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6 **seeps**

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39 **Nest guarding behaviour of a temperate wrasse differs between sites off Mediterranean CO<sub>2</sub>**  
40 **seeps**

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61

62 **Abstract**

63 Organisms may respond to changing environmental conditions by adjusting their behaviour (i.e.,  
64 behavioural plasticity). Ocean acidification (OA), resulting from anthropogenic emissions of carbon  
65 dioxide (CO<sub>2</sub>), is predicted to impair sensory function and behaviour of fish. However, reproductive  
66 behaviours, and parental care in particular, and their role in mediating responses to OA are presently  
67 overlooked. Here, we assessed whether the nesting male ocellated wrasse *Symphodus ocellatus* from  
68 sites with different CO<sub>2</sub> concentrations showed different behaviours during their breeding season. We  
69 also investigated potential re-allocation of the time-budget toward different behavioural activities  
70 between sites. We measured the time period that the nesting male spent carrying out parental care,  
71 mating and exploring activities, as well as changes in the time allocation between sites at ambient  
72 (~400 µatm) and high CO<sub>2</sub> concentrations (~1000 µatm). Whilst the behavioural connectance (i.e.,  
73 the number of linkages among different behaviours relative to the total amount of linkages) was  
74 unaffected, we observed a significant reduction in the time spent on parental care behaviour, and a  
75 significant decrease in the guarding activity of fish at the high CO<sub>2</sub> sites, with a proportional re-  
76 allocation of the time budget in favour of courting and wandering around, which however did not  
77 change between sites. This study shows behavioural differences in wild fish living off volcanic CO<sub>2</sub>  
78 seeps that could be linked to different OA levels, suggesting that behavioural plasticity may  
79 potentially act as a mechanism for buffering the effects of ongoing environmental change. A  
80 reallocation of the time budget between key behaviours may play a fundamental role in determining  
81 which marine organisms are thriving under projected OA.

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## 87 **1. Introduction**

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

97 Increased uptake of anthropogenic CO<sub>2</sub> by the oceans, namely ocean acidification, represents  
98 one important global process that can affect the survival, calcification, growth, development and  
99 reproduction of marine organisms as well as alter the behaviour of fish (Cattano, et al., 2018; Gaylord  
100 et al., 2015; Heuer & Grosell, 2014; Nagelkerken & Munday, 2016; Kroeker et al., 2013). Several  
101 studies have reported fish behavioural effects and sensory disruptions due to elevated CO<sub>2</sub> conditions  
102 involving olfaction and audition functions, lateralization, activity levels, learning and decision  
103 making (Munday et al., 2009; Dixson et al., 2010; Simpson et al., 2011). The reported behavioural  
104 effects on some coral reef fish have recently been questioned (Clark et al., 2020), and a large debate  
105 over experiments' reproducibility in fish behavioural studies is presently underway (Clark et al.,  
106 2020; Munday et al., 2020; Williamson et al., 2020). Although fish are efficient acid-base regulators  
107 (Esbaugh et al., 2012; Pörtner et al., 2004), an increase in the energy used to maintain acid-base  
108 balance due to high CO<sub>2</sub>/low pH conditions may affect the amount of energy available for other  
109 activities, including reproduction (Ishimatsu et al., 2008; Sokolova et al., 2012).

110 To date, the few laboratory studies on the effect of elevated CO<sub>2</sub> on reproduction have mainly  
111 focused on parental allocation to offspring and sperm production, suggesting variable and species-  
112 specific responses (Heuer & Grosell, 2014; Faria et al., 2018). Indeed, the cost of reproduction should

113 also encompass behavioural activities such as mate choice and acquisition (courting), nest building  
114 and defence, and parental care (Gillooly & Baylis, 1999; Husak & Swallow, 2011). In fish species  
115 that invest heavily in parental care, changes in the amount of energy for some behavioural activities  
116 under altered environmental conditions could lead parents to adjust their behaviour, ultimately  
117 affecting reproductive success. Changes in reproductive behaviours were documented under varying  
118 salinity (Mary et al., 2001), oxygen (Jones & Reynolds, 1999; Lissåker & Kvarnemo, 2006; Reeb et  
119 al., 1984) and temperature (Skolbekken & Utne-Palm, 2001) levels. However, shifts in behavioural  
120 activities during reproduction are critically understudied in relation to the effects of high CO<sub>2</sub>  
121 concentrations on fish and other marine organisms (Gaylord et al., 2015; Nagelkerken & Munday,  
122 2016).

123 A few studies investigated ocean acidification (OA) effects on the reproductive behaviour of  
124 fish both in controlled laboratory conditions and in the wild. In a laboratory experiment, Sundin et al.  
125 (2017) did not detect any CO<sub>2</sub> effect on the nest building activity, courtship and fanning of the three-  
126 spined stickleback *Gasterosteus aculeatus* (Linnaeus 1758). Similarly, no CO<sub>2</sub> effects were detected  
127 on the time males of two-spotted goby *Gobiusculus flavescens* (Fabricius, 1779) spent on differential  
128 parental care behaviours, with this male investment being mostly affected by increased temperature  
129 in a 2×2 (CO<sub>2</sub> × T) factorial experiment carried out in lab conditions (Lopes et al., 2020). In the first  
130 study carried out in the wild so far, no differences in many mating behaviours were detected, but the  
131 number of pair spawning events with females by the nesting male ocellated wrasse *Symphodus*  
132 *ocellatus* was reduced by almost two thirds in areas at high CO<sub>2</sub> levels near volcanic seeps (Milazzo  
133 et al., 2016). However, nesting male paternity was maintained (Milazzo et al., 2016).

134 Here, we used well-established CO<sub>2</sub> gradients off seeps at Vulcano and Panarea Islands (Sicily,  
135 Italy) (Goffredo et al., 2014; Aiuppa et al., 2020), to investigate the potential effect of high seawater  
136 CO<sub>2</sub> concentrations on the behavioural activities of the nesting male *Symphodus ocellatus*. The  
137 potential time budget re-allocation between reproductive activities, with specific focus on parental  
138 care activities (e.g., fanning, nest maintenance, guarding and chasing) was also investigated.

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

146           Altered CO<sub>2</sub> concentrations may affect mating and parental care behaviours as well as the fish  
147 species performance in different ways, such as via impaired decision-making, behavioural disruption  
148 or altered energetic balance (Cattano et al., 2018; Heuer & Grosell, 2014; Nagelkerken & Munday,  
149 2016). In this specific context, we build on previous field experiments carried out along the Vulcano  
150 gradient that observed unaltered O<sub>2</sub> consumption and size at hatching of embryos from nests exposed  
151 to ambient and high CO<sub>2</sub> conditions (Cattano et al., 2016). Here we expect that to increase offspring  
152 survival and mitigate potential adverse CO<sub>2</sub> effects on embryos, the nesting male ocellated wrasse  
153 might increase the amount of time spent on parental care, altering behaviours such as guarding and  
154 chasing off nest predators and sex competitors (i.e., nest defence) or fanning laid eggs (i.e., increasing  
155 oxygen supply for embryos). To support this hypothesis, we assessed the potential OA effects on the  
156 ocellated wrasse behaviour, assessing their time spent for parental care, mating and exploring  
157 activities in two sites along the Vulcano pH/pCO<sub>2</sub> gradient. We expect that any change in a given  
158 behavioural activity under elevated CO<sub>2</sub> would result in a time/energy compensation towards other  
159 behaviours aimed at maintaining the species fitness.

160           To achieve these goals, we recorded the number of male competitors (i.e. sneaker and satellite  
161 males), females and potential egg predators visiting each nest, as between-site differences in these  
162 variables may ultimately affect the nesting male behaviour (Alonzo & Warner, 2000). We also  
163 characterized the ocellated wrasse behaviour through Behavioural Network Graphs, and evaluated

164 potential differences in the time spent by nesting males on parental care, mating and exploring  
165 behaviours in multiple nests at ambient-CO<sub>2</sub> (400 μatm pCO<sub>2</sub>) and high CO<sub>2</sub> sites (1100 μatm pCO<sub>2</sub>).

## 166 **2. Materials and methods**

### 167 *2.1 Study site and carbonate chemistry*

168 This study was conducted in four different sampling surveys during the breeding season, which  
169 lasts from May to July (Lejeune, 1985; Taborsky et al., 1987), and specifically on: 18-20 May 2012,  
170 7-12 June 2013, 29 May-7 June 2014, 1-9 July 2018. Fish behaviour was observed at two CO<sub>2</sub> seeps  
171 at ambient temperature off the islands of Vulcano (Italy) and Panarea (Italy), both belonging to the  
172 Aeolian Islands Archipelago (NE Sicily, Italy). In these CO<sub>2</sub> sites, ocellated wrasse nests are naturally  
173 exposed to present-day and high-CO<sub>2</sub> conditions (electronic supplementary material, table S1). The  
174 high CO<sub>2</sub> and the ambient-CO<sub>2</sub> nesting sites in Vulcano Island were at approximately 450 m and  
175 approximately 800 m distance from the main seeping area, respectively (see Boatta et al., 2013;  
176 Aiuppa et al., 2020). The nesting sites located off the Island of Panarea were positioned close (i.e.,  
177 <5 m apart) and at a >50 m distance from a caldera at 10 m depth (Aliani et al., 2010; Goffredo et al.,  
178 2014). Gas emissions in both shallow seeps are 97–99% CO<sub>2</sub> (Boatta et al 2013; Aliani et al 2010).  
179 Seawater carbonate chemistry was characterized daily on several visits at the two nesting sites along  
180 the Vulcano Island gradient in 2012 (n = 22), 2013 (n = 24), 2014 (n = 12), 2018 (n = 6) and at  
181 Panarea in 2014 (n = 5; see electronic supplementary material, Table S1 for details). A 556 MPS YSI  
182 (Yellow Springs, USA) multiparametric probe was used to measure salinity and pH and temperature.  
183 The sensor was calibrated using the same procedure carried out in the same site study by Milazzo et  
184 al. (2016). Total Alkalinity (TA) was calculated from the Gran function applied to pH variations from  
185 4.2 to 3.0, from the slope of the curve HCl volume versus pH. TA measurements were corrected using  
186 standards provided by A. G. Dickson (Scripps Institution of Oceanography, USA; batch 99 and 102).  
187 The pCO<sub>2</sub> levels were calculated from pH<sub>NBS</sub>, TA, temperature and salinity with the free-access CO<sub>2</sub>

188 SYS package (Pierrot et al., 2006), using the constants of Roy et al. (Roy et al., 1993) and Dickson  
189 (Dickson, 1990).

## 190 2.2 *Study species*

191 *Symphodus ocellatus* is a widespread Mediterranean coastal wrasse found in shallow rocky and  
192 seagrass habitats (Taborsky et al., 1987). During the breeding season (May-July; Lejeune, 1985;  
193 Taborsky et al., 1987), large colourful nesting males (81–95 mm, TL) build algal nests where they  
194 attract small females to spawn (35–75 mm, total length, TL) and compete with two alternative male  
195 reproductive types to fertilize the eggs (Warner & Lejeune, 1985). During the breeding season the  
196 nesting males go through multiple nest cycles (each lasting on average 8–10 days) (Lejeune, 1985),  
197 during which they build and defend a nest, spawn for 3–4 days, and provide obligate care for 3–5  
198 days before hatching. Females are non-territorial, do not participate in nest-building or parental care,  
199 and visit multiple nests where they lay dozens of eggs by brushing their genital papillae against algae  
200 (Taborsky et al., 1987). Two other types of males, i.e., the satellite and the sneaker (Taborsky et al.,  
201 1987), attempt to parasitically spawn in the nests of the dominant male (Fiedler, 1964; Lejeune, 1985;  
202 Šoljan, 1930; Taborsky et al., 1987). Sneakers (35–60 mm, TL) hover around various nests and try  
203 to join the female and nesting male during spawning, releasing large quantities of viable sperm  
204 without providing parental care activity (Alonzo & Warner, 2000). Satellite males (61–80 mm, TL)  
205 help nesting males in reducing sneaking events and courting females, getting a share of paternity as  
206 a result (Stiver & Alonzo, 2013). Nesting males also actively defend the nest from potential egg  
207 predators which are often congeneric and other fish potentially preying on eggs in nests (mostly other  
208 wrasse species; Lejeune et al. 1985; Taborsky et al., 1987; Alonzo, 2004).

## 209 2.3 *Video collection*

210 The behavioural observations were collected through replicated 10-min videos on different  
211 surveys over the study period. Nests of *S. ocellatus* were randomly selected among those in the



212 spawning phase and filmed in ambient (n = 16) and high CO<sub>2</sub> (n = 15) conditions off Vulcano and  
213 Panarea. The same nest (and nesting male) was filmed only once, with observations typically  
214 occurring on the same days for both ambient and high CO<sub>2</sub> nests along the CO<sub>2</sub> gradients. All of the  
215 nests were filmed in rocky habitat at 3–4 m depth between 10:00 a.m. and 4:00 p.m., when light  
216 intensity and spawning activity were highest (Milazzo et al., 2016). Each replicate of 10 min video  
217 were made with a GoPro 3 camera placed on a tripod at one meter away from the nest. Water visibility  
218 (always exceeding 10–15 m) and tidal ranges (between 20 and 40 cm) were similar in the two nesting  
219 sites along the gradients off Vulcano and Panarea Islands.

#### 220 2.4 Preliminary analyses

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

228 Given that the nesting male behaviour (e.g. guarding and chasing activities) may be affected by  
229 the presence of fish potentially preying on eggs in ocellated nests, we recorded the maximum number  
230 of fish egg predators (i.e. *Coris julis*, *Symphodus mediterraneus*, *Symphodus roissali*, *Symphodus*  
231 *tinca* and *Thalassoma pavo*) within a 1-m radius from the nest in both CO<sub>2</sub> sites for each 10-min  
232 video (n= 16 at ambient pCO<sub>2</sub>, n=15 at high pCO<sub>2</sub>). No event of egg predation was recorded in any  
233 video. No other potential egg predators (e.g. invertebrates) were observed in proximity to the nest in  
234 all the videos analysed.

235 In addition, the densities of the egg predator species were recorded in the two nesting sites at ambient  
236 and high pCO<sub>2</sub> using standard linear transects (Harmelin-Vivien et al. 1985) performed on June 2013

237 (n=6 for each site). Each transect was randomly positioned on rocky substrata covered by brown  
238 macroalgae, at 1-3 meters depth and was conducted by an observer swimming for 25 m while  
239 counting all the fish encountered within 1 m either side of the transect line (50 m<sup>2</sup>) (Harmelin-Vivien  
240 et al. 1985). Counts were conducted in the morning from 09.00 to 14.00 h, to avoid potential within-  
241 day variability which is particularly evident for coastal labrid (Willis et al. 2006). Water visibility  
242 exceeded 20 m for all counts.

### 243 *2.5 Behavioural analyses*

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

253 Behavioural network graphs or kinematic diagrams were constructed to describe the overall  
254 organizational pattern (e.g., frequencies of occurrence from one activity to another, the time spent  
255 performing each behaviour or time-budget, and the density of linkage in the network or connectance  
256 (*sensu* Martinez, 1992) of the ocellated wrasse following the different steps proposed in Brockmann  
257 (1984). After the definition of a general ethogram (as described before), the number of times (or  
258 occurrences) that one behaviour followed another were calculated to build a matrix (*transition matrix*;  
259 *sensu* Brockmann, 1984). This matrix was used to create two behavioural network graphs for each  
260 CO<sub>2</sub> site, showing the behavioural flow. The different behavioural activities were represented by  
261 nodes whose size represents the average time spent in that particular behaviour. The connections

262 between nodes are represented by links (or edges), whose width represents the specific number of  
263 occurrences between single activities (e.g from fanning to guarding). The behavioural network graphs  
264 were represented using the package igraph in R software (Csárdi & Nepusz, 2005). The connectance  
265 – i.e., the number of linkages between different activities divided by the total amount of linkages  
266 possible – was measured to assess the potential differences in the density of interactions of the  
267 organizational pattern (represented by the network graph) between the two nesting sites at ambient  
268 and high CO<sub>2</sub>.

## 269 *2.6 Statistical analyses*

270 We preliminary used linear models (lms) to assess the differences between sites in the log + 1  
271 transformed maximum and total number of sneakers and females, as well as in the number of egg  
272 predators (as log+1 transformed sum of maximum number of each species) considering “Nesting site”  
273 as a fixed factor with two levels (high CO<sub>2</sub> and ambient CO<sub>2</sub>). Similarly, potential differences in the  
274 presence or absence of satellite males between nesting sites were assessed by a binomial generalized  
275 linear-model (B-GLM), and comparing the reduction in deviance from the null model using a  
276 likelihood ratio-test.

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

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

283 To test the CO<sub>2</sub> effect on the time spent by the nesting males on behavioural categories and  
284 activities we used the multivariate linear models with the package mvabund (function manylm; Wang,  
285 Naumann et al., 2012) based on resampling (n=999). This procedure takes into account the correlation  
286 between response variables, thus improving the power of the statistical test (Wang et al., 2012;

287 Warton, 2011), and allows us to test both the multivariate and univariate hypothesis. As the data was  
288 comprised of behavioural categories and activities composition, a Centered Log Ratio (CLR)  
289 transformation was performed. (Boogaart & Tolosana-Delgado, 2013).

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

### 299 **3. Results**

300 The maximum and the total number of sneakers, females and egg predators, as well as the  
301 presence or absence of satellite males recorded in the nests (for each 10-min video) at Vulcano and  
302 Panarea Islands showed no differences between the two sites at high and ambient CO<sub>2</sub> (Table 1 and  
303 electronic supplementary material, Fig. S1). Similarly, the density of egg predators (no. of individuals  
304 50 m<sup>-2</sup>) was similar between sites (Table S3, Fig. S2).

305 The Behavioural Network Graphs (Fig. 1) showed no differences in the relative number of  
306 linkages (connectance) between behavioural activities observed in the two sites at different CO<sub>2</sub> levels  
307 (Table 1; Fig. S3 in supplementary material).

308 The overall time spent on the behavioural categories (i.e., parental care, mating and exploring)  
309 by the nesting males was not significantly different between sites when approached in a multivariate  
310 context (Table 1). However, the univariate analysis showed that the time nesting males displayed  
311 parental care activity was significantly lower at the high CO<sub>2</sub> site ( $281.73 \pm 52.01$  sec; mean  $\pm$  95%

312 Confidence Interval, CI) compared to the ambient conditions ( $378.19 \pm 51.16$  sec; Table 1; Fig. 2).  
313 In contrast, no significant differences were found in the time spent on the behavioural categories  
314 “mating” and “exploring”, between the nesting sites at ambient and high CO<sub>2</sub> (Table 1; Fig. 2).

315 Total time spent on the behavioural activities showed significant differences between CO<sub>2</sub> sites  
316 (Table 1). Univariate analyses performed on each activity showed that time spent guarding led the  
317 overall differences between CO<sub>2</sub> nesting sites (Table 1), being on average 152 sec. ( $\pm 40.84$  CI) and  
318 53.53 sec. ( $\pm 18.04$  CI) in ambient and high CO<sub>2</sub> sites, respectively (Fig. 3). No differences between  
319 high and ambient CO<sub>2</sub> nesting sites were observed when considering the other behavioural activities  
320 (Table 1; Fig. 3).

321 The maximum number of sneakers, females and egg predators did not affect (both in overall  
322 and univariate tests) the time spent on the behavioural categories and activities, which in turn were  
323 affected by the presence/absence of the satellite male (Table 1 and Table S4 in Supplementary  
324 material). Particularly, the presence of satellite males significantly increased the time that the nesting  
325 male spent on parental care independently from the CO<sub>2</sub> sites considered (Table 1 and Table S4 in  
326 Supplementary material). Similarly, the presence of the satellite significantly reduced the time spent  
327 on courting behaviour in both the nesting sites at ambient and high CO<sub>2</sub> (Table 1 and Table S3 in  
328 Supplementary material).

#### 329 **4. Discussion**

330 This study investigated the potential differences between sites in the behaviour of a wild wrasse  
331 breeding off two volcanic CO<sub>2</sub> seeps in the Western Mediterranean Sea. Our findings show that the  
332 ocellated wrasse nesting male significantly reduced the total time spent on parental care at nests  
333 exposed to high CO<sub>2</sub> compared with nests at ambient CO<sub>2</sub> levels. Guarding behaviour significantly  
334 decreased in nesting sites at high CO<sub>2</sub> levels, whilst other parental care activities (i.e., fanning, nest  
335 maintenance and chasing), mating (courting and spawning), and exploring behaviours (wandering  
336 around), did not show any differences between the two nesting sites. As a consequence of the reduced

337 guarding at nests in the high CO<sub>2</sub> site, nesting males re-allocated the time budget toward different  
338 behaviours, such as courting and wandering around (although they did not significantly differ  
339 between sites).

340 Previous OA studies in laboratory and natural conditions have considered only a small number  
341 of behavioural activities at the same time (Milazzo et al., 2016; Sundin et al., 2017; Lopes et al.,  
342 2020), with this likely underestimating the potential energy/time reallocation towards other keys  
343 behaviours. Aquaria experiments showed no differences in some activities such as fanning, courtship  
344 and nest building in the three-spined stickleback *Gasterosteus aculeatus* (Sundin et al., 2017) and in  
345 the two-spotted goby *Gobiusculus flavescens* (Lopes et al., 2020). Similarly, no effects of elevated  
346 CO<sub>2</sub> on the time spent in courtship behaviour and on the number of chasing events were observed for  
347 the ocellated wrasse at the Vulcano CO<sub>2</sub> seep site (Milazzo et al., 2016).

348 Due to the multiple types of behaviours considered in this study during the breeding season  
349 of the ocellated wrasse, we used a behavioural network graph to represent the overall behavioural  
350 patterns of this species at the two nesting sites. Despite a different reallocation of the time budget was  
351 found between sites, a similar number of interactions among different behaviours emerged by the  
352 analysis of the connectance, thus suggesting that the ocellated wrasse nesting male is able to maintain  
353 the same overall number of connections among different behavioural activities independently to the  
354 different level of CO<sub>2</sub> at the two nesting sites. However some interesting patterns emerged when  
355 looking at behavioural differences between sites at category (i.e., parental care, mating, exploring  
356 behaviours) and activity (i.e., fanning, guarding, nest maintenance, chasing, spawning, fertilization,  
357 and wandering around) levels, also considering the potential effects of conspecifics and other egg  
358 predators in the nesting sites.

359 The presence/absence or the abundance of females, sneakers, and satellite males did not differ  
360 between nesting sites exposed to different CO<sub>2</sub> levels. There is evidence that the abundance of female  
361 and accessory males around nests may affect in different ways the behaviour of the ocellated wrasse  
362 nesting male during the reproductive season (e.g. Alonzo & Warner, 2000; Alonzo, 2004). For

363 instance, the presence and the abundance of females at a nest can make it more attractive to sneaker  
364 males, consequently requiring the nesting male to allocate more time to guarding activity to minimize  
365 the risk of sperm competition (Alonzo & Warner, 2000; Milazzo et al., 2016). Here we found that all  
366 the behavioural responses considered were unaffected by the number of females and sneaker males  
367 around nests. In addition to this and as expected, the presence of the satellite significantly increased  
368 the time spent by the nesting male on parental care. This was independent from the CO<sub>2</sub> site  
369 considered and in accordance with theoretical predictions and previous empirical studies (Houston et  
370 al. 2005; Seki et al. 2007; Kokko & Jennions 2008; but see Alonzo & Heckman, 2010) reporting an  
371 increase of the male care as a function of the reduced risk of sperm competition, which in turn is  
372 favoured by the presence of the satellite males (Stiver & Alonzo, 2013). When the satellite was  
373 present, we specifically observed a significant reduction of the courtship, which this being consistent  
374 with previous work on the same species, supporting evidence that nesting males partnered with a  
375 satellite may invest less in courtship when helped by satellites in doing so (Stiver & Alonzo, 2013;  
376 Nugent et al., 2016). In this and other previous studies, the presence of the satellite did not affect the  
377 time spent for the guarding behaviour (Taborsky 1994; Stiver & Alonzo, 2013).

378         The significant reduction of the guarding behaviour observed in the nesting sites at high CO<sub>2</sub>  
379 concentration, might be a consequence of a lower risk of egg predation in the ocellated wrasse nests  
380 by other labrid species, as predicted in previous studies on different fish species (Ongarato & Snucins,  
381 1993; Gravel & Cooke, 2009-2013). However, our findings showed that the density and the maximum  
382 number of egg predator species were similar between the two nesting sites and did not affect any  
383 behavioural category and activity of the ocellated wrasse nesting male.

384         Given that, we speculate the observed decline of the amount of time spent guarding by nesting  
385 male ocellated wrasse under elevated CO<sub>2</sub> levels may be related either to a slower decision-making  
386 (Dixson et al., 2010; Ferrari et al., 2012; Jutfelt et al., 2013; Munday et al., 2010) or to a deliberate  
387 tendency of the nesting male to invest in other activities to ensure fitness (Faria et al., 2018). Although  
388 these mechanisms were not tested in this study, an altered decision-making ability under OA could

389 decrease the behavioural performance of the ocellated wrasse nesting male when facing multiple tasks  
390 (e.g., courting, guarding nests and chasing competing sneakers or nest predators) (Cattano et al., 2018;  
391 Nagelkerken & Munday, 2016). Previous observations for the same species suggest that when the  
392 risk of sperm competition is high under natural conditions, behavioural adjustments like increased  
393 guarding activity may occur to avoid further sneaker males approaching the nest (Alonzo & Warner,  
394 2000). A similar response was observed in the nesting damselfish *Chromis chromis*, whose males  
395 switch from courting to parental care activities when the perceived risk of cuckoldry is high in the  
396 wild (Mascolino et al., 2016).

397         Previous evidence using transplantation experiments of ocellated wrasse embryos revealed no  
398 differences in the O<sub>2</sub> consumption between embryos developing in nests exposed to ambient and high  
399 CO<sub>2</sub> conditions (Cattano et al., 2016). Here we found no differences in the fanning activity between  
400 the two nesting sites, with this rejecting our initial hypothesis of an increased fanning behaviour (i.e.  
401 increased oxygenation for embryos in the nest environment) to mitigate potentially adverse effects  
402 on embryo development and metabolism and thus guarantee offspring performance in the high CO<sub>2</sub>  
403 nesting sites.

404         Our findings might rather support a behavioural shift under elevated CO<sub>2</sub>, when a significant  
405 reduction in the proportion of time spent on guarding translated into a time budget reallocation  
406 towards other activities (e.g., courting and wandering around) increasing proportionally, but not  
407 differing between different CO<sub>2</sub> sites. According to previous studies, a lower time allocation to mate  
408 guarding may increase the risk of sperm competition as the probability of sneak spawns could be  
409 higher (Alonzo & Warner, 2000). At the same time, the observed proportional increase of time spent  
410 on courtship by the nesting male may favour pair spawns. However, here we found no differences in  
411 the spawning time between CO<sub>2</sub> sites, while elevated CO<sub>2</sub> levels did not affect the number of the  
412 ocellated wrasse sneak spawns but significantly reduced the number of pair spawns between nesting  
413 sites exposed to different CO<sub>2</sub> levels (Milazzo et al., 2016). This suggest that sneaker males may not  
414 take advantage from a potentially impaired dominant male behaviour. At present, the specific role of



415 the wandering around activity is not well established, as nesting males may leave the nest for several  
416 purposes (Taborsky et al. 1987).

417 Overall, by showing that OA will decrease parental care and guarding activity in the *S.*  
418 *ocellatus* nesting male, this study may contribute to emphasize the behaviour as a first strategy by  
419 which this widespread Mediterranean wrasse responds to environmental change. The present findings  
420 – along with previous research assessing the mating behaviour, the reproductive success, and the  
421 abundance of the Mediterranean ocellated wrasse along a CO<sub>2</sub> gradient – suggest this species may  
422 maintain fitness and population persistence under OA conditions (Milazzo et al., 2016; Cattano et al.,  
423 2016; Mirasole et al., 2020). Future research efforts should also consider the fundamental link  
424 between species behaviour and population responses under changing environmental conditions, for  
425 instance deepening the different processes and mechanisms underpinning tolerance to elevated CO<sub>2</sub>  
426 in marine populations, including transgenerational effects (Schunter et al., 2016; Tsang et al., 2020;  
427 Petit-Mart et al., 2021).

#### 428 **Credit author statement**

429 Spatafora Davide: Conceptualization, Methodology, Validation, Formal analysis, Investigation,

430 Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization

431 Quattrocchi Federico: Methodology, Formal analysis, Data Curation, Writing – Review

432 Cattano Carlo: Conceptualization, Methodology, Investigation, Writing – Review

433 Badalamenti Fabio: Conceptualization, Methodology, Validation, Formal analysis, Investigation,

434 Writing - Review, Supervision

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#### 437 **Declaration of Interest Statement**

438 The authors declare that they have no known competing financial interests or personal relationships  
439 that could have appeared to influence the work reported in this paper.

#### 440 **Tables and Figures captions**

441 **Table 1.** Summary of statistical analyses on the number of females and sneakers (total and maximum  
442 number), presence/absence of satellites males, maximum number of egg predators, connectance and  
443 behavioural time (category and single activities). Comparisons between the two nesting sites at high  
444 and ambient CO<sub>2</sub> are reported. The results for the time spent for different behaviours are presented as  
445 multivariate (overall test) and univariate analyses (in brackets). Only the significant effect of the  
446 presence/absence of satellite was reported for all analyses. Significant results are in bold. Full  
447 analyses are reported in Table S3.

448 **Fig.2.** Bar plots (mean ± CI) of the time nesting male spent on each behavioral category (e.g., parental  
449 care, mating and exploring) in the two different nesting sites at high and ambient CO<sub>2</sub>. The asterisk  
450 indicates significant differences ( $p < 0.05$ ).

451 **Fig.3.** Bar plots (mean ± CI) of the time nesting male spent on activities within each behavioural  
452 category in the two different nesting sites at high and ambient CO<sub>2</sub>. The asterisk indicates significant  
453 differences ( $p < 0.05$ ).

454

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