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Interaction strength between different grazers and macroalgae mediated by ocean acidification over warming gradients.

Macroalgae-herbivore interactions in changing oceans

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Abstract

Since the past century, rising CO₂ levels have led to global changes (ocean warming and acidification) with subsequent effects on marine ecosystems and organisms. Macroalgae-herbivore interactions have a main role in the regulation of marine community structure (top-down control). Gradients of warming prompt complex non-linear effects on organism metabolism, cascading into altered trophic interactions and community dynamics. However, not much is known on how will acidification and grazer assemblage composition shape these effects. Within this context, we aimed to assess the combined effects of warming gradients and acidification on macroalgae-herbivore interactions, using three cosmopolitan species, abundant in the Iberian Peninsula and closely associated in nature: the amphipod *Melita palmata*, the gastropod *Gibbula umbilicalis*, and the green macroalga *Ulva rigida*. Under two CO₂ treatments ($\Delta\text{CO}_2 \simeq 450 \mu\text{atm}$) across a temperature gradient (13.5, 16.6, 19.9 and 22.1 °C), two mesocosm experiments were performed to assess grazer consumption rates and macroalgae-herbivore interaction, respectively. Warming (Experiment I and II) and acidification (Experiment II) prompted negative effects in grazer's survival and species-specific differences in consumption rates. *M. palmata* was shown to be the stronger grazer per biomass (but not per capita), and also the most affected by climate stressors. Macroalgae-herbivore interaction strength was markedly shaped by the temperature gradient, while simultaneous acidification lowered thermal optimal threshold. In the near future, warming and acidification are likely to strengthen top-down control, but further increases in disturbances may lead to bottom-up regulated communities. Finally, our results suggest that grazer assemblage composition may modulate future macroalgae-herbivore interactions.

45 Key-words: warming, acidification, macroalgae-herbivore interaction, stressor gradient,
46 interaction strength.

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Introduction

Anthropogenic CO₂ accumulates in the atmosphere, retaining infrared radiation and rising temperatures of both terrestrial and ocean ecosystems. As a result, global average sea surface temperature increased approximately 0.1 °C per decade during the last 40 years (Taboada and Anadón, 2012) and a further 2-5 °C increase in mean sea surface temperature is predicted by 2100 (IPCC, 2013). Biologically, temperature affects metabolic rates by altering biochemical reactions' kinetics and energies. According to basic metabolic theory, all organisms possess a survival thermal window, where increasing temperature increases reaction rates until an optimal level is reached (Angilletta, 2009). Beyond this threshold, further increases in temperature cause physiological stress (e.g. protein denaturation), resulting in a steep decline in metabolic rates and consequently in biological processes (see Fig. 1 in Kingsolver, 2009) such as growth, development and feeding activity (Angilletta, 2009; Kingsolver, 2009; Mertens et al., 2015). In fact, due to differential effects on autotrophic and heterotrophic metabolisms, metabolic theory of ecology (MTE) dictates that producers will show a weaker response to temperature than consumers (Yvon-Durocher et al., 2010). Thus, as individual changes lead to community effects (Mertens et al., 2015), small increases in temperature are predicted to strengthen top-down control by herbivores on marine primary producers, as a consequence of higher herbivore consumption rates relative to macroalgal production (Eklöf et al., 2012; O'Connor, 2009). Furthermore, it is also possible that marine communities find an equilibrium by balancing herbivore grazing and algal biomass growth, through compensatory mechanisms which allow for community stabilization (Connell and Ghedini, 2015).

The dissolution of atmospheric CO₂ into the oceans alters seawater carbonate system and the associated drop in pH levels is known as ocean acidification. Since the past

century, atmospheric CO₂ concentrations increased to approximately 400 µatm and are predicted to reach 1000 µatm by the end of this century, with a corresponding drop of 0.14-0.35 units in mean ocean pH (IPCC, 2013). Calcifying animals and algae are deemed as the most imperiled marine organisms due to deregulation of acid-base processes, decrease in calcification rates and/or increase in calcium carbonate dissolution, and hypercapnic growth inhibition (Byrne, 2011; Koch et al., 2013; Kroeker et al., 2011). Non-calcifying organisms register quite different responses across taxa (Connell and Russell, 2010; Kroeker et al., 2010; Poore et al., 2013), since some species of crustaceans and fish show aptitude to partially offset the negative effects of acidification through mechanisms of acid-base compensation, changes in metabolism, energy reallocation and/or increase in mobility (Larsen et al., 1997; Melzner et al., 2009; Widdicombe and Spicer, 2008; Wood et al., 2008). For seaweeds, dissolved CO₂ is normally used as a substrate for photosynthesis, and non-calcareous species may benefit from increases of this resource (Koch et al., 2013). Nevertheless, some species, e.g. *Ulva rigida*, are unaffected by increased CO₂, as present day carbon concentrations already saturate its photosynthetic and growth physiological processes (Rautenberger et al., 2015). Identically to warming effects, ocean acidification may affect species interaction strength due to differential sensitivities to pH changes, potentially cascading into alterations in species competition dynamics and trophic interactions (Falkenberg et al., 2013; Hepburn et al., 2011; Kroeker et al., 2012).

To date, most research performed examining climate change impacts of marine systems have used multi-level single stressor (Connell et al., 2013; Falkenberg et al., 2013; O'Connor, 2009), straightforward two-stressors/two-levels (2 x 2) (Connell and Russell, 2010; Gaitán-Espitia et al., 2014; Veteli et al., 2002), or single species experimental designs (see Wernberg et al., 2012 for a review). However, climate change

will create new complex physicochemical scenarios characterized by multi-levels of stressors interacting. It is critical to improve our ecological knowledge on the potential combined effects of global stressors not only on different trophic levels, but also on interacting communities to strengthen our capacity in predicting future impacts of climate change on marine ecosystems (Poore et al., 2013). Whilst an increase in top-down control is likely under warming alone (López-Urrutia et al., 2006; O'Connor, 2009), forecasting impacts on macroalgae-herbivore interactions elicited by temperature and acidification effects is far more complex, given the non-linear variation in organism metabolic rates across stressor gradients (Mertens et al., 2015). Further increasing temperature (Mertens et al., 2015) and/or exposure to multiple stressors (Ghedini et al., 2015a) are reported to overcome the compensatory/grazing metabolic threshold, leading to shifts in community regimes, from top-down (herbivore-regulated) to bottom-up (algae- or nutrient-regulated) systems. Moreover, studies comparing mono-specific herbivore populations *versus* mixed herbivore populations under climate change scenarios are scarce (Alsterberg et al., 2013), but essential to understand community effects on natural systems. Within this context, here we studied how distinct CO₂ concentrations across a temperature gradient would differently impact the survival and consumption rates of two common herbivore species from the rocky intertidal; the non-calcifying amphipod species *Melita palmata* (Montagu, 1804), and the calcifying gastropod species *Gibbula umbilicalis* (da Costa, 1778). Additionally, we tested how these climate stressors would alter algal biomass of an abundant rocky intertidal algal species, *Ulva rigida* (Agardh, 1823). Finally, we hypothesized that community dynamics, i.e. macroalgae-herbivore interaction strength, would be interactively modified by temperature and CO₂. Simultaneously, we investigated if grazer species

identity may also play a role in shaping community response, due to differential effects on grazers' survival and consumption rates.

Methods

Study species

Gastropods are considered efficient grazers (Jernakoff and Nielsen, 1997), generally grazing larger algal portions than amphipods (Morrissey, 1988). We used the calcifying gastropod *Gibbula umbilicalis* as a large grazer species and the non-calcifying amphipod *Melita palmata* as a small-bodied grazer (~1/10 biomass of *G. umbilicalis*) to assess the joint effects of different climate change stressors in macroalgae-herbivore interactions. Both macroinvertebrate species are common grazers from the western Atlantic intertidal rocky coast of the Iberian Peninsula. They can be found associated to mixed macroalgal beds, including large accumulations of *Ulva spp.*, searching for food and refuge during low intertidal. The green seaweeds from the *Ulva* genus are very common opportunistic primary producer in shallow coastal waters and tidal pools in rocky shore communities (Aníbal et al., 2007).

The three species (i.e. *G. umbilicalis*, *M. palmata* and *U. rigida*) were collected on the same date at Viana do Castelo (North of Portugal, 41°41'44"N, 8°51'2"W), and were later separated by hand in the laboratory. To select our experimental gradient of temperatures, we registered water temperature from the sampling location through two continuous temperature data loggers (Tidbit V2 Onset HOB0®) installed at two mid-shore rockpools, during the month of August, 2013 (date of this study). Daily average temperature in the seawater of these rockpools ranged from 14.96 ± 0.72 °C to 17.80 ± 2.11 °C (mean $\pm$ SD) depending on night/day phase, with 13.28 °C and 25.23 °C recorded as the absolute minimum and maximum values, respectively. This large

gradient of temperatures in rock pools from this region results from a combination of frequent summer upwelling events (Lemos and Pires, 2004), and prolonged exposure to the sun without water exchange during low tides. As we were interested in investigating realistic interactions between pH and temperature, CO₂ concentrations followed the “business-as-usual” predicted scenario for 2100 (IPCC, 2013) and mean experimental temperatures were chosen within the thermal limits of *G. umbilicalis* (Evans 1948), *U. rigida* (Steffensen 1976) and *M. palmata* (Obenat et al. 2006). We used adult individuals of amphipods (0.96 ± 0.19 cm, mean length measured from the tip of the head to the telson) and gastropods (1.05 ± 0.18 cm, mean length measured from the tip of the operculum to the furthest point in the horizontal plane).

Experimental mesocosm setting

Experimental mesocosm (closed system) consisted of eight transparent PVC tanks (approx. 100 L, 50 x 50 x 40 cm, hereafter referred as treatment tanks) filled with seawater and subjected to eight crossed treatment combinations of two CO₂ levels: $\sim 380 \pm 30$ μ atm (ambient) and $\sim 830 \pm 45$ μ atm (increased), and four temperature levels (mean $\pm$ SD, gradient): 13.5 ± 2.0 °C (low), 16.1 ± 2.0 °C (midlow), 19.9 ± 3.0 °C (midhigh), and 22.1 ± 2.0 °C (high) (see Figure S1). As mean rock pool temperature was approximately 16 °C (see also Cacabelos et al. 2013 for confirmation of similar values), midlow treatment was acknowledged as mean natural temperature conditions. Temperature was controlled through titanium aquarium heaters, maintained and adjusted by AT Control devices (Aqua Medic®). Seawater pH was maintained by manipulating seawater carbonate chemistry, constantly monitored and registered with Aqua Medic pH electrodes. Each treatment tank (n = 8) connected to a separated PVC header tank (100 L of capacity, n = 8), where the incorporation of enriched CO₂ /

normal air was performed. To adjust pH, a pH-stat system (Aqua Medic®, AT Control) injected certified CO₂-enriched air (Air Liquide) through solenoid valves to down regulate pH, and normal air to up regulate pH. Seawater carbonate chemistry was calculated based on, temperature, salinity, total alkalinity (assessed spectrophotometrically at 595 nm) and pH_t measurements (Sarazin et al., 1999) (Table S1). As such, pH_t was quantified through a Metrohm pH meter (826 pH mobile, Metrohm, Filderstadt, Germany) connected to a glass electrode (Schott IoLine, SI analytics, ± 0.001), which was calibrated with 2-aminopyridine-HCl (AMP) and TRIS-HCl (TRIS) seawater buffers following Dickson et al. (2007). Total carbonate and pCO₂ (Table S1) were calculated using CO2SYS software (Lewis and Wallace, 1998), with equilibrium constants (Mehrbach et al., 1973) refitted by Dickson and Millero (1987). Abiotic conditions between header and treatment tanks were secured using submersible pumps (570 l h⁻¹), with water permanently exchanged to maintain pH treatments, using one submersible pump (3000 l h⁻¹) and gravitational force. Within each treatment tank, water exchange was enhanced through water tubing (smaller submersible pump 570 l h⁻¹) that assured all abiotic variable conditions were similar between cylinders.

Each treatment tank contained mechanical and biological filtering, as well as twelve transparent cylinders (D 0.33 x H 0.10 m, ~2 l, 0.085 m² cylinder base area), i.e. experimental units, consisting of four different grazer levels (G: *Gibbula umbilicalis*, M: *Melita palmata*, G+M: both species together, and C: controls with no grazers, n = 3) interspersed through the treatment tank (Figure S1). The top of each cylinder was covered with a 1.0 mm mesh to avoid animals from escaping, while still enabling water circulation and light entry. Nutrients (1 ml of 42.50 g l⁻¹ NaNO₃ solution and 1 ml of 10.75 g l⁻¹ Na₂HPO₄ solution per liter of seawater) were added to each tank every two days, and water was renewed manually when salinity increased to 36 (35.5 ± 1.0 PSU;

mean $\pm$ SD, $n = 8$). Temperature, pH and salinity were monitored three times a day with the use of a data-logger. The experimental setup was placed inside a shadow greenhouse under natural light and photoperiod, with consequently reduced incident light inside, mimicking commonly found subsaturating light conditions on rocky pools (Guidone et al., 2012; O'Connor, 2009).

Experiment I: Warming and acidification effects on grazers consumption rates

We performed a two-day (i.e. 48h) experiment to assess grazer consumption rates for mono-specific and mixed grazer combinations (O'Connor, 2009). This experiment was preceded by a 24h algae-free period for grazer acclimatization (algae were acclimated in separate cylinders) to avoid the influence of past diet and ensure algae consumption during the experiment (Swanson & Fox, 2007). We used 3-replicated cylinders with four levels of grazers (density in cylinder = $0.009 \text{ ind} / \text{m}^2$): *G. umbilicalis* (hereafter G, $n = 10$), *M. palmata* (hereafter M, $n = 10$), both species together (hereafter G+M, $n = 5 + 5$), and control (hereafter C, no grazers). At the end of the 24 hour acclimatization period, algae were spun 25 revolutions in a salad spinner to remove excess water and $\sim 3.015 \pm 0.041 \text{ g}$ (mean $\pm$ SD, density in cylinder = $0.028 \text{ g algae} / \text{m}^2$) of algal blotted wet weight (bww) were inserted in each cylinder. While in the cylinders, algae were strapped to weights and left swinging inside the cylinder, mimicking natural rock pool conditions. After the 48 hour grazing trial, portions were removed, blotted and weighted again as above. Dead animals were recorded at the end of the experiment.

Experiment II: Warming and acidification effects on interaction strength

We performed a longer (seven-day or 168 hours) experiment with new animals and algal portions to assess differences in per capita macroalgae-herbivore interaction

strength, i.e. individual grazer pressure per algal biomass growth ratio. A 24h period of acclimatization with algae was performed (Eklöf et al., 2015), and at the beginning of the experiment, algae were added, mimicking natural conditions where seaweed is abundant (Cacabelos et al., 2013; O'Connor, 2009). Three-replicated cylinders were used, with 6 grazers for each level of grazer treatment (density in cylinder = 0.014 ind / m²): G (n = 6), M (n = 6), G+M (n = 3 + 3) and control (C, no grazers). Grazer numbers were reduced to avoid excessive competition for habitat and food. At the beginning of the experiment, $\sim 7.030 \pm 0.025$ g (mean $\pm$ SD, density in cylinder = 0.012 g algae / m²) of blotted wet weight portions of algae were strapped to weights and inserted in each cylinder. After seven days, algae were retrieved, blotted and weighed. The number of dead animals was recorded at the end of the experiment.

Data Analysis

Survival rates in both experiments were assessed according to the number of living *G. umbilicalis* and *M. palmata* individuals (and the sum of both in the case of G+M treatment) present at the end of each experiment, and transformed into survival rate (%) per cylinder. Changes in algal biomass were estimated through the difference of initial and final values of algal wet biomass in experiments. Consumption rates in Experiment I was calculated using the following formula based on Taylor and Brown (2006):

$$\text{Consumption} = (T_i * (C_f / C_i) - T_f) / (n_{\text{bio}} * t),$$

Where T_i is the initial algal bww, T_f is the final algal bww, C_i is the initial control algal bww, C_f is the final control algal bww, n_{bio} is the added biomass of living grazers in each cylinder at the end of the experiment and t is the time elapsed in the experiment

(2 days). Thus, consumption rates are expressed in grams of algae consumed per invertebrate biomass per day. Biomass was extrapolated from a referenced length-weight regression based on *M. palmata* individuals' total length (Grilo et al., 2009; Pardal et al., 2002). Likewise, *G. umbilicalis* biomass was extrapolated by measuring the longest vertical axis and fitted in a referenced length-weight regression (Robinson et al., 2010).

In Experiment II, in addition to survival rates and changes in algal biomass, alterations in community dynamics, i.e. grazing pressure and simultaneous macroalgal growth, were also analyzed. To assess the strength of macroalgae-herbivore interaction we used the Dynamic Index (Berlow et al., 1999; Mertens et al., 2015; O'Connor, 2009; Wootton and Emmerson, 2005) modified from Wootton (1997), with the following formula:

$$DI = \ln (N / D) / (n * t),$$

where DI is the Dynamic Index, N is algal wet biomass with grazers, D is the algal wet biomass on treatment without grazers, n is the number of living grazers in the cylinder, and t is the known period of time elapsed (i.e. seven days). This index measures interaction strength, i.e. the absolute value of daily per capita interaction strength, accounting for differences in algal growth rates with and without herbivores (O'Connor 2009). Negative interaction strength values implicate that one species reduces the abundance of the other species; therefore, lower values indicate stronger interactions (i.e. high grazer pressure). This index translates survival rates and metabolic alterations provoked by climate change stressors into an ecological response (O'Connor 2009). Compared to other methods, DI does not assume equilibrium between algae and

grazers, which is convenient for relatively short experiments as ours (Berlow et al., 1999).

Changes in survival rates, algal biomass, consumption rates and DI were analyzed separately through a distance-based permutational multivariate analysis of variance (PERMANOVA, Anderson 2001). We calculated distance resemblance matrices using Euclidean dissimilarity measures based on untransformed data. PERMANOVA was run with 4999 permutations to obtain p values under unrestricted permutation of raw data (Anderson 2001), using CO₂ (2 levels; ambient and increased), temperature (4 levels: low, midlow, midhigh and high) and grazers (4 levels: G, M, G+M and C) as fixed orthogonal factors ($n = 3$). The permutational approach was used because the data were non-normally distributed even after transformations (Wernberg et al., 2008). Only significant effects ($p < 0.05$) were further investigated through a series of pair-wise comparisons using the appropriate terms in the model (Anderson et al. 2008). All the multivariate analyses were performed with PRIMER 6 & PERMANOVA+ package.

Results

Experiment I: Warming and acidification on grazer consumption rates

After 48h, survival rates differed significantly depending on temperature and grazer treatments (Table 1). Specifically, survival rates decreased from lower (13.5 °C and 16.1 °C) to higher (19.9 °C and 22.1 °C) temperatures ($p < 0.001$, Figure 1, see Table S2 for pair-wise comparisons). Concerning grazer treatments, survival rates were comparatively higher in G (90%), and lower in M (~40%), while G+M registered intermediate survival rates (~70 %) (Figure 1, Table S2).

Algal biomass was significantly altered by temperature and the interaction between CO₂ and grazers (Table 1, Figure 2). Thus, at high temperature (22.1 °C) algal biomass loss was reduced compared to biomass loss reported for the two lower temperature levels, 13.5 °C and 16.1 °C (Figure 2a, Table S3). G registered higher biomass loss than M, and increased CO₂ amplified these effects (Figure 2b, Table S3). In grazer control treatments, i.e. C, algal biomass increased: 0.1 g (from initial 3 g) per day $\approx$ 3 % per day.

Consumption rates were significantly affected by the triple interaction between CO₂, temperature and grazer (Table 1). Multiple pairwise comparisons revealed that consumption rates in M were always higher than in G and G+M, regardless of temperature or CO₂ (Figure 3, Table S4). Comparing CO₂ treatments, at 13.5 °C, G+M displayed lower consumption rates in increased CO₂ (Figure 3, Table S4). Under ambient CO₂ (Figure 3a), G and G+M consumption rates dropped across the temperature gradient, whereas M consumption rates increased (Figure 3, Table S4). Lastly, under increased CO₂, temperature did not different consumption rates in any grazer treatment (Figure 3b, Table S4).

Experiment II: Warming and acidification effects on interaction strength

After 7 days (i.e. 168h), survival rates were significantly affected by variations in CO₂ and the interaction between temperature and grazers (Table 2). Lower survival rates were reported under increased CO₂ (SNK test, $p < 0.001$, Figure 4a). Moreover, survival rates also dropped with increasing temperature in all grazer treatments (Figure 4b, Table S5). Specifically, all grazer treatments showed higher survival rates at 13.5 °C

than at 19.9 °C and 22.1 °C. Comparing grazer treatments, G generally showed significantly higher survival rates (Figure 4b, Table S5).

Algal biomass in Experiment II was significantly affected by a triple interaction of CO₂, temperature and grazer (Table 2, Figure 5). Temperature and CO₂ showed no effect on grazer control treatments, which consistently revealed a ~20 % increase (from initial 7 g, 0.2g increase per day $\approx$ 3 % per day; 3% per day * 7 days $\approx$ 20 %) in algal biomass at the end of 7 days (Table S6). Algal biomass loss peaked at 19.9 °C under ambient CO₂, while increased CO₂ led to higher algal biomass loss at 16.1 °C (Table S6). Moreover, extreme temperatures (13.5 °C and 22.1 °C) consistently registered algal biomass growth, in contrast to algal loss verified in intermediate temperatures (16.1 °C and 19.9 °C). Under ambient CO₂ and 13.5 °C, *Ulva rigida* growth was higher in M compared to G+M (SNK test, $p < 0.05$, Table S6).

The Dynamic Index (DI) was interactively affected by CO₂ and temperature (Table 2, Figure 6). Thus, under increased CO₂ a temperature increase from 13.5 °C to 16.1 °C led to an overall DI minimum, i.e. largest top-down control (SNK test, $p < 0.01$, Table S7), while at ambient CO₂ no significant effects in DI were detected (Figure 6). Under increased CO₂, further temperature increase (16.1 °C to 19.9 °C and 22.1 °C) led to a consecutive increase in DI values, i.e. a reduction of top-down interaction strength (Figure 6, Table S7). Conversely, under ambient CO₂, the strongest top-down interaction (i.e. low DI, high grazer pressure) occurred at 19.9 °C. Once again this significant trend was reverted at 22.1 °C, and DI values indicating bottom-up control were registered (SNK test, $p < 0.001$; Figure 6, Table S7). No grazer treatment effects were observed in the DI index (Table 2).

Discussion

Our results showed that grazers' survival and consumption rates were differently affected by warming and acidification. Additionally, temperature showed immediate effects (Experiment I, two days) on survival rates, whereas acidification-related effects emerged later on (Experiment II, seven days). When co-occurring, stressor impacts were additive and independent, hence no interactive climate stressor effects were found on survival rates. Increased CO₂, i.e. hypercapnia-linked, mortality is known to occur as a consequence of prolonged animal inactivity and/or starvation due to metabolic suppression, which explains why CO₂ effects were only detected after seven days (Kurihara et al., 2008; Langenbuch and Pörtner, 2004; Spicer et al., 2007). Thus, these results strengthen the claim that warming is the strongest stressor, outweighing acidification effects on animal survival (see Wernberg et al. 2012 for a review). the relatively high mortality verified was likely caused by lower animal physiological tolerances to fixed stressor (e.g. temperature) levels in comparison to variable stressor levels (Benedetti-Cecchi et al., 2006; Vasseur et al., 2014). Contrary to natural fluctuating conditions (i.e. where stressor levels vary over time), our experimental conditions comprised of fixed stressor treatments (i.e. constant thermal and pH conditions with low intra-treatment variance), which likely contributed to increased physiological stress and mortality (Stillman, 2002).

Herbivore performance analysis also revealed striking differences between both grazers tested. Molluscs are usually considered more sensitive to warming, and especially acidification, than crustaceans (Harvey et al., 2013). In theory, due to naturally higher basal metabolism (see Peck et al., 2009), *M. palmata* may be closer to its metabolic peak (i.e. "optimal" level) than *G. umbilicalis*, thus overcoming the optimal metabolic threshold with shorter increases in stressors. Consumption rates as impacts on algal biomass were similar to those reported by Giannotti and McGlathery

(2001) for gastropod (around 0.01-0.02 grams of algae per individual and day) and by Cruz-Rivera and Hay (2001) for amphipods (around 0.004-0.01 grams of algae per individual and day) grazing on *Ulva* sp. Thus, *G. umbilicalis* showed higher impact on algae biomass, with natural temperature (16.1 °C) and increased CO₂ amplifying algal biomass loss. Nevertheless, we found that *M. palmata* is actually a stronger grazer per biomass than *G. umbilicalis*, likely due higher metabolic rates. *G. umbilicalis* inherently higher biomass also mathematically justifies why consumption rates per biomass in mixed species treatments closely matched those reported for *G. umbilicalis*. Thus, according to our results, increases in temperature (but also in CO₂) will benefit *G. umbilicalis* by lowering *M. palmata* survival, while increasing grazing pressure on algal biomass, as predicted by the metabolic theory of ecology (MTE; Kingsolver, 2009).

Concerning abiotic effects on seaweed biomass, our results are in line with previous research, reporting no acidification effects on *U. rigida* growth (Rautenberger et al., 2015), but differ from the previously reported temperature dependence for the genus *Ulva* (Steffensen, 1976). In our study, controls (only algae, no grazers) showed similar algal biomass increase under warming and acidification conditions, in both experiments (~3 % growth per day). As such, mean algal growth rate reported here is lower than expected under perfect light intensity laboratorial conditions (20 % growth per day in Rautenberger et al., 2015). However, it is similar to results verified in field experiments, as well as for laboratorial settings mimicking subsaturating light conditions (e.g. ~5 % growth per day in Guidone et al., 2012; see also O'Connor, 2009). We suggest that the lack of algal response to climate stressors and the algal growth rates registered here were caused by comparatively reduced solar irradiation (Rautenberger et al., 2015). This reduction was a direct consequence of the greenhouse set-up housing, further amplified perhaps by the use of a 1 mm mesh on the experimental cylinders (to prevent grazers

from escaping). Moreover, warming-related algal growth may have been potentially concealed by undetected temperature-induced algal decomposition (Schiel et al., 2004).

After seven days (Experiment II), algal biomass changes in treatments including grazers showed the expected profile across the temperature gradient (Kingsolver, 2009; Mertens et al., 2015). However, maximum algal biomass loss also depended on CO₂ conditions. Although interaction strength may increase with higher temperature and CO₂, as shown by previous studies (Alsterberg et al., 2013; O'Connor, 2009), the response patterns are indeed more complex than a direct linear antagonistic or synergistic effect (Ghedini et al., 2015a). These non-linear CO₂-dependent responses to temperature gradients were clearly displayed in the dynamic index profiles. As it is, grazing pressure increased over increasing temperature, until survival rates decreased to the point of soothing grazer pressure on macroalgae (i.e. overcoming the community stability threshold), which significantly weakened macroalgae-herbivore interactions, and ultimately shifted community dominance. As expected, herbivores were shown to be more susceptible to changes in temperature than primary producers (López-Urrutia et al., 2006). Moreover, increased CO₂ stimulated community response by accelerating the interaction strength profile across warming scenarios. Thus, as predicted by Ghedini et al. (2015a), the occurrence of simultaneous stressors lead to a bottom-up controlled community earlier in the temperature gradient.

The observed warming and acidification effects were consistent in the three different combinations where grazers were used, i.e. previously detected grazer identity effects for other parameters were concealed in the dynamic index analyses. Thus, our results did not support the claim that grazing activity by different grazer species would modify the strength of top-down control on seaweeds (O'Connor 2009). Most likely our election of the herbivores, without a clearly defined stronger grazer (i.e. *M. palmata* was

the stronger grazer per biomass, but produced a comparatively smaller impact on algal biomass, and was also the most sensitive to the climate stressors) was the main cause for the verified grazer homogeneity. Moreover, our experimental set-ups ran through relatively short time spans (up to seven days), so extrapolating or generalizing conclusions to natural systems may be inaccurate. However, it is important to note that studies that were prolonged for longer periods of time (two to five weeks), generally led to stronger interactions between climate change stressors (e.g. Alsterberg et al., 2013; Christensen et al., 2011; Vasseur et al., 2014). Furthermore, the strength of macroalgal-herbivore interactions is reported to increase from 11 to 17 days (Ghedini et al., 2015b; Mertens et al., 2015; O'Connor, 2009). Therefore, we suggest that grazer identity effects may still arise in the future ocean as a consequence of starkly different survival and consumption rates.

Future climate change is expected to modify the fundamental top-down control exerted by herbivore species feeding on macroalgae (Eklöf et al. 2012, this study). Warming and acidification can interact and play a major role in differentially driving rocky intertidal communities' structure and functioning. Extreme increases in both stressors, or higher frequencies of extreme weather events (Kroeker et al., 2011), can largely affect the survival rates of typical grazers. Taking advantage of lower thermal sensitivity, opportunistic algal species like *U. rigida* might grow freely, disrupt ecosystem equilibrium, and promote ecological shifting from top-down to bottom-up regulated communities (Connell and Russell, 2010; Veteli et al., 2002). Conversely, moderate increases in acidification or warming might lead to higher grazing pressure as long as these stressors do not exceed metabolic grazers' thermal and acid-base limits (Alsterberg et al., 2013; O'Connor, 2009), and lower algal biomass as a consequence of stronger top-down control (i.e. increased interaction strength). Thus, understanding

whether macroalgal and herbivore responses to different climate change stressors are synchronous is fundamental to assess the future strength of top-down control in marine systems. We also suggest that the characteristics and composition of intertidal grazer assemblages may play a relevant role in macroalgae-herbivore interactions, inducing differences in grazing pressure on macroalgae, mainly due to species-specific differences in survival and algal consumption rates. Longer exposures in mesocosm and field experiments focusing on the combined effects of different climate stressor gradients, and using diverse assemblages of herbivores and macroalgal species, are needed to underpin forecasts on how macroalgae-herbivore interactions will be affected by global change in the future.

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Figures & Tables

Figure 1. Survival rates of *G. umbilicalis* and *M. palmata* individuals present (% , mean +SE) at the end of Experiment I. Data is displayed according to significant factors (non-significant factor are averaged, see Table 1): a) temperature (low: 13.5 °C, midlow: 16.5 °C, midhigh: 19.9 °C, high: 22.1 °C), b) grazer treatments (G: *G. umbilicalis*, M: *M. palmata*, G + M: *G. umbilicalis* + *M. palmata*). Different letters represent significant differences.

Figure 2. Changes in mean (+ SE) algal biomass (*U. rigida* g, blot wet weight per day) in the end of Experiment I. Data is displayed relative to significant factors (non-interactive factors are averaged, see Table 1): a) temperature (low: 13.5 °C, midlow: 16.5 °C, midhigh: 19.9 °C, high: 22.1 °C), and b) CO₂ (ambient CO₂: 380 µatm, increased CO₂: 830 µatm) and grazer treatments (G: *G. umbilicalis*, M: *M. palmata*, G + M: *G. umbilicalis* + *M. palmata*, C: No grazers). Different letters represent significant differences.

Figure 3. Effects on mean (+SE) herbivore consumption rates in the end of Experiment I. Data is displayed relative to significant factors (non-interactive factors are averaged, see Table 1): a) grazer (G: *G. umbilicalis*, M: *M. palmata*, G + M: *G. umbilicalis* + *M. palmata*) and CO₂ treatments (ambient CO₂: 380 µatm, increased CO₂: 830 µatm), and b) grazer (G: *G. umbilicalis*, M: *M. palmata*, G + M: *G. umbilicalis* + *M. palmata*) and temperature treatments (low: 13.5 °C, midlow: 16.5 °C, midhigh: 19.9 °C, high: 22.1 °C). Results expressed as grams of algae (algae biomass) per milligrams of grazers

(grazer biomass) contained in the treatment, per day. Different letters represent significant differences.

Figure 4. Survival rates of *G. umbilicalis* and *M. palmata* individuals present (% , mean + SE) at the end of Experiment II. Data is displayed relative to significant factors (non-interactive factors are averaged, see Table 2): a) CO₂ (ambient CO₂: 380 µatm, increased CO₂: 830 µatm), and b) temperature (low: 13.5 °C, midlow: 16.5 °C, midhigh: 19.9 °C, high: 22.1 °C) and grazer treatments (G: *G. umbilicalis*, M: *M. palmata*, G + M: *G.umbilicalis* + *M. palmata*). Different letters represent significant differences.

Figure 5. Effects of temperature (low: 13.5 °C, midlow: 16.5 °C, midhigh: 19.9 °C, high: 22.1 °C) and grazer treatments (G: *G. umbilicalis*, M: *M. palmata*, G + M: *G.umbilicalis* + *M. palmata*, C: No grazers) on mean (± SE) algal growth (*U. lactuca* g, blot wet weight) at a) ambient CO₂ (380 µatm) and b) increased CO₂ (830 µatm) in the end of Experiment II. Different letters represent significant differences.

Figure 6. Effects of temperature (low: 13.5 °C, midlow: 16.5 °C, midhigh: 19.9 °C, high: 22.1 °C) and CO₂ treatments (ambient CO₂: 380 µatm, increased CO₂: 830 µatm) on the Dynamic Index (DI, mean ± SE) as a strength measure of the herbivore-macroalgae interaction (Experiment II). Data is displayed according to significant factors (non-significant factor is averaged, see Table 2). Different letters and numbers represent significant differences.

Table 1. Summary of PERMANOVA for the effects of temperature (T, 4 levels: low, midlow, midhigh, high), CO₂ (2 levels: ambient and increased), and grazer (G, 3 levels: *G. umbilicalis*, *M. palmata*, *G. umbilicalis* + *M. palmata*) treatments on grazer survival and consumption rates for Experiment I. DF: Degrees of freedom, MS: Mean squares. Significant values are in bold ($p < 0.05$).

<i>Grazer survival rate</i>					<i>Algal biomass</i>				<i>Consumption rate</i>			
Source of variation	DF	MS	F	<i>P</i> (perm)	DF	MS	F	<i>P</i> (perm)	DF	MS	F	<i>P</i> (perm)
CO ₂	1	7133.5	14.911	0.0004	1	0.1700	11.852	0.0018	1	0.0002	3.7689	0.0596
T	3	13368	27.944	0.0002	3	0.0410	2.8572	0.0470	3	0.0001	1.2986	0.2862
G	2	3059.4	6.3952	0.0052	3	0.8490	59.190	0.0002	2	0.0102	241.45	0.0002
CO ₂ x T	3	538.84	1.1263	0.3466	3	0.0285	1.9904	0.1318	3	0.0001	2.4633	0.0762
CO ₂ x G	2	397.38	0.8307	0.4386	3	0.0497	3.4651	0.0256	2	0.0001	1.5419	0.2124
T x G	6	1315.6	2.7500	0.0242	9	0.0226	1.5745	0.1414	6	0.0001	2.0158	0.0814
CO ₂ x T x G	6	145.32	0.3038	0.9324	9	0.0110	0.7697	0.6466	6	0.0001	3.2616	0.0106
Residuals	48	478.40			64	0.0143			48	0.0000		
Total	71				95				71			

Table 2. Summary of PERMANOVA for the effects of temperature (T, 4 levels: low, midlow, midhigh, high), CO₂ (2 levels: ambient and increased), and grazer (3 levels: *G. umbilicalis*, *M. palmata*, *G. umbilicalis* + *M. palmata*) treatments on grazer survival rate, algal growth and Dynamic Index for Experiment II. DF: Degrees of freedom, MS: Mean squares. Significant values are in bold ($p < 0.05$).

Source of variation	<i>Grazer survival rate</i>				<i>Algal biomass</i>				<i>Dynamic Index</i>			
	DF	MS	F	<i>P</i> (perm)	DF	MS	F	<i>P</i> (perm)	DF	MS	F	<i>P</i> (perm)
CO ₂	1	22.22	0.0465	0.8202	1	0.0010	0.1223	0.7204	1	0.3155	8.0122	0.0042
T	3	4544.4	9.5116	0.0002	3	0.4468	52.9910	0.0002	3	0.8288	21.046	0.0002
G	2	16289	34.093	0.0002	3	0.2097	24.8660	0.0002	2	0.0346	0.8784	0.4376
CO ₂ x T	3	277.78	0.5814	0.6246	3	0.2306	27.3470	0.0002	3	0.8202	20.826	0.0002
CO ₂ x G	2	72.222	0.1511	0.8544	3	0.0316	3.7510	0.0154	2	0.0499	1.2681	0.2886
T x G	6	833.33	1.7442	0.1294	9	0.0546	6.4809	0.0002	6	0.0325	0.8250	0.5646
CO ₂ x T x G	6	394.44	0.8256	0.547	9	0.0447	5.2981	0.0006	6	0.0445	1.1306	0.3576
Residuals	48	477.78			64	0.0084			48	0.0394		
Total	71				95				71

Highlights

- Warming and acidification elicited negative effects in grazer's survival rates.
- Macroalgae-herbivore interaction strength was shaped by temperature gradient.
- Acidification shifts thermal optimal metabolic threshold to lower temperatures.
- Grazer identity may modulate macroalgae-herbivore interactions.