

## RESEARCH ARTICLE

# Effects of elevated carbon dioxide and temperature on locomotion and the repeatability of lateralization in a keystone marine mollusc

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## ABSTRACT

Recent work has shown that the behaviour of marine organisms can be affected by elevated  $P_{\text{CO}_2}$ , although little is known about the effect of multiple stressors. We therefore investigated the effect of elevated  $P_{\text{CO}_2}$  and temperature on locomotion and behaviour during prey searching in the marine gastropod *Concholepas concholepas*, a predator characteristic of the southeastern Pacific coast. Movement duration, decision time, route finding and lateralization were measured using a T-maze tank with a prey positioned behind a barrier. Four treatments, representing present day and near-future scenarios of ocean acidification and warming were used in rearing the individuals for 6 months. Regardless of the treatment, no significant differences were found in relative and absolute lateralization before and after exposure for 6 months. However, relative lateralization was not repeatable for animals tested after 6 months at elevated  $P_{\text{CO}_2}$  at both experimental temperatures, whereas it was repeatable in individuals kept at the present day level of  $P_{\text{CO}_2}$ . We suggest that these effects may be related to a behavioural malfunction caused by elevated  $P_{\text{CO}_2}$ . Movement duration, decision time and route finding were not repeatable. However, movement duration and decision time increased and route finding decreased in elevated  $P_{\text{CO}_2}$  (at 15°C), suggesting that elevated  $P_{\text{CO}_2}$  has negative effects on the locomotor and sensory performance of *C. concholepas* in the presence of a prey odour, thereby decreasing their ability to forage efficiently.

**KEY WORDS:** Ocean acidification, Temperature, Gastropod locomotion, Lateralization, Route finding, *Concholepas concholepas*

## INTRODUCTION

Ocean acidification (OA) and global warming (GW) are issues of increasing concern (Doney, 2010) owing to their key role in global change. Although early studies have focused on calcifying organisms as the main species that may be affected by ocean acidification (Hofmann et al., 2010; Orr et al., 2005), more recent work has shown that the physiological and behavioural performances of marine fish and invertebrates may also be directly affected (Briffa et al., 2012; Byrne, 2011). The exposure to elevated  $P_{\text{CO}_2}$  associated with OA could impair sensory performance, anti-predator responses, decision-making, foraging,

learning and homing of many marine species (Briffa et al., 2012; Nagelkerken and Munday, 2016). In fish, the malfunctioning of behaviours resulting from high  $P_{\text{CO}_2}$  exposure has been linked to a disruption of the function of the neurotransmitter GABA-A, (Nilsson et al., 2012). Other suggested mechanisms underlying the impact of acidification are a significant decrease in brain aerobic potential, as observed in sharks (Rosa et al., 2016). Moreover, the increase in seawater temperatures associated with GW is expected to impact many physiological processes and ecological interactions (Pörtner and Farrell, 2008), and to affect a number of behavioural traits (Nagelkerken and Munday, 2016). This suggests that the evaluation of the impact of global changes on the behaviour and physiology of marine organisms needs to consider multiple stressors (e.g. GW and OA). Understanding multiple stressor interactions is a key step for a better assessment of the ecological repercussions of climate change (Byrne and Przeslawski, 2013; Hale et al., 2011; Nagelkerken and Munday, 2016).

Elevated  $P_{\text{CO}_2}$  and temperature have been shown to affect the movement behaviour and activity of various marine species (Briffa et al., 2012; Nagelkerken and Munday, 2016). Although marine fishes can be considered as a model group for OA because much of the research on the behavioural effects of OA has been carried out on them (Nagelkerken and Munday, 2016), some recent evidence has shown that elevated  $P_{\text{CO}_2}$  levels can also affect the behaviour of some invertebrate species. For example, OA was shown to alter the antipredator behaviour of jumping snails (Watson et al., 2014), king scallops (Schalkhauser et al., 2013) and squid (Spady et al., 2014), the righting time and predator perception of the muricid gastropod *Concholepas concholepas* (Manríquez et al., 2013, 2014, 2016), the decision-making, locomotor activity and ability to locate an odour source in hermit crabs (de la Haye et al., 2011, 2012), and the swimming activity of the decapod *Metapenaeus joyneri* (Dissanayake and Ishimatsu, 2011). These behavioural changes may have far-reaching ecological consequences in a rapidly changing environment (Nagelkerken and Munday, 2016). Therefore, further studies on the effect of elevated  $P_{\text{CO}_2}$  levels (along with other stressors such as temperature) on the movement behaviour of key species are fundamental in order to increase our predictive ability on the effect of climate change on marine ecosystems.

Lateralization, defined as the tendency to have a preferred side for turning, limb use or localization of function on one side of the body (Frasnelli et al., 2012; Vallortigara and Rogers, 2005) is one of the behavioural traits that was recently shown to be affected by both elevated  $P_{\text{CO}_2}$  and temperature levels, in marine fishes (Domenici et al., 2014). Behavioural lateralization has been studied extensively in vertebrates and was shown to provide a number of advantages in cognitive tasks in general and specifically in collective behaviour, escaping and multitasking behaviours (Vallortigara and Rogers, 2005). Lateralization may also be a feature of relatively simple brains and indeed, recent evidence shows that behavioural

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asymmetries can occur in invertebrates, suggesting that lateralization of the nervous system can confer advantages at the individual level in invertebrates as well (Frasnelli, 2013; Frasnelli et al., 2012). This highlights the notion that important traits such as odour-liking and odour-aversion displacements in marine invertebrates such as mollusc gastropods can reflect the existence of asymmetries in the nervous system (Frasnelli, 2013; Frasnelli et al., 2012). Although recent studies have shown that the lateralization of various species of fish at the individual and population level is disrupted by exposure to elevated  $P_{\text{CO}_2}$  levels (Domenici et al., 2012, 2014; Jutfelt et al., 2013) as well as by hypoxia and increased temperature (Domenici et al., 2014; Lucon-Xiccato et al., 2014), nothing is known about the effect of these environmental factors on lateralization in invertebrates.

The temporal stability of a phenotypic trait within individuals relative to differences in that trait among individuals is known as trait repeatability and it is an important characteristic that is receiving increasing attention (Bell et al., 2009; Killen et al., 2016). Most previous studies on repeatability have been carried out within the context of evolutionary processes in ecology. However, as argued by Killen et al. (2016), the extent to which environmental variables will affect trait repeatability (i.e. across-context repeatability) will play a fundamental role in the plastic responses of species to climate change. Previous work has shown that environmental factors can affect trait repeatability in both marine (Briffa and Greenaway, 2011) and terrestrial organisms (Brommer, 2013). However, little is known about how trait repeatability is affected by elevated  $P_{\text{CO}_2}$  levels (Killen et al., 2016).

Here, we investigate the combined effect of long-term exposure to elevated  $P_{\text{CO}_2}$  and temperature levels on locomotion and behaviour (including lateralization) of a marine gastropod, the Chilean abalone *Concholepas concholepas* (Bruguière 1789). This species is a keystone rocky shore predator characteristic of the southeastern Pacific Ocean coast (Castilla, 1999; Castilla and Paine, 1987). Individuals present a number of morphological asymmetries that might potentially affect lateralization. These include the position of the siphon that brings seawater with external signals to the osphradium and ctenidium both situated in the anterior left (anteriorly on the left side) (Huaquín, 1966; Huaquín and Garrido, 2000; Maldonado, 1965) and the shape of the shell with the umbo situated on the left. In *C. concholepas*, as in other gastropods, locomotion associated with predator–prey interactions involves bilateral pedal crawling by muscular contractions waves and the secretion of a mucous sheet (Denny, 1980). Previous studies based on Y-maze experiments have shown that juvenile *C. concholepas* use chemical cues originated from prey and predator to orientate their displacement (Manríquez et al., 2013). After long-term (5 months) exposure to elevated  $P_{\text{CO}_2}$  levels, small juveniles of this species lost the ability to avoid a predator cue, but maintained their capacity to perceive cues originated from prey items (Manríquez et al., 2014). Although the sensory response of *C. concholepas* to a prey is unaffected by elevated  $P_{\text{CO}_2}$  levels, it is not known if such levels affect the locomotion and behaviour (e.g. time to cover a given distance, route finding) associated with prey detection. *Concholepas concholepas* is an economically and ecologically important component of the rocky intertidal and subtidal communities along the Chilean coast (Castilla 1988, 1999). As a result, it is likely to be exposed to daily fluctuations of environmental conditions. Although this may make it relatively tolerant to short-term changes, its response to longer term changes would be more relevant for its potential response to climate change.

Ocean temperatures and  $P_{\text{CO}_2}$  are predicted to increase in most regions of the world by the end of the year 2100 (IPCC, 2104; Meinshausen et al., 2011). The rise of atmospheric  $\text{CO}_2$  caused by human activity is the main source of the Earth's energy imbalance driving GW (von Schuckmann et al., 2016) and the increment of the  $\text{CO}_2$  flux from the atmosphere to the ocean driving OA (Doney et al., 2009). Regionally, the subtropical coast of western South America is expected to respond to GW in various ways. While GW may intensify the upwelling winds (Bakun, 1990; Garreaud and Falvey, 2009) and La Niña events (Cai et al., 2015) producing a regional cooling, more frequent El Niño-like conditions as a response to the increased concentration of greenhouse gases (Timmermann et al., 1999) will be characterized by extreme warm periods (Cai et al., 2014). During the strong El Niño event of 1997–1998, the coastal waters off northern Chile were anomalously warm ( $+3^\circ\text{C}$  anomalies) and supersaturated in  $\text{CO}_2$  (Torres et al., 2003). We took into account this scenario to determine the treatments to test the potential response of *C. concholepas* to global change characterized by the intensification of extreme events (Easterling et al., 2000).

Using long-term exposure (6 months) we manipulated water temperature and  $P_{\text{CO}_2}$  levels to determine their synergistic effects on various locomotor and behavioural traits (i.e. movement duration, decision time, route finding and lateralization) in juvenile *C. concholepas* in a T-maze tank with a prey item positioned behind a barrier at the end of a runway, as well as on the across-context repeatability of each trait. We hypothesized that the locomotion and behaviour of small juveniles of this species will be negatively affected after exposure to the combination of elevated  $P_{\text{CO}_2}$  and temperature levels.

## MATERIALS AND METHODS

### Animals and experimental treatments

Small juvenile *C. concholepas* (~1.5 cm in length, approximately 3 months of age, benthic life) were collected in rocky intertidal platforms of southern Chile ( $39^\circ45'51''\text{S}$ ;  $73^\circ23'54''\text{W}$ ) dominated by barnacle stands and the mussels *Semimytilus algosus* and *Perumytilus purpuratus*. The individuals were then transported to Coquimbo (northern-central Chile;  $29^\circ57'58''\text{S}$ ;  $71^\circ21'17''\text{W}$ ) where all the experiments were conducted. In the laboratory, 40 juveniles were reared in a Plexiglass aquarium ( $18\times30\times17$  cm,  $\text{high}\times\text{length}\times\text{width}$ ) with running seawater ( $1.5$  litre  $\text{h}^{-1}$ ) and fed *ad libitum* with small live specimens of *S. algosus* for 1.5 months (i.e. 45 days). During this period, the aquarium was semi-immersed in a sea table with running seawater to maintain the temperature at  $\sim 14.8\pm 0.19^\circ\text{C}$  and  $\text{CO}_2$  level at  $506.68\pm 16.90$   $\mu\text{atm}$   $P_{\text{CO}_2}$  (mean  $\pm$  s.e.). After this 1.5 month period, locomotor and behavioural traits were evaluated using a two-way T-maze runaway (Fig. S1). All individuals were tested in the same conditions at which they were kept during the initial rearing period [pH 7.79 at  $25^\circ\text{C}$  compared with *in situ* (i.e. actual) pH of 7.93]. Prior to the T-maze experiments, *C. concholepas* were deprived of food for 2 days in order to standardize hunger levels. After the behavioural observations in the T-maze, 10 individuals per group were haphazardly assigned to one of four treatments (see below). Individuals in each treatment show no significant difference in shell size at the beginning of the treatment period (one-way ANOVA, d.f.=3,  $F=0.33$ ;  $P=0.80$ ; mean shell length= $1.55\pm 0.042$  cm; mean  $\pm$  s.e.).

The measurements and the behavioural observations in the T-maze were repeated in the same individuals after ~6 months (treatment phase; 178 days) of acclimation at the following four conditions: (1)  $15^\circ\text{C}$  and present day  $500$   $\mu\text{atm}$   $P_{\text{CO}_2}$  levels [15P

(control),  $N=9$ ]; (2) 15°C and future 1400  $\mu\text{atm } P_{\text{CO}_2}$  levels (15F,  $N=9$ ); (3) 19°C and present day 500  $\mu\text{atm } P_{\text{CO}_2}$  levels (19P,  $N=8$ ); and (4) 19°C and future 1400  $\mu\text{atm } P_{\text{CO}_2}$  (19F,  $N=10$ ). A total of 36 individuals (9, 9, 10 and 8 per treatment, respectively) out of the initial 40 were tested after the 6 month period; 4 individuals were discarded because they had escaped from the rearing bottles. Treatment levels were nominal. Actual values based on seawater chemistry and temperature records are reported in Table 1. The low  $P_{\text{CO}_2}$  level was chosen as representative of the current-day level in coastal areas near the collection site (Torres et al., 2013) whereas the high  $P_{\text{CO}_2}$  level was chosen to represent conditions predicted under worst-case scenarios for the beginning of the next century (Meinshausen et al., 2011). Seawater chemistry analysis (Table 1) gives values of current day  $P_{\text{CO}_2}$  used here, which are within the range of those found in nature by *C. concholepas*. Indeed, throughout the year, at the site of collection, *C. concholepas* experience temperatures in the range of 10–16°C with summer temperature ranging from 14 to 16°C, and  $P_{\text{CO}_2}$  ranging from 200 to 650  $\mu\text{atm}$  (Torres et al., 2013). The control temperatures (15±0.5°C) were chosen to match the average high temperatures near the collection site during the austral summer months (Torres et al., 2013) while the elevated temperature (19°C) was chosen to approximate the temperature increase (+4°C) predicted for the beginning of the next century under scenario RCP8.5 (IPCC, 2014), although this level of warming (+4°C) is likely to occur sooner as a result of extreme events such as El Niño periods (Easterling et al., 2000). During the treatment phase, snails were individually maintained in 2 litre plastic bottles (each snail within its own bottle), sealed with a plastic lid (with a small hole to allow tube access) filled with 1.5 litre of 1  $\mu\text{m}$  filtered seawater (FSW) conditioned at the required  $P_{\text{CO}_2}$  level. The system in which the experimental individuals were reared was not recirculating. Only the water in the sea-tables in which the rearing bottles were semi-immersed was recirculated, meaning that any chemicals present in the sea-tables were never in contact with the seawater within each bottle. Conditioned FSW from the appropriate header tank (see below) was then used to replace the seawater of each bottle every second day.

Each individual was reared during the 6 months of treatment within its own bottle, thus providing a means of replication. However, the system did not include as many tanks with controlled temperature as the rearing bottles. Based on the terminology suggested by Cornwall and Hurd (2016), the system used had two mixing tanks associated with each experimental sea table (one set at 15°C and the other at 19°C). In these 200 litre tanks, a mix between the seawater and the corresponding gases took place. Every 48 h, the seawater in each individual tank (plastic bottles) was replaced using the seawater taken from the corresponding mixing tank. During the time between seawater exchanges, the required  $P_{\text{CO}_2}$  in the bottles was maintained by the bubbling of the required gases as for the corresponding mixing tank. This experimental array created the four contrasting  $P_{\text{CO}_2}$  treatments conditions (Table 1) with independent experimental units that corresponded to the individual plastic bottles. This design is a modified version of the designs described by Cornwall and Hurd (2016); all the bottles share a mixing tank but at the same time each tank has its own and continuous supply of pre-mixed gases. However, our experimental design also included temperature as a second experimental variable. This is a potential limitation of our methods, since it does not allow us to distinguish between the treatment effect (temperature) and a potential tank effect. At the end of the experiments all the surviving individuals were returned to the collection site.

### Seawater acidification and carbonate system determination in the equilibrated seawater

Conditioned FSW with the two  $P_{\text{CO}_2}$  levels was generated in four polyethylene (230 litre) header tanks. Each bottle was filled with conditioned FSW, using a tube inserted within each bottle, fitted with an air stone through which a continuous stream of either air (~400  $\mu\text{atm } \text{CO}_2$ ) or enriched  $\text{CO}_2$  air (~1200  $\mu\text{atm } \text{CO}_2$ ) was bubbled through the water. Enriched  $\text{CO}_2$  air was produced by blending air and pure  $\text{CO}_2$  using mass flow controllers (Aalborg, model GFC) (Torres et al., 2013). During the rearing phase, seawater parameters (i.e. pH, temperature, salinity and total alkalinity) were measured twice a week in seawater samples obtained from the rearing aquarium (Table 1). In the treatment phase, the parameters were measured twice a week in water samples taken from the bottles before the water change took place. In the rearing phase, these measurements represent the final carbonate speciation due the continuous stream of air and the cumulative effect on the carbonate system of respiration, calcification and ammonium excretion associated with the metabolism of both the snails and the food (*S. algosus*) between water samples in a running seawater system. However, in the treatment phase, these measurements represent the final carbonate speciation due the continuous stream of either air or enriched  $\text{CO}_2$  air and the cumulative effect on the carbonate system of the 48 h of respiration, calcification and ammonium excretion in a still-water system. The pH measurements were made in a closed 25 ml cell, thermostatically controlled at 25.0°C, with a Metrohm 713 pH meter (input resistance >10<sup>13</sup> Ohm) and a glass fixed ground-joint diaphragm electrode with Pt1000 (Metrohm 6.0257.000, Aquatrode Plus) calibrated with standard Tris buffer in synthetic sea water.

Tris buffer was prepared at salinity 35, rigorously following the standard operating procedures outlined by Dickson and Goyet (1994). The pH of this buffer was calculated to be 8.089 pH units at 25.0°C and salinity 35 on the total hydrogen ion scale (Dickson and Goyet, 1994). We checked the accuracy of this buffer against pH certified Tris buffers (using hydrogen electrode system at 25°C) obtained from Andrew Dickson's laboratory (Nemzer and Dickson, 2005). We discarded any Tris buffer batch that differed from the Dickson Tris buffer by more than 0.003 pH units.

Total alkalinity (AT) was determined by potentiometric titration in an open cell, according to Haraldsson et al. (1997). The accuracy was controlled against a certified reference material supplied by Andrew Dickson (Scripps Institution of Oceanography). The correction factor was  $\approx 1.002$ , corresponding to a difference of <5  $\mu\text{mol kg}^{-1}$ . Each sample was analysed using 2 or 3 replicates. Temperature and salinity were measured using a CTD (Ocean Seven 305). The pH, AT and hydrographic data were used to calculate the rest of the carbonate system parameters at *in situ* temperature (pH,  $P_{\text{CO}_2}$ ,  $[\text{CO}_3^{2-}]$  and the saturation state of  $\Omega$  aragonite and calcite), using CO2SYS software (Lewis et al., 1998) set with Mehrbach solubility constants (Mehrbach et al., 1973) refitted by Dickson and Millero (1987).

### Shell length

Shell length was measured in all individuals before and after the 6 month treatment, to test whether  $P_{\text{CO}_2}$  and/or temperature had an effect on growth, which could potentially modulate any effect on locomotion.

### T-maze set-up and lateralization

The experimental apparatus was based on a design used previously to investigate behavioural lateralization in fish (Domenici et al.,

**Table 1. Mean ( $\pm$ s.e.) conditions of seawater used to maintain juvenile *Concholepas concholepas* during the rearing and treatment phases**

	Temperature (°C)	pH at 25°C	pH <i>in situ</i>	AT ( $\mu\text{mol kg}^{-1}$ )	$P_{\text{CO}_2}$ <i>in situ</i> ( $\mu\text{atm}$ )	$[\text{CO}_3^{2-}]$ <i>in situ</i> ( $\mu\text{mol kg}^{-1}$ SW)	Salinity	$\Omega$ Calcite	$\Omega$ Aragonite
1.5 month rearing phase									
15P	14.85 (0.19)	7.79 (0.02)	7.93 (0.03)	2283.14 (4.28)	506.68 (16.90)	124.43 (4.74)	34.24 (0.07)	2.98 (0.11)	1.91 (0.07)
6 month treatment phase									
15P	15.72 (0.12)	7.71 (0.02)	7.85 (0.02)	1865.37 (52.31)	539.45 (9.74)	91.66 (4.74)	34.53 (0.03)	2.19 (0.11)	1.41 (0.07)
15F	15.62 (0.09)	7.35 (0.01)	7.47 (0.01)	1942.44 (42.65)	1462.74 (25.37)	42.39 (1.86)	34.61 (0.04)	1.01 (0.04)	0.65 (0.03)
19P	19.15 (0.10)	7.65 (0.03)	7.73 (0.03)	1692.22 (69.71)	651.33 (25.54)	77.67 (6.06)	34.55 (0.03)	1.86 (0.15)	1.21 (0.09)
19F	19.27 (0.05)	7.40 (0.01)	7.47 (0.02)	1906.12 (42.35)	1445.91 (33.21)	47.99 (2.09)	34.61 (0.10)	1.15 (0.05)	0.75 (0.03)

15P, present day levels (500  $\mu\text{atm}$ ) of  $\text{CO}_2$  at 15°C; 15F, future levels (1400  $\mu\text{atm}$ )  $\text{CO}_2$  at 15°C; 19P, present day levels (500  $\mu\text{atm}$ ) of  $\text{CO}_2$  at 19°C; 19F, future levels (1400  $\mu\text{atm}$ ) of  $\text{CO}_2$  at 19°C. The elevated  $P_{\text{CO}_2}$  is based on the rate of change in pH predicted by the most extreme scenario (RCP8.5 scenario) of atmospheric  $\text{CO}_2$  for the beginning of the next century. See Meinshausen et al. (2011) for further details.

2014). This consisted of a two-way T-maze runaway (Fig. S1) which allowed scoring of the turning direction (i.e. left or right) of each individual in 8 consecutive runs. To account for any possible asymmetry in the set-up, tests were carried out alternately on the two ends of the runway. The T-maze tank was made of black Plexiglass (60×29×10 cm, length×width×height) with a runaway in the middle section (25×4 cm, length×width). At both ends of the runaway and 5 cm ahead of the side walls, a black Plexiglass barrier (10×12.5 cm, width×height, 0.3 cm thick) was located. Forty prey items (the mussel *Semimytilus algosus*) were positioned inside a plastic cylindrical cage behind the barrier to attract *C. concholepas* (Manríquez et al., 2013), because elevated  $P_{\text{CO}_2}$  is known to have no effect on prey detection in *C. concholepas* (Manríquez et al., 2014). The prey items were removed after each run, and a new group of prey items was positioned in the opposite end of the runaway. Between consecutive runs (of the same individual) and also between individuals, the seawater in the T-mazes was replaced and the tank was carefully cleaned with tap water to remove any remains of previously formed mucous track left by *C. concholepas* that potentially could affect turning direction. The T-maze was filled with 5 litres of FSW to obtain a water depth of 3.5 cm. The seawater used during the detour test had the same  $P_{\text{CO}_2}$  level and temperature as the holding tanks.

Turning preference (i.e. bias in left or right turns) was assessed using the relative lateralization index ( $L_R$ , ranging from  $-100$  to  $+100$ ), indicating complete preference for left or right turning, respectively, using: [(turn to the right–turn to the left)/(turn to the right+turn to the left)]×100. The strength of lateralization (irrespective of its direction) was then assessed using the absolute lateralization index ( $L_A$ ), ranging from 0 (an individual that turned in equal proportion to the right and to the left) to 100 (an individual that turned right or left on all 8 trials) (Bisazza et al., 1998; Domenici et al., 2012).

At the beginning of the T-maze measurements, one single individual was positioned in the mid-point of the runaway (i.e. 12.5 cm from the end of the runaway) with its head oriented toward the end of the runaway bearing the prey for an adjustment period of 10 min using two PVC tube stoppers. After this 10 min period, the PVC tube stoppers were removed and the animals were allowed to move towards the barrier. To prevent disturbances, a semi-transparent piece of Plexiglass (35×14 cm, width×length) was used to cover the entire runaway. At the end of the runaway and in front of the barrier, a decision zone was defined (i.e. a 4×5 cm area delimited by the barrier and the exit of the runaway, Fig. S1). A mirror positioned above the tank was used to make all the observations. The turnings were considered left or right when the entire snail had crossed the left or right edge of the decision zone

(Fig. S1). All the experimental trials in the detour test ended with *C. concholepas* successfully reaching the prey.

### Locomotion and route finding

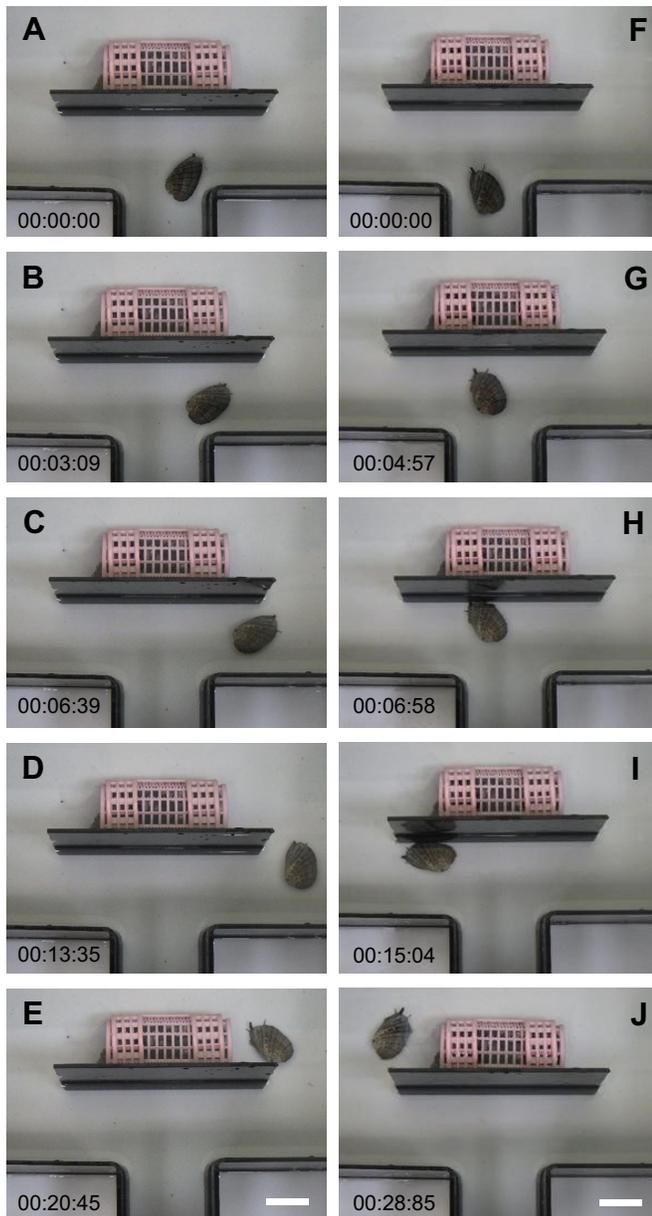
A set of locomotor and behavioural traits were scored while the snail was crawling through the runaway. The total time spent by the snail to cover the distance from the midpoint of the runaway (i.e. at the beginning of the trial) to the decision zone in the eight runs summed together, was defined as movement duration ( $t_M$ ). Therefore,  $t_M$  is an index of the average speed (i.e. a low  $t_M$  indicates a high speed) at which the snails crawled while covering the distance between the centre of the T-maze and the decision zone (Fig. S1). The total time (i.e. the sum of the time spent in the 8 runs) that each snail spent in the decision zone was defined as decision time ( $t_D$ ). Route finding (RF) was assessed by scoring the number of runs out of the total (i.e. as a proportion out of 8) within which the snail made no contact with the Plexiglass barrier at the end of the runaway before turning, but rather crawled directly around the barrier in order to reach the prey (see Fig. 1A–E). Hence RF ranges from 0 to 1 where 1 implies the highest route finding performance. Examples of route finding with and without contact with the Plexiglass barrier are shown in Fig. 1.

### Statistics

Parametric statistics were used unless the distribution was not normal (based on D'Agostino–Pearson test for normality), in which case non-parametric tests or rank-transformations were used. For relative lateralization, a mean  $L_R$  near zero indicates that a given sample of the population is neither left- nor right-biased in its turning tendency (Bisazza et al., 2000). Significant sample-level departures from unbiased turning ( $L_R=0$ ) were assessed by one-sample two-tailed  $t$ -tests performed on the mean values of  $L_R$  (Bisazza et al., 2000; Zar, 1984).

The strength of lateralization (irrespective of its direction) at the individual level was assessed by comparing each treatment's distribution with the proportions of absolute lateralization expected from random choice based on a binomial distribution [with 8 repetitions and  $p$  (probability to turn left)= $q$  (probability to turn right)=0.5] using a  $G$ -test.

Repeatability was tested between the trials before (first trial) and after treatment (second trial), using a Pearson's correlation (Spearman in the case of non-normal distribution). Repeatable variables were defined as those with a significant positive correlation between the first and second trial (Killen et al., 2016; Klueen and Brommer, 2013). Lateralization,  $t_D$ ,  $t_M$  and RF before and after treatment were compared using a paired- $t$ -test (Wilcoxon test for non-normal distribution). Two-way ANOVAs, followed by a *post hoc* test (Tukey test) were used to compare the variables before



**Fig. 1. Photographic records of two juvenile *Concholepas concholepas* crawling near the barrier at one end of a T-maze.** Turns without and with contact with the barrier in the decision zone are shown in two sequences (A–E and F–J, respectively). Time (min:s:1/100 of s) of each frame relative to the first frame (time=0) is indicated in the bottom left of each panel. In both cases (E and J, respectively), individuals ended up near the cage (containing the prey) behind the barrier. Scale bars: 2.5 cm. These sequences are for the purpose of illustration and were taken without a cover on the runway. Pictures were taken with a still camera Ricoh WG-30 and a digital chronometer for the timing.

and after treatment. A rank-transformation using aligned rank transform (Wobbrock et al., 2011) was used if the distribution was not normal.

## RESULTS

### Shell length

Mean values of shell length after 6 months of treatment were: 15P (control),  $3.55 \pm 0.09$ ; 15F,  $3.42 \pm 0.20$ ; 19P,  $3.94 \pm 0.05$ ; and 19F,  $3.73 \pm 0.08$  cm. Shell length of all samples was not different from a normal distribution (D'Agostino test,  $P > 0.05$  in all cases). Two-way

ANOVA comparing individuals after 6 months of treatment, showed that temperature had a positive effect on shell length (d.f.=1,  $F=7.9$ ,  $P < 0.01$ ), while  $P_{CO_2}$  had no effect (d.f.=1,  $F=1.80$ ,  $P=0.18$ ) and the interaction was also not significant (d.f.=1,  $F=0.07$ ,  $P=0.79$ ). *Post hoc* comparisons (Tukey test) showed significant differences between 15°C and 19°C groups at present day  $P_{CO_2}$  levels (15P vs 19P:  $P < 0.05$ ); however, comparisons between all other variables showed no significant differences.

### Relative lateralization ( $L_R$ )

$L_R$  of all samples was not different from a normal distribution (D'Agostino test,  $P > 0.05$  in all cases). For each treatment,  $L_R$  data did not differ from the theoretical mean value of zero (Bisazza et al., 2000) ( $P > 0.05$  in all cases), implying that none of the samples showed a turning bias at the population level. No difference between the  $L_R$  before and after the 6 month treatment was found for 15P ( $P=0.59$ ,  $t_8=0.55$ ), 15F ( $P=0.49$ ,  $t_8=0.72$ ), 19P ( $P=0.10$ ,  $t_7=1.87$ ) or 19F ( $P=0.24$ ,  $t_9=1.24$ ).

$L_R$  was repeatable for 15P (Pearson  $r=0.98$ ,  $P < 0.0001$ ) and 19P (Pearson's  $r=0.96$ ,  $P < 0.001$ ), but not for 15F (Pearson's  $r=0.08$ ,  $P > 0.5$ ) or 19F (Pearson's  $r=0.11$ ,  $P > 0.5$ ), suggesting that any turning bias at the individual level was maintained in control  $P_{CO_2}$  levels regardless of temperature, whereas it was lost after individuals were exposed to elevated  $P_{CO_2}$  levels for 6 months (Fig. 2). Two-way ANOVA comparing individuals after 6 months of treatment, showed that relative lateralization was not affected by temperature (d.f.=1,  $F=0.17$ ,  $P=0.68$ ) nor by  $P_{CO_2}$  level (d.f.=1,  $F=0.75$ ,  $P=0.39$ ) and no interaction effect was found (d.f.=1,  $F=0.002$ ,  $P=0.96$ ) (Fig. 3A).

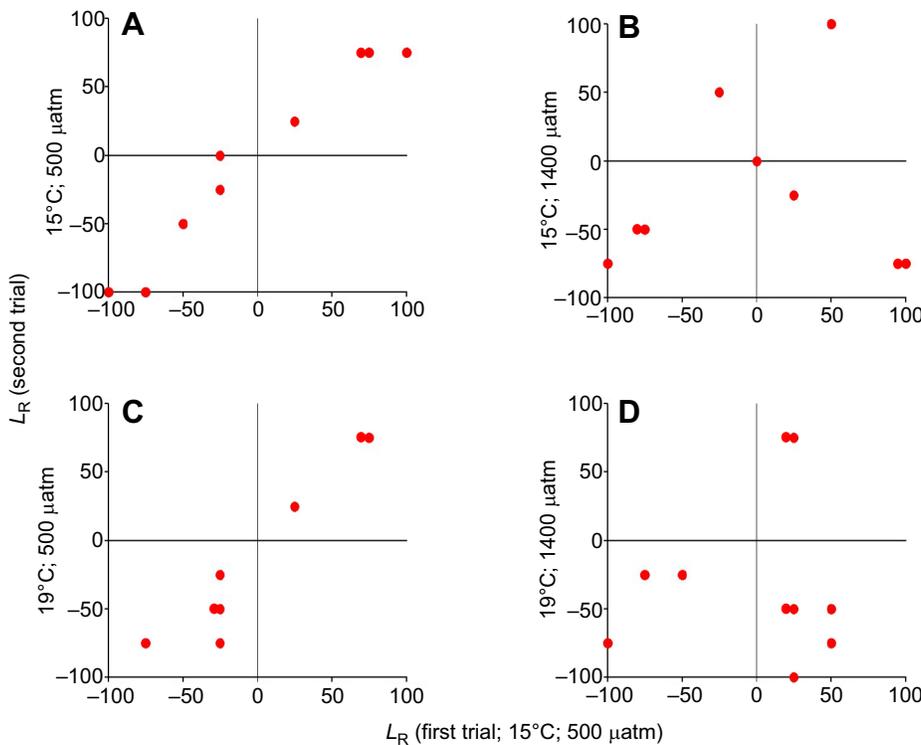
### Absolute lateralization ( $L_A$ )

$L_A$  of all samples were not different from a normal distribution (D'Agostino test,  $P > 0.05$  in all cases). As for  $L_R$ , no significant differences in the absolute lateralization before and after treatment was found in all cases; for 15P ( $P=0.59$ ,  $t_8=0.55$ ), 15F ( $P=0.56$ ,  $t_8=0.61$ ), 19P ( $P=0.10$ ,  $t_7=1.87$ ) and 19F ( $P=0.26$ ,  $t_9=1.20$ ). Overall, in 46% of the trails, snails turned in a consistent direction in  $\geq 7$  out of 8 runs. Therefore,  $L_A$  was relatively high in all cases (ranging from 43.7 to 61.1) since individuals had the tendency to turn in the same direction in successive runs. Accordingly, snails of all groups (before and after treatment) exhibited a significantly higher preference for making either left or right turns than expected by chance (*G*-test:  $P < 0.001$  in all cases, d.f.=4), except for 19F before the 6 month treatment ( $P=0.055$ ).

Absolute lateralization was repeatable for 15P (Pearson's  $r=0.9058$ ,  $P < 0.001$ ), 15F (Pearson's  $r=0.69$ ,  $P=0.04$ ), borderline significant for 19P (Pearson's  $r=0.70$ ,  $P=0.05$ ), but not significant for 19F (Pearson's  $r=-0.2450$ ,  $P=0.49$ ). The measurements of  $L_A$  for the present day conditions (15P and 19P) reflect those of  $L_R$  for the present day  $P_{CO_2}$  conditions. For 15F, significant repeatability for  $L_A$  and not for  $L_R$  implies that individuals tend to maintain their tendency to turn in a given direction, although the side of the turning bias was not necessarily the same as before the treatment. Two-way ANOVA for absolute lateralization showed no effect of  $P_{CO_2}$  (d.f.=1,  $F=0.003$ ,  $P=0.96$ ) nor of temperature (d.f.=1,  $F=0.015$ ,  $P=0.90$ ), and the interaction effect was not significant (d.f.=1,  $F=0.12$ ,  $P=0.73$ ) (Fig. 3B).

### Locomotion

Both movement duration ( $t_M$ ) and decision time ( $t_D$ ) were not different from a normal distribution (D'Agostino test,  $P > 0.05$  in all cases). No differences in the  $t_M$  and  $t_D$  before and after treatment



**Fig. 2. Repeatability of relative lateralization of *C. concholepas* analysed using correlations.** x-axis=first trial; y-axis=second trial after the 6 month treatment. The first trial was always carried out in present conditions (500  $\mu\text{atm CO}_2$ ; 15°C). Relative lateralization was repeatable when the second trial was for (A) 15P (500  $\mu\text{atm CO}_2$ ; 15°C; Pearson's  $r=0.98$ ,  $P<0.0001$ ,  $N=9$ ), (C) 19P (500  $\mu\text{atm CO}_2$ ; 19°C; Pearson's  $r=0.96$ ,  $P<0.001$ ,  $N=8$ ), but not for 15F (1400  $\mu\text{atm CO}_2$ ; 15°C; Pearson's  $r=0.08$ ,  $P>0.5$ ,  $N=9$ ) and (D) 19F (1400  $\mu\text{atm CO}_2$ ; 19°C; Pearson's  $r=0.11$ ,  $P>0.5$ ,  $N=10$ ). Data with the same x- and y-values of relative lateralization are shown as two adjacent data points.

were found in 15P ( $t_M$ ,  $P=0.10$ ,  $t_8=0.45$ ;  $t_D$ ,  $P=0.10$ ,  $t_8=1.87$ ) and 19P ( $t_M$ ,  $P=0.06$ ,  $t_7=2.19$ ;  $t_D$ ,  $P=0.92$ ,  $t_7=0.10$ ). However, both were significantly different before and after treatment in 15F ( $t_M$ ,  $P=0.013$ ,  $t_8=3.150$ ;  $t_D$ ,  $P<0.001$ ,  $t_8=5.46$ ) and in 19F ( $t_M$ ,  $P=0.02$ ,  $t_9=2.809$ ;  $t_D$ ,  $P=0.027$ ,  $t_9=2.64$ ). Therefore, in the control  $P_{\text{CO}_2}$  level, locomotor traits did not change at the sample level after a 6 month period, whereas exposure to an elevated  $P_{\text{CO}_2}$  level increased both movement duration and decision time (i.e. decreased speed of locomotion). None of the locomotor traits were repeatable; therefore, individuals did not retain these performance values in the long term.

Two-way ANOVA showed that  $P_{\text{CO}_2}$  had a significant effect on  $t_M$  (d.f.=1,  $F=7.57$ ,  $P=0.01$ ), while temperature had no effect (d.f.=1,  $F=1.56$ ,  $P=0.22$ ) and the interaction was also not significant (d.f.=1,  $F=0.75$ ,  $P=0.39$ ). *Post hoc* comparisons for  $t_M$  (Tukey test) showed that 19P differed from 19F ( $P=0.016$ ), while all other comparisons were not significant (Fig. 4A). Two-way ANOVA shows that  $P_{\text{CO}_2}$  had a significant effect on  $t_D$  (d.f.=1,  $F=34.32$ ,  $P<0.001$ ), while temperature had no effect (d.f.=1,  $F=1.29$ ,  $P=0.26$ ) and the interaction was also not significant (d.f.=1,  $F=0.60$ ,  $P=0.44$ ). *Post hoc* comparisons for  $t_D$  (Tukey test) showed significant differences between 15P and 15F ( $P<0.001$ ), and 19P and 19F ( $P=0.001$ ), while all other comparisons were not significant (Fig. 4B).

### Route finding

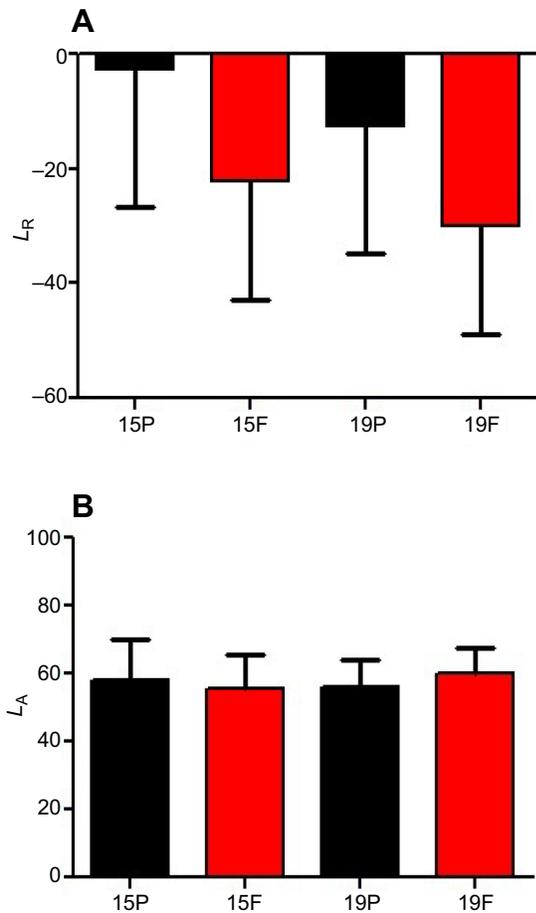
Route finding data were not different from a normal distribution in all groups (D'Agostino test,  $P>0.05$  in all cases), except for 15P (after-treatment trial,  $P=0.002$ ). RF was found to be significantly different before and after treatment in 15F ( $P<0.001$ ,  $t_8=5.96$ ), while all other comparisons yielded non-significant results: 15P ( $P=0.59$ , Wilcoxon  $W=-7$ , d.f.=8), 19P ( $P=0.89$ ,  $t_7=0.14$ ) and 19F ( $P=0.56$ ,  $t_9=0.60$ ). RF was not repeatable in any of the four treatments (Pearson test in all cases, except 15P, for which the Spearman test was used).

Two-way ANOVA on aligned ranked data (Wobbrock et al., 2011) showed that RF was significantly affected by  $P_{\text{CO}_2}$  level (d.f.=1,  $F=4.88$ ,  $P=0.03$ ) as well as by temperature (d.f.=1,  $F=5.96$ ,  $P=0.02$ ), and their interaction was significant (d.f.=1,  $F=18.38$ ,  $P<0.001$ ). *Post hoc* comparisons (Tukey test) within each temperature, showed significant differences between 15P and 15F ( $P<0.001$ ) but not between 19P and 19F ( $P=0.54$ ). Within each  $P_{\text{CO}_2}$  level, *post hoc* comparisons showed differences between 15F and 19F ( $P<0.01$ ) but not between 15P and 19P ( $P=0.2$ ) (Fig. 4C).

### DISCUSSION

The results show that a number of locomotor and behavioural traits of *C. concholepas* are affected by long-term exposure to elevated  $P_{\text{CO}_2}$ , while temperature has a modulating effect only in some cases. This is in line with previous work on the effect on multiple stressors on the lateralization behaviour of fish (Domenici et al., 2014). *C. concholepas* is an intertidal and subtidal species and therefore may experience a relatively wide range of temperatures; hence, it may be quite tolerant to elevated temperatures. Repeatability is affected by elevated  $P_{\text{CO}_2}$  only for relative lateralization (Fig. 2). However, no effects were detected on relative lateralization at the sample level, as mean relative lateralization appears to be unaffected by the treatments (Fig. 3A).

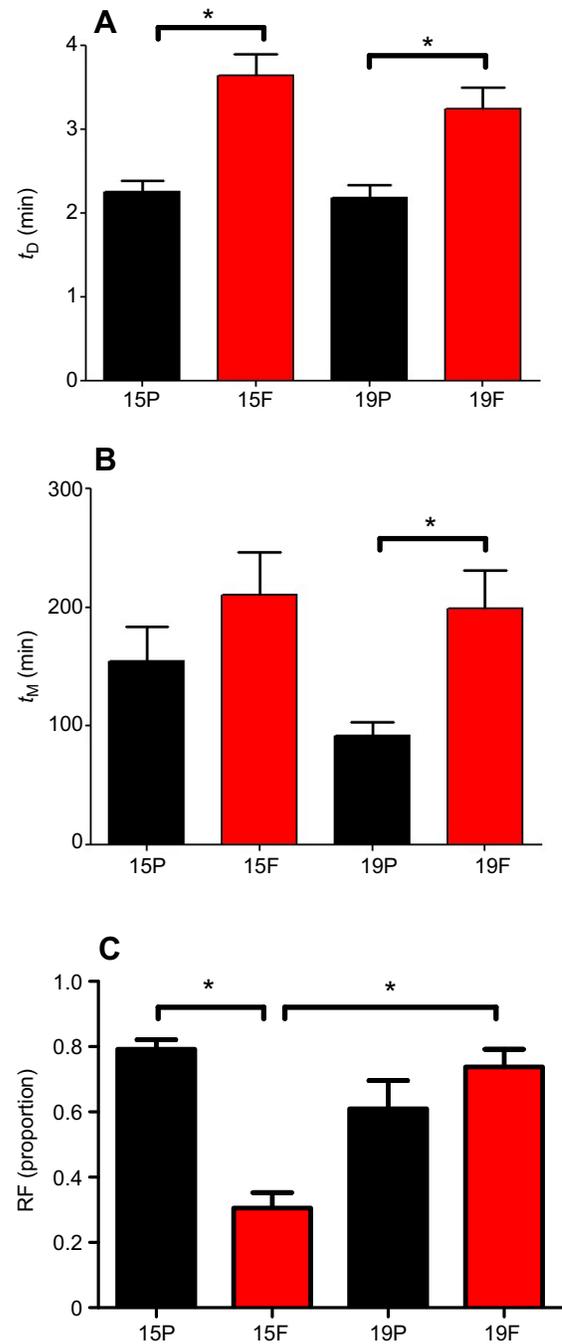
Interestingly, at the sample level, snails do not exhibit a significant turning bias in any of the treatments, although they show a non-significant tendency to turn left (Fig. 3A). Although previous work on the same species has demonstrated a side-bias in righting behaviour associated with morphological asymmetries, i.e. the projection of the umbo to the left in all individuals impedes righting in that direction (Manríquez et al., 2013), *C. concholepas* in captivity were observed to be able to turn in any direction if a barrier is positioned between them and a prey item (P.H.M., personal observations). In line with these observations, no directional bias in turning was shown by the present results. Long-term exposure to



**Fig. 3. Relative and absolute lateralization of *C. concholepas* after the 6 month treatment.** No significance differences were found in (A)  $L_R$  or (B)  $L_A$  among treatments (two-way ANOVA). 15P,  $N=9$ ; 15S,  $N=9$ ; 19P,  $N=8$ ; and 19F,  $N=10$ . Data are means $\pm$ s.e.

elevated  $P_{CO_2}$  did not have an effect on the lack of turning bias at the population level. The absence of a turning bias during prey searching at the population level might be advantageous by randomizing the movements and therefore the distributions of the individuals.

Although individuals of *C. concholepas* do not show a significant directional bias at the population level, they are significantly lateralized at the individual level: individuals tend to turn either left or right in most runs, more often than would be expected by random choice. This strong individual-level bias is in line with their significant tendency to retain their lateralization level in the long term (6 months) when tested in control water (i.e. significant repeatability, Fig. 2). However, elevated  $CO_2$  interferes with this tendency, and snails tested after 6 months in elevated  $P_{CO_2}$ , lost (not reversed) their individual turning bias (hence individuals that had a high proportion of right turns in the first trial at control levels, lost this patterns after 6 months in elevated  $P_{CO_2}$  when they may show higher proportion of left or right turns, see Fig. 2). As in previous works on fish and molluscs, it is possible that this behavioural effect caused by elevated  $P_{CO_2}$  levels is related to interference with the neurotransmitter function (Nilsson et al., 2012; Watson et al., 2014). Previous work on fish (Domenici et al., 2012) showed that absolute lateralization was decreased by short-term exposure (4 days) to elevated  $P_{CO_2}$  levels and suggested that such loss of lateralization may increase vulnerability, given that lateralization enhances



**Fig. 4. Decision time, movement duration and route finding of *C. concholepas* after the 6 month treatment.** Asterisks indicate significant differences in (A)  $t_D$ , (B)  $t_M$  and (C) RF resulting from the *post hoc* test within temperature or within  $P_{CO_2}$ . 15P,  $N=9$ ; 15F,  $N=9$ ; 19P,  $N=8$ ; and 19F,  $N=10$ . Data are means $\pm$ s.e.

performance in a number of cognitive tasks and anti-predator behaviours (Vallortigara and Rogers, 2005). Here, absolute lateralization was not affected by exposure to elevated  $P_{CO_2}$ . Therefore, it could be argued that elevated  $P_{CO_2}$  levels will not affect the vulnerability of this species through lateralization effects. However, the effect of elevated  $P_{CO_2}$  levels on across-context repeatability might have repercussions on the fitness of individuals if the control-level lateralization was optimized for each specific individual.

Neither movement duration nor decision time were repeatable traits. However,  $P_{CO_2}$  level (but not temperature) had a significant effect on both traits. Movement duration was affected by elevated  $P_{CO_2}$  only at 19°C, which could, in part, be explained by the higher activity (shorter movement duration) likely induced by high temperature at the control  $P_{CO_2}$  level, although the effect of temperature on movement duration was not significant. Generally speaking, high  $P_{CO_2}$  levels appear to slow down the snails, which could be related to negative effects on both locomotion and sensory performance. Elevated  $P_{CO_2}$  levels appear to have an even stronger effect on decision time, since the latter is affected at both temperatures. This may be due to the additive negative effect on various potential components of decision time such as cognitive, sensory and locomotor performance as found in other organisms (Briffa et al., 2012). It is unlikely that the effect of  $P_{CO_2}$  on locomotion is related to the effect of treatment on shell length, since the latter is not affected by  $P_{CO_2}$ . However, despite the fact that temperature affects shell length (with higher temperature resulting in larger shells), temperature did not affect  $t_M$  or  $t_D$ . It is therefore possible that the effect of temperature on shell length is not sufficient to cause differences in displacement speed. The larger size of individuals kept at higher temperature suggests that growth in *C. concholepas* increases even with temperatures (i.e. 19°C) that are higher than the maximum typically recorded in their natural habitat (15–16°C) (Navarro and Torrijos, 1994; Torres et al., 2013), suggesting that *C. concholepas* has tolerance for a wide range of temperatures. Previous work has shown that the scope for growth and net growth efficiency in *C. concholepas* were higher during the warm seasons (summer and spring), compared with the winter and the fall (Navarro and Torrijos, 1994). Our results suggest that this trend to increase growth with temperature continues up to 19°C.

All the experimental trials in the detour test ended with *C. concholepas* reaching the prey successfully. This suggests that the ability to eventually locate the prey was not affected by the experimental conditions as found by a previous study (Manríquez et al., 2014). However, the relatively high proportion of trials in which the snail made contact with the barrier in elevated  $P_{CO_2}$  levels at 15°C suggests a negative effect of elevated  $P_{CO_2}$  on perception of the barrier, or more simply, a decreased ability to follow the most direct path towards the prey scent coming from either sides of the barrier. Furthermore, although little is known about the visual performance in gastropods, most gastropod species have some capacity for using their eyes to keep on a straight course, and a few have a very basic capacity for pattern recognition and landmark navigation (Land and Nilsson, 2006). The negative effect of elevated  $P_{CO_2}$  levels on eyesight through effects on the retinal reaction has been reported in fish (Chung et al., 2014). Thus, it is possible that near-future  $P_{CO_2}$  levels might impair the capacity of other marine organisms such as molluscs to respond visually not only to fast events such as predator attacks (Allan et al., 2013), but also to take the most direct route towards a prey without contact with a barrier.

Furthermore, it is possible that other senses (in addition to, or instead of, vision) that may be potentially involved in minimizing the path length to reach the prey, were negatively affected by the elevated  $P_{CO_2}$  in our experiments. These may include mechanical reception of the barrier as well as chemoreception of the precise direction of prey odour from either sides of the barrier. Chemoreception in gastropods is mediated by the osphradium, an external sensory organ containing neuro-epithelial cells which monitor the physiochemical properties of the surrounding seawater (Karnik et al., 2012). Although previous work shows that predator, but not prey, odour detection is affected by

elevated  $P_{CO_2}$  in *C. concholepas* (Manríquez et al., 2014), it is possible that elevated  $P_{CO_2}$  may decrease the chemosensory performance without eliminating it completely. This would explain why *C. concholepas* does eventually reach the prey in all the treatments used here, although its performance in elevated  $P_{CO_2}$  is low in terms of time to reach the prey and route finding.

Temperature modulated the effect of  $P_{CO_2}$  levels on route finding, since elevated  $P_{CO_2}$  did not affect RF at 19°C. Furthermore, at 19°C, RF was higher at elevated  $P_{CO_2}$  than in the control, suggesting that the effects of elevated  $P_{CO_2}$  levels on the route finding of *C. concholepas* are complex and may be compensated by elevated temperatures. Elevated temperatures were found to mitigate the effects of elevated  $P_{CO_2}$  in various marine organisms, for example in sea urchins (Byrne et al., 2013) and in fish (Domenici et al., 2014). Route finding was not repeatable, implying that the ability to reach the most direct route towards the prey is not temporally stable in the long-term, unlike lateralization.

### Conclusions and perspectives

This study is the first to investigate the potential impacts of ocean acidification on lateralization and route finding in a predatory gastropod and to show that elevated  $P_{CO_2}$  can affect the across-context repeatability of behavioural performance in marine organisms. During the early benthic ontogeny, *C. concholepas* can be found in rocky intertidal habitats (Guisado and Castilla, 1983; Moreno et al., 1993), where it naturally experiences daily fluctuations in pH,  $P_{CO_2}$ , total alkalinity and temperature (Torres et al., 2013, 1999) and therefore it may have evolved mechanisms to partially cope with these environmental stressors. Although *C. concholepas* may be quite tolerant to daily fluctuations in environmental factors, our results showed that it is affected by long-term and chronic exposure to increased  $P_{CO_2}$  and this effect is partly modulated by temperature. Long-term exposure to high  $P_{CO_2}$  and temperature that are in line with those expected at the end of the century is arguably relevant for what might be the response of *C. concholepas* to climate change, as it has been suggested for other organisms (Melzner et al., 2009). Future work may also consider the additional effect of parental exposure (Nagelkerken and Munday, 2016). Previous work has shown that elevated  $P_{CO_2}$  levels negatively affect their capacity to recognize water-borne predator cues (Manríquez et al., 2014), and the present study suggests that near-future  $P_{CO_2}$  levels could have further consequences for the locomotion and sensory performance of *C. concholepas* and other similar species when searching for prey. The direct effect of temperature was not significant, although temperature did show a modulating effect. The present study suggests that increased  $P_{CO_2}$  levels rather than temperature maybe the main stressor driving behavioural modifications in *C. concholepas* along the Chilean coasts and this may therefore, at least potentially, affect species interactions and ecological processes (Nagelkerken and Munday, 2016).

Further studies are required to determine the effects of the decreased locomotion performance and route finding on the success of this keystone species in the field and the impact this has on their interactions with prey and predator populations. The marine gastropod *C. concholepas* is a keystone predator species characteristic of rocky intertidal and subtidal communities of the southeastern Pacific Ocean coast (Castilla, 1999; Castilla and Paine, 1987). Since in this system the presence or absence of this species drives the community structure (Castilla, 1999), the negative effects of ocean acidification on locomotion traits associated with prey-finding may cause cascading effects beyond those described at the individual level such as predator–prey population dynamics and community structure. Furthermore, our results on the effect of

elevated  $P_{CO_2}$  levels on the repeatability of relative lateralization open up the question as to whether or not other behavioural traits affected by elevated  $P_{CO_2}$  in various marine organisms (Briffa et al., 2012; Leduc et al., 2013; Nagelkerken and Munday, 2016) may also lose their repeatability after exposure to elevated  $P_{CO_2}$ . As advocated by Killen et al., (2016), our current lack of knowledge regarding how trait repeatability is affected by elevated  $P_{CO_2}$  levels is a crucial factor limiting our ability to predict how aquatic organisms will evolve in response to ocean acidification.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

P.D. and P.H.M. conceived and designed the study. P.H.M. performed the behavioural experiments. R.T. analysed the water samples contributed with reagents, materials and analysis equipment. P.D. analysed the data. P.D. drafted the manuscript, P.H.M. and R.T. contributed to revisions.

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#### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.151779.supplemental>

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