Seagrass (Posidonia oceanica) seedlings in a high-\(\text{CO}_2\) world: from physiology to herbivory

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Under future increased \(\text{CO}_2\) 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 \(\text{CO}_2\) 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 \(p\text{CO}_2\) 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 \(p\text{CO}_2\) 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.

Seagrass meadows are of utmost importance for marine coastal systems. These photosynthetic organisms form the basis of diverse coastal webs having important ecological functions and offering crucial services for humans\(^1\). As seagrasses are highly productive, they act as a carbon sink\(^2,4\) and, as ecosystem engineers, they provide substratum for epiphytic plants and animals to settle\(^2\) and support coastal and offshore fisheries\(^6\). However, seagrasses are presently suffering a global loss associated to numerous human activities\(^7,8\).

Early life stages of plants play a crucial role in providing a new genetic variation pool necessary for adaptation to future environmental changes\(^9-11\). 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\(^12\). 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\(^13\). Furthermore, seeds and seedlings provide a key opportunity to colonize new habitats\(^14\), 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 \(\text{CO}_2\) concentrations have increased between 1.3% and 3.4% y\(^{-1}\) over the last decade\(^15,16\), and projections based on current emission rates estimate that atmospheric concentrations would increase up to 800 ppm by the end of the century\(^17\) and up to 1500–2100 ppm between 2100 and 2200\(^18,19\). Plants with C3 metabolism

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are predicted to benefit by this increase in CO₂ concentrations in two ways. First, increasing CO₂ availability increases carbon fixation rates²⁰,²¹. Secondly, a higher pCO₂ will reduce photorespiration because of the higher diffusion of CO₂, increasing the efficiency of carbon uptake²²,²³ and overall photosynthesis⁴⁴. As a result of the increased carbon assimilation, studies in terrestrial plants show increases in biomass, non-structural carbohydrates and C:N ratios²⁵. Due to the absorbing potential of the ocean (30–40% of anthropogenic CO₂ released to the atmosphere³⁶), seawater would increase its pCO₂, with more CO₂ available for photosynthetic organisms. Since most seagrasses have a C3 photosynthetic metabolism, they are predicted to benefit by the increase in CO₂ availability²¹. Indeed, even though most seagrass species have carbon-concentrating mechanisms, they seem to be limited by the current CO₂ concentration, as increased CO₂ availability in the water enhances photosynthesis³⁷–³⁹. 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)³⁰,³¹. Besides, there are no conclusive patterns about long-term effects of elevated pCO₂ in carbon budget or chemical composition in most seagrasses³²–³⁵.

Furthermore, beyond plant productivity effects, changes in CO₂ concentrations may also modify the capacity of plants to resist or tolerate herbivory³⁶. 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)³⁷,³⁸. Indeed, plant nutritional quality as well as chemical defenses are key resistance traits in controlling plant consumption by herbivores (e.g. decreasing nutritional quality)³⁷,³⁸. Increased plant nutritional quality as well as chemical defenses (e.g. phenolic compounds) in terrestrial plants, with consequences for plant-herbivore interactions⁴⁰,⁴¹. Nevertheless, the scarce available literature in seagrasses suggest a decrease, rather than an increase, in the concentration of phenolic compounds with increased pCO₂²,⁴³. Furthermore, the consequences of CO₂-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 emerge⁴⁴–⁴⁶.

In addition, environmental changes in resource availability, such as high CO₂ concentrations, may affect the trade-offs in resource allocation between growth and secondary metabolism in plants⁴⁷,⁴⁸ 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 pCO₂ on the performance of seagrass seedlings is important as they represent a particularly vulnerable period experiencing high mortality rates⁴⁹. Furthermore, herbivore pressure exerted on seedlings has critical effects on plant populations⁵⁰, shaping composition and structure of plant communities⁵¹. Because of the critical ecological importance of early life stages and the likely physiological differences with adult stages, the effects of future high pCO₂ in plant early life stages require specific examination.

In this study we hypothesized that under increased CO₂ 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 CO₂ 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 pCO₂ on seedling survivorship and their palatability to herbivores. To our knowledge, this is the first study examining the effects of increased CO₂ 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 CO₂ will influence the performance of seagrass seedlings is critical for evaluating the consequences of future CO₂ increases on seagrass populations.

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 pCO₂ (Supplementary material Fig. S1, Table 1). While there were no differences in AF (control pCO₂: 0.64 ± 0.025, high pCO₂: 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 pCO₂ treatments (Table 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 1).

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 pCO₂ treatment after 60 days (Fig. 1, Table 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 pCO₂ treatments was found in total root length after 90 days (Fig. 1, Table 1). CO₂ 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 pCO₂ was almost 2-fold higher than control seedlings, while there where no differences between treatments for leaf and root biomass (Fig. 2, Table 3).

Seedling chemical traits. The δ¹³C in leaves of seedlings from the high pCO₂ treatment (−20.5 ± 1.19 ‰) was significantly lower than in control plants (−16.4 ± 1.39 ‰), (Table 3).

Carbon content increased in leaves and roots when compared to the beginning of the experiment, but differences related to CO₂ treatments were only observed in seeds, which had a higher C content under high pCO₂ (Fig. 3 Table 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 CO₂-enriched plants when compared to controls (Fig. 3, Table 3). CO₂ 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. 4, Table 3). The increase in CO₂ 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. 4, Table 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).

**Herbivore feeding experiment.** Sea urchins consumed a significantly higher amount of fresh leaf tissue biomass from leaves grown under high pCO₂ in comparison to control pCO₂ conditions (Wilcoxon signed-ranks paired test, $z = 2.78$, n = 19, P = 0.004, Fig. 5).

**Discussion**

Early life seagrass stages could benefit under the future elevated CO₂ predicted scenarios. Our results show that, in general, seedling photosynthetic performance was enhanced under elevated pCO₂ 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 CO₂ 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 HCO₃⁻ rather than CO₂[aq], a future increase in CO₂[aq] may change this ratio. The higher ETRmax observed after 60 days of experiment suggests a greater ability to transfer electrons under high CO₂ conditions.

### Table 1.

Mean, standard error and results of repeated measures ANOVA in photosynthetic parameters and Tukey HSD test (H: HighCO₂, C: Control CO₂, 60: 60 days 90: 90 days, n = 7).

| Variable                        | Control CO₂       | High CO₂        | Source | DF | DFError | MS    | MSError | F     | P     |
|---------------------------------|-------------------|-----------------|--------|----|---------|-------|---------|-------|-------|
| Maximum Quantum Yield           | 0.708 (0.012)     | 0.682 (0.179)   | CO₂    | 1  | 12      | 0.00  | 0.00    | 1.10  | 0.314 |
|                                 | 0.712 (0.008)     | 0.705 (0.008)   | Time   | 1  | 12      | 0.00  | 0.00    | 2.28  | 0.157 |
|                                 |                   |                 | Time x CO₂ | 1 | 12  | 0.00  | 0.00    | 0.74  | 0.406 |
| Alfa                            | 0.179 (0.007)     | 0.144 (0.009)   | CO₂    | 1  | 12      | 0.00  | 0.00    | 0.61  | 0.452 |
|                                 | 0.186 (0.006)     | 0.15 (0.008)    | Time   | 1  | 12      | 0.01  | 0.00    | 27.32 | <0.001 |
|                                 |                   |                 | Time x CO₂ | 1 | 12  | 0.00  | 0.00    | 0.02  | 0.899 |
| Maximum electron transport rate  | 12.15 (0.92)      | 9.11 (0.462)    | CO₂    | 1  | 12      | 36.66 | 3.69    | 9.93  | <0.008 |
| (ETRmax)                        | 16.73 (0.99)      | 9.11 (0.419)    | Time   | 1  | 12      | 198.68| 4.11    | 48.31 | <0.001 |
|                                 |                   |                 | Time x CO₂ | 1 | 12  | 36.60 | 4.11    | 8.90  | <0.001 |
| Saturation Irradiance (Ek)      | 68.35 (4.68)      | 64.34 (3.56)    | CO₂    | 1  | 12      | 762.10| 197.00  | 3.87  | 0.0728 |
|                                 | 90.65 (5.9)       | 62.91 (3.03)    | Time   | 1  | 12      | 1764.60| 79.40   | 22.23 | <0.001 |
|                                 |                   |                 | Time x CO₂ | 1 | 12  | 985.70| 79.40   | 12.41 | <0.004 |

Table 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 CO₂ (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.
Interestingly, seeds of *Posidonia* spp seedlings have photosynthetic activity that enhances seedling growth. 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. Sucrose is the principal end-product of leaf photosynthesis and the higher content found in our study is thus likely resulting from the increased photosynthetic activity during the

### Table 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 | DFErr | MS   | MSError | 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    | 23.39| 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    | 17.31| 0.029   | 602.5   | <0.0001| 0 < 25 < 60 < 90 |
|                   | Time x CO$_2$ | 3 | 36 | 0.009| 0.029   | 0.32    | 0.811  |               |
| Total 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 2. Mean dry Biomass (g) of leaves, seeds and roots of seedlings growing under high CO$_2$ (black) or control (grey) conditions.](image-url)

Error bars indicate standard error. Asterisk indicates statistically significant differences between treatments.
early development of the seedlings. In general, higher CO₂ availability increases photosynthetic activity in seagrasses, which sometimes translates into increases in aboveground biomass or growth. However, this increase in photosynthesis and thus in carbon incorporation is not always allocated to aboveground growth. In our study, seedlings under high pCO₂ 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 or changes in the chemical composition of below and aboveground tissues.

In this study, seeds exhibited lower sucrose content in the control treatment, which suggests that seedlings growing under high pCO₂ 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 CO₂ availability. In seedlings, this is particularly important since seeds store and supply carbon and nutrients to the seedling during the first year of its life. Increased carbon reserves and biomass of the seed would benefit seedling survival and resilience to stressful conditions, especially in seagrasses such as Posidonia 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.

### Table 3. Results of one way ANOVAs in plant traits and Tukey HSD test (I: Initial, H: High CO₂, C: Control CO₂, 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 |               |
|        | Sucrose (%)  | 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      | 0.015 | 0.02   | 6.837 | 0.01  | C < H         |
|        | Biomass (g)  | 2  | 12      | 0     | 0      | 0.031 | 0.863 |               |
|        | Sucrose (%)  | 1  | 12      | 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.** 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 CO₂ (black) or control (grey) conditions. Error bars indicate standard error and different letters indicate statistically significant differences across treatments (Tukey).
survival\textsuperscript{58}, which are key features of a successful population expansion process. Particularly in \textit{P. oceanica} in which flowering frequency varies greatly spatially and among years (0–26%)\textsuperscript{59} with a low reproductive success (3–11% of seedlings available for establishment) mainly due to seed predation\textsuperscript{60}.

Despite the cost of less carbon available for growth, carbon allocation to defense and storage often results in higher survivorship of organs and individuals\textsuperscript{61}. Secondary metabolites are associated with defense mechanisms in plants (e.g. feeding deterrence\textsuperscript{62,63}) being for some herbivores more determinant of their preference than other attributes such as carbohydrates or fibers\textsuperscript{64}. According to the resource availability hypothesis (RAH\textsuperscript{65}) plants grown under high resource availability will invest less in defense components than plants grown under limited resource environments. Therefore, seagrasses grown under elevated nutrient availability (usually a limiting resource\textsuperscript{66}) often decrease the production of chemical defenses such as phenols\textsuperscript{67,68}. Being carbon-based compounds, most of the studies in terrestrial plants\textsuperscript{25} and some species of macroalgae\textsuperscript{69} have found increases in phenolic compounds with elevated CO\textsubscript{2} availability. Yet, because CO\textsubscript{2} is also a resource that can greatly limit primary production in seagrasses\textsuperscript{28,52,70}, we may expect a decrease in phenolics (rather than the increase often observed in terrestrial plants) under high CO\textsubscript{2} scenarios, following RAH. Indeed both a decrease\textsuperscript{42,43} as well as no changes\textsuperscript{45}, but never an increase, in phenolic compounds have been reported in seagrass leaves growing under elevated CO\textsubscript{2} conditions. While we did not find significant changes in phenol content in leaves associated with CO\textsubscript{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\textsuperscript{71,72}. 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\textsubscript{2}). Seeds from seedlings of the control treatments had significantly higher phenol content than those from the increased pCO\textsubscript{2} treatment, which were

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**Figure 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\textsubscript{2} (black) or control (grey) conditions. Error bars indicate standard error. Asterisk indicates statistically significant differences between treatments.

**Figure 5.** Percentage of fresh weight consumed by herbivores from leaf tissue of seedlings growing under high CO\textsubscript{2} (black) or control (grey) conditions. Error bars indicate standard error. Asterisk indicates statistically significant differences between treatments.
bigger (higher biomass), with higher carbon content and more stored sucrose. Having more resources (i.e. CO₂) 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.

A decrease in nutritional quality (as a decrease in nitrogen or increased C/N content) in response to high CO₂ has been commonly observed in terrestrial25,26 and marine plants32,33,38, and it has been attributed to a dilution of nitrogen due to increased leaf growth35, increases in leaf carbohydrates and structural material, higher plant internal nitrogen requirements36,37 and/or reductions in protein concentrations38. Some studies in Zostera noltei also found a lower N content under high pCO₂ conditions together with a lower nitrate uptake29. This reduced nitrate uptake could be the reason for the lower nitrogen content observed in leaves in the high pCO₂ 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. The reduced nutritional quality observed in seagrass leaves could have consequences for herbivores that may compensate this low nutritional quality by increasing their feeding rates80,81.

Unexpectedly, in our study, leaves with lower nitrogen content were preferred by sea urchins, whereas herbivores typically prefer tissues with higher N content42,43. However, N content also includes nitrogen in insoluble forms and alkaloids44, 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 herbivores45, increasing leaf toughness46 or decreasing the preference for high carbon-fiber plant species47. 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 equivalents48. 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 CO₂ concentrations43,49,50, and consequently, may have modified the palatability of the tissues.

One of the biochemical traits that changed with higher pCO₂ availability was sucrose content in leaves, which may have enhanced plant palatability. In insects, for instance, sugars increase stimulation to taste49 and can mask the deterrent effect of other compounds50. Additionally, we performed the feeding experiments only with the sea urchin P. lividus, whereas different herbivore species may have responded differently to CO₂-driven changes in plant chemical composition45,46 or epiphyte abundance or composition since it is expected that fleshy epiphytes may increase51 and calcareous epibionts would decrease their abundance under low pH conditions52. While we only performed the feeding experiments under ambient CO₂ water conditions, studies to date with adult sea urchins do not suggest strong changes in feeding rates94,95, nor in preferences (S.R. Fitzpatrick, personal communication) under ambient vs. high pCO₂ conditions.

In summary, the results of our experiment suggest that seedlings of P. oceanica might perform better under a high CO₂ 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.

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 (17 °C) with UV-filtered seawater for approximately one month until the initiation of the experiment.

Experimental design and setup. To evaluate the effect of CO₂ availability on P. oceanica seedlings, seawater was aerated with a mix of air and pure CO₂ gases using Mass Flow Controllers (Aalborg, USA) in order to obtain experimental CO₂ values of actual (ca.500 ppm hereafter control treatment) and future oceanic conditions (ca. 1550 ppm, hereafter high pCO₂ treatments). Seventeen seedlings of homogeneous size (control pCO₂: 0.759 ± 0.007 g wet mass seedling⁻¹, high pCO₂: 0.758 ± 0.010 g wet mass seedling⁻¹) were assigned to each of the seven replicate 9-L aquaria with control or high pCO₂ 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 CO₂ 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 (17 °C). At the same time, two replicate water samples (50 cc) from each aquarium were taken for dissolved inorganic carbon (DIC) and Total Alkalinity (AT). Water samples were fixed with supersaturated HgCl₂ (Merck, Analar) to avoid biological activity and changes in AT conditions. AT values were obtained by double endpoint titration to pH 4.45 and 4.41 (NBS scale) with HCl (Fixanalan®) according to Dickson Sop 3b (version 3.01), using a Titrando 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® with dissociation constants (K1 and K2) according
to Millero et al.97 and KHSO₄ dissociation constant after Dickson98. 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 pCO₂ 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 seagrass99. 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 × irradiance × 0.5 × AF100. ETR values were plotted against the incident absorbed PAR and the photosynthetic quantum efficiency (φ) 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 0.01 mm) in three seedlings per aquarium. 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 60°C to determine biomass of leaves, roots and seed of each one.

**Seedling chemical traits.** Effects of CO₂ enriched seawater in the inorganic carbon intake of leaves, were analyzed using stable isotope ratios. Leaves of four seedlings per aquarium were dried (60°C for 48 hours), ground and treated with HCl-tumes (37%, 12–24h) to remove carbonates101. 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:

\[
\delta X = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000
\]

where X is ¹³C, and R is the corresponding ratio of ¹³C/¹²C.

Commercial CO₂ was used as working standard and two internal standards with δ¹³C = −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 ± 0.1‰ for δ¹³C.

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 (~80°C), 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. 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.

Carbon and nitrogen content in leaves, seeds and roots 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 24h and were determined with spectrophotometer (Hitachi, U-2900) following a modified Folin-Ciocalteu method using caffeic acid as standard (modified from Bolser et al.102). Non-structural carbohydrates in leaves (sucrose), and seeds and roots (sucrose and starch) were measured using methodology described by Invers66. 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 24h 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 leaf sample (see de los Santos et al.103, modification from Van Soest et al.104). The amount of NDF in each sample was obtained by difference in dry biomass and is referred as ‘fiber content’ hereafter.

**Feeding experiment.** In order to examine how biochemical changes due to increased CO₂ 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 pCO₂ treatments were used in a two-choice experiment. The feeding assay was performed in an indoor seawater flow-through system (i.e. ambient CO₂ conditions) and
a light:dark photoperiod of 12:12 h. Similar-sized sea urchins (4.98 ± 0.66 cm, 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 lactuca ad libitum. Individual urchins were placed in cages of 225 cm² covered with a 1 cm mesh and offered similar amounts of fresh tissue clean of epiphytes (c.a. 3–4 leaves from one seedling) from control and high CO₂ treatments. Control cages without herbivores were used to measure any potential changes in leaf tissue not related to grazing. The weight changes in the controls were used to correct the autogenic changes in the feeding replicates. The corrected consumption was calculated as:

\[
\text{Treatment}_{\text{Initial(FW)}} = (\text{Control}_{\text{Final(FW)}}/\text{Control}_{\text{Initial(FW)}}) - \text{Treatment}_{\text{Final(FW)}}
\]

The experiment consisted of 20 replicates and ended when approximately 50% of initial material was consumed. Following the procedures of previous feeding behavior experiments⁴⁵, replicates in which all the offered samples were either totally consumed or fully intact were not considered in the statistical analysis.

**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 CO₂ 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.

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Author Contributions

G.H., L.R., L.B., F.T. and J.T. conceived the experiment. G.H., L.R., and L.B., conducted the experiment. A.D. conducted the isotope analysis. F.T., J.T. and CMD contributed with reagents/material/labatory equipment. G.H. L.R and F.T. prepared the manuscript. All authors reviewed and contributed to the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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