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Ocean acidification affects fish spawning but not paternity at CO₂ seeps

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28 **Abstract**

29 *Fish exhibit impaired sensory function and altered behaviour at levels of ocean acidification*
30 *expected to occur due to anthropogenic carbon dioxide emissions this century. We provide the first*
31 *evidence of the effects of ocean acidification on reproductive behaviour of fish in the wild. Satellite*
32 *and sneaker male ocellated wrasse (*Symphodus ocellatus*) compete to fertilize eggs guarded by*
33 *dominant nesting males. Key mating behaviours like dominant male courtship and nest defence did*
34 *not differ between sites with ambient versus elevated CO₂ concentrations. Dominant males did,*
35 *however, experience significantly lower rates of pair spawning at elevated CO₂ levels. Despite the*
36 *higher risk of sperm competition found at elevated CO₂, we also found a trend of lower satellite*
37 *and sneaker male paternity at elevated CO₂. Given the importance of fish for food security and*
38 *ecosystem stability, this study highlights the need for targeted research into the effects of rising*
39 *CO₂ levels on patterns of reproduction in wild fish.*

40

41 **Introduction**

42 Ongoing ocean acidification is an inevitable consequence of continued atmospheric CO₂
43 emissions and is expected to have profound effects on marine biodiversity and ecosystem function
44 [1]. Coping with the physiological stress of ocean acidification has metabolic costs that can impair
45 reproduction in a range of marine organisms, including fish [2,3]. Elevated CO₂ also causes striking
46 behavioural and sensory disruptions in fish, involving loss of lateralization and of learning ability,
47 reversal of olfactory and auditory functions, and impaired decision-making [4-8]. This
48 neurosensory impairment may have consequences on marine communities as increased levels of
49 CO₂ can cause juvenile fish to be attracted by predator odors they would normally avoid [5] or by
50 sounds of unfavorable habitats for settlement [7]. These effects can be rapidly reversed by
51 reducing GABA-A receptor activity [8], highlighting a link between increasing seawater CO₂ levels
52 and brain function in fish [9].

53 Successful reproduction will be essential for the persistence of marine populations as seawater
54 CO₂ levels continue to rise [10]. When cinnamon anemone fish (*Amphiprion melanopus*) parents
55 are exposed to high-CO₂ conditions their offspring do not show behavioural impairment, however
56 their potential for behavioural acclimation across generations is not fully restored, as in many
57 instances their escape performance is negatively affected [11]. Similarly, other studies showed
58 minimal potential for transgenerational acclimation in the tropical damselfish *Acanthochromis*

59 *polyacanthus* [12] and in several other tropical fish chronically exposed to elevated CO₂ at volcanic
60 seeps [13]. In aquaria, simulated ocean acidification increases the reproductive output of females
61 (e.g., egg production) in some fish [2,14] but not others [15]. Intense sperm competition has
62 resulted in male behavioural adaptations that may be affected by rising CO₂ levels. At present,
63 mating system structure and reproductive behaviour are critically understudied in relation to the
64 effects of high CO₂ on fish and other marine organisms [16]. Given the complex sexual behaviour
65 of many fish in the wild, there are concerns that mating behaviour and reproductive success might
66 be affected by ongoing rising CO₂ levels. The present study is the first to investigate this issue in
67 fish exposed to high CO₂ levels in their natural habitat.

68 We used a gradient in CO₂ near volcanic seeps off Vulcano Island (Italy) [17] to examine mating
69 behaviour and fertilization success of the ocellated wrasse *S. ocellatus* (figure 1). Ocellated wrasse
70 ‘dominant males’ build nests and provide parental care to eggs, attract females for pair spawning,
71 chase off sexual competitors (‘satellite’ and ‘sneaker’ males) [18], and sometimes refuse to mate
72 when other males are prevalent [19]. Satellite males cooperate with dominant males to attract
73 females and drive away sneakers, but they also engage in sneak fertilization of the eggs [20,21].
74 Sneaker males, which release more sperm per spawn than dominant nesting males or satellites, do
75 not cooperate or care and only attempt to sneak spawn [19]. Thus high levels of sperm
76 competition occur in the wild [20]. We filmed multiple wrasse nests at present-day Ambient-CO₂
77 conditions (~400 μatm pCO₂) and High-CO₂ conditions (~1100 μatm pCO₂) [22] (Electronic
78 Supplementary table S1), recording the number of male competitors and females visiting each
79 nest (i.e. nest composition), assessing dominant males courtship, nest defense against male
80 competitors and spawning disruptions. We counted the number of pair-spawns (involving one
81 dominant male with a female) and sneak-spawns (involving accessory males). To assess male
82 fertilization success we genotyped fin clips and embryos from nests exposed to Ambient and High-
83 CO₂ levels for paternity tests. Finally, to examine potential population differences in fish response
84 we compared nest composition, mating behaviour, and spawning at nesting sites exposed to
85 present-day Ambient-CO₂ conditions at Vulcano Island with those at Cala Isola, more than 150 km
86 away (see Methods).

87 Given that ocean acidification may affect reproduction, cause sensorial disruptions and impair
88 decision-making in fish, our hypotheses were that elevated CO₂ levels would affect ocellated
89 wrasse reproductive behaviour, reducing dominant male pair spawns and their fertilization
90 success due to increased sperm competition by accessory males.

91

92 **Methods**

93 **Carbonate chemistry at study sites.** Seawater carbonate chemistry was characterized daily on
94 several visits at two nesting sites off Vulcano Island in 2012 (n=11) and 2013 (n=12) and at Cala
95 Isola in 2012 (n=14) (see Electronic Supplementary table S1 for details).

96 A 556 MPS YSI (Yellow Springs, USA) multiparametric probe was used to measure salinity and pH
97 and temperature (°C). The sensor was calibrated using NBS scale standard buffers and then soaked
98 in seawater for one hour. For each site, average pH was calculated from hydrogen ion
99 concentrations before reconvertng back to pH values. Water samples for total alkalinity (TA) were
100 filtered through 0.2µm pore size filters, poisoned with 0.05 ml of 50% HgCl₂ to avoid biological
101 alteration, and then stored in the dark at 4° C. Three replicates were analyzed at 25° C using a
102 titration system (Mettler Toledo, Inc.). The pH was measured at 0.02 ml increments of 0.1 N HCl.
103 Total alkalinity was calculated from the Gran function applied to pH variations from 4.2 to 3.0,
104 from the slope of the curve HCl volume versus pH. Total alkalinity measurements were corrected
105 using standards provided by A.G. Dickson (batch 99 and 102). The pCO₂ levels were calculated
106 from pH_{NBS}, TA, temperature and salinity with the free-access CO₂ SYS package [23], using the
107 constants of Roy et al. [24] and Dickson [25].

108

109 **Study species and mating behaviour.** *Symphodus ocellatus* is a widespread wrasse in the rocky
110 subtidal of the Mediterranean Sea with an annual breeding season lasting from late April to July
111 [18,26]. Spawning occurs in the nest and involves small females (35-75 mm, Total Length) and
112 three alternative male reproductive types that compete to fertilize the eggs [27]. Fertilization is
113 external. Large, dominant males (81-95 mm, TL) [26] build nests with pieces of algae [28], court
114 females, and provide parental care ensuring oxygenation of the eggs by fanning, and actively
115 defending the nest from egg predators and other competing males. Females are non-territorial
116 and often swim in small groups with other females, do not participate in nest-building or parental
117 care, and during the spawning phase of a nesting cycle they visit several nests to lay a portion of
118 their eggs by brushing their genital papillae against algae. Smaller breeding males, called sneakers
119 (35-60 mm, TL), hover around various nests and try to join the female and dominant male during
120 spawning. Medium sized satellite males (61-80 mm, TL), cooperate with dominant males to reduce
121 sneaker male spawning and they also help court females and sneak spawn, getting a share of

122 paternity in the nest as a result [21,27]. Throughout the breeding season, each dominant male
123 completes several nesting cycles (each of which involves nest construction, spawning and parental
124 care) [18], lasting on average 8-10 days [26]. Females prefer nests where other females are
125 present or have recently spawned (i.e., mate-choice coping) [29], and with fewer sneakers present
126 [30].

127

128 **Study sites, behavioural and statistical analyses.** On 8-12 May 2012 and 7-12 June 2013, we
129 observed fish reproductive behaviour at two sites off Vulcano Island (NE Sicily, Italy), where
130 ocellated wrasse nests were exposed to present-day seawater pCO₂ conditions and those
131 predicted by the end of this century (High-CO₂ 38°25.184'N, 14°57.696'E; Ambient-CO₂
132 38°25.248'N, 14°57.8533'E) (Electronic Supplementary table S1). The High CO₂ and the Ambient
133 CO₂ nesting sites were at ~450 m and ~800 m distance from the main seeping area, respectively.
134 On 16-20 May 2012, an additional nesting site exposed to ambient CO₂ at Cala Isola (38°12.341'N
135 13°15.490'E; NW Sicily, Italy), 150 km from Vulcano Island, was investigated to assess inter-
136 population differences in mating behaviour under present-day pCO₂ conditions.

137 Nests of *S. ocellatus* were randomly selected among those in the spawning phase (which occur
138 over 3-4 days) of the nest cycle and filmed in Ambient (n=14) and High-CO₂ (n=18) conditions off
139 Vulcano and in ambient conditions at Cala Isola (n=10). The same nest (and dominant male) was
140 filmed only once, with observations typically occurring on the same days for both Ambient and
141 High CO₂ nests along the Vulcano gradient. The 42 total behavioural observations were collected
142 on different dives over the study period (16 days). All the nests considered were at 3-4 m depth in
143 rocky habitat covered by brown macroalgae. Our behavioural analyses took place when light
144 intensity and spawning activity were highest (i.e., from 10.00 to 16.00 hour). Water visibility
145 (always exceeding 10-15 m) and tidal ranges (between 20 and 40 cm) were similar in the two
146 nesting sites along the gradient off Vulcano island and in the additional nesting site exposed to
147 ambient CO₂ at Cala Isola. Each nest was filmed for 10 minutes using a GoPro 3.0 camera placed on
148 a tripod 1 m away from the nest. Five nests and dominant males from each nesting site at Vulcano
149 Island were marked for subsequent genetic analyses (see below). Details on the standard length,
150 weight, and age (from otoliths) of five dominant males belonging to nests exposed to high CO₂
151 conditions and of eight dominant nesting males from Ambient CO₂ nests are reported in the
152 Electronic Supplementary table S2.

153 To assess the 'nest composition' we subdivided each 10-min video into 15-sec. frames. For
154 each frame we recorded both the maximum number (MaxN) of females, sneakers and satellites
155 and the total number (TN) of females and sneakers participating in reproduction or visiting within
156 1 m of the nest. MaxN is a conservative estimate to avoid repeated measurements of the same
157 individual, by recording the maximum number of individuals appearing at the same time in a single
158 frame. TotN is a cumulative estimate of all individuals recorded within each frame in a 10-min
159 video. In this case the same individual could be repeatedly counted. TotN was not assessed for the
160 satellite males, as these were the same within a single video and therefore expressed as MaxN
161 only. For each nest we recorded the number of spawns by females 10 min^{-1} , the percent of time
162 the dominant nesting male spent courting females 10 min^{-1} , the number of times the dominant
163 male chased sneakers 10 min^{-1} , and the number of dominant male spawn disruption 10 min^{-1} .

164 Exploratory data analysis following Zuur et al. [31] revealed issues of heteroscedasticity of
165 variances, structuring of residuals and/or limited ranges of data values in many of the planned
166 analyses. Analytical models were therefore selected on a test by test basis using inspection of
167 residuals and, where applicable, AIC values as criteria. To control the type I error rate attendant
168 with the number of tests involved in the study, planned contrasts of intercept coefficients were
169 used to compare factor levels where pairwise testing was required, and when appropriate the
170 robustness of influence from marginal p values ($0.01 < p < 0.05$) was confirmed by bootstrapping
171 confidence intervals. All tests were conducted using R ver. 3.2.1 [32].

172 Differences in the number of accessory males (MaxN and TotN sneakers; MaxN satellites) and
173 the number of females (MaxN and TotN females) between the two nesting sites (High CO_2 and
174 Ambient CO_2) and between the two different ambient conditions populations were modelled by
175 generalised least squares (GLS) fitting using maximum likelihood. The same approach was used to
176 test effects of nesting site (fixed factor: High CO_2 and Ambient CO_2) and satellite males (fixed
177 factor: satellites present or absent) upon the number of spawns by females 10 min^{-1} , the
178 proportion of male time spent in courtship and the number of chases performed. In all cases F-
179 tests with Type III sums of squares were used to generate p values for main effects and
180 interactions. The number of dominant nesting male spawning disruptions was only exceptionally
181 greater than one, and was therefore analysed as a binary response by binomial family GLM with
182 chi-squared likelihood ratio tests used to generate p values.

183 We also recorded the number of spawns involving one dominant male with a female (pair
184 spawns), and the spawns involving both dominant and accessory males (sneak spawns). GLS by

185 maximum likelihood was again used to determine whether the number of spawns differed
186 between types (pair and sneak), nesting sites (Ambient and High CO₂) and nests with or without
187 satellites.

188

189 **Genetic analyses and paternity assignments.** For paternity analyses, we focused on the
190 developmental stage most likely to be from the same day as the behavioural observation. Since
191 spawning tends to occur over a few days (3-4 d) and it takes 3-5 days for eggs to hatch [26]
192 depending on seawater temperature, we expected larvae to hatch at the recorded temperature
193 value about 80 hours after spawning. Therefore, we waited 2.5 days (about 60 hours) to sample
194 nests, fertilised eggs and marked dominant males and used only larvae with pigmented eyes
195 (which usually form around 60 hours after fertilization) for subsequent genetic analyses.

196 Specifically, five of the dominant males that had been observed from each nesting site were
197 captured by SCUBA diving. Fin clips were collected and fin fragments were preserved in 80%
198 ethanol for later paternity analyses. Nests were also collected to allow a comparison of the
199 number and size of eggs laid between the two nesting sites (n=200). Individual egg surface (mm²)
200 was measured using digital photography and the open access software ImageJ was used to
201 estimate diameter to the nearest 0.01 mm. Wilcoxon rank sum tests (with continuity correction in
202 the case of egg surface area) were used to determine whether the number of eggs and their
203 surface area differed between the two nesting sites (High CO₂ and Ambient CO₂).

204 A sub-sample of embryos developed to the pigmented eye stage (n=60) was collected from
205 each nest, preserved in 80% ethanol and sent to University of Arizona Genetics Core (Tucson, USA)
206 for genotyping. DNA from 10 dominant nesting males and 585 eggs from 10 nests (n=5 at Ambient
207 and n=5 at High CO₂ conditions) was extracted using magnetic bead mediated robotic extraction
208 (Verde Labs Genomic DNA Extraction Chemistry on a Biosprint96 Extraction Robot). All samples
209 were amplified using six microsatellite loci developed for *S. ocellatus* (Soc1017, Soc1063, Soc1109,
210 Soc1198, Soc3121, Soc3200), and previously used for paternity assignment in this species [20].
211 Primer lengths were modified to allow all six loci to be used in combination in a single PCR
212 reaction. DNA was amplified using a DNA Engine Tetrad[®] 2 Thermal Cycler from Biorad set at the
213 following parameters: 94°C (120s); 15 cycles of 94°C (30s), 60-54°C (30s, 60°C on first cycle,
214 decreasing by 0.5°C for each subsequent cycle), 72°C (90s); 23 cycles of 94°C (30s), 54°C (30s), 72°C
215 (90s); 72°C (10 minutes). PCR product was run for fragment analysis on an Applied Biosystem 3730
216 DNA Analyzer, visualized and scored using the standard protocol for Genemarker software from

217 Softgenetics using a newly created bin-set. Peaks were then evaluated and scored visually by two
218 observers blind to sample identity. Paternity was assigned to eggs based on strict exclusion; eggs
219 that had at least one mismatch to the putative father were left unassigned to maintain a
220 conservative estimate of the fertilization success of dominant males (see Alonzo & Heckman [20]
221 for a comparison of different parentage assignment methods using these loci in *S. ocellatus*). Eggs
222 were included in the analysis only if they could be compared to the putative father at three or
223 more loci ($N = 505$ of 585 eggs were included in the final analysis; 62 were excluded due to low
224 levels of DNA extracted and poor amplification, and 18 because they could not be compared to
225 the putative father at three or more loci). We first analysed the paternity data (e.g. the number of
226 eggs assigned to the nesting male versus not assigned to the nesting male) using a logistic
227 regression fit by maximum likelihood with nest as a random effect due to significant
228 overdispersion of the data [33] and CO₂ condition (Ambient versus High) treated as a fixed effect
229 (using glmer from the lme4 package in R) [32,34]. Given the small sample size ($n=5$ nests per
230 condition), to reduce the possibility of a type I error, we also fit the model using a bootstrap
231 method following Warton & Hui [33].

232

233 **Results**

234 **Nest composition, nest attractiveness and egg characteristics at different CO₂ levels.** The
235 maximum number (MaxN) of satellites, sneakers and females visiting the nests at Vulcano Island
236 showed no differences between nesting sites exposed to different CO₂ levels (table 1; Electronic
237 Supplementary figure S1a). Similarly, the total number (TotN) of sneakers and the total number of
238 females recorded in 10-min videos did not differ between nests exposed to end-of-century and
239 ambient CO₂ (table 1; Electronic Supplementary figure S1b). Female spawning rate was also
240 unaffected by CO₂ levels (Electronic Supplementary figure S2 and table S3). Thus there were no
241 differences in nest attractiveness to ocellated wrasse females under different CO₂ levels (table 1;
242 Electronic Supplementary figure S2, Electronic Supplementary table S3). No differences in any of
243 these variables were recorded at nesting sites exposed to present-day Ambient-CO₂ conditions at
244 Vulcano Island with those at Cala Isola (Electronic Supplementary table S4). Also, there were no
245 differences in the size or number of eggs laid between nesting sites (Table 1; Electronic
246 Supplementary figure S3 and figure S4), as the Ambient-CO₂ nests had an average of 17833 (± 3275 ,
247 S.E., $n=5$) eggs and the High-CO₂ nests had 18621 (± 3156 , S.E., $n=5$) eggs.

248

249 **Behavioural interactions of the dominant nesting male.** The percent time that dominant males
250 spent courting females did not differ significantly between High-CO₂ (15.8% ±2.1; S.E., n= 18) and
251 Ambient sites (21.1% ±3.3; S.E., n= 14, table 1; figure 2a). The number of times that dominant
252 males behaved aggressively towards accessory males was also similar between nesting sites (table
253 1; figure 2b) with 12.6 (±3.9 S.E., n= 12) versus 16.6 (±3.1 S.E., n= 7) chasing events 10 min⁻¹ at
254 High-CO₂ compared with Ambient-CO₂ nests respectively. The disruption of dominant male
255 spawning did not differ significantly between nesting sites (table 1; Electronic Supplementary
256 figure S5). Similarly, no population differences in the mating behaviour of the dominant males
257 were recorded between nests exposed to present-day Ambient-CO₂ conditions at Vulcano Island
258 and Cala Isola (table 1).

259
260 **Mating competition and dominant male paternity.** Along the Vulcano CO₂ gradient, we found
261 that the number of pair spawns (a spawning event involving only a female and a dominant male)
262 were significantly lower at High-CO₂ (SpxSI interaction term, Table 1; figure 3; Electronic
263 Supplementary table S5), whilst sneak spawns (a spawning event involving both dominant and
264 accessory males) did not differ significantly between sites (SpxSI interaction, table 1; figure 3;
265 Electronic Supplementary table S5). Spawning by satellite males accounted for the 12.5% and 17%
266 of the total sneak spawns in the High-CO₂ (n=56) and the Ambient (n=47) nesting sites
267 respectively, with the remainder involving sneaker males. There were no differences in the
268 number of pair versus sneak spawns at High CO₂ nests (Contrast: pair = sneak, P =0.777; Electronic
269 Supplementary table S5), while – as expected based on earlier studies of this species [20]– the
270 number of pair spawns was higher than that of sneak spawns in the Ambient nests (contrast: pair
271 > sneak, P =0.002; Electronic Supplementary Table S5) (figure 3). Such differences in pair and
272 sneak spawning were not recorded between nests exposed to Ambient-CO₂ levels at Vulcano
273 Island and Cala Isola (Electronic Supplementary table S4).

274 Genetic analyses revealed that all of the dominant males experienced extra-pair paternity;
275 they sired 58.2% of the embryos (39.6-80.0%; n = 5) at High CO₂ nests, and 38.3% of the embryos
276 (19.6-50.9%; n = 5) at nests exposed to ambient conditions (Electronic Supplementary figure S6).
277 The logistic regression fit using maximum likelihood to predict the probability an egg is sired by the
278 nesting male found a significant effect of nest condition on nesting male paternity (z value -2.248,
279 P= 0.0245). However the pattern of increased siring success of the dominant males at higher CO₂

280 levels was not statistically significant after using a bootstrapping method to address the potential
281 for Type I errors, given the small sample size (bootstrap P value = 0.07).

282

283 **Discussion**

284 Here we show that dominant nesting males had approximately one third the number of pair
285 spawns at nests exposed to elevated CO₂ compared with nests at ambient CO₂ levels,
286 demonstrating a clear effect of rising CO₂ on the ocellated wrasse reproductive behaviour.
287 However, other dominant males' behaviours like female courtship and nest defence from intra-
288 specific (i.e., inter-male) competition were unaffected under elevated CO₂ conditions. Although
289 ocean acidification increased the risk of sperm competition in the ocellated wrasse, sneaker males
290 were unable to benefit from impaired dominant male mating as revealed by paternity tests,
291 indicating that there was probably no net loss of reproductive success for dominant nesting males.

292 Investigations into the effects of rising CO₂ effects on fish reproduction are in their infancy; the
293 few studies to date showed mixed responses when simulating ocean acidification in aquaria
294 [2,14,15]. Laboratory studies of the effects of rising pCO₂ levels in aquaria are augmented with
295 work at natural analogues for ocean acidification as they improve ecological realism, although it is
296 more difficult to determine dose-response relationships than in experiments that control
297 variations in carbonate chemistry and great care is needed to consider the possible effects of
298 confounding factors [35]. We used sites that were as similar as possible chemically (e.g. salinity,
299 alkalinity) and physically (substratum, wave exposure, currents, light levels, tidal range, depth,
300 temperature) with the one key difference being pCO₂ levels; as such this is the first study to
301 document effects of ocean acidification on fish mating behaviour in the wild.

302 We found no differences in nest attractiveness to females at different CO₂ levels [36]. Female
303 *Symphodus ocellatus* base their spawning decisions primarily on the absence of sneakers at the
304 nest, high mating activity and the mate choice of other females [36], rather than on dominant
305 male phenotype (size and colour patterns) or behaviour (ability in nest defence from intra-specific
306 competitors or courtship) [20,36]. A high number of females at a nest can in turn make it more
307 attractive to sneakers, therefore increasing the risk of sperm competition and affecting the
308 reproductive success of the dominant male [36]. We found no differences in the size or number of
309 eggs laid between nesting sites. Laboratory work on the reproductive performance of the
310 cinnamon anemone fish revealed increased egg production at elevated CO₂ – which is thought to
311 be due to stimulation of the hypothalamo-pituitary-gonadal (HPG) axis [3] – with no differences in

312 egg size at ambient (420 μatm) and end-of-century CO_2 (1032 μatm) levels, but a significant
313 decrease in egg size at moderate CO_2 (584 μatm) level, suggesting a CO_2 dose dependent
314 investment strategy of females [2]. The effect appears to be species-specific, since, as in our study,
315 in the three-spined stickleback *Gasterosteus aculeatus* egg size did not differ when exposed to
316 ambient and end-of-century CO_2 levels [14].

317 Dominant males were actively engaged in female courtship and in aggression against the
318 sneakers and satellite males at both nesting sites, however we found that the levels of seawater
319 pCO_2 expected by the end of this century did not significantly affect these dominant male
320 behaviours, which are key in ensuring its spawning success [18]. Occasionally dominant male
321 spawning was disrupted when a sneaker male caused the female to leave a nest without laying
322 eggs, and sometimes accessory males were successful in spawning alone with a female. However
323 the frequency of spawning disruptions of the dominant male did not differ between nesting sites.
324 In laboratory tests, four-day exposure to elevated CO_2 can affect both juvenile and adult fish
325 behaviour [4,16,37] and field work off Papua New Guinea has shown that chronic ocean
326 acidification disrupts the behaviour of sedentary fish at CO_2 seeps, such as those that hide in
327 anemones or coral colonies [13]. As reported in studies elsewhere, we observed that dominant
328 nesting male ocellated wrasse stayed within a small home range (10s of meters) and attended
329 individual nests for periods of 8-10 days [18] during the breeding season. Females – often
330 travelling in small groups – and sneaker males were more mobile (100s of meters). Thus the
331 dominant males near to CO_2 seeps experienced chronic exposure to ocean acidification whereas
332 more mobile males and females will have experienced acute effects. High CO_2 levels affect
333 behavioural lateralization, visual assessment and cognition in several fish species [8,9,37-39].
334 Work on escape behaviour has shown that ocean acidification affects decision-making time [8,9]
335 when fish try to escape predation [38]. Therefore, neuro-sensory impairment may be important
336 when dominant male ocellated wrasse have to make a quick decision about either to spawn or to
337 chase away sexual competitors, since these behaviours are key in ensuring the fertilization success
338 of dominant males [18]. Consistently, dominant nesting males had lower pair spawns at nests
339 exposed to elevated than ambient CO_2 levels, but sneak spawning was not affected. Although
340 ocellated wrasse sneaker males have a higher gonadal investment and release more sperm per
341 spawn than dominant or satellites males [19], genetic analyses revealed a trend towards increased
342 dominant male paternity at elevated CO_2 levels. The fact that a near significant trend was detected
343 for increased dominant male paternity at the high CO_2 , despite the low numbers of nests available

344 for study, suggests that there may be an important biological effect in evidence. Given the
345 previously observed variation among nests in both sperm competition and dominant nesting male
346 paternity [20], it is possible that larger sample sizes would pin down these differences in
347 reproductive success. In addition, it is also plausible that ocean acidification differentially affects
348 sperm mobility in the three types of male wrasse; a number of studies have shown that ocean
349 acidification affects sperm in some invertebrates [40,41] although no impact has yet been found in
350 fish [42].

351

352 In summary, our observations are the first to document effects of rising seawater CO₂
353 concentrations on the sexual behaviour of fish in the wild. We expected that increased sperm
354 competition due to ocean acidification would reduce dominant male reproductive success.
355 However, genetic paternity tests showed a 20% increase in their paternity at high CO₂ sites,
356 suggesting that sneaker and satellite mates were at a disadvantage. These results raise questions
357 around potential effects of increasing CO₂ levels on gametogenesis and sperm competition, on
358 embryonic development and on survival of different males' descendants. Work on the effects of
359 ocean acidification on the reproductive fitness of marine fish must be a priority given their
360 importance for ecosystem stability and for food security and livelihoods in coastal communities.

361

362 **References**

- 363 1. Wittmann AC, Portner H-O. 2013 Sensitivities of extant animal taxa to ocean acidification. *Nat.*
364 *Clim. Change* **3**, 995–1001.
- 365 2. Miller GM, Watson S-A, McCormick MI, Munday PL. 2013 Increased CO₂ stimulates
366 reproduction in a coral reef fish. *Glob. Change Biol.* **19**(10), 3037–3045.
- 367 3. Heuer RM, Grosell M. 2014 Physiological impacts of elevated carbon dioxide and ocean
368 acidification on fish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **307**, 1061–1084.
- 369 4. Munday PL, Dixon DL, Donelson JM, Jones GP, Pratchett MS, Devitsina GV, Døving KB. 2009
370 Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proc.*
371 *Natl. Acad. Sci. USA* **106**, 1848–1852.
- 372 5. Munday PL, Dixon DL, McCormick MI, Meekan M, Ferrari MC, Chivers DP. 2010
373 Replenishment of fish populations is threatened by ocean acidification. *Proc. Natl. Acad. Sci.*
374 *USA* **107**, 12930–12934.
- 375 6. Dixon DL, Munday PL, Jones GP. 2010 Ocean acidification disrupts the innate ability of fish to
376 detect predator olfactory cues. *Ecol. Lett.* **13**, 68–75.

- 377 7. Simpson SD, Munday PL, Wittenrich ML, Manassa R, Dixson DL, Gagliano M, Yan HY. 2011
378 Ocean acidification erodes crucial auditory behaviour in a marine fish. *Biol. Lett.* **7**, 917–920
379 (2011).
- 380 8. Domenici P, Allan BJM, McCormick MI, Munday PL. 2012 Elevated carbon dioxide affects
381 behavioural lateralization in a coral reef fish. *Biol. Lett.* **8**, 78–81.
- 382 9. Nilsson GE, Dixson DL, Domenici P, McCormick MI, Sørensen C, Watson SA, Munday PL 2012
383 Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter
384 function. *Nature Clim. Change* **2**, 201–204.
- 385 10. Sunday JM, Calosi P, Dupont S, Munday PL, Stillman JH, Reusch TBH. 2014 Evolution in an
386 acidifying ocean. *Trends Ecol. Evol.* **29**(2), 117–125.
- 387 11. Allan BJM, Miller GM, McCormick MI, Domenici P, Munday PL. 2014 Parental effects improve
388 escape performance of juvenile reef fish in a high-CO₂ world. *Proc. R. Soc. B.* **281**, 20132179.
- 389 12. Welch MJ, Watson S-A, Welsh JQ, McCormick MI, Munday PL. 2014 Effects of elevated CO₂ on
390 fish behaviour undiminished by transgenerational acclimation. *Nature Clim. Change* **4**(12),
391 1086–1089.
- 392 13. Munday PL, Cheal AJ, Dixson DL, Rummer JL, Fabricius KE 2014 Behavioural impairment in reef
393 fishes caused by ocean acidification at CO₂ seeps. *Nature Clim. Change* **4**, 487–492.
- 394 14. Schade FM, Clemmesen C, Wegner KM. 2014 Within- and transgenerational effects of ocean
395 acidification on life history of marine three-spined stickleback (*Gasterosteus aculeatus*) *Mar.*
396 *Biol.* **161**(7), 1667–1676.
- 397 15. Forsgren E, Dupont S, Jutfelt F, Amundsen T. 2013 Elevated CO₂ affects embryonic
398 development and larval phototaxis in a temperate marine fish. *Ecol. Evol.* **3**(11), 3637–3646.
- 399 16. Nagerlkerken I, Munday PL. 2016 Animal behaviour shapes the ecological effects of ocean
400 acidification and warming: moving from individual to community-level responses. *Glob.*
401 *Change Biol.* **22**(3), 974–989.
- 402 17. Boatta F, D’Alessandro W, Gagliano AL, Liotta M, Milazzo M, Rodolfo-Metalpa R, Hall-Spencer
403 JM, Parello F. 2013 Geochemical survey of Levante Bay, Vulcano Island (Italy) and its suitability
404 as a natural laboratory for ocean acidification studies. *Mar. Poll. Bul.* **73**, 485-494.
- 405 18. Taborsky M, Hudde B, Wirtz P. 1987 Reproductive behavior and ecology of *Symphodus*
406 (*Crenilabrus*) *ocellatus*, a European Wrasse with 4 types of male behavior. *Behaviour* **102**, 82-
407 118.
- 408 19. Alonzo SH, Warner RR. 2000 Allocation to mate guarding or increased sperm competition in a
409 Mediterranean wrasse. *Am. Nat.* **156**, 266–275.
- 410 20. Alonzo SH, Heckman KL. 2010 The unexpected but understandable dynamics of mating,
411 paternity and paternal care in the ocellated wrasse. *Proc. R. Soc. B.* **277**, 115–122.
- 412 21. Stiver KA, Alonzo SH 2013 Does the risk of sperm competition help explain cooperation
413 between reproductive competitors? A study in the ocellated wrasse (*Symphodus ocellatus*).
414 *Am. Nat.* **181**(3), 357–368.
- 415 22. Meinshausen M, *et al.* 2011 The RCP greenhouse gas concentrations and their extensions from
416 1765 to 2300. *Clim. Change* **109**, 213–241.

- 417 23. Pierrot DE, Wallace DWR. 2006 MS Excel Program Developed for CO₂ System Calculations.
 418 ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National
 419 Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee.
- 420 24. Roy RN, Roy LN, Vogel KM, Porter-Moore C, Pearson T, Good CE, Millero FJ, Campbell DM.
 421 1993 The dissociation constants of carbonic acid in seawater at salinities 5 to 45 and
 422 temperatures 0 to 45° C. *Mar. Chem.* **4**, 249–267.
- 423 25. Dickson AG. 1990 Standard potential of the reaction: $\text{AgCl(s)} + 1/2 \text{H}_2\text{(g)} = \text{Ag(s)} + \text{HCl(aq)}$, and
 424 the standard acidity constant of the ion HSO_4^- in synthetic seawater from 273.15 to 318.15 K. *J.*
 425 *Chem. Thermodyn.* **22**, 113–127.
- 426 26. Lejeune P. 1985 Le comportement social des Labride's mediterrane'ens: etude ecoethologique
 427 des comportements reproducteur et sociaux des Labridae mediterraneens des genres
 428 *Symphodus* (Rafinesque 1810) et *Coris* (Lacepede 1802). *Cah. Ethol. Appl.* **5**, 1–208.
- 429 27. Warner RR, Lejeune P. 1985 Sex change limited by parental care: a test using four
 430 Mediterranean labrid fishes, genus *Symphodus*. *Mar. Biol.* **87**, 89–99.
- 431 28. Sinopoli M, Cattano C, Chemello R, Timpanaro A, Timpanaro V, Gristina M. 2015 Nest building
 432 in a Mediterranean wrasse (*Symphodus ocellatus*): are the algae used randomly chosen or
 433 actively selected? *Mar. Ecol.* **36**(4), 942–949.
- 434 29. Alonzo SH. 2008 Female mate choice copying affects sexual selection in wild populations of the
 435 ocellated wrasse. *Anim. Behav.* **75**(5), 1715–1723.
- 436 30. Alonzo SH, Warner RR. 2000 Dynamic games and field experiments examining intra- and inter-
 437 sexual conflict: explaining counter-intuitive mating behavior in a Mediterranean wrasse,
 438 *Symphodus ocellatus*. *Behav. Ecol.* **11**(1), 56–70.
- 439 31. Zuur AF, Ieno EN, Elphick CS. 2010 A protocol for data exploration to avoid common statistical
 440 problems. *Methods Ecol. Evol.* **1**, 3–14.
- 441 32. R Core Team. 2016 R: A language and environment for statistical computing. R Foundation for
 442 Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>.
- 443 33. Warton DI, Hui FKC. 2011 The arcsine is asinine: the analysis of proportions in ecology. *Ecology*
 444 **92**(1), 3–10
- 445 34. Bates D, Maechler M, Bolker B, Walker S. 2015 Fitting Linear Mixed-Effects Models Using lme4.
 446 *J. Stat. Softw.* **67**(1), 1–48.
- 447 35. Cornwall CE, Hurd, CL. 2016 Experimental design in ocean acidification research: problems and
 448 solutions. *ICES J. Mar. Sci.* **73**(3), 572–581.
- 449 36. Alonzo SH. 2004 Uncertainty in territory quality affects the benefits of usurpation in a
 450 Mediterranean wrasse. *Behav. Ecol.* **15**(2), 278–285.
- 451 37. Jutfelt F, Bresolin de Souza K, Vuylsteke A, Sturve J. 2013 Behavioural disturbances in a
 452 temperate fish exposed to sustained high-CO₂ levels. *PLoS ONE* **8**(6), e65825.
- 453 38. Chivers DP, McCormick MI, Nilsson GE, Munday PL, Watson SA, Meekan MG, Mitchell MD,
 454 Corkill KC, Ferrari MC. 2014 Impaired learning of predators and lower prey survival under
 455 elevated CO₂: a consequence of neurotransmitter interference. *Glob. Change Biol.* **20**(2), 515–
 456 522.

- 457 39. Chung W-S, Marshall NJ, Watson S-A, Munday PL, Nilsson GE. 2014 Ocean acidification slows
458 retinal function in a damselfish through interference with GABA_A receptors. *J. Exp. Biol.* **217**,
459 323–326.
- 460 40. Havenhand JN, Schlegel P. 2009 Near-future levels of ocean acidification do not affect sperm
461 motility and fertilization kinetics in the oyster *Crassostrea gigas*. *Biogeosciences* **6**, 3009–3015.
- 462 41. Caldwell GS, Fitzer S, Gillespie CS, Pickavance G, Turnbull E, Bentley MG. 2011 Ocean
463 acidification takes sperm back in time. *Invert. Reprod. Dev.* **55**, 217–221.
- 464 42. Frommel AY, Stiebens V, Clemmesen C, Havenhand J. 2010 Effect of ocean acidification on
465 marine fish sperm (Baltic cod: *Gadus morhua*). *Biogeosciences* **7**, 5859–5872.

466

467 **Ethics.**

468 All experiments were carried out in accordance with institutional and national (law 116/1992)
469 guidelines concerning the use of animals in research.

470

471 **Data accessibility.**

472 All data and supplementary material are deposited in Dryad digital repository
473 (doi:10.5061/dryad.3vk01).

474

475 **Author's contributions.**

476 MM, CC, and JMH-S conceived the project; MM, CC, DS, MG, and MS collected the data; RRM
477 performed the carbonate chemistry analyses; MM, CC, SHA, AF and KAS analysed the data; MM
478 and CC wrote the initial draft of the manuscript; all authors contributed to the final version. The
479 order of authors from 3 to 9 was determined alphabetically.

480

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482 The authors declare no competing financial interests.

483

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492

493

494 **Figure captions**

495

496 **Figure 1.** A dominant male ocellated wrasse ready to spawn with the female
497 in a nest off Vulcano Island.

498

499 **Figure 2.** Mating behaviours of dominant males at the sites with different CO₂ levels. (a) Average
500 time spent (%) by the dominant male in courting females and (b) average number of dominant
501 male nest defence (chasing) events 10 min⁻¹. In both cases there are no differences between High
502 CO₂ vs Ambient nesting sites. Error bars ± 1 Standard Error.

503

504 **Figure 3.** Number of dominant male spawns (pair spawns) and sneak spawns at High and Ambient
505 CO₂ nests. Means with different letters (a, b) were significantly different in the pair-wise planned
506 contrasts (see also Table 1). Error bars ± 1 Standard Error.

507

508

Table 1. Summary of statistical analysis of nest composition and success, reproductive output, mating behaviour and spawning of ocellated wrasse (see methods for test details)

Nest composition		df	t	P
MaxN	Satellites	1,31	0.820	0.418
	Sneakers	1,31	1.568	0.125
	Females	1,31	0.885	0.488
TotN	Sneakers	1,31	0.771	0.445
	Females	1,31	0.778	0.441
Nest success		df	F	p
N. of spawns by females (SI x SA interaction)		1,28	12.041	0.002
			Satellite present ($t= 1.259, P= 0.218$)	
			Satellite absent ($t= 4.107, P<0.001$)	
Eggs (reproductive output)		df	W	p
N. of eggs/nest		1,8	11	0.841
Surface		1,398	21066	0.352
Behavioural interactions of the dominant nesting male		df	F	p
Courtship (%time)		1,28	1.829	0.187
N. of chasing events against accessory males		1,15	0.479	0.499
Inter-male competition		df	Chisq	p
Dominant nesting male spawn disruption		1	0.547	0.459
		df	F	p
Number of spawns (SP x SI interaction)		1,63	8.928	0.004
			Pair ($t= 3.282, P= 0.002$)	
			Sneak ($t= 0.515, P= 0.609$)	

Only comparisons between the two nesting sites exposed to different CO₂ conditions (i.e., High CO₂ vs Ambient CO₂ nesting sites) off Vulcano island are reported (see electronic supplementary Tables for full analyses and for inter-population analyses between ambient nesting sites). When an interaction term was significant, the results of pair-wise planned contrasts between High CO₂ vs Ambient levels are reported in brackets. SI: Nesting Site (with two levels: High CO₂, Ambient); SA: Satellite (with two levels: present, absent); SP: Spawn Type (with two levels: Pair spawn, Sneak spawn). Significant results are in bold.