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Differential response to ocean acidification in physiological traits of *Concholepas concholepas* populations

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ABSTRACT

Phenotypic adaptation to environmental fluctuations frequently occurs by preexisting plasticity and its role as a major component of variation in physiological diversity is being widely recognized. Few studies have considered the change in phenotypic flexibility among geographic populations in marine calcifiers to ocean acidification projections, despite the fact that this type of study provides understanding about how the organism may respond to this chemical change in the ocean. We examined the geographic variation in CO_2 seawater concentrations in the phenotype and in the reaction norm of physiological traits using a laboratory mesocosm approach with short-term acclimation in two contrasting populations (Antofagasta and Calfuco) of the intertidal snail *Concholepas concholepas*. Our results show that elevated pCO_2 conditions increase standard metabolic rates in both populations of the snail juveniles, likely due to the higher energy cost of homeostasis. Juveniles of *C. concholepas* in the Calfuco (southern) population showed a lower increment of metabolic rate in high-pCO_2 environments concordant with a lesser gene expression of a heat shock protein with respect to the Antofagasta (northern) population. Combined these results indicate a negative effect of ocean acidification on whole-organism functioning of *C. concholepas*. Finally, the significant Population × pCO_2 level interaction in both studied traits indicates that there is variation between populations in response to high-pCO_2 conditions.

1. Introduction

Phenotypic acclimatization to short-term environmental fluctuations should frequently occur by preexisting plasticity and underlying standing genetic variation (see Lande, 2009). Phenotypic flexibility is a particular kind of phenotypic plasticity and is defined as the possible selective advantage of those individuals that can show continuous but reversible changes in behavior, physiology and morphology in response to rapidly changing environmental conditions in timescales shorter than a lifetime (Piersma and Drent, 2003). Paleoclimate data provided evidence that wild populations rarely experienced such huge and rapid changes in environmental variables such as temperature, salinity or pH (Caldeira and Wickett, 2005; Hönisch et al., 2012). For instance, paleoclimate evidence about global warming and ocean acidification (OA) event over the past 300 MY suggest similarities with contemporaneous extinction and evolutionary turnover. However, none of these past events parallels the rapidity of the current increase of CO_2 release and chemical changes in seawater (Hönisch et al., 2012). Thus, rapid evolution of phenotypic plasticity may be necessary to prevent extinction of species subjected to sudden environmental changes such as the present event of anthropogenic OA. This may be especially threatening to calcifying organisms, given that effects on calcification may delay developmental rates at critical early life stages and could restrict opportunities in many species for larval dispersal and/or changes in geographical range (Fabry et al., 2008; Kroeker et al., 2010).

Many morphological, life-history, and metabolic traits show signs of phenotypic flexibility (Lardies et al., 2011; Pigliucci and Preston, 2004). Furthermore, geographic variations in life-history and metabolic traits among populations are ubiquitous among ectotherms (see Gilchrist and Huey, 2004; Lardies et al., 2011). Physiological variation within the life history of an individual can have profound implications for fitness (Lardies and Bozinovic, 2006; Ricklefs and Wikelski, 2002), since physiological maintenance costs are a large component of animal

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energy budgets (Angilletta, 2009; Sibly and Calow, 1986). Different effects of the environment on the phenotype of different populations result in variation in reaction norms and a significant population (genotype) × environment interaction (Pigliucci, 2005). Genotype × environment interactions are the type of genetic variation required for the evolution of phenotypic flexibility (Lardies and Bozinovic, 2008; Via and Lande, 1985). The significance of phenotypic plasticity as a major component of geographic variation of biological responses among populations is now being widely recognized, as a consequence, it became one of the main topics in physiological studies (e.g. Angilletta, 2009; Piersma and Gils, 2011) and are particularly relevant in the perspective of elucidating how physiological differences among organisms may affect their responses to ocean acidification, with cascading ecological consequences (Chown and Gaston, 2008; Helmuth et al., 2005). Unfortunately, few studies have considered the variations in phenotypic flexibility among geographic populations of marine calcifying organisms in response to OA.

It is believed that OA levels projected for the near future will have an impact on every level of organization of biological systems: from molecular to ecosystemic (Fabry et al., 2008; Byrne, 2011). One of the molecular responses that are activated in a cell under temperature or acidification stress is the heat shock proteins response (HsP), an event of genetic activation that occurs in the cells in response to abnormal stressful temperatures or acidosis (Cummings et al., 2011; Hofmann, 2005). The genes that encode for Heat-Shock Proteins (HsPs) are highly conserved and have been found in all studied species (Feder and Hofmann, 1999). Among HsP families the group in the 70-kDa size range (HsP70) is the most extensively studied because of its prominent response to stresses (see reviews in Feder and Hofmann, 1999). The phenotypic expression of these protein patterns could explain not only differences in fitness but also the geographical distribution of the organisms (Hofmann and Todgham, 2010; Sorte and Hofmann, 2005). Recent studies in natural systems have shown that the patterns of expression in HsPs exhibit phenotypic flexibility related to the thermal history (Arias et al., 2011; Hammond and Hofmann, 2010). However, the patterns of expression of HsPs to different natural scenarios of pCO_2 are practically unknown, only recent evidence showed that sea urchin larvae reared under elevated CO_2 displayed compromised expression of HsP70 (see O'Donnell et al., 2009). Furthermore, the synthesis, degradation and replacement of these proteins imply an increase in energetic costs to the organism (Hartl and Hayer-Hartl, 2002; Sorensen and Loeschcke, 2002). Therefore, the pCO_2 levels experienced by different populations, in their respective marine habitats, could condition trade-offs between traits or constraint of HsP expressions. Furthermore, phenotypic plasticity during development may provide a temporary response from this stress or there is a potential for further adaptation to changing ocean chemistry (Hofmann and Todgham, 2010).

In this study, we examined the effects of pCO_2 variation on metabolic rate and in the expression of a gene belonging to the HSP70 family in a marine snail, the carnivorous gastropod *C. concholepas* (Brugière 1789), which is an economically and ecologically important component of the rocky intertidal and sub-tidal communities along the Chilean coast (Castilla, 1999). *C. concholepas*, has a shell with variable proportion of carbonate phases along the ontogeny: in juvenile stages calcite dominate (ca. 75%) over aragonite (ca. 25%), and the proportion of aragonite increased larval and post-settlement stages (Ramajo et al., 2013). Due to the broad geographic distribution of *C. concholepas*, it has been subjected to use in ecological and evolutionary research (i.e. Cardenas et al., 2009; Manríquez et al., 2009, 2012; Poulin et al., 2002). However, we are not aware of any study examining physiological trait plasticity among populations under projected pCO_2 scenarios. Our study had the following two objectives: (1) to evaluate, experimentally, the effect of different pCO_2 concentrations in seawater on oxygen consumption and expression of a stress related gene in juveniles of *C. concholepas*; and (2) to evaluate geographic variation in environmental variables (carbonate system parameters) as a source of variation in phenotype (phenotypic flexibility) and in the reaction norm.

2. Material & methods

2.1. Study sites and animals

Two regions along the coast of Chile were selected with inherent differences in physical and chemical properties of seawater (Fig. 1a). In Chile, sea surface temperature decreases from north to south with clear seasonal variability between regions: (i) Northern (23°38′S), has shown an annual average mean temperature of 17.03 °C ± 1.23 SD (Lagos et al., 2008), while (ii) Southern (39°42′S) has shown an average mean temperature of 11.2 °C ± 1.10 SD (Manríquez et al., 2012). Fluctuations in sea surface temperature (SST) along the Chilean coast are driven by wind-induced upwelling of cold waters (Strub et al., 1998; Fig. 1b). In addition, the input of river discharge into the coastal ocean also promotes a strong gradient along the coast (see Davila et al., 2002; Fig. 1c), characterized by an absolute lack of river discharge into Antofagasta Bay and surrounding areas in northern Chile; while the southern region (Valdivia), has high river discharges into the Chilean coastal ocean (Fig. 1c). Because river discharges represent one of the most important sources of alkalinity and other chemical elements into coastal waters (Bakker et al., 1996; Ternon et al., 2000), these differences may be reflected in chemical properties of seawater influencing the study regions (see Table 1). In addition to the SST latitudinal gradient and input of freshwater along the Chilean coast, there is large variation in carbonate system parameters both at the local as regional and biogeographical scales (Mayol et al., 2012; Torres et al., 2011). Particularly, there is a step (abrupt) gradient in air–sea CO_2 fluxes, changing from CO_2-outgassing in coastal upwelling areas (20°S–37°S) to CO_2-sink in non–upwelling areas (37°S–42°S) (Torres et al., 2011). As such, our study sites encompass the major transitions in SST, river input and seawater pCO_2 along the Chilean coast, thus providing an adequate environmental template to test for responses of CO_2 sensitive traits on marine invertebrates inhabiting these shores. In this study, we compiled a published data in order to provide a picture of chemical variations of seawater in northern Chile (Table 1). In general, the area encompassing from 22 to 24°S showed pCO_2 ranges from 237 to 637 μatm, with pH levels in the surface waters very close to 8.0 during the upwelling season (Torres et al., 2002). However, inside the Antofagasta bay, where the individuals for the study were collected, pH@25 °C levels ranges from 8.0 to 8.1, with CO_2 fugacity levels of 300, which suggest a seawater pCO_2 of ca. 650 μatm (assuming an atmospheric $CO_2 \approx 352$, Torres et al., 2002). For southern Chile (Calfuco) as part of an ongoing research program about carbonate systems variability monitoring, we performed discrete daily samples of temperature (resolution = 0.0006 °C) and salinity (conductivity resolution = 0.001 mS/cm) using an Idronaut CTDO (Model Ocean Seven 304 CTD Logger) placed in shallow subtidal from January 2011 to early 2012 (Torres et al., 2013). Simultaneous water samples were collected to determine pH variations trough the potentiometric method (total scale) with using a Metrohm 713 pH meter connected to a combined electrode (double juncture), calibrated using buffers Tris (pH = 8.089) and 2-aminopiridine (pH = 6.786) at 25 °C using a temperature controlled water bath. For Total Alkalinity (A_T) samples were collected using borosilicate glass bottles (Corning 500-mL) from the shallow subtidal and alkalinity was measured using automated potentiometric titration (Haraldsson et al., 1997) calibrated with certified Dickson alkalinity standards. The resulting pCO_2 and saturation states for calcite (Ω_c) and aragonite (Ω_a) were estimated from measured values of temperature, salinity, pH and A_T using a CO_2 SYS software (Pierrot et al., 2006). Thus, both compiled chemical data for northern Chile as the discrete time series data for Southern Chile were processed following the same protocols (see Torres et al., 2002, 2011; Torres et al., 2013).

Juveniles (0.5 to 1.5 cm of peristomal length) of *C. concholepas* were collected at low tides from natural stand from rocky shores at southern (Calfuco; n = 23), and northern (Antofagasta; n = 23) Chile during the austral spring (December) of 2010 (Fig. 1a). Individuals were

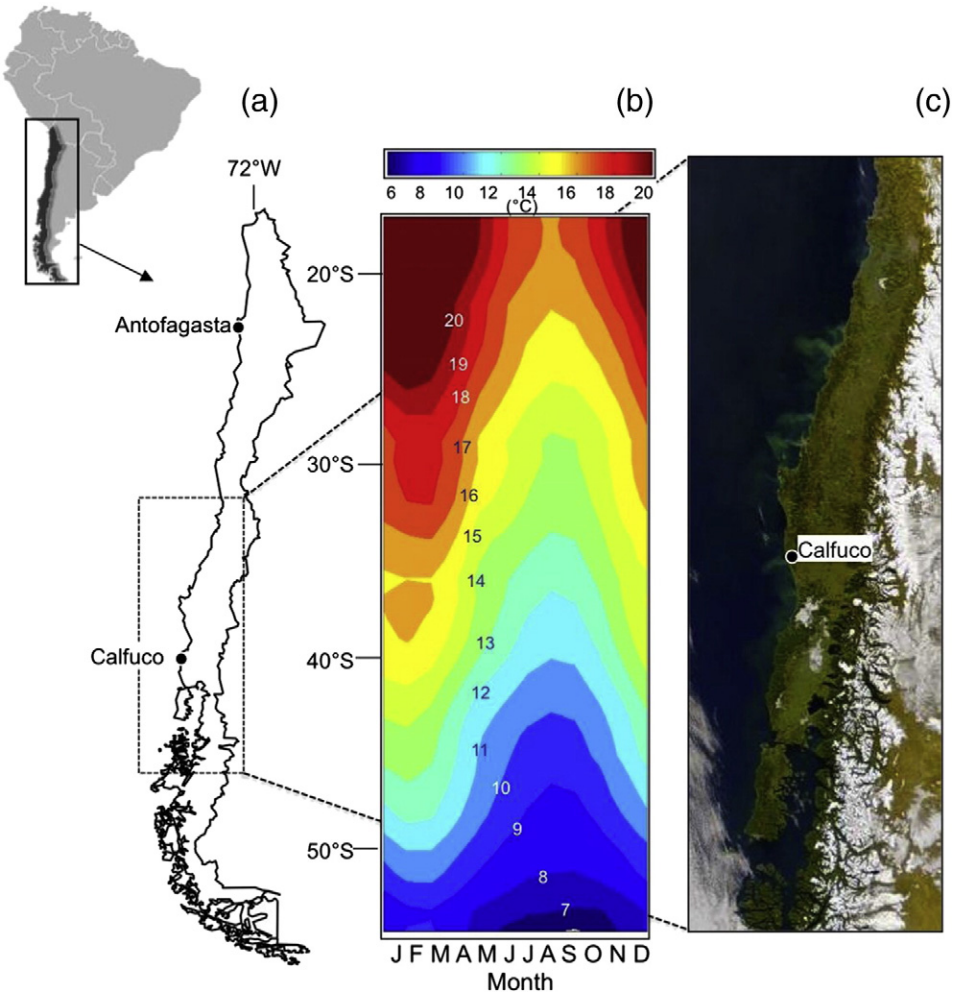

Fig. 1. Location of the study sites at the Northern (Antofagasta) and Southern (Calfuco) Chile coasts (a); Gradient in sea surface temperature (SST) along the Chilean coast; numbers indicate temperature isolines (historical dataset available at http://www.podacc.gov) (b); Plumes of river outflow along the central and southern Chile coasts visible by SeaWiFS image central Chile (image provided by the SeaWiFS Project. NASA/Goddard Space Flight Center and Orbimage; http://eoimages.gsfc.nasa.gov/images).

transported to the laboratory and after 12 days of acclimation (14 °C and 12 L:12D for temperature and photoperiod, respectively) in running seawater at natural pCO_2 level (approximately 380 ppm of CO_2). *C. concholepas* were fed during the acclimation using fresh small mussels (*Perumytilus purpuratus*) collected from field.

2.2. pCO_2 sea-water equilibration and laboratory experimental mesocosms

Juveniles of *C. concholepas* from both populations were marked and exposed for 72 h to seawater bubbled with technical grade air–CO_2 gas mixtures representing two nominal levels of CO_2 (natural = 380 ppm, and high-pCO_2 = 1500 ppm). A sample of 23 individuals of *C. concholepas* from both populations was incubated, 15 individuals for metabolic measurements (i.e. to construct individual reaction norm) and 8 individuals for HsP70 analyses in natural pCO_2 level (which were sacrificed after first incubation). First, individuals of both populations were maintained simultaneously for 72 h in natural levels of CO_2. After acclimation in natural pCO_2 conditions, snails were newly fed with *P. purpuratus* by 48 h and starved 24 h (i.e. 72 h) in natural levels of CO_2. Then snails from both populations were exposed to the increased pCO_2 level during the next 72 h. Oxygen consumption measurements for 15 juvenile snails were recorded in each pCO_2 level after 72 h incubation and HsP70-like gene expression was realized after metabolic measurements in each treatment. Each individual was maintained separately in 0.5 L plastic exposure containers, which were filled with the appropriate (either natural or high pCO_2) preconditioned seawater. During the 72 h of incubation, the seawater in each of the aquaria was replaced daily with fresh UV and filtered

Table 1
Latitudinal coordinates of the study sites, historical[a] averages of Sea Surface Temperatures (SST), and summary (mean ± 1 SD) of carbonate systems parameters measured in situ; pCO_2 = CO_2 partial pressure in seawater, Ω = saturation state for calcite and aragonite. ND = no data available.

Region	Site	Latitude (south)	SST (°C) historical[a]	SST (°C) in situ	Salinity (psu)	pH@25 °C	Total alkalinity A_T (meq × kg^{-1})	pCO_2 (ppm)	$\Omega_{calcite}$	$\Omega_{aragonite}$
Northern	Antofagasta	23°31′	18.70 ± 0.27	18.73[e]	34.53[e]	8.0–8.1[b]	2275–2325[b,c]	237–637[b,c]	4.7[e]	3.1[e]
Southern	Calfuco	39°46′	13.60 ± 0.10	13.09	32.80	7.8–8.2[d]	1750–2300[d]	200–750[e]	ND	1.0–3.7[e]

[a] Available at www.podacc.gov.
[b] Data from Torres et al., 2002.
[c] Data from Torres et al., 2011.
[d] Data from Torres et al., 2013.
[e] Data from Ramajo et al., submitted.

seawater which has been conditioned to the appropriate pCO_2 level. Gases mixtures were specially prepared by INDURA Chile (www.indura.cl). The gas tubes were properly connected to CO_2-manometer to adjust the level of CO_2-bubbling into a 170 L header tank containing natural seawater pumped directly from the surface waters (ca. 1 m in depth) at Calfuco Marine Lab (39°42′S, Valdivia, Southern Chile). The resulting levels of pCO_2 and carbonate system parameters are shown in Table 2. Inside the head tank three water samples were taken per day and analyzed for pH, alkalinity determination and additional carbonate system parameters as described previously. In general, the high-CO_2 condition showed the expected increase in pCO_2, decreased pH and undersaturated conditions for aragonite saturation state ($\Omega < 1$). Since the proportion of aragonite in the shell carbonates of the juvenile of *C. concholepas* is ca. 43% (Ramajo et al., 2013), the experimental scenarios include conditions for shell dissolution and physiological stress upon *C. concholepas*.

2.3. Metabolic measurements

Oxygen consumption (O_2 mg $\times$ L^{-1}) was measured at a constant temperature of 14 ± 1 °C which is common during later spring and observed within the latitudinal range of the species (Thiel et al. 2007). Metabolic rate was measure individually in an acrylic respirometric chamber for 15 juvenile snails of each population after incubation in each pCO_2 treatment. Seawater used for metabolism measurement was UV and filtered seawater equilibrated to natural and high pCO_2, respectively. We used an optic fiber oxygen-meter (Microx TX3, PreSens, Germany), with diameter tips of 20–50 um. Zero calibration was performed using a Na_2O_3S solution (0% saturation) and 100% was calibrated using air-bubbled seawater. Temperature was stabilized using a temperature-controlled waterbath (JioTech, Co). Then, 24 h before measurements, individuals were maintained under starvation in UV and filtered seawater. Individual measurements lasted for at least 60 min per individual, eliminating the first five minutes to remove the possibility of potential stress of manipulation.

2.4. HsP70-like expression analysis

After incubation period, 8 individuals exposed to natural level of pCO_2 of each population were used for HsP70-like analyses. Similarly, at end of incubation in high level of pCO_2, the tissues of 8 snails of each population were utilized for molecular response analyses. RNA total was extracted from 50 mg of pedal muscle of the juvenile *C. concholepas* using the TRIzol® Reagent (Invitrogen™, USA) method following the manufacturer's instructions. For cDNA synthesis, 1 μg of total RNA treated with DNAse I (RQ1, Promega, USA) was reverse transcribed with random hexamer primers using the Improm II reverse transcriptase (Promega, USA), according to the manufacturer's instructions. Real time (RT)-PCR was performed using the SYBR Green Master Mix (Quantace Co.) and the Gene-Mx3000P® detection system (Stratagene Co.) as described by Arias et al. (2011). Briefly, the PCR mixture (25 μl) contained 2.0 μl of 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, 30 s; 55 or 56 °C, 30 s; and 72 °C, 40 s, followed by a melting cycle from 55 °C to 95 °C. For the amplification of a HsP70 family related gene (HsP70-like) primers used were: Hsp70 F: 5′GTGGGGGTTTTCCAGCAT3′ and Hsp70 R: 5′ CGATGAGACGCTCGGTGT3′ (Tm: 55 °C; amplicon of 102 nt). For 18S fragment amplification primers were: 18S F: 5′CCCGTCGCTACTAC CGATTG3′ and 18S R: 5′TGGTCATCTTCCCAGCAACAT3′ (Tm: 56 °C; amplicon of 94 nt). Reaction specificities were tested with melt gradient dissociation curves and electrophoresis gels of each PCR product. Also, PCR products were cloned and sequenced and used for BLAST confirmation of specificity. All experiments were performed with 8 biological (per treatment) and two technical replicates. Relative gene expression calculations were conducted as described by Arias et al. (2011), an accurate ratio between the expression of the gene of interest (GOI: Hsp70-like) and the housekeeping (HK: 18S) gene was calculated according to equation: $2^{-(\Delta CtGOI\text{-}HK)}$. Then, gene expression levels were normalized to the average value in individuals of normal pCO_2.

Table 2
Carbonate Systems parameter (mean ± SD); recorded in natural and CO_2-equilibrated seawater used in incubation in Calfuco (southern Chile). Four replicated samples were collected at the start, 14 and 45 h of incubation.

Carbonate system parameters	Natural seawater (380 ppm of CO_2)	CO_2 equilibrated seawater (1500 ppm of CO_2)
pCO2 at in situ temperature (μatm)	382.02 ± 14.583	1539.3 ± 238.34
pH at in situ temperature	8.050 ± 0.007	7.506 ± 0.064
pH@25 °C	7.879 ± 0.003	7.368 ± 0.059
Total alkalinity (mmol/kg seawater)	2191.94 ± 42.507	2222.7 ± 1.895
Ω Calcite at in situ temperature	3.360 ± 0.101	1.123 ± 0.155
Ω Aragonite at in situ temperature	2.143 ± 0.068	0.717 ± 0.099

2.5. Statistical analyses

The population sources $\times$ pCO_2 levels (hereafter P $\times$ pCO_2) interaction in the metabolic rate was assessed as follows: the interaction term was computed to determine variation in P $\times$ pCO_2in response to normal or high pCO_2 levels, according to the population upon which natural selection could operate (Via and Lande, 1985). Interaction terms were also computed for metabolism and *HsP*70-like gene expression. A mixed-model two-way analysis of variance (ANCOVA) (Fry, 1992) was performed to determine the effects of source population and pCO_2 level on mass-specific metabolism, using body mass as covariate. Prior to all statistical analyses, data were examined for assumptions of normality and homogeneity of variance using Kolmogorov–Smirnov and Levene tests, respectively. All statistical analyses were conducted using a SPSS 12.0 software (Apache Software Foundation, Somers, New York), where a level $p < 0.05$ was used to reject the null hypothesis. Data are presented as mean ± standard error of the mean (S.E.M.). The independent variables were (1) source population, designated as a fixed factor (represent extreme populations along the environmental gradient), and (2) pCO_2 level designated as the fixed factor (levels are predetermined). The P $\times$ pCO_2 interaction term was also designated as a random factor. A two-way mixed model ANOVA was used to test the effect of pCO_2 levels and source population (i.e. factors) in *HsP*70-like qPCR (i.e. response variable).

3. Results

3.1. Metabolic rate

Oxygen consumption was higher in the high-pCO_2 condition but also was affected significantly by the population source (Fig. 2 and Table 3). A significant P $\times$ pCO_2 interaction contributed to the observed variance for metabolic rate (Fig. 2) (Two-way ANCOVA, $F_{1,52} = 4.20$; $P = 0.046$). This interaction was evident from the intersecting reaction norms of the analyzed trait (Fig. 2). Mass-specific metabolic rate in juveniles of *C. concholepas* was significantly lower in the Calfuco population than in Antofagasta population in the high-pCO_2 level (0.42 ± 0.16 and 0.72 ± 0.23 mg O_2 L h^{-1} g^{-1}, respectively). Nevertheless, metabolic rate between populations was similar in natural pCO_2 levels (0.17 ± 0.030 and 0.23 ± 0.045 mg O_2 L h^{-1} g^{-1} for Calfuco and Antofagasta, respectively). A post hoc comparison evidenced that individuals of *C. concholepas* from the two studied population has similar metabolic rate under natural conditions (Scheffe test; DF = 52; Error Between MS = 1137.3; $P = 0.998$), all the remaining pairwise comparison between population and across pCO_2 levels revealed significant differences in metabolism (Scheffe test, $P < 0.030$).

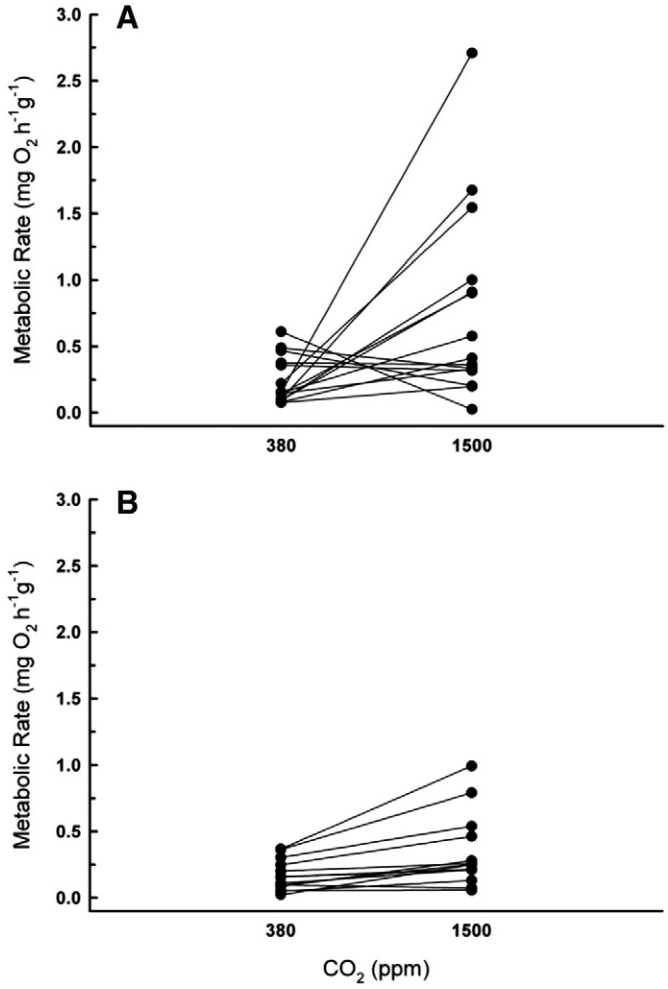

Fig. 2. Reaction norms plots for metabolic rates of juvenile individuals of *Concholepas concholepas* collected from the northern (Antofagasta) (A) and southern (Calfuco) (B) regions. Individuals were reared first for 72 h at below normal (380 ppm) and then for high levels of pCO_2 (1500 ppm). Each line connects an individual measured in both pCO_2 conditions. The slope of each reaction norm is proportional to the individual's phenotypic plasticity in the studied trait.

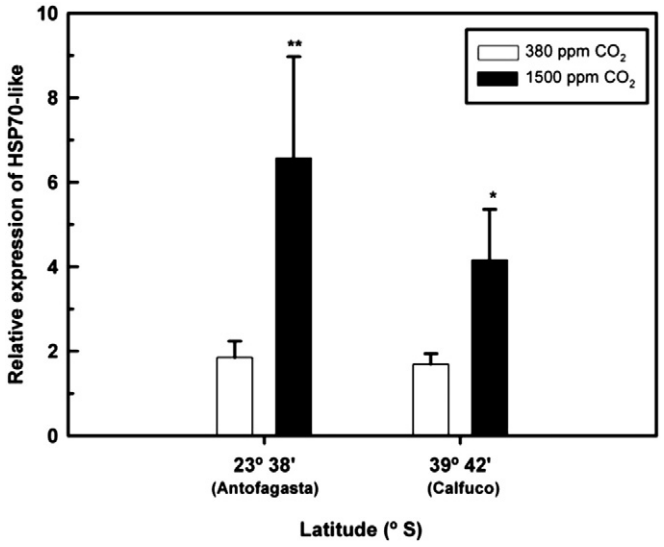

Fig. 3. quantitative Real-Time PCR of *HsP70*-like gene (Mean ± SD) recorded in northern and southern populations of juveniles of *Concholepas concholepas* incubated for 72 h at natural and increased pCO_2 levels. (*) Represents significant differences between pCO_2 treatments and (**) shows significant differences between localities (mean ± S.E.M.) in *HsP*70-like/18 s expression.

3.2. HsP70-like gene expression

The relative expression the *HsP70*-like transcripts in the juveniles of the *C. concholepas* in the natural pCO_2 levels were of 1.85 ± 0.39 and 1.69 ± 0.25 for Antofagasta and Calfuco, respectively (Fig. 3). In the case of high-pCO_2 condition this relation was 6.57 ± 2.4 and 4.16 ± 1.20 for Antofagasta and Calfuco populations, respectively. A two-way ANOVA to evaluate differences in *HsP70*-like expression considering as factors, population and CO_2 levels, showed a significant a significant effect of the interaction term ($F_{1,25} = 10.21$; $P = 0.015$) and the main factors (two-way ANOVA; $F_{1,25} = 49.06$, $P < 0.0001$ and $F_{1,25} = 3.98$, $P = 0.048$ for population and pCO_2 levels, respectively) (see Fig. 3 and Table 3). A post hoc comparison evidenced that individuals of *C. concholepas* from the two studied population has similar *Hsp70*-like expression under natural conditions (Scheffe test; DF = 25; Error Between MS = 568.8; $P = 0.915$), all the remaining pairwise comparison between population and across pCO_2 levels revealed significant differences in *HsP70*-like expression (Scheffe test, $P < 0.022$).

4. Discussion

Our results show that elevated pCO_2 concentrations in seawater resulted in higher standard metabolic rates and *HsP70* family related gene expression in juvenile snails, likely due to the higher energy cost of homeostasis. In general terms, the observed reaction norm in *C. concholepas* metabolism when exposed to increased pCO2 levels were steep for the Antofagasta (northern) population and almost flat for Calfuco (southern) population. Since the deviation from the flat line parallel to the environmental axis (i.e., pCO_2) represent the degree of phenotypic plasticity our results indicate an increased ability of *C. concholepas* juveniles inhabiting in northern Chile for plastic adjustment of their metabolism to increased pCO_2 levels in seawater. However, this pattern of reaction norms was rather complex in both studied populations, with some individuals evidencing increased, decreased or stable metabolic response across pCO_2 conditions. Crossed-reaction norms indicate that there is standing genetic variation for plasticity in metabolic rate within and between populations, as corroborated by the significant Population × pCO_2 level interaction in factorial analyses.

Table 3
Concholepas concholepas. Summary of two-way ANCOVA and two-way ANOVA for comparison between populations (Antofagasta and Calfuco) and pCO_2 treatments (natural and high-pCO_2 level) in metabolic rate and HsP70-like expression, respectively.

Response	Effect	Degrees of freedom	MS	F	p
Metabolic rate	Population	1	5926.0	5.2104	0.0266
	pCO_2 level	1	57,838.8	50.854	0.0000
	Population × pCO_2 level	1	4772.1	4.196	0.0456
	Error	52	1137.3		
HsP70-like expression	Population	1	47,218.3	49.062	0.0001
	pCO_2 level	1	3748.9	3.984	0.0048
	Population × pCO_2 level	1	9622.4	10.212	0.0154
	Error	25	948.1		

Similar conclusion can be expanded for the reaction norm in *HsP70*-like expression of *C. concholepas*, also indicating the presence of populational variation in plasticity in response to high-pCO_2 levels. Similar results have been reported in an oyster (Beniash et al., 2010), a brittlestars (Christensen et al., 2011; Wood et al., 2008), Antarctic bivalves (Cummings et al., 2011) and sea urchin larvae (Stumpp et al., 2011) (but see Form and Riebesell, 2012). Juvenile snails collected from northern Chile showed increased metabolism (until 4× in average) and *HsP*70-like gene expression when exposed to high-pCO_2 conditions. These results indicate a negative effect of ocean acidification (i.e. stress) on whole-organism functioning of *C. concholepas* over relatively short terms (days) that may be energetically difficult to maintain over longer time periods.

Environmental and population sources tended to affect metabolic rate to a similar extent (Byrne, 2011; Niewiarowski, 2001). The significant population × pCO_2 levels interaction in both studied traits suggest that there is populational variation (as representing of unknown standing genetic variation) for metabolic flexibility in response to increased pCO_2 levels in seawater. This observation was likely due to phenotypic flexibility in individuals collected at Calfuco, an important estuarine area in southern Chile. Most river plumes are acidic relative to the receiving ocean, and river water is mixed extensively over the continental shelf (Salisbury et al., 2008). Furthermore, it has recently been demonstrated that most ocean and coastal sites are indeed characterized by natural variation in seawater chemistry which is demonstrating that in some particular habitats, resident organisms are already experiencing pH regimes projected for the year 2100 (Hofmann et al., 2011). This can be the case of the Calfuco site due to continuous river discharges (see Table 1).

An adjustment in metabolic rate and *HsP*70-like expression to the present pCO_2 conditions could be of particular importance for the estuarine populations of *C. concholepas* inhabiting the more acidic habitat (i.e. Calfuco), since these populations showed the lowest degree of plasticity (i.e., almost flat reaction norms in the individual responses measured at normal and high-pCO_2 levels) We agree with the suggestions that the degree of phenotypic flexibility may vary among populations associated to the presence of geographical gradients in environmental parameters (Bronikowski and Arnold, 1999; Lardies et al., 2011). Thus, the population of *C. concholepas* inhabiting a more variable pH habitat (i.e. Calfuco) showed a less plastic response due probably to acclimatization to this environmental variable (see Fig. 2).

The rationale to explain the above results is that; 1) theoretically, the costs of maintenance in ectotherms (i.e. standard metabolic rate) may have important effects on the quantity of energy available for activity and reproduction (Angilletta, 2009; Sibly and Calow, 1986), which is lower in Calfuco population, and 2) the synthesis, degradation and replacement of these proteins also imply an increase in energetic costs of the organisms (Hartl and Hayer-Hartl, 2002; Sorensen and Loeschcke, 2002) which may increase in the high-pCO_2 condition (Cummings et al., 2011 and this study). Therefore, the higher energetic costs in snails of the Antofagasta population exposed to high-pCO_2 in seawater may not cope with a long-term increase in environmental pCO_2 resulting in a lower fitness in individuals. Implicit in the covariation between metabolic rate and HsP70 gene expression is that between-population variations could result in higher fitness in individuals of different populations when exposed to stress condition (i.e., high pCO_2). In summary, these results suggest that *C. concholepas* occurring in the Calfuco area might be pre-adapted to natural pH variability in its habitat. This result is in agreement with Manríquez et al. (2013) report that juveniles of *C. concholepas* collected from Calfuco showed a slight but nonsignificant increase in metabolic rates as increased pCO_2 levels in seawater. A similar result was reported recently in the metabolic rate and energy budget of the sea urchin *Strongylocentrotus droebachiensis* in response to low pH conditions due to pCO_2 increment in seawater (Stumpp et al., 2011). However, pCO_2 variation is only one of the processes that affect estuaries and coastal pH (Waldbusser et al., 2013), the eutrophication and respiration by heterotrophic organisms also increase acidity levels (Duarte et al., 2013), simultaneous inputs of fresh water in the basins (Salisbury et al., 2008) and deposition of acidic compounds such as sulfur and nitrogen (Amaral et al., 2012) modify the pH of the coastal zones. In addition, to the role of these environmental factors driving variation in pH levels in the coastal ocean, other uncontrolled local stressors that could be present only in Calfuco waters (southern Chile) may also promote the increased variability in the responses of individuals translocated from Antofagasta. Whether these circumstances apply to our experiment require further study but, in general, the best performance of *C. concholepas* individuals collected in Calfuco may also fit the local vs. foreign criterion that is used as evidence of local adaption (Kawecki and Ebert, 2004; Kelly et al., 2011).

Metabolic rate and *HsP*70-like gene expression in *C. concholepas* from Antofagasta were significantly plastic in response to both pCO_2 conditions, whereas the same traits were less plastic in southern population (i.e. Calfuco). Thus, geographical variation in the degree of plasticity for these traits has been demonstrated within a single species. It is important to note that we cannot determine whether phenotypic flexibility is absent in the Calfuco population, since it is possible that experimental conditions of lower or higher CO_2 could produce significant plasticity. Furthermore, there are many potential environmental factors that could differ between studied populations apart of water chemistry, for example sea surface temperature. Nevertheless, early juveniles may be vulnerable to skeletal dissolution because *C. concholepas* precipitate a significant proportion (43%) of the more soluble aragonite instead calcite juvenile stages (see Ramajo et al., 2013) although warming may diminish the negative impact of acidification on calcification (Byrne, 2011). Recently, we found that the trend of calcite:aragonite ratio precipitated in the shell of in juveniles of *C. concholepas* showed a significant increase from north to south, which in inverse to the trend in sea water temperature along the Chilean coast (Ramajo et al. submitted). In the present, there are controversial results about the interactive, additive or antagonistic effects of pH decrease and sea water temperature increase, and other human-induced stressors (Crain et al., 2008; Kroeker et al., 2010, 2013; Byrne, 2011, Therefore, it is necessary to understand the interactive effects of ocean acidification and ocean warming to make adaptive interpretations of marine invertebrates.

There is an inherent limitation in scope between the time-scale of the incubation period regarded in our study with the scales at which is projected that the event of OA will take place in nature. This simplistic view is common in short term studies about OA effects, and tend to disregard relevant responses that organism may express in the incoming decades like local adaptation that may also modulate the ecological relevance of the organisms upon the ecosystem functioning (but see Dupont et al., 2012). In spite these discrepancies, such experiments do provide insights into stressor tolerance levels and in some marine invertebrates realized rates of acclimation to hypercapnia has been reported to occur especially fast (Calosi et al., 2013; Pörtner et al., 2011), it is likely that observed differences in metabolic rate are the end-product of local adaptation. Several empirical studies have also demonstrated that exposure to a given environmental condition does not necessarily provide an organism with a performance advantage in that environment relative to those organisms that have not experienced it (e.g., Matson et al., 2012; Stillwell and Fox, 2005). Having in mind the above described limitations; we suggest that short-term measures of metabolic responses to high-pCO_2 conditions are expected to indicate the sensitivity of species to ocean acidification. Our results evidenced that the less severe response to high-pCO2 levels of the population that is naturally exposed to higher pH variations (i.e., Calfuco), suggesting the acclimation potential for the species. However, or results cannot exclude that individuals of the other population (Antofagasta), if exposed for a longer term to the same pH variation would express similar responses to OA. Thus, may be also expected that our species model may be able to acclimate to increasing ocean acidification, and after to adapt through natural selection (e.g., Russell and Connell, 2009).

Natural selection has been effective to rescue population from environmental stress. However, evolutionary rescue requires adequate population abundance and phenotypic and corresponding underlying genetic variation. Our results suggest that acclimation ability vary greatly both within and between populations and that understanding such variations will be critical for predicting the impacts of ocean acidification. Broadly distributed species, such as *C. concholepas*, may also have genotypic variability to facilitate resilience to face environmental changes (see Cardenas et al., 2009). Growing evidence from CO_2 perturbation experiments suggests that several taxa might react quite sensitively to ocean acidification; others seem to be surprisingly tolerant (see Byrne, 2011; Kroeker et al., 2010). However, there is little mechanistic understanding on what physiological traits are responsible for the observed differential sensitivities among species and populations (Fabry et al., 2008; Pörtner, 2008).

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