Geographic variation in acid–base balance of the intertidal crustacean *Cyclograpsus cinereus* (Decapoda, Grapsidae) during air exposure

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In intertidal poikilotherms with wide geographic distribution, physiological variations are ubiquitous, due to phenotypic plasticity and/or individual geographic variation. Using the grapsid crab, Cyclograpsus cinereus *as a study model, acclimatization differences in respiratory physiology were evaluated among populations along the Chilean coast, covering a latitudinal gradient of about 2000 km. This species inhabits the supratidal zones and, therefore, is subject to constant immersion and emersion periods, producing physiological acidification due to CO_2 retention, mainly in the branchial cavity. Individuals of six populations were collected along the coastline of Chile and were exposed to air for different time periods in the laboratory. The following parameters were measured: pH, Ca^{2+}, Cl^- and haemolymphatic lactate dehydrogenase (LDH) enzyme activity. Populations from lower latitudes were significantly different from those from central and southern Chile, with a higher haemolymphatic pH variation and higher Ca^{2+} level, along with lower levels of Cl^- and LDH enzyme activity. This indicates that the populations from lower latitudes, which are subject to higher air temperatures during emersion, have a higher homeostatic capacity during emersion periods than those of intermediate and higher latitudes. This response seems to be determined by genetic bases due to adaptation to the local environment.*

Keywords: emersion, latitudinal gradient, respiration, intertidal, Chile, lactate dehydrogenase, LDH, acidosis

INTRODUCTION

Transition of aquatic organisms to terrestrial systems is one of the most important landmarks in the evolutionary history of animals (Truchot, 1990). In this context, intertidal animals are of great use in understanding this process, due to the fact that they are constantly exposed to both aquatic and semi-terrestrial conditions by tidal cycles. This process generates ecological, anatomical and physiological necessities that some groups, such as decapod crustaceans have been able to respond to (Morris, 2002; Greenaway, 2003; Lardies *et al.*, 2011).

Exposure to the air produces a decrease in the ability of aquatic organisms to capture O_2 efficiently, and many crustaceans have developed different strategies that allow them to overcome these limitations, for example annexed respiratory structures such as decalcified locomotive appendages in some intertidal species (Stillman, 2000; Vargas *et al.*, 2010), the ability to maintain bimodal respiration (Henry, 1994; Greenaway, 2003; Lardies *et al.*, 2011), or, as in low latitudes, pulmonary structures that render them completely terrestrial (Farrelly & Greenaway, 1994; Halperin *et al.*, 2000). In this last group, studies regarding the effect of air exposure have been

carried out to measure parameters such as desiccation, hypoxia and temperature (Pellegrino, 1984; Jensen & Armstron, 1991; Hofmann & Somero, 1995; Full 1984; O'Mahoney & Lagos *et al.*, 2011).

In marine aquatic crustaceans, the collapse of gills during emersion periods challenges both O_2 capture and CO_2 excretion, producing acidification of haemolymph, and therefore, an increase in enzymes such as lactate dehydrogenase (LDH). This enzyme catalyses the conversion of lactate, and is involved in the final step of anaerobic glicolysis. It is an efficient indicator of physiological stress due to environmental conditions (Varley & Greenaway, 1992; Astete-Espinoza & Cáceres 2000; Morris, 2002). This alteration in the acid–base balance is regulated by the 'strong ion differences' (SID) (Varley & Greenaway, 1992; Luquet & Ansaldo, 1997), which produce a change in pH that activates homeostatic mechanisms (Henry & Wheatly, 1992; Wheatly & Henry, 1992; Lagos & Cáceres, 2008; Vargas *et al.*, 2010).

Crustaceans also carry out active regulation of acid–base balance by ionic exchange with the environment through the gills (Cl^- for HCO_3^- and Na^+ for H^+), but crabs exposed to the air cannot perform this regulation. It has been suggested that some intertidal organisms could be using dissolution of exoskeleton $CaCO_3$ in to HCO_3^- and Ca^{2+} as a compensatory mechanism during air exposure; $CaCO_3$ is highly soluble and sensitive to pH changes and its dissolution can be quantified

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through changes in the concentration of Ca^{2+} in the haemolymph (Henry et al., 1981; Henry & Wheatly, 1992; Luquet & Ansaldo, 1997; Henry, 2001). This phenomenon has been observed in molluscs and porcellanid crustaceans, where the concentration of Ca^{2+} in the exoskeleton decreases in relation to an increase in haemolymph during air exposure of more than two hours (Henry et al., 1981; Lagos & Cáceres, 2008; Montecinos et al., 2009; Vargas et al., 2010).

Nevertheless, the effects of air exposure are not the same in the different environments that these organisms inhabit, due to differences caused by humidity and/or temperature. For example, inhabiting lower latitudes is associated with higher physiological costs than higher latitudes (Lardies et al., 2011). This is because different populations of the same species, distributed along a wide latitudinal range, live under condition gradients that may vary significantly, producing phenotypic variations at different levels of environmental pressure (Lardies & Castilla, 2001; Ricklefs & Wikelski, 2002; Mizera & Meszéna, 2003; Lardies & Bozinovic, 2008). This phenotypic plasticity may cause effects on metabolism, body size (Laptikhovsky, 2006; Lardies & Bozinovic, 2006; Lardies et al., 2010; Monaco et al., 2010) and also the ability to capture O_2 in a bimodal manner, from both water and air (Lardies et al., 2011). Nevertheless, variation of acid–base balance during air exposure has not been studied using geographical gradients to see if this phenomenon behaves in an analogous way to the metabolism of individuals.

Our goals are: (1) to evaluate spatial variation of acid–base balance; and (2) to evaluate how acid–base regulation may compromise respiration. We will use the intertidal crab Cyclograpsus cinereus (Decapoda: Grapsidae) as a study model, in a latitudinal gradient of 2000 km along the Chilean coastline. This crustacean lives under boulders of the supratidal zone and spends most of the tide cycle out of the water (Bahamonde & López, 1969). We will estimate acid–base equilibrium during emersion periods of six populations of C. cinereus using variables associated with respiratory physiology: pH, Ca^{2+}, Cl^- and haemolymphatic lactate dehydrogenase (LDH) enzyme activity.

MATERIALS AND METHODS

Study sites

Samples of Cyclograpsus cinereus adult male crabs, in inter-molt stage C (Moriyasu & Mallet, 1986; Luquet & Ansaldo, 1997), were collected by hand in rocky intertidal zones at six localities along the Chilean coastline during spring months: Arica ($18°28'$S $70°18'$W), Antofagasta ($23°38'$S $70°24'$W), La Serena ($29°54'$S $71°15'$W), El Quisco ($33°23'$S $71°4'$W), Caleta Lenga ($36°45'$S $70°10'$W) and Valdivia ($39°48'$S $73°14'$W). The localities used correspond to zones that are subject to different environmental temperatures (see Figure 1) (for more details on superficial ocean and air temperatures, see Table 1). At each site, approximately 100 individuals were collected and transported in thermally isolated containers to the Animal Physiology Laboratory of Universidad Católica de la Santísima Concepción. In the laboratory, samples from all populations were acclimatized in seawater tanks at $13 \pm 0.5°$C, with salt levels of 30 psu for a period of 96 h (Chen & Chia, 1997), before the experimental trials.
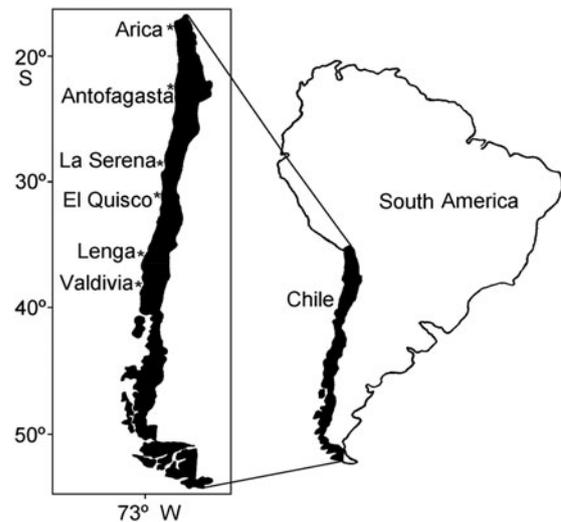

Fig. 1. Location of six study sites for individuals of Cyclograpsus cinereus collected along the coast of Chile. Sites were grouped to the following geographical regions: Arica–Antofagasta, northern region; La Serena–El Quisco, central region; and Lenga–Valdivia, southern region.

Table 1. Latitudinal gradient information of the six study sites along the Chilean coast. Sea surface temperatures (SST) and air temperature (AT) (data from SHOA, 2006 and FAO, 1985, respectively). Temperature is presented as the annual average, with annual average minimum and maximum temperatures in parentheses.

Locality	SST (°C)	AT (°C)
Arica	18.5 (16.2, 24.3)	18.9 (15.3, 22.9)
Antofagasta	17.5 (15.6, 20.1)	17.0 (13.3, 22.9)
La Serena	15.6 (13.5, 17.6)	14.8 (11.2, 18.9)
El Quisco	13.8 (11.7, 16.5)	13.6 (9.8, 17.8)
Caleta Lenga	12.9 (10.5, 15.6)	12.2 (7.1, 16.7)
Valdivia	12.8 (9.5, 14.9)	11.0 (6.1, 16.0)

Laboratory analysis

To evaluate the effect of air exposure on the measured variables, crabs were subjected to different air exposure times: 0 (completely submerged organisms), 6, 15, 60, 120, 240 and 360 min (Luquet & Ansaldo, 1997). Zero minutes of air exposure is considered control treatment. Three to five individuals for each treatment were placed in individual 250 ml containers with damp sand (using seawater) to avoid desiccation. Containers were then placed inside a refrigerated chamber in a PolyScience® (PolyScience PPO7R-20, PolyScience, USA) thermal bath for the rest of the experiment, defined by the exposure time, at the same temperature as the one used for acclimatization.

At the end of each experimental period, individual samples of haemolymph were collected by cephalothoraxic puncture using tuberculin syringes (1 ml). Immediately after extraction, samples were processed and the following parameters measured: (a) pH (NBS scale) using a pHmeter (HANNA® model 1332; Hanna Instruments S.L.; Spain) (b) Ca^{2+} concentration in haemolymph using the method described by Moorehead & Biggs (1974); (c) Cl^- concentration in haemolymph using the method described by Schales & Schales (1941); and (d) lactate dehydrogenase (LDH) activity using the method described by Klin (1970).

Statistical analysis

The sample localities were grouped into three geographic zones, because the nearest locality pairs did not present significant differences among the variable responses and phenotypic traits analysed (one way ANOVA; $P > 0.001$). The three biogeographic regions were: Arica and Antofagasta represent the northern region; La Serena and El Quisco represent the central region; and Caleta Lenga and Valdivia represent the southern region.

Before statistical analysis, variance homogeneity for the data were tested using the Hartley Fmax test (Sokal & Rohlf, 1997). To evaluate if there is any significant difference between each exposure time for each variable measured, an ANCOVA was used for each zone, using zone and air exposure as factors and body mass as a covariable. The statistically significant level considered was 0.05 (Zar, 1996). Differences between groups (*a posteriori* comparison) were evaluated using a Tukey HSD test (Sokal & Rohlf, 1997). All analyses were carried out using the STATISTICA® program, v.6.0 (StatSoft, USA) for Windows® operating system. Data are presented as a mean plus or minus standard error ($X \pm$ EE).

RESULTS

Mean value for body mass of sampled organisms for each zone was 0.25 ± 0.008 g for the north zone, 0.688 ± 0.033 g for the central zone and 1.130 ± 0.109 g for the south zone. The body mass of the three zones varied significantly ($F_{1,303} = 327.18$; $P < 0.05$) (Tukey *a posteriori* $P < 0.05$).

Haemolymph parameters

Mean values for pH were independent from body mass of the organisms ($F_{1,71} = 1.43$; $P = 0.235$). The pH was significantly different between different air exposure times and geographic zones. Initial times were significantly lower in pH values than final air exposure times ($F_{5,71} = 15.99$; $P < 0.05$), northern organisms separated from central and southern organisms ($F_{2,71} = 215.069$; $P < 0.05$) (Tukey *a posteriori* $P < 0.05$). The highest variations were observed in the north zone, where the lowest value was found in the control group (6.54 ± 0.04) and the highest value was found after 120 min of air exposure ($7.14 \pm 0,12$) (Tukey *a posteriori* $P < 0.05$) (Figure 2A). No significant interaction was observed between exposition time and locality ($F_{10,71} = 1.29$; $P = 0.254$). In individuals from the northern zone, the lowest pH values were found in organisms under immersion (air exposure time of 0) with a subsequent alkalinization that stabilized after 60 min of air exposure (see Figure 2A).

Mean values for haemolymphatic Ca^{2+} were determined independently from the body mass of organisms ($F_{1,89} = 0.06$; $P = 0.801$). Within this parameter an interaction between locality and time was observed. Organisms from the northern zone after 60 min of air exposure presented the highest values in regard to the other experimental groups (Figure 2B) (Tukey *a posteriori* $P < 0.001$). The lowest values for Ca^{2+} were obtained at the initial time in comparison to other exposition times (Tukey *a posteriori* $P < 0.001$).
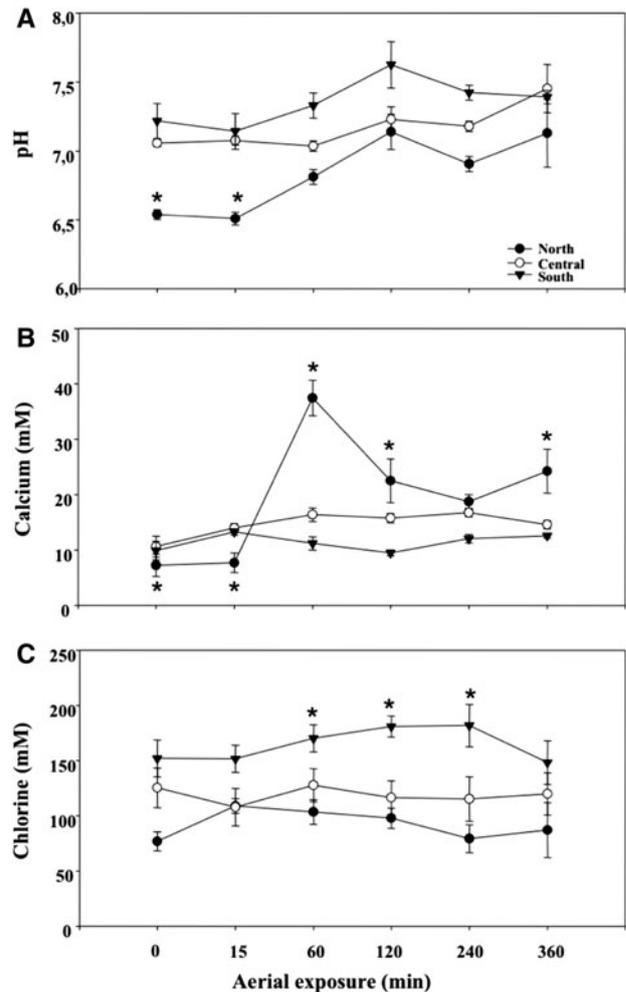

Fig. 2. Haemolymphatic values measured in the intertidal crab *Cyclograpsus cinereus* over several times of air exposure in north (Arica and Antofagasta), central (La Serena and El Quisco) and south (Lenga and Valdivia) zones of Chilean coast (mean $\pm$ SE): (A) variations in haemolymphatic pH; (B) Ca^{2+} haemolymphatic concentrations; (C) Cl^- haemolymphatic concentrations. Significant differences among regions are highlighted by asterisks (for statistics see Results). Zero minutes indicate control treatment (no air exposure).

Mean values for haemolymphatic Cl^- were independent from body mass of organisms ($F_{1,89} = 0.33$; $P = 0.563$). Concentrations on haemolymphatic Cl^- differed significantly between the three geographic zones, with the highest values observed in the southern zone ($F_{2,89} = 13.41$; $P < 0.001$) (Tukey *a posteriori* $P < 0.05$). No differences were observed between exposition times or for geographic and time interaction ($F_{5,89} = 0.54$; $P = 0.74$ for time factor and $F_{10,89} = 0.69$; $P = 0.72$ for factor interaction) (Figure 2C).

Enzyme activity

Values for LDH enzyme activity were independent from body mass of individuals. ($F_{1,71} = 0.008$; $P = 0.929$). The LDH enzyme activity in haemolymph was significantly different between analysed geographic zones ($F_{2,71} = 41.58$; $P < 0.001$). Among central and south zones was found the highest LDH activity, nevertheless these zones did not show significant differences (Tukey *a posteriori* $P > 0.05$). In contrast, the lowest values were found in the north zone (see
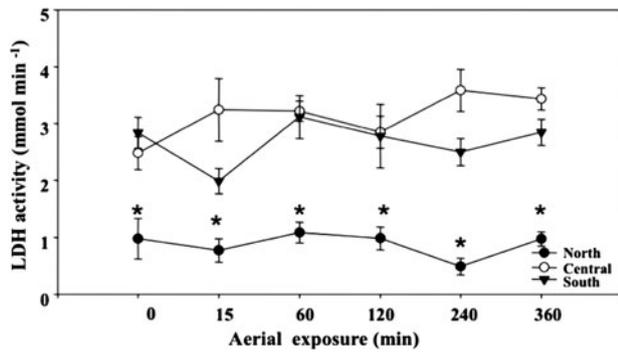
Fig. 3. Haemolymphatic values of LDH activity measured in the intertidal crab *Cyclograpsus cinereus* over several times of air exposure in north (Arica and Antofagasta), central (La Serena and El Quisco) and south (Lenga and Valdivia) zones of Chilean coast (mean $\pm$ SE). Significant differences among regions are highlighted by asterisks (for statistics see Results). Zero minutes indicate control treatment (no air exposure).

Figure 3), (Tukey *a posteriori* $P < 0.05$). No significant differences were found between air exposure times or for the geographic zone and time interaction ($F_{5,71} = 0.740$; $P = 0.590$ for time and ANCOVA $F_{10,71} = 1.18$; $P = 0.720$ for factor interaction, respectively). The LDH and other parameters show differences which were associated with the origin of the individuals, with a clear separation between organisms from lower latitudes with those of intermediate and higher latitudes.

DISCUSSION

Many environmental factors can be selective agents for organisms, and temperature is one of the most important ones, as it affects processes that range from the molecular to the ecosystem level (Fischer & Fiedler, 2002; Pörtner *et al.*, 2006). The clear differential pattern found for *Cyclograpsus cinereus* between the north and central–south regions (Table 1), indicated that temperature, both aquatic and air, may act as a key selective agent that marks physiological respiratory differences for this species in both zones (Camus, 2001; Lardies *et al.*, 2010, 2011; Lagos *et al.*, 2011). In this study, the effect of air exposure on acid–base balance was evaluated in different populations of *C. cinereus* subjected to different climatic temperature regimes (see Thiel *et al.*, 2007). Different patterns were found in respiratory parameters between populations of the northern region and those from the central–south regions which are consistent with patterns reported for this species in other physiological and life history traits (Lardies *et al.*, 2010, 2011).

Variations in haemolymphatic pH for *C. cinereus* clearly separated the northern population from the central and southern ones. In individuals from the northern region, the lowest pH values were found in organisms under immersion (air exposure time of 0) with a subsequent alkalinization that stabilized after 60 min of air exposure (see Figure 2A). This indicates that this population has a high ability to maintain adequate acid–base regulation during air respiration periods (Henry *et al.*, 1981). This physiological response has been described in crustaceans such as *Petrolisthes laevigatus* (Decapoda: Porcellan) and *Cardisoma carnifex* (Decapoda: Gecarcinidae), which suffer an increase in PCO_2 and a decrease in pH during immersion in comparison to emersion

periods (Morris & Adamezewska, 1996; Lagos & Cáceres, 2008).

Variation in Ca^{2+} concentrations were larger for individuals of the north region, which is associated with the ability to use $CaCO_3$ reservoirs from the soluble exoskeleton to obtain Ca^{2+} and HCO_3^- when pH lowers, thus maintaining internal acid–base balance (Henry *et al.*, 1981; Gunthorpe *et al.*, 1990; Lagos & Cáceres, 2008; Montecinos *et al.*, 2009; Vargas *et al.*, 2010). On the other hand, absence of variations in Ca^{2+} in populations in the central and south regions do not indicate any mobilization of exoskeletal $CaCO_3$, at least under the conditions of this study. One of the described mechanisms that crustaceans use for acid–alkali regulation is modification of strong ion difference (SID), which is the difference between the sum of strong cations and anions, mainly Cl^- and Na^+ (Randall *et al.*, 2001). In crustaceans such as *Chasmagnathus granulata*, a large increase in Cl^- has been observed within the first 2 h of air exposure. In this case, the high SID of this time frame indicated a high capacity to regulate this variable that regulates pH (Luquet & Ansaldo, 1997). The lack of variability of haemolymphatic Cl^- concentration in air exposure is an indicator of the fine adjustement of *Cyclograpsus cinereus* to semi-terrestrial habitats; however, the higher concentration of Cl^-, along with null conversion of Ca^{2+} variation in organisms from high latitudes might indicate that *C. cinereus* uses different strategies in different geographic regions. This means that populations from high latitudes regulate pH more efficiently by using a SID mechanism, whereas those from lower latitudes would dissolve $CaCO_3$ from the exoskeleton. This shows a higher independence of these organisms in aquatic environments, because haemolymphatic Ca^{2+} concentrations occur in terrestrial crustaceans where acid–alkali regulation using gills is not a feasible option (Henry *et al.*, 1981; Innes *et al.*, 1986; Lagos & Cáceres, 2008).

Since *C. cinereus* is a poikilotherm, its metabolism is conditioned to environmental temperature, which follows a decreasing pattern towards colder areas (see Table 1 and Thiel *et al.*, 2007), meaning that populations from high latitudes have a lower metabolic rate than those of lower latitudes (Vernberg, 1959; Osovitz & Hofmann, 2007; Lardies *et al.*, 2011; Whiteley *et al.*, 2011). The higher response capacity in low latitude populations is reflected in the fact that they have a lower LDH activity, showing that under emersion conditions they do not have to resort to alternative anaerobic energy capture, which is necessary in intermediate and higher latitudes in general. In populations from high latitudes, where LDH activity is higher, this may be a sign that under the same conditions, these populations must resort to anaerobic energy capture due to their lower capacity to maintain effective air respiration. This is in contrast to higher latitude populations, which have the ability to breathe directly from the air (Lardies *et al.*, 2011), as has been described also for *Cyclograpsus lavauxi* (Innes *et al.*, 1986; Waldron *et al.*, 1986).

In general, a latitudinal pattern has been reported in regard to the ability that crustaceans have to maintain adequate respiration under air conditions; pulmonated crustaceans and amphibians are found at low latitudes, and these characteristics tend to disappear towards higher latitudes. Several species of brachyuran and anomuran possess physiological modifications such as decreased gill area and the development of accessory respiratory structures such as lungs and highly vascularized gill chambers that allow them to occupy terrestrial and semi-terrestrial habitats, as well as the ability to

survive prolonged emersion periods (Innes *et al.*, 1986; Waldron *et al.*, 1986; Farrely & Greenaway, 1994; Stillman, 2000; Vargas *et al.*, 2010).

Variation observed in acid-balance and, specifically, differences in LDH activity, in *C. cinereus* may be determined by acclimatization and/or adaptation (i.e. genetic basis). Studies carried out in the fish *Fundulus heteroclitus* indicate that there are differences in both concentration and activity of LDH enzyme, and also in the transcription speed in the LDH locus (Crawford & Powers, 1992). Populations inhabiting high latitudes have a high rate of LDH–RNA transcription, which results in a high enzyme concentration. This seems to be a genetically-controlled evolutionary adaptation mechanism, and not a direct acclimatization effect. This also has a larger effect on the enzyme, which is an important mechanism for adaptation affecting diverse physiological processes (Paynter *et al.*, 1991; Schulte *et al.*, 2000). This should take place in *C. cinereus,* given the almost null response of the LDH enzyme activity in low latitude populations. When organisms are faced with temperature change, they may maintain their physiological rates using three different strategies: (a) quantitative, involving changes in substrate and enzyme concentration; (b) qualitative, where protein variants with different thermal properties are used; and (c) modulation, which changes the environment where the protein is to minimize temperature impact (Yang & Bielanoski, 2000; Hochachka & Somero, 2002). Therefore, comparative studies between species or different populations, like this study, are useful to determine evolutionary adaptation; nevertheless, since these crustaceans are intertidal, acclimatization rates would be relatively quick (Willmer *et al.*, 2000) and individuals from all populations were placed in a common environment. Therefore, the difference in the parameter measures are most probably due to local adaptation and may correspond to heritable traits. Nevertheless, results from transgenerational studies are needed to confirm that this variability is due to genetic differences among populations.

Oceanic uptake of anthropogenic carbon dioxide (CO_2) is altering the seawater chemistry of the oceans with consequences for organisms and ecosystems (Fabry *et al.*, 2008). High quantities of CO_2 in oceans (i.e. hypercapnia) are producing a calcium carbonate saturation horizon to shoals in many regions, particularly in high latitudes (Gatusso & Hanson, 2011). Hypercapnia can impact marine organisms both via decreased calcium carbonate ($CaCO_3$) saturation, which affects calcification rates, and via disturbance to acid–base physiology. *Cyclograpsus cinereus* show significant geographic variability in response to hypercapnia that indicates low buffering capacity in southern regions, the projected most-impacted population, and a 'natural' higher buffering capacity in low latitude populations (but see Pane & Barry, 2007). Evidence suggests that there is now a critical need to test the physiological consequences of ocean acidification, while integrating into the analysis the variability among geographic populations.

COMPLIANCE

This study complies with current Chilean legislation regarding the collection and treatment of invertebrates.

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