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Genetic diversity of structural species, and stability of populations and ecosystems

Sónia Isabel Rodrigues Aldeia Sanches Massa

Doutoramento em Ciências Biológicas
Especialidade em Ecologia Molecular

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ABSTRACT

Anthropogenic actions are responsible for changes in the environment with irreversible consequences on ecosystems worldwide. The positive effect of greater biodiversity in the stability of communities, stability or productivity of ecosystems and resistance to disturbances such as disease and invasion is often debated, but usually accepted. However, the influence of the genetic component of biodiversity on higher levels of biological organization remains poorly examined.

Some empirical data have shown that the genetic composition of key plant populations can have a strong effect at the level of the community and ecosystem. In ecosystems dominated by one or a few species, such as seagrass meadows or algae stands, the loss of genetic diversity resulting from habitat loss and population fragmentation of that structural species may have consequences on the overall biodiversity and function of the community .

In this study we used a key-species of the intertidal system of the Ria Formosa to combine field studies and manipulative experiments in order to assess the relationship between genetic diversity and the stability (resilience or resistance) of populations. We have determined the sub-lethal temperature of *Z. noltei* in the Ria Formosa to be approx.. 38ºC, and assessed the physiological response of *Z. noltei* shoots from two distinct populations under high temperature stress conditions, as well as the gene expression variations at distinct steps of the stress treatment. We also showed that survival of *Z noltei* shoots can be favoured by higher levels of genotypic and allelic richness, after suffering a diatom bloom.

Keywords: *Zostera noltei*, diversity-stability, high temperature stress, photosynthesis, gene expression, genetic diversity, global warming.

RESUMO

As ações antropogénicas são responsáveis por alterações ambientais com consequências irreversíveis nos ecossistemas de todo o Mundo. O efeito benéfico de uma maior biodiversidade na estabilidade de comunidades, estabilidade e produtividade dos ecossistemas e resistência a perturbações como doenças são frequentemente debatidos mas, em geral, aceites. Contudo, a influência da componente genética na biodiversidade em níveis superiores de organização biológica permanece pouco estudada.

Alguns dados empíricos mostraram que a composição genética de espécies-chave pode ter um forte efeito nas comunidades e no ecossistema. Em ecossistemas dominados por uma ou mais espécies, como pradarias de ervas marinhas, a perda da diversidade genética resultante da perda do habitat e da fragmentação da população de tais espécies estruturais pode ter consequências na biodiversidade global e função da comunidade.

Neste estudo foram utilizadas espécies-chave do sistema intertidal da Ria Formosa através da associação de estudos de campo e experiências manipulativas, com o intuito de estabelecer a relação entre a diversidade genética e a estabilidade das populações (resiliência ou resistência). Foi determinada a temperatura sub-letal de *Z. noltei* na Ria Formosa, de aproximadamente 38ºC, e avaliada a resposta fisiológica de *Z. noltei* de duas populações distintas sob condições de *stress* de elevadas temperaturas bem como das variações de expressão génica em etapas distintas do stress. Foi ainda demonstrado que a sobrevivência da *Z. noltei* pode ser favorecida por níveis mais elevados de riqueza genotípica e alélica após um *bloom* de diatomáceas.

Palavras-chave: *Zostera noltei*, diversidade-estabilidade, *stress* de alta temperatura, fotossíntese, expressão génica, diversidade genética, aquecimento global.

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1. INTRODUCTION

1.1 The study organism, a seagrass

Seagrasses are perennial marine angiosperms, belonging to four plant families (Posidoniaceae, Zosteraceae, Hydrocharitaceae, or Cymodoceaceae), of the order Alismatales, issued from terrestrially evolved clonal plants that adapted back to the marine environment. Phylogenetic reconstructions showed their group is paraphyletic, supporting multiple events of return to the marine environment (Les et al. 1997; Hemminga and Duarte 2000). Fossil records and present day distribution suggest seagrasses have never exceeded 60 species worldwide, representing less than 0.02% of flowering plants (Les and Philbrick 1993). This low speciation rate has been attributed to the lack of barriers to gene flow in the marine environment (Vermeij 1987; Palumbi 1994), allowing for large-scale dispersion, as well as to the adaptation of pollination to the aquatic habitats, precluding the co-evolution with pollinators that is thought to be responsible for high co-speciation in insects and terrestrial angiosperms (vanderHage 1996; Les et al. 1997).

Seagrasses have long and narrow, ribbon-like leaves that compose shoots, which are connected by horizontal and/or vertical rhizomes. Reproduction can be either clonal (vegetative growth producing new clonal units called ramets – Fig. 1) or sexual, mediated by flower and seed production (producing novel genetic combinations, or genets). In the aquatic environment, spores and pollen are passively transported by water (Denny and Shibata 1989; Serrao et al. 1996; Ackerman 1997; Engel et al. 1999) lowering the efficiency of fertilization compared to that mediated by animals (Cox 1983; Barrett et al. 1993). The dominance of clonal growth in numerous meadows may also be responsible for the low evolutionary rate in seagrasses (Aires et al. 2011; Arnaud-Haond et al. 2012). Inbreeding depression can occur in seagrasses (Price and

Waser 1979; Billingham et al. 2007) as the dispersal of seeds and pollen is limited (Alberto et al. 2005), thus neighbouring plants are often closely related, increasing the probability of mating between clones, siblings and/or parent-offspring (Waycott et al. 2009).

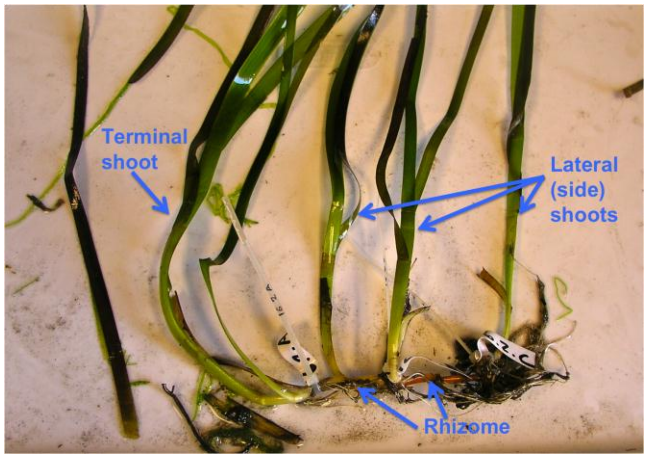

Figure 1: *Z. marina* growing through clonal reproduction. Photo shows only one multi-locus genotype (genet), composed of several shoots (ramets) connected by the same rhizome.

Seagrasses often grow in large meadows that resemble underwater grasslands, which can be monospecific or mixed beds with more than one species. As they are photosynthetic organisms, their growth is limited to the photic zone of shallow coastal habitats, usually in sandy or muddy bottoms. Individual growth, maximal shoot density and reproductive output usually occurs during the summer (Reusch et al. 2005). Seagrasses are ecosystem engineers, as they modulate the availability of resources to other species (Lawton 1994) by slowing down water motion, increasing sedimentation and stabilizing the seabed with their roots and rhizomes, generating nursery areas, shelter and food for many invertebrate and fish species (Duarte and Cebrian 1996). They are one of the main primary producers in the aquatic environment, and are also an essential carbon sink, accounting for about 12% of the total oceanic carbon storage (Duarte and Cebrian 1996). Seagrasses are also key biological sentinels, as changes in their distribution or biomass reflect changes in the environment (Orth et al. 2006).

There are four seagrass species, native to Europe, occurring in the Mediterranean Sea, namely *Posidonia oceanica*, *Cymodocea nodosa*, *Zostera marina*

and *Zostera noltei. Z. noltei* is distributed along the coasts of Europe, the Canary Islands and North Africa, from the Baltic Sea to Mauritania (den Hartog 2003).

Figure 2: Distribution range of *Z. noltei. In* Encyclopedia of Life

In the Mediterranean Sea, it can be found in shallow, sheltered and muddy habitats such as brackish lagoons, bays and small harbours (Fig. 3), often in mixed beds with *C. nodosa* or with *Z. marina* (Meinesz and Simonian 1983; Loques et al. 1990; Ribera et al. 1997; Menendez et al. 2002; Plus et al. 2003; Pergent-Martini et al. 2005).

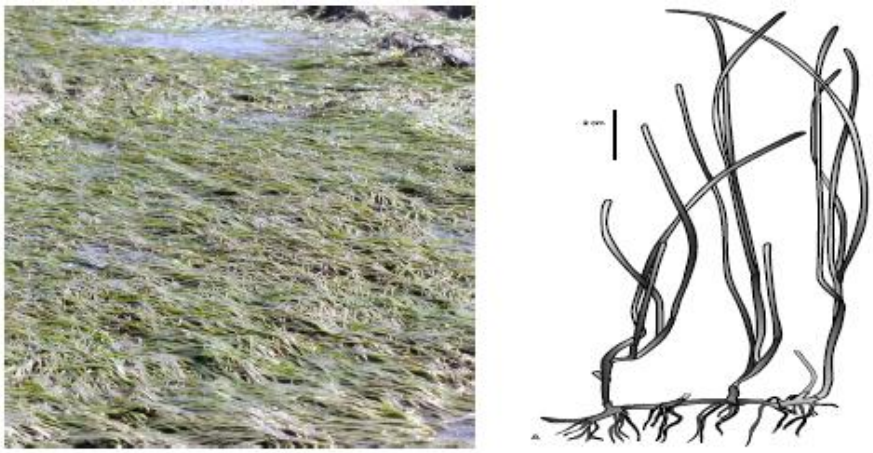

Figure 3: Photograph of a seagrass meadow and drawing of a seagrass. *in* European Seagrasses: an introduction to monitoring and management, Chapter 1: The four European seagrass species

Z. noltei is the dominant seagrass species of the intertidal habital of the Ria Formosa (Silva et al. 2005), a coastal lagoon on the South coast of Portugal. This is a warm distributional margin for temperate seagrasses in the genus *Zostera*. Extinction

has occurred in several meadows of the closely related species *Z. marina* (Guimaraes et al. 2012) since they were studied by Billingham and colleagues (Billingham et al. 2003; Billingham et al. 2007). The heat waves observed in 2003 that also affected other *Z. marina* populations throughout Europe (Reusch et al. 2005; Reusch 2006), might have contributed to these local extinctions. These extinct meadows were genetically unique and highly differentiated (Diekmann and Serrao 2012), and had locally adapted features (Billingham et al. 2007).

1.2 Ecosystem perturbations

Anthropogenic actions are responsible for changes in the environment with irreversible consequences on ecosystems worldwide. The most common causes are pollution, habitat loss and degradation (especially from water diversion for agriculture), introduction of non-indigenous species and human predation (Noss and Murphy 1995) (Fig. 4). The increasing occupation of coastal areas by humans has significantly altered the habitats, causing degradation of estuaries and coastal seas due to overexploitation, nutrient and sediment runoff, invasive species, hydrological and physical alterations and commercial fishing (reviewed in Gray 1997). Ocean acidification is currently a great concern for marine habitats. As the uptake of carbon dioxide from the atmosphere increases, the pH of the ocean decreases due to the accumulation of hydrogen ions, leading to a decrease in carbonate ions that are essential to the calcification process of the skeletons and shells of several marine organisms. Ocean pH is expected to decrease by 0.3-0.4 units, relative to the pre-industrial era, by the end of the century (Feely et al. 2009).

Figure 4: List of anthropogenic action that lead to the degradation of coastal habitats and their relative significance in each marine habitat type. *In* United Nations Environment Programme

1.2.1 Climate change

Climate change, particularly the expected temperature rise predicted by IPCC, is one of the biggest threats forecasted, expected to induce changes in distribution ranges and high levels of mortality, and raising concerns as to the survival of many species. In temperate regions of the North Atlantic, sea surface temperature (SST) is expected to increase by 0.2°C per decade, while global average surface warming is estimated to be between 2 and 4ºC for the 21st century (IPCC 2007). The situation is thus more alarming for coastal areas than for the open ocean, particularly in shallow water habitats like the intertidal where temperature regimes are more variable and can reach limiting values (Helmuth et al. 2006a; Pearson et al. 2009) Shifts in range distributions have already

been reported as lethal temperatures for some organisms may be reached during low tide periods (Wethey 2002; Zacherl et al. 2003; Mieszkowska et al. 2006b).

The intertidal habitats are characterized by their extreme environmental conditions, with daily variations of light, salinity, desiccation and temperature. During low tide, the temperature in isolated pools is more influenced by air temperature and irradiance than water temperature, leading to extremely high temperatures, especially during the summer. These severe temperature/desiccation conditions have been shown to cause biomass loss in numerous seagrass meadows. In South Australia, a large-scale dieback of 12,717 ha of intertidal and subtidal seagrasses occurred due to the heat waves of the end of the summer of 1993 (Seddon et al. 2000). In Indonesia, above-ground biomass of the mixed seagrass bed of *Thalassia hemprichii* and *Enhalus acoroides* decreased as low tide exposure during spring tides switched from night (January - June) to day (July – December) (Erftemeijer and Herman 1994).

As a consequence of the seagrass mortality at an estimated rate of 110 km^2/year since 1980, approx.. 29% of all known seagrass biomass has disappeared since they were first recorded in 1879, in an estimated total of 51,000 km^2. Mortality rate has been increasing as well, rising from 0.9% before 1940 to 7% after 1990. In approx.. 60% of the cases, it was either due to direct impact of coastal development and dredging activities or indirect impacts from declining water quality (reviewed in Waycott et al. 2009).

1.3 Photosynthetic efficiency

One of the first symptoms of heat stress is the inhibition of photosynthesis (Fig. 5), as high temperature stress can cause severe damage in the photosynthetic apparatus (Berry and Bjorkman 1980; Camejo et al. 2005; Guo et al. 2006; Hassan 2006).

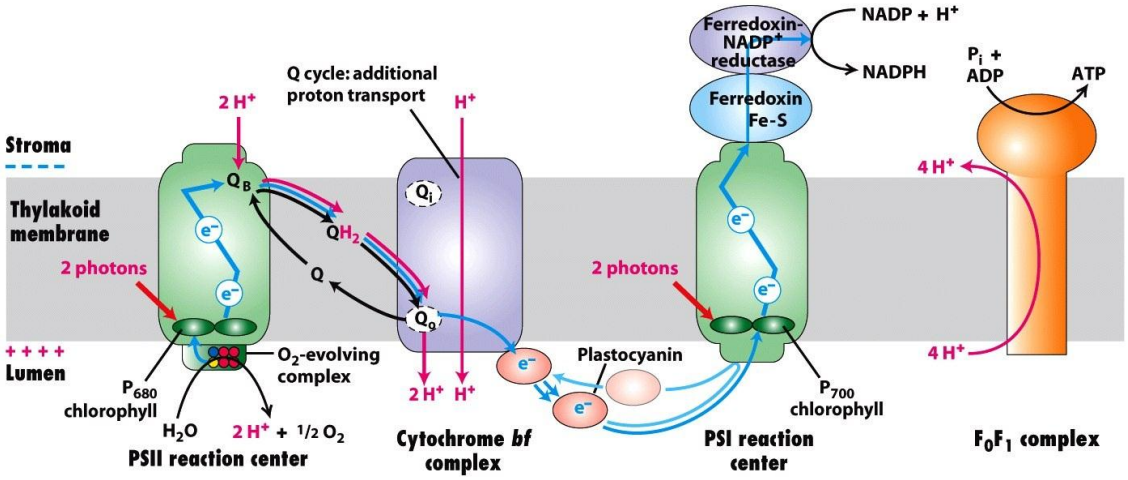

Figure 5: Light-dependent reactions of photosynthesis at the thylakoid membrane. *In* Molecular Cell Biology, Sixth Edition © 2008 W.H. Freeman and Company

Chlorophyll fluorescence has been shown to be a reliable method of determining the physiological condition of photosynthetic organisms and its interrelation with photosynthetic activity was first observed and described by Kautsky and Hirsch (1931). It is especially useful for *in situ* measurements, as it is a very fast and sensitive, non-invasive, non-destructive method that allows verification of short-term responses to various environmental stresses, such as light quality (Ralph and Burchett 1995; Ralph 1996; Ralph 1999b), UV radiation (Dawson and Dennison 1996; Flanigan and Critchley 1996), desiccation (Seddon and Cheshire 2001) and toxics such as herbicides (Haynes et al. 2000; Ralph 2000). It allows monitoring the regeneration of the photosynthetic apparatus when the stress factor is removed.

Previous studies examining the relationship between chlorophyll fluorescence and photosynthetic performance, as measured by oxygen evolution, have demonstrated that pulse amplitude modulation (PAM) fluorometry is a valid proxy for photosynthetic production of *Z. noltei* in a light range between 35 and 490 µmol photons m^{-2} s^{-1} (PAR) (Silva and Santos 2004). The photochemical efficiency of photosystem II (PSII) is determined by assessment of several fluorescence parameters. The initial fluorescence

(F_0) represents the fluorescence when the PSII reaction centres are open and the overall condition of the PSII reaction centres. An increase in F_0 is attributed to photodamage, while a decrease in F_0 is believed to reflect photoprotection (Demmig and Bjorkman 1987). Maximal fluorescence F_m is proportional to the amount of reaction centres closed due to stress. A reduced F_m indicates photosynthetic downregulation and closure of PSII reaction centres associated with plant stress. F_v, the variable fluorescence, is defined as the difference of maximal and minimal fluorescence ($F_v = F_m - F_0$) of dark-adapted leaves (Ball et al. 1995; Roháček 2002) and the ratio between light-induced variable and maximum fluorescence of dark-adapted leaves (F_v/F_m) is an indicator of the potential photochemical efficiency PSII. The higher the F_v values with the corresponding F_0 and F_m, the better the performance of the photosynthetic apparatus (Roháček 2002). The F_v/F_m parameter is often used to assess plant resistance to different stress factors, as it usually decreases after the stress is applied, which may reflect certain disorders in the electron transport chain (ETC) of PSII (Venediktov et al. 1999; Roháček 2002).

The influence of high temperature in photosynthesis has been previously studied in several terrestrial plants (Cui et al. 2006; Guo et al. 2006; Yang et al. 2006) and in seagrasses (Terrados and Ros 1995; Ralph 1999b; Campbell et al. 2006; Cui et al. 2006).

1.4 Gene expression

In addition to reducing the photosynthetic yield and increasing photoinhibition in plants, high temperature stress also induces changes in gene expression, as a way to maintain cellular homeostasis. An increase in the synthesis of heat-shock proteins in response to high temperatures is expected, as it is usually the first response to any kind of stress.

With the development of molecular techniques such as DNA extraction, amplification and sequencing in the recent past, there has been a significant increase in the amount of genetic markers available in the form of allozyme, mitochondrial DNA, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite, single nucleotide polymorphism (SNP), and expressed sequence tag (EST) (Liu and Cordes 2004).

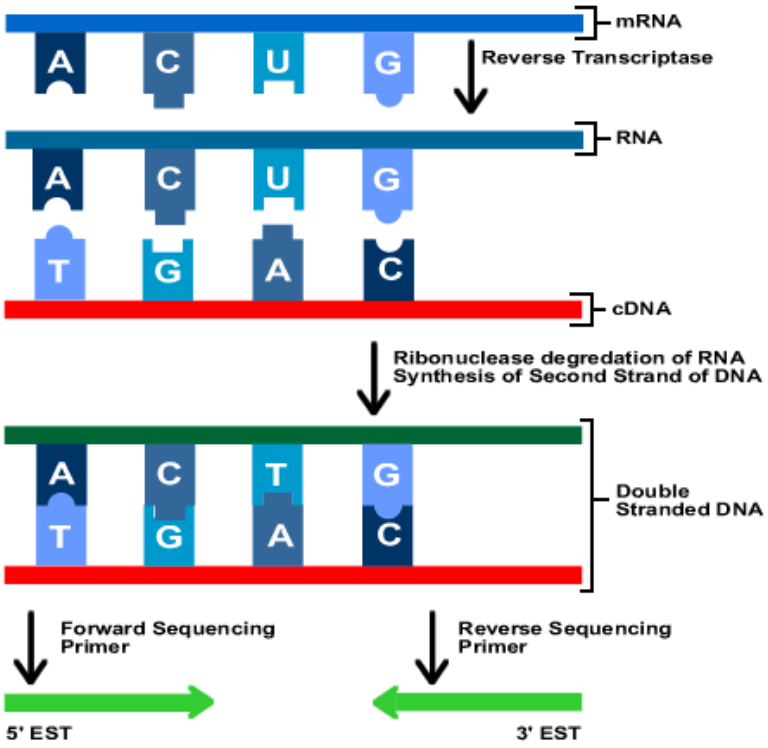

Figure 6. An overview of how ESTs are generated. *In* NCBI.

ESTs are produced by the sequencing of single-read cDNA clones reverse transcribed from a bulk mRNA pool (Fig. 6). They provide a snapshot of gene expression at a given time which allows for gene expression profiling in different tissues and/or conditions, but are also used for the identification of expressed genes by comparison with annotated sequences in online databases, cDNA microarrays and genome mapping (Rudd 2003; Bouck and Vision 2007).

The mRNA collection of a cell contains the genes that are actively being transcribed at a given moment, thus it may not contain all genes that a cell is able to express and certainly not all at the same ratio. A pool of housekeeping genes is ubiquitously expressed in every cell and the remaining genes may be present or absent in a cDNA library depending on whether they were required for that specific developmental stage or biotic/abiotic challenge or not. However, the absence of an EST does not mean the gene is not present in the genome or is not being expressed; it just may have not been measurable. To minimize the effect of uneven representation of some genes, both oligofingerprinting and normalization/subtraction of libraries can be used (Rudd 2003).

1.5 The diversity-stability debate

Although there is still much debate regarding the relationship between diversity and stability, most studies point to a positive effect of higher biodiversity (i. e. species diversity) in the stability of communities, stability or productivity of ecosystems (Tilman 1996; Hector et al. 1999; Lehman and Tilman 2000), and resistance to disturbances such as disease and invasion (Knops et al. 1999) or drought (Tilman and Downing 1994). Although the relationship between species diversity and some components of ecosystem stability has been extensively studied (reviewed in Schlapfer and Schmid 1999; McCann 2000), the influence of the genetic diversity – which reflects the evolutionary potential of species - remains poorly understood.

1.6 Genotypic diversity: a specific level of diversity in clonal organisms

Microsatellites, or simple sequence repeats, are repeated sequences of nucleotides. Since they are typically located in neutral, non-coding regions of the DNA, they have high mutation rates, allowing for the differentiation of phenotypically

identical organisms with clonal reproduction like seagrasses. In diploid entities there are two alleles (two forms of a gene) for each *locus* (specific location of a gene in the chromosome). If the two alleles are the same, the individual is homozygous for that *locus*; if they are different, the individual is heterozygous (Fig. 7). By analysing a sufficient number of microsatellite *loci* per individual, it is possible to identify distinct genotypes that otherwise do not differ in coding regions of the genome. However, in order to safely assign two individuals to the same Multi Locus Genotype (MLG) one must first verify the probability of finding identical MLGs resulting from distinct sexual reproductive events and the probability of somatic mutations or scoring errors (reviewed in Arnaud-Haond et al. 2007).

Box 1 Glossary

Number of ramets (N): total number of modular units of the same genetic individual

Number of genets (G): total number of multi-locus genotypes or lineages;

Genotypic richness (R): proportion of different genets in each sample:

$R = (G - 1)/(N - 1)$. (Dorken & Eckert 2001)

Allelic richness (A): total number of alleles;

Heterozygosity (H): the fraction of individuals in a population that are heterozygous for a particular locus. Comparing observed (H_o) with expected (H_e) heterozygosities allows to infer departures from equilibrium;

Genetic diversity: the genetic component of species diversity; the *proxy* for genetic diversity can vary between studies (total number of alleles, mean number of alleles, heterozygosity, etc).

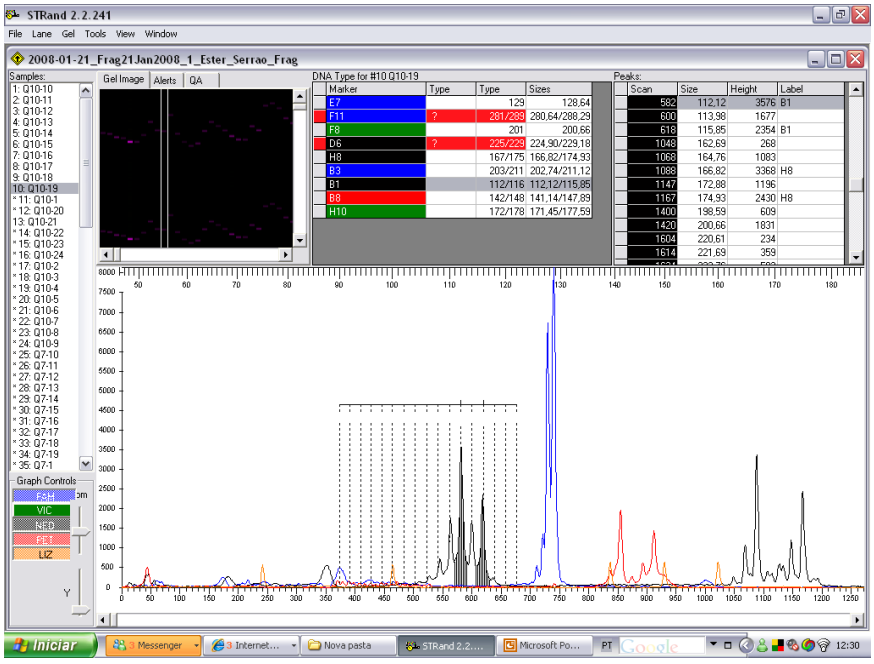

Figure 7: Electrofluorogram of four *Z. noltei* microsatellite *loci*. Highlighted are the two alleles for locus B1, in black; in blue is locus E7 (homozygote); locus B8 in red (heterozygote) and locus H8 in black aswell (heterozygote), in the far right.

Vegetative growth can have an important role in the dispersal of the species. Disturbance affects clonal reproduction, depending on the relative importance of modes of propagation (sexual versus clonal) and the dispersal characteristics of the propagules; these effects determine genotypic richness. If disturbance generates and disperse fragments, genotypic diversity decreases with perturbation; if the vegetative propagation is insensitive to perturbation, genotypic diversity initially increases (Coffroth and Lasker 1998).

Habitat loss and fragmentation causes loss of genetic diversity by elimination of genotypes and alleles (Diaz-Almela et al. 2007; Diekmann and Serrao 2012), putting populations at risk of extinction due to their reduced adaptive potential and to the fixation of deleterious alleles by genetic drift and elevated inbreeding, as a consequence of small effective population sizes (Diaz-Almela et al. 2007; Provan et al. 2008). On the other hand, old and large seagrass clones can have higher survival rates than small ones,

probably due to a lasting adaptation to the local conditions, clonal integration and foraging capacity – as larger clones can reach a higher number of micro-habitats in which they can successfully thrive (Diaz-Almela et al. 2007; Arnaud-Haond et al. 2010; Arnaud-Haond et al. 2012). In this case, a population may benefit from expanding by clonal reproduction and maintaining low genetic diversity, just as long as the main large clone is resilient to future environmental changes.

Recent literature on seagrasses (Hughes and Stachowicz 2004; Reusch et al. 2005; Ehlers et al. 2008) sometimes confounds genetic (allelic richness or heterozygosity level) and genotypic diversity (Box 1). Such studies demonstrated a relationship between resistance or resilience to perturbations with genotypic diversity but not with genetic diversity.

1.7 Aim and outline of the thesis

The main objective of this thesis was to better understand the link between genetic diversity and resistance to environmental stress, as well as the response of a key-species of the intertidal habitat of the Ria Formosa, *Z. noltei*, to the present and future challenge of warming. Research goals involved accessing the effect of high temperature stress in (i) photosynthetic efficiency and (ii) gene expression, as well as determining the influence of genetic diversity in the resistance and resilience to heat-stress conditions.

Chapter I shows the determination of the sub-lethal temperature of *Z. noltei* shoots in the Ria Formosa, by testing four different temperatures in individuals from two distinct populations, in tanks with simulated tide conditions.

Chapter II evaluated the influence of high temperature stress in gene expression, by determining the evolution of gene expression at different time steps of the heat-shock treatment using ESTs (expressed sequence tags).

In Chapter III we assessed the phenotypic variability in the response to heat-shock of distinct genotypes of *Z. noltei*, by submitting a pre-genotyped group of genets from two different populations to the pre-determined sub-lethal temperature and analysed photosynthetic efficiency in each one of them.

Finally, in Chapter IV we analysed the influence of genetic diversity in the resistance and resilience of *Z. noltei* to stress, by pre-defining different combinations of genotypic and allelic richness and comparing survival after stress.

CHAPTER I: TEMPERATURE TOLERANCE AND SURVIVAL OF INTERTIDAL POPULATIONS OF THE SEAGRASS *ZOSTERA NOLTEI* (HORNEMANN) IN SOUTHERN EUROPE (RIA FORMOSA, PORTUGAL).

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Abstract

The dwarf seagrass *Zostera noltei* is an important primary producer in Atlantic coastal ecosystems from Mauritania to southern Norway and the Mediterranean Sea. Sessile intertidal organisms existing at the interface between marine and terrestrial environments may be particularly vulnerable to environmental change. In this study we asked how near to thermal tolerance limits natural populations of *Z. noltei* are in the Ria Formosa coastal lagoon system in southern Portugal. We recorded the maximum temperatures in the Ria Formosa during the 2007 summer, and conducted experiments to determine the sub-lethal temperature of *Z. noltei* shoots sampled at two sites located at different tidal heights. Mortality rates and photosynthetic performance were recorded within a range of heat shock temperatures between 35 and 41ºC. Survival was recorded ≤ 37ºC, while higher temperatures led to a sudden drop in photosynthetic capacity followed by mortality (shoot loss) that occurred more rapidly with increasing temperatures. At 39ºC and above, the rate of shoot mortality in both sites was close to 100%, occurring between 5 and 13 days after the heat shock. Survival was ca. 95% and 90% at 35ºC and 37ºC, respectively. From these results for *Z. noltei* populations in the Ria Formosa we estimated sub-lethal temperature to be approximately 38ºC for *Z. noltei*, close to the maximum of 36°C recorded in the summer 2007. Considering predicted trajectories in the coming decades, these results raise concern as to the future viability of intertidal *Z. noltei* populations near the southernmost edge of their distribution.

Predictions for climate change scenarios along temperate regions of the North Atlantic forecast sea surface temperature (SST) to increase by 0.2°C per decade (IPCC 2007), with temperatures increasing more rapidly near coastal areas than in the open

ocean. The impact of such changes in marine ecosystems is, however, likely to be magnified in intertidal habitats (Helmuth et al. 2006a), where shifts in range distributions have already been reported (Zacherl et al. 2003; Mieszkowska et al. 2006b). Complex patterns of temperature variation may occur in the intertidal, and lethal temperatures for some organisms may be reached during low tide periods (Wethey 2002). Climate change will have major impacts when affecting species that play key roles in ecosystem function, as is the case for seagrasses (Duarte 2002). The seagrass *Zostera noltei* is one such key player in ecosystem functioning, in sheltered soft bottom habitats along the Eastern North Atlantic.

Seagrasses are key-species in coastal systems, contributing most of the primary production, while also providing nursery areas and food for many invertebrate and fish species. In intertidal ecosystems, cycles of immersion and emersion mean that seagrass meadows are exposed to a wide range of environmental variation in, e.g., temperature, desiccation and solar radiation. Water temperature in small isolated pools during low tide is primarily a function of air temperature and irradiance, and can therefore, reach extremely high values, especially during summer.

In the Ria Formosa, a coastal lagoon on the South coast of Portugal, *Z. noltei* dominates the intertidal zone (Silva et al. 2005) and is one of the main primary producers in this area. For the sister species *Zostera marina* in the Ria Formosa local population extinction has been observed during the past five years (EAS, personal observation) in two meadows previously studied (Billingham et al. 2003; Billingham et al. 2007). The factors responsible for this local disappearance are unknown, but it correlates with the warmest summers experienced throughout Europe, including Southern Portugal, and which have strongly affected other *Z. marina* populations (Reusch et al. 2005; Reusch 2006). In common with *Z. marina* at higher latitudes

(Reusch et al. 2005), individual growth, maximal shoot density and reproductive output of *Z. noltei* in the Ria Formosa occurs mostly during the summer (Alexandre et al. 2005; Peralta et al. 2005), in the same window of time when patches of local mortality are commonly observed (J. Silva pers. comm.), coinciding with sites most exposed to desiccation during the warmest annual temperatures. There is therefore concern for persistence of the species in these habitats in the context of predicted climate change.

Photosynthesis is a heat-sensitive process, thought to be primarily due to heat labile components associated with PSII (Berry and Bjorkman 1980; Weis and Berry 1987; Rokka et al. 2000), and can provide evidence of heat stress before other symptoms are detected. Chlorophyll fluorescence has been shown to be a reliable method of determining the physiological condition of photosynthetic organisms (Beer et al. 1998; Silva and Santos 2004), since its relationship with photosynthetic activity was first observed (Kautsky and Hirsch 1931b). Rapid, non-invasive measurements using Pulse Amplitude Modulated (PAM) fluorometry have been successfully applied in several studies of seagrass species (Beer et al. 1998; Bjork et al. 1999; Schwarz et al. 2000; Schwarz and Hellblom 2002) and PAM fluorometry has been shown to be a valid proxy for photosynthetic production of *Z. noltei* except at photoinhibitory irradiances, where photorespiration is likely responsible for deviations from expectations based on gas exchange (Beer et al. 1998; Silva and Santos 2004). The available evidence for various seagrass species suggests that capacity to tolerate light and/or temperature stressors may be more important in setting vertical distributional limits on the shore than desiccation tolerance (Bjork et al. 1999; Ralph 1999b).

Here we report the capacity of *Zostera noltei* to resist and recover from heat shock during simulated low tide exposures in one of the warmest parts of its range, the Ria Formosa Natural Park in southern Portugal. Experimental stress exposures were

based on some sparse water temperature data (unpublished) available for the Ria Formosa, and confirmed later on with daily records in natural *Z. noltei* stands. Shoot mortality rates and photosynthetic performance were monitored over a time course of several days following the stress. The natural thermal environment during summer was recorded by logging the temperature in an intertidal *Z. noltei* meadow in the Ria Formosa, at three different depth levels, over a period of ca. 7 weeks in order to record the maximum temperatures *Z. noltei* currently experiences.

In situ temperature profiles.

Temperature loggers (DS1921G Thermochron® iButton®, +/- 1ºC accuracy, 0.5ºC resolution) were placed at three heights from the upper to lower limits in natural *Z. noltei* meadows at Ramalhete (Ria Formosa) to record summer temperatures. At each of the three heights, two iButtons® were placed 2 cm below the surface (in the sediment) and two above surface (near *Z. noltei* leaves). Therefore, four replicate measurements were planned per tidal height. However, some iButtons® were lost during the course of the experiment such that in the sediment at medium and low tide levels respectively only two and one data series could eventually be collected. Temperature data were recorded every 60 minutes from the 30th of July to the 12th of September of 2007, which spans the period when temperatures usually reach their annual maxima .

Heat shock experiments

Z. noltei plants were collected in sets of several shoots connected by one rhizome in April 2007 at two sites in the Ria Formosa: Ramalhete (37º 00' 18'' N. 7º 58' 01'' W) and Praia de Faro (37º 00' 15'' N. 7º 59' 16'' W). Since the size of *Z.*

noltei clones in the Ria Formosa may reach several meters (Diekmann et al. 2005), plants were collected haphazardly across the meadow with a minimum distance of 10 meters between samples in order to minimize clonal repetition. The populations sampled were representative of a range of tidal exposure conditions that can be met throughout the Ria Formosa. The meadow from Ramalhete is exposed to air for up to 6 hours during spring low tides, whereas Praia de Faro is lower in the intertidal, and plants do not become completely dry, but remain covered by residual, very shallow water during daily low tide periods.

Samples of *Z. noltei* were placed in small plastic aquaria (approx. 2.5 L) re-planted in approximately 10 cm sediment from the Ria Formosa, in an outdoor tank (1.5 x 1.5 m, maximum seawater volume 225 L) with continuous seawater flow and artificially simulated tides. Plants were acclimated for approximately one month before heat shock tests were performed at 4 temperatures between 35 and 41ºC. For each temperature treatment, 20 individual shoots from each site were transferred into one individual small plastic aquarium (approx. 2.5 L) and re-planted in sediment, under approximately 5 cm aerated seawater. Additionally, one aquarium per site was similarly prepared with 20 individual shoots, to act as a control for the temperature stress. Both control aquaria were kept in the tank with continuous water flow and simulated tides during the course of the experiments. The heat-shock was applied for 3 h, to mimic a low tide exposure, by warming the water with thermostatic heaters until the temperatures to be tested (35, 37, 39 or 41 ºC) were reached. Temperatures were chosen on the basis of unpublished data, and compared afterwards with data obtained from iButtons® showing 36°C as maximum temperature in 2007 (here detailed) and 38°C as maximal temperature in July 2008 in order to ensure that they were appropriate proxies for predicted increases due to climate change. After the heat shock, ambient seawater

was gradually added (approx. flow rate 0.3L/min) to the small aquaria to return the temperature to its initial level. The small aquaria with the shoots were then transferred back to the large tank with simulated tides.

The physiological effects of heat shock were assessed from chlorophyll fluorescence measurements of Fv/Fm, the maximum quantum yield of photosystem II (PSII), using a portable fluorometer (FMS2, Hansatech, UK). Measurements were performed over time for each shoot in the four temperature treatments and in both controls. Prior to fluorescence measurements, leaves were dark-adapted for a minimum of 10 minutes, after which the minimum (Fo) and maximum (Fm) fluorescence yield of open and closed PSII reaction centers before and after a saturating light pulse, respectively, were determined. Fv/Fm was measured periodically after the heat shock in all shoots, until they had recovered or died (determined by the brownish-black discoloration of the leaves or loss of them). The survey was stopped in the treatments that still had surviving shoots after approximately 3 weeks, when significant mortality was no longer occurring and most of the remaining shoots had reached Fv/Fm values that did not differ significantly from those in controls.

Mean and SE of the Fv/Fm values for every available shoot were estimated for each time step, treatment and population. We performed a factorial ANOVA (STATISTICA 7.0 © StatSoft, Inc.) to address three specific questions at particular points in time: i) are any differences detectable between the initial Fv/Fm ratio of plants from both sites (by comparing values before experiments, at t=0, performing an ANOVA among treatments and sites); ii) How is photosynthetic efficiency affected by heat-shock conditions (by comparing values before mortality peaks, at t=24 hours, performing an ANOVA among treatments and sites); iii) Is survival different among treatments, and do the survivors recover the same photosynthetic efficiency (comparing

percentage survival and Fv/Fm values of survivors, controls and sites, using ANOVA at the end of the experiment).

In both populations, an increase in water temperature led to a decrease in all fluorescence parameters, suggesting a high thermal sensitivity of primary photochemical processes in *Z. noltei* shoots. At 41ºC, in both populations, all shoots survived the first 3 days of experiment but after that there was a clear drop in survival eventually leading to 100% shoot mortality for both sites (Figure 1a and 1b). At 39ºC, for both populations, shoot survival was > 95% until the 5th day, at the 7th day there was a sudden drop and mortality reached 25% (Figure 1a and 1b). Over 90% of the shoots were dead by the 17th day and by the end of the survey there was only one surviving shoot from Praia de Faro (Figure 1a and 1b) in the 39ºC temperature treatment. Shoots from Praia de Faro had a survival rate of at least 95% at both 35 and 37ºC (Figure 1a), similar to values for Ramalhete (94% and 80% survival at 35ºC and 37ºC, respectively) (Figure 1b).

The initial mean ratio of shoot Fv/Fm was not significantly different between Ramalhete and Praia de Faro [factorial ANOVA, $p>0.05$, $n=20$ (except for treatments Praia de Faro control, Praia de Faro at 41ºC and Ramalhete at 41ºC, for which $n=19$)], and ranged from 0.692 to 0.775 in Ramalhete, and from 0.741 to 0.799 in Praia de Faro (Figure 1c and 1d). 24 hours after the heat-shock, Fv/Fm was significantly different between treatments (factorial ANOVA, $p=0.000$). The lowest Fv/Fm values were recorded on the 3rd day for Praia de Faro (0.575) (Figure 1c) and on the 7th day for Ramalhete (0.665) (Figure 1d), followed by a gradual increase of Fv/Fm in surviving shoots to their initial level, despite the fact that shoot mortality was still occurring (Figure 1a and 1b).

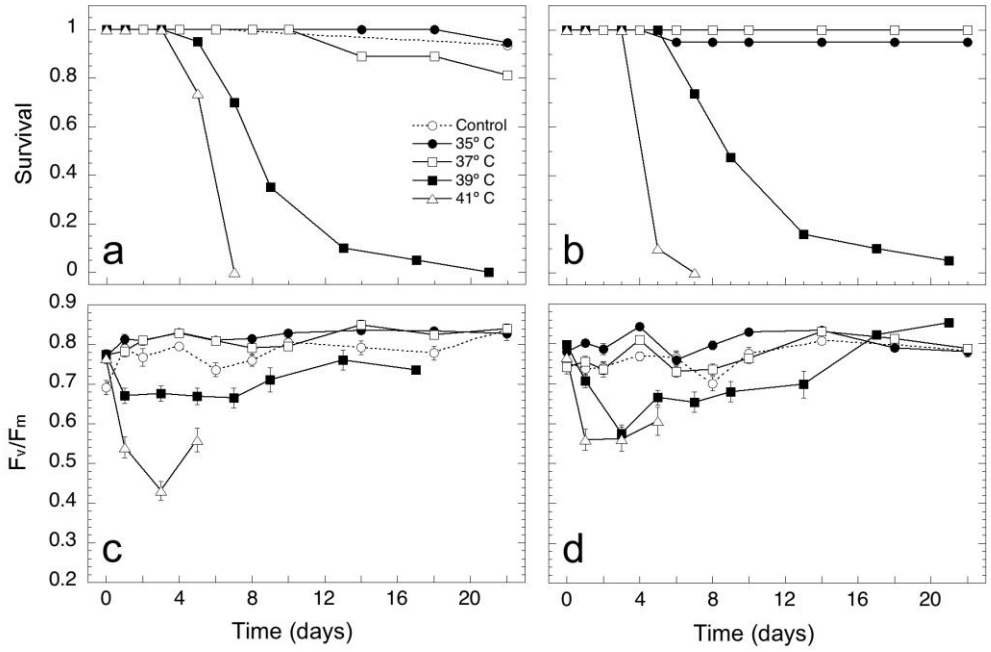

Figure 1. Survival rate (a, b) and Fv/Fm (c, d) for *Z. noltei* shoots from the two sampled sites, Praia de Faro and Ramalhete, at 35, 37, 39 and 41ºC, and in controls (no heat shock applied). Values for Fv/Fm are means ± SE (initial n = 20).

The sensitivity of shoots to a single short exposure of > 37°C can be compared with the maximum temperature observed and predicted in this part of the species range. Temperatures in natural stands recorded from July 30th to September 12th, 2007 at Ramalhete are shown in figure 2. Tidal heights for that period are also shown in figure 2. Temperature in intertidal meadows in Ria Formosa usually ranges from 15 to 35ºC (Alexandre et al. 2004); the data collected this summer shows that sediment/surface temperatures in *Z. noltei* stands routinely reached 30ºC at low tide in August, with even higher temperatures during spring tides (Fig. 2). However, the most exposed plants are more subject to air than to water temperature during low tide, and the plants covered by a thin layer of water may be exposed to even higher temperatures linked to the effect of irradiance (cf. Fig 2a and b), with temperatures measured in the canopy of *Z. noltei*

reaching a maximum of 36ºC, while upper sediment maxima were on average 5ºC lower. It is noteworthy that temperature regimes in these intertidal ecosystems vary not only temporally but also between sites within the same habitat and even between different parts of the same 'individual' (i.e., clone).

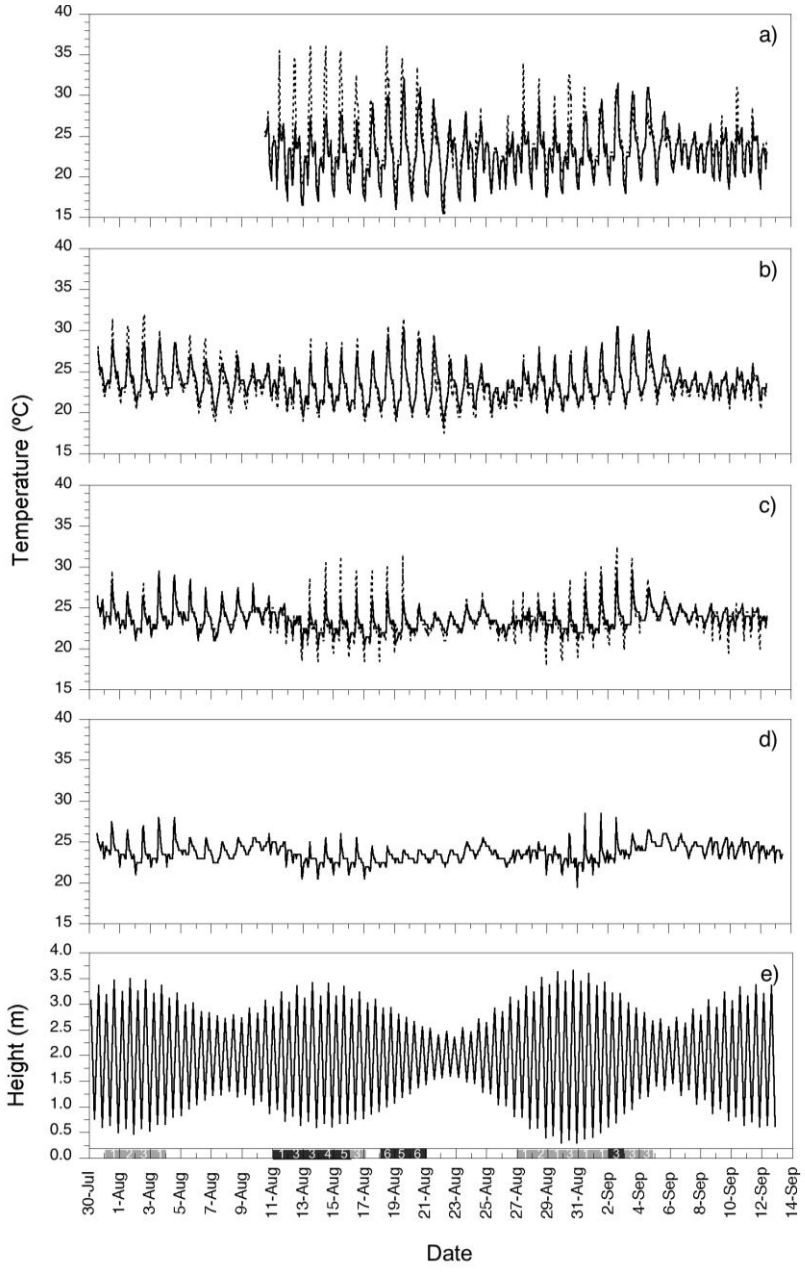

Figure 2: Temperature data recorded in the *Z. noltei* meadow in Ramalhete, once every 60 minutes, from 30/07/2007 to 12/09/2007: a) upper intertidal canopy, b) upper intertidal sediment, c) mid intertidal sediment and d) lower intertidal sediment. Replicate measurements were recorded at each tidal height/microhabitat (full and dotted lines), with the exception of the lower intertidal sediment. Panel e)

shows tide heights for Barra Faro-Olhão for the period between 30-07-2007 and 12-09-2007 . Maximum temperature registered each day is shown in three ranges in the colour bar below the graph, as well as the number of hours it reached temperatures above 30ºC.

Changes in global average surface warming due to rising greenhouse gas concentrations lead to expect an increase of at least 2-4.5ºC to be reached within the next 100 years for air temperature, and 1-3ºC for sea surface temperature (IPCC 2007). Although no data are available for coastal and lagoon seawater, intertidal organisms that are exposed in air or covered by shallow films of water at low tide are likely to be more influenced by air than sea temperature changes. This suggests that in the next century the physiological tolerance of *Z. noltei* may be reached in the warmest habitats of its range, such as southern shallow coastal lagoons, where they are exposed to high air temperatures at low tide. This is particularly true as we applied a single heat shock of three hours, whereas predicted increases in temperature would imply longer, and multiple, exposures to extreme temperatures. Although the Ria Formosa is not at the edges of the distribution for this species, it might be one of the most extreme environments this plant has to cope with. This shallow coastal lagoon indeed reaches extreme temperatures and irradiances during the summer (Silva and Santos 2003; Alexandre et al. 2004) and the greatest mortality for *Z. noltei* has been recorded in the summer (J. Silva, pers. com.), coinciding with the season for greatest shoot production and reproductive output (Alexandre et al. 2005), as for the sister species *Z. marina* during this season (Reusch et al. 2005). It is thus during the main growth and reproductive season of *Z. noltei* that events of local mortality occur in sites under most extreme tidal exposure conditions, suggesting that the present temperatures occurring in the Ria Formosa during summer are already close to the physiological limits of this species. Therefore, the predicted increase of about 0.2ºC per decade in the global

temperature for the next two decades (IPCC 2007) may be especially harmful for *Z. noltei* in many habitats and may affect its distributional range. Local extinction of this species in the Ria Formosa would have a considerably negative effect on the ecosystem, with potential consequences on the very economically important bivalve production in this coastal lagoon.

Our results demonstrate the sensitivity of *Z. noltei* to slight increases in water temperature as it approaches its physiological limits, as even an increase of 2ºC causes an increase in shoot mortality from 5-20% to almost 100%. The limiting temperature for *Z. noltei* shoots in the Ria Formosa for a three hour heat-shock seems to be slightly above 37ºC, as mortality was respectively 0% and approx. 19% of all shoots used in this temperature treatment in Praia de Faro and Ramalhete. Also, at the end of the experiment photosynthetic efficiency of the surviving plants did not differ among treatments, (factorial ANOVA on Fv/Fm values, $p>0.05$, either using all surviving shoots or using the same sample size for all treatments, *i.e.* n=12) but differed significantly between populations (factorial ANOVA, $p=0.000$, n=12), revealing a difference in the physiology of plants from different sites after a heat shock and two months in common-garden culture conditions. The observed population differences in photosynthetic efficiencies following recovery suggest that there may be local adaptation or phenotypic acclimation to specific sites within the coastal lagoon. One month acclimation period in "common-garden" conditions should have limited/buffered short-term phenotypic differences, and a significant difference between populations after this period, plus about one extra month in common experimental conditions therefore suggests a genetically-based effect in the ability to recover from the stress. This would support the hypothesis of local adaptation in these populations. Despite the small distance scales separating populations inside the Ria Formosa, outbreeding

depression, one possible consequence of local adaptation, has been reported between populations of the congeneric species *Zostera marina* also within this coastal lagoon (Billingham et al. 2007). In the *Zostera noltei* populations studied here, the most obvious difference in selective pressure between sites is the level and duration of exposure at each site during low tide. Accordingly, plants at Praia de Faro are exposed to emersion-stress less often (and for shorter periods) and may therefore be expected to experience lower selective pressure for temperature stress resistance than plants at Ramalhete. It remains to be seen if other factors (e.g., population size, genetic diversity) may play a role in generating the observed differences. Whatever the ultimate cause, this difference raises interesting questions about the possible roles of genetic composition, phenotypic plasticity, demographic factors and/or selective processes in shaping the diversity and distribution of *Z. noltei* along its distribution range, and particularly in extreme conditions such as those encountered in the Ria Formosa.

According to various models and scenarios, global temperature is expected to increase between 2.0 and 4.5 ºC, with a best estimate of about 3ºC, by the year 2100 (IPCC 2007). This study suggests that this predicted increase may have consequences on the persistence of *Z. noltei*, and on the ecosystem based on this habitat structuring species, in the warmer habitats of their ranges, as shown here in a shallow coastal lagoon in southern Europe.

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CHAPTER II: EXPRESSED SEQUENCE TAGS FROM HEAT-SHOCKED SEAGRASS *ZOSTERA NOLTEI* (HORNEMANN) FROM ITS SOUTHERN DISTRIBUTION RANGE.

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Abstract

Predicted global climate change threatens the distributional ranges of species worldwide. We identified genes expressed in the intertidal seagrass *Zostera noltei* during recovery from a simulated low tide heat-shock exposure. Five Expressed Sequence Tag (EST) libraries were compared, corresponding to four recovery times following sub-lethal temperature stress, and a non-stressed control. We sequenced and analyzed 7,009 sequence reads from 30 min, 2 h, 4 h and 24 h after the beginning of the heat-shock (AHS), and 1,585 from the control library, for a total of 8,594 sequence reads. Among 51 Tentative UniGenes (TUGs) exhibiting significantly different expression between libraries, 19 (37.3%) were identified as 'molecular chaperones' and were over-expressed following heat-shock, while 12 (23.5%) were 'photosynthesis TUGs' generally under-expressed in heat-shocked plants. A time course analysis of expression showed a rapid increase in expression of the molecular chaperone class, most of which were heat-shock proteins; which increased from 2 sequence reads in the control library to almost 230 in the 30 min AHS library, followed by a slow decrease during further recovery. In contrast, 'photosynthesis TUGs' were under-expressed 30 min AHS compared with the control library, and declined progressively with recovery time in the stress libraries, with a total of 29 sequence reads 24 h AHS, compared with 125 in the control. A total of 4,734 TUGs were screened for EST-Single Sequence Repeats (EST-SSRs) and 86 microsatellites were identified.

Keywords: EST library; high temperature stress; abiotic stress response; climate change.

1. Introduction

Seagrasses are key-species in coastal ecosystems, as important primary producers providing food, nursery and shelter for many marine species, but are declining worldwide (Orth et al. 2006; Waycott et al. 2009); changes attributed to anthropogenic activities and climate change (Duffy 2006; Orth et al. 2006). The dwarf seagrass *Zostera noltei* dominates the intertidal habitats of the coastal lagoon system Ria Formosa, in southern Portugal, where at low tide this species occurs either in shallow intertidal pools or completely emersed. The predicted increase in sea surface temperatures (SST) of 0.2ºC/decade (IPCC 2007) is raising concerns as to the ability of this species to survive the expected global warming, particularly since intertidal habitats are more affected by air temperature than SST. Previous work suggested that the current temperature in shallow intertidal pools is already very close to the temperature tolerance of approximately 38ºC determined for *Z. noltei* in the Ria Formosa (Massa et al. 2009), with records showing temperatures as high as 36ºC in the summer of 2007. Plants that occur in the upper intertidal can be exposed for as long as 6 hours during spring tides, and temperature in these small intertidal pools have reached 38ºC in low wind/high sun and air temperature situations (S. Massa, personal observation).

When higher plants are exposed to temperatures higher than required for optimal growth, a cellular adaptive response is activated to maintain cellular homeostasis under stress, resulting in increased synthesis of heat-shock proteins (HSP) and reduced production of other metabolic proteins (Arya et al. 2007). Heat-shock can affect macromolecular synthesis, levels of cations, states of protein phosphorylation, metabolic pathways and cytoskeleton networks (Schlesinger 1990). Heat stress has been shown to reduce photosynthetic yield in tropical seagrasses and increase photoinhibition (Campbell et al. 2006). It also reduces chlorophyll levels, reducing light (energy)

absorption in chloroplasts for photosynthesis, and affects the levels of protective enzymes against oxidative stress, such as ascorbate peroxidase (APX) and superoxide dismutase (SOD) (Almeselmani et al. 2006; Cui et al. 2006). All of this suggests that the response to heat stress is a complex process involving a large number of genes.

As genomic tools become more accessible for a wide range of non-model organisms, they can be applied by evolutionary ecologists to address questions concerning adaptation to novel or stressful environments, in order to clarify whether changes in the phenotype of independent populations confronted with similar environmental challenges are adaptive or a result of random genetic drift (Bull et al. 1997; de Visser et al. 2004; Woods et al. 2006). Differences in individual responses to adverse conditions affect fitness through selection, and may ultimately influence the survival of populations and potential shifts in species distribution ranges (Travers et al. 2010).

As of February 5th, 2011 there were 13,434 marine seagrass EST sequences reads in NCBI, from only 2 species, of which 10,345 are from the closely related species *Zostera marina* and 3,089 from *Posidonia oceanica*, and none for *Z. noltei.* This study aims to identify genetic markers and genes expressed in the intertidal seagrass *Z. noltei* during recovery from heat-shock exposure, simulating a low-tide event. A description of transcriptional changes in *Z. noltei* in response to heat-shock is needed to study the impacts of temperature stress and to explore the adaptation potential of this species in the face of climate change. (e.g. molecular markers including SSR and SNPs to identify loci potentially under selection, and gene sequence information to develop qPCR assays for functional studies of target genes). Moreover, an increased coverage of the transcriptome of seagrasses in general will allow the identification of crucial clusters

of genes systematically involved in response to warming, providing a basis for comparative studies of gene evolution in seagrasses.

2. Material and methods

2.1. Culture conditions and stress treatment

Z. noltei plants were collected in the spring of 2007 at 4 sites in the Ria Formosa (Ramalhete, Praia de Faro, Olhão and Portimão) in several distant cores with natural sediment, and placed in an outdoor tank (1.5 x 1.5 m, maximum seawater volume 225 L) with ambient light conditions, continuous seawater flow from the Ria Formosa and simulated tides for acclimation during approx. 4 weeks. In high tide situation, water level in the tank was up to its maximum capacity, and every day at 10 a.m. the water level was slowly decreased until roughly two hours later only a 2 cm layer of water (approximately) remained above the sediment, mimicking a low tide situation. At 4 p.m., ambient seawater was gradually added to increase the water level back to high tide situation. This process was repeated again starting at 10 p.m. as tides in the Ria Formosa are semidiurnal. Previous work had determined the sub-lethal temperature for *Z. noltei* shoots in Ria Formosa to be slightly above 37ºC (Massa et al. 2009), and so a single heat-shock of 37.5±0.5 ºC was then applied for four hours, the approximate average duration of low tide exposure in this part of *Z.noltei* distributional range, during a simulated low tide situation as previously described, between 10 am and 2 pm. After the heat-shock, ambient seawater was gradually added to the tank to lower the temperature to its initial value of approx. 22ºC. Sampling of inner leaves of randomly picked shoots (on average, 59 x approx. 20 shoots per time step) from all sites and cores for RNA extraction occurred at 30 minutes, 2, 4 and 24 hours after the beginning of the heat-shock. Each group of approximately 20 shoots was put in a tube and immediately

frozen in liquid nitrogen for later processing. A sample of non-stressed plants was also collected to be used as a control (blank).

2.2. RNA preservation and storage

At the end of the first three samplings (plus the control), samples were taken to the lab in liquid nitrogen and immediately freeze-dried for at least 48 hours, and preserved at –80ºC until extraction. The same procedure was performed after the 24 hour sampling.

2.3. cDNA library construction

Samples were transferred into a tube with a tungsten sphere and ground at 30 g for 10 minutes. RNA extraction was performed using Qiagen and GE Healthcare extraction kits (Germany). RNA quality was verified using 3µL of RNA extraction on denaturing agarose gels and quantification was performed with a spectrophotometer at 260 and 280 nm.

RNA extractions were treated with the Macherey-Nagel NucleoSpin RNAII kit for DNAse I digestion. All cDNA libraries were constructed with purified mRNA using Dynabeads® mRNA purification kit (Invitrogen); each sample was fractionated by column chromatography and the highest quality fractions were pooled together and directionally cloned into pDORN 222, using CloneMiner™ cDNA Library Construction Kit (Invitrogen). Electrocompetent cells were transformed by electroporation and then grown in SOC medium. After plasmid extraction, sequence data were obtained using M13-21 primer (5'-TGTAAAACGACGGCCACT-3´), amplified by the following programme: denaturation step (96ºC 1'), followed by 35 cycles of denaturation (96ºC 20''), annealing (55ºC 10'') and elongation (60ºC 4'). The cloned cDNAs were 5′-end-sequenced using Big Dye 3.1 chemistry and ABI 3130XL capillary sequencers.

2.4. Sequence processing and EST assembling

Each library was treated independently to allow comparison of the response to stress over time. An average of 1,719 clones were sequenced for each of the five treatments (30 min, 2h, 4h, 24h and control) and analysed with the ESTragon program (developed and routinely used in the Max Planck Institute for Molecular Genetics) for removal of low quality (Chou and Holmes 2001) and/or contaminated sequence reads from each dataset before alignment, with a cut-off length of 100 bp. All vector-clipped and high quality sequence reads were submitted to dbEST within GenBank [accession numbers HO214335-HO215643 for the control library, HO215644-HO217278 for 30 minutes, HO217279-HO218949 for 2 hours, HO218950-HO220485 for 4 hours and HO220486-HO222020 for 24 hours]. Processed sequence reads were then assembled into contigs to cluster the individual sequence reads to represent unique transcripts using TGICL (Pertea et al. 2003). Unique sequences, either singletons or contigs, will from now on be referred to as Tentative UniGenes (TUGs). Blastx analysis was performed with Blast2GO® (V 2.4.4) (Conesa et al. 2005; Gotz et al. 2008) against NCBI non redundant (nr) protein database, using a preliminary E-value of 10^{-3} as cutoff, to maximise the number of annotation terms obtained. Blastx results were then mapped and annotated against the Gene Ontology (GO) database, according to three different categories: Biological Process (BP), Molecular Function (MF) and Cellular Component (CC). Annotation parameters were as follows: E-value Hit filter $1.0E^{-6}$, annotation cutoff 55, GO Weight 5. Fisher's Exact Tests were performed to test for significance of differential expression in each heat-shock library *versus* the control library.

2.5. Homology searching

Individual stress libraries were screened by local Blastn against a database consisting of a pooled assembly of all stress treatment libraries (i.e., 30 min, 2 h, 4 h stress, and 24 h recovery) to identify homologues and estimate their expression levels. In this case, when two or more TUGs from individual stress libraries had the same top blast hit against this database, they were considered the same TUG. In the case of the control library that was not included in the database, we applied a stringent E-value cut-off of < $1.0E^{-100}$ when considering homologous consensus TUG sequences.

TUGs with significantly different expression levels over the time-course of the stress treatment, or between stressed and non-stressed samples, were identified with the IDEG6 (Identification of Differentially Expressed Genes) web tool (Romualdi et al. 2003), using the Audic and Claverie method (Audic and Claverie 1997) to compare, after normalization, the number of sequence reads/TUG in each library (significant threshold of $\alpha = 0.05$, corrected for multiple testing with [(m+1)/2m]). Only TUGs with a total of at least 10 sequence reads from all libraries were used in the expression analysis, in an attempt to control for random sequencing effects in small TUGs. The analysis resulted in a total of 51 TUGs with significantly different expression in at least one pairwise library comparison. These were then screened using Blastn against Dr. Zompo's EST databases for *Z. marina* and *P. oceanica* to find interspecific matches (Reusch et al. 2008; Wissler et al. 2009) with an bit score cutoff value of 200.

2.6. Microsatellite identification

All TUGs from all 5 libraries were screened for single sequence repeats (EST-SSRs) or microsatellites using MsatCommander (Faircloth 2008), for all motifs with two to six nucleotides length and a minimum of six repeat units.

3. Results and Discussion

3.1. EST sequencing determination

A total of 8,594 clones from all libraries were sequenced, resulting in 7,799 high quality sequence reads. After screening for cloning relics and contamination, a total of 113 reads were removed, leaving a total of 7,686 successful reads. Clustering (TGICL) resulted in 170 contigs for the 30 min library, 174 contigs for the 2h library, 151 contigs for the 4h library, and 145 contigs for both the 24h and the control libraries. Average percentage of sequence reads with a successful blast against NCBI non redundant (nr) protein database was 82%, of successfully mapped sequences 70% and successfully annotated against the GO database was 56%. Detailed information for each library can be found in table 1.

Table 1: Summary of the sequence read analysis for each heat-shock library (30 min, 2h, 4h and 24h) and the control (blank).

Library	**30 min**	**2h**	**4h**	**24h**	**control**
Total number of clones sequenced	1,724	1,795	1,756	1,734	1,585
Number of high quality sequences	1,657 (96.1%)	1,698 (94.6%)	1,553 (88.4%)	1,563 (90.1%)	1,328 (83.8%)
Total number of successful sequences	1,635 (94.8%)	1,671 (93.1%)	1,536 (87.5%)	1,535 (88.5%)	1,309 (82.6%)
Number of contigs[a]	170	174	151	145	145
Number of clones included in the contigs	733 (44.8%)	700 (41.9%)	479 (31.2%)	439 (28.6%)	511 (39.0%)
Average contig length	755 bp	783 bp	737 bp	736 bp	682 bp
Number of singletons[b]	902 (55.2%)	971 (58.1%)	1,057 (68.8%)	1,096 (71.4%)	798 (61.0%)
Tentative UniGenes (TUGs) (a+b)	1,072	1,145	1,208	1,241	943
Number of TUG with no ORF	2 (0.18%)	4 (0.35%)	1 (0.08%)	1 (0.08%)	13 (1.38%)
Number of TUGs with a successful blast	907 (84.6%)	985 (86.0%)	1,030 (85.3%)	1,031 (83.1%)	690 (73.2%)
Number of successfully mapped TUGs	745 (69.5%)	824 (72.0%)	832 (68.9%)	914 (73.7%)	628 (66.6%)
Number of successfully annotated TUGs	600 (56.0%)	639 (55.8%)	650 (53.8%)	730 (58.8%)	527 (55.9%)

The main protein families found in our data were the *HSP70* protein family, the protein kinase domain family and the chlorophyll a-b binding protein family (Table 2). The reduction in the number of sequence reads of the *HSP70* protein family between the stress and the control libraries was very clear, while the number of chlorophyll a-b binding proteins was roughly the same among libraries.

Table 2: Main protein families. All families with at least 10 sequence reads in one of the libraries.

	30 min	2h	4h	24h	control
(Hsp70 protein)	28	31	16	13	1
(Protein kinase domain)	16	23	19	20	12
(Chlorophyll A-B binding protein)	16	15	16	15	16
(RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain))	11	8	8	8	5
(Cytochrome P450)	6	2	10	2	4

3.2. Identification of differential expression

Out of the 51 TUGs with significantly different expression between libraries and a total of at least 10 sequence reads, 26 (51%) had a matching hit with *Z. marina*, 16 (31%) of which also had a match with *P. oceanica* (Annex I). Most of the highest-scoring matches were photosynthesis-related TUGs but also included 2 heat-shock cognate protein 80 TUGs, a stress-induced phosphoprotein, a glyceraldehyde-3-phosphate dehydrogenase, a flavoprotein, a 70 kDa peptidyl-prolyl isomerase, a glutamine synthetase and one TUG with no significant Blastx hit, suggesting that these TUGs are highly conserved across the seagrass species, probably due to their fundamental role in cellular pathways. The remaining 13 TUGs (25%) had no meaningful matches. These 51 TUGs were divided into 3 groups according to their function: 'molecular chaperones', 'photosynthesis TUGs' and 'other TUGs of interest', which will be discussed further below.

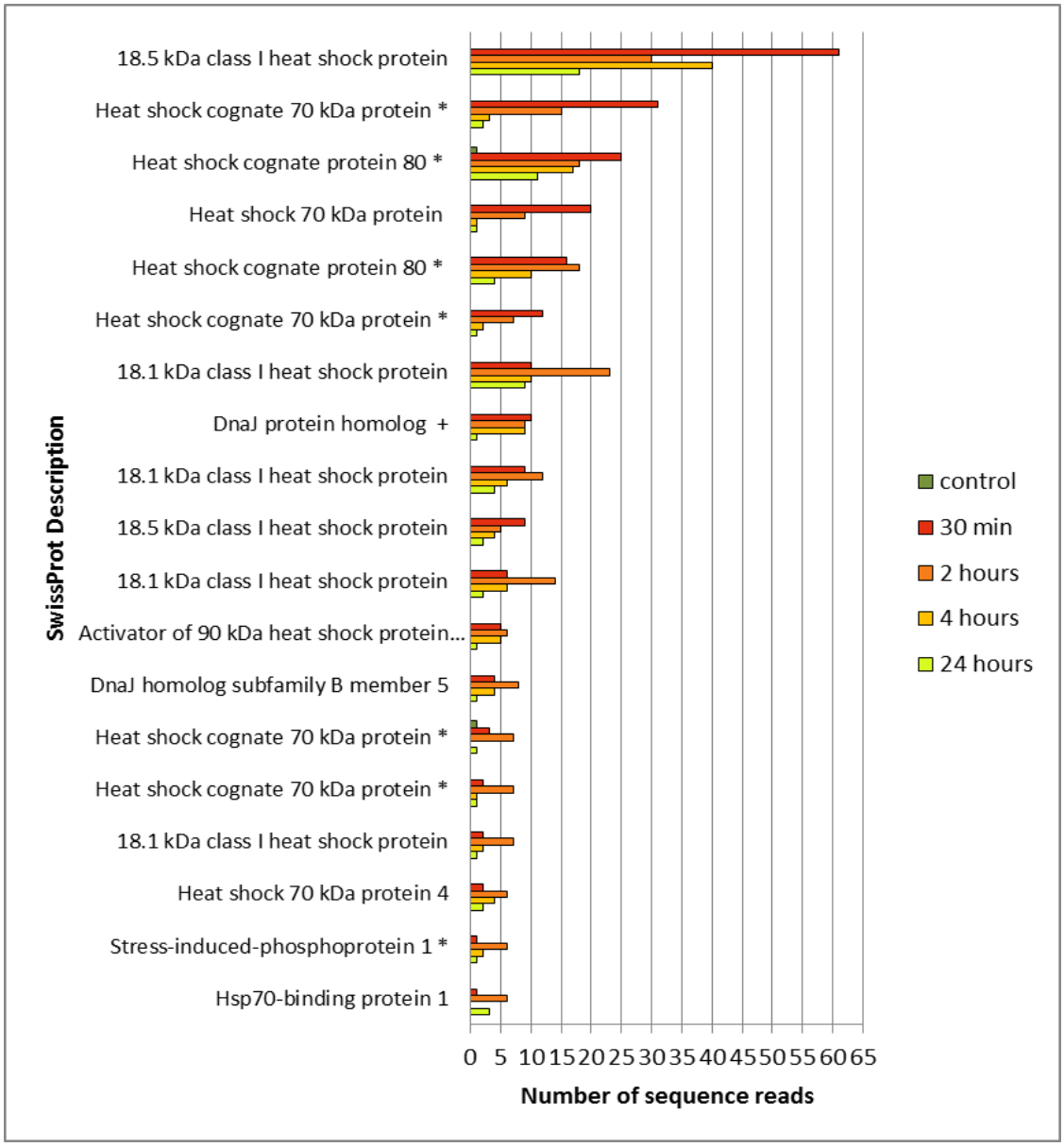

Figure 1: Number of sequence reads of each 'molecular chaperone' TUG with significantly different expressions in each library. SwissProt descriptions were used to identify each TUG. The number of sequence reads for the control library is shown in white, stress libraries are as follows: 30 minutes in black, 2h in dark grey, 4h in medium grey and 24h in light grey. It is also shown when a corresponding hit was found in Dr. Zompo database for *Zostera marina* (*) and *Posidonia oceanica* (+).

3.3. Molecular chaperones

Among 943 TUGs from the control library, only two heat-shock protein TUGs were found, and 61 heat-shock protein TUGs were found in the combined stress libraries,

from a total of 4,666 TUGs. When the number of sequence reads was taken into consideration, IDEG found 9 of these heat-shock protein TUGs to have a significantly different expression between libraries. 'Molecular chaperones' include not only these 9 heat-shock protein TUGs but also 10 other TUGs, like heat-shock cognate proteins and DnaJ homologues, and were over-expressed in the stress libraries. While the control library showed only 2 sequence reads, these increased rapidly at the beginning of the stress, showing a total of 229 sequence reads at 30 minutes, and slowly decreased thereafter to 213 at 2 hours, 126 at 4 hours and 66 at 24 hours (Fig. 1). The response was very fast, with significant difference being recorded as soon as 30 minutes after the beginning of the heat-shock.

A total of eight sequences (5 contigs and 3 singletons) encoding small heat shock proteins (*sHSPs*) were identified in our dataset based on pfam database searches of predicted ORFs. ORF prediction was performed using the OrfPredictor tool (Min et al. 2005) available at http://proteomics.ysu.edu/tools/OrfPredictor.html. The resulting ORFs were used to identify Pfam domains on the UFO webserver (http://ufo.gobics.de/submission, (Meinicke 2009)). All 8 sequences had start sites and encoded a predicted full-length *sHSP* with an *HSP20*/alpha crystalline domain (PF00011). In two separate cases a singleton and contig were found to have identical protein sequences, but variable 3' UTRs. The other 3 contigs and one singleton encoded unique proteins. Searches for signal peptides targeting the proteins to the chloroplast or mitochondrion were negative, and no transmembrane domains were identified, suggesting that these proteins are putatively cytoplastic. Heat-shock proteins are highly conserved among species; their main role is to protect and repair protein structures, preventing the denaturation process or promoting the proper refolding of denatured proteins (Schlesinger 1990). Since protein conformation is also important in

normal conditions, most HSPs are present in all cells and tissues even in the absence of stress factors such as elevated temperatures (Krishna 2004). Heat-shock proteins are also involved in the apoptotic pathways, as irreparably damaged cells will eventually be eliminated when stress conditions are too severe (Beere 2005). Previous work has shown that mortality will eventually occur (Massa et al, unpubl.). After 24 hours the response appeared to be complete as Fisher's Exact Test showed no further differences between 24h expression and the control library (Annex 2), suggesting a return to normal gene expression 24 hours after the heat-shock.

The other 10 TUGs categorized as 'molecular chaperones' include DnaJ-like proteins, which stimulate the ATPase activity of *HSP70* protein (Cheetham and Caplan 1998), that catalyse the folding of proteins and the assembly of protein complexes (Cyr et al. 1994; Govind et al. 2009) and heat-shock cognate (Hsc) 70 and 80 (Fig 1). Although HS cognates are constitutively expressed, it has been shown that *HSC70* is required for Heat-Shock Factor 1 to become activated and target expression of appropriate genes during heat stress (Ahn et al. 2005). Molecular chaperones are important during both unstressed and stressful conditions, as they not only assist in the *de-novo* folding of denatured proteins but also in conformational changes that affect function, while they also transport unfolded proteins across membranes and plasmodesmata (Aoki et al. 2002; Genevaux et al. 2007). The stress-induced phosphoprotein 1, that we also included in this group of 'molecular chaperones', was first described in *Saccharomyces cerevisiae* where it was implicated in mediating the heat-shock response of some *HSP70* genes (Nicolet and Craig 1989).

Our results are in contrast with findings for the closely related species *Z. marina* (Reusch et al. 2008) where no over-expression of heat-shock proteins was observed in the EST library constructed for elevated growth temperature. However, this may reflect

the differences in the organismal biology and ecology of this species in comparison with intertidal *Z. noltei* (the *Z. marina* populations used were subtidal and therefore exist in an environment with considerably lower amplitudes and extreme values of temperature fluctuation). The major aim of the *Z. marina* study was to investigate sub-lethal growth temperature (25ºC; 2ºC higher than an expected summer maximum) rather than heat shock *per se*. If a minor heat-shock response was induced, it may have subsided by the time of sampling, as our results show that the response may be over between 4 and 24 h after the end of the shock.

Reusch and colleagues suggested that the lack of heat-shock protein expression in *Z. marina* might be explained by the fact that permanently submerged seagrasses, as was the case with *Z. marina* in their experiments, are seldom subject to rapid temperature fluctuations because of the 'buffered' aquatic environment, lending contrast to terrestrial environments where the presence of heat-shock proteins genes is a common response to temperature stress (Reusch et al. 2008). Our results demonstrate that for intertidal marine angiosperms like *Z. noltei*, the geographic distribution of which extends to warmer latitudes where *Z. marina* is strictly subtidal, temperature in small intertidal pools is indeed more influenced by air than by sea temperatures; the former reach levels very close to the sub-lethal temperature of *Z. noltei* shoots during summer in the Ria Formosa (Massa et al. 2009). The regular and rapid increase of temperature in small intertidal pools, together with potential desiccation, may therefore require the maintenance of a heat stress response in dwarf eelgrass more similar to terrestrial angiosperms than subtidal ones.

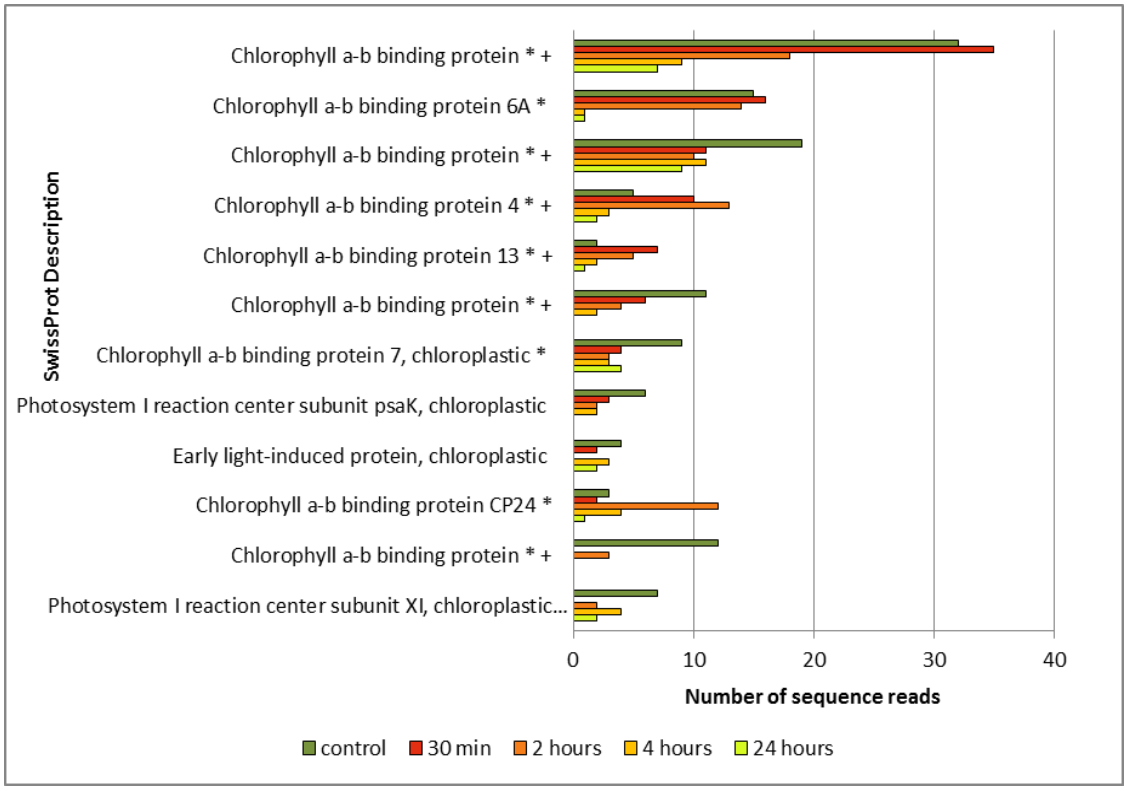

Figure 2: Number of sequence reads of each photosynthesis-related TUG with significantly different expressions in each library. SwissProt descriptions were used to identify each TUG. The number of sequence reads for the control library is shown in white, stress libraries are as follows: 30 minutes in black, 2h in dark grey, 4h in medium grey and 24h in light grey. It is also shown when a corresponding hit was found in Dr. Zompo database for *Zostera marina* (*) and *Posidonia oceanica* (+).

3.4. Photosynthesis TUGs

We found 12 TUGs with photosynthetic function and with significantly different expression between libraries, which were overall under-expressed in the stress libraries. The control library showed a total of 125 sequence reads, which decreased to 96 after 30 min, 86 after 2h, 44 after 4h, and reached a minimum of 29 after 24h (Fig. 2). Most of the TUGs encode for chlorophyll a-b binding proteins that transfer energy between photosystem antennae and reaction centers, and suggests a general down-regulation of photosynthetic activity. This is consistent with physiological studies showing that the photosynthetic yield of *Z. noltei* decreases immediately after a heat-shock (Ralph 1998;

Massa et al. 2009). The photosynthetic machinery is integrated and composed of many subunits making it energetically expensive for the cell to produce under stress (Govind et al. 2009). Therefore, the down-regulation of TUGs related to photosynthetic components under heat-shock, such as highly abundant chlorophyll a-b binding proteins, could reveal a trade-off that helps maintain energy balance. However, since previous work has shown that about half of the plants will eventually die following a similar heat-shock (Massa et al. 2009), under-expression of photosynthesis TUGs may also be a consequence of apoptosis.

An exception to the general pattern in light-harvesting proteins was the up-regulation after 2 h of a CP24 protein (ELIP/psbS family; Figure 2). Members of this protein family are thought to be important in non-photochemical quenching of excess light energy and protection against photo-oxidative stress (Hutin et al. 2003).

3.5. Other TUGs of interest

A number of other TUGs with diverse functions were also found to have significantly different expressions among libraries (Fig. 3).

Ubiquitin

Ubiquitin is a highly conserved small protein, involved in the selective degradation of many short-lived proteins in eukaryotic cells as they are targeted for degradation by covalent ligation to ubiquitin. Ubiquitin-mediated degradation of regulatory proteins plays important roles in the control of numerous processes, including cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis. The ubiquitin system has been implicated in the immune response, development, and programmed cell death (Johnson et al. 1992; Hershko and

Ciechanover 1998). Ubiquitin TUGs were over-expressed in the stress libraries, suggesting that heat-stress may have damaged some proteins, which were targeted for proteolysis.

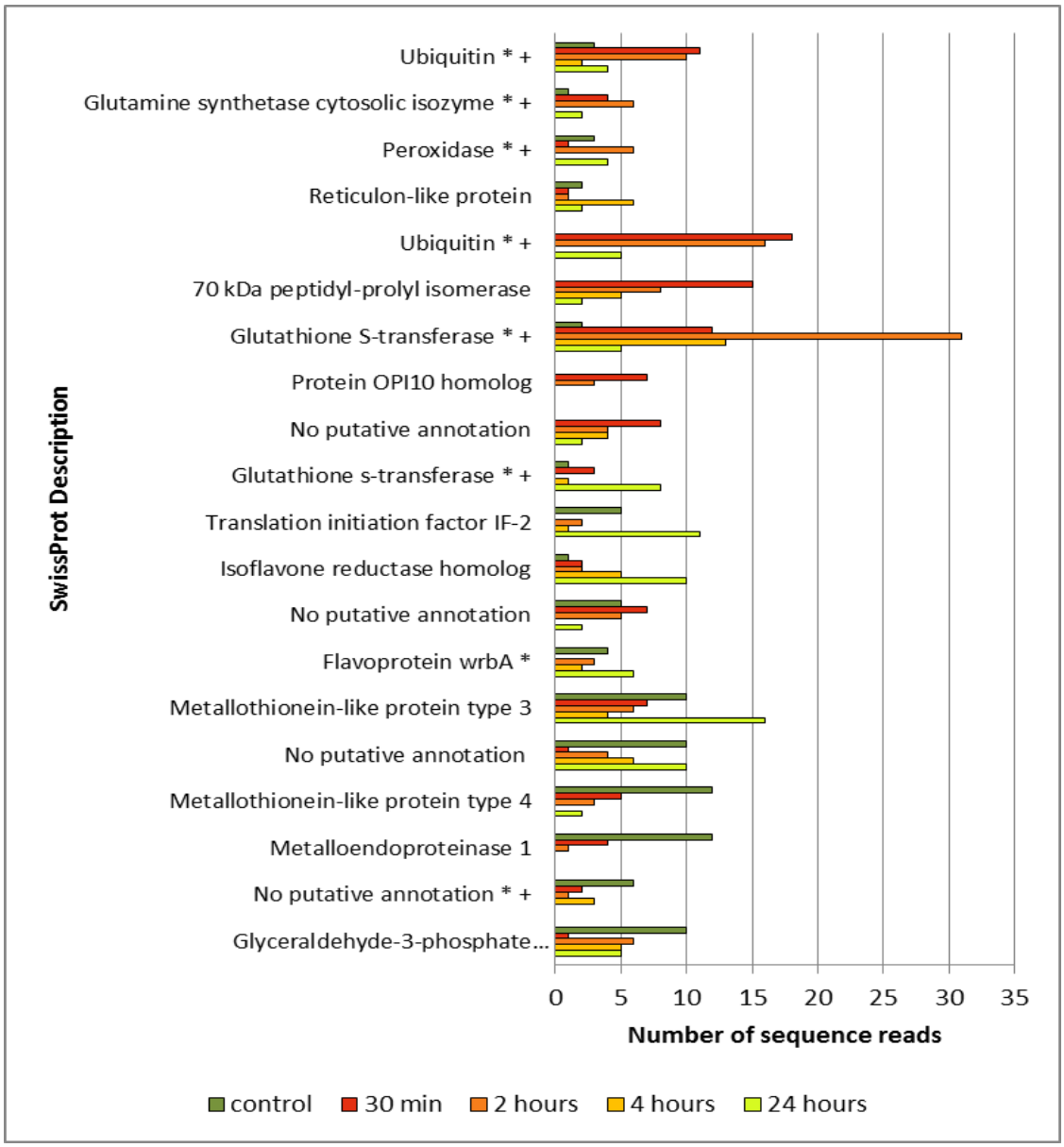

Figure 3: Number of sequence reads of other TUGs of interest with significantly different expressions in each library. SwissProt descriptions were used to identify each TUG. The number of sequence reads for the control library is shown in white, stress libraries are as follows: 30 minutes in black, 2h in dark grey, 4h in medium grey and 24h in light grey. It is also shown when a corresponding hit was found in Dr. Zompo database for *Zostera marina* (*) and *Posidonia oceanica* (+).

Glutamine synthetase

Another TUG that was also slightly elevated in the stress libraries was glutamine synthetase (GS), involved in the assimilation of nitrogen and a biomarker of plant metabolism, indicating nutrient deficiency under stress conditions (Ferrat et al. 2003). Higher plant GS in roots functions in the primary assimilation of ammonia from the soil. In leaves, GS is also responsible for the reassimilation and detoxification of the large amounts of ammonia lost during photorespiration (Keys et al. 1978). Accumulation of nitrogen may be explained by the lack of use of this nutrient for growth during stress conditions, as was observed in *Z. marina* during reduced light conditions (Vanlent et al. 1995).

Response to oxidative stress and/or cellular detoxification

The classical plant peroxidases are a well-studied group of heme-containing enzymes that utilize either H_2O_2 or O_2 to oxidize a wide variety of substrates (Yoshida et al. 2003). In the majority of plant species investigated they occur as distinctive isoenzymes which can be constitutive or induced in response to external factors such as wounding, stress and attack by pathogens (Veitch 2004). Peroxidase showed an over-expression in the middle of the heat-shock (2 hours) when compared to the remaining libraries.

The glutathione S-transferase (*GST*) super-family of genes encode enzymes that catalyse a number of distinct glutathione-dependent reactions: in addition to their ability to catalyse the formation of conjugates, *GST* can also serve as peroxidases and isomerases. They play a critical role in protecting against electrophiles and products of oxidative stress and cellular detoxification generally, suggesting that they are part of an adaptive response to chemical stress (Hayes and Pulford 1995). This TUG was also

over-expressed in the stress libraries, suggesting that GST may also participate in the response to temperature stress.

Plant metallothioneins are involved in heavy metal tolerance and detoxification and might also be involved in regulation of cellular availability of required heavy metals, namely copper and zinc (Robinson et al. 1993; Moisyadi and Stiles 1995) and were under-expressed in the stress libraries, suggesting cell energy may be switched from heavy metal detoxification to heat stress response .

Reticulon-like protein

Reticulons are proteins that have been found predominantly associated with the endoplasmic reticulum in yeast and mammalian cells. Although reticulon-like proteins have been identified in plants, very little is known about their cellular localization and functions, but have been shown to be associated with the endoplasmic reticulum in *Arabidopsis thaliana* (Nziengui et al. 2007). Reticulon-like proteins showed a slight variation among stress libraries, being under-expressed during the beginning of the heat-shock (30 minutes and 2 hours libraries), suggesting a decrease in a hypothetical housekeeping gene during high stress conditions.

Peptidyl-prolyl isomerase

Peptidyl prolyl isomerases are protein folding catalysts (Kruse et al. 1995; Schmid 1995), whose function is the cis-trans isomerization of peptidyl-prolyl bonds, a relevant conformational change that is rate limiting. Most, but not all, peptide bonds are connected in the trans conformation during biosynthesis at the ribosomes, and this conformation is also found in the native structure of most peptide bonds (Gothel and Marahiel 1999). The over-expression of these TUGs in the stress libraries may suggest

an increase in protein biosynthesis, which is possibly related to the production of stress-response molecules such as heat-shock proteins.

Isoflavone reductase homolog

Isoflavone reductase (*IFR*) is an enzyme specific to isoflavonoid biosynthesis, a pathway which is mainly found in the *Leguminosae* (angiosperms). It catalyses a NADPH-dependent reduction involved in the biosynthesis of important and related phenylpropanoid-derived plant defense compounds (Franca et al. 2001). Interestingly, this enzyme was over-expressed in the 24 hours library, which may reflect a delayed response to the stress.

Flavoprotein WrbA

The flavoprotein WrbA, originally described as a tryptophan (*W*) *r*epressor-*b*inding protein in *Escherichia coli*, has recently been shown to exhibit the enzymatic activity of a NADH: quinone oxidoreductase to maintain of a supply of reduced quinine, and having a possible role in stress response (Andrade et al. 2007). WrBA was under-expressed in the first stress library (30 minutes), which suggests this enzyme might be inhibited by high temperatures.

Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) is a nucleic-acid-binding protein involved in glycolysis and in the Calvin cycle (Plaxton 1996; Wedel and Soll 1998; Barber et al. 2005) and was under-expressed under stress conditions, suggesting an inhibition of important metabolic pathways during high temperature stress. *GAPDH*

exhibits high temperature sensitivity (Mair et al. 2005) and the inhibition of this enzyme affects the photosynthetic process, by causing a secondary photoinhibitory response in PSII (Ralph 1999b) that may be the cause of the decrease in photosynthetic activity. It can also reduce energy generation if glycolysis is inhibited and ATP levels decrease.

Table 3: EST-SSRs found in all TUGs.

	Number of repeat units											
Motif	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**Total**
AC		2	1									3
AG	6	2	2	1		1	1	1	1	1	1	17
AT	8	7	2	1	1	2		1		1		23
AAC	1											1
AAG	7	8	1	1			1	1				19
AAT	4											4
ACC		1	1									2
ACT	1											1
AGC	1											1
AGG	2	2										4
ATC	1	1										2
CCG	2											2
AATG					1							1
AATT		1										1
AGAT	1											1
ATCC		1										1
AACATG	1											1
AATCAC	1											1
AGATGG	1											1
Total	31	19	7	3	1	1	2	3	1	2	1	86

4. Conclusions

This study reports the first transcriptomic dataset for *Z. noltei*, focusing on response to high temperature stress. Understanding the molecular basis of traits of interest has been hindered by a lack of genomic resources for this species, and this study has provided a considerable dataset covering the transcriptional response to heat-stress. Almost 8,600 sequences reads were produced from all libraries, which resulted in over 3,000 annotated TUGs. As expected, an important part of the TUGs with significantly

different expressions between libraries were known to be stress-related, mostly heat-shock proteins, which were over-expressed in stress libraries and are commonly induced by high temperature stress. Also, photosynthetic activity seems to be affected by heat-shock, as important enzymes are thermally inhibited and cells redirect their energy towards defense strategies. The heat-shock response in *Z. noltei* is very fast, showing significant differences from unstressed plants as soon as 30 minutes after the beginning of the stress, causing an increase of heat-shock protein TUGs and an inhibition of some of the TUGs with photosynthetic function. A total of 86 microsatellites were also identified, and in future work may be used to develop genetic markers in candidates TUGs related to the response to heat stress.

5. Acknowledgments:

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CHAPTER III: VARIABLE PHYSIOLOGICAL RESPONSE TO TEMPERATURE STRESS AMONG CLONES OF THE SEAGRASS SPECIES ZOSTERA NOLTEI (HORNEMANN)

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Final revisions before submission

Abstract

IPCC predictions of a 0.2ºC increase in sea surface temperature per decade are raising concerns as to the survival of the seagrass *Zostera noltei* in the southern part of its distribution. This work focused on the variance of the response to heat-shock of distinct *Z. noltei* genets from two sites of the Southern part of its distribution range (Ria Formosa, Portugal) where temperature naturally reaches a sub-lethal level. We explored the influence of low tide exposure length, by comparing two different tidal depths across two meadows. The plants were strongly affected physiologically by high temperature stress, a drop in photosynthetic efficiency was observed in stressed plants within 48 hours after the heat-shock, and this was significantly different among genets throughout the course of the experiment. The origin of the genets, as sites or tidal heights, did not affect physiological comparisons but significantly influenced survival. By the end of the experiment, plants originated from meadows under higher temperature stress survived better. The difference in physiological response and survival could not be linked to allele clusters at microsatellites.

Introduction

Seagrass beds are highly productive ecosystems, providing food and shelter for many associated species, improving habitat quality, and acting as important carbon sinks (Duarte and Cebrian 1996). Intertidal seagrasses are exposed to daily fluctuations of several environmental factors like solar irradiance, salinity, desiccation and temperature, which can be a major cause of biomass loss in seagrass meadows (Erftemeijer and Herman 1994; Walker and Cambridge 1995; Seddon and Cheshire 2001). Although seagrasses have evolved physiological and anatomical mechanisms to cope with these

extreme conditions across wide distribution ranges, the recent climate changes and IPCC predictions for global warming scenarios along temperate regions of the North Atlantic forecast sea surface temperature (SST) to increase by 0.2°C per decade (IPCC 2007), with temperatures increasing faster near coastal areas than in the open ocean. The temperature increase may, in some parts of each species distributional range, come as a threat to the persistence of their populations as their physiological tolerance to high temperatures may be surpassed. As a result, the geographical distribution of the species might be altered because of decreased survival of populations that occupy the thermal limits (Bulthuis 1983; McMillan 1984; Ralph 1998; Billingham et al. 2003; Reusch et al. 2005), restricting their distribution or displacing it toward more temperate regions.

The impact of such changes in marine ecosystems is likely to be magnified in intertidal habitats (Helmuth et al. 2006b), where shifts in range distributions have already been reported (Zacherl et al. 2003; Mieszkowska et al. 2006b) and sub-lethal extreme temperatures have been recorded in summer in *Z. noltei* beds (Massa et al. 2009). In intertidal habitats complex patterns of temperature variation may occur, and lethal temperatures may be more easily reached during low tide conditions (Massa et al. 2009; Pearson et al. 2009).

Z. noltei is the key-species in many intertidal marshy habitats such as the Ria Formosa in southern Europe, where temperature in small pools during low tide is often more influenced by air than by sea temperatures, already reaching alarming values up to 36ºC during the summer for as long as 6 hours per day (Massa et al. 2009). High temperature stress can cause severe damage in the photosynthetic apparatus, and inhibition of photosynthesis is one of the first symptoms of heat stress (Berry and Bjorkman 1980; Camejo et al. 2005; Guo et al. 2006; Hassan 2006). Thermal stress affects photosynthesis productivity and seagrass morphology and mortality (Cui et al.

2006; Massa et al. 2009) showing a reduction of net photosynthetic rate in several species detectable through gene expression switches (Massa et al. 2011). Signs of thermal stress at temperatures above 35ºC in temperate seagrasses include increased respiration and photosynthetic enzyme breakdown (Bulthuis 1983; Ralph 1998; Dekov et al. 2000).

Climate change will have most accute impacts when affecting species that play major roles in ecosystem function, as structuring seagrass species (Duarte 2002). Although a marginal increase in temperature will drive *Z. noltei* to its sub-lethal limit in the southern part of its range (Massa et al. 2009), the possible existence of physiological or genetic adaptations even in a small subset of the populations may allow the persistence of the population despite temperature increase. In this study we therefore aimed at testing for the existence and extent of differential phenotypic or genetic adaptations of clonal lineages, of *Z. noltei* from natural meadows growing at different tide exposures in distinct regions of the Ria Formosa.

Material and methods

Z. noltei plants were collected in January and February 2008 at two sites in Ria Formosa, southern Portugal: Ramalhete (37º 00' 18'' N, 7º 58' 01'' W) and Fontes Santas (37º 02' 16'' N, 7º 47' 31'' W). In each site, 30 ramets, each comprising a set of 10 or more shoots connected by one rhizome, were collected haphazardly in each one of four 2 x 10 m quadrats (two in the upper intertidal and two in the lower intertidal) separated by 35 m along both vertical and horizontal distances. The height on the shore differed by 75 cm between the upper and lower quadrats. These 240 ramets were labeled and replanted in natural sediment from the field, where they were all acclimated in a tank with running seawater and simulated tides. From each ramet 1 or 2 shoots were

used for genotyping with 9 microsatellite loci (Coyer et al. 2004b). Genomic DNA extraction was performed using a standard CTAB extraction procedure (Doyle and Doyle 1988).

After genotyping, 10 distinct genets from each site and depth, discriminated using the program GenClone (Arnaud-Haond and Belkhir 2007), were selected to be used in the experiment. Each of these genets was planted in two aquaria (of approx. 2.5 L each) with sediment from the field. Each had 2 sets of 3 shoots: one set of 3 shoots was used for chlorophyll fluorescence measurements, and the other as a control for survival without manipulation. One aquarium was placed in a 225 L tank to be subjected to a sub-lethal heat-shock, and the other aquarium was placed in another 225 L tank to be used as a control for the heat-shock. After acclimating the plants for 21 days in outdoor tanks with running seawater from the Ria Formosa and artificial tide occurring progressively every 12 hours, a 37,5ºC heat-shock (1º C more than the maximum temperature recorded in the Ria Formosa in summer, (Massa et al. 2009)) was applied for 3 hours in conditions mimicking a low tide situation. After the heat-shock, ambient seawater was gradually added to the tank to lower the temperature to its initial level of approx. 20 ºC as the water level increased for high tide. Approximately 4 weeks after the first heat-shock, a second heat-shock was performed as the one described above to mimic a new spring tide. The response to stress was recorded in terms of shoot survival and photosynthetic activity and was followed until stabilization could be ensured, for approximately 70 days. Phenotypic plasticity in response to stress was therefore analyzed for genets collected at distinct sites and depths.

The physiological effects of heat-shock were assessed from chlorophyll fluorescence measurements of Fv/Fm, to estimate the photochemical efficiency of photosystem II (PSII), using a portable fluorometer (FMS2, Hansatech, UK).

Chlorophyll fluorescence has been shown to be a reliable method of determining the physiological condition of photosynthetic organisms since its relation with photosynthetic activity was first observed and described in the early 1930s (Kautsky and Hirsch 1931a). It is especially useful for *in situ* measurements as it is a very fast and sensitive, non-invasive, non-destructive method that provides information about the photosynthetic efficiency of photosystem II (PSII) and allows verification of short-term responses to various kinds of environmental stress, such as light quality (Ralph and Burchett 1995; Ralph 1996; Ralph 1999a), UV radiation (Dawson and Dennison 1996; Flanigan and Critchley 1996), desiccation (Seddon and Cheshire 2001), and herbicides (Haynes et al. 2000; Ralph 2000); it also shows the regeneration of the photosynthetic apparatus when the stress factor is removed.

Prior to fluorescence measurements, leaves were dark-adapted for a minimum of 10 minutes, after which the minimum (Fo) and maximum (Fm) fluorescence yield of open and closed PSII reaction centers before and after a saturating light pulse, respectively, were determined. The first chlorophyll fluorescence measures were taken 24 hours before the heat-shock on 6 randomly chosen genets of each depth and population. To minimize the variability induced by light conditions that vary throughout the day, or photoinhibition and recovery of the plants, genets from each site and depth were divided into 3 groups to standardize measurement time every day between 1pm and 3 pm on consecutive days, starting 24 h after the heat-shock (AHS).

Mean and SE of the Fv/Fm values for every available shoot were estimated for each time step, treatment, depth and population, and genet mortality was recorded. We performed ANOVA (STATISTICA 7.0 © StatSoft, Inc.) to address five specific questions: i) are any differences detectable between the initial Fv/Fm ratio of plants from both sites, or Fv/Fm values at 24 and 48 h (by comparing values before

experiments, at t=0, performing a factorial ANOVA among treatments and sites); ii) are any differences detectable between photosynthetic efficiency of plants grown at different depths (by comparing Fv/Fm values of plants from the upper intertidal with plants from the lower intertidal, performing a repeated-measures ANOVA among treatments, sites and depths); iii) how is photosynthetic efficiency affected by heat-shock conditions (by comparing Fv/Fm values over time, performing a repeated measures ANOVA among treatments and sites); iv) is photosynthetic efficiency different among MGLs (by comparing Fv/Fm values throughout the experiment, performing a repeated measures ANOVA among MLGs); v) is survival significantly different between upper and lower quadrats in the same site (by comparing survival values at the end of the experiment, performing a factorial ANOVA among sites and depths). We compared survival at the end of the experiment among sites and between upper and lower plants for each site, by using tests for proportions.

Spatial genetic structure was analysed using distinct *multilocus* genotypes with Genetix 4.0 package (Belkhir et al. 1996-2004); Nei's gene diversity (1987) estimation of expected heterozygosity (H_E) was used to assess genetic diversity within populations. Deviations from Hardy-Weinberg equilibrium were tested using 1000 permutations to assess deviations from zero of the inbreeding coefficient (F_{IS}). Clonal parameters were estimated using GenClone 2.0 (Arnaud-Haond and Belkhir 2007). Genotypic richness, R = [N genotypes – 1]/[N samples – 1] and mean number of alleles per locus, Â, were estimated. Clonal subrange (Alberto et al. 2005) was estimated using the central coordinates of each quadrat, to estimate the spatial range beyond which clonality may no longer affect spatial genetic structure (Harada and Iwasa 1996; Harada et al. 1997). We also performed a factorial correspondence analysis using Genetix 4.0 (Belkhir et al. 1996-2004) to look for genetic effects on survival by comparing both populations and

subdividing them further according to their status at the end of the experiment (alive or dead).

Results:

Genetic composition of meadows and synthetic assemblages

Genotypic richness was lower (0.28) in Ramalhete than in Fontes Santas (0.50). Allelic richness was similar between sites (Table 1 and 2). Clonal sub-range extended to the maximum distance between quadrats in Ramalhete (approx. 50 m), while in Fontes Santas it was restricted within each quadrat (Table 1). Significant genetic differentiation was detected between sites ($p < 0.05$), but not among upper and lower quadrats of each site (Table 2). Factorial correspondence analysis (Fig. 2) accounted for 51.9 (axis 1) and 28.5% (axis 2) of the variance, showing genetic proximity of individuals from the same site. It also suggests genetic similarity of the individuals from Ramalhete that did not survive the heat-shock (towards the upper left on Fig. 2).

Table 1: Summary of genetic diversity analysis. Values are shown, for each site, of total number of genotypes (G), genotypic richness (R), mean number of alleles per locus (Â) and clonal subrange.

Site		G	R	Average	Â	Average	Clonal Subrange
Ramalhete	**Lower**	19	0,32723	**0,2818**	6,3333	**6,1111**	49,4975
	Upper	14	0,2364		5,8889		
Fontes Santas	**Lower**	24	0,4182	**0,5000**	6,1111	**6,4445**	0
	Upper	33	0,5818		6,7778		

Survival

Survival was clearly affected by the heat-shock in both populations, although this outcome was delayed as there was no shoot mortality in the first 10-14 days. Then a drop in survival rates was observed in shoots from both locations, yet stronger in the group from Fontes Santas (Fig. 1c), until stabilization at approx. 80% survival in shoots

from both sites around day 34. After the second heat-shock there was another drop in survival rates, with larger differences among sites. By the end of the experiment shoot survival in the assemblages from Ramalhete was approx. 65% whereas only about 40% of the shoots from Fontes Santas survived (Fig. 1c). Controls had high survival, similar between Ramalhete (85%) and Fontes Santas (95%), corresponding to one dead shoot per site.

Table 2: Summary of F_{ST} analysis.

F_{ST}	Upper FS	Lower R	Upper R
Lower FS	0,0082	**0,0219**	**0,0306**
Upper FS		0,0056	**0,0128**
Lower R			0,0037

Fontes Santas (FS) and Ramalhete (R)

Comparing among intertidal height levels within site revealed no consistent differences; final survival was 60% for upper intertidal and 70% for lower intertidal plants from Ramalhete, whilst 50% for upper intertidal and 30% for lower intertidal plants from Fontes Santas. Final survival was significantly different between shocked and non-shocked plants in Fontes Santas, but not in Ramalhete or between sites either in stress or control treatments (Table 3).

Photosynthetic activity

The analysis of Fv/Fm values across the course of the experiment (repeated measure ANOVA) showed a significant effect of the daily group plants were inserted in, suggesting an influence of changing sunlight conditions on these measurement and supporting the choice to measure a subset of plants from both sites, depth and treatment each day under the same conditions for a reliable comparison.

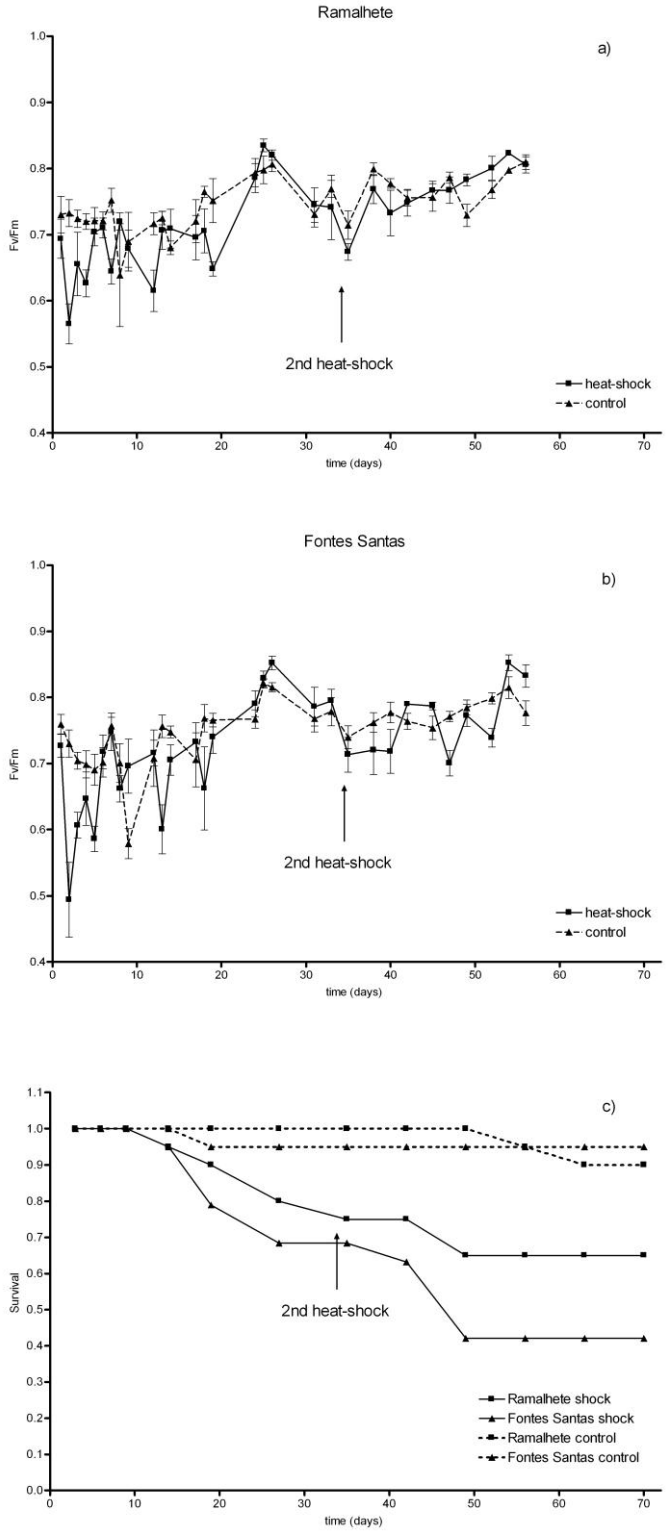

Figure 1. Fv/Fm for *Z. noltei* shoots from Ramalhete (a) and Fontes Santas (b) and survival rate for the shoots of the two sampled sites (c), both in heat-shock and in control conditions (no heat-shock applied). Values for Fv/Fm are means ± SE.

Table 3: Summary of statistical analysis.

	Effect	p-value
Initial Fv/Fm (factorial ANOVA)	Site	0,319
	Depth	0,139
	treatment	0,577
Fv/Fm 24h (factorial ANOVA)	Site	0,350
	Depth	**0,037**
	Treatment	0,208
Fv/Fm 48h (factorial ANOVA)	Site	0,322
	Depth	0,323
	Treatment	**0,000**
Fv/Fm over time (repeated measures ANOVA)	Site	0,907
	Depth	0,057
	Treatment	**0,006**
	Fv/Fm	**0,000**
Final survival (t-test)	Site (shock)	0,155
	Site (control)	0,592
	Shock vs Control	R: 0,333 FS: **0,039**
Genotypic richness (t-test)	Site	0,145
Mean number of alleles/locus (t-test)	Site	0,493

The initial mean Fv/Fm was not significantly different between Ramalhete and Fontes Santas (factorial ANOVA, $p>0.05$, $n=12$, Table 3), and ranged from 0.626 to 0.862 in Ramalhete, and from 0.625 to 0.886 in Fontes Santas (Fig. 1a and 1b). About 24 hours after the first heat-shock there was a significant difference between Fv/Fm of plants originating from upper and lower intertidal (factorial ANOVA, $p<0.05$, $n=48$, Table 3), yet not between heat-shocked and control plants. A significant difference between shocked and non-shocked plants was first observed 48 hours after the heat-shock, when the lowest mean Fv/Fm values were recorded in both sites; they were 0.601 for Ramalhete (Fig. 1a) and 0.512 for Fontes Santas (Fig. 1b), and significantly different between sites (factorial ANOVA, $p<0.05$, $n=47$, Table 3). After this low point, Fv/Fm values gradually started to increase back to their initial levels despite the fact that shoot mortality was still occurring (Fig. 1). The same pattern was also observed

after the second heat-shock, with a drop in Fv/Fm on the following days and a slow recovery to normal values by the 47th day.

Repeated measures ANOVA over the course of the experiment, revealed no significant difference in the evolution of Fv/Fm values of plants from the upper and the lower intertidal (repeated measures ANOVA, $p>0.05$, n= 48, Table 3), but Fv/Fm values were significantly different among genets (repeated measures ANOVA, $p=0.00$, n= 48, in both cases, Table 3).

Discussion

This study shows variance between genotypes (i.e., clones) in susceptibility to stress, by demonstrating that distinct seagrass genotypes respond differently to heat shock. A temperature stress mimicking the sub-lethal temperature of *Z. noltei* (Massa et al. 2009) showed a drop of photosynthetic efficiency and of survival in plants from two meadows, confirming increased mortality and decrease in expression of photosyntesis related genes at such temperatures (Massa et al. 2011). Most importantly, variance in the response of different genotypes to heat shock, was revealed in both survival and photosynthetic activity changes. Such differential responses could derive from adaptation to local environmental conditions in distinct sites and shore level or to differential selection on distinct genetic backgrounds.

After heat shock, plants from the upper shore, more exposed to high temperatures during low tides, had higher photosynthetic efficiency than plants from a permanently submerged area, although significant differences between stressed *versus* control plants were first observed 48 hours after the heat-shock. This suggests a slightly delayed response than previously reported for the same species, where the effect of high temperature stress was detectable (i.e., significant) 24 hours after the heat-shock (Massa

et al. 2009). Such delays fit the observed response of *Z. noltei* to heat-shock at the gene expression level, that necessarily precedes the physiological effects and was significantly altered as early as 30 minutes after the heat-shock (Massa et al. 2011). In this former study, there was a clear drop in expression of photosynthesis-related genes explaining the subsequent decreased photosynthetic efficiency. The photosynthetic machinery is integrated and composed of many subunits making it energetically expensive for the cell to produce them under stress (Govind et al. 2009). The decrease in photosynthetic efficiency may therefore result of a trade-off maintaining energy balance until environmental conditions return to normal, when photosynthesis levels can return to their typical state. Photosynthetic efficiency recovered even though some plants eventually died, and despite the continuously significant effect across the repeated measurements until the end of the experiment .The site where plants were collected did not affect the photosynthetic response at any time point during the course of the experiment, and the difference in photosynthetic efficiency among plants from different levels on the shore disappeared after 48 hours. These results support the existence of phenotypic plasticity and acclimation to heat stress at different tide height rather than a genetically based difference in photosynthetic efficiency. Plants from different habitats changed their phenotype in response to the new conditions, reaching a common average response, despite initial differences and despite differences between individual clones in their plasticity.

A longer term influence of the origin of plants on their response to heat shock was however revealed when comparing susceptibility to heat shock among sites, independently of their tide height. Temperature recordings during the summer of 2007, showed that the Ramalhete site was indeed exposed to higher temperatures during low tide, as high as 36ºC (Massa et al. 2009), whilst in Fontes Santas the maximum recorded

was 33,5ºC (data not shown), whereas sites were otherwise chosen to be similar in terms of tidal levels and slope. In agreement with the natural temperature regimes at these sites, individual clones from Fontes Santas had lower survival to heat-shock, although not significantly different from Ramalhete, but this was likely due to limited statistical power as experiments had to be standardized for a common sample size of only 11 genotypes per site and depth (11 being the lowest number of distinct MLG found in one sampling location, lower Ramalhete). Additionally, heat shock reduced final mortality in Fontes Santas but not in Ramalhete (relative to controls), indicating a site effect in susceptibility to heat shock. The differential effect of heat shock on plants from these distinct origins may therefore either be due to local differences in adaptation to higher temperatures, as resilience was higher where plants are recurrently exposed to temperatures closer to their sub-lethal tolerance (in Ramalhete). Another hypothesis is differential adaptive potential of different genetic backgrounds. This is plausible because sites are genetically differentiated (low but significant), and have radically different genotypic compositions. The more resilient plants were from the site with lower genotypic richness (by almost a half) and thus with the most spatially spreading genotypes, exhibiting a clonal sub-range of at least 50 m. This appears to contradict experimental results on the closely related species *Z. marina, which* showed that higher genotypic richness was associated with higher resistance (Hughes and Stachowicz 2004) and resilience (Reusch et al. 2005; Ehlers et al. 2008). Yet experiments in the present work were precisely set with a standardized level of genotypic richness (11 MLG) in order to focus on phenotypic plasticity or differential adaptation of plants from distinct origins and to avoid possible confounding effects due to variable genotypic richness. Our results obtained fit observations in natural populations of *Posidonia oceanica* (Diaz-Almela et al. 2007) where survival under stress was higher in meadows

with larger clones and consequent lower genotypic and allelic richnesses. Survival was proposed to be favoured by clonal integration, and/or by stronger phenotypic plasticity of larger and likely older clones (Diaz-Almela et al. 2007). Here, the standardized number of ramets used in the experiments for each distinct genet leads to favour the hypothesis of a stronger plasticity of larger genotypes. This hypothesis is also supported by extreme cases of large and ecologically successful genotypes, as the very extensive and old clones of *Posidonia oceanica* (Arnaud-Haond et al. 2012) or the single genotype of *Cymodocea nodosa* encountered over ca. 50 km (Alberto et al. 2001), as well as the meadow of *Z. marina* in the Baltic Sea dominated by one large clone (Reusch et al. 1999).

Conclusion:

Here we show strong variance in the response of different genotypes of the seagrass *Zostera noltei* to heat-shock in one of the warmest parts of its distribution range, the Ria Formosa. Plants from locations under contrasting stress regimes (different sites and heights on the shore) had differential physiological and survival responses to heat shock. This reveals differential susceptibility of genotypes from distinct environmental backgrounds that can be attributed to either phenotypic acclimation or differential selection on distinct genetic background. Both hypotheses may explain the results observed here, as the less susceptible plants originate from the most stressful environment in terms of temperature. Results also suggest enhanced fitness of larger clones and additional experiments are needed to further test this hypothesis. In the framework of global seagrass decline, meadows formed by such clones may deserve special attention but are missed in management plans classically focusing on conservation of diversity.

CHAPTER IV: ENTANGLED EFFECTS OF GENOTYPIC AND GENETIC DIVERSITY ON THE RESISTANCE AND RESILIENCE OF THE SEAGRASS ZOSTERA NOLTEI TO DIATOM INVASION.

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Abstract

The relationship between species diversity and components of ecosystem stability has been extensively studied, whilst the influence of the genetic component of biodiversity on lower levels of biological organization remains poorly understood. Here we manipulated both genotypic and allelic richness of the seagrass *Zostera noltei*, in order to explore their respective influences on the resistance of the experimental population to stress. Our results show a positive influence of both allelic and genotypic richness on the resistance of meadows to environmental perturbations. They also show that at the low genotypic (i.e. clonal) richness levels hitherto used in previous experimental approaches, the effect of genotypic and allelic richness cannot be disentangled.

Altogether, those results emphasize the need to acknowledge and take into account the interdependency of both genotypic and allelic richness in experimental designs attempting to estimate their importance alone or in combination. Differential mortality at increased genotypic and allelic richness demonstrates the positive influence of allelic richness, possibly combined with genotypic richness, on the survival of the experimental populations. These results, on the key species of one of the most threatened coastal ecosystem worldwide, seagrass meadows, support the need to better take into account genetic diversity in management plans.

Keywords: allelic richness, genotypic richness, *Zostera noltei*, biotic stress, seagrass.

Background

The relationship between diversity and ecosystem stability has been explored since the 1950's, as the number and type of species was expected to determine the specific traits of the ecosystem (Macarthur 1955). Yet the relationship between higher diversity and higher population/ecosystem stability is still subject to debate (Duarte 2000) as empirical evidence does not universally support it, although most studies point towards some positive, but variable, effects of higher diversity on stability (Ives and Carpenter 2007; Jiang and Pu 2009; Loeuille 2010; van Ruijven and Berendse 2010; Aragon et al. 2011; Isbell et al. 2011; Latta et al. 2011). Recently, the debate on the relationship between diversity and ecosystem stability has intensified, fuelled by concern about the consequences of worldwide biodiversity loss and extinction on ecosystems (Lehman and Tilman 2000; McCann 2000; Tilman 2001; Duffy 2002; Lhomme and Winkel 2002; Lambers et al. 2004; Ives and Carpenter 2007; Laikre 2010; Cardinale et al. 2011).

Resolving the relationship between biodiversity and ecosystem function is not simple because several components of biodiversity can affect ecosystem functioning, even when considering only diversity at the species level. These components include species richness (the number of species), species evenness (their relative abundance), species composition (their taxonomic or functional nature) and non-additive effects (their interactions) as well as the spatial and temporal variations of those patterns (Symstad et al. 2003). Stability also has two components: resistance, the ability to withstand disturbance, and resilience, the ability to recover back to an equilibrium state after disturbance (Pimm 1984). A number of studies have concluded that greater biodiversity (i. e. species richness) enhances the stability of communities, the stability or productivity of ecosystems (McNaughton 1977; Tilman 1996; Hector et al. 1999;

Lehman and Tilman 2000; Reich et al. 2001; Tilman et al. 2001; Balvanera et al. 2006; Tilman et al. 2006; Cardinale et al. 2011), the resistance to disturbances such as disease and invasion (Knops et al. 1999; Fargione and Tilman 2005) or drought (Tilman and Downing 1994). In a slightly parallel line of work, functional richness (number of different plant functional types) and composition has also been shown to increase stability (Hooper and Vitousek 1997; Tilman et al. 1997; Hector et al. 1999; Naeem et al. 1999; Symstad 2000; Dukes 2001; Leps et al. 2001; Reich et al. 2004; Romanuk et al. 2010).

When dealing with ecosystems based on one structural species, its genetic diversity may have a similar effect on resilience and resistance of the population as species richness does on ecosystem resilience and resistance. Genetic diversity is one of the three main components of biodiversity recognized by the Convention for Biodiversity as a priority target for conservation measures, yet it is still largely neglected in management plans (Laikre et al. 2010). Genetic diversity is thought to reflect the evolutionary potential of species, as the genome encodes the information necessary not only to survive and reproduce in the current environment, but also the potential to adapt to changing or alternative environments (Hall and Malik 1998; Duffy 2006). In strongly declining and threatened populations with critically depleted genetic diversity, both reduced adaptive potential and the possible fixation of deleterious alleles by genetic drift due to small effective population sizes (Provan et al. 2008) can affect the long term survival capacity of populations and species (Pearson et al. 2009). Some empirical studies have shown that the genetic composition of key plant populations can have a strong effect at the level of the community and ecosystem (Neuhauser et al. 2003; Whitham et al. 2003) and even enhance diversity of associated species (Wimp et al. 2004). Yet, little empirical evidence has been gathered thus far to demonstrate the

general influence of this component of biodiversity on resistance and resilience of populations or ecosystems, even when these are critically threatened or on the edge of extinction, which may explain its widespread neglect in most management plans.

Ecosystems dominated by one or a few species, such as seagrass meadows or algae stands, are particularly vulnerable, because the loss of genetic diversity resulting from habitat loss and population fragmentation of key-species (Diaz-Almela et al. 2007) may have extended consequences on the overall biodiversity and function of the community (Hughes and Stachowicz 2004; Reusch et al. 2005). Seagrass meadows provide habitat for fish, shellfish and algal species, particularly during the developmental stages of these organisms; improve water quality by absorbing dissolved nutrients, as well as stabilizing sediments and minimizing resuspension. Seagrass meadows represent an important carbon-sink in the oceans, accounting for about 12% of the total oceanic carbon storage (Duarte and Cebrian 1996). These essential and emblematic ecosystems are however threatened and declining worldwide (Waycott et al. 2009), and elucidating both the extrinsic and intrinsic factors influencing their decline or resistance, including the genetic components, is a priority.

Recent experimental studies on seagrasses (Hughes and Stachowicz 2004; Reusch et al. 2005; Ehlers et al. 2008) suggested the importance of genotypic richness, the proportion of clonal lineages, or "distinct genetic individuals" in clonal organisms (Arnaud-Haond et al. 2007), on the resistance or resilience to perturbations in stands of *Zostera marina*. Similar results were observed at a local scale in natural stands (Hughes and Stachowicz 2009). Yet the combined influence of genetic diversity *sensu stricto* (i.e. heterozygosity or allelic richness), was not specifically tested for. A lack of correlation between heterozygosity and genotypic richness was put forward by some authors (Reusch et al. 2005) to interpret the results as a non-confounded effect of

genotypic richness *per se*. Besides, contrasting results reported in natural meadows of *Posidonia oceanica* (Diaz-Almela et al. 2007; Arnaud-Haond et al. 2010) suggest an inverse relationship of stability with genotypic richness and a possible influence of genetic diversity (as heterozygosity and mean number of alleles). Hence, further research on the importance of genetic diversity on the stability of seagrass meadows should attempt to clarify the respective effects of both components by dissociating them. In this study, we tested experimentally the relationship between genetic diversity and the resistance of experimental assemblages of *Zostera noltei*, a key-species of the intertidal ecosystem of Ria Formosa, to i) test for the level of interdependency of genotypic and allelic richness and ii) test for their respective or combined influence on the resistance and potential for recovery (resilience) of experimental populations.

Box 1 Glossary of terms used in this experimental design

Genotypic richness (G): total number of multi-locus genotypes or lineages (here within each subplot);

Genetic diversity: the genetic component of diversity; the *proxy* for genetic diversity can vary between studies (total number of alleles, mean number of alleles, heterozygosity, etc). In this study we use :

Allelic richness (A): total number of alleles (here within each subplot).

Material and Methods

In order to test for the existence of a relationship between allelic and/or genotypic richness and the resistance or resilience to stress conditions, synthetic assemblages of *Zostera noltei* shoots were setup. Each plot (four in total) contained nine assemblages

(subplots, the experimental unit) crossing three levels of genotypic richness (3, 6 and 9 genotypes) with three levels of allelic richness (low, medium and high, for each level of genotypic richness). These crossed levels where defined after *a priori* genotyping of a large number of shoots collected the field, and examination of the possible combinations of genotypic and allelic richness from those samples (Table 1). Below we provide the details for each step of this process up to the setup of each plot of nine subplots with increasing allelic and genotypic richness.

Sample preparation

A total of 376 plants with at least 10 shoots connected by one rhizome were collected in March 2009 from a natural meadow in the Ria Formosa, Portugal, at the channel of Ramalhete, and acclimated in a tank with running seawater and simulated tides for approximately 2 weeks. Each plant was tagged and one or two shoots used for DNA extraction using a standard CTAB extraction procedure (Doyle and Doyle 1988) and genotyped for 9 microsatellite markers (Coyer et al. 2004a; Diekmann et al. 2005).

Pre-selection of genotypes for the experimental setup

Based on the list of 376 genotypes obtained, the distinct genets were recognized based on their multi locus genotypes assessed with the 9 microsatellite markers following (Arnaud-Haond et al. 2007). The multi-locus genotypes were then used to virtually generate one thousand combinations of multi-locus genotypes (MLGs) for each of three genotypic richness levels (3, 6 and 9 MLGs) using a computer routine written for that purpose (S. Arnaud-Haond, available on request). The thousand combinations obtained for each level of genotypic richness were then sorted for their levels of allelic richness. Frequency distributions of allelic richness levels were drawn for the combinations

generated for each genotypic richness level. (Fig. 1) The ability to standardize allelic richness (as total number of alleles for all loci) for each level of genotypic richness was explored, and three values of A were selected to correspond to minimum, medium and maximum levels (Table 1). One combination of each pair of genotypic and allelic richness levels was then selected to set each subplot.

Table 1: Total number of alleles in each possible combination of genotypic and allelic richness in the experimental design.

		Genotypic Richness		
		3 MLGs	**6 MLGs**	**9 MLGs**
Allelic Richness	**Minimum**	16	25	31
	Medium	25	31	41
	Maximum	31	41	47

Experimental setup

Each subplot was set up with a standardized initial density of 27 shoots split into 9 sets of 3 shoots each and planted in individual small vases (approx. 2.5 L) with sediment from the Ria Formosa. Specifically, subplots with a genotypic richness of 3 MLGs had 3 sets of 3 shoots from each one of the 3 genets; subplots with a genotypic richness of 6 MLGs had a mixture of 3 shoots from 3 genets and 6 shoots from the other 3 genets split in two fragment of 3 shoots; and subplots with a genotypic richness with 9 MLGs had sets of 3 shoots from each one of the 9 different genets. Four replicates of such experimental units of 9 subplots/vases were setup and randomly distributed in a shared aquaculture tank of approx. $1m^3$ with running seawater pumped from the Ria Formosa, simulating regular tides.

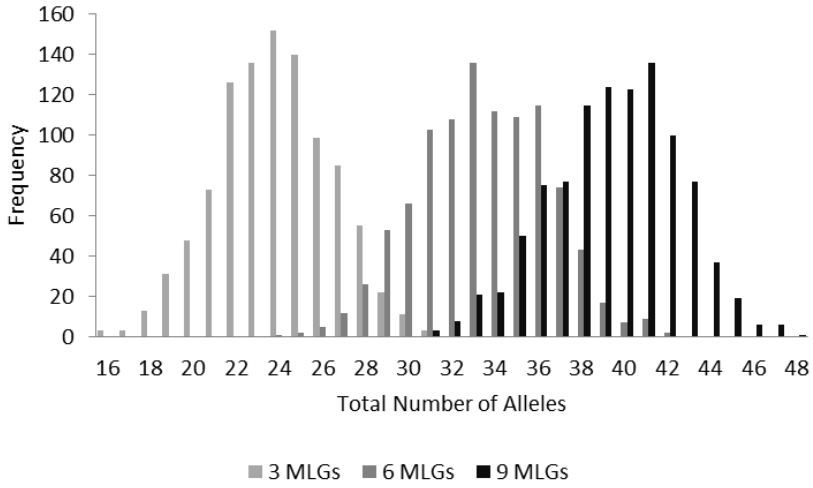

Figure 1. Frequency distribution of allelic richness (in total number of alleles) across the three levels of genotypic richness (3 MLGs in light gray, 6 MLGs in medium-dark gray and 9 MLGs in black).

Stress treatment

Although the initial goal was to apply a temperature shock, the tank where the experimental plots were setup suffered a diatom bloom two weeks after the assemblage of the combinations, leading to significant seagrass mortality. The diatoms formed an epiphytic layer over the leaves, causing them to die of hypoxia. Sub-lethal stress and some mortality was observed only in this tank, and no comparable loss was recorded in any of the other two tanks where other experiments with *Z. noltei* plots had been setup, supporting the role of the diatom bloom, unique to this tank, as responsible for the sublethal stress and partial mortality. The resistance of the plants was monitored after the end of the bloom, approximately 40 days later, by counting the remaining shoots in each subplot. Resilience, as the capacity to recover, was estimated following the same parameter every two weeks for ten months (data not shown).

Statistical analysis

The effect of genotypic richness was initially meant to be assessed by two-way ANOVA. Yet the impossibility to set subplots with standardized levels of allelic richness for each of the three level of genotypic diversity explored (see results section) limited the pertinence and power of this analysis. We therefore assessed genotypic richness effects by a one-way ANOVA on shoot density at the first count after the algal bloom and at the last count taken eleven months later, performed at allelic richness A=31, the only level of A represented in all three genotypic richness levels, and by t-test when aimed at comparing only two means, i.e., for A=25 (between 3 and 6 MLGs) and A=41 (between 6 and 9 MLGs). The effect of allelic richness was also assessed by one-way ANOVA on shoot density at both moments previously described, performed at each genotypic richness level individually. In order to illustrate results expected when controlling and measuring only one of those two parameters, regressions were also performed on the three levels of genotypic richness ignoring allelic richness differences and the effect of allelic richness was assessed by regression for each genotypic richness treatment. Finally, the role of allelic and genotypic richness in the performance of the plants along the various stages of the experiment was evaluated through multiple linear regression by evaluating the model: $Y = b0 + b1R + b2A + b3R*A$ using stepwise regression. ANOVA and regressions were constructed using STATISTICA (STATISTICA 7.0, StatSoft, Inc.).

Results

Genotyping of the 376 collected clones returned a total of 343 complete genotypes at all loci and 164 distinct MLG. Allelic richness in one thousand possible combinations of 3, 6 and 9 genotypes ranged from 16 to 31 at G=3, 24 to 42 at G=6, and

31 to 48 at G=9, with hardly any overlapping between the minimum and the maximum levels. A strong correlation between genotypic and allelic richness at those levels was found ($r = 0.904$, $p < 0.001$), as across 1000 combinations, Â was 23.93 ± 2.63 for G=3, 33.44 ± 2.97 for G=6 and 39.39 ± 2.96 for G=9, and specific low, medium and high levels of allelic richness had therefore to be defined independently for each MLG level. As a result, identical levels of allelic richness could not be standardized for the three genotypic richness but five levels corresponding to 16, 25, 31, 41 and 47 alleles were chosen to distribute low, medium and high levels among genotypic richness levels as detailed in Table 1. As a consequence, combined effects of allelic and genotypic diversities could not be simply disentangled through ANOVA analysis, and the compared effects of both were analysed simultaneously.

The first shoot count, taken approximately 40 days after the diatom bloom, ranged from 2 to 15. The mean number of shoots increased for both increasing MLG (7.67 for G=3, 9.75 for G=6 and 10.75 for G=9, n=9) and increasing Â (7.5 when A=16, n=4; 7.625 when A=25, n=8; 9.25 when A=31, n=12; 10.75 when A=41, n=8; 12.5 when A=47, n=4). These results show a trend towards higher survival for both higher allelic and genotypic richness.

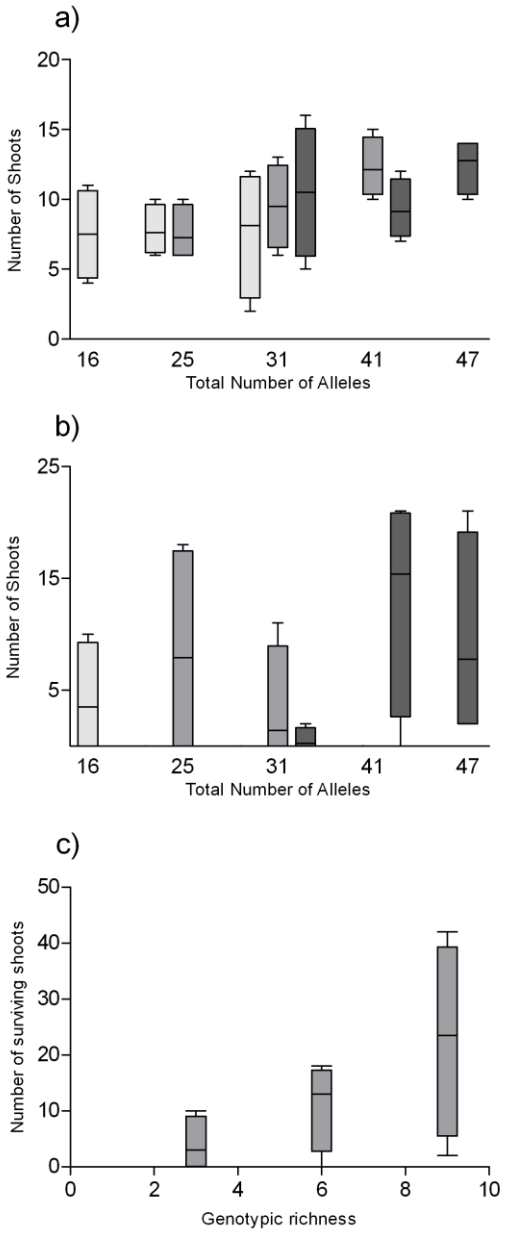

Figure 2. Combined effect of allelic and genotypic richness on survival. Mean shoot density for the five levels of allelic richness (16, 25, 31, 41 and 47) in the three genotypic richness levels (3 MLGs in light gray, 6 MLGs in medium-dark gray and 9 MLGs in dark gray) in the first count (a) and the last count (b). Total number of surviving shoots for each level of genotypic richness when ignoring the hidden effect of allelic richness (c). All values are represented by 25th and 75th percentile and minimum and maximum values.

When analysing the influence of genotypic richness while ignoring the parallel increasing levels of allelic richness (i.e. merging within each MLG class all levels of Â), results supported a significant effect (p=0.0439, Table 2) on resistance of experimental populations. When the effect of genotypic richness was compared at similar levels of allelic richness among them (specifically Â=25 for MLG 3 and 6, Â=31 for all MLG and Â=41 for MLG 6 and 9; Fig 2), no significant trend was recorded at low levels of Â (A=25 with p =0.623; A=31 with p=0.624) and the only significant, effect corresponded

to A=41 (p=0.034), suggesting an inverse relationship with higher genotypic richness inducing lower survival (Table 2). The effect of allelic richness did not significantly affect survival or recovery within any of the three levels of genotypic richness tested individually (Table 2).

Table 2: Summary of ANOVA and t-test analysis results.

Source of Variation	**Degrees of freedom**	**P value**
Two-way ANOVA of allelic and genotypic richness (first count)		
G	2	**0,0439**
A	2	0,1234
Interaction	4	0,5021
Two-way ANOVA of allelic and genotypic richness (last count)		
G	2	**0,0436**
A	2	0,5990
Interaction	4	**0,0193**
One-way ANOVA of allelic richness for each genotypic richness level individually (first count)		
3MLG	2	0,992
6MLG	2	0,051
9MLG	2	0,362
One-way ANOVA of allelic richness for each genotypic richness level individually (last count)		
3MLG	2	0,123
6MLG	2	0,232
9MLG	2	0,097
One-way ANOVA for A=31		*first count (resistance)*
R	2	0,629
One-way ANOVA for A=31		*last count (resistance)*
R	2	0,469
t-Test: Paired Two Sample for Means (for identical A)		*first count (resistance)*
3 and 6 MLGs	3	0,624
6 and 9 MLGs	3	**0,035**
t-Test: Paired Two Sample for Means (for identical A)		*last count (resistance)*
3 and 6 MLGs	3	0,066
6 and 9 MLGs	3	0,184

Simple regression analysis however showed a highly significant (p=0.007) overall relationship between allelic richness and resistance in terms of shoot density at 40 days after stress but not for resilience, measured as number of shoots after 11 months (p=0.368 ; Table 3). Stepwise multiple regression (backward as forward) showed a significant, positive effect of allelic richness (p = 0.03) on the rate of survival, but no influence of genotypic richness nor of its interaction with allelic richness (p > 0.05), on

survival throughout the experiment. The positive effect of allelic richness on survival was particularly strong following the heat shock ($p = 0.002$), and significant ($p = 0.02$) for 10 months after the heat shock.

Table 3. Summary of simple and multiple regression analysis.

Source of variation	**p-level**
Simple regression for shoot density vs. mean A (first count - resistance)	
Intercept	**0,036**
A	**0,007**
Multiple regression: Backwards stepwise regression for shoot density (first count - resistance)	
Intercept	**0,027**
A	**0,002**
R	-
R*A	-

Discussion

Confounding effects of genotypic and allelic richness

This study makes a first attempt to test for the effect and interaction of allelic richness, a component of genetic diversity that has not been manipulated in previous studies. The first important result is the relationship between genotypic and allelic richness at the low levels of genotypic richness typically used thus far in similar experiments. Indeed, results reported here show that it is unrealistic to dissociate the effect of allelic and genotypic richness on the whole experimental setup, as their high correlation prevented the setup of standardized levels of allelic richness for the three levels of genotypic richness. The simulation of 1000 random combinations of allelic richness for each level of genotypic richness showed a clear correlation between these two ($r = 0.904$, $p < 0.001$) that precludes the dissociation of their respective effects. Such a strong correlation is particularly expected when allelic richness levels are ignored, in the absence of a deliberate attempt to reach comparable levels across different genotypic richness.

These results point to an underlying effect of allelic richness in previously reported relationships between genotypic richness and resistance (Hughes et al. 2004; Hughes and Stachowicz 2004) or resilience (Reusch et al. 2005; Ehlers et al. 2008) to perturbations, as the two cannot be disentangled at low levels of genotypic richness. In fact, overlooking the allelic richness effect in our experiment, would also suggest an enhanced resistance with increased genotypic richness, similar to reports from previous studies (Hughes and Stachowicz 2004; Reusch et al. 2005; Ehlers et al. 2008). The lack of relationship between genotypic richness and heterozygosity was tested for in some cases to ensure that any observed effect was due to genotypic richness (i.e. clonal richness) rather than heterozygosity as a measure of genetic diversity *sensu stricto* (Reusch et al. 2005), but allelic richness, a more accurate indicator of the evolutionary potential of a population (Widmer and Lexer 2001; Leberg 2002), was not disclosed in these experiments.

From a theoretical point of view it is interesting to differentiate the respective effects of allelic richness, an estimate of the number of genetic variants per locus, which is meaningful for all organisms, from those of genotypic richness as an estimate of the number or proportion of genetically distinct individuals, or unique allele combinations, which is only relevant for organisms capable of clonal propagation. Our simulation analysis indicates that it is possible to dissociate effects of allelic and genotypic richness at much higher levels of genotypic richness than those typically used in experimental assessments. Indeed, the levels of genotypic richness that must be tested to allow separation of these effects are close to the levels recorded in natural meadows (up to 90% of sampling units for this species (Diekmann et al. 2005)). These levels are so high, that they are hardly amenable to experimental test. When analysing shoot density at the only comparable levels of allelic richness, no significant trends emerge for an

effect of genotypic richness except a significantly negative influence detected in one case. Experimental results reported here point towards a positive effect of allelic richness even when the effect of genotypic richness is not detectable or unclear. Future experiments should systematically report both these components to avoid confounding effects, and should ideally attempt to separate both levels of allelic and genotypic richness.

Overall positive effect of genetic diversity on resistance to diatom invasion

This study supports a positive effect of genetic diversity on shoot survival immediately after perturbation. Algal blooms are increasingly reported and forecasted to intensify worldwide (Hallegraeff 1993; Bushaw-Newton and Sellner 1999 (on-line); Heisler et al. 2008; Anderson et al. 2010), linked to human-induced coastal eutrophication, generating severe consequences particularly in terms of hypoxia and associated mortality events (Tiffany et al. 2006; Anderson et al. 2008). Global warming is expected to affect growth and life regimes of diatoms (Wiltshire and Manly 2004; Bopp et al. 2005; Esparza-Alvarez et al. 2007; Laird et al. 2007; Bucolo et al. 2008; Aydin et al. 2009; Malkin et al. 2009; Piontek et al. 2009; Ros et al. 2009), and several studies have already reported the negative effect of diatoms and other epiphytes on seagrasses (Schanz and Asmus 2003; Hasegawa et al. 2007; Lee et al. 2007), which may experience mortality due to suffocation by excessive growth of associated epiphytes and macroalgae. Epiphyte biomass is commonly very low in natural meadows of *Z. noltei* (Lebreton et al. 2009). This experimental study provides a first record of the negative effect of diatom blooms on a key-species of coastal ecosystems, the impact of which can be buffered at higher levels of genotypic and/or allelic richness. Diatoms grow on the leaves and may even fully cover them, preventing them from capturing light and

eventually leading to the death of the shoots (Howard and Short 1986; Hughes et al. 2004). The percentage of shoot loss in the tank that suffered the algal diatom bloom exceeded 50% in all cases (65.23% ± 11.79%), revealing sub-lethal stress and mortality. Surrounding tanks were not affected by diatom blooms nor did they have unusual mortality (S. Massa, personal observation).

The genetic component of diversity in the diversity-stability debate.

At the first count, 40 days after the diatom bloom, a tendency toward a higher shoot survival was observed at maximum allelic richness, especially when the number of genotypes was medium/high (6 or 9). When analysing all allelic richness levels while dissociating the three classes of genotypes, the significance of the relationship between survival and richness supports two classical hypotheses underlying conservation genetics studies, i) the positive effect of the genetic component of diversity as estimated through allelic richness on resistance of populations, and ii) that a large enough set of neutral markers delivers reliable estimates of the level of polymorphism for the whole genome, including those genes potentially submitted to differential selective pressure (Hansson and Westerberg 2002; Vali et al. 2008).

Biodiversity has been shown to enhance the ecosystem's ability to cope with stress (Mulder et al. 2001) but ecosystems depending on one key engineering species, as is the case for intertidal meadows of *Z. noltei* in the Ria Formosa, may therefore be particularly dependent on the genetic diversity of that key species. Our results are consistent with the insurance hypothesis or the redundancy effect frequently referred to in the diversity-stability debate (Yachi and Loreau 1999; McCann 2000). They also support the expected positive influence of genetic diversity on the future persistence of populations and species, a cornerstone of conservation genetics. Even though the

insurance hypothesis does not infer that diversity per se promotes stability, increasing diversity raises the probability of different responses to perturbations to be present in the population or community. It therefore increases the odds that some members will have the capacity to cope with the stress, thereby enhancing the ecosystem's ability to buffer perturbations. Low genetic diversity populations at stressful edges may thus be less capable to adapt despite higher selective pressures (Pearson et al. 2009).

Ecosystems may contain functional redundancy whenever species are capable of replacing each other. While some may decrease their contribution to ecosystem functions in face of environmental changes, others may increase theirs, thereby compensating losses (Naeem and Li 1997; Naeem 1998; Yachi and Loreau 1999). The functional redundancy hypothesis can also operate at the level of genotypic and genetic diversity, especially when key-species are concerned. Hence, higher clonal (i.e. genotypic richness) and genetic (allelic richness) diversity may also be expected to increase the likelihood that the population will display a broader range of responses to variable conditions, displaying a higher phenotypic diversity for key traits, and therefore higher population stability. Previous studies suggested that genotypic richness can have a positive effect on population stability, when traits displayed by distinct genotypes play an analogous role to species diversity. More genotypically diverse populations show enhanced resilience to different disturbances, settling success or density compared to less diverse communities (Hughes and Stachowicz 2004; Gamfeldt et al. 2005; Reusch et al. 2005; Reusch 2006; Ehlers et al. 2008). In the same way as each species in the community contributes with its unique use of resources and response to perturbations, different genotypes that may seem functionally redundant under some circumstances may fill different roles under changing conditions (Hughes and Stachowicz 2004).

In terms of implications for conservation genetics, our results support the correlation between genotypic richness and resistance or resilience by previous studies (Hughes et al. 2004; Hughes and Stachowicz 2004; Ehlers et al. 2008), although these previous interpretations may be partly attributable to a hidden treatment, that of increasing allelic richness with increasing genotypic richness. Overall, this study clearly showsthe positive influence of the genetic component of biodiversity, as estimated through allelic richness, on resistance to perturbations.

Conclusions:

This study reports the first experimental manipulation of both genotypic and allelic richness in a structural species, in an attempt to dissociate the effect of both parameters on its demographic response to stress. Our results show that the two effects cannot be disentangled at low levels of genotypic richness, yet support a positive effect of allelic richness on the resistance of populations to environmental stress. This study underlines the importance of genetic diversity for the persistence of populations, an issue of particularly great concern when affecting key-species of an ecosystem as seagrasses, and the importance to take this parameter into account in management strategies.

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6. CONCLUSIONS

These studies showed the role of genetic diversity in mediating responses to perturbations. This topic was approached by applying sub-lethal temperatures as a source of perturbation that is relevant for future climatic predictions, and assessed in terms of survival, physiological photosynthetic efficiency and gene expression of *Z. noltei*, a key-species of intertidal marsh zones throughout Europe, here assessed in the Ria Formosa, Portugal.

The response to heat-stress is activated very early on, with significant changes in photosynthetic yield being observed 24h-48h after the stress was applied and in gene expression as early as 30 minutes into the stress treatment. While photosynthetic efficiency drops after stress, accompanied by a drop in expression of genes with photosynthetic functions, expression of heat-shock proteins and chaperones is increased, in order to protect and repair protein structures. The molecular response is also very short, since gene expression returns to normal approx. 24h hours after the heat-shock, while the recovery of the photosynthetic apparatus takes longer, as efficiency only returns to normal a few days after the stress.

High temperature affects several stages of plant development. It may slow down or totally inhibit seed germination and affect photosynthesis and respiration (reviewd in Wahid et al. 2007). It has also been shown to enhance expression of a variety of heat shock proteins and other stress-related proteins (Feder and Hofmann 1999; Iba 2002), as well as the production of reactive oxygen species (Howarth and Skot 1994; Almeselmani et al. 2006). However, the damage to the photosynthetic apparatus due to protein denaturation and even the complete separation of the PSII reaction centres from the light harvesting complex in extreme thermal stress conditions is probably the most significant and irreversible effect (Ralph 1998). Our results showed that some plants

eventually die even after the overall photosynthetic yield returns to normal, which has also been suggested in other plant species (Berry and Bjorkman 1980; Ralph 1998; Campbell et al. 2006).

It is somewhat expected that, if high temperature conditions remain, plants will have the ability to build up a tolerance and eventually become acclimated to the stress and develop a way to continue to grow. Acclimation over an extended period of time can also lead to cellular responses including changes in cell cycle, metabolism and physical condition that could enable a greater heat tolerance in plants, similar to acclimation mechanisms that have been observed in this species when facing physical injuries (Peralta et al. 2005). The experiment on inter-individual variation in sensitivity to heat shock however suggested here the existence of either acclimation capacity enhancing resistance, or of a genetically based capacity, as survival was enhanced in one site of the Ria Formosa compared to the other. Progressive acclimation linked to global warming may enhance the temperature tolerance of populations for a longer period of time than anticipated on the basis of our experiments. Selection of genetic variants showing improved survival capacity if existing may allow a longer survival of local populations, although secondary consequences of trade-off imposed by a strong selective pressure on a particular character (i.e. resistance to temperature) are hard to anticipate on ecological and evolutionary time scales.

This work took an unexpected deviation from its original plan when one of our experimental setups suffered a diatom outbreak. Diatom blooms are a recurring phenomenon in coastal areas with several documented negative impacts on seagrasses, as they cover the leaves and reduce their capacity to capture light (Howard and Short 1986; Hughes et al. 2004), and are expected to increase since global warming will likely affect their life regimes (Wiltshire and Manly 2004; Esparza-Alvarez et al. 2007; Lee et

al. 2007; Piontek et al. 2009; Ros et al. 2009). Our results first confirmed the lethal effect of diatom bloom on coastal engineer species, but also showed that survival may be favoured by higher genetic diversity levels, as genetically distinct individuals may offer distinct responses to environmental, providing a broader range of coping mechanisms and resulting in increased likelihood of persistence of the population. In ecosystems that rely on one or few key-species, such as seagrass beds, the genetic diversity of that species may be as important as high species richness in a multi-specific habitat in providing a broader range of responses to environmental changes, which has often been suggested to have a positive effect on the stability of populations and ecosystems. There is also some evidence that a single genotype may dominate an entire meadow, suggesting higher survival for well-adapted, high fitness, large older clones (Diaz-Almela et al. 2007; Arnaud-Haond et al. 2012). This leads to question the most important part of a trade-off between an optimal strategy based on a set of few genotypes with high fitness due to large phenotypic plasticity, yet necessarily impoverished in terms of genetic diversity *sensu stricto*, and a scenario where highest allelic richness linked to more numerous or at least unrelated genotypes enhances the population resistance and resilience. The optimal strategy likely lies in between the two extremes, leaving space to the development of dominance of large clones capable of high phenotypic plasticity in populations or groups of populations where they would be distinct enough genetically to ensure the persistence of genetic diversity and the ability to form new combination through even scarce events of sexual reproduction.

Many fish, gastropods and molluscs rely on seagrass beds to provide shelter and food, as well as a safe environment for reproduction. Seagrass beds are also essential carbon sinks and are of economic importance due to local bivalve production. The latter, however, is also a major threat to survival of *Z. noltei* populations in the Ria

Formosa, as clam diggers uproot plants from the sediment and destroy rhizomes whilst collecting bivalves, causing pertinent biomass losses.

This work also showed that temperature is already reaching the sub-lethal limits for this species in this part of its distributional range, where mortality has been observed in the summer (S. Massa, *personal observation*) which is normally the season with greater shoot production and reproductive output, and where a recent survey has shown that biomass coverage of *Z. noltei* has decreased up to 75% in the past three decades (Cunha et al. 2012). Our results also point to high sensitivity to temperature increases when this close to its physiological threshold of approximately 38°C, as an increase of only 2ºC raises mortality from 5-20% to almost 100%. Although 38°C could be at a first glance considered as extremely elevated, it is sometimes already recorded in intertidal pools where *Z. noltei* is found in the Ria Formosa in Southern Portugal (S. Massa, personal observation). Considering the IPCC predictions, these results therefore lead to forecast either a range shift toward northern latitudes in the forthcoming decades, or a switch of the genetic composition of *Z. noltei* populations toward more resistant genotypes, if they exist. In the former case the local extinction of meadows in the Southern part of the species range would pile up with the worldwide decline of seagrasses (Waycott et al. 2009).

High temperature stress has been shown to cause biomass loss and/or species range shifts worldwide. Several studies have documented different responses to oceanic warming, like changes in the migration patterns of squid in the English Channel (Sims et al. 2001); shifts in abundance and distribution in plankton assemblages in the North Sea and English Channel (Southward et al. 1995; Beaugrand et al. 2002); north and north-eastern range extensions of several marine gastropods from the south of Britain (Mieszkowska et al. 2006a) and in Australian fish species (Morrongiello et al. 2011), as

well as changes in the community dynamics of intertidal invertebrates in the North West Atlantic (Barry et al. 1995; Bertness et al. 1999; Sagarin et al. 1999) and of coral reef fish species due to coral bleaching (Pratchett et al. 2008).

Even though IPCC predictions are somewhat controversial and not completely accepted, the situation in the Ria Formosa is already alarming for this species, regardless of any (more) global warming yet to come. Genetic diversity is one of the components of biodiversity recognized by the Convention for Biodiversity and may have an important role to play in the survival of this species, so it should therefore be taken into account in management plans and future - maybe inevitable - transplantation efforts for recolonization.

6.1 Future perspectives

Our results open a broad range of possibilities in the study of *Z. noltei*'s response to stress. Although it is a logistical challenge, it would be interesting to recreate the experiment of manipulation of genetic diversity at a larger scale, if possible using high temperature stress, in order to potentially identify the main driver of the resistance to perturbation (and finally disentangle genotypic and allelic richness) and also apply the scheme to field conditions, which we unfortunately were not successful at even after several attempts.

Further work could explore the potential connection between the EST-SSRs and heat-shock genes, providing new insight to the polymorphism in coding regions of the genome. Such polymorphism studied on the different sites used in the inter-individual resistance experiment may help elucidating the question of a possible distinct genetic backgrounds leading to differential ability of clones to cope with higher temperature.

In order to evaluate the distinct scenario of local extinction and range shift, acclimation or genetic shift of local populations, further screening of the genome and mapping of polymorphism in natural meadows (space) and in survival experiments (time) would be required. With the development of new techniques such as the RAD tags, further studies could include a rapid, cost effective, high resolution genotyping of restriction site associated DNA polymorphisms throughout the whole genome and even association of a given phenotype – such as resistance to high temperature, by using the genotypes that survived the heat-shock experiments - with particular genetic markers or identifying regions that exhibit significant differentiation between environmentally distinct populations (either extremely different in the latitude of origin or in tide height and desiccation exposure for example) as a sign of natural selection (Hohenlohe et al. 2010a; Hohenlohe et al. 2010b). Once a cluster of genes involved in temperature-resistance genes would be identified, the comparison of genotypes of both large, successful genets with those that survived heat-shock experiments and the ones that didn't for those loci, would allow evaluating the possibility of a relationship between fitness and heat tolerance. Besides, it may help elucidating the high phenotypic plasticity of larger clones by identifying key clusters of genes or regulation factors allowing the persistence of a particular genetic individual in very distinct environmental conditions .Such screening may be applied to the whole distributional range of *Z. noltei* to try to identify a pattern between temperature and genotype distribution and use this to estimate range shifts or local extinctions correlated to global warming predictions.

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