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Plasticity and trade-offs in physiological traits of intertidal mussels subjected to freshwater-induced environmental variation

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ABSTRACT: Environmental gradients play an important role in shaping geographic variability in coastal marine populations. Thus, the ability of organisms to cope with these changes will depend on their potential to acclimatize, or adapt, to these new environmental conditions. We investigated the spatial variability in biological responses shown by *Perumytilus purpuratus* mussels collected from 2 intertidal areas experiencing contrasting freshwater input influences (river-influenced vs. marine conditions). To highlight the role of plasticity and adaptive potential in biological responses, we performed a reciprocal-transplant experiment and measured relevant phenotypic traits including mortality, growth, calcification, metabolism, and chemical composition of the shell periostracum. We determined that mussels exposed to river-influenced conditions had increased metabolic rates and reduced growth rates, as compared to mussels experiencing marine conditions ($p < 0.05$). While the energy investment strategies of the 2 local populations resulted in similar net calcification rates, these rates decreased significantly when mussels were transplanted to the river-influenced site. Stressful conditions at the river-influenced site were evidenced by decreased survivorship across treatments. Freshwater inputs modify the organic composition of the shell periostracum through a significant reduction in polysaccharides. Although our field experiment did not identify specific environmental factors underlying these contrasting phenotypic changes, the results imply that plasticity plays a strong role when *P. purpuratus* is exposed to some combination of natural (e.g. salinity) and anthropogenic influences (e.g. pollution), and that the lack of exposure to freshwater may promote less tolerant mussels with greater potential for local adaptation.

KEY WORDS: Calcification · Shell periostracum · Metabolism · Local adaptation · Central Chile · *Perumytilus purpuratus*

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INTRODUCTION

Variable environmental conditions may impose divergent selection pressures, as evidenced by the fact that different populations evolve specific physiological characteristics to maximize the fitness of genotypes in their native habitats (Kawecki & Ebert 2004). Local adaptation in marine systems, which involves

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several biological processes in response to both environmental (e.g. temperature, salinity, wave actions; Struhsaker 1968, Sokolova & Pörtner 2001, Sokolova & Boulding 2004) and biotic forces (e.g. predation or food availability; Trussell 2000), has been documented on a broad range of spatial scales, from latitudinal gradients (e.g. Sokolova & Pörtner 2001), to regional (hundreds of km; e.g. Trussell 2000) and mesoscales (tens of km; e.g. Johannesson et al. 1997). However, in some cases, particularly when comparing populations from strongly differing environments, evidence of local adaptation has not been found (e.g. Geber & Eckhart 2005). In fact, transplant experiments have actually shown greater fitness in foreign populations than in native populations (e.g. Galloway & Fenster 2000), which possibly indicates that some populations may be prevented from reaching adaptive optima (Hereford 2009).

Temperate nearshore ecosystems are characterized by large fluctuations in environmental variables such as temperature, pH, dissolved oxygen, and salinity. This is the result of multiple drivers, including impacts from watershed processes, nutrient inputs, and changes in ecosystem structure and/or metabolism, among others (Duarte et al. 2013). While all of these drivers are important for nearshore environments that receive significant riverine run-off, the impacts of river discharges are largely understudied in the coastal zone (Dagg et al. 2004). Freshwater input creates regional differences in salinity on the sea surface and alters chemical and biological properties of seawater by adding calcium ions, organic and inorganic carbon, nutrients, and increasing alkalinity (Dagg et al. 2004, Borges & Gypens 2010, Duarte et al. 2013). Alterations in the chemical composition of river water have the capacity to modify the expected concentrations of inorganic carbon species in estuarine waters (when freshwater and seawater are mixed), altering the pH of receiving coastal waters (Aufdenkampe et al. 2011). High CO_2 levels that result from organic matter respiration (Frankignoulle et al. 1998, Borges et al. 2005) reduce pH in river plumes and enhance regional acidification processes on different temporal scales (Waldbusser & Salisbury 2014). Such impacts will be more pronounced under future climate projections, particularly with changes in precipitation patterns (Portmann et al. 2009), modification of land use (Pérez et al. 2015), eutrophication processes (Aufdenkampe et al. 2011), and increase of atmospheric CO_2 (Feely et al. 2008).

Both salinity (Wrange et al. 2014) and pH (Kroeker et al. 2013) control physiological processes in marine and estuarine species. Thus, habitats influenced by river run-off provide an excellent opportunity to examine the effects of these environmental stressors on the biological responses of sessile organisms. Recent experimental studies have reported that individuals that regularly experience high environmental variability in salinity (Wrange et al. 2014) and partial pressure of CO_2 (pCO_2) levels (Pansch et al. 2014, Thomsen et al. 2013, Basso et al. 2015) in their native habitat were more tolerant to environmental stress than individuals inhabiting more stable environments, which demonstrates the role of potential local adaptation. However, the effect of riverine discharge on intertidal organisms, and their capacity to acclimate or adapt to these variable environments, is poorly understood.

To date, few studies have investigated how river discharges affect the biological responses of calcifying marine organisms and their ability to acclimate or potentially adapt to these variable environments. Therefore, we conducted reciprocal-transplant experiments on the mussel *Perumytilus purpuratus* between 2 geographically separated (ca. 10 km apart) populations on the central coast of Chile. One location was persistently influenced by discharge from the Maipo River, and the other location only received marine water influences. The aim of the study was to test whether mussel populations that inhabit marine and river-influenced environments are potentially adapted to local environmental conditions. We estimated the organisms' reaction norms for several morphological, physiological, and life-history traits of *P. purpuratus*.

MATERIALS AND METHODS

Species and study sites

Perumytilus purpuratus (Lamarck 1819) is an intertidal mussel that is a dominant competitor in the mid-intertidal zone (Alvarado & Castilla 1996). It forms dense, 3-dimensional matrices providing habitat for several benthic invertebrates (Tokeshi et al. 1989), thereby acting as an important ecosystem engineer (Prado & Castilla 2006). *P. purpuratus* has a pelagic larval development (>1 mo), with potential for long-distance dispersal (O'Connor et al. 2007). In central Chile, the settlement of *P. purpuratus* occurs synchronously across sites separated by less than 30 km, which resembles the scale of fluctuations in coastal upwelling by way of modulating larval dispersal (Lagos et al. 2007). This mesoscale larval dispersal, and the genetic homogeneity of mussels described for central Chile (23–28°C, Briones et al. 2013), indicates a low potential for genetic differentiation

between mussel populations inhabiting the central coast of Chile. In this region, 2 sites, separated by approximately 10 km, were chosen for the reciprocal-transplant experiment (Fig. 1).

The first location, San Antonio (33° 34′ S), is influenced by freshwater inputs from the Maipo River plume (Piñones et al. 2005, Vargas et al. 2006). In particular, the waters surrounding this site are characterized by increased turbidity signatures, representing >75% of the turbidity (water-leaving radiance) occurring at the river mouth (see Fig. 1; Pérez et al. 2015). The historical mean flow of the Maipo River is 90 m^3 s^{-1}. Higher flows occur during the austral spring–summer season, mainly due to ice melting (Meza et al. 2012). During the study period, the annual mean river flow was 46.3 ± 30.9 m^3 s^{-1} and was characterized by increased input of nutrients and dissolved inorganic and organic carbon associated with agricultural and industrial uses along the river catchment (Pérez et al. 2015). Thus, this river-influenced site is only affected by the influx of terrestrial material during austral winter periods (June–July). As a result, the river-influenced site has more refractory terrestrial particulate organic matter during winter months (Pérez et al. 2015). In addition, the coastal waters receiving discharge from the Maipo River experience a fluctuation in salinity between 32 and 33.5 psu (Vargas et al. 2006) and 7.76 pH units (Pérez et al. 2015).

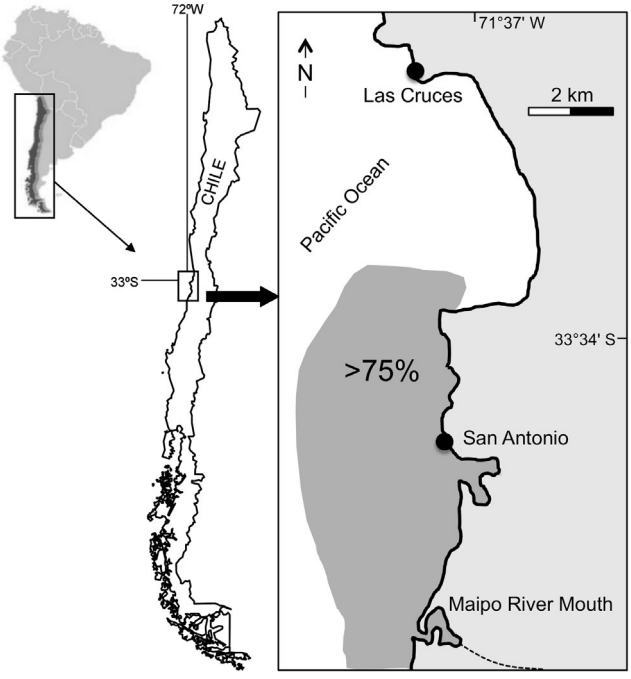

Fig. 1. Two study locations (black dots) on the central coast of Chile where mussels *Perumytilus purpuratus* were subjected to the reciprocal-transplant experiment. The darker gray shading along the coast indicates the river plume extension of the Maipo River encompassing surface water that has a turbidity signal (water-leaving radiance) representing at least a 75% of the turbidity recorded in the river mouth (see Pérez et al. 2015)

The second location, Las Cruces (33° 30′ S), is free of any significant freshwater discharge, and water maintains a salinity consistently higher than 33.5 and a pH of 7.86 units (Pérez et al. 2015). Large differences in chlorophyll concentration occur in the studied areas. The highest concentration of chlorophyll *a* (chl *a*, up to 30 mg m^{-3}), including pico-, nano-, and microplankton, was associated with the plume of the Maipo River. However, at the marine site, the chl *a* concentration was dominated by the small-sized pico- and nanophytoplankton (Vargas et al. 2006).

Beginning in May 2011, environmental conditions at both experimental sites, San Antonio and Las Cruces, were sampled every week. Sea surface temperature (SST), surface salinity, and oxygen concentration were measured using a CTDO (Hydronaut OCEAN SEVEN 304). For pH_T measurements (pH in total scale), 3 water samples were collected and analyzed within 60 min of collection, using a Metrohm 826 pHMobile Meter connected to a combined electrode (double juncture), and calibrated with TRIS buffers (pH = 8.089) at 25°C using a thermo-regulated water bath. The estimated error for pH measurement was 0.006 pH at the river-influenced site (Pérez et al. 2015). For total alkalinity (A_T) analyses, discrete water samples were collected using borosilicate glass bottles (Corning, 500 ml), poisoned using mercuric chloride ($HgCl_2$, 0.2 cm^3 of a 50% saturated solution), and sealed with Apiezon L grease for transportation to the laboratory. In the laboratory, samples were stored in cool, dark conditions until analysis, which was within 3 mo of collection. Three to 5 seawater subsamples of each bottle were used to estimate A_T using automated potentiometric titration (Haraldsson et al. 1997). Partial pressure of CO_2 and saturation states (Ω) for calcite and aragonite were estimated from the averaged values of pH_T, A_T, and SST using CO2SYS software (Pierrot et al. 2006) that used dissociation constants from Mehrbach et al. (1973), refits by Dickson & Millero (1987), and $KHSO_4$ (Dickson 1990).

Experimental procedures

Individuals of *P. purpuratus* were collected at low tide from mussel beds distributed across the intertidal zone of San Antonio and Las Cruces in May 2011. Subsequently, organisms were transferred to the lab-

oratory (Estación Costera de Investigaciones Marinas in Las Cruces, Chile) and maintained in trays with aerated seawater that was collected at each study location. Sixty healthy individuals of similar maximum shell length size (13.31 ± 0.03 mm) and buoyant weight (0.2148 ± 0.0088 g) were selected per site (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m553p093_supp.pdf) and marked using bee tags glued onto the shell (Fig. S1 in the Supplement). After these procedures, 30 tagged individuals, plus 50 additional mussels (not labeled and of variable size), were placed together in running seawater and allowed to re-attach to each other through byssal thread production. Small clusters of mussels formed, which helped reduce the loss of mussels when they were returned to the experimental field sites. All mussels were placed inside experimental cages that were built from plastic Vexar mesh boxes (10 × 10 × 5 cm [W × L × H], aperture 5 mm), protected with a stainless steel box of similar dimensions, and anchored to the rocks using stainless steel bolts (see Fig. S1). Individual mussels were assigned randomly to experimental treatments: the auto-transplant treatment included mussels whose origin and destination sites were the same (i.e. individuals from the marine site remained at the marine site), while transplant treatments included mussels that were moved from their origin site to the destination site (i.e. populations from the marine site were transplanted to the river-influenced site and vice versa). In total, 8 cages were randomly assigned to 4 experimental treatments (auto-transplant and transplant).

The reciprocal-transplant experiment was performed from May to October 2011, and lasted for a total of 154 d. During these months, the mean river flow was low (32.96 ± 8.4 $m^3 s^{-1}$), which represented a reduction of ca. 48% in relation to the high flow season (e.g. Meza et al. 2012). During the experiment, cages were checked after 56 d (July 2011). All cages were removed from each experimental site, transported to the laboratory, and maintained in aerated seawater that was collected at each site. We recorded maximum shell length, buoyant weight, and mussel mortality across treatments. Subsequently, the individuals were returned to the field for the remaining 98 d (October 2011). During the experiment, approximately 35% of *P. purpuratus* individuals lost their labels, likely due to wave action, sediment abrasion, or traction from byssal threads of other individuals inside the box. Between the first and second experimental periods, 1 experimental box (mussels from the auto-transplanted treatment at the river-influenced site) was lost due to rough weather conditions.

Biological responses of mussels

Metabolic rates, measured as oxygen consumption, were quantified at the end of the experiment using 7 randomly selected individuals per treatment. Before these measurements, mussels were returned to the laboratory and acclimated at 14°C for 7 d. In the last 72 h before measurements, individuals were maintained free of food supply in UV-filtered seawater and with natural photoperiod. The temperature during measurements was controlled using a chiller (BOYU, Model L075). Oxygen consumption rate was measured using an oxygen optode connected to a PreSens Microx TX3 temperature-compensated oxygen meter with a tip diameter of 140 µm. Prior to measurements, oxygen micro-sensors were calibrated with a solution of Na_2O_3S at 5% and aerated water for the values 0 and 100% air saturation at 14°C, respectively (Storch et al. 2011). Oxygen consumption rates are reported as consumption per gram of animal buoyant weight (i.e. mg O_2 g^{-1} h^{-1}).

Growth rate, net calcification rate, and survivorship were recorded after 56 d and at the end of the experiment. Growth rates (mm d^{-1}) were estimated from changes in the maximum shell length between the 2 experimental periods. Net calcification was calculated using the buoyant weight technique (Davies 1989). Buoyant weight was converted into dry weight, and then calcification rates were calculated as the change in dry weight between the 2 sampling points (56 and 154 d after the beginning the experiment) and normalized to the initial weight per month (mg $CaCO_3$ g^{-1} d^{-1}). Thus, this net calcification estimate includes the dissolution rates of shells, and corresponds to the net amount of calcium carbonate, organic matrix, and inorganic carbon deposited by the animal over time (Rodolfo-Metalpa et al. 2011).

Shell periostracum morphology and organic composition

The morphology and structure of the periostracum was assessed by analyzing the outer surface of intact shell samples of *P. purpuratus* specimens with scanning electron microscopy (SEM) using a Zeiss Auriga SEM. Prior to observation, samples were carbon coated using a Hitachi UHS evaporator. The chemical composition of the periostracum was analyzed with infrared (IR) spectroscopy. For IR analyses, the outer surface of intact shell samples (near the growth shell margin) were pressed against an attenuated total reflectance (ATR) diamond crystal window, and

the IR spectra were recorded at a 2 cm^{-1} resolution for more than 100 scans using a Fourier transform infrared (FTIR) spectrometer (model 6200, JASCO Analytical Instruments). The amounts of water, protein, sulfate, carbonate, polysaccharides, and lipids were estimated from the absorption peak areas associated with the characteristic molecular group of each component (e.g. O–H: water, C–H: lipids or fatty acids, amide: proteins, C–O: carbonates, S–O: sulfates, COC: sugars/polysaccharides). Calculated peak areas of the main bands were normalized to the total area of the spectrum (Rodríguez-Navarro et al. 2013). ATR–FTIR can be also used to determine the thickness of coatings after calibration with samples of known thickness. We used the periostracum thickness, measured in a cross-section of shells with an SEM, to determine a calibration curve relating the thickness of this coating and the normalized carbonate signal (CO_3%) in ATR–FTIR spectra. This calibration curve (thickness [µm] = −78.6 CO_3% + 16.2; r^2 = 0.98, n = 4) was used to calculate the periostracum thickness for the remaining specimens.

Data analysis

We evaluated differences in initial conditions between individuals in each population in terms of buoyant weight, wet weight, and shell length using 1-way ANOVA. We used the paired *t*-test for comparing variation in carbonate system parameters between study sites, and Pearson r correlation testing for temporal association between these environmental parameters. To determine whether study sites can be differentiated in terms of spatial–temporal variability, we used a multivariate ANOVA (MANOVA) with a reclassification test (discriminant function analysis, DFA; see below) using *in situ* measured parameters (pH_T, SST, salinity, and A_T). We performed a comparison of growth, net calcification, and oxygen consumption rates between mussels exposed to different treatments of the reciprocal transplant using 2–way ANOVA with the individual mussels as replicates and the treatment as fixed factors. We carried out multiple comparisons using *a posteriori* Tukey's HSD test. We regarded the mussels inside the same cage as replicates because they were allowed to re-attach themselves in order to mimic the grouped tridimensional structure that mussels produce in nature. While we are aware that this decision could imply a pseudo-replicated design, we used several experimental cages to spread the risk of losing a portion of mussels, and not for strict statistical purposes. We used chi-squared analysis to assess the significance of survivorship (counts) across experimental treatments at the end of the experiment. We used Pearson's correlation analyses of peaks contributing to the IR spectra of the outer shell surface to provide information about the relationship between the main organic components of the periostracum and the shell carbonate. The carbonate shell signal provided information about the thickness and/or degree of coverage of the shell by the periostracum. Periostracum compositional parameters determined from IR spectra of the outer shell surface were used to develop a quadratic DFA (Discriminant Function Analysis) to determine whether changes in periostracum composition can be used to discriminate among samples from corresponding treatments of the reciprocal-transplant experiment, and to examine the reclassification success of the mussels from those treatments. The reclassification success was cross-validated using bootstrapping. The compositional data from mussels in the auto-transplant treatment was used as a training data set to evaluate the reclassification of the mussels subjected to the transplant treatment. Finally, 2-way ANOVA was used to identify which particular periostracum chemical component explained the variations observed in the DFA multivariate analyses. Prior to all statistical analyses, data were transformed when normality and homogeneity of variance assumptions were not satisfied (Shapiro-Wilks and Levene tests). Values are reported throughout as means ± SE. Tests were performed using JMP software (version 9.0.1).

RESULTS

Environmental conditions of study sites

The river-influenced site (San Antonio) showed, on average, significantly higher SST and pCO_2 and lower values of pH, salinity, and saturation states of aragonite (Ω_a) and calcite (Ω_c), in comparison with the marine site (Las Cruces) (Table 1). Despite the variability over time (Fig. 2), these differences were temporally persistent (i.e. salinity was always lower at San Antonio than at Las Cruces). With the exception of salinity and alkalinity (Pearson r: 0.095 and 0.019, respectively, $p > 0.05$), the remaining environmental variables were temporally correlated between study sites (Pearson r: SST = 0.908; pH = 0.917; pCO_2 = 0.892; Ω_a = 0.933; Ω_c = 0.934; $p < 0.001$ in all cases). Additionally, multivariate analysis indicated that spatiotemporal variability in pH, SST, salinity, and

alkalinity showed a significant discriminatory power between study sites (MANOVA: Wilks' $\lambda = 0.218$; $p = 0.001$). Reclassification analysis indicated that 100% of the samples could be successfully classified to their collection site.

Biological responses of mussels

Mussels from the river-influenced site had higher metabolic rates (0.278 ± 0.036 mg O_2 g^{-1} h^{-1}) than mussels from the marine site (0.086 ± 0.019 mg O_2 g^{-1} h^{-1}; Tukey HSD test: $p = 0.039$). The oxygen consumption rate of individuals transplanted from the river-influenced to the marine site decreased significantly (0.121 ± 0.022 mg O_2 g^{-1} h^{-1}), converging to metabolic rates compatible with those observed in mussels at the marine site (Tukey HSD test: $p = 0.996$). Conversely, no changes were observed in the metabolic rate of individuals transplanted from the marine to the river-influenced site (0.089 ± 0.012 mg O_2 g^{-1} h^{-1}; Tukey HSD test: $p = 0.917$, Fig. 3a). These patterns of variability led to significant differences in metabolism between both local populations (origin: $F_{1,26} = 16.94$, $p < 0.001$, Table 2) and in the interaction term ($F_{1,26} = 7.53$, $p = 0.012$, Table 2).

After 154 d, growth rates of individuals auto-transplanted at the marine site were significantly higher (0.0114 ± 0.0009 mm d^{-1}) than those of mussels auto-transplanted at the river-influenced site (0.0054 ± 0.0008 mm d^{-1}; Tukey HSD test: $p = 0.0006$, Fig. 3b). However, mussels transplanted from the marine to the river-influenced site had 64% lower growth rates (0.0041 ± 0.0005 mm d^{-1}) than mussels auto-transplanted at the marine site (Tukey HSD test: $p = 0.0001$). In contrast, mussels transplanted from the river-influenced to the marine site increased their growth rates (0.0106 ± 0.0007 mm d^{-1}) to a level similar with organisms originally growing in the marine site (Tukey HSD test; $p = 0.944$, Fig. 3b). A similar pattern was observed at 56 d (Fig. S2 in the Supplement). Thus, both the origin (marine and river-influenced population) and destination location (auto-transplant and transplant treatments) are relevant factors in the variation in the growth rate of *Perumytilus purpuratus* in the experiment, as depicted by a significant interaction term in the ANOVA model applied at both 56 d ($F_{1,167} = 24.22$, $p < 0.001$, Table S2 in the Supplement) and 154 d ($F_{1,113} = 33.09$, $p < 0.001$, Table 2) of the experiment.

Net calcification rates of *P. purpuratus* mussels showed significant differences, depending on the origin and destination site of mussels at 56 d ($F_{1,169} = 22.7$, $p < 0.001$, Table S2) and 154 d ($F_{1,118} = 12.96$, $p < 0.001$, Table 2). After 154 d, no significant differences were found between the auto-transplanted individuals at the marine (0.0040 ± 0.0004 mg $CaCO_3$ g^{-1} d^{-1}) and river-influenced sites (0.0027 ± 0.0005 mg $CaCO_3$ g^{-1} d^{-1}; Tukey HSD test; $p = 0.418$). However, a significant 2-fold reduction in net calcification rate was observed in individuals transplanted from the marine to the river-influenced site (0.0018 ± 0.0003 mg $CaCO_3$ g^{-1} d^{-1}), in comparison with individuals from the marine site (Tukey HSD test: $p = 0.011$). Finally, mussels transplanted from the river-influenced to the marine site showed net calcification rates similar to those recorded in mussels auto-transplanted at the marine site (river-influenced to marine site: 0.0042 ± 0.0004 mg $CaCO_3$ g^{-1} d^{-1}; Tukey HSD test; $p = 0.953$; Fig. 3c). A similar pattern was also observed at 56 d (Fig. S2).

Mussel survival was dependent on the treatment to which they were exposed ($\chi^2 = 24.327$, df = 1, $p < 0.001$). Relative survivorship of mussels in auto-transplanted treatments, at both the marine (98%) and river-influenced sites (81%), was high. However, after 154 d, marine individuals transplanted to the river-influenced site showed a significantly lower level of survivorship (46%). Mussels transplanted from the river-influenced to the marine site showed survivorship levels (96%) similar to the auto-transplanted individuals (Fig. 3d).

Shell periostracum morphology and organic composition

SEM analyses of the outer surface of a *P. purpuratus* shell showed that it is covered homogenously by the

Table 1. Mean (± 1 SD) temporal variation in carbonate system parameters recorded at each study site during the reciprocal-transplant experiment with mussels *Perumytilus purpuratus* (May to September 2011). A summary of the paired comparison between sites is presented (**bold** text shows significant p-values at $\alpha = 0.05$). pH_T: pH in total scale; SST: sea surface temperature; A_T: total alkalinity

Parameter	Marine site	River-influenced site	Paired *t*-test	p
pH_T (25°C)	7.79 ± 0.05	7.76 ± 0.06	4.14	**0.002**
SST (°C)	12.50 ± 0.37	12.30 ± 0.43	3.29	**0.011**
Salinity	34.26 ± 0.089	33.47 ± 0.37	6.39	**<0.0001**
A_T (µmol kg^{-1})	2280 ± 15	2290 ± 19	−1.35	0.210
pCO_2 (µatm)	488 ± 70	531 ± 81	−3.92	**0.003**
$\Omega_{calcite}$	2.98 ± 0.32	2.77 ± 0.36	5.13	**<0.0001**
$\Omega_{aragonite}$	1.90 ± 0.21	1.77 ± 0.23	5.42	**<0.0001**

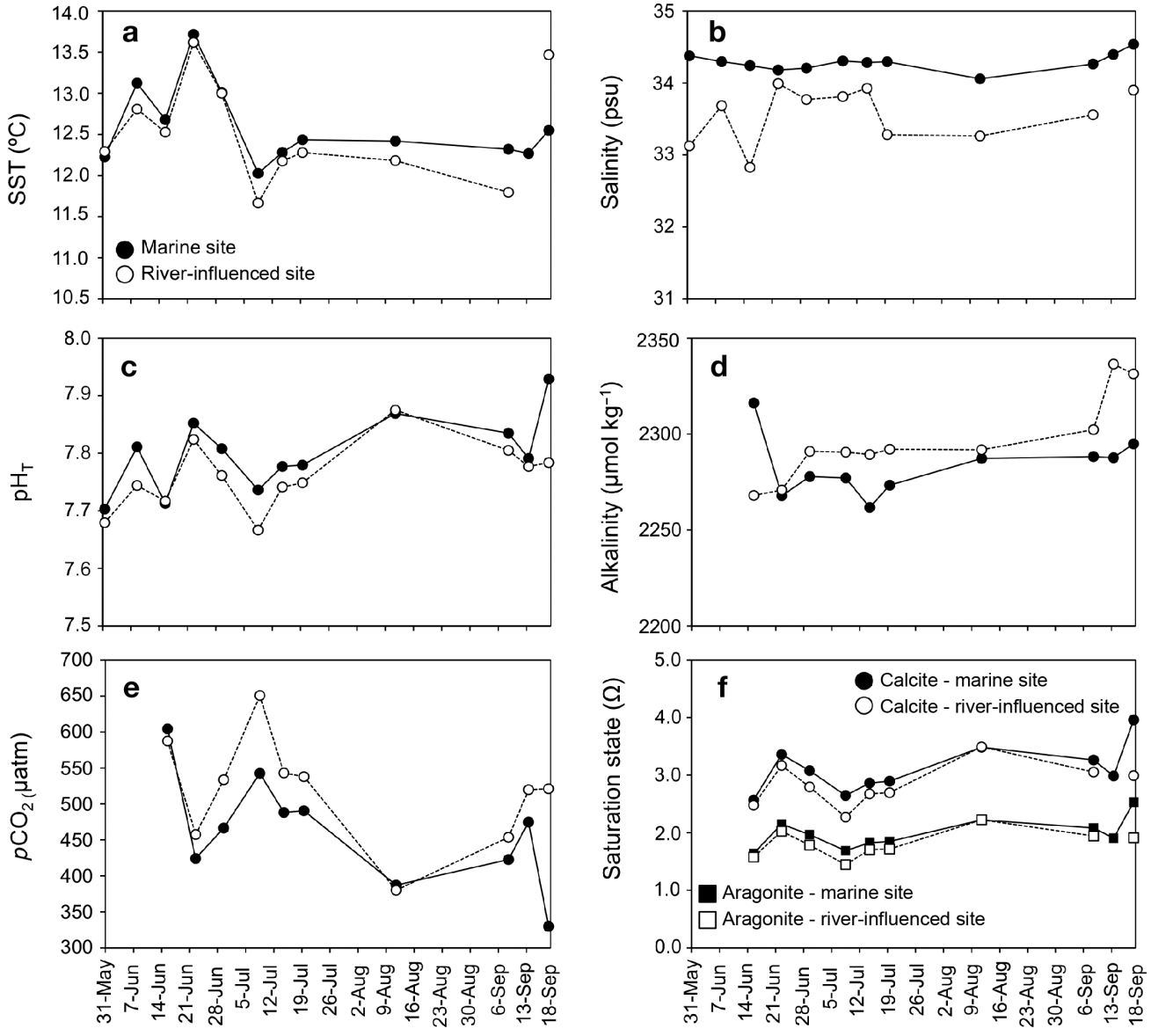

Fig. 2. Time series of environmental conditions in 2011: (a) sea surface temperature (SST); (b) salinity; (c) pH in total scale (pH$_T$); (d) alkalinity (µm kg^{-1}); (e) pCO_2 (µatm); and (f) Ω calcite and aragonite measured at the 2 experimental sites (marine and river-influenced) during the reciprocal-transplant experiment with mussels *Perumytilus purpuratus*

periostracum, which has an inner vesicular structure (Fig. S3 in the Supplement). The less organized part of the shell carbonates is a thin aragonite fibrous layer between the periostracum and the nacre layer that comprises most of the shell thickness. The average thickness of the periostracum estimated from the carbonate signal was ca. 6 microns. The IR spectra of the shell outer surface show amide/protein bands, lipids, sulfates, and polysaccharide bands, which indicates that the periostracum is a proteinous layer rich in sulfated polysaccharides. Additionally, the underlying shell mineral contributes with a band centered at around 1410 cm^{-1} and a peak at 855 cm^{-1}, characteristic of aragonite. In all specimens, the most intense bands were those associated with proteins and polysaccharides, although their relative intensity changed among treatments (Fig. 4). Particularly, specimens influenced by the marine environment showed more intense polysaccharide bands than those that were exposed to river-influenced conditions, which indicates that the periostracum had a partial loss, or secreted less, of this component under river-influenced conditions (Table 3). The presence of relatively stronger carbonate bands in some specimens ex-

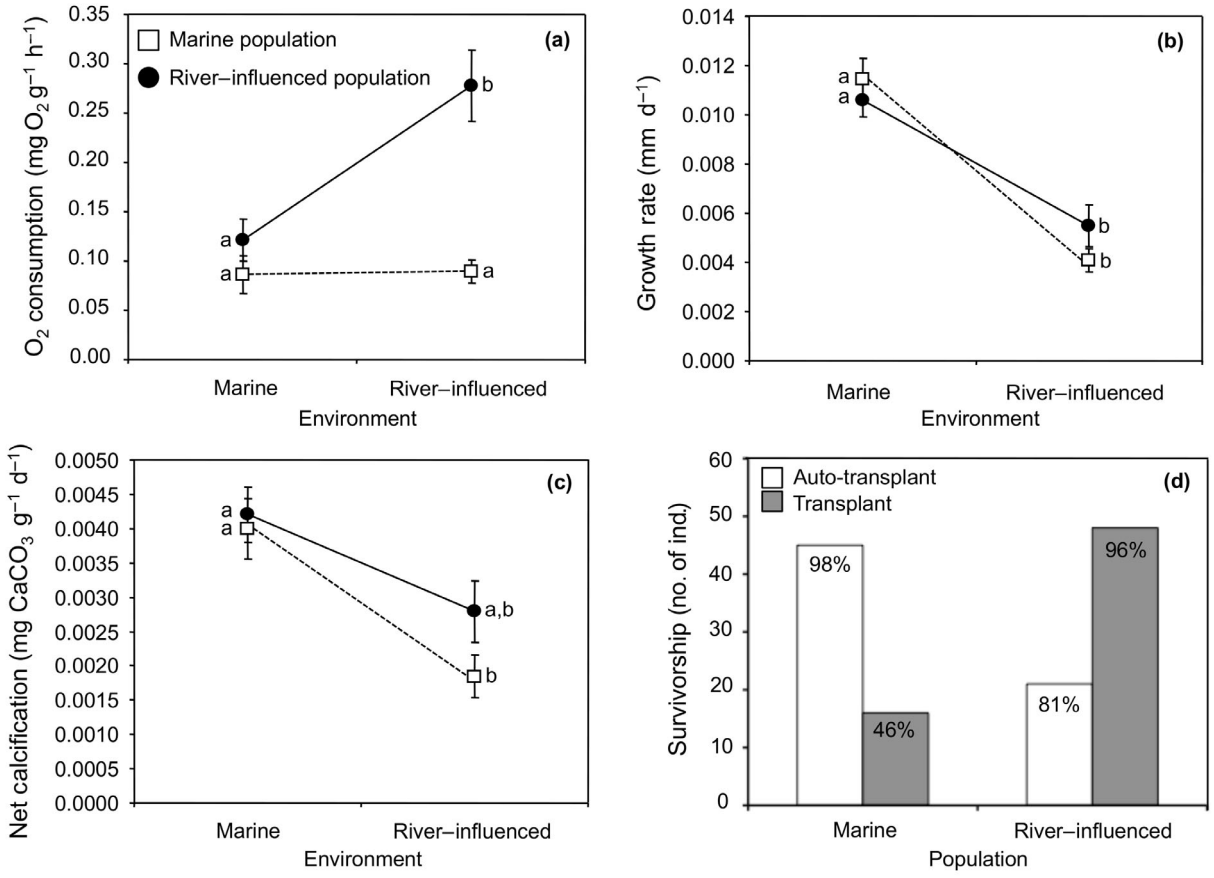

Fig. 3. (a) Metabolic rates measured as oxygen consumption (mg O_2 g^{-1} h^{-1}), (b) growth rate (mm d^{-1}), (c) net calcification rate (mg $CaCO_3$ g^{-1} d^{-1}), and (d) survivorship (individuals and %) of mussels *Perumytilus purpuratus* exposed to marine and river-influenced conditions under a reciprocal-transplant experimental design. Data are means ± SE. Different letters beside each symbol indicate significant differences between experimental treatments evaluated using the Tukey HSD test as a post hoc comparison. Auto-transplant: origin and destination sites were the same; transplant: mussels were moved from their origin site to the destination site

posed to river-influenced conditions indicates that their periostracum is thinner, or has a lower degree of coverage over the shell surface, than that for specimens auto-transplanted or transplanted to the marine site. Additionally, Pearson correlation analyses showed that the intensity of the carbonate IR absorption bands was negatively and strongly correlated with proteins (Pearson $r = -0.784$, $p < 0.001$) and polysaccharides (Pearson $r = -0.501$, $p = 0.002$). This negative correlation is due to the fact that the underlying shell carbonate becomes more exposed and contributes more to the IR spectra as the thickness, or the degree of coverage, of the periostracum (mainly composed of proteins and polysaccharides) decreases.

Table 2. Effects on metabolic, growth, and calcification rates in *Perumytilus purpuratus* mussels depending on origin (marine or river-influenced) and destination site (auto-transplant or transplant treatments) after 154 d. **Bold** text shows significant p-values at $\alpha = 0.05$

Biological responses	Source	df	MS	*F*	p
O_2 consumption (mg O_2 g^{-1} h^{-1})	Origin site (OS)	1	0.747	16.94	**<0.001**
	Destination site (DS)	1	0.185	4.2	0.052
	OS × DS	1	0.332	7.53	**0.012**
	Error	23	0.044		
	Total	26			
Growth rate (mm d^{-1})	OS	1	0.0001	0.19	0.667
	DS	1	0.0006	0.87	0.353
	OS × DS	1	0.0022	33.09	**<0.001**
	Error	113	0.0007		
	Total	116			
Net calcification rate (mg $CaCO_3$ g^{-1} d^{-1})	OS	1	0.0008	2.07	0.152
	DS	1	0.0003	0.85	0.359
	OS × DS	1	0.0051	12.96	**<0.001**
	Error	115	0.0004		
	Total	118			

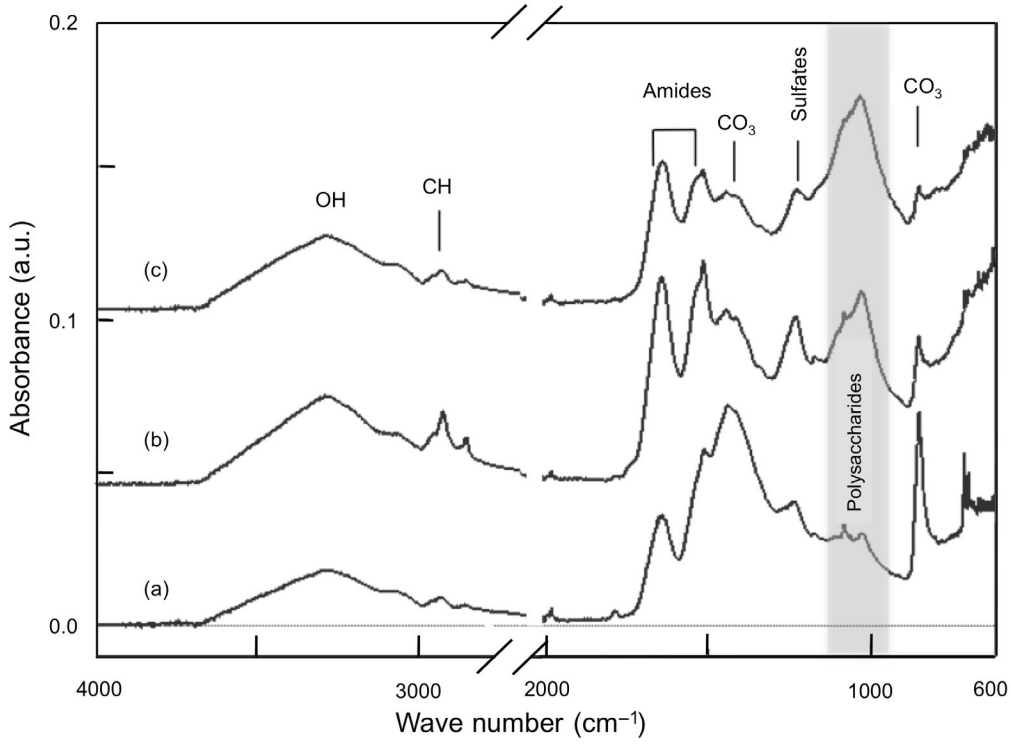

Fig. 4. Attenuated total reflectance – Fourier transform infrared (ATR–FTIR) spectra of the outer surface of the *Perumytilus purpuratus* shell showing peaks associated with proteins, polysaccharides, sulfates, carbonates, and lipids composing the periostracum and carbonate peaks from the underlying shell mineral. Lines represent individuals with (a) a thin periostracum showing strong carbonate bands, (b) a normal periostracum, and (c) a periostracum containing a high amount of polysaccharides

The DFA based on periostracum chemical composition showed a significant segregation between experimental treatments, which provides evidence of changes in the concentration of these compounds with the exposure of the organisms to contrasting environmental conditions (Wilks' $\lambda = 0.66$, $p = 0.034$, Fig. 5). In addition, the 75% confidence ellipses depict a strong overlapping pattern in the periostracum composition in mussels transplanted from the marine to the river-influenced site, converging to a similar ellipse pattern for the mussels auto-transplanted at the river-influenced site (Fig. 5). In contrast, the periostracum composition in mussels transplanted from the river-influenced to the marine site did not overlap with the ellipses of mussels raised at their origin site. There was also a low overlap relative to the ellipses describing the organic composition of mussels raised at the destination marine site (Fig. 5). Thus, these changes in the periostracum composition were evident in the transplanted mussels, of which only 22% on average were correctly reclassified, while up to 65% on average of the auto-transplanted mussels were successfully assigned to their corresponding origin sites (Table 4). Exploratory analyses using a combination of other periostracum components (proteins and lipids) and shell carbonate (CO_3) did not show any significant segregation patterns among experimental treatments (Fig. S4 in the Supplement). Two-way ANOVA determined that transplanted *P. purpuratus* modified the intensity of the carbonate (CO_3)

Table 3. Normalized intensity of main absorption bands of attenuated total reflectance – Fourier transform infrared (ATR–FTIR) spectra of the outer shell surface of *Perumytilus purpuratus* from different experimental sites. These parameters were used to characterize the composition of the shell periostracum. Auto-transplant: origin and destination sites were the same; transplant: mussels were moved from their origin site to the destination site

Compound	Marine site		River-influenced site	
	Auto-transplant	Transplant	Auto-transplant	Transplant
CO_3	0.499 ± 0.173	1.008 ± 0.338	0.996 ± 0.382	0.383 ± 0.130
Polysaccharides	7.370 ± 1.303	4.396 ± 0.177	4.823 ± 0.306	5.360 ± 0.451
Proteins	22.872 ± 2.082	19.760 ± 2.366	18.877 ± 2.562	21.927 ± 1.350
Lipids	3.395 ± 1.168	2.994 ± 0416	2.983 ± 0.704	4.513 ± 1.480

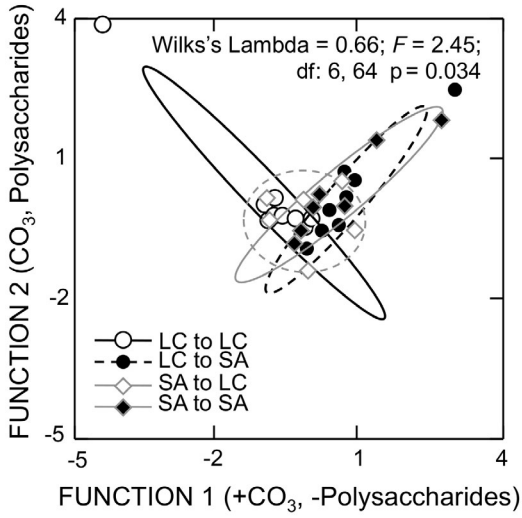

Fig. 5. Discriminant function analysis generated using relative levels (absorbance values from FTIR) of carbonate signals (CO_3) in combination with polysaccharides recorded on the shell periostracum of *Perumytilus purpuratus* for each treatment after 154 d of the cross-reciprocal experiment. Also shown is the summary of multivariate ANOVA. LC: Las Cruces, the marine site; SA: San Antonio, the river-influenced site

signal and that of polysaccharides on their shell periostracum at the destination site (Table 5). These modifications were stronger in mussels auto-transplanted and transplanted to the river-influenced site, in comparison to individuals who had the marine site as their origin and destination site (auto-transplanted and transplanted treatments). Polysaccharides were affected in the opposite way. The lowest band intensities were observed for *P. purpuratus* individuals exposed to the river-influenced site, while the most intense polysaccharide bands were observed in mussels exposed to the marine environment. Concentrations of proteins and lipids were similar in the periostracum of specimens from marine and river-influenced sites, and they were not modified by the destination site after the reciprocal-transplant experiment (see Tables 3 & 5).

DISCUSSION

The 2 habitats studied showed consistent differences in environmental variables, potentially affecting the life history and performance of the intertidal mussel *Perumytilus purpuratus*. Recently, using MODIS satellite imagery, the spatial influence of the Maipo River plume along the San Antonio coast and the lack of river influences at the Las Cruces site have been characterized (Pérez et al. 2015). These authors also described the variability in carbonate system parameters (salinity, pH, pCO_2, dissolved inorganic carbon) for the river-influenced site, which falls within the values observed in our study. Under these subtle, but persistent, environmental differences between study sites, we found significantly increased metabolism, reduced growth rates, and increased mortalities in the mussels raised at the river-influenced site. This clearly suggests the occurrence of stressful conditions at that site. However, as discussed below, in field conditions, the environmental (i.e. salinity, pH) and anthropogenic drivers (i.e. pollution) affecting coastal ecosystems make it difficult to identify the specific mechanisms underlying the biological differences observed in the local mussel populations that we studied. Therefore, our results highlight the potential relevance of river-induced environmental variation upon coastal habitats and the species inhabiting this area.

Environmental variables, with the exception of salinity and alkalinity, were temporally correlated between study sites, suggesting the shared influence of environmental processes operating on scales larger than the distance among sites (e.g. upwelling, Lagos et al. 2005). For instance, during the study period, the SST was variable between sites, but remained relatively stable throughout time (ca. 13°C). However, between June and July (austral winter), we observed a substantial decrease in SST with an increase of pCO_2 in seawater, which led to low pH in waters at both sites. Our results also suggest a spatial

Table 4. Results of re-classification analysis using discriminant function analysis (see 'Materials and methods' for details) applied over the organic components (sugars and carbonate) recorded on the shell periostracum of mussels *Perumytilus purpuratus*. **Bold**: number of individuals correctly assigned to the treatments applied in a reciprocal-transplant experiment

Origin site	Destination site	Treatment	Classified as:				
			Marine to:		River-influenced to:		% correct classification
			Marine	River-infl.	Marine	River-infl.	
Marine	Marine	Auto-transplant	**5**	0	3	1	56
	River-influenced	Transplant	1	**4**	0	4	44
River-influenced	Marine	Transplant	3	5	**0**	2	0
	River-influenced	Auto-transplant	0	1	1	**6**	75

Table 5. Two-way ANOVA summary evaluating differences in organic compounds recorded using FTIR analysis on the shell periostracum of mussels *Perumytilus purpuratus* subjected to a reciprocal transplant. **Bold** text shows significant p-values at $\alpha = 0.05$

Compound	Origin site		Destination site		Interaction	
	$F_{1, 33}$	p	$F_{1, 33}$	p	$F_{1, 33}$	p
CO_3	0.06	0.807	4.63	**0.039**	0.04	0.844
Polysaccharides	1.06	0.311	5.22	**0.029**	2.51	0.122
Proteins	0.19	0.665	2.17	0.150	0.01	0.988
Lipids	0.26	0.615	0.78	0.383	0.27	0.608

decoupling between sites in terms of the underlying processes driving variations in salinity and alkalinity. For instance, at the end of the experiment, we observed an increase in alkalinity at the river-influenced site, which corresponds with the onset of the seasonal increase in river flows and suggests a strong spatial differentiation in the processes affecting alkalinity at this site. This increase in alkalinity can be associated with the increased exportation of the compound that alters the biogeochemical properties of the rivers (Raymond & Cole 2003), which is linked to industrial and agricultural land-use changes, as in the case of the Maipo River (Pérez et al. 2015). Because our field experiment was performed during the reduced river flow season, with less freshwater discharge, it can be expected that these environmental differences between sites would become exacerbated for periods of increased river discharge at the coast.

Rivers are an important link between terrestrial and coastal ecosystems. The flux of elements and materials introduced into the Maipo River through run-off could be discharged at the shore, inducing spatial variability in the physiological responses observed in *P. purpuratus*. For instance, Pizarro et al. (2010) detected high concentrations of heavy metals in the Maipo River, and high levels of As, Hg, Pb, and phenanthrene were detected in soft parts of the bivalve *Mesodesma donacium* inhabiting the discharge area (Diaz et al. 2008). High concentrations of heavy metals dissolved in seawater have significant impacts on the physiology and fitness of bivalves (e.g. Sonawane 2015). However, in several species, lethal doses of heavy metals (Bryant et al. 1984) and physiological stress of mussels (Tedengren et al. 1999) are reduced at lower salinities. This suggests that low salinity due to freshwater discharge may confer resistance to benthic organisms when confronting negative effects of metals in seawater. In addition, low salinity gradients correlate with low pH values. Several studies have reported that pH levels can change the speciation of metals, modifying their interactions with marine organisms (Millero et al. 2009, Roberts et al. 2013), and thus the toxicity level of heavy metals and their effects on growth and physiology (Bibby et al. 2008). However, heavy metals, as well as their interaction with salinity and pH, were not explored in the present study. Thus, the biological responses observed in both mussel populations after the experiment may have resulted from a combination of both environmental (i.e. salinity) and anthropogenic (i.e. pollution) drivers. In addition, river discharges generate variability in the nutrients (Yin et al. 2000) that, together with light and vertical mixing, affect the primary productivity of the coastal habitat receiving the river discharges (Lu & Gan 2015). Variability of nutrients is important, because food availability may confer resistance to stressful conditions such as salinity (Fernández–Reiriz et al. 2005) or pH (Melzner et al. 2011, Ramajo et al. 2015, 2016). However, in the study area, high variability in the concentration of chl *a* has been reported for the Maipo River plume and the marine site, with major differences in terms of the body size of the plankton groups and food concentration (Vargas et al. 2006). Finally, in addition to these environmental variations modulating eco-physiological responses in benthic organisms, biological interactions may also affect our field observations. For instance, phenotypic plasticity or changes in shell thickness of *P. purpuratus* mussels has been associated with the presence of predators in intertidal habitats (Manriquez et al. 2013). We currently lack information about biotic differences between the study sites. However, it is also possible that an increase in abundance of crab predators at the river-influenced site is due to the ability of crustaceans to compensate for acidosis by increasing bicarbonate supplied by the dissolution of the exoskeleton under low pH conditions (Spicer et al. 2007). The above studies and our observations suggest that disentangling the relative importance of river-induced environmental variability and interactions with anthropogenic components and/or biotic forces upon marine habitats is a complex task. Therefore, additional studies considering these stressors are needed for a better understanding of their relative roles in driving the ecophysiological responses of marine organisms.

In addition to complexities associated with river influences on coastal habitats, we found significant differences in the subset of biological responses

measured in the 2 local populations of *P. purpuratus*. For instance, mussels native to the river-influenced site presented metabolic rates 3 times higher than those growing at sites unaffected by the discharge. The higher metabolic rates of mussels at the river-influenced site, combined with lower growth rates, suggest that this population is affected by stressful conditions incurring additional energetic costs to maintain homeostasis. Changes in seawater salinity are considered among the most important environmental stressors contributing to organism distribution in intertidal zones (Wrange et al. 2014). Mollusks are generally osmo-conformers, and their resistance to salinity is based on impeding the water–salt exchange with the external medium through hermetization of the mantle cavity (Berger & Kharazova 1997). Since bivalves are unable to perform extracellular osmoregulation, their internal osmotic concentration changes rapidly with changes in external salinities (Landes et al. 2015), which impacts additional physiological responses such as respiration rates that, for instance, increase linearly with decreasing salinity for the bivalve *Mytilus edulis* (Stickle & Sabourin 1979). Also, fluctuations in salinity and consequent alterations in the intracellular osmolality have important effects on several traits, including filtration, growth, size, survival, development, and behavior (e.g. Almada-Villela 1984, Gruffydd et al. 1984, Hamer et al. 2008). For instance, *M. edulis* (Malone & Dodd 1967), coralline algae (King & Schramm 1982), and coccolithophorids (Paasche et al. 1996) showed rapid calcification rates in high-salinity environments. By modifying Ca^{2+} concentrations and total inorganic carbon (Hofmann et al. 2009), salinity may also impact the energy budgets (Hamer et al. 2008) and biomineralization processes of marine calcifiers (Dickinson et al. 2013). In addition to salinity and temperature changes, increased carbon dioxide (CO_2) and low pH levels also affect the performance of marine calcifiers (Kroeker et al. 2013) and physiological responses, such as metabolic rates (Basso et al. 2015). In CO_2 vents (i.e. Ischia Island, Italy), higher metabolic rates were detected in polychaetes (Calosi et al. 2013) and the bivalve species *Pinna nobilis* (Basso et al. 2015) in response to naturally elevated CO_2 conditions. These field observations are in agreement with an increase in metabolic rates under experimental exposure to high-CO_2 conditions for several benthic taxa, including echinoderms (Wood et al. 2010), gastropods (Lardies et al. 2014), and bivalves (Beniash et al. 2010, Lannig et al. 2010, Duarte et al. 2015). We also found higher variation in the metabolic rates among individuals from the river-influenced site than in mussels from the marine population. A similar result has been reported for the bivalve *P. nobilis* at the lowest pH levels under field conditions (Basso et al. 2015). Pistevos et al. (2011) showed that intra-population variability should not be treated as 'noise' because a portion of this variation actually provides important information about underlying genetic diversity and the potential adaptation of populations.

Metabolic upregulation in mussels and other marine invertebrates involves important energetic costs (Wood et al. 2008, Thomsen & Melzner 2010, Lardies et al. 2014), thereby affecting the energy available for maintenance and other physiological functions. This could explain the higher mortalities and the low growth rates observed in mussels raised at the river-influenced site (both auto-transplanted and transplanted treatments), in comparison with mussels raised in marine conditions. Studies on 'estuarine acidification' demonstrate that low pH levels lead to high mortality in oysters *Saccostrea glomerata*, which is attributed to acid-induced shell degradation (Dove & Sammut 2007). In similar freshwater-influenced areas, recent studies have indicated that both oysters and gastropods are less abundant and are small in size at sites characterized by low pH levels (Amaral et al. 2011), which leads to corrosion and reduced shell strength, thereby increasing the susceptibility of oysters to be preyed upon (Amaral et al. 2012). These results indicate that spatial variability in physical-chemical variables typically observed in coastal areas affected by freshwater river discharges may also generate spatial patterns in benthic populations and communities, both along estuarine and in river-plume influenced coastal areas (Salisbury et al. 2008, Amaral et al. 2011). However, the absence of statistically significant differences in calcification rates between river-influenced and marine *P. purpuratus* populations (comparing auto-transplant treatments) could be due to the existence of trade-offs between metabolic and calcification rates. Our results suggest that mussels inhabiting the river-influenced site were successful in maintaining calcification rates (under low salinity and pH conditions) with re-allocation of energy from other physiological processes (i.e. increasing metabolic rate), which further suggests that lower calcification rates are not necessarily an inevitable consequence of pH-related environmental stress (Findlay et al. 2011).

In our study, both mussel populations (river-influenced and marine) responded differently to being transplanted to a different environment. Marine mussels transplanted to the river-influenced site

experienced a significant increase in mortality, and low growth and calcification rates. These observations suggest reduced phenotypic plasticity. Therefore, more stable conditions at the marine site have the potential to promote local adaptation in the studied intertidal mussel populations. Furthermore, metabolic rates of marine-influenced mussels remained unchanged after experiencing river-influenced conditions for more than 5 mo, thus indicating a lack of plasticity in their response to variable environmental conditions and supporting the notion of potential for local adaptation to their settlement habitats. In contrast, river-influenced mussels transplanted to the marine site underwent a reduction in their metabolic rates, which was accompanied by an increase in growth and calcification rates. These plastic responses are expected when organisms, already affected by variable and stressful conditions (natural and anthropogenic) in their native habitats, experience a shift in environmental conditions (see Duarte et al. 2015). These observations indicate that the river-influenced mussel populations, unlike marine populations, are acclimated to variable environmental conditions and therefore possess the capacity for rapid and reversible phenotypic transformation in physiology (e.g. adjusting the metabolic rate), with remarkable implications for other physiological responses (i.e. growth and calcification rates). Phenotypic plasticity (i.e. environmentally induced changes in physiological, morphological, or life-history traits, see Stearns 1989) is likely to play an important role in the ability of species to cope with uncertain environmental changes (Gienapp et al. 2008), buffering against selection and, thus, reducing opportunities for the evolution of local adaptations (Miller et al. 2009).

The mussel periostracum is mainly composed of quinone-tanned proteins, chitin, a polysaccharide, muco-polysaccharides, and lipids (Hillman 1961, Peters 1972, Nakayama et al. 2013), which makes this coating waterproof (Taylor et al. 1969). The shell periostracum acts as a protective organic layer that prevents mineral dissolution in acidic waters (Ries et al. 2009, Tunnicliffe et al. 2009, Thomsen et al. 2010, Rodolfo-Metalpa et al. 2011), which can play a very important role in confronting stressful conditions from freshwater acidic inputs (Duarte et al. 2013, Waldbusser & Salisbury 2014). Recent studies have shown partial loss of periostracum and/or fractures in bivalves exposed to corrosive experimental and field conditions (e.g. Thomsen et al. 2010, Gazeau et al. 2013). However, there are no reports about how shifts in natural environmental conditions alter or damage the periostracum. We found that the *P. purpuratus* periostracum composition is affected significantly by changes in environmental conditions when the mussel is transplanted to river-influenced conditions, resulting in periostracum loss (i.e. enhancing the CO_3 signal) and a reduction of the amount of polysaccharide in this organic coating, thus rendering shell carbonate more vulnerable to corrosion. An opposite pattern was observed for mussels transplanted to marine conditions. Thus, our results suggest that changes observed in net calcification rates (for mussels in transplanted treatments) could be related to changes in the amount and/or composition of the periostracum, which plays a key role in protecting shells from dissolution in low (Waldbusser et al. 2011) and under-saturated carbonate conditions (Gazeau et al. 2013). Lastly, the protective role of the periostracum is likely to be especially important in the fully aragonitic shell, such as that of *P. purpuratus* (Taylor et al. 1969), due to the fact that aragonite is a carbonate polymorph more soluble than calcite (Mucci 1983). However, other factors, such as crystal size or organic matrix content, also determine the solubility of the minerals (Harper 2000). The results indicate that the periostracum thickness (inversely related to the CO_3 signal) and composition (amount of polysaccharides) play an important role in the observed adaptive biological responses (plasticity) to the transplant treatments. Additionally, experimental studies have shown that low pH conditions have an impact on mantle gene expression related to shell and periostracum formation (Hüning et al. 2013). Their results showed some trade-offs, including a decrease in the expression of several genes involved in metabolism and calcification (chitinase), which were compensated with the over-expression of tyrosinase, the gene involved in the organic matrix and periostracum formation. Our results show the importance of the organismal energy budget in the reparation and formation of the periostracum layer, which is likely to explain the lack of statistically significant differences between river-influenced and marine populations in the organic composition of the periostracum, in contrast with strong changes when the organisms were transplanted.

The results presented here demonstrated that local populations of *P. purpuratus* experiencing different environmental conditions show high phenotypic variation in calcification, growth, and metabolic rates, as evidenced by intraspecific variability in the response of populations to persistent freshwater inputs. The differences between populations are probably due to local adaptation and acclimation to the environment, most likely seen in stable and less pol-

luted marine habitats. On one hand, marine mussels transplanted to the river-influenced site decreased their growth and calcification rates and, most importantly, experienced increased mortality rates, which likely reflects the costs of the local adaptation process. On the other hand, the variation in metabolic rates and periostracum organic composition showed that individuals had a different response when transplanted from the river-influenced to the marine site unaffected by the river, which suggests that *P. purpuratus* is acclimatized to the river-influenced conditions and the response in its biological processes is related to phenotypic plasticity. In a similar reciprocal experiment, oysters originating at sites with the lowest salinity demonstrated the potential for local adaptation within an estuary, along with increased tolerance and survival (Bible & Sanford 2016). These results imply that populations exposed to higher environmental variability could be more resilient to future climate projections (e.g. decreased precipitation regime, changes in land use, eutrophication, ocean acidification) than their counterparts inhabiting less variable environments. Climate change projections along the Chilean coast suggest a reduction in freshwater inputs (CEPAL 2012), which may imply a corresponding reduction in freshwater-induced responses or phenotypic plasticity in coastal populations. An increasing dominance of marine conditions along the Chilean coast in the future may lead to increased local adaptation in mussel populations, thus compromising their ability to cope, for instance, with changes in pH due to stochastic increases in ocean acidification common in upwelling ecosystems (Feely et al. 2008). Lastly, recognizing that different local populations employ different strategies (i.e. adaptation or phenotypic plasticity) to cope with changing environmental conditions, and accepting that this will have different ecological and evolutionary implications, will enable us to better understand and predict the impacts of changes in precipitation patterns and the resulting freshwater inputs upon coastal populations.

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