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Ocean acidification exacerbates the effects of paralytic shellfish toxins on the fitness of the edible mussel *Mytilus chilensis*

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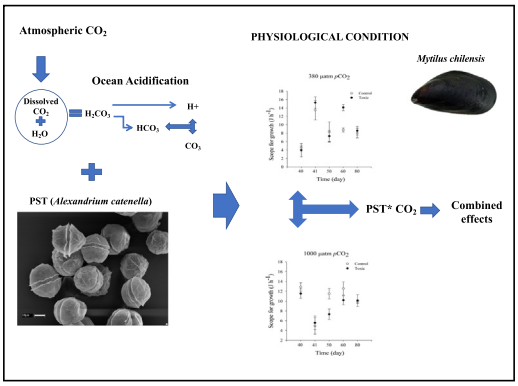
HIGHLIGHTS

- The association between pCO2 - PST impacts negatively of the physiology of *M. chilensis*
- The association between pCO2 and PST may also result in indirect effect on mussel fitness.
- The inhibition of energy acquisition by PST may negatively impact mussel fitness.

GRAPHICAL ABSTRACT



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ABSTRACT

High latitudes are considered particularly vulnerable to ocean acidification, since they are naturally low in carbonate ions. The edible mussel *Mytilus chilensis* is a common calcifier inhabiting marine ecosystems of the southern Chile, where culturing of this species is concentrated and where algal blooms produced by the toxic dinoflagellate *A. catenella* are becoming more frequent. Juvenile *Mytilus chilensis* were exposed to experimental conditions simulating two environmental phenomena: pCO_2 increase and the presence of paralytic shellfish toxins (PST) produced by the dinoflagellate *Alexandrium catenella*. Individuals were exposed to two levels of pCO_2: 380 μatm (control condition) and 1000 μatm (future conditions) over a period of 39 days (acclimation), followed by another period of 40 days exposure to a combination of pCO_2 and PST. Both factors significantly affected most of the physiological variables measured (feeding, metabolism and scope for growth). However, these effects greatly varied over time, which can be explained by the high individual variability described for mussels exposed to different environmental conditions. Absorption efficiency was not affected by the independent effect of the toxic diet; however, the diet and pCO_2 interaction affected it significantly. The inhibition of the physiological processes related with energy acquisition by diets containing PST, may negatively impact mussel fitness, which could have important consequences for both wild and cultured mussel populations, and thus, for socioeconomic development in southern Chile.

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1. Introduction

According to the current trend of increasing atmospheric pCO_2, surface ocean pCO_2 could increase to levels near 1000 μatm by 2100 (Caldeira and Wickett, 2003; IPCC, 2013), changing the content of dissolved inorganic carbon (DIC) of the ocean as well as its speciation, including increments in the hydrogen cations and ion bicarbonate, and the reduction of ion carbonate concentration in seawater (Feely et al., 2004).

Although pCO_2 is not an isolated driver, it can act together with other environmental drivers such as temperature, salinity, and oxygen content (Breitberg et al., 2015; IPCC, 2013; Kavousi et al., 2016; Piggott et al., 2015). Effects of such interactions have been observed for numerous groups of marine organisms (e.g. corals, macroalgae, oysters, mussels, crabs, and sea urchins), with a trend for greater sensitivity in early life stages (Al-Janabi et al., 2016; Campanati et al., 2016; Gobler and Talmage, 2014; Kavousi et al., 2016). The combined effect of two or more factors can have significant impacts on various processes such as homeostasis, metabolism, growth, reproduction, and calcification (Azevedo et al., 2015; Ban et al., 2014; Navarro et al., 2016a; Boyd et al., 2018).

Marine mussels belong to a group of calcifier bivalves that have been studied in the context of the impacts of acidification and climate change (Fabry et al., 2008). Navarro et al. (2013) and Duarte et al. (2014) described the effects of high levels of pCO_2 on juvenile mussels (*Mytilus chilensis*) from populations in southern Chile, concluding that growth rate, net calcium deposition rate and total weight decreased significantly when mussels were exposed to high levels of pCO_2. These effects may be explained by the changes in dissolved inorganic carbon (DIC) speciation induced by an increase in pCO_2 (e.g. reduction of the ion carbonate concentration) or by hypercapnia, which leads to intra - and extracellular acidosis, interfering with physiological processes (Duarte et al., 2014; Kurihara, 2008; Raven et al., 2005).

Dinoflagellates represent a cosmopolitan group of phytoplankton, some of which produce toxins and can form harmful algal blooms (HABs) in different geographical areas (Hallegraeff, 2004). Future increased ocean acidification may exacerbate the toxic threat posed by the toxic dinoflagellate *Alexandrium catenella*, especially when combined with nutrient limitation (Tatters et al., 2013). Suspension-feeding organisms, especially bivalves, exposed to toxic dinoflagellate blooms ingest these microalgae and accumulate paralytic shellfish toxins (PST) in their tissues. Toxic dinoflagellate blooms are detrimental to aquatic organisms (Shumway and Cucci, 1987; Gainey and Shumway, 1988a; Marsden and Shumway, 1992; Bricelj et al., 1996; Bricelj and Shumway, 1998), and several physiological and behavioural effects, such as reductions in ingestion, metabolism, and growth rates, have been described in marine copepods and bivalves exposed to diets containing PST (Colin and Dam, 2007; Li et al., 2002). The presence of PST toxins in southern Chile is associated with blooms of the dinoflagellate *Alexandrium catenella* and represents a serious risk to human health. Harmful algae blooms of this dinoflagellate have occurred with almost annual frequency in the most southerly regions of Chile (Castro: 42°36′ S, 73°57′ W; Punta Arenas: 53°10′ S, 70°56′ W) and have recently extended northwards, reaching the coast near Valdivia (39°53′ S, 73°26′ W). *Mytilus chilensis* is an important species for aquaculture, with a landing of ca. 300,000 tons per year in Chile (SERNAPESCA, 2015). It is found between the Tirúa River (38° 20 S) and the Straits of Magellan (53° 28′S; 70° 47′W), occupying extensive subtidal and intertidal zones, and plays an important ecological role that partially determines the structure of the associated macrofaunal community (Duarte et al., 2006; Hernández and González, 1976). Due to its broad latitudinal distribution, *M. chilensis* is exposed to diverse environmental conditions, including exposure to HABs, making this species useful for evaluating how various environmental drivers affect physiological processes. *Mytilus chilensis* is a suitable model species for the study of biological impacts of high pCO_2 levels (Navarro et al., 2013) and the presence of PST in the sea, because previous studies have shown that both of these environmental drivers (individually) can affect to this bivalve (Molinet et al., 2010; Navarro et al., 2013).

This study evaluates the combined effects of two environmental drivers that could occur simultaneously in southern Chile, namely a pCO_2 increase (associated with global climate change) and the presence of PST, on the physiological performance (feeding, metabolism and growth) of juvenile *M. chilensis*. We hypothesize that the combined effects of these two factors decreases the fitness of *M. chilensis* expressed in terms of the energy available for growth processes (scope for growth).

2. Materials and methods

2.1. Collection and acclimation of mussels

Mussels (*Mytilus chilensis*) were collected (May 2012) from suspended cultures in Huelmo Bay, Puerto Montt, southern Chile (41°40′ S, 73°02′ W) and transported to the laboratory under controlled temperature conditions. Specimens with a shell length range of 2.4–2.9 cm were selected; these were all juveniles in the sense that they were not yet capable of reproduction. In preparation for physiological measurements, mussels were divided into two groups and acclimated for 39 days in 20 aquaria (4 L), each containing 9 individuals, one group exposed to 380 μatm pCO_2 (control), the other to 1000 μatm pCO_2 (predicted future condition, IPCC, 2013). Temperature was maintained at 14 °C by partially immersing the aquaria in thermostatically controlled water baths. Salinity was maintained at 30, and all experimental mussels were fed continuously (peristaltic pump) with the microalga *Isochrysis galbana* (ca 1.5 mg L^{-1}). Seawater was changed daily with water pre-equilibrated to the above conditions.

2.2. Diets and experimental design

The dinoflagellate *Alexandrium catenella* (strain ACC02) was cultured in filtered seawater (0.45 μm) at 14 °C and a 14:10 photoperiod (light: dark), using L1 culture medium (Guillard, 1975). According to Velásquez and Navarro (2014), strain ACC02 has an average toxin concentration of 10.3 ± 0.91 fmoles STX eq. $cell^{-1}$. The microalga *Isochrysis galbana* was cultured in filtered seawater (0.45 μm) at 25 °C and a 14:10 photoperiod (light: dark), using f/2 culture medium (Guillard, 1975).

Following 39 days of acclimation to the two pCO_2 treatments, mussels were exposed for 40 days to two combinations of pCO_2 and PST-containing diets. For each treatment (380 μatm pCO_2 + control diet; 380 μatm pCO_2 + toxic diet; 1000 μatm pCO_2 + control diet; 1000 μatm pCO_2 + toxic diet) 5 mussels were placed in each of 5 replicate aquaria. The control diet was 100% *I. galbana*, and the toxic diet consisted of 70% *A. catenella* and 30% *I. galbana* (by weight). Both diets were delivered continuously through a peristaltic pump (Masterflex 7524). In all treatments the diet offered represented a daily contribution of 4% of the soft tissue weight of the mussel biomass in the tank, calculated as follows:

Mean dry weight of a mussel = 130 mg; 4%dry weight = 5.2 mg

Dry weight of 10^6*A.catenella cells* = 6.19 mg

Dry weight of 10^6*I.galbana cells* = 0.032 mg

Weight of *A.catenella cells* added per day per mussel = 0.7×5.2 mg = 3.64 mg $\left(\equiv 0.59 \times 10^6 \text{cells} \right)$

Weight of *I.galbana cells* added per day per mussel = 0.3×5.2 mg = 1.56 mg $\left(\equiv 49.1 \times 10^6 \text{cells} \right)$

Seawater in the mussel holding tanks was replaced daily by gradual introduction of water from the equilibriation tanks (see below), maintaining the appropriate diet and pCO_2 levels.

2.3. Seawater equilibration system

Three polyethylene header tanks (250 L, referred to here as equilibration tanks) were filled with filtered (1 μm) seawater taken from the subtidal zone at Calfuco (39°44′S, 73°23′W) and adjusted to two pCO_2 levels, current atmospheric levels (approx. 380 ppm) and the worst case scenario for the end of the century (approx. 1000 ppm) by bubbling air or an air–CO_2 mixture (Torres et al., 2013). During the experiments, water pH and total alkalinity were monitored in each tank every 3 d. All pH measurements were done in a closed 25 mL thermostatic cell at 25.0 °C with a Metrohm 713 pH meter (input resistance $>10^{13}$ Ω, 0.1 mV sensitivity and nominal resolution 0.001 pH units) and a glass combined double junction Ag/AgCl electrode (Metrohm model 6.0219.100) calibrated with 8.089 Tris buffer at 25 °C; pH values are reported using the total hydrogen ion scale (DOE, 1994). The overall uncertainty in the measured pH values was estimated by Torres et al. (1999) as 0.006 pH for surface waters (pH near 8) and <0.009 pH for very acid waters (pH 7.2). Temperature and salinity were measured in the equilibration tanks with a small conductivity-temperature-depth instrument (Ocean Seven 305 Plus CTD, www.idronaut.it). Total Alkalinity (A_T) was determinate in seawater samples by automatic titration with HCl (Haraldsson et al., 1997) using CRM from Andrew Dickson Laboratory to constrain analytical uncertainties; Based in the results of 2017 Inter-laboratory Comparison of CO_2 Measurements (coordinated. Emily Bockmon and Andrew Dickson, unpublished data) we calculate that the difference between our analysis (Carbonate System Laboratory at CIEP) and Scripps Institution of Oceanography (CRM Batches 162 and 164) were approximately 0.1% which is considerate adequate (Bockmon and Dickson, 2015).

The pH, total alkalinity (A_T), phosphate (Strickland and Parsons, 1972), dissolved silicate (Strickland and Parsons, 1972), and hydrographic data were used to calculate the remaining carbonate system parameters and the saturation state of seawater with respect to aragonite and calcite using CO2SYS software (Lewis and Wallace, 1998), set with Mehrbach solubility constants (Mehrbach et al., 1973), as refitted by Dickson and Millero (1987).

2.4. Measurement of physiological variables

On each sampling date (days 40, 41, 50, 60, and 80) one mussel was taken at random from each replicate aquarium for each treatment, i.e. 5 mussels per treatment, for physiological measurements. Mussels reached a PST concentration of 128.0 μg STXeq./100 g tissue at the end of the experiment at 380 μatm pCO_2 and 85.6 μg STXeq./100 g tissue at 1000 μatm pCO_2.

Clearance rate (CR) was measured in a static system in which the decrease in particle concentration in each experimental chamber resulting from feeding activity was monitored periodically (Widdows, 1985). Before the measurement period, the experimental specimens were allowed to acclimate for 30 min in the chamber, after which the algal cells constituting the appropriate diet (1.5 mg L^{-1}) were added. All experiments were conducted at 14 °C and salinity 30. Each experimental mussel was placed in an experimental chamber (0.5 L), one mussel per chamber, and the decrease in the number of particles was measured after 30 min using a particle counter (Beckman Z2) fitted with a 100 μm aperture tube. The consumed cells were then replaced to reset the cell concentration to the initial value, and the clearance rate ($L\ h^{-1}$) for the 30 min period calculated (Coughlan, 1969). This procedure was repeated 7 times and a mean value of clearance rate determined, representing an overall clearance rate over a period of approximately 4 h. At no time was the particle concentration in the chamber allowed to fall below 60% of the initial value. No pseudofaeces were produced with the food concentration used. During each set of measurements, a chamber without mussels was used as a control for each treatment to account for natural changes in particle concentration (growth or algae sedimentation). The organic ingestion rate was calculated as the product of clearance rate and the organic content of the diet.

Absorption efficiency (AE) was estimated by the ratio method of Conover (1966). Faeces were collected from each experimental mussel after clearance rate measurements were completed. Samples of food and faeces were retained on pre-ashed, pre-weighed Whatman GF/C filters (1.2 μm pore size), rinsed with ammonium formate (3%), dried to constant weight at 100 °C, weighed, combusted at 450 °C for 3 h in a muffle furnace and weighed again to determine the organic and inorganic fractions. Absorption rate was calculated as the product of organic ingestion rate and absorption efficiency.

Immediately after the clearance rate measurements were made, ammonia excretion (VNH_4-N) and oxygen uptake (VO_2) were determined on the same individuals used for clearance rate determinations. Individual mussels were placed in sealed glass beakers (0.13 L) containing filtered (0.45 μm) seawater. During each measurement set, one additional beaker containing filtered seawater, but without mussels, was used as a control. After 2 h, water samples (5 mL) were taken from each beaker for the determination of ammonia excretion using the colorimetric method of Solórzano (1969) as modified by Widdows (1985). Oxygen concentration was determined by the micro-Winkler titration method (Ohle, 1953) and the oxygen uptake by each mussel was calculated as the difference between the oxygen content of the water in the control and experimental beakers over a period of 2 h.

2.5. Scope for Growth (SFG)

Measurements of the energy available for growth, termed scope for growth (SFG), provide a rapid and quantitative assessment of the energy status of the bivalve (Widdows, 1985). Scope for growth was calculated after converting VO_2, VNH_4-N and absorbed organic matter from the diet to energy equivalents ($J \cdot h - 1$): 1 mL O_2 = 19.9 J; 1 μg NH_4-N = 0.0249 J (Elliott and Davison, 1975) and 1 mg of organic material from the diet = 21 J (McLusky, 1989).

2.6. Statistical analyses

The prediction that the combined effects of pCO_2 and PST decreases the fitness of *M. chilensis* expressed in terms of energy acquisition was tested by means of random-intercept linear mixed models (LMM) computed separately for each dependent variable. The models included diet, time, and pCO_2 as fixed and fully crossed factors, and the identification of each aquarium as a random factor influencing the intercept (i.e. baseline level) of the model. The aquarium was included as random factor in order to account for the repeated measurements conducted over time. In addition, we modelled the temporal autocorrelation of errors as an autoregressive (AR1) process. The effects parameters were estimated by means of maximum likelihood. Residual-by-quantile, density, and residual-by-fitted value plots were graphically inspected as model diagnostics. Moreover, we assessed the degree of autocorrelation of errors by means of Auto-Correlation Function (ACF) plots. These plots showed minimum degrees of autocorrelation of residuals, always $r < 0.2$ (Pearson Product Moment correlation). Marginal and conditional coefficients of determination (pseudo-R^2) were computed as estimators of variance accounted for the fixed factors and the entire model, respectively (Nakagawa and Schielzeth, 2013). Each LMM was further analysed by means of a Wald-test analysis of variance (ANOVA). After a significant term was detected in the ANOVA, we computed paired comparisons between the marginal means (i.e. averaging across the levels of the other factors). In the cases of statistically significant interactions, the paired comparisons were conducted for a given factor within each level of the other factor involved in the interaction. Tukey's multiplicity adjustment was used to reduce the Type-I error rate. All statistical analyses were conducted in the R programming environment version 3.5.0 (R Core Team, 2018) – the *ggplot2, dplyr, nlme, MuMIn,* and *emmeans* R packages were required to complete the statistical analyses.

Table 1
Characteristics (mean ± SD) of seawater used to maintain *Mytilus chilensis* individuals during the experimental period in the 250 L tanks equilibrated with air (approx. 380 μatm pCO_2) or air – CO_2 mixture (approx. 1000 μatm pCO_2). TA = total alkalinity; $[CO_3^{2-}]$ = carbonate ion concentration; Ω_{ca} = omega calcite; Ω_{ar} = omega aragonite. From Duarte et al. (2014).

CO_2 system variables	Experimental pCO_2 levels (μatm)	
	380	1000
pH in situ (pH units)	8.06 ± 0.03	7.67 ± 0.03
Salinity (psu)	31.79 ± 1.88	31.87 ± 1.87
TA (μmol kg^{-1})	2156 ± 86	2142 ± 93
pCO_2 in situ (μatm)	371 ± 23	977 ± 60
$[CO_3^{2-}]$ in situ (μmol kg^{-1})	129 ± 14	58 ± 7
Ω_{ca}	3.1 ± 0.3	1.4 ± 0.2
Ω_{ar}	2.0 ± 0.2	0.9 ± 0.1

3. Results

3.1. Experimental seawater system

The physical and chemical characteristics of the experimental seawater are summarized in Table 1, and were previously described in a parallel study on the calcification rate of mussels (Duarte et al., 2014). Values for salinity were very similar at both pCO_2. The pH, carbonate ion concentration and the saturation state of seawater with respect to aragonite decreases with increasing pCO_2.

Table 2
Summary of ANOVA on six physiological variables in mussels exposed to two levels of diet (controls or toxic), pCO_2 (380 and 1000), and sampled over time of exposure (after 40, 41, 50, 60, and 80 days) in a fully orthogonal experiment. Degrees of freedom (DF) are provided for the numerator and denominator (num and den, respectively) of each F-ratio.

Source of variation	DF		Clearance rate		Absorption efficiency		Absorption rate	
	Num	Den	F	*p*	F	*p*	F	*p*
Diet	1	6	30.519	<0.01	3.21	0.09	0.17	0.69
Time	4	64	1.387	0.248	8.02	<0.01	5.92	0.00
pCO_2	1	16	0.049	0.827	46.64	<0.01	0.95	0.34
Diet:Time	4	64	13.653	<0.01	2.72	0.04	3.46	0.01
Diet:pCO_2	1	16	0.005	0.945	12.70	<0.01	6.16	0.03
Time:pCO_2	4	64	1.527	0.205	17.68	<0.01	26.44	<0.01
Diet:Time:pCO_2	4	64	2.052	0.098	4.47	<0.01	1.17	0.33

	DF		Ammonia excretion		Oxygen consumption		Scope for growth	
	Num	Den	F	*p*	F	*p*	F	*p*
Diet	1	16	6.25	0.02	0.07	0.80	0.03	0.87
Time	4	64	6.80	<0.01	2.31	0.07	4.09	0.01
pCO_2	1	16	0.01	0.93	19.28	<0.01	0.85	0.37
Diet:Time	4	64	5.93	<0.01	9.56	<0.01	1.94	0.12
Diet:pCO_2	1	16	38.11	<0.01	0.02	0.91	4.46	0.05
Time:pCO_2	4	64	2.95	0.03	16.61	<0.01	25.19	<0.01
Diet:Time:pCO_2	4	64	0.99	0.42	1.24	0.30	1.48	0.22

3.2. Physiological parameters

3.2.1. Clearance rate

The effect of the toxic diet on clearance rate was negative and depended on the sampling time, these effects remained relatively similar between pCO_2 treatments (Fig. 1). The ANOVA (Wald test) conducted on the linear mixed model (LMM) showed a statistically significant interaction between diet and time (Table 2). This interaction was given by the statistically significant and negative effects of the toxic diet that were observed at days 40 and 41 (−0.61 [0.07] L h^{-1} and −0.26 [0.07] L h^{-1}, respectively, mean [standard error of the mean]); but that became non-significant after 60 days (Table S1). The fixed factors in the LMM accounted for ca. 56% of the variation in clearance rate; the entire model explained ca. 62% of the variation in this variable.

3.2.2. Absorption efficiency

Absorption efficiency was dependent on sampling time, diet, and pCO_2 (Fig. 2). This observation was supported by the LMM and ANOVA, which showed a statistically significant interaction between the three factors (Table 1). According to this second-order interaction, we observed statistically significant effects of the toxin at days 40 and 60 on the absorption efficiency of the mussels exposed to 380 μatm (−9.2 [4.04] % and 16.5 [4.04] %), respectively (Table S1). On the other side, we detected statistically significant effects of the toxic diet at days 50 and 80 (−14.1 [4.04] % and −11.1 [4.04] %, respectively) of mussels exposed to 1000 μatm (Table S1). The fixed factors accounted for ca. 71% of the variation in absorption efficiency. The same proportion of variance was explained by the entire model, suggesting a negligible effect of sampling units on the variable of interest.

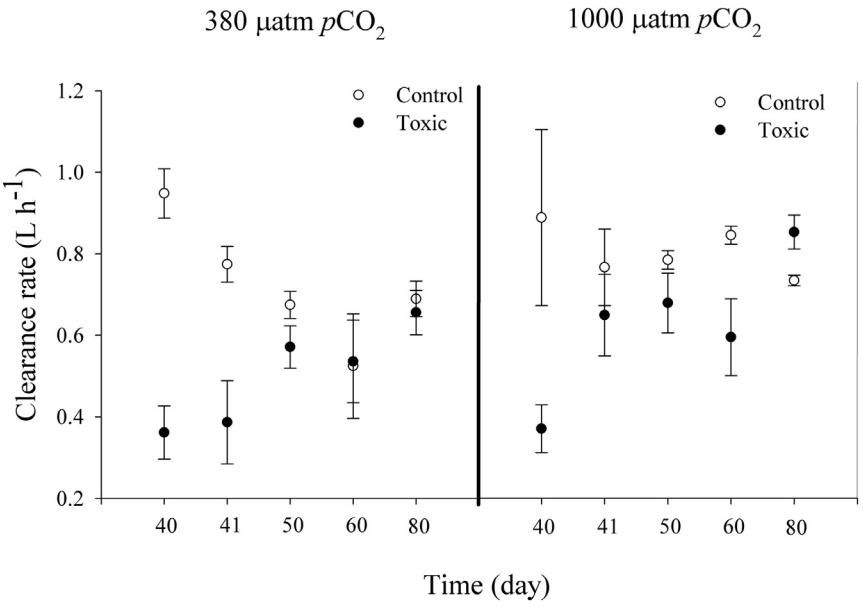

Fig. 1. Clearance rate in *Mytilus chilensis* juveniles exposed to toxic (PST) and control diets at 380 (a) and 1000 (b) μatm pCO_2. Values represent mean ± standard error.

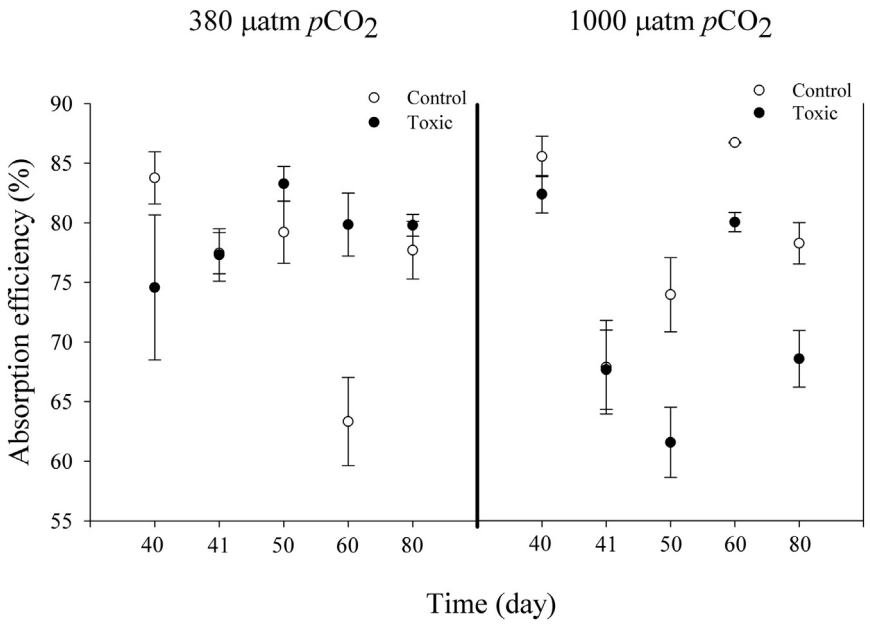

Fig. 2. Absorption efficiency in *Mytilus chilensis* juveniles exposed to toxic (PST) and control diets at 380 (a) and 1000 (b) µatm pCO_2. Values represent mean ± standard error.

3.2.3. Absorption rate

This variable showed temporal patterns that depend on the diet and pCO_2 treatments. Moreover, the effects of pCO_2 and diet on absorption rate were interdependent (Table 1; Fig. 3). First, the temporal variation in the effects of the toxin was due to the significant decrease (−0.21 [0.06] mg h^{-1}, averaging across pCO_2 treatments) detected at day 50 of exposure; no other statistically significant effect of diet was observed (Table S1). Second, the time-dependent effect of pCO_2 was supported by an initial increase in absorption rate (0.44 [0.07] mg h^{-1} day 40) and a subsequent decrease (−0.44 [0.07] mg h^{-1} day 41) in this variable as a result of the treatment (Table S1). Finally, and averaging across sampling times, the diet by pCO_2 interaction was explained by contrasting effects of the diet under 380 µatm (non-significant) and 1000 µatm (negative) on absorption rate (Table S1). The fixed factors of the LMM explained a 62% of the variation in absorption rate. The entire model accounted for the same proportion of variation.

3.2.4. Ammonia excretion

The temporal patterns of ammonia excretion varied between diet treatments and between pCO_2 treatments (Table 1; Fig. 4). Similar to absorption rate, the diet and pCO_2 treatments interactively affected ammonia excretion (Table 1). A statistically significant diet by time interaction was supported by the significant increase in ammonia excretion observed at day 40 of experimentation (10.4 [4.2] µg NH_4-N h^{-1}) averaged across diet treatments (Table S1). Non-significant decreases were observed at days 41 and 50, and significant decreases observed

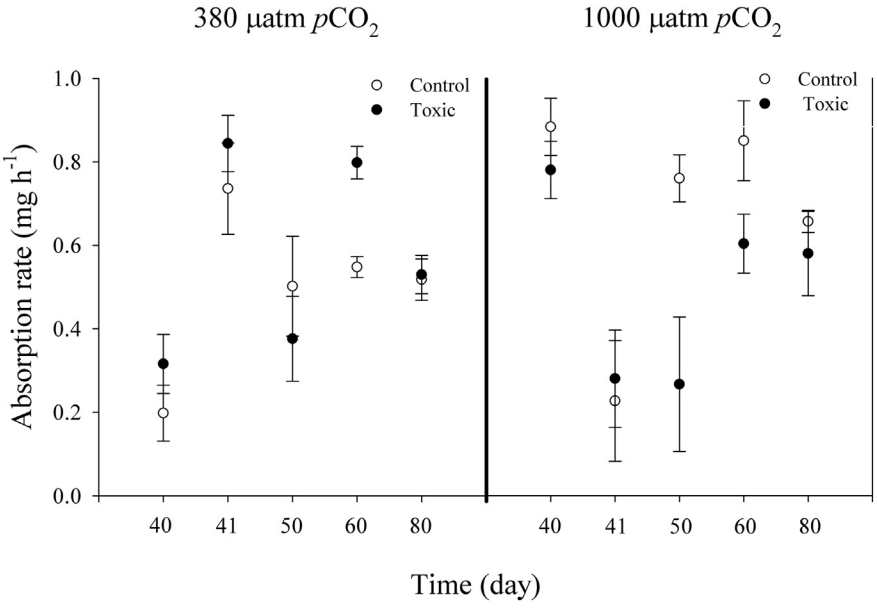

Fig. 3. Absorption rate in *Mytilus chilensis* juveniles exposed to toxic (PST) and control diets at 380 (a) and 1000 (b) µatm pCO_2. Values represent mean ± standard error.

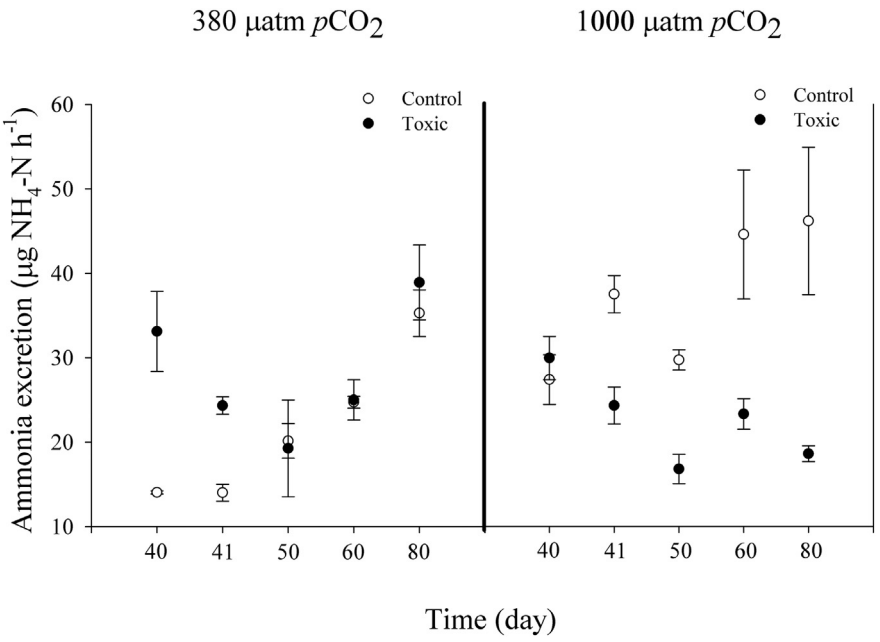

Fig. 4. Ammonia excretion in *Mytilus chilensis* juveniles exposed to toxic (PST) and control diets at 380 (a) and 1000 (b) µatm pCO_2. Values represent mean ± standard error.

at days 60 and 80 (−12.6 [4.2] and −14.7 [4.2] µg NH_4-N h^{-1}, respectively) (Table S1). The temporal variation in the responses to pCO_2 was due to the fact that the marginal effect (i.e. averaged across diets) of this treatment was observed only at day 80 of exposure (−10.2 [4.2] µg NH_4-N h^{-1}) (Table S1). Lastly, we detected a pCO_2-dependent switch in the response of ammonia excretion to the diet: averaged across times, the toxic diet had a significantly positive effect on the excretion of the mussels exposed to 380 µatm, but a negative effect on those exposed to 1000 µatm (7.6 [2.9] and −18.2 [2.9] µg NH_4-N h^{-1}, respectively) (Table S1). The fixed factors in this model accounted for a 60% of the variation in ammonia excretion, whereas the entire model accounted for ca. 62% of the variation in this response.

3.2.5. Oxygen consumption

Oxygen consumption varied over time, depending on the exposure to toxic diet and also on the pCO_2 treatment (Table 1; Fig. 5). Averaged across pCO_2 treatments, the marginal effect of the toxic diet was statistically significant at days 40 and 80 of exposure (0.01 [0.006] and −0.02 [0.006] mL h^{-1}, respectively) (Table S1). On the other side, and averaging across diet treatments, the marginal effect of pCO_2 changed from non-significant (until 50 days) to significantly negative at days 60 and 80 of exposure to the enhanced pCO_2 (−0.04 [0.006] and −0.02 [0.006] mL h^{-1}, respectively; Table S1). Diet and pCO_2, as fixed factors, explained the 57% of the variation in oxygen consumption, and the entire model explained the 69% of the variation in this metric.

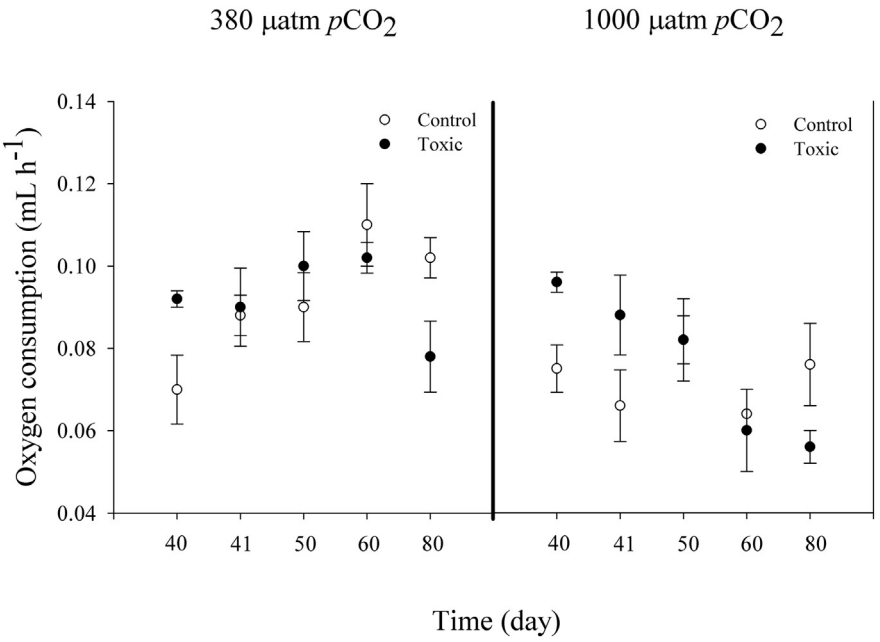

Fig. 5. Oxygen uptake in *Mytilus chilensis* juveniles exposed to toxic (PST) and control diets at 380 (a) and 1000 (b) µatm pCO_2. Values represent mean ± standard error.

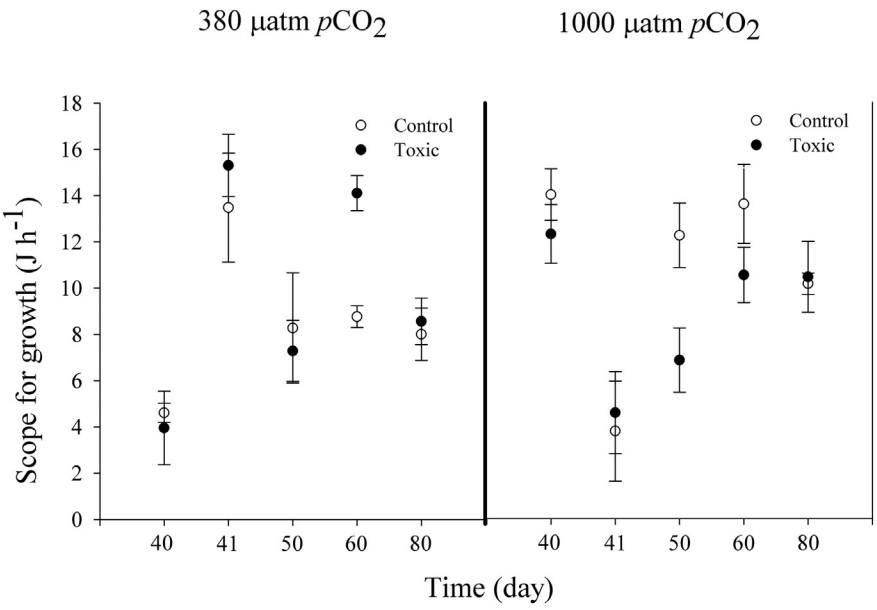

Fig. 6. Scope for growth in *Mytilus chilensis* juveniles exposed to toxic (PST) and control diets at 380 (a) and 1000 (b) µatm pCO_2. Values represent mean ± standard error.

3.2.6. Scope for growth

Scope for growth exhibited variable pattern over time, which was dependent on the pCO_2 treatments (Table 1; Fig. 6). This interaction was caused by the significant and positive effect on scope for growth detected at day 40, which switched to negative at day 41 (7.95 [1.2] and −9.14 [1.2] J h^{-1}, respectively; Table S1), and then increases remained statistically non-significant until the end of the experiment (Table S1). The diet and pCO_2 treatments interactively affected scope for growth (Table 1). The statistical model explained ca. 61% of the variation in scope for growth (both, fixed factors and entire model).

4. Discussion

Various physiological responses have been described in marine invertebrates to a pCO_2 increase, either alone or in combination with other environmental drivers (Fernández-Reiriz et al., 2012; Le Moullac et al., 2016; Wang et al., 2015), but there are no published studies of the combined effects of PST and high pCO_2 on the physiology of suspension-feeding bivalves. Wang et al. (2015) found that increasing temperature significantly reduced clearance rate of the mussel *Mytilus coruscus*, but elevated levels of pCO_2 together with a temperature increase did not show a combined effect. This is consistent with Range et al. (2014), who observed that clearance rate in *Mytilus galloprovincialis* was unaffected by a pCO_2 increase. Fernández-Reiriz et al. (2012) showed that observed seawater pCO_2 (pH reduced by 0.3 and 0.6 units, relative to the natural pH levels of Ria Formosa lagoon) had no effect on feeding (clearance and ingestion rates) in *M. galloprovincialis* juveniles. Similarly, in our study pCO_2 did not affect clearance rate during the acclimation (39 days) and the experimental period (from day 40 to 80). Other studies have described negative effects of high pCO_2 levels on clearance rate in bivalves. Navarro et al. (2016a) found that clearance rate of *M. chilensis* was significantly affected by pCO_2 and temperature, but without interactions between these drivers. Fernández-Reiriz et al. (2011) observed a reduction in feeding in the clam *Ruditapes decussatus* at the highest experimental pCO_2 used (3702 µatm). Similarly, Xu et al. (2016) recorded significantly lower feeding values in *R. philippinarum* exposed to high pCO_2 levels than in clams at ambient pCO_2.

The variable physiological responses of marine invertebrates to changes in pCO_2 levels described in the literature are probably attributable to the wide range of natural environmental conditions that individuals are exposed in their habitats. Since organisms live periods or its entire life at high pCO_2 levels, they have to develop tolerance to pCO_2 variability (Widdicombe et al., 2009) or adequately synchronize its development with the natural variability of pCO_2. According to Lardies et al. (2014), phenotypic adaptation to environmental fluctuations frequently occurs as a result of pre-existing plasticity, whose role as a major component of variation in physiological diversity is generally recognized. In the case of our study, the experimental mussels were collected from Huelmo Bay, 50 km west of Reloncaví Sound, an area characterized by events of salinity drops which decreases Omega and eventually turning estuarine water undersaturated with respect to calcium carbonate during the cold and rainy winter season (Alarcon et al., 2015). During the warm period phytoplankton productivity cause a seasonal drop in surface water pCO_2 (Torres et al., 2011) resulting in highly supersaturated surface water with respect calcium carbonate. Most of the juvenile mussels development takes place under condition of high temperature, omega and food availability (phytoplankton). However, intraseasonal and interannual climatic and oceanographic variability processes are well known modulator this seasonal pattern (e.g. climatic anomalies as rainy/dry periods or changes in meteorological or hydrographical processes that control the mixing and advection of fresh water in the estuary) changing the timing, intensity or synchronization between low salinity/temperature rise/food availability. The adaptation to such environmental variability could partially explain the lack of statistically significant differences in some physiological responses at contrasting pCO_2 levels. The significant interaction between diet and time was related to the initial negative effect and the subsequent recovery of the clearance rate, behavior that has been described for different species of bivalves exposed to diets containing PST (Navarro and Contreras, 2010; Wildish et al., 1998). After one day of exposure to the toxic diet (day 41), *M. chilensis* showed a recovery in feeding activity similar to that described for juveniles and adults of the same species fed with *Alexandrium catenella* (Navarro et al., 2011; Velásquez and Navarro, 2014). Conversely, oysters, clams, and scallops are highly sensitive to PST toxins, which induce a reduction in suspension-feeding (Bricelj et al., 1996; Gainey and Shumway, 1988b; Lassus et al., 2004; Navarro et al., 2016b). However, the mussel *Perna canaliculus* showed no reduction in clearance rate when exposed to the toxic dinoflagellate *Alexandrium tamarense* for four days (Contreras et al., 2012). There is considerable evidence that the feeding

response of marine bivalves to toxic dinoflagellates, be it inhibition, an increase, or no effect, is species-specific (Bricelj et al., 2005; Contreras et al., 2012; Hégaret et al., 2007; Leverone et al., 2007).

Our data showed that the factor pCO_2 affected significantly absorption efficiency in *M. chilensis*. Similarly, Navarro et al. (2013, 2016a) reported that elevated pCO_2 levels (1000, 1200 μatm) significantly reduced absorption efficiency in *M. chilensis*, suggesting deficiencies in the functioning of the digestive system under hypercapnia. Le Moullac et al. (2016) found that absorption efficiency in the oyster *Pinctada margaritifera* showed no significant differences in response to different levels of pCO_2 (426, 1198, and 3667 μatm pCO_2), temperature (22, 26, 30, and 34 °C) or the interaction between the two factors. Fernández-Reiriz et al. (2012) found a significant increase in absorption efficiency in *M. galloprovincialis* caused by elevated pCO_2 which may be related to the optimization of certain digestive enzymes (amylase, glucosidase, and peptidase) under conditions of reduced pH (Areekijseree et al., 2004; Wojtowicz, 1972), facilitating nutrient absorption. In our study, the absorption efficiency was not affected by the treatment of the toxic diet independently, however the interaction diet and pCO_2 affected significantly absorption efficiency. Navarro and Contreras (2010) observed a decrease in absorption efficiency in a different population of the same species during the first days of exposure to a diet containing 50% *A. catenella*. Fernández-Reiriz et al. (2013) found a significant reduction in absorption efficiency when razor clams (*Tagelus dombeii*) from a non-PST exposure field site were fed a diet containing PST. Absorption rate was also dependent of the interaction pCO_2 and diet, with significant lower values in mussels fed toxic diet at the higher level of pCO_2 (1000 μatm), but not significant different values were observed at the control level of pCO_2 (380 μatm).

It has been described that ammonia excretion increased significantly at high pCO_2 in *M. galloprovincialis* and *R. philippinarum*, as a result of the enhancement of protein metabolism which contributed to intracellular pH regulation (Fernández-Reiriz et al., 2012; Michaelidis et al., 2005; Xu et al., 2016). Regarding paralytic shellfish toxin (PST), a significant effect on the excretion rate of *M. chilensis* has also been described by Navarro and Contreras (2010), who associated this response with the capacity of the mussels to degrade the PST toxin, which is a rich source of nitrogen (Pérez, 1998). The present study shows that excretion rate was not affected by high pCO_2 in *M. chilensis*. However, the interaction between diet and pCO_2, diet and time and time and pCO_2 affected significantly the ammonia excretion of *M. chilensis*, showing the importance to include not only a single driver, but multiple drivers on the climate change studies (Boyd et al., 2018).

Different responses in oxygen uptake have been described in various bivalves exposed separately to high pCO_2 and diets containing PST. Fernández-Reiriz et al. (2012) found that acidified seawater ($\Delta pH = -0.6$) had no effect on oxygen uptake in *M. galloprovincialis* juveniles. In contrast, Navarro et al. (2016a) described a decrease in oxygen uptake when *M. chilensis* was exposed to high pCO_2, similar to Wang et al. (2015), who reported a significant effect of pH, temperature, and their interaction on oxygen uptake by *M. coruscus.* Our results showed that oxygen consumption was affected by pCO_2 and also by the interactions between pCO_2 and diet and time and diet, suggesting a more complex response of *M. chilensis* when exposed to more than a single environmental driver. Shumway et al. (1985) found a reduction in oxygen uptake in the giant scallop *Placopecten magellanicus* and the clam *Spisula solidissima* exposed to diets containing PST, even when there was no decrease in suspension-feeding activity. In contrast, the presence of STX in the diet and its accumulation in the tissues of *M. chilensis* did not affect oxygen uptake in the present study and also according Navarro and Contreras (2010) and Navarro et al. (2014) for individuals from two populations of the clam *Tagelus dombeii* which were exposed to similar concentrations of *A. catenella* (ca. 210.000 cells L^{-1}).

Although scope for growth in *M. chilensis* was positive for each combination of pCO_2 and PST, the diet and pCO_2 treatments interactively affected scope for growth, reducing significantly the amount of energy allocated to growth. Other authors have obtained similar results for other bivalve species when considering only pCO_2 increases, e.g. the oyster *Pinctada margaritifera* is unaffected by high pCO_2 (Le Moullac et al., 2016). Likewise, Wang et al. (2015) recorded positive SFG values at different combinations of temperature and pH in the mussel *M. coruscus*. In contrast, Xu et al. (2016) found that elevated pCO_2 in the seawater resulted in lower SFG for the clam *R. philippinarum*. The presence of PST in the diet significantly affected the SFG of mussels (*M. chilensis*) from Huelmo Bay, which responded in a similar manner to individuals from other populations of this species (Navarro et al., 2014; Navarro and Contreras, 2010). The initial decrease in SFG of *M. chilensis* exposed to PST was attributable to a lower clearance rate, which is consistent with results for other filter feeders (Lassus et al., 2004; Li et al., 2002). The recovery in scope for growth after exposure confirms the resistance of *M. chilensis* to harmful algae blooms events, demonstrating its ability to incorporate *A. catenella* into its diet (Bricelj et al., 2005; Fernández-Reiriz et al., 2008). A significant effect of the factor time and/or of the interaction of time with the other fixed factors was found for most of the physiological variables measured (feeding, metabolism, scope for growth), which can be explained by the high individual variability described for mussels exposed to different environmental conditions.

These findings are important considering the ecological and commercial importance of *M. chilensis* in the austral zone of Chile, where today over 280,000 tons are produced annually in suspended culture, and where harmful algal blooms are highly frequent.

5. Conclusions

The association between pCO_2 and paralytic shellfish toxin (PST) may result in an indirect effect on mussel fitness. In fact, this study showed an inhibition of the processes associated with the acquisition of energy in mussels exposed to a diet containing PST. Therefore, the indirect effect of the high levels of pCO_2 combined with paralytic shellfish toxins (PST) on mussel physiology, limits our understanding to know if this population will be able to adapt to the worst atmospheric scenarios projected for the next century, and points out the need to investigate the indirect effect that CO_2 would have on marine organisms.

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