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# The energetic physiology of juvenile mussels, *Mytilus chilensis* (Hupe): The prevalent role of salinity under current and predicted $pCO_2$ scenarios☆

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ABSTRACT

As a result of human activities, climate forecasts predict changes in the oceans $pCO_2$ and salinity levels with unknown impacts on marine organisms. As a consequence, an increasing number of studies have begun to address the individual influence of $pCO_2$ and salinity but much remains to be done to understand their combined effects on the physiology and ecology of marine species. Our study addressed this knowledge gap by measuring the influence of current and predicted levels of $pCO_2$ (380 and 1200 ppm, respectively) and salinity (20, 25 and 30 psμ) on the energetic physiology of juvenile mussels (*Mytilus chilensis*) from the south-eastern Pacific region. Our results indicate that a reduced salinity caused a significant reduction in clearance rate, absorption efficiency and scope for growth of this species. Meanwhile, an increase in $pCO_2$ levels caused a reduction in excretion rates and interacted significantly with salinity in the rate of oxygen uptake measured in the mussel. These results suggest that potential changes in salinity might have a direct role on the physiology of *M. chilensis*. The effect of $pCO_2$, although less prevalent among the variables measured here, did interact with salinity and is also likely to alter the physiology of this species. Given the ecological and economic importance of *M. chilensis*, we call for further studies exploring the influence of $pCO_2$ across a wider range of salinities.



## 1. Introduction

As result of human activities, various oceanic and coastal conditions are being increasingly modified (IPCC, 2014) with uncertain consequences for marine organisms. A prime example of this is the raise of anthropogenic $CO_2$ levels in the atmosphere and its absorption by the ocean (Houghton, 1996, 2001). Consistently higher $CO_2$ levels in the ocean are already reducing seawater pH, the concentration of $CO_3^{-2}$, and the saturation state of calcium carbonate minerals (Orr et al., 2005; Feely et al., 2009). The acidification process has the potential to directly affect marine calcifying organisms (e.g. Caldeira and Wickett, 2003; Orr et al., 2005; Kleypas et al., 2006; Fabry et al., 2008; Nienhuis et al., 2010; Gazeau et al., 2010). For instance, calcification rates in

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adult and juvenile Pacific oysters *Crassostrea gigas* and mussel *Mytilus edulis* and in juvenile *M. chilensis* exposed to 2100 and 1200 $pCO_2$, respectively, all linearly declined with an increase in $pCO_2$ levels (Gazeau et al., 2007; Duarte et al., 2014). In addition, a $CO_2$ increase has been shown to indirectly affect the physiology, growth, reproductive success and behaviour of many other marine animals (Arnold et al., 2009; Widdicombe and Spicer, 2008; Briffa et al., 2012; Navarro et al., 2013; Clements and Hunt, 2015; Lardies et al., 2017).

Oceanic climate scenarios also predict other changes such as ice melting and a growing frequency of storms related to climate change phenomena (Groisman et al., 1999; IPCC, 2014) which are expected to promote a reduction of salinity levels in surface waters of many areas. Such changes in salinity should *a priori* be expected to influence feeding, respiration, growth and reproduction of bivalve species (Widdows, 1985; Navarro, 1988; Chandran and Damodaran, 2000). For example, filter feeders living in estuarine habitats have been shown to significantly reduce their feeding and locomotor activities (Widdows, 1985; Navarro, 1988; Chandran and Damodaran, 2000). Moreover, low salinities (e.g. 18 psμ) have also been linked to negative scopes for growth in species like the large mussel *Choromytilus chorus* in the southeastern Pacific littoral (Navarro, 1988). Despite a relatively large number of studies addressing physiological responses of marine invertebrates to salinity, fewer studies have addressed these physiological changes in the context of other alterations (such as $pCO_2$) simultaneously taking place in coastal habitats.

Growing evidence suggests that environmental drivers can act in a combined manner (synergistic, additive or antagonistic effects; Gooding et al., 2009; Byrne, 2011), challenging our ability to predict the response of marine animals. Although scarce, some studies have started to address the interactive effects of $pCO_2$ and salinity levels (e.g. Waldbusser et al., 2011; Dickinson et al., 2012, 2013; Cole et al., 2016). For example, in scenarios of increased $pCO_2$ levels, Dickinson et al. (2012) showed that reduced salinities had negative effects on the survival, growth and calcification rates of juvenile Eastern oysters, *Crassostrea virginica*. In similar $pCO_2$ scenarios, Waldbusser et al. (2011) showed that increased salinities seemed to ameliorate the negative effects of $pCO_2$ effects on calcification rates, probably due to the higher saturation state of the waters with higher salinity levels.

The aim of this study was to assess the combined effects of variable $pCO_2$ and salinity levels on the energetic physiology of a widespread southeastern Pacific mussel, the "Chilean mussel" (*Mytilus chilensis* Hupe). This species is found along a broad latitudinal range and across several habitats, from marine to brackish water conditions (Krapivka et al., 2007). Given the natural exposure of *M. chilensis* to such a broad range of conditions, we propose as a null hypothesis that this species is tolerant to these two stressors and its energetic physiology is not affected by predicted climate scenarios in which high $pCO_2$ and low salinity levels are expected to occur. The potential response (or lack of) of *M. chilensis* to such scenarios has implications for coastal communities in which this species forms extensive subtidal and intertidal beds. These beds play key roles that parallel the ones played by some species in the northern hemisphere (e.g. *Mytilus edulis*; Commito and Dankers, 2001; Gutiérrez et al., 2003). Hence, we argue that the response of *M. chilensis* to $pCO_2$ and salinity will have implications for the physical habitat and the structure of associated macrofaunal communities (Quijón et al., 1996; Duarte et al., 2006). In addition, given its status as one of the main aquaculture species in the region (over 200,000 tons. per year), the response of *M. chilensis* to the stressors studied here may also have indirect social and economic implications (Navarro et al., 2013).

## 2. Materials and methods

### 2.1. Collection and acclimation of mussels

All the mussels required for the experiments were obtained from an aquaculture operation located in Castro Bay, Chiloé Island (42°29′ S; 73°44′55 W). Only juvenile mussels (~15 mm shell length, ~350 mg fresh weight) were retained and brought to the laboratory (Laboratorio Costero de Calfuco) in coolers with chilled conditions. The mussels were then placed in plastic containers (30 cm diameter, 40 cm height) and kept in filtered seawater (0.1 μm) at 13.1 ± 0.01 °C, 33 psμ and 8.10 ( ±0.01) pH. Acclimation continued for two weeks in which mussels were regularly fed (four times a day) with *Isochysis galbana* (~25 × 106 cel $L^{-1}$). This food concentration corresponds to near 0.8 mg $L^{-1}$ dry weight, which is within the natural range found in the bays of southern Chile (0.6–1.2 mg $L^{-1}$ dry weight) (Navarro and Jaramillo, 1994).

### 2.2. Experimental setup

Experimental mussels were randomly assigned to six separate treatments: 380 ppm (current) and 1200 PPM (predicted $pCO_2$ conditions for years 2070–2110; see Meinshausen et al., 2011), each of which was associated to 20, 25 and 30 psμ of salinity. The latter (25 and 30 psμ) represent the lowest and highest salinities measured in areas of Chiloé Island where *M. chilensis* is currently found (Navarro and Jaramillo, 1994). 20 psμ represents lower salinity conditions registered in estuarine zones close to river discharges and where is still possible to find *M. chilensis* populations (Navarro and Jaramillo, 1994). Four replicates per treatment containing seven experimental animals each were used. To generate the two $CO_2$ levels, experimental microcosms were adapted following Navarro et al. (2013): To obtain a $pCO_2$ of 380 ppm, pure atmospheric air was bubbled into a 250 L header tank. To obtain a $pCO_2$ of 1200 ppm, we blended dry air with pure $CO_2$ to the target concentration using mass flow controllers (http://www.aalborg.com) for air and $CO_2$. That blend was then bubbled into a 250 L header tank. Dry and clean air was generated by compressing atmospheric air (117 psi) using an oil-free air compressor. To obtain the three proposed salinities (20, 25 and 30 psμ) seawater was diluted using distilled water. For each experimental container, the seawater was replaced on a daily basis with seawater previously equilibrated in each 250 L header tank (see above).

Over the duration of the experiments, water temperature and salinity were measured every day with an Ocean Seven 305 Plus CTD (www.idronaut.it). Total alkalinity was measured every three days by automatic potentiometric open-cell titration using HCl 0.05 N and the titration system described in Haraldsson et al. (1997). The accuracy of $A_T$ analysis was controlled using A. Dickson Lab $A_T$ reference seawater. pH measurements were taken every three days in a closed 25 ml cells thermostated at 25.0 °C using a Metrohm 713 pH meter (input resistance>1013 X, 0.1 mV sensitivity, and 0.001 pH units nominal resolution) and a glass combined double junction Ag/AgCl electrode (Metrohm model 6.0219.100) calibrated with 8.089 Tris buffer at 25 °C. Values of pH are reported on the total hydrogen ion scale (DOE, 1994). Values of pH, temperature, salinity and $A_T$, were used to estimate the remaining parameters of the carbonate system and the saturation stage (Ω) of aragonite and calcite using CO2SYS software (Lewis and Wallace, 1998) and Mehrbach solubility constants (Mehrbach et al., 1973) refitted by Dickson and Millero (1987) (see Table 1).

### 2.3. Physiological parameters

In the laboratory, the physiological parameters described below

**Table 1**
Sea water characteristics (mean ± SD) used to maintain *M. chilensis* during the experiments. TA = Total Alkalinity; $[CO_3^{2-}]$ = Carbonate Ion Concentration; $\Omega_{ca}$ = Omega Calcite; $\Omega_{ar}$ = Omega Aragonite.

| | 380 ppm | | | 1200 ppm | | |
|---|---|---|---|---|---|---|
| | 20 psu | 25 psu | 30 psu | 20 psu | 25 psu | 30 psu |
| pH *in situ* (pH units) | 8175 (0,034) | 8121 (0,034) | 8073 (0,034) | 7733 (0,041) | 7685 (0,040) | 7643 (0,039) |
| TA (μmol $Kg^{-1}$) | 1287,6 | 1609,4 | 1931,3 | 1277,4 | 1596,7 | 1916,1 |
| $pCO_2$ *in situ* (μatm) | 191,3 (15,3) | 257,8 (20,5) | 333,8 (26,3) | 590,7 (49,9) | 785,8 (64,8) | 1000,4 (80,9) |
| $[CO_3^{2-}]$ *in situ* (μmol $Kg^{-1}$) | 85,5 (6,8) | 109,4 (8,6) | 134,1 (10,5) | 34,6 (3,6) | 44,8 (4,5) | 55,7 (5,5) |
| Ωca | 2,24 (018) | 2,78 (0,22) | 3,30 (0,26) | 0,91 (0,09) | 1,14 (0,12) | 1,37 (0,14) |
| Ωar | 1,36 (0,11) | 1,73 (0.14) | 2,10 (0,16) | 0,55 (0,06) | 0,71 (0,07) | 0,87 (0,09) |

were measured from separate mussels chosen randomly from control and experimental tanks (n = 24) and maintained consistently at 16 °C (two different analyses were never conducted over a same specimen). All these physiological measurements were conducted over the course of a 60-day period.

### 2.3.1. Clearance rate and absorption efficiency

Clearance rate (CR) was estimated following Bayne et al. (1985)'s methodology. Each experimental mussel from each salinity and $CO_2$ treatment was placed in an individual glass container containing 0.4L of seawater. Each of these experimental containers was matched with three additional control containers containing seawater at each salinity and $CO_2$ level but no mussels. After 1 h of acclimatization, all the containers received a standard microalgae diet ($25 \times 10^6$ cells/L), and an initial 20 mL water sample was collected to measure the amount of suspended particles using a Beckman Z2 Particle Counter fitted with a 100 μm orifice tube. The same measurement was conducted again every 30 min for 4 h (particle depletion was assumed to be linear), re-establishing the amount of food consumed immediately after each measurement. Clearance rate (CR) was calculated using the following equation:

$$CR = 0.4\ L \times (LnC_1 - LnC_2) / time\ (h)$$

where $C_1$ and $C_2$ correspond to the particle concentration in control and experimental containers, respectively.

Absorption efficiency was estimated following Conover (1966)'s ratio method, which calculates the fraction of organic matter absorbed by an organism. Faeces from each of the experimental animals used for CR estimations were collected and filtered through Whatman GF/C 25 mm filters previously weighted and washed with an isotonic ammonia formate solution. Individual filters were stored in petri dishes and subsequently dried (100 °C, 48 h), weighted, burned (450 °C, 3 h) and re-weighted to estimate the organic fraction of the faeces. Using information on diet and faeces organic contents, absorption efficiency (AE) was estimated as follows:

$$AE = (D\text{-}F) / ([1\text{-}F] \times D)$$

where D represents the proportion of organic matter in the diet and F represents the proportion of organic matter in the faeces.

### 2.3.2. Excretion rate and oxygen uptake

To estimate the proportion of absorbed energy that is subsequently lost as excretion products (Bayne et al., 1985), experimental individuals from each salinity and $CO_2$ condition were placed in 100 ml incubation chambers and matched again with controls without organisms. After 2 h, a 5 ml sample was collected from each chamber and analysed for ammonia excretion using Solórzano (1969)'s methodology. A calibration curve was prepared with known concentrations of $NH_4$-N solution, measuring all the samples at a 640 nm wavelength in an Optizen Pop uv/vis spectrophotometer. Excretion rates (ER) were calculated as follows:

$$ER = (28 \times X \times V) / t$$

where X corresponds to the reading in μM according to the $NH_4$-N calibration curve, V stands for experimental volume (L) and t equals time (h).

To estimate oxygen uptake ($VO_2$), individuals from each $CO_2$ and salinity levels were placed in incubation chambers embedded into a Haake temperature-controlled (16 °C) water bath. Oxygen uptake was measured using a Fiber Optic Oxygen Transmitter (FIBOX 3, PreSens) and oxygen sensor spots (PreSens GmbH, Regensburg, Germany) attached to the inner wall of the chambers. Oxygen concentrations were measured at the beginning and the end of the incubation time (1 h). Values of oxygen uptake were expressed as millilitres of $O_2$ $h^{-1}$.

### 2.3.3. Scope for growth

A quantitative assessment of the energy available for growth and the energetic status of the organism were calculated as scope for growth (SFG). To measure SFG, physiological measurements were converted to energy equivalents (Joules, J): 1 ml $O_2$ = 19.9 J; 1 μg $Nh_4$-N = 0.0249 J (Elliot and Davison, 1975) and 1 mg organic matter (*I. galbana*) = 18.75 J (Whyte, 1987). SFG was calculated using Widdows (1985)'s equation:

$$SFG = A - (R + E)$$

where A = absorption ($Jh^{-1}$), R = respiration ($Jh^{-1}$) and E = Excretion ($Jh^{-1}$).

### 2.3.4. Statistical analyses

To avoid potential pseudoreplication problems, measurements conducted on the four mussels within each replicate were averaged before being used for data analyses. The influence of salinity, $CO_2$ levels and their interaction upon the physiological measurements conducted on *M. chilensis* was assessed with two-way ANOVAs followed by Tukey's *a posteriori* HSD tests. For each comparison, the choice of parametric analyses was based on the assessment of normality and homocedasticity using the Kolmogorov-Smirnov and Levene's tests, respectively (no assumption violations were detected in any of the analyses). For all the analyses, $p < 0.05$ was used as a statistical threshold value. All analyses were conducted using Statistica 7.0 software (Stat. Soft. Inc., USA). In addition to the plot of means (+SE), data is also displayed in boxplot format in an Appendix of this document.

## 3. Results

### 3.1. Clearance rate and absorption efficiency

The two-way ANOVAs showed that salinity level had a significant influence on the clearance rate and the absorption efficiency of *Mytilus chilensis*. Meanwhile, the influence of $pCO_2$ level or the interaction between salinity and $pCO_2$ levels were in both cases non-significant (Table 2). Post-hoc comparisons indicated that mussels exposed to a 20 psμ salinity had clearance rates significantly lower (near half) than those exposed to 25 or 30 psμ (~0.28 L $ind^{-1}h^{-1}$), regardless of $pCO_2$ levels (Fig. 1A). With regards to absorption efficiency, post-hoc comparisons showed that mussels exposed to 30 psμ exhibited absorption efficiencies significantly (near 25%) higher than those exposed to the two lower salinity levels (~0.56–0.59%), regardless of $pCO_2$ levels (Fig. 1B).

### 3.2. Excretion rate and oxygen uptake

Salinity and $pCO_2$ had a significant influence on excretion rates of *M. chilensis* but the interaction of these two variables was not significant (Table 2, Fig. 2A). The excretion rates were significantly higher at 380 ppm (mean ~3.3 μg $NH_4$-N $ind^{-1}h^{-1}$) than 1200 ppm (~2.6 μg $NH_4$-N $ind^{-1}h^{-1}$) (Fig. 2A). The excretion rates decreased significantly and almost linearly with the increase in salinity (from ~4.1 μg $NH_4$-N $ind^{-1}h^{-1}$ at 20 psμ to 3.0 and 1.8 μg $NH_4$-N $ind^{-1}h^{-1}$ at 25 and 30 psμ, respectively). Oxygen uptake was significantly influenced by salinity but not by $pCO_2$ levels, whereas the interaction of these two variables was significant (Table 2). At 380 ppm, oxygen uptake was highest at 20 psμ (~0.075 mL $ind^{-1}h^{-1}$) and decreased significantly and linearly up to near half that rate (~0.035 mL $ind^{-1}h^{-1}$) at 30 psμ. At 1200 ppm, post-hoc comparisons indicated that oxygen uptake was significantly higher at 20 psμ (~0.070 mL $ind^{-1}h^{-1}$) than at the two higher salinities (~0.052 mL $ind^{-1}h^{-1}$) (Fig. 2B).

### 3.3. Scope for growth (SFG)

The two-way ANOVA showed that salinity, but not $pCO_2$, had a significant influence on scope for growth of *M. chilensis* (Table 2; Fig. 3). Regardless of the $pCO_2$ level, scope for growth was negative at 20 psμ (−0.39 J $ind^{-1}h^{-1}$ in both cases) and positive and

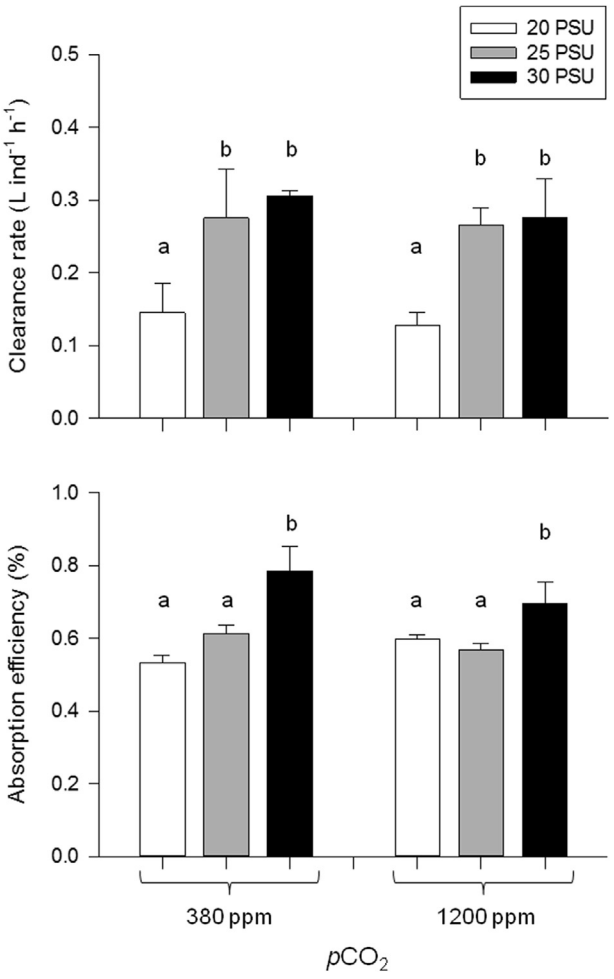


**Fig. 1.** Mean (+S.E.) clearance rate (A) and absorption efficiency (B) in mussels exposed to distinct $CO_2$ and salinity levels. Different letters on top of bars stand for significant differences ($p < 0.05$) detected by Tukey's post-hoc HSD tests.

**Table 2**
Summary of two-way ANOVAs assessing the influence of $CO_2$, salinity and their interaction upon each of the physiological (dependent) variables identified below. Significant P-values are in highlighted in bold.

| Dependent variable | Source of variation | DF | MS | F | P |
|---|---|---|---|---|---|
| Clearance rate (CR) | $CO_2$ | 1 | 0.0023 | 0.688 | 0.418 |
| | Salinity | 2 | 0.0563 | 16.954 | **<0.001** |
| | $CO_2$ x Salinity | 2 | 0.0002 | 0.064 | 0.938 |
| | Error | 18 | 0.0033 | | |
| Absorption efficiency (AE) | $CO_2$ | 1 | 0.0035 | 0.557 | 0.465 |
| | Salinity | 2 | 0.0716 | 11.337 | **0.001** |
| | $CO_2$ x Salinity | 2 | 0.0125 | 1.985 | 0.166 |
| | Error | 18 | 0.0063 | | |
| Excretion rate (ER) | $CO_2$ | 1 | 3.3906 | 11.101 | **0.004** |
| | Salinity | 2 | 9.9483 | 32.570 | **<0.001** |
| | $CO_2$ x Salinity | 2 | 0.4540 | 1.486 | 0.253 |
| | Error | 18 | 0.3054 | | |
| Oxygen uptake (OU) | $CO_2$ | 1 | 0.0001 | 0.658 | 0.428 |
| | Salinity | 2 | 0.0017 | 23.299 | **<0.001** |
| | $CO_2$ x Salinity | 2 | 0.0003 | 4.115 | **0.034** |
| | Error | 18 | 0.0001 | | |
| Scope for growth (SFG) | $CO_2$ | 1 | 0.6995 | 2.774 | 0.113 |
| | Salinity | 2 | 11.9817 | 47.520 | **<0.001** |
| | $CO_2$ x Salinity | 2 | 0.4621 | 1.833 | 0.189 |
| | Error | 18 | 0.2521 | | |

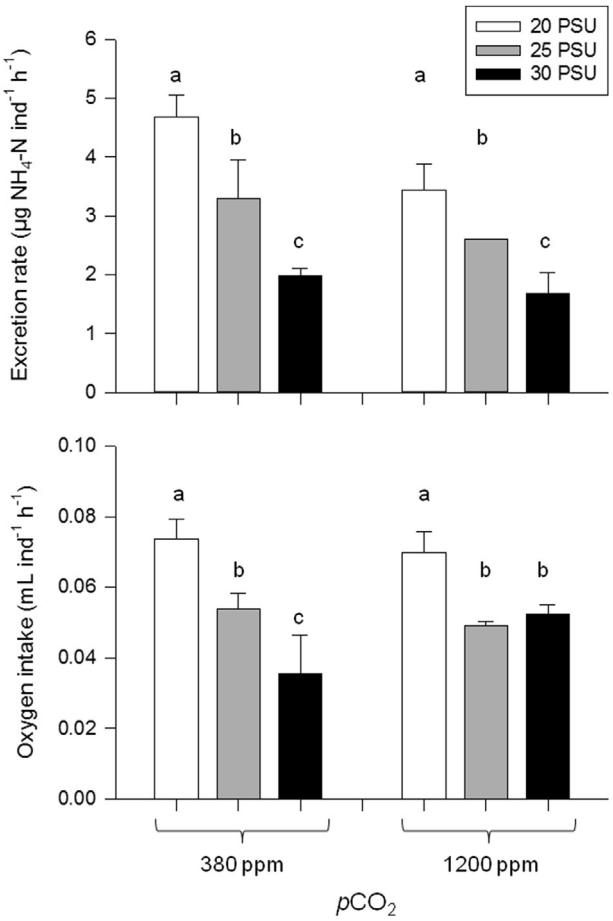


**Fig. 2.** Mean (+S.E.) excretion rate (A) and oxygen uptake (B) in mussels exposed to distinct $CO_2$ and salinity levels. Different letters on top of bars stand for significant differences ($p < 0.05$) detected by Tukey's post-hoc HSD tests.

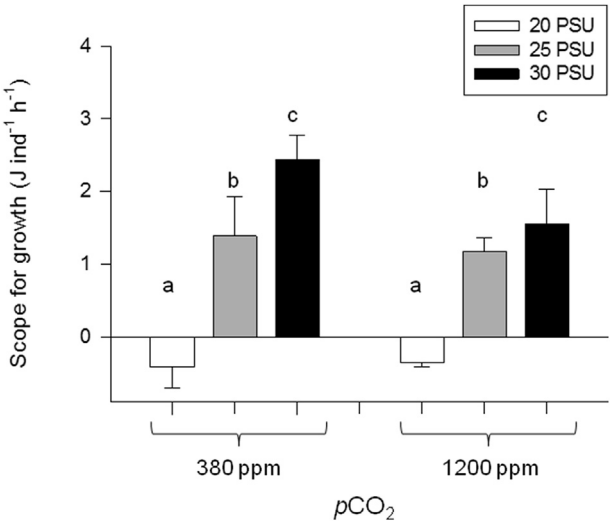


**Fig. 3.** Mean (+S.E.) scope for growth in mussels exposed to distinct $CO_2$ and salinity levels. Different letters on top of bars stand for significant differences ($p < 0.05$) detected by Tukey's post-hoc HSD tests.

increasingly higher (>1 J $ind^{-1}h^{-1}$) at 25 and 30 psµ. Post-hoc comparisons indicated that pairwise differences among the three salinity levels were significant ($p < 0.05$) at both $pCO_2$ levels (Fig. 3).

## 4. Discussion

Marine coastal bivalves are exposed to tidal cycles that regularly modify the salinity and pH of their surrounding waters (e.g. Navarro and González, 1998; Gazeau et al., 2013). The range of these changes can be gradually modified by anthropogenic activities (IPCC, 2014) and have the potential to affect biological traits in ways not yet understood (e.g. Matozzo et al., 2013; Velez et al., 2016). Although considerable research has addressed the individual effects of salinity and pH on the physiology of bivalves, little has been done on the study of the combined effects of these stressors (see Dickinson et al., 2012; Velez et al., 2016). This gap is relevant in the light of climate scenarios projecting large inputs of freshwater from the melting of pole caps and the acidification process already taking place in many coastal regions. Our aim was to assess the combined influence of salinity and $pCO_2$, and based on our results we reject our null hypothesis that the energetic physiology of this species is unaffected by the combined variation of these two stressors. Our results indicate that a 10 ppt decrease in salinity triggers a clear physiological response in *Mytilus chilensis*, meanwhile an increase in $pCO_2$ alter at least one variable and significantly interact with salinity on another one.

### 4.1. The strong influence of salinity

Mussel clearance rates dropped by over 40% at the lowest salinity level (20 psµ). This reduction was likely mediated by a more extended closure of valves, a condition that has already been reported in scallops such as *Argopecten irradians* (Palmer, 1980) and *Argopecten purpuratus* (Navarro and González, 1998), the mussel *Choromytilus chorus* (Navarro, 1988) and the clam *Ruditapes philippinarum* (Velez et al., 2016). Similarly, lower water salinities prompted the lowest estimates of absorption efficiency in the mussels. This latter result is coincident with previous studies in several other species of bivalves (e.g. Navarro and González, 1998; Gardner and Thompson, 2001). Exceptions include mussels of the genus *Perna*, which offer less consistent results. Similar to our study, Wang et al. (2011) reported a significant decrease in the absorption efficiency of *Perna viridis* when exposed to low salinity levels. However, Resgalla et al. (2007) found that a reduction in the absorption efficiency in *Perna perna* was caused by both lower (15 psµ) and also higher (40 psµ) salinity levels than those studied here. We argue that the wider range of salinities used on those studies may account for at least some of these effects in the latter species.

Salinity did also have a significant effect on *Mytilus chilensis* excretion rates: an increase in salinity levels caused an almost linear decline on excretion rates. This suggests that a reduction in salinity is able to regulate cell volume by the breakdown of amino acids as intracellular isosmotic regulators or, alternatively, by moving the amino acids to extracellular fluids (see Garton and Berg, 1989; Navarro and González, 1998; Wang et al., 2011). Although measurements at the cellular level were not conducted in the present study, this potential mechanism is consistent with a study conducted on a conspecific bivalve by Livingstone et al. (1979). These latter authors showed that an increase in excretion rates in *M. edulis* was linked to an increase in amino acid concentration in the hemolymph resulting from a change in salinity. Our results therefore adhere to what has been documented in the literature: changes in excretion rates in response to salinity have also been observed in other bivalves, including the scallop *Argopecten purpuratus* (Navarro and González, 1998) and the mussel *Perna viridis* (Wang et al., 2011).

In the present study, the highest oxygen consumption rates were detected in mussels exposed to the lowest salinity level (20 psµ). However, the role of salinity in this case cannot be

unambiguously distinguished due to a significant interaction between salinity and $pCO_2$ levels. Oxygen consumption has been indirectly linked to energetic costs due to the corresponding osmoregulation process that must take place with the intake of oxygen, in this case, at low salinity levels. This result, gathered from mussels maintained at current and projected $pCO_2$ levels (380 ppm), is similar to the one gathered in previous studies using the scallop *Argopecten purpuratus* (Navarro and González, 1998) but contradicts the ones using the mussel *Perna viridis* (Wang et al., 2011). In that latter study, oxygen consumption rates increased at higher salinity values, which did not occur here at any of the $pCO_2$ scenarios. Higher oxygen uptake and related energetic costs are therefore expected to occur in current or projected scenarios in which salinity levels are low. Such low salinities already occur at habitats that could be considered near the edge of the distribution of this species (e.g. areas with heavy freshwater runoff, as in some Chiloé locations), or in forecasted conditions in which freshwater input due to ice melting is expected to be more common.

Scope for growth is a variable that reflects the balance between energy acquisition and energy expenses, and is considered a useful proxy for the study of the effects of natural or anthropogenic stressors on the condition of an individual (Widdows, 1985). In the present study, this index was lowest (negative) at the lowest salinity level regardless of $pCO_2$ levels. The negative scope for growth was likely related to the higher energy invested in oxygen uptake and ammonia excretion while the lowest values of clearance rates and absorption efficiencies were simultaneously recorded. This is consistent with fairly similar results gathered from others species exposed to relatively low salinities, including the mussels *Perna viridis* (Wang et al., 2011), *Choromytilus chorus* (Navarro, 1988), *Mytilus edulis* (Widdows, 1985) and the scallop *Argopecten purpuratus* (Navarro and González, 1998). The reduction of the scope for growth of juvenile *M. chilensis* by 35% at 25 psμ and 119% at 20 psμ, compared to current "control" conditions, implies potentially severe ecological impacts for this species. Likewise, a reduced scope for growth may negatively affect the development and sustainability of mussel farming operations (see below).

### 4.2. The influence of $pCO_2$ and its interaction with salinity

Previous studies have shown clear physiological and behavioural responses to projected increases in $pCO_2$ levels, even though those responses have varied widely among species and among populations of a same species (e.g. Ries et al., 2009; Range et al., 2011; Clements and Hunt, 2015; Duarte et al., 2015). Our study shows that the influence of $pCO_2$ levels on three physiological variables (clearance rates, absorption efficiency and scope for growth) was non-significant. However, the influence of $pCO_2$ cannot be disregarded as, indeed, this variable had a significant (negative) effect on excretion rates, and did also interact with salinity on the rates of oxygen uptake measured. Below, we weight the results obtained here against the literature available for coastal bivalves, primarily mussel species, from the south Pacific region and elsewhere.

Published studies on clearance rates indicate that an increase in $pCO_2$ levels causes either a reduction (Fernández-Reiriz et al., 2011) or no measurable change on the clearance rates of the mussel *Mytilus galloprovincialis*. In previous studies using *Mytilus chilensis* the exposure of this species to high $pCO_2$ levels has also resulted in clearance rates that were lower than those estimated from current $pCO_2$ levels (Navarro et al., 2013, 2016). With regards to absorption efficiency, the conclusions of previous studies addressing the influence of $pCO_2$ are less consistent. An increase in $pCO_2$ levels caused an increase in absorption efficiency in the mussel *M. galloprovincialis* (Fernandez-Reiriz et al., 2012), a decrease in *M. chilensis* (Navarro et al., 2013, 2016) and only negligible (non-significant) changes in the clam *Ruditapes philippinarum* (Xu et al., 2016) and the pearl oyster *Pinctada margaritifera* (Le Moullac et al., 2016). Only further studies may shed light on what causes these differences (e.g. species-specific responses, interactions with other variables not considered in this or those studies).

The minor influence of $pCO_2$ levels on the scope for growth of the mussels was particularly surprising considering what has been reported in the literature for the same species, *M. chilensis*. In previous studies an increase in $pCO_2$ levels significantly reduced the scope of growth in other populations of this species (Navarro et al., 2013, 2016). Similar results were found on a study of populations of the American oyster *Crassostrea virginica* from the western North Atlantic region (Beniash et al., 2010). To the best of our knowledge, only one other study has found that the effects of $pCO_2$ levels on scope for growth were not significant: Le Moullac et al. (2016) working on the pearl oyster *Pinctada margaritifera* in the French Polynesia. Although the influence of $pCO_2$ on scope for growth cannot be disregarded considering the existing evidence (cf. Navarro et al., 2013, 2016), the lack of a clear effect is in sharp contrast with the severe influence salinity had on this important variable.

The potential impact of increased $pCO_2$ levels was evident on the excretion rates of the mussels. This projected $pCO_2$ increase (1200 ppm) caused a significant decline on the excretion rates of the mussels, regardless of the also significant effects caused by salinity variations. A decrease in ammonia excretion by the mussel *M. chilensis* when exposed to high $pCO_2$ levels is likely associated to a reduction of protein metabolism, probably in response to intracellular pH regulation (Fernández-Reiriz et al., 2005; Thomsen and Melzner, 2010). We speculate that such reduction in protein metabolism may be related to the concentration or movement of intra- or extra-cellular aminoacids (see Livingstone et al., 1979; Wang et al., 2011), at a level (cell-molecular) that goes beyond the scope of this study. A reduction in the excretion rates of the mussels when exposed to projected $pCO_2$ levels suggest a negative scenario for this and potentially other bivalves with similar physiology. Filter feeders living in less variable habitats (i.e. naturally exposed to narrower ranges of $pCO_2$ levels) would be the populations most likely to respond and become negatively affected by long-term variations in ocean pH (Vargas et al., 2017). Similarly, organisms living in habitats with naturally high $pCO_2$ levels already should be expected to be better acclimatized to changes in $pCO_2$ levels and therefore should be less affected by pH variations (Widdicombe et al., 2009; Sunday et al., 2011; Lardies et al., 2014; Vargas et al., 2017).

As indicated above, $pCO_2$ levels did also significantly interact with salinity on the measurements of oxygen uptake. Although the response of the mussels to the lowest salinity level (20 psμ) was almost identical at current and projected $pCO_2$ levels, at the higher salinities levels (25 and 30 psμ) oxygen uptake values differed in response to the different $pCO_2$ level. The variable responses of mussels like *M. chilensis* to the combined changes in $pCO_2$ and salinity levels, in terms of oxygen uptake or other variables are difficult to explain. We hypothesize that this breadth of variation may be explained by the range of conditions individuals mussels are naturally exposed to in their local habitats (Duarte et al., 2015; Vargas et al., 2017). Most species are exposed to changes in pH and salinity due to their position along estuarine gradients and the regular tidal, semilunar or seasonal cycles. In fact, this applies to most locations in Chiloé Island, where experimental mussels came from, due to continuous river discharges and melting of ice from fjords zone (Vargas et al., 2017). Intuitively we expected that these mussels would be tolerant to the variations in salinity used in the experiments (see Duarte et al., 2014). However, our results indicate

otherwise and suggest that although mussels can cope with some variation (decline) in salinity this seems a rather limited ability. We therefore stress the value of further *in situ* studies addressing physiological variation with respect to $pCO_2$ levels across an even wider range of salinities.

The results of this study, in the context of predicted scenarios associated to climate change have broad ecological and economic implications. The separate or combined effects of changes in salinity and $pCO_2$ on the energetic physiology of *Mytilus chilensis* have indirect ramifications to other coastal species and habitats. *M. chilensis* is widely distributed over coastal and estuarine bottoms along the Chilean coasts (Brattström and Johansen, 1983; Krapivka et al., 2007) forming dense beds on rocky shores, tidal flats (Duarte et al., 2006) and subtidal sedimentary bottoms (Quijón et al., 1996). *M. chilensis* is a main prey item for several vertebrate and invertebrate predators (e.g. Curelovich et al., 2016), and play a critical role as an ecosystem engineer that provides biogenic habitat to sedimentary communities (Duarte et al., 2006). *M. chilensis* also sustains a growing aquaculture industry in southern Chile (e.g. Marambio et al., 2012), considered the world third largest producer of farmed mussels (annual production in excess of 200,000 tons). Potential reductions in e.g. scope for growth affecting broodstocks of cultivated or natural populations of *M. chilensis*, could negatively affect the offspring and the reproductive success of this species. As such, further studies on $pCO_2$ and salinity forecasted changes are critical to better understand the response of this species to long-term climate-related changes, and to prepare for it.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.06.053.

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