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ECOSYSTEM ECOLOGY – ORIGINAL RESEARCH

Seagrass-driven changes in carbonate chemistry enhance oyster shell growth

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Abstract

Quantifying the strength of non-trophic interactions exerted by foundation species is critical to understanding how natural communities respond to environmental stress. In the case of ocean acidification (OA), submerged marine macrophytes, such as seagrasses, may create local areas of elevated pH due to their capacity to sequester dissolved inorganic carbon through photosynthesis. However, although seagrasses may increase seawater pH during the day, they can also decrease pH at night due to respiration. Therefore, it remains unclear how consequences of such diel fluctuations may unfold for organisms vulnerable to OA. We established mesocosms containing different levels of seagrass biomass (*Zostera marina*) to create a gradient of carbonate chemistry conditions and explored consequences for growth of juvenile and adult oysters (*Crassostrea gigas*), a non-native species widely used in aquaculture that can co-occur, and is often grown, in proximity to seagrass beds. In particular, we investigated whether increased diel fluctuations in pH due to seagrass metabolism affected oyster growth. Seagrasses increased daytime pH up to 0.4 units but had little effect on nighttime pH (reductions less than 0.02 units). Thus, both the average pH and the amplitude of diel pH fluctuations increased with greater seagrass biomass. The highest seagrass biomass increased oyster shell growth rate (mm day^{-1}) up to 40%. Oyster somatic tissue weight and oyster condition index exhibited a different pattern, peaking at intermediate levels of seagrass biomass. This work demonstrates the ability of seagrasses to facilitate oyster calcification and illustrates how non-trophic metabolic interactions can modulate effects of environmental change.

Keywords Climate refugia · Marine macrophytes · Ocean acidification · Facilitative interaction · Non-trophic interaction

Introduction

Direct, indirect, trophic and non-trophic interactions among species act simultaneously to structure ecological communities. Non-trophic interactions, such as those exerted by foundation species in biogenic habitats, have fundamental community-wide implications because they alter the physical and chemical properties of the surrounding environment and influence the performance of sympatric species (Dayton 1972; Angelini et al. 2011). Accordingly, foundation species are essential to the amelioration of environmental stress (Stachowicz 2001; Jurgens and Gaylord 2018). Therefore, quantifying the strength of these non-trophic interactions is critical to understanding how organisms respond to climate change and to scaling up projected impacts to community and ecosystem levels (Hale et al. 2011; Sunday et al. 2017).

Oceans are changing as a consequence of the absorption of atmospheric carbon dioxide (CO_2) produced by human

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activities. The term "ocean acidification" (OA) describes the consequences of ongoing influx of CO_2 into seawater, which induces changes in the seawater carbonate system, including reductions in pH and carbonate ion (CO_3^{-2}) concentration (Caldeira and Wickett 2003). OA is predicted to significantly impact species and the ecology of marine communities (Gaylord et al. 2015; Nagelkerken and Connell 2015; Jellison and Gaylord 2019). In particular, OA is a major threat to calcifying taxa that precipitate calcium carbonate ($CaCO_3$) shells or skeletons, as the decrease in CO_3^{-2} concentrations drives an accompanying decline in $CaCO_3$ saturation state, with associated negative effects on growth, performance, and survival of many of such species (e.g., Kroeker et al. 2013).

Marine macrophytes (i.e., seagrasses and seaweeds) have been proposed to create "refugia" from OA in coastal areas (Nielsen et al. 2018) due to their capacity to influence the seawater carbonate system. They remove dissolved inorganic carbon (DIC) through photosynthetic activity, which shifts the equilibrium of inorganic carbon constituents and increases local pH and CO_3^{-2} concentration (Hendriks et al. 2014; Krause-Jensen et al. 2016). However, such elevations in mean values of pH and CO_3^{-2} are often accompanied by larger diel fluctuations promoted by light-driven cycles between net photosynthesis and net respiration (Hofmann et al. 2011), and these fluctuations are expected to be larger in the future (Pacella et al. 2018). Whether such enhanced fluctuations also affect associated calcifying organisms remains incompletely known, with some studies suggesting positive effects (Silbiger and Sorte 2018; Wahl et al. 2018) and others not (Cornwall et al. 2013). Further work examining consequences of strong fluctuations in pH and associated carbonate chemistry parameters is, therefore, warranted (Barry et al. 2013). This need is especially acute given that fluctuations occur in a variety of habitats (Kwiatkowski et al. 2016; Pacella et al. 2018; Kapsenberg and Cyronak 2019) and often produce minima lower than those predicted for mean global pH under future scenarios of OA.

Seagrasses are foundation species present in many coastal areas worldwide (Short et al. 2001), and some species have been shown to naturally influence calcification in calcareous macroalgae (Semesi et al. 2009; Barry et al. 2013), while also offsetting OA effects in manipulative experiments (Bergstrom et al. 2019). However, such non-trophic metabolic interactions remain underexplored in temperate seagrasses (e.g., *Zostera marina*), and little research has tested for effects of seagrasses on the calcification and growth of organisms at trophic levels above that of basal macroalgae. The few temperate-system studies that have been conducted on seagrass impacts on bivalves (see Groner et al. 2018; Lowe et al. 2019; Spencer et al. 2019) have reported variable results including cases of negligible benefit to the calcifiers. A potential contributor to these inconsistent outcomes may be that prior studies have tended to use factorial designs that compare presence/absence of seagrass. Such approaches cannot resolve non-linear responses that might arise along a gradient of seagrass abundance (Wahl et al. 2018).

Here, we investigated whether seagrass-induced increases in the amplitude of diel pH fluctuations (and associated carbonate chemistry parameters) affected the growth of juvenile and adult oysters, using multiple levels of seagrass biomass to manipulate pH conditions. Oysters, along with other bivalves and calcifying organisms, often inhabit or grow adjacent to seagrass meadows (Trimble et al. 2009; Smith et al. 2009), and oyster farms are often located in their proximity (Padilla 2010). Indeed, protection and/or restoration of seagrasses have been suggested as a possible strategy for oyster growers to mitigate against threats of ocean acidification to shellfish production (Tan and Zheng 2020). We hypothesized that higher seagrass biomass would increase the amplitude of diel pH fluctuations in seawater, and thereby benefit calcification in oysters by decreasing aqueous DIC during the day, which increases pH (Figs. 1a-–c). Alternatively, seagrass biomass might decrease oyster growth. In this latter scenario, the tendency for greater seagrass biomass to promote higher magnitude diel fluctuations in pH might impose nighttime pH minima due to net respiration that drops below critical thresholds of physiological stress (Fig. 1a, b). Such stressful conditions for oysters, which in this scenario would be induced at very high levels of seagrass biomass, could potentially outweigh the beneficial effects of higher time-averaged pH (Fig. 1c).

Materials and methods

Study species

The seagrass *Zostera marina,* commonly known as eelgrass, is a widely distributed species in the northern hemisphere that occupies a wide range of habitat conditions from intertidal to shallow subtidal zones, and from sheltered to exposed areas. Extensive natural monocultures of this temperate seagrass occur throughout most of its distribution, and these beds are critical habitat and a basis of coastal food webs (Moore and Short 2006). The Pacific oyster, *Crassostrea gigas*, is native to Japan but is distributed worldwide as a result of being widely grown in aquaculture since the last century (Padilla 2010). Although non-native, we selected *C. gigas* for this study both because it is frequently cultivated near seagrass beds (Ferriss et al. 2019), and because it can serve as a model species for understanding how calcifying organisms might be influenced by seagrasses. More than 95% of the shell weight in *C. gigas* consists of biologically precipitated $CaCO_3$ deposited mainly as low-magnesium

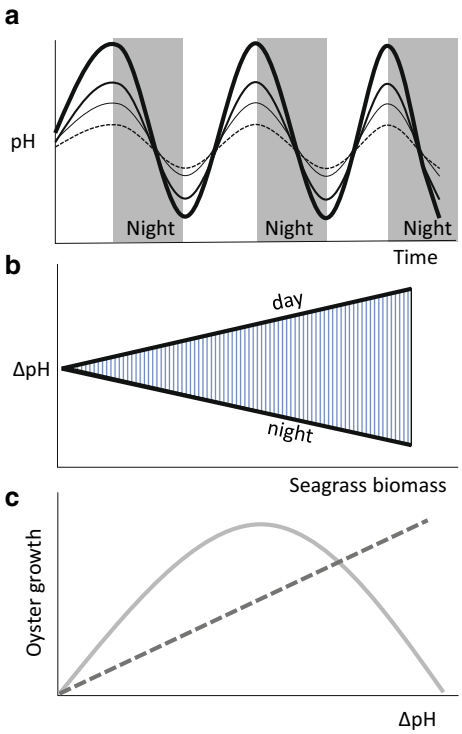

Fig. 1 Hypotheses of seagrass (*Zostera marina*) driven changes in seawater carbonate chemistry and oyster (*Crassostrea gigas*) performance. **a** Expected temporal fluctuation of pH with increasing levels of seagrass biomass (solid lines where increasing thickness represents increasing biomass) and a control treatment with no seagrass (dashed line). **b** Expected pH fluctuation and pH maxima in a gradient of seagrass biomass during the day (with an increase in peak pH due to photosynthetic activity) and during the night (with lower pH minima due to respiration effects). **c** Two alternative hypotheses regarding how an increase of seagrass biomass might affect oyster growth through its effects on pH; a monotonically positive facilitative interaction (dashed line) or a non-linear hump-shaped interaction (solid line) due to negative effects of the highest-amplitude pH fluctuations which concomitantly generate the lowest pH minima

calcite with small inclusions of aragonite (Stenzel 1963; Taylor et al. 1969).

Sampling and experimental setup

To examine the effects of seagrass on the growth of juvenile and adult oysters, an outdoor mesocosm experiment was conducted using a flow-through seawater system at the Bodega Marine Laboratory (BML) of the University of California, Davis in Bodega Bay, California, USA. In April 2018, a cohort of 2-month-old *C. gigas* oysters, ranging from 6.07 to 7.09 mm in length, (hereafter juveniles) and a cohort of 1-year-old *C. gigas* oysters, ranging from 26.8 to 53.8 mm in length (hereafter adults) were sourced from the Hog Island Oyster Company (Humboldt Bay, California, USA). These cohorts were produced from captive broodstock and reared under common conditions before they were transported on ice to BML, where they were maintained in flowing seawater at 12 °C until the beginning of the experimental trials. During this time, oysters received ambient food concentrations (a mix of diatoms *Navicula* spp. 1–10%, *Odontella* spp. 1–5%, *Skeletonema* spp. 1–10%, *Fragilaria* spp. 1–10% and other detritus 60–90%) as found in the flow-through seawater drawn from coastal waters immediately adjacent to the laboratory.

Experiments were conducted on the juvenile oysters in early April 2018, and on the adults in late May 2018. Both experiments were conducted during periods of similar oceanographic conditions (Bodega Ocean Observing Node 2018). For these experiments, the oysters were placed in mesocosms along with specified biomasses of the seagrass, *Z. marina*, collected and processed as follows. Non-reproductive terminal shoots of seagrass were gathered by hand from the low intertidal zone of Bodega Harbor (California, USA) during low tide and immediately transported in cool seawater to BML. In the laboratory, shoots were carefully cleaned to remove epiphytes using a cotton pad. Following established methods (Hughes et al. 2009; DuBois et al. 2019), leaves were trimmed to 35 cm, and rhizomes were cut to 6 cm (to standardize above- and below-ground biomass). Seagrass shoots were transplanted into plastic flowerpots (5 shoots per 0.5 L pot; dimensions = 8 × 8 × 9 cm, L × W × H, respectively), each filled with natural sediments from the same seagrass meadow sieved through a 1 mm mesh. After potting, all plants were held in the same outdoor tank supplied with flow-through seawater for a 15-day acclimation period, before they were assigned randomly to the different experimental treatments.

The treatments consisted of five different levels of increasing shoot density, thus generating a gradient of seagrass biomass, where 4 replicate tanks of 200 L (dimensions = 70 × 40 × 70 cm, L × W × H, respectively) were used per treatment: controls (with no seagrass), low seagrass biomass (with 2 pots with plants, 57 shoots per m^{-2}), medium seagrass biomass (with 4 pots with plants, 115 shoots per m^{-2}), high seagrass biomass (with 9 pots with plants, 259 shoots per m^{-2}) and very high seagrass biomass (with 18 pots with plants, 517 shoots per m^{-2}). Shoot densities were selected based on annual natural shoot density values in nearby areas (Huntington and Boyer 2008; Ha and Williams 2018). In each tank, more pots were included with only natural sediments to equalize the amount of sediment in all tanks. The medium biomass treatment was not used for the trial with adult oysters. All pots were uniformly distributed across the bottom of the tanks. All tanks were randomly distributed across two rows oriented north–south, avoiding auto-shading so that all tanks received the same sunlight exposure throughout the experiment. PAR light measurements, made on the bottom of each tank (LICOR Li-193

spherical underwater quantum sensor), showed light levels above the threshold established for the maximum photosynthetic rate of *Z. marina*, ~ 200 µmol photons m^{-2} s^{-1} (Beer et al. 1998). Running seawater was supplied to each tank from the same source at a rate of 25 ml s^{-1} to mimic natural conditions of average flow in nearby areas (Worcester 1995). Seawater was supplied into one side of the tank and drained through the opposite side following the longest axis of the tank so that water flow had a constant direction and no obvious stagnant regions were present. During the experimental trials, temperatures in the tanks ranged between 9 and 13 °C with typical diel fluctuations of ~ 2 °C. Salinity ranged from 32.68 to 34.21, with diel fluctuations of ~ 0.1. Dissolved oxygen concentration (measured with MiniDOT sensors, PME, Vista, CA, USA and Aanderaa Data Instruments AS, whose factory calibrations were verified in air-bubbled seawater assuming 100% oxygen saturation) ranged between 8.6 and 17.24 mg L^{-1} with typical diel fluctuations of ~ 0.6 mg L^{-1} in the control treatments (with no seagrass) to ~ 5 mg L^{-1} in the highest seagrass biomass. Every 2 days, detached seagrass leaves and any new epiphytes on the leaves and tank walls were removed. Epiphytes from the leaves were gently removed by gliding two fingers along the blades to avoid any abrasion, and epiphytes from the tank walls were removed using a household cleaning sponge.

For the juvenile trial, 30 oysters were deployed into each mesocosm tank. In each tank, oysters were placed into small, mesh-sided plastic cages with separate compartments so that each oyster could be individually tracked (Fig. 1ESM). These cages were then suspended in the middle of the tank and at the height of the seagrass canopies. The juvenile oyster experimental trial lasted 30 days. For the adult trial, 5 oysters were deployed into each tank. Oysters were individually labeled with a numbered plastic bee tag on the shell and haphazardly placed (not attached) on a horizontal plastic tile suspended at the height of the seagrass canopies. The adult oyster experimental trial lasted 10 days, which was sufficient to detect differences in growth using the calcein staining method (see "Oyster traits" below). Morphological measurements of oysters were made at the start of the experimental trials (see detailed methods below). At the end of the experimental trials, live and dead oysters were counted to quantify survival and brought into the laboratory for further measurements.

To verify that biomass values across the multiple treatments remained distinct from each other throughout the trials, and that plants retained similar health conditions, seagrass shoot density and growth were measured in all tanks. Shoot density was measured in each tank at the beginning and end of the experimental trials. To estimate shoot-specific growth, all seagrass leaves were punched with a hypodermic needle, 2 cm above the meristem within the sheath 7 days before the end of the experimental trials (Zieman 1974; Short 1987). At the end of the experimental trials, all shoots were harvested, and maximum leaf growth was calculated per shoot (cm day^{-1}).

Monitoring of seawater carbonate system

Regular monitoring of pH, temperature and salinity conditions was conducted in each tank and on the incoming seawater throughout the experimental trials. Monitoring occurred daily and twice per day, at 6:00 am (before sunrise) and at 2:00 pm (during sunlight exposure) local time to target pH minima and maxima, respectively. Sampling times were selected a priori based on analyses of 1 week of data from three autonomous sensor packages employing Honeywell Durafet pH sensors. One sensor package was deployed in a tank with the highest seagrass biomass treatment. The other two sensor packages were deployed at 1 m depth during low tide in Bodega Harbor, one within a seagrass meadow with similar shoot density of the highest seagrass biomass treatment, and the other in a non-vegetated area. In all cases, the temporal pH series showed that the pH minima occurred around 6:00 am, and pH maxima around 2:00 pm (Fig. 2ESM). Therefore, these sampling times were kept consistent through both experimental trials and represent a conservative measure of diel pH range. Values of pH, temperature and salinity were recorded in the tanks using a multiparameter meter (Yellow Springs Instruments Professional Plus Multiparameter meter, YSI ProPlus). The pH meter on the YSI ProPlus (0.2 mV resolution) was calibrated on the total scale using discrete water samples (Easley and Byrne 2012, $g' = 69.44$, $E_0 = 457.98$; $r^2 = 0.96$; $P < 0.005$, $n = 190$). Discrete water samples were acquired from each replicate tank at the beginning and at the end of each experimental trial for analysis of pH and also total alkalinity (A_T). In addition, a 24 h cycle of discrete water samples was completed during the adult oyster experimental trial in which each tank was sampled every 3 h beginning at 8:00 am.

Seawater pH of the discrete water samples was determined on the total scale with a high-precision spectrophotometer (Ocean Optics Jaz Spectrophotometer EL200) using unpurified m-cresol purple dye (Clayton and Byrne 1993). Water samples were kept in a temperature-controlled water bath (Thermo Scientific, Precision Microprocessor Controlled 280 Series) at 25 °C before analysis to minimize temperature-induced errors in absorbance measurements. The spectrophotometer was validated by analyzing TRIS buffer revealing that the system was accurate to within 0.005 pH units. The Excel macro, CO2SYS, was used to calculate in situ pH values using in situ salinity and temperature measurements. Seawater A_T was measured using open-cell titration with triplicate samples (Metrohm 855 Robotic Titrosampler, Metrohm, USA) using 0.1 N HCl (Fisher Chemical) diluted to a nominal concentration of 0.0125 M.

Acid concentration was calibrated by analyzing Certified Reference Material (CRM Batch 170) from A. Dickson's laboratory before each titration session. For each set of triplicate analyses, the median value was considered. Instrumental precision, calculated from 20 analyses of CRM Batch 170 over the course of the study, was SD < 5 µmol kg^{-1}. Calcite saturation state (Ω_c) and pCO_2 were calculated from pH and the mean A_T from all discrete water samples taken in each tank per experimental trial (Table 1). Seawater with a saturation state greater than one indicates a thermodynamic tendency of calcium carbonate to precipitate while seawater with a saturation state less than one favors dissolution (Zeebe and Wolf-Gladrow 2001; however, see Ries et al. 2016). Carbonate system calculations were performed using the *seacarb* R package (Gattuso et al. 2019) and assuming published values for constants K_1 and K_2 (Lueker et al. 2000), K_f (Perez and Fraga 1987), and K_s (Dickson 1990).

Oyster traits

Shell growth in juvenile oysters was assessed by measuring the change in shell surface area. Photos of each individual were taken before and after the experimental trial with a camera facing down at a fixed height to the shell surface and the projected shell area was quantified using image analysis software (ImageJ v1.8, NIH). Note that projected area quantified in this fashion can differ from the actual surface area of a contoured shell; our analysis focuses on the former. Relative change in shell area was estimated by subtracting the initial area from the final area and then dividing by the initial area. After photography, juvenile oysters were dried in an oven at 80 °C for 24 h and weighed for dry weight. A subset of 10 juvenile oysters per treatment ($n = 50$) were dissected, and somatic tissue was separated from the shell and weighed for dry weight. Then, the proportion of organic matrix in the oyster shell was calculated by subtracting ash-free dry weight (500 °C for 8 h) from the pre-ashed dry shell weight and dividing by the latter. In the remaining oysters ($n = 550$), somatic tissue mass was estimated by subtracting ash-free dry weight (500 °C for 8 h) from the pre-ashed total oyster weight (including tissue), correcting for the average proportion of shell organic matrix per treatment.

Shell growth in adult oysters was assessed by calcein staining (Moran 2000; Mahé et al. 2010). All individuals were marked with fluorochrome calcein (Sigma Aldrich, France) 1 day before the experimental trial. A stock solution containing 6.25 g L^{-1} of stain powder in filtered seawater (0.1 µm) was dissolved over 12 h at 20 °C and pH 6. This solution was then diluted with more seawater to a final concentration of 150 mg L^{-1} in which oysters were immersed for 90 min. Before being immersed, oysters were lightly scrubbed and rinsed to remove epibionts from the shell surface. After immersion, specimens were rinsed again with seawater and placed in the treatment tanks for the experimental trial. At the end of the experimental trial, adult oysters were dissected, and somatic tissue was separated from the shell, dried at 80 °C for 24 h and weighed for dry weight. To observe the calcein stain, the inner edge of the upper valve of each oyster shell was photographed after excitation with 450–480 nm light under a stereomicroscope. The distance from the calcein stain mark to the most external edge was measured at 15 points along the contour of each shell using

Table 1 Seawater carbonate chemistry parameters

Juveniles experimental trial												
Seagrass treatment	pH_T				A_T (µmol kg^{-1})				Calcite saturation state (Ω_c)			
	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max
Incoming water	7.93	0.11	7.79	8.25	2263	3	2258	2266	2.65	0.76	1.82	5.01
Control	8.00	0.14	7.77	8.32	2265	3	2260	2274	3.16	1.02	1.75	5.86
Low biomass	8.03	0.16	7.75	8.34	2264	5	2250	2270	3.35	1.11	1.69	6.05
Medium biomass	8.04	0.16	7.75	8.32	2265	3	2258	2271	3.37	1.11	1.67	5.74
High biomass	8.06	0.17	7.73	8.37	2266	2	2261	2271	3.57	1.24	1.59	6.24
Very high biomass	8.07	0.18	7.73	8.43	2266	3	2261	2270	3.64	1.29	1.62	6.82
Adults experimental trial												
Incoming water	7.90	0.08	7.83	8.14	2271	5	2264	2280	2.42	0.54	2.00	4.11
Control	7.93	0.13	7.83	8.32	2270	5	2263	2279	2.71	0.95	1.98	5.76
Low biomass	7.95	0.15	7.83	8.37	2270	5	2259	2279	2.83	1.11	2.01	6.27
High biomass	7.99	0.16	7.84	8.36	2269	7	2250	2279	3.07	1.23	2.01	6.81
Very high biomass	8.00	0.14	7.82	8.42	2270	0	2269	2270	3.12	1.07	1.94	6.16

Juvenile and adult oysters were held in treatments with no seagrass (control) and 3–4 levels of increasing seagrass biomass

pH_T pH in the total scale, A_T total alkalinity

image-analysis software (ImageJ v1.8, NIH) and averaged to one measure of shell growth per oyster. No bias from autofluorescence was detected using shells that had not been stained and calcein-marked dead shells. The condition index in juvenile and adult oysters was calculated as the ratio of dry somatic tissue weight to total dry weight (shell and somatic tissue) multiplied by 100 (Davenport and Chen 1987).

Data analysis

The effects of seagrass biomass on mean pH, pH diel fluctuations, pH during the day, and pH during the night were assessed using simple linear regressions with average shoot density per tank as the independent variable. For these analyses, the pH values measured throughout the experimental trial were averaged by tank. The relative oyster shell growth, proportion of shell organic matrix, somatic tissue weight, shell weight and condition index of juvenile and adult oysters were analyzed using linear mixed models. Average shoot density per tank was used as a fixed factor and tank as a random factor, which allowed us to account for the non-independence of samples within tanks. The linear effect of shoot density on traits in oysters and the potential for a quadratic relationship were tested, including a covariate of oyster total weight (shell and somatic tissue) for non-relative variables. All models were fitted by maximum likelihood estimation using the *lme4* R package (Bates et al. 2014). All models were compared with a null model and the best model was chosen based on AIC and by performing a Chi-square test to assess model significance ($P < 0.05$). Normality and homoscedasticity were assessed via visual estimation of trends of model residuals. A metric of oyster shell thickness was computed as the ratio of shell weight to shell surface area (measured at the end of the experimental trials). We then tested whether estimated shell thickness was related to shell growth. Data analysis was performed in R Statistical Software Environment (2019).

Results

Experimental conditions

The different biomass treatments remained non-overlapping throughout both experimental trials despite the increase in shoot density in all tanks (Fig. 3ESMa, c). Seagrass leaves grew at the same rate in all treatments in each experimental trial, 0.72 cm day^{-1} ± 0.08 SE in the juvenile trial and 1.93 cm day^{-1} ± 0.04 SE in the adult trial (Fig. 3ESMb, d). Seagrass treatments established a clear gradient of pH, pCO_2, bicarbonate ion (HCO_3^-), carbonate ion (CO_3^{-2}), dissolved inorganic carbon (DIC), and calcite saturation state (Ω_c), each correlated with seagrass biomass (Table 1 and Table 2ESM). Alkalinity (A_T) remained invariant across treatments (Table 1 and Table 2ESM). These trends were confirmed during the 24 h cycle (Table 3ESM). For simplicity, we hereafter refer to seawater carbonate chemistry in terms of pH.

In both experimental trials, pH ranged from 7.73 to 8.43 (Table 1), with diel pH fluctuations ranging from 0.01 to 0.4 pH units (Table 1; Fig. 2a, b). Higher seagrass biomass promoted larger fluctuations in pH in both trials (Juveniles: $r^2 = 0.81$; df = 18; $P < 0.001$; Adults: $r^2 = 0.73$; df = 14; $P < 0.001$), and also a higher mean pH (Juveniles: $r^2 = 0.68$; df = 18; $P < 0.001$; Adults: $r^2 = 0.71$; df = 14; $P < 0.001$) (Table 1; Fig. 2a, b). These fluctuations were mostly driven by the increase of pH during the day (Juveniles: $r^2 = 0.75$; df = 18; $P < 0.001$; Adults: $r^2 = 0.72$; df = 14; $P < 0.001$), as at night, all pH values were similar among treatments with just a slight decline in pH minima with seagrass biomass in the juvenile trail ($r^2 = 0.61$; df = 18; $P < 0.001$), but not in the adult oyster trial ($r^2 = 0.01$; df = 14; $P = 0.64$) (Table 1; Fig. 2c, d).

Oyster traits

Oysters exhibited an overall survival rate of 96.5% in the juvenile trial and 100% in the adult trial. Despite the variability found among individual oysters within the treatments, relative shell growth of juvenile and adult oysters increased significantly with seagrass biomass ($P < 0.01$; Table 1 ESM; Fig. 3a, b). The proportion of shell organic matrix in juveniles also increased significantly with seagrass biomass ($P = 0.03$; Table 1 ESM). Growth rates ranged from 0 to 0.66 mm^2 day^{-1} in juveniles, and 0–0.18 mm day^{-1} in adults. Shell weight at the end of the experiment was not significantly correlated with seagrass biomass either in juveniles or adults ($P > 0.05$; Table 1 ESM, Fig. 4ESM). In contrast, somatic tissue weight at the end of the experiment was significantly related to seagrass biomass in juveniles ($P = 0.04$; Table 1 ESM, Fig. 4ESM) and adults ($P < 0.01$; Table 1 ESM, Fig. 4ESM), where the best model fitting our data included the quadratic term of shoot density. The condition index was also significantly related to seagrass biomass in both juvenile ($P = 0.04$; Table 1 ESM; Fig. 3c) and adult oysters ($P = 0.03$; Table 1 ESM; Fig. 3d), and the best model also included the quadratic term of shoot density. The ratio of shell weight to shell area (an index of shell thickness) declined with increasing shell growth in juvenile oysters ($P < 0.01$; Table 1 ESM; Fig. 4a). This effect was not significant for adult oysters ($P = 0.86$; Table 1 ESM; Fig. 4b).

Discussion

Results of this study indicate that higher seagrass biomass increased mean pH and the amplitude of diel pH fluctuations in seawater carbonate chemistry, and that these changes were

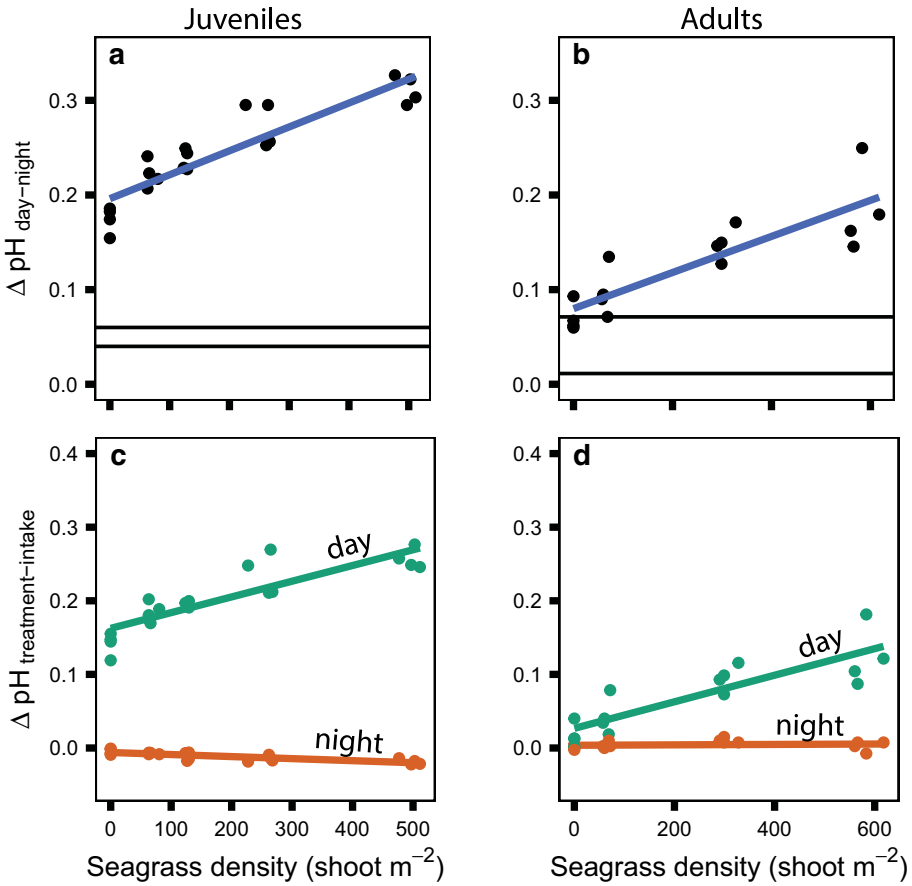

Fig. 2 Fluctuation of pH in experimental treatments. Solid lines correspond to simple linear regressions using shoot density as predictor. Upper panels: average diel variation of pH per tank across the seagrass (*Zostera marina*) biomass gradient. The analogous range of pH fluctuation (min and max) in the intake water is shown for comparison by black horizontal lines. **a** Juvenile oysters trial ($r^2=0.81$; $P<0.001$, $n=20$); **b** adult oysters trial ($r^2=0.73$; $P<0.001$, $n=20$). Lower panels: average diel variation of the difference between tank pH and the intake seawater pH, during the night (orange) and during the day (green) across the seagrass biomass gradient. Seagrass shoot density values are averaged from the initial and final shoot density data in each tank. **c** juvenile oysters trial (Day: $r^2=0.75$; $P<0.001$, $n=20$; Night: $r^2=0.61$; $P<0.001$, $n=20$); **d** adult oysters trial (Day: $r^2=0.72$; $P<0.001$, $n=16$; Night: $r^2=0.01$; $P=0.64$, $n=16$)

associated with elevated oyster performance, particularly shell growth. Seawater pH increased more during daytime hours than it decreased during nighttime. Higher seagrass biomass increased oyster shell growth monotonically, however, somatic tissue weight and the condition index peaked at intermediate levels of seagrass biomass (i.e., low and medium treatment levels). Our results indicate that the strength of a non-trophic metabolic interaction between a photosynthetic habitat-forming species and a calcifying organism can be modulated by functional traits of the habitat (i.e., shoot density or biomass) and could help to mitigate the decrease in seawater pH due to global environmental change.

Understanding the causes and consequences of coastal environmental variability in seawater carbonate chemistry is crucial for predicting future consequences of OA. Previous studies have quantified diel pH fluctuations driven by marine macrophytes (e.g., Krause-Jensen et al. 2016 for seaweed; e.g., Buapet et al. 2013; Hendriks et al. 2014 for seagrass) and natural diel pH fluctuations of up to 1 unit have been described (Middelboe and Hansen 2007). However, the importance of these fluctuations in biological processes and what aspect of the variability (e.g., mean, minimum, range) organisms respond to is still unclear. In this study, diel pH fluctuations seem mostly driven by net photosynthetic activity (rather than net respiration) as seagrass metabolism during the day affected pH to a greater extent than at night. As a consequence, effects of mean pH versus pH fluctuations are difficult to separate. Nevertheless, the mesocosm results reported here confirm that seagrass facilitates calcification of oyster shells, in contrast to prior findings that have been inconsistent in showing evidence of positive effects of seagrass on oyster growth (Groner et al. 2018; Lowe et al. 2019).

It is widely acknowledged that in marine systems, calcification depends on external seawater carbonate chemistry (Thomsen et al. 2015; Waldbusser et al. 2015). Here, as seagrass biomass increased, shell growth in juvenile oysters increased up to 40% relative to controls and nearly 5% in the more slowly growing adults. These findings suggest that facilitative effects of seagrasses over bivalves can arise not only from their tendency to trap and foster higher particulate food concentrations within their confines (Peterson et al. 1984), but also through their capacity to change the seawater carbonate system.

The effects of seagrass biomass on the somatic tissue weight and condition index of oysters differed from the response observed in shell growth, in that the former two responses peaked at intermediate seagrass biomass levels. The smaller somatic tissue weight and condition index observed in the higher seagrass biomass treatment suggest

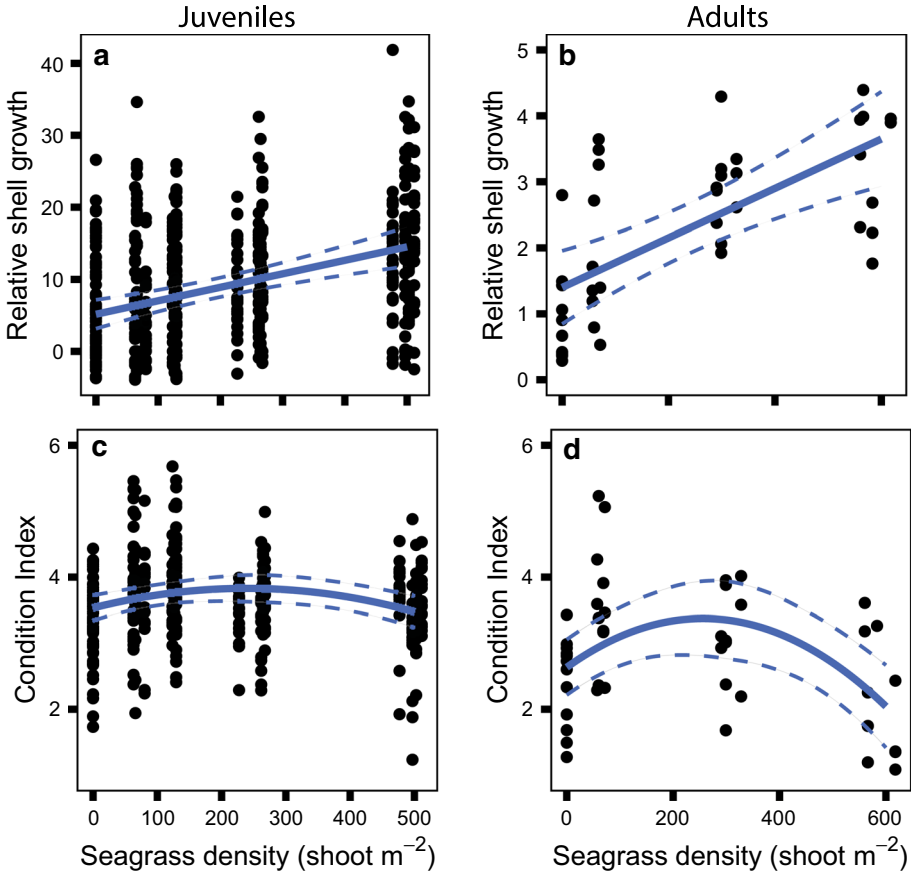

Fig. 3 Relative shell growth (upper panels) and condition index (lower panels) of oysters (*Crassostrea gigas*) as a function of seagrass (*Zostera marina*) biomass. Seagrass shoot density values are averaged from the initial and final shoot density data in each tank. Solid lines correspond to the prediction of a linear mixed model using shoot density as fixed effect. Dashed lines represent the 95% confidence intervals around the fitted values. **a**, **c** Juvenile oysters trial ($n = 120$ per treatment); **b**, **d** adult oysters trial ($n = 25$ per treatment). Statistical details can be seen in text and Table 1 ESM

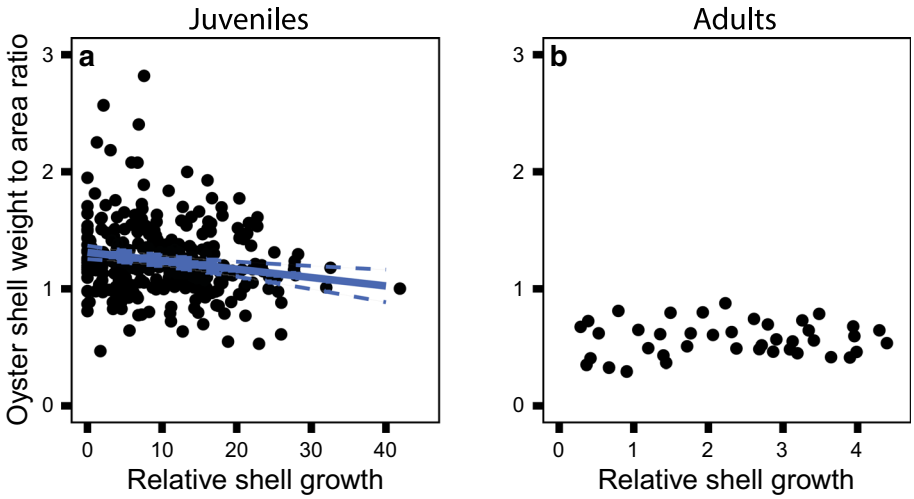

Fig. 4 Ratio of shell weight to surface area vs. shell growth of oysters (*Crassostrea gigas*). The ratio of shell weight to surface area was calculated as an index of shell thickness. Solid lines correspond to the prediction of the linear mixed model. Dashed lines represent the 95% confidence intervals around the fitted values. **a** Juvenile oysters trial ($n = 120$ per treatment); **b** adult oysters trial ($n = 25$ per treatment). Statistical details can be seen in text and Table 1 ESM. No statistically significant trend was apparent for adults

that our results on oyster shell growth were not biased by a higher availability of food trapped within the canopy in the higher seagrass biomass treatment. Upon first consideration, these contrasting responses between shell and tissue growth might suggest an energetic trade-off. In particular, exposure to more extreme low pH conditions might create conditions that increase the energetic costs of calcification. Under unfavorable carbonate conditions, bivalves invest more resources into maintaining the acid–base balance required for calcification, which can reduce the energy available for protein synthesis and somatic tissue growth (Thomsen et al. 2015; Pan et al. 2015). The same pattern has been seen in other groups of mollusks, which prioritize energy for shell formation over other physiological processes in extreme experimental OA conditions (Carey et al. 2016; Sigwart et al. 2016). However, in contrast to our initial expectations, an increase in

seagrass biomass did not translate into lower pH values at night. Thus, although oysters in the high biomass treatment were exposed to greater variability in pH, it seems unlikely that these oysters experienced greater energetic challenges because pH minima were similar across treatments.

Rather, our results support an alternative hypothesis that shell growth and somatic tissue growth were uncoupled and not coincident in time (Hilbish 1986). Bivalve shell growth responds more quickly to seawater chemistry as shells have lower organic content and only have partial dependence on metabolic carbon (Nair and Robinson 1998). In contrast, tissue growth is more sensitive to food availability and patterns of energy storage and utilization (Gabbott 1976). In this scenario, shell growth might respond rapidly to the influence of seagrass-mediated carbonate chemistry, whereas tissue growth might be minimally influenced by water chemistry. It is also possible that somatic tissue growth might have been reduced in treatments with higher seagrass biomass as a result of changes in the quantity of food available for oysters. Denser seagrass canopies can reduce intensities of turbulent mixing, which can encourage suspended food resources that are denser than seawater to settle faster out of the water column. Additional investigations are needed to investigate whether such trophic pathways might contribute to reduced tissue growth in areas of high seagrass biomass.

Fast-growing mollusks often have thinner shells (Kemp and Bertness 1984). In this study, although growth in oyster shells was enhanced by seagrass biomass, newly formed portions of shell were apparently thinner. In spite of this trend, our estimates of shell thickness were assessed by changes in shell mass, and thus do not consider possible changes in the mineralogy or density of new shell produced during periods of faster growth. Both possibilities have been suggested as mechanisms to counteract negative effects of low pH on growth in some calcifiers (Swezey et al. 2017; Martinez et al. 2019), and such changes can have important ecological implications (e.g., risk of predation by drilling gastropods) and should be further studied (Sanford et al. 2014; Barclay et al. 2019).

Previous studies have shown facilitative effects of different marine macrophytes on the growth of calcifying organisms, mostly algae, corals and foraminifera (Semesi et al. 2009; Unsworth et al. 2012; Barry et al. 2013), although others have shown deleterious or no effects (Cornwall et al. 2013; Pettit et al. 2015; Roleda et al. 2015). Overall, it appears that the role of marine macrophyte communities as potential OA refugia depends on the balance between positive effects during the day, due to net photosynthesis, and negative effects during the night, due to net respiration (Krause-Jensen et al. 2016). These fluctuations are strongly scale-dependent: spatial effects of macrophytes on pH have been described over scales ranging from the diffusive boundary layer (mm to cm) (Hurd 2015; Hendriks et al. 2017) to the habitat scale (meters to km) (Manzello et al. 2012; Unsworth et al. 2012; Kwiatkowski et al. 2016; Wahl et al. 2018). In addition, the effects of macrophytes on pH have been described on temporal scales ranging from hours (as in this study) to seasons (Krause-Jensen et al. 2016). Most of these effects are dependent on light availability and the macrophyte biomass to water volume ratio, and so, daylength and current flow have important influences on the overall pH dynamics (Barry et al. 2013; Koweek et al. 2018; James et al. 2020). Our study, despite keeping constant flow conditions across treatments, demonstrates a facilitative interaction of seagrasses over oyster calcification while mimicking seagrass biomass and pH fluctuations in nearby areas (Shukla et al. 2011), where pH within and outside seagrass was also similar at night (Fig. 2 ESM). We conducted these experiments during the seagrass growth season because the magnitude of the pH fluctuations tends to be larger (Hendriks et al. 2014). Nonetheless, seagrasses, as perennial species, are expected to continue to facilitate oyster growth during the colder winter months if photosynthesis effects exceed respiration (Duarte and Cebrián 1996). However, in the field, the strength of seagrass biomass effects on pH fluctuations can be masked by other ecological processes that affect seagrasses or calcifying organisms abundances such as herbivory or predation (Lowe et al. 2019). Thus, the contrast between our results and those from previous seagrass-bivalve-calcification studies could be due to context specificities, such as spatially limited effects of seagrasses over oysters (Lowe et al. 2019); or the species of bivalve (Spencer et al. 2019), as different species can show different growth rates and could imply a change in the strength of the seagrass-calcification interaction. Future studies in natural communities should compare in situ calcification of different species and pH regimes at a variety of spatial and temporal scales to assess the influence of seagrass biomass in different hydrodynamic conditions and habitat mosaics.

The definition of OA refugia is based on the amelioration of unfavorable conditions of low pH, or the capacity to enhance acclimatization or adaptation to low pH values (Kapsenberg and Cyronak 2019). Our study supports the hypothesis that seagrass beds could operate as OA refugia based on their capacity to increase pH, and highlights that the refuge can be mediated by seagrass shoot density or biomass with a strong temporal component dictated by diel fluctuations. Therefore, natural variability of seawater carbonate chemistry, promoted by net photosynthetic effects in seagrasses, might help mitigate episodes of extreme low pH (i.e., upwelling events). Moreover, given that the photosynthetic performance of seagrasses is expected to increase in a higher CO_2 world (Koch et al. 2013), seagrasses may mitigate further long-term OA effects, assuming other detrimental effects (i.e., warming, eutrophication) do not reduce their populations. Thus, this carbon ecosystem service from

seagrasses has important implications for conservation and management in coastal areas and also supports growing interest in the potential for seagrasses and other marine macrophytes to be used in integrated multi-trophic oyster aquaculture (Clements et al. 2018).

In conclusion, our results demonstrate that seagrasses can facilitate the shell growth of co-occurring calcifying organisms in association with changes in the carbonate system of seawater that bolster calcification. This study suggests that non-trophic metabolic interactions, here mediated by increased seagrass shoot density or biomass, could potentially help alleviate the consequences of human-induced environmental change on calcifying organisms. As such, this study contributes to understanding CO_2 dynamics in coastal vegetated areas and supports the hypothesis that marine macrophytes can create local OA refugia.

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Author contribution statement AMR, BG, TMH and ES conceived and designed methodology. AMR, JDS, PS, MW and AN collected the data. AMR, BG and ES analyzed the data. AMR led the writing of the manuscript. All the authors contributed critically to the drafts and gave final approval for publication.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval All applicable institutional and/or national guidelines for the care and use of animals were followed.

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