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$\delta^{13}C$ and $\delta^{18}O$ signatures from sea urchin skeleton: importance of diet type in metabolic contributions

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ABSTRACT: The incorporation of $\delta^{13}C$ and $\delta^{18}O$ from respired CO_2 to tests and whole calcified tissues (WCT) of the sea urchin *Lytechinus variegatus* was investigated in 120 individuals to elucidate the influence of diet type on the calcification process. Sea urchins were raised during 4 mo in controlled seawater tanks using 3 different diets ($\delta^{13}C$; $\delta^{18}O$) (means ± SE): seagrass (−10.2 ± 0.1‰; −15.2 ± 0.3‰), red macroalgae (−17.8 ± 1.4‰; −21.6 ± 0.8‰), and a formulated diet (−21.5 ± 0.04‰; −25 ± 0.4‰). Individuals fed the formulated and red macroalgae diets were depleted in both $\delta^{13}C$ and $\delta^{18}O$ compared to those fed seagrass. The isotope composition of skeletons mirrored that of organic carbon ($\delta^{13}C_o$) and $\delta^{18}O$ in diets, but contributions of dietary carbon were higher for urchins fed formulated (40.2 ± 1.2% in test and 34.8 ± 0.4% in WCT) or macroalgae diets (40.38 ± 1.1% in test and 32.1 ± 0.4% in WCT) than for those fed seagrass (29.1 ± 1% in test and 29.9 ± 0.7% in WCT), concurrently with greater growth rates and distinctive rates of fractionation. Differences between test and WCT contributions among diets could not be explained by patterns of biomass allocation across calcified structures (i.e. similar test to spines+lantern ratios for all diets). This suggests that individuals fed red macroalgae or formulated diets increased the amounts of dietary carbon going into their tests, whereas individuals fed seagrass did not. Overall, we identified diet type as a new factor in the process of carbonate deposition that could influence species' responses to changes in ocean chemistry. We suggest that the reconstruction of sea urchin paleodiets might also be possible from fossil records. Test structures may be particularly useful for this purpose, because they accumulate high contributions of metabolic carbon that enhance the detection of differences in the isotopic signatures of diets.

KEY WORDS: *Lytechinus variegatus* · Metabolic CO_2 · Biological calcification · Stable isotopes · Seagrass

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INTRODUCTION

Biocalcification is of central importance in the biology of a wide range of marine organisms and ecosystems from planktonic algae to corals, seagrasses, and rhodoliths (see review by Hofmann et al. 2010). The amount of carbon accumulated in carbonate-rich sediments and rocks by marine organisms is estimated to be ~10^7 Pg and to comprise over 99.9% of the stored carbon on Earth (Pidwirny 2006). Echino-

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derms have a standing stock of ~0.25 Pg and global production rates (0.102 Pg C yr^{-1}; Lebrato et al. 2010) that are similar to coral reefs (0.108 Pg C yr^{-1}; Iglesias-Rodriguez et al. 2002) and exceed that of other benthic producers such as *Halimeda* bioherms (0.048 Pg C yr^{-1}; Iglesias-Rodriguez et al. 2002). Seawater acidification resulting from CO_2 emissions (ocean uptake of ~16.5 10^3 Pg CO_2 yr^{-1}; Feely et al. 2008) reduces the availability of CO_3^{-2} ions and the saturation state of $CaCO_3$ ($\Omega = [CO_3^{-2}] \times [Ca^{+2}] / K_{sp}$); where K_{sp} is the apparent solubility product) and may increase the dissolution of carbonate structures (Isaji 1995, Raven et al. 2005, Atkinson & Cuet 2008). These changes in ocean chemistry may severely impact carbonate-producing organisms with important roles in ecosystem functioning (Hall-Spencer et al. 2008, Hofmann et al. 2010, Fabricius et al. 2011) and alter their contribution to the global marine carbon budget. Organisms accumulating high-magnesium calcite, such as echinoderms, may be particularly vulnerable to acidification, because the saturation state of water with respect to high magnesium calcite is much lower than that of calcite or aragonite (Morse et al. 2006, Andersson et al. 2008).

Stable oxygen and carbon isotope measurements of biogenic carbonates have become standard tools for reconstructing past oceanographic and climatic variability (Smith et al. 1997, Spero et al. 1997) and addressing the environmental history and physiological requirements of organisms (Schwarcz et al. 1998, Gao et al. 2001, Høie et al. 2003). $\delta^{18}O$ and $\delta^{13}C$ signatures in biogenic carbonates are controlled to different degrees by physical and biological factors (McConnaughey 1989a). ^{18}O:^{16}O ratios are fundamentally regarded as a function of seawater abundance and the mineralization temperature (e.g. T in °C = 20.6 − 4.38[$\delta^{18}O_{ar} - \delta^{18}O_w$] for the foraminifer *Hoeglundina elegans*; Grossman & Ku 1986) and have promoted numerous calibrations in foraminifera, mollusks, and corals (e.g. Epstein et al. 1953, Weber & Woodhead 1972, Grossman & Ku 1986). ^{13}C:^{12}C ratios are also influenced by temperature (Romanek et al. 1992), but in living organisms are additionally controlled by the availability of dissolved inorganic carbon (DIC) in seawater and physiological processes such as respiration and photosynthetic rates (Swart 1983, Schwarcz et al. 1998, Gao et al. 2001). Kinetic effects due to discrimination against heavier isotopes during hydration and hydroxylation of CO_2 can deplete isotope signatures by up to 4‰ and 10 to 15‰ for $\delta^{18}O$ and $\delta^{13}C$, respectively (McConnaughey 1989a,b). But accurate interpretation of data is still possible if the offset from isotopic equilibrium at the temperature range of interest is known (e.g. Leder et al. 1996, Heikoop et al. 2000). In contrast, the incorporation of metabolically-derived carbon into the carbonate structures of foraminifera, mollusks, or fish is reported to have $\delta^{13}C$ deviations of ~5‰ compared to inorganic aragonite precipitation (Grossman & Ku 1986, Thorrold et al. 1997) and provides valuable information about the physiological processes regulating carbon uptake (Spero & Williams 1988). Photosynthetic organisms such as most corals tend to have higher $\delta^{13}C$ signatures in the skeleton due to preferential use of ^{12}C during photosynthesis (Swart 1983, McConnaughey 1989a). In contrast, incorporation of respired CO_2 lowers $\delta^{13}C$ signatures of carbonate structures because body biomass is depleted in $\delta^{13}C$ relative to seawater (McConnaughey 1989a, Høie et al. 2003).

There is some evidence that rates of somatic growth may be important in determining $\delta^{13}C$ signatures and the proportion of metabolically derived carbon in hard parts (up to 28 to 32% in fish otoliths: Kalish 1991, Høie et al. 2003). In addition, biogenic aragonite in fish otoliths has been suggested to reflect dietary shifts among trophic levels with distinctive $\delta^{13}C$ values (Kalish 1991, Schwarcz et al. 1998). For herbivorous species, experimental results for the land snail *Helix aspersa maxima* raised on C3 or C4 plant diets ($\delta^{13}C$ = −27.49 and −11.7, respectively), and a mix of both (C3 + C4) also evidenced a clear dietary effect on the isotopic composition of the snail shells ($\delta^{13}C$ = −13.7, −6.8, and −8.4, respectively, for the C3, C4, and mixed diet) and up to 8.8‰ variability in $\Delta^{13}C$ (isotopic fractionation) values (Metref et al. 2003). However, because diets may have both distinctive $\delta^{13}C$ signatures and nutritional qualities (i.e. varying levels of insoluble fiber, proteins, lipids, and carbohydrate contents), the influence of isotope composition may be confounded by differences in the incorporation of metabolic CO_2 into skeletons resulting from variable demands for growth. This may be relevant in benthic foragers such as sea urchins in which growth rates are tightly controlled by diet type (e.g. Frantzis & Grémare 1992, Fernandez & Boudouresque 2000) of variable $\delta^{13}C$ values, and this limits our ability to predict how such organisms may react to changes in water chemistry as well as possible changes in future contributions to biogeochemical cycles. In addition, understanding these mechanisms may also benefit future investigations of the dietary habits of fossil organisms (e.g. Quade at al. 1995, Zazzo et al. 2000).

Aside from their role in the biogeochemical cycle of marine carbonates (Dupont et al. 2010, Lebrato et al. 2010), sea urchins also play major ecological roles in the control of macrophyte communities and in the transfer of biomass and energy to higher trophic levels (e.g. Heck & Valentine 1995, Shears & Babcock 2003, Prado et al. 2007). For instance, the variegated sea urchin *Lytechinus variegatus*, an abundant species in the northern Gulf of Mexico (Heck & Valentine 1995), feeds on a variety of food items with distinctive $\delta^{13}C$ signatures, including seagrasses (Greenway 1976, Heck & Valentine 1995, Valentine & Heck 2001), macroalgae (Cobb & Lawrence 2005, Souza et al. 2008), and benthic animals (e.g. mussels, crustaceans, and epibionts, Skelenar 1994, Cobb & Lawrence 2005). We investigated patterns of $\delta^{13}C$ and $\delta^{18}O$ signatures in the mineral fraction of *L. variegatus* tests and whole calcified skeletons (WCT: tests, spines, teeth, and Aristotle's lantern), reared at constant temperature (23°C) from juvenile to adult stages on 2 types of natural and 1 formulated diet specifically developed for *L. variegatus* (Hammer et al. 2012). More specifically, we evaluated the effect of diet on the $\delta^{13}C$ and $\delta^{18}O$ signatures in sea urchin tests and during growth from juvenile to adult stages, and we provide estimates of dietary carbon contribution to carbonate structures.

MATERIALS AND METHODS

Collection of sea urchins and initial acclimation period

Small *Lytechinus variegatus* (1.5 to 2 cm; n = 150) were collected in mid-February 2009 from a shallow *Thalassia testudinum* bed located at the end of the Tyndall peninsula in the Saint Andrew's Bay estuary, adjacent to Panama City, Florida, USA (30° 7′ 20.57″ N, 85° 41′ 16.32″ W). In an aerated cooler, the urchins were transferred to a large indoor tank (500 l) at the Dauphin Island Sea Lab. Seawater was pumped from Mobile Bay, Alabama, filtered, and recirculated in an environmentally controlled lab. Experimental conditions were 32 ppt, 23°C (median annual field values, kept constant to avoid confounding effects on isotopic signatures), >200 ppt alkalinity and pH 8.1 to 8.3, and low dissolved nutrients (NO_3: $<1.6 \times 10^{-4}$ M; NO_2: 0 to 4.3×10^{-5} M; NH_4: 0 to 1.2×10^{-6} M). The experimental room was maintained on a 12:12 h light:dark photoperiod (see Hammer et al. 2004). Nutrient and alkalinity levels were monitored daily and water changes made, with appropriate quantities (<200 g) of sodium bicarbonate (Arm and Hammer®) added periodically into the main system of recirculating water (ca. 2000 l) to maintain the desired levels.

To standardize the stable isotope composition of field-collected individuals during the initial acclimation period, sea urchins were fed daily from mid-February until the end of March with storable food blocks prepared with thalli of the green alga *Ulva* sp. that were collected from rocks in Mobile Bay, dried, ground, and mixed with water and agar (10 g of algae plus 2 g of agar per 100 ml of seawater). The tank was cleaned daily from excess food and feces.

Experimental design and diet treatments

Ten 75 indoor l tanks connected to the same system of recirculating water were deployed. Seawater and photoperiod conditions were the same as during the initial acclimation period. Within each tank, 12 sea urchins were placed within individual containers (to eliminate conspecific interactions), and 4 ind. were randomly assigned to 1 of 3 diets: seagrass, red macroalgae, or a formulated diet, for a total of 120 ind. in the experiment. The containers consisted of a 50 × 10 cm rigid plastic 3 mm mesh cylinder attached to a PVC base that kept the cylinder upright. Each container was fitted with an aerator. Water samples were haphazardly collected from the different tanks throughout the experiment in order to identify possible cross-contamination of particulate organic matter (POM), and they showed POM signatures (means ± SE = −18.45 ± 0.45‰ and 5.93 ± 0.26‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively; Prado et al. 2012) closer to macroalgae than to seagrass or formulated diets, which suggested that there was no cross-contamination.

For the natural diets, sea urchins were fed green leaves of the seagrass *Thalassia testudinum* and 2 types of foliose red algae (*Grauteloupia* sp. from April to mid-June and *Palmaria palmata* from mid-June to July). Shoots of *T. testudinum* and fronds of the red alga *Grauteloupia* sp. were collected weekly from a site in Big Lagoon, Florida (30° 18′ 32.47″ N, 87° 22′ 55.54″ W), and from a rocky area adjacent to the Mobile Bay ferry landing at Fort Morgan, Alabama (30° 13′ 54.44″ N, 88° 0′ 55.70″ W). Samples were kept alive within aerated tanks in the wet lab. The *Grauteloupia* sp. sampled was consumed at high rates by *Lytechinus variegatus* (P. Prado pers. obs.), although it may be the first occurrence of the invasive *G. turuturu* (J. M. Lopez-Bautista pers. comm.). The alga's local life cycle is unknown, and it disappeared

suddenly in mid-June, necessitating replacement with *P. palmata* from a commercial supplier (Atlantic Mariculture) during the last 6 wk of the experiment. We selected *P. palmata* because it has a sheet-like morphology that is easy to manipulate and has similar protein and carbohydrate content as *Grauteloupia* sp. *P. palmata* was received sun-dried but recovered a fresh texture when hydrated. Epiphytes attached to leaves of *T. testudinum* and fronds of *Grauteloupia* sp. were carefully removed by scraping with a razor blade before being offered to urchins, and no epiphytes were present in commercial algae.

The formulated diet consisted of an extruded, nutrient-rich diet with vegetal and animal ingredients designed to maximize sea urchin growth (see Hammer et al. 2012 for details on composition). The diet was embedded in agar (10 g pellets plus 2 g of agar per 100 ml of seawater) to minimize disintegration and cross-contamination across treatments (see above).

The diets were supplied *ad libitum*. Food remains and feces were removed from tanks and containers daily, and the food in the containers was replaced with fresh material.

Sea urchin growth

At the end of each month (i.e. March to July), the test diameters of sea urchins were measured with calipers to the nearest mm and then weighed to the nearest 0.1 g. Then 3 ind. from each tank, 1 on each experimental diet, were separated and stored at −20°C for later dissection and determination of stable isotope contents in the mineral fraction of skeletons (our present study), and in soft tissues including muscle, gonad, and gut, as well as test (Prado et al. 2012). For individuals sacrificed monthly, cumulative growth rates were estimated as the increase in size and weight between the beginning of the experiment and the time of death (i.e. measures repeated 1 to 4 times depending on the time of death). Hence, cumulative growth incorporated the entire influence of the experimental diet, in the way that stable isotope composition of animals integrates long-term dietary patterns.

Stable isotope analyses

Diets

The composition of $\delta^{13}C_o$ (the organic carbon fraction) and the $\delta^{13}C_i$ (the inorganic carbon fraction), and $\delta^{18}O$ within diets was investigated to elucidate the incorporation of carbon and oxygen sources into the urchin hard parts. Samples of epiphyte-free seagrass and *Grauteloupia* sp. for $\delta^{13}C_o$ analyses were taken weekly from the aerated tanks and stored at −20°C. For the *Palmaria palmata* and the nutrient-rich formulated diets (which were not subjected to environmental change), 5 replicate samples were collected throughout the experimental period and stored at −20°C. Later, samples were dried to a constant weight at 70°C and ground to a fine powder with a mortar and pestle, and then a subsample from the homogenized mixture was weighed (~2 to 2.5 mg for vegetal tissues; ~1 to 1.3 mg for urchin tissues and the nutrient-rich formulated diet), packed into tin capsules, and stored in a desiccator. All diet samples analyzed for $\delta^{13}C_o$ were corrected for lipid content by applying values obtained by Prado et al. (2012) to the formulas given by Post et al. (2007). Diets were investigated for $\delta^{13}C_i$ and $\delta^{18}O$ values from the remaining dried material. For seagrass and *Grauteloupia* sp., we homogenized 5 g dry weight (DW) of sample from each time collection and then took 5 aliquots of 0.5 g DW and stored them in 2 ml glass vials. For *P. palmata* and the formulated diet, 5 aliquots of 0.5 g DW were also taken and stored from the general remaining material.

Sea urchins

From the initial acclimation period, 10 ind. (n = 5 for test and tissues, and 5 for WCT analyses) were stored at −20°C for later dissection and determination of organic $\delta^{13}C$ in soft tissues and test organic matrix (Prado et al. 2012) as well as for $\delta^{13}C_i$ and $\delta^{18}O$ in tests and WCT. During the following 4 mo, 1 ind. per experimental diet and tank (i.e. N_{total} = 3 ind. × 10 tanks × 4 mo) was removed for stable isotope analyses, immediately after growth measurements at the end of each month. Individuals from Tanks 1 to 5 were assigned for test $\delta^{13}C_i$ and $\delta^{18}O$ and stable isotope analyses in specific tissues (Prado et al. 2012), whereas individuals from Tanks 6 to 10 were analyzed for WCT. Tests and WCT were dried to a constant weight at 70°C, weighted, ground to a fine powder with a mortar and pestle, stored into 2 ml glass vials and sent untreated for analytical determination.

Determination of $\delta^{13}C_i$ and $\delta^{18}O$ abundances in diets and sea urchin tests and WCT was conducted with a Thermo Finnigan Delta V ratio mass spectrometer equipped with a Kiel III device for auto-

mated analysis of carbonates (this device uses a common acid bath of phosphoric acid at 90°C into which the sample carbonate is dropped) at the Geological Science Department at the University of Alabama (Tuscaloosa). $\delta^{13}C$ in DIC in seawater was analyzed at the University of California Davis Stable Isotopes Facility. The values are expressed in δ notation (δ‰) compared to the international standard, Vienna Pee-Dee Belemnite (VPDB), which constitutes the reference point on the scale. Because the supply of this material is exhausted, the abundances of carbon and oxygen isotopes of carbonates are expressed relative to VPDB by calibration with the NBS-19 calcite standard, with consensual values of +1.95 and −2.2‰ relative to VPDB for $\delta^{13}C$ and for $\delta^{18}O$, respectively. The reproducibility of the NBS-19 standard was ±0.10‰ for both C and O. We also expressed our results by calculating the difference in carbon isotopic composition between tests and WCT signals and the diets ($\Delta^{13}C$).

Diet samples were analyzed for $\delta^{13}C_o$ contents with a Thermo Finnigan Delta V advantage mass spectrometer connected to a Costech 4010 elemental analyzer through a Thermo Finnigan Conflo III interface at the Biochemical, Mass spectral, Stable Isotope Analytical Facility at University of Alabama (Tuscaloosa) with an analytical precision of ±0.15‰.

Water samples

Samples (n = 3 replicates) were collected once at the end of the experiment from the reservoir tank that recirculated water to the 10 replicate aquaria with sea urchins. Alkalinity and pH were constant at 200 ppt and 8.1 to 8.3, respectively, which suggests small fluctuations in DIC availability throughout the experiment. For $\delta^{13}C$ DIC determination, 1.5 ml of water was transferred into exetainers (2 ml glass vials, Labco Limited), filled completely with 0.5 ml of $ZnCl_2$ (50% w/v) and kept refrigerated. Water samples for $\delta^{18}O$ were unfortunately damaged during transportation, and this isotopic signature could not be determined.

Dietary carbon contributions

The contribution of dietary carbon to the growth of the skeleton was estimated with the mass balance equation used by Metref et al. (2003) for shells of pulmonate gastropods, modified for seawater media and calcite precipitation:

$$\delta^{13}C_{\text{Test (Calcite)}} = \delta^{13}C_m\,(X) + \delta^{13}C_w\,(1 - X) + 0.9‰ \quad (1)$$

where $\delta^{13}C_m$ is the isotopic composition of metabolic CO_2 (i.e. $\delta^{13}C_o$ of diets plus the isotopic enrichment for each diet in the test organic matrix (*Thalassia testudinum*: −1.01‰, red macroalgae: 0.26‰, formulated diet: 0.61‰), obtained from Prado et al. (2012) using values for the last month of the experiment to allow for isotopic equilibrium; X is the fraction of metabolic CO_2 incorporated by sea urchins; $\delta^{13}C_w$ is the isotopic composition of seawater (~1.1 to 1.4‰ in superficial waters of the Gulf of Mexico; Grossman & Ku 1986); and 0.9‰ is the isotopic offset between bicarbonate and calcite precipitated in isotopic equilibrium (Rubinson & Clayton 1969). To ensure that isotopic equilibrium between diets and sea urchin carbonate was reached, only test values for the last month of the experiment were used for calculations.

Analyses of data

Dietary differences in the composition of $\delta^{13}C_o$, $\delta^{13}C_i$, and $\delta^{18}O$ were investigated with 1-way analysis of variance (ANOVA). Patterns of $\delta^{13}C_i$ and $\delta^{18}O$ signatures in sea urchin tests and WCT were investigated with a 2-way factorial ANOVA (Diet: 3 levels, fixed factor; Month: 4 levels, fixed factor). Differences in cumulative growth of individuals for each diet and month were investigated with 2-way repeated-measures ANOVA. The influence of diet type in the percent contribution of dietary carbon and in the diet-carbonate fractionation in test and WCT was assessed with a 1-way ANOVA, using data obtained in the last month of the experiment to guarantee that isotopic equilibrium was not compromised. The patterns of biomass allocation into tests versus the other 2 calcareous structures (i.e. ratios of test to spines+lantern) were investigated using a 1-way ANOVA. The assumptions of normality (chi-squared test) and homogeneity of variances (Cochran's test) were not always achieved by transformation, and in those instances, the significance level was set at $p < 0.01$ to minimize the risk of making a type I error (Underwood 1981). SNK post hoc analyses were conducted to investigate significant groupings. All ANOVAs were performed using Statistica v.7 software.

Linear correlation was used to investigate (1) the association among $\delta^{13}C_o$, $\delta^{13}C_i$, and $\delta^{18}O$ signatures within diets; and between (2) dietary isotope signatures ($\delta^{13}C_o$, $\delta^{13}C_i$, and $\delta^{18}O$; mean values per month)

and $\delta^{13}C_i$ and $\delta^{18}O$ signatures in sea urchin tests and WCT (mean values) to assess whether possible differences among sea urchin treatments were caused by metabolic carbon and oxygen contributions. In the particular case of red macroalgae, the month of June was the combination of both algal species, whereas for the other months, only *Grauteloupia* sp. (March to May) or *Palmaria palmata* (July) was used. The relationships were tested for both dietary $\delta^{13}C_o$ and $\delta^{13}C_i$ to identify all the possible sources of metabolized carbon. Estimates of $\delta^{13}C$ fractionation across diet treatments were also calculated for tests and WCT using $\delta^{13}C$ values for the last month of the experiment (except for the macroalgae diet, for which June values were used instead to compensate $\delta^{13}C$ variability due to species changes) in order to allow for isotopic equilibrium.

RESULTS

$\delta^{13}C_o$, $\delta^{13}C_i$, and $\delta^{18}O$ in diets

Lipid-corrected values (means ± SE) for dietary organic carbon ($\delta^{13}C_o$) were lower in the formulated diet (−21.58 ± 0.05‰), the red macroalgae *Grateloupia* sp. (−20.53 ± 0.53‰), and in the agar-incorporated *Ulva* diet (−18.86 ± 0.02‰) than in the red macroalga *Palmaria palmata* (−13.10 ± 0.06‰) and seagrass *Thalassia testudinum* (−10.21 ± 0.13‰) (Prado et al. 2012). In contrast, signatures of dietary $\delta^{13}C_i$ were similar in the 3 experimental diets (seagrass: −12.26 ± 0.25; red macroalgae: −11.41 ± 0.36; formulated: −11.82 ± 0.20) but differed significantly from the starter diet (i.e. the agar-incorporated *Ulva* diet; −2.93 ± 0.07) supplied in March prior to the initiation of the experiment (df = 3, F = 322.6, $p < 0.0001$). $\delta^{18}O$ signatures were different for all diets (df = 3, F = 438.84, $p < 0.0001$), with higher values in the agar-incorporated *Ulva* diet (−1.68 ± 0.08), followed by the seagrass diet (−15.19 ± 0.33), the red macroalgae (−21.62 ± 0.81), and the formulated diet (−24.95 ± 0.42).

Overall values of dietary $\delta^{13}C_i$ and $\delta^{18}O$ were positively correlated (df = 19, r = 0.898; $p < 0.0001$). In fact, with the exception of the agar-incorporated *Ulva* diet (df = 4, r = 0.847; $p > 0.05$), significant relationships were observed in independent samples of seagrass (df = 4, r = 0.878; $p < 0.05$), red macroalgae (df = 4, r = 0.998; $p < 0.001$), and the formulated diet (df = 4, r = 0.903; $p < 0.05$). A significant association was also found between $\delta^{13}C_o$ and $\delta^{18}O$ signatures in experimental diets (df = 14, r = 0.950; $p < 0.0001$).

$\delta^{13}C_i$ and $\delta^{18}O$ in tests and WCT

$\delta^{13}C_i$ signatures of sea urchins fed on the seagrass diet were significantly higher than those fed on red macroalgae and the formulated diet, and greater effects were also observed in tests compared to WCT (Table 1a,b, Fig. 1). During the 2 first months of the experiment, $\delta^{13}C_i$ signatures of individuals increased for those fed the seagrass diet, and decreased, to varying degrees, for those fed the formulated diet and macroalgae (i.e. significant Diet × Month interaction; see Fig. 1).

$\delta^{18}O$ signatures showed similar dietary effects to those of $\delta^{13}C_i$, with significantly higher values in individuals fed the seagrass diet, intermediate for those fed the red macroalgae, and lowest for those fed the formulated diet (Table 1c,d, Fig. 1). However, temporal trends evidenced a significant decrease for all treatment diets through the experiment (Fig. 1).

Diet–urchin relationships and fractionation rates

We found significant positive associations between $\delta^{13}C_o$ in diets (including the agar-incorporated *Ulva* diet in March; n = 20) and $\delta^{13}C_i$ in sea urchin tests (Fig. 2A) and WCT (Fig. 2B), whereas inorganic $\delta^{13}C$ in diets were unrelated (Fig. 2C,D). For $\delta^{18}O$ of sea urchins, we observed positive relationships with diet $\delta^{18}O$ for tests (Fig. 2E) and WCT (Fig. 2F).

$\Delta^{13}C$ values (means ± SE) between $\delta^{13}C_o$ and carbonate signatures displayed significant differences among diets in both tests (df = 2, F = 325.26, $p < 0.0001$) and WCT (df = 2, F = 1255.39, $p < 0.0001$). $\Delta^{13}C$ was lowest in seagrass (Test: 8.7 ± 0.12‰; WCT: 8.3 ± 0.05‰), intermediate in red macroalgae (Test: 12.9 ± 0.13‰; WCT: 14.2 ± 0.13‰), and highest in formulated diet (Test: 14.7 ± 0.27‰; WCT: 16.9 ± 0.16‰), in accordance with the extent of differences between $\delta^{13}C$ in DIC, the $\delta^{13}C$ signatures in diets, and the degree of mixing.

Diet effects on sea urchin growth

Values of cumulative growth showed significantly higher size and weight increments in individuals fed the formulated diet, followed by those fed red macroalgae, and were lowest for those fed seagrass (Table 2a,b, Fig. 3). The largest increments in test diameter occurred in April and May and then

Table 1. *Lytechinus variegatus.* 2-way analysis of variance (ANOVA) showing the effects of each diet (SG: seagrass, RM: red macroalgae, and FOR: nutrient-dense formulated diet) and time in the patterns of (a,b) $\delta^{13}C_i$ ($\delta^{13}C$ in the inorganic component) and (c,d) $\delta^{18}O$ in tests and whole calcified tissues (WCT). Significant results in **bold** (level of statistical significance: $p < 0.05$ and $p < 0.01$ for non transformable data, ns: not significant). In Student-Newman-Keuls post hoc test (SNK), significant groupings for each Diet and Month are indicated

ANOVA Source of variance	df	(a) $\delta^{13}C_i$ in test MS	*F*	p	(b) $\delta^{13}C_i$ in WCT MS	*F*	p
Diet	2	102.96	842.83	**0.001**	21.74	260.08	**0.001**
Month	3	0.542	4.437	**0.008**	0.229	2.74	0.053
Diet × Month	6	1.882	15.41	**0.001**	0.787	9.41	**0.001**
Error	48	0.122			0.083		
		Cochran's *C*: ns SNK: SG >RM > FOR	Cochran's *C*: **0.183** SNK: SG > RM > FOR				
		(c) $\delta^{18}O$ in test			(d) $\delta^{18}O$ in WCT		
Diet	2	1.174	30.58	**0.001**	0.402	14.21	**0.001**
Month	3	0.535	13.93	**0.001**	1.017	35.94	**0.001**
Diet × Month	6	0.086	2.254	0.054	0.025	0.885	0.513
Error	48	0.038			0.028		
		Cochran's *C*: ns SNK: SG > RM > FOR	Cochran's *C*: **0.382** SNK: SG > RM > FOR				

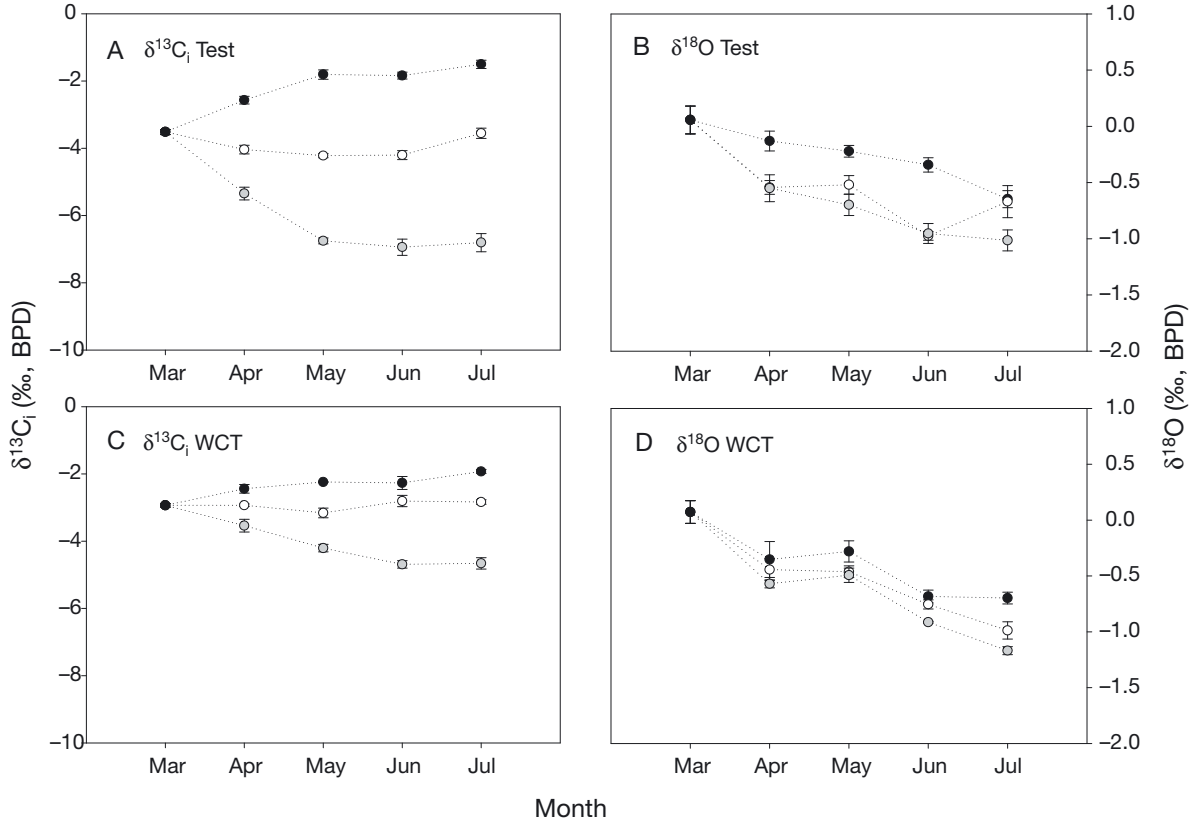

Fig. 1. *Lytechinus variegatus.* $\delta^{13}C_i$ ($\delta^{13}C$ in the inorganic component) and $\delta^{18}O$ composition of (A,B) tests and (C,D) whole calcified tissues (WCT: test, spines, teeth, and Aristotle's lantern) of urchins fed (●) seagrass, (○) red macroalgae, or (⊙) formulated diet, during each month of the experiment. Means ± SE. BPD: PeeDee Belemite

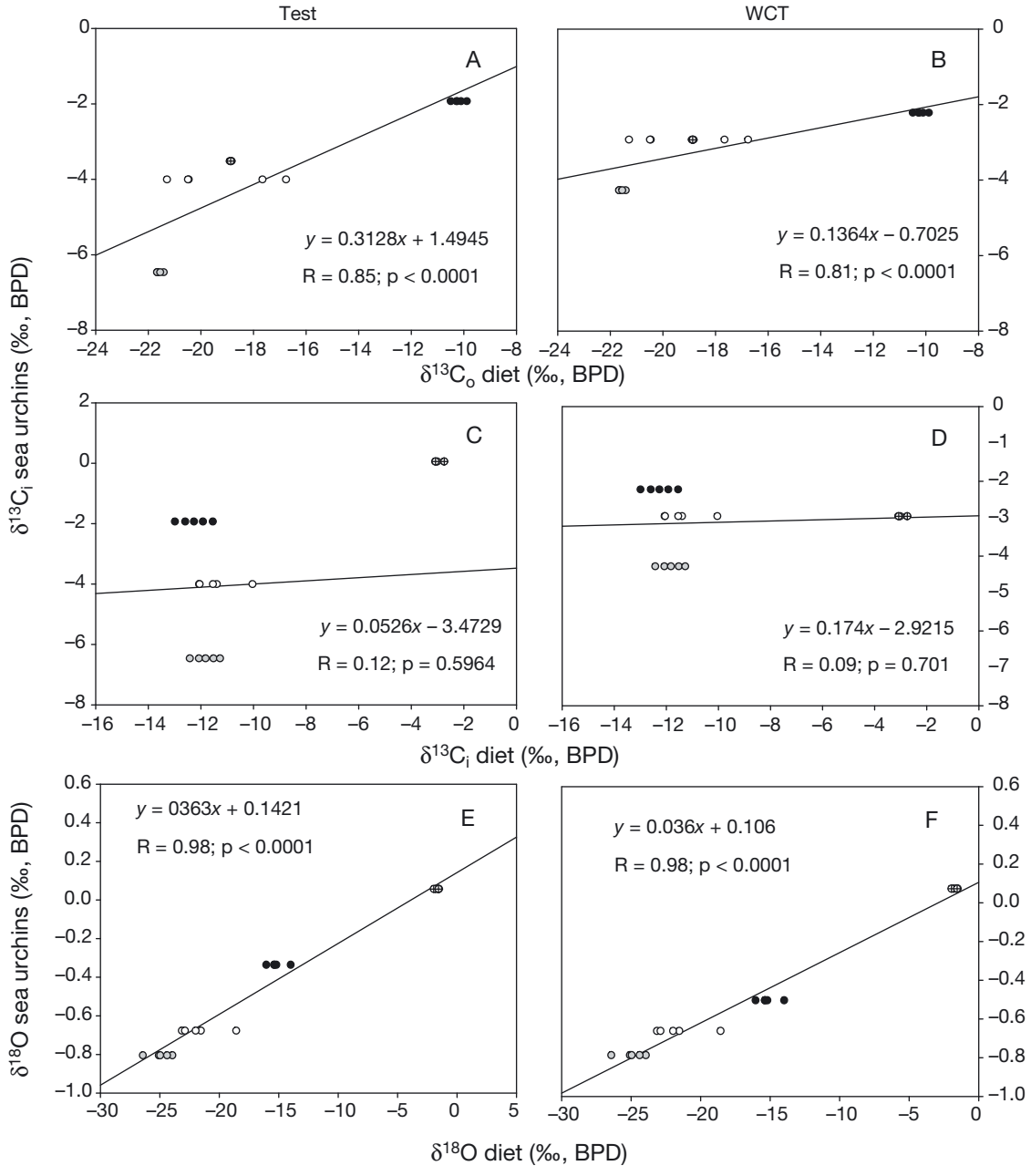

Fig. 2. *Lytechinus variegatus.* Linear correlations between: (A,B) $\delta^{13}C_o$ ($\delta^{13}C$ in the organic component of diets, viz. *Ulva* sp. incorporated in agar, seagrass, red macroalgae, and formulated extruded diet) and mean $\delta^{13}C_i$ ($\delta^{13}C$ in the inorganic component) in tests and whole calcified tissues (WCT); (C,D) $\delta^{13}C_i$ in diets and $\delta^{13}C_i$ in tests and WCT; and (E,F) $\delta^{18}O$ in diets and mean $\delta^{18}O$ in tests and WCT. BPD: Peedee Belemnite

decreased towards a plateau during the last 2 mo of the experiment, particularly with the seagrass diet (i.e. significant Month and Diet × Month interactions). In contrast, the largest increase in body weight occurred in July, coinciding with gonad development and the reproductive period, and was also higher in individuals fed the formulated and red macroalgae diets (see Fig. 3).

Dietary contribution to sea urchin carbonate and biomass allocation in calcareous structures

Individuals fed different diets were exposed to the same conditions with values of $\delta^{13}C$ in DIC of 1.22 ± 0.01 (mean ± SE), which are similar to those reported by Grossman & Ku (1986) for shallow waters in the northwestern Gulf of Mexico.

Table 2. *Lytechinus variegatus.* Repeated measures analysis of variance (ANOVA) showing the cumulative growth of individuals for each experimental diet (SG: seagrass, RM: red macroalgae, and FOR: nutrient-dense formulated diet). Increment in (a) test diameter (cm) and (b) sea urchin body wet weight (g). Significant results in **bold** (level of statistical significance: $p < 0.005$ and $p < 0.001$ for non transformable data, ns: not significant). In Student-Newman-Keuls post hoc test (SNK), significant groupings for each Diet and Month are indicated

ANOVA Source of variance	df	(a) Cumulative test growth MS	*F*	p	(b) Cumulative body growth MS	*F*	p
Diet	2	16.56	472.97	**0.001**	8991.9	162.29	**0.001**
Month	3	9.113	242.41	**0.001**	20953.6	463.67	**0.001**
Diet × Month	6	0.697	18.53	**0.001**	1423.2	31.49	**0.001**
Error	81	0.038			45.2		
		Cochran's *C*: ns			Cochran's *C*: ns		
		SNK (Diet): FOR > RM > SG			SNK (Diet): FOR > RM > SG		
		SNK (Month): July > June > May > April			SNK (Month): July > June > May > April		

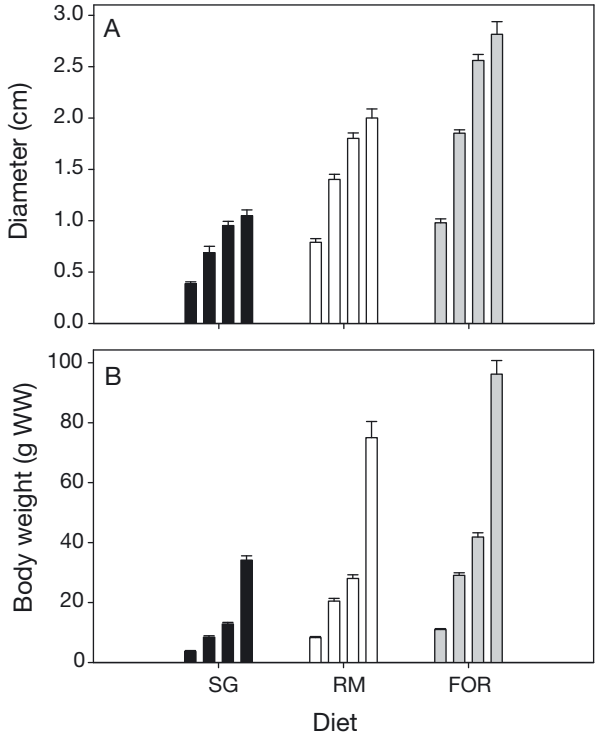

Fig. 3. *Lytechinus variegatus.* Cumulative growth of sea urchins fed each diet (SG: seagrass; RM: red macroalgae; and FOR: formulated) during each experimental month (i.e. April to July). Increment in (A) test diameter and (B) total body wet weight (g WW). Mean + SE

Estimates of carbon contributions using $\delta^{13}C$ of tests during the last experimental month (i.e. July) and the mean $\delta^{13}C$ composition of diets throughout the experimental period showed significantly lower values (df = 2, $F = 34.13$, $p < 0.0001$) for individuals fed seagrass (29.1 ± 1%) and similar values for those fed red macroalgae (40.2 ± 1.2%) and formulated diet (40.38 ± 1.1%; Fig. 4). In contrast, estimates using the $\delta^{13}C$ of WCT indicated lower contributions of red macroalgae (32.1 ± 0.4%) and formulated diets (34.8 ± 0.4%), but not of seagrass (29.9 ± 0.7%), although differences were significant for all diets (df = 2, $F = 18.53$, $p < 0.0001$; Fig. 4).The ratios between the biomass allocated to tests and the sum of that allocated to the other 2 calcified structures (i.e. spines and lantern) displayed little variation among diets (0.78 ± 0.04 in seagrass, 0.84 ± 0.05 for macroalgae, and 0.80 ± 0.09 for the formulated diet) and were not significantly different (df = 2, $F = 0.218$, $p > 0.05$). Yet, individuals fed the seagrass diet allocated almost all of their body weight to carbonate structures (up to 97.8%), whereas those fed red macroalgae and formulated diets allocated smaller proportions to carbonate (87.4 and 89.5%, respectively) and invested the remaining biomass into soft tissues (see Fig. 4).

DISCUSSION

Diet type can be traced in the $\delta^{13}C$ and $\delta^{18}O$ signatures of sea urchin hard parts. We found significant differences in $\delta^{13}C$ signatures of sea urchins fed each type of diet, with more depleted values in the test and WCT of individuals feed formulated and macroalgae diets and more enriched in those fed the seagrass diet; for $\delta^{18}O$, a similar pattern was observed. We propose that CO_2 contributions from the diet (up to 40.4% of the carbon in tests and up to 34.8% in WCT) are mirrored by the skeletons. Variability in the carbon contributions among diets (4.7 to 11% increases) may also be the result of differences in their nutritional quality, which caused differential

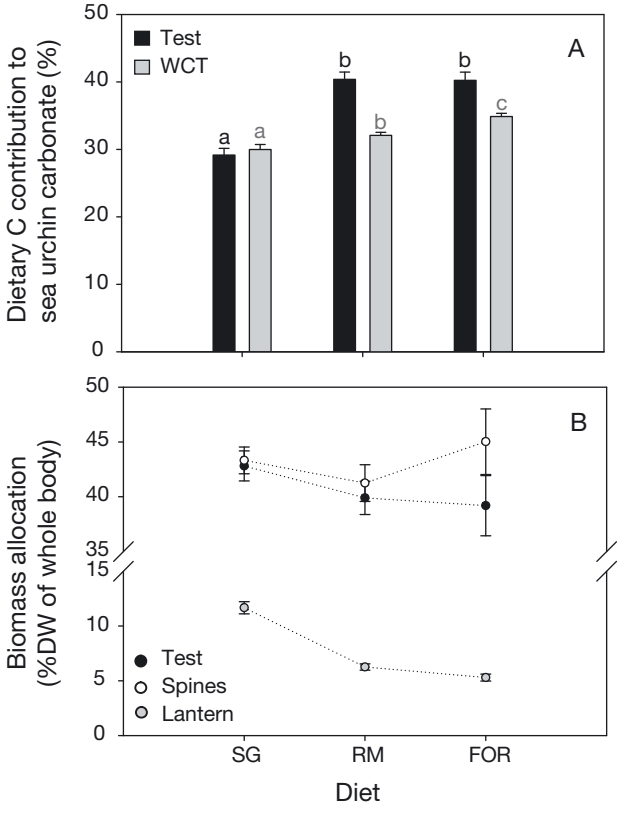

Fig. 4. *Lytechinus variegatus.* (A) Carbon contributions from each diet (seagrass, red macroalgae, and formulated) to the carbonate structure (tests and whole calcified tissues, WCT) of sea urchins. Estimates were obtained from $\delta^{13}C$ values at the end of the experiment (see 'Materials and methods'). Significant differences among diets are indicated with different letters. (B) Biomass allocation (in % of total dry weight, DW) in the carbonate structures included in WCT (i.e. test, spines, and lantern) used for calculating the ratio of test to spines+lantern. Means ± SE

growth among urchins that required enhanced metabolic contributions. In addition, our results further suggest that sea urchins do not evenly compensate for the higher demands across carbonated tissues, and that test structures may play a central role in mediating metabolic contributions.

Nutritional effects in isotope incorporation and growth rates

Sea urchins fed the seagrass diet (−10.2 ± 0.13‰ for $\delta^{13}C_o$ and −15.19 ± 0.33 for $\delta^{18}O$) had higher isotopic signatures than those fed red macroalgae (−17.2 ± 0.66‰ for $\delta^{13}C_o$ and −20.72 ± 0.81 for $\delta^{18}O$) and the formulated diet (−21.58 ± 0.05‰ for $\delta^{13}C_o$ and −24.95 ± 0.42 for $\delta^{18}O$). This strong positive association between $\delta^{13}C_o$ and $\delta^{18}O$ signatures both in diets and in sea urchins (r = 0.85 and 0.98, respectively) indicates that significant amounts of metabolic products are being used in the formation of the mineral skeleton. In contrast, signatures of $\delta^{13}C_i$ in diets did not show a significant association with sea urchin signatures despite dual investigation with $\delta^{18}O$, possibly because ash content in diets is lower compared to the organic fraction (Prado & Heck 2011) or may be more difficult to be assimilated by organisms (e.g. Johnston & Lasenby 1982). Since a large number of benthic calcifying organisms such as suspension feeders draw their food from the water column, the nutritional constraints of diet type — rather than seasonal periods of food availability or overall constraints in food supply (e.g. MacDonald & Thompson 1985, Thompson & Nichols 1988, Gao et al. 2001, Seitz et al. 2005) — in skeletal $\delta^{13}C$ signatures may have been overlooked. In terrestrial gastropods, there is evidence that shell $\delta^{13}C$ is not constant and may vary by up to 7.4‰ depending on the source of ingested food, including possible effects due to differences in growth rates (Metref et al. 2003). In sea urchins, differences were less pronounced (up to 4.5% between the formulated and the seagrass diet), but growth rates were also influenced by nutritional quality (~66 and 26% higher growth in urchins fed the formulated and the macroalgae diet, respectively, than in those fed seagrass), suggesting that enhanced growth may have increased the demand for metabolic carbon toward calcified structures. In fact, tests of individuals fed red macroalgae and formulated diets were more depleted in $\delta^{13}C$ relative to WCT, which is indicative of higher metabolic effects (McConnaughey 1989a, Høie et al. 2003).

For $\delta^{18}O$, our results contrast with patterns in previous studies reporting no influence of metabolic processes on the signatures of biogenic carbonates (Epstein et al. 1953, Weber & Woodhead 1972, Grossman & Ku 1986), and reinforce the idea that this effect may depend on the feeding habits of the organisms being investigated. Yet, the deviation between expected and observed values of $\delta^{18}O$ in test and WCT is small, which also suggests that the majority of the oxygen must still come from the water. $\delta^{18}O$ of DIC frequently ranges between ca. −0.3 and +1 (Grossman & Ku 1986, Kalish 1991, Leder et al. 1996), which at a temperature of 23°C, results in water-corrected $\delta^{18}O$ values between −2 and 0‰ in biogenic carbonates (see review by Kalish 1991), overlapping the range of variation observed in

sea urchin treatments in July (from −0.65 to −1.17‰ in seagrass and the formulated diet, respectively). Although the selection mechanism of water versus respired material has not yet been identified, it is possible that the enzyme carbonic anhydrase, which discriminates against the heavy carbon isotope in the cell cytoplasm (McConnaughey 1989a), favors the incorporation of less abundant metabolic CO_2 molecules more depleted in $\delta^{13}C$. Alternatively, dietary $\delta^{18}O$ may also be incorporated from water molecules resulting from metabolic processes, which may explain some of the variability in the observed patterns of the 2 isotopes.

Dietary carbon contributions among diets and calcified structures

Our values (means ± SE) for dietary carbon contributions, from 29.1 ± 1 to ca. 40% in tests and from 29.9 ± 0.7 to 34.8 ± 0.4% in WCT, are lower than the 57 and 62% reported for terrestrial gastropods fed C3 and C4 plants (Metref et al. 2003). Yet, the range of variability in dietary carbon contributions to tests was double that observed by Metref et al. (2003), whereas that for WCT was the same (i.e. ca. 11 and 5% in tests and WCT, respectively), suggesting greater plasticity in the dietary response. Given that water $\delta^{13}C$ was only investigated at the end of the experiment, it is possible that monthly differences in respired CO_2 resulting from growth and monthly removal of animals from the tanks might have influenced the water signature to some extent. However, this should have affected all individuals in the same way, and thus the relative differences among treatments observed at the end of the experiment should still be robust. In addition, our values for $\delta^{13}C$ (1.2‰) are representative of the 1.1 to 1.4‰ range reported by Grossman & Ku (1986) for superficial waters of the Gulf of Mexico. Differences in carbon contribution among diet treatments may at first seem low. However, when considered together with growth rate increments, red macroalgae and formulated diets increased the allocation of dietary carbon by ca. 1.9 to 2.6 times in WCT and by 2.5 to 2.9 times in tests, compared to individuals fed the seagrass diet. Overall, differences in organic carbon contributions to skeleton formation might be explained by variability in absorption rates (22.7, 46.5, and 58%, respectively, for seagrass, red macroalgae, and the formulated diet) and in the nutritional quality of diets (Hammer et al. 2012, Prado et al. 2012).

In addition to differences among diet treatments, higher contributions of metabolic carbon in test versus WCT were also observed that could not be attributed to variability in biomass allocation to tests relative to that of the whole calcified structures. Weber & Raup (1966) examined patterns of variation in $\delta^{13}C$ and $\delta^{18}O$ in sea urchins and found that tests were greatly depleted in $\delta^{13}C$ compared to spines (by ca. 2.6‰ in *Lytechinus variegatus*), which displayed values more similar to other marine invertebrates such as mollusks or foraminifera. Similarly, our results show more depleted values in test than in WCT including spines (by ~1‰ across all treatments in July), but in addition, important effects of diet type were observed. Tests and WCT of individuals fed the seagrass diet showed similar signatures and carbon contributions, whereas tests of individuals fed red macroalgae and formulated diets were more depleted and drew higher contributions of carbon from the diet compared to individuals fed seagrass. For $\delta^{18}O$, our values showed no isotopic equilibrium at the end of the experiment, but Weber & Raup (1966) also found slightly more depleted values in tests compared to spines (by ca. 0.5‰ in the same species). This inconsistency in the lag time to isotopic equilibrium between $\delta^{18}O$ and $\delta^{13}C$ is possibly the result of their different contributions to biogenic carbonate, in agreement with commonly reported patterns of metabolic disequilibrium (Grossman & Ku 1986, McConnaughey 1989a, Kalish 1991, Thorrold et al. 1997).

Seawater contributions

The physiological model for biological calcification involves the transport of diffusible CO_2, formed by reaction of abundant bicarbonate in ambient water (>85% of DIC) with a protons' gradient of ~1 pH unit across the membrane, into the cell cytoplasm where the enzyme carbonic anhydrase catalyzes its reaction with water molecules to non-diffusible HCO^-_3 and $CO^=_3$ that react with Ca^{+2} ions driven into the cell by a Ca^{+2}/H^+ ATPase and precipitate as $CaCO_3$ (McConnaughey 1989b). The isotopic composition of sea urchins reflected the mixing of bicarbonate ($\delta^{13}C$ of DIC was ~1.2‰ while that of atmospheric CO_2 is ~ −8‰; Wahlen 1994) and dietary signatures to variable extents, that resulted in different fractionations depending on diet type, supporting the idea that there is a need to incorporate this variability in trophic models (Prado et al. 2012). Mechanisms for discrimination between CO_2 types (i.e. ambient water versus meta-

bolically derived) are unknown, but enzymatic activity might be controlled by CO_2 concentration (Fukuzawa et al. 1990). Our results suggest that increased metabolic rates might result in decreased uptake of inorganic carbon from seawater availability (from 70.9 to 59.1%). On the one hand, the availability of an alternative source of carbon to that of DIC may involve that changes in ocean chemistry could not have a straightforward effect in the calcification rates of echinoderms, although carbonate structures may equally start dissolving at saturation states <1 (Isaji 1995). On the other hand, because a large fraction of echinoderm carbon may come from their diet, this would imply that a significant amount (e.g. 30 to 40%) of their global annual inorganic carbon production of 0.102 Pg yr^{-1} (Lebrato et al. 2010) is not being removed directly from seawater and must be accounted for in their contributions to the functioning of biogeochemical cycles (Lebrato et al. 2010).

To conclude, we provided evidence that $\delta^{13}C$ and $\delta^{18}O$ signatures in sea urchin skeletons, particularly tests, are significantly influenced by diet type. The isotopic composition of sea urchins mirrored dietary signatures, but the further influence of diet quality accounted for further depleted $\delta^{13}C$ values in fast-growing individuals fed the formulated diet with high nutrient content (Hammer et al. 2012). Enhanced growth rates in urchins fed the formulated and the macroalgae diet (by ~66 and 26%, respectively, compared to the seagrass diet), received higher contributions of metabolic carbon than urchins fed seagrass, particularly in tests. Therefore, our results also suggest that the $\delta^{13}C$ and $\delta^{18}O$ composition of sea urchin skeletons and their potential diets could be used as tracers for paleo-dietary reconstruction (see Quade at al. 1995, Zazzo et al. 2000), at least during periods or seasons with stable temperature conditions. This could be particularly helpful for understanding geographical distributions of seagrass ecosystems in ancient seas (relatively rare in the fossil record due to their poor preservation potential; Ivany et al. 1990) and to elucidate co-evolutionary traits and adaptation of different species to seagrass habitats. Given the significant contribution of dietary carbon to skeleton formation (30 to 40%) and the potential of diet type to increase by up to ca. 2.6- to 3-fold the overall amount of metabolic carbon incorporated (i.e. growth deposition and the effect of metabolic contribution), our results point out the need for similar reassessments in other benthic consumers such as crustaceans and gastropods that are also of relevance to the global marine $CaCO_3$ cycle.

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