

Ocean acidification affects predator avoidance behaviour but not prey detection in the early ontogeny of a keystone species

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ABSTRACT: In a series of laboratory Y-maze experiments, we investigated the directional response of juveniles of the muricid snail *Concholepas concholepas*, a rocky-shore keystone predator characteristic of the southeastern Pacific coast, in displaying prey detection and predator escape behaviour after 5 mo rearing under contrasting levels of $p\text{CO}_2$: 390 (present-day), 700 and 1000 $\mu\text{atm CO}_2$ (as expected in near-future scenarios of ocean acidification [OA]). Regardless of the experimental condition, juveniles significantly chose the Y-maze arm containing prey. In general terms, the directions of the displacement paths of the snails in the Y-maze were straight from the starting point to the final position, where the prey items were offered. Moreover, juveniles reared at present-day concentrations and 700 $\mu\text{atm CO}_2$ significantly avoided displacement in the Y-maze arm receiving predator odours. This predator-avoidance behaviour was disrupted in juveniles reared at 1000 $\mu\text{atm CO}_2$. In most cases, displacements recorded under such conditions were sinuous, and the trial individuals did not significantly choose the predator-free arm. In contrast, displacement paths recorded for snails reared at present-day concentrations and at 700 $\mu\text{atm CO}_2$ were straight from the initial to the final position, with displacements mostly ending near the prey and in the arm free of cues associated with predation. The loss of responses to a natural predator under high $p\text{CO}_2$ levels in the early ontogeny of *C. concholepas* may result in ecologically deleterious decisions by this keystone species. We conclude that the negative effects of OA on the chemosensory behaviour of keystone species could have negative consequences for community dynamics.

KEY WORDS: Y-maze · Chemoreception · Decision-making · Early ontogeny · Mucous trail · pH · *Concholepas concholepas*

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INTRODUCTION

Changes in seawater chemistry (e.g. changes in pH and carbonate ion values) in the marine environment are expected to take place under scenarios

of ocean acidification (OA) (Orr et al. 2005). OA, as a consequence of rising global CO_2 levels, is expected to have negative impacts primarily on marine shell-forming organisms (Gazeau et al. 2007, Fabry et al. 2008, Salisbury et al. 2008). Most

research on OA has focused largely on the process of calcification and the physiological trade-offs employed by calcifying organisms to support the building of calcified structures (Kleypas et al. 2006, Gazeau et al. 2007, Ries et al. 2009) or on developmental-larval mortality (Kurihara & Shirayama 2004, Kurihara et al. 2007, Dupont et al. 2008, Havenhand et al. 2008, Findlay et al. 2009). However, there is growing evidence that OA can also affect behavioural traits (Bibby et al. 2007, Munday et al. 2009a,b, 2010, de la Haye et al. 2012, Leduc et al. 2013). In freshwater snails, the reduction of environmental calcium blocks long-term memory formation (Dalesman et al. 2011), which likely depends on sensory structures such as the osphradium. In molluscs, the osphradium is a chemoreceptor organ involved in predator and prey detection behaviour (Brown & Noble 1960, Huaquín & Garrido 2000). The Ca^{2+} ion concentration in seawater (Brewer et al. 1975) is considered constant and higher than in continental water (UNEP 2001). The high level of dissolved Ca in seawater ($0.413 \text{ gCa kg}^{-1}$ at salinity 35, or $10.3 \text{ mmol kg}^{-1}$) relative to the small changes due to CaCO_3 dissolution in the ocean (ΔCa is ca. 0.1 mmol kg^{-1} ; Brewer et al. 1975) suggests that in saline marine waters the effect of acidification on predator avoidance is not plausible due to effects of Ca ion levels on osphradium functioning. However, recent studies have demonstrated that predicted future CO_2 levels might alter sensory responses and behaviour of marine fishes (Nilsson et al. 2012, Jutfelt et al. 2013, Chivers et al. 2014). These studies suggest that in fish, high CO_2 levels impair neurotransmitter functions through interference of acid–base regulatory mechanisms with the function of the GABA-A neurotransmitter receptors. Therefore, it is expected that GABA-A neurotransmitter receptors could be affected in marine invertebrates as they are affected in marine fishes.

The marine snail *Concholepas concholepas* is a carnivorous predator that uses chemical cues to perceive its prey, predators and conspecifics (Huaquín & Garrido 2000, Manríquez et al. 2013a). Since *C. concholepas* is an important component of intertidal and shallow subtidal communities, where it is a keystone species (Castilla 1999), the success of its early ontogeny could have consequences for the entire community. In the clownfish *Amphiprion percula*, a combination of field and laboratory-based experiments have shown that CO_2 -rich seawater alters the behaviour of juveniles and their survival during recruitment to adult populations

(Munday et al. 2009a,b, 2010). However, to date, little information is available about the potential consequences of ocean acidification on similar behavioural traits in marine invertebrates (Bibby et al. 2007). The super-saturation of CaCO_3 is a normal characteristic of surface seawater (Wattenberg & Timmerman 1936, Cloud 1962, McIntyre & Platford 1964, Lyakin 1968, Chave & Suess 1970, Cohen & Holcomb 2009). However, under a future scenario of CO_2 -rich seawater with altered physico-chemical properties, it is expected that marine species will be exposed to changed conditions of carbonate ions and thus experience stress (Pörtner 2008). The underlying mechanistic explanation for this stress is metabolic depression under reduced pH, acidification of body fluids (hypercapnia) and changes in extracellular acid–base balance conditions (Reipschläger & Pörtner 1996, Michaelidis et al. 2005). Such changes can slow down or hinder several critical physiological or neurological processes at the individual level with potential consequences at higher levels of organisation.

Laboratory experiments with small individuals of *Concholepas concholepas* have demonstrated that shell colour in this species is determined by the colour of the consumed prey and, in turn, is an effective mechanism for reducing visual predation by crabs such as *Acanthocyclus hassleri* (Manríquez et al. 2009). Moreover, Y-maze experiments designed to test whether or not individuals placed at the starting position modify their displacement depending on the presence of prey or predators located at the other end of the maze indicated that *C. concholepas* uses water-borne cues both to detect prey and deploy strong anti-predator behaviour (Manríquez et al. 2013a). This suggests that passive and active behavioural traits play an important role during the early ontogeny of this species. However, the consequences of stressful conditions, such as those associated with OA, on behavioural responses associated with prey and predator perception by *C. concholepas* are still largely unknown. A recent study (Manríquez et al. 2013b) has demonstrated that OA disrupts self-righting speed in presence or absence of the predatory crab *A. hassleri* but does not disrupt net shell growth in *C. concholepas*. Consequently, in the present study, we hypothesised that under near-future conditions, in CO_2 -rich seawater associated with OA, the behavioural responses of *C. concholepas* to prey and natural predators will be affected as a consequence of being exposed to seawater with different chemical properties.

MATERIALS AND METHODS

Early benthic stages of *Concholepas concholepas* were obtained either as competent larvae (ca. 0.18 cm in size, Fig. 1a) collected from inner nearshore waters ca. 0.1 to 0.5 km from the coastline (Manríquez & Castilla 2011) or as newly settled individuals (0.5 cm in size, Fig. 1b) collected from rocky intertidal habitats. Competent larvae of *C. concholepas* were collected at El Quisco (central Chile, 33° 39' S) from foam slicks using a heavy-duty swimming pool cleaning net (800 μm mesh) connected to an aluminium telescopic pole, while the sampling boat was immobile alongside the foam slick (Manríquez & Castilla 2011, Manríquez et al. 2012). Newly settled individuals of

C. concholepas were collected from rocky intertidal habitats in Antofagasta (northern Chile, 23° 30' S) and Calfuco (southern Chile, 39° 46' S). Once in the laboratory, competent larvae were induced to metamorphose in the presence of small specimens of the mussel *Perumitylus purpuratus* (Manríquez et al. 2004, 2008, 2009) in conditioned seawater at acidification levels of ca. 390 (present-day), 700 and 1000 μatm CO_2 . The post-metamorphic individuals, together with the newly settled individuals, were reared for 5 mo at present-day, 700 and 1000 μatm CO_2 and fed *ad libitum* with *P. purpuratus* (this is hereafter referred to as the acidification phase).

A seawater acidification unit was designed to provide conditioned seawater at the 3 acidification

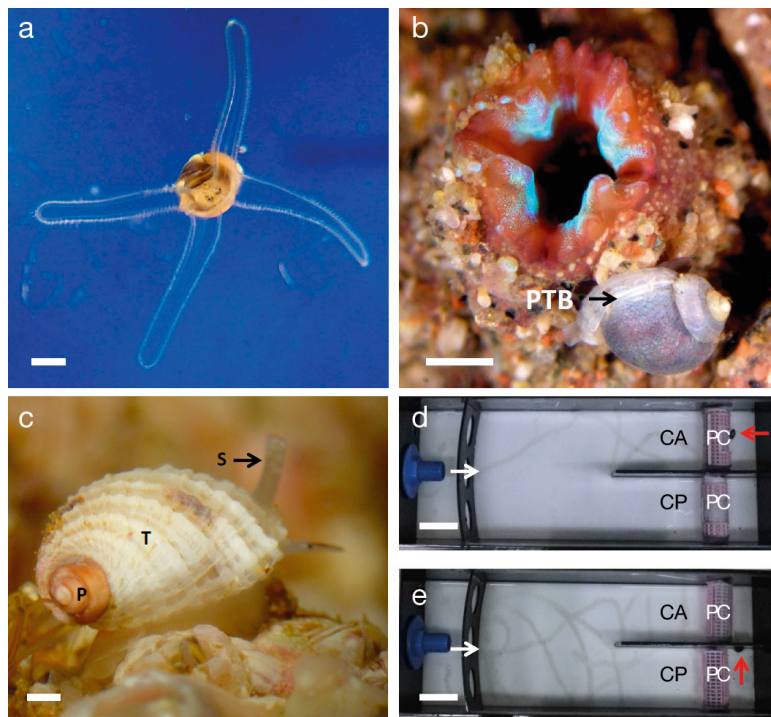


Fig. 1. *Concholepas concholepas*. Photographs of (a) a newly captured free-swimming competent larva showing expanded velar lobes, (b) a newly settled individual crawling past the inhalant siphon of an intertidal ascidian *Pyura chilensis*, showing the protoconch-teloconch boundary (PTB), (c) a juvenile individual after ca. 5 mo of rearing and showing the protoconch (P) deeply enclosed in the first teloconch (T) whorl as well as the expanded siphon responsible for sucking water in the mantle cavity, and (d,e) the displacement paths made by juvenile individuals in the Y-maze experiments in response to cues from the predatory crab *Acanthocyclus hassleri* after being reared for 5 mo (d) at present-day levels of $p\text{CO}_2$ and (e) at 1000 μatm CO_2 . In (d) and (e), the white and red arrows show the position of the trial snails at the beginning and at end of the experiments respectively. Plastic hair rollers used as cages and filled with mussels were used as source of prey cues (PC) in both Y-maze arms. One arm received seawater from a header tank with 30 individuals of the predatory crab *A. hassleri* (CP), and the other received seawater from a control header tank maintained without crabs (CA).

Scale bars (a–c) = 1000 μm ; (d,e) = 4.5 cm

levels. Three large (230 l), independent plastic reservoir tanks were filled with filtered (0.5 μm) seawater (FSW). The seawater was continuously bubbled with either ambient air or premixed CO_2 -enriched air precisely following the method described by Navarro et al. (2013), Torres et al. (2013) and Manríquez et al. (2013b). The 2 high $p\text{CO}_2$ levels were chosen to represent conditions predicted under worst-case scenarios for the end of the 21st century and the beginning of the next one (Meinshausen et al. 2011). Every day, the reservoir tanks were topped up with treated FSW, and once a week, the tanks were cleaned and the total water content of each reservoir tank was replaced with fresh seawater. Normally, after 5 to 12 h of vigorous CO_2 -air mixture bubbling (with the air flow ranging between 2000 and 3000 ml min^{-1}), the seawater $p\text{CO}_2$ reached the reported mean values (Table 1). Seawater samples were taken from each reservoir tank 3 times per week to assure consistency in carbonate parameter measurements. The pure air bubbling through the 250 l container, which yielded a $p\text{CO}_2$ of ca. 390 μatm , was used as the control for CO_2 -enriched treatments. Air was blended with pure CO_2 using mass flow controllers (MFCs, www.aalborg.com) to produce 2 CO_2 -enriched mixtures that yielded seawater $p\text{CO}_2$ of ~ 700 and ~ 1000 μatm after ~ 12 h of bubbling the CO_2 -air mixture through the seawater reservoir tanks. During the experiment, seawater

Table 1. *Concholepas concholepas*. Average (± 1 SE) conditions of the seawater used to maintain individuals during the 5 mo rearing period (February to July 2011; December 2011 to May 2012): pH, total alkalinity, partial pressure of CO₂, carbonate ion concentration, saturation states of the water regarding aragonite and calcite minerals ($\Omega_{\text{aragonite}}$ and Ω_{calcite} respectively). The different experimental levels of pCO₂ (390: present-day, 700: end of 21st century, 1000: beginning of 22nd century) in the mesocosms and in the rearing containers were achieved and maintained during the entire experimental period by active injection of CO₂ and air. The treatment levels were based on the rate of change predicted by the most extreme scenario (RCP8.5 scenario) of atmospheric CO₂. See Meinhausen et al. (2011) for further details

Treatment (pCO ₂ level)	pH <i>in situ</i>	pH (at 25°C)	Total alkalinity ($\mu\text{mol kg}^{-1}$)	pCO ₂ <i>in situ</i> (μatm)	[CO ₃ ²⁻] <i>in situ</i> (mmol kg ⁻¹ SW)	Temperature (°C)	Ω aragonite	Ω calcite
February–July 2011								
390	8.046 (0.009)	7.870 (0.009)	2206.959 (14.110)	393.169 (9.813)	134.250 (3.290)	13.382 (0.380)	2.076 (0.051)	3.267 (0.079)
700	7.812 (0.008)	7.645 (0.011)	2196.530 (12.470)	712.634 (11.555)	83.178 (2.419)	13.235 (0.350)	1.286 (0.038)	2.023 (0.059)
1000	7.683 (0.011)	7.524 (0.013)	2208.808 (13.562)	992.975 (22.327)	64.347 (2.310)	13.313 (0.342)	0.995 (0.036)	1.565 (0.056)
December 2011–May 2012								
390	8.057 (0.057)	7.911 (0.055)	2254.09 (37.653)	389.273 (62.026)	153.987 (15.340)	15.049 (1.785)	2.377 (0.236)	3.713 (0.366)
700	7.857 (0.039)	7.711 (0.042)	2256.637 (38.322)	655.071 (67.315)	102.803 (6.199)	14.970 (1.755)	1.588 (0.094)	2.482 (0.143)
1000	7.734 (0.046)	7.596 (0.047)	2251.718 (41.420)	895.752 (107.627)	80.201 (6.209)	15.229 (1.842)	1.240 (0.095)	1.937 (0.147)

pH, temperature, salinity and total alkalinity were measured in each reservoir every 3 d to determine the actual carbonate system speciation in the equilibrated water. Clean dry air was generated by compressing atmospheric air (117 psi) using an oil-free air compressor and passing it through particle filters (1 μm). The clean air flow was set to 5 l min⁻¹ for both treatments using air MFCs. Downstream of the air MFCs, it was mixed with pure CO₂ gas, the flow of which was manually adjusted to produce air-CO₂ mixtures with pCO₂ levels in dry air of 700 μatm and 1000 μatm , respectively. The air-CO₂ mixture pCO₂ levels were measured using a CO₂ analyser (QUBIT SYSTEMS S151 CO₂ Analyser calibrated with CO₂-free air and a standard CO₂-air mixture of 1110 μatm provided by INDURA). The pH measurements were made in a closed 25 ml cell thermostatically controlled at 25°C using a Metrohm 713 pH meter (input resistance 10⁻¹³ Ohm, 0.1 mV sensitivity and nominal resolution 0.001 pH units) and a glass combined double junction Ag/AgCl electrode (Metrohm model 6.0219.100) calibrated with 8.089 Tris buffer (DOE 1994) at 25°C; pH values are therefore reported on the total hydrogen ion scale (DOE 1994). Temperature and salinity were measured using an Ocean Seven 305 Plus CTD. The total alkalinity was measured using the method of Haraldsson et al. (1997). The pH, total alkalinity and hydrographic data were used to calculate the rest of the carbonate system parameters (pCO₂ and DIC) and the saturation stage of $\Omega_{\text{aragonite}}$ using CO2SYS software (Lewis & Wallace 1998) set with Mehrbach solubility constants (Mehrbach et al. 1973) refitted by Dickson & Millero (1987). Discrepancies between the pCO₂ (estimated using a gas CO₂ analyser) in dry air-CO₂ gas blends

that were injected into tanks filled with FSW and the resulting FSW pCO₂ (estimated from pH and AT measures) after bubbling are discussed by Torres et al. (2013).

During the acidification phase, all individuals of *Concholepas concholepas* were maintained in closed Plexiglas aquaria (30 cm long, 18 cm wide and 17 cm high) filled with 7.5 l of 0.5 μm FSW conditioned at the required pCO₂ level. Conditioned seawater, from the appropriate reservoir tank, was then used to replace each of the exposure aquariums once a day. To ensure that pCO₂ levels remained stable within the Plexiglas aquaria, between each daily water change, the aquaria were closed with a plastic lid with a small hole to allow tube access. Inside each exposure aquarium, the end of each tube was fitted with an air-stone through which a continuous stream of either air (390 μatm CO₂) or enriched CO₂ air (700 or 1000 μatm) was bubbled. A small hole of 1.0 cm in diameter made in the lateral wall of the aquariums and above the water level served as an aeration vent.

Twelve competent larvae of *C. concholepas* were metamorphosed and then assigned to each CO₂ level. However, high mortalities in post-settler individuals were recorded at 700 and 1000 CO₂ levels during the first week after metamorphosis. After that, during the 5 mo of rearing, no mortalities were recorded in the surviving individuals. As a consequence of the low number of post-metamorphosed individuals of *C. concholepas* available for the Y-maze experiments, each individual was used in 2 or 3 trials (Table 2). In contrast, during the 5 mo of rearing, no mortality was recorded in individuals collected as settlers, and those individuals were only used once in each trial. As a control for present-day

Table 2. *Concholepas concholepas*. Summary of individuals reared for 5 mo under the 3 experimental $p\text{CO}_2$ levels, number of Y-maze trials and the number of times that those individuals were used in the trials to evaluate their responses to prey and predator chemical cues. The trial individuals were collected in the field as competent larvae (central Chile) or newly settled (northern and southern Chile, pooled data) and then reared in the laboratory for 5 mo under 3 different $p\text{CO}_2$ levels. As a consequence of the low number of individuals available, depending on the availability, they were used 1, 2 or 3 times in each trial, which is indicated in the table with the corresponding superscript number. Present-day (field) samples corresponding to juvenile individuals were collected in the field 2 to 3 mo after the collection of the other specimens with sizes similar to those achieved after 5 mo of laboratory rearing. Average sizes represent those measured after 5 mo of rearing and correspond to the sizes when the trials were conducted

Experimental condition	No. of specimens	Average size (SD) (cm)	No. of Y-maze trials	
			Prey	Predator
Competent larvae (Central Chile)				
Present-day	12	14.89 (3.10)	12 ¹	24 ²
700 $\mu\text{atm CO}_2$	8	14.34 (2.53)	16 ²	24 ³
1000	5	15.75 (0.44)	15 ³	15 ³
Present-day (field)	24	14.83 (1.49)	24 ¹	24 ¹
Newly settled (N Chile)				
Present-day	13	18.60 (3.88)	13 ¹	26 ²
700 $\mu\text{atm CO}_2$	17	18.49 (2.66)	17 ¹	34 ²
1000	12	18.56 (3.34)	12 ¹	24 ²
Present-day (field)	24	18.43 (1.23)	24 ¹	24 ¹
Newly settled (S Chile)				
Present-day	15	17.32 (3.94)	15 ¹	15 ¹
700 $\mu\text{atm CO}_2$	15	17.71 (4.55)	15 ¹	15 ¹
1000	14	17.00 (4.62)	14 ¹	14 ¹
Present-day (field)	16	17.26 (3.78)	16 ¹	16 ¹

conditions, juvenile individuals of *Concholepas concholepas* with sizes similar to those achieved at the end of the acidification phase (Fig. 1c) were collected from rocky intertidal habitats in the same 3 localities (Table 2).

Individuals obtained from competent larvae and those collected as newly settled individuals were considered to be theoretically inexperienced or experienced, respectively, with respect to benthic predators. After the acidification process, their directional responses to prey (*Perumitylus purpuratus*) and the predatory crab (*Acanthocyclus hassleri*) were evaluated in Y-maze experiments.

The experimental design consisted of 8 Y-mazes (black lateral walls, black lid and white floor, Fig. 1d,e) run simultaneously with the arm of each maze receiving seawater from a separate header tank. In the first experimental series, the consequences of contrasting $p\text{CO}_2$ levels on the capacity of *Concholepas concholepas* to detect the position of prey in the Y-mazes was investigated. During the entire rearing period, *Perumitylus purpuratus* were used as prey; thus, all individuals of *C. concholepas*

were experienced with this type of prey. At the beginning of the trials, the prey (ca. 50 mussels) were placed inside plastic hair rollers (used as cages) near the seawater inlet, randomly assigned to one of the arms of the Y-maze. In the second experimental series, the consequences of contrasting $p\text{CO}_2$ levels on the capacity to perceive the presence of predators was investigated. In this experimental series, individuals of *P. purpuratus* (ca. 50 mussels) were used as prey and offered inside plastic hair rollers near the seawater inlet in both arms of the Y-mazes (Fig. 1d,e). Thirty individuals of the predatory crab *Acanthocyclus hassleri* were placed in one of the header tanks, and the other header tank was maintained without crabs and served as a control. For both experiments, the test individuals were placed at the starting position for an acclimatization period of 1 h, the trials were conducted for 15 h, and the final position of the snails in the Y-mazes was recorded. To standardise hunger, all trial specimens were starved for 12 h before the experiment. The displacement of *C.*

concholepas, as in other gastropods, occurs by muscular contraction on a mucous sheet secreted by the anterior part of the foot. Therefore, the displacement paths were evident as fine sediment stuck to the mucous track along the white Y-maze floor. This allowed us to complement information regarding the final position of the test snails in the Y-mazes with information about the number of visited arms and length of the displacement paths (DP) under the different experimental conditions. A displacement path complexity index was computed by dividing the linear distance between the initial and final positions of the trial snails in the Y-mazes by the DP length (Davies & Beckwith 1999). An index value of 1 describes a straight line, with increasingly lower values describing increasingly sinuous paths.

We used Pearson's chi-squared test (χ^2) of independence to test if the distribution frequency of visited arms (i.e. 1 or 2, receiving or not prey and predator cues) differed among the treatments or among ontogenetic stages (i.e. competent larvae or newly settled individuals). The frequencies of visited arms across $p\text{CO}_2$ treatments did not differ between north-

ern and southern populations, either in the case of chemical cues from the prey ($\chi^2 = 3.727$, $df = 3$, $p = 0.293$) or from the predator ($\chi^2 = 0.037$; $df = 3$; $p = 0.998$). Thus, for posterior analysis, the responses of both populations were pooled. When significant differences were found among $p\text{CO}_2$ levels, we compared the standardised residual with the critical value as a post hoc test. Snails showing no responses were not included in the test. A 1-way ANOVA followed by a post hoc Turkey's test was used to test for the differences in displacement path complexity index between treatments.

RESULTS

In the first experimental series (i.e. prey cues), most of the DP ended up in the arm in which the mussels were offered (Fig. 2a,b). Moreover, no significant consequences of the experimental CO_2 level on the behavioural capacity of *Concholepas concholepas* to detect the position of the prey in the Y-mazes were found. This pattern of detecting the position of the mussels in the Y-maze was not significantly affected by the $p\text{CO}_2$ level when the trial snails were reared from competent larvae ($\chi^2 = 3.61$; $df = 3$; $p = 0.307$; Fig. 2a) or newly settled individuals ($\chi^2 = 9.932$; $df = 3$; $p = 0.019$; Fig. 2b). In this series, most experimental snails visited only 1 Y-maze arm. Regardless of whether the experiments were conducted with individuals reared from newly settled individuals or from competent larvae, in the presence of mussels, the *C. concholepas* significantly favoured one arm, and no significant differences were detected among different $p\text{CO}_2$ levels (Table 3). This pattern of visiting only 1 arm across levels of $p\text{CO}_2$ was similar between trial individuals when comparing individuals collected as competent larvae with those collected as newly settled individuals ($\chi^2 = 0.434$; $df = 3$; $p = 0.933$). Regardless of the $p\text{CO}_2$ level, the average displacement path indices were close to 1, denoting that the paths were almost a straight line from the starting point to the final position (Table 4). This pattern was similar across $p\text{CO}_2$ for experimental individuals reared from competent larvae ($F_{3,68} = 0.67$; $p = 0.574$) but varied significantly across $p\text{CO}_2$

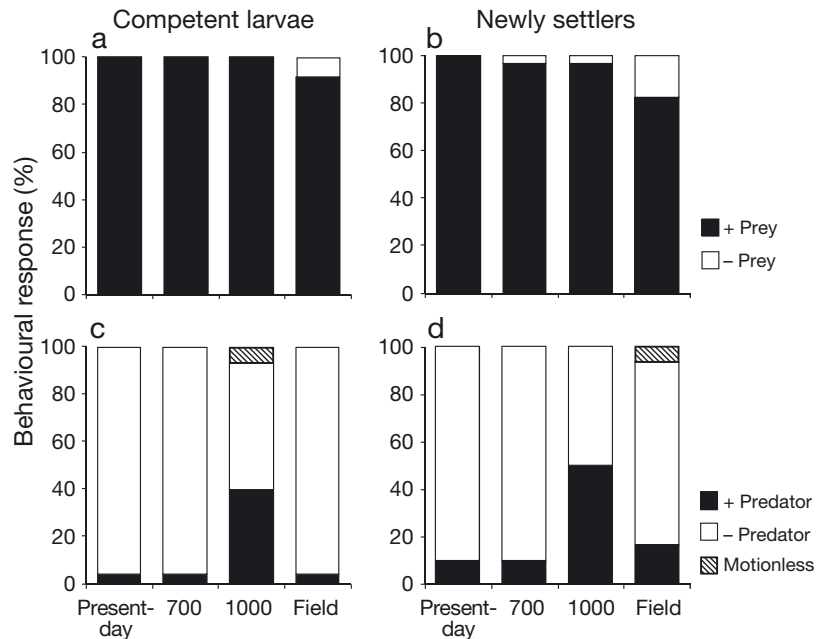


Fig. 2. *Concholepas concholepas*. Effect of different $p\text{CO}_2$ levels on the behavioural response of experimental individuals collected as competent larvae (left) and newly settled individuals (right) in response to (a, b) prey and (c, d) predator cues and compared to wild-caught individuals (field). Before the experiments, the individuals were reared for 5 mo at present-day, 700 and 1000 μatm CO_2 . Wild-caught individuals had sizes matching those achieved after 5 mo of laboratory rearing. Three behavioural responses were considered: snail displacement directed toward (+) or avoiding (-) the prey or predators and amotionless response in the starting position of the Y-maze

levels in any case of the newly settled individuals (northern Chile: $F_{3,65} = 3.64$; $p < 0.01$ and southern Chile: $F_{3,57} = 3.37$; $p < 0.05$).

In the second experimental series, when chemical cues of the predatory crabs *Acanthocycclus hassleri* flowed into 1 arm of the Y-maze, individuals of *Concholepas concholepas* reared at present-day (390 μatm CO_2) or 700 μatm CO_2 , and those collected as small juveniles in the field, exhibited a significant tendency for avoiding the arm receiving predatory crab cues (Fig. 2c,d). Motionless snails in the starting position of the Y-maze were only recorded for individuals reared from competent larvae at 1000 μatm CO_2 (Fig. 2c) and those collected as small recruits from the field (Fig. 2d). However, this response was never recorded in individuals reared for 5 mo from competent larvae or newly settled individuals at 390 and 700 μatm CO_2 (Fig. 2c,d). The pattern of avoiding the predatory crab was significantly affected by the $p\text{CO}_2$ level in the experimental snails reared from competent larvae ($\chi^2 = 22.742$; $df = 6$; $p < 0.0001$; Fig. 2c) and newly settled individuals ($\chi^2 = 48.940$; $df = 6$; $p < 0.0001$; Fig. 2d). Individuals of *C. concholepas* reared at 1000 μatm CO_2 were not able to dis-

Table 3. *Concholepas concholepas*. Number of individuals visiting 1 or 2 Y-maze arms after being reared for 5 mo, either from competent larvae or newly settled individuals, under 3 experimental levels of $p\text{CO}_2$ and in response to the presence of prey and predator chemical cues. Data obtained for small field-collected individuals with sizes similar to those achieved after 5 mo of laboratory rearing are also included. When significant differences were found, we compared the standardized residual with the critical value as a post hoc test. Different letters below the number of visits to 1 or 2 arms indicate significant differences between treatments. For the first test, significance must be interpreted with caution because the χ^2 approximation is probably invalid due the strong trend of snails to select only one of the arms across all $p\text{CO}_2$ treatments

Ontogenetic stage at collection	— Laboratory reared —			Field collected Present-day
	Present-day	700 $\mu\text{atm CO}_2$	1000 $\mu\text{atm CO}_2$	
Competent larvae				
Prey chemical cues				
1 visited arm	12	16	15	23
2 visited arms	0	0	0	1
Motionless	0	0	0	0
$\chi^2 = 1.82$; df = 3; $p > 0.5$				
Predator chemical cues				
1 visited arm	23	24	2	22
2 visited arms	1	0	12	2
Motionless	0	0	1	0
$\chi^2 = 57.144$; df = 6; $p < 0.001$				
Newly settled (Northern + Southern)				
Prey chemical cues				
1 visited arm	26	28	24	40
2 visited arms	2	4	4	5
Motionless	0	0	0	0
$\chi^2 = 0.781$; df = 6; $p > 0.05$				
Predator chemical cues				
1 visited arm	35	43	6	34
2 visited arms	5	5	32	6
Motionless	2	1	0	2
$\chi^2 = 77.413$; df = 6; $p < 0.001$				

criminate between the Y-maze arms, increasing up to ca. 40% the frequency of visits to the Y-maze receiving predatory crab cues. Significant differences were found in the number of visited arms in individuals reared from competent larvae (Table 3, $\chi^2 = 57.115$; df = 6; $p < 0.001$) and newly settled individuals (Table 3, $\chi^2 = 77.413$; df = 6; $p < 0.001$). At 1000 μatm , for both groups of *C. concholepas*, post hoc tests indicated that a significant number ($p < 0.001$) of snails visited both Y-maze arms during the experiment (Table 3). The paths recorded for specimens reared at present-day CO_2 and 700 $\mu\text{atm CO}_2$ and for field-collected individuals were straighter than those recorded at 1000 $\mu\text{atm CO}_2$ (Fig. 1d). However, most of the paths recorded at 1000 $\mu\text{atm CO}_2$ were long and sinuous (Fig. 1e). This was supported by the significantly lower average DP indices measured in indi-

viduals reared from newly settled individuals (northern Chile: $F_{3,65} = 3.64$, $p < 0.01$, and southern Chile: $F_{3,54} = 150.61$, $p < 0.01$) and competent larvae ($F_{3,83} = 551.60$, $p < 0.01$) at 1000 $\mu\text{atm CO}_2$ compared with the other experimental conditions (Table 4).

DISCUSSION

In agreement with a previous study (Manríquez et al. 2013a), our results show that post-metamorphic larvae and juveniles of *Concholepas concholepas* show displacements orientated by cues associated with prey and predation. During the entire rearing period, the experimental individuals of *C. concholepas* were fed ad libitum with *Perumitylus purpuratus*, the same prey item used in the Y-maze detection experiments. This suggests that the long experimental exposure to this food item, for ca. 5 mo, may involve the formation of a long-term memory, a characteristic trait present in marine gastropods and known as ingestive conditioning (Wood 1968, Hall et al. 1982). Our results suggest that the ingestive conditioning or the capacity to perceive chemical cues associated with the prey eaten prior to the test was not affected by seawater acidification. Since wild-collected juveniles of *C. concholepas* were re-

moved from a habitat where mussels were the main food item, it can also be argued that ingestive conditioning might explain the response found in those individuals that significantly chose the Y-maze arm with this prey item. The predator avoidance behaviour detected in the present study, with the exception of the responses observed in the highest- CO_2 treatment, agrees with a previous study showing that juveniles of *C. concholepas* respond to chemical cues associated with the predatory crab *Acanthocyclus hassleri* (Manríquez et al. 2013a,b). In molluscs, the osphradium is the specialised sensory region, and in *C. concholepas*, it is located at the base of the incurrent siphon and acts as a chemoreceptory organ (Huaquín & Garrido 2000). This highlights the importance in this species of chemo-detection in acquiring information regarding the presence and position of

Table 4. *Concholepas concholepas*. Displacement path indices recorded for individuals reared for 5 mo, either from competent larvae or newly settled, in 3 experimental levels of CO₂ and in response to the presence of prey and predator chemical cues. Data obtained for small field-collected individuals with sizes similar to those achieved after 5 mo of laboratory rearing are also included. All values are averages (± 1 SD). The path complexity indices were computed by dividing the lineal distance between the initial and final position of the trial specimen by the total path length. Index values close to 1 correspond to a straight line, and those close to 0 correspond to long and sinuous paths. Path complexity indices were compared by 1-way ANOVA, and similar superscripts indicate lack of significant differences between treatments (Tukey post hoc test; $p > 0.05$)

Locality	Laboratory reared			Field collected Present-day (field)
	Present-day	700 μ atm CO ₂	1000 μ atm CO ₂	
Newly settled (Northern Chile)				
Prey chemical cues				
Path index	0.91 (0.03) ^{a,c}	0.89 (0.03) ^b	0.90 (0.03) ^{a,b}	0.92 (0.02) ^c
$F_{3,65} = 3.64$; $p < 0.01$				
Predator chemical cues				
Path index	0.92 (0.03) ^a	0.91 (0.03) ^a	0.50 (0.09) ^b	0.93 (0.05) ^a
$F_{3,106} = 385.06$; $p < 0.01$				
Competent larvae (Central Chile)				
Prey chemical cues				
Path index	0.93 (0.02) ^a	0.92 (0.02) ^a	0.93 (0.02) ^a	0.93 (0.04) ^a
$F_{3,68} = 0.67$; $p = 0.574$				
Predator chemical cues				
Path index	0.93 (0.03) ^a	0.91 (0.04) ^a	0.50 (0.05) ^b	0.91 (0.03) ^a
$F_{3,83} = 551.60$; $p < 0.01$				
Newly settled (Southern Chile)				
Prey chemical cues				
Path index	0.90 (0.03) ^{a,b}	0.93 (0.03) ^{a,b}	0.92 (0.04) ^b	0.90 (0.01) ^a
$F_{3,57} = 3.37$; $p < 0.05$				
Predator chemical cues				
Path index	0.97 (0.05) ^a	0.95 (0.02) ^a	0.55 (0.09) ^b	0.91 (0.05) ^a
$F_{3,54} = 150.61$; $p < 0.01$				

food and predators in the surrounding area. The present study illustrates that predator detection in *C. concholepas* is a behavioural trait modified by elevated $p\text{CO}_2$ levels associated with OA. The present study does not provide evidence regarding the underlying mechanism involved in the failure to detect predators. However, our results suggest that CO₂-rich seawater may have properties that produce, through an unknown mechanism, a reduction in the detection response. In freshwater snails, the Ca conditions in which the specimens were reared affected their ability to form a long-term memory, which likely depends on sensory structures such as the osphradium (Dalesman et al. 2011). The response of osphradial neurons in *C. concholepas*, as in other gastropod molluscs (Kamardin et al. 1999), almost certainly relies on GABA-A neurotransmitters. Therefore, it seems to be quite probable that, as has

been described in fishes (Nilsson et al. 2012, Chivers et al. 2014), changes in CO₂ levels might affect neurotransmitter receptors associated with predator perception in marine invertebrates, such as *C. concholepas*.

In our study, at present-day concentrations and 700 μ atm CO₂, the inexperienced (i.e. collected as competent larvae) and experienced individuals of *Concholepas concholepas* (i.e. collected as individuals with a few weeks of benthic life) were able to display predator-avoidance behaviour. This agrees with a previous report suggesting that this behaviour could be an innate trait in *C. concholepas* (Manríquez et al. 2013a). However, the riskier behaviour of failure to avoid one of their natural predators under the most CO₂-enriched conditions (1000 μ atm CO₂) suggests that OA may erode this important behavioural trait during the early ontogeny of this keystone species. In our study, together with effects on predator avoidance, we found that, in the presence of the predatory crab at present-day concentrations and 700 μ atm CO₂, experienced and inexperienced individuals of *C. concholepas* displayed straighter displacement paths than those individuals reared at 1000 μ atm CO₂. Thus, individuals of *C. concholepas* reared at high CO₂ levels (1000 μ atm) exhibit

poorer orientation skills than individuals reared at present-day concentrations and 700 μ atm CO₂. Locomotion by gastropod molluscs involves the secretion of an energetically costly pedal mucous path (Davies et al. 1990). Therefore, the longer displacement paths recorded at 1000 μ atm CO₂ suggest that OA might have not only negative consequences on detecting both the appropriate food and the presence of the predatory crab *Acanthocyclus hassleri* during their early ontogeny but also an energetic cost. In agreement with studies in juvenile fishes (Munday et al. 2010, Dixson et al. 2010, Ferrari et al. 2011), our study shows clear similarities. In both cases, juvenile fishes and snails are less repelled by predator cues at elevated CO₂ levels. Differing from our results that show no effect of 700 μ atm CO₂ on predator detection, studies on fish show effects at these CO₂ levels (Munday et al. 2010). This suggests that juvenile fishes are

more sensitive to elevated CO_2 than marine snails. The fact that some trial snails remained motionless in the starting position of the Y-mazes agrees well with similar results recorded with small individuals of *C. concholepas* reared in the presence of restrained crabs (Manríquez et al. 2013a). However, in the present study, this behaviour was only recorded when the trial snails were exposed to predatory cues after 5 mo of rearing under the highest CO_2 levels. In another study, developed to assess the consequences of CO_2 levels on self-righting times in the presence of predatory crabs, the fastest responses were observed in the treatments that included predator cues, and motionless *C. concholepas* were never recorded (Manríquez et al. 2013b). This suggests that the anti-predator response displayed by *C. concholepas* (i.e. avoidance displacement or motionless) can be modified by the CO_2 level during rearing.

In the present study, settlement and metamorphosis was 100% successful under the 3 conditioned seawater conditions (data not shown). However, the severe consequences of the exposure of *Concholepas concholepas* competent larvae to elevated $p\text{CO}_2$ levels (i.e. 700 and 1000 $\mu\text{atm CO}_2$) during the first week of post metamorphic survival suggests that stresses experienced during the competent larval period can potentially carry-over effects that influence subsequent benthic post-metamorphic stages. This issue is part of an ongoing research project using *C. concholepas* as biological model (led by P. H. M.), and it has been recorded in other species with complex life histories (Hettinger et al. 2012).

The consequences of OA on dynamic patterns and ecological impact have been investigated using a high-resolution multi-year dataset (Wootton et al. 2008). The present study suggests that OA will have direct effects, by reducing calcification, and indirect effects, arising from the web of species interactions. In agreement, studies reporting negative consequences of OA on auditory capacity and predator-avoidance behaviour have been recorded in marine fishes (Munday et al. 2010, Simpson et al. 2011). A recent review made mainly for fishes suggests important effects of OA on olfactory-mediated behaviour in freshwater and marine ecosystems (Leduc et al. 2013). Moreover, a negative effect of elevated CO_2 levels on predator-prey interactions has also been recently reported for fishes (Allan et al. 2013). However, a study conducted with the intertidal gastropod *Littorina littorea* reported an increased avoidance response in acidified seawater (Bibby et al. 2007). To our knowledge, the results presented here represent the first report of negative consequences of OA on preda-

tor avoidance in a marine gastropod species. Since *Concholepas concholepas* plays an important role as a keystone species in intertidal and subtidal communities in Chile (Castilla 1999), and given the importance of chemical cues in the sea, our results suggest that $p\text{CO}_2$ levels expected by the end of the 21st century and beginning of the next one could have negative consequences for species interactions. However, the results of the present study should take into account recent findings showing that self-righting, an adaptive trait evolved to reduce lethal predatory attacks in the early ontogeny of *C. concholepas*, will be positively affected by high $p\text{CO}_2$ levels (Manríquez et al. 2013b), which also implies a major disruption of the predator-prey interactions. The apparent contradictory consequences of OA on behavioural traits of *C. concholepas* found under laboratory conditions (Manríquez et al. 2013b, present study) highlight the fact that predictions about the consequences of OA on community dynamics based only on controlled, single-species laboratory experiments can be limited. Moreover, how the consequences of OA on the disruption of the predator-prey interaction in *C. concholepas* will both change with the snail size and affect the predators of this species is still unknown. This highlights the need to complement laboratory (i.e. organism physiology and behaviour approaches) experiments with field research to demonstrate that effects observed under laboratory conditions do, in fact, affect the individual's fitness in nature, thus allowing a better understanding of the consequences of OA on populations, ecosystem functions and biodiversity that will aid in the development of policy recommendations for adapting to OA.

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