Ocean acidification affects fish spawning but not paternity at CO_2 seeps

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Abstract

Fish exhibit impaired sensory function and altered behaviour at levels of ocean acidification expected to occur due to anthropogenic carbon dioxide emissions this century. We provide the first evidence of the effects of ocean acidification on reproductive behaviour of fish in the wild. Satellite and sneaker male ocellated wrasse (*Symphodus ocellatus*) *compete to fertilize eggs guarded by dominant nesting males. Key mating behaviours like dominant male courtship and nest defence did not differ between sites with ambient versus elevated CO_2 concentrations. Dominant males did, however, experience significantly lower rates of pair spawning at elevated CO_2 levels. Despite the higher risk of sperm competition found at elevated CO_2, we also found a trend of lower satellite and sneaker male paternity at elevated CO_2. Given the importance of fish for food security and ecosystem stability, this study highlights the need for targeted research into the effects of rising CO_2 levels on patterns of reproduction in wild fish.*

Introduction

Ongoing ocean acidification is an inevitable consequence of continued atmospheric CO_2 emissions and is expected to have profound effects on marine biodiversity and ecosystem function [1]. Coping with the physiological stress of ocean acidification has metabolic costs that can impair reproduction in a range of marine organisms, including fish [2,3]. Elevated CO_2 also causes striking behavioural and sensory disruptions in fish, involving loss of lateralization and of learning ability, reversal of olfactory and auditory functions, and impaired decision-making [4-8]. This neurosensory impairment may have consequences on marine communities as increased levels of CO_2 can cause juvenile fish to be attracted by predator odors they would normally avoid [5] or by sounds of unfavorable habitats for settlement [7]. These effects can be rapidly reversed by reducing GABA-A receptor activity [8], highlighting a link between increasing seawater CO_2 levels and brain function in fish [9].

Successful reproduction will be essential for the persistence of marine populations as seawater CO_2 levels continue to rise [10]. When cinnamon anemone fish (*Amphiprion melanopus)* parents are exposed to high-CO_2 conditions their offspring do not show behavioural impairment, however their potential for behavioural acclimation across generations is not fully restored, as in many instances their escape performance is negatively affected [11]. Similarly, other studies showed minimal potential for transgenerational acclimation in the tropical damselfish *Acanthochromis* *polyacanthus* [12] and in several other tropical fish chronically exposed to elevated CO_2 at volcanic seeps [13]. In aquaria, simulated ocean acidification increases the reproductive output of females (e.g., egg production) in some fish [2,14] but not others [15]. Intense sperm competition has resulted in male behavioural adaptations that may be affected by rising CO_2 levels. At present, mating system structure and reproductive behaviour are critically understudied in relation to the effects of high CO_2 on fish and other marine organisms [16]. Given the complex sexual behaviour of many fish in the wild, there are concerns that mating behaviour and reproductive success might be affected by ongoing rising CO_2 levels. The present study is the first to investigate this issue in fish exposed to high CO_2 levels in their natural habitat.

We used a gradient in CO_2 near volcanic seeps off Vulcano Island (Italy) [17] to examine mating behaviour and fertilization success of the ocellated wrasse *S. ocellatus* (figure 1). Ocellated wrasse 'dominant males' build nests and provide parental care to eggs, attract females for pair spawning, chase off sexual competitors ('satellite' and 'sneaker' males) [18], and sometimes refuse to mate when other males are prevalent [19]. Satellite males cooperate with dominant males to attract females and drive away sneakers, but they also engage in sneak fertilization of the eggs [20,21]. Sneaker males, which release more sperm per spawn than dominant nesting males or satellites, do not cooperate or care and only attempt to sneak spawn [19]. Thus high levels of sperm competition occur in the wild [20]. We filmed multiple wrasse nests at present-day Ambient-CO_2 conditions (~400 µatm pCO_2) and High-CO_2 conditions (~1100 µatm pCO_2) [22] (Electronic Supplementary table S1), recording the number of male competitors and females visiting each nest (i.e. nest composition), assessing dominant males courtship, nest defense against male competitors and spawning disruptions. We counted the number of pair-spawns (involving one dominant male with a female) and sneak-spawns (involving accessory males). To assess male fertilization success we genotyped fin clips and embryos from nests exposed to Ambient and High-CO_2 levels for paternity tests. Finally, to examine potential population differences in fish response we compared nest composition, mating behaviour, and spawning at nesting sites exposed to present-day Ambient-CO_2 conditions at Vulcano Island with those at Cala Isola, more than 150 km away (see Methods).

Given that ocean acidification may affect reproduction, cause sensorial disruptions and impair decision-making in fish, our hypotheses were that elevated CO_2 levels would affect ocellated wrasse reproductive behaviour, reducing dominant male pair spawns and their fertilization success due to increased sperm competition by accessory males.

Methods

Carbonate chemistry at study sites. Seawater carbonate chemistry was characterized daily on several visits at two nesting sites off Vulcano Island in 2012 (n=11) and 2013 (n=12) and at Cala Isola in 2012 (n=14) (see Electronic Supplementary table S1 for details).
A 556 MPS YSI (Yellow Springs, USA) multiparametric probe was used to measure salinity and pH and temperature (°C). The sensor was calibrated using NBS scale standard buffers and then soaked in seawater for one hour. For each site, average pH was calculated from hydrogen ion concentrations before reconverting back to pH values. Water samples for total alkalinity (TA) were filtered through 0.2µm pore size filters, poisoned with 0.05 ml of 50% $HgCl_2$ to avoid biological alteration, and then stored in the dark at 4° C. Three replicates were analyzed at 25° C using a titration system (Mettler Toledo, Inc.). The pH was measured at 0.02 ml increments of 0.1 N HCl. Total alkalinity 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. Total alkalinity measurements were corrected using standards provided by A.G. Dickson (batch 99 and 102). The $p\mathrm{CO}_2$ levels were calculated from $\mathrm{pH}_{\mathrm{NBS}}$, TA, temperature and salinity with the free-access CO_2 SYS package [23], using the constants of Roy et al. [24] and Dickson [25].

Study species and mating behaviour. *Symphodus ocellatus* is a widespread wrasse in the rocky subtidal of the Mediterranean Sea with an annual breeding season lasting from late April to July [18,26]. Spawning occurs in the nest and involves small females (35-75 mm, Total Length) and three alternative male reproductive types that compete to fertilize the eggs [27]. Fertilization is external. Large, dominant males (81-95 mm, TL) [26] build nests with pieces of algae [28], court females, and provide parental care ensuring oxygenation of the eggs by fanning, and actively defending the nest from egg predators and other competing males. Females are non-territorial and often swim in small groups with other females, do not participate in nest-building or parental care, and during the spawning phase of a nesting cycle they visit several nests to lay a portion of their eggs by brushing their genital papillae against algae. Smaller breeding males, called sneakers (35-60 mm, TL), hover around various nests and try to join the female and dominant male during spawning. Medium sized satellite males (61-80 mm, TL), cooperate with dominant males to reduce sneaker male spawning and they also help court females and sneak spawn, getting a share of paternity in the nest as a result [21,27]. Throughout the breeding season, each dominant male completes several nesting cycles (each of which involves nest construction, spawning and parental care) [18], lasting on average 8-10 days [26]. Females prefer nests where other females are present or have recently spawned (i.e., mate-choice coping) [29], and with fewer sneakers present [30].

Study sites, behavioural and statistical analyses. On 8-12 May 2012 and 7-12 June 2013, we observed fish reproductive behaviour at two sites off Vulcano Island (NE Sicily, Italy), where ocellated wrasse nests were exposed to present-day seawater pCO_2 conditions and those predicted by the end of this century (High-CO_2 38°25.184'N, 14°57.696'E; Ambient-CO_2 38°25.248'N, 14°57.8533'E) (Electronic Supplementary table S1). The High CO_2 and the Ambient CO_2 nesting sites were at ~450 m and ~800 m distance from the main seeping area, respectively. On 16-20 May 2012, an additional nesting site exposed to ambient CO_2 at Cala Isola (38°12.341'N 13°15.490'E; NW Sicily, Italy), 150 km from Vulcano Island, was investigated to assess inter-population differences in mating behaviour under present-day pCO_2 conditions.

Nests of *S. ocellatus* were randomly selected among those in the spawning phase (which occur over 3-4 days) of the nest cycle and filmed in Ambient (n=14) and High-CO_2 (n=18) conditions off Vulcano and in ambient conditions at Cala Isola (n=10). The same nest (and dominant male) was filmed only once, with observations typically occurring on the same days for both Ambient and High CO_2 nests along the Vulcano gradient. The 42 total behavioural observations were collected on different dives over the study period (16 days). All the nests considered were at 3-4 m depth in rocky habitat covered by brown macroalgae. Our behavioural analyses took place when light intensity and spawning activity were highest (i.e., from 10.00 to 16.00 hour). Water visibility (always exceeding 10-15 m) and tidal ranges (between 20 and 40 cm) were similar in the two nesting sites along the gradient off Vulcano island and in the additional nesting site exposed to ambient CO_2 at Cala Isola. Each nest was filmed for 10 minutes using a GoPro 3.0 camera placed on a tripod 1 m away from the nest. Five nests and dominant males from each nesting site at Vulcano Island were marked for subsequent genetic analyses (see below). Details on the standard length, weight, and age (from otoliths) of five dominant males belonging to nests exposed to high CO_2 conditions and of eight dominant nesting males from Ambient CO_2 nests are reported in the Electronic Supplementary table S2.

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

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

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

We also recorded the number of spawns involving one dominant male with a female (pair spawns), and the spawns involving both dominant and accessory males (sneak spawns). GLS by maximum likelihood was again used to determine whether the number of spawns differed between types (pair and sneak), nesting sites (Ambient and High CO_2) and nests with or without satellites.

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

Specifically, five of the dominant males that had been observed from each nesting site were captured by SCUBA diving. Fin clips were collected and fin fragments were preserved in 80% ethanol for later paternity analyses. Nests were also collected to allow a comparison of the number and size of eggs laid between the two nesting sites (n=200). Individual egg surface (mm^2) was measured using digital photography and the open access software ImageJ was used to estimate diameter to the nearest 0.01 mm. Wilcoxon rank sum tests (with continuity correction in the case of egg surface area) were used to determine whether the number of eggs and their surface area differed between the two nesting sites (High CO_2 and Ambient CO_2).

A sub-sample of embryos developed to the pigmented eye stage (n=60) was collected from each nest, preserved in 80% ethanol and sent to University of Arizona Genetics Core (Tucson, USA) for genotyping. DNA from 10 dominant nesting males and 585 eggs from 10 nests (n=5 at Ambient and n=5 at High CO_2 conditions) was extracted using magnetic bead mediated robotic extraction (Verde Labs Genomic DNA Extraction Chemistry on a Biosprint96 Extraction Robot). All samples were amplified using six microsatellite loci developed for *S. ocellatus* (Soc1017, Soc1063, Soc1109, Soc1198, Soc3121, Soc3200), and previously used for paternity assignment in this species [20]. Primer lengths were modified to allow all six loci to be used in combination in a single PCR reaction. DNA was amplified using a DNA Engine Tetrad® 2 Thermal Cycler from Biorad set at the following parameters: 94°C (120s); 15 cycles of 94°C (30s), 60-54°C (30s, 60°C on first cycle, decreasing by 0.5°C for each subsequent cycle), 72°C (90s); 23 cycles of 94°C (30s), 54°C (30s), 72°C (90s); 72°C (10 minutes). PCR product was run for fragment analysis on an Applied Biosystem 3730 DNA Analyzer, visualized and scored using the standard protocol for Genemarker software from

Softgenetics using a newly created bin-set. Peaks were then evaluated and scored visually by two observers blind to sample identity. Paternity was assigned to eggs based on strict exclusion; eggs that had at least one mismatch to the putative father were left unassigned to maintain a conservative estimate of the fertilization success of dominant males (see Alonzo & Heckman [20] for a comparison of different parentage assignment methods using these loci in *S. ocellatus*). Eggs were included in the analysis only if they could be compared to the putative father at three or more loci (*N* = 505 of 585 eggs were included in the final analysis; 62 were excluded due to low levels of DNA extracted and poor amplification, and 18 because they could not be compared to the putative father at three or more loci). We first analysed the paternity data (e.g. the number of eggs assigned to the nesting male versus not assigned to the nesting male) using a logistic regression fit by maximum likelihood with nest as a random effect due to significant overdispersion of the data [33] and CO_2 condition (Ambient versus High) treated as a fixed effect (using glmer from the lme4 package in R) [32,34]. Given the small sample size (n=5 nests per condition), to reduce the possibility of a type I error, we also fit the model using a bootstrap method following Warton & Hui [33].

Results

Nest composition, nest attractiveness and egg characteristics at different CO_2 levels. The maximum number (MaxN) of satellites, sneakers and females visiting the nests at Vulcano Island showed no differences between nesting sites exposed to different CO_2 levels (table 1; Electronic Supplementary figure S1a). Similarly, the total number (TotN) of sneakers and the total number of females recorded in 10-min videos did not differ between nests exposed to end-of-century and ambient CO_2 (table 1; Electronic Supplementary figure S1b). Female spawning rate was also unaffected by CO_2 levels (Electronic Supplementary figure S2 and table S3). Thus there were no differences in nest attractiveness to ocellated wrasse females under different CO_2 levels (table 1; Electronic Supplementary figure S2, Electronic Supplementary table S3). No differences in any of these variables were recorded at nesting sites exposed to present-day Ambient-CO_2 conditions at Vulcano Island with those at Cala Isola (Electronic Supplementary table S4). Also, there were no differences in the size or number of eggs laid between nesting sites (Table 1; Electronic Supplementary figure S3 and figure S4), as the Ambient-CO_2 nests had an average of 17833 (±3275, S.E., n= 5) eggs and the High-CO_2 nests had 18621 (±3156, S.E., n= 5) eggs.

Behavioural interactions of the dominant nesting male. The percent time that dominant males spent courting females did not differ significantly between High-CO_2 (15.8% ±2.1; S.E., n= 18) and Ambient sites (21.1% ±3.3; S.E., n= 14, table 1; figure 2a). The number of times that dominant males behaved aggressively towards accessory males was also similar between nesting sites (table 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 High-CO_2 compared with Ambient-CO_2 nests respectively. The disruption of dominant male spawning did not differ significantly between nesting sites (table 1; Electronic Supplementary figure S5). Similarly, no population differences in the mating behaviour of the dominant males were recorded between nests exposed to present-day Ambient-CO_2 conditions at Vulcano Island and Cala Isola (table 1).

Mating competition and dominant male paternity. Along the Vulcano CO_2 gradient, we found that the number of pair spawns (a spawning event involving only a female and a dominant male) were significantly lower at High-CO_2 (SpxSI interaction term, Table 1; figure 3; Electronic Supplementary table S5), whilst sneak spawns (a spawning event involving both dominant and accessory males) did not differ significantly between sites (SpxSI interaction, table 1; figure 3; Electronic Supplementary table S5). Spawning by satellite males accounted for the 12.5% and 17% of the total sneak spawns in the High-CO_2 (n=56) and the Ambient (n=47) nesting sites respectively, with the remainder involving sneaker males. There were no differences in the number of pair versus sneak spawns at High CO_2 nests (Contrast: pair = sneak, P =0.777; Electronic Supplementary table S5), while – as expected based on earlier studies of this species [20] – the number of pair spawns was higher than that of sneak spawns in the Ambient nests (contrast: pair > sneak, P =0.002; Electronic Supplementary Table S5) (figure 3). Such differences in pair and sneak spawning were not recorded between nests exposed to Ambient-CO_2 levels at Vulcano Island and Cala Isola (Electronic Supplementary table S4).

Genetic analyses revealed that all of the dominant males experienced extra-pair paternity; they sired 58.2% of the embryos (39.6-80.0%; n = 5) at High CO_2 nests, and 38.3% of the embryos (19.6-50.9%; n = 5) at nests exposed to ambient conditions (Electronic Supplementary figure S6). The logistic regression fit using maximum likelihood to predict the probability an egg is sired by the nesting male found a significant effect of nest condition on nesting male paternity (z value -2.248, P= 0.0245). However the pattern of increased siring success of the dominant males at higher CO_2

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

Discussion

Here we show that dominant nesting males had approximately one third the number of pair spawns at nests exposed to elevated CO_2 compared with nests at ambient CO_2 levels, demonstrating a clear effect of rising CO_2 on the ocellated wrasse reproductive behaviour. However, other dominant males' behaviours like female courtship and nest defence from intra-specific (i.e., inter-male) competition were unaffected under elevated CO_2 conditions. Although ocean acidification increased the risk of sperm competition in the ocellated wrasse, sneaker males were unable to benefit from impaired dominant male mating as revealed by paternity tests, indicating that there was probably no net loss of reproductive success for dominant nesting males.

Investigations into the effects of rising CO_2 effects on fish reproduction are in their infancy; the few studies to date showed mixed responses when simulating ocean acidification in aquaria [2,14,15]. Laboratory studies of the effects of rising pCO_2 levels in aquaria are augmented with work at natural analogues for ocean acidification as they improve ecological realism, although it is more difficult to determine dose-response relationships than in experiments that control variations in carbonate chemistry and great care is needed to consider the possible effects of confounding factors [35]. We used sites that were as similar as possible chemically (e.g. salinity, alkalinity) and physically (substratum, wave exposure, currents, light levels, tidal range, depth, temperature) with the one key difference being pCO_2 levels; as such this is the first study to document effects of ocean acidification on fish mating behaviour in the wild.

We found no differences in nest attractiveness to females at different CO_2 levels [36]. Female *Symphodus ocellatus* base their spawning decisions primarily on the absence of sneakers at the nest, high mating activity and the mate choice of other females [36], rather than on dominant male phenotype (size and colour patterns) or behaviour (ability in nest defence from intra-specific competitors or courtship) [20,36]. A high number of females at a nest can in turn make it more attractive to sneakers, therefore increasing the risk of sperm competition and affecting the reproductive success of the dominant male [36]. We found no differences in the size or number of eggs laid between nesting sites. Laboratory work on the reproductive performance of the cinnamon anemone fish revealed increased egg production at elevated CO_2 – which is thought to be due to stimulation of the hypothalamo-pituitary-gonadal (HPG) axis [3] – with no differences in egg size at ambient (420 µatm) and end-of-century CO_2 (1032 µatm) levels, but a significant decrease in egg size at moderate CO_2 (584 µatm) level, suggesting a CO_2 dose dependent investment strategy of females [2]. The effect appears to be species-specific, since, as in our study, in the three-spined stickleback *Gasterosteus aculeatus* egg size did not differ when exposed to ambient and end-of-century CO_2 levels [14].

Dominant males were actively engaged in female courtship and in aggression against the sneakers and satellite males at both nesting sites, however we found that the levels of seawater pCO_2 expected by the end of this century did not significantly affect these dominant male behaviours, which are key in ensuring its spawning success [18]. Occasionally dominant male spawning was disrupted when a sneaker male caused the female to leave a nest without laying eggs, and sometimes accessory males were successful in spawning alone with a female. However the frequency of spawning disruptions of the dominant male did not differ between nesting sites. In laboratory tests, four-day exposure to elevated CO_2 can affect both juvenile and adult fish behaviour [4,16,37] and field work off Papua New Guinea has shown that chronic ocean acidification disrupts the behaviour of sedentary fish at CO_2 seeps, such as those that hide in anemones or coral colonies [13]. As reported in studies elsewhere, we observed that dominant nesting male ocellated wrasse stayed within a small home range (10s of meters) and attended individual nests for periods of 8-10 days [18] during the breeding season. Females – often travelling in small groups – and sneaker males were more mobile (100s of meters). Thus the dominant males near to CO_2 seeps experienced chronic exposure to ocean acidification whereas more mobile males and females will have experienced acute effects. High CO_2 levels affect behavioural lateralization, visual assessment and cognition in several fish species [8,9,37-39]. Work on escape behaviour has shown that ocean acidification affects decision-making time [8,9] when fish try to escape predation [38]. Therefore, neuro-sensory impairment may be important when dominant male ocellated wrasse have to make a quick decision about either to spawn or to chase away sexual competitors, since these behaviours are key in ensuring the fertilization success of dominant males [18]. Consistently, dominant nesting males had lower pair spawns at nests exposed to elevated than ambient CO_2 levels, but sneak spawning was not affected. Although ocellated wrasse sneaker males have a higher gonadal investment and release more sperm per spawn than dominant or satellites males [19], genetic analyses revealed a trend towards increased dominant male paternity at elevated CO_2 levels. The fact that a near significant trend was detected for increased dominant male paternity at the high CO_2, despite the low numbers of nests available for study, suggests that there may be an important biological effect in evidence. Given the previously observed variation among nests in both sperm competition and dominant nesting male paternity [20], it is possible that larger sample sizes would pin down these differences in reproductive success. In addition, it is also plausible that ocean acidification differentially affects sperm mobility in the three types of male wrasse; a number of studies have shown that ocean acidification affects sperm in some invertebrates [40,41] although no impact has yet been found in fish [42].

In summary, our observations are the first to document effects of rising seawater CO_2 concentrations on the sexual behaviour of fish in the wild. We expected that increased sperm competition due to ocean acidification would reduce dominant male reproductive success. However, genetic paternity tests showed a 20% increase in their paternity at high CO_2 sites, suggesting that sneaker and satellite mates were at a disadvantage. These results raise questions around potential effects of increasing CO_2 levels on gametogenesis and sperm competition, on embryonic development and on survival of different males' descendants. Work on the effects of ocean acidification on the reproductive fitness of marine fish must be a priority given their importance for ecosystem stability and for food security and livelihoods in coastal communities.

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Ethics.

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

Data accessibility.

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

Author's contributions.

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

Competing financial interests.

The authors declare no competing financial interests.

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Figure captions

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

Figure 2. Mating behaviours of dominant males at the sites with different CO_2 levels. (a) Average time spent (%) by the dominant male in courting females and (b) average number of dominant male nest defence (chasing) events 10 min^{-1}. In both cases there are no differences between High CO_2 vs Ambient nesting sites. Error bars ± 1 Standard Error.

Figure 3. Number of dominant male spawns (pair spawns) and sneak spawns at High and Ambient CO_2 nests. Means with different letters (a, b) were significantly different in the pair-wise planned contrasts (see also Table 1). Error bars ± 1 Standard Error.

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 CO_2 conditions (i.e., High CO_2 vs Ambient CO_2 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 CO_2 vs Ambient levels are reported in brackets. SI: Nesting Site (with two levels: High CO_2, Ambient); SA: Satellite (with two levels: present, absent); SP: Spawn Type (with two levels: Pair spawn, Sneak spawn). Significant results are in bold.