



# Effects of tidal emersion and marine heatwaves on cuttlefish early ontogeny

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## Abstract

Anthropogenic climate change has increased the frequency and intensity of extreme weather events, such as marine heatwaves (MHW). They exert a strong influence over the structure and functioning of marine ecosystems, considering temperature is one of the most critical environmental factors affecting marine life. Additionally, intertidal habitats are ecologically challenging ecosystems, and inhabiting organisms need to possess the necessary mechanisms to adapt to the periodic fluctuations in physical characteristics across tidal cycles. To assess the effect of simulated MHWs (categories I and IV) and low tide conditions (emersion) on the early development of the common cuttlefish *Sepia officinalis*, the development time and hatching success were evaluated, as well as the antioxidant enzymatic machinery, lipid peroxidation, HSP70 and total ubiquitin concentrations. Embryonic development time decreased significantly with temperature, but tidal emersion held no significant impact on development time or hatching success. Superoxide dismutase activity levels were significantly increased with temperature but lowered under emersion conditions. Glutathione-S-transferase activity significantly increased with temperature, while glutathione peroxidase activity was significantly enhanced under emersion. Catalase activity, lipid peroxidation, HSP70 content and total ubiquitin content were not affected by any of the treatments. These findings suggest that while development time is greatly conditioned by temperature, *S. officinalis* embryos are remarkably resilient to emersion conditions. Moreover, the simulated marine heatwaves did not elicit any sub-lethal oxidative stress-related effects, suggesting that such temperatures were still within the optimum range of the cuttlefish thermal window of aerobic performance.

**Keywords** Cuttlefish · Embryogenesis · Marine heatwaves · Emersion · Oxidative stress

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## Introduction

Over the past centuries, human-induced climate change has affected ecosystems worldwide, with significant implications for their services (Chiabai et al. 2018; Gulev et al. 2021; Redlin and Gries 2021). Anthropogenic greenhouse gas forcing, in particular, has been deemed the main factor influencing the increase of seawater surface temperatures (SSTs), with future SSTs projected to increase by up to 5.7 °C above pre-industrial values by the end of the century (Eyring et al. 2021; Gulev et al. 2021; Lee et al. 2021). Along with long-term warming, the frequency and intensity of extreme temperature events have been increasing as a byproduct of anthropogenic climate change (Perkins et al. 2012). Marine heatwaves (MHWs) are an example of these climatic extremes, defined as prolonged discrete warm water events characterized by their duration, intensity and spatial extent (Hobday et al. 2016, 2018). MHWs strongly

influence marine ecosystems by affecting the development time, metabolism, survival and reproduction of organisms, and as a consequence of rising greenhouse gases emissions, the intensity and duration of such events have increased by 54% since the last century, being expected to continue to rise in the future (Oliver et al. 2018, 2019).

Alongside, intertidal environments represent some of the most challenging environments in the coastal marine systems, subject to rapid and periodic fluctuations in physical characteristics across tidal cycles (Helmuth et al. 2006; Teixeira et al. 2013). Low tides expose resident organisms to thermal stress, aerial emersion and desiccation, all of which influence their physiological state, growth and reproduction (Aguilera and Rautenberger 2011; Freire et al. 2011). As such, shallow water and intertidal organisms must display the necessary adaptive strategies to successfully thrive in such dynamic habitats (Teixeira et al. 2013). Two relevant mechanisms involved in dealing with these adversities are the heat shock response (HSR) and the anti-oxidative system (Dong et al. 2008; Teixeira et al. 2013; Axenov-Gribanov et al. 2014; Hawkins et al. 2016). The HSR prevents the unfolding of protein structures as a result of temperature fluctuations, hypoxia or excessive irradiation, and is characterized by the synthesis of heat shock proteins (HSPs) (Tomanek 2010; Yamashita et al. 2010; Logan and Somero 2011). The anti-oxidative system includes a group of antioxidant enzymes that act towards the detoxification of reactive oxygen species (ROS), and oxygen derivatives that are overproduced under the environmental fluctuations typical of intertidal conditions (Lesser 2011). Toxic effects of ROS are indicative of oxidative stress and range from deleterious effects on proteins and DNA to a mechanism of cellular injury termed lipid peroxidation (LPO), which targets membrane-associated lipids and is quantified by assessing the levels of the terminal product malondialdehyde (MDA) (Abele and Puntarulo 2004; Lesser 2006; Bardaweel et al. 2018; Ighodaro and Akinloye 2018). The enzymes acting against ROS include superoxide dismutase (SOD), which converts the superoxide radical  $O_2^-$  into hydrogen peroxide ( $H_2O_2$ ); catalase (CAT) and glutathione peroxidase (GPx), which prevent the accumulation of  $H_2O_2$  in cells; and glutathione-S-transferase (GST), which deactivates secondary metabolites originating from oxidative stress (Abele and Puntarulo 2004; Lesser 2006; Belcheva and Chelomin 2011; Teixeira et al. 2013; Rosa et al. 2014, 2016; Ighodaro and Akinloye 2018).

The common cuttlefish (*Sepia officinalis*) is an ecologically and commercially important cephalopod species (Wang et al. 2003; Jereb and Roper, 2005; Jereb et al 2015). It is a semelparous organism characterized as having an active nekton-benthic lifestyle and hatchlings with direct development (Guerra 2006). *S. officinalis* is predominantly found on sandy and muddy substrates of the continental

shelf, from the shoreline (2–3 m depth) to depths of approximately 200 m (Jereb and Roper, 2005; Guerra 2006; Jereb et al 2015). Previous studies have shown that *S. officinalis* possesses remarkable abiotic tolerance, not only to variations in salinity but also environmental fluctuations in ocean  $O_2$  partial pressure ( $pO_2$ ) and  $CO_2$  partial pressure ( $pCO_2$ ) (Feely et al. 2008; Melzner et al. 2013; Rosa et al. 2013; Hu 2016; Moura et al. 2019). Consequently, it has a wide distributional range, covering the North West coast of Africa, the Mediterranean Sea and the Eastern North Atlantic (Guerra 2006). The temperature range of *S. officinalis* spans from 10 to 30 °C, which is a key factor in mediating the reproductive processes of the species, as individuals undergo seasonal migrations from deeper to shallower waters where mating and spawning occur (Reid et al. 1998; Guerra 2006; Pierce et al. 2010; Bonnaud et al. 2013). Eggs are laid at depths rarely deeper than 40 m, and embryos develop within egg capsules, which act as protection against desiccation and as a barrier to gases diffusion (Boletzky 1986; Reid et al. 1998; Bonnaud et al. 2013).

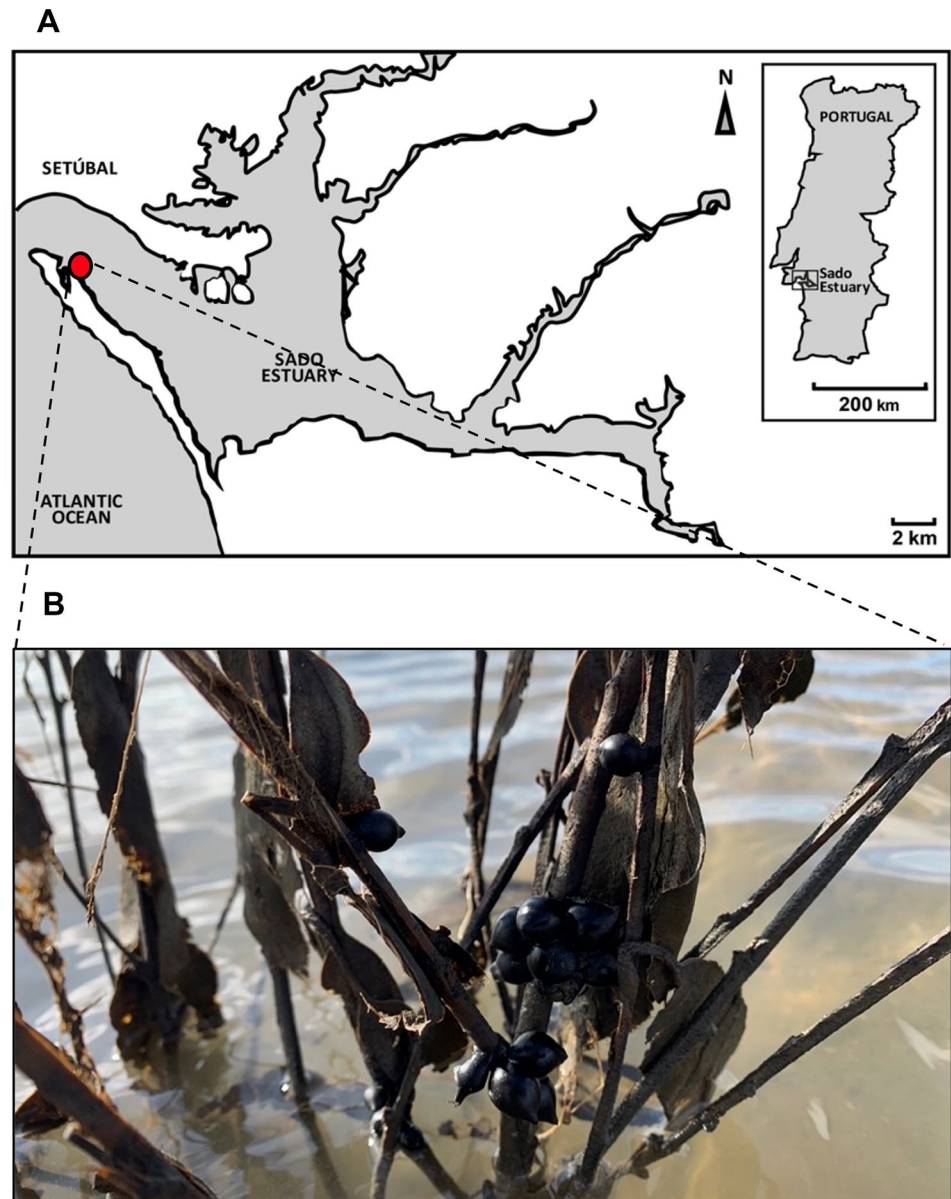
To the best of our knowledge, there are no studies addressing the biological effects of realistic MHW conditions on *S. officinalis* early ontogeny. This study aims to fill this knowledge gap and to bring a novel insight about our understanding on how cephalopod early stages are affected by tidal emersion—an abiotic characteristic of the shallow waters where cuttlefish spawning can take place (Fig. 1).

## Methods

### Egg collection and laboratory acclimation

During early October 2020, recently spawned egg clusters of common cuttlefish (*S. officinalis*) were collected during low tide in the Sado estuary (west coast of Portugal; 38°29'18.42"N; 8°53'15.12"W; Fig. 1). Subsequently, eggs ( $N=720$ ) were transported (under controlled conditions) to Laboratório Marítimo da Guia (MARE-ULisboa, Cascais, Portugal). Upon arrival, eggs from different clutches were evenly distributed across eighteen rectangular-shaped experimental tanks ( $n=40$  per tank), to control for the developmental stage. Three semi-open life support systems (LSS, 92.4 L total volume each) housed 6 experimental tanks (5L volume) each, being supplied with mechanically filtered (down to 5  $\mu$ m) and UV-sterilized natural seawater (NSW). Photoperiod was maintained according to prevailing natural conditions (15 h/9 h, light/dark cycle), via T8 LED overhead illumination. The LSS were equipped with biological (ouriço®, Fernando Ribeiro Lda, Portugal) and mechanical (protein skimmer Reef SkimPro, TMC Iberia, Portugal) filtration. Water temperature was independently controlled by a custom-made digital STC-3000

**Fig. 1** Location of the sampling location in Sado estuary, west coast of Portugal ( $38^{\circ}29'18.42''$  N;  $8^{\circ}53'15.12''$  W; red square; panel A). Panel B shows cuttlefish eggs under emersion, during low tide, in the sampling site



temperature-controlling system. Upon demand, temperature was increased through submerged digital thermostats (V2Therm, 200 W, TMC Iberia, Portugal) or decreased using seawater chillers (Hailea, HC-250A, Guangdong, China). Temperature, pH, salinity and dissolved oxygen (DO) were monitored daily, using a portable pH/mV meter (pHEnomenal<sup>®</sup> pH 1100 H, VWR), a digital refractometer (HI98319, Hanna Instruments) and a portable DO meter (DO 220 M, VWR), respectively. Total ammonia, nitrite and nitrate levels were monitored daily and kept below detectable levels. Laboratory acclimation was performed during a period of 8 days, so that the eggs are exposed to experimental conditions for at least two-thirds of their embryogenesis.

### Experimental exposure

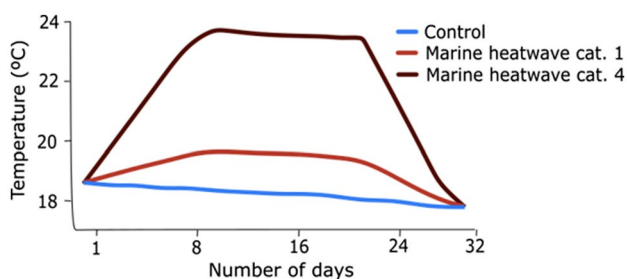
After the acclimation period, the eggs were randomly distributed across six experimental treatments (two treatments per LSS, each treatment comprising three replicates each; Supplemental Figure 1):

- (i) Control I (Control temperature, Immersion);
- (ii) Control E (Control temperature, Periodic Emersion);
- (iii) HW1I (MHW category I scenario, Immersion);
- (iv) MHW1E (MHW category I scenario, Periodic Emersion);
- (v) MHW4I (MHW category IV scenario, Immersion) and

- (vi) MHW4E (MHW category IV scenario, Periodic Emersion).

A thirty-year (1989–2019) dataset for SST in Sado estuary (Setúbal, Portugal) was acquired from the program Climate Data Record (CDR) of the National Oceanic and Atmospheric Administration (NOAA) (Banzon et al. 2016). The R package *heatwaveR* (Schlegel & Smit 2018) was used to determine the average duration (12 days), the maximum temperatures registered (19.7 and 23.8 °C, corresponding to a MHW categories I and IV, respectively), and the climatology (control) values (average of 18.2 °C). This package calculates and displays MHWs according to the definition by Hobday et al. (2018), wherein a moderate MHW (category I) occurs as SSTs surpass the 90th percentile compared with local historical climatology, and an extreme MHW (category IV) occurs as SSTs exceed four times that threshold (the respective projections for the different experimental treatments are shown in Fig. 2).

For the immersion scenarios ( $n = 3$ , Control I, MHW1I and MHW4I), replicate tanks were left submerged throughout the entire experimental period. Regarding the periodic emersion scenarios ( $n = 3$ , Control E, MHW1E and MHW4E) exposure to simulated high/low tide environment was performed via periodic oscillations (two high-low tide cycles per day) in seawater level, accomplished through an automatic control system (Profilux 3.1 N, GHL, Kaiserslautern, Germany) connected to height submerged pumps, which created and controlled high/low tide conditions on a daily basis. The emersion period followed a semi-diurnal tide cycle, characteristic of the Portuguese coast, which emerges cuttlefish during three hours, twice a day. LSS specifications and abiotic control were performed as previously described (i.e., laboratory acclimation). The eggs were exposed to experimental conditions for 30 days. Upon hatching, development time from collection of the eggs from the estuary was registered, and hatching success was calculated as the number of live hatchlings divided by the total number of fertilized eggs.



**Fig. 2** Marine heatwave (MHW) treatments over the course of the exposure period

## Biochemical analyses

### Sample preparation

After the 30-day exposure, cuttlefish were collected upon hatching (Control I,  $n = 89$ ; Control E,  $n = 97$ ; MHW1 I,  $n = 94$ ; MHW1 E,  $n = 94$ ; MHW4 I,  $n = 101$ ; MHW4 E,  $n = 90$ ), transferred to 2-mL Eppendorfs, instantly frozen at  $-80$  °C and stored for biochemical analyses. Hatchling samples were homogenized in 3 mL of phosphate-buffered saline solution (PBS) (pH 7.4) (140 mM NaCl, 3 mM KCl, 10 mM disodium phosphate ( $\text{Na}_2\text{HPO}_4$ ), 2 mM potassium phosphate ( $\text{KH}_2\text{PO}_4$ )), using an electrical tissue grinder (Warsley, Techn Germany). The homogenates were divided by two 1.5 mL microtubes, all of which were centrifuged (15 min at  $10,000\times g$ , at 4 °C) (VWR, Hitachi Koki Co., Ltd). Subsequently, the supernatant was transferred to 1.5 mL microtubes and frozen ( $-20$  °C) until further analyses. Enzymatic activity and protein concentration were measured according to the supernatant fraction of the samples. In subsequent assays, each sample was run in duplicate.

### Total protein content

To measure the concentration of total protein per sample, the method described by Bradford (1976) was used and optimized for 96-well microplates (Greiner Bio-One, Germany). A calibration curve was built using bovine serum albumin (BSA) (Sigma-Aldrich, USA) as a standard. The stock solution (4.0 mg/mL) was prepared in PBS and then serial dilutions were performed to obtain a standard curve (0–4.0 mg/mL). To carry out the assay, 20  $\mu\text{L}$  of protein standard or sample were added to 180  $\mu\text{L}$  of Bradford reagent in each well of 96-well microplates (Greiner Bio-One, Austria). The absorbance at 595 nm was recorded using a microplate reader (BioTek Synergy HTX multi-mode reader, USA). The results were used to express the analyzed biomarkers per mg of total protein.

### Glutathione S-transferase (GST) activity

GST activity was determined according to the method described by Habig et al. (1974) and adapted for 96-well microplates (Greiner Bio-One, Germany). In this assay, 1-chloro-2,4-dinitrobenzene (CDNB) was used as substrate, and enzymatic activity was measured according to the formation of a conjugate between CDNB and reduced L-glutathione (GSH). A reagent mix solution was prepared, consisting of 20 mM GSH (Sigma-Aldrich, USA), 100 mM CDNB solution (Sigma-Aldrich, USA) and 9.8 mL of PBS. To perform the assay, 180  $\mu\text{L}$  of the substrate solution were added to each well of a 96-well microplate (Greiner Bio-One, Austria), followed by adding 20  $\mu\text{L}$  of GST standard or



sample. The absorbance at 340 nm was recorded every minute, for 6 min, using a microplate reader (BioTek Synergy HTX multi-mode reader, USA). The variation in absorbance per minute ( $\Delta A_{340}/\text{min}$ ) was determined for each sample, and the reaction rate was determined using the CDNB molar extinction coefficient of  $0.0053 \mu\text{M}^{-1} \text{cm}^{-1}$ .

### Glutathione peroxidase (GPx) activity

GPx activity was determined according to the method described by Lawrence and Burk (1976) and adapted for 96-well microplates (Greiner Bio-One, Germany). An assay buffer [50 mM  $\text{KH}_2\text{PO}_4$  buffer (pH 7.4) (Sigma-Aldrich); 5 mM ethylenediaminetetraacetic acid (EDTA) (Riedel-Haën)] and a co-substrate mixture [4 mM sodium azide ( $\text{NaN}_3$ ) (Sigma-Aldrich); 1 mM beta nicotinamide adenine dinucleotide phosphate ( $\beta$ -NADPH) (Sigma-Aldrich); 4 U/mL glutathione reductase (Sigma-Aldrich, USA); 4 mM GSH (Sigma-Aldrich, USA)] were prepared. To perform the assay, assay buffer (120  $\mu\text{L}$ ) was added to each well, followed by 50  $\mu\text{L}$  of the co-substrate mixture and 20  $\mu\text{L}$  of sample or standard. To start the reaction, 20  $\mu\text{L}$  of a cumene hydroperoxide solution (Sigma-Aldrich, USA) was added to each well, and the microplates were lightly shaken. The absorbance (340 nm) was read at 25 °C, for 6 min, using a microplate reader (BioTek Synergy HTX multi-mode reader, USA). Variations in absorbance per minute ( $\Delta A_{340}/\text{min}$ ) were estimated for each sample. To determine GPx activity, the molar extinction coefficient ( $3.73 \text{mM}^{-1} \text{cm}^{-1}$ ) for  $\beta$ -NADPH was used.

### Superoxide dismutase (SOD) activity

SOD activity was determined according to the procedure by Sun et al. (1988), adapted for 96-well microplates. To perform the assay, 200  $\mu\text{L}$  of buffer (50 mM  $\text{KH}_2\text{PO}_4$  buffer (pH 8.0), 10  $\mu\text{L}$  of 3 mM EDTA (Riedel-Haën)), 10  $\mu\text{L}$  of 3 mM xanthine solution (Sigma-Aldrich), 10  $\mu\text{L}$  of 0.75 mM nitroblue tetrazolium (NBT) (Sigma-Aldrich) and 10  $\mu\text{L}$  of sample were added to each well of a 96-well microplate (Greiner, Bio-One, Austria). Next, 10  $\mu\text{L}$  of 100 mU xanthine-oxidase (XOD) was added to each well to initiate the reaction. The absorbance at 550 nm was read at 25 °C, for 15 min, using a microplate reader (BioTek Synergy HTX multi-mode reader, USA). Variations in absorbance per minute ( $\Delta A_{550}/\text{min}$ ) were estimated for each sample, and the enzyme inhibition percentage (normalized by total protein) was determined.

### Catalase (CAT) activity

To determine CAT activity, the method followed Beers and Sizer (1952), adapted for 96-well microplates. A solution of

50 mM  $\text{KH}_2\text{PO}_4$  buffer containing  $\text{H}_2\text{O}_2$  [0.0036% (w/w)] (Sigma-Aldrich, USA) was prepared. To perform the assay, 7  $\mu\text{L}$  of sample and 193  $\mu\text{L}$  of the buffer- $\text{H}_2\text{O}_2$  solution were added to each well of a 96-well microplate (Greiner, Bio-One UV-Star, Austria). The absorbance at 240 nm was read at 25 °C, at 42-s intervals, for a total of 2 min and 48 s, using a microplate reader (BioTek Synergy HTX multi-mode reader, USA). Variations in absorbance per minute ( $\Delta A_{240}/\text{min}$ ) were estimated for each sample. To determine CAT activity, the molar extinction coefficient for  $\text{H}_2\text{O}_2$  of  $0.04 \mu\text{M}^{-1} \text{cm}^{-1}$  was used.

### Lipid peroxidation (LPO)

LPO was measured by quantifying MDA concentration, following the method described by Ohkawa et al. (1979) (Pannunzio and Storey 1998) and adapted to 96-well microplate (Greiner, Bio-One, Austria). To perform the assay, 5  $\mu\text{L}$  of sample were added to each microtube, followed by 45  $\mu\text{L}$  of PBS, 12.5  $\mu\text{L}$  of 8.1% sodium dodecyl sulfate (SDS) (Merck), 93.5  $\mu\text{L}$  of 20% trichloroacetic acid (TCA) (Panreac), 93.5  $\mu\text{L}$  of 1% thiobarbituric acid (TBA) (Sigma-Aldrich, USA) and 50.5  $\mu\text{L}$  of milli-Q water. The microtubes were vortexed for 1 min, and the caps were pierced. Each microtube was incubated (100 °C) for 10 min, using a digital thermoblock (D130 Series, Labnet International). After the incubation, the microtubes were briefly placed on ice to cool. An additional 62.5  $\mu\text{L}$  of milli-Q water was added to each microtube. Subsequently, 150  $\mu\text{L}$  (in duplicate) was added to microplate wells. The absorbance was read (530 nm) using a microplate reader (BioTek Synergy HTX multi-mode reader, USA). To quantify lipid peroxidation in samples, a 1  $\mu\text{M}$  MDA stock solution (Merck) was used to construct a calibration curve (0–0.1  $\mu\text{M}$ ).

### Heat shock protein (HSP70) content

HSP70 content was determined through a method of indirect Enzyme-Linked Immunosorbent Assay (ELISA), as described by Njemini et al. (2005). To carry out the assay, 100  $\mu\text{L}$  of standard or sample was added to each well of a 96-well microplate (Greiner, Bio-One, Microton, High Binding Austria), and the microplates were left to incubate overnight at 4 °C. After the incubation, the microplates were washed three times with PBS-Tween-20 (PanReac Appli-Chem, ITW Reagents). After preparing a 1% BSA blocking solution in PBS, 200  $\mu\text{L}$  of it was added to each well, and an incubation of 2 h at room temperature took place. The microplates were washed as mentioned previously. The primary antibody solution (anti-HSP70, Acris Antibodies) (0.5  $\mu\text{g}/\text{mL}$ ) was prepared by performing a dilution in PBS with BSA (1%). Then, 100  $\mu\text{L}$  of the primary antibody solution was added to each well, after which the microplates

were left to incubate for 90 min at 37 °C. The microplates were washed again, and 100 µL of a conjugated secondary antibody solution (anti-mouse IgG, Fc specific, alkaline phosphatase-conjugated, Sigma-Aldrich, USA) were added to each well after the proper dilution (1:1000) of the solution in BSA (1%). The microplates were, once again, incubated for 90 min at 37 °C and washed afterwards. Following the previous step, 100 µL of alkaline phosphatase substrate solution (100 mM NaCl (Panreac, Spain), 100 mM Tris-HCl (Sigma-Aldrich, USA), 50 mM MgCl<sub>2</sub> (Sigma-Aldrich, USA), 27 mM p-nitrophenyl phosphate, disodium salt hexahydrate (PnPP) (pH 8.5) (Sigma-Aldrich, USA)) were added to each microplate well, after which they were left to incubate for 30 min at room temperature. This step was followed by the addition of 100 µL of stop solution (3 M NaOH) (Panreac, Spain) to each well. The absorbance was read, at 405 nm, using a microplate reader (BioTek Synergy HTX multi-mode reader, USA). To determine the HSP70 concentration in each sample, a calibration curve (0–2 µg/mL) was prepared.

### Total ubiquitin (Ub) content

Ubiquitin content was determined by an indirect ELISA, as described by Pegado et al. (2020). To perform the assay, 100 µL of standard or sample was added to each well of a 96-well microplate (Greiner, Bio-One, Microlon, High Binding Austria). Microplates were left to incubate overnight at 4 °C, after which they were washed three times with PBS-Tween-20 (PanReac AppliChem, ITW Reagents). Following the previous step, 200 µL of a 1% BSA blocking solution prepared in PBS was added to each well, and an incubation of 2 h at room temperature took place. Afterwards, microplates were washed as mentioned previously. A primary antibody solution (Ub antibody (P4D1): sc-8017, Santa Cruz Biotechnology) (0.5 µg/mL) was prepared by performing a dilution in PBS with BSA(1%). Next, 100 µL of the primary antibody solution was added to each microplate well, after which they were left to incubate for 90 min at 37 °C. The microplates were washed again, and 100 µL of a conjugated secondary antibody solution (anti-mouse IgG, Fc specific, alkaline phosphatase-conjugated, Sigma-Aldrich, USA) was added to each well after diluting (1:1000) the solution in BSA (1%). The microplates were incubated for 90 min at 37 °C and washed once again as described previously. Then, 100 µL of alkaline phosphatase substrate solution (100 mM NaCl (Panreac, Spain), 100 mM Tris-HCl (Sigma-Aldrich, USA), 50 mM MgCl<sub>2</sub> (Sigma-Aldrich, USA), 27 mM PnPP (pH 8.5) (Sigma-Aldrich, USA)) were added to each well, and the microplates were left to incubate for 30 min at room temperature. This step was followed by the addition of 100 µL of stop solution (3 M NaOH) (Panreac, Spain) to each well. The absorbance was measured, at 405 nm, using a

microplate reader (BioTek Synergy HTX multi-mode reader, USA). To quantify total ubiquitin content per sample, a calibration curve (0–0.8 µg/mL) was prepared using standard purified ubiquitin (Ubiquitin-Proteasome Biotechnologies).

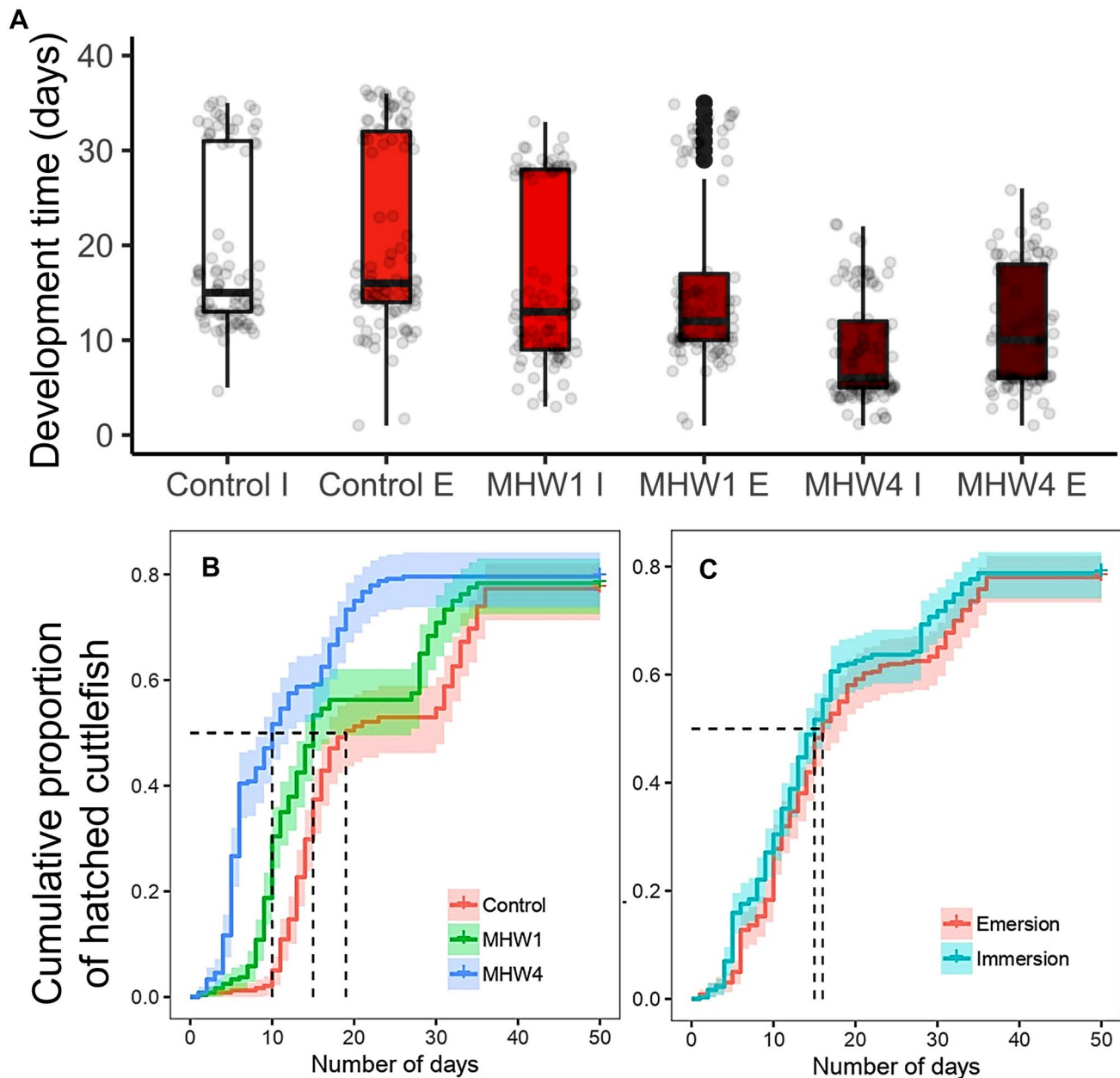
### Statistical analyses

To infer the influence of treatments on both stress-related biomarkers and development time, linear mixed-models (glmmTMB, from the package ‘glmmTMB’) (LMM) with a Gaussian distribution were employed. Treatments were set as a factor with 6 levels, and replicates as a random factor with 18 levels (3 replicates per treatment). Type II Wald chi-square tests were performed on every model, to evaluate the effect of the different treatments on all response variables. Model residuals were analyzed for assumptions of normality and homoscedasticity. Outliers consisting of values superior to 10 times the value of the upper quartile were removed. Significance levels were set at  $p < 0.05$ , and  $p$ -values were adjusted via Tukey corrections. An analysis was performed to determine the effects of emersion and MHWs on survival, and a Cox proportional hazards regression model (‘coxph’, from the package ‘survival’) was fitted to successful hatchings (0/1) and embryonic development time. Temperature was set as a three-level factor and emersion as a two-level factor (co-variates). The assumptions of proportional hazards of the ‘coxph’ models (proportional hazards, no over-influential observations and linearity of co-variates) were not held (Schoenfeld test, represents scaled Schoenfeld residuals over time). Subsequently, a ‘survdiff’ function was fitted, followed by post hoc multiple comparisons (‘pairwise\_survdiff’). Statistical analyses were done in RStudio (version 2021.09.1).

## Results

### Development time and survival

Cuttlefish exhibited a significantly shorter embryonic development under increased temperatures (Control vs. MHW1 condition, *LMM*  $d.f. = 555$ ,  $t$  ratio = 4.138,  $p = 0.0001$ ; Control vs. MHW4 condition, *LMM*  $t$  ratio = 11.033,  $p < 0.0001$ ). On the other hand, tidal-related treatments had no significant effect on development time (Wald chi-squared test,  $d.f. = 1$ ,  $\chi^2 = 3.15$ ,  $p = 0.0762$ ) (Fig. 3; Supplemental Table 1). Hatching success significantly increased in the MHW1 (Cox Model,  $p = 0.0078$ ) and MHW4 ( $p < 0.0001$ ) when compared to control conditions (Fig. 3). Hatching success increased under immersion, but an opposite trend was observed under emersion. However, tidal emersion had no significant effect on hatching success (Cox Model,  $p = 0.2$ ).



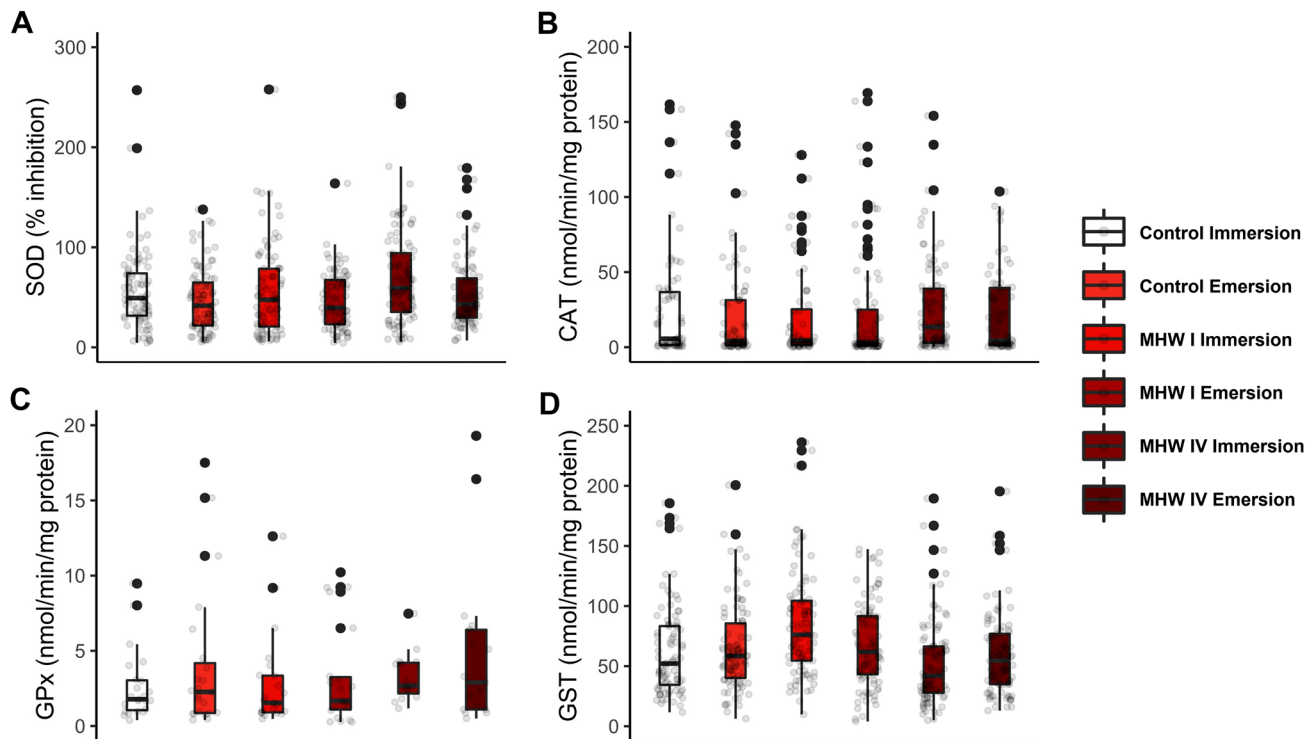
**Fig. 3** Development time of cuttlefish (*Sepia officinalis*) embryos under the different treatments (relative to the day of the first hatching) (A), and cumulative proportions of hatched eggs under different marine heatwave (MHW) (B) and immersion (C) treatments. Boxplots illustrate the upper and lower quartile, and medians are

displayed by an inside bar. Outliers outside the inter-quartile range are displayed by black dots, individual observations are displayed through gray dots. *MHW I* marine heat wave category I, *MHW IV* marine heat wave category IV, *E* emersion, *I* immersion

### Biochemical responses

SOD activity levels increased significantly from the control to the MHW4 conditions (LMM,  $d.f. = 519$ ,  $t$  ratio =  $-2.438$ ,  $p = 0.04$ ), and were observed to significantly decrease when exposed to tidal emersion (Wald chi-squared test,  $d.f. = 1$ ,  $\chi^2 = 9.56$ ,  $p = 0.0020$ ) (Fig. 4). CAT activity levels showed no significant differences among temperature

and tidal-related treatments (Wald chi-squared test,  $d.f. = 2$ ,  $\chi^2 = 0.62$ ,  $p = 0.7325$  and  $d.f. = 1$ ,  $\chi^2 = 0.01$ ,  $p = 0.9345$ , respectively). There were no significant differences in GPx activity levels among MHW treatments (Wald chi-squared test,  $d.f. = 2$ ,  $\chi^2 = 1.66$ ,  $p = 0.4367$ ). However, they were significantly increased by tidal-related treatments (Wald chi-squared test,  $d.f. = 1$ ,  $\chi^2 = 3.99$ ,  $p = 0.046$ ). There was a significant increase in the activity of GSTs under MHW1 when



**Fig. 4** SOD (A), CAT (B), GPx (C) and GST (D) activity levels in cuttlefish (*Sepia officinalis*) embryos under the different treatments. Boxplots illustrate the upper and lower quartile, and medians are displayed by an inside bar. Outliers outside the inter-quartile range

are displayed by black dots, individual observations are displayed through gray dots. *MHW I* marine heat wave category I, *MHW IV* marine heat wave category IV, *E* emersion, *I* immersion

compared to Control (LMM,  $d.f. = 550$ ,  $t$  ratio =  $-3.261$ ,  $p = 0.0149$ ) and MHW4 ( $t$  ratio =  $5.907$ ,  $p < 0.0001$ ). Yet, no significant changes were observed among tidal-related treatments (Wald chi-squared test,  $d.f. = 1$ ,  $\chi^2 = 1.05$ ,  $p = 0.3058$ ).

LPO levels in *S. officinalis* embryos, expressed as MDA concentration, are shown in Fig. 5. No significant variations in LPO were observed among all treatments (Wald chi-squared test,  $d.f. = 2$ ,  $\chi^2 = 2.31$ ,  $p = 0.3144$  and  $d.f. = 1$ ,  $\chi^2 = 0.00$ ,  $p = 0.9807$  for MHW and tidal-related treatments, respectively). HSP70 levels revealed no significant differences under the different MHW scenarios and tidal-related treatments (Wald chi-squared test,  $d.f. = 2$ ,  $\chi^2 = 0.04$ ,  $p = 0.9794$  and  $d.f. = 1$ ,  $\chi^2 = 1.15$ ,  $p = 0.2837$ , respectively). A similar trend was observed in Ub levels (Wald chi-squared test,  $d.f. = 2$ ,  $\chi^2 = 2.49$ ,  $p = 0.2877$  and  $d.f. = 1$ ,  $\chi^2 = 0.77$ ,  $p = 0.3787$ , respectively).

## Discussion

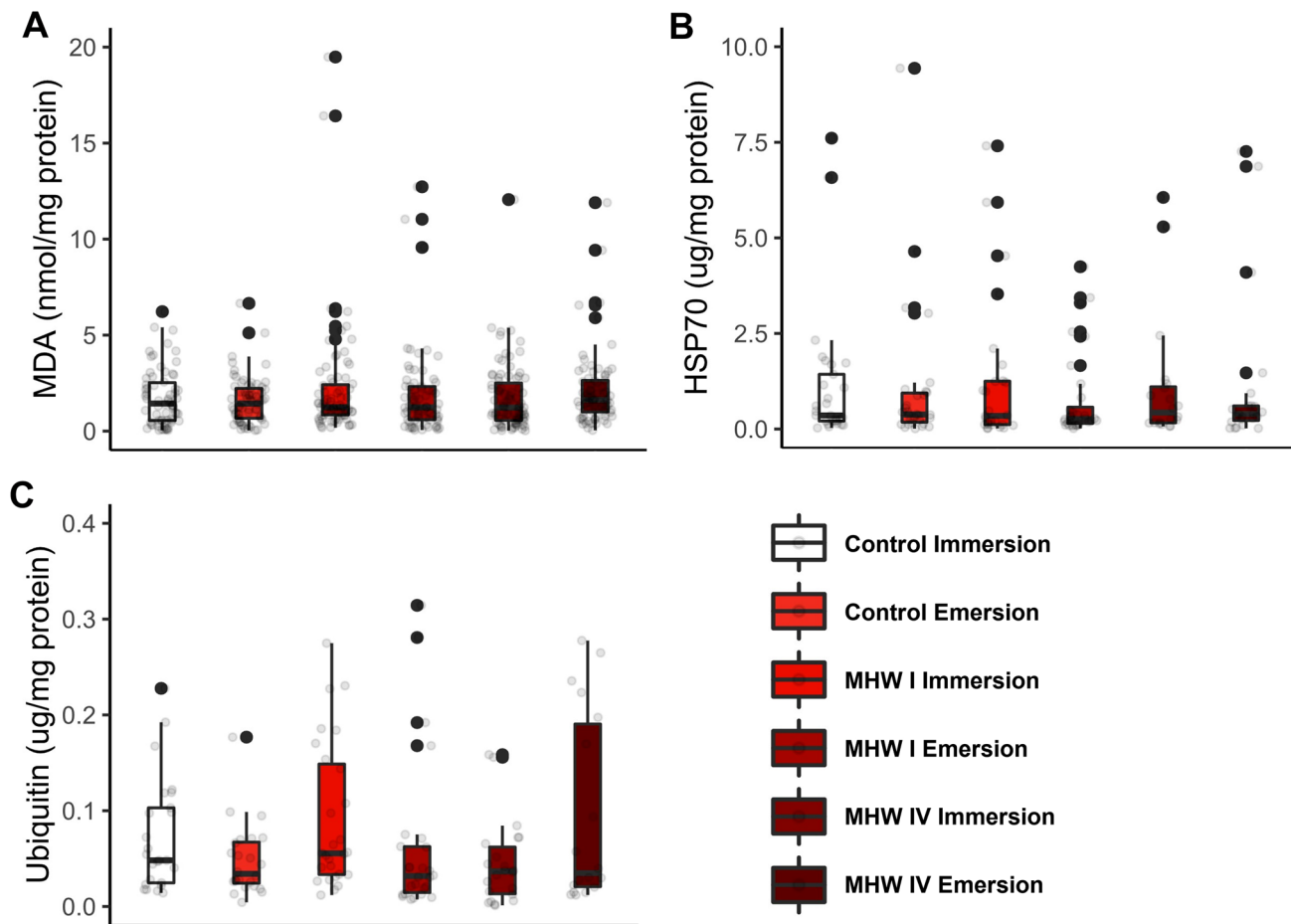
### Development time and survival

In the present study, the development time of *S. officinalis* embryos decreased under warming conditions

(Fig. 3A). In fact, elevated temperatures are known to promote increased metabolic rates in ectotherms (Moran and Woods 2007; Rosa and Seibel 2008; Pimentel et al. 2012), and faster early development (Pimentel et al. 2012; Rosa et al. 2013). Concomitantly, there is a drop in  $pO_2$  levels within eggs, which has also been established as a trigger for early hatching in embryos (Moran and Woods 2007; Rosa et al. 2013).

On other hand, periodic exposure to emersion had no significant impact on development time, showing that *S. officinalis* early stages are tolerant to the effects of air exposure. In intertidal habitats, air exposure is a major stressor to inhabiting organisms, leading to hypoxia and thermal stress (Aguilera and Rautenberger 2011; Teixeira et al. 2013). Yet, *S. officinalis* intertidal spawning grounds (such as those in Sado estuary; Fig. 1) are characterized by significant daily (and seasonal) fluctuations in key abiotic factors (e.g., temperature, salinity, UV radiation), which might confer the required phenotypic tolerance to the simulated emersion periods. It is also worth noting that, in Sado estuary, *S. officinalis* spawning not only occur during spring and summer, but can also extend until late Autumn (Rosa, pers. observation); such extended spawning period also highlights the great phenotypic plasticity of *S. officinalis* early life stages.





**Fig. 5** MDA (A), HSP70 (B) and Ub (C) levels in cuttlefish (*Sepia officinalis*) embryos under the different treatments. Boxplots illustrate the upper and lower quartile, and medians are displayed by an inside bar. Outliers outside the inter-quartile range are displayed by black

dots, individual observations are displayed through gray dots. *MHW I* marine heat wave category I, *MHW IV* marine heat wave category IV, *E* emersion, *I* immersion

Concomitantly, hatching rates increased with temperature during immersion (Fig. 3; Supplemental Table 1). Such results may suggest that the temperature conditions during MHW1 and MHW4 are still within the optimum thermal range for aerobic performance. According to the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis, the range of active thermal tolerance is limited by the “pejus” temperatures, and warming or cooling beyond these leads to a decrease in the ability to perform aerobically, compromising growth and survival (Pörtner and Knust 2007; Pörtner et al. 2017). Therefore, MHW1 and MHW4 with immersion conditions did not seem to exceed such physiological thresholds. Moreover, emersion itself had no significant impact on hatching success (Fig. 3C), highlighting again the high tolerance of cuttlefish embryos to such environmental conditions.

### Biochemical responses

MHW conditions led a significant increase in SOD activity (Fig. 4), which might be expected because higher temperatures lead to a higher mitochondrial activity, thus creating more ROS that are catalyzed by SOD (Lesser 2006; Rosa et al. 2012; Wang et al. 2018; Mariana and Badr 2019). Other studies have also shown that  $O_2^-$  production increases under exposure to other-type of stressors, namely xenobiotics and pollutants, reinforcing SOD as a relevant antioxidant defense (Lesser 2006; Caeiro et al. 2017). Considering that production of ROS directly relates to the concentration of  $O_2$ , the significant decreases in SOD activity observed in embryos exposed to air (Fig. 4) suggest a reduced production of the enzyme’s substrate as a result of lower oxygen availability for aerobic metabolism (Lesser 2006; Teixeira et al.

2013). The activity of CAT, the enzyme that completes the ROS-detoxifying chain reaction initiated by SOD, was not influenced by either the MHW or the emersion treatments (Fig. 4), further reinforcing the tolerance of embryos to oxidative stress-inducing conditions. GPx activity increased significantly when embryos were subjected to emersion (Fig. 4). This is in accordance with previous research, as the subsequent reoxygenation state drives the formation of H<sub>2</sub>O<sub>2</sub>, which GPx removes from cells as a means to oxidize GSH (Lesser 2006; Rosa et al. 2014). The few changes in enzymatic activity observed in the present study may also suggest that embryos possess other means of maintaining homeostasis, such as alterations of general energetic metabolism and a high tolerance to hypoxia (Wolf et al. 1985; Pimentel et al. 2012; Rosa et al. 2013). Hypoxia-tolerant organisms can downregulate their metabolism into a hypometabolic state, thus balancing their ATP demand and supply pathways, allowing them to survive under short-term hypoxic conditions (Rosa and Seibel 2008, 2010; Rosa et al. 2013; Teixeira et al. 2013).

The present study also showed no significant changes in MDA levels among treatments (Fig. 5). The low levels of MDA, and thus LPO, can be indicative of a low amount of oxidative stress or of the antioxidant system working properly. Similar to MDA, HSP70 and Ub also showed no significant changes among treatments (Fig. 5). All these biochemical findings corroborate the idea of a great phenotypic plasticity of *S. officinalis* early life stages. This is also supported by the high number of influential data points, which reflect high inter-individual variability. Interestingly, outliers in biochemical responses were consistently from the same individuals, suggesting that they exhibit high intrinsic variation in antioxidant responses. They also support the notion that when certain abiotic stressors are frequently encountered (here in the dynamic intertidal zones), the organisms develop a type of desensitization against such stressor(s), thus leading to a less drastic biological response or even an absence of it (Reid et al. 1998).

Summing up, *S. officinalis* is an ecologically and commercially important species that is considered a viable alternative resource in the context of the ongoing decline in finfish stocks, and this study showed *S. officinalis* early life stages are well adapted to fluctuating tidal conditions and MHWs. Nonetheless, it is worth noting that MHWs are expected to increase in frequency, duration and intensity in the near future (Hobday et al. 2016, 2018). Therefore, the impacts of repeated and longer extreme temperature events should be investigated. More research on predicting how the common cuttlefish will adapt (or not) in the oceans of tomorrow of paramount importance.

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**Data availability** Data will be made available upon request.

**Code availability** Data will be made available upon request.

## Declarations

**Conflict of interest** All authors declare no conflicts of interest.

**Ethical approval** All the procedures were approved by the FCUL Animal Welfare Committee (ORBEA FCUL) and the Portuguese General-Directorate for Food and Veterinarian Contacts (DGAV) of the Portuguese Government, according to National (Decreto-Lei 113/2013) and the EU legislation (Directive 2010/63/EU) on the protection of animals used for scientific purposes (within the framework of MAR2020—MAR-01.04.02-FEAMP-0007).

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