Title: Bottom-up effects on biomechanical properties of the skeletal plates of the sea urchin *Paracentrotus lividus* (Lamarck, 1816) in an acidified ocean scenario

Valentina Asnaghi [1*], Marie Collard [2], Luisa Mangialajo [3], Jean-Pierre Gattuso[4,5], Philippe Dubois[2]

[1] Department of Earth, Environment and Life Science, DiSTAV, University of Genoa, Italy

[2] Laboratoire de Biologie marine CP160/15, Université Libre de Bruxelles, av F.D. Roosevelt, 50, B-1050 Bruxelles, Belgium

[3] Université de Nice-Sophia Antipolis, EA 4228 ECOMERS, Nice, France,

[4] Sorbonne Université, CNRS, Laboratoire d'Océanographie de Villefranche, 181 chemin du Lazaret, F 06230, Villefranche-sur-mer, France

[5] Institute for Sustainable Development and International Relations, Sciences Po, 27 rue Saint Guillaume, F75007, Paris, France

*corresponding author: valentina.asnaghi@unige.it

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Authors contribution

VA, LM, JPG, PD conceived of or designed study; VA, MC performed research; VA analyzed data, VA, MC, PD wrote the paper.

Abstract

Sea urchins, ecologically important herbivores of shallow subtidal temperate reefs, are considered particularly threatened in a future ocean acidification scenario, since their carbonate structures (skeleton and grazing apparatus) are made up of the very soluble high-magnesium calcite, particularly sensitive to a decrease in pH. The biomechanical properties of their skeletal structures are of great importance for their individual fitness, because the skeleton provides the means for locomotion, grazing and protection from predators. Sea urchin skeleton is composed of discrete calcite plates attached to each other at sutures by organic ligaments. The present study addressed the fate of the sea urchin *Paracentrotus lividus* (Lamarck, 1816) skeleton in acidified oceans, taking into account the combined effect of reduced pH and macroalgal diet, with potential cascading consequences at the ecosystem level. A breaking test on individual plates of juvenile specimens fed different macroalgal diets has been performed, teasing apart plate strength and stiffness from general robustness,. Results showed no direct short-term effect of a decrease in seawater pH nor of the macroalgal diet on single plate mechanical properties. Nevertheless, results from apical plates, the ones presumably formed during the experimental period, provided an indication of a possible diet-mediated response, with sea urchins fed the more calcified macroalga sustaining higher forces before breakage than the one fed the non-calcified algae. This supports the need of longer term experiments to observe substantial differences on skeletal plate structure.

48 **Introduction**

49 Sea urchins are important calcifiers in shallow subtidal areas of temperate regions and play a key
50 ecological role in these ecosystems being generally the most effective benthic herbivores and
51 controlling, through their grazing activity, the dynamic, structure and composition of macroalgal
52 assemblages (Jangoux and Lawrence 1982; Ruitton et al., 2000; Bulleri et al., 2002; Privitera et al.,
53 2008; Bonaviri et al., 2011). Their skeleton, spines and grazing apparatus are made of high-
54 magnesium calcite, a form of calcium carbonate that is particularly vulnerable to dissolution under
55 low pH conditions (Andersson et al., 2008; Hermans et al., 2010). For this reason, sea urchins have
56 long been regarded as particularly threatened by the ongoing decrease of pH and calcium carbonate
57 saturation states of the oceans, referred to as ocean acidification (Kurihara and Shirayama 2004;
58 Dupont et al., 2010; Byrne et al., 2011).

59 Echinoid skeleton is made up of discrete ossicles located in the dermis. Each ossicle consists of a
60 three-dimensional network of mineralized trabeculae, the stereom, delimiting an internal and
61 complementary network filled by connective tissue, the stroma (Dubois and Chen 1989). The
62 perforated calcite plates are attached to each other at sutures by ligaments that wrap around calcite
63 rods, thus sewing together adjacent plates. Trabeculae project from one plate into holes in the adjacent
64 plates, thus interlocking the plates (Moss and Meehan 1967). These processes ensure a relative
65 rigidity of the test. The stereom consists of high-magnesium calcite and of 0.1% (w/w) organic
66 material (the intrastereomic organic matrix; e.g. Weiner 1985). Sutural ligaments among plates
67 strengthen sea urchin skeleton (Kidwell and Baumiller 1990) and, on the basis of histological and
68 morphological evidence, these ligaments may be interpreted as “stress-breakers” that evenly
69 distribute stresses and thus contribute to the structural integrity of echinoid skeletons (Moss and
70 Meehan 1967). The strengthening role of sutural ligaments is different according to size, age, diet
71 and *taxa* (Ellers et al., 1998). Sutural ligaments are known to reinforce urchin skeletons under natural
72 loads such as the action of crab claws, apical or lateral forces from waves and forces generated when
73 an urchin wedges itself in a crack (Ellers et al., 1998).

74 The biomechanical properties of skeletal structures have a great importance for individual fitness
75 (Currey 1989; Meyers et al., 2008) because skeletons provide the means for locomotion, grazing and
76 protection from predators. Few studies have investigated the skeletal biomechanical properties of
77 echinoderms with respect to ocean acidification (reviewed in Dubois 2014; Collard et al., 2016; Dery
78 et al., 2017). Sea urchin spines and test plates are differently affected by seawater acidification.
79 Spines, more vulnerable, showed reduced fracture forces at reduced pH also in short term studies
80 (Holtmann et al., 2013; Dery et al., 2017; Emerson et al., 2017) while plates were not impacted by
81 acidified conditions in short and long term experiments (Holtmann et al., 2013; Moulin et al., 2015;
82 Collard et al., 2016).
83 Only few studies focused on the mechanical resistance of the whole test under low pH conditions.
84 Byrne et al. (2014) reported a reduced crushing force in live *Tripneustes gratilla* juveniles grown
85 from metamorphosis at pH_{NBS} 7.6 (0.5 pH units below control) but this was attributed to differences
86 in urchin size. A decrease in test robustness, also related to test size, was observed on *Paracentrotus*
87 *lividus* and *Diadema africanum* juveniles kept under reduced pH (7.6 pH_{NBS}) for 100 days, compared
88 to control conditions (8.0 pH_{NBS}; Rodriguez et al., 2017). Similarly, in juvenile *Paracentrotus lividus*
89 maintained for one month at pH_T 7.7 (0.4 pH_T units below control), the test was less robust (in terms
90 of resistance to an increasing crushing load, tested on dried skeletons) than at higher pH_T (7.84, 7.89,
91 8.09; Asnaghi et al., 2013). These differences in test robustness were mirrored by diet-related
92 differences (calcified vs. non-calcified macroalgae) in skeletal composition (particularly Mg/Ca ratio;
93 Asnaghi et al., 2013, 2014), suggesting that diet is another potentially relevant source for bicarbonate
94 uptake (in addition to possible uptake from the seawater). In Asnaghi et al. (2013), the crushing test
95 was performed on entire dry preserved juvenile sea urchins, where organic material and ligaments
96 were still present. This dried organic material is flexurally stiff relative to the calcite plates and might
97 provide tensile reinforcement to the skeleton (Ellers et al., 1998), even if performing the test on dried
98 ligaments could have resulted in biased resistance compared to fresh organisms (Ellers et al., 1998).

In the present study, the fate of sea urchin skeletons in acidified oceans has been addressed, teasing apart plate strength and stiffness from general robustness of the test, due to calcium carbonate plates and organic material associated. We performed a breaking test, using a motorized load frame (Instron 5543 tensile tester), on individual plates detached from the skeleton and cleaned of organic material in juvenile sea urchins fed different diets. The individuals were part of the experiment presented in Asnaghi et al. (2013, 2014). The individuals tested in this study were not previously tested for whole test strength to avoid any confounding factors such as cracks or breaks.

Juvenile sea urchins were treated for one month under four pH levels and fed one of three species of macroalgae with variable carbonate content (i.e. calcified vs. not-calcified). Since sea urchin skeletons grow both by the accretion of calcite at the edges and faces of the plates and by the addition of new plates at the apex resulting in gradual migration of initially apical plates adorally during growth (Deutler 1926; Märkel 1975), we can assume that in our juvenile sea urchins the largest part of the most apical plates were formed during the experimental period, while ambital ones were already formed before the experiment (as proposed by Collard et al., 2015). Consequently, we expected different responses to lowered pH, in terms of individual plate strength and stiffness, for plates under formation (apical) and for already formed ones (ambital), and according to the different macroalgal diets.

Materials and methods

Experimental set-up

The experimental set-up was thoroughly described in Asnaghi et al. (2013). Briefly, a total of 144 4-month old juveniles of *Paracentrotus lividus* (Lamarck, 1816), provided by a sea urchin hatchery in Camogli (NW Mediterranean Sea, Italy), with an average test diameter of 5.8 mm (± 0.1 standard error, SE), were randomly selected and transferred to the Laboratoire d'Océanographie de Villefranche (NW Mediterranean Sea, France) where the experiment was performed in July 2011.

Four pH_T levels, corresponding to future pCO_2 conditions chosen according to best practices (Barry et al., 2010) and three scenarios were used: (1) present day, pCO_2 = 390 µatm ($pH_T \approx 8.1$, control); (2) optimistic scenario (SRES scenario B1), pCO_2 = 550 µatm ($pH_T \approx 8.0$); (3) realistic scenario (midway between SRES scenario AB1 and A2), pCO_2 = 750 µatm ($pH_T \approx 7.8$) and (4) pessimistic scenario (A1F1), pCO_2 = 1000 µatm ($pH_T \approx 7.7$). pCO_2 was controlled by bubbling pure-CO_2, according to best practices for ocean acidification experiments (Riebesell et al., 2010).

Unfiltered seawater, pumped from a depth of 10 m, was continuously supplied to four 200 l header tanks and bubbled with air. pH was continuously monitored by a pH-stat system (IKS, Karlsbad, Aquastar) and small amounts of pure CO_2 were added to keep pH at the desired level. Manipulated seawater from the four header tanks was delivered to experimental units at a rate of about 6 l h^{-1} in an open system.

The experiment was performed within a thermostatic chamber kept at 22°C. Irradiance values in the aquaria were maintained at about 215 µmol photons $m^{-2}s^{-1}$, with a 12:12 h L:D photoperiod. Juvenile sea urchins were fed three different macroalgal species: a calcified species, *Ellisolandia elongata* (J.Ellis & Solander) K.R.Hind & G.W.Saunders, 2013 (previously known as *Corallina elongata*) and two non-calcified species *Dictyota dichotoma* (Hudson) J.V.Lamouroux, 1809 and *Cystoseira amentacea* (C.Agardh) Bory de Saint-Vincent, 1832. Macroalgae were collected prior to the start of the experiment and kept for at least one week in distinct aquaria at the same pH levels as the sea urchins. Feeding was *ad libitum* with macroalgae from the corresponding pH level.

Six juvenile sea urchins were placed in each of the 24 experimental units (2 independent replicates for each combination of pH and diet - see details of experimental set-up and sea water parameters in Asnaghi et al., 2013). The experiment lasted one month. At the end of the experiment, all specimens were measured, air-dried and stored pending future analyses.

Mechanical test

For the mechanical test, a subset of 24 sea urchins (one from each experimental unit, representing an independent replicate) was selected. First of all, a cleaning protocol was set up in order to remove the

150 soft tissues surrounding the skeleton without damaging the stereom and allow separation of the plates.
151 Different exposure times were tested in order to select the best cleaning protocol and effectiveness of
152 the different methods was checked under a scanning electron microscope (SEM). The best procedure
153 for cleaning plates without damaging their structure consisted in placing entire dried urchins in 2.5%
154 (v:v) sodium hypochlorite for 1 h to detach the spines and Aristotle's lantern, rinsing 3 times in MilliQ
155 water and air drying for 1 h. Tests devoid of spines and lantern were further cleaned for 30 min in
156 1% (v:v) sodium hypochlorite, rinsed 3 times in MilliQ water and air-dried overnight.
157 Single interambulacral plates were separated under a stereo-microscope; 3 apical (presumably formed
158 during the experimental period) and 3 ambital plates (already formed before the beginning of the
159 experiment) for each specimen were used for biomechanical measurements (Figure 1a).
160 Top and lateral view photographs of each sea urchin plate were obtained with a Nikon Coolpix 995
161 3Mpi under a binocular lens. Photographs were analyzed, using the software ImageJ, to measure
162 tubercle areas (A), which is the area over which the force was actually applied (Figure 1b) and
163 effective length (L_e), which is the height to the edge of the tubercles (Figure 1c). These parameters
164 are necessary for Strain and Stress calculations (see equations below).
165 Due to the small size and flatness of both apical and ambital plates of juvenile sea urchins, an *ad hoc*
166 system, halfway between compression test and three-point bending test, was created to measure the
167 fracture force. Individual cleaned and detached sea urchin plates were placed on a metal stand with a
168 groove in the middle and the mechanical test was carried out using a second metal block fixed on the
169 load frame of the force stand, Instron 5543 tensile tester, at a speed of 0.1 mm min^{-1}, applying constant
170 compression till breakage (Figure 1d). Displacement and force were recorded at a frequency of 10
171 Hz. For each plate, the Bluehill software (Instron) provided information about force at fracture (F_{max})
172 and displacement (ΔL).
173 Stiffness was measured through Young's modulus (E), a quantity used to characterize materials and
174 defined as the ratio of the stress along an axis over the strain along that same axis.
175

Strain and Stress were calculated for each plate, using the following equations:

Strain: $\sigma = F/A$

Stress: $\varepsilon = \Delta L/L_e$

where F: force at fracture, A: tubercle area, ΔL: displacement, L_e: effective length.

The Young's modulus (E) was calculated as the slope between two points of the final linear part of the curve, in this case the maximum force and the 100th point before that, using the following formula:

$$E = \frac{\sigma_{max} - \sigma_{100\text{th point}}}{\varepsilon_{max} - \varepsilon_{100\text{th point}}} \text{ (Pa)}$$

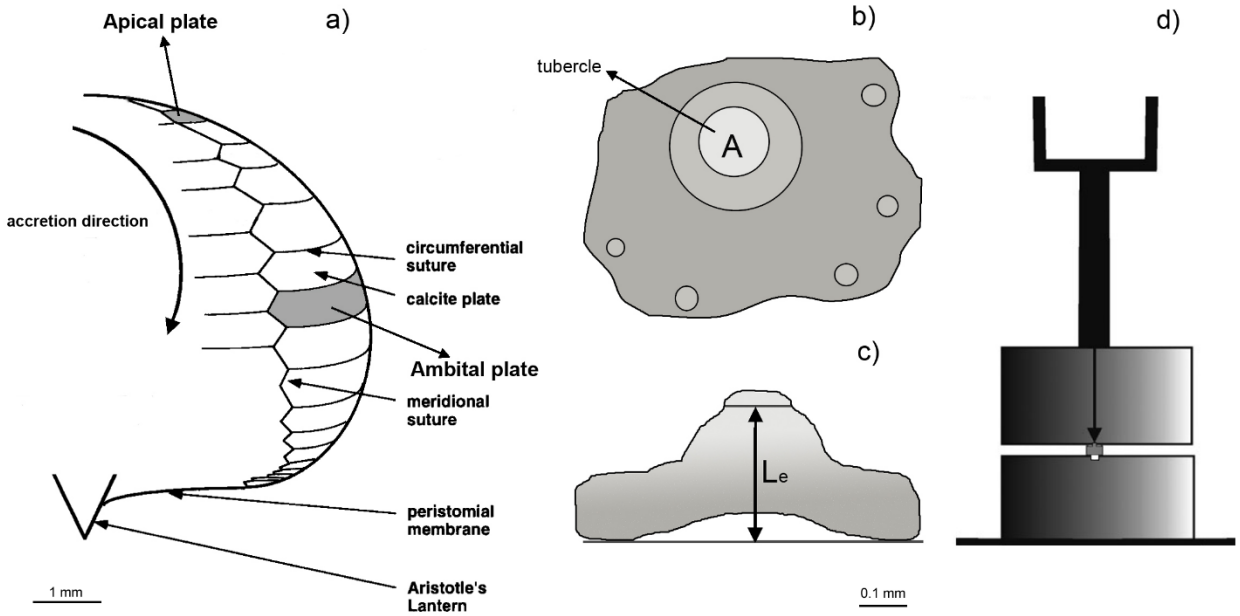

Figure 1: Schematic representation of a) interambulacral plates, apical and ambital, and suture geometry with corresponding scale bar at the bottom (modified from Ellers et al., 1998; b) upper and c) lateral view of one plate, in order to perform tubercle area (A) and effective length (L_e) measurements, with corresponding scale bar at the bottom; d) simple *ad hoc* compression device composed by two metal blocks, the lower, where the plate should be placed, with a groove and the upper fixed on the load frame (modified from Collard et al., 2016)

194 **Data analyses**

195 In order to assess the effect of pH level, algal diet and their interaction on apical and ambital plates
196 strain (F_{max}/A) and stiffness (Young's modulus, square root transformed data), a crossed ANOVA
197 design was applied, using 'pH' and 'diet' as fixed crossed factors. Since multiple observations were
198 performed on each specimen, a random effect *specimen* nested in the interaction (pH * diet) was
199 added in order to account for the dependency structure in the data.
200 Normality and homogeneity of variance were verified for both the considered response variables
201 (F_{max}/A and $\sqrt{E}$) using Shapiro-Wilk test and Bartlett tests, respectively. All statistical analyses were
202 performed using the software R (R Core Team 2014).
203

204 **Results**

205 No significant effect of the interaction between the two fixed factors, pH and diet, was observed in
206 strain (fracture force/surface on which the force is applied) or in stiffness neither for ambital nor for
207 apical plates. Moreover, no significant difference according to factors seawater pH or diet singularly
208 were detected in ambital plates (Tables 1, 2). Similarly, no effect of seawater pH or diet was
209 evidenced in apical plates strain and stiffness (Tables 1, 2).
210 Both strain and stiffness of ambital plates (Fig. 2 a, c) showed more homogeneous values among
211 treatments compared to apical ones (Fig. 2 b, d). Apical plates stiffness slightly decreased according
212 to the pH decrease for *E. elongata* and *C. amentacea*, but not in *D. dichotoma*, where the trend seems
213 to be the opposite (Fig. 2b), even if differences are not statistically significant. A weak gradient of
214 apical plates strain values according to the algal diet can be observed, *i.e.* gradual decrease of the
215 strain value for sea urchins fed *E. elongata*, *C. amentacea* and *D. dichotoma,* respectively (Figure
216 2d).
217

Table 1: ANOVA results table for strain data (F_{max}/A) of ambital and apical plates under factors pH and Diet and their interaction. The random effect *specimen* nested in the interaction (pH x* diet) is considered in order to account for the dependency structure in the data

	AMBITAL PLATES					**APICAL PLATES**				
	Df	SS	MS	F	signif.	Df	SS	MS	F	signif.
pH	3	2. 8	0.95	0.01	0.998	3	271	90.2	0.16	0.918
Diet	2	51.1	25.55	0.38	0.694	2	3798	1898.9	3.47	0.065
pH * diet	6	641	106.84	1.58	0.236	6	529	88.1	0.16	0.983
***specimen*(pH * diet)**	12	776.5	67.71	2.91	0.004	12	6567	547.3	3.32	0.001
Residuals	48	1116	23.24			48	7906	164.7		

Table 2: ANOVA results table on square root transformed Young's modulus data (√E) of ambital and apical plates under factors pH and Diet and their interaction. The random effect *specimen* nested in the interaction (pH x* diet) is considered in order to account for the dependency structure in the data

	AMBITAL PLATES					**APICAL PLATES**				
	df	SS	MS	F	signif.	Df	SS	MS	F	signif.
pH	3	0.22	0.07	0.88	0.481	3	0.23	0.08	0.61	0.618
Diet	2	0.03	0.01	0.13	0.884	2	0.38	0.19	1.46	0.270
pH * diet	6	0.30	0.05	0.63	0.708	6	1.74	0.29	2.23	0.112
***specimen*(pH * diet)**	12	0.94	0.08	2.00	0.045	12	1.52	0.13	2.20	0.029
Residuals	48	2.09	0.04			48	3.11	0.06		

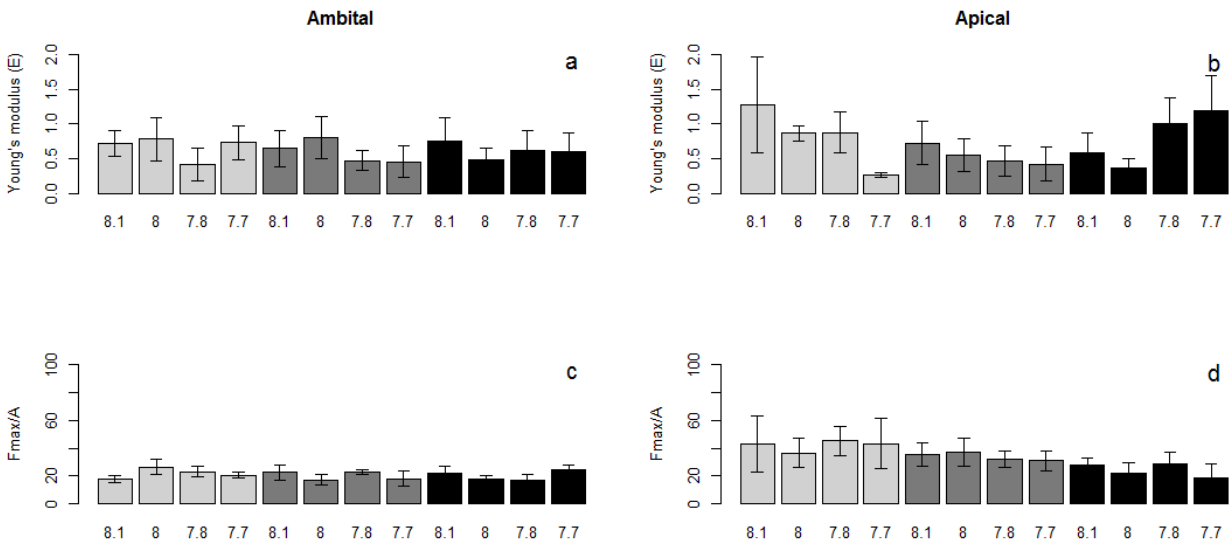

Figure 2: Barplots of Young's modulus (E), upper panels, and strain data (F_{max}/A), lower panels, measured for ambital (a, c) and apical (b, d) plates under different sea water pH (x axis) and different macroalgal diets (light gray= *E. elongata*, gray= *C. amentacea*, dark gray= *D. dichotoma*). Error bars: standard error.

Discussion

Ocean acidification caused by anthropogenic carbon dioxide emissions is known to pose major threats for marine organisms, particularly calcifying ones (*e.g.* Kroeker et al., 2010; Riebesell et al., 2010), since their calcium carbonate structures are potentially susceptible to dissolution in acidic waters (Orr et al. 2005). Sea urchins are important marine calcifiers, playing a relevant ecological role in temperate ecosystems (Jangoux and Lawrence 1982; Ruitton et al., 2000; Bulleri et al., 2002; Privitera et al., 2008; Bonaviri et al., 2011). Among calcifying organisms, highly calcified sea urchins are expected to be the echinoderms more affected by reduced pH, with differences at species, population and stage level (reviewed in Dupon et al., 2010).

Early life stages are acknowledged to be the most sensitive to ocean acidification (Dupont et al., 2010; Moulin et al., 2011; Byrne et al., 2013; Stumpp et al., 2013), but also post-metamorphic phases (juveniles and adults) showed to be affected, mainly in terms of survival and growth, both from laboratory experiments (e.g. Shirayama and Thornton 2005; Byrne et al., 2011; Albright et al., 2012;

246 Asnaghi et al., 2013, 2014; Moulin et al., 2015; Collard et al., 2016), and from *in situ* records in
247 naturally acidified areas (only on adults, e.g. Hall-Spencer et al., 2008; Bray et al., 2014; Collard et
248 al., 2016). Moreover, different studies investigated coelomic fluid regulatory capacities in adult sea
249 urchins exposed to hypercapnic conditions, reporting contrasting results about their ability to partially
250 or fully compensate extracellular pH (Stumpp et al., 2012; Collard et al., 2013, 2014; Calosi et al.,
251 2013; Kurihara et al., 2013; Moulin et al., 2014, 2015) and prevent skeletal dissolution at low pH
252 (reviewed by Dery et al., 2017).
253 Only few studies addressed the combined effect of reduced pH and macroalgal diet on sea urchins,
254 highlighting potential cascading consequences at the ecosystem level (Johnson and Carpenter, 2012;
255 Asnaghi et al., 2013; 2014). Macroalgae exhibit a broad range of responses to ocean acidification
256 (Hall-Spencer et al., 2008; Nelson, 2009; Martin and Gattuso, 2009; Connell and Russell, 2010;
257 Cornwall et al. 2011, 2017; Poore et al., 2013), mainly linked to their calcium carbonate content
258 (Cornwall et al., 2014; James et al, 2014) and inorganic carbon physiology (Cornwall et al., 2017).
259 The loss of calcium carbonate and tissue modification in macroalgal thalli caused by reduced pH may
260 enhance their palatability to grazers (Duarte et al., 2016; Rich et al., 2018, Rodriguez et al., 2018),
261 leading to potential shifts in the ecosystem equilibrium under an acidified scenario.
262 Different algal feedings, quantity and species of macroalgae available as food are known to directly
263 affect somatic and gonadal production in sea urchins, both from field and laboratory studies (e.g.
264 Ebert 1968; Lawrence 1975; Lilly 1975; Vadas 1977; Larson et al., 1980; Lawrence and Lane 1982;
265 Thompson 1983, 1984; Privitera et al., 2008). Food shortage (i.e. the lack of macroalgal biomass for
266 feeding) have been reported to cause modifications in plastic resource allocation (Ebert 1980; Haag
267 et al., 2016), differences in mechanical properties of sea urchin spines (Moureaux and Dubois 2012)
268 and, in an acidified scenario, reduction of the buffer capacity of the coelomic fluid to compensate the
269 decrease of external pH (Collard et al., 2013).
270 In the present study, the combined effect of pH level and macroalgal diet was investigated in terms
271 of sea urchin plates biomechanical properties, showing no direct short-term effect of seawater pH nor

272 diet on single plate strain and stiffness, in agreement with previous laboratory studies (Holtmann et
273 al., 2013; Moulin et al., 2015; Collard et al., 2016).
274 A field study, instead, highlighted a role of the diet in mediating sea urchins biomechanical properties,
275 showing that test plates from *P. lividus* living in tide pools mainly covered by encrusting calcareous
276 algae exhibit a higher fracture force than test plates of sea urchins living in pools containing erected
277 non-calcifying algae (Collard et al., 2016). Similar role of calcifying macroalgae in strengthen *P.*
278 *lividus* juvenile tests has been shown by Asnaghi et al. (2013) on specimens exposed to the same
279 experimental conditions of the present study, but it is not easy to disentangle the role of plates,
280 ligaments and calcified locking structures in providing test robustness.
281 Results from the present study showed more homogeneous responses of the ambital plates (the ones
282 already formed before the start of the experiment) in terms of strain and stiffness (Fig. 2 a, c), while
283 apical plates, the one presumably formed during the one-month experimental period, showed more
284 variable values, with strain data suggesting a possible diet-mediated response, maybe visible on a
285 longer term (Fig. 2 b, d): sea urchins fed the more calcified macroalga (*E. elongata*) sustained higher
286 fracture force than the one fed the non-calcified algae (*C. amentacea* and *D. dichotoma)*. The high
287 variability at the level of the specimen (Table 1 and 2) may have masked patterns on the short term.
288 Mechanical properties can be affected by changes in the growth rate (e.g. Moureaux et al., 2010),
289 which may affect the three-dimensional morphology or density of the stereom (Smith 1980).
290 Alternatively, the structural properties of the material itself may be affected by the formation of
291 imperfections during the $CaCO_3$ precipitation (e.g. Moureaux et al., 2011).
292 Sea urchins from the present experiment showed different growth rates according to their diet.
293 *Ellisolandia elongata* allowed for a faster growth rate of the sea urchins compared to that of the ones
294 fed *Cystoseira amentacea* and *Dictyota dichotoma.* (Asnaghi et al., 2014). This could be linked to the
295 supply of calcium by calcified algae (Powell et al., 2010) rather than to the carbonate ions, which are
296 not transported through cell membranes, or bicarbonate ions which are available in high concentration
297 in seawater from which they are readily absorbed (from this source; Collard et al., 2014).

In the present study, the choice to use juveniles specimens, characterized by higher growth rates compared to adults, was driven by the possibility to observe potential modifications linked to pH and diet treatments on a short time scales (one month). Indeed, a diet-medieted decrease in growth and test robustness in these *P. lividus* juveniles under acidified conditions was proven (Asnaghi et al., 2013), even if not mirrored by substantial modification of test thickness and single plates biomechanical properties. Rodriguez et al. (2017) reported significantly thinner test plates in juvenile *Paracentrotus lividus* and *Diadema africanum* kept for 100 days in one tank at low pH conditions compared to the ones in the control tank, that may have led to less robust test, even if several other sources of variability were present in the experimental design.

Those considerations suggest that, in order to observe substantial differences on skeletal plate structure, it is necessary to perform longer term experiments (Dupont et al., 2010; Hendricks et al., 2010), taking into particular account feeding conditions, that are frequently neglected in ocean acidification studies on post-metamorphic individuals (Dubois et al., 2014).

The impact of algal diet on sea urchin test resistance to breakage is particularly relevant in the context of global change. Coralline algae are renowned impacted by ocean acidification, while non-calcified algae will be most likely favored in future oceans (Hall-Spencer et al., 2008; Porzio et al., 2011; Koch et al., 2013; Sunday et al., 2017). As a consequence, some sea urchin species might be affected by the expected change in macroalgal diet availability, growing slower and producing a less robust skeleton, with potential relevant cascading consequences on their ability to graze, move, search for shelters and defend from predation and hydrodynamics.

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