Absence of cellular damage in tropical newly hatched sharks (*Chiloscyllium plagiosum*) under ocean acidification conditions

Ana Rita Lopes^{1,2} · Eduardo Sampaio¹ · Catarina Santos¹ · Ana Couto¹ · Maria Rita Pegado¹ · Mário Diniz² · Philip L. Munday³ · Jodie L. Rummer³ · Rui Rosa¹

Received: 30 January 2018 / Revised: 7 March 2018 / Accepted: 8 March 2018 / Published online: 27 March 2018
© Cell Stress Society International 2018

Abstract

Sharks have maintained a key role in marine food webs for 400 million years and across varying physicochemical contexts, suggesting plasticity to environmental change. In this study, we investigated the biochemical effects of ocean acidification (OA) levels predicted for 2100 ($p\text{CO}_2 \sim 900 \mu\text{atm}$) on newly hatched tropical whitespotted bamboo sharks (*Chiloscyllium plagiosum*). Specifically, we measured lipid, protein, and DNA damage levels, as well as changes in the activity of antioxidant enzymes and non-enzymatic ROS scavengers in juvenile sharks exposed to elevated CO_2 for 50 days following hatching. Moreover, we also assessed the secondary oxidative stress response, i.e., heat shock response and ubiquitin levels. Newly hatched sharks appear to cope with OA-related stress through a range of tissue-specific biochemical strategies, specifically through the action of antioxidant enzymatic compounds. Our findings suggest that ROS-scavenging molecules, rather than complex enzymatic proteins, provide an effective defense mechanism in dealing with OA-elicited ROS formation. We argue that sharks' ancient antioxidant system, strongly based on non-enzymatic antioxidants (e.g., urea), may provide them with resilience towards OA, potentially beyond the tolerance of more recently evolved species, i.e., teleosts. Nevertheless, previous research has provided evidence of detrimental effects of OA (interacting with other climate-related stressors) on some aspects of shark biology. Moreover, given that long-term acclimation and adaptive potential to rapid environmental changes are yet experimentally unaccounted for, future research is warranted to accurately predict shark physiological performance under future ocean conditions.

Keywords Carbon dioxide · CO_2 · Elasmobranchs · Antioxidant systems · Heat shock response · Oxidative damage

Introduction

Atmospheric carbon dioxide (CO_2) concentrations have risen above 400 ppm for the first time in over 800,000 years (Lüthi

et al. 2008), and, unless anthropogenic CO_2 emissions are curtailed, are predicted to reach ~ 900 ppm by the end of the twenty-first century (Gattuso and Hansson 2011; Pörtner et al. 2014). Almost one third of the anthropogenically originated CO_2 is absorbed by the oceans, which has already led to a 0.1 unit drop in seawater pH from the pre-industrial period to the present days (Gattuso and Hansson 2011; Pörtner et al. 2014). These changes in seawater chemistry are underpinned by a net increase of hydrogen (H^+) and bicarbonate (HCO_3^-) ions and a decrease in carbonate ions (CO_3^{2-}), a phenomenon known as ocean acidification (OA) (Caldeira and Wickett 2005; Ridgwell and Zeebe 2005, Zeebe and Ridgwell 2011). By 2100, assuming a “business-as-usual” scenario (~ 900 ppm CO_2), the continuous CO_2 uptake is expected to elicit a further 0.13–0.42 pH drop (Gattuso and Hansson 2011; Pörtner et al. 2014).

Marine biota can be negatively impacted by OA in numerous ways, including changes in calcification (Orr et al. 2005), development (Kroeker et al. 2010; Wittmann and Pörtner 2013), growth rates, behavior (Clements and Hunt 2014;

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12192-018-0892-3>) contains supplementary material, which is available to authorized users.

✉ Ana Rita Lopes
arlobes@fc.ul.pt

¹ MARE - Marine and Environmental Sciences Centre, Laboratório Marítimo da Guia, Faculdade de Ciências da Universidade de Lisboa, Avenida Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal

² UCIBIO, REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Quinta da Torre, 2829-516 Caparica, Portugal

³ ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, QLD 4811, Australia

Nagelkerken and Munday 2015), and mortality (Baumann et al. 2012; Stiasny et al. 2016; Sswat et al. 2018). Physiologically, OA disrupts the acid-base balance of marine organisms, and even though marine fishes are known to compensate for internal pH disturbances, the assumption that such compensation will confer broad CO₂ tolerance could be overestimated (Heuer and Grosell 2014a). In fact, greater acid-base regulation may have other downstream consequences, such as reduced metabolic performance, and concurrent alterations in protein activities and functions (Burnett 1997; Gattuso and Hansson 2011; Heuer and Grosell 2014b). Specifically, elevated CO₂ induces oxidative stress due to (a) the parallel increase of H⁺ ions (extra and intracellular acidosis) and (b) the reaction of CO₂ with nitrogen reactive species, e.g., peroxynitrite, both of which contribute to an overproduction of reactive oxygen species (ROS) (Dean 2010; Feder and Hofmann 1999; Sampaio et al. 2018). Moreover, intracellular acidosis could also lead to the release of chelated transition metals (e.g., iron), which will in turn generate hydroxyl radicals through the Fenton reaction and potentially cause membrane-associated, protein, and DNA damage (Dean 2010; Hu et al. 2015; Sampaio et al. 2018; Stohs and Bagchi 1995; Tomanek et al. 2011).

To cope with naturally occurring oxidative stress, marine organisms harbor a wide set of regulatory mechanisms, including enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Lesser 2006). Moreover, a complementary antioxidant defense mechanism is provided by the diffusion of low-molecular-weight ROS scavengers (e.g., ascorbic and uric acids, carotenoids, reduced glutathione, and amino acids) (Bartosz 2003). Prior studies have demonstrated that these compounds are particularly prevalent in chondrichthyans, suggesting that low-molecular-weight scavengers are the main antioxidant defense in opposition to the enzymatic machinery in teleosts (Rudneva 1997). In response to acute physical and chemical stressors, marine organisms also generate heat shock proteins (HSPs) in order to repair and refold denatured proteins (Tomanek 2010; Tomanek et al. 2011). As a final line of defense, when HSPs fail to maintain functional protein conformation, ubiquitin targets irreversibly damaged proteins so they can be permanently eliminated (Bond et al. 1988; Hanna et al. 2007).

Recent studies show that the antioxidant defense system of teleost fishes under OA conditions sometimes fails to prevent oxidative damage, translating into chain reactions in increased skeletal and tissue deformities and decreased metabolic, growth, and survival rates in some species (Frommel et al. 2014; Pimentel et al. 2014, 2015; Silva et al. 2016; Stiasny et al. 2016). However, antioxidant defense responses to OA vary with ecological and life-history traits, such as life-stage, life-strategy, and phylogeny (Doney et al. 2012; Hofmann et al. 2010). Sharks represent one of the oldest taxa of marine

vertebrates and have maintained an important role in the structure of marine food webs throughout their evolutionary history (Baum et al. 2003). Indeed, these cartilaginous fishes have survived over the last 400 million years (Lund and Grogan 2004), coping with changes in the seawater chemistry from above 3000 CO₂ ppm during the Devonian period to below 300 CO₂ ppm in the pre-industrial era (see Fig. 1 in Rummer and Munday 2016). But now, the questions pertain to their acclimation to CO₂ concentrations consistently above 400 ppm levels (NOAA 2017) and increasing at a rate faster than ever recorded (Lüthi et al. 2008). Recent studies have shown that, although the performance of shark embryos appears mostly unaffected by OA-relevant conditions, there is evidence of OA-induced effects on the growth, metabolism, and behavior of later life stages of some species (see review in Rosa et al. 2017). For example, Pistevos et al. (2015) found significant changes in the Port Jackson shark behavior with elevated CO₂ levels. Similarly, Dixon et al. (2010) demonstrated that exposure to elevated CO₂ can significantly impair the feeding behavior of the smooth dogfish. On the other hand, Heinrich et al. (2014, 2016) found no effect of near-future CO₂ levels on the respiratory physiology and behavior of the epaulette shark.

Considering previous results in other species and taking into account the variable outcomes reported for sharks, we aimed to investigate sharks' capacity to handle oxidative stress under OA conditions. Thus, here, we investigated the oxidative stress-related responses of recently hatched tropical sharks (whitespotted bamboo shark *Chiloscyllium plagiosum*) to OA conditions. After 50 days exposure to CO₂ levels predicted for 2100 under a business-as-usual scenario ($p\text{CO}_2 \sim 900 \mu\text{atm}$), we measured membrane-associated, protein, and DNA damage levels, as well as changes in the activity of individual antioxidant enzymes and non-enzymatic molecules, i.e., primary antioxidant defenses. Moreover, we gauged the secondary oxidative stress response, by quantifying heat shock response and ubiquitin levels. Ultimately, we discussed if the variety of physicochemical contexts experienced during shark evolution (Ridgwell and Zeebe 2005) could allow these animals to successfully offset OA-induced oxidative stress.

Materials and methods

Ethics statement

Experimental procedures used in this research were in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. They were reviewed and approved by the animal ethics committee ORBEA ± Animal

Welfare Body of FCUL (Statement 5/2016) and by the National Veterinary Medicines Directorate (DGAV).

Sharks acquisition and lab acclimation

Whitespotted bamboo shark eggs (*C. plagiosum*) were hand-collected by local fishers between June and July 2016 in the area of Lungsod Ng Cebu (Philippines; around 10°11'N 123°58'E) and transported to the Laboratório Marítimo da Guia aquaculture facilities (LMG, Cascais, Portugal) through a certified commercial supplier (TMC - Iberia). Upon arrival, eggs were suspended by strings in a 200-L recirculating aquaculture system until hatching at average ambient temperature (26 °C) and pCO₂ (400 µatm). Contrary to our previous studies with a closely related shark species (*C. punctatum*; Rosa et al. 2014, 2016a, b), where we were able to expose shark embryos to high CO₂ levels from the beginning of the embryogenesis, *C. plagiosum* embryos arrived at our facilities in a more advanced developmental stage. As a result, exposure to elevated CO₂ only started after hatching. Each newly hatched shark ($n = 10$) was transferred to individual opaque tanks (50 L) and randomly allocated to one of the experimental conditions: control (~390 µatm CO₂, $n = 5$) or elevated CO₂ (~890 µatm CO₂, $n = 5$) for 50 days, according to relevant levels expected for 2100 (Pörtner et al. 2014). Individual tanks were coupled within recirculation aquaculture systems (RAS) filled with 0.35-µm filtered (Harmsco, USA) and UV-irradiated (V2ecton 600, TMC Iberia, Portugal) natural seawater, pumped directly from the sea. Water quality in each RAS was ensured by biological (Ouriço®, Fernando Ribeiro Lda, Portugal), mechanical (Glass wool, Fernando Ribeiro Lda, Portugal), and physical filtration (V2 Skim Pro 450, TMC Iberia, Portugal), as well as an additional UV irradiation (V2ecton 300, TMC Iberia, Portugal). Water quality was maintained by protein skimmers (Schuran, Jülich, Germany), wet-dry filters (BioBalls), and 30-W UV sterilizers (TMC, Chorleywood, UK). Each RAS worked in a semi-closed system, with a continuous low-flow exchange system, allowing a daily 50% water exchange. Ammonia, nitrite, and nitrate levels were daily monitored by means of colorimetric tests (Profi Test, Salifert, Holland) and kept within safe levels. The CO₂ level in each tank was controlled and maintained by continuous monitoring of seawater pH. A pH probe (GHL, Germany) in each tank was connected to an automated controller (Profilux 3.1, GHL, Germany) that adjusted pH values every 2 s. The appropriate seawater pH was achieved in each mixing tank ($n = 5$), in which pH was downregulated by the injection of a certified CO₂ gas mixture (Air Liquid, Portugal), via solenoid valves (Etopi, Portugal), and upregulated by the injection of atmospheric air. Additionally, a daily manual monitoring of seawater pH (SevenGo pro SG8, Mettler Toledo) was performed to adjust system set-points, as necessary. Seawater carbonate system speciation (see

Supplementary Table S1) was calculated weekly from total alkalinity (determined spectrophotometrically at a wavelength of 595 nm) according to Sarazin et al. (1999) and pH measurements, using pH total scale. Total dissolved inorganic carbon (CT), pCO₂, bicarbonate concentration, and aragonite saturation levels were calculated using the CO2SYS software (Lewis and Wallace 1998), with dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987). Temperature was regulated using electronic heaters. Temperature and salinity were monitored daily using a thermometer (TFX 430, WTW GmbH, Germany) and a refractometer (V2, TMC Iberia, Portugal), respectively. The systems were illuminated with white fluorescent lamps in a 12 h/12 h (light/dark) photoperiod. Sharks were fed daily to satiation with shrimp, kingfish, and squid. After the exposure period, organisms were sacrificed with MS222 and tissue samples were immediately frozen at -80 °C until further analyses.

Biochemical analyses

Preparation of tissue extracts

Muscle, liver, and gills samples ($n = 5$ per treatment) were homogenized (Ultra-Turrax, Staufen, Germany) in 2 mL phosphate-buffered saline solution (PBS, pH 7.4: 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.47 mM KH₂PO₄). Subsequently, homogenates were centrifuged (14,000×g for 20 min at 4 °C) and the supernatant fraction transferred to new microtubes (1.5 mL) and frozen (-80 °C) until further analyses. Each sample was run in triplicate (technical replicates), and the enzyme results were normalized to total protein content, as described by Bradford (1976).

Cellular, protein, and DNA damage

Lipid peroxidation (LPO), an indicator of cellular oxidative damage, was determined by malondialdehyde (MDA) quantification, a by-product of lipid damage, according to the thiobarbituric acid reactive substance (TBARS) assay (Uchiyama and Mihara 1978). A total of 10 µL of the sample was added to 45 µL of monobasic sodium phosphate buffer (50 mM), followed by the addition of 12.5 µL of sodium dodecyl sulfate (8.1%), 93.5 µL of trichloroacetic acid (20%, pH 3.5), and 93.5 µL of thiobarbituric acid (1%), to a 1-mL microtube. A volume of 50.5 µL of Milli-Q ultrapure water was added to this mixture, being subsequently mixed for 30 s and incubated in boiling water (100 °C) for 10 min. The resulting mixture was placed on ice for 3 min to lower the temperature. Afterwards, 62.5 µL of Milli-Q ultrapure water and 312.5 µL of *n*-butanol pyridine (15:1 v/v) were added and microtubes centrifuged at 2000×g for 5 min. The supernatant fraction (150 µL) was added to 96-well microplates, and absorbance was read at 532 nm in a microplate reader (Bio-Rad,

Benchmark, USA). Malondialdehyde concentrations were calculated based on a calibration curve (0–0.3 μM) using MDA bis (dimethyl acetal) standards.

Protein carbonylation (i.e., protein modification and damage) was determined spectrophotometrically through an enzyme-linked immunosorbent assay (ELISA) method, according to Alamdari et al. (2005). Briefly, 50 μL of each sample was added into the well and the platelet to incubate overnight at 4 °C. Afterwards, microplates were washed (3 $\times$) with PBS-TWEEN (0.1%) and incubated for 45 min at room temperature with 250 μL of a 0.05 M DNPH solution (2,4-dinitrophenylhydrazine) adjusted to pH 6.2. After the incubation period, plates were washed with PBS-TWEEN: ethanol (5 $\times$, 1:1) and PBS-TWEEN (3 $\times$). Blocking solution (200 μL BSA) was added, and after another incubation period (90 min at 37 °C), microplates were washed with PBS-TWEEN (3 $\times$). Primary antibody (1 $\mu\text{g}/\text{ml}$) was added into the wells (50 μL : anti-DNP, Acris, USA), and microplates were allowed to incubate for another 90 min at 37 °C. After another washing procedure (3 $\times$ with PBS-TWEEN), 50 μL of diluted (1 $\mu\text{g}/\text{ml}$) secondary antibody (alkaline phosphatase-conjugated anti-mouse IgG, Fab specific, Sigma-Aldrich, USA) was added to each well, and microplates were incubated again for 90 min (37 °C). After a final washing procedure, 100 μL substrate solution (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) was added to each well and, after 30 min, the reaction was stopped by adding 50 μL of sodium hydroxide (NaOH, 3 M). The absorbance was read at 405 nm in a microplate reader (Bio-Rad, Benchmark, USA).

8-hydroxy-2'-deoxyguanosine (8-OHdG), as a measure of DNA damage (i.e., DNA strand break or base mismatches), was assessed through an ELISA method, according to Maclouf et al. (1987) and Shen et al. (2007). Each sample (100 μL) was added to each well in a 96-well microplate and allowed to incubate overnight at 4 °C. After 24 h, plates were washed (3 $\times$) with PBS-TWEEN and incubated for 90 min at room temperature with the blocking solution (200 μL BSA). After another washing procedure (3 $\times$), microplates were incubated overnight with primary antibody (anti-OHdG, clone 15 A3, Sigma-Aldrich, Germany). On the subsequent day, microplates were washed to remove non-linked antibody and allowed to incubate for 90 min at 37 °C with the secondary antibody (alkaline phosphatase-conjugated anti-mouse IgG, Fab specific, Sigma-Aldrich, USA). After a final washing procedure, plates were incubated at room temperature for 30 min with the substrate (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA). Finally, the reaction was stopped by adding 100 μL of 3 M NaOH, and the absorbance was read at 405 nm (Bio-Rad, Benchmark, USA).

Antioxidant enzyme activities and total antioxidant capacity

Superoxide dismutase (SOD: EC 1.15.1.1) inhibition was determined based upon the method described by McCord and

Fridovich (1969). Each sample (0.5 mL) was added to a polystyrene cuvette with 1.4 mL of reaction mix (deionized water, 50 mM potassium phosphate, 0.1 mM ethylenediaminetetraacetic acid, 0.01 mM cytochrome c, 0.05 mM xanthine, pH 7.8). Subsequently the reaction was initiated by adding 0.5 mL of xanthine oxidase. The absorbance was read during 15 min at 550 nm using a UV-visible spectrophotometer (Ultrospec 2100 pro, Amersham BioSciences, UK).

Catalase (CAT) activity was assessed based on the method described by Johansson and Borg (1988). Briefly, 20 μL of each sample, 100 μL of 100 mM potassium phosphate, and 30 μL of methanol were added to a 96-well microplate, which was promptly shaken and incubated for 20 min. Afterwards, 30 μL of potassium hydroxide (10 M KOH) and 30 μL of purpald (34.2 mM in 0.5 M HCl) were added to each well, and the plate was shaken and incubated for another 10 min. Subsequently, 10 μL of potassium metaperiodate (65.2 mM in 0.5 M KOH) was added to each well, and a final incubation was performed for 5 min. Enzymatic activity was measured spectrophotometrically at 540 nm, using a microplate reader (Bio-Rad, Benchmark, USA). Formaldehyde concentration of the samples was calculated based on a calibration curve (from 0 to 75 μM formaldehyde), followed by the calculation of the CAT activity of each sample, where one unit of catalase is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C. The results were expressed in relation to total protein content ($\text{nmol min}^{-1} \text{mg}^{-1} \text{protein}$).

Glutathione peroxidase (GPx: EC 1.11.1.9) activity was determined through an adaptation of the method described by Lawrence and Burk (1976) to 96-well microplates. Briefly, 20 μL of each sample, 120 μL of 50 mM phosphate buffer (pH 7.6), 50 μL of co-substrate mixture (0.8 mM β -NADPH, 4 mM glutathione, 4 U/mL glutathione reductase, and 4 mM sodium azide), and 20 μL of 15 mM cumene hydroperoxide ($\text{C}_9\text{H}_{12}\text{O}_2$) were added to each well, and the absorbance was read every minute for 6 min at 340 nm (Bio-Rad, Benchmark, USA). The GPx activity was determined using the β -NADPH coefficient extinction, and results given in $\text{nmol min}^{-1} \text{mg}^{-1} \text{protein}$.

Aconitase (EC 4.2.1.3) activity was determined spectrophotometrically according to Morrison (1954) and adapted to 96-well microplates. Each sample (20 μL) and 115 μL of 100 mM Tris-HCl buffer, 20 μL of 20 mM manganese sulfate, 10 μL of 5.4 mM NADP^+ , 10 μL of 143 U/mL isocitric dehydrogenase enzyme (IsoDH) and 20 μL of activation solution (100 mM Tris-HCl, 50 mM L-cysteine, and 1 mM ammonium ferrous sulfate, pH 7.4) were added to each well. Then, the reaction was initiated by adding 10 μL of 2 mM citric acid, and the absorbance was read at 340 nm each minute for 25 min (Bio-Rad, Benchmark, USA). Aconitase activity was achieved by measuring the formation of β -NADPH during the catalysis of isocitrate to α -ketoglutarate.

Total antioxidant capacity (TAC) was determined according to Kambayashi et al. (2009). Briefly, a total of 10 μL of

each sample was added to each well in a 96-well microplate. Afterwards, 10 μL of 90 μM myoglobin, 150 μL of 600 μM ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)], and 40 μL of 500 μM hydrogen peroxide were added to the wells, and the microplates were incubated at room temperature for 5 min. Absorbance was read at 410 nm (Bio-Rad, Benchmark, USA), and TAC was calculated from a calibration curve, based on a series of Trolox (0–0.3 mM).

Protein repair and removal mechanisms

Heat Shock Protein 70 (HSP70) content was assessed through an ELISA according to Njemini et al. (2005). Each sample (20 μL) was diluted in 980 μL phosphate-buffered saline (PBS). Afterwards, 100 μL of each diluted sample was added to 96-well microplates (Microloan 600, Greiner, Germany) and incubated overnight at 4 °C. After 24 h, microplates were washed (3 $\times$) using PBS containing 0.05% TWEEN 20. Afterwards, 100 μL of blocking solution [1% bovine serum albumin (BSA)] was added to each well, and microplates were incubated for 90 min at 37 °C. Then, 50 μL of primary antibody (5 $\mu\text{g mL}^{-1}$ in 1% BSA: anti-HSP70/HSC70, Acris, USA) was added to each well. After another incubation period (overnight at 4 °C), microplates were washed (3 $\times$) to remove non-linked antibodies. A second antibody [alkaline phosphatase-conjugated anti-mouse IgG (Fab specific, Sigma-Aldrich, USA)] was used by adding 50 μL (1 $\mu\text{g mL}^{-1}$) to each well, and then the microplate was incubated for 90 min at 37 °C. After an additional washing procedure, 100 μL of substrate (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) was added to each well and incubated for 30 min at room temperature. Finally, 50 μL of stop solution (3 M NaOH) was added to each well.

Ubiquitin (Ub) content was assessed through an ELISA. Each sample (100 μL) was added to 96-well microplates (Microloan 600, Greiner, Germany) and incubated overnight at 4 °C. After 24 h, microplates were washed (3 $\times$) using PBS containing 0.05% TWEEN 20. Afterwards, 100 μL of blocking solution [1% bovine serum albumin (BSA)] was added to each well and microplates were then incubated for 90 min at 37 °C. Consequently, 50 μL of primary antibody (P4D1, sc-8017, HRP conjugate, Santa Cruz, USA) was added to each well. After another incubation period (overnight at 4 °C), microplates were washed (3 $\times$) to remove non-linked antibodies, and 100 μL of substrate (TMB/E, Temecula California, Merck Millipore) was added to each well and incubated for 30 min at room temperature. Afterwards, 100 μL of stop solution (1 M HCL) was added to each well. Absorbances were read at 405 and 415 nm, respectively, using a microplate reader (Bio-Rad, Benchmark, USA). The HSP and Ub content were calculated from the calibration curve, based on serial dilutions of purified HSP70 active protein

(0–2000 $\mu\text{g mL}^{-1}$, ACRIS, USA) and purified ubiquitin (0–1 $\mu\text{g mL}^{-1}$, UbqBio, E-1100, USA), respectively.

Statistical analyses

Generalized linear models (GLMs) were used to infer significant differences between CO₂ treatments and across the tissues analyzed. All data followed a normal distribution, with the assumptions of homogeneity of variances and normality confirmed by visual inspection. In a first approach, tissue (three levels: muscle, liver, gills) was used as an explanatory variable to find organ-specific patterns for each specific dependent variable (oxidative damage: LPO, protein carbonylation, and 8-OHdG; oxidative stress: SOD, CAT, GPx, aconitase and TAC; protein repair and removal: HSP and Ub), for all the samples combined (i.e., control and high CO₂). Afterwards, CO₂ (two levels: control and high CO₂) was used as an explanatory variable to identify the effects of CO₂ within each tissue type. All statistical analyses were performed using R Studio (R Development Core Team 2017).

Results

No mortalities occurred in either the control or elevated CO₂ treatment during the experiment. Lipid damage (i.e., LPO) exhibited significant differences among tissues, with lower levels in the gills and muscle when compared to the liver (both $p < 0.001$, GLM analysis in Table S2; Fig. 1A). However, no effects of elevated CO₂ were found within tissues ($p > 0.05$, GLM analysis in Table S5). Protein carbonylation levels also varied among tissues, with significantly lower values in the liver compared to muscle ($p < 0.05$, GLM analysis in Table S2; Fig. 1B). Moreover, elevated CO₂ decreased protein carbonylation in the gills ($p < 0.05$, GLM analysis in Table S5; Fig. 1B). Regarding DNA damage, the liver displayed significantly lower 8-OHdG levels than the other two tissues ($p < 0.001$, GLM analysis in Table S2; Fig. 1C). Furthermore, there was an increase in 8-OHdG levels in the liver with high CO₂ exposure ($p < 0.05$, GLM analysis in Table S5; Fig. 1C).

Regarding the antioxidant enzymatic machinery, SOD activity did not exhibit any significant differences among tissues ($p > 0.05$, GLM analysis in Table S3). Within the tissues, elevated CO₂ resulted in a decrease in SOD activity in the liver ($p < 0.05$, GLM analysis in Table S6; Fig. 2A), but a twofold increase in activity within the muscle ($p < 0.05$, GLM analysis in Table S6; Fig. 2A). CAT activity significantly varied among tissues, with lower levels detected in the gills when compared to the muscle and liver ($p < 0.001$ and $p < 0.05$, respectively, GLM analysis in Table S3; Fig. 2B). Within tissues, elevated CO₂ caused a decrease in CAT activity in the liver ($p < 0.05$; GLM analysis in Table S6; Fig. 2B). Elevated CO₂ also led to

Fig. 1 Impact of high CO₂ exposure on levels of **A** LPO, **B** protein carbonyl, and **C** 8-OHdG levels in *C. plagiosum* tissues. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentiles, and the whiskers indicate the highest and

lowest values of the results. Different letters represent significant differences between tissues, while asterisks (*) represent significant differences within tissues. GLM analyses described in Supplemental Tables S2 and S5

diminishing GPx activity in the liver ($p < 0.001$, GLM analysis in Table S6; Fig. 2C) and gills ($p < 0.05$, GLM analysis in Table S6; Fig. 2C), but an opposite trend was observed in the muscle ($p < 0.05$, GLM analysis in Table S6; Fig. 2C). Aconitase activity was significantly lower in the gills and liver when compared to the muscle (both $p < 0.001$, GLM analysis in Table S3; Fig. 2D). Elevated CO₂ also elicited a tissue-specific negative effect on aconitase activity in the gills ($p < 0.05$, GLM analysis in Table S6; Fig. 2D), whereas no changes were observed in the liver and muscle (both $p > 0.05$, GLM analysis in Table S6; Fig. 2D). With respect to the non-enzymatic antioxidant response, TAC levels were significantly higher in the gills when compared to the muscle ($p < 0.05$) and liver ($p < 0.001$, GLM analysis in Table S3; Fig. 2E). In addition, elevated CO₂ increased TAC levels in the muscle ($p < 0.05$, GLM analysis in Table S6; Fig. 2E).

HSP levels were significantly higher in the gills and lower in the liver than in the muscle ($p < 0.001$, GLM analysis in Table S4; Fig. 3A). Elevated CO₂ reduced HSP levels in the gills ($p < 0.05$, GLM analysis in Table S7; Fig. 3A). Lastly, ubiquitination (Ub levels) was higher in the muscle when compared to the liver and gills (both $p < 0.05$, GLM analysis in Table S4; Fig. 3B). Elevated CO₂ increased Ub content within the gills ($p < 0.05$), while an opposite trend was observed in the liver ($p < 0.001$, GLM analysis in Table S7; Fig. 3B).

In summary, elevated CO₂ levels elicited macromolecular damage only in the form of increased 8-OHdG in the liver, while SOD, CAT, and GPx activities decreased in the same tissue. In parallel, elevated CO₂ elicited an increase in SOD, GPx, and TAC within the muscle, whereas aconitase decreased in the gills. Furthermore, HSP levels declined with elevated CO₂ in the gills, while Ub levels augmented. Lastly, Ub levels decreased within the liver.

Discussion

In the present study, OA conditions expected for the end of this century did not result in significant oxidative damage in *C. plagiosum*, via lipid peroxidation (i.e., membrane-associated lipid damage) or protein carbonylation (i.e., oxidative modification of proteins). Our previous study with a closely related shark species (*C. punctatum*) detected some detrimental effects (i.e., neurooxidative damage and MDA production in the muscle) on shark early development due to elevated CO₂ (Rosa et al. 2014, 2016a, 2016b). However, in those experiments, animals were exposed to elevated CO₂ during the entire embryonic development (> 200 days) and the pCO₂ levels used were substantially higher (~ 1500 μ atm) than the ones currently used. In contrast to the previous study, here, we observed different tissue-specific biochemical responses of antioxidant enzymes in

Fig. 2 Impact of high CO₂ exposure on levels of **A** SOD, **B** CAT, **C** GPx, **D** aconitase activities, and **E** TAC in *C. plagiosum* tissues. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentiles, and the whiskers indicate the highest and

lowest values of the results. Different letters represent significant differences between tissues, while asterisks (*) represent significant differences within tissues. GLM analyses described in Supplemental Tables S3 and S6

juvenile *C. plagiosum* exposed to elevated CO₂ for 50 days following hatching.

Specifically, we found that SOD, CAT, and GPx decreased in the liver under elevated CO₂, which translated into increasing DNA damage. It is also worth noting that DNA damage found within the liver was lower when compared with muscle and gills although, as a detoxifying organ (e.g., biotransformation of xenobiotics), the liver is naturally more prone to oxidative stress (Rudneva et al. 2014). Nevertheless, cells harbor an effective DNA repair mechanism, comprising specialized enzymes, to overhaul DNA strand breaks or base mismatches (Hoeijmakers 2009; Sejersted et al. 2009). Furthermore, SOD and GPx activities, as well as non-enzymatic ROS scavengers, were upregulated in the muscle under elevated CO₂ conditions, successfully preventing any sort of damage. This increase in TAC levels may be linked to an upregulation of the naturally high urea levels that occur in sharks (Rudneva 1997). These molecules are known to be produced in the liver (via ammonium transformation) and retained by other tissues, playing a role both in osmoregulation and as a non-enzymatic complement to the antioxidant system, aiding in the prevention of oxidative damage (Rudneva 1997; Rudneva et al. 2014). Moreover, a significant pattern towards decreasing CAT, GPx, and aconitase levels

was detected in the gills under elevated CO₂ conditions. When sharks experience high concentrations of hydrogen peroxide (H₂O₂), gill diffusion rates increase in order to remove excess ROS (López-Cruz et al. 2012). Thus, this decrease in H₂O₂ could lead to decreased antioxidant enzymatic response, as observed in the present study, i.e., decreased GPx and CAT levels (Filho and Boveris 1993; López-Cruz et al. 2012; Rudneva 1997). Gills are also known to accumulate high levels of urea (Wood et al. 2013); therefore, naturally occurring levels may have been sufficient to prevent damage.

Regarding protein repair and elimination, significant differences between CO₂ treatments were found solely in the gills. Specifically, elevated CO₂ led to a decrease in HSP levels, while Ub levels increased. We reason that the heat shock response may have been insufficient in ensuring optimal protein function and conformation in this tissue, and therefore, ubiquitin specifically targeted those proteins to be eliminated. Nevertheless, activation of these secondary antioxidant defenses rarely occurred, which may be attributed to the co-occurring high levels of trimethylamine *N*-oxide (TMAO) in elasmobranchs (MacLellan et al. 2015). As a chemical chaperone, TMAO is energetically less expensive than HSP (molecular chaperones) synthesis, acting therefore as a first line in protein repair and refolding (Kumar 2009).

Fig. 3 Impact of high CO₂ exposure on protein repair and removal, namely, **A** HSP and **B** Ub levels in *C. plagiosum* tissues after 50 days exposure to high CO₂. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentiles, and

the whiskers indicate the highest and lowest values of the results. Different letters represent significant differences between tissues, while asterisks (*) represent significant differences within tissues. GLM analyses described in Supplemental Tables S4 and S7

Overall, our results suggest that recently hatched *C. plagiosum* can be resilient to OA-induced ROS that may be prevalent in a future ocean, as a consequence of CO₂ dissolution in seawater leading to chemical chain reactions (Dean 2010; Feder and Hofmann 1999; Sampaio et al. 2018), through multiple biochemical and physiological pathways. Long-lived species, such as most sharks, typically display comparatively lower ROS formation that shorter-lived species, presumably to prevent constant detoxification and repairing actions, and insufficient energetic expenditure (Pamplona and Constantini 2011). Sharks also possess specialized compensatory mechanisms that prevent oxidative damage, e.g., direct diffusion of excess ROS through the gills (López-Cruz et al. 2012). Notwithstanding, resistance to oxidative stress in *C. plagiosum* seems to be strongly underpinned by the mainly molecular-based (i.e., urea, ascorbic acid) antioxidant system (Rudneva 1997) and its capacity to eradicate ROS and prevent cellular damage (Rudneva 1999).

The evolution of structure complexity in agents of the antioxidant system, from molecules to enzymatic proteins, can be associated with phylogenetic position in the tree of life (Rudneva 1997). In contrast to ancient cartilaginous fishes, relatively newly evolved teleost fishes possess an enzyme-based antioxidant system (Filho and Boveris 1993; Solé et al. 2009). Thus, given the effects of elevated CO₂ found in some teleost fishes (Baumann et al. 2012; Pimentel et al. 2014; Stiasny et al. 2016), our findings could suggest that molecules, instead of complex enzymatic

proteins, effectively deal with OA-elicited ROS formation in sharks. Mechanistically, as high CO₂-associated stress is attributed to increased H⁺ concentrations, we reason that it is likely that relatively unstable molecules (especially urea), are able to (i) incorporate H⁺ quickly and/or (ii) yield a neutralizing electron to ROS (highly reactive scavenger) (Wang et al. 1999). Moreover, beyond the naturally high concentrations of urea in shark fluids and tissues, serving as both the main molecular antioxidant agent and as an osmolyte (Rudneva et al. 2014), its synthesis is also energetically less costly than that of the protein analogs (Kumar 2009). Thus, paradoxically, shark species could be potentially more resilient to future ocean acidification conditions than more derived teleost fishes because of their conserved antioxidant systems based on non-enzymatic antioxidants.

It should also be taken into account that, beyond their inherent capacity to buffer ROS formation, sharks might be able to adapt in parallel to gradually increasing CO₂, before the latter reaches the levels projected for the end of this century (Sunday et al. 2014). Further research is warranted to accurately predict shark physiological performance under future ocean conditions taking into account their adaptive potential over the timescale at which CO₂ is rising in the ocean.

Acknowledgments We would like to thank Eduarda Pinto for the fundamental technical support during the preparation of the experimental setup and shark acclimation.

Funding The Portuguese Foundation for Science and Technology (FCT) supported this work through the project grant PTDC/AAG-GLO/1926/2014 and Programa Investigador FCT 2013 to R.R. FCT also supported this work through (i) the strategic project UID/MAR/04292/2013 granted to MARE and (ii) PhD grants to ARL (SFRH/BD/97070/2013), ES (SFRH/BD/131771/2017), CS (SFRH/BD/117890/2016), and MRP (SFRH/BD/111691/2015). Both PLM and JLR are supported by funding from the Australian Research Council (ARC) Centre of Excellence for Coral Reef Studies.

Compliance with ethical standards

Competing of interests The authors declare that they have no conflicts of interest.

References

- Alamdari DH, Kostidou E, Paletas K, Sarigianni M, Konstas AG, Karapiperidou A, Koliakos G (2005) High sensitivity enzyme-linked immunosorbent assay (ELISA) method for measuring protein carbonyl in samples with low amounts of protein. *Free Radic Biol Med* 39:1362–1367
- Bartosz G (2003) Total antioxidant capacity. In: Spiegel HE, Nowacki G, Hsiao K-J (eds) *Advances in clinical chemistry*, vol 37. Academic press, California, pp 220–272
- Baum JK, Myers RA, Kehler DG, Worm B, Harley SJ, Doherty PA (2003) Collapse and conservation of shark populations in the Northwest Atlantic. *Science* 299:389–392
- Baumann H, Talmage SC, Gobler CJ (2012) Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. *Nat Clim Chang* 2:38–41
- Bond U, Agell N, Haas AL, Redman K, Schlesinger MJ (1988) Ubiquitin in stressed chicken embryo fibroblast. *J Biol Chem* 263:2384–2388
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Burnett LE (1997) The challenges of living in hypoxic and hypercapnic aquatic environments. *Integr Comp Biol* 37:633–640
- Caldeira K, Wickett ME (2005) Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *J Geophys Res* 110:C09S04
- Clements JC, Hunt HL (2014) Influence of sediment acidification and water flow on sediment acceptance and dispersal of juvenile soft-shell clams (*Mya arenaria* L.). *J Exp Mar Biol Ecol* 453:62–69
- Dean JB (2010) Hypercapnia causes cellular oxidation and nitrosation in addition to acidosis: implications for CO₂ chemoreceptor function and dysfunction. *J Appl Physiol* 108:1786–1795
- Dickson A, Millero F (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep-Sea Res* 34:1733–1743
- Dixon DL, Munday PL, Jones GP (2010) Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. *Ecol Lett* 13:68–75
- Doney SC, Ruckelshaus M, Duffy JE, Barry JP, Chan F, English CA, Galindo HM, Grebmeier JM, Hollowed AB, Knowlton N, Polovina J, Rabalais NN, Sydeman WJ, Talley LD (2012) Climate change impacts on marine ecosystems. *Annu Rev Mar Sci* 4:11–37
- Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* 61:243–282
- Filho DW, Boveris A (1993) Antioxidant defences in marine fish—II. Elasmobranchs. *Comp Biochem Physiol C* 106:415–418
- Frommel AY, Maneja R, Lowe D, Pascoe CK, Geffen AJ, Folkvord A, Piatkowski U, Clemmesen C (2014) Organ damage in Atlantic herring larvae as a result of ocean acidification. *Ecol Appl* 24:1131–1143
- Gattuso J-P, Hansson L (2011) Ocean acidification: background and history. In: Gattuso J-P, Hansson L (eds) *Ocean acidification*. Oxford University Press, Oxford, pp 1–17
- Hanna J, Meides A, Zhang DP, Finley D (2007) A ubiquitin stress response induces altered proteasome composition. *Cell* 129:747–759
- Heinrich DDU, Rummer JL, Morash AJ, Watson S-A, Simpfendorfer CA, Heupel MR, Munday PL (2014) A product of its environment: the epaulette shark (*Hemiscyllium ocellatum*) exhibits physiological tolerance to elevated environmental CO₂. *Conservation Physiology* 2(1):cou047–cou047
- Heinrich DDU, Watson S-A, Rummer JL, Brandl SJ, Simpfendorfer CA, Heupel MR, Munday PL (2016) Foraging behaviour of the epaulette shark *Hemiscyllium ocellatum* is not affected by elevated CO₂. *ICES J Mar Sci: J Conseil* 73(3):633–640
- Heuer RM, Grosell M (2014) Physiological impacts of elevated carbon dioxide and ocean acidification on fish. *Am J Physiol—Regul Integr Comp Physiol* 307:R1061–R1084
- Hoeijmakers JHJ (2009) DNA damage, aging and cancer. *N Engl J Med* 361(15):1475–1485
- Hofmann GE, Barry JP, Edmunds PJ, Gates RD, Hutchins DA, Klinger T, Sewell MA (2010) The effect of ocean acidification on calcifying organisms in marine ecosystems: an organism-to-ecosystem perspective. *Annu Rev Ecol Evol Syst* 41:127–147
- Hu M, Li L, Sui Y, Li J, Wang Y, Lu W, Dupont S (2015) Effect of pH and temperature on antioxidant responses of the thick shell mussel *Mytilus coruscus*. *Fish Shellfish Immunol* 46:573–583
- Johansson LH, Borg LA (1988) A spectrophotometric method for determination of catalase activity in small tissue samples. *Anal Biochem* 174(1):331–336
- Kambayashi Y, Binh NT, Asakura HW, Hibino Y, Hitomi Y, Nakamura H, Ogino K (2009) Efficient assay for total antioxidant capacity in human plasma using a 96-well microplate. *J Clin Biochem Nutr* 44:46–51
- Kroeker KJ, Kordas RL, Crim RN, Singh GG (2010) Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. *Ecol Lett* 13:1419–1434
- Kumar R (2009) Role of naturally occurring osmolytes in protein folding and stability. *Arch Biochem Biophys* 49:1–6. <https://doi.org/10.1016/j.abb.2009.09.007>
- Lawrence RA, Burk RF (1976) Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun* 71:952–958
- Lesser MP (2006) Oxidative stress in marine environments: biochemistry and physiological ecology. *Annu Rev Physiol* 68:253–278
- Lewis E, Wallace DWR (1998) CO₂SYS-Program developed for the CO₂ system calculations. Report ORNL/CDIAC-105
- López-Cruz RI, Dafre AL, Filho DW (2012) Oxidative stress in sharks and rays. In: D Abele Vázquez-Medina JP, Zenteno-Savín T (eds) *Oxidative stress in aquatic ecosystems*. Wiley-Blackwell, Oxford, pp 157–163
- Lund R, Grogan ED (2004) The origin and relationships of early Chondrichthyes. In: Musick JA, Carrier JC, Heithaus MR (eds) *Biology of sharks and their relatives*. CRC Press Inc, Boca Raton, pp 3–31
- Lüthi D, Le Floch M, Bereiter B, Blunier T, Barnola J-M (2008) High-resolution carbon dioxide concentration record 650,000–800,000 years before present. *Nature* 453:379–382
- MacLellan RJ, Tunnah L, Barnett D, Wright PA, MacCormack T, Currie S (2015) Chaperone roles for TMAO and HSP70 during hyposmotic stress in the spiny dogfish shark (*Squalus acanthias*). *J Comp Physiol B* 185:729–740
- MacIouf J, Grassi J, Pradelles P (1987) Development of enzyme-immunoassay techniques for measurement of eicosanoids. In:

- Walden TL, Hughes HN (eds) Prostaglandin and lipid metabolism in radiation injury. Springer, New York, pp 355–364
- McCord JM, Fridovich I (1969) Superoxide dismutase an enzymic function for erythrocyte (hemocuprein). *J Biol Chem* 244:6049–6055
- Mehrbach C, Culbertson C, Hawley J, Pytkowicz R (1973) Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol Oceanogr* 18:897–907
- Morrison J (1954) The activation of aconitase by ferrous ions and reducing agents. *Biochem J* 58:685–692
- Nagelkerken I, Munday PL (2015) Animal behaviour shapes the ecological effects of ocean acidification and warming: moving from individual to community-level responses. *Glob Chang Biol* 22:974–989
- Njemini R, Lambert M, Demanet C, Mets T (2005) Heat shock protein 32 in human peripheral blood mononuclear cells: effect of aging and inflammation. *J Clin Immunol* 25:405–417
- NOAA (2017) Trends in atmospheric carbon dioxide. **Global Greenhouse Gas Reference Network**
- Orr JC, Fabry VJ, Aumont O, Bopp L, Doney SC, Feely RA, Gnanadesikan A, Gruber N, Ishida A, Joos F, Key RM, Lindsay K, Maier-Reimer E, Matear R, Monfray P, Mouchet A, Najjar RG, Plattner G-K, Rodgers KB, Sabine CL, Sarmiento JL, Schlitzer R, Slater RD, Totterdell IJ, Weirig M-F, Yamanaka Y, Yool A (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* 437:681–686
- Pamplona R, Constantini D (2011) Molecular and structural antioxidant defenses against oxidative stress in animals. *Am J Physiol Regul Integr Comp Physiol* 301:R843–R863
- Pimentel M, Pegado MR, Repolho T, Rosa R (2014) Impact of ocean acidification in the metabolism and swimming behavior of the dolphinfish (*Coryphaena hippurus*) early larvae. *Mar Biol* 161:725–729
- Pimentel MS, Faleiro F, Diniz M, Machado J, Pousão-Ferreira P, Peck MA, Pörtner HO, Rosa R (2015) Oxidative stress and digestive enzyme activity of flatfish larvae in a changing ocean. *PLoS One* 10:e0134082
- Pistevos JC, Nagelkerken I, Rossi T, Olmos M, Connell SD (2015) Ocean acidification and global warming impair shark hunting behaviour and growth. *Sci Rep* 5:16293
- Pörtner H-O, Karl DM, Cheung WWL, Lluch-Cota SE, Nojiri Y, Schmidt DN, Zavialov PO (2014) Ocean systems. In: Field CB, Barros VR, Dokken DJ, Mach KJ, Mastrandrea MD (eds) *Climate change 2014: impacts, adaptation and vulnerabilities. part A: global and sectorial aspects contribution of working group II to the fifth assessment report of the intergovernmental panel on climate change*. Cambridge, United Kingdom and New York, USA, pp 411–484
- Ridgwell A, Zeebe RE (2005) The role of the global carbonate cycle in the regulation and evolution of the earth system. *Earth Planet Sci Lett* 234:299–315
- Rosa R, Baptista M, Lopes VM, Pegado MR, Paula JR, Trübenbach K, Leal MC, Calado R, Repolho T (2014) Early-life exposure to climate change impairs tropical shark survival. *Proc R Soc Lond B Biol Sci* 281:20141738
- Rosa R, Paula JR, Sampaio E, Pimentel M, Lopes AR, Baptista M, Guerreiro M, Santos C, Campos D, Almeida-Val VMF, Calado C, Diniz M, Repolho T (2016a) Neuro-oxidative damage and aerobic potential loss of sharks under elevated CO₂. *Mar Biol* 163:119
- Rosa R, Pimentel M, Galan JG, Baptista M, Lopes VM, Couto A, Guerreiro M, Sampaio E, Castro J, Santos C, Calado C, Repolho T (2016b) Deficit in digestive capabilities of bamboo shark early stages under climate change. *Mar Biol* 163:1–5
- Rosa R, Rummer JL, Munday PL (2017) Biological responses of sharks to ocean acidification. *Biol Lett* 13:20160796
- Rudneva II (1997) Blood antioxidant system of black sea elasmobranch and teleosts. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 118:255–260
- Rudneva II (1999) Antioxidant system of Black Sea animals in early development. *Comp Biochem Physiol C Toxicol Pharmacol* 122:265–271
- Rudneva II, Dorokhova II, Skuratovskaya EN, Kuz'minova NS (2014) Comparative studies of hepatic and blood biomarkers in three species of black sea elasmobranchs. *Int J Mar Sci* 4:1–14
- Rummer JL, Munday PL (2016) Climate change and the evolution of reef fishes: past and future. *Fish Fish* 18:22–39
- Sampaio E, Lopes AR, Francisco S, Paula JR, Pimentel M, Maulvault AL, Repolho T, Grilo TF, Pousão-Ferreira P, Marques A, Rosa R (2018) Ocean acidification dampens physiological stress response to warming and contamination in a commercially-important fish (*Argyrosomus regius*). *Sci Total Environ* 618:388–398
- Sarazin G, Michard G, Prevot F (1999) A rapid and accurate spectroscopic method for alkalinity measurements in seawater samples. *Water Res* 33:290–294
- Sejersted Y, Aasland AL, Bjørås M, Eide L, Saugstad OD (2009) Accumulation of 8-oxoguanine in liver DNA during hyperoxic resuscitation of newborn mice. *Pediatr Res* 66(5):533–538
- Shen J, Deininger P, Hunt JD, Zhao H (2007) 8-hydroxy-2'-deoxyguanosine (8-OH-dG) as a potential survival biomarker in patients with nonsmall-cell lung cancer. *Cancer* 109:574–580
- Silva CSE, Novais SC, Lemos MFL, Mendes S, Oliveira AP, Gonçalves EJ, Faria AM (2016) Effects of ocean acidification on the swimming ability, development and biochemical responses of sand smelt larvae. *Sci Total Environ* 563–564:89–98
- Solé M, Rodríguez S, Papiol V, Maynou F, Cartes JE (2009) Xenobiotic metabolism markers in marine fish with different trophic strategies and their relationship to ecological variables. *Comparative Biochemistry and Physiology, Part C* 149:83–89
- Sswat M, Stiasny MH, Jutfelt F, Riebesell U, Clemmesen C (2018) Growth performance and survival of larval Atlantic herring, under the combined effects of elevated temperatures and CO₂. *PLoS One* 13(1):e0191947
- Stiasny MH, Mittermayer FH, Sswat M, Voss R, Jutfelt F, Chierici M, Puvanendran V, Mortensen A, Reusch TBH, Clemmesen C (2016) Ocean acidification effects on Atlantic cod larval survival and recruitment to the fished population. *PLoS One* 11:e0155448
- Stohs S, Bagchi D (1995) Mechanisms in the toxicity of metal ions. *Free Radic Biol Med* 18(2):321–336
- Sunday JM, Calosi P, Dupont S, Munday PL, Stillman JH, Reusch TBH (2014) Evolution in an acidifying ocean. *Trends Ecol Evol* 29:117–125
- Tomanek L (2010) Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. *J Exp Biol* 213:971–979
- Tomanek L, Zuzow MJ, Ivanina AV, Beniash E, Sokolova IM (2011) Proteomic response to elevated P_{CO2} level in eastern oysters, *Crassostrea virginica*: evidence for oxidative stress. *J Exp Biol* 214:1836–1844
- Uchiyama M, Mihara M (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem* 86:271–278
- Wang X, Wu L, Aouffen M, Mateescu M-A, Nadeau R, Wang R (1999) Novel cardiac protective effects of urea: from shark to rat. *Br J Pharmacol* 128:1477–1484
- Wittmann AC, Pörtner H-O (2013) Sensitivities of extant animal taxa to ocean acidification. *Nat Clim Chang* 3:995–1001
- Wood CM, Liew HJ, Boeck GD, Walsh PJ (2013) A perfusion study of the handling of urea analogues by the gills of the dogfish shark (*Squalus acanthias*). *PeerJ* 1:e33
- Zeebe RE, Ridgwell A (2011) Past changes in ocean carbonate chemistry. In: Gattuso J-P, Hansson L (eds) *Ocean acidification*. Oxford University Press, Oxford, New York, pp 21–37