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Original Article

Ocean warming and elevated carbon dioxide: multiple stressor impacts on juvenile mussels from southern Chile

Jorge M. Navarro[1,2]*, Cristian Duarte[3,4], Patricio H. Manríquez[5], Marco A. Lardies[6], Rodrigo Torres[7], Karin Acuña[1], Cristian A. Vargas[8], and Nelson A. Lagos[9]

[1]*Instituto de Ciencias Marinas y Limnológicas, Laboratorio Costero de Calfuco, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile*
[2]*Centro Fondap de Investigación de Ecosistemas Marinos de Altas Latitudes (IDEAL), Valdivia, Chile*
[3]*Departamento de Ecología y Biodiversidad, Facultad de Ecología y Recursos Naturales, Universidad Andres Bello, Santiago, Chile*
[4]*Center for the Study of Multiple-drivers on Marine Socio-Ecological System (MUSELS), Universidad de Concepción, Concepción, Chile*
[5]*Laboratorio de Ecología y Conducta de la Ontogenia Temprana (LECOT), Centro de Estudios Avanzados en Zonas Áridas (CEAZA), Av. Ossandón 877, Coquimbo, Chile*
[6]*Facultad de Artes Liberales and Facultad de Ingeniería y Ciencias, Universidad Adolfo Ibáñez, Santiago, Chile*
[7]*Centro de Investigación en Ecosistemas de la Patagonia (CIEP), Coyhaique, Chile*
[8]*Laboratorio de Funcionamiento de Ecosistemas Acuáticos (LAFE), Departamento de Sistemas Acuáticos, Facultad de Ciencias Ambientales, Universidad de Concepción, Concepción, Chile*
[9]*Centro de Investigación e Innovación para el Cambio Climático, Universidad Santo Tomás, Santiago, Chile*

**Corresponding author: tel: + 56 63 2221556; fax: + 56 63 2221455; e-mail: jnavarro@uach.cl*

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The combined effect of increased ocean warming and elevated carbon dioxide in seawater is expected to have significant physiological and ecological consequences at many organizational levels of the marine ecosystem. In the present study, juvenile mussels *Mytilus chilensis* were reared for 80 d in a factorial combination of two temperatures (12 and 16°C) and three pCO_2 levels (380, 700, and 1000 μatm). We investigated the combined effects of increasing seawater temperature and pCO_2 on the physiological performance (i.e. feeding, metabolism, and growth). Lower clearance rate (CR) occurred at the highest pCO_2 concentration (1000 μatm) compared with the control (380 μatm) and with the intermediate concentration of pCO_2 (700 μatm). Conversely, CR was significantly higher at 16°C than at 12°C. Significant lower values of oxygen uptake were observed in mussels exposed to 1000 μatm pCO_2 level compared with those exposed to 380 μatm pCO_2. Scope for growth (SFG) was significantly lower at the highest pCO_2 concentration compared with the control. Mussels exposed to 700 μatm pCO_2 did not show significantly different SFG from the other two pCO_2 treatments. SFG was significantly higher at 16°C than at 12°C. This might be explained because the experimental mussels were exposed to temperatures experienced in their natural environment, which are within the range of thermal tolerance of the species. Our results suggest that the temperature rise within the natural range experienced by *M. chilensis* generates a positive effect on the processes related with energy gain (i.e. feeding and absorption) to be allocated to growth. In turn, the increase in the pCO_2 level of 1000 μatm, independent of temperature, adversely affects this species, with significantly reduced energy allocated to growth (SFG) compared with the control treatment.

Keywords: high CO_2, multiple stressors, mussels, ocean warming, scope for growth, thermal window.

Introduction

Fossil fuel burning and land use change are the dominant causes of the increase in atmospheric CO_2 concentration from pre-industrial levels (ca. 280 μatm) reaching current values of ca. 400 μatm (IPCC, 2013). Atmospheric CO_2 concentration increased at an average rate of 2.0 ± 0.1 μatm $year^{-1}$ during 2002–2011, being higher than any

previous decade since direct atmospheric concentration measurements began in 1958 (IPCC, 2013). According to the current tendency, it is expected that by the end of this century atmospheric CO_2 concentration could reach 700 μatm and will exceed 1500 μatm between the years 2100 and 2200 (Caldeira and Wickett, 2003; Pörtner *et al.*, 2004; IPCC, 2013). Approximately 30% of atmospheric CO_2 dissolves in the ocean, resulting in reduction of pH and the availability of carbonate ions, which are essential to calcium carbonate deposition in calcifier organisms. According to the current trend of increasing CO_2 concentration, ocean acidification (OA) will decrease from 0.2 to 0.4 pH units by 2100, together with a reduction of 50% of carbonate ions (Caldeira and Wickett, 2003; Feely *et al.*, 2004). OA is not an isolated stressor, yet acts together with other environmental shifts such as ocean warming (Meier, 2006; IPCC, 2013). Sea surface temperature has increased by ~0.6°C during the last 50 years (Walther *et al.*, 2002), and it is expected that it will continue to rise between 1 and 4°C by the end of the century (IPCC, 2013).

Growing evidence shows that many environmental stressors can act synergistically, affecting many physiological processes of marine organisms (Schiedek *et al.*, 2007; Gooding *et al.*, 2009). Increased CO_2 concentration in seawater has significant physiological and ecological consequences at many organizational levels of the marine ecosystem (Fabry *et al.*, 2008). A wide range of sensitivities to projected rates of OA exist within and across groups of marine organisms (e.g. corals, oysters, mussels, crabs, and sea urchins), with a trend for greater sensitivity in early life stages (Vargas *et al.*, 2013; Gobler and Talmage, 2014). However, key uncertainties remain in our understanding of the impacts on organisms, life histories, and ecosystems. Most results describe that marine organisms are affected by high pCO_2 levels by disturbances in acid–base regulation, respiration, metabolism, growth rates, reproduction, and calcification (Pörtner, 2008; Widdicombe and Spicer, 2008; Navarro *et al.*, 2013; Duarte *et al.*, 2014; Gazeau *et al.*, 2014).

Because many marine organisms live close to their thermal compensatory capacity (Somero, 2002), the increase in temperature associated with global climate change is expected to impact all physiological processes, survival, and many ecological interactions (Godbold and Solan, 2013). Biological response to rising temperature may vary within and among species, and even across ontogenetic stages (Harley *et al.*, 2006). Seawater temperature is shown to have strong effects on the physiological performance (Pörtner, 2010; Hiebenthal *et al.*, 2013) and reproduction (Navarro *et al.*, 2000; Philippart *et al.*, 2003) of benthic organisms. Recent studies show that the effects of increased CO_2 could be modified by a rise in temperature (Gooding *et al.*, 2009; Findlay *et al.*, 2010). The interaction of elevated seawater CO_2 and extreme temperatures is described to narrow the thermal tolerance window of an organism exposed to high CO_2 levels (Pörtner, 2008; Pörtner and Farrel, 2008). Metzger *et al.* (2007) found that at elevated seawater CO_2 concentrations, the upper thermal tolerance limits were reduced by several Celsius degrees in crustaceans. High temperature and elevated CO_2 lead to a significant decrease in shell hardness in the oyster *Crassostrea virginica* and the clam *Mercenaria mercenaria*, suggesting major changes in their biomineralization processes (Ivanina *et al.*, 2013). However, early juveniles and adults may be vulnerable to skeletal dissolution, although warming may diminish the negative impact of acidification on calcification (Byrne, 2011). In a recent study, we found that the net rate of calcium deposition in juveniles of *Mytilus chilensis* was not significantly affected by temperature, but was negatively affected by high levels of CO_2 (Duarte *et al.*, 2014).

In high-latitude coastal areas of South America, the edible mussel *M. chilensis* forms extensive subtidal and intertidal beds that play an important ecological role, affecting the community structure of the associated macrofauna (Duarte *et al.*, 2006). *Mytilus chilensis* is an important species in Chile for aquaculture activities, with a landing of ca. 300 000 tons per year. These facts make this bivalve species a key group of marine invertebrates for studying the biological impacts of the interaction of elevated pCO_2 levels and global warming on their physiology and growth. In a previous study, Navarro *et al.* (2013) described the effects of high levels of pCO_2 for another population of *M. chilensis* of southern Chile, concluding that growth rate might be significantly reduced under medium-term exposure to elevated pCO_2 levels.

In the present study, juvenile individuals of the mussel *M. chilensis* were reared in a factorial combination of two temperatures (12 and 16°C) and three pCO_2 levels (380, 700, and 1000 μatm). We analysed the combined effects of increasing seawater temperature and pCO_2 on the physiological performance (i.e. feeding, metabolism, and growth) of *M. chilensis*. We hypothesized that the sensitivity of juvenile mussel *M. chilensis* exposed to elevated levels of pCO_2 increases with rising temperature.

Material and methods

Juvenile individuals of *M. chilensis* were collected from suspended cultures (permanently submerged) at Huelmo Bay, Puerto Montt (41°67′S, 73°03′W), during the 2011 winter season (sea surface temperature = 11.5°C; pH 8.09 at the day of collection) and transported to the laboratory under chilled conditions. Mussels of approximately equal length (24.2 ± 0.4 mm SE) and dry tissue weight (33.4 ± 1.6 mg SE) were selected. Before the experiments, mussels were acclimated for 2 weeks in aquaria with circulating seawater (temperature = 12–13°C and salinity 32–33 psu) and fed daily with the microalgae *Isochrysis galbana*.

Experimental set-up

Following the acclimation period, experimental mussels were transferred to the aquaria and exposed to six different treatments at the combination of two temperatures (12 and 16°C) and three pCO_2 levels (380, 700, and 1000 μatm), for a period of 80 d. Each treatment was replicated four times, and each replicate contained five experimental animals, which were identified using bee tags. The experimental temperatures were within the range of thermal tolerance of the species; 12°C represents the average and 16°C the extreme highest value for the area although this was last recorded in a narrow temporal window during summer (Navarro and Jaramillo, 1994). Experimental pCO_2 concentrations were selected to match current day and expected near-future levels according to the predicted values by the IPCC (2013), based on the extreme scenario (RCP8.5) of atmospheric CO_2. Two thermo-regulated baths were prepared for each experimental temperature (12 and 16°C) consisting each of 12 plastic 3.5 l tanks (four replicates for each pCO_2 treatment). The different pCO_2 concentrations were obtained using a mesocosm system, following Navarro *et al.* (2013) and Torres *et al.* (2013). Briefly, for 380 μatm, pure atmospheric air was bubbled into experimental containers. For 700 and 1000 μatm, dry air was blended with pure CO_2 to each CO_2 concentration using mass flow controllers (MFCs, www.aalborg.com) for air and CO_2. During the experiments, water pH and total alkalinity were monitored every 3 d in equilibration tanks. pH measurements were done in closed 25 ml cells thermostated at 25°C using a Metrohm 713 pH meter, calibrated with 8.089 Tris-buffer at 25°C. Temperature and salinity

were measured in the equilibration tanks using a small CTD (Ocean Seven 305 Plus CTD, www.Idronaut.it). Total alkalinity was measured using the method of Haraldsson *et al.* (1997). Resulting hydrographic data were used to calculate the rest of the carbonate system parameters and the saturation stage of Omega Aragonite and Calcite using CO_2SYS software (Lewis and Wallace, 1998) set with Mehrbach solubility constants (Mehrbach *et al.*, 1973) refitted by Dickson and Millero (1987). All experimental individuals were fed with a daily amount of food (*I. galbana*) equal to 5% of the body weight. An open system, consisting of a stock suspension of the microalgae and a multichannel peristaltic pump (Masterflex 7524), was used to deliver the suspension directly to each experimental aquarium. Food concentration was maintained within a range similar to that found in the natural southern Chilean environment throughout the year (ca. 0.8–1.5 mg l^{-1} dry weight). Seawater was gently changed every day, with the corresponding pCO_2 level from the seawater acidification system.

Physiological parameters

Physiological measurements (below) were done on 120 individual mussels taken randomly from each experimental aquarium of the six treatments (two temperatures and three pCO_2 concentrations), starting on day 0 (initial exposure) and continuing every 20 d along a period of 80 d.

Feeding experiments

Clearance rate (CR) of mussels exposed to the experimental temperature and CO_2 combinations (six treatments) was estimated in a static system homogenized by aeration using a food concentration of 25×10^6 cells *I. galbana* l^{-1}. One juvenile mussel was taken randomly from the replicates of each treatment and placed in an experimental chamber (0.4 l). Subsequently, the decrease in the number of particles was monitored every 30 min over a period of 3 h, using an Elzone 180XY particle counter, equipped with a tube of 120-μm aperture. A control aquarium without mussels was used to discard the sedimentation of particles and the CR (l h^{-1} $mussel^{-1}$) was calculated according to Coughlan (1969). Organic ingestion rate was calculated as the product of the CR and the organic material contained in the diet.

After CR measurements were completed, absorption efficiency (AE) was estimated by determining the organic and inorganic content of the food and the faeces following the ratio method of Conover (1966). Samples of food and faeces were filtered through pre-washed, preweighed, 25-mm glassfibre filters. Filters were rinsed with isotonic ammonium formate, dried to a constant weight at 100°C, weighed, combusted at 450°C for 3 h, and weighed again to estimate the organic and inorganic fraction contained in the food and faeces. Absorption rate was calculated as the product of the organic ingestion rate and AE.

Ammonia excretion and oxygen uptake

Ammonia excretion and oxygen uptake were determined immediately after the CR measurements were made. Individual mussels were placed in sealed glass beakers (0.14 l) containing filtered (0.45 μm) seawater. One additional beaker containing filtered seawater but without mussels was used as a control. All beakers were filled with the corresponding experimental pCO_2 levels (380, 700, and 1000 μatm) and maintained at 12 and 16°C by submerging them in two thermostatic water baths. After 2 h, water samples (5 ml) from each beaker were taken and analysed for ammonia–nitrogen by the phenol-hypochlorite colorimetric method of Solórzano (1969). Values for excretion rate were expressed in microgram of NH_4-N. The oxygen uptake by each mussel was estimated by the difference between the oxygen contained in the control and that contained in the experimental beaker over the period of 2 h and analysed by the micro-Winkler method, modified by Ohle (1953), which uses titration to determine dissolved oxygen in the water.

Scope for growth

Scope for growth (SFG) was calculated after converting all the physiological rates to energy equivalents (J h^{-1}): 1 ml O_2 = 19.9 J; 1 μg NH_4-N = 0.0249 J (Elliot and Davison, 1975), and 1 mg of organic material of *I. galbana* = 18.75 J (Whyte, 1987).

Statistical analyses

To avoid pseudo-replication problems, the measured physiological processes were averaged for five mussels of each replicate. A two-way ANOVA was used to estimate the effects of temperature and pCO_2 concentrations on different physiological measurements. When the analyses did not show significant interactions, multiple comparisons were carried out using Tukey's *a posteriori* HSD test on each factor that showed significant differences (Underwood, 1997). Before analyses, normality and homoscedasticity of the data were tested using Kolmogorov–Smirnov and Bartlett tests, respectively. All the analyses were performed using the program Statgraphics Plus 5.1.

Results

Experimental seawater system

The physical and chemical characteristics of the experimental seawater are summarized in Table 1, and were previously described by Duarte *et al.* (2014) in a parallel study on the calcification rate of mussels. The experimental seawater showed very similar values for salinity at all the temperature/pCO_2 combinations. There was a constant decreasing trend of pH, carbonate contents, and aragonite with the increase in the pCO_2 concentrations at both experimental temperatures.

Physiological parameters

CR of *M. chilensis* (Figure. 1a) was significantly affected by pCO_2 and temperature (two-way ANOVA; pCO_2: $F_{1,12} = 7.72$, $p < 0.01$; temperature: $F_{1,12} = 30.17$, $p < 0.001$). Lower values of CR occurred at the highest pCO_2 concentration (1000 μatm) compared with the control (380 μatm) and with the intermediate concentration of pCO_2 (700 μatm). Conversely, CR was significantly higher at 16°C. The interaction between both factors did not have a significant effect ($p > 0.05$) on the CR (temperature * pCO_2: $F_{1,12} = 0.13$, $p > 0.05$). A similar pattern was found for the organic ingestion rate, where the highest level of pCO_2 had a negative effect on this physiological process, while a rise in temperature produced an increase in the organic ingestion rate (Figure. 1b).

AE was not affected by temperature; while under the higher concentration of pCO_2 (1000 μatm), it was significantly lower than in the control treatment (380 μatm CO_2). Individuals maintained at 700 μatm CO_2 exhibited intermediate values, without stating significant differences with the two other treatments (two-way ANOVA; temperature: $F_{1,12} = 3.00$, $p > 0.05$; pCO_2: $F_{2,12} = 4.10$, $p < 0.05$; temperature * pCO_2: $F_{2,12} = 0.77$, $p > 0.05$; Figure. 2a). The absorption rate was affected significantly both by pCO_2 and by temperature, but not for the interaction of both factors. It was significantly lower at the highest pCO_2 concentration compared with the other two treatments; while between 380 and 700 μatm pCO_2, there were no

Table 1. Seawater characteristics (mean ± SD) used to maintain *M. chilensis* during the experimental period [see Material and methods for details on the temperature and CO_2 values used in the study; from Duarte *et al.* (2014)].

	Experimental treatments: temperature and CO_2 concentrations					
	12°C	**16°C**	**12°C**	**16°C**	**12°C**	**16°C**
CO_2 system parameters	**380 µatm (current)**		**700 µatm (2050 year)**		**1000 µatm (2100 year)**	
pH *in situ* (pH units)	8.057 ± 0.026	7.996 ± 0.026	7.835 ± 0.035	7.776 ± 0.034	7.673 ± 0.030	7.618 ± 0.030
Salinity (psu)	31.79 ± 1.88	31.80 ± 1.66	31.81 ± 1.90	31.83 ± 1.72	31.87 ± 1.87	31.88 ± 1.66
TA (µmol kg^{-1})	2156.44 ± 86.24		2148.63 ± 90.76		2142.19 ± 93.23	
pCO_2 *in situ* (µatm)	371.35 ± 23.52	439.54 ± 27.63	656.91 ± 50.44	772.64 ± 58.46	977.56 ± 60.52	1141.90 ± 69.19
$[CO_3^{2-}]$ *in situ* (µmol kg^{-1})	129.48 ± 14.65	131.08 ± 14.74	82.45 ± 11.24	83.97 ± 11.32	58.47 ± 7.92	59.95 ± 8.00
Ω_{ca}	3.14 ± 0.33	3.19 ± 0.33	1.99 ± 0.25	2.04 ± 0.25	1.42 ± 0.18	1.46 ± 0.18
Ω_{ar}	1.99 ± 0.22	2.04 ± 0.22	1.27 ± 0.17	1.31 ± 0.17	0.90 ± 0.12	0.93 ± 0.12

TA, total alkalinity; $[CO_3^{2-}]$, carbonate ion concentration; Ω_{ca}, omega calcite; Ω_{ar}, omega aragonite.

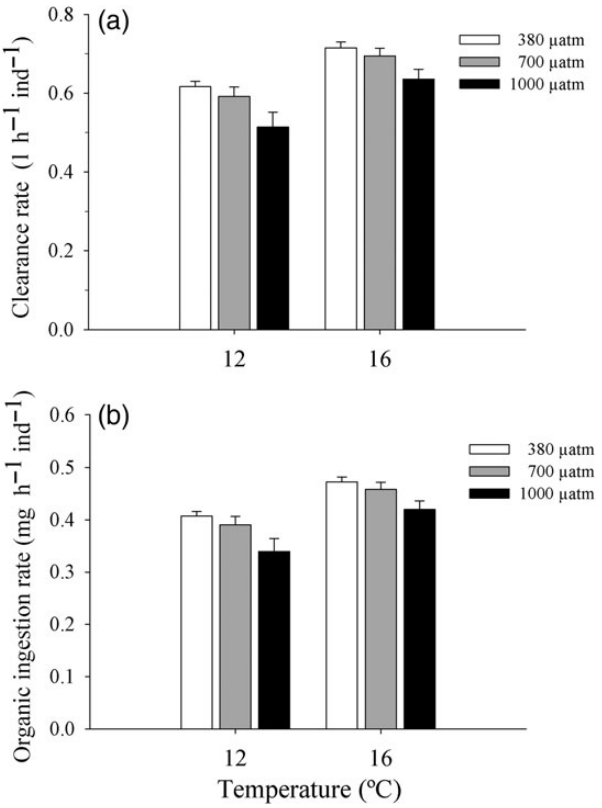

Figure 1. CR (a) and organic ingestion rate (b) of juvenile individuals of the mussel *M. chilensis* exposed to different combinations of temperature and pCO_2 for 80 d. Values are means ± standard error.

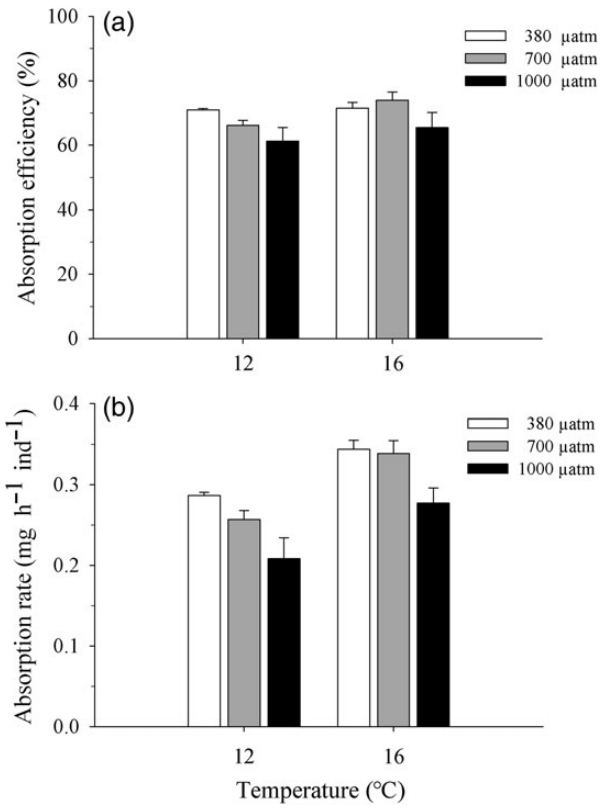

Figure 2. AE (a) and absorption rate (b) of juvenile individuals of the mussel *M. chilensis* exposed to different combinations of temperature and pCO_2 for 80 d. Values are means ± standard error.

significant difference. The absorption rate was significantly higher in mussels exposed to 16°C. (two-way ANOVA; pCO_2: $F_{2,12} = 11.20$, $p < 0.05$; temperature: $F_{1,12} = 28.00$, $p < 0.01$; temperature * pCO_2: $F_{2,12} = 0.28$, $p > 0.05$; Figure. 2b).

The excretion rate of *M. chilensis* was not affected by pCO_2 concentration; however, it was affected significantly by temperature, with higher values at 16°C (two-way ANOVA; pCO_2: $F_{2,12} = 0.77$, $p > 0.05$; temperature: $F_{1,12} = 5.32$, $p < 0.05$; temperature * pCO_2: $F_{2,12} = 0.84$, $p > 0.05$; Figure. 3a).

Oxygen consumption was significantly affected by pCO_2, but not by temperature or by the temperature * pCO_2 interaction (two-way ANOVA; pCO_2: $F_{2,12} = 6.10$, $p < 0.01$; temperature: $F_{1,12} = 0.031$, $p > 0.05$; temperature * pCO_2: $F_{2,12} = 0.26$, $p > 0.05$). Significantly lower values of oxygen uptake were observed in mussels exposed to 1000 µatm pCO_2 compared with those exposed to 380 µatm pCO_2. Individuals maintained at 700 µatm pCO_2 exhibited intermediate values of oxygen uptake, without stating significant differences with the two other treatments (Figure. 3b).

Two-way ANOVA showed a significant effect of the factors pCO_2 ($F_{1,12} = 4.38, p < 0.05$) and temperature ($F_{1,12} = 15.58, p < 0.001$) on the SFG of *M. chilensis*. However, the interaction between both factors did not have a significant effect ($F_{1,12} = 0.59$, $p > 0.05$).

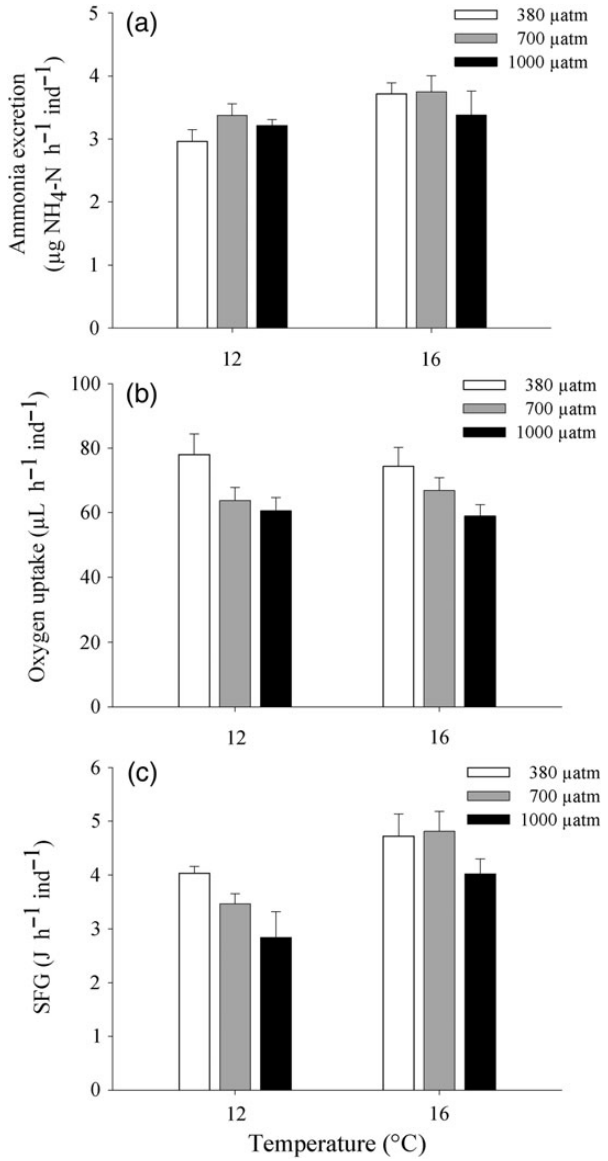

Figure 3. Ammonia excretion (a), oxygen uptake (b) and SFG (c) of juvenile individuals of the mussel *M. chilensis* exposed to different combinations of temperature and pCO_2 for 80 days. Values are means ± standard error.

SFG was significantly lower at the highest pCO_2 concentration (1000 µatm) compared with the control (380 µatm). Mussels exposed to 700 µatm pCO_2 did not show significantly different values of SFG from the other two pCO_2 treatments. SFG was significantly higher at 16°C than at 12°C (Figure. 3c).

Discussion

Our results showed that the rise in temperature from 12 to 16°C in a scenario of elevated pCO_2 (1000 µatm pCO_2) did not increase the sensitivity of the physiological processes measured in *M. chilensis*. On the contrary, it was observed that the increase in temperature induced a positive effect on the physiological processes associated with increased energy. In turn, the increase of pCO_2 adversely affected this species by reducing the energy intake, which resulted in significantly lower values of SFG at 1000 µatm pCO_2 at both temperatures, compared with 380 µatm pCO_2.

Contrasting results have been described in previous studies on the combined effects of warming and elevated pCO_2 levels on different groups of marine invertebrates. Catarino *et al.* (2012) demonstrated that ocean warming and acidification antagonistically interacted on oxygen uptake of the sea urchin *Paracentrotus lividus*. Byrne and Przeslawski (2013) reviewed the combined effect of ocean warming and high pCO_2 levels in >20 species of invertebrates. Their results showed that antagonistic effects were common, where ocean warming reduces the negative effects of pCO_2, unlike synergies, which were less common. Previous studies have shown that feeding activity in molluscs can increase within the thermal window of each species and drastically reduced when the temperature exceeds the limit of thermal tolerance (Navarro *et al.*, 2002; Peck *et al.*, 2009). Wang *et al.* (2015) found that increasing temperature significantly reduced CR of the mussel *Mytilus coruscus*, but elevated levels of pCO_2 did not show an additive or antagonistic effect with temperature. In our study, temperature positively affected CR, increasing from 12 to 16°C at all experimental pCO_2 levels. This can be explained by the experimental temperature range used, which is within the thermal window (9–16°C) of the studied population of *M. chilensis* (Navarro and Jaramillo, 1994). The negative effect of elevated concentrations of pCO_2 on the feeding rate of *M. chilensis* observed in our study has been also described for other species of bivalves. Fernandez-Reiriz *et al.* (2011) observed a reduction in the feeding rate in the clam *Ruditapes decussatus* at the highest experimental pCO_2 concentration (pH 7.48). Navarro *et al.* (2013) also described negative effects of elevated levels of pCO_2 (pH 7.57) on the feeding rate of the mussel *M. chilensis*.

There are few studies on the combined effects of ocean warming and high pCO_2 levels on AE of marine organisms. Zhang *et al.* (2015) found that AE of the gastropod *Nassarius conoidalis* was not significantly affected at the beginning of the experimental period (day 2) by temperature, pCO_2 level, nor the interaction between temperature and pCO_2 levels. However, on day 30, the temperature negatively affected AE when this gastropod was exposed at the highest pCO_2 concentration. Wang *et al.* (2015) described low values for AE (20–45%) on the mussel *M. coruscus* with no significant differences between three different pH levels (8.1, 7.7, and 7.3). Our results showed that pCO_2 levels significantly affected the AE of *M. chilensis*, but this effect was not registered for neither temperature nor the interaction between temperature and pCO_2. In a previous study, Navarro *et al.* (2013) reported that elevated pCO_2 levels (pH 7.57) reduced significantly the AE of *M. chilensis*, indicating possible deficiencies in the functioning of the digestive system under conditions of seawater acidification.

Similar to other studies on marine bivalves (Bayne and Newell, 1983; Velasco and Navarro, 2005), ammonia excretion of *M. chilensis* represented a low amount of the energy absorbed, with a minimal impact on the energy balance. Ammonia excretion was only affected by temperature, and the increase observed in mussels exposed to the highest temperature (16°C) could be explained by the larger amount of food ingested and absorbed under these conditions. Navarro *et al.* (2013) found similar values for ammonia excretion in juveniles of *M. chilensis* exposed to a wide range of pCO_2 levels (7.91–7.57).

There is evidence of the capability of marine organisms to acclimate the metabolic rate within certain range of temperature (thermal window), being a species-specific response (Pörtner *et al.*, 2005, Pörtner, 2008, 2010; Ezgeta-Balic *et al.*, 2011). However, at high temperatures, beyond the range of thermal tolerance of a species, a

drastic decline occurs in oxygen consumption and an increase in the anaerobic metabolic pathways (Jansen *et al.*, 2007). In our study, oxygen consumption was not affected by temperature. This can be explained because the mussels were exposed to a temperature range that the animals experience in their natural environment. However, temperatures over the thermal window naturally could have a negative impact on oxygen consumption of *M. chilensis*. Several studies have described metabolic depression in different species of marine organisms at elevated pCO_2 concentrations, caused by the low capacity to compensate for disturbances in extracellular ion and acid–base status (Michaelidis *et al.*, 2005; Pörtner, 2008). Our study showed a depletion of oxygen consumption when mussels were exposed to the highest pCO_2 level, suggesting that *M. chilensis* also is not able to cope with high pCO_2 levels in the seawater.

Different physiological processes related with gain and expenditure of energy largely depend on an organism's environmental temperature (Bayne and Newell, 1983). Populations that can maintain their aerobic capacity at warmer temperatures have a higher thermal tolerance, and are thereby predicted to persist longer as the temperature increases (Pörtner, 2001; Gardiner *et al.*, 2010). SFG represents the integration of all these processes varying as a function of temperature (Ezgeta-Balic *et al.*, 2011). Wang *et al.* (2015), studying the combined effects of seawater temperature and pH on the mussel *M. coruscus*, described positive values of SFG for all treatments, with no significant effects of pH. However, temperature showed a significant effect on the SFG, being reduced from 25 to 30°C. Ezgeta-Balic *et al.* (2011) recorded negative values of SFG for the mussel *Modiolus barbatus* when temperature rises from 26 to 28°C. Anestis *et al.* (2010), when studying the response of the mussel *Mytilus galloprovincialis* to increasing seawater temperature, found that the SFG values became negative at temperatures higher than 24°C, associated with a reduction in CR. Contrary to these authors, the present study showed a significant positive effect of temperature on SFG at all pCO_2 levels. This might be explained because the experimental mussels were exposed to temperatures experienced in their natural environment, which are within the range of thermal tolerance of the species although the highest temperature (16°C) is recorded in a narrow temporal window during summer (Navarro and Jaramillo, 1994). The increase of the SFG observed in the present study matches well with the significant increase of CR and absorption rate from 12 to 16°C. Oxygen uptake was not affected by increasing temperature, suggesting that metabolic activity is maintained constant within the thermal window of *M. chilensis*. Similar conclusions were reported by Findlay *et al.* (2010) to explain the lack of differences between the growth rates of *Semibalanus balanoides* exposed to a range of temperatures experienced in their natural environment. The zone of the Reloncaví Sound, located 50 km east from the place where the experimental mussels were collected (Bay of Huelmo), has been described as an environment with a low calcium carbonate saturation and even "corrosive" when occurring in waters with low salinity and alkalinity (Alarcón *et al.*, 2015). This can explain the lack of significant differences in some of the physiological responses between the 380 and 700 pCO_2 levels, suggesting that mussels from this area might be pre-adapted to natural pH variability.

There are still few studies on the combined effect of pCO_2 with other environmental stressors. Gazeau *et al.* (2014) found that somatic and shell growth of *M. galloprovincialis* did not appear very sensitive to OA and warming during most of the experiment. However, growth was significantly reduced after summer in the lowered pH treatment. According to these authors, there is a progressive insufficiency in acid–base regulation capacity, which was also consistent with shell net dissolution observed in the mussels (Gazeau *et al.*, 2014). Other studies described that growth might be significantly reduced under medium- and long-term exposure to elevated pCO_2 levels (Beniash *et al.*, 2010; Navarro *et al.*, 2013; Duarte *et al.*, 2014). Both shell and soft tissue growth of the oyster *C. virginica* were reduced when exposed to high pCO_2 levels (pH 7.5; Beniash *et al.*, 2010). Similarly, Duarte *et al.* (2014) found that net rate of calcium deposition and total weight of the mussel *M. chilensis* were not significantly affected by temperature, but were negatively affected by high pCO_2 levels. Our results suggest that a rise in temperature within the natural range experienced by juveniles *M. chilensis* generates a positive effect on the processes related with energy gain from the environment (i.e. feeding and absorption) to be allocated for growth. In turn, the increase in 1000 μatm pCO_2, independent of temperature, adversely affects this species, with significantly reduced energy allocated to growth (SFG) compared with the control treatment (380 μatm pCO_2). Our results are interpreted as answers to short-term toxicity challenges with temperature and CO_2 as environmental factors. On the basis of these, inferences about environmental changes that would slowly occur over the next 100–200 years must be made with appropriate caution. This is because animals used for testing would vary in geno- and phenotypes.

The effect of elevated pCO_2 on marine organisms can be negative (Gazeau *et al*, 2014), reduced, or absent in the presence of high temperature (Pörtner and Farrel, 2008; Duarte *et al.*, 2014). This is because the effect of warming will depend on the experimental temperature range and thermal window of the species under study. Thus, the studies related to the combined effect of ocean warming and elevated concentrations of pCO_2 must consider the thermal windows of each species to avoid contradictory interpretations. Future experiments with different levels of temperatures should be designed in a way that will allow greater predictive power, selecting temperature treatments that will be physiologically realistic to the animal's thermal windows and to future climate scenarios.

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