Limited behavioural effects of ocean acidification on a Mediterranean anemone goby (*Gobius incognitus*) chronically exposed to elevated CO_2 levels

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Abstract

A reciprocal transplant experiment was carried along a volcanic CO_2 gradient to evaluate the anti-predator responses of an anemone goby species exposed to Ambient (~380 µatm) and High (~850 µatm) CO_2 sites. Overall, the anemone gobies displayed largely unaffected behaviors under High-CO2 conditions suggesting an adaptive potential of *Gobius incognitus* to ocean acidification (OA). This is also supported by its 3-fold higher density recorded in the field under High CO2. However, while fish exposed to ambient conditions showed an expected reduction in the swimming activity in the proximity of the predator between the pre- and post-exposure period, no such changes were detected in any of the other treatments, all of which involved exposure to high CO2. With this suggesting an OA effect on the goby antipredator strategy. Our findings contribute to the ongoing debate over realistic predictions of the impacts of expected increased CO_2 concentration on fish.

Keywords: Behaviour; Gobiidae; Predation; Shelter use; Anemone; Global change; Risk assessment; CO_2 seeps

1. Introduction

Anthropogenic CO_2 emissions in the atmosphere are responsible of the ongoing ocean carbonate chemistry changes and pH drop, a process known as ocean acidification (OA) (Doney et al., 2009, Orr et al., 2005;). This might cause a range of effects on marine organisms, potentially leading to population, community and ecosystem changes and affecting a range of key processes (e.g. competition, predation and habitat provisioning) regulating ecosystem structure and function (Gaylord et al., 2015; Sunday et al., 2017; Milazzo et al., 2019; Cattano et al., 2020). In this frame, one way that organisms may respond to High CO_2/Low pH conditions is through the rapid modification of their behaviour (Briffa et al., 2012; Nagelkerken and Munday, 2016), potentially allowing them to avoid risks, relocate in preferred habitats and increase survival and fitness (Goldenberg et al., 2018; Nagelkerken & Munday, 2015). To date, most of the studies looking for potential OA effects on species behaviour involving predator-prey dynamics, reproduction, homing, and habitat choice have been conducted on selected model species, and under controlled laboratory conditions (e.g., Cattano et al., 2018 for a review), whilst behavioural experiments carried out in the wild, encompassing the large variability of the natural environments, are largely overlooked (but see Nagelkerken et al., 2015; Milazzo et al., 2016; Spatafora et al., 2021).

The ability to detect and avoid predators is a key mechanism to ensure prey survival (Houston et al., 1993), and many laboratory experiments have showed an impaired ability of fish to distinguish between predator and non-predator olfactory cues under elevated CO_2 conditions (pCO_2) expected to occur by the next few decades (e.g., Cattano et al., 2019; Dixson et al., 2010; Ferrari et al., 2011; Munday et al., 2010; Porteus et al., 2018). Among the sensory mechanisms used to detect risk-associated cues, chemosensory has been widely considered in studies on predation risk assessment under OA conditions both for tropical and temperate fish species (e.g. Dixson et al., 2010; Porteus et al., 2018; Williams et al. 2019) and resulted in impaired olfactory ability. Other sensorial impairments involving auditory and visual systems due to increased CO_2 levels have been also observed (e.g. Chung et al., 2014; Ferrari et al., 2010, 2012; Simpson et al., 2011; Radford et al., 2021; Rossi et al., 2016, 2018). In addition, some studies documented altered lateralization in fish exposed in the short-term to elevated CO_2 concentrations (Domenici et al., 2012; Jutfelt et al., 2013; Näslund et al., 2015), or increased activity levels and boldness leading prey venturing further from shelters heedless of the predator presence (Cattano et al. 2019; Munday et al., 2013). However, other studies reported no changes in chemosensory reception ability of prey (Jutfelt & Hedgärde, 2013; Sundin et al., 2017), unaltered or decreased activity levels (e.g. Duteil et al., 2016; Porteus et al., 2018; Sundin et al., 2013, 2017; Sundin & Jutfelt, 2016) and increased or unvaried amount of time spent sheltering (e.g. Näslund et al., 2015; Rossi et al., 2015) under elevated CO_2 conditions. Overall, such heterogeneity in anti-predator responses suggests that elevated CO_2 levels may have species-specific effects, likely depending on potential adaptations to local environmental conditions (Vargas et al., 2017), with experiments showing that detrimental effects of stable elevated CO_2 on fish anti-predator responses may be reduced under natural diel pCO_2 fluctuations (Jarrold et al. 2017).

In this context, it may be often difficult to extrapolate from laboratory studies to natural conditions, as these are generally too short-term to reveal how organisms may adapt/acclimatise, or use steady pCO2 levels (which are unrealistic) and organisms that are separated from their communities. In this context, field experiments conducted along natural CO_2 vents where fish are chronically exposed to elevated and variable CO_2 concentrations, can be particularly useful for assessing the potential ability of fish to acclimatize and adapt to future ocean acidification conditions (REF). These analogues provide opportunities to assess distribution patterns in the wild (Hall-Spencer et al., 2008), the ability for physiological and behavioural adaptation/acclimatisation of organisms in responses to elevated pCO2 levels (REF vedi tu) as well as the importance of natural variability in carbonate chemistry in affecting the distribution of species (Small et al., 2015). Few attempts have been made to control fish behavioural responses along volcanic CO_2 gradients experiencing fluctuations of CO_2 in shallow waters, (Cattano et al., 2016). In this regard, *in situ* experiments carried out in the same study area of ours, have suggested impaired escape responses in a goby fish (Nagelkerken et al., 2016) as well as effects on reproductive behaviors in wrasses (Milazzo et al., 2016; Spatafora et al., 2021). By contrast, *ex situ* OA experiments on the predator recognition ability of both gabazine-treated and -untreated *Symphodus ocellatus* post-settlers living off CO_2 seeps was unaffected (Cattano et al., 2017). Such mixed responses emphasize the strong need of further studies focusing on behavioural responses under varying CO_2 levels.

Here, we used the anemone goby *Gobius incognitus* (Kovačić & Šanda, 2016) living off natural CO_2 vents to investigate potential behavioural alteration caused by OA. This small territorial benthic fish represents an ideal candidate for *in situ* OA experiments due to its very limited home range, that ensures the spatial segregation between groups of individuals living in adjacent areas naturally characterized by different pCO_2 conditions. Moreover, the strict association between *G. incognitus* and the sea anemone *Anemonia viridis* (Forsskål, 1775), used as shelter in case of threats (Kovačić & Šanda, 2016; Nagelkerken et al., 2015; Tiralongo et al., 2020), allows to use the sheltering time as a clearly measurable variable linked to the anti-predator behavior. In addition to sheltering, the anemone goby can show alternative antipredator strategies like an escaping ("fleeing") and 'freezing' behaviors as observed in similar species (Larson & McCormick, 2005; McCormick & Larson, 2007).

Given the limited range of movement of this species, we hypothesize that *G. incognitus* adults experiencing long-term exposure to elevated CO_2 concentrations may show a behavioural tolerance resulting in unaltered antipredator behaviour. To achieve our goal, we carried out an *in-situ* reciprocal transplant experiment along the Vulcano Island natural CO_2 gradient (Southern Italy) to evaluate whether gobies from OA and ambient conditions show behavioural effects when experiencing a predatory threat. Specifically, the use of a reciprocal transplant approach in this study allowed us to investigate whether there is evidence for local adaptation and/or plasticity in antipredator responses to long and short- term natural exposure to elevated CO2 conditions representative of future OA. Since both the number of predators and the availability of shelters may influence the behaviour of the study species (Nagelkerken et al., 2015), before conducting the experiment we quantified the densities of predators and anemones, as well as the association between the goby and the anemone in the two different CO_2 sites.

2. Materials and methods

2.1 Study species

G. incognitus (Kovačić and Šanda, 2016) (Fig. 1a) is widespread in Mediterranean shallow coastal waters, mostly preferring rocky (gravel, cobbles, boulders and bedrock) and sand substrata, specifically when mixed with rocky bottoms (Kovačić and Šanda, 2016; Tiralongo et al., 2020). Its presence from the western to the eastern part of the basin has been often confused with the congeneric *Gobius bucchichi* (Steindachner, 1870), which is actually only reported from the eastern part of the Adriatic Sea and from the northern Ionian Sea (Albania) (Kovačić & Šanda, 2016). This suggests that previous studies carried out in the present study area and using *G. bucchichi* (Nagelkerken et al., 2015; Mirasole et al., 2020) probably dealt with *G. incognitus* (Tiralongo et al., 2020). To avoid a species misidentification, we conservatively used a DNA barcoding approach (see Table S1 and Fig. S2 in Supplementary data) to clarify the taxonomic identity of the species, by analysing the identity of two specimens accidentally captured by local fishers with benthic traps in the study site. The analysis (Supplementary data, Table S1 and Fig. S2) identified the study species as *G. incognitus*.

2.2 Study site

The experiment was carried out in shallow water off the CO_2 vent of Levante Bay (Vulcano Island, Aeolian Archipelago, Italy) during two sampling surveys on May 17th– June the 2nd 2019, and 9–18 September 2019. In this area, the presence of submerged CO_2 seep systems generates a CO_2/pH gradient that runs parallel to the coast (Boatta et al., 2013) and represents a natural laboratory for investigating the effects of OA on marine species and communities (Aiuppa et al., 2021). Gas composition is dominated by CO_2 (97-99 %) while other gases, such as H_2S, rapidly decrease with distance from the vent (Boatta et al., 2013). Behavioural observation of G. *incognitus* were conducted at two sites, where carbonate chemistry was extensively characterized in previous studies (Boatta et al., 2013; Milazzo et al., 2016; Aiuppa et al., 2021), and here designated as Ambient (A, present-day conditions: ~380 µatm, pCO_2) and High CO_2 (H), the latter being consistent with projected end-of-century conditions for the atmosphere and ocean surface (~850 µatm pCO_2; Meinshausen et al. 2011). Average carbonate chemistry from the study period is reported in Table S2 in Supplementary data. A fixed Total Alkalinity of 2500 µmol kg^{-1} was assumed for this study and used to calculate pCO_2 using the software CO_2SYS (Pierrot et al., 2006) with dissociation constants for carbonate from Mehrbach et al. (1973) refitted by Dickson and Millero (1987), and for KSO_4 from Dickson (1990).

2.3 Experiment set-up

2.3.1 Patterns of fish, anemone and predator densities and of fish-anemone association

Density of *G. incognitus* and *A. viridis* were visually quantified by scuba diving in replicated belt transects (3m long and 1m wide) positioned at 1-2 meters depth on mixed sandy/rocky substrates (Ambient, n=15; High-CO_2, n=15). At the same time, fish/anemone association (i.e., the percentage of gobies observed at a distance of <5 cm from an anemone) was recorded in the two sites under different CO_2 conditions (A and H). Density of *Serranus scriba*, a goby predator (e.g. Moreno-López et al., 2002), was also visually assessed in replicate 10 x 2 m belt transects at 1-3 meters depth on mixed sandy/rocky bottoms in the two CO_2 condition sites (Ambient, n=19; High-CO_2, n=22).

2.3.2 Reciprocal transplant experiment to assess fish antipredator behaviour

Individuals of *G. incognitus* were gently collected from Ambient and High CO_2 sites between 1- and 5-meters depth using a hand net and placed within 10-L containers kept underwater. To assess the effects of elevated CO_2 on risk assessment of *G. incognitus* individuals and their responses when exposed to different CO_2 conditions, fifteen fish individuals were haphazardly selected and transplanted from the High-CO_2 site to the Ambient site and *vice-versa* (treatments HA and AH, respectively). Fifteen individuals were replaced in the original site (treatments HH and AA) to control the translocation effect. Each translocation was performed by placing fish in a 10-L plastic container (30 cm height and 27 cm diameter) with 10 holes on each side to ensure water flow-through and oxygenation. The containers were fixed to the sea bottom at 50 cm depth. Before testing, fish were kept for 96 h in the containers for acclimation at each condition in the field, a sufficient time to observe OA-induced behavioural effects in some coral reef fishes (e.g. Munday et al., 2010). To control for a potential effect of fish size on displayed behaviour, standard length (SL, cm) of each fish used in the transplant experiment was measured from digital photographs, using a ruler as a reference with the ImageJ software (Schneider et al., 2012). To assess fish behaviour we used six experimental arenas (L:80×H:20×W:40 cm) opened at the base and fixed to sandy bottom (Fig. 1c). The experimental arenas were divided into three compartments: an experimental compartment (40 cm × 40 cm) at the middle of each arena and two predator compartments (20 cm × 40 cm) on both sides (Fig. S1 in Supplementary data). Each arena was covered on every side with a net (0.6 mm mesh size) to ensure visual isolation of the fish from the surrounding environment. Two anemones were placed in the right or left side of each experimental compartment to provide a suitable shelter for the experimental fish (Fig. 1c). The anemones were collected from each CO_2 site at a depth of 1-3 meters (and kept in the original CO_2 condition). After 96-hours of acclimation, fish from each CO_2 treatment were individually transferred into the experimental arenas and acclimated for 10 min before the behavioural trials. A video camera (Apeman A80) was fixed on the middle-top (~30 cm from the anemone) of the cage to record the behaviour of the experimental fish. *G. incognitus* behaviour was assessed using a standard continuous focal sampling procedure (Martin & Bateson, 1993). Behavioural observations followed a well-established protocol adopted previously for other fish species (Ferrari et al., 2010, 2012; Holmes & McCormick, 2010), which consisted in recording goby responses before and after the presentation of a predator (i.e. predator stimulus). Specifically, for each replicate a 4-min pre-stimulus presentation period was followed by a 4-min post-stimulus presentation period during which each fish was exposed to the visual and olfactory cues of an adult painted comber *S. scriba* (Linneus, 1758). Specifically, a total of four individuals of *S. scriba* (two for each treatment, average SL: 27.3 cm) belonging to Ambient CO_2 waters were used for this experiment and haphazardly assigned to the different CO_2 treatments. Each predator was placed in a transparent plastic tank (39 x 19.6 x 16 cm) with two of the six sides (i.e. the upper and the one oriented to the prey) opened and covered with a net (0.5 cm mesh size), thus permitting visual as well as olfactory contact between prey and predator (Fig. 1c). The tank containing the predator was haphazardly assigned and gently introduced in one of the two predator compartments during a 60-sec stimulus introduction period between the two observation periods (i.e., pre- and post-stimulus). Since the predator was haphazardly placed into either the left or right predator compartment (in order to remove any confounding effect of the arena side), this affected the position of the shelter relative to the predator (since the anemone couldn't be moved). This "Shelter position" was therefore either classed as "Close" (distance between predator compartment and anemone: ~5 cm apart) or "Far" (distance between predator compartment and anemone: ~35 cm apart) from the predator side (see Fig. S1 in the supplementary material for an explanation).

During the pre- and the post-stimulus periods we measured: (i) the activity level, as the amount of time (sec) spent by the fish swimming in the entire experimental compartment; (ii) the time spent (sec) by the fish actively swimming or resting at <20 cm from the predator compartment (i.e. the *predator zone*) and at >20 cm from the predator compartment (*no predator zone*) (Fig. S1 in Supplementary data); (iii) the shelter use, as the total time the fish spent near the anemone (i.e. less than 5 cm); (iv) the minimum approach distance, as the mean smallest distance (cm) of the fish centroid from the shelter (*A. viridis*). Fifteen videos were not analysed as they did not allow clear identification of fish behaviours (e.g. the position of the animal with respect to the camera was at times not optimal). Thus, a total of forty-five videos were used (n=10 in AA, n=11 in AH, n=12 in HA and n=12 in HH). All observations were conducted between 09:00 and 12:00 and between 15:00 and 19:00. Replicates were temporally interspersed between the different CO_2 sites to avoid the possibility of biases that may arise from confounding or correlation between treatments (Hurlbert, 1984).

Figure 1. The anemone goby *Gobius incognitus* (a). A typical association between *G. incognitus* and *Anemonia viridis* (b). The experimental arena used for the behavioural trials (c), with a schematic representation of the experiment showing the goby *G. incognitus*, the shelters (two *A. viridis* individuals) and the predator *Serranus scriba* (bottom right).

2.4 Experimental design and statistical analyses

Differences in fish, anemone (shelter) and predator log-transformed abundance between the different CO_2 sites (as a fixed orthogonal factor with two levels: Ambient (A) and High CO_2 (H)), were tested using one-way analysis of variance (ANOVA) (Kaufmann & Schering, 2007). Differences in the proportion of goby fish (with respect to all the individuals censused in Ambient and High CO_2 sites) associated with anemones among Ambient and High CO_2 sites were assessed using a binomial generalized linear model (Dobson, 1990). The significance of the association was obtained using the analysis of deviance test (Zuur et al., 2007).

Potential differences in experimental fish standard length (cm) (log transformed) between the CO_2 sites were tested using a one-way ANOVA, with “CO_2 treatment” as a fixed factor with four levels: (AH, HA, AA and HH treatment). Potential differences in each behaviour between “CO_2 treatment” (fixed factor with four levels: AH, HA, AA and HH), “Predator stimulus” (fixed factor with two levels: pre- and post-stimulus), “Shelter position” (fixed factor with two levels: Close and Far from predator side) and their interaction were assessed through linear mixed models (lmer). Specifically, five linear mixed models were performed for each log-transformed (+1) response variable: 1) activity level in the entire experimental compartment (sec), 2) activity level in the predator zone (sec), 3) activity level in the no predator zone (sec), 4) shelter use (sec) 5) minimum approach distance (cm). Specifically, for the activity level measured in both predator and no predator zone, the fixed factor “Shelter position” was replaced with “Shelter presence” (fixed factor with two levels: presence and absence of the shelter) as the two response variables were considered separately. For each lmer, the identity of the experimental individuals (ID –factor with forty-five levels: pair 1 to 45) was used as a random effect to resolve the issue of violation of independence assumption (Zuur et al., 2009) since the observations were repeated on the same individuals during the pre and post stimulus presentation. The significance of the fixed terms in the model was tested using the F-test with the Satterthwaite approximation for degree of freedom and the F statistic (‘lmerTest’ package in R). In addition, to assess whether fish displayed changes in their anti-predator strategy (e.g. freezing behavior) between the different CO_2 treatments, we used a linear model (lm) with “CO_2 treatment” and “Shelter presence” as fixed factors. In this case, random ID effects were not included in the analysis as the observation was considered only during the post-stimulus period. For each statistical analysis, residuals were checked for the model assumptions. All the analyses were performed using the R software version 1.3.1073 (RStudio Team, 2020) and the lme4 package for R for linear mixed effect modelling (Bates et al., 2015).

3. Results

The densities of gobies and anemones were significantly higher at the High CO_2 than the Ambient CO_2 site with on average 1.46 (±0.25 SE) and 0.73 (±0.08 SE) goby individuals m^{-2}, and 10.71 (±0.88 SE) and 5.78 (±0.57 SE) anemone individuals m^{-2}, respectively (Table 1; Fig. 2a-b). The percentage of fish associated with anemones did not significantly differ between sites, as we observed an association of 84.2% (± 5.41 SE) at High CO_2 and of 87.2% (±5.07 SE) at the Ambient CO_2 (Table 1; Fig. 2c) site. The density of the predator *S. scriba* was similar between the two sites with a mean value of 1.05 (± 0.18 SE) individuals 20 m^{-2} in the Ambient and 1.00 (± 0.17 SE) individuals 20 m^{-2} in the High CO_2 site (Table 1; Fig. S3 in Supplementary data).

In the transplant experiment, no difference in the mean standard length (cm) of *G. incognitus* was observed between the different CO_2 treatments (AA: 6.04 ± 0.18SE; AH: 6.16± 0.36 SE, HA: 6.72 ± 0.20 SE; HH: 6.47± 0.23 SE) (Table 1; Fig. S4 in Supplementary data). The activity level in the entire experimental arena was not affected by the different CO_2 treatments, presentation periods (*pre*- and *post-stimulus)* and shelter position (close and far from the predator), as well as by their interactions. (Table 1; Fig. 3A). However, activity level in the predator zone was significantly different between *pre*- and *post-stimulus* periods among the different CO_2 treatments (CO_2 treatments x Predator stimulus; Table 1). A post hoc test performed on the interaction term showed that in the AA treatment the mean time spent swimming by *G. incognitus* individuals was significantly lower during the *post-stimulus* period than the pre-stimulus period (8.2 sec ± 3.64 SE vs 31 sec ±5.27 SE in *pre-stimulus*,

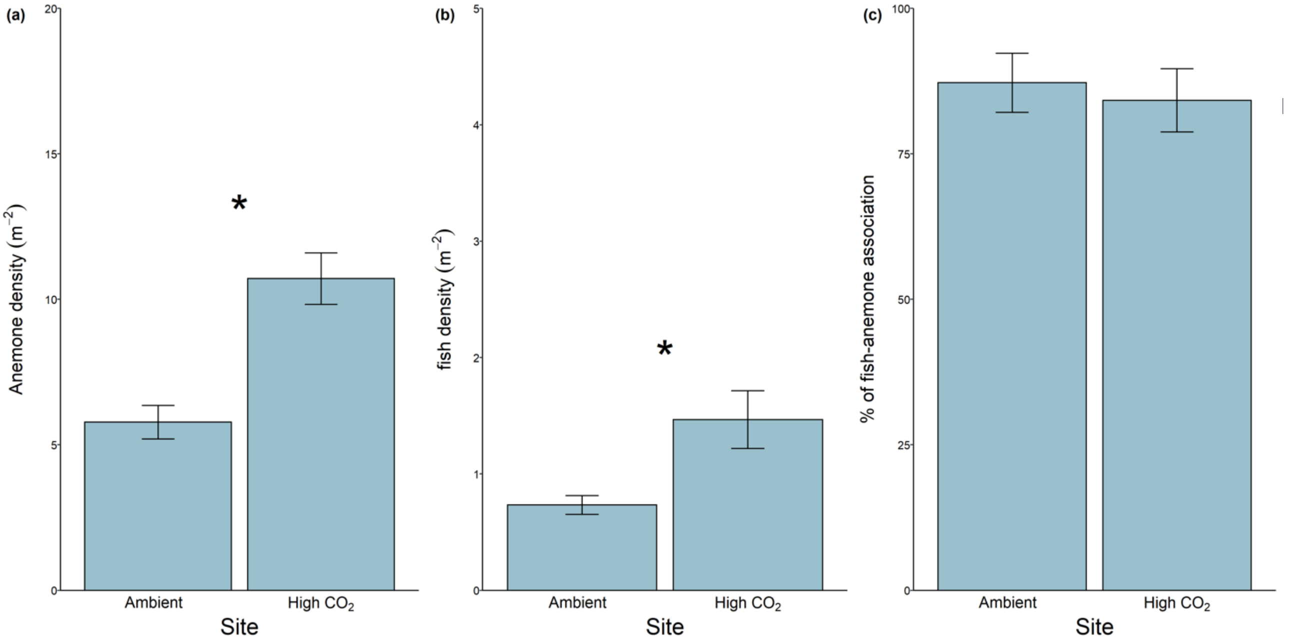

Table 1; Fig. 3B). By contrast, no difference in the same response variable was observed for the other CO_2 treatments between Predator stimulus (AH, HA and HH; $p > 0.05$; Table1; Fig. 3B). Finally, the average time spent actively moving by *G. incognitus* individuals in the predator zone was significantly higher in the presence of the anemone (16.5 sec ± 2.65 SE) than in its absence (11.71 sec ± 2.75 SE). However, this result was not related to the different CO_2 treatments and to the predator presentation (Table 1). Activity level in the no predator zone was similar between pre- and post-stimulus periods ("CO_2 treatments x Predator stimulus" interaction; Table 1; Fig. S5 in Supplementary data).

During the post-stimulus period, the percentage of time individuals of *G. incognitus* spent resting in the predator zone ranged from 29% (in the HH treatment) to 45 % (in AA), with this behaviour showing no differences between the four CO_2 treatments considered (Table 1), and gobies spending the rest of time resting or actively moving far in the no-predator zone (see pie charts in Fig. 3B).

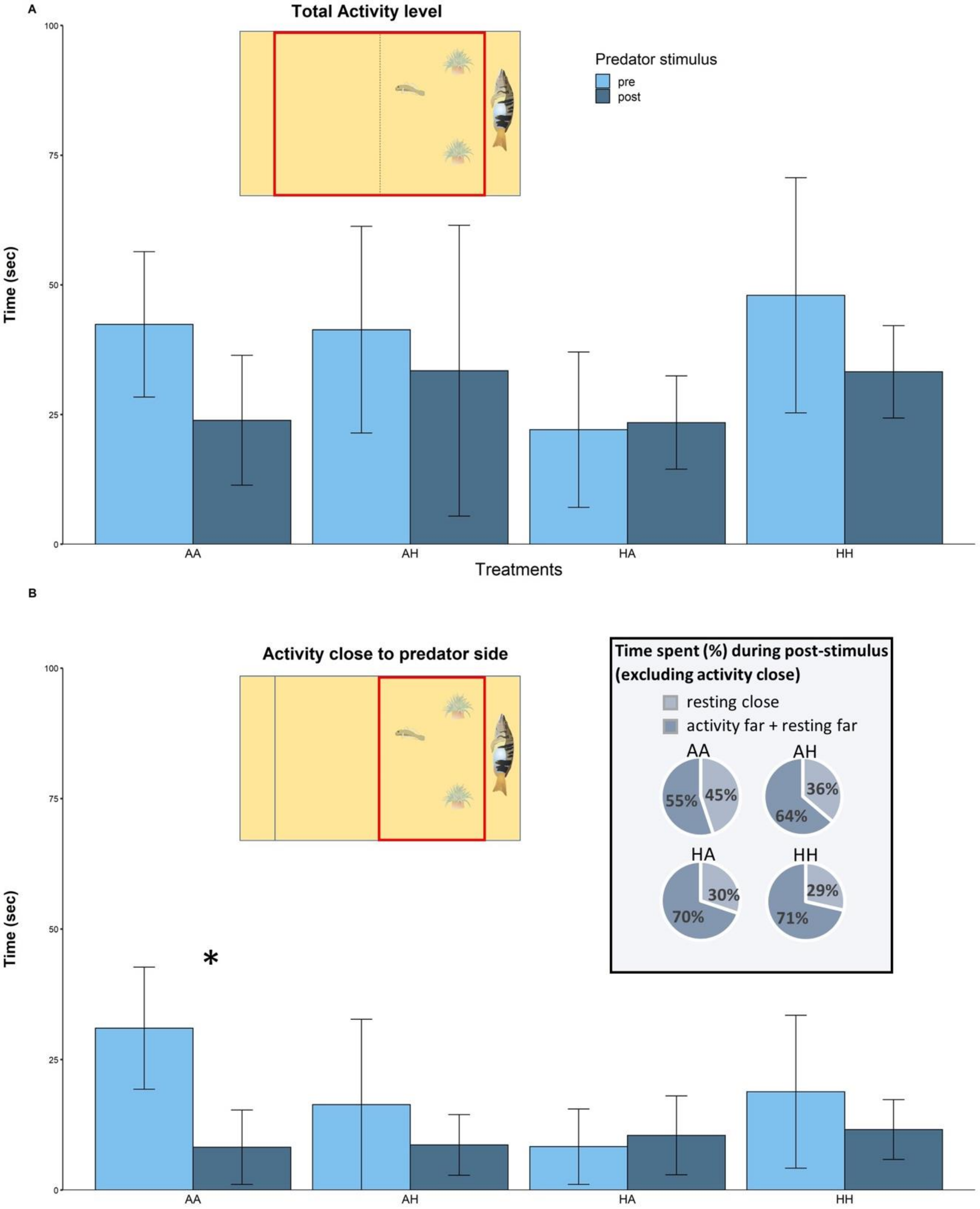

Figure 3. Average (±SE) activity levels (sec) in the entire experimental arena (a), and in the predator zone (b) displayed by *G. incognitus* before (pre-stimulus) and after (post-stimulus) the presentation of the predator *S. scriba* in the different CO_2 treatments. Pie charts in the panel b show the percentage of time gobies spent "resting in the predator zone" and the total time they spent actively moving or resting in the no predator zone ("activity + resting") during the post-stimulus period. The asterisk indicates significant differences at p-level = 0.05.

No differences in the shelter use (Fig. 4A) and the minimum approach distance (Fig. 4B) were found between the CO_2 treatments when considering the two stimulus presentation periods, the two shelter positions, as well as their interactions (Table 1).

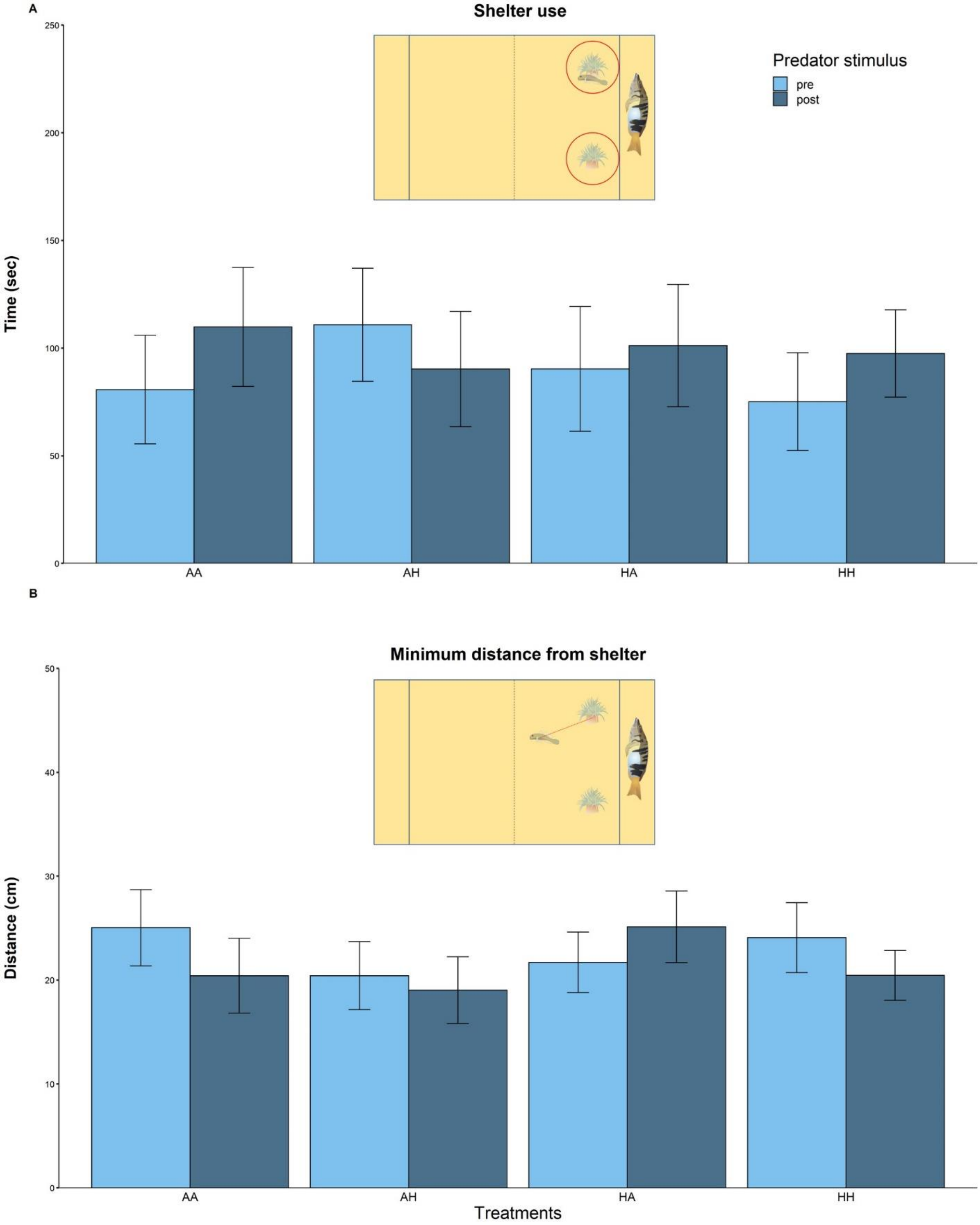

Figure 4. Average (±SE) shelter use (sec) (a) and minimum approach distance (cm) (b) displayed by individuals of *G. incognitus* before (pre-stimulus) and after (post-stimulus) the presentation of the predator *S. scriba* in the different CO_2 treatments.

Table 1 Summary results of the statistical analyses performed for the field patterns and transplant experiments in the two CO_2 sites (i.e., High CO_2 vs. Ambient CO_2 sites). Comparisons between the two sites exposed to different CO_2 conditions for densities and association, and among the different CO_2 treatments (AA, AH, HA, HH) for fish length are reported. Results of fish antipredator behaviour (fixed and random parts) in the different CO_2 treatments (CO_2), during the pre- and post-stimulus predator presentation period (Predator stimulus) and the shelter position (Shelter) are reported. Degree of freedom (df), F-tests (F), and probability levels (p) are provided, and significant effects are reported in bold.

Fish, anemone and predator density and association	df	F	p
Fish density (goby)	**1**	**9.72**	**0.004**
Anemone density	**1**	**20.41**	**< 0.001**
Predator density	1	0.09	0.796
Fish-anemone association	1	0.62	0.439

Fish length	df	F	p
Total length	3	1.872	0.14

Fish antipredator behaviour

Activity level in the entire experimental compartment

	Fixed parts		
	df	F	p
CO_2 treatment	3	0.76	0.522
Predator stimulus	1	3.64	0.064
Shelter	1	0.52	0.477
CO_2 treatment x Predator stimulus	3	2.77	0.055
CO_2 treatment x shelter	3	0.29	0.829
Predator stimulus x Shelter	1	0.00	0.999
CO_2 treatment x Predator stimulus x Shelter	3	0.25	0.864
	Random parts		
ID	Variance = 0.45		

Activity level in the predator zone

	Fixed parts		
	df	F	p
CO_2 treatment	3	1.15	0.341
Predator stimulus	1	3.45	0.071
Shelter presence	**1**	**4.24**	**0.047**
CO_2 treatment x Predator stimulus	**3**	**4.96**	**0.005**
	AA pre – AA post (t-ratio = -3.98, p = 0.007)		
	AA pre – HA pre (t-ratio = 3.43, p = 0.022)		
CO_2 treatment x shelter	3	1.11	0.359
Predator stimulus x Shelter	1	1.45	0.236
CO_2 treatment x Predator stimulus x Shelter	3	1.71	0.180
	Random parts		
ID	Variance = 0.75		

Activity level in the no predator zone (Fig. S5 in Supplementary material)

	Fixed parts		
	df	F	p
CO_2 treatment	3	1.76	0.172
Predator stimulus	1	0.00	0.955
Shelter presence	1	2.70	0.109
CO_2 treatment x Predator stimulus	3	1.66	0.192
CO_2 treatment x shelter	3	1.30	0.288
Predator stimulus x Shelter	1	3.67	0.063
CO_2 treatment x Predator stimulus x Shelter	3	1.21	0.322
	Random parts		

	ID	Variance = 0.28		
Resting in the predator zone (during post stimulus period; pie chart in Fig. 3B)				
		df	F	p
	CO$_2$ treatment	3	0.10	0.961
	Shelter presence	1	2.86	0.094
	CO$_2$ treatment x shelter presence	3	1.65	0.195
Shelter use				
		Fixed parts		
		df	F	p
	CO$_2$ treatment	3	0.17	0.918
	Predator stimulus	1	0.99	0.325
	Shelter	1	0.25	0.621
	CO$_2$ treatment x Predator stimulus	3	1.61	0.204
	CO$_2$ treatment x shelter	3	2.36	0.087
	Predator stimulus x Shelter	1	0.08	0.784
	CO$_2$ treatment x Predator stimulus x Shelter	3	0.42	0.743
		Random parts		
	ID	Variance = 0.85		
Minimun distance from shelter				
		Fixed parts		
		df	F	p
	CO$_2$ treatment	3	0.23	0.875
	Predator stimulus	1	0.46	0.502
	Shelter	1	0.55	0.462
	CO$_2$ treatment x Predator stimulus	3	0.38	0.769
	CO$_2$ treatment x shelter	3	2.03	0.126
	Predator stimulus x Shelter	1	1.77	0.192
	CO$_2$ treatment x Predator stimulus x Shelter	33	0.49	0.690
		Random parts		
	ID	Variance = 0.37		

4. Discussion

This study represents one of the first attempts in natural conditions to assess the potential impacts of ocean acidification on antipredator responses of fish acutely and chronically exposed to elevated CO_2 concentrations. Mediterranean goby fish and anemone densities were greater under High CO_2 than Ambient conditions, whilst the fish-anemone per cent association was similar between sites. We show that both short and long-term exposure to the expected end of century-pCO_2 levels did not affect most of the behaviours investigated in this goby species, with only the activity level measured close to the predator in the AA treatment being significantly lower during the predator presentation stimulus. Our results suggest that the ability of gobies to tolerate reduced pH/elevated CO_2 environment may be the result of behavioural adjustments (e.g. change in antipredator strategy) combined with the potential benefits of OA-indirect effects (e.g. habitat provisioning, food availability).

Swimming activity is one of the behavioural measurements most frequently used in studies aimed at investigating the OA effects on predator detection ability in fish (Cripps et al., 2011; Ferrari et al., 2011, 2012; Munday et al., 2013). The majority of previous studies conducted in laboratory conditions reported increased (see Briffa et al., 2012; Cattano et al., 2018; Draper and Weissburg, 2019; Nagelkerken and Munday, 2016 for reviews) or unvaried activity levels (e.g. Clark et al., 2020; Duteil et al., 2016; Sundin et al., 2013; Sundin & Jutfelt, 2016;) in fish exposed to elevated CO_2 concentrations in the short-term. Our findings, showing no difference between CO_2 treatments (AA, AH, HA, HH) in the amount of time the fish spent actively swimming in the entire arena (total activity levels) before and after the predator presentation, are in line with these studies failing to detect effects of elevated CO_2 on activity levels of fish. This suggests that they can maintain their perception of predators, although their specific behavioural strategy may be altered.

When we look at the amount of time *G. incognitus* individuals spent swimming in the half side of the arena near the predator, a clear reduction of activity during the *post-stimulus* period than the pre-stimulus period was observed only for the AA treatment (i.e., when fish were collected from the site at Ambient CO_2 and replaced in the same site). Inversely, in the High CO_2 (HH) and the reciprocal transplants Ambient-High CO_2 treatments (i.e., HA and AH), *G. incognitus* individuals spent a similar amount of time actively moving close to the predator side between the pre- and post- stimulus periods. This reduction of the activity level in proximity of the predator displayed by Ambient CO_2 fish may be related either to an increased resting time in the predator zone which can be interpreted as a "freezing behaviour" or to an increase in the time spent far from the predator, both reducing the risk to be predated. The freezing behaviour is a fundamental component of the antipredator response of many goby species (Smith et al., 1989), and represents a tactic aimed at increasing the chance of not being recognized by the predator (Paul et al., 2018; Smith, 1989; Manassa & McCormick, 2012). In addition to freezing behaviour, other response types such as fleeing, have been observed in threatened fish. Our findings reporting significative difference in activity levels between the pre- and post-stimulus period only in the AA treatment, suggest that fish that have experienced OA-effects may prefer different antipredator strategy (i.e. spending more time far from the predator). However, our findings, reporting no differences in the duration of the resting time/freezing during the post- stimulus periods between the different CO_2 treatments (but only between the pre and post-stimulus periods), do not support previous findings showing that fish under elevated CO_2 conditions display altered freezing behaviour when exposed to a predatory risk (e.g. Näslund et al., 2015; Porteus et al., 2018).

Since predators' density in the environment may affect the behaviour, and ultimately the population density of a given prey (Kusch et al. 2004; Nagelkerken et al., 2015; Ferrari et al., 2017), we expected that a different number of predators may lead to different predation risk levels with consequences on *G. incognitus* along the Vulcano CO_2 gradient. However, our observations and previous studies (Mirasole et al., 2017; Cattano et al., 2017) reported similar densities of the predator *Serranus scriba* between the two CO_2 sites. If we assume that the extent of predation risk may be solely inferred from the abundance of predators, our results suggest that the behavioural responses observed in this study are not driven by intrinsic differences in the number of predators between CO_2 sites. However field patterns data suggest that the abundance of anemone gobies (the prey) does differ between collection sites. In this regard, we suggest that specific experiments should be designed to correctly understand the relative importance of predator and prey abundances and behaviours in determining predation risk under different CO2 levels.

Similarly, other potential factors such as the different availability of shelters (i.e., anemones) the fish had experienced at their original (collection) site may potentially affect the goby responses (e.g. the ability to use the shelter). In this regard, goby fish from the different CO_2 sites exhibited a similar amount of time spent in the shelter (both when the anemones were placed far or close to the predator) before and after the predatory stimulus presentation. Consequently, our results suggest that any changes in behaviour cannot be related to the different density of anemones from the original site of collection. Previous experiments conducted both in laboratory (e.g. Cattano et al., 2019; Munday et al., 2013, 2014) and in the wild conditions (i.e., natural CO_2 seeps; Nagelkerken et al., 2015) have documented a reduced sheltering time under High CO2 conditions. However, no difference in the sheltering behaviour of the temperate fish species *Gasterosteus aculeatus* was observed between elevated (1000 μatm) and Ambient (400 μatm) pCO_2, with this suggesting responses of the fish to increased CO_2 levels may be species-specific (Näslund et al., 2015). Interestingly we also found no differences in the minimum approach distance from the anemone among goby individuals before and after the predator presentation in the two CO_2 sites. Such finding is consistent with a study showing that the distance from the shelter kept by the dottyback *Pseudochromis fuscus* was not affected by elevated seawater CO_2 concentrations (Cripps et al., 2011). By contrast, some evidence reported a lower risk perception (i.e. an increased distance ventured from the shelter) under elevated CO_2 conditions in different tropical fish species (Cattano et al., 2019; Munday et al., 2010, 2012).

We also observed a greater density of the sea anemone *A. viridis* (i.e., the goby shelter) at the elevated CO_2 site, and no differences in fish-anemone association between the Ambient and High CO_2 sites. Previous studies in the same location suggested that elevated CO_2 conditions, proximity to the vent site and the related habitat effect (e.g., increased macroalgae and sandy substrates) might be larger than that of other CO_2-mediated effects such as the escape behaviour of *Gobius bucchichi* (*cfr Gobius incognitus*) (Nagelkerken et al., 2015). Here we add on these observations, suggesting that the higher densities of *G. incognitus* under elevated CO_2 levels are related to the higher density of the sea anemone *A. viridis*, hence supporting one of the most uncontroversial responses of coastal fishes to OA, i.e. the changing habitat provisioning effect (Sunday et al., 2017; Cattano et al., 2020). In this regard, previous studies conducted along the Levante Bay gradient in Vulcano Island suggested that increased pCO_2 may boost abundance/size, photosynthesis of zooxanthellae algae, respiration (Suggett et al., 2012), trophic flexibility (e.g., a higher autotrophic/heterotrophic ratio) (Horwitz et al., 2015), and the mechanisms by which *A. viridis* acclimate to chronic exposure to ocean acidification conditions (Urbarova et al., 2019).

We might also speculate that the increased abundance of the goby fish in the elevated CO2 site can be related to a higher food availability under this condition. G. incognitus mainly prey on small crustaceans (Pölzer & Patzner, 2000; Tiralongo et al., 2020). Previous experiments in Vulcano and other CO 2 seep sites do suggest that a reduced abundance of OA-sensitive species is often compensated by an increased abundance of OA-tolerant small crustaceans (Kroeker et al., 2011; Fabricius et al., 2014; Milazzo et al., 2019). However, this food provisioning hypothesis has not been tested in this study and eventually needs further confirmation with targeted research (but see Nagelkerken et al., 2017).

In summary, our findings show that most of the anti-predator responses of *G. incognitus* are largely unaffected by elevated CO_2. When looking at the activity level recorded near the predator, we show that fish experiencing chronic (HH, HA treatments) or short-term (AH treatment) exposure to elevated CO_2 levels exhibited a similar response between the pre- and post-predator presentation periods. This suggests that OA may affect the use of specific antipredator strategy without altering their ability to perceive the presence of the predator.

Overall, the behavioural tolerance recorded under elevated CO_2 conditions, combined with the higher population density of *G. incognitus* in the High CO_2 site may indicate behavioural plasticity and adaptative potential of the species to environmental change (e.g. Allan et al., 2014; Calosi et al., 2016; Kang et al. 2022; Petit-Mart et al., 2021). The fish ability to survive in a reduced pH/elevated CO_2 environment (e.g. close to CO_2 vents) and to maintain viable populations will depend upon their biophysical environmental requirements and on their ability to tolerate, acclimate and eventually adapt to ocean changing conditions (Munday et al. 2013; Sunday et al. 2014). However, long-term adaptation does not occur directly but might be the result of mechanisms of behavioural adjustments (and physiological acclimatization) (Petit-Mart et al., 2021). Therefore, individuals of *G. incognitus* living near CO_2 seeps could potentially benefit from OA-indirect effects (e.g. habitat provisioning, food availability), which in turn may have also supported behavioural mediated tolerance of the species to an elevated CO_2 environment. Further explanations may encompass the presence of some mechanisms alleviating the impact of elevated pCO_2 on the behaviour of this goby species when acutely or chronically exposed to OA. For instance, the individuals could experience in these systems large diel pCO_2 variability (Jarrold et al., 2017) or compensate the sensorial impairment due to high CO_2 concentrations by using multiple senses to detect their predators (Draper & Weissburg, 2019). Elevated CO_2 may affect differently the single sensory modalities (visual and olfaction), and one of the two senses used to detect predator cues may compensate for the lacking response to the other (Goldenberg et al., 2018).

Behavioural mechanisms underlying potential local adaptation to OA are still little known, particularly in fish chronically exposed to high pCO_2 levels in the wild. One interesting follow-up question resulting from this study is whether behavioural-induced acclimatization/adaptation to OA conditions can mitigate, or even outplay, negative OA effects at population level, as seems to be the case for this goby. Therefore, future research needs to assess mechanisms involved in plasticity and adaptation of marine species to ongoing OA and the relative importance of its direct (behavioural responses) and indirect (e.g., altered food and habitat provisioning) effects.

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Authors’ contributions

DS and MM: conceptualized and designed the work. DS, GT, JD, GQ and GA: conducted the field survey. DS and FQ analyzed the data; DS wrote the initial draft of the manuscript with the help of MM and CC. All authors contributed to the last version of this MS.

Declaration of interests

The authors declare that they have no conflict of interest.

Supplementary data

Supplementary data to this article can be found online at XXX.

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