Plasticity and inter-population variability in physiological and life-history traits of the mussel *Mytilus chilensis*: A reciprocal transplant experiment

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**A B S T R A C T**

Geographically widespread species must cope with environmental differences between habitats. Information concerning geographic variations in response to climate variability is critical because many morphological, life-history and physiological traits show variation across space. Reciprocal transplant experiments have shown to be relevant to evaluate the role of phenotypic plasticity and potential local adaptation in ecophysiological responses when coping with environmental variability. In this study, we characterize through reciprocal transplant experiments the reaction norms of morphological, biochemical, physiological and life-history traits between two intertidal populations of the socioeconomically important mussel *Mytilus chilensis*, inhabiting contrasting local environments (estuarine vs coastal habitats). We found a gradient in phenotypic plasticity with plastic trait responses in metabolic, ingestion and clearance rates, and in Hsp70 gene expression, and some traits with responses more canalized as growth and calcification rates. This emphasizes that responses not only vary across different local populations but also in different traits in *M. chilensis*, thus it is difficult to establish an overall trend of the responses at integrated organismal level. Moreover, the synergistic interaction of factors such as salinity and carbonate system parameters evaluated make it necessary to study the response at the population level with emphasis on benthic species important in aquaculture. Finally, field studies such as this one are useful for documenting the patterns of traits variation that occur in nature, identifying possible causes of such variation, and generating testable hypotheses for future controlled experiments.

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1. Introduction

Geographically widespread species must cope with environmental differences between habitats. This ability may be achieved by genetic differentiation, phenotypic flexibility and/or local adaptation (Blanckenhorn, 1997; Lardies and Bozinovic, 2008; Lardies et al., 2011; Gaitán-Espitia et al., 2014). Information concerning geographical variations in response to ocean acidification (OA), salinity and temperature are critical because many morphological, life-history and metabolic traits show variation across space (Lardies and Bozinovic, 2008; Aguilera et al., 2013; Lardies et al., 2014); an issue often attributed to organismal adaptation over environmental gradients or ecological transitions (Miner et al., 2005).

It is broadly accepted that to evaluate evolutionary changes, populations within species should be compared as well as individuals within breeding populations. As a consequence, little attention has been paid to interpopulation variation despite its potential use as an approach to study changes in performance between local environments (Angilletta et al., 2002; Dupont and Thorne, 2009; Ramajo et al., 2016). The natural variability in eco-physiological traits between populations may...
reflect differences in their environment and/or phenotypic plasticity that can be increased by the differences in environmental parameters (Pascoal et al., 2012; Melatunan et al., 2013; Lardies et al., 2014). Tools from evolutionary ecology, such as reciprocal transplant experiments, have shown to be relevant in evaluating the role of phenotypic and genetic components of trait adaptation in heterogeneous environments in different populations of organisms (Stekkens et al., 2012). For example, in a reciprocal transplant in a marine snail Trussell (2000) found that shell traits followed a counterregression pattern, reporting the existence of life-history trade-offs associated with increased shell production, and concluded that geographic differences are likely induced by water temperature and/or abundance of a predator. On the other hand, in some studies the role of plasticity and adaptive potential in physiological responses in reciprocal transplants experiments have revealed in some studies changes in physiological responses showing differential reaction norm depending on the populations and their exposure to different environmental factors (Holland and Butlin, 2010; Mayfield et al., 2012; Burford et al., 2014; Ramajo et al., 2016).

In this regard, estuarine zones generally have corrosive waters and greater variability in physical-chemical parameters compared to coastal waters because they have lower absolute concentrations of carbonate, in the same way that acidification is often stronger in coastal areas than in the open oceans (Feely et al., 2010). The exchange of water – atmosphere CO2 is only one of the processes affecting the pH and the carbonate systems of estuaries (Miller et al., 2009), eutrophication and heterotrophy also increase acidity levels (Kemp et al., 2005; Borges and Gypens, 2010), and simultaneous inputs of fresh water in the basins (Salisbury et al., 2008). Additionally, freshwater can carry acid components of nitrogen and sulfur types (Mason, 1989; Baker et al., 1991). Therefore, when transplanting animals inhabiting areas with different environmental scenarios (i.e. coastal versus estuarine) it becomes informative to evaluate the potential effects of changes in water salinity and carbonate chemistry and how these changes have altered the genetic component on the morphological, life-history and physiology traits of the calcifying populations inhabiting the contrasting environments (see Calosi et al., 2013). Interpopulational changes in physiology, growth rate, calcification and molecular responses (e.g. Heat shock genes/proteins), that are activated in a cell under stress (Clark and Peck, 2009), are scarcely known in marine invertebrates and trade-offs and/or constraint of phenotypic traits could modulate the responses to stress or limit further adaptation of coastal populations to the changing ocean. The study of this group of physiological and molecular traits, is useful to evaluate strategies that organisms use to cope with contrasting environmental conditions, which could be linked to metabolic costs or restrictions that determine how might be the effects on organisms of a potential change in environmental factors (e.g. carbonates systems).

The mussel Mytilus chilensis (Hupé, 1854) is a native species distributed from central Chile (ca. 38°S) to southern Patagonia (ca. 53°S), inhabiting depths from low intertidal to 25 m (Navarro et al., 2016). This mussel’s species has an economic importance for the Chilean aquaculture industry (Navarro et al., 2013) and have ecological relevance in terms of their role in providing shell habitats in benthic mussel bed reefs and in recycling shell material (Brattström and Johansen, 1983). A recent experimental mesocosm study has shown a negative role of increased pCO2 in seawater on the physiology of M. chilensis collected from shallow subtidal habitats in the inner sea of the Chiloé Island located at ca. 43°S. However, high abundances and occurrence of this species also take place in estuarine habitats along central and southern Chile (e.g., see, Jaramillo et al., 1992a, 1992b), suggesting that local populations would display physiological adjustments in sensitive traits to cope with these environmental changes. In this study we characterized, through reciprocal transplants, the reaction norms of morphological, biochemical (i.e. Hsp70 gene expression), physiological and life-history traits between an estuarine and coastal population. The aim of this study was to determine the extent of populational phenotypic plasticity of mussel populations to provide insight into adaptation to local environmental conditions.

2. Material and methods

2.1. Study sites and environmental measurements

Experiments were conducted between two sites: Mancera Island (39°53′ 38.4°S; 73°23′03.9°W) and Metri (41°35′39.30°S; 72°42′17.20°W) (Fig. 1a–c). These sites represent two different coastal conditions (Fig. 1b): Mancera (hereafter: estuarine site) is located inside the estuarine portion of the Valdivia river, where there is a continuous input of freshwater.

At estuarine site local hydrographic conditions are characterized by partially mixed circulation, which is widely affected by tidal cycles and intense river runoff, especially during the rainy season (Aguilera et al., 2013). Intio estuary seawater pH varied between 7.723 and 8.023 (Aguilera et al., 2013), sea surface temperature (SST) ranged between 10.1 and 18.7 °C (Garcés-Vargas et al., 2013), while salinity varied in Macera Island between 18.9 and 33.9 psu (Garcés-Vargas et al., 2013). Furthermore, in winter and spring the estuary showed a temperature inversion which was associated with a large surface heat loss and subsurface advection of warm waters from the adjacent ocean to into the estuary that is not mixed with the surface due to intense stratification by salinity (Garcés-Vargas et al., 2013).

In contrast, although it may also be influenced by freshwater input by the Reloncavi fjord, the Metri site (hereafter: coastal site) shows a dominance of marine seawater penetrating from the Ancud gulf into the Reloncavi basin (Lara et al., 2010, see Table 1 and Fig. 1c). Reloncavi fjord the level regarding to calcium carbonate saturation state (Ω) ranged between 1.18 and 1.52 and 1.87–2.43 for aragonite and calcite, respectively. In the same area SST ranged between 11.2 and 20.5 °C, and salinity varied between 23.7 and 30.0 psu (Alarcón et al., 2015). No relationship it is observed between the tidal variations and the oceanographic variables measured in this basin (Alarcón et al., 2015).

To characterize environmental differences between study sites discrete measurements of the carbonates components were performed at the beginning, middle (20 and 40 days) and end of the experiment (63 days), and also including monthly composites of satellite imagery of chlorophyll-a (Chl-a) and fluorescence (nFLH) from the Moderate Resolution Imaging Spectroradiometer (MODIS). The MODIS images were processed using NASA’s software Sea-viewing Wide Field-of-View Sensor (SeaWiFS) Data Analysis System and following high-resolution (1000 m) options. A detailed description of processing options including atmospheric corrections and flags can be found in Saldías et al. (2012). We took discrete samples of temperature, salinity and oxygen in situ using a CTDO (Idronaut Ocean seven 304). In addition, seawater pH was determined in situ using a Metrohm pH-meter (model pH mobile 826), connected to a combined electrode (double junction), calibrated using buffers Tris (pH = 8.089) and 2-Aminopiridine (pH = 6.786) (Torres et al., 2013). Total Alkalinity (TA) was determined using discrete water samples, collected using borosilicate glass bottles (Corning 500–mL), and poisoned with mercuric chloride (0.2 cm3 of a 50% saturated solution). The stopper was sealed with Apiezon® L–grease for transportation to the laboratory and stored in cool-dark conditions until analysis. Measurement of TA was done using automated potentiometric titration (Haraldsson et al., 1997). Using CO2SYS software, pCO2 and saturation states for calcite (1Tc) and aragonite (1Ta) were estimated from pH, AT, salinity and SST (Lewis and Wallace, 1998) using solubility constants (Mehrbach et al., 1973) retitled by Dickson and Millero (1987).

2.2. Transplant experiments

During 2012 we performed two reciprocal experiments, an experiment was performed during the austral winter, obtaining a major
number of mussels across treatments that allowed us to test for variability across sites in biological responses (see below). We collected juvenile *M. chilensis* (16–17 mm) maximum shell length, size that is not reproductive in both locations (Oyarzun et al., 2011), from natural mussel beds. Then, individuals were transported to Calfuco Laboratory, University Austral of Chile and kept in seawater source at 14 °C, with constant air bubbling and ca. 28–29 psu for two days. We measured maximum shell length (TL), total weight (TW) and buoyant weight (BW), as proxy of shell weight following the procedure described by Palmer (1982) (hereafter shell weight) using a digital caliper (©Mitutoyo) and analytical balance (©Shimadzu), respectively. Mussels (*n* = 120) were selected from each study site. All individuals were marked using bee-tags glued to the shell for posterior individual characterization. Before the installation in the field, health statuses of the experimental mussels were checked. Finally, 119 and 115 individuals from estuarine site and coastal site respectively, were considered as healthy mussels (i.e. siphon activity). Randomly, 50% of the total mussels were assigned to each treatment: auto-transplanted within the same site of collection (source) and cross-transplanted to the other study site (destination). Approximately 15 mussels were enclosed within mesh-plastic cages (10 L × 10 W × 3H cm, 0.5 cm mesh aperture, *n* = 4 cages per treatment). We use the reciprocal transplant protocol used by Ramajo et al. (2016). The field experiment lasted for 63 days, with the cages being retired in September 2012.

After the collection of the experiment cages, all mussels were transported to the Calfuco Laboratory, University Austral of Chile, and placed in aquaria with sea water, at 14 °C and 28–29 psu. At the laboratory, several morphological and physiological traits were tested and compared between field treatments: growth rate, calcification rate, metabolic rate, ingestion rate, clearance rate and the relative expression of the gene coding for Heat-shock Protein (HsP70). Supporting Information (hereafter: S) 1, describes the sample size across the treatment combinations for each biological response measured. On the other hand, during summer 2012, a preliminary experiment was performed; some of the experimental cages were damaged, or pulled out by tourist-visitors. Thus, the few replicates available only allowed us to make an observation using optical and electronic microscopes (see below SEM analysis in methods and Supplementary data).

### 2.3. Scaling relationships

To explore the geographic variability in total and shell weight across sites, we describe the scaling relationships between total weight and buoyant weight with the maximum shell length of the mussels (i.e., allometric scaling), which has the general form: \( WT = aM^b \) in the case of mussel total weight (WT). On log-log scale, this scaling relationship can be estimated using a simple linear regression and estimating the parameters slope (a) and intercept (b).
2.4. Net calcification rate (CR) and growth rate (GR)

The net calcification rates in all organisms were estimated from changes in their buoyant weight (i.e., underwater weight) using an analytical balance (Shimadzu, 0.0001 g precision), following the methodology validated successfully by M. chilensis (Duarte et al., 2014), using the buoyant weight as a proxy measure of shell production (see Palmer, 1982).

Also we evaluated differences in maximum shell length (mm) using a precision scale (Mitutoyo, 0.01 mm) to estimate the growth rate, which were expressed as the difference between the measurements recorded before and after the experiment divided by the number of days of exposure in the field.

2.5. Ingestion rate (IR) and clearance rate (CR)

Immediately after completion of the transplant period (63 days), 10 individuals from each site (auto and cross) were used to determine clearance and ingestion rates. We used 300 mL polycarbonate bottles previously cleaned with HCl with three replicates per treatment. Mussels were incubated by 4 h, at a temperature of 14 °C (±1 °C) and salinity of 28 psu. For these experiments, we added Isochrysis galbana at a concentration of 20,000 cell mL⁻¹. After incubation we took a subsample of 200 mL each from each bottle, which was filtered through a GF/F filter of 0.7 µm mesh-size. The filter was stored in an aluminum envelope at −20 °C until analysis of Chlorophyll concentration. Filters were placed for 20 h in acetone 10% for the extraction of pigments, and were then analyzed through a spectrophotometric fluoroprobe. Clearance (CIR) and Ingestion (IR) rates were estimated through the measurement of chlorophyll-a, according to Frost (1972) modified by Marin et al. (1986).

2.6. Metabolic rate (MR)

Mussels were left in aquaria for 48 h with no food and then oxygen consumption (mgO₂ x h⁻¹) was measured using a Sensys Mini Oxy-4 respirometer. To quantify the MR, 8 replicates of each treatment were placed individually in respirometric chambers filled with 113 mL of seawater of 28 psu salinity and oxygen-saturated through air bubbling before starting measuring. The measurements were performed at a controlled temperature of 14 °C using an automated temperature chiller. In each chamber, dissolved oxygen was quantified every 15 s for 120 min. Sensors were previously calibrated in anoxic water using an Na₂SO₃ saturated solution and water 100% saturated with oxygen. The same respirometric chambers were used as controls, but without animals inside, under the same experimental conditions (the control never had a reduction of the oxygen concentration higher than 3% of measurements). Each oxygen reduction due to background noise was never had a reduction of the oxygen concentration higher than 3% of measurements.

2.7. HsP70 relative expression

Total RNA was extracted with the Trizol® (InvitrogenTM) method from a mixture of the shellfish tissues (excluding stomach) following the manufacturer's instructions. RNA samples were then cleaned up using the RNAeasy® mini kit (Qiagen). For cDNA synthesis, 1 µg of total RNA was treated with DNase I (RQ1, Promega) and then reverse transcribed with random hexamers using the ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer's instructions. Real Time PCR was performed using the Brilliant® SYBR® Green QPCR Master Reagent Kit (Agilent Technologies) and the Eco Real Time-PCR detection system (illumina®) as described by Arias et al. (2010). The PCR mixture (15 µL) contained 2 µL of template cDNA (diluted 1:10) and 140 nM of each primer. Amplification was performed under the following conditions: 95 °C for 10 min, followed by 35 cycles of 94 °C, 30 s; 60 °C, 30 s; and 72 °C, 30 s. At the end of PCR amplification all products were subjected to a melt cycle from 55 to 95 °C. Primers used were as described by Dutton and Hofmann (2009) for Mytilus galloprovincialis. Ef1-α was used as a housekeeping gene (HK) and HsP70 as the gene of interest (GOI). Gene expression levels were calibrated to the average value of the treatment with less expression to obtain a calibrated ΔCt. A previous standard quantification curve with serial dilutions of PCR products was constructed for each gene to calculate amplification efficiency (E) according to the equation: \( E = 10^{-\frac{1}{\text{slope}}} - 1 \) (Poupin et al., 2007).

This value was then used to obtain an accurate ratio between the gene of interest (GOI) and the housekeeping (HK) gene expression, using the equation:

\[
\frac{(1 + E_{\text{GOI}})(ct\ GOI - ct\ GOI\ calibrated)}{(1 + E_{\text{HK}})(ct\ HK - ct\ HK\ calibrated)}
\]

Efficiency values for Hsp70 and Ef1-α during the standard curve calibration were 1.09 and 1.07, respectively, and in both cases \( r^2 \) values were over 98% (Bustin et al., 2009).

In all samples expressions of the HK were similar (standard deviation 1.3; One-way ANOVA: \( F_{(3,19)} = 0.3, P < 0.05 \)) and the reaction specificities were tested with melt gradient dissociation curves and electrophoresis geles (agarose 2% of each PCR product). Expression was not detected in the NTC samples or in the control cDNA-samples without reverse transcriptase enzyme. All experiments were performed with at least 4 biological and 2 technical replicates by treatment.

2.8. Electronic microscope scanning (SEM) observations

Using the reduced data set available after the reciprocal transplant performed during February–April 2012, we made observations at several optical and scanning electronic microscope (SEM) resolutions of the surface and transversal section of the umbo area, the central area of the shell where the transplantation procedure was evident, and in the new growth area at the shell edge of the mussel valves. Measurements of the periostracum and shell thickness were performed in order to explore changes in shell and periostracum before and after the reciprocal transplant experiment.

2.9. Statistical analyses

Paired t-tests were used to test for differences of physico-chemical parameters between sites. We test for differences in total (TW) and buoyant weight (BW) between sites using analysis of covariance (ANCOVA), with maximum shell length as covariate. We tested for differences in slopes (b) and intercepts (a) using Least Square Means (LSM) estimation where LSM corresponds to the fitted value of TW or BW at the mean value of the covariate. These analyses were implemented using the general linear model procedure in MINITAB software.

All the previous biological responses (GR, CR, CIR, IR, MR, HsP70) were analyzed using two-way ANOVA, with the source (estuarine and coastal) and destination sites (i.e., auto- or cross- transplanted in the corresponding estuarine and coastal site) as main fixed factors. Differences between groups (a posteriori comparison) were evaluated using a Tukey HSD test (Zar, 1999; Sokal and Rohlf, 1995). Data were transformed to meet ANOVA assumptions, particularly for the CR and MR variables, using Arcsin and reciprocal transformation, respectively. The relationship between shell and periostracum thickness estimated through SEM analysis was evaluated using Pearson product-moment correlation implemented in R platform 2.15.0 (r Development Core Team, 2009).
3. Results

The individuals used in the experiment showed no significant differences in maximum shell length between locations (estuarine site – coastal site) before the transplant experiment (One-way ANOVA; F(1, 232) = 0.21, P > 0.648, see S1).

3.1. Differences in physical-chemical conditions of the study sites

Both sites exhibited contrasting environmental conditions, being salinity significantly different between sites. Estuarine site showed a mean salinity of 14.64 ± 7.8 psu, while coastal site presented a high mean salinity with values near to 26.29 ± 4.5 psu (t-paired test: P < 0.05). Alkalinity (AT) also exhibited significant differences between both study, where estuarine site presented significantly lower AT values 1468 ± 526 μmol kg⁻¹ than coastal site 1818 ± 293 μmol kg⁻¹ at (t-paired test: P = 0.0063). Other parameters such as pH, SST, Ω Calcite and Ω Aragonite also exhibited significant differences between sites (t-paired test: P > 0.05), but undersaturation values of aragonite (Ω₉ₐ) were registered at estuarine site (Table 1).

The MODIS images of Chl-a revealed contrasting phytoplankton concentration in both sites and also conditions during July and September 2012. Both sites were characterized with higher Chl-a biomass during the spring period (September; Fig. 2b–d), whereas winter conditions presented reduced Chl-a concentration and thus constitute a direct food supply for the mussel population. As described, M. chilensis collected from both study sites did not show significant differences in shell length at the beginning of the experiment. However, the average total and buoyant weight of the mussels showed significant differences between sites: juvenile mussels collected from the estuarine site evidenced a significant increase in shell weight compared to mussels of the same size collected from the coastal site (Table 2).

The geographic differences in total and shell weight of M. chilensis were also evident through the allometric relationships. At the initial stage of the experiment (Fig. 3 A,C; but see Fig. S2), the scaling relationship of total weight with shell length showed between-sites differences both in slopes and intercepts (see regression parameter estimation and ANCOVA in Table 2). In addition, when comparing between sites, total weight (shell length, LSM = 1.214 mm ± 0.036 SD; in log scale) showed that mussels from the estuarine site had a significant increase in total weight with respect to those mussels of the same shell size but raised at the coastal site (see Table 2, LSM comparison). When examining the scaling relationship of shell weight versus shell length, we found differences between-sites in the intercepts but similar slopes (see regression parameter estimation and ANCOVA, Table 2). To compare shell weight LSM values between sites, mussels from the estuarine site showed a significant increase in shell weight with respect to mussels of the same shell size collected from the coastal site (see Table 2, LSM comparison). At the end of the experiment (Fig. 3 b,d; but see Fig. S2), both total shell weights only showed significant differences in the intercept of the scaling relationships (see ANCOVA, Table 2). Regarding shell weight, when comparing mussels at the mean value of the shell length (LSM = 1258 mm ± 0.036 SD, in log scale), we found significant differences among all available combinations of source/destination sites, and those mussels transplanted from the coastal site to the estuarine site showed a significant increase in shell weight when compared to those auto-transplanted in the source coastal site (Me-Au) (see Fig. 2d, and corresponding LSM comparison in Table 2).

After 63 days, growth rate (GR) in juveniles of M. chilensis, measured as change in shell length, showed no differences between sites where the specimens were transplanted (Two way ANOVA, P > 0.05, see Fig. 4a). However, the differences in GR were expressed according to the origin of each population (Two way ANOVA, P = 0.010; Table 3).
Table 2

Mytilus chilensis. Parameter estimation using regression analysis for comparing the slopes (β) and intercepts (α) describing the scaling relationships (log-transformed data) between total and buoyant weight (as proxy of shell weight) with maximum shell length of juvenile mussels for each study site at the initial and final phases of the reciprocal transplant experiment. n = sample size; r² = coefficient of determination of the corresponding regression model. Below each parameter estimation is showed the ANCOVA summary (F-ratio(DF source, error); p-value) testing for homogeneity (Ho: βi = βj) and separated slopes (Ho: αi = αj) between locations and location/destination combinations (Mtrie = Coastal site = Me; Mancera = Estuarine site = Ma). In bold are depicted significant p-values for each ANCOVA. Least Square Mean (LSM) estimation was used to compare the fitted value of total and shell weight at the mean value of the maximum shell length as covariate (value at initial: 1.214 mm ± 0.036 SD and final = 1.258 mm ± 0.036 SD, indicated by arrows in Fig. 2) between source/destination combinations. Different superscripts represent significant differences (p < 0.05) in LSM in total or shell weight at the mean value of the shell length among combination of source/destination sites.

<table>
<thead>
<tr>
<th>Experimental phase</th>
<th>Variable</th>
<th>Source - destination</th>
<th>Parameter estimation</th>
<th>LSM comparison</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Intercept (α ± SE)</td>
<td>Slope (β ± SE)</td>
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<tr>
<td>Initial</td>
<td>Total weight</td>
<td>Coastal site</td>
<td>−3.78 ± 0.125***</td>
<td>2.880 ± 0.103***</td>
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<td>Estuarine site</td>
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<td>2.369 ± 0.158***</td>
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<td></td>
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<td></td>
<td>F(2, 223) = 398.2; p &lt; 0.001</td>
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<tr>
<td></td>
<td>Shell weight</td>
<td>Coastal site</td>
<td>−4.655 ± 0.207***</td>
<td>3.073 ± 0.245***</td>
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<tr>
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<td>Estuarine site</td>
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<td>F(2, 223) = 125.64; p &lt; 0.001</td>
<td>F(1, 223) = 2.98; p = 0.086</td>
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<tr>
<td>Final</td>
<td>Total weight</td>
<td>Me − Me</td>
<td>−3.59 ± 0.19***</td>
<td>2.73 ± 0.15***</td>
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<tr>
<td></td>
<td></td>
<td>Ma − Me</td>
<td>−3.79 ± 0.18***</td>
<td>2.90 ± 0.14***</td>
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<td></td>
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<td>Ma − Ma</td>
<td>−2.96 ± 0.14***</td>
<td>2.34 ± 0.11***</td>
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<td>F(3, 88) = 390.36; p &lt; 0.0001</td>
<td>F(3, 88) = 4.36; p = 0.160</td>
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<td>Shell weight</td>
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<td>Ma − Ma</td>
<td>−4.47 ± 0.33***</td>
<td>2.93 ± 0.26***</td>
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<td>F(3, 88) = 79.84; p &lt; 0.0001</td>
<td>F(3, 88) = 2.36; p = 0.10</td>
</tr>
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</table>

*** = p < 0.0001 in the parameter fitting and corresponding regression model.
ND = not included in ANCOVA because no data available for an adequate linear regression adjustment.

Therefore, the genetic component is strongly associated with the differences in growth rates (0.0275 ± 0.012 mm • day⁻¹) for mussels of coastal origin and a lower growth rate (0.0220 ± 0.010 mm • day⁻¹) for mussels of estuarine origin. Thus, the source factor for these mussels is a relevant factor in the variation of the CR in M. chilensis. The same pattern was found in the calcification rate, where M. chilensis showed differences between populations of different sources (P < 0.001; Two way ANOVA; Table 3). Additionally, significant differences were found depending on the destination location to which the different populations were transplanted (P = 0.02; Two way ANOVA; Table 3) (see Fig. 4b). Overall mussels from coastal sites showed lower rates of net calcification (0.601 ± 0.35 μg • day⁻¹), while populations originating from the estuarine site maintained a constant calcification rate 1.132 ± 0.51 μg • day⁻¹.

Feeding performance of M. chilensis showed similar variability patterns in clearance and ingestion rates; higher clearance and ingestion rates were observed at the coastal sites in both the auto and cross-transplanted mussels (Fig. 4c,d). Also, significant and lower clearance...
and ingestion rates were observed in the foreign mussels cross-transplanted to the estuarine site. These changes were evidenced by a significant effect of the source habitat and the destination site on the feeding performance of the studied mussels (see Table 3).

The metabolic rate, measured as oxygen consumption, showed a significant increase in individuals collected at the estuarine site followed by the mussels collected at the coastal site (Fig. 4e), but with significant differences between both source sites (Table 3). Individuals transplanted to the corresponding destination sites showed a similar and significant decrease in the metabolic rates, maintaining the differences between local populations (Table 3).

The gene expression of HsP70 of M. chilensis showed a complex pattern of variability between source and destination sites: low levels of HsP70 expression were evidenced in mussels inhabiting the coastal site, showing the largest increase in HsP70 expression when mussels were transplants to the estuarine site, converging to the expression recorded for the local mussels at this site (Fig. 4f). In addition, mussels raised at the estuarine site reduced their HsP70 gene expression to levels similar to those recorded for the mussel raised at the coastal site, thus leading to a significant source-destination interaction (Table 3). The interaction between factors is evidenced in the cross of lines (see Fig. 4f), indicating that occurs a Source population × Environment interaction in the HsP70 gene expression.

The individuals of M. chilensis subjected to the experimental reciprocal transplant evidenced changes on the shell surface. After reciprocal experiment of February–April 2012, the periostracum layer of individuals growing at the estuarine site were eroded or almost absent, while mussels raised at the coastal site showed a surface layer covering all the mussel valves (Fig. 5a). Consistent with these observations, the mussels coming from the coastal site also showed an eroded periostracum, particularly in the umbo area, after being translocated.

**Table 3**  
*Mytilus chilensis*. Summary of two-way ANOVA results comparing biological responses of juvenile mussels among source populations (Metri = Coastal site and Mancera = Estuarine site) and the treatment (auto (Au)- and cross-transplanted (T)) applied depending on destination site in a reciprocal transplants experiment. Significant p-values are showed in bold.

<table>
<thead>
<tr>
<th>Biological response</th>
<th>Source (S)</th>
<th>DF (source, error)</th>
<th>MS</th>
<th>F-ratio</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate (mm day⁻¹)</td>
<td>Source (S)</td>
<td>1.99</td>
<td>0.00176</td>
<td>6.983</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>Destination</td>
<td>1.99</td>
<td>0.000156</td>
<td>0.618</td>
<td>0.434</td>
</tr>
<tr>
<td></td>
<td>S × D</td>
<td>1.99</td>
<td>0.000371</td>
<td>1.470</td>
<td>0.228</td>
</tr>
<tr>
<td>Buoyant weight rate (µg day⁻¹)</td>
<td>Source (S)</td>
<td>1.98</td>
<td>4.555</td>
<td>24.243</td>
<td>0.001</td>
</tr>
<tr>
<td>(calcification rate)</td>
<td>Destination</td>
<td>1.98</td>
<td>1.021</td>
<td>5.433</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>S × D</td>
<td>1.98</td>
<td>0.000</td>
<td>0.002</td>
<td>0.960</td>
</tr>
<tr>
<td>Clearance rate (mL Ind⁻¹ h⁻¹)</td>
<td>Source (S)</td>
<td>1.36</td>
<td>7105.3</td>
<td>16.682</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Destination</td>
<td>1.36</td>
<td>35,152.8</td>
<td>82.531</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>S × D</td>
<td>1.36</td>
<td>175.3</td>
<td>0.411</td>
<td>0.525</td>
</tr>
<tr>
<td>Ingestion rate (mg Chl Ind⁻¹ h⁻¹)</td>
<td>Source (S)</td>
<td>1.36</td>
<td>99,160.7</td>
<td>29.847</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Destination</td>
<td>1.36</td>
<td>256,611.4</td>
<td>77.238</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>S × D</td>
<td>1.36</td>
<td>1858.5</td>
<td>0.559</td>
<td>0.459</td>
</tr>
<tr>
<td>Metabolic rate (mg O₂ Ind⁻¹ h⁻¹)</td>
<td>Source (S)</td>
<td>1.28</td>
<td>56,929</td>
<td>32.962</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Destination</td>
<td>1.28</td>
<td>36,833</td>
<td>21.326</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>S × D</td>
<td>1.28</td>
<td>0.286</td>
<td>0.286</td>
<td>0.597</td>
</tr>
<tr>
<td>HsP70-relative gene expression</td>
<td>Source (S)</td>
<td>1.16</td>
<td>0.0056</td>
<td>0.0009</td>
<td>0.975</td>
</tr>
<tr>
<td></td>
<td>Destination</td>
<td>1.16</td>
<td>2.6770</td>
<td>0.7635</td>
<td>0.395</td>
</tr>
<tr>
<td></td>
<td>S × D</td>
<td>1.16</td>
<td>4.3396</td>
<td>5.7499</td>
<td>0.029</td>
</tr>
</tbody>
</table>

**Fig. 4.** *Mytilus chilensis*. Mean (±1 SD) in the Growth Rate (A), Calcification Rate (B), Clearance Rate (C), Ingestion Rate (D), Metabolic Rate (E) and HsP70 gene relative expression (F) recorded in juvenile mussels after experiencing an in situ reciprocal transplant experiment between a population from coastal site (black circle) and an population from estuarine site (black triangle), sites (Metri and Mancera, respectively).
and raised under the environmental conditions of the estuarine site. However, individuals lacking periostracum at the estuarine site evidenced a strong production of this organic layer when raised under the environmental conditions at the coastal site (Fig. 5a). Observation using SEM evidenced that the translocated mussels showed erosion over the surface of the valves, which was also evident in the transversal microstructure of the shell and depicted by a reduction in shell thickness after the translocation to the coastal site (Fig. 5b; but see Fig. S3, S4, S5 and S6). Inversely, an increase in shell thickness was observed in the individuals translocated to the estuarine site. The periostracum thickness also varies among localities, showing an increase in the mussels translocated to the coastal site (Fig. 5c; but see Fig. S2, S3, S4 and S5). These patterns of variation in shell and periostracum thickness by juvenile individuals of *Mytilus chilensis* evidenced an inverse but significant relationship (Fig. 5d).

4. Discussion

Phenotypic plasticity in *M. chilensis* plays an important role because it allows organisms to face environmental variability through morphological and physiological changes and these changes can have both short and long-term effects, ranging from an immediate developmental response to evolutionary adaptation over a number of generations. In *M. chilensis* the specific response of traits and their magnitude may depend on the environmental history operating on their native habitats (see also Duarte et al., 2014). The physical-chemical environment along the Chilean coast is extremely diverse (Torres et al., 2011; Ramajo et al., 2013; Ramajo et al., 2015). Our reciprocal transplant between populations experiencing differential environmental variability showed that some of the measured traits exhibit large phenotypic plasticity (i.e. metabolic rate, gene expression of HsP70, ingestion and clearance rate) while other traits may be less sensitive to the environment (i.e. growth rate and calcification rate), in which it has been experimentally shown that genetic components have a major contribution in shaping juvenile phenotypes that environmental changes in carbonate systems in this species (Duarte et al., 2014). The showed patterns of inter-population variability suggest that major differences in salinity between the estuarine and coastal sites can explain results (Navarro and Gonzalez, 1998; Navarro et al., 2006; Chaparro et al., 2008). In addition, salinity operates in a synergistic way with carbonate system parameters (Dickinson et al., 2012, 2013), which are among the main factors that explain the phenotypic flexibility in coastal populations, and these factors may vary hourly/daily and seasonally in estuarine sites (Ramajo et al., 2016).

The two study sites despite being separated in a small spatial scale (i.e. approximately 190 km), differ abruptly in environmental factors, these differences being mainly due to the input of fresh water from the nearby river and due to the carbonate cycle inside the estuaries (Salisbury et al., 2008; Feely et al., 2010; Waldbusser and Salisbury, 2014). At the estuarine site, levels of salinity, total alkalinity and aragonite saturation (*Ω* < 1) were lower and showed significant differences compared to coastal site. Our results support studies linking genetic variation to the physiological responses of calcifying organisms (Sunday et al., 2011), since the organisms of the estuarine site always had heavier shells and lower growth rate (see Fig. 3 and Table 2). Thus, indicating that both genetic and environmental components could influence phenotype in the shells of the mussels with an impact on the energy budget to maintain other physiological rates (Hamer et al., 2008; Dickinson et al., 2013). Some studies suggest that for low values of *Ω*, shell formation is less favorable mainly during early ontogeny of mollusk because shell production is energetically expensive (Barton et al., 2012; Hahn et al., 2012). In the same vein, metabolic rate in *M. chilensis* showed a higher metabolism in the estuarine site, which may be also related to stress associated with osmoregulation and with other environmental conditions (Sokolova et al., 2012). That is, maintaining constant growth rate lead to increased energy consumption in those animals that were exposed to the estuarine site. Range et al. (2011) suggest that in seawater with low levels of carbonate (*Ω* and *Ω*′), bivalves hardly maintain

![Fig. 5. Mytilus chilensis.](image)
their growth rates. Although in this study we found levels of $\Omega_a$ below 1 in the estuarine site, this condition did not change the growth rate of the mussels. Our results suggest that for mussels facing conditions of environmental variability in the estuarine site, local adaptation and/or traits trade-off arise as important mechanisms for the adjustment of growth rate (see Lardies et al., 2014). However, our limited temporal resolution of environmental data cannot allow for a direct interpretation about their role on the measured physiological responses of studied mussels. Nevertheless, several studies have evidenced the role of environmental fluctuations in the native habitats upon the organismal responses (Calosi et al., 2013; Ramajo et al., 2016). For example, it has been shown in estuarine environments that hourly salinity variation produce stressful events in molluscs with a cumulative lethal effects and nonlinear responses (Montery et al. 2014). The previous suggests that without an adequate characterization at the organism’s scale of the environment can underestimate the physiological impacts on organismal performance of the marine invertebrates (Helmut et al., 2010).

Previous results suggest that organisms display some mechanisms of protection (i.e. periostracum) that differentially regulate the process of calcification in the two populations, allowing an increase of carbonates despite the lower availability (Michaelidis et al., 2005). Bivalves of the auto-transplant in the estuarine site maintained the same rates of calcification at the expense of forming a distinctive periostracum (see supporting information Figs. S3, S4, S5, S6). Electronic microscope data indicate that there are significant changes in the periostracum thickness, which depend on the exposure site; individuals transplanted in the estuarine site showed abrupt changes with a coroded periostracum and a thick shell when compared to the site of origin. The increase in the thickness of the shell entails trade-offs with periostracum regeneration, which can be very expensive in energetic terms to repair at low pH for *Mytilus* spp. (Rodolfo-Metalpa et al., 2011). Therefore, we suggest that individuals maintain an energetic trade-off as they invest more energy in producing their shells and less energy in repairing the damaged periostracum.

Ries et al. (2009) found that the snail *Crepipatella fornicata* decalci- fies its shell in waters with aragonite saturation values ($\Omega_a$) above 1.5, suggesting that for some marine invertebrates, the relation of environmental variables and calcification rate is non-linear, it is highly likely that the calcification rate operates at an optimal level of aragonite saturation. The trade-off between the formation of the shell and periostracum would be produced by the limited energy budget in individuals of *M. chilensis* at the estuarine site reflected in low food ingestion and higher metabolic rates. Mussels auto-transplanted in estuarine site maintained their shells in good conditions during the experiment, but the periostracum was eroded. On the other hand, individuals transplanted to estuarine site recovered the periostracum. The correlation between the thickness of the shell and periostracum revealed interpopulation variability of *M. chilensis* when confronting the influence of estuarine waters, showing that mussels of the estuarine site conserve the weight of their shells. It has been found that the composition of the carbonates (i.e. percentage of calcite/aragonite) can vary in different populations underlying potential environmental causes; likewise, Ramajo et al. (2015), suggest that shell that consists primarily of aragonite, are more susceptible to corrosion in coastal waters which are influ- enced by river discharges. Carbonate and organic composition of the shell of *M. chilensis* play an important role over its potential dissolution by corrosive waters.

Our results indicate that for individuals maintained in the estuarine site the clearance and ingestion rates were lower compared to bivalves maintained to the coastal site, therefore having less efficiency in the capture of food, a pattern found in bivalves exposed to corrosive waters (Liu and He, 2012; Vargas et al., 2013; Navarro et al., 2013). However, despite the differences in the concentration of chlorophyll between sites, which are higher in estuarine site, clearance rates of individuals maintained at the estuarine site were significantly lower compared to individuals from the coastal site. Many coastal bivalves are seldom exposed to environmental hypercapnia in intertidal habitats, upwelling coastal zones, estuaries, oxygen minimum zones and in general feeding performance have been reported to decrease with the increase in pCO$_2$ and changes in the carbonates system parameters (Melzner et al., 2009; Vargas et al., 2015; Navarro et al., 2016) as well as with salinity decline (Navarro and Gonzalez, 1998; Sara et al., 2008, Chaparro et al., 2008). Furthermore, the observed differences in feeding behavior of individuals exposed to different sites also may also be affected by the quality of the food (Velasco and Navarro, 2002). Feeding rates in the estuarine site could be related to increased seston concentration that can be a mechanism responsible for the regulation of ingestion rate to avoid saturation of the ctenidia and labial palps (Iglesias et al. 1996; Velasco and Navarro, 2002). Recently, it has been reported that clearance and ingestion rates are slightly higher in laboratory maintained juveniles of *M. chilensis* (Navarro et al., 2016), which could be subject to the controlled conditions of carbonate system, temperature and feeding in which they were exposed the organisms, whose optimal conditions are directly linked to feeding rates (see also Navarro et al., 2013). Alternatively, in the coastal site where the food supply is more variable on a temporal scale, clearance and ingestion rates were higher and this increase could be explained as a mechanism for maximizing food uptake during periods of limited food availability (Beneish et al., 2010) suggest that *Crassostrea virginica* increased metabolism due to an increase in pCO$_2$, and this is related to the additional energy costs associated with acid-base regulation. Moreover, Pörtner et al. (2004) suggest an increased metabolic rate is due to the effects of a decrease in extracellular pH, which decreases the rate of ion exchange of H$^+$, and may have a cost of up to 50% of the metabolism under extreme conditions, even more when it is variable as in estuarine sites where the system of carbonates together with the effects of salinity vary in the tidal cycle. However, metabolic responses are species specific and can be expressed in different ways to environmental variations (see Thomsen and Melzner, 2010; Cummings et al., 2011; Navarro et al., 2013).

Factors that trigger increased gene/protein expression of HsP$_{70}$ have been well studied in invertebrates (Rossi and Snyder, 2001; Dunphy et al., 2012; Wang et al., 2013; Yang et al., 2013). Our results show that increases in the expression of HsP$_{70}$ gene in *M. chilensis* individuals that have been cross-transplanted, evidenced that animals are adapted to their origin site in relation to the natural variability of the carbonate system that occurs in different places of the habitat. Crossed-reaction norms indicate that there is standing genetic variation for plasticity in HsP$_{70}$ expression between populations, as corroborated by the significant Source × destination interaction in factorial analyses. A reduced expression in the individuals auto-transplanted to the estuarine site, indicates that there was local acclimatization to exposure of freshwater inputs. Synthesis of HsP$_{70}$ might be acclimated to face environmental conditions that can vary to the conditions in which animals are pre-adapted. In energetic terms the constitutive expression of this gene demands a high energetic cost, such that its function is to protect cells from stress, avoiding partial loss of protein structure; being very common to find diminishing of ATP in cells subjected to stress, it can be compensated with a high metabolic rate (Sokolova et al., 2012; Sokolova, 2013).

Contrasting results between the two analyzed populations allowed us to conclude that most of the physiological traits studied are...
controlled by an environmental component showing phenotypic plasticity (i.e. feeding rate, Hsp70 gene expression, metabolic rate), while other life-history traits, are probably affected largely by the genetic component of the population (i.e. growth rate). Similar results were obtained by Navarro et al. (2013) and Duarte et al. (2014), suggesting that for M. chilensis the effects of OA may vary between different populations. Although the mortality is overestimated by losses of individuals during storms and bad weather conditions in autotransplant treatments in estuarine site, we can ensure that mortality was not significantly different between treatments transplant vs autotransplant of individuals coastal site. However, organisms transplanted from estuarine site had lower mortality rates compared to organisms transplanted to estuarine site (see Supplementary material S1). In our study, individuals transplanted to estuarine sites had higher costs for maintenance (Thomson and Melzner, 2010; Lardies et al., 2014; Ramajo et al., 2016), however the presence of the metabolic up-regulation, implies a lower mortality than expected. The strategy used by different populations (i.e. adaptation or acclimatization) has different ecological and evolutionary implications that contribute to better understand the predictions made about the impact of climate change in coastal populations.

Finally, our results showed that in some physiological traits, different populations may vary in plasticity, and the costs of maintaining growth rates for the populations that were transplanted to an estuarine site were higher, which were paid for by an increase in metabolic rate and the reduction of ingestion and clearance rate due to salinity decline as been described for the mitilid Choromytilus chorus (Navarro, 1988). Changes observed in phenotypic traits against estuarine acidification of two populations of juveniles M. chilensis using reciprocal transplants show that mussel traits respond in different directions, making it difficult to predict responses to the current scenario in ocean acidification. Indeed, coastal, upwelling and estuarine environments naturally experience large CO2 fluctuations, however long-term anthropogenic-induced changes to carbon chemistry are likely to induce a substantial non-linear amplification of future CO2 variability (McNeil and Sasse, 2016). Moreover, the synergistic interaction with factors such as salinity make it necessary to study the response at the population level with emphasis on benthic species important in aquaculture.

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