Reproductive trade-offs in a temperate reef fish under high pCO₂ levels


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ABSTRACT

Fishes are currently facing novel types of anthropogenic stressors that have never experienced in their evolutionary history, such as ocean acidification. Under these stressful conditions, energetically costly processes, such as reproduction, may be sacrificed for increased chances of survival. This trade-off does not only affect the organism itself but may result in reduced offspring fitness. In the present study, the effects of exposure to high pCO₂ levels were tested on the reproductive performance of a temperate species, the two-spotted goby, Gobiusculus flavescens. Breeding pairs were kept under control (≈600 μatm, pH ≈ 8.05) and high pCO₂ levels (≈2300 μatm, pH ≈ 7.60) conditions for a 4-month period. Additionally, oxidative stress and energy metabolism-related biomarkers were measured. Results suggest that reproductive activity is stimulated under high pCO₂ levels. Parental pairs in the simulated ocean acidification conditions exhibited increased reproductive output, with 50% more clutches and 44% more eggs per clutch than pairs under control conditions. However, there was an apparent trade-off between offspring number and size, as larvae of parental pairs under high pCO₂ levels hatched significantly smaller, suggesting differences in parental provisioning, which could be related to the fact that these females produce more eggs. Moreover, results support the hypothesis of different energy allocation strategies used by females under high pCO₂ conditions. These changes might, ultimately, affect individual fitness and population replenishment.

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1. Introduction

Reproduction is a very energetically expensive process due to the time and energy parents invest towards producing offspring. In many coastal reef fish, while males typically expend large amounts of energy on courtship, nest defense, and offspring care (Gillooly and Baylis, 1999; Mackereth et al., 1999; Cox et al., 2010), females may have a greater influence on the offspring phenotype by altering the number, size, energy content, or yolk biochemical composition of their eggs, depending on the available energy to invest in offspring (Bernardo, 1996).

Due to the associated physiological costs, fish reproduction generally occurs within a narrow range of environmental conditions, set to favour offspring. However, variation in the environmental conditions experienced by parents may influence their body condition, potentially affecting offspring phenotype (Leatherland et al., 2010; Meylan et al., 2012). In addition to the natural ecological stressors, such as predation and habitat availability, fishes are currently facing novel types of anthropogenic stressors that are varying in ways outside their evolutionary history (Sih et al., 2011), such as climate change. Under these circumstances, energetically costly processes such as reproduction may be sacrificed for increased chances of survival. This trade-off may affect the organism, as well as induce changes in offspring fitness, with consequences for population replenishment. The predicted increase in temperature is well known to affect reproductive processes from gamete development, spawning, to embryogenesis and hatching (reviewed by Pankhurst and Munday, 2011). However, the impacts of climate change are not limited to increasing temperature. Rising atmospheric CO₂ levels are also driving an increased CO₂ ocean uptake, with concomitant changes in the carbonate chemistry of seawater (Caldeira and Wickett, 2003; Orr et al., 2005; Gattuso et al., 2015). It is generally assumed that adult fish can maintain their internal pH within an optimal range and are therefore relatively tolerant to changes in ambient CO₂ concentrations (Ishimatsu et al., 2004, 2008; Melzner et al., 2009), suggesting that effects of exposure to high CO₂ can be compensated. Still, compensation can be metabolically costly and may reduce the amount of energy available for other critical processes, such as reproduction (Pörtner et al., 2004; Kroeker et al., 2010). The exposure to specific stressors, including environmental stress, may increase individuals' internal imbalance, with subsequent negative
consequences on a variety of biological processes and systems (i.e. molecular and metabolic disruptions, cellular damage), which can impair the physiological condition of an organism (Depledge, 1993; Metcalfe and Alonso-Alvarez, 2010). To address these changes, biomarkers associated with stress-related biochemical responses have been widely applied as early-warning tools to assess an individual’s condition (Van der Oost et al., 2003; Wu et al., 2005; Silva et al., 2016; Alves et al., 2016; Kühnhold et al., 2017).

To date, only few studies have examined the potential effects of exposure to high CO2 in fish reproductive performance, but have yielded contradictory results. While Miller et al. (2013) described positive effects (more clutches and more eggs) of elevated CO2 on reproductive performance in the cinnamon anemonefish, Amphiprion melanopus, in a posterior study on the same species, no effect of high CO2 on reproduction was detected (Miller et al., 2015). For another anemonefish, A. percula, Welch and Munday (2016) demonstrated an increased reproductive output. However, for a close related species, the damselfish Acanthochromis polyacanthus, the authors reported decreased number of clutches produced under high CO2. On a temperate species, the three-spined sticklebacks, Gasterosteus aculeatus, Schade et al. (2014) provided evidence of increased clutch size and increased survival and growth rates in juveniles. These mixed results reinforce the need for further investigation on the impacts of future ocean acidification scenarios on the reproductive success of marine species.

In the present study, the effects of exposure to high CO2 levels were tested on the reproductive performance of a temperate species, the two-spotted goby, Gobiusculus flavescens. Breeding pairs were kept under control (∼600 μatm, pH∼ 8.05) and high pCO2 (∼2300 μatm, pH∼ 7.60) conditions for a 4-month period, which included the breeding season and 1 month of CO2 conditioning before breeding begun. Reproductive performance was evaluated by measuring the number of clutches produced by breeding pairs, the average number of eggs, average egg area, larval size at hatching, and adult body condition. Additionally, oxidative stress and energy metabolism-related biomarkers were measured for both males and females, as increased stress due to exposure to high CO2 may increase metabolic costs, and affect sexes differently.

2. Material and methods

2.1. Study species and collection

The two-spotted goby, Gobiusculus flavescens, is a small (adult 35–55 mm), sexually dimorphic, marine fish that lives along the coast of Europe, from Portugal to northern Norway (Miller, 1986). It typically lives for only 1 year (Johnsen, 1945), but can reproduce repeatedly during the breeding season (Mobley et al., 2009), which in the present study area can last from February to July. It is a substrate brooder, with males providing all parental care, for a period of 1–3 weeks depending on water temperature (Bjelvenmark and Forsgren, 2003; Skolbekken and Utne-Palm, 2001). According to the literature, the two-spotted goby exhibits sexual size dimorphism, with males on average slightly larger than females (Pélabon et al., 2003; Forsgren et al., 2004). However, the species may show some size dependent plasticity, as another recent study has reported a population showing a reversal of this trend, with males on average being smaller than females (Utne-Palm et al., 2015).

Adult males and females G. flavescens were collected by SCUBA diving at the Arrábida Marine Park, Portugal (38° 28’N; 8° 59’W), during early March 2016. Fish were immediately transported to the laboratory and transferred to a 100 L tank with a continuous supply of recirculating seawater, and left for one week to recover from handling. Subsequently, breeding pairs were randomly assigned to 35 L tanks (one pair per tank). All individuals were weighed (wet weight; ww) and standard length and total length (mm) measured immediately before being placed into treatment tanks, ensuring that there was an even distribution of weights and sizes among treatment groups. Nine breeding pairs were randomly assigned to each of the two treatment groups - control (∼ 600 μatm, pH∼ 8.05) and high pCO2 (∼2300 μatm, pH∼ 7.60) - and maintained at those conditions until the end of the breeding season, in July. Within sex, weight and size did not differ between control and high pCO2 treatments (males, weight: F1,16 = 0.03, p = 0.86; males, standard length: F1,16 = 0.00, p = 1.01; females, weight: F1,16 = 0.36, p = 0.55; females, standard length: F1,16 = 0.00, p = 1.01). Furthermore, within each treatment, initial weight and size did not differ between males and females (control, weight: F1,16 = 3.24, p = 0.09; control, standard length: F1,16 = 3.45, p = 0.08; high, weight: F1,16 = 3.53, p = 0.08; high, standard length: F1,16 = 2.29, p = 0.15).

Pairs were maintained under temperature and salinity conditions matching conditions at the field site (~16 °C, 35 PSU), with a summer light cycle of 14h light: 10h dark simulated using fluorescent lights, and fed with Artemia nauplii twice a day.

2.2. pCO2 treatment

The high pCO2 treatment was chosen as an extreme condition, which is not expected to occur in the open ocean until 2300 (Hartin et al., 2016); however, the two-spotted goby inhabits nearshore regions frequently exposed to CO2 fluctuations, where pCO2 values up to 1900 μatm have been recorded during seasonal upwelling events (Oliveira et al., 2012). The amplifying effects of anthropogenic ocean acidification can result in pCO2 levels greater than those projected to occur in open ocean environments (Melzner et al., 2013; Shaw et al., 2013).

Artificial seawater used in the experiments was adjusted to a salinity of 35 by blending a commercial salt mixture (Tropic Marin®) with filtered freshwater (reverse osmosis system). pHCO2 in the high pCO2 treatment (pHCO2 ~7.6) was slowly adjusted over a 3-week period to the desired level, by CO2 injection into a 2001 sump, regulated with a pH-controller (Tunze Aquarientechnik, Germany). A second 200 L sump was maintained at ambient pCO2 levels (pHCO2 ~8.0). Each sump, equipped with biological, mechanical, chemical, and ultraviolet filtration, supplied seawater into nine 35 L tanks at a flowrate of ~700 mL.min⁻¹. Rearing tanks were seeded with a clear glass lid to limit CO2 exchange with the atmosphere. Seawater pH, on NBS scale (pH(NBS)), was measured twice daily, using a portable, hand-held pH meter (SevenGo Pro pH/Ion, Mettler Toledo) and glass electrode (InLab1413 SB, Mettler Toledo) calibrated with certified reference materials for NBS consisting of pH(NBS) 4 and 7 buffer solutions (Mettler Toledo, two-point calibration). Temperature and salinity were also measured twice daily, using the same portable meter. Oxygen levels were maintained above 90% saturation by the mixing action of the diffusion pumps in the sumps. Samples for total alkalinity (TA) determination were weekly collected from experimental tanks, placed in air-tight containers without air space, stabilized by mercuric chloride poisoning and kept at +4 °C until further analysis. Analyses were performed using automated Gran titrations, with certified reference material supplied by A. Dickson (Scripps Institutions of Oceanography, San Diego). pCO2 was calculated from the in situ temperature, TA and pH, using the carbonic acid dissociation constants given by Millero et al. (2006) and the CO2 solubility coefficient of Weiss (1974). Estimated seawater parameters are shown in Table 1.

2.3. Experimental design and data collection

Breeding pairs were provided with a PVC pipe (10 cm long, Ø1.3 cm) as a shelter and breeding substrate. Each pipe was lined inside with a removable acetate sheet, where spawning females could attach their eggs. The presence of egg clutches was checked daily and in the presence of clutches, the acetate sheet with the clutch was carefully
removed from the nest, placed in a Petri dish with sea water, photographed to assess the number of eggs laid, and gently placed back into the nest. Parental males seemed not to be affected by the short-time removal of the clutch. A random sample of 10 eggs was also taken, and photographed under a stereomicroscope directly after removal from the clutch, to calculate egg area (to the nearest 0.01 mm²).

Reproductive output, per clutch, was calculated by multiplying the total number of eggs in the clutch by the average individual egg area for that clutch, to give an overall area (mm²) of eggs, and therefore an estimate of maternal investment per clutch. The day before hatching, egg clutches were transferred to small rearing boxes (~50 mL), with a mesh bottom to allow water recirculation, and placed in the same tank as the parental pair. At hatching, a sample of 10–15 larvae was collected, larvae euthanized using an overdose of MS222, and immediately photographed under a stereomicroscope. Larvae standard length was determined to the nearest 0.01 mm. All photographs were analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

At the end of the experiment, all adult individuals were weighed (ww) and measured (standard and total length) for determination of Fulton’s K condition factor (body condition), using the formula 

\[
K = 100 \times \frac{W}{L^3}
\]

where W is the wet weight in grams and L is the standard length in centimeters. Afterward, fish were euthanized by rapid cooling and immediately stored at −80°C for subsequent biomarkers analysis.

2.4. Biomarker analysis

Muscle from 35 fish, 17 males and 18 females, was dissected for biochemical measurements. Visceral content, as well as egg sacs, were discarded to avoid biochemical variations, since most of females were pregnant by the time they were sacrificed. Approximately 0.5 g of muscle (ww) was homogenized, using a mechanical homogenizer (Ystral X10/25, Germany), in a 1:12 proportion (m:v) of potassium-phosphate buffer (0.1 M, pH 7.4). To further analyze the presence of the superoxide anion radical (O₂⁻) [reactive oxygen species (ROS) proxy], an additional 0.05 g (± 0.01) of tissue was separated from the total muscle available. Oxidative stress (superoxide anion, superoxide dismutase, catalase), oxidative damage (DNA damage, lipid peroxidation) and energy metabolism (lactate dehydrogenase, isocitrate dehydrogenase, carbohydrates, lipids, proteins and electron transport system) protocols were conducted following Silva et al. (2016, 2017) procedures. All parameters were determined in triplicate, together with blanks (potassium-phosphate buffer 0.1 M, pH 7.4), using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Vermont, USA).

2.5. Data analysis

Validity of assumptions that underlie the data analysis was verified and data were standardized and log(x + 1) transformed, when applicable. A one-way ANOVA tested for differences in fish size and weight, within sex and treatment, at the start and end of the experiment, and an ANCOVA was used to compare body condition of adults between treatments, at the start and end of experiment, with weight as the dependent variable and length as the covariant. A Chi-square test for independence was used to compare the total number of clutches produced in control and high CO2 treatments. A one-way ANOVA was then used to compare the average number of clutches per reproductive pair between treatments. The mean number of eggs per clutch and average egg area per clutch were analyzed with nested ANOVAs, with parental pair nested within CO2 treatment. For egg area, the average of the 10 measurements per clutch was used as the unit of replication in the analysis. A nested ANOVA, with parental pair nested within CO2 treatment, was used to test for effects of exposure to high CO2 on size at hatching. The effect of CO2 treatment on biochemical parameters was addressed by a two-way ANOVA, using the sex and treatment as factors, followed by Tukey Unequal N HSD post-hoc test for multiple comparisons.

Statistical tests were performed using Statistics13.0™ software. All data is presented as mean ± SD, and the significance level was set at p ≤ 0.05.

2.6. Ethics statement

This study was performed in strict accordance with the recommendations of the Animal Care and Use Committee of ISPA - Instituto Universitário, and undertaken under the supervision of an accredited expert in laboratory animal science (following FELASA category C recommendations). Permission for capturing fish at the field site was granted by the Instituto da Conservação da Natureza e das Florestas (ICNF).

3. Results

Table 1

<table>
<thead>
<tr>
<th>pCO2 condition</th>
<th>pH&lt;sub&gt;H&lt;/sub&gt;</th>
<th>T (°C)</th>
<th>Salinity</th>
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<td>Control</td>
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Fish weight (0.73 ± 0.15 g) and size (3.80 ± 0.20 cm) at the start of the experiment did not differ between treatments (weight: F<sub>1,46</sub> = 0.19, p = 0.669; standard length: F<sub>1,32</sub> = 0.01, p = 0.935). Eight of nine pairs reproduced in control treatment, while all nine pairs reproduced in the high CO2 treatment (the non-breeding pair from the control treatment was excluded from the reproductive output analysis). Over the breeding season, control pairs of two-spotted goby produced a total of 26 clutches, while pairs under high CO2 conditions produced a total of 40 clutches, but this 50% increase was non-significant (χ² = 2.97, p = 0.085). Likewise, there was a trend towards more clutches per pair in the high CO2 treatment (4.44 ± 2.00 vs. 2.89 ± 1.69), although also non-significant (Fig. 1a; F<sub>1,16</sub> = 3.16, p = 0.09).

Embryonic duration did not change with increasing CO2, varying from 10 to 11 days. The average number of eggs per clutch increased with increasing CO2 (Fig. 1b; F<sub>1,46</sub> = 15.60, p < 0.001), and differed among parental pairs (F<sub>3,46</sub> = 4.43, p < 0.001). The control group produced 531 ± 254 eggs per clutch, while breeding pairs under high CO2 produced, on average, almost 44% more eggs per clutch (763 ± 253). Egg area did not differ between control and high CO2 treatments (0.43 ± 0.03 vs. 0.44 ± 0.04 mm²) (Fig. 1c; F<sub>1,46</sub> = 0.343, p = 0.561), but the overall reproductive output (mean egg area*-number of eggs) was 40% higher under high CO2 conditions (Fig. 1d; F<sub>1,46</sub> = 11.90, p < 0.001), and differed among parental pairs (F<sub>3,46</sub> = 4.17, p < 0.001).

Despite the lack of difference in egg area between treatments, pairs under high CO2 produced significantly smaller larvae at hatching (Fig. 1e; F<sub>1,207</sub> = 16.26, p < 0.001), and larval size differed among parental pairs (F<sub>6,207</sub> = 4.92, p < 0.001). In the control treatment larvae hatched with 2.89 ± 0.21 mm, while under high CO2 larvae hatched with 2.79 ± 0.21 mm.

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The higher reproductive output under high CO2 conditions did not appear to come at a cost to adult body condition, as no significant difference in Fulton’s K body condition factor between treatments was detected (0.94 ± 0.09 vs. 0.92 ± 0.09, for control and high CO2 treatments, respectively) (F(1,31) = 0.43, p = 0.517). Moreover, within each sex, no differences in weight and size between treatments were detected by the end of the breeding season (males, weight: F(1,16) = 0.26, p = 0.61; males, standard length: F(1,16) = 0.002, p = 0.96; females, weight: F(1,16) = 0.56, p = 0.47; females, standard length: F(1,16) = 0.10, p = 0.75). However, by the end of the experiment, females from each treatment were significantly smaller and weighting less than males (control, weight: F(1,16) = 18.11, p < 0.001; control, standard length: F(1,16) = 13.08, p = 0.002; high, weight: F(1,16) = 14.75, p = 0.002), likely highlighting females’ greater reproductive effort throughout the breeding season.

The reactive oxygen species (ROS) levels, measured by means of superoxide anion production, differed between CO2 treatment and sex (pCO2 treatment: F(1,30) = 4.877, p = 0.035; sex: F(1,30) = 15.301, p < 0.001), with females from control conditions presenting lower ROS levels when compared to control and high CO2 exposed males (Fig. 2a). There was, however, no significant interaction between the two factors (F(1,30) = 0.423, p = 0.521), contrary to the observed for the levels of lipid peroxidation (LPO), where exposure to high levels of CO2 affected sexes differently (F(1,30) = 5.752, p = 0.023). Despite the lack of a treatment or sex effect on LPO levels (CO2 treatment: F(1,30) = 2.877, p = 0.100; sex: F(1,30) = 0.749, p = 0.349) (Fig. 2b), there was a tendency for lower levels in females under high CO2 levels. Other than ROS levels, no significant differences were detected between treatments for the remaining measured biomarkers. On the other hand, differences between sexes were more commonly observed, with males presenting significant higher levels of CAT (F(1,30) = 4.314, p = 0.046), SOD (F(1,30) = 4.580, p = 0.041) and ETS (F(1,30) = 4.512, p = 0.042), and a similar tendency (not significant) for the other biomarkers, except for carbohydrates (Fig. S1 and Table S1 – Supplementary material).

4. Discussion

Results suggest that reproductive activity of the two-spotted goby is stimulated under high CO2 levels. Parental pairs in the simulated ocean acidification conditions exhibited increased reproductive output, with 50% more clutches and 44% more eggs per clutch than pairs under control conditions. These results contrast with the ones by Forsgren et al. (2013) on the same species, where elevated CO2 did not affect number of eggs. However, in that study, authors exposed adult fish to treatment shortly before spawning, whereas in the present study fish were exposed to elevated CO2 conditions before the onset of the breeding season, potentially affecting processes such as gametogenesis. Moreover, the elevated CO2 condition in that study was lower than the pCO2 levels in the present study (1400 μatm vs. 2300 μatm, respectively), which may explain the different results. However, other studies exposing adult fish to lower pCO2 levels than the ones used in the current study have also reported increased reproductive activity. The temperate marine three-spined stickleback Gasterosteus aculeatus, acclimated for a 3-month period to control (∼ 400 μatm) and
The tropical damselfish *Amphiprion melanopus* exposed to ~1000 μm for a 9-month period, which included the breeding season, produced 67% more eggs per clutch compared to fish exposed to moderate *p*CO$_2$ levels (~430 μm and 580 μm) (Miller et al., 2013); interestingly, in a posterior study on the same species, no effect of high CO$_2$ on reproduction was detected (Miller et al., 2015). The authors argued that these conflicting results may be related to differences in the time required to acclimate to captivity, as in the former study fish were studied in their first year of captivity, while in the later reproductive performance was evaluated in the second year of captivity. Supporting the results from the current study is another experimental work on another tropical species - *Amphiprion percula* exposed to ~650 and ~900 μm for a 9-month period, which also included the breeding season, produced 47–56% more eggs than control breeding pairs (Welch and Munday, 2016). In contrast, in the same study, but on another damselfish, *Acanthochromis polypoacanthus*, the authors reported decreased reproductive output with increasing CO$_2$ with approximately one-third as many clutches produced in high CO$_2$ compared with controls (Welch and Munday, 2016). In the first study conducted in the wild, Milazzo et al. (2016) found no differences in the number of eggs laid by the ocellated wrasse *Symphodus ocellatus* between nests, along a CO$_2$ gradient near volcanic seeps. Altogether, these contrasting results suggest a highly variable response of the reproductive performance of different fish species under high CO$_2$ and highlight the need for further investigation on this matter.

Contrary to the above-mentioned species, the two-spotted goby is a semelparous, short-living species, which typically lives for 1–1.5 years, and has only 1 breeding season, reproducing repeatedly during that season. In face of a stressor, semelparous species have no opportunity to postpone reproduction, or future opportunities to offset low quality progeny, as iteroparous species might have (Love and Williams, 2008). With only a single breeding episode, semelparous species may have evolved strategies to persist and cope with ecological stressors without compromising reproduction and offspring success (Wingfield and Sapolsky, 2003). There are however very few studies addressing effects of parental stress on offspring success in semelparous species and, to our knowledge, our current study is the first addressing the impacts of exposure to high CO$_2$ on a fish species that breeds only once in their lifetime. Sopinka et al. (2014) showed, for the first time in a wild semelparous fish species, the sockeye salmon, that mothers exposed to a stressor (repeated chase) appear to possess buffering strategies that protect progeny, as egg size and early offspring survival were not affected. Although results of our current study suggest the existence of a trade-off between reproductive output and offspring size at hatch, predictions on offspring survival and success cannot be made. Future studies should aim at addressing how a stressful environment experienced by parents will affect future generations. Transgenerational plasticity (TGP) could act as an effective mechanism to buffer populations against rapid environmental changes (e.g. Salinas and Munch, 2012), and despite the experimental evidence that suggests a high potential for adaptive transgenerational plasticity in fish under ocean warming and acidification conditions (Donelson et al., 2011; Miller et al., 2012; Salinas and Munch, 2012; Murray et al., 2014; Shama and Wegner, 2014), there is still much work to do on this respect.

Increased reproductive output observed in the two-spotted goby was not explained by differences in female size or weight between treatments, as they were evenly distributed at the start and end of the experiment, and it was neither associated with changes in adult body condition (Fulton’s K), which is in accordance with other studies reporting changes in reproductive output, but no effect of high CO$_2$ on adults’ body condition (Miller et al., 2013, 2015; Welch and Munday, 2016). This could be explained by the fact that fish were provided with food *ad libitum* throughout the experiment, which allowed them to maintain their body and physiological condition despite an increased reproductive effort, also justifying the observed lack of differences in energy reserves. In the wild, however, it is not likely that food will be fully available, and it is expected that facing different stressors (either abiotic or biotic), reproduction will come at a physiological cost, for both females (egg production) and males (parental care). Moreover, in the wild, organisms require more energy for other activities, such as foraging and predator avoidance, which may involve other energetic trade-offs. Donelson et al. (2010) tested the effects of both water temperature and food supply on the damselfish *Acanthochromis polypoacanthus* and found that both affected the reproductive output, with fewer pairs breeding at elevated water temperatures and no pairs reproducing at either of the higher temperatures on the lower quantity diet. However, to our knowledge, all studies to date on effects of simulated ocean acidification have been conducted on fish fed to satiation in laboratory conditions, and future work should investigate whether variation in food supply will influence the effects of exposure to high CO$_2$ on reproductive performance.

Although there was no effect of high CO$_2$ on egg size, an apparent trade-off between offspring number and size was observed, as larvae of parental pairs under high CO$_2$ levels hatched significantly smaller, suggesting differences in parental provisioning which could be related to the fact that these females produce more eggs. Maternal influences are typically more prevalent than paternal ones (Bernardo, 1996). Despite the well-developed acid-base regulatory system in fish, it is expected that compensation of the increased metabolic costs associated to acid-base regulation in a high CO$_2$ environment (Pörtner et al., 2004; Ishimatsu et al., 2008) would increase physiological costs, particularly, the potential for oxidative stress. However, this was not evident in our results. The present results only revealed a tendency for the decrease of LPO in females from the high CO$_2$ treatment, which is in accordance to a recent study on the impacts of high CO$_2$ on oxidative stress of early life stages of another fish species, the sand smelt, *Atherina presbyter* (Silva et al., 2016). It has been hypothesized that the exposure to stressors, such as high CO$_2$, may change the composition and relative proportion of fatty acids in fish (e.g., Frommel et al., 2011; Díaz-Gil et al., 2015), and in invertebrates (e.g., Timmins-Schifflmann et al., 2014; Valles-Regino et al., 2015), which may contribute to reduce cellular membrane vulnerability to damage and thus, explain lower LPO levels under stressful conditions. In general, the slight (yet not significant) reduction of LPO in females could be explained by the energy available for reproduction. This scenario could be supported by the increased reproductive output of females from the high CO$_2$ treatment, which is in accordance to the experiment, where more energy was available for reproduction.

**Fig. 2.** Oxidative stress related parameters measured in muscle of males and females of *Gobiusculus flavescens* after 4 months of exposure to control (600 μm) and high (2500 μm) CO$_2$ levels. a) Reactive oxygen species (superoxide anion); b) Lipid peroxidation. Results are expressed as mean ± S.D.; different letters indicate statistical differences between females of different treatments.

Fig. 2.
significant) increase of ROS in reproductively active females is not compensated by an increase of the studied antioxidant enzymes. These results seem to sustain the hypothesis of a different energy allocation strategy by females under high CO2 conditions, likely favoring reproductive success rather than protection against cellular damage, that overall seems to be occurring (decreased LPO) via eventual non-costly metabolically events, such as membrane fatty acid composition changes when faced with stress (e.g., Frommel et al., 2011; Díaz-Gil et al., 2015). In a recent study, Jordão et al. (2016) detected altered lipid storage patterns on Daphnia magna and, without any association with differences in energy intake, hypothesized the occurrence of specific mechanisms such as endocrine disruption in stress conditions. However, caution must be taken when interpreting this type of biomarkers’ data and its relevance from an ecological perspective once in the present study only the end of the reproductive season was taken into consideration and greater differences could have been observed earlier in time.

Despite the larger influence of maternal provisioning, there is also the potential for male parents to affect offspring phenotype through nest tending in species with paternal care (Green and McCormick, 2005), as the two-spotted goby. Higher energy requirement associated with elevated oxidative stress levels have also been reported in males prior to spawning periods mostly due to parental care (Smith and Wootton, 1995; Gravel et al., 2010; Zolderdo et al., 2016), which is also present in this species. Although time spent in paternal activities (e.g. egg aeration, and removal of dead eggs) was not quantified, there was no apparent difference in activity between treatments, and nest abandonment was never observed. On this matter, a recent study by Sundin et al. (2017) provided evidence of unaltered reproductive behaviours (nest building activities, courtship behaviours, fanning) in the three-spined stickleback Gasterosteus aculeatus exposed to 1000 μM. In the present study, despite no evident effects of the treatment to the exposed males, it can be noticed that males have generally higher ROS levels than females. It is also noted that, under control conditions, LPO levels are lower for males. These difference point to a generally more efficient antioxidant system (reducing lipid damage) which here is seen in higher CAT and SOD activities in males that might explain the higher ETS.

Whether smaller size at hatch will have consequences for larval growth and survival later in the ontogeny was not tested here and would require further investigation. In general, laboratory studies have provided evidence that fast growth during larval life enhances survival, as larvae are better able to capture food, resist starvation, and avoid predators (Miller et al., 1988; Bailey and Houde, 1989). In this sense, it can be hypothesized that the smaller offspring produced by parents under high CO2 will face higher mortality rates throughout the larval stage. However, other studies have found evidence of compensatory growth (e.g. Gagliano and McCormick, 2007; Donelson et al., 2009), indicating that nongenetic parental effects do not permanently limit offspring’s phenotype. It would be valuable for future studies to address the impacts of smaller size at hatching on offspring survival rates, under several scenarios, including food limitation and other stressors.

The mechanisms explaining different reproductive performances under high CO2 scenarios remains unclear. It is hypothesized that changes in the endocrine pathways associated to reproduction are responsible for altered reproductive output (Pankhurst and Monday, 2011), but it could also be related with GABA-A receptor function, which has been suggested to be implicated in the main behavioural changes observed in fish under ocean acidification scenarios (review by Tresguerres and Hamilton, 2017), but it also plays an important role in mediating secretions of reproduction related hormones (Trudeau et al., 2000; Zohar et al., 2010). Future studies should aim at examining hormonal profiles in fish exposed to high CO2 and test the involvement of GABA-A receptor.

Overall, this study makes a useful contribution in a research field that has been recognized as a knowledge gap (Heuer and Grosell, 2014; Milazzo et al., 2016). Results reveal clear effects of exposure to high CO2 on the reproductive success of the two-spotted goby, and support the hypothesis of different energy allocation strategies used by females under high CO2 conditions.

Authors’ contributions

AF conceived the project; AF, AL, CS collected data; AF, CS, SN, ML analyzed data; AF, AL, CS, SN, ML, EG contributed to writing the manuscript. All authors have approved the final article.

Conflicts of interest

Ana Faria, Ana Lopes, Cátia Silva, Sara Novais, Marco Lemos, Emanuel Gonçalves declare that we have no proprietary, financial, professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2018.02.027.

References


