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Intertidal pool fish *Girella laevifrons* (Kyphosidae) shown strong physiological homeostasis but shy personality: The cost of living in hypercapnic habitats

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ABSTRACT

Tide pools habitats are naturally exposed to a high degree of environmental variability. The consequences of living in these extreme habitats are not well established. In particular, little it is known about of the effects of hypercanic seawater (i.e. high pCO_2 levels) on marine vertebrates such as intertidal pool fish. The aim of this study was to evaluate the effects of increased pCO_2 on the physiology and behavior in juveniles of the intertidal pool fish *Girella laevifrons*. Two nominal pCO_2 concentrations (400 and 1600 μatm) were used. We found that exposure to hypercapnic conditions did not affect oxygen consumption and absorption efficiency. However, the lateralization and boldness behavior was significantly disrupted in high pCO_2 conditions. In general, a predator-risk cost of boldness is assumed, thus the increased occurrence of shy personality in juvenile fishes may result in a change in the balance of this biological interaction, with significant ecological consequences.

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1. Introduction

Tidal pools are complex habitats, exposed to strong environmental variability but somewhat predictable changes resulting from interactions between physico-chemical and biological processes (Romanuk and Kolasa, 2002; Vogt et al., 2006; Pulgar et al., 2006, 2015; Jellison et al., 2016). Early studies aimed to characterize these processes reported relevant changes in temperature, pH and dissolved gasses (Morris and Taylor, 1983), which have major implication upon the distribution and abundance of intertidal biota organisms (Huggett and Griffiths, 1986). Intertidal fish inhabiting these tide pools are naturally exposed to a high degree of stress, due to fluctuations in temperature, salinity, oxygen, pH, and pCO_2 levels (Challener et al., 2011; Shaw et al., 2013). For example, the pH in tide pools can vary daily and seasonally (Morris and Taylor, 1983; Moulin et al., 2011; Davis et al., 2013; Jellison et al., 2016) with values ranging from 8.60 down to 7.36 and up to >2000 μatm pCO_2 (Challener et al., 2011). For instance, in the coast of California, the pH of intertidal pools can highly variable in 24 h, indeed can fall to at least 7.2 in the night and low-tide (Jellison

et al., 2016). Due to the high pCO_2 levels in tidal pools, the resident and transient fish species inhabiting there may be naturally exposed to hypercanic conditions. The recent awareness in ocean hypercapnia or acidification due to the increased concentration of atmospheric carbon dioxide poses an new interesting theorethical and methodological framework to explore the effects of dissolved CO_2 on different biological responses (physiological and behavioural) in marine species inhabiting tidal pools, which have been relatively poorly addressed.

Mostly, the effects of high pCO_2 levels on fishes has been focused on the physiology (Langenbuch and Pörtner, 2003; Strobel et al., 2012; Enzor et al., 2013; Forsgren et al., 2013; Munday et al., 2016), sensory systems (Munday et al., 2009a, 2009b; Dixson et al., 2010) and behavior (Domenici et al., 2012, 2014; Jutfelt et al., 2013; Welch et al., 2014; Nagelkerken and Munday, 2016; Regan et al., 2016). Also, several studies have evaluated the potential effects of elevated pCO_2 on life history traits of fish (Hurst et al., 2013; Frommel et al., 2013, 2016; Baumann et al., 2012; Stiasny et al., 2016). It is important to emphasize that fish can have compensatory mechanisms to face high pCO_2 levels (Portner et al., 2004; Melzner et al., 2009), although this might have some physiological consequences, especially on early life stages (e.g. with high metabolic demands) (Brauner and Baker, 2009; Pörtner and Farrell, 2008). In general, these studies involve the manipulation of pCO_2 according to global projection of increased carbon dioxide in seawater. But, in the case of intertidal

pool, the hypercapnic conditions correspond to conditions that can be naturally experienced by the individuals inhabiting these environments. Thus, ocean acidification research put the conceptual and theorethical tools to explore the biological responses that may be displayed by these juveniles under low pCO_2 conditions that will experience in, for instance, the subtidal habitat when recruit into the adult population.

Along the Chilean coast the intertidal fish *Girella laevifons* (Kyphosidae, Tschudi) show high abundances in tide pools during the early stage of their development and they have been recognized as an ecologically relevant species in the intertidal rock community (Pulgar et al., 2006, 2015). Previous studies have shown that this species may modify its physiology facing to changes in physical characteristics, as for example with temperature conditions (Pulgar et al., 2006). Until now, the effects of the elevated pCO_2 levels on the physiology and behavior of intertidal fish have not been addressed. We hypothesized that the natural exposure of the juvenile individuals of the intertidal rock pools *G. laevifrons* to variable pCO_2 levels may increase its tolerance (behavioural and physiological) to elevated pCO_2 contents. We tested these hypotheses by rearing *G. laevifrons* juveniles at low and elevated pCO_2 levels during 45 days and, under an integrative approach, measuring the Oxygen Consumption and Absorption Efficiency (physiological traits) and Lateralization and the Boldness in exploring new objects (behavioural traits). The results of this study will contribute to a better understanding of the effects of hypercapnia that naturally occur in these typical environments of the intertidal rocky shore.

2. Materials and methods

2.1. Animal collection

Juvenile of *G. laevifrons* ($n = 45$, 32.65 mm $\pm$ 0.46 SD) standard length (SL) were captured at Las Cruces (33°29′ S; 71°37′ W), central Chile during the 2015 autum season. All animals were obtained from upper intertidal pools using BZ-20 anaesthetic. The animals were immediately deposited into sea water with constant aeration and then transported to the laboratory (see Pulgar et al., 2006, 2015) (Laboratorio de Bioingeniería, Universidad Adolfo Ibañez, Santiago). Before the begin of the experiments, individuals were kept in 6 aquaria (9 L, 5 fishes per aquarium) for 7 days in running seawater (ca. 14 °C), with natural photoperiod and fed daily with TetraMarine® saltwater granules (2 g per aquarium). Similar conditions were kept during experimental period.

2.2. Experimental set-up

After the aclimatization period, fish were randomly assigned to aquarium and exposed to two pCO_2 treatments (see above): low pCO_2 concentration (ca. 400 µatm of pCO_2) and high pCO_2 (ca. 1600 µatm of pCO_2). The exposure period lasted for 45 days. Each treatment was replicated three times, and each replicate (i.e. each aquarium) contained five experimental animals. To achieve the two different pCO_2 levels in the seawater (Table 1), we used laboratory-based experimental CO_2-equilibration system (see Torres et al., 2013; Duarte et al., 2014;

Lardies et al., 2014). In brief, in the low treatment, pure atmospheric air was bubbled into experimental aquaria. In order to obtain a high 1600 µatm, we blended dry air with pure CO_2 to the target concentration using mass flow controllers (MFCs) (Aalborg ®) for air and CO_2. This blend was then bubbled into the corresponding containers (see Torres et al., 2013). The seawater of each aquarium was replaced every 2 d using pre-equilibrated seawater from two header tanks (see Duarte et al., 2015). The high pCO_2 level resulted in a corresponding drop ~0.5 pH units (i.e. pH ~7.5), while the low pCO_2 scenario yield a pH level of ~8.0 units (see Table 1). These pCO_2 levels in seawater were selected based on the global current scenarios (Caldeira and Wickett, 2003; Orr et al., 2005; Meinshausen et al., 2011; Doney et al., 2012) and the range of pH daily variation registered in tidal pools in general (see Davis et al., 2013; Jellison et al., 2016) and in the tidal pools of the study area (unpublished data).

At the experiments, water pH and total alkalinity were monitored every 4 days. Samples for pH were collected in 50 mL syringes and immediately transferred to a 25 mL thermostated cell at 25.0 $\pm$ 0.1 °C for standardization, using a Metrohm® pH–meter with a glass combined double junction Ag/AgCl electrode (Metrohm model 6.0258.600) calibrated with standard buffer Metrohm® of pH 4 (6.2307.200), pH 7 (6.2307.210) and pH 9 (6.2307.220). The pH values are reported on the NBS scale. Samples for AT were poisoned with 50 µL of saturated $HgCl_2$ solution and stored in 500 mL borosilicate bottles (Pyrex, Corning®) with ground-glass stoppers lightly coated with Apiezon L® grease and stored in the dark at room temperature. Additionally, temperature and salinity were monitored during incubations by using a portable Salinometer (Salt6+, Oakton®, accuracy: $\pm$ 1% and $\pm$ 0.5 °C, respectively). AT was determined using the open-cell titration method (Dickson et al., 2007) using an automatic Alkalinity Titrator (Model AS-ALK2 Apollo SciTech) equipped with a combination pH electrode (8102BNUWP, Thermo Scientific, USA) and temperature probe (Star ATC, Thermo Scientific, USA) connected to a pH–meter (Orion Star A211, Thermo Scientific, USA). All samples were analyzed at 25 °C ($\pm$ 0.1 °C) with temperature regulated using a water-bath (Lab Companion CW-05G). Accuracy was controlled against a certified reference material (CRM, supplied by A. Dickson, UCSD, USA) and the AT repeatability was 2–3 µmol kg^{-1} on average. Temperature and salinity data were used to calculate the rest of the carbonate system parameters (e.g. pCO_2 in µatm, CO_3^{2-} in µM kg^{-1} seawater). Analyses were performed using CO2SYS software for MS Excel (Pierrot et al., 2006) set with Mehrbach solubility constants (Mehrbach et al., 1973) refitted by Dickson and Millero (1987). The KHSO4 equilibrium constant determined by Dickson (1990) was used for all calculations (Table 1).

2.3. Physiological measurements

2.3.1. Oxygen consumption

At the end of the experiment, fishes were left in aquarium for 24 h with no food (Chabot et al., 2016). Then, we measured (four times) oxygen consumption (mg O$_2$ h^{-1} g^{-1}) using a Presens Mini Oxy-4 respirometer (see Gaitán-Espitia et al., 2014). Seawater utilized during oxygen consumption estimations was previously filtered and sterilized with UV radiation. To quantify the oxygen consumption, the experimental animals of each replicate were used and placed individually in a closed respirometric chambers filled with 131 mL of sea water with the corresponding µatm of pCO_2 levels from the seawater acidification system (see above), $\approx$ 32 psu salinity, and oxygen-saturated through air bubbling (15–20 min) before starting measuring. The measurements were performed at a controlled temperature of 14 °C using an automated temperature chiller. In each chamber, dissolved oxygen was quantified every 15 s for 45 min. Oxygen consumption was estimated using a temperature compensated Microx optic fiber O$_2$-meter (Presens Inc., DE) connected to a recirculating water bath by a flow-through cell housing (Presens Inc., DE). The optic fiber was calibrated in a solution saturated with Sodium Sulfite (Na$_2$O$_3$S, 0% air saturation) and in aerated seawater

Table 1
Seawater characteristics (mean $\pm$ SE) used to maintain *G. laevifrons* during the experimental period (see Materials and methods for details on the estimation of the mean values of each parameter).

CO$_2$ system parameters	Experimental treatments (nominal levels µatm of pCO_2 in seawater)	
	400 (low)	1600 (control)
pH at 25 °C (pH unit)	8.014 $\pm$ 0.022	7.572 $\pm$ 0.023
Salinity (psu)	32.182 $\pm$ 0.072	32.153 $\pm$ 0.073
Temperature (°C)	14.197 $\pm$ 0.050	14.281 $\pm$ 0.033
TA (µmol kg^{-1})	2250.266 $\pm$ 9.066	2297.299 $\pm$ 39.494
CO$_3$ (µM kg^{-1} SW)	149.344 $\pm$ 9.824	47.882 $\pm$ 2.178
pCO_2 (µatm)	374.884 $\pm$ 33.455	1559.282 $\pm$ 24.167

(100% air saturation, ASW), checking for sensor drift before and after each trial. Sensors were previously calibrated in anoxic water, using a saturated solution of Na_2SO_3 and in water 100% saturated with oxygen using bubbled air. The same respirometric chambers were used as controls, but without animals inside, under the same experimental conditions (the control never had a decayment of the oxygen concentration higher than 3% of measurements). Each oxygen decayment due to background noise was deducted from the individual measurements performed in the experimental chambers. Finalized the oxygen consumption measurements the pH of treated seawater inside the respirometric chamber was measured with using a Metrohm® pH–meter with a glass combined double junction Ag/AgCl electrode (Metrohm model 6.0258.600) calibrated with standard buffer Metrohm®, and pH values obtained did not show significant variation before and after experimental time.

2.4. Absorption efficiency (AE)

Absorption efficiency was measured using Conover's (1966) method, based on the relationship between organic and inorganic matter values of ingested food and fecal material. This method assumes that the absorption affects only the organic portion of the food. Absorption efficiency was then calculated according to the following equation: where:

$$AE = [(F' - E') / (1 - E')F'] \times 100.$$

AE = absorption efficiency expressed as a percentage, F' = the proportion of organic matter in the food, and E' = the proportion of organic matter in the feces.

In order to obtain fecal pellets, two fishes from each replicate (and each treatment, i.e. 400 and 1600 µatm of pCO_2) were maintained in individual aquariums for 24 h in similar conditions (i.e. seawater characteristics and food amount) to that described before. The feces generated to 24 h in each replicate were collected with a pipete and frozen until subsequent laboratory analysis. Samples of food and feces were filtered through pre-ashed, preweighed Whatman GF/F filters (47 mm diameter), rinsed with destilled water, dried to a constant weight at 60 °C, weighed, combusted at 450 °C for 4 h; and weighed again to estimate the organic and inorganic fractions.

2.5. Behavioural experiments

A total of 16 individuals of *G. laeviforns* (SL: 32.8 mm ± 2.73 SD) were used in behavioural experiments, which were carried out in the same environmental conditions of temperature, salinity, light, pCO_2 and subjected to observational procedures in behavioural experiments. The seawater was replaced after each experimental fish was evaluated (i.e. 20 runs per fish) using the pre-equilibrated seawater from header tanks (see above).

2.6. Lateralization

The behavioural lateralization of *G. laeviforns* was evaluated using a T-maze (according to Domenici et al., 2012; Jutfelt et al., 2013). Briefly, this divice consisted of a glass tank (L 45 × W 30 × H 15 cm), with a runway in the middle (L 10 × W 5 cm). The runway was delimited by two glass tanks (L 20 × W 10 × H 10 cm), which separated both left and right areas. A single fish was introduced in a T-maze runway and after a acclimation time (5 min), the fish was gently moved to the starting point of the runway and the direction of its turn (i.e. right or left, relative lateralization) at the end of the runway and the time of lateralization (s) used by the fish in displaying these directional movements (i.e. time needed for a fish to select the side) were registered during a period of 3 min. To account for any possible asymmetry in the set up, tests were carried out alternately on the two ends of the runway (see Bisazza et al., 1998). Twenty consecutive runs were observed for each fish. Score of the turning direction was used to estimate the degree of lateralization relative index (LR, from − 100 to + 100, indicating complete preference

for left and right turning; Bisazza et al., 1998, Domenici et al., 2012). Water in the T-maze was 4 cm deep and was replaced after each fish was evaluated. In all trails the fish never stopped swimming.

2.7. Boldness in exploring novel object

To evaluate the fear of novelty (Jutfelt et al., 2013, but see Toms et al., 2010) in *G. laevifrons* exposed to different levels of pCO_2, the novel object test was used. This method is well established to estimate the boldness and curiosity in fish (Sneddon et al., 2003; Von der Emde and Fetz, 2007). The fishes were left to settle individually in an aquarium (L 45 × W 30 × H 15 cm) with a start position marked on the middle and bottom area and a delimited area (5 cm^2) at the upper left corner of the glass tank. After five acclimation minutes, the fish was gently maneuvered to the starting position and simultaneously the novel object was introduced inside the delimited area in the upper left corner of the aquarium. In each trial, the time spent by the fish until go into the delimited area containing the object time to entry (s), the time spent investigating the object (s) and the number of new entries (counts) to the object area was recorded during a period of 5 min. The fish that not go into the object during the time of each trial was registered as zero.

2.8. Data analysis

The physiological responses of *G. laevifrons* exposed to the two pCO_2 treatments (400 and 1600 µatm of pCO_2) were analized by ANCOVA including the wet weight as covariate and ANOVA models. Differences in the oxygen consumption of individuals exposed to the pCO_2 treatments were analyzed by ANCOVA, including the wet weight as covariate ($F_{(1, 23)} = 0.47$ $p = 0.04$). We used a nested mixed model including the replicated (aquarium) of each treatment as a random effect to account for the likelihood that individuals samples from a replicate aquarium may be more similar to each other than individuals sampled from other aquarium. The absorption efficiency of *G. laevifrons* exposed to the two pCO_2 treatments were compared using one-way ANOVA.

The relative lateralization and time of lateralization were compared between treatments using one-way ANOVA. In fish exposed to low pCO_2 conditions, the boldness in exploring novel object was evaluated using one sample *t*-test regarding the hypothesis $\mu_{pCO2-1600} = 0$ vs $\mu > 0$ (where $\mu_{pCO2-1600}$ = average in time to entry, time investigating the object and number of entries). This allow for a relative comparison with fish exposed to high pCO_2 conditions because their lack of any behavioural response (i.e. $\mu_{pCO2-400} = 0$). The normality and homocedasticity of the data were tested using the Kolmogorov–Smirnov and Bartlett tests, respectively (Zar, 1999). All analyses were carried out using the program Minitab v14.

3. Results

Seawater parameters where experimental animals were maintained during the experiments are summarized in Table 1). (Mean ± SE) pH values (at 25 °C) of the two nominal treatments (i.e. 400 and 1600 µatm of pCO_2) were 8.01 ± 0.13 and 7.57 ± 0.13, respectively (Table 1). The carbonate contents showed a corresponding decreased with an increase in pCO_2, whereas salinity (≈ 32 psu), temperature (≈ 14 °C) and total alkalinity (≈ 2270 µmol kg^{-1}) remained similar between the two pCO_2 treatments.

3.1. Physiological measurements

The juveniles individuals of *G. laevifrons* exposed to hypercapnic conditions (i.e. 1600 µatm of pCO_2) showed an oxygen consumption lower (0.190 mg O_2 h^{-1} g^{-1} ± 0.01 SE) than to those exposed to low pCO_2 (0.215 mg O_2 h^{-1} g^{-1} ± 0.01 SE), but these differences were not significant between treatments (ANCOVA: $F_{(1, 4)} = 1.04$, $p = 0.366$; Fig. 1a). The individuals samples (fish) from each replicate
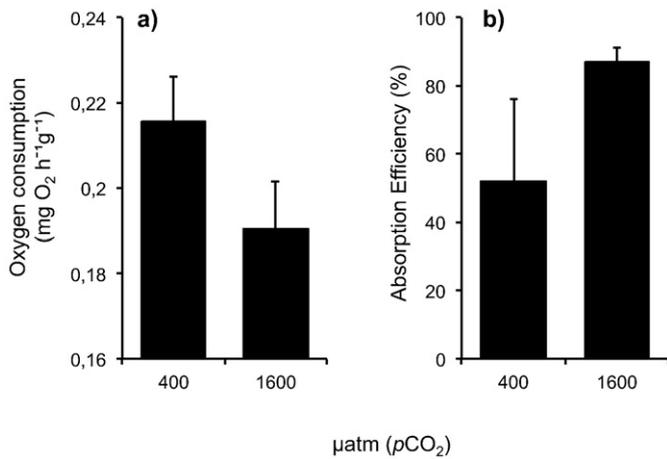
Fig. 1. (a) Oxygen consumption and (b) absorption efficiency (%) of individuals of *G. laevifrons* exposed to contrasting pCO$_2$ levels (400 and 1600 μatm of pCO$_2$). Bars correspond to means ($+$1EE).

(aquarium) also showed similar oxygen consumption respect to fish sampled from others aquarium and treatments (ANCOVA, aquarium (pCO$_2$ treatment): $F_{(4, 19)} = 0.83$, $p = 0.52$). On the other hand, absorption efficiency (AE) showed a subtle increment under hypercaninc conditions (86.82% $\pm$ 4.44 SE), but it was not significantly different respect to the low pCO$_2$ treatment (52.07% $\pm$ 23.9 SE) (one way ANOVA: $F_{(1, 5)} = 2.03$, $p = 0.22$; Fig. 1b).

3.2. Behavioural experiments

Individuals of *G. laevifrons* showed a significant disrupts on lateralization behavior. We recorded that individuals exposed to low pCO$_2$ conditions turned to the right (27.5 $\pm$ 13.3 SE), which is opposed to the behavior displayed by individuals exposed to hypercapnic conditions (-17.5 ± 14.2 SE) (one way ANOVA: $F_{(1,15)} = 5.32$, $p = 0.037$; Fig. 2 a, b, c). The average time of lateralization of *G. laevifrons* did not differ significantly between the two pCO$_2$ treatments (one way ANOVA: $F_{(1,15)} = 0.44$, $p = 0.51$; Fig. 3). However, in the novel object experiments, the 100% of the individuals of *G. laevifrons* exposed to hypercapnic conditions showed a total absence of behavior associated to boldness. Boldness was only evidenced by fish exposed to low pCO$_2$ concentration showing a relative fast time to entry to the delimited area of the new object (2.24 s $\pm$ 0.87 SE; one sample t-test: $t = 2.39$;
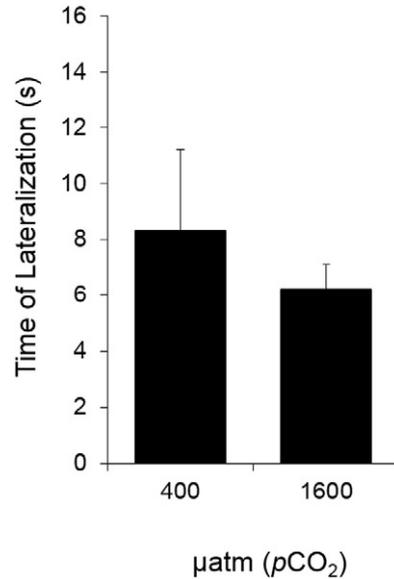

Fig. 3. Time of Lateralization (s) of the individuals of *G. laevifrons* exposed to contrasting pCO$_2$ levels (400 and 1600 μatm of pCO$_2$). Bars correspond to means ($+$1EE).

$p = 0.038$;), a long period of time was spent in investigating the object (22.22 s $\pm$ 5.35 SE; $t = 3.77$, $p = 0.010$) and the number of new entries (2.16 count $\pm$ 0.86 SE; $t = 2.48$; $p = 0.028$) to the delimited area.

4. Discussion

4.1. Physiological responses

In this study, we interpret the hypercaninc conditions as a situation that can be experienced by juvenile fishes inhabiting intertidal pool. Thus, our approach is different in rationale but methodologically similar to previous studies aimed to evaluate the effect of pCO$_2$ in seawater. In particular, we found that the assessed physiologic traits (i.e. oxygen consumption and absorption efficiency) of the rockfish *Girella laevifrons* do not shown variation when exposed to contrasting μatm of pCO$_2$ conditions in seaweater but with significant and clear-cut differences in behavior.

Previous studies carried out to evaluate the effects of high pCO$_2$ levels on metabolic rate have shown contrasting results, showing in some cases increases (Enzor et al., 2013; Rummer et al., 2013) or not
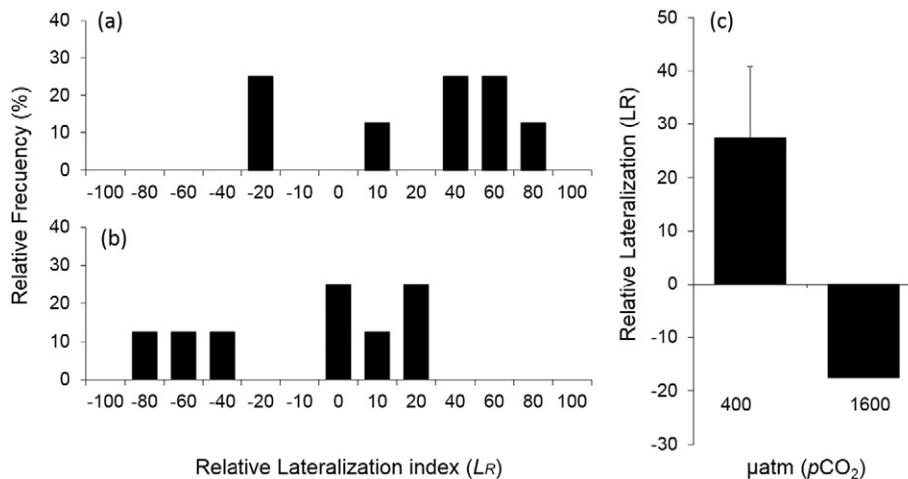

Fig. 2. (a) Relative frequency distributions of LR of the individuals of *G. laevifrons* exposed to 400 ppm, (b) and 1600 ppm. Positive and negative values indicate right and left turns, respectively. The extreme values of [100] indicate fish that turned in the same direction on all 20 trails. (c) Relative lateralization of *G. laevifrons* exposed to contrasting pCO$_2$ levels (400 and 1600 μatm of pCO$_2$). Bars correspond to means ($+$1EE).

effects (Strobel et al., 2012; Couturier et al., 2013; Munday et al., 2014). Increasing evidence suggests that organism associated with environments that naturally present high pCO$_2$ levels may have physiological and metabolic adaptations and consequently to be better acclimatized to ocean acidification (e.g., Cummings et al., 2011; Widdicombe et al., 2009; Duarte et al., 2015). In this context, the natural hypercapnic conditions occurring in tide pools (e.g. Challener et al., 2011; McNeil and Sasse, 2015; Shaw et al., 2013; Jellison et al., 2016) could explain why the oxygen consumption of *G. laevifrons* was not affected by the high pCO$_2$ levels. Despite that we do not have data on the pCO$_2$ content in the study tidal pools, we registered pH values similars to those registered in the treatment with high pCO$_2$ content (≈ 7.5), indicating that high pCO$_2$ levels as those used in this study and observed in other tidal pools can be occur in the site where the experimental animals were collected.

On the other hand, the effects of high pCO$_2$ on absorption efficiency in marine organisms has been poorly documented (e.g. Navarro et al., 2013), and this is the first study in evaluating the impact of pCO$_2$ variations on the absorption efficiency on an intertidal fish species. A recent study in an invertebrate marine species showed that exposure to elevated pCO$_2$ levels negativelly affected the food absorption efficiency (Navarro et al., 2013) and it was suggested that this negative effect would be a deficiency in the functioning of the digestive systems. In this study, the exposure to hypercapnic conditions did not affect the absorption efficiency in *G. laevifrons*. However, due to the low number of replicates ($n = 3$) we find that statistical power ($1 - \beta$) of the test was 0.197, which potentially increased Type II error ($\beta = 0.803$); thus this lack of differences in AE between treatments must be interpreted with caution. Notwithstanding, it is important to emphasize that although no statistical significant differences were observed, the absorption efficiency showed a clear tendency to increase in fishes experiencing hypercanic conditions in seawater (i.e. 1600 μatm of pCO$_2$). These results could also (as indicated above) indicate an acclimatation of this species to high pCO$_2$ (Wootton et al., 2008; Hall-Spencer et al., 2008; Thomsen et al., 2010, 2013. Duarte et al., 2014; Lardies et al., 2014).

It is important to emphasize here, that the unchanged oxygen consumption and absorption efficiency of *G. laevifrons* exposed to high pCO$_2$ levels not necessary preclude that other physiological changes occurred (Couturier et al., 2013). For example, Deigweiher et al. (2008) demonstrated that the exposure of the fish *Zoarces viviparous* to hypercapnia altered the regulatory gill machinery, but without measurable effect on its metabolic rate. We do not know if this kind of compensatory physiological changes occur in this intertidal rockfish, but it has been shown that fish have an efficient ion regulatory mechanisms that allow them to compensate pCO$_2$ environmental and internal pH (e.g. acid-base regulation, osmoregulatory and cardiorespiratory control) (Perry and Gilmour, 2002, 2006; Portner et al., 2004; Munday et al., 2009a, 2009b). Other possible explanation is that the highly variable environments (i.e. physical characteristics) where inhabit this fish could explain the no effects of high pCO$_2$ on the physiological studied traits, as has been shown in others species (Duarte et al., 2015; Lardies et al., 2014).

4.2. Behavioural responses

Our results show that exposure to high pCO$_2$ levels disrupted the lateralization and boldness in *G. laevifrons*. Previous studies have shown that lateralization is a fundamental determinant of fish behavior (Domenici et al., 2012, 2014; Dadda et al., 2010), and may be potentially related to the different specialization of contralateral brain structures (Bisazza et al., 1998) and to an increase in the cognitive performance (Vallortigara and Rogers, 2005; Bisazza et al., 2000). Furthermore, this behavior is thought to minimize the decision time and also to avoid simultaneous initiation of compatible responses when the animals facing tasks that require directional responses (Vallortigara and Rogers, 2005).

The juvenile fish exposed to low pCO$_2$ treatment were highly lateralized to right, but those exposed to hypercapnic conditions appears to disrupt significantly this behavior, because the animals in this treatment turned to the left. Similar results were found by Domenici et al. (2012), who demonstrated that pCO$_2$ increase affected the behavioural lateralization in larvae of the coral reef fish *Neopomacentrus azysron* and the temperate stickleback fish *Gasterosteus aculeatus* (Jutfelt et al., 2013; Lai et al., 2015). In addition, Domenici et al. (2014) demonstrated that elevated pCO$_2$ treatment the turning of damselfish, *Pomacentrus wardi* was reversed (i.e., from right to the left side). In contrast, Jutfelt and Hedgärde (2015) found no effect of high pCO$_2$ treatment on any behaviors such as activity, emergence from shelter, relative and absolute lateralization on Atlantic cod *Gadus morhua*. Previous studies have shown that any changes in the lateralization are likely to decrease the performance of the population (see Munday et al., 2012; Domenici et al., 2012; Briffa et al., 2012; Jutfelt et al., 2013). In addition, various fitness advantages have been correlated with a lateralized (i.e. showing a side preference), behavior (Dadda et al., 2010; Dadda and Bisazza, 2006), and stronger lateralization correlates with an increased escape reactivity. In consequence, the disrupted lateralization in *G. laevifrons* produced by hypercapnic conditions could result in negative effects on the whole population of this intertidal fish. On the other hand, the increased of pCO$_2$ levels did not affect the time of lateralization in *G. laevifrons*. Although of high pCO$_2$ levels has been demonstrated affect lateralization in fishes, the decision time for any direction (i.e. left or right) in this behavior test it had not been registered so far.

The pCO$_2$ increase has been shown to exert a strong effect on others behavior traits in fishes (Welch et al., 2014; Jutfelt et al., 2013; Hamilton et al., 2014; Lai et al., 2015; Regan et al., 2016) and others marine invertebrate (Spady et al., 2014). Recently, previous studies has been detected in the early life stages of several fish species both tropical and temperate environment exposed to high pCO$_2$ concentrations, including effects on boldness, exploratory behavior, activity levels and scape performance (Ferrari et al., 2011; Jutfelt et al., 2013; Lai et al., 2015; Faleiro et al., 2015; Dadda et al., 2010). For example, Jutfelt et al. (2013) demonstrated that the 20 day of exposure to high pCO$_2$ levels, the fish also spend five less times investigating a novel object than fish maintained under low pCO$_2$ levels. These authors also demonstrated that hypercania decreased boldness and curiosity in the fish *Gasterosteus aculeatus*, and reduced the escape time in the 40 days of exposure. Furthermore, Hamilton et al. (2014) demonstrated that the rockfish *Sebastes diploproa* increased their time spent in darkness when exposed to elevated pCO$_2$ levels, also indicating reduced boldness. Similary, Faleiro et al. (2015) demonstrated that in hypercapnic conditions, adult's seahorses *Hippocampus guttulatus* spent less time in activity and more time resting. In this study, in the novel object test, fishes exposed to high CO$_2$ conditions showed a total absence of behavior associated to boldness and curiosity. These results suggest that elevated pCO$_2$ would have a direct effect on brain function of the juvenile individuals of this specie. The behavioural inhibition (i.e., novel object test) might have detrimental consequences, because an individual might not respond in an appropriate manner to a fearful situation and this might have severe negative effects (Sneddon et al., 2003). In general, a predator-risk cost of boldness is assumed, thus the increased occurrence of shy personality or lack of boldness recorded in juvenile fish of *Girella laevifrons* may change the balance in this biological interaction, with significant ecological consequences, not only to population level if not for the whole community. For this particular species, exposure to a predator could be negative if the fish react in a similar manner to how they responded to a novel object. On the other hand, the display of boldness under low pCO$_2$ conditions may be ecologically relevant during the displacement from the tidepool to the subtidal habitat by the juvenile fishes where will be exposed to increased vulnerability and predatory risk. However, the implications of these effects on the fitness of this species have not yet been tested.

Behavior serves as the link between physiological and ecological processes (Weber and Spieler, 1994; Scott and Sloman, 2004). For example, many researchers have proposed using behavioural indicators in fish for ecologically relevant monitoring of pollution (e.g. Atchison et al., 1987). In our study, fish behavior may be proposed as an indicator of ocean acidification organismal impacts. It is well known, that the performance of normal behavior by individual fish follows specific physiological sequences, which are triggered by external stimuli acting via neural networks (Weber and Spieler, 1994; Scott and Sloman, 2004) and this interaction finally determine variation in life-history.

5. Conclusions

This study shows that increased of pCO_2 levels adversely affects behavior but not physiological traits of *G. laevifrons*. The highly variable environments where inhabit *G. laevifrons* could explain the no effects of high pCO_2 levels on the studied physiological traits. However, we do not rule out possible effects on other physiological traits in this species. The negative effects of increased pCO_2 on behavior of this intertidal fish can have significant ecological consequences, mainly in the tidal pools habitats where this group is dominant in terms of abundance and biomass. These changes could affect not only in a population level, if not also on the entire community. Finally, it is importan to emphasize here, that the projected increase of the CO_2 emmsions could increase the already high pCO_2 content of these environement, exacerbating the hypercapnic conditions of these habitats.

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