

# Effects of ocean warming and acidification on the early benthic ontogeny of an ecologically and economically important echinoderm

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**ABSTRACT:** The sea urchin *Loxechinus albus* is a benthic shallow water coastal herbivore and an exploited natural resource. This study evaluated the consequences of projected near-future ocean acidification (OA) and warming (OW) for small juveniles of this species. Individuals were exposed for 7 mo to contrasting  $p\text{CO}_2$  (~400 and 1200  $\mu\text{atm}$ ) and temperature (~16 and 19°C) levels. We compared grazing rates during the first 2 mo of rearing. After an additional period (2 to 7 mo), we compared body size change (in terms of diameter, and wet and buoyant weight), self-righting, dislodgement resistance, foraging speeds, test dissolution rate, oxygen consumption and strength of structural integrity. Regardless of the temperature, urchins reared under present-day  $p\text{CO}_2$  grazed preferentially on algae also reared under present-day  $p\text{CO}_2$  conditions. However, urchins reared under elevated  $p\text{CO}_2$  at both temperatures exhibited no grazing preference. Other traits such as growth rate in terms of diameter, vertical foraging speed and tenacity were not affected significantly by  $p\text{CO}_2$ , temperature and the interaction between them. However, growth rate in terms of wet weight, metabolism and dissolution rate of empty urchin tests was significantly affected by temperature and  $p\text{CO}_2$  but not by the interaction between them. At 16°C, self-righting was faster for individuals reared at elevated  $p\text{CO}_2$  but no differences were found at 19°C. We conclude that OA and OW may disrupt some early benthic ontogenetic traits of this species and thus have negative ecological and economic consequences. However, most traits will be not threatened by the 2 investigated stressors.

**KEY WORDS:** Growth · Grazing rate · Tenacity · Structural integrity · Self-righting · Foraging speed · *Loxechinus albus*

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## INTRODUCTION

Predicted near-future changes in ocean pH (ocean acidification; OA) and temperature (ocean warming; OW) will likely have an impact on the biodiversity of marine ecosystems, and particularly those organ-

isms that rely on calcification processes (Byrne 2011). Studies investigating the individual and combined effects of OA and OW on marine invertebrates have described a wide variety of sensitive metabolic, morphological and behavioural traits (Findlay et al. 2010, Hofmann et al. 2010, Walther et

al. 2010, Byrne 2011, Reyes-Nivia et al. 2013, Wolfe et al. 2013, Paganini et al. 2014, Clements & Hunt 2015, Nagelkerken & Munday 2016). When these effects are negative and occur during the early ontogeny of an organism, the local extinction risk for populations of the species may increase. This is supported by experimental evidence that highlights the high degree of susceptibility to changes in  $p\text{CO}_2$  and temperature throughout early ontogeny, beginning with gametes and continuing through larval development to juvenile stages (Kurihara 2008, Ross et al. 2011).

Echinoderms are important components of the marine environment, often functioning as ecosystem engineers and keystone species (Hughes 1994, Lessios et al. 2001, Hereu et al. 2008, Steneck 2013). Investigations of physiological responses in echinoderms to OA and OW are very common in the literature, showing a range in stage- and species-specific responses in terms of fertilisation, larval development, survival, gonad development, growth, skeletal mechanical properties and calcification. Observations reported in the literature include: a compromising of fertilisation through temperature, but not high  $p\text{CO}_2$ , in the sea urchin *Heliocidaris erythrogramma* (Byrne et al. 2009); a reduction in larvae size of the urchin *Tripneustes gratilla* at high  $p\text{CO}_2$  levels regardless of the rearing temperature (Sheppard-Brennand et al. 2010); a reduction in larval development and growth in larvae of the urchin *Strongylocentrotus purpuratus* (Stump et al. 2012); a mitigation of the negative effects of high  $p\text{CO}_2$  by warming in larvae of the urchin *Heliocidaris tuberculata* (Byrne et al. 2013); increased growth rates of the lecithotrophic larvae of the sea star *Crossaster papposus* under high  $p\text{CO}_2$  (Dupont et al. 2010); negative effects of high  $p\text{CO}_2$  and warming on the physiology and gonad developmental of adult urchin *Echinometra* sp. A (Uthicke et al. 2014); and the absence of effects of high  $p\text{CO}_2$  and warming on the skeletal mechanical properties of the urchin *Paracentrotus lividus* (Collard et al. 2016).

The importance of animal behaviour when considering the pervasive impacts of climate change has recently been highlighted (Nagelkerken & Munday 2016). In ecosystem engineers such as sea urchins, changes in behavioural traits including grazing rate (Dean et al. 1984, Harrold & Reed 1985), self-righting (Kleitman 1941), foraging speed (Tertschnig 1989) and tenacity (Tuya et al. 2007) may have important impacts on distribution patterns and ecosystem functioning. However, to our knowledge, no studies incorporating both physiological (survival, growth,

calcification) and behavioural (grazing, predator avoidance) responses in sea urchins to OA and OW have been published.

The edible sea urchin *Loxechinus albus* (known locally as the Chilean red sea urchin) is an important benthic herbivore found along the Pacific coast of South America from Isla Lobos de Afuera in Peru ( $6^\circ\text{S}$ ) to the southern end of South America in Tierra del Fuego ( $55^\circ\text{S}$ ) (Vásquez 2001, Molinet et al. 2012). In Chilean shallow water coastal ecosystems, this species is a target for local fishers (Vásquez et al. 1984, Guisado & Castilla 1987, Castilla 1990, Vásquez & Donoso 2013), and represents one of the largest urchin fisheries in the world (Andrew et al. 2002). Due to its association with kelp beds, the overexploitation of this fishery may have negative consequences for kelp forest communities through altered grazing rates affecting the density of kelps such as *Macrocystis pyrifera* in protected coastal areas (Buschmann et al. 2004). The wide geographic distribution of this sea urchin, which inhabits depths ranging from the intertidal down to 100 m (Molinet et al. 2012), suggests that it has the physiological capacity to cope with environmental variation. However, past attempts to use hatchery-produced juvenile *L. albus* to restock depleted areas have highlighted a sensitivity to environmental conditions in terms of predation, starvation and dislodgement at this life stage (Bustos et al. 1991, González & Jerez 2001, Guzmán et al. 2009). In fact, available information indicates that recruitment of *L. albus* takes place in microhabitats that support mechanical protection of juveniles, such as in shallow-water rocky crevices (Castilla 1990), in areas containing established adult conspecifics (González & Jerez 2001) or in tide pools dominated by the black sea urchin *Tetratypygus niger* (Stotz et al. 1992). As such, juvenile *L. albus* are an excellent model for exploring the synergistic effects of OA and OW in coastal ecosystems.

In this study, we used a multi-stressor experimental approach to examine the physiological, morphological and behavioural responses of small juvenile *L. albus* to elevated levels of temperature ( $\sim 16$  vs.  $19^\circ\text{C}$ ) and  $p\text{CO}_2$  ( $\sim 400$  vs.  $1200 \mu\text{atm}$ ). The higher  $p\text{CO}_2$  level represents the worst-case scenario for the beginning of the next century (Meinshausen et al. 2011), while the higher temperature represents a warming of  $3^\circ\text{C}$ , as observed in coastal waters of northern Chile during intense El Niño events (Torres et al. 2003) that could be the signature of climate change in the South Pacific Eastern Boundary Current (Timmermann et al. 1999).

## MATERIALS AND METHODS

### Juvenile production

Early benthic stages of *Loxechinus albus* were produced under laboratory conditions in an experimental hatchery facility at the Universidad Católica del Norte, Coquimbo, Chile (29° 58' S, 71° 21' W). Adults were collected from nearby shallow subtidal zones. Larvae were generated from manipulated fertilisations in which eggs and sperm from 10 adult individuals were used following standard protocols described for the species (Bustos et al. 1991). Larvae were raised in mass cultures at densities of approximately 1 ml<sup>-1</sup>, fed with a mixed diet of microalgae (*Chaetoceros gracilis* and *Isochrysis galbana*) at cell densities of 2 to 4 × 10<sup>3</sup> ml<sup>-1</sup> until they reached settlement competency, and induced to settle on biofilms covering polycarbonate sheets. Prior to the experiments, early settler (0.15–0.20 cm diameter) and small juvenile individuals (>0.2 cm in diameter) were reared in 5000 l containers with running seawater. Early settler-stage urchins grazed on biofilms until they reached the small juvenile stage, when their diet was switched to fresh fronds of the green macroalgae *Ulva* sp. and later to the brown macroalgae *Lessonia berteroana*.

### CO<sub>2</sub> mixing system

A flow-through CO<sub>2</sub> mixing system, as described by Torres et al. (2013), was used to manipulate *p*CO<sub>2</sub> levels in aquaria. Briefly, mass flow controllers (Aalborg®, model GFC) were used to precisely mix CO<sub>2</sub>-free air with pure CO<sub>2</sub> gas, which was then bubbled into 4 large (230 l), independent plastic reservoir tanks filled with 1.0 µm filtered seawater (FSW). From these reservoirs, treated seawater was delivered to 20 l equilibration vessels immersed in temperature-controlled water tables (~16 and 19°C) using an electric pump. Within each equilibration vessel, treated seawater was mixed with additional mixed gas using a Mazzei® Venturi injector (model 484) and then delivered to 4 temperature-controlled aquaria containing the experimental urchins. Carbonate system parameters, total alkalinity, pH, temperature and salinity were quantified twice weekly in each rearing aquarium (Table 1). Total alkalinity (*T<sub>A</sub>*) was measured using an automated open cell titration system, described in detail by Haraldsson et al. (1997) and the accuracy was verified using certified reference material (CRM) supplied by Andrew Dickson

(Scripps Institution of Oceanography, San Diego, USA). Seawater pH was measured inside a 25 ml closed cell at 25°C using a pH meter (Metrohm 713) with a glass combined double junction Ag/AgCl electrode (Metrohm, 6.0219.100) calibrated with 8.089 Tris buffer (DOE [US Department of Energy] 1994) at 25°C. Values of pH were reported using the total hydrogen ion scale (DOE 1994). Temperature and salinity were measured using an in-line conductivity-temperature sensor (Ocean Seven, 304 Plus). The pH, total alkalinity and hydrographic data were used to calculate the rest of the carbonate system parameters (*p*CO<sub>2</sub> and DIC) and the saturation stage of Ω aragonite using CO2SYS software (Lewis & Wallace 1998) set with Mehrbach solubility constants (Mehrbach et al. 1973) refitted by Dickson & Millero (1987).

### Experimental rearing

During October 2014, 3 mo after fertilisation, 80 small juvenile urchins with an average (±SD) diameter of 0.66 cm (0.07, Fig. 1a) were removed from the rearing conditions and randomly assigned to 4 experimental treatments. Plastic tubing connected to an electric water pump was used to move the treatment FSW from each reservoir tank to the experimental units located in the 2 temperature-controlled water tables (~16 and 19°C). In each experimental treatment (n = 4), the urchins were divided into 4 groups and each group was assigned to a separate replicate plexiglass aquarium (Fig. 1; 30 cm in length and 17 cm in width and height) filled with 7.5 l of 1.0 µm FSW conditioned to the required *p*CO<sub>2</sub> levels, and placed in the temperature-controlled water tables. The rate of water delivery from the equilibration vessel to the experimental aquaria was controlled by a plastic drip-feed system, set to deliver at a rate of 1 l h<sup>-1</sup> to ensure the renewal of the total seawater of each aquarium 3.2 times per day. Each replicate aquarium was covered with a plastic lid pierced by the drip-feed system. The treatment air supply was delivered to each aquarium by flexible silicone tubing, entering the aquarium through a hole 2 cm in diameter and 11.5 cm above the base. At the end of tube was an air-stone through which a continuous stream of either air (400 µatm CO<sub>2</sub>) or enriched CO<sub>2</sub> air (1200 µatm) was bubbled. The same hole was used as an overflow to maintain the seawater level in the aquaria. Inside each replicate aquarium, a plastic cage made with 0.5 × 0.5 cm mesh was subdivided into 16 cells, each measuring 3 × 3 cm. Five urchins

Table 1. Average ( $\pm$  SE) conditions of the seawater (SW) used to maintain small juvenile *Loxechinus albus* (reared for 7 mo) and fragments of *Ulva* sp. (reared for 12 d for the grazing preference and algal nutritional experiments) under contrasting temperature and  $p\text{CO}_2$  levels. Present-day and elevated  $p\text{CO}_2$  levels are based on the rate of change in pH predicted by the most extreme scenario (RCP8.5) of atmospheric  $\text{CO}_2$  for the beginning of the next century. See Meinshausen et al. (2011) for further details

	pH at 25°C (pH units)	Temperature (°C)	$T_A$ ( $\mu\text{mol kg}^{-1}$ )	$p\text{CO}_2$ in situ ( $\mu\text{atm}$ )	$[\text{CO}_3^{2-}]$ in situ ( $\mu\text{mol kg}^{-1}$ SW)	Salinity (PSW)	$\Omega$ calcite	$\Omega$ aragonite
Natural seawater <sup>a</sup>	7.88 (0.02)	17.29 (0.16)	2291.13 (3.08)	489.30 (26.87)	157.10 (6.84)	34.24 (0.03)	3.77 (0.16)	2.43 (0.11)
<b>Expt. Small juveniles</b>								
Present-day $p\text{CO}_2$ (~16°C)	7.88 (0.01)	16.00 (0.09)	2262.47 (3.07)	430.29 (13.94)	154.63 (4.16)	34.35 (0.03)	3.70 (0.10)	2.38 (0.07)
Elevated $p\text{CO}_2$ (~16°C)	7.57 (0.01)	16.04 (0.10)	2262.68 (2.81)	983.17 (24.07)	80.19 (1.74)	34.29 (0.03)	1.92 (0.04)	1.24 (0.03)
Present-day $p\text{CO}_2$ (~19°C)	7.92 (0.02)	19.15 (0.06)	2269.14 (2.88)	470.96 (22.74)	166.37 (5.94)	34.34 (0.07)	4.00 (0.14)	2.60 (0.09)
Elevated $p\text{CO}_2$ (~19°C)	7.63 (0.01)	19.12 (0.05)	2264.41 (3.32)	944.86 (28.16)	94.29 (2.64)	34.38 (0.02)	2.26 (0.07)	1.47 (0.04)
<b>Expt. Grazing preference</b>								
Present-day $p\text{CO}_2$ (~16°C)	7.84 (0.02)	15.03 (0.24)	2233.56 (6.98)	453.56 (21.13)	136.18 (4.18)	34.14 (0.01)	3.26 (0.10)	2.09 (0.06)
Elevated $p\text{CO}_2$ (~16°C)	7.45 (0.02)	14.96 (0.24)	2246.58 (15.95)	1281.83 (51.22)	58.85 (2.34)	33.99 (0.05)	1.41 (0.06)	0.91 (0.04)
Present-day $p\text{CO}_2$ (~19°C)	7.86 (0.03)	18.30 (0.11)	2211.97 (29.50)	486.98 (35.49)	142.78 (10.16)	34.15 (0.05)	3.43 (0.25)	2.22 (0.16)
Elevated $p\text{CO}_2$ (~19°C)	7.48 (0.02)	18.37 (0.07)	2187.79 (32.78)	1310.02 (55.21)	63.15 (3.87)	34.14 (0.03)	1.52 (0.09)	0.98 (0.06)
<b>Expt. Algal nutritional quality</b>								
Present-day $p\text{CO}_2$ (~16°C)	7.78 (0.00)	15.29 (0.15)	2163.23 (68.26)	523.59 (15.51)	116.34 (4.74)	34.26 (0.05)	2.79 (0.11)	1.79 (0.07)
Elevated $p\text{CO}_2$ (~16°C)	7.42 (0.04)	15.28 (0.17)	2160.06 (33.78)	1354.20 (97.77)	53.05 (5.33)	34.23 (0.01)	1.27 (0.13)	0.82 (0.08)
Present-day $p\text{CO}_2$ (~19°C)	7.77 (0.04)	18.44 (0.23)	2240.34 (57.96)	638.90 (85.99)	119.72 (7.86)	34.37 (0.08)	2.87 (0.19)	1.86 (0.12)
Elevated $p\text{CO}_2$ (~19°C)	7.50 (0.03)	18.61 (0.16)	2200.39 (100.18)	1262.19 (31.34)	66.41 (7.54)	34.20 (0.04)	1.60 (1.18)	1.03 (0.12)

<sup>a</sup>The parameters of the natural seawater used to renew the reservoir tanks during the rearing period were measured twice a week in near-shore water samples collected during daylight hours and therefore the average temperature did not include day/night temperature variations

were assigned to random cells within the cages and no 2 adjacent cells were occupied, ensuring that the urchins did not have to compete with each other for algae. The investigated traits (see following sections) were then measured at different times during the experimental period following a temporal sequence (Fig. 1).

The experiments were conducted at different levels within the mesocosm setup (the mesocosms consist of a series of water tables stacked one above the other due to space limitations) and therefore differences in the light regime between the levels may be responsible for the different photosynthetic performances and for the slight difference in seawater parameters between the experiments.

## Measured traits

**Growth and survival.** The growth and survival of the small juvenile urchins were evaluated over experimental periods of 6 and 7 mo, respectively. Maximum diameter and both wet and buoyant weights (as a proxy for calcification) were measured at the beginning and end of the rearing period. The differences in these variables were then converted to percent change over time. Diameters of small individuals (<1 cm) were measured using a stereomicroscope equipped with an ocular micrometer, while larger individuals were measured using a digital calliper. All weight measurements were quantified using an analytical balance (Adam AFA180 LC, 0.1 mg precision).

**Test dissolution rate.** To determine whether any of the 4 seawater treatments used in the experiments was corrosive to the naked urchin test material, dissolution rates of empty tests of *L. albus* were estimated by weighing (dry weight) the test ( $n = 4$  per treatment) at the beginning and the end of 2 mo exposure to the treatment conditions. The empty tests were placed within the same type of rearing cages but maintained in a different aquarium. The changes in the weight were converted to percent change over time.

**Grazing rate.** The amount of food consumed by each urchin in each treatment was evaluated during the first 2 mo of rearing. Each urchin was offered a single fragment of fresh green algae, *Ulva* sp. (~0.15 g), per week. Three additional fragments per treatment were placed in a cell without an urchin as a control for natural changes in algal weight. *Ulva* sp. is the primary food item

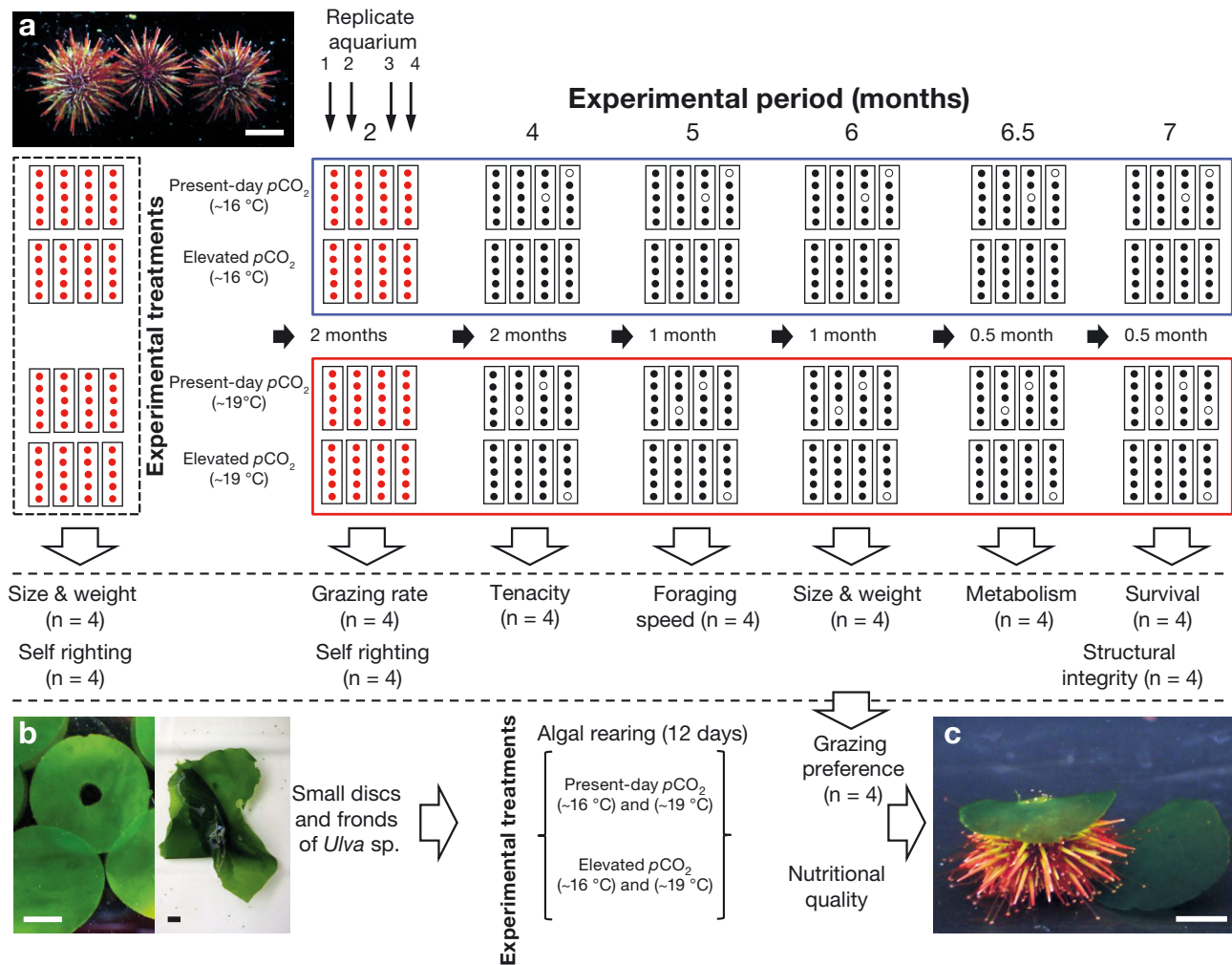


Fig. 1. (Top) Placement of replicate aquaria across the ocean acidification (OA) and warming (OW) experimental treatments used in this study before assignment to the experimental treatments at approximately 16°C (blue box) and 19°C (red box), and (middle) their allocation to measurements of the investigated traits. The circles within each replicate aquarium depict the small juvenile urchins *Loxechinus albus* (inset a) with which repeated grazing rate (red circles) or other measurements (black circles) were made. Open circles: dead urchins; n: number of aquaria. (Bottom) Rearing of algal *Ulva* sp. discs with and without a small hole (inset b, left) and fronds (inset b, right) under the same experimental conditions for the grazing preference experiment (inset c). Scale bars are (a) 0.3 cm and (b,c) 0.5 cm

consumed and efficiently assimilated by juvenile individuals of *L. albus* in their natural environment (Vásquez 2001, González et al. 2008). To estimate feeding rate, the algal fragments were weighed at the beginning and end of each week for the entire experimental period. Wet weight changes in the control fragments were used to calibrate the changes in weight that occurred in the presence of urchins.

**Self-righting.** Self-righting success and self-righting times of the small juvenile urchins ( $n = 80$ ) were evaluated in the absence of any predatory cues, a few hours before their assignment to the experimental treatments. Each individual was placed in a small glass chamber (0.15 l) immersed in a water bath to

maintain the temperature at the corresponding rearing temperature. To improve adherence, the bottom of the chamber was covered with a strip of a rough-textured rubber surface (3M™ Safety-Walk™ tape). Each urchin was placed in the chamber for 15 min of acclimatisation and then the urchin was placed upside down in the middle of the chamber. Self-righting time was defined as the time needed by the individual to completely return to its normal upright position. The total time elapsed from the moment that the urchins were placed upside down to their return to the normal upright position (self-righting time) was measured using a digital stopwatch. A maximum of 5 min was allowed per individual. The

same measurements were repeated after rearing for 2 mo under the experimental conditions using 5 individuals from each aquaria. Self-righting times of >5 min were categorised as failures.

**Tenacity.** The ability to adhere to a solid surface was measured in 4 or 5 urchins removed from each aquarium. The measurements were made after rearing for 4 mo in a specially designed plexiglass chamber described in detail by Manríquez et al. (2016). The chamber consisted of a rectangular box (16 × 8 × 7.6 cm; length × width × height), along with 2 plexiglass flaps joined at 90° to each other and suspended from the top of the chamber from the axis created by the intersection of the 2 flaps. The longer of the 2 flaps (11.8 cm) hung vertically in the chamber and the shorter (5.2 cm) parallel to the base of the chamber. A vertical force applied to the horizontal flap resulted in the rotation of the vertical flap, applying a horizontal force across the base of the chamber. The chambers were filled with 0.3 l of seawater. An individual urchin was positioned on the base of the chamber in contact with the vertical flap and temporary plastic barriers were used to prevent it from moving away. A 3 × 3 cm section of rough-textured rubber surface (3M™ Safety-Walk™ tape) was fixed to the surface of the base where the urchin was positioned to aid urchin adherence. The urchin was given 20 min to attach itself to the base of the chamber, after which the barriers were removed and the dislodgement force applied. A vertical force was carefully applied with a digital push-dynamometer (PCE FM50) on the left side of the horizontal flap generating a horizontal force at the base of the vertical flap that pushes against the test urchin. The push-dynamometer is able to measure a maximum force of 49 N (~5 kg), and the force applied increased at a constant rate throughout the trial until dislodgement occurred. The dislodgement force required to obtain the complete displacement from the surface was standardised by the oral surface area, which was estimated by measuring the diameter of each urchin.

**Foraging speed.** The success and velocity of horizontal and vertical foraging was measured 5 mo after the start of the experiment in 3 or 4 urchins, depending on the number surviving, removed from each aquarium. Both variables were measured in a 5 l flat-bottom plastic jar (23.2 × 19.3 cm; height × diameter) filled with 1.0 μm FSW. The sea urchins were starved for 24 h at the corresponding  $p\text{CO}_2$  and temperature prior to the experiments. To induce movement of the urchin, a mesh bag containing approximately 10 g of the algae *Ulva* sp. was suspended at the water surface. An individual urchin was placed in the centre of

the bottom of the jar and the time required to reach (1) the wall of the jar and then (2) the upper section of the jar at the level of the mesh bag, 20 cm from the bottom, were recorded. To account for differences in urchin sizes, the distance travelled was expressed as a fraction of body size (diameter) and speed was calculated as the time required to cross that distance (min). Foraging success was measured as the capacity of the urchins to reach the wall and then the upper section of the jar, and expressed as a percentage based on the total number of trials ( $n = 16$ ).

**Grazing preference.** Grazing preference was measured 6 mo after the start of the experimental rearing using a randomly selected group of 4 small juveniles from each experimental treatment, one removed from each of 4 of the 5 replicate aquaria. Each individual was placed inside a one l plastic jar filled with 1.0 μm FSW along with 2 small discs of *Ulva* sp. (each 1 cm in diameter) but with different pre-experimental conditioning. The algae were maintained for 12 d under the same temperature conditions as the test group of urchins, but with one group of *Ulva* sp. maintained at present-day  $p\text{CO}_2$  conditions and the other at elevated  $p\text{CO}_2$  conditions (see Table 1). The jars were immersed in either a 16 or 19°C water table to maintain temperature and a plastic air-line was used to deliver treated air via an air stone to the top of the water column. Algal discs were weighed at the beginning of the experiment and again 72 h later. The algal discs were visually differentiated by a small hole (0.2 cm in diameter) made in the middle of the disc, randomly applied to 1 of the 2 discs at the beginning of each experimental run (Fig. 1b). Since direct and continuous observations were not made during the experiments, differences in the weight loss of the algal discs were used to determine grazing preference. To control for weight loss over the experimental period not associated with grazing, a similar number of algal discs were also maintained under the same experimental conditions but in the absence of urchins. The weight loss in these discs was used to adjust the final measurements of weight loss due to grazing (Roa 1992). All the urchins were starved for 24 h prior to the experiments to standardise hunger levels. In order to describe the seawater carbonate chemistry parameters during the experiment, pH and total alkalinity were analysed in 250 ml samples of seawater collected at 4 different times (Days 0, 4, 6 and 12) from each of the experimental jars and averaged for each treatment combination (Table 1).

**Algal nutritional quality.** To measure the effect of temperature and  $p\text{CO}_2$  levels on food quality, 15 fronds of *Ulva* sp. were maintained for 12 d inside

plastic jars (5 l) with running FSW under the 4 seawater treatments. The jars were exposed to the ambient austral fall photoperiod (12 h light:12 h dark cycle) during this period. To each jar, 1.0  $\mu\text{m}$  FSW was delivered at a rate of 1 l h<sup>-1</sup> and a continuous stream of either air (400  $\mu\text{atm}$  CO<sub>2</sub>) or enriched CO<sub>2</sub> air (1200  $\mu\text{atm}$ ) was bubbled through an air-stone. After the rearing period, the fronds were dried, ground and mixed, and the organic carbon and nitrogen contents were analysed using a LECO TruSpec<sup>®</sup> Micro CHN analyser at the Centro de Investigacion en Ecosistemas de la Patagonia, Coyhaique (CIEP), Chile. The analysis included the entire fronds without distinction between new and old growth tissues. The seawater parameters were measured every 3 d during the experimental period (12 d) (Table 1).

**Oxygen consumption.** Oxygen consumption was measured 26 wk after the beginning of the experiments in a randomly selected group of 8 urchins from each treatment (i.e. 2 urchin from each aquarium) using 0.45  $\mu\text{m}$  FSW equilibrated at the same temperature and  $p\text{CO}_2$  levels used during the urchin rearing. Metabolic rates were measured for individual urchins in either a 35 ml glass respirometry chamber (individuals smaller than 1 cm) or a 70 ml chamber (individuals larger than 1 cm). A fibre-optic oxygen meter (Fibox, PreSens) was used for all oxygen consumption measurements. Calibration was performed using a Na<sub>2</sub>O<sub>3</sub>S solution (0% saturation) and using air-bubbled seawater (100% saturation). Temperature was stabilised using a refrigerated and heating water bath circulator (Lab Companion RW-2025G). Individuals were starved in 1.0  $\mu\text{m}$  FSW for 24 h prior to the measurements. Individual measurements lasted for at least 60 min, with the first 5 min removed to avoid possible manipulation effects. Special care was taken to prevent oxygen levels from dropping below 70% of air saturation. Background respiration was determined by measuring oxygen consumption without an urchin in the chamber, and this was subtracted from the experimental oxygen consumption rates.

**Structural integrity of the urchin test.** After 7 mo of experimental rearing, the force required to cause a failure in the structural integrity of the urchin test was measured in all the surviving individuals (see Table 2). Each urchin was positioned over a plastic surface with the oral side facing down and a vertical force was carefully applied to the aboral side with a digital push-dynamometer (PCE FM50) until the urchin broke. During the measurements the contact area between the probe and the aboral side was 0.8 cm<sup>2</sup>. The minimum breaking force was measured and standardised by the wet weight of each urchin. All the in-

dividuals were tested out of the water with individuals exposed to air for 3 min prior to testing in order to standardise the effect of desiccation on the propagation of the structural failure through the urchin test.

**Statistical analyses.** This study used a clumped segregated experimental design with inter-dependent replicates within treatments (Cornwall & Hurd 2015). In detail, 4 header tanks were used to obtain the required  $p\text{CO}_2$  conditions to supply treated seawater to each group of 4 aquaria bearing the 4 groups of 5 experimental individuals. Therefore, multiple individuals ( $n = 5$ ) were housed within 1 experimental aquarium, with 4 separate replicate aquaria per treatment. This design was used due to a combination of logistical constraints and the need to identify the experimental individuals within the experimental tanks to allow repeated assessment of some of the investigated traits for the same individuals through time (see 'Grazing rate' above). Treatment levels were maintained by diffusing pre-mixed treatment gas directly into the experimental tanks; therefore, we assumed that the conditions within each experimental tank were homogeneous and not affected by the presence of other live or empty tests. However, the individuals sampled in the same aquarium were not considered as true replicates. The main effects of the factors  $p\text{CO}_2$  (present-day vs. elevated  $p\text{CO}_2$  levels) and temperature (~16 vs. 19°C) on the studied variables (i.e. test dissolution rate, self-righting, structural integrity, tenacity, foraging speed, algal nutritional quality and oxygen consumption) were tested using 2-way mixed-effects model ANOVA (Pinheiro & Bates 2000) with temperature and pH as the fixed effects and cage as the random effect. This was followed by a Tukey post hoc test when a significant interaction was observed. The effects of temperature and  $p\text{CO}_2$  levels on feeding rate (20 urchins) over time (2 mo) were analysed by a 2-factor repeated measures ANOVA followed by a Bonferroni post hoc test. Time (d) was treated as the within-subject factor and temperature (2 levels, fixed) and  $p\text{CO}_2$  (2 levels, fixed) were treated as between-sub-

Table 2. Mean percentage survival ( $\pm$ SE; n) of small juvenile *Loxechinus albus* after 7 mo rearing under contrasting  $p\text{CO}_2$  and temperature levels

Treatment	Survival of small juveniles
~16°C / present-day $p\text{CO}_2$	90 (5.77; 4)
~16°C / elevated $p\text{CO}_2$	100 (0; 4)
~19°C / present-day $p\text{CO}_2$	85 (5.00; 4)
~19°C / elevated $p\text{CO}_2$	90 (5.77; 4)

ject factors. The replicates were the aquaria ( $n = 4$ ) using the mean value of the individual urchin as a datum to measure the corresponding traits. The assumption of sphericity was checked by Mauchly's test. Data transformations were optimised using Box-Cox power transformations when required. The size and self-righting time of the urchins before being assigned to the treatments was compared using 1-way ANOVA. Urchin grazing preferences were assessed using a paired  $t$ -test (Zar 1999). All statistical analyses were performed using R Statistical Software (R Core Team 2016).

## RESULTS

### Survival and growth

Mean survival of small juvenile *Loxechinus albus* individuals over the rearing period (7 mo) was always  $\geq 85\%$  and no mortality attributable to the experimental conditions was observed (Table 2). Mean maximum diameter ( $\pm$ SE;  $n$ ) of the juveniles at the beginning of the experiments was 0.66 cm ( $0.07$ ; 80) and no significant differences in the maximum urchin diameter were detected between individuals before they were assigned to the different experimental treatments (1-way ANOVA;  $F_{3,77} = 2.94$ ,  $p > 0.05$ ).

The specific growth rate of small juveniles in terms of changes in maximum diameter was not significantly affected by temperature or  $p\text{CO}_2$  (Fig. 2a). The specific growth rate in terms of changes in wet weight was significantly affected by temperature ( $F_{1,12} = 5.583$ ,  $p = 0.036$ ) and  $p\text{CO}_2$  ( $F_{1,12} = 5.676$ ,  $p = 0.035$ ) (Table 3, Fig. 2b), with no significant interaction present between the 2 factors ( $F_{1,12} = 4.063$ ,  $p = 0.068$ , Table 3). Changes in buoyant weight were not affected by  $p\text{CO}_2$  (Fig. 2c).

### Test dissolution rate

Dissolution rates of naked tests of *L. albus* maintained for 2 mo under the treatment conditions were

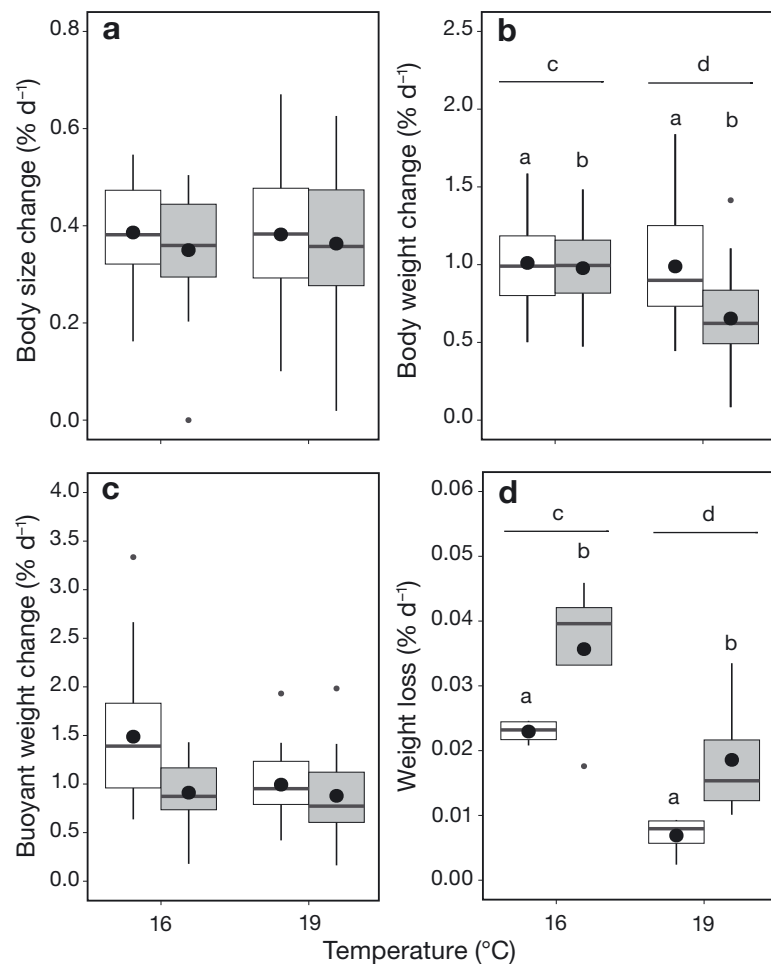


Fig. 2. Specific growth rates in terms of (a) diameter, (b) wet weight and (c) buoyant weight measured in small juvenile urchins *Loxechinus albus* reared for 6 mo under contrasting  $p\text{CO}_2$  (open bars: present-day; filled bars: elevated levels) and temperature levels. (d) Changes in dry weight of naked tests after maintenance for 2 mo under the same conditions. The boundaries of the boxes represent the 25th and 75th percentiles and the lines above and below the box indicate the 10th and 90th percentiles; solid line: median; black dot: mean. The points beyond the lines are outliers. Different letters above the plots represent significant differences

significantly affected by temperature ( $F_{1,8} = 14.551$ ,  $p = 0.005$ ) and  $p\text{CO}_2$  ( $F_{1,8} = 8.097$ ,  $p = 0.022$ ) (Table 3, Fig. 2d).

### Grazing rate

Mean urchin grazing rates ( $\pm$ SE) were greater at  $19^\circ\text{C}$  ( $9.8 \pm 0.3 \text{ mg d}^{-1}$ ) than at  $16^\circ\text{C}$  ( $7.2 \pm 0.3 \text{ mg d}^{-1}$ ), regardless of the  $p\text{CO}_2$  level (Fig. 3). At  $16^\circ\text{C}$ , the grazing rate was 33% higher at present-day levels than at elevated  $p\text{CO}_2$  levels. However, at  $19^\circ\text{C}$  the grazing rate was 22% higher at elevated  $p\text{CO}_2$  levels



Table 3. Results of 2-way mixed-effects model ANOVAs on the effect of temperature and  $p\text{CO}_2$  levels on several traits in *Loxechinus albus* measured at different times during the duration of the study. See 'Materials and methods' for details. Statistically significant relationships ( $p \leq 0.05$ ) are indicated in **bold**

Factors in living urchins	df	<i>F</i>	<i>p</i>
<b>Growth rate (diameter)</b>			
$p\text{CO}_2$	1, 12	0.576	0.463
Temperature	1, 12	0.002	0.968
$p\text{CO}_2 \times \text{Temperature}$	1, 12	0.051	0.826
Intercept	1, 57	372.984	
<b>Growth rate (wet weight)</b>			
$p\text{CO}_2$	1, 12	5.676	<b>0.035</b>
Temperature	1, 12	5.583	<b>0.036</b>
$p\text{CO}_2 \times \text{Temperature}$	1, 12	4.036	0.068
Intercept	1, 57	586.572	
<b>Calcification (buoyant weight)</b>			
$p\text{CO}_2$	1, 12	4.393	0.058
Temperature	1, 12	2.045	0.178
$p\text{CO}_2 \times \text{Temperature}$	1, 12	1.282	0.280
Intercept	1, 57	0.003	
<b>Self-righting success</b>			
$p\text{CO}_2$	1, 12	4.180	0.064
Temperature	1, 12	2.126	0.171
$p\text{CO}_2 \times \text{Temperature}$	1, 12	1.951	0.188
Intercept	1, 57	0.001104	0.974
<b>Self-righting time</b>			
$p\text{CO}_2$	1, 14	14.620	<b>0.002</b>
Temperature	1, 14	0.224	0.644
$p\text{CO}_2 \times \text{Temperature}$	1, 14	3.869	0.069
Intercept	1, 57	5.231	
<b>Foraging speed (horizontal)</b>			
$p\text{CO}_2$	1, 8	0.001	0.994
Temperature	1, 8	1.176	0.310
$p\text{CO}_2 \times \text{Temperature}$	1, 8	9.345	<b>0.016</b>
Intercept	1, 43	1205.389	
<b>Foraging speed (vertical)</b>			
$p\text{CO}_2$	1, 8	0.949	0.359
Temperature	1, 8	0.371	0.559
$p\text{CO}_2 \times \text{Temperature}$	1, 8	2.772	0.135
Intercept	1, 39	186.403	
<b>Tenacity</b>			
$p\text{CO}_2$	1, 12	2.277	0.157
Temperature	1, 12	0.027	0.872
$p\text{CO}_2 \times \text{Temperature}$	1, 12	2.985	0.110
Intercept	1, 57	186.403	
<b>Structural integrity</b>			
$p\text{CO}_2$	1, 8	5.2	0.053
Temperature	1, 8	20.1	<b>0.002</b>
$p\text{CO}_2 \times \text{Temperature}$	1, 8	12.5	<b>0.008</b>
Intercept	1, 61	470387.600	
<b>Oxygen consumption</b>			
$p\text{CO}_2$	1, 12	15.805	<b>0.002</b>
Temperature	1, 12	8.962	<b>0.011</b>
$p\text{CO}_2 \times \text{Temperature}$	1, 12	1.647	0.224
Intercept	1, 16	1029.676	
<b>Factor in dead tests</b>			
Dissolution rate (dry weight)			
$p\text{CO}_2$	1, 8	8.097	<b>0.022</b>
Temperature	1, 8	14.551	<b>0.005</b>
$p\text{CO}_2 \times \text{Temperature}$	1, 8	0.027	0.873
Intercept	1, 8	94.560	

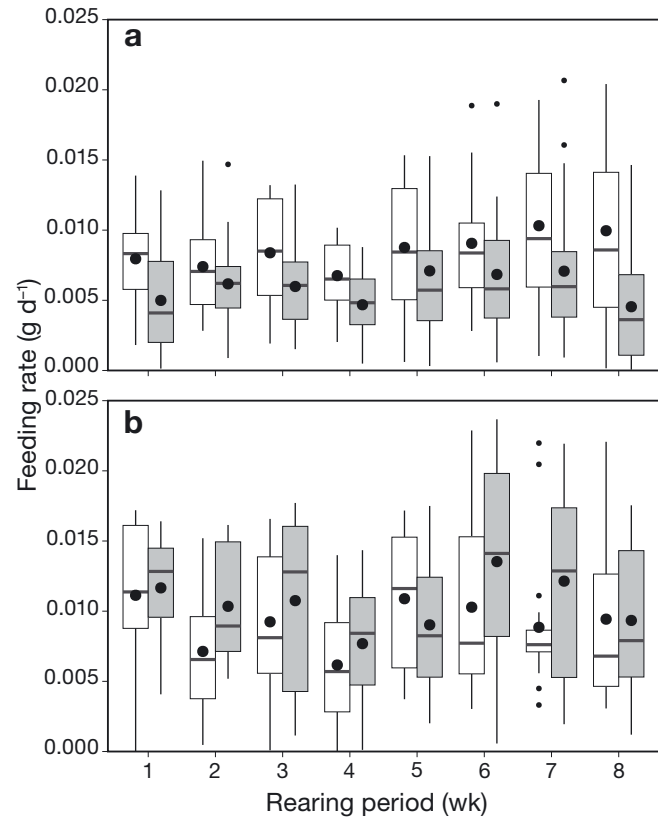


Fig. 3. Mean feeding rates of small juvenile urchins *Loxechinus albus* ( $\pm$ SE) measured over a rearing period of 2 mo under approximately (a) 16°C and (b) 19°C and present-day (open bars) and elevated (filled bars) levels of  $p\text{CO}_2$ . See Fig. 2 for description of box information

than at present-day levels. There was a significant effect of temperature and the interaction between temperature and  $p\text{CO}_2$  over time on the grazing rate (between subjects, Table 4). Moreover, the repeated measure on each unit indicated significant effects of both time and the interaction between time and temperature (within subjects, Table 4). Elevated temperatures had the overall effect of significantly increasing grazing rates regardless of the  $p\text{CO}_2$  level.

### Grazing preference

After 12 h, urchins were observed with both control and treated algal discs on the upper body surface. However, just 1 disc was observed on the oral body surface at the end of the experimental period (72 h, Fig. 1c). At this time, clear signs of grazing were observed as discontinuities along the edges of the algal discs, while no signs of grazing were observed on algal discs maintained without urchins. At both 16 and 19°C, urchins reared under present-day  $p\text{CO}_2$

Table 4. Repeated measures ANOVA results comparing feeding rates in small juvenile *Loxechinus albus* individuals reared for 2 mo at 2 temperatures (15 and 19°C) and 2  $p\text{CO}_2$  levels (400 and 1200  $\mu\text{atm}$ ). Statistically significant relationships ( $p \leq 0.05$ ) are indicated in **bold**

	df	SS	MS	F	p
<b>Within subject analysis</b>					
Time	7	23.547	3.364	5.755	<b>&lt;0.001</b>
Time $\times$ Temperature	7	9.165	1.309	2.240	<b>&lt;0.05</b>
Time $\times p\text{CO}_2$	1	7.911	7.911	1.933	>0.05
Time $\times$ Temperature $\times p\text{CO}_2$	7	3.980	0.569	0.973	>0.05
<b>Between subject analysis</b>					
Temperature	1	38.135	38.135	13.859	<b>&lt;0.005</b>
$p\text{CO}_2$	1	5.282	5.282	1.920	>0.05
Temperature $\times p\text{CO}_2$	1	22.652	22.652	8.232	<b>&lt;0.05</b>

levels exhibited a strong preference for algal discs cultivated at present-day  $p\text{CO}_2$  over those cultivated under elevated  $p\text{CO}_2$  levels. On average, grazing rates were 21 and 27 times higher (16 and 19°C, respectively) on algal discs cultivated under present-day  $p\text{CO}_2$  compared to those cultivated under elevated  $p\text{CO}_2$  levels (16°C: paired  $t$ -test,  $p < 0.001$ , Fig. 4a; 19°C: paired  $t$ -test,  $p < 0.001$ , Fig. 4b). For urchins reared under elevated  $p\text{CO}_2$  levels, no grazing preferences were observed at either 16 (Fig. 4c) or 19°C (Fig. 4d).

### Algal nutritional quality

C/N ratios were significantly affected by temperature with a significant interaction present between temperature and  $p\text{CO}_2$  (Table 5). There was a significant effect of  $p\text{CO}_2$  level at 16°C, where the higher  $p\text{CO}_2$  levels resulted in a higher C/N ratio (mean C/N of 8.5 at present-day  $p\text{CO}_2$  levels compared to 9.5 at elevated  $p\text{CO}_2$  levels; Tukey post hoc test,  $p < 0.005$ ). At the elevated temperature, the C/N ratio was significantly higher than at the lower temperature ( $p < 0.001$ ) but it was significantly higher at the

Table 5. Results of the 2-way ANOVAs on the effect of temperature and  $p\text{CO}_2$  levels on the C/N ratio of fragments of *Ulva* sp. reared for 12 d under the contrasting conditions. Statistically significant relationships ( $p \leq 0.05$ ) are indicated in **bold**

	df	SS	MS	F	p
<b>C/N ratio</b>					
Temperature	1	2.471	2.471	18.61	<b>0.003</b>
$p\text{CO}_2$	1	0.000	0.000	0.00	1.000
Temperature $\times p\text{CO}_2$	1	7.257	7.257	54.66	<b>&lt;0.001</b>
Residual	8	1.062	0.133		

lower  $p\text{CO}_2$  level (Tukey post hoc test,  $p < 0.005$ ). However, the magnitude of this difference (mean C/N of 9.3 at elevated  $p\text{CO}_2$  levels compared to 9.7 at present-day  $p\text{CO}_2$  levels) was smaller than that observed at 16°C (Fig. 5).

### Self-righting

Self-righting was observed in all individuals at the beginning of the experimental period. Mean self-righting time ( $\pm$ SE) measured at the beginning of the acidification-warming experiment in small juveniles of *L. albus* was 1.06 min ( $\pm 0.04$ ;  $n = 80$ ) and no significant differences were detected between the trial individuals assigned to the 4 experimental treatments (1-way ANOVA,  $F_{3,76} = 1.32$ ). After 2 mo rearing, the occurrence of self-righting success ( $\leq 5$  min) was always  $>72\%$ . When considering only individuals that were

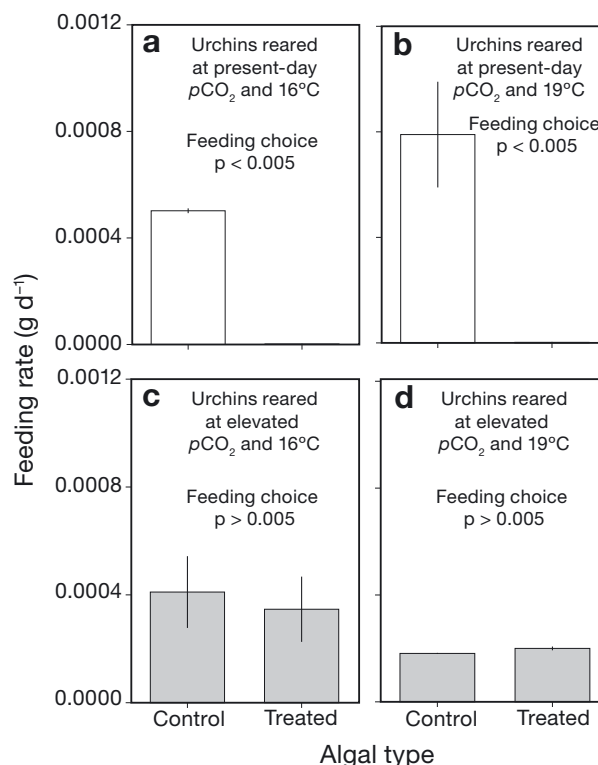


Fig. 4. Feeding preference of *Loxechinus albus* measured as the mean ( $\pm$ SE) grazing rates exhibited by individuals reared at (a,b) present-day (control urchins, open bars) and (c,d) elevated (treated urchins, filled bars)  $p\text{CO}_2$  levels and approximately (a,c) 16°C and (b,d) 19°C on control and treated algae *Ulva* sp. Control algae were grown at  $\sim 16^\circ\text{C}$  and present-day  $p\text{CO}_2$  levels, and treated algae were grown at  $\sim 19^\circ\text{C}$  and/or elevated  $p\text{CO}_2$  levels.  $p$ -values were obtained from paired  $t$ -tests

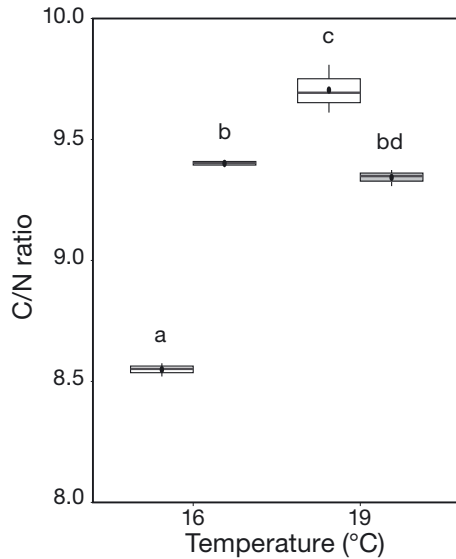


Fig. 5. *Ulva* sp. C/N ratios (% of wet weight) within individuals exposed to contrasting temperature and  $p\text{CO}_2$  levels (open bars present-day and filled bars elevated levels). Different letters above the plots represent significant differences. See Fig. 2 for description of box information

able to successfully self-right, there was a significant effect of  $p\text{CO}_2$ , with faster self-rightings occurring at elevated  $p\text{CO}_2$  than at present levels ( $F_{1,14} = 14.620$ ,  $p = 0.002$ , Table 3). There was no significant effect of temperature alone or of the interaction between temperature and  $p\text{CO}_2$  on self-righting time (Fig. 6a).

### Tenacity

There were no statistically significant effects of temperature,  $p\text{CO}_2$ , or the interaction between them on *L. albus* tenacity (Fig. 7a, Table 3).

### Foraging speed

The horizontal extent of foraging differed between treatments, with varying proportions of juvenile urchins reaching the edge of the container. Differences were greatest at 16°C, with 100% and 63% of urchins reaching the container edge at present-day and elevated  $p\text{CO}_2$ , respectively. At 19°C, 94% and 88% of urchins reached the container edge at present-day and elevated  $p\text{CO}_2$  levels, respectively. On the other hand, the pattern of vertical displacement shows higher proportions of juvenile urchins reaching the upper section of the jars at present-day  $p\text{CO}_2$  at both rearing temperatures. While there were no

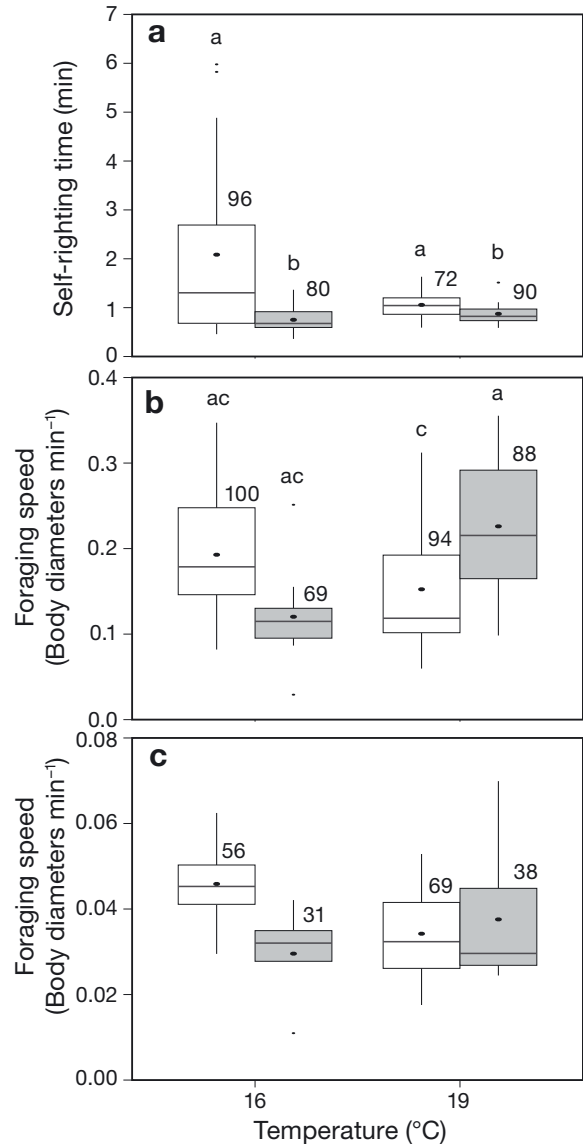


Fig. 6. (a) Self-righting time of small juvenile urchins *Loxechinus albus* reared for 2 mo under contrasting  $p\text{CO}_2$  and temperature levels. (b) Horizontal foraging speed and (c) vertical foraging speed measured in small juvenile urchins reared for 5 mo under contrasting temperature and  $p\text{CO}_2$  levels (open bars present-day and filled bars elevated levels). Percentages above each bar represent numbers of urchins able to successfully self-right (a), reach the wall of the jars (b), or reach the upper section of the jars (c), respectively. Different letters above the plots represent significant differences. See Fig. 2 for description of box information

significant effects of either temperature or  $p\text{CO}_2$  on horizontal speed, there was a significant effect of the interaction between  $p\text{CO}_2$  and temperature ( $F_{1,8} = 9.345$ ,  $p = 0.016$ , Table 3). Post hoc testing found that horizontal speeds were significantly slower ( $p = 0.027$ ) at high  $p\text{CO}_2$  and 16°C but the situation was

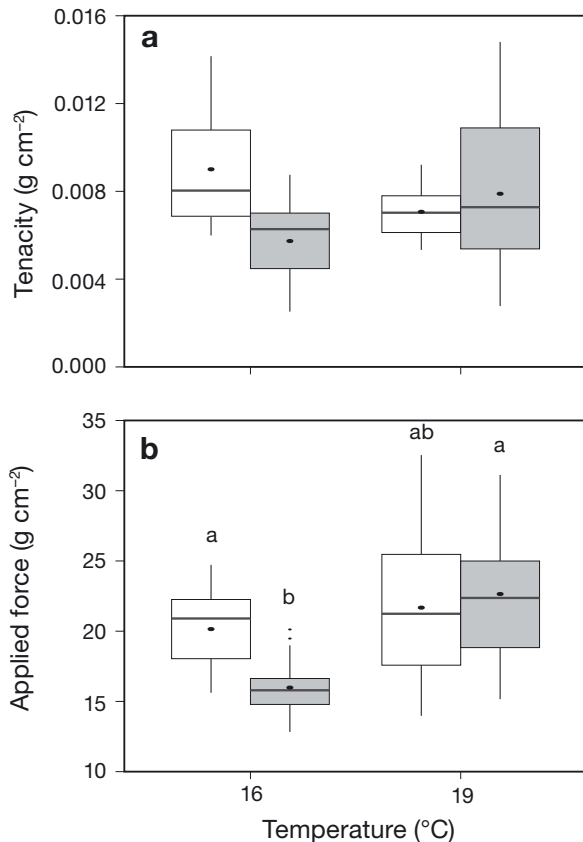


Fig. 7. (a) Tenacity and (b) structural integrity of small juvenile urchins *Loxechinus albus* reared for 4 and 7 mo, respectively, under contrasting temperature and  $p\text{CO}_2$  levels (open bars present-day and filled bars elevated levels). Different letters above the plots represent significant differences. See Fig. 2 for description of box information

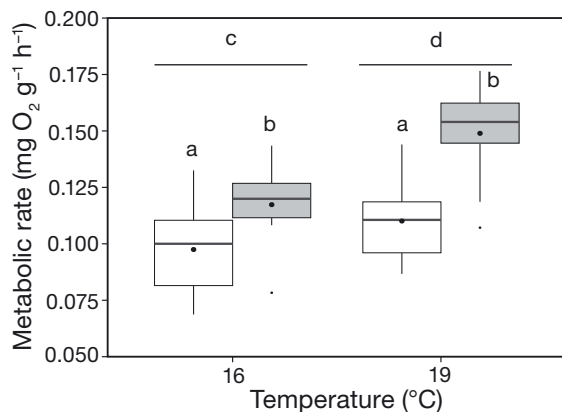


Fig. 8. Metabolism of small juvenile urchins *Loxechinus albus* measured after rearing for 6 mo under contrasting temperature and  $p\text{CO}_2$  levels (open bars present-day and filled bars elevated levels). All the measurements were conducted in seawater conditioned to the same temperature and  $p\text{CO}_2$  levels used during urchin rearing. Different letters above the plots represent significant differences. See Fig. 2 for description of box information

reversed at 19°C, with horizontal speeds significantly faster ( $p = 0.029$ ) at high  $p\text{CO}_2$  (Fig. 6b). No significant effects of either factor on vertical speeds were detected (Fig. 6c, Table 3).

### Structural integrity of the urchin test

There was a significant effect of temperature ( $F_{1,8} = 20.100$ ,  $p = 0.002$ , Table 3), and of the interaction between temperature and  $p\text{CO}_2$  ( $F_{1,8} = 12.500$ ,  $p = 0.008$ , Table 3), but no significant effect of  $p\text{CO}_2$  alone on the amount of force required to cause the failure of structural integrity. The post hoc tests indicated that urchin tests maintained at 16°C and high  $p\text{CO}_2$  were significantly weaker ( $p < 0.001$ ). Urchin tests maintained at high  $p\text{CO}_2$  were also significantly weaker at 16°C compared to 19°C ( $p = 0.037$ , Fig. 7b).

### Oxygen consumption

Metabolic rates were significantly higher under elevated  $p\text{CO}_2$  conditions ( $F_{1,12} = 15.805$ ,  $p = 0.002$ ) and higher temperatures ( $F_{1,12} = 8.962$ ,  $p = 0.011$ ). (Table 3, Fig. 8).

## DISCUSSION

### Risk of predation

Large body size and resistance to the loss of structural integrity reduces predation vulnerability in the early benthic ontogeny of urchins (Miller & Emlet 1999). Reductions in body size or structural integrity in response to OA and OW would suggest that the vulnerability of an urchin to predation would increase under the projected future ocean climate. While we found no evidence of OA and OW causing changes in body size in *Loxechinus albus*, we did find evidence for reduction in the structural integrity of the urchin test at high  $p\text{CO}_2$  levels and at present-day temperatures. Our findings disagree with a previous study using a different urchin species, *Paracentrotus lividus*, that reported no effects of OA and OW on the mechanical properties of either whole adult individuals or *ex vivo* ambital and apical plates (Collard et al. 2016). However, it should be noted that while Collard et al. (2016) made their measurements in seawater, structural integrity in the present study was measured in air, which may have altered the mechanical properties of the ligament joining the test

plates (Ellers et al. 1998). Therefore, the reduced structural integrity of small *L. albus* found in the present study might be an artefact of reduced ligament strength and not represent the consequences of the investigated stressors on the skeleton of the individuals. In conclusion, the effects of OA and OW on predation risk derived from the reduction in the structural integrity of the urchin test must be considered with caution.

The discrepancy between no effects of OA on both body size and calcification (buoyant weight) and negative effects of OA and OW on the dissolution rate of empty tests indicates the importance of the tissue surrounding an urchin test. This concurs with published information highlighting that the loss of material from a dead test skeleton is not relevant for living urchins which have mechanisms to counteract the effects of near-future  $p\text{CO}_2$  levels (DuBois 2014).

Urchin survival may also be influenced by their ability to move through their environment (foraging), the strength with which they can maintain their position, particularly in areas with high flow (tenacity), and the time required to recover from physical disturbance (self-righting time). Responses to OA and OW, such as reduced foraging speeds and tenacity, or increased self-righting time, would likely increase the vulnerability of *L. albus* to predation in their natural environment. While the present study found no effect of OA and OW on foraging speed and tenacity, we did find evidence that the self-righting time was reduced in *L. albus* under elevated  $p\text{CO}_2$  conditions at 16°C, although no differences were found at 19°C at either  $p\text{CO}_2$  level. A similar response of self-righting behaviour to OA and OW was shown in the early ontogeny of a marine snail (Manríquez et al. 2013, 2016). An upside-down urchin is at greater risk to predation; thus reducing the time required to complete this behaviour may increase the likelihood of surviving being overturned by physical disturbance (e.g. waves) or falling upside-down during typical hatchery restocking efforts. Faster self-righting at elevated  $p\text{CO}_2$  does not support the assumption that stressful conditions will negatively affect behavioural traits with consequent negative impacts on distribution patterns and ecosystem functioning. The metabolic rate of *L. albus* increased at elevated  $p\text{CO}_2$ , which suggests that this  $p\text{CO}_2$  level will require a higher energy cost for homeostasis than present-day conditions. This agrees well with previous studies of echinoderms and other invertebrate species that show increasing metabolic rates in response to elevated  $p\text{CO}_2$  (Wood et al. 2008, Beniash et al. 2010, Lardies et al. 2014).

Two of the experiments reported here identified significant interactions between temperature and  $p\text{CO}_2$  (i.e. horizontal foraging speed and structural integrity), highlighting the utility of multifactorial experiments to reveal more of the complexity of organismal responses to environmental change. Within the context of OA and OW, evidence from this work suggests that higher ocean temperatures may mitigate the overall impact of near-future elevated  $p\text{CO}_2$  levels. In the zone where *L. albus* brood stock were collected (29° to 30° S), the mean near-shore sea surface temperature is 16°C and the mean summer temperature is 18.2°C. This suggests that this particular population in northern central Chile (29° S) is infrequently exposed to chronic temperatures as high as 19°C. By rearing urchins at 19°C, it is possible that their sensitivity to high  $p\text{CO}_2$  may have been reduced. Unfortunately, we cannot determine at present whether the reported interaction between OA and OW are additive (the sum of the individual stressor effects) or synergistic (greater than the sum of the individual stressor effects) due to the limited number of temperature or  $p\text{CO}_2$  levels used in this study.

### Grazing and algal preference

While no drastic effects of OA and OW on the survival of small juvenile *L. albus* were detected, grazing rates and algal preferences were found to differ between treatments. These changes may help explain the reduced growth, in terms of wet weight, of small juvenile *L. albus* reared under elevated  $p\text{CO}_2$ . Duarte et al. (2016) recently described higher grazing rates on algae exposed to elevated  $p\text{CO}_2$  as an efficient compensating mechanism for the low nutritional quality of the algae, but this was not found in the present study. Instead, grazing rates were reduced under elevated  $p\text{CO}_2$  and urchins showed a preference for *Ulva* sp. grown under present-day  $p\text{CO}_2$  conditions. Work by Gordillo et al. (2001) demonstrated enhanced growth and decreased net photosynthesis rates, soluble proteins and internal C content, with a constant C/N ratio, in *U. rigida* cultivated under elevated  $p\text{CO}_2$  levels. Such physiological changes may be interpreted as a reduction in the nutritional quality of the alga (Mercado et al. 1999), which is critical for the growth of herbivorous invertebrates (Mattson 1980). To fulfil nutritional requirements, grazing must be optimised to preferentially select algae with high nutritional content (Duarte et al. 2016). This could explain both the low grazing rates of *L. albus* recorded under high  $p\text{CO}_2$  levels

and the clear grazing preference for higher quality *Ulva* sp. (in terms of a lower C/N ratio) cultivated under present-day  $p\text{CO}_2$ . Further, this suggests the possibility that natural populations of *L. albus* may be threatened not just directly by the  $p\text{CO}_2$  and temperature, but indirectly via lower quality food resources. This is particularly important if we take into account that small juvenile individuals of *L. albus* feed mainly on *Ulva* sp., algae with a higher assimilation efficiency than brown algae (González et al. 2008). A recent field study has found a resilient shift from sea urchin to fish as the primary grazers under naturally elevated  $p\text{CO}_2$  levels (Baggini et al. 2015). Depending on how the changes described here affect interactions between *L. albus* and other species (i.e. prey and predators), near-future climate change may induce unknown indirect effects on marine communities within its distribution.

Recent information suggests that the localised hydrodynamic conditions created by interactions between an organism and the flow of seawater may be important in ameliorating the negative effects of OA on calcifying organisms (Cornwall et al. 2014). Evidence indicates that carbonate chemistry along a surface is conducive to higher net calcification in low-flow conditions, where the boundary layer is thicker than in higher-flows that have a thinner boundary layer (Hurd et al. 2011). In our study, urchins and algae were reared in running seawater, but it is unlikely that flow speeds were high enough to prevent the formation of thick boundary layers. As a result, the conditions used in the present study may not accurately mimic the hydrodynamic conditions found in the natural habitat of the urchins. As such, the consequences of OA on calcification or test dissolution of *L. albus* could be less severe if the effect of the boundary layer and flow velocity is taken into account, though this is beyond the limits of the present study.

### Implications for management

Restocking of *L. albus* in Chile relies on releasing hatchery-generated small juvenile urchins (1 to 7 cm in diameter) into natural environments. Juveniles are produced from gametes using standardised practices and fed diets consisting of both micro- and macroalgae (Castilla 1990, Bustos et al. 1991, Zamora & Stotz 1994, Bustos & Olave 2001). However, there have been no published attempts to follow the acclimatisation of the young *L. albus* after restocking (i.e. growth and survival), though the little information

that is available suggests high losses of these hatchery-generated individuals (González & Jerez 2001, Guzmán et al. 2009). In semi-commercial aquaculture species such as *L. albus* and others, there is a chance that the results derived from manipulative experiments may guide future manipulative practices in order to minimise the harmful effects of near-future elevated  $p\text{CO}_2$  and temperature levels. Such an approach has been successfully implemented in the Oregon oyster hatchery aquaculture, where the stakeholders have identified key bottlenecks in the life cycle of the Pacific oyster *Crassostrea gigas* exposed to natural increases in  $p\text{CO}_2$  levels and taken measures to improve seawater chemistry during early larval development (Barton et al. 2012). We conclude that future hatchery-based efforts to artificially restock *L. albus* populations should aim to produce more robust juvenile urchins by exerting more control over temperature and  $p\text{CO}_2$  conditions within the hatchery environment. Further, this strategy should also include similar treatment of the algae used as food for the urchins based on our findings regarding urchin grazing preference.

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