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Combined effect of pCO_2 and temperature levels on the thermal niche in the early benthic ontogeny of a keystone species

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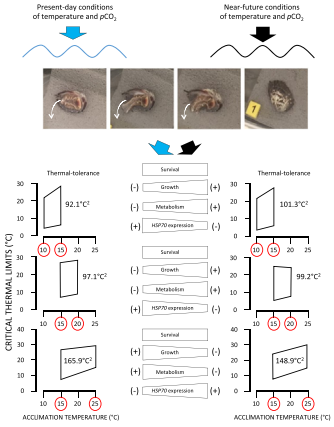
HIGHLIGHTS

- At 20 °C at both pCO_2 levels similar thermal tolerance polygons were obtained.
- Acidification expanded the thermal tolerance polygons at 10 °C.
- Acidification reduced the thermal tolerance polygons at 25 °C.
- Regardless of the pCO_2 levels, growth increased from 10 °C to 20 °C acclimation
- Regardless of the pCO_2 levels, growth was reduced when individuals were acclimated to 25 °C

GRAPHICAL ABSTRACT

Laboratory experiments examined the effect of near-future changes in temperature (cooling and warming) and elevated pCO_2 levels (acidification) on small juveniles of the keystone predator species *Concholepas concholepas* using self-righting success as end points for thermal tolerance. Survival, metabolism and *HSP70* transcription were also investigated. Extreme cooling and warming under near-future pCO_2 levels reduced the partial thermal tolerance polygons, growth and metabolism whilst increased *HSP70* transcription in comparison to present-day conditions. Although survival was not affected, the results indicate that near-future conditions of cooling or warming may potentially disrupt the performance and narrow the distribution of this species with potential ecological and economic consequences.



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ABSTRACT

We evaluated the effects of projected, near future ocean acidification (OA) and extreme events of temperature (warming or cooling) on the thermal tolerance of *Concholepas concholepas*, a coastal benthic keystone species. Three separate trials of an experiment were conducted by exposing juvenile *C. concholepas* for 1 month to one of two contrasting pCO_2 levels (~500 and ~1200 μatm). In addition, each pCO_2 level was combined with one of four temperature treatments. The control was 15 °C, whilst the other temperatures were 10 °C (Trial 1), 20 °C

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(Trial 2) and 25 °C (Trial 3). At the end of each trial, we assessed Critical Thermal maximum (CTmax) and minimum (CTmin) via self-righting success, calculated partial thermal tolerance polygons, measured somatic growth, determined transcription of Heat Shock Proteins *70* (*HSP70*) and measured oxygen consumption rates. Regardless of pCO_2 level, *HSP70* transcript levels were significantly higher in juveniles after exposure to extreme temperatures (10 °C and 25 °C) indicating physiological stress. Oxygen consumption rates increased with increasing temperature from 10 °C to 20 °C though showed a decrease at 25 °C. This rate was not affected by pCO_2 or the interaction between temperature and pCO_2. Juveniles exposed to present-day and near future pCO_2 levels at 20 °C showed similar thermal tolerance polygonal areas; whilst changes in both CTmin and CTmax at 25 °C and 10 °C caused narrower and broader areas, respectively. Temperature affected growth, oxygen consumption and *HSP70* transcription in small juvenile *C. concholepas*. Exposure to elevated pCO_2 did not affect thermal tolerance, growth or oxygen consumption at temperatures within the thermal range normally experienced by this species in northern Chile (15-20 °C). At elevated pCO_2 conditions, however, exposure to warmer (25 °C) or colder (10 °C) temperatures reduced or increased the thermal area, respectively. This study demonstrates the importance of examining the thermal-tolerance edges to better understand how OA and temperature will combine to physiologically challenge inter-tidal organisms.

1. Introduction

Since the beginning of the industrial revolution, increasing atmospheric CO_2 concentrations have caused two important phenomena in the World's oceans: an increase in seawater temperature, known as ocean warming (OW), and a decrease in seawater pH, known as ocean acidification (OA) (Levitus et al., 2005; Caldeira and Wickett, 2003). At the local scale, however, these general trends can be more complex with coastal waters experiencing periods of cooling (OC) as well as warming. For example, from 1979 to 2006 in the north-central coast of Chile, waters cooled at a rate of 0.2 °C per decade, linked to the intensification of the South Pacific anticyclone as a consequence of global warming (Falvey and Garreaud, 2009). During the same period, there was an increased frequency of episodic warming events due to El Niño-like conditions in response to increasing concentrations of greenhouse gases (Timmermann et al., 1999). This suggests that marine invertebrates inhabiting oceanographically dynamic areas such as the north-central coast of Chile will need to cope with a complex environment of fluctuating temperatures along with changing seawater pH levels in the future. In order to predict the likely impact of these variable environmental conditions on key marine organisms, we need a greater understanding of the combined effects of warming and acidification on organism performance.

The critical thermal minimum and maximum (CTmin, CTmax) of organisms are commonly measured to assess the lower and upper limits of the temperature tolerance window. Beyond these temperatures, performance and activity rapidly decrease and chances of mortality increase (Huey and Stevenson, 1979). In marine ectotherms, those two temperatures are thresholds beyond which organisms are unable to supply sufficient oxygen to metabolically demanding tissues (Pörtner, 2010). Determining CTmax and CTmin for organisms at different exposure temperatures provides the endpoint data needed to construct CTM-polygons (areas, $°C^2$ of cold and warm temperature tolerance at two or more exposure temperatures). These polygons define the fundamental (thermal) niche of a species (Bennett and Beitinger, 1997; Magnuson et al., 1979) or the intrinsic thermal tolerance zone (i.e. tolerance independent of previous thermal acclimation history) as well as the upper and lower acquired tolerance zones (i.e. tolerance gained through acclimation) (Beitinger and Bennett, 2000) needed to gain a cause-and-effect understanding of how temperature impacts trophodynamic structure and function (Pörtner, 2008) including fluctuations in the size and productivity of populations (Eme and Bennett, 2009). Thermal tolerance polygons not only provide a useful physiological metric to compare species (Beitinger and Bennett, 2000; Eme and Bennett, 2009; Lattuca et al., 2018) but have also been used to construct partial dynamic temperature tolerance polygons for sea urchins acclimated to different temperatures and fractions of dissolved or aqueous carbon dioxide in seawater (hereafter pCO_2 levels) (Manríquez et al., 2019a).

The muricid *Concholepas concholepas* (known in Chile as "loco") is the main target of local fishers (Castilla, 1999; Leiva and Castilla, 2002). Moreover, this species is a keystone rocky shore predator (Castilla, 1988; Castilla, 1999; Castilla and Paine, 1987) characteristic of the southeastern coast of the Pacific Ocean (Manríquez and Castilla, 2018). The current bio-geographical distribution of *C. concholepas* extends from Islas Lobos de Afuera in Perú (6° S) to Cape Horn (55° S) in southern Chile (Stuardo, 1979; Castilla and Guiñez, 2000). Across this ~50° range in latitudes, this species is likely to face a myriad of temperature and pCO_2 levels (Vargas et al., 2017) and to have developed the physiological capacity to cope with such large environmental variation. Thermal anomalies linked with El Niño conditions do not seem to affect adult populations of benthic invertebrates including *C. concholepas* inhabiting the south-central Chilean coast (Castilla and Camus, 1992; Moreno et al., 1998). However, this is not the case in northern Chile (18° S to 21°S) where warming due to the 1982/83 El Niño was associated with reductions in the population size of adult *C. concholepas* (Castilla and Camus, 1992; Soto, 1986). It is important to highlight, however, that those reductions may also have been caused by increased fishing effort occurring in the years following that particular 1982/83 El Niño (Bustamante and Castilla, 1987; Castilla and Camus, 1992). Moreover, failures in the recruitment of *C. concholepas* to intertidal areas of southern Chile were associated with changes in wind patterns in response to El Niño events (Moreno et al., 1998).

The capacity and time needed for self-righting has been used as a reliable stable, mechano-behavioral trait that can be used as an indicator of pCO_2 and thermal stress in *C. concholepas* (Manríquez et al., 2016). Similarly, upregulation of *HSP70*-like proteins has proven to be a useful molecular indicator of thermal stress and population-specific differences in acclimation capacity of *C. concholepas* to pCO_2 (Lardies et al., 2014). Despite this apparent broad physiological tolerance of *C. concholepas*, OA has been demonstrated to disrupt its ability to detect predators (Manríquez et al., 2014), reduce growth in terms of peristomal lenght, wet mass and buoyant weight (Manriquez et al., 2016), and increase the speed of self-righting (Manríquez et al., 2016), but has no effect on shell thickness (Manriquez et al., 2016). Moreover, the combined effects of OA and OW significantly altered the lateralization of small juveniles (Domenici et al., 2017). However, no previous study has incorporated both organismal- (survival, growth, calcification and critical thermal range) and cellular- (*HSP70*) level responses to changes in temperature and pCO_2 levels on benthic stages of this keystone species.

The present study examined the combined impacts of exposure to increased or decreased temperature at contrasting pCO_2 levels on the survival, growth (i.e. shell length, buoyant weight and wet mass), metabolism (i.e. oxygen consumption rates), thermal tolerance window (i.e. CTmin and CTmax via self-righting success) and thermal stress response (HSP transcription) in small juvenile *C. concholepas*. Prior to the

present study, the only available information for *C. concholepas* suggests that elevated pCO_2 levels induce changes in the relative expression of *HSP70*-like genes and increase the metabolic rate of northern and southern populations of this species (Lardies et al., 2014). In contrast to the study by Lardies et al. (2014), our current study aims to relate the thermal response of this species to global stressors such as pCO_2 and temperature based on the real expression of heat shock proteins (*HSP70*). By conducting three standard trials, we examined four temperatures (10 °C, 15 °C, 20 °C and 25 °C) and two pCO_2 (~500 μatm and ~1200 μatm) levels (Table 1). We chose 15 °C and 20 °C as they represent the annual mean temperature during the austral winter and summer months, respectively, at the site from which the test organisms were collected (Manríquez et al., 2018) while 10 °C and 25 °C represent extreme cold and warm temperatures, respectively. The 5 °C warming treatment represents the expected mean temperature increase for coastal waters of northern Chile during intense El Niño events (Torres et al., 2003) and also represents the amount of warming projected to occur by 2100 from climate change in the South Pacific Eastern Boundary Current (Timmermann et al., 1999). The, 5 °C cooling treatment represents the extent of cooling projected over a longer period (the next 250 years) for northern Chile (Falvey and Garreaud, 2009). In terms of pCO_2 levels, the higher level (~1200 μatm) represents the worst-case scenario for atmospheric CO_2 levels at the beginning of the next century (Meinshausen et al., 2011; IPCC, 2014) and the control level (~500 μatm) is the average coastal values in the collection region at Antofagasta (Vargas et al., 2017). Our trials were performed on small juvenile *C. concholepas* (<2.5 cm), because they are not only the most abundant (Manríquez et al., 2009; Manríquez and Castilla, 2018) but also the most vulnerable life stage of this species in mid-low rocky intertidal and shallow subtidal habitats; juveniles are susceptible to being attacked by visual predators and dislodged by wave action. Repeatedly conducting trials making individual-level measurements on groups of different juveniles provided a better population-level estimate of thermal niche (Gvoždík, 2018) and how it may be modified under near future levels of pCO_2 and temperature. Our hypothesis was that elevated, chronic pCO_2 levels along with either warming or cooling will have a synergistic effect on the thermal physiology of *C. concholepas* collected in northern Chile, altering their thermal stress response in comparison to present-day levels.

Table 1
Average (± SE) treatment conditions of the seawater used to maintain small juvenile *Concholepas concholepas* during the acclimation and then under the exposure period (60 days) in which they were reared under contrasting and chronic temperature and pCO_2 levels. Present and high pCO_2 levels are based on rate of change in pH projected under a "business-as-usual" scenario (RCP8.5) of atmospheric CO_2 for the beginning of the next century. See Meinshausen et al., 2011 for further details.

Treatments	pH at 25 °C (pH units)	Temperature (°C)	AT (μmol kg^{-1})	pCO_2 in situ (μatm)	$[CO_3^{2-}]$ in situ (μmol kg^{-1}SW)	Salinity	Ω calcite	Ω aragonite
10 °C vs. 15 °C, (Trial 10 °C)								
Acclimation period								
Natural seawater	7.71 (±0.02)	15.23 (±0.13)	2222.05 (±28.66)	637.59 (±35.76)	106.04 (±7.06)	34.48 (±0.08)	2.54 (±0.17)	1.63 (±0.11)
Exposure period								
Natural seawater	7.81 (±0.02)	13.36 (±0.13)	2272.86 (±6.72)	458.48 (±33.40)	132.42 (±5.81)	34.04 (±0.06)	3.17 (±0.14)	2.03 (±0.09)
10P	7.75 (±0.01)	10.42 (±0.15)	2204.49 (±17.34)	460.68 (±14.31)	113.19 (±3.72)	34.55 (±0.06)	2.70 (±0.09)	1.72 (±0.06)
10F	7.40 (±0.03)	10.22 (±0.16)	2192.97 (±21.25)	1195.15 (±68.96)	53.08 (±4.16)	34.52 (±0.10)	1.27 (±0.10)	0.81 (±0.06)
15P	7.73 (±0.01)	15.03 (±0.06)	2169.96 (±37.59)	585.95 (±20.09)	107.69 (±4.43)	34.24 (±0.06)	2.58 (±0.11)	1.66 (±0.07)
15F	7.41 (±0.02)	15.09 (±0.04)	2080.93 (±40.16)	1299.40 (±62.46)	53.28 (±3.66)	34.38 (±0.04)	1.28 (±0.09)	0.82 (±0.06)
15 °C C vs. 20 °C, (Trial 20 °C)								
Acclimation period								
Natural seawater	7.95 (±0.02)	17.03 (±0.25)	2198.49 (±27.77)	350.35 (±19.06)	170.19 (±7.06)	34.54 (±0.05)	4.07 (±0.17)	2.63 (±0.11)
Exposure period								
Natural seawater	8.06 (±0.02)	17.99 (±0.31)	2259.64 (±3.88)	327.36 (±45.37)	201.55 (±13.20)	34.47 (±0.03)	4.83 (±0.32)	3.12 (±0.21)
15P	7.83 (±0.01)	15.55 (±0.06)	2227.87 (±11.95)	538.49 (±37.31)	126.93 (±4.19)	34.43 (±0.07)	3.04 (±0.10)	1.95 (±0.06)
15F	7.45 (±0.01)	15.22 (±0.04)	2144.46 (±27.86)	1342.63 (±73.25)	55.18 (±2.26)	34.61 (±0.11)	1.32 (±0.05)	0.85 (±0.03)
20P	7.88 (±0.01)	20.32 (±0.06)	2145.78 (±27.24)	543.48 (±33.25)	136.88 (±4.19)	34.57 (±0.13)	3.28 (±0.10)	2.14 (±0.06)
20F	7.52 (±0.01)	20.46 (±0.06)	2183.45 (±34.31)	1412.11 (±77.66)	66.74 (±2.87)	34.21 (±0.09)	1.61 (±0.07)	1.04 (±0.04)
15 °C vs. 25 °C, (Trial 25 °C)								
Acclimation period								
Natural seawater	8.02 (±0.04)	17.46 (±0.55)	2265.08 (±4.62)	294.98 (±25.02)	205.47 (±9.84)	34.35 (± 0.13)	4.92 (±0.24)	3.18 (±0.15)
Exposure period								
Natural seawater	7.91 (±0.04)	15.10 (±0.20)	2268.44 (±6.08)	381.86 (±37.32)	162.37 (±12.24)	34.63 (± 0.03)	3.88 (±0.29)	2.49 (±0.19)
15P	7.79 (±0.02)	15.17 (±0.06)	1956.97 (±116.74)	455.31 (±36.88)	109.42 (±7.43)	34.66 (± 0.13)	2.62 (±0.18)	1.68 (±0.11)
15F	7.47 (±0.02)	15.13 (±0.07)	2137.29 (±50.66)	1163.46 (±65.88)	61.12 (±4.14)	34.38 (± 0.14)	1.46 (±0.10)	0.94 (±0.06)
25P	7.94 (±0.01)	24.42 (±0.20)	2172.74 (±27.42)	509.48 (±32.69)	160.11 (±7.43)	34.17 (± 0.16)	3.90 (±0.16)	2.55 (±0.11)
25F	7.59 (±0.02)	24.46 (±0.16)	2096.23 (±86.97)	1183.37 (±65.35)	80.46 (±4.58)	34.58 (± 0.28)	1.94 (±0.11)	1.28 (±0.07)

Carbonate system parameters (i.e. total alkalinity, Ω calcite, Ω aragonite, pH), temperature and salinity were quantified once a week in each experimental treatment on three randomly chosen experimental units (replicates). The parameters of the natural seawater used to renew the reservoir tanks during the experiments were measured twice a week in near-shore water samples collected during daytime hours and therefore the average temperature did not include day/night temperature variations. 10P, present-day levels (500 μatm) of CO_2 at 10 °C; 10F, future levels (~1200 μatm) CO_2 at 10 °C; 15P, present-day levels (~500 μatm) of CO_2 at 15 °C; 15F, future levels (~1200 μatm) CO_2 at 15 °C; 20P, present-day levels (500 μatm) of CO_2 at 20 °C; 20F, future levels (~1200 μatm) of CO_2 at 19 °C; 25P, present day levels (~500 μatm) of CO_2 at 25 °C; 25F, future levels (~1200 μatm) of CO_2 at 25 °C.

2. Methods

2.1. Collection of the study organisms

Small juvenile *Concholepas concholepas* were collected from low rocky intertidal habitats at Caleta Coloso in northern Chile, Antofagasta (23°45′S; 70°27′W) and transported to the laboratory at Coquimbo (29° 57′S; 71° 21′W) where all OA × OW/OC work was conducted. Prior to each trial (n = 3), juveniles were acclimated to laboratory conditions for 1 to 2 months (see below). They were maintained in flow-through seawater tanks at the average temperature measured in the field at the collection site (16 °C; June to November, Manríquez PH unp. data) and fed ad libitum with their preferred prey item, the mussel *Semimytilus algosus* (Méndez and Cancino, 1990). The trials were conducted with *C. concholepas* collected in two sampling campaigns to match the recruitment season at Antofagasta: November 2017 (first and second trials) and June 2018 (third trial). Due to time and space constraints, the first two trials (15 °C vs. 20 °C and 15 °C vs. 25 °C) were conducted in series. Hence, the individuals of the second trial had a longer laboratory acclimation period (2-mo) and a larger initial size (Table 2). In each of the trials, however, growth, thermal tolerance and thermal stress responses were always assessed after a 1-mo exposure to OA and OW/OC while oxygen consumption rate was always assessed after a 2-mo exposure. Although the three trials included the same (15 °C) control temperature, they were considered independent trials and the data were not pooled.

2.2. CO_2 mixing system

A flow-through CO_2 mixing system similar to that described by Torres et al. (2013) was used to manipulate pCO_2 levels in the rearing tanks. This system was complemented with two, independent temperature-controlled seawater units (for further details see González et al., 2018; Leiva et al., 2018; Manríquez et al., 2019a). Briefly, mass flow controllers (Aalborg ®, model GFC) were used to blend ambient air with pure CO_2 gas to obtain CO_2-enriched air to approximately 1200 μatm for the future pCO_2 condition and 500 μatm for the present day condition, which was then bubbled into four large (230-L), independent plastic reservoirs filled with 1.0 μm filtered seawater (FSW).

Table 2
Average (± SE; N) peristomal length (mm), wet mass (g) and buoyant weight (g) of the small juvenile *Concholepas concholepas* at the beginning of each trial along with the one-way ANOVA testing for differences between individuals assigned to the four treatment conditions in the three traits. Statistically significant results ($P < 0.05$) are indicated in **bold**.

Source	Size (± SE; N)	DF	MS	F	*P*
Trial 10 °C					
Peristomal length	15.569 (± 0.132; 48)	3	0.1816	0.205	0.892
Residuals		44	0.8840		
Wet mass	0.706 (± 0.018; 48)	3	0.0085	0.505	0.681
Residuals		44	0.0169		
Buoyant weight	0.225 (± 0.006; 48)	3	0.0007	0.367	0.777
Residuals		44	0.0016		
Trial 20 °C					
Peristomal length	10.188 (± 0.100; 48)	3	0.3183	0.693	0.561
Residuals		44	0.4591		
Wet mass	0.193 (± 0.006; 48)	3	0.0005	0.333	0.802
Residuals		44	0.0015		
Buoyant weight	0.058 (± 0.002; 48)	3	2.659×10^{-5}	0.199	0.897
Residuals		44	1.336×10^{-4}		
Trial 25 °C					
Peristomal length	19.705 (± 0.125; 48)	3	1.2435	1.730	0.175
Residuals		44	0.7186		
Wet mass	1.207 (± 0.024; 48)	3	0.0323	1.233	0.309
Residuals		44	0.0262		
Buoyant weight	0.389 (± 0.008; 48)	3	0.0059	1.873	0.148
Residuals		44	0.0032		

From these reservoirs, treated FSW was delivered to 20-L equilibration tanks to adjust water temperature and then further transported to each experimental unit (replicate) at a flow rate of ~1 L h^{-1}. The carbonate system parameters, i.e. total alkalinity (TA), pH, temperature and salinity were quantified in triplicate once a week in each experimental treatment on three randomly chosen tanks per treatment (Table 1). Total alkalinity was measured using an automated, open-cell titration system, described by Haraldsson et al. (1997) and the accuracy was verified using certified reference material (CRM) supplied by Andrew Dickson (Scripps Institution of Oceanography, San Diego, USA). The correction factor was approximately 1.002, corresponding to a difference of <5 μmol kg^{-1}. Seawater pH was measured inside a 25 mL closed cell at 25 °C using a pH meter (Metrohm 713) with a glass combined double junction Ag/AgCl electrode (Metrohm, 6.0219.100) calibrated using 8.089 Tris buffer (DOE [US Department of Energy], 1994) at 25 °C. Values of pH are reported on the total hydrogen ion scale (DOE, 1994). Temperature and salinity were measured using an Idronaut Ocean Seven CTD (model 304 Plus). The pH, TA, temperature and salinity data were used to calculate pCO_2 and CO_3^{2-} and seawater saturation stages for calcite and aragonite using CO_2SYS program for Microsoft Excel (Lewis and Wallace, 1998) set with Mehrbach solubility constants (Mehrbach et al., 1973) as refitted by Dickson and Millero (1987).

2.3. Experimental rearing

After the acclimation period to the laboratory, 76 small juvenile *C. concholepas* were randomly placed into one of four exposure treatments. Due to space and time constraints, trials were conducted in series, each lasting 1-mo and with the same control temperature (15 °C) but with different exposure temperatures (10 °C, 20 °C or 25 °C). The control temperature represented the annual average at the collection site. After juveniles were transferred to their conditioning tanks, water temperature was increased or decreased from ambient by ~1 °C day^{-1} until the treatment level was reached. In each trial at each temperature, juveniles were exposed to either ~500 μatm or ~1200 μatm CO_2 representing present (P) and near-future (F) conditions within each temperature level (Table 1). Each experimental unit consisted of an individual held in a plastic container filled with 0.6 L of FSW conditioned to the required pCO_2 level, and placed in a temperature-controlled water bath. Each experimental unit was covered with a plastic lid pierced with a drip-feed system to supply treatment FSW at a rate of ~1 L h^{-1} allowing 40 renewals per day. The treatment air supply was delivered to each experimental unit by silicone tubing, entering the rearing container through a second hole in the lid. A standard 1–200 μL pipette tip was inserted at the end of the tube. To maintain the required pCO_2 levels in the replicate containers, a continuous stream of either air (~500 μatm CO_2) or enriched CO_2 air (~1200 μatm) was bubbled through the pipette. Plastic gang valves were used to control the amount of air delivered to each experimental unit. The experimental unit had an outflow hole (~0.4 cm diameter) in the side of the container at ~8 cm above the base. In order to keep the outflow holes just above the water level in the water bath, the experimental units were placed on raised glass platforms. The small juvenile in each experimental unit was housed inside a spherical cage (~10 cm in diameter) to ensure that it was always below the water surface. The experimental units were maintained under a natural light regime (12:12 h light: dark and without control for gradual changes at dawn and dusk). Moreover, juveniles were transferred into a new, clean experimental unit (at the corresponding temperature and pCO_2 level) one time per week. Dead mussels were replaced by fresh ones as needed at least once a week. At the end of the exposure period (1-mo), all the juveniles were measured and 12 individuals were randomly chosen from each treatment and used for the thermal tolerance measurements (see below). *HSP70* measurements were made on the remained seven juveniles from each treatment, which were rapidly deep frozen in liquid nitrogen and stored at −80 °C.

2.4. Survival and growth

Peristomal length (i.e. the maximal length at the shell aperture), wet mass and buoyant weight (as a proxy for shell calcification or dilution) of juvenile *C. concholepas* were measured at the start and end of each trial. After a 1-mo exposure to treatment conditions, growth was measured for each individual for which thermal tolerance and metabolism was subsequently measured (see below). Specific growth rate (percentage change per day) in terms of size, wet mass and buoyant weight was calculated. Peristomal length was measured using a digital Vernier calliper (0.01 mm precision). Juvenile mass was measured using an analytical balance (Adam AFA180 LC, 0.1 mg precision). Survival was constantly monitored for all individuals in each treatment in each trial until metabolic (oxygen consumption) rates were measured after 2-mo of treatment exposure.

2.5. Real-time quantitative PCR (RT-qPCR) analysis of HSP70 transcription

After 1-mo of treatment exposure, *HSP70* transcriptional level was measured in the complete soft tissues of juvenile *C. concholepas* maintained under the treatments described above (n = 7 per treatment). Total RNA was extracted from soft tissue of each individual using the TRIzol® Reagent (Invitrogen™, USA) method following the manufacturer's instructions. The RNA obtained was quantified with an Epoch spectrophotometer (BioTek, USA) and its intactness was verified by visual inspection of integrity of 28S and 18S rRNA bands in denaturing formaldehyde/agarose gel electrophoresis stained with SYBR® Safe (Invitrogen™, USA). Reverse transcription (RT) of RNAs from tissues was carried out with a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) and oligo-p $(dT)_{15}$ primer according to the manufacturer's protocol. The RT of RNAs was done in equi-proportions (i.e., from equal quantity of RNA) within all compared samples from each experimental series.

In order to isolate *HSP70* sequences from *C. concholepas*, primers were designed using inducible *HSP70* sequences from seven species of aquatic gastropods (GenBank Acc. No. HQ225739). Primers were 5′ ACATCGATGCCAACGGTATCC3′ (forward) and 5′-GCGTCGTTCACCATGC GCTCGA-3′ (reverse). PCR reaction contained 5 μL of cDNA and 0.2 μM (final concentration) of each primer, in a final volume of 20 μL. The initial denaturing time was 3 min, followed by 30 PCR cycles of 94 °C, 40 s; 55 °C, 40 s; and 72 °C, 40 s; and a final extension of 3 min at 72 °C. A 130-bp PCR product was obtained, purified and sequenced by the Sequencing Service of Macrogen Inc. (Korea). From obtained sequence primers for RT-qPCR reaction were designed with Primer Express v3.0 software (Applied Biosystems, USA) to have melting temperatures of 58 °C to 60 °C and generate PCR products of 111 bp. For *HSP70* the forward primer was 5′ATGCGCTCGATCTCCTCCTT3′; and the revers 5′TGCCAACGGTA TCCTCAACG3′. *EF-1* was used as endogenous control in order to normalize experimental results. This gene was previously validated as a housekeeping gene for *C. concholepas* by Chávez-Mardones et al. (2013). For *EF-1*, the forward primer was 5′TGACAGTTCAGAGCGACGAC3′; and the revers 5′CATCAAGTCGGTGGAGAAGC3′. RT-qPCR assays were performed in triplicate using Eco™ Real-Time PCR System (Illumina, San Diego, CA, USA). The 20 μL-volume reaction consisted of 10 μL 2× Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Rockford, IL, USA), 2 μL cDNA and 0.3 μM (final concentration) of each primer. Initial denaturation time was 10 min at 95 °C, followed by 40 PCR cycles of 95 °C, 15 s and 60 °C, 1 min. After the PCR cycles, the purity of the PCR product was checked by the analysis of its melting curve; the thermal profile for melting curve analysis consisted of denaturation for 15 s at 95 °C, lowered to 55 °C for 15 s and then increased to 95 °C for 15 s with continuous fluorescence readings. By serial dilution of cDNA, RT-qPCR efficiency was set to be between 95 and 110%. Efficiency of *HSP70* amplification was similar to that of the housekeeping gene (determined by slope calculation), so the comparative C_T method (also called the $\Delta\Delta C_T$ method; Livak and Schmittgen, 2001) was applied for relative quantification of *HSP70*. Experiments included seven biological replicates and three technical replicates per treatment.

2.6. Thermal tolerance

After 1-mo of exposure to each treatment, the Critical Thermal minimum (CTmin) and the Critical Thermal maximum (CTmax) was determined using the Critical Thermal Methodology (CTM) (Lutterschmidt and Hutchison, 1997). To determine CTmin (n = 6) and CTmax (n = 6), a juvenile was placed into a 1-L plastic beakers (10 × 12 cm) suspended within a 30-L insulated bath connected to a temperature-controlled, circulating water bath (Lab Companion RW-2025G). During the CTmin/CTmax trials, individuals were exposed to a constant rate of warming or cooling of ~0.05 °C min^{-1} (~3 °C h^{-1}; see Table S1 in the supplementary material) and continuously observed until they reached the end-point (see below). We chose this rate of change in temperature to emulate that recorded inside intertidal pools that remain as pockets of seawater when the tide ebbs at the collection site (Manríquez PH unp. data). Juveniles were placed individually in another 1-L plastic beaker filled with FSW at the corresponding temperature and pCO_2 levels and acclimated for 5 min under vigorous aeration with the corresponding mix of air and CO_2 to maintain the corresponding treatment levels. After each 1 °C change in water temperature, self-righting success and time to self-right was measured. Temperature and pH were monitored using a digital thermometer (PCE-T390 4-channel digital thermometer) and a portable pH-meter (Metrohm 913), respectively.

For the self-righting exercise, juveniles were carefully placed upside down in the middle of the containers at the edge of a sloping Plexiglas surface covered with a Safety Walk® 3 M anti-slip tape. Hence, the plane of the shell aperture was inclined by 30° with respect to the horizontal plane. This ensured that all individuals were in the same initial position and exposed to a similar degree of difficulty to self-right at the beginning of the CTmin/CTmax trials (Fig. 1a–b). Inability to self-right within 20 min was considered as the end-point since it involves a relatively simple procedure (Manríquez et al., 2016, 2017). Those individuals that were unable to self-right but displaying feet movement were given a second chance to self-right at the next measurement temperature. The CTmax and CTmin end-points were defined as the temperatures at which individuals first lost the ability to self-right within 20 min and could not self-right after being tested again at three successive (colder or warmer) temperatures. Time to self-right was measured with a digital stopwatch. After the end-point, each individual was returned to the treatment conditions and their capacity to recover was evaluated after 10 min and 2 days. Recovery was based on their ability to adhere to the substratum, self-right, and move their foot during a 20-min observation period. Two months after the end of the trials, all surviving individuals were returned to the collection site.

2.7. Partial thermal tolerance polygons

Upper and lower thermal tolerance for present-day and elevated pCO_2 and temperature exposure levels was calculated as the mean of the CTmax and CTmin, respectively. Partial thermal tolerance polygons were constructed for *C. concholepas* by connecting the CTmin and CTmax with CTM regressions to produce a quadrilateral figure. The area of each partial polygon was expressed in units of $°C^2$ with a 95% confidence interval (CI), which was calculated using the 95% CI of each CTmax/CTmin value. The area of the polygon was partitioned into three distinct zones (each also expressed with their 95% CI): an intrinsic tolerance zone (ITZ), bounded by upper and lower acquired tolerance zones (UAAZ and LATZ, respectively). These areas were obtained by dividing the polygons with horizontal lines originating at the intersection of the CTM regressions with vertical lines connecting the CTmin and CTmax values at each exposure temperature (Fangue and Bennett, 2003; Dabruzzi et al., 2012; Lattuca et al., 2018).

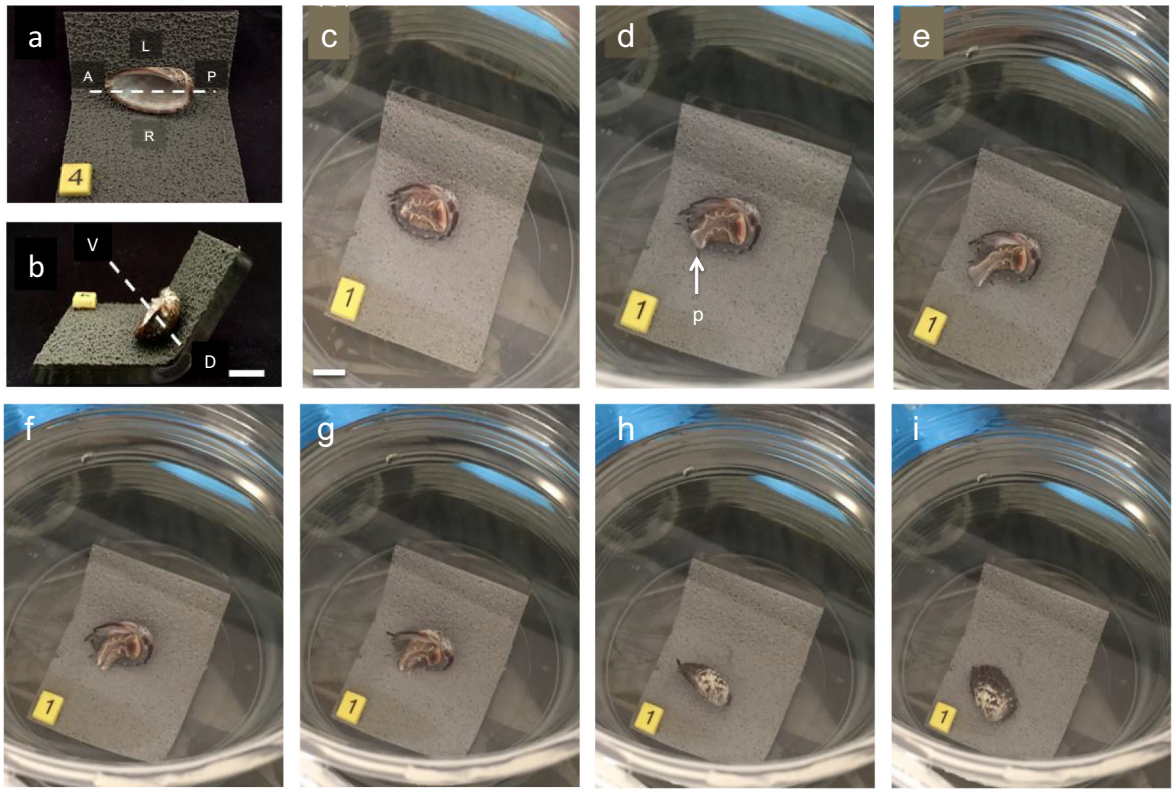

Fig. 1. *Concholepas concholepas*. Photographs of the experimental sloping Plexiglas surface (a–b) used in the self-righting measurements and the sequence showing the self-righting behavior of a small juvenile (c–i). In (a) a frontal view of the Plexiglas surface covered with a Safety Walk® 3M® anti-slip tape used to place the experimental individual at the beginning of the measurements. In (b) the experimental individual is view from its posterior end with the plane of the shell aperture parallel to the inclined plane in 30° with respect to the horizontal plane. In (b) and (c) the dashed line depicts the anterior-posterior and dorsal-ventral axes, respectively. A: anterior; P: posterior; L: left; R: right; V: ventral, D: dorsal. In (d) p: propodium. Scale bar is 1 cm.

2.8. Metabolic (oxygen consumption) rate

After a 2-month exposure to each treatment, the metabolic (oxygen consumption) rate of seven individuals from each treatment was measured. Those individuals were randomly chosen from those previously used in the CTM measurements. Selected individuals were unfed for 24 h in 1.0 μm FSW prior to the measurements. Individuals were placed within 50- or 100-mL glass respiration chambers (depending on the size of the individual) filled with 0.45 μm FSW at the respective treatment temperature and pCO_2 level. Oxygen sensor spots were fixed to the inner surface of the chambers with acetic acid curing silicone rubber. Respiration chambers were semi-immersed in an opaque Plexiglas box connected to a water bath set at the treatment temperature to ensure stable temperature conditions during measurements. The Plexiglass box was opaque to avoid changes in the metabolic rate associated with changes in artificial light (Manríquez et al., 2019b). A fiber-optic oxygen meter (Fibox, PreSens) was used for all oxygen consumption measurements. The fiber was placed perpendicularly to the outside surface of the bottle and the fluorescent signal of the sensor spots was read from outside the bottle. The outer surface of the chamber where the spot was located was just above the water level. A two-point calibration was performed using 0% (saturated Na_2O_3S solution) and 100% (air-bubbled seawater) O_2 saturation. Temperature was stabilized using a refrigerating and heating water bath circulator (Lab Companion RW-2025G). Each measurement lasted for at least 180 min, with the first 15 min removed to avoid possible effects of manipulation on metabolic rate. During this period, oxygen consumption rates were measured every 30–60 min to assess the rate of decline in oxygen concentration. To control for differences in body mass, oxygen consumption rates are reported as consumption per gram of body mass (g) and time (h). Care was taken to prevent oxygen levels from dropping below 70% of saturation. Background respiration was determined by measuring oxygen consumption in 3 similar chambers maintained without an individual, and this average value was subtracted from the experimental oxygen consumption rates. To avoid stress, during oxygen consumption measurements no mixing within the chambers was considered.

2.9. Statistical analysis

Peristomal length and weight (wet mass and buoyant) at the start of each trial was compared among treatments using a one-way ANOVA. Two-way ANOVAs, followed by a post hoc test (Tukey test), were used to evaluate the effect of pCO_2 and temperature on the specific growth rate, *HSP70* transcription, CTmin and CTmax (self-righting), and metabolic (oxygen consumption) rate. Normality of residuals was tested using the Shapiro-Wilk test and homogeneity of variances was tested using Levene test. Data analysis was conducted using R software (version 3.5.1; R Core Team, 2016). If the data were not normally distributed, the factors were examined after an aligned rank transformation (ART, Wobbrock et al., 2011).

3. Results

3.1. Survival and growth

At the beginning of each trial, the size (peristomal length) and weight (wet mass and buoyant) of small juvenile *C. concholepas* were not significantly different among the individuals assigned to the four treatment groups (Table 2). However, as a consequence of collecting juveniles later in the settlement season and due to the longer acclimation

period in the laboratory, individuals at the start of the second trial (15 °C vs. 25 °C) were larger than those at the start of the other trials (Table 2).

There was no mortality during the acclimation and exposure periods at 10 °C, 15 °C and 20 °C. At 25 °C, three individuals died during the third week of the exposure (~8% mortality). Compared to the control (15 °C), specific growth rate in size and wet mass increased significantly at warmer (20 °C and 25 °C) and decreased at cooler (10 °C) temperatures irrespective of pCO_2 level (Fig. 2; Table 3). The only significant effect of pCO_2 level was an increase in specific growth rate in length and wet mass, but not buoyant weight, under future (higher pCO_2) conditions in the 25 °C trial (Table 3; Fig. 2f). The interaction between pCO_2 and temperature was only significant in the 20 °C trial for one measure (buoyant weight) (Table 3). Within all temperature treatments, there was no significant effect of pCO_2 on specific growth rate (Fig. 2) except for wet mass in the 25 °C trial (Fig. 2; Table 3).

3.2. HSP70 transcriptional levels

In general, exposures to low (10 °C) or high temperatures (20 °C and 25 °C) significantly increased *HSP70* levels in small juvenile *C. concholepas* when compared to 15 °C; but the effect of pCO_2 depended on temperatures/trial (Table 4; Fig. 3). The pCO_2 level or its interaction with temperature did not significantly affect *HSP70* levels within the 10 °C trial (Table 4; Fig. 3a). Within the high-temperature trials (20 °C or 25 °C), pCO_2 significantly affected *HSP70* levels, but in opposite ways (Table 4; Fig. 3b, c). In comparison to low pCO_2, *HSP70* levels at high pCO_2 were elevated within the 20 °C trial, while these levels were lowered within the 25 °C trial. Also within the 25 °C trial, a significant interaction of temperature and pCO_2 on *HSP70* levels was observed; with a significant lower induction of transcripts in juveniles maintained under the highest temperature (25 °C) and high pCO_2, compared with those maintained at the same temperature but in present-day pCO_2 conditions (Table 4; Fig. 3c).

3.3. Thermal tolerance

Self-righting response in small juvenile *C. concholepas* was achieved in four consecutive phases. First, the foremost part of the foot or propodium elongated, next, it made contact with the substratum and pulled the upside-down individual to a position in which the major axis reached an angle that allowed the individuals to roll upright due to its own weight (Fig. 1, c–i). Failure to self-right occurred when the individual was not able to elongate the propodium or when the propodium was not able to touch the substratum. During CTmax and CTmin measurements, the average time to self-right at the exposure temperature was ~4 to 5 min across all treatments. As temperature increased (or decreased) above (or below) the exposure temperature, the movement of the foot decreased and then ceased completely at the end-point (self-righting failure).

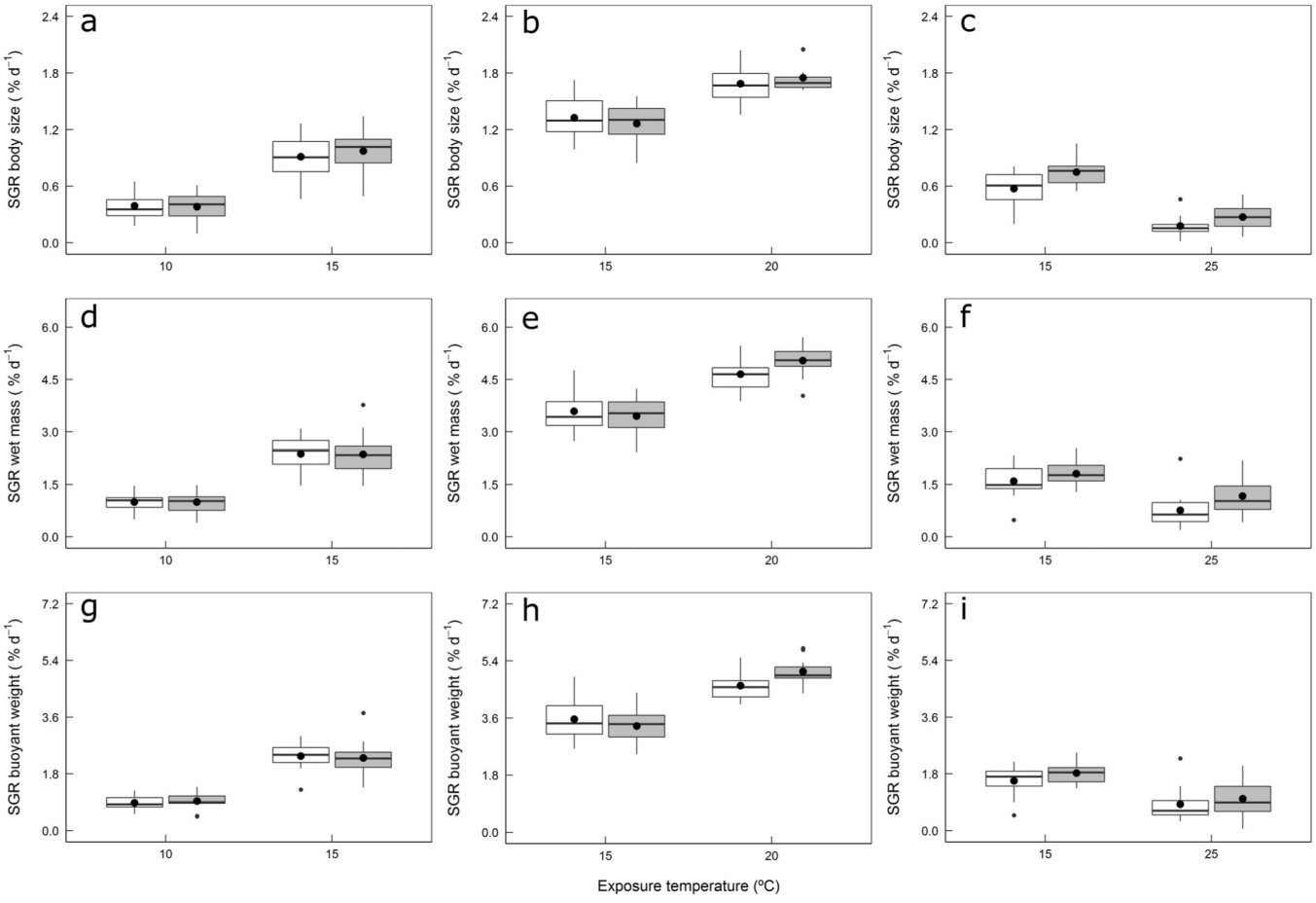

Fig. 2. The effect of different pCO_2 and temperature levels on specific growth rates in terms of size (a–c), wet mass (d–f) and buoyant weight (g–h) measured in small juvenile *Concholepas concholepas* during a 1 mo exposure period to contrasting pCO_2 (open bars: present-day or ~500 μatm; filled bars: elevated levels or ~1200 μatm) and temperature (10–25 °C) levels over a series of three experimental trials. Data are from the first (left panels), second (middle panels) and third (right panels) trials. Box plots display the 25th and 75th percentile (box), the median (solid line), the mean (block dot), the 10th and 90th percentiles (whiskers) and outliers (dots above or below whiskers). See Table 2 for further details of the analysis.

Table 3
Two-way ANOVA on row (†) or aligned rank transformed data (††) evaluating the effect 1-mo exposure period under contrasting temperature and pCO_2 levels (see Table 1 for details) on the specific growth rate in terms of size (length), and weight (wet and buoyant) of small juvenile *Concholepas concholepas* in the three trials. Statistically significant results ($P < 0.05$) are indicated in **bold**.

Source	DF	MS	F	*P*	Comparison
Trial 10 °C					
Peristomal length (†)					
Temperature	1	3.693	89.829	$\mathbf{3.4 \times 10^{-12}}$	15 > 10
pCO_2	1	0.008	0.203	0.654	
Temperature × pCO_2	1	0.015	0.364	0.549	
Residuals	44	0.041			
Wet mass (†)					
Temperature	1	22.641	106.470	$\mathbf{2.52 \times 10^{-13}}$	15 > 10
pCO_2	1	0.000	0.002	0.963	
Temperature × pCO_2	1	0.000	0.002	0.646	
Residuals	44	0.213			
Buoyant weight (†)					
Temperature	1	24.529	137.070	$\mathbf{4.15 \times 10^{-15}}$	15 > 10
pCO_2	1	0.000	0.000	0.993	
Temperature × pCO_2	1	0.038	0.213	0.646	
Residuals	44	0.213			
Trial 20 °C					
Peristomal length (††)					
Temperature	1	5764.083	73.952	$\mathbf{5.65 \times 10^{-11}}$	20 > 15
pCO_2	1	30.083	0.145	0.706	
Temperature × pCO_2	1	90.750	0.440	0.511	
Residuals	44	492.244			
Wet mass (†)					
Temperature	1	21.236	73.253	$\mathbf{6.45 \times 10^{-11}}$	20 > 15
pCO_2	1	0.194	0.669	0.4177	
Temperature × pCO_2	1	0.829	2.860	0.0979	
Residuals	44	12.756			
Buoyant weight (†)					
Temperature	1	22.913	76.637	$\mathbf{3.42 \times 10^{-11}}$	20 > 15
pCO_2	1	0.153	0.511	0.4784	
Temperature × pCO_2	1	1.321	4.418	**0.0413**	
Residuals	44	0.290			
Trial 25 °C					
Peristomal length (†)					
Temperature	1	2.275	100.156	$\mathbf{6.52 \times 10^{-13}}$	15 > 25
pCO_2	1	0.217	9.566	**0.0034**	F > P
Temperature × pCO_2	1	0.019	0.874	0.355	
Residuals	44	0.023			
Wet mass (††)					
Temperature	1	3675.00	29.243	$\mathbf{2.48 \times 10^{-6}}$	15 > 25
pCO_2	1	1244.33	6.908	**0.0117**	F > P
Temperature × pCO_2	1	184.08	0.907	0.346	
Residuals	44	508.176			
Buoyant weight (††)					
Temperature	1	3468.00	26.605	$\mathbf{5.71 \times 10^{-6}}$	15 > 25
pCO_2	1	456.33	2.350	0.132	
Temperature × pCO_2	1	6.75	0.033	0.857	
Residuals	44	530.942			

F and P represent future (~1200 μatm) and present (~500 μatm) pCO_2 levels, respectively.

Table 4
Two-way ANOVA evaluating the effect of a 1-mo exposure period under contrasting temperature and pCO_2 levels (see Table 1 for details) on the relative transcriptional levels of *HSP70* in small juvenile *Concholepas concholepas*. Statistically significant results ($P < 0.05$) are indicated in **bold**.

Source	DF	MS	F	*P*	Comparison
Trial 10 °C					
Temperature	1	1.202	9.235	**0.0065**	10 > 15
pCO_2	1	0.025	0.189	0.6688	
Temperature × pCO_2	1	0.202	1.554	0.2270	
Residuals	20	0.130			
Trial 20 °C					
Temperature	1	1.453	6.217	**0.0211**	20 > 15
pCO_2	1	5.261	22.51	**0.0001**	F > P
Temperature × pCO_2	1	0.051	0.220	0.6437	
Residuals	20	0.234			
Trial 25 °C					
Temperature	1	5.797	81.16	$\mathbf{1.8 \times 10^{-8}}$	Within 15: P = F Within 25: P > F
pCO_2	1	0.293	4.804	**0.0463**	Within P: 25 > 15 Within F: 25 > 15
Temperature × pCO_2	1	0.868	12.15	**0.0023**	
Residuals	20	0.071			

F and P represent future (~1200 μatm) and present (~500 μatm) pCO_2 levels, respectively.

The CTmin was, on average, between ~4 and ~9 °C (Fig. 4) and consistently increased with increasing acclimation/exposure temperature (Table 5; Fig. 4). In the second and third trials, no significant effect of the pCO_2 on CTmin was found (Table 5). The only significant effect of pCO_2 on CTmin was found at the lowest temperatures (Trial 1) (Table 5). The CTmax was between ~20 and 27 °C and was unaffected by exposure temperature and pCO_2 in the 20 °C trial (Table 5). However, in the same trial the interaction was significant (Table 5; Fig. 4). In the 25 °C and 10 °C trials, CTmax was significantly greater at the warmer temperature (Table 5; Fig. 4). No significant effect of pCO_2 nor an interaction between pCO_2 and temperature was found for CTmax (Table 5). After the CTmin and CTmax tests, full recovery was observed in all individuals and not mortality was recorded until the individuals were returned to the field two month later.

3.4. Partial thermal tolerance polygons

For each combination of CTmin and CTmax trial, the partial thermal tolerance zone (i.e. the total polygonal area, TPA), the area of the upper and lower acquired thermal tolerance zones (UATZ, LATZ) at the corresponding pCO_2 and temperature of the small juvenile *C. concholepas* are shown in Fig. 5. Considering mean values of CTmin and CTmax, TPA of *C. concholepas* exposed at the two different temperatures and pCO_2 levels was similar between present-day and future pCO_2 levels with 97.1 °C^2 (95% CI = 89.7–104.5 °C^2) and 99.2 °C^2 (95% CI = 90.6–107.7 °C^2), respectively, in the 15 °C–20 °C trial. When acclimated to 15 °C and 25 °C, the TPA was ~13% larger for individuals exposed to present-day pCO_2 levels (165.9 °C^2; 95% CI = 122.3–209.4 °C^2) in comparison to individuals at future pCO_2 levels (148.4 °C^2; 95% CI = 109.2–187.6 °C^2). In the 10 °C–15 °C, the TPA of individuals under near-future pCO_2 levels (101.3 °C^2, 95% CI = 87.7–114.8°C^2) was larger than those of individuals under present-day pCO_2 levels (92.1 °C^2, 95% CI = 76.1–108.1°C^2). In contrast to 10 °C and 20 °C trials, the TPA of *C. concholepas* in the 25 °C was ~10.5% smaller for individuals exposed to near-future pCO_2 levels when compared to individuals at present pCO_2 levels (see Table S2 in the supplementary material).

Considering the areal units of °C^2, *C. concholepas* displayed the largest ITZ, in relation to the TPA, under present-day (92.4%) and future (94.4%) levels of pCO_2 when the acclimation range was 15 °C and 20 °C. This was a consequence of the low ability to acquire additional cold (3.9–5%) and heat (1.7–2.5%) tolerance, respectively, through acclimatization to temperature and/or pCO_2 levels. On the other hand, when the acclimation range was 15 °C and 25 °C, the smallest ITZ was recorded under present-day (71.4%) and future (57.3%) pCO_2 levels due to heat (71.4–57.3%) and cold (22.6–27%) tolerance gained through acclimatization to temperature and/or pCO_2 levels.

3.5. Metabolic (oxygen consumption) rate

The metabolic rate of small juvenile *C. concholepas* was significantly increased with increasing temperature from 10 °C to 20 °C though showed a decrease at the highest temperature (25 °C). However, the metabolic rate was not affected by pCO_2 or the interaction between both factors (Table 6; Fig. 6). Metabolic rates measured at 25 °C was significant reduced in comparison to 15 °C (Table 6; Fig. 6).

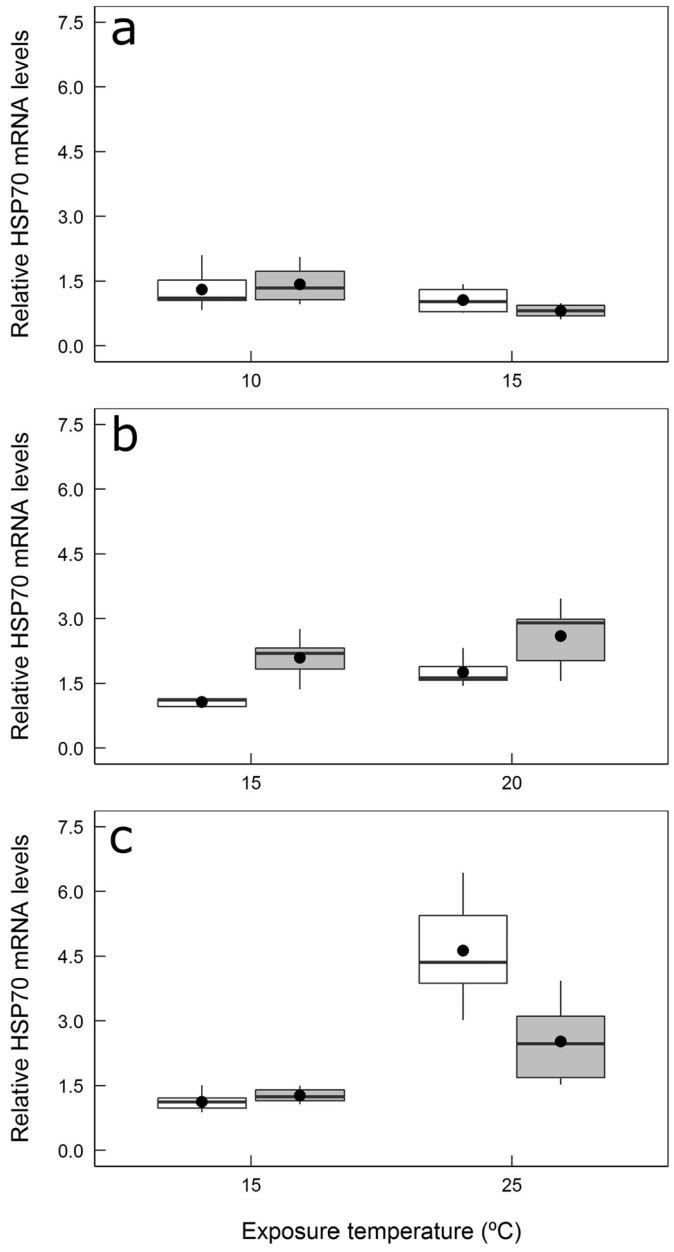

Fig. 3. *HSP70* relative transcriptional levels in small juvenile *Concholepas concholepas* after a 1-mo exposure period under contrasting pCO_2 and temperature levels. Experiment with juveniles exposed in three experimental series to (a) 10 °C; (b) 20 °C; and (c) 25 °C; each at present-day (open bars) and near future pCO_2 conditions (filled bars), are depicted. See Table 4 for further details of the analysis. See Fig. 2 for description of box plot information.

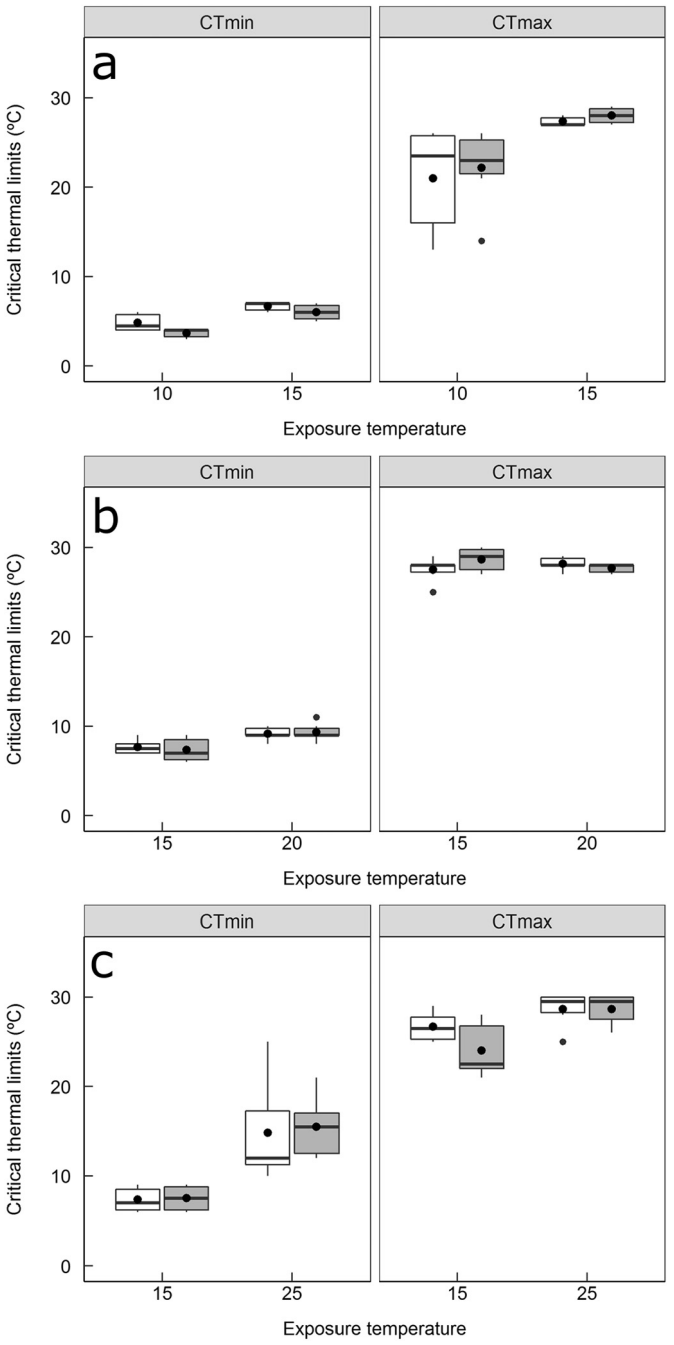

Fig. 4. The effect of different pCO_2 and temperature levels on CTmin and CTmax of small juvenile *Concholepas concholepas* after a 1-mo exposure to contrasting pCO_2 (open bars: present-day; filled bars: elevated levels) and temperature levels exposed in a series of three experimental trials: (a) 10 °C; (b) 20 °C; and (c) 25 °C. See Table 5 for details of the analysis. See Fig. 2 for description of box plot information.

4. Discussion

Understanding how multiple stressors such as temperature and acidification affect tolerance limits is fundamental to assess how future climate-driven conditions may cause physiological stress in marine organisms (Byrne, 2011). In the present study, we examined the combined effects of exposure to different (colder and warmer) temperature and elevated pCO_2 levels (acidification), on survival, growth (i.e. shell length, buoyant weight and wet mass), metabolism (i.e. oxygen consumption rates), thermal tolerance window (i.e. CTmin/CTmax via self-righting success) and thermal stress response (*HSP* transcription) in small juveniles of the keystone species *C. concholepas*. Regardless of the trial, the average trait response at 15 °C was different in comparison to responses at 10 °C, 20 °C and 25 °C. This suggests that, regardless of the initial size and the extension of the acclimation period, the trait responses at the control temperature in the three trials were similar and represents a stable response at that specific condition. Our results indicate that the survival of *C. concholepas* from a low-latitude population (~23°) was not impaired at any of the treatment conditions. A few individuals (approx. 8%), however, died during the acclimation period at 25 °C. Small juvenile *C. concholepas* are found in pools in low intertidal habitats where temperatures at ebb tide reach 25 °C in northern Chile at Antofagasta (Pulgar et al., 2007). Those high temperatures, however, are only short-term acute exposures as flood tides a few hours later reduce water temperatures. Small juvenile *C. concholepas* confined to tide pools hide below boulders likely as a thermal refuge until flood tides

Table 5
Two-way ANOVA on row (†) or aligned rank transformed data (††) evaluating the effect of an exposure period of a 1-mo under contrasting temperature and pCO_2 levels (see Table 1 for details) on CTmin and CTmax of small juvenile *Concholepas concholepas*. Statistically significant results ($P < 0.05$) are indicated in **bold**.

Source	CTmin					CTmax				
	DF	MS	F	*P*	Comparison	DF	MS	F	*P*	Comparison
Trial 10 °C (††)						(††)				
Temperature	1	770.67	43.09	**2.14×10^{-6}**	15 > 10	1	864.00	65.41	**9.84×10^{-8}**	15 > 10
pCO_2	1	294.00	7.27	**0.01**	P > F	1	60.17	1.28	0.27	
Temperature × pCO_2	1	10.67	0.21	0.66		1	0.17	0.003	0.95	
Residuals	20					20				
Trial 20 °C (†)						(††)				
Temperature	1	18.38	17.64	**0.0004**	20 > 15	1	48.17	0.89	0.36	Within 15: P = F Within 25: P = F
pCO_2	1	0.04	0.04	0.84		1	32.67	0.60	0.45	Within P: 20 = 15 Within F: 20 = 15
Temperature × pCO_2	1	0.38	0.36	0.55		1	228.17	5.22	**0.03**	
Residuals	20	1.04				20				
Trial 25 °C (†)						(††)				
Temperature	1	360.4	28.25	**3.35×10^{-5}**	25 > 15	1	486.00	15.09	**0.0009**	25 > 15
pCO_2	1	1.0	0.08	0.78		1	112.67	2.19	0.153	
Temperature × pCO_2	1	0.4	0.03	0.87		1	121.50	2.39	0.137	
Residuals	20	12.8				20				

F and P represent future (~1200 μatm) and present (~500 μatm) pCO_2 levels, respective.

restore cooler ocean temperature. In contrast to the natural environment, individuals in our laboratory study were chronically exposed to 25 °C. Based on mortality and the rates of growth and oxygen consumption, 25 °C appears to be a sub-optimally warm temperature for small juvenile *C. concholepas*. The significant increase in growth rate with 5 °C increases in exposure temperature between 10 and 20 °C observed for the low-latitude population studied here and the results of a previous study (Manríquez et al., 2016) using small juveniles from a high-latitude population (Valdivia, Chile, ~73°S) suggest some degree of local adaptation to prevailing temperatures. This study observed no increase in growth rate with warming from 15 to 19 °C (Manríquez et al., 2016). At Antofagasta, the species is exposed to warmer waters and smaller fluctuations in pCO_2 than at Valdivia (Vargas et al., 2017). This suggests that low-latitude populations of *C. concholepas* at Antofagasta are more warm-adapted than the higher-latitude population and, therefore, less threatened by further increases in temperature. Therefore, in term of survival and growth, small juveniles of this low-latitude population at Antofagasta are expected to cope well with near-future warming or cooling within the range of the natural (e.g. daily, seasonal) variability (between 15 °C and 20 °C) and with near-future increase in pCO_2. Changes in mean specific growth rate between 10 °C and 20 °C recorded in our study were not unexpected since rates of somatic growth and ontogenetic development increase with increasing temperatures in well-fed ectotherms within their tolerable thermal window (Atkinson, 1994; Sibly and Atkinson, 1994). Moreover, the negative effects of temperatures near 10 °C and beyond 25 °C, are in line with expected negative effects on growth and other important traits when temperatures are beyond the thermal niche determined by CTmin and CTmax (Pörtner, 2008; Pörtner and Peck, 2011).

At the time of and location of collection of juveniles used in the present study, the pH (standardized to 25 °C) of shallow subtidal waters was between 7.63 (~916 μatm pCO_2) and 7.71 (~631 μatm pCO_2) with an average (± SE; N) of 7.77 (± 0.01; 27 (743 μatm pCO_2)). These values are within the range of the present-day conditions used in our trials (Table 1). On the other hand, at the same site during ebb tide, seawater pH of small tide pools ranged from 7.33 (~847 μatm pCO_2) to 8.47 (~960 μatm pCO_2) with an average (± SE; N) of 7.75 (± 0.07; 27, (~804 μatm pCO_2)) (Manríquez PH unp. data). Therefore, juveniles in tide pools in northern Chile at Antofagasta are exposed to extremely low levels of pHs during short periods of time (<2 h) at ebb tides. Moreover, shallow, sub-tidal waters can also have extremely low values of pH during upwelling events (Vargas et al., 2017). Along the Chilean coast, the mean duration of cold events (e.g. during windy periods favouring upwelling) ranged from 8.79 to 15.73 days within the 1997–2008 period (Tapia et al., 2009). Particularly, within the Bay of Antofagasta where the small *C. concholepas* were collected, the mean duration of cold events ranged from 8.94 to 11.15 days (Tapia et al., 2009). It was recently suggested, however, that global warming will increase alongshore winds off Chile, leading to an intensification of upwelling and year-round decrease in nearshore sea surface temperatures (Falvey and Garreaud, 2009; Oerder et al., 2005). This suggests that northern populations of *C. concholepas* inhabiting low intertidal habitats are not chronically exposed to the high pCO_2 levels as those applied in this study to depict near-future pH conditions. However, it is expected that under near-future scenarios of upwelling intensification the potential negative consequences of more frequent events of upwelled waters (with low temperatures and highly saturated with pCO_2) can occur in this and other similar species.

Juveniles of *C. concholepas* are often exposed to acute pulses of high pCO_2 (low pH) and our results are, therefore, in line with the Beneficial Acclimation Hypothesis (BAH, Leroi et al., 1994). The BAH predicts that animals acclimated to a particular environment have enhanced performance (fitness advantage) at those environment conditions compared to animals acclimated to other environmental conditions (Leroi et al., 1994). In post-settlement juveniles, the specific growth rate of *C. concholepas* decreases with increasing body size, particularly after individuals reach 1 cm in size (Manríquez et al., 2008). The growth rate differences among the trials with differently-sized individuals (e.g. small individuals at 20 °C compared to larger individuals at 10 °C and 25 °C), therefore, may not only be due to treatments conditions but also due to differences in body size (Table 3; Fig. 2). We attempted to account for this by calculating specific growth rate (% per day). The fact that only slight differences in the specific growth rates were observed among the trials at 15 °C suggests that observed differences are mainly due to treatment effects.

It is important to take into account that small juveniles *C. concholepas* might also experience acute short-term air exposure during which they may be exposed to higher temperatures when inhabiting mid-low rocky intertidal habitats become isolated from the subtidal system during ebb tides. Therefore, the potential effects of near-future temperature and pCO_2 levels in intertidal organism such as *C. concholepas* are complex and future studies should also consider the additional effect of air exposure. In our study, the seawater was under-saturated with respect of aragonite (Ω aragonite) at the high pCO_2 treatment in the three trials at 15 °C and in the third trial at 10 °C. The buoyant weight of the experimental individuals, as proxy for net calcification, was not significantly affected by the high pCO_2 levels. Moreover, the natural seawater used in our study, as a proxy of the

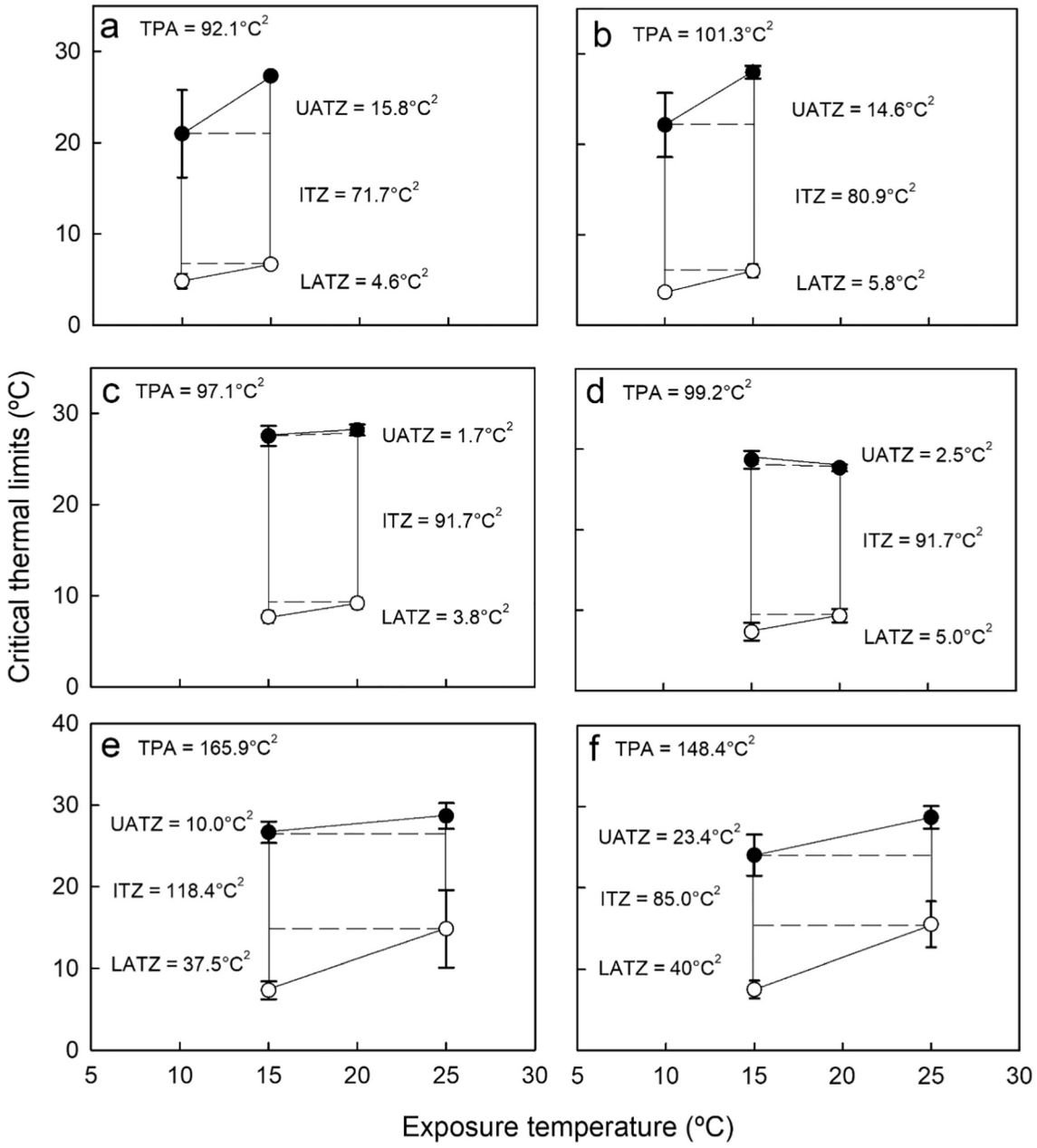

Fig. 5. The effect of different pCO_2 levels and temperature on the partial thermal tolerance polygons for small juvenile *Concholepas concholepas* after a 1-mo exposure to current-day (left panels) and elevated pCO_2 levels (right panels) during three experimental trials; first (a–b, 10 °C), second (c–d, 20 °C) and third (e–f, 25 °C). In all panels open and filled circles represent CTmax and CTmin measurements, respectively (mean and their confidence intervals (CI)). The bottom and top solid lines represent the CTmax and CTmin regression lines. The partial thermal tolerance zone (PTZ) is divided into an intrinsic tolerance zone (ITZ) and into upper and lower acquired tolerance zones (UATZ, LATZ). The total polygonal area (TPA) corresponds to the sum of UATZ, ITZ and LATZ. For further details see Table S2 in the supplementary material.

situation in nature, was always saturated with respect of aragonite and calcite. Therefore, our findings suggest that small juvenile *C. concholepas* have the ability to maintain calcification even at elevated pCO_2 levels, when the seawater was saturated for calcite but not for aragonite, and in consequence was corrosive for the experimental individuals in those treatments. The absence of negative effects of low saturation states on calcification (buoyant weight) is in agreement with similar results reported in the literature for *C. concholepas* (Manríquez et al., 2013) and other invertebrates (Ries et al., 2009; Manzello, 2010; Rodolfo-Metalpa et al., 2011; Duarte et al., 2015). This suggests that the effects of OA on net calcification in *C. concholepas* and other calcifying species are more complex than expected.

Regardless of pCO_2 level, CTmin of *C. concholepas* shifted in individuals exposed to different temperatures. The average annual temperature in shallow subtidal waters in Antofagasta are 15 °C and 20 °C as it represents the annual mean temperature during the austral winter and summer months (Manríquez et al., 2018). This suggests that increments above (20 °C or 25 °C) or reductions (10 °C) below 15 °C move the small individuals beyond the thermal comfort zone. Since no effects of pCO_2 levels on CTmin were found, this suggests that *C. concholepas* used in the present study were more sensitive to changes in temperature than pCO_2 (at the levels of these factors tested here). The significant effect of exposure temperatures below (15 °C) or above (20 °C) on CTmax suggest that temperatures that are expected under near-future conditions of cooling (10 °C) or warming (25 °C) in northern or central-south Chile might have negative effects on the thermal tolerance of this species. On the other hand, increments in temperature from 15 °C to 20 °C, which are within the range of temperatures to

Table 6
Two-way ANOVA on row (†) or aligned rank transformed data (††) evaluating the effect of a 2-mo exposure period under contrasting temperature and pCO_2 levels (see Table 1 for details) on the oxygen consumption rate of small juvenile *Concholepas concholepas* in the three experimental trials. Statistically significant results ($P < 0.05$) are indicated in **bold**.

Source	DF	MS	F	*P*	Comparison
Trial 10 °C (††)					
Temperature	1	1485.1	33.542	$\mathbf{3.2 \times 10^{-6}}$	10 < 15
pCO_2	1	0.500	0.005	0.943	
Temperature × pCO_2	1	4.500	0.046	0.831	
Residuals	28	79.409			
Trial 20 °C (††)					
Temperature	1	984.142	28.301	$\mathbf{1.8 \times 10^{-5}}$	20 > 15
pCO_2	1	0.143	0.001	0.96567	
Temperature × pCO_2	1	82.286	1.137	0.29670	
Residuals	24	60.869			
Trial 25 °C (†)					
Temperature	1	0.015	8.013	**0.00903**	25 < 15
pCO_2	1	0.001	0.455	0.50598	
Temperature × pCO_2	1	0.001	0.439	0.51355	
Residuals	25	0.002			

which small juvenile *C. concholepas* are exposed in shallow subtidal habitats in Antofagasta have different effects on CTmin and CTmax; CTmin increased (i.e. reduced tolerance to low temperatures) but CTmax (i.e. tolerance to warm temperatures) was not affected. Since no mortality was recorded at the end of the measurements and two weeks after the tests, we conclude that small juvenile *C. concholepas* can tolerate acute exposure to temperatures far beyond their maximal and minimal habitat temperature.

The shape of each species' CTM-polygon reflects its thermal niche (Bennett and Beitinger, 1997). Therefore, CTM-polygons may be potentially useful in several important areas of research such as to understand population fluctuations (Eme and Bennett, 2009) and to provide a baseline for thermal aquaculture condition (Das et al., 2005). In our study, the observed increment of ~9% in the partial thermal area of juveniles exposed to 15 °C and 10 °C suggests that in near-future cooling scenarios thermal stress to low temperatures is expected when *C. concholepas* experiences near-future conditions of elevated pCO_2. Regional cooling associated with both the intensification of winds that favour upwelling and the ENSO cold-phase (La Niña) is expected to affect the central and northern Chilean coast (17–27° S, Falvey and Garreaud, 2009). On the other hand, under chronic exposure to 15 °C and 20 °C, the thermal area was roughly the same under present and near-future conditions of pCO_2. This suggests that, within the thermal range in which small juvenile *C. concholepas* inhabit in northern Chile, changes in pCO_2 levels will not affect the thermal area. The high values for CTmax (between ~28 °C and 30 °C) suggest that juvenile *C. concholepas* are well adapted to cope with the increased environmental temperatures projected to occur from climate change within the water bodies along the Chilean coast. This finding may have been expected based on the large range of latitude (from 6° S to 55° S) and abiotic factors inhabited by this species. However, the observed reduction of ~13% in the thermal area for small juveniles exposed to 15 °C and 25 °C, was mainly a consequence of a reduction in the upper tolerance limit (measured after an exposure to elevated pCO_2 and temperature). This suggests that small juvenile *C. concholepas* experiencing near-future conditions of elevated pCO_2 will suffer thermal stress under projected levels of warming. In a future climate, therefore, *C. concholepas* and other similar species, will not be able to tolerate warming of ~4 °C above the maximal annual temperatures (central and southern Chile) or cooling of ~5 °C below (northern Chile) the annual average conditions in Antofagasta (~16 °C). Although most studies calculating thermal tolerance polygons to assess how acclimation temperatures affect the ranges in tolerable temperatures have been conducted on marine and freshwater fish (Bennett and Beitinger, 1997; Eme and Bennett, 2009; King and Sardella, 2017; Moyano et al., 2017; but see Manríquez et al., 2019a on marine invertebrates). We suggest that the use of thermal tolerance polygons along physiological and morphological trait responses is adequate to investigate how temperature and pCO_2 levels might affect the performance of marine organisms of various taxa in near future scenarios.

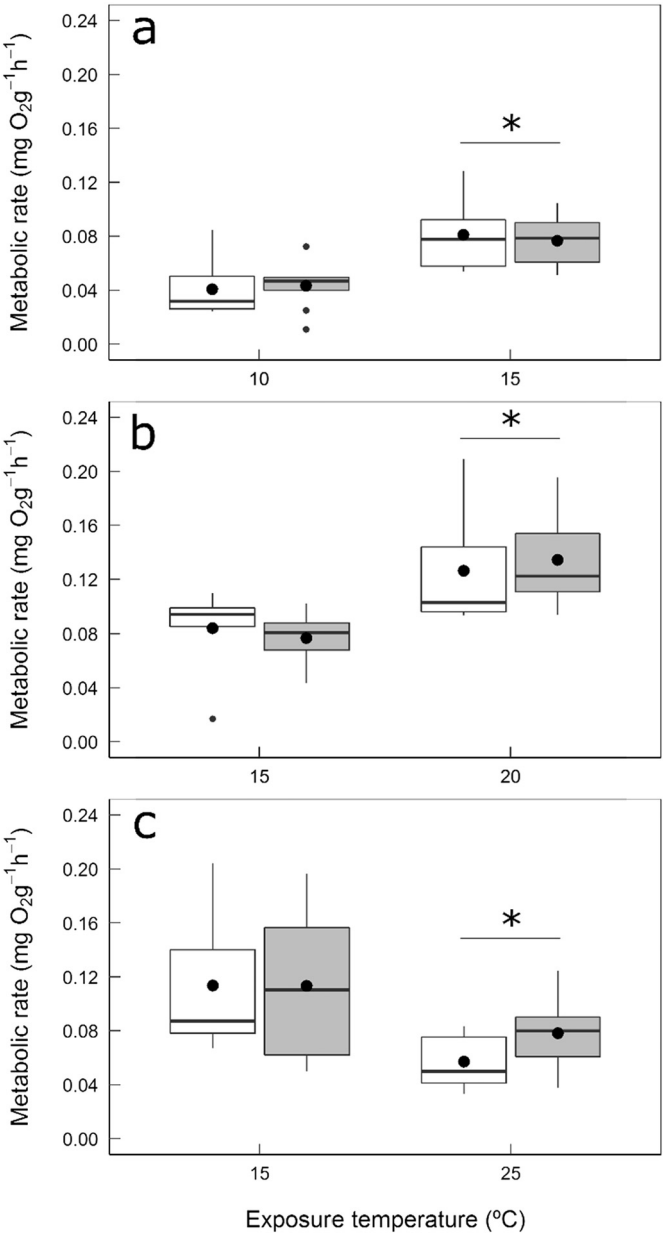

Fig. 6. Metabolic (oxygen consumption) rates of small juvenile *Concholepas concholepas* measured after a 2-mo exposure to two levels of temperatures and pCO_2 in three experimental trials: (a) 10 °C; (b) 20 °C; and (c) 25 °C. An asterisk above the bars depicts a significant ($P < 0.05$) effect of temperature (see Table 6). The box plots are described elsewhere (Fig. 2).

Measuring oxygen consumption rates is a relevant proxy for the metabolic response of organisms and, when measured across a wide range of temperatures, can provide valuable information on the shape of thermal performance windows (Pörtner, 2001; Pörtner, 2002). In agreement with expected responses for ectotherm organisms (Markle and Kozak, 2018), oxygen consumption rate of small juvenile *C. concholepas* increased with increasing treatment temperature but only from 10 °C to 20 °C. At 25 °C, a reduction in the oxygen consumption rate indicates that *C. concholepas* experienced metabolic stress. This temperature is ~2 °C warmer than individuals of this species might

experience during short periods (summer ebb tides) at the collection site (Manríquez et al., 2018). The reduction of the metabolic rate in response to warming has been described previously in the literature in marine gastropods as a mechanism to improve energy conservation (Marshall and McQuaid, 2011). Therefore, this metabolic depression in combination with potential increased costs associated with upregulated HSP transcription are likely mechanisms behind the reduction in weight- and length-specific growth rates of small juveniles at this temperature. It seems that juvenile *C. concholepas* that inhabit the northern end of its geographic range lack the ability to acclimate to 25 °C which suggests that this is the temperature threshold above which profound decrements in fitness would occur with consequences for survival (Bernardo and Spotila, 2006). Local adaptation of this species to prevailing temperatures is possible considering the wide bio-geographic range of *C. concholepas*, which ranges from Islas Lobos de Afuera in Perú (6° 27′S) to Cape Horn in southern Chile (55° 43′S). Biogeographical patterns in temperature adaptation have been described in other gastropods such as abalones based on population-specific structure and function of cytosolic malate dehydrogenases (cMDHs), an enzyme involved in heat-resistance (Dahlhoff and Somero, 1993). The absence of significant effect of pCO_2 on oxygen consumption rates in *C. concholepas* determined in the present study suggests that chronic exposure to near-future pCO_2 levels across temperatures that they might experience in shallow subtidal habitats (15 °C to 25 °C) in northern Chile (23° S) will not have a significant effect on the metabolic response of juveniles of this species.

Both low (10 °C) and moderately high (20 °C) temperatures induced moderate physiological stress in small juvenile *C. concholepas* as shown by the small (but significant) changes in induction of *HSP70*. Under the cooling scenario, the increase in pCO_2 level did not add stress to this snail; however, under the moderate warming scenario, the near-future pCO_2 level significantly generated more physiological stress. On the other hand, exposure to the highest temperature (25 °C) produced marked physiological stress, as showed by a 3-fold *HSP70* induction; but when combined with near-future pCO_2 reduced significantly *HSP70* levels. These results suggest that combined effects of the highest temperature with near-future pCO_2 levels produced an extreme stress situation, which may have increased ATP demand for maintenance (including protein reparation by HSPs) to a level that may have exceeded ATP supply by aerobic metabolism. This hypothesis is in line with the observed metabolic depression induced by the exposure to the highest temperature in juvenile *C. concholepas*. The energetic cost associated with transcription, synthesis and chaperoning activity of HSPs can contribute substantially to cellular energy demands (Somero, 2002; Sharma et al., 2010). This has been empirically demonstrated by in vitro inhibition of energetic metabolic pathways in blood cells from rainbow trout *Oncorhynchus mykiss* that decreased ATP by 79%; which in turn reduced *HSP70* mRNA expression after heat shock (Currie et al., 1999). Therefore, energy limitations may prevent HSPs overexpression even in the presence of cellular stress. We have previously observed this in juvenile *C. concholepas*, in which starvation decreased the content of stored energy substrates as well as their ability to synthesize *HSP70* after thermal stress by low and high temperatures (Jeno and Brokordt, 2014). Thus, overall our results suggest that under a scenario of the combined effects of high temperature (25 °C) and near-future pCO_2 levels, *HSP70* would not be able to accomplish their functions, such as the reparation of other functionally important proteins.

Species with extensive latitudinal distributions generally experience a larger range of thermal conditions and are expected to have broader physiological tolerance than species with more narrow latitudinal distribution (Bozinovic et al., 2011). Species-specific association between ranges in environmental temperatures and physiological tolerance have been found in many taxa including fish, insects, birds, amphibian and invertebrates (Addo-Bediako et al., 2000; Pörtner et al., 2001; Clusella-Trullas et al., 2011). The species examined in this study (Chilean false abalone, *C. concholepas*) has a latitudinal extent of 50° (from 6 to 56° S) and, in shallow, subtidal habitats, this species could experience a wide range in average temperatures (e.g. from 6 °C to 20 °C). In the present study, we used small juvenile individuals from a northern population of *C. concholepas* (23° S). In this geographical area, the small juvenile *C. concholepas* inhabiting shallow subtidal habitats are exposed to a thermal range from 15 °C to 20 °C (Manríquez et al., 2018), and theoretically more warm-adapted that southern populations which are normally exposed to colder temperatures. Therefore, our study suggests that if thermal stress associated with climate change in this area (cooling) exceeds the limits of evolutionary local adaptation *C. concholepas* populations in northern Chile will become susceptible to local extension risk. In fact, a regional cooling in a global warming scenario along the central and northern Chile (17°–37°S) has been reported in the literature (Falvey and Garreaud, 2009); therefore, cooling can be a potential thermal stressful scenario for this or other similar species in that geographical extension. The same can be valid for more southern populations in which the predicted warming (Yáñez et al., 2017) also exceeds the limits of evolutionary local adaptation. However, this species has a long pelagic larval dispersion phase of ~4 mo (DiSalvo, 1988) that might prevent local extinction by the colonization from a population locally acclimated to less stressful conditions. However, genetic studies show a spatial pattern of isolation by distance in conjunction with a genetic structure (Cardenas et al., 2015). This suggest that depending on the isolation level, some populations can be more susceptible to local extension risk if near future changes in temperature exceed the threshold for the species adaptability.

5. Conclusion and future perspectives

We conclude that near-future levels of pCO_2 and temperature will likely result in a physiological-based reduction in overall thermal performance of small juveniles of this keystone species. Newly arrived settlers (~0.2 cm) and small juvenile *C. concholepas* (<3 cm) in low rocky intertidal and shallow subtidal habitats (Moreno et al., 1993a, 1993b; Manríquez et al., 2008; Manríquez et al., 2009) are commonly exposed to air during ebb tides. In the case of benthic invertebrates inhabiting shallow coastal areas, the extent of aerial exposure is important to consider when drawing conclusions on potential impacts of climate change (Whiteley, 2011). Therefore, the effects of global stressors such as ocean warming (OW) and pCO_2 (OA) might be more complex and drastic than expected for small individuals of this species inhabiting rocky intertidal habitats. In fact, it has been suggested that episodes of mortality of intertidal organisms such mussels will be more likely at some high-latitude 'hot spots' well within the latitudinal range of a species rather than at low-latitudes, due to the mosaic pattern of thermal stress along the coast (Helmuth et al., 2002). Therefore, regional patterns of tidal regime and local pattern of wave splash can overwhelm large-scale climate in driving patterns of body temperature in intertidal organisms. This highlights the need to develop more environmentally realistic experiments to characterize organismal responses to OA, OW and OC for species that inhabit intertidal habitats and are regularly exposed to increased variability of pCO_2 and temperature levels.

CRediT authorship contribution statement

Patricio H. Manríquez: Conceptualization, Funding acquisition, Resources, Formal analysis, Writing - original draft, Writing - review & editing. **María Elisa Jara:** Resources. **Claudio P. González:** Resources, Formal analysis. **María Isabel Díaz:** Resources. **Katherina Brokordt:** Conceptualization, Formal analysis, Writing - review & editing. **María Eugenia Lattuca:** Conceptualization, Writing - original draft, Writing - review & editing.**Myron A. Peck:** Conceptualization, Funding acquisition, Writing - review & editing. **Katharina Alter:** Writing - review & editing. **Stefano Marras:** Conceptualization, Writing - review & editing. **Paolo Domenici:** Conceptualization, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.137239.

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