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**DEFENSE STRATEGIES AGAINST HERBIVORY IN SEAGRASSES**

Gema Hernán Martínez

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*A todas las gotas*

*a cada una de las olas*

*que han contribuido a formar este mar*

*A mi familia*

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# Contents

# ABSTRACT

Herbivory is a key ecological process that regulates the composition and structure of plant communities and determines the energy transferred from primary producers to upper trophic levels. Plants have evolved a suite of defense strategies to avoid or resist herbivory. Tolerance strategies reduce the impact of herbivory in plant fitness (e.g., increased belowground reserves), and resistance strategies reduce preference or performance of the herbivore (e.g., low nutritional quality, high fiber content). These strategies are based on morphological (e.g., toughness) and chemical traits (e.g., phenolic compounds) and can be expressed regardless of the risk of herbivory (constitutively) or in response to herbivore damage (induced). In addition, defense strategies may shift under different environmental scenarios (e.g. higher resource availability often drives a lower investment in resistance). Herbivory in marine systems can be greater than in terrestrial ecosystems, and it can have particularly important consequences when it is exerted upon habitat-forming plants.

Seagrasses are key foundation species dominating shallow coastal areas and providing numerous and critical ecosystem services to humans. Given their ecological and socio-economic relevance, understanding plant-herbivore interactions in these systems is crucial since changes in herbivore populations can result in important disturbances in these ecosystems. The main purpose of this thesis is to understand the effect of changes in environmental factors in plant defense strategies against herbivory and how these changes affect the palatability of the plant, and thus herbivore behavior.

Nutrient availability stands out for its effects on chemical and morphological plant defense traits. Plants under high nutrient environments in fertilization experiments and regions of higher nutrient availability (i.e. latitudinal comparison) exhibited higher nutritional quality and lower fiber content, both of which can increase their vulnerability to consumption. Interestingly, effects of nutrients on secondary compounds were absent or inconsistent. Simulated herbivory had clear effects on both morphological and chemical plant defense traits, however the two species studied differed in their responses. While in *Posidonia oceanica,* herbivory induced the production of resistance traits (e.g. fiber, secondary metabolites), in *Zostera marina* there was no induction of resistance traits, and on the contrary, simulated herbivory reduced their tolerance and resistance. As a result of the changes in traits exhibited by the plants, herbivores preferred the more nutritious repeatedly clipped leaves of *Z. marina* and the less chemically defended and more nutritious unclipped leaves of *P. oceanica*.

The environmental changes related to global climate change that I analyzed in this thesis (i.e. increased $CO_2$ and temperature), had important effects on defense strategies and susceptibility to grazers of *P. oceanica* seedlings. The increased $pCO2$ of seawater enhanced plant photosynthetic activity, leading to higher carbohydrate reserves in the seeds, which are the main storage tissue of the seedling. Although the increase in $CO_2$ decreased leaf nutritional quality (i.e. leaf nitrogen), plants growing under high $CO_2$ were preferred by the herbivores, possibly due to their increase in sucrose content or perhaps other chemical or structural characteristics that were not analyzed. In contrast to $CO_2$, the increase in temperature produced

clear negative effects on seedlings; increasing mortality and respiration resulting in greater use of seed reserves. Furthermore, warming reduced leaf fiber, which increased herbivore preference for warmed plants, and thus resulted in a decreased resistance to herbivory. These results illustrate the potential additive or counteractive effects that herbivory could have on determining the effects of environmental changes in seagrass ecosystems.

The research presented in this thesis contributes to identify the mechanisms that drive the changes in defense strategies against herbivory due to changes in environmental factors. Particularly, how these mechanisms change under different environmental conditions and how changes in traits associated with resistance to herbivores determine the vulnerability of plants to herbivory, highlighting the importance of assessing the effects of environmental factors on species interactions.

# RESUMEN

El herbivorismo es un proceso ecológico clave que regula la composición y estructura de las comunidades de plantas y determina la transferencia de energía de productores primarios al resto de la cadena trófica. Las plantas han desarrollado diversos mecanismos de defensa para evitar o resistir el herbivorismo. Entre ellos están las estrategias tolerancia, que disminuyen el efecto del herbivorismo en la vitalidad de la planta (ej. acumulación de reservas en tejidos subterráneos) y las estrategias de resistencia cuyo objetivo es evitar el consumo (ej. aumento del contenido en fibra). Estas estrategias se basan en características morfológicas (ej. dureza) y químicas de las plantas (ej. defensas químicas) y pueden expresarse de forma continua (constitutivas) o en respuesta al daño por herbívoros (inducidas). El herbivorismo en el medio marino puede ser mayor que en sistemas terrestres y puede tener importantes consecuencias cuando afecta a especies formadoras de hábitat

Las fanerógamas marinas son especies fundadoras dominantes en zonas someras costeras que nos proporcionan múltiples e importantes servicios. Debido a su relevancia ecológica y socioeconómica, es crucial profundizar en el conocimiento de las interacciones planta-herbívoro en estos ecosistemas pues existen cada vez más ejemplos que indican que cambios en las poblaciones de herbívoros han supuesto importantes perturbaciones en dichos ecosistemas. El propósito principal de esta tesis es entender cómo cambios en factores ambientales determinan la variación de las estrategias de defensa y palatabilidad de la planta, y por tanto el comportamiento de los herbívoros.

La disponibilidad de nutrientes destaca por sus efectos sobre las características químicas y morfológicas de las plantas ya que aumenta el valor nutritivo y disminuye el contenido en fibras de las hojas tanto en experimentos de fertilización como en regiones con mayor disponibilidad de nutrientes, lo cual las puede hacer más vulnerables al consumo por herbívoros. La simulación del daño por herbívoros afecta a las estrategias de defensa de las plantas de forma diferente en las dos especies estudiadas. Mientras que en *Posidonia oceanica* se induce la producción de compuestos de resistencia, en *Zostera marina* no hay inducción disminuyendo además su resistencia y tolerancia. Esto se traduce en que los herbívoros prefieren las hojas más nutritivas repetidamente recortadas de *Z. marina* y las hojas sin recortar con menos fibras y más nutrientes de *P. oceanica*.

Los cambios ambientales relacionados con el cambio global analizados en esta tesis (aumento del $CO_2$ y de la temperatura), tienen importantes efectos en las plántulas de *P. oceanica.* El aumento del $CO_2$ disuelto aumenta la actividad fotosintética de la planta y con esto las reservas de carbohidratos de las semillas. A pesar de que el aumento de $CO_2$ disminuye la calidad nutricional de las hojas, éstas fueron las preferidas por los herbívoros, posiblemente debido al aumento de sacarosa o por otras características no analizadas en las plántulas. Al contrario que el aumento de $CO_2$, el incremento de la temperatura produce efectos claramente negativos aumentando la mortalidad, la respiración y uso de las reservas de la semilla en estas plántulas. Además, disminuye el contenido en fibras de las hojas reduciéndose la resistencia frente al herbivorismo y aumentando por tanto la preferencia por herbívoros. Estos resultados

muestran los potenciales efectos aditivos que el herbivorismo puede suponer en los impactos de los cambios ambientales en las poblaciones de plantas marinas.

La investigación presentada en esta tesis contribuye a entender los mecanismos que influyen en los cambios de las estrategias de defensa frente al herbivorismo. Principalmente, en cómo estos mecanismos cambian bajo diferentes condiciones ambientales y como los cambios en las características asociadas a resistencia frente a herbívoros determinan la vulnerabilidad de la planta frente al herbivorismo. Además, destaca la importancia de evaluar los efectos de los cambios ambientales sobre las interacciones entre especies.

# RESUM

L' herbivorisme és un procés ecològic clau que regula la composició i l'estructura de les comunitats de plantes i determina la transferència d'energia de productors primaris a la resta de la cadena tròfica. Les plantes han desenvolupat diversos mecanismes de defensa per evitar o resistir l'herbivorisme. Entre ells destaquen les estratègies tolerància, que disminueixen l'efecte de l'herbivorisme en la vitalitat de la planta (ex. acumulació de reserves en teixits subterranis) i les estratègies de resistència, l'objectiu de les quals evitar el consum (ex. augment del contingut en fibra). Aquestes estratègies es basen en característiques morfològiques (ex. duresa) i químiques (ex. defenses químiques) de les plantes i poden expressar-se de forma contínua (constitutives) o en resposta al dany per herbívors (induïdes). L'herbivorisme en el medi marí pot ser major que en sistemes terrestres i pot tenir conseqüències especialment importants quan afecta a espècies formadores d'habitat

Les fanerògames marines són espècies fundadores dominants en zones costaneres i que ens proporcionen múltiples i importants serveis ecosistèmics. Com a conseqüència de la seva rellevància ecològica i socioeconòmica, aprofundir en el coneixement de les interaccions planta-herbívor en aquests ecosistemes és crucial, ja que existeixen cada vegada més exemples que indiquen que canvis en les poblacions d'herbívors han suposat importants pertorbacions en aquests ecosistemes. El propòsit principal d'aquesta tesi és entendre com canvis en factors ambientals determinen la variació de les estratègies de defensa i la palatabilitat de la planta, i per tant el comportament dels herbívors.

La disponibilitat de nutrients destaca pels seus efectes sobre les característiques químiques i morfològiques de les plantes ja que augmenta el valor nutritiu i disminueix el contingut en fibres de les fulles tant en experiments de fertilització com en regions amb major disponibilitat de nutrients, la qual cosa les pot fer més vulnerables al consum per herbívors. La simulació del dany per herbívors afecta a les estratègies de defensa de les plantes de forma diferent en les dues espècies estudiades. Mentre que en *Posidonia oceanica* s'indueix la producció de compostos de resistència, en *Zostera marina* no hi ha inducció, disminuint a més la seva resistència i tolerància. Això es tradueix en què els herbívors prefereixen les fulles més nutritives repetidament retallades de *Z. marina* i les fulles sense retallar amb menys fibres i més nutrients de P. *oceanica*.

Els canvis ambientals relacionats amb el canvi global analitzats en aquesta tesi (augment del $CO_2$ i de la temperatura), tenen importants efectes en les plàntules de *P. oceanica*. L'augment del $CO_2$ dissolt augmenta l'activitat fotosintètica de la planta i amb això les reserves de carbohidrats de les llavors. Tot i que l'augment de $CO_2$ disminueix la qualitat nutricional de les fulles, van ser aquestes les preferides pels herbívors, possiblement a causa de l'augment de sacarosa o per altres característiques no analitzades en les plàntules. Contràriament als efectes observats amb l'augment de $CO_2$, l'increment de la temperatura produeix efectes clarament negatius; augmentant la mortalitat, la respiració i l'ús de les reserves de la llavor en aquestes plàntules. A més disminueix el contingut en fibres de les fulles, reduint-se la resistència enfront de l'herbivorisme i augmentant per tant la preferència per herbívors. Aquests resultats mostren

els potencials efectes additius que l'herbivorisme pot suposar en els impactes dels canvis ambientals en les poblacions de plantes marines.

La recerca presentada en aquesta tesi contribueix a entendre els mecanismes que influeixen en els canvis de les estratègies de defensa enfront de l'herbivorisme. Principalment, en com aquests mecanismes canvien sota diferents condicions ambientals i com els canvis en les característiques associades a resistència enfront d'herbívors determinen la vulnerabilitat de la planta enfront de l'herbivorisme. A més, destaca la importància d'avaluar els efectes dels canvis ambientals sobre les interaccions entre espècies.

# List of abbreviations and acronyms

| | |
|---|---|
| α | Photosynthetic quantum efficiency |
| AF | Leaf Absorbance |
| At | Total alkalinity |
| AVHRR | Advanced Very High Resolution Radiometer |
| BB | Bodega Bay |
| BC | British Columbia |
| $\delta^{13}C$ | Carbon isotopic signature |
| CB | Coos Bay |
| CCH | Compensatory Continuum Hypothesis |
| CCS | California Current System |
| CNBH | Carbon Nutrient Balance Hypothesis |
| DIC | Dissolved inorganic carbon |
| DW | Dry weight |
| EK | Saturation irradiance |
| ESI | Electrospray Ionization |
| ETR | Electron transport rates |
| ETRmax | Maximum electron transport rates |
| FW | Fresh weight |
| GDBH | Growth Differentiation Balance Hypothesis |
| GRM | Growth Rate Model |
| HK | Hakai |
| Ic | Compensation Irradiance |
| Ik | Saturation Irradiance |
| LHDH | Latitudinal Herbivory-Defense Hypothesis |
| LRM | Limiting Resource Model |
| MS | Mass spectrometry |
| MX | Mexico |
| NDF | Neutral detergen fiber |
| NMR | Nuclear Magnetic Resonance |
| ODH | Optimal Defense Hypothesis |
| ODT | Optimal defense theory |
| PAR | Photosynthetically active radiation |
| $pCO_2$ | Partial pressure of carbon dioxide |

| | |
|---|---|
| **ppt** | Parts per thousand |
| **QU** | Quebec |
| **R** | Respiration |
| **RAH** | Resource Availability Hypothesis |
| **RLC** | Rapid life curves |
| **SD** | San Diego |
| **SE** | Standard Error |
| **SLA** | Specific leaf Area |
| **sp.** | Species |
| **SST** | Sea surface temperature |
| **TD** | Test Diameter |
| **UPLC** | Ultra-Performance Liquid Chromatography |
| **US** | United States |
| **UV** | Ultra violet |
| **w/** | With |
| **WA** | Washington |
| **Y** | Maximum quantum yield |
| **YB** | Yaquina Bay |

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# 1 Introduction and Objectives

## 1.1 Herbivory: a key process regulating plant communities

Plants are the primary producers of many ecosystems. They are the basis on which communities and food webs are assembled. Hence, understanding the processes and factors that determine plant abundance and distribution is crucial for having a general ecological perspective of ecosystem functioning. Herbivory is a key ecological process that determines the fluxes of energy and matter from primary producers to upper trophic levels (McNaughton *et al.*, 1989; Hulme, 1996). Herbivores regulate plant community composition and structure through different processes. For example, through feeding selectivity, herbivores can disproportionately affect growth rates, mortality, or reproduction of plant species (Maron & Crone, 2006; Wood *et al.*, 2016). Furthermore, herbivory has great effects on plant evolution through plant-herbivore coevolution (see below) and through the selection pressure that herbivores exert on plant propagules (e.g., seedlings; Barton & Hanley, 2013).

Changes in plant abundance or physical structure are particularly important when involving habitat-forming species. As ecosystem engineers, these species structure the community and are key to the resilience of the ecosystem through their influence on ecosystem processes and associated organisms (Romero *et al.*, 2014). They modify the landscape and reduce physical and/or biotic stress creating a suitable habitat for many species (Jones *et al.*, 1997). Many of these habitat-forming species are marine organisms such as kelps, corals and seagrasses, and the relationships between herbivores and these primary producers are critical for the ecosystem (Bruno & Bertness, 2000; Stachowicz, 2001; Burkepile & Hay, 2008). Unbalances in plant-herbivore interactions such as overgrazing can lead to dramatic changes in foundation species structure and abundance, and these effects cascade through the food web affecting directly (e.g., loss of feeding resource) and indirectly (e.g., increased predation risk) all the associated species (Watanabe & Harrold, 1991; Pagès *et al.*, 2012; Christianen *et al.*, 2014; Vergés *et al.*, 2016).

Whether consumers (top-down control) or resources (bottom-up control) are the primary factors influencing the regulation of populations is a topic of scientific analysis. The bottom-up hypothesis assumes that communities are limited by resources hence, a particular

trophic level is limited by the availability of the one below. Resource (e.g., light, nutrients) availability is an important factor determining plant growth and defense (see below). In this regard, herbivores play a crucial role influencing bottom-up mechanisms through nutrient cycling in marine environments via excretion of nutrients (Allgeier *et al.*, 2013). The top-down control perspective postulates that a trophic level is limited by the one above, thus herbivores are most often limited by predators, and plants are controlled by herbivore populations (Hairston *et al.*, 1960). Studies have found that most populations are regulated by both top-down and bottom-up processes, being their relative magnitude context-dependent (Burkepile & Hay, 2006; Littler *et al.*, 2006; Burghardt & Schmitz, 2015). In marine ecosystems, the loss of predators due to overfishing has led to a predominance of top-down (i.e. herbivore) effects through trophic cascades, especially in coastal benthic communities (Shurin *et al.*, 2002; Halpern *et al.*, 2006; Heck & Valentine, 2007; Poore *et al.*, 2012; Burkholder *et al.*, 2013). Hence, herbivore pressure in marine plants could be more intense than in terrestrial plants (Valentine & Duffy, 2006; Poore *et al.*, 2012; Bakker *et al.*, 2016). However, the impact of herbivory is highly variable ranging from 0 to 100% of biomass removal due to bottom-up (e.g., plant palatability; Cebrian *et al.* 2009) or top-down effects (e.g., herbivore size and metabolism; Borer *et al.* 2005). In addition, the relative effects of top-down and bottom-up forcing may shift due to anthropogenic factors such as removal of top-predators (Estes *et al.*, 2011), eutrophication (Östman *et al.*, 2016), or environmental changes (Jochum *et al.*, 2012).

### 1.1.1 Herbivory in seagrasses

Seagrasses are habitat-forming species with important ecological functions that provide numerous and critical ecosystem services to humans. Seagrasses are the basis of many coastal food webs, and due to the high primary productivity and the biomass buried in the meadows, they also act as an important carbon stock (Fourqurean *et al.*, 2012; Mazarrasa *et al.*, 2015). Being a foundation species, they provide suitable habitat for many organisms, epiphytes who settle on their leaves, or fish and crustaceans who find refuge in seagrass meadows (Nordlund *et al.*, 2016). Moreover, they attenuate wave height (Infantes *et al.*, 2012), reducing coastal erosion (Fonseca & Koehl, 2006), they increase water clarity, and contribute to the removal of pathogens (Lamb *et al.*, 2017). Early studies indicated that seagrasses had lower nutritional quality than terrestrial plants (Duarte, 1990) and it was therefore suggested that these ecosystems suffered

low grazing pressure. However, more recent studies have found that seagrasses have higher nitrogen and phosphorus contents than terrestrial plants (Cebrian & Lartigue, 2004; Cebrian *et al.*, 2009). Moreover, seagrasses share some similar traits with terrestrial grasses consistent with adaptations to grazing such as basal meristems or belowground storage (Heck & Valentine, 2006; Valentine & Duffy, 2006; Gordon & Prins, 2008). In fact, plant biomass lost through herbivory is 5-10 times greater in marine systems (Bakker et al. 2016), and there are many instances of major impacts of herbivores on seagrasses (Preen, 1995; Tomas *et al.*, 2005a; Prado *et al.*, 2007; Ruíz *et al.*, 2009; Fourqurean *et al.*, 2010; Lal *et al.*, 2010; Heithaus *et al.*, 2014). Nevertheless, plant-herbivore interactions and plant defense strategies have been less explored in seagrasses than in terrestrial plants. Hence, due to their key roles in coastal ecosystems, the feeding pressure they undergo and the knowledge gaps in relation to their defense strategies against herbivory, seagrasses are an important group in which to study plant-herbivore interactions.

## 1.2 Plant-herbivore interactions

The diversity of herbivores reflects the diversity of plants and feeding opportunities within the same plant, with some herbivores only feeding on particular plant groups (e.g., trees) or species, or on particular plant parts (e.g., leaves). Given the wide range and complexity of interactions between plants and herbivores, achieving a general understanding of how herbivores affect communities and ecosystems is challenging.

Herbivory is a bidirectional relationship and has led to a coevolution of herbivores and plants (Becerra, 2007; Futuyma & Agrawal, 2009; Agrawal *et al.*, 2012) that modules ecosystem functioning (Loeuille *et al.*, 2002). The "battle" of plants and herbivores has led to the evolution of diverse strategies to reduce or overcome herbivore damage in plants (Rosenthal & Kotanen, 1994; Strauss & Agrawal, 1999). These strategies involve different plant traits that confer a fitness benefit against herbivore damage (Karban & Baldwin, 1997) and are heritable and influenced by both environment and genetic constrains.

### 1.2.1 Plant defense strategies

Being plants sessile organisms which are often the main food source in many ecosystems, they are forced to defend themselves against consumers and pathogens. Plant defense strategies can be classified into four main mechanisms: escape, associative defense, resistance

and tolerance. Table 1 summarizes the main plant traits associated with the different defense mechanisms:

| ESCAPE | ASSOCIATIVE | RESISTANCE | | TOLERANCE |
|---|---|---|---|---|
| **Spatial:** | | **Physical:** | **Chemical:** | |
| Seed production | Toxic endophytes | Mineral crystals | N,P content | Belowground biomass |
| Stolon elongation | Grow w/ unpalatable sp. | Wax and lattices | Mineral crystals | Belowground reserves |
| **Temporal:** | Mutualism w/ predators | Spines, thorns, trichomes | Toxic latices | Photosynthetic activity |
| Seed dormancy | Toxic epiphytes | Toughness | Fibers | Growth rate |
| Synchronous phenology | | Fibers | Secondary metabolites | |

**Table 1. 1.** Traits related to plant defense strategies against herbivory

*Escape*

Plants can avoid herbivory through spatial and temporal escape. Growing in locations with difficult access such as rock outcrops, plants can reduce the chances of being found and eaten by herbivores. In addition, these populations may serve as a source of propagules to sink populations (Milchunas & Noy-Meir, 2002). In marine environments, rugose habitats such as crevices, barnacle or polychaete tubes, provide a refuge from grazing for algal spores or germlings, and once they have reached a specific size, become less susceptible to grazing (Lubchenco, 1983; Wahl & Hoppe, 2002). Likewise, structurally complex landscapes such as coral reefs (Vergés *et al.*, 2011a) or depth and shore exposure (Underwood & Jernakoff, 1984; Witman, 1987; Vergés *et al.*, 2012) can be an anti-herbivore refuge for macroalgae. Furthermore, growth form and architectural plasticity can also be a form of escape by limiting herbivore accessibility having for example, decumbent or erect canopies (Briske, 1996). Phenological escape through seasonality in plant growth or reproduction can also be a mechanism of escape from herbivore damage (Haavisto & Jormalainen, 2014). Algal species without other defense mechanisms (i.e. chemical, morphological) which are usually ephemeral species (e.g.,*Ulva*) rely on temporal escape through early reproduction and on spatial escape by colonizing areas with reduced grazing due to local disturbance (Raffaelli & Hawkins, 1999). The

unpredictability of flowering on the seagrass *Posidonia oceanica* has been suggested to be an escape strategy against grazers (Jahnke *et al.*, 2015a)

### *Associative defense*

Plants can receive indirect protection through association with other organisms. Grazing-sensitive plants can find a refuge living around unpalatable plants. Such is the case of the chemical protection provided by unpalatable epiphytic algae (e.g., *Dictyota sp*) growing over more palatable species (e.g., *Sargassum furcatum*; Wahl & Hay 1995; Pereira *et al.* 2010) or palatable species growing under the protective canopy of unpalatable shrubs (Saiz & Alados, 2012). The benefit of this association is based on the assumption that the growth reduction resulting from competition with epiphytes or shrubs for light and nutrients is less than the growth reduction resulting from the potential herbivory (Hay, 1986). In addition to this mechanism, plants can also obtain indirect defense by forming mutualistic relationships with predators (which will presumably reduce herbivory pressure), for instance by attracting them with volatile compounds (Heil, 2008), providing a suitable habitat for them (Janzen, 1966; Thaler, 1999), or forming endophytic symbiosis with toxic organisms (White *et al.*, 2014).

### *Resistance*

Plants have evolved other mechanisms that reduce the feeding preference or performance of herbivores (Fritz & Simms 1992). Once plants cannot escape herbivory, structural or morphological defenses can discourage consumption. In fact, leaf structural traits are a good predictor of feeding preference (Peeters, 2002). Components of plant surface such as spines (Gowda, 1996), trichomes, or thorns (Milewski *et al.*, 1991), all serve as deterrents against many herbivores. Likewise, leaf toughness is a good determinant of herbivory (Coley, 1983; Howlett *et al.*, 2001). Toughness affects penetration of plant tissues and increases breaking strength. Even though it is considered a physical characteristic, leaf toughness is achieved by chemical compounds, such as fibers (i.e. lignins, cellulose), produced and accumulated in the plants at different levels (molecules, cell walls). In addition, fibers reduce the digestibility to herbivores (Lincoln, 1993) and fiber content, being a C-based compound, is inversely correlated to leaf nutritional quality (de los Santos *et al.*, 2012). Moreover, some components that confer toughness such as lignin can bind to nutrients such as proteins, hindering their absorption (Hagerman & Butler, 1991). Hence, it can be difficult to discern the

exact role of the mechanical properties of plants since they also have a chemical effect. In addition to fiber compounds, minerals such as silica ($SiO_2$) or calcium oxalate ($CaC_2O_4$) can affect digestion and erode mouthparts (Lucas, 2000). Silica is accumulated in grasses and can be induced by herbivore damage (Hartley *et al.*, 2016). In the marine environment, silicon (Si) deposition in the red alga *Saccharina japonica* seems to have a protective role in wounded tissues (Mizuta & Yasui, 2012). Heavy calcification in marine algae together with chemical compounds contribute to herbivore deterrence (Paul & Hay, 1986; Schupp & Paul, 1994). Seagrasses also exhibit internal and external deposits of aragonite crystals (Enríquez & Schubert, 2014) but their effectiveness as feeding deterrents in seagrasses remains unexplored.

Chemical defenses are secondary metabolites; organic compounds that are typically not involved in the essential photosynthetic and metabolic activities (i.e. growth, development, reproduction) of the plant, although some of these compounds also contribute to primary metabolism (e.g., terpenoids; see below). Secondary metabolites are considered to play an important role in defense against herbivores (Bennett & Wallsgrove, 1994; Nykänen & Koricheva, 2004; Lambers *et al.*, 2008a), through their activity as disruptive chemicals making tissues difficult to digest or unpalatable (e.g., tannins), as protective chemicals making tissues toxic (e.g., alkaloids), and as modifiers of feeding behavior (e.g., volatile compounds; Rosenthal & Berenbaum, 1992). Based on their biosynthetic origin, plant secondary metabolites are divided into three groups: (1) terpenoids, (2) alkaloids and sulphur-containing compounds, and (3) flavonoids and phenolic and polyphenolic compounds (Crozier *et al.*, 2006a).

Terpenoids contribute to primary metabolism as pigments and electron carriers in photosynthesis, as hormones regulating growth and development (i.e. steroids), and are also involved in other chemical processes. For instance, latex, resins and other terpenoids accumulated in glandular trichomes act as insect repellents. Volatile terpenoids attract pollinators and predators in mutualism and associative defense (Blande & Glinwood, 2016). In addition, they have important functions as damage-induced compounds priming induced defense responses in plants. The volatile terpenoids released by damaged plants induce defense in neighbor plants (Frost *et al.*, 2008; Schrader & Bohlmann, 2015).

Alkaloids are nitrogen-containing compounds whose main function is defense against herbivores and pathogens through toxicity (McKey, 1974; Matsuura & Fett-Neto, 1996).

Flavonoids are the most numerous phenolic compounds, they accumulate in the epidermis and are involved in UV protection (Ferrat *et al.*, 2003). Phenolic acids such as tannins reduce plant palatability due to their flavor. In addition, tannins bind to proteins affecting herbivore nutrient absorption and they can inactivate digestive enzymes hindering digestion (Crozier *et al.*, 2006b). Marine macrophytes produce a variety of secondary metabolites but not as many nitrogen-containing compounds (i.e. alkaloids) as terrestrial plants (McClintock & Baker, 2001; Zidorn, 2016). Seagrass chemical defense compounds include flavonoids, phenolic compounds, and terpenoids (reviewed in Zidorn 2016) although they have been less studied than those of seaweeds (e.g., Paul & Hay, 1986; Duffy & Hay, 1990; Schupp & Paul, 1994; Toth & Pavia, 2007; Sabeena Farvin & Jacobsen, 2013). However, recent works diminish the importance of these compounds against other plant traits (e.g., morphology) as predictors of herbivore susceptibility in terrestrial systems (Carmona *et al.*, 2011).

Nutritional quality can be an important determinant of susceptibility to herbivory in plants (Ngai & Jefferies, 2004) and of ecosystem trophic structure (Cebrian *et al.*, 2009). Herbivores need to fulfill a determined requirement of nitrogen (Mattson, 1980). However, plants have low N contents since in most ecosystems there is a limited availability of N and P (Güsewell, 2004). Thus, increases in N or P can improve the palatability and nutritional quality of plants, and thus herbivore preference and performance (Valentine & Heck, 2001; De Bruyn *et al.*, 2002; Hemmi & Jormalainen, 2002; Prado *et al.*, 2010). On the other hand, low nutritional quality can stimulate a compensatory feeding behaviour, increasing feeding rates (Cruz-Rivera & Hay, 2000; Valentine & Heck, 2001), although this may increase herbivore exposure to predators and therefore, risk of mortality (Anholt & Werner, 1998).

Even though resistance strategies are quite effective, herbivores can evolve to overcome them by adapting their phenology, modifying mouth parts, and through detoxification *via* chemical modification and metabolization of phenolic compounds (Dearing & Cork, 1999; Salminen *et al.*, 2004) or through suppression of induction of plant defenses (reviewed in Karban & Agrawal, 2002; Kant *et al.*, 2015).

*Tolerance*

Once herbivores have overcome all the previous defenses, plants can mitigate the negative effects of herbivore damage in fitness through tolerance mechanisms (Rosenthal &

Kotanen, 1994). Increasing photosynthetic activity is a tolerance mechanism (Strauss & Agrawal, 1999; Tiffin, 2000) that can be achieved through different pathways (e.g., increase in Rubisco levels, delays in leaf senescence; Stowe *et al.*, 2000). Storing reserves during herbivore damage and reallocating those resources to reproduction or biomass regrowth after herbivory is commonly cited and it is the most studied mechanism (Tiffin, 2000). Furthermore, many experiments report increases in leaf growth as a compensatory response after leaf loss by herbivory in terrestrial plants and marine macrophytes (McNaughton, 1983; Hawkes & Sullivan, 2001; Moran & Bjorndal, 2005; Vergés *et al.*, 2008; Sanmartí *et al.*, 2014). Compensatory growth can be coupled to the utilization of stored reserves (van Staalduinen & Anten, 2005; Sanmartí *et al.*, 2014), and thus a reduction of root growth (Stevens *et al.*, 2008), or a lower allocation to structural defense such as reducing leaf toughness (Tiffin, 2000).

Plants have evolved this suite of strategies against herbivory expressed in a mixed defense strategy which involves tolerance and resistance mechanisms (Carmona & Fornoni, 2013). The evolution of tolerance or resistance strategies seems to be shaped and constrained by plant evolutionary history (e.g., phylogenetic constrains; Fine *et al.*, 2006), ecological interactions (e.g., specialist and/or generalists herbivores; Carmona & Fornoni 2013) and abiotic factors ( e.g., nutrient availability; Stowe *et al.*, 2000), being thus the degree of tolerance and resistance context-dependent.

In addition, these defenses can be permanently present in the plant (constitutive) or produced upon recognition of a threat or damage (induced). Constitutive defense requires a constant energy investment in production of defense compounds, but it is particularly advantageous when the herbivore attack is not predictable. Induction reduces the probability of herbivores to adapt to plant defenses (Karban *et al.*, 1997) while also reducing the costs of defense production when herbivore attack is predictable (Karban, 2011). Inductive defenses are usually chemical or morphological resistance traits, although they can also entail tolerance traits (e.g., increase in photosynthetic activity). Indeed, many studies in terrestrial plants (reviewed in Karban & Baldwin 1997; Nykänen & Koricheva 2004) and marine macrophytes (Toth & Pavia, 2007; Vergés *et al.*, 2008; Flöthe & Molis, 2013; Tomas *et al.*, 2015) have found an induction of defenses after herbivore damage, although it is difficult to draw a general conclusion about which defense mechanism will be induced in response to herbivore damage since inducibility is

affected by many factors (e.g., rate, duration and type of damage, season, abiotic factors; Toth & Pavia 2007; Vergés *et al.* 2008; Lemoine, Barrat-Segretain & Roy 2009; Flöthe & Molis 2013; Martínez-Crego *et al.* 2015).

### 1.2.2   Plant defense theories

Numerous hypotheses have been posited to explain within and among species variation in defense strategies against herbivory (tolerance and resistance). The different hypotheses also try to discern what the predictors for the exhibited defenses are and how plants deal with fitness costs.

#### *Biochemical Coevolutionary Theory*

The vast diversity of herbivores and their feeding specificity represent a selective force in plant evolution. Despite the arsenal of plant defenses, herbivores have evolved diverse mechanisms to feed on plants (Karban & Agrawal, 2002), leading to a reciprocal evolutionary change. The Biochemical Coevolutionary Theory (Ehrlich & Raven, 1964) suggests a coevolutionary process which starts by a change in plant phenotype through mutation or recombination (e.g., secondary compounds) which modifies plant palatability and thus reduces herbivore consumption until herbivores evolve resistance to the new phenotype (Berenbaum, 1983). More recent works confirm a long-term evolutionary history between plants and insects with the radiation of insects related to the diversification of plants (Futuyma & Agrawal, 2009; Johnson & Rasmann, 2011) as a result of many different biotic (e.g., competition, mutualism) and biogeographic (e.g., vicariance) processes (Suchan & Alvarez, 2015). In this regard, the effects of habitat fragmentation and climate change resulting in shifts in species distributions can modify the interactions between plant and herbivores, and thus their coevolution (Leimu *et al.*, 2012).

Most theories in plant resistance assume that defense has a cost, directly in terms of resource allocation, or indirectly in terms of ecological costs by affecting mutualistic relationships of the plant (Agrawal *et al.*, 2000; Heil, 2002; Koricheva, 2002; Strauss *et al.*, 2002). The interactions between resource availability or uptake, resource loss to herbivores, and plant resistance strategies are dynamic in space and time, complex and poorly understood.

## 1. Introduction and Objectives

*Optimal Defense Hypothesis*

The optimal defense hypothesis (ODH) considers how plants evolve allocating defenses which are costly while maximizing fitness. Such allocation will be the result of several factors such as the risk of the attack, the value of the plant part, and how the plant leads with the cost of this defense (McKey, 1974, 1979; Rhoades, 1979). The investment in plant defense related to the likelihood of a plant or plant part to be damaged has been stated in the Plant Apparency Theory (Feeny, 1976). According to this theory, plants that are more apparent (e.g. bigger, more abundant, perennial) should invest in quantitative defenses (e.g., cellulose, lignin) which reduce digestibility and whose effectiveness is concentration-dependent (i.e. higher concentration leads to higher toxicity). On the other hand, plants that are not easily found (e.g. ephemeral, rare) should invest in qualitative defenses (e.g., alkaloids); these are toxins present in small concentrations which are effective against generalist or unadapted herbivores. However, more recent studies in terrestrial plants have found that qualitative defenses are more effective against specialist herbivores and chemical defenses are equally effective against generalists and specialists (see review by Smilanich *et al.*, 2016). Regarding the fitness value of different plant parts, the Optimal Defense Theory (ODT) states that plant parts that contribute more to plant fitness are better defended (McKey, 1974, 1979; Rhoades, 1979). This idea has been supported by different works (see review by Stamp 2003) showing that reproductive tissues or younger parts are usually better defended.

One final consideration within ODH is the cost of defenses. After all, in the absence of cost, selection is expected to favor the most defended genotype (Moles, 1994). Because protection is costly, chemical defenses should be reduced in the absence of herbivores and increased when plants are subject to attack (Rhoades, 1979). Although there is a lag time where the plant is "unprotected", the induction of defenses is a well-documented cost-saving strategy (Karban & Baldwin, 1997; Agrawal & Karban, 1999; Zangerl, 2003; Arnold *et al.*, 2008; Vergés *et al.*, 2008) showing the adaptative benefits when defenses are costly to implement and there is spatial or temporal variability on the attack (Karban *et al.*, 1999; Rasmann & Agrawal, 2011). Induction may be initiated in response to signaling cues such as herbivory-induced plant volatiles or internally by signaling molecules (Frost *et al.*, 2008; Zheng & Dicke, 2008). The allocation cost of phenotypic defense implies that allocation to defense reduces the allocation to

other needs (e.g., growth, reproduction; Rhoades 1979).

*Resource availability-related hypotheses*

Another group of hypotheses focuses on how abiotic resources influence the production of plant defenses and tolerance strategies.

A general model is the one proposed by the Resource Availability Hypothesis (RAH) or Growth Rate Hypothesis (GRH; Coley, Bryant & Chapin 1985), which states that defense strategies are determined by the growth rate of the plant, which is in turn determined by the resources available. The main assumption is that growth rates are limited by available resources. The model predicts that high-resource environments favor fast-growing plants which have less constitutive defenses but are more plastic in defense, and thus have a reversible commitment to defense (i.e. inducibility), while low-resource environments favor slow-growing plants with long-lived tissues that invest more in constitutive defenses since replacement of tissue lost to herbivory is costlier in these environments. Numerous works have examined the validity of this hypothesis in terrestrial plants, and thirty years after its proposal many studies confirm its predictions (Endara & Coley 2011)

An earlier hypothesis is the Carbon-Nutrient Balance Hypothesis (CNBH). This hypothesis represents a model explaining how variations in the availability of carbon and nutrients in the environment influence the phenotypic expression of plant resistance defenses (i.e. secondary metabolites; Bryant *et al.*, 1983; Tuomi *et al.*, 1988). The assumptions of this hypothesis are that under high nutrient availability, carbon is allocated to growth and carbon surplus will be allocated to defense or storage. CNBH predicts that the carbon/nitrogen ratio in plants determines which secondary metabolites will be synthesized. Plants with high C/N due to for example, C accumulation in stems, will produce carbon-based defenses, while plants with a low C/N are more likely to produce nitrogen-based toxins (e.g, alkaloids). Plants can change their defenses in response to changes in C/N levels if they have a high phenotypic plasticity in defense (e.g., *Fabaceae*). This hypothesis has shown some limitations in the outcome of their predictions [e.g., not applicable to high dynamic metabolites and terpenoids, reviewed by Hamilton *et al.*, 2001].

In a similar approach, the Growth Differentiation Balance Hypothesis (GDBH) states that

plant defenses are the result of a tradeoff between growth-related processes (i.e. cell division and elongation) and differentiation-related processes (i.e. specialization and maturation; Herms & Mattson 1992) over a range of environmental conditions (Loomis, 1932). This hypothesis assumes that growth and chemical defenses compete for photosynthetic resources, and thus carbon allocation to all functions cannot be met simultaneously (Lorio, 1986). The GDBH accounts for tradeoffs between growth and defense over a resource availability gradient. In environments where resources (e.g., water, nutrients) limit growth and photosynthesis, internal resources (e.g., carbon) will be allocated to growth. As resource availability increases, growth is more constrained than photosynthetic activity which is less sensitive to for instance nutrient and water deficit (Loomis, 1932, 1953), hence these carbon compounds can instead be allocated to carbon based secondary metabolites (e.g., phenolics, tannins). In resource-rich environments where the demands for growth are met, carbon is allocated to growth of meristems, decreasing the activity of secondary metabolism. Hence, fast-growing plants are predicted to contain lower levels of secondary metabolites and vice versa. Although this has been cited as the most mature hypothesis in plant defense, very few studies have specifically tested this hypothesis and they exhibit contradictory results (Glynn *et al.* 2007; but see Massad, Dyer & Vega C. 2012; Hattas, Scogings & Julkunen-Tiitto 2017).

On the other hand, plants can offset herbivory damage in plant fitness by compensation mechanisms such as increasing leaf production to counterbalance leaf loss through herbivory (Briske, 1996; Valentine & Heck, 1999; Vergés *et al.*, 2008), or by overcompensation, such as by increasing seed production or seed chemical defense to increase reproductive rates (Paige, 1992; Agrawal, 2000; McArt *et al.*, 2013). At low levels of herbivory, fitness can be maintained (i.e. compensated) or increased (i.e. overcompensated) through different mechanisms. There are three models that try to predict how resource availability affects plant tolerance strategies: The Compensatory Continuum Hypothesis (CCH) suggests that plants growing under less stressful conditions will have more resources to recover from (herbivory) damage (Maschinski & Whitham, 1989). On the contrary, the Growth Rate Model (GRM) suggests that plants growing under stressful conditions (e.g., low resources) at the time of herbivore damage may have a higher capacity to increase growth (compensatory growth) since they were growing below their maximum growth rate (Hilbert *et al.*, 1981). However, probably the most accurate model

predicting the effects that resource availability has on tolerance strategies is the Limiting Resource Model (LRM) (Hawkes & Sullivan, 2001; Wise & Abrahamson, 2008; Hay *et al.*, 2011) which suggests that under high resource conditions, plants can better tolerate and/ or compensate herbivory if all the limiting nutrients are available and particularly when the acquisition or use of this resource is affected by herbivory.

### 1.2.3  Effects of environmental factors in plant defenses

The complexity of all the factors that determine the production or selection of the different defense strategies and the associated cost will be influenced by changes in environmental factors, as they may alter plant response to herbivory by modifying plant metabolism and resource availability.

#### *Latitude*

Latitudinal gradients involve a variation in abiotic (e.g., daylight duration, nutrients, temperature) and biotic (e.g., species diversity, evolutionary history) conditions and have important effects in many plant traits (e.g., leaf nutritional quality, productivity). Furthermore, it has been hypothesized that the intensity of biotic interactions also increases towards lower latitudes (Dobzhansky, 1950; MacArthur, 1972; Schemske *et al.*, 2009) although there is much controversy surrounding this hypothesis (Lambers *et al.*, 2002; Hillebrand, 2004). Intensified species interactions also imply increases in herbivory damage, hence according to the Latitudinal Herbivory-Defense Hypothesis (LHDH), plants are hypothesized to have a higher investment in defense at lower latitudes. Linked to the LHDH, studies have found that plants are less palatable (e.g., higher toughness, lower nitrogen and phosphorus leaf content; Reich & Oleksyn, 2004) and better defended (e.g., higher content in secondary compounds) towards lower latitudes (Coley & Aide 1991; Rasmann & Agrawal 2011; but see Moles *et al.*, 2011; Anstett *et al.*, 2016a). This pattern has been observed in salt-marsh plants (e.g., Pennings *et al.* 2007; Ho & Pennings 2013) and seaweeds (Bolser & Hay, 1996), but not so clearly in terrestrial plants (Adams & Zhang, 2009; Moles *et al.*, 2011). On the other hand, latitudinal variations in tolerance strategies (Lehndal & Agren, 2015) and differences between constitutive and induced defense have been much less explored (Wieski & Pennings, 2014; Anstett *et al.*, 2016b), and remain unexamined for seagrasses.

# 1. Introduction and Objectives

## *Human-induced changes in environmental factors*

Human activity has led to many important changes in the environment and the transformation of ecosystems. The conversion of forests into croplands linked to the use of fertilizers has increased the total of nitrogen available (Sarukhan & Whyte, 2005). The burning of carbon-rich fossil fuels to produce energy has added $CO_2$ to the atmosphere (IPCC, 2014). Some of this $CO_2$ is absorbed by the oceans changing carbon biochemistry and increasing seawater acidity (Doney *et al.*, 2012). Furthermore $CO_2$ is heat-trapping gas which has led to the increase of the global temperature and changing weather patterns with increased extreme events (IPCC, 2014). All these changes in abiotic factors have led to changes in the ecosystems.

Most studies about plant defense and environmental factors are related to resource availability. Until recently, few studies have explored the effects of anthropogenic stressors in plant defense, except for eutrophication. The number of experiments assessing the effects of increased $CO_2$ and temperature in relation to plant physiology and defense against herbivory in terrestrial systems outnumbers by far the studies in marine systems.

## *Nutrient availability*

As explained above, according to the RAH, CCH and LRM, increases in resources, especially limiting resources, are expected to reduce the allocation of plants to constitutive defense strategies. Indeed, increased nutrient availability (i.e. nitrogen and phosphorus) increases nutritional quality of both terrestrial and marine plant leaves (Olsen & Valiela, 2010; Tomas *et al.*, 2015; Yuan *et al.*, 2015) and can change plant morphology by decreasing belowground biomass (i.e. roots and rhizomes; Reynolds & D'Antonio, 1996; Lee & Dunton, 2000; Olsen & Valiela, 2010) and by increasing specific leaf area ($cm^2/gr$) (Knops & Reinhart, 2000; Lee *et al.*, 2004; Sardans *et al.*, 2006), which is inverse of plant toughness (Paul *et al.*, 2012). All these changes would decrease the capacity of plants to resist and tolerate consumption (see Table 1). Increased nutrient availability also appears to decrease carbon-based secondary compounds such as phenolic compounds (Koricheva *et al.*, 1998; Throop & Lerdau, 2004; Fine *et al.*, 2006; Massad & Dyer, 2010) that together with the higher nutritional quality (i.e. higher nutrient content) could benefit herbivore performance. In terrestrial plants and seagrasses, carbohydrate storage seems to be lower under high nutrient availability (Bloom *et al.*, 1985a; Invers *et al.*, 2004; Tomas *et al.*, 2011; Martinez-Crego *et al.*, 2014). However, few

studies have assessed the effects of nutrient availability in defense induction, finding inconsistent responses (Ibarra-Obando *et al.*, 2004; Tomas *et al.*, 2015).

*CO$_2$ availability*

The increase of atmospheric $CO_2$ is expected to reach 800 ppm by the end of the century (IPCC, 2014). Due to the absorbing capacity of the oceans (Solomon *et al.*, 2009), seawater also undergoes increases in $pCO_2$, and thus both terrestrial and marine plants will be exposed to higher $CO_2$ concentrations. This higher $CO_2$ concentration may benefit plants by enhancing photosynthetic activity through an increase of the substrate of carbon fixation metabolism (i.e. $CO_2$), consequently increasing carbon fixation rates (Ehleringer, 2005) and thus carbon assimilation. Moreover, a higher $pCO_2$ will reduce photorespiration because of the higher diffusion of $CO_2$, increasing the efficiency of carbon uptake (Bowes *et al.*, 1971; Ehleringer, 2005) and overall net photosynthesis (Long *et al.*, 2004). In addition, these physiological changes may affect plant defense strategies against herbivory. For instance, various studies have found that high $CO_2$ availability increases carbohydrate content and/or biomass in belowground tissues due to increased carbon fixation (e.g., Nie *et al.*, 1995; Palacios & Zimmerman, 2007; Campbell & Fourqurean, 2013; Takahashi *et al.*, 2016) although this response is not so clear in seagrasses (Ow *et al.*, 2015, 2016; Tomas *et al.*, 2015). Furthermore, higher $CO_2$ availability changes plant morphology (e.g., number of leaves, leaf size, number of trichomes; Farnsworth, Ellison & Gong 1996; Pritchard *et al.* 1999; Bidart-Bouzat *et al.* 2005) and reduces leaf nutritional quality (e.g., C/N; Huang *et al.*, 2015) likely by nitrogen dilution due to the increase in growth rates (reviewed in Stiling & Cornelissen 2007; Massad & Dyer 2010). However, in seagrasses studies report decreases (Jiang *et al.*, 2010; Alexandre *et al.*, 2012; Campbell & Fourqurean, 2013) or no change (Arnold *et al.*, 2014; Martinez-Crego *et al.*, 2014; Tomas *et al.*, 2015) in leaf nutrient content in response to higher $CO_2$ availability. Moreover, although secondary metabolites (carbon-based compounds) increase in response to higher $CO_2$ (Peñuelas *et al.*, 1997; Stiling & Cornelissen, 2007; Massad & Dyer, 2010), the scarce experiments analyzing seagrass secondary metabolites in response to increased $CO_2$ have shown again inconclusive results that are not in accordance with terrestrial plant responses; i.e. a reduction (Arnold *et al.*, 2012, 2014) or no change of phenolic compounds (Tomas *et al.*, 2015) have been reported with changes in $CO_2$. Likewise, nitrogen-based secondary compounds (e.g., alkaloids) have been less studied and their content

could decrease as a result of changes in plant C/N according to the CNBH (Massad & Dyer, 2010).

*Warming*

Due to human activities, global mean temperature has increased together with the frequency of heat waves and extreme climatic events. In fact, global mean temperature is expected to increase between 1ºC and 4ºC by the end of the century (IPCC, 2014). Warming increases metabolic rates in plants and herbivores (Clarke, 2006). Increased temperature may change or disrupt (due to changes in distribution) the strength of plant-herbivore interactions (O'Connor, 2009; Carr & Bruno, 2013) since thermal tolerance ranges and sensitivity may differ between species (Voigt *et al.*, 2003). Increased temperature could also lead to phenological mismatches (e.g., leaf maturity, larvae feeding; Rosenblatt & Schmitz, 2016) or increase the number of herbivore life cycles completed in one season, overall increasing herbivore damage (DeLucia *et al.*, 2012).

In aquatic macrophytes, increased warming is predicted to enhance growth and germination, unless the temperature reaches threshold values above which these responses decline (reviewed by Dhir, 2015). Furthermore, warming seems to reduce plant capacity to tolerate herbivory by decreasing carbohydrate reserves (Zvereva & Kozlov, 2006; Koch *et al.*, 2007), which has been attributed to a higher demand of stored resources due to increased respiration. Resistance to herbivory seems to differ between terrestrial plants and marine macrophytes in relation to warming. While leaf nutritional quality (i.e. nitrogen content) is unaffected by warming in terrestrial plants (Zvereva & Kozlov, 2006), in marine macrophytes it appears to diminish (Kaldy, 2014; Zhang *et al.*, 2016a). Furthermore, in terrestrial plants, phenolic compounds tend to decrease with warming (Sudatti *et al.*, 2011), while terpenoids increase (Zvereva & Kozlov, 2006). In addition, since toxicity of plant secondary compounds has proven to be temperature-dependent, warming could also increase their toxicity and thus reduce the capacity of herbivores to cope with these compounds (Kurnath *et al.*, 2016). Very few studies have assessed the effects of warming in secondary compounds of seagrasses.

## 1.3  Objectives

The aim of this thesis is to increase our understanding of the mechanisms that influence seagrass-herbivore interactions, which I approach by investigating the variations in defense

strategies of seagrasses and how they regulate feeding behavior. Herbivory is a crucial process regulating community structure and dynamics. Thus, understanding the mechanisms that regulate plant-herbivore interactions increases our knowledge on how communities are structured, having important implications for management and conservation. With this thesis I attempt to fill the main gaps of knowledge outlined in this introduction; identify what traits can be related to defense mechanisms in seagrasses, examine how herbivores respond to resistance traits in seagrasses, and quantify the effects of environmental changes on defense mechanisms.

Following the structure under the Marine Ecology PhD program from the University of the Balearic Islands (UIB), this thesis is structured in six sections. The first section is this general introduction with the objectives of the thesis. The second section is a description of the general methodology and experimental approaches followed in this thesis. The third section includes the results of the thesis (which are presented as four chapters; see below). The last two sections are the general discussion of the thesis followed by the conclusions.

The results section consists of four chapters that focus on how environmental and / or biotic factors produce changes in seagrass traits related to plant defense strategies against herbivory and how herbivores respond to changes in seagrass traits. Each chapter has consisted of a series of manipulative experiments; exposure of seagrasses to experimental treatments, and feeding experiments with herbivores. Each chapter constitutes a separate article in preparation, in revision, or published in a peer reviewed journal (Q1).

**Chapter 1:**

*"Latitudinal patterns of productivity and response to herbivory in a marine habitat-forming species"*

In this chapter we aim to assess the changes on plant traits related to tolerance and resistance against herbivory in response to latitude and the potential induction and compensatory responses to different herbivory rates. Moreover, we evaluate how changes in plant traits affect herbivore preference.

**Chapter 2:**

*"Resource availability and herbivory damage do not interact to modulate plant defense strategies"*

The objective of this chapter is to investigate how increased resource availability affects tolerance and resistance seagrass traits to herbivory and how the induction of defenses can be modified by resource availability. Likewise, we test how changes in plants traits translate to changes in plant palatability for the main vertebrate and invertebrate herbivore of the seagrass species studied.

**Chapter 3:**

*"Seagrass (Posidonia oceanica) seedlings in a high-$CO_2$ world: from physiology to herbivory"*

In this chapter we assess how increased $p$$CO_2$ availability affects seedling physiology and plant traits related to defense strategies against herbivory and how $CO_2$-driven changes in plants traits modify plant palatability.

**Chapter 4:**

*"Future warmer seas: increased stress and susceptibility to grazing in seedlings of a marine habitat-forming species"*

The focus of this chapter is to assess the changes on seedling physiology and traits related to tolerance and resistance against herbivory in response to increased ocean temperature, and how warming-induced changes in plant traits affected herbivore preference. Furthermore, we evaluate how feeding behavior of herbivores may change due to warming.

# 2 General Methodology

The goals addressed in this thesis required different methodological approaches. This section provides a general overview of the methods applied in the thesis. Particular methods used to assess the specific objectives and specific study sites are described in section 3 in the methods section of each chapter.

## 2.1 Experimental approaches

In order to study how seagrass defense strategies respond to changes in environmental factors and herbivory, I performed a suite of manipulative experiments with two temperate seagrass species, the Mediterranean endemic species *Posidonia oceanica*, and the circumglobal species *Zostera marina*.

### 2.1.1 Field experiments

The effects of herbivore pressure and environmental conditions (i.e. latitude and nutrient availability) were tested in field experiments since they recreate better natural conditions and some factors (i.e. latitude) are more adequately studied in field experiments.



**Figure 2.1**. a) Field setup with intertidal plots of *Zostera marina* in Oregon, USA; b) Subtidal *Posidonia oceanica* clipped plot; c) Intertidal *Zostera marina* clipped plot

Marked fixed plots were set at similar depths avoiding edges of the meadow and patchy areas and randomly assigned to the different experimental treatments. In order to avoid or minimize transport of resources among rhizomes, plots were set 3-4 m apart from each other (Fig. 2.1.a.).

The simulation of herbivore pressure was performed by clipping the leaves inside the experimental plots as well as around the margin surrounding the plot mimicking the grazing exerted by the main herbivores in the studied shallow meadows (Fig.2.1.b,c). The control treatment corresponded to the ambient herbivory which was naturally low in all field sites, while the moderate and high herbivory treatment consisted on clipping a percentage of the blade length. Removal of leaf canopy was quantified as related to the maximum leaf length in the ambient plots (average of 5 plants measured per plot).

### 2.1.2 Mesocosm experiments

In situ manipulation of environmental variables such as temperature and $CO_2$ availability are quite challenging. Thus, most of the studies on warming effects on seagrasses have been conducted in chambers (e.g., Kaldy, 2014), tanks/aquaria (e.g., Palacios & Zimmerman, 2007; Nejrup & Pedersen, 2008) or mesocosms with closed systems (e.g., Koch *et al.*, 2007). We have performed the manipulative experiments in aquaria in temperature-controlled facilities at IMEDEA. This was the most optimal approach in order to be able to conduct studies with newly emerged seedlings in controlled temperature conditions that allowed us to obtain a thorough understanding of the effects of warming and increased $CO_2$ on seagrasses. We performed these experiments with seedlings because seagrass seedling ecology has been almost unexplored, and particularly in relation to defense strategies against herbivores. Furthermore, seedlings are individual organisms while adult seagrasses are connected through rhizomes whose dissection could generate stress. Moreover, seedlings are important life stages in plant populations since the selection pressure that herbivores exert on seedlings is an important evolutionary pressure (Barton & Hanley, 2013) and has critical effects on composition and structure of plant communities (Watkinson, 1997; Barton & Hanley, 2013).

**Figure 2.2**. Aquaria with *P. oceanica* seedlings in a) $CO_2$ addition experiment (Chapter 3) and b) warming experiment (Chapter 4) within the temperature-controlled rooms at IMEDEA.

Beach-stranded fruits of *P.oceanica* were collected from Palma Bay (Balearic Islands, Spain) and transported in seawater at ambient temperature to the laboratory. Seeds were extracted from fruits and kept in aquaria at constant temperature until germination. Newly germinated seedlings were randomly selected for the experiments. Each treatment was conducted with a 25L-replicate aquaria with (Fig.2.2.b) or without (Fig.2.2.a) substratum, aerated seawater, and a recirculating pump. All aquaria and seedlings were gently cleaned of epiphytes to avoid confounding factors of epiphyte colonization in plants (i.e. light and nutrient competition). Aquaria were filled with filtered seawater from Palma Bay which was changed or refilled once a week. Temperature and light intensities of each aquarium were recorded throughout the experiment using data loggers (Onset Hobo). Salinity was controlled every three days and the photoperiod was maintained along the experiments. Specifics on the $CO_2$ system and temperature maintenance can be found in the respective chapters.

## 2.2 Plant analyses

In order to assess the effects of the experimental treatments on plant biochemical traits, we performed a series of chemical analyses. As tolerance traits we analyzed carbon, nitrogen, phosphorus and carbohydrate contents in belowground tissues, while the resistance traits that we examined were carbon, nitrogen, phosphorous, total and specific phenolic compounds, and fiber content of leaves.

Pooled plant material of adult shoots or seedlings of each replicate was freeze-dried and ground to a fine powder for analyses. Carbon and nitrogen content in leaves, seeds, rhizomes

and roots were analyzed using a Carlo-Erba CNH elemental analyzer. Phosphorous content was analyzed as in Fourqurean *et al.*, (1992) using certified standard beech leaves (CRM No. 100). Sucrose of leaves and carbohydrate reserves (sucrose and starch) in rhizomes, seeds and roots were measured using methodology described by Invers *et al.*, (2002). Sucrose and other soluble sugars were obtained after three sequential extractions with 95% (v/v) ethanol at 80°C for 15 min. The remaining pellet of roots and seeds was dissolved in 0.1N NaOH for 24 h at room temperature for starch extraction. Soluble sugars and starch contents of extracts were determined by spectrophotometry using an anthrone assay with sucrose as standard. Neutral detergent fiber content (NDF) was measured in 25–30 mg of sample [see de los Santos *et al.*, (2012)]. The amount of fiber in each sample was obtained from the difference in mass, and expressed as fiber percentage in dry biomass.

Total phenols (total Folin phenols) were extracted from ca. 4 mg of ground tissue with 1.5 mL of methanol 50% (v/v) for 24 h and were determined with spectrophotometry following a modified Folin-Ciocalteu method using caffeic acid as standard (modified from Bolser *et al.*, 1998). Additionally, we calculated total phenol content as the sum of specific phenolic compounds (see below), since the Folin method has known limitations, such as lower sensitivity (Blum, 1997) and interference of organic matter (e.g., sugars, Benlloch-Tinoco *et al.*, 2015).

Specific phenolic compounds were extracted from powdered leaf samples (0.1-0.2 g) with MeOH (3-5mL x 3) and the organic extracts obtained were suspended in $H_2O$. Separation and identification of compounds was performed using an Ultra-Performance Liquid Chromatography (UPLC) instrument (ACQUITY, Waters) fitted with UPLC BEH C18 column (1.7 µm, 2.1 x 50 mm, Waters) at 25 °C with photodiode array detector at 300-450 nm tandem with a mass spectrometer (Xevo G2 Q-TOF, Waters) equipped with an electrospray ionization (ESI) source operating in negative mode. Samples were injected using a mobile phase A (0.1% of formic acid) and solvent B (100% acetonitrile) in a linear gradient with constant flow (0.4 mL/min): 0.0-0.5 min 90.0% A (isocratic), 0.50-3.50 55% A (linear), 3.50-4.20 0% A (linear), 4.20-4.80 90.0%A (linear), 4.80-5.50 90.0% A (isocratic). The optimized MS conditions were as follows: capillary voltage of 3000 V, cone voltage of 20 V, source temperature of 120 °C, desolvation temperature of 350 °C, and desolvation gas (nitrogen) flow rate of 850 L/h. For UPLC-MS/MS analysis the parameters were: source temperature 150 °C, capillary voltage 3000 V,

cone voltage 20 V, trap collision energy 20-40 eV, mass scan range m/z 50-1200 Da. The phenolic compounds were identified by their retention time and molecular masses with MassLynx 4.0 ChromaLynx Application Manager software (Waters). Quantification of phenolic compounds was performed by external calibration curves, using a reference compound selected based on the principle of structure-related target analyte/standard (chemical structure and functional group) and all determinations were performed in triplicate.

## 2.3 Herbivore feeding experiments

In order to assess how changes in plant traits affect plant palatability, we performed a suite of herbivore feeding experiments (some in the field and some in the laboratory; see below) with different herbivores that naturally feed on the seagrass species studied. To assess the effect on feeding palatability on *P. oceanica* different series of feeding experiments were performed with the sea urchin *Paracentrotus lividus* and with the fish *Sarpa salpa*. In the experiments conducted with *Z. marina* the herbivore selected to perform the feeding experiments was the isopod *Pentidotea resecata*. The feeding experiments were performed with fresh leaf tissue or reconstructed agar-food and all the experiments ended when approximately 50% of the initially offered material was consumed. In each replicate of each experiment, herbivores were offered similar amounts of young leaf tissue clean of epiphytes. The experiments consisted of 15 to 30 replicates and in order to measure any potential changes in leaf tissue not related to grazing, control cages protected from herbivores were used to correct for autogenic changes in area prior to the statistical analyses. The corrected consumption was calculated as:

$$\text{Treatment}_{\text{Initial (FW)}} * (\text{Control}_{\text{Final (FW)}} / \text{Control}_{\text{Initial (FW)}}) - \text{Treatment}_{\text{Final (FW)}}$$

Following the procedures of previous feeding behavior experiments in seaweeds and seagrasses (e.g., Bolser *et al.*, 1998; Tomas *et al.*, 2005b; Vergés *et al.*, 2007), replicates where all the offered leaf material was consumed or remained intact were not considered in the statistical analyses.

## 2.3.1  Field experiments

In order to examine how changes in *P. oceanica* defense traits modify plant palatability, we performed a series of feeding trials with herbivorous sea urchins and fish at the end of the experiment.

Similar-sized sea urchins of the species *Paracentrotus lividus*, the main invertebrate herbivore on *P. oceanica* meadows, were collected and kept in cages of 225 cm$^2$ covered with a 1 cm mesh. These cages were placed in the experimental sites in P. oceanica dead matte (natural substratum formed by this seagrass species) and sand protected from currents (Fig. 2.3.a.). Sea urchins were maintained in the cages for a period of 48 hours and fed with *Ulva sp. ad libitum* previous to the performance of the feeding experiments. Replicates of two (for two-choice experiments) tethered leaves (from the different treatments) attached with a clothes pin were placed inside the cages (Fig. 2.3.b.).
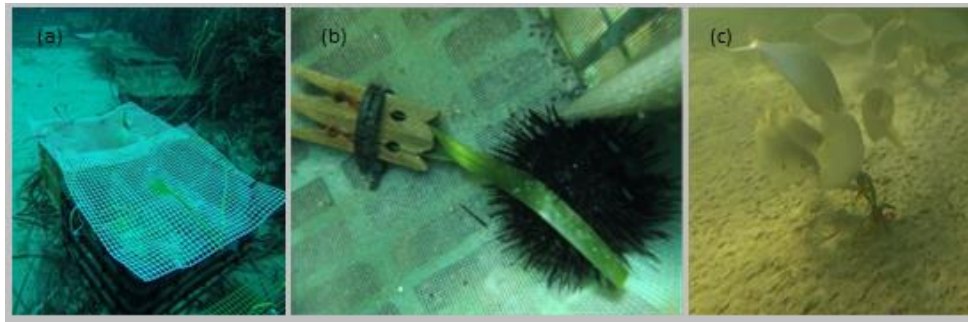


**Figure 2.3**. Feeding experiments with *P. oceanica* (chapter 2); a) cages with sea urchins; b) two-choice with sea urchin; c) two-choice with fish.

The feeding experiments with fish were performed in a third site, a shallow bay in the middle of Palma Bay that harbors a *Cymodocea nodosa* meadow and where large groups of *S. salpa* are present. In sandy patches and at least 3 m away from *the C. nodosa* meadow, replicates of two (for two-choice experiments) pegs with tethered leaves (from the different treatments) attached with a clothes pin and a cable tie ca. 10 cm above the sediment were offered to the fish (Fig. 2.3.c.).

## 2.3.2   Laboratory experiments
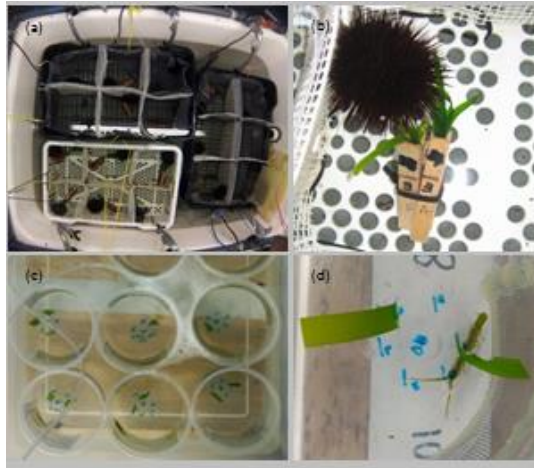


**Figure 2.4**. a) Sea urchins in cages in flow through system (chapters 3, 4); b) two-choice experiment with sea urchin; c) Isopods in cups in aerated tanks (chapter 1); d) two-choice experiment with isopod

### *Experiments with fresh plant material*

When performing feeding trials in the laboratory, we used similar sized sea urchins of the species *Paracentrotus lividus*, which were kept in individual cages of 225 cm$^2$ covered with a 1 cm mesh in a seawater flow-through system (Fig. 2.4.a) under controlled light conditions (12/12 h) and fed with *Ulva sp. ad libitum* at least 24h prior to the start of the experiments. When assessing palatability of *Zostera marina*, similar sized isopods of the species *Pentidotea resecata* were acclimated for 24h in food deprivation prior to the start of the experiments, being kept in individual containers (500mL plastic cups with two parallel windows) inside tanks with aerated seawater (Fig. 2.4.c) under controlled temperature and light conditions (12/12h).

*Experiments with reconstructed food*
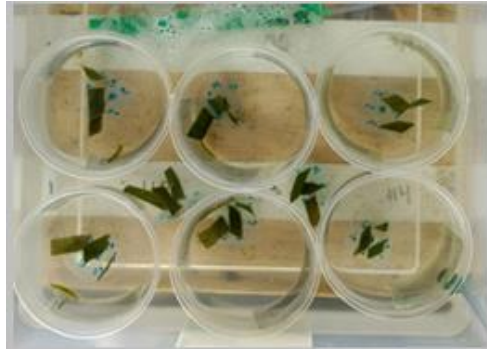


**Figure 2.5**. Isopods in cups in aerated tanks with agar-based food (chapter 1)

In order to examine if the feeding patterns observed were driven by mechanical-structural or chemical traits, in Chapter 1 (i.e. latitudinal comparison) we performed a feeding experiment using agar-based artificial food. One gram of agar was added to 25 mL of boiling distilled water, the mix was chilled while stirring until reaching approximately 60ºC (to avoid sample degradation by temperature). Then, 2 g of powdered plant material from a pool of leaves (young leaves from 3-4 shoots without apical and basal parts) were added to the mix. The mixture was then poured onto a thin plastic window screen and flattened. Once the agar mix was solid, it was cut into rectangles of 1 x 3.5 cm and offered to the herbivores following the same procedure as with fresh leaf tissue (Fig. 2.5.). Consumption was measured by counting the number of mesh squares that were cleared of food and calculated as percentage of area eaten. Control tethered rectangles placed out of the reach of herbivores were used to correct for changes in area.

# 3 Results

## 3.1 Chapter 1: Latitudinal patterns of response to herbivory in a marine habitat-forming species

### 3.1.1 Abstract

Plant-herbivore interaction strength generally increases with decreasing latitude as a function of species diversity. Consequently, according to the Latitudinal Herbivory-Defense Hypothesis (LHDH) plants at lower latitudes are predicted to be better defended than high latitude conspecifics. Because environmental conditions and resource availability also change with latitude and the cost of defense strategies trades-off with other important functions (e.g., growth), allocation to defenses can change with latitude. In fact, higher resource availability decreases allocation to constitutive defenses (Resource Availability Hypothesis; RAH) and when limiting resources are provided, allocation to tolerance defenses increases (Limiting Resource Model; LRM). Moreover, differences in herbivore pressure may change plant defense responses. Studies trying to test the LHDH yield contradictory results due to the lack of studies covering broad latitudinal ranges and the use of inconsistent methods. Therefore, our aim was to assess the changes on plant traits related to tolerance and resistance strategies against herbivory in response to latitude, the potential induction of defense responses to different herbivory rates and how herbivore-driven changes in plant traits affect palatability. To this end we conducted simulated herbivory experiments across the east Pacific coast (from Canada to Mexico) and Quebec with the circumglobal seagrass species *Zostera marina*. Almost all the chemical and morphological traits measured responded to latitude, but we did not find support for the LHDH, since plants did not exhibit higher defenses at lower latitudes. Following the LRM, seagrasses from mid-northern latitudes (which undergo higher nutrient inputs from upwelling) exhibited higher tolerance capacity via higher content of belowground reserves. Similarly, and in accordance with the RAH, plants from upwelling sites allocated less to constitutive resistance traits exhibiting no signs of induction to herbivore damage, and thus a potential higher susceptibility to grazing. Our results highlight the great importance that latitude with its underlying abiotic factors (i.e. temperature, nutrient availability, day length) has on plant traits.

**3.1.2  Introduction**

One of the most recognized patterns in ecology is the increase in diversity of species towards lower latitudes (reviewed by Hillebrand, 2004). As a result of this latitudinal diversity gradient among other factors (Thompson 1988; Menge 2003; Louthan *et al.* 2015), the intensity of biotic interactions also increases towards lower latitudes (Dobzhansky 1950; MacArthur 1972; Schemske *et al.* 2009; Roslin *et al.* 2017). Biotic interactions such as herbivory strongly influence plant fitness and distribution patterns (Wisz *et al.* 2013) and, consequently, plants have evolved diverse strategies to tolerate and resist herbivory (Nunez-Farfan *et al.* 2007). Tolerance strategies (e.g. regrowth) mitigate the negative effects of herbivory in plant fitness (Rosenthal & Kotanen 1994) whereas resistance strategies (e.g. chemical defenses, nutritional quality) reduce the feeding preference or performance of herbivores (Fritz & Simms 1992).

Due to the increased strength of trophic interactions, and thus herbivory, towards the equator, it has been proposed that plants are better defended towards lower latitudes (Latitudinal-Herbivory-Defense Hypothesis, LHDH; Coley & Aide 1991; Johnson & Rasmann 2011). Indeed, many studies in salt marshes show that plants suffer higher herbivory at lower latitudes (e.g., Pennings & Silliman, 2005; Schemske *et al.*, 2009; Wieski & Pennings, 2014), and that such plants exhibit lower palatability resulting from lower nitrogen content, higher toughness, or higher content of secondary compounds (e.g., Pennings *et al.*, 2001; Siska *et al.*, 2002; Ho & Pennings, 2013). However, the LHDH remains a topic of controversy (Anstett *et al.* 2016b), and recent reviews have found weak support for this hypothesis (Moles *et al.* 2011; Zhang *et al.* 2016) pointing to other explanatory mechanisms (e.g. optimal defence theory; Kooyers *et al.* 2017)) rather than herbivory pressure *per se* on those latitudinal gradients. It has been pointed out that the solution to this controversy requires both new large-scale investigations encompassing broad latitudinal geographic areas, as well as the use of consistent comparable methods (see Schemske *et al.* 2009; Anstett *et al.* 2016b), as well as examining traits that are truly associated with susceptibility to herbivory and plant defense (Carmona *et al.* 2011), particularly regarding the induction of chemicals (Anstett *et al.* 2016a) .

Moreover, most studies exploring the LHDH have focused on herbivore damage and resistance strategies, whereas latitudinal patterns in tolerance strategies have been largely unexplored (Anstett *et al.* 2016b). The few existing tolerance studies have assessed the effects on compensatory growth, and have yielded inconsistent results. Wieski & Pennings (2014) did

not observe latitudinal differences in compensatory growth in a saltmarsh species (*Iva frutescens*) in response to herbivory, which the authors attributed to similar regrowth responses driven by different selective pressures (i.e. leaf loss by freezing at high latitudes, and herbivore damage at low latitudes). On the other hand, the perennial herb *Lythrum salicaria* exhibited an increased tolerance towards higher latitudes. However, the higher leaf length in response to herbivory found in northern populations was attributed to timing of sampling since northern populations start their growing season earlier (Lehndal & Agren 2015). Importantly, in addition to regrowth, plants exhibit several other tolerance traits against herbivory (e.g., stored belowground reserves), yet their variations have not been examined in a latitudinal context.

Resources allocated to defense can trade-off with growth and reproduction (Bazzaz & Grace 1997; Strauss *et al.* 2002) thus, resource availability greatly influences the expression of defensive traits. The Resource Availability Hypothesis (RAH; Coley, Bryant & Chapin 1985) proposed that plants in resource-rich environments will allocate less to constitutive resistance since tissue lost through herbivory can be easily replaced, and invest in induction of secondary metabolites. Regarding tolerance strategies, the Limiting Resource Model (Wise & Abrahamson, 2007) describes an increase in plant tolerance if limiting resources are provided and herbivore damage does not hamper the acquisition or use of resources. Since environmental resources (e.g., light, nutrients) change with latitude, the defense strategies that plants use to cope with herbivory are also likely to change with latitude. Furthermore, the production of defensive traits can be induced, that is, expressed in response to herbivory damage (Agrawal & Karban 1999), or constitutive, i.e. expressed regardless of the risk of attack, and some studies have detected a trade-off between both strategies. Thus, plants investing in high constitutive resistance may not increase their production of defences after damage (Kempel *et al.* 2011; Rasmann *et al.* 2015). Yet generalities of the trade-off between constitutive and induced defenses and how it may change with latitude remain elusive; some studies have detected higher constitutive defenses at lower latitudes versus higher induction at higher latitudes (Anstett *et al.* 2016a) while other studies found the opposite pattern (Moreira *et al.* 2014b).

Beyond environmental factors, the suite of defense strategies that plants express against herbivory involve different traits that are heritable and influenced by genetic constrains (e.g., genetic variation; (O'Reilly-wapstra *et al.* 2002; Andrew *et al.* 2007; Anstett *et al.* 2015) modulating the expression of these traits. In fact, reduction of genotypic richness or increased

inbreeding reduce plant resistance to herbivory (Du *et al.* 2008; Moreira *et al.* 2014a). Thus it is also important to consider these variations especially in wide range studies. Furthermore, the level of herbivory pressure (i.e., duration and/or intensity) can shift plant defense responses. While some traits may be expressed under high herbivory rates (e.g., induction of secondary metabolites; Dostálek *et al.*, 2016), others are induced under moderate pressure (e.g., compensatory growth; Ruiz-R *et al.*, 2008). However, few studies have considered these differences in relation to latitude.

Plant-herbivore interactions can have profound effects in ecosystems, especially when involving foundation species, because the effects of herbivory upon them has both direct (e.g. loss of feeding resources) and indirect (e.g. loss of refuge) impacts on associated species as well as on ecosystem properties (e.g. nutrient cycling; Ellison *et al.*, 2005; Silliman *et al.*, 2013). As foundation species, seagrasses create critical coastal ecosystems, increasing habitat structural complexity and thus providing settlement, refuge and food for a wide variety of species (Willlams & Heck 2001; Nordlund *et al.* 2016) as well as contributing through many ecosystem services (e.g., carbon burial, reduction of coastal erosion, increase of water clarity (Nordlund *et al.* 2016).

The aim of this study was to investigate the latitudinal patterns on plant traits related to tolerance and resistance against herbivory and the potential inductive responses to different herbivory rates. We hypothesized that plants from regions with more resources available (e.g. nutrients via upwelling) will allocate more resources to tolerance (following LRM), having lower constitutive resistance (following RAH), but higher inducibility of secondary metabolites under grazing pressure. Consequently, we expect that herbivores will be deterred by plants suffering higher herbivory rates. We examined these questions by performing a simulated herbivory experiment with the seagrass *Zostera marina*, a dominant foundation species in estuaries and coastal areas across the northern hemisphere (Short *et al.* 2010), as it provides a perfect opportunity to examine patterns in plant defense along latitudinal gradients. To this end we conducted a simulated herbivory experiment across the US west coast, Mexico and Quebec to 1) examine the influence of latitude on plant defense strategies against herbivory 2) asses the effects of latitude and herbivory in these strategies and their trade-offs and 3) investigate how herbivore-driven changes in plant traits affect palatability through a series of feeding assays with a common seagrass grazer.

### 3.1.3  **Materials and methods**

Simulated herbivory experiments were performed at ten study sites (nine of them along the eastern coast of the Pacific Ocean) ranging approximately 20 degrees in latitude (52º–32°), from Calvert in Canada to Ensenada in Mexico (Fig. 3.1.1). This latitudinal gradient is negatively correlated with mean sea surface temperature (SST; Fig.3.1.1.a) while nutrient (i.e. $NO_3$) concentrations follow a bell-shape distribution (Fig. 3.1.1.b).

The abiotic factors of the study sites are affected by the variability of the California Current System (CCS). The CCS extends from the North Pacific Current (~50°N) to Baja California (~25°N), where the nearshore equatorward winds drive the upwelling of cold nutrient-rich deep waters. The CCS is therefore associated with an increase of dissolved nitrate concentration where coastal upwelling occurs (e.g., up to 34 mmol/L at Yaquina Bay, Oregon; Sigleo *et al.* 2005) and a lower surface temperature (SST; Fig.3.1.1b). The annual cycle of coastal wind stress displays two distinct regimes (Fig. S.1.1). In the southern sites (San Diego, and Ensenada) , the annual cycle features a weak upwelling season in early spring (Schwing & Mendelssohn, 1997). In contrast, in the central sites (San Francisco and Bodega Bay), the greatest upwelling season, which is more intense than further south, occurs from April-July (Fig., S.1.1). Additionally, alongshore variations of upwelling are also driven by the interaction of the coastal current and the coastline/bathymetric features. Likewise, local sources of nutrients from river plumes along the U.S. coast can be particularly important in determining nutrient inputs; such as the freshwater discharge of the Columbia River (Washington site) which in summer is advected southwards and offshore (Barnes *et al.*, 1972). Additionally, along the sampling range the tidal currents are stronger in the northern sites (Fig. S.1.2.)

The experiment started the first week of May 2015 (ca. 2 - 4 weeks before known maximum peak of leaf growth) and was maintained for a total of 10 to 11 weeks (i.e. ending in early July 2015) in order to be able to capture potential changes in carbon reserves in belowground tissues as well as in leaf growth. The plots were 4m$^2$ and were set in shallow subtidal (0.5 to 1m at low tide) monospecific *Zostera marina* beds. The experimental treatments consisted on three simulated herbivory levels; control (i.e. natural low levels), moderate (i.e. removal of 40% of maximum leaf length), and high (removal of 80% of maximum leaf length), and plants were clipped every two weeks throughout the experimental period. Maximum leaf

length was quantified as the average maximum leaf length of 5 plants measured per plot.

There were 5 replicate plots per treatment (total 15 plots) randomly distributed in homogeneous meadows avoiding the edges of the meadow or patchy areas (see methodology section). The separation between plots was ca. 2m, and the treatments were randomly assigned to each plot.
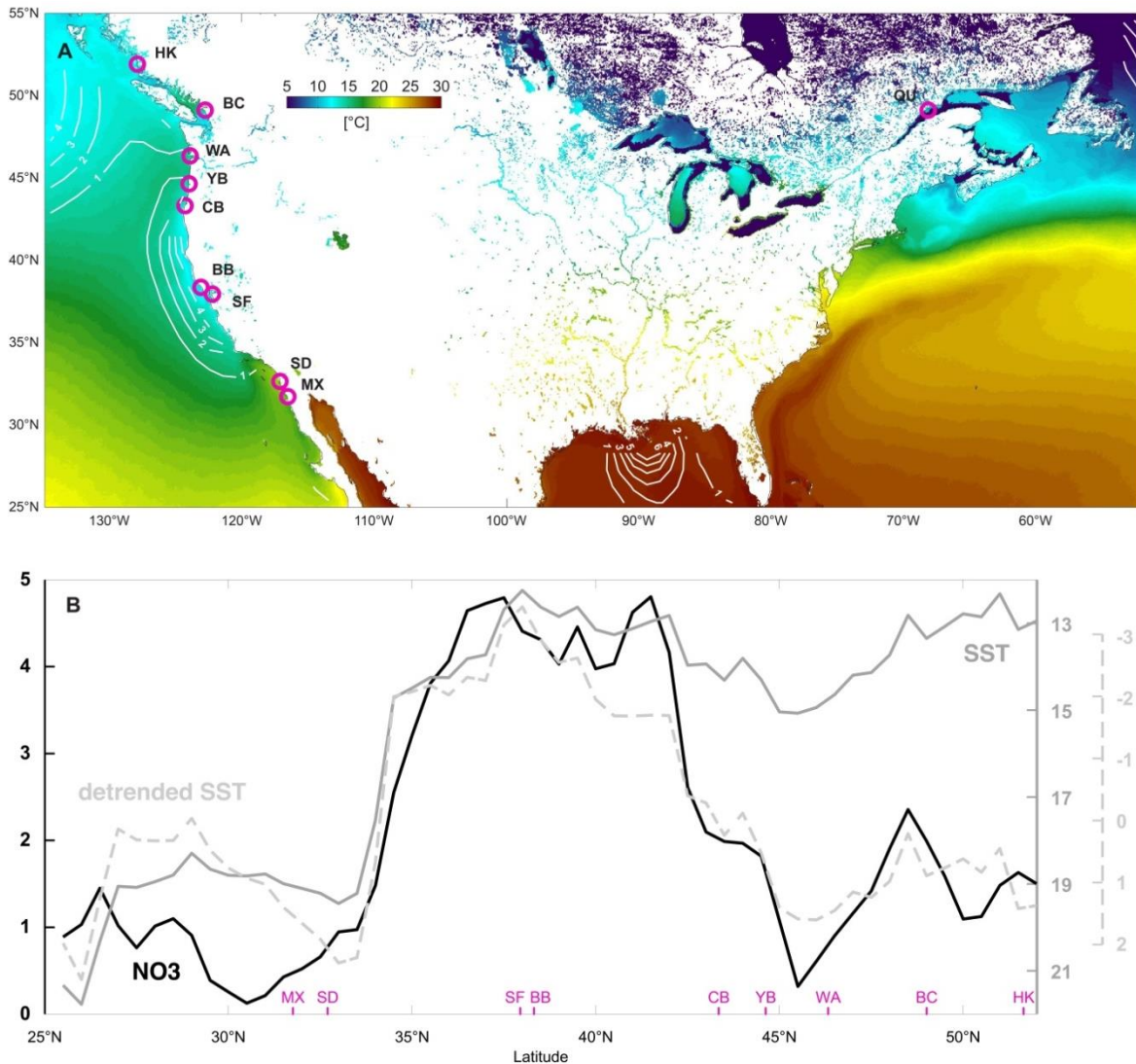


**Figure 3.1.1.** (A) Mean May-June-July SST derived from the 1982-2008 AVHRR data set. White contours indicate mean May-June-July $NO_3$ (μmol/L) from the 1878-2012 World Ocean Atlas data set. The location of the study sites, Calvert (Hakai, Canada; HK), Baie St. Ludger (Quebec, Canada; QU), Tsawwaseen (British Columbia, Canada; BC), Willapa (Washington, United States; WA), Yaquina Bay (Oregon, US; YB), Coos Bay (Oregon, US; CB), West side Regional Park (Bodega Bay, California, US; BB), Point Molate (San Francisco, California, US; SF), Coronado (San Diego, California, US; SD) and Punta Banda Estuary (Ensenada, Baja California, Mexico; MX) is indicated with magenta circles. (B) Comparison of the May-June-July $NO_3$ (μmol/L; black), with the May-June-July SST (ºC; grey). The dashed grey line (detrended SST) corresponds to the difference between the SST at each latitude and the North-South SST gradient (linear trend of -0.29 ºC per degree of latitude). Note the reverse axis for the SST and the detrended SST.

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## 3. Results

### *Plant size traits*

Leaf growth was measured in five to ten shoots per plot at the end of the experiment by punching the leaves following the method of Zieman, 1974. Leaf width, maximum leaf length, number of leaves, total leaf area, leaf biomass, specific leaf area (SLA; cm$^2$/g), sheath area and biomass, and rhizome biomass and number of internodes were measured at the end of the experiment. We considered growth rates, leaf biomass, number of leaves and rhizome biomass as tolerance traits and SLA a resistance trait.

### *Plant chemical traits*

Regarding traits related to herbivory, we considered carbon, sucrose and starch contents of rhizomes as tolerance traits, whereas specific and total phenolic compounds, nitrogen and fiber contents of leaves were considered as resistance traits.

Specific phenolic compounds, nitrogen, carbon, sucrose, and fiber content of young leaves (the three newest leaves formed) and nitrogen, carbon, sucrose and starch content of rhizomes were analyzed from samples collected at the end of the experiment. Pooled plant material of five to ten shoots per plot collected at the end of the experiment. Plants were cleaned of epiphytes, ultrafrozen (-80ºC), freeze-dried, and ground to a fine powder to determine the concentration of chemical traits in young leaves and rhizomes (see thesis methodology section).

Phenolic compounds and flavonoids were extracted from powdered leaf samples (0.1-0.2 g) with MeOH: H$_2$O (3mL x 3) under maceration followed by vacuum evaporation. The organic extracts obtained were suspended in H$_2$O to 100 ppm. Separation and identification of compounds was performed as explained in the methodology section of this thesis with the following modifications: single components were identified by their UV, mass spectra, retention times, accurate molecular masses and comparison with standards (purity of the compounds determined by NMR, ≥95%) with the MassLynx 4.1ChromaLynx Application Manager software (Waters). The quantification of phenolic compounds was performed by external calibration curves, using a reference compound: rosmarinic acid (RA), apigenin-7-sulfate (APS), diosmetin-7-sulfate (DS), luteolin-7-O-β-glucoside (LUTG), luteolin-7-sulfate (LUTS). For those compounds for which no standard was available, a reference compound was selected based on the principle of

structure-related target analyte/standard (chemical structure and functional group). Hence, for luteolin-7,3'-disulfate (LUTS2) and luteolin-7-O-β-(6''-malonyl)glucoside (LUTMG) the calibration curves of luteolin-7-sulfate (LUTS) and luteolin-7-O-β-glucoside (LUTG) were used, respectively. The sum of LUTS, LUTS2, LUTG, LUTMG was calculated as the total Flavonoids (TF) content. The sum of flavonoids, RA and APS was calculated as the total phenolic compounds (TPC).

### *Genetic traits*

Genetic data were extracted from Duffy et al. (in prep). Genotypic richness, allelic richness and inbreeding were assessed from 20 shoots per site genotyped at 24 microsatellite loci using standard methods (J. Olsen and J. Stachowicz unpublished data).

### *Herbivore feeding assays*

To examine the effect of herbivory-driven changes on herbivore feeding behavior we conducted a series of three-choice feeding experiments with isopods (*Pentidotea resecata*) of similar size (CB = 27.55 ± 0.6mm length, YB = 26.88 ± 0.79mm; One-way ANOVA: $F_{1/78}$=0.466, p=0.497) from two sites, i.e. Coos Bay and Yaquina Bay, maintained at 15ºC (Mean summer SST at those sites). *P. resecata* is an invertebrate grazer commonly found in *Z. marina* seagrass beds along the Central and North Pacific coast. Isopods were acclimated for 24h prior to the start of the experiments, being kept in individual containers in food deprivation ($500cm^3$ plastic cups with two parallel windows and the top covered with a 1.5 mm mesh) inside tanks with aerated seawater under controlled light conditions (12/12h).

We performed a three choice experiment (i.e. offering plants from the control, moderate and high herbivory treatments see methodology section) with 20 replicates. To avoid confounding factors such as within tissue differences in structural (Enríquez, 2005) or chemical traits (McKey, 1979; Cronin & Hay, 1996), the tissue offered was clipped from the second leaf avoiding basal and apical tissue, and the length of all the pieces offered was similar (ca. between 2 and 4 cm depending on the trial). Additionally, in order to examine if the feeding patterns observed were driven by mechanical-structural or chemical traits, we performed the equivalent three-choice feeding experiment but using agar-base artificial food (see methodology section). The experiment consisted of 20 (for YB) and 15 (for CB) replicates. All the experiments ended when approximately 50% of the initially offered material was consumed. Following the

procedures of previous feeding behavior experiments in seaweeds and seagrasses (e.g., Bolser *et al.*, 1998; Vergés *et al.*, 2007; Tomas *et al.*, 2015), replicates where all the offered leaf material was consumed or remained intact were not considered in the statistical analyses.

**Statistical analyses**

The effect of simulated herbivory on plant traits across the latitudinal gradient was examined while controlling for genetic co-variates using univariate General Linear Models (GLM). Before fitting the GLMs to our response variables, we first explored *i*) the correlation between latitude and co-variates to avoid co-linearity in the model fitting and *ii*) correlation among our response variables to reduce the number of GLMs to fit. We performed pairwise correlations between each of the predictor variables (mean May-June-July SST, latitude, genotypic richness, allelic richness and inbreeding) and among all the response variables using Pearson correlation coefficients (Fig. S.1.3). As expected, we identified a strong negative correlation between latitude and SST, while genotypic richness and inbreeding were latitude independent but positively correlated between them and allelic richness positively correlated with genotypic richness. We therefore discarded STT as co-variate and included genotypic richness into the analysis. In order to reduce the number of response variables, we also performed a Principal Component Analysis (PCA) with all the response variables (Fig. S.1.4) and selected 12 response variables to test our hypothesis (i.e. SLA, Number of leaves, growth rate, rhizome biomass per internode, leaf biomass, leaf nitrogen, carbon and sucrose content, rhizome nitrogen, carbon, sucrose and starch content, leaf fiber content and specific phenolic compounds, except LUTG and LUTMG).

The main goal of our work was to statistically test the interaction effect between latitude and herbivory treatment (i.e. control, moderate and high herbivory) and the effect of latitude in defense traits while controlling for genetic co-variates (i.e. genotypic richness) using the GLM. However, the main effect of latitude and treatment were also interpreted when there was no significant effect of the interaction. Since some latitudinal responses may follow a non-linear pattern defining an optimal at mid-latitudinal ranges, we also included a quadratic term of latitude and its interaction with treatment as predictive variables in the GLM. We reduced the full GLM of each response variable (with all fixed factors and interactions) using a stepwise selection procedure to find the minimum adequate model, that is, the one with the lowest

Akaike information criterion (AIC) value. The coefficients, standard errors, and *p*-values of fixed effects in all minimum adequate models were estimated trough the iteratively reweighted least squares using the glm function of the R-package (R Core Team, 2007). All models were fitted considering a Gaussian distribution of errors. The distribution of the residuals (errors) was checked for normality and an adequate natural logarithm- (ln) or square root- (sqrt) transformation was applied to response variables when the distribution of the residuals strongly deviated from normality.

The results of the feeding experiments were performed using Hotelling tests as in Prince, LeBlanc & Maciá (2004) without transformation of the variables. Post hoc analyses were performed with Tukey multiple comparisons of means.

### 3.1.4 Results

*Latitudinal and treatment responses of tolerance traits*

All tolerance traits responded to latitude following quadratic or linear distributions and generally decreased under the high herbivory treatment (except for rhizome starch content) or were affected by the interaction of both factors (i.e. latitude and high herbivory; Fig. 3.1.2, Table 3.1.1). We found statistically significant independent effects of latitude and clipping treatment in leaf growth rate, but not a significant interaction. Leaf growth rate was lower at intense herbivory rates compared to controls and moderate clipping and followed a non-linear response with latitude, exhibiting a pronounced optimal at mid-latitudes (Fig.3.1.2). In accordance with these results and considering the correlation of growth rate with other variables such as maximum leaf length, leaf width and sheath area (Fig. S.1.3, S.1.4), we also observed higher values of these variables at intermediate latitudes (Fig. S.1.5), indicating a general trend of larger morphological traits at mid-latitudes. Leaf biomass also exhibited an interactive effect of latitude with the high herbivory treatment; only high levels of herbivory significantly decreased aboveground biomass and these effects were weakest in the southern sites. The number of leaves per shoot was also lower in the high herbivory treatment and did not change with latitude.

## 3. Results

Leaf sucrose content as well as rhizome biomass and sucrose content also followed a quadratic distribution with latitude. These three variables were significantly lower in the high herbivory treatment whereas rhizome starch was unaffected by simulated herbivory and increased linearly with latitude (Fig. 3.1.2). Moreover, both rhizome nitrogen and carbon contents responded differently to herbivory along latitude. While C content increased linearly with latitude and was lower under the high herbivory treatment, and particularly at higher latitudes (i.e. significant latitude x herbivory treatment interaction), N content had an optimum at low-mid latitudes, and it was particularly affected by high herbivory at higher latitudes, where it increased (Table 3.1.1, Fig. 3.1.2). Finally, the number of leaves as well as rhizome sucrose and carbon contents tended to increase with genotypic richness (Table 3.1.1).

*Latitudinal and treatment responses of resistance traits*

All resistance traits responded significantly to latitude except for total phenolic compounds, whereas only SLA and leaf N responded to the high herbivory treatment (Fig. 3.1.3, Table 3.1.2). SLA increased linearly with latitude and also increased under high herbivory. Both leaf fiber and leaf carbon exhibited a U-quadratic distribution along latitude, but whereas carbon content did not respond to simulated herbivory, there was a marginal interaction between latitude and the high herbivory treatment for fiber content ($P = 0.057$), exhibiting a marginal induction at higher latitudes. On the other hand, nitrogen content exhibited the highest contents at mid-latitudes and underwent a substantial increase in response to high herbivory, being this response stronger towards higher latitudes. Secondary compounds (i.e. phenols) were not induced by leaf clipping while they were affected by latitude. LUTS2, DS (and by correlation also total flavonoids; Fig. S.1.3, S.1.4, S.1.5), exhibited a U-quadratic distribution, while LUTS, RA, and due to their correlation also LUTG and LUTMG (Fig. S.1.3, S.1.4, S.1.5) were slightly higher at mid-latitudes. LUTG and LUTMG were present in very low concentrations and absent in some samples (Table 3.1.2, Fig. 3.1.3).

The main effects of genotypic richness were statistically significant for all the secondary metabolites but did not exhibit any consistent trends (Table 3.1.2).
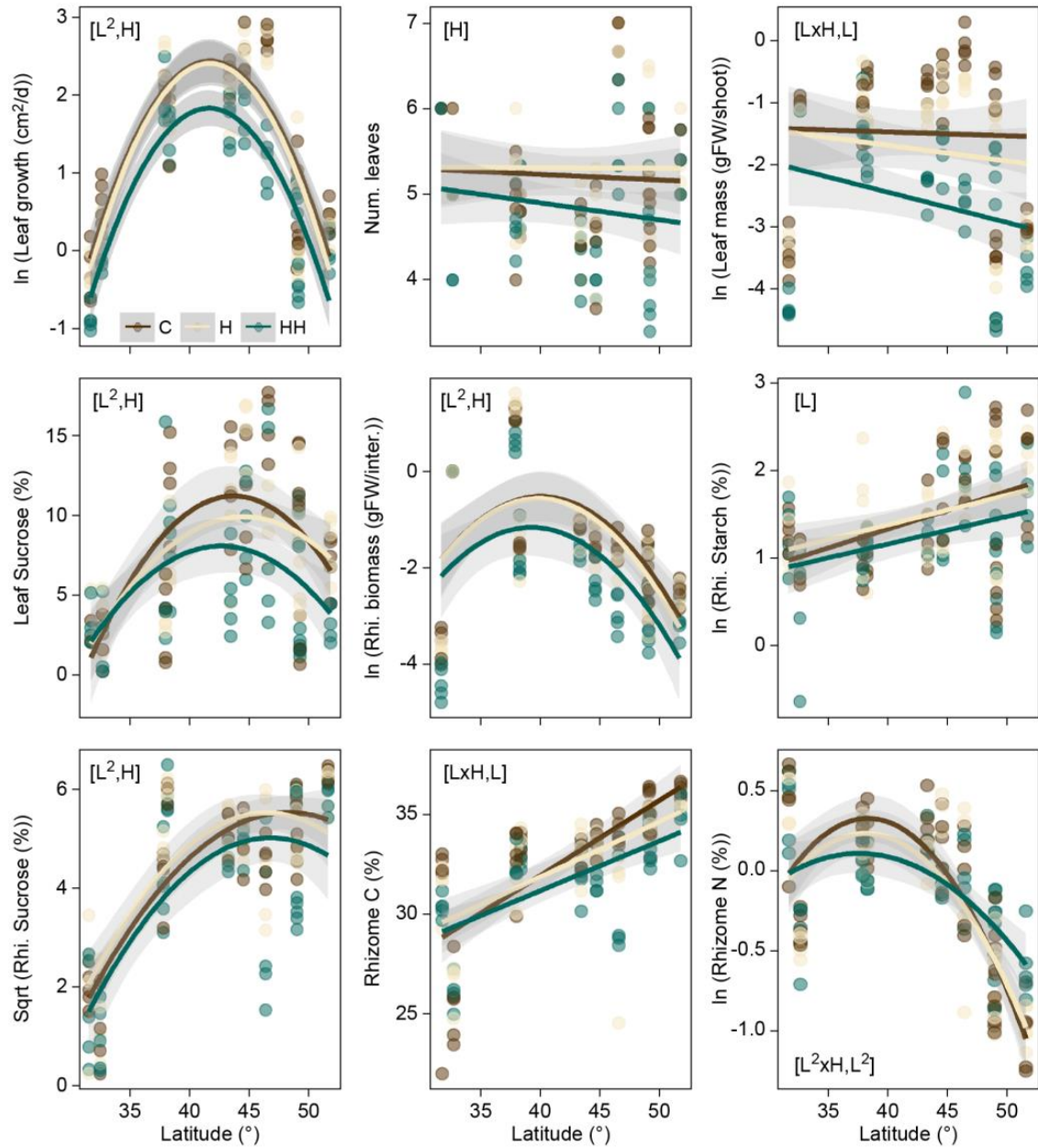
**Figure 3.1.2.** Distribution of tolerance traits along the latitudinal gradient. Grey bands indicate 95% confidence boundaries for the control (C; dark brown), moderate (H; beige) and high (HH; blue) herbivory treatment fitted curves (linear and quadratic models). Letters indicate statistical differences due to latitude (L), herbivore treatment (H), and interactions (L x H). Rhi= rhizome, ln=napierian logarithm, sqrt=square root.
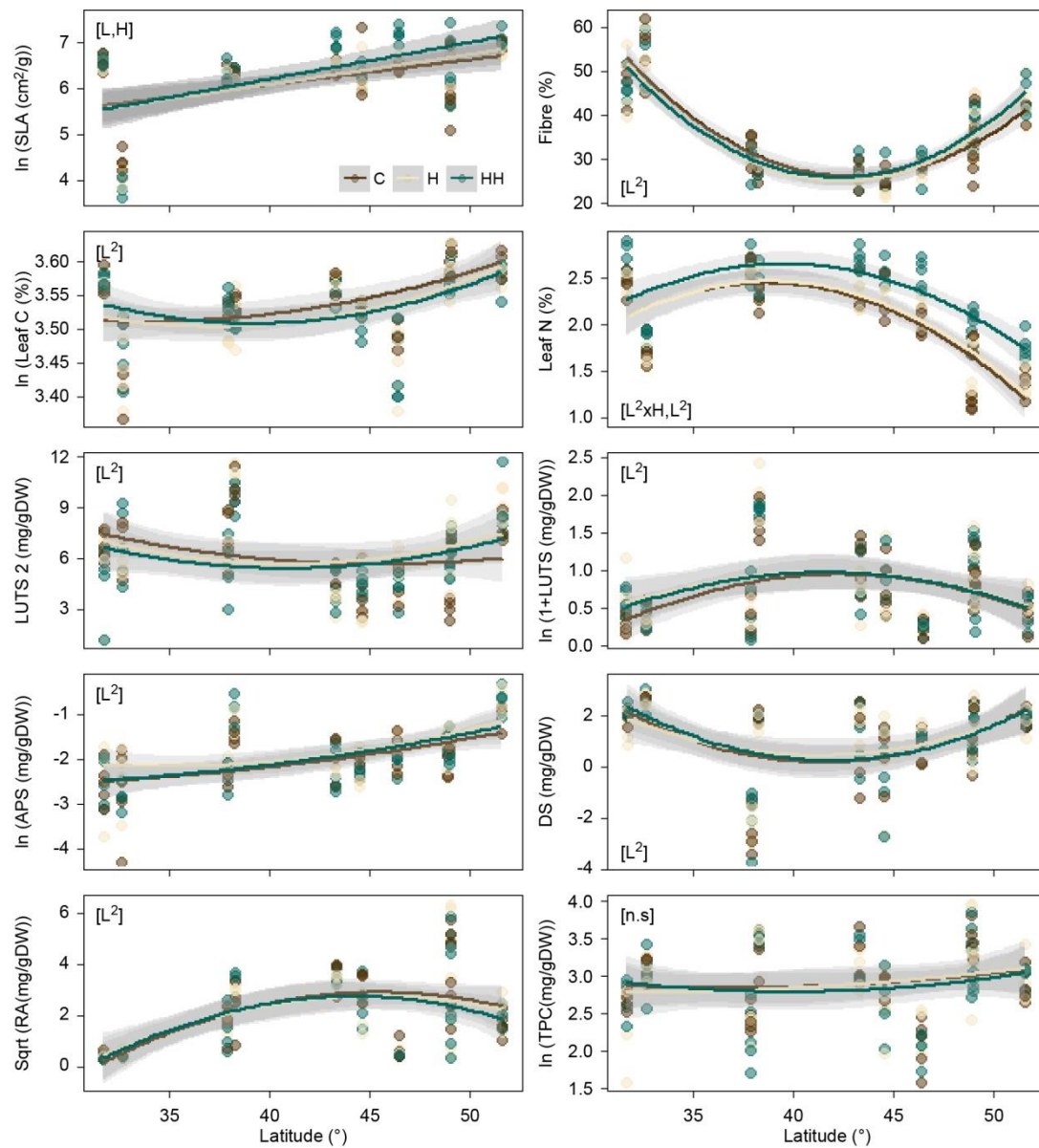
**Figure 3.1.3.** Distribution of resistance traits along the latitudinal gradient. Grey bands indicate 95% confidence boundaries for the control (C), moderate (H) and high (HH) herbivory treatment fitted curves (linear and quadratic models). Letters indicate statistical differences due to latitude (L), herbivore treatment (H), and interactions (L x H). LUTS1= luteolin-7-sulfate, LUST2= luteolin-7,3'-disulfate, APS= apigenin-7-sulfate, DS= diosmetin-7-sulfate, RA= rosmarinic acid, TPC= total phenolic compounds, SLA= specific leaf area.

# 3. Results

**Table 3.1.1**: Results of GLM on tolerance traits. Intercept (i) and slopes of latitude ($\beta$lat), herbivory simulation moderate ($\beta$H) and high level ($\beta$HH), quadratic latitude ($\beta$lat$^2$), genotypic richness ($\beta$G.R), and the interactions of latitude with both herbivory simulation levels ($\beta$ lat*H, $\beta$ lat*HH; $\beta$ lat2*H, $\beta$ lat2*HH ; $\beta$ NO3*H, $\beta$ NO3*HH). Ln and Sqrt indicate napierian logarithm and square root transformation respectively. Est, estimate; SE, standard error and p-value (P)

| | | i | $\beta_{lat}$ | $\beta_H$ | $\beta_{HH}$ | $\beta_{lat}^{2}$ | $\beta_{G.R.}$ | $\beta_{lat*H}$ | $\beta_{lat*HH}$ | $\beta_{lat}^{2}{}_{*H}$ | $\beta_{lat}^{2}{}_{*HH}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Est. | -51.556 | 2.630 | -0.090 | -0.598 | -0.032 | | | | | |
| **Ln (Growth (cm2/d))** | SE | 2.432 | 0.122 | 0.109 | 0.109 | 0.002 | | | | | |
| | P | **<0.001** | **<0.001** | 0.412 | **<0.001** | **<0.001** | | | | | |
| | Est. | 9.647 | -0.322 | 0.093 | -0.406 | 0.004 | 2.403 | | | | |
| **Number of leaves** | SE | 4.506 | 0.208 | 0.158 | 0.158 | 0.003 | 0.667 | | | | |
| | P | **0.034** | 0.124 | 0.558 | **0.011** | 0.137 | **<0.001** | | | | |
| | Est. | -41.013 | 1.969 | 0.763 | 1.539 | -0.024 | | -0.025 | -0.065 | | |
| **Ln (Leaf biomass (grDW/shoot))** | SE | 4.524 | 0.225 | 1.347 | 1.347 | 0.003 | | 0.032 | 0.032 | | |
| | P | **<0.001** | **<0.001** | 0.572 | 0.255 | **<0.001** | | 0.430 | **0.047** | | |
| | Est. | -40.603 | 2.037 | -0.095 | -0.688 | -0.026 | | | | | |
| **Ln (Rhizome biomass (grFW/internode))** | SE | 5.739 | 0.288 | 0.257 | 0.257 | 0.004 | | | | | |
| | P | **<0.001** | **<0.001** | 0.712 | **0.009** | **<0.001** | | | | | |
| | Est. | -103.438 | 5.263 | -0.552 | -1.924 | -0.061 | | | | | |
| **Leaf sucrose (%)** | SE | 18.648 | 0.937 | 0.836 | 0.836 | 0.012 | | | | | |
| | P | **<0.001** | **<0.001** | 0.510 | **0.023** | **<0.001** | | | | | |
| | Est. | 0.078 | 0.029 | | | | | | | | |
| **Ln (Rhizome starch(%))** | SE | 0.330 | 0.008 | | | | | | | | |
| | P | 0.813 | **<0.001** | | | | | | | | |
| | Est. | -59.482 | 2.854 | 0.127 | -0.434 | -0.033 | 3.816 | | | | |
| **Sqrt (Rhizome sucrose (%))** | SE | 5.801 | 0.268 | 0.203 | 0.203 | 0.003 | 0.858 | | | | |
| | P | **<0.001** | **<0.001** | 0.534 | **0.035** | **<0.001** | **<0.001** | | | | |
| | Est. | -38.174 | 2.798 | 4.233 | 5.034 | -0.030 | 7.492 | -0.110 | -0.147 | | |
| **Rhizome C (%)** | SE | 12.778 | 0.588 | 2.987 | 2.987 | 0.007 | 1.876 | 0.071 | 0.071 | | |
| | P | **0.003** | **<0.001** | 0.159 | 0.094 | **<0.001** | **<0.001** | 0.126 | **0.040** | | |
| | Est. | -11.727 | 0.628 | 2.080 | 7.089 | -0.008 | | -0.111 | -0.376 | 0.001 | 0.005 |
| **Ln (Rhizome N (%))** | SE | 2.679 | 0.135 | 3.764 | 3.764 | 0.002 | | 0.189 | 0.189 | 0.002 | 0.002 |
| | P | **<0.001** | **<0.001** | 0.582 | 0.062 | **<0.001** | | 0.559 | **0.049** | 0.546 | **0.041** |

*Herbivore responses to simulated herbivory-driven changes in plant palatability*

Isopods from both Coos Bay and Yaquina Bay consumed significantly higher amounts of leaf biomass from the high herbivory treatment when compared to the control treatment in the three-choice experiments. Furthermore, when offered agar-reconstructed food, the responses of the isopods were similar (Fig.3.1.3).
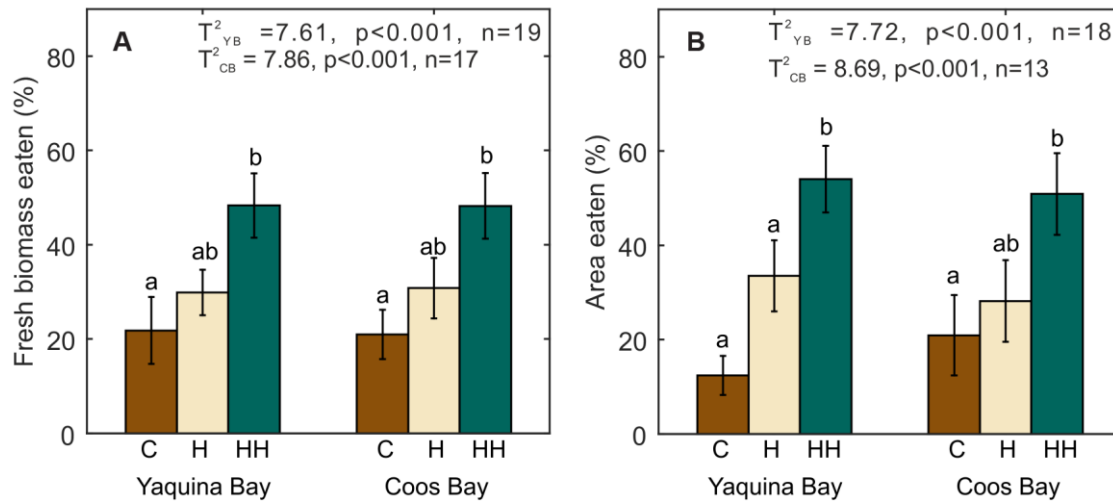


**Figure 3.1.4.** (A) Mean percentage of fresh biomass and (B) agar-based reconstructed food eaten by isopods of plant material from different experimental treatments. Plants and isopods were collected in Yaquina bay and Coos bay and fed seagrass from their respective bays. Error bars indicate standard error, and different letters indicate statistically significant differences across treatments (Tukey). Hotelling test results, T2 from samples collected in Yaquina Bay (T2YB) and Coos Bay (T2CB), p-value (p), and number of replicates (n) shown.

## 3. Results

**Table 3.1.2:** Results of GLM on resistance traits. Intercept (i) and slopes (β) for the fixed: latitude ($\beta_{lat}$), herbivory simulation moderate ($\beta_H$) and high level ($\beta_{HH}$), quadratic latitude ($\beta_{lat}^2$), genotypic richness ($\beta_{G.R}$) and the interactions of latitude with both herbivory simulation levels ($\beta_{lat*H}$, $\beta_{lat*HH}$), and quadratic latitude with both herbivory simulation levels ($\beta_{lat^2*H}$, $\beta_{lat^2*HH}$). Ln and Sqrt indicate napierian logarithm and square root transformation respectively. SLA= specific leaf area, LUTS= luteolin-7-sulfate, LUST2= luteolin-7,3'-disulfate, APS= apigenin-7-sulfate, DS= diosmetin-7-sulfate, RA= rosmarinic acid and TPC= total phenolic compounds. Est, estimate; SE, standard error and p-value (P)

| | | i | $\beta_{lat}$ | $\beta_H$ | $\beta_{HH}$ | $\beta_{lat}^2$ | $\beta_{G.R.}$ | $\beta_{lat^2*H}$ | $\beta_{lat^2*HH}$ |
|---|---|---|---|---|---|---|---|---|---|
| **Ln (SLA(cm2/g))** | **Est.** | -402.461 | 23.011 | 23.176 | 178.163 | | | | |
| | **SE** | 175.923 | 4.097 | 62.600 | 62.600 | | | | |
| | **P** | 0.024 | **<0.001** | 0.712 | **0.005** | | | | |
| **Fibre (%)** | **Est.** | 483.644 | -21.156 | -5.250 | -6.424 | 0.248 | -7.759 | 0.003 | 0.004 |
| | **SE** | 28.152 | 1.300 | 3.560 | 3.560 | 0.016 | 4.159 | 0.002 | 0.002 |
| | **P** | **<0.001** | **<0.001** | 0.143 | 0.074 | **<0.001** | 0.064 | 0.144 | 0.057 |
| **Ln (LeafC (%))** | **Est.** | 4.190 | -0.036 | | | 0.000 | | | |
| | **SE** | 0.246 | 0.012 | | | 0.000 | | | |
| | **P** | **<0.001** | **<0.001** | | | **0.002** | | | |
| **Leaf N (%)** | **Est.** | -9.900 | 0.641 | -0.069 | -0.141 | -0.008 | | 0.000 | 0.000 |
| | **SE** | 1.405 | 0.070 | 0.227 | 0.227 | 0.001 | | 0.000 | 0.000 |
| | **P** | **<0.001** | **<0.001** | 0.761 | 0.536 | **<0.001** | | 0.644 | **0.046** |
| **Ln (1+LUTS(mg/g))** | **Est.** | -13.109 | 0.622 | | | -0.007 | 1.398 | | |
| | **SE** | 2.922 | 0.135 | | | 0.002 | 0.432 | | |
| | **P** | **<0.001** | **<0.001** | | | **<0.001** | **0.002** | | |
| **LUTS 2 (mg/gDW)** | **Est.** | -54.341 | 2.518 | | | -0.032 | 13.129 | | |
| | **SE** | 10.834 | 0.501 | | | 0.006 | 1.603 | | |
| | **P** | **<0.001** | **<0.001** | | | **<0.001** | **<0.001** | | |
| **Ln (APS(mg/gDW))** | **Est.** | -19.301 | 0.712 | | | -0.008 | 2.572 | | |
| | **SE** | 2.795 | 0.129 | | | 0.002 | 0.414 | | |
| | **P** | **<0.001** | **<0.001** | | | **<0.001** | **<0.001** | | |
| **Ln (DS(mg/gDW))** | **Est.** | 50.550 | -2.344 | | | 0.028 | -2.603 | | |
| | **SE** | 8.430 | 0.390 | | | 0.005 | 1.247 | | |
| | **P** | **<0.001** | **<0.001** | | | **<0.001** | **0.039** | | |
| **Sqrt (RA(mg/gDW))** | **Est.** | -21.022 | 1.024 | | | -0.011 | | | |
| | **SE** | 6.256 | 0.314 | | | 0.004 | | | |
| | **P** | **0.001** | **0.001** | | | **0.005** | | | |
| **Sqrt (TPC(mg/gDW))** | **Est.** | 2.646 | | | | 0.000 | | | |
| | **SE** | 0.164 | | | | 0.000 | | | |
| | **P** | **<0.001** | | | | 0.112 | | | |

### 3.1.5 Discussion

Latitude influenced all the seagrass traits examined, yet only one of the resistance traits (leaf toughness measured as SLA) actually increased towards lower latitudes, as we would have expected from the latitude herbivory defense hypothesis (LHDH). In contrast, most plant defense traits appeared to be greatly influenced by latitude, but following quadratic distributions rather than a linear one. Indeed, most tolerance traits exhibited higher values at mid latitudes (bell-shaped), while higher values at extreme latitudes (U-shape) were observed for many resistance traits. Simulated herbivory at moderate levels had no effects in any of the defense or size traits measured. On the other hand, intense herbivory negatively affected tolerance, especially at the northern populations. Furthermore, high herbivory also decreased resistance, which increased plant vulnerability to consumers

Most tolerance traits were more strongly expressed at mid latitudes (ca. 35ºN - 42ºN), coincident with the region of strong upwelling (Schwing & Mendelssohn, 1997). Upwelling brings nutrient-rich waters which also have higher $pCO_2$(Feely *et al.*, 2008), thus providing resources that generally limit photosynthesis growth and overall seagrass productivity (Invers *et al.*, 2001; Beer *et al.*, 2002; Lee *et al.*, 2007; Borum *et al.*, 2016). In accordance with the Limiting Resource Model (Wise & Abrahamson, 2005), we observe an increase in tolerance when limiting resources such as nutrients and $CO_2$ are provided. In consequence, plants at mid latitudes are better prepared to tolerate herbivory or other disturbances that can remove aboveground biomass such as harsher hydrodynamic conditions from tidal currents or wave action. Interestingly, in the central range of our study (37ºN-47ºN), higher resource availability appears to translate into leaf growth and increased above and belowground biomass.). Interestingly, while temperature has been suggested as an important factor controlling seagrass productivity (Clausen *et al.*, 2014), our results are in accordance with the processes suggested for kelps (Graham *et al.*, 2016), where higher resource (i.e. nutrient and $CO_2$) availability together with optimum temperature and light conditions are likely the main drivers of higher seagrass productivity at mid latitudes. On the other hand, towards the northern sites, where nitrogen availability is lower (Fig. 3.1.1.) carbon mainly accumulates into carbohydrate reserves in the belowground tissue (i.e. starch, sucrose, carbon), rather than being invested in plant growth. Such differences in carbon investment may be due to a different selective pressure occurring at higher latitudes such as

overwintering due to longer winter periods, lower temperatures or ice cover (Robertson & Mann, 1984; Clausen *et al.*, 2014).

The production of resistance traits also appears to be strongly influenced by available resources (RAH), since plants from the upwelling-influenced mid latitudes tended to have lower constitutive resistance defenses such as higher nitrogen content in leaves. Experimental increases of nutrient availability usually lead to higher nutrient content in plant tissues (e.g., Tomas *et al.*, 2015; Yuan *et al.*, 2015), and decreases in fiber production (Johnson *et al.* 2001, Chapter 2). Furthermore, we would expect a negative linear relationship between the concentrations of phenolic compounds and latitude (i.e. LHDH; Rasmann & Agrawal, 2011; Moreira *et al.*, 2014b; Anstett *et al.*, 2016b) and we would also expect an induction in the production of chemical defenses in response to herbivory (REF). Yet, we did no observe either of those phenomena. In fact, in our system the production of chemical defenses appears to be largely driven by resource availability rather than herbivory pressure. Indeed, higher nutrient and $p$CO$_2$ availabilities often reduce phenolic contents of seagrass leaves (e.g. Goecker *et al.*, 2005; Tomas *et al.*, 2011; Arnold *et al.*, 2012, 2014), and we found that the most abundant flavonoid compounds (i.e. luteolin-7,3'-disulfate,LUTS2; diosmetin-7-sulfate, DS; and apigenin-7-sulfate, APS) were lower in the region where upwelling occurs. Such lower investment in chemical (e.g. phenols) and structural (e.g. fibre) defenses and higher nutritional quality typically enhance plant susceptibility to herbivory (e.g., Lucas, 2000; Valentine & Heck, 2001; Ngai & Jefferies, 2004), and thus would make mid-latitude plants more vulnerable to being consumed.

Not all chemical compounds, however, followed the U-shaped pattern of lower abundance at mid latitudes. Minority flavonoids such as the glucoside derivates of luteolin (LUTG) and luteolin-7-sulfate (LUTS), as well as the phenolic compound rosmarinic acid, exhibited the opposite pattern being slightly higher at mid-latitudes. These compounds protect plants against pathogens (Vergeer & Develi, 1997; Widmer & Laurent, 2006) and herbivores (Petersen, 2003), but also against UV-light (Falcone Ferreyra *et al.*, 2012; Žemlička *et al.*, 2014), and their higher abundance may be a response to higher radiation levels at these latitudes (Fig.S.1.6).

The only resistance trait that followed the predictions of the LHDH was specific leaf area (SLA), with southern populations having tougher leaves (i.e. lower SLA; Paul *et al.*, 2012). While

this pattern may respond to higher grazing pressure in the southern sites (which can exhibit strong consumption by brant geese; e.g. Jorgensen et al. unpublished), it may also be related to stronger hydrodynamic regimes of northern sites (Fig.S.1.2), but also suggest a need for a higher leaf expansion in order to increase their photosynthetic capacity (Enriquez & Sand-Jensen, 2003). Additionally, lower SLA may be related to higher temperature stress that intertidal plants suffer during low tides in southern sites (Ramírez-Valiente *et al.*, 2014; McAfee *et al.*, 2016). In fact, we observed that high herbivory rates made plants more tender rather than tougher, so environmental variables rather than herbivory are more likely to be the drivers of latitudinal patterns in leaf toughness.

In addition to making seagrasses more tender, plants under simulated high herbivory also exhibited higher leaf nitrogen content than in the moderate and control treatments. Lower nitrogen content in the latter could be attributed to an effect of nitrogen "dilution" resulting from higher leaf growth rates (Alcoverro *et al.*, 1997), or to a stimulation of nitrogen uptake due to defoliation under high herbivory (Jaramillo & Detling, 1988; Valentine *et al.*, 2004). Interestingly, the effect of intense herbivory in increasing nitrogen pools was stronger at the northern sites, and this may be a consequence of a higher nitrogen uptake efficiency of those plants (Elser *et al.*, 2007) or to higher nitrogen internal requirements (Reich & Oleksyn, 2004) in northern latitudes under defoliation stress.

Regardless of the mechanism driving the increase in SLA and nutrients under high herbivory, this decrease in resistance would make plants even more attractive to grazers. Indeed, herbivores are known to perform "cultivation" or "gardening" grazing whereby they maintain certain species or plant tissues that optimize their foraging (e.g., Bjorndal, 1985; Preen, 1995; Hata & Kato, 2006; Gordon & Prins, 2008). While we simulated grazing that would typically be performed by a larger herbivore (e.g. waterfowl, fish, turtles), smaller crustaceans such as amphipods and isopods can also remove significant amounts of seagrass when present at high densities (Reynolds *et al.*, 2014Kollars and Tomas personal observations), and we observed that this herbivory-driven decrease in resistance did in fact make plants more consistently susceptible to being consumed by isopods. Indeed, isopods from both populations preferred the clipped leaves with a tendency towards the most clipped, which were also the more nutritious (i.e. high leaf nitrogen content), more tender (i.e. higher SLA) and which had not

exhibited any induction of phenolic compounds or other resistance traits (i.e. fibers). The fact that isopods exhibited the same preferences when we performed the agar-base feeding experiment (i.e. when the physical structure of the leaves was removed) suggests that likely both nutritional quality and structural traits are driving feeding behavior of this herbivore.

In addition to reducing plant resistance, simulated herbivory also reduced some tolerance traits (e.g., growth rate, rhizome biomass) even at the upwelling-influenced sites, suggesting that herbivory affected the use or acquisition of some limiting resources (LRM; Wise & Abrahamson, 2007). Unlike terrestrial plants, seagrasses preferentially absorb nitrogen through their leaves (Touchette & Burkholder, 2000a). Thus, a reduction in leaf area may have hindered nutrient acquisition (Hay *et al.*, 2011). Moreover, with a lower leaf area, plants would also have a lower area to perform photosynthesis, thus also hampering C fixation. Indeed, sucrose, starch and carbon content of leaf and rhizomes and growth rate were lower under high herbivory simulation. Furthermore, in our experiment, the reduction of leaf biomass under simulated high herbivory was stronger towards northern latitudes, which is likely related to the higher SLA (i.e. lower leaf density) of these plants. On the other hand, rhizomes of plants from the high herbivory treatment at higher latitudes contained more nitrogen, likely due to the lower investment of nitrogen in leaf biomass production. Moreover, these rhizomes also had a stronger reduction of their carbon content towards northern latitudes, which could be due to an investment of carbon in nitrogen assimilation (Touchette & Burkholder, 2000b).

Plants of the intense herbivory treatment did not enhance their growth to compensate for leaf loss, and they in fact exhibited lower number of leaves per shoot, lower rhizome biomass and lower sucrose content in both leaf and rhizome. This negative impact of herbivory and lack of compensatory responses has been observed in other studies and has been attributed to increased stress after repeated defoliation (Thayer *et al.*, 1984; Hudgeons *et al.*, 2007). Interestingly, plants undergoing the moderate herbivory treatment had similar growth rates, leaf mass, number of leaves and carbohydrate content in above and belowground tissues than control plants, even though these plants have a 40% reduction in leaf length. Thus, plants under moderate treatments were able to compensate leaf loss through herbivory without decreasing carbohydrate reserves, probably by increasing photosynthetic activity (Strauss & Agrawal, 1999; Tiffin, 2000). This suggests that the eelgrass populations studied are highly tolerant to moderate

herbivory pressures likely due to the selective pressure that herbivory, mainly by waterfowl (Rivers & Short, 2007), has had in these populations, but they are not adapted to high herbivory. Yet, warming-driven range expansion of tropical grazers (and therefore herbivory pressure) into temperate areas is already occurring and is predicted to increase (Vergés *et al.*, 2014a; Hyndes *et al.*, 2016). Furthermore, migration patterns of waterfowl species are being altered with climate and degradation of foraging habitats (Ward *et al.*, 2005) and may shift spatial patterns of grazing pressure (Kollars *et al. in prep*). These new scenarios of higher grazing pressure could have strong detrimental effects on temperate seagrasses, particularly for the most sensitive northern latitudes, not only by reducing plant standing stock but also by reducing further resistance (to those herbivores and other consumers present in the community) as well as the capacity to tolerate this disturbance. The results of this experiment highlight the importance of understanding the context-specificity of vulnerability to herbivory and the main drivers of plant defense strategies.

## 3. Results

## 3.2  Chapter 2: Resource availability and herbivory damage do not interact to modulate plant defense strategies

### 3.2.1  Abstract

Herbivory is a critical ecological and evolutionary driver of plant traits (e.g. plant defenses). It regulates the composition of plant communities, changes plant architecture and abundance, and it is particularly significant in altering community structure when herbivores consume foundation species such as seagrasses. Increased resource availability is predicted to decrease constitutive resistance defenses while enhancing allocation to induced defense (Resource availability Hypothesis), and increase tolerance when all the limiting resources are provided (Limited Resource Model). Furthermore, differences in herbivore pressure may shift defense responses by inducing different resistance traits or changing allocation between tolerance and resistance. However, resource availability and herbivore pressure have scarcely been examined concurrently.  Thus, our aim is to understand how plant defense traits respond to herbivory and nutrient availability, what the effects are in palatability to different herbivores within the community, and which mechanisms are involved in plant changes. To this end we performed an *in situ* experiment in which we simulated three herbivory levels and two resource (i.e. nutrient) availability conditions in a factorial design using the endemic Mediterranean seagrass *Posidonia oceanica*. We also explored the effects of simulated-herbivory and nutrient fertilization in leaf palatability by a series of feeding experiments with its two main herbivores.

Simulated herbivory induced resistance traits (e.g. phenols), but the magnitude of this effect was not always linear, with some traits being higher under moderate herbivory. We found no interactive effects of nutrient fertilization and herbivory simulation. On the other hand, following the RAH, nutrient fertilization decreased constitutive resistance. Likewise, as stated in the LRM, fertilization did not increase plant allocation to tolerance due to resource limitation since P seemed unavailable for plants. Both herbivores responded similarly to plant changes which affected plant palatability, exhibiting a clear preference for nutrient-enriched plants as well as for non-clipped plants.

### 3.2.2 Introduction

Herbivory is a key ecological process that regulates the flow of energy to upper trophic levels and the composition, abundance and distribution of plant communities (Huntly, 1991; Wood *et al.*, 2016). Herbivory has strong effects on plant fitness and can be an important pathway of biomass and nutrient loss in plants (Cebrian, 1999). Unlike biomass lost through senescence, there is not nutrient resorption from leaves lost by herbivory, and plants can lose twice as much nitrogen and phosphorus through this process (Chapin *et al.*, 2002). Plants have evolved diverse strategies against herbivory; tolerance strategies that reduce the impact of herbivory on plant fitness (e.g., increased belowground resources), and resistance strategies that reduce the preference or performance of herbivores (e.g., phenolic compounds; Fritz & Simms, 1992; Strauss & Agrawal, 1999). Plant allocation to these different strategies is determined by a tradeoff between benefits (i.e. better defense against herbivores) and costs (i.e. less resources available for growth and/or reproduction; Bazzaz *et al.*, 1987). Although initially thought otherwise (Meijden *et al.*, 1988), recent studies suggests that plants present mixed tolerance-resistance defense mechanisms (Agrawal, 2011; Carmona & Fornoni, 2013), but that a trade-off exists between both strategies whereby selection for increased resistance can lead to decreased tolerance (Fineblum & Rausher, 2002). In addition, these defenses can be either constitutive (i.e. expressed regardless of the herbivory pressure suffered by the plant) or induced (i.e. produced in response to herbivory damage; Rausher *et al.* 1993; Agrawal & Karban 1999; Vergés *et al.* 2008; McArt *et al.* 2013). Induced defenses are thought to be favored by selection for being more effective and for minimizing costs, since they are produced only when needed, providing thus more resources for growth and reproduction when there is no herbivory damage (Herms & Mattson, 1992).

As an explanation of how resources influence plant allocation to defense, the Resource Availability Hypothesis (RAH) postulates that species adapted to resource-rich environments have higher growth rates and have less constitutive defenses but higher inducible defenses. On the contrary, species adapted to environments with low resource availability will have lower growth rates and invest more in constitutive defense strategies (Coley *et al.*, 1985; Endara & Coley, 2011). This hypothesis is further supported by studies that have indeed found that plants with higher induced resistance invest less in constitutive resistance (Kempel *et al.*, 2011; Moreira

*et al.*, 2014b; Rasmann *et al.*, 2015). Similarly, high resource availability should also allow plants to better tolerate and/or compensate herbivory. In this regard, the Limiting Resource Model (LRM; Wise & Abrahamson 2005) highlights that the plant will become more tolerant to herbivory when the increase of resources enhances, directly or indirectly, the availability of a resource that is limiting. However, when herbivory damage affects the use or acquisition of an alternate resource plants under high nitrogen availability would not enhance their investment in tolerance. Indeed, previous studies showed that plants and algae under high nutrient environmental conditions may not increase their allocation to tolerance when the reduction of photosynthetic biomass due to herbivore consumption impairs carbon acquisition (Hay *et al.*, 2011; Hattas *et al.*, 2017). In marine macrophytes (i.e. seagrasses and algae), blades do not only perform photosynthesis but also absorb nutrients from the water column (Romero *et al.*, 2006). Thus, higher rates of herbivory may not only result in higher limitation of carbon fixation but also of nutrient acquisition.

In addition to resource availability, plant defense strategies against herbivores can respond to different consumption levels. Indeed, herbivore intensity and duration change plant defense responses, with some traits only being induced under intense herbivory (e.g., secondary metabolites, mineral crystals; Vergés *et al.* 2008; Dostálek *et al.* 2016; Hartley, DeGabriel & Cooke 2016), while others tend to be induced under moderate herbivory (e.g., compensatory growth; Ruiz-R, Ward & Saltz 2008; Vergés *et al.* 2008; Sanmartí *et al.* 2014). Therefore, increased resource availability (when limiting resources are considered) could minimize the costs of defense under different levels of herbivory pressure. Yet, there is a dearth of studies assessing the interactive effects of resource availability and herbivory pressure on plant defense strategies.

Importantly, the consequences of resource availability and herbivory pressure on plants may extend beyond the individual species of herbivore, as we often find several herbivore species feeding on the same plant species. Indeed, herbivory damage exerted by one guild or group of herbivores has great effects on other herbivores within the same community due to changes in plant community structure or abundance (Foster *et al.*, 2014), plant architecture (Mopper *et al.*, 1991; Rudgers & Whitney, 2006) or plant defenses (Delphia, Mescher & De Moraes 2007; Ramirez & Eubanks 2016). This is particularly relevant when herbivory is exerted upon foundation species that are the base of ecosystems such as terrestrial forests, coral reefs,

or kelp beds since, in these ecosystems, grazing-mediated changes cascade through the food web having profound ecological impacts such as loss of feeding resources or refuge (Terborgh *et al.*, 2001; Steneck *et al.*, 2002; Ellison *et al.*, 2005; Lilley & Schiel, 2006; Pagès *et al.*, 2012).

Seagrasses are one of these important foundation species, forming extensive meadows which are a key component of coastal ecosystems and that perform important functions within ecosystems. Due to their high primary productivity and structural complexity, they provide food and shelter for many marine species (Jackson *et al.*, 2015) and through their facilitative effects they enhance biodiversity of associated species (Bruno & Bertness, 2000). Moreover, they protect the shore against coastal erosion, stabilize the substrate, increase water clarity (Nordlund *et al.*, 2016) and remove pathogens from seawater (Lamb *et al.*, 2017).

Recent studies have found a tropicalization of marine ecosystems (Vergés *et al.*, 2014b; Hyndes *et al.*, 2016) that together with the predicted enhance in herbivore feeding rates, due to higher seawater temperature (Carr & Bruno, 2013), may increase the feeding pressure in seagrass meadows. Likewise, increases in nutrient concentrations in seawater are commonly observed in coastal waters. Thus, understanding how herbivory-driven changes in plant traits affect other herbivores in the same community or how increases in nutrient availability due to eutrophication can affect trophic interactions has important implications for management and conservation of seagrass ecosystems.

In this study we aim to unravel the complexity of predictions that are posed by the resource availability hypotheses in relation to tolerance and resistance strategies against herbivory considering the effect of different levels of herbivory damage. In addition, we assess how herbivore damage inflicted by one specific herbivore affects another herbivore species within the community. Specifically, we tested the hypothesis that under simulated herbivory, especially in oligotrophic environments in which plants would have high constitutive defenses, plants will not induce the production of defenses related to resistance as much as under high availability of resources (i.e. nutrients). Thus, the same levels of herbivory under different nutrient conditions will cause different responses on resistance strategies, which will modify their palatability. In addition, by increasing the resources available (i.e. nutrients) plants will invest more towards tolerance and compensatory strategies. Finally, we hypothesized that the induction of resistance defenses and the capacity of plants to tolerate herbivory may differ with

herbivory pressure and may affect differently to other herbivores within the same community. To this end we performed an *in situ* manipulative experiment in which we simulated different levels of fish herbivory and two resource (i.e. nutrient) availability conditions in a factorial experiment with *Posidonia oceanica*, a dominant seagrass species inhabiting an oligotrophic environment (i.e. the Mediterranean Sea), and assessed the effects on size and plant traits related to tolerance and resistance against herbivory. We also explored what the species-specific effects of simulated herbivory and nutrient-driven changes were on leaf palatability by a series of feeding experiments with the two main herbivores in *P. oceanica* meadows; the sea urchin *Paracentrotus lividus* and the fish *Sarpa salpa.*

### 3.2.3 Materials and methods

#### *Study sites and Experimental design*

To test the combined and independent effects of nutrient availability and herbivore pressure on resistance and tolerance strategies against herbivores and the induced changes in seagrass palatability, 24 plots of 1 x 1 m were installed in two *P. oceanica* meadows North (A= 39º29'52''N, 2º32'15''E) and South (B= 39º27'53''N, 2º43'56''E) of Palma Bay at depths of 6.6 ± 0.8 m at both sites. Mean initial density of the plots was 416.5 ± 7 shoots/ m$^2$ and there was no difference neither between sites (Three-way ANOVA: F$_{(1/72)}$ = 0.146; P = 0.7), treatments (Three-way ANOVA: F$_{(10/72)}$ = 0.67; P = 0.75), or plots (Three-way ANOVA: F$_{(1/72)}$ = 0.38; P = 0.54).

The effects of herbivore pressure (C=ambient, H=moderate and HH= high) and nutrient enrichment (A=ambient and N=enriched) were tested in a factorial design resulting in 6 experimental treatments randomly assigned along the 24 plots having thus 4 replicates per treatment. In order to avoid nutrient contamination and minimize nutrient transport among rhizomes, plots were set 3.5-4 m apart from each other. The experiment started in May 2014 and lasted for 4 months coinciding with the period of maximum fish herbivory (Tomas *et al.*, 2005b; Prado *et al.*, 2007) and the growing season of *P. oceanica* (Alcoverro *et al.*, 1995). In order to have samples comparable in time and confirm the effectiveness of the fertilizing treatment, reference (T1) samples were collected in June (i.e. ca. one month after the beginning of the experiment) and at the end of the experiment (T2) at both sites.

In addition, we performed feeding choice experiments with different herbivores (fish and sea urchins; see below). These experiments were conducted during August and September.

*Experimental treatments*

Nutrients were added in the sediment by introducing 1g fertilizing bars (COMPO ®) homogeneously distributed across the plot, in a concentration of 100 g/m$^2$/month (N:P:K molar ratio= 13:6:10). Plots were fertilized once a month and as perturbation control, non-fertilized plots were perturbed by perforating the sediment using plastic bars with a similar size to the fertilization bars. The nutrient loading rate selected was above the plant requirements (Alcoverro *et al.*, 1997, 2000) and below the concentrations in which negative effects from toxicity have been previously reported (Invers *et al.*, 2004). Porewater samples were collected at the end of the experiment. Upon collection, samples were filtered (0.22μm), put on ice, and brought to the laboratory for analyses. The fertilization method significantly increased the concentrations of nitrite, nitrate, ammonium and phosphate in the nutrient-added treatments (Supplementary material, Table S.3.1).

Herbivory simulation was performed once a month by clipping the leaves mimicking the grazing performed by the main vertebrate consumer of *P. oceanica* in shallow meadows, the fish *S.salpa* (Tomas *et al.*, 2005b; Prado *et al.*, 2007). Clipping was performed inside the experimental plots as well as around a 15 cm margin surrounding the plot. The control treatment (C) corresponded to the ambient herbivory, which was naturally low in both sites and was previously evaluated through a tethering experiment (relative fresh biomass consumed in 3 days, A= 0.08±0.066%; B= 0.08±0.048%). In addition, no bite marks were observed on the control plots during the experiment nor on the shoots collected for analysis or for feeding experiments. The moderate herbivory treatment (H) consisted on trimming 40% of the blade length (i.e. ca. to 60 cm in length), while the high herbivory treatment (HH) consisted on performing ca. 80% removal (i.e. leaves were cut to ca. 20 cm in length).

*Herbivore feeding behavior experiments*

In order to examine how changes in plant defense traits due to changes in nutrient availability and herbivory pressure modify plant palatability, we performed a series of two-choice feeding trials with herbivorous sea urchins and fish at the end of the field experiment.

## 3. Results

Similar-sized sea urchins (4.93± 0.18 cm, one-way ANOVA: F site $_{(1/409)}$ = 1.13; P = 0.29; F experiment $_{(1/403)}$ = 1.81; P = 0.08) of the species *Paracentrotus lividus*, the main invertebrate herbivore on *P. oceanica* meadows, were collected and kept in cages of 225 cm$^2$ covered with a 1 cm mesh. These cages were placed in the experimental sites (A and B), in *P. oceanica* dead matte (natural substrate formed by this seagrass species) protected from currents. Sea urchins were maintained in the cages for a period of 48 hours and fed with *Ulva sp. ad libitum* prior to starting the feeding experiments. Replicates of two tethered leaf fragments (from two different treatments) attached with a clothes pin were placed inside the cages. To avoid confounding factors such as within tissue differences in structural (Enríquez, 2005) or chemical traits (McKey, 1979; Cronin & Hay, 1996), the fragments offered were clipped from the young leaves avoiding basal and apical parts.

Considering the low fish herbivory pressure measured and observed in the experimental sites (see above), the feeding experiments with fish were performed at a third site, a shallow bay in the middle of Palma Bay where *Cymodocea nodosa* is present and which harbors large groups of the herbivorous fish *S. salpa*. Two-choice feeding trials were setup in a similar fashion than for sea urchins. In sandy patches and at least 3 m away from the *C. nodosa* meadow, replicates of two pegs with the tethered leaves (from two different treatments) attached with a clothes pin and a cable tie ca. 10 cm above the sediment were offered to the fish.

In each replicate, sea urchins or fish were offered similar amounts of young leaf tissue (ca. 15-20 cm long) clean of epiphytes. The experiments consisted of 25-30 replicates and ended when approximately 50% of initial material was consumed. Following the procedures of previous feeding behavior experiments in seagrasses (e.g., Tomas *et al.* 2015), replicates in which all the offered samples were either totally consumed or fully intact were not considered in the statistical analysis. Consumption was calculated as leaf area eaten of each tissue relative to the total amount of leaf area eaten in the assay. In order to measure any potential changes in leaf tissue not related to grazing, control cages protected from herbivores were used to correct for autogenic changes in area prior to the statistical analyses. Since we found no changes in area in these controls, that data was not used.

## 3. Results

### *Plant size traits and epiphyte load*

Leaf growth was measured in three shoots per plot at the end of the experiment by punching the leaves following the method of Zieman (1974). Leaf width, maximum leaf length, number of leaves, total leaf area, leaf biomass, specific leaf area (SLA; calculated as fresh leaf area by leaf dry mass), and percentage of necrotic surface were measured at the end of the experiment (T2) on these same shoots. Epiphytes were removed from both sides of each leaf by gentle scraping with a glass slide, the water with the epiphytes was filtered and salt was removed by rinsing with distilled water. Filters were dried (60 °C for 48h) and weighed, along with the corresponding dried leaves, and epiphyte dry mass was standardized by shoot dry mass and shoot leaf area to estimate epiphyte load.

### *Plant chemical traits*

Regarding traits related to herbivory, we considered total phenolic compounds, nitrogen, sucrose, phosphorous and fiber contents of leaves as resistance traits, whereas the carbon, sucrose, starch, nitrogen and phosphorous contents of rhizomes were considered tolerance traits.

Nitrogen, carbon and phosphorous content of both young leaves and rhizomes and sucrose and starch of rhizomes were analyzed from samples collected at the midst of the experiment (45 days from the beginning; T1) and at the end of the experiment (T2). Sucrose, fiber and phenol content in young leaves were analyzed only from samples collected at the end of the experiment (T2). Pooled plant material of 3 shoots per plot collected at T1, and 8 shoots per plot collected at T2 were cleaned of epiphytes, ultrafrozen (-80ºC), freeze-dried, and ground to a fine powder for further analyses of chemical traits in young leaves (the newest two leaves formed) and rhizomes.

Carbon and nitrogen content in young leaves and rhizomes were analyzed using a Carlo-Erba CNH elemental analyzer (EA1108). Total phenols were extracted from ca. 4 mg of ground tissue with 1.5 mL of methanol 50% for 24 h and were determined with spectrophotometer (Hitachi, U-2900) following a modified Folin-Ciocalteu method using caffeic acid as standard (modified from Bolser *et al.*, 1998). Non-structural carbohydrates in young leaves (sucrose), and rhizomes (sucrose and starch) were measured using methodology described by Invers *et al.*,

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(2004). Neutral detergent fiber content (NDF) was measured in 25–30 mg of leaf sample (see de los Santos *et al.*, 2012, modification from Van Soest *et al.*, 1991). The amount of NDF in each sample was obtained by difference in dry biomass and is referred as 'fiber content' hereafter.

### *Statistical analyses*

The effect of nutrient availability and simulated herbivory were analyzed by linear mixed effects models in which site was considered as a random effect. The models contrasted different combinations of two fixed effects, nutrient availability (i.e. ambient and nutrient addition) and herbivory level (i.e. control, moderate and high) independently or interacting. For dependent variables analyzed in two different times (i.e. T1, T2) during the experimental period, time was added as a fixed effect since we may expect differences in those variables with time due to plant phenology or length of the experimental treatment. Models were selected by their AIC value, calculated as the sum of the model deviance and twice the number of parameters (Akaike´s information criterion; Burnham & Anderson 2002). The model with the lowest AIC was taken as the best compromise between model adequacy (i.e. residual deviance) and model complexity (i.e. number of estimated parameters). All dependent variables were checked for normality with the Shapiro-Wilk test and transformed when needed. *Post-hoc* analyses were performed using Tukey´s HSD (honest significant difference) test.

The analyses of two-choice feeding experiments were performed using a Wilcoxon signed-ranks paired test since data were not normal even after transformation. The overall effect of herbivory and nutrient addition on herbivore feeding preference was analyzed through meta-analysis. The effect size was measured as the standardized mean difference using Hedges' d (bias corrected; Hedges 1981) and confidence intervals were obtained iteratively via the Q-profile method (Viechtbauer, 2007). The differences in effect size were assessed with a Random effects model since plants came from two different sites and sea urchins and fish were different in each experiment. Models were fitted by restricted maximum likelihood. Meta-analysis considered experiments performed with sea urchins or fish individually, as well as combining both herbivores.

### 3.2.4  Results

***Responses of plant size traits and epiphyte load***

Increased nutrient availability reduced leaf biomass (Mean ± SE; 11 ± 2%) while it had no significant effects on any other size traits measured (Fig.3.2.1, Table 3.2.1). Both levels of simulated herbivory reduced all the size traits measured except mean leaf width and leaf growth, for which there were no significant effect. Clipped shoots had significantly lower (H = 18±3%; HH = 51±2%) leaf biomass, leaf area (H = 32±2%; HH =6 1±3%), maximum leaf length (H = 42±2%; HH = 66±1%), and SLA (H = 18±1%; HH = 22±3%) than controls. Additionally, plants under the moderate clipping treatment exhibited a higher number of leaves (12±6% higher) when compared to the control and the high herbivory treatments, which had a similar number of leaves (Fig.3.2.2, Table 3.2.1). Necrosis was significantly lower both in the moderate (59±1% lower) and the high clipping treatment (85±7%) than in the control treatment, while epiphyte load increased with clipping. The biomass of epiphytes per leaf area almost doubled in both clipping treatments, and epiphyte load (i.e. g epiphyte / g seagrass) also increased ca. 36% under higher nutrient availability (Fig.3.2.1, Table 3.2.1).

***Responses of plant chemical traits***

Both nutrient addition and simulated herbivory modified plant chemical traits, while there were no interactive effects of those two factors. Clipped shoots exhibited lower nutrient content than unclipped ones. Nitrogen content of moderate and high herbivory treatments was lower than controls for both rhizome (H=21±5%; HH= 25±5%) and leaves (H=23±3%; HH= 31±3%) and, consequently, both tissues had higher C/N (H=27±9%; HH= 33±9% and H=30±4%; HH= 42±5%, respectively) than controls. Similarly, phosphorous content in leaves was lower in the simulated herbivory treatments (H=27±6%; HH= 30±3%) and was also marginally lower in rhizomes compared to controls. In addition, simulated herbivory increased rhizome sucrose content (H=14±2%; HH= 40±6% higher than controls) and also increased leaf sucrose under the moderate herbivory level (19±4% higher than C and HH). Plant clipping also induced the production of fibers (H=9±2%; HH= 5±0.04%) and phenols (H=9±1%; HH=20±1%; Fig.3.2.2, Tables 3.2.2 and 3.2.3).
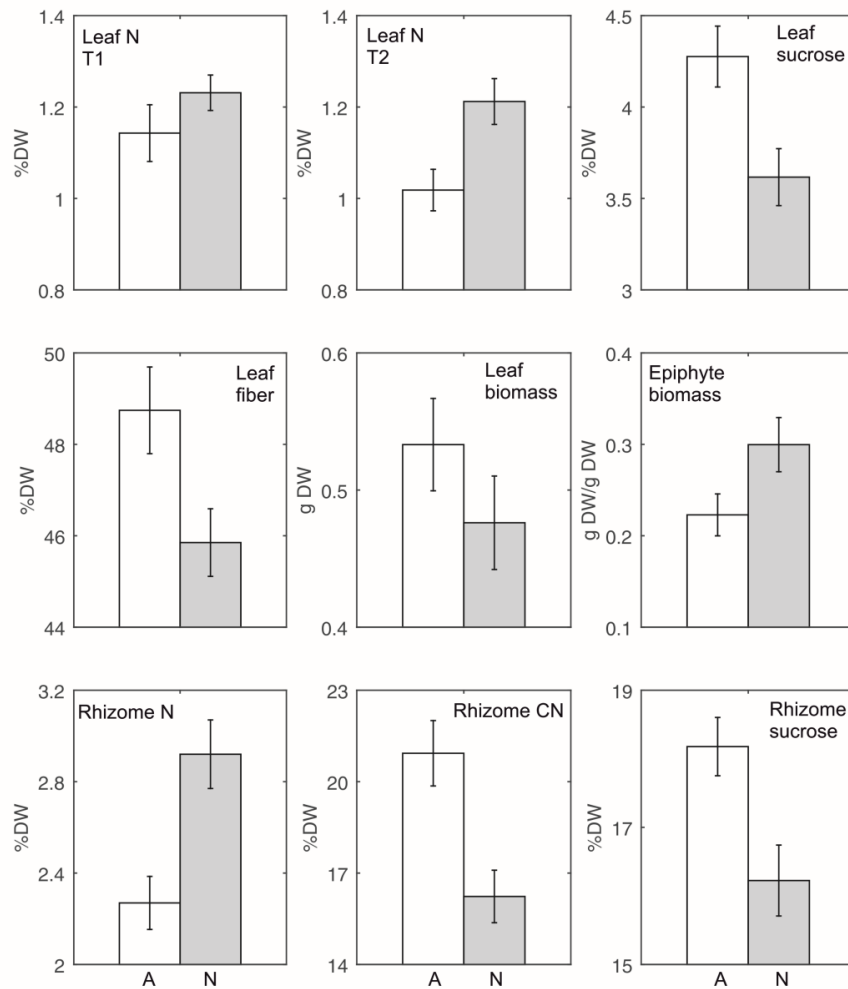
**Figure 3.2.1.** Significant effects of increased nutrient availability on plant traits. Mean contents of plant traits from plants grown at the ambient (A; white) and increased nutrient availability (N; grey). Error bars represent standard error (n=24 for Leaf sucrose, fiber and biomass, and epiphyte biomass; n = 48 for Leaf N and rhizome N, CN and sucrose).

Nutrient fertilization produced a reduction in rhizome and leaf sucrose content (11±4% and 15±2% respectively) and in leaf fiber content (6±1%; Fig.3.2.1, Table 3.2.3). Additionally, plant rhizomes from fertilized plots had lower C/N content (23±1%; Fig.3.2.1, Table 3.2.2) than control plots and both rhizomes and leaves had higher N content (29±4% and 13±6% respectively Fig.3.2.1, Table 3.2.3). Furthermore, there was an interactive effect of nutrient availability and time. Leaf N content of plants growing under ambient conditions strongly decreased during the experimental period (10±2% decrease) in comparison to plants growing under nutrient enriched conditions (2%±0.3 decrease). Finally, P in leaves and N in rhizome increased (12±4% higher than initial samples) during the experiment, while C (0.7±0.2%), C/N (17±1%) and carbohydrate reserves of rhizomes (sucrose=11±4%; starch=37±2%) decreased (Fig. S1, Tables 3.2.2 and 3.2.3) through time.

**Table 3.2.1**. Results of mixed effects models on size traits. Intercept (i) and slopes (β) for the fixed: nutrient addition ($\beta_N$), herbivory simulation moderate ($\beta_H$) and high level ($\beta_{HH}$), and the random site ($\beta_{residual}$) effects on size traits. Log indicates logarithmic transformation of data. Est, estimate; SE, standard error; Var, variance; SD, standard deviation and p-value (P)

| Trait | Fixed | $i$ | $\beta_N$ | $\beta_H$ | $\beta_{HH}$ | Random | $i_{site}$ | $\beta_{residual}$ |
|---|---|---|---|---|---|---|---|---|
| **Leaf biomass (gr DW)** | Est. | 0.683 | -0.057 | -0.115 | -0.336 | Var. | 0.000 | 0.008 |
| | SE | 0.025 | 0.025 | 0.031 | 0.031 | SD | 0.000 | 0.087 |
| | P | **<0.001** | **0.028** | **<0.001** | **<0.001** | | | |
| **Log (Leaf area (cm$^2$))** | Est. | 2.220 | -0.044 | -0.159 | -0.410 | Var. | 0.000 | 0.006 |
| | SE | 0.024 | 0.023 | 0.028 | 0.028 | SD | 0.013 | 0.078 |
| | P | **<0.001** | 0.061 | **<0.001** | **<0.001** | | | |
| **Log (Maximum leaf length (cm))** | Est. | 1.835 | | -0.231 | -0.462 | Var. | 0.000 | 0.003 |
| | SE | 0.013 | | 0.019 | 0.019 | SD | 0.000 | 0.053 |
| | P | **<0.001** | | **<0.001** | **<0.001** | | | |
| **Mean width (cm)** | Est. | 0.956 | | 0.002 | -0.023 | Var. | 0.000 | 0.001 |
| | SE | 0.009 | | 0.011 | 0.011 | SD | 0.005 | 0.032 |
| | P | **<0.001** | | 0.870 | 0.053 | | | |
| **Leaf number** | Est. | 5.083 | | 0.490 | -0.250 | Var. | 0.032 | 0.398 |
| | SE | 0.202 | | 0.223 | 0.223 | SD | 0.180 | 0.631 |
| | P | **<0.001** | | **0.033** | 0.268 | | | |
| **SLA (cm$^2$/gr)** | Est. | 255.494 | | -45.279 | -57.060 | Var. | 181.600 | 630.900 |
| | SE | 11.412 | | 8.881 | 8.881 | SD | 13.480 | 25.150 |
| | P | **0.006** | | **<0.001** | **<0.001** | | | |
| **Log (Leaf growth (cm$^2$/day))** | Est. | -0.157 | | 0.072 | -0.044 | Var. | 0.002 | 0.012 |
| | SE | 0.044 | | 0.039 | 0.039 | SD | 0.048 | 0.111 |
| | P | 0.077 | | 0.074 | 0.264 | | | |
| **Leaf necrosis (% cm$^2$)** | Est. | 16.436 | | -9.664 | -13.937 | Var. | 0.000 | 37.350 |
| | SE | 1.528 | | 2.161 | 2.161 | SD | 0.000 | 6.110 |
| | P | **<0.001** | | **<0.001** | **<0.001** | | | |
| **Epiphyte biomass (g. epi./g. Leaf)** | Est. | 0.132 | 0.077 | 0.129 | 0.144 | Var. | 0.002 | 0.012 |
| | SE | 0.044 | 0.032 | 0.039 | 0.039 | SD | 0.042 | 0.110 |
| | P | 0.065 | **0.020** | **0.002** | **0.001** | | | |
| **Epiphyte biomass (g. epi./cm$^2$ leaf)** | Est. | 0.001 | | 0.001 | 0.001 | Var. | 0.000 | 0.000 |
| | SE | 0.000 | | 0.000 | 0.000 | SD | 0.000 | 0.001 |
| | P | **0.006** | | **<0.001** | **<0.001** | | | |
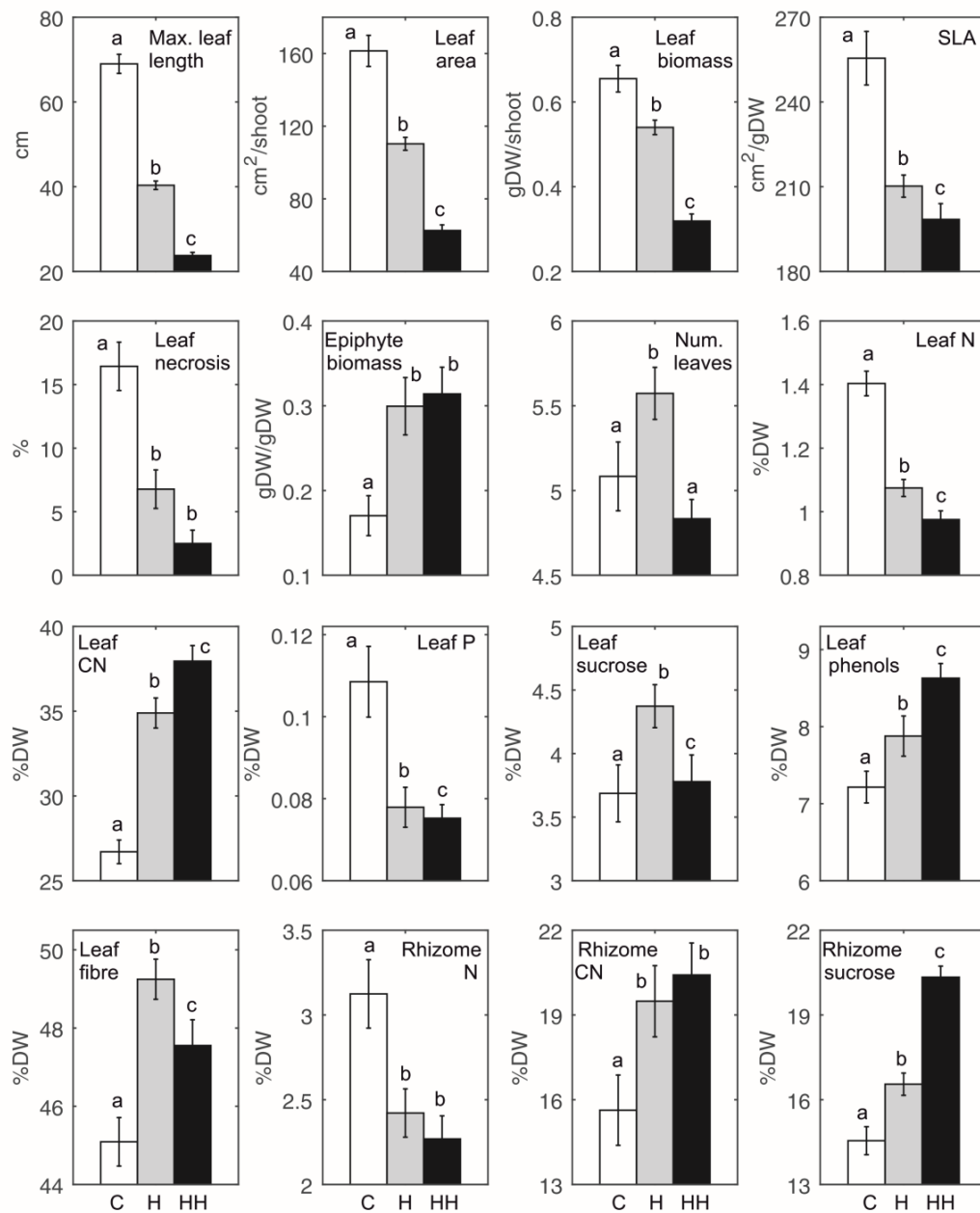
**Figure 3.2.2.** Significant effects of simulated herbivory on plant traits. Mean contents from plants grown at the control (C; white), moderate (H; grey) and high (HH; black) herbivory treatments. Error bars represent standard error (n=16 for all traits except for: leaf and rhizome N and C/N, rhizome sucrose and leaf phosphorus in which n = 32). Different letters indicate statistically significant differences across treatments (Tukey).

**Table 3.2.2.** Results of mixed effects models on rhizome chemical traits. Intercept (i) and slopes (β) for the fixed: nutrient addition ($\beta_N$), herbivory simulation moderate ($\beta_H$) and high level ($\beta_{HH}$), time ($\beta_t$) and the random site (βresidual) effects on rhizome chemical contents. Log and Sqrt indicate logarithmic and square root transformation respectively. Est, estimate; SE, standard error; Var, variance; SD, standard deviation and p-value (P)

| | Rhizome | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Log (Nitrogen (%)) | | | Sqrt (Carbon (%)) | | | Log (C/N) | | | Log (Phosphorus (%)) | | | Sucrose (%) | | | Log (Starch (%)) | | |
| **Fixed** | Est. | SE | P | Est. | SE | P | Est. | SE | P | Est. | SE | P | Est. | SE | P | Est. | SE | P |
| *I* | 0.441 | 0.090 | 0.105 | 6.479 | 0.022 | **<0.001** | 1.182 | 0.087 | **0.032** | -0.816 | 0.085 | **0.022** | 14.561 | 0.732 | **0.003** | 0.574 | 0.022 | **<0.001** |
| $\beta_N$ | 0.105 | 0.023 | **<0.001** | -0.021 | 0.011 | 0.056 | -0.108 | 0.023 | **<0.001** | | | | -1.903 | 0.397 | **<0.001** | | | |
| $\beta_H$ | -0.105 | 0.028 | **<0.001** | | | | 0.105 | 0.029 | **<0.001** | -0.096 | 0.055 | 0.084 | 2.000 | 0.488 | **<0.001** | | | |
| $\beta_{HH}$ | -0.132 | 0.028 | **<0.001** | | | | 0.130 | 0.028 | **<0.001** | -0.101 | 0.054 | 0.067 | 5.743 | 0.485 | **<0.001** | | | |
| $\beta_t$ | -0.058 | 0.023 | **0.014** | 0.025 | 0.011 | **0.024** | 0.061 | 0.023 | **0.010** | -0.085 | 0.044 | 0.060 | 1.971 | 0.397 | **<0.001** | 0.189 | 0.031 | **<0.001** |
| **Random** | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | |
| $i_{site}$ | 0.015 | 0.122 | | 0.001 | 0.028 | | 0.014 | 0.118 | | 0.011 | 0.103 | | 0.678 | 0.824 | | 0.000 | 0.000 | |
| $\beta_{residual}$ | 0.013 | 0.112 | | 0.003 | 0.053 | | 0.013 | 0.112 | | 0.047 | 0.216 | | 3.694 | 1.922 | | 0.022 | 0.150 | |

**Table 3.2.3**. Results of mixed effects models on leaf chemical traits. Intercept (i) and slopes (β) for the fixed: nutrient addition ($\beta_N$), herbivory simulation moderate ($\beta_H$) and high level ($\beta_{HH}$), time ($\beta t$) and their interactions (marked with asterisk), and the random site ($\beta$residual) effects on leaf chemical contents. Est, estimate; SE, standard error; Var, variance; SD, standard deviation and p-value (P)

| | Leaf | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Nitrogen (%) | | | Carbon (%) | | | C/N | | | Phosphorus (%) | | | Sucrose (%) | | | Fibre (%) | | | Phenols (%) | | |
| **Fixed** | Est. | SE | P | Est. | SE | P | Est. | SE | P | Est. | SE | P | Est. | SE | P | Est. | SE | P | Est. | SE | P |
| *i* | 0.104 | 0.032 | 0.103 | 36.252 | 0.246 | 0.001 | 28.674 | 3.152 | **0.042** | -0.970 | 0.040 | **0.001** | 0.592 | 0.025 | **<0.001** | 46.538 | 0.498 | **<0.001** | 7.215 | 0.289 | **<0.001** |
| $\beta_N$ | 0.075 | 0.023 | **0.002** | | | | -4.057 | 2.327 | 0.187 | | | | -0.075 | 0.025 | **0.004** | -2.893 | 0.488 | **<0.001** | | | |
| $\beta_H$ | -0.131 | 0.023 | **<0.001** | | | | 10.259 | 1.607 | **<0.001** | -0.134 | -4.245 | **<0.001** | 0.082 | 0.030 | **0.009** | 4.1556 | 0.598 | **<0.001** | 0.662 | 0.304 | **0.035** |
| $\beta_{HH}$ | -0.186 | 0.023 | **<0.001** | | | | 14.707 | 1.607 | **<0.001** | -0.140 | -4.434 | **<0.001** | 0.013 | 0.030 | 0.673 | 2.462 | 0.598 | **<0.001** | 1.414 | 0.304 | **<0.001** |
| $\beta_t$ | 0.035 | 0.023 | 0.134 | 0.290 | 0.171 | 0.093 | -1.504 | 1.607 | 0.352 | -0.052 | -2.003 | **0.048** | | | | | | | | | |
| $\beta_{N*H}$ | -0.023 | 0.033 | 0.483 | | | | -0.593 | 2.273 | 0.795 | | | | | | | | | | | | |
| $\beta_{N*HH}$ | 0.029 | 0.033 | 0.376 | | | | -4.359 | 2.273 | 0.059 | | | | | | | | | | | | |
| $\beta_{N*t}$ | -0.068 | 0.033 | **0.041** | | | | 3.248 | 2.273 | 0.157 | | | | | | | | | | | | |
| $\beta_{H*t}$ | 0.029 | 0.033 | 0.372 | | | | -3.464 | 2.273 | 0.131 | | | | | | | | | | | | |
| $\beta_{HH*t}$ | 0.006 | 0.033 | 0.854 | | | | -2.078 | 2.273 | 0.363 | | | | | | | | | | | | |
| $\beta_{N*H*t}$ | 0.051 | 0.046 | 0.268 | | | | -0.165 | 3.215 | 0.959 | | | | | | | | | | | | |
| $\beta_{N*HH*t}$ | 0.043 | 0.046 | 0.352 | | | | -0.952 | 3.215 | 0.768 | | | | | | | | | | | | |
| **Random** | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | | Var. | SD | |
| $i_{site}$ | 0.002 | 0.039 | | 0.091 | 0.302 | | 17.291 | 4.158 | | 0.002 | 0.043 | | 0.000 | 0.000 | | 0.020 | 0.140 | | 0.074 | 0.273 | |
| $\beta_{residual}$ | 0.002 | 0.046 | | 0.701 | 0.837 | | 10.334 | 3.215 | | 0.016 | 0.126 | | 0.007 | 0.085 | | 2.859 | 1.691 | | 0.741 | 0.861 | |

## 3. Results

### *Herbivore feeding behavior experiments*

Both nutrient and herbivory-induced changes affected plant palatability. Overall, sea urchins and fish exhibited similar feeding patterns with a clear preference for leaves grown under control herbivory treatment, as well as for leaves from the nutrient-fertilized treatment. These responses were consistent regardless of the site of origin of the leaf tissue. There was only one two-choice feeding experiments with contrasting responses between both herbivores. When offered moderate (HA) and high herbivory (HHA) leaves that had not undergone nutrient fertilization, sea urchins preferred high herbivory plants, while fish preferred the opposite. When these plant tissues were fertilized, the prior preference disappeared and both herbivores preferred leaves from the moderate herbivory treatment (Fig. 3.2.4).
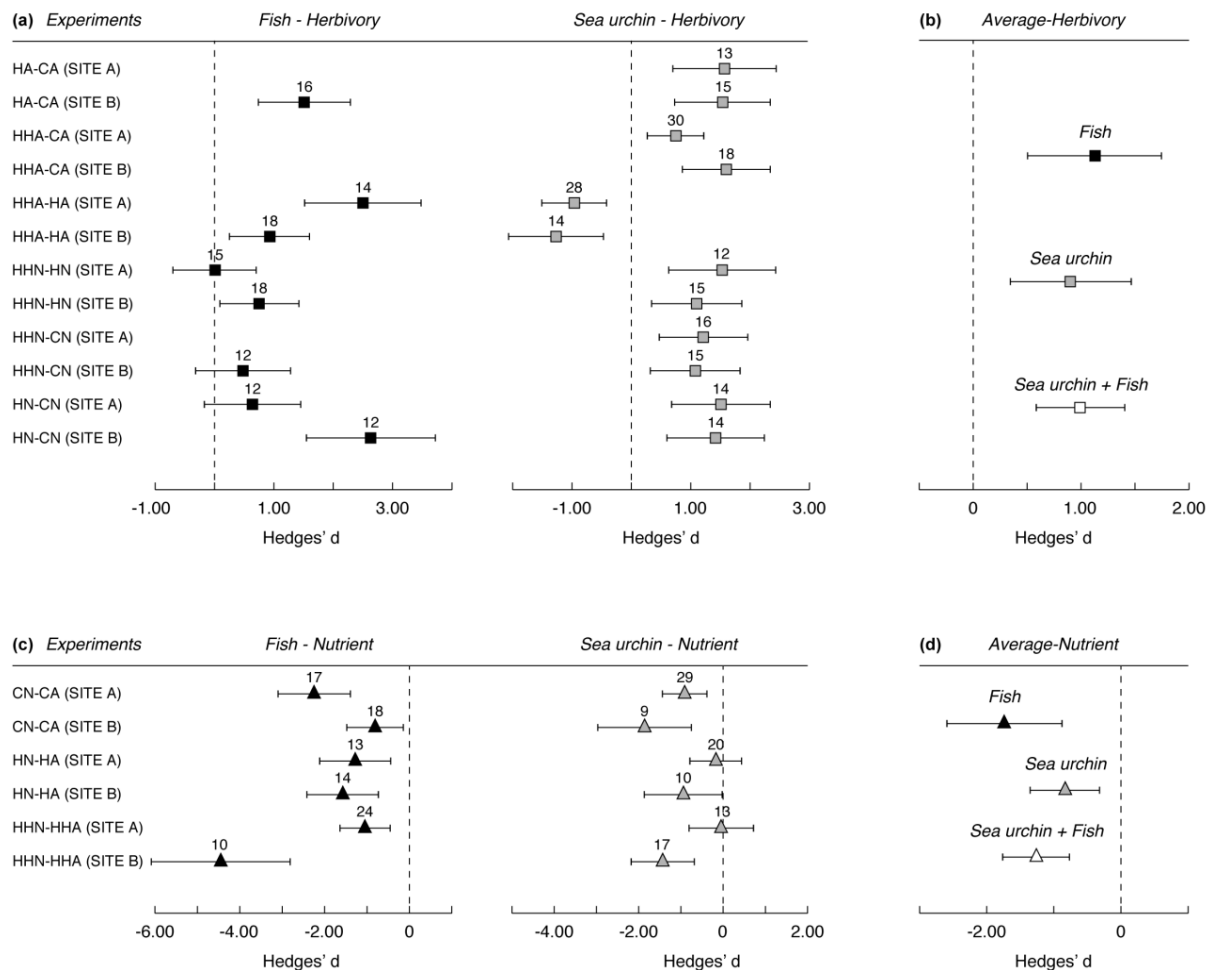


**Figure 3.2.4.** Individual Hedges'd effect sizes (a,b,d,e) and overall Hedges'd effect sizes (c, f) mean and 95% confidence intervals of herbivory-driven (squares; a,b,c) nutrient-driven (triangles; d,e,f) changes in feeding preference of fishes (a,d) and sea urchins (b,e). Numbers above symbols indicate the number of replicates in each feeding experiment.

### 3.2.5 Discussion

Nutrient addition reduced investment in constitutive resistance traits as predicted by the RAH, and it also reduced investment in tolerance. Unexpectedly, we found no interactive effects of nutrient availability and simulated herbivory. In fact, higher nutrient availability did not enhance the induction of resistance traits under herbivory, as stated in the RAH (Endara & Coley, 2011). Similarly, we did not observe an increased allocation to tolerance traits under nutrient fertilization (regardless of whether the plants were submitted to herbivory or not), probably due to the existence of a limiting resource, as suggested by the LRM. Furthermore, simulated herbivory modified plant defense traits inducing resistance defenses and enhancing the accumulation of belowground reserves, without compensatory growth. However, not all traits responded linearly with herbivory pressure, and some tolerance and resistance trait responses were strongest at the intermediate herbivory level. Both nutrient addition and clipping independently modified plant traits that altered plant palatability, with both herbivores responding similarly: sea urchins and fish preferred leaves from the nutrient-fertilized treatment (which were both more nutritious and tender) while they were deterred by clipped plants (which were more chemically and structurally defended).

Our results provide evidence supporting the decrease in constitutive resistance traits with higher resource (i.e. nutrient) availability (i.e. RAH) and of how this enhances susceptibility to being consumed. Even though a reduction in secondary compounds (e.g. phenols) is one of the most common responses to higher nutrients observed in terrestrial plants (reviewed by Endara & Coley 2011) and seagrasses (e.g. Goecker, Heck & Valentine 2005; Tomas *et al.* 2011; Martínez-Crego *et al.* 2016; but see Tomas *et al.*, 2015), we did actually not detect changes in phenolic compounds. On the other hand, our study species did however follow RAH predictions when considering fiber content, a response which has also been observed in terrestrial plants (Johnson *et al.*, 2001). Such decrease in fibers may enhance plant susceptibility to herbivory since fibers deter consumers by reducing tissue digestibility (Klumpp & Nichols, 1983; Sanson, 2006) and by increasing leaf toughness. In addition to modifying fiber content, the increase in external sources of nutrients enhanced nitrogen (but not phosphorous) content in plant tissues. The higher nutritional quality can increase herbivore performance (Minkenberg & Ottenheim, 1990; De Bruyn *et al.*, 2002; Hemmi & Jormalainen, 2002) and can be an important determinant

of susceptibility to herbivory due to an increased preference by consumers (e.g., Cebrian 1999; Valentine & Heck 2001; Ngai & Jefferies 2004). Indeed, the addition of nutrients strongly modified plant palatability, as plants growing under the nutrient addition treatment were consistently preferred by both herbivores over non-fertilized plants. This suggests that the increase in tissue tenderness and digestibility (resulting from lower fiber content), as well as nutritional quality (via increase in nitrogen) were the main drivers of feeding behavior.

In addition to reducing resistance to herbivory, nutrient addition also reduced above and belowground carbohydrate reserves (i.e. sucrose) which would imply a lower capacity for plants to invest in regrowth after damage, potentially further exacerbating negative impacts of grazing that would result from higher palatability. Such carbohydrate reduction may have resulted from the plant's requirements for carbon skeletons needed for nitrogen assimilation (Touchette & Burkholder, 2000b; Invers *et al.*, 2004), and it is not an uncommon response of plants to nutrient additions (Bloom *et al.*, 1985a). Interestingly, plant phosphorus content did not increase despite the increase of P in porewater, which suggests that P may have not been available for the plant due to adsorption to sediment particles (Stumm & Morgan, 1996) or the carbonate matrix of the sediment (Short, 1987). Hampered P absorption by seagrass roots likely resulted in P limitation which may have hindered the allocation to tolerance traits under herbivory pressure, as suggested by the LRM (Wise & Abrahamson, 2008).

Herbivory simulation induced plant resistance to herbivory through enhanced production of leaf phenolic compounds and fibers as well as the decrease in leaf nutrient (N and P) content. On the other hand, nutrient fertilization did not enhanced the induction of resistance traits (e.g. fibers, nitrogen content) in response to herbivore damage contrary to the predictions of the RAH (Endara & Coley, 2011). In fact, the few studies exploring this interaction (i.e. nutrients and herbivory) in seagrasses have not found an enhanced induction of defense in response to herbivore damage under increased nutrient availability (Tomas *et al.*, 2015). In addition to inducing the production of chemical defenses (i.e. phenols) and fiber, which are common responses for many terrestrial and aquatic plants, herbivory also enhanced leaf structural defense by reducing SLA ($cm^2 \cdot g^{-1}$), which is inversely correlated to leaf toughness (Paul *et al.*, 2012) and leaf thickness (Enríquez, 2005). The decrease in SLA is likely related to clipping the apical part of the leaf which is usually thinner and thus with higher SLA (Enríquez, 2005). This

induced resistance from clipping produced a deterrent effect in both herbivores, although the specific mechanisms driving feeding behavior may have differed in several instances between fish and sea urchins. For example, we detected a difference in preference between fish and sea urchins when considering clipped plants (i.e. moderate vs. high herbivory) under ambient nutrient conditions. Sea urchins preferred the high herbivory leaves, which while they were lower in fiber, they were higher in phenols, and both lower in sucrose and nitrogen contents. Fish, on the other hand, preferred the moderate herbivory plants, which were higher in nitrogen and sucrose, lower in phenols, but higher in fiber content. This difference in feeding behavior suggests that under ambient nutrient conditions, phenolic compounds exert a higher deterrent effect on fish while sea urchins are more sensitive to fiber content. When nutrients were added, however, preference in sea urchins shifted and both herbivores preferred the moderate herbivory plants. Higher nitrogen and generally lower fiber of fertilized tissues may have reduced or compensated for the deterrent effect of fiber under high nutrient conditions for sea urchins. Additionally, this difference in tissue preference could have been driven by changes in other traits modifying palatability that we did not analyze such as nitrogen-based compounds (e.g., alkaloids; Massad & Dyer 2010). Although in our feeding experiments we did not consider the differences in epiphyte load or tissue age, clipping of the oldest fragment of the leave decreased leaf necrotic tissue and increased epiphyte load due to reduction of shelf-shading (Borowitzka *et al.*, 2006). Thus, changes in tissue age and epiphyte load may also influence the preference of sea urchins and fish in natural conditions (Boudouresque & Verlaque, 2001; Tomas *et al.*, 2005a; Vergés *et al.*, 2011b; Marco-Méndez *et al.*, 2012, 2015).

In addition to modifying plant abundance and palatability, the reduction of leaf surface by defoliation may also hinder N absorption through leaves, hampering allocation to tolerance (Hay *et al.*, 2011). Indeed, in our study, simulated herbivory reduced leaf N and P contents as well as rhizome N, which unlike in other studies (Vergés *et al.*, 2008; Sanmartí *et al.*, 2014), could not be attributed to mobilization of internal resources for compensatory growth. However, the reduction in leaf area did not seem to affect carbon fixation since clipped plants accumulated sucrose in belowground tissues. The higher sucrose content of rhizomes can also be associated with the lower need of C for nitrogen assimilation. This contradicts previous works in which carbohydrate reserves were reduced due to invested in regrowth (Thayer *et al.*, 1984; Vergés *et*

*al.*, 2008; Sanmartí *et al.*, 2014). While we did not detect a compensatory growth response under high herbivory, we did however observe a marginally significant increase in growth in the moderate treatment. Moreover, these shoots had a higher number of leaves with higher sucrose content, which may be linked to a compensatory increase in photosynthetic activity (Lunn, 2008). According to previous studies, seagrasses can increase leaf growth and biomass in response to grazing (e.g., Moran & Bjorndal 2005; Vergés *et al.* 2008) until repeated cropping increases stress beyond a tolerable threshold (Thayer *et al.*, 1984). In our study plants appear to invest in trying to deter further loss of leaf area (through increase in resistance traits) and storing available carbon for future tolerance rather than to generate extra photosynthetic tissue, suggesting a possible tradeoff between the different strategies.

Overall, while the seagrass *P. oceanica* responded as predicted by the RAH to external sources of nutrients reducing their constitutive resistance, increased nutrient availability did not enhance induced resistance or tolerance in fertilized plants, likely due to the existence of limiting resources, as predicted by the LRM. On the other hand, seagrasses exhibited a mixed defense strategy under herbivory pressure with induction of tolerance (i.e. increased number of leaves and sucrose content) and resistance traits (i.e. phenolic compounds, fiber). However, some defense traits did not respond linearly to herbivory pressure being higher at moderate levels of clipping (e.g. number of leaves). Thus, resource availability and herbivore damage can change plant traits influencing the feeding patterns of different herbivores within the same community. This is highly relevant in systems with one or few dominant primary producers which are the main food source for all the associated herbivores in the community. Furthermore, herbivore damage in temperate systems will likely become more relevant as tropical consumers expand polewards (Vergés *et al.*, 2014a, 2014b) and exert stronger pressure on seagrass beds and algal forests (e.g., Heck *et al.* 2015; Hyndes *et al.* 2016; Vergés *et al.* 2016).

## 3.3 Chapter 3: Seagrass (*Posidonia oceanica*) seedlings in a high-CO2 world: from physiology to herbivory.

### 3.3.1 Abstract

Under future increased $CO_2$ concentrations, seagrasses are predicted to perform better as a result of increased photosynthesis, but the effects in carbon balance and growth are unclear and remain unexplored for early life stages such as seedlings, which allow plant dispersal and provide the potential for adaptation under changing environmental conditions. Furthermore, the outcome of the concomitant biochemical changes in plant-herbivore interactions has been poorly studied, yet may have important implications in plant communities. In this study we determined the effects of experimental exposure to current and future predicted $CO_2$ concentrations on the physiology, size and defense strategies against herbivory in the earliest life stage of the Mediterranean seagrass *Posidonia oceanica*. The photosynthetic performance of seedlings, assessed by fluorescence, improved under increased $pCO_2$ conditions after 60 days, although these differences disappeared after 90 days. Furthermore, these plants exhibited bigger seeds and higher carbon storage in belowground tissues, having thus more resources to tolerate and recover from stressors. Of the several herbivory resistance traits measured, plants under high $pCO_2$ conditions had a lower leaf N content but higher sucrose. These seedlings were preferred by herbivorous sea urchins in feeding trials, which could potentially counteract some of the positive effects observed.

### 3.3.2 Introduction

Seagrass meadows are of utmost importance for marine coastal systems. These photosynthetic organisms form the basis of diverse coastal food webs having important ecological functions and offering crucial services for humans (Orth *et al.*, 2006a). As seagrasses are highly productive, they act as a carbon sink (Fourqurean *et al.*, 2012; Pergent *et al.*, 2014; Mazarrasa *et al.*, 2015) and, as ecosystem engineers, they provide substratum for epiphytic plants and animals to settle (Borowitzka & Lethbridge, 1989) and support coastal and offshore fisheries (Jackson *et al.*, 2015). However, seagrasses are presently suffering a global loss associated to numerous human activities (Boudouresque *et al.*, 2009; Waycott *et al.*, 2009).

## 3. Results

Early life stages of plants play a crucial role in providing a new genetic variation pool necessary for adaptation to future environmental changes (Hughes *et al.*, 2008; Jump *et al.*, 2009; Kramer & Havens, 2009). In seagrasses this is a critical life stage since, in some species, sexual reproduction is very variable in space and time and in general, there is a very low rate of seedling establishment (Orth *et al.*, 2006b). Although some seagrass species rely mainly on clonal reproduction, sexual reproduction provides genetic diversity which could make seagrass populations more resilient and resistant to future changes (Jahnke *et al.*, 2015b). Furthermore, seeds and seedlings provide a key opportunity to colonize new habitats (Ridley, 1930), and under the current scenarios of anthropogenic threats, this strategy may be particularly relevant for allowing the establishment of seagrass into more suitable environments.

Atmospheric $CO_2$ concentrations have increased between 1.3% and 3.4% $y^{-1}$ over the last decade (Canadell *et al.*, 2007; Le Quéré *et al.*, 2009), and projections based on current emission rates estimate that atmospheric concentrations would increase up to 800 ppm by the end of the century (IPCC, 2013) and up to 1500-2100 ppm between 2100 and 2200 (Wigley *et al.*, 1996; Pörtner *et al.*, 2004). Plants with C3 metabolism are predicted to benefit by this increase in $CO_2$ concentrations in two ways. First, increasing $CO_2$ availability increases carbon fixation rates (Giordano *et al.*, 2005; Koch *et al.*, 2013). Secondly, a higher $pCO_2$ will reduce photorespiration because of the higher diffusion of $CO_2$, increasing the efficiency of carbon uptake (Bowes *et al.*, 1971; Ehleringer, 2005) and overall photosynthesis (Long *et al.*, 2004). As a result of the increased carbon assimilation, studies in terrestrial plants show increases in biomass, non-structural carbohydrates and C:N ratios (Robinson *et al.*, 2012). Due to the absorbing potential of the ocean (30-40% of anthropogenic $CO_2$ released to the atmosphere (Houghton, 2007), seawater would increase its $pCO_2$, with more $CO_2$ available for photosynthetic organisms. Since most seagrasses have a C3 photosynthetic metabolism, they are predicted to benefit by the increase in $CO_2$ availability (Koch *et al.*, 2013). Indeed, even though most seagrass species have carbon-concentrating mechanisms, they seem to be limited by the current $CO_2$ concentration, as increased $CO_2$ availability in the water enhances photosynthesis (Invers *et al.*, 2001; Beer *et al.*, 2002; Borum *et al.*, 2016). However, how this predicted increase in photosynthesis would translate into increase in plant performance or abundance remains unclear although long term experiments and studies in CO2 vents found increased productivity and density respectively at

low pH (7.6 and 7.3; Hall-Spencer *et al.*, 2008; Cox *et al.*, 2015). Besides, there are no conclusive patterns about long-term effects of elevated $p$CO$_2$ in carbon budget or chemical composition in most seagrasses (Campbell & Fourqurean, 2013; Russell *et al.*, 2013; Apostolaki *et al.*, 2014; Takahashi *et al.*, 2016).

Furthermore, beyond plant productivity effects, changes in CO$_2$ concentrations may also modify the capacity of plants to resist or tolerate herbivory (Strauss & Agrawal, 1999). Plants have developed diverse mechanisms against herbivory; their tolerance strategies reduce the impact of herbivory on plant fitness (e.g., increasing carbon storage to regrow after damage) and their resistance strategies reduce the feeding preference or performance of the herbivore (e.g., decreasing nutritional quality; Fritz & Simms, 1992; Strauss & Agrawal, 1999). Indeed, plant nutritional quality as well as chemical defenses are key resistance traits in controlling plant consumption by herbivores (Fritz & Simms, 1992; Hay, 2009). Increased $p$CO$_2$ often decreases nutritional quality (increasing C/N; Jiang *et al.*, 2010; Takahashi *et al.*, 2016) ) and increases chemical defenses (e.g., phenolic compounds) in terrestrial plants, with consequences for plant-herbivore interactions (Zvereva & Kozlov, 2006; Stiling & Cornelissen, 2007). Nevertheless, the scarce available literature in seagrasses suggest a decrease, rather than an increase, in the concentration of phenolic compounds with increased $p$CO$_2$ (Arnold *et al.*, 2012, 2014). Furthermore, the consequences of CO$_2$-driven changes in seagrass defense strategies, as well as the consequences for plant-herbivore interactions remain poorly studied, and so far no clear general patterns emerge (Burnell *et al.*, 2013; Arnold *et al.*, 2014; Martinez-Crego *et al.*, 2014; Tomas *et al.*, 2015).

In addition, environmental changes in resource availability, such as high CO$_2$ concentrations, may affect the trade-offs in resource allocation between growth and secondary metabolism in plants (Bryant *et al.*, 1983; Herms & Mattson, 1992) with significant ecological costs (e.g., outcome of interactions with herbivores, pathogens or competitors) that might be difficult to predict *a priori*. Understanding the effects of elevated $p$CO$_2$ on the performance of seagrass seedlings is important as they represent a particularly vulnerable period experiencing high mortality rates (Lambers *et al.*, 2008b). Furthermore, herbivore pressure exerted on seedlings has critical effects on plant populations (Watkinson, 1997), shaping composition and structure of plant communities (Barton & Hanley, 2013). Because of the critical ecological

importance of early life stages and the likely physiological differences with adult stages, the effects of future high $p$CO$_2$ in plant early life stages require specific examination.

In this study we hypothesized that under increased CO$_2$ availability seagrass seedlings 1) would increase their incorporation of carbon and perform better and therefore, 2) become less palatable for herbivores and thus, less preferred. To test this, we experimentally assessed the effects of increased CO$_2$ concentrations predicted for the end of 21st century in newly emerged seedlings of *P. oceanica* on several morphological and physiological responses. Additionally, we estimated the effects of high $p$CO$_2$ on seedling survivorship and their palatability to herbivores. To our knowledge, this is the first study examining the effects of increased CO$_2$ availability on early life stages of seagrasses and the implications for interactions with herbivores. Given the importance of early life history changes in providing potential for adaptation and colonization to new environments, understanding how increased CO$_2$ will influence the performance of seagrass seedlings is critical for evaluating the consequences of future CO$_2$ increases on seagrass populations.

### 3.3.3 Materials and Methods

#### *Fruit collection and seed germination*

Beach-stranded fruits of *Posidonia oceanica* were collected in Palma Bay (Mallorca, Balearic Islands, Western Mediterranean) during May 2013 and transported to the laboratory in a cooler with seawater. Seeds were extracted from the fruits and maintained in aquaria at constant temperature (17ºC) with UV-filtered seawater for approximately one month until the initiation of the experiment.

#### *Experimental design and setup*

To evaluate the effect of CO$_2$ availability on *P. oceanica* seedlings, seawater was aerated with a mix of air and pure CO$_2$ gasses using Mass Flow Controllers (Aalborg, USA) in order to obtain experimental CO$_2$ values of actual (ca.500 ppm hereafter control treatment) and future oceanic conditions (ca. 1550 ppm, hereafter high $p$CO$_2$ treatments). Seventeen seedlings of homogeneous size (control $p$CO$_2$: 0.759 ± 0.007 g wet mass seedling$^{-1}$, high $p$CO$_2$: 0.758 ± 0.010 g wet mass seedling$^{-1}$; one-way ANOVA: $F_{(1/12)}$ = 0.015; P = 0.908) were randomly assigned to each of the seven replicate 9-L aquaria with control or high $p$CO$_2$ treatments and maintained in

these conditions for 90 days in 14:10h (light: dark) light cycle. In order to maintain pH conditions and to avoid changes in other parameters of carbonate systems (i.e. alkalinity) seedlings were grown without substrate. Aquariums were cleaned and re-filled every 7 days with filtered seawater (10 μm plus UV filter) and $CO_2$ pre-treated seawater to maintain stable salinity levels and water quality.

### *Water conditions*

Two discrete pH samples (total scale) were taken once a week from each aquarium and analyzed by spectrophotometric method under controlled temperature (17ºC). At the same time, two replicate water samples (50 cc) from each aquarium were taken for dissolved inorganic carbon (DIC) and Total Alkalinity ($A_T$). Water samples were fixed with supersaturated $HgCl_2$ (Merck, Analar) to avoid biological activity and changes in $A_T$ conditions. $A_T$ values were obtained by double endpoint titration to pH 4.45 and 4.41 (NBS scale) with HCl (Fixanal®) according to Dickson Sop 3b (version 3.01), using a Tritando 808 and Aquatrode plus (Metrohm®). The accuracy of measurements was checked against certified reference seawater (CRM, Batch 101, Dickson Scripps Institution of Oceanography, San Diego, USA). Salinity was measured daily (Hanna Instruments) and maintained at 36 psu while light and temperature were continuously recorded using HOBO data loggers (Onset®). Carbonate system parameters were estimated using CO2SYS (Pierrot *et al.,* 2006) with dissociation constants (K1 and K2) according to Millero *et al.* (2006) and $KHSO_4$ dissociation constant after Dickson (1990). A summary of experimental treatment conditions is shown in Supplementary material Table S1.

### *Seedling photosynthetic traits*

Photosynthetic measurements were performed by pulse amplitude modulated (PAM) fluorometry (Walz, Effeltrich, Germany) on the seedlings after 60 and 90 days of exposition to control and high $p$CO$_2$ conditions. First, the maximum quantum yield on dark-adapted seedlings was determined in three seedlings per aquarium by applying a saturating light pulse in the second leaf of each seedling after a 5-min period of dark-adaptation. To reduce variability within seedlings, all measurements were made approximately 2 cm above the leaf meristem. Effective quantum yield was measured after 10 s-exposures to 0, 11, 36, 72, 82, 140, 231, 300 and 455 μmol m$^{-2}$ s$^{-1}$ photon flux densities to obtain Rapid Light Curves (RLCs) in the same dark-adapted seedlings. Leaf absorbance (AF) was measured by placing 1-4 layers of leaves in front of the PAR

sensor instrument and recording the percentage light absorbed by the seagrass(Beer *et al.*, 1998). AF was calculated as 1- exp (-α) where α is the slope of the linear correlation of the ln of the light transmitted against the number of leaf layers. Electron Transport Rates (ETR) from the RLC data were calculated as ETR = yield x irradiance x 0.5 x AF (Beer *et al.*, 2001). ETR values were plotted against the incident absorbed PAR and the photosynthetic quantum efficiency (α) was calculated as the slope of the linear part of the light response curve and the saturation irradiance (Ek) as the division of ETRmax by the initial slope. The maximum electron transport rate (ETRmax) and the maximum quantum yield (Y) were calculated as the maximum ETR and effective quantum yield (ΔF/Fm') of each ETR-PAR curve.

### *Seedling size and mortality*

Leaf width of the second leaf, maximum leaf length, total root length and number of leaves and roots of each seedling were measured at the beginning of the experiment and after 25, 60 and 90 days with the exception of root length that was only measured at the beginning and at the end of the experiment (90 days) to avoid damage. Leaf thickness was measured in the second leaf at the mid-point of their length with a precision caliper (resolution 0.01mm) in three seedlings per aquaria. Seedling mortality was calculated as the percentage of seedlings dead after 90 days relative to the initial number of seedlings placed in each replicate aquarium. A seedling was considered dead when all leaves were shed from the sheath or necrotic. After 90 days, five seedlings were randomly selected from each experimental aquarium and dried for 48 h at 60ºC to determine biomass of leaves, roots and seed of each one.

### *Seedling chemical traits*

Effects of $CO_2$ enriched seawater in the inorganic carbon intake of leaves, were analyzed using stable isotope ratios. Leaves of four seedlings per aquaria were dried (60ºC for 48 hours), ground and treated with HCL fumes (37%, 12-24h) to remove carbonates (Hedges & Stern, 1984). Stable isotopes signatures were analyzed from 0.5 mg in a NC1500 elemental analyzer (Carlo Erba, Milan, Italy) combined with a Delta Plus XL isotope ratio mass spectrometer (ThermoQuest, Bremen, Germany). Isotope ratios in samples were calculated as:

$$\delta X = [(R_{sample}/R_{standard} - 1)] \times 1000$$

where X is $^{13}C$, and R is the corresponding ratio of $^{13}C/^{12}C$.

## 3. Results

Commercial $CO_2$ was used as working standard and two internal standards with $\delta^{13}C$ -30.63‰ and -11.65 ‰ (Vienna- PDB, V-PDB) were used for the isotopic analyses. For carbon, 22 internal standards (organic and inorganic material) ranging from -49.44 ‰ to +28.59 ‰ (V-PDB) were contrasted with the IAEA international references NBS-28, NBS-29, NBS-20 (carbonates) and NBS-22, IAEA-CH-7, IAEA-CH-6 (organic material). The precision, calculated after correction of the mass spectrometer daily drift, was $\pm$ 0.1‰ for $\delta^{13}C$.

Regarding seedling traits related to herbivory, we considered total phenolic compounds, fiber, nitrogen content (% dry weight, DW), sucrose content (%DW) and C/N of leaves as resistance traits, whereas the number of leaves, and the carbon (% DW), sucrose and starch (%DW) content of seeds and roots were considered tolerance traits.

Pooled plant material (ca. 6 seedlings) of each experimental aquarium was ultrafrozen (-80ºC), freeze-dried, and ground to a fine powder to determine the concentration of carbon, nitrogen, fiber, sucrose and total phenols in leaves and carbon, nitrogen, starch and sucrose in seeds and roots and total phenols in seeds (see general methodology). In order to have an initial reference on carbon and nitrogen contents, 6 samples of seedlings (pooled plant material of ca. 6 seedlings each) were freeze-dried at the beginning of the experiment.

### *Feeding experiment*

In order to examine how biochemical changes due to increased $CO_2$ availability modify plant palatability, we performed a feeding assay with herbivorous sea urchins. After 90 days of treatment, four seedlings from each experimental aquarium of both $pCO_2$ treatments were used in a two-choice experiment. The feeding assay was performed in an indoor seawater flow-through system (i.e. ambient $CO_2$ conditions) and a light:dark photoperiod of 12:12h. Similar-sized sea urchins (4.98$\pm$ 0.66 cm test diameter (TD), one-way ANOVA: $F_{(1/22)}$ = 1.695; P = 0.21) of the species *Paracentrotus lividus*, the main invertebrate herbivore on *P. oceanica* meadows, were acclimated for a period of 48 hours and fed with *Ulva sp.* ad libitum. The experiment consisted of 20 replicates and Individual urchins were offered similar amounts of leaf tissue clean of epiphytes (c.a. 3-4 leaves from one seedling, see general methodology).

### Statistical analyses

Differences in initial wet weights between treatments as well as seedling mortality and size of sea urchins were analyzed using a one-way ANOVA analysis. Plant size traits were analyzed using repeated measures ANOVA analyses with time (days) as within-subject factor and $pCO_2$ treatment (high and control) as the between subject factor. The effects of experimental treatments on plant chemical traits (leaf total phenol content, leaf thickness, leaf fiber content, non-structural carbohydrates of leaves, seeds and roots, and biomass of leaves, seed and roots) obtained at the end of the experiment were analyzed by means of one-way ANOVA tests. Carbon and nitrogen contents of leaf, seed and roots were also compared with the initial samples with a one-way ANOVA. Total phenolic content of leaves and seeds was analyzed with Kruskal-Wallis rank sum test as the data were not normal even after transformation. The mean value of each aquarium was used as replicate for all the above-mentioned analyses. The analysis of two-choice experiments was performed using a Wilcoxon signed-ranks paired test. *Post hoc* analyses were performed with Tukey multiple comparisons of means. Data were checked for normality with the Saphiro-Wilk test and homogeneity of variances with the Bartlett test. ANOVAs were conducted without transformation of the variables since data followed normality and homocedasticity.

### 3.3.4 Results

### Seedling photosynthetic traits

ETRmax and saturation irradiance (Ek) were significantly higher (27% and 24% respectively) after 60 days of treatment in the leaves of seedlings grown under high $pCO_2$ (Supplementary material Fig. S1, Table 3.3.1). While there were no differences in AF (control $pCO_2$: 0.641 ± 0.025, high $pCO_2$: 0.628 ± 0.08, one-way ANOVA: $F_{(1/12)}$ = 0.023, P = 0.89), maximum quantum yield (Y) nor in the photosynthetic quantum efficiency (α) between $pCO_2$ treatments (Table 3.3.1). Furthermore, no differences in photosynthetic parameters were evident after 90 days between treatments, with ETRmax and Ek decreasing in both treatments (Supplementary material Fig. S1, Table 3.3.1).

## 3. Results

**Table 3.3.1.** Mean, standard error and results of repeated measures ANOVA in photosynthetic parameters and Tukey HSD test (H: High$CO_2$, C: Control $CO_2$, 60: 60 days 90: 90 days, n=7).

| Variable | Control CO2 | | High CO2 | | Source | DF | DF error | MS | MS error | F | P | Tukey´s tests |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 60 days | 90days | 60 days | 90days | | | | | | | | |
| Maximum Quantum Yield | 0.708 (0.012) | 0.682 (0.179) | 0.712 (0.008) | 0.705 (0.008) | $CO_2$ | 1 | 12 | 0.00 | 0.00 | 1.10 | 0.314 | |
| | | | | | Time | 1 | 12 | 0.00 | 0.00 | 2.28 | 0.157 | |
| | | | | | Time x $CO_2$ | 1 | 12 | 0.00 | 0.00 | 0.74 | 0.406 | |
| Alfa | 0.179 (0.007) | 0.144 (0.009) | 0.186 (0.006) | 0.15 (0.008) | $CO_2$ | 1 | 12 | 0.00 | 0.00 | 0.61 | 0.452 | |
| | | | | | Time | 1 | 12 | 0.01 | 0.00 | 27.32 | **<0.001** | 90<60 |
| | | | | | Time x $CO_2$ | 1 | 12 | 0.00 | 0.00 | 0.02 | 0.899 | |
| Maximum electron transport rate (ETRmax) | 12.15 (0.92) | 9.11 (0.462) | 16.73 (0.99) | 9.11 (0.419) | $CO_2$ | 1 | 12 | 36.66 | 3.69 | 9.93 | **0.008** | C<H |
| | | | | | Time | 1 | 12 | 198.68 | 4.11 | 48.31 | **<0.001** | 90<60 |
| | | | | | Time x $CO_2$ | 1 | 12 | 36.60 | 4.11 | 8.90 | **<0.001** | C90=H90<C60<H60 |
| Saturation Irradiance (Ek) | 68.35 (4.68) | 64.34 (3.56 | 90.65 (5.9) | 62.91 (3.03) | $CO_2$ | 1 | 12 | 762.10 | 197.00 | 3.87 | 0.0728 | |
| | | | | | Time | 1 | 12 | 1764.60 | 79.40 | 22.23 | **<0.001** | 90<60 |
| | | | | | Time x $CO_2$ | 1 | 12 | 985.70 | 79.40 | 12.41 | **0.004** | C90=H90<C60<H60 |
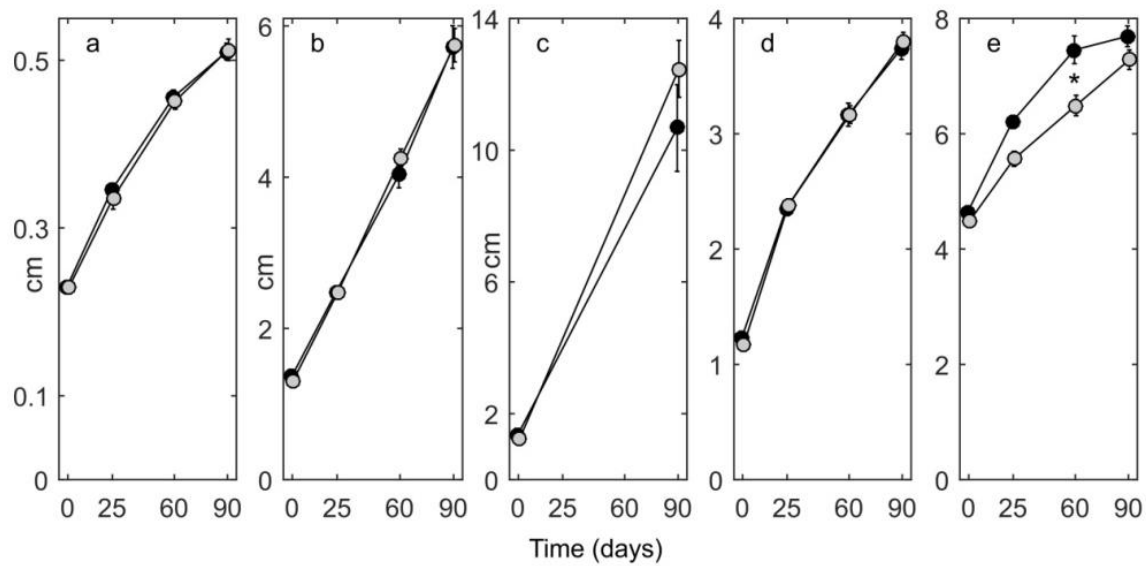
**Figure 3.3.1.** Leaf width (a), maximum leaf length (b), root length (c), number of roots (d), and number of leaves (e) measured in seedlings growing under high $CO_2$ (black) or control (grey) conditions, at the beginning of the experiment and after 25, 60 and 90 days. Total root length (c) was only measured at the beginning and after 90 days of experiment. Error bars indicate standard error. Asterisk indicates statistically significant differences between treatments

### Seedling size and mortality

While initial seedling size (i.e. leaf width, maximum leaf length, number of leaves, number of roots, and total root length) was similar between treatments, the number of leaves was significantly higher in seedlings from the high $pCO_2$ treatment after 60 days (Fig.3.3.1, Table 3.3.2). Leaf width, maximum leaf length and number of roots did not differ between treatments despite substantial growth along the experimental period. No significant difference between $pCO_2$ treatments was found in total root length after 90 days (Fig.3.3.1, Table 3.3.1). $CO_2$ concentrations did not affect the mortality of seedlings (control $pCO2$: 7.6 ± 2.79%, high $pCO2$: 8.4 ± 3.1%, one-way ANOVA: $F_{(1/12)}$ = 0.04, P = 0.84). Seed biomass under high $pCO_2$ was almost 2-fold higher than for control seedlings, while there were no differences between treatments for leaf and root biomass (Fig.3.3.2, Table 3.3.3).

**Table 3.3.2.** Results of Repeated Measures ANOVA in morphometric parameters, and Tukey HSD test (H: HighCO$_2$; C: Control CO$_2$; 0: beginning experiment; 25: 25 days; 60: 60 days; 90: 90 days, n=7).

| Variable | Source | DF | DF error | MS | MS error | F | P | Tukey´s tests |
|---|---|---|---|---|---|---|---|---|
| Leaf Width | CO$_2$ | 1 | 12 | 0 | 0.001 | 0.12 | 0.733 | |
| | Time | 3 | 36 | 0.219 | 0 | 514.37 | **<0.0001** | 0<25<60<90 |
| | Time x CO$_2$ | 3 | 36 | 0 | 0 | 0.18 | 0.909 | |
| Maximum Leaf Length | CO$_2$ | 1 | 12 | 0.034 | 0.333 | 0.1 | 0.754 | |
| | Time | 3 | 36 | 51.38 | 0.1 | 525.88 | **<0.0001** | 0<25<60<90 |
| | Time x CO$_2$ | 3 | 36 | 0.05 | 0.1 | 0.54 | 0.661 | |
| Number of Leaves | CO$_2$ | 1 | 12 | 4.153 | 0.31 | 13.4 | **0.003** | C60<H60 |
| | Time | 3 | 36 | 23.392 | 0.126 | 184.99 | **<0.0001** | 0<25<60<90 |
| | Time x CO$_2$ | 3 | 36 | 0.426 | 0.126 | 3.29 | **0.032** | C60<H60 |
| Number of Roots | CO$_2$ | 1 | 12 | 0.001 | 0.055 | 0.02 | 0.923 | |
| | Time | 3 | 36 | 17.314 | 0.029 | 602.5 | **<0.0001** | 0<25<60<90 |
| | Time x CO$_2$ | 3 | 36 | 0.009 | 0.029 | 0.32 | 0.811 | |
| Root Length | CO$_2$ | 1 | 6 | 0.8 | 3.99 | 0.18 | 0.674 | |
| | Time | 3 | 23 | 740.3 | 4.5 | 162.9 | **<0.0001** | 0<90 |
| | Time x CO$_2$ | 3 | 23 | 6.5 | 4.5 | 1.43 | 0.244 | |
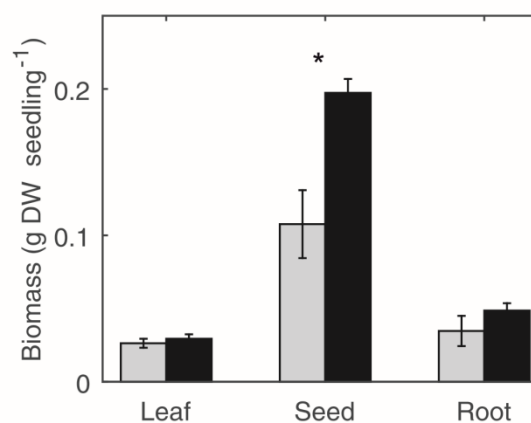


**Figure 3.3.2.** Mean dry Biomass (g) of leaves, seeds and roots of seedlings growing under high CO$_2$ (black) or control (grey) conditions. Error bars indicate standard error. Asterisk indicates statistically significant differences between treatments.

***Seedling chemical traits***

The δ13C in leaves of seedlings from the high *pCO₂* treatment (-20.5±1.19 δ‰) was significantly lower than in control plants (-16.4 ±1.39 δ ‰), (Table 3.3.3).

Carbon content increased in leaves and roots when compared to the beginning of the experiment, but differences related to $CO_2$ treatments were only observed in seeds, which had a higher C content under high *pCO₂* (Fig.3.3.3 Table 3.3.3). The nitrogen content of seeds decreased and that of roots increased throughout the experiment and did not differ between experimental treatments. Conversely, leaf nitrogen content was ca. 17% lower in the high *pCO₂* when compared to the control, which increased by 13% throughout the experimental period. Hence, the leaf C/N ratio was 14% higher in $CO_2$-enriched plants when compared to controls (Fig.3.3.3, Table 3.3.3). $CO_2$ enrichment resulted in higher (more than 30%) content of sucrose in seeds and roots, while no significant changes were detected between treatments in starch content. Sucrose content in leaves was almost two fold higher in the increased *pCO₂* treatment compared to the non-enriched (Fig.3.3.4, Table 3.3.3). The increase in $CO_2$ availability did not affect the total phenolic content (Kruskal Wallis test , $\chi^2$=0.102, df=1, P = 0.749) nor the fiber content (Fig.3.3.4, Table 3.3.3) of leaves, while it significantly decreased (39%) phenolic content in the seeds (Kruskal Wallis test, $\chi^2$= 6.208, df=1, P = 0.013).
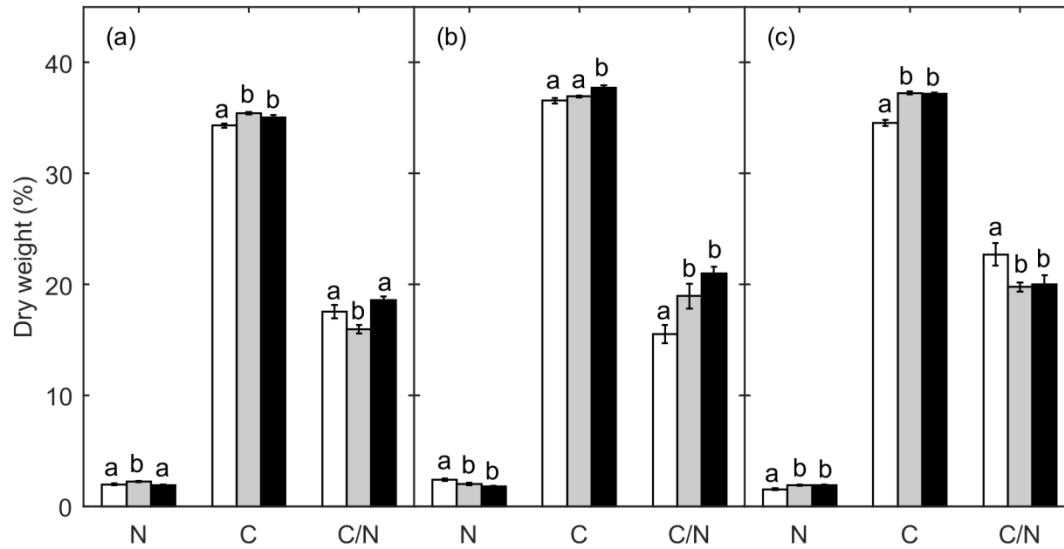
**Figure 3.3.3.** Percentage of dry weight in Nitrogen (N), Carbon (C), and Carbon Nitrogen ratio (C/N), in leaves (a), seeds (b) and roots (c) of seedlings at the beginning of the experiment (white) or after growing under high $CO_2$ (black) or control (grey) conditions. Error bars indicate standard error and different letters indicate statistically significant differences across treatments (Tukey).
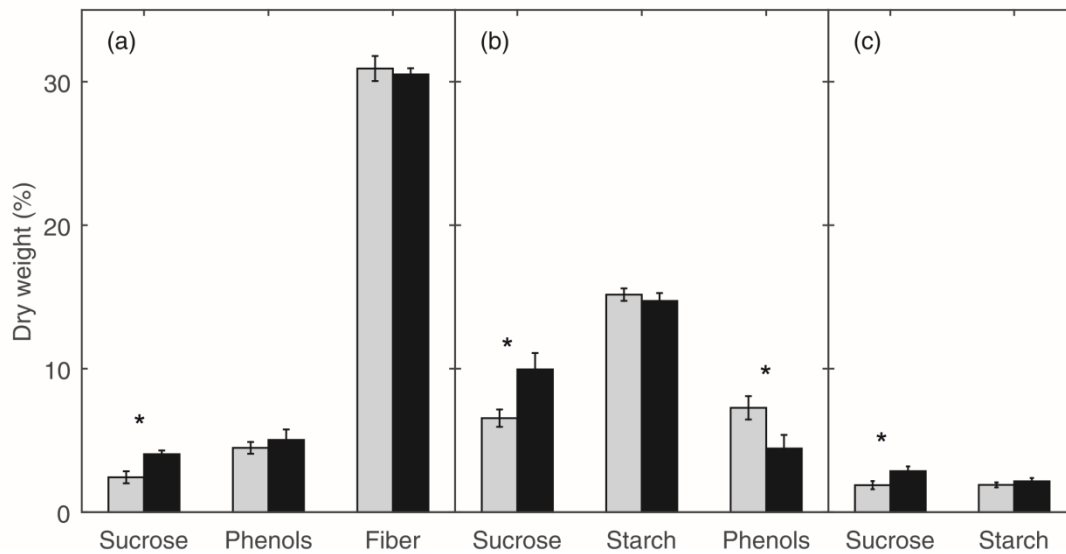


**Figure 3.3.4.** Percentage of dry weight in sucrose, starch, total phenol content (Phenols), and fiber in leaves (a), seeds (b) and roots (c) of seedlings growing under high $CO_2$ (black) or control (grey) conditions. Error bars indicate standard error. Asterisk indicates statistically significant differences between treatments.

**Table 3.3.3.** Results of one way ANOVAs in plant traits and Tukey HSD test (I: Initial, H: HighCO$_2$, C: Control CO$_2$, n=7).

| Tissue | Plant Traits | DF | DF error | MS | MS error | F | P | Tukey´s tests |
|---|---|---|---|---|---|---|---|---|
| Leaf | C (%DW) | 2 | 17 | 2.115 | 0.235 | 8.995 | **0.002** | I <H=C |
| | N (%DW) | 2 | 17 | 0.218 | 0.023 | 9.591 | **0.002** | I=H<C |
| | C/N | 2 | 17 | 12.09 | 1.37 | 8.824 | **0.002** | C<H=I |
| | Sucrose (%DW) | 1 | 12 | 9.072 | 0.848 | 10.7 | **0.007** | C<H |
| | Fiber (%DW) | 1 | 12 | 0.582 | 3.306 | 0.18 | 0.682 | |
| | Thickness (cm) | 1 | 12 | 0 | 0 | 0.031 | 0.863 | |
| | Biomass (g) | 2 | 12 | 0 | 0 | 0.27 | 0.768 | |
| | δ13C | 1 | 12 | 59.49 | 1.047 | 56.83 | **<0.001** | H<C |
| Seed | C (%DW) | 2 | 17 | 2.285 | 0.264 | 8.673 | **0.002** | I=C<H |
| | N (%DW) | 2 | 17 | 0.569 | 0.072 | 7.875 | **0.004** | H=C<I |
| | C/N | 2 | 17 | 49.24 | 5.22 | 9.429 | **0.002** | I<C=H |
| | Sucrose (%DW) | 1 | 12 | 40.42 | 5.83 | 6.94 | **0.022** | C<H |
| | Starch (%DW) | 1 | 12 | 0.682 | 1.711 | 0.398 | 0.54 | |
| | Biomass (g) | 2 | 12 | 0.015 | 0.02 | 6.837 | **0.01** | C<H |
| Root | C (%DW) | 2 | 17 | 14.71 | 0.24 | 61.19 | **<0.001** | I<H=C |
| | N (%DW) | 2 | 17 | 0.25 | 0.028 | 8.957 | **0.002** | I<H=C |
| | C/N | 2 | 17 | 17.034 | 3.975 | 4.286 | **0.031** | H=C<I |
| | Sucrose (%DW) | 1 | 12 | 3.324 | 0.626 | 5.31 | **0.04** | C<H |
| | Starch (%DW) | 1 | 12 | 0.214 | 0.388 | 0.55 | 0.472 | |
| | Biomass (g) | 2 | 12 | 0 | 0 | 0.794 | 0.474 | |

***Herbivore feeding experiment***

Sea urchins consumed a significantly higher amount of fresh leaf tissue biomass from leaves grown under high $p$CO$_2$ in comparison to control $p$CO$_2$ conditions (Wilcoxon signed-ranks paired test, Z =2.78, n = 19, P = 0.004, Fig.3.3.5).

**Table 3.3.3.** Results of one way ANOVAs in plant traits and Tukey HSD test (I: Initial, H: High$CO_2$, C: Control $CO_2$, n=7).

| Tissue | Plant Traits | DF | DFerror | MS | MSerror | F | P | Tukey´s tests |
|--------|--------------|----|---------|-----|---------|---|---|---------------|
| Leaf | C (%DW) | 2 | 17 | 2.115 | 0.235 | 8.995 | **0.002** | I <H=C |
| | N (%DW) | 2 | 17 | 0.218 | 0.023 | 9.591 | **0.002** | I=H<C |
| | C/N | 2 | 17 | 12.09 | 1.37 | 8.824 | **0.002** | C<H=I |
| | Sucrose (%DW) | 1 | 12 | 9.072 | 0.848 | 10.7 | **0.007** | C<H |
| | Fiber (%DW) | 1 | 12 | 0.582 | 3.306 | 0.18 | 0.682 | |
| | Thickness (cm) | 1 | 12 | 0 | 0 | 0.031 | 0.863 | |
| | Biomass (g) | 2 | 12 | 0 | 0 | 0.27 | 0.768 | |
| | δ13C | 1 | 12 | 59.49 | 1.047 | 56.83 | **<0.001** | H<C |
| Seed | C (%DW) | 2 | 17 | 2.285 | 0.264 | 8.673 | **0.002** | I=C<H |
| | N (%DW) | 2 | 17 | 0.569 | 0.072 | 7.875 | **0.004** | H=C<I |
| | C/N | 2 | 17 | 49.24 | 5.22 | 9.429 | **0.002** | I<C=H |
| | Sucrose (%DW) | 1 | 12 | 40.42 | 5.83 | 6.94 | **0.022** | C<H |
| | Starch (%DW) | 1 | 12 | 0.682 | 1.711 | 0.398 | 0.54 | |
| | Biomass (g) | 2 | 12 | 0.015 | 0.02 | 6.837 | **0.01** | C<H |
| Root | C (%DW) | 2 | 17 | 14.71 | 0.24 | 61.19 | **<0.001** | I<H=C |
| | N (%DW) | 2 | 17 | 0.25 | 0.028 | 8.957 | **0.002** | I<H=C |
| | C/N | 2 | 17 | 17.034 | 3.975 | 4.286 | **0.031** | H=C<I |
| | Sucrose (%DW) | 1 | 12 | 3.324 | 0.626 | 5.31 | **0.04** | C<H |
| | Starch (%DW) | 1 | 12 | 0.214 | 0.388 | 0.55 | 0.472 | |
| | Biomass (g) | 2 | 12 | 0 | 0 | 0.794 | 0.474 | |



**Figure 3.3.5.** Percentage of fresh weight consumed by herbivores from leaf tissue of seedlings growing under high $CO_2$ (black) or control (grey) conditions. Error bars indicate standard error. Asterisk indicates statistically significant differences between treatments.

### 3.3.5   Discussion

Early life seagrass stages could benefit under the future elevated $CO_2$ predicted scenarios. Our results show that, in general, seedling photosynthetic performance was enhanced under elevated $pCO_2$ levels during the initial phases of seedling development, leading to increased sucrose content of leaves, roots and seeds, and an overall increase in carbon storage. These positive effects could translate into having more resources stored to resist or recover from stressful conditions. On the other hand, increased $CO_2$ availability led to biochemical changes in leaves that resulted in shifts in the palatability of this tissue.

While currently the major source of photosynthetic inorganic carbon uptake in *P. oceanica* seems to be in form of $HCO_3^-$ rather than $CO_{2[aq]}$, a future increase in $CO_{2[aq]}$ may change this ratio (Invers *et al.*, 2001, 2002). The higher ETRmax observed after 60 days of experiment suggests a greater ability to transfer electrons under high $CO_2$ conditions. Interestingly, seeds of *Posidonia* spp seedlings have photosynthetic activity that enhances seedling growth (Celdran *et al.*, 2015). While we did not measure the photosynthetic activity of the seed, a higher $CO_2$ availability could have also increased photosynthesis in this organ, potentially contributing to a higher total photosynthetic activity when compared to seedlings from other species or adults.

As demonstrated by the $\delta^{13}C$ values in our study, seedlings from high $pCO_2$ treatments exhibited reduced $CO_2$ fractionation, suggesting that seedlings growing under present $CO_2$ are likely $CO_2$-limited. In addition, leaves from the increased $pCO_2$ treatment had a higher content of sucrose, an effect that has been also found in other studies with adult seagrasses (Jiang *et al.*, 2010). Sucrose is the principal end-product of leaf photosynthesis (Lunn, 2008); and the higher content found in our study is thus likely resulting from the increased photosynthetic activity during the early development of the seedlings. In general, higher $CO_2$ availability increases photosynthetic activity in seagrasses (Apostolaki *et al.*, 2014; Cox *et al.*, 2015; Ow *et al.*, 2015; Takahashi *et al.*, 2016) which sometimes translates into increases in aboveground biomass or growth (Jiang *et al.*, 2010; Russell *et al.*, 2013; Takahashi *et al.*, 2016). However this increase in photosynthesis and thus in carbon incorporation is not always allocated to aboveground growth (Campbell & Fourqurean, 2013; Apostolaki *et al.*, 2014; Tomas *et al.*, 2015). In our study, seedlings under high $pCO_2$ did not allocate carbon to changes in aboveground size at the end of

the experimental period but rather to maintain or slow the decrease of seed biomass. Similarly, adult seagrasses can also exhibit an increase in belowground biomass (Palacios & Zimmerman, 2007) or changes in the chemical composition of below and aboveground tissues (Campbell & Fourqurean, 2013; Tomas *et al.,* 2015).

In this study, seeds exhibited lower sucrose content in the control treatment, which suggests that seedlings growing under high $p$CO$_2$ had a lower consumption of sucrose from seeds or that sucrose was produced through photosynthesis, mobilized to belowground tissues and stored in seeds. This effect of increased non-structural carbohydrates in belowground tissues has been also found in adult seagrasses under experimental increase in CO$_2$ availability (Jiang *et al.,* 2010; Campbell & Fourqurean, 2013). In seedlings, this is particularly important since seeds store and supply carbon and nutrients to the seedling during the first year of its life (Balestri *et al.*, 2009). Increased carbon reserves and biomass of the seed would benefit seedling survival and resilience to stressful conditions, especially in seagrasses such as *P. oceanica* in which the buoyant fruits disperse to new habitats away from the original meadow. Having more resources to tolerate or resist adverse light and temperature conditions or damage by herbivores would likely improve seedling establishment and survival(Moles *et al.,* 2004), which are key features of a successful population expansion process. Particularly in *P. oceanica* in which flowering frequency varies greatly spatially and among years (0-26%; Balestri, 2004) with a low reproductive success (3-11% of seedlings available for establishment) mainly due to seed predation (Balestri & Cinelli, 2003).

Despite the cost of less carbon available for growth, carbon allocation to defense and storage often results in higher survivorship of organs and individuals (Bloom *et al.,* 1985b). Secondary metabolites are associated with defense mechanisms in plants (e.g. feeding deterrence; Hay & Steinberg, 1992; Bennett & Wallsgrove, 1994) being for some herbivores more determinant of their preference than other attributes such as carbohydrates or fibers (Buchsbaum *et al.,* 1984). According to the resource availability hypothesis (RAH; Coley *et al.,* 1985)) plants grown under high resource availability will invest less in defense components than plants grown under limited resourced environments. Therefore, seagrasses grown under elevated nutrient availability (usually a limiting resource; Invers *et al.,* 2004) often decrease the production of chemical defenses such as phenols (Goecker *et al.,* 2005; Tomas *et al.,* 2011).

Being carbon-based compounds, most of the studies in terrestrial plants (Robinson *et al.*, 2012) and some species of macroalgae (Celis-Plá *et al.*, 2015) have found increases in phenolic compounds with elevated $CO_2$ availability. Yet, because $CO_2$ is also a resource that can greatly limit primary production in seagrasses (Zimmerman *et al.*, 1997; Invers *et al.*, 2001, 2002), we may expect a decrease in phenolics (rather than the increase often observed in terrestrial plants) under high $CO_2$ scenarios, following RAH. Indeed both a decrease (Arnold *et al.*, 2012, 2014) as well as no changes(Tomas *et al.*, 2015), but never an increase, in phenolic compounds have been reported in seagrass leaves growing under elevated $CO_2$ conditions. While we did not find significant changes in phenol content in leaves associated with $CO_2$ availability, we did observe it in seeds. Since defense has a cost, not all plant parts are equally defended, as they contribute differently to fitness (McKey, 1979; Rhoades, 1979). Seeds have multiple important functions (e.g., carbon storage, nutrient supply and photosynthesis) which are critical for seedling survival. Thus, seeds may be an organ whose defense is prioritized under resource-limited conditions (e.g., present-day levels of $CO_2$). Seeds from seedlings of the control treatments had significantly higher phenol content than those from the increased $pCO_2$ treatment, which were bigger (higher biomass), with higher carbon content and more stored sucrose. Having more resources (i.e. $CO_2$) available in the environment may have decreased the investment of carbon on seed defense towards favoring the storage of other more rapidly available carbon-based compounds such as sucrose (Endara & Coley, 2011) .

A decrease in nutritional quality (as a decrease in nitrogen or increased C/N content) in response to high $CO_2$ has been commonly observed in terrestrial (Bezemer & Jones, 1998; Robinson *et al.*, 2012) and marine plants (Jiang *et al.*, 2010; Campbell & Fourqurean, 2013; Takahashi *et al.*, 2016), and it has been attributed to a dilution of nitrogen due to increased leaf growth (Bazzaz, 1997), increases in leaf carbohydrates and structural material, higher plant internal nitrogen requirements (Luo *et al.*, 1994; Yin, 2002) and/or reductions in protein concentrations (Rogers *et al.*, 1996). Some studies in *Zostera noltei* also found a lower N content under high $pCO_2$ conditions together with a lower nitrate uptake (Alexandre *et al.*, 2012). This reduced nitrate uptake could be the reason for the lower nitrogen content observed in leaves in the high $pCO_2$ treatment in this study, which would not be related to a dilution of nitrogen by increased growth since there were no differences in leaf biomass at the end of the experiment.

## 3. Results

The reduced nutritional quality observed in seagrass leaves could have consequences for herbivores that may compensate this low nutritional quality by increasing their feeding rates (Cruz-Rivera & Hay, 2000; DeLucia *et al.*, 2012).

Unexpectedly, in our study, leaves with lower nitrogen content were preferred by sea urchins, whereas herbivores typically prefer tissues with higher N content (Mattson, 1980; Cebrian & Duarte, 1998; Pierce & Berry, 2011). However, N content also includes nitrogen in insoluble forms and alkaloids (Mattson, 1980), and does not necessarily reflect availability and quality for herbivores. In addition, factors other than nitrogen content may also be influencing the palatability of seagrass to herbivores.

Leaf fiber content may reduce the preference of grazers by reducing the digestibility to herbivores (Lincoln, 1993), increasing leaf toughness (Coley, 1983) or decreasing the preference for high carbon-fiber plant species (Mariani & Alcoverro, 1999). The neutral detergent fiber method measures most of the groups of structural constituents of plant cells (e.g., cellulose, lignin, hemicellulose). Yet, not all the components are similar in terms of production costs and defensive properties. Lignin provides better structural and chemical defensive properties than cellulose, which has half the biosynthesis cost in glucose equivalents (Williams *et al.*, 1987). Therefore, even though we did not detect differences in the fiber content between treatments, we cannot rule out that the relative composition of chemical components of the fiber could have differed under high $CO_2$ concentrations (Cotrufo & Ineson, 2000; Newman *et al.*, 2003; Arnold *et al.*, 2014), and consequently, may have modified the palatability of the tissues.

One of the biochemical traits that changed with higher $pCO_2$ availability was sucrose content in leaves, which may have enhanced plant palatability. In insects, for instance, sugars increase stimulation to taste (Bernays & Chapman, 1994) and can mask the deterrent effect of other compounds (Cocco & Glendinning, 2012). Additionally, we performed the feeding experiments only with the sea urchin *P. lividus*, whereas different herbivore species may have responded differently to $CO_2$-driven changes in plant chemical composition (Bezemer & Jones, 1998; Tomas *et al.*, 2015) or epiphyte abundance or composition since it is expected that fleshy epiphytes may increase (Rodolfo-Metalpa *et al.*, 2010) calcareous epibionts would decrease their abundance under low pH conditions (Cox *et al.*, 2015). While we only performed the feeding experiments under ambient $CO_2$ water conditions, studies to date with adult sea urchins

do not suggest strong changes in feeding rates (Brown *et al.*, 2014; Carey *et al.*, 2016), nor in preferences (S.R. Fitzpatrick, personal communication) under ambient vs. high $p$CO$_2$ conditions.

In summary, the results of our experiment suggest that seedlings of *P. oceanica* might perform better under a high CO$_2$ scenario. The enhanced photosynthetic activity and carbon fixation increased the amount of resources available for storage, which would benefit these early life stages to resist or recover from stress. Yet, positive effects might be counterbalanced by changes in grazing pressure due to increased palatability, although allocation of resources to tolerance could allow seedlings to survive and persist to shifts in herbivory pressure.

## 3.4 Chapter 4: Future warmer seas: increased stress and susceptibility to grazing in seedlings of a marine habitat-forming species

### 3.4.1 Abstract

Increases in seawater temperature are expected to have negative consequences for marine organisms. Beyond individual effects, species-specific differences in thermal tolerance are predicted to modify species interactions and increase the strength of top-down effects, particularly in plant-herbivore interactions. Shifts in trophic interactions will be especially important when affecting habitat-forming species such as seagrasses, as the consequences on their abundance will cascade throughout the food web. Seagrasses are a major component of coastal ecosystems offering important ecosystem services, but are threatened by multiple anthropogenic stressors, including warming.

The mechanistic understanding of seagrass responses to warming at multiple scales of organization remains largely unexplored, especially in early life stages such as seedlings. Yet these early life stages are critical for seagrass expansion processes and adaptation to climate change. In this study we determined the effects of a three-month experimental exposure to present and predicted mean summer SST of the Mediterranean Sea (25ºC, 27ºC, and 29ºC) on the photophysiology, size and ecology (i.e. plant-herbivore interactions) of seedlings of the seagrass *Posidonia oceanica*. Warming resulted in increased mortality, leaf necrosis and respiration as well as lower carbohydrate reserves in the seed, the main storage organ in seedlings. Aboveground biomass and root growth were also limited with warming, which could hamper seedling establishment success. Furthermore, warming increased the susceptibility to consumption by grazers, likely due to lower leaf fiber content and thickness. Our results indicate that warming will negatively affect seagrass seedlings through multiple direct and indirect pathways: increased stress, reduced establishment potential, lower storage of carbohydrate reserves, and increased susceptibly to consumption. This work provides a significant step forward in understanding the major mechanisms that will drive the capacity of seagrass seedlings to adapt and survive to warming, highlighting the potential additive effects that herbivory will have on ultimately determining seedling success.

### 3.4.2 Introduction

Sea surface temperatures have globally increased since the 20th century, and are projected to increase between 0.6 to 1.5 ºC in the next 40 years (IPCC, 2013), especially in tropical and Northern hemisphere subtropical regions (IPCC, 2014). Warming poses a threat to marine biota and their ecosystems (Oviatt, 2004; Díaz-Almela *et al.*, 2009; Lejeusne *et al.*, 2010). Increased temperature can affect the metabolism, life cycle, and behavior of organisms (Pörter & Farrell, 2008). Furthermore, it may also cause poleward shifts in species distribution (Perry *et al.*, 2005; Parmesan, 2006; Vergés *et al.*, 2014b, 2016), particularly for high mobility species (e.g., fishes), but expansion will be hindered for low mobility and sessile species (Sunday *et al.*, 2015).

The effects of warming in habitat-forming species (e.g., corals, seagrasses, kelps) further changes landscape structure and community composition with ecosystem-level implications due to the impacts on the associated species (Wernberg *et al.*, 2013; Hyndes *et al.*, 2016). Seagrasses are ecosystem engineers forming important coastal habitats along temperate and tropical regions. They have key ecological roles and provide important ecosystem services, increasing water clarity (De Boer, 2007), attenuating wave height (Infantes *et al.*, 2012), acting as a carbon sink (Fourqurean *et al.*, 2012) and providing nursery habitats and feeding grounds for many species (Jackson *et al.*, 2015).

Seagrasses have an optimal growth temperature range between 12-26 ºC and 23-32 ºC for temperate and tropical species, respectively (Lee *et al.*, 2007). Although some species are more tolerant to temperature changes than others (e.g., *Halodule wrightii,* Koch *et al.*, 2007; *Halophila ovalis* Ralph, 1998), most physiological and growth parameters in seagrasses exhibit thermal performance curves (Walker & Cambridge, 1995; Pedersen *et al.*, 2016) with a threshold temperature after which seagrass performance declines (e.g., Nejrup & Pedersen, 2008). Among the negative effects of increased temperature, are that seagrasses exhibit a decrease in photosynthesis and growth (e.g., Marsh *et al.*, 1986; Terrados & Ros, 1995; Collier *et al.*, 2011), and increased mortality (e.g., Nejrup & Pedersen, 2008; Massa *et al.*, 2009). Indeed, past warming periods have been associated with a decline of some seagrass populations (Oviatt, 2004; Díaz-Almela *et al.*, 2009; Marbà & Duarte, 2010).

Clonal growth is generally the dominant process for meadow expansion in seagrasses (Tomlinson, 1974; Migliaccio *et al.*, 2005), yet sexual reproduction such through seed fertilization

and seedling establishment is crucial for enabling long distance dispersal and for providing the genetic variation necessary for future adaptation to environmental changes. Furthermore, higher genetic diversity allows greater resilience to disturbance, including warming (Reusch *et al.*, 2005; Ehlers *et al.*, 2008). Thus, understanding the effects that warming may have on these early life stages becomes imperative. In fact, to date there are no studies that provide an in-depth mechanistic understanding of the effects of warming on newly emerged seedlings of a marine habitat-forming species.

In addition to changes in plant physiology, ecological interactions such as herbivory may shift the responses of plant communities to warming (Post & Pedersen, 2008). Understanding the link between ecological and physiological responses to global warming of key species and their interactions is crucial for a successful mitigation of impacts. Increased temperature strengthens plant-herbivore interactions and might promote a stronger top-down control of plant performance due to changes in primary production and herbivore preferences and/or feeding rates (O'Connor, 2009; Gutow *et al.*, 2016; West & Post, 2016).  Herbivory is especially intense in marine ecosystems (Poore *et al.*, 2012), yet few studies have investigated the impacts of warming in relation to plant-herbivore interactions in these systems (e.g., Burnell *et al.*, 2013; Garthwin *et al.*, 2014). The effects of temperature on herbivory processes are complex and may cause important shifts in community structure (Blake & Duffy, 2012; Eklöf *et al.*, 2012), particularly when habitat-forming and facilitator species are being consumed (Bruno *et al.*, 2003; Halpern *et al.*, 2007). Indeed, evidence of this critical process is the recent collapse of algal forests and associated biota due to warming-driven increases in herbivory in the Mediterranean and Australia (Vergés *et al.*, 2014a, 2016).

Plants have evolved diverse mechanisms to tolerate (e.g., increased carbon storage) or resist (e.g., phenolic compounds) herbivory (Fritz & Simms, 1992; Strauss & Agrawal, 1999). The importance of phenolic compounds against herbivory is widely recognized (Nykänen & Koricheva, 2004; Lambers *et al.*, 2008a), and warming decreases their production in terrestrial plants (Zvereva & Kozlov, 2006). The identification and quantification of specific phenolic compounds in seagrasses has been scarcely studied (Heglmeier & Zidorn, 2010; Grignon-Dubois *et al.*, 2011; Zidorn, 2016) and the responses of secondary compounds to warming remain unexplored.

Importantly, defense often changes across plant life stages (Elger *et al.*, 2009) and herbivory can be the main cause of mortality during early development (Moles & Westoby, 2014). Thus, the strong selection pressure that herbivores exert on seedlings is an important evolutionary pressure (Barton & Hanley, 2013), and has critical effects on composition and structure of plant communities (Watkinson, 1997; Barton & Hanley, 2013). However, to our knowledge, studies on the effects of increased temperature on seagrass early life stages and the consequences on plant-herbivore interactions have yet not been performed.

Here we focus on the responses of seedlings of *P. oceanica*, an endemic seagrass species, which is a major carbon sink among seagrasses (Lavery *et al.*, 2013) and inhabits a region (the Mediterranean Sea) that is especially vulnerable to warming (Christensen & Hewitson, 2007). In this study we conducted a three-month experiment to assess how photophysiology, size, biochemical traits, susceptibility to herbivory and survivorship of *P. oceanica* seedlings are affected by increased seawater temperatures predicted for the end of this century. Our work uses an integrative approach that encompasses multiple scales of study (from photophysiology to herbivory) to explore the consequences of warming in ecological communities.

### 3.4.3   Materials and methods
#### *Experimental design and conditions*

*P. oceanica* fruits were collected from Palma Bay (Balearic Islands, Spain) during May 2013 and transported in seawater at ambient temperature to the laboratory. Seedlings (approximately three-months old) that had been kept in aquaria at constant temperature, salinity and light intensity (21 ºC, 36.5±1 ppt, 36.07±1.98 µmol·m$^{-2}$·s$^{-1}$) with a recirculating pump in aerated seawater from Palma Bay and 14h: 10h (light: dark) photoperiod were used in this experiment.

We experimentally manipulated seawater temperature to reach three values: 25 ºC as a control treatment (the summer mean SST in the Mediterranean sea over the last 20 years (1982-2012); Shaltout & Omstedt, 2014), used as control treatment; 27 ºC (temperature in the range of mean summer SST forecasted for the end of this century; Shaltout & Omstedt, 2014) and 29 ºC (temperature in the range of mean summer SST forecasted for next century in the Mediterranean Sea; Shaltout & Omstedt, 2014). Treatment temperatures were reached after an

acclimation period in which temperature was increased 1 ºC per week and seedlings were exposed to the treatment temperatures for three months. Each treatment was conducted in 10 replicate 25L-aquaria containing 30 randomly selected seedlings, commercial aquarium gravel as substratum, aerated seawater, a heater, and a recirculating pump. All aquaria and seedlings were gently cleaned of epiphytes once a week and refilled with seawater to maintain water level after cleaning (≈ 1 % of aquaria volume). Temperatures and light intensities of each aquarium were recorded every 15 min. during the acclimation period and throughout the experiment using data loggers (Onset Hobo). Salinity was controlled every three days and oxygen saturation was measured at the end of the experiment to control for any changes in oxygen solubility. The photoperiod was maintained at 14h: 10h (light: dark) and there were no differences between treatments in light intensity (Kruskal Wallis: df= 2, $\chi^2$=0.235, p=0.89), salinity (Kruskal Wallis: df= 2, $\chi^2$=1.56, p=0.45) nor oxygen saturation (Kruskal Wallis: df= 2, $\chi^2$=0.9, p=0.64; Supplementary material, Table S.4.1.).

### *Size and mortality*

To assess changes in seedling size we measured: total wet weight, leaf width and thickness of the second leaf, maximum leaf length, total leaf area, number of leaves, percentage of necrotic surface on each leaf, total root number, root length, and biomass (dry weight, DW) of leaves, roots, rhizomes and seeds at the end of the experiment. All measurements were made at the beginning and at the end of the experiment on a total of six seedlings per aquarium. Leaf growth was estimated as the new leaf area produced per day in three of six seedlings per aquaria by punching the leaves two weeks before finalizing the experiment following the method of Zieman (1974). In addition, in order to be able to compare biomass through time, 20 seedlings were oven-dried (60 ºC, 48 h) at the beginning of the experiment to measure the biomass of leaves, roots, rhizomes and seeds. The above / belowground biomass ratio was calculated as the ratio of total leaf biomass to total belowground biomass (rhizomes, seeds, and roots).

Seedling mortality was calculated as the percentage of dead seedlings (seedlings with no blades or all of them necrotic) at the end of the experiment relative to the initial number of seedlings contained in each replicate aquarium.

## 3. Results

### *Seedling photophysiology*

Effects of increased temperature in seedling photophysiology were examined at the end of the experiment in photosynthesis-irradiance (P-I) curves, net photosynthesis at saturating irradiance, post-illumination respiration, and photosynthetic pigment content. P-I curves were produced for seedlings from the 25 ºC and 29 ºC treatments. Oxygen was measured with optical oxygen sensors ("optode", PreSens DP-PSt3) and a PreSens Oxy 4 four-channel fibre-optic oxygen meter at the end of experiment. Seven seedlings of each 25 ºC and 29 ºC aquaria were gently cleaned of epiphytes and individually incubated at the same treatment temperatures in chambers (300 mL). Photosynthetic and respiration rates were measured on the whole seedling at one light intensity (15, 30, 60, 80, 100, 120, 130, 150 or 180 µmol photon m$^{-2}$ s$^{-1}$) during 60 to 120 min. to avoid bubble formation at high irradiances, whereas respiration was measured under 80 min of darkness. Seedlings were randomly assigned to one of the measured light intensities or darkness.

After performing P-I curves, seedlings were ultra-frozen, freeze-dried and weighed to normalize $O_2$ rates to whole seedling dry weight. Normalized $O_2$ rates were plotted against irradiance and curve parameters estimated through a nonlinear regression fitted with non-linear least squares procedure and using the model equation of Jassby & Platt (1976):

$$P = Pmax \, (\tanh \alpha \cdot I / Pmax) + R,$$

where Pmax (mgO2 gDW $^{-1}$ h$^{-1}$) is the maximum photosynthetic rate, $\alpha$, the slope of the linear part of the curve, is the photosynthetic efficency (mgO$_2$ gDW $^{-1}$ h$^{-1}$/ µmol photon m$^{-2}$ s$^{-1}$) and I is the photon flux density (µmol photon m$^{-2}$ s$^{-1}$). The irradiance at which photosynthesis is saturated ($I_k$) was estimated from the intersection of the initial slope with Pmax ($I_k$ = Pmax/$\alpha$). The compensation irradiance ($I_c$) at which photosynthesis equals respiration was determined using the ratio R/$\alpha$, R being dark respiration.

Two seedlings of each aquarium were incubated at the three treatment temperatures in chambers (300 mL) at saturating irradiance to measure net photosynthesis, (mgO$_2$/gr (DW)·h) for 120 min and after that 80 min at dark to measure post-illumination respiration (mgO$_2$/gr (DW)·h).

Leaf photosynthetic pigment content (µgChl·(g FW)$^{-1}$) was analyzed for two seedlings per

aquaria from $1cm^2$ of the middle part of the blade from the leaves 2 and 3 as in Dennison (1990).

### *Seedling biochemical traits*

The effects of increased temperature were measured on several plant biochemical traits. Carbon, nitrogen, and carbohydrate contents were analyzed for seeds, rhizome and roots as a measurement of tolerance traits. Carbon, nitrogen, phosphorous, total and specific phenolic compounds, and fiber content of leaves as well as leaf thickness were quantified as defense traits against consumers. Pooled plant material (ca. 15 seedlings) of each experimental aquarium was freeze-dried and ground to a fine powder for further analyses (see general methodology). In addition, a total of 36 seedlings from the initial population were freeze-dried in six groups of six pooled seedlings at the beginning of the experiment to compare with final measurements. Additionally, we calculated total phenol content as the sum of specific phenolic compounds (see below), since the Folin method has known limitations, such as lower sensitivity (Blum, 1997) and interference of organic matter (e.g., sugars, Benlloch-Tinoco *et al.*, 2015).

Specific phenolic compounds were extracted from powdered leaf samples (0.2 g) with MeOH (5mL x 3) and the organic extracts obtained were subjected to C18 cartridge solid phase extraction (SPE) using MeOH:$H_2O$ (1:9 and 2:8) as solvents. Separation and identification of compounds was performed using an UPLC-MS (see methodology) and a solution of quercetin was added for a final concentration of 500 μg/mL of extract and 3 μg/mL of quercetin (internal standard). Quantification of phenolic compounds was performed by external calibration curves, using a reference compound (i.e. chicoric acid) selected based on the principle of structure-related target analyte/standard (chemical structure and functional group). Quercetin was used as internal standard, and all determinations were performed in triplicate.

### *Herbivore feeding assays*

To examine the effect of temperature on herbivore feeding behavior we conducted two feeding choice experiments with sea urchins maintained at 25 ºC (25.08 ± 0.06 ºC, Mean ± SE), 27 ºC (27.07 ± 0.06 ºC) and 29ºC (29.08 ± 0.06 ºC), in accordance with the seedling treatment temperatures. Similar sized sea urchins (25 ºC = 5.3 ± 0.07 cm Test Diameter (TD), 27 ºC = 5.2 ± 0.06 cm TD, 29 ºC = 5.2 ± 0.06 cm TD; One-way ANOVA: F=0.89, df=2, p=0.42) of the species

*Paracentrotus lividus*, the main invertebrate herbivore in the Mediterranean Sea, were acclimated to their treatment temperatures through increasing the water by 0.5 ºC every four days. Experiments were performed in flow-through system under controlled light conditions (12/12 h; see general methodology) and fed with *Ulva* sp. ad libitum throughout the acclimation period.

In the three-choice experiment, similar amounts of leaf tissue clean of epiphytes (c.a. 3-4 leaves from one seedling) from the seedlings grown at the three treatment temperatures (25 ºC, 27 ºC, 29 ºC) were simultaneously offered to the sea urchins. Additionally, considering the tendency of preference observed in the three-choice experiment (see results) and significant differences in plant defense traits between 25 ºC and 29 ºC (see results), a two-choice experiment was performed with sea urchins kept at extreme treatment temperatures (25 and 29 ºC). Both experiments (i.e., three and two choice) consisted of 25 replicates per treatment

### *Statistical analyses*.

Data were checked for normality with the Saphiro-Wilk test and homogeneity of variances with the Bartlett test. Experimental conditions (i.e., salinity, oxygen concentration, light intensity), as well as the effects of experimental treatments on plant carbohydrates (sucrose in leaves, sucrose and starch content in rhizomes, seeds and roots), leaf phosphorous content, leaf fiber content, photosynthetic parameters (Net P, Gross P and Respiration), biomass parameters (biomass of leaf, rhizome, seed and root, and above: belowground biomass ratio) and specific phenolic compounds were analyzed by means of Kruskal Wallis test since data were not normal even after data transformation. In all plant trait analyses the mean value of each aquarium was used as a replicate (n=10 for each treatment). Size traits (leaf width, thickness, maximum leaf length, whole seedling wet weight, total root length, number of roots, leaf thickness, width, number of leaves and total leaf area) were analyzed with repeated measures ANOVA with time (days) as the within-subject factor and temperature treatment (25ºC, 27ºC, 29ºC) as the between subject factor. Carbon, nitrogen, total leaf phenol content, Chl a and b leaf content, leaf necrosis, leaf surface growth, and sea urchin size were analyzed with a one way ANOVA test. All the ANOVAs were conducted without transformation of the variables. Differences in photosynthetic parameters derived from the P-I curves were analyzed by means of a single sample t-test. The results of the two-choice feeding experiments were analyzed with

a Wilcoxon´s matched paired test and the analyses of the three-choice experiments were performed using Hotelling tests as in Prince *et al.*, (2004). *Post hoc* analyses were performed with Tukey multiple comparisons of means for parametric data and Dunn tests multiple comparisons were performed for non-parametric data.

### 3.4.4   Results

#### *Size and mortality*

The size parameters measured (seedling wet weight, maximum leaf length, leaf width and thickness, total leaf area, number of leaves, number of roots and total root length) were similar between treatments at the beginning of the experiment (Fig. 3.4.1, Table 3.4.1). Seedling wet weight, leaf width, thickness and total leaf area significantly increased with time in all the treatments and maximum leaf length only increased (17%) in the 25ºC treatment (Fig.3.4.1, Table 3.4.1). In addition, leaves and roots almost doubled their biomass when compared to initial samples; the rhizome being the tissue that grew most (four times more biomass than initial samples; Fig.3.4.1, Table 3.4.2). Warming up to 29 ºC resulted in a 20 % lower seed biomass, 5 % lower leaf thickness, 26 % less leaves, 30 % lower total leaf area and 18 % lower total root length when compared to the 25 ºC treatment (Fig.3.4.1, Table 3.4.1, Tukey). Seedlings reared at 29 ºC also exhibited lower leaf biomass when compared to the 25 ºC treatment, and the above /belowground ratio was higher in the control (25 ºC = 0.368±0.028) treatment when compared to the other treatments (27 ºC = 0.283 ±0.019, 29 ºC = 0.289±0.023) and all were higher than the initial samples (i= 0.23±0.016, Kruskal Wallis test, $\chi^2$=15.86, df=3, P = 0.001). In contrast, leaf surface growth (cm$^2$/day) was not significantly affected by temperature (25 ºC = 0.33±0.027, 27 ºC = 0.29±0.022, 29 ºC = 0.27±0.01; One-way ANOVA, $F_{2/27}$ = 2, P=0.155).

Mortality was two-fold higher in the warmest treatment (13±0.4%) when compared to 25 ºC (6 ±0.3%) and almost three times higher when compared to the 27 ºC (5±0.3%; one-way ANOVA, $F_{(1/28)}$ = 7.67; P = 0.01, Tukey test: 25 ºC =27 ºC <29 ºC). Similarly, necrosis was almost 2 fold higher in the warmest treatment when compared to the control treatment (25 ºC=4.8±0.45%, 27 ºC= 5.9±0.42%, 29 ºC= 9.1±0.43%, Table 3.4.2)
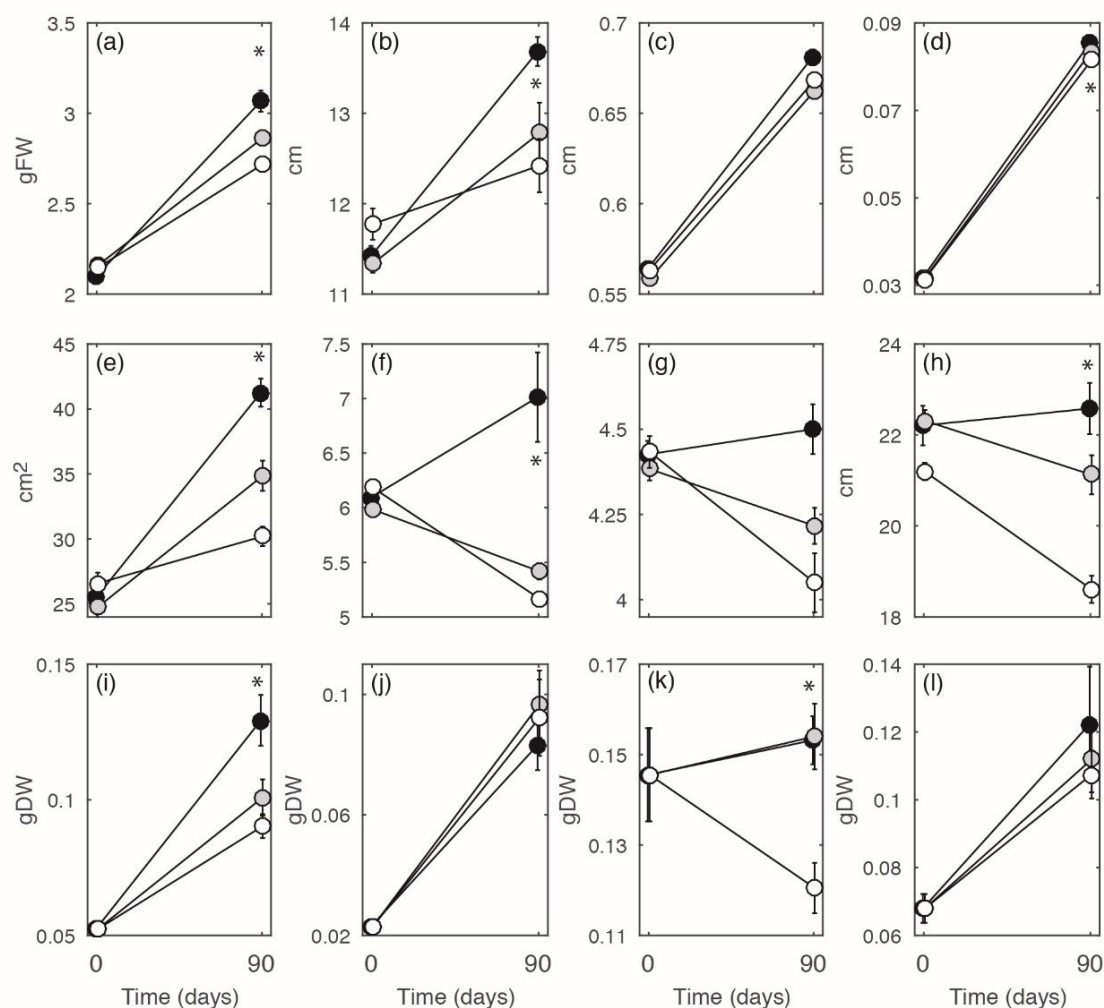
**Figure 3.4.1.** Mean (a) Total wet weight, (b) maximum leaf length, (c) width, (d) thickness , ( e) total leaf area, (f) number of leaves (g) and roots, (h) total root length and biomass of (i) leaf, (j) rhizome, (k) seed and (l) root of seedlings growing at 25 ºC (black), 27 ºC (grey) and 29 ºC (white) at the beginning and at the end of the experiment. Error bars indicate standard error and asterisk indicates statistically significant differences among treatments.

### *Seedling photophysiology*

*P. oceanica* seedlings exhibited typical P-I response curves (Supplementary material Fig. S1), with photosynthetic rates increasing linearly (initial slope, α) under limiting irradiances until reaching the maximum photosynthetic rate (Pmax). P–I curves exhibited a good fit (P-I 25 ºC; $R^2$ =0.96, Sum of squares error, SSE=0.28. P-I 29 ºC; $R^2$ =0.95, SSE=0.41) to the adapted hyperbolic tangent model and no photoinhibition was observed.

Plants grown at 29 ºC and 25 ºC did not exhibit differences in either Pmax (T = 0.23, P = 0.82), Ik (T = 1.17, P = 0.25), α (T = 0.14, P = 0.89), or in Ic (T = 0.095, P = 0.93; Table S.4.2).

Likewise, there were no differences in net photosynthesis ($\chi^2$=5.14, P = 0.07; Table S.4.2), chlorophyll a (one-way ANOVA, $F_{(2/27)}$ = 0.25; P = 0.779), or in chlorophyll b content (one-way ANOVA, $F_{(2/27)}$ = 0.51; P = 0.608) among treatments. On the other hand, post-illumination respiration was significantly higher by 14.4% in the warmest treatment in comparison to the 25ºC treatment (Table S.4.2).

**Table 3.4.1.** Results of Repeated Measures ANOVA in size parameters, and Tukey HSD test measured in the 25 ºC, 27 ºC and 29 ºC treatments (n = 10) at the beginning (0 days; $t_0$) and at the end of the experiment (90 days; $t_{90}$).

| Variable | Souce | DF | DF error | MS | MS error | F | p | Tukey test |
|---|---|---|---|---|---|---|---|---|
| Wet weight (g) | Temperature | 2 | 27 | 0.11 | 0.09 | 1.25 | 0.302 | |
| | Time | 1 | 27 | 8.43 | 0.04 | 195.20 | **<0.001** | $t_0< t_{90}$ |
| | Temp X Time | 2 | 27 | 0.20 | 0.04 | 4.74 | **0.042** | $25t_0=27t_0=29t_0$ $<29t_{90}=27t_{90}<25t_{90}=27t_{90}$ |
| Maximum Leaf Length (cm) | Temperature | 2 | 27 | 1.50 | 3.48 | 0.43 | 0.654 | |
| | Time | 1 | 27 | 31.64 | 56.21 | 15.20 | **0.041** | $25t_0<29t_{90}$ |
| | Temp X Time | 2 | 27 | 3.28 | 2.08 | 1.57 | 0.226 | |
| Width (cm) | Temperature | 2 | 27 | 0.00 | 0.00 | 1.11 | 0.345 | |
| | Time | 1 | 27 | 0.18 | 0.00 | 589.26 | **<0.001** | $t_0<t_{90}$ |
| | Temp X Time | 2 | 27 | 0.00 | 0.00 | 0.90 | 0.420 | |
| Thickness (mm) | Temperature | 2 | 27 | 0.00 | 0.00 | 3.14 | 0.059 | |
| | Time | 1 | 27 | 0.09 | 0.00 | 5491.65 | **<0.001** | $t_0<t_{90}$ |
| | Temp X Time | 2 | 27 | 0.00 | 0.00 | 2.08 | **0.026** | $25t_0=27t_0=29t_0$ $<29t_{90}=27t_{90}<25t_{90}=27t_{90}$ |
| Total leaf area (cm$^2$) | Temperature | 2 | 27 | 130.89 | 39.61 | 3.31 | **0.028** | $29<25$ |
| | Time | 1 | 27 | 1452.2 | 28.7 | 50.59 | **<0.001** | $t_0<t_{90}$ |
| | Temp X Time | 2 | 27 | 185.6 | 28.7 | 6.47 | **<0.001** | $25t_0=27t_0=29t_0$ $<29t_{90}=27t_{90}<25t_{90}=27t_{90}$ |
| Number of leaves | Temperature | 2 | 27 | 4.99 | 1.99 | 2.51 | 0.100 | |
| | Time | 1 | 27 | 0.74 | 1.68 | 0.44 | 0.513 | |
| | Temp X Time | 2 | 27 | 5.13 | 1.68 | 3.06 | **0.039** | $25t_0=27t_0=29t_0=$ $29t_{90}=27t_{90}<25t_{90}$ |
| Number of Roots | Temperature | 2 | 27 | 0.26 | 0.31 | 0.85 | 0.441 | |
| | Time | 1 | 27 | 0.38 | 0.10 | 3.92 | 0.058 | |
| | Temp X Time | 2 | 27 | 0.26 | 0.10 | 2.71 | 0.085 | |
| Total Root Length (cm) | Temperature | 2 | 27 | 33.22 | 14.32 | 2.32 | **0.028** | $29<25$ |
| | Time | 1 | 27 | 19.30 | 3.00 | 6.43 | 0.141 | |
| | Temp X Time | 2 | 27 | 10.98 | 3.00 | 3.66 | **0.043** | $25t_0=27t_0=29t_0=$ $29t_{90}=27t_{90}<25t_{90}=27t_{90}$ |

***Seedling biochemical traits***

The increase of temperature (i.e., 29 ºC) resulted in a 29 % lower starch content in the seeds compared to the 25 ºC treatment. Conversely, starch content in rhizome and sucrose content in roots were higher (39 % and 24 %, respectively) in the 29 ºC treatment than in the control treatment (Fig. 3.4.2 Table 3.4.2).

Nitrogen content significantly decreased (44±4.2 %) whereas carbon content increased (6±1.4 %) from the beginning to the end of the experiment in all plant parts. Consequently, when compared to initial values, roots doubled their C/N in all treatments. C/N in seeds and rhizomes increased at 25 ºC (44.5 % and 67.2 % respectively) and 27 ºC (37.1 % and 62.3 %). Seeds from the 29 ºC treatment had a higher content of nitrogen and phosphorous (24 % and 34 % respectively) at the end of the experiment than seeds of the 25 ºC treatment (Fig. 3.4.2, Table 3.4.2). Also, roots in the warmest treatment had a 31 % higher phosphorus content than roots from seedlings grown at control temperature. Leaf fiber content was significantly lower in the warmest treatment when compared to the control (25 ºC = 42.2±0.76 %, 27 ºC = 41.7±1.45 %, 29 ºC = 38.5±0.9 %; Table 3.4.2). Total Folin phenolic compounds were 14 % higher in seeds from the warmest treatment compared to the seeds growing at 25 ºC and there were no effects of temperature on total phenols of leaves, rhizomes or roots (Fig. 3.4.2, Table 3.4.2). The specific phenolic compounds found in leaves were all derivatives of the cinnamic acid; chicoric acid, 4-methyl chicoric acid, *p*-coumaroylcaffeoyltartaric acid, *p*-coumaroyltartaric acid and bis-*p*-coumaroyltartaric acid (Supplementary material, Table S.4.3, Table S.4.4, Figure S.4.2), and their concentrations did not differ amongst temperature treatments.
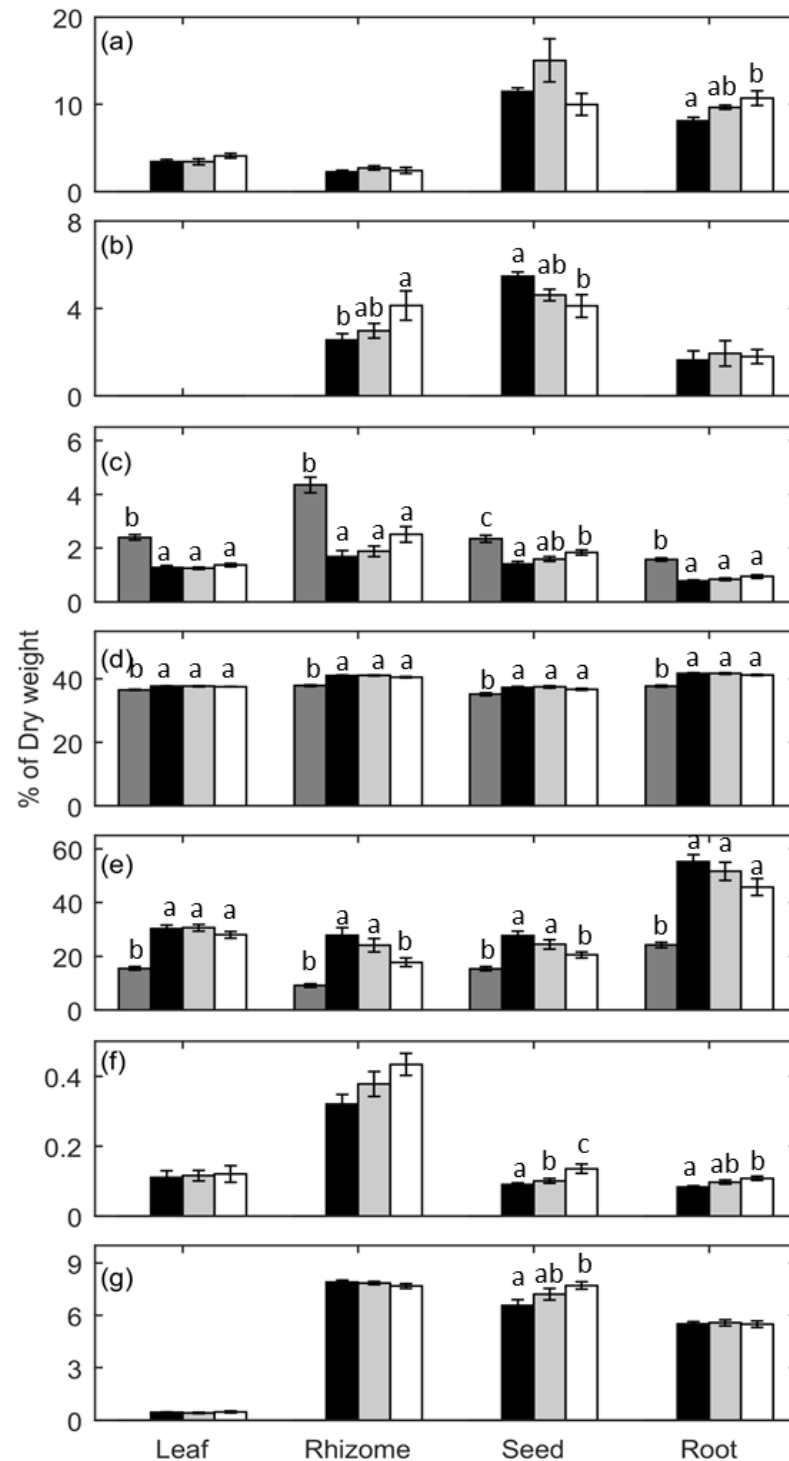
**Figure 3.4.2.** Mean content (in % DW) of (a) sucrose, (b) starch, (c) nitrogen, (d) carbon, (e) carbon:nitrogen ratio, (f) phosphorus, and (g) total Folin phenols in leaves, rhizome, seeds and roots of seedlings growing under 25 ºC (black), 27 ºC (grey), 29 ºC (white) conditions and initial (dark grey) contents. Error bars indicate standard error and different letters indicate statistically significant differences across treatments or with initial contents (Tukey).

**Table 3.4.2.** Results of one-way Anova and Kruskal Wallis test in plant traits and Tukey HSD and Dunn test measured in the 25 ºC , 27 ºC and 29 ºC treatments (n = 10). Carbon (C), Nitrogen (N) and C/N ratio were also compared with initial (i) contents.

| Tissue | Trait | DF | DF-error | MS | MS-error | F-ratio | Chi-Squared | P | Tukey/Dunn |
|---|---|---|---|---|---|---|---|---|---|
| Leaf | Necrosis (%) | 2 | 27 | 50.90 | 11.24 | 4.53 | | **0.020** | 25<29 |
| | N (%DW) | 3 | 33 | 2.31 | 0.05 | 46.11 | | **<0.001** | 25=27=30<i |
| | C (%DW) | 3 | 33 | 2.34 | 0.19 | 12.46 | | **<0.001** | i<25=27=29 |
| | C/N | 3 | 33 | 390.65 | 13.73 | 28.46 | | **<0.001** | i<25=27=30 |
| | Folin phenols (%DW) | 2 | 27 | 0.01 | 0.02 | 0.47 | | 0.631 | |
| | Fiber (%DW) | 2 | | | | | 8.74 | **0.008** | 29<25 |
| | Sucrose (%DW) | 2 | | | | | 4.29 | 0.117 | |
| | P (%DW) | 2 | | | | | 1.23 | 0.542 | |
| | Biomass (g) | 3 | | | | | 38.79 | **<0.001** | i<27=29<25=27 |
| Rhizome | N (%DW) | 3 | 33 | 11.51 | 0.63 | 18.28 | | **<0.001** | 25=27=30<i |
| | C (%DW) | 3 | 33 | 17.78 | 0.34 | 51.63 | | **<0.001** | i<25=27=30 |
| | C/N | 3 | 33 | 548.20 | 48.00 | 11.42 | | **<0.001** | i=29<27=25 |
| | Folin phenols (%DW) | 2 | 27 | 0.14 | 0.14 | 1.02 | | 0.375 | |
| | Sucrose (%DW) | 2 | | | | | 3.00 | 0.223 | |
| | Starch (%DW) | 2 | | | | | 7.81 | **0.020** | 25<29 |
| | P (%DW) | 2 | | | | | 5.71 | 0.058 | |
| | Biomass (g) | 3 | | | | | 35.55 | **<0.001** | i<25=27=29 |
| Seed | N (%DW) | 3 | 33 | 1.34 | 0.11 | 12.56 | | **<0.001** | 25=27<29=27<i |
| | C (%DW) | 3 | 33 | 8.64 | 1.11 | 7.78 | | **<0.001** | i<25=27=30 |
| | C/N | 3 | 33 | 234.80 | 20.20 | 11.63 | | **<0.001** | i=29<27=25 |
| | Folin phenols (%DW) | 2 | 27 | 3.28 | 0.88 | 3.74 | | **0.037** | 25<29 |
| | Sucrose (%DW) | 2 | | | | | 4.33 | 0.115 | |
| | Starch (%DW) | 2 | | | | | 9.65 | **0.008** | 29=27<25 |
| | P (%DW) | 2 | | | | | 9.67 | **0.008** | 25<27<29 |
| | Biomass (g) | 3 | | | | | 11.01 | **0.039** | 29<i=25=27 |
| Root | N (%DW) | 3 | 33 | 1.05 | 0.03 | 36.78 | | **<0.001** | 25=27=29<i |
| | C (%DW) | 3 | 33 | 28.61 | 0.54 | 52.63 | | **<0.001** | i<25=27=30 |
| | C/N | 3 | 33 | 1494.54 | 78.27 | 19.09 | | **<0.001** | i<27=29=25 |
| | Folin phenols (%DW) | 2 | 25 | 0.02 | 0.25 | 0.08 | | 0.921 | |
| | Sucrose (%DW) | 2 | | | | | 9.32 | **0.009** | 25<27=29 |
| | Starch (%DW) | 2 | | | | | 2.49 | 0.288 | |
| | P (%DW) | 2 | | | | | 8.55 | **0.014** | 25<29 |
| | Biomass (g) | 3 | | | | | 25.07 | **<0.001** | i<25=27=29 |

*Herbivore feeding assay*

Sea urchins maintained at 25 ºC consumed significantly higher amounts of leaf biomass from the 29 ºC treatment in the three-choice experiment, and sea urchins at 27 ºC and 29 ºC also tended to prefer plants grown at 29 ºC, although we did not detect statistical differences. In the two-choice experiment sea urchins both reared at 25 ºC and at 29 ºC consumed about double the amount of leaf tissue from seedlings grown at 29 ºC compared to those at 25 ºC (Fig.3.4.3).
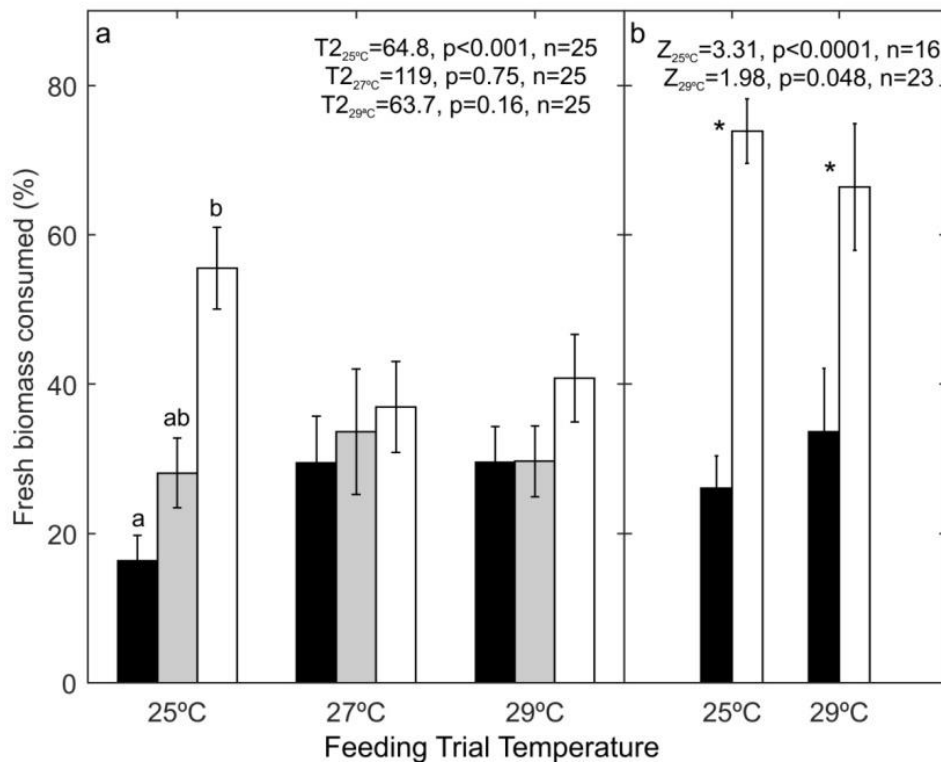


**Figure 3.4.3.** Mean percentage of fresh leaf biomass consumed from seedlings growing under 25 ºC (black), 27 ºC (grey), 29 ºC (white) conditions by sea urchins kept at 25 ºC, 27 ºC and 29 ºC in (a) the three-choice experiment and (b) in the two-choice experiment. Error bars indicate standard error, asterisk indicates statistically significant differences between treatments and different letters indicate statistically significant differences across treatments (Tukey). Hotelling test results, $T^2$ at 25 ºC ($T^2_{25ºC}$), 27 ºC ($T^2_{27ºC}$) and 29 ºC ($T^2_{29ºC}$), Wilcoxon test results Z at 25 ºC ($Z_{25ºC}$) and 29 ºC ($Z_{29ºC}$), p-value (p), and number of replicates (n) shown

**3.4.5 Discussion**

Our results indicate that under a future scenario of warming, early life stages of the temperate seagrass *Posidonia oceanica* will be negatively affected by increasing temperatures both directly through increased stress, as well as indirectly through enhanced susceptibility to consumers. Indeed, higher temperatures (29 ºC) increased metabolic demands (i.e., respiration rate), causing stress and leading to lower photosynthetic biomass, lower carbohydrate reserves in the seeds, a smaller root system, higher mortality, and higher palatability thus increasing susceptibility to grazing and enhancing top-down control.

Respiration is a more temperature-sensitive metabolic process than photosynthesis (Allen *et al.*, 2005) and it typically increases with temperature in most temperate seagrasses (Marsh *et al.*, 1986; Lee *et al.*, 2007). On the other hand, photosynthesis follows a thermal performance curve increasing with temperature up to a maximum temperature where it diminishes (e.g., Marsh *et al.*, 1986; Pedersen *et al.*, 2016), and loss of photosynthetic pigments is sometimes observed (e.g., Niu *et al.*, 2012). We did not observe changes in photosynthetic capacity, nor in pigment content at higher temperatures, while respiration increased at 29 ºC. This increase in respiration as a result of metabolic demands, likely led to the subsequent signs of stress observed in the seedlings (i.e. increased leaf necrosis and seedling mortality). Indeed, seedlings of the temperate *Zostera marina* and *Z. japonica* exhibit sublethal symptoms, such as increased leaf necrosis, bleaching (Abe *et al.*, 2008, 2009) or decreased growth (Niu *et al.*, 2012) under warming. Furthermore, the lower leaf biomass observed at 29 ºC could also be attributed to a decrease in leaf formation rates or an increase in leaf mortality due to necrosis and consequent leaf shedding. Even though the photophysiology of newly emerged *P. oceanica* seedlings appears to be rather tolerant to temperatures up to 29 ºC, the increased leaf necrosis and mortality that we observed suggests a similar lethal temperature limit to the one reported in adult plants for this species (Augier *et al.*, 1980; Olsen *et al.*, 2012).

Warming also led to a lower production of smaller and thinner (in terms of thickness) leaves, and consequently, lower leaf biomass, which is consistent with responses to increased temperature in other temperate adult seagrasses (Bintz *et al.*, 2003; Nejrup & Pedersen, 2008; Shafer *et al.*, 2008) and young seedlings (Niu *et al.*, 2012). Warming generally decreases leaf growth (whether measured as surface or biomass) past a threshold temperature in seagrass

adult plants (Walker & Cambridge, 1995; Bintz *et al.*, 2003; Koch *et al.*, 2007) and seedlings (Niu *et al.*, 2012). We did not detect such effect of decreased growth, and this could be for various reasons. In our experiment we may not have reached the extreme threshold temperature, or, given that we did observe a decrease in leaf thickness, measuring leaf growth in terms of biomass may have led to different results. Additionally, seedlings perhaps favor development of leaves (and thus do not lower leaf growth under stress) as it is critical that they have an adequate leaf system to become independent of the seeds once these are no longer providing resources.

Higher temperatures affected the chemical traits and morphology of belowground tissues. Rhizomes, roots and seeds accumulate starch and sucrose, which are key storage compounds of plants (Tetlow *et al.*, 2004) that provide critical reserves to resist or recover from stressful conditions (e.g. Gacia *et al.*, 2012). In our study, higher temperatures appear to increase the consumption of starch reserves in seeds, which are the overall major contributor to carbon reserve storage in seedlings (Balestri et al., 2009). Therefore, seedlings under increased temperatures would have less carbohydrate reserves stored in seeds, which could hamper their ability to resist or tolerate stressors. On the other hand, we also observed differences in carbohydrate storage patterns between belowground tissues, which could be related to plant responses to stress due to changes between supply and demand for fixed carbon (Farrar *et al.*, 2000). Carbohydrate reserves in seagrass rhizomes appear to be unaffected (Zimmerman *et al.*, 1989; Salo & Pedersen, 2014) or to decrease (Koch *et al.*, 2007) under warming, which has been attributed to increases in demand of stored carbohydrates due to higher respiration rates, and thus lower net photosynthesis (Marsh *et al.*, 1986). While we do see lower reserves in the seeds of our study, rhizomes of plants under warming contained more starch, and roots from the warmest treatment exhibited a higher content of sucrose. Such an increase in the sucrose content of roots is likely being supplied from the seeds, but we cannot discard that the source may have been the leaves (although leaf sucrose content was similar among treatments). Similarly, starch stored in seeds may have been mobilized to rhizomes to support the increased metabolic demands (Williams & Farrar, 1990) of these tissues under warming. Additionally, changes within the tissue, such as modification of carbohydrates breakdown rates with temperature (Gu *et al.*, 2012), may have also contributed to this increase in roots and rhizomes.

Increased temperature led to lower total root length (see also Niu *et al.*, 2012). Roots and root hairs, particularly, enable seedlings to anchor and establish onto new substrates (Infantes *et al.*, 2011; Alagna *et al.*, 2015). Furthermore, roots are an important absorptive area, not only in adult seagrasses (Hemminga, 1998), but also in newly emerged seedlings (Statton *et al.*, 2014) since some nutrients are preferably assimilated from porewater through the roots rather than from water column through the leaves (Touchette & Burkholder, 2000a). Hence, a smaller root system in seagrass seedlings could limit the anchoring capacity and nutrient acquisition of seedlings, potentially compromising seedling establishment and survival under warming.

Nitrogen and phosphorus are quantitatively the most important nutrients in seagrass growth (Elser *et al.*, 2007). In terrestrial plants, warming appears not to affect (see review by Zvereva & Kozlov, 2006) or reduce nitrogen content (Doiron *et al.*, 2014) in plant tissues. The few studies available for adult seagrasses have found an increase in leaf C/N with temperature, which has been linked to an increase in shoot growth and a reduction of the N pools even under stressful temperatures (Kaldy, 2014). In young seedlings, the seed provides nutrients to the rest of the plant during the first 6-8 months of development (Balestri *et al.*, 2009). In our experiment, N and P in seeds and P content in roots exhibited the strongest decrease at the lowest temperature (25 ºC). Seedlings from this treatment had higher leaf biomass and total root length, and thus the nitrogen and phosphorus of the seed may have been devoted to the development and/or maintenance of these tissues, leading to an overall lower seed nutrient content.

In addition to the direct negative effects that we have observed with warming on seedling traits, warming may further stimulate stronger top-down control of seedlings by modifying plant-herbivore interactions. Optimal defense theory (ODT) postulates that tissues or life stages contributing more to plant fitness are better defended (McKey, 1979; Rhoades, 1979). Evidence of ODT for seagrasses can be found when comparing leaves of different ages as. For example, in adult shoots of *P. oceanica* the younger and more metabolically active leaves are better defended than old ones (Cariello & Zanetti, 1979). Furthermore, leaves of seedlings have a higher concentration of chicoric acid (a feeding deterrent against sea urchins; Zanetti, 1979) than leaves from adult plants of *P. oceanica* (Cuny *et al.*, 1995; Haznedaroglu & Zeybek, 2007).

Warming tends to decrease the content of leaf phenols in terrestrial plants (Zvereva & Kozlov, 2006; Stark *et al.*, 2015) and seaweeds (Mannino *et al.*, 2016), and it has been attributed to their polymerisation into other chemical forms (Koivikko *et al.*, 2008). We did not observe changes in phenol content with temperature of seedling tissues except in seeds, which exhibited higher phenol content at 29 ºC compared to controls. Seeds are a critical organ for seedling fitness, providing nutrients and carbohydrate storage (Balestri *et al.*, 2009) and performing photosynthesis (Celdran *et al.*, 2015), and thus may be preferentially defended in comparison to other tissues, following ODT, especially under conditions of stress such as warming. Seagrass seeds can suffer consumption from multiple generalist consumers such as fishes, crustaceans, and sea urchins (e.g. Fishman & Orth, 1996; Nakaoka, 2002; Orth *et al.*, 2002, Tomas personal observation), and higher investment of chemical defenses would decrease their susceptibility to being consumed. In addition to their function as feeding deterrents, phenols may offer further protection to seeds against environmental stressors (e.g., UV radiation, heavy metals; Ferrat *et al.*, 2003; Zidorn, 2016).

As quantitative defenses, fibers also provide resistance against herbivory by reducing the digestibility to herbivores (Lincoln, 1993), contributing to increasing mechanical strength and leaf toughness (Coley, 1983), and reducing the preference for high fiber tissues (Mattson, 1980; Mariani & Alcoverro, 1999). As for many other metabolic processes, there is an optimum temperature for fiber formation (Rowell & Stout, 2006; Zhou *et al.*, 2009), and environmental conditions (such as temperature) during growth influence the structural (microfibrillar angle, crystallinity, defects) and chemical composition of fibers (Bourmaud *et al.*, 2013). All these traits influence the mechanical behavior of the fibers and, as a consequence, their function of defense against herbivores (Lucas, 2000). Accordingly, the decrease in fiber production and the lower thickness observed in leaves growing under increased temperatures likely influenced leaf palatability, enhancing their susceptibility to herbivores.

Indeed, we found a tendency of increased seedling leaf palatability with increasing temperature. While we did not detect statistically significant preferences in the three-choice experiments conducted at 27 ºC and 29 ºC, we observed a tendency by sea urchins to consume less of the tissue grown at 25 ºC (around 20-30 % of the total amount consumed) than plants growing at higher temperatures (i.e., in all experiments consumption of tissue grown at

temperatures higher than 25 ºC encompassed 70 to 80 % of the total consumption) in both the three-choice and two-choice experiments.

This feeding behavior is in concordance with observed changes in plant traits that influence palatability, e.g., fiber content and leaf thickness decreased from 25 ºC to 27 ºC and 29 ºC. Overall, feeding preferences (i.e., preference towards high temperature tissue) were consistent throughout the different water temperatures at which sea urchins were feeding (although they were more conspicuous in sea urchins kept at 25 ºC), indicating that feeding preference of sea urchins is not remarkably affected by warming. Higher palatability under warming (presumably from loss of structural defenses) would likely be synergistic with the increasing feeding rates of herbivores due to increased metabolic requirements at higher temperatures (e.g., Burnell *et al.*, 2013; Lemoine *et al.*, 2014), therefore making plants further susceptible to grazing impact. Thus, under future warming scenarios, seagrass seedlings will not only undergo negative direct effects on their physiology that lead to higher mortality and reduced reserves to resist stressful conditions, but also become more susceptible to grazing as top-down control of seedlings strengthens. Together, these effects could seriously compromise seagrass seedling recruitment success and survival. Through hindering the provisioning of new genetic material, and thus their adaptive potential as well as the ability for colonizing new areas, warming would endanger seagrass populations.

# 4 General Discussion

The results of this thesis have been discussed in detail in each chapter. Therefore, this discussion aims to summarize the general findings and comment on some knowledge gaps and possibilities for future research.

Many studies have focused on herbivory in order to explain part of the complexity underlying ecosystem functioning. Most of this work has been performed in terrestrial plants. Nevertheless, despite the physiological adaptations and different selective pressures of marine plants to underwater life, the general ecological theories on plant defense against herbivory explain seagrass responses to changes in resource allocation, cost of defense, and risk of defense. It is now widely accepted that plants present a mixed defense strategy based in tolerance and resistance traits (Carmona & Fornoni, 2013) and some studies before this thesis have explored some of the tolerance and resistance traits present in seagrasses. This thesis provides a step forward in better understanding the mechanisms that drive changes in resistance and tolerance traits, and focuses largely on how these mechanisms change under different environmental conditions. Moreover, this thesis also explores how the changes in defense mechanisms determine plant susceptibility to herbivory.

## 4.1 Resource availability and plant defenses against herbivory

Seagrasses are vascular plants that evolved from terrestrial plants and recolonized the sea (Larkum *et al.*, 2006). Submerged life has led to differences in the acquisition resources, such as absorption of nutrients by the leaves (Touchette & Burkholder, 2000a) and differences in the type and availability of limiting resources such as light, nutrients or $CO_2$. In the first chapter of this thesis we explore the effects of resource availability as an underlying factor of latitude. Productivity of kelps appears to be driven by SST, seawater nutrients, and irradiance (Graham *et al.*, 2016). Our study with the circumglobal seagrass *Zostera marina* indicates similar trends. Indeed, eelgrass was generally larger, with higher growth rate and more carbohydrate resources in mid- high-latitude ranges, coincident with upwelling of nutrient-rich waters and longer daylight duration during the experimental period. In addition to being an important factor in plant productivity, resource availability drives allocation patterns to defense strategies, by

increasing allocation to tolerance (i.e. Limiting Resource Model (LRM) ; Maschinski & Whitham, 1989; Wise & Abrahamson, 2005) or induced resistance (i.e. Resource Availability Hypothesis (RAH); Coley *et al.*, 1985). In fact, as explained in the introduction of this thesis, many defense-related theories explore the effects of resource availability on plant defense against herbivory. Thus, both in chapters one and two we delve into the relation of resource availability (e.g. nutrients) and defense strategies. In both chapters we find that seagrasses respond to higher resource availability by decreasing their investment in constitutive resistance traits (e.g., fiber) in addition to increasing their nitrogen content, in accordance with the predictions of the RAH (Coley *et al.*, 1985). On the other hand, the results of the two studies differed regarding plant allocation to tolerance defenses under higher nutrient availability. In mid-latitudinal ranges, where nutrient-rich waters in the upwelling areas were likely providing all the necessary nutrient resources, *Z. marina* increased allocation to tolerance exhibiting higher rhizome biomass, sucrose and nitrogen content. However, in chapter two, fertilization may have not provided all the limiting resources needed for *P. oceanica* since phosphorus may have been trapped by particles hindering absorption by plant roots (Touchette & Burkholder, 2000a). Indeed, and as predicted by the LRM(Wise & Abrahamson, 2007), which suggests that when a limiting resource is lacking there is no increased tolerance, we found no allocation of resources to tolerance in this experiment, even when leaf removal rates were high. Regarding belowground storage, we observed different responses in the two experiments. According to previous studies (Hay *et al.*, 2011), the reduction of leaf surface would hinder C fixation and nitrogen absorption, limiting the allocation of resources to tolerance. Whereas in the experiment with *Zostera marina* high herbivory levels reduced belowground biomass and stored resources (i.e. sucrose, carbon), in *P. oceanica* high herbivory levels induced storage of sucrose in rhizomes. Furthermore, in the intermediate herbivory level there was an increase in leaf sucrose and in the number of leaves per shoot. Plants in both experiments responded to simulated herbivory by undergoing changes in many traits that resulted in changes in palatability. However, the two species responded differently. While herbivore damage in *Z. marina* increased leaf nutritional quality (i.e. nitrogen content) and did not induce the production of resistance traits such as phenol and fibers, in the experiment with *Posidonia oceanica*, simulated herbivory did induce the production of resistance traits. These changes in plant traits led to different herbivore preferences; while the most preferred *Z. marina* plants were those that had been repeatedly clipped (which were more

nutritious and were not defended), herbivores preferred the more nutritious and less chemically and structurally defended unclipped plants of *P. oceanica*.

The different responses in defense strategies can be the related to the different life strategies of both species. *P. oceanica* being a k-strategist has longer lived meristems and slower growth than Z. marina. Moreover, *P. oceanica* inhabits an oligotrophic sea. Hence, the cost of tissue replacement in this species is likely higher than in *Z. marina,* increasing then the investment of *P. oceanica* in resistance strategies in response to herbivore damage.

Resource availability has a strong influence on seagrass defensive traits, decreasing plant resistance and also modifying tolerance. However, both herbivory pressure and limiting resources modulate and can shift plant defense responses, and this suite of strategies appear to be species-specific. Since usually there are multiple herbivores feeding on the same plant, changes in herbivore pressure due to variations in herbivore populations or feeding rates may indirectly affect other herbivores within the same community by modifying plant traits.

## 4.2   Human-mediated environmental changes and plant defenses against herbivory

Since increased nutrient availability has been discussed in the previous section, here I will discuss the two last chapters of the thesis which focus on two global climate change topics: seawater acidification (or carbonification) and warming. Increases in $CO_2$ availability and temperature triggered opposite responses regarding plant physiology and defense traits. As explained in chapter three, seagrasses are carbon limited due to the lower availability of dissolved inorganic carbon in seawater (Koch *et al.*, 2013). Accordingly, increased carbon availability enhanced photosynthetic activity reducing the utilization of carbohydrate reserves from the seeds and therefore enhanced their capacity to tolerate or resist further stress such as defoliation by herbivory. Furthermore, seedlings from the $CO_2$-enriched treatment allocated the excess carbon to accumulating carbon-based defenses (i.e. phenolic compounds) in the seeds. The fact that phenolic compounds were stored in seeds rather than in leaves highlights their importance to plant fitness, as the Optimal Defense Theory postulates (McKey, 1979; Rhoades, 1979). Additionally, and as found in other studies, the leaves of these seedlings exhibited lower nitrogen content (Zvereva & Kozlov, 2006), which could reduce preference and performance of herbivores towards these tissues (Valentine & Heck, 2001; Hemmi & Jormalainen, 2002),  but that on the other hand could also stimulate compensatory feeding, increasing feeding rates

(Cruz-Rivera & Hay, 2000). While $CO_2$ would appear to benefit seagrass seedlings with increased tolerance capacity and presumably less palatable tissues, we actually observed that seedlings from the high $CO_2$ treatment were preferred by herbivores, despite that most of the leaf traits that we analyzed would suggest higher palatability of plants growing under control conditions (i.e. higher nitrogen). This preference for high $CO_2$ plants indicates that other factors are also contributing to plant palatability, such as specific fiber composition, leaf breaking strength (Lucas, 2000) or sucrose content (Le Gall & Behmer, 2014); and sucrose was indeed higher in the high $CO_2$ treatment.

In contrast to increased $CO_2$, warming negatively affected seagrass seedlings. Due to increased metabolic demands, seedlings had higher respiration rates and lower reserves in seeds. Additionally, these plants had lower constitutive resistance (i.e. fiber, thickness) potentially increasing their susceptibility to herbivory. Indeed, herbivores preferred leaves from seedlings under high temperature, and this would likely further exacerbate the negative effects of warming in seagrasses.

The results of these last two chapters emphasize the importance of using integrative approaches in the study of herbivore defenses in seagrasses since changes in plant traits may result in unexpected feeding preferences. Furthermore, the results of these experiments highlight the potential of additive effects that herbivory will have in ultimately determining plant success under different stressors.

## 4.3 Knowledge gaps and further research

### *Chemical traits*

Secondary compounds are important drivers of herbivore feeding preferences (e.g., Hay & Steinberg, 1992; Steinberg, 2006; Lattanzio *et al.*, 2009). Additionally, they have important protective functions such as protection against pathogens, UV-radiation or preventing growth or germination of competitors (Lattanzio *et al.*, 2009). Furthermore, in terrestrial plants, airborne cues (e.g., volatile organic compounds) attract pollinators and prime inducible defense after herbivore damage in neighbor plants (Cape, 2003; Frost *et al.*, 2008). Seagrasses seem to have less secondary compounds than terrestrial plants (Zidorn, 2016). According to the Apparency Theory (Feeny, 1976) a lower investment in qualitative defenses (e.g., alkaloids) is related to the

higher size or abundance of the plants that make them more apparent and easily found. Indeed, seagrasses typically form extensive meadows, and many seagrass species reach big sizes and are in general easy to find by herbivores. Being thus apparent species, we would expect that they may invest more in quantitative defenses (e.g., fibers) which are effective against all herbivores, generalist or specialists (Feeny, 1976) and less in qualitative defenses. Indeed, seagrasses have low contents and variety of alkaloids (Zidorn, 2016). However, there are few studies exploring both qualitative and quantitative defenses in seagrasses.

Few works have explored water-borne cues in marine macrophytes (Toth & Pavia, 2000; Yun *et al.*, 2012), showing that these cues tend to prime resistance responses on macrophytes. Water-borne cues could be equivalent to volatile compounds in terrestrial plants, which have multiple functions. In addition to priming plant inductive responses (Frost *et al.*, 2008), volatile compound also attract pollinators (Farré-Armengol *et al.*, 2015) and herbivore predators (Peñuelas & Llusia, 2004) and examining how water-borne cues are involved in some of these ecological processes is indeed an interesting way forward.

In order to forecast the impacts of future environmental and biotic changes related to climate change (e.g., herbivore range shifts due to warming, increased $CO_2$) in seagrass ecosystems, we need to explore new topics on plant-herbivore interactions linking chemical and ecological knowledge, since most of plant communication and defense relies on chemical cues (Blande & Glinwood, 2016) and those may change in response to environmental changes (Zavala *et al.*, 2013).

### *Herbivory and demographic processes*

Herbivory processes in early life stages and its interference with sexual reproduction have been thoroughly studied in terrestrial plants (Johnson *et al.*, 2009, 2015). Early life stages in plants (e.g. seedlings) are a very vulnerable period in which the plant loses the protection of the seed coat and has not achieved the strength of the adult. Studies exploring plant-herbivore interactions in early life stages (i.e. seeds and seedlings) have illustrated the critical ecological and evolutionary effects of herbivore pressure on these stages (Barton & Hanley, 2013). In fact, it is in these phases in which most of plant mortality typically occurs (Lambers *et al.*, 2008b). Furthermore, plant propagules are critical in providing a new genetic variation pool and in contributing to species expansion processes. Therefore, the outcome of the plant-herbivore

interactions in these stages will have the most critical effects on plant populations (Watkinson, 1997) shaping composition and structure of plant communities (Barton & Hanley, 2013), being crucial in plant demographic processes as well as in plant evolution (Barton & Boege, 2017).

The process of sexual reproduction is highly variable in terms of frequency and success among seagrass species (Orth *et al.*, 2006b). Studies have found that seagrass seeds can suffer high predation pressure (Nakaoka, 2002; Orth *et al.*, 2006b, 2007) and flowers are also consumed (Vergés *et al.*, 2007b; van Tussenbroek & Muhlia-Montero, 2013). In my thesis I have examined how different abiotic factors related to climate change may modify plant-herbivore interactions on seagrass seedlings, but the effects of herbivory on seagrass seedling defense traits and survival remain scarcely explored. Thus, I have examined this topic during my thesis in an experiment on the effects of simulated herbivory in *P. oceanica* seedlings resulting in a work which is now in preparation but not included here.

Furthermore, herbivory in terrestrial plants can induce compensatory responses such as increased flowering (e.g., Freeman *et al.*, 2003; Huhta *et al.*, 2009; Brody & Irwin, 2012; Schiestl *et al.*, 2014). While there are still few works on this topic on seagrasses, existing evidence suggest that herbivory pressure may be a mechanism of flowering induction (Planes *et al.*, 2011). The impact of herbivory on seagrass demography in relation to sexual reproduction and its evolutionary consequences remains almost unexplored and its understanding is crucial for management of seagrass populations.

### *Herbivore responses*

In the puzzle of plant-herbivore interactions most works analyze changes in plant traits or allocation to different strategies. However, understanding how herbivores respond to these changes is the other half of the puzzle. There are many factors involved in the responses of herbivores to plant traits. For example, different herbivore species or guilds may respond differently to changes plant traits (e.g., Peeters, 2002; Caldwell *et al.*, 2015), and this also may be the case in seagrasses (Vergés *et al.*, 2007a; Tomas *et al.*, 2015; Martínez-Crego *et al.*, 2016). Thus, changes in some plant traits may affect differently the community of herbivores within the same ecosystem. Moreover, as illustrated in all the chapters of this thesis, it is important to consider feeding behavior in order to extrapolate conclusions on how changes in plant traits effectively determine herbivore damage.

As explained in the introduction, the coevolution of herbivores and plants illustrates the importance of their relationship. Like plants, herbivores have also evolved mechanisms to obtain food despite the arsenal of plant defenses (Karban & Agrawal, 2002), since plant traits greatly affect herbivore performance (Kant *et al.*, 2015). A challenging step forward in the outcome of this thesis could be linking the changes on plant traits and herbivore preference to herbivore performance. Some works have focused on this topic (e.g., Hemmi & Jormalainen, 2002; Jormalainen *et al.*, 2005; Tomas *et al.*, 2011; Robinson *et al.*, 2012); however, a mechanistic understanding of the drivers of these changes remains to be obtained.

*Meta-analytical reviews*

There are many works analyzing herbivore defenses in plants. Specific topics such as effects of environmental changes in defense traits, natural patterns in defense or the effect of different traits in plant palatability need to be summarized in order to get a general idea and a better understanding of general patterns. Thus, in a manuscript in preparation not included in this thesis, I am using metanalytical methods to assess the effect of increased $CO_2$ and warming in plant traits and photophysiology. The use of systematic reviews and specifically meta-analytical methods allow us to aggregate information from individual studies leading to more robust conclusions. In addition, meta-analysis results can be generalized to larger populations and also allows us to investigate the presence of publication bias. It is challenging to follow objective criteria with standardized methods when individual studies have incomplete data or different methodologies. However, by increasing the number of studies included in the meta-analysis we increase the robustness of our estimates. Therefore, research efforts need to be made in increasing underexplored topics and summarizing existing knowledge.

*Plant-herbivore interactions: insights into ecosystem responses*

There is a compelling need for using integrative approaches in plant studies especially when forecasting the effects of future environmental changes. Many works on this topic focus on plant responses to these changes (e.g., Campbell *et al.*, 2006; Arnold *et al.*, 2012; Campbell & Fourqurean, 2013; Russell *et al.*, 2013; Borum *et al.*, 2016; Cox *et al.*, 2016; Takahashi *et al.*, 2016). Yet, herbivores and plant-herbivore interactions play a crucial role in plant life and in community structure (Gordon, 2006; Poore *et al.*, 2012), especially when affecting habitat-forming species and thus the study of plant-herbivore interactions needs to be included, as it

provides critical insight into the responses of ecological communities to future changes or stressors. Moreover, the effects of global change on herbivores modifies the strength of top-down processes in the ecosystem (O'Connor, 2009; Burrows *et al.*, 2011; West & Post, 2016), highlighting the need of considering plant-herbivore interactions when forecasting the effects of global change in ecosystem structure. Indeed, recent studies about effects of global change in seagrasses are already integrating this plant-herbivore interaction approach (e.g., Burnell *et al.*, 2013; Garthwin *et al.*, 2014; Tomas *et al.*, 2015).

# 5 Conclusions

Nutrient availability has important effects on the chemical and morphological traits of seagrasses. High nutrient availability when all limiting resources are provided increases seagrass tolerance traits. However, when a limiting resource is lacking or herbivory induces the limitation of an alternative resource, tolerance diminishes in accordance with the limiting resource model. In addition, high resource availability increases nutritional quality and reduces fiber content reducing constitutive resistance as predicted by the resource availability hypothesis. This lower constitutive resistance can make these plants more vulnerable to consumption by herbivores since high nutrient availability did not enhance the induction of resistance or tolerance traits in response to herbivory damage.

The simulation of herbivory damage has clear effects in both chemical and morphological plant traits. However, the two species studied varied in their responses. In *Posidonia oceanica*, herbivory damage induced the production of resistance traits such as fiber or secondary metabolites, at all herbivory levels. In *Zostera marina* herbivory pressure did not induce resistance traits reducing in fact its resistance in some populations. Herbivores responded to these changes in plant traits by preferring the more nutritious repeatedly clipped leaves of *Z. marina* and the uncut leaves of *P. oceanica* which had fewer fibers and more nutrients. Plants of *Z. marina* were highly tolerant to moderate herbivory pressures likely due to the selective pressure that herbivory, mainly by waterfowl, has had in the populations studied. In *P. oceanica* plants under moderate herbivory exhibited some tolerance traits such as the increase leaf number and leaf sucrose, thus suggesting a mixed defense strategy in this species.

Latitudinal patterns greatly influence plant defense traits due to underlying factors such as nutrient $CO_2$ and light availability. Northern eelgrass populations exhibited a higher sensitivity to herbivory which can increase the vulnerability of these populations to due to changes in the community of herbivores or the increase in feeding rates expected by warming.

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## 5. Conclusions

The increase in dissolved $CO_2$ concentrations in seawater increases the photosynthetic activity of *P. oceanica* seedlings and its carbohydrate reserves in seeds. In addition, it diminishes the nutritional quality of the leaves making them predictably less preferred by herbivores. However, leaves under acidification were preferred by herbivores, suggesting that sucrose leaf content, which was higher in these seedlings, can be an important factor affecting feeding preference. Therefore, positive effects of higher CO2 on seagrass may be counteracted by enhanced feeding pressure on these plants.

Warming produced clear negative effects on seedlings, increasing mortality and respiration, and leading to increased use of seed reserves. In addition, warming decreased seedling resistance to herbivores by decreaseing fiber production, which made the plants more vulnerable to herbivory. Thus, negative effects of warming on seagrass may be further enhanced by enhanced susceptibility to being consumed.

The results of this thesis highlight the importance of considering species interactions when assessing the effects of changes in environmental factors on plant populations. Herbivore pressure can counterbalance the positive effects observed or represent an increased vulnerability associated with the negative effects exhibited by marine plants in response to changes in environmental factors.

# 6 References

Abe M, Kurashima A, Maegawa M (2008) High water-temperature tolerance in photosynthetic activity of *Zostera marina* seedlings from Ise Bay, Mie Prefecture, central Japan. *Fisheries Science*, **74**, 1017–1023.

Abe M, Yokota K, Kurashima A, Maegawa M (2009) High water temperature tolerance in photosynthetic activity of Zostera japonica Ascherson & Graebner seedlings from Ago Bay, Mie Prefecture, central Japan. *Fisheries Science*, **75**, 1117–1123.

Adams JM, Zhang Y (2009) Is there more insect folivory in warmer temperate climates? A latitudinal comparison of insect folivory in eastern North America. *Journal of Ecology*, **97**, 933–940.

Agrawal AA (2000) Overcompensation of plants in response to herbivory and the by-product benefits of mutualism. *Tend in plant science*, **5**, 309–313.

Agrawal AA (2011) Current trends in the evolutionary ecology of plant defence. *Functional Ecology*, **25**, 420–432.

Agrawal AA, Karban R (1999) Why induced defenses may be favored over constitutive strategies in plants. In: *The ecology and evolution of inducible defenses* (eds Tollrian R, Harwell CD), pp. 45–61. Princeton University Press, Princeton.

Agrawal AA, Rudgers JA, Botsford LW et al. (2000) Benefits and constraints on plant defense against herbivores : Spines influence the legitimate and Iillegitimate flower visitors of Yellow star thistle Centaurea solstitialis L. (Asteraceae). *The Southwestern Naturalist*, **45**, 1–5.

Agrawal AA, Hastings AP, Johnson MTJ, Maron JL, Salminen J (2012) Insect herbivores drive real-time ecological and evolutionary change in plant populations. *Science*, **338**, 113–116.

Alagna A, Vega Fernndez T, Giovanni DA, Magliola C, Mazzola S, Badalamenti F (2015) Assessing *Posidonia oceanica* seedling substrate preference: An experimental determination of seedling anchorage success in rocky vs. sandy substrates. *PLoS ONE*, **10**, 1–15.

## 6. References

Alcoverro T, Duarte CM, Romero J (1995) Annual growth dynamics of Posidonia oceanica: Contribution of large-scale versus local factors to seasonality. *Marine Ecology-Progress Series*, **120**, 203–210.

Alcoverro T, Romero J, Duarte CM, Lopez NI (1997) Spatial and temporal variations in nutrient limitation of seagrass *Posidonia oceanica* growth in the NW Mediterranean. *Marine Ecology-Progress Series*, **146**, 155–161.

Alcoverro T, Manzanera M, Romero J (2000) Nutrient mass balance of the seagrass *Posidonia oceanica*: The importance of nutrient retranslocation. *Marine Ecology Progress Series*, **194**, 13–21.

Alexandre A, Silva J, Buapet P, Björk M, Santos R (2012) Effects of CO2 enrichment on photosynthesis, growth, and nitrogen metabolism of the seagrass *Zostera noltii* . *Ecology and Evolution*, **2**, 2625–2635.

Allen AP, Gillooly JF, Brown JH (2005) Linking the global carbon cycle to individual metabolism. *Functional Ecology*, **19**, 202–213.

Allgeier JE, Yeager LA, Layman CA (2013) Consumers regulate nutrient limitation regimes and primary production in seagrass ecosystems. *Ecology*, **94**, 521–529.

Andrew RL, Wallis IR, Harwood CE, Henson M, Foley WJ (2007) Heritable variation in the foliar secondary metabolite sideroxylonal in Eucalyptus confers cross-resistance to herbivores. *Oecologia*, **153**, 891–901.

Anholt BR, Werner EE (1998) Predictable changes in predation mortality as a consequence of changes in food availability and predation risk. *Evolutionary Ecology*, **12**, 729–738.

Anstett DN, Nunes KA, Baskett C, Kotanen PM (2016a) Sources of controversy surrounding latitudinal patterns in herbivory and defense. *Trends in Ecology and Evolution*, **31**, 789–802.

Anstett DN, Chen W, Johnson MTJ (2016b) Latitudinal gradients in induced and constitutive resistance against herbivores. *Journal of Chemical Ecology*, **42**, 772–781.

Apostolaki ET, Vizzini S, Hendriks IE, Olsen YS (2014) Seagrass ecosystem response to long-term high CO2 in a Mediterranean volcanic vent. *Marine Environmental Research*, **99**, 9–15.

Arnold TM, Tanner CE, Rothen M, Bullington J (2008) Wound-induced accumulations of

condensed tannins in turtlegrass, *Thalassia testudinum*. *Aquatic Botany*, **89**, 27–33.

Arnold T, Mealey C, Leahey H, Miller WA, Hall-Spencer JM, Milazzo M, Maers K (2012) Ocean acidification and the loss of phenolic substances in marine plants. *PLoS ONE*, **7**, e35107.

Arnold T, Freundlich G, Weilnau T, Verdi A, Tibbetts IR (2014) Impacts of groundwater discharge at Myora Springs (North Stradbroke Island, Australia) on the phenolic metabolism of eelgrass, *Zostera muelleri* , and grazing by the Juvenile Rabbitfish, Siganus fuscescens. *PLoS ONE*, **9**, e104738.

Augier H, Robert P, Maffre R (1980) Étude du régime thermique annuel des eaux au niveau des peuplements de phanérogames marines de la baie de Port-Cros (îles d´hyeres, Mediterranee, France). *Trav. Sci. Parc Natl Port-Cros*, **6**, 69–131.

Bakker ES, Wood KA, Pagés JF et al. (2016) Herbivory on freshwater and marine macrophytes: A review and perspective. *Aquatic Botany*, **135**, 18–36.

Bakun A (1990) Global climate change and instensification of coastal ocean upwelling. *Science*, **247**, 198–201.

Balestri E (2004) Flowering of the seagrass *Posidonia oceanica* in a north-western Mediterranean coastal area: temporal and spatial variations. *Marine Biology*, **145**, 61–68.

Balestri E, Cinelli F (2003) Sexual reproductive success in *Posidonia oceanica* . *Aquatic Botany*, **75**, 21–32.

Balestri E, Gobert S, Lepoint G, Lardicci C (2009) Seed nutrient content and nutritional status of *Posidonia oceanica* seedlings in the northwestern Mediterranean Sea. *Marine Ecology Progress Series*, **388**, 99–109.

Barnes CA, Duxbury AC, Morse B. (1972) Circulation and selected properties of the Columbia River effluent at sea. In: *The Columbia River Estuary and Adjacent Ocean Waters* (eds Pruter AT, Alverson DL), pp. 41–80. University of Washington Press, Seattle, WA.

Barton KE, Boege K (2017) Future directions in the ontogeny of plant defence: understanding the evolutionary causes and consequences. *Ecology Letters*, 403–411.

Barton KE, Hanley ME (2013) Seedling-herbivore interactions: Insights into plant defence and regeneration patterns. *Annals of Botany*, **112**, 643–650.

## 6. References

Bazzaz FA (1997) Allocation of resources in plants: state of the science and critical questions. In: *Plant Resource Allocation* (eds Bazzaz FA, Grace J), pp. 1–30. Elsevier.

Bazzaz FA, Grace J (1997) *Plant Resource Allocation* (eds Bazzaz FA, Grace J). Elsevier.

Bazzaz F a, Chiariello NR, Coley PD, Pitelka LF (1987) Allocating resources to reproduction and defense. *BioScience*, **37**, 58–67.

Becerra JX (2007) The impact of herbivore–plant coevolution on plant community structure. *Proceedings of the National Academy of Sciences*, **104**, 7483–7488.

Beer S, Vilenkin B, Weil A, Veste M, Susel L, Eshel A (1998) Measuring photosynthetic rates in seagrasses by pulse amplitude modulated (PAM) fluorometry. *Marine Ecology Progress Series*, **174**, 293–300.

Beer S, Björk M, Gademann R, Ralph P (2001) Measurements of photosynthetic rates in seagrasses. In: *Global Seagrass Research Methods* (eds Short F, Coles RG), pp. 183–198. Elsevier B.V., Amsterdam.

Beer S, Bjork M, Hellblom F, Axelsson L (2002) Inorganic carbon utilisation in marine angiosperms (seagrasses). *Functional Plant Biology*, **29**, 349–354.

Benlloch-Tinoco M, Carranza-Concha J, Camacho MM, Martinez-Navarrete N (2015) Production of raisins and its impact on active compounds. In: *Processing and Impact on Active Components in Food*, 1st edn (ed Preedy V), pp. 181–187. Elsevier Inc., Oxford, UK.

Bennett BYRN, Wallsgrove RM (1994) Secondary metabolites in plant defence mechanisms. *New Phytologist*, **72**, 617–633.

Berenbaum M (1983) Coumarins and Caterpillars: A case for coevolution. *Evolution*, **37**, 163–179.

Bernays EA, Chapman RF (1994) *Host-plant selection by phytophagous insects*. Chapman & Hall, New York, 312 pp.

Bezemer TM, Jones TH (1998) Plant-insect herbivore interactions in elevated atmospheric CO2: Quantitative analyses and guild effects. *Oikos*, **82**, 212–222.

Bidart-Bouzat MG, Mithen R, Berenbaum MR (2005) Elevated CO2 influences herbivory-induced

## 6. References

defense responses of *Arabidopsis thaliana*. *Oecologia*, **145**, 415–424.

Bintz JC, Nixon SW, Buckley BA, Granger SL (2003) Impacts of temperature and nutrients on coastal lagoon plant communities. *Estuaries*, **26**, 765–776.

Bjorndal KA (1985) Nutritional ecology of sea turtles. *Copeia*, **3**, 736–751.

Blake RE, Duffy JE (2012) Changes in biodiversity and environmental stressors influence community structure of an experimental eelgrass *Zostera marina* system. *Marine Ecology Progress Series*, **470**, 41–54.

Blande JD, Glinwood R (2016) *Deciphering Chemical Language of Plant Communication* (eds Blande JD, Glinwood R). Springer, 326 pp.

Bloom AJ, Chapin FS, Mooney HA (1985a) Plants-an economic analogy. *Annual Review of Ecology and Systematics*, **16**, 363–392.

Bloom AJ, Chapin FS, Mooney HA (1985b) Resource limitation in plants- an economic analogy. *Annual Review of Ecology and Systematics*, 363–392.

Blum U (1997) Benefits of citrate over EDTA for extracting phenolic acids from soils and plant debris. *Journal of Chemical Ecology*, **23**, 347–362.

De Boer WF (2007) Seagrass-sediment interactions, positive feedbacks and critical thresholds for occurrence: A review. *Hydrobiologia*, **591**, 5–24.

Bolser RC, Hay ME (1996) Are tropical plants better defended? Palatability and defenses of temperate vs. tropical seaweeds. *Ecology*, **77**, 2269–2286.

Bolser RC, Hay ME, Lindquist N (1998) Chemical defenses of freshwater macrophytes against crayfish herbivory. J Chem Ecol 24:1639–16. *Journal of chemical ecologyof che*, **24**, 1639–1658.

Borer AET, Seabloom EW, Shurin JB, Anderson KE, Blanchette CA, Cooper SD, Halpern BS (2005) What determines the strength of a trophic cascade? *Ecology*, **86**, 528–537.

Borowitzka MA, Lethbridge RC (1989) Seagrass epiphytes. In: *Seagrasses: With Special Reference to the Australasian Region* (eds Larkum AWD, McComb AJ, Shepherd SA), pp. 458–499. Elsevier Ltd, Amsterdam.

## 6. References

Borowitzka MA, Lavery P, van Keulen M (2006) Epiphytes of Seagrasses. In: *Seagrasses: Biology, Ecology and Conservation* (eds Larkum A, Orth RJ, Dua), pp. 441–461. Springer.

Borum J, Pedersen O, Kotula L, Fraser MW, Statton J, Colmer TD, Kendrick GA (2016) Photosynthetic response to globally increasing $CO_2$ of co-occurring temperate seagrass species. *Plant, Cell & Environment*, **39**, 1240–1250.

Boudouresque CF, Verlaque M (2001) Ecology of Paracentrotus lividus. *Developments in Aquaculture and Fisheries Science*, **32**, 177–216.

Boudouresque CF, Bernard G, Pergent G, Shili A, Verlaque M (2009) Regression of Mediterranean seagrasses caused by natural processes and anthropogenic disturbances and stress: A critical review. *Botanica Marina*, **52**, 395–418.

Bourmaud A, Morvan C, Bouali A, Placet V, Perré P, Baley C (2013) Relationships between micro-fibrillar angle, mechanical properties and biochemical composition of flax fibers. *Industrial Crops and Products*, **44**, 343–351.

Bowes G, Ogren WL, Hageman RH (1971) Phosphoglycolate production catalyzed by ribulose diphosphate carboxylase. *Biochemical and biophysical research communications*, **45**, 716–722.

Briske DD (1996) Strategies of plant survival in grazed systems : A functional interpretation. In: *The Ecology and Management of Grazing Systems* (eds Hodgson J, Illius AW), pp. 37–67. CAB International.

Brody AK, Irwin RE (2012) When resources don't rescue: Flowering phenology and species interactions affect compensation to herbivory in *Ipomopsis aggregata*. *Oikos*, **121**, 1424–1434.

Brown MB, Edwards MS, Kim KY (2014) Effects of climate change on the physiology of giant kelp, *Macrocystis pyrifera*, and grazing by purple urchin, *Strongylocentrotus purpuratus*. *Algae*, **29**, 203–215.

Bruno JF, Bertness MD (2000) Habitat modification and facilitation in benthic marine communities. *Marine Community Ecology*, 201–218.

Bruno JF, Stachowicz JJ, Bertness MD (2003) Inclusion of facilitation into ecological theory.

## 6. References

*Trends in Ecology and Evolution*, **18**, 119–125.

De Bruyn L, Scheirs J, Verhagen R (2002) Nutrient stress, host plant quality and herbivore performance of a leaf-mining fly on grass. *Oecologia*, **130**, 594–599.

Bryant JP, Chapin FS, Klein DR (1983) Carbon nutrient balance of boreal plants in relation to vertebrate herbivory. *OIKOS*, **40**, 357–368.

Buchsbaum R, Valiela I, Swain T (1984) The role of phenolic compounds and other plant constituents in feeding by Canada geese in a coastal marsh. *Oecologia*, **63**, 343–349.

Burghardt KT, Schmitz OJ (2015) Influence of plant defenses and nutrients on trophic control of ecosystems. In: *Trophic Ecology: Bottom-Up and Top-Down Interactions across Aquatic and Terrestrial Systems* (eds Hanley TC, La Pierre KJ), pp. 203–232. Cambridge University Press.

Burkepile DE, Hay ME (2006) Herbivore vs. nutrient control of marine primary producers: context-dependent effects. *Ecology*, **87**, 3128–39.

Burkepile DE, Hay ME (2008) Herbivore species richness and feeding complementarity affect community structure and function on a coral reef. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 16201–16206.

Burkholder DA, Heithaus MR, Fourqurean JW, Wirsing A, Dill LM (2013) Patterns of top-down control in a seagrass ecosystem: Could a roving apex predator induce a behaviour-mediated trophic cascade? *Journal of Animal Ecology*, **82**, 1192–1202.

Burnell OW, Russell BD, Irving AD, Connell SD (2013) Eutrophication offsets increased sea urchin grazing on seagrass caused by ocean warming and acidification. *Marine Ecology Progress Series*, **485**, 37–46.

Burnham KP, Anderson DR (2002) *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*, Second edn, Vol. 172 (eds Burnham KP, Anderson DR). Springer, 488 pp.

Burrows MT, Schoeman DS, Buckley LB et al. (2011) The pace of shifting climate in marine and terrestrial ecosystems. *Science*, **334**, 652–655.

Caldwell E, Read J, Sanson GD (2016) Which leaf mechanical traits correlate with insect herbivory among feeding guilds? *Annals of botany*, **117**, 349–361.

## 6. References

Campbell JE, Fourqurean JW (2013) Effects of in situ CO2 enrichment on the structural and chemical characteristics of the seagrass *Thalassia testudinum*. *Marine Biology*, **160**, 1465–1475.

Campbell SJ, McKenzie LJ, Kerville SP (2006) Photosynthetic responses of seven tropical seagrasses to elevated seawater temperature. *Journal of Experimental Marine Biology and Ecology*, **330**, 455–468.

Canadell JG, Le Quéré C, Raupach MR et al. (2007) Contributions to accelerating atmospheric CO2 growth from economic activity, carbon intensity, and efficiency of natural sinks. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 18866–18870.

Cape JN (2003) Effects of airborne volatile organic compounds on plants. *Environmental Pollution*, **122**, 145–157.

Carey N, Harianto J, Byrne M (2016) Sea urchins in a high CO2 world: partitioned effects of body-size, ocean warming and acidification on metabolic rate. *The Journal of Experimental Biology*, **219**, 1178–1186.

Cariello L, Zanetti L (1979) Distribution of Chicoric acid during leaf development of *Posidonia oceanica*. *Botanica Marina*, **22**, 359–360.

Carmona D, Fornoni J (2013) Herbivores can select for mixed defensive strategies in plants. *New Phytologist*, **197**, 576–585.

Carmona D, Lajeunesse MJ, Johnson MTJ, Carolina N (2011) Plant traits that predict resistance to herbivores. *Functional Ecology*, **25**, 358–367.

Carr L a, Bruno JF (2013) Warming increases the top-down effects and metabolism of a subtidal herbivore. *PeerJ*, **1**, 1–15.

Cebrian J (1999) Patterns in the fate of production in plant communities. *The American Naturalist*, **154**, 449–468.

Cebrian J, Duarte CM (1998) Patterns in leaf herbivory on seagrasses. *Aquatic Biology*, **60**, 67–82.

Cebrian J, Lartigue J (2004) Patterns of herbivory and decomposition in aquatic and terrestrial ecosystems. *Ecological Monographs*, **74**, 237–259.

Cebrian J, Shurin JB, Borer ET, Cardinale BJ, Ngai JT, Smith MD, Fagan WF (2009) Producer nutritional quality controls ecosystem trophic structure. *PLoS ONE*, **4**, 1–5.

Celdran D, Lloret J, Verduin J, van Keulen M, Marín A (2015) Linking seed photosynthesis and evolution of the Australian and Mediterranean seagrass genus Posidonia. *Plos One*, **10**, e0130015.

Celis-Plá PSM, Hall-Spencer JM, Horta PA, Milazzo M, Korbee N, Cornwall CE, Figueroa FL (2015) Macroalgal responses to ocean acidification depend on nutrient and light levels. *Frontiers in Marine Science*, **2**.

Chapin FS, Matson PA, Mooney HA (2002) *Principles of Terrestrial Ecosystem Ecology*, First edn, Vol. 21. Springer, 436 pp.

Christensen JH, Hewitson B (2007) Regional climate projections. In: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, Vol. 11 (eds Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL), pp. 847–940. Cambridge, United Kingdom and New York, NY, USA.

Christianen MJ a, Herman PMJ, Bouma TJ et al. (2014) Habitat collapse due to overgrazing threatens turtle conservation in marine protected areas. *Proceedings of The Royal Socienty BThe Royal Society*, **281**, 20132890.

Clarke A (2006) Temperature and the metabolic theory of ecology. *Functional Ecology*, **20**, 405–412.

Clausen KK, Krause-Jensen D, Olesen B, Marbà N (2014) Seasonality of eelgrass biomass across gradients in temperature and latitude. *Marine Ecology Progress Series*, **506**, 71–85.

Cocco N, Glendinning JI (2012) Not all sugars are created equal: some mask aversive tastes better than others in an herbivorous insect. *Journal of Experimental Biology*, **215**, 1412–1421.

Coley PD (1983) Herbivory and defensive characteristics of tree species in lowland tropical forest. *Ecological Monographs*, **53**, 209–234.

Coley PD, Aide TM (1991) Comparison of herbivory and plant defenses in temperate and tropical

broad-leaved forests. In: *Plant-Animal Interactions : Evolutionary Ecology in Tropical and Temperate Regions* (eds Price PW, Lewinsohm TM, Fernandes GW, Benson WW), pp. 25–49. Wiley, New York.

Coley PD, Bryant JP, Chapin FS (1985) Resource availability and plant antiherbivore defense. *Science (New York, N.Y.)*, **230**, 895–899.

Collier CJ, Uthicke S, Waycott M (2011) Thermal tolerance of two seagrass species at contrasting light levels: Implications for future distribution in the Great Barrier Reef. *Limnology and Oceanography*, **56**, 2200–2210.

Cotrufo MF, Ineson P (2000) Does elevated atmospheric $CO_2$ concentrations affect wood decomposition? *Plant and Soil*, **224**, 51–57.

Cox TE, Schenone S, Delille J, Diaz-Castañeda V, Alliouane S, Gattuso JP, Gazeau F (2015) Effects of ocean acidification on *Posidonia oceanica* epiphytic community and shoot productivity. *Journal of Ecology*, **103**, 1594–1609.

Cox TE, Gazeau F, Alliouane S, Hendriks IE, Mahacek P, Le Fur A, Gattuso JP (2016) Effects of in situ $CO_2$ enrichment on structural characteristics, photosynthesis, and growth of the Mediterranean seagrass *Posidonia oceanica* . *Biogeosciences*, **13**, 2179–2194.

Cronin G, Hay ME (1996) Within-plant variation in seaweed palatability and chemical defenses: optimal defense theory versus the growth-differentiation balance hypothesis. *Oecologia*, **105**, 361–368.

Crozier A, Clifford MN, Ashihara H (2006a) *Plant secondary metabolites occurrence, structure and role in the human diet* (eds Crozier A, Clifford MN, Ashihara H). Blackwell Publishing Ltd., 353 pp.

Crozier A, Jaganath IB, Clifford MN (2006b) Phenols, Polyphenols and Tannins: An Overview. In: *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet* (eds Crozier A, Clifford MN, Ashihara H), p. 372. Blackwell Publishing Ltd.

Cruz-Rivera E, Hay ME (2000) Can quantity replace quality? Food choice, compensatory feeding, and fitness of marine mesograzers. *Ecology*, **81**, 201–219.

Cuny P, Serve L, Jupin H, Boudouresque C (1995) Short communication: Water soluble phenolic

compounds of the marine phanerogam *Posidonia oceanica* in a Mediterranean area colonised by the introduced chlorophyte *Caulerpa taxifolia*. *Aquatic Botany*, **52**, 237–242.

Dearing MD, Cork S (1999) Role of detoxification of plant secondary compounds on diet breadth in a mammalian herbivore, *Trichosurus vulpecula*. *Journal of Chemical Ecology*, **25**, 1205–1219.

DeLucia EH, Nabity PD, Zavala JA, Berenbaum MR (2012) Climate change: Resetting plant-insect interactions. *Plant Physiology*, **160**, 1677–1685.

Dennison W (1990) Chlorophyll content. In: *Seagrass research methods* (eds Phillips RC, McRoy P), pp. 83–85. UNESCO, Paris.

Dhir B (2015) Status of aquatic macrophytes in changing climate: A perspective. *Journal of Environmental Science and Technology*, **8**, 139–148.

Díaz-Almela E, Marbà N, Martínez R, Santiago R, Duarte CM (2009) Seasonal dynamics of *Posidonia oceanica* in Magalluf Bay (Mallorca, Spain): Temperature effects on seagrass mortality. *Limnology and Oceanography*, **54**, 2170–2182.

Dickson AG (1990) Thermodynamics of the dissociation of boric acid in synthetic seawater from 273.15 to 318.15 K. *Deep Sea Research Part A. Oceanographic Research Papers*, **37**, 755–766.

Dobzhansky T (1950) Evolution in the Tropics. *American Scientist*, **38**, 209–221.

Doiron M, Gauthier G, Lévesque E (2014) Effects of experimental warming on nitrogen concentration and biomass of forage plants for an arctic herbivore. *Journal of Ecology*, **102**, 508–517.

Doney SC, Ruckelshaus M, Emmett Duffy J et al. (2012) Climate change impacts on marine ecosystems. *Annual Review of Marine Science*, **4**, 11–37.

Dostálek T, Rokaya MB, Maršík P, Rezek J (2016) Trade-off among different anti-herbivore defence strategies along an altitudinal gradient. *AoB plants*, **8**.

Du D, Winsor JA, Smith M, DeNicco A, Stephenson AG (2008) Resistance and tolerance to herbivory changes with inbreeding and ontogeny in a wild gourd (Cucurbitaceae). *American Journal of Botany*, **95**, 84–92.

## 6. References

Duarte C (1990) Seagrass nutrient content. *Marine Ecology Progress Series*, **67**, 201–207.

Duffy JE, Hay ME (1990) Seaweed adaptations to herbivory. *BioScience*, **40**, 368–375.

Ehleringer JR (2005) The influence of atmospheric CO2, temperature, and water on the abundance of C3/C4 taxa. In: *A History of Atmospheric CO2 and its Effects on Plants, Animals and Ecosistems* (eds Ehleringer JR, Cerling TE, Dearing MD), pp. 214–231. Springer, New York.

Ehlers A, Worm B, Reusch TBH (2008) Importance of genetic diversity in eelgrass *Zostera marina* for its resilience to global warming. *Marine Ecology Progress Series*, **355**, 1–7.

Ehrlich PR, Raven RJ (1964) Butterflies and plants : A study in coevolution. *Evolution*, **18**, 586–608.

Eklöf JS, Alsterberg C, Havenhand JN, Sundbäck K, Wood HL, Gamfeldt L (2012) Experimental climate change weakens the insurance effect of biodiversity. *Ecology letters*, **15**, 864–72.

Elger A, Lemoine DG, Fenner M, Hanley ME (2009) Plant ontogeny and chemical defence: Older seedlings are better defended. *Oikos*, **118**, 767–773.

Ellison AM, Bank MS, Clinton BD et al. (2005) Loss of foundation species : consequences for the structure and dynamics of forested ecosystems. *Frontiers in the ecology and the environment*, **3**, 479–486.

Elser JJ, Bracken MES, Cleland EE et al. (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecology Letters*, **10**, 1135–1142.

Endara M-J, Coley PD (2011) The resource availability hypothesis revisited: a meta-analysis. *Functional Ecology*, **25**, 389–398.

Enriquez S, Sand-Jensen K (2003) Variation in light absorption properties of *Mentha aquatica* L. as a function of leaf form: Implications for plant growth. *International Journal of Plant Sciences*, **164**, 125–136.

Enríquez S (2005) Light absorption efficiency and the package effect in the leaves of the seagrass *Thalassia testudinum*. *Mar Ecol Prog Ser*, **289**, 141–150.

## 6. References

Enríquez S, Schubert N (2014) Direct contribution of the seagrass *Thalassia testudinum* to lime mud production. *Nature communications*, **5**, 3835.

Estes JA, Terborgh J, Brashares JS et al. (2011) Trophic downgrading of planet Earth. *Science (New York, N.Y.)*, **333**, 301–306.

Falcone Ferreyra ML, Rius SP, Casati P (2012) Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Frontiers in plant science*, **3**, 222.

Farnsworth EJ, Ellison a. M, Gong WK (1996) Elevated CO2 alters anatomy, physiology, growth, and reproduction of red mangrove (*Rhizophora mangle* L.). *Oecologia*, **108**, 599–609.

Farrar J, Pollock C, Gallagher J (2000) Sucrose and the integration of metabolism in vascular plants. *Plant Science*, **154**, 1–11.

Farré-Armengol G, Filella I, Llusià J, Peñuelas J (2015) Relationships among floral VOC emissions, floral rewards and visits of pollinators in five plant species of a Mediterranean shrubland. *Plant ecology and evolution*, **148**, 90–99.

Feely RA, Sabine CL, Hernandez-Ayon JM, Ianson D, Hales B (2008) Evidence for upwelling of corrosive "acidified" water onto the continental shelf. *Science*, **320**, 1490–1492.

Feeny P (1976) Plant apparency and chemical defense. In: *Recent Advances in Phytochemistry* (eds Wallace JW, Mansell RL), pp. 1–40. Plenum Press, New York.

Ferrat L, Pergent-Martini C, Roméo M (2003) Assessment of the use of biomarkers in aquatic plants for the evaluation of environmental quality: application to seagrasses. *Aquatic Toxicology*, **65**, 187–204.

Fine PVA, Miller ZJ, Mesones I et al. (2006) The growth–defense trade-off and habitat specialization by plants in Amazonian forests. *Ecology*, **87**, 150–162.

Fineblum WL, Rausher MD (2002) Tradeoff between resistance and tolerance to herbivore damage in a morning glory. *Nature*, **377**, 517–520.

Fishman JR, Orth RJ (1996) Effects of predation on *Zostera marina* L. seed abundance. *Journal of Experimental Marine Biology and Ecology*, **198**, 11–26.

Flöthe CR, Molis M (2013) Temporal dynamics of inducible anti-herbivory defenses in the brown

seaweed *Ascophyllum nodosum* (Phaeophyceae). *Journal of Phycology*, **49**, 468–474.

Fonseca MS, Koehl M a R (2006) Flow in seagrass canopies: The influence of patch width. *Estuarine, Coastal and Shelf Science*, **67**, 1–9.

Foster CN, Barton PS, Lindenmayer DB (2014) Effects of large native herbivores on other animals. *Journal of Applied Ecology*, **51**, 929–938.

Fourqurean J, Zieman J, Powell G (1992) Relationships between porewater nutrients and seagrasses in a subtropical carbonate environment. *Marine Biology*, **114**, 57–65.

Fourqurean J, Manuel S, Coates K, Kenworthy W, Smith S (2010) Effects of excluding sea turtle herbivores from a seagrass bed: Overgrazing may have led to loss of seagrass meadows in Bermuda. *Marine Ecology Progress Series*, **419**, 223–232.

Fourqurean JW, Duarte CM, Kennedy H et al. (2012) Seagrass ecosystems as a globally significant carbon stock. *Nature Geoscience*, **5**, 505–509.

Francis AP, Currie DJ (2003) A globally consistent richness-climate relationship for angiosperms. *The American naturalist*, **161**, 523–536.

Freeman RS, Brody AK, Neefus CD (2003) Flowering phenology and compensation for herbivory in *Ipomopsis aggregata*. *Oecologia*, **136**, 394–401.

Fritz RS, Simms EL (1992) *Plant Resistance to Herbivores and Pathogens, ecology, evolution, and genetics.* (eds Fritz RS, Simms EL). Univ. of Chicago Press, Chicago.

Frost CJ, Mescher MC, Carlson JE, De Moraes CM (2008) Plant defense priming against herbivores: getting ready for a different battle. *Plant physiology*, **146**, 818–824.

Futuyma DJ, Agrawal AA (2009) Macroevolution and the biological diversity of plants and herbivores. *Proceedings of the National Academy of Sciences*, **106**, 18054–18061.

Gacia E, Marba N, Cebrián J, Vaquer-Sunyer R, Garcias-Bonet N, Duarte CM (2012) Thresholds of irradiance for seagrass *Posidonia oceanica* meadow metabolism. *Marine Ecology Progress Series*, **466**, 69–79.

Le Gall M, Behmer ST (2014) Effects of protein and carbohydrate on an insect herbivore: The vista from a fitness landscape. *Integrative and comparative biology*, **54**, 942–954.

## 6. References

Garthwin RG, Poore AGB, Vergés A (2014) Seagrass tolerance to herbivory under increased ocean temperatures. *Marine Pollution Bulletin*, **83**, 475–482.

Giordano M, Beardall J, Raven J a (2005) CO2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. *Annual review of plant biology*, **56**, 99–131.

Glynn C, Herms DA, Orians CM, Hansen RC, Larsson S (2007) Testing the growth-differentiation balance hypothesis: Dynamic responses of willows to nutrient availability. *New Phytologist*, **176**, 623–634.

Goecker ME, Heck KL, Valentine JF (2005) Effects of nitrogen concentrations in turtlegrass *Thalassia testudinum* on consumption by the bucktooth parrotfish *Sparisoma radians* . *Marine Ecology Progress Series*, **286**, 239–248.

Gordon IJ (2006) *Large Herbivore Ecology , Ecosystem Dynamics and Conservation* (eds Danell K, Duncan P, Bergstrom R, Pastor J). Cambridge University Press, New York, 449-467 pp.

Gordon IJ, Prins HHT (2008) *The Ecology of browsing and grazing* (eds Gordon IJ, Prins HHT). Springer.

Gowda JH (1996) Spines of *Acacia tortilis*: What do they defend and how? *Oikos*, **77**, 279–284.

Graham MH, Fox MD, Hamilton SL (2016) Macrophyte productivity and the provisioning of energy and habitat to nearshore systems. In: *Marine Macrophytes as Foundation species* (ed Olafsson E), pp. 133–160. CRC Press.

Grignon-Dubois M, Rezzonico B, Alcoverro T (2011) Regional scale patterns in seagrass defences: Phenolic acid content in *Zostera noltii*. *Estuarine, Coastal and Shelf Science*, **114**, 18–22.

Gu J, Weber K, Klemp E et al. (2012) Identifying core features of adaptive metabolic mechanisms for chronic heat stress attenuation contributing to systems robustness. *Integrative Biology*, **4**, 480.

Güsewell S (2004) N:P ratios in terrestrial plants: Variation and functional significance. *New Phytologist*, **164**, 243–266.

Gutow L, Petersen I, Bartl K, Huenerlage K (2016) Marine meso-herbivore consumption scales faster with temperature than seaweed primary production. *Journal of Experimental Marine Biology and Ecology*, **477**, 80–85.

## 6. References

Haavisto F, Jormalainen V (2014) Seasonality elicits herbivores' escape from trophic control and favors induced resistance in a temperate macroalga. *Ecology*, **95**, 3035–3045.

Hagerman A, Butler L (1991) Tannins and lignins. In: *Herbivores: Their Interactions with Secondary Plant Metabolites*, 2nd edn, Vol. I (eds Rosenthal GA, Berenbaum MR), pp. 355–388. Academic Press.

Hairston N, Smith F, Slobodkin L (1960) Community structure, population control, and competition. *The American Naturalist*, **94**, 421–425.

Hall-Spencer JM, Rodolfo-Metalpa R, Martin S et al. (2008) Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature*, **454**, 96–99.

Halpern BS, Cottenie K, Broitman BR (2006) Strong top-down control in Southern California kelp forest ecosystems. *Science*, **312**, 1230–1232.

Halpern BS, Silliman BR, Olden JD, Bruno JP, Bertness MD (2007) Incorporating positive interactions in aquatic restoration and conservation. *Frontiers in Ecology and the Environment*, **5**, 153–160.

Hamilton JG, Zangerl AR, DeLucia EH, Berenbaum MR (2001) The Carbon-Nutrient Balance Hypothesis: Its rise and fall. *Ecology Letters*, **4**, 86–95.

Hartley SE, DeGabriel JL, Cooke J (2016) The ecology of herbivore-induced silicon defences in grasses. *Functional Ecology*, **30**, 1311–1322.

Hata H, Kato M (2006) A novel obligate cultivation mutualism between damselfish and Polysiphonia algae. *Biology Letters*, **2**, 593–596.

Hattas D, Scogings PF, Julkunen-Tiitto R (2017) Does the Growth Differentiation Balance Hypothesis explain allocation to secondary metabolites in Combretum apiculatum , an African savanna woody species? *Journal of Chemical Ecology*, **43**, 153–163.

Hawkes C V., Sullivan JJ (2001) The impact of herbivory on plants in different resource conditions: a meta-analysis. *Ecology*, **82**, 2045–2058.

Hay ME (1986) Associational plant defenses and the maintenance of species diversity: turning competitors into accomplices. *The American Naturalist*, **128**, 617–641.

## 6. References

Hay ME (2009) Marine chemical ecology: chemical signals and cues structure marine populations, communities and ecosystems. *Annual Review of Marine Science*, **1**, 193–212.

Hay ME, Steinberg PD (1992) The chemical ecology of plant–herbivore interactions in marine versus terrestrial communities. In: *Herbivores: Their Interactions with Secondary Plant Metabolites* (eds Rosenthal GA, Berembaum MA), pp. 371–413. Elsevier.

Hay KB, Poore AGB, Lovelock CE (2011) The effects of nutrient availability on tolerance to herbivory in a brown seaweed. *Journal of Ecology*, **99**, 1540–1550.

Haznedaroglu MZ, Zeybek U (2007) HPLC determination of chicoric acid in leaves of *Posidonia oceanica*. *Pharmaceutical Biology*, **45**, 745–748.

Heck KL, Valentine JF (2006) Plant–herbivore interactions in seagrass meadows. *Journal of Experimental Marine Biology and Ecology*, **330**, 420–436.

Heck KL, Valentine JF (2007) The primacy of top-down effects in shallow benthic ecosystems. *Estuaries and Coasts*, **30**, 371–381.

Heck KL, Fodrie FJ, Madsen S, Baillie CJ, Byron DA (2015) Seagrass consumption by native and a tropically associated fish species: Potential impacts of the tropicalization of the northern Gulf of Mexico. *Marine Ecology Progress Series*, **520**, 165–173.

Hedges L V (1981) Distribution theory for Glass 's estimator of effect size and related estimators. *American educational research association*, **6**, 107–128.

Hedges JI, Stern JH (1984) Carbon and nitrogen determinations of carbonate-containing solids. *Limnology and Oceanography*, **29**, 657–663.

Heglmeier A, Zidorn C (2010) Secondary metabolites of *Posidonia oceanica* (Posidoniaceae). *Biochemical Systematics and Ecology*, **38**, 964–970.

Heil M (2002) Ecological costs of induced resistance. *Current Opinion in Plant Biology*, **5**, 345–350.

Heil M (2008) Indirect defence via tritrophic interactions. *New Phytologist*, **178**, 41–61.

Heithaus MR, Alcoverro T, Arthur R et al. (2014) Seagrasses in the age of sea turtle conservation and shark overfishing. *Frontiers in Marine Science*, **1**, 1–6.

## 6. References

Hemmi A, Jormalainen V (2002) Nutrient enhancement increases performance of a marine herbivore via quality of its food alga. *Ecology*, **83**, 1052–1064.

Hemminga MA (1998) The root/rhizome system of seagrasses: An asset and a burden. *Journal of Sea Research*, **39**, 183–196.

Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. *The Quarterly Review of Biology*, **67**, 283–335.

Hilbert DW, Swift DM, Detling JK, Dyer MI (1981) Relative growth rates and the grazing optimization hypothesis. *Oecologia*, **51**, 14–18.

Hillebrand H (2004) On the generality of the latitudinal diversity gradient. *American Naturalist*, **163**, 192–211.

Ho CK, Pennings SC (2013) Preference and performance in plant-herbivore interactions across latitude-A study in U.S. Atlantic salt marshes. *PLoS ONE*, **8**, 1–11.

Houghton RA (2007) Balancing the global carbon budget. *Annual Review Of Earth And Planetary Sciences*, **35**, 313–347.

Howlett BG, Clarke AR, Madden JL (2001) The influence of leaf age on the oviposition preference of *Chrysophtharta bimaculata* (Olivier) and the establishment of neonates. *Agricultural and Forest Entomology*, **3**, 121–127.

Huang W, Houlton BZ, Marklein AR, Liu J, Zhou G (2015) Plant stoichiometric responses to elevated CO2 vary with nitrogen and phosphorus inputs: Evidence from a global-scale meta-analysis. *Scientific reports*, **5**, 18225.

Hudgeons JL, Knutson AE, Heinz KM, DeLoach CJ, Dudley TL, Pattison RR, Kiniry JR (2007) Defoliation by introduced *Diorhabda elongata* leaf beetles (Coleoptera: Chrysomelidae) reduces carbohydrate reserves and regrowth of *Tamarix* (Tamaricaceae). *Biological Control*, **43**, 213–221.

Hughes AR, Inouye BD, Johnson MTJ, Underwood N, Vellend M (2008) Ecological consequences of genetic diversity. *Ecology Letters*, **11**, 609–623.

Huhta AP, Rautio P, Hellström K, Saari M, Tuomi J (2009) Tolerance of a perennial herb, *Pimpinella saxifraga*, to simulated flower herbivory and grazing: Immediate repair of injury

or postponed reproduction? *Herbaceous Plant Ecology: Recent Advances in Plant Ecology*, 235–245.

Hulme PE (1996) Herbivory, plant regeneration, and species coexistence. *Journal of Ecology*, **84**, 609–615.

Huntly N (1991) Herbivores and the dynamics of communities and ecosystems. *Annual Review of Ecology and Systematics*, **22**, 477–503.

Hyndes GA, Heck KL, Vergés A et al. (2016) Accelerating tropicalization and the transformation of temperate seagrass meadows. *BioScience*, **66**, 938–948.

Ibarra-Obando SE, Heck KL, Spitzer PM (2004) Effects of simultaneous changes in light, nutrients, and herbivory levels, on the structure and function of a subtropical turtlegrass meadow. *Journal of Experimental Marine Biology and Ecology*, **301**, 193–224.

Infantes E, Orfila A, Bouma TJ, Simarro G, Terrados J (2011) *Posidonia oceanica* and *Cymodocea nodosa* seedling tolerance to wave exposure. *Limnology and Oceanography*, **56**, 2223–2232.

Infantes E, Orfila A, Simarro G, Terrados J, Luhar M, Nepf H (2012) Effect of a seagrass (*Posidonia oceanica*) meadow on wave propagation. *Marine Ecology Progress Series*, **456**, 63–72.

Invers O, Zimmerman RC, Alberte RS, Pérez M, Romero J (2001) Inorganic carbon sources for seagrass photosynthesis: An experimental evaluation of bicarbonate use in species inhabiting temperate waters. *Journal of Experimental Marine Biology and Ecology*, **265**, 203–217.

Invers O, Tomas F, Of B, Science M (2002) Potencial effect of increased global CO2 availability on the depth distribution of the seagrass *Posidonia oceanica* (L.) Delile: A tentative assessment using a carbon balance model. *Bulletin of Marine Science*, **71**, 1191–1198.

Invers O, Kraemer GP, Pérez M, Romero J (2004) Effects of nitrogen addition on nitrogen metabolism and carbon reserves in the temperate seagrass *Posidonia oceanica* . *Journal of Experimental Marine Biology and Ecology*, **303**, 97–114.

IPCC (2013) Climate Change 2013: The Physical Science Basis. Summary for Policymakers. *Ipcc*, 1–29.

## 6. References

IPCC (2014) *IPCC,2014: Climate Change 2014: Synthesis Report.Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (eds Pachauri RK, Meyer LA). Geneva, 151 pp.

Jackson EL, Rees SE, Wilding C, Attrill MJ (2015) Use of a seagrass residency index to apportion commercial fishery landing values and recreation fisheries expenditure to seagrass habitat service. *Conservation Biology*, **29**, 899–909.

Jahnke M, Pagés JF, Alcoverro T, Lavery PS, Mcmahon KM, Procaccini G (2015a) Should we sync? Seascape-level genetic and ecological factors determine seagrass flowering patterns. *Journal of Ecology*, **103**, 1464–1474.

Jahnke M, Olsen JL, Procaccini G (2015b) A meta-analysis reveals a positive correlation between genetic diversity metrics and environmental status in the long-lived seagrass *Posidonia oceanica* . *Molecular Ecology*, **24**, 2336–2348.

Janzen DH (1966) Coevolution of mutualism between ants and acacias in Central America. *Evolution*, **20**, 249–275.

Jaramillo V, Detling JK (1988) Grazing history, defoliation, and competition: effects on shortgrass production and nitrogen accumulation. *Ecology*, **69**, 1599–1608.

Jassby AD, Platt T (1976) Mathematical formulation of the relationship between photosynthesis and light for phytoplankton. *Limnology and Oceanography*, **21**, 540–547.

Jiang ZJ, Huang XP, Zhang JP (2010) Effects of $CO_2$ enrichment on photosynthesis, growth, and biochemical composition of seagrass *Thalassia hemprichii* (Ehrenb.) Aschers. *Journal of Integrative Plant Biology*, **52**, 904–913.

Jochum M, Schneider FD, Crowe TP, Brose U, O'Gorman EJ (2012) Climate-induced changes in bottom-up and top-down processes independently alter a marine ecosystem. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **367**, 2962–2970.

Johnson MTJ, Rasmann S (2011) The latitudinal herbivory-defence hypothesis takes a detour on the map. *New Phytologist*, **191**, 589–592.

Johnson CR, Reiling BA, Mislevy P et al. (2001) Effects of nitrogen fertilization and harvest date on yield, digestibility, fiber, and protein fractions of tropical grasses. *Journal of Animal*

## 6. References

*Science*, **79**, 2439–2448.

Johnson MTJ, Smith SD, Rausher MD (2009) Plant sex and the evolution of plant defenses against herbivores. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 18079–18084.

Johnson MTJ, Campbell SA, Barrett SCH (2015) Evolutionary interactions between plant reproduction and defense against herbivores. *Annual Review of Ecology, Evolution, and Systematics*, **46**, 191–213.

Jones CG, Lawton JH, Shachak M (1997) Positive and negative effects of organisms as physical ecosystem engineers. *Ecology*, **78**, 1946–1957.

Jormalainen V, Honkanen T, Vesakoski O, Koivikko R (2005) Polar extracts of the brown alga *Fucus vesiculosus* (L.) reduce assimilation efficiency but do not deter the herbivorous isopod *Idotea baltica* (Pallas). *Journal of Experimental Marine Biology and Ecology*, **317**, 143–157.

Jump AS, Marchant R, Peñuelas J (2009) Environmental change and the option value of genetic diversity. *Trends in Plant Science*, **14**, 51–58.

Kaldy JE (2014) Effect of temperature and nutrient manipulations on eelgrass *Zostera marina* L. from the Pacific Northwest, USA. *Journal of Experimental Marine Biology and Ecology*, **453**, 108–115.

Kant MR, Jonckheere W, Knegt B et al. (2015) Mechanisms and ecological consequences of plant defence induction and suppression in herbivore communities. *Annals of Botany*, **115**, 1015–1051.

Karban R (2011) The ecology and evolution of induced resistance against herbivores. *Functional Ecology*, **25**, 339–347.

Karban R, Agrawal AA (2002) Herbivore offense. *Annual Review of Ecology and Systematics*, **33**, 641–664.

Karban R, Baldwin IT (1997) *Induced responses to herbivory* (eds Karban R, Baldwin IT). The University of Chicago Press, Chicago, 330 pp.

Karban R, Agrawal AA, Mangel M (1997) The benefits of induced defenses against herbivores. *Ecology*, **78**, 1351–1355.

## 6. References

Karban R, Agrawal AA, Thaler JS, Adler LS (1999) Induced plant responses and information content about risk of herbivory. *Trends in Ecology and Evolution*, **14**, 443–447.

Kempel A, Schädler M, Chrobock T, Fischer M, van Kleunen M (2011) Tradeoffs associated with constitutive and induced plant resistance against herbivory. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 5685–5689.

Klumpp DW, Nichols PD (1983) Nutrition of the southern sea garfish *Hyporhamphus melanochir*: gut passage rate and daily consumption of two food types and assimilation of seagrass components*. *Marine Ecology Progress Series*, **12**, 207–216.

Knops JMH, Reinhart K (2000) Specifin leaf area along a nitrogen fertilization gradient. *The America Midlan Naturalist*, **144**, 265–272.

Koch MS, Schopmeyer S, Kyhn-Hansen C, Madden CJ (2007) Synergistic effects of high temperature and sulfide on tropical seagrass. *Journal of Experimental Marine Biology and Ecology*, **341**, 91–101.

Koch M, Bowes G, Ross C, Zhang XH (2013) Climate change and ocean acidification effects on seagrasses and marine macroalgae. *Global Change Biology*, **19**, 103–132.

Koivikko R, Eränen JK, Loponen J, Jormalainen V (2008) Variation of phlorotannins among three populations of *Fucus vesiculosus* as revealed by HPLC and colorimetric quantification. *Journal of chemical ecology*, **34**, 57–64.

Kollars NM, Henry AK, Boyer K et al. Reciprocal linkages between temperate seagrasses and waterfowl can guide joint conservation. *in prep*.

Kooyers NJ, Blackman BK, Holeski LM (2017) Optimal defense theory explains deviations from latitudinal herbivory defense hypothesis. *Ecology*, **98**, 1036–1048.

Koricheva J (2002) Meta-analysis of sources of variation in fitness costs. *Ecology*, **83**, 176–190.

Koricheva J, Larsson S, Haukioja E, Keinanen M (1998) Regulation of woody plant secondary metabolism by resource availability: Hypothesis testing by means of meta-analysis. *Oikos*, **83**, 212–226.

Kramer AT, Havens K (2009) Plant conservation genetics in a changing world. *Trends in Plant Science*, **14**, 599–607.

## 6. References

Kurnath P, Merz ND, Dearing MD (2016) Ambient temperature influences tolerance to plant secondary compounds in a mammalian herbivore. *Proceedings of the Royal Society of London B: Biological Sciences*, **283**.

Lal A, Arthur R, Marbà N, Lill AWT, Alcoverro T (2010) Implications of conserving an ecosystem modifier: Increasing green turtle (*Chelonia mydas*) densities substantially alters seagrass meadows. *Biological Conservation*, **143**, 2730–2738.

Lamb JB, Water JAJM Van De, Bourne DG, Altier C (2017) Seagrass ecosystems reduce exposure to bacterial pathogens of humans, fishes, and invertebrates. **355**, 731–733.

Lambers JHR, Clark JS, Beckage B, Hille J, Lambers R, Clark JS, Beckage B (2002) Density-dependent mortality and the latitudinal gradient in species diversity. *Nature*, **417**, 732–735.

Lambers H, Stuart Chapin III F, Pons TL (2008a) *Plant Physiological Ecology*, Second edn. Springer, New York, 605 pp.

Lambers H, Chapin FS, Pons TL (2008b) Life cycles: Environmental influences and adaptations. In: *Plant Physiological ecology*, second edn (eds Lambers H, Chapin III F, Pons T), pp. 375–402. Springer, New York.

Larkum AWD, Orth RJ, Duarte CM (2006) *Seagrasses: biology, ecology and conservation*. 691 pp.

Lattanzio V, Kroon PA, Quideau S, Treutter D (2009) Plant phenolics - secondary metabolites with diverse functions. In: *Recent Advances in Polyphenol Research*, Vol. 1, pp. 1–35.

Lavery PS, Mateo MA, Serrano O, Rozaimi M (2013) Variability in the carbon storage of seagrass habitats and its implications for global estimates of blue carbon ecosystem service. *PLoS ONE*, **8**, e73748.

Lee K, Dunton KH (2000) Effects of nitrogen enrichment on biomass allocation, growth, and leaf morphology of the seagrass. *Marine Ecology Progress Series*, **196**, 39–48.

Lee KS, Short FT, Burdick DM (2004) Development of a nutrient pollution indicator using the seagrass, *Zostera marina*, along nutrient gradients in three New England estuaries. *Aquatic Botany*, **78**, 197–216.

Lee K-S, Park SR, Kim YK (2007) Effects of irradiance, temperature, and nutrients on growth dynamics of seagrasses: A review. *Journal of Experimental Marine Biology and Ecology*, **350**,

144–175.

Lehndal L, Agren J (2015) Latitudinal variation in resistance and tolerance to herbivory in the perennial herb *Lythrum salicaria* is related to intensity of herbivory and plant phenology. *Journal of Evolutionary Biology*, **28**, 576–589.

Leimu R, Muola A, Laukkanen L, Kalske A, Prill N, Mutikainen P (2012) Plant-herbivore coevolution in a changing world. *Entomologia Experimentalis et Applicata*, **144**, 3–13.

Lejeusne C, Chevaldonné P, Pergent-Martini C, Boudouresque CF, Pérez T (2010) Climate change effects on a miniature ocean: the highly diverse, highly impacted Mediterranean Sea. *Trends in Ecology & Evolution*, **25**, 250–260.

Lemoine DG, Barrat-Segretain M-H, Roy A (2009) Morphological and chemical changes induced by herbivory in three common aquatic macrophytes. *International Review of Hydrobiology*, **94**, 282–289.

Lemoine NP, Burkepile DE, Parker JD (2014) Variable effects of temperature on insect herbivory. *PeerJ*, **2**, e376.

Lilley SA, Schiel DR (2006) Community effects following the deletion of a habitat-forming alga from rocky marine shores. *Oecologia*, **148**, 672–681.

Lincoln DE (1993) The influence of plant carbon dioxide and nutrient supply on susceptibility to insect herbivores. *Vegetatio*, **104–105**, 273–280.

Littler MM, Littler DS, Brooks BL (2006) Harmful algae on tropical coral reefs: Bottom-up eutrophication and top-down herbivory. *Harmful Algae*, **5**, 565–585.

Loeuille N, Loreau M, Ferrière R (2002) Consequences of plant-herbivore coevolution on the dynamics and functioning of ecosystems. *Journal of theoretical biology*, **217**, 369–381.

Long SP, Ainsworth E, Rogers A, Ort DR (2004) Rising atmospheric carbon dioxide: plants FACE the future. *Annual review of plant biology*, **55**, 591–628.

Loomis WE (1932) Growth–differentiation balance vs. carbohydrate– nitrogen ratio. *American Society for Horticultural Science Proceedings*, **29**, 240–245.

Loomis WE (1953) Growth and differentiation—an introduction and summary. In: *Growth and*

## 6. References

*Differentiation in Plants* (ed Loomis WE), pp. 1–17. Ames: Iowa State College Press.

Lorio PL (1986) Growth-differentiation balance: A basis for understanding southern pine beetle-tree interactions. *Forest Ecology and Management*, **14**, 259–273.

de los Santos CB, Brun FG, Onoda Y, Cambridge ML, Bouma TJ, Vergara JJ, Pérez-Lloréns JL (2012) Leaf-fracture properties correlated with nutritional traits in nine Australian seagrass species: implications for susceptibility to herbivory. *Marine Ecology Progress Series*, **458**, 89–102.

Louthan AM, Doak DF, Angert AL (2015) Where and when do species interactions set range limits? *Trends in Ecology and Evolution*, **30**, 780–792.

Lubchenco J (1983) Littornia and Fucus: Effects of herbivores, substratum heterogeneity, and plant escapes during succession. *Ecology*, **64**, 1116–1123.

Lucas P (2000) Mechanical defences to herbivory. *Annals of Botany*, **86**, 913–920.

Lunn EJ (2008) Sucrose metabolism. In: *Encyclopedia of life sciences.* John Wiley & Sons, Chichester.

Luo Y, Field CB, Mooney HA (1994) Predicting responses of photosynthesis and root fraction to elevated CO2: interactions among carbon, nitrogen, and growth. *Plant, Cell & Environment*, **17**, 1195–1204.

MacArthur RH (1972) *Geographical Ecology: Patterns in the distribution of species*. Princeton University Press, 510-533 pp.

Mannino AM, Vaglica V, Cammarata M, Oddo E (2016) Effects of temperature on total phenolic compounds in *Cystoseira amentacea* (C. Agardh) Bory (Fucales, Phaeophyceae) from southern Mediterranean Sea. *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology*, **150**, 152–160.

Marbà N, Duarte CM (2010) Mediterranean warming triggers seagrass (*Posidonia oceanica*) shoot mortality. *Global Change Biology*, **16**, 2366–2375.

Marco-Méndez C, Prado P, Heck K, Cebrián J, Sánchez-Lizaso J (2012) Epiphytes mediate the trophic role of sea urchins in Thalassia testudinum seagrass beds. *Marine Ecology Progress Series*, **460**, 91–100.

## 6.   References

Marco-Méndez C, Ferrero-Vicente LM, Prado P, Heck KL, Cebrián J, Sánchez-Lizaso JL (2015) Epiphyte presence and seagrass species identity influence rates of herbivory in Mediterranean seagrass meadows. *Estuarine, Coastal and Shelf Science*, **154**, 94–101.

Mariani S, Alcoverro T (1999) A multiple-choice feeding-preference experiment utilising seagrasses with a natural population of herbivorous fishes. *Marine Ecology Progress Series*, **189**, 295–299.

Maron JL, Crone E (2006) Herbivory: effects on plant abundance, distribution and population growth. *Proceedings of the Royal Society Biological sciences*, **273**, 2575–2584.

Marsh JA, Dennison WC, Alberte RS, Alberte S (1986) Effects of temperature on photosynthesis and respiration in eelgrass (*Zostera marina* L.). *Journal of Experimental Marine Biology and Ecology*, **101**, 257–267.

Martinez-Crego B, Olive I, Santos R (2014) CO2 and nutrient-driven changes across multiple levels of organization in *Zostera noltii* ecosystems. *Biogeosciences*, **11**, 7237–7249.

Martínez-Crego B, Arteaga P, Ueber A, Engelen AH, Santos R, Molis M (2015) Specificity in mesograzer-induced defences in seagrasses. *Plos One*, **10**, e0141219.

Martínez-Crego B, Arteaga P, Tomas F, Santos R (2016) The role of seagrass traits in mediating *Zostera noltei* vulnerability to mesograzers. *Plos One*, **11**, e0156848.

Maschinski J, Whitham TG (1989) The continuum of plant responses to herbivory: the influence of plant association, nutrient availability and timing. *The American Naturalist*, **134**, 1–19.

Massa SI, Arnaud-Haond S, Pearson GA, Serrão EA (2009) Temperature tolerance and survival of intertidal populations of the seagrass *Zostera noltii* (Hornemann) in Southern Europe (Ria Formosa, Portugal). *Hydrobiologia*, **619**, 195–201.

Massad TJ, Dyer L a. (2010) A meta-analysis of the effects of global environmental change on plant-herbivore interactions. *Arthropod-Plant Interactions*, **4**, 181–188.

Massad TJ, Dyer LA, Vega C. G (2012) Costs of defense and a test of the Carbon-Nutrient Balance and Growth-Differentiation Balance Hypotheses for two co-occurring classes of plant defense. *PLoS ONE*, **7**.

Matsuura H, Fett-Neto AG (1996) Plant alkaloids: main features, toxicity, and mechanisms of

action. In: *Plant Toxins* (eds Gopalakrishnakone P, Carlini CR, Ligabue-Braum R), pp. 1–15. Springer.

Mattson WJJ (1980) Herbivory in relation to plant nitrogen content. *Annual Review of Ecology and Systematics*, **11**, 119–61.

Mazarrasa I, Marbà N, Lovelock CE et al. (2015) Seagrass meadows as a globally significant carbonate reservoir. *Biogeosciences*, **12**, 4993–5003.

McAfee D, Cole VJ, Bishop MJ (2016) Latitudinal gradients in ecosystem engineering by oysters vary across habitats. *Ecology*, **97**, 929–939.

McArt SH, Halitschke R, Salminen JP, Thaler JS (2013) Leaf herbivory increases plant fitness via induced resistance to seed predators. *Ecology*, **94**, 966–975.

McClintock JB, Baker BJ (2001) *Marine Chemical Ecology* (eds McClintock JB, Baker BJ). CRC Press.

McKey D (1974) Adaptive patterns in alkaloid physiology. *The American Naturalist*, **108**, 305–320.

McKey D (1979) The distribution of secondary compounds within plants. In: *Herbivores: Their Interaction with Secondary Plant Metabolites* (eds Rosenthal GA, Janzen DH), pp. 55–133. Academic Press, New York.

McNaughton SJ (1983) Compensatory plant growth as a response to herbivory as a response to herbivory. *Oikos*, **40**, 329–336.

McNaughton SJ, Oesterheld M, Frank DA, Williams KJ (1989) Ecosystem-level patterns of primary productivity and herbivory in terrestrial habitats. *Nature*, **341**, 142–4.

Meijden E Van Der, Wijn M, Verkaar HJ (1988) Defence and regrowth, alternative plant strategies in the struggle against herbivores. *Oikos*, **51**, 355–363.

Menge B (2003) The overriding importance of environmental context in determining the outcome of species-deletion experiments. In: *The Importance of Species: Perspecitives on Expendability and Triage* (eds Kareiva P, Levin SA), pp. 16–43. Princeton University Press, New Jersey, U.S.

## 6. References

Migliaccio M, De Martino F, Silvestre F, Procaccini G (2005) Meadow-scale genetic structure in *Posidonia oceanica*. *Marine Ecology Progress Series*, **304**, 55–65.

Milchunas DG, Noy-Meir I (2002) Grazing refuges, external avoidance of herbivory and plant diversity. *Oikos*, **99**, 113–130.

Milewski  a V, Young TP, Madden D (1991) Thorns as induced defenses.Experimental evidence. *Oecologia.*, **86**, 70–76.

Millero FJ, Graham TB, Huang F, Bustos-Serrano H, Pierrot D (2006) Dissociation constants of carbonic acid in seawater as a function of salinity and temperature. *Marine Chemistry*, **100**, 80–94.

Minkenberg OPJ., Ottenheim JJG. (1990) Effect of leaf nitrogen content of tomato plants on preference and performance of a leafmining fly. *Oecologia*, **83**, 291–298.

Mizuta H, Yasui H (2012) Protective function of silicon deposition in *Saccharina japonica* sporophytes (Phaeophyceae). *Journal of Applied Phycology*, **24**, 1177–1182.

Moles S (1994) Trade-offs and constrains in plant-herbivore defense theory: a life-history perspective. *Oikos*, **71**, 3–12.

Moles AT, Westoby M (2014) What do seedlings die from and what are the implications for evolution of seed size? *OIKOS*, **106**, 193–199.

Moles AT, Westoby M, Moles AT, Westoby M, Westoby M (2004) Seedling survival and seed size: a synthesis of the literature. *Society*, **92**, 372–383.

Moles AT, Bonser SP, Poore AGB, Wallis IR, Foley WJ (2011) Assessing the evidence for latitudinal gradients in plant defence and herbivory. *Functional Ecology*, **25**, 380–388.

Mopper S, Maschinski N, Cobb N, Whitham TG (1991) A new look at habitat structure: consequences of herbivore-modified plant architecture. In: *Habitat structure* (eds Bell SS, McCoy ED, Mushinsky HR), pp. 260–280. Chapman & Hall, London.

Moran KL, Bjorndal KA (2005) Simulated green turtle grazing affects structure and productivity of seagrass pastures. *Marine Ecology Progress Series*, **305**, 235–247.

Moreira X, Abdala-Roberts L, Parra-Tabla V, Mooney KA (2014a) Positive effects of plant

genotypic and species diversity on anti-herbivore defenses in a tropical tree species. *PLoS ONE*, **9**.

Moreira X, Mooney KA, Rasmann S, Petry WK, Carrillo-Gavilán A, Zas R, Sampedro L (2014b) Trade-offs between constitutive and induced defences drive geographical and climatic clines in pine chemical defences. *Ecology Letters*, **17**, 537–546.

Nakaoka M (2002) Predation on seeds of seagrasses Zostera marina and Zostera caulescens by a tanaid crustacean *Zeuxo* sp. *Aquatic Botany*, **72**, 99–106.

Nejrup LB, Pedersen MF (2008) Effects of salinity and water temperature on the ecological performance of *Zostera marina*. *Aquatic Botany*, **88**, 239–246.

Newman JA, Abner ML, Dado RG, Gibson DJ, Brookings A, Parsons AJ (2003) Effects of elevated $CO_2$, nitrogen and fungal endophyte-infection on tall fescue: Growth, photosynthesis, chemical composition and digestibility. *Global Change Biology*, **9**, 425–437.

Ngai JT, Jefferies RL (2004) Nutrient limitation of plant growth and forage quality in Arctic coastal marshes. *Journal of Ecology*, **92**, 1001–1010.

Nie G, Hendrix DL, Webber AN, Kimball BA, Long SP (1995) Increased accumulation of carbohydrates and decreased photosynthetic gene transcript levels in wheat grown at an elevated $CO_2$ concentration in the field. *Plant physiology*, **108**, 975–983.

Niu S, Zhang P, Liu J, Guo D, Zhang X (2012) The effect of temperature on the survival, growth, photosynthesis, and respiration of young seedlings of eelgrass *Zostera marina* L. *Aquaculture*, **350–353**, 98–108.

Nordlund LM, Koch EW, Barbier EB, Creed JC (2016) Seagrass ecosystem services and their variability across genera and geographical regions. *PLoS ONE*, **11**, 1–23.

Nunez-Farfan J, Fornoni J, Valverde P (2007) The evolution of resistance and tolerance to herbivores. *Annual Review of Ecology Evolution and Systematics*, **38**, 541–566.

Nykänen H, Koricheva J (2004) Damage-induced changes in woody plants and their effects on insect herbivore performance : a meta-analysis. *Oikos*, **104**, 247–268.

O'Connor MI (2009) Warming strengthens an herbivore-plant interaction. *Ecology*, **90**, 388–398.

## 6. References

O'Reilly-wapstra JM, Mcarthur C, Potts BM (2002) Genetic variation in resistance of *Eucalyptus globulus* to Marsupial browsers. *International Association for Ecology*, **130**, 289–296.

Olsen YS, Valiela I (2010) Effect of sediment nutrient enrichment and grazing on turtle grass Thalassia testudinum in Jobos Bay, Puerto Rico. *Estuaries and Coasts*, **33**, 769–783.

Olsen YS, Sánchez-Camacho M, Marbà N, Duarte CM (2012) Mediterranean seagrass growth and demography responses to experimental warming. *Estuaries and Coasts*, **35**, 1205–1213.

Orth RJ, Jr KLH, Tunbridge DJ (2002) Predation on seeds of the seagrass *Posidonia australis* in Western Australia. *Marine Ecology Progress Series*, **244**, 81–88.

Orth R, Carruthers T, Dennison W et al. (2006a) A global crisis for seagrass ecosystems. *Bioscience*, **56**, 987–996.

Orth R, Harwell M, Inglis G (2006b) Ecology of seagrass seeds and seagrass dispersal processes. In: *Seagrasses: Biology, Ecology and Conservation*, 1st edn (eds Larkum A, Orth RJ, Duarte CM), pp. 111–133. Springer.

Orth RJ, Kendrick GA, Marion SR (2007) *Posidonia australis* seed predation in seagrass habitats of Two Peoples Bay, Western Australia. *Aquatic Botany*, **86**, 83–85.

Östman Ö, Eklöf J, Eriksson BK, Olsson J, Moksnes PO, Bergström U, Cao Y (2016) Top-down control as important as nutrient enrichment for eutrophication effects in North Atlantic coastal ecosystems. *Journal of Applied Ecology*, **53**, 1138–1147.

Oviatt C (2004) The changing ecology of temperate coastal waters during a warming trend. *Journal*, **27**, 895–904.

Ow YX, Collier CJ, Uthicke S (2015) Responses of three tropical seagrass species to CO2 enrichment. *Marine Biology*, **162**, 1005–1017.

Ow YX, Vogel N, Holtum JAM, Flores F, Uthicke S (2016) Nitrate fertilisation does not enhance $CO_2$ responses in two tropical seagrass species. *Scientific Reports*, Under review.

Pagès JF, Farina S, Gera A, Arthur R, Romero J, Alcoverro T (2012) Indirect interactions in seagrasses: fish herbivores increase predation risk to sea urchins by modifying plant traits. *Functional Ecology*, **26**, 1015–1023.

## 6. References

Paige KN (1992) Overcompensation in response to mammalian herbivory: From mutualistic to antagonistic interactions. *Ecological society of America*, **73**, 2076–2085.

Palacios SL, Zimmerman RC (2007) Response of eelgrass *Zostera marina* to CO2 enrichment: Possible impacts of climate change and potential for remediation of coastal habitats. *Marine Ecology Progress Series*, **344**, 1–13.

Parmesan C (2006) Ecological and evolutionary responses to recent climate change. *Annual Review of Ecology and Systematics*, **37**, 637–669.

Paul V, Hay M (1986) Seaweed susceptibility to herbivory: chemical and morphological correlates . *Marine Ecology Progress Series*, **33**, 255–264.

Paul GS, Montagnini F, Berlyn GP, Craven DJ, van Breugel M, Hall JS (2012) Foliar herbivory and leaf traits of five native tree species in a young plantation of Central Panama. *New Forests*, **43**, 69–87.

Pedersen O, Colmer TD, Borum J, Zavala-Perez A, Kendrick GA (2016) Heat stress of two tropical seagrass species during low tides - impact on underwater net photosynthesis, dark respiration and diel in situ internal aeration. *New Phytologist*, **210**, 1207–1218.

Peeters PJ (2002) Correlations between leaf constituent levels and the densities of herbivorous insect guilds in an Australian forest. *Austral Ecology*, **27**, 658–671.

Pennings SC, Silliman BR (2005) Linking biogeography and community ecology : Latitudinal variation in plant – herbivore interaction strength. *Ecology*, **86**, 2310–2319.

Pennings SC, Siska EL, Bertness MD (2001) Latitudinal differences in plant palatability in Atlantic coast salt marshes. *Ecology*, **82**, 1344–1359.

Pennings SC, Zimmer M, Dias N et al. (2007) Latitudinal variation in plant-herbivore interactions in European salt marshes. *Oikos*, **116**, 543–549.

Peñuelas J, Llusia J (2004) Plant VOC emmisions: making use of the unavoidable. *Trends in Ecology and Evolution*, **19**, 402–404.

Peñuelas J, Estiarte M, LLusia J (1997) Carbon- based secondary compounds at elevated CO2. *Photosynthetica*, **33**, 313–316.

## 6. References

Pereira RC, Bianco EM, Bueno LB, De Oliveira MAL, Pamplona OS, Da Gama BAP (2010) Associational defense against herbivory between brown seaweeds. *Phycologia*, **49**, 424–428.

Pergent G, Bazairi H, Bianchi CN et al. (2014) Climate change and Mediterranean seagrass meadows: A synopsis for environmental managers. *Mediterranean Marine Science*, **15**, 462–473.

Perry AL, Low PL, Ellis JR, Reynolds JD (2005) Climate change and distribution shifts in marine fishes. *Science*, **308**, 1912–1915.

Petersen M (2003) Rosmarinic acid. *Phytochemistry*, **62**, 121–125.

Pierce NE, Berry A (2011) The Herbivore's dilemma: Never enough nitrogen. In: *In the Light of Evolution: Essays from the Laboratory and Field* (eds Losos JB, Quammen D, Browne J), pp. 121–133. Roberts & Co.

Pierrot D, Lewis E, Wallace DW. (2006) *MS Excel Program Developed for CO2 System CalculationsProgram developed for CO2 system calculations. ORNL/CDIAC-105a.* Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee.

Planes S, Raventos N, Ferrari B, Alcoverro T (2011) Fish herbivory leads to shifts in seagrass *Posidonia oceanica* investments in sexual reproduction. *Marine Ecology Progress Series*, **431**, 205–213.

Poore AGB, Campbell AH, Coleman RA et al. (2012) Global patterns in the impact of marine herbivores on benthic primary producers. *Ecology Letters*, **15**, 912–922.

Pörter HO, Farrell AP (2008) Ecology, physiology and climate change. *Science*, **322**, 690–692.

Pörtner HO, Langenbuch M, Reipschläger A (2004) Biological impact of elevated ocean CO2 concentrations: Lessons from animal physiology and earth history. *Journal of Oceanography*, **60**, 705–718.

Post E, Pedersen C (2008) Opposing plant community responses to warming with and without herbivores. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 12353–12358.

## 6. References

Prado P, Tomas F, Alcoverro T, Romero J (2007) Extensive direct measurements of *Posidonia oceanica* defoliation confirm the importance of herbivory in temperate seagrass meadows. *Marine Ecology Progress Series*, **340**, 63–71.

Prado P, Romero J, Alcoverro T (2010) Nutrient status, plant availability and seasonal forcing mediate fish herbivory in temperate seagrass beds. *Marine Ecology Progress Series*, **409**, 229–239.

Preen A (1995) Impacts of dugong foraging on seagrass habitats: observational and experimental evidence for cultivation grazing. *Marine Ecology Progress Series*, **124**, 201–213.

Prince JS, LeBlanc WG, Maciá S (2004) Design and analysis of multiple choice feeding preference data. *Oecologia*, **138**, 1–4.

Pritchard SG, Rogers HH, Prior SA, Peterson CM (1999) Elevated CO2 and plant structure: A review. *Global Change Biology*, **5**, 807–837.

Le Quéré C, Raupach MR, Canadell JG et al. (2009) Trends in the sources and sinks of carbon dioxide. *Nature Geoscience*, **2**, 831–836.

R Core Team (2007) R: a language and environment for statistical Computing.

Raffaelli D, Hawkins S (1999) *Intertidal Ecology* (eds Raffaelli D, Hawkins S). The Nederlandskluwer Academic Publishers.

Ralph PJ (1998) Photosynthetic response of laboratory-cultured *Halophila ovalis* to thermal stress. *Marine Ecology Progress Series*, **171**, 123–130.

Ramírez-Valiente JA, Valladares F, Sánchez-Gómez D, Delgado A, Aranda I (2014) Population variation and natural selection on leaf traits in cork oak throughout its distribution range. *Acta Oecologica*, **58**, 49–56.

Rasmann S, Agrawal A a. (2011) Latitudinal patterns in plant defense: Evolution of cardenolides, their toxicity and induction following herbivory. *Ecology Letters*, **14**, 476–483.

Rasmann S, Chassin E, Bilat J, Glauser G, Reymond P (2015) Trade-off between constitutive and inducible resistance against herbivores is only partially explained by gene expression and glucosinolate production. *Journal of Experimental Botany*, **66**, 2527–2534.

## 6. References

Rausher MD, Iwao K, Simms EL, Ohsaki N, Hall D (1993) Induced Resistance in *Ipomoea Purpurea*. *Ecology*, **74**, 20–29.

Reich PB, Oleksyn J (2004) Global patterns of plant leaf N and P in relation to temperature and latitude. *Proceedings of the National Academy of Sciences*, **101**, 11001–11006.

Reusch TBH, Ehlers A, Ha A (2005) Ecosystem recovery after climatic extremes enhanced by genotypic diversity.

Reynolds HL, D'Antonio C (1996) The ecological significance of plasticity in root weight ratio in response to nitrogen: Opinion. *Plant and Soil*, **185**, 75–97.

Rhoades DF (1979) Evolution of plant chemical defense against herbivores. In: *Herbivores, Their Interactions with Secondary Plant Metabolites: The chemical participants* (eds Rosenthal G, Janzen D), p. 353. Academic Press, Nwe York.

Ridley HN (1930) *The dispersal of plants throughout the world*. Reeve & Company, Ashford, U.K., 744 pp.

Rivers DO, Short FT (2007) Effect of grazing by Canada geese *Branta canadensis* on an intertidal eelgrass *Zostera marina* meadow. *Marine Ecology Progress Series*, **333**, 271–279.

Robertson AI, Mann KH (1984) Disturbance by ice and life-history adaptations of the seagrass Zostera marina. *Marine Biology*, **80**, 131–141.

Robinson E a, Ryan GD, Newman JA (2012) A meta-analytical review of the effects of elevated CO 2 on plant – arthropod interactions highlights the importance of interacting environmental and biological variables. *New Phytologist*, **194**, 321–336.

Rodolfo-Metalpa R, Lombardi C, Cocito S, Hall-Spencer JM, Gambi MC (2010) Effects of ocean acidification and high temperatures on the bryozoan *Myriapora truncata* at natural CO2 vents. *Marine Ecology*, **31**, 447–456.

Rogers G, Milham P, Gillings M, Conroy J (1996) Sink strength may be the key to growth and nitrogen responses in N-deficient wheat at elevated CO2. *Functional Plant Biology*, **23**, 253–264.

Romero J, Lee K, Marta P, Mateo MA, Alcoverro T (2006) Nutrient dynamics in sSeagrass ecosystems. In: *Seagrasses: Biology, Ecology and Conservation* (eds Larkum A, Orth R,

## 6. References

Duarte C), pp. 227–254. Springer.

Romero GQ, Gonçalves-Souza T, Vieira C, Koricheva J (2014) Ecosystem engineering effects on species diversity across ecosystems: A meta-analysis. *Biological Reviews*, **90**, 877–890.

Rosenblatt AE, Schmitz OJ (2016) Climate change, nutrition, and bottom-up and top-down food web processes. *Trends in Ecology and Evolution*, **31**, 965–975.

Rosenthal GA, Berenbaum MR (1992) *Herbivores: their interactions with secondary metabolites*, 2nd edn (eds Rosenthal GA, Berenbaum MR). Academic Press, New York, 492 pp.

Rosenthal JP, Kotanen PM (1994) Terrestrial plant tolerance to herbivory. *Trends in Ecology & Evolution*, **9**, 145–148.

Rowell RM, Stout HP (2006) Jute and kenaf. In: *Handbook of Fibre Chemistry*, 3rd edn (ed Lewin M), pp. 405–452. CRC Press, Boca Raton.

Rudgers JA, Whitney KD (2006) Interactions between insect herbivores and a plant architectural dimorphism. *Journal of Ecology*, **94**, 1249–1260.

Ruíz JM, Pérez M, Romero J, Tomas F (2009) The importance of herbivory in the decline of a seagrass (*Posidonia oceanica*) meadow near a fish farm: an experimental approach. *Botanica Marina*, **52**, 449–458.

Ruiz-R N, Ward D, Saltz D (2008) Leaf compensatory growth as a tolerance strategy to resist herbivory in *Pancratium sickenbergeri*. *Plant Ecology*, **198**, 19–26.

Russell BD, Connell SD, Uthicke S, Muehllehner N, Fabricius KE, Hall-Spencer JM (2013) Future seagrass beds: Can increased productivity lead to increased carbon storage? *Marine Pollution Bulletin*, **73**, 463–469.

Sabeena Farvin KH, Jacobsen C (2013) Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. *Food Chemistry*, **138**, 1670–1681.

Saiz H, Alados CL (2012) Changes in semi-arid plant species associations along a livestock grazing gradient. *PLoS ONE*, **7**, 1–9.

Salminen JP, Lahtinen M, Lempa K, Kapari L, Haukioja E, Pihlaja K (2004) Metabolic modifications of birch leaf phenolics by an herbivorous insect: Detoxification of flavonoid aglycones via

glycosylation. *Zeitschrift fur Naturforschung - Section C Journal of Biosciences*, **59**, 437–444.

Salo T, Pedersen MF (2014) Synergistic effects of altered salinity and temperature on estuarine eelgrass (*Zostera marina*) seedlings and clonal shoots. *Journal of Experimental Marine Biology and Ecology*, **457**, 143–150.

Sanmartí N, Saiz L, Llagostera I, Pérez M, Romero J (2014) Tolerance responses to simulated herbivory in the seagrass *Cymodocea nodosa*. *Marine Ecology Progress Series*, **517**, 159–169.

Sanson G (2006) Biomechanics of Browsing and Grazing. *American Journal of Botany*, **93**, 1531–1545.

Sardans J, Peñuelas J, Rodà F (2006) Plasticity of leaf morphological traits, leaf nutrient content, and water capture in the Mediterranean evergreen oak *Quercus ilex subsp. ballota* in response to fertilization and changes in competitive conditions. *Ecoscience*, **13**, 258–270.

Sarukhan J, Whyte A (2005) *Ecosystems and human well-being: Synthesis* (eds Sarukhan J, Whyte A). Island Press, Washington , D.C., 137 pp.

Schemske DW, Mittelbach GG, Cornell H V, Sobel JM, Roy K (2009) Is there a latitudinal gradient in the importance of biotic interactions? *Annual Review of Ecology Evolution and Systematics*, **40**, 245–269.

Schiestl FP, Kirk H, Bigler L, Cozzolino S, Desurmont GA (2014) Herbivory and floral signaling: Phenotypic plasticity and tradeoffs between reproduction and indirect defense. *New Phytologist*, **203**, 257–266.

Schrader J, Bohlmann J (2015) *Biosynthesis and biological functions of terpenoids in plants.*, Vol. 148 (eds Schrader J, Bohlmann J). Springer, 470 pp.

Schupp PJ, Paul VJ (1994) Calcium carbonate and secondary metabolites in tropical seaweeds : Variable effects on herbivorous fishes. *Ecology*, **75**, 1172–1185.

Schwing FB, Mendelssohn R (1997) Increased coastal upwelling in the California Current System. **102**, 3421–3438.

Shafer DJ, Wyllie-Echeverria S, Sherman TD (2008) The potential role of climate in the distribution and zonation of the introduced seagrass *Zostera japonica* in North America.

## 6. References

*Aquatic Botany*, **89**, 297–302.

Shaltout M, Omstedt A (2014) Recent sea surface temperature trends and future scenarios for the Mediterranean Sea. *Oceanologia*, **56**, 411–443.

Short FT (1987) Effects of sediment nutrients on seagrasses: literature review a n d mesocosm experiment. *Aquatic Botany*, **27**, 41–57.

Shurin JB, Borer ET, Seabloom EW et al. (2002) A cross-ecosystem comparison of the strength of trophic cascades. *Ecology Letters*, **5**, 785–791.

Sigleo AC, Mordy CW, Stabeno P, Frick WE (2005) Nitrate variability along the Oregon coast : Estuarine-coastal exchange. **64**, 211–222.

Silliman BR, Mccoy MW, Angelini C, Holt RD, Griffin JN, Koppel J Van De (2013) Consumer fronts, global change, and runaway collapse in ecosystems. *Annual Review of Ecology, Evolution, and Systematics*, **44**, 503–538.

Siska EL, Pennings SC, Buck T., Hanisak MD (2002) Latitudinal variation in palatability of salt-marsh plants: which traits are responsible? *Ecology*, **83**, 3369–3381.

Smilanich AM, Fincher RM, Dyer LA (2016) Does plant apparency matter? Thirty years of data provide limited support but reveal clear patterns of the effects of plant chemistry on herbivores. *New Phytologist*, **210**, 1044–1057.

Van Soest PJ, Robertson JB, Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of dairy science*, **74**, 3583–3597.

Solomon S, Plattner G-K, Knutti R, Friedlingstein P (2009) Irreversible climate change due to carbon dioxide emissions. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 1704–9.

van Staalduinen M a, Anten NPR (2005) Differences in the compensatory growth of two co-occurring grass species in relation to water availability. *Oecologia*, **146**, 190–199.

Stachowicz JJ (2001) Mutualism, facilitation, and the structure of ecological communities. *BioScience*, **51**, 235–246.

## 6. References

Stamp N (2003) Out of the quagmire of plant defense hypotheses. *The Quarterly review of biology*, **78**, 23–55.

Stark S, Väisänen M, Ylänne H, Julkunen-Tiitto R, Martz F (2015) Decreased phenolic defence in dwarf birch (*Betula nana*) after warming in subarctic tundra. *Polar Biology*, **38**, 1993–2005.

Statton J, Kendrick GA, Dixon KW, Cambridge ML (2014) Inorganic nutrient supplements constrain restoration potential of seedlings of the seagrass, *Posidonia australis*. *Restoration Ecology*, **22**, 196–203.

Steinberg PD (2006) Effects of quantitative and qualitative variation in phenolic compounds on feeding in three species of marine invertebrate herbivores. **120**, 221–237.

Steneck RS, Graham MH, Bourque BJ, Corbett D, Erlandson JM, Estes JA, Tegner MJ (2002) Kelp forest ecosystems: biodiversity, stability, resilience and future. *Environmental Conservation*, **29**.

Stevens MT, Kruger EL, Lindroth RL (2008) Variation in tolerance to herbivory is mediated by differences in biomass allocation in aspen. *Functional Ecology*, **22**, 40–47.

Stiling P, Cornelissen T (2007) How does elevated carbon dioxide (CO2) affect plant–herbivore interactions? A field experiment and meta-analysis of CO2 -mediated changes on plant chemistry and herbivore performance. *Global Change Biology*, **13**, 1823–1842.

Stowe KA, Marquis RJ, Hochwender CG et al. (2000) The evolutionary ecology of tolerance to consumer damage. *Annual Review of Ecology and Systematics*, **31**, 565–595.

Strauss SY, Agrawal AA (1999) The ecology and evolution of plant tolerance to herbivory. *Trends in Ecology and Evolution*, **14**, 179–185.

Strauss SY, Rudgers JA, Lau JA, Irwin RE (2002) Direct and ecological costs of resistance to herbivory. *Trends in Ecology and Evolution*, **17**, 278–285.

Stumm W, Morgan JJ (1996) *Aquatic Chemistry: Chemical Equilibria and Rates in Natural Waters*, 3rd edn. Wiley, New York.

Suchan T, Alvarez N (2015) Fifty years after Ehrlich and Raven, is there support for plant-insect coevolution as a major driver of species diversification? *Entomologia Experimentalis et Applicata*, **157**, 98–112.

## 6. References

Sudatti DB, Fujii MT, Rodrigues SV, Turra A, Pereira RC (2011) Effects of abiotic factors on growth and chemical defenses in cultivated clones of *Laurencia dendroidea* J. Agardh (Ceramiales, Rhodophyta). *Marine Biology*, **158**, 1439–1446.

Sunday JM, Pecl GT, Frusher S et al. (2015) Species traits and climate velocity explain geographic range shifts in an ocean-warming hotspot. *Ecology Letters*, **18**, 944–953.

Takahashi M, Noonan SHC, Fabricius KE, Collier CJ (2016) The effects of long term in-situ CO2 enrichment on tropical seagrass communities at volcanic vents. *ICES Journal of Marine Sciences*, **73**, 876–886.

Terborgh J, Lopez L, Nuñez P et al. (2001) Ecological meltdown in predator-free forest fragments. *Science*, **294**, 1923–1926.

Terrados J, Ros JD (1995) Temperature effects on photosynthesis and depth distribution of the seagrass *Cymodocea nodosa* (Ucria) Ascherson in a Mediterranean coastal lagoon: the Mar Menor (SE Spain). *Marine Ecology*, **6**, 133–144.

Tetlow IJ, Morell MK, Emes MJ (2004) Recent developments in understanding the regulation of starch metabolism in higher plants. *Journal of Experimental Botany*, **55**, 2131–2145.

Thaler JS (1999) Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature*, **399**, 686–688.

Thayer GW, Bjorndal KA, Ogden JC, Williams SL, Zieman FC (1984) Role of larger herbivores in seagrass community. *Estuaries*, **7**, 351–376.

Thompson JN (1988) Variation in Interspecific Interactions. *Annual Review of Ecology and Systematics*, **19**, 65–87.

Throop HL, Lerdau MT (2004) Effects of nitrogen deposition on insect herbivory: implications for community and ecosystem processes. *Ecosystems*, **7**, 109–133.

Tiffin P (2000) Mechanisms of tolerance to herbivore damage: What do we know? *Evolutionary Ecology*, **14**, 523–536.

Tomas F, Turon X, Romero J (2005a) Effects of herbivores on a *Posidonia oceanica* seagrass meadow: importance of epiphytes. **287**, 115–125.

## 6. References

Tomas F, Turon X, Romero J (2005b) Seasonal and small-scale spatial variability of herbivory pressure on the temperate seagrass *Posidonia oceanica*. *Marine Ecology Progress Series*, **301**, 95–107.

Tomas F, Abbott JM, Steinberg C, Balk M, Williams SL, Stachowicz JJ (2011) Plant genotype and nitrogen loading influence seagrass productivity, biochemistry, and plant-herbivore interactions. *Ecology*, **92**, 1807–17.

Tomas F, Martinez-Crego B, Hernan G, Santos R (2015) Responses of seagrass to anthropogenic and natural disturbances do not equally translate to its consumers. *Global Change Biology*, **21**, 1–10.

Tomlinson PB (1974) Vegetative morphology and meristem dependence - The foundation of productivity in seagrasses. *Aquaculture*, **4**, 107–130.

Toth GB, Pavia H (2000) Water-borne cues induce chemical defense in a marine alga (*Ascophyllum nodosum*). *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 14418–14420.

Toth GB, Pavia H (2007) Induced herbivore resistance in seaweeds: a meta-analysis. *Journal of Ecology*, **95**, 425–434.

Touchette BW, Burkholder JM (2000a) Review of nitrogen and phosphorus metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology*, **250**, 133–167.

Touchette BW, Burkholder JAM (2000b) Overview of the physiological ecology of carbon metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology*, **250**, 169–205.

Tuomi J, Niemelä P, Stuart Chapin III F, Bryant JP, Sirén S (1988) Defensive responses of trees in relation to their carbon/nutrient balance. In: *Mechanisms of Woody Plant Defenses Against Insects: search for pattern* (eds Mattson WJ, Levieux J, Bernard-Dagan C), pp. 52–72. Springer-Verlag, Berlin.

van Tussenbroek BI, Muhlia-Montero M (2013) Can floral consumption by fish shape traits of seagrass flowers? *Evolutionary Ecology*, **27**, 269–284.

Underwood AJ, Jernakoff P (1984) The effects of tidal height, wave-exposure, seasonality and

rock-pools on grazing and the distribution of intertidal macroalgae in New South Wales. *Journal of Experimental Marine Biology and Ecology*, **75**, 71–96.

Valentine J, Duffy JE (2006) The central role of grazing in seagrass ecology. In: *Seagrasses: Biology, Ecology and Conservation* (eds Larkum A, Orth R, Duarte C), pp. 463–501. Springer.

Valentine J, Heck K (1999) Seagrass herbivory:evidence for the continued grazing of marine grasses. *Marine Ecology Progress Series*, **176**, 291–302.

Valentine JF, Heck KL (2001) The role of leaf nitrogen content in determining turtlegrass (*Thalassia testudinum*) grazing by a generalized herbivore in the northeastern Gulf of Mexico. *Journal of experimental marine biology and ecology*, **258**, 65–86.

Valentine JF, Blythe EF, Madhavan S, Sherman TD (2004) Effects of simulated herbivory on nitrogen enzyme levels, assimilation and allocation in *Thalassia testudinum*. *Aquatic Botany*, **79**, 235–255.

Vergeer LHT, Develi A (1997) Phenolic acids in healthy and infected leaves of *Zostera marina* and their growth-limiting properties towards *Labyrinthula zosterae*. *Aquatic Botany*, **58**, 65–72.

Vergés A, Becerro M, Alcoverro T, Romero J (2007a) Experimental evidence of chemical deterrence against multiple herbivores in the seagrass *Posidonia oceanica*. *Marine Ecology Progress Series*, **343**, 107–114.

Vergés A, Becerro M a, Alcoverro T, Romero J (2007b) Variation in multiple traits of vegetative and reproductive seagrass tissues influences plant-herbivore interactions. *Oecologia*, **151**, 675–86.

Vergés A, Pérez M, Alcoverro T, Romero J (2008) Compensation and resistance to herbivory in seagrasses: induced responses to simulated consumption by fish. *Oecologia*, **155**, 751–60.

Vergés A, Vanderklift MA, Doropoulos C, Hyndes GA (2011a) Spatial patterns in herbivory on a coral reef are influenced by structural complexity but not by algal traits. *PLoS ONE*, **6**.

Vergés A, Alcoverro T, Romero J (2011b) Plant defences and the role of epibiosis in mediating within-plant feeding choices of seagrass consumers. *Oecologia*, **166**, 381–90.

Vergés A, Tomas F, Ballesteros E (2012) Interactive effects of depth and marine protection on predation and herbivory patterns. *Marine Ecology Progress Series*, **450**, 55–65.

## 6. References

Vergés A, Steinberg PDT tropicalization of temperate marine ecosystems: climate-mediated changes in herbivory and community phase shifts, Hay ME et al. (2014a) The tropicalization of temperate marine ecosystems: climate-mediated changes in herbivory and community phase shifts. *Proceedings of The Royal Socienty B*, **281**, 1–10.

Vergés A, Tomas F, Cebrian E et al. (2014b) Tropical rabbitfish and the deforestation of a warming temperate sea. *Journal of Ecology*, **102**, 1518–1527.

Vergés A, Doropoulos C, Malcolm HA et al. (2016) Long-term empirical evidence of ocean warming leading to tropicalization of fish communities, increased herbivory, and loss of kelp. *Proceedings of the National Academy of Sciences*, **113**, 13791–13796.

Viechtbauer W (2007) Confidence intervals for the amount of heterogeneity in meta-analysis. *Statistics in Medicine*, **26**, 37–52.

Voigt W, Perner J, Davis AJ et al. (2003) Trophic levels are differentially sensitive to climate. *Ecology*, **84**, 2444–2453.

Wahl M, Hay ME (1995) Associational resistance and shared doom: effects of epibiosis on herbivory. *Oecologia*, **102**, 329–340.

Wahl M, Hoppe K (2002) Interactions between substratum rugosity , colonization density and periwinkle grazing efficiency. *Marine Ecology Progress Series*, **225**, 239–249.

Walker DI, Cambridge ML (1995) An experimental assessment of the temperature responses of two sympatric seagrasses, *Amphibolis antarctica* and *Amphibolis griffithii*, in relation to their biogeography. *Hydrobiologia*, **302**, 63–70.

Ward DH, Reed A, Sedinger JS, BLack JM, Derksen D V., Castelli PM (2005) North American Brant: Effects of changes in habitat and climate on population dynamics. *Global Change Biology*, **11**, 869–880.

Watanabe JM, Harrold C (1991) Destructive grazing by sea urchins *Strongylocentrotus spp.* in a central California kelp forest: potential roles of recruitment, depth, and predation. *Marine Ecology Progress Series*, **71**, 125–141.

Watkinson AR (1997) Plant population dynamics. In: *Plant Ecology*, 2nd edn (ed Crawley MJ), pp. 359–400. Blackwell Scientific Publications, Oxford.

## 6. References

Waycott M, Duarte CM, Carruthers TJB et al. (2009) Accelerating loss of seagrasses across the globe threatens coastal ecosystems. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 12377–81.

Wernberg T, Smale DA, Tuya F et al. (2013) An extreme climatic event alters marine ecosystem structure in a global biodiversity hotspot. *Nature Climate Change*, **3**, 78–82.

West DC, Post DM (2016) Impacts of warming revealed by linking resource growth rates with consumer functional responses. *Journal of Animal Ecology*, **85**, 671–680.

White JF, Torres MS, Johnson H, Irizarry I, Tadych M (2014) A functional view of plant microbes: Endosymbiotic systems that enhance plant growth and. In: *Advances in Endophytic Research* (eds Verma VC, Gange AC), pp. 425–440. Springer.

Widmer TL, Laurent N (2006) Plant extracts containing caffeic acid and rosmarinic acid inhibit zoospore germination of *Phytophthora spp.* pathogenic to *Theobroma cacao*. *European Journal of Plant Pathology*, **115**, 377–388.

Wieski K, Pennings S (2014) Latitudinal variation in resistance and tolerance to herbivory of a salt marsh shrub. *Ecography*, **37**, 763–769.

Wigley TML, Richels R, Edmonds JA (1996) Economic and environmental choices in the stabilization of atmospheric CO2 concentrations. *Nature*, **379**, 240–243.

Williams JHH, Farrar JF (1990) Control of barley root respiration. *Physiologia Plantarum*, **79**, 259–266.

Williams K, Percival F, Merino J, Mooney H a (1987) Estimation of tissue construction cost from heat of combustion and organic nitrogen content. *Plant Cell and Environment*, **10**, 725–734.

Willlams SL, Heck KL (2001) Seagrass community ecology. In: *Marine Community Ecology* (eds McNeely DL, Bertness MD, Gaines SD, Hay ME), pp. 317–337. Sinauer Associates, Inc., Sunderland, MA.

Wise MJ, Abrahamson WG (2005) Beyond the compensatory continuum: Environmental resource levels and plant tolerance of herbivory. *Oikos*, **109**, 417–428.

Wise MJ, Abrahamson WG (2007) Effects of resource availability on tolerance of herbivory: a review and assessment of three opposing models. *The American Naturalist*, **169**, 443–454.

## 6. References

Wise MJ, Abrahamson WG (2008) Applying the limiting resource model to plant tolerance of apical meristem damage. *The American naturalist*, **172**, 635–47.

Wisz MS, Pottier J, Kissling WD et al. (2013) The role of biotic interactions in shaping distributions and realised assemblages of species: Implications for species distribution modelling. *Biological Reviews*, **88**, 15–30.

Witman JD (1987) Subtidal coexistence: storms, grazing, mutualism, and the zonation of kelps and mussels. *Ecological Monographs*, **57**, 167–187.

Wood KA, O'Hare MT, Mcdonald C, Searle KR, Daunt F, Stillman RA (2016) Herbivore regulation of plant abundance in aquatic ecosystems. *Biological Reviews*.

Wright S, Keeling J, Gillman L (2006) The road from Santa Rosalia: a faster tempo of evolution in tropical climates. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 7718–7722.

Yin X (2002) Responses of leaf nitrogen concentration and specific leaf area to atmospheric CO2 enrichment: A retrospective synthesis across 62 species. *Global Change Biology*, **8**, 631–642.

Yuan ZY, Chen HYH, Bay T (2015) Negative effects of fertilization on plant nutrient resorption. *Ecology*, **96**, 373–380.

Yun HY, Engelen AH, Santos RO, Molis M (2012) Water-borne cues of a non-indigenous Seaweed mediate grazer-deterrent responses in native seaweeds, but not vice versa. *PLoS ONE*, **7**.

Zangerl AR (2003) Evolution of induced plant responses to herbivores. *Basic and Applied Ecology*, **103**, 91–103.

Zavala JA, Nabity PD, DeLucia EH (2013) An Emerging Understanding of Mechanisms Governing Insect Herbivory Under Elevated CO(2). *Annual review of entomology*, 79–97.

Žemlička L, Fodran P, Lukeš V et al. (2014) Physicochemical and biological properties of luteolin-7-O-β-d- glucoside (cynaroside) isolated from *Anthriscus sylvestris* (L.) Hoffm. *Monatshefte fur Chemie*, **145**, 1307–1318.

Zhang P, Bakker ES, Zhang M, Xu J (2016a) Effects of warming on *Potamogeton crispus* growth and tissue stoichiometry in the growing season. *Aquatic Botany*, **128**, 13–17.

## 6. References

Zhang S, Zhang Y, Ma K, Shefferson R (2016b) Latitudinal variation in herbivory: hemispheric asymmetries and the role of climatic drivers. *Journal of Ecology*, **104**, 1089–1095.

Zheng S-J, Dicke M (2008) Ecological genomics of plant-insect interactions: from gene to community. *Plant physiology*, **146**, 812–817.

Zhou Q, Wang YH, Xu NY, Zhang CX, Zhou ZG, Chen BL (2009) Effects of air temperature on enzyme activities of cotton plants related to saccharide metabolism of cotton fiber. *Ying Yong Sheng Tai Xue Bao*, **20**, 149–156.

Zidorn C (2016) Secondary metabolites of seagrasses (Alismatales and Potamogetonales; Alismatidae): Chemical diversity, bioactivity, and ecological function. *Phytochemistry*, **124**, 5–28.

Zieman J (1974) Methods for the study of the growth and production of Turtlegrass, *Thalassia Testudinum* Konig. *Aquaculture*, **4**, 139–143.

Zimmerman RC, Smith RD, Alberte RS (1989) Thermal acclimation and whole-plant carbon balance in *Zostera marina* L. (eelgrass). *Journal of Experimental Marine Biology and Ecology*, **130**, 93–109.

Zimmerman RC, Kohrs DG, Steller DL, Alberte RS (1997) Impacts of CO2 Enrichment on productivity and light requirements of Eelgrass. *Plant physiology*, **115**, 599–607.

Zvereva EL, Kozlov M V. (2006) Consequences of simultaneous elevation of carbon dioxide and temperature for plant-herbivore interactions: a metaanalysis. *Global Change Biology*, **12**, 27–41.

# 7 Appendix

## 7.1 Supplementary Material Chapter 1: Latitudinal patterns of response to herbivory in a marine habitat-forming species
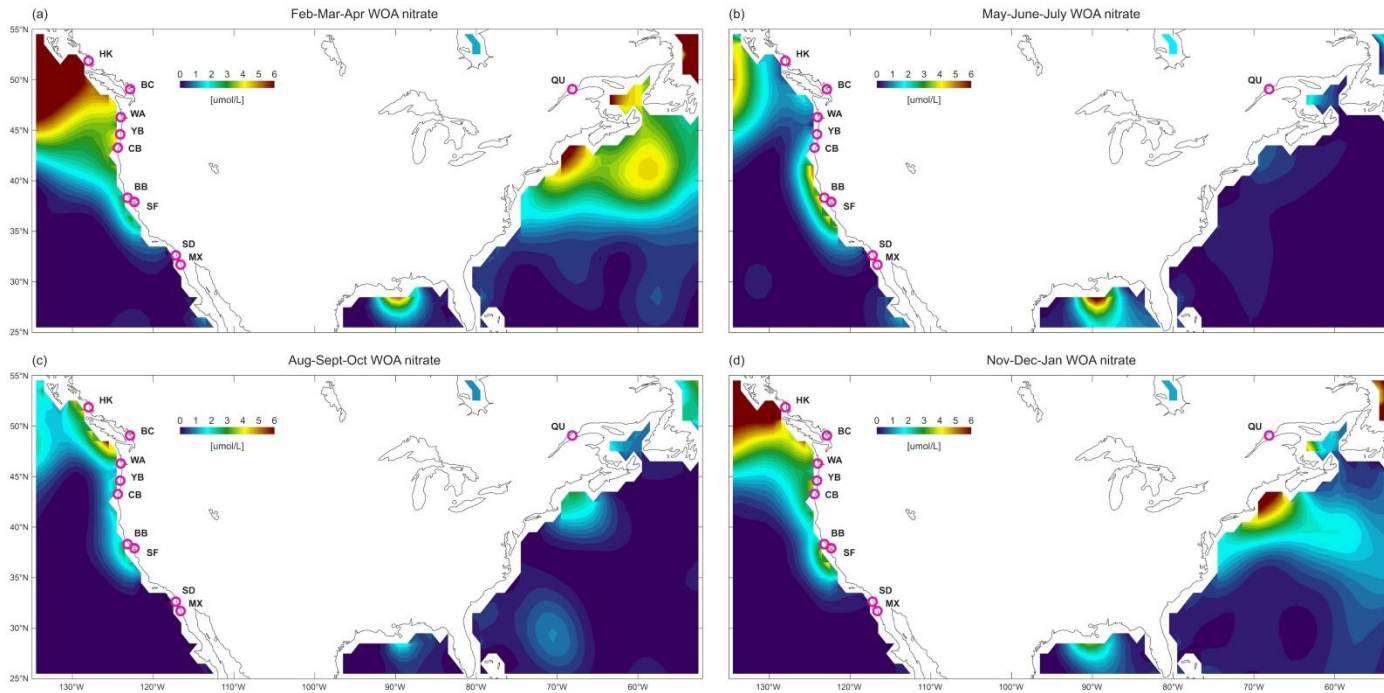


**Figure S.1.1**: Mean (a) February- March- April, (b) May-June-July, (c) August-September-October, (d) November-December-January nitrate concentration (μmol/L) from the 1878-2012 World Ocean Atlas data set. Study sites, Calvert (Hakai, Canada; HK), Baie St. Ludger (Quebec, Canada; QU), Tsawwaseen (British Columbia, Canada; BC), Willapa (Washington, United States; WA), Yaquina Bay (Oregon, US; YB), Coos Bay (Oregon, US; CB), West side Regional Park (Bodega Bay, California, US; BB), Point Molate (San Francisco, California, US; SF), Coronado (San Diego, California, US; SD) and Punta Banda Estuary (Ensenada, Baja California, Mexico; MX) are shown.
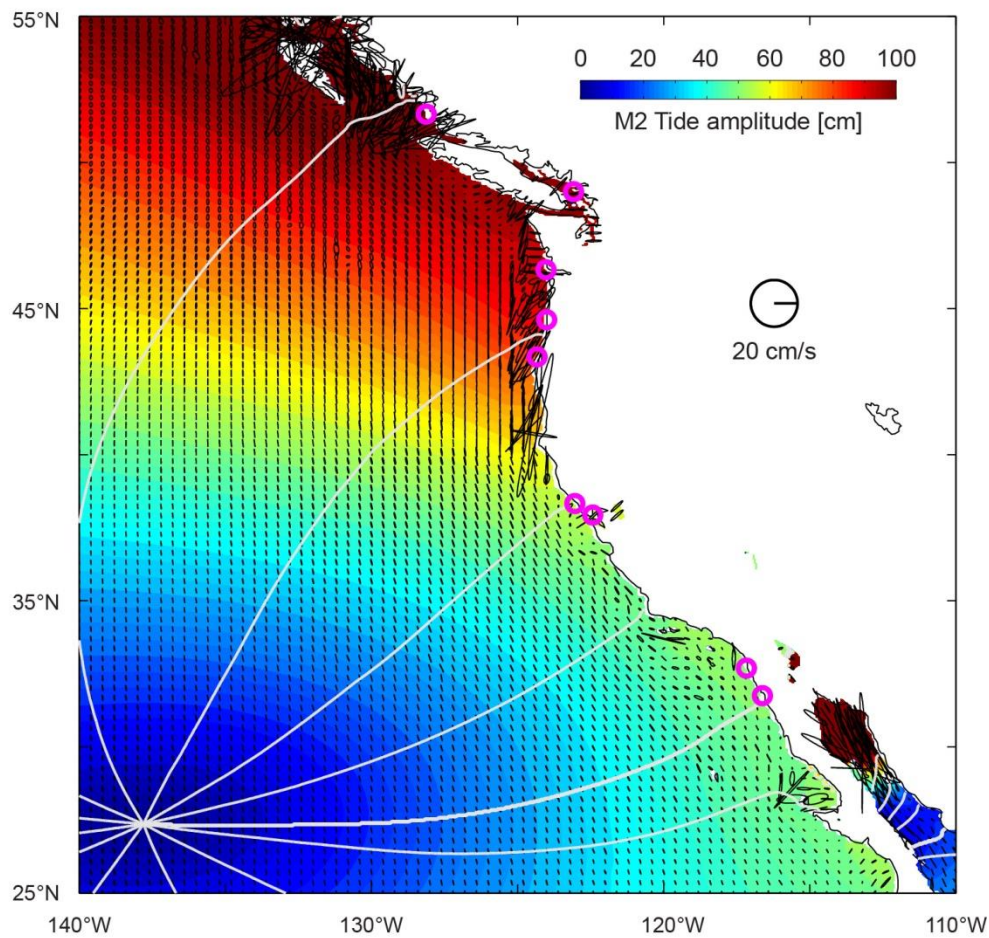
**Figure S.1.2**: Amplitude (color) and phase (white contours differing by 1 hour) of the main (M2) tidal component from the TPX0.7 data set and its associated current tidal ellipses (black ellipses) along the eastern Pacific.
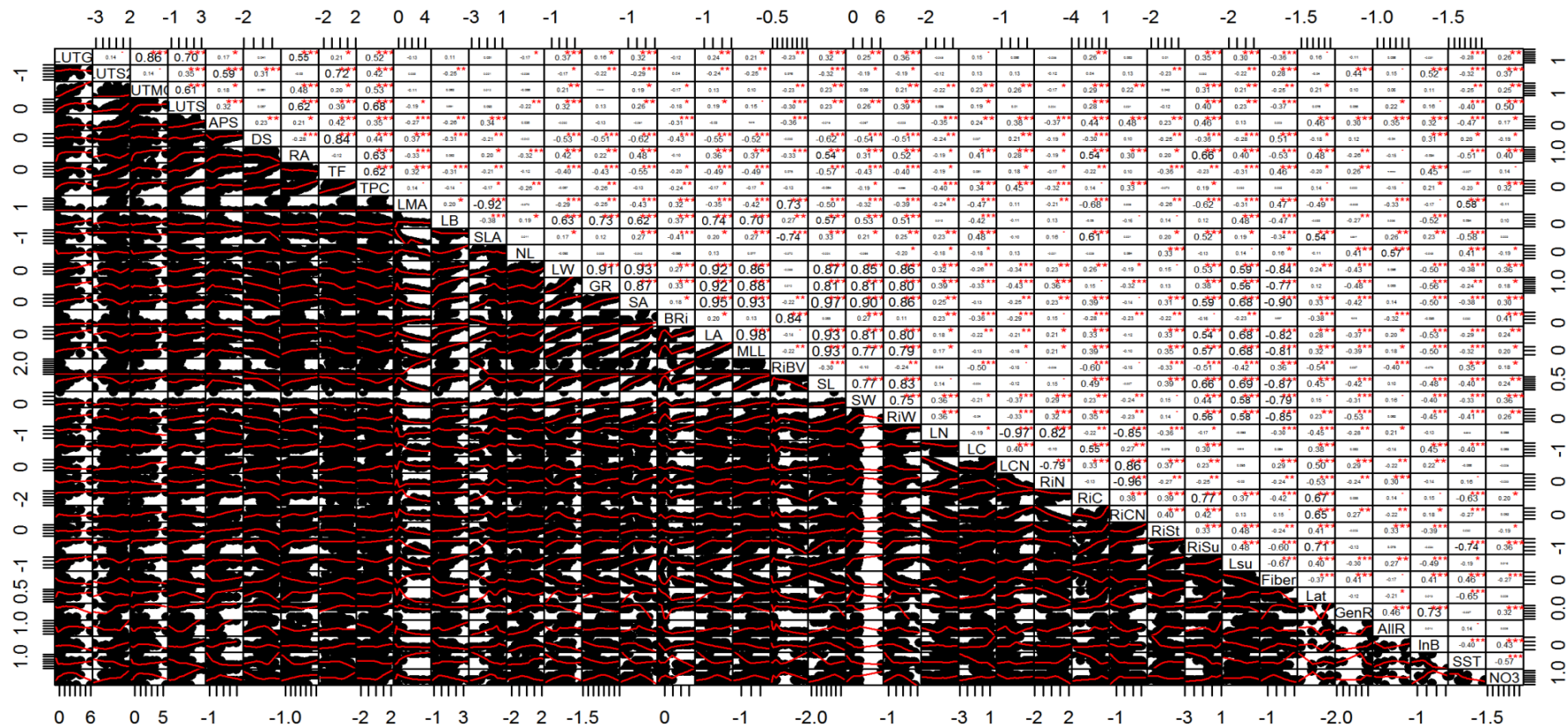
**Figure S.1.3.:** Correlation matrix of Flavonoids (LUTG,LUTS2,LUTMG,LUTS,APS,DS), Rosamrnic acid (RA), Total falvonoids (TF), total phenolic compounds (TPC), leaf mass area (LMA), leaf biomass (LB), specific leaf area (SLA), number of leaves (NL), leaf width (LW), growth rate (GR), sheath area (SA), biomass of rhizome per internode (BRi), leaf area (LA), maximum leaf length (MLL), Biomass per volume of rhizome (RiBV), sheath length (SL), sheath width (SW), Rhizome width (RiW), leaf nitrogen content (LN), leaf carbon content (LC), leaf C/N (LCN), rhizome nitrogen content (RiN),  rhizome carbon content (RiC), rhizome C/N (RiCN), rhizome starch content (RiSt),  rhizome sucrose content (RiSu), leaf sucrose content (LSu), leaf fiber (Fiber), latitude(Lat), genotypic richness (GenR), allelic richness (AllR), inbreeding (InB) and mean May-June-July sea surface temperature (SST) and nitrate (NO3). On top, absolute value of the pairwise correlations plus the result of the Pearson correlation test as stars. On bottom, bivariate scatterplots with fitted line in red.
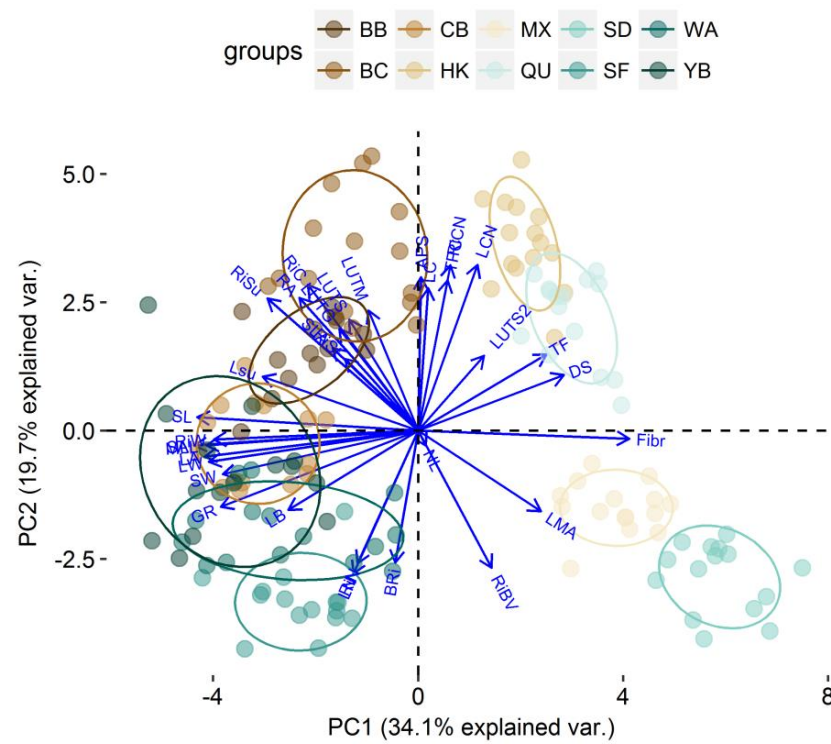
**Figure S.1.4.:** Principal Component Analysis. Arrows represent the loading of the variables. Dots represent the score of each replicate on PC1 and PC2.
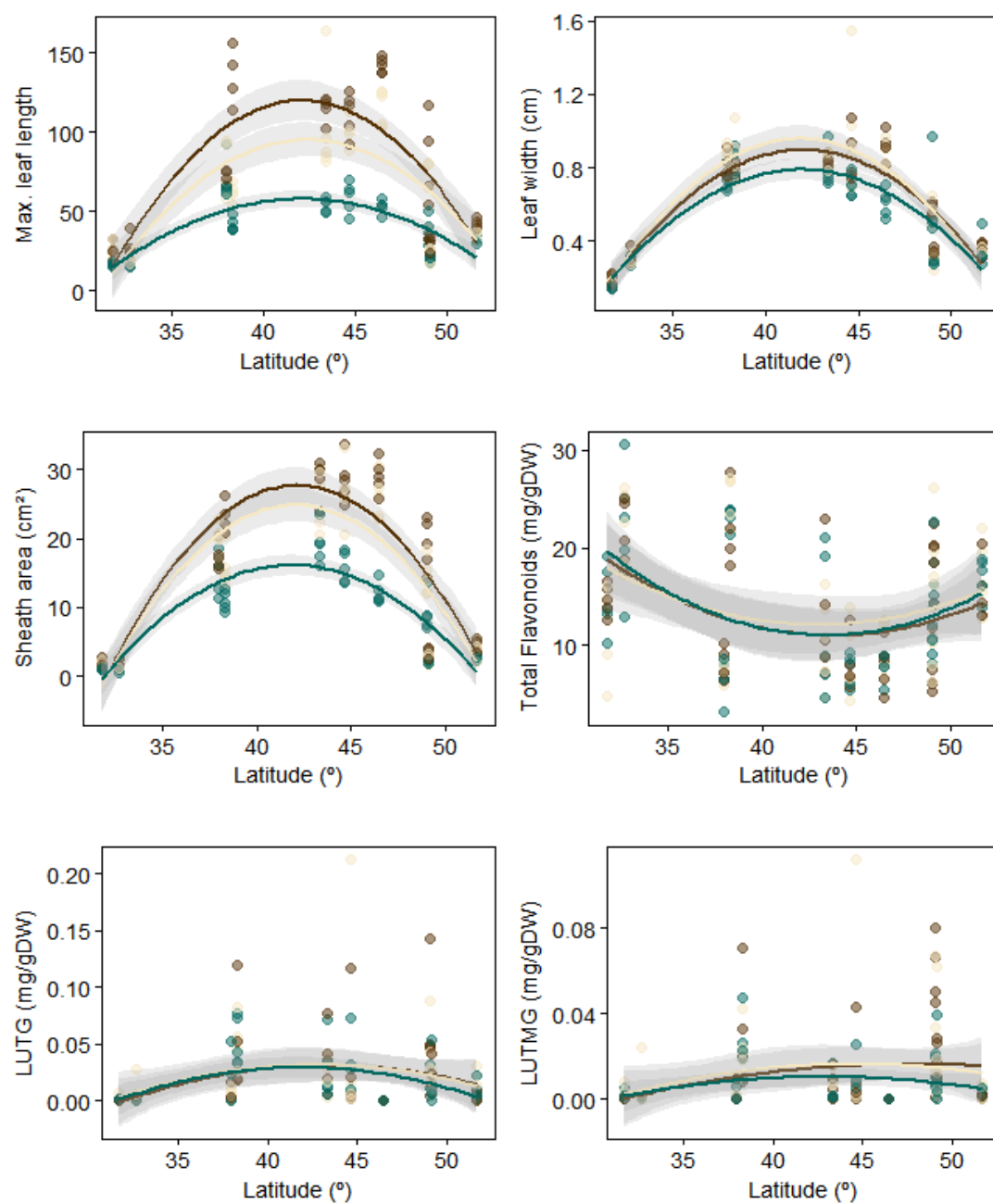
**Figure S.1.5.** Distribution of traits along the latitudinal gradient. Grey bands indicate 95% confidence boundaries for the control (C; dark brown), moderate (H; beige) and high (HH; green) herbivory treatment fitted curves (linear and quadratic models).
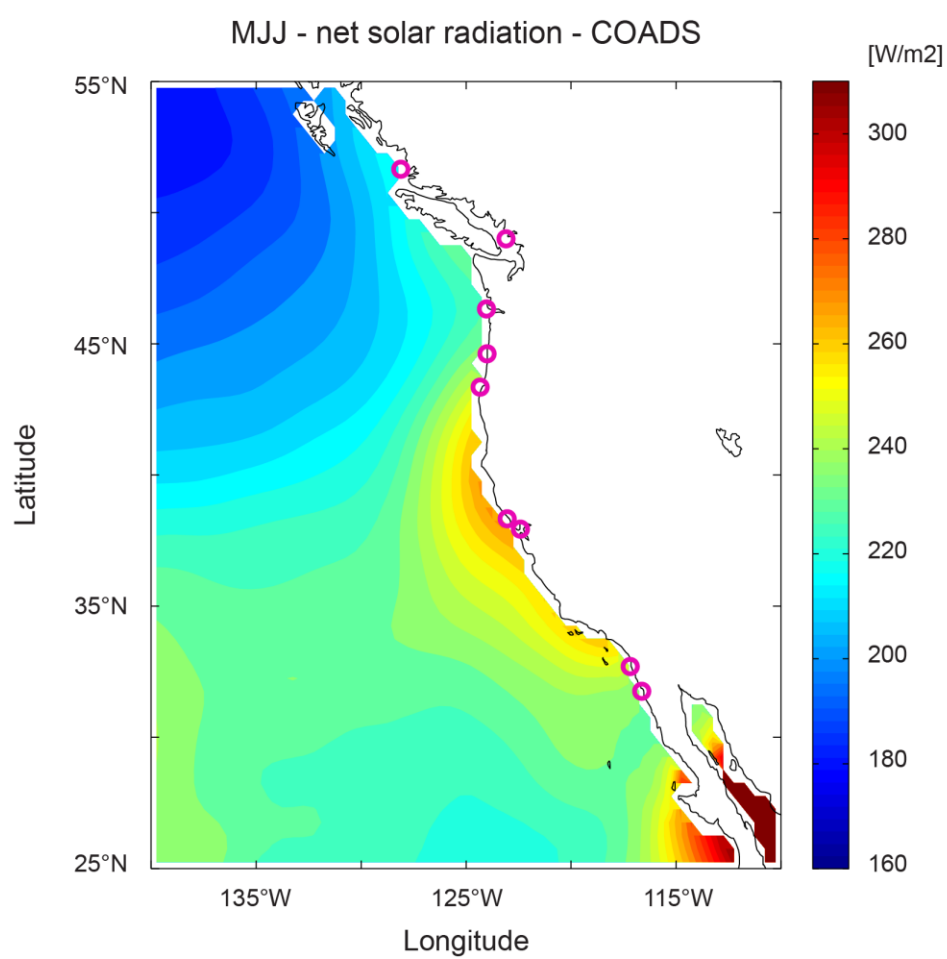
**Figure S.1.6.:** Mean May-June-July net surface solar radiation from the COADS data set in W/m2

## 7.2 Supplementary Material Chapter 2: Resource availability and herbivory damage do not interact to modulate plant defense strategies

**Table S.2.1**. Results of linear models on porewater nutrient concentrations (μM). Intercept (i) and slopes (β) for the fixed nutrient addition ($\beta_N$), herbivory simulation medium ($\beta_H$) and high level ($\beta_{HH}$), site ($\beta_{site}$) and their interactions (marked with asterisk). Log indicates log-transformed variables. Est, estimate; SE, standard error and p-value (P).

| Fixed effects | Nitrate+Nitrite | | | Ammonium | | | Ln (nitrite) | | | Ln (Phosphate) | | | Nitrate | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Est. | SE | P | Est. | SE | P | Est. | SE | P | Est. | SE | P | Est. | SE | P |
| *i* | 1.185 | 0.144 | **<0.001** | 4.950 | 0.976 | **<0.001** | -2.998 | 0.204 | **<0.001** | -0.105 | 0.229 | 0.651 | 1.134 | 0.132 | **<0.001** |
| $\beta_N$ | 1.270 | 0.204 | **<0.001** | 6.654 | 1.380 | **<0.001** | 1.354 | 0.289 | **<0.001** | 1.467 | 0.323 | **<0.001** | 1.103 | 0.187 | **<0.001** |
| $\beta_H$ | 0.326 | 0.204 | 0.122 | -3.640 | 1.380 | 0.014 | -0.340 | 0.289 | 0.251 | -0.064 | 0.323 | 0.846 | 0.406 | 0.187 | 0.050 |
| $\beta_{HH}$ | 0.165 | 0.204 | 0.425 | -1.127 | 1.380 | 0.422 | -0.269 | 0.289 | 0.361 | 0.084 | 0.323 | 0.798 | 0.212 | 0.187 | 0.267 |
| $\beta_{site}$ | 0.082 | 0.204 | 0.690 | -1.123 | 1.380 | 0.424 | 0.194 | 0.289 | 0.509 | -0.197 | 0.323 | 0.547 | 0.070 | 0.187 | 0.710 |
| $\beta_{N*H}$ | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| $\beta_{N*HH}$ | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| $\beta_{N*site}$ | 0.305 | 0.288 | 0.300 | -1.345 | 1.952 | 0.497 | -0.409 | 0.408 | 0.326 | -0.036 | 0.457 | 0.938 | 0.366 | 0.264 | 0.179 |
| $\beta_{H*site}$ | -0.306 | 0.288 | 0.298 | 2.383 | 1.952 | 0.234 | 0.302 | 0.408 | 0.467 | 0.197 | 0.457 | 0.670 | -0.382 | 0.264 | 0.161 |
| $\beta_{HH*site}$ | -0.376 | 0.288 | 0.204 | 0.013 | 1.952 | 0.995 | 0.089 | 0.408 | 0.830 | -0.364 | 0.457 | 0.434 | -0.385 | 0.264 | 0.158 |
| $\beta_{N*H*site}$ | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| $\beta_{N*HH*site}$ | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
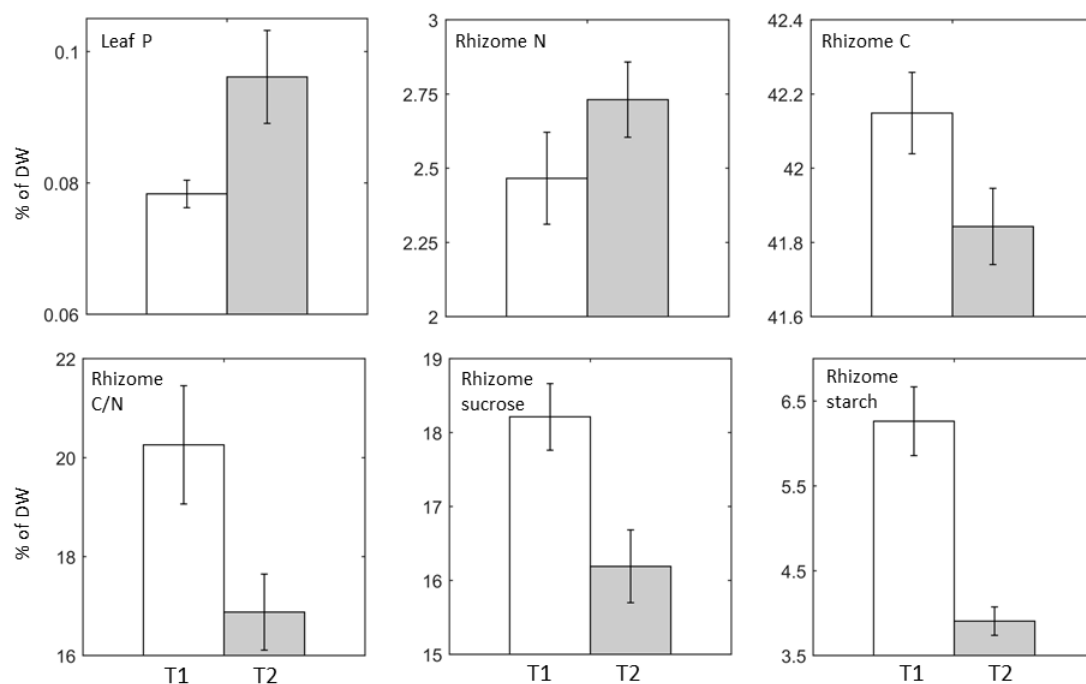
**Figure S.2.1.** Significant effects of time on plant traits. Mean contents of plant traits from plants measured at T1 (45 days after beginning the experiment; white) and T2 (90Days; grey). Error bars represent standard error (n=48).

## 7.3 Supplementary Material Chapter 3: Seagrass (*Posidonia oceanica*) seedlings in a high-CO2 world: from physiology to herbivory

**Table S.3.1**. Experimental conditions. Average (±SE) water conditions and carbonate system parameters of experimental aquaria (with seedlings, n = 7) and reference aquarium (without seedlings, n = 1) during incubations of seedlings of *P. oceanica* exposed to control and high $CO_2$ conditions for 90 days.

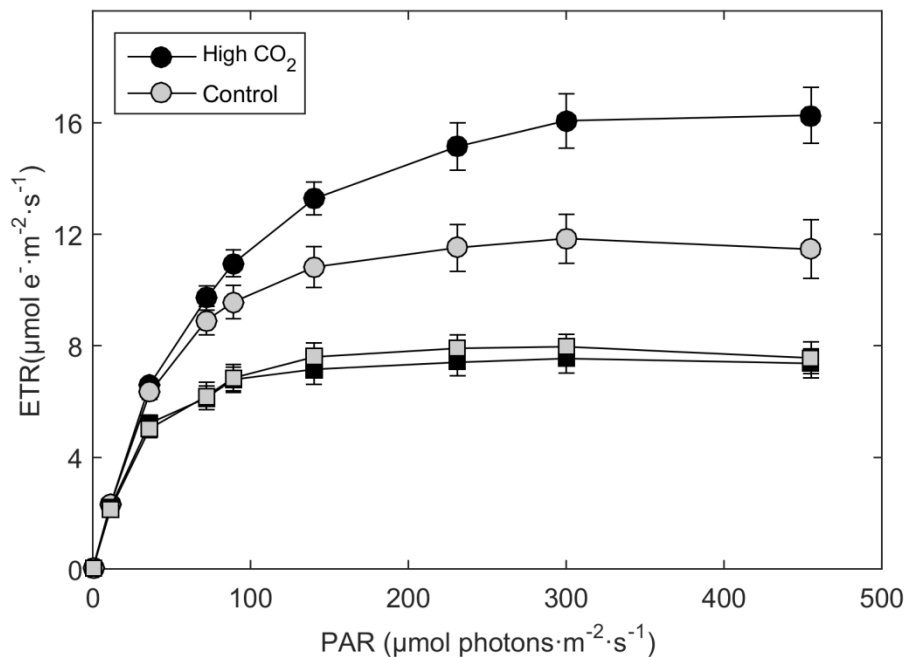| Variable | Control CO$_2$ | High CO$_2$ |
|---|---|---|
| Temperature (ºC) | 16.36 ⯑ 0.53 | 16.19 ⯑ 0.75 |
| Light (µmol s$^{-1}$ m$^{-2}$) | 71.07 ⯑ 5.32 | 73.59 ⯑ 5.69 |
| pH$_T$ (17ºC) | 8.06 ⯑ 0.02 | 7.61 ⯑ 0.02 |
| A$_T$ (mmol/kgSW) | 2984 ⯑ 29 | 2971 ⯑ 40 |
| $p$CO$_2$ (µatm) | 535 ⯑ 21 | 1574 ⯑ 47 |
| [CO$_3^{2-}$] (mmol/kgSW) | 233 ⯑ 7 | 97 ⯑ 5 |
| [HCO$_3^-$] (mmol/kgSW) | 2337 ⯑ 41 | 2743 ⯑ 29 |
| Ω $_{aragonite}$ | 3.54 ⯑ 0.11 | 1.47 ⯑ 0.07 |
| Ω $_{calcite}$ | 5.48 ⯑ 0.17 | 2.27 ⯑ 0.10 |

**Figure S.3.1. ETR-light curves**. Electron transport rate estimated from Rapid Light Curves performed in seedlings growing under high $CO_2$ (black) or control (grey) conditions after 60 (circles) and 90 (squares) days of experiment. Error bars indicate standard error.

## 7.4 Supplementary Material Chapter 4: Future warmer seas: increased stress and susceptibility to grazing in seedlings of a marine habitat-forming species

**Table S.4.1.** Mean and standard error (SE) values of temperature (Cº), salinity (ppt), light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$) and Oxygen saturation (%sat) measured for the different experimental treatments (25ºC, 27ºC and 29ºC) after the acclimation period (n = 10 aquaria per treatment).

| Treatment | Temperature (Cº) | | Salinity (ppt) | | Light (umol/m2·s1) | | O2 (%sat) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| 25ºC | 25.18 | 0.12 | 37.02 | 0.10 | 51.85 | 0.65 | 79.89 | 0.64 |
| 27ºC | 27.28 | 0.07 | 37.07 | 0.10 | 50.72 | 0.61 | 79.84 | 1.03 |
| 29ºC | 29.32 | 0.05 | 37.00 | 0.09 | 51.35 | 0.86 | 78.55 | 1.13 |

**Table S.4.2.** Mean±SE of photosynthetic parameters, results of Kruskal Wallis test for post-illumination Respiration (R) and net photosynthesis (Pnet) Dunn test measured in the 25ºC, 27ºC and 29ºC treatments (n=10). Results of T-student test in Maximum photosynthesis (Pmax), Photosynthetic efficiency (α), saturating irradiance (Ik) and compensation irradiance (Ic) Tukey HSD test measured in the 25ºC and 29ºC treatments.

| Variable | Treatment | | | DF | chi-squared | P | Dunn |
|---|---|---|---|---|---|---|---|
| | 25ªC | 27ºC | 29ºC | | | | |
| Pnet | 0.389 ± 0.02 | 0.268 ± 0.006 | 0.347 ± 0.01 | 2 | 5.143 | 0.076 | |
| R | -0.204 ± 0.028 | -0.139 ± 0.012 | -0.233 ± 0.025 | 2 | 7.661 | **0.0216** | 25=27<29 |
| | | | | | t- student | | |
| Pmax | 0.838 ± 0.051 | | 0.808 ± 0.116 | 67 | 0.234 | 0.816 | |
| alpha | 0.011 ± 0.002 | | 0.011 ± 0.002 | 67 | 0.141 | 0.888 | |
| Ik | 89.377 ± 15.171 | | 65.647 ± 13.508 | 67 | 1.167 | 0.247 | |
| Ic | 11.565 ± 5.097 | | 12.017 ± 3.755 | 67 | 0.095 | 0.925 | |

**Table S.4.3**. Mean± standard error (mg/g DW) of specific phenolic compounds analyzed in leaves of seedlings grown at 25ºC, 27ºC and 29ºC and results of Kruskal Wallis test (n = 10).

| Phenolic compound | 25ºC | 27ºC | 29ºC | df | chi-squared | p |
|---|---|---|---|---|---|---|
| p-Coumaroyl tartaric acid | 0.19±0.021 | 0.322±0.122 | 0.216±0.023 | 2 | 1.272 | 0.529 |
| Chicoric acid | 8.486±0.927 | 14.719±5.866 | 9.225±0.594 | 2 | 1.332 | 0.514 |
| p-Coumaroyl caffeoyl tartaric acid | 0.42±0.066 | 0.63±0.149 | 0.399±0.047 | 2 | 1.127 | 0.569 |
| 4-methyl Chicoric acid | 1.042±0.152 | 1.073±0.757 | 1.125±0.086 | 2 | 1.304 | 0.521 |
| bis-p-Coumaroyl tartaric acid | 0.153±0.017 | 0.236±0.057 | 0.158±0.011 | 2 | 2.942 | 0.23 |
| Total | 10.291±1.166 | 17.61±6.941 | 11.123±0.733 | 2 | 1.463 | 0.481 |

**Table S.4.4**. HRESIMS (-) spectroscopic data for the identified phenolic compounds in the UPLC-MS analysis from leaf extracts of *Posidonia oceanica* seedlings.

| Phenolic compound | Rt (min) | Formula | Observed [M-H]⁻ ion (*m/z*) | MS/MS fragments (*m/z*) |
|---|---|---|---|---|
| p-Coumaroyl tartaric acid (**1**) | 1.99 | $C_{13}H_{12}O_8$ | 295.0449 | 163.0394, 149.0082 |
| Chicoric acid (**2**) | 2.47 | $C_{22}H_{18}O_{12}$ | 473.0718 | 311.0404, 293.0296 |
| p-Coumaroyl caffeoyl tartaric acid (**3**) | 2.82 | $C_{22}H_{18}O_{11}$ | 457.0770 | 295.0454, 293.0297, 277.0350 |
| 4-methyl Chicoric acid (**4**) | 2.96 | $C_{23}H_{20}O_{12}$ | 487.0891 | 325.0570, 293.0307 |
| bis-p-Coumaroyl tartaric acid (**5**) | 3.14 | $C_{22}H_{18}O_{10}$ | 441.0829 | 295.0456, 277.0347 |
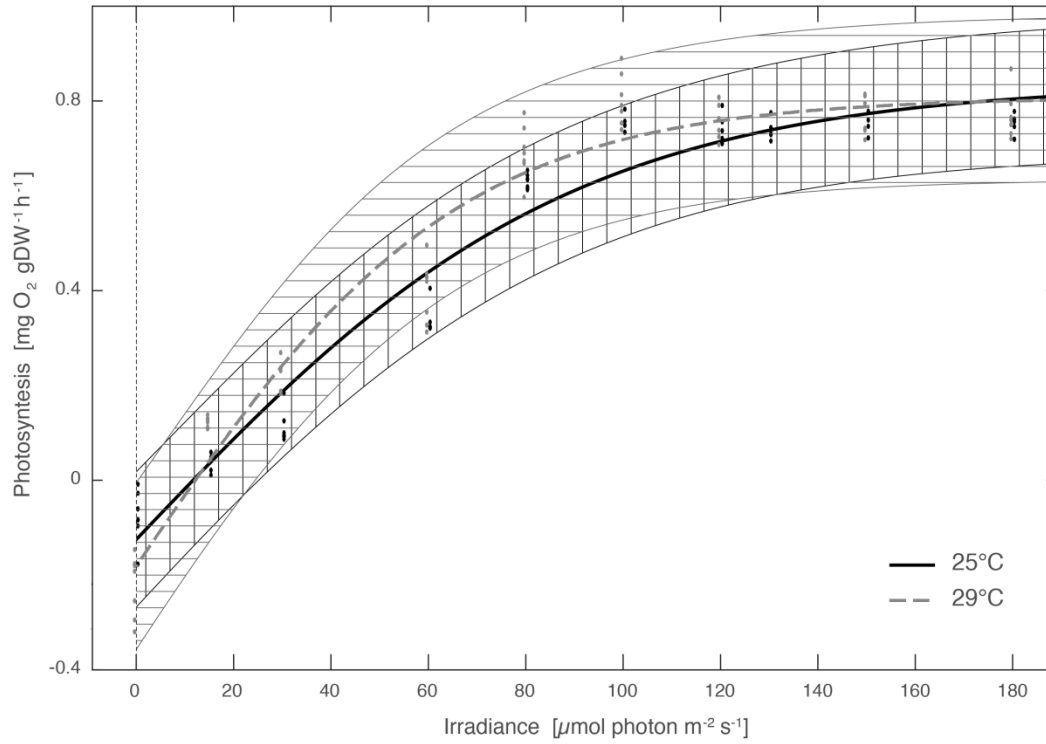
**Figure S.4.1.** Gross Photosynthesis-Irradiance curve of whole seedlings grown at 25ºC (black line) and 29ºC (dashed grey line). Points have been displaced for clarity. Stripped bands indicate 95% confidence boundaries for 25ºC (vertical) and 29ºC (horizontal) fitted curves.
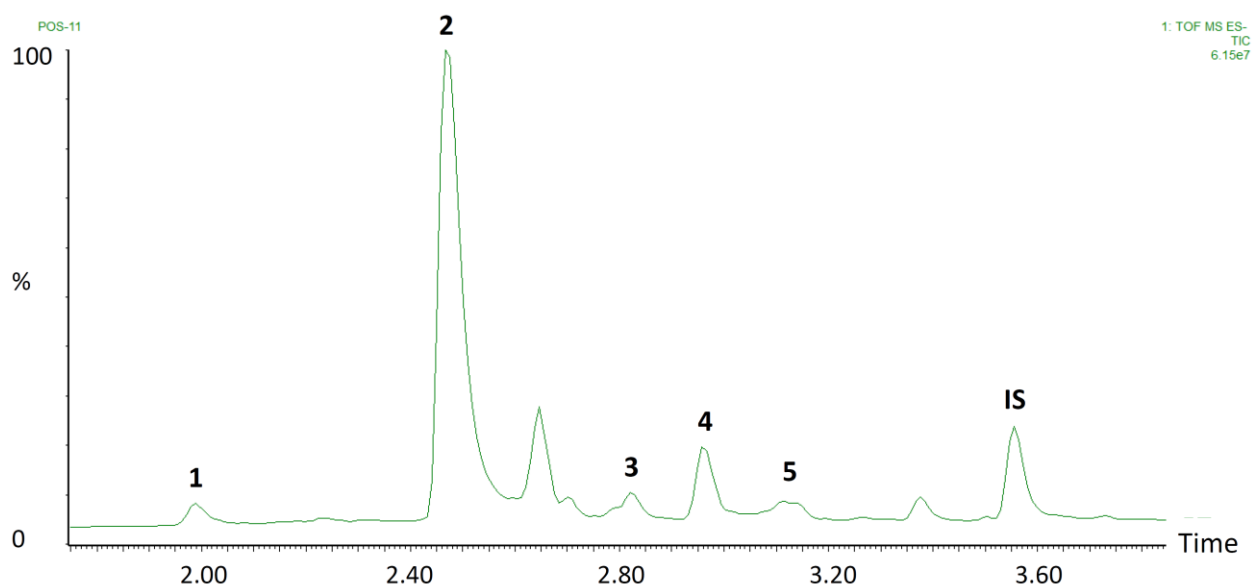
**Figure S.4.2.** TIC (total ion current) chromatogram obtained by UPLC-MS from leaf extracts of *Posidonia oceanica* seedlings. Retention times of (1) *p*-coumaroyltartaric acid, (2) chicoric acid, (3) *p*-coumaroycaffeoyltartaric acid, (4) chicoric acid methyl ether (5) di-*p*-coumaroyltartaric acid and (IS) internal standard, Quercetin shown in the figure.