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ROLE OF BEHAVIOUR IN MARINE ORGANISMS: POTENTIAL EFFECTS UNDER FUTURE OCEAN CONDITIONS

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Role of behaviour in marine organisms: potential effects under future ocean conditions

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List of papers

Published papers:

- Spatafora, D., Massamba N'Siala, G., Quattrocchi, F., Milazzo, M., Calosi, P. (2020). Plastic adjustments of biparental care behaviour across embryonic development under elevated temperature in a marine ectotherm. Ecology and Evolution. DOI: 10.1002/ece3.7902

Papers under review:

- Spatafora, D., Quattrocchi, F., Cattano, C., Badalamenti, F., Milazzo, M. (2021). Nest guarding behaviour of a temperate wrasse differs between sites off Mediterranean CO_2 seeps. Science of the Total Environment (Under review)

- Spatafora, D., Cattano, C., Aglieri, G., Quattrocchi, F., Turco, G., Quartararo, G., Dudemain J., Milazzo, M. (2021). Ocean acidification and behavioural response to predation risk in the Mediterranean goby (*Gobius incognitus*) living along a vulcanic CO_2 gradient off Vulcano Island. Marine Environmental Research (Under review)

Summary

Over the last 250 years, the intensive burning of fossil fuels along with industrial processes and land uses (e.g. clearing forests and agriculture) has contributed to an increase in atmospheric CO_2 from approximately 280 to 410 ppm, with a further increase (from 730 to 1020 ppm) projected by the end of this century. About 30% of the anthropogenic CO_2 has been absorbed by the ocean, with a consequent decrease of the ocean's surface pH causing a phenomenon better known as Ocean Acidification (OA). The average pH of the surface ocean has declined from 8.2 by 0.1 units since pre-industrial times as a result of CO_2 emissions and a further reduction of 0.3–0.5 pH units is expected to occur by the 2100.

This increased concentration of atmospheric CO_2 has driven an increase in atmospheric and oceanic temperatures enhanced at a rate of ~ 0.2°C per decade in the past 30 years. These rapid changing ocean conditions in pCO_2 and temperature are considered two of the major threats to marine biodiversity, leading to changes in the distribution, physiology and behaviour of marine organisms, with potential consequences in community and ecosystem functioning and structure. Despite the increasing interest and amount of literature on this topic, the effects of OA and ocean warming (OW) on marine fauna is difficult to predict, especially because a wide range of impacts have been found across different life stages-and species suggesting that tolerance thresholds to such stressors can vary among life stages experienced by an organism or even between species. In this regard, an increased number of studies has been conducted to better understand the mechanisms by which species can cope with these rapid environmental changes.

The first response of animals to a changing environment is predominantly through modification of their behaviour. To date, only a few climate change biology studies have considered behavioural plasticity as a way that animals can adjust their performance under rapid climate change, especially for marine ectotherms.

The general objective of this thesis was to evaluate the effects of ocean warming and acidification on different aspects of behaviour in marine ectotherms. To achieve this aim I investigated the behavioural responses of two marine fish and one invertebrate, through field-based and laboratory experiments.

In **Chapter 2** of this thesis, I assessed the plasticity of parental care investment under elevated temperatures in a gonochoric marine annelid with bi-parental care, *Ophryotrocha labronica*, and investigated its role in maintaining the reproductive success of this species in a warming ocean. I measured the time individuals spent carrying out parental care activities across three phases of embryonic development, as well as the hatching success of the offspring as a proxy for reproductive success, at control (24°C) and elevated (27°C) temperature conditions. Under elevated temperature we observed: (i) a significant decrease in total parental care activity, underpinned by a decreased in male and simultaneous parental care activity, in the late stage of embryonic development; and ii) a reduction of hatching success, that was however not significantly related to changes in parental-care activity levels. These findings, along with the observed unaltered somatic growth of parents and decreased brood size, suggest that potential cost-benefit trade-offs between offspring survival (i.e. immediate fitness) and parents somatic condition (i.e. longer-term fitness potential) may occur under ongoing ocean warming. Finally, our results suggest that plasticity in parental care behaviour is a mechanism able to partially mitigate the negative effects of temperature-dependent impacts.

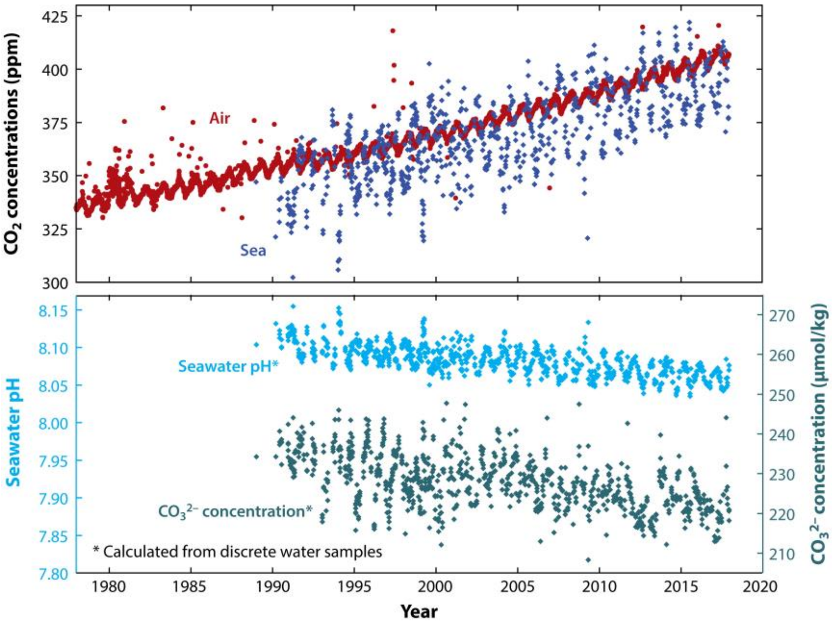
Chapter 3 provides one of the first evidence of the effect of elevated CO_2 on the behaviours of a coastal wrasse in the wild. Here, I assessed whether the nesting male ocellated wrasse Symphodus ocellatus from sites with different CO_2 concentrations showed different behaviours during their breeding season. I also investigated potential re-allocation of the time-budget toward different behavioural activities between sites. I measured the time period that the nesting male spent carrying out parental care, mating and exploring activities, as well as changes in the time allocation between sites at ambient (~400 µatm) and high CO_2 concentrations (~1000 µatm). Whilst the behavioural connectance (i.e., the number of linkages between different behaviours relative to the total amount of linkages possible) was unaffected, I observed a significant reduction in the time spent on parental care behaviour, and a significant decrease in the guarding activity of fish at the high CO_2 sites, with a proportional re-allocation of the time budget in favour of courting and wandering around, which did not change between sites.

This study shows behavioural differences in wild fish living off volcanic CO_2 seeps that could be linked to different OA levels, suggesting that behavioural plasticity may potentially act as a mechanism for buffering the effects of ongoing environmental change. A reallocation of the time budget between key behaviours may play a fundamental role in determining which marine organisms are thriving under projected OA.

Chapter 4 represents one of the few attempts to assess the effect of increasing CO_2 on the response of fish to visual and chemical risk cues in natural conditions. More specifically, I carried out an *in situ* experiment along a volcanic CO_2 gradient to evaluate anti-predator responses of a benthic fish with a tiny home range and chronically exposed to OA conditions. We used individuals from ambient (~400 µatm) and elevated (800-1000 µatm) CO_2 sites, and reciprocally transplanted fish from these conditions. I investigated the possible effects of CO_2 on swimming activity, shelter use and the minimum approach distance between shelter and prey, before and after exposure to a common predator. Interestingly, we did not detect acute and long-term CO_2 effects for most of the behaviours investigated, but swimming activity in the proximity of the predator. In this context, fish reared at ambient pCO_2 level and replaced in this condition responded to the predatory threat sensibly reducing their activity in proximity to the predator. This response was not evident for the other treatments, therefore further suggesting tolerance of this species to elevated CO_2. This is also supported by the 3-fold higher density of *G. incognitus* in this condition. Overall, behavioural plasticity displayed by fish in our experiment suggests an adaptive potential of this goby species to the High-CO_2 environment. This study may contribute to the ongoing debate over realistic predictions of the impacts of expected increased CO_2 concentration on fish.

Chapter 1 - General Introduction

Humans are rapidly changing the Earth's climate, and scientific research is significantly improving knowledge and understanding of the current and future potential impacts of climate change on the global environment. Since the industrial revolution, the total concentration of the anthropogenic carbon dioxide (CO_2) in the atmosphere has risen greatly in the last 250 years as a consequences of human activities (industry, increasing fossil fuel combustion and deforestation). At present, atmospheric CO_2 has already reached more than 410 parts per million (ppm) with levels that are nearly 50% higher than pre-industrial atmospheric concentrations (Doney et al., 2020). The current levels and the rapid growth rates of this gas have never been recorded in the past 55 million years of the geological record (Gingerich, 2019) (see Fig. 1). Four pathways were used for climate modelling and research by the IPCC Assessment Report (AR). During the fifth Assessment Report (AR5) the Intergovernmental Panel on Climate Change (IPCC) adopted four Representative Concentration Pathways (RCPs) – a greenhouse gas concentration (not emissions) trajectory – to describe different future climate conditions which are expected to occur depending on the volume of greenhouse gases emitted in the years to come. The scenarios represent the high (RCP8.5, RCP6.0), medium (RCP4.5) and low (RCP2.6) atmospheric CO_2 concentration pathways of the IPCC's Fifth Assessment Report (Stocker et al., 2013; see Fig. 2).



Doney SC, et al. 2020.
Annu. Rev. Environ. Resour. 45:83–112

Figure 1. Trends in surface (<50 m) ocean carbonate chemistry calculated from observations obtained at the Hawai'i Ocean Time-series (A) The upper panel shows the linked increase in carbon dioxide (CO_2) in the atmosphere (red points) and surface ocean (blue points), both presented in terms of CO_2 concentration in air (ppm). The bottom panel shows a decline in seawater pH (light blue points, primary y-axis) and carbonate ion (CO_3^{2-}) concentration (green points, secondary y-axis). Ocean chemistry data were obtained from the Hawai'i Ocean Time-series Data Organization & Graphical System. Figure adapted from Jewett & Romanou, originally created by Dwight Gledhill, NOAA.

The rise in atmospheric carbon dioxide (CO_2) concentration, resulting from anthropogenic activities, is responsible for the increase in the dissolved state of this gas in the oceans. It is also well understood that additional CO_2 in the seawater, results in a shift in seawater acid-base balance toward more acidic, lower pH conditions which is considered a phenomena known as Ocean Acidification (OA) (Caldeira & Wickett, 2003). Atmospheric CO_2 levels are predicted to continue to increase from current levels (~400 µatm), to approximately 600-700 µatm by 2050 and up to ~1000 µatm by 2100 (Meinshausen et al. 2011; Fig 2). By the year 2100, the increased CO_2 in the sea surface, will result in the decreased availability of carbonate ions and a further reduction in pH by 0.3–0.5 units under the RCP8.5 scenario (Fig 2) (Caldeira and Wickett 2005; IPCC 2016; Gooding et al. 2009, Pörtner et al. 2014).

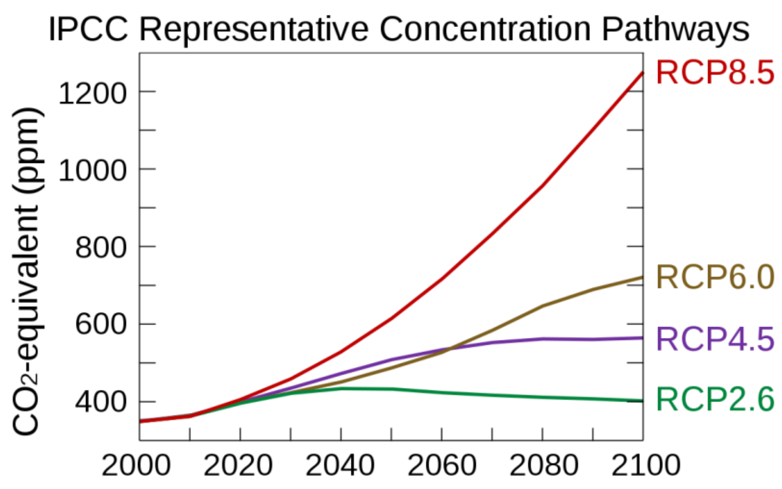


Figure 2. All forcing agents' atmospheric CO_2-equivalent concentrations (in parts-per-million-by-volume (ppm)) according to the four RCPs used by the fifth IPCC Assessment Report to make predictions.

The huge increase of greenhouse gas concentrations (e.g. CO_2) in the atmosphere is responsible of almost 100% of the observed temperature increase over the last 50 years (e.g. Stocker et al. 2013). Global warming is the observed and projected increases in the average temperature of Earth's atmosphere and oceans (Kumar,2018). The Earth's average temperature has raised by 0.6° Celsius in the 20th century and the projection of current trends as represented by a different scenarios reports temperature increases of about 3° to 5 °C by the year 2100 (Collins et al. 2013; Fig. 3).

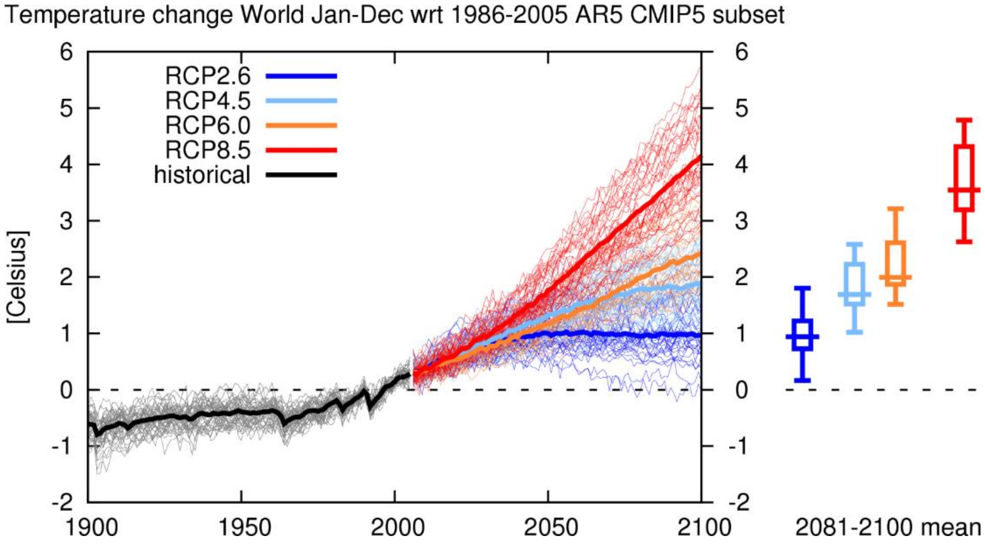
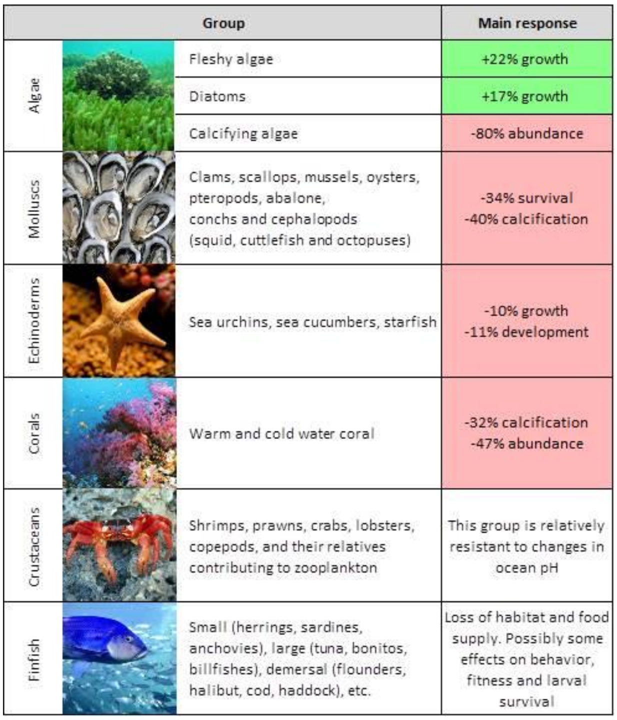


Figure 3. Global temperature change (mean and one standard deviation as shading) relative to 1986–2005 for the RCP scenarios run by CMIP5. The box plots (mean, one standard deviation, and minimum to maximum range) are given for 2080–2099 for CMIP5 (colours). Source : KNMI.

In addition to this, IPCC projections suggest a global (up to) four-fold increase in oceanic pCO_2 by 2100, with marine organisms experiencing much larger extreme CO_2 levels across the twenty-first century (McNeil & Sasse, 2016). These changes in temperature and ocean carbonate chemistry are considered two of the greatest threats to marine biodiversity (Kleypas et al. 1999; Doney et al. 2009), leading to changes in the physiological performance of individual organisms, which will in turn alter biotic interactions, community structure, and ecosystem functioning.

Effects of Ocean Acidification (OA) on marine organisms

Experimental studies assessing the potential impacts of increasing CO_2 and ocean acidification on marine organisms have recently started to be well appreciated. Within the last two decades, a growing number of studies have investigated the variety of organismal responses to predicted levels of CO_2in the marine environment (Royal Society 2005). Acidification effects include changes at different levels of biological organization from cellular metabolism, organism physiology, sensorial perception until population and community levels (Gattuso & Hansson, 2011; Doney et al., 2012; Hoegh-Guldberg & Bruno, 2010). However, studies have reported differences in organisms' responses to CO_2 changes highly suggesting variable sensitivity among and within taxa (Fig. 4).



Group		Main response
Algae	Fleshy algae	+22% growth
	Diatoms	+17% growth
	Calcifying algae	-80% abundance
Molluscs	Clams, scallops, mussels, oysters, pteropods, abalone, conchs and cephalopods (squid, cuttlefish and octopuses)	-34% survival -40% calcification
Echinoderms	Sea urchins, sea cucumbers, starfish	-10% growth -11% development
Corals	Warm and cold water coral	-32% calcification -47% abundance
Crustaceans	Shrimps, prawns, crabs, lobsters, copepods, and their relatives contributing to zooplankton	This group is relatively resistant to changes in ocean pH
Finfish	Small (herrings, sardines, anchovies), large (tuna, bonitos, billfishes), demersal (flounders, halibut, cod, haddock), etc.	Loss of habitat and food supply. Possibly some effects on behavior, fitness and larval survival

Figure 4. Summary of effects of ocean acidification among key taxonomic groups. The main responses are represented in percent changes, which could be either positive (green) or negative (red). Source: Adapted from Kroeker et al. 2013.

This heterogeneity in organisms' responses to OA, as evidenced by many meta-analyses (Dupont et al. 2010; Hendriks & Duarte, 2010; Kroeker et al. 2010, Wittman & Pörtner 2013; Kroeker et al., 2013; Cattano et al., 2018; Dupont et al. 2021) suggests that it is unlikely to act in a uniform manner as variation exists in marine organism responses and resilience (Harvey et al. 2013).

On the other hand, quantitative reviews have allowed some generalizations on species ability to acclimate or adapt to OA. Some taxa and specific traits have been classified as being potentially more vulnerable than others (Whiteley, 2011; Doney et al., 2012; Cattano et al., 2018). For example, calcifying organisms are considered more susceptible to ocean acidification as this process will impair their capacity to produce calcified skeletons due to increased energy costs for calcification (Pörtner et al., 2004; Hofmann et al., 2010; Doney et al., 2012), or suffer significant reductions in their calcification rates (Fabry et al., 2008; Whiteley, 2011). Inversely, some species, are able to cope with the effects of OA through different mechanisms such as acid–base regulation (Heisler, 1984; Larsen et al. 1997; Claiborne and Evans 1992), energy reallocation, active mobility and metabolism (McDonald et al. 2009; Widdicombe and Spicer 2008; Whiteley, 2011). Although some organisms, such as fish and crustaceans, possess more efficient acid–base regulation compared to many invertebrate taxa (Widdicombe and Spicer 2008; Ishimatsu et al., 2005; Melzner et al., 2009), a growing number of studies demonstrated altered calcification rate (e.g. otolith in lobster, crabs and fish; see Checkley et al. 2009; Munday et al. 2011) and metabolic process (Rummer et al 2013; Pimental et al., 2014) when exposed to high pCO_2 levels. In addition, recent laboratory and field-based experiments have showed that elevated CO_2 may disrupt a wide variety of sensory and behavioural pathways in the nervous systems of fish, molluscs and crustaceans which are related to the neurotransmitter GABA-A (Nilsson et al., 2012; Heuer & Grossell 2014; Tresguerres & Hamilton 2017). The resulting alteration of function in the GABA-A receptor due to elevated CO_2 levels causes striking behavioural and sensory disruptions in animals with consequences on impaired neurosensory function, boldness and activity and altered risk perception (Fig. 5) (Nilsson et al., 2012; Watson et al., 2014; Porteus et al., 2018).

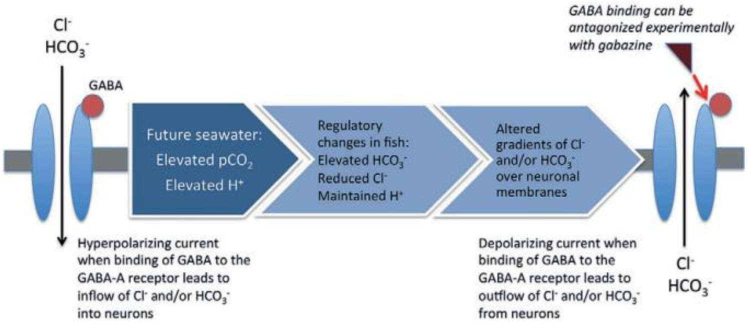


Figure 5. Schematic representation of high CO_2 effects on GABA-A receptor functions. Normally GABA neurotransmitters determine the inflow of Cl^- and HCO^{3-} through GABA receptor causing membrane hyperpolarization and inhibited neural activity. High pCO_2 levels determine altered transmembrane gradient of Cl^- and HCO^{3-} leading to excitatory responses due to GABA-A receptor depolarization. These abnormal effects are reversed by gabazine, an antagonist of GABA neurotransmitter. (source: Nilsson et al. 2012).

On the other hand, recent studies have also documented low or no evident effect of elevated CO_2 on fish behaviour (Clark et al., 2020; Raby et al., 2018, Sundin et al., 2017), suggesting at least that variability exists in fish behavioural responses to OA. These contradictory behavioural responses of fish under elevated CO_2 levels have resulted ina large debate over experiments' reproducibility in fish behavioural studies (Clark et al., 2020; Munday et al., 2020; Williamson et al., 2020).

By now, the majority of studies on the effects of high levels of CO_2 on behaviour have reported varied responses in marine species that role of local adaptation or adaptive phenotypic plasticity in modulating the effects of altered CO_2 conditions (Kroeker et al., 2013; Vargas et al., 2017). Furthermore, these studies are mostly conducted in laboratory conditions and limited to short-term CO_2 exposure experiments (Cattano et al., 2018), which may potentially underestimate the ability of organisms to acclimate and adapt to predicted ocean acidification in the long term (Wittmann and Pörtner, 2013).

Effects of Ocean Acidification (OA) at the ecosystem levels

Ecosystems are shaped by complex interactions between species and their environment. Studies examining how ocean acidification will affect composition and structure of communities and functioning of ecosystems have received increasing recent attention (Gaylord et al., 2015). Ecological community are regulated by complex interactions between organisms (e.g. predation, competition, mutualism and parasitism) which are affected by the environmental condition (Draper & Weissburg, 2019; Nagelkerken & Munday, 2016). Since species are part of interactive communities, there are likely going to be "losers" (those intolerant taxa with comparatively strong negative responses) and "winners" (those tolerant taxa with comparatively less severe or even positive responses) in ecological communities under future OA conditions (Doney et al. 2009). Therefore, a major challenge remains to better understand both how individual species respond to pH variation among other environmental complexities and how those responses will cascade through community interactions in natural ecosystems (Doney et al. 2009, Hofmann et al. 2010, Turley and Gattuso 2012). The majority of studies using natural gradients in carbonate chemistry underlined that ocean acidification increases primary producer biomass and decreases taxonomic diversity (Hall-Spencer et al. 2008; Fabricius et al. 2011, 2014; Inoue et al. 2013; Enochs et al. 2015; Cattano et al., 2020), although many species are able to survive in high-CO_2 conditions. For example, Fabricius et al. (2011) documented a decrease in taxonomic diversity resulting in a shift from a highly diversified and complex hard coral community to a single massive coral species in three different shallow carbon dioxide seeps in Papua New Guinea (Fig. 6). Similar results were reported by Inoue et al. (2013) who observed a replacement from hard coral habitats to dense soft coral populations in a medium-pCO_2 site (~ 850 µatm) off Iwotorishima Island (Japan). A reduction in habitat complexity from calcified to non-calcified habitats along with decreased diversity of associated fish was observed in the sites at elevated CO_2 (up to ~ 1000 µatm) site off Shikine Island (Japan) (Cattano et al., 2020).

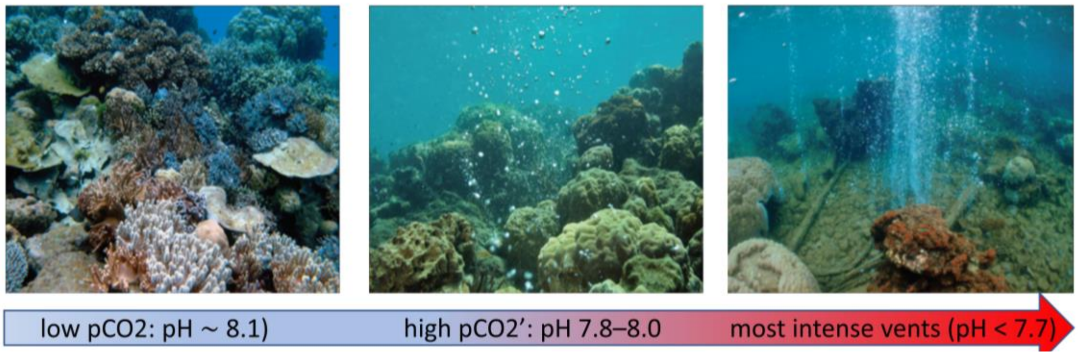


Figure 6. Progressive loss of diversity and structural complexity with increasing pCO$_2$ at three cool and shallow volcanic carbon dioxide seeps in Papua New Guinea (Fabricius et al., 2011). Figures' sources: Fabricius et al., 2011

Several studies have demonstrated that OA affects community composition of planktonic communities (Bach et al., 2017; Taucher et al., 2017) with cascading impacts on the productivity of the entire food web. For instance, OA may selectively favour an increase in the growth rates of larger versus smaller phytoplankton species (Wu et al., 2014; Bach & Taucher, 2019), leading to increased trophic transfer of energy to marine animals by shortening food chains (Sommer et al., 2015) and promoting production in higher trophic levels. At natural CO_2 seep in the North Pacific Ocean, the large chain-forming diatom *Biddulphia biddulphiana* greatly increases in abundance as CO_2 increases along a seawater concentration gradient. Along this same gradient, the abundance of calcified grazers such as gastropods and sea urchins decreased (Harvey et al., 2019). This observation suggests that OA can alter the food-web structure and ecosystem productivity by shifting the community composition of primary producers. Evidence from CO_2 seeps also documented shifts in habitat-forming species and consequent reshuffling of species at community-level or imbalanced function at ecosystem-level (e.g., Vizzini et al. 2017; Milazzo et al. 2019; Aiuppa et al., 2021). For example, an experiment conducted along CO_2 gradients off Vulcano Island revealed a clear decrease of the vermetid reefs complexity via a reduction in the reef-building species density due to low pH/elevated CO_2 conditions (Milazzo et al. 2019).

Despite the obvious caution needed to avoid confounding factors, experiments conducted in natural CO_2 vents, may represent an important approach to better understand which organisms and processes are most resilient to OA and to assess potential risks for marine populations, communities and ecosystems (Aiuppa et al., 2021; Petit-Mart et al., 2021).

Effects of Ocean Warming on organisms and ecosystems

Ongoing ocean warming (OW) is expected to affect marine ecosystems at all levels of biological organization (Abram et al., 2017). OW has important biological effects that may influence whole marine ecosystems and all of their constituent species from microorganisms to algae to top predators (Brierley et al., 2009; Yao & Somero, 2014). Changes in temperature condition can primarily influence biochemical reactions scaling up to influence growth, development, and other life history traits, ultimately affecting an organism's fitness (McNamara et al. 2011; Gillooly et al. 2001, 2002; Angilletta, 2009) (Fig. 7).

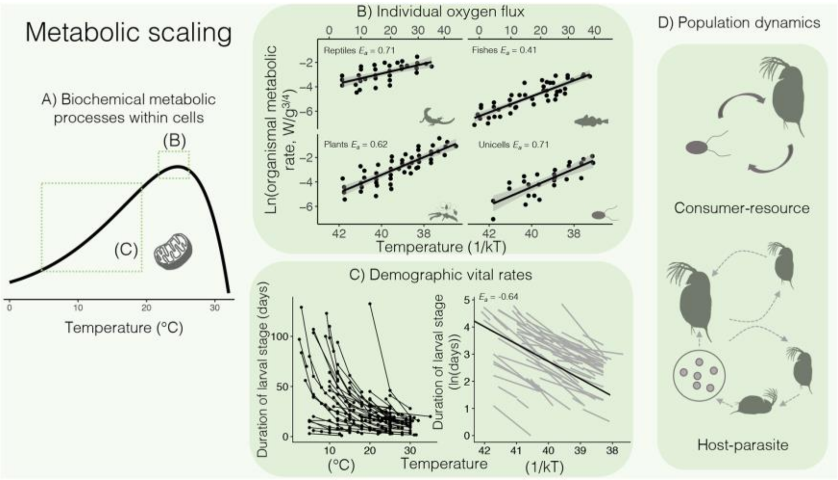


Figure 7. Effect of temperature on: metabolic process within cells(A); individual oxygen flux of different organisms (B); demographic vital rates (C); population dynamics (D).

A range of marine biological responses have already been observed in response to ocean warming including hypoxia (Pörtner and Knust 2007), coral bleaching (Hoegh-Guldberg et al. 2007), species range shifts (Parmesan and Yohe 2003; Root et al. 2003), changes to phenology (Walther et al. 2002), and reduced organism body size (Daufresne et al. 2009). However, the biological importance of rising temperature varies within and among species and its effect depends on the different ontogenetic stages which are differentially susceptible to environmental stress (Harley et al., 2006; Yao & Somero, 2014; Hoegh-Guldberg & Bruno 2010). Deleterious effects of elevated temperature have been observed in juveniles and the larval stage of many marine organisms (Pechenik 1989; Gagliano et al., 2007). An example of that can be found in certain planktonic larval stages which are particularly susceptible to thermal effects (Pechenik 1989) as well as for young benthic stages of many organisms which are more vulnerable to stress than are adults (Foster, 1971). Rising temperature may also have detrimental effects on embryo survival and growth of many marine organism as previously reported in several studies (e.g. Rosa et al., 2012; Byrne et al., 2009; Webb et al., 2007).

Since the majority of marine organisms live close to their optimal temperature (Hughes et al., 2003), increases in water temperature may have important consequences for their performance and survival. This is the case, for example, of many reef-building corals, which are very susceptible to temperature changes and have suffered severe bleaching and mortality as a consequence of warm episodes (Hughes et al. 2003; McWilliams et al. 2005). Other studies reported a reduction in phytoplankton abundance caused by a significant increase in sea surface temperature with consequences to the upper trophic levels (Richardson & Schoeman, 2004).

The effects of rising sea temperature on marine fish are pronounced and varied. Many physiological effects of higher temperature are reported in fishes leading to reductions in aerobic scope (e.g. Farrell, 2002; Johansen & Jones, 2011; Munday et al., 2009) as well as changes in swimming performance (Rome, 2007) and sensory performance (Szabo et al., 2008). As reported for the effects of OA, higher temperatures also cause changes in behaviours such as activity rates (Biro et al., 2010) and interactions between predators and their prey (Freitas et al., 2007; Grigaltchik et al., 2012; Allan et al., 2015; Ferrari et al. 2015; Nagelkerken et al., 2015). OW may also affect reproductive behaviour as observed in many fish species (Kvarnemo, 1994; Hopkins et al., 2011; Miller et al., 2015).

Many traits show plastic responses to temperature in order to buffer negative effects of suboptimal temperatures on physiology. This is particularly true for ectothermic animals, which, in contrast to endotherms, do not typically maintain a constant body temperature through homeostatic processes with body temperature instead conforming to that of the surrounding environment (Abram et al., 2017). However, ectotherms have evolved to use physiological and behavioural adjustments in order to thermoregulate, thus coping with temperature changes (Abram et al., 2017; Nagelkerken & Munday, 2016). In this direction, behavioural plasticity may be one of the most powerful ways that animals can adjust to rapid climate change (Wong & Candolin, 2015). Plastic thermoregulatory behaviours can improve an organism's survival and reproduction if behavioural modification are linked to measures of performance, such as nutrition or energy gain. Many ectotherms, for example, can exhibit plastic thermoregulatory behaviours such as moving to warmer or cooler locations (Huey and Berrigan 2001) or adjusting the timing of breeding to location to maintain closer to optimal body temperature for biological functions (Huey and Berrigan 2001). Parental care behaviour is another trait that can vary with changes in environmental temperature. In several species, parental care is necessary to maintain appropriate temperatures to ensure the correct development and survival of offspring (van Iersel 1953; AlRashidi et al. 2010). For example, in three-spined sticklebacks (*Gasterosteus aculeatus*), male parents increase parental care investment (e.g. by fanning) at higher temperatures to overcome the greater metabolic demand of embryos due to increased temperatures (van Iersel 1953; Candolin et al. 2008; Hopkins et al. 2011). Additionally, the relationship between temperature and parental care can be complicated by trade-offs between self-maintenance and parental care. For example, a greater metabolic demand due to increased temperature can lead parents to devote less energy to parental care activities in order to favour self-maintenance, thus enhancing their chances of survival but reducing the quality of their offspring (Ardia et al. 2010; Wiley & Ridley, 2016)

Temperature can also have important implications at ecosystem levels. Global warming can indirectly influence entire communities that are being invaded by range-extending species (Galaiduk et al., 2013; Galasso et al. 2015) or affecting the strength of the existing ones (e.g. Milazzo et al. 2013; Wernberg et al. 2013; Alexander et al. 2016). For example, Milazzo et al. (2013) observed habitat displacement of a local cool-water species by a range-extending warm-water fish species. In this direction, variation in both the local distribution and predation rate of key species (as discussed before) are particularly important, due to the large effects on marine communities (Sanford 1999; Harley 2011; Nogués-Bravo & Rahbek 2011; Bonaviri et al., 2017).

For all the reason explained above, the importance of the role of temperature in biology combined with the increasingly unpredictable weather and temperature fluctuations due to climate change makes it imperative to understand how organisms cope with changes in temperature (Easterling et al., 2000).

Aims and structure of the thesis

The overall aim of my thesis was to assess the role of the behaviour in marine ectotherms, as a potential strategy adopted by species to cope with rapid environmental changes. To achieve this goal, I used different experimental approaches (i.e. lab-based experiments, field-based observations and translocation experiments) and differentstudy species (one invertebrate and two marine fish species) to investigate behavioural responses of marine ectotherms to ocean warming and acidification.

The specific aims of my dissertation are detailed in the following chapters, whose general contents are the following. Specifically, I investigated the effect of elevated temperature (+ 3 °C, RCP 8.5, IPCC 2014) on the parental care behaviour in a gonochoric marine annelid with bi-parental care, *Ophryotrocha labronica* (Chapter 2). The following two chapters dealt with the effects of OA on reproductive and anti-predator behaviour respectively in two temperate fish species (Chapter 3: *Symphodus ocellatus* and Chapter 4: *gobius incognitus*) living off a volcanic CO_2 seep site (Vulcano island, Italy). Finally, concluding remarks and potential future directions are presented in Chapter 5.

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Chapter 2 - Plastic adjustments of biparental care behaviour across embryonic development under elevated temperature in a marine ectotherm

Introduction

Environmental temperature has ubiquitous effects on all aspects of organismal biology (Angilletta, 2009; Hochachka & Somero, 2002). This is particularly true for ectothermic animals, whose body temperature conforms to that of the surrounding environment and depends mainly upon external heat sources (Abram et al., 2017). In these organisms, the relationship between thermosensitivity and thermoregulatory capacity in variable environments governs the evolution of a wide range of behavioural, physiological, and life-history traits, finally determining their overall fitness (Przeslawski et al., 2008; Munday et al., 2009; Abram et al., 2017). Phenotypic plasticity, i.e. the capacity of a given genotype to produce a range of phenotypes under varying environmental conditions, is a key mechanism that allows ectotherms to cope with rapid thermal changes (Schlichting & Pigliucci, 1998). Depending on the effect of plasticity on individual fitness, plasticity can be defined as adaptive, if it improves a genotype's fitness when environmental conditions change, or neutral, if fitness is not affected (Ghalambor et al., 2007). Alternatively, plasticity can even be maladaptive if its expression decreases fitness (Schlichting & Pigliucci, 1998). Most research on thermal plasticity has been focused on physiological and life history traits, with only more recently an increasing number of studies considering the importance of behavioural traits for terrestrial and aquatic ectotherms (e.g. Huey et al., 2012; Nagelkerken & Munday, 2016; Abram et al., 2017). An even wider knowledge gap exists for marine organisms' ability to adjust specific fitness-related behavioural responses when submitted to a thermal change. This paucity of information is particularly evident for parental care activities (Dick et al., 1998; Brante et al., 2003; Hopkins et al., 2011).

In species exhibiting parental care, variation in temperature conditions - far from their optimal thermal range - may alter the energetic investment required by parents to effectively perform such activity (Johnston & Bennett, 1996). As a result, parents may incur trade-offs between behavioural and physiological processes, and thus between parental care and cell repair, homeostasis, feeding, and growth, that can ultimately affect organismal fitness (Stearns, 1992; Roff, 2002; Ardia et al., 2009). For examples, a greater metabolic demand due to increased temperature can cause parents to devote less energy to parental care activities in order to favour self-maintenance (e.g. cell repair and mass loss avoidance), thus enhancing their chances of survival and as well as future breeding attempts (Wiley & Ridley, 2016). Alternatively, the maintenance of parental care and reproductive performance at a higher temperature may divert resources away from somatic maintenance (e.g. growth) (Donelson et al., 2010), with possible consequences for survival and life-span fitness (Edward & Chapman, 2011). Changes in the amount of energy invested by parents caring for eggs and self-maintenance due to intrinsic (e.g. age, health) and extrinsic factors (e.g. environmental conditions, predation) could also be a strategy adopted by species to favour future reproduction at the expense of current reproduction, as early postulated by *William's principle* (1966) and later supported by Carlisle (1982). Optimal parental behaviour can also be indirectly affected *via* temperature-dependent changes in embryos' development rate, size and number (St Mary et al., 2004; Angilletta et al., 2006). Commonly, variation in clutch size has been shown to affect the amount of energy invested in parental care activity in fish (Van Iersel, 1953; Coleman et al., 1985) and invertebrates (Rauter & Moore, 2004; Smiseth & Moore, 2004; Fernández & Brante, 2003). Larger broods require a greater parental care investment (e.g. by fanning) to guarantee embryo development, likely because the lower surface/volume ratio of egg masses may cause a lower rate of oxygen diffusion especially in their centre (Fernández et al., 2002). In addition to this, the increasing metabolic needs of embryos across developmental stages may alter the amount of energy/time allocated by parents for care activities (Baeza & Fernández, 2002; Dick et al., 1998; Green & McCormick, 2005).

Thermal changes can also have an asymmetric effect on parental care investment in iteroparous species with biparental care, due to differential impacts on care-giving timing and duration provided by each sex (Vincze et al., 2017; AlRashidi et al., 2011). Several theoretical models have been proposed to explain the conflict that occurs in biparental care regarding the level of investment that each parent provides (Houston and Davies 1985; McNamara et al., 1999-2003). According to one early model, also known as the "no negotiation model", if one parent provides significantly less care due to a change in the environment (e.g. rising temperature) or due to changes in life-history traits (e.g. brood size), the other partner may modify its effort independently of the effort adopted by the first parent (Houston and Davies 1985). Inversely, as predicted by more recent models (i.e. "the negotiation models"), one parent may adjust its level of parental investment in relation to the decrease in parental care provided by its partner (e.g. McNamara et al., 1999, 2003; Johnstone & Hinde, 2006). Under such circumstances, the partner may have different options: 1) to abandon altogether the care of the offspring in favour of future longer-term reproductive opportunities; 2) to reduce its parental care effort; or 3) to increase its parental care effort (Johnstone & Hinde, 2006; McNamara et al., 2003). To date, despite numerous factors are known to affect biparental care patterns, such as mating system, developmental mode, and brood size (Houston & McNamara, 2002; Olson et al., 2008), the effect of raising temperatures on parental investment in species exhibiting biparental care remains poorly understood or completely overlooked, especially when concerning aquatic invertebrates.

In this study, we assessed the role of behavioural plasticity in mediating, or exacerbating, climate-related impacts on organismal fitness using the marine annelid *Ophryotrocha labronica* (Eunicida, Dorvilleidae, La Greca & Bacci, 1962). *Ophryotrocha labronica* (max length = 4 mm) is a gonochoric species occurring in a variety of temporally and spatially fluctuating coastal habitats across the globe

(Simonini et al., 2009). Females reproduce several times over an extended breeding period (defined as semi-continuous reproduction), spanning approximately between 83 and 16.5 d at 14.5 and 28°C, respectively (Åkesson 1976). Females lay their eggs in characteristic tubular masses after a period of courtship with a male (Prevedelli & Simonini, 2001). Immediately before spawning, the couple move side by side emitting a loose jelly into which eggs and spermatozoa, which are almost immotile, are extruded; this behaviour being known as pseudocopulation (Lorenzi et al., 2018; Paxton and Åkesson, 2010). The tubular egg masses are formed before the surfaces of the egg mass harden. When individuals are isolated into pairs, *O. labronica* provide bi-parental cares to ensure the cleanliness and oxygenation of the eggs mass (Paxton & Åkesson, 2007). However, at higher densities, males can mate with multiple females, abandoning their partner at any time after the fertilization of one mass of eggs to breed with another female, ending up caring for only one of the egg masses fertilized (Sella & Bona, 1993; Picchi & Lorenzi, 2019). In both cases, females are considered the main caregivers and are constrained to parental care duties, while males can adjust their parental care effort at different densities to maximise mating opportunities (Picchi & Lorenzi, 2019). Parental cares is necessary for the survival of the brood, as exemplified by the observation that eggs usually degenerate if parents are removed before embryos are completely developed (Paxton & Åkesson, 2007). Parental care enhances oxygenation of eggs and consists of active movements of the parents' bodies in close contact with the outer or internal surface of the tubular mass (Paxton & Åkesson, 2007). In addition, parents periodically clean the surface of the eggs mass with grazing-like movements of their jaws, thought to prevent the proliferation of fungi, protozoans, and bacteria (Sella 1991; Paxton & Åkesson, 2007). Parental care is provided until the embryos break free of the egg mass casing (Paxton & Åkesson, 2007), and its duration depends on the eggs' developmental time, which generally decreases under increasing temperatures: approximatelybetween 3 and 9 d at 30 and 18°C, respectively (Åkesson, 1976, Massamba-N'Siala unpublished data).

To achieve our goal, we first investigated the occurrence of changes in parental care in response to elevated temperatures in this marine annelid, and then assessed whether thermal plasticity contributes to maintaining individuals' reproductive success. In particular, we exposed independent groups of *O. labronica* parents together with their spawned egg masses to control (24°C) and elevated (+ 3°C, RCP 8.5, IPCC 2014) temperature conditions, and measured the amount of time spent by parents (individually and together) carrying out parental care activities. Then, we assessed whether variation in the time dedicated to parental care affected offspring hatching success, which was used as a proxy for parental fitness. Temperature is a major abiotic factor triggering plastic responses in *O. labronica* (Åkesson 1976; Prevedelli and Simonini 2001, Chakravarti et al., 2016; Gibbin et al, 2017a.b, Massamba N'Siala et al. 2012, 2014, Jarrold et al., 2019). In this species, increasing temperatures induce physiological adjustments that underlie higher growth and reproductive rates, as well as reduced developmental times, age to sexual maturity, fecundity per reproductive events (brood size), and lifespan (Prevedelli and Simonini 2001; Massamba N'Siala et al. 2012). Living at a greater pace of life may divert energy from parental care behaviours, which consist of energetically demanding activities (e.g., Green & McCormick, 2005; Baeza & Fernández, 2002). As a consequence, we expect parents to decrease the time spent to care for the offspring in favour of their self-maintenance, with negative implications for the reproductive success for the specific breeding event. This decrease in parental care investment may also be favoured by a reduction in brood size expected under increased temperature (e.g. Fernández et al., 2000).

In addition, to more accurately characterised the role of each parent in caring for the brood and assess whether their parental investment is differently affected by elevated temperature, we compared the time spent separately by each parent, as well as simultaneously, in taking care of the egg mass at the two temperature conditions tested. Based on the previous observation on sex-specific behavioural patterns in *O. labronica* and specifically that males are less strictly bounded by parental duties (Kokko and Jennions, 2012; Picchi & Lorenzi, 2019), we expect that parental care activities will be more likely reduced or completely dropped by the male when compared to the female. Under these conditions, the decline in the male investment of caring for the eggs may leave the female with two main options: (1) maintaining her parental care effort in favour of her short-term reproductive success, but with potential costs for her self-maintenance, or (2) reducing her investment in parental care to the benefit of her self-maintenance, but at the detriment of her short-term reproductive success.

Materials and Methods

Specimens' collection and maintenance

Ophryotrocha labronica specimens used in this study are descendants of approx. 60 individuals collected in the Gela harbour (Sicily, Italy: 37°040N, 14°130E) as described by Massamba-N'Siala et al. (2011), and then transferred to the Marine Eco-Evolutionary Physiology laboratory at the University of Québec in Rimouski (QC, Canada). Individuals were divided into four glass bowls (70 mm diameter, 30 mm height) and reared for approximately six generations in artificial seawater (Aquarium Sea Salt Mixture, Instant Ocean®, Blacksburg, VA, USA) at constant temperature (24 ± 0.5 °C; mean ± SD), salinity (35 ± 2), pH (8.05 ± 0.1), and 12:12 light:dark photoperiod.

Experimental set up and design

To assess changes in *O. labronica* parental care behaviour in response to elevated temperatures, sexually mature females and males (Fig. 1a, b) were first randomly selected from the laboratory cultures to form 48 pairs (F0 generation), which were kept at the same conditions of previous exposure. Each pair was placed in one of the wells (34 mm diam., 20 mm height) of a 6-well culture plate (Costar, VWR, Radnor, PA, USA) until the first egg mass production. When F1 individuals reached sexual maturity, 34 pairs were formed by crossing sexually matured males and females randomly chosen from different broods in order to avoid inbreeding. Each pair was isolated in one removable well and randomly assigned to one of two temperature conditions (17 pairs per condition): control (24°C) and elevated (27°C) temperature. The former temperature condition represents an average summer temperature (June-September) experienced by this species in the location where individuals were originally collected (Massamba-N'Siala et al., *pers. comm.*), whilst the latter temperature condition represented a mean + 3°C of temperature increase expected by the end of the 21st century scenarios following the Representative Concentration Pathway (RCP) 8.5 of the Intergovernmental Panel on Climate Change (IPCC, 2014). The elevated temperature condition was reached from control conditions progressively (1°C h^{-1}) (Massamba-N'Siala et al., 2012) using a temperature incubator (MLR-352H-PA, Panasonic Healthcare Co. Ltd, Tokyo, Japan). Stable thermal conditions and a 12 Light: 12 Dark regimes were achieved by placing the culture plates in two incubators. Each plate was kept on separate shelves and cyclically moved to another shelf to remove the effect of the position in the incubator on our observations. To reduce evaporation, plates were covered with a breathable seal (Aeraseal, Alpha Laboratories Ltd, Eastleigh, UK). Throughout the experiment, individuals were daily fed *ad libitum* with minced spinach (Massamba-N'Siala et al., 2012) to avoid food-limiting conditions, which can affect parental care behaviour (Carlisle, 1982; Arcese and Smith, 1988). Water changes were performed daily to prevent undesired fermentations and the accumulation of excreta, whilst maintaining stable oxygen levels (always > 70 %).

Determination of parental care activity

Video recording for parental care activities was performed with a digital camera (14 MP, Omax, Bucheon, South Korea) mounted on a light microscope (MS5, Leica, StGallen, Switzerland). During video recording, temperature conditions were maintained constant by immersing the experimental plate inside a water bath heated by two aquarium heaters (100 W Hydor, Sacramento, CA, USA). To ensure homogenous heat distribution, a submersible water pump (Koralia nano 900, Hydor, Sacramento, CA, USA) was placed inside each water bath. Temperature was recorded continuously using a high accuracy J/K input thermocouple thermometer (HH802U, OMEGA, Laval, QC, Canada, ± 0.1°C), while salinity was checked before and after video recording with a refractometer (DD H_2Ocean, MOPS, Hamilton, ON, Canada, ± 1.0 unit).

F1 pairs were checked several times on a daily basis. Whenever a female laid her first egg mass (Fig. 1c), the well with the pair was moved in the system for video recording of parental care. Since the time frame of parental activities could change depending on the temperature condition, we divided the period of egg development (from spawning to hatching) into three phases representing specific stages of embryo development comparable between temperature conditions (Fig. 1d - f). We referred to Phase 1 (Fig. 1d) as the time between the deposition of the egg mass and the first emergence of jaws in the embryos (Paxton, 2004). During this period, eggs had a roundish shape and a homogeneous yellow colour. Phase 2 (Fig. 1e) was defined as the time between the end of Phase 1 and the embryos' full body development. During this time, embryos changed from an elongated egg-shape to the final shape observed in hatchlings. At the end of this phase, embryos started to be able to actively move within the brood pouch giving start to Phase 3 (Fig. 1f). This last phase ended when the larvae hatched, i.e. when they broke free out of the envelope that protected them during the entire duration of development (Fig. 1g) (Oyarzun & Strathmann, 2011).

Ophryotrocha labronica parental care behaviour was assessed using a standard continuous focal sampling procedure (Martin & Bateson, 1993). We grouped into a single category of parental care activity all the behaviours identified by Paxton & Akesson (2007) (Table 1). Parental care activities were recorded for 30-min every 3 h until the end of Phase 3, specifically during an average time of 6 d at 24°C and 4 d at 27°C. Then, for each pair, we randomly selected one video corresponding to each of the three developmental phases previously identified, thus obtaining 1800 sec of recording for each phase that was used to monitor parental care behaviours. To explore whether temperature affected how parental investment was divided between sexes, we measured the individual contribution of each sex to parental care activity, which was defined as the proportion of time spent by parents performing parental care activity alone with their body in close contact with the egg mass (Picchi & Lorenzi. 2019). These time variables were defined as TF for the female and TM for the male. In addition, we measured the proportion time spent simultaneously by both parents caring for the eggs (defined as TS) and the cumulative contribution of TF, TM and TS, defined as the proportion of total time for parental care activity (TT).

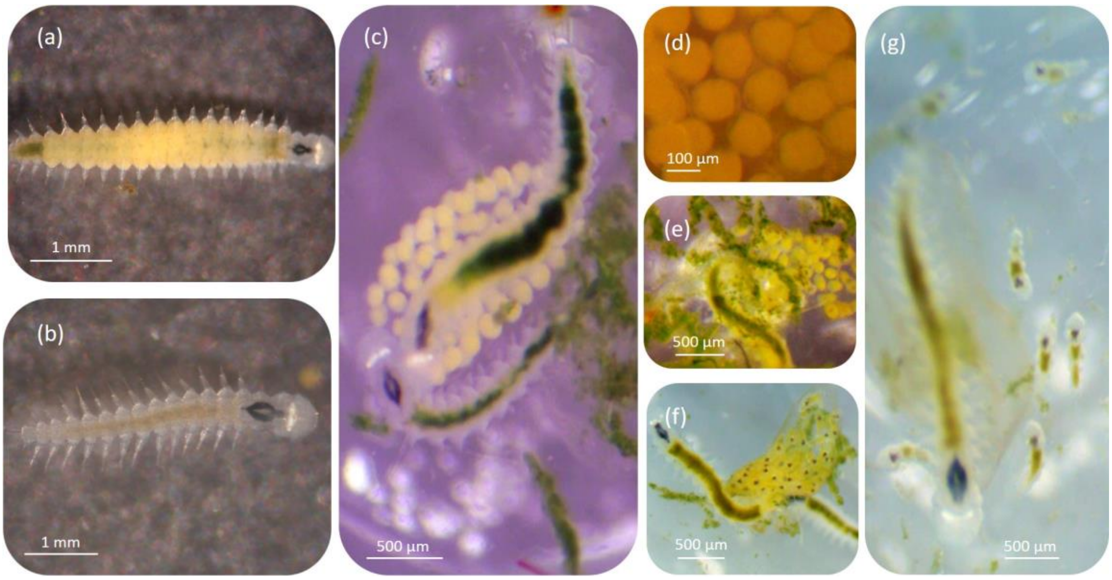

Figure 1 Adult female (a) and male (b) of Ophryotrocha labronica in dorsal view and during parental care activities (c). The stages that identified the start and end of the three phases of embryonic development considered in our study are also shown: phase 1 (d), phase 2 (e), and phase 3 (f), as well as the hatching moments (g, end of phase 3).

Table 1 Description of all parental care behaviours in *Ophryotrocha labronica*	
Parental care behaviours	Cleaning and oxygenation by scratching or brushing the body on the eggs mass
	Cleaning the egg mass from debrides with jaws movements
	Parents in close contact with the eggs accompanied or not by clear peristaltic contractions

Determination of reproductive success and life history traits

Hatching success was measured as the number of juveniles that hatched successfully over the total number of eggs spawned. The count was performed by singularly moving each hatchling from the well to another well using a Pasteur pipette. Three life-history traits were also considered to help control for other factors potentially influencing parental care investment: brood size, body size, and growth rate of both male and female. The number of eggs spawned by a female was used as a proxy for brood size (N = 34), which is known to affect parental care behaviours (Rauter & Moore, 2004). Specifically, digital photos of each egg mass were taken at first deposition using the digital camera mounted on the microscope, and the number of eggs was counted using the software ImageJ (Schneider et al., 2012). Given that parental care activity is mainly carried out through active movements of the parents' body over the eggs mass, we also measured female and male body size by counting the number of chaetigers (metameric segments bearing bristles) at the time of spawning and hatching of the larvae (Massamba-N'Siala et al., 2012). This trait is known to be sensitive to thermal variations in *O. labronica* (Massamba-N'Siala et al., 2012), and it is commonly positively correlated with brood size in females (Berglund, 1991). Finally, we measured parents' growth rate as the number of chaetigers added *per* day from the day females produced the egg mass to the end of parental care activity. Temperature-dependent changes in growth rate are expected in *O. labronica* (Massamba-N'Siala et al., 2012), and may divert energy away from parental care functions (Stearns, 1992).

Statistical analyses

Effect of temperature and brood size on parental care activity

A set of preliminary analyses were performed to explore the effects of (i) seawater temperature on brood size, growth rate and body size of parents and of (ii) parental growth rate and brood size on the proportional time of parental care (see statistical analyses and results in Appendix S1 and S2 in Supporting Information). Only brood size significantly decreased at the elevated temperature (Table A1; Fig. A1 in Appendix S1 in Supporting Information), and showed a positive relation with total time for parental care activity (TT): binomial generalized mixed model (B-GLMM); Appendix S2, Table A3a and Fig. A2. Therefore, to separate the effect of brood size from the effect of temperature on parental care behaviours, we calculated the 'residual index' (Jakob et al., 1996) by extracting the regression residuals from the previous B-GLMM between the proportion of TT and brood size, which represented the times of parental care activity controlled for brood size. We then assessed the effects of temperature ("Temp" – fixed factor with two levels: 24 and 27°C), embryo developmental phase ("Phase" – fixed factor with three levels: Phase 1, 2, 3), and their interaction on the 'residual index' using a generalized least squares model (GLS; nlme package) (Pinheiro & Bates, 2000). GLS was used since no significant differences were found when comparing it with the linear mixed model (LMM) considering 'Pairs' as random factor (Likelihood Ratio Test LRT = $8.93e^{-08}$, $p = 1.00$). Female and male body size was not included in all final analyses because its effect was always found not to be significant ($p > 0.05$). Pairwise comparisons among least square means for levels of factors were performed with Tukey's test by using the "lsmeans" package (Lenth, 2016). All analyses were performed using the R software version 3.3.0 (R Core Team, 2016).

Effect of temperature and parental care activity on reproductive success

The effect of “Temp”, Total TT (used as continuous covariate and measured as the sum of TT measured at each phase: i.e. Total TT = TT(Phase 1) + TT(Phase 2)+ TT(Phase 3)), and their interaction, on hatching success was analysed with a Poisson distribution generalized linear model tests (P-GLM). Brood size was used as an offset variable to scale the model because the quantification of hatching success was based on the total number of eggs spawned.

Effect of temperature on the sex-related division of parental care

Given that the proportion of TF, TM, and TS decreased significantly with the reduction of the brood size (Appendix S2, Table A3b-d in Supporting Information), we tested the effect of temperature on these descriptors by taking into account the effect of brood size, using the same procedure adopted for the proportion of TT. Specifically, we extracted the regression residuals from B-GLMMs between each descriptor and brood size. The term “Pairs” was initially included as a random factor, but it was never significant (TF: LRT = 8.93e^{-08}, p = 1.00; TM: LRT = 8.93e^{-08}, p = 1.00; TS: LRT =8.93e-^{08}, p = 1.00). Thus, we used three GLSs models, one for each descriptor’s residual index, to test for the effect of the factors “Temp”, “Phase”, and their interactions on the proportion of TF, TM, and S. Post-hoc pairwise comparisons using Tukey’s test (“lsmeans” package) were also performed to assess the significant interaction between levels of factors.

Results

Effect of temperature on parental care activity

The proportion of total time for parental care activity (TT) ranged between 0.83 ± 0.04 (mean ± SE) at 27°C and 0.95 ± 0.02 at 24°C, in Phase 3 (Fig. 2). Only in Phase 3, the proportion of TT was significantly lower for pairs reared at 27°C compared to those at 24°C ($t_{(96)} = 3.26$; $p = 0.02$; Table 2 and Fig. 2), while it was comparable in Phase 1 and 2 ($p > 0.05$; Table 2 and Fig. 2). Differences in the proportion of TT during different phases of embryonic development within the same temperature condition were observed only at 27 °C, more specifically between Phase 2 and 3 ($t_{(96)} = 3.72$; $p = 0.004$) and between Phase 1 and 3 ($t_{(96)} = 3.06$; $p = 0.03$) (Fig. 2; Table A4 in Appendix S3 in Supporting Information). No differences in the proportion of TT were found between phases of embryonic development at 24°C.

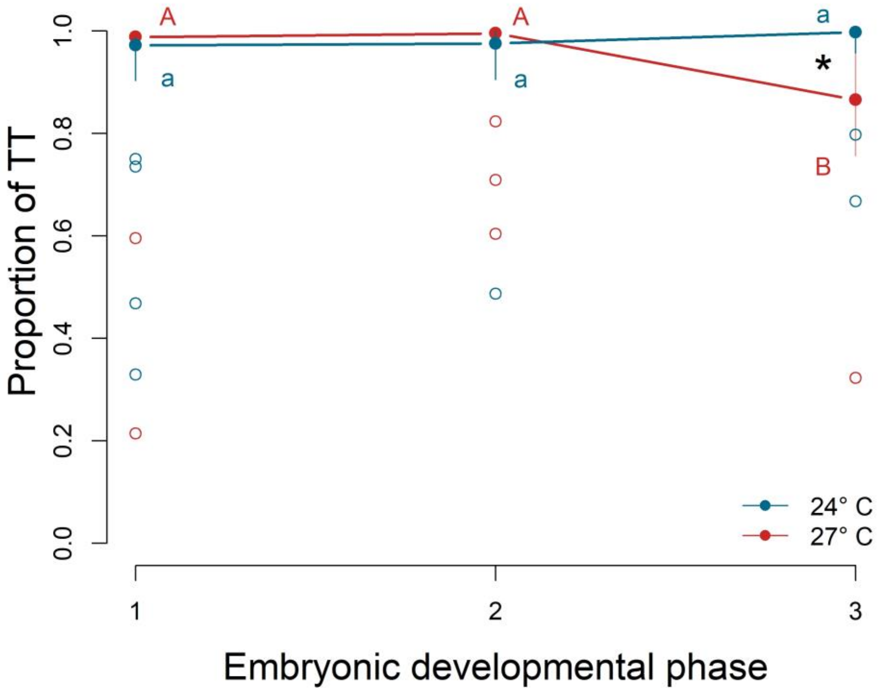

Figure 2. Relationship between phase of embryonic development and the proportion of the total time (TT) spent by parents carrying out parental care activity in the marine annelid O. labronica measured at 24 (blue dots and blue line) and 27°C (red dots and red line). Solid dots represent the median, top and bottom vertical whiskers represent quartiles, and empty dots indicate outliers. Capital and lower-case letters represent significant differences (p < 0.05) between different phases of the embryonic development for the elevated and control temperature conditions, respectively. Asterisk (*) indicates significant differences (p < 0.05) between temperature conditions within the same phase of embryonic development.

Effect of temperature and parental care activity on reproductive success

Hatching success decreased significantly from 89 % to 81 % at 24°C and at 27°C, respectively (Fig. 3). Temperature was the only factor significantly affecting hatching success ($\chi^2_{(1)} = 5.58$; p = 0.02; Table 2), while Total TT and its interaction with temperature did not have any significant effect on this trait (Table 2).

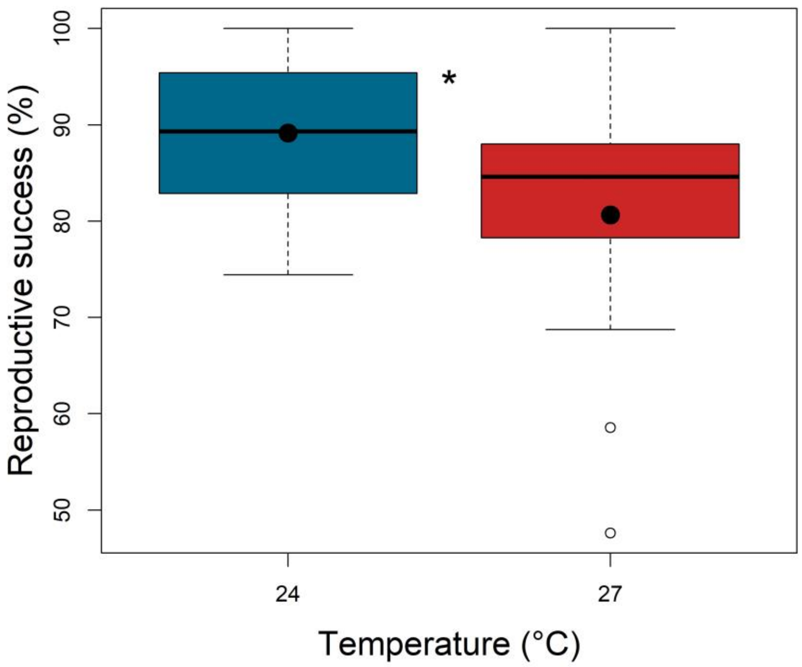

Figure 3. Effect of temperature on the reproductive success of O. labronica. Mean values of the reproductive success (%) at 24°C and 27°C are reported as black dots. The median (horizontal dark line in each box), quartiles (top and bottom of box), and the 0.05 and 0.95 quantiles (vertical whiskers) are shown for each group. An asterisk (*) indicates significant differences (p < 0.05) between temperature conditions.

Effect of temperature on the sex-related division of parental care

Overall, the proportion of the time spent carrying out parental cares separately by the female (TF), male (TM), and the two partners simultaneously (TS) significantly change along the different phases of embryonic development differently depending on the temperature conditions tested, as shown by the presence of significant interactions between "Temp" and "Phase" (TF: $p < 0.01$; TM, $p < 0.001$; S, $p < 0.001$) (Table 2; Table A5 in Appendix S3 in Supporting Information for the pair-wise results). In details, a significant decrease in the proportion of TF was observed between Phase 2 and 3 at 27°C ($t_{(96)} = 3.21$; $p = 0.02$), while no differences were found at 24°C (Fig. 4a; Table A5 in Appendix S3 in Supporting Information). The proportion of TF within a given phase of egg development did not differ with temperature ($p > 0.05$; Table 2; Fig. 4a).

The proportion of TM was significantly lower at 27°C (0.3 ± 0.08; mean ± SE) than 24°C (0.69 ± 0.09) in the third phase of egg development ($t_{(96)}$ = -3.44; p = 0.01; Table 1; Fig. 4b). In addition, at 27 C, this trait was significantly lower at Phase 3 compared to Phase 2 ($t_{(96)}$ = 2.98; p = 0.04), whilst trait values at Phase 1 and Phase 3 were comparable (Fig. 4b; Table A5 in Appendix S3 in Supporting Information). Contrarily, the proportion of TM increased significantly from Phase 1 to Phase 2 ($t_{(96)}$ = -2.94; p = 0.046), as well as from Phase 1 to Phase 3 ($t_{(96)}$ = -4.07; p = 0.001) at 24°C.

Finally, the proportion of TS was significantly lower at 27°C (0.21 ± 0.07) when compared to 24°C (0.62 ± 0.09), but only at Phase 3 ($t_{(96)}$ = -4.16; p = 0.001; Table 2; Fig. 4c). In addition, it decreased significantly from Phase 2 to Phase 3 ($t_{(96)}$ = 3.79; p = 0.003) at 27°C, while was comparable between Phase 1 and 2, as well as between Phase 1 and 3 (Fig. 4c; Table A5 in Appendix S3 in Supporting Information). By contrast, the proportion of TS showed the tendency to increase significantly from Phase 1 to Phase 2 ($t_{(96)}$ = -2.92; p = 0.049) and from Phase 1 to Phase 3 at 24°C ($t_{(96)}$ = -4.44; p = 0.0003), Phase 2 and 3 showing comparable results.

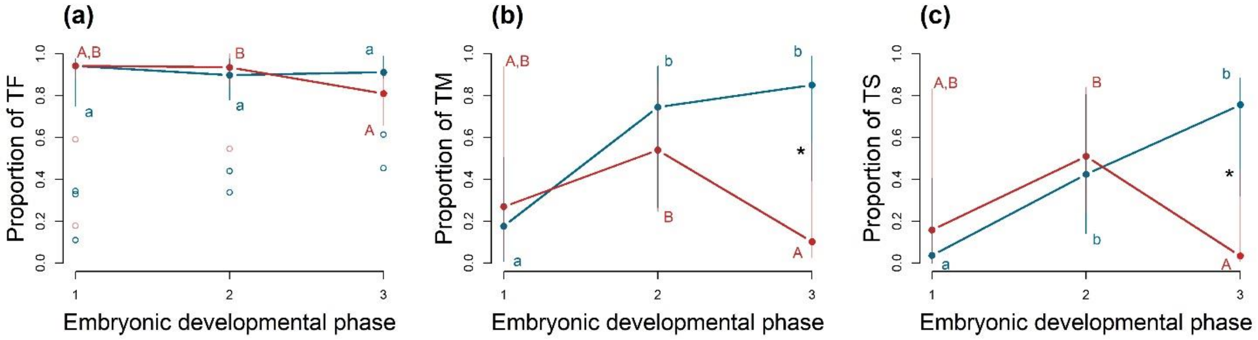

Figure 4. Relationship between phase of embryonic development and time spent by (a) the female (TF), (b) the male (TM) and both parents simultaneously (TS) of O. labronica carrying out parental care activity at 24 (blue) and 27°C (red). Solid dots represent the median, top and bottom vertical whiskers represent quartiles, and empty dots indicate outliers. Capital and lower-case letters represent significant differences (p < 0.05) between phases of embryonic development for the elevated and control temperature conditions, respectively. Asterisks (*) indicate significant differences (p < 0.05) between temperature conditions within the same phase of embryonic development.

Table 2. Summary of statistical analyses for the effect of temperature (Temp) on the proportion of the total times spent for parental care activity (TT), relative contribution of the proportion of TT on hatching success, proportion of the total times spent for parental care activity by the female (TF) and male (TM) in isolation and simultaneously (TS) in the marine annelid O. labronica. Only comparisons between the two temperature conditions within each phase of embryonic development are reported for the analysis of the proportion of TT, TF, TM and TS (see Tables A4 and A5 in Appendix S3 in Supporting Information for all pairwise comparisons). Degree of freedom (df), Wald chi-square (χ2), and probability levels (p) are provided. Significant effects are reported in bold and the results of pairwise contrasts are indicated within brackets.

PARENTAL CARE ACTIVITY FOR TT				
		df	**χ^2**	**p**
Proportion of TT	Temp	1	0.01	0.9137879
	Phase	2	3.62	0.1637082
	Temp * Phase	**2**	**16.67**	**0.0002395**
		Phase 1 (t = -1.93; p = 0.39) Phase 2 (t = -1.52; p = 0.65) **Phase 3 (t = 3.26; p = 0.019)**		

HATCHING SUCCESS				
		df	**χ^2**	**p**
	Temp	**1**	**5.58**	**0.018**
	TT	1	0.52	0.471
	Temp*TT	1	1.94	0.163

PARENTAL CARE ACTIVITY FOR TF, TM, AND TS				
		df	**χ^2**	**p**
Proportion of TF	Temp	1	0.01	0.93
	Phase	2	2.58	0.275
	Temp*Phase	**2**	**10.53**	**0.005**
		Phase 1 (t = 1.03; p = 0.908) Phase 2 (t = -2.57; p =0.539) Phase 3 (t = -1.33; p = 0.115)		
Proportion of TM	Temp	1	0.02	0.879
	Phase	**2**	**8.19**	**0.017**
	Temp*Phase	**2**	**18.50**	**< 0.0001**
		Phase 1 (t = 2.50; p = 0.134) Phase 2 (t = 0.67; p = 0.985) **Phase 3 (t = -3.44; p = 0.011)**		
Proportion of TS	Temp	1	0.02	0.892
	Phase	**2**	**8.19**	**0.012**
	Temp*Phase	**2**	**18.50**	**< 0.0001**
		Phase 1 (t = 2.77; p = 0.071) Phase 2 (t = 1.15; p = 0.858) **Phase 3 (t = -4.16; p = 0.001)**		

Discussions

Our study is among the few investigating thermal plasticity in parental care behaviours in marine invertebrates with biparental care systems, and its role in affecting organisms' reproductive success within a climate change context. Whether organisms will be able to adjust or adapt to ongoing ocean warming is a central question in global change biology (Calosi et al., 2016; Shama, 2015; Chakravarti et al., 2016, Donelson et al. 2018). Behavioural plasticity provides an organism with an immediate tactical response to rapidly changing conditions, thus representing the first barrier of defence against the negative impacts of climate changes (Kearney et al., 2009; Sih et al., 2011; Walther et al., 2002). Here we show that, in the marine annelid *Ophryotrocha labronica*, exposure to an elevated temperature can reduce the total time spent by parents caring for their brood, as well as the time of simultaneous parental care, during the last phase of embryonic development. These responses seem to be driven by a reduction of the time spent by the male in performing parental care activities in the third phase. Interestingly, this behavioural plasticity is not related to the parents' short-term fitness, measured as hatching success, despite the fact that the latter was negatively affected by the exposure to the elevated temperature tested.

The reduction in parental care observed only during the third phase of development at the highest temperature tested may be explained by the existence of cost-benefit trade-offs between the parental care investment and offspring fitness (Wrinkler, 1987). Evolutionary theory on parental care predicts that selection favours the evolution of parental care strategies when the costs of providing care (e.g. higher energetic demand, reduced parental survival or future reproduction) do not outweigh its benefits (i.e. higher offspring survival and quality) (Wrinkler, 1987; Clutton-Brock, 1991; Pike & Wen-san Huang, 2013; Klug & Bonsall, 2014). Accordingly, organisms may have evolved multiple behavioural responses able to guarantee that the overall beneficial nature of their parental care strategy is maintained also under stressful conditions, such as rapid thermal changes. In *Ophryotrocha* *labronica*, this condition may have been achieved through the fine-tuning of parental care behaviours during embryo development. For example, by evolving temperature-independent parental care behaviours at those stages of embryonic development, specifically from cleavage to gastrulation, that in some marine invertebrates are more vulnerable to the negative effects of temperature (Andronikov, 1975; Cossins & Bowler 1987; Kinne & Kinne, 1962): i.e. the first and second phase in *O. labronica*. Therefore, by evolving a less strict tie with the offspring at a given stage, i.e. the third phase, when embryos are more developed and able to actively moving inside the egg mass case. The latter strategy may allow for a reduction in parental care investment at the elevated temperature, enabling parents to cope with the increased energetic demand their incur in, without negatively affecting offspring's survival. From a mechanistic perspective, the greater energetic demand commonly experienced by ectotherms at higher temperatures as a result of increased cell kinetics (Angilletta, 2009; Hochachka & Somero, 2002) may be the consequence of having to allocate more energy to fuel cells maintenance, repair, and other costly whole-organism functions (Schaffer, 1974; Stearns, 1992). This increased cost may be likely sustained at 27°C during the third phase of embryonic development in *O. labronica*, or cumulatively up to this phase. On the contrary, reproductive performance and parental care activities at the control condition may have not resulted in additional costs associated with parental investment and, consequently, in the necessity to alter parental care behaviour along the eggs' development.

Several studies on aquatic ectotherms have shown a negative correlation between time spent by parents caring for their offspring *versus* parental investment in self-maintenance. Marconato et al. (1993), for example, found that somatic conditions (body weight) of males of the river bullhead *Cottus gobio* (Linnaeus, 1758) declined proportionally with the time spent undertaking parental care activities. Similarly, in the marine mantis shrimp *Pullosquilla thomassini* (Manning, 1978), a species exhibiting biparental care, a reduction in body mass was detected as a consequence of increased parental activity of the male partner, probably to compensate for the absence of the other caregiver (Wright & Caldwell, 2015). In our study, the lack of changes either in growth rate or body size at maturity of parents due to a temperature increase – although we could not estimate other metrics of body condition – suggest that *O. labronica* may have the ability to release energy for somatic maintenance that benefits current adult performance at the advantage of future reproduction (Martins & Wright, 1993; Roff, 2002), ultimately maximizing parental fitness on a longer term under the novel thermal condition (Nagelkerken & Munday, 2016). In our study, we are unable to demonstrate the existence of the trade-off between short-term and longer-term fitness, as well as its relationship with thermal plasticity in parental care behaviours. However, we know that the first reproductive events (1-3) provide the greatest contribution in defining the population growth rate of *O. labronica* at high temperatures (Prevedelli & Simonini, 2001). Given the positive relationship commonly found between female body size and fecundity in this species (Berglund 1991; Prevedelli et al. 2006; Thornhill et al. 2009), a relatively higher investment in self-maintenance under increasing temperatures may increase chances for longer-term fitness, and thus indirectly result in greater fitness at the population level.

The production of smaller broods may have favoured the reduction in parental care investment. In many marine invertebrates, in fact, larger brood contains a higher proportion of eggs located deep in the clutch, thus requiring more ventilation in order for oxygen to reach the centre of the egg mass (Cohen & Strathmann, 1996; Strathmann & Strathmann, 1995; Baeza & Fernández, 2002; Fernández et al., 2000). Accordingly, a smaller amount of eggs in the clutch, as observed in *O. labronica* at the elevated temperature, would require less care, thus allowing parents to preserve energy for self-maintenance, repair, growth, and future reproductive investments, as postulated by the *Parental Investment Theory* (Williams, 1966; Sargent & Gross, 1986). On the other hand, the increase in parental investment when broods are larger can be explained by the increased fitness value that larger broods represent (Galvani & Coleman, 1998). In our study, we indeed find a positive relationship between brood size and all four measurements of parental effort, a result that is consistent with experimental observations showing an increase in maternal and paternal care investment in larger broods in the congeneric hermaphroditic annelid *Ophryotrocha diadema (*Åkesson, 1976) (Picchi & Lorenzi 2019).

Interestingly, the proportion of total time spent by parents in caring for their offspring in *O. labronica* does not increase with the progression of embryonic development, as documented in other marine ectotherms as a strategy to sustain the higher energetic demand of growing embryos (Green & McCormick, 2005; Baeza & Fernández, 2002). Neither we observe an overall trend of decreasing parental care activity across developmental stages as found in other aquatic species, where embryos gained the ability to self-ventilate towards the end of development (Dick et al., 1998). This variety of responses suggests that more than one strategy exists in marine invertebrates for parental care investments across development.

Regarding our second research aim, we did not find any significant relationship between the time spent for parental care activity and the hatching success under the elevated temperature condition. We report a moderate, but significant, 7 % reduction in reproductive success compared to the control condition. However, this change is not related to the thermal plasticity of parental care activity observed in response to exposure to an elevated temperature. Hopkins et al. (2011) reported that a negative effect of elevated temperature on reproductive success was accompanied by an increase of parental activity in the three-spined stickleback *Gasterosteus aculeatus* (Linnaeus 1758), but the authors did not formally test for the presence of a relationship between these two traits. Similarly, to our study, no apparent relationship between reproductive success and total parental care at elevated temperature was observed in the burying beetle *Nicrophorus orbicollis* (Fabricius, 1775) (Ong, 2019).

Therefore, hatching success may be independent from limited changes in the amount of care embryos receive from the parents.

Finally, we found a sex-related contribution to the care of the eggs at the elevated temperature. In particular, males' parental investment was less than a half of that provided by females in the last phase of egg development at 27°C, when compared to our control conditions when male contribution represented 20% of that of the female. The existence of sex-specific behavioural patterns in the genus *Ophryotrocha* was also demonstrated by Picchi & Lorenzi (2019), who found that parental care was a female-biased behaviour both in *O. labronica* and the hermaphroditic *O. diadema*. In addition, they observed that males were less constrained by parental duties and invested more effort (e.g. increased motility) to increase mating opportunities, especially at higher densities (Picchi & Lorenzi, 2019). We may conclude that sex-biased plasticity can also be induced by factors other than density, such as conditions of thermal stress tested in our study. More in general, our results are in line with several studies, almost exclusively conducted on birds, showing that increased temperatures affected investment patterns in species with biparental care, with the dominant protector (i.e. the female in our study) and the subordinate one (i.e. the male) responding differently to this environmental challenge (Wiley & Ridley, 2016; Vincze et al., 2016). In addition, females' parental investment is neither affected by temperature or by the reduced males' parental care under elevated temperature. This is in accordance with the "No negotiation" model, according to which one parent alters its investment in the offspring independently from the level of investment of its partner (Houston and Davies 1985). The absence of a negotiation strategy in this species may be due to the differences in costs and benefits of parental care between sexes. In fact, males of this species appear to have much more fitness advantages by engaging in multiple mating events than undertaking parental care activity, whilst for females it appears more advantageous to maintain parental care investment to maximize their fitness (Picchi & Lorenzi, 2019). Altogether, the significant reduction of males' care activities and the simultaneous contribution of males and females to parental care activities appear to be responsible for the general decrease of total parental care activity during the late phase of embryonic development at elevated temperatures. Manipulative experiments, monitoring parental care behaviour of one parent in response to the removal of its partner, would help to more definitively confirm the existence of these patterns of biparental care in this species under elevated temperature.

In summary, our findings showed that ocean warming will exert negative effects on the reproductive success of *O. labronica*. However, this species appears to have evolved a parental care strategy that enables it to maintain a positive cost-benefit trade-off between parents and offspring, with potential benefits for parents' individual and species fitness (e.g. successive reproductive events) under elevated temperatures. This suggests that plasticity in parental care behaviour is a mechanism that can partially mitigate the negative effects of temperature-dependent impacts; however, how this mechanism will play out along the life span of individuals, and thus contribute to population level responses in the longer term, is still to be determined. Nonetheless, our results contribute to the ongoing debate on the role and limits of behavioural plasticity as a coping strategy to buffer the impact of rapid environmental change.

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Supporting Information

Chapter 2 - Plastic adjustments of biparental care behaviour across embryonic development under elevated temperature in a marine ectotherm

APPENDIX S1

Effect of temperature on brood size, body size, and growth rate

Statistical analyses

A set of preliminary analyses on a number of life-history traits (i.e. brood size, body size, and growth rate) was performed to support the interpretation of the results on the effect of temperature on parental care investment. Specifically, the combined effect of temperature and female body size, used as covariate, on brood size was analysed using a Poisson-GLM (P-GLM) corrected for overdispersion using quasi-Poisson GLM (QP GLM). P-GLM was also used to assess the effect of temperature on male and female body size. Similarly, a P-GLM with total days used as an offset variable was performed to assess the effect of temperature on the growth rate of both parents.

Results

Only brood size was significantly reduced under the elevated temperature (Table A1; Fig. A1a), while temperature had no effect on growth rate and body size of both parents (Table A1; Fig. A1b-e).

Table A1 *Sequential analysis of deviance based on Wald chi-squared test for the effect of temperature (Temp) on different life-history traits (brood size, parents' body size and growth rate) in the marine annelid O. labronica. Degree of freedom (df), Chisq (χ2) and probability levels (p) are provided (significant effects: p < 0.05).*

PARENTAL TRAITS				
		df	**χ^2**	**p**
Brood size	**Temp**	**1**	**8.06**	**0.005**
	Female body size	1	2.41	0.120
	Temp * Female body size	1	2.31	0.128
Female body size	Temp	1	0.002	0.964
Male body size	Temp	1	0.49	0.484
Female growth rate	Temp	1	0.39	0.531
Male growth rate	Temp	1	0.74	0.391

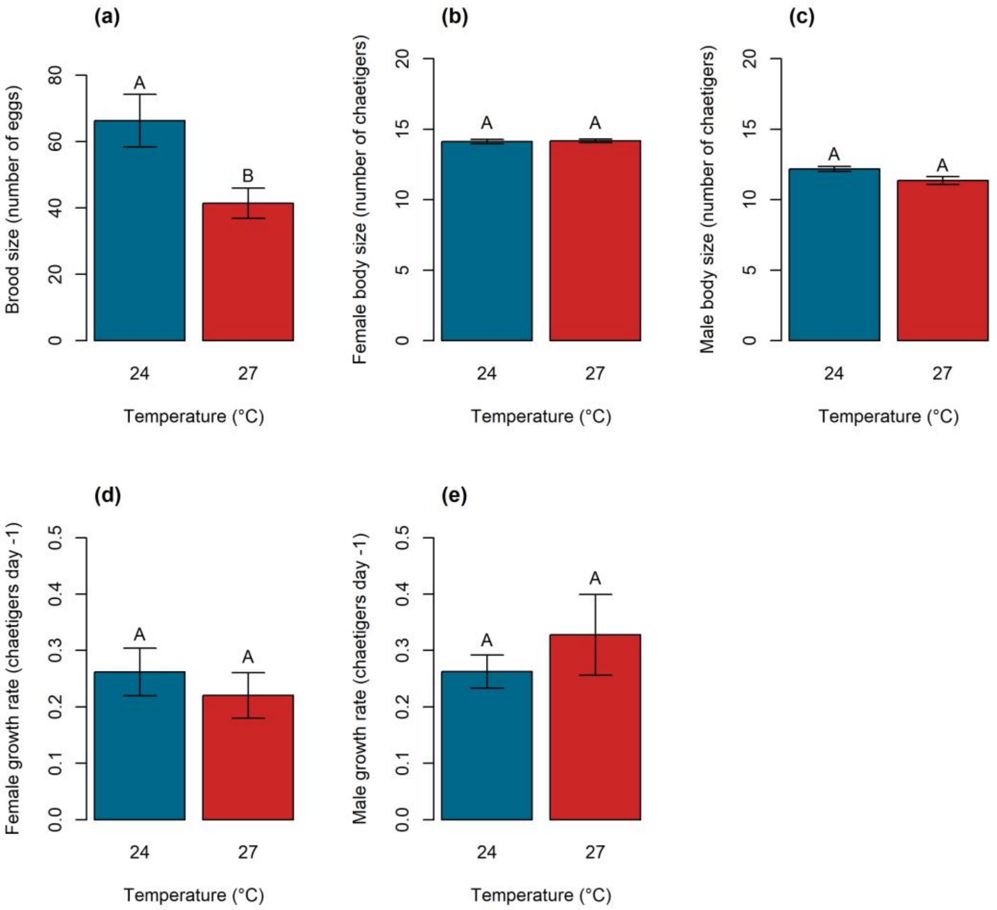


Figure A1 *Effect of temperature on (a) brood size, (b) female body size, (c) male body size, (d) female growth rate, and (e) male growth rate in the marine annelid O. labronica. Mean (±S.E.) are reported. Different capital letters indicate significant differences between temperature conditions (*$\alpha = 0.05$*).*

APPENDIX S2

Relation between parents growth rate, brood size and parental care behaviour

Statistical analyses

Preliminary analyses were performed to assess the role of parental growth rate and brood size in affecting the proportional time of parental care. In particular, to test the relationships between parental growth rate (log transformed) and the proportion of parental care time provided by each sex (defined TF for the female and TM for the male) in each embryo developmental phase (“Phase” – fix factor with three levels: Phase 1, 2, 3) at the two temperature conditions (“Temp” – fix factor with two levels: 24 and 27°C), we used a linear model test, separately for each sex.

The relationship between the proportion of total time for parental care activity (TT) and brood size was tested using a binomial generalized mixed model (B-GLMM) with lme4 package for R (Bates et al., 2015). Similarly, three B-GLMMs were used to test the proportion of the time spent carrying out parental cares by each descriptor (TF, TM and TS) and brood size. The identity of the experimental pairs ("Pair" –factor with thirty-six levels: pair 1 to 36) was used as random factor (random intercept model), as observations were repeated on the same pair along the three phases of embryonic development, thus violating the assumption of independence.

Results

No significant effect of parental care on both female and males' growth rate were observed (Table A2). In addition, we found that the proportion of TT, the proportion of TF and TM decreased significantly with the reduction of the brood size (Table A3; Fig. A2)

Table A2 *Parameters estimation of the linear regressions ran to test the effect of the proportion of time spent by single parents for parental care (TF and TM) on both female and males' growth rate at the control and elevated temperature condition in the marine annelid O. labronica. Estimate (Est.), Standard Error (S.E.), t-value (t) and probability levels (p) are provided (significant effects: p < 0.05).*

		Est.	S.E.	t	p
Female's growth rate	(Intercept)	-0.1249	0.3978	-0.314	0.7561
	Phase 1	0.1109	0.1723	0.644	0.5253
	Phase 2	-0.2649	0.2295	-1.155	0.2587
	Phase 3	0.5194	0.3837	1.354	0.1875
	27 °C	0.6977	0.4989	1.398	0.1738
	Phase 1 * 27°C	0.6481	0.3452	1.878	0.0717
	Phase 2 * 27°C	-0.8383	0.5186	-1.616	0.1181
	Phase 3 * 27°C	-0.564	0.4352	-1.296	0.2064
Male's growth rate	(Intercept)	0.2017235	0.6239449	0.323	0.749
	Phase 1	-0.0134682	0.2701731	-0.05	0.961
	Phase 2	-0.0006363	0.3598707	-0.002	0.999
	Phase 3	0.0415466	0.6018673	0.069	0.945
	27 °C	-0.5081616	0.7824254	-0.649	0.522
	Phase 1 * 27°C	-0.2988153	0.5413387	-0.552	0.586
	Phase 2 * 27°C	0.6428327	0.8134289	0.79	0.437
	Phase 3 * 27°C	0.25699	0.6826344	0.376	0.71

Table A3 *Anova results for the relationship between the proportion of the total time for parental care activity (TT) (a), separate and simultaneous contribution of female and male to parental care activity (TF, TM and TS respectively) (b-d), and brood size in the marine annelid O. labronica. Degree of freedom (df), Wald Chisq (χ2) and probability levels (p) are provided (significant effects: p < 0.05).*

		df	χ^2	*p*
(a)	**Proportion of TT**	1	24.99	**< 0.0001**
(b)	**Proportion of TF**	1	4626.1	**< 0.0001**
(c)	**Proportion of TM**	1	10.99	**0.0009**
(d)	**Proportion of TS**	1	12.16	**0.0005**

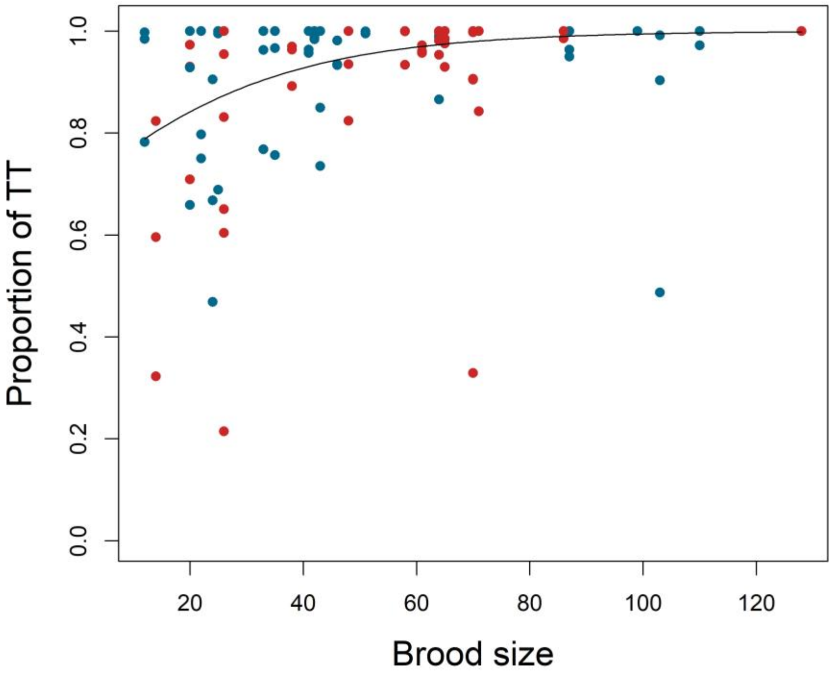


Figure A2 Relationship between brood size and proportion of the total time (TT) spent by individuals of the marine annelid O. labronica, carrying out parental care activity measured at 24 (blue dots) and 27°C (red dots). The black line represents the fitted value of the glmm model.

APPENDIX S3

Effect of temperature on parental care activity (pairwise comparisons)

Table A4 Pair-wise results for the significant interaction between temperature and phase of embryonic development for the proportion of the total time spent for parental care activity in the marine annelid *O. labronica*. Degree of freedom (df), t-ratio(t) and probability levels (p) are provided and significant effects ($p < 0.05$). Significance levels are based on the adjusted p-values from Tukey's HSD.

Total time Parental care		**df**	***t***	**p**
Within Temperature	**24 °C**			
	Phase 1 – Phase 2	96	-1.07	0.893
	Phase 2 – Phase 3	96	-1.07	0.892
	Phase 1 – Phase 3	96	-2.14	0.277
	27 °C			
	Phase 1 – Phase 2	96	-0.66	0.986
	Phase 2 – Phase 3	**96**	**3.72**	**0.004**
	Phase 1 – Phase 3	**96**	**3.06**	**0.034**
Within Phase	**Phase 1**	96		
	27°C – 24°C	96	-1.93	0.392
	Phase 2	96		
	27°C – 24°C	96	-1.52	0.650
	Phase 3	96		
	27°C – 24°C	**96**	**3.26**	**0.019**
Other contrasts	27°C, Phase 1 – 24°C, Phase 2	96	-0.86	0.955
	27°C, Phase 1 – 24°C, Phase 3	96	0.21	0.999
	27°C, Phase 2 – 24°C, Phase 3	96	-0.45	0.998
	24°C, Phase 1 – 27°C, Phase 2	96	-2.59	0.110
	24°C, Phase 1 – 27°C, Phase 3	96	1.13	0.869
	24°C, Phase 2 – 27°C, Phase 3	96	2.20	0.250

Table A5 Results for the pair-wise comparison for significant interactions between the terms “Temp” and “Phase” for the proportion of the total times spent for parental care activity by the female (TF) and male (TM) in isolation and simultaneously (TS) in the marine annelid *O. labronica*. Degree of freedom (df), t-ratio (t), probability levels (p) are provided and significant effects (p < 0.05). Significance levels are based on the adjusted p-values from Tukey’s HSD.

PARENTAL CARE ACTIVITY FOR TF, TM, AND S			df	t	p
Proportion of TF	**Within Temperature**	**24°C**			
		Phase 1 – Phase 2	96	0.10	1
		Phase 2 – Phase 3	96	-1.05	0.898
		Phase 1 – Phase 3	96	-0.95	0.931
		27°C			
		Phase 1 – Phase 2	96	-0.57	0.993
		Phase 2 – Phase 3	**96**	**3.21**	**0.022**
		Phase 1 – Phase 3	96	2.65	0.097
	Within Phase	**Phase 1**			
		27°C – 24 °C	96	1.03	0.908
		Phase 2			
		27°C – 24°C	96	1.69	0.539
		Phase 3			
		27°C – 24°C	96	-2.57	0.115
	Other contrasts	27°C, Phase 1 – 24°C, Phase 2	96	1.13	0.868
		27°C, Phase 2 – 24°C, Phase 3	96	0.64	0.988
		27°C, Phase 1 – 24°C, Phase 3	96	0.07	1
		24°C, Phase 1 – 27°C, Phase 2	96	1.59	0.604
		24°C, Phase 2 – 27°C, Phase 3	96	-1.52	0.654
		24°C, Phase 1 – 27°C, Phase 3	96	-1.62	0.589
Proportion of TM	**Within Temperature**	**24°C**			
		Phase 1 – Phase 2	**96**	**-2.94**	**0.046**
		Phase 2 – Phase 3	96	-1.13	0.868
		Phase 1 – Phase 3	**96**	**-4.07**	**0.001**
		27°C			
		Phase 1 – Phase 2	96	-1.11	0.877
		Phase 2 – Phase 3	**96**	**2.98**	**0.041**
		Phase 1 – Phase 3	96	1.87	0.425
	Within Phase	**Phase 1**			
		27°C – 24°C	96	2.50	0.134
		Phase 2			
		27°C – 24°C	96	0.67	0.985
		Phase 3			
		27°C – 24°C	**96**	**-3.44**	**0.011**
	Other interactions	27°C, Phase 1 – 24°C, Phase 2	96	-0.44	0.9980
		27°C, Phase 2 – 24°C, Phase 3	96	-0.46	0.9975
		27°C, Phase 1 – 24°C, Phase 3	96	-1.56	0.6242
		24°C, Phase 1 – 27°C, Phase 2	**96**	**3.61**	**0.0063**
		24°C, Phase 2 – 27°C, Phase 3	96	-2.31	0.2009
		24°C, Phase 1 – 27°C, Phase 3	96	0.63	0.9887
Proportion of TS	**Within Temperature**	**24°C**			
		Phase 1 – Phase 2	**96**	**-2.92**	**0.049**
		Phase 2 – Phase 3	96	-1.52	0.656
		Phase 1 – Phase 3	**96**	**-4.44**	**0.0003**
		27°C			
		Phase 1 – Phase 2	96	-1.29	0.786
		Phase 2 – Phase 3	**96**	**3.79**	**0.003**
		Phase 1 – Phase 3	96	2.50	0.134
	Within Phase	**Phase 1**			
		27°C – 24°C	96	2.77	0.071
		Phase 2			
		27°C – 24°C	96	1.15	0.858
		Phase 3			

	27°C – 24°C	**96**	**-4.16**	**0.001**
Other interactions	27°C, Phase 1 – 24°C, Phase 2	96	-0.15	1
	27°C, Phase 2 – 24°C, Phase 3	96	-0.36	0.999
	27°C, Phase 1 – 24°C, Phase 3	96	-1.66	0.561
	24°C, Phase 1 – 27°C, Phase 2	**96**	**4.07**	**0.001**
	24°C, Phase 2 – 27°C, Phase 3	96	-2.65	0.096
	24°C, Phase 1 – 27°C, Phase 3	96	0.27	1

Chapter 3 - Nest guarding behaviour of a temperate wrasse differs between sites off Mediterranean CO_2 seeps

Introduction

One way that organisms can respond to changing environmental conditions is through adjustments in their behaviour, representing an immediate strategy to increase their chances of survival and individual fitness (Candolin & Wong, 2012; Sih, 2013). Such changes to environmental conditions are often related to human activities, and may involve a set of behavioural responses of individual species including relocation, habitat choice, movement, reproduction, foraging and antipredator strategies (Tuomainen & Candolin, 2011). Adaptive behavioural plasticity may allow species to maintain or mitigate the negative effect of a given stressor (Pigliucci, 2001; Van Kleunen & Fischer, 2005; Wong & Candolin, 2015). However, these behavioral responses can also be maladaptive if they reduce the fitness of the individuals in the new environment (Pigliucci, 2001).

Increased uptake of anthropogenic CO_2 by the oceans, namely ocean acidification, represents one important global process that can affect the survival, calcification, growth, development and reproduction of marine organisms as well as alter the behaviour of fish (Cattano, et al., 2018; Gaylord et al., 2015; Heuer & Grosell, 2014; Nagelkerken & Munday, 2016; Kroeker et al., 2013). Several studies have reported fish behavioural effects and sensory disruptions due to elevated CO_2 conditions involving olfaction and audition functions, lateralization, activity levels, learning and decision making (Munday et al., 2009; Dixson et al., 2010; Simpson et al., 2011). The reported behavioural effects on some coral reef fish have recently been questioned (Clark et al., 2020), and a large debate over experiments' reproducibility in fish behavioural studies is presently underway (Clark et al., 2020; Munday et al., 2020; Williamson et al., 2020). Although fish are efficient acid-base regulators (Esbaugh et al., 2012; Pörtner et al., 2004), an increase in the energy used to maintain acid-base balance due to high CO_2/low pH conditions may affect the amount of energy available for other activities, including reproduction (Ishimatsu et al., 2008; Sokolova et al., 2012).

To date, the few laboratory studies on the effect of elevated CO_2 on reproduction have mainly focused on parental allocation to offspring and sperm production, suggesting variable and species-specific responses (Heuer & Grosell, 2014; Faria et al., 2018). Indeed, the cost of reproduction should also encompass behavioural activities such as mate choice and acquisition (courting), nest building and defence, and parental care (Gillooly & Baylis, 1999; Husak & Swallow, 2011). In fish species that invest heavily in parental care, changes in the amount of energy for some behavioural activities under altered environmental conditions could lead parents to adjust their behaviour, ultimately affecting reproductive success. Changes in reproductive behaviours were documented under varying salinity (Mary et al., 2001), oxygen (Jones & Reynolds, 1999; Lissåker & Kvarnemo, 2006; Reebs et al., 1984) and temperature (Skolbekken & Utne-Palm, 2001) levels. However, shifts in behavioural activities during reproduction are critically understudied in relation to the effects of high CO_2 concentrations on fish and other marine organisms (Gaylord et al., 2015; Nagelkerken & Munday, 2016).

A few studies investigated ocean acidification (OA) effects on the reproductive behaviour of fish both in controlled laboratory conditions and in the wild. In a laboratory experiment, Sundin et al. (2017) did not detect any CO_2 effect on the nest building activity, courtship and fanning of the three-spined stickleback *Gasterosteus aculeatus* (Linnaeus 1758). Similarly, no CO_2 effects were detected on the time males of two-spotted goby *Gobiusculus flavescens* (Fabricius, 1779) spent on differential parental care behaviours, with this male investment being mostly affected by increased temperature in a 2×2 ($CO_2 \times T$) factorial experiment carried out in lab conditions (Lopes et al., 2020). In the first study carried out in the wild so far, no differences in many mating behaviours were detected, but the number of pair spawning events with females by the nesting male ocellated wrasse *Symphodus* *ocellatus* was reduced by almost two thirds in areas at high CO_2 levels near volcanic seeps (Milazzo et al., 2016). However, nesting male paternity was maintained (Milazzo et al., 2016).

Here, we used well-established CO_2 gradients off seeps at Vulcano and Panarea Islands (Sicily, Italy) (Goffredo et al., 2014; Aiuppa et al., 2020), to investigate the potential effect of high seawater CO_2 concentrations on the behavioural activities of the nesting male *Symphodus ocellatus*. The potential time budget re-allocation between reproductive activities, with specific focus on parental care activities (e.g., fanning, nest maintenance, guarding and chasing) was also investigated.

The breeding behaviour of this species has been widely described (Lejeune, 1985; Taborsky et al., 1987). Nesting males build nests with algae (Sinopoli et al., 2014), attract several females for pair spawns through courtship and provide parental care to embryos until hatching. Parental care activity consists of a number of behavioural activities adopted by the nesting male to guarantee embryos' development (Lejeune, 1985). Male care behaviour includes nest defence or chasing of egg predators and other competing males (e.g., sneakers and satellite males), nest-maintenance and embryos' oxygenation by fanning through pectoral fins movements (Lejeune, 1985).

Altered CO_2 concentrations may affect mating and parental care behaviours as well as the fish species performance in different ways, such as via impaired decision-making, behavioural disruption or altered energetic balance (Cattano et al., 2018; Heuer & Grosell, 2014; Nagelkerken & Munday, 2016). In this specific context, we build on previous field experiments carried out along the Vulcano gradient that observed unaltered O_2 consumption and size at hatching of embryos from nests exposed to ambient and high CO_2 conditions (Cattano et al., 2016). Here we expect that to increase offpring survival and mitigate potential adverse CO_2 effects on embryos, the nesting male ocellated wrasse might increase the amount of time spent on parental care, altering behaviours such as guarding and chasing off nest predators and sex competitors (i.e., nest defence) or fanning laid eggs (i.e., increasing oxygen supply for embryos). To support this hypothesis, we assessed the potential OA effects on the ocellated wrasse behaviour, assessing their time spent for parental care, mating and exploring activities in two sites along the Vulcano pH/pCO_2 gradient. We expect that any change in a given behavioural activity under elevated CO_2 would result in a time/energy compensation towards other behaviours aimed at maintaining the species fitness.

To achieve these goals, we recorded the number of male competitors (i.e. sneaker and satellite males), females and potential egg predators visiting each nest, as between-site differences in these variables may ultimately affect the nesting male behaviour. We also characterized the ocellated wrasse behaviour through Behavioural Network Graphs, and evaluated potential differences in the time spent by nesting males on parental care, mating and exploring behaviours in multiple nests at ambient-CO_2 (400 µatm pCO_2) and high CO_2 sites (1100 µatm pCO_2).

Materials and methods

Study site and carbonate chemistry

This study was conducted in four different sampling surveys during the breeding season, which lasts from May to July (Lejeune, 1985; Taborsky et al., 1987), and specifically on: 18-20 May 2012, 7-12 June 2013, 29 May-7 June 2014, 1-9 July 2018. Fish behaviour was observed at two CO_2 seeps at ambient temperature off the islands of Vulcano (Italy) and Panarea (Italy), both belonging to the Aeolian Islands Archipelago (NE Sicily, Italy). In these CO_2 sites, ocellated wrasse nests are naturally exposed to present-day and high-CO_2 conditions (electronic supplementary material, table S1). The high CO_2 and the ambient-CO_2 nesting sites in Vulcano Island were at approximately 450 m and approximately 800 m distance from the main seeping area, respectively (see Boatta et al., 2013; Aiuppa et al., 2020). The nesting sites located off the Island of Panarea were positioned close (i.e., <5 m apart) and at a >50 m distance from a caldera at 10 m depth (Aliani et al., 2010; Goffredo et al., 2014). Gas emissions in both shallow seeps are 97–99% CO_2 (Boatta et al 2013; Aliani et al 2010).

Seawater carbonate chemistry was characterized daily on several visits at the two nesting sites along the Vulcano Island gradient in 2012 (n = 22), 2013 (n = 24), 2014 (n = 12), 2018 (n = 6) and at Panarea in 2014 (n = 5; see electronic supplementary material, Table S1 for details). A 556 MPS YSI (Yellow Springs, USA) multiparametric probe was used to measure salinity and pH and temperature. The sensor was calibrated using the same procedure carried out in the same site study by Milazzo et al. (2016). Total Alkalinity (TA) was calculated from the Gran function applied to pH variations from 4.2 to 3.0, from the slope of the curve HCl volume versus pH. TA measurements were corrected using standards provided by A. G. Dickson (Scripps Institution of Oceanography, USA; batch 99 and 102). The pCO_2 levels were calculated from pH_{NBS}, TA, temperature and salinity with the free-access CO_2 SYS package (Pierrot et al., 2006), using the constants of Roy et al. (Roy et al., 1993) and Dickson (Dickson, 1990).

Study species

Symphodus ocellatus is a widespread Mediterranean coastal wrasse found in shallow rocky and seagrass habitats (Taborsky et al., 1987). During the breeding season (May-July; Lejeune, 1985; Taborsky et al., 1987), large colourful nesting males (81–95 mm, TL) build algal nests where they attract small females to spawn (35–75 mm, total length, TL) and compete with two alternative male reproductive types to fertilize the eggs (Warner & Lejeune, 1985). During the breeding season the nesting males go through multiple nest cycles (each lasting on average 8–10 days) (Lejeune, 1985), during which they build and defend a nest, spawn for 3–4 days, and provide obligate care for 3–5 days before hatching. Females are non-territorial, do not participate in nest-building or parental care, and visit multiple nests where they lay dozens of eggs by brushing their genital papillae against algae (Taborsky et al., 1987). Two other types of males, i.e., the satellite and the sneaker (Taborsky et al., 1987), attempt to parasitically spawn in the nests of the dominant male (Fiedler, 1964; Lejeune, 1985;

Šoljan, 1930; Taborsky et al., 1987). Sneakers (35–60 mm, TL) hover around various nests and try to join the female and nesting male during spawning, releasing large quantities of viable sperm without providing parental care activity (Alonzo & Warner, 2000). Satellite males (61–80 mm, TL) help nesting males in reducing sneaking events and courting females, getting a share of paternity as a result (Stiver & Alonzo, 2013). Nesting males also actively defend the nest from potential egg predators which are often congeneric and other fish potentially predating eggs in nests (mostly other wrasse species; Lejeune et al. 1985; Taborsky et al., 1987).

Video collection

The behavioural observations were collected through replicated 10-min videos on different surveys over the study period. Nests of *S. ocellatus* were randomly selected among those in the spawning phase and filmed in ambient (n = 16) and high CO_2 (n = 15) conditions off Vulcano and Panarea. The same nest (and nesting male) was filmed only once, with observations typically occurring on the same days for both ambient and high CO_2 nests along the CO_2 gradients. All of the nests were filmed in rocky habitat at 3–4 m depth between 10:00 a.m. and 4:00 p.m., when light intensity and spawning activity were highest (Milazzo et al., 2016). Each replicate of 10 min video were made with a GoPro 3 camera placed on a tripod at one meter away from the nest. Water visibility (always exceeding 10 –15 m) and tidal ranges (between 20 and 40 cm) were similar in the two nesting sites along the gradients off Vulcano and Panarea Islands.

Preliminary analyses

The total number of females, sneaker males and satellite males participating in the reproduction or visiting the nest at a < 1m distance was evaluated for each 10-min video to exclude their potential effect in influencing the behaviour of the nesting male ocellated wrasse. The total number is a cumulative estimate of all individuals, therefore this metric could be overestimated. To avoid this, we also recorded the maximum number of females and sneakers appearing at the same time in a single frame during the video (Milazzo et al., 2016). Since no more than one satellite male was observed in each nest within all the videos, this was expressed as presence or absence only.

Given that the nesting male behaviour (e.g. guarding and chasing activities) may be affected by the presence of fish potentially predating eggs in ocellated nests, we recorded the maximum number of fish egg predators (i.e. *Coris julis, Symphodus mediterraneus, Symphodus roissali, Symphodus tinca* and *Thalassoma pavo)* within a 1-m radius from the nest in both CO_2 sites for each 10-min video (n= 16 at Ambient pCO2, n=15 at High pCO_2). No event of egg predation was recorded in any video. No other potential egg predators (e.g. invertebrates) were observed in proximity to the nest in all the videos analysed.

In addition, the densities of the egg predator species were recorded in the two nesting sites at ambient and high pCO_2 using standard linear transects (Harmelin-Vivien et al. 1985) performed on June 2013 (n=6 for each site). Each transect was randomly positioned on rocky substrata covered by brown macroalgae, at 1-3 meters depth and was conducted by an observer swimming for 25 m while counting all the fish encountered within 1 m either side of the transect line (50 m^2) (Harmelin-Vivien et al. 1985). Counts were conducted in the morning from 09.00 to 14.00 h, to avoid potential within-day variability which is particularly evident for coastal labrid (Willis et al. 2006). Water visibility exceeded 20 m for all counts.

Behavioural analyses

The nesting male ocellated wrasse behaviour was assessed using a standard continuous focal sampling procedure (Martin & Bateson, 1993) using the VLC software (VideoLAN; https://www.videolan.org/vlc/index.it.html). After video analyses, the behaviourof *S. ocellatus* nesting males were described through a species ethogram and were assigned as activities to three major categories: i) parental care (fanning, guarding, nest maintenance and chasing); ii) mating (spawning and fertilization); iii) exploring behaviour (wandering around); (see Table S2 in Supplementary material for the detailed description of the behavioural activities). For each nest, we recorded the time (sec) spent by the nesting male on all the activities performed during the 10-min video observations.

Behavioural network graphs or kinematic diagrams were constructed to describe the overall organizational pattern (e.g., frequencies of occurrence from one activity to another, the time spent performing each behaviour or time-budget, and the density of linkage in the network or connectance (*sensu* Martinez, 1992) of the ocellated wrasse following the different steps proposed in Brockmann (1984). After the definition of a general ethogram (as described before), the number of times (or occurrences) that one behaviour followed another were calculated to build a matrix (*transition matrix*; *sensu* Brockmann. 1984). This matrix was used to create two behavioural network graphs for each CO_2 site, showing the behavioural flow. The different behavioural activities were represented by nodes whose size represents the average time spent in that particular behaviour. The connections between nodes are represented by links (or edges), whose width represents the specific number of occurrences between single activities (e.g from fanning to guarding). The behavioural network graphs were represented using the package igraph in R software (Csárdi & Nepusz, 2005). The connectance – i.e., the number of linkages between different activities divided by the total amount of linkages possible – was measured to assess the potential differences in the density of interactions of the organizational pattern (represented by the network graph) between the two nesting sites at ambient and high CO_2.

Statistical analyses

We preliminary used linear models (lms) to assess the differences between sites in the log + 1 transformed maximum and total number of sneakers and females, as well as in the number of egg predators (as log+1 transformed sum of maximum number of each species) considering “Nesting site” as a fixed factor with two levels (high CO_2 and ambient CO_2). Similarly, potential differences in the presence or absence of satellite males between nesting sites were assessed by a binomial generalized linear-model (B-GLM), and comparing the reduction in deviance from the null model using a likelihood ratio-test.

A linear model (lm) was also modelled for each egg predator species (censused by standard underwater transects) to test differences in their density (log+1 transformed) between “Nesting site” as a fixed factor.

Potential differences in the relative number of linkages between behavioural activities (i.e., connectance) in the two nesting sites were analysed with a Poisson distribution generalized linear model tests (P-GLM) with the log-link function.

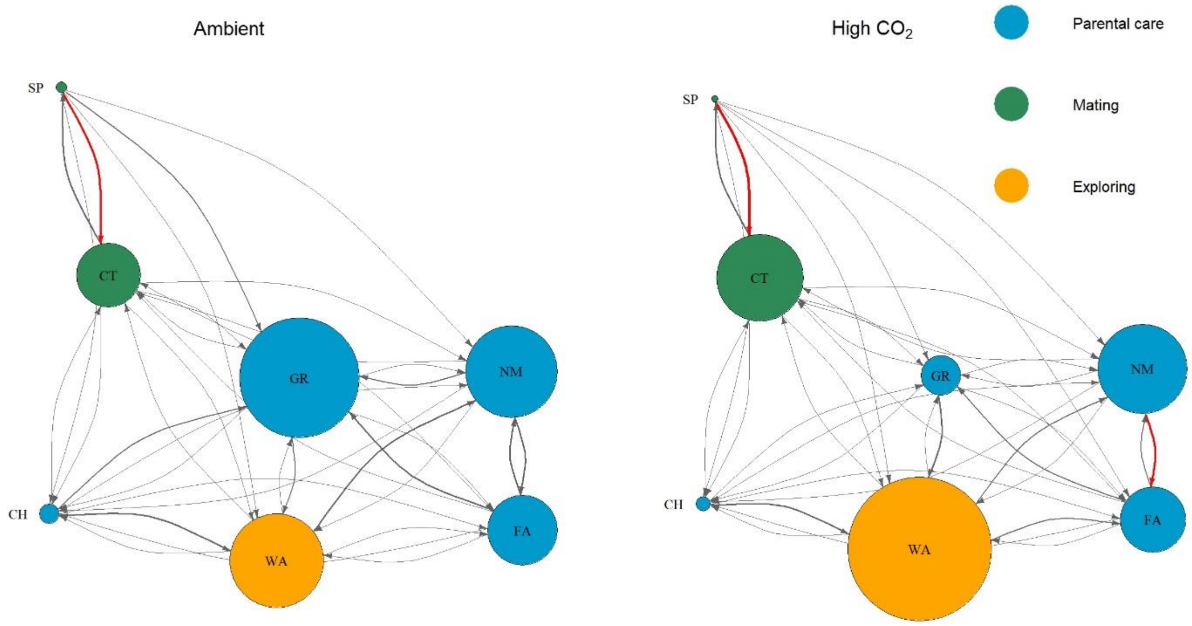
To test the CO_2 effect on the time spent by the nesting males on behavioural categories and activities we used the multivariate linear models with the package mvabund (function manylm; Wang, Naumann et al., 2012) based on resampling (n=999). This procedure takes into account the correlation between response variables, thus improving the power of the statistical test (Wang et al., 2012; Warton, 2011), and allows us to test both the multivariate and univariate hypothesis. As the data was comprised of behavioural categories and activities composition, a Centered Log Ratio (CLR) transformation was performed. (Boogaart & Tolosana-Delgado, 2013).

Multivariate linear model approach was used to test the effect of “Nesting site” (fixed factor with two levels: High CO_2 and Ambient CO_2) on: 1) the amount of time spent on the three behavioural categories (parental care, mating and exploring) 2) the different activities within the behavioural categories (fanning, guarding, nest maintenance and chasing for parental care; courting and spawning for mating; wandering around for exploring). We considered “satellites” (two levels: presence and absence) and “egg predators” (three levels: no predators, one predator and more than one predator, since the maximum numbers of predators ranged from 0 to 3) as factors, and the maximum number of sneakers and females as covariates in all analyses, which were performed using the R software version 3.3.0 (RCore, 2016).

Results

The maximum and the total number of sneakers, females and egg predators, as well as the presence or absence of satellite males recorded in the nests at Vulcano and Panarea Islands showed no differences between the two sites at high and ambient CO_2 (Table 1 and electronic supplementary material, Fig. S1). Similarly, the density of egg predators (no. of individuals 50 m^{-2}) was similar between sites (Table S3, Fig. S2).

The Behavioural Network Graphs (Fig. 1) showed no differences in the relative number of linkages (connectance) between behavioural activities observed in the two sites at different CO_2 levels (Table 1; Fig. S3 in supplementary material).



The overall time spent on the behavioural categories (i.e., parental care, mating and exploring) by the nesting males was not significantly different between the two nesting sites when approached in a multivariate context taking in consideration the correlation between response variables (Table 1). However, the univariate analysis showed that the time nesting males displayed parental care activity was significantly lower at the high CO_2 site (281.73 ± 52.01 sec; mean ± Confidence Interval, CI) compared to the control condition (378.19 ± 51.16 sec; Table 1; Fig. 1). By contrast, no significant differences were found in the time spent on the behavioural categories “mating” and “exploring”, between the nesting sites at ambient and high CO_2 (Table 1; Fig. 1).

The overall time spent on the behavioural categories (i.e., parental care, mating and exploring) by the nesting males was not significantly different between sites when approached in a multivariate context (Table 1). However, the univariate analysis showed that the time nesting males displayed parental care activity was significantly lower at the high CO_2 site (281.73 ± 52.01 sec; mean ± 95% Confidence Interval, CI) compared to the ambient conditions (378.19 ± 51.16 sec; Table 1; Fig. 2). In contrast, no significant differences were found in the time spent on the behavioural categories “mating” and “exploring”, between the nesting sites at ambient and high CO_2 (Table 1; Fig. 2).

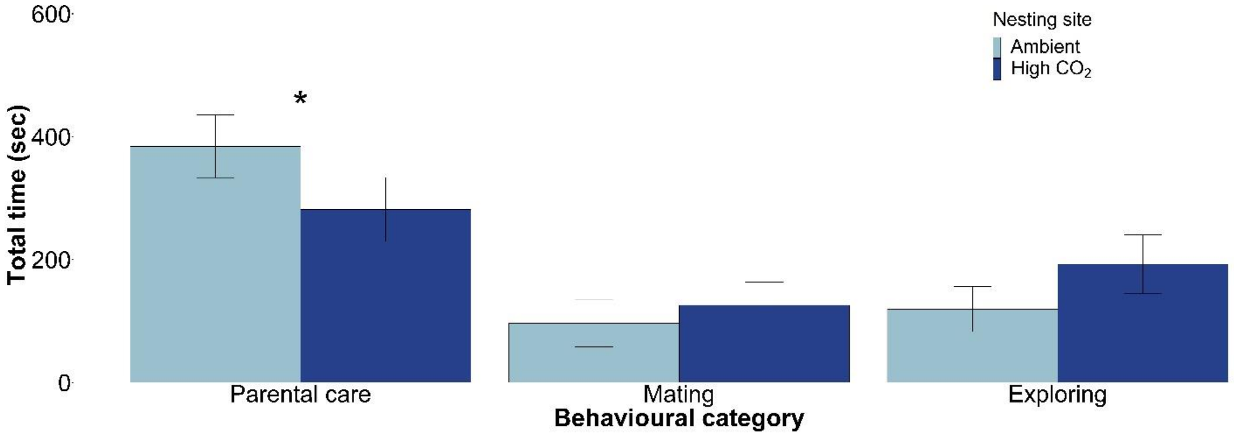


Figure2 Bar plots (mean ± CI) of the time nesting male spent on each behavioral category (e.g., parental care, mating and exploring) in the two different nesting sites at high and ambient CO_2. The asterisk indicates significant differences (at $P < 0.05$).

Total time spent on the behavioural activities showed significant differences between CO_2 sites (Table 1). Univariate analyses performed on each activity showed that time spent guarding led the overall differences between CO_2 nesting sites (Table 1), being on average 152 sec. (±40.84 CI) and 53.53 sec. (±18.04 CI) in ambient and high CO_2 sites, respectively (Fig. 3). No differences between high and ambient CO_2 nesting sites were observed when considering the other behavioural activities (Table 1; Fig. 3).

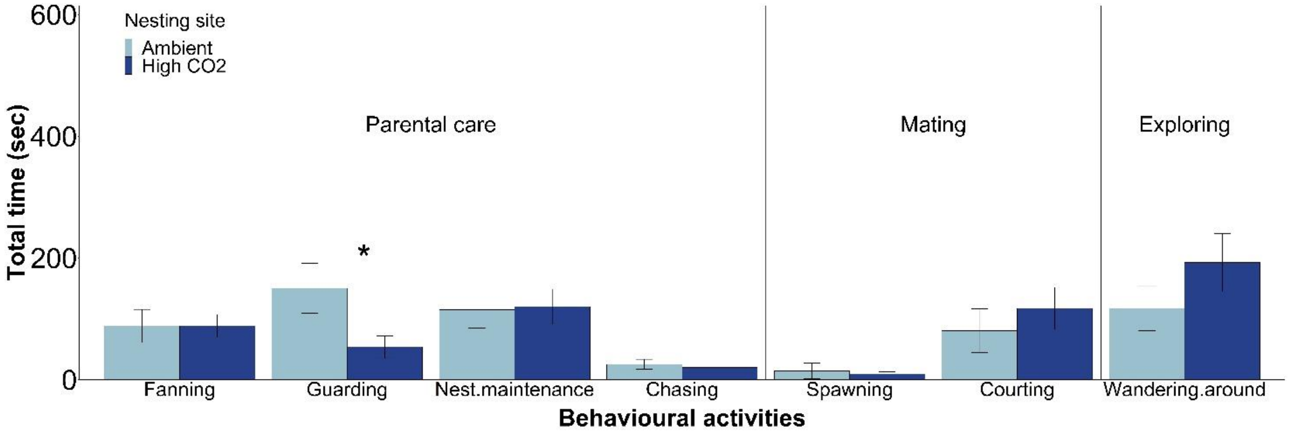


Figure 3 Bar plots (mean ± CI)) of the time nesting male spent on activities within each behavioural category in the two different nesting sites at high and ambient CO_2. The asterisk indicates significant differences (at $P < 0.05$).

The maximum number of sneakers, females and egg predators did not affect (both in overall and univariate tests) the time spent on the behavioural categories and activities, which in turn were affected by the presence/absence of the satellite male (Table 1 and Table S4 in Supplementary material). Particularly, the presence of satellite males significantly increased the time that the nesting male spent on parental care independently from the CO_2 sites considered (Table 1 and Table S4 in Supplementary material). Similarly, the presence of the satellite significantly reduced the time spent on courting behaviour in both the nesting sites at ambient and high CO_2 (Table 1 and Table S3 in Supplementary material).

Table 1 Summary of statistical analyses on the number of females and sneakers (total and maximum number), presence/absence of satellites males, maximum number of egg predators, connectance and behavioural time (category and single activities). Comparisons between the two nesting sites at high and ambient CO_2 are reported. The results for the time spent for different behaviours are presented as multivariate (overall test) and univariate analyses (in brackets). Only the significant effect of the presence/absence of satellite was reported for all analyses. Significant results are in bold. Full analyses are reported in Table S3.

Number of females, sneakers, satellites and egg predators		df	F	p
Maximum number	Females	1	0.07	0.7975
	Sneakers	1	0.39	0.539
	Egg predators	1	0.78	0.3856
Presence/absence	Satellites	1	1.68	0.205
Total number	Females	1	1.42	0.2427
	Sneakers	1	0.62	0.4391
Connectance		**df**	**χ^2**	**p**
		1	0.37	0.544
Behavioural category		**Df**	**F**	**p**
Overall test		29	7.58	0.058
Satellite (presence/absence)		**24**	**27.07**	**0.002**
Univariate tests	**Parental care (Nesting sites: F =7.03, p = 0.034) (satellite: F = 26.74, p = 0.002)** Mating (Nesting sites: F = 1.05, p = 0.376) Exploring (Nesting sites: F =1.39, p = 0.376)			
Behavioural activities		**Df**	**F**	**p**
Overall test		**29**	**18.11**	**0.019**
Satellite (presence/absence)		**26**	**34.03**	**0.002**
Univariate tests	Fanning (Nesting sites: F = 0.29, p = 0.913) **Guarding (Nesting sites: F = 12.27, p = 0.013)** Nest maintenance (Nesting sites: F = 0.05, p = 0.913) Chasing (Nesting sites: F = 2.44, p = 0.40) Spawning (Nesting sites: F = 0.18, p = 0.913) Courting (Nesting sites: F = 2.73, p = 0.400) **(satellite: F = 10.59, p = 0.02)** Wandering around (Nesting sites: F = 3.82, p = 0.287)			

Discussion

This study investigated the potential differences in the behaviour of a wild wrasse breeding between sites off two volcanic CO_2 seeps in the Western Mediterranean Sea. Our findings show that the ocellated wrasse nesting male significantly reduced the total time spent on parental care at nests exposed to high CO_2 compared with nests at ambient CO_2 levels. Guarding behaviour significantly decreased in nesting sites at high CO_2 levels, whilst other parental care activities (i.e., fanning, nest maintenance and chasing), mating (courting and spawning), and exploring behaviours (wandering around), did not show any differences between the two nesting sites. As a consequence of the reduced guarding at nests in the high CO_2 site, nesting males re-allocated the time budget toward different behaviours, such as courting and wandering around (although they did not significantly differ between sites).

Previous OA studies in laboratory and natural conditions have considered only a small number of behavioural activities at the same time (Milazzo et al., 2016; Sundin et al., 2017; Lopes et al., 2020) thus underestimating potential energy/time reallocation towards other keys behaviours. Aquaria experiments showed no differences in some activities such as fanning, courtship and nest building in the three-spined stickleback *Gasterosteus aculeatus* (Sundin et al., 2017) and in the two-spotted goby *Gobiusculus flavescens* (Lopes et al., 2020). Similarly, no effects of elevated CO_2 on the time spent in courtship behaviour and on the number of chasing events were observed for the ocellated wrasse at the Vulcano CO_2 seep site (Milazzo et al., 2016).

Due to the multiple types of behaviours considered in this study during the breeding season of the ocellated wrasse, we used the behavioural network graphs to represent the overall behavioural pattern of this species at the two nesting sites. Despite a different reallocation of the time budget was found between sites, a similar number of interactions among different behaviours emerged by the analysis of the connectance, thus suggesting that the ocellated wrasse nesting male is able to maintain the same number of overall connections among the different behavioural activities independently to the different level of CO_2 at the two nesting sites. Interestingly, we show a reduction of the time spent on parental care activity at nests exposed to high CO_2, with this being mainly due to a 65% reduction of the guarding activity (i.e., representing approximatively 16% out of the total 600 sec time of observation). When considering the remaining activities included in the parental care category (e.g., fanning and chasing), in the mating or exploring categories, no differences between different CO_2 levels were observed.

The presence/absence or the abundance of females, sneakers, and satellite males did not differ between nesting sites exposed to different CO_2 levels. There is evidence that the abundance of female and accessory males around nests may affect in different ways the behaviour of the ocellated wrasse nesting male during the reproductive season (e.g. Alonzo, 2004). For instance, the presence and the abundance of females at a nest can make it more attractive to sneaker males, consequently requiring the nesting male to allocate more time to guarding activity to minimize the risk of sperm competition (Alonzo, 2004; Milazzo et al., 2016). Here we found that all the behavioural responses considered in this study were unaffected by the number of females and sneaker males around nests. In addition to this and as expected, the presence of the satellite significantly increased the time spent by the nesting male on parental care. This was independent from the CO_2 site considered and in accordance with theoretical predictions and previous empirical studies (Houston et al. 2005; Seki et al. 2007; Kokko & Jennions 2008; but see Alonzo & Heckman, 2010) reporting an increase of the male care as a function of the reduced risk of sperm competition, which in turn is favoured by the presence of the satellite males (Stiver & Alonzo, 2013). When the satellite was present, we specifically observed a significant reduction of the courtship, which this being consistent with previous work on the same species, supporting evidence that nesting males partnered with a satellite may invest less in courtship when helped by satellites in doing so (Stiver & Alonzo, 2013; Nugent et al., 2016). In this and other previous studies, the presence of the satellite did not affect the time spent for the guarding behaviour (Taborsky 1994; Stiver & Alonzo, 2013).

The significant reduction of the guarding behaviour observed in the nesting sites at high CO_2 concentration, might be also a consequence of a lower risk of egg predation in the ocellated wrasse nests by other labrid species, as predicted in previous studies on different fish species (Ongarato & Snucins, 1993; Gravel & Cooke, 2009-2013). However, our findings show that the density and the maximum number of egg predator species were similar between the two nesting sites and did not affect any behavioural category and activity of the ocellated wrasse nesting male.

Given that, we speculate the observed decline of the amount of time spent guarding by nesting male ocellated wrasse under elevated CO_2 levels might be related either to a slower decision-making (Dixson et al., 2010; Ferrari et al., 2012; Jutfelt et al., 2013; Munday et al., 2010) or to a deliberate tendency of the nesting male to invest in other activities to ensure fitness (Faria et al., 2018). Although mechanisms were not tested in this study, an altered decision-making ability under OA could decrease the behavioural performance of the ocellated wrasse nesting male when facing multiple tasks (e.g., courting, guarding nests and chasing competing sneakers or nest predators) (Cattano et al., 2018; Nagelkerken & Munday, 2016). Previous observations for the same species suggest that when the risk of sperm competition is high under natural conditions, behavioural adjustments like increased guarding activity may occur to avoid further sneaker males approaching the nest (Alonzo & Warner, 1999). A similar response was observed in the nesting damselfish *Chromis chromis*, whose males switch from courting to parental care activities when the perceived risk of cuckoldry is high in the wild (Mascolino et al., 2016).

Previous evidence using transplantation experiments of ocellated wrasse embryos revealed no differences in the O_2 consumption between embryos developing in nests exposed to ambient and high CO_2 conditions (Cattano et al., 2016). Here we found no differences in the fanning activity between the two nesting sites, with this rejecting our initial hypothesis of an increased fanning behaviour (i.e. increased oxygenation for embryos in the nest environment) to mitigate potentially adverse effects on embryo development and metabolism to guarantee offspring performance in the high CO_2 nesting site.

Our findings might rather support a behavioural shift under elevated CO_2, when a significant reduction in the proportion of time spent on guarding translated into a time budget reallocation towards other activities (e.g., courting and wandering around) increasing proportionally, but not differing between different CO_2 sites. According to previous studies, a lower time allocation to mate guarding may increase the risk of sperm competition as the probability of sneak spawns could be higher (Alonzo & Warner, 2000). At the same time, the observed proportional increase of time spent on courtship by the nesting male may favour pair spawns. However, here we found no differences in the spawning time between CO_2 sites, while elevated CO_2 levels did not affect the number of the ocellated wrasse sneak spawns but significantly reduced the number of pair spawns between nesting sites exposed to different CO_2 levels (Milazzo et al., 2016). This suggest that sneaker males may not take advantage from a potentially impaired dominant male behaviour. At present, the specific role of the wandering around activity is not well established, as nesting males may leave the nest for several purposes (Taborsky et al. 1987).

Overall, by showing that OA will decrease parental care and guarding activity in the *S. ocellatus* nesting male, this study may contribute to emphasize the behaviour as a first strategy by which this widespread Mediterranean wrasse responds to environmental change. The present findings along with previous research assessing the mating behaviour, the reproductive success, and the abundance of the Mediterranean ocellated wrasse along a CO_2 gradient suggest this species may maintain fitness and population persistence under OA conditions (Milazzo et al., 2016; Cattano et al., 2016; Mirasole et al., 2020). Future research efforts should also consider the fundamental link between species behaviour and population responses under changing environmental conditions, for instance deepening the different processes and mechanisms underpinning tolerance to elevated CO_2 in marine populations, including transgenerational effects (Schunter et al., 2016; Tsang et al., 2020; Petit-Mart et al., 2021).

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Supplementary material

Chapter 3: Nest guarding behaviour of a temperate wrasse differs between sites off Mediterranean CO_2 seeps

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Figure S2. Barplot of the connectance between the behavioural activities in the two nesting sites at ambient and high CO_2.

Supplementary References

Table S1 Summary of the carbonate chemistry measured during the reproductive season of S. ocellatus in the two nesting sites at high and ambient CO_2 of Vulcano Island (years 2012, 2013, 2014 and 2018) and Bottaro, Panarea Island (year 2014). Multiple measurements of Salinity, Temperature (T°C), Total Alcalinity (TA), pH and Aragonite and calcite saturation states (Ω) were made daily in each nesting site (between 10:00-16:00 h) both on different days before and on the same day of the behavioural observations.

Data	**Locality**	**Site**	**n**	**Salinity (± SE)**	**T °C (± SE)**	**TA**	**pH (± SE)**	**pCO_2 (± SE)**
May-12	Vulcano	Amb. CO_2	11	38.12 (0.02)	19.59 (0.13)	2607	8.15 (0.01)	479.60 (18.96)
May-12	Vulcano	High CO_2	11	38.15 (0.02)	19.38 (0.12)	2581	7.82 (0.06)	1273.50 (244.32)
June-13	Vulcano	Amb. CO_2	12	38.14 (0.02)	19.54 (0.12)	2528	8.18 (0.01)	421.13 (14.55)
June-13	Vulcano	High CO_2	12	38.15 (0.02)	19.97 (0.09)	2545	7.83 (0.05)	1179.62 (152.77)
June-14	Vulcano	Amb. CO_2	3	38	23.46	2520	8.08 (0.01)	389.82 (12.80)
June-14	Vulcano	High CO_2	3	38	23.64	2527	7.72 (0.11)	1134.27 (351.34)
July-14	Vulcano	Amb. CO_2	3	38	26.05	2520	8.09 (0.02)	375.76 (19.99)
July-14	Vulcano	High CO_2	3	38	26.27	2501	7.69 (0.09)	1188.34 (297.72)
June-18	Vulcano	Amb. CO_2	3	38.1	24.05	2532	8.14 (0.01)	483.65 (18.29)
June-18	Vulcano	High CO_2	3	38.1	24.21	2501	7.83 (0.06)	1101.38 (153.03)
July-14	Panarea	Amb. CO_2	3	38.1	28.88 (0.02)	2500	8.16 (0.06)	453.93 (69.81)
July-14	Panarea	High CO_2	2	38.1	28.97 (0.18)	2500	7.82 (0.02)	1184.11 (68.96)
							Ambient (n= 35)	**High CO_2 (n=34)**
						Overall pCO_2	434 (± 18.69)	1177 (± 23.96)

Table S2 List of the the 8 behavioural activities grouped in the three categories (parental care, mating and exploring) observed in during the breeding season of S. ocellatus at the study sites.

Categories	Behaviours	Detailed Behavioural description
Parental care	Fanning (FA)	Rhythmic pectoral oscillations of high amplitude with compensatory movements of the tip of the dorsal fin (Taborsky et al., 1987).
	Guarding (GR)	The nesting male is hovering above the nest and swims next to it assuming a parallel position to the exterior part of the nest.
	Nest maintenance (NM)	The nesting male pushes the nest with the snout and head to compress the algal layers and to insert new algae in the nest. The algae's pieces can be collected by the male several meters away from the nest and then transported by mouth to the nest. The male adopts a position that is almost perpendicular to the nest (Lejeune and Voss 1979, 1980; Lejeune 1985) and presses the algae in the nest with strong strokes of the caudal fin.
	Chasing (CH)	Swimming towards a fish which swims away very fast (for several meters). It frequently occurs when an intruder male (sneaker) or a potential egg predator enters the nest or during agonistic interactions (Potts 1974; Helas et al. 1982).
Mating	Spawning (SP)	Male spawning occurs after female lays eggs by rubbing the belly in the nest with circular movements (Potts 1974; Voss 1976; Helas et al. 1982; Lejeune 1985). This behaviour lasts just a few seconds (Helas et al., 1982) where male comes very close above the female before spawning, often making direct body contact.
	Courting (CT)	The nesting male leaves quickly the nest and then return to the nest performing a U-turn movement with the tail beating at high amplitude to lead the female to the nest ("nest showing"). This behavior is generally followed by a series of exaggerate spawning movement performed in the nest ("courtship spawning"), (Taborsky et al., 1987).
Exploring	Wandering around (WA)	The nesting male exploring the habitat around the nest usually swims several meters away from the nest.

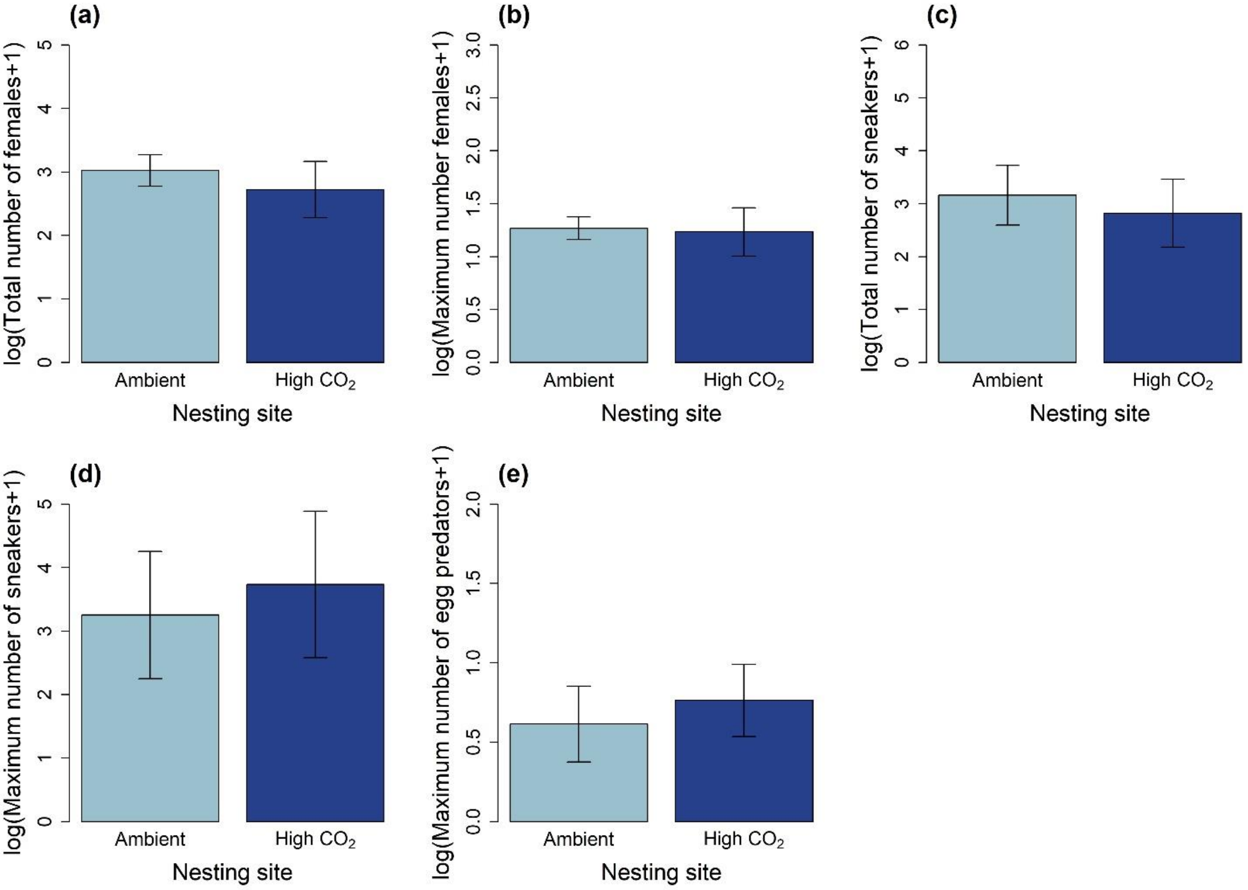

Figure S1 Barplots (mean ± CI) of the total and the maximum number of females (a-b), sneakers (c-d) and the maximum number of satellities (e)egg predators (e) visiting the ocellated wrasse nest at a < 1m distance of ocellated wrasse in the two nesting sites at ambient and high CO_2.

Table S3 Summary of statistical analyses on the density (no. of individuals 50 m-2) of each egg predator species, between the two nesting sites at high and ambient CO_2 *are reported. Significant results are in bold. Symphodus mediterraneus was not included since no individuals of this species were found in both* CO_2 *sites.*

Egg predator species	**df**	**F**	**p**
Coris julis	1	1.6061	0.2338
Symphodus roissali	1	0.142	0.7142
Symphodus tinca	1	2.9889	0.114
Thalassoma pavo	1	3.2026	0.1038

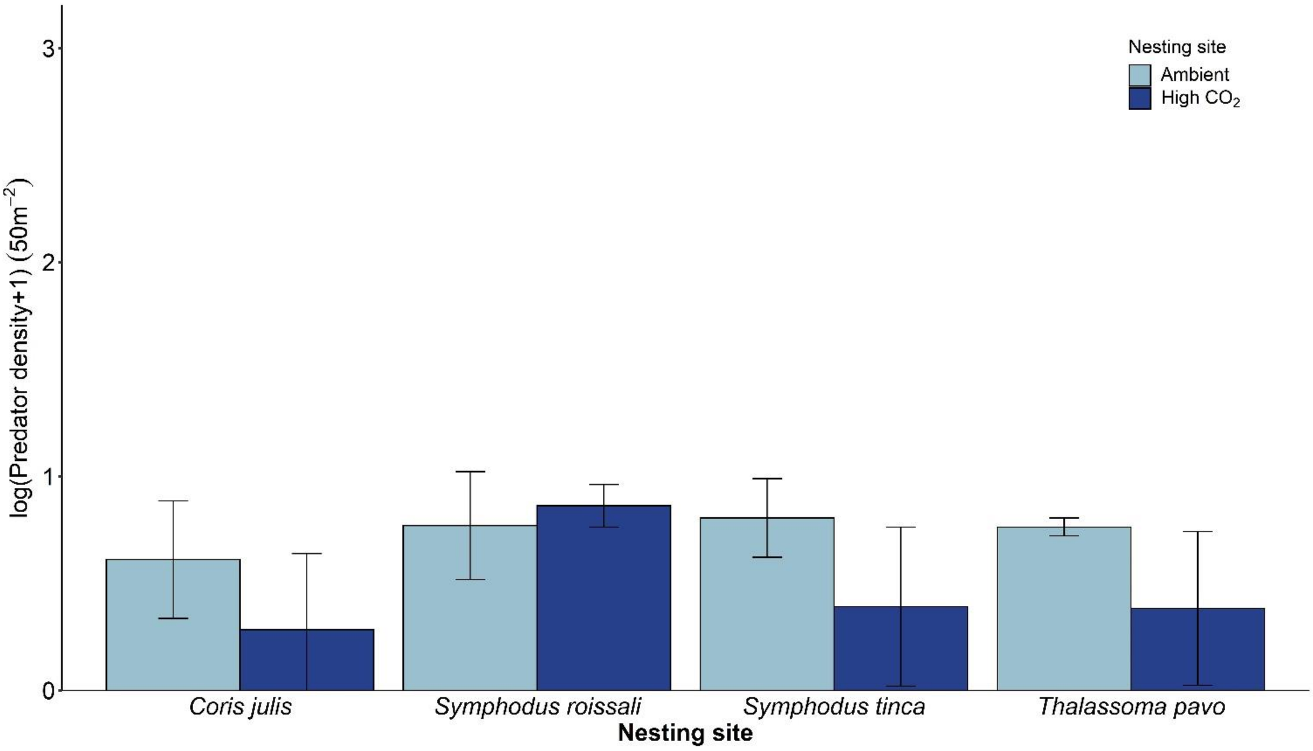

Figure S2 Barplots (mean ± CI) of density (50 m^{-2}) of each potential egg predator species visiting the ocellated wrasse nest at a < 1m distance in the two nesting sites at ambient and high CO_2.

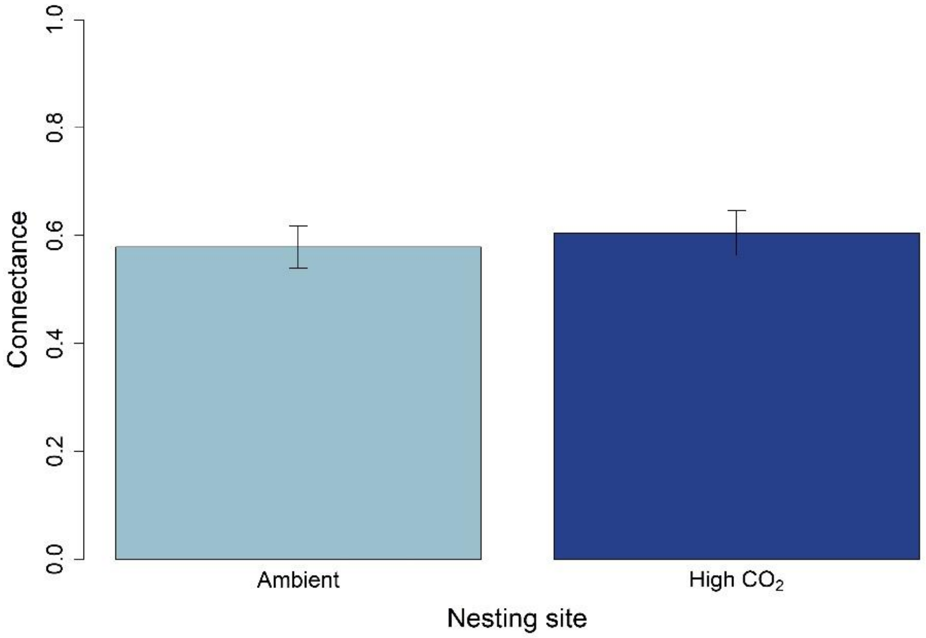

Figure S3 Barplot (mean ± CI) of the connectance between the behavioural activities in the two nesting sites at ambient and high CO_2.

Table S4 Summary of statistical analysis of behavioural time (category and single activities). All the comparisons between the two nesting sites at high and ambient CO_2 are reported. The effects of the maximum number (MaxN) of sneakers and females (as covariates) as well as the number of egg predators (pred) and the presence/absence (P/A) of the satellites were reported for all analyses. The results for the behavioural time are presented as multivariate (overall test) and univariate analyses. Significant results are in bold.

Behavioural category			**df**	**F**	**p**
Overall test		Nesting sites	29	7.58	0.058
		Pred	27	1.15	0.734
		MaxN of sneakers	26	5.07	0.119
		MaxN of females	25	3.24	0.287
		P/A satellite	**24**	**27.07**	**0.002**
		Nesting sites × Pred	**22**	**1.56**	**0.609**
		Nesting sites × MaxN of sneakers	21	0.55	0.783
		Nesting sites × MaxN of females	20	7.74	0.060
		Nesting sites × P/A satellite	19	2.02	0.430
Univariate tests	Parental care	Nesting sites	**1**	**7.03**	**0.034**
		Pred	**1**	**0.12**	**0.895**
		MaxN of sneakers	1	4.21	0.111
		MaxN of females	1	1.5	0.419
		P/A satellite	**1**	**26.74**	**0.002**
		Nesting sites × Pred	**1**	**0.96**	**0.678**
		Nesting sites × MaxN of sneakers	1	0.40	0.795
		Nesting sites × MaxN of females	1	0.47	0.504
		Nesting sites × P/A satellite	1	0.23	0.650
	Mating	Nesting sites	1	1.05	0.376
		Pred	1	0.06	0.729
		MaxN of sneakers	1	2.44	0.215
		MaxN of females	1	2.08	0.345
		P/A satellite	1	4.26	0.098
		Nesting sites × Pred	1	0.86	0.68
		Nesting sites × MaxN of sneakers	1	0.26	0.795
		Nesting sites × MaxN of females	1	5.65	0.071
		Nesting sites × P/A satellite	1	1.51	0.460
	Exploring	Nesting sites	1	1.39	0.376
		Pred	1	0.83	0.729
		MaxN of sneakers	1	0.023	0.881
		MaxN of females	1	0.518	0.480
		P/A satellite	1	0.659	0.419
		Nesting sites × Pred	1	0.24	0.784
		Nesting sites × MaxN of sneakers	1	0.04	0.835
		Nesting sites × MaxN of females	1	4.99	0.077
		Nesting sites × P/A satellite	1	1.10	0.538

Behavioural activities			**Df**	**F**	**p**
Overall test		**Nesting sites**	29	18.11	**0.019**
		Pred	27	8.86	**0.209**
		MaxN of sneakers	26	8.79	0.253
		MaxN of females	25	6.02	0.479
		P/A satellite	**24**	**34.03**	**0.002**
		Nesting sites × Pred	**22**	**4.92**	**0.667**
		Nesting sites × MaxN of sneakers	21	3.19	0.797
		Nesting sites × MaxN of females	20	8.30	0.291
		Nesting sites × P/A satellite	19	6.22	0.440
Univariate tests	Fanning	Nesting sites	1	0.29	0.928
		Pred	1	2.96	0.363
		MaxN of sneakers	1	0.14	0.808
		MaxN of females	1	0.001	0.994
		P/A satellite	1	1.49	0.404
		Nesting sites × Pred	1	0.21	0.998
		Nesting sites × MaxN of sneakers	1	0.11	0.990
		Nesting sites × MaxN of females	1	2.76	0.494
		Nesting sites × P/A satellite	1	0.31	0.923
	Guarding	**Nesting sites**	**1**	**12.27**	**0.009**
		Pred	**1**	**1.35**	**0.788**

	MaxN of sneakers	1	4.58	0.228
	MaxN of females	1	1.84	0.667
	P/A satellite	1	3.34	0.230
	Nesting sites × Pred	1	0.226	0.998
	Nesting sites × MaxN of sneakers	1	1.26	0.822
	Nesting sites × MaxN of females	1	0.25	0.873
	Nesting sites × P/A satellite	1	2.75	0.506
Nest Maintanance	Nesting sites	1	0.05	0.928
	Pred	1	0.72	0.934
	MaxN of sneakers	1	1.69	0.648
	MaxN of females	1	2.70	0.529
	P/A satellite	1	6.15	0.111
	Nesting sites × Pred	1	1.57	0.763
	Nesting sites × MaxN of sneakers	1	0.20	0.982
	Nesting sites × MaxN of females	1	0.47	0.873
	Nesting sites × P/A satellite	1	0.00	0.983
Chasing	Nesting sites	1	2.44	0.397
	Pred	1	2.96	0.363
	MaxN of sneakers	1	1.40	0.648
	MaxN of females	1	0.01	0.994
	P/A satellite	1	6.19	0.111
	Nesting sites × Pred	1	0.03	0.998
	Nesting sites × MaxN of sneakers	1	1.39	0.822
	Nesting sites × MaxN of females	1	0.14	0.873
	Nesting sites × P/A satellite	1	0.03	0.983
Spawning	Nesting sites	1	0.18	0.928
	Pred	1	0.08	0.934
	MaxN of sneakers	1	1.69	0.648
	MaxN of females	1	0.73	0.856
	P/A satellite	1	0.31	0.56
	Nesting sites × Pred	1	3.22	0.334
	Nesting sites × MaxN of sneakers	1	0.04	0.99
	Nesting sites × MaxN of females	1	0.88	0.815
	Nesting sites × P/A satellite	1	1.27	0.761
Courting	Nesting sites	1	2.73	0.387
	Pred	1	0.72	0.934
	MaxN of sneakers	1	3.00	0.414
	MaxN of females	1	1.19	0.772
	P/A satellite	**1**	**13.55**	**0.011**
	Nesting sites × Pred	**1**	0.11	0.998
	Nesting sites × MaxN of sneakers	1	0.50	0.954
	Nesting sites × MaxN of females	1	3.51	0.409
	Nesting sites × P/A satellite	1	2.30	0.561
Wandering around	Nesting sites	1	3.82	0.279
	Pred		0.70	0.934
	MaxN of sneakers	1	0.32	0.808
	MaxN of females	1	0.03	0.994
	P/A satellite	1	3.84	0.23
	Nesting sites × Pred	1	0.50	0.987
	Nesting sites × MaxN of sneakers	1	0.03	0.99
	Nesting sites × MaxN of females	1	1.66	0.696
	Nesting sites × P/A satellite	1	1.02	0.761

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Chapter 4 - Limited behavioural effects of ocean acidification on a Mediterranean goby (*Gobius incognitus*) chronically exposed to elevated CO_2 levels

Introduction

Anthropogenic CO_2 emissions in the atmosphere are responsible of the ongoing carbonate chemistry changes and pH drop in the ocean, a process known as Ocean Acidification (OA) (Orr et al., 2005; Doney et al., 2009). This might lead to a range of effects on marine organisms, which could cause population, community and ecosystem changes (Gaylord et al., 2015). Indeed, key processes (e.g. competition, predation, habitat provisioning) regulating ecosystem structure and function may be affected by these changing environmental conditions (Gaylord et al., 2015; Sunday et al., 2017).

In this study we focus on fish predator-prey interactions under elevated CO_2 concentrations (Briffa et al., 2012; Nagelkerken & Munday, 2016; Draper & Weissburg, 2019). The ability to detect and avoid predators is one of the most important mechanisms to ensure survival, and previous research have reported that ocean pCO_2 levels expected to occur by the next few decades may lead to impaired ability of fish to distinguish between predator and non-predator olfactory cues (e.g., Dixson et al., 2010; Ferrari et al., 2011; Munday et al., 2010). Among the sensory mechanisms used to detect risk-associated cues, chemosensory has been widely considered in studies on predator risk assessment under OA conditions both for tropical and temperate fish species (e.g. Porteus et al., 2018; Dixson et al., 2010) and resulted in impaired olfactory ability. Despite the majority of chemosensory studies on predator-prey interactions, other sensorial impairments involving auditory and visual systems due to increased CO_2 levels have been also elucidated (e.g. Ferrari et al., 2010, 2012; Simpson et al., 2011). In addition, several studies documented that exposure to elevated CO_2 may alter fish lateralization (Domenici et al., 2012; Jutfelt et al., 2013; Näslund et al., 2015), increase activity and boldness, with potential prey venturing further from shelters (Cattano et al. 2019; Munday et al., 2013). However, other studies have shown no changes in chemosensory reception ability of prey (Cattano et al., 2017;

Jutfelt & Hedgärde, 2013; Sundin et al., 2017), unaltered or decreased activity (e.g. Porteus et al., 2018; Duteil et al., 2016; Sundin et al., 2017; Sundin et al., 2013; Sundin & Jutfelt, 2016) and increased or unvaried sheltering behaviour (e.g. Näslund et al., 2015; Rossi et al., 2015) under elevated CO_2. Overall, such heterogeneity in responses may underline that elevated CO_2 levels may have species-specific effects, likely depending on potential adaptations to local conditions (Vargas et al., 2017). For instance, Jarrold et al. (2017) suggested that detrimental effects of stable elevated CO_2 on anti-predator response maybe reduced by the natural daily pCO_2 fluctuations. Similarly, experiments conducted in natural CO_2 gradients (e.g., CO_2 seeps) may contribute to highlight the ability of fish to acclimate and adapt to predicted OA conditions in the long term (Wittmann & Pörtner, 2013; Calosi et al., 2013; Petit-Mart et al., 2021).

Here, we experimentally evaluated whether chronically exposed fish to OA and reference conditions off a volcanic CO_2 seep showed behavioural effects when experiencing the presence of a predator. To this purpose, we carried out an in-situ reciprocal transplant experiment along the Vulcano Island natural CO_2 gradient (Southern Italy), using the Mediterranean anemone goby *Gobius incognitus* (Kovačić & Šanda, 2016). This species is a territorial benthic fish, showing facultative association to anemones and characterized by a very limited home range (Tiralongo et al., 2020; Kovačić & Šanda, 2016; Nagelkerken et al., 2015). We used the Mediterranean anemone goby as a species model to investigate potentiality for fish behavioural adaptation or acclimation in response to chronic long-term exposure to elevated CO_2 levels. In addition to the manipulative experiment, we preliminary assessed *G. incognitus* density, through visual census surveys carried out at the elevated and ambient CO_2 sites. Finally, since both the number of predators and the availability of shelter (anemone) may influence the behaviour of the study species (Nagelkerken et al., 2015), we also quantified the predator and anemone densities as well as the association between the goby and the anemone in the two different CO_2 sites.

Given the limited range of movement of this species, we hypothesize that *G. incognitus* adults under long-term exposure to different level of CO_2 at a volcanic seep may show behavioural tolerance when individuals were moved from sites at high to ambient CO_2 and viceversa. Therefore, we expected that fish will show unaltered antipredator behaviour with swimming activity, shelter use and distance from the shelter being unaffected.

Materials and Methods

Study species

Gobius incognitus (Kovačić and Šanda, 2016) (Fig. 1a) is a widespread species in Mediterranean shallow coastal waters. 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 most of the previous studies on *G. bucchichi*, including a couple of previous studies in the study site (Nagelkerken et al., 2015; Mirasole et al., 2020) probably deal with *G. incognitus* (Tiralongo et al., 2020). To avoid a species misidentification, we conservatively used a DNA barcoding approach (Supplementary methods) to clarify the taxonomic identity of our study species. Our results (Table S1 and Figure S1 in Supplementary Materials) identified the study species as *G. incognitus*.

This goby occurs on all kinds of rocky substrata (gravel, cobbles, boulders and bedrock) and on sand only when it is mixed with the rocky substrates (Kovačić and Šanda, 2016; Tiralongo et al., 2020). As well as reported for *G. bucchichi (Abel, 1960),* this species can live in facultative association with the symbiotic sea anemone *Anemonia viridis* (Forsskål, 1775) (Fig. 1b) hiding amongst its tentacles when threatened (Kovačić, 2020; Patzner, 2005).

Study site

The experiment was carried out in the shallow CO_2 vent located in Levante Bay (Vulcano Island, Aeolian Archipelago, Italy) during two sampling surveys on 17 May–02 June 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 mainly 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 in two sites, whose carbonate chemistry was extensively characterized in many previous studies (Boatta et al., 2013; Milazzo et al., 2016; Aiuppa et al., 2021), designated as Ambient (A, present-day conditions: ~400 μatm, pCO_2) and high CO_2 (H), this latter showing end-of-century CO_2 conditions (~1000 μatm pCO_2; IPCC 2016). Average (±S.E.) carbonate chemistry (temperature, °C; Salinity; $pH_{total;}$ pCO_2, μatm) from the study period are reported in Table S2, where a fixed Total Alkalinity of 2500 μmol kg^{-1} (not measured in this study) was used for calculation with the software CO_2SYS (Pierrot et al., 2006) using the dissociation constants for carbonate from Mehrbach et al. (1973) refitted by Dickson and Millero (1987), and for KSO_4 from Dickson (1990).

Experiment set-up

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 bottoms, (Ambient, n=15; high-CO_2, n=15). At the same time, to determine whether CO_2 had a direct effect on the fish-anemone association, the number of anemones which had a goby nearby (at a distance of less than 6 centimeters), were recorded in the two sites at different CO_2 conditions (A and H). Density of *Serranus scriba* (predator) in the two CO_2sites were visually assessed in replicate 10 x 2 belt transects at 1-3 meters depth on mixed sandy/rocky bottoms (Ambient, n=19; high-CO_2, n=22).

Reciprocal transplant experiment to assess fish antipredator behaviour

In each site exposed to different CO_2 levels, 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 undewater. To assess the effects of elevated CO_2 on visual and chemical cues risk assessment of G. *incognitus* individuals, and their responses when exposed to different CO_2 conditions, fish specimens were transplanted from the high-CO_2 site to the ambient site and *vice-versa* (treatments HA and AH, respectively; 15 fish for each condition). To control the translocation effect, some specimens were replaced into the original site (treatments HH and AA; 15 fish for each condition). Each translocation was performed by placing fish (15 individuals for each treatment) 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 a rope suspended from the sea bottom at 50 cm depth. Fish were kept for 96 h in the containers for acclimation at each condition in the field before testing. To control for a potential effect of fish size on displayed behaviours, standard length (SL, cm) of each fish individual used in the transplant experiment was calculated using a digital photograph, and a ruler as a reference by image analyses using the ImageJ software (Schneider et al., 2012). To assess the behaviour of the fish we used 6 arenas (L:60×H:40×W:20 cm) opened at the base and fixed to sandy bottom (Fig. 1c). Each arena was covered on every side with a black net (0.06 cm mesh size) to ensure visual isolation of the fish from the surrounding environment. Fifteen anemones were collected from each CO_2 site at a depth of 1-3 meters (and kept in the original CO_2 condition) and two of them were randomly placed in the right or left side of each arena to provide a suitable shelter for the experimental fish (Fig. 1c). After the 96-h acclimation period, fish kept in the different CO_2 treatments, were individually transferred into the experimental arenas and acclimated for 10 min before each behavioural trial started. A video camera (Apeman A80) was fixed on the middle-top (~30 cm from the anemone) of the cage to allow behavioural recording. *Gobius incognitus* antipredator 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) and consisted of a 4-min pre-stimulus presentation period 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 *Serranus scriba* (Linneus, 1758). The predator was placed in a clear 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) (Fig. 1c). The tank containing the predator, was gently and randomly introduced from the left or the right end of the arena during a 60-sec stimulus introduction period between the two observation periods (i.e., pre- and post-stimulus).

The following response variables were collected during the pre- and the post-stimulus period: (i) activity level, measured as the amount of time (sec) spent by the focal fish swimming in the entire arena; (ii) time spent by the fish actively swimming or resting in each side of the arena close and far from predator position; (iii) shelter use, measured as the total time the fish spent in anemone proximity (i.e. less than 5 cm); (iv) minimum approach distance, as the mean smallest distance (cm) of the fish centroid from the shelter (*A. viridis*). Some of the video collected (14 in total) did not allow a clear identification of fish behaviours (e.g. the position of the animal respect to the camera was at times not optimal) and were excluded for the subsequent analysis. Thus, a total of forty-six video (n=11 in AA, 12 in AH, 13 in HA and 11 in HH) were used.

Figure 1 (a) the Mediterranean anemone fish Gobius incognitus; (b) a typical association between G. incognitus and Anemonia viridis. (c) the experimental arena used for the behavioural trials, 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).

Experimental design and statistical analysis

Differences in fish, anemone and predator abundance (shelter) between the different CO_2 sites (fixed orthogonal factor with 2 levels: Ambient (A) and High CO_2 (H)), were tested using one-way analysis of variance (ANOVA). Differences in the proportion of goby fish (with respect to all the individuals censused in Ambient and High CO_2 sites) associated with anemone between the two CO_2 sites were assessed using a binomial generalized linear model. The significance of the association was obtained using the analysis of deviance test.

Potential differences in experimental fish standard length (cm) 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). Fish standard length was not included in subsequent behavioural analyses because it was not significant different between the CO_2 treatments ($p > 0.05$, see results section). Potential differences in each behavioural trait between “CO_2 treatment” (fixed factor with 4 levels: AH, HA, AA and HH) and “Time Period” (fixed factor with 2 levels: pre- and post-stimulus) and “Shelter position” (fixed factor with two levels: Close and Far from predator side) and their interaction were assessed through a linear mixed model (lmer). Specifically, five Gaussian linear mixed models with log-transformation (except for the minimum approach distance) were performed for each response variable: 1) total activity levels (sec), 2) activity level close to the predator side (sec), 3) activity level far from predator side (sec), 4) differences in shelter use (sec) 5) minimum approach distance (cm). Specifically, for the activity measured close and far to the predator side, the fixed factor “Shelter position” was replaced with “Shelter presence” (fixed factor with two levels: presence and absence of the shelter) as we only considered a specific sector of the arena. For each lmer, the identity of the experimental individuals (ID –factor with forty-six levels: pair 1 to 46) was used as a random effect allowing us to introduce a correlation structure in the model, as the observations were repeated on the same individuals during the pre and post stimulus presentation. The significance of the fixed terms in the model were tested using the F-test with the Satterthwaite approximation for degree of freedom and the F statistic ('lmerTest' package in R).. All analyses were performed using the R software version 1.3.1073 (RStudio Team, 2020), and the lme4 package for R (Bates et al., 2015) for linear mixed effect modelling. Pairwise comparisons among least square means for levels of factors were performed with Tukey’s test by using the “lsmeans” package (Lenth, 2016). All analyses were performed using the R software version 3.3.0 (R Core Team, 2016).

Results

The densities of gobies and anemones (no. of individuals $3m^{-2}$) at the high CO_2 site were significantly higher than the Ambient CO_2 site with on average (± 95% Confidence Interval, CI.) 4.4 (±1.45 CI) and 2.2 (±0.48 CI) goby individuals, and 32.13 (±5.18 CI) and 17.3 (±3.37 CI) anemone individuals, respectively (Table 1; Fig. 2a-b). The percentage of fish associated with anemones did not differ between sites, as we observed an association of 84.2% (± 10.61 CI) at High CO_2 and of 87.2% (± 9.94 C.I.) at the Ambient CO_2 (Table 1; Fig. 2c). The density of the predator *S. scriba* (no. of individuals $20m^{-2}$) was similar between the two sites with a mean value of 1.05 (± 0.35 C.I.) in the Ambient and

1.00 (± 0.34 C.I.) in High CO_2 site (CO_2 effect: $F_{1,39} = 0.04$; $p = 0.835$; Fig. S2 in Supplementary Materials).

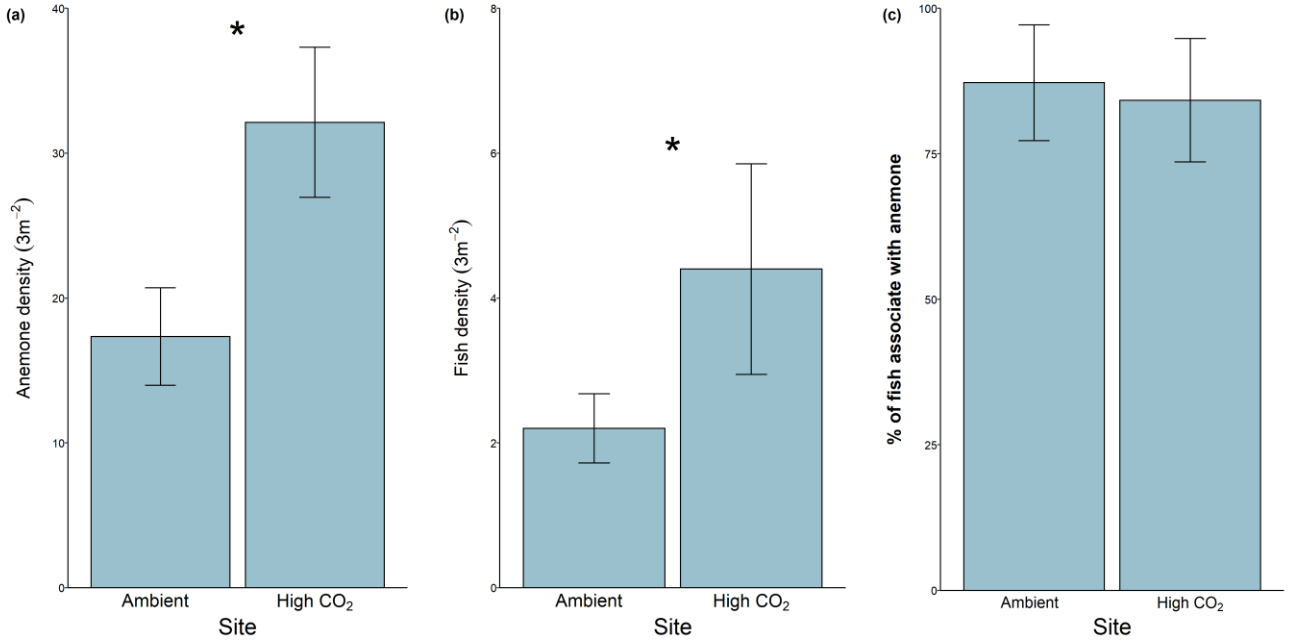

Figure 2 Bar plots (mean ± C.I.) of density (3 m-2) of A. viridis (a), G. incognitus (b) and the percentage of adult goby fish associated with anemones (c) in the two different CO_2 sites at Ambient and High CO_2.The asterisk indicates significant differences (at $P < 0.05$).

In the transplant experiment, no difference in the mean (± 95% C.I.) standard length (cm) of *G. incognitus* was observed between the different CO_2 treatments (AA: 6.04 ± 0.52; AH: 6.15± 0.99, HA: 6.66 ± 0.52; HH: 6.47± 0.64) (Table 1; Fig. S3 in Supplementary Materials). The total activity levels was not affected by the different CO_2 treatments, presentation periods (*pre*- and *post-stimulus)* and shelter position (close and far from the predator) and their interactions ("CO_2 treatments x Time period" interaction; Table 1; Fig. 3A). However, activity levels close to the predator side were significantly different between *pre- and post-stimulus* among the different CO_2 treatment ("CO_2 treatments x Time period" interaction; Table 1). Particularly, fish observed in the AA treatment significantly decreased the time spent actively moving in the arena during the *post- stimulus* period (Table 1; Fig. 3B). No significative difference in this response variable between pre- and post-stimulus was observed in the other CO_2 treatment (Table 1; Fig. 3B). During the post-stimulus period, the individuals of *G. incognitus* spent a similar amount of time resting close to the predator side (45 % of the total amount) and resting or actively moving far from predator side (considered together, 55% of the total amount) (see pie charts in Fig. 3B). On the other hand, the percentage of time resting or actively moving far from predator side (on average about 70%) was higher than the percentage of time spent resting close to the predator side in the other CO_2 treatments (about 32%) (see pie charts in Fig. 3B). Activity levels far from predator side were similar between pre- and post-stimulus presentation periods ("CO_2 treatments x Time period" interaction; Table 1; Fig. S4 in Supplementary Materials). Finally, no differences in the shelter use (Table 1; Fig. 4A) and the minimum approach distance (Table 1; Fig. 4B) were found when considering the two stimulus presentation periods, the CO_2 treatments and their interaction.

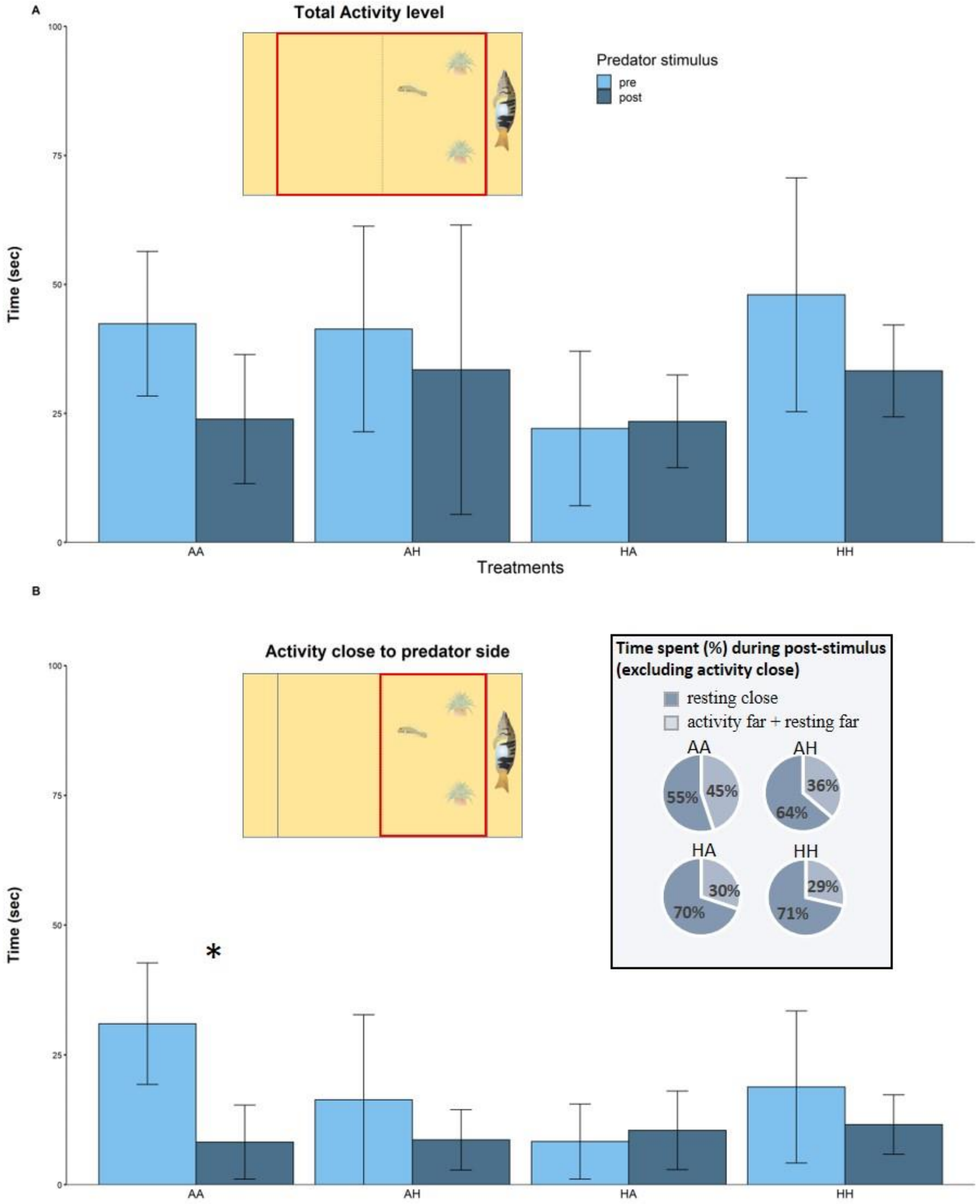

Figure 3 Mean values (±CI) of: total activity level (panel A), and activity close to predator side (panel B) for individuals of G. incognitus before (pre-stimulus) and after (post-stimulus) the presentation of the predator S. scriba in the different CO_2 *treatments. Panel B: Pie charts show the percentage of time individuals of goby spent resting close to the predator side ("resting close") and the total time spent actively moving or resting far from predator side ("activity+resting") during the post-stimulus period. The asterisk indicates significant differences. (at* $p < 0.05$*).*

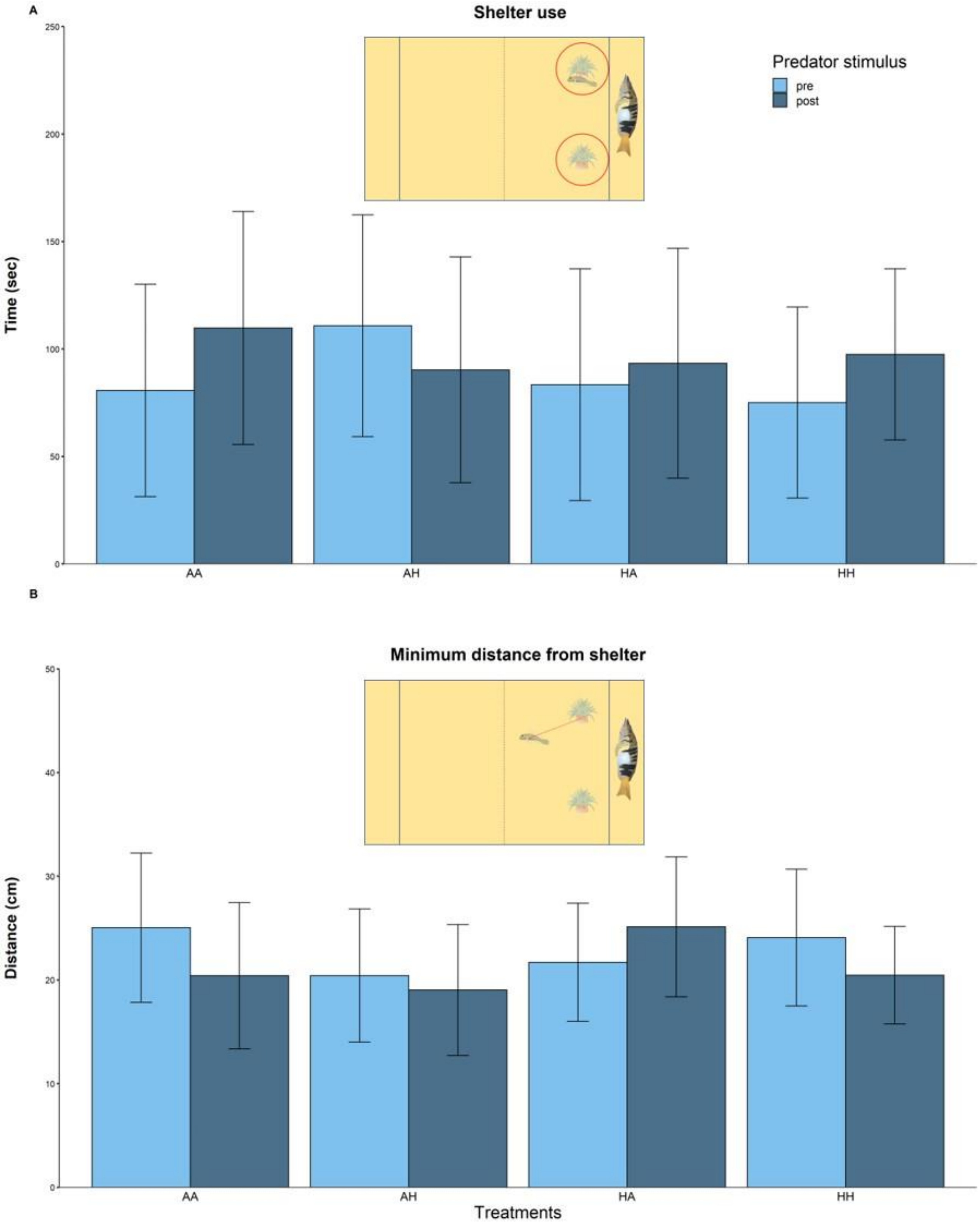

Figure 4 Mean (±CI) values of: shelter use (Panel A) and minimum approach distance (Panel B) displayed by individuals of G. incognitus before (pre-stimulus) and after (post-stimulus) the presentation of the predator S. scriba in the different CO_2 treatments.

Table 1 Summary results of the statistical analyses performed for the field patterns and transplant experiments in the two CO$_2$ sites. Comparisons between the two sites exposed to different CO$_2$ conditions (i.e., high CO$_2$ versus ambient CO$_2$ nesting sites) for densities and association, and between the different CO$_2$ treatments (AA, AH, HA, HH) for fish length are reported. Results relatively to fish antipredator behaviour in the different CO$_2$ treatments (CO$_2$), during pre- and post-stimulus predator presentation period (Time period) and shelter position (Shelter) are reported (ID as random factor, not reported in the table). Degree of freedom (df), F-tests (F), chi-square (χ^2) and probability levels (p) are provided, and significant effect are reported in bold.

Fish, anemone and predator density and association	**df**	**F**	**p**
Fish density (goby)	**1**	**20.82**	**< 0.001**
Anemone density	**1**	**9.72**	**0.004**
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		**df**	**F**	**p**
Total activity level	CO$_2$ treatment	3	0.76	0.522
	Time period	1	3.64	0.064
	Shelter	1	0.52	0.477
	CO$_2$ treatment x Time period	3	2.77	0.055
	CO$_2$ treatment x shelter	3	0.29	0.829
	Time period x Shelter	1	0.00	0.999
	CO$_2$ treatment x Time period x Shelter	3	0.25	0.864
Activity close to predator side	CO$_2$ treatment	3	1.15	0.341
	Time period	1	3.45	0.071
	Shelter	**1**	**4.24**	**0.047**
	CO$_2$ treatment x Time period	**3**	**4.96**	**0.005**
		AA pre – AA post (t-ratio = -3.98, p = 0.007)		
	CO$_2$ treatment x shelter	3	1.11	0.359
	Time period x Shelter	1	1.45	0.236
	CO$_2$ treatment x Time period x Shelter	3	1.71	0.180
Activity far from predator side	CO$_2$ treatment	3	1.76	0.172
	Time period	1	0.00	0.955
	Shelter	1	2.70	0.109
	CO$_2$ treatment x Time period	3	1.66	0.192
	CO$_2$ treatment x shelter	3	1.30	0.288
	Time period x Shelter	1	3.67	0.063
	CO$_2$ treatment x Time period x Shelter	3	1.21	0.322
Shelter use	CO$_2$ treatment	3	0.17	0.918
	Time period	1	0.99	0.325
	Shelter	1	0.25	0.621
	CO$_2$ treatment x Time period	3	1.61	0.204
	CO$_2$ treatment x shelter	3	2.36	0.087
	Time period x Shelter	1	0.08	0.784
CO$_2$ treatment x Time period x Shelter3	CO$_2$ treatment x Time period x Shelter	3	0.42	0.743
Minimun distance from shelter	CO$_2$ treatment	3	0.23	0.875
	Time period	1	0.46	0.502
	Shelter	1	0.55	0.462
	CO$_2$ treatment x Time period	3	0.38	0.769
	CO$_2$ treatment x shelter	3	2.03	0.126
	Time period x Shelter	1	1.77	0.192
	CO$_2$ treatment x Time period x Shelter	33	0.49	0.690

Discussion

This study represents one of the first attempts to assess the potential impacts of ocean acidification on antipredator response of fish acutely and chronically exposed to elevated CO_2 concentrations in the wild. Analysis of patterns of abundances suggests a significant increase of fish and anemones under High CO_2 conditions compared to the Ambient site, whilst the fish-anemone association was similar between sites. Importantly, we show that both short and long-term exposure to expected end of century-pCO_2 levels did not affect most of the behaviours investigated in this species, but activity levels measured close to the predator.

Swimming activity is one of the behavioural measurements most frequently used in studies aimed at investigating the effect of OA conditions on predator detection ability in fish (Cripps et al., 2011; Ferrari et al., 2011, 2012; Munday et al., 2013). The majority of previous short term-OA studies conducted in laboratory conditions reported increased activity levels in fish under elevated CO_2 concentrations and exposed to a predator, indicating a lower perception of predation risk (see Briffa et al., 2012; Cattano et al., 2018; Draper and Weissburg, 2019; Nagelkerken and Munday, 2016 for reviews). In presence of a predetor, differences in activity levels were also observed in fish exposed to long-term elevated CO_2 off natural CO_2 seeps, which showed an increased activity for some species, whilst others revealed a decreasing response when compared with fish from reference sites (Munday et al., 2014). In this study, we found no difference between the different CO_2 treatments (AA, AH, HA, HH) in the amount of time fish spent actively swimming in the entire arena (total activity) before and after the predator presentation. This finding is consistent with several studies failing to detect effects of elevated CO_2 on activity levels of adult fish (e.g. Clark et al., 2020; Duteil et al., 2016; Sundin et al., 2013; Sundin & Jutfelt, 2016). However, when we look at the amount of time *G. incognitus* individuals spent swimming close to the predator, a clear reduction of activity was observed in presence of the predator compared to the pre-stimuls period for the AA treatment (i.e., when the fish were collected from the site with Ambient condition 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. This reduction in the activity levels in the AA treatment may represent an antipredator strategy adopted by this species to reduce their risk to be predated. In fact, in a risky situation due to the presence of a predator (i.e. during post stimulus) individuals of *G. incognitus* may potentially respond with a reduction of the activity (or an increase of resting) interpreted as a "freezing type" behaviour, or instead moving far from predator. This freezing behavior is well described for several goby species (Smith et al., 1989) exposed to a predatory risk in natural condition and may represent a strategy to increase the chance of not being recognized by the predator (Paul et al., 2018; Smith et al., 1989; Larson & McCormick, 2005; McCormick & Larson, 2007; Manassa & McCormick, 2012). In this study, fish from the AA treatment reduced their activity levels in the proximity to the predator side that was equally allocated (about 50%) between the percentage of time fish remains immobilized (resting) in the sector of the arena close to the predator tank and the percentage of time fish spent (actively moving or resting) in the sector far away from the predator. This suggests that individuals of *G. incognitus* may independently choose between freezing in proximity of the predator or moving far from it, thus reducing predation risk. However, in the other CO_2 treatments (AH, HA and HH) the fish mostly prefer to stay away from predator rather than exhibiting a "freezing" behaviour, as instead observed in fish reared in ambient condition (AA). These results support previous findings showing that fish under elevated CO_2 conditions display bold behaviours (Munday et al. 2010, 2012, 2013 Cattano et al., 2019) and avoid freezing strategy (e.g. Porteus et al., 2018; Näslund et al., 2015). Nevertheless, individuals of *G. incognitus* living in proximity of the CO_2 vents appear to be adapted, rather than acclimatized, to elevated pCO_2. This deducibile from the fact that once removed from elevated pCO_2, their anti-predator behaviour do not return to a 'normal' state: i.e. comparable with that of individuals from ambient CO_2 areas (AA). These results, along with an increased density of fish under acidified condition, may suggest that, despite the unwillingness of adopting the freezing strategy, other potential antipredator mechanisms (e.g. moving far away from the predator) may favour survival in fish chronically exposed to elevated CO_2.

The density of predators in the environment may affect the behavior, and ultimately the population density of a given prey (Kusch et al. 2004; Nagelkerken et al., 2015; Ferrari et al., 2017). Along the Vulcano CO_2 gradient, a different number of predators may lead to different predation risk levels with consequences on *G. incognitus* density in the two CO_2 sites. 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. This suggests that the behavioural responses observed in this study may not be driven by intrinsic-differences in predation risk between CO_2 sites.

Other factors like the different availability of shelters (e.g. anemones) in the two CO_2 sites may potentially affect responses. In this regard, goby fish from the different CO_2 sites exhibited 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. This finding contrasts with previous long-term CO_2 exposure experiments conducted both in the laboratory (e.g. Cattano et al., 2019; Munday et al., 2013) and in fish living in natural CO_2 seeps (Munday et al., 2014; Nagelkerken et al., 2015). However, similarly to our results, no differences in sheltering behaviour was observed in a temperate fish *Gasterosteus aculeatus* under elevated pCO_2 (1000 µatm), suggesting that a response of fish to increased CO_2 levels may not be ubiquitous among fish (Näslund et al., 2015). Interestingly, our experiment also documented no differences in the minimum approach distance from the anemone among *G. incognitus* individuals measured before and after the predator presentation in the different CO_2 treatments. Such finding is consistent with a study showing that the distance from the shelter kept by the damselfish *Pseudochromis fuscus* was not affected by elevated seawater CO_2

concentration (Cripps et al., 2011). By contrast, recent evidences reported lower risk perception under elevated CO_2 conditions in terms of increased distance ventured from shelter both in tropical fish species (Cattano et al., 2019; Munday et al., 2010, 2012).

Here, we found a greater density of the sea anemone *Anemonia 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 and proximity to the vent site along the pH/pCO_2 gradient enabled shifts towards an increased cover of habitats main utilized by *Gobius bucchichi* (as *G. incognitus* in this study)*;* and that this habitat effects might be larger than that of other CO_2-mediated effects such as the escape behaviour (Nagelkerken et al., 2015). Here we add on these observations, revealing that the higher densities of *G. incognitus* we recorded under elevated CO_2 levels are related to the higher density of the sea anemone *A. viridis*, hence supporting one of the most uncontroversial response of coastal fishes to OA, i.e. the changing habitat provisioning effect (Sunday et al., 2017; Cattano et al., 2020). In this regard, previous researches 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).

In addition to this, we speculate that the increased abundance of the goby fish in the elevated CO_2 site may 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 showed that most of the anti-predator responses of *Gobius incognitus* were largely unaffected by elevated CO_2. When looking at activity levels recorded close to the predator, we show that fishes experiencing chronical (HH, HA treatments) or short-term (AH treatment) exposure to elevated CO_2 levels exhibited a similar response between pre- and post-predator presentation. However, the maintenance of this impaired antipredator behaviour when individuals living under elevated CO_2 condition were moved to smbient CO_2, may suggest that specific behaviour (e.g., freezing) may be not relevant for the species survival from predation, and that other mechanisms could be at play under OA conditions.

Overall, the absence of evident effects under elevated CO_2 conditions, combined with the higher population density of *G. incognitus* in this site, may indicate behavioural plasticity and adaptive potential of the species toenvironmental change (e.g. Allan et al., 2014; Calosi et al., 2016; Petit-Mart et al., 2021). The fish's 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). Thus, individuals of *G. incognitus* living near CO_2 seeps could benefit from the indirect effect of OA (e.g. habitat provisioning, food availability) which in turn may have favoured species tolerance to a reduced pH environment through behaviour acclimatization. Additional mechanisms also exist that could help explain the largely unaffected behaviour of this goby fish when exposed to ocean acidification." 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 by using multiple senses to detect its 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 the lack of response to the other (Goldenberg et al., 2018).

Behavioural mechanisms underlying the adaptation to ocean acidification are still little known in wild fish populations. In this direction, this study underlines the priority to improve the amount of research carried out in natural CO_2 vents, which may contribute towards improving our ability to predict the mechanisms involved in the short and long-term adaptation of marine species to ocean acidification.

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Supplementary material

Chapter 3: Ocean Acidification alters the reproductive behavior of a nesting wrasse: evidence from in situ observations off temperate CO_2 seeps

List of contents:

Supplementary methods

DNA Barcoding of the study species

A very small fin clip piece was carefully collected from two specimens of *Gobius* sp. from the study site, without killing or seriously damaging the animals, and preserved in 97% ethanol at -20C. The DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN), following the manufacturers' protocol. A ~167bp fragment of mitochondrial 12S rRNA was amplified using the "tele02" primer pair (Taberlet et al., 2018). The PCR mix had a total volume of 20 µl, composed by 10 µl Amplitaq Gold Master Mix (Thermo Fisher Scientific), 0.16 µl BSA, 1 µl of 5 µM forward primer, 1 µl of 5 µM reverse primer, 10 ng of eDNA template and 5.84 µl of molecular biology grade water. The thermocycler profile included an initial denaturing step of 94 ˚C for 10 min, 35 cycles of 94 ˚C 1 min, 54 ˚C 1 min and 72 ˚C 1 min and a final extension step of 72 ˚C for 5 minutes. The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN) and sequenced in a ABI 3730XLs.

Sequences were edited with BioEdit v.7.2 (Hall et al., 2011), and genetic divergence (Kimura-2-parameter, K2P) with four *G. bucchichi* 12S rRNA sequences (kindly provided by Dr. Radek Šanda) was calculated in MEGAX (Kumar et al., 2018). The K2P genetic distance between our specimens and *G. bucchichi* ranged from 7.97% to 8.15% (Table S1), clearly separating the two species. A Neighbour-joining tree estimated using the K2P distance is reported in Figure S1. The obtained study species' 12S rRNA sequences were deposited in GenBank (Acc. Numbers: MT903914-15).

Table S1 Kimura-2-parameter (K2P) genetic divergence among the study species and Gobius bucchichi

▨	**J90_Tele02_F *G. bucchichi***	**J92_TELE02_F *G. bucchichi***	**RK2_TELE02_F *G. bucchichi***	**RK46_TELE02_F *G. bucchichi***	**MT903914.1 *G. incognitus***	**MT903915.1 *G. incognitus***
J90_TELE02_F *G. bucchichi*	▨					
J92_TELE02_F *G. bucchichi*	0.00855	▨				
RK2_TELE02_F *G. bucchichi*	0.00000	0.00855	▨			
RK46_TELE02_F *G. bucchichi*	0.00000	0.00855	0.00000	▨		
MT903914.1 *G. incognitus*	0.07970	0.08148	0.07970	0.07970	▨	
MT903915.1 *G. incognitus*	0.07970	0.08148	0.07970	0.07970	0.00000	▨

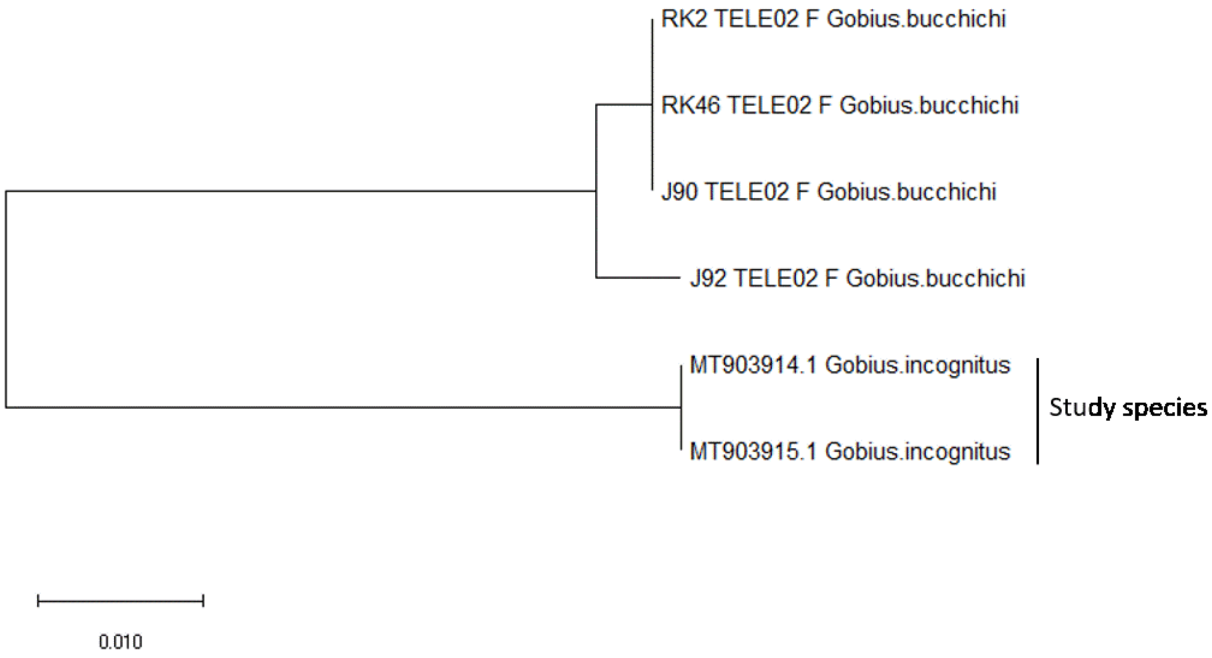

Figure S1 Neighbour-joining tree estimated using the K2P model of sequence evolution on the 12S rRNA sequences of Gobius bucchichi and the species object of the present study.

Table S2 Summary of the carbonate chemistry measured at the two nesting sites with different pCO$_2$ conditions (high pCO$_2$ and ambient pCO$_2$) of Vulcano Island (year 2019). Multiple measurements of Salinity, Temperature (T°C), pH and pCO$_2$ were made daily in each nesting site (between 10:00-17:00 h) both on different days before and on the same day of the behavioural observations. A fixed Total Alkalinity of 2500 µmol kg^{-1} (not measured in this study) was used for calculation with the software CO$_2$SYS

Data	Locality	Site	Salinity	T °C	pH	pCO$_2$
June-19	Vulcano	Amb. CO$_2$	37.8	23.14	8.112	341.6
June-19	Vulcano	Amb. CO$_2$	37.7	23.42	8.066	390.0
July-19	Vulcano	Amb. CO$_2$	37.8	23.27	8.056	401.1
July-19	Vulcano	High CO$_2$	37.8	23.19	7.853	702.1
June-19	Vulcano	High CO$_2$	37.8	23.58	7.695	1063.2
July-19	Vulcano	High CO$_2$	37.7	23.36	7.823	761.0
					Ambient (n= 3)	High CO$_2$ (n=3)
				Overall pCO$_2$(mean ± SE)	377.57 (±18.27)	842.1(±111.85)

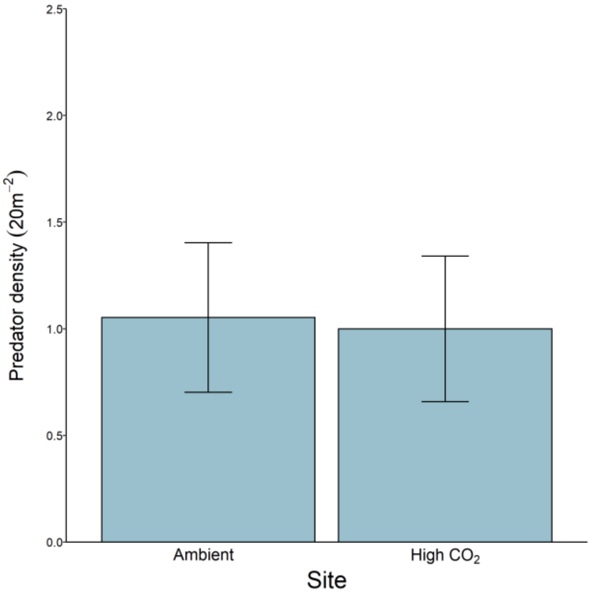

Figure S2 Bar plots of the average density (± CI) of the predator Serranus scriba in the two different CO$_2$ sites at Ambient and High CO$_2$.

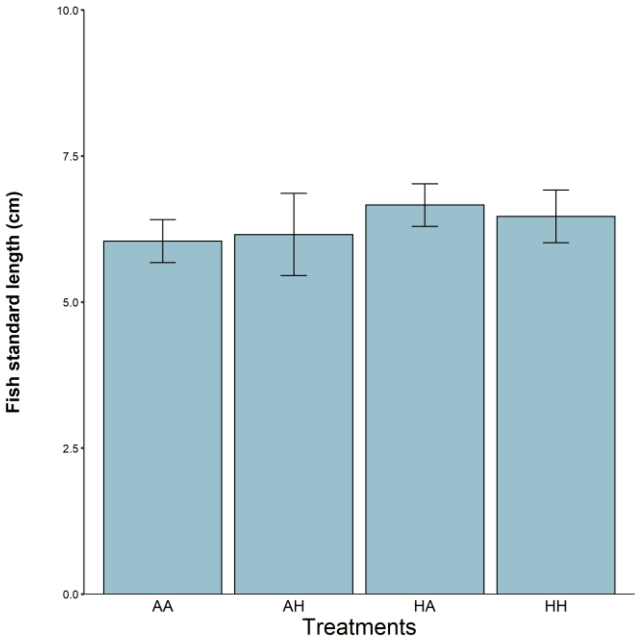

Figure S3 Bar plots of the average length (±CI) of G. incognitus individuals between the different CO_2 *treatments (AA, AH, HA, HH).*

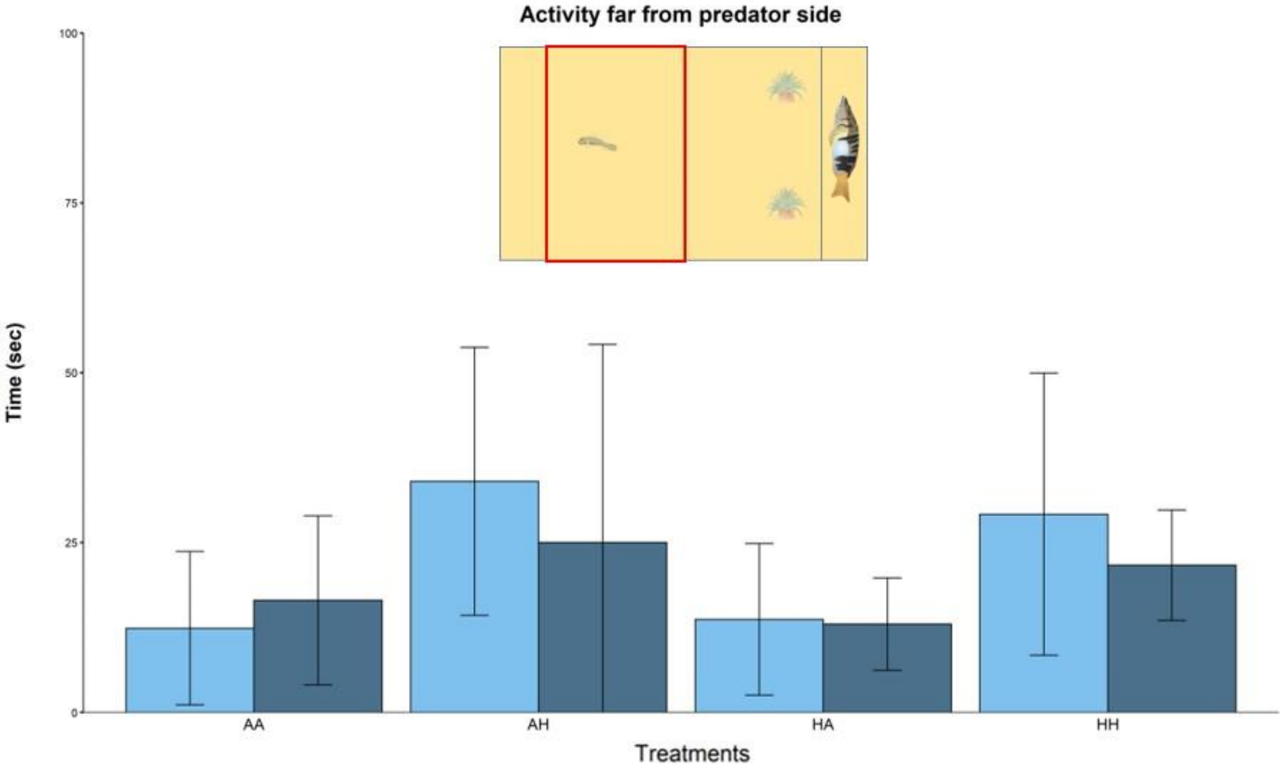

Figure S4 Mean values (± C.I.) of: activity close to predator side for individuals of G. incognitus before (pre-stimulus) and after (post-stimulus) the presentation of the predator S. scriba in the different CO_2 *treatments.*

Supplementary References

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Chapter 5 – Concluding remarks and future directions

By examining different species models and using lab and field experiments, this thesis contributes to the ongoing debate over the role of behaviour in marine ectotherms as a coping strategy in response to rapid environmental change. To date, the existing literature focussing on this topic give mixed results underlining the difficulty to make general considerations on species ability to respond to ocean warming and acidification. This heterogeneity suggests that the responses to rapidly environmental change are species-specific and may depend on the different species sensitivity or to local adaptations to habitat conditions. The overall aim of this dissertation was to improve our knowledge on the potential effect of OW and OA on the behaviour of marine invertebrate and fish focussing on different aspects (e.g. reproduction, embryonic development, prey-predator interactions) through laboratory (i.e. for *O. labronica*) and field-based experiment (*S. ocellatus* and *G. incognitus*).

In this context, increasing our knowledge on how behaviour thathelp different species to face with future ocean conditions is a high priority to better understand species ability to adapt to future ocean conditions.

In the second chapter I attempted to fill a knowledge gap on whether plasticity in parental care behaviours can mediate responses to elevated temperature in a widespread marine invertebrate. My findings showed that ocean warming will exert negative effects on the reproductive success of *O. labronica*. However, my results also suggest that under increased temperatures, this species may have evolved a behavioural strategy resulting in a reduction of energy/time spent for caring the eggs during a stage when embryos require less parental duties (i.e. the third phase of development). This reallocation of energy, also favoured by the reduction of the brood size under elevated temperature, might enable this species to maintain a positive cost-benefit trade-off between parents and offspring, with potentially benefit for parents' individual (i.e. growth) and species fitness (e.g. successive reproductive events) under increased temperatures. Understanding, whether organisms are able to cope with different temperatures is becoming crucial because climate change is generating increasingly unpredictable and extreme weather patterns. In fact, organisms that are unable to cope with these environmental changes may suffer decreased fitness, impacting the ability of populations to persist (DeWitt et al. 1998). In this context, the second chapter of this thesis furthers the understanding of how parental care contributes to plastic strategies to cope with the challenges that arise in changing environments. Future investigation on behavioural plasticity should incorporate transgenerational experiment (i.e. plasticity across generations) which may be crucial to better understand long-term evolutionary adaptation of the species to novel environments. Therefore, here I made an attempt in contributing to fill the wider knowledge gap existing for marine ectotherms' ability to adjust specific reproductive-related behavioural responses (i.e. parental care) to cope with rapid environmental change. For these reasons, this thesis also identifies a critical need to increase the number of experiments focussing on behavioural traits, which may be the most powerful ways that animals can adjust to rapid climate change (Wong & Candolin, 2015).

Chapters 3 and 4 of this thesis were addressed to increase our knowledge on fish behavioural response to OA through field-based experiments. To date, most of the studies investigating the effect of high CO_2 on fish behaviour has been conducted under controlled laboratory conditions and showed highly variable sensitivities among species, potentially underestimating the possible future impact of this environmental change. Working on natural CO_2 vents, as I did in the two field-based experiments assessing behavioural responses of fish to OA, may represent an important approach to better understand longer-term and community-level responses under future projected conditions. However, at the present only very few experiments have been conducted in such locations (Munday et al. 2014; Nagelkerken et al. 2015; Cattano et al. 2016; Milazzo et al. 2016), while the majority of OA studies on fish and other taxa consisted in laboratory experiments which may potentially underestimate the complex species interactions and community structure (Wittmann & Pörtner, 2013; Calosi et al., 2013; Petit-Mart et al., 2021). In this direction, much progress has been made in designing experiments to assess how elevated pCO_2 can alter fish and invertebrate behaviour, to date we are still far from understanding adaptive capacity to OA (Munday et al. 2013; Abram et al., 2016). However, the use of different approaches in designing experiments (e.g. laboratory, mesocosm and natural analogues) may represent an opportunity to integrate various types of data in order to better forecast effects of future climate on marine organisms and communities (Nagelkerken & Munday, 2016).

The results of the Chapter 3 showed that the ocellated wrasse nesting males significantly reduced the total time spent for parental care, and specifically the guarding behaviour at nests exposed to high CO_2 compared with nests at ambient-CO_2 levels. Despite this, a time budget reallocation between guarding and specific behaviours such as courting and wandering around was found in this species. These results show for the first time OA effects on multiple behaviours and underline the role of behavioural adjustments as potential mechanisms to buffer the impacts of ongoing environmental changes. These findings diverge from what was observed in previous experiments carried out in aquaria showing no effect of high levels of CO_2 on all the reproductive behaviour investigated (e.g. fanning, courtship and nest building) (Sundin et al., 2017; Lopes et al., 2020) further adding on previous evidence that behavioural responses to OA can be species-specific.

In the Chapter 4, I showed that most of the behaviours investigated in a common goby fish with a tiny home range translocated to different CO_2 conditions and exposed to combined stimuli (i.e. the odour and the sight) of a predator did not differ among treatments. Moreover, I observed also differences in the density patterns of *G. incognitus* in the elevated CO_2 sites, which were by far more abundant under this condition. Overall, these results suggest the existence of long-term adaptation of this species to the high CO_2 environments (e.g. Allan et al., 2014; Calosi et al., 2016; Petit-Mart et al., 2021). In a more general context, investigating behavioural responses (e.g. antipredator behaviour) of fish living under reduced pH/high-CO_2 environment (e.g. off volcanic seep sites) may contribute to better understand how marine species might adapt to environmental conditions under projected ocean scenarios. Although forecasting responses of marine organisms to ongoing environmental change may be difficult, this thesis highlights the need for a better understanding of the role of behaviour in shaping individuals and species' responses to changing environmental conditions. Indeed, behavioural adjustments can also influence evolutionary processes, by determining which individual can survive and reproduce under changing conditions, and thereby altering selection acting on specific traits. I speculate such behavioural changes can have long-term effects on the viability and evolution of populations.

As the first response by animals to a changing environment is predominantly through modification of their behaviour (Tuomainen & Candolin, 2011), the downstream consequences of the observed behavioural and activity effects are expected to affect both ecological performance (e.g. related to predator-prey dynamics, reproduction, homing, habitat choice and recruitment success) and therefore to affect the outcomes at community level (e.g. Nagelkerken and Munday 2016). In this direction, my findings may indicate that the observed behavioural adjustments involving important reproductive behavior (i.e. parental care) or predator-prey interaction may lead to increased organisms tolerance, therefore enhancing the ability of a species to persist in a given community.

In this context, mechanisms of acclimation and adaptation may have significant consequences for how marine organisms will respond to future ocean condition (Sunday et al. 2014). However, some studies suggest that organisms adaptation in a rapid changing environment may be too slow for long-lived species (Pörtner et al. 2014). Although much progress has been made in designing experiments to assess how elevated pCO_2 and temperature can alter fish and invertebrate performance, quantitative generalisations on the role of behaviour in modulating organisms responses are not yet possible, given the small number of experiments carried out on tolerant, low mobile species. For instance, as here presented species exhibiting small home ranges and chronically exposed to high CO_2 levels, could display behavioural adjustments, that may further favour potential acclimation ability and adaptation. Future research efforts should be addressed to such species model, assessing behaviours and unlike previous laboratory investigations, be aimed at describing their behaviour in wild conditions. This kind of investigations might contribute to a deeper understanding of fish responses to rising CO_2 and temperature, highlighting if populations chronically exposed to high-CO_2 conditions show plasticity mechanisms that could encompass epigenetics and transgenerational effects, ultimately enhancing species tolerance.

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