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Original Article

Physiological responses of juvenile Chilean scallops (*Argopecten purpuratus*) to isolated and combined environmental drivers of coastal upwelling

Laura Ramajo [1,2,3,4]*, Carolina Fernández[1,3], Yolanda Núñez[5], Paz Caballero[2], Marco A. Lardies[1,3], and María Josefina Poupin[6,7]

[1]*Departamento de Ciencias, Facultad de Artes Liberales, Universidad Adolfo Ibáñez, Avenida Diagonal Las Torres 2640, Peñalolén, Santiago, Chile*
[2]*Centro de Estudios Avanzados en Zonas Áridas, Avenida Ossandón 877, Coquimbo, Chile*
[3]*Center for the Study of Multiple-drivers on Marine Socio-Ecological Systems (MUSELS), Universidad de Concepción, Chile*
[4]*Centro de Investigación e Innovación para el Cambio Climático (CiiCC), Avenida Ejército 146, Santiago, Chile*
[5]*Facultad de Ciencias Biológicas, Pontificia Universidad de Chile (PUC), Av Libertador Bernardo O'Higgins 340, Santiago, Chile*
[6]*Laboratorio de Bioingeniería, Facultad de Ingeniería and Ciencias, Universidad Adolfo Ibáñez, Avenida Diagonal Las Torres 2640, Peñalolén, Santiago, Chile*
[7]*Center of Applied Ecology and Sustainability (CAPES), Av Libertador Bernardo O'Higgins 340, Santiago, Chile*

**Corresponding author: tel: + 56 51 2673259; e-mail: laura.ramajo@ceaza.cl.*

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Coastal biota is exposed to continuous environmental variability as a consequence of natural and anthropogenic processes. Responding to heterogeneous conditions requires the presence of physiological strategies to cope with the environment. Ecosystems influenced by upwelling endure naturally cold, acidic and hypoxic conditions, nevertheless they sustain major fisheries worldwide. This suggests that species inhabiting upwelling habitats possess physiological adaptations to handle high environmental variability. Here, we assessed the impact of the main upwelling drivers (temperature, pH and oxygen) in isolation and combined on eco-physiological responses of Chilean scallop *Argopecten purpuratus*. *A. purpuratus* responded to hypoxia by increasing their metabolic performance to maintain growth and calcification. Calcification was only affected by pH and increased under acidic conditions. Further, *A. purpuratus* juveniles prioritized calcification at the expense of growth under upwelling conditions. Increasing temperature had a significant impact by enhancing the physiological performance of *A. purpuratus* juveniles independently of oxygen and pH conditions, but this was associated with earlier and higher mortalities. Our results suggest that *A. purpuratus* is acclimated to short-term colder, acidic and hypoxic conditions, and provide important information of how this species responds to the heterogeneous environment of upwelling, which is significantly relevant in the climatic context of upwelling intensification.

Keywords: environmental drivers, environmental heterogeneity, global change, metabolism, multi-stressor, physiology, tolerance, upwelling

Introduction

Coastal oceans experience elevated environmental variability in temperature, pH, salinity, oxygen, food supply, and nutrients conditions (Duarte *et al.*, 2013). Consequently, resident biota of coastal habitats face a combination of multiple environmental drivers that impact a large number of eco-physiological processes (Lachkar, 2014; Gunderson *et al.*, 2016). In particular, coastal areas impacted by permanent (or seasonal) upwelling are subjected to a high variability as a consequence of the biogeochemical properties of upwelled waters. Deeper upwelled waters are

colder and present lower oxygen saturations (hypoxic/anoxic concentrations) and higher CO_2 concentrations (acidic) in comparison to surface coastal waters (Feely *et al.*, 2004; Lachkar, 2014). Still, even under such physiologically stressful conditions, upwelling ecosystems are highly productive and support the largest fisheries worldwide (Bakun, 1990). This makes upwelling ecosystems a particularly relevant interesting and valuable research models to assess the biological strategies that evidence the tolerance (and resistance) to multiple environmental drivers such as oxygen, pH, or temperature (Lagos *et al.*, 2016; Ramajo, Marbá, *et al.*, 2016).

Multiple studies, on a large amount of different marine species, have observed different levels of adaptation, resistance, and vulnerability to natural environmental conditions occurring in upwelling systems, river-influenced habitats, or CO_2 vents ecosystems. Coastal species (or populations) can be affected positive or negatively by changes in the environmental conditions showing increased or decreased growth (Ramajo, Marbá, *et al.*, 2016; Ramajo, Prado, *et al.*, 2016) and calcification rates (Heinemann *et al.*, 2012; Ramajo, Prado, *et al.*, 2016). Also, some species present increased metabolic rates or metabolic depression (Ramajo, Prado, *et al.*, 2016; Osores *et al.*, 2017), changes in periostracum thickness (Ramajo, Marbá, *et al.*, 2016; Ramajo, Prado, *et al.*, 2016; Osores *et al.*, 2017), differential patterns in shell carbonate precipitation (Ramajo *et al.*, 2015), oxidative stress (Bednaršek *et al.*, 2018), decreased fertilization success (Boch *et al.*, 2017), or even better gonadal development (Vilchis *et al.*, 2005). These studies demonstrate that the direction and the magnitude of the physiological impacts due to the environmental variability are species-specific (Kroeker *et al.*, 2013; Boch *et al.*, 2017), but also evidence the existence (or the absence) of physiological (i.e. up-metabolic regulation), mineralogical (i.e. changes in organic periostracum composition), molecular responses [i.e. up-regulation of heat shock proteins and chitin synthase (CHS)], or evolutionary mechanisms (i.e. higher phenotypic plasticity) that help to cope with environmental conditions such as low pH, hypoxia, or increasing temperatures (Cummings *et al.*, 2011; Hendriks *et al.*, 2015; Ramajo, Marbá, *et al.*, 2016; Ramajo, Pérez-León, *et al.*, 2016). Most of these studies concluded that the display of these physiological and genetical mechanisms, as well as the phenotypic plasticity expression, are a consequence of evolutionary processes such as acclimation or local adaption (Hendriks *et al.*, 2015; Ramajo, Marbá, *et al.*, 2016; Ramajo, Prado, *et al.*, 2016; Vargas *et al.*, 2017). Furthermore, food availability is as a major factor that provides the energy required to express these great spectra of the energetically costly biological mechanisms displayed under environmental changes (Ramajo, Pérez-León, *et al.*, 2016; Brown *et al.*, 2018). Hence, it should be expected that species residing in variable and high productivity habitats have better opportunities to handle with current stressful environmental conditions or future climate forecasts (Ramajo, Pérez-León, *et al.*, 2016; Vargas *et al.*, 2017).

Also, coastal systems are highly vulnerable to the additional environmental changes imposed by anthropogenic forces, mainly due to their reduced buffer capacity to regulate additional changes (Gruber, 2011; Duarte *et al.*, 2013). Surface seawater had the ability to regulate the excess of CO_2 of upwelled waters by consuming carbonate ions (CO_3^{-2}) and other bases (Fraginoulle, 1994; Egleston *et al.*, 2010). Nevertheless, under hypoxic conditions, as seawater CO_2 concentration increases, the capacity to absorb additional CO_2 gets reduced (Egleston *et al.*, 2010; Gobler and Baumann, 2016). Decreased seawater buffering capacity is extremely relevant in upwelling systems, especially because the frequency and intensity of upwellings are predicted to increase as a consequence of changes in wind patterns due to the increase in atmospheric temperatures (Bakun 1990; Sydeman *et al.*, 2014) or by poleward shifts in major atmospheric high-pressure Hadley cells (Lu *et al.*, 2007; Rykaczewski *et al.*, 2015). Recent studies show an increase in the duration and magnitude of upwelling events has already been reported for two of the major world upwelling systems, the Humboldt (HCS) and California Current Systems (Belkin, 2009; Falvey and Garreaud, 2009; Lima and Wethey, 2012; Aravena *et al.*, 2014; Jacob *et al.*, 2018). Consequently, the marine species currently living at upwelling-influenced habitats will be affected by intensified environmental conditions in both, average and variance, in pH, oxygen, and temperature conditions. These conditions will be also exacerbated by intensified and more frequent El Niño–Southern Oscillation (ENSO) events (Cai *et al.*, 2014). Hence, we are facing an immediate requirement to understand which are the physiological impacts and the biological mechanisms that coastal species show to counteract the current (and predicted) environmental conditions of their habitats.

Argopecten purpuratus, the northern Chilean scallop, is one of the most important and successful marine resources cultured along HCS. This species is a filter feeding bivalve inhabiting a geographic area ranging from Panama (10°N) to northern-central Chile (30°S) (Avendaño, 1993) which holds significant socio-economic importance along the Peruvian and Chilean coasts (Yáñez *et al.*, 2017). In northern Chile, Tongoy Bay (30°15′S 71°34′W) is the principal area where *A. purpuratus* is intensively cultured with >3330 tons obtained in 2016 (SERNAPESCA, 2016). Tongoy Bay is a closed bay influenced seasonally by one of the most important and active upwelling centres of the Chilean coast, Lengua de Vaca Point (PLV, 30°18′S) (Strub *et al.*, 1998; Figueroa and Moffat, 2000; Aravena *et al.*, 2014). During spring–summer seasons, favourable-upwelling winds generate the rise of deeper upwelled waters that increase the variability in temperature (18–13°C), pH (8.1–7.6), and oxygen (7–0.5 mlL^{-1}) (Torres *et al.*, 1999; Torres and Ampuero, 2009). In addition, ENSO enhances this variability with important impacts on the *A. purpuratus* fishery (Mendo and Wolff, 2003).

To date, several efforts have been made to improve and extend *A. purpuratus* fisheries by evaluating the physiological impacts of many biotic and abiotic variables such as salinity, temperature, diet, and depth (Martínez *et al.*, 1995, 2000; Navarro and Gonzalez, 1998; Avendaño *et al.*, 2008). Also, pH or hypoxia in combination with temperature (Brokordt *et al.*, 2015; Aguirre-Velarde *et al.*, 2016; Lagos *et al.*, 2016; Lardies *et al.*, 2017) and changes in the food availability impacts (Ramajo, Marbá, *et al.*, 2016) have been studied in the context of climate change. Most studies agree with the great tolerance and resistance of *A. purpuratus* to a broad range of environmental conditions, mainly explained by the historical environmental conditions of the habitats where this species is living (Lagos *et al.*, 2016; Ramajo, Marbá, *et al.*, 2016). Nevertheless, important gaps of knowledge still exits about this species such as, what are the nature of the interactions (additive, antagonist, or synergistic) of the multiple drivers co-occurring and co-varying during upwelling and their physiological impacts.

Based on that, the aim of this study is to assess, for first time on the Chilean scallop *A. purpuratus*, which are the physiological and genetical impacts of the main upwelling drivers of the upwelling-influenced habitat where is cultured. Our study

complements and improves the results of previous studies by investigating the nature of the interactions among temperature, pH, and oxygen by using a laboratory full-factorial design. The information obtained from this study contributes to complete the missing information required to understand the physiological strategies that make *A. purpuratus* highly tolerant and resistant to the permanent and stressful environmental conditions of upwelling. These results would provide more reliable information that support more precise predictions for its fishery in the future climate context of upwelling and ENSO intensification.

Methods

Animal collection

On July 2017, ~200 juvenile scallops, with sizes no higher than 20 mm length and without gonads presence, from wild populations were provided by the company INVERTEC S.A. located at Tongoy Bay (30°16′S 71°35′W). The organisms along with seawater from Tongoy Bay were transported in insulated containers, to the laboratory (Universidad Adolfo Ibáñez, Santiago de Chile) to conduct the experiment. During the acclimation period (12 days), organisms were fed with an adequate food supply (i.e. superior to 2% of the dry weight (DW), see Ramajo, Marbá, *et al.*, 2016) using a blend of phytoplankton, zooplankton, amino-acids, vitamins, and minerals (Reef Blizzard-O, Brightwell Aquatics, Catawiss, PA) and maintained at 14°C (seawater temperature recorded when juvenile scallops were collected), pH (NBS scale) near 8.0 and saturated oxygen concentrations (>90%). Before the experiment started, 192 healthy juvenile scallops were visually checked to avoid the use of organisms with shell damage by spionid polychaete *Polydora* sp. (Radashevsky and Cárdenas, 2004). Although sexual maturation for this species occurs at sizes higher than 33 mm (see Cañas *et al.*, 1984), the absence of gonads was also checked and confirmed visually before experiment started. Finally, selected experimental juvenile scallops were labelled with numbered bee tags glued onto the shells for future identification, and both the initial size (maximum length, mm) and the initial buoyant weight (mg) were determined for all them.

Register of environmental variability of Tongoy Bay

For 2016 and 2017, temperature (°C) and dissolved oxygen (% saturation) conditions of Tongoy Bay were measured by high-frequency sensors located at CEAZAMET buoy in Tongoy Bay at 10 m depth (see www.ceazamet.cl). Temperature and oxygen data were registered by a CTD WQM (WetLabs) measuring every 15 min. A low-frequency record of pH conditions (once a week) of Tongoy Bay was initiated on August 2017. Weekly pH (NBS scale) measurements were conducted by collecting water samples at 10 m depth by using a NISKIN bottle (5 l volume). pH water samples were analysed within 60 min after collection and measured by using a combined electrode (double juncture) connected to a Metrohm 826 pH Mobile-metre and calibrated with commercial buffers (Metrohm®) maintained at 25°C using a temperature-controlled water bath.

In summary, during 2016 and 2017, Tongoy Bay showed a mean temperature of 14.35 ± 0.25°C (s.e.) with maximum and minimum values ranged between 19.68 ± 0.40 and 10.66 ± 0.20°C, respectively (Figure 1a–c). However, longer time series (12 years) have reported for Tongoy Bay maximum temperatures of 18°C (Aravena *et al.*, 2014).

Furthermore, Tongoy Bay presented an average oxygen saturation of 79.19% (±2.41 s.e.) with important reductions when upwelling is active. For instance, the upwelling activation produced a mean reduction of 32.19% (±1.20) in the oxygen concentrations comparing with conditions of upwelling absence (Figure 1d–f). Previous studies assessing pH variability near to study area (Lengua de Vaca Point, PLV) registered pH values near 7.7 during upwelling season (Torres *et al.*, 1999; Torres and Ampuero, 2009). These water characteristics also impact the pH conditions of Tongoy Bay. Indeed, in terms of pH, we found that during 2017 (from August to December) Tongoy Bay showed mean pH values of 7.85 ± 0.03 units and a high pH variability where pH ranged between 8.17 and 7.67 (Figure 1g–i).

Experimental design

Different levels of the experimental treatments selected for this study were established by incorporating ecologically relevant conditions of the habitat where *A. purpuratus* organisms live and are cultured. In addition, experimental levels used also coincide with previous studies (Lagos *et al.*, 2016; Ramajo, Marbá, *et al.*, 2016) to complement and improve the information missing to date. Based on that, temperature experimental levels selected corresponded to the mean and maximum temperatures registered for Tongoy Bay (14°C vs. 18°C) (Aravena *et al.*, 2014) (Figure 1). Due to a lack of historical pH time-series for Tongoy Bay, pH experimental levels were based on previous studies (Torres and Ampuero, 2009). These values were corroborated by the recent low-frequency pH series at Tongoy Bay (since August 2017) (see Figure 1). Thus, we applied a reduction near 0.4 pH units in the acidified treatment with respect to the control pH treatment (see Figure 1). Temperature and pH experimental values also coincide with the experimental levels used by previous studies that assessed the impact of future ocean acidification and temperature on *A. purpuratus* (see Lagos *et al.*, 2016; Ramajo, Marbá, *et al.*, 2016). In terms of oxygen, we applied an oxygen reduction of almost 35% between control treatment (i.e. normoxia, >80%) and hypoxic treatment (≈45%), similar to observed at Tongoy Bay for 2017 (see Figure 1). The simultaneous effects of temperature, pH, and oxygen were evaluated by using a full-factorial design. Hence, juvenile scallops were exposed to a total of eight different experimental treatments where oxygen concentration (*Hypoxic Treatment*) and pH conditions (*Acidified Treatment*) were decreased isolated or in combination (*Hypoxic-Acidified Treatment*) at 14 and 18°C and compared to control conditions (*Control Treatment:* normoxic and high pH conditions at 14 or 18°C) (see Table 1). Each experimental treatment contained three-independent replicate 9 l aquaria (24 aquaria total) with eight juvenile scallops randomly selected with similar initial size (19.61 ± 0.11 mm, one-way ANOVA, $p = 0.265$).

Indices of thermal variability for Guanaqueros Bay (30°11′57′S 71°28′45′W), the nearest bay to Tongoy Bay (≈15 km distance), shows that cooling events imposed by the activation of PLV upwelling centre have an average duration of 13.1 days (±3.1 *SD*) (Tapia *et al.*, 2009). We used this information and we applied an experimental duration of 11 days in our design. Eleven days match with the temporal range that study site (Tongoy Bay) would present colder, acidic, and hypoxic conditions imposed by upwelling (see Tapia *et al.*, 2009).

Oxygen and pH and levels during the experiment were manipulated by bubbling seawater with a mix of air, CO_2, and N_2 gasses using Mass Flow Controllers (Aalborg, USA). Temperature was manipulated by using two-independent chillers (BOYU, Model L075).

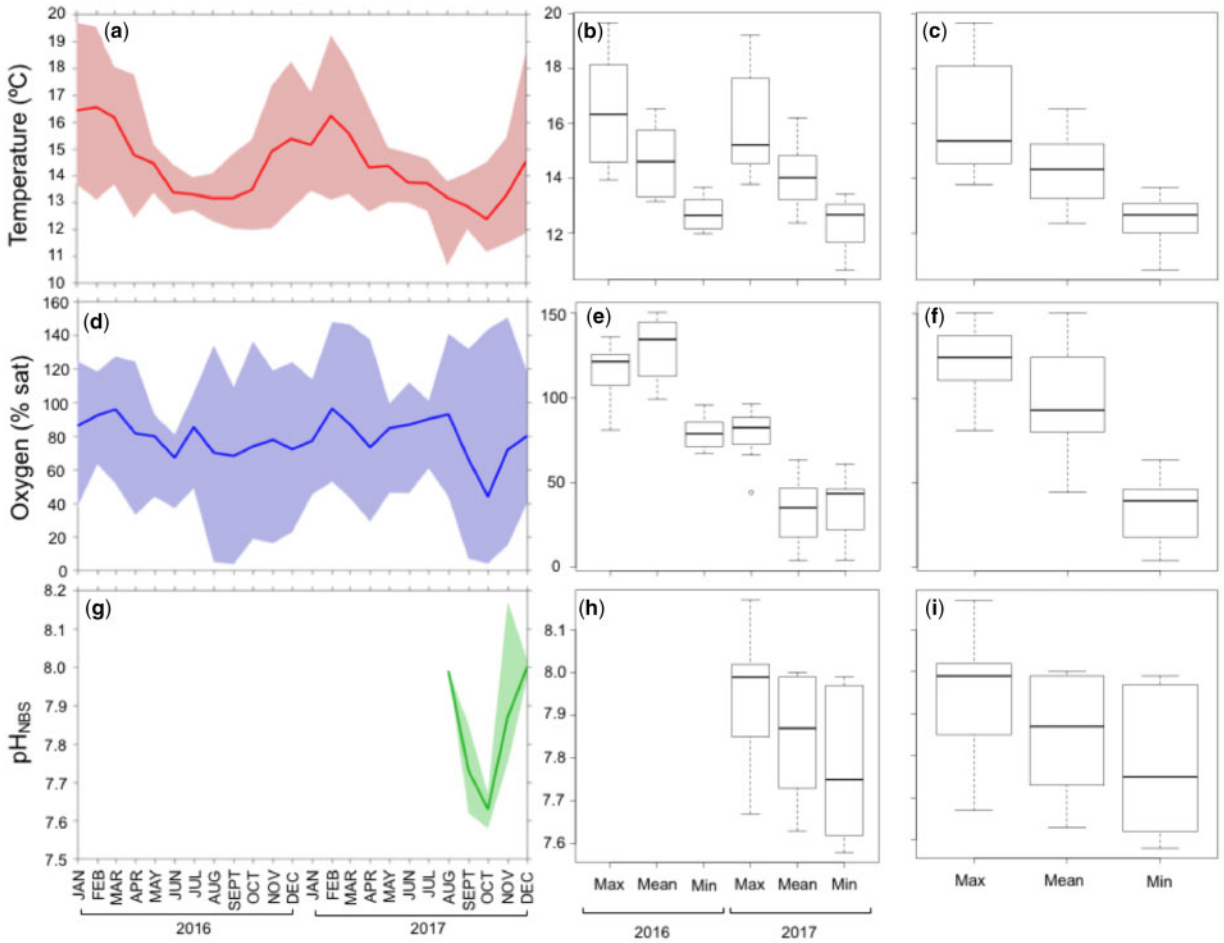

Figure 1. Environmental time series registered at Tongoy Bay for 2016 and 2017. (a) Temperature (°C), (d) Oxygen saturation (%), and (g) pH_{NBS} (25°C) at 10 m depth. Lines represent monthly averages. Shadows show the monthly average range for each variable. Maximum, mean, and minimum average values are shown for 2016, 2017, and both years together for (b, c) Temperature (°C), (e, f) Oxygen (% sat), and (h, i) pH_{NBS} (25°C).

Table 1. Measured and estimated experimental conditions recorded in the different experimental treatments.

Measured

Treatments		pH_{NBS} (25°C)	Temperature (°C)	Oxygen (% sat)	A_T (µmol kg^{-1})
14°C	Control	8.10 (0.03)	14.11 (0.03)	79.85 (0.53)	2276 (7)
	Hypoxic	8.02 (0.02)	13.98 (0.07)	43.14 (0.85)	2279 (11)
	Acidified	7.58 (0.03)	13.95 (0.04)	77.03 (0.85)	2267 (5)
	Hyp-Acid	7.67 (0.01)	14.00 (0.05)	44.02 (0.00)	2298 (13)
18°C	Control	8.11 (0.03)	17.74 (0.02)	81.34 (0.21)	2271 (12)
	Hypoxic	8.05 (0.04)	17.79 (0.01)	42.10 (0.85)	2269 (6)
	Acidified	7.62 (0.00)	17.60 (0.01)	81.66 (0.53)	2285 (4)
	Hyp-Acid	7.72 (0.02)	17.64 (0.02)	45.81 (0.32)	2271 (1)

Estimated

Treatments		pH_{NBS} (*in situ*)	pCO_2 (µatm)	$\Omega_{Calcite}$	$\Omega_{Aragonite}$
14°C	Control	8.22 (0.01)	330 (13)	4.1 (0.1)	2.6 (0.1)
	Hypoxic	8.16 (0.01)	399 (14)	3.6 (0.1)	2.3 (0.0)
	Acidified	7.70 (0.02)	1276 (53)	1.4 (0.1)	0.9 (0.0)
	Hyp-Acid	7.79 (0.00)	1027 (10)	1.7 (0.1)	1.0 (0.0)
18°C	Control	8.19 (0.02)	364 (15)	4.2 (0.1)	2.8 (0.1)
	Hypoxic	8.14 (0.03)	425 (30)	3.8 (0.2)	2.5 (0.1)
	Acidified	7.63 (0.07)	1324 (12)	1.6 (0.3)	1.0 (0.2)
	Hyp-Acid	7.80 (0.01)	1009 (33)	1.9 (0.1)	1.2 (0.0)

Data are means (± s.e.). Salinity was stable at 34 (psu). Estimated parameters were calculated by using CO2SYS software.
Hyp-Acid, hypoxic-acidified treatment.

During the experiment, organisms were fed daily with the same type of food and amount utilized during acclimation period (see above). To maintain water quality and stable salinity levels (34‰), the aquaria were cleaned every 2 days and re-filled with filtered (5 µm plus UV filter) seawater, pre-treated with the corresponding temperature, pH, and oxygen experimental conditions of the treatment.

Experimental conditions and parameter measurements

Discrete pH (NBS scale) measurements during the experiment were taken every day. Every 2 days, and before water changes, two replicate water samples per aquarium were collected for Total Alkalinity (A_T) and fixed with supersaturated $HgCl_2$. A_T was measured using automatic titration (open-cell method) with HCl (Fixanal®) and double endpoint titration to pH 4.45 and 4.41 (NBS scale) following Dickson Sop 3 b (version 3.01) with a 808 Tritando and Aquatrode plus (Metrohm®). The accuracy of measurements was checked against certified reference seawater (Batch 155) supplied by the Scripps Institution of Oceanography in San Diego, CA.

Oxygen and temperature values were recorded every day in all aquaria using a Dissolved Oxygen meter (Model HI98193, HANNA®). Salinity for each aquarium was recorded using a digital salinometer (Eutech-Salt-6). Carbonate system parameters were estimated from the average values of salinity, pH_{NBS}, A_T, and temperature using CO2SYS software (Pierrot *et al.*, 2006) and applying dissociation constants from Mehrbach *et al.* (1973) refitted by Dickson and Millero (1987) and $KHSO_4$ (Dickson, 1990). Average experimental carbonate water conditions (measured and estimated) for all experimental treatments are reported in Table 1.

Metabolic rates

Metabolic rates were evaluated by measuring oxygen consumption rates and determined on four individual scallops per replicate aquarium for each treatment. Experimental organisms were incubated individually in 0.067 l respirometric chambers at its corresponding experimental temperature (14 or 18°C), which was controlled by an automated temperature chiller (BOYU, Model L075). Oxygen consumption measurements were obtained by using water equilibrated to the corresponding pH and oxygen treatments. Before measurements, individuals were exposed to inanition during 48 h in UV-treated filtered in seawater with temperature, pH, and oxygen of corresponding treatments. Metabolic rate for each individual was measured by using an optical fibre system (Presens Mini Oxy-4 Respirometer, PreSens, Regensburg, Germany), which quantified dissolved oxygen every 15 s for 60 min. Before measurements, sensors were calibrated with a solution of Na_2O_3S at 5% and aerated water for the values 0 and 100% air saturation, respectively. Oxygen consumption rates were standardized to individual DW ($mgO_2\ h^{-1}\ g^{-1}$). DW of each experimental scallop was estimated from the maximum length by using the allometric relation described by Uribe *et al.* (2008).

Mortality, growth, and calcification rates

Mortality was evaluated daily and dead organisms were retired from their respective aquarium to avoid water deterioration. After 11 days, growth rates and net calcification rates were determined. Growth rates correspond to the difference in maximum length between two consecutive experimental sampling events divided by the number of days elapsed ($mm\ d^{-1}$). The net calcification rate was calculated using the buoyant weight technique (Davies, 1989), where buoyant weight was converted into shell DW using the seawater density of experimental seawater (salinity = 34%, temperature = 14 or 18°C) and the density of calcite ($2.71\ g\ cm^{-3}$) (see Ramajo, Marbá, *et al.*, 2016). Net calcification rates were calculated as the change in shell DW between 0 and 11 days and normalized to the initial shell DW of the individuals ($mgCaCO_3\ g^{-1}\ d^{-1}$).

Stress-related gene expression

After that oxygen consumption, growth, and calcification rates were determined; the effects of the studied environmental changes were assessed in the expression of genes related to stress. First, the tissues of all juvenile scallops were excised and immediately frozen at −80°C until RNA extraction. Total RNA extraction was performed using the gills of 5–6 scallops per treatment (replicates) by using the Trizol® method (Invitrogen™, Carlsbad, CA) following the manufacturer instructions. Then, RNA was treated with DNAse I (RQ1, Promega). cDNA was synthesized, using 1 µg of total RNA and random hexamers and the ImProm IITM Reverse Transcription System (Promega), following the manufacturer instructions.

Real-time PCR was performed using the Brilliant® SYBR® Green QPCR Master Reagent Kit (Agilent Technologies) and the Eco Real Time-PCR detection system (Illumina®) as described by Arias *et al.* (2011) using two technical replicates for each sample. The PCR mixture (10 µl) contained 4.75 µl of template cDNA (diluted 1/10) and 140 nM of each primer. Amplification was performed under the following conditions: 40 cycles of 95°C, 30 s; Melting temperature for 30 s; and 72°C, 40 s. At the end of PCR amplification all products were subjected to a melt cycle from 55 to 95°C. The Primers used are described in Supplementary Table S1. *ACTIN* and *EF1a* were used as housekeeping genes. Both reference genes were highly stable and essentially the same expression patterns were obtained with both. According to RefFinder (Xie *et al.*, 2012) the most stable reference gene was *EF1a*, and consequently the results shown are those obtained normalizing with this gene. Normalization of the genes of interest (GOI), *FERRITIN* and *HSP70*, was performed according to equation: $2^{-(\Delta ctGOI-ctHK)}$. Then, gene expression levels were normalized to the average value of the *Control Treatment* (i.e. control pH and normoxia at 14°C).

Data analysis

We analysed the differences in oxygen consumption rates, growth rates, net calcification rates, mortality, and the relative expression stress-related gene expression (*HSP70* and *FERRITIN*) by using a three-way factorial ANOVA, where temperature, pH, and oxygen were fixed as factors with their two respective levels (i.e. 2 × 2 × 2, eight treatments). When a significant interaction between factors was registered (i.e. growth rate), the effect of each factor was analysed independently at each level of the other factor by using a one-way ANOVA following by a Tukey pairwise comparison.

When no significant interaction among factors was recorded, a Student's *t*-test was used to resolve differences between treatments. Prior to the statistical analyses, data were log or square-transformed (i.e. oxygen consumption, mortality and protein expression) to satisfy the assumptions of normality and homogeneity of variance. This was verified by using the Shapiro–Wilk and

Levene tests. All analyses were performed by using JMP software for OS X (Version 9.0.1).

To test the nature of the interactions among factors (additive, synergistic, or antagonistic), the relative response ratio (RR) of oxygen consumption rates, growth rate, calcification rate, and mortality for each single (and combined) variable was calculated by performing bootstrapping analyses following the same statistical procedures of Egea *et al.* (2018). Bootstrapping analyses were obtained by re-sampling 2000 values of the original data for each biological parameter using the "bootES" package (Gerlanc and Kirby, 2015) of R 3.3.4 software (R Core Team, 2013).

Results

Metabolic rates

No interactive effects among temperature, pH, and oxygen concentrations were observed in the metabolic rates of juveniles of *A. purpuratus* (Table 2). However, different temperature and oxygen concentrations generated significant changes in the metabolic rates (Table 2). At 14°C, juveniles exposed to the *Hypoxic Treatment* presented 1.8 times higher metabolic rates (1.21 ± 0.11, ± s.e.) than individuals exposed to normoxia (0.67 ± 0.20, ± s.e.) ($p < 0.05$) (Figure 2a, Table 2). On the contrary, when experimental juvenile scallops were exposed to a reduction in pH conditions (isolated and combined with lower oxygen concentrations), no changes in the metabolic rates were observed ($p > 0.05$) (Figure 2a). At 18°C, similar metabolic rates were observed among experimental treatments ($p > 0.05$), but were always superior to that observed in the *Control Treatment* at 14°C ($p < 0.05$) (Figure 2b). At 18°C, in scallops exposed to the combined effect of decreasing pH and low oxygen concentrations (*Hypoxic-Acidified Treatment*), metabolic rates were significantly higher than at 14°C ($p < 0.05$) (Figure 2a and b, Table 2).

Growth rates

Growth rates were affected by seawater temperature, oxygen concentration, and pH seawater conditions; however, no interaction among the three factors was found (Table 2). At 14°C, scallops exposed to the isolated effect of decreasing oxygen concentration (*Hypoxic Treatment)* and pH (*Acidified Treatment*) showed similar growth rates to juvenile scallops exposed to normoxic and control pH conditions ($p > 0.05$) (*Control Treatment*) (Figure 2c,

Table 2. Effect of temperature, pH, and oxygen concentration on metabolic rates, growth rates, net calcification rates, and total mortality in juvenile scallops of *A. purpuratus* after 11 days of experimental conditions during which temperature, oxygen, and pH levels were modified in isolation and combined.

Response	Source	DF	SS	*F*	*p*-Value
Metabolic rate (mg O_2 h^{-1} g^{-1})	Temperature (*T*)	1	0.206	11.95	**0.003**
	pH	1	0.062	3.61	0.076
	Oxygen (O_2)	1	0.133	7.70	**0.014**
	T × pH	1	0.008	0.489	0.494
	T × O_2	1	0.000	0.00	0.951
	pH × O_2	1	0.030	1.76	0.203
	T × pH × O_2	1	0.040	2.31	0.148
	Error	16	0.276		
	Total	23	0.756		
Growth rate (mm d^{-1})	Temperature (*T*)	1	0.555	12.02	**0.003**
	pH	1	0.017	0.38	0.549
	Oxygen (O_2)	1	0.008	0.17	0.684
	T × pH	1	0.758	16.40	**0.001**
	T × O_2	1	0.356	7.69	**0.014**
	pH × O_2	1	1.116	24.14	**0.000**
	T × pH × O_2	1	0.067	1.46	0.245
	Error	16	0.740		
	Total	23	3.617		
Net calcification rate (mg $CaCO_3$ d^{-1} g^{-1})	Temperature (*T*)	1	0.251	0.04	0.851
	pH	1	124.213	17.91	**0.001**
	Oxygen (O_2)	1	2.798	0.40	0.534
	T × pH	1	1.282	0.18	0.673
	T × O_2	1	16.456	2.37	0.143
	pH × O_2	1	0.509	0.07	0.789
	T × pH × O_2	1	6.372	0.92	0.352
	Error	16	110.949		
	Total	23	262.831		
Mortality (%)	Temperature (*T*)	1	65.536	32.11	**<0.0001**
	pH	1	3.887	1.90	0.187
	Oxygen (O_2)	1	1.887	0.92	0.351
	T × pH	1	0.887	0.41	0.531
	T × O_2	1	0.279	1.12	0.305
	pH × O_2	1	2.289	0.28	0.716
	T × pH × O_2	1	0.279	0.13	0.716
	Error	16	32.658		
	Total	23	107.653		

Bold numbers indicate significant *p*-values at $\alpha = 0.05$.

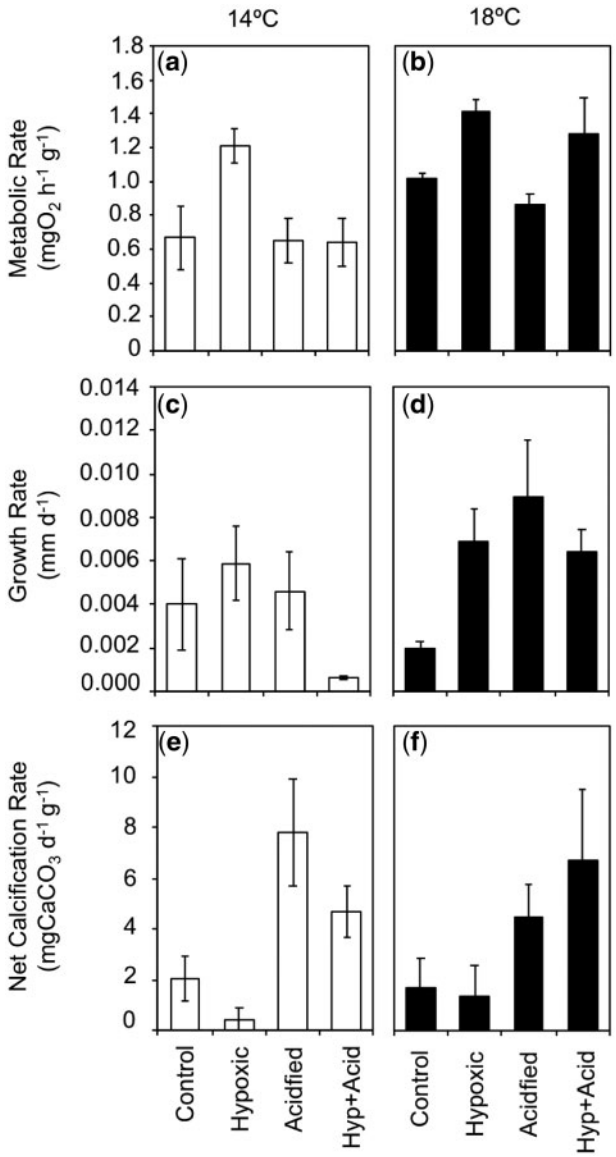

Figure 2. Metabolic rates ($mgO_2\ h^{-1}\ g^{-1}$) (a, b), growth rates ($mm\ d^{-1}$) (c, d), and net calcification rates ($mgCaCO_3\ g^{-1}\ d^{-1}$) (e, f) of juvenile scallops *A. purpuratus* exposed for 11 days to a full factorial design where temperature, pH, and oxygen concentrations were modified. Data are means ± s.e.

Supplementary Table S2). On the contrary, scallops grown under the combined effect of lower pH and decreased oxygen concentrations (*Hypoxic-Acidified Treatment*) presented a sharp drop in their growth rates (near 85% less, $p < 0.05$) (Figure 2c, Supplementary Table S2). At 18°C, juvenile scallops exposed to combined (*Hypoxic-Acidified Treatment*) and isolated changes in pH (*Acidified Treatment*) and oxygen (*Hypoxic Treatment*) showed a significant increase in their growth rates in comparison to normoxic and non-acidified conditions ($p < 0.05$) (*Control Treatment*) (Figure 2d; Supplementary Table S2).

Net calcification rates

Net calcifications rates were only affected by changes in the pH conditions (*Acidified Treatment*), and no interaction with oxygen and temperature was observed (Table 2). Juvenile scallops exposed to lower pH conditions (*Acidified Treatment*) presented significant increases (as much as fourfold) in their net calcification rates at 14°C, but not at 18°C (Figure 2e and f).

Mortality

Mortality was only affected by temperature conditions (Table 2). Mortality at 18°C showed an increase as the experiment progressed. At 18°C and under control pH and normoxic conditions (*Control Treatment*) we registered the first mortality event at day 8 (Figure 3a), while when pH and oxygen concentrations were decreased in isolation (*Acidified Treatment* and *Hypoxic Treatment*, respectively) and combined (*Hypoxic-Acidified Treatment*), these mortality events appeared earlier (Figure 3b–d). On average, juvenile scallops exposed to higher temperatures (18°C) presented significantly higher mortality (Figure 4) in all treatments in comparison to 14°C. At equal temperature treatment (both 14 and 18°C), no significant differences in mortality were observed among the treatments where pH and oxygen concentrations were modified in isolation and combined ($p > 0.05$) (Figure 4).

Stress-related gene expression

It is well documented that FERRITIN and HSP proteins are suitable markers of stress in marine invertebrates, since they are highly expressed under adverse environmental conditions (Feder and Hofmann, 1999; English and Storey, 2003; Theil, 2003; Zapata *et al.*, 2009; Lardies *et al.*, 2014; Aguilera *et al.*, 2016). Here, no significant differences were observed for *HSP70* and *FERRITIN* gene expression in *A. purpuratus* juveniles exposed for 11 days to isolated and combined changes in temperature, pH, and oxygen concentration (Figure 5a and b; Supplementary Table S3).

Relative RRs

No clear trend in RRs in those treatments where temperature, pH, and oxygen concentrations were combined was observed to compare to single factor treatments (Table 3). With one exception, where oxygen and pH at 14°C had an antagonist effect on the growth rates of juvenile *A. purpuratus*, changes in pH (acidified), temperature (higher), and oxygen (hypoxia) had additive effects on metabolic, growth, and calcification rates after 11 experimental days (see Table 3).

Discussion

Determining how species will respond to future global warming, ocean acidification, or ocean de-oxygenation implies understanding the biological mechanisms and strategies that coastal species display to handle with the natural variations in temperature, pH, and oxygen concentration occurring in their native habitats. But also, it is of extreme relevance to determine the nature of the interactions of multiple environmental drivers on the organism's physiology. Here, for first time in the Chilean scallop *A. purpuratus*, it was assessed the physiological and genetical impacts of isolated and combined changes in temperature, pH, and oxygen. In summary, the results of the experiment showed a significant antagonistic effect between pH and oxygen concentration on *A. purpuratus* growth rates at lower temperatures (upwelling conditions). For the rest of experimental conditions, pH and oxygen showed additive effects on the multiple physiological responses

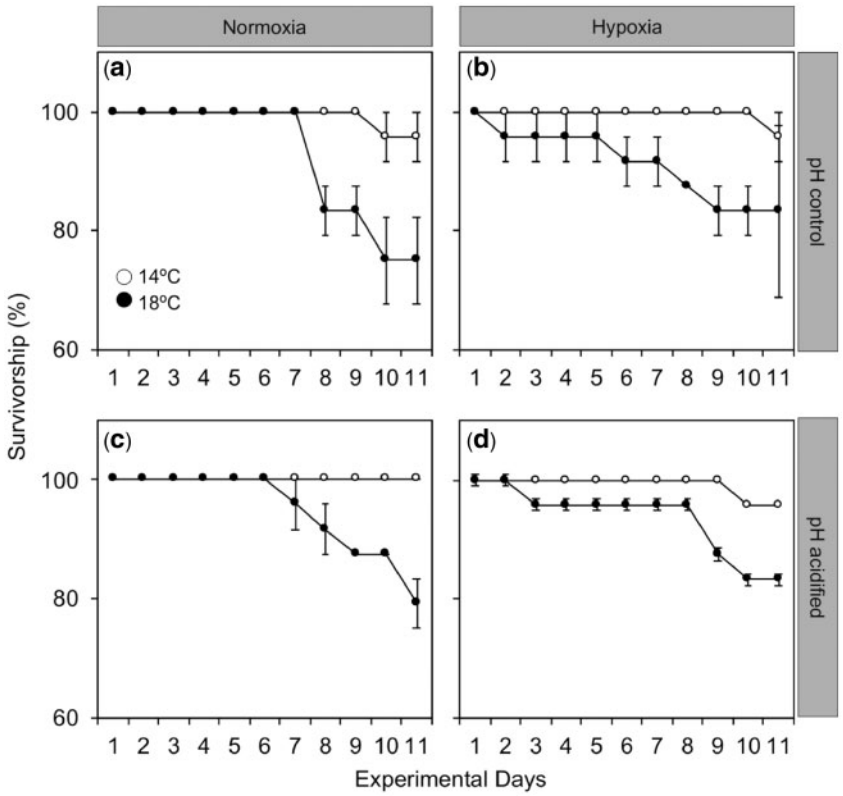

Figure 3. Mean (±s.e.) survivorship (per day) of juvenile scallops *A. purpuratus* among Control (a), Hypoxic (b), Acidified (c), and Hypoxic-Acidified (d) treatments at 14 and 18°C.

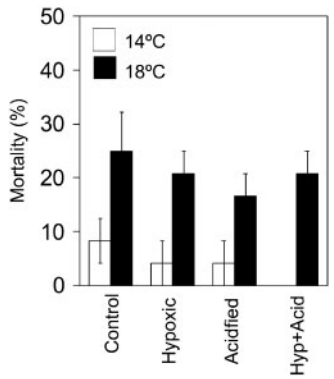

Figure 4. Mean mortality (%) of juvenile scallops *A. purpuratus* per treatment at the end of the experiment. Data are means ± s.e.

studied. Moreover, temperature had an additive effect on growth, calcification, and metabolic rates under isolated and combined acidified and hypoxic experimental conditions, with a significant impact on the fitness of *A. purpuratus*.

Temperature is a key environmental driver that induces changes in the majority of the biological processes and establishes the geographical range of marine species (Kordas *et al.*, 2011). In isolation or combined with temperature, pH could enhance, or modify many of these physiological responses (Kroeker *et al.*, 2013). In addition, a decrease in oxygen concentrations expose marine biota to important physiological threats (Vaquer-Sunyer and Duarte, 2008) by increasing mortality and amplifying the effect of other environmental drivers such as temperature or pH (Diaz and Rosenberg, 1995; Pörtner, 2010).

Effect of hypoxia

Metabolic adaptations are crucial to respond a decrease in oxygen concentrations in marine habitats (Hagerman, 1998). Oxygen consumption rates of *A. purpuratus* juveniles were affected by the oxygen concentration and temperature regimes, but with different impacts. At 14°C, we observed an increase in oxygen consumption rates (almost twofold) under hypoxic conditions in comparison to normoxic conditions. Nevertheless, at 18°C, elevated oxygen consumption rates were only influenced by increased temperatures and not seawater oxygen concentration. This result is in agreement with previous studies (Artigaud *et al.*, 2015; Aguirre-Velarde *et al.*, 2016; Jeppesen *et al.*, 2018) that show temperature's ability to impact the magnitude and direction of the physiological responses to conditions such as hypoxia and highlights the significant role that this driver has in comparison to others (Le Moullac *et al.*, 2007).

For many species, responding to hypoxia generates metabolic depression (Pörtner *et al.*, 2005) impacting over multiples biological processes such as growth and calcification rates (Chan *et al.*, 2012; Leung and Cheung, 2018). However, elevated metabolic rates, mediated by hyperventilation, increased cardiac rates or changes in respiratory pigments, are common adaptive responses showed by bivalve species to cope with acute hypoxic conditions (Das and Stickle, 1993; Burnett and Stickle, 2013; Gurr *et al.*, 2018). Maintaining the oxygen delivery under hypoxic conditions allowed *A. purpuratus* juveniles sustain similar growth and

calcification rates. Calcification is one of the biological processes consuming major amounts of energy (Palmer, 1992), thus when species show elevated (or unalterable) calcification rates under stressful conditions, sometimes the apparition of physiological trade-offs is favoured (Wood *et al.*, 2008). In these particular cases, food supply and the available energy budget (i.e. high metabolic rates) are vital to avoid negative physiological impacts and trade-offs (Ramajo, Marbá, *et al.*, 2016; Ramajo, Pérez-León, *et al.*, 2016). The results observed when *A. purpuratus* juveniles were exposed to hypoxic conditions indicate that this species could have the ability to tolerate short-term and relatively low concentrations of oxygen. Indeed, the low value of the critical oxygen saturation point (P_cO_2) (near to 24%), even at higher temperatures, point to *A. purpuratus* as a good oxy-regulator species (see Aguirre-Velarde *et al.*, 2016). Our results agree with other studies that show mollusk species as highly tolerant to hypoxia (Vaquer-Sunyer and Duarte, 2008), specifically if they are naturally exposed to low and variable oxygen concentrations in their habitats (Steckbauer *et al.*, 2015; Jeppesen *et al.*, 2018).

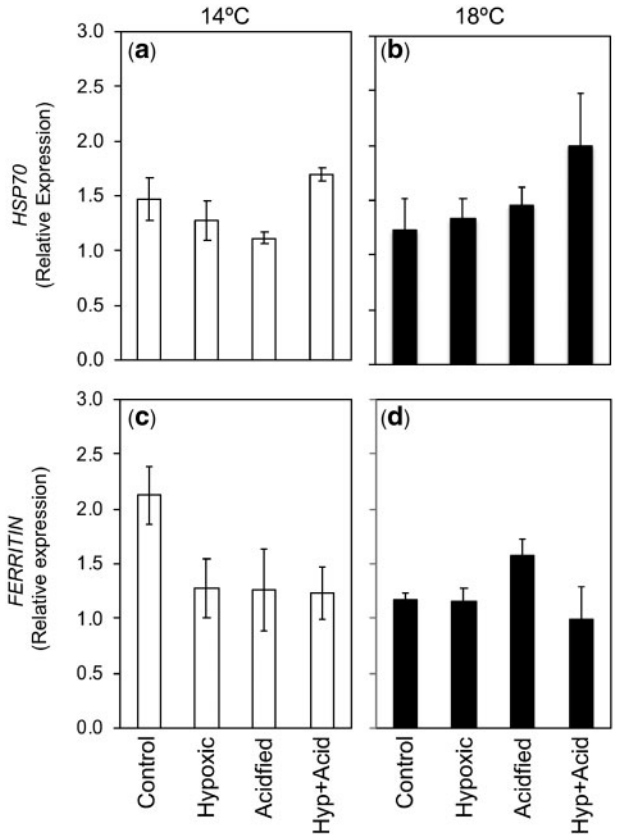

Figure 5. Relative gene expression of *HSP70* (a, b) and *FERRITIN* (c, d) on gill tissues of *A. purpuratus* juveniles after 11 days under isolated and combined changes in temperature, pH, and oxygen concentration. Data are means of 5–6 replicates per treatment ± s.e.

Effects of pH

Metabolic down- and up-regulation have been reported as biological mechanisms that marine species use to deal with field and experimental acidic conditions (Thomsen and Melzner, 2010; Hendriks *et al.*, 2015; Ramajo, Marbá, *et al.*, 2016). In our study, as observed by Lardies *et al.* (2017), acidic conditions did not impact the oxygen consumption rates of *A. purpuratus* juveniles at either experimental temperature. Nevertheless, a metabolic up-regulation for *A. purpuratus* has also been recorded under low pH conditions (see Ramajo, Marbá, *et al.*, 2016). The differences and similarities found among these two studies and ours might be attributed to the duration of the experiments. While our study and Lardies *et al.* (2017) exposed the organisms to a short experimental duration of lower pH (11 and 18 days, respectively), the experiment performed by Ramajo and co-authors lasted 30 days. The duration of pH changes to which species are exposed is a key factor (Kroeker *et al.*, 2013), mainly because many species show different physiological responses when coping with short- or long-term low pH periods (Form and Riebesell, 2012; Sui *et al.*, 2016).

pH significantly impacts the growth and shell formation processes of marine calcifying species (Kroeker *et al.*, 2013). Previous studies on *A. purpuratus* showed higher growth and calcification rates under long-term acidified conditions in comparison to

Table 3. Relative RRs on metabolic rates, growth rates, and net calcification rates of *A. purpuratus* juveniles exposed to isolated and combined changes in temperature, pH, and oxygen concentration.

		Biological response		
Driver		**Metabolic rate**	**Growth rate**	**Net calcification rate**
Single Temperature (high)		+35.2% (47.0, 22.2)	−0.2% (−0.8, 0.1)	−12.3% (257, 191.1)
Single pH (low)		−1.8% (−24.0, 24.0)	+0.1% (−0.4, 0.6)	+605.2% (249.5, 1319.6)
Single Oxygen (low)		+53.5% (18.1, 109.1)	+0.3% (−0.2, 0.8)	−145% (379.2, 45.4)
Combined Temperature (high)	Observed	+19.6% (−4.8, 43.7)	+0.5% (0, 0.9)	+239.9% (−12.8, 544)
+ pH (low)	Expected	+33.4% (−12.2, 81.4)	−0.1% (−1.2, 0.7)	+592.9% (−7.5, 1510.7)
	Effect	Additive	Additive	Additive
Combined Temperature (high)	Observed	+74.8% (49.0, 98.1)	+0.3% (−0.2, 0.9)	−56.9% (−370.8, 167.1)
+ Oxygen (low)	Expected	+88.7% (29.9, 166.5)	+0.1% (−1, 0.9)	−157.6% (122.2, 236.5)
	Effect	Additive	Additive	Additive
Combined pH (low)	Observed	−2.6% (−21.2, 22.5)	−0.3% (−0.8, −0.1)	+280.3% (41.9, 738.7)
+ Oxygen (low)	Expected	+51.7% (−5.9, 133.1)	+0.4% (−0.6, 1.4)	+459.9% (628.8, 1365)
	Effect	Additive	Antagonist	Additive
Combined pH (low)	Observed	+64.9% (24.1, 115.4)	+0.3% (−0.2, 0.7)	+451.5% (128.0, 1274.3)
+ Oxygen (low)	Expected	+86.9% (5.9, 190.5)	+0.2% (−1.4, 1.5)	+447.6% (371.8, 1556.1)
+ Temperature (high)	Effect	Additive	Additive	Additive

Adjusted bootstrap means (95% confidence intervals) of observed and expected additive responses (null model) are shown.

control pH conditions. These higher growth and calcification rates were supported by elevated metabolic and ingestion rates, the over-expression of CHS and the increment in the periostracum production that avoided shell dissolution (Ramajo, Marbá, *et al.*, 2016). Although in our study, CHS and periostracum analyses were not performed, we also registered an elevated net calcification rate under low pH conditions at 14°C. However, no associated changes in growth rates were observed. Higher calcification rates without growth changes could indicate that under short-term periods of acidic conditions (as observed during upwelling) and an equal energy budget (no changes in metabolic rates), *A. purpuratus* juveniles prioritize shell calcification over shell growth. Indeed, maintaining extracellular pH independently of environmental conditions is crucial to avoid hypercapnia and its subsequent lethal effects (Hendriks *et al.*, 2015). Thus, enhancing calcification seems to be a valuable biological mechanism to avoid the negative effects of shell dissolution and hypercapnia imposed by acidic conditions (Cummings *et al.*, 2011; Waldbusser *et al.*, 2015; Ramajo, Marbá, *et al.*, 2016).

At coastal habitats influenced by upwelling, pH co-varies with temperature. Under low pH conditions, increasing temperatures can have additive, synergistic, or antagonistic effects on several physiological processes (Pörtner and Farrell, 2008). This variability makes difficult to quantify the precise nature of the interactions that temperature shows with other environmental drivers, as well as extrapolate among species (Basso *et al.*, 2015). In our study, temperature and pH showed additive effects on metabolic, growth, and calcification rates. Metabolic rates were higher at 18°C than at 14°C under acidic conditions sustaining higher growth rates, but unchanged calcification rates. Temperature, like pH, has also a significant effect on bio-mineralization and calcification processes by determining the type of mineral secreted (Ramajo *et al.*, 2015) or the shell microstructure (width, thickness, and the angle of crystals) (Milano *et al.*, 2017). At 18°C, in our experiment, calcification rates maintained unmodified under acidic conditions, which could be related to the fact that this species does not have physiological strategies to cope with acidic conditions (Ramajo, Marbá, *et al.*, 2016, this study) under high temperatures. It this relevant to note that, at Tongoy Bay, low pH (or hypoxic) conditions under high temperatures are not commonly registered. This confirms the good acclimation of Chilean scallops to the set of specific environmental conditions imposed by upwelling (cold, acidic, and hypoxic). These results have significant implications in the context of future climate change and provide us valuable information about how this species might be affected by the future climate scenarios where is expected that intensified upwellings will be coupled with more frequent ENSO events (see Bednaršek *et al.*, 2018).

Combined effects of oxygen and pH

Both hypoxia and low pH conditions constrain organisms' physiology being mediated by thermal conditions (Pörtner *et al.*, 2005; Tripp *et al.*, 2017; Fontanini *et al.*, 2018). Indeed, we observed that at 14°C, pH and oxygen had an interactive (antagonist) effect on the *A. purpuratus* growth, while at 18°C the effect was additive. This interaction variability has been previously recorded for other species inhabiting upwelling systems (Bednaršek *et al.*, 2018; Boch *et al.*, 2017), but more importantly it is indicating that the consequences of combined hypoxic and acidic conditions are usually more severe than those observed for individual drivers (Sui *et al.*, 2016). In our study, *A. purpuratus* juveniles at 14°C and short-term (11 days) hypoxic and acidic conditions maintained unchanged their metabolic rates, in agreement with other species that inhabit upwelling systems (see Steckbauer *et al.*, 2015). Under similar energy budget, we detected a trade-off between growth and calcification (a significant decrease in growth rates was accompanied by no changes in calcification) corroborating our previous hypothesis about that *A. purpuratus* under acidic conditions, and independently of oxygen concentrations, favour shell calcification which could avoid a potential hypercapnia (see above). At 18°C, this trade-off between growth and calcification was not evident, probably due to the higher metabolic rates that allowed maintain similar or increased growth and calcification rates. These results (at 14°C and 18°C) corroborate the important link between the organism's energy budget and the expression of physiological trade-offs and the expression of biological mechanisms to cope with environmental conditions. However, a better performance in metabolic, growth, and calcification rates under hypoxic and acidic conditions and increasing temperatures had an additional cost in the fitness of *A. purpuratus* (discussed below).

Effect of temperature on mortality

In our study, after exposing *A. purpuratus* juveniles for 11 days to the average (14°C) and maximum temperatures (18°C) that this species experiences in its native habitat (Tongoy Bay), significant and higher mortalities were recorded at 18°C than at 14°C. Independently of pH and oxygen concentrations, mortality was almost duplicated at 18°C (>20%) than at 14°C (near 10%) with a clear trend to increase over time. The tropical/sub-tropical origin of *A. purpuratus* makes this species, by definition, highly adapted to high temperatures (Waller, 1969), indeed higher larval survivorship and elevated landings during El Niño events at Peruvian coasts have been registered (Arntz *et al.*, 2006). However, in other geographical areas of the HCS where this species is found, high mortality events have been attributed to high temperatures (Tarazona *et al.*, 2007). The differences found between our study and previous ones may be attributed to the important role of thermal exposure history which establishes if a species is warm or cold-adapted (Araújo *et al.*, 2013; Gaitán-Espitia *et al.*, 2014). For example, it is known that calcifying species, such as corals, living under upwelling conditions are less tolerant to increasing temperatures than those inhabiting non-upwelling areas (D'Croz and Maté, 2004). Similarly, our results revel that the specific *A. purpuratus* population living at Tongoy Bay, which has historically been influenced by upwelling conditions, seems to be better adapted to colder than warmer conditions.

Relative gene expression

HSP70 and Ferritin protein expression (and their coding genes) confers tolerance to multiple environmental drivers such as temperature, pH, anoxia/hypoxia, or heavy metals (Feder and Hofmann, 1999; Kregel, 2002; Larade and Storey, 2004; Zapata *et al.*, 2009). Previous studies on *A. purpuratus* have shown that under elevated temperatures and hypoxic conditions, *HSP70* gene expression is increased in different ontogenic stages (Brokordt *et al.*, 2015). Until now the expression of *HSP70* and FERRITIN genes on *A. purpuratus* under acidic conditions has not been evaluate on *A. purpuratus*. In our study, we did not observe any

statistically significant changes in the *HSP70* and *FERRITIN* gene expression across experimental treatments after 11 days. Two hypotheses (that are not necessarily opposite) can be suggested to explain these results: (i) there were rapid gene responses that we could not detect under our experimental settings (Clark and Peck, 2009), and/or (ii) *A. purpuratus* juveniles retrieved from an upwelling zone are tolerant to changes in pH, temperature, and oxygen in agreement with what has been observed for the other physiological responses studied. Indeed, Brokordt *et al.* (2009) reported no changes in the expression of stress proteins which was attributed to acclimatization or adaption processes for *A. purpuratus*.

In summary, a good physiological performance and fitness were observed under experimental conditions that simulated upwelling conditions. These observations confirm that *A. purpuratus* juveniles are acclimated to short-term colder, acidic, and hypoxic conditions (Aguirre-Velarde *et al.*, 2016; Ramajo, Marbá, *et al.*, 2016). In addition, our study shows the significant role that upwelling conditions have to govern the magnitude and direction of physiological responses and the fitness of *A. purpuratus*, by favouring the expression of trade-offs. Although our study has limitations in terms of the short exposure time, it provides novel physiological knowledge for this social-economically and ecologically important species of the HCS. Future studies need to consider long-term effects in the context of intensified upwelling and more frequent ENSO events. Nevertheless, our results provide a more reliable information that should allow develop better management practices for this species and its industry to the future climate conditions.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

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