Effects of ocean acidification on the swimming ability, development and biochemical responses of sand smelt larvae

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HIGHLIGHTS
• Exposure to high pCO2 has several impacts on fish early life stages.
• Behavioural, morphometric and biochemical approaches were used to assess impacts.
• Critical swimming speed was unaffected by high pCO2, contrary to larval development.
• High pCO2 leads to increased oxidative stress and differential energy allocation.

ABSTRACT
Ocean acidification, recognized as a major threat to marine ecosystems, has developed into one of the fastest growing fields of research in marine sciences. Several studies on fish larval stages point to abnormal behaviours, malformations and increased mortality rates as a result of exposure to increased levels of CO2. However, other studies fail to recognize any consequence, suggesting species-specific sensitivity to increased levels of CO2, highlighting the need of further research. In this study we investigated the effects of exposure to elevated pCO2 on behaviour, development, oxidative stress and energy metabolism of sand smelt larvae, Atherina presbyter. Larvae were caught at Arrábida Marine Park (Portugal) and exposed to different pCO2 levels (control: ~600 μatm, pH = 8.03; medium: ~1000 μatm, pH = 7.85; high: ~1800 μatm, pH = 7.64) up to 15 days, after which critical swimming speed (Ucrit), morphometric traits and biochemical biomarkers were determined. Measured biomarkers were related with: 1) oxidative stress — superoxide dismutase and catalase enzyme activities, levels of lipid peroxidation and DNA damage, and levels of superoxide anion production; 2) energy metabolism — total carbohydrate levels, electron transport system activity, lactate dehydrogenase and isocitrate dehydrogenase enzyme activities. Swimming speed was not affected by treatment, but exposure to increasing levels of pCO2 leads to higher energetic costs and morphometric changes, with larger larvae in high pCO2 treatment and smaller larvae in medium pCO2 treatment. The efficient antioxidant response capacity and increase in energetic metabolism only registered at the medium pCO2 treatment may indicate that at higher pCO2 levels the capacity of larvae to restore their internal balance can be impaired. Our findings illustrate the need of using multiple approaches to explore the consequences of future pCO2 levels on organisms.

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1. Introduction

Ocean acidification is recognized as an important driver of change in biological systems (Hoegh-Guldberg and Bruno, 2010; Doney et al., 2012), and has developed into one of the fastest growing fields of research in marine sciences (Rudd, 2014). The continuous uptake of additional atmospheric CO₂ is expected to cause severe changes in seawater chemistry, and depending on the emission trajectory, CO₂ partial pressure (pCO₂) levels are projected to exceed 900 ppm by 2100 (pH decreases of approximately 0.4 units) in an open ocean scenario (IPCC, 2013). In coastal environments, the effects of ocean acidification may be even greater and more variable due to eutrophication (Borges and Gypens, 2010; Cai et al., 2011) and upwelling events (Feely et al., 2008; Lachkar, 2014).

Most studies on the impacts of ocean acidification have focused on marine calcifying invertebrates, particularly molluscs and Cnidaria (Heuer and Grosell, 2014) due to their calcium carbonate dependence (Orr et al., 2005). Less attention has traditionally been given to fish, but in the past few years an impressive body of studies on the effects of ocean acidification has been published, covering a wide range of species. The early life stages have been particularly studied as they are most likely to be affected by increasing pCO₂, due to poorly developed mechanisms of physiological regulation (Brauner, 2008). Results point towards disturbances across a wide range of sensory systems and neurological functions, like olfaction (Dixson et al., 2010), vision (Chung et al., 2014), lateralization (Domenici et al., 2012), hearing (Simpson et al., 2011), learning (Ferrari et al., 2012), and activity levels (Munday et al., 2010). These disturbances will presumably have substantial impacts on predator-prey interactions (Domenici et al., 2012; Ferrari et al., 2012) and orientation abilities (Siebeck et al., 2015), and may ultimately lead to increased mortality rates (Munday et al., 2010). Recent studies have also detected impacts on survival, growth (Baumann et al., 2012) and development (Munday et al., 2009a), as well as on physiological and biochemical responses (Pimentel et al., 2015) of fish early life stages.

Despite these significant impacts, other studies fail to find any dramatic consequence of high pCO₂ on larval development and survival (e.g. Munday et al., 2011) or behaviour (e.g. Jutfelt and Hedgarde, 2013), suggesting species-specific levels of sensitivity to a changing environment. It is still unclear what justifies different species sensitivities to ocean acidification, but it might be related to the spawning mode of fishes (Munday et al., 2009a) or metabolic rates (Pane and Barry, 2007). Additional experimentation across a wide range of fish species with contrasting life histories and habitats will help identifying the factors associated with relative sensitivity to ocean acidification, as this will be critical for assessing the impacts on marine biodiversity and ecosystem function (Fabry et al., 2008).

In this study, we investigated the impact of ocean acidification on the early life stages of sand smelt (Atherina presbyter). Sand smelt is one of the two species of Atherinidae in the north-eastern Atlantic Ocean (Whitehead et al., 1986). It lives inshore, occasionally entering the coastal waters of where sand smelt inhabits, where pCO₂ values up to 1170 μatm have been recorded under upwelling conditions (Cabecadas and Oliveira, 2005). The high pCO₂ treatment was chosen as an extreme condition, but with the amplifying effects of anthropogenic ocean acidification (Melzner et al., 2013), future pCO₂ during upwelling events could easily exceed 1800 μatm.

Artificial seawater adjusted to a salinity of 34.5‰ was used in the experiments by blending a commercial salt mixture (Tropic Marin®) with filtered freshwater. Seawater was diffused with ambient air (control) or CO₂ in a 200-L sump to achieve the required pH, controlled by two pH controllers (DuoPro, SG23). Diffusion pumps on each sump kept oxygen levels above 90% saturation in all treatments. Samples for total alkalinity
(TA) determination were collected on a weekly basis from each system. Analyses of TA 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). Errors associated with pCO₂ calculations were estimated to be ± 10 µatm (accumulate errors on TA and pH). Estimated seawater parameters are shown in Table 1.

2.2. Larvae

Shoals of Atherina presbyter larvae were collected at Portinho da Arrábida, Portugal (8° 28’48” N | 8° 58’59” W), in July 2014, using a 1 mm mesh hand net. Larvae were transferred to 35-L aquariums with a continuous supply of recirculating seawater, and left for 2 days to acclimate from transport and handling effects. Larvae were then randomly assigned to a control, medium or high pCO₂ treatment group and exposed to these different acidification conditions between 7 and 15 days. Preliminary tests on sand smelt larvae showed that a minimum exposure period of 7 days was needed to induce behavioural changes. Larvae were reared under temperature and salinity matching field conditions, and a summer light cycle of 14 h light:10 h dark was simulated with fluorescent lights. Larvae were daily fed with Artemia nauplii ad libitum with exception of the acclimation period and the U₉₀ test day, to avoid some potential influence on performance.

2.3. Critical swimming speed (U₉₀)

In the end of the exposure period, fish were randomly chosen from each aquarium of the different treatments and tested for U₉₀. A total of 120 individuals, 40 per treatment, with final total length (TL) sizes ranging between 10 mm to 21 mm, were tested. Speed was measured following the protocols of Stobutzki and Bellwood (1994, 1997), using a swimming chamber adapted from Faria et al. (2009). Briefly, larvae were transferred to the swimming chamber, 1 larva per lane, and maintained for 5 min at a slow flow speed of 1.5 cm s⁻¹. Water velocity was increased by 1.5 cm s⁻¹ every 2 min until larvae could no longer swim against the current, adjusting to methods from Faria and Gonçalves (2010). Calculation of U₉₀ followed Brett (1964): U₉₀ = U + (t / t₁ + U), where U is the penultimate speed that a fish could maintain; U₁ is the velocity increment; t is the swimming time in the final velocity increment; and t₁ is the time interval for each velocity increment (2 min).

Individuals that displayed symptoms of stress, such as clinging to the sides, during this acclimatization period were removed and replaced. Chamber water temperatures varied between 17 and 19.1 °C.

Control water (i.e. seawater not treated with additional CO₂) was used in all the tests. However, preliminary tests suggested that there was no difference in behaviour when larvae were tested in control or treatment water. Moreover, previous studies (Munday et al., 2010) have shown that behavioural impairment caused by exposure to elevated CO₂ lasts for several days and is not affected by testing fish in CO₂-treated water versus control water. After the test, individuals were immediately placed on an ice-cold plastic keeper to slow down the metabolism and photographed under a dissecting stereo microscope for morphometric analysis, using Image-J (v1.48; U. S. National Institutes of Health, Bethesda, Maryland). Each individual was then weighed, transferred to a microtube and stored at −80 °C until biomarker analysis. A total of 120 individuals, 40 per treatment, were used (Table S1).

2.4. Morphometric analysis

For each larva the following morphometric traits that are indicators of development in larval fishes (Jones and McCormick, 2002; Chambers et al., 2014) were measured (Fig. S1): standard length (SL); total length (TL); anal height (HA); body depth (BD); head length (HL); dorsal height (HD); and caudal peduncle (CP) (Table S1).

2.5. Biomarker analysis

A total of 20 replicates per treatment were used for the following measurements (Table S1). Each larva was homogenized in 1 mL of potassium-phosphate buffer (0.1 M, pH 7.4), using an electrical homogenizer. Part of the homogenate was separated into different microtubes to further measure: protein concentration, electron transport system (ETS) activity, levels of LPO, DNA damage and total carbohydrates (CBH) levels. The rest of the homogenate was then centrifuged for 20 min, at 10.000 g (4 °C), to obtain the post-mitochondrial supernatant (PMS). The PMS was used for the evaluation of SOD and CAT activities. To obtain more concentrated samples, a different set of 6 organisms per treatment was used for LDH and IDH activity measurements. These organisms were homogenized in 300 µL of potassium-phosphate buffer (0.1 M, pH 7.4). To quantify superoxide anion (O₂⁻) levels, as a measure of ROS, 10 organisms per treatment were weighed and used for this purpose (Table S1).

All enzymatic activities and levels of mentioned parameters were determined in quadruplicate using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Vermont, USA). In all assays, potassium-phosphate buffer (0.1 M, pH 7.4) was used as blank.

For total protein quantification of each replicate homogenate and PMS fraction, bovine γ-globulin (BCG, Sigma-Aldrich, USA) was used as standard protein following the Bradford method, using 96-well flat bottom plate (Bradford, 1976). Absorbance was read at 600 nm and results expressed in mg of protein mL⁻¹.

2.6. Oxidative stress biomarkers

SOD activity was measured following McCord and Fridovich (1969) method, adapted to microplate (Lima et al., 2007). This procedure uses the reaction between cytochrome C and superoxide radicals generated by the complex xanthine-xanthine oxidase, resulting in a reduction of cytochrome C that can be measured by reading the absorbance at 550 nm for 10 min. SOD activity was expressed in U mg of protein⁻¹ using a SOD standard of 1.5 U mL⁻¹, where 1 U represents the amount of enzyme in the sample that causes 50% inhibition of cytochrome C reduction.

CAT activity measurements were based on the consumption of substrate (H₂O₂), optimizing the protocol of Clairborne (1985). The decrease in the substrate was followed at 240 nm for 1 min. CAT activity was expressed in nmol min⁻¹ mg of protein⁻¹, using a molar extinction coefficient of 40 M⁻¹ cm⁻¹.

The determination of O₂⁻ levels, as ROS, was conducted by following the method of Drossos et al. (1995). The presence of O₂⁻ was determined

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Total alkalinity (TA), CO₂ partial pressure (pCO₂), pH, salinity (S) and temperature (T) in the three treatments (control, medium and high). Values are presented as mean ± standard-deviation (SD).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2485.0 ± 58.29</td>
</tr>
<tr>
<td>Medium</td>
<td>2488.1 ± 63.69</td>
</tr>
<tr>
<td>High</td>
<td>2654.3 ± 111.10</td>
</tr>
</tbody>
</table>
by the capacity of the radicals present to reduce cytochrome C, which can be measured at 550 nm. Using an extinction coefficient of $1.9 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, the amount of O$_2$ produced was calculated and expressed in $\mu$mol O$_2$ mg protein$^{-1}$.

LPO levels were determined by measuring the content of thiobarbituric acid reactive substances (TBARS), following Ohkawa et al. (1979) and Bird and Draper (1984). After the reaction with TBA (2-thiobarbituric acid), absorbance was read at 535 nm and LPO levels expressed as nmol TBARS mg of protein$^{-1}$, using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

DNA damage (strand breaks) was evaluated following the DNA alka-line precipitation assay (Olive, 1988) adapted from Lafontaine et al. (2000). After the precipitation of SDS associated nucleoproteins and intact genomic DNA, the single and double stranded damaged DNA in the supernatant were coupled with Hoesch dye (1 $\mu$g mL$^{-1}$ bis-benzimide, Sigma-Aldrich) and the levels of damaged DNA were determined by measuring fluorescence using an excitation/emission wavelength of 360/460 nm. Results were expressed as $\mu$g of DNA mg of protein$^{-1}$, using calf thymus DNA as standard to extrapolate DNA concentration.

### 2.7. Energy metabolism related biomarkers

The determination of LDH activity was assessed using the method described and adapted by Vassault (1983) and Diamantino et al. (2001), respectively. LDH activity was measured as the efficiency of this enzyme to convert pyruvate to lactate in the presence of NADH, which results in NADH oxidation and consequent decrease in absorbance. The absorbance was read at 340 nm for 5 min. Results were expressed as nmol min$^{-1}$ mg protein$^{-1}$, using a molar extinction coefficient of $6.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

The IDH activity was measured following Ellis and Goldberg (1971) with the adaptations of Lima et al. (2007). The decarboxylation of isocitrate by IDH promotes the conversion of NADP$^+$ to NADPH. The activity of IDH is then determined with the increase in NADPH measured according to a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nmol min$^{-1}$ mg protein$^{-1}$.

The total carbohydrate content was measured at 492 nm according to De Coen and Janssen (1997), using a reaction of 5% phenol with $\text{H}_2\text{SO}_4$ (95–97%), with glucose as standard solution. The results were expressed as $\text{mJ mg protein}^{-1}$.

ETS activity in the mitochondria is a measurement of the cellular energy consumed (oxygen consumption rate) and can be determined following the method described by De Coen and Janssen (1997). NADPH and INT (p iodo-nitro-tetrazolium) solution were mixed with the samples and absorbance was read at 490 nm over a 3-minute period. The oxygen consumption was then calculated using a stoichiometric relationship: 2 $\mu$mol of formazan formed = 1 $\mu$mol of oxygen consumed. The oxygen consumption rate was then converted into the energetic equivalent of 484 kJ mol$^{-1}$ O$_2$ for average carbohydrate, lipid, and protein consumption combinations (Gnaiger, 1983). Results were expressed as $\mu$J h$^{-1}$ mg protein$^{-1}$.

### 2.8. Data analysis

One-way analysis of variance (ANOVA) was applied to address the effect of pCO$_2$ treatment (control, medium and high) on $U_{\text{crit}}$ and biomarker analysis followed by Tukey post-hoc test for multiple comparisons of group means for comparison between treatments (Zar, 2010). When assumptions of normality and homoscedasticity were not met, Kruskal-Wallis was applied followed by Games-Howell post-hoc test (Zar, 2010).

In addition, the morphometric traits were analyzed using a multivariate analysis of variance (MANOVA) in order to compare multivariate sample means, followed by a significance test involving individual dependent variables separately (namely, Tukey post-hoc test). Also, the validity of assumptions that underlie the data analysis was verified. Furthermore, influence of size (TL) on $U_{\text{crit}}$ was addressed by a linear regression followed by a Pearson's correlation to evaluate the strength of the relationship (Zar, 2010). A similar procedure was performed to evaluate the association between biomarker parameters and morphometric traits. Additionally, a principal component analysis (PCA) for morphometric traits was performed to emphasize the correlations and possible patterns between the variables (TL, HA, BD, HD, HL, CP) and the treatments. The principal components (PC1 and PC2) provide information on the most meaningful parameters, which describe a whole data set affording data reduction with minimum loss of original information. Although the results concerning the first two components were presented, the others were also analyzed.

Calculations were performed using Sigma Plot software (v11.0) and IBM SPSS Statistics 22. PCA was performed with CANOCO version 4.5 package (Ter Braak and Smilauer, 1998). Results were considered significant at $P < 0.05$.

### 3. Results

#### 3.1. Critical swimming speed, $U_{\text{crit}}$

$U_{\text{crit}}$ did not differ significantly among treatments ($F_{2,117} = 1.631; P = 0.200$), with speeds ranging from 1.6 to 18 cm s$^{-1}$ (Fig. 1). A weak but positive significant relationship between $U_{\text{crit}}$ and TL was detected ($r = 0.258; P = 0.004$).

#### 3.2. Morphometry

The results achieved by the Pearson correlation analysis showed that all the morphometric traits presented a significant association between all treatments (Table S5). Furthermore, the results of MANOVA demonstrated that, with the exception of control and medium treatments for HA and control and high treatments for BD, all morphometric variables were significantly affected by pCO$_2$ treatments (Tables S2 and S3). On the other hand, the PCA showed that 95.1% of total variance was explained by the two principal components (Fig. 2). PC1 expressed 89.1% of total variance, which is the most significant response regarding the separation between pCO$_2$ treatments. This demonstrates that the morphometric parameters HL (especially), SI, TL, HD and HA increase with increasing acidification, reinforcing the faster growth in the higher acidification treatment and demonstrating a separation of responses between treatments (Fig. 2). Thus, there was a trend for higher lengths in...
the high pCO₂ treatment in opposition with lower ones in the medium pCO₂ treatment with morphometric parameters.

3.3. Biomarker analysis — oxidative stress

Sand smelt larvae exposed to different acidification conditions revealed statistical significant differences in their oxidative stress status (Kruskal-Wallis, P < 0.05) (Fig. 3).

Levels of ROS, measured by means of superoxide anion production, were significantly lower in organisms exposed to the medium pCO₂ treatment, compared to larvae reared in control pCO₂ conditions (Tukey; P = 0.030; Fig. 3a), as well as a significant decrease in DNA damage levels (Games-Howell, P = 0.002; Fig. 3d).

Regarding the high pCO₂ treatment, there was a significant reduction in the enzymatic activity of SOD (Games-Howell, P = 0.001; Fig. 3b) and CAT (Games-Howell; P = 0.002; Fig. 3c) in comparison with the control pCO₂ treatment. Nonetheless, and although no differences were observed in DNA damage levels compared to control pCO₂ conditions (Fig. 3d), the LPO values were significantly lower in the high pCO₂ treatment (Games-Howell; P = 0.011; Fig. 3e).

3.4. Biomarker analysis — energy metabolism

Regarding energy metabolism, results showed that at high pCO₂ levels there was a significant increase in LDH (F(2,15) = 4.537; P = 0.029; Fig. 4a) and ETS (χ²(2) = 17.38; P = 0.001; Fig. 4c) activity values, but also on carbohydrate levels (F(2,55) = 28.322; P = 0.000; Fig. 4d). Nonetheless, no effects on IDH activity were observed with the acidification treatments (F(2,15) = 2.631; P = 0.105; Fig. 4b).

3.5. Biomarker analysis — correlations

Pearson's correlation analysis showed significant positive and negative correlations between the measured biomarkers (Table S4), with SOD being positively correlated with CAT (r = 0.36; P = 0.005), but negatively correlated with ROS (r = −0.397; P = 0.002) and DNA damage (r = −0.624; P = 0.000). From the energy metabolism related endpoints, a positive correlation between ETS and LDH (r = 0.327; P = 0.032) and between ETS and CBH levels (r = 0.669; P = 0.000) was observed (Table S4).

4. Discussion

A wide range of environmental factors, such as salinity, temperature, dissolved O₂ and CO₂ are broadly known as capable of influencing the physiological homeostasis of marine organisms (Shin et al., 2014; Hernando et al., 2015; Stapp et al., 2015), as a result of a compensatory response of acid-base regulation (Heuer and Grosell, 2014). These changes can be related with neuronal (Nilsson et al., 2012) and behavioural changes (Munday et al., 2010, 2012; Dixon et al., 2010; Cripps et al., 2011; Simpson et al., 2012; Domenici et al., 2012; Ferrari et al., 2012), compromising the organism's development and survival (Fabry et al., 2008). Behavioural changes induced by altered processes in the brain may be expressed within the time needed to induce them, which could reflect the essential physiological changes required to compensate a CO₂ acidosis (Chivers et al., 2014). For this reason, molecular and biochemical measurements, in addition to behavioural studies, are promising and determinant to assess the impacts of ocean acidification across species, life stages and environments.

In the present study, U_crit was not affected by exposure to a high pCO₂ environment, ranging from 2.9 to 18.0 cm s⁻¹, over a final size range of 10.3 to 21.0 mm (TL). These speeds were within the range of speeds that Faria et al. (2014) reported for sand smelt larvae. For all treatments, variability, at any size, was large, as has been observed in other studies on other species (Fisher et al., 2005b; Faria et al., 2009, 2014; Faria and Gonçalves, 2010), suggesting that additional factors (other than size) can lead to differences in behaviour.

The lack of effects of high pCO₂ on swimming abilities has already been reported for other species, such as the Atlantic cod Gadus morhua (Melzner et al., 2009; Maneja et al., 2013), mahi-mahi Coryphaena

Fig. 2. Biplot from principal component analysis integrating all measured morphometric traits measured in sand smelt larvae reared in three pCO₂ treatments [C — control (~600 μatm); M — medium (~1000 μatm); H — high (~1800 μatm)]. HA = anal height; BD = body depth; HL = head length; HD = dorsal height; CP = caudal peduncle; TL = total length.
hippurus (Bignami et al., 2014), cobia Rachycentron canadum (Bignami et al., 2013), and the Atlantic herring Clupea harengus (Maneja et al., 2015), suggesting that swimming-related physiological performance of larvae may not be substantially affected by projected future $p$CO$_2$ levels. However, a word of caution is needed as the lack of an apparent effect might be related to differences in experimental approaches.

Contrary to $U_{crit}$, a treatment effect was detected concerning larval morphology. Results reveal larger larvae in high $p$CO$_2$ levels and smaller larvae in the medium $p$CO$_2$ level. Although heterogeneous growth in fish is reported to be a common phenomenon, the mechanisms associated to these differences, and even differential strategies to face this stress, are still not clearly understood (Fernandes and Volpato, 1993). The reduction in somatic growth detected at medium $p$CO$_2$ treatment may be associated with high costs of internal homeostasis maintenance, functioning as an indicative of sub lethal physiological effects (Pörtner et al., 2004; Fabry et al., 2008; Amiard-Triquet, 2009; Timmins-Schiffmann et al., 2014). Reduced growth after exposure to higher levels of CO$_2$ was reported for other organisms, such as the shrimp Pandalus borealis and the mussel Mytilus edulis (Bechmann et al., 2011), the sea urchin Strongylocentrotus droebachiensis (Chan et al., 2015), and the estuarine fish Menidia beryllina (Baumann et al., 2012). A decrease in fish size may be associated with a reduction in protein synthesis, essential for energy demanding metabolic activities, such as growth and reproduction (Fabry et al., 2008). In the future,

**Fig. 3.** Oxidative stress related parameters measured in sand smelt larvae reared in three $p$CO$_2$ treatments [C — control (~600 μatm); M — medium (~1000 μatm); H — high (~1800 μatm)]. (a) Superoxide anion (ROS) levels; (b) superoxide dismutase activity; (c) catalase activity; (d) DNA damage levels; (e) lipid peroxidation levels. Results are expressed as mean ± SD. Bars that do not share a letter are significantly different (Games-Howell or Tukey, $P < 0.05$).
addressing tools such as RNA/DNA ratio may provide insight into these energetic trade-offs in larval fish (Buckley et al., 1999; Pepin et al., 1999; Franke and Clemmesen, 2011).

Conversely, larvae reared in the highest $p$CO$_2$ treatment, presented larger sizes. This different organism response might reflect a threshold where there is a shift in the organism's strategy, while the defenses can no longer cope with the higher stress input. Examples can be seen in other studies reporting larger size-at-age of larvae reared under increasing acidification scenarios. Mahi-mahi Coryphaena hippurus larvae exhibited larger size-at-age and faster developmental rates when reared at 770 and 1460 μatm $p$CO$_2$, but differences in larval size were only evident till 8 days post hatching (Bignami et al., 2014). Chambers et al. (2014) also reported increased size-at-age in larval flounder Paralichthys dentatus reared at 1808 and 4714 μatm $p$CO$_2$ but only during the first 14 days post hatch. Orange clownfish Amphiprion percula larvae reared at 550 and 1030 μatm $p$CO$_2$ were larger and heavier at settlement (Munday et al., 2009c). The authors argue that in laboratory experiments, the costs of acid-base balance could be compensated or even over compensated by the higher amounts of prey provided in captivity — resulting in higher levels of energy intake, in comparison with the expected in natural habitat, where food is limiting. Thomsen et al. (2013) provided evidence that this could be the case with juvenile mussels Mytilus edulis, which tolerate high ambient $p$CO$_2$ when food supply is abundant. Towle et al. (2015) also showed that coral species may buffer acidification effects by increasing feeding rates and lipid content. The present $p$CO$_2$ treatment results seem to agree with the assumptions of higher energy intake for the lowest pH given that: 1) larger sizes indicate higher growth rates, which are energy costly; 2) ETS and LDH activity levels, which were positively correlated, were significantly elevated, showing that the organisms had high cellular energy consumption rates and high demands of energy seen by the change to the anaerobic metabolism; 3) the increased metabolism did not result in consumption of carbohydrate reserves. Therefore, given this high energy consumption (with an increase in the carbohydrate levels), the larger sizes of the organisms, and the fact that all larvae from the different treatments were given the same amount of Artemia, it can be hypothesized that organisms exposed to high $p$CO$_2$ levels might in fact be feeding more than in the other treatments as a response to this stress, while the medium treatment organisms, due to lesser stress, may be using their energy reserves to face antioxidant defenses, diverting it from somatic growth in a different strategy. Moreover, one cannot exclude that other mechanisms underlying these effects may also be explained by other approaches rather the ones here addressed by this biomarker approach, that may in future studies be complemented by non-hypothesis driven integrative tool such as omics, which may also unveil non-monotonic responses/strategies (Lemos et al., 2010).

The oxidative stress analysis related to high $p$CO$_2$ treatment may also indicate enzymatic inactivation, since both enzyme levels (SOD and CAT) presented lower values in comparison with control and medium $p$CO$_2$ treatments. This pattern is also not surprising since high levels of stress are known to increase free radicals (superoxide anion and others), which can cause enzymatic inactivation. A study with flatfish larvae, Solea senegalensis, associating hypercapnia with present (18 °C) and warmer temperatures (+4 °C), reported similar results related with the apparent absence of CAT activity (Pimentel et al., 2015). These authors suggested that early life stages do not possess a fully developed antioxidant defense system, capable of avoiding peroxidation damages. Although oxidative stress is widely associated with formation...
and accumulation of LPO (Wdziczak et al., 1982; Filho et al., 1993; GutterIDGE, 1995; NegrE-Salvayre et al., 2010; Vinagre et al., 2012; Fonseca et al., 2014; cabECinhas et al., 2015), the results of this study showed, on the contrary, a decrease in the levels of LPO. Recent studies have demonstrated that elevated pCO2 scenarios can alter the relative proportions of fatty acids in fish larvae and oysters (Diaz-Gil et al., 2014; Timmins-Schiffmann et al., 2014), which might help to explain the present results. Timmins-Schiffmann et al. (2014) detected the presence of lower levels of highly unsaturated fatty acids, which are very sensitive to oxidative stress damage. These reduced levels may not only protect cell membranes, but also cellular membranes from damage caused by ROS (Pamplona et al., 2002), which may also explain the progressively lower LPO levels from control to higher pCO2 treatment in the study.

In contrast to the high pCO2 treatment, in the medium pCO2 scenario, higher activities of SOD were observed, along with lower levels of ROS and DNA damage. SOD acts to convert and decrease superoxide anion levels within the cell, which explains the negative correlation between SOD and ROS, meaning that the higher the activity of SOD, the lower the ROS levels. This prominent SOD activity also may have prevented oxidative damage on DNA, as can be seen by the significant lower levels of DNA damage and the negative correlation between both endpoints. These energy requirements for defense mechanisms may result in less energy available for other important functions, as somatic growth and reproduction, which may contribute to explain the smaller sizes of larvae in the medium pCO2 treatment.

Future ocean acidification conditions may demand higher energy allocation in marine organisms to ensure physiological homeostasis maintenance. Although the majority of the research in this field demonstrate limited or even an absence of impacts at the organism's level, most still fail to show what happens at the cellular level and how metabolic regulation may act to compensate internal fluctuations originated by stressful conditions. In the present work, although the impact of ocean acidification at the behaviour endpoint was not displayed, morphological and biochemical measurements were key to understand the mechanisms underlying the apparent resilience in this species. These results find support in Pan et al. (2015) who correlated higher amounts of ATP allocation in the sea urchin Strongylocentrotus purpuratus to protein synthesis and ion regulation in acidified conditions, with also limited impacts displayed at the organism level. These metabolic changes associated with a wide range of stressful factors acting together in nature, rise important questions related with energy metabolism associated with a wide range of stressful factors, with also limited impacts displayed at the organism level. These energy requirements for defense mechanisms may result in less energy available for other important functions, as somatic growth and reproduction, which may contribute to explain the smaller sizes of larvae in the medium pCO2 treatment.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.04.091.

References


