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SHORT COMMUNICATION

Adaptive variability to low-pH river discharges in *Acartia tonsa* and stress responses to high $_pCO_2$ conditions

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Abstract

Environmental transitions leading to spatial physical–chemical gradients are of ecological and evolutionary interest because they are able to induce variations in phenotypic plasticity. Thus, the adaptive variability to low-pH river discharges may drive divergent stress responses [ingestion rates (IR) and expression of stress-related genes such as *Heat shock protein 70* (*Hsp70*) and *Ferritin*] in the neritic copepod *Acartia tonsa* facing changes in the marine chemistry associated to ocean acidification (OA). These responses were tested in copepod populations inhabiting two environments with contrasting carbonate system parameters (an estuarine *versus* coastal area) in the Southern Pacific Ocean, and assessing an *in situ* and 96-h experimental incubation under conditions of high pressure of CO_2 ($_pCO_2$ 1200 ppm). Adaptive variability was a determining factor in driving variability of copepods' responses. Thus, the food-rich but colder and corrosive estuary induced a traits trade-off expressed as depressed IR under *in situ* conditions. However, this experience allowed these copepods to tolerate further exposure to high $_pCO_2$ levels better, as their IRs were on average 43% higher than those of the coastal individuals. Indeed, expression of both the *Hsp70* and *Ferritin* genes in coastal copepods was significantly higher after acclimation to high $_pCO_2$ conditions. Along with other recent evidence, our findings confirm that adaptation to local fluctuations in seawater pH seems to play a significant role in the response of planktonic populations to OA-associated conditions. Facing the environmental threat represented by the inter-play between multiple drivers of climate change, this biological feature should be examined in detail as a potential tool for risk mitigation policies in coastal management arrangements.

Introduction

Geographically widespread species must cope with environmental differences among habitats. This ability can, in principle, be achieved by genetic differentiation and/or phenotypic flexibility (Blanckenhorn 1997; Lardies *et al.* 2008). Information concerning geographic variations in response to ocean acidification (OA; Feely *et al.* 2004; Caldeira & Wickett 2003) is critical because many morphologic, life-history and metabolic traits show variation

across space (Lardies *et al.* 2008, 2014; Aguilera *et al.* 2013); an issue often attributed to organismal adaptation over environmental gradients or ecological transitions (Levins 1968). Given its global coverage and the accelerated rate of change associated with OA (Doney & Schimel 2007; Dore *et al.* 2009), it is expected that the interactions between it and other environmental drivers reducing seawater pH, such as the case of upwelling areas (Feely *et al.* 2008) or low-pH river discharges (Waldbusser & Salisbury 2014), results in complex environmental conditions for marine biota (Boyd & Hutchins 2012). Freshwater discharges are typically characterized by low pH in comparison to the adjacent ocean, mainly due to riverine dissolved inorganic carbon (DIC) fluxes resulting from land weathering (Aufdenkampe *et al.* 2011) and terrestrial organic carbon fluxes (Cai *et al.* 2011). These fuel microbial respiration in both riverine and the adjacent coastal waters, increasing CO_2 levels and reducing seawater pH (Borges & Gypens 2010). For marine invertebrates facing stressful conditions such as those associated with estuarine acidification (corrosive waters), local adaptation and phenotypic plasticity involving indeed traits trade-off arise as important mechanisms for adjustment of their physiology, morphology and life history because it allows them to improve the energy budget. So, despite the highly variable nature of these coastal ecosystems marine copepods support 70–90% of zooplankton biomass (Kiørboe 2011) and exert key roles in marine functioning (Rivkin & Legendre 2002) and biogeochemistry (Mauchline 1998).

Physical–chemical changes in seawater resulting from OA processes may induce copepod inactivity/dormancy, a short-term (*i.e.* hours to days) lethargy which reduce oxygen demands (McAllen *et al.* 1999; Richmond *et al.* 2006). However, this metabolic depression may also be expressed as a reduction of the feeding performance, compromising the ingestion rates and thus, affecting finally other biological energy-dependent processes, such as survival, reproduction and growth. In this respect, different observations have shown reduced clearance and ingestion rates of marine invertebrates under high CO_2/low pH conditions (Fernández-Reiriz *et al.* 2011; Stumpp *et al.* 2011a,b; Barton *et al.* 2012; Navarro *et al.* 2012; Range *et al.* 2013; Vargas *et al.* 2013). The synthesis of stress-related proteins is another mechanism of stress response, which on the contrary to the lethargy-type, involves an energetically active strategy to compensate injurious metabolic compounds derived from stressful conditions (Southgate *et al.* 1985). Among such compounds, heat shock proteins (Hsps) and ferritin are intensively synthesized by invertebrates under adverse environmental conditions (Feder & Hofmann 1999; Theil 2003).

Ferritin is a metal chelator protein ubiquitously distributed among living species, and plays a key role in iron metabolism (Harrison & Arosio 1996). Its ability to sequester this element gives ferritin the dual functions of detoxification and iron reserve, and, by decreasing the intra-cellular free iron pool, ferritin prevents the formation of highly toxic hydroxyl radicals *via* the iron-catalysed Fenton reaction (Harrison & Arosio 1996). This protein has been related to different stress responses in marine organisms, such as anoxia and metabolic acidosis (English & Storey 2003), and copper exposure (Zapata *et al.* 2009; Götze *et al.* 2014). By contrast, Hsp70 is a protein induced in marine organisms by a variety of stressful conditions, not only heat shock and the presence of contaminants but also the occurrence of pathogens and pathophysiological conditions related to inflammation and acidification (Cheng *et al.* 2007; Cummings *et al.* 2011; Lardies *et al.* 2014). In copepods, elevated *Hsp70* synthesis has been observed under high temperatures in shallow water environments (Voznesensky *et al.* 2004; Rhee *et al.* 2009; Aruda *et al.* 2011), whereas synthesis of *Ferritin* increases in response to anoxia in *Acartia tonsa* (Nilsson *et al.* 2013). Either through passive or active mechanisms adopted to tolerate adverse environmental conditions, potential negative impacts are expected in the fitness of coastal marine populations associated with OA processes.

Adaptive variations due to low-pH river discharges may drive divergent stress responses in copepods facing OA conditions. We tested this hypothesis by considering specific ingestion rates (IRs) and expression of stress-related genes (*i.e. Hsp70* and *ferritin*) in *A. tonsa* as responses to high CO_2/low pH conditions. In order to address this question, we characterized environmental factors (temperature, salinity, pH, food) and the *in situ* IR of *A. tonsa* inhabiting two geographically distant and differently influenced river locations along an estuarine–coastal gradient in the Southern Pacific Ocean. After a mid-term acclimation period (96 h), we then conducted a common garden experiment, in which we modified the CO_2 levels according to scenarios of predicted levels of OA proposed by IPCC (2007). Finally, we again estimated IR as well as the relative gene expression of *Hsp70* and *Ferritin* in response to these elevated CO_2 levels.

Material and Methods

Study area

The study was conducted in the highly river-influenced Corral Bay in the Southern Pacific Ocean, off the coast of Central-Southern Chile (39°50′ S, 73°25′ W). Local coastal area involves a salt wedge estuary whose hydrography

is strongly affected by tidal cycles and intense river runoff flowing into the estuarine system. Along the coast where the bathymetry reaches 25–30 m in depth, river runoff tends to move northward driven mostly by the coastal wind shear (Strub *et al.* 1998; FIP 2002). This propitiates conspicuous variations in seawater total alkalinity, pH and $_pCO_2$ along the coast (Torres *et al.* 2013).

Characterization of *in situ* physical–chemical and food conditions

Characterization of *in situ* oceanographic conditions (temperature, salinity, seawater pH, and food conditions) was carried out during the spring and summer seasons of 2011 and 2012 at two locations along the north axis of the estuarine system of the bay: a location in the inner estuarine area, behind the estuarine front (hereafter 'estuarine'), and another location in the northernmost portion of the system with lower influence of freshwater runoff (hereafter 'coastal') (Aguilera *et al.* 2013; Table 1A). Each location was sampled twice in summer and twice again in spring, each time during three subsequent flood tides (*i.e.* each 24 h). Salinity and temperature profiles were recorded by using a small Conductivity, Temperature, Depth Sensor (Ocean Seven 305 Plus, www.idronaut.it) from a depth of 1 to 12 m, although the reported data correspond to a mean value for the upper 7 m depth of the water column. Seawater was also collected at 7 m depth with a 10-l Niskin bottle. Subsamples were stored in borosilicate bottles without air bubbles and transported to the field lab where pH was measured within 2 h of collection. The pH was measured in a closed 25-ml cell thermostat at 25.0 ± 0.18 °C using a Metrohm 713 pH meter (input resistance >1.013 Ω, 0.1 mV sensitivity and nominal resolution 0.001 pH units) and a glass combined double junction Ag/AgCl electrode (Metrohm model 6.0219.100) calibrated with 8.089 Tris buffer at 25 °C. Therefore, pH values are reported over the total hydrogen ion scale (Dickson & Goyet 1994). A subsample was used to estimate food availability in terms of food carbon availability. For this purpose water was sieved through a 150-μm sieve and food was classified into six functional groups: chain-forming (CFD), pinnate (PD) and centric diatoms (CD), dinoflagellates (DIN), and small and large nanoflagellates (<5 and >5 μm NF, respectively).

Copepod sampling

Plankton samples were collected from the upper 7 to 12 m depth at each location by means of vertical hauls

Table 1. (A): Details of seasonal field surveys performed to characterize spatial hydrographic variability as well as copepod isotopic composition and adult body size in the river-influenced study area in the Southern Pacific Ocean. Locations were sampled during spring (Sprg) and summer (Sum) between 2010 and 2012. Isotopic signal represents the isotopic composition ($\delta^{15}N$ and $\delta^{13}C$, n = 8) of copepods during this study, which is included as to supplementing conjecture about differential degree of exposure to river influence in the chemistry of the water column. Table also shows the body size of adult females. (B): Statistical results of spatial comparison of environmental conditions and *in situ* ingestion rates (IRs) of *Acartia tonsa* during the study.

(A)

location	co-ordinates	campaigns	isotopic signal of copepods	copepod size (mm)
estuarine	39°88′8″ S	Sum2010	$\delta^{15}N = 8.5 \pm 0.5$*	1.074 ± 0.07
	73°40′7″ W	Sum2010	$\delta^{13}C = -20.1 \pm 0.1$**	
		Sprg2011		
		Sprg2012		
coastal	39°67′9″ S	Sum2010	$\delta^{15}N = 10.5 \pm 0.4$*	1.051 ± 0.07
	73°38′8″ W	Sum2010	$\delta^{13}C = -19.1 \pm 0.1$**	
		Sprg2011		
		Sprg2012		

(B)

statistical comparison factor	variable	*t*-value	df	F-ratio	P-value
location	temperature	−33	1, 24	6.0	0.001
	salinity	−14	1, 24	1.8	0.001
	seawater pH	−12	1, 24	49	0.001
	food availability	3.7	1, 24	1.5	0.001
	IR	−5.3	1, 24	1.1	0.001

*, ** = Differences statistically significant and highly significant, respectively, according to Mann–Whitney *U*-test ($P < 0.05$).

with a WP2 net of 200-μm mesh size equipped with a non-filtering 1-l cod-end. Within 2 h of collection, up to 200 undamaged, mature and healthy females of *Acartia tonsa* were sorted under an OLYMPUS SZ51 stereomicroscope, transferred to new beakers and stored at *in situ* temperature until setting up of the experiments. In addition, up to 50 copepod females were preserved each time in 10% formalin for body length (BL) determination. BL was converted to body mass with the length–weight regressions cited by Uye (1982) and to body carbon assuming a specific-C content of 45% (Kiørboe & Nielsen 1994). In order to evaluate the freshwater influence on local populations collected at both sites, another fraction of the plankton samples was immediately frozen at −20 °C after sampling for determination of their stable isotope composition ($\delta^{13}C$ and $\delta^{15}N$). Samples were processed according to Bunn *et al.* (1995) before mass spectrometry analysis (VG Micromass 602C equipment), considering the international standards Vienna Pee Dee Belemnite (PDB) and atmospheric N_2 as reference materials for carbon and nitrogen, respectively. The relative importance of the contribution of terrestrial river-carried C and N to the marine planktonic system was assessed by applying a two-source mixing model (Bianchi 2007).

In situ ingestion rates

After each sampling (twice in spring and summer, three consecutive flood tides), adult females from each sampling station were sorted and pipetted into 600-ml borosilicate acid-washed bottles containing ambient water with natural <150 μm food assemblages. Three control bottles without copepods and three bottles containing six adult females of *Acartia tonsa* were incubated at *in situ* conditions for 24 h, and gently mixed periodically to minimize cell settling. Subsamples (60 ml) of all control bottles were immediately preserved with 2% Lugol's solution for phytoplankton counts, and a subsample was preserved in glutaraldehyde (6.0% weight/volume in 0.2 μm pre-filtered seawater) for counts of nanoflagellates (T_0). At the end of a 24-h incubation period, subsamples were taken from all bottles and preserved, as mentioned above, for cell counts. Nanoflagellates and large microplankton cells were counted, measured and their volumes converted to carbon units. For more details see Aguilera *et al.* (2013). IRs measured as cell removal were calculated following Frost (1972) as modified by Marín *et al.* (1986) and expressed as specific IRs by considering adult body mass (day^{-1}). This IR estimation based on cell removal is a common technique for measuring clearance and ingestion rates in planktonic organisms (Vargas & González 2004).

Acclimation and common garden test of pCO_2 effects on copepod responses

After estimations of *in situ* IR, copepods from each location were acclimated to high pCO_2 levels (1200 μatm) and to controlled temperature, salinity and food conditions for 96 h before conducting new incubation experiments to estimate IRs. For this purpose, copepods (estuarine and coastal) were transferred to new beakers, fed daily with *c.* 70 μg $C \cdot l^{-1}$ of *Isochrysis* sp. and incubated at *c.* 14 °C, in 0.2 μm filtered seawater with salinity 33 and equilibrated at *c.* 1200 ppm CO_2 for 96 h. The temperature was maintained at 14 °C, chosen because this is similar to that at the base of the thermocline throughout the study area. A salinity value of 33 was maintained during acclimation since it was frequently observed in both locations despite temporal variations during the study (Aguilera *et al.* 2013). Food conditions were supplied *ad libitum* so as to not limit copepod ingestion whereas pH was modified in agreement with the IPCC CO_2 scenario for 2100 (IPCC 2007). In order to obtain the specific pH conditions, the incubation water was exchanged daily with water from equilibration tanks in which CO_2 concentration was modified by mixing the seawater with air containing different CO_2 concentrations, as described by Findlay *et al.* (2008). Air/CO_2 mixtures were produced using a bulk flow technique, whereby known flows of dry air (obtained by compressing atmospheric air to 117 psi and passing it through a 1 mm particle) and ultra-pure (*i.e.* research grade) CO_2 gas were supplied, *via* a mass flow controller (MFC), and mixed before equilibration with seawater. Air flow in the MFC was set manually to 5 $l \cdot min^{-1}$ and CO_2 flow was set manually to 4.25 $ml \cdot min^{-1}$ in order to produce a CO_2 treatment of approximately 1200 ppm. The CO_2 of the blended gas was monitored to allow fine regulation of CO_2 through MFCs to reach each target pCO_2 in seawater. Other carbonate parameters (total alkalinity, salinity, and temperature) were also monitored daily as mentioned above.

After the 96-h acclimation period to these conditions a 24-h experiment was performed to estimate IR as previously detailed. An additional stock of animals was maintained under similar conditions to the experimental ones to avoid mortality constraints for further statistical analysis.

RNA isolation, cDNA synthesis and gene expression analysis

Once incubation for IR estimations under high pCO_2 conditions had finished, adult females were cleaned with filtered seawater and then carefully concentrated into cryovials at −80 °C until molecular analysis. A pool of *Acartia tonsa* adults (100–120) from each location was used to extract total RNA with the Trizol™ (Invitrogen, Waltham,

MA, USA) method, following the manufacturer's instructions, and using an electronic pestle. For cDNA synthesis, 1 μg total RNA was treated with DNAse I (RQ1; Promega, Madison, WI, USA) and then reverse-transcribed with random hexamers using the ImProm-II™ Reverse Transcription System (Promega), according to the manufacturer's instructions. Reverse transcription-quantitative PCR (RT-qPCR) was performed using a Brilliant™ SYBR™ Green QPCR Master Reagent Kit (Agilent Technologies, Santa Clara, CA, USA) and the Eco Real Time-PCR detection system (Illumina, San Diego, CS, USA) as described by Arias *et al.* (2011) for three replicates of each sample. The RT-qPCR mixture (15 μl) contained 2 μl template cDNA and 140 nM of each primer. Amplification was performed under the following conditions: 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. At the end of RT-qPCR amplification all products were subjected to a melt cycle from 55 to 95 °C. The primers used (*Hsp*, *ferritin* and β-*actin*) were described by Nilsson *et al.* (2013). Relative gene expression of *Hsp70* and *ferritin* (gene of interest, GOI) was normalized to the level of the housekeeping β-*actin* gene (HK) according to the equation $2^{-(\Delta ctGOI\text{-}ctHK)}$, as described by Lardies *et al.* (2014). Then, gene expression levels were normalized to the average value of the treatment with lower expression (estuarine). In all samples, expressions of the HK were similar (varying by no more than two cycles among samples) and the reaction specificities were tested with melt gradient dissociation curves and electrophoresis gels (agarose 2% of each RT-qPCR product).

Data analysis

Seasonal (spring and summer) environmental and biological data were considered together to perform spatial comparisons, as the main focus of this study was to evaluate the effect of spatial environmental variability on copepod traits (*i.e.* adaptive variability). In this way, the working hypothesis could be validated or refuted depending on whether or not location was a statistically significant factor in determining variability in copepod responses in both *in situ* and laboratory observations.

The effect of location upon the variability of oceanographic parameters and *in situ* IR was assessed through the parametric Student's *t*-test for cases in which normal distribution (Kolmogorov–Smirnov) and homoscedasticity (Hartley test) assumptions were satisfied. Otherwise, the non-parametric Mann–Whitney *U*-test was applied to evaluate these potential spatial variations. Furthermore, as environmental factors simultaneously affect copepod ingestion, a generalized linear model (GLM) was formulated with significantly correlated variables to better determine the specific role of each environmental factor in modulating the IRs of planktonic copepods in the study area. As for *in situ* spatial comparisons, a similar statistical approach was adopted to verify changes in experimental conditions maintained during the common garden test of CO_2 (temperature, salinity, food and parameters of the carbonate system). Normality and equal variances for *ferritin* and *Hsp70* expression data were tested through the Anderson–Darling normality and Leven's tests, respectively. Differences in the means of IRs and relative gene expression of *ferritin* and *Hsp70* after the common garden test of CO_2 were tested using a one-way analysis of variance (ANOVA) or Mann–Whitney test depending upon whether the assumption of normality was satisfied.

Results

Environmental factors and *in situ* ingestion rates

In situ temperature, salinity, seawater pH and food availability showed substantial alongshore spatial differences also occurred (Fig. 1, left panel). The statistical comparison of the observed spatial environmental variability is shown in Table 1B. From the estuarine to the coastal location, temperature increased from 13.7 ± 0.4 to 16.6 ± 0.2 °C, respectively (Fig. 1A). Showing a smaller range of fluctuation, salinity also increased towards the coastal section, from 32.1 ± 0.2 to 33.3 ± 0.3 (Fig. 1C). Sharp spatial differences also occurred in seawater pH (Δ = 0.275 pH units) with a more acidic estuary (7.739 ± 0.022 pH units) in relation to the more oceanic coastal site (8.014 ± 0.015 pH units) (Fig. 1E). The size of copepods sampled under these conditions did not vary significantly between locations whereas food availability, in terms of micro-phytoplankton biomass, showed a significant inverse pattern of variability in relation to the other environmental factors; such that food availability was lower in the coastal site (Fig. 1G). Under these field conditions, mean *in situ* IR showed significant differences, with lower values occurring in the estuary (1.88 ± 0.7 day^{-1}) than in the coastal section (4.3 ± 1.07 day^{-1}) (Fig. 1I). Temperature, salinity, pH and food biomass were all correlated with IR, such that the GLM explained a significant proportion (r^2 = 0.5) of IR variability under *in situ* conditions where pH was the main environmental factor driving this variability (r^2 = 0.3).

Testing pCO_2 effects on IR

Variations of experimental conditions maintained during copepod acclimation to common garden test of CO_2 as well as IR of copepods acclimated to these conditions are shown in Fig. 1 (right panel). Common garden temperature was successfully kept stable, with small variations ranging between 13.2 ± 0.4 and 13.1 ± 0.5 °C (Fig. 1B). Salinity

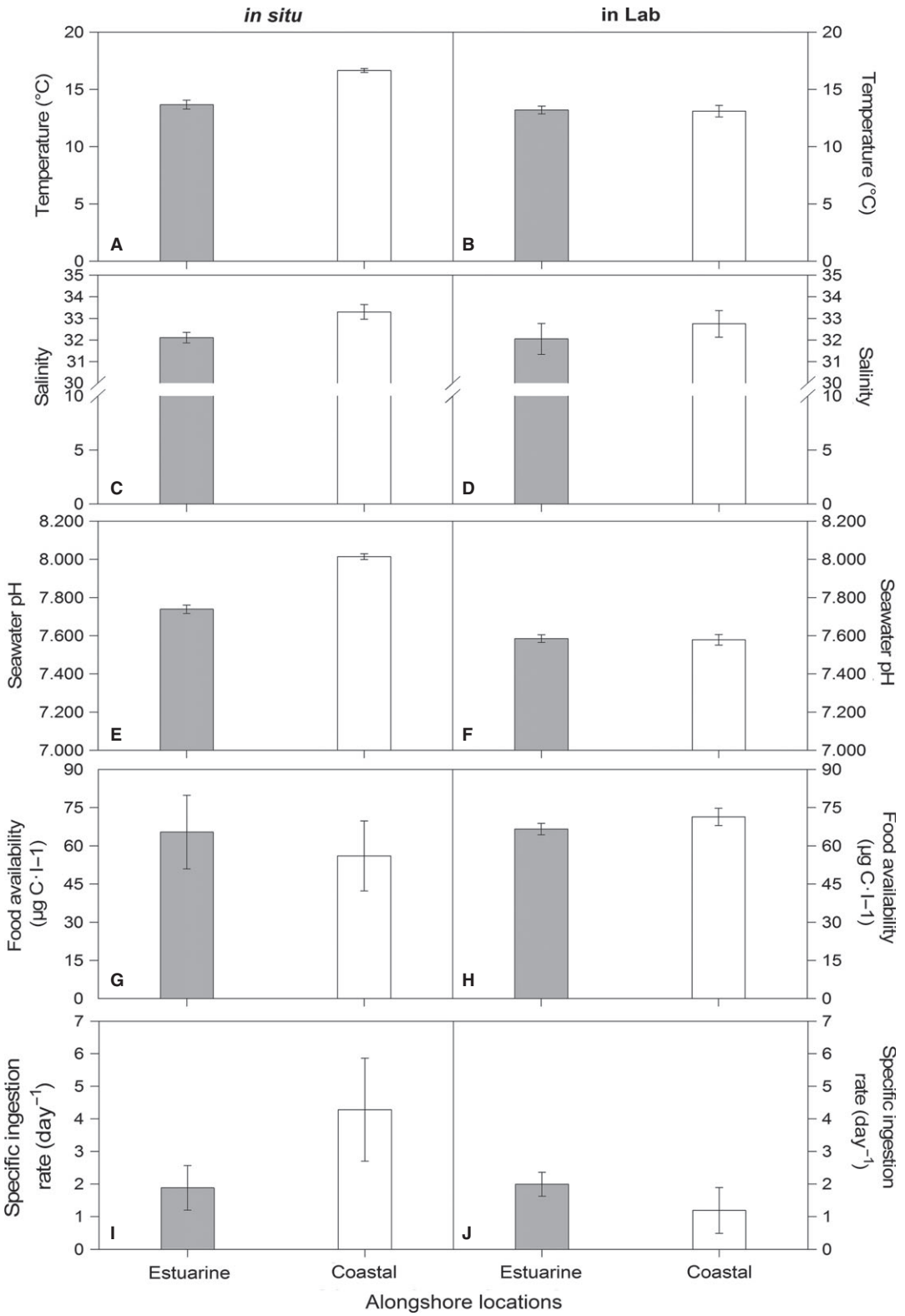

Fig. 1. Variations of environmental conditions and IRs of *Acartia tonsa* in the two alongshore locations of the study area: temperature (A–B), salinity (C–D), seawater pH (E–F), food availability (G–H) and specific ingestion rates (I–J). Left panel corresponds to *in situ* conditions whereas the right panel shows in Lab variations during experiments with high CO_2 conditions ($_PCO_2$ 1200 µatm). Vertical bars denote mean values (±SD).

Table 2. Mean values (±SD) of experimental factors during common garden experiments carried out during summer (1) and spring (2) seasons, conducted to evaluate ingestion rates of *Acartia tonsa* under high CO_2 levels (*c.* 1200 ppm CO_2). Below appear the statistical results of the Mann–Whitney *U* (M–W) test applied to verify variations of these conditions between experiments conducted with copepods from the two alongshore locations.

Location	Experiment	Temperature (°C)	Salinity	pCO_2 (µatm)	TA (µmol·kg^{-1})	Ω Ar
estuary	1	13.2 ± 0.5	32.1 ± 0.4	1255 ± 26	2270 ± 15	0.85 ± 0.03
	2	13.2 ± 0.2	32.0 ± 0.2	1256 ± 93	2243 ± 17	0.82 ± 0.04
coastal	1	13.0 ± 0.5	33.0 ± 0.4	1382 ± 39	2259 ± 31	0.77 ± 0.08
	2	13.2 ± 0.6	32.0 ± 0.2	1279 ± 20	2261 ± 41	0.82 ± 0.08
M–W test factor : location						
U		<1	1.2	2.2	<1	1.5
df		2, 18	2, 18	2, 18	2, 18	2, 18
P-value		>0.05	>0.05	>0.05	>0.05	>0.05

TA, total alkalinity.

during incubations was maintained at around 33 (Fig. 1D), whereas smaller variations in seawater pH (<0.01 pH units) occurred between incubations with estuarine (7.585 ± 0.02) and coastal copepods (7.578 ± 0.03) (Fig. 1F). Food availability for experiments with Estuarine and Coastal copepods, in terms of micro-phytoplankton biomass, was maintained at *c.* 70 µg C·l^{-1}, with variations between 67 ± 2 and 71 ± 3 µg C·l^{-1} respectively (Fig. 1H). The statistical evaluation of these variations indicated that experimental conditions were similar for copepods from both locations ($P > 0.05$; Table 2).

After 96 h acclimation to the above conditions, IR varied between 1.99 ± 0.4 and 1.19 ± 0.7 day^{-1} (Fig. 1J) and was significantly lower at the coastal location ($t = 1,24$; $P = 0.01$). Furthermore, statistical analysis of the IR anomaly, computed as the ratio between the laboratory and *in situ* IR (Fig. 2A), revealed that the feeding performance of coastal copepods was significantly lower under high CO_2 conditions ($t = 1, 24$; $P = 0.001$).

Testing pCO_2 effects on the expression of stress-related genes

The results of the RT-qPCR of the *ferritin* and *Hsp70* genes in *Acartia tonsa* individuals inhabiting the two different locations are shown in Fig. 2B. The relative gene expression of *ferritin* varied from 1.04 ± 0.4 to 3.27 ± 1.1 in the estuarine and coastal locations, respectively. Similarly, *Hsp70* gene expression increased from 1.09 ± 0.6 in the estuarine location to 2.31 ± 0.24 in the coastal one (Fig. 2B). A parametric ANOVA indicated that both *ferritin* ($F_{1,5} = 11.96$; $P = 0.026$) and *Hsp70* ($F_{1,5} = 12.09$; $P = 0.025$) relative gene expression values were significantly higher in coastal copepods.

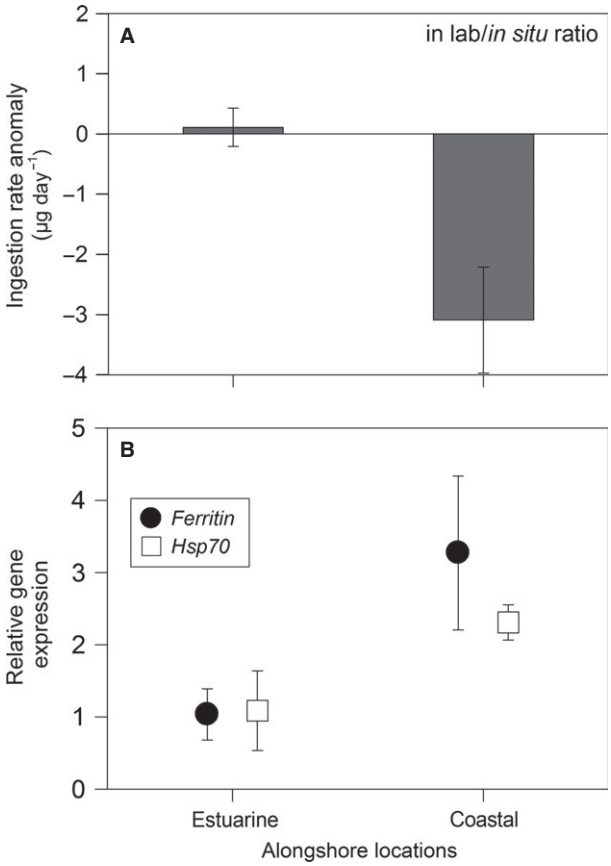

Fig. 2. (A) Ingestion rate (IR) anomalies of *Acartia tonsa* computed as differences between IRs in laboratory (high CO_2) and *in situ* conditions. (B) Reverse transcription-quantitative PCR of *ferritin* and *heat shock protein 70* (*Hsp70*) genes of *A. tonsa* adults from populations inhabiting two different alongshore locations. Gene expression is presented in units relative to the expression of the β-*actin* housekeeping gene.

Discussion

River's plumes occupy a significant proportion of the coastal edge at mid-latitudes, as is the case for this study area (Saldías *et al.* 2012), and whose freshwater footprint tends to dissipate as it moves away from the estuary. Thus, the physical and chemical conditions of seawater

change and generate variable, but overall contrasting, environmental conditions along the coast. In the case of this estuarine system in Southern Chile, this results in geographic differentiation over a local spatial scale, with more acidic, less saline and colder waters almost permanently at the inner estuarine location, especially behind the estuarine front at Corral Bay, giving rise to a plankton retention area (Vargas *et al.* 2003). This situation is confirmed by the results of the isotopic analysis, which revealed a stronger terrigenous signature in the estuarine copepods (Table 1A). This type of river-induced mesoscale (<200 km) perturbation of the physical–chemical conditions of seawater ultimately leads to important consequences for plankton dynamics in estuarine systems. In fact, these ecosystems act as plankton retention areas (Rogers 1940; Chen *et al.* 1997), determining much of the variability in morphology, physiology and life-history traits of local planktonic populations (Aguilera *et al.* 2013).

Along with spatial variations in salinity and pH, the local environmental setting also showed geographical differences in food availability (*i.e.* micro-phytoplankton biomass) which was higher in the estuarine location. Meanwhile, food composition, whose biomass was contributed up to 70% by CFD, NF <5 μm and NF >5 μm, was independent of sampling site (please see Fig. S1, Supporting Information). On the contrary, IR was lower in this location and was negatively associated with pH and with salinity (GLM, $r^2 = 0.5$). There seems to be a trade-off involving a depression in the feeding rates of copepods in an adequate nutritional environment, but under stressing conditions of pH and salinity (*i.e.* adaptive variability). The salinity results probably include residual effects of lower salinities experienced by copepods during ebb tides, which were also captured by statistical analysis. This is because subtle salinity variations observed during this flood-tide based sampling (Δ1.4) are smaller published short-term salinity reductions (Δ6) able to induce slower feeding activity in *Acartia tonsa* (Calliari *et al.* 2008). Furthermore, substantial differences of *c.* 0.3 pH units were observed between the coastal and estuarine locations, which, despite their transient nature, may equally represent sublethal stress for coastal grazers, given the logarithmic scale of pH and their potential interactions with salinity-induced osmotic balance (Henry & Wheatly 1992). Can this recent life history permeate the physiological rates and then modulate the copepod responses to sustained conditions of low pH/high CO_2 associated with OA?

To tackle this question, after *in situ* IR estimations, copepods were acclimated for 96 h under controlled environmental conditions in the laboratory. During this acclimation period, experimental conditions were similarly maintained for the incubations of copepods from both locations (Table 2), meaning that they experienced the same level of experimental stress and thus that any potential divergence in subsequent biological responses could have been driven by previous stimulus in the field (*i.e.* maternal effect or environmental history). After experimental acclimation, feeding responses were spatially divergent and estuarine copepods showed higher IRs than did coastal ones. Moreover, the anomalies in IRs, computed as the ratio between in laboratory and *in situ* IRs (Fig. 2A), revealed that the experimental feeding response of coastal copepods decreased dramatically compared with the *in situ* conditions ($t = 1, 24$; $P = 0.0001$). This depression in the feeding activity of coastal copepods may be associated with inactivity and/or dormancy, a behavior adopted by copepods under stressful or adverse conditions, in this case high CO_2/low pH. In this sense, McAllen *et al.* (1999) showed that the rocky littoral copepod *Tigriopus brevicornis* triggers a dormant stage under low oxygen conditions. Likewise, several metabolic rates of *A. tonsa* tend to be slower in response to both low oxygen levels and low temperature (Richmond *et al.* 2006). This type of metabolic depression may thus also impact upon feeding activity, leading to a depression in IR. As field measurements demonstrated that salinity and seawater pH were important drivers for *in situ* IR variability and as both locations were clearly different taking into account the estuary notably more acidic or corrosive. This recent exposure to low pH in the field could have allowed the estuarine copepods to tolerate further conditions of high CO_2/low pH better during the common garden experiment. Such rapid achievement of resistance has been observed in copepods facing drastic changes in salinity (Lee & Petersen 2002) or acidity conditions in freshwater environments (Kawecki & Ebert 2004), findings that highlight the role of maternal effects and adaptation potential (Räsänen *et al.* 2003; Baker *et al.* 2004; Sarnelle & Wilson 2005) in driving tolerance to the stress that we observed.

Although we did not directly measure dormancy stress response to experimental conditions, for example through estimations of oxygen consumption rates (Nielsen *et al.* 2007), a physiological process like this could explain the observed reductions in the feeding activity of *A. tonsa*. Indeed, gene expression of stress-related proteins does support our assumptions about divergent stress responses among populations. To the best of our knowledge, these are the first results reporting OA drivers (*i.e.* CO_2, pH) as a stressor for the expression of stress proteins in copepods (Lauritano *et al.* 2012), and they are also consistent with published values of stress-related gene expression in other calanoid copepods (Rhee *et al.* 2009; Nilsson *et al.* 2013). After mid-term incubations at high CO_2 levels the relative expression of both

the *ferritin* and *Hsp70* genes was higher in coastal than estuarine copepods (Fig. 2B). As for IR, maternal effects and environmental history probably account for much of the variability observed in copepod responses. Ferritin is induced at higher levels due either to increased levels of iron or reactive oxygen species, for example when invertebrates experience anoxia (Larade & Storey 2004). Therefore, it is possible that changes in the marine chemistry associated with OA may induce some oxidative stress that is able to damage macromolecules in cells. By contrast, Hsp70, which is induced due to a broad range of stressors (Lauritano *et al.* 2012), including pH stress (see Lardies *et al.* 2014), acts as a molecular chaperone to enable the proper folding of a wide range of cellular proteins (Taipale *et al.* 2010). In terms of bioenergetic budgets, our findings indicate critical consequences under OA scenarios, because, contrary to dormant-stress responses; synthesis of stress proteins implies a direct energetic cost. We previously showed that not only IR but especially egg production rates of *A. tonsa* showed an inverse negative relationship with seawater pH ($r^2 = 0.6$, $P < 0.05$; Aguilera *et al.* 2013). In that study, small brood sizes and reduced egg production rates were observed in the more acidic estuary. Those findings can now be more clearly understood as stressful conditions associated with OA drivers may compromise biological performance of copepods not only through dormancy-induced reductions in feeding activity, but also through the redirection of energy towards the expression of stress-related genes and proteins, which is highly energetically costly (see Lardies *et al.* 2014). As a consequence, other metabolic energy-dependent processes such as growth and reproduction also experience a decrease (Richmond *et al.* 2006).

Genetic differentiation and/or phenotypic flexibility allow geographically widespread species to cope with environmental differences among habitats. Information concerning geographic variations in response to OA is critical because many life-history traits show variations across space (Aguilera *et al.* 2013; Vargas *et al.* 2013; Lardies *et al.* 2014). As we have confirmed in this study, adaptation to local fluctuations in seawater pH seems to exert a significant role upon the response of planktonic populations to conditions associated with OA. This may be a key feature of the coastal marine biota to face drivers of climate change, a question that should be deeply examined as a tool for environmental mitigation policies in coastal management arrangements (Duarte *et al.* 2013). Further studies considering for instance, geographical gradients and transgenerational approaches are thus necessary in order to understand better the biological consequences of different OA drivers on marine physical–chemical conditions.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (A) Specific IR of *A. tonsa* on (B) biomass of six functional groups contributing to food availability under *in situ* conditions: chain forming (CFD), pinnate (PD), and centric diatoms (CD), dinoflagellates (DIN), and small and large nanoflagellates (<5 and >5 μm NF, respectively).