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Riverine discharges impact physiological traits and carbon sources for shell carbonate in the marine intertidal mussel *Perumytilus purpuratus*

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Abstract

Anthropogenic modification watersheds and climate change have altered export from fluvial systems causing changes to the carbonate chemistry of *river-influenced* near shore environments. To determine the possible effects of riverine discharges on the mussel *Perumytilus purpuratus*, we performed in situ transplant experiments between *river-influenced* and *open coastal* habitats with contrasting seawater carbonate chemistries (i.e., pCO_2, pH, Ω_{ar}) across four regions covering a wide latitudinal range (32°55′S–40°10′S). The *river-influenced* habitats selected for transplant experiments were different than *open coastal* habitats; with higher pCO_2 (354–1313 μatm), lower pH (7.6–7.9) and Ω_{ar} values (0.4–1.4) than in *open coastal* area. Growth, calcification, metabolism were measured in a reciprocal transplant experiment to determine physiological responses associated with *river-influenced* sites and non-influenced control sites. Growth and calcification rates were higher in *river-influenced* habitats; however the organisms in this area also had lower metabolic rates, possibly due to enhanced food supply from river systems. Further analysis of carbon isotopic composition ($\delta^{13}C$) indicated that the relative contribution of seawater dissolved inorganic carbon (DIC) to the carbonate shells of *P. purpuratus* was much higher than respiratory carbon. Nevertheless, *P. purpuratus* incorporated between 7% and 26% of metabolic carbon in the shell depending on season. There was a strong, significant relationship between $\delta^{13}C_{POC}$ and $\delta^{13}C_{Tissue}$, which likely influenced the isotopic composition of the shell carbon.

The coastal zone, which extends seaward from the land to the inner continental shelves, represents a small fraction of the total ocean surface (~ 7%) (Gattuso et al. 1998; Laruelle et al. 2010). Significant environmental and economic value is contained within the globally small coastal zone. However, the importance of this oceanic region stems not only from the goods and services it provides (Costanza et al. 1997), but also from its contribution to global biogeochemical cycling of carbon and nutrients (Crossland et al. 2005). Coastal ecosystems are very biologically productive and concentrate a significant fraction of marine biodiversity (Miller et al. 2009) supporting roughly 10–30% of total global marine primary productivity and ~ 90% of all global fisheries (Gattuso et al. 1998; Wollast 1998; Crossland et al. 2005). These dynamic environments are influenced by terrestrial, atmospheric and oceanic processes providing many challenges to understanding these systems (Cai 2011; Canuel et al. 2012).

Industrialization has increased the atmospheric CO_2 levels by roughly 30% since the preindustrial era (e.g., Feely et al. 2004; Sabine et al. 2004b; Orr et al. 2005; Feely et al. 2008) with about a third of this anthropogenic CO_2 being dissolved in the world's oceans (Sabine et al. 2004a; Sabine and

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Feely 2007). The increase in atmospheric pCO_2 has altered the global marine carbonate chemistry system by altering the distribution of reactive inorganic carbon species thus reducing pH values ($\sim$ 0.1 unit since the pre-industrial) and the saturation state for calcium carbonate minerals (Ω) (e.g., Feely et al. 2004; Orr et al. 2005; Gazeau et al. 2007; Fabry et al. 2008; Harris et al. 2013). While the open ocean has experienced acidification in lock step with atmospheric CO_2 concentrations, coastal areas can be acidification hot-spots due to additive effects of atmospheric CO_2 with other environmental drivers such as freshwater discharge and/or coastal upwelling (Gattuso et al. 1998; Salisbury et al. 2008; Duarte et al. 2013; Harris et al. 2013; Waldbusser and Salisbury 2014). Moreover, anthropogenic alteration of river basins has further influenced the natural export of water, nutrients, and carbon to estuarine and coastal marine ecosystems (e.g., Beman et al. 2005; Raymond et al. 2008; Savage et al. 2010; Regnier et al. 2013; Wheatcroft et al. 2013). The carbonate chemistry of riverine-influenced near shore environments is therefore affected by lower salinity and resultant decreased alkalinity, eutrophication and resultant production/respiration cycles, and terrestrial particulate organic carbon loading and resultant changes in respiration (Gattuso et al. 1998; Salisbury et al. 2008; Kelly et al. 2011).

The changes noted above in seawater carbonate chemistry can affect the physiology of marine organisms (e.g., Berge et al. 2006; Kleypas et al. 2006; Fabry et al. 2008; Kroeker et al. 2013; Chambers et al. 2014; Waldbusser et al. 2015b). One particularly sensitive physiological trait is biocalcification in marine invertebrates; many of which are common to rocky shore areas (e.g., Gazeau et al. 2007, 2013; Kurihara et al. 2007, 2008). In near-shore coastal areas associated with rivers, riverine discharge may lower saturation state, due to lower salinity and increasing acidity, often leading to $CaCO_3$ undersaturation in river dominated coastal areas (e.g., Salisbury et al. 2008; Aufdenkampe et al. 2011; Duarte et al. 2013; Waldbusser and Salisbury 2014). Decreases in calcium carbonate saturation make biocalcification more energetically expensive (Waldbusser et al. 2013, 2015a) and decreasing saturation states lowers carbonate mineral precipitation rate (Burton and Walter 1987).

It is well documented that during calcification, organisms incorporate both dissolved inorganic carbon (DIC) and respiratory carbon (McConnaughey and Gillikin 2008), and the stable isotope of carbon in $CaCO_3$ can be used to determine the relative contribution of these different carbon sources (McConnaughey et al. 1997). For many marine invertebrates the environmental (DIC) comprises the bulk fraction of the total shell carbonate (Lorrain et al. 2004; McConnaughey and Gillikin 2008). Recent estimates however, for some bivalve species (Gillikin et al. 2007; Wanamaker et al. 2007; Gillikin et al. 2009), have shown that relative amount of respiratory carbon incorporated into the shell may reach up to 35% (Gillikin et al. 2009) depending on ontogeny, growth rate, available food, different habitats and environmental conditions (Lorrain et al. 2004; Wanamaker et al. 2007; Gillikin et al. 2009; Lartaud et al. 2010; Waldbusser et al. 2013).

In this article, we evaluate the relative contribution of DIC sources (environmental vs. metabolic) for shell formation of a local mussel population (*P. purpuratus*). Furthermore, we test whether exposure to coastal waters influenced by riverine discharge affects differentially the growth, calcification, and metabolism in local mussel populations through an in situ transplant experiment between *riverine-influenced* and *open coastal* habitats. The results of both sets of measurements illuminate how changes in coastal hydrology can compound or mitigate other climate change related stressors, such as ocean acidification.

Materials and methods

Study area

The present study was conducted in the coastal zone of central-southern Chile (32°55′–40°10′S) close to the Maipo, Rapel, Biobío, and Valdivia Rivers (i.e., henceforth *rivers*) (Fig. 1). The watersheds feeding these rivers range in area between 10,275 and 24,264 km^2 (Piñones et al. 2005; Pizarro et al. 2010) and whose regional climate vary latitudinally from temperate Mediterranean with a long dry season (summer, mostly January to February) in the north, to temperate rain in the south (winter, mostly July to August) (DGA-Dirección General de Aguas 2004*a,b,c,d,e*). As a result of this regional climate variability, monthly precipitation (2.4 ± 6.2 to 130.4 ± 93.8 mm) and river discharges (46.3 ± 28.3 to 720.3 ± 451.1 m^3 s^{-1}) increase to the southern part of this study area, and river water temperature (17.6 ± 4.1 to 13.9 ± 3.2°C) decrease to the southern (Pérez et al. 2015). Furthermore, riverine concentrations of dissolved nutrients (orthophosphate, PO_4^{3-}: 13.35 ± 7 to 0.07 ± 0.13 μmol L^{-1}; nitrate + nitrite, NO_3^- + NO_2^-: 86.3 ± 22.3 to 3.3 ± 3 μmol L^{-1}) and dissolved carbon (dissolved organic carbon, DOC: 213.1 ± 46.1 to 32.4 ± 18.9 μM; DIC: 4097.6 ± 647.7 to 789.2 ± 153.8 μM) decreases southward, mostly associated with different river land uses and carbonate rock dominated watershed (Pérez et al. 2015).

We selected two rocky shoreline habitats associated with each of the four major river mouths: a *river*-influenced area (i.e., area influenced for river discharge) and *open coastal* area (i.e., area not influenced of river discharge) to conduct our study. The spatial definition of these habitats was based on the use of high resolution MODIS imagery (500 m) of normalized water-leaving radiance at 555 nm (nLw555) which is often used as a proxy for water column turbidity and therefore provided a means of tracking the path and spatial variation in riverine discharge (Saldías et al. 2012) (Fig. 1). Here we used daily nLw555 images for each *watershed* (adjacent coast to the river basins) for producing average composites of river plume influence. All MODIS images were processed

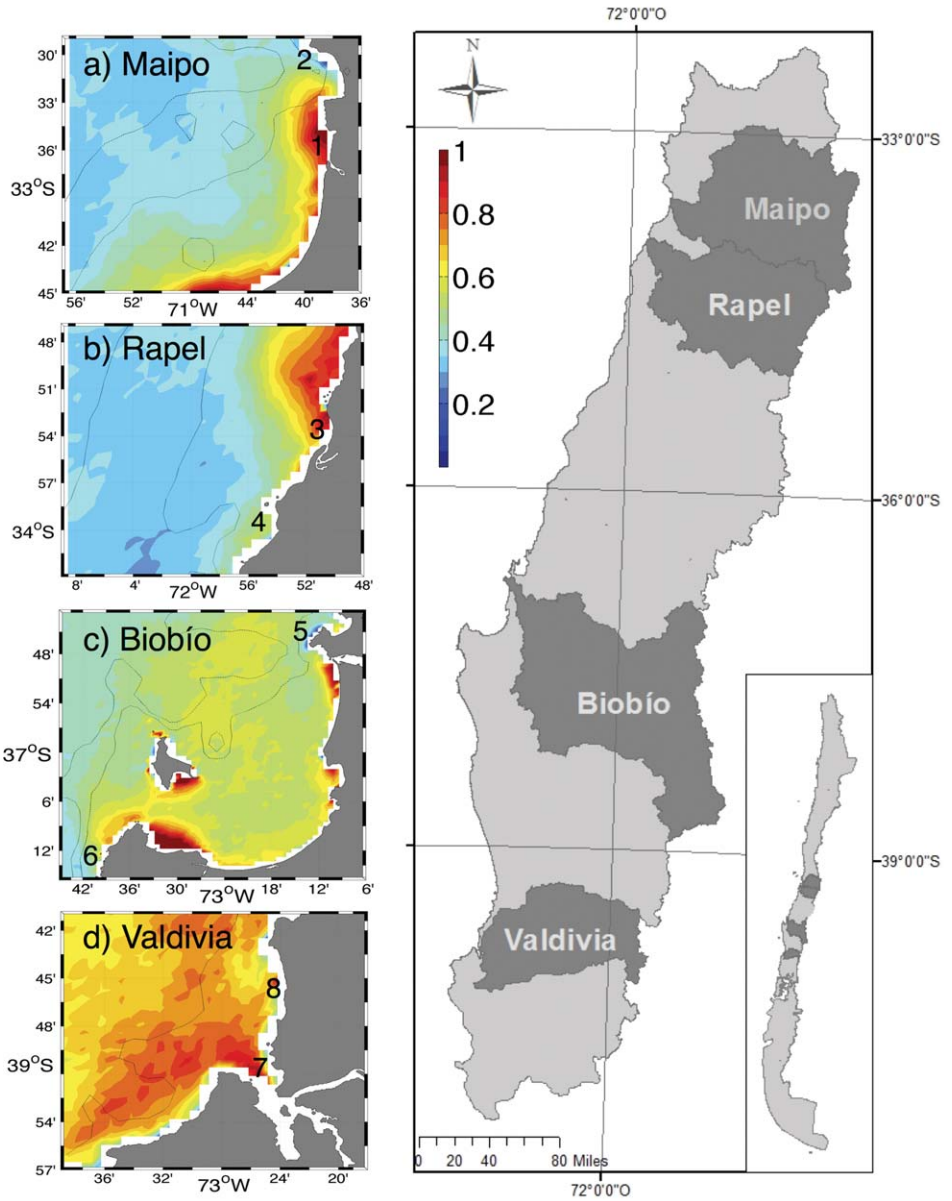

Fig. 1. Map showing the locations of transplant experiments in adjacent coastal area to of (**a**) Maipo, (**b**) Rapel, (**c**) Biobío and (**d**) Valdivia *river*. Numbers represent the location of experiments, (1) *river-influenced* areas and (2) *open coastal* areas. In addition, composites of nLw555 are presented for case. These composites have been normalized by the average values at the corresponding river mouth, so that they represent relative fields (percentage) of turbid plume influence with respect to the river mouth. Finally, the 100 m and 200 m isobaths are also included (black lines) to show the main variability of the shelf bottom topography. For Valdivia, the 50 m isobaths is shown as the 100 m and 200 m isobaths are further offshore than the domain in consideration.

using NASA's software SeaDAS (SeaWIFS Data Analysis System). A more detailed explanation regarding processing options including atmospheric corrections and flags can be found in Saldías et al. (2012). Finally, the nLw555 fields were normalized by the average intensity of pixels corresponding to the location of the river mouths to produce relative coastal maps of river plume influence the fraction of plume signal with respect to the river mouth (Fig. 1).

Environmental characterization

Seawater samples were collected from each *river-influenced* and *open coastal* habitat associated with each *river* for environmental characterization. Samples from *river-influenced* areas were collected monthly from September 2011 to June 2012, coinciding with the collection of mussels for the isotopic analysis on tissue and shells. In contrast, samples from the *open coastal* habitats were collected only during July of

2012, due to logistical problems. Surface water measurements of salinity and temperature in each habitat were conducted with a CTD (Model Ocean Seven 304) during each sampling. All pH are reported on the total scale (pH_T), measured with an Metrohm 713 pH meter connected to a combined electrode, and calibrated using a tris and 2-aminopyridine buffers at 25.0 ± 0.1°C (pH 8.089 and 6.786; Clayton and Byrne 1993; Doe - U. S. Dept. of Energy 1994). For measurements of total alkalinity (A_T), water samples were collected in borosilicate glass bottles and poisoned with mercuric chloride, and the analysis was performed using automated potentiometric titration (Haraldsson et al. 1997). For the *river-influenced* habitats, the total alkalinity, pCO_2 and aragonite saturation state (Ω_{Ar}) were calculated from the pH, DIC (described below), salinity and temperature data using CO2SYS (Lewis and Wallace 1998). For the *open coastal* habitats, DIC, pCO_2 and Ω_{Ar} were calculated from the pH, total alkalinity, salinity, and temperature data. The carbonic acid constants used were those of Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

Mass concentration and isotopic composition of seawater carbon

For measurements of DIC, particulate organic carbon (POC) and $\delta^{13}C$; 40 mL samples were collected from the *river-influenced* sites of each *river* between September 2011 and June 2012 with a sterile syringe and filtered through a Swinnex® filter holder (25 mm) containing a pre-combusted (4–5 h at 450°C) 0.7 μm GF/F filter. The samples were collected directly in 40 mL I-CHEM® 200 Series glass vials, preventing the formation of any bubbles. Because we placed a higher priority on measuring the $\delta^{13}C$ rather than the concentration of DIC to get accurate estimates of shell carbon sources, we did not treat our DIC samples with mercuric chloride since this has been shown to could alter $\delta^{13}C$ estimates (Vargas et al. 2013). Instead we chose to filter seawater samples intended for the isotopic analysis of DIC and preservation in cool conditions according to recommendations made by Li and Liu (2011). We also used butyl rubber septa to further prevent diffusion of CO_2 and, therefore, unwanted fractionation of the DIC samples during storage and refrigerated all samples at 5°C until analysis (i.e., within 30 d from collection). For the analysis of the concentration and $\delta^{13}C$ of POC, 0.5–2.5 L was filtered through pre-combusted GF/F filters (4–5 h at 450°C), dried at ~ 60°C for 24 h, and held in a desiccator until later being twice analyzed on an OI Analytical TIC-TOC Analyzer Model 1030 interfaced with a Finnegan Mat Delta Plus isotope ratio mass spectrometer for analysis by continuous flow (CF-IRMS). The first analysis was to determine the concentration of DIC and DOC (or POC) in water samples, and the second analysis was to the $\delta^{13}C$ isotope of carbon pool in question. Data were normalized using internal standards. The analytical precision was 2% for the DIC and POC measurements, and ± 0.2‰ for the $\delta^{13}C$ measurements. All analyses of mass concentration and isotopic composition were performed in the G. G. Hatch Stable Isotope Laboratory, at the University of Ottawa, Canada.

Study organism

This study is focused in *P. purpuratus* (Lamarck, 1819), a marine mussel common to rocky intertidal habitats ranging from the tropical latitudes of Ecuador to the Strait of Magellan at the southern tip of Chile and then extending northward along the Atlantic coast of Argentina to Santa Cruz (Osorio and Bahamonde 1968). *P. purpuratus* play a key ecological role in rocky shore environment giving habitat for numerous others intertidal organisms (Gutiérrez et al. 2003) as well as providing an important link between primary producers and upper trophic levels (Range et al. 2011). Shell thickness in *P. purpuratus* can be negatively affected by population density, which is expected for such a gregarious species with high intraspecific competition (Briones et al. 2014). (L. Ramajo pers. comm).

Isotopic analyses of mussel tissue and shell

For the analysis of $\delta^{13}C$ in mussel tissue and shell, we randomly selected two live *P. purpuratus* individuals (~ 24 cm) every other month at low tide from *river-influenced* areas of each *river* in September and November 2011 as well as in January, March, and May 2012. Soft tissue was extracted from each individual with a scalpel, stored in a micro-centrifuge tube, and then frozen (−20°C). Before being analyzed, samples were dried to 60°C for 24 h and homogenized with a mortar, exposed to HCl fumes to remove any carbonate or shell material accidentally removed during the tissue extraction process, and finally stored in a desiccator. All shell samples were cleaned, then washed with distilled water, and dried in the air and pre-combusted to 360°C for 24 h after which a scalpel was used to obtain a subsample of outer shell margin (i.e., the most recently formed shell material). The subsamples were ground with an agate mortar, weighed and stored in a microcentrifuge tube. The isotopic composition of tissue samples were made by analysis of CO_2 generated by combustion on an Elemental Isotope Cube Elemental Analyser followed by analysis in CF-IRMS, with an analytical precision ± 2‰. The isotopic composition of shell samples were analysed in continuous flow on the Thermo Finnigan GasBench copupled to an IRMS; however, shell samples also further analysed for their oxygen isotopic signal in the same machine ($\delta^{18}O_{Shell}$). The percent contribution of carbon to formation of the *P. purpuratus* shell was calculated from the carbon isotope data according to McConnaughey et al. (1997):

$$\delta^{13}C_{Shell} = R\delta^{13}C_{Resp} + (1-R)\delta^{13}C_{DIC} + \Delta \quad (1)$$

where R is percentage of inorganic carbon from respiration (assuming the rest is solely from DIC), $\delta^{13}C_{Resp}$ is isotopic signal of tissue and is used as a proxy the isotopic composition of respiratory carbon (McConnaughey et al. 1997;

Lorrain et al. 2004; Gillikin et al. 2007), $\delta^{13}C_{DIC}$ is isotopic composition of environmental DIC, $\delta^{13}C_{Shell}$ is isotopic signal from shell and Δ is the isotopic enrichment that occurs during the precipitation of calcite or aragonite relative to ambient seawater DIC (Romanek et al. 1992; McConnaughey et al. 1997; Gillikin et al. 2006a; McConnaughey and Gillikin 2008). Recent studies on *P. purpuratus* have shown that shell mineral composition is based completely on aragonite with no calcite present in the shell (L. Ramajo pers. comm). We therefore used an enrichment factor $2.7 \pm 0.6‰$ in this study as the fractionation between DIC and aragonite (Romanek et al. 1992).

Field experiment

In situ transplant experiments were carried out in the rocky shore habitats associated with the Maipo, Rapel, Biobío and Valdivia Rivers (*rivers*) (Fig. 1). *P. purpuratus* are excellent organisms for conducting in-situ transplant experiments because it is possible to re-locate and keep them caged without interfering with their feeding (Shelmerdine 2007). Near each *river* we collected mussels from two local populations experiencing different environmental conditions: (1) *river-influenced* habitats and (2) *open coastal* habitats, which were subjected to an in-situ reciprocal transplant experiment. Juvenile individuals of *P. purpuratus* were carefully removed with a scraper from the rocky shore of each selected local habitat at low tide and transferred to the laboratory where individuals with shell length of 5–7.5 mm were selected for the experiments. All specimens were acclimatized in the laboratory by being immersed for 24 h in seawater whose salinity was $\sim$ 33. Following this period of acclimatization, the total shell length, buoyant and total wet weights of all individuals were measured and then each individual was marked using bee tags. Total shell length was recorded using a digital calliper (CD-6" CSX Mitutoyo, precision $\pm$ 0.01 mm). Buoyant and wet weight was recorded with a digital balance (Shimadzu AUX120) with precision of $\pm$ 0.0001 g following procedures described by Palmer (1982). Fouling organisms were removed on the selected individuals to prevent errors due the entrapment of air within the closed valves during measurements of the buoyant weight.

We used a combination of plastic mesh and stainless steel cages for all transplant experiments. The plastic mesh cages were 70 mm long, 70 mm wide, and 35 deep, with a 4 mm mesh size and sat inside a stainless steel cage of rough similar dimensions, to protect the mussels and to provide more secure anchorage to the rocky substrate. Four cages were installed at each habitat (*river-influenced* and *open coastal*) near each *river* with 30 individuals being housed in each cage. Two of the cages contained auto-transplanted organisms (i.e., from the same site or the *control*) while the others two cages housed individuals that had been translocated between habitats (*transplanted*, Fig. 2). Thus there were a

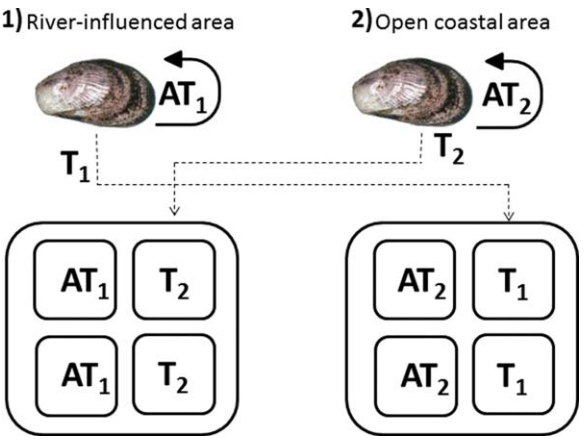

Fig. 2. Schematic diagram of the in situ transplant experimental design. AT, auto-transplanted (control); T, transplanted. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

total of eight cages deployed near each *river*, four cages (two *control* and two *transplanted*) in *river-influenced* habitat and four cages (two *control* and two *transplanted*) in *open coastal* habitat, for a total of 120 individual mussels for each habitat (2 *control* $\times$ 2 *transplanted* $\times$ 30 individuals = 120 per habitat). Each experiment remained installed from 98 d to 125 d, between July of 2012 and March of 2013, according to watershed. At the termination of the experiment, the cages were removed and transferred to laboratory where the size and mass of individuals were measured as described previously.

For metabolic measurements, four to nine individuals were randomly chosen from each cage (controls and translocated). One organism was introduced individually into acrylic respirometric chamber and the oxygen consumption was measured with a fiber-optic oxygen meter (Microx TX3, PreSens, Germany) calibrated with a solution of sodium sulphite (Na_2SO_3) (as the zero O_2 calibrant) and 100% air-saturated seawater. The first few minutes of each measurement were not included in the slope estimates to rule out any lingering effects of handling stress (Lardies et al. 2014). The metabolism was expressed as oxygen consumption (mg) per time (h) and wet weight (g). Unfortunately, logistical problems prevented these measurements from being made on mussels from the Biobío River. Growth (μm ind. d^{-1}) and net calcification rates (mg ind. d^{-1}) were calculated from differences in shell length and buoyant weight of each mussel, respectively (Palmer 1982; Kleypas et al. 2006; Gazeau et al. 2010).

Statistical analysis

Spearman correlation was used to test the relationships between $\delta^{13}C_{Shell}$ and $\delta^{18}O_{Shell}$ to evaluate the kinetic effect over the vital effect (i.e., when the kinetic effect is more important than vital effect, this relationship is positive). Then, $\delta^{13}C_{Shell}$ was correlated with $\delta^{13}C_{Tissue}$ and $\delta^{13}C_{DIC}$ to explore the potential carbon sources for carbonate shell, and

Table 1. Mean environmental characteristics of *river-influenced* and *open coastal* areas for each *river*. For the *open coastal* areas, characterization was based only in one sampling on July 2012, whereas for *river-influenced* areas, it was based over an annual scale study (Pérez et al. 2015).

	Maipo		Rapel		Biobío		Valdivia	
	River-influenced	Open coastal	River-influenced	Open coastal	River-influenced	Open coastal	River-influenced	Open coastal
Salinity (psu)	33.27	34.22	33.97	33.97	31.66	33.71	25.20	33.20
Temperature (°C)	11.67	11.58	10.84	10.77	12.12	11.87	10.95	13.20
pH	7.765	7.866	7.675	7.717	7.773	7.805	7.905	7.929
Alkalinity (μmol/kg)	2308	2278	2259	2278	2213	2239	985	2196
DIC (μmol/kg)	2264	2198	2242	2249	2175	2180	948	2095
pCO_2 (μatm)	1094	832	1313	1196	1047	958	354	702
Ω_{ar}	1.47	1.83	0.75	0.82	0.9	1.01	0.42	1.33

finally, we correlated $\delta^{13}C_{Tissue}$ and $\delta^{13}C_{POC}$ for evaluate the influence of POC over the metabolic carbon (i.e., respired).

Differences in isotopic composition between habitats in the various carbon and oxygen pools ($\delta^{13}C_{DIC}$, $\delta^{13}C_{POC}$, $\delta^{13}C_{Shell}$, $\delta^{18}O_{Shell}$) as well as percent-respired carbon incorporated in the shell among *river-influenced* areas were examined by one-way analysis of variance (ANOVA) or Kruskal–Wallis when the normality assumption was not satisfied. In this analysis, we regarded the bi-monthly data collected in *river-influenced* areas as within-site replicates given low temporal correlation indicated a high degree of statistical independence. Factorial analysis of variance was used to test differences in growth, net calcification and metabolic rates of mussels from different *rivers* (i.e., adjacent coastal region of Maipo, Rapel, Biobío, and Valdivia Rivers), *habitats* (i.e., origin of mussel collected, from *river-influenced* and *open coastal* areas), *transplants* (i.e., mussels subjected to autotransplant and transplant cross treatments), and interactions among these primary factors. The assumptions of normality and homoscedasticity were satisfied in all analyses. We also performed Tukey's test as a posterior pairwise comparison between significant main effects on these data; however, these significance tests must be interpreted with caution given that the interaction terms between the independent factors were often significant.

Results

Environmental conditions

Average composites of MODIS nLw555 show the mean river plume influence in adjacent rocky shore sites during the complete period of each field experiments (Fig. 1). These composites images represent the average spatial distribution of plume influence with respect to the river mouth, and consequently, they should be interpreted with caution as single plume events may differ from the average plume propagation pattern. In most cases, the riverine discharge affected each *river-influenced* habitat, with varying the influence degree among *rivers*. The *river-influenced* area adjacent to the Maipo *river* had over 90% the signal of the river mouth. In contrast, the *open coastal* area had a reduced (~ 50%) river-related turbidity signature (Fig. 1a). Similar patterns were presented in adjacent to Rapel *river*; the experiments located in *river-influenced* area were highly impacted by the presence of the river flow/plume, whereas the experiment placed in the *open coastal* area presented decreased NLw555 signal from the river mouth (Fig. 1b). Both experiments conducted near the Biobío *river* did not show a major influence of river discharge (Fig. 1c). The plume was, on average, preferentially attached to the coast south of the river mouth (Fig. 1c). Values of nLw555 about 0.6 all over the adjacent continental shelf also suggest that turbid plume waters could have been highly dispersive with high spatial variability (Fig. 1c). For the last case, the turbid plume signal of Valdivia River had the highest values of nLw55 oriented in the southwest direction, as a typical buoyant plume with anticyclonic rotation and spreading mostly attached to the coast (Fig. 1d). However, high values (~60%) of nLw555 from the river mouth are all over the domain by which a dominant average pattern is hard to identify (Fig. 1d). In fact, the *open coastal* areas presented a turbid plume signature of around 70% the signal of the mouth, by which clear contrasting river plume conditions are not necessarily expected (Fig. 1d).

The environmental and carbonate chemistry parameters for *river-influenced* and *open coastal* areas are provided in Table 1 for each *river*. The salinity ranged from 25.2 to 34.22, and in the *river-influenced* areas experiencing less saline waters; reflected in the turbid plume data above. Coastal water temperatures varied between 10.77°C and 13.20°C across all *rivers*, with a slight difference between *river-influenced* and *open coastal* areas. The pH varied between 7.675 and 7.929, and the *river-influenced* areas experienced the lowest pH values for all *rivers*. Alkalinity (~ 2200–2300 μmol/kg) and DIC (~ 2100–2260 μmol/kg) showed similar

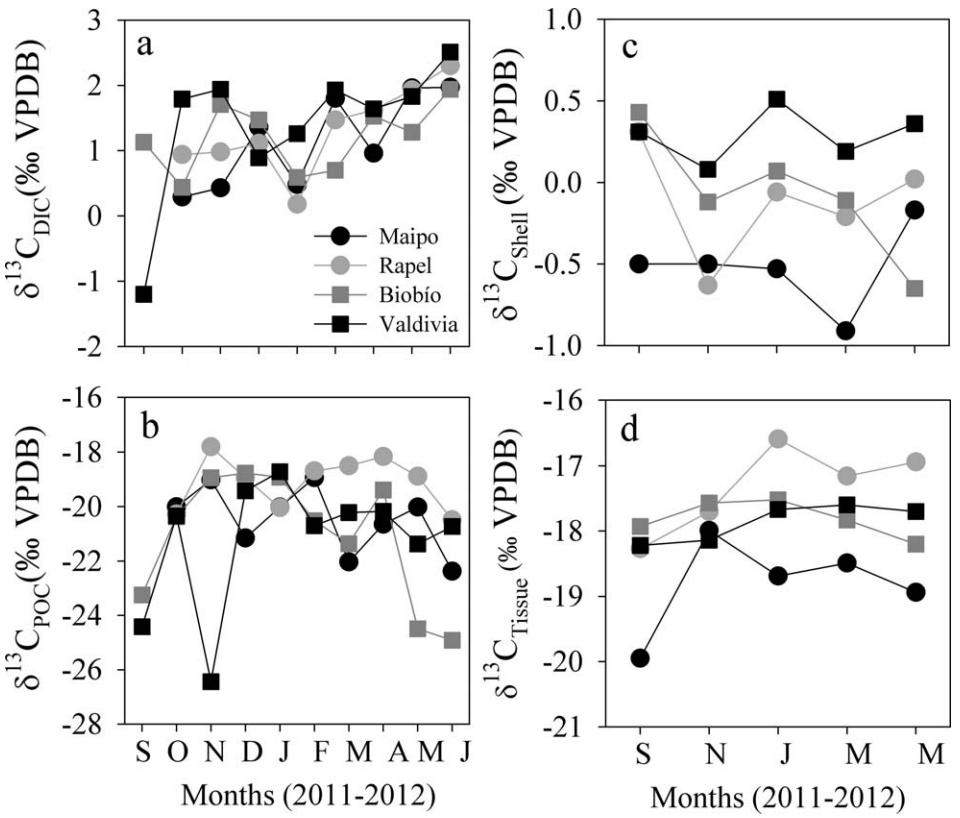

Fig. 3. Isotopic composition of (**a**) DIC and (**b**) POC from environmental, and isotopic composition of inorganic carbon from (**c**) shell and (**d**) tissue of *Perumytilus purpuratus*, for period between September 2011 and June 2012 in *river-influenced* areas.

values among *rivers*, with the exception of the *river-influenced* area in Valdivia. The pCO_2 fluctuated between 354 and 1196 μatm across all sites, and in the most cases, was higher at *river-influenced* areas where values were above the average atmospheric concentration of $\sim$ 400 μatm. Ω_{ar} ranged from 0.42 to 1.83 across all sites and the lowest values ($\Omega_{ar} < 1$) were mostly observed in *river-influenced* habitats as well.

Isotopic composition

Seawater $\delta^{13}C_{DIC}$ ranged between -1.2‰ and 2.5‰ in *river-influenced* areas (Fig. 3a) and exhibited minor seasonal variability; becoming moderately more enriched during the autumn months. The $\delta^{13}C_{DIC}$ varied similarly among *river-influenced* habitats, with no statistically significant differences (Kruskal–Wallis, df = 3, $H = 1.5$, $p > 0.05$). $\delta^{13}C_{POC}$ oscillated from -26.4‰ to -17.8‰ in *river-influenced* areas (Fig. 3b), and showed seasonal variability with values more depleted in autumn-winter. Nonetheless, mean $\delta^{13}C_{POC}$ were significantly different among all *river-influenced* habitats (Kruskal–Wallis, df = 3, $H = 8.74$, $p < 0.05$).

The $\delta^{13}C$ of *P. purpuratus* shell from *river-influenced* areas ranged from -0.91‰ to 0.51‰ (Fig. 3c) and did not exhibit any seasonal pattern in all studied areas. Mean $\delta^{13}C_{Shell}$ values were however significantly different among *river-influenced* habitats (ANOVA, $F_3 = 6$, $p < 0.01$) and showed a latitudinal enrichment in the isotopic signature southward. Indeed, $\delta^{13}C_{Shell}$ in *river-influenced* areas of Maipo *river* had more depleted values (-0.5 ± 0.3‰), whereas Valdivia *river* presented the most enriched values (0.3 ± 0.2‰). $\delta^{18}O_{Shell}$ of *P. purpuratus* oscillated between -2.3‰ and -0.4‰ (data not shown), isotopic signal was significantly different between *river-influenced* areas (ANOVA, $F_3 = 6.2$, $p = 0.005$), depleted southward. $\delta^{13}C_{Tissue}$ of *P. purpuratus* from *river-influenced* areas varied between -20‰ and -16.6‰ (Fig. 3d), and did not show a seasonal pattern for any of the studied *rivers*. Mean $\delta^{13}C_{Tissue}$ were significantly different among *river-influenced* areas (ANOVA, $F_3 = 6.82$, $p < 0.01$), and the most depleted values of $\delta^{13}C_{Tissue}$ were observed in mussels living near the Maipo *river* (-18.8 ± 0.7‰).

Variations in $\delta^{13}C_{Shell}$ of individuals of *P. purpuratus* did not show any temporal correlation with variations in $\delta^{18}O_{Shell}$ ($p > 0.05$, $n = 20$, *r*-spearman = -0.35), $\delta^{13}C_{Tissue}$ ($p > 0.05$, $n = 20$, *r*-spearman = 0.3) or $\delta^{13}C_{DIC}$ ($p > 0.05$, $n = 20$, *r*-spearman = 0.13). On the contrary, $\delta^{13}C_{Tissue}$ were positively correlated with $\delta^{13}C_{POC}$ ($p < 0.05$, $n = 18$, *r*-spearman = 0.53).

Isotope model

Model estimates indicated that environmental DIC was the major source of carbon to the formation of *P. purpuratus* shells (Fig. 4). The estimated percentage of respired inorganic carbon

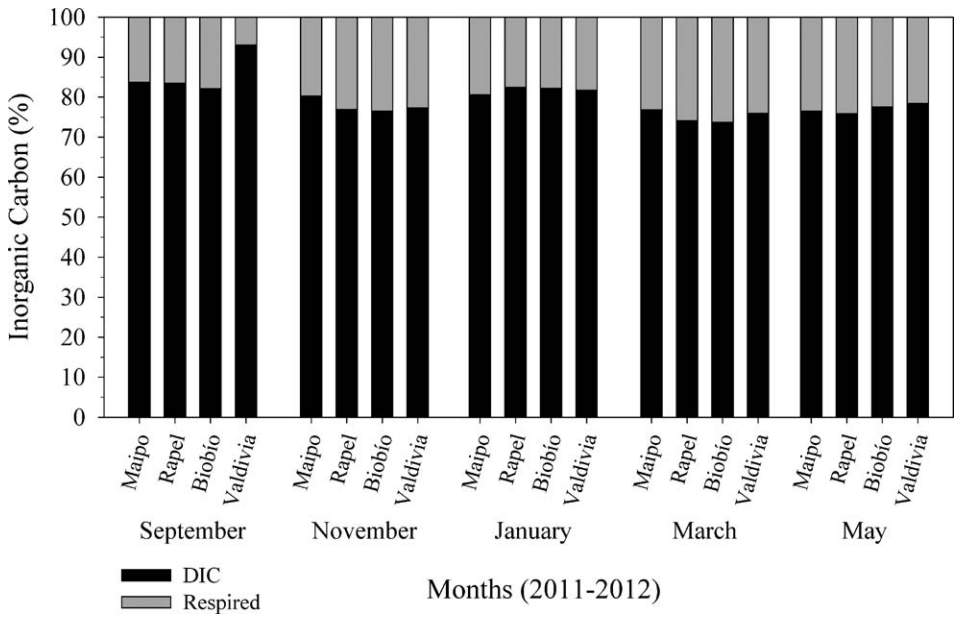

Fig. 4. Estimated percent of inorganic carbon incorporated into shell from respired carbon (grey colour) and DIC (black colour), between September 2011 and May 2012 in *river-influenced* areas.

incorporated in the shell of *P. purpuratus* ranged between 7% and 26.3% and there were no statistically significant differences in percent contribution among *river-influenced* habitats from the different *rivers* (Kruskal–Wallis, $H_3 = 0.47$, $p > 0.05$). The proportion of carbon source in the shell of *P. purpuratus* was variable across the sampling period, however, did not exhibit any seasonal pattern. The environmental source (DIC) was higher in September and lower in May (Fig. 4).

Field experiments

Growth rates of *P. purpuratus* varied among *rivers* (adjacent coastal region of Maipo, Rapel, Biobío, and Valdivia Rivers), *habitats* (collected from *river-influenced* and *open coastal* areas) and whether or not they were *transplanted* (auto-transplanted organisms, from the same *habitat*, and *transplanted* organisms, translocated between *habitats*). Furthermore, there was a significant interaction between all primary factors in our factorial analysis (*river* × *habitat* × *transplant* interaction in Table 2; Fig. 5a–d). The largest growth rates of mussels were recorded near the Biobío *river*, with the lowest values were recorded at the Maipo *river* (i.e., Biobío > Rapel > Valdivia > Maipo; Tukey's test, $p < 0.05$). Regardless, in nearly all cases mussels in *river-influenced habitats* grew better than those in *open coastal* habitats regardless of whether they were transplanted or not (Tukey's, $p < 0{,}05$; Fig. 5a–d). The *habitat* × *transplant* interaction term was not significant indicating that the differences in growth rates between local populations coming from different habitats are reversed when the mussels are transplanted (Table 2; Fig. 5a–d).

Net calcification rates of *P. purpuratus* showed similar patterns of spatial variation recorded for the growth rates, varying significantly among *rivers*, *habitats*, and whether or not the organisms were transplanted (i.e., *transplants*; Table 2). Similarly, we have found the interactive influence of the three sources of variation on the calcification of the studied mussels species (i.e., *river* × *habitat* × *transplant* interaction in Table 2; Fig. 5e–h). The differences in calcification among *rivers* were mainly characterized by the largest rates recorded in mussels collected in Biobío *river* and lowest in the Maipo and Valdivia *river* (i.e., Biobío > Rapel > Valdivia = Maipo; Tukey's test; Fig. 5e–h). Similar to growth rates, calcification rates in mussels raised at the *river-influenced habitats* were significantly greater than mussels raised in the *open coastal habitats* (Tukey's test, $p < 0.005$). Calcification rates of *transplanted* mussels to *river-influenced habitats* were higher than transplanted mussels to *open coastal habitats* (Tukey's, $p < 0.05$, Fig. 5e–h). As in the case of growth rates, the *habitat* × *transplant* interaction term was not significant evidencing that the spatial patterns in calcification rates of the studied local populations are also reversed when the mussels are exposed to the contrasting environmental conditions (Table 2; Fig. 5e–h).

Metabolic rates also vary spatially but the differences were dominated by variation in *river* and *transplant*; however, interaction between factors was not statistically significant (Table 2). In general, mussels raised in the Valdivia *river* respired at a higher rate relative to *P. purpuratus* raised in Maipo and Rapel *rivers* (Tukey's, $p < 0.05$, Fig. 5i–k). The

Table 2. Analysis of variance on patterns of spatial variation in growth, calcification and metabolic rates of intertidal mussel *Perumytilus purpuratus**.

Response	Source of variation	DF	SS	MS	*F*	*p*
Growth	River	3	23.26	6.07	121.40	**<0.001**
	Habitat	1	2.66	0.97	19.42	**<0.001**
	Transplant	1	17.37	11.19	223.70	**<0.001**
	River × habitat	3	2.23	0.25	5.04	**0.002**
	River × transplant	3	4.08	1.14	22.87	**<0.001**
	Habitat × transplant	1	0.01	0.06	1.22	0.269
	River × habitat × transplant	3	1.53	0.51	10.18	**<0.001**
	Error	603	30.17	0.05		
Calcification	River	3	100.23	26.04	127.15	**<0.001**
	Habitat	1	11.92	6.86	33.50	**<0.001**
	Transplant	1	131.92	74.54	364.01	**<0.001**
	river × habitat	3	13.54	2.06	10.07	**<0.001**
	River × transplant	3	14.51	3.02	14.73	**0.000**
	Habitat × transplant	1	0.26	0.00	0.02	0.891
	River × habitat × transplant	3	6.31	2.10	10.27	**<0.001**
	Error	602	123.27	0.21		
Metabolism	River	2	2.34	1.00	10.78	**<0.001**
	Habitat	1	0.13	0.14	1.47	0.228
	Transplant	1	1.43	1.25	13.41	**<0.001**
	River × habitat	2	0.11	0.06	0.66	0.517
	River × transplant	2	0.28	0.13	1.35	0.266
	Habitat × transplant	1	0.03	0.02	0.26	0.609
	River × habitat × transplant	2	0.04	0.02	0.23	0.795
	Error	89	8.28	0.09		

*Analysis on metabolism data include only Maipo, Rapel and Valdivia *river*. In bold are depicted significant *p*-values.

transplant effect also influenced the metabolic rates with a significantly greater metabolic rate at the *open coastal habitats* (Fig. 5i–k).

Discussion

Inorganic carbon source in shell formation in *P. purpuratus*

There has been a long interest in using the isotopic composition of mollusc shells as a proxy of environmental conditions under which they are produced (Gillikin et al. 2009; Poulain et al. 2010; Beirne et al. 2012; Walther and Rowley 2013). While $\delta^{18}O$ of deposited carbonate generally occurs in equilibrium with $\delta^{18}O$ of dissolved oxygen in aquatic systems (Sharp 2007), the $\delta^{13}C$ of mollusc shell carbonates rarely are in equilibrium with seawater DIC (e.g., McConnaughey and Gillikin 2008; Lartaud et al. 2010; Santos et al. 2012). As well documented elsewhere, our results showed that the $\delta^{13}C$ of *P. purpuratus* shells were not in equilibrium with the $\delta^{13}C$ of environmental DIC (McConnaughey and Gillikin 2008), and the changing contribution of metabolic carbon over time and space limits their use as environmental recorders (Pfister et al. 2011). While this well-known vital effect on shell $\delta^{13}C$ is generated by metabolic processes (McConnaughey 1989), we did not find any significant positive correlation between $\delta^{18}O_{Shell}$ and $\delta^{13}C_{Shell}$ in the *P. purpuratus* thus excluding a kinetic isotope effect underlying this vital effect (McConnaughey 1989; Lorrain et al. 2004). To date, in fact, most studies of shell carbonate isotopes in marine molluscs have not shown kinetic effects, but do show vital effects from the inclusion of metabolic CO_2 into the shell because the carbonate is deposited in a semi-enclosed space (Lorrain et al. 2004; McConnaughey and Gillikin 2008).

Environment variability commonly influences the metabolic processes of living organisms through modulation of the composition and amount of food being supplied (Klein et al. 1996; Lartaud et al. 2010; Beirne et al. 2012) or environmental stressors. Although the environmental DIC is the main source of carbon used in shell formation by *P. purpuratus*, approximately one quarter of their shell carbon comes from a respiratory pathway (i.e., 23.1–26.3%). The abiotic precipitation of aragonite from seawater DIC should be result in a fractionation of $2.7‰ \pm 0.6$ enriched in $\delta^{13}C$ (Romanek et al. 1992). However, the average difference between the $\delta^{13}C$ of shell and DIC was $-1.24‰ \pm 0.95$, suggesting that it the incorporation of depleted metabolic carbon during shell formation (Sharp 2007; Bouillon et al. 2011).

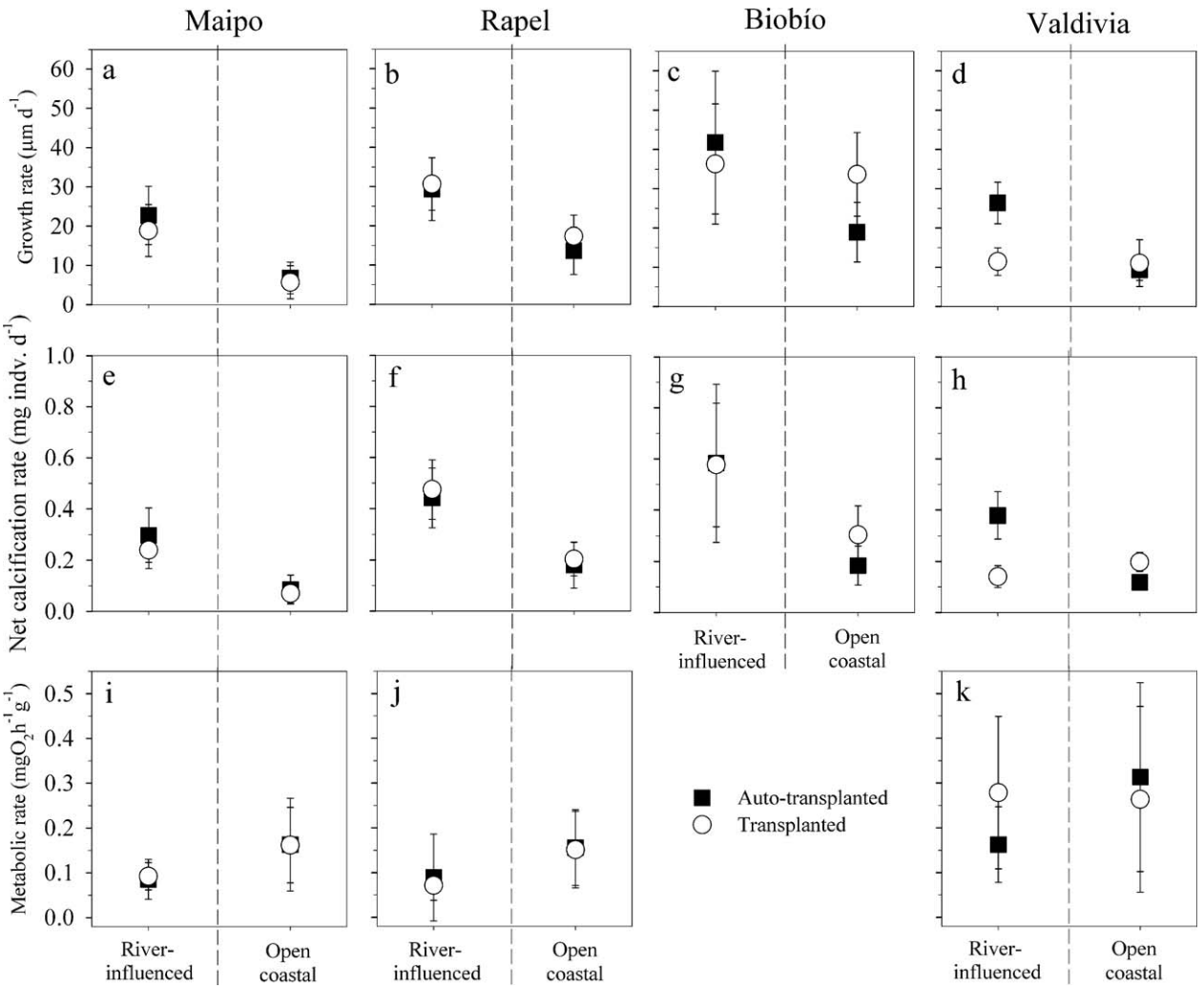

Fig. 5. Summary results observed in the transplants experiments, growth rates (μm d^{-1}) (**a**, **b**, **c**, **d**), net calcification rates (mg d^{-1}) (**e**, **f**, **g**, **h**) and metabolic rates (mgO_2 h^{-1} g^{-1}) (**i**, **j**, **k**, **l**) of *Perumytilus purpuratus* in Maipo, Rapel, Biobío and Valdivia *river*, respectively. For mussel auto-transplanted (square solid) and transplanted (circles open).

The relative incorporation of different carbon sources into mollusc shell can vary among species (Beirne et al. 2012), and be further influenced by distinct factors within a species (Lorrain et al. 2004; Lartaud et al. 2010; Waldbusser et al. 2013). Wanamaker et al. (2007) showed that an increase in the uptake of metabolic carbon in shell formation by *Mytilus edulis* was influenced by variations in salinity; possibly due to increased salinity levels inhibiting the activity of carbonic anhydrase, which catalyses transitions between bicarbonate to CO_2. Food availability is another important environmental factor influencing $\delta^{13}C_{Shell}$ (Lorrain et al. 2004; Lartaud et al. 2010). While we lack direct measures of food availability, the $\delta^{13}C_{POC}$ helps to provide some insights. The $\delta^{13}C_{POC}$ in *river-influenced* habitats reflected a marine enriched signature, however $\delta^{13}C_{POC}$ was depleted periods of high rainfall and river discharge, suggesting more terrestrial organic carbon supply to the mussels (Kendall et al. 2001; Ogrinc et al. 2008; Bianchi and Bauer 2011). This environmental variability in $\delta^{13}C_{POC}$ is indeed reflected in the $\delta^{13}C_{Tissue}$ of the mussel. Therefore, it is possible to infer that a greater proportion of metabolic carbon in the *P. purpuratus* shells might have been a consequence of food availability and thus higher respiration rates (Putten et al. 2000; Lorrain et al. 2004; Lartaud et al. 2010). Growth rates and vital functions in bivalves are well known to change seasonally with food availability. The dynamics noted here suggest changes in abundance and composition of food supply could affect calcification processes of *P. purpuratus*, and limit the utility of $\delta^{13}C_{Shell}$ as a recorder of environmental variability free of vital effects (as reviewed in McConnaughey and Gillikin 2008).

Impact of riverine discharges in physiological traits

Freshwater discharges from rivers to the coastal ocean can modify the biogeochemical characteristics of proximal habitats to the river mouth, through for example, modifications

to carbonate chemistry (e.g., high pCO_2, low pH and low Ω_{ar}) (Salisbury et al. 2008; Borges and Gypens 2010; Aufdenkampe et al. 2011; Duarte et al. 2013; Pérez et al. 2015). Data published by Pérez et al. (2015), and our results confirm that *river-influenced* areas typically possess higher pCO_2, lower pH, and reduced $CaCO_3$ saturation state, in comparison to *open coastal* areas, not proximal to rivers (with the exception of Valdivia, Table 1). Since our study focused on *river-influenced* areas (i.e., mussel collection, isotopic analysis, chemical analysis, etc.) and *open coastal* areas only were visited for the transplant experiment (due to logistical issues), the temporal resolution of the *open coastal* water chemistry was relatively poor (i.e., only one sampling July 2012). The use of annually averaged MODIS images however confirmed the different levels of riverine influence between both study habitats. Nevertheless, more detailed temporal resolution of discrete samples for the transplant experiments would have been ideal; we will however note major findings below.

Our hypothesis postulates that organisms exposed to riverine discharges would have lower growth and calcification rates than those from *open coastal areas*. However, our hypothesis was rejected since most organisms exposed to riverine discharges (in both cases, e.g., *controls* and *transplants*), showed higher growth and net calcification rate than organisms in *open coastal* areas. The potential stress generated by riverine-based acidification due to elevated pCO_2, low pH and lower Ω_{ar} in *river-influenced* areas were not reflected in growth and net calcification rates measured here. While it may be possible that acidification was not significant enough to elicit a response, it may also be that other factors are driving the pattern in organismal response.

Increased the food supply or energy availability may offset some acidification stress (e.g., Wood et al. 2008; Waldbusser et al. 2010, 2013; Melzner et al. 2011; Gazeau et al. 2013; Thomsen et al. 2013). Rivers contribute particulate organic carbon (POC) to adjacent coastal areas (Bauer et al. 2013), providing a potential food subsidy. We therefore posit that organisms located in *river-influenced* areas can be exposed to elevated food supply countering possible effects of elevated CO_2. Previous *P. purpuratus* specimens from the Biobío and Valdivia regions showed higher clearance and ingestion rates in organisms from *river-influenced* areas, than those from *open coastal* locations, suggesting a possible food/energy subsidy (Parra 2013).

Metabolic rate provides insights into energy is allocated to maintenance or other physiological processes (Brown et al. 2004; Calosi et al. 2013), and will vary as a function of many environmental factors (e.g., Wood et al. 2008; Beniash et al. 2010; Lannig et al. 2010; Thomsen et al. 2013; Lardies et al. 2014). For example, when pCO_2 is elevated, metabolic rate may increase due to the high energetic demand required maintaining the homeostasis, until metabolic depression occurs at very elevated CO_2 values (Beniash et al. 2010; Lannig et al. 2010; Thomsen et al. 2013; Lardies et al. 2014). According to Thomsen et al. (2013), Adult mussels maintained growth and calcification rates in elevated pCO_2 conditions when food supply is abundant (Thomsen et al. 2013), and therefore, the maintenance energy costs are satisfied (Sunday et al. 2013). The increased POC at *river-influenced* sites and organismal responses therefore suggest that any of the following: (1) food is offsetting any river-dominated acidification impacts, (2) mussels in the *open coastal* environment are food limited in general, (3) the *river-influenced* acidification is not strong enough to impact this mussel species. We do however note that these studies were conducted on adult specimens, and we cannot extrapolate to larval stages, nor potential impacts on individuals preparing for reproduction.

Conclusion

In summary, the main source of carbon used in the formation of *P. purpuratus* shells comes from environmental DIC, as noted elsewhere. Nevertheless, $\delta^{13}C_{Shell}$ is in disequilibrium with $\delta^{13}C_{DIC}$ due to vital effects and therefore cannot be reliably used as a proxy of environmental conditions. Our results suggest that adult *P. purpuratus* can tolerate pH/pCO_2 variability in the coastal domain (with increased food), since organisms exposed to riverine discharges did not exhibit a negative response to acidification stress associated with elevated pCO_2, low pH and low Ω_a. The acidification resistance we found may have been offset by an energy subsidy related to the elevated food particle concentration typically observed in these areas. We however lack information on the early stages of larvae of this species and genus. In situ transplant experiments are relatively scarce in the literature, and Chilean coast provides a natural laboratory to undertake such studies, due to multiple natural drivers for changing carbonate systems occurs (i.e., river discharges, upwelling areas, glacier melting, etc.). Nevertheless, more studies are needed, including more intensive environmental monitoring, the consideration of multiple-stressors (e.g., temperature, food supply, salinity, and pollutants), and integration across multiple life-history stages all of which can provide additional information to understand the complex biological responses of marine organisms to ultimately be able to understand how natural populations will respond under the synergistic scenarios of local and global acidification process.

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