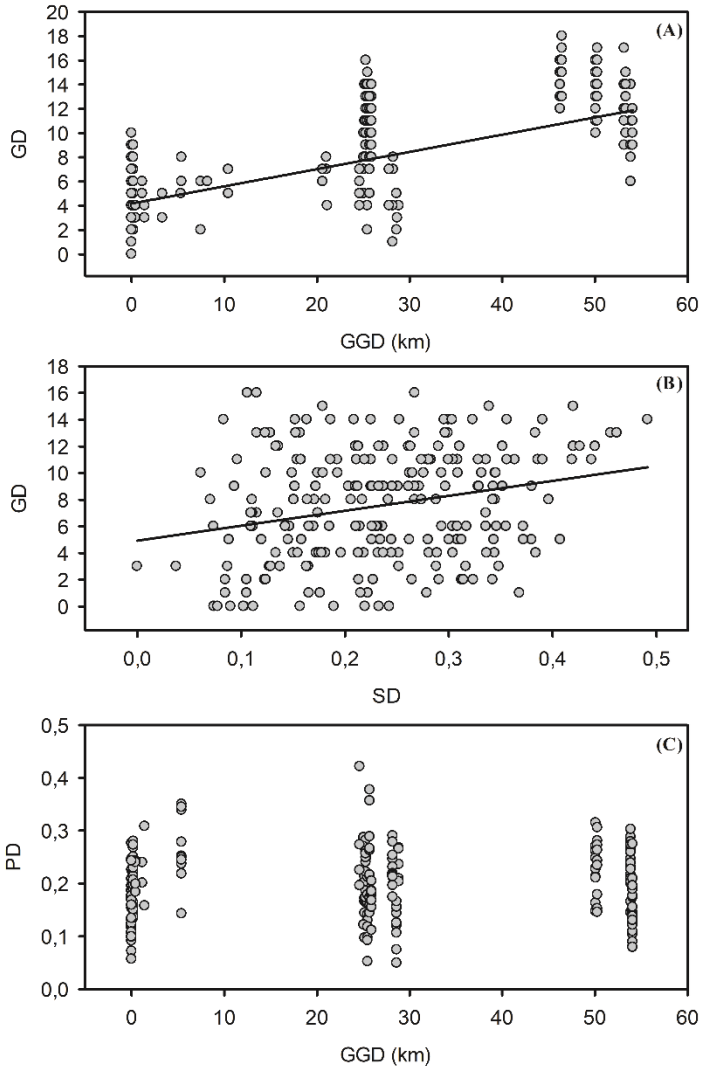

Fig. 3.4. Mantel tests between genetic (GD), phenotypic (PD), geographic (GGD) and sedimentary (SD) distances for hybrids between *Spartina maritima* and *S. densiflora* from the Gulf of Cadiz (Southwest Iberian Peninsula) (N = 22-26). Regression equations: (A) y = 0.1421x + 4.1831, R^2 = 0.461, *P*-value = 0.010, (B) y = 11.202x + 4.9277, R^2 = 0.066, *P*-value = 0.010; (C) *P*-value = 0.060.

In contrast, *S. maritima* exhibited greater inter-population genetic differentiation between estuaries than *S. densiflora*. The weak population structure of *S. densiflora* agrees with its invasive status and associated bottlenecks resulting from founding effects (Dlugosch and Parker 2008, Brzyski et al. 2014). Additionally, establishment of these invading populations is more recent than the native populations of *S. maritima* that can survive for decades,

or even centuries, in non-successional marshes (Castellanos et al. 1998), allowing natural selection, neutral mutations and genetic isolation to act longer on these native populations. Moreover, the genetic isolation between *S. maritima* populations may be stronger than between *S. densiflora* populations due to their lower sexual reproduction. *S. densiflora* produces many viable seeds (Kittelson and Milton 1997, Nieva et al. 2001) that can be transported by tides and currents floating on water (Xiao et al. 2016) and by birds (Vivian-Smith and Stiles 1994). Invasive *S. densiflora* introduced along the Atlantic Coast of North America also showed low genetic differentiation among populations (Castillo et al. 2018).

Our genetic analyses using cpDNA and nuclear SSR markers confirmed the hybrid origin of every tussock identified as such in the field and the reciprocal crosses that occurred in the Guadiana, Piedras and Tinto-Odiel Estuaries. In relation to the 8 SSR loci analyzed, most of the alleles present in the parental species were also present in their hybrids (27 out of 31 of *S. maritima* alleles and 22 out of 24 of *S. densiflora* alleles), while the 48% of the alleles of *S. maritima* and 63% of *S. densiflora* were shared between both parental species. Consequently, the hybrids contained greater number of different alleles than both parental species separately. Hybridization is expected to increase the number of alleles at all loci (Long 1991) while non-inherited alleles may be associated to changes occurred during hybridization such as chromosomal rearrangement, gene loss or changes in genome size (Baack and Rieseberg 2007), but we cannot reject possible technical bias in detecting some alleles. Thus, the hybrids showed higher inter-individual genetic diversity within populations than their parents, except in the Piedras Estuary population. The studied hybrids are sterile, each hybrid tussock being the product of independent hybridization events (Castillo et al. 2010a). Gene flow within populations tends to homogenize the genetic differences between individuals (Slatkin 1987, Lenormand 2002), which does not occur in these sterile hybrids as it could occur in the parental species, especially in *S. densiflora* as mentioned

above. Moreover, the fact that hybrids whose seed parent was *S. maritima* inherited all its genetic material from unreduced ovules (Castillo et al. 2010a) could explain that, globally, hybrids inherited more alleles from *S. maritima* than from *S. densiflora*. Nonetheless, no direct relationship was found between the maternal origin and the proximity to the genotype of *S. maritima*.

Both parental species and their hybrids showed clear spatial genetic structure. The genetic structure of the hybrids was revealed by their higher genetic differentiation among populations (55%) than within populations (45%) at the estuary level, which was the consequence of the genetic structure of their parental species. The tussocks from the Guadiana Estuary for every taxon were clearly differentiated from those sampled in the Piedras and Tinto-Odiel Estuaries, which are located closer to each other along the coast. Thus, the spatial genetic structure of the hybrids was directly related to geographic distances among populations. The total or partial reproductive isolation by distance predicts the increase of genetic differentiation with geographic distance due to reduction or lack of gene flow between populations (Wright 1943, Baack et al. 2015). Sloop et al. (2011) observed that hybrids between the native *Spartina foliosa* and the invasive *Spartina alterniflora* in San Francisco Bay also showed a marked genetic structure due to limitations in seeds and pollen dispersal. The GD of the hybrids analyzed in our study was also directly related to the differences in their sedimentary environments among marsh habitats that may have imposed selective pressures on the parental species, favoring isolation by environmental processes (Wang and Bradburd 2014). Thus, for example, *S. maritima* growing in low marshes starts its flowering earlier in the year than *S. densiflora* (B. Gallego-Tévar, personal observation). Similar observations were made in wild barley where the spatial population structure was determined not only by geographic isolation, but also by differences in environmental conditions such as elevation, temperature and rainfall (Hübner et al. 2009). In our case, plants from Guadiana Estuary were colonizing in sediments with lower conductivities and at lower elevations than in Piedras and Tinto-Odiel Estuaries.

The hybrids from Guadiana Estuary were the only hybrids showing transgressive tiller height, while the hybrids in Piedras and Tinto-Odiel Estuaries were taller than *S. maritima* but not different from *S. densiflora*. This was probably related to the recorded greater genetic differentiation between the parental species in the Guadiana Estuary, as greater genetic distances between parents have been frequently related to the development of heterosis leading hybrid vigor in their hybrids (Ali et al. 1995, Reif et al. 2003, Krystkowiak et al. 2009, Stelkens and Seehausen 2009, Pandey et al. 2018). Along with differences in parental genotypes among populations, sedimentary environment differences in Guadiana Estuary (lower conductivity and elevation) may also contribute to the heterosis shown by the hybrids. Different studies in hybrids such as those on the genera *Ipomomsis* (Campbell and Waser 2007), *Artmisia* (McArthur et al. 1998) and *Iris* (Arnold et al. 2012) proved that the phenotype and the fitness of hybrids can be strongly influenced by the environment in which they develop (reviewed in Arnold and Martin, 2010).

The three taxa of *Spartina* had shorter leaves in Guadiana than in the other estuaries and higher tiller density and smaller number and area of leaves than in the Piedras Estuary. But also, all taxa exhibited greater width of leaves in Piedras Estuary, where the lower pH was registered, with respect to the other estuaries and lower LAI than in Tinto-Odiel. Although the influence of the genotype cannot be ruled out, these differences among the 3 estuaries could be marked by the environment since both *Spartina maritima* and *S. densiflora* are species with high reported phenotypic plasticity in contrasted environments (Castillo et al. 2005a, 2014, 2016). In fact, *S. maritima* presented taller tillers in Tinto-Odiel Estuary where the sediment redox potential was lower, which has been previously described in the species (Castillo et al. 2005a).

When all phenotypical traits were taken into account, genetic, geographical and sedimentary environmental distances in hybrid populations among estuaries did not relate to their vegetative phenotypic distance, although PD and GD were close to positive correlation (P = 0.060). When polygenic

inheritance of traits is operating, correlation between GD and PD decreases as the number of loci involved in the regulation of the phenotypic traits increases (Burstin and Charcosset 1997, Lefebvre et al. 2001). In hybrid taxa, genotype-phenotype association is even more complex since gene expression can be non-additive (Meyer et al. 2007, Bassene et al. 2009) and the molecular mechanisms that regulate gene expression are not completely identified (Tirosh et al. 2009, Yoo et al. 2014, Bird et al. 2018). In *Spartina*, hybridization and allopolyploidy were shown to be accompanied by substantial non-additive parental expression patterns in both controlled (Chelaifa et al. 2010) and natural conditions (Ferreira de Carvalho et al. 2017). Our results describe phenotypic patterns reflecting parental dominance, additivity and over-dominance, making complex the genotype-phenotype relationship.

3.5 Conclusions

Populations of sterile exotic *Spartina* hybrids between the native *S. maritima* and the invasive *S. densiflora* in the Gulf of Cadiz (Southwest Iberian Peninsula) are establishing hybrid zones developing a spatial genetic structure inherited from both parental species. The hybrid populations with greater genetic differentiation are those more spatially separated from each other and that are present in more contrasted sedimentary environments, revealing respective isolation processes by distance and by environment. The hybrids in the Guadiana Estuary were the most genetically differentiated and with the highest transgressive behavior in terms of tiller height. However, the relationship between the population genetic structure of these hybrids and their phenotype is complex and no correlation pattern was found between the molecular markers and the set of recorded vegetative morphological traits. This study provides new data on the dynamics of exotic hybrid invasions, integrating effects of the genetic, phenotypic, geographical and environmental scenario with hybridization following invasion of a halophyte community by an exotic plant species.

CHAPTER IV

Phenotypic plasticity of polyploid plant species promotes transgressive behavior in their hybrids

CAPÍTULO 4: La plasticidad fenotípica de las especies vegetales poliploides promueven el comportamiento transgresivo en sus híbridos

Resumen

La hibridación es un proceso frecuente que da lugar a importantes consecuencias evolutivas, pero hay una falta de estudios sobre las relaciones de la variabilidad de las respuestas de las especies vegetales parentales a factores ambientales y las respuestas de sus híbridos a nivel fenotípico. Para ello, diseñamos un experimento en el que expusimos a dos híbridos recíprocos de *Spartina*, *S. maritima x densiflora* y *S. densiflora x maritima,* y sus especies parentales a cuatro tratamientos de salinidad durante 30 días. Los principales objetivos fueron comparar la actuación de los híbridos con la de sus parentales, distinguir la herencia fenotípica actuando en los híbridos, y analizar las relaciones entre la variabilidad de las respuestas de los padres y las respuestas de los híbridos a la salinidad. Se caracterizaron las respuestas y el grado de variabilidad de 37 variables foliares. Ambos híbridos presentaron mayor tolerancia a la salinidad que sus padres, mostrando su mayor porcentaje de caracteres transgresivos a ambos extremos del gradiente de concentración salina. Cuando los parentales exhibieron una respuesta más plástica para un determinado carácter, hubo mayor probabilidad de que el híbrido desarrollase un comportamiento transgresivo para ese carácter. Este hallazgo respalda un nuevo enfoque a ser aplicado en el desarrollo artificial de híbridos de interés agrícola.

CHAPTER 4: Phenotypic plasticity of polyploid plant species promotes transgressive behavior in their hybrids

Abstract

Hybridization is a frequent process that leads to relevant evolutionary consequences, but there is a lack of studies regarding the relationships of the variability of the response of parental plant species to environmental gradients and the responses of their hybrids at a phenotypic level. We designed an experiment in which we exposed two reciprocal cordgrass hybrids, *Spartina maritima x densiflora* and *S. densiflora x maritima*, and their parental species to four salinity concentrations for 30 days. The main objectives were to compare the performance of the hybrids with that of their parents, to distinguish the phenotypic inheritance operating in the hybrids, and to analyze the relationships between the variability in the responses of the parents and the responses of their hybrids to salinity. We characterized the responses and the degree of variability for 37 foliar traits. Both hybrids presented greater salinity tolerance than their parents, showing their highest percentage of transgressive traits at both extremes of the salinity gradient. When the parental plants themselves showed a more plastic response for a given trait, there was a greater chance that their hybrid developed a transgressive behavior for this trait. This finding supports a new focus to be applied for the artificial development of vigorous hybrid crops.

4.1 Introduction

Hybridization is a frequent process in both plants and animals that leads to relevant evolutionary and ecological consequences (Arnold 1992). The ecological performance of hybrids depends on the expression of genes that control traits related to their stress tolerance and fitness (Chen 2013). Thus, the novel genotypes obtained by hybridization commonly exhibit phenotypic traits with intermediate values between both parents due to an additive genetic control when the traits are controlled by a large number of genes that act independently, as well as similar to parental species as a product of a dominant inheritance (Favre and Karrenberg 2011). But hybrids can also display another phenotypic inheritance that produces phenotypic traits outside the ranges of variability of both parental species, showing transgressive phenotypes as a product of heterosis or hybrid vigor (Rieseberg et al. 1999). In wild invasive hybrids, fixed heterosis leads to an increase of invasiveness (Ellstrand and Schierenbeck 2000) as they may be fitter than the parental species and able to colonize, establish and tolerate more extreme environments (Vilà et al. 2000, Castillo et al. 2010a, Hovick and Whitney 2014, Parepa et al. 2014). In cultivated hybrids, heterosis is being applied in crop production to develop more vigorous and better performing cultivars (Fu et al. 2014). The molecular mechanisms underlying heterosis have been subject of long deliberations (Chen 2010, 2013, Baranwal et al. 2012). In addition to the genomic mechanisms, recent studies have revealed the importance of epigenetic changes in key genes regulating fitness-related traits in hybrids (Salmon et al. 2005, Ni et al. 2009). Additionally, there is evidence that a greater genetic differentiation between parents results in greater heterosis in their hybrids (East 1936, Chen 2010).

On the other hand, hybridization has been associated with high phenotypic plasticity (Ainouche and Jenczewski 2010, Te Beest et al. 2012, Cara et al. 2013) that also contributes to the enhanced ability of hybrids to occupy wide ecological ranges. Phenotypic plasticity can be adaptive when it is

regulated by heritable mechanisms (Matesanz et al. 2010) and, therefore, evolve itself as an independent functional trait. In hybrids and allopolyploids, variations in the expected additive or parental-like phenotypic plasticity are controlled by changes in genes expression both at transcriptional and post-transcriptional levels (Jackson and Chen 2011). Furthermore, phenotypic plasticity can be also regulated at an epigenetic level (Parepa et al. 2014). However, phenotypic inheritance and phenotypic plasticity are both regulated at genetic and epigenetic levels, but there are a lack of studies regarding the relationships of the variability on the response of parental species to environmental gradients and the inheritance at work in their hybrids.

Cordgrasses (former genus *Spartina* Schreb.) are halophytes with a wide distribution all around the world (Strong and Ayres 2013). All *Spartina* species are polyploids (Ainouche et al. 2012) and hybridization has repeatedly occurred between them, playing an important role in their evolution (Ainouche et al. 2009). Recently, two first generation (F1) reciprocal hybrids between the native European *Spartina maritima* (Curtis) Fernald (2n = 6x = 60) from low salt marshes and the invasive South American *Spartina densiflora* Brongn. (2n = 7x = 70) from middle marshes have been described on the Gulf of Cadiz (Southwest Iberian Peninsula). *Spartina maritima x densiflora* has ca. 65 chromosomes, *S. maritima* as maternal species and appears in low marshes, whereas *S. densiflora x maritima* has ca. 95 chromosomes, *S. densiflora* as mother and colonizes middle marshes (Castillo et al. 2010a). Both F1 hybrids are sterile and show some transgressive behaviors such as higher growth rates and taller tillers than their parental species (Castillo et al. 2010a). Given the differences in performance of *S. maritima*, *S. densiflora* and their hybrids, these taxa constitute a good model for studying how parental responses along an environmental gradient determine the phenotypic inheritance operating in their hybrids. With this aim, we designed a glasshouse experiment in which *S. maritima x densiflora*, *S. densiflora x maritima* and both parental species were exposed to four salinities (from freshwater to hypersalinity). Their middle-term responses

and degree of variability were characterized by the measurement of 37 distinct plant traits. The main objectives were:(1) to compare the performance of the hybrids at different salinities with that of their parents by evaluating the magnitude of the different traits and their variability; (2) to distinguish the phenotypic inheritance operating in the hybrids; and (3) to analyze the relationships between the variability in the responses of the parents and the responses of their hybrids. Our hypothesis was that the *Spartina* hybrids would outperform their parental species showing greater fitness, especially at the extreme salinities, due to transgressive segregation. Additionally, we postulated that traits showing greater variability in the parents would lead to a higher number of transgressive responses in the hybrids given the greater possibilities of advantageous combinations.

4.2 Material and Methods

Plant collection and experimental design

Spartina densiflora x maritima (2n = ca. 65) is the result of the fecundation of the reduced ovule of *S. densiflora* (2n = 7x = 70) by a reduced gamete of *S. maritima* (2n = 6x = 60), whereas *S. maritima x densiflora* (2n = ca.95) has *S. maritima* as a maternal species contributing with its total genome (unreduced gamete) and a reduced gamete of *S. densiflora* (Castillo et al. 2010a). Below ground biomass (BGB) of 5 different individuals of *Spartina maritima* (*Sm*), *S. densiflora* (*Sd*) and both of their hybrids (*Smxd* and *Sdxm*) were collected from natural populations in the Special Area of Conservation San Bruno Marsh (Guadiana River Estuary, see *Chapter 2*) in November 2015. Plants were collected at least 2 m away from each other to ensure sampling discrete individuals. *Spartina maritima* and the hybrid *S. maritima x densiflora* were collected from the low marsh and *S. densiflora* and *S. densiflora x maritima* from the middle marsh, where they were more abundant. The plant material was transported to the greenhouse facility of the University of Seville where it was

cleaned and trimmed, with roots removed. Rhizomes were then weighed and potted in 16 cm diameter x 15 cm high pots using expanded perlite (Comercial Projar S.A., Valencia, Spain) as a substrate. Rhizomes weights were as similar as possible between taxa, being 4±1 g for *S. maritima* due to its long and sparse rhizomes, 9±1 g for *S. densiflora* due to their short and dense rhizomes and intermediate (7±1 g) for their hybrids.

The pots were arranged in groups of 6 in 38.5 cm wide x 53.5 cm long x 5.0 cm deep black plastic trays, and then submerged in water with liquid fertilizer (Naturplant, Fertilizantes Orgánicos Melguizo, S.L., Seville, Spain) to a depth of 4 cm for acclimatization and growth for four months. Subsequently, five replicates of each taxon were randomly placed in four salinity treatments ranging from freshwater to hyper salinity (0.5, 10, 20 and 40 ppt salinity) using sea salt Instant Ocean ® (Aquarium Systems Inc., Mentor, Ohio USA) plus 20% Hoagland's nutrient solution which was changed weekly. The same five genotypes (individuals) of each taxon were divided into four pieces of rhizomes from which experimental plants were obtained for the four saline treatments. Opaque plastic black bags were used to cover those areas of the trays not occupied by pots in order to prevent algae proliferation in the solution. The highest salinity treatments were established by increasing salinity by 10 ppt each week until reaching the final concentration for avoiding osmotic shock. The experiment lasted 30 days in April-May 2016 in the glasshouse with a controlled temperature of 21 – 25ºC and natural sunlight.

Data collection

Plant mechanistic and functional traits were measured after 30 days of salinity treatments to assess medium-term responses of the *Spartina* hybrids and their parental species to different salinity levels. Foliar measurements were always conducted for the youngest totally unfolded adult leaf to avoid differences due to the ontogeny of leaves. Every plant trait was recorded on or in the leaves of the studied *Spartina* taxa since we expected to see significant changes across

the experimental salinity gradient because the leaf (the organ of photosynthesis and transpiration) is highly sensitive to salt stress (Suárez 2011).

Leaf morphology. Leaf area was calculated as the triangle area obtained with the leaf base width and its length, both measured using a ruler. Specific Leaf Area (SLA, m^{-2} g^{-1}) was calculated as the ratio between the leaf area and its dry weight (Garnier et al. 2001). Sub-replicates of 3 leaves per plant were conducted for these measurements. Leaf area and SLA may change with salinity as morphological acclimations to salt stress (Castillo et al. 2014).

Leaf biochemistry. Leaf samples for biochemical analyses were always collected around noon. Leaf Water Content (LWC) was determined for one leaf per plant as LWC (%) = (FW − DW) × 100 / FW, where FW was the fresh weight and DW was the dry weight after oven-drying samples at 80 °C for 48 h (Castillo et al. 2007)

Free proline content in leaves was recorded as an indicator of salt stress (Grewell et al. 2016). It was determined for one leaf per plant following the procedure presented in Bates et al. (1973) (see Appendix 4.A).

Malondialdehyde (MDA) is a product of lipid peroxidation, so its leaf content was recorded as an indicator of oxidative stress (Meloni et al. 2003). Leaf MDA content was measured for one leaf per plant according to the method described in Dhindsa et al. (1981) (see Appendix 4.B).

Leaf pigments were measured for one leaf per plant according to the method described in Arnon (1949) and Lichtenthaler (1987) (see Appendix 4.C).Photosynthetic pigments have been used to indicate the effects of salt stress in the photosynthetic apparatus of *Spartina* species (Castillo et al. 2014, Grewell et al. 2016). The ratios Chl (*a*+*b*):Car and Chl *a* :Chl *b* were calculated. Additionally, the relative content (%) of the non-photosynthetic anthocyanin pigments was also recorded measuring the absorbance at 530 nm using the same spectrophotometer (Mancinelli et al. 1975, Khlestkina et al. 2014). Anthocyanin accumulation may provide antioxidant and photoprotection functions, and act

as a dehydration-tolerance mechanism under salt stress (Chalker-Scott 1999, Lee and Collins 2001, Gould et al. 2002)

Dry leaf tissue from one leaf per plant was ground to pass through a No. 4 mesh sieve prior to measurement of total carbon (C) and nitrogen (N) content using a Perkin Elmer 2400 CHNS/O analyzer (Perkin Elmer, Waltham, MA, USA). C:N ratio was calculated as an indicator of salt stress (Yousef and Sprent 1983).

Salt excretion. In order to measure the salt exudation rate from leaves, two flag leaves per plant were marked and rinsed with deionized water to remove salt exuded previously. After 48 h, a known area of the marked leaves was introduced in a vial with 3 ml of deionized water and shaken to dissolve all salt accumulated on the leaf surface. Then, dissolved salts were measured with a conductivity portable meter (Crison-522, Hach Lange Spain S.L.U, Barcelona, Spain) and excretion rate was calculated.

Chlorophyll fluorescence. Light and dark-adapted Chl fluorescence were measured for 5 leaves per plant at sunrise (at 12 ºC, 60% air relative humidity and a photosynthetic photon flux density (PPFD) of 20 µmol photon m^{-2} s^{-1}) and at noon (at 26 ºC, 45% air relative humidity, and PPFD of 1450 µmol photon m^{-2} s^{-1}) with a portable modulated fluorimeter (FMS-2, Hansatech Instruments Ltd, Norfolk, England) using leaf clips for dark adaptation for 30 minutes. Chl fluorescence parameters were measured according to Maxwell and Johnson (2000) and Schreiber et al. (1986) (see Appendix 4.D). In addition, delayed Chl fluorescence was measured using a modular optical imaging system (NightSHADE LB985, Berthold Technologies GmBh & Co., Baden-Württemberg, Germany) to quantify the post-illumination luminescence emitted by Chl *a*, mainly by PSII that is an indicator of its photochemistry (reviewed in Jursinic, 1986). Chl fluorescence is a useful tool to assess the effects of salt stress on the photosynthetic apparatus (Castillo et al. 2005b).

Gas exchange. Gas exchange measurements were obtained by using an infrared gas analyzer in an open system (LI-6400, Li-COR Inc., Nebraska, USA)

and using a Clark type oxygen electrode (Leaflab 2 System, Hansatech Instruments Ltd, Norfolk, England). The first was used to determine net photosynthesis rate (A), stomatal conductance to CO_2 (G_s) and intracellular CO_2 concentration (C_i) at fixed 400 ppt CO_2 concentration, 15-20 ºC, 36,5 ± 1,3 % relative humidity, a PPFD of 1000 µmol m^{-2} s^{-1} and a flow rate set to 350 µmol s^{-1} within 2 hours of solar noon. Water use efficiency (WUE; mmol CO_2 mol H_2O^{-1}) was calculated from simultaneous measures of photosynthesis rate and stomatal conductance. The oxygen electrode was employed to measure maximum photosynthesis rate (A_{max}) by providing a saturated atmosphere of CO_2 with a 1 M carbonate / bicarbonate buffer (pH 9.0) at PPFD of 1200-1400 µmol photon m^{-2} s^{-1} and 25 ° C. Net photosynthesis rate and Gs are optimal indicators of the effects of salt stress on CO_2 fixation and the water use, respectively (Chaves et al. 2009).

Leaf growth. Apical leaf growth (mm day^{-1}) is a measure of plant fitness. It was quantified by applying red permanent sealer to base of the youngest leaf and the top of 3 tillers per plant to measure the separation between the two plants parts 48 hours later (Castillo et al. 2014).

Data analyses

Phenotypic inheritance. Inheritance operating in the hybrids *S. maritima x densiflora* and *S. densiflora x maritima* were analyzed for the above-mentioned 37 plant traits related to their response to salt stress. Three types of phenotypic inheritance were distinguished (Favre and Karrenberg 2011). First, dominant inheritance (D) was considered when a hybrid showed a trait similar to one of its parents (D-*Sm* for *S. maritima*; D-*Sd* for *S. densiflora*); parental codominance (D-*Sm*,*Sd*) was considered when a trait of a hybrid was similar to both parents. Second, parental additivity (I) was recorded when a trait for a hybrid was intermediate between significantly different values of its parents. The third mechanism, transgressive segregation (T), corresponded with a trait of a hybrid being different from both parental species (outperforming both parental species

at least in +/-5 %). The three inheritance types were quantified for each variable and at each salinity level for both hybrids at the population level (*S. maritima x densiflora* at low marshes and *S. densiflora x maritima* at middle marshes), and for each population at the individual level.

Trait variability and fitness. Inter-treatment trait variability index was used as a general indicator of trait variability among salinity levels and individuals for a given taxon. It was calculated for each taxon including all salinity treatments as the ratio (in percentage) of the difference between the maximum (X_{max}) and the minimum (X_{min}) values of a given trait divided by the maximum (Valladares et al. 2006).

$$\text{Inter. Trait Var.} = [(X_{max} - X_{min})/X_{max}] * 100$$

Mean intra-population trait variability index indicated the intrinsic variability between individuals of the same population, since the same genotypes (individuals) of each taxon were used at each salinity treatment. It was calculated as the arithmetic mean (n = 4 salinity treatments) of the ratios (in percentage) of the differences between the maximum (x_{max}) and the minimum (x_{min}) values divided by the maximum of a given trait for a taxon in a certain salinity (Castillo et al. 2018).

$$\text{Intra. Trait Var.} = \frac{\sum_{i=1}^{4}[(x_{max} - x_{min})/x_{max}] * 100}{4}$$

Phenotypic plasticity index (PPI) for a given trait and taxon was obtained after subtracting the mean intra-population trait variability from the inter-population trait variability. In this way, just the variability associated with trait difference related to salt stress were obtained, removing the variation due to intrinsic individual differences that seems to be especially relevant for hybrid

and polyploid taxa (Te Beest et al. 2012) such as the four studied *Spartina* (Ainouche et al. 2012).

Fitness (%) was calculated for each taxon at each salinity as the population average of the percentages (x_i) of six key physiological functional traits and growth (F_v/F_m, Φ_{PSII} at both sunrise and noon, A, and leaf growth) in relation to the maximum population value (x_{max}) at any salinity (Dodd 2005).

$$\text{Fitness} = \sum_{i=1}^{5} \frac{x_i * 100}{x_{max}}$$

Statistics. Deviations of all data were calculated as the standard error of the mean (SE). All statistical analyses were carried out using Sigma-Plot for Windows version 14.0 applying a significance level (α) of 0.05 for every analysis. Two-way analysis of variance (ANOVA) with taxon and salinity as grouping factors were conducted to compare mean values for each plant trait and the fitness, and one-way ANOVA with taxon as a grouping factor to compare the three trait variability indexes. Prior to the use of the parametric models, data series were tested for normality with the Kolmogorov-Smirnov's test and for homogeneity of variance with the Levene's test. When an ANOVA was significant, Tukey's honestly significant difference (HSD) test was used as post hoc analysis. When homogeneity of variance or normality was not achieved, means were compared using a Kruskal-Wallis nonparametric ANOVA, with Bonferroni-Dunn's test as post hoc analysis. Pearson correlation coefficient and linear regression between inter-treatment and intra-population trait variability and the phenotypic plasticity index of the different traits for each taxon were calculated to analyze the relationships between trait variability among and within taxa. Correlation and regression analyses were also applied to explore the association between phenotypic inheritance and parental trait variability, calculating the relationships between the number of hybrid

individuals with transgressive or parental dominated traits and the variability indexes for the parental species for each trait.

4.3 Results

Phenotypic inheritance

A total of 33 out of the 37 evaluated plant traits changed with salinity for at least one taxon, while 35 traits showed differences between taxa in at least one salinity treatment level (see Appendix 4.E).

The parental species showed differences in the traits measured at the different salinity treatments. *Spartina densiflora* presented greater leaf size and LWC than *S. maritima* that showed higher SLA. Regarding the biochemical traits, *S. maritima* had a higher proline, C and N foliar content, and shoed higher salt excretion rate than *S. densiflora* in the presence of salt (10, 20 and 40 ppt salinity). However, leaf C:N ratio of *S. densiflora* was ca. 2 times higher than that of *S. maritima.* The pigments content (chlorophylls, carotenoids and anthocyanin) of *S. maritima* exceeded that of *S. densiflora* only at hypersalinity. Differences in Chl fluorescence between both species occurred mainly at the extremes of the salinity gradient (0.5 and 40 ppt), except for the higher F_0 at sunrise in all treatments and higher Φ_{PSII} at noon of *S. densiflora* at 10 ppt salinity. At hypersalinity, *S. maritima* showed higher Φ_{PSII} and F_v/F_m at sunrise and *S. densiflora* greater qP at sunrise and NPQ and F_m at sunrise and noon. At freshwater, *S. maritima* exhibited higher luminescence and F_0 and *S. densiflora* higher F_m at noon. Regarding the gas exchange, *S. maritima* showed higher WUE at 20 ppt salinity and higher A_{max} at 20-40 ppt salinity than *S. densiflora*. Finally, the apical growth of *S. densiflora* was maximum at 10 ppt salinity, being always superior to the constant growth rate of *S. maritima* (see Appendix 4.E).

The phenotypic inheritances at the population level of both hybrids for every trait and salinity are listed in Appendix 4.F. The traits dominated by the

parental *S. densiflora* were at least 30% greater at freshwater than at any elevated salinity concentration for *S. maritima x densiflora*. On the contrary, they were at least 40% higher at hypersalinity than at the rest of the salinity levels for *S. densiflora x maritima*. At lower salinity concentrations (0.5 and 10 ppt), both hybrids had a similar percentage of traits dominated by *S. densiflora*, but *S. densiflora x maritima* exhibited a higher dominance by *S. densiflora* than *S. maritima x densiflora* at higher salinity ranges (20 and 40 ppt). No particular traits were dominantly characteristic of *S. maritima* for the *S. densiflora x maritima* hybrid at freshwater (5% for *S. maritima x densiflora*) or for *S. maritima x densiflora* at hypersalinity (5% for *S. densiflora x maritima*). The dominance of *S. maritima* trait responses was higher for *S. maritima x densiflora* than for *S. densiflora x maritima* at the intermediate treatments (5% vs. 3% at 10 ppt salinity; 16% vs. 5% at 20 ppt salinity). The traits dominated by both parents (parental codominance) were lower at the extremes of the salinity gradient than at intermediate salinities (Fig. 4.1).

The traits showing intermediate values between both parents were between 11-19% for both hybrids at every salinity, except for *S. maritima x densiflora* at hypersalinity (43%) (Fig. 4.1).

Transgressive traits were more abundant at freshwater and hypersalinity than at the intermediate salinity levels (Fig. 4.1). At the population level, transgressive traits for *S. maritima x densiflora* were leaf C:N, A and A_{max} at freshwater, non-photochemical quenching (NPQ) (at sunrise) and maximum quantum efficiency of the Photosystem II (PSII) photochemistry (F_v/F_m) (at noon) at 10 ppt salinity, quantum efficiency of PSII (Φ_{PSII}) (at noon) at 20 ppt, and Φ_{PSII} (at noon), and G_s, C_i and WUE at hypersalinity. Transgressive traits at the population level for *S. densiflora x maritima* were G_s and A_{max} at freshwater, G_s at 10 ppt, and proline at hypersalinity. At the individual level, every hybrid showed a unique transgressive profile (see Appendix 4.G). Both hybrids had individuals with the same 9 transgressive traits at freshwater, 8 transgressive traits at 10 ppt salinity, 6 at 20 ppt, and 4 at hypersalinity. Six traits (SLA, LWC

and 4 traits related to the Chl fluorescence at sunrise) did not show any transgressive individual at any salinity. F_0 and Φ_{PSII} (at noon), A_{max} and G_s were the traits showing more transgressive individuals (> 14 indiv.).

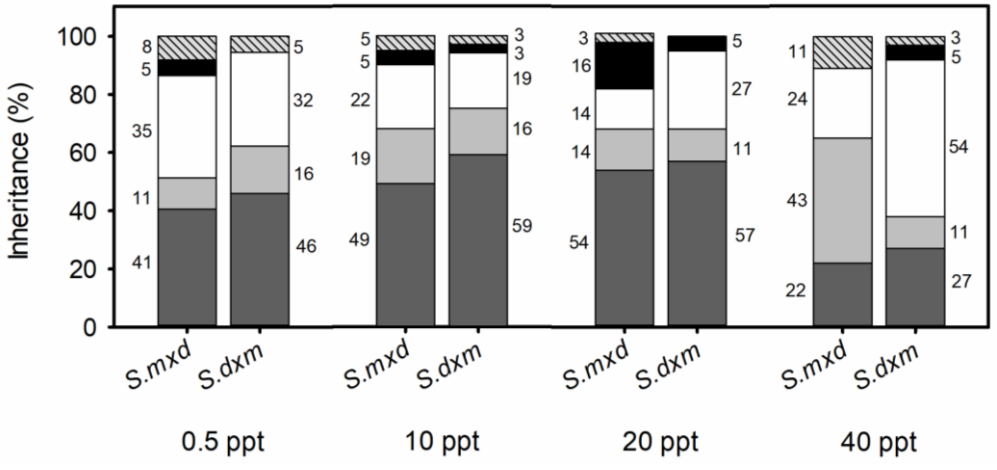

Fig. 4.1. Percentage of different phenotypic inheritances for 37 foliar traits for the hybrids *Spartina maritima x densiflora* (*Smxd*) and *S. densiflora x maritima* (*Sdxm*) at 0.5, 10, 20 and 40 ppt salinity. Inheritance types: parental codominance (dark gray); parental additivity (light gray); parental dominance of *S. densiflora* (white); parental dominance of *S. maritima* (black); transgressive (striped).

Traits variability

Average inter-treatment variability for every trait (*Sm:*61 ± 4%, *Smxd:*56 ± 5%, *Sdxm:*51 ± 4%, *Sd:*51 ± 4%), average intra-population variability (*Sm:*35 ± 4%, *Smxd:*34 ± 3%, *Sdxm:*32 ± 3%, *Sd:*30 ± 3%) and average phenotypic plasticity (*Sm:*26 ± 2%, *Smxd:*22 ± 2%, *Sdxm:*19 ± 2%, *Sd:*20 ± 2%) were similar for all taxa (One-way ANOVA: $P > 0.05$), except for PPI being higher for *S. maritima* than *S. densiflora x maritima* (One-way ANOVA:$H = 8.03$, $n = 37$, $P < 0.05$). Some leaf biochemical traits (such as free proline content and salt excretion rate), some Chl fluorescence and gas exchange traits, and leaf growth were the most variable traits (Appendix 4.H).

Inter-treatment and intra-population trait variability were positively correlated among every taxon. Additionally, PPI was also positively correlated among all taxa, except between *S. densiflora* and *S. maritima* (Table 4.1). PPI for initial fluorescence (F_0) and F_v/F_m (at noon) and F_0 and maximal fluorescence (F_m) (at sunrise) was higher for *S. maritima* than *S. densiflora*, and PPI for photochemical quenching (qP) (at noon) and Chl *b* content was higher for *S. densiflora* than *S. maritima* (Appendix 4.H).

Within each taxon, inter-treatment, intra-population trait variability and PPI correlated with each other, except PPI that was independent of intra-population trait variability for *S. maritima* (Table 4.1); the main traits determining this lack of correlation were NPQ (at sunrise), Ci and WUE, since they showed high PPI and low intra-population variability, and leaf length, leaf area, NPQ (at noon) and leaf growth that showed low PPI and high intra-population variability (Fig. 4.2).

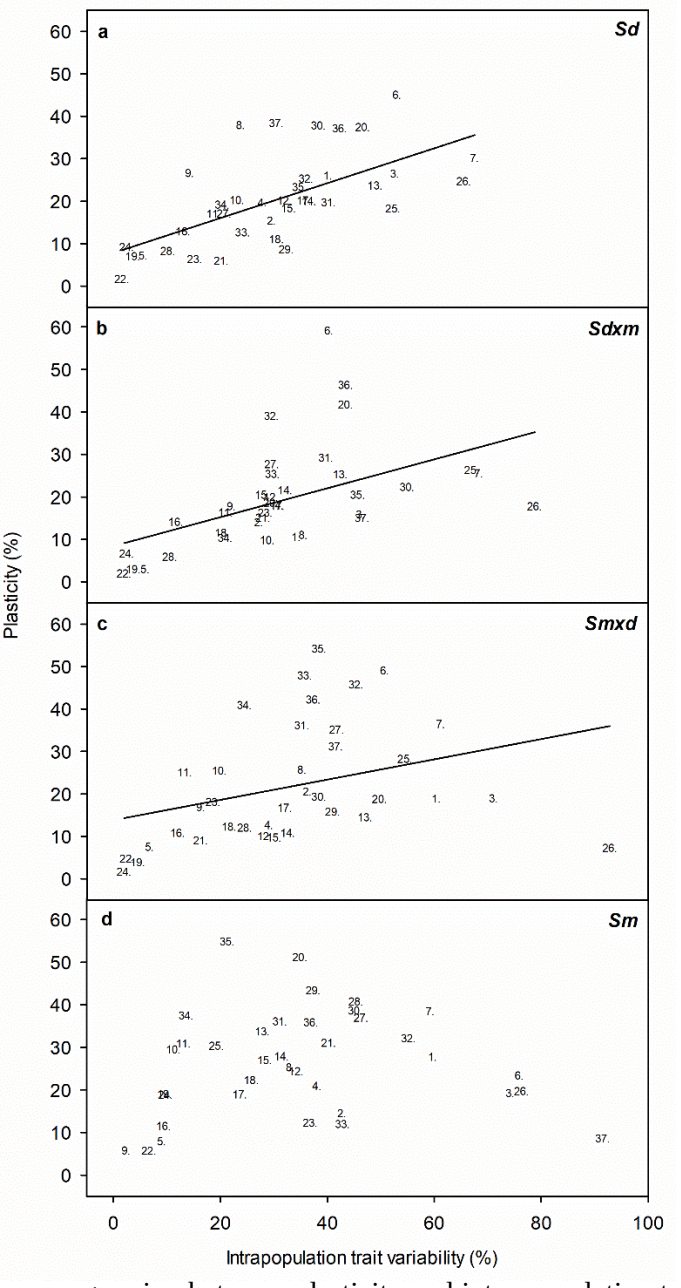

Fig. 4.2. Linear regression between plasticity and intra-population trait variability for 37 foliar traits measured in *S. densiflora* (*Sd*; a), *S. densiflora x maritima* (*Sdxm*; b), *Spartina maritima x densiflora* (*Smxd*; c) and *Spartina maritima* (*Sm*; d) at 0.5, 10, 20 and 40 ppt salinity. Linear regression models: *Sd*:y = 10.098 + 0.9964 x (R = -0.64, P < 0.001); *Sdxm*:y = 8.574 – 1.336 x (R = -0.91, P < 0.001); *Smxd*:y = 13.745 + 1.242 x (R = -0.87, P < 0.001). Traits: **1.** Leaf length **2.** Leaf width **3.** Leaf area **4.** Specific Leaf Area **5.** LWC **6.** Salt excretion rate **7.** Proline **8.** Malondialdehyde **9.** Leaf C **10.** Leaf N **11.** Leaf C:N **12.** Chl *a* **13.** Chl *b* **14.** Chl *a*+*b* **15.** Car **16.** Chl:Car **17.** Chl *a*:Chl *b* **18.** Anthocyanin **19.** qP (sunrise) **20.** NPQ (sunrise) **21.** F_0 (sunrise) **22.** F_v/F_m (sunrise) **23.** F_m (sunrise) **24.** ΦPSII (sunrise) **25.** qP (noon) **26.** NPQ (noon) **27.** F_0 (noon) **28.** F_v/F_m (noon) **29.** F_m (noon) **30.** ΦPSII (noon) **31.** Luminiscence **32.** A **33.** Gs **34.** Intercellular CO_2 concentration (Ci) **35.** WUE **36.** A_{max} **37.** Leaf apical growth.

Table 4.1. Pearson correlation coefficients and *P*-values for inter-treatment trait variability (Inter), intra-population trait variability (Intra) and phenotypic plasticity measured for 37 functional traits in 4 taxa (*Sdxm*, *S. densiflora x maritima Smxd*, *S. maritima x densiflora*; *Sd*, *S. densiflora*; *Sm*, *Spartina maritima*).

	Intra (*Sdxm*)	Plasticity (*Sdxm*)	Inter (*Smxd*)	Intra (*Smxd*)	Plasticity (*Smxd*)	Inter (*Sd*)	Intra (*Sd*)	Plasticity (*Sd*)	Inter (*Sm*)	Intra (*Sm*)	Plasticity (*Sm*)
Inter (*Sdxm*)	**0.913** **3.15E-15**	**0.806** **1.79E-09**	**0.85** **2.81E-11**	**0.809** **1.39E-09**	**0.544** **0.001**	**0.918** **1.32E-15**	**0.903** **2.09E-14**	**0.728** **3.35E-07**	**0.699** **0.000001**	**0.59** **0.0001**	**0.418** **0.001**
Intra (*Sdxm*)		**0.495** **0.002**	**0.806** **1.8E-09**	**0.866** **4.74E-12**	**0.379** **0.021**	**0.876** **1.30E-12**	**0.904** **1.78E-14**	**0.629** **0.00003**	**0.672** **0.00001**	**0.593** **0.0001**	**0.355** **0.031**
Plasticity (*Sdxm*)			**0.642** **0.00002**	**0.467** **0.004**	**0.611** **0.0001**	**0.685** **0.00001**	**0.613** **0.0001**	**0.639** **0.0001**	**0.515** **0.001**	**0.395** **0.015**	**0.377** **0.022**
Inter (*Smxd*)				**0.876** **1.19E-12**	**0.744** **1.28E-07**	**0.83** **2.13E-10**	**0.818** **6.59E-10**	**0.657** **0.00001**	**0.775** **1.83E-08**	**0.681** **0.00001**	**0.414** **0.011**
Intra (*Smxd*)					**0.331** **0.046**	**0.85** **2.97E-11**	**0.899** **3.95E-14**	**0.575** **0.0002**	**0.784** **9.36E-09**	**0.75** **9.08E-08**	0.312 0.0603
Plasticity (*Smxd*)						**0.448** **0.005**	**0.355** **0.031**	**0.489** **0.002**	**0.431** **0.008**	0.295 0.0765	**0.379** **0.021**
Inter (*Sd*)							**0.943** **2.84E-18**	**0.857** **1.36E-11**	**0.715** **6.66E-07**	**0.633** **0.0001**	**0.375** **0.022**
Intra (*Sd*)								**0.636** **0.0001**	**0.704** **0.000001**	**0.611** **0.0001**	**0.389** **0.017**
Plasticity (*Sd*)									**0.566** **0.0003**	**0.519** **0.001**	0.266 0.112
Inter (*Sm*)										**0.879** **8.03E-13**	**0.535** **0.001**
Intra (*Sm*)											0.0675 0.691

Relationships between phenotypic inheritance and trait variability

The number of individuals with transgressive inheritance for a given trait for each hybrid increased together with the PPI of both parental species (Pearson correlation coefficient, $P < 0.05$, n = 37). On the other hand, the number of transgressive individuals for *S. densiflora x maritima* increased also with the intra-population trait variability of *S. densiflora*, being independent of that of *S. maritima*. The number of transgressive individuals of *S. maritima x densiflora* was independent of the intra-population traits variability of both parents (Table 4.2; Fig. 4.3). The lack of correlation between the number of hybrids with transgressive traits and the intra-population trait variability of their parental species were due to some highly transgressive traits with low intra-population trait variability in both parents (F_0 and Φ_{PSII} (at noon), A_{max} and G_s), and to some traits with high intra-population variability in *S. densiflora* (proline content) and *S. maritima* (leaf length, leaf area, NPQ (at noon) and leaf growth) with low number of individuals with transgressive traits for both hybrids (Fig. 4.3). These traits of *S. maritima* were included in those breaking the correlation between the intra-population trait variability and the PPI of *S. maritima* (Figs. 4.2 and 4.3).

The number of hybrid individuals with traits dominated by any of the parents was independent of the intra-population trait variability and the PPI of each parent, except the individuals of *S. densiflora x maritima* with traits dominated by *S. densiflora* that decreased together with the PPI of *S. densiflora* (Table 4.2).

Table 4.2. Pearson correlation coefficients and *P*-values for intra-population trait variability (Intra) and phenotypic plasticity measured for 37 foliar traits in the parental taxa (*Sd, S. densiflora*; *Sm, Spartina maritima*) in relation with the number of hybrid (*Sdxm, S. densiflora x maritima*; *Smxd, S. maritima x densiflora*) individuals with transgressive behavior (T) or dominated by one of the parental species (D) for each trait. Significant correlations (P < 0.05) are marked in bold.

	# T (*Sdxm*)	# T (*Smxd*)	# D-*Sd* *(Sdxm)*	# D-*Sd* (*Smxd*)	# D-*Sm* (*Sdxm*)	# D-*Sm* (*Smxd*)
Intra (*Sd*)	**0.373** **0.023**	0.249 0.137	0.153 0.366	0.187 0.269	-0.00868 0.959	0.0117 0.945
Plasticity (*Sd*)	**0.429** **0.008**	**0.380** **0.020**	**-0.331** **0.046**	-0.231 0.169	-0.186 0.271	-0.231 0.17
Intra (*Sm*)	0.321 0.0526	0.197 0.243	-0.174 0.303	-0.136 0.423	0.0532 0.755	0.0735 0.666
Plasticity (*Sm*)	**0.377** **0.022**	**0.516** **0.001**	-0.04 0.814	-0.0468 0.783	-0.154 0.362	-0.169 0.317

Fitness

The fitness was 80 ± 1% for *Spartina maritima x densiflora* and 76 ± 1% for *S. densiflora x maritima* at freshwater (0.5 ppt salinity), being significantly higher than for the rest of the salinity treatments, except for *S. densiflora x maritima* at 10 ppt salinity. Fitness at 10 ppt salinity was higher than at hypersalinity (40 ppt salinity) for *S. maritima x densiflora* and higher than 20 ppt salinity for *S. densiflora x maritima*. For the parental species, the fitness of *S. densiflora* was higher at freshwater (76 ± 2%) than at hypersalinity (61 ± 1%) and higher at 10 ppt (79 ± 1%) than at 20 ppt (71 ± 1%) and 40 ppt, while *S. maritima* showed its maximum fitness at 20 ppt salinity (63 ± 2%), being higher than at freshwater (53 ± 3%) and hypersalinity (56 ± 2%). *S. maritima x densiflora*, *S. densiflora x maritima* and *S. densiflora* showed ca. 15% higher fitness than *S. maritima* at every treatment, except at hypersalinity. At hypersalinity, both parents showed similar fitness and lower than those of both hybrids (Two-way ANOVA, taxa x salinity: $F_{9,79}$= 8.500, P < 0.001; Fig. 4.4).

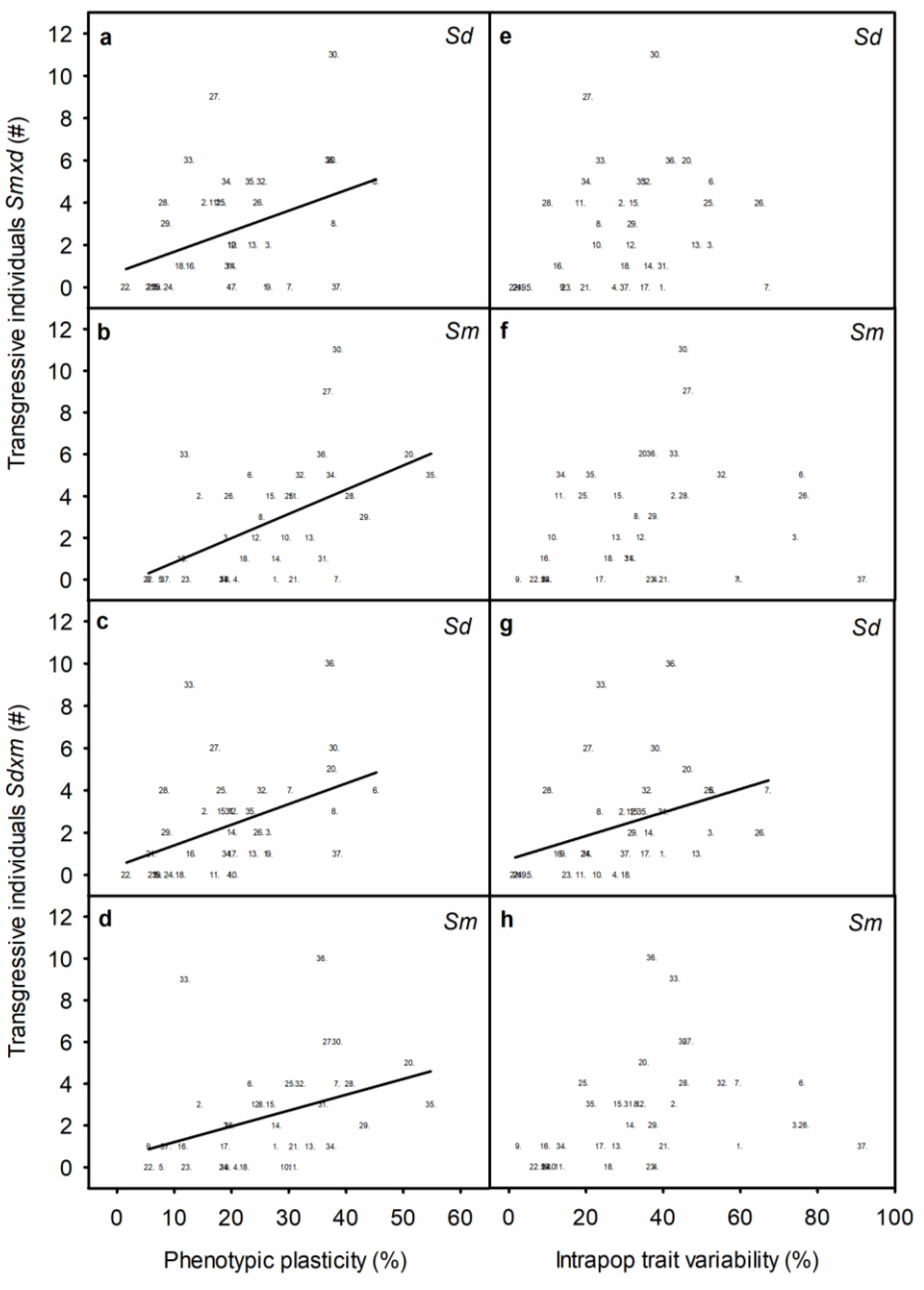

Fig. 4.3. Linear regression between phenotypic plasticity and intra-population trait variability of parental species versus the number of hybrid individuals with transgressive traits for 37 foliar traits (listed in Fig. 4.2) measured in *Spartina maritima* (*Sm*), *S. densiflora* (*Sd*), and their hybrids *Spartina maritima x densiflora* (*Smxd*) and *S. densiflora x maritima* (*Sdxm*) at 0.5, 10, 20 and 40 ppt salinity. Linear regression models: a:$y = 0.720 + 0.0976\ x$ (R = 0.38, $P < 0.05$); b:$y = -0.344 + 0.116\ x$ (R = -0.52, $P < 0.001$); c:$y = 0.440 + 0.0981\ x$ (R = 0.43, $P < 0.01$); d:$y = 0.760 + 0.0551\ x$ (R = -0.37, $P < 0.05$); g:$y = 0.453 + 0.0756\ x$ (R = 0.38, $P < 0.05$).

4.4 Discussion

Our results show that the phenotypic inheritance of hybrids is determined by a complex combination of different processes. Ploidy level, maternal effects and the phenotypic plasticity of the parental species are all influential processes. Also, the biochemical, physiological and morphological responses of hybrids related to their phenotypic inheritances are modulated by the abiotic environment.

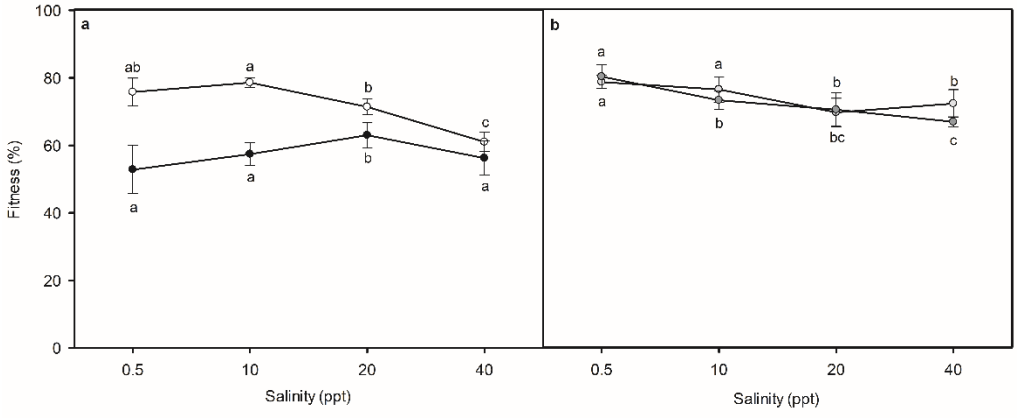

Fig. 4.4. Percentage of fitness (measured as the mean of 6 fitness-related traits) of *Spartina maritima* (●), *S. densiflora* (○) (a) and their hybrids *S. maritima x densiflora* (◉) and *S. densiflora x maritima* (◉) (b) at 0.5, 10, 20 and 40 ppt salinity. Values are mean ± SD (n = 5).

Traits variability

Inter-treatment trait variability increased together with both intra-population trait variability and phenotypic plasticity for all taxa, confirming that both are components of the former. Moreover, intra-population trait variability and phenotypic plasticity increased together for *S. densiflora* and both hybrids, so both components of trait variability seem to be regulated by the same mechanisms in these three taxa. Castillo et al. (2018) found the same positive relation between phenotypic plasticity and intra-population trait variability for

invasive populations of *S. densiflora* in North America. Intra-population trait variability itself is the expression of the variability between the different genotypes of the population, and phenotypic plasticity may change through genetic and epigenetic mechanisms (Salmon et al. 2005, Crispo 2008) and therefore be inherited. In fact, both hybrids exhibited phenotypic plasticities that varied among traits like the phenotypically plasticities of both parental species, pointing to the heritability of phenotypic plasticity. On the contrary, the phenotypic plasticity of traits exhibited by the parental species did not show correlation between both taxa, reflecting independent evolution processes in response to contrasted environments since *S. maritima* was sampled from low marshes and *S. densiflora* from middle marshes. On the other hand, no relationship was found between intra-population trait variability and phenotypic plasticity for *S. maritima*, which is consistent with phenotypic plasticity being a target of natural selection that also may evolve itself with environmental changes (Pigliucci 2001). The variables that broke the correlation between intra-population variability and phenotypic plasticity for *S. maritima* were different from those breaking the correlation between the phenotypic plasticity of *S. maritima* and *S. densiflora*, showing the contrasted responses of the parental species to salinity. In this sense, it has been described that invasive taxa usually show high phenotypic plasticity that allows them to colonize highly diverse and changing environments, favoring their invasive ability (Richards et al. 2006, Caño et al. 2008, Drenovsky et al. 2012). Thus, high phenotypic plasticity (40%) in response to salinity was reported for four invasive populations of *S. densiflora* from a broad latitudinal gradient along the west coast of North America (Grewell et al. 2016), similar to our inter-population trait variability (calculated the same way) for *S. densiflora* (51%). Also, hybridization has been related to high levels of phenotypic plasticity that increases the amplitude of the niche occupied by these taxa (Ainouche and Jenczewski 2010, Te Beest et al. 2012, Cara et al. 2013). Thus, we found relatively high phenotypic plasticity for both *Spartina* hybrids and for the invasive *S. densiflora* with an ancient hybrid origin

(Fortune et al. 2008). However, native *S. maritima* showed similar levels of phenotypic plasticity as did both of its hybrids and *S. densiflora*, which may be related to the highly fluctuating salinity levels and other stress factors in the abiotic environment in their low salt marsh habitat (Contreras-Cruzado et al. 2017) since high environmental variability frequently leads to high adaptive phenotypic variability (Schlichting 1986, Schmid 1992, Steinger et al. 2003). High and heritable phenotypic plasticity was found for the stable species *Fallopia japonica* (Houtt.) Ronse Decr. in relation to the hybrid *F. x bohemica* (Chrtek & Chrtková) J.P. Bailey, which was attributed to epigenetic changes between clones (Parepa et al. 2014).

Phenotypic inheritance

Dominant inheritance and parental additivity were recorded for both *Spartina* hybrids. *S. densiflora x maritima* showed more dominant characters from its maternal species at high salinity levels, whereas *S. maritima x densiflora* inherited more dominant traits from its maternal species at low salinity and also especially at 20 ppt salinity, where *S. maritima* presented its greatest fitness. Maternal effect, frequent in first-generation hybrids as those studied in this work, may affect gene expression promoting differences between hybrids that can be crucial for the divergent evolution of their tolerance to abiotic stress (Burgess and Husband 2004, Kimball et al. 2008, Favre and Karrenberg 2011).

Both *Spartina* hybrids showed their highest percentage of foliar transgressive traits at both extremes of the salinity gradient (freshwater and hypersalinity), whereas the trait responses resulting from the codominance of both parental species predominated at intermediate salinity levels. Transgressive traits in relation to salt tolerance have been previously reported in several hybrid taxa such as the hybrids between *Silene dioica* (L.) Clairv. and *S. latifolia* Poir. (Favre and Karrenberg 2011) and the hybrid sunflower *Helianthus paradoxus* Heiser (Lexer et al. 2003b).

Hybridization between native and invasive *Spartina* species has been frequent: The highly invasive and plastic allododecaploid *Spartina anglica* C.E. Hubb. (2n = 12x = 120) arose after the introduction of *Spartina alterniflora* Loisel. (2n = 6x = 60) from the Atlantic Coast of North America in European marshes, where it hybridized with *S. maritima* (2n = 6x = 60) forming firstly two different and independent sterile F1 hybrids, the vigorous *Spartina x townsendii* H. & J. Groves (2n = 6x = 60; 2n = 9x = 90) in England (the hybrid predecessor of *S. anglica*) and *Spartina x neyrautii* Foucaud (2n = 6x = 60) in France. Additionally, introduced *S. alterniflora* in San Francisco Bay hybridized with *Spartina foliosa* Trin. (2n = 6x = 60), native to California, forming a hybrid swarm of very plastic, invasive and fertile plants (2n = 6x = 60) (Strong and Ayres, 2013). On the other hand, the ancient hybrid *S. densiflora* from South America hybridized with *S. foliosa* also in San Francisco Bay forming the reciprocal sterile hybrids *S. densiflora x foliosa* (2n = 6.5x = 65) and *S. alterniflora x foliosa* (2n = 9.5x = 95) that outperformed their parental species for different traits at extreme levels of salinity (Pakenham-Walsh et al. 2010). In the same way, introduced *S. densiflora* in Southwest Iberian Peninsula hybridized with *S. maritima* to form both hybrids studied in this work.

Comparing both hybrids, *S. maritima x densiflora* was transgressive in a greater number of foliar traits than *S. densiflora x maritima*, which may be related to the different ploidy level of both hybrids (*S. maritima x densiflora*:2n = 95 chromosomes and *S. densiflora x maritima*:2n = 65 chromosomes, following Castillo et al. 2010). A higher number of chromosomes and ploidy level usually leads to increased invasiveness through heterosis (Comai 2005, Pandit et al. 2014). Also, heterosis has been previously reported to be different among reciprocal hybrids with the same ploidy level, which have been related to parent-of-origin effects in *Arabidopsis* (Miller et al. 2012) and/or dosage effects in *Zea mays* L. (Yao et al. 2013). Transgressive traits for both *Spartina* hybrids were mainly related to gas exchange and Chl fluorescence, especially the efficiencies of PSII. Ni et al. (2009) observed that epigenetic alterations of

the circadian rhythm in allotetraploids between *Arabidopsis thaliana* (L.) Heynh. and *A. arenosa* (L.) Lawalrée gave rise to increased photosynthetic efficiency leading to higher growth rates and heterosis.

Parental divergence and heterosis

At the molecular level, heterosis is driven by non-additive expression of some key genes regulated genetically (dominance, overdominance and pseudo-overdominance) or epigenetically (reviewed in Chen 2013). In general, higher levels of heterosis in hybrids are found when there is a greater genetic difference between the parental taxa (East 1936, Chen 2010). For example, heterosis of *A. thaliana* crosses have been attributed to their parents showing their optimal performance at different environmental conditions that combined beneficially in the offspring (Kang 1997). *S. maritima* inhabits in low salt marshes, with optimum growth observed at intermediate levels of salinity (Naidoo et al. 2012), whereas *S. densiflora* growth is optimum at low salinity (Grewell et al. 2016). Hexaploid *Spartina* species (including *S. maritima*) colonize low marshes, whereas tetraploids are high marsh species (Ainouche et al. 2009). The heptaploid *S. densiflora* is of hybrid origin (Fortune et al. 2008) deriving from a hexaploid species and a (maternal) tetraploid ancestor that diverged sometimes 6-10 MYA (Rousseau-Gueutin et al. 2015). Additionally, the lack of correlation between the phenotypic plasticity of *S. maritima* and *S. densiflora* also suggest differentiation between the two parents, probably favoring the heterosis of their hybrids.

But our study goes further, demonstrating that when the parents themselves show a more plastic response for a given trait, there is a greater chance that the hybrid will develop a transgressive behavior for this trait. This relationship seems to be mediated by epigenetic changes since both phenotypic plasticity and heterosis are regulated at an epigenetic level (Parepa et al. 2014) and important epigenetic changes recorded after the hybridization between

Spartina taxa have been associated with high levels of phenotypic plasticity (Salmon et al. 2005, Parisod et al. 2009). On the other hand, the number of transgressive hybrids for a given trait in our study was independent of the intra-population trait variability of both parents, except for *S. densiflora x maritima* and its maternal species. Maternal effect may determine heterosis in hybrids due to maternal influence in the regulation of gene expression at transcriptional and post-transcriptional levels (Guo et al. 2003, Auger et al. 2004, Mosher et al. 2009). The maternal effect on transgressive traits recorded for *S. densiflora x maritima* was supported by a decrease in its number of traits dominated by *S. densiflora* with increasing phenotypic plasticity of *S. densiflora*.

The more frequent foliar transgressive traits of the hybrids at both extremes of the salinity gradient (freshwater and hypersalinity) coincided with their higher fitness in relation to one or both parents. Thus, both *Spartina* hybrids showed high tolerance to salt stress, presenting their maximum fitness at freshwater with a slight decrease with increasing salinity. Both hybrids exhibited higher fitness than *S. maritima* from freshwater to hypersalinity and higher than *S. densiflora* at hypersalinity. Previous studies have found that mechanistic physiological traits related to fitness such as gas exchange and Chl fluorescence, as well as overall growth decreased for *S. maritima* at both freshwater and high salinities (Naidoo et al. 2012), whereas *S. densiflora* growth and performance responses were severely limited at hypersalinity and trait responses were optimum in brackish conditions (Grewell et al. 2016). These studies add to the thinking that polyploidy and hybridization may lead to the expression of novel phenotypes with increased fitness (Jackson 2017).

4.5 Conclusions

The greater tolerance of both *Spartina* hybrids to salinity than their parental species highlights the relevance of heterosis in hybridization processes. These hybrids currently maintain limited distribution in the salt marshes of the Southwest Iberian Peninsula due to their infertility (Castillo et al. 2010a), but if a chromosomal duplication occurs in the hybrids, the new allopolyploids could become fertile (Dobzhansky 1933, Rieseberg 2001) and lead to a permanent heterosis fixation (Iehisa and Takumi 2012) increasing their capacity of invasion. Allopolyploidization has been previously documented in the genus *Spartina* for *S. anglica*, a polyploid of hybrid origin that is highly invasive (Huskins 1930, Thompson 1991, Ainouche et al. 2004). Given the consequences of hybridization for increased invasiveness, the eradication of the studied *Spartina* hybrids is an urgent concern for conservation and recovery of tidal wetland plant communities.

The characteristics of the parental species that determine heterosis in their hybrids are poorly understood. Our study reveals new data on the direct relationship between phenotypic plasticity of parents and transgressive responses of hybrids. Our results are relevant to understand the important adaptive role of interspecific hybridization in natural and potentially invasive populations. These findings in the *Poaceae* family, which include agriculturally important species such as wheat and barley, support a new focus to be applied for the artificial development of vigorous hybrid crops.

CHAPTER V

Changes on the functional traits of phosphoenolpyruvate carboxylase following hybridization in C4 halophytes

CAPÍTULO 5: Cambios en rasgos funcionales de la enzima fosfoenolpiruvato carboxilasa tras la hibridación en halófitas C4

Resumen

La hibridación interespecífica es un mecanismo evolutivo importante asociado a la invasividad de las especies vegetales, pero se conoce poco de su efecto en las actividades enzimáticas como respuesta a factores ambientales estresantes. Los taxones vegetales que resultan de la hibridación natural suponen una oportunidad para el estudio de mecanismos evolutivos *in situ*. Analizamos los efectos de la salinidad en rasgos mecanicistas de la enzima fosfoenolpiruvato carboxilasa (PEPC), que controla la fotosíntesis eficiente en plantas C4, en dos taxones híbridos de *Spartina maritima* y *S. densiflora* en comparación con sus especies parentales. También se registraron los distintos tipos de herencia que dieron lugar a los diferentes comportamientos híbridos. Las especies parentales mostraron estrategias contrastadas a nivel de la PEPC para hacer frente a la salinidad. El óptimo fisiológico de la nativa *S. maritima* fue de 10 a 40 g/L de salinidad, con constante y alto contenido de PEPC y actividad específica, en contraste con el menor óptimo de salinidad a 0.5 y 10 g/L para la exótica *S. densiflora*, cuyo máximo nivel de actividad específica coincidió con la alta activación inducida por luz de la PEPC. Ambos híbridos mostraron niveles constantes de actividad específica de la PEPC desde agua dulce a hipersalinidad y exhibieron mayor tasa de fotosíntesis neta en agua dulce que sus padres. *S. maritima x densiflora* presentó tres caracteres funcionales relacionados con la PEPC diferentes a ambos parentales debido a la segregación transgresiva, siendo el único taxón capaz de aumentar el nivel de activación de la PEPC (IC_{50}) en oscuridad y en luz a alta salinidad. Por el contrario, *S. densiflora x maritima* mostró la mayoría de los rasgos intermedios entre sus especies parentales. Los resultados de este estudio revelan que los tipos de herencia, operando de manera diferente en híbridos recíprocos, determina caracteres funcionales claves condicionando su respuesta a factores ambientales.

CHAPTER 5: Changes on the functional traits of phosphoenolpyruvate carboxylase following hybridization in C4 halophytes

Abstract

Interspecific hybridization is a relevant evolutionary mechanism linked to the invasiveness of plant species, but little is known about its effect on enzymatic activities in response to environmental stress factors. Plant taxa resulting from natural hybridization represent an opportunity for the study of evolutionary mechanisms *in situ*. We analyzed the effects of salinity on key mechanistic traits of phosphoenolpyruvate carboxylase (PEPC) enzyme that drives efficient photosynthesis in C4 plants, for two hybrid taxa derived from *Spartina maritima* and *S. densiflora* in comparison with their parental species. The type of inheritances that led to different hybrid behaviors were also recorded. Parental species showed contrasted strategies at the PEPC level to cope with salinity. Native *S. maritima* showed its physiological optimum at 10 to 40 ppt salinity, with constant high PEPC content and specific activity, in contrast to the lower salinity optimum of 0.5 and 10 ppt for the alien *S. densiflora*, where highest levels of PEPC specific activity coincided with high light-induced activation of PEPC. Both hybrids showed constant PEPC specific activity from fresh water to hypersalinity and exhibited higher net photosynthesis rates in fresh water than their parents. *S. maritima x densiflora* presented three functional PEPC-related traits different from its both parental species due to transgressive segregation, being the only taxon able to increase its PEPC activation level (IC_{50}) in darkness and light at high salinity. On the contrary, *S. densiflora x maritima* showed most PEPC-related traits intermediate between its parental species. The results of this study reveal that inheritance types operating differently in reciprocal hybrids determine key functional traits conditioning their ecological performance.

5.1 Introduction

Interspecific hybridization is a frequent and relevant evolutionary mechanism (Arnold 1992, 1997). Plant taxa resulting from natural hybridization represent an opportunity for the study of evolutionary mechanisms *in situ* (Abbott 1992, Ellstrand and Schierenbeck 2000). The anthropogenic movement and introduction of plant species to new environments has provided increased opportunities for hybridization to occur, and has led to the recognition of hybridization as an evolutionary pathway to increased invasiveness of plant species (Ellstrand and Schierenbeck 2000, Schierenbeck and Ellstrand 2009). Plant hybrid taxa may be successful invaders due to their greater tolerance to novel or extreme environmental conditions than resident flora as a result of the process of heterosis or 'hybrid vigor' that leads to transgressive traits (Comai 2005, Chen 2013). Heterosis takes place just in one generation that is even more rapidly than most rapid adaptive evolution processes in response to local environments, speeding up evolution. The invasion of fertile taxa may be facilitated by rapid evolution as a response to local conditions (Molina-Montenegro et al. 2018). However, in the case of first generation (F1) infertile hybrids this process is not possible. In this case, the evolutionary changes in the expression of different traits occur directly due to the hybridization process and contribute to the evolution of their invasiveness. In this context, greater genetic distances between parents have been related to the development of heterosis in their hybrids (Stelkens and Seehausen 2009), so local adaptation of parental species to contrasted habitats may promote hybrid vigor. In addition to a non-additive inheritance by which the characters of the hybrids are different from both parents, when hybridization occurs, other mechanisms can influence functional and mechanistic traits of the hybrids and thus their ecological performance. Hybrids can also show parental-like traits by the dominance of one or both parental species or intermediate values between the two parent

species when an additive inheritance takes place (Favre and Karrenberg 2011, Chen 2013, Fridman 2015).

Mechanistic traits with clear physiological functions can underlie and explain broad patterns of evolution and ecology in plants (Brodribb 2017). Despite the importance of hybridization as an evolutionary mechanism and its wide distribution among plants, little is known about its consequences for key enzymatic processes involved in carbon fixation during photosynthesis in non-model hybrids that establish and grow in natural environments. In contrast, many studies of enzymatic activity have been performed for synthetic commercial hybrids of species with agricultural value in relation to their greater fitness (Bhatt et al. 1979, Joshi et al. 1986, De Gara et al. 2000) and their tolerance to different environmental factors such as pests (Kluge et al. 2017), drought (Silva et al. 2017), salt stress (Meloni et al. 2003), deficiency of nutrients (López-Millán et al. 2000) or extreme temperatures (Payton et al. 2001, Yuan et al. 2011).

One of the main enzymes involved in photosynthesis of plants with C-4 photosynthetic pathway is the phosphoenolpyruvate carboxylase (PEPC; EC4.1.1.31), which has a key role in atmospheric CO_2 fixation as it rapidly shuttles CO_2 from mesophyll cells and concentrates it in bundle sheath cells (Osborne and Saack 2012, Hatch 1992). PEPC is an ubiquitous cytosolic enzyme in higher plants that catalyzes the reaction of irreversible β-carboxylation by which phosphoenolpyruvate (C-3) in the presence of HCO_3^- and Mg^{2+} produces oxaloacetate (C-4) and inorganic phosphate (Pi) (Chollet et al. 1996). PEPC has evolved polyphyletically, having originated several times independently, and changes in the kinetic and regulatory properties of the enzyme and the expression of C-4 PEPC genes have occurred during the evolution of C-4 photosynthesis (Gowick and Westhoff, 2011). Previous studies have shown that hybrids of maize, a C-4 crop species, presented high tolerance to abiotic stresses such as drought and salinity related to less inhibited expression of the PEPC (Nemat Alla and Hassan 2012, Sicher et al. 2015).

Salinity is one of the main abiotic factors determining the distribution of plant species in coastal ecosystems (Callaway et al. 1990, Pennings et al. 2005); as well as a threat for agricultural output all around the world (Flowers and Yeo 1995, Munns 2002, Shahbaz and Ashraf 2013). Physiological and biochemical changes induced by salinity are fundamental for the salt tolerance of species (Zhu 2001, Munns and Tester 2008). Maintaining PEPC activity at increasing concentrations of salinity is essential for the tolerance of C-4 halophytes to the saline environments in which they inhabit (Meinzer and Zhu 1999, Leisner et al. 2010). Salt stress may also affect photosynthesis by giving rise to a reduction in carbon fixation in order to limit water uptake under low water potential conditions, and effect of specific ions concentration or changes in the activity of essential enzymes in photosynthetic plant tissues (Munns 1993, Zhu 2003, Leisner et al. 2010). The evolution of salt tolerance seems to be dominated in angiosperms by independent gains near the tips of the phylogeny in many families, showing high evolutionary lability (Moray et al. 2015). The emergence of transgressive hybrids with high salinity tolerances fits within this evolutionary model.

In the salt marshes of the Gulf of Cadiz (southwest Iberian Peninsula), the presence of two genetically different halophytic C-4 hybrids between the native small cordgrass *Spartina maritima* (Curtis) Fernald (Poaceae; 2n = 6x = 60) and the invasive South American neophyte *Spartina densiflora* Brongn. (2n = 7x = 70) provides an opportunity to examine the consequences of hybridization on PEPC activity and its tolerance to salt stress. One of these hybrids is *S. maritima x densiflora* (2n = ca. 95) that is present at low marshes close to its maternal species, *S. maritima,* from which it inherits two chromosomic sets (unreduced gamete). The other is *S. densiflora x maritima* (2n = ca. 65) which is abundant at middle elevations along the intertidal gradient as is its maternal species. Transgressive traits related to hybrid vigor have been previously described for these hybrids in relation to their taller tillers and greater growth rates in the field. The presence of these hybrids have been reported in

three estuaries along the coast of the Gulf of Cadiz, being less than ten individuals in the estuaries of Tinto, Odiel and Piedras Rivers and hundreds of them in the Guadiana Estuary. These exotic hybrid cordgrasses have colonized salt marsh habitats affected by natural and artificial fresh water runoff and can grow all along the entire intertidal elevation gradient. In contrast, *S. maritima* is absent from higher elevation marsh zones and *S. densiflora* is absent from lower elevations (Castillo et al. 2000, 2010a). Additionally, both *Spartina* hybrids are sterile (Castillo et al. 2010a). *Spartina* species are anemophilous and their seeds are dispersed by tides and currents floating on water (Xiao et al. 2016). Other studies have described that hybrids between *Spartina alterniflora* Liosel., *Spartina foliosa* Trin. and *S. densiflora* showed higher salinity tolerance than their parental species (Lee et al. 2016), but nothing is known about the PEPC activity of any *Spartina* hybrid or any other wild hybrid.

The aim of this study was to analyze the effects of salinity on main functional traits related to PEPC for the exotic hybrids *S. maritima x densiflora* and *S. densiflora x maritima* in relation to their parental species, and the inheritances that lead to different hybrid behaviors. With this aim, an experiment was designed in which four taxa (the two hybrids and their two parental *Spartina* species) were subjected to four salinity treatments from fresh water to hypersalinity in order to investigate: (1) PEPC specific activity and *in vivo* phosphorylation degree of the enzyme in extracts of light- and dark-acclimated leaves; (2) the inheritance of main functional traits related to PEPC to elucidate the relation of the performance between the hybrids and their parents; (3) net photosynthesis rate (A) at ambient conditions, and maximum net photosynthesis rate (A_{max}) in a CO_2 saturated atmosphere, in order to study the carbon fixation at different CO_2 concentrations; and (4) the content of free proline in leaves as an indicator of salt stress (Barnett and Naylor 1966, Bates et al. 1973). The fitness of the studied cordgrasses, growing in salt marshes where salinity is one of the main abiotic stressors, would notably depend on the tolerance of their C-4 photosynthetic pathway, and particularly of PEPC, to osmotic stress. Thus, the

inheritance behavior of traits related to PEPC functioning in the hybrids in comparison with their parental species may be crucial for their invasive ability. We hypothesized that the parental species would present specific responses in PEPC traits since they colonize contrasted habitats along the intertidal gradient and that their hybrids would show greater tolerance to salt stress than both parents through an enhanced performance of PEPC activity as 'hybrid vigor' has been reported for other functional traits in these hybrids (Castillo et al. 2010a).

5.2 Material and Methods

Plant collection and experimental design

Below-ground biomass (rhizomes and attached roots) of 5 individuals of each taxon (*Spartina maritima*, *Spartina densiflora*, *S. maritima x densiflora* and *S. densiflora x maritima*) were collected in the San Bruno Marsh (Guadiana River Estuary, see *Chapter 2*) in November 2015. The plant material was obtained from both extremes of the intertidal gradient: *S. maritima* and the hybrid *S. maritima x densiflora* from the low marsh and *S. densiflora* and *S. densiflora x maritima* from the middle-high marsh, where they are more abundant (Castillo et al. 2010a) (Fig. 5.1). Experimental design is detailed in *Chapter 4*.

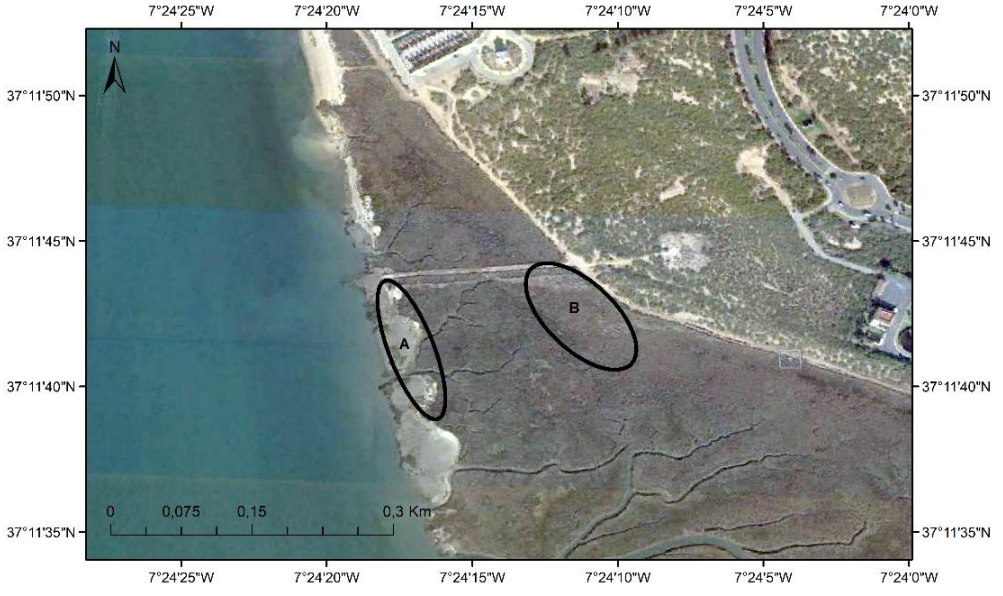

Fig. 5.1 Aerial image (Source: Google Earth 2018 [online]; Image date: 18-11-2017 [Accessed 3 July 2018]) of San Bruno Marsh (Guadiana Estuary, Southwest Iberian Peninsula) indicating the sampling points for (A) *Spartina maritima* and the hybrid *S. maritima x densiflora* at a low marsh area, and (B) *Spartina densiflora* and *S. densiflora x maritima* in a middle marsh area.

PEPC activity, inhibition by L-malate and total proteins

Plants were exposed to 2 h of direct sunlight at midday and they were also kept in the dark for 2 h (n = 3) since PEPC in C-4 plants is regulated by light dependent phosphorylation (Vidal and Chollet 1997) that increases the affinity with the allosteric activator glucose-6-phosphate and decreases the sensitivity to the inhibitor L-malate (Echevarría et al. 1994). Light-acclimated leaves from the different salinity treatments (0.5, 10, 20 and 40 ppt), and dark- acclimated leaves from fresh water (0.5 ppt) and hypersalinity (40 ppt), were immediately frozen and stored at -20 ºC. To extract PEPC, 0.2 g fresh leaf tissue were ground in a chilled mortar with 1 ml extraction buffer containing 0.1 M Tris-HCl pH 7.5, 20% (v/v) glycerol, 1 mM EDTA, 10 mM $MgCl_2$ and 14 mM β-mercaptoethanol. The homogenate was centrifuged at 15,000 g for 2 min and

the supernatant was used immediately to determine the activity and sensitivity of PEPC to L-malate, as described below. PEPC activity was measured spectrophotometrically at the optimal and suboptimal pH values of 8.0 and 7.3 respectively, using the NAD-malate dehydrogenase coupled assay at 2.5 mM phosphoenolpyruvate (PEP) (Echevarría et al. 1994). Assays were initiated by addition of an aliquot of crude extract (n=3). An enzyme unit (U) was defined as the amount of PEPC that catalyzes the carboxylation of 1 μmol of PEP min^{-1} at pH 8 and 30º C. Malate sensitivity was determined at suboptimal pH 7.3 in the presence or absence of various concentrations of L-malate (IC_{50}, 50% inhibition of initial PEPC activity by L-malate). A high IC_{50} is related to a high degree of PEPC phosphorylation (Echevarría et al. 1994). Total protein amounts were determined using the method of Bradford (1976), with bovine serum albumin (BSA) as standard and PEPC activity was expressed as units per g of protein (specific activity).

Inheritance

Inheritance of seven functional traits related to the enzyme PEPC operating for the hybrid taxa were analyzed: (1) PEPC specific activity; (2) level of activation of PEPC by phosphorylation for illuminated leaves (IC_{50} light); (3) level of activation of PEPC for dark-acclimated leaves (IC_{50} darkness); (4) change in the level of activation of PEPC for illuminated leaves in relation to dark-acclimated leaves (IC_{50} light vs. darkness); (5) change in the level of activation of PEPC from fresh water to hypersalinity for illuminated leaves (IC_{50} 0.5 ppt vs. 40 ppt light); (6) change in the level of activation of PEPC from fresh water to hypersalinity for dark-acclimated leaves (IC_{50} 0.5 ppt vs. 40 ppt darkness); (7) compensatory mechanism (increasing IC_{50} to compensate low amounts of PEPC). A dominant inheritance (D) was attributed when the trait for one hybrid was equal to one of the parents (D-Sm for *S. maritima*; D-Sd for *S. densiflora*); parental codominance (D-Sm,Sd) when it was the same as both parents; parental

additivity (I) when it was intermediate between both parents; and transgressive segregation (T) when it was different to both parental species. These inheritances were quantified as percentages for each hybrid taxon.

Photosynthesis rate, proline content and growth rate

Photosynthesis measurements were obtained by using an infrared gas analyzer (IRGA) in an open circuit (LI-6400, Li-COR Inc., Nebraska, USA) and a Clark type oxygen electrode (Leaflab 2 System, Hansatech Instruments Ltd, Norfolk, England). The IRGA was used to determine the net photosynthesis rate (A) at fixed 400 ppt CO_2 concentration, 15-20 ºC, 36 ± 1% relative humidity, a PPFD of 1000 μmol photon m^{-2} s^{-1} and a flow rate set to 350 μmol s^{-1}. The oxygen electrode was employed to measure maximum photosynthesis rate (A_{max}) by providing a saturated atmosphere of CO_2 with a 1 M carbonate / bicarbonate buffer (pH 9.0) at 25 °C. In this way, A_{max} was recorded when the CO_2 concentration mechanism of the PEPC did not influence photosynthesis since there was no limitation of CO_2 at the bundle sheath cells.

Free proline content in leaves was recorded as an indicator of salt stress. It was determined following the method in Bates et al. (1973). Fresh leaf weight of 0.5 g was homogenized in 10 ml of 3% sulfosalicylic acid and centrifuged at 13.000 rpm for 5 min. Two milliliters of supernatant were combined with 2 ml acid-ninhydrin and 2 ml glacial acetic acid and boiled 1 hour at 100 ºC in a bath. Reaction was stopped in ice and 2 ml of toluene was added to each sample. The upper toluene phase was obtained to read its absorbance at 517 nm on a spectrophotometer (Hitachi U-1900, Gemini BV, Güeldres, Netherlands) using toluene as a blank. The concentration of proline was calculated from a standard curve of L-proline. Proportional weights and volumes were used when sample weights were insufficient (< 0.5 g).

Fitness was measured as leaf elongation rate (mm day^{-1}) that was quantified by applying red permanent sealer to the base of the youngest leaf and

to the top of 3 tillers per plant to measure the separation between the two marked plant parts 48 hours later (Castillo et al. 2014). Apical growth is usually related to vegetative growth and has been previously used as a measure of plant fitness (García and Ehrlén 2002).

Statistical analyses

Statistical analyses were carried out using Sigma-Plot for Windows version 13.0 (Systat Software, San Jose, CA). A significance level (α) of 0.05 was applied for every analysis. Prior to use of parametric models, data series were tested for normality with the Kolmogorov-Smirnov's test and for homogeneity of variance with the Levene's test. Every physiological and biochemical variable measured was analyzed using two-way analysis of variance (ANOVA) with taxa and salinity as grouping factors. Significant test results were followed by Tukey's honestly significant difference (HSD) tests for post hoc analyses. Differences between IC_{50} in dark and light conditions were assessed for every taxon using a Student's t-test. The best regression model and the coefficient of regression (R) between plant traits (dependent variables) versus salinity (independent variable) were conducted for every taxon.

5.3 Results

PEPC specific activity, inhibition by L-malate and total protein content

Differences in PEPC specific activity and IC_{50} were found among taxa at different salinity concentrations and among salinity concentrations for the same taxa after 30 days of treatment (two-way ANOVA salinity *x* taxa, PEPC: $F_{9,34} = 4.8$, $P < 0.001$; IC_{50}: $F_{9,34} = 3.6$, $P < 0.01$). At fresh water (0.5 ppt salinity), PEPC specific activity was less than the half for *S. maritima* than for all other taxa, and no significant difference was found among *S. densiflora* and both hybrids. On the contrary, both hybrids showed similar PEPC specific activity than *S.*

maritima and they duplicated the activity recorded for *S. densiflora* at 20 and 40 ppt salinity, whereas all taxa showed the same PEPC specific activity at intermediate salinity level of 10 ppt (Fig. 5.2A). PEPC specific activity for *S. maritima* showed a marked increase (+168%) when salinity increased from 0.5 to 10 ppt, being steady at the higher salinity level. On the contrary, PEPC activity for *S. densiflora* decreased with salinity (-36%) (Fig. 5.2B) being higher at 0.5 and 10 ppt than at 20 and 40 ppt. No relationship was found between PEPC specific activity and salinity concentration for either hybrid taxon, showing a mean of 4.97 ± 0.80 µmol min^{-1} mg^{-1} for *S. maritima x densiflora* and 5.40 ± 0.30 µmol min^{-1} mg^{-1} for *S. densiflora x maritima* without significant difference between them (Tukey test, $P > 0.05$) (Fig. 5.2B).

The value of the PEPC *in vivo* phosphorylation degree (IC_{50}) for light-acclimated leaves was 60-80% higher for *S. densiflora* than for the rest of the taxa at fresh water, being similar for *S. maritima* and the hybrid *S. maritima x densiflora* and intermediate between its parental species for *S. densiflora x maritima*. At the intermediate salinity levels (10 and 20 ppt), both hybrid taxa showed intermediate IC_{50} values between the higher value of *S. densiflora* and the lower value of *S. maritima*, except at 10 ppt salinity where IC_{50} values for *S. maritima x densiflora* and *S. maritima* were the same. At hypersalinity, both hybrid taxa showed similar IC_{50} values to those measured for *S. densiflora,* and higher IC_{50} values than *S. maritima* (Fig. 5.2C). IC_{50} decreased 74% at higher salinities for *S. densiflora* and increased 36% with salinity for *S. maritima x densiflora* (Fig. 5.2D). IC_{50} was independent of salinity for *S. maritima* and *S. densiflora x maritima* (Fig. 5.2D).

IC_{50} for dark-acclimated leaves was similar for all taxa at 0.5 ppt salinity, while it was higher for *S. maritima x densiflora* than the rest of taxa (60% higher than *S. maritima* and 40% higher than *S. densiflora x maritima*), except *S. densiflora*, at 40 ppt salinity. The hybrid *S. maritima x densiflora* was the only taxa that increased its IC_{50} with increasing salinity in dark conditions, from 0.28 ± 0.06 mM at 0.5 ppt salinity to 0.50 ± 0.08 mM at 40 ppt salinity (two-way

ANOVA salinity *x* taxa, $F_{3,16} = 2.5$, $P < 0.05$; Tukey test, $P < 0.05$). No significant difference was found for IC_{50} in dark versus light conditions for *S. maritima*, or for *S. maritima x densiflora* grown in fresh water or in hypersaline conditions (Tukey's test: *S. maritima*, *P* (0.5 ppt salinity) = 0.921, *P* (40 ppt) = 0.206; *S. maritima x densiflora*, *P* (0.5 ppt) = 0.914, *P* (40 ppt) = 0.988), nor for *S. densiflora x maritima* at hypersaline levels (Tukey's test, $P = 0.264$). In contrast, IC_{50} values for illuminated leaves were 73% and 35% higher than those in dark conditions for *S. densiflora* in fresh and hypersaline solutions respectively, and 44% for *S. densiflora x maritima* in fresh water, showing that the enzyme was activated by light-depended phosphorylation (Table 1).

The total amount of proteins was similar for all taxa at 0.5 and 40 ppt salinity concentrations. *S. maritima* had 41% more total proteins than *S. maritima x densiflora* at 10 ppt salinity, and both parental species had ca. 50% more total proteins than *S. densiflora x maritima* at 20 ppt salinity (two-way ANOVA salinity x taxa, $F_{9,34} = 3.1$, $P < 0.01$) (Fig. 5.2E). The total amount of proteins increased with salinity for both parental species (Fig. 5.2F).

Table 5.1. *In vivo* phosphorylation state of PEPC determined by the L-malate test (IC_{50}) for *Spartina maritima, Spartina densiflora* and their hybrids at 0.5 and 40 ppt salinity at dark and light conditions. Values are mean ± SEM (n = 3-4).

Taxa	Salinity (ppt)	IC_{50} (mM) (light)	IC_{50} (mM) (dark)	*t-test*
S. maritima	0.5	0.19 ± 0.02	0.20 ± 0.01	t = 0.106 *P* = 0.921
	40	0.23 ± 0.02	0.20 ± 0.01	t = -1.506 *P* = 0.206
S. maritima x densiflora	0.5	0.27 ± 0.04	0.28 ±0.06	t = 0.116 *P* = 0.914
	40	0.50 ± 0.08	0.50 ± 0.08	t = 0.0154 *P* = 0.988
S. densiflora x maritima	0.5	0.48 ± 0.04	0.27 ± 0.01	**t = -4.999 *P* < 0.01**
	40	0.37 ± 0.02	0.30 ± 0.05	t = -1.299 *P* = 0.264
S. densiflora	0.5	1.17 ± 0.27	0.34 ± 0.03	**t = -3.013 *P* < 0.05**
	40	0.57 ± 0.05	0.37 ± 0.06	**t = -2.648 *P* = 0.05**

Significant results for Student t-test for dependent samples between light and dark conditions are marked in bold.

Inheritance

Spartina maritima was the dominate parental species influencing most of the functional traits related to PEPC activity of *S. maritima x densiflora* (D-Sm = 40%) (Fig. 3A), whereas *S. densiflora* was the dominate parental influence (D-Sd) on 20% of its traits, 13% were equal to both parents, and 7% were intermediate between the parental species (Table 2; Fig. 3B). *Spartina maritima x densiflora* was the only taxon that presented an increase in the phosphorylation degree of PEPC with salinity (IC_{50}) both in dark and light conditions, compensating the drop in the amount of PEPC (Fig. 5.2). These innovative behaviors were a product of transgressive segregation since they were not found in any of its parental species (T = 20%) (Table 2; Fig. 3A). Most of the PEPC traits of hybrid taxon *S. densiflora x maritima* were equal to both parents (D-Sm,Sd = 40%) and 20% showed intermediate values compared to its parental species. Our results indicate this hybrid inherited 27% of PEPC-related traits from *S. maritima* and 13% from *S. densiflora* (Table 2; Fig. 3B).

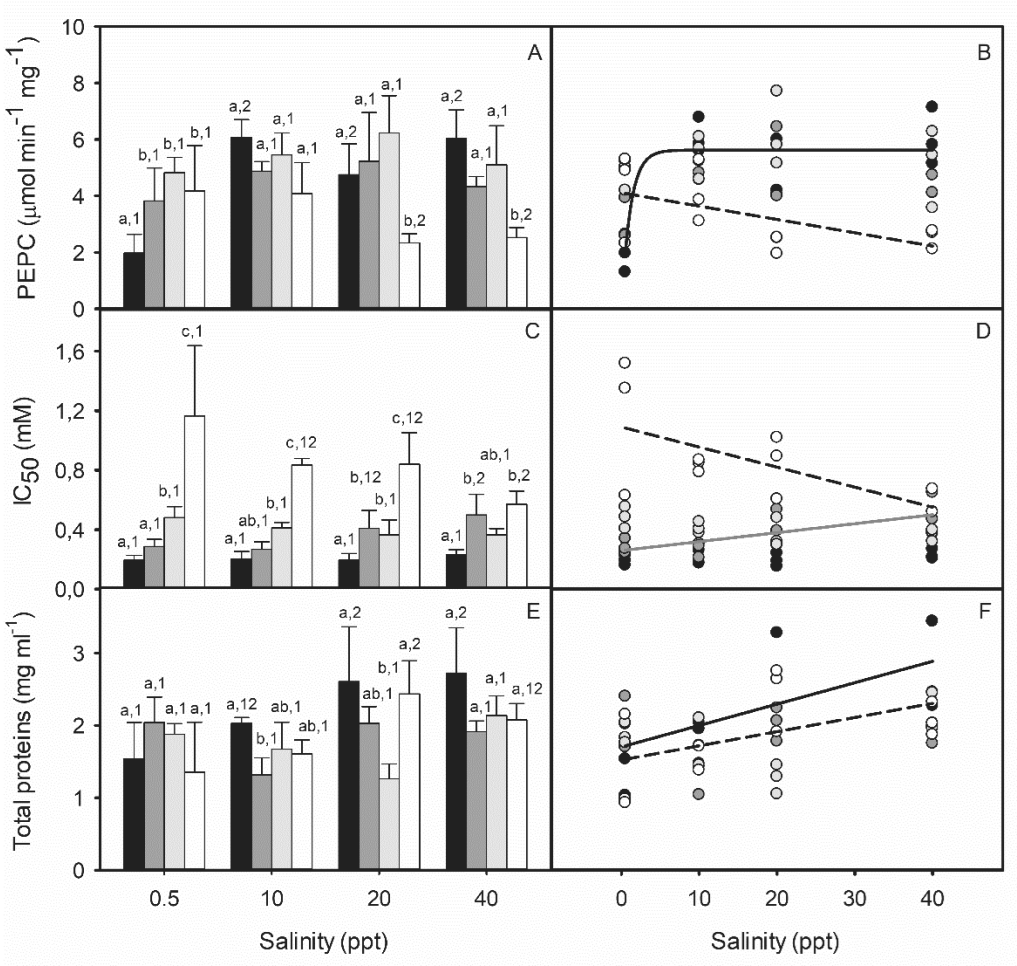

Fig. 5.2 PEPC specific activity (A, B) and *in vivo* phosphorylation state of PEPC determined by L-malate (IC_{50}) (C, D), and total proteins content (E, F) in crude extracts of illuminated leaves of *Spartina maritima, Spartina densiflora* and their hybrids in different salinity treatments (0.5, 10, 20 and 40 ppt). *S. maritima* = black, *S. maritima x densiflora* = dark grey, *S. densiflora x maritima* = light grey, *S. densiflora* = white columns, dash line. Values are mean ± SD (n = 3). Different letters indicate significant differences among taxa for the same salinity treatment; different numbers indicate significant differences among salinity treatments for the same taxon (ANOVA, $P < 0.05$). Regression equations: (B) Sm: $y = 5.623 * (1 - 0.421^{x})$, $R = 0.87$, $P < 0.001$; Sd: $y = 4.112 - 0.074\ x$, $R = -0.584$, $P < 0.05$; (D) Smxd: $y = 0.259 + 0.006\ x$, $R = 0.71$, $P < 0.01$; Sd: $1.091 - 0.014\ x$, $R = -0.66$, $P < 0.05$; (F) Sm: $y = 1.702 + 0.030\ x$, $R = 0.66$, $P < 0.05$; Sd: $y = 1.454 + 0.199 \ln(x)$, $R = 0.60$, $P < 0.05$.

Table 5.2. Inheritances for hybrids between *Spartina maritima* and *Spartina densiflora* for seven traits related to the enzyme PEPC: (1) level of activation of PEPC by phosphorylation for illuminated leaves (IC_{50} light); (2) level of activation of PEPC for dark-acclimated leaves (IC_{50} darkness); (3) change in the level of activation of PEPC for illuminated leaves in relation to dark-acclimated leaves (IC_{50} light vs. darkness); (4) change in the level of activation of PEPC from fresh water to hypersalinity for illuminated leaves (IC_{50} 0.5 ppt vs. 40 ppt light); (5) change in the level of activation of PEPC from fresh water to hypersalinity for dark-acclimated leaves (IC_{50} 0.5 ppt vs. 40 ppt darkness); (6) PEPC specific activity; (7) compensatory mechanism (increasing IC_{50} to compensate low amounts of PEPC) at 0.5, 10, 20 and 40 ppt NaCl. D = parental dominance, Sm = *S. maritima*, Sd = *S. densiflora* (D-Sm = black, D-Sd = white, D-Sm,Sd = light grey), I = parental additivity (dark grey), T = transgressive (striped).

	Spartina maritima x densiflora				*Spartina densiflora x maritima*			
PEPC trait / salinity	**0.5 ppt**	**10 ppt**	**20 ppt**	**40 ppt**	**0.5 ppt**	**10 ppt**	**20 ppt**	**40 ppt**
IC_{50} light	D–Sm	D–Sm	I	D–Sd	I	I	I	D–Sm,Sd
IC_{50} darkness	D–Sm,Sd	-	-	D–Sd	D–Sm,Sd	-	-	D–Sm,Sd
IC_{50} light vs. darkness	D–Sm	-	-	D–Sm	D–Sd	-	-	D–Sm
IC_{50} 0.5 ppt vs. 40 ppt light		T				D–Sm		
IC_{50} 0.5 ppt vs. 40 ppt darkness		T				D–Sm,Sd		
PEPC specific activity	D–Sd	D– Sm,Sd	D–Sm	D–Sm	D–Sd	D–Sm,Sd	D–Sm	D–Sm
Compensatory mechanisms		T				D–Sm,Sd		

Photosynthesis rate, proline content and growth rate

Net photosynthesis rate (A) and A_{max} were higher than 35% for both hybrids than for their parental species at the lowest 0.5 ppt salinity level, except for A that was no statistically different between *S. densiflora* and *S. densiflora x maritima* (Fig. 4A, C). Net photosynthesis values were not different for the parental species at any salinity treatment and among every taxon at 10, 20 and 40 ppt salinity. Net photosynthesis rate decreased at higher salinities for both hybrids (two-way ANOVA, salinity x taxa: $F_{9,34} = 3.0$, $P < 0.01$) (Fig. 4A), decreasing linearly in the case of *S. maritima x densiflora* (Fig. 4B). A_{max} was ca. 45% higher for *S. maritima* than the rest of the taxa at 20 ppt salinity. *S. maritima x densiflora* showed intermediate values (5.94 ± 0.85 µmol O_2 m^{-2} s^{-1}) between the higher values of *S. maritima* (10.09 ± 2.04 µmol O_2 m^{-2} s^{-1}) and the lower values of *S. densiflora* (3.73 ± 0.22 µmol O_2 m^{-2} s^{-1}) and *S. densiflora x maritima* (2.68 ± 0.30 µmol O_2 m^{-2} s^{-1}) at 40 ppt salinity. A_{max} decreased at higher salinities for all taxa (two-way ANOVA, salinity x taxa: $F_{9,64} = 10.7$, $P < 0.001$) (Fig. 4C). This decrease of A_{max} with increasing salinity was linear for both hybrids, whereas *S. maritima* showed its highest value at 20 ppt salinity and *S. densiflora* at 10 ppt (Fig. 4D). No difference was found between A and A_{max} at different salinity levels for all taxa, except for higher A_{max} than A at 20 ppt salinity for *S. densiflora x maritima* (two-way ANOVA A/A_{max} *x* salinity, *S. densiflora x maritima*: $F_{3,23} = 13.8$, $P < 0.01$) (Fig 4A, C).

Free proline content increased linearly with salinity for all taxa (two-way ANOVA salinity *x* taxa, $F_{9,64} = 5.4$, $P < 0.001$) (Fig 4E, F). All taxa accumulated the same amount of free proline under fresh water conditions, but *S. maritima* accumulated at least 35% more proline than the rest of the taxa at 10, 20 and 40 ppt salinity. The hybrid *S. maritima x densiflora* had 85, 75 and 68% less proline at 40 ppt hypersalinity than *S. maritima, S. maritima x densiflora* and *S. densiflora* respectively (Fig 4E).

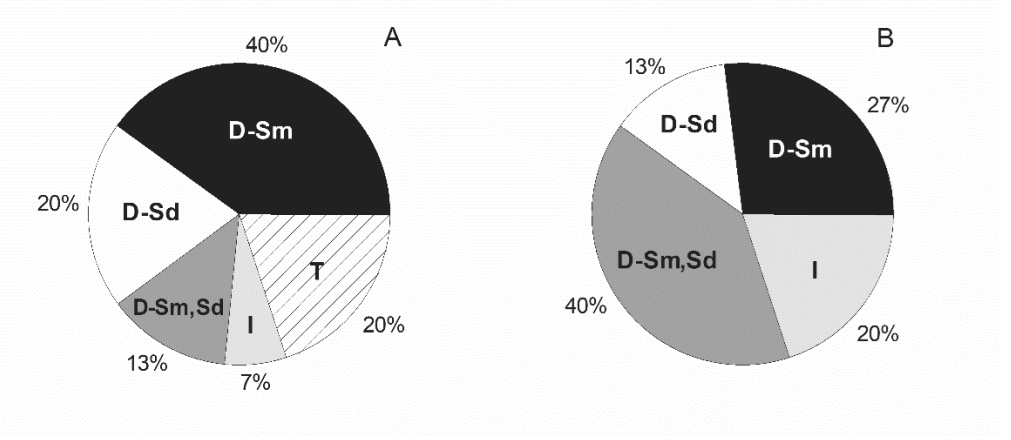

Fig. 5.3 Inheritances for the hybrids (A) *Spartina maritima x densiflora* and (B) *S. densiflora x maritima* for seven traits related to the enzyme PEPC at 0.5, 10, 20 and 40 ppt NaCl. D = parental dominance, Sm = *S. maritima,* Sd = *S. densiflora* (D-Sm = black, D-Sd = white, D-Sm,Sd = light grey), I = parental additivity (dark grey), T = transgressive (striped).

Leaf elongation rate was the lowest for *S. maritima* and the highest for *S. densiflora* between 0.5 and 20 ppt salinity, with both hybrids showing intermediate values. At 40 ppt, the hybrids showed similar growth rates than *S. densiflora* and still higher (+77%) than *S. maritima* (Fig. 4G). Leaf elongation rate of *S. maritima* and its both hybrids did not change between salinity treatments, whereas *S. densiflora* showed its maximum at 10 ppt, decreasing 58% at higher salinities (Fig. 4H).

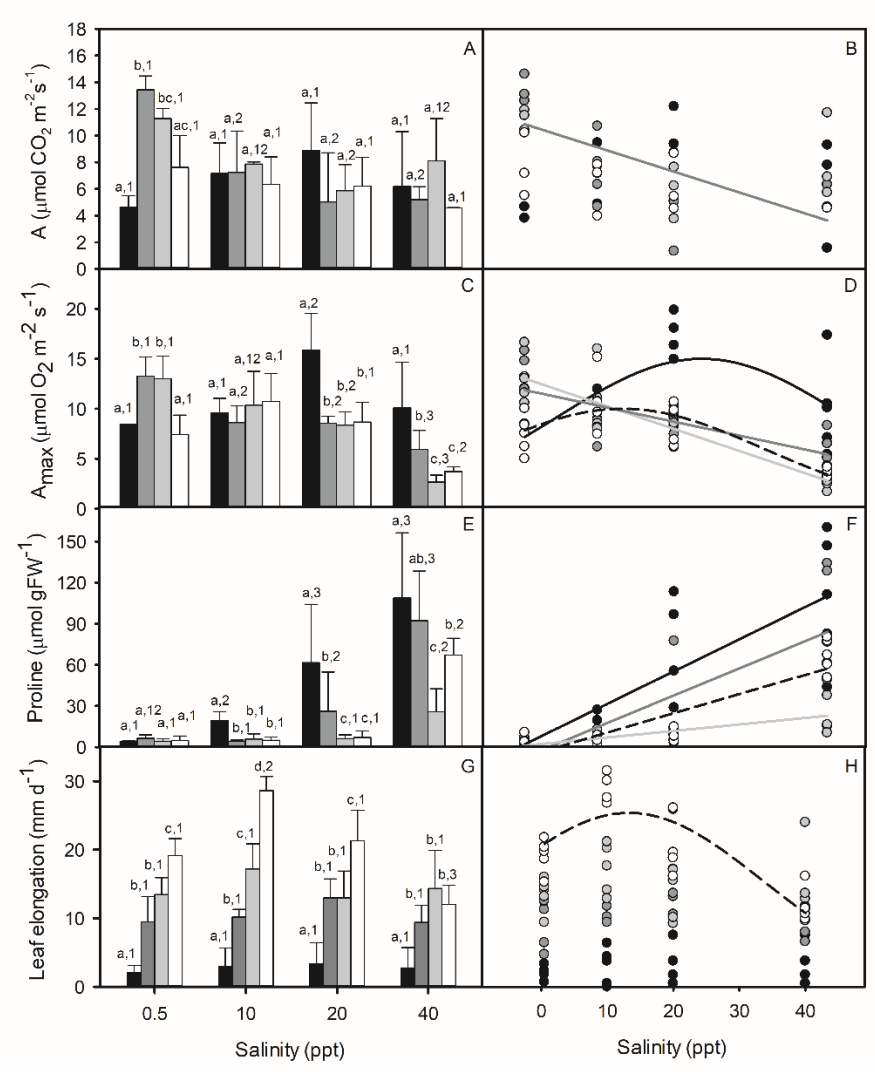

Fig. 5.4 Net photosynthesis rate (A) (A, B) measured using an infrared gas analyzer, maximum photosynthesis rate (A_{max}) (C, D) measured using Clark type oxygen electrode at a CO_2 saturated atmosphere, proline content (E, F) and leaf elongation rate (G, H) for *Spartina maritima*, *Spartina densiflora* and their hybrids in different salinity treatments (0.5, 10, 20 and 40 ppt). *S. maritima* = black, *S. maritima x densiflora* = dark grey, *S. densiflora x maritima* = light grey, *S. densiflora* = white. Values are mean ± SD (n = 3-5). Different letters indicate significant differences among taxa for the same salinity treatment; different numbers indicate significant differences among salinity treatments for the same taxa (ANOVA, P < 0.05). FW, fresh weight. Regression equations: (B) Smxd: y =10.948 - 0.183 x, $R = 0.673$, $P < 0.05$; (D) Sm: y = 15.001 exp (-0.5*((x-23.619)/19.050)^2), $R = 0.648$, $P < 0.01$; Smxd: y =11.954 - 0.162 x, $R = 0.791$, $P < 0.0001$; Sdxm: y =13.156 - 0.259 x, $R = 0.888$, $P < 0.0001$; Sd: y = 10.008 exp(-0.5 * ((x - 13.232) / 18.295)^2, $R = 0.797$, $P < 0.001$; (F) Sm: y = 0.664 + 2.737 x, R=0.807, $P < 0.0001$; Smxd: y = -8.521 + 2.314 x, $R = 0.822$, $P < 0.0001$; Sdxm: y = 0.790 + 0.552 x, $R = 0.685$, $P < 0.001$; Sd: y = -7.803 + 1.631 x, $R = 0.873$, $P < 0.0001$; (H) Sd: y = 25.363 exp(-0.5 * ((x - 13.287) / 20.403)^2, $R = 0.810$, $P < 0.001$.

5.4 Discussion

The phosphoenolpyruvate carboxylase (PEPC) is the key enzyme that promotes rapid CO_2 fixation and increased efficiency of photosynthetic metabolism in plants with a C-4 photosynthetic pathway that colonize dry and saline habitats all around the world (Ward et al. 1999, Nelson et al. 2004, Nayyar and Gupta 2006). Our study analyzes different mechanistic traits of PEPC for two exotic hybrids and their common parental species in response to environmental salinity concentrations ranging from fresh water to hypersalinity. Our results showed that all of the studied taxa were subjected to increasing physiological stress as indicated by rises in free proline content in their plant tissue at higher salinity. These results support and add insight to findings of research on the growth and functional traits responses of *Spartina densiflora* to salinity (Castillo et al. 2005b; Grewell et al. 2016), and the contrasted responses of *Spartina* hybrids formed in San Francisco Bay in experimental salinity regimes in comparison with their parental species (Lee et al. 2016). Allocation of plant resources to proline accumulation provides a compatible osmolyte to maintain favorable cellular water relations, and can prevent cellular damage in response to salinity stress (Kavi-Kishor and Sreenivasulu, 2014). Moreover, A_{max} (recorded at saturating CO_2 conditions) was similar than A (recorded at ambient CO_2 conditions) for every taxon at every salinity level, except for *S. densiflora x maritima* at 20 ppt salinity, indicating that the C-4 metabolism allowed maximum intracellular carbon concentrations at ambient conditions. Similar results were obtained by Mateos-Naranjo et al. (2010) for *S. densiflora* finding equal photosynthesis rates at different air CO_2 concentrations (380 and 700 ppm), which was attributed to the PEPC carboxylation capacity that enables the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) to operate near its maximum activity, requiring less Rubisco for the same photosynthesis rates (Evans and von Caemmerer 2000).

Parental responses to salinity

The PEPC specific activity of *S. maritima* increased with increasing salinity keeping constant IC_{50} values, indicating higher amounts of PEPC at salinity concentrations higher than fresh water, which allowed *S. maritima* to keep constant net photosynthesis rates (A) along the whole salinity gradient. This increase in the amount of PEPC with salinity is in accordance with the expression of the PEPC gene in the low marsh cordgrass *Spartina alterniflora* being induced at 4 ppt. However, its expression in *S. alterniflora* decreased sharply at 12 ppt (Courtney et al. 2016), which contrasts with *S. maritima* maintaining high PEPC amounts even at hypersalinity. At the Mediterranean Basin, *S. maritima* colonizes low marshes where salinity increases to ca. 34 ppt during the summer drought (Contreras-Cruzado et al. 2017). In this habitat, keeping high photosynthetic levels is crucial for maintaining positive carbon balances being exposed to solar radiation just for a few hours a day due to long flooding periods (Castillo et al. 2000). A decrease of net photosynthesis rate for *S. maritima* in fresh water as that recorded in our study was also previously recorded (Naidoo et al. 2012), which may be related with the fact that PEPC activity is lower at this low salinity. On the other hand, PEPC phosphorylation degree (IC_{50}) for illuminated leaves of *S. maritima* was low and similar to dark-acclimated leaves at every salinity, denoting that the enzyme was not activated by phosphorylation (Echevarría et al. 1990). This lack of light-induced activation of PEPC for *S. maritima* may be related to its low marsh habitat, where photo-periods are short and radiation intensity is low due to long flooding periods with turbid waters (Castillo et al. 2000).

Both PEPC specific activity and IC_{50} at light conditions decreased at higher salinities for *Spartina densiflora*. Still, a slight increase in the amount of PEPC with salinity might have occurred since the fall for the PEPC specific activity (39%) was lower than for the IC_{50} (51%) and the total protein content increased with salinity. However, this increase in the PEPC amount was not

enough to compensate the marked fall in its activation by phosphorylation mediated by light. Contrary to our results and those of Álvarez et al. (2010), Mateos-Naranjo et al. (2010) did not find differences in PEPC activity nor IC_{50} for *S. densiflora* with increasing salinity. The recorded decrease in PEPC specific activity coincided with a tendency to decline in A with salinity (from 7.6 ± 1.4 μmol CO_2 m^{-2} s^{-1} at 0.5 ppt to 4.6 ± 0.01 μmol CO_2 m^{-2} s^{-1} at 40 ppt). Castillo et al. (2005b) recorded a decrease in A and growth for *S. densiflora* at hypersalinity. In our study, A_{max} (recorded at CO_2 saturation) showed a pronounced decrease at higher salinities, so the recorded decrease in A seems to be related not only to the decrease in PEPC activity but also to the limitation of other photosynthetic processes at higher salinity concentrations. In this context, different studies have found that the enzyme Rubisco is negatively affected by a greater extent than PEPC by salinity (Osmond and Greenway 1972; Meinzer and Zhu 1999). On the other hand, IC_{50} in illuminated leaves increased in relation to dark-acclimated leaves both at 0.5 and 40 ppt, indicating light-induced activation of PEPC (Echevarría et al. 1990). This light-induced activation of PEPC, recorded previously by Álvarez et al. (2010) for *S. densiflora*, was much more pronounced at fresh water (262%) than at hypersalinity (63%). This behavior may be the result of the adaptation of *S. densiflora* to medium-high marsh environments subjected to long emerge periods and high radiation intensities (Castillo et al. 2008b).

Stressful environments such as salt marshes expose plant species to strong selective pressures that can drive evolutionary changes even at an enzymatic level (Meng and Wu, 2017). In this sense, local adaptation in the form of an ecotype for PEPC activity was described for *S. densiflora* from high latitudes that was able to compensate low activation by phosphorylation of the PEPC with higher amounts of the enzyme at fresh water conditions (Álvarez et al. 2010). In view of our results about PEPC functioning, the parental species showed contrasted strategies at the PEPC level to cope with salinity, reflecting their actual occupation of distinct habitats along the intertidal gradient. *Spartina*

maritima showed its physiological optimum throughout a wide salinity range from 10 to 40 ppt, maintaining constant PEPC specific activity (due to high enzyme contents) and photosynthesis rates. In contrast, the salinity optimum for *S. densiflora* was between 0.5 and 10 ppt, where it presented its highest levels of PEPC specific activity (coinciding with high light-induced activation of PEPC) and photosynthetic rates. Together, these results clearly show contrasted enzymatic responses of the parental species coming from different salt marsh habitats.

Responses of hybrids to salinity

Both *Spartina* hybrids showed constant PEPC specific activity along the salinity gradient. At both salinity extremes, the hybrids presented similar values to the parent that showed higher PEPC specific activity (*S. densiflora* at low salinity and *S. maritima* at high salinity). Despite keeping constant PEPC specific activity, both hybrids were negatively affected by salt stress since their A and A_{max} decreased with increasing salinity, pointing to negative effects of salt stress on other photosynthetic processes rather than carbon fixation by PEPC. Additionally, both hybrids exhibited higher A at fresh water than their parental species that did not relate exclusively to PEPC activity, nor to stomatal conductance, since the same tendency was observed for the A_{max} in a saturated CO_2 atmosphere. These higher net photosynthesis rates for the hybrids in relation to their parental species at fresh water may be related to enhanced Rubisco activity due to higher Rubisco contents (Leisner et al. 2010), as maximum photosynthesis rate has been reported to be generally limited by the *in vivo* maximum rate of Rubisco carboxylation (von Caemmerer 2000). Higher A for both hybrids than for their parental species at fresh water would be a benefit during the rainy season under Mediterranean climate and close to natural and artificial fresh water sources during great part of the year. In fact, the presence of both hybrids natural populations coincides with areas under the influence of water runoffs from adjacent coastal dunes (in Odiel, Piedras and

Guadiana Marshes) and with locations close to pipes pouring fresh water into salt marsh channels coming from sewage treatment plants (in Tinto and Guadiana marshes) (Gallego-Tévar, personal observation). However, both hybrids showed higher net photosynthesis rates in fresh water than both parents, but this was not reflected in higher fitness (recorded as leaf elongation rate) since photosynthetic assimilates are not only diverted to growth but also to carbohydrate reserves (mainly stored in rhizomes in *Spartina* species).

*Spartina maritima x densiflor*a was the only taxon able to increase its IC_{50} at light and high salinities, compensating the decrease in the amount of PEPC. As a result, it was able to keep similar PEPC specific activity at every salinity. Moreover, *S. maritima x densiflora* was also the only taxon increasing its IC_{50} in darkness at high salinity, which would allow it to accumulate carbon when emerged during nocturnal low tides ensuring the supply of malate to the bundle-sheath cells during the following light period (García-Mauriño et al. 2003). Therefore, the total annual photosynthetic carbon assimilation may be higher for *S. maritima x densiflora* than for its parental species due to carbon store during nocturnal low tides, maintaining positive carbon balances even during stomatal enclosure and illuminated submerged periods. The induction of IC_{50} at darkness by salts has been described for *Sorghum vulgaris* Pers. (García-Mauriño et al. 2003), being explained as an induction to a type of CAM metabolism present in some halophytic CAM species and reminiscent in some C-4 plants (Winter and Willert 1972, Baur et al. 1992, Li and Chollet 1994, Taybi et al. 2000). Thus, *S. maritima x densiflora* presented three functional PEPC-related traits different from both parental species due to transgressive segregation (T = 20% of the seven analyzed traits). Greater tolerance to abiotic factors for hybrids than for their parental species have been previously reported in relation to traits product of transgressive segregation (e.g. Lexer et al. 2003b; Ayres et al. 2004; Hall et al. 2006). On the other hand, *S. maritima x densiflora* did not exhibit light-induced PEPC activation, neither at fresh water nor at hypersalinity, like its maternal species, both colonizing low marshes with short photo-periods and low

radiations levels when flooded (Castillo et al. 2010a). In this sense, the inheritance of 20% of the PEPC functional traits studied in *S. maritima x densiflora* was dominated by *S. densiflora* and 40% was dominated by its maternal species *S. maritima*. This high maternal influence on the inheritance of its hybrid may be related to *S. maritima* adding its whole genome (diploid gametes) to the hybrid (Castillo et al. 2010a), and from maternal effects arising from interspecific interaction between cytoplasmic and nuclear genomes (Iida et al. 2013). The better performance of the hybrid relative to that of *S. maritima* at low salinities may be related to the influence of *S. densiflora*. Environmental sources of phenotypic variation in the hybrids due to the parental environments cannot be ruled out since environmental conditions of the salt marshes colonized by the parental cordgrasses may provoke differential survival of developing gametophyte (pollen grains and ovules) genotypes and differential postfertilization gene expression in the sporophyte (Mazer and Gorchov, 1996).

Spartina densiflora x maritima was also able to keep similar PEPC specific activity and IC_{50} values along the whole salinity gradient. Light-induced activation of the PEPC occurred at low salinity as for its maternal species *S. densiflora*, while it was absent at hypersalinity as for *S. maritima.* This hybrid presented most PEPC-related traits intermediate between its parental species, being 60% similar or intermediated to both parents, 27% dominated by *S. maritima*, and only 13% dominated by its maternal species. This intermediate behavior resulted in keeping high PEPC specific activity at low salinities at rates similarly to *S. densiflora* and at high salinities as *S. maritima*. Intermediate values of inherited traits are frequent in F1 hybrids when a large number of genes acts independently giving rise to an additive inheritance that may be modified in the following hybrids generations if other non-additive mechanisms, such as heterosis, occur (Stelkens and Seehausen, 2009; Rieseberg et al. 1999; Mallet, 2007). Later-generation hybrids have not been found for hybrids between *S. maritima* and *S. densiflora* as their F_1 hybrids are sterile (Castillo et al. 2010a), but fertility can be achieved if chromosomal doubling occurs which have been

previously reported for this genus driving to the formation of the highly invasive allopolyploid *Spartina anglica* C.E. Hubb. (Marchant 1968, Baumel et al. 2001).

However, both hybrids showed intermediate growth rates between their parents in our greenhouse experiment. Their phenotypic inheritances at the level of a key enzyme in C4 plants that changed depending on the salinity of the growth medium may be related to their greater growth rates in comparison with their parental species in field conditions under tidal influence (Castillo et al. 2010a).

Our results on quantitative trait differences between the hybrids and their parental species are in agreement with genetic studies showing that deviation from parental additivity and transgressively expressed genes are important following hybridization, as it was the case of *S. anglica* (Chelaifa et al. 2010). Thus, Lee et al. (2016) reported that hybrids between *Spartina alterniflora*, *S. foliosa* and *S. densiflora* showed higher salinity tolerance than their parental species, and Favre and Karrenberg (2011) found that different inheritances (intermediate, parental-like or transgressive) were responsible of different tolerance of *Silene* hybrids to drought and shade stress. In this sense, hybrids of maize showed higher tolerance to salinity due to the role of C-4 enzymes such as PEPC (Nemat Alla and Hassan 2012). Histone modifications play important roles in regulating the expression of C-4 photosynthetic genes (Li et al. 2017) and hybridization often results in dramatic structural genome reconfigurations, including epigenetic changes, that control gene expression (Koroma et al. 2011). These epigenetic changes after hybridization seem to be related to increased salt tolerance in hybrids (Zeng et al. 2015). More genetic and epigenetic studies are needed to reveal the mechanisms leading to the production of hybrids with enhanced PEPC functionality in relation to different environmental stress factors such as salinity. In this sense, genetic studies using an RNA-sequencing approach (Bell et al. 2013) may shed light on the molecular mechanism driving gene expression after the hybridization between *S. maritima* and *S. densiflora*.

Applied to crop species, this knowledge may facilitate and enhance the design of genetically modified C-3 plants, such as rice and wheat, incorporating the C-4 photosynthetic pathway (Kajala et al. 2011, Bachir et al. 2017). Moreover, the production of hybrids between C-4 species, or between C-3 plants with C-4-induced metabolism (Evans and von Caemmerer 2000), may be an advantage for their salinity tolerance if transgressive responses are selected.

The parental species, the European native *S. maritima* and the invasive *S. densiflora*, have been growing together for decades in some marshes where the hybrids have formed, flowering every year from two years of age (Castillo and Figueroa 2009). In this context, the presence in the Iberian salt marshes of transgressive *Spartina* hybrids with better photosynthetic performances than their parental species along the whole salinity gradient opens the possibility of a new sympatric speciation. As Castillo et al. (2010a) reported these invasive hybrids colonize a wider intertidal range, showing taller shoots and higher growth rates, than their parental species. Thus, once established in the stressful environment of the salt marshes, these sterile F1 hybrids perform better than their parental species, being able to retain the colonized space and to expand by rhizomes to the surrounding sediments while producing many unviable seeds. As in the case of the sterile hybrid *Spartina townsendii* H. et J. Groves becoming fertile by allopolyploidization (hybridization followed by genome multiplication) as in *S. anglica*, the studied hybrids may go through an allopolyploidization process leading to substantial epigenetic variation. This epigenetic reorganization would be additive to the process experienced during hybridization, consolidating the first and allowing this new invasive *Spartina* species to express novel phenotypes with increased fitness (Jackson 2017). In fact, the parental heptaploid species *S. densiflora* seems to have an ancestral hybrid origin (Fortune et al. 2008) that likely contributes to its high phenotypic plasticity and ability to invade a wide range of different habitats (Castillo et al. 2014). On the other hand, the studied *Spartina* taxa presented different amounts and activation levels of PEPC depending on the salinity of the growth medium

in our greenhouse experiment. Since salinity also changes spatially and temporally in the salt marshes colonized by these cordgrasses (Contreras-Cruzado et al. 2017), our results suggest the possibility of balancing selection mechanisms, such as spatial and temporal heterogeneity in selection and antagonistic pleiotropy, operating in natural populations (Mojica et al. 2012).

5.5 Conclusions

Our results showed that two closely related species of the Genus *Spartina*, the European native *S. maritima* and the invader *S. densiflora*, presented specific responses at the enzymatic level in relation to the functioning and regulation of PEPC along a salinity gradient from freshwater to hypersalinity. Moreover, F1 reciprocal hybrids of these *Spartina* species showed clear maternal effects. Additionally, these hybrids also presented high levels of non-additive inheritance of traits related to PEPC functioning, including some transgressive traits for *S. maritima x densiflora*.

CHAPTER VI

Effects of salinity and inundation on native *Spartina foliosa*, invasive *S. densiflora* and their hybrid *S. densiflora x foliosa* from San Francisco Bay: (I) Parental species

CAPÍTULO 6. Efectos de la salinidad y la inundación en la nativa Spartina foliosa*, la invasora* S. densiflora *y su híbrido* S. densiflora x foliosa *en la Bahía de San Francisco: (I) Especies parentales.*

Se espera que el aumento del nivel del mar asociado al cambio climático incremente el periodo de inundación permanente y la salinidad de las marismas mareales. En este contexto, el estudio de la respuesta de las halófitas nativas e invasoras al aumento del nivel del mar resulta fundamental para mantener su estado de conservación en el futuro. Con este objetivo, llevamos a cabo un experimento de invernadero en mesocosmos en el que la especie nativa *Spartina foliosa* y la invasora *S. densiflora* procedentes de la Bahía de San Francisco (EEUU) fueron expuestas a distintas combinaciones de diferentes profundidades de inundación permanente (4.5, 35.5 and 55 cm) y diferentes salinidades (0.5, 10, 20 and 40 ppt). Se midieron 38 caracteres morfológicos, bioquímicos y ecofisiológicos diferentes. Ambas especies mostraron diferentes síndromes en sus respuestas a la combinación de estrés combinado por inundación y salinidad. La nativa *S. foliosa* se comportó como una especie tolerante al estrés, mostrando un conjunto de respuestas que le permitieron mantener una tasa de crecimiento baja y caracterizada por una alta tasa de expansión lateral (mayor número de tallos). En cambio, la invasora *S. densiflora* actuó como una especie de crecimiento rápido exhibiendo altos niveles de producción de espiguillas y de biomasa aérea (en tallos más largos) con bajo estrés, mostrando mecanismos de tolerancia al estrés abiótico insuficientes para prevenir la caída de fitness a mayores salinidades y profundidades de inundación. Según nuestros resultados, la inundación permanente afectará más a la productividad de las especies de *Spartina* que la salinidad, limitando ambos factores la invasividad de *S. densiflora* al reducir su producción de semillas, factor clave para su propagación.

CHAPTER 6. Effects of salinity and inundation on native Spartina foliosa, *invasive* S. densiflora *and its hybrid* S. densiflora x foliosa *from San Francisco Bay: (I) Parental species*

Sea Level Rise (SLR) associated with climate change is expected to intensify the period of permanent submersion and salinity in coastal tidal wetlands. In this context, the study of the responses of native and invasive halophytic plant species to SLR is essential to maintain their conservation status. With this aim, we conducted a mesocosm experiment in which the combinations of different inundation depths (4.5, 35.5 and 55 cm) and different salinities (0.5, 10, 20 and 40 ppt) were imposed on the native species *Spartina foliosa* and the invasive *S. densiflora* from San Francisco Bay (USA). We measured 38 morphological, biochemical and ecophysiological traits. Both cordgrass species exhibited different syndromes in their response to the combination of inundation and saline stress. The native *S. foliosa* behaved as a stress-tolerant species, showing a set of responses that allowed it to maintain a low and relatively constant growth rate characterized by a high lateral expansion rate (tiller production). In contrast, the invasive *S. densiflora* acted as a rapid-growth species exhibiting high levels of floret production and AGB allocation (in long tillers) with low stress, showing tolerance mechanisms to abiotic stress insufficient to prevent the fall of fitness at higher salinities and inundation depths. Our study revealed that permanent inundation will affect more the productivity of *Spartina* species than salinity. Both abiotic factors would limit the invasiveness of *S. densiflora* by reducing its seed production, a key factor for its propagation.

6.1 Introduction

Salinity and inundation are among the most important abiotic drivers of the distribution and abundance of plant species within tidal salt marshes (Pennings et al. 2005; Engels and Jensen, 2010). Variation in the salinity of tidewater and the depth, duration and frequency of inundation reflect differences in distance from oceanic tidal source, local hydrogeomorphology, fluctuations in tidal ranges, precipitation and evapotranspiration rate and freshwater river inflow. These complex interacting factors lead to a range of different intertidal habitats along the hydrologic gradient of coastal marshes that will together play a role in the response of wetland vegetation to sea level rise (Bertness et al. 1992, Morris et al. 2002, Crain and Bertness 2006). As a result of these factors, halophytes in salt marshes are subjected to a wide range of abiotic stress levels, and depending on their position along the stress gradient, they can be exposed to stress levels close to their tolerance limits (Adam 1990). It is expected that accelerating rates of sea level rise (SLR) associated with climate change will modify the patterns of salinity and inundation in tidal marshes (IPCC 2015). In this context, salinity and permanent inundation depth are increasing in salt marshes where sediment accretion will not compensate SLR, intensifying abiotic stress levels on halophytes (Stralberg et al. 2011) and threatening intertidal habitat with extreme submergence by the end of the century (Thorne et al. 2018). Hence, studies on the response of plant species to varying levels of salinity and inundation stress imposed by SLR are essential for the preservation of wetlands (Tabot and Adams 2012). In tidal wetlands, it is especially important to understand how SLR will limit or extend the role of invasive species known to function as ecosystem engineers that can alter habitat with cascading ecological effects on resident biota (Crooks et al. 2002).

Halophytes by definition have developed morphological, anatomical and physiological adaptations that support stress tolerance or avoidance mechanisms to complete their life cycles in saline and inundated environments, yet the degree

of these adaptations and effectiveness of the tolerance strategies varies among species (Flowers and Colmer 2015, Mishra and Tanna 2017). Therefore, the nature and degree of osmotic and oxidative stress responses should also vary by species as SLR progressively imposes greater salinity and flooding stress (including sediment anoxia and related soil phytotoxins) with constant to more frequent and deeper inundation by saline tidewater across tidal elevations. Responses to these stresses by halophytic species can negatively influence their photosynthetic capacity, modify biomass allocation patterns and increase maintenance costs as they activate tolerance mechanisms such as stomatal closure, metabolic inhibition, limitation of nutrient uptake to detoxify and maintain osmoregulation or ion homeostasis through production of osmoprotectants, and development of specialized structures such as salt glands. The need to exclude, excrete or compartmentalize toxic ions requires energy otherwise available for biomass production to be directed to antioxidant enzymatic activities and the production and accumulation of carbon-rich osmolyte contents in plant tissue. In turn, these processes are reflected in accelerated senescence, low growth rates and diminished biomass accumulation (Pezeshki 2001, Munns and Tester 2008). When combined, inundation and saline stresses can be synergistic with enhanced negative effects on marsh vegetation (Spalding and Hester 2007, Janousek and Mayo 2013, Barrett-Lennard and Shabala 2013). Even moderate stress levels can activate common stress responses in plant species, inducing responses that reduce physiological activity and can thereby increase the plant's vulnerability to an additional environmental stress (Syvertsen and García-Sanchez 2014). While halophytes serve as model organisms for studies on the effects of salinity and flooding on tolerance of agricultural crops to improve stress tolerance, the direct and indirect interactions of combined osmotic and flooding stresses on halophytes are poorly understood (Flowers and Colmer 2015).

Biological invasions of plant species well beyond their native ranges are a significant component of global environmental change resulting from

anthropogenic breakdown of barriers to species dispersal (Vitousek et al. 1997). In natural ecosystems such as salt marshes, the interactive effects of the changing environmental conditions derived from SLR on native versus invasive plant species remains unclear (Hellmann et al. 2008, Parker et al. 2011). Some studies suggest that invasive species can benefit from the effects of climate change (Dukes and Mooney 1999, Loebl et al. 2006, Vilà et al. 2007). Other authors point out that the effects may differentiate between species given the complexity of the interaction between the new environmental conditions and the invasive species (Rahel and Olden 2008, Hellmann et al. 2008). In order to elucidate how invasive plants will respond to environmental changes, it is fundamental to study the responses of invasive species through a functional trait framework (Drenovsky et al. 2012). In general, invasive species are able to colonize environments significantly different from their native range by exhibiting traits that provide them the capacity to tolerate heterogonous abiotic conditions (Dukes and Mooney 1999) frequently developing high phenotypic plasticity (Richards et al. 2008, Nicotra et al. 2010, Wetson et al. 2012, Castillo et al. 2014, Grewell et al. 2016).

In this study, we evaluated the variation in effects of salinity, inundation depth and their interaction on native and invasive halophyte congeners in the context of SLR. With this aim, our study model focused on two cordgrass species (Genus *Spartina;* Poaceae) that colonize different habitats along the intertidal gradient in Californian salt marshes: 1) *Spartina densiflora* Brongn. is native from the southwest coast of South America and invasive on the Pacific Coast of North America (Bortolus 2006) and 2) *Spartina foliosa* Trin., the native cordgrass endemic to California. *S. densiflora* shows low genetic diversity and high phenotypic plasticity in its invaded range in North America (Castillo et al. 2014, 2016, 2018, Grewell et al. 2016) where it has become highly invasive in middle elevation salt marshes, brackish tidal wetlands and along tidal brackish to near freshwater rivers (Strong and Ayres 2013). In the San Francisco Estuary, *S. densiflora* may co-occur with *S. foliosa* (Ayres et al. 2003) where it colonizes

low salt marshes subjected to long flooding periods and anoxic sediments (Hinde 1954, Mahall and Park 1976). In this situation, hybridization processes have been described between the two species, being able to act both *S. foliosa* and *S. densiflora* as seed parent (Ayres et al. 2008).

To evaluate the performance of native *S. foliosa* and invasive *S. densiflora* along increasing salinity and inundation gradients, we designed and conducted an experiment to study the response of both under controlled environmental conditions in a glasshouse in Davis, California (USA). Our experiment also included the hybrid *S. densiflora x foliosa*, whose comparison with its parental species is reported *Chapter 7*. The species were randomized within aquatic mesocosms and subjected to the combination of four salinity treatments from freshwater to hypersalinity (0.5, 10, 20 and 40 ppt) crossed with three constant inundation depths (4.5, 35.5, and 55.0 cm deep) to assess response to predicted hypoxic effects of permanent deep flooding. We tracked growth and phenological development. After 31 days, we measured 38 biochemical, physiological and morphological plant traits to characterize the responses of both species. We hypothesized: 1) *S. foliosa* would be more tolerant than *S. densiflora* to flooding stress given its adaptation to low marsh habitats; 2) both cordgrass species would present high tolerance to salinity; and 3) the interaction between salinity and flooding stress would provoke particularly high stress levels on both species.

6.2 Material and Methods

Experimental design

Plants of *Spartina foliosa* were collected from low to middle brackish tidal marshes in the Carquinez Straits - North Bay (38°01'17"N 122°09'20"W), *Spartina densiflora* was collected from middle elevation brackish tidal marsh at Corte Madera Creek - Central Bay (37º56’27” N, 122º31’2” W) as well as the

hybrid *S. densiflora x foliosa* from low to middle marshes at Corte Madera Creek, region of the expansive San Francisco Estuary (California, USA) (see *Chapter 2*). At the Aquatic Weed Research Facility of the University of California (Davis, USA), rhizomes were separated from aerial tillers and roots, and cleaned to obtain similar-size experimental individuals according to the growth form of each taxon. On 3 March 2017, rhizomes were transplanted to pots (15 cm diameter x 17.5 cm height) with bottom drainage holes using sterile sand as substrate and grown until the beginning of the experiment. The base of the pots was kept flooded to sub-irrigate with 5 cm deep freshwater and nutrients were added as 10 ml of 40% Hoagland's nutrient solution pipetted onto the sediment surface of each pot once per week. Plants of each taxon were randomly assigned to experimental treatments. To avoid osmotic shock in the plants, the higher saline treatments were obtained increased 10 ppt salinity per week to gradually ramp up their exposure to the target experimental salinity level. After salinity conditioning, on 8 May 2017, plants were arranged in randomized complete block design within treatments nested within sixteen 500L (1.3 m x 0.8 m x 0.6 m) plastic mesocosms (Rubbermaid, Atlanta, GA). A split-plot full factorial experiment was designed in which the factor salinity was assigned at the different mesocosms as the main plot in a randomized complete block design and the factor inundation to the subplots with each of 3 taxa, including the parental species and the hybrid *S. densiflora x foliosa* (n = 4 plants per treatment) nested within each subplot. The salinity treatments ranged from freshwater to hypersalinity (0.5, 10, 20 and 40 ppt) and were created using sea salt Instant Ocean ® (Aquarium Systems Inc., Mentor, OH) plus 20% Hoagland's nutrient solution and Eco Pond Clear biological product (Grow More Inc., Gardena, CA) for reducing algae proliferation. Three permanent inundation treatments (shallow inundation (SI: 4.4 cm deep from the base of the pots), intermediate inundation (II: 35.5 cm deep) and deep inundation (DI: 55.0 cm deep)) were established by placing the plants on top of concrete block platforms at different heights inside each tank (Appendix 6.A). Glasshouse conditions were

maintained with controlled air temperature (21-25 ºC) and a 12 h daily photoperiod set with high intensity discharge lights (GE Lucalox LU1000/ ECO HPS 1000 W, PARsource, Petaluma, CA). The photon flux density measured by a LI-COR LI-250A light meter (LI-COR Inc., Lincoln, NE) was 500 μmol m^{-2} s^{-1} at the canopy level. The experiment was carried out for 31 days prior to initiation of live response measurements and harvest of biomass (initial and final display of the greenhouse in Appendix 6.B).

Pre-harvest Measurements

Phenology. The number of flowering tillers of each experimental plant was counted weekly in order to monitor the differences in the phenology and reproductive potential of the two *Spartina* taxa under the different flooding and salinity treatments.

Leaf gas exchange. Prior to harvest, measurements of net photosynthesis rate (A) and stomatal conductance (Gs) were carried out using a LI-COR 6400 portable infrared CO_2 analyzer (LI-COR Biosciences, Lincoln, NE, USA) in differential mode and in an open circuit. The CO_2 concentration inside the chamber was fixed at 400 μmol mol^{-1}, photon flux density was 1000 μmol m^{-2} s^{-1} (actinic light source 90% red and 10% blue) and flow rate 400 μmol s^{-1}. Before taking each measurement, one leaf per plant was wiped with a tissue wet with distilled water to remove the salt accumulated on the surface and left to dry. Each individual measurement was the mean of three subreplicates separated by a 10-second interval beginning when conditions stabilized inside the chamber. All measurements were conducted on sunny days (13-17 June 2017) within the two hours around solar noon. Intrinsic water use efficiency (WUE) was calculated as the ratio of A to Gs (Osmond et al. 1980).

Root and rhizome porosity. Aerenchyma presence, or porosity, of roots and rhizomes was determined following the method described in Kercher and Zedler (2004). Fragments of live roots and rhizomes of 5 cm length were gently

dried with a tissue and weighed to the nearest 0.001 g. The air volume in the plant fragments was then immediately extracted and substituted by water with a vacuum pump connected to a 250-ml flask with 200 ml of distilled water running for 5 min. Finally, the piece of root or rhizome was superficially dried with a tissue and weighed again. Porosity (%) was calculated as the difference between initial and final weight in relation to the initial weight.

Plant Tissue Chemistry. Fresh leaf tissue (0.5 – 2.0 g per plant) was collected prior to harvest and immediately frozen for later analyses of photosynthetic pigments and proline.

Post-harvest Evaluations

Biomass and growth. At the end of the experiment, the below-ground biomass (BGB) of each tussock was separated into roots and rhizomes, and the above-ground biomass (AGB) into leaves, tillers and inflorescences (inflorescences). Dry weights (DW; g) were obtained after oven-drying each fraction of biomass at 70 ºC for 48 h. The proportion (%) of each organ in relation to BGB or AGB and the AGB : BGB ratio were calculated (Castillo et al. 2008a). Root mass ratio (RMR) was also calculated as the proportion of roots (DW) in relation to the total clump biomass (Martin and Hine 2008). The number of tillers (live, dead and flowered) was counted for each clump at the beginning and at the end of the experiment. Tiller relative growth rate (TGR, tillers tillers^{-1} yr^{-1}) was calculated as the difference between the number of final and initial tillers divided by the number of experiment days (Castillo et al. 2010a). Lengths of tillers were measured for 5 adult tillers of each tussock at the end of the experiment. The final number of flowering tillers per plant was counted at harvest. As a measure of reproductive fitness (Kulheim et al. 2002), flowers were separated and the number of florets per plant was determined from the average number of florets per inflorescence for each taxa at each treatment (n = 1 inflorescence per plant) and the number of inflorescences per individual (n = 4 plant per treatment).

Leaf morphology. All leaf measurements were conducted on flag leaves (first unfolded adult leaf from the apical leaf) to avoid effects due to the leaf ontogeny. Leaf adaxial rolling was calculated as the percentage reduction in leaf width after rolling (Premachandra et al. 1993). Specific Leaf Area (SLA, m^{-2} g^{-1}) was determined for three leaves per plant as the ratio between the leaf area and its DW (Garnier et al. 2001). Leaf area was calculated using WinFOLIA (Regent Instruments Inc., Saint-Foy, Quebec, Canada) and DW was obtained after oven-drying the leaves at 70 ºC for 48 hours. Leaf Water Content (LWC) was obtained for one leaf per plant and LWC was calculated on the total leaf mass of the entire plant as LWC (%) = (FW − DW) × 100 / FW, where FW is the fresh weight (Castillo et al. 2007).

Leaf pigments, carbon and nitrogen content. Plant tissue samples (from frozen samples) were extracted in 80 % aqueous acetone. The extracts were centrifuged and supernatants were used for the determination of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids (Car) content from their absorbance at 664, 647 and 470 nm (Arnon 1949, Lichtenthaler 1987) using a spectrophotometer (Beckman DU-64, Beckman Coulter, Inc., Brea, CA, USA). Additionally, dry leaf tissue was ground to pass through a No. 40 mesh screen for nitrogen (N), carbon (C), Na, total non-structural carbohydrates (TNC) and glycinebetaine analyses. Total leaf N and C content were determined using a Perkin Elmer 2400 CHNS/O analyzer (Perkin Elmer, Waltham, MA, USA). The ratio C : N was calculated.

Leaf chemical stress. Free proline content in leaves was determined following the procedure in Bates et al. (1973). Glycinebetaine content in leaves was estimated as quaternary ammonium compounds following the protocol in Grieve and Grattan (1983). Leaf Na was measured using a sodium electrode on dry-ashes samples that were dissolved in 1 M HCl (Grewell et al. 2016). The leaf sodium (Na) exudation rate was measured following the protocol in Christman et al. (2009).

Subterranean resource storage. The total content of C, N and the C: N index of rhizomes were obtained as reported above for leaves. Rhizomes were analyzed for TNC using a modified enzymatic digestion procedure as detailed by Swank et al. (1982), followed by spectrophotometric assay for reducing sugars (Nelson 1944).

Statistical analyses

All statistical analyzes were performed using IBM SPSS V. 20 for Windows, applying a significance level (α) of 0.05 for every analysis. Data series were tested for homoscedasticity using the Levene's test and the traits SLA, leaf C, leaf C: N, leaf Na, proline, Gs and rhizome C: N were transformed using the function 1/x to meet the assumption of homogeneity of variances for parametric tests. Plant traits were classified into seven functional groups: (1) growth and biomass, (2) leaf morphology, (3) leaf pigments, C and N contents, (4) leaf chemical stress, (5) subterranean resource storage, (6) Root and rhizome porosity, and (7) leaf gas exchange. In order to avoid type I error (Scheiner 2001), the means of the dependent variables of each trait group were firstly compared using the multivariate analysis of variance (MANOVA) and Pillai's Trace to evaluate the significance of the factors: taxon (*S. densiflora* and *S. foliosa*), salinity (0.5, 10, 20 and 40 ppt) and inundation (shallow, intermediate and deep inundation). Once the multivariate significance was confirmed, the main univariate differences were evaluated for each functional plant trait with General Lineal Models (GLMs) and Bonferroni-Dunn's test as post hoc analysis. Principal Components Analysis (PCA) were conducted for both species independently in order to investigate the relationships between traits and to reduce the number of plant traits to characterize the response syndromes to abiotic stress. PCA was carried out analyzing the correlation matrix with 25 maximum iterations for convergence without rotation to extract independent PC factors with eigenvalues > 1. The PCA factors obtained for the response traits

of each species were correlated with salinity (ppt) and inundation (cm deep) treatments.

6.3 Results

Salinity, inundation, and their interaction had a significant effect on growth and biomass allocation traits (salinity: $F_{33,183} = 3.341$, $p < 0.001$, inundation: $F_{22,120} = 5.561$, $p < 0.001$, salinity x inundation: $F_{66,384} = 1.170$, $p < 0.001$), leaf morphology (salinity: $F_{9,192} = 6.483$, $p < 0.001$, inundation: $F_{6,126} = 5.907$ $p < 0.001$, salinity x inundation: $F1_{18,192} = 2.015$, $p < 0.05$), leaf pigments, C and N contents (salinity: $F_{24,198} = 7.518$, $p < 0.001$, inundation: $F_{16,130} = 6.085$, $p < 0.001$, salinity x inundation: $F_{48,414} = 1.483$, $p < 0.05$), leaf chemical stress (salinity: $F_{12,189} = 21.638$, $p < 0.001$, inundation: $F_{8,124} = 8.124$, $p < 0.001$, salinity x inundation: $F_{24,256} = 1.888$, $p < 0.01$) and subterranean resource storage (salinity: $F_{15,210} = 5.592$, $p < 0.001$, inundation: $F_{10,138} = 5.744$, $p < 0.001$, salinity x inundation: $F_{30,360} = 1.924$, $p < 0.01$). Root and rhizome porosity was affected just by inundation ($F_{4,144} = 2.412$, $p < 0.05$) and foliar gas exchange just by salinity ($F_{12,192} = 3.141$, $p < 0.001$) (MANOVA, Appendix 6.C).

Growth and biomass allocation. GLMs for plant traits related to growth and biomass revealed that *S. densiflora* had ca. 3 times more AGB than *S. foliosa* in all treatments, except under DI at 10 ppt salinity ($F_{6,60} = 2.412$, $p < 0.05$). AGB of both species decreased ca. 70% from SI to DI, *S. foliosa* showing similar AGB under SI and II, whereas AGB of *S. densiflora* linearly decreased with inundation depth ($F_{2,60} = 12.392$, $p < 0.001$). The effect of salinity on AGB was only significant for *S. densiflora* that reduced 40% its AGB ($F_{3,60} = 3.069$, $p < 0.05$) (Fig. 6.1A). However, the percentage of leaves, tillers and inflorescences biomass of the AGB was no affected by salinity for *S. densiflora*. By contrast, the proportion of leaves of *S. foliosa* was halved at hypersalinity under DI ($F_{6,60} = 2.699$, $p < 0.05$), and the percentage of inflorescences biomass was reduced 70% at hypersalinity independently of inundation ($F_{3,60} = 4.623$, $p < 0.01$) in

favor of tillers biomass that was maximum (63% of AGB) at 40 ppt ($F_{3,60}$ = 9.827, $p < 0.001$). In relation to inundation effects, it was observed the same decrease in inflorescences ($F_{2,60}$ = 5.398, $p < 0.01$) and increase in tillers biomass ($F_{2,60}$ = 25.505, $p < 0.001$) for both species and the mentioned reduction in leaves for *S. foliosa* with stress. Thus, *S. foliosa* changed from having 10% more proportion of leaves ($F_{6,60}$ = 2.699, $p < 0.05$) and the same of tillers than *S. densiflora* in the absence of stress (SI-0.5 ppt) to not significant differences in leaves and 20% more proportion of tillers ($F_{6,60}$ = 2.507, $p < 0.05$) at maximum stress (DI-40 ppt). The percentage of inflorescences biomass went from being similar for both species at 0.5 ppt to higher for *S. densiflora* (16 ± 2%) than *S. foliosa* (3 ± 2%) at 40 ppt ($F_{3,60}$ = 4.623, $p < 0.01$) (Appendix 6.D).

Regarding BGB, inundation depth reduced it by ca. 10% for both species ($F_{2,60}$ = 25.550, $p < 0.001$) (Fig. 6.1B). *Spartina densiflora* BGB doubled that of *S. foliosa*, being the percentage of roots biomass higher for *S. foliosa* (48 ± 1% versus 41 ± 1% for *S. densiflora*) ($F_{1,60}$ = 368.803, $p < 0.001$) and therefore rhizomes biomass higher for *S. densiflora* ($F_{1,60}$ =18.351, $p < 0.001$) (Appendix 6.D). AGB: BGB ratio was halved at hypersalinity in relation to freshwater ($F_{3,12}$ = 17.444, $p < 0.001$), as well as under DI in relation to SI ($F_{2,60}$ = 32.158, $p < 0.001$), being generally higher for *S. densiflora* (0.72 ± 0.04) than *S. foliosa* (0.60 ± 0.04) ($F_{1,60}$ = 8.3605, $p < 0.01$) (Fig. 6.1 C). On the contrary, RMR was lower for *S. densiflora* (0.24 ± 0.01) than *S. foliosa* (0.31 ± 0.01) ($F_{1,60}$ = 22.142, $p < 0.001$), increasing ca. 30% under DI in relation to SI ($F_{2,60}$ = 8.344, p = 0.001) (Fig. 6.1D). Tillers length was more affected by inundation depth (47% reduction) ($F_{2,60}$ = 28.290, $p < 0.001$) than salinity (38%) ($F_{3,12}$ = 44.141, $p < 0.001$) for *S. foliosa* while both factors reduced 30% *S. densiflora* tillers length, always remaining *S. densi*flora taller than *S. foliosa* ($F_{1,60}$ = 1,4.197, $p < 0.001$) (Fig. 6.1E). TGR decreased with increasing salinity for *S. densiflora* from 0.257 ± 0.064 to -0.007 ± 0.150 tillers tillers^{-1} yr^{-1}, showing *S. foliosa* its maximum (0.291 ± 0.089) at 10 ppt, and minimum at 40 ppt (-0.047 ± 0.172). TGR was also negatively affected by inundation, except at hypersalinity for both species

and 10 ppt for *S. foliosa* ($F_{6,60} = 2.816$, $p < 0.05$). *S. foliosa* showed higher TGR than *S. densiflora* (Fig. 6.1F). In contrast, *S. densiflora* exhibited greater pre-dispersion reproductive fitness (number of florets per plant) than *S. foliosa*, except under DI since it decreased with inundation depth for *S. densiflora* from 2904 ± 325 to 611 ± 123 florets, matching the lower and constant values of *S. foliosa* ($F_{2,60} = 7.844$, $p < 0.001$) (Fig. 6.1G).

***Leaf morphology*.** The only interspecific difference in leaf morphological traits was observed for the greater leaf rolling of *S. densiflora* (25 ± 1%) than *S. foliosa* (14 ± 1%). *S. densiflora* doubled and *S. foliosa* (except under DI) increased up to 6 times their leaf rolling with increasing salinity, however, it decreased for both species with increasing inundation, except at freshwater for both species and hypersalinity for *S. densiflora* ($F_{6,57} = 2.461$, $p < 0.05$) (Fig. 6.1H). A general reduction of 20% was recorded for SLA at hypersalinity ($F_{3,13} = 3.834$, $p < 0.05$) (Fig. 6.1I) and LWC did not show any significant change (Appendix 6.D)

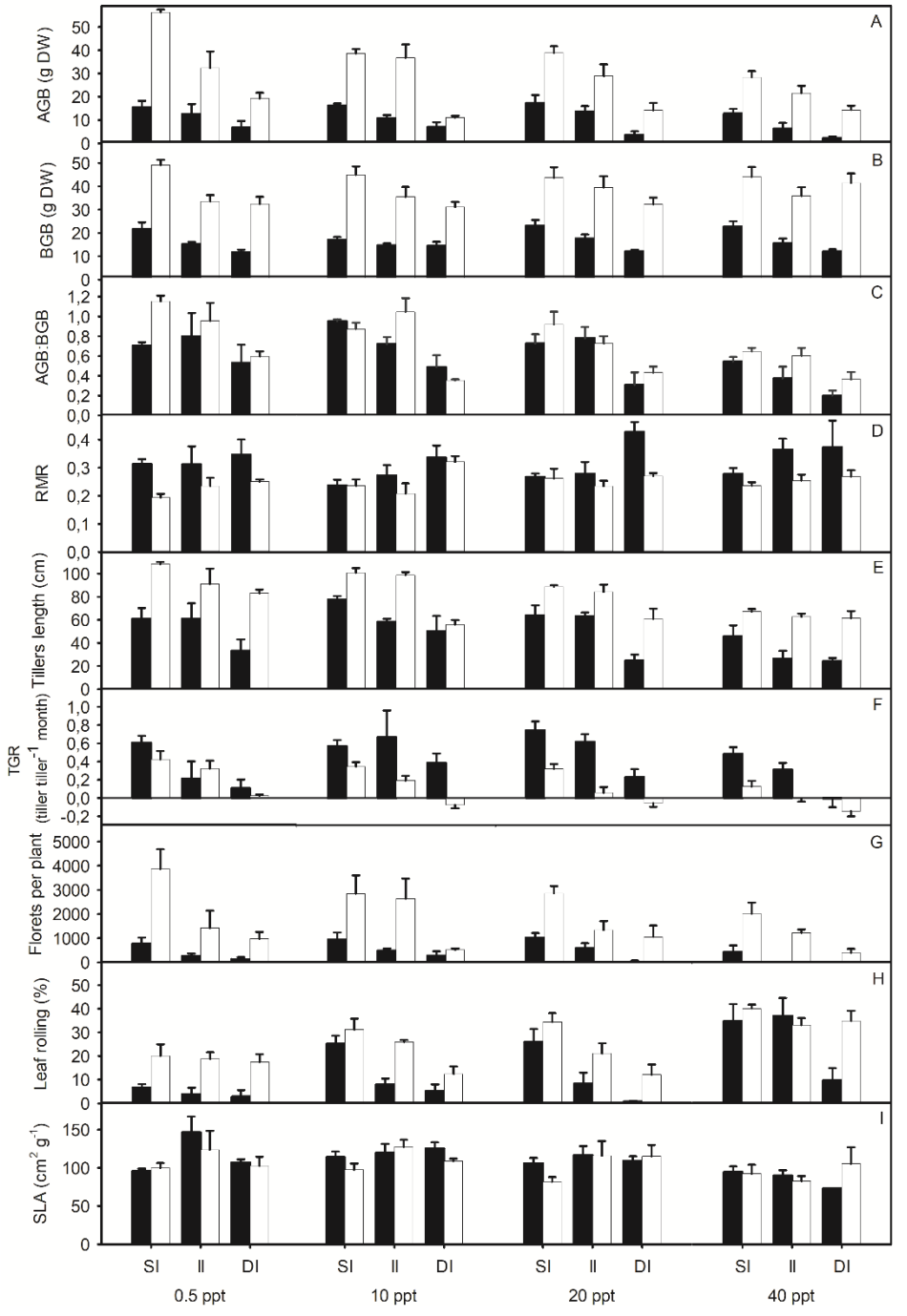

Fig. 6.1. Growth and biomass allocation and leaf morphology: above-ground biomass (AGB), below-ground biomass (BGB), AGB : BGB ratio, root mass ratio (RMR), tillers length, tiller growth rate (TGR), number of florets per plant, leaf rolling and specific leaf area (SLA) for *Spartina foliosa* (black bars) and *S. densiflora* (white bars) under different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (SI, shallow inundation (4.4 cm deep); II, intermediate inundation (35.5 cm deep); DI, deep inundation (55.0 cm deep)). Values are mean ± SE (n = 4).

Leaf pigments, C and N contents. Both salinity and inundation caused a decrease in Chl *a*, Chl *b* and Car of 30, 50 and 20% for *S. densiflora* and 52, 62 and 47%, for *S. foliosa* respectively, but the content of Chl *a* did not vary with inundation depth for *S. densiflora* (GLM, $p < 0.05$). As a consequence, the greater general pigmentary content of *S. foliosa* than *S. densiflora* was not significant at extreme stress levels (Chl *a*: $F_{1,60} = 12.266$, $p = 0.001$; Chl *b*: $F_{1,60} = 21.755$, $p < 0.001$; Car: $F_{1,60} = 25.209$, $p < 0.001$) (Fig. 6.2 A, B, C). On the contrary, *S. densiflora* presented higher Chl : Car ratio ($F_{1,60} = 18.847$, $p < 0.001$) and Chl *a* : Chl *b* under DI ($F_{2,60} = 4.625$, $p < 0.05$) and at high salinity ($F_{3,60} = 4.011$, $p < 0.05$) than *S. foliosa.* Chl : Car ratio decreased 10% and Chl *a* : Chl *b* increased 25% with saline stress for both species ($F_{3,12}$, $p < 0.001$). As for the pigmentary content, foliar C content decreased with both salinity ($F_{3,12} = 61.642$, $p < 0.001$) and inundation ($F_{2,59} = 19.021$, $p < 0.001$), while N content varied just with inundation ($F_{2,59} = 5.040$, $p < 0.05$) (Fig. 6.2 D, E). The reduction of leaf C was slight (5% with salinity and 2% with inundation), so the reduction of 14% leaf N derived in the increment in C : N ratio with inundation ($F_{2,60} = 4.405$, $p < 0.05$). Despite changes among treatments, *S. densiflora* maintained higher leaf C content (436.2 ± 0.9 mg g^{-1}) and lower N content (20.1 ± 0.3 mg g^{-1}) than *S. foliosa* (422.7 ± 0.9 and 23.2 ± 0.3 mg g^{-1}, respectively) (leaf C: $F_{1,59} = 105.39$, $p < 0.0001$; leaf N: $F_{1,59} = 41.41$, $p < 0.0001$) (Fig. 6.3 A, B, C).

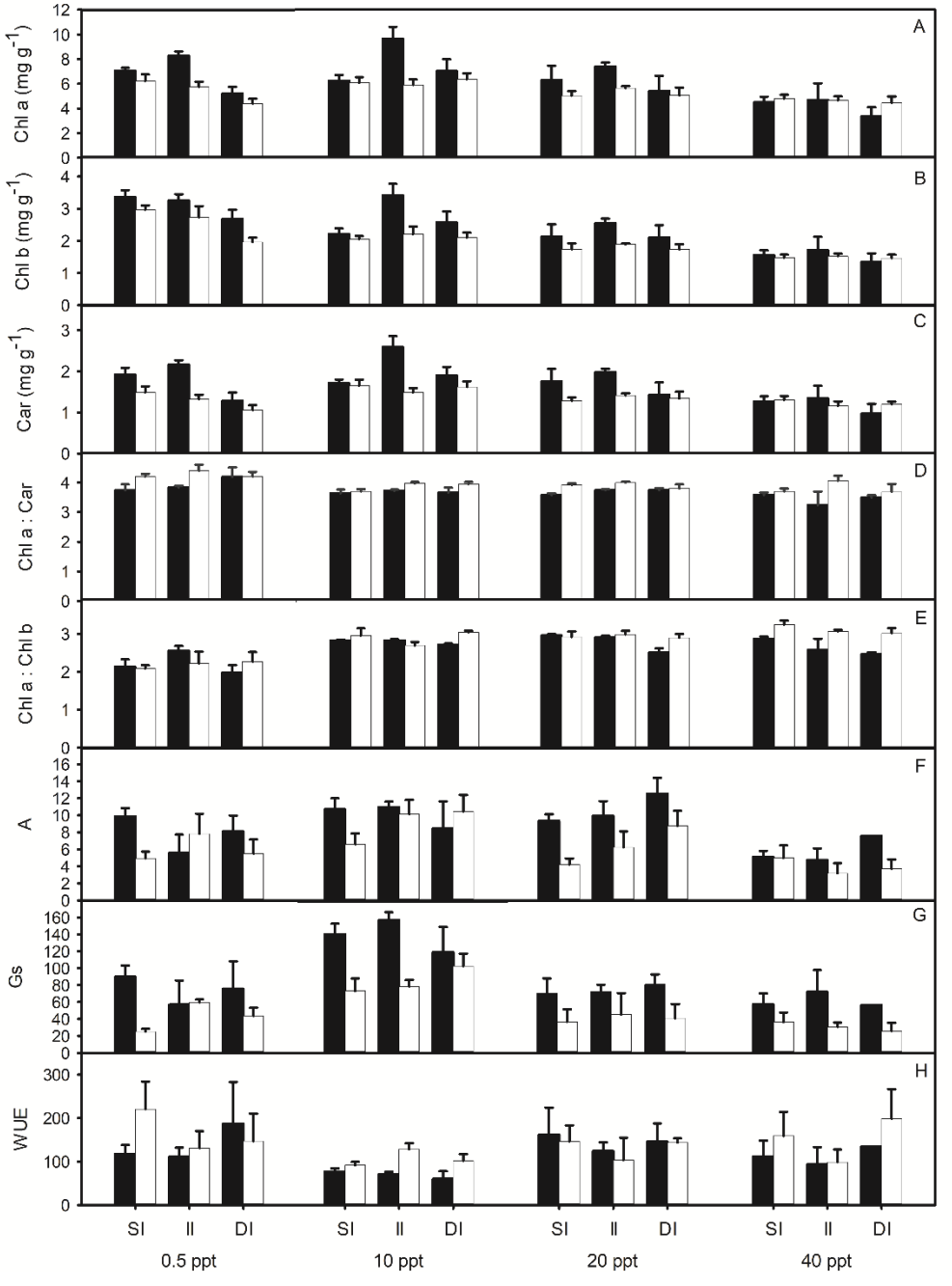

Fig. 6.2. Leaf photosynthetic pigments and gas exchange traits: chlorophyll *a* (Chl. *a*), chlorophyll *b* (Chl. *b*), carotenoids (Car), Chl a : Car, Chl *a* : b, net photosynthesis rate (A), stomatal conductance (Gs) and water use efficiency (WUE) for *Spartina foliosa* (black bars) and *S. densiflora* (white bars) under different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (SI, shallow inundation (4.4 cm deep); II, intermediate inundation (35.5 cm deep); DI, deep inundation (55.0 cm deep)). Values are mean ± SE (n = 4).

Leaf chemical stress. Leaf Na content increased with NaCl concentration 2.5 times for *S. densiflora* and 5 times for *S. foliosa* at 40 ppt. Interspecific differences were found just under DI, varying from being higher for *S. densiflora* (6.0 ± 0.5 mg g^{-1}) than *S. foliosa* (3.8 ± 0.4 mg g^{-1}) at 0.5 ppt-DI to being lower for *S. densiflora* (13.3 ± 0.6 mg g^{-1}) than *S. foliosa* (28.3 ± 0.04 mg g^{-1}) at 40 ppt-DI ($F_{6,60}$ = 3.477, $p < 0.001$) (Fig. 6.4A). Although both species increased leaf Na excretion rate with salinity, *S. foliosa* showed a 50 and 70% higher excretion rate than *S. densiflora* at 20 and 40 ppt, respectively ($F_{3,54}$ = 7.365, $p < 0.001$). In general, Na excretion was greater under DI for both taxa ($F_{2,54}$ = 7.644, p = 0.001) (Fig. 6.4B). Both salinity and inundation induced increments in glycinebetaine and proline leaf contents. Proline content increased with salinity for both species ($F_{3,60}$ = 4.570, $p < 0.01$), but only increased under DI in relation to SI for *S. foliosa* ($F_{2,60}$ = 5.926, $p < 0.01$). Glycinebetaine content was ca. 25% higher for *S. foliosa* than *S. densiflora* ($F_{1,57}$ = 83.837, $p < 0.001$) and proline content was also greater for *S. foliosa* at all salinities under DI and at all inundations at freshwater (Fig. 6.4 C, D).

Subterranean resource storage. Contrasted responses were obtained for rhizomes carbon content decreasing 10% with increasing salinity ($F_{3,12}$ = 17.624 $p < 0.001$) and increasing 2% with inundation ($F_{2,60}$ = 4.865, $p < 0.05$) for both species (Fig. 6.3D). Nitrogen content in rhizomes increased with salinity under SI and decreased with inundation at 40 ppt ($F_{6,60}$ = 5.096, $p < 0.001$) (Fig. 6.3E). The higher rhizomes C content and the lower N content of *S. densiflora* in relation to *S. foliosa* ($F_{1,60}$, $p < 0.001$) resulted in 2 times higher C : N ratio under all treatments ($F_{1,60}$ = 601.498, $p < 0.001$) (Fig. 6.3F). Inundation reduced 65% rhizomes TNC for *S. densiflora* and 30% for *S. foliosa*, resulting in higher TNC content of *S. densiflora* (52.6 mg g^{-1}) than *S. foliosa* (28.5 mg g^{-1}) under DI ($F_{2,60}$ = 3.640, $p < 0.05$) (Fig. 6.3G,H).

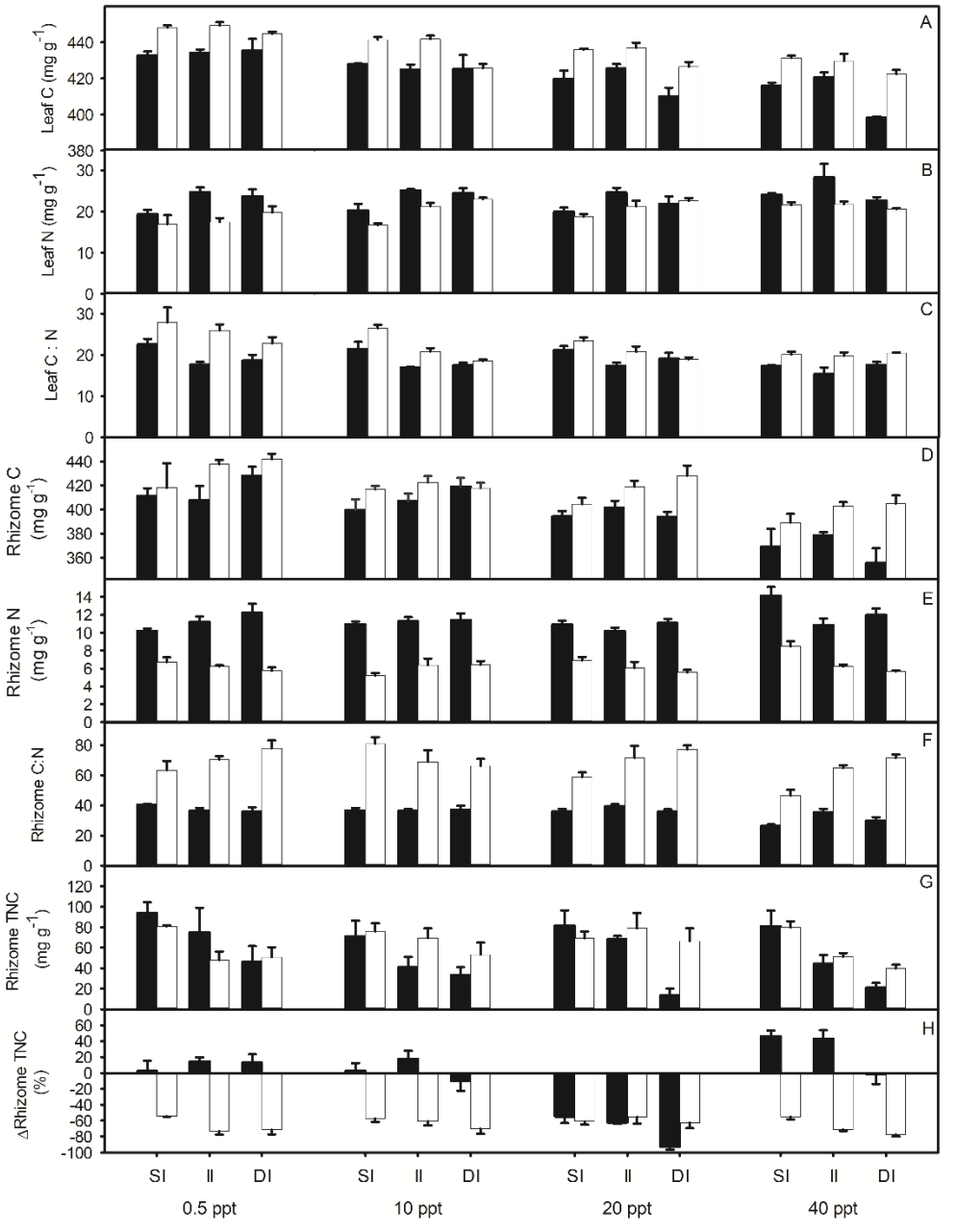

Fig. 6.3. Leaf and rhizome tissue nutrient responses: carbon (C) and nitrogen (N) concentrations and C : N ratio for leaves and rhizomes and total non-structural carbohydrates (TNC) and change in total non-structural carbohydrates (TNC) during the experiment in the rhizomes of *Spartina foliosa* (black bars) and *S. densiflora* (white bars) at different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (SI, shallow inundation (4.4 cm deep); II, intermediate inundation (35.5 cm deep); DI, deep inundation (55.0 cm deep)). Values are mean ± SE (n = 4).

Root and rhizome porosity. Rhizome porosity was independent of inundation depth and it was higher for *S. foliosa* (34 ± 3%) than *S. densiflora* (6 ± 1%) ($F_{1,60}$ = 84.253, p < 0.001) (Fig. 6.4E). Root porosity increased with inundation depth for *S. densiflora* (from 42 to 55%) and at both extremes of the inundation gradient for *S. foliosa* (SI = 49, II = 31 and DI = 45%), *S. densiflora* exhibiting higher root porosity than *S. foliosa* under II and DI ($F_{2,60}$ = 5.250, p < 0.01) (Fig. 6.4F).

Leaf gas exchange. A strong influence of salinity was observed on A ($F_{3,13}$ = 6.110, p < 0.01) for both species (Fig. 6.2F). The drop of A coincided with reductions in Gs ($F_{3,14}$ = 6.395, p < 0.01) (*S. densiflora*: r = +0.538, p < 0.0001; *S. foliosa*: r = +0.635, p < 0.0001), Chl *a* (*S. densiflora*: r = +0.322, p < 0.05; *S. foliosa*: r = +0.423, p < 0.01), Car (*S. densiflora*: r = +0.311, p < 0.05; *S. foliosa*: r = +0.421, p < 0.01) and Chl *b* for *S. foliosa* (r = +0.398, p = 0.01). Thus, A showed maximum values at intermediate salinities (10 ppt), dropping 20% in relation to 0.5 ppt and 50% in relation to 40 ppt. In general, *S. foliosa* presented 30% higher A and 50% Gs than *S. densiflora* ($F_{1,53}$, p < 0.05) (Fig. 6.2G). WUE did not show significant differences between species nor treatments (Fig. 6.2H).

F-statistics and P-values of all the GLMs for the 36 traits measured are summarized in Appendix 6.E.

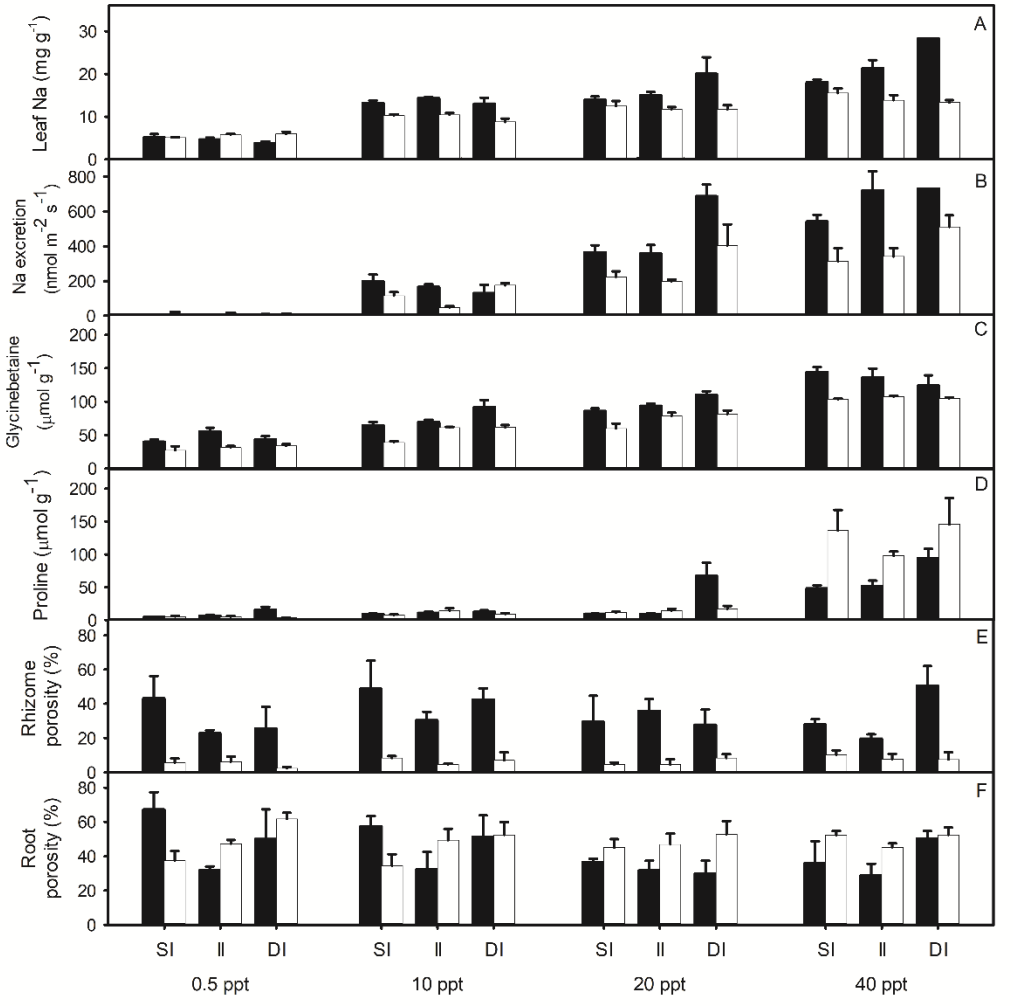

Fig. 6.4. Chemical stress and porosity responses: Na content, Na excretion rate, glycinebetaine and proline contents for leaves and rhizome and root porosity of *Spartina foliosa* (black bars) and *S. densiflora* (white bars) at different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (SI, shallow inundation (4.4 cm deep); II, intermediate inundation (35.5 cm deep); DI, deep inundation (55.0 cm deep)). Values are mean ± SE (n = 4).

Relationships between plant traits and abiotic factors

The PCA for *S. foliosa* traits yielded ten factors, explaining 86.7% of variance. PC1-*Sf* explained 31.1% of variance and it was positively correlated with growth and biomass traits (AGB, percentage of inflorescences biomass, AGB : BGB, tillers length, TGR and florets per plant), pigments contents (Chl *a*, Chl

b, Car and Chl *a*+*b*), leaf C content and subterranean resource storage traits (content and increment of rhizomes TNC). PC1-*Sf* was negatively correlated with the percentage of tillers biomass and leaf chemical stress traits (leaf Na, glycinebetaine and proline contents, and Na excretion) and also with salinity (r = -0.705, $p < 0.001$, n = 48) and inundation depth (r = -0.498, $p < 0.001$, n = 48). The second factor (PC2-*Sf*; explaining 14.5% of variance) was positively correlated with BGB and leaf rolling, and it increased with salinity (r = +0.526, $p < 0.001$, n = 48) but decreased with inundation depth (r = -0.524, $p < 0.001$, n = 48) (Fig. 6.5).

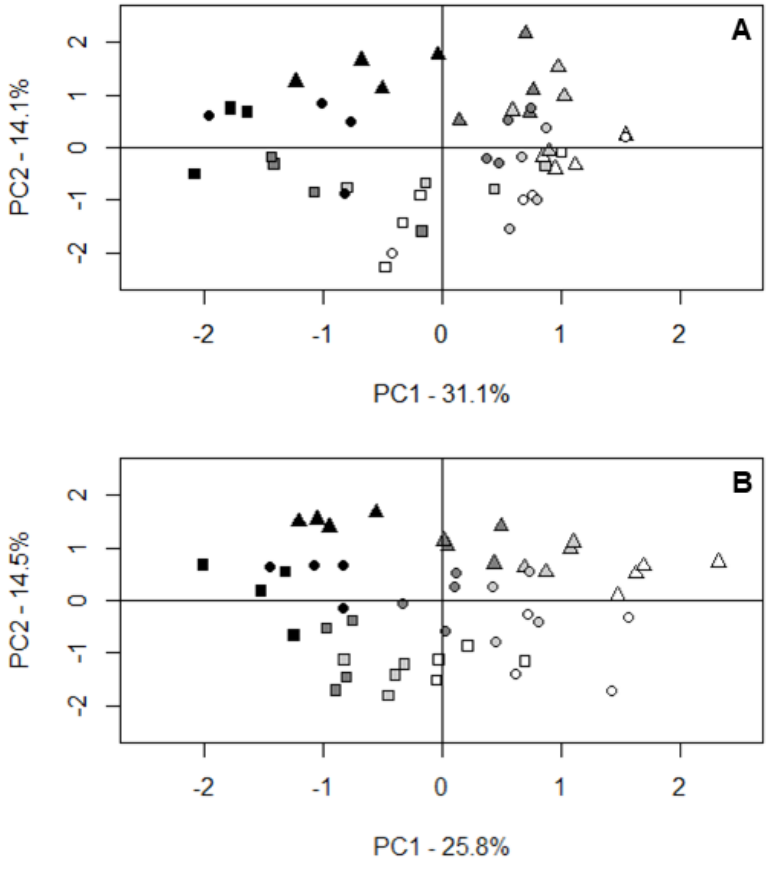

Fig. 6.5. Principal Components Analysis (PCA) plot for *Spartina foliosa* (A) and *Spartina densiflora* (B) responses (n = 35 traits) under different salinities (0.5 ppt, white; 10 ppt, light grey; 20 ppt, dark grey; 40 ppt, black) and inundation depths (shallow (4.4 cm deep), triangle; intermediate (35.5 cm deep), circle; deep (55.0 cm deep), square).

Ten factors were also determined from the PCA for *S. densiflora* traits, explaining 83.3% of the total variance. PC1-*Sd* (explaining 25.8% of variance) was positively correlated with growth and biomass traits (AGB, AGB : BGB, tillers length, TGR and florets per plant), pigments contents (Chl *b*, Car, Chl *a*+*b*), leaf C content and leaf C : N ratio, and negatively with leaf N content and leaf chemical stress traits (leaf Na, glycinebetaine and proline contents and Na excretion). PC1-*Sd* decreased with increasing salinity ($r = -0.809$, $p < 0.001$, $n = 48$) and inundation depth ($r = -0.494$, $p < 0.001$, $n = 48$). PC2-*Sd* explained 14.1% of variance, being positively correlated with the percentage of inflorescences biomass, BGB and leaf rolling and negatively with tillers biomass. PC2-*Sd* increased with salinity ($r = +0.467$, $p < 0.001$, $n = 48$) and decreased with inundation depth ($r = -0.739$, $p < 0.001$, $n = 48$) (Fig. 6.5, Appendix 6.F).

6.4 Discussion

Our study focused on the comparative analysis that the effect of SLR will have on native and invasive cordgrass species in relation to changes in the patterns of salinity and waterlogging. According to our results, some plant traits varied only with one stress factor but the majority changed in the same direction when increasing both salinity and flooding depth with some being intensified by their interaction. Only three of the recorded plant traits changed in opposite ways to both stress factors (Table 6.1).

Responses to salinity

Salinity was the only abiotic factor affecting A, Gs, SLA and leaf Na content for both species. The reduction in A at freshwater and hypersalinity in relation to brackish conditions (10 ppt salinity) and the higher A values for *S. foliosa* than *S. densiflora* at every salinity coincided with what was previously reported

for these cordgrasses (i.e. Pearcy and Ustin 1984, Grewell et al. 2016). The falls of A were greater at hypersalinity (-50%) than at freshwater (-20%), which is consistent with these species being facultative halophytes (Phleger 1971, Pearcy and Ustin 1984, Nieva et al. 2001, Castillo et al. 2005b). The above-mentioned decreases in A were related to lower Gs, keeping similar WUE. Total or partial stomatal closure is a frequent consequence of high salt stress to minimize water loss (Parvaiz and Azooz 2013) and moderate Gs reductions at freshwater have also been recorded for other C4 halophytes (Ball and Farquhar 1984, Pearcy and Ustin 1984, Maricle and Lee 2006). Nevertheless, the falls in Gs (-48% at freshwater and -60% at hypersalinity) were higher than those in A what is frequent in plants with C4 metabolism since they accumulate C compounds in bundle sheath cells that allow them to make photosynthesis during stomatal closure (Hatch 1992). Chl *a* and Car contents showed also a direct correlation with A for both species. Photosynthetic pigment contents are frequently reduced under salt stress (Parida and Das 2005) as it was previously found for *S. densiflora* in North America (Grewell et al. 2016) and *S. maritima* in South Africa (Adams and Bate 1995). Chlorophyll content is commonly used as an indicative to the ability to photosynthesize as they are the main pigments responsible for photosynthesis (Janousek and Mayo 2013). In this sense, *S. foliosa* exhibited higher Chl and Car contents than *S. densiflora* except at hypersalinity, which was reflected in higher A. Moreover, *S. foliosa* showed also lower Chl : Car and Chl *a* : Chl *b* ratios than *S. densiflora*, denoting a greater proportion of accessory pigments related to higher ability to protect the photosynthetic apparatus (Demmig-Adams and Adams 1996).

Another factor that was only affected by salinity was the decrease in SLA for both taxa, probably related to the accumulation of salts in the tissue (Brugnoli and Björkman 1992). In addition, leaf Na content increased with salinity, being higher for *S. foliosa* (even showing higher Na exudation rates) than for *S. densiflora*. Despite the deleterious effect of Na in plant tissue (Yeo

et al. 1985), some halophytes are capable to accumulate high Na contents via compartmentalization into vacuoles (Geissler et al. 2009).

Responses to inundation depth

Inundation was the only abiotic factor determining BGB traits and floret production. BGB and rhizome TNC decreased, and RMR and roots porosity increased under deeper inundation for both species. *Sporobolus virginicus* (L.) Kunth also reduced its BGB with increasing flooding, being independent of salinity (Naidoo and Mundree 1993). *S. foliosa* exhibited higher proportion of root biomass and 5 times higher rhizome porosity than *S. densiflora*, and *S. densiflora* showed higher total BGB, greater root porosity and rhizome TNC under DI. Keeping high root biomass when inundated is especially important because flooding frequently reduces nutrients uptake rate (Pezeshki 2001). In relation to the changes in root porosity, aerenchyma development is one of the main responses to flooding stress, facilitating oxygen diffusion to roots (Armstrong and Gaynard 1976, Pezeshki 2001). Rhizome porosity also contributes to tolerate anoxia (Armstrong et al. 1996, Sharma et al. 2008b), being probably less plastic than root porosity in the process of flooding acclimation since rhizomes have less turnover than roots. This would limit the acclimation capacity of *S. densiflora* to flooding stress as reported for *Spartina patens* (Aiton) Muhl (Burdick 1989). Regarding rhizomes TNC, inundation stress frequently causes a decrease in the transport of carbohydrates to the BGB, but TNC reserve is key to obtain energy by anaerobic fermentation (Chen and Qualls 2003, Chen et al. 2013) in situations of hypoxia (Sharma et al. 2008a, 2008b). Inundation stress also induced a reduction in floret production for *S. densiflora* (from 2904 ± 325 to 611 ± 123 floret per plant), while it remained low for *S. foliosa* (417 ± 67 floret per plant) so that both species did not show differences at DI. Many species show a reduction in the production of seeds when exposed to prolonged inundation (i. e. Blom et al. 1990; Boem et al. 1996).

It was remarkable that both species kept similar A values under increasing inundation depth, the submergence of their photosynthetic tissues would shrink their carbon fixation through light and CO_2 limitations (Castillo et al. 2000). However, at intermediate salinities, both species showed a tendency to increase A at deeper inundations independently of their pigment contents and Gs, which can be interpreted as physiological compensation mechanism to submersion by higher A probably related to higher Rubisco activity reflected in higher leaf N contents (Zhang et al. 2016).

Combined effects of salinity and inundation

The rest of the recorded plant traits were generally affected by both saline and inundation stress, but only *S. densiflora* suffered a significant reduction of AGB with increasing salinity whereas AGB of *S. foliosa* was always low and independent of salinity. The allocation to tillers biomass increased and that of inflorescences decreased with increasing stress levels for both species, and *S. foliosa* also reduced its higher allocation to leaves at hypersalinity and DI. A reduction in the number of leaves with stress has been found for several wetland species under flooding (Begum et al. 2006) as well as at high salinities (Gulzar et al. 2003), which generally contributes to the reduction of biomass production (Chapman and Lemaire 1996, Redondo-Gómez et al. 2007). Tiller length and TGR have been used as indicators of vegetative fitness (Pyšek and Richardson 2008, Castillo et al. 2010a, Lee et al. 2016), both being influenced by saline and flooding stress in our experiment. Tiller length was always higher for *S. densiflora* than *S. foliosa*, which would enable the invasive species to keep a higher proportion of aerial tissues out water, facilitating oxygen transport below ground (Naidoo and Mundree 1993), light collection (Burdick et al. 2001) and CO_2 fixation (Castillo et al. 2005a). In contrast, TGR was higher for *S. foliosa* than *S. densiflora*, reflecting higher lateral expansion rates. In this sense, *S. foliosa* was able to keep positive TGR even under DI (except at hypersalinity),

while *S. densiflora* presented negative values. Reduction in tiller production induced by salinity and inundation has been previously observed for *S. densiflora* (Di Bella et al. 2014) as well as for other wetland species (Naidoo and Mundree 1993; Adams and Bate 1995). In addition, the combined effect of both stresses led to the maximization of leaf chemical stress indicators. *S. foliosa* showed higher glycinebetaine content than *S. densiflora* that exhibited higher content of proline at hypersalinity and DI. The accumulation of organic solutes under salt stress results in an increase of osmolarity maintaining cell turgor, enzyme and membrane integrity (Kavi-Kishor et al. 2005, Ashraf and Foolad 2007). Proline content decreased under flooded conditions for *S. alterniflora* and *S. patens* (Naidoo et al. 1992), but it is known to be promoted in other species under inundation, with the role of detoxifying free radicals (Yan et al. 1996, Yordanova and Popova 2007). As in our study, different N-containing compounds (NCC), among them proline and glycinebetaine, accumulated in leaves of salt and flood stressed plants with functions related to osmoregulation, storage of N, detoxification and enzyme protection (Parida and Das 2005; Chen et al. 2010). In relation to foliar C content, it has been found to be reduced in the halophyte *Aster tripolium* L. under saline stress (Geissler et al. 2009) and in *Ricinus communis* L. under inundation (Gadallah 1995). Consistently to this, lower C : N ratio was found for both species with increasing salinity and inundation depth. It is remarkable that no variations were observed in leaf C, N nor C : N ratio with inundation depth at hypersalinity, nor with salinity at DI, since the extreme stress of one of these abiotic factors masked the response to the other factor (Ritchie 2000, Harley and Helmuth 2003).

An opposite variation was found for leaf rolling, rhizome C content, and C : N ratio when increasing salinity and reducing inundation depth or *vice versa*. For example, leaf rolling reduces transpiration and minimizes leave exposure to light avoiding photoinhibition (Heckathorn and DeLucia 1991, Kadioglu and Terzi 2007) and it increased with salinity (when *S. foliosa* equaled the greater leaf rolling of *S. densiflora*) and decreased under deeper inundation. These plant

traits responding in opposite ways when exposed to individual stresses would limit species tolerance to the interaction between those abiotic factors whose interaction results in intermediate and non-optimal responses for any of both stresses (Visser et al. 2016).

Plant response syndromes to salinity and inundation depth

According to our results, the native halophyte *S. foliosa* from low to middle marshes and the invasive *S. densiflora* from middle marshes of the Pacific Coast of North America exhibited contrasting responses to salinity and inundation and the combination of both abiotic factors, which were related to the different salt marsh habitats from they were sampled. The syndrome of *S. foliosa* along broad gradients of salinity and inundation depth was characterized by keeping relatively low AGB with high allocation to leaves and prioritizing asexual reproduction in the form of production of new lateral tillers over sexual reproduction, maintaining high photosynthesis rates (except at hypersalinity) related to high photosynthetic pigments and stomatal conductance, high N contents accumulating glycinebetaine in leaves (that increased with both stresses), developing great and constant rhizome porosity and high root biomass (that increased together with their porosity with inundation depth) and keeping high quality of subterranean reserves (low rhizome C : N ratio) (Table 6.1). This combination of plant traits would enable *S. foliosa* to colonize and expand on bare mud flats of low salt marshes with anoxic sediments exposed to long submersion periods and short photoperiods, where *S. foliosa* behaves as a primary colonizer (Hinde 1954, Mahall and Park 1976). *Spartina foliosa* was particularly impacted by the interaction between high salinities (20-40 ppt) and deeper inundations (DI), where its biomass allocation to leaves, leaf and rhizome C content and rhizome TNC decreased distinctly at the same time that its floret set was inhibited. Hypoxia under saline conditions may cause serious ion imbalances related to diminished growth rates (Barrett-Lennard and Shabala

2013). These responses portray native *S. foliosa* as a slow-growing and stress-tolerant species (Grime 1977, 2006).

By contrast, the syndrome of *S. densiflora* was typified as rapid-growth in the absence of stress, high sexual reproduction, great AGB and BGB (specially rhizomes with low porosity) accumulation with tall tillers, and high leaf rolling, root porosity and proline content in leaves under extreme conditions (hypersalinity and/or DI) as expenses of spending its below-ground reserves (Table 6.1). Thus, the invasive *S. densiflora* behaved as a fast-growing species with high competitive ability and able to take advantage of low stress conditions. However, it also shows mechanisms that allow it to tolerate some saline and waterlogging stress. This high versatility of *S. densiflora* matches with its reported high phenotypic plasticity (Castillo et al. 2014, 2018, Grewell et al. 2016), but also its invasiveness given the advantage to act as an opportunistic species under low stress episodes and as a relative stress-tolerant species under high stress conditions.

6.5 Conclusions

For all the above, in view of the intensification of salinity and inundation depth in salt marshes by the SLR foreseen with climatic change (Stralberg et al. 2011, Parker et al. 2011, IPCC 2015), established populations of both cordgrass species would reduce their vegetative and sexual reproductive fitness. *S. foliosa* would be endangered in nowadays low marshes due to increasing permanent inundation depths, but it could response reaching upper elevations in the intertidal gradient by lateral expansion, having its capacity to colonize new locations limited by its constant low floret production. On the other hand, *S. densiflora* is likely to reduce its invasiveness since it will accumulate less biomass and produce fewer florets, which is key in the spread of its invasive populations (Kittelson and Milton 1997, Nieva et al. 2001).

Table 6.1. Summary of responses of 38 plant traits of *Spartina foliosa* and *S. densiflora* to salinity and inundation gradients and their interaction, indicating the species showing higher general mean values. Increase, light grey; Decrease, dark grey; "-" = no significant difference.

Plant traits	**Species**	**Salinity** (0.5 -> 40 ppt)		**Inundation depth** (4.4 -> 55.0 cm)		**Salinity x inundation**	
		S. foliosa	*S. densiflora*	*S. foliosa*	*S. densiflora*	*S. foliosa*	*S. densiflora*
Leaf biomass (% AGB)	**Sf > Sd**	-	-	-	-	under DI and 40 ppt	-
Tiller biomass (% AGB)	Sf < Sd	↑	-	↑	↑	with salinity under II and DI and with inundation at 10, 20 and 40 ppt	with inundation at 10, 20 and 40 ppt
Inflorescence b. (% AGB)	Sf < Sd	↓	-	↓	↓	-	-
Above-ground biomass	Sf < Sd	-	↓	↓	↓	with inundation at 20 and 40 ppt	with salinity under SI and II and with inundation at all salinities
Root biomass (% BGB)	**Sf > Sd**	-	-	-	-	-	-
Rhizome biomass (% BGB)	Sf < Sd	-	-	-	-	-	-
Below-ground biomass	Sf < Sd	-	-	↓	↓	-	-
AGB : BGB ratio	Sf < Sd	↓	↓	↓	↓	-	-
Root mass ratio	**Sf > Sd**	-	-	↑	↑	-	-
Tiller length	Sf < Sd	↓	↓	↓	↓	-	-

Tiller growth rate	**Sf > Sd**	↓	-	↓	↓	with salinity at all inundation depths and with inundation at 0.5, 10 and 20 ppt.	with inundation at 0.5, 10 and 20 ppt
Floret production	Sf < Sd	-	-	-	↓	-	-
Leaf rolling	Sf < Sd	↑	↑	↓	↓	Increase with salinity at all inundation depths and decrease with inundation at 10 and 20 ppt	Increase with salinity under SI and II and decrease with inundation at 10, 20 and 40 ppt
Leaf water content	-	-	-	-	-	-	-
Specific leaf area	-	↓	↓	-	-	-	-
Chl *a* content	**Sf > Sd**	↓	↓	Max. under II	-	-	-
Chl *b* content	**Sf > Sd**	↓	↓	↓	↓	with salinity at all inundation depths and with inundation at 0.5 and 10 ppt. with salinity under II and DI and with inundation at 0.5 ppt	with salinity at all inundation depths and with inundation at 0.5 and 10 ppt. with salinity under II and DI and with inundation at 0.5 ppt
Carotenoids content	**Sf > Sd**	↓	↓	↓	↓		
Chl *a* + *b* content	**Sf > Sd**	↓	↓	Max. under II	-	-	-
Chl *a* : Carotenoids ratio	Sf < Sd	↓	↓	-	-	-	-
Chl *a* : Chl *b* ratio	Sf < Sd	↑	↑	↓	-	-	-
Leaf C content	Sf < Sd	↓	↓	↓	↓	-	-
Leaf N content	**Sf > Sd**	-	-	↑	↑	with salinity under II and SI and with inundation at all salinities	with salinity under II and SI and with inundation at all salinities
Leaf C : N ratio	**Sf > Sd**	-	-	↓	↓	with salinity under II and SI and with inundation at 10 ppt	with salinity under II and SI and with inundation at 10 ppt
Leaf Na content	-	↑	↑	-	-	increase with salinity under all inundation depths	increase with salinity under all inundation depths and decrease with inundation at 0.5 ppt

Na excretion rate	**Sf > Sd**	↑	↑	↑	↑	with salinity at all inundation depths and with inundation at 20 and 40 ppt.	with salinity at all inundation depths and with inundation at 20 and 40 ppt.
Glycinebetaine content	**Sf > Sd**	↑	↑	↑	↑	with salinity at all inundation depths and with inundation at 10 and 20 ppt.	with salinity at all inundation depths and with inundation at 10 and 20 ppt.
Proline content	Sf < Sd	↑	↑	↑	-	-	-
Rhizome C content	Sf < Sd	↓	↓	↑	↑	-	-
Rhizome N content	**Sf > Sd**	↑	↑	-	-	Increase with salinity under SI and decrease with inundation at 40 ppt	Increase with salinity under SI and decrease with inundation at 40 ppt
Rhizome C : N ratio	Sf < Sd	↓	↓	↑	↑	Decrease with salinity under SI and increase with inundation at 40 ppt	Decrease with salinity under SI and increase with inundation at 40 ppt
Rhizome TNC	-	-	-	↓	↓	-	-
Δ rhizome TNC	-	-	-	↓	↓	-	-
Root porosity	Sf < Sd	-	-	↑	↑	-	-
Rhizome porosity	**Sf > Sd**	-	-	-	-	-	-
Net photosynthesis rate	**Sf > Sd**	↓	↓	-	-	-	-
Stomatal conductance	**Sf > Sd**	↓	↓	-	-	-	-
Water Use Efficiency	-	-	-	-	-	-	-

CHAPTER VII

Effects of salinity and inundation on native *Spartina foliosa*, invasive *S. densiflora* and their hybrid *S. densiflora x foliosa* from San Francisco Bay: (II) the hybrid

CAPÍTULO 7: Efectos de la salinidad y la inundación en la nativa S. foliosa*, la invasora* S. densiflora *y su híbrido* S. densiflora x foliosa *en la Bahía de San Francisco: (II) El híbrido.*

La hibridación entre especies vegetales nativas e invasoras frecuentemente promueve la invasividad de los descendientes por a la adquisición de mayor tolerancia a factores ambientales relacionada con la heterosis o 'vigor híbrido', y/o mayor plasticidad fenotípica. La relación de la respuesta de los híbridos al cambio climático y el tipo de herencia de estos supone una oportunidad para analizar los cambios adaptativos asociados a este fenómeno, desde el punto de vista evolutivo. En el caso de los híbridos de ecosistemas costeros, el aumento del nivel del mar asociado al cambio climático hará que estén sometidos a mayores periodos de sumersión y salinidad del agua. En el híbrido, *Spartina densiflora x foliosa*, entre la nativa *Spartina foliosa* y la invasora *S. densiflora* en la Bahía de San Francisco (EEUU), la transgresividad en determinados caracteres funcionales dio lugar a una mayor tolerancia que sus parentales al efecto combinado de inundación y salinidad, que estudiamos en un experimento de invernadero. Esta transgresividad aumentó en caracteres claves que fueron los que más variaron entre tratamientos en los padres. Los caracteres transgresivos en el híbrido fueron más numerosos bajo estrés por inundación que a altas salinidades, siendo la inundación el factor que más afectó negativamente a las especies parentales. Nuestros resultados indican que el aumento de salinidad y la inundación asociados a la subida del nivel del mar producto del cambio climático aumentarán la ventaja competitiva del híbrido exótico *S. densiflora x foliosa* sobre ambos padres.

CHAPTER 7: Effects of salinity and inundation on native Spartina foliosa, invasive S. densiflora and its hybrid S. densiflora x foliosa from San Francisco Bay: (II) the hybrid

Hybridization between native and invasive plant species frequently promotes the invasiveness of the offspring by the acquisition of greater tolerance to environmental factors due to heterosis or 'hybrid vigor' and/or higher levels of phenotypic plasticity. The relationship between the response of hybrids to climate change and their type of inheritance is an opportunity to analyze the adaptive changes associated with this global phenomenon, from the evolutionary point of view. In the case of hybrids from coastal ecosystems, the sea level rise associated with climate change will make them, and their parental species, to be subjected to longer periods of submergence and higher salinity. For the exotic hybrid *Spartina densiflora x foliosa*, between the native *Spartina foliosa* and the invasive *S. densiflora* in the San Francisco Bay (USA), the transgressivity in certain functional traits resulted in a greater tolerance than their parents to the combined effect of waterlogging and salinity. This transgressivity was related to the higher trait variability shown by the parents and was more important under inundation stress, this being the factor that most negatively affected the parental species. Our findings show that the increase in salinity and inundation associated with sea level rise product of climate change will increase the competitive superiority of the exotic hybrid *S. densiflora x foliosa* over its parental species.

7.1 Introduction

Sea level rise (SLR) is one of the main effects of the current process of global warming and it is primarily produced by both the thermal expansion of the ocean and the melting of polar glaciers and ice sheets (Titus et al. 1991, Rahmstorf 2007, IPCC 2015). As a consequence of SLR, the period of submersion of the species present in the coastal ecosystems increase, as well as the salinity to which they are subjected by the intrusion of seawater inland (Sutter et al. 2015, Thorne et al. 2018). In tidal marshes, the expected increase of inundation and salinity will modify the spatial pattern and magnitude of plant species, given their importance in the configuration of the ecological niche and the biotic interaction among species (Bertness and Callaway 1994, Pennings et al. 2005). Thus, it is expected that the stress that both factors, both independently and combined, produce on plant species leads to a decrease in the productivity of coastal wetlands (Janousek and Mayo 2013, Watson et al. 2017).

In this context of global environmental changes, plant species with a greater tolerance to changes in environmental factors and broader ecological niches will be decisive in the future configuration of ecosystems (Thuiller et al. 2005). This is the case of invasive plant hybrids since interspecific hybridization including an invasive species as a parent frequently results in an increase in invasiveness (Ellstrand and Schierenbeck 2000, Hovick and Whitney 2014) related to greater tolerance to abiotic factors and competitive ability than their parents. The higher performance of hybrids, called heterosis or 'hybrid vigor' (Rieseberg et al. 1999), is a type of non-additive inheritance controlled genetic and epigenetically, by which hybrids develop transgressive traits for which they exceed both parental species (Chen 2013). In plants, hybrid vigor is associated to a complex of physiological and morphological superior traits that lead to high vegetative and reproductive fitness (Lippman and Zamir 2007). However, hybrids can also show certain traits that are intermediate to both parents as a result of a parental additivity, as well as equal to one of the parents related to a

dominant inheritance (Bassene et al. 2009, Favre and Karrenberg 2011). The enhanced performance of invasive hybrids is therefore conditioned by the predominance of transgressive traits but the processes giving rise to heterotic hybrids is still under discussion (Baranwal et al. 2012, Chen 2013).

The ability of invasive hybrids and allopolyploids to occupy a broad ecological niche is also related to their greater phenotypic plasticity (Ainouche and Jenczewski 2010, Te Beest et al. 2012, Cara et al. 2013). Phenotypic plasticity can be considered as a singular trait since it has the ability to evolve itself and may be inherited (Matesanz et al. 2010). As for the rest of functional traits, the improved phenotypic plasticity of the hybrids with respect to the parents is related to non-additive inheritance as a consequence of modifications in gene expression both at the genetic and epigenetic level (Jackson and Chen 2011, Parepa et al. 2014). Then, based on the study of the phenotypic inheritance in hybrids, it is possible to relate the characteristics of the parental species that lead to a more transgressive and / or more plastic response in the hybrids.

For all this, the study of hybrids is an opportunity from the evolutionary point of view to document adaptive changes in response to climate change (Hoffmann and Sgrò 2011, Taylor et al. 2015). Some studies have assessed the effects of increasing salinity (Karrenberg et al. 2006, Favre and Karrenberg 2011, Lee et al. 2016) and waterlogging (Waldren et al. 1988, Boers and Zedler 2008) on plant hybrids finding patterns of enhanced tolerance but, to our knowledge, the combined effect salinity and inundation on coastal ecosystems hybrids has not been analyzed. To address this question we performed an experiment in the genus of polyploid halophyte grasses *Spartina*, in which interspecific hybridization is a common process (summarized in Strong and Ayres 2013). Some of the *Spartina* hybrids and allopolyploids have been described as transgressive for different traits such as growth, tillers height or tolerance to environmental factors (Aïnouche et al. 2004, Hall et al. 2006, Castillo et al. 2010a, Lee et al. 2016). In addition, high phenotypic plasticity has been observed for the allopolyploid *S. anglica* C. E. Hubbard (Thompson 1991)

and species with hybrid origin *S. densiflora* Brongn (Grewell et al. 2016, Castillo et al. 2018). In the San Francisco Bay, one of the observed hybridization processes occurs between the invasive South American *S. densiflora* and the native *Spartina foliosa* Trin. (Ayres et al. 2008). These hybrids exceed their two parental species in their tolerance to salinity by showing certain transgressive traits such as production of aboveground biomass or quantity of seeds (Lee et al. 2016). However, no viable seeds have been found in the hybrid *S. densiflora x foliosa* (Ayres et al. 2008, Lee et al. 2016) although it is not ruled out that there had happened an allopolyploidization process that leaded to fertility given its high spread along the Bay (Strong and Ayres 2013). At present, both the hybrids *S. densiflora x foliosa* and the invasive parent *S. densiflora* are close to extinction due to eradication efforts in the San Francisco Bay (Rohmer et al. 2012, Strong and Ayres 2013).

To analyze the responses of this hybrid to salinity and inundation in relation to its parental species, an experiment was designed in which the three taxa were subjected to different salinity (from fresh water to hypersalinity) and inundation (from 4.5 to 55.0 cm deep) levels. Mid-term response of different morphological, biochemical, ecophysiological and fitness-related traits were recorded after 31 days of exposure to the treatments. We investigated (1) the main morphological, biochemical and physiological responses to salinity and inundation and the combination of both factors of the hybrid *S. densiflora x foliosa* (2) the phenotypic inheritance leading to the performance of the hybrid (3) inter-treatment and intra-population trait variability, and phenotypic plasticity in the response of the hybrid and its parental species and their relation to the phenotypic inheritance in the hybrid, and (4) the resulting vegetative and reproductive fitness of *S. densiflora x foliosa* and both parental species. Our hypothesis was that the hybrids would present higher fitness related to higher phenotypic plasticity and greater number of transgressive traits outperforming both parents at extreme levels of salinity and inundation.

7.2 Material and Methods

'*Experimental design'* and *'pre- and post-harvest measurements'* are listed in *Chapter 6.*

Phenotypic inheritance

We determined the phenotypic inheritance for the 36 recorded plant traits in response to salinity and inundation for the hybrid *S. densiflora x foliosa*. Four different phenotypic inheritances were determined at the population level for each variable and treatment combination (N = 12): 1) A dominant inheritance (D) was attributed when a given trait for the hybrid was the same as one of the parental species (D-*Sf* for *S. foliosa*; D-*Sd* for *S. densiflora*); 2) Parental codominance (D-*Sf,Sd*) when it was equal to both parental species; 3) Parental additivity (I) when the trait value was in-between both parents, being the values for both parents different; and 4) Transgressive segregation (T) when the value exceeded the higher or lower value of both parents (Favre and Karrenberg 2011). At the individual level, the number of hybrids with heterotic response (outperforming both parental species) was computed for each plant trait and treatment combination.

Plant trait variability

Inter-treatment trait variability index was calculated for each taxon as the relation of the difference between the maximum (X_{max}) and the minimum (X_{min}) values of a given plant trait divided by its maximum, when all the values of the different treatment combinations were included (Valladares et al. 2006). Inter-treatment trait variability index was considered as a general indicator of trait variability among treatments and individuals for each taxon.

$$\text{Inter. Trait Var.} = \frac{(X_{max}\text{-}X_{min})*100}{X_{max}}$$

Mean intra-population trait variability index was calculated for each taxon as the arithmetic average (n = 12 inundation x salinity treatments) of the relation between the difference between the maximum (x_{max}) and the minimum (x_{min}) values divided by the maximum of a given plant trait at each salinity x inundation combination (Castillo et al. 2018). Intra-population trait variability was used as an intrinsic indicator of trait variation within populations.

$$\text{Intra. Trait Var.} = \frac{\sum_{i=1}^{12}[(x_{max}-x_{min})/x_{max}]*100}{12}$$

Since intra-population variations in plant traits may be important due to the differences between individuals in polyploid taxa such as *Spartina* (Ainouche et al. 2012) and especially in sterile hybrids (Te Beest et al. 2012), phenotypic plasticity index (PPI) was determined after subtracting the intra-population component to the inter-treatment trait variability. Thus, the trait variability in PPI was only related to the responses to the application of the inundation and salinity treatments.

Fitness

Reproductive and vegetative fitness were measured for the three *Spartina* taxa at the different treatment combinations as the mean percentage in relation to the maximum value of fitness-related traits. Vegetative fitness was calculated using AGB (mg DW month^{-1}) (Sakai et al. 1989, Arriola and Ellstrand 1997), while the total number of florets produced per individual was used for reproductive fitness (Castillo et al. 2010a).

$$\text{Fitness}_i\ (\%) = \frac{x_i*100}{x_{max}}$$

, where x_i was the value of AGB or the total number of florets per individual for the treatment combination i and x_{max} was the maximum value for that fitness trait at every treatment combination. In order to evaluate the

production of mature seeds of the hybrids, the florets were pressed with a fingertip to check if they contained any seeds inside (Castillo et al. 2010a).

Statistical analyses

Every statistical analysis was performed with IBM SPSS V. 20 for Windows using a significance level (α) of 0.05. Graphs were obtained with Sigma-Plot 14.0 for Windows. The homogeneity of variances of all the data sets were tested using Levene's Test and transformations using square root and 1 / x were performed if homoscedasticity was not reached. A protected procedure of variance analysis was conducted to avoid type I errors, for which the significance of salinity, inundation and taxon factors was first tested by multivariate analysis of variance (MANOVA) (Scheiner 2001). When multivariate significance was confirmed, the main univariate differences of the hybrid biochemical, morphological and physiological responses to salinity and inundation and their relationships to its parental species were assessed using General Lineal Models (GLMs) and Bonferroni-Dunn's test as post hoc analysis. We evaluated the significance of the factors salinity (0.5, 10, 20 and 40 ppt) and inundation depth (shallow, intermediate and deep inundation) for the hybrid, and we added the taxon factor (*S. densiflora x foliosa*, *S. densiflora* and *S. foliosa*) in order to determine the different phenotypic inheritances. The relationships among traits of *S. densiflora x foliosa* were analyzed using Principal Components Analysis (PCA) so independent PC factors with eigenvalues > 1 were extracted, evaluating convergence of the correlation matrix with maximum 25 iterations without rotation. Two-way analysis of variance (ANOVA) with salinity and inundation as grouping factors were carried out to compare the average number of transgressive traits and hybrid individuals with transgressive responses as dependent variables. Moreover, three-way ANOVA with taxon, inundation depth and salinity as grouping factors were conducted for trait variability indexes, and vegetative and

reproductive fitness. The relationships between PCA factors, salinity, inundation depth, and vegetative and reproductive fitness as well as between intra-population and inter-treatment trait variability and phenotypic plasticity indexes for the different traits for each taxon were calculated using Pearson correlation coefficient (r).

7.3 Results

All experimental hybrid plants had 65 chromosomes and were therefore diploid hybrids whose seed parent was most likely *S. densiflora* (Ayres et al. 2008).

Responses of Spartina densiflora x foliosa *to salinity and inundation*

The responses of both parental species of the hybrid *S. densiflora x foliosa* to salinity and inundation recorded in this experiment are reported *Chapter 6*.

The set of biochemical, morphological and physiological responses of the *Spartina* taxa were affected by salinity (MANOVA, Pillai's trace = 2.295, $F_{102,189} = 6.030$, $P < 0.001$) and inundation MANOVA, (Pillai's trace = 1.416, $F_{68,124} = 4.424$, $P < 0.001$) and presented differences between taxa (MANOVA, Pillai's trace = 1.915, $F_{68,124} = 41.199$, $P < 0.001$). Leaf rolling, rhizome TNC content and increment, leaf Na, Na excretion, proline and glycinebetaine contents, and Chl *a* : Chl *b* ratio of the hybrid increased with increasing salinity. Glycinebetaine content was higher at DI than II and SI at salinities equal and higher than 20 ppt. Na excretion was close to be significantly higher at DI compare with SI ($P = 0.05$). On the other hand, LWC, Chl *b*, Car, Chl *a* : Car ratio, rhizome porosity and rhizome C content decreased at higher salinities, being independent of inundation depth. In relation to the gas exchange of the hybrid, G_s was reduced at 20 and 40 ppt salinity and WUE increased at 20 ppt, while A did not change significantly among salinity treatments (Figs. 7.1-7.4). SLA and the leaf N increased only with increasing inundation depth, while

BGB, the proportion of leaves in the AGB and leaf C: N ratio decreased with increasing inundation depth (Figs. 7.1-7.4).

The combined increase of salinity and waterlogging reduced tiller length and TGR of *S. densiflora x foliosa* The percentage of inflorescences biomass in the AGB was also reduced with the maximization of both stresses, although it did not change with increasing inundation depth at lower salinities (0.5 and 10 ppt). On the contrary, the percentage of tillers biomass in the AGB increased with increasing both abiotic stresses, although it did not change between salinities at SI. On the other hand, some plant traits responded in an opposite way to salinity and inundation. Thus, the percentage of rhizomes biomass in the AGB and their N content increased with salinity and decreased with inundation depth, while the percentage of roots biomass in BGB, AGB : BGB ratio, Chl *a*, Chl *a* + *b* and rhizome C : N ratio decreased with increasing salinity and increased together with inundation depth. Leaf C concentration decreased with increasing salinity and was maximum at II (Figs. 7.1-7.4). F-statistics and P-values for the GLM of each univariate analyses are listed in Appendix 7.A.

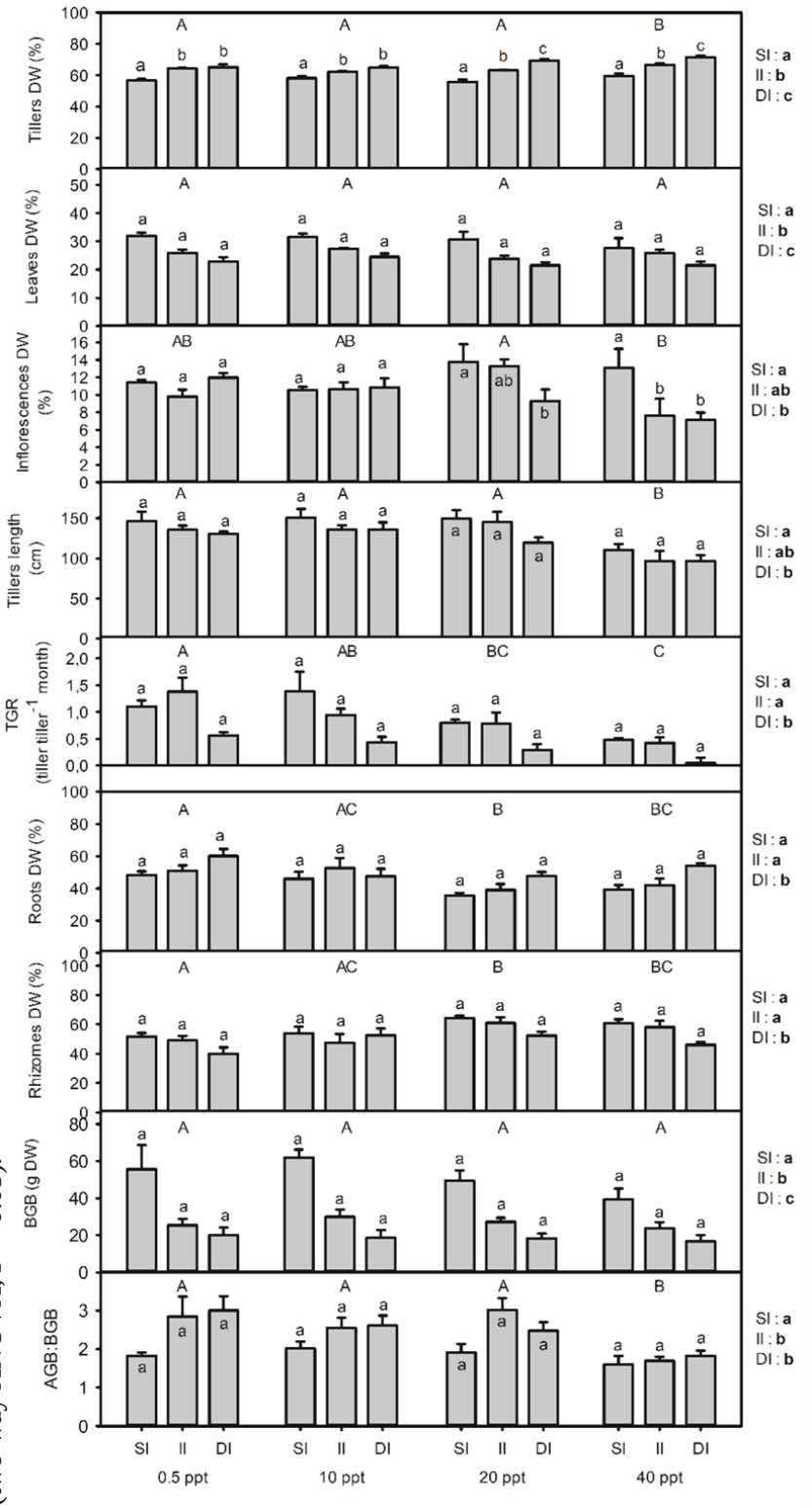

Fig. 7.1. Growth and biomass allocation: tillers, leaves and inflorescences proportions of AGB, tillers length, tiller growth rate (TGR), roots and rhizomes proportions of below-ground biomass (BGB), BGB, AGB : BGB ratio for *Spartina densiflora x foliosa* under different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (SI, shallow inundation; II, intermediate inundation; DI, deep inundation). Values are mean ± SE (n = 4). Capital letters indicate significant differences among salinities; bold letters indicate significant differences among inundation treatments and lowercase simple letters indicate differences among inundation treatments within salinities (two-way ANOVA, $P < 0.05$).

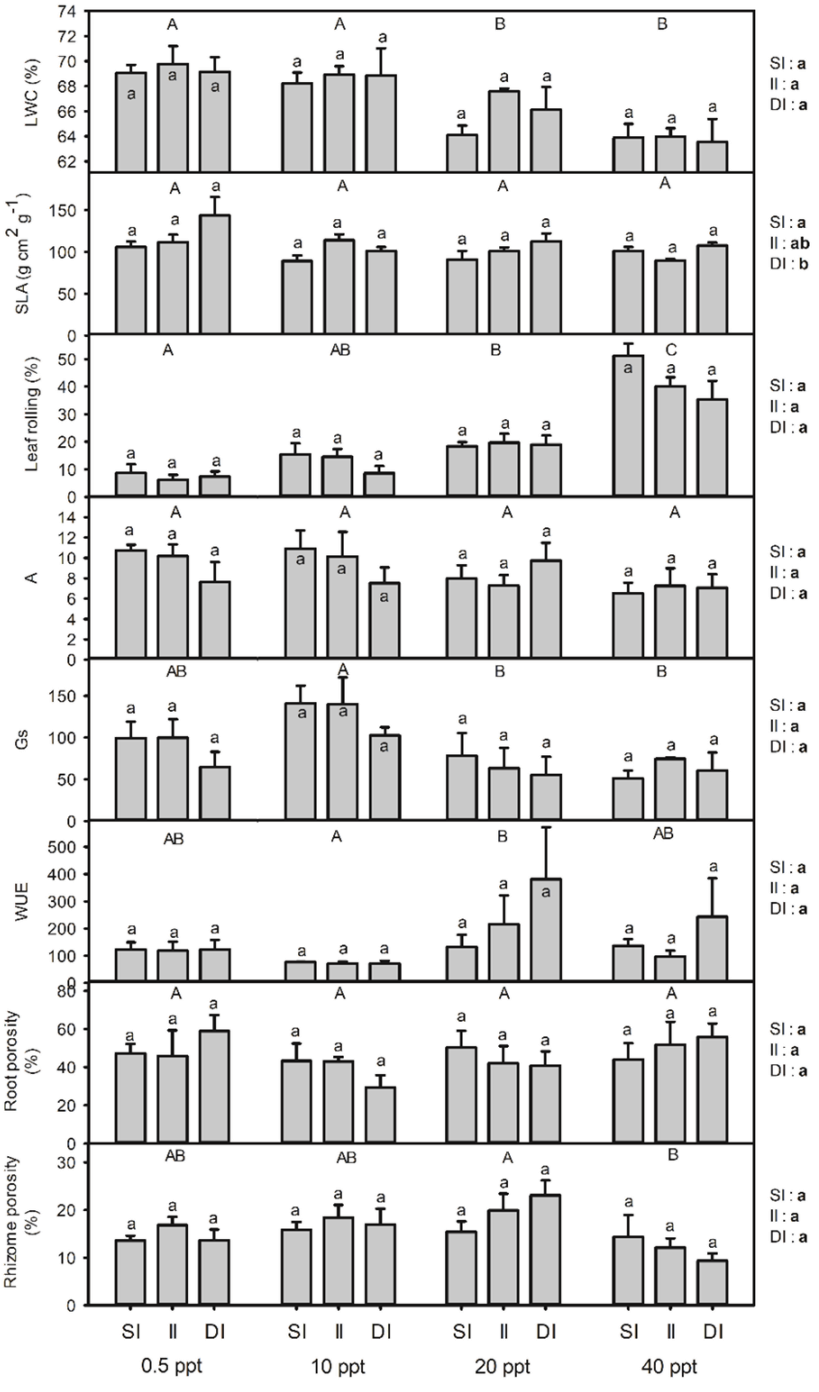

Fig. 7.2. Leaf morphology, gas exchange and porosity responses: leaf water content (LWC), leaf rolling, specific leaf area (SLA), net photosynthesis rate (A), stomatal conductance (Gs) and water use efficiency (WUE), root and rhizome porosity for *Spartina densiflora* x *foliosa* under different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (SI, shallow inundation; II, intermediate inundation; DI, deep inundation). Values are mean ± SE (n = 4). Capital letters indicate significant differences among salinities; bold letters indicate significant differences among inundation treatments and lowercase simple letters indicate differences among inundation treatments within salinities (two-way ANOVA, $P < 0.05$).

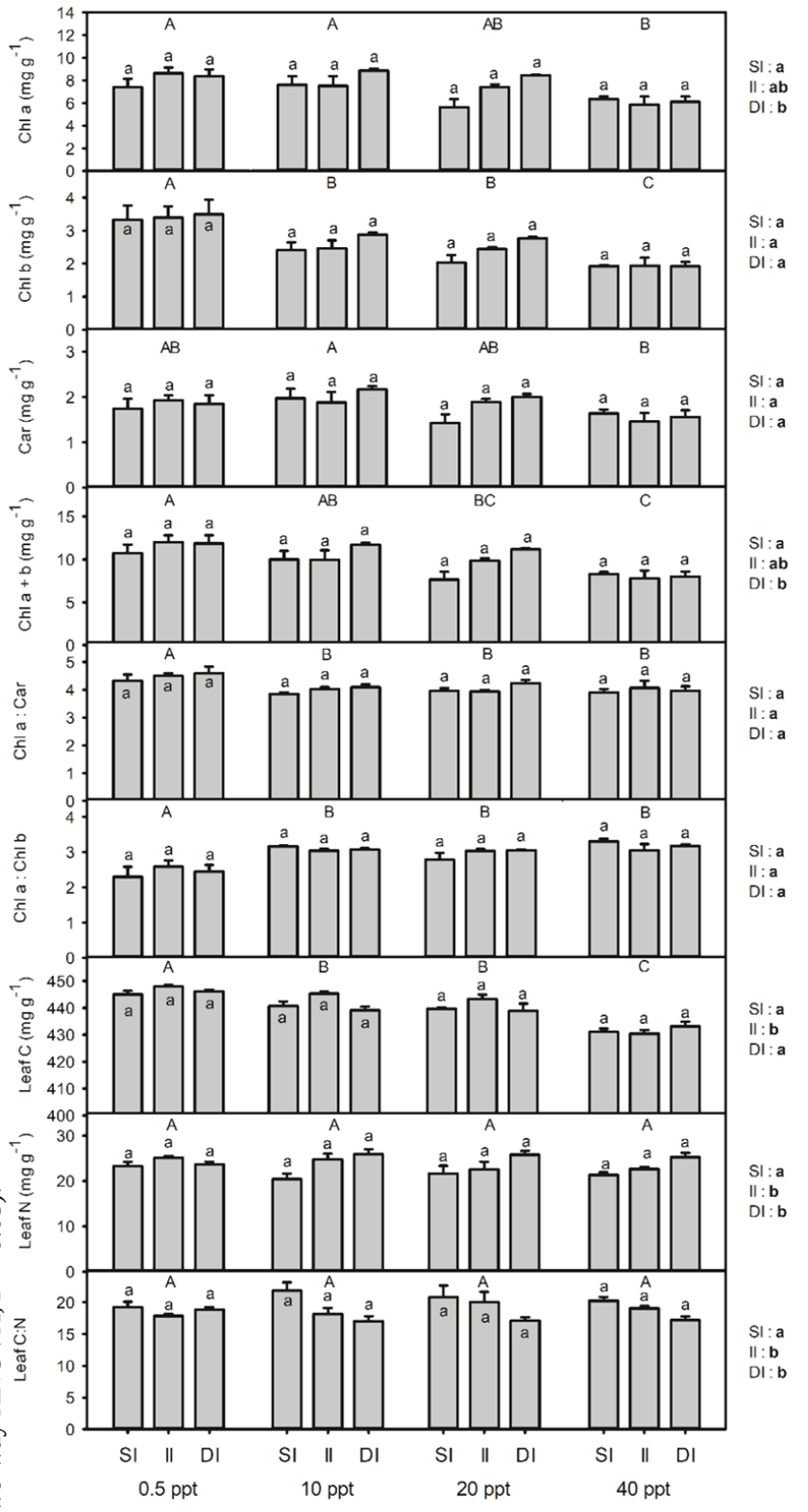

Fig. 7.3. Leaf photosynthetic pigments, C and N contents: chlorophyll *a* (Chl. *a*), chlorophyll b (Chl. *b*), carotenoids (Car), Chl *a* + *b*, Chl *a* : Car, Chl *a* : *b*, carbon (C) and nitrogen (N) concentrations and C : N ratio for leaves of *Spartina densiflora x foliosa* under different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (SI, shallow inundation; II, intermediate inundation; DI, deep inundation). Values are mean ± SE (n = 4). Capital letters indicate significant differences among salinities; bold letters indicate significant differences among inundation treatments and lowercase simple letters indicate differences among inundation treatments within salinities (two-way ANOVA, $P < 0.05$).

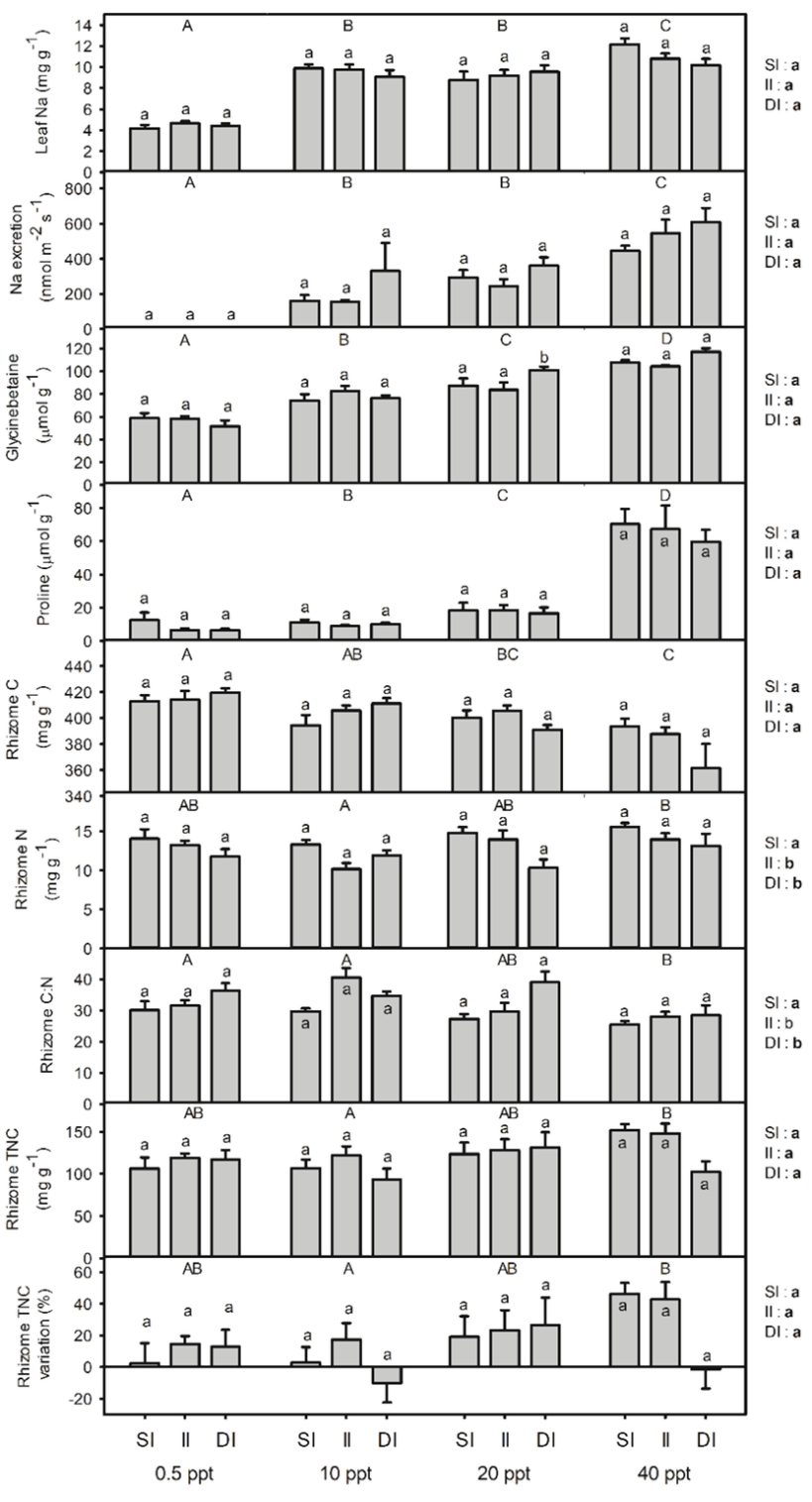

Fig. 7.4. Chemical stress and rhizome and subterranean resource storage: Na content, Na excretion rate, glycinebetaine and proline contents for leaves and carbon (C) and nitrogen (N) concentrations and C : N ratio for rhizomes of *Spartina densiflora x foliosa* under different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (SI, shallow inundation; II, intermediate inundation; DI, deep inundation). Values are mean ± SE (n = 4). Capital letters indicate significant differences among salinities; bold letters indicate significant differences among inundation treatments and lowercase simple letters indicate differences among inundation treatments within salinities (two-way ANOVA, $P < 0.05$).

Ten factors were found for the responses of the hybrid to salinity and inundation from the PCA, explaining 84% of the variance. The first factor (PC1-H; explaining 27.8% of the variance) was positively correlated with photosynthetic pigments concentrations (Chl *a*, Chl *b* and Chl *a* + *b*), LWC and rhizome and leaf C contents, and negatively with leaf rolling and chemical leaf responses (leaf Na, Na excretion, glycinebetaine and proline contents). PC1-H increased together with the vegetative fitness (AGB) ($r = +0.282$, $P < 0.05$, $n = 48$) and with reproductive fitness (number of florets per plant) ($r = +0.298$, $P < 0.05$, $n = 48$), and decreased with increasing salinity ($r = -0.941$, $P < 0.0001$, $n = 48$). The second factor (PC2-H; explaining 15.2% of the variance) was positively correlated with the percentage of tiller biomass in AGB and leaf nitrogen, and negatively with percentage of leaf biomass in the AGB, BGB, tillers length and leaf C : N ratio. PC2-H decreased with vegetative ($r = -0.729$, $P < 0.001$, $n = 48$) and reproductive fitness ($r = -0.537$, $P < 0.0001$, $n = 48$) and increased together with inundation depth ($r = +0.855$, $P < 0.0001$, $n = 48$), (Fig. 7.5, Appendix 7.B).

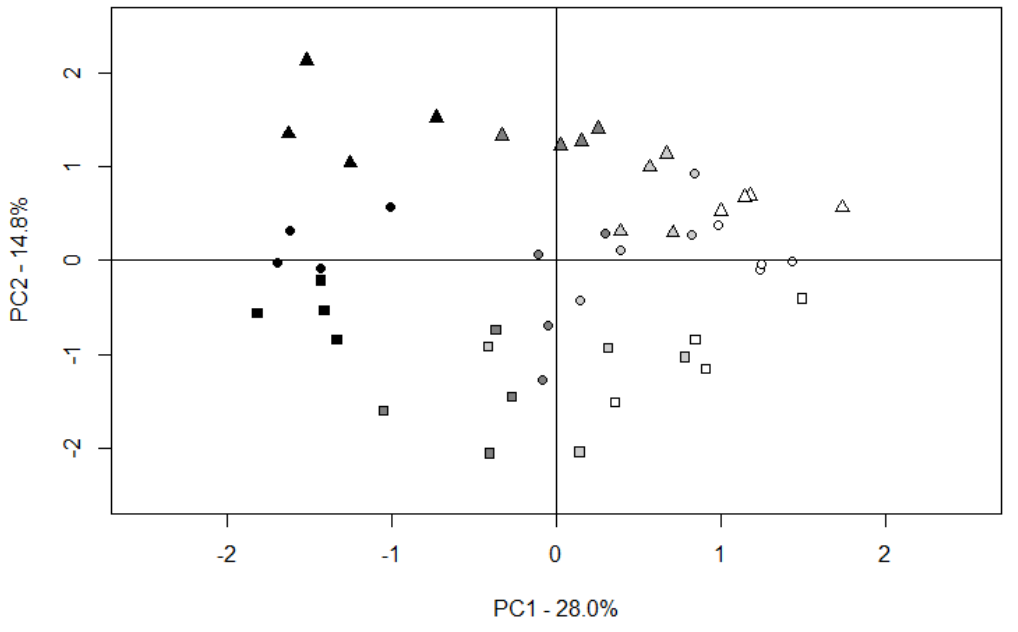

Fig. 7.5. Principal Components Analysis (PCA) plot for *Spartina densiflora x foliosa* responses (n = 36 traits) at different salinities (0.5 ppt, white; 10 ppt, light grey; 20 ppt, dark grey; 40 ppt, black) and inundation depths (shallow (4.4 cm deep), triangle; intermediate (35.5 cm deep), circle; deep (55.0 cm deep), square).

Phenotypic inheritance

The recorded responses of *S. densiflora x foliosa* to salinity and inundation stress were 36% product of the dominance of both parents, 17% the dominance of *S. foliosa*, 17% due to the dominance of *S. densiflora*, 3% were intermediate between both parental species and 26% transgressive (Fig. 7.6, Appendix 7.C). At the population level, the proportion of transgressive traits of the hybrid did not vary significantly between salinities and it was greater under DI (35%) than at II and SI (18 and 24%, respectively) (Two-way ANOVA, inundation: $F_{2,11}$ = 8.535, P <0.02). The opposite tendency was found for the traits dominated by both parents that were lower under DI (26%) than at SI (46%), showing a mid-value under II (37%) (Two-way ANOVA, inundation: $F_{2,11}$ = 14.067, P <0.01).

The number of individuals with transgressive traits was higher at SI (34%) than at II (21%) and DI (24%) (Two-way ANOVA, inundation: $F_{2,11}$ = 11.239, P <0.01) and it did not change among salinities. The traits for which the majority of hybrid individuals were transgressive were AGB : BGB (100%), change in rhizomes TNC content (100%), tillers length (98%), RMR (79%), TGR (67%) and rhizomes TNC content (63%). On the contrary, C content of leaves and rhizomes, and LWC were the only non-transgressive traits for any of the hybrid plants (Appendix 7.D).

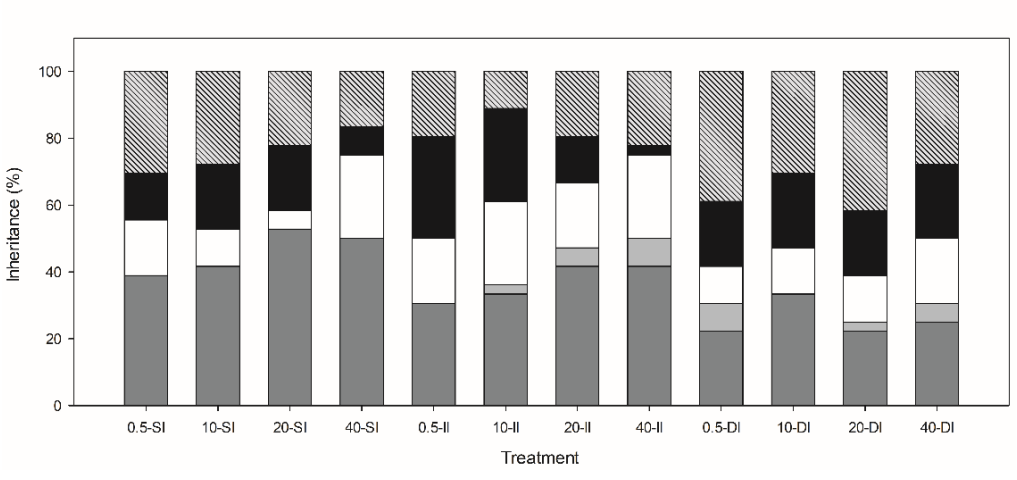

Fig. 7.6. Percentage of different phenotypic inheritance of the hybrid *Spartina densiflora x foliosa* for 36 traits measured at different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (4.4, 35.5 and 55.0 cm deep). Inheritance mechanisms include: parental codominance (dark gray); parental additivity (light gray); parental dominance of *S. densiflora* (white); parental dominance of *S. foliosa*(black); transgressive (striped).

Plant trait variability

The hybrid *S. densiflora x foliosa* showed an average inter-treatment trait variability of 64 ± 4%, average intra-population variability of 32 ± 3% and phenotypic plasticity of 32 ± 2% for the 36 key traits measured in its response to salinity and inundation treatments. No significant differences were found between these results and those obtained for its parental species for average inter-treatment variability (*Sf:* 76 ± 4%, *Sd:* 65 ± 4%), nor for the average intra-population trait variability (*Sf:* 38 ± 3%, *Sd:* 34 ± 3%) or average phenotypic plasticity (*Sf:* 38 ± 2%, *Sd:* 32 ± 2%). However, the phenotypic plasticity of *S.*

foliosa was significantly higher than that of *S. densiflora* (One-Way ANOVA, $F_{2,105} = 3.478$, $P < 0.05$) (Appendix 7.E).

The inter-treatment variability of the hybrid positively correlated with its phenotypic plasticity and intra-population trait variability (Pearson coefficient, $P < 0.0001$, $n = 36$), since both are components of the former, and its intra-population variability was positively related to its phenotypic plasticity ($r = 0.461$, $P < 0.01$, $n = 36$). However, the analyses of trait variability in the parents showed that intra-population variability and phenotypic plasticity were independent for both species. On the other hand, the three indicators of trait variability positively correlated within the three taxa independently (Pearson coefficient, $P < 0.0001$, $n = 36$). The traits that were transgressive for a higher number of hybrid plants were those that presented a greater phenotypic plasticity for the parent *S. densiflora* and higher inter and intra-population trait variability for *S. foliosa* (Pearson coefficient, $P < 0.05$, $n = 36$). Also, the number of hybrid plants with traits product of the dominance of *S. densiflora* was negatively related to the phenotypic plasticity of *S. densiflora* while they were independent in the case of the dominance and phenotypic plasticity of *S. foliosa* (Table 7.1).

Table 7.1. Pearson correlation coefficients and P-values between intra-population (Intra) and inter-treatment (Inter) trait variability and phenotypic plasticity (PP) of *S. densiflora x foliosa* (*Sdxf*), *S. densiflora* (*Sd*) and *S. foliosa* (*Sf)*; and the number of hybrid individuals with transgressive (T) and *S. densiflora* (D-Sd) and *S. foliosa* (D-Sf) dominant phenotypic inheritance for 36 traits measured at different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (4.4, 35.5 and 55.0 cm deep).

Intra-population trait variability

	Sd	*Sdxf*
Sd	**0.794**	**0.890**
	<0.0001	**<0.0001**
Sf		**0.817**
		<0.0001

S. densiflora x foliosa

	Intra	PP
Inter	**0.911**	**0.785**
	<0.0001	**<0.0001**
Intra		**0.461**
		<0.0001

Inter-treatment trait variability

	Sd	*Sdxf*
Sd	**0.874**	**0.900**
	<0.0001	**<0.0001**
Sf		**0.83**
		<0.0001

S. densiflora

	Intra	PP
Inter	0.904	0.636
	<0.0001	**<0.0001**
Intra		0.245
		0.150

Phenotypic plasticity

	Sd	*Sdxf*
Sd	**0.643**	**0.722**
	<0.0001	**<0.0001**
Sf		**0.652**
		<0.0001

S. foliosa

	Intra	PP
Inter	**0.914**	**0.653**
	<0.0001	**<0.0001**
Intra		0.290
		0.0859

	T	D-Sd	D-Sf
Inter (*Sd*)	0.193	-0.090	0.112
	0.26	0.602	0.514
Intra (*Sd*)	0.0293	0.0756	0.16
	0.865	0.661	0.352
PP (*Sd*)	**0.384**	**-0.340**	-0.034
	0.0207	**0.0424**	0.846
Inter (*Sf*)	**0.357**	-0.178	0.0249
	0.0327	0.299	0.885
Intra (*Sf*)	**0.422**	-0.269	-0.037
	0.0103	0.112	0.832
PP (*Sf*)	0.0532	0.083	0.127
	0.758	0.632	0.46
Inter (*Sdxf*)	0.159	-0.080	0.122
	0.354	0.645	0.479
Intra (*Sdxf*)	0.103	-0.040	0.162
	0.548	0.819	0.346
PP (*Sdxf*)	0.188	-0.112	0.020
	0.273	0.516	0.909

Fitness

Vegetative fitness (AGB) of the hybrid was lower at hypersalinity than at the rest of saline treatments (0.5-20 ppt), falling with increasing salinity from 48 to 14%. Similarly, inundation depth induced a reduction in it vegetative fitness falling from 58 to 28% from SI to DI. Nonetheless, the hybrid presented greater vegetative fitness than both parental taxa at all treatments. Fitness fall with salinity was from 23 to 14% for *S. densiflora* while it did not change for *S. foliosa*. The parents also reduced their vegetative fitness with inundation from 26 to 9% for *S. densiflora* and 10 to 3% for *S. foliosa* (Three-way ANOVA, taxa: $F_{2,143} = 243.550$, $P < 0.001$; taxa x salinity: $F_{6,143} = 4.797$, $P < 0.001$; taxa x inundation: $F_{6,143} = 8.397$, $P < 0.001$) (Fig. 7.7 A-C).

Reproductive fitness (number of florets per plant) of the hybrid was also lower at hypersalinity (29%) than at the rest of the salinities (55%) and decreased with increasing inundation stress (from 61 to 36%). In relation to the parental species, reproductive fitness of the hybrids was higher than for both parents at every treatment combination. Thus, the decrease of reproductive fitness of the parental species was 30 to 17% and 5 to 2% with salinity and 41 to 11% and 11 to 2% with inundation for *S. densiflora* and *S. foliosa*, respectively (Three-way ANOVA, salinity: $F_{2,140} = 7.110$, $P < 0.001$, inundation: $F_{2,140} = 89.056$, $P < 0.001$, taxa: $F_{3,140} = 25.884$, $P < 0.001$) (Fig. 7.7 D-F). However, every floret of the hybrid was empty without containing mature seeds.

Differences in vegetative and reproductive fitness at harvest are shown in Appendix 7.F.

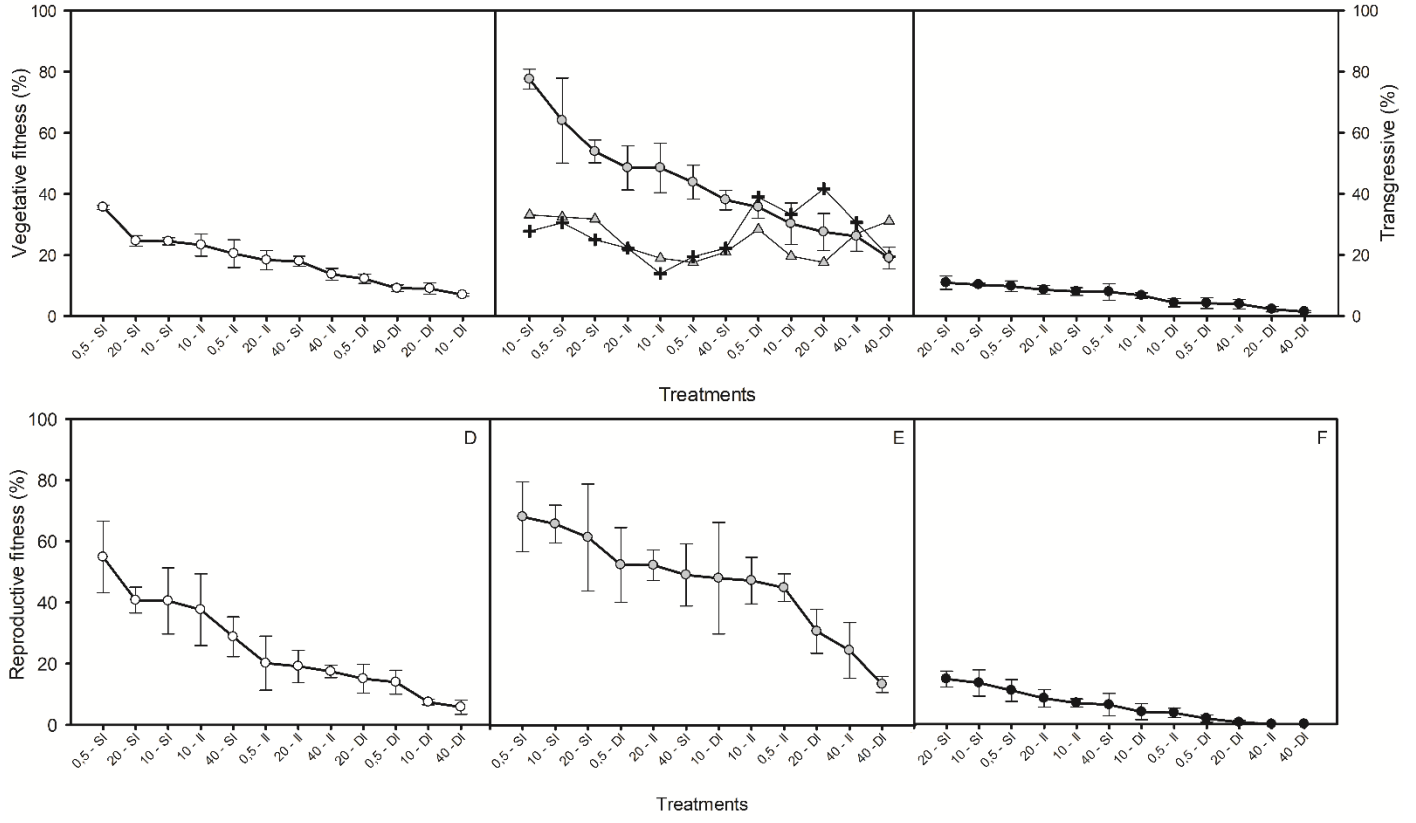

Fig. 7.7. Percentage of vegetative (A,B,C) and reproductive (D,E,F) fitness (measured as AGB and florets per individual, respectively) of the hybrid *S. densiflora x foliosa* (◍) and its parental species *S. densiflora* (○), *Spartina foliosa* (●) at different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (SI, shallow inundation (4.4 cm deep); II, intermediate inundation (35.5 cm deep); DI, deep inundation (55.0 cm deep)). Values are mean ± SEM (n = 4). Percentage of individuals with transgressive responses (△) and percentage of transgressive traits at the population level (✚) for 36 key functional responses are represented in (B).

7.4 Discussion

Responses of Spartina densiflora x foliosa *to salinity and inundation*

From the two environmental factors (salinity and inundation) to which the hybrid *S. densiflora x foliosa* was subjected, salinity was the most influential in its response since the first factor of the PCA (PC1-H) was correlated to salinity and explained two times the variance (30%) of the PC2-H (15%) that correlated to inundation. A greater number of plant traits in the hybrid were affected by salinity (30) than by inundation (19).

The response to salinity of *S. densiflora x foliosa* was characterized by an increase in leaf rolling, which is related to the reduction of water loss under water stress (Yeo et al. 1991). Nonetheless, it did not prevent the fall of LWC with salinity, probably as a consequence of the reduction of water uptake due to the decrease in soil water potential (Munns 2002, Rodríguez et al. 2005). Additionally, saline stress induced an increase of the reserves of rhizomes (higher TNC), which contribute to maintain cell metabolism generating energy by anaerobic fermentation under adverse conditions (Chen and Qualls 2003, Martínez-Vilalta et al. 2016). However, rhizome C content was reduced with increasing salinity, so structural C compounds decreased which may be related to their greater mineralization rate at high concentration of Na (Nelson et al. 1996). As expected, traits related to leaf chemical stress (leaf Na, Na excretion rate, proline and glycinebetaine) increased with saline stress. Some halophytes accumulate Na in vacuoles and / or excrete it ameliorating its cellular toxicity (Bradley and Morris 1991, Geissler et al. 2009), while proline and glycinebetaine act as organic osmolytes (Ashraf and Foolad 2007). Despite the reduction of stomatal conductance and leaf pigmentary content (Chl *a*, Chl *b*, Car and Chl *a* : Car) with salinity, the photosynthesis rate of the hybrid did not vary among salinities, showing a high efficiency in the use of water (WUE). The

independence of A to Gs and the pigmentary content has been previously observed in other species of *Spartina* and related to a probable higher activity of Rubisco enzyme (Castillo et al. 2005b, Zhang et al. 2016). Also, the reduction in the Chl *a*: Car ratio with salinity may have acted as a photoprotection mechanism in hypersalinity, as particular carotenoids mediate the dissipation of excess of energy in the antenna complex of photosystems (Demmig-Adams and Adams 1996).

On the other hand, *S. densiflora x foliosa* reduced its BGB and the proportion of leaves in the AGB, and increased its SLA and its leaf N content (with a consequent reduction of leaf C : N) with increasing inundation depth. Lower BGB reduces the oxygen request under flooding conditions, increasing its diffusion from the greater proportion of AGB (Naidoo and Mundree 1993). On the other hand, the decrease in the proportion of leaves in the AGB may have been related to a greater degradability of leaves than tillers under water (Terry and Tilley 1964, Osaki et al. 1991). In fact, SLA increased with inundation which has been linked to lower content of recalcitrant compounds and higher degradability (Vaieretti et al. 2005). The increase in foliar N compounds in flooding-tolerant plants has been associated to the ability of using nitrate as an electron acceptor instead to oxygen in anoxic conditions, exhibiting higher activities of nitrate reductase and production of amino-acids (García-Novo and Crawford 1973). It is remarkable that rhizome porosity only decreased at deeper inundations from 20 to 40 ppt salinity and root porosity did not respond clearly to inundation. The increase of roots aerenchyma is a frequent response to flooding stress that enhances the diffusion of oxygen to the BGB (Pezeshki 2001) and has been described for different *Spartina* species such as *S. patens* (Aiton) Muhl, *S. alterniflora* Loisel, the allopolyploid *S. anglica* (Burdick 1989, Maricle and Lee 2002) and both parental species (see *Chapter 6*).

As a consequence of the combination of both stressors, the hybrid reduced its tiller height, TGR and the proportion of inflorescences, and increased the proportion of tillers in the AGB. Salinity and inundation

frequently reduces vegetative and reproductive development (Munns 1993, Pezeshki 2001, Parvaiz and Azooz 2013). Generally, salinity affects more to the growth of stems than roots (Djanaguiraman et al. 2006), showing inundation the opposite trend (Naidoo and Mundree 1993). Thus, AGB: BGB ratio of the hybrid decreased with salinity, whereas it increased with inundation. This was not the only opposite response to both stress factors since the proportion of rhizomes in the BGB of *S. densiflora x foliosa* and their N content increased at higher salinities and decreased under deeper inundation. On the contrary, the proportion of roots in the BGB increased with inundation and decreased at higher salinities, responding to the greater demand of oxygenation of the BGB underwater (Pezeshki 2001) since roots were more porous than rhizomes. Like for the rest of the pigments, salinity reduced the content of leaf Chl *a* while inundation increased its concentration contributing to maintain its photosynthesis rate under flooding stress (Janousek and Mayo 2013).

Phenotypic inheritance, trait variability and fitness

The phenotypic inheritance of *S. densiflora x foliosa* in relation to its parental species was characterized by the prevalence of traits similar to both parents at the same time (36%) over those traits showing intermediate responses between the parental species (3%). This recorded parental additivity by which hybrids present intermediate gene expression (or similar if both parents show similar values) is expected in F1 hybrids (Bassene et al. 2010, Chen 2013). But deviations from the midparent values can occur if there are non-additive expression processes such as dominance or overdominance (Hochholdinger and Hoecker 2007, Baranwal et al. 2012, Chen 2013). An example of this kind of inheritance is the dominance of one of the two parents' phenotype that, in our case, occurred in the same percentage of traits (17%) for the two parental species. This was probably related to the fact that *S. densiflora x foliosa* receive a similar genetic load from both species (gametes n = 35 chromosomes from *S.*

densiflora and n = 30 chromosomes from *S. foliosa*) (Ayres et al. 2008). Guo et al. (2003) reported that maternal effects on gene expression of hybrid maize were only manifested in reciprocal crosses when the maternal genomic contribution compared to the paternal increased from 1 : 1 to 2 : 1.

Another type of non-additive genetic expression in hybrids is heterosis or hybrid vigor, in which epigenetic processes seem to have a relevant role (Salmon et al. 2005, Ni et al. 2009, Groszmann et al. 2013). The percentage of transgressive traits of *S. densiflora x foliosa* was the second most frequent type of inheritance (27%). Transgressive traits related to higher fitness were also found in different hybrids of the salt marsh species *Iris brevicaulis* Walker and *Iris fulva* Ker-Gawler (Johnston et al. 2004), as well as for *Typha x glauca*, a hybrid between native *T. latifolia* L. and introduced *T. angustifolia* L. in North America (Bunbury-Blanchette et al. 2015). In *Spartina*, responses outperforming both parents at different salinities have been observed for different genotypes of *S. densiflora x foliosa* (Lee et al. 2016) as well as for the hybrids between *Spartina maritima* and *S. densiflora* in the Iberian Peninsula (Castillo et al. 2010a, Gallego-Tévar et al. 2018b). In our study, the transgressivity of the hybrid was directly related to the phenotypic plasticity of *S. densiflora* and the intra-population trait variability of *S. foliosa*, pointing out that the trait variability of the parents individually (both within population (intra-population) and as a consequence of environmental factors (plasticity)) may determine the heterosis of their hybrid offspring. Gallego-Tévar et al. (2018b) recorded that phenotypic plasticity of both parents and intra-population trait variability of *S. densiflora* increased together with the number of transgressive hybrids between native *S. maritima* and invasive *S. densiflora* in Southwest Iberian Peninsula. Moreover, in the same way as in this study, maternal effect on phenotypic inheritance was observed as the number of traits product of the dominance of *S. densiflora* was also negatively related to the intra-population variability of the seed parent.

In relation to phenotypic plasticity of the hybrid, it was expected to be higher than its parental species, since genetic and epigenetic changes that occur in hybridization promote the increase of phenotypic plasticity and adaptation to different environments (Cara et al. 2013). However, the phenotypic plasticity of *S. densiflora x foliosa* (33%) was not different to both parents, and *S. foliosa* showed a greater value (38%) than the ancestral hybrid (Fortune et al. 2008) *S. densiflora* (32%). The three *Spartina* taxa are polyploid which is also a factor that stimulates high phenotypic plasticity (Ainouche and Jenczewski 2010, Te Beest et al. 2012) but *S. foliosa* is the one with the lowest ploidy level (*S. foliosa* 2n = 60, *S. densiflora* 2n = 70, hybrid 2n = 65). The traits that showed higher plasticity for *S. foliosa*, exceeding those values of *S. densiflora* and the hybrid, were the increased accumulation of Na in leaves and the reductions of A, percentage of leaves of the AGB and chlorophylls content under high levels of combined abiotic stress. This point to the greater plasticity was probably a passive plasticity (Kurashige and Callahan 2007). Additionally, phenotypic plasticity was not related to intra-population trait variability for both parental species but it was for the hybrid, indicating that phenotypic plasticity may evolve itself in a changing environment (Pigliucci 2001) and probably it has not yet passed enough time for this phenomenon in the case of the hybrid.

For all the above, the maintenance of a greater vegetative and reproductive fitness than both parental species observed at all treatments seemed to be more related to the abundance of transgressive traits than the development of high phenotypic plasticity. Most of the *Spartina* hybrid individuals (> 50%) were transgressive in their tiller length, TGR, rhizome TNC content and increment, AGB:BGB, and lower RMR than its parental species. The combination of these traits would render a hybrid phenotype with advantage in waterlogging conditions since taller tillers and the maintenance of a greater proportion of biomass out water (AGB : BGB) would facilitate light collection over the water surface (Castillo et al. 2005a, Gioria and Osborne 2014) and oxygen diffusion to roots under anoxic conditions (Naidoo and Mundree 1993).

Also, higher rhizome TNC content is a reservoir that allows to supply the demand of growth and respiration under stress conditions when the assimilation of carbon is scarce, and they also have an immediate role in abiotic tolerance as intermediary metabolites, osmolyte, and substrates for transport (Martínez-Vilalta et al. 2016). It was remarkable that, in regarding flooding stress, a higher number of the transgressive traits was recorded under DI than at SI and II, while there was a lower number of hybrid individuals with transgressive traits, indicating that relatively few individuals were transgressive for many different traits. As a consequence, the reduction of vegetative fitness (AGB) of the hybrid with inundation was lower than for both parents. Thus, inundation depth affected in a greater extent to the fitness of *S. densiflora* (-65%) and *S. foliosa* (-70%) than the hybrid (-50%). One of the variables that was only higher for the hybrid than for the both parents at maximum inundation depth (DI) was the proportion of roots in the BGB, which would contribute to the maintenance of nutrient uptake and root oxygenation in flooding conditions (Pezeshki 2001). Furthermore, the hybrid exhibited higher content of foliar photosynthetic pigments than both parents only at DI which may have contributed to maintain constant values of A. Net photosynthesis rate can otherwise decrease under flooding stress by factors such as stomatal enclosure, disturbance in photosynthate transport and/or reduction in leaf area or chlorophyll concentration (Mutava et al. 2015). The development of higher number of transgressive traits under extreme abiotic conditions were also found in hybrids between *S. densiflora* and *S. maritima* at hypersalinity (Gallego-Tévar et al. 2018b). In our study, *S. densiflora x foliosa* did not show greater number of transgressive individuals or traits at hypersalinity. In fact, the decline of its vegetative fitness with salinity was similar to *S. densiflora* (-40%).

The greater transgressivity of *S. densiflora x foliosa* to inundation was also observed in its reproductive fitness (number of florets produced per individual). While it decreased in a similar extent with inundation (-41%) and salinity (-47%) for the hybrid, its parents were affected in a greater extent by

inundation (-82% *S. foliosa* and -76% *S. densiflora*) than salinity (-60% *S. foliosa* and -43% *S. densiflora*). In any case, the reproductive fitness of the hybrid was much higher than that of *S. densiflora* and especially *S. foliosa* that maintained low and constant floret production. However, *Spartina densiflora x foliosa* is sterile (Ayres et al. 2008, Lee et al. 2016), which was also observed in our study by the absence of mature seeds inside the florets. According to our results, if fertility is achieved by chromosomal doubling in the genome of *S. densiflora x foliosa* (Abbott 1992, Mallet 2007), the resulting allopolyploid would likely exhibit a spread ability even greater than its invasive parent *S. densiflora* (Kittelson and Milton 1997, Nieva et al. 2001, Castillo et al. 2010a) at a wide range of salinities and inundation regimes.

7.5 Conclusions

The comparative study of morphological, biochemical and physiological responses of the exotic hybrid *S. densiflora x foliosa* and its both parental species to the combined effects of salinity and inundation has shown a greater tolerance of the hybrid to both factors. Thus, the transgressivity found for certain functional traits of *S. densiflora x foliosa*, related to the greater trait variability of its parents, led to a greater vegetative and reproductive fitness of the hybrid at all abiotic conditions. Especially important was the development of transgressivity under high inundation stress, which was the factor more negatively affecting the fitness of both parents. The sterility of this hybrid is a limitation to its spread ability whereas, once established, it is highly competitive particularly due to the high and rapid development of AGB. The possibility of acquiring fertility by chromosomal doubling, together with the increase in inundation and salinity associated with SLR, will intensify the competitive superiority of the hybrid over its parents. In view of these circumstances, this exotic hybrid should be promptly eradicated from the San Francisco Bay.

CHAPTER VIII

Realized niche and spatial pattern of native and exotic halophyte hybrids

CAPÍTULO 8: NICHO REALIZADO Y PATRÓN ESPACIAL DE HÍBRIDOS NATIVOS Y EXÓTICOS DE HALÓFITAS

Resumen

La hibridación interespecífica es un mecanismo evolutivo importante y frecuente entre taxones vegetales, pero hay una falta de estudios de campo que analicen los cambios en el nicho realizado y en los patrones de zonación de híbridos nativos y exóticos en relación a sus especies parentales. En el presente estudio examinamos las principales características sedimentarias de los nichos realizados de híbridos nativos de *Sarcocornia*, híbridos exóticos de *Spartina* y sus especies parentales en 14 marismas de 4 estuarios del Suroeste de la Península Ibérica, así como el nivel de interacción entre los taxones vegetales y su patrón de zonación a lo largo del gradiente mareal. Los híbridos nativos de *Sarcocornia* y los exóticos de *Spartina* mostraron diferentes amplitudes de hábitat, patrones de zonación y comportamientos ecológicos que sus especies parentales. Mientras que los híbridos nativos y fértiles de *Sarcocornia* exhibieron una amplitud de nicho realizado similar a la de sus especies parentales y compartieron la marisma con otras especies, los híbridos exóticos y estériles de *Spartina* mostraron amplitudes de nichos realizados menores que sus especies parentales (probablemente debido a sus limitaciones de dispersión) pero no compartieron su espacio ocupado con otras halófitas. Así, mientras que los híbridos nativos juegan un papel estructurador, los híbridos exóticos de *Spartina* parecen estar teniendo un papel perturbador en el patrón nativo de zonación, lo cual podría ser agravado si estos híbridos se volvieran fértiles. A la vista de nuestros resultados, se deberían tomar medidas a la mayor brevedad posible con objeto de erradicar ambos híbridos de *Spartina* en el Golfo de Cádiz.

CHAPTER 8: REALIZED NICHE AND SPATIAL PATTERN OF NATIVE AND EXOTIC HALOPHYTE HYBRIDS

Abstract

Interspecific hybridization is an important and common evolutionary mechanism, but field-based evaluations of changes in realized niches and zonation patterns of native and exotic hybrids relative to those of their parental plant species are rare. Would native hybrids forming hybrid zones between their parental species show realized niches similar to that of their parents, whereas would exotic hybrids show larger realized niches than their parents, and alter zonation patterns of native species? To address these questions, we examined key sediment characteristics in plots representing realized niches of native *Sarcocornia* hybrids, invasive *Spartina* hybrids and parental species in 14 salt marshes from four estuaries in the Gulf of Cadiz, Southwest Iberian Peninsula. In one representative marsh, the presence of plant taxa relative to intertidal plant zonation was recorded. Results documented that native and fertile hybrids of *Sarcocornia* had similar realized niche dimensions as their parental species and co-occurred with other plant species, supporting community diversity. However, exotic sterile hybrids of *Spartina* had realized niche dimensions lower than those of their parental species and occurred in monocultures. The native hybrids played a community structuring role, whereas the exotic *Spartina* hybrids were a disruptive influence that changed native halophyte zonation pattern and decreased diversity. This negative functional role could intensify if the sterile hybrids evolve and become fertile. Our study suggests the ecological niche dimension concept is an important tool for understanding species roles in ecosystems, incorporating many ideas from the individual to ecosystem levels.

8.1 Introduction

The classical view of ecological niches describes the niche as environmental conditions within which a species can maintain a population in the long term without immigration (MacArthur 1972, Schoener 1989). The study and modelling of species niches has been widely used to predict their potential spatial distribution relative to their environmental requirements and physiological stress thresholds (Grinnell 1917, Qiao et al. 2017). A contemporary view of the niche concept includes focus on its utility for evaluation of functional ecological roles resulting from factors such as dispersion ability and species interactions within communities (Chase and Leibold 2003, Soberón and Peterson 2005).

Biological invasion by alien plant species is one of the primary threats to biological diversity (Ehrenfeld 2010, Vilà et al. 2011). Invasions also provide an opportunity to improve understanding of how evolution and ecology of plant species influence changes in the environment via organism-driven niche construction or alteration, and the distribution of species within communities (Odling-Smee et al. 2003, Turner et al. 2014). Interspecific hybridization is an important evolutionary mechanism and it is common between evolutionarily related plant species (Arnold 1997). Hybridization between native and alien species can intensify the impacts of biological invasions (Todesco et al. 2016). The phenotype of hybrids is the result of different inheritance mechanisms that give rise to contrasted performance of hybrids (Chen 2013). Different studies have linked hybridization with increasing invasiveness that can be the result of non-additive inheritance mechanisms leading to novel and beneficial genotypes, increasing genetic variation and fixed heterosis (Vilà et al. 2000, Ellstrand and Schierenbeck 2000) and conferring superior phenotypic traits to hybrids (Rieseberg and Wendel 1993, Comai 2005). This way, hybridization leads to a genetic change in the population that may cause a niche shift (breaking the phylogenetic niche conservatism), which often favors the invasive ability and

tolerance to environmental factors of the new genotype in comparison to their parents (Hovick et al. 2011, Thornton and Murray 2014). However, there is a lack of field studies analyzing if these genetic and phenotypic differences between native and exotic hybrids in relation to their parental species are reflected in significant changes in their realized niches and how this would affect zonation patterns (Hovick et al. 2011). With climate change, hybridization risk between introduced and native species may even be increased when climatic ranges of the potential hybridizing species overlap (Dehnen-Schmutz 2011).

Salt marshes are model ecosystems in which to study the environmental abiotic and biotic factors influencing the realized niche of species and the establishment of ecological zonation, since their plant communities with low species diversity are organized in bands parallel to the tidal line due to marked environmental gradients over short distances (Adam 1990). The study of environmental factors determining distribution and zonation patterns of halophytes is fundamental to determining the adequacy of conservation actions under a sea-level rise scenario associated with climate change that increases plant physiological stress levels in response to increased inundation (Pearson and Dawson 2003). In the marshes of the Gulf of Cadiz (Southwest Iberian Peninsula), the formation of hybrids of *Sarcocornia* between two native species and between a native and an invasive species of *Spartina* have been described (Figueroa et al. 2003; Castillo et al. 2010). The native fertile hybrids derived from *Sarcocornia fruticosa* (L.) A.J. Scott (family: Chenopodiaceae) from high marshes that hybridize with *Sarcocornia perennis* (Mill.) A.J. Scott from low marshes show additive traits (Castroviejo and Lago 1992, Figueroa et al. 2003). The exotic sterile hybrids are a consequence of different hybridization processes between the native European cordgrass *Spartina maritima* (Curtis) Fernald (family: Poaceae) from low marshes and the invasive South American *Spartina densiflora* Brongn. Both species act as maternal species resulting in the two hybrids *S. maritima x densiflora* in low elevation marshes and *S. densiflora x maritima* in middle marshes. These hybrids show some key transgressive traits

such as taller shoots and higher growth rates (Castillo et al. 2010a). This scenario is suitable for the comparison of the realized niches of exotic and native hybrids and their role in the establishment of the ecological zonation in relation to their parental species. With these aims, we examined: (1) the main sediment characteristics of the realized niches of the native *Sarcocornia* hybrids, the invasive *Spartina* hybrids and their parental species in 14 marshes of four estuaries; and (2) the level of interaction between plant taxa and the zonation pattern along the intertidal gradient of native and invasive hybrids and their parental species in a model salt marsh where these taxa were all well represented. Our hypothesis was that the native *Sarcocornia* hybrids would exhibit traits and occupy a realized niche similar to that of their parental species, occupying intermediate elevations between their two parents and structuring the plant zonation along the tidal gradient. In contrast, the exotic hybrids with transgressive traits would show larger realized niches than their parents, being able to alter the elevational zonation pattern occupied by the native species.

8.2 Material and Methods

Study sites

Our study was carried out in 14 salt marsh sites from four estuaries in the Gulf of Cadiz, Southwest Iberian Peninsula: 2 sites in the Estuary of Tinto River (Huelva, Spain; 37º12' - 37º17'N, 6º50' - 6º55' W), 8 sites in the Estuary of Odiel River (Huelva, Spain; 37º08'–37º20'N, 6º57'–7º02'W), 2 sites in the Estuary of Piedras River (Huelva, Spain; 37º12'–37º18'N, 7º12'–7º06'W), 1 site in the Estuary of Guadiana River (San Bruno Marsh, Huelva, Spain; 37º10'–37º16'N, 7º28'–7º16'W), and 1 site in the Estuary of Ria Formosa (Algarve, Portugal; 36º57–37º10'N, 7º28'–8º02'W). The native *Sarcocornia* hybrids were present in 4 sites (3 in the Odiel Estuary and 1 in the Guadiana Estuary) and the exotic *Spartina* hybrids in 4 sites (1 in the Tinto Estuary, 1 in the Odiel Estuary, 1 in the Piedras Estuary and 1 in the Guadiana Estuary) (see Appendix 8.A,

Location map). The physical environment and typical plant zonation pattern of the marshes of the Gulf of Cadiz are described in *Chapter 2* (see Appendix 8.B, plant zonation).

A salt marsh known locally as 'San Bruno', located at the Guadiana River Estuary was selected as a model ecosystem to evaluate spatial relationships of the native and invasive hybrid taxa and their parental species as well as the rest of species of the halophyte community, because it presents the typical zonation pattern of the salt marshes of the Gulf of Cadiz and includes a unique abundance of the hybrids and their parental species. Therefore, San Bruno Marsh was an ideal location for the characterization of the sediment environment relative to the plant community. This community includes hybrids of *Sarcocornia* and *Spartina* and their four parental species along the intertidal elevational gradient, from the lowest distribution limit of the marsh vegetation to the upper marsh ecotone transition to adjacent coastal dunes.

Regional scale: Realized niches

Environmental characteristics. We defined realized niche as the ability of a taxa to occupy habitat through resistance of competitive exclusion within occupied communities. We characterized the realized niches of the native *Sarcocornia* hybrids derived from *S. perennis* and *S. fruticosa*, the exotic *Spartina* hybrids of *S. maritima* and *S. densiflora*, and their parental species by sampling sediment variables where they were present. The salt marsh sediments (niche axes) within areas where these taxa were present were sampled for analyses in 2009 and 2016 in the Tinto Marshes, in 1997, 2001, 2005, 2007, 2009, 2010, 2013, 2015 and 2016 in the Odiel Marshes, in 2016 in the Piedras Marshes, in 2003 and 2016 in the Guadiana Marshes, and in 2016 in the Ria Formosa Marshes (Contreras-Cruzado et al. 2017). (see Appendix 8.A, Location Map; Appendix 8.C includes additional information about sampling plots within various study sites).

All sediment characteristics were measured or sampled from randomly chosen 0.5 x 0.5 m monospecific plots for the focal taxa (see Appendix 8.C for details). Sampling was executed during low tidal cycles in winter (November - January). Sediments for most variables were measured or collected at surface to –5cm depth. However, sediment redox potential (Eh) was also recorded at - 5 to -10 cm in The Guadiana Marshes. Sediment Eh was determined in the field with a portable meter and an electrode system (Crison pH/mV p-506), with recorded data being the mean of three sub-sample measurements. Elevation relative to SHZ was recorded *in situ* by using a Leica NA 820 theodolite (Singapore) with a resolution of 2 cm. Reference points were determined in relation to measurements of tidal extremes (Ranwell et al. 1964). Sediment samples were collected in 250 ml sealed vials and the pH of interstitial water of the sediment was recorded in the laboratory (pH/redox Crison with the electrode M-506) after adding distilled water to the sediment (1:1, v/v). Sediment salinity of the interstitial water was measured as electrical conductivity (conductivity meter, Crison-522) in the laboratory after adding distilled water to the sediment (1:2, v/v following Curado et al. 2014). Sediment water content (%) was recorded in the laboratory as the difference between fresh (ca. 100 g) and dry weights of samples. Dry weight (DW) was obtained after drying the sediments in an oven at 80 °C for 48 h (Castillo et al. 2016).

The proportional minimum to maximum value interval of each measured sediment variable that coincided with the presence of a specific taxon was calculated as the percentage of the data interval for each sediment variable relative to the cumulative minimum – maximum interval of that variable recorded from sampling where any of the focal taxa were present. The total mean dimension of the realized niche in relation to the sediment environment was calculated for every taxon as the arithmetic mean of its previously calculated intervals for every sediment variable.

Local Scale: Realized Niches

Sediment environment. In a more detailed study, the sediment variables reported above were also recorded in Jan 2016 in the San Bruno Marsh every 10 m along three randomly established altitudinal transects perpendicular to the tidal line set up from the lower to the upper distribution limit of the salt marsh vegetation. These transects were between 215-240 m long and they were separated by ca. 50 m. These data on the sediment environment were then related to the percent cover of each halophyte species present along the intertidal gradient.

Plant zonation pattern. To evaluate the spatial zonation patterns of focal taxa, absolute cover of each plant species (%) was measured along the same elevational transects where the sediment environment was recorded in the San Bruno Marsh. The presence of every plant taxon was annotated continuously along these transects. Parental taxa were identified following (Valdes et al. 1987). The exotic *Spartina* hybrids were distinguished from their parental species according to the size of the invaginations in the adaxial side of their leaves, described as intermediate between parental species, and their taller stems compared to their parental species (Castillo et al. 2010a). Castillo et al. (2010a) described that the *Spartina* hybrid colonizing low marshes (*S. maritima x densiflora*) had *S. maritima* as the maternal species and the hybrid colonizing middle and high marshes (*S. densiflora x maritima*) had *S. densiflora* as its maternal species. In this sense, Sloop et al. (2009) found highly local seedling dispersal, near the maternal plants, even in the tidally dynamic mudflats of San Francisco Bay. Due to the difficulty to distinguish between the cryptic *Sarcocornia* hybrids and their parental species based on field phenotypes, chromosome counts were carried out to discern between the hybrid taxa and their parental species. *S. perennis* presented a chromosomal number of 2n = 2x = 18 and *S. fruticosa* 2n = 8x = 72 (Valdes et al. 1987). The plants that presented chromosomal numbers of 2n = 45 and 54 were described as hybrids (Castroviejo

and Lago 1992, Figueroa et al. 2003). Chromosome counts were carried out following Bailey and Stace (1992). Spatial coincidence (%) between taxa was calculated for every intertidal transect as the percentage of each taxon coinciding with other taxon in relation to its total absolute cover. Total value for every pair of taxa was calculated as the arithmetic mean of their spatial coincidence for the three transects.

Statistical analysis

All statistical analyses were conducted using Sigma-Plot for Windows (version 12.0, Systat Software Inc., IL, US), except for the Canonical Correspondence Analyses (CCA) and the Monte Carlo permutation test that were conducted using 'vegan' package of R-software (R core team, 2016). Deviations were calculated as the standard error of the mean (SE). A significance level (α) of 0.05 was applied for every analysis. Prior to use of the parametric models, data series were tested for normality with the Kolmogorov-Smirnov's test and for homogeneity of variance with the Levene's test. Pearson correlation coefficient and linear regression between sediment variables and plant cover were conducted in order to explore the characterization of the realized niche dimension for each taxon. The mean of the sediment characteristics (using sites as replicates), the total dimension of the realized niche in all marshes under study (using the partial dimension of the niche for every sedimentary variable as replicates) and the spatial coincidence of pairs of taxa in the San Bruno Marsh (with the three recorded transects as replicates) were compared by one-way analysis of variance (ANOVA) using the taxon as the grouping variable. When an ANOVA was significant, Tukey's honestly significant difference (HSD) test was used as post hoc analysis. When homogeneity of variance or normality was not achieved, means were compared using a Kruskal-Wallis nonparametric ANOVA, with Bonferroni-Dunn's test as post hoc analysis. Canonical Correspondence Analyses (CCA) was carried out using a full model to test the significance of the relationship between all the environmental variables

measured and the presence/absence matrix of *Sarcocornia* and *Spartina* taxa to determine main sediment characteristics describing the realized niche of each taxon at different sites. Three sites in the Odiel Estuary, one site in the Tinto Estuary, one site in the Piedras Estuary, and one site in the Guadiana Estuary were included in the CCA. These are sites where paired samples for every sedimentary characteristic and plant species presence were recorded for the same sampling points. There was a unimodal distribution of species in relation to measured sediment characteristics reflecting elevational stress gradients imposed by tidal processes within the salt marshes. Monte-Carlo permutation tests (999 permutations) were performed for assessing significance of the canonical correlation coefficients.

8.3 Results

Regional scale: Characterization of realized niches

The *Spartina* hybrids invaded marsh elevations between +1.82 and +3.31 m SHZ, colonizing 56% of the total elevation interval occupied by all taxa (49% for *S. maritima x densiflora* and 24% for *S. densiflora x maritima*). *Spartina maritima* occupied 74% of the total elevation interval (between +1.41 and +3.39 m SHZ) and *Spartina densiflora* 88% (between +1.43 and +3.77 m SHZ). The *Sarcocornia* hybrids were present in 32% of the total elevation interval (between +2.47 and +3.33 m SHZ). *Sarcocornia perennis* was present in 43% (between +2.16 and +3.32 m SHZ) and *Sarcocornia fruticosa* was present in 57% of the total elevation interval (between +2.56 and +4.09 m SHZ). The lower distribution limit of *S. maritima*, *S. densiflora*, both *Spartina* hybrids and *S. perennis* was below the Mean High Water Neaps (+2.44 m SHZ). The lower distribution limit of the *Sarcocornia* hybrids coincided with the Mean High Water Neaps. *S. densiflora* and *S. fruticosa* were able to colonize elevations even above the Highest Astronomical Tide (HAT) (+3.71 m SHZ), while *S.*

densiflora x maritima, the *Sarcocornia* hybrids, *S. maritima* and *S. perennis* had their upper distribution limits close to the Mean High Water Springs (+3.37 m SHZ). Mean marsh elevation was lower for *S. maritima* than for *S. densiflora* and *S. densiflora x maritima*, showing no significant difference with *S. maritima x densiflora*. The mean elevation colonized by the *Sarcocornia* hybrids was higher than for *S. perennis* and lower than for *S. fruticosa* (Fig. 8.1A, Table 8.1).

The sediment pH interval occupied by *S. maritima x densiflora* was 17% of the total pH interval, 19% for *S. densiflora x maritima* (changing between 6.2 and 7.4), 25% for *S. maritima* (5.9-7.6) and 100% for *S. densiflora* (1.7-8.3). The interval of pH was 28% for the *Sarcocornia* hybrids (6.5-8.3), being similar than for their parental species. The mean pH for both *Spartina* hybrids was lower than for *S. maritima* and higher than for *S. densiflora*. No significant differences were found for sediment mean pH among the *Sarcocornia* taxa (Fig. 8.1B, Table 8.1).

The electrical conductivity of interstitial sediment water was measured as an interval of 40% for *S. maritima x densiflora* and 45% for *S. densiflora x maritima* (varying between 11.4 and 38.3 mS cm^{-1}), 59% for *S. maritima* (8.3-40.1 mS cm^{-1}) and 66% for *S. densiflora* (2.0-38.0 mS cm^{-1}). The interval of conductivity for the *Sarcocornia* hybrids was 65% (4.7-40.1 mS cm^{-1}), 38% for *S. perennis* (9.7-30.3 mS cm^{-1}) and 100% for *S. fruticosa* (2.0-56.4 mS cm^{-1}). Mean electrical conductivity was higher for *S. densiflora x maritima* than for its parental species. In contrast, the hybrids of *Sarcocornia* colonized sediments with lower mean conductivity than *S. fruticosa*, with *S. perennis* showing intermediate values (Fig. 8.1C, Table 8.1).

Interstitial sediment water content interval was 65% for *S. maritima x densiflora* and 38% for *S. densiflora x* maritima, 57% for *S. maritima* and 92% for *S. densiflora*. The *Sarcocornia* hybrids occupied an interval of sediment water content of 45%, being 67% for *S. perennis* and 73% for *S. fruticosa*. None significant difference was found in the mean water content of the sediments colonized by different taxa (Fig. 8.1D, Table 8.1).

The interval of sediment Eh occupied by *S. maritima x densiflora* was 34% and 24% for *S. densiflora x maritima* (varying between -41 and +235 mV), 77% for *S. maritima* (between -350 and +267 mV) and 73% for *S. densiflora* (between -130 and +456 mV). The *Sarcocornia* hybrids coincided with a sediment Eh interval of 38% (between -27 and +277 mV), with 45% for *S. perennis* (between -87 and +277 mV) and 25% for *S. fruticosa* (between +56 and +254 mV). The mean Eh of the sediments colonized by *S. maritima* was lower than for *S. densiflora*, with both *S. maritima x densiflora* and *S. densiflora x maritima* associated with intermediate values. The hybrids of *Sarcocornia* and *S. fruticosa* colonized sediments with similar Eh, being higher than those colonized by *S. perennis* (Fig. 8.1E, Table 8.1).

The mean dimension of the realized niche was higher for *S. densiflora* than for both exotic hybrids, with *S. maritima* showing intermediate values. All *Sarcocornia* taxa showed similar realized niche dimensions (Table 8.1). *Spartina* and *Sarcocornia* hybrids showed similar realized niche dimensions (ANOVA, $F = 0.985$, $P = 0.402$).

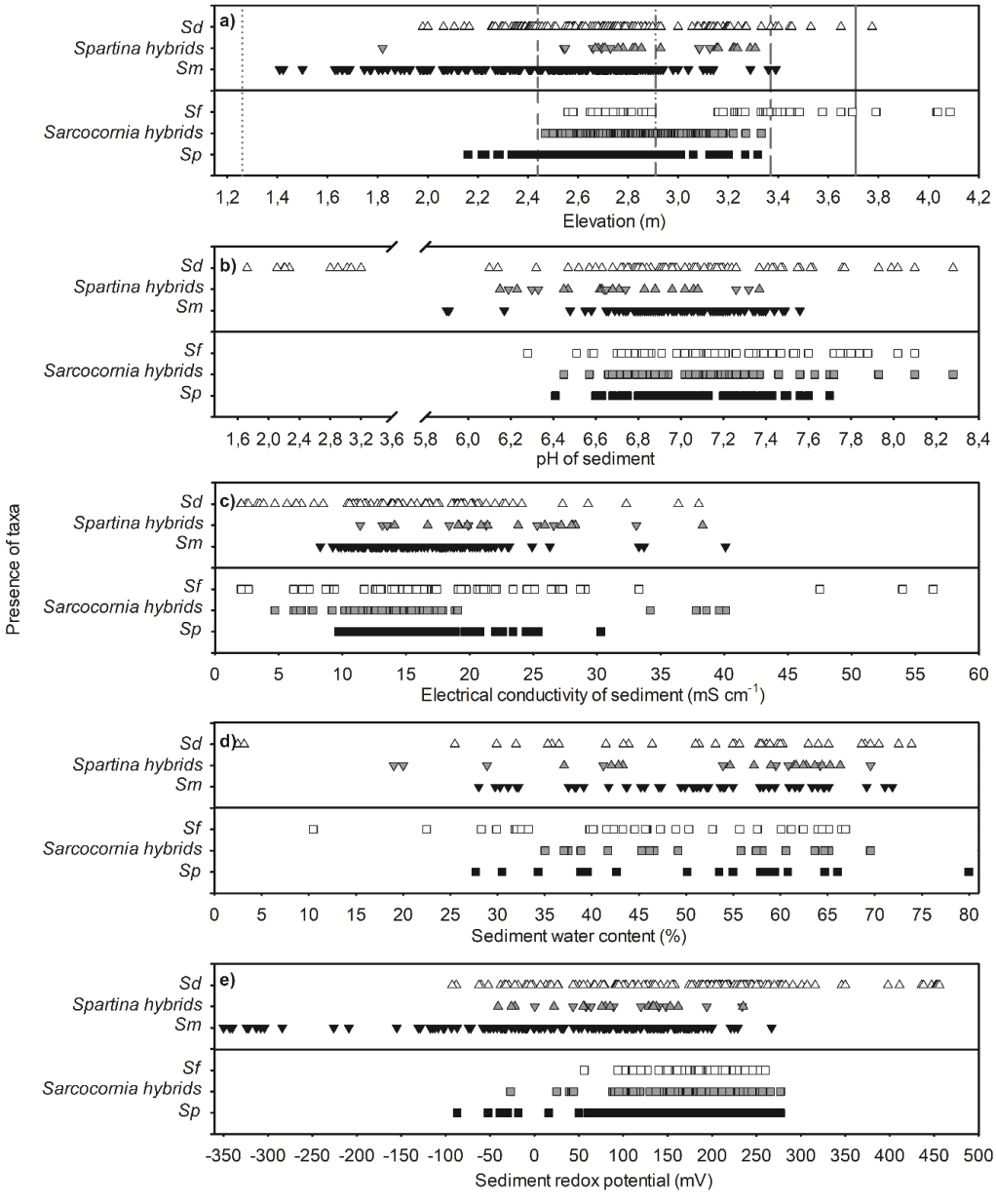

Fig. 8.1 Realized niche indicated by measuring presence of various focal species (*Spartina maritima x densiflora* (inverted grey triangle), *S. densiflora x maritima* (upright grey triangle), *S. maritima*, *S. densiflora*, *Sarcocornia* hybrids, *S. perennis* and *S. fruticosa* along environmental gradients (niche axes) relative to a) marsh surface elevation over Spanish Hydrographic Zero (SHZ) (m), b) sediment pH, c) electrical conductivity of sediment (mS cm^{-1}), d) sediment water content at low tide (%) and e) sediment redox potential (Eh) (mV). Vertical lines in a) indicate: Mean Low Water Neaps (.....), Mean High Water Neaps (_ _), Mean High Water (___ . . _), Mean High Water Springs (___ ___) and Highest Astronomical Tide (____).Species: Sm, *S. maritima*; Sd, *S. densiflora*; Sp, *S. perennis*; Sf, *S. fruticosa.*

Table 8.1 Percentage for every taxon of the total interval covered by all taxa for marsh elevation (m above Spanish Hydrographic Zero), sediment pH, electrical conductivity (mS cm^{-1}), water content (%) and redox potential (mV). Values for measured sediment characteristics (mean ± SE). Habitat amplitude for *Spartina maritima, Spartina densiflora, Sarcocornia perennis, Sarcocornia fruticosa* and their hybrids in the salt marshes of the Gulf of Cadiz, Southwest Iberian Peninsula. Habitat amplitude represents the arithmetic mean of all measured environmental habitat intervals for each plant taxa. Different letters in superscripts indicate significant differences between plant taxa for every sediment characteristic and habitat amplitude (ANOVA or Kruskal-Wallis test, $P < 0.05$, n = 10-212; n.s., non-significant)

Taxon	Elevation	pH	Conductivity	Water content	Redox potential	Habitat amplitude
S. densiflora	88% / 2.68 ± 0.03^{a}	100% / 6.5 ± 0.1^{a}	66% / 15.4 ± 0.8^{a}	92% / 51 ± 3^{a}	73% / 169 ± 12^{a}	79 ± 7^{a}
S. densiflora x maritima	24% / 2.94 ± 0.06^{b}	19% / 6.7 ± 0.1^{ab}	45% / 23.4 ± 1.6^{b}	38% / 56 ± 3^{a}	24% / 86 ± 19^{ab}	30 ± 5^{b}
S. maritima x densiflora	49% / 2.70 ± 0.13^{abc}	17% / 6.7 ± 0.1^{ab}	40% / 20.3 ± 2.4^{ab}	65% / 46 ± 7^{a}	34% / 109 ± 23^{ab}	41 ± 8^{b}
S. maritima	74% / 2.49 ± 0.03^{c}	25% / 7.0 ± 0.0^{b}	59% / 16.3 ± 0.5^{a}	57% / 50 ± 2^{a}	77% / 44 ± 11^{b}	58 ± 9^{ab}
ANOVA or Kruskal-Wallis test	H = 32.3 d.f = 3 $P < 0.001$	H = 10.9 d.f = 3 $P < 0.001$	H = 22.6 d.f = 3 $P < 0.001$	n.s.	H = 52.8 d.f = 3 $P < 0.001$	F = 8.0 d.f = 3 $P < 0.005$
S. fruticosa	57% / 3.18 ± 0.06^{a}	28% / 7.2 ± 0.0^{a}	100% / 18.1 ± 1.3^{a}	73% / 46 ± 3^{a}	25% / 183 ± 5^{a}	57 ± 14^{a}
Sarcocornia hybrids	32% / 2.84 ± 0.02^{b}	28% / 7.1 ± 0.1^{a}	65% / 14.8 ± 1.1^{b}	45% / 51 ± 3^{a}	38% / 171 ± 6^{a}	42 ± 7^{a}
S. perennis	43% / 2.69 ± 0.02^{c}	20% / 7.1 ± 0.2^{a}	38% / 15.1 ± 0.5^{ab}	67% / 52 ± 3^{a}	45% / 136 ± 7^{b}	43 ± 8^{a}
Kruskal-Wallis test	H = 80.9 d.f = 2 $P < 0.001$	n.s.	H = 24.9 d.f = 2 $P < 0.001$	n.s.	H = 11.4 d.f = 2 $P < 0.005$	n.s.

The first 2 axes of the CCA explained 82.9 % of the total variance of the data relating environmental variables to taxa presence. Axes 1 was strong and positively correlated with sediment Eh and elevation, and Axes 2 was positively correlated with sediment conductivity and negatively with pH (Fig. 8.2, Table 8.2). According to a Monte-Carlo permutation test these 2 axes were significant at $P < 0.001$. Axes 3, 4 and 5 only represented 17.1% of total variance (Table 8.2). The realized niche of *S. maritima* was characterized by low elevations and low Eh, whereas *S. fruticosa* showed the opposite response. Both *Spartina* hybrids showed high positive correlation with conductivity and negative correlation with pH, and the contrary occurred with the *Sarcocornia* hybrids. *S. densiflora* and *S. perennis* were situated at the center of the CCA diagram with none of the sediment variables dominating the characterization of their realized niches (Fig. 8.2, Table 8.2).

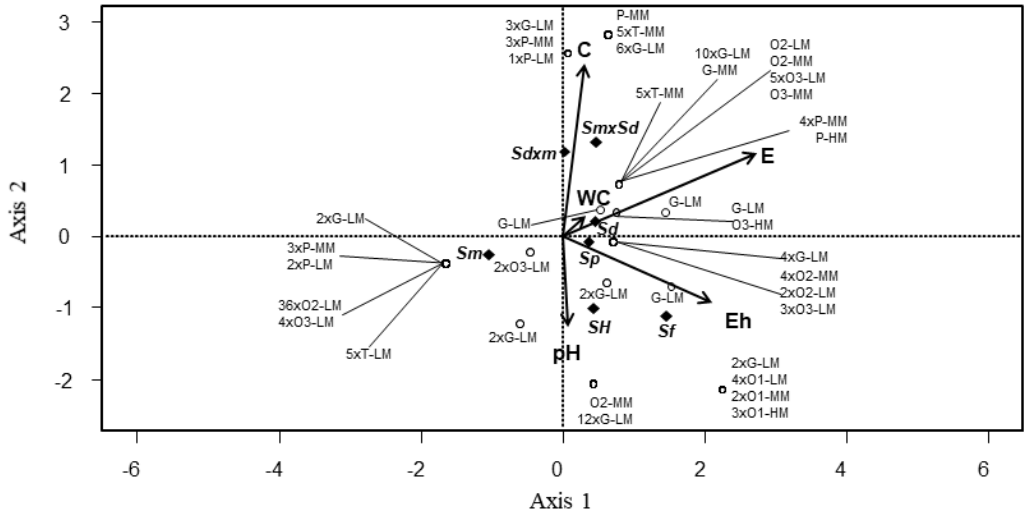

Fig. 8.2 Ordination diagram of a Canonical Correspondence Analysis (CCA) with species presence (black diamonds), sites (open circles) and sediment variables (arrows). The first two CCA axes accounted for 82.9% of the cumulative variance. Species: Smxd, Sdxm, *Spartina* hybrids, Sm, *S. maritima*; Sd, *S. densiflora*; SH, *Sarcocornia* hybrids; Sp, *S. perennis*; Sf, *S. fruticosa*. Sedimentary variables are: WC, water content; C, electrical conductivity; Eh, redox potential; E, elevation

Table 8.2 Eigenvalues, proportion of explained variance and regression coefficients for canonical axes against standardized variables for ordination of plant species abundance relative to sediment characteristics and marsh elevations. Values less than 0.05 have been omitted. Marsh elevations reported are relative to Spanish Hydrographic Zero (SHZ)

	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5
Eigenvalue	+0.41	+0.22	+0.12	+0.01	+0.002
Explained variance	+0.53	+0.30	+0.15	+0.01	+0.003
Spartina densiflora	+0.18	+0.20	-0.16	-	-
S. densiflora x maritima	+0.26	+0.97	-0.27	-0.18	-0.05
S. maritima x densiflora	-	+0.97	+0.54	+0.33	-
Spartina maritima	-0.70	-0.15	-	-	-
Sarcocornia fruticosa	+1.09	-0.59	-0.43	-	+0.06
Sarcocornia hybrids	+0.50	-0.15	+0.63	-0.09	-
Sarcocornia perennis	+0.22	-0.27	-0.09	+0.09	-0.09
Sediment pH	+0.09	-0.69	-0.26	-	+0.67
Sediment water content	+0.10	+0.17	-0.28	-0.77	-0.55
Sediment conductivity	+0.07	+0.74	-0.07	-0.47	+0.47
Sediment redox potential	+0.72	-0.22	+0.65	+0.08	-0.07
Marsh elevation	+0.77	+0.42	-0.38	+0.28	-

Local scale zonation pattern: The San Bruno case study

Results from the detailed study at San Bruno Marsh revealed that sediment Eh at surface and depth were positively correlated with each other, and with marsh elevation (Fig. 8.3). Sediment pH decreased with increasing conductivity and water content, and was also positively correlated with the marsh elevation (Fig. 8.3). Conductivity and water content were highly correlated in a positive direction. Both variables reached their maximum at intermediate elevations, and decreased markedly at higher elevations (Fig. 8.3).

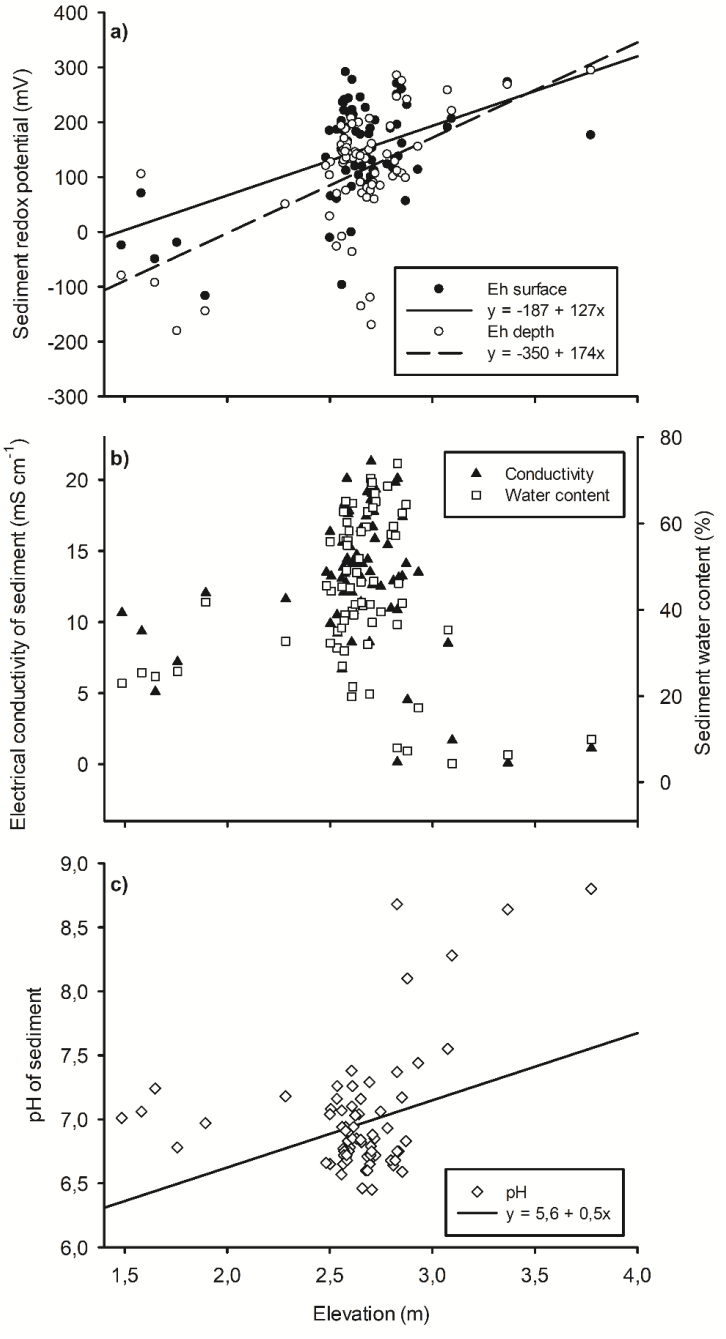

Fig. 8.3 Relationships between marsh elevation (m over SHZ) and a) redox potential (Eh) (mV) at surface and depth, b) electrical conductivity (mS cm^{-1}), water content (%) and c) pH of sediments.

Every analyzed *Sarcocornia* plant with a prostrate growth-form in low marsh elevations consistently had the same 2n = 18 chromosome numbers, corresponding to *S. perennis*. The *Sarcocornia* plants with a complete to partial-erect growth form within low-middle and middle-high marshes had chromosome counts of 2n = 45 and 54, corresponding to hybrids of *Sarcocornia*. Finally, totally erect plants of *Sarcocornia* colonizing the high marsh close to the ecotone with the coastal dunes had 2n = 72 chromosomes, corresponding to *S. fruticosa*.

The elevation distribution and absolute cover of all plant species recorded along study transects are shown in Figure 2.4. *Atriplex portulacoides* L. was the most abundant species at San Bruno Marsh, with 33% absolute cover recorded mainly at middle elevations. *S. densiflora* was present at 29% cover, mostly in high marsh areas. The *Sarcocornia* hybrids comprised 28% of the vegetation cover, and were mostly in middle intertidal elevations. Their cover tended to decrease at higher sediment Eh at depth (r = -0.244, P < 0.07, n = 55). *S. perennis* and *S. fruticosa* were present at 8 and 7% cover in low and high marsh areas, respectively. The cover of *S. fruticosa* increased with the distance to the main channel (r = +0.366, P < 0.01) and sediment water content (r = +0.346, P < 0.01, n = 55). *Spartina maritima* was present in lower elevations and its absolute cover decreased at higher elevations (r = -0.295, P < 0.05, n = 55) and higher Eh at surface (r = -0.360, P < 0.01, n = 55). The cover of *S. densiflora* was positively correlated with the distance to the main channel (r = +0.568, P < 0.001, n = 55), sediment conductivity (r = +0.281, P < 0.05, n = 55) and water content (r = +0.309, P < 0.05, n = 55). Together both *Spartina* hybrids accounted for 4% cover in the plant community. The cover of both hybrids was not related with any sediment characteristics (Pearson correlation coefficient, P > 0.05, n = 19-36). *Spartina maritima x densiflora* was present at 1% cover in 3 discrete bands at low elevations, and *S. densiflora x maritima* occurred at 3% cover, and was distributed over 6 discrete areas at high elevations. *Arthrocnemum*

macrostachyum and *Suaeda vera* were present in the highest elevations in and only accounted for low absolute cover at 1 and 0.3%, respectively (Fig. 8.4).

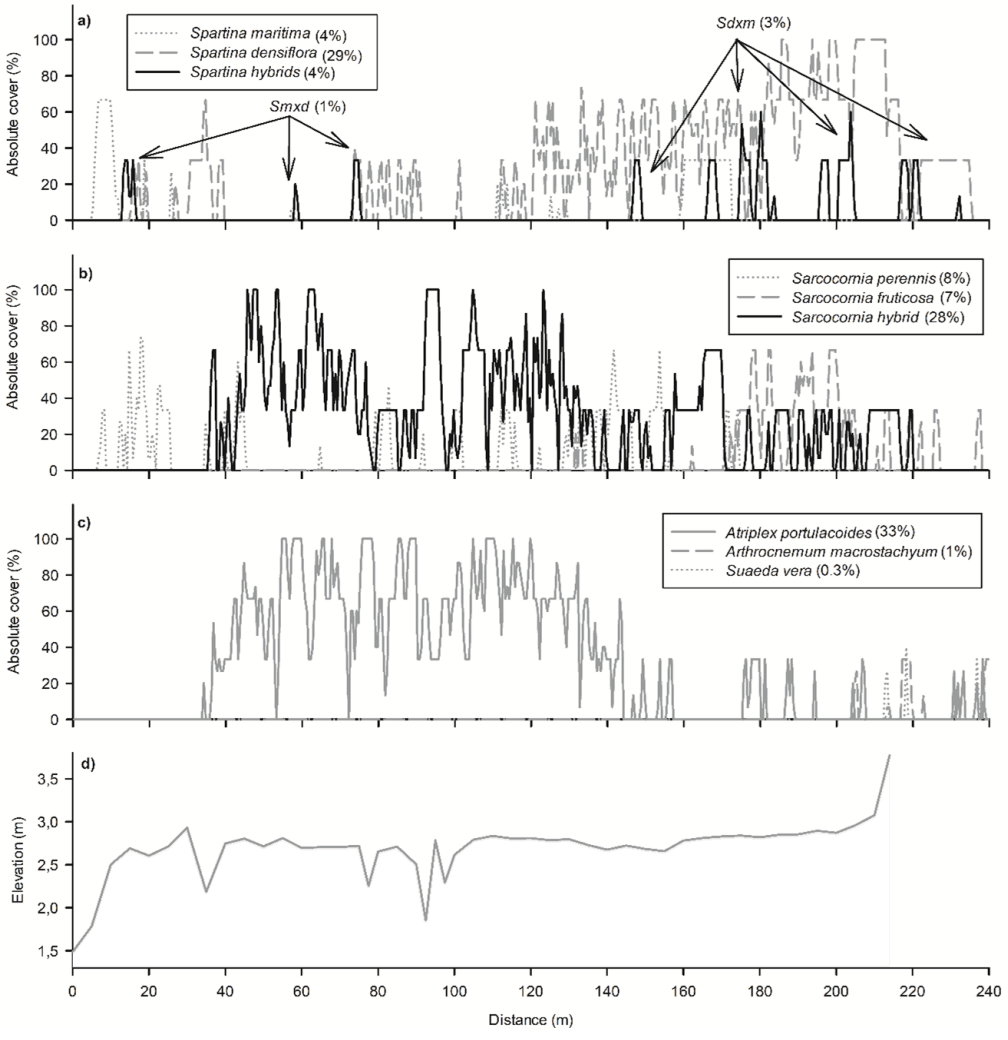

Fig. 8.4 Relationship between a-c) absolute cover for different halophyte taxa and distance to the lower distribution limit of vegetation (n = 3 transects). d) Marsh elevation (m over SHZ) from transect data recorded in San Bruno Marsh in relation to distance.

Spartina maritima x densiflora did not co-occur with any other taxa. *S. densiflora x maritima* showed only a total spatial coincidence with other taxa of 7 ± 1% of its total cover, varying between 1% cover with the *Sarcocornia* hybrids to 2% cover when associated with *S. densiflora* and *S. fruticosa*. A total

of 43 ± 26% of the cover of *S. maritima* was recorded where the species co-occurred with at least one other plant taxa, and *S. densiflora* coincided spatially with other plant taxa at 52 ± 2% of its absolute cover. The maximum co-occurrence of the *Sarcocornia* hybrids was 50 ± 6% with *A. portulacoides*, resulting in 75 ± 10% of its cover coexisting with other taxa. *S. perennis* appeared with other taxa in 29 ± 4% of its cover, and *S. fruticosa* shared its spatial distribution mostly with *S. densiflora* (68 ± 6%) (Table 8.3).

8.4 Discussion

In this study, we illuminate differences in the realized niche breadths among native and alien invasive species and their hybrids and their influence on spatial zonation patterns in multiple salt marshes of the Southwest Iberian Peninsula. Niche segregation is thought to enable the coexistence of closely related plant taxa. When introduced species mate and hybridize with nearby native flora, niche segregation relative to environmental conditions can influence the frequency and direction of introgressive hybridization though mating isolation does not always occur (Pieper et al. 2018). Studies of *Spartina* invasions have noted that in cases where there is realized niche overlap among progenitor species and their hybrid taxa, the native taxa may be at greater risk of extinction through competitive displacement from hybrids than from the alien parental species alone (Strong and Ayres 2013). For these reasons, improved understanding of the realized niche of invasive species is an important step towards recognizing the impacts of invasions on salt marsh and other ecosystems.

	Spartina densiflora	*Spartina densiflora x maritima*	*Spartina maritima x densiflora*	*Spartina maritima*	*Sarcocornia fruticosa*	*Sarcocornia* hybrids	*Sarcocornia perennis*	*Atriplex portulacoides*	*Arthrocnemum macrostachyum*	*Suaeda vera*	Total
Spartina densiflora	-	0	0	0	17 ± 7	19 ± 2	1 ± 1	14 ± 3	1 ± 0	0	52 ± 2
Spartina densiflora x maritima	2 ± 2	-	0	0	2 ± 1	1 ± 1	0	2 ± 2	0	0	7 ± 1
Spartina maritima x densiflora	0	0	-	0	0	0	0	0	0	0	0 ± 0
Spartina maritima	0	0	0	-	0	24 ± 24	16 ± 10	3 ± 2	0	0	43 ± 26
Sarcocornia fruticosa	68 ± 6	2 ± 1	0	0	-	8 ± 8	0	3 ± 1	0	3 ± 3	84 ± 7
Sarcocornia hybrids	19 ± 3	0	0	5 ± 5	1 ± 1	-	0	50 ± 6	0	0	75 ± 10
Sarcocornia perennis	5 ± 3	0	0	8 ± 4	0	1 ± 1	-	15 ± 8	0	0	29 ± 4
Atriplex portulacoides	12 ± 3	0	0	0	1 ± 0	44 ± 6	4 ± 2	-	0	0	61 ± 5
Arthrocnemum macrostachyum	32 ± 24	0	0	0	0	0	0	0	-	4 ± 4	36 ± 21
Suaeda vera	14 ± 14	0	0	0	19 ±19	0	0	19 ± 19	33 ± 33	-	85 ± 19

Table 8.3 Percentages of co-occurrence of focal halophyte taxa within San Bruno Marsh in the Guadiana River Estuary, southwest Iberian Peninsula. The percentages of the total cover of the taxa and the total % cover of each taxa coinciding with other taxa are reported in the rows. Values are mean ± SE (n = 3 transects).

Realized niche and zonation of native hybrids

In our study, the lower elevational distribution limit of the *Sarcocornia* hybrids coincided with the MHWN (+2.44 m SHZ) and their realized niche was characterized by sediment Eh levels that were always higher than -50 mV, without anoxia stress (Wildish et al. 2001). Thus, sediment Eh for the *Sarcocornia* hybrids did not differ from that of *S. fruticosa* colonizing high marshes, and both were higher than for *S. perennis* at low marshes. In this sense, Figueroa et al. (2003) described that *S. perennis* facilitated the colonization of its F1 hybrid on oxygenated sediments after promoting accretion (Castellanos et al. 1994). In view of these results, the lower distribution limit of the native hybrids of *Sarcocornia* seemed to be related to the level of sediment anoxia.

The upper distribution limit of the *Sarcocornia* hybrids coincided with the MHWS (+3.37 m SHZ) and a marked decrease in both soil water content and interstitial sediment salinity. This may be explained by the change in the texture of the sediments from fine-texture sediments at middle marshes to sandy sediments with lower water and salt retention capacity at high marshes (Muñoz-Vallés et al. 2015). The absence of the hybrids of *Sarcocornia* from sandy, brackish and drier sediments in the San Bruno Marsh coincided with their realize niche being characterized by soil water content at levels higher than 34%, similar to the conditions recorded for their parental species *S. perennis*. Davy et al. (2006) studied *S. perennis* at 29 English salt marshes and found similar results with the species occurring where soil water content was >25%. Thus, the upper elevational distribution limit of the native *Sarcocornia* hybrids seemed to be directly limited by their low tolerance to well-drained sandy sediments and/or, indirectly, by interspecific competition with species more tolerant of the high marsh environment. In this sense, the abundance of the *Sarcocornia* hybrids decreased at higher elevations where they co-occurred with more abundant species such as their parental species *S. fruticosa* and invasive *S. densiflora*.

In agreement with our results, other studies conducted in European marshes described *S. perennis* as a low marsh species colonizing non-anoxic sediments (Davy et al. 2006) and *S. fruticosa* as a high marsh species colonizing sediments with low flooding influence (Álvarez et al. 2000). In this study, the occurrence of the native *Sarcocornia* hybrids under intermediate levels of both recorded sediment characteristics and intertidal elevations relative to their parental species, but with similar realized niche dimensions, documents the existence of a continuum of different *Sarcocornia* taxa along almost the entire intertidal gradient. The location and high abundance of the native hybrids in the middle marsh suggests their role as structuring taxa and as a 'bridge' between their parental species which occur below and above them in low and high marsh. This structuring role was reflected in the formation of stable patches throughout the middle marsh (Figueroa et al. 2003). Here, these native hybrids coexist with *A. portulacoides*, the co-dominant species in this habitat, at a maximum spatial coincidence of 50 ± 6% cover. *A. portulacoides* has been described as achieving maximum biomass production in middle intertidal elevation zones (Crooks et al. 2002), where it has high competitive ability (Dormann et al. 2000). Despite their similar realized niche dimension, the *Sarcocornia* hybrids at 28% absolute cover were more abundant than their parental species that were limited to 8% and 7% cover for *S. perennis* and *S. fruticosa* respectively in the San Bruno Marsh. This is because the +2.47 and +3.33 m SHZ elevation interval in which the hybrids were present was 89% wider in this marsh with respect to the total length of the intertidal habitat, in contrast to 8% lower and 3% higher elevations colonized by their parental species. This situation is common in many intertidal wetlands where extensive middle marsh elevation zones are bordered by narrow fringing bands of low and high tidal salt marshes.

In view of our results, the *Sarcocornia* taxa in the studied marshes have formed a stable hybrid zone that seems to be related to the fitness of the hybrids being bounded by their parents thus occupying an intermediate environment (Buggs, 2007). As in our study, hybridization between native species of the

genus *Sarcocornia* in South Africa (mainly in the *S. natalensis* (Bunge ex Ung.-Sternb.) A.J. Scott, *S. pillansii* (Moss) A.J. Scott clade and in S. *capensis* (Moss) A.J. Scott) gave rise to hybrids that occupied different positions along the intertidal gradient according to their contrasted tolerances to different abiotic factors (Steffen et al. 2015). In contrast with our results, the native hybrid *Helianthus paradoxus* (*H. annuus* × *H. petiolaris*) was able to colonize salt marshes in New Mexico from where both of its parental species were excluded due to their lower salinity tolerance (Lexer et al. 2003a). This is probably related to different inheritance mechanisms operating in the hybridization processes being additive for adaptive traits in individuals with intermediate characters between their parents (Taylor and Rowland 2010) and transgressive segregation of adaptive characters resulting in hybrids that occupy ecological niches different from those of their parents (Rieseberg et al. 1999).

Realized niche and zonation of exotic hybrids

The lower distribution limits for both *Spartina* hybrids (*S. maritima x densiflora*: +1.82 m SHZ; *S. densiflora x maritima*: +2.67 m SHZ) were at higher elevations than those of their parental species. The lowest elevation for *S. maritima* was +1.41 m SHZ, tolerating long flooding periods and high anoxia levels. By comparison, the lowest elevation observed for *S. densiflora* was +1.43 m SHZ for biannual populations and +2.00 m SHZ for perennial populations due to its low tolerance to anoxic sediments (Castillo et al. 2000). *S. densiflora* tussocks in the more stressful abiotic environment of the low marshes die off after flowering limiting plants in this habitat to biannual lifespan. In contrast, perennial populations are established and persist at higher elevations along the intertidal gradient where soil redox conditions are more benign (Castillo and Figueroa 2009).

On the other hand, the upper distribution limits observed for both *Spartina* hybrids were at lower elevations than those of their parental species (*S.*

maritima x densiflora: +3.13 m SHZ; *S. densiflora x maritima*: +3.31 m SHZ), being the upper distribution limit of *S. maritima* (+3.39 m SHZ) determined by interspecific competition (Castellanos et al. 1994) and that of *S. densiflora* (+3.67 m SHZ) by the upper distribution limit of the salt marshes close to the ecotone with the coastal dunes.

In fact, both hybrids of *Spartina* colonized where levels of pH, salinity and elevation were less than where their parental species occur. The hybrids also occurred at an intermediate interval of sediment Eh relative to their parental species, which was reflected in lower realized niche dimensions for the hybrids. This limitation in the distribution of both *Spartina* hybrids contrasted with a broader tolerance to the abiotic environment along the intertidal gradient than their parental species recorded by Castillo et al. (2010a) in a transplant experiment, indicating a dispersion limitation in these sterile hybrids. The invasive *S. densiflora* showed the widest realized niche dimension, occupying ca. 80% of the total interval covered by every taxon. Invasive plant species show high levels of tolerance to extreme factors (Cadotte and Lovett-Doust 2001, Sutherland 2004, Bernez et al. 2006), and successfully colonize and spread in dynamic environments quite different from their native range (Dukes and Mooney 1999). This may give rise to a niche shift of the invasive species in the introduced range (Broennimann et al. 2007). This niche shift may take place in the realized niche if it is related to a decrease in competition, predation or parasitism, or in the fundamental niche when a genetic change related to founder effects, adaptation or hybridization occurs (Wiens and Graham 2005, Pearman et al. 2008).

Hybridization has been highly related to an increase of invasiveness when heterosis occurs resulting in a genetic improvement conferring hybrid vigor (Ellstrand and Schierenbeck 2000). The novel genetic combinations of hybrids may lead to new responses to abiotic factors and therefore to an expansion of the realized niche in relation to their parental species (Duchoslav et al. 2010, Thornton and Murray 2014). In this sense, the invasive hybrids of

Spartina alterniflora Loisel and *Spartina foliosa* Trin. have been described as having numerous transgressive traits in relation to their parents (Ayres et al. 2003, 2004). Their ecological impact in the plant communities in the San Francisco Estuary has been significant, and mainly due to their great tolerance to the abiotic environment and their ability to spread rapidly and widely by seeds (Ayres et al. 2003, 2004, Hall et al. 2006). In contrast, the limitation in the spread of transgressive *Spartina* hybrids in the Gulf of Cadiz (occupying only 1% of the San Bruno Marsh for *S. maritima x densiflora* and 3% for *S. densiflora x maritima*) may be related with their inability to produce viable seeds (Castillo et al. 2010a). Thus, they have been relegated to discrete bands near their maternal species. These hybrids also have a very low percentage of coincidence with other species (0% for *S. maritima x densiflora*; 7% for *S. densiflora x maritima*) in relation to their parents (51% for *S. densiflora;* 43% for *S. maritima*) and the rest of analyzed taxa. This result suggests both *Spartina* hybrids may have high competitive abilities related to transgressive traits such as tall shoots and high growth rates (Castillo et al. 2010a), totally excluding other halophytes from their colonized areas and altering the native zonation pattern.

The fact that the studied hybrids of *Spartina* formed narrow discrete spaces between their parental species as hybrid 'tension zones' points to the fitness of the hybrids being lower than that of the parental species probably due to their infertility. This situation could change if the *Spartina* hybrids become fertile through allopolyploidy, and achieve fitness greater than their parental species (Buggs, 2007). As in our study, the sterile hybrids between *S. densiflora* and *S. foliosa* in San Francisco Bay (Ayres et al. 2008) mostly grow in isolated patches in middle marshes nearer *S. densiflora*; however, they have high tolerance to abiotic factors such as salinity (Lee et al. 2016). Fertility can be achieved when chromosomal doubling occurs among hybrids resulting in a new allopolyploid species (Abbott 1992). This process was reported in the genus *Spartina* when the sterile first-generation hybrid between the native *S. maritima*

and the invasive *S. alterniflora* gave rise to a fertile and successful allopolyploid hybrid, *Spartina anglica*, C. E. Hubb. in England (Huskins 1930, Guenegou et al. 1988) that had rapidly expanded to many locations around the world (Aïnouche et al. 2004, Strong and Ayres 2009). Superior competitive abilities have been also described in the invasive hybrid *Typha x glauca* Godron that competitively dominates its two parental species in North America (Waters and Shay 1992, Kuehn and White 1999) as well as other native species (Travis et al. 2010, Larkin et al. 2012b, Farrer and Goldberg 2014). Thus, hybridization followed by polyploidization have been related to habitat shifts (Donkpegan et al. 2017).

8.5 Conclusions

Our study shows that the niche dimension concept may be an important tool to analyze species roles in the ecosystems, incorporating many ideas from the individual to the ecosystem levels. Thus, our approach to the niche provides a framework to explore the function of plant hybrid taxa in plant communities in relation to their parental species, examining the ways hybrids respond to their environment (i.e. their requirements) and reciprocally, how they impact the environment (i.e. through biotic interactions such as interspecific competition). Our results point to fertile and sterile, and native and exotic hybrids carrying out contrasted responses to the abiotic environment and developing different roles in how plant communities assemble, which may be relevant to the biological evolution of hybrids.

CHAPTER IX

The role of exotic and native hybrids in the ecological succession of salt marshes

CAPÍTULO 9: EL PAPEL DE LOS HÍBRIDOS EXÓTICOS Y NATIVOS EN LA SUCESIÓN ECOLÓGICA DE MARISMAS SALADAS

Resumen

El estudio del desarrollo de la sucesión ecológica es clave para el éxito de la conservación y la restauración de los ecosistemas. Procesos de tolerancia a ambientes estresantes e interacciones entre especies determinan las diferentes etapas de la sucesión, por lo que el análisis tanto de las características bióticas como abióticas de los ecosistemas en el tiempo resulta fundamental. En este sentido, especies con alta tolerancia al estrés y/o potencial competitivo, como especies invasoras o híbridos transgresivos, pueden adquirir un papel relevante en el desarrollo de la sucesión. Nosotros estudiamos el papel de los híbridos nativos de *Sarcocornia* y exóticos e invasores de *Spartina* en el desarrollo de la sucesión ecológica en marismas del Golfo de Cádiz. Para ello se registraron las principales características sedimentarias y la estructura de la vegetación en marismas costeras sucesionales y no sucesionales durante aprox. 12 años. Los híbridos nativos e invasivos desempeñaron diferentes papeles en la sucesión ecológica. Los híbridos nativos de Sarcocornia aumentaron su abundancia en marismas sucesionales y no sucesionales a lo largo del tiempo sin inhibir el desarrollo de la sucesión, ya que no impidieron la colonización por parte de otras especies nativas. Además, la propagación de los híbridos nativos de *Sarcocornia* coincidió con la disminución en la distribución de la especie invasora *Spartina densiflora*. Por el contrario, los híbridos invasores de *Spartina* inhibieron el desarrollo de la sucesión al superar competitivamente a otras halófitas nativas en la marisma no sucesional, mientras que no estuvieron presentes en la marisma sucesional donde la especie parental *S. densiflora* acaba de comenzar su invasión.

CHAPTER 9: THE ROLE OF EXOTIC AND NATIVE HYBRIDS IN THE ECOLOGICAL SUCCESSION OF SALT MARSHES

Abstract

The knowledge of the development of ecological succession is key for integrated successful conservation and restoration of ecosystems. Processes of tolerance to stressful environments and interactions between species determine the different stages of succession, so the analysis of both abiotic and biotic characteristics of ecosystems over time become fundamental. In this sense, species with high expected tolerance to stress and/or competitive ability, such as invasive species or transgressive hybrids, may acquire a relevant role in the development of ecological succession. We studied the role of native *Sarcocornia* and exotic and invasive *Spartina* hybrids on the development of ecological succession in salt marshes of the Gulf of Cadiz (SW Iberian Peninsula). Main sedimentary characteristics and vegetation structure were recorded in successional and non-successional coastal salt marshes during ca. 12 years. Native and invasive hybrids played different roles in ecological succession. Native *Sarcocornia* hybrids increased their abundance in successional and non-successional marshes over time without inhibiting the development of succession since they did not prevent the colonization by other native species. Furthermore, the spread of native *Sarcocornia* hybrids coincided with the decrease in the distribution of invasive *Spartina densiflora*. On the contrary, invasive *Spartina* hybrids inhibited the development of succession by outcompeting other halophytes in the non-successional marsh, whereas they were not present in the successional marsh where the parental species *S. densiflora* has just started its invasion.

9.1 Introduction

Ecological succession may be described as an orderly, and therefore foreseeable, process by which ecosystems develop (Odum 1969). Our knowledge about the development of ecological succession is key for integrated successful conservation and restoration of ecosystems (Rosenberg and Freedman 1984). At the early stages of ecological succession, the main conditioners of plant colonization are the tolerance of the species to stressful environmental factors followed by the occurrence of facilitation processes, while biotic relations such us competition and inhibition progressively become dominant at the later stages (Connell and Slatyer 1977, Maestre et al. 2009). Therefore, to study the development of succession it is necessary to analyze both abiotic and biotic aspects of the system over time.

In this context, species with high tolerance to environmental stress or with high competitive potential, such as invasive species (Vilà and Weiner 2004), may acquire a relevant role in the temporal configuration of ecosystems (Prach and Walker 2010). Invasive plant species with high tolerance to extreme abiotic factors and high dispersion ability can be primary colonizers driving the beginning of the succession (Mack and D'Antonio 1998). Other invasive plants with high competitive capacity can inhibit the development of succession (Luke-Flory and Clay 2010, Catford et al. 2012). Thus, plant community composition and the functional plant traits involved are crucial for the progress of ecological succession.

Once introduced, invasive species can hybridize with pre-existing native species giving rise to exotic hybrids and diluting the native genotype (Huxel 1999). Hybridization is a frequent process in plant communities with an important role in evolution (Arnold et al. 2012). There are many native hybrid plants that use to form hybrid zones close to the habitats of their parental species (Buggs 2007). These hybrid taxa may show transgressive traits related to 'hybrid vigor' or heterosis that confer them higher fitness than their parental

species (Fridman 2015). The invasiveness of exotic hybrids is frequently enhanced in relation to the development of heterosis that confer them greater stress tolerance and competitiveness than parental species (Ellstrand and Schierenbeck 2000, Ellstrand 2009). Nevertheless, little is known about the role of hybrids in the development of ecological succession and, to our knowledge, there are no long-term studies comparing the effects of native and exotic hybrids on ecological succession.

Coastal salt marshes are suitable ecosystems for the study of ecological succession, since successional development is relatively fast and it is related to the positive balance between inputs (accretion) and outputs (erosion) of organic and inorganic materials that promote marsh vertical growth over time (Allen and Pye 1992). In addition, the low number of plant species colonizing salt marshes due to high stress level related to tidal flooding (Silvestri et al. 2005) makes it easier to study the development of succession than in more complex and diverse ecosystems. Accretion in salt marshes depends on numerous factors such as variations in sea level, geomorphology and the development of vegetation (Stevenson et al. 1986, Nyman et al. 2006, Neubauer 2008, Fagherazzi et al. 2012). Hence, salt marshes are classified in succesional and non-succesional from the perspective of their geomorphology and physiography and their influence in the velocity of ecological succession development (Castellanos et al. 1998). On the other hand, changes in marsh elevation condition the sedimentary environment, influencing environmental factors such as redox potential, pH or salinity of interstitial pore water (Curado et al. 2014, Gallego-Tévar et al. 2018a). Sedimentary environment is a main factor in the establishment of plant species according to their abiotic tolerance (Ungar 1998). Once established, species can promote the colonization of other species by ameliorating environmental conditions leading to facilitation processes (Bruno et al. 2003). As the succession progresses, environmental conditions become milder and competition among species preponderate (Dormann et al. 2000).

In salt marshes of the Gulf of Cadiz (Southwest Iberian Peninsula), reciprocal sterile F1 hybrids between the native cordgrass *Spartina maritima* (Curtis) Fernald and the invasive South American neophyte *Spartina densiflora* Brongn. present certain transgressive traits that confer them hybrid vigor, playing a disturbing role of native plant zonation (Gallego-Tévar et al. 2018a). Both exotic hybrids show limited distribution due to their sterility and are distributed close to their maternal species, so that *S. maritima x densiflora* shares low marshes with the primary colonizer *S. maritima* and *S. densiflora x maritima* with *S. densiflora* at low-middle invaded marshes (Castillo et al. 2010a, Gallego-Tévar et al. 2018a). In these same salt marshes, native hybrids have been described between natives *Sarcocornia perennis* (Mill.) A. J. Scott and *Sarcocornia fruticosa* (L.) A. J. Scott. The distribution and presence of these native *Sarcocornia* hybrids is abundant, establishing a hybrid zone between the low marsh habitat of S. *perennis* and the high marsh habitat of *S. fruticosa* (Figueroa et al. 2003, Gallego-Tévar et al. 2018a). The colonization of the hybrid *S. perennis x fruticosa* (2n = 45) occurs through facilitation of the maternal species *S. perennis* that promotes accretion inducing favorable conditions (higher potential redox) for the establishment of the hybrid that later grows displacing its seed parental (Figueroa et al. 2003). Thus, the presence of native and exotic hybrids in the same ecological context of the salt marshes in the Gulf of Cadiz allowed us to study their roles in the development of ecological succession.

The main objective of this study was to analyze the role of native and invasive plant hybrids on the development of ecological succession. With this aim, we analyzed the development of succession through studying in a long-term work (ca. 12 years) main sedimentary variables (elevation, pH, redox potential and electrical conductivity) and vegetation structure (cover and degree of coincidence of the different taxa) in successional and non-successional coastal salt marshes colonized by native *Sarcocornia* hybrids and exotic *Spartina* hybrids. Our hypothesis was that the native hybrids establishing a

stabilized hybrid zone would promote the usual development of the ecological succession, while their high competitive potential would make the exotic hybrids to perform a disturbing role, inhibiting succession development and reducing biodiversity.

9.2 Material and Methods

Studied hybrid taxa

The native hybrids analyzed were the hybrids between the Chenopodiaceae species *Sarcocornia perennis* (Mill.) A. J. Scott (2n = 18) from low marshes and *Sarcocornia fruticosa* (L.) A. J. Scott (2n = 8x = 72) from high marshes (Castroviejo and Lago 1992, Figueroa et al. 2003). Different crossing between these two species gives rise to hybrids with different ploidy levels (2n = 45 and 54) that show an intermediate phenotype and occupy an intermediate habitat in relation to both parent species (Gallego-Tévar et al. 2018a). These hybrids have been described in different marshes of the Guadiana and the Tinto-Odiel Estuaries (Figueroa et al. 2003, Curado et al. 2014, Gallego-Tévar et al. 2018a).

The exotic hybrids were the reciprocal hybrids between the native cordgrass species *Spartina maritima* (Curtis) Fernald. (2n = 6x = 60) and its congener the invasive from South America *S. densiflora* Brongn. (2n = 7x = 70) (Castillo et al. 2010a). The hybrid *S. maritima x densiflora* (2n = 9.5x = 95) shares habitat with its seed parent *S. maritima* in low marshes, while *S. densiflora x maritima* (2n = 6.5x = 65) is found in middle marshes next to *S. densiflora* (Castillo et al. 2010a, Gallego-Tévar et al. 2018a). The phenotype of these *Spartina* hybrids is characterized by presenting intermediate characters, others similar to one or both parents and also transgressive traits that confer them ‘hybrid vigor’ (Castillo et al. 2010a, Gallego-Tévar et al. 2018a). The *Spartina* hybrids of the Gulf of Cadiz have been described in the Guadiana, Piedras and Tinto-Odiel Estuaries (see *Chapter III*).

Study sites

This study was carried out in Don Claudio Marsh in Tinto-Odiel Estuary (37º13'N, 6º57'W) and in San Bruno Marsh in Guadiana Estuary (37º11'N, 7º24'W) both on the Atlantic Coast of Southwest Iberian Peninsula (Gulf of Cadiz) (Appendix 9.A, see *Chapter 2*).

Don Claudio Marsh is a well-drained successional intertidal lagoon in which vegetation has established from tussocks of the primary colonizer *Spartina maritima.* This species has facilitated the establishment of *Sarcocornia perennis* by favoring the accretion in the center of its centrifugally expanding tussocks (Castellanos et al. 1994). Then, *S. perennis* expanded from the center to the periphery of the tussocks and gave rise to facilitation by hybridization with the species *Sarcocornia fruticosa*, located at peripheral high marsh zones. The resulting F1 hybrid *S. perennis x fruticosa* (2n = 45) is stablished at the beginning at the center of the tussocks. This *Sarcocornia* hybrid and also the species *Atriplex portulacoides* (L.) Allen begun to colonize this marsh area between 1990 and 1997 (Figueroa et al. 2003). Young clumps of *S. densiflora* have been recently observed in the lowest areas of bare sediment (personal observation), pointing to the beginning of the invasion in this marsh.

By contrast, San Bruno Marsh is a non-successional area located on the left bank of the main channel of Guadiana River in which clear bands of vegetation are established parallel to the tidal line. Thus, *S. maritima* and *S. perennis* predominate in the lower part of the marsh, the hybrids between *S. perennis* and *S. fruticosa* and *A. portulacoides* in the lower part of the middle marsh, the invasive *Spartina densiflora* and *S. fruticosa* are dominant in the subsequent zone of middle marshes, and *Arthrocnemum macrostachyum* (Moric.) C. Koch and *Suaeda vera* Forssk. ex J.F.Gmel. are the most abundant species in the highest area of the middle marsh (Gallego-Tévar et al. 2018a). The two reciprocal hybrids between *S. maritima* and *S. densiflora* are present in this salt marsh close to their seed parents at low and low-middle elevations, respectively (Castillo et al. 2010a, Gallego-Tévar et al. 2018a).

This system of successional and another non-successional marsh areas colonized by native and invasive hybrids allowed to analyze and to compare the role of these hybrids on the development of ecological succession.

Sedimentary environment

The sampling was adapted to the different physiography of the studied marsh areas (described above). In order to explore changes in the physiography of the marshes, elevation relative to Spanish Hydrographic Zero (SHZ) was measured at the center and the periphery of 16 permanent plots coinciding with initial vegetation tussocks in Don Claudio Marsh, where vegetation was structured in patches, in November 2007 and January 2018 and every 5 m in a representative transect perpendicular to the tidal line in San Bruno Marsh, where vegetation was organized in bands parallel to the tidal line, in April 2003 and January 2016. Accretion rate was calculated as the difference in elevation for a given location divided by the difference in years (cm yr^{-1}). Additionally, marsh elevation, redox potential at surface (0-5 cm deep) and depth (5-10 cm), pH and electrical conductivity of the interstitial water for the main halophytes were measured in Don Claudio (n = 5 – 20 plots per taxon) and in San Bruno (n = 3 – 20 plots per taxon). The analysis of the sedimentary variables was carried out grouping them in each marsh location by vegetation zones: (1) *Spartina maritima* and *Sarcocornia perennis* (*Sm-Sp*) zone; (2) *Sarcocornia hybrids* and *Atriplex portulacoides* (*HSar-Ap*) zone; and (3) *Spartina densiflora* (*Sd*) zone All the samplings took place at the low tide time of the cycle.

Elevation relative to SHZ and sediment redox potential were recorded *in situ* in the field. Measurements of elevation were obtained with a Leica NA 820 theodolite (Singapore) with a resolution of 2 cm and defining reference points with measurements of tidal extremes (Ranwell et al. 1964). Sediment Eh was calculated as the mean of three measures at the same location by using an electrode system (Crison pH/mV p-506). At the same time, sediment samples were collected in 250 ml sealed containers for the determination of pH and

conductivity in the laboratory. After adding distilled water to the sediment samples, pH (1:1, v/v) was determined using a pH/redox Crison with the electrode M-506 and conductivity (1:2, v/v) was obtained with a conductivimeter Crison-522 (Curado et al. 2014).

Vegetation structure

The presence of the different plant taxa were measured continuously along perpendicular transects to the tidal line at the same time than the sedimentary at both marsh locations. Transects covered 291 m long in Don Claudio Marsh (n = 16 tussocks each including between 14and 20 m long transects) and 274 m long in San Bruno Marsh (n = 3 marsh areas each including between 214 and 240 m long transects). Absolute cover (%) was calculated as the total length occupied by a given taxon with respect to the total length of transects. The percentage of coincidence was calculated as the length in which a taxon coincided spatially with another taxon with respect to the total length of occupation of the former.

Statistical analyses

All the statistical analyses were performed using Sigma-Plot for Windows (version 12.0, Systat Software Inc., IL, US), using a significance level (α) of 0.05. Before the application of parametric analyses, the data sets were tested for normality with the Shapiro-Wilk's test and for homoscedasticity with Levene's test. In the case of one of the two assumptions of parametric analyses were not met, data transformations (inverse, square root or logarithm) were conducted and, when they were insufficient, non-parametric tests were carried out. The variation in elevation between the two sampling years were compared using the Mann-Whitney U-test for each marsh location. Elevation, sediment redox potential, pH and conductivity were compared with two-way ANOVA, using year and vegetation zone as grouping factors and Bonferroni t-test as post-hoc

analysis. Since vegetation samplings were made at the same points in both years for the two marsh locations, comparisons of absolute vegetation cover and percentage of coincidence were performed with a two-way repeated measures ANOVA using year and vegetation zones as grouping factors and Holm-Sidak test as post-hoc analysis.

9.3 Results

Sedimentary environment

Sedimentation led to general increases in elevation of both studied marshes (Mann Whitney U-tests, Don Claudio Intertidal Lagoon: U = 296, $P < 0.01$; San Bruno Channel Bank: U = 681, $P < 0.001$). Sedimentation was higher in Don Claudio Intertidal Lagoon (1.9 ± 0.3 cm yr^{-1}) than in San Bruno Channel Bank (1.0 ± 0.1 cm yr^{-1}). In Don Claudio, accretion was lower at the center of the tussocks (0.8 ± 0.3 cm $year^{-1}$) than at their periphery (2.8 ± 0.5 cm $year^{-1}$) (Mann-Whitney U Statistic= 27, P < 0.001). Two new drain tidal channels were formed from 2003 to 2016 in San Bruno (Fig. 1). Elevation was revealed as an indicator variable of change in the sedimentary environment, since the rest of the abiotic variables correlated with it. This correlation was positive except for the pH in Don Claudio and the conductivity in San Bruno (Table 1). On the other hand, redox potential at surface and depth were positively correlated, while conductivity was negatively correlated with pH and Eh at depth in both marshes. The relationship between pH and Eh was positive in San Bruno and negative in Don Claudio (Table 1).

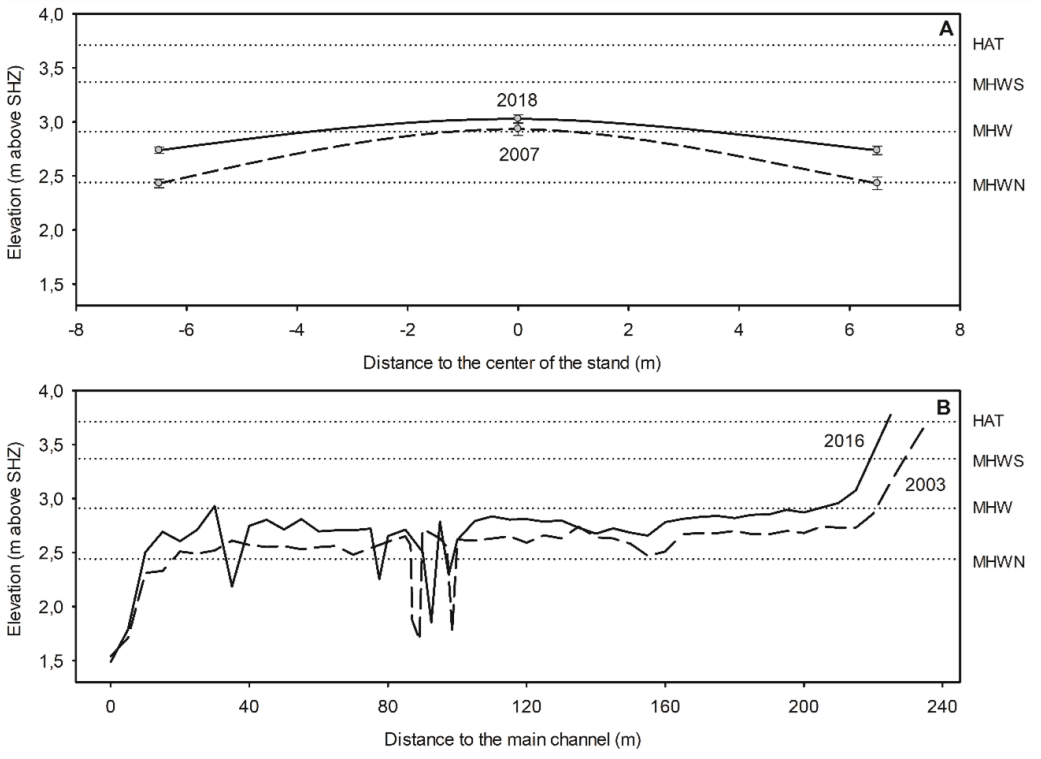

Fig. 9.1. Marsh elevation in (A) Don Claudio Intertidal Lagoon, and (B) in San Bruno Channel Bank over time along perpendicular transects to the tidal line. Tidal levels: MHWN: Mean High Water Neaps, MHW: Mean High Water, MHWS: Mean High Water Spring, HAT: High Astronomical Tides.

Sediment redox potentials did not change over time in the *Sm-Sp* zones at surface and depth and in *Sd* zones at depth of both marsh areas (Two-Way ANOVA, $P > 0.05$). However, redox potential at surface increased over time from -34 to +62 mV in the *Sd* zone in Don Claudio (Two-Way ANOVA, $F_{2,54} = 5.901$, $P < 0.01$) and it decreased from +246 to +156 mV in the *Sd* zone, and from +206 to +155 mV and +217 to +121 mV at surface and depth, respectively, in the *HSar-Ap* zone in San Bruno (Two-Way ANOVA, Eh surface: $F_{1,92} = 6.238$; Eh depth: $F_{1,92} = 4.071$, $P < 0.05$). Regarding vegetation zones, sediment Eh was significantly lower in *Sd* zone than in the rest of the vegetation zones at surface (Two-Way ANOVA, $F_{2,54} = 33.288$, $P < 0.001$) and than the *HSar-Ap* zone at depth in Don Claudio (Two-Way ANOVA, $F_{2,54} = 5.462$, $P < 0.01$). In San Bruno, *Sm-Sp* showed lower sediment Eh than the other vegetation areas, both at surface and depth (Two-Way ANOVA, Eh surface: $F_{2,92} = 13,205$; Eh

depth: $F_{2,92} = 7.962$, $P < 0.001$) (Table 9.1).

Table 9.1. Marsh elevation (m above Spanish Hydrographic Zero (SHZ)), redox potential (mV) at two sediment depths, electrical conductivity and pH of sediment interstitial pore water over time in Don Claudio Intertidal Lagoon (Odiel Estuary) and in San Bruno Channel Bank (Guadiana Estuary). Significant differences between years are marked in bold.

Don Claudio Marsh

	Sm-Sp		*HSar-Ap*		*Sd*		Two-way ANOVA, *zone x year*
	2007	*2018*	*2007*	*2018*	*2007*	*2018*	
Elevation (m above SHZ)	2.80 ± 0.02	2.82 ± 0.02	2.91 ± 0.03	2.93 ± 0.02	2.41 ± 0.03	2.46 ± 0.01	$F_{2,178} = 0.268$, $P = 0.765$
Redox potential (mV) 0-10 cm	+192 ± 13	+147 ± 23	+197 ± 25	+164 ± 8	**-34 ± 23**	**+62 ± 26**	**$F_{2,54} = 5.901$, $P < 0.01$**
Redox potential (mV) 10-20 cm	+171 ± 30	+101 ± 49	+198 ± 29	+171 ± 8	+14 ± 43	+88 ± 32	$F_{2,54} = 1.574$, $P = 0.217$
Conductivity (mS cm^{-1})	**14.6 ± 0.4**	**22.7 ± 1.6**	**14.0 ± 0.4**	**17.4 ± 0.7**	**17.5 ± 0.4**	**20.2 ± 0.7**	**$F_{2,54} = 219.838$, $P < 0.001$**
pH	**7.0 ± 0.0**	**6.8 ± 0.0**	**7.1 ± 0.1**	**6.9 ± 0.1**	**7.1 ± 0.1**	**6.7 ± 0.1**	**$F_{2,54} = 549.443$, $P < 0.001$**

San Bruno Marsh

	Sm-Sp		*HSar-Ap*		*Sd*		Two-way ANOVA, *zone x year* / t-test
	2003	*2016*	*2003*	*2016*	*2003*	*2016*	
Elevation (m above SHZ)	**2.29 ± 0.16**	**2.59 ± 0.06**	2.69 ± 0.01	2.66 ± 0.02	2.76 ± 0.01	2.72 ± 0.03	**$F_{2,90} = 6.156$, $P < 0.01$**
Redox potential (mV) 0-10 cm	+79 ± 42	+82 ± 37	**+206 ± 18**	**+155 ± 15**	**+246 ± 4**	**+156 ± 11**	**$F_{1,92} = 6.238$, $P < 0.05$**
Redox potential (mV) 10-20 cm	+40 ± 61	+74 ± 31	**+217 ± 18**	**+121 ± 16**	+185 ± 24	+107 ± 21	**$F_{1,92} = 4.071$, $P < 0.05$**
Conductivity (mS cm^{-1})	-	12.3 ± 1.1	-	12.5 ± 0.6	**12.2 ± 0.5**	**16.3 ± 0.7**	**$t_{45} = -5.021$, $P < 0.001$**
pH	-	7.1 ± 0.1	-	6.9 ± 0.1	**6.5 ± 0.1**	**6.9 ± 0.1**	**$t_{45} = -5.311$, $P < 0.001$**

Sedimentary pH in Don Claudio was close to neutrality, decreasing slightly over time in all vegetation zones (-0.2 for *Sm-Sp* and *HSar-Ap* zones and -0.4 for *Sd* zone) (Two-Way ANOVA, $F_{2,54} = 549.443$ $P < 0.001$). In San Bruno, pH increased from 6.5 to 6.9 in the *Sd* zone (t-Student test, $t_{45} = -5{,}311$ $P < 0{,}001$). Among vegetation zones, pH was more acidic for the *Sd* zone compared to the rest of vegetation zones in Don Claudio in 2018 (Table 9.1).

Electrical conductivity of sediment interstitial water increased over time in all vegetation zones. The increases in conductivity were 55%, 25% and 15% in the *Sm-Sp*, *HSar-Ap* and *Sd* zones in Don Claudio, respectively (Two-Way ANOVA, $F_{2,54} = 219.838$, $P < 0.001$), and 35% in the *Sd* zone in San Bruno (t-Student test, $t_{45} = -5{,}021$ $P < 0{,}001$). In Don Claudio, conductivity was greater in the *Sd* zone than in the rest of the zones in 2007, while it was higher in *Sm-Sp* zone than *Sd* and this higher than *HSar-Ap* in 2018. Higher conductivity was registered in the *Sd* zone than in the rest of the zones in San Bruno in 2016 (One-Way ANOVA, $F_{6,62} = 8.638$, $P < 0.001$) (Table 9.1).

Vegetation structure

Mean absolute vegetation cover in Don Claudio increased from 21% in 2007 to 28% in 2018 (Two-Way RM ANOVA, $F_{1,60} = 46.540$, $P < 0.001$), and it remained constant ca. 13% in San Bruno from 2003 to 2016 (Two-Way RM ANOVA, $F_{1,16} = 0.114$, $P > 0.05$). In these periods, the absolute covers of the *Sarcocornia* hybrids and *A. portulacoides* increased (from 61% to 71% and from 18 to 59%, respectively) and that of *S. perennis* decreased (from 22% to 4%) in Don Claudio (Two-Way RM ANOVA, $F_{4,60} = 23.081$, $P < 0.001$). In San Bruno, the absolute cover also increased for the *Sarcocornia* hybrids (from 17% to 29%) and decreased for *S. densiflora* (from 37% to 29%) (Two-Way RM ANOVA, $F_{8,16} = 2.914$, $P < 0.05$). In Don Claudio Marsh, *Spartina maritima* and *Suaeda maritima* (L.) Dumort exhibited lower absolute covers than *S. perennis*, the *Sarcocornia* hybrids and *A. portulacoides* in 2007, but *Spartina*

maritima, *Suaeda maritima* and *S. perennis* showed the same cover among them and lower than *Sarcocornia* hybrids and *A. portulacoides* in 2018. In San Bruno Marsh, *Spartina maritima*, the *Spartina* hybrids, *S. fruticosa*, *A. macrostachyum* and *S. vera* showed the lowest absolute covers in 2003, followed by *S. perennis* (which was not different from *S. maritima* and *S. fruticosa*), the *Sarcocornia* hybrids (which was not different from *S. fruticosa*), and *A. portulacoides* and *S. densiflora* that showed the highest covers. In 2016, *S. maritima*, *S. perennis*, the *Spartina* hybrids, *S. fruticosa*, *A. macrostachyum* and *S. vera* presented similar absolute covers and lower than those of *S. densiflora*, the *Sarcocornia* hybrids and *A. portulacoides* (Fig. 9.2).

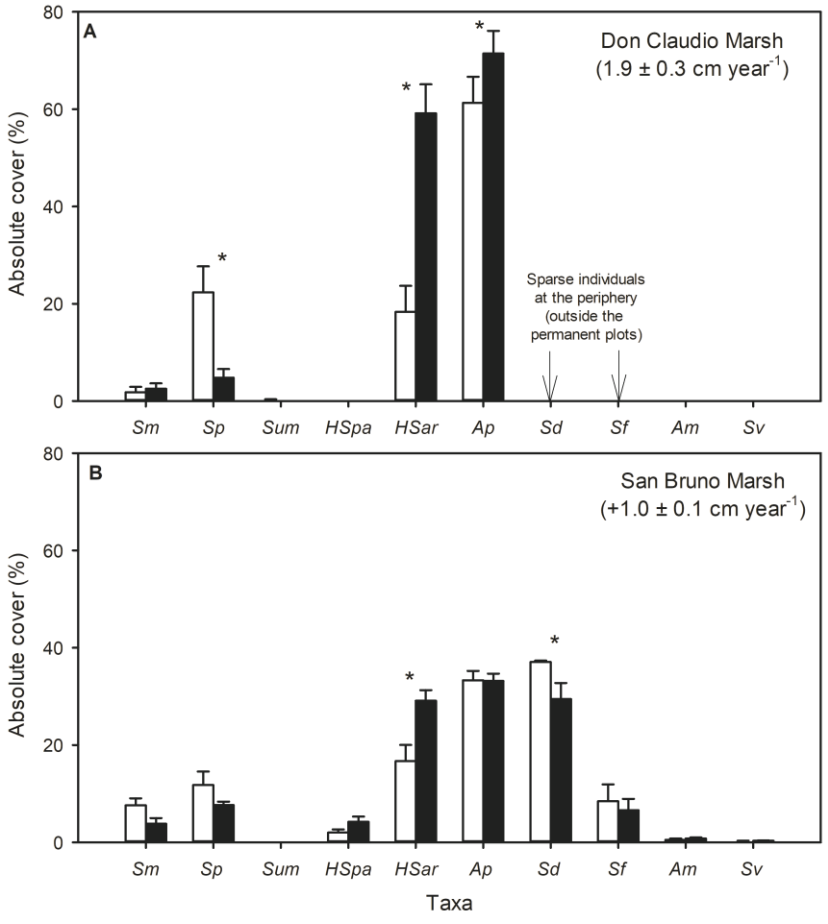

Fig. 9.2. Mean absolute cover of plant taxa colonizing (**A)** Don Claudio Intertidal Lagoon (n = 16) in 2007 (white bars) and 2018 (black bars), and (**B)** San Bruno Channel Bank (n = 3) in 2003 (white bars) and 2016 (black bars). Taxa from lower to higher elevations along the intertidal frame: *Sm*, *Spartina maritima*; *Sp*, *Sarcocornia perennis*;

Sum, *Suaeda maritima*; H*Spa*, *Spartina* hybrids; H*Sar*, *Sarcocornia* hybrids; *Ap*, *Atriplex portulacoides*; *Sd*, *Spartina densiflora*; *Sf*, *Sarcocornia fruticosa*; *Am*, *Arthrocnemum macrostachyum*, *Sv: Suaeda vera.* Asterisks mark significant differences between years (ANOVA for Taxon x Year interaction, $P < 0.05$). Accretion rates are in brackets.

Mean percentage of coincidence of different taxa increased over time in Don Claudio from 53% to 78% (Two-Way RM ANOVA, $F_{1,45} = 62.414$, $P < 0.001$) and it did not change in San Bruno (Two-Way RM ANOVA, $F_{1,12} = 0.116$, $P > 0.050$), where it remained ca. 50 %. The increase in the percentage of coincidence in Don Claudio was related to an increase in the overlap of *S. perennis* (from 41% to 76%), the *Sarcocornia* hybrids (from 46% to 74%) and *A. portulacoides* (from 25% to 62%) with other taxa. *S. maritima* showed 100% of co-occurence with other taxa in both years. Thus, the coincidence of *S. maritima* with other taxa in Don Claudio was always greater than that of the other taxa. Also, the *Sarcocornia* hybrids percentage of coincidence (45%) was higher than that of *A. portulacoides* (25%) in 2007 (Two-Way RM ANOVA, $F_{3,45} = 4.348$, $P < 0.001$). By contrast, in San Bruno, the coincidence of *S. maritima* increased from 9% to 43% while the *Spartina* hybrids decreased drastically their co-occurrence with other taxa from 62% to 6% from 2003 to 2016. In 2003, all the taxa showed a coincidence without significant differences, except the higher percentage of *S. fruticosa* (99%) than *S. maritima* (9%), *S. perennis* (30%) and *A. portulacoides* (40%), and the higher percentage of the *Sarcocornia* and *Spartina* hybrids (67% and 62%, respectively) than *S. maritima*. In 2016, the species in San Bruno also showed a similar percentage of coincidence except the lower value of the *Spartina* hybrids (6%) than *S. fruticosa* (83%), the *Sarcocornia* hybrids (74%) and *A. portulacoides* (61%), and the higher value of *S. fruticosa* than *S. perennis* (29%) (Two-Way RM ANOVA, $F_{3,12} = 5.732$, $P < 0.001$) (Fig. 9.3).

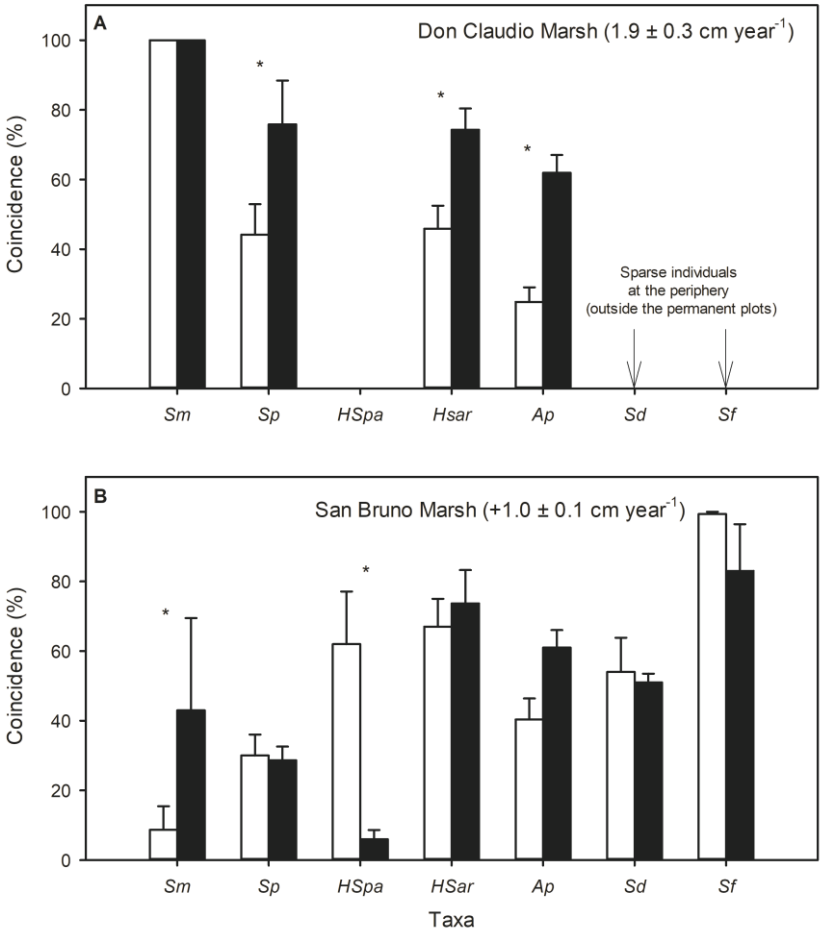

Fig. 9.3. Percentage of coincidence of halophytes in (**A)** Don Claudio Intertidal Lagoon (n = 16) in 2007 (white bars) and 2018 (black bars), and (**B)** in San Bruno Channel Bank (n = 3) in 2003 (white bars) and 2016 (black bars). Taxa from lower to higher elevations along the intertidal frame: *Sm: Spartina maritima, Sp: Sarcocornia perennis, Sum: Suaeda maritima,* HSpa*: Spartina* hybrids, HSar: *Sarcocornia* hybrids, *Ap: Atriplex portulacoides, Sd: Spartina densiflora, Sf: Sarcocornia fruticosa, Am: Arthrocnemum macrostachyum, Sv: Suaeda vera.* Asterisks mark significant differences between years (ANOVA for Taxon x Year interaction, $P < 0.05$). Accretion rates are in brackets.

9.4 Discussion

Our study focused on successional changes over time in biotic and abiotic characteristics of two different salt marshes with native and exotic hybrids as

center taxa. Containing both marshes the native hybrid between *Sarcocornia perennis* and *S. fruticosa*, the presence of the exotic hybrids between native *Spartina maritima* and invasive *S. densiflora* in one of them gave rise to a different successional development, also marked by differences in the sedimentary environment.

Both studied marsh zones with different marsh physiography presented different sedimentary dynamics revealed by contrasted accretion rates, being almost double (1.9 ± 0.3 cm yr^{-1}) in Don Claudio Lagoon than in San Bruno Marsh (1.0 ± 0.1 cm yr^{-1}). This difference seemed to be mainly related to the different physiography of the studied marshes, since Don Claudio is a successional intertidal lagoon (Castellanos et al. 1994) and San Bruno an intertidal channel bank and geomorphology a conditioning factor of different sedimentary dynamics (Fagherazzi et al. 2012). The recorded difference in accretion rate explain the successional nature of Don Claudio Marsh, where drastic vegetation changes have occurred during the last decades (Castellanos et al. 1994, Figueroa et al. 2003) in contrast to the non-successional dynamics of San Bruno Marshes with slower vegetation development, as revealed by the analysis of aerial photographs (see Appendix 9.B). On the other hand, the increase in elevation over time was greater in the lower parts of both marshes (peripheries of the vegetation tussocks in Don Claudio and *Sm-Sp* area in San Bruno) than at higher elevations. Higher sedimentation rates at low marshes have been generally related to greater accumulation of inorganic matter, while at high marshes, where tidal flow is shorter, sedimentary accumulation is lower and mainly organic (Bricker-Urso et al. 1989, Neubauer 2008).

Although changes in elevation in the different vegetation zones were only significant for the *Sm-Sp* zone in San Bruno, they exhibited variations in Eh, pH and conductivity. Thus, sediment redox potential decreased in vegetation zones at the higher elevations of San Bruno (*Ap-HSar* and *Sd* zones), where the accumulation and decomposition of organic matter at the surface is usually higher (Neubauer 2008) consuming oxygen from the sediment atmosphere. On

the contrary, sediment Eh increased in *Sd* zone at the lowest elevations in Don Claudio, which may be related to the oxygenation of sediments through the aerenchyma of *S. densiflora* roots and rhizomes (Castillo et al. 2000) as reported previously for *Typha domingensis* Pers. (Aldridge and Ganf 2003).

The above-mentioned reduction of sediment Eh in San Bruno coincided with an increase in pH *Sd* zone, which may be explained by the consumption of protons occurring in many reduction reactions (Patra and Mohanty 1994, Kashem and Singh 2001). However, pH decreased slightly in all the vegetation's zones in Don Claudio, which was probably due to the organic accumulation and matter decomposition that liberates protons acidifying the sediments (Curtis 1987). On the other hand, the generalized increase of salinity (recorded as electrical conductivity) in all vegetation zones of both locations could have been related to the increase of mean sea level between years since even slight interannual rise of mean sea level may affect factors that control productivity in coastal marshes, such as salinity (Morris et al. 2002). The calculation of the sea level rise rate with historical data between 1961 and 2010 is 4.02 mm yr^{-1} in the Gulf of Cadiz (NOAA 2018).

The reported changes in the sedimentary environment were concomitant with changes in the halophytic vegetation. The native *Sarcocornia* hybrids significantly increased their absolute cover over time in both locations. This increase in Don Claudio (+40%) coincided with a decrease in the cover of the parental species *S. perennis* (-18%), which is in agreement with the facilitation by hybridization process and further spread of *Sarcocornia perennis x fruticosa* described by Figueroa et al. (2003). According to this process, *S. perennis x fruticosa* occupied the center of *S. perennis* tussocks facilitated by the accretion at the center of the tussocks and it expanded centrifugally from the center to the periphery growing over *S. perennis*. Furthermore, this increase of the *Sarcocornia* hybrids in Don Claudio did not prevent the spread of *A. portulacoides* (+10%), another representative native species of the marshes of the Gulf of Cadiz that gets its maximum biomass and cover in middle marshes (Álvarez et al. 2000). In San Bruno, the increase in absolute cover of the

Sarcocornia hybrids (+12%) coincided with a decrease of the invasive *S. densiflora* (-8%) and a high spatial coincidence between them. Thus, the development of this native hybrids seemed to control the invasion of *S. densiflora*, having a positive effect on the conservation of native vegetation communities. *Spartina densiflora* is a highly invasive species in salt marshes of the Southwest Iberian Peninsula and the Pacific Coast of North America (Strong and Ayres 2013), where it reduces diversity of the invaded ecosystems forming mono-specific prairies (Nieva et al. 2001). Similar to our results, an effect of control of the *S. densiflora* invasion by a well-established population of native chenopods was recorded by Curado et al. (2018). By contrast, Abbas et al. (2014) carried out a greenhouse experiment of competition between the abundant chenopod species at middle marshes *A. portulacoides* and *S. densiflora* and concluded that the presence of the native species did not prevent the establishment of the invasive cordgrass.

In relation to the exotic hybrids, the increment of absolute cover of the *Spartina* hybrids in San Bruno (+2%) was not statistically significant, as expected given their current infertility limiting their spread ability (Castillo et al. 2010a, Gallego-Tévar et al. 2018a). However, an important drop of the percentage of coincidence with other species was recorded for these exotic hybrids indicating that their competitive capacity is developed over time, being able to displace neighboring halophytes (Gallego-Tévar et al. 2018a). Numerous invasive species can eliminate native species with which they coexist due to competitive exclusion (Vilà and Weiner 2004), altering the evolutionary development of the native ecosystem (Mooney and Cleland 2001). In this case, the tendency to spread asexually by rhizomes of the exotic hybrids and their new establishment from independent hybridization events together with the decrease of the species with which they coexisted (*S. maritima* at low marshes and *S. densiflora* at low-middle marshes) would also support their high capacity for competitive exclusion. In contrast, in Don Claudio Intertidal Lagoon where the ecological succession is not altered by any invasive taxon, since *S. densiflora* is still invading depressed unvegetated mudflats, the percentage of coincidence

increased for all taxa, except for the primary colonizer *Spartina maritima* that was 100% both years since its presence was residual. Throughout succession, plant species tend to reduce their degree of aggregation and to coalesce, increasing their coincidence in space (Brereton 1971). Russell et al. (1985) observed that the overlap among species was related to elevation in a study of two salt marshes in England, being higher at a higher and more mature marsh than at a young marsh. In addition, we found an overall increase of the absolute cover of all taxa in Don Claudio, also revealing the progress of succession. The ecological theory says that the early stages of the succession are conditioned mainly by species tolerance to environmental factors and by facilitation processes (Bruno et al. 2003). The maintenance of the vegetation cover in San Bruno would be related with other biotic relationships such as competition that prevail in more mature stages when environmental conditions become milder (Maestre et al. 2009) and the system tend to stability (Connell and Slatyer 1977).

9.5 Conclusions

Our study in a successional salt marsh location with native hybrids and in a non-successional marsh location colonized by native and exotic hybrids showed that both types of hybrids played different roles in ecological succession. Despite the different sedimentary dynamics observed between both marsh types, the native *Sarcocornia* hybrids increased their abundance in both locations over time without inhibiting the development of succession since they did not prevent the colonization by other native species such as *A. portulacoides* at similar elevations, *S. perennis* at lower elevations and *Suaeda maritima* at disturbed patches without perennials cover. Moreover, the spread of the native hybrids coincided with the decrease in the distribution of invasive *S. densiflora*. Therefore, in addition to their structuring role of the local zonation pattern (Gallego-Tévar et al. 2018a), these native hybrids tolerated the presence of other taxa in the development of succession. In contrast, exotic and invading Spartina hybrids inhibited the development of succession by outcompeting other halophytes decreasing their percentage of coincidence with other species over time.

CHAPTER X

Maternal-switching with climate change modify formation of invasive *Spartina* hybrids

CAPÍTULO 10: CAMBIO MATERNO CON CAMBIO CLIMÁTICO MODIFICA LA FORMACIÓN DE HÍBRIDOS INVASORES DE SPARTINA

Resumen

El cambio climático puede inducir cambios temporales, espaciales o de comportamiento en las especies, de manera que solo algunas especies pueden adaptarse a las nuevas condiciones climáticas. En el caso concreto de las especies invasoras, se espera que serán promovidas en un contexto de cambio global, dada su alta tolerancia a factores ambientales y plasticidad fenotípica. Una vez en el rango invadido, estas especies pueden hibridar con las especies nativas introduciendo así su genotipo en la biota nativa. Sin embargo, no hay mucha información sobre los efectos que el cambio climático tendrá en este proceso de invasión por hibridación. Nosotros evaluamos el establecimiento histórico de los híbridos recíprocos entre la nativa *Spartina maritima* y la invasora *S. densiflora* en el Golfo de Cádiz (Suroeste de la Península Ibérica) y lo relacionamos con los cambios climáticos durante el periodo 1955-2017. Obtuvimos que, según su datación basada en su tasa de expansión lateral, el establecimiento de *S. maritima x densiflora* y *S. densiflora x maritima* en el Golfo de Cádiz ha ocurrido en los últimos dos siglos y ha estado relacionado con cambios en la temperatura y la precipitación durante la floración de las especies parentales, con un efecto antagónico en ambos híbridos. Así, *S. densiflora x maritima* se ha establecido en años con primaveras-veranos suaves cuando la floración de *S. maritima* se alargó y su producción de polen fue mayor coincidiendo con el principio de la floración de *S. densiflora.* Además, el establecimiento de este híbrido se relacionó con mayores precipitaciones en primavera-verano, probablemente debido a la reducción de salinidad en marismas medias. Sin embargo, *S. maritima x densiflora* se estableció principalmente en primaveras-veranos cálidos en los que la proporción polen:óvulo de *S. maritima* fue menor favoreciendo la polinización por *S. densiflora*. Como consecuencia de la promoción de *S. maritima x densiflora* con el cambio climático, la especie nativa y en peligro de extinción *S. maritima* se vería amenazada, ya que ambos taxones comparten el mismo hábitat y el híbrido muestra un potencial competitivo notablemente más alto.

CHAPTER 10: MATERNAL-SWITCHING WITH CLIMATE CHANGE MODIFY FORMATION OF INVASIVE SPARTINA HYBRIDS

Abstract

Climate change can induce temporary, spatial or behavioral changes in species, so that only some species can adapt to the new climatic conditions. In the case of invasive species, it is expected that they will be promoted in a context of global change, given their high tolerance to environmental factors and phenotypic plasticity. Once in the invaded range, these species can hybridize with native species thus introducing their genotype in the native biota. However, the effects that climate change will have on this process of invasion by hybridization remain unclear. We evaluated the historical establishment of the reciprocal hybrids between the native *Spartina maritma* and the invasive *S. densiflora* in the Gulf of Cadiz (SW Iberian Peninsula) and we related it to climatic changes during the period 1955-2017. Our results showed that, according to their dating based on their rate of lateral expansion rates, the establishment of *S. maritima x densiflora* and *S. densiflora x maritima* in the Gulf of Cadiz has occurred in the last two centuries and has been related to changes in air temperature and rainfall during the flowering periods of their parental species, with antagonist impacts on both hybrids. Thus, the hybrid *S. densiflora x maritima* has been established in years with mild ends of spring and beginning of summer when the flowering of *S. maritima* lengthened and its pollen production was higher, and it coincided with the beginning of the flowering period of *S. densiflora*. Moreover, the establishment of this hybrid was related to higher spring/summer rainfalls, probably due to the reduction in salinity in middle marshes. However, the hybrid *S. maritima x densiflora*, was established mainly in warmer spring/summers in which the proportion of pollen:ovule of *S. maritima* was reduced favoring its pollination by *S. densiflora*. As a consequence of the promotion of *S. maritima x densiflora* with climate change, the native and endangered species *S. maritima* would be threatened, as both taxa share the same habitat and the hybrid shows a remarkably higher competitive potential.

10.1 Introduction

New climatic conditions induced by global warming can lead to alterations of different biological processes in both plant and animal species (Parmesan 2006). These changes include temporal, spatial or behavioral adjustments of species, such as changes in distribution range, phenological or physiological characteristics (Bellard et al. 2012). In this context, it is expected that many species will not be able to adapt to the new conditions, leading to a generalized increase of the extinction rate (Urban 2015). In contrast, species with specific life-history traits that confer them high capacity to adapt to environmental changes, such as invasive species, are likely to be promoted in the new climatic scenario (Dukes and Mooney 1999, Vilà et al. 2007).

Biological invasions and their drastic alteration of natural ecosystems are widespread and have long been recognized as a significant component of global environmental change (Vitousek et al. 1997, 2017). Understanding how environmental change factors are influencing fundamental biological processes is imperative for conservation and management of natural ecosystems. The continued spread of non-native plant species, synergistic with accelerating global environmental changes, poses significant challenges for understanding how natural ecosystems and the native plant communities will be altered (Drenovsky et al. 2012). Once introduced, one way by which invasive species displace or exclude native species is through hybridization and introgression (Rhymer and Simberloff 1996), diluting or assimilating the native genotype (Huxel 1999). Global change is expected to increase the likelihood of hybridization between native and invasive species (Muhlfeld et al. 2014), both by the increment in the introduction of species, and the changes in their ranges so that sympatry between divergent species may increase (Garroway et al. 2010, Hoffmann and Sgrò 2011). However, studies on the biological processes underlying the increase in interspecific hybridization related to climate change are limited (Becker et al. 2013, Muhlfeld et al. 2014).

In this context of global environmental changes, many invasive plants are clonal organisms (Liu et al. 2006, Keser et al. 2014), and their longevity is an important demographic trait for understanding the life history, population dynamics, ecology and evolutionary fitness of plant species, (Harper 1977, Schmid 1990, Silvertown 1991). Long-lived plant clones have been documented in various aquatic and wetland ecosystems (Santamaría 2002). The asexual reproduction of clonal growth results in size expansion of genets and increased fitness as greater floral production in larger clones increases the potential for outcrossing and sexual reproduction (Barrett 2015). Extreme longevity of clonal species allows genets to persist through periods with environmental conditions when sexual reproduction is rare or precluded. Clonality is a survival strategy of plant species that may support longevity at millennial timescales and through rapid global environmental changes (Bricker et al. 2018).

Cordgrasses (genus *Spartina*) are an adequate model for the study of hybridization between native and invasive species since there are different documented examples of interspecific hybridization after the introduction of one species in the native range of another (Strong and Ayres 2013). These halophytic grasses, typical of tidal marshes, exhibit both sexual and clonal reproduction by rhizomes (Bortolus 2006, Castillo and Figueroa 2009), forming clones that can remain for long periods of time (Castellanos et al. 1998, Travis and Hester 2005). *Spartina* are protogynous species (Davis et al. 2004), whose flowering period occurs in the warm season (late spring-summer) and is controlled by environmental factors such as temperature or photoperiod (Ranwell 1967, Seneca and Blum 1984, Gray et al. 1991, Thompson 1991). In the Gulf of Cadiz (Southwest Iberian Peninsula), native *Spartina maritima* (Curtis) Fernald and invasive *Spartina densiflora* Brongn. from the East Coast of South America have hybridized giving rise to two reciprocal hybrids (Castillo et al. 2010a). According to the predictions of climate change models, these cordgrass populations will be subjected to increases in temperature and decreases in precipitation that will be more accentuated during the summer season (Anaya-Romero et al. 2015, IPCC 2015). In order to know how these climatic changes

are affecting the process of hybridization between native and invasive species, we carried out a study in which we dated different clones of *S. maritima x densiflora* and *S. densiflora x maritima* based on their size and lateral expansion rates in three estuaries of the Gulf of Cádiz. Then, their establishment dates were related to the historical data of rainfall and temperature. Our hypothesis was that the increase in temperature and decrease in rainfall associated with climate change would induce changes in the reproductive traits of *S. maritima* and *S. densiflora*, altering the process of hybridization between both species, since both parental species show high levels of phenotypic plasticity in response to changing environmental conditions (Castillo et al. 2005b, 2014, 2018, Grewell et al. 2016)

10.2 Material and Methods

Study area and taxa

This work was conducted in the estuaries of the Rivers Tinto-Odiel, Piedras and Guadiana, along the Atlantic Coast of Southwest Iberian Peninsula (Gulf of Cadiz). Location, climate, physical environment and marsh vegetation of these estuaries of the Gulf of Cadiz are described in *Chapter 2*. Some predictions of climate change in this area indicate temperature increases of 2.8-6.1 ºC for minimum temperature and 3.3-7.2 ºC for maximum temperature, as well as a decrease of 12-32% rainfall by 2100, in winter-summer respectively (Anaya-Romero et al. 2015).

Tussocks of the exotic hybrids between the native European Cordgrass *S. maritima* (2n = 6x = 60) and the invasive *S. densiflora* (2n = 7x = 70) have been observed in these three estuaries. Two different reciprocal hybrids have been described, one whose seed parent is *S. densiflora* (*S. densiflora x maritima*, 2n = 6.5x = 65) that colonizes middle marshes where its maternal species is more frequent, and the other is *S. maritima x densiflora* (2n = 9.5x = 95, by unreduced gamete of *S. maritima*) whose habitat is low marshes as well as its seed parent *S. maritima*. These *Spartina* hybrids are particularly abundant in the

Guadiana Estuary (+100) while they are very rare (< 10 tussocks) in the Tinto-Odiel and Piedras Estuaries (Castillo et al. 2010a). Both *Spartina* hybrids exhibit certain transgressive traits that confer them hybrid vigor (Gallego-Tévar et al. 2018b), playing an altering role on the typical zonation pattern of these salt marshes (Gallego-Tévar et al. 2018a). Nowadays, the current sterility of these exotic hybrids limits their expansion but fertility acquisition by allopolyploidization is a frequent process in *Spartina* hybrids (Strong and Ayres 2013). The flowering period for *S. maritima* has been described between May and July and for *S. densiflora* between June and December (Valdes et al. 1987). The tussocks of these hybrid modular plants were considered genets (clones) that were composed by units of rhizome and aerial shoots or tillers (ramets).

Lateral expansion rate of Spartina *tussocks*

Lateral expansion rate (cm yr^{-1}) by rhizomes of the tussocks of both *Spartina* hybrids was calculated as the diameter increment of individual tussocks in a given period; recording their diameter twice in different dates. For this purpose, the diameters of 5 tussocks of *S. densiflora x maritima* and 24 tussocks of *S. maritima x densiflora* were measured in Guadiana Estuary (37º10'–37º16'N, 7º16'–7º28'W) in February 2005. Also, the diameters of one tussock of *S. densiflora x maritima* and 2 tussocks of *S. maritima x densiflora* in Piedras Estuary (37º12'–37º18'N, 7º06'–7º12'W) in March 2005 (Appendices 10.A, 10.B). In order to compare the lateral expansion rates of both hybrids with their parental species, we also recorded the diameter of 13 tussocks of *S. maritima* in January and September 2000 and of 7 tussocks of *S. densiflora* in February 1997 and June 1999 at the East of Bacuta Island in Odiel Marshes (37º13'41''N, 6º57'41''W), and of other 10 tussocks of *S. densiflora* in October 1996 and September 2000 at the left bank of the main channel of the Tinto-Odiel Estuary (37º13'32''N, 6º57'06''W) (Appendix 10.B). All the diameters of the individuals of both hybrids and both parental species were remeasured in May 2018.

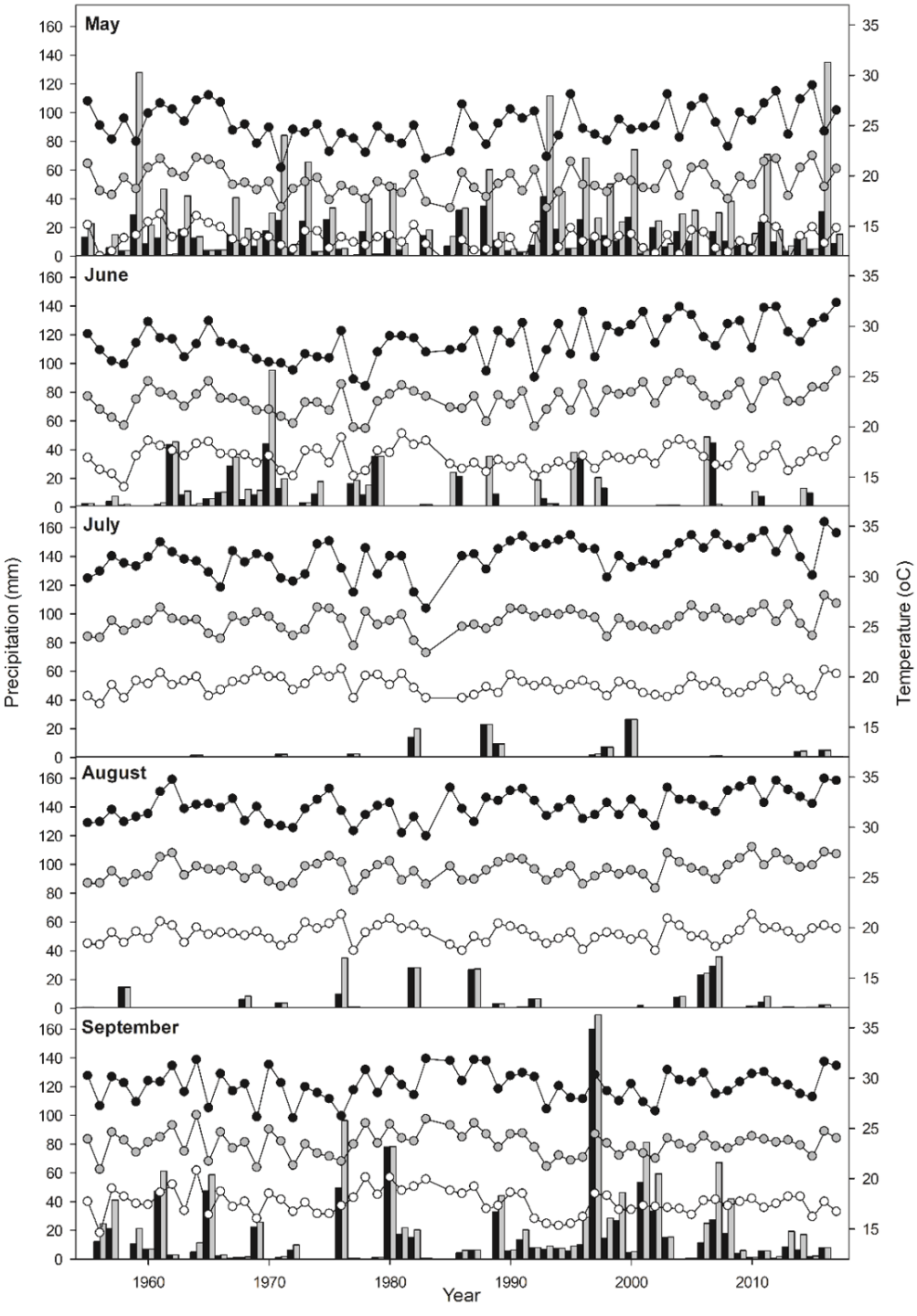

Fig. 10.1. Mean monthly precipitation (black bars), maximum daily precipitation (grey bars), mean maximum temperatures (black circles), mean average temperatures (grey circles) and mean minimum temperatures (white circles) in May, June, July, August and September from 1955 to 2017 in Southwest Iberian Peninsula.

Estimation of Spartina *hybrids age*

The major diameter of tussocks of *S. maritima x densiflora* were recorded in the estuaries of Tinto-Odiel (n = 3 tussocks), Piedras (n = 4 tussocks; all known tussock of this hybrid in this estuary) and Guadiana (n = 18 tussocks) in May 2018. The major diameter of *S. densiflora x maritima* tussocks was measured in the estuaries of Tinto-Odiel (n = 2 tussocks; all known tussock of this hybrid in this estuary), Piedras (n = 1 tussocks; all known tussock of this hybrid in this estuary) and Guadiana (n = 205 tussocks) in May 2018. The seed parent of measured hybrids in Tinto-Odiel and Piedras Estuaries were determined by Castillo et al. (2010b) and *Chapter III*. In Guadiana, measured *Spartina* hybrids at the low marshes were identified as *S. maritima x densiflora* and those at middle marshes as *S. densiflora x maritima* following Castillo et al. (2010a).

The ages (in yr) of individual tussocks of both *Spartina* hybrids were estimated as the ratio between the tussock major diameter (cm) and its lateral expansion rate (cm yr^{-1}). Lateral expansion rate by rhizomes was recorded during periods longer than 10 years for both hybrids, integrating the environmental variability that hybrid tussocks were exposed to during significant periods of their actual life span. Our growth model considered that recorded integrated lateral expansion rates were constant during the whole life span of the studied tussocks, resulting in constant radial growth of the clone for uncrowded, density independent individual plants. In this sense, Dennis, Civille, and Strong (2011) showed that factors intrinsic to *Spartina* tussocks dominated the effects of large scale abiotic factors on clone growth, resulting in constant radial growth over time. As in our study, previous works have estimated the age of clonal herbs also using the size of the genet and its growth rate (de Witte and Stöcklin 2010)

Relationships between Spartina *hybrids establishment and meteorological conditions*

The year of establishment of every hybrid tussock was inferred from its estimated age. Then, the annual number of established tussocks for every *Spartina* hybrid (*S. maritima x densiflora*, N = 25 tussocks; *S. densiflora x maritima*, N = 208 tussocks) was related to the meteorological conditions of every year of establishment. Meteorological monthly data (mean rainfall, maximum daily rainfall, mean of minimum, average and maximum temperatures) from 1955 to 2017 were obtained from the meteorological station of the city of Huelva (37º15'35.02"N, 6º56'55.37''W). This period of meteorological data is the maximum available in the Spanish State Meteorological Agency (AEMET 2018). Specifically, monthly meteorological data for May, June, July, August and September were used in our analyses since these are the months in which the flowering and fruiting time of *S. maritima* (May-September) and *S. densiflora* (June-December) may coincide in the Gulf of Cadiz (Valdes et al. 1987).

Additionally, we studied the genet dynamics of the historical establishment of *Spartina* hybrids in a model marsh known locally as San Bruno (Guadiana Estuary, 37º10'-37º16'N, 7º28'-7º16'W), where the greatest density of hybrids (hundreds) has been observed. With this aim, the diameter of each tussock and its spatial distribution (using a Garmin Oregon 550t decametric GPS (Garmin Ltd, KA, USA)) was recorded for both *Spartina* hybrids in a total area of 28 ha in May 2018. The year of establishment of each hybrid tussock was estimated as reported above. Hybrid tussocks were classified according to their decade of establishment and represented on the corresponding aerial color photograph for each decade obtained from those available for the period 1943-2018 in the photo libraries of the Spanish National Geographical Institute (IGN 2018) and the Andalusian Institute of Statistics and Cartography (IECA 2018).

Statistical analyses

All the analyses were applied with a significance level (α) of 0.05 and they were conducted using the software Sigma-Plot for Windows (version 12.0, Systat Software Inc., IL, US). Data series were verified for normality with the Shapiro-Wilk's test and for homoscedasticity with Levene's test, before the application of parametric analyses. In cases when data transformations (inverse, square root or logarithm) were insufficient to meet assumptions of the parametric models, non-parametric tests were conducted. The lateral expansion rates of *S. densiflora*, *S. maritima* and their hybrids were compared using one-way analysis of variance (ANOVA) on ranks, using taxa as grouping factor and Dunn's test as post-hoc analysis. Diameter and estimated age of both *Spartina* hybrids were compared using Mann-Whitney U-test and Student's t-test for impendent samples, respectively. To explore the relationships between the establishment of both *Spartina* hybrids and meteorological conditions, linear correlations (Pearson coefficient) between the numbers of annually established hybrids and monthly meteorological variables, and linear regressions between numbers of annually established hybrids and mean maximum temperature were carried out.

10.1 Results

The lateral expansion rate of *Spartina maritima x densiflora* (21 ± 2 cm yr^{-1}) was similar to that of its seed parent *S. maritima* (44 ± 6 cm yr^{-1}) and higher than that of *S. densiflora x maritima* (4 ± 2 cm yr^{-1}), which presented a similar lateral expansion rate to its seed parent *S. densiflora* (5 ± 0.3 cm yr^{-1}) (Kruskal-Wallis, H = 47.421, $P < 0.001$; Dunn's test, $P < 0.05$) (Appendix 10.A, 10.B).

Tussock diameters of *S. maritima x densiflora* and *S. densiflora x maritima* were 314 ± 55 cm and 238 ± 9 cm, respectively (Mann-Whitney test, U = 2284, P > 0.05). Accordingly, with their lateral expansion rates, those diameters corresponded to their establishment taking place 17 ± 3 yr ago for *S. maritima x densiflora* and 54 ± 2 yr ago for *S. densiflora x maritima* (t-test, t = -6.204, P < 0.001). The estimated date of establishment of the oldest tussock of *S. maritima x densiflora* was 1974 and the youngest was established in 2016 (Fig. 10.2A), while the oldest and the youngest tussocks of *S. densiflora x maritima* were established in 1813 and 2007, respectively (Fig. 10.2B).

Mean average and maximum temperatures in June, July and August increased over time in the period 1955-2017 (Pearson correlation, $P < 0.01$). The highest monthly average and maximum temperatures were recorded in 2017 for June, in 2015 for July and in 2016 for August (Fig. 10.1). However, mean average and maximum rainfall did not show a significant relationship with time for the aforementioned period (Pearson correlation, $P > 0.05$). For June, the highest average rainfall (two times greater than the next higher value) was registered in 1970, with the previous and following years being more humid than the average, while in 7 of the last 13 years there was no precipitation. July and August were generally very dry, with some years reaching precipitations of 25 and 35 mm, respectively (Fig. 10.1).

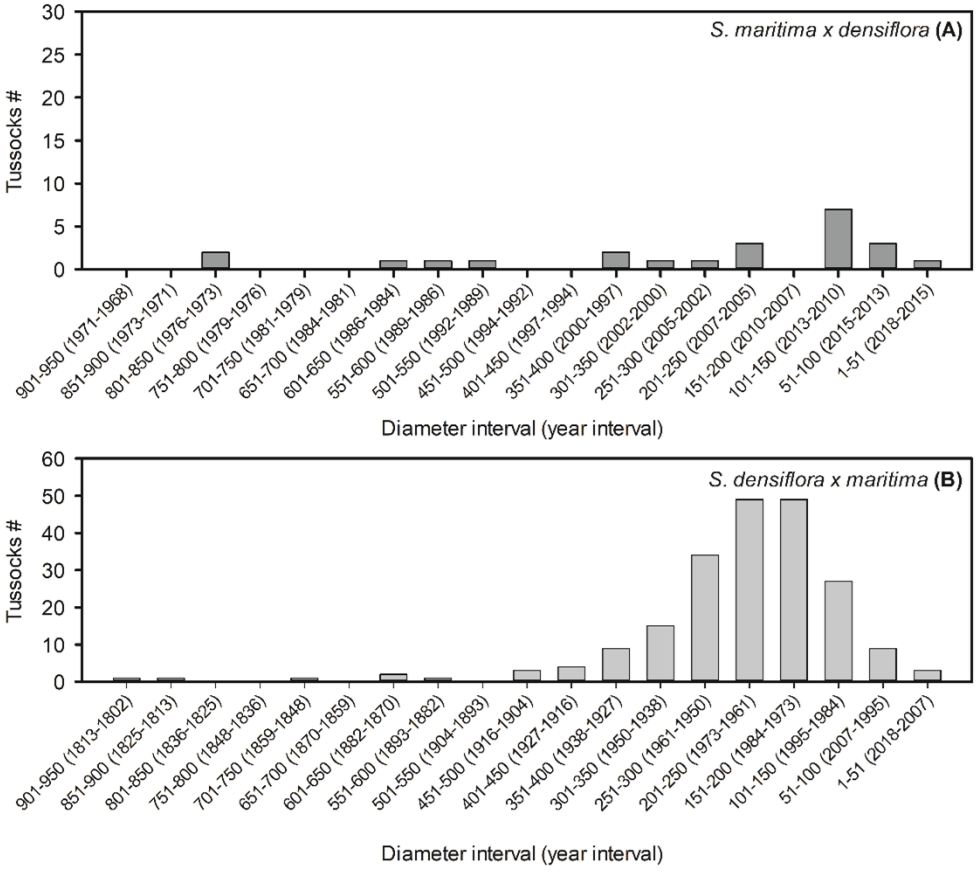

Fig. 10.2. Number of tussocks for different size classes (diameters in cm) of the hybrids *Spartina maritima x densiflora* (n = 22) (A) and *S. densiflora x maritima* (n = 208) (B) in the estuaries of the Tinto-Odiel, Piedras and Guadiana Rivers (Southwest Iberian Peninsula). The years of establishment estimated according to their lateral expansion rates by rhizomes are indicated in parentheses.

The number of annually established tussocks of *S. maritima x densiflora* between 1955 and 2017 was positively correlated with mean maximum temperature in June, mean average and maximum temperatures in July, and mean average temperature in August (Table 10.1, Fig. 10.3). On the contrary, the annual number of established tussocks of *S. densiflora x maritima* was negatively correlated with mean average and maximum temperatures in June and mean maximum temperature in July and August (Table 10.1, Fig. 10.3). In all cases, the slopes of the negative regressions between the numbers of established tussocks of *S. densiflora x maritima* and maximum monthly temperatures were higher than the positive slopes for *S. maritima x densiflora* (Fig. 10.3). Additionally, the annual number of established tussocks of *S. densiflora x maritima* increased together with mean average and maximum rainfall in June. Moreover, both *Spartina* hybrids showed a negative correlation between them regarding the annual number of established tussocks. No correlation was found between the number of established hybrids and any of the monthly meteorological parameters in May or in September (Table 10.1).

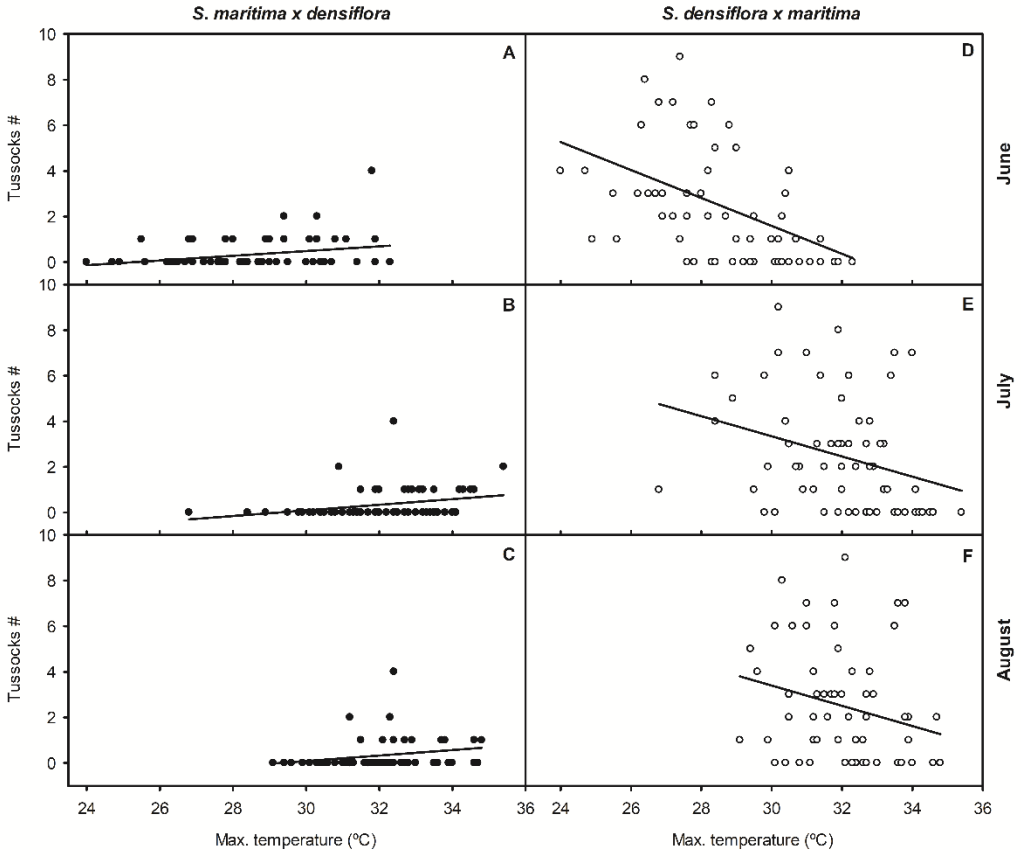

Fig. 10.3. Relationships between the number of annually established tussocks of *Spartina maritima x densiflora* (black symbols) and *S. densiflora x maritima* (white symbols) and mean maximum temperatures on the Gulf of Cadiz (Southwest Iberian Peninsula) in June (A, D), July (B, E) and August (C, F) from 1955 to 2017. Regression equations (N = 61-62): (A) y = -2.612 + 0.103 x, $R = 0.28$, $P < 0.05$; (B) y = -3.605 + 0.123 x, $R = 0.30$, $P < 0.05$; (C) y = -3.537 + 0.121 x, $R = 0.25$, $P = 0.05$; (D) y = 19.957 – 0.612 x, $R = 0.47$, $P < 0.0001$; (E) y = 16.617 + 0.442 x, $R = 0.30$, $P < 0.05$; (F) y = 16.717 + 0.444 x, $R = 0.25$, $P < 0.05$.

In the model marsh of San Bruno (Guadiana Estuary), the number of tussocks identified as *S. densiflora x maritima* in middle marshes (205 tussocks) was much higher than that of *S. maritima x densiflora* in low marshes (18 tussocks). According to the estimated ages of these tussocks, the first hybrid to be established was *S. densiflora x maritima* (ca. year 1813) while the first tussock of *S. maritima x densiflora* did not appear until 1984. Since 2007, no novel tussocks of *S. densiflora x maritima* have been established, while the latest tussock of *S. maritima x densiflora* dates back to 2016 (Fig. 10.4).

Table 10.1. Pearson correlation coefficient (upper digit) and *P*-values (lower digit) between the number of annually established of tussocks of *Spartina maritima x densiflora* (*Smxd*) and *Spartina densiflora x maritima* (*Sdxm*), and mean monthly maximum and average rainfall, and mean minimum, average and maximum temperature in Southwest Iberian Peninsula in May, June, July and August from 1955 to 2017. N = 61-62. Significant correlations are marked in bold.

			May		
	Max. Rainfall	Rainfall	Min T^a	Average T^a	Max T^a
Tussocks # (*Smxd*)	0.147 0.255	0.164 0.204	0.148 0.252	0.213 0.097	0.217 0.090
Tussocks # (*Sdxm*)	0.016 0.904	0.005 0.972	0.136 0.290	-0.074 0.569	-0.181 0.158
			June		
	Max. Rainfall	Rainfall	Min T^a	Average T^a	Max T^a
Tussocks # (*Smxd*)	-0.071 0.588	-0.117 0.369	0.012 0.924	0.205 0.110	**0.283** **0.026**
Tussocks # (*Sdxm*)	**0.321** **0.012**	**0.310** **0.015**	-0.001 0.992	**-0.330** **0.009**	**-0.471** **0.000**
			July		
	Max. Rainfall	Rainfall	Min T^a	Average T^a	Max T^a
Tussocks # (*Smxd*)	0.210 0.101	0.189 0.142	0.068 0.598	**0.264** **0.038**	**0.300** **0.018**
Tussocks # (*Sdxm*)	-0.025 0.846	-0.001 0.995	0.215 0.094	-0.155 0.230	**-0.302** **0.017**
			August		
	Max. Rainfall	Rainfall	Min T^a	Average T^a	Max T^a
Tussocks # (*Smxd*)	0.055 0.669	0.070 0.594	0.176 0.171	**0.260** **0.041**	0.246 0.054
Tussocks # (*Sdxm*)	0.022 0.869	0.001 0.997	0.046 0.723	-0.165 0.200	**-0.253** **0.047**

	September				
	Max. Rainfall	Rainfall	Min T[a]	Average T[a]	Max T[a]
Tussocks # (*Smxd*)	-0.052 0.688	-0.056 0.665	-0.131 0.310	0.001 0.992	0.100 0.437
Tussocks # (*Sdxm*)	-0.082 0.525	-0.116 0.369	0.242 0.058	0.090 0.486	-0.047 0.717

	Tussocks # (*Sdxm*)
Tussocks # (*Smxd*)	**-0.289** **0.023**

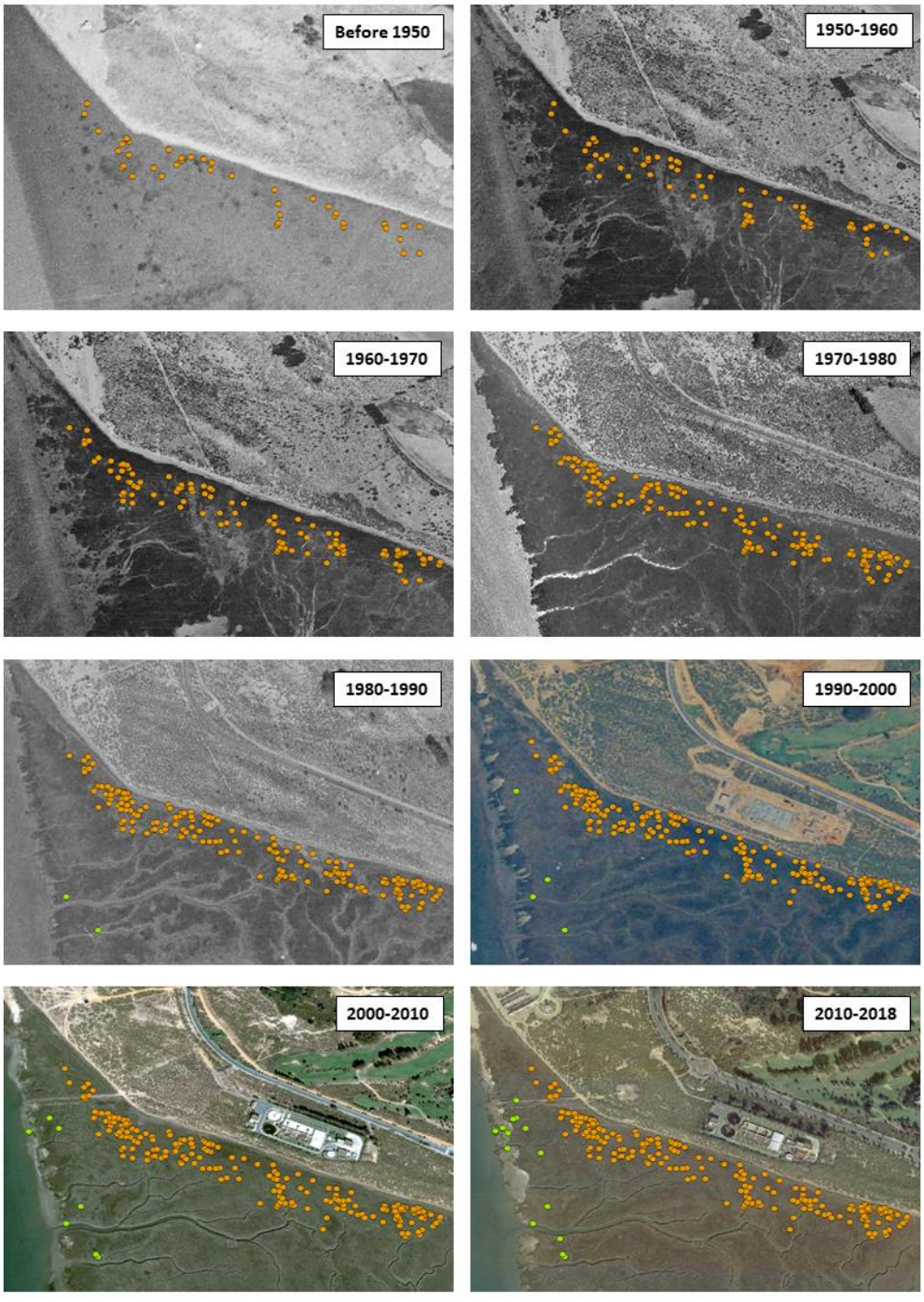

Fig. 10.4. Historical spatial distribution of the hybrids *Spartina maritima x densiflora* (green symbols) and *S. densiflora x maritima* (orange symbols) in San Bruno Marsh (Guadiana Estuary, Southwest Iberian Peninsula), before 1950 (aerial photography 1945), 1950-1960, 1960-1970 (a. p. of 1956), 1970-1980 (a. p. of 1973), 1980-1990 (a. p. of 1984), 1990-2000 (a. p. of 1996), 2000-2010 (a. p. of 2005) and 2010-2018 (a. p. of 2017).

10.2 Discussion

Our study focused on the dynamics of establishment of the reciprocal hybrids between native *Spartina maritima* and invasive *S. densiflora* provides new insights on the effects of climate change on the interactions between native and invasive species and on the origin of exotic hybrid plants.

The lateral expansion rates of the hybrids *Spartina maritima x densiflora* and *S. densiflora x maritima* were different from each other and similar to those of their corresponding seed parents, revealing the maternal influence on this plant trait. Maternal effect is a relevant process in gene expression of hybrids (Videvall et al. 2016) such as for growth characteristics of *Seneceio jacobea x aquaticus* (Kirk et al. 2005) or the hybrids between transgenic *Brassica napus* L. and wild *B. juncea* (L.) Vassiliĭ Matveievitch (Di et al. 2009). For the reciprocal hybrids *S. maritima x densiflora* and *S. densiflora x maritima*, maternal effects have been previously observed in their response to different salinities (Gallego-Tévar et al. 2018b, and *Chapter V*). The recorded greater expansion rate of native *Spartina maritima* was consistent with its growth form in 'guerrilla' (*sensu* Lovett Doust and Lovett Doust, 1982) in which asexual lateral spreading by long rhizomes predominates over sexual reproduction (Castellanos et al. 1994). In contrast, invasive caespitose *S. densiflora* exhibited short rhizomes, growing in ‘phalanx’ (*sensu* Lovett Doust and Lovett Doust, 1982) and forming dense tussocks (Castillo et al. 2010b), with its dispersion occurring mainly by seeds (Kittelson and Milton 1997). The lateral expansion rate recorded in this study for *S. maritima* was in the range of that reported previously by Figueroa et al. (2003) (38 cm yr^{-1}) and by Castillo and Figueroa (2009) (26 cm yr^{-1}) in Odiel Marshes. Similarly, the recorded lateral expansion rate for *S. densiflora* was comparable to those reported by Kittelson and Milton (1997) and Nieva et al. (2005) (ca. 8 cm yr^{-1}). The lateral expansion rates of both hybrids may be explained by their growth forms in relation to their parental species. Thus, *S. maritima x densiflora* expanded laterally more rapidly and with

longer rhizomes than *S. densiflora x maritima*. Both hybrids formed dense turfs of tillers within their tussocks like *S. maritima*, but more clearly differentiated and of bigger sizes, so that the turfs of tillers within hybrid tussocks presented similar growth form than individuals tussocks of *S. densiflora*, but with taller tillers. The bare spaces within the hybrid turfs usually constituted intertidal ponds or were occupied by other halophytes (Appendix 10.C; Gallego-Tévar, personal observation), while the turfs of tillers within tussocks were rarely colonized by other halophytes (Gallego-Tévar et al. 2018a).

As a consequence of the greater lateral expansion rate of *S. maritima x densiflora* in relation to *S. densiflora x maritima*, its tussocks were dated younger, despite having both hybrids similar sizes. When the number of annually established hybrid tussocks (1955-2017), according to their estimated ages, was related to meteorological conditions, correlations were found for both hybrids with mean maximum monthly temperature in June, July and August. These were the only months for which significant increases in temperatures were registered in the analyzed time period, being also these months the common period of flowering for both parental species (Valdes et al. 1987). The above-mentioned increase in air temperature during the end of spring and the beginning of summer was consistent with the predictions of climate change models for the Mediterranean region (Giorgi and Lionello 2008, Giannakopoulos et al. 2009, Kovats et al. 2014). The correlations between the number of established tussocks and mean maximum monthly temperatures were positive for *S. maritima x densiflora* and negative for *S. densiflora x maritima*. Therefore, an alternation in the establishment of both hybrids in relation to changing climatic conditions was observed, so that *S. maritima x densiflora* establishment increased and *S. densiflora x maritima* decreased in warmer spring-summers, and *vice versa*.

The effects of meteorological conditions (and climatic conditions in the long term) on the formation of each of the *Spartina* hybrids may be related to alterations in the flowering dynamic of *S. maritima* phenology. Air temperature

is an important factor in the induction of flowering in grasses (Cooper and Calder 1964, Heide and Heide 1994). Both *S. maritima* and *S. densiflora* flowers during the end of spring and the beginning of summer when increasing temperatures and photoperiods co-occur and both cordgrasses are protogynous, with the pistil emerging earlier than the stamens starting from upper flowers in the inflorescences to their bottom (Davis et al. 2004). In mild years during May-July, as those frequently recorded in the first decades of the twentieth century, *S. maritima* lengthened its flowering starting in May and being prolonged until the end of July, and *S. densiflora* started its flowering in July. In these meteorological conditions similar to those of 2018, *S. maritima* finished its flowering exerting all its stamens, showing high pollen : ovule ratio (Fig. 10.5) . Thus, it would be expected that the hybrid of which *S. densiflora* is the seed parent (*S. densiflora x maritima*) would be the most abundant, as recorded in our study. There would be a greater chance of matching the last stamens (pollen) of *S. maritima* to the first pistils (stigma) of *S. densiflora*, when its stamens have not yet been exerted, than *vice versa*. In contrast, during warm flowering periods, as those recorded mainly at the end of the twentieth century and the beginning of the twenty-first century as 2017, the flowering of *S. maritima* finished early and abruptly in July so that the stamens of some inflorescences did not get to be exerted while the pistils were already exerted, which was reflected in low pollen : ovules ratio for *S. maritima* (Fig. 10.5).

Thus, the result of low pollen : ovule ratio of *S. maritima* during warm flowering periods would result, as recorded in our study, in a higher formation of the hybrid *S. maritima x densiflora*. On the other hand, the broad flowering period of *S. densiflora* from June to December reported by Valdes et al. (1987) and Castillo and Figueroa (2009) in the Gulf of Cadiz and (Bortolus, 2006) in different regions worldwide would be related to the high phenotypic plasticity of this cordgrass in response to contrasted environmental conditions (Nieva et al. 2001, Castillo et al. 2014, 2018, Grewell et al. 2016). Seneca and Blum (1984) observed that air temperature was the main factor controlling the

flowering of *Spartina alterniflora* Loisel., blooming at 22-26 ºC, while *Spartina foliosa* Trin. flowered in a wider temperature range being less dependent on this factor. This increase in the flowering of *S. alterniflora* with temperature was mainly associated to high carbon assimilation, although photoperiod-temperature direct induction was not ruled out. Also, mild temperatures in spring and early summer are known to delay flowering of the allopolyploid *Spartina anglica* C. E. Hubb (Ranwell 1967, Gray et al. 1991, Thompson 1991). Another factor that would favor the formation of the hybrid *S. maritima x densiflora* at low marshes is the fact that *S. densiflora* in this stressful habitat flowers normally earlier than at middle marshes (Nieva et al. 2005), facilitating the pollination of *S. maritima*.

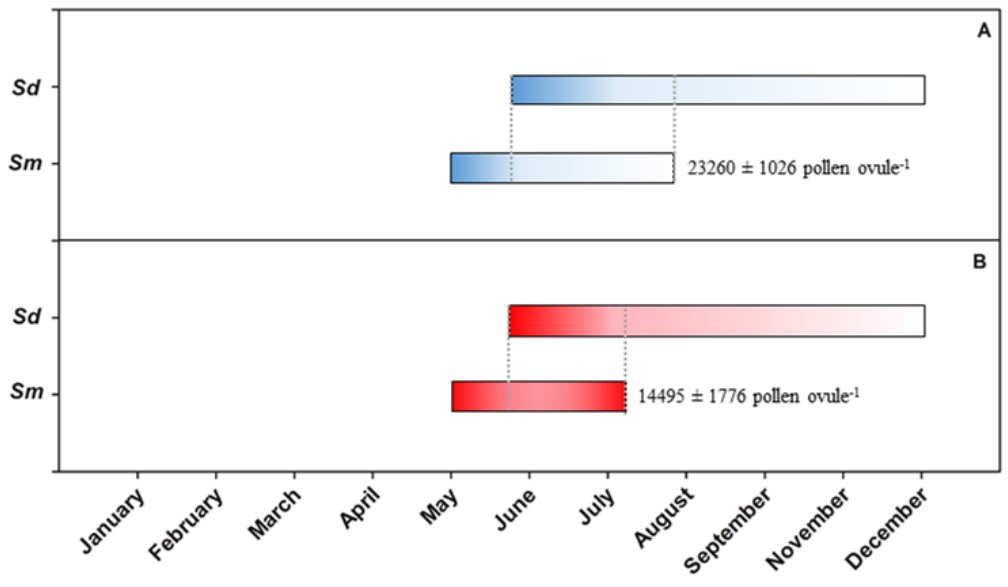

Fig. 10.5. Flowering periods of native *Spartina maritima* (*Sm*) and invasive *S. densiflora* (*Sd*) in (A) 2018 (a mild flowering period) and (B) 2017 (a warm flowering period). Darker color represents the predominance of pistils in the inflorescence and lighter color is the predominance of stamens. The pollen : ovule ratio of *S. maritima* is indicated nearby its flowering bars (paired Student t-test, $t = 8.472$, $P < 0.001$, $df = 4$). The flowering period of *S. densiflora* was obtained from Valdes et al. (1987) and Castillo and Figueroa (2009). Data provided by Infante et al. (University of Huelva), see Appendix 10.D for methods.

The number of annually established tussocks of *S. densiflora x maritima* was also higher in the years with higher rainfall in June. Published accounts on the effects of climate change on flowering phenology have found no direct relationships between high rainfalls and change in flowering of grasses (Cleland et al. 2006, Sherry et al. 2007), including cordgrasses such as *S. alterniflora* and *Spartina patens* (Aiton) Muhl. (Charles and Dukes 2009). However, an increase in rainfall during the beginning of the summer would lead to a reduction in soil salinity in salt marshes (Burdick et al. 2001), which would favor the development of *S. densiflora* (Castillo et al. 2005b, 2014, 2018, Grewell et al. 2016) and *S. maritima* (Naidoo et al. 2012). High salinities can reduce flowering of halophytes (Flowers et al. 1986, Ventura et al. 2014). In this sense, *S. densiflora* and *S. foliosa* from San Francisco Bay reduced their production of inflorescences at hypersalinity in relation to lower salinities (B. Gallego-Tévar et al. unpublished data). In our study, rainfall was only related to the establishment of the hybrid *S. densiflora x maritima* in middle marshes, which is consistent with the fact that the highest salinities during the dry season (summer) are reached in middle marshes (Contreras-Cruzado et al. 2017). Thus, according to our results, *S. densiflora x maritima* hybrids were formed mostly between 1961-1984 coinciding with the years of lower maximum temperatures and higher rainfall of the period 1955-2017.

Climate change models predict increases in maximum temperatures and decreases in rainfall in the Mediterranean basin especially in summer (Giorgi and Lionello 2008, Giannakopoulos et al. 2009, Kovats et al. 2014, IPCC 2015), so we predict a reduction of the formation of the hybrid *S. densiflora x maritima* in favor of *S. maritima x densiflora*.

10.3 Conclusions

In view of our results, the rise in just a few degrees in air maximum temperature during the flowering periods of *S. maritima* and *S. densiflora* increases the probabilities of establishment of the hybrid *S. maritima x densiflora* in relation to *S. densiflora x maritima*. *S. maritima x densiflora* was formed mainly with mean maximum temperature in June higher than 29 ºC, whereas *S. densiflora x maritima* with mean maximum temperature in June lower than 31 ºC. The predictions of maximum temperature increase with climate change in summer in Southern Iberian Peninsula range from +2.5 ºC in 2040 to +7.2 ºC in 2100 (Anaya-Romero et al. 2015), so a marked intensification in the maternal switch in the formation of *Spartina* hybrids is expected. The increase of the appearance of *S. maritima x densiflora* with climate change would pose a greater threat to the native species *S. maritima*, since this hybrid grows rapider, taller and in the same habitat than its seed parent (Castillo et al. 2010a). This competitive pressure would be added to the one exerted by sea level rise on low marsh halophytes (Schile et al. 2014) such as *S. maritima*, which is included in different European red lists as that one from the South of Iberian Peninsula (Cabezudo et al. 2005).

CHAPTER XI

General discussion

CHAPTER XI: GENERAL DISCUSSION

This chapter presents a synthesis of the contributions that this PhD thesis provides to the previous knowledge on native *Sarcocornia* and exotic and invasive *Spartina* hybrids from the point of view of their role in the functioning and the structure of coastal marshes.

Phenotypic inheritance of exotic hybrids in response to stress

Our results showed that biochemical, physiological and morphological responses of hybrids to the environment is the product of their phenotypic inheritance. Different processes such as ploidy level, maternal effects and the phenotypic plasticity of the parental species and the hybrids theirself have revealed as significant in our analyses on exotic *Spartina* hybrids.

Different authors have shown that hybrids can exhibit phenotypic characters similar to one or both parents (dominance), intermediate (additivity) or different to both parents (transgressivity) (Bassene et al. 2010, Fridman 2015). Both in the study of the responses to salinity of the reciprocal hybrids between native *S. maritima* and invasive *S. densiflora* in Souwthest Europe and to the combination of salinity and inundation of *S. densiflora x foliosa* in Southwest North America, we recorded the different phenotypic inheritance types cited above. One kind of dominant inheritance in hybrids that can be crucial for the divergent evolution of the tolerance to abiotic stress is the prevalence of the maternal phenotype or maternal effect (Burgess and Husband 2004, Kimball et al. 2008, Favre and Karrenberg 2011). Our studies showed maternal effects on the response of *Spartina* hybrids to abiotic stress, especially for PEPC-related traits, of *S. maritima x densiflora* while they were less abundant in *S. densiflora x maritima* and not observed in *S. densiflora x foliosa*. The greater genomic contribution of the maternal species in the case of *S. maritima x densiflora* (2:1) compared to

the other two studied hybrids (1:1) probably explained this difference between taxa, as reported in other hybrids (Guo et al. 2003).

Another type of non-additive genetic expression in hybrids is heterosis or 'hybrid vigor', in which the response of hybrids is transgressive compared to parents. Transgressive traits in relation to tolerance to stress have been reported in several hybrid taxa (Ellstrand and Schierenbeck 2000) such as those between native *T. latifolia* L. and introduced *T. angustifolia* L. in North America (Bunbury-Blanchette et al. 2015) or the exotic hybrids *Senecio squalidus* L. (James and Abbott 2005) and *Fallopia x bohemica* Chrtek and Chrtkova in Europe (Parepa et al. 2014). Our studies have brought new contributions to the knowledge of heterosis in hybrids, obtaining that the development of transgressive traits in hybrids is: (1) greater at the extremes of abiotic stress gradients, when the parents suffer greater reductions in fitness; and (2) directly related with trait variability of their parents. Thus, both hybrids between *S. maritima* and *S. densiflora* showed their highest percentage of transgressive traits at both extremes of the salinity gradient (fresh water and hypersalinity) and *S. densiflora x foliosa* under deep inundation (55 cm deep). In addition, many other studies have revealed that heterosis is directly related to genetic difference between parental taxa (East 1936, Ali et al. 1995, Reif et al. 2003, Stelkens and Seehausen 2009, Pandey et al. 2018). But our studies went further, demonstrating that when the parents themselves show a more plastic response for a given trait, there is a greater chance that the hybrid will develop a transgressive behavior for this trait. Maternal effect was also observed in the relationship between parental plasticity and phenotypic inheritance in hybrids by finding negative relationships between phenotypic plasticity of *S. densiflora* and the percentage of traits product of the dominance of the maternal species in *S. densiflora x maritima* and *S. densiflora x foliosa*. Moreover, the development of transgressive traits in the reciprocal *Spartina* hybrids depended on the ploidy level and/or on the maternal origin (Miller et al. 2012, Yao et al. 2013, Pandit et

al. 2014) so that *S. maritima x densiflora* presented greater proportion of transgressive traits in its response to salinity than *S. densiflora x maritima*.

By the development of advantageous traits, hybridization may lead to the expression of novel phenotypes with increased fitness (Johnston et al. 2004, Jackson 2017). In fact, both the *Spartina* hybrids from the Iberian Peninsula (*S. densiflora x maritima* and *S. maritima x densiflora*) and from San Francisco Bay (*S. densiflora x foliosa*) displayed higher tolerances to the abiotic factors imposed, showing higher fitness than parental species. Another factor that may contribute to enhanced performance of hybrids at extreme environmental conditions is the promotion of higher phenotypic plasticity (Ainouche and Jenczewski 2010, Te Beest et al. 2012, Cara et al. 2013). However, the maintenance of high fitness in our studies was more related to the abundance of transgressive traits than the development of high phenotypic plasticity since we did not find differences in plasticity between the hybrids and their parental species. The different *Spartina* taxa analyzed are all polyploids which is also a factor that may stimulate high phenotypic plasticity (Ainouche and Jenczewski 2010, Te Beest et al. 2012). In addition, some of the variables that showed high plasticity for both studied native parental species (*S. maritima* and *S. foliosa*) were indicators of high stress, pointing to passive plasticity (Kurashige and Callahan 2007). We conclude that the evolution of phenotypic plasticity in the studied sterile and exotic *Spartina* hybrids was probably in an early stage as they were the only taxa in which it was related to intrinsic variability (intra-population trait variability) and plasticity may evolve independently over time (Pigliucci 2001).

Ecological role of native and exotic hybrids

The tolerance to environmental factors resulting from phenotypic and genotypic characteristics of the hybrids determines their ecological niches and, ultimately, the roles in the zonation pattern and in the development of ecological succession. The analysis of the role of the native hybrids between *Sarcocornia*

perennis and *S. fruticose* in the current spatial configuration of coastal salt marshes (zonation) and their change over time (succession) revealed that they played a structuring role. The presence of the native hybrids at intermediate levels of abiotic environment relative to their parental species, but with similar realized niche dimensions, documented the existence of a continuum of different *Sarcocornia* taxa along the intertidal gradient, acting as a 'bridge' between the low marsh halophyte *S. perennis* and *S. fruticosa* in high marshes. Moreover, high degree of coexistence between the hybrid and the most predominant native species at middle marshes, *Atriplex portulacoides,* was found (Crooks et al. 2002). However, *Sarcocornia* hybrids were more abundant than their parents because middle marsh habitat is the most frequent. The stable hybrid zone formed by *Sarcocornia* taxa in the studied marshes seemed to be related to the fitness of the hybrids (Buggs, 2007). Other studies have related the ecological performance of native hybrids to their fitness obtaining similar results as in our study. Thus, intermediate phenotypic traits and occupied habitat between parents has been found for *Sarcocornia* taxa from South Africa (Steffen et al. 2015) but also higher ecological amplitude is observed in native *Helianthus* hybrids in North America (Lexer et al. 2003a). The structuring role of the *Sarcocornia* hybrids was maintained throughout succession so that their significant increase in plant cover facilitated by the parental *S. perennis* (Figueroa et al. 2003) did not prevent the colonization of other native species. The presence of *Sarcocornia* hybrids was even related to the decrease of the cover of invasive *S. densiflora*. Similar to our results, an effect of control of the *S. densiflora* invasion by a well-established population of native *Sarcocornia* taxa was recorded by Curado et al. (2018) in Odiel Marshes.

On the other hand, a disrupting role of plant zonation and ecological succession was revealed for the exotic hybrids between native *Spartina maritima* and invasive *S. densiflora*. Our results showed that their niche was lower than that of both parent species. This was probably related to their inability to produce viable seeds (Castillo et al. 2010a), so they are relegated to

discrete bands near their maternal species forming 'tension zones'. In fact, previously reported increase of invasiveness by hybridization (Ellstrand and Schierenbeck 2000) leading to an enlargement of realized niche in relation to the parents (Duchoslav et al. 2010, Thornton and Murray 2014) has been frequently associated to high ability to spread by seeds (Ayres et al. 2003, 2004, Hall et al. 2006). However, the altering role of the zonation pattern by the studied *Spartina* hybrids was evidenced by the low percentage of co-occurrence with other native species, suggesting that they have the ability to outcompete other halophytes due to transgressive traits such as tall tillers and high growth rates (Castillo et al. 2010a). Our study on plant succession showed that this competitive capacity is acquired over time, significantly reducing the percentage of coincidence with other species in a decade, although their plant cover advanced relatively slowly. Invasive species reduce the cover of native species with which they cohabit due to competitive exclusion (Vilà and Weiner 2004), altering the evolutionary development of the native ecosystem (Mooney and Cleland 2001). As a result, in marshes where the exotic hybrids were present, vegetation cover of the rest of the species did not increased and neither did their percentages of coincidence with other species, as it would have been expected during the development of coastal marsh succession (Brereton 1971, Russell et al. 1985). As it has been mentioned in several chapters of this PhD thesis, this competitive ability restrained by the sterility of the *Spartina* hybrids puts attention on the possibility of becoming fertile through allopolyploidy, achieving much greater fitness than their parental species and high dispersion capacity. This phenomenon has previously taken place in the genus *Spartina*, giving rise to the highly invasive species *S. anglica* (Huskins 1930, Guenegou et al. 1988) that is threating the conservation status of many salt marshes around the world (Ainouche et al. 2004, Strong and Ayres 2009).

At the regional scale, the ecological role of hybrids may also be conditioned by processes such as reproductive isolation or adaptive evolution. In these sense, marked genetic structure have been described in other hybrids

due to limitations in seeds and pollen dispersal (Sloop et al. 2011). In our study on the genetic structure of the three populations of the exotic *Spartina* hybrids in different estuaries of the Gulf of Cadiz, we observed that the Guadiana Estuary population was the most genetically and environmentally differentiated, in addition to be the furthest and where the hybrids were more abundant (Castillo et al. 2010a). Thus, reproductive isolation by distance (Wright 1943, Baack et al. 2015) and isolation by environment (Wang and Bradburd 2014) processes seemed to play a role in the development of these hybrids. Our results showed that the development of transgressive characters in the field occurred only in the Guadiana population, probably related to the greater genetic distances found between parents (i.e. Ali et al. 1995, Pandey et al. 2018) and/or to sedimentary differences as hybrid fitness may be environment dependent (reviewed in Arnold and Martin 2010).

Hybridization and global change

Climate change is modifying climatic characteristics at a rate that many species are expected to not be able to adapt to the new conditions, leading to a generalized increase in the extinction rate (Parmesan 2006, Urban 2015). In salt marshes, accelerating rates of sea level rise (SLR) associated with climate change will modify the patterns of salinity and inundation (IPCC 2015), intensifying abiotic stress levels on halophytes (Stralberg et al. 2011). Our studies have shown that *Spartina* exotic hybrids will tolerate better than the parental species the intensification of these factors (discussed above). Taxa with high capacity to adapt to environmental changes may be promoted in the new climatic scenario (Dukes and Mooney, 1999; Vilà et al. 2007). The hybrid *S. densiflora x foliosa* and its parental species showed different syndromes to face the combination of increasing salinity and waterlogging. *Spartina densiflora* acted as a rapid-growth species in the absence of stress and *S. foliosa* as a slow-growing and stress-tolerant species (Grime 1977, 2006), while the hybrid maintained higher fitness than its parents at all conditions. The response of the

parents was in accordance with the adaptation of *S. foliosa* to low salt marshes with anoxic sediments exposed to long submersion periods with salty waters, and with the high invasiveness of *S. densiflora* able to take advantage of low stress conditions but also showing mechanisms to tolerate some degree of saline and flooding stress. The hybrid *S. densiflora x foliosa* would take advantage of the combination of both syndromes and the development of transgressivity, being highly competitive along the intertidal gradient in the foreseeable climate change scenario in San Francisco Bay (Stralberg et al. 2011, Schile et al. 2014).

On the other hand, we have found that the increase in temperature and precipitation associated to climate change (Anaya-Romero et al. 2015, IPCC 2015) have effects on the hybridization process. Our study on the establishment of *Spartina* reciprocal hybrids between native *S. maritima* and invasive *S. densiflora* revealed an alternation in the establishment of both hybrids in relation to changing climatic conditions. *Spartina maritima x densiflora* establishment increased and *S. densiflora x maritima* decreased in warmer spring-summers, and *vice versa*. Global change is expected to increase the likelihood of hybridization between native and invasive species (Muhlfeld et al. 2014), both by the increased introduction of species, and by the changes in their ranges so that sympatry between divergent species may increase (Garroway et al. 2010, Hoffmann and Sgrò 2011). However, empirical studies on the biological processes underlying the increase in interspecific hybridization related to climate change are limited and focused on animal taxa (Becker et al. 2013; Muhlfeld et al. 2014; Ryan et al. 2009). In this sense, we observed that the above-mentioned effects of meteorological conditions on the formation of each of the *Spartina* hybrids was probably related to alterations in the phenological flowering dynamics of the parental species, as air temperature is an important factor in the induction of flowering in grasses (Cooper and Calder 1964, Heide and Heide 1994). Different pollen : ovule ratios were found for *S. maritima* in different climatic years promoting the formation of *S. densiflora x maritima* in mild spring-summers, and *S. maritima x densiflora* in warmer years. Other

factor to consider is the broad flowering period of *S. densiflora* (Valdes et al. 1987, Castillo and Figueroa 2009) that would be related to the high phenotypic plasticity of this invasive cordgrass in response to contrasted environmental conditions (Castillo et al. 2014, 2018; Grewell et al. 2016; Nieva et al. 2001). In addition to the increase of temperature, higher rainfall in summer was related to the formation of the hybrid *S. densiflora x maritima*, probably due to a reduction in soil salinity (Burdick et al. 2001) favoring the development and folowering of *S. densiflora* (Castillo et al. 2005, 2014, 2018; Grewell et al. 2016) and *S. maritima* (data not shown) at middle marshes (Contreras-Cruzado et al. 2017). Maternal switch in the formation of *Spartina* hybrids with climate change leading to an increase in formation of *S. maritima x densiflora* would pose greater threat to the native species *S. maritima*, since this invasive hybrid grows rapider, taller and in the same habitat than its seed parent (Castillo et al. 2010a).

Future research

This PhD thesis has responded to the questions raised four years ago about the ecological role of plant hybrids in salt marshes but, at the same time, it opens other interesting questions to be addressed in the future.

For example, ecophysiological studies on tolerance to environmental factors have not been carried out for the native *Sarcocornia* hybrids. The comparison of abiotic tolerance between the native and the exotic hybrids would be interesting to explain the differences between the structuring role of the former and the altering role of the later. Given the intermediate phenotype of the *Sarcocornia* hybrids between their parental species (Figueroa et al. 2003, Gallego-Tévar unpublished data), we hypothesize that intermediate phenotypic traits between both parents would be the dominant inheritance type in these hybrids.

Another possible line of research is the application of the advances in the knowledge of heterosis or ‘hybrid vigor’ found in this PhD thesis on species with agricultural interest. In the last century, hybrid varieties showing heterosis

have been increasingly used for many of the most important crops worldwide, improving agricultural performance (Duvick 1999, Fu et al. 2014, Hochholdinger and Baldauf 2018). Although recent advances in the mechanisms controlling heterosis have been addressed (Hochholdinger and Hoecker 2007, Chen 2013), many aspects still remain poorly understood (Baranwal et al. 2012) with particular difficulty in polyploid species (Washburn and Birchler 2014).

To better understand the development of transgressive traits promoting heterosis in the studied hybrids, we could analyze the relationship between phenotypic inheritance and gene expression (Bassene et al. 2009) by performing transcriptome analyses (Ferreira de Carvalho et al. 2017). On the other hand, epigenetic changes seem to have a relevant role in the development of heterosis in hybrids (Chen 2013, Groszmann et al. 2013). For this, epigenetic analysis such as the study of DNA methylation degree (Zhao et al. 2007) or histone modifications (He et al. 2010), would be an adequate complement to the advances obtained in this PhD thesis.

CONCLUSIONS

1. Populations of sterile reciprocal *Spartina* hybrids between native *Spartina maritima* and invasive *Spartina densiflora* in the Gulf of Cadiz are establishing hybrid zones with a **spatial genetic structure** inherited from both parental species, responding to processes of isolation by distance and by environment.

2. The hybrids *Spartina maritima x densiflora* and *Spartina densiflora x maritima* show greater **tolerance to salinity** than their parental species, highlighting the relevance of heterosis in the hybridization process. The development of transgressive traits was higher at the extremes of the salinity gradient and was directly related to phenotypic plasticity of the parental species.

3. Specific responses in relation to the functioning and regulation of phosphoenolpyruvate carboxylase (**PEPC**) **along a salinity gradient** are developed by the hybrids *Spartina maritima x densiflora* and *Spartina densiflora x maritima*. These hybrids show clear maternal effects and present non-additive inheritance, being transgressive traits related to PEPC functioning only found for *Spartina maritima x densiflora*.

4. In view of the intensification of **salinity and inundation** depth in salt marshes by the sea level rise, hybridizing species ***Spartina foliosa*** **and** ***Spartina densiflora*** from San Francisco Bay would reduce their vegetative and sexual reproductive fitness. *Spartina foliosa* would be endangered in low marshes due to increasing permanent inundation, being able to reach upper elevations by lateral expansion but its spread would be limited by its low floret production. *Spartina densiflora* would reduce its invasiveness accumulating less biomass and producing fewer florets.

5. The **exotic hybrid** *Spartina densiflora x foliosa* from San Francisco Bay are more tolerant to the combined effects of **salinity and inundation** than its both parental species, revealed by greater vegetative and reproductive fitness at all abiotic conditions. The number of transgressive traits of the hybrid was higher at extreme inundation. The number of transgressive individuals for a given trait increased with the phenotypic plasticity of the parental species for that trait.

6. Native *Sarcocornia* hybrids and exotic *Spartina* hybrids in the Gulf of Cadiz carry out contrasted responses to the abiotic environment and they performe different roles in how plant communities assemble. The native hybrids play a structuring role on the **zonation pattern** and the exotic hybrids a disruptive role limited by their sterility.

7. Native *Sarcocornia* hybrids increase their abundance over time in different salt marshes of the Gulf of Cadiz without inhibiting the developing of **ecological succession**, coinciding with increase of native species and reduction of invasive *Spartina densiflora*. In contrast, exotic and invading *Spartina* hybrids inhibit the development of succession by outcompeting other halophytes.

8. **Climate change** is associated to maternal switch in the formation of *Spartina* hybrids in the Gulf of Cadiz. The rise in just a few degrees in maximum air temperature during the flowering periods of *Spartina maritima* and *Spartina densiflora* increases the probability of formation and establishment of the hybrid *Spartina maritima x densiflora* and a reduction in the establishment of *Spartina densiflora x maritima*. The endangered and native *Spartina maritima* will be more affected by this maternal switch since it shares habitat with highly competitive *Spartina maritima x densiflora*.

9. Exotic *Spartina* hybrids have an invasive capacity limited by their sterility, but they display a high pre-dispersal reproductive fitness (production of florets). If a chromosomal duplication occurs in these hybrids, as previously reported in the genus, the new allopolyploid species could become fertile and lead to heterosis fixation, increasing their invasiveness. For this reason, the **eradication** of the studied *Spartina* hybrids is an urgent task for the management, conservation and restoration of tidal wetland plant communities in the Gulf of Cadiz and San Francisco Bay.

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Appendix 3.A. F-statistic and *P*-values of two-way ANOVAs for phenotypic traits and sedimentary factors of populations from three different estuaries (Tinto-Odiel, Piedras and Guadiana Estuaries) and taxa (*Spartina maritima, S. densiflora* and their hybrids) as fixed factors, and their corresponding interactions. Significant differences are marked in bold.

		Taxa	Estuary	Taxa x Estuary
Phenotypic traits	Number of leaves	**$F_{2,52}$ = 16.141; *P* < 0.001**	**$F_{2,52}$ = 3.789; *P* < 0.05**	$F_{4,52}$ = 1.292; *P* = 0.285
	Number of dead leaves	**$F_{2,52}$ = 39.658; *P* < 0.001**	$F_{2,52}$ = 1.318; *P* = 0.276	$F_{4,52}$ = 1.664; *P* = 0.172
	Leaves per tiller (cm^{-1})	**$F_{2,52}$ = 72.866; *P* < 0.001**	**$F_{2,52}$ = 9.811; *P* < 0.001**	**$F_{4,52}$ = 6.129; *P* < 0.001**
	Leaf width (cm)	**$F_{2,52}$ = 18.629; *P* < 0.001**	**$F_{2,52}$ = 8.040; *P* < 0.001**	$F_{4,52}$ = 2.360; *P* = 0.065
	Leaf length (cm)	**$F_{2,52}$ = 44.320; *P* < 0.001**	**$F_{2,52}$ = 3.387; *P* < 0.05**	$F_{4,52}$ = 2.013; *P* = 0.106
	Leaf area (cm^2)	**$F_{2,52}$ = 13.546; *P* < 0.001**	$F_{2,52}$ = 2.318; *P* = 0.109	**$F_{4,52}$ = 3.418; *P* < 0.05**
	SLA ($m^2 \cdot g^{-1}$)	**$F_{2,52}$ = 15.398; *P* < 0.001**	**$F_{2,52}$ = 4.147; *P* < 0.05**	$F_{4,52}$ = 1.366; *P* = 0.259
	Leaf rolling (%)	**$F_{2,52}$ = 28.105; *P* < 0.001**	$F_{2,52}$ = 2.339; *P* = 0.101	$F_{4,52}$ = 0.235; *P* = 0.917
	Tiller diameter (mm)	$F_{2,52}$ = 1.558; *P* < 0.221	**$F_{2,52}$ = 3.462; *P* < 0.05**	**$F_{4,52}$ = 2.784; *P* < 0.05**
	Tiller length (cm)	**$F_{2,52}$ = 37.133; *P* < 0.001**	**$F_{2,52}$ = 3.177; *P* < 0.05**	**$F_{4,52}$ = 4.407; *P* < 0.01**
	Tillers density (cm^{-2})	**$F_{2,52}$ = 18.753; *P* < 0.001**	**$F_{2,52}$ = 4.244; *P* < 0.05**	$F_{4,52}$ = 0.371; *P* = 0.828
	LAI	$F_{2,52}$ = 2.771; *P* = 0.072	**$F_{2,52}$ = 4.437; *P* < 0.05**	$F_{4,52}$ = 1.618; *P* = 0.184
Sedimentary factors	pH	$F_{2,51}$ = 0.0259; *P* = 0.974	**$F_{2,51}$ = 12.403; *P* < 0.001**	$F_{4,51}$ = 0.205; *P* = 0.935
	Conductivity (mS cm^{-1})	$F_{2,51}$ = 2.863; *P* = 0.066	**$F_{2,51}$ = 9.348; *P* < 0.001**	$F_{4,51}$ = 0.514; *P* = 0.726
	Water content (%)	$F_{2,51}$ = 0.151; *P* = 0.860	$F_{2,51}$ = 0.673; *P* = 0.514	$F_{4,51}$ = 1.356; *P* = 0.262
	Redox potential (mV)	$F_{2,51}$ = 0.761; *P* = 0.472	**$F_{2,51}$ = 12.827; *P* < 0.001**	$F_{4,51}$ = 2.188; *P* = 0.083
	Elevation (m above SHZ)	**$F_{2,51}$ = 16.070; *P* < 0.001**	**$F_{2,51}$ = 28.913; *P* < 0.001**	**$F_{4,51}$ = 6.626; *P* < 0.001**

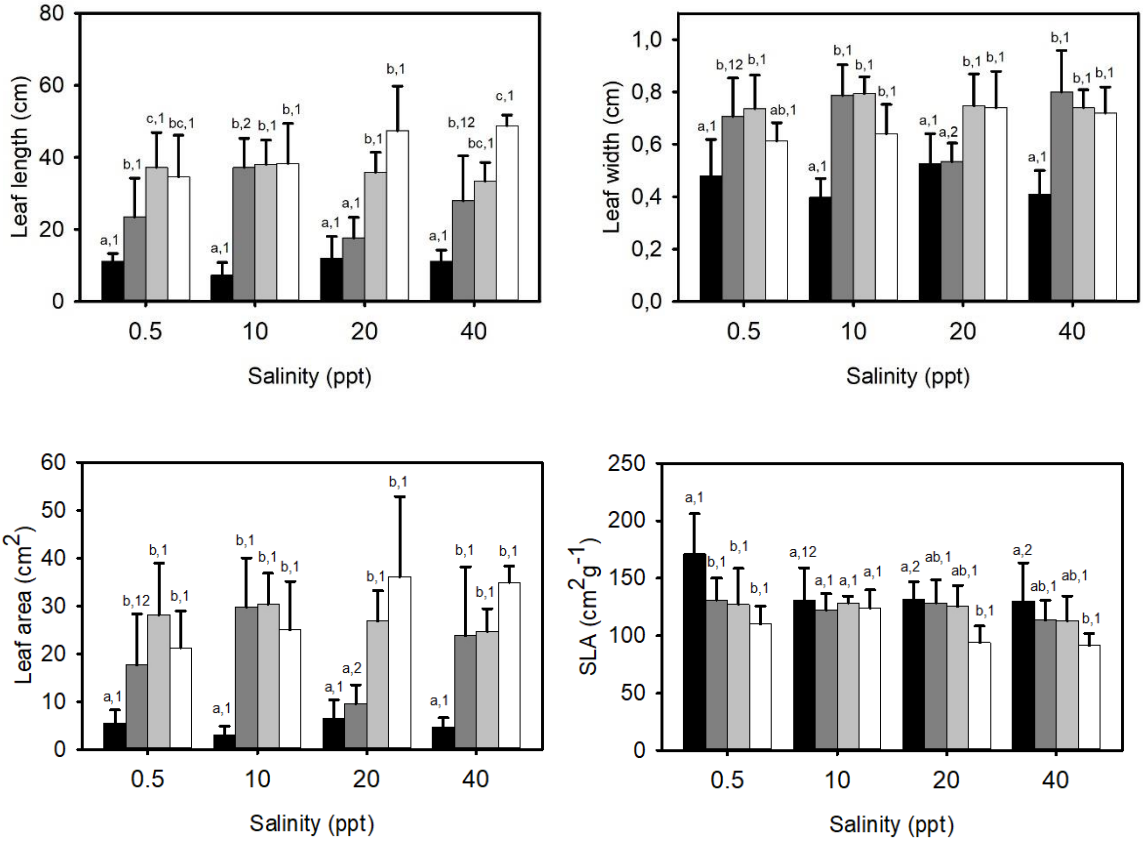
Appendix 4.A. A 0.5 g sample of fresh leaf tissue was homogenized in 10 ml of 3% sulfosalicylic acid and centrifuged at 13.000 rpm for 5 min. Two milliliters each of supernatant, acid-ninhydrin and glacial acetic acid were combined and boiled 1 h at 100 ºC in a bath. The reaction was stopped in ice and 2 ml of toluene was added to each sample. The upper toluene phase was obtained to read its absorbance at 517 nm on a spectrophotometer (Hitachi U-1900, Gemini BV, Güeldres, Netherlands) using toluene as a blank. The concentration of free proline was calculated from a standard curve of L-proline. Proportional weights and volumes were used when samples weights were lower than 0.5 g.

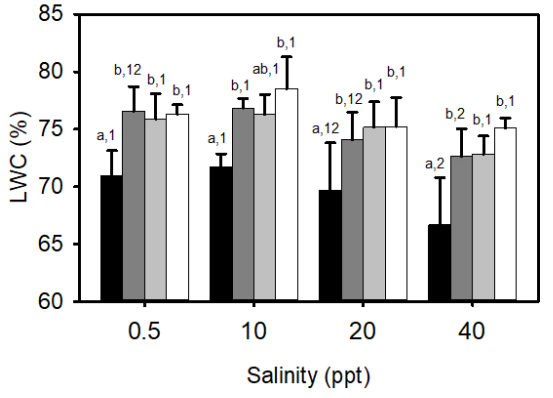
Appendix 4.B. A volume of 1 ml of 0.1% trichloroacetic acid (TCA) was added to 0.2 g of ground, frozen leaf samples. The homogenate was centrifuged for 5 min at 10,000g and 4ºC. An aliquot of the supernatant (0.4-0.5 ml) was mixed with the same volume of 20% TCA, 0.5% thiobarbituric acid (TBA), 0.01% butylated hydroxytoluene. The final volume of each sample was divided into 3 sub-replicates, heated at 95ºC in a bath for 60 min, and then cooled down immediately with ice to stop the reaction. The samples were centrifuged for 5 min at 10,000 g and the absorbance of the supernatant was measured at 532 nm for determination of MDA content and the non-specific absorption at 600 nm using a spectrophotometer (GeneQuant 1300, GE Healthcare, Little Chalfont, UK). MDA concentration ($\varepsilon = 155\ mM^{-1}\ cm^{-1}$)

Appendix 4.C. Leaf pigments were extracted using a proportion of 0.2 g of leaf FW (from one leaf per plant) and 5 ml aqueous acetone 80% and anthocyanins with a proportion of 0.2 g FW and 3 ml of 1% HCl / 80% methanol. The last extracts were incubated 24 h at 4 ºC. All extracts were centrifuged and supernatants were used for measurement of the photosynthetic pigments chlorophyll (Chl) *a*, Chl *b* and carotenoids (Car) (mg g-1 FW) at 647, 664 and 470 nm in a spectrophotometer (Hitachi U-1900, Gemini BV, Güeldres, Netherlands)

Appendix 4.D. Initial fluorescence (F_0) in the dark-adapted state was measured using a PPFD<0.05 μmol photon m^{-2} s^{-1} for 1.8 μs, too small to induce significant physiological changes in the plant. Maximal fluorescence (F_m) was recorded after a saturating light pulse of 15.000 μmol photon m^{-2} s^{-1}. Variable fluorescence ($F_v = F_m - F_0$) and maximum quantum efficiency of the Photosystem II (PSII) photochemistry (F_v/F_m) were calculated to quantify photoinhibition. The same leaf section of each leaf was used to measure light–adapted parameters. Steady state fluorescence yield (F_s) was recorded after adapting plants to ambient light conditions (with full sunlight of 1,150 μmol photon m^{-2} s^{-1}). A saturating actinic light pulse of 15,000 μmol photon m^{-2} s^{-1} for 0.7 s was then used to produce the maximum fluorescence yield (F_m') by temporarily inhibiting PSII photochemistry. Quantum efficiency of PSII ($\Phi PSII = (F_m' - F_s)/F_m'$] was calculated. Photochemical quenching (qP) and non-photochemical quenching (NPQ) were calculated from parameters obtained in both dark and light-adapted states.

Appendix 4.E. 37 foliar traits for *Spartina maritima*, *S. densiflora* and their two hybrids in 0.5, 10, 20 and 40 ppt salinity. *S. maritima* (black); *S. maritima x densiflora* (dark grey); *S. densiflora x maritima* (light grey); *S. densiflora* (white). Values are mean ± SD (n = 3- 5). Different letters indicate significant differences among taxa for the same salinity treatment; different numbers indicate significant differences among salinities for the same taxon (two-way ANOVA, salinity x taxa, $P<0.05$, n = 3-5). Traits: **1.** Leaf length **2.** Leaf width **3.** Leaf area **4.** Specific Leaf Area **5.** LWC **6.** Salt excretion rate **7.** Proline **8.** Malondialdehyde **9.** Leaf C **10.** Leaf N **11.** Leaf C:N **12.** Chl *a* **13.** Chl *b* **14.** Chl *a*+*b* **15.** Car **16.** Chl:Car **17.** Chl *a*: Chl *b* **18.** Anthocyanin **19.** qP (sunrise) **20.** NPQ (sunrise) **21.** F_0 (sunrise) **22.** F_v/F_m (sunrise) **23.** F_m (sunrise) **24.** ΦPSII (sunrise) **25.** qP (noon) **26.** NPQ (noon) **27.** F_0 (noon) **28.** F_v/F_m (noon) **29.** F_m (noon) **30.** ΦPSII (noon) **31.** Luminiscence **32.** A **33.** Gs **34.** Intercellular CO_2 concentration (Ci) **35.** WUE **36.** A_{max} **37.** Leaf apical growth.

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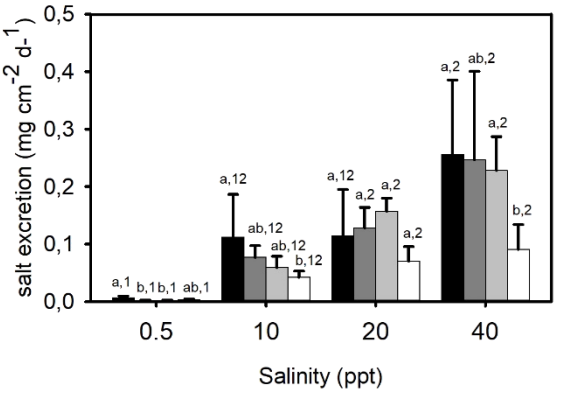
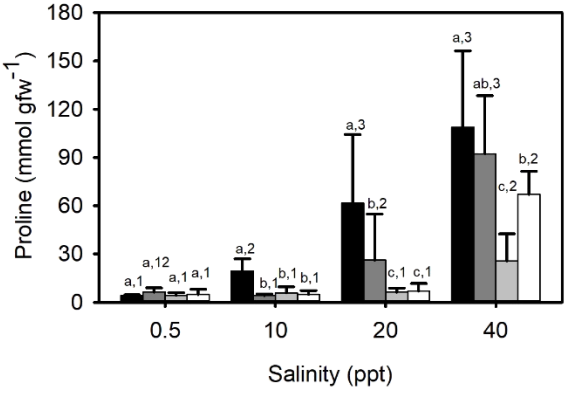
Salinity (ppt)


salt excretion (mg cm-2 d-1)
Salinity (ppt)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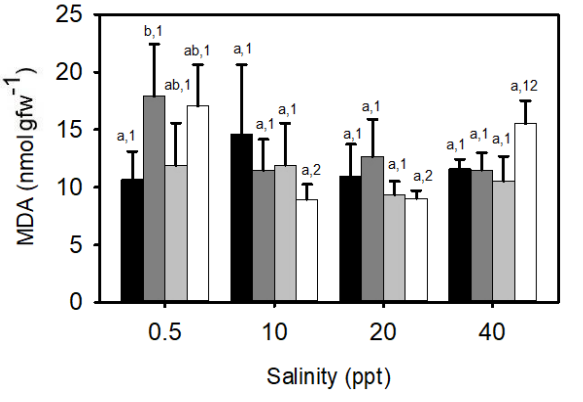
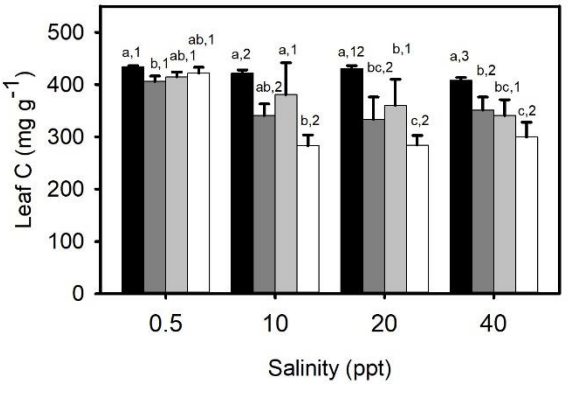
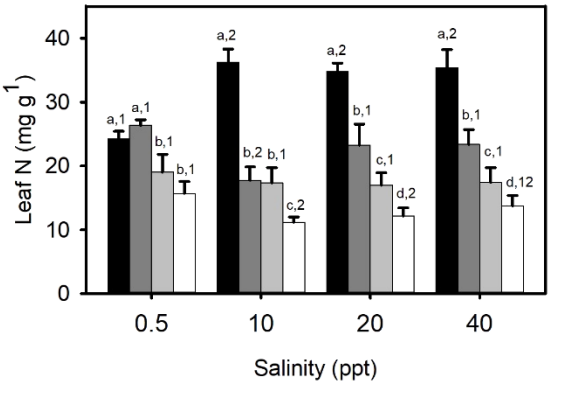
Salinity (ppt)


MDA (nmol gfw-1)
Salinity (ppt)


Leaf C (mg g-1)
Salinity (ppt)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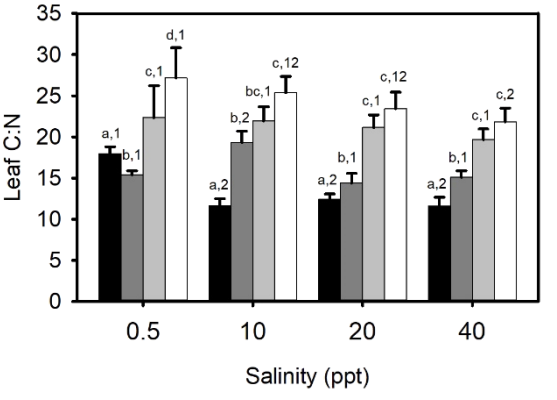
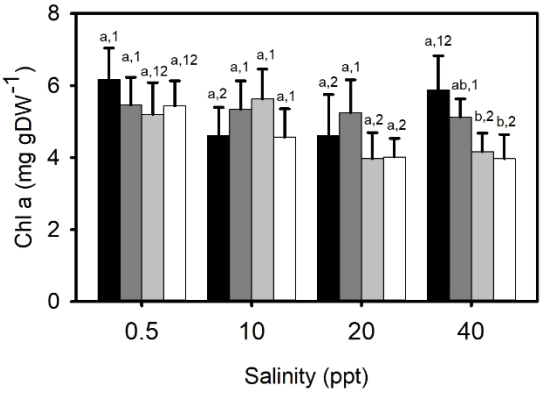
Salinity (ppt)


Leaf C:N
Salinity (ppt)


Chl a (mg gDW-1)
Salinity (ppt)
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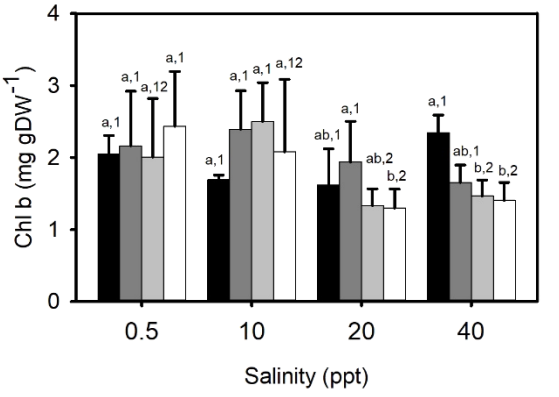
10
20
40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Salinity (ppt)
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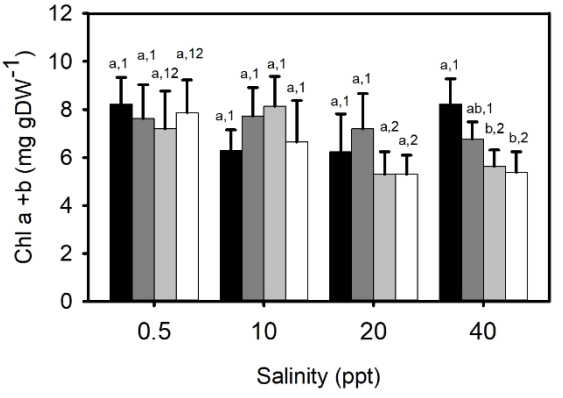
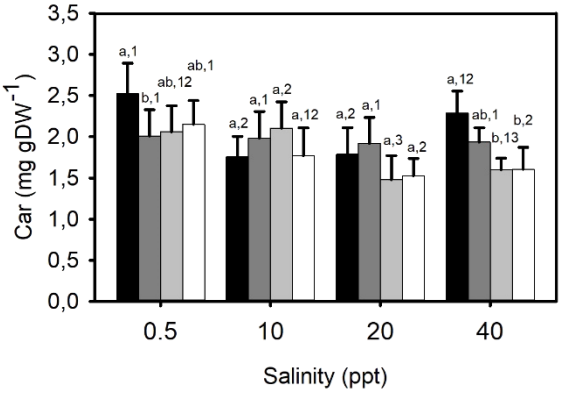
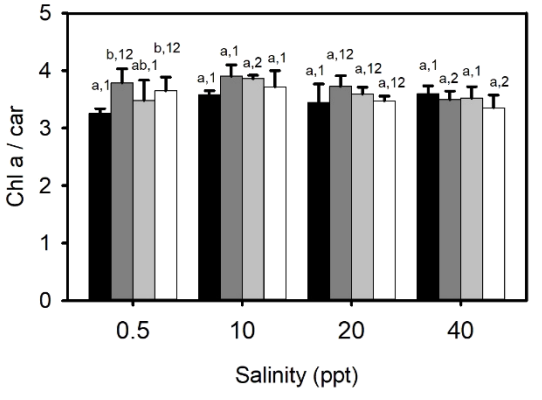
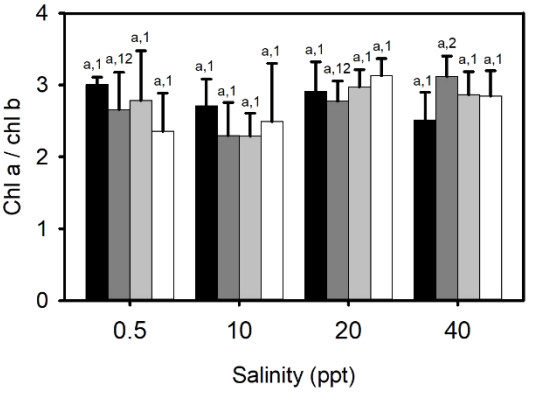
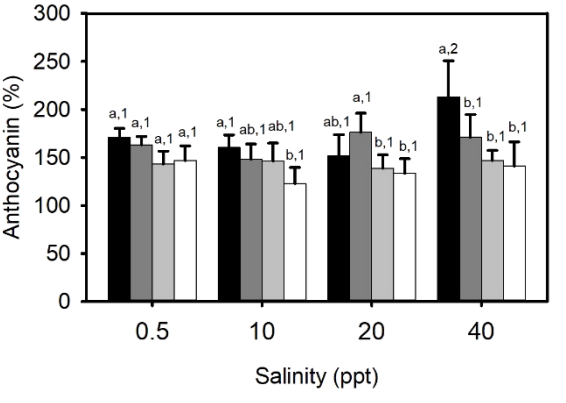
10
20
40

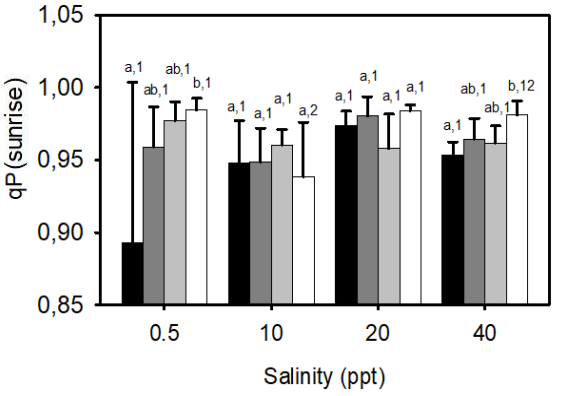

Chl a +b (mg gDW-1)
Salinity (ppt)
0.5
10
20
40

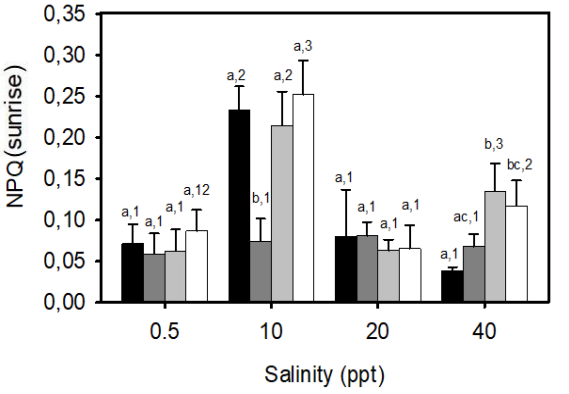

Car (mg gDW-1)
Salinity (ppt)
0.5
10
20
40

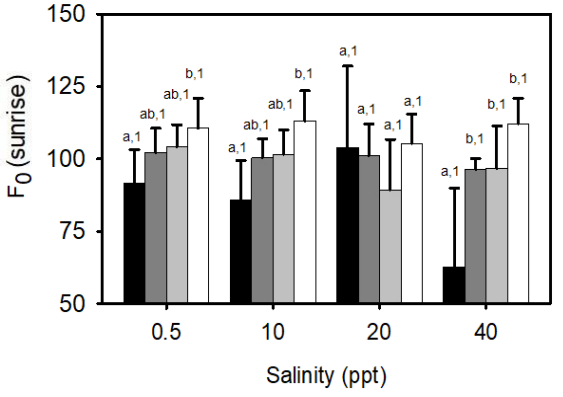

Chl a / car
Salinity (ppt)
0.5
10
20
40

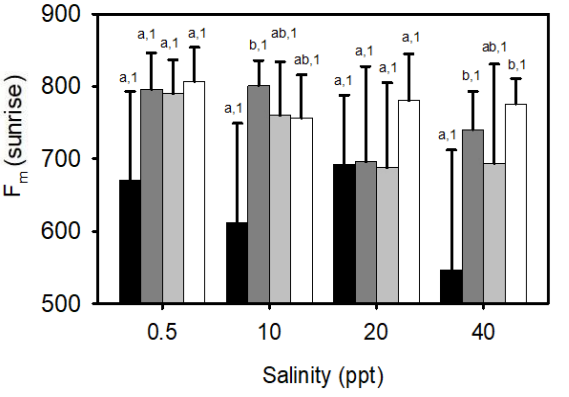

Chl a / chl b
Salinity (ppt)
0.5
10
20
40

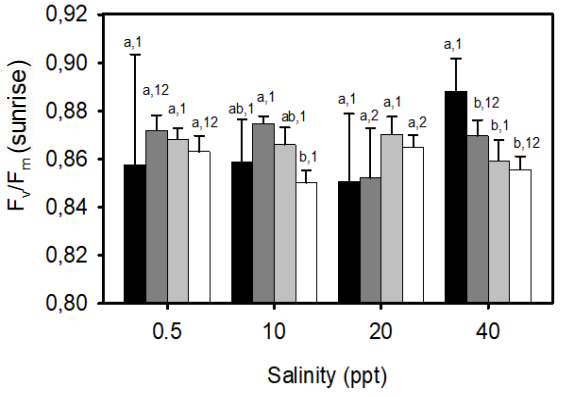

Anthocyanin (%)
Salinity (ppt)
0.5
10
20
40

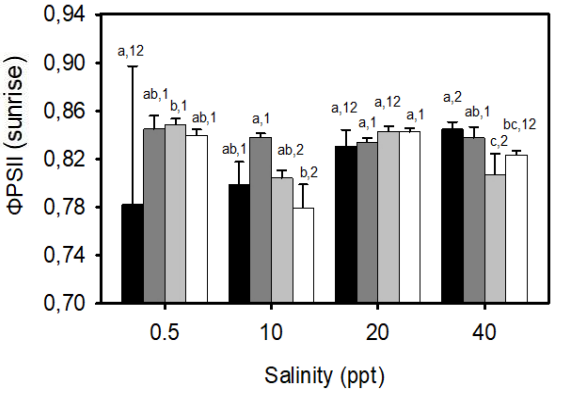

qP (sunrise)
1,05
1,00
0,95
0,90
0,85
0.5
10
20
40
Salinity (ppt)

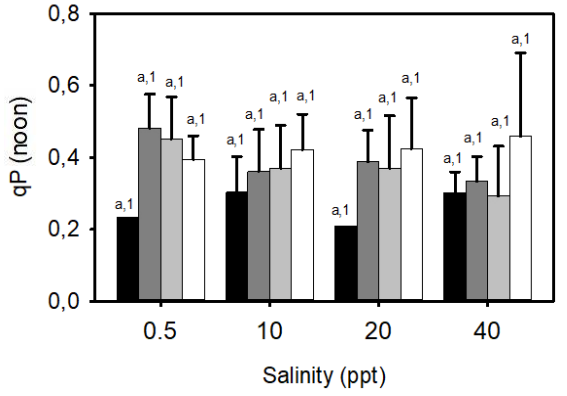

NPQ (sunrise)
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
0.5
10
20
40
Salinity (ppt)

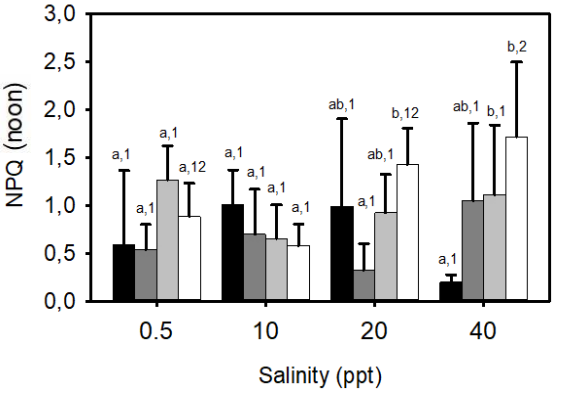

F0 (sunrise)
150
125
100
75
50
0.5
10
20
40
Salinity (ppt)

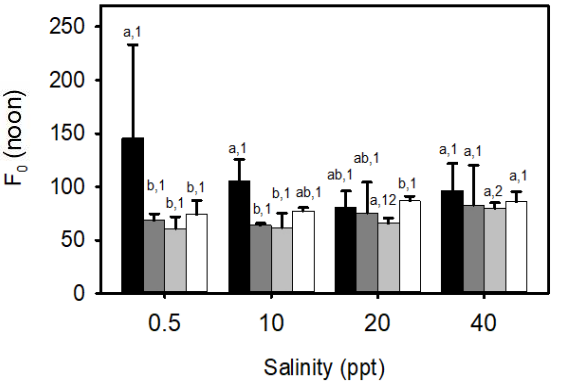

Fm (sunrise)
900
800
700
600
500
0.5
10
20
40
Salinity (ppt)

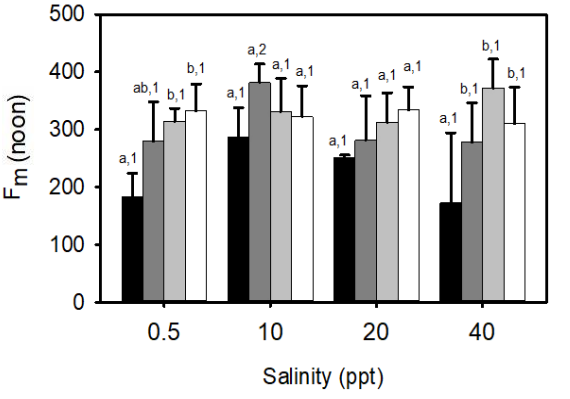

Fv/Fm (sunrise)
0,92
0,90
0,88
0,86
0,84
0,82
0,80
0.5
10
20
40
Salinity (ppt)

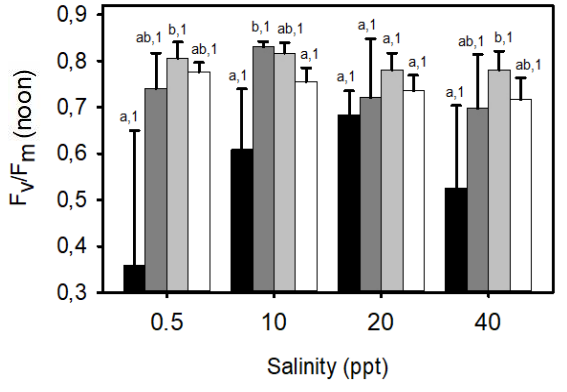

ΦPSII (sunrise)
0,94
0,90
0,86
0,82
0,78
0,74
0,70
0.5
10
20
40
Salinity (ppt)

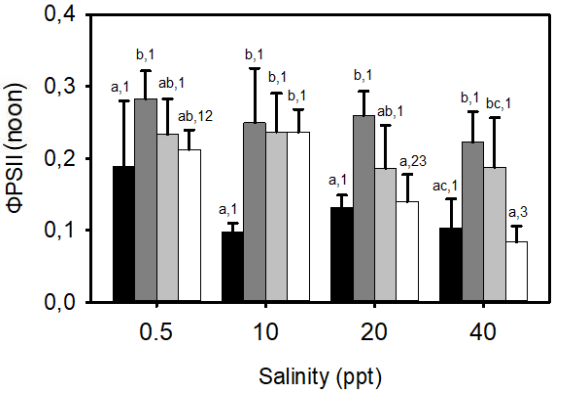

qP (noon)
0,0
0,2
0,4
0,6
0,8
0.5
10
20
40
Salinity (ppt)

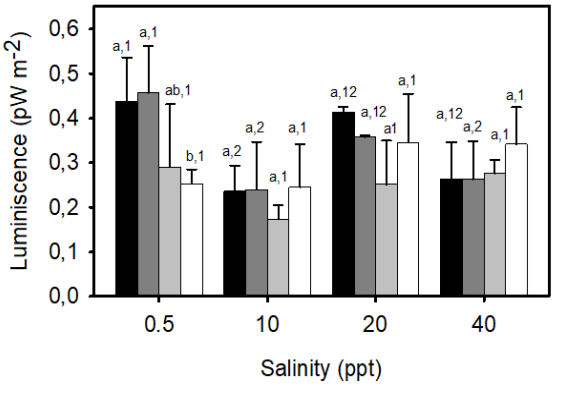

NPQ (noon)
0,0
0,5
1,0
1,5
2,0
2,5
3,0
0.5
10
20
40
Salinity (ppt)

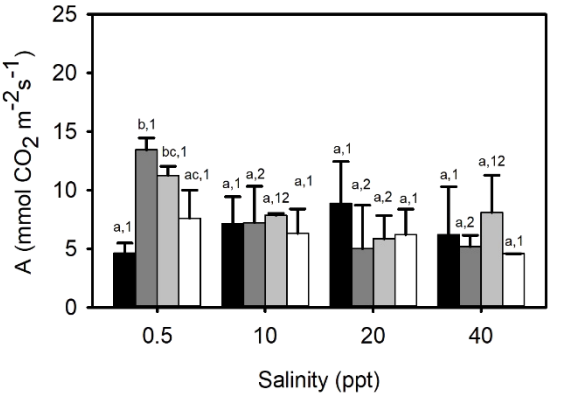

F0 (noon)
0
50
100
150
200
250
0.5
10
20
40
Salinity (ppt)

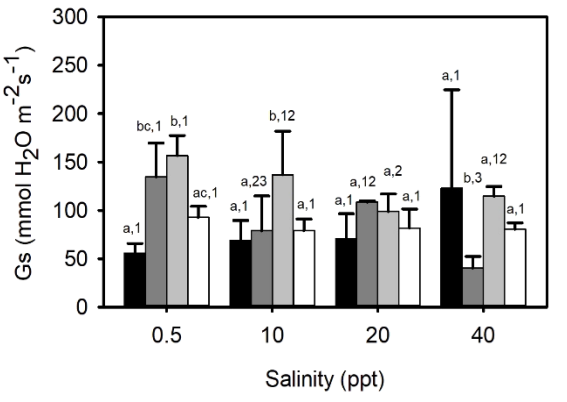

Fm (noon)
0
100
200
300
400
500
0.5
10
20
40
Salinity (ppt)

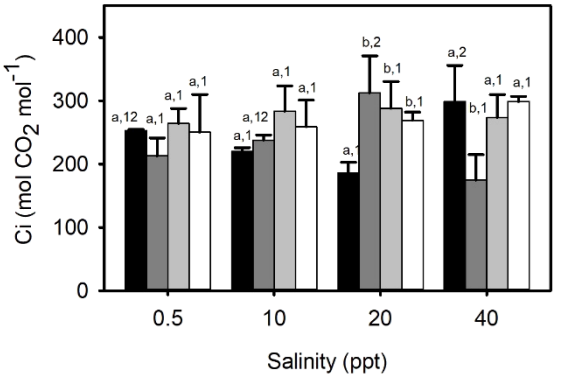

Fv/Fm (noon)
0,3
0,4
0,5
0,6
0,7
0,8
0,9
0.5
10
20
40
Salinity (ppt)

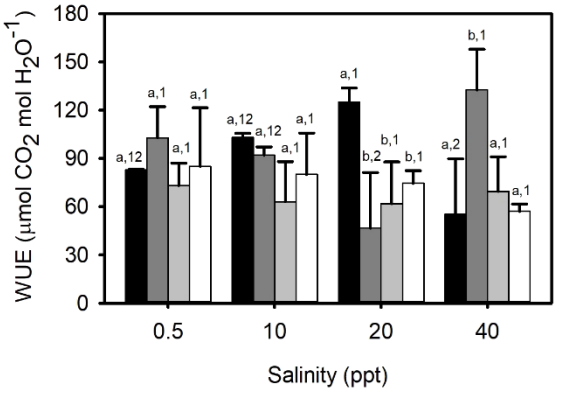

ΦPSII (noon)
0,0
0,1
0,2
0,3
0,4
0.5
10
20
40
Salinity (ppt)

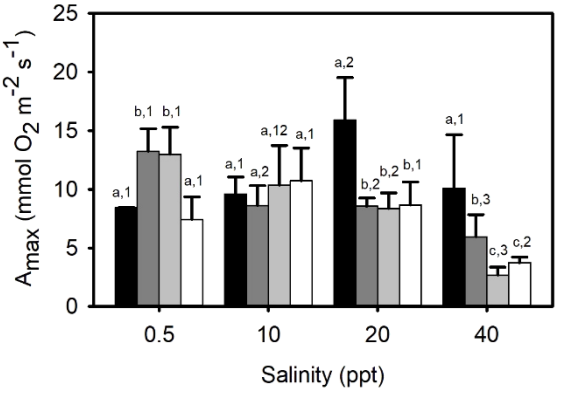

Luminiscence (pW m-2)
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0.5
10
20
40
Salinity (ppt)

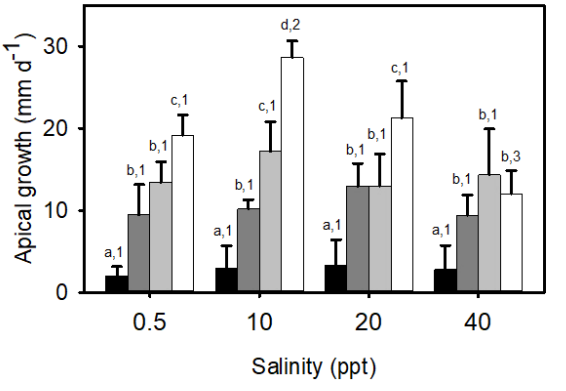

A (mmol CO_2 $m^{-2}s^{-1}$)
Salinity (ppt)
0.5
10
20
40


Gs (mmol H_2O $m^{-2}s^{-1}$)
Salinity (ppt)
0.5
10
20
40


Ci (mol CO_2 mol^{-1})
Salinity (ppt)
0.5
10
20
40


WUE (µmol CO_2 mol H_2O^{-1})
Salinity (ppt)
0.5
10
20
40


A_{max} (mmol O_2 m^{-2} s^{-1})
Salinity (ppt)
0.5
10
20
40


Apical growth (mm d^{-1})
Salinity (ppt)
0.5
10
20
40

Appendix 4.F. Inheritance mechanisms for the hybrids *Spartina maritima x densiflora* (Smxd) and *S. densiflora x maritima* (Sdxm) for 37 foliar traits in 6 different categories: (1) leaf morphological traits, (2) leaf biochemistry and salt excretion, (3) pigment contents, (4) chlorophyll fluorescence, (5) gas exchange, and (6) growth at 0.5, 10, 20 and 40 ppt salinity. Parental species: *S. maritima* (Sm); *S. densiflora* (Sd). Inheritance mechanisms: parental dominance (D; Sd, orange; Sm, green); parental additivity (I; white); transgressive (T; red). The number of individuals with transgressive trait is indicated in brackets (two-way ANOVA, salinity x taxa, $P < 0.05$, $n = 3$-5).

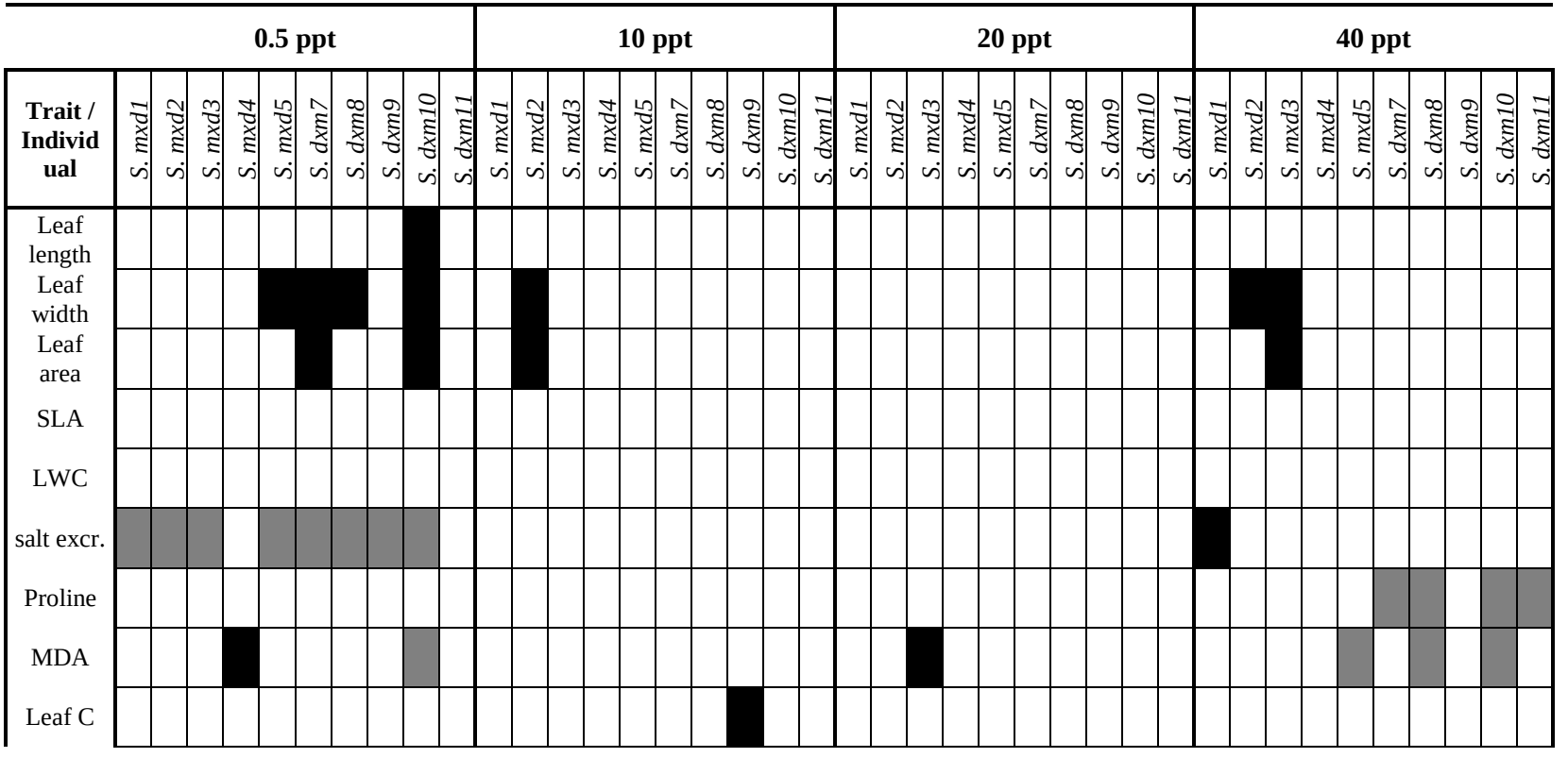
Trait / Salinity	0.5 ppt		10 ppt		20 ppt		40 ppt		ANOVA
	S.mxd	*S.dxm*	*S.mxd*	*S.dxm*	*S.mxd*	*S.dxm*	*S.mxd*	*S.dxm*	
Leaf morphological traits									
Leaf length	D - *Sd*	D - *Sd* (1)	D - *Sd*	D - *Sd*	D - *Sm*	D - *Sd*	I	D - *Sd*	Taxa, F = 74.50 P< 0.001 Treatment, n.s Interaction, F = 3.04 P< 0.001
Leaf width	D - *Sd* (1)	D - *Sd* (3)	D - *Sd* (1)	D - *Sd*	D - *Sm*	D - *Sd*	D - *Sd* (2)	D - *Sd*	Taxa, F = 29.07 P< 0.001 Treatment,n.s Interaction, F = 3.01 P< 0.005
Leaf area	D - *Sd*	D - *Sd* (2)	D - *Sd* (1)	D - *Sd*	D - *Sm*	D - *Sd*	D - *Sd* (1)	D - *Sd*	Taxa, F = 57.59 P< 0.001 Treatment, n.s Interaction, F = 3.343 P< 0.001
SLA	D - *Sd*	D - *Sd*	D - *SdSm*	D - *SdSm*	I	I	I	I	Taxa, F = 9.11 P< 0.001 Treatment, F = 4.18 P< 0.01 Interaction, n.s
Some leaf contents and salt excretion									
LWC	D - *Sd*	D - *Sd*	D - *Sd*	I	D - *Sd*	D - *Sd*	D - *Sd*	D - *Sd*	Taxa, F = 26.63 P< 0.001 Treatment, F = 10.03 P< 0.001 Interaction, n.s
Salt excretion rate	D - *Sd* (4)	D - *Sd* (4)	I	I	D - *SdSm*	D - *SdSm*	I (1)	D - *Sm*	Taxa, n.s Treatment, H = 55.88 P< 0.001
Proline	D - *SdSm*	D - *SdSm*	D - *Sd*	D - *Sd*	I	D - *Sd*	I	T (4)	Taxa, F = 21.51 P< 0.001 Treatment, F = 97.44 P< 0.001 Interaction, F = 5.45 P< 0.001
MDA (malondialdehyde)	D - *Sd* (1)	D - *SdSm* (1)	D - *SdSm*	D - *SdSm*	D - *SdSm* (1)	D - *SdSm*	D - *SdSm* (1)	D - *SdSm* (2)	Taxa, n.s Treatment, F = 3.55 P< 0.05

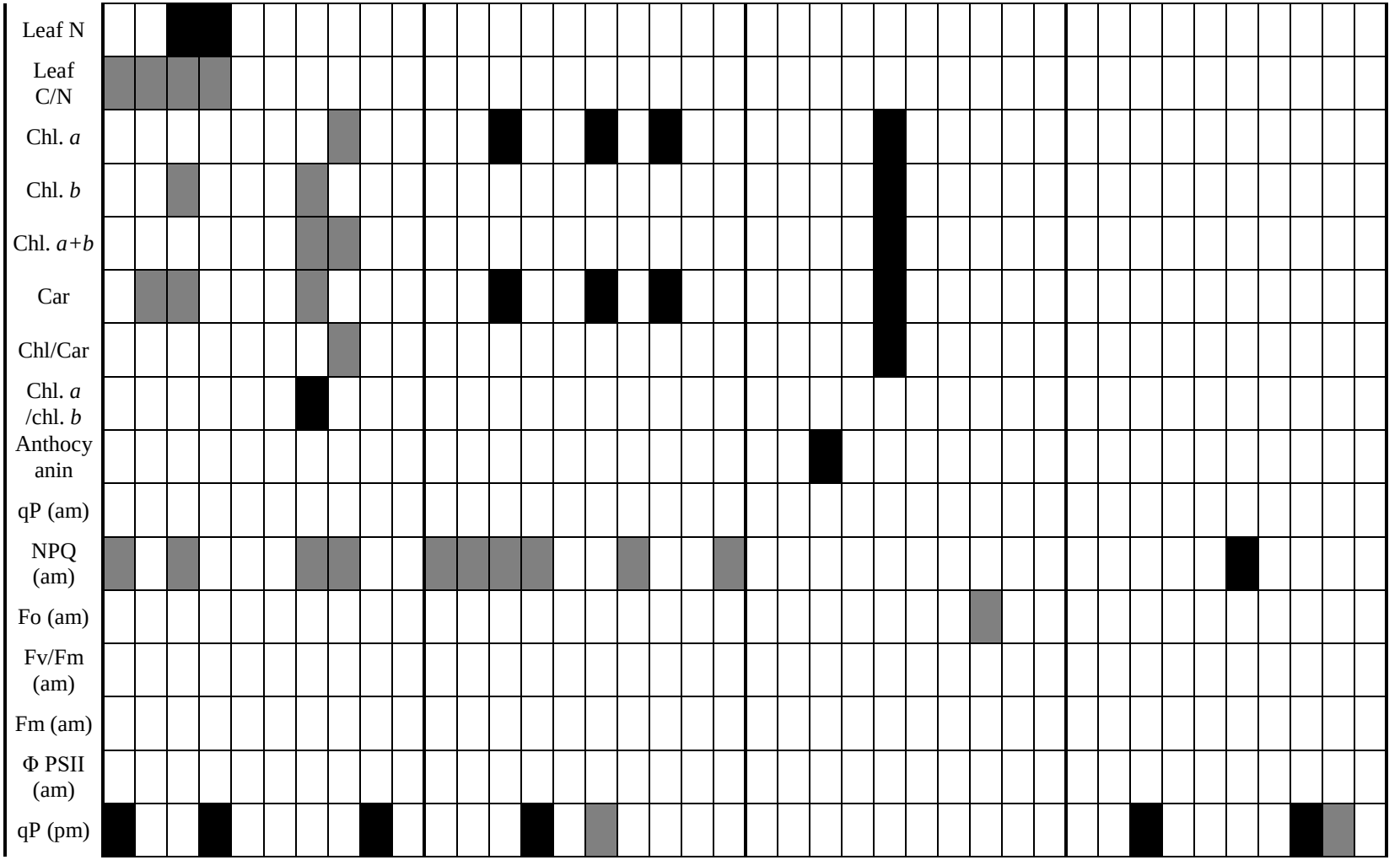
									Interaction, F = 2.46 P< 0.05
Leaf Carbon	D - *Sd*	D - *SdSm*	I	D - *Sm* (1)	D-*Sd*	D-*Sm*	I	D-*Sd*	Taxa, H = 29.07 P< 0.001 Treatment, H = 22.84 P< 0.001
Leaf Nitrogen	D - *Sm* (2)	D - *Sd*	I	I	I	I	I	I	Taxa, F = 264.82 P< 0.001 Treatment, n.s Interaction, F = 15.85 P< 0.001
Leaf C/N	T (4)	I	I	D - *Sd*	I	D - *Sd*	I	D - *Sd*	Taxa, F = 226.05 P< 0.001 Treatment, F = 24.72 P< 0.001 Interaction, F = 14.26 P< 0.001
Pigments contents									
Chl. a	D - *SdSm*	D - *SdSm* (1)	D - *SdSm* (1)	D - *SdSm* (2)	D - *SdSm* (1)	D - *SdSm*	I	D - *Sd*	Taxa, F = 5.28 P< 0.01 Treatment, F = 6.99 P< 0.001 Interaction, F = 2.34 P< 0.05
Chl. b	D - *SdSm* (1)	D - *SdSm* (1)	D - *SdSm*	D - *SdSm*	D - *Sm* (1)	I	I	D - *Sd*	Taxa, n.s Treatment, F = 8.56 P< 0.001 Interaction, F = 2.29 P< 0.05
Chl. a + b	D - *SdSm*	D - *SdSm* (2)	D - *SdSm*	D - *SdSm*	D - *SdSm* (1)	D - *SdSm*	I	D - *Sd*	Taxa, F = 3.41 P< 0.05 Treatment, F = 7.68 P< 0.001 Interaction, F = 2.49 P< 0.05
Carotenoids	D - *Sd* (2)	D - *SdSm* (1)	D - *SdSm* (1)	D - *SdSm* (2)	D - *SdSm* (1)	D - *SdSm*	I	D - *Sd*	Taxa, F = 4.85 P< 0.01 Treatment, F = 10.38 P< 0.001 Interaction, F = 2.68 P< 0.05
Chl/Car	D - *Sd*	I (1)	D - *SdSm*	D - *SdSm*	D - *SdSm* (1)	D - *SdSm*	D - *SdSm*	D - *SdSm*	Taxa, F = 5.41 P< 0.01 Treatment, F = 6.70 P< 0.001 Interaction, n.s
Chl. a/Chl. b	D - *SdSm*	D - *SdSm* (1)	D - *SdSm*	D - *SdSm*	D - *SdSm*	D - *SdSm*	D - *SdSm*	D - *SdSm*	Taxa, n.s Treatment, H = 11.09 P< 0.01
Anthocyanins	D - *SdSm*	D - *SdSm*	I	I	D - *Sm* (1)	D - *Sd*	D - *Sd*	D - *Sd*	Taxa, F = 17.62 P< 0.001 Treatment, F = 5.74 P< 0.01 Interaction, F = 2.182 P< 0.01

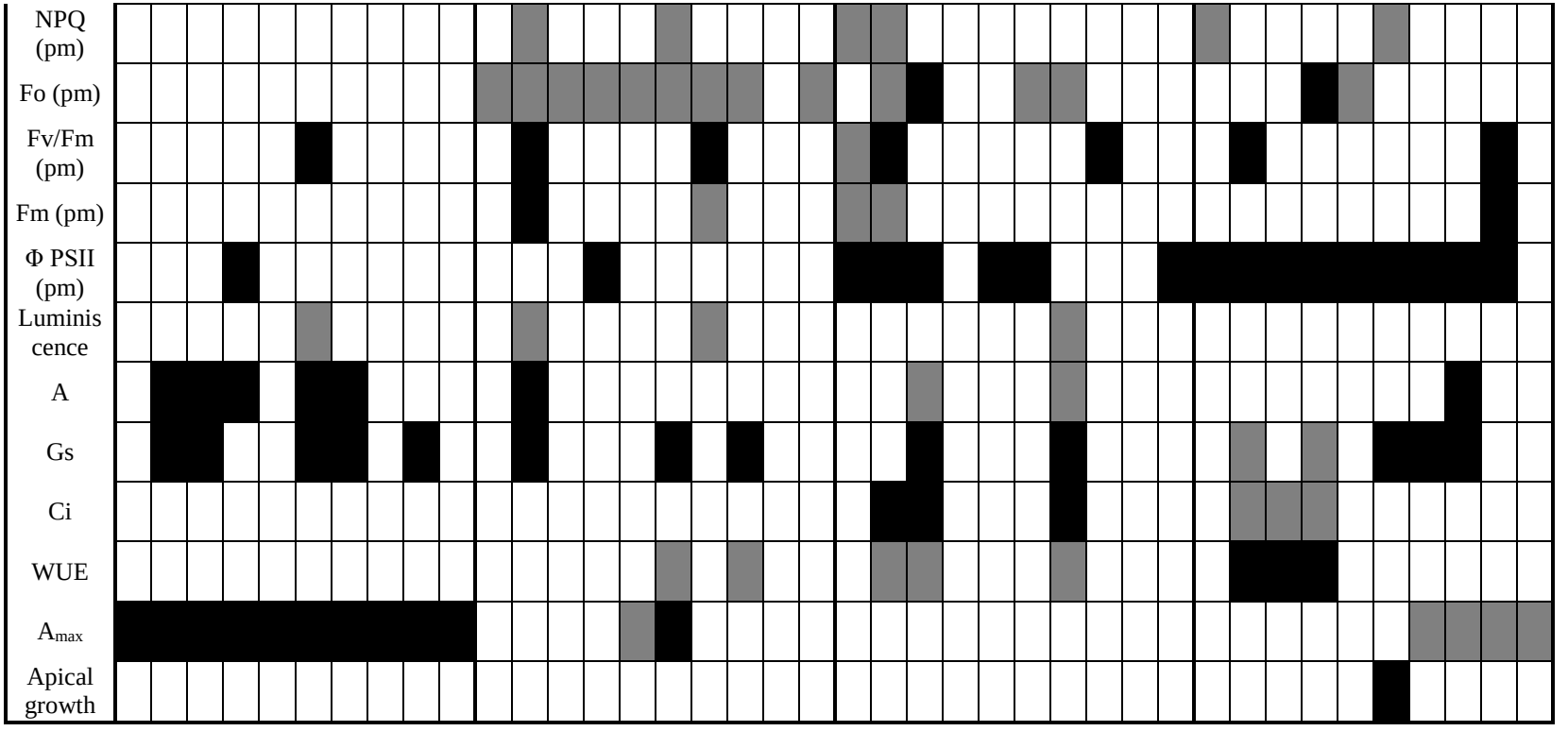
Chlorophyll fluorescence									
qP (sunrise)	I	I	D - *SdSm*	D - *SdSm*	D - *SdSm*	D - *SdSm*	I	I	Taxa, H = 11.68 P< 0.01 Treatment, H = 14.50 P< 0.01
NPQ (sunrise)	D - *SdSm* (2)	D - *SdSm* (2)	T (4)	D - *SdSm* (2)	D - *SdSm*	D - *SdSm*	I	D - *Sd* (1)	Taxa,F = 12.94 P< 0.001 Treatment, F = 55.51 P< 0.001 Interaction, F = 8.19 P< 0.001
Fo (sunrise)	I	I	I	I	D - *SdSm*	D - *SdSm* (1)	D - *Sd*	D - *Sd*	Taxa, H = 24.82 P< 0.001 Treatment, n.s
Fm (sunrise)	D - *SdSm*	D - *SdSm*	D - *Sd*	D - *SdSm*	D - *SdSm*	D - *SdSm*	D - *Sd*	I	Taxa, F = 8.89 P< 0.001 Treatment, n.s Interaction, n.s
Fv/Fm (sunrise)	D - *SdSm*	D - *SdSm*	D - *Sm*	D - *SdSm*	D - *SdSm*	D - *SdSm*	D - *Sd*	D - *Sd*	Taxa, H = 12.88 P< 0.01 Treatment, n.s
PS2 (sunrise)	D - *SdSm*	D - *Sd*	D - *Sm*	D - *SdSm*	D - *SdSm*	D - *SdSm*	I	D - *Sd*	Taxa, n.s Treatment, F = 25.65 P< 0.001
qP (noon)	D - *SdSm* (2)	D - *SdSm* (1)	D - *SdSm* (1)	D - *SdSm* (1)	D - *SdSm*	D - *SdSm*	D - *SdSm* (1)	D - *SdSm* (1) (1)	Taxa, H = 9.53 P< 0.05 Treatment, n.s
NPQ (noon)	D - *SdSm*	D - *SdSm*	D - *SdSm* (1)	D - *SdSm* (1)	D - *Sm* (2)	D - *SdSm*	I (1)	D - *Sd* (1)	Taxa, F = 4.11 P< 0.05 Treatment, n.s Interaction, F = 2.86 P< 0.05
Fo (noon)	D - *Sd*	D - *Sd*	D - *Sd* (5)	D - *Sd* (4)	D - *SdSm* (1) (1)	D - *Sm* (2)	D - *SdSm* (1) (1)	D - *SdSm*	Taxa, H = 27.23 P< 0.001 Treatment, n.s
Fm (noon)	I	D - *Sd*	D - *SdSm* (1)	D - *SdSm* (1)	D - *SdSm* (2)	D - *SdSm*	D - *Sd*	D - *Sd* (1)	Taxa, F = 9.83 P< 0.001 Treatment, F = 2.89 P< 0.05 Interaction, n.s
Fv/Fm (noon)	D - *SdSm*	D - *Sd* (1)	T (1)	D - *SdSm* (1)	D - *SdSm* (1) (1)	D - *SdSm* (1)	D - *SdSm* (1)	D - *Sd* (1)	Taxa, H = 35.12 P< 0.001 Treatment, n.s
PS2 (noon)	D - *Sd* (1)	D - *SdSm*	D - *Sd* (1)	D - *Sd*	T (4)	D - *SdSm* (2)	T (5)	D - *Sm* (4)	Taxa, F = 20.01 P< 0.001 Treatment, F = 9.07 P< 0.001 Interaction, F = 2.00 P= 0.055
Luminiscence	D - *Sm*	I (1)	D - *SdSm* (1)	D - *SdSm* (1)	D - *SdSm*	D - *SdSm* (1)	D - *SdSm*	D - *SdSm*	Taxa, F = 2.95 P< 0.05 Treatment, F = 6.11 P<

								0.01 Interaction, n.s
Gas exchange								
A	T **(3)**	D - *Sd* (2)	D - *SdSm* (1)	D - *SdSm*	D - *SdSm* **(1)**	D - *SdSm* **(1)**	D - *SdSm*	D - *SdSm* (1)	Taxa, n.s Treatment, F = 4.30 P< 0.05 Interaction, F = 3.04 P< 0.05
Gs	D - *Sd* (2)	T **(3)**	D - *SdSm* (1)	T **(2)**	D - *SdSm* (1)	D - *SdSm* (1)	T (2)	D - *SdSm*	Taxa, F = 6,95 P< 0.001 Treatment, n.s Interaction, F = 3.35 P< 0.01
Ci	D - *SdSm*	D - *SdSm*	D - *SdSm*	D - *SdSm*	D - *Sd* **(2)**	D - *Sd* (1)	T **(3)**	D - *SdSm*	Taxa, F = 4.31 P< 0.05 Treatment, n.s Interaction, F = 4.67 P< 0.001
WUE	D - *SdSm*	D - *SdSm*	D - *SdSm*	D - *SdSm* **(2)**	D - *Sd* **(2)**	D - *Sd* **(1)**	T **(3)**	D - *SdSm*	Taxa, F = 4.34 P< 0.05 Treatment, n.s Interaction, F = 4.78 P< 0.001
A max	T **(5)**	T **(5)**	D - *SdSm* **(1)**	D - *SdSm* (1)	D - *Sd*	D - *Sd*	I	D - *Sd* **(4)**	Taxa, F = 11.69 P< 0.001 Treatment, F = 46.63 P< 0.001 Interaction, F = 10.69 P< 0.001
Growth									
Apical growth	I	I	I	I	I	I	D - *Sd*	D - *Sd* (1)	Taxa, F = 95.69 P< 0.001 Treatment, F = 8.86 P< 0.001 Interaction, F = 4.96 P< 0.001

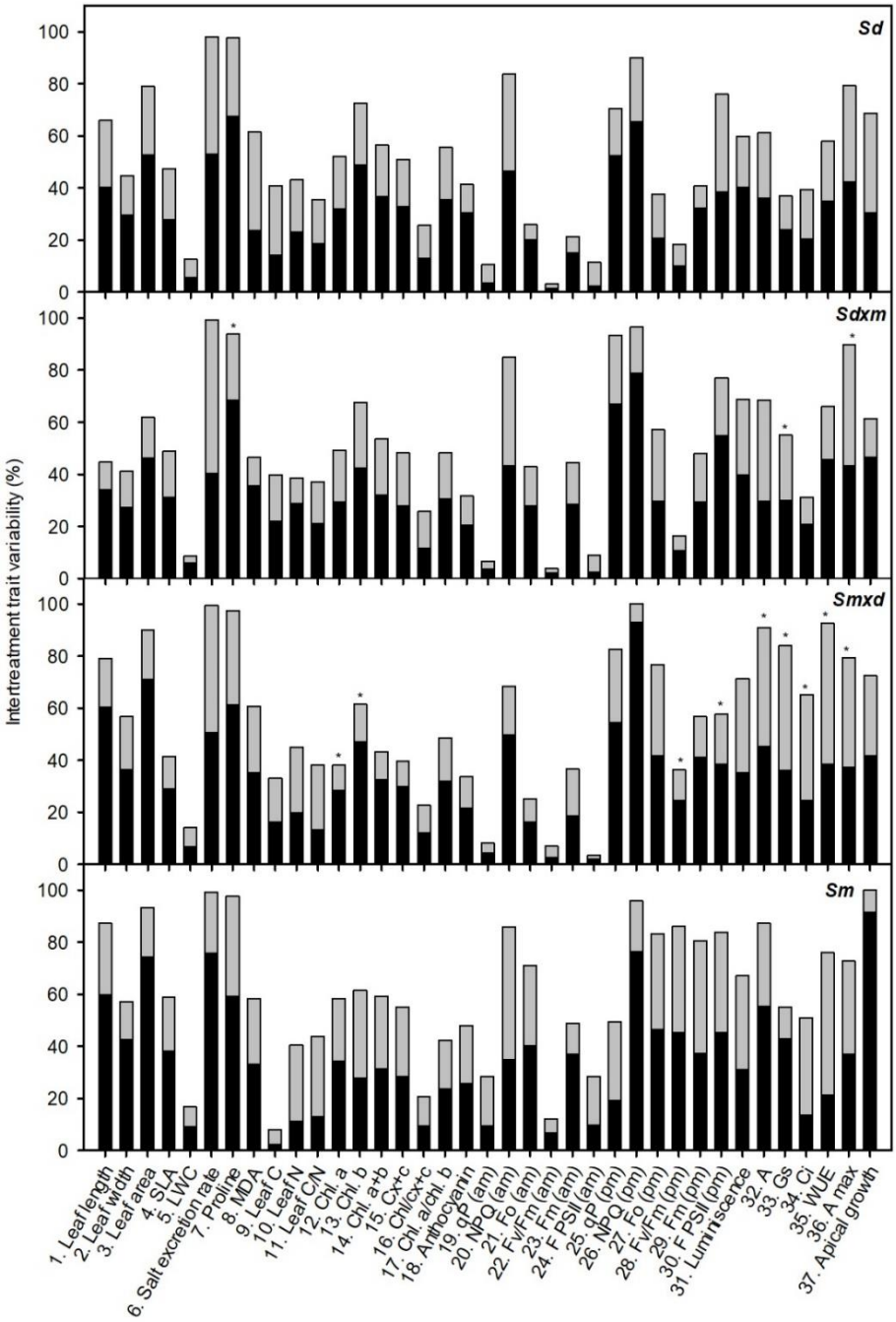
Appendix 4.G. Transgressive profile of *Spartina maritima x densiflora* (Smxd) and *S. densiflora x maritima* (Sdxm) individuals (n = 5) at 0.5, 10, 20 and 40 ppt salinity for 37 foliar traits. Black, values over maximum values of parental species; Grey, values below minimum values of parental species. The total number of transgressive individuals for a given trait and the total number of transgressive traits for a given individual are indicated. Am = measured at sunrise, pm = measured at noon.



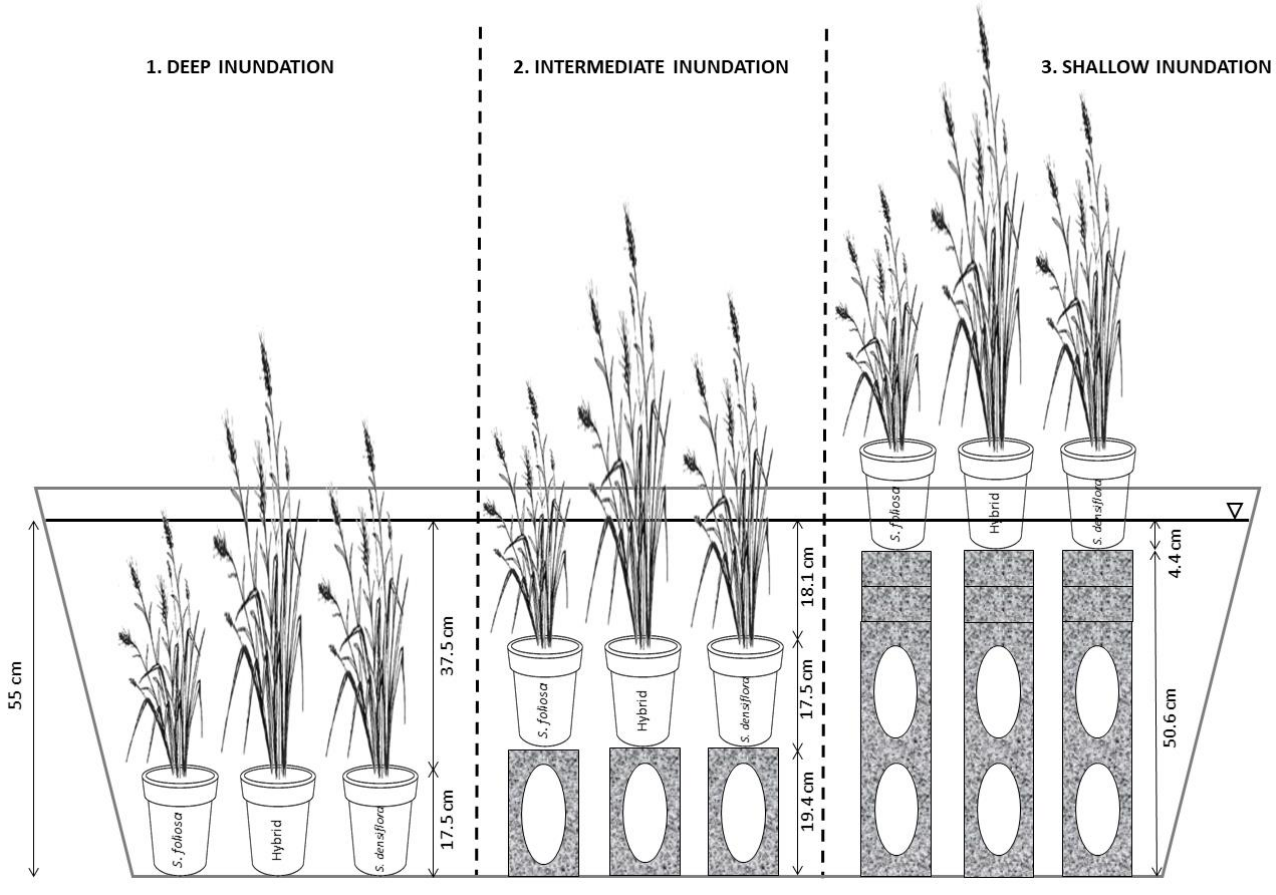

Leaf N
Leaf C/N
Chl. a
Chl. b
Chl. a+b
Car
Chl/Car
Chl. a /chl. b
Anthocy anin
qP (am)
NPQ (am)
Fo (am)
Fv/Fm (am)
Fm (am)
Φ PSII (am)
qP (pm)


NPQ (pm)
Fo (pm)
Fv/Fm (pm)
Fm (pm)
Φ PSII (pm)
Luminis cence
A
Gs
Ci
WUE
A_{max}
Apical growth

Appendix 4.H. Intrapopulation trait variability (black), phenotypic plasticity (gray) and interpopulation trait variability (bar length) for 37 foliar traits measured in *Spartina maritima* (Sm), *S. densiflora* (Sd) and their hybrids *S. maritima x densiflora* (Smxd) and *S. densiflora x maritima* (Sdxm) in 0.5, 10, 20 and 40 ppt salinity. The traits with a transgressive behavior at the population level are marked with an asterisk.



Appendix 6.A. Design of the 16 tanks (4 salinity treatments x 4 replicates) with the arrangement of the different inundation levels (SI, shallow inundation (4.4 cm deep); II, intermediate inundation (35.5 cm deep); DI, deep inundation (55.0 cm deep)) for *S. foliosa*, *S. densiflora* and *S. densiflora x foliosa* individuals. Plant heights are scaled using the average initial heights of the three species. The comparison of the results of the experiment in the hybrid in relation to the parents is shown in Chapter 7.



Appendix 6.B. Display of the experiment in the greenhouse (A) on the first day after setting up the experimental conditions of salinity and inundation and (B) 31 days later when the measurements of the response variables and the collection of the plant material began.

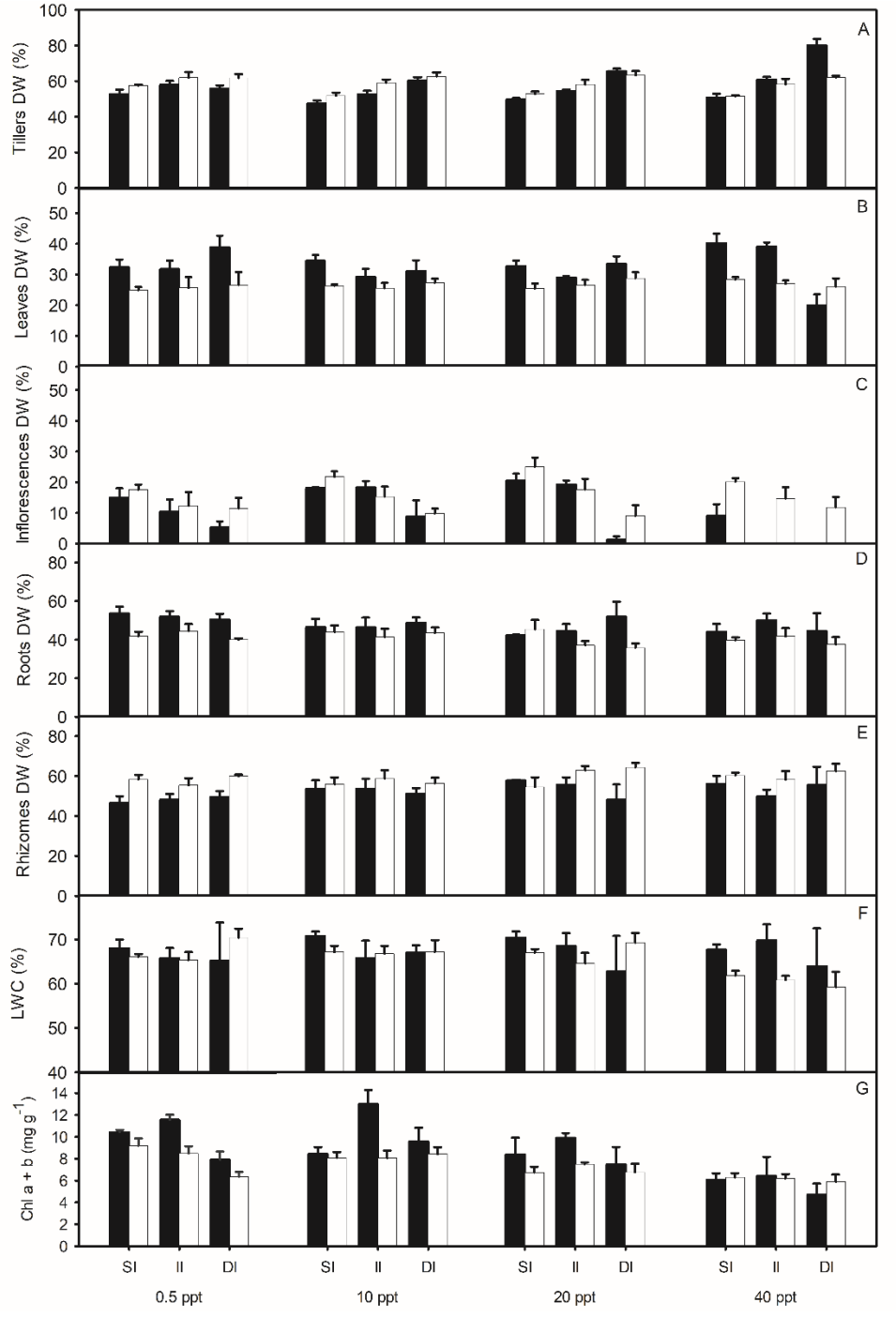
Appendix 6.C. F-statistics and Pillai's trace from MANOVAs for seven trait response groups for the factors species (*Spartina foliosa* and *S. densiflora*), salinity (0.5, 10, 20 and 40 ppt) and inundation depth (4.4, 35.5 and 55.0 cm deep) and their interactions, including main effect and model degrees of freedom (DF). Significant values ($p < 0.05$) are marked in bold.

MANOVA

	Factors	Pillai's Trace	F	DF	*p*
Biomass allocation and growth	Salinity	1.13	3.34	33,183	**< 0.0001**
	Species	0.86	34.32	11,59	**< 0.0001**
	Inundation	1.01	5.56	22,12	**< 0.0001**
	Salinity * Inundation	1.36	1.71	66,384	**< 0.01**
	Species * Inundation	0.71	2.99	22,12	**< 0.0001**
	Salinity * Species	0.90	2.37	33,183	**< 0.001**
	Salinity * Species * Inundation	1.19	1.44	66,384	**< 0.05**
Leaf morphology	Salinity	0.70	6.48	9,192	**< 0.0001**
	Species	0.35	10.89	3,62	**< 0.0001**
	Inundation	0.44	5.91	6,126	**< 0.0001**
	Salinity * Inundation	0.48	2.01	18,192	**< 0.05**
	Species * Inundation	0.03	0.32	6,126	0.93
	Salinity * Species	0.12	0.89	9,192	0.54
	Salinity * Species * Inundation	0.23	0.87	18,192	0.62
Leaf pigments, C and N content	Salinity	1.43	7.52	24,198	**< 0.0001**
	Species	0.78	28.14	8,64	**< 0.0001**
	Inundation	0.86	6.08	16,130	**< 0.0001**
	Salinity * Inundation	0.88	1.48	48,414	**< 0.05**
	Species * Inundation	0.44	2.31	16,130	**< 0.01**
	Salinity * Species	0.68	2.43	24,198	**< 0.001**
	Salinity * Species * Inundation	0.72	1.18	48,414	0.20
Subterranean resource storage	Salinity	0.86	5.59	15,210	**< 0.0001**
	Species	0.97	404.55	5,68	**< 0.0001**
	Inundation	0.59	5.74	10,138	**< 0.0001**
	Salinity * Inundation	0.69	1.92	30,360	**< 0.01**
	Species * Inundation	0.53	4.94	10,138	**< 0.0001**
	Salinity * Species	0.70	4.27	15,210	**< 0.0001**
	Salinity * Species * Inundation	0.47	1.25	30,360	0.18
Root and rhizome porosity	Salinity	0.09	1.15	6,144	0.34
	Species	0.58	48.94	2,71	**< 0.0001**
	Inundation	0.13	2.41	4,144	0.05
	Salinity * Inundation	0.11	0.73	12,144	0.72
	Species * Inundation	0.14	2.63	4,144	**< 0.05**
	Salinity * Species	0.09	1.15	6,144	0.34
	Salinity * Species * Inundation	0.18	1.21	12,144	0.28

Leaf chemical stress	Salinity	1.74	21.64	12,189	< **0.0001**
	Species	0.69	34.34	4,61	< **0.0001**
	Inundation	0.48	4.95	8,124	< **0.0001**
	Salinity * Inundation	0.60	1.89	24,256	< **0.01**
	Species * Inundation	0.17	1.47	8,124	0.17
	Salinity * Species	0.71	4.90	12,189	< **0.0001**
	Salinity * Species * Inundation	0.45	1.36	24,256	0.13
Gas exchange	Salinity	0.49	3.14	12,192	< **0.01**
	Species	0.19	3.71	4,62	< **0.01**
	Inundation	0.12	0.98	8,126	0.46
	Salinity * Inundation	0.31	0.92	24,260	0.58
	Species * Inundation	0.11	0.92	8,126	0.50
	Salinity * Species	0.21	1.18	12,192	0.30
	Salinity * Species * Inundation	0.41	1.24	24,260	0.21

Appendix 6.D. Proportion of tillers, leaves and inflorescences DW of the AGB and roots and rhizomes DW of the BGB, leaf water content (LWC) and leaf chlorophyll *a* + *b* content of *Spartina foliosa* (black bars) and *S. densiflora* (white bars) at different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (SI, shallow inundation (4.4 cm deep); II, intermediate inundation (35.5 cm deep); DI, deep inundation (55.0 cm deep)). Values are mean ± SE (n = 4).



Appendix 6.E. F-statistic, *P*-values General Linear Models with species (T), salinity (S) (0.5, 10, 20 and 40 ppt) and inundation (I) (4.4, 35.5 and 55.0 cm deep) treatments as fixed factors, and their corresponding interactions, for biochemical, physiological and morphological traits of *Spartina densiflora* and *Spartina foliosa*. Significant differences are marked in bold. *Data transformed using 1/x function.

	Plant traits	Species (T)		Salinity (S)		T × S		Inundation (I)		T × I		S × I		T × S × I	
		$F_{1,60}$	P	$F_{3,12}$	P	$F_{3,60}$	P	$F_{2,60}$	P	$F_{2,60}$	P	$F_{6,60}$	P	$F_{6,60}$	P
Biomass allocation and growth	Leaf biomass (% AGB)	**33.94**	**<0.0001**	0.69	0.58	0.68	0.57	0.91	0.41	1.91	0.16	**4.69**	**<0.0001**	**2.70**	**<0.05**
	Tiller biomass (% AGB)	1.60	0.21	**5.68**	**<0.0001**	**9.83**	**<0.0001**	**66.87**	**<0.0001**	**5.40**	**<0.01**	**5.66**	**<0.0001**	**2.51**	**<0.05**
	Inflorescence biomass (% AGB)	**17.06**	**<0.0001**	**19.39**	**<0.0001**	**4.62**	**<0.01**	**25.50**	**<0.0001**	0.70	0.50	1.30	0.27	0.48	0.82
	AGB (g)	**203.32**	**<0.0001**	**10.23**	**<0.01**	**3.07**	**<0.05**	**69.45**	**<0.0001**	**12.39**	**<0.0001**	1.80	0.11	**2.64**	**<0.05**
	Root biomass (% BGB)	**18.35**	**<0.0001**	1.10	0.39	0.53	0.67	0.06	0.94	1.15	0.32	0.59	0.74	0.79	0.58
	Rhizome biomass (% BGB)	**18.35**	**<0.0001**	1.10	0.39	0.53	0.67	0.06	0.94	1.15	0.32	0.59	0.74	0.79	0.58
	BGB (g)	**368.80**	**<0.0001**	0.84	0.50	0.25	0.86	**26.55**	**<0.0001**	1.00	0.37	1.05	0.40	1.33	0.26
	AGB : BGB ratio	**8.36**	**<0.01**	**17.44**	**<0.0001**	0.86	0.47	**32.16**	**<0.0001**	0.70	0.50	0.55	0.77	1.50	0.20
	Root Mass Ratio	**22.14**	**<0.0001**	1.28	0.33	1.16	0.33	**8.34**	**<0.0001**	0.96	0.39	0.70	0.65	0.77	0.60
	Tiller length (cm)	**104.20**	**<0.0001**	**44.14**	**<0.0001**	1.84	0.15	**28.29**	**<0.0001**	0.12	0.89	1.69	0.14	1.62	0.16
	TGR (tillers tillers^{-1} yr^{-1})	**9.00**	**<0.01**	**8.17**	**<0.01**	**6.28**	**<0.0001**	**26.38**	**<0.0001**	0.16	0.85	1.93	0.09	**2.82**	**<0.05**
	Floret production (#)	**59.78**	**<0.0001**	**19.66**	**<0.0001**	0.68	0.57	**27.47**	**<0.0001**	**7.84**	**<0.0001**	1.29	0.28	1.10	0.37
Leaf morphology	Leaf rolling (%)	**43.58**	**<0.0001**	**19.64**	**<0.0001**	0.48	0.70	**26.78**	**<0.0001**	0.99	0.38	**2.81**	**<0.05**	**2.46**	**<0.05**
	Leaf Water Content (%)	1.37	0.25	1.25	0.33	1.52	0.22	0.79	0.46	1.95	0.15	0.38	0.89	0.44	0.85
	SLA (cm^2 g^{-1})*	2.35	0.13	**3.83**	**<0.05**	0.20	0.90	3.10	0.05	0.45	0.64	1.17	0.34	0.87	0.52
	Chl *a* (mg g^{-1})	**12.27**	**<0.0001**	**9.96**	**<0.01**	**3.26**	**<0.05**	**9.28**	**<0.0001**	**4.99**	**<0.01**	1.55	0.18	0.74	0.62
	Chl *b* (mg g^{-1})	**21.76**	**<0.0001**	**19.80**	**<0.0001**	1.97	0.13	**7.15**	**<0.01**	1.72	0.19	**2.57**	**<0.05**	0.69	0.66

Leaf pigments, C and N content	Carotenoids (mg g^{-1})	**25.21**	**<0.0001**	**8.06**	**<0.01**	**3.47**	**<0.05**	**8.65**	**<0.0001**	**7.14**	**<0.01**	1.55	0.18	0.88	0.52
	Chl *a* + *b* (mg g^{-1})	**15.18**	**<0.0001**	**12.14**	**<0.0001**	**3.03**	**<0.05**	**9.12**	**<0.0001**	**4.05**	**<0.05**	1.80	0.11	0.70	0.65
	Chl *a* : Carotenoids ratio	**18.85**	**<0.0001**	**6.90**	**<0.01**	0.44	0.73	1.06	0.35	2.22	0.12	0.34	0.91	0.99	0.44
	Chl *a* : Chl *b* ratio	**10.46**	**<0.01**	**17.15**	**<0.0001**	**4.01**	**<0.05**	2.60	0.08	**4.26**	**<0.05**	2.12	0.06	0.62	0.71
	Leaf C (mg g^{-1})*	**105.39**	**<0.0001**	**61.64**	**<0.0001**	0.85	0.47	**19.02**	**<0.0001**	0.38	0.69	2.01	0.08	2.10	0.07
	Leaf N (mg g^{-1})	**41.41**	**<0.0001**	2.75	0.09	2.04	0.12	**18.25**	**<0.0001**	**5.04**	**<0.01**	**2.74**	**<0.05**	0.48	0.82
	Leaf C : N ratio*	**22.32**	**<0.0001**	1.82	0.20	0.88	0.46	**7.97**	**<0.0001**	**4.40**	**<0.05**	**3.10**	**<0.05**	0.66	0.68
Leaf chemical stress	Leaf Na (mg g^{-1})*	2.40	0.13	**204.39**	**<0.0001**	**16.91**	**<0.0001**	0.20	0.82	0.03	0.97	1.36	0.25	**3.48**	**<0.01**
	Na excretion (nmol m^{-2} s^{-1})	**30.96**	**<0.0001**	**157.21**	**<0.0001**	**7.31**	**<0.0001**	**7.64**	**<0.01**	0.70	0.50	**3.80**	**<0.01**	1.09	0.38
	Glycinebetaine content (µmol g^{-1})	**83.84**	**<0.0001**	**246.73**	**<0.0001**	1.86	0.15	**6.68**	**<0.01**	1.08	0.35	**3.41**	**<0.01**	1.10	0.37
	Proline content (µmol g^{-1})*	**60.87**	**<0.0001**	**5.00**	**<0.05**	**13.26**	**<0.0001**	**6.49**	**<0.01**	2.57	0.08	2.02	0.08	1.07	0.39
Subterranean resource storage	Rhizome C (mg g^{-1})	**41.13**	**<0.0001**	**17.62**	**<0.0001**	1.99	0.12	**4.87**	**<0.05**	1.04	0.36	0.90	0.50	1.53	0.19
	Rhizome N (mg g^{-1})	**508.62**	**<0.0001**	**5.66**	**<0.05**	0.88	0.46	2.73	0.07	2.85	0.07	**5.10**	**<0.0001**	1.17	0.33
	Rhizome C : N ratio*	**601.50**	**<0.0001**	**17.66**	**<0.0001**	**3.25**	**<0.05**	**5.35**	**<0.01**	**3.36**	**<0.05**	**5.26**	**<0.0001**	1.81	0.11
	Rhizome TNC (mg g^{-1})	2.99	0.09	1.93	0.18	2.34	0.08	**24.28**	**<0.0001**	**3.64**	**<0.05**	0.94	0.47	0.96	0.46
	Δ rhizome TNC (%)	3.81	0.06	1.92	0.18	2.34	0.08	**24.26**	**<0.0001**	**3.51**	**<0.05**	0.95	0.47	0.97	0.45
Porosity	Root porosity (%)	**4.22**	**<0.05**	1.10	0.39	2.09	0.11	**4.47**	**<0.05**	**5.25**	**<0.01**	0.44	0.85	1.84	0.11
	Rhizome porosity (%)	**84.25**	**<0.0001**	0.83	0.50	0.56	0.64	1.50	0.23	0.97	0.39	1.13	0.35	1.18	0.33
Gas exchange	A (µmol CO_2 m^{-2} s^{-1})	**10.75**	**<0.01**	**6.11**	**<0.01**	1.29	0.29	0.95	0.39	1.34	0.27	1.11	0.37	1.29	0.28
	Gs (mmol H_2O m^{-2} s^{-1})*	**5.42**	**<0.05**	**6.40**	**<0.01**	0.82	0.49	1.80	0.18	1.55	0.22	0.98	0.45	2.15	0.06
	WUE (µmol CO_2 µmol H_2O^{-1})	1.09	0.30	0.52	0.68	1.10	0.36	0.41	0.67	0.89	0.42	0.43	0.86	0.96	0.46

Appendix 6.F. Factor loadings of the individual variables obtained by Principal Component Analysis (PCA) on traits of native *Spartina foliosa* (PC-*Sf*) and invasive *Spartina densiflora* (PC-*Sd*) exposed to different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (4.4, 35.5 and 55.0 cm deep). Correlations between the PCA and plant traits with factor loadings > ± 0.600 are marked in bold.

	PC-Sf1	*PC-Sf2*	*PC-Sf3*	*PC-Sf4*	*PC-Sf5*	*PC-Sf6*	*PC-Sf7*	*PC-Sf8*	*PC-Sf9*	*PC-Sf10*	*PC-Sd1*	*PC-Sd2*	*PC-Sd3*	*PC-Sd4*	*PC-Sd5*	*PC-Sd6*	*PC-Sd7*	*PC-Sd8*	*PC-Sd9*	*PC-Sd10*
Tiller length	**0.852**	0.318	0.090	0.008	0.140	-0.092	0.028	0.006	-0.090	0.038	**0.824**	0.225	-0.061	-0.093	0.081	0.031	-0.090	0.287	-0.043	0.068
Inflorescences biomass	**0.850**	0.260	0.120	-0.049	0.120	-0.065	0.088	0.049	-0.099	0.212	0.267	**0.734**	-0.057	0.108	0.003	-0.385	-0.264	-0.162	0.070	0.126
Proline content	**-0.850**	0.189	0.055	-0.215	0.050	0.037	0.017	-0.051	0.099	0.005	**-0.669**	0.384	0.083	-0.210	-0.011	0.190	0.061	0.133	-0.047	0.203
AGB:BGB	**0.847**	0.305	0.110	0.067	0.006	-0.106	-0.004	-0.013	0.102	-0.065	**0.771**	0.337	0.001	-0.022	0.318	-0.019	-0.089	0.145	-0.091	0.145
AGB	**0.783**	0.527	-0.101	0.079	0.137	-0.022	0.054	0.041	-0.001	-0.061	**0.773**	0.512	-0.050	0.001	0.084	0.126	-0.002	0.109	-0.071	0.166
Chl. *a* + *b*	**0.715**	-0.448	0.400	0.063	0.005	0.141	0.209	0.107	0.016	-0.098	**0.676**	-0.230	0.474	0.244	-0.282	0.089	0.214	-0.134	-0.044	0.166
Chl *b*	**0.708**	-0.537	0.158	-0.067	-0.033	0.074	0.225	0.111	0.059	-0.156	**0.785**	-0.321	0.209	-0.066	-0.095	0.192	0.256	-0.204	0.009	0.222
Leaf Na	**-0.706**	0.418	0.417	-0.189	0.121	0.037	0.000	0.091	0.023	0.152	**-0.707**	0.524	0.147	0.094	0.006	0.002	0.090	0.154	0.069	-0.019
Na excretion	**-0.703**	0.362	0.251	-0.019	0.233	0.312	0.027	0.076	0.117	0.036	**-0.774**	0.169	0.068	0.232	-0.145	0.172	-0.074	-0.177	0.103	0.128
Chl *a*	**0.697**	-0.401	0.481	0.111	0.019	0.162	0.197	0.102	-0.001	-0.073	0.542	-0.156	0.561	0.382	-0.350	0.023	0.167	-0.081	-0.068	0.117
Tillers biomass	**-0.693**	-0.208	0.239	-0.341	0.262	-0.093	0.146	-0.105	0.064	-0.336	-0.140	**-0.797**	-0.025	-0.068	0.041	0.086	0.071	0.074	-0.131	0.243
Leaf C	**0.682**	-0.351	-0.351	0.292	-0.033	-0.072	-0.002	-0.183	-0.043	0.093	**0.860**	-0.029	-0.115	-0.209	0.209	0.038	-0.029	0.142	-0.033	0.018
Glycinebetaine	**-0.682**	0.351	0.301	0.251	0.096	0.314	0.062	0.113	0.005	0.037	**-0.857**	0.279	0.182	0.003	0.041	-0.064	0.101	0.088	-0.091	0.206

Carotenoids content	**0.678**	-0.322	0.559	0.125	0.042	0.127	0.151	0.058	0.016	-0.099	0.389	-0.030	0.590	0.496	-0.438	0.025	0.029	0.037	-0.088	0.050
Δ rhizome TNC	**0.661**	0.497	-0.191	0.158	0.046	0.097	-0.027	-0.010	0.226	-0.374	0.250	0.273	0.591	0.280	0.404	-0.116	0.110	-0.101	0.376	-0.065
Rhizome TNC	**0.661**	0.497	-0.191	0.158	0.046	0.097	-0.027	-0.010	0.226	-0.374	0.250	0.273	0.591	0.280	0.404	-0.116	0.110	-0.101	0.376	-0.065
Floret production	**0.658**	0.406	-0.138	-0.105	0.286	0.034	0.166	-0.042	-0.094	0.303	**0.600**	0.560	0.014	0.097	-0.053	-0.074	-0.220	0.015	-0.006	-0.018
TGR	**0.649**	0.200	-0.004	-0.185	-0.061	-0.112	-0.141	0.140	-0.058	0.315	**0.713**	0.312	0.163	-0.168	0.160	0.143	-0.107	-0.008	-0.129	-0.248
Leaf C:N	0.396	0.149	-0.398	-0.514	-0.006	0.506	-0.055	-0.086	-0.167	-0.067	**0.699**	0.197	-0.335	-0.093	-0.327	0.173	0.104	-0.027	0.242	-0.133
Leaf N	-0.281	-0.174	0.283	0.554	0.009	**-0.610**	-0.033	0.035	0.221	0.110	**-0.625**	-0.250	0.373	0.098	0.394	-0.187	-0.065	-0.003	-0.226	0.177
Rhizome C:N	0.591	-0.262	0.118	-0.167	0.171	0.121	-0.357	-0.518	0.189	0.105	0.183	-0.256	**-0.683**	0.398	-0.276	-0.228	-0.030	0.079	0.210	0.065
Rhizome C	0.573	-0.384	-0.215	-0.106	-0.439	-0.054	-0.260	0.058	0.170	-0.004	0.372	-0.568	-0.438	0.155	0.231	-0.191	-0.019	0.062	0.094	-0.050
BGB	0.409	**0.625**	-0.287	0.211	0.303	0.097	0.098	0.131	-0.154	-0.015	0.238	**0.616**	-0.150	-0.011	-0.399	0.283	0.114	0.113	-0.055	0.088
Gs	0.385	-0.010	0.438	-0.240	-0.280	0.069	-0.528	0.396	-0.051	-0.002	0.093	-0.118	0.095	0.176	-0.076	**-0.651**	0.309	0.151	0.072	-0.324
SLA	0.362	-0.431	0.200	0.381	-0.005	-0.260	0.181	-0.153	-0.299	0.117	0.169	-0.376	0.235	0.039	0.060	-0.029	0.230	**0.614**	-0.173	0.206
A	0.335	-0.097	0.288	-0.557	-0.093	0.148	-0.128	0.370	0.297	0.251	0.142	-0.480	0.272	0.270	0.050	0.063	-0.234	0.385	0.035	-0.010
Root porosity	0.312	0.153	-0.167	-0.490	0.253	-0.377	0.023	0.112	0.161	-0.030	-0.332	-0.382	-0.045	-0.106	0.199	0.196	-0.230	0.140	0.388	0.107
Chl *a* : Car	0.283	-0.437	-0.313	-0.117	-0.157	0.387	0.313	0.192	-0.143	0.030	0.357	-0.334	-0.158	-0.314	0.252	-0.031	0.362	-0.332	0.118	0.108
LWC	0.266	0.332	-0.034	0.110	0.271	-0.461	-0.055	0.268	0.297	0.045	0.353	-0.351	0.021	0.308	0.141	0.325	-0.431	0.141	-0.035	-0.246
Chl *a* : Chl *b*	0.171	0.344	0.585	0.360	0.159	0.333	0.009	0.024	-0.208	0.105	-0.548	0.344	0.284	0.402	-0.230	-0.196	-0.136	0.213	-0.098	-0.165

Rhizome porosity	0.054	0.245	-0.075	-0.397	0.135	-0.459	-0.048	0.080	-0.564	-0.069	-0.156	0.144	-0.105	0.071	0.086	0.106	0.223	0.374	**0.614**	0.233
Root biomass	0.045	-0.556	-0.261	0.197	**0.650**	0.105	-0.239	0.273	0.012	-0.012	0.267	-0.156	0.489	**-0.637**	-0.279	-0.175	-0.271	0.080	0.183	0.024
Rhizome biomass	-0.045	0.556	0.261	-0.197	**-0.650**	-0.105	0.239	-0.273	-0.012	0.012	-0.267	0.156	-0.489	**0.637**	0.279	0.175	0.271	-0.080	-0.183	-0.024
WUE	-0.083	-0.162	-0.383	-0.135	0.089	0.074	**0.698**	0.020	0.372	0.340	-0.152	-0.205	0.137	0.451	0.013	0.529	-0.350	-0.245	0.197	0.117
Leaves biomass	-0.096	-0.035	-0.452	0.501	-0.481	0.197	-0.293	0.078	0.034	0.188	-0.261	-0.134	0.133	-0.089	-0.063	0.541	0.355	0.174	0.066	-0.562
Leaf Rolling	-0.099	**0.625**	-0.030	0.444	-0.002	0.306	-0.114	0.013	0.081	0.100	-0.230	**0.733**	-0.110	-0.239	-0.136	0.180	0.112	0.138	0.094	0.086
Rhizome N	-0.385	0.152	-0.251	0.164	-0.436	-0.145	0.283	**0.611**	-0.117	-0.169	-0.108	0.194	**0.678**	-0.364	0.394	0.185	0.057	-0.048	-0.152	-0.094
RMR	-0.530	-0.563	-0.265	0.078	0.445	0.113	-0.176	0.162	-0.104	0.020	-0.421	-0.422	0.363	-0.430	-0.455	-0.140	-0.118	-0.055	0.204	-0.050

Appendix 7.A. F-statistic, *P*-values General Linear Models with salinity (S) (0.5, 10, 20 and 40 ppt) and inundation (I) (4.4, 35.5 and 55.0 cm deep) treatments as fixed factors, and their corresponding interactions, for biochemical, physiological and morphological traits of the hybrid *Spartina densiflora x Spartina foliosa*. Significant differences are marked in bold. *Data transformed using 1/x function.

	Plant traits	Salinity (S)		Inundation (I)		S × I	
		$F_{3,6}$	P	$F_{2,6}$	P	$F_{6,36}$	P
Biomass allocation and growth	Leaf biomass (% AGB)	1.842	0.157	**22.18**	**<0.001**	0.5	0.804
	Tiller biomass (% AGB)	**9.233**	**<0.001**	**88.94**	**<0.001**	**2.551**	**<0.05**
	Inflorescence biomass (% AGB)	2.867	0.05	**4.188**	**<0.05**	**2.692**	**<0.05**
	Root biomass (% BGB)	**6.039**	**<0.01**	**7.434**	**<0.01**	1.169	0.344
	Rhizome biomass (% BGB)	**6.039**	**<0.01**	**7.434**	**<0.01**	1.169	0.344
	BGB (g)	1.885	0.15	**41.55**	**<0.001**	0.794	0.581
	AGB : BGB ratio *	**7.641**	**<0.001**	**9.622**	**<0.001**	0.559	0.760
	Root Mass Ratio	**3.141**	**<0.05**	1.32	0.28	1.098	0.382
	Tiller length (cm)	**13.01**	**<0.001**	**4.205**	**<0.05**	0.455	0.836
	TGR (tillers tillers^{-1} yr^{-1})	**10.89**	**<0.001**	**16.61**	**<0.001**	1.057	0.406
Leaf morphology	Leaf rolling (%)	**57.59**	**<0.001**	2.855	0.071	1.312	0.277
	Leaf Water Content (%)	**12.76**	**<0.001**	1.004	0.376	0.404	0.872
	Specific Leaf Area (cm^2 g^{-1}) *	2.35	0.089	**4.321**	**<0.05**	1.345	0.263
Gas exchange	Net photosynthesis rate (µmol CO_2 m^{-2} s^{-1})	1.873	0.152	0.483	0.621	0.912	0.497
	Stomatal conductance (mmol H_2O m^{-2} s^{-1})	**5.391**	**<0.01**	0.585	0.563	0.67	0.674
	Water Use Efficiency (µmol CO_2 µmol H_2O^{-1})	**2.963**	**<0.05**	0.045	0.956	0.399	0.875
Porosity	Root porosity (%)	1.319	0.284	0.006	0.994	0.767	0.601
	Rhizome porosity (%)	**4.673**	**<0.01**	0.612	0.548	1.07	0.399
Leaf pigments, C and N content	Chl *a* (mg g^{-1})	**8.091**	**<0.001**	**4.423**	**<0.05**	1.692	0.151
	Chl *b* (mg g^{-1})	**18.35**	**<0.001**	1.907	0.163	0.489	0.812
	Carotenoids (mg g^{-1})	**4.148**	**<0.05**	1.518	0.233	1.17	0.344
	Chl *a* + *b* (mg g^{-1})	**11.44**	**<0.001**	**3.998**	**<0.05**	1.358	0.258
	Chl *a* : Carotenoids ratio	**7.411**	**<0.001**	1.965	0.155	0.318	0.924
	Chl *a* : Chl *b* ratio	**16.99**	**<0.001**	0.158	0.854	1.042	0.415
	Leaf C (mg g^{-1})	**55.94**	**<0.001**	**4.135**	**<0.05**	1.868	0.113
	Leaf N (mg g^{-1})	0.462	0.71	**11.25**	**<0.001**	1.681	0.154
	Leaf C : N ratio	0.229	0.875	**9.348**	**<0.001**	1.373	0.252

Leaf chemical stress	Leaf Na (mg g^{-1})*	**93.15**	**<0.001**	0.786	0.463	1.51	0.20
	Na excretion (nmol m^{-2} s^{-1})	**37.96**	**<0.001**	3.209	0.052	0.71	0.64
	Glycinebetaine content (µmol g^{-1})	**88.45**	**<0.001**	1.482	0.241	**2.62**	**<0.05**
	Proline content (µmol g^{-1}) *	**38.09**	**<0.001**	1.55	0.226	1.114	0.374
Subterranean resource storage	Rhizome C (mg g^{-1})	**11.63**	**<0.001**	0.442	0.646	1.772	0.134
	Rhizome N (mg g^{-1})	**3.377**	**<0.05**	**8.117**	**<0.001**	1.51	0.203
	Rhizome C : N ratio*	**5.786**	**<0.01**	**8.043**	**<0.001**	2.32	0.054
	Rhizome TNC (mg g^{-1})	**3.095**	**<0.05**	2.33	0.112	1.588	0.179
	Δ rhizome TNC (%)	**3.095**	**<0.05**	2.33	0.112	1.588	0.179

Appendix 7.B. Factor loadings obtained by Principal Component Analysis (PCA) for plant traits of *Spartina densiflora x foliosa* (PC-H) exposed to different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (4.4, 35.5 and 55.0 cm deep), and relationships for each factor with salinity, inundation depth, and vegetative and reproductive fitness (Pearson correlation coefficient (r) and P values, n = 48).

	PC-H1	PC-H2	PC-H3	PC-H4	PC-H5	PC-H6	PC-H7	PC-H8	PC-H9	PC-H10
Leaf Carbon content	**0.868**	-0.148	0.098	-0.008	0.011	0.013	-0.084	0.071	-0.169	0.190
Chl *b* content	**0.830**	0.111	0.014	-0.031	-0.320	-0.005	0.152	0.098	0.316	-0.041
Chl. *a* + *b* content	**0.795**	0.248	-0.101	0.285	-0.232	0.112	0.074	0.084	0.345	0.029
Chl *a* content	**0.717**	0.297	-0.150	0.418	-0.171	0.160	0.030	0.071	0.334	0.062
Leaf Water content	**0.700**	0.022	-0.174	-0.074	0.148	0.215	0.050	0.019	-0.014	-0.087
Rhizome Carbon content	**0.669**	-0.223	0.115	0.155	0.019	0.198	-0.033	0.141	-0.062	-0.314
Leaf Rolling	**-0.864**	0.026	0.000	0.037	0.039	0.102	0.072	0.150	0.080	-0.203
Proline content	**-0.847**	0.120	0.031	-0.121	-0.170	0.106	0.089	0.280	0.109	-0.028
Glycinebetaine content	**-0.807**	0.335	-0.176	0.283	-0.030	0.080	0.120	0.084	-0.098	0.068
Na excretion	**-0.756**	0.364	-0.142	0.191	-0.035	-0.075	-0.022	-0.002	-0.015	-0.152
Leaf Na content	**-0.722**	0.077	-0.113	0.345	0.289	-0.066	0.107	-0.043	0.027	-0.085
Tiller biomass (% AGB)	-0.162	**0.827**	0.206	0.085	0.034	-0.180	-0.212	0.144	0.000	0.101
Leaf Nitrogen content	0.257	**0.712**	-0.181	0.116	-0.307	0.239	0.088	0.089	-0.370	0.048
Leaf C:N ratio	-0.141	**-0.730**	0.157	-0.089	0.283	-0.320	-0.113	-0.113	0.369	-0.016
Below-ground biomass (BGB)	-0.032	**-0.744**	-0.218	-0.089	0.018	0.420	-0.034	-0.104	-0.064	0.270
Leaf biomass (% AGB)	0.044	**-0.678**	-0.403	-0.258	0.042	-0.038	0.422	-0.015	0.128	0.055
Tiller length	0.490	**-0.680**	0.200	0.272	0.058	0.040	-0.285	-0.156	-0.163	0.042
Tillers growth rate (TGR)	0.481	-0.558	-0.126	-0.034	0.017	0.206	0.136	0.077	-0.189	0.092
AGB : BGB	0.564	0.145	0.486	0.196	0.057	-0.327	-0.176	0.133	-0.119	-0.080
Carotenoids content	0.554	0.230	-0.331	0.572	-0.055	0.237	-0.066	0.049	0.308	0.119
Rhizome C:N ratio	0.458	0.363	0.287	0.111	0.580	-0.006	0.297	-0.232	0.046	-0.026
Chl *a* : Carotenoids ratio	0.410	0.147	0.463	-0.424	-0.254	-0.191	0.286	0.075	0.067	-0.158

Root biomass (% BGB)	0.406	0.537	-0.210	-0.507	0.249	0.174	-0.176	-0.047	-0.018	-0.064
Stomatal conductance	0.400	-0.191	-0.484	0.135	0.410	-0.229	0.067	0.347	-0.195	-0.145
Net photosynthesis rate	0.376	-0.151	-0.128	0.150	0.320	-0.215	0.195	0.468	-0.203	0.385
Specific Leaf Area	0.362	0.346	0.493	-0.298	0.122	0.182	-0.042	0.154	-0.177	0.043
Rhizome porosity	0.267	0.102	-0.165	0.373	0.187	0.106	0.110	-0.237	0.020	-0.251
Inflorescences biomass (% AGB)	0.215	-0.379	0.276	0.261	-0.126	0.377	-0.297	-0.229	-0.203	-0.265
Root porosity	-0.078	-0.013	0.040	-0.226	0.272	-0.018	**-0.674**	0.193	0.357	0.205
Root Mass Ratio	-0.133	0.306	-0.534	**-0.610**	0.154	0.392	0.029	-0.108	0.076	0.021
Water Use Eficiency	-0.182	0.301	0.433	0.023	-0.175	0.038	0.188	-0.400	-0.029	0.526
Rhizome Nitrogen content	-0.342	-0.415	-0.216	-0.110	**-0.604**	0.083	-0.289	0.241	-0.092	-0.015
Rhizome TNC	-0.399	-0.176	0.585	0.039	0.211	0.517	0.151	0.260	0.121	-0.006
Δ rhizome TNC	-0.399	-0.176	0.585	0.039	0.211	0.517	0.151	0.260	0.121	-0.006
Rhizome biomass (% BGB)	-0.406	-0.537	0.210	0.507	-0.249	-0.174	0.176	0.047	0.018	0.064
Chl *a* : Chl *b* ratio	-0.543	0.203	-0.215	0.523	0.302	0.194	-0.206	-0.049	-0.072	0.128
Salinity (r)	**-0.941**	0.188	-0.030	0.111	-0.011	-0.019	-0.014	0.109	0.068	-0.072
P-value	**<0.0001**	0.201	0.839	0.452	0.943	0.897	0.922	0.462	0.645	0.627
Inundation (r)	0.149	**0.855**	0.186	0.037	-0.002	-0.172	-0.099	-0.045	0.053	-0.024
P-value	0.313	**<0.0001**	0.205	0.803	0.987	0.241	0.505	0.762	0.722	0.872
Vegetative fitness (r)	**0.393**	**-0.716**	0.099	0.115	0.070	0.150	-0.100	0.072	-0.080	0.020
P-value	**0.006**	**<0.0001**	0.505	0.435	0.635	0.307	0.500	0.628	0.588	0.892
Reproductive fitness (r)	**0.298**	**-0.537**	0.128	0.044	0.064	**0.368**	**-0.321**	-0.167	-0.061	0.079
P-value	**0.040**	**<0.0001**	0.386	0.766	0.665	**0.010**	**0.026**	0.256	0.679	0.592

Appendix 7.C. Phenotypic inheritance in the hybrid *S. densiflora x foliosa* (*Sdxf*) for 36 traits measured at different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (4.4, 35.5 and 55.0 cm deep). Parental dominance (D); parental additivity (I); transgressive (T); *S. densiflora* (*Sd*); *S. foliosa* (*Sf*). Three-way ANOVA, salinity *x* inundation *x* taxa, $P < 0.05$, n = 4.

	0.5			10			20			40		
	SI	*II*	*DI*	*SI*	*II*	*DI*	*SI*	*II*	*DI*	*SI*	*II*	*DI*
Tiller biomass	D-Sf	D-Sd	T+	D-Sd	D-SdSf	T+	D-SdSf	D-SdSf	T+	D-SdSf	D-SdSf	I
Leaf biomass	D-Sf	D-Sd	D-Sd	D-Sf	D-SdSf	D-Sd	D-SdSf	D-SdSf	T-	d-Sd	D-Sd	D-SdSf
Inflorescence b.	D-SdSf	D-SdSf	D-SdSf	T-	D-Sd	D-SdSf	D-SdSf	D-SdSf	D-Sd	D-SdSf	I	I
Root biomass	D-SdSf	D-SdSf	T+	D-SdSf	D-Sf	D-SdSf	D-Sf	D-SdSf	D-Sf	D-SdSf	D-SdSf	T+
Rhizome b.	D-SdSf	D-SdSf	T-	D-SdSf	D-Sf	D-SdSf	D-Sf	D-SdSf	D-Sf	D-SdSf	D-SdSf	T-
BGB	D-Sd	D-Sd	I	T+	D-Sd	D-Sf	D-Sd	I	I	D-Sd	I	D-Sf
AGB : BGB	T+	T+	T+	T+	T+	T+	T+	T+	T+	T+	T+	T+

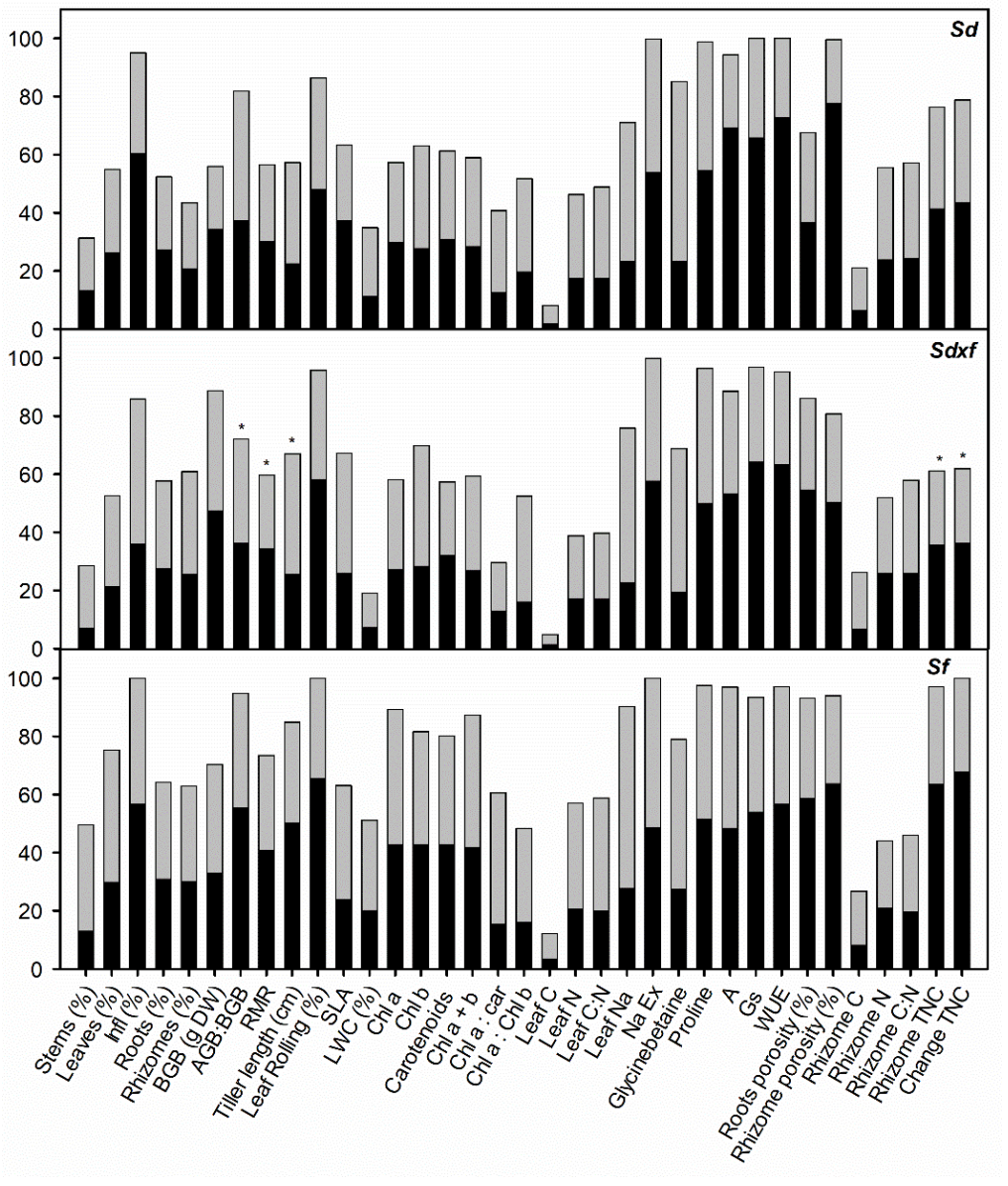
rmr	D-Sd	T-	T-	T-	D-Sd	T-	T-	T-	T-	T-	T-	T-
Tiller length	T+	T+	T+	T+	T+	T+	T+	T+	T+	T+	T+	T+
TGR	T+	T+	T+	T+	D-Sf	D-Sf	D-Sf	D-Sf	T+	D-SdSf	T+	D-SdSf
Leaf Rolling	D-Sf	D-Sf	D-Sf	D-Sf	D-Sf	D-SdSf	D-Sf	D-Sd	D-Sd	T+	D-SdSf	D-Sd
SLA	D-SdSf	D-SdSf	D-Sd	D-Sd	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-Sd
LWC	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf
Chl *a*	D-SdSf	D-Sf	T+	D-SdSf	I	T+	D-SdSf	D-Sf	T+	D-Sd	D-SdSf	T+
Chl *b*	D-SdSf	D-Sf	T+	D-SdSf	D-Sd	D-Sf	D-SdSf	D-SdSf	T+	D-SdSf	D-SdSf	D-SdSf
Carotenoids	D-SdSf	D-Sf	T+	D-SdSf	D-Sd	D-Sf	D-SdSf	D-Sf	T+	D-SdSf	D-SdSf	D-Sd
Chl. *a* + *b*	D-SdSf	D-Sf	T+	D-SdSf	D-Sd	T+	D-SdSf	D-Sf	T+	D-Sd	D-SdSf	T+
Chl *a* : Car.	D-Sd	D-Sd	T+	D-SdSf	D-SdSf	D-Sd	D-SdSf	D-SdSf	T+	D-SdSf	D-Sd	D-Sd
Chl *a* : Chl *b*	D-SdSf	D-Sd	D-Sd	D-SdSf	D-Sd	D-Sd	D-SdSf	D-Sd	D-Sd	D-SdSf	D-Sd	D-Sd
Leaf C	D-Sd	D-Sd	D-Sd	D-Sd	D-Sd	T+	D-Sd	D-Sd	T+	D-Sd	D-Sd	T+
Leaf N	T+	D-Sf	D-Sf	D-Sf	D-Sf	D-Sf	D-SdSf	D-SdSf	T+	D-SdSf	D-Sd	D-Sf
Leaf C:N	T-	D-Sf	D-Sf	D-Sf	D-Sf	D-SdSf	D-SdSf	D-Sd	D-SdSf	D-Sd	D-Sd	D-Sf
Leaf Na	T-	D-Sf	I	D-SdSf	D-Sd	D-Sd	T-	D-Sd	D-Sd	D-Sd	D-Sd	D-Sd
Na excretion	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-Sf	T+	D-SdSf	D-Sd	D-Sd	D-Sf	I	D-Sf
Glycinebetaine	T+	D-Sf	D-Sf	D-Sf	D-Sf	D-Sf	D-Sf	D-SdSf	D-Sf	D-Sd	D-Sd	D-SdSf
Proline	T+	D-SdSf	I	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-Sd	D-SdSf	D-SdSf	D-SdSf	D-SdSf
A	D-Sf	D-Sd	D-SdSf	D-Sf	D-SdSf	D-SdSf	D-Sf	D-SdSf	D-SdSf	D-SdSf	D-Sf	D-SdSf
Gs	D-Sf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-Sf	D-SdSf	D-Sf	D-SdSf	D-SdSf	D-Sf
WUE	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-Sf	D-SdSf	D-SdSf	D-SdSf	D-SdSf
Root porosity	D-Sd	D-SdSf	D-SdSf	D-SdSf	D-SdSf	T-	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-Sd	D-SdSf
Rhizome porosity	D-Sd	D-SdSf	D-SdSf	D-Sd	D-SdSf	D-Sd	D-SdSf	I	D-SdSf	D-SdSf	D-SdSf	D-Sd
Rhizome C	D-SdSf	D-Sf	D-Sf	D-Sf	D-SdSf	D-SdSf	D-SdSf	D-SdSf	D-Sf	D-Sd	D-SdSf	D-Sf
Rhizome N	T+	T+	D-Sf	T+	D-Sf	D-Sf	T+	T+	D-Sf	D-Sf	T+	D-Sf
Rhizome C:N	T-	D-Sf	D-Sf	T-	D-Sf	D-Sf	T-	T-	D-Sf	D-Sf	T-	D-Sf
Rhizome TNC	D-SdSf	T+	T+	T+	T+	T+	T+	T+	T+	T+	T+	T+
Δ rhizome TNC	T+	T+	T+	T+	T+	T+	T+	T+	T+	T+	T+	T+

Appendix 7.D. Number of individuals of *S. densiflora x maritima* (n=4) with transgressive response for 36 traits measured at each treatment combination of salinities (0.5, 10, 20 and 40 ppt) and inundation depths (SI, shallow inundation (4.4 cm deep); II, intermediate inundation (35.5 cm deep); DI, deep inundation (55.0 cm deep)). Percentage of individuals are marked in bold.

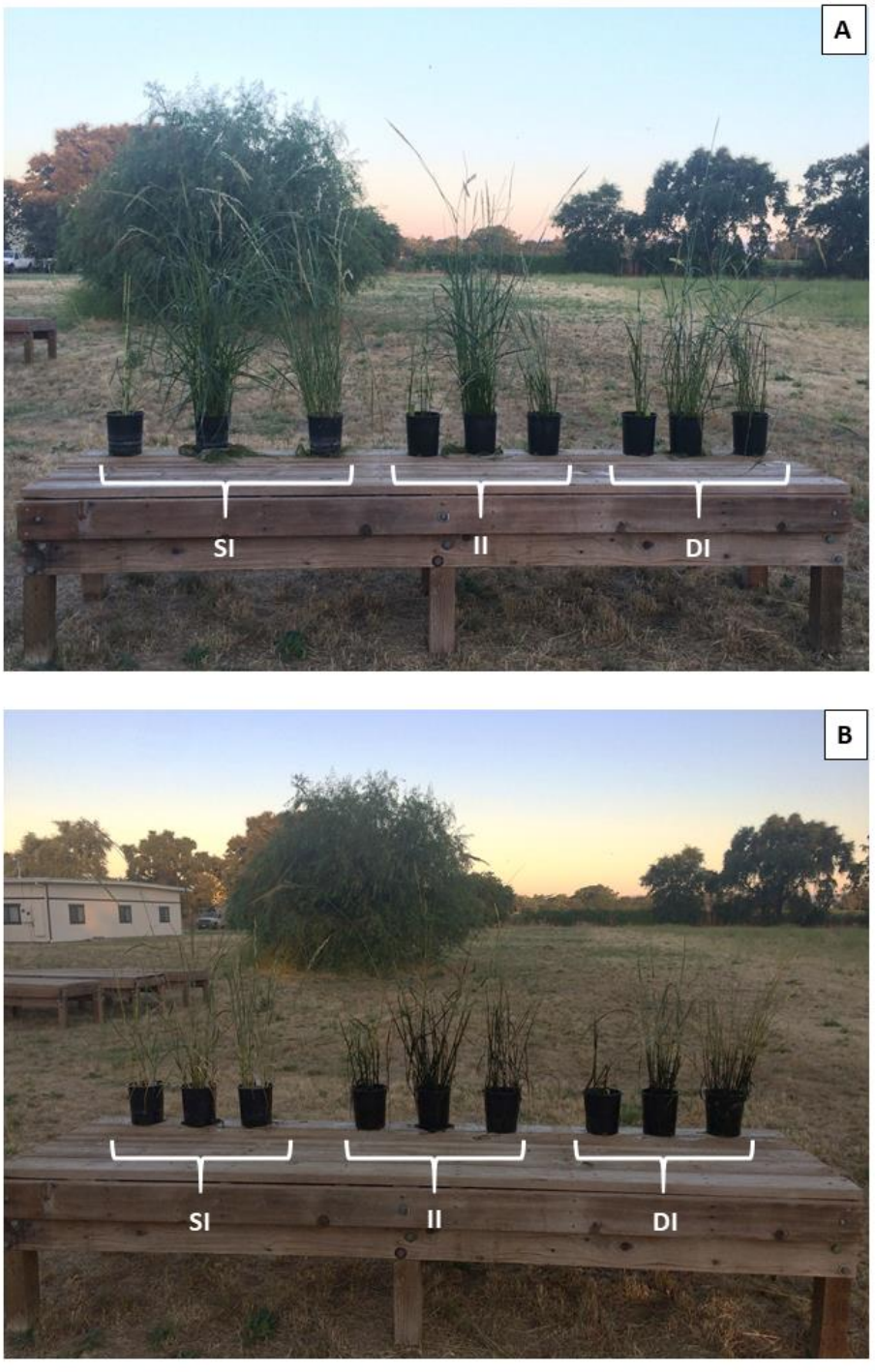
	0.5SI	10SI	20SI	40SI	**SI**	0.5II	10II	20II	40II	**II**	0.5DI	10DI	20DI	40DI	**DI**	**0.5**	**10**	**20**	**40**	Total
AGB : BGB	4	4	4	4	**100**	4	4	4	4	**100**	4	4	4	4	**100**	**100**	**100**	**100**	**100**	**100**
Tiller length	4	4	4	4	**100**	4	4	4	3	**94**	4	4	4	4	**100**	**100**	**100**	**100**	**92**	**98**
Δ rhizome TNC	4	4	4	4	**100**	4	4	4	4	**100**	4	4	4	4	**100**	**100**	**100**	**100**	**100**	**100**
RMR	2	2	4	4	**75**	2	2	4	4	**75**	4	4	4	2	**88**	**67**	**67**	**100**	**83**	**79**
TGR	3	4	3	4	**88**	4	0	1	4	**56**	3	1	3	2	**56**	**83**	**42**	**58**	**83**	**67**
Rhizome TNC	1	2	1	4	**50**	0	3	3	4	**63**	4	1	3	4	**75**	**42**	**50**	**58**	**100**	**63**
Rhizome N	3	3	4	0	**63**	2	0	3	3	**50**	0	0	1	1	**13**	**42**	**25**	**67**	**33**	**42**
Chl *a*	2	3	0	4	**56**	1	0	0	0	**6**	3	3	0	1	**44**	**50**	**50**	**0**	**42**	**35**
Rhizome C:N	3	2	4	0	**56**	2	0	3	2	**44**	0	0	1	1	**13**	**42**	**17**	**67**	**25**	**38**
Leaf Na	1	1	3	2	**44**	0	1	3	2	**38**	0	0	1	3	**25**	**8**	**17**	**58**	**58**	**35**
Na excretion	0	0	0	0	**0**	2	0	1	0	**19**	4	1	0	1	**38**	**50**	**8**	**8**	**8**	**19**
Chl. *a* + *b*	1	3	0	4	**50**	1	0	0	0	**6**	3	3	0	1	**44**	**42**	**50**	**0**	**42**	**33**
Chl *b*	2	2	0	1	**31**	1	0	0	0	**6**	2	0	0	1	**19**	**42**	**17**	**0**	**17**	**19**
A	0	1	0	1	**13**	0	2	0	2	**25**	1	0	0	2	**19**	**8**	**25**	**0**	**42**	**19**
Proline	2	2	2	0	**38**	0	0	1	0	**6**	0	0	0	2	**13**	**17**	**17**	**25**	**17**	**19**
Glycinebetaine	3	2	0	0	**31**	0	2	0	0	**13**	2	0	0	0	**13**	**42**	**33**	**0**	**0**	**19**
Gs	1	1	1	0	**19**	1	1	1	0	**19**	0	0	0	2	**13**	**17**	**17**	**17**	**17**	**17**
Root porosity	0	0	2	1	**19**	2	0	0	1	**19**	0	1	0	1	**13**	**17**	**8**	**17**	**25**	**17**
Carotenoids	0	2	0	1	**19**	0	0	0	0	**0**	2	1	0	1	**25**	**17**	**25**	**0**	**17**	**15**
SLA	2	1	1	0	**25**	0	0	0	0	**0**	2	1	0	0	**19**	**33**	**17**	**8**	**0**	**15**
Chl *a* : Car.	1	0	1	1	**19**	0	0	0	1	**6**	1	0	1	1	**19**	**17**	**0**	**17**	**25**	**15**
BGB	2	3	1	0	**38**	0	0	0	0	**0**	0	1	0	0	**6**	**17**	**33**	**8**	**0**	**15**
Leaf Rolling	2	2	0	2	**38**	0	0	0	0	**0**	0	0	0	1	**6**	**17**	**17**	**0**	**25**	**15**
Root biomass	0	0	2	1	**19**	0	1	0	0	**6**	1	1	0	0	**13**	**8**	**17**	**17**	**8**	**13**
Leaf biomass	0	0	0	3	**19**	0	0	1	0	**6**	0	0	2	0	**13**	**0**	**0**	**25**	**25**	**13**

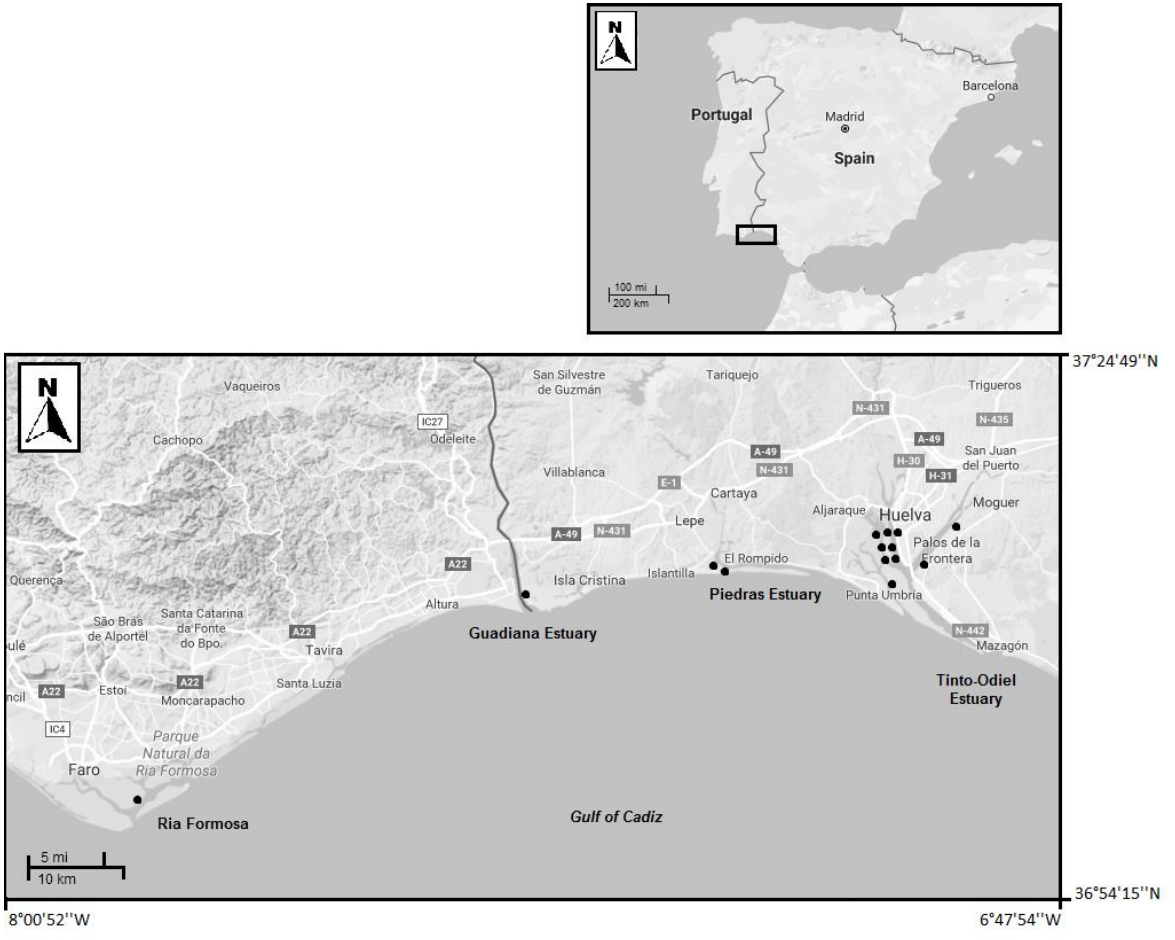
Leaf N	3	0	2	0	**31**	0	0	0	0	**0**	0	0	0	1	**6**	**25**	**0**	**17**	**8**	**13**
Leaf C:N	3	0	2	0	**31**	0	0	0	0	**0**	0	0	0	1	**6**	**25**	**0**	**17**	**8**	**13**
Rhizome b.	0	0	2	1	**19**	0	2	0	0	**13**	1	0	0	0	**6**	**8**	**17**	**17**	**8**	**13**
Tiller biomass	0	1	1	3	**31**	0	0	0	1	**6**	0	0	0	0	**0**	**0**	**8**	**8**	**33**	**13**
Inflorescences b.	0	4	1	0	**31**	0	0	0	0	**0**	0	0	0	0	**0**	**0**	**33**	**8**	**0**	**10**
Chl *a* : Chl *b*	2	0	1	0	**19**	0	2	0	0	**13**	0	0	0	0	**0**	**17**	**17**	**8**	**0**	**10**
WUE	0	0	0	0	**0**	0	0	1	0	**6**	0	0	1	1	**13**	**0**	**0**	**17**	**8**	**6**
Rhizome por.	0	0	0	1	**6**	0	0	0	0	**0**	0	0	0	0	**0**	**0**	**0**	**0**	**8**	**2**
LWC	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	**0**	**0**	**0**	**0**	**0**
Leaf C	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	**0**	**0**	**0**	**0**	**0**
Rhizome C	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	**0**	**0**	**0**	**0**	**0**
	34	**36**	**34**	**34**		**20**	**19**	**23**	**24**		**30**	**20**	**20**	**28**						
	0.5SI	10SI	20SI	40SI		0.5II	10II	20II	40II		0.5DI	10DI	20DI	40DI						

Appendix 7.E. Intra-population trait variability (black), phenotypic plasticity (gray) and inter-population trait variability (bar length) for 36 foliar measured in *Spartina densiflora* (*Sd*), *S. densiflora x foliosa* (*Sdxf*) and *S. foliosa* (*Sf*) at different salinities (0.5, 10, 20 and 40 ppt) and inundation depths (4.4, 35.5 and 55.0 cm deep). The traits with a transgressive behavior at the population level are marked with an asterisk.

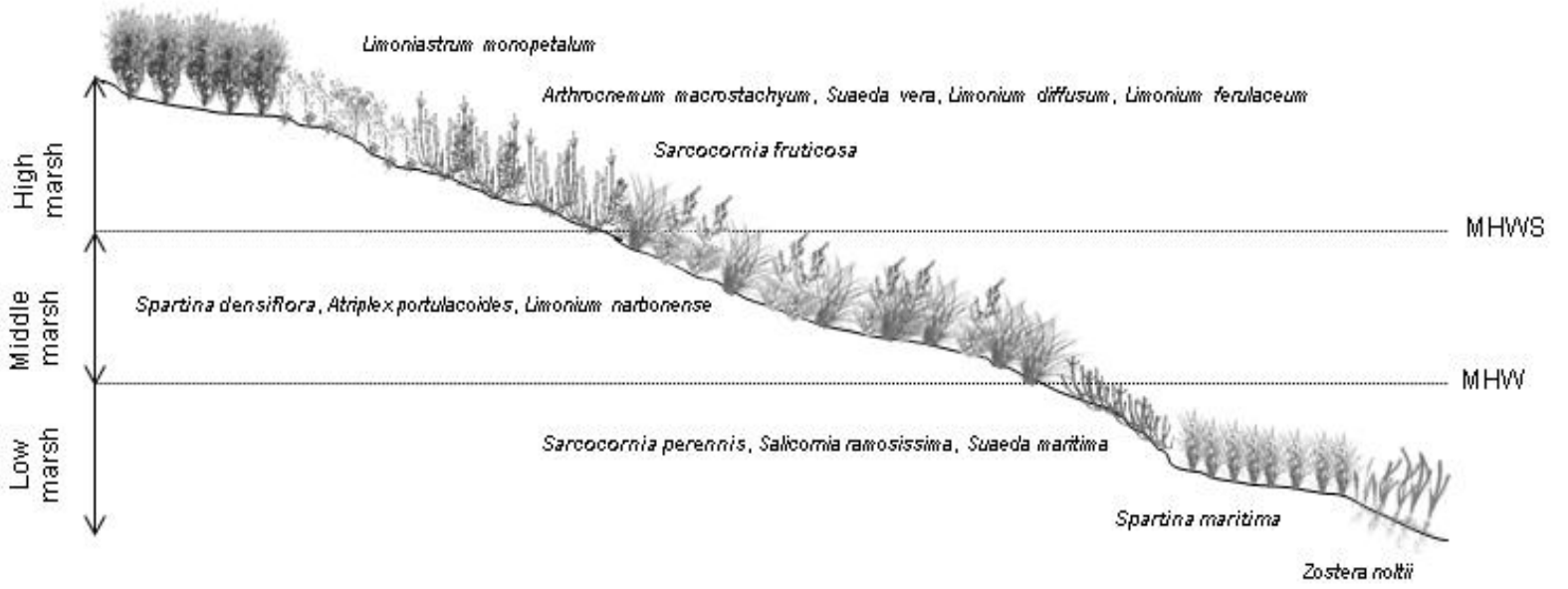


Appendix 7.F. Pictures of individuals of *S. foliosa*, *S. densiflora x foliosa* and *S. densiflora* (order for every treatment combination) after 44 days exposed to (A) 0.5 ppt and (B) 40 ppt NaCl, and shallow (SI), intermediate (II) and deep inundation (DI) treatments.





Appendix 8.A. Location map showing primary study areas at the Iberian Peninsula, and 14 sampled marshes (●) within four estuaries along the coast of the Gulf of Cádiz



Appendix 8.B. Schematic drawing of typical zonation pattern along the intertidal gradient of the Gulf of Cadiz, showing the most characteristics halophyte species (parental species studied in this work are marked in bold). MHW, Mean High Water; MHWS, Mean High Water Spring

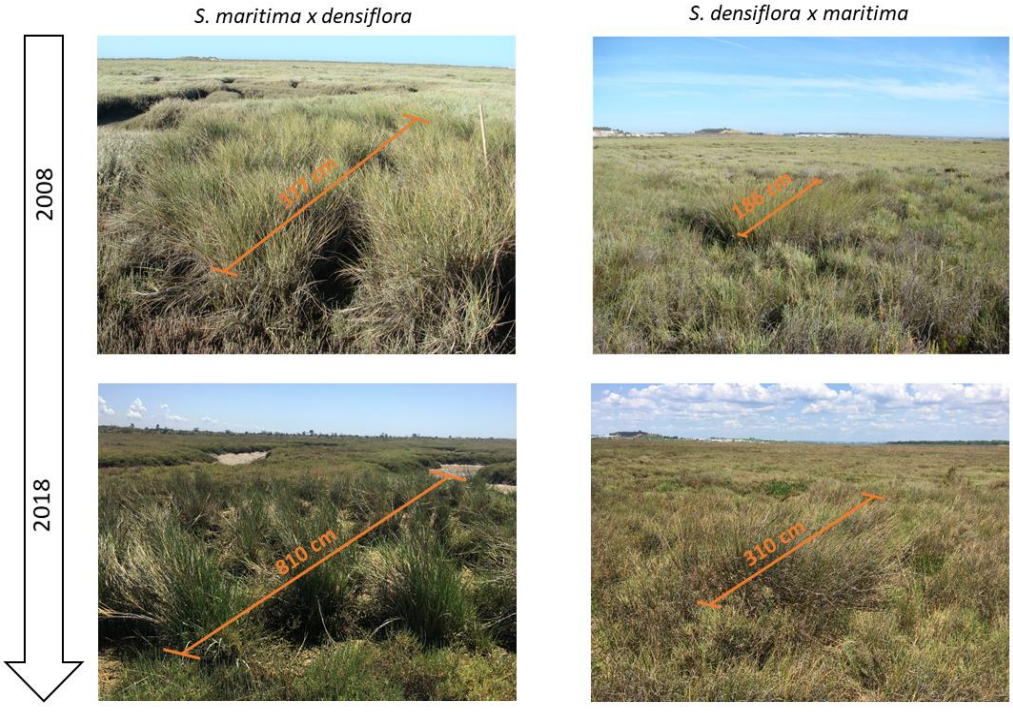
Appendix 8.C. Number of marshes, rivers' estuaries, and number of monospecific plots (0.5 x 0.5 m) of *Sarcocornia perennis*, *Sarcocornia fruticosa*, *Spartina maritima*, *Spartina densiflora* and their hybrids where pH, electrical conductivity, water content and redox potential of sediments, and marsh elevation, were recorded since 1997 to 2016

Taxa	# Marshes	Estuaries	pH	Electrical conductivity	Water content	Redox potential	Marsh elevation
Sarcocornia perennis	7	Odiel, Guadiana, Ria Formosa	65	87	17	115	166
Sarcocornia hybrids	4	Odiel, Guadiana	56	56	18	99	133
Sarcocornia fruticosa	10	Odiel, Piedras, Guadiana, Ria Formosa	59	64	30	67	46
Spartina maritima	7	Tinto, Odiel, Piedras, Guadiana	115	114	52	157	211
S. maritima x densiflora	3	Odiel, Piedras, Guadiana	9	9	9	10	9
S. densiflora x maritima	3	Tinto, Piedras, Guadiana	15	15	15	16	18
Spartina densiflora	12	Tinto, Odiel, Piedras, Guadiana	86	85	36	125	152

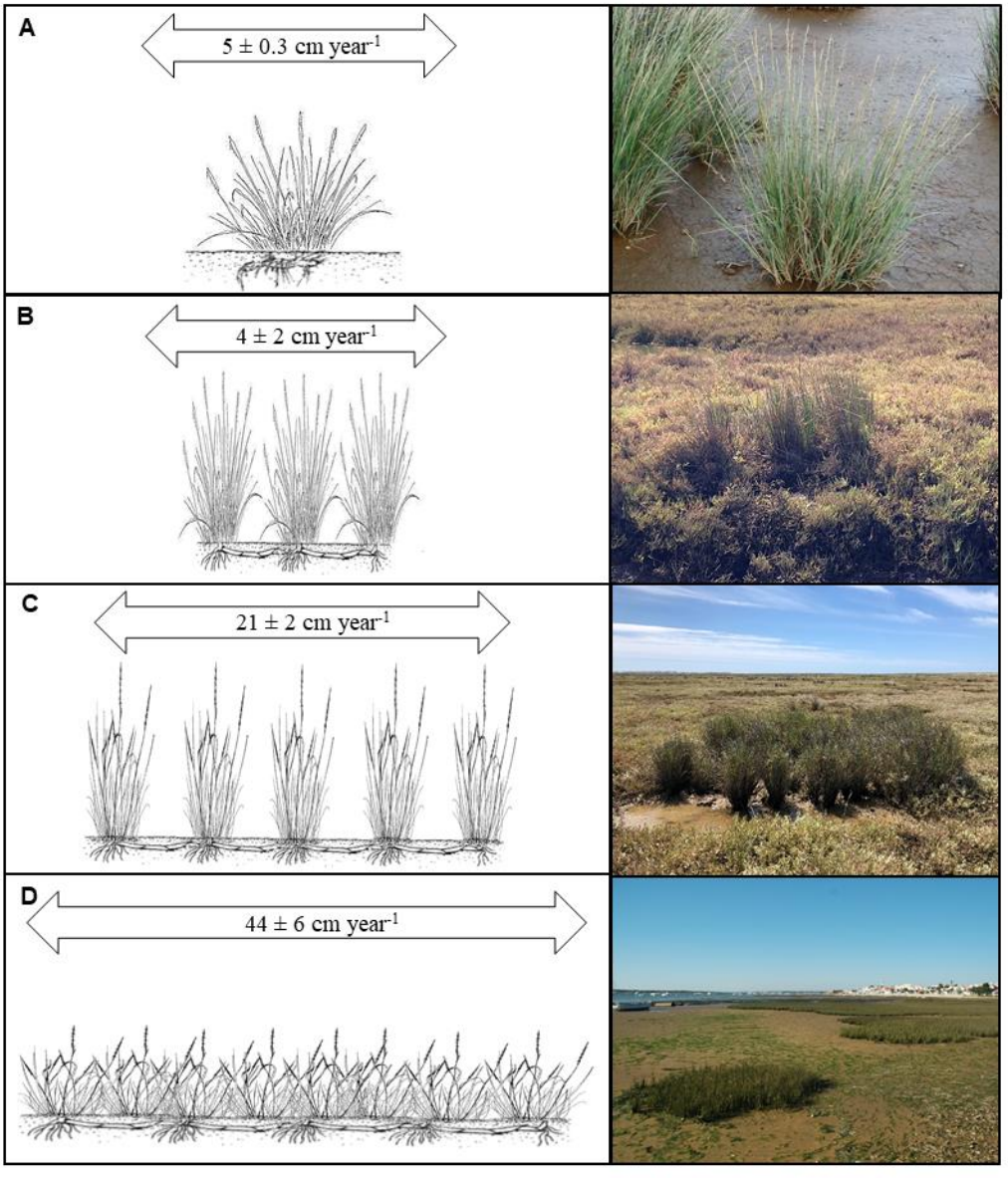
Appendix 10.A. Tussocks used for the calculation of the lateral expansion rate by rhizomes of the hybrids between *Spartina maritima* and *S. densiflora*, indicating their estuary, the main accompanying species, the date for the first diameter measure (the diameter of all tussocks was re-measured on 17-18 May 2018), and their lateral expansion rate (cm yr^{-1}). Species: *Sd = Spartina densiflora*; *Sm = Spartina maritima*; *Sp = Sarcocornia perennis*; *Sf = Sarcocornia fruticosa*; *Ap = Atriplex portulacoides*; *Spxf = Sarcocornia* hybrid between *S. perennis* and *S. fruticosa*.

S. densiflora x maritima				*S. maritima x densiflora*			
Estuary	**Main accompanying species**	**Date of 1st measurement**	**Lateral exp. rate**	**Estuary**	**Main accompanying species**	**Date of 1st measurement**	**Lateral exp. rate**
Piedras	*Sd*	04/03/2005	12	Piedras	*Spxf*	04/03/2005	43
Guadiana	*Sd*	20/01/2003	3	Piedras	*Spxf*	04/03/2005	54
Guadiana	*Sd*	20/01/2003	2	Guadiana	*Sm*	15/02/2006	27
Guadiana	*Sd*	20/01/2003	4	Guadiana	*Sp*	15/02/2006	17
Guadiana	*Sd*	20/01/2003	1	Guadiana	*Sp*	15/02/2006	18
Guadiana	*Sd*	20/01/2003	3	Guadiana	*Sp*	15/02/2006	21
				Guadiana	*Sp*	15/02/2006	16
		Mean ± SEM	**4 ± 2**	Guadiana	*Sp*	15/02/2006	19
				Guadiana	*Sf*	15/02/2006	25
				Guadiana	*Sf*	15/02/2006	25
				Guadiana	*Sf*	15/02/2006	35
				Guadiana	*Sf*	15/02/2006	34
				Guadiana	*Ap*	15/02/2006	23
				Guadiana	*Ap*	15/02/2006	21
				Guadiana	*Ap*	15/02/2006	15
				Guadiana	*Ap*	15/02/2006	18
				Guadiana	*Ap*	15/02/2006	25
				Guadiana	*Sd*	15/02/2006	9
				Guadiana	*Sd*	15/02/2006	15
				Guadiana	*Sd*	15/02/2006	8
				Guadiana	*Sd*	15/02/2006	6
				Guadiana	*Spxf*	15/02/2006	6
				Guadiana	*Spxf*	15/02/2006	19
				Guadiana	*Spxf*	15/02/2006	10
				Guadiana	*Spxf*	15/02/2006	25
				Guadiana	*Spxf*	15/02/2006	27
						Mean ± SEM	**21 ± 2**

Appendix 10.B. Size differences of one individual of *S. maritima x densiflora* and one of *S. densiflora x maritima* from Piedras Estuary between 2008 and 2018. The diameters of each individual and year are indicated in each picture. Images are not scaled.



Appendix 10.C. Schematic models of growth forms, field pictures and lateral expansion rates by rhizomes for tussocks of *Spartina densiflora* (n = 17) (A), *S. densiflora x maritima* (n = 6) (B), *S. maritima x densiflora* (n = 26) (C) and *S. maritima* (n = 13) (D).



Appendix 10.D. The duration of the flowering period of *S. maritima* was evaluated for 20 tussocks during a warm flowering period in 2017 (mean temperature 24.1 ± 1.7 ºC and maximum temperature 30.6 ± 2.1 ºC for May-July) and for 5 tussocks during a mild flowering period in 2018 (mean temperature 21.4 ± 1.6 ºC and maximum temperature 27.2 ± 1.7 ºC for May-July) in Odiel Marshes. The duration of the flowering period of *S. densiflora* was obtained from Valdes et al. (1987) and Castillo & Figueroa (2009).

The number of pollen grains per anther of *S. maritima* was calculating by extracting 5 anthers from each of 2 tussocks in two different locations in July 2017, staining them in a mix of a few drops of cotton blue lactophenol solution in 1.5 ml of water, taking 3 aliquots of 10 µl and counting pollen grains on a microscope slide. The total number of pollen grains per anther was calculated as product of the mean pollen concentration in the aliquots (n = 3) per the total volume of pollen suspension. The number of pollen grains per anther has been found to be constant in the Poaceae family (Prieto-Baena et al. 2003). Subsequently, the calculated value of pollen grains per anther was used for the calculation of the pollen:ovule ratio of *S. maritima* in 25 spikelets chosen randomly from each of 20 inflorescences collected at random from 5 tussocks in 2 locations in Odiel Marshes in July 2017 (warm flowering period) and July 2018 (mild flowering period). For this propose the number of spikelets with exerted stamens and the number of spikelets per inflorescence were counted during anthesis. Then, the number of exerted stamens per inflorescence was estimated as product of the number of spikelets with exerted stamens per the number of spikelets per inflorescence and by 3 stamens per spikelet. The number of pollen grains per inflorescence was calculated for 2017 and 2018 as the product of the number of pollen grains per anther and the number of exerted stamens per inflorescence. Finally, pollen:ovule ratio was calculated as the quotient of the number of pollen grains per inflorescence and the number of spikelets per inflorescence, since the *Spartina* Genus presents an uniovular carpel and the number of seminal primordia per inflorescence corresponded to the number of spikelets on each inflorescence. Pollen:ovule ratio for *S. maritima* was compared between 2017 and 2018 using Student's t-test for paired samples.

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