Physiological and histopathological impacts of increased carbon dioxide and temperature on the scallops *Argopecten purpuratus* cultured under upwelling influences in northern Chile

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ABSTRACT

In addition to the increase in temperature occurring in the world’s oceans, new evidences suggest a tendency for an increase in upwelling-favorable winds, bringing to surface cold and high pCO₂ corrosive waters. Changing temperature and pCO₂ conditions may have significant implications for the shellfish farming socio-ecological system. In order, to setup the basis for understand the impact of both environmental variables, in this study, we investigated the combined effects of changing temperature and pCO₂ on the physiological rates and histopathology of scallops *Argopecten purpuratus* farmed in Tongoy Bay, an area permanently influenced by coastal upwelling. Juvenile scallops were reared at two pCO₂ levels (400 and 1000 μatm) and two temperatures (14 and 18 °C). After 18 d of experimental exposure, growth, metabolic and clearance rates increased significantly at high temperature but independent of pCO₂ level, indicating a positive effect of warming on the physiological processes associated with energy acquisition. However, ingestion rates of scallops showed a synergistic interactive effect when exposed to both stressors. Increased pCO₂ also impacts the health of *A. purpuratus* through atrophy in the digestive gland. These results suggest that, the presence of trade-offs in energy allocation during upwelling-induced stress (low temperature and high pCO₂) can impact growth, metabolism, ingestion rates and health status of scallops cultured in Tongoy Bay. But, less severe response to high pCO₂ levels, suggest that natural variability in upwelling areas may promote acclimation and adaptation potential in this farmed scallops.

Keywords:
Ocean acidification
Histopathology
Metabolism
Food ingestion
Multiple stressor impacts
Aquaculture

1. Introduction

Coastal oceans are arguably one of the most important ecosystems of the planet and impacted by human activities in the ocean. Global environmental stressors, such as Ocean Warming (OW), Ocean Acidification (OA) and Deoxygenation (DO) are undergoing pronounced change in the world’s coastal oceans today. There is growing concern with respect to the combined effects (thereby synergistically, additively or antagonistically) of these environmental stressors on the coastal marine biota. During the last decades, there is growing interest about the need to understand the interplay between environmental and socio-economic drivers in marine resource management (Cooley and Doney 2009; Perry et al. 2010). Essentially, humans’ adaptation capacities based on a better understanding of the combined impacts of multiple global and local drivers on marine ecosystems services as food provision through fisheries and aquaculture activities will facilitate the success of our societies in establishing sustainable pathways for the future (Gattuso et al., 2015).

In Chile, coastal shellfish aquaculture activities are focused on bivalves (e.g. oysters, mussels, scallops), and gastropods (e.g. snails). From 1990, shellfish aquaculture has experienced an annual average growth rate of ~18%. Shellfish farming accounted with ca. 265,000 tons in 2013, or 14% of total biomass and Chile is the third and second largest producer of scallops and mussels in the world,
respectively (www.sernapesca.cl). Although the industry has thrived in the past decade, global drivers such as climate, market variability, and anthropogenic activities provide major challenges in sustaining the future of the aquaculture sector in our coastal ocean. This is of particular concern in the case of valuable commercially exploited species such as the scallop Argopecten purpuratus. A recent crash in the scallop production has been recorded in Chile, where the number of scallop farms became closed due to market and environmental drivers, with concomitant reduction in national production from 20.000 tons in year 2000 to 5000 tons in year 2013 (Lagos et al. 2016).

Eastern Boundary Upwelling Ecosystems, located off the mid-latitude western coasts of the U.S., South America, Iberia and Africa, are predicted to be one of the most increasingly stressed marine ecosystems under multiple-stressors (e.g. ocean acidification, warming and deoxygenation; Gruber, 2011). Upwelling ecosystems account for only 1.9% of the world's oceans, but provide an estimated 23% of global fisheries (Kudela et al. 2005; Chavez et al. 2008), being the Humboldt Current System the most biologically productive ecosystem of world's coastal oceans (Thiel et al. 2007). These areas are characterized by strong seasonal winds that promote the upwelling of cold and CO2 super-saturated subsurface waters. Climatic projections suggest an increase in the intensification of upwelling events due to the change in the intensity and patterns of wind that favor the upwelling (Sydeman et al. 2014). In northern Chile, upwelling dynamics occur year around, with foci located at major point or headlands of the coastline. In particular, a strong latitudinal gradient in SST anomalies located at 30°S is associated with the coastal area of Punta Lengua de Vaca (PLV) (Figueras and Moffat 2000). This coastal area at the PLV upwelling system might result in under-saturated subsurface waters with respect to aragonite ($\Omega_{arag} < 1$) and in lower pH in nearshore and bays areas (Torres et al. 2011), characteristics previously expected to occur in the open ocean until the year 2100 in a scenario of ocean acidification (Orr et al. 2005; Gruber et al. 2012). Coastal waters with low levels of carbonate saturation state are corrosive, challenging calcification of shells and skeletons of marine organisms (Fabry et al. 2008; Beniash et al. 2010; Duarte et al. 2015). Furthermore, large upwelling areas are located northward of 37°S (in northern (18–30°S) and central Chile (30°–37°S)) showing SST anomalies that varies between 4 and 7 °C, whereas in latitudes above 37°S, SST varies between 2 °C to 4 °C (Lagos et al. 2008; Aravena et al. 2014). The Chilean scallop farming industry in northern Chile is localized near to these upwelling centres and has been regularly affected by events of larval and adult mortality, potentially driven by the effect of corrosive upwelling waters. The environmentally induced phenotypic change, namely, phenotypic plasticity has been recognized as an important strategy for maximizing or maintaining fitness in an organism that inhabits variable environments (Bouvet et al. 2005; Pigliucci 2005; Schlichting and Pigliucci 1998; Via and Lande 1985). These notions, suggest that may be expected that populations of A. purpuratus that inhabit waters influenced by coastal upwelling, namely, subject to environmental heterogeneity in pH, temperature and oxygen concentration may be “naturally” acclimated to this environmental variation in temperature and pCO2 (i.e., phenotypic plasticity) probably as end product of local adaptation (see Lardies et al. 2014).

In this study we evaluate the combined effects of temperature and pCO2 on scallops Argopecten purpuratus cultured under the influence of coastal upwelling. A. purpuratus represents an important species for aquaculture, since it is the most cultivated mollusk species in the north of Chile (over 5000 tons in 2013) with its main production zone is localized in Tongoy Bay (von Brandt et al. 2006; SERNAPESCA 2013). A. purpuratus is a relatively well studied species regarding the effect of environmental variables on physiological processes (see Navarro and Jaramillo, 1994, Navarro and González, 1998; Labarta et al. 1997; Uriarte et al. 2004; Martínez et al. 1995, 2000; Fernández-Reiriz et al., 2005; Soria et al., 2007, 2011; Ramajo et al. 2016a) and the impacts of increased pCO2 and temperature on shell properties of this species (Lagos et al. 2016). However, no studies have addressed how combined effects of these climate stressors will affect the physiological rates and histopathology of this scallop species. Thus, in this study we evaluate through laboratory experiments the effect of cold temperatures and increased pCO2 conditions, which commonly occur during upwelling events influencing the study area (i.e. Tongoy Bay) upon growth rates and metabolic and feeding performance of A. purpuratus.
2. Materials and methods

2.1. Study site and animal collection

Juvenile individuals of *A. purpuratus* (< 40 mm in size (LT)) were collected from culture ropes (2 m of depth) in Tongoy Bay (30°12'S, 71°34'W) located in northern Chile (Fig. 1). Thus, the juvenile scallops experienced during its early life stages the influence of Punta Lengua de Vaca upwelling center which waters penetrate into the Tongoy Bay seasonally (i.e. spring and summer) when winds coming from the south, transport Equatorial subsurface waters (ESSW) with high salinity, high nutrients content, low temperature, low pH, and low oxygen content to the same scallop farming area (Rutillant 1993; Uribe and Blanco 2001; Lagos et al. 2016).

These experimental scallops are produced in hatchery from broodstock of the same Tongoy Bay. After collection, the scallops were transported in chilled conditions to the Calcufo Coastal Laboratory of the Universidad Austral de Chile (LCC, ca. 39°S, Valdivia) and placed in plastic containers (30 cm in diameter and 40 cm in height). To acclimatize them to experimental conditions, the scallops were kept for 5 days and fed daily with microalgae *Tetraselmis* spp. (~65 × 10⁶ cell/ml) before the beginning of the experiments. In addition, during the day of the scallop collection, 17 juvenile individuals (hereafter natural control) were immediately preserved for histological procedures (see below).

2.2. Seawater acidification system

After the acclimation period, the scallops were randomly assigned to one of four treatments: (Angilletta et al., 2004) 14 °C and 400 μatm pCO₂ (ambient condition), (Angilletta, 2009) 14 °C and 1000 μatm pCO₂ (upwelling condition), (Aravena et al., 2014) 18 °C and 400 μatm pCO₂ (warming and upwelling relaxation), (Beniash et al., 2010) 18 °C and 1000 μatm pCO₂ (ocean acidification and warming) (Table 1), for a period 18 days which is larger time than two complete upwelling cycles in northern Chile (see Strub et al. 1998; Garreaud et al. 2011). Each treatment was replicated five times and each replicate contained four scallops (N = 80). The experimental animals were identified using bee tags. We used a semi-automatic system for long-term seawater carbonate chemistry manipulation (Torres et al. 2013; Duarte et al. 2014; Navarro et al. 2013; Manríquez et al. 2013; Vargas et al. 2013; Duarte et al. 2015). The experimental temperatures represent the average surface value (14 °C) and alternatively the maximum surface value (18 °C) of the study area (Uribe and Blanco 2001; Pérez et al. 2012; Aravena et al. 2014). The pCO₂ levels were defined considering the mean range reported for this coastal embayment by Torres & Ampuero (2009) and Vargas et al. (2017). The temperatures were adjusted and maintained using external chillers. To obtain the different CO₂ concentrations, the experimental containers were adjusted following the methodology described by Navarro et al. (2013). Briefly, for 400 ppm, pure atmospheric air was bubbled into experimental containers; for 1000 ppm, we blended dry air with pure CO₂ to each target concentration using mass flow controllers (MFCs, www.aalborg.com) for air and CO₂ then this blend was bubbled into the corresponding container. Dry and clean air was generated by compressing atmospheric air (117 psi) using an oil-free air compressor. The experimental containers were constantly bubbled with the corresponding CO₂ concentration. During the experiment, total alkalinity (TA) of the water was monitored every 4 days (two replicates) and pH, temperature, salinity of the water was monitored every day in each aquaria (i.e. replicate) and each header tank (Navarro et al. 2013). Seawater was changed every day, with the corresponding pCO₂ levels from the seawater acidification head-tank following the methodology proposed by Navarro et al. (2013). pH_{1988} and total alkalinity (A_{1}) was monitored on day 2, 6, 10, 14 and 18 for carbonate systems estimates. pH samples were collected in 50 mL syringes and immediately transferred to a 25 mL thermostatted cell at 25.0 ± 0.1 °C for standardization, with a pH meter Metrom® using a glass combined double junction Ag/AgCl electrode (Metrohm model 6.0258.600) calibrated with standard calibration buffer Metrom® 4 (6.2307.200), 7 (6.2307.210) y 9 (6.2307.220). pH values are reported on the NBS scale. Samples for A_{1} were poisoned with 50 μL of saturated HgCl₂ solution and stored in 500 mL borosilicate BOD bottles with ground-glass stoppers lightly coated with Apiezon L® grease and kept in darkness at room temperature. Temperature and salinity were monitored during incubations by using a salinometer Eco Sense EC 300, YSI A_{1} was determined using the open-cell titration method (Dickson et al. 2007), by using an automatic Alkalinity Titrator Model AS-ALK2 Apollo SciTech. The AS-ALK2 system is equipped with a combination pH electrode (8102BINUWP, Thermo Scientific, USA) and temperature probe for temperature control (Star ATC probe, Thermo Scientific, USA) connected to a pH meter (Orion Star A211 pH meter, Thermo Scientific, USA). All samples were analyzed at 25 °C (± 0.1 °C) with temperature regulation using a water-bath (Lab Companion CW-05G). The accuracy was controlled against a certified reference material (CRM, supplied by Andrew Dickson, Scripps Institution of Oceanography, San Diego, USA) and the A₁ repeatability averaged 2–3 μmol kg⁻¹. Temperature and salinity data were used to calculate the rest of carbonate system parameters (e.g. pCO₂, CO₂⁻³) and the saturation stage of Omega Aragonite (Ω_{arag}). Analyses were performed using CO2SYS software for MS Excel (Pierrot et al. 2006) set with Mehrbach solubility constants (Mehrbach et al. 1973) refitted by Dickson and Millero (1987). The KHCO₃ equilibrium constant determined by Dickson (1990) was used for all calculations.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Average (+ SE) conditions of carbonate system parameters during experiments conducted with juvenile scallops: pH (NBS scale), Total Alkalinity (TA in μmol kg⁻¹), partial pressure of CO₂ (levels of pCO₂ in seawater in μatm), carbonate ion concentration (CO₂⁻³ in μmol kg⁻¹), saturation state of the water with respect to aragonite minerals (Ω_{arag}).</th>
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<tbody>
<tr>
<td></td>
<td><strong>Low (400 μatm)</strong></td>
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<td></td>
<td><strong>14 °C</strong></td>
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<tr>
<td>Temperature (°C)</td>
<td>14.13 ± 0.19</td>
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<tr>
<td>Salinity (PSU)</td>
<td>34.48 ± 1.78</td>
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<tr>
<td>pH_{1988}</td>
<td>8.058 ± 0.017</td>
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<tr>
<td>Total alkalinity (TA)</td>
<td>1580.41 ± 206.40</td>
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<tr>
<td>CO₂⁻³</td>
<td>86.54 ± 10.15</td>
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<tr>
<td>Ω_{aragonite}</td>
<td>1.32 ± 0.15</td>
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<tr>
<td>Ω_{calcite}</td>
<td>2.07 ± 0.24</td>
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2.3. Physiological rates

2.3.1. Growth rate

Growth rates of juvenile individuals of *A. purpuratus* were estimated from changes in the Total Weight (TW) (to the nearest 0.01 mg) of the scallops (Palmer 1982). For all measurements, each scallop was carefully removed from the water, immediately weighed under the water (buoyant weight = BW), then gently blotted, and weighed in air (TW).

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Furthermore we estimated biomass increase (i.e. metabolically active tissue) as the change in the relation between (TW−BW) during the time of the experiment.

2.3.2. Standard metabolic rate (SMR)
Scallops were left in aquaria for 24 h with no food after exposition time. Then, we measured oxygen consumption (mg O₂ × h⁻¹ g⁻¹) using a Presens Mini Oxy-4 respirometer (see Gaitán-Espitia et al. 2017). To quantify the SMR, animals of each replicate were used and placed individually in respirometric chambers filled with 805 mL of seawater with the corresponding level. Bottles were immersed in a container with 200 mL from each bottle, which was system used for maintaining temperature and CO₂ levels and temperature of each treatment (see above). In addition 33 psu salinity and oxygen-saturated seawater through air bubbling were added before starting measuring. The measurements were performed at a controlled temperature of 14 °C and 18 °C using an automated temperature chiller. In each chamber, dissolved oxygen was quantified every 15 s for 55 min. Sensors were previously calibrated in anoxic water, using a saturated solution of Na₂SO₃ and in water 100% saturated with oxygen using bubbled air. The same respirometric chambers were used as controls, but without animals inside, under the same experimental conditions (the control never had a decayment of the oxygen concentration higher than 3% of the measurements). Each oxygen decay due to background noise was deducted from the individual measurements performed in the experimental chambers. The first and the last 5 min were discarded in order to avoid possible disturbances when the scallops were placed in respirometric chambers. Thus, oxygen estimations are the average of the remaining 45 min of measurements. Before and after each measurement, buoyant weight of scallops were recorded using an analytical balance (ADAM AFA-180LC with precision ± 0.001 mg). The average of both body mass measurements was used in the statistical analyses. Slopes were calculated by the decrease of oxygen in the chamber per hour and normalized to fresh mass to get respiration rates in units of mg O₂ L⁻¹ h⁻¹.

2.3.3. Ingestion rate (IR) and clearance rate (CR)
Immediately after completion of the experiment period (i.e. 18 days), we determined clearance and ingestion rates. We used 1000 mL polycarbonate bottles previously cleaned with HCl. Individuals belonging to each experimental group were individually sorted into 1000 mL acid-washed polycarbonate bottles filled with filtered seawater (1 μm, salinity of 33‰) and equilibrated with the respective CO₂ treatment. Three control bottles without A. purpuratus and three experimental bottles with each individual for one four treatments were incubated for approximately 2 h, and periodically rotated by hand to avoid particle sedimentation. Prior to the experiment juvenile body mass (mb) was determined with an analytical balance (± 0.01 mg) in order to standardize our clearance and ingestion rate estimates in g⁻¹ h⁻¹. For these experiments, we added Tetrasselmis spp. at a concentration of ~32,000 cell/mL⁻¹, to supply food at saturation level. Bottles were immersed in a container with flow-through seawater system used for maintaining temperature fluctuation during a given experiment within one degree. After incubation we took a sub-sample of 200 mL from each bottle, which was filtered through a GF/F filter of 0.75 μm mesh-size. The filter was stored in an aluminum envelope at −20 °C until analysis of Chlorophyll (Chl-a) concentration. Chl-a was dark extracted in acetone 95% before measurement on a TD 6700 Turner Fluorometer (Strickland and Parsons 1968). Clearance (CR) and Ingestion (IR) rates were estimated through the measurement of Chl-a depletion and cell removal, according to Frost (1972) modified by Marin et al. (1986). For all experiments, there were no significant differences in food availability among treatments. No mortality effects were found on juveniles scallops in any of the experimental treatments, which indicates that the projected increase of pCO₂ levels and temperature had chronic, but not lethal, effects on these organisms.

2.4. Histopathological measurements

2.4.1. Histopathological analysis
Tissues for histology analysis were excised from scallops exposed to the different treatments of pCO₂ and temperature (N = 40) and from those 17 scallops representing natural control conditions. The tissues excised included gills, digestive gland and mantle. The tissue samples were fixed for 24 h. in Davidson’s fluid, transferred to 70% ethanol, and sent to the Histopathology Laboratory of the Universidad Católica del Norte (Coquimbo, Chile). They were routine processed for histology, 5 μm sections were cut, and stained with Harris Hematoxilyn and Eosin. The slides were analyzed using a Zeiss Axiosumar plus microscope.

2.4.2. Pathological conditions
The prevalence, percentage of individuals presenting each parasite and condition was assessed for A. purpuratus from the four experimental treatments and the natural controls. For the two most prevalent indicators observed (ricketsials-like organisms, RLOs, and digestive gland atrophy), also the intensity of infection was assessed. For the RLOs this was accomplished counting all the inclusions of these parasites from the whole 5 μm thick section. The mean intensity of infection was calculated dividing the total number of parasites through the total number of infected hosts. The intensity of digestive gland atrophy was assessed registering the percentage of this condition when present, and the mean percentage was calculated. Prevalence, infection intensity and mean intensity of infection were calculated following Bush et al. (1997).

2.5. Statistical analyses

To avoid pseudo-replication problems, the variables measured were averaged for the four scallops of each replicate. Two-way ANOVA were used to evaluate whether temperature and CO₂ levels and the interaction between factors affected growth rate (increase in TW and Biomass increase with time), ingestion and clearance rate (Zar, 1999). A two-way analysis of covariance (ANCOVA) was performed to determine the effects of temperature and pCO₂ level on mass-specific metabolism, using body mass as covariate. When the analysis showed significant interactions, a one-way ANOVA was carried out for each factor separately in each level from the other factor, followed by Tukey’s a posteriori HSD test. When the analysis 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). Buoyant weight increases were expressed in g per scallop per day. All analyses were carried out using Statistica version 7.0. Assumptions of normality and homoscedasticity of the one-way ANOVA were evaluated using the Kolmogorov–Smirnov and Burtlett tests, respectively (Sokal and Rohlf, 1995). Finally, we used an exact binomial calculation to estimate the 2-tail probability that the prevalence of conditions evaluated in the histological analysis of scallop tissues was higher or lower than expected by chance (p = 0.5) in n independent trials or scallops observations (Zar 1999).

3. Results

3.1. Physiological rates

3.1.1. Growth rate
Growth rate of the scallops increased significantly in treatment with higher temperature compared with individuals maintained at low temperature, 14 °C (Two way ANOVA, F₁,₁₉ = 24.40; P < 0.0000), but were not influenced by increased pCO₂ levels within each temperature treatment (F₁,₁₉ = 0.97; P = 0.340) (Fig. 2a). Although, increased growth rate was also recorded in those scallops reared at the treatment with low temperature and high pCO₂ levels, representing a typical ‘upwelling’ condition, these do not lead to a significant
interaction term (Temperature × pCO2; F1,19 = 3.89; P = 0.066) (Fig. 2a). Otherwise, biomass, which represents metabolically active tissue, showed an significant increase in scallops exposed to low pCO2 treatments (Two way ANOVA; F1,19 = 3.17; P = 0.045), but were not influenced by temperature treatments (F1,19 = 0.92; P = 0.991) (Fig. 2b), and also leading to non-significant interaction between treatments (Fig. 2b).

3.1.2. Ingestion and clearance rates

The scallops A. purpuratus significantly increased their clearance rates when exposed to the high temperature (18 °C), with a ~ 35% increase recorded in individuals exposed to high temperature/low pCO2 combination (Two-way ANOVA, F1,17 = 7.68, P = 0.015) (Fig. 2c). But, non-significant changes were observed in clearance rates in relation to pCO2 levels for scallops at each temperature conditions, although there was a subtle increase from low to high temperatures treatments (~175 ml g⁻¹ h⁻¹ to 206 ml g⁻¹ h⁻¹), respectively (Two-way ANOVA, F1,17 = 0.003; P = 0.990) (Fig. 2c).

The interaction between temperature and pCO2 levels affected significantly the ingestion rates A. purpuratus juveniles (Two-way ANOVA, F1,17 = 6.72; P = 0.021) (Fig. 2d). The scallops exposed to the combination of high-pCO2/low temperature treatment (‘upwelling condition’) presented significant higher ingestion rate than organisms exposed to the resto of treatments combinations (Fig. 2d). Non-significant differences were found between levels of temperature (14 °C and 18 °C), and neither for high and low pCO2 treatments in particles ingestion. Scallops maintained at low temperature and high pCO2 raised the ingestion rate over 20% relative to low pCO2 conditions (high pCO2: 1.91 ± 0.23 mg Chl a g⁻¹ h⁻¹; low pCO2 pH: 1.49 ± 0.27 mg Chl a g⁻¹ h⁻¹) (Fig. 2d).

Fig. 2. Biological responses (mean ± S.E.) of Argopecten purpuratus reared at two temperatures and two nominal levels of CO2: (a) Growth; (b) Biomass increase; (c) Metabolic; (d) Ingestion and (e) Clearance rates. Different letters (a,b) represents significant differences after a post hoc Tukey Test between pCO2 or temperature treatments.
3.1.3. Standard metabolic rate (SMR)

Metabolic rate of *A. purpuratus*, measured as oxygen consumption increased significantly at the high temperature condition (Two-way ANCOVA, $F_{1,17} = 7.29; P = 0.017$) (Fig. 2e). Despite that oxygen consumption in scallops was higher in both high pCO$_2$ treatments, these differences were not significant ($F_{1,17} = 3.35; P = 0.088$). Furthermore, a non-significant Temperature $\times$ pCO$_2$ interaction was observed for metabolic rate (Fig. 2e) (Two-way ANCOVA, $F_{1,17} = 0.45; P = 0.515$). Mass-specific metabolic rate in juveniles of *A. purpuratus* was lower in the low temperature/low-pCO$_2$ level treatment than in low temperature/high-pCO$_2$ level combination (0.73 ± 0.19 and 0.97 ± 0.37 mg O$_2$ h$^{-1}$ g$^{-1}$, respectively). Metabolic rate of individuals exposed to high temperature and high pCO$_2$ level reached the highest values observed in the experiment (1.60 ± 0.40 mg O$_2$ h$^{-1}$ g$^{-1}$), almost doubling oxygen consumption of scallops exposed to low temperature conditions (Fig. 2e).

3.2. Histopathological measurement

3.2.1. Histopathological analysis

The main affected tissue in juvenile scallops was the digestive gland, showing degenerative alterations of the digestive tubules (Fig. 3a normal tubule) and secondary (transport) tubules across treatments (Fig. 3b,c). This alteration was evidenced as a desquamation of the epithelial cells (Fig. 3b), flattening the epithelium, and thus in some extreme cases leaving only the presence of the basal lamina (Fig. 3c). The secondary tubule atrophy, although present, showed a low intensity across all treatments. Although statocysts had not specifically been sampled for, they appeared located close to the pedal ganglia and the digestive gland (Fig. 3g, right and left statocysts from natural control scallops). The left statocyst showed no alteration in the experimental scallops (Fig. 3h), however, the right statocyst showed irregular outlines, due to epithelial desquamation in the high pCO$_2$ condition at both levels of temperature (Fig. 3i).

3.2.2. Pathological conditions

The prevalence of parasites and pathological conditions is shown in Table 2. The rickettsiales–like organisms (RLOs) were detected as basophilic, ovoid-spherical inclusions in epithelial cells of the digestive tubules (Fig. 3a), residing in otherwise completely normal digestive cells. RLOs showed low prevalence across the experimental treatments (Fig. 3b,c). This alteration was evidenced as a desquamation of the epithelial cells (Fig. 3b), flattening the epithelium, and thus in some extreme cases leaving only the presence of the basal lamina (Fig. 3c). The secondary tubule atrophy, although present, showed a low intensity across all treatments. Although statocysts had not specifically been sampled for, they appeared located close to the pedal ganglia and the digestive gland (Fig. 3g, right and left statocysts from natural control scallops). The left statocyst showed no alteration in the experimental scallops (Fig. 3h), however, the right statocyst showed irregular outlines, due to epithelial desquamation in the high pCO$_2$ condition at both levels of temperature (Fig. 3i).

4. Discussion

Our results indicate that under rise in pCO$_2$ from 400 to 1000 μatm in a scenario of low temperature, which may resemble the influence of upwelling conditions operating in Tongoy Bay, did not affect most physiological rates measured in juveniles of *A. purpuratus*, except for a significant increase in about of 40% of the ingestion rate at the high pCO$_2$ scenario. Nevertheless, physiological processes were sensitive to changes in temperature, which induced a positive effect on the physiological processes associated with increased energy acquisition. Specifically, our factorial experiment showed that the environmental drivers studied, temperature and pCO$_2$ operate in an additive way upon the physiological performance of *A. purpuratus* from Tongoy Bay. Nevertheless, the current study also suggests that under experimental conditions the combination of low temperature across both pCO$_2$ conditions may reduce the health of *A. purpuratus*. Histopathological assessment of the soft tissues of juvenile scallops exposed to acidification and temperature treatments, showed damage, particularly in digestive gland, but without mortality. In addition, though not fully assessed, it also suggests the role of high pCO$_2$ on statocyst volume.

Temperature profoundly affects growth and its underlying processes in ectotherms (Angilletta 2009; Somero 2010), rising temperature leads to increasing rates of biochemical-physiological processes (e.g. metabolic and heart rate) and life-history characteristics (Gillooly et al. 2001; Willmer et al. 2005). Temperature increases the growth rate of scallops, an expected pattern because temperature increases growth and body size in marine invertebrates (see Sidar et al., 1999; Woods et al. 2003; Angiletta et al. 2004). The increase of growth rate with temperature increment has been demonstrated previously in *A. purpuratus* for populations from Chile and Perú (see Martínez and Pérez 2003; Thébault et al. 2008; Soria et al. 2011; Lagos et al. 2016). Water temperature is the environmental factor most often cited as influencing bivalve reproduction (see Saxty 1968; Wolff 1998; Navarro et al. 2016). Nevertheless, we detected a significant increase in biomass of the scallops (i.e., metabolically active tissue) raised under low pCO$_2$ levels. Thus, juveniles scallops experiencing high pCO$_2$ conditions, probably due to higher energetic cost of homeostasis, decrease the tissue mass (see Lardies et al. 2014). Recently, we reported a reduction in shell length and growth in juveniles of *A. purparatus* from Tongoy Bay under high pCO$_2$ treatments (Lagos et al. 2016). Reduced growth under acidified conditions has been reported also for other scallops species (e.g. Talmage and Gohler 2009; White et al. 2013). A similar pattern was informed in the bivalve *Mytilus galloprovincialis* where high pCO$_2$ significantly reduced growth rate at low temperature but this effect gradually decreased with warming (Kroeker et al. 2014). Negative impacts on biomass under low pH could be explained because energy allocation is being diverted away to other key physiological processes such as calcification, acid–base balance, reproduction, and immune function (Wood et al. 2008; Findlay et al. 2009; Lagos et al. 2016). Nevertheless, previous studies report that juveniles of *A. purpuratus* maintained between 10 °C and 18 °C showed no effect in growth rate and their scope for growth (Gonzalez et al. 2002). The origin of experimental populations may explain this contradictory results, whereas the juveniles scallops used in our experiment were obtained from Tongoy Bay (northern Chile, 30° 12′ S), these authors collected scallops from southern Chile (41° 54′ S), thus suggesting the role of a possible acclimatization and/or adaptation of local populations to environmental factors experienced in their native habitats. Temperature regimes vary widely along the Chilean coast and specifically in > 6 °C between these two localities (Thiel et al. 2007; Lagos et al. 2008). Thus, geographical variation in the degree of plasticity and/or potential for local adaptation for these traits could be present within *A. purpuratus* species along the Chilean coast and partially may explain the differences in biological responses observed between local population of *A. purpuratus*.

There are few studies on combined effects of temperature and pCO$_2$ on metabolism and performance of marine invertebrates (Queirós et al., 2015). For instance, Navarro et al. (2016) found that the metabolic rate was not affected by temperature but detected an increase in metabolism on the farmed mussels *Mytilus chilensis* when exposed to low pH level.
On the contrary, in our study temperature increased significantly the metabolic rate in scallops but did not vary across pCO2 level. This result suggests a major role for warmer temperatures, which could potentially offset the reduction in energy budget originated by ocean acidification (see Byrne and Przeslawski 2013, Kroeker et al. 2013, Duarte et al. 2014; Navarro et al. 2016). Navarro et al. (2000) found that oxygen uptake and scope for growth were not affected in A. purpuratus by temperatures in a range between 16 °C and 20 °C. However, these experimental temperatures are higher to the average conditions (e.g., 12–14 °C, Figueroa and Moffat 2000; Lagos et al. 2016) dominating

![Histopathological effect of two temperatures and two nominal levels of pCO2 on tissues of Argopecten purpuratus (Stain H & E). A: Normal digestive tubules, composed of digestive cells (DC) and basophilic cells (arrowhead), L: lumen of digestive tubule. RLO inclusions present in digestive cells (arrows). B & C: Digestive gland atrophy, flat epithelium (arrowheads), desquamating cells (dsq. cells), *: basal lamina. D: Unknown organism in lumen (L) of secondary tubule. Ep: epithelium of secondary tubule. E & F: Hemocytic infiltration (*), E in gill within distended Gill filaments (GF). F: in digestive gland (DG), and in male gonad (MG). G: Statocysts in natural control from Tongoy Bay, left statocyst (LS) and right statocyst (RS). In the interior the statoconia (*) can be observed, in the left statocyst (**) it forms a mass filling it completely, in the right statocyst it only occupies about 25% of the volume. Part of the pedal ganglion visible on the upper part of the photograph. H: perfectly normal looking left statocyst from the low pCO2/high temperature treatment. I: Right statocyst from the high pCO2/high temperature treatment, were desquamation of the epithelium is observed (arrows).]
during upwelling processes influencing Tongoy Bay. Similar results were found in the scallop *Pecten maximus* where the respiration rate was not affected in juveniles displaying tolerance to levels of pH ranging between 8.2 and 7.6 (Sanders et al. 2013). It is important to emphasize that in our experiment, as well as in the Sanders et al. (2013) study, food availability was not restricted. In addition to temperature, food supply seems to play a major role modulating organismal responses of *A. purpuratus* by providing the energetic means to support the physiological cost imposed by ocean acidification stress (Ramajo et al. 2016a).

Our results can be discussed in the framework of natural pCO$_2$ variability in coastal upwelling areas influencing scallop farming and the potential for local adaptation in these regions (Vargas et al. 2017). This can be rationalized because the scallops were exposed to a pH and temperature range that the animals experience in their natural environment (see Torres and Ampuero 2009; Lagos et al. 2016; Vargas et al. 2017). Coastal upwelling modulates the physical and chemical properties of seawater over large areas of the coastal ocean. In relation to the current study, the Pt. Lenga de Vaca upwelling center increases CO$_2$-fluxes and promotes strong reductions in pH (pH ~ 7.6) in Tongoy Bay and the surrounding area (Torres and Ampuero 2009; Torres et al. 2011) and adds also a high variability in sea surface temperature (see Aravena et al. 2014). That is, the moderate effect found on physiological performance of juvenile individuals of *A. purpuratus* from Tongoy Bay is likely due to the presence of phenotypic plasticity leading to increased tolerances which is characteristic of organisms that inhabit heterogeneous environments (Via et al. 1995). Most upwelling zones are acidic compared to the coastal and open ocean (Hofmann et al. 2011). Furthermore, it has been demonstrated that most upwelling zones are indeed characterized by natural variations in seawater chemistry, suggesting that in some particular habitats, resident organisms are already experiencing pH regimes projected for the year 2100 (Hofmann et al. 2011; Vargas et al. 2017). This can be the case of the Tongoy Bay due to the presence of a semi-permanent upwelling (Vargas et al. 2017). Our results evidenced that a less severe response to high pCO$_2$ levels of the scallops that are naturally exposed to higher pH and temperature variability, suggesting the acclimatization and adaptation potential for this species. This moderate effect of temperature and high pCO$_2$ has also been reported in other marine invertebrates that inhabit highly variable environments on terms of pH and temperature (see Lardies et al. 2014; Collard et al. 2016; Vargas et al. 2017). We observed an increase of scallop metabolism in high pCO$_2$ at both temperatures. This up-regulation observed in metabolic rate of scallops under high pCO$_2$ scenarios has also been observed in others bivalves species exposed to low pH conditions, and may be a requirement for maintaining intracellular pH, and cellular homeostasis (e.g., Cummings et al. 2011; Lardies et al. 2014; Ramajo et al. 2016a), which is consistent with the metabolic costs of mechanisms developed by calcifiers to cope with acidic conditions (Ramajo et al. 2016b; Hendriks et al. 2015). Metabolic up-regulation to face stressful environments is energetically costly and may impose limits to the energy available to sustain other physiological activities like growth or ingestion rate, generating trade-offs between phenotypic traits (Wood et al. 2008; Deigweber et al. 2010; Lardies et al. 2014; Navarro et al. 2016).

Studies analyzing the effect of high pCO$_2$ on bivalve species have shown negative effects on feeding activities, such as clearance and ingestion rates, possibly due to deficiencies in the functioning of the digestive and filtering systems (Fernández-Reiriz et al. 2011; Navarro et al. 2013; Vargas et al. 2013; Vargas et al. 2015). Reduced feeding under ocean acidification and warming conditions results in lower survival, size, and calcification (Dupont & Throndyke, 2009; Byrne & Przeslawski 2013). In contrast, in our study juvenile scallops showed a significant increase of ingestion rate at high pCO$_2$/low temperature treatment (i.e., upwelling conditions) and clearance rate was increased as expected in higher temperatures without effect of pH. It is evident from our results and the other available studies that high pCO$_2$ increases juvenile feeding of scallop species. Our findings are consistent with results reported for other scallops such as *Chaetomus nobilis* and *Pecten maximus* under low pH treatments (Wenguang & Maoxian, 2012; Sanders et al., 2013) and in the juveniles of *A. purpuratus* under different scenarios of pH and food availability (Ramajo et al. 2016a). Navarro et al. (2000) show that *A. purpuratus* can actively regulate clearance rate and does not simply switch between feeding and non-feeding states. A different pattern have been reported in other mollusk species for the Chilean coast, for instance in newly hatched larvae of the gastropod *Concholepas concholepas* and juvenile stages of the mussel *Perumytilus purpuratus*, whose clearance rates decreased between 15 up to 70% under high pCO$_2$ respect to control conditions (Vargas et al. 2013; Vargas et al. 2015). On the other hand, the maintenance of scallop biomass across treatments (see Fig. 2b) may require high food ingestion at the same time that more energy needs to be canalized to the shell calcification process (see Lagos et al. 2016). In upwelling areas the exposure of the organisms to low temperatures is concomitant with high-pCO$_2$, which would depress the saturation state for carbonate below the level derived from high pCO$_2$ alone (Feely et al. 2008). The previous is because the kinetics of dissolution/solubility of carbonate shells increases at low temperatures (Morse et al. 2007). These “upwelling conditions” experienced by scallops in our experimental treatments (i.e. low temperature and high pCO$_2$) are already experienced by scallop farmed in Tongoy Bay, hence an adaptation to local environmental conditions trough phenotypic plasticity may be part of the responses expressed by this scallop population. It is important to maintain a perspective about the several organismal responses as an integrated phenotype because low pH regimes can significantly affect calcification process in *A. purpuratus*, which becomes enhanced under low
temperatures such as those occurring during an active upwelling phase (Lagos et al. 2016; Vargas et al. 2017). Thus, the presence of trade-offs in energy allocation during stress induced by upwelling processes is present at least in terms of calcification, growth rate and metabolism. Therefore, studies that aim to improve our understanding about the mechanisms that govern bivalve aquaculture species in terms of calcification, growth and physiological performance in upwelling zones are urgently needed.

For A. purpuratus the most striking histopathological effect was on the digestive gland integrity, which consisted in disintegrating and sloughing of digestive cells. Many stressors can generate atrophied digestive tubules, such as pollution (Sysa et al. 1997), food availability, saline and thermal stress (Carella et al. 2015). We measured that natural only a small proportion of scallops collected from the farm (natural control) showed reconstituting tubules morphology (Mathers 1976, Henry et al. 1991, Mathers et al. 1979, Sysa et al. 1997), which was atrophied (60%) in scallops subjected to the experimental conditions. This implies a reduction of digestive tubules available for accomplishing intracellular digestion, which may alter an adequate digestion during the exposure time. Clearly, this pattern of tubules atrophy in scallops across treatments may be associated with differences between the natural diet from Tongoy Bay and the artificial food used during the experiment. It has been reported for juvenile bivalve mussels that food availability can outweigh ocean acidification, as shown for Mytilus edulis (Thomsen et al. 2013), Pecten maximus (Sanders et al. 2013) and Argopecten purpuratus (Ramajo et al., 2016a, b). This latter study was undertaken in a population of juvenile scallops from the same population as this study and consisted in a 30-day exposure to different pH and food concentrations (Ramajo et al. 2016a) similar to those used in our incubations. Thus, an explanation of this positive response could be the existence of a greater proportion of healthy digestive tubules, providing the capacity to digest the available food, and the amelioration of ocean acidification effects trough increased temperature.

Hemocytic infiltration is an inflammatory response to pathogens, chemicals (such as pollutants) or physical cell injury (De Vico and Carella 2012; Carella et al. 2015). High prevalences but non-significant gill hemocytic infiltration were observed in the experimental treatments, respect to the natural control group. Gill had highest prevalence in the high temperature treatment (i.e., independent of pCO2 level). Mytilus edulis showed highest hemocytes infiltration when exposed to high temperature conditions, but was absent under high pCO2 treatments (Mackenzie et al. 2014). In addition, two parasites or symbionts were found in this study, a prokaryote, rickettsiales-like organism (RLO) and an unidentified protistan. Rickettsiales-like organisms (RLO) have been described as basophilic intracellular inclusion bodies in the gills or digestive gland of most bivalve molluscs (Bower et al. 1994), usually not causing any harm. They have also been described from digestive gland of adult cultivated A. purpuratus from Caldera, and the bays of Guanaqueros and Tongoy (Lohrmann 2009). In this study RLOs were most prevalent in the natural control group than in the experimental treatment, but with similar mean intensity. RLOs prevalence have though been linked to serious mortalities in some bivalves such as in the scallop Pecten maximus (Legall et al. 1988), giant clams Hippopus hippopus (Norton et al. 1993) and clams Venerupis rhomboides (Villalba et al. 1999). Although, RLOs have always been detected at low prevalence and intensity of infection in A. purpuratus, with no host hemocyte responses, is suggested that a harmless parasite can become a pathogen under the influence of as climate stressors that can enhance the virulence and/or the parasite pathogenicity (Burge et al. 2014).

In scallops, statocytes are sacs formed by a ciliated epithelium with a single statolith and a loose statocysta filling about 25% up to 100% of the sac, lie close to the pedal ganglion, are asymmetrical and function as gravity receptor, which epithelium development, cell types and cilia allow to differentiate the left and right statocytes (Cragg and Nott 1977). In our study the right statocyst observed in scallop exposed to the high pCO2 treatment suggests that the statocysta have been partially lost due to increased pCO2 or acidification conditions. Observation on scallops farmed at Tongoy bay (natural control), evidenced a right statocyst less developed and partially filled with statocysta, as has been described for other pectinids (e.g., Von Buddenbrock 1915). In addition, this right statocyst of the high pCO2 treatment, regardless of the temperature, showed some desquamation of the epithelium. It is not clear if this loss of integrity of the right statocyst epithelium could impair the gravity perception of the scallops, as only removal of the left one seemed to cause harm in other pectinids (Buddenbrock 1915).

Recently, Schalkhauser et al. (2013) demonstrate that Pecten maximus reduce their clapping performance when exposed to low temperature and increased pCO2 conditions suggesting potential link between the impact on the escape behaviour of scallops and the reduced epithelial integrity of the statocyst observed in our study.

There are several possible reasons to explain the moderate effect in A. purpuratus of ocean acidification and in scallops in general; firstly, the main mineral component of the shell is entirely of calcite a less soluble form of carbonates (Gazeau et al. 2013) and also a secretion of a thicker periostracum has been reported involving higher levels of polysaccharides under OA conditions as shell protection mechanism in juveniles (Ramajo et al. 2016a). Secondly, seawater remained in all treatments in our experiments above the calicite saturation state (Ωcalicite > 1). Lastly, Scanes et al. (2014) propose that swimming capacity of scallops which produce elevations of CO2 in the haemolymph and alterations in acid-base status and is another possible reason for the moderate effects of ocean acidification on scallops when compared to the negative physiological effects widely reported in oysters and mussels which are sessile mollusks. The previous is applicable for wild scallops in natural stocks; nevertheless, this mechanism is potentially less plausible in conditions of suspended culture where density in the lantern net is high (i.e., from 200 to 25 scallops per lantern level in Tongoy Bay farm), which reduces the opportunities for swimming in juvenile scallops. However, the underlying mechanism by which high pCO2 impacts moderately scallops physiology remains unclear for us.

Although our treatment of low temperature and high pCO2 or “upwelling conditions” did not evidence strong effect on the studied physiological traits, it is still uncertain the potential effect of future ocean acidification upon biological responses measured. One of the main problems in order to evaluate the effect of ocean acidification in marine organisms inhabiting these regions is the lack of information regarding to potential scenarios of ocean acidification for eastern boundary regions. There is no consensus of how the effects of warming and ocean acidification will interact (e.g. additive, synergistic, or antagonist) to influence the physiology and performance and health of mollusks (e.g. Findlay et al. 2009, Goedig et al. 2009, Comeau et al. 2010, Byrne 2011, Kroeker et al. 2013, Byrne and Przeslowski 2013; Duarte et al. 2014; Lagos et al. 2016; Navarro et al. 2016). Although high pCO2 significantly reduced growth rate of scallops at 14 °C, this effect is not significant at temperatures of 18 °C, showing how moderate warming can ameliorate the effects of OA through temperature effects on both physiology and histopathology. However, these scenarios of progressive acidification and warming may be not fully applicable to highly productive upwelling ecosystems, where the projected intensification of upwelling events due to the change in the intensity and patterns of wind (Sydeman et al. 2014) forecast more extreme upwelling events with cold subsurface waters, which are supersaturated with dissolved CO2 during longer periods. Therefore, the consideration of this kind of climatic scenarios in multistressor experiments is urgently needed for shellfish aquaculture species cultivated in upwelling zones in such as, for instance North America, Iberia and Africa.

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References


Blackwell, Carlton.