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ORIGINAL PAPER

Octopus crawling on land: physiological and biochemical responses of *Octopus vulgaris* to emersion

Janina Leonie Röckner[1,2] · Vanessa M. Lopes[1] · José Ricardo Paula[1,4] · Maria Rita Pegado[1] · Martim Costa Seco[1] · Mário Diniz[3,5] · Tiago Repolho[1,4] · Rui Rosa[1,4]

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Abstract

Cephalopods are well known for their cognitive capabilities and unique behavioural repertoires. Yet, certain life strategies and behaviours are still not fully understood. For instance, coastal octopuses have been documented (mainly through citizen science and TV documentaries) to occasionally leave the water and crawl in intertidal areas. Yet, there is a complete lack of knowledge on this behaviour's physiological and biochemical basis. Within this context, this study aimed to investigate, for the first time, physiological (routine and maximum metabolic rates and aerobic scope) and biochemical (i.e., antioxidant enzymes activities, heat shock protein and ubiquitin levels, DNA damage, lipid peroxidation) responses of the common octopus, *Octopus vulgaris*, to emersion. The octopuses' physiological performance was determined by measuring metabolic rates in different emersion treatments and biochemical markers. The size-adjusted maximum metabolic rates (*MMRadj*) of octopuses exposed to 2:30 min of air exposure followed by re-immersion did not differ significantly from the *MMRadj* of the chased individuals (control group). Yet, most biochemical markers revealed no significant differences among the different emersion treatments. Our findings showed that *O. vulgaris* could tolerate exposure to short-term emersion periods due to an efficient antioxidant machinery and cellular repair mechanisms. Alongside, we argue that the use of atmospheric air through the mucus-covered gills and/or cutaneous respiration may also help octopus withstand emersion and crawling on land.

Keywords Tidepools · Air exposure · *Octopus vulgaris* · Metabolism · Oxidative stress

Introduction

Located on rocky intertidal shores, tidepools are unique shallow-water habitats where dramatic fluctuations in abiotic parameters occur daily (Metaxas and Scheibling 1993; Denny and Gains 2007). The temperatures can vary up to 15 ºC within the same tidal cycle (Huggett and Griffiths 1986; Metaxas and Scheibling 1993); salinity can increase between 5 and 25 units above the basal levels (Morris and Taylor 1983; Metaxas and Scheibling 1993). The partial pressure of CO_2 (pCO_2) increases during the night due to a combination of the cessation of photosynthesis, tidepool inhabitants' respiration and isolation (Truchot and Duhamel-Jouve 1980). Concomitantly, the rise in pCO_2 causes a rise in hydrogen ion concentration in the water, thus decreasing pH (Wurts and Durborow 1992). Dissolved oxygen concentrations can vary from oxygen-saturated water to hypoxic and even complete anoxic pools (Seibel and Childress 2000). Therefore, species inhabiting tide pools (and other intertidal habitats) need to be well adapted to these rapidly changing

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✉ Janina Leonie Röckner
janina.roeckner@gmail.com

1 Laboratório Marítimo da Guia, Faculdade de Ciências, MARE, Marine and Environmental Sciences Centre/ ARNET, Aquatic Research Network, Universidade de Lisboa, Cascais, Portugal

2 Faculty of Mathematics and Natural Sciences, GEOMAR, Helmholtz Centre for Ocean Research Kiel, Christian-Albrechts-University of Kiel, Kiel, Germany

3 Applied Molecular Biosciences Unit, Department of Chemistry, School of Science and Technology, UCIBIO, NOVA University Lisbon, 2819-516 Caparica, Portugal

4 Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, Lisbon, Portugal

5 Associate Laboratory i4HB, Institute for Health and Bioeconomy, NOVA School of Science and Technology, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal

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environments, and some of them may even withstand air exposure (emersion) for short periods. For instance, the epaulette shark *Hemiscyllium ocellatum* is known to occur in shallow coral reef waters and occasionally can be fully exposed to air in intertidal habitats. It can survive a certain period of time on land by suppressing neuronal metabolic activity (Renshaw et al. 2002). Alongside, many intertidal species possess an enhanced antioxidant machinery since the transition between hypoxia/anoxia and reoxygenation increases the risk of an overproduction of reactive oxygen species (ROS) (Hermes-Lima and Zenteno-Savín 2002; Storey and Storey 2004; Teixeira et al. 2013; Giraud-Billoud et al. 2019). ROS are free radicals produced as a by-product during cellular aerobic metabolism, which can easily react with other molecules (Turrens 2003). Organisms can compensate for the constantly produced ROS by synthesising antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione-*S*-transferase (GST) (Hermes-Lima and Zenteno-Savín 2002; Storey and Storey 2004; Romero et al. 2007; Teixeira et al. 2013). In this sense, and depending on the extent of environmental stress, higher levels of ROS need to be compensated for by a higher antioxidant defence capacity (Romero et al. 2007).

Although some studies evaluate the impact of hypoxia on cephalopod physiology (e.g., Rosa and Seibel 2008, 2010a, b), there is a lack of knowledge on the tolerance to air emersion in coastal octopuses. They are opportunistic and voracious predators, primarily active during crepuscular periods and at night at subtidal depths (Hanlon and Messenger 2018). Still, some also developed a specific hunting strategy in intertidal areas: crawling out of the water to forage during low tide. This behaviour has been described in several octopus' species worldwide, mainly through citizen reports and media coverage (see Supplemental Table 1). The common octopus, *Octopus vulgaris,* can be found in rock pools in the intertidal zone (Mangold 1983), and it is also known to display this foraging behaviour (Norman 2000). In fact, Aristotle noted that "*the octopus is the only mollusc that ventures on to dry land; it walks by preference on rough ground"* (Aristotle 350 BC see, Thompson 1910; Balme and Gotthelf 1991). Also, Wodinsky (1971) described that "O. vulgaris leave the water, climb on exposed rocks, pull various immobile gastropods (such as *Nerita* spp.) off the rocks, return to the water and feed on them." Additionally, some octopus species in captivity show regular escape behaviour that can result in being outside of water for a sufficient amount of time (Lee 1875; Anderson and Martin 2002; Wood and Anderson 2004).

Although it has been observed for different octopus' species worldwide, no scientific attempt has been made to understand how these marine animals can tolerate air exposure. Without access to oxygen in a terrestrial environment, physiological adaptations are necessary to reduce the risks resulting from anoxic conditions. The common octopus presents suitable morphologies, such as a large gill surface-to-volume ratio, cutaneous respiration, and short diffusion distances from the water to the blood (Houlihan et al. 1982; Eno 1987; Madan and Wells 1996). When oxygen availability is limited, *O. vulgaris* can slow down their heart rate to decrease the mean aorta pressure and change the blood flow to improve the unloading of the hemocyanin at lower internal pO^2-levels (Houlihan et al. 1982). To cover their adenosine triphosphate (ATP) demand at low oxygen levels, octopuses can also enhance anaerobic metabolism using their arginine phosphate storage and opine pathways (Gäde 1976; Pörtner 1994).

Octopus do run the risk of an ROS overproduction after crawling on land. Like other coastal species that are exposed to low tides (Almeida et al. 2005), re-submersion causes an oxygen boost and can enhance ROS production. Therefore, it is suspected that octopus do need an efficient antioxidant mechanism to be able to perform this emersion behaviour.

Within this context, this study aimed to investigate, for the first time, physiological (routine and maximum metabolic rates, aerobic scope and facultative aerobic scope) and biochemical (antioxidant enzymes activities, heat shock protein and ubiquitin levels, DNA damage, lipid peroxidation) responses of the common octopus, *Octopus vulgaris* (in the mantle, gills and digestive gland), to emersion. Prior to the laboratory work, a survey among fishermen from Cascais (Portugal) was conducted to determine how frequent is the foraging behaviour associated to emersion in the local population of *O. vulgaris*.

Material and methods

Fishermen survey

To ensure the veracity of the reported cases (i.e., octopuses crawling out of the water), a survey (see Supplemental Table 2) among 20 fishermen around Cascais area (Portugal) was performed. Fishermen were asked if they had seen octopuses in the tidepools or also crawling on land, during daytime or at night.

Specimen collection and housing

The specimens of *O. vulgaris* were collected in Cascais (38°41′22.4″ N 9°26′18.0″ W) and Figueira da Foz, Portugal (40°09′03.4″ N 8°52′36.5″ W). With the help of local fishermen, the octopuses were caught in pots close to the shore between June 2020 and September 2020. A total of 20 specimens weighing between 100 and 835 g were collected. Since previous studies suggest that the sex of immature octopuses does not significantly alter aerobic scope (Valverde and

García García 2004), 9 females and 10 males from Cascais, 1 female from Figueira da Foz, were used. All specimens chosen for the experiment were under a weight limit that in accordance to the weight-to-maturity results of Lourenço (2014) are immature. Upon arrival at the aquaculture facilities of Laboratorio Marítimo da Guia (Cascais, Portugal), they were individually transferred and placed into separate opaque 20 L tanks to prevent territorial fights. Six of these tanks were placed in another 2 m × 2 m tank to collect the outflowing water of the 20 L aquaria and directing it to a 200 L sump passing through a 0.2 µm mesh filter, bacteria matured bioballs assuring biological filtration (Ouriço® bioballs, Fernando Ribeiro, Portugal) and a protein skimmer (Reef SkimPro, TMC Iberia, Portugal) before a pump forces the water through a chiller (Hailea, HC-250A, Guangdong, China) entering each 20 L tank to produce a constant water flow that provides two complete water exchanges per hour in the keeping aquaria. The collected *O. vulgaris* specimens were kept in this semi-closed aquatic system during the acclimation period. Throughout the acclimation period, the individuals were in well aerated water secured by air stones placed in the sump, with stable salinity values supported by a water-dripping system from the facility's main seawater reservoir. Feeding was performed daily with blue mussels (*Mytilus edulis*). The specimens included in this study were used in experimental treatments after being kept in the facility for at least 48 h. Specimens were considered acclimated when they accepted and ate food they were given.

A 12 h:12 h (light:dark cycle) photoperiod was used throughout the acclimation period. Seawater physiochemical parameters were monitored daily. Natural seawater was 1 µm filtered and UV (Vecton 400, TMC Iberia, Portugal) sterilised, with salinity being maintained at 35 ± 1 (refractometer V2, TMC Iberia, Portugal). Temperature was kept at 18.5 ± 0.5 °C (WTW Multi 3510 IDS, WTW GmbH, Germany) through seawater chilling systems (Hailea, HC-250A, Guangdong, China) and pH was maintained at 8.1 ± 0.1 (pHenomenal® pH 1100 H, VWR). Total ammonia (Tropic Marin Ammonia/Ammonium Test Kit, TMC Iberia, Portugal), nitrite and nitrate (Tropic Marin Nitrite/Nitrate Combo Test Kit, TMC Iberia, Portugal) were monitored and maintained within recommended levels (Iglesias et al. 2006).

Respirometry

Oxygen consumption rates (OCR) were measured via an intermittent flow through respirometry setup (FirestingO_2, PyroScience GmbH, Germany), following established respirometry methods (Clark et al. 2013; Roche et al. 2013; Rummer et al. 2016). In detail, Plexiglas respirometry chambers (volume: 4.7 L or 11.3 L for specimens above 500 g) (Loligo Systems, Tjele, Denmark) were partially covered with opaque black plastic to simulate a den and limit the visual stimuli. The lids of the chambers had an additional barrier in front of the tube exits to prevent the specimens from blocking them. Each chamber had two exits on each side. Tygon tubing (VWR international, LLC) was used to connect one exit of each chamber to a recirculation pump that created a stable waterflow (~ 300 mL/min). Another Tygon tube led the seawater from the pump through the optode sensor back into the chambers at the cross-opposite exit, to prevent stagnation, while the respirometry had no seawater income through the flush. The remaining tube going into the chambers transferred well aerated and filtered (0.5 mm) seawater from the flush-pump of the reservoir. To prevent pressure from building up, a tube on the opposite side of the incoming flush was placed above the water surface, functioning as a drainage.

OCR were measured using intermittent flow respirometry, including flush periods that completely exchanged the seawater therein (Forstner 1983; Steffensen 1989; Paula et al. 2022). The oxygen concentration values of the passing seawater collected from the optode sensor and a temperature probe in the seawater bath was processed in a FirestingO_2 interface and through the computer software Pyro Oxygen Logger (PyroScience GmbH, Germany). All measurements were carried out at atmospheric pressure at the same time of the day. Oxygen consumption was measured while the specimen was fully submerged, with both chamber lids closed and the flush pump turned off, i.e., the same water was recirculating between the sensor and the chamber. The setup was cleaned with hydrogen peroxide after each trial.

Experimental design

Three treatments and two control-groups were used [$n = 5$ per treatment: "Emersion", "5 min recov" and "24 h recov", $n = 5$ in the "Chase control" and $n = 5$ in the "Immersion" control, $n = 20$ specimens in total], to study the octopuses' tolerance to air exposure. For ethical reasons the number of specimens was kept as low as possible. Since the octopuses of the "Chase control" were not sacrificed for enzyme analysis, they were used again for the "24 h recov" treatment. To avoid bias potentially caused by food digestion, the specimens were fasted 20–24 h before measuring OCR, here used as a proxy for aerobic metabolism (Wells et al. 1983). Due to the lack of information about the extent of the hunting-related emersion periods in *O. vulgaris*, the information provided by a video of a presumably red octopus *Octopus rubescens* (JV Fitzgerald marine reserve, California, USA), walking from one tidepool over land to another tidepool was used (see Supplemental Table 1). The animal was fully emersed and walked on land for 2 min and 30 s. This time length was used for the performed experimental treatments.

At the start of the respirometry trials, the specimens were weighed following the procedure of Onthank (2008) and

lured in one of the chambers while keeping handling to a minimum. The cycle started with a 20-min flush period followed by a 1-min waiting period and, depending on the animal's size, either a 3-min (> 500 g), 5-min (249–430 g), 8-min (250–320 g) or 10-min (< 200 g) measurement period. Since some specimens were recorded within the same trial, the waiting period was set in accordance to the heaviest octopus. In the course of the experiment, the size classes were adjusted to reduce the risk of oxygen levels dropping under the set limit. The oxygen consumption was measured during a period of 24 h. Among all treatments, all specimens' standard metabolic rates (SMR) were recorded using the mean of the lowest 10% oxygen consumption rates of the 24-h stay. Throughout this time, the set flush period kept the oxygen levels in the chambers above ~ 35% air saturation values to have no influence of low oxygen values that are known to alter metabolic rates of *O. vulgaris* (Valverde and García García 2005). After the 24-h measurement, one of the following treatments was carried out.

The first treatment ("Emersion") included opening the chamber to let the seawater run out without allowing the octopus to escape. Two lids of the chamber that can be either opened or fully sealed served as a barrier to prevent the specimen from climbing out. After 2:30 min of air exposure, the specimen was sacrificed and tissues were sampled. The time was taken when the octopus's gills were not covered with seawater anymore.

The second treatment entailed removing the seawater in the chamber and keeping the octopus in that emersion condition for 2:30 min. Afterwards, the chamber was refilled with seawater, all air bubbles were carefully removed by tilting the chamber in the bath and the lids were sealed for a 5-min (recovery) measurement ("5 min recov"). After this period, the individuals were sacrificed and tissue samples were taken. The slope (created by oxygen consumption) after re-submersion was noted for maximum metabolic rate (MMR) comparison.

The third treatment was focused on the recovery values after emersion. Following the emersion period (2:30 min), the octopus was re-submersed, and oxygen consumption was measured for another 24 h of recovery ("24 h recov"). Following this period, the specimens were sacrificed and tissue samples were obtained.

The first control ("Chase control") was carried out to quantify elevated metabolic rates under exhaustion. This control served as a reference to be able to better evaluate the impact that the emersion treatment has on the oxygen consumption rates of the octopuses. This procedure was a modified version of the chase protocol described by Rummer et al. (2016) for fishes. More specifically, after a 24-h SMR measurement, the octopus was taken out of the chamber and put into a tank with seawater of its Acclimation aquatic system. The octopus was continually chased for 5 min by hand which included occasionally manipulating the specimens to prevent them from attaching to the bottom or walls. The chase always resulted in the specimens to ink while jetting away from the hand showing typical behaviour of octopuses fleeing from a predator (Neill and Cullen 1974). As soon as the octopus inked, it was transferred to another tank with seawater coming from their Acclimation aquatic system. Subsequently, the octopus was placed back and sealed into the chamber and oxygen consumption was measured for another 24 h. The first slope, resulting from a 5-min oxygen consumption measurement, was taken as the maximum metabolic rate (MMR). For simplicity, the rates recorded in this control and the "5 min recov" treatment will be referred to as MMR although it cannot be certain that the maximum is reached after 5 min of chasing or the emersion treatment mentioned before.

The second control treatment ("Immersion") included the 24-h SMR measurement, immediately followed by specimen sacrifice and tissue sampling. This control served as a reference of an untreated and unstressed octopus.

Data analysis

The software Aquaresp and Pyro Oxygen Logger recorded parameters related to oxygen consumption. Respirometry data were converted to a spreadsheet and analysed using the package respR (Harianto et al. 2019) in R (Version 1.4.1717, R Core Team 2021). To determine the SMR, every slope of the first 24-h trials were evaluated fitting a linear regression between starting and ending oxygen concentration of every measurement period. Only those linear models with a slope value below 0 and an R^2 value of more or equal to 0.8 were used. The lowest 10% of the oxygen consumption rates were taken to calculate a mean that represents respiration rates of a resting and non-starving octopus. This mean was used in an equation that included the volume of the respirometry chamber in litre, the tubing minus the volume of the octopus (where 1 kg of octopus was assumed to be equivalent to 1 kg of water and hence to 1 L of seawater) and the wet weight of the octopus in kg resulting in the SMR (µmol/g/h).

To determine the maximum metabolic rate (MMR), the OCR of the first measurement after the "5 min recov" treatment and "Chase control" was taken (µmol/g/h). Due to scaling effects, all metabolic rates (SMR and MMR) were normalised to the same size. The adjusted SMR (*SMRadj*) and MMR (*MMRadj*) were normalised to 400 g using the size dependency of cephalopods described by Seibel (2007). The equation includes the mass specific oxygen consumption rates (M_{O2}), the normalisation constant (b_0) and the body mass (M) to the power of the intraspecific scaling coefficient (b).

$$M_{O_2} = b_0 M^b \left(\mu\text{mol } O_2/\text{g/h}\right)$$

The aerobic scope (*AS*) was determined by subtracting the *SMRadj* from the *MMRadj*. To determine the proportion between the *SMRadj* and the *MMRadj*, the maximum was divided by the routine metabolic rate, which resulted in the so called factorial metabolic rate (*facAS*).

To investigate the recovery period (from the emersion exposure), the slopes of the second 24-h measurement after the treatment of "24 h recov" and the "Chase control" were evaluated with the same criteria as the SMR (Rate > 0; $R^2 \geq 0.8$) except for the first and highest slope, which was included in the recovery analysis in all cases. Recovery time was quantified with a modified Herrmann and Enders (2000) method. Due to a different size of dataset used in this study, instead of using the last 30 oxygen consumption values, the median of the second half of the slopes was used for a first approximation of standard metabolism. Then the consecutive moving averages were calculated and compared to the median plus 10% of the median. The first three consecutive moving averages that were less than the latter value set the first approximated start of the routine phase. Then the median of the slopes after the first of the three consecutive moving averages was determined. Comparing this second median in the same manner again with the consecutive moving averages leads to the quantification of the time it took to recover. The first of the three consecutive moving averages that were less than the second median plus 10% of the second median was considered as the first value of the routine phase after the treatment "24 h recov" and the "Chase control".

To avoid compromising the enzymatic responses due to a prolonged anaesthetic procedure (at least 15 min, Fiorito et al. 2015), tissue samples were collected immediately after the respirometry runs. Due to the risk of losing the immediate effect of the air exposure and, therefore, the overall purpose of the sampling, we were not able to anaesthetise the octopuses before sacrifice. Specimen's brain was mechanically destroyed by highly skilled operators (Fiorito et al. 2015), and after confirming death (cessation of circulation), the gills, the digestive gland and the mantle muscle were collected, put on dry ice and frozen (at -80 °C) for further analyses.

Sample preparation

Tissue (gills, the digestive gland and mantle) samples were transferred to microtubes (1.5 mL) and homogenised in 1 mL of phosphate-buffered saline solution (PBS; pH = 7.42). The PBS solution was prepared with 0.14 M NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4 and milliQ H_2O and pH adjusted to 7.4. Samples were homogenised with an Ultra-Turrax (Staufen, Germany) followed by centrifugation at 14,000×*g* (20 min, 4 °C). The supernatant fraction was transferred into a new microtube and stored at -80 °C until further analysis.

Protein content and antioxidant enzymatic activities

To normalise the individual biomarker's results, in each sample, the total protein was quantified following the Bradford assay (Bradford 1976). The protocol was adapted for a 96-well microplate and using bovine serum albumin (BSA) as standard to construct a calibration curve by serial dilutions of a stock solution containing 2 mg/mL BSA. Superoxide dismutase (SOD) activity was carried out by following the method of McCord and Fridovich (1969), optimised for 96-well microplates. Each microplate well (Greiner Bio-One, Germany) was filled with 200 µL potassium phosphate buffer solution (50 mM K_3PO_4, Sigma-Aldrich, Germany; pH ~ 8.0). Then, 10 µL each of ethylenediaminetetraacetic acid (EDTA), xanthine, nitroblue tetrazolium-diformazan (NBT) and sample were added to each well. Blanks were also added to the microplate wells in triplicate. Lastly, 10 µL xanthine oxidase was added to start the reaction and after 10 min of incubating at room temperature, the microplate was read at 560 nm, every minute, during 20 min (Synergy HTX, BioTek, USA). The reducing absorption of NBT-diformazan was used to quantify SOD activity. Results were expressed as units per mg of total protein.

Catalase (CAT) activity was determined as described by Johansson and Borg (1988). In summary, standards were prepared using a formaldehyde stock solution (4.5 M) diluted in sample buffer (Potassium phosphate 25 mM, pH = 7.0; 1 mM EDTA; 0.1% BSA) to obtain a standard curve ranging from 0 to 75 µM. Next, the wells of a 96-well microplate (Greiner Bio-One, Germany) were filled with 100 µL of diluted assay buffer (potassium phosphate 100 mM, pH = 7.0), 30 µL of methanol and 20 µL of either the sample or the standard. Two wells were used as a blank by adding 20 µL of PBS (pH = 7.4). Then the reaction was initiated by adding 20 µL of 0.035 M hydrogen peroxide to each well. Subsequently, the plate was covered with aluminum foil and incubated at room temperature for 20 min. Then, 30 µL of 10 M potassium hydroxide and 30 µL purpald (49,99 mg purpald 34.2 mM in 40 µL of 0.5 M HCl) were pipetted into each well. The plate was covered and incubated under agitation, using a shaker, for another 10 min at room temperature. Afterwards, 10 µL of potassium periodate (65.2 mM in 0.5 M KOH) was added to the microplate wells followed by 5-min-incubation on a shaker, at room temperature. At the end of the incubation period, the absorbance was read at 540 nm (Synergy HTX, BioTek, USA). CAT activity was determined by quantifying the formaldehyde concentration

within each sample. Activity results were expressed as nmol/min/mg total protein.

Glutathione-*S*-transferase (GST) activity was carried out using a method first described by Habig et al. (1974) and adapted for 96-well microplates. The first reaction mixture was prepared, containing 9.8 mL of phosphate-buffered saline (PBS, pH = 7.42), 0.1 mL of glutathione (GSH) and 0.1 mL of 1-chloro-2,4-dinitrobenzene (CDNB). Then 20 µL of either the sample or distilled water (blank) were added into each well of the microplate (Greiner Bio-One, Germany). Subsequently, 180 µL of the reaction mixture was added to each well and the increase in absorbance was read at 340 nm, every minute, for 6 min using a microplate reader (Synergy HTX, BioTek, USA). Activity results were expressed as µg/min/mg of total protein.

Heat shock proteins

Heat shock protein 70 (HSP70) content was determined according to an indirect ELISA technique (Crowther 2008) and carried out as described by Maulvault et al. (2019). Thus, 50 µL of each sample or standard was pipetted into the wells of a 96-well microplate (Microlon 600 High-binding, Greiner Bio-One, Germany) and left incubating overnight at 4 °C. Subsequently, the microplate was washed two times with PBS-Tween 20 (0.05%) and the pH was adjusted to 7.4, followed by adding 100 µL of a blocking solution (1% BSA) to each well. After 90 min of incubation at room temperature the microplate was washed two times with PBS-Tween 20 (0.05%) and 50 µL of the primary antibody (anti-HSP_{70}/HSC_{70}, Origene, USA) was added into each well. Microplates were incubated overnight at 4 °C and then washed again two times with PBS-Tween 20 (0.05%). Then, 50 µL of the second antibody, alkaline phosphate-conjugated anti-mouse IgG (Fc specific, Sigma-Aldrich, USA) was added into the microplate wells and left incubating for 90 min at 37 °C. Afterwards, microplates were washed with PBS-Tween 20 (0.05%) and 50 µL of substrate (2.7 mM p-Nitrophenyl Phosphate, 100 mM trizma hydrochloride, 100 mM sodium chloride, 5 mM magnesium chloride in deionised ultrapure water, pH = 8.5) was added into each well. After 30 min of incubation at room temperature, 3 M sodium hydroxide (NaOH) was added to stop the reaction and the absorbance was read at 405 nm with a microplate reader (Synergy HTX, BioTek, USA). The HSP_{70} concentration was then determined using a calibration curve, based on a serial dilution of purified HSP_{70} active protein (0 to 2000 µg mL^{-1}, Origene, USA).

Ubiquitin content

The ubiquitin content was quantified by ELISA as described previously by Lopes et al. (2018). Thus, 50 µL of each sample or standard was added to the wells of the 96-well microplate (Microlon 600 High-binding, Greiner, Germany) and was left incubating overnight at 4 °C. Then, the plate was washed twice with PBS-Tween 20 (0.05%), 50 µL of the blocking solution (1% BSA) was added into each well and the microplate was incubated at 37 °C for 90 min. Afterwards, the microplate was washed again two times with PBS-Tween 20 (0.05%) and 50 µL of the primary antibody (P4D1, sc-8017, HRP conjugate, Santa Cruz, USA) was added into each well. Subsequently after microplate incubation overnight at 4 °C, they were washed with PBS-Tween 20 (0.05%). Then, 50 µL of substrate (TMB/E, Merck Millipore, USA) was added and the microplates incubated for 30 min at room temperature. At the end of the incubation period, 50 µL of a stop solution (1 M hydrogen chloride, HCL) was added into each well and the absorbance was read at 405 nm (Synergy HTX, BioTek, USA). Lastly, the values were determined using a calibration curve which was previously prepared by serial dilutions of standard purified ubiquitin (0 to1 µgmL-1, UbpBio, E-1100, USA).

Lipid peroxidation

Lipid peroxidation (LPO) was determined by measuring the amount of malondialdehyde (MDA) present in the samples, following the method described by Ohkawa et al. (1979) and adapted to a 96-well microplate. To perform the assay, 5 µL of sample were added to each microtube (1.5 mL), followed by 45 µL of PBS, 12.5 µL of 8.1% sodium dodecyl sulfate (SDS) (Merck, Germany), 93.5 µL of 20% trichloroacetic acid (TCA) (Panreac, Spain), 93.5 µL of 1% thiobarbituric acid (TBA) (Sigma-Aldrich, USA) and 50.5 µL of milli-Q water. The microtubes were then 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 period, the microtubes were briefly placed on ice to cool. Additional, 62.5 µL of milli-Q water were added to each microtube. Subsequently, 150 µL (in duplicate) were taken and added to a 96-well microplate (Greiner, Bio-One, Germany). The absorbance was read at 532 nm using a microplate reader (Synergy HTX, BioTek, USA). To quantify lipid peroxidation (MDA content) in samples, a 1 µM MDA stock solution (Merck, Germany) was used to construct a calibration curve ranging from 0 to 0.1 µM. MDA results were expressed as nmol/mg of total protein.

DNA damage

DNA damage was determined by ELISA by quantifying 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Maclouf et al. 1987; Shen et al. 2007). In brief, 50 µL of the sample was added to each well of a 96-well microplate (Microlon 600

High-binding, Greiner, Germany) and incubated overnight at 4 °C. Then, the microplate was washed with PBS-Tween (0.05%; pH = 7.4) two times, 100 µL of the blocking solution bovine serum albumin (1% BSA) was added into each well and the microplate incubated at room temperature for 90 min. After, a new washing step was performed as referred previously with PBS-Tween 20 (0.05%), followed by adding 50 µL of the primary antibody (anti-OHdG, clone 15 A3, Sigma–Aldrich, Germany) into each well. After new incubation overnight at 4 °C, the microplate was washed again as referred before and 50 µL of the second antibody (alkaline phosphate-conjugated anti-mouse IgG, Fc specific, Sigma-Aldrich, USA) was added to the microplate wells. Then, the microplate was left to incubate at 37 °C for 90 min. Subsequently, the unlinked antibodies were washed out with PBS-Tween 20 (0.05%), 50 µL of alkaline phosphate substrate (2.7 mM p-Nitrophenyl Phosphate, 100 mM trizma hydrochloride, 100 mM sodium chloride, 5 mM magnesium chloride in deionised ultrapure water, pH = 8.5) was added to each well and the microplate incubated for 30 min at room temperature. The reaction was stopped by adding 50 µL of a stop solution (3 M NaOH) to the microplate wells and the sample´s absorbance was read at 405 nm (Synergy HTX, BioTek, USA).

Total antioxidant capacity

Total antioxidant capacity (TAC) was determined according to Kambayashi et al. (2009). To perform the assay, a 96-well microplate (Greiner, Bio-One, Germany) was filled with 10 µL of either sample or standards. Then, 10 µL of 90µM myoglobin (Sigma-Aldrich, Germany) was pipetted into each well followed by adding 150 µL of 600 µM ABTS and 40 µL of 500µM hydrogen peroxide. After 5 min incubation, the microplate was read at 410 nm, (Synergy HTX, BioTek, USA). The total antioxidant capacity of the samples was calculated using the calibration curve and the results expressed as nmol/ mg total protein. Absorbance was read at 410 nm, and TAC was calculated from a calibration curve, based on a series of Trolox (0–0.3 mM).

Statistical analysis

Generalised linear models (GLM) with Gaussian family were used to determine effects of air exposure on the specimen's oxygen consumption rates compared to maximum metabolic levels of chased *O. vulgaris* represented by the "Chase control". The values of *SMRadj*, *MMRadj*, *AS*, *facAS* and recovery time were set as response variables and the treatment groups were set as predictor variables. The recovery time was tested fitting a Gaussian GLM to the data of the "Chase control" and the "24 h recov" treatment. For the analysis of the biochemical response a Gamma (link = log) GLM was fit to the enzyme activity (SOD, Catalase and GST), protein content (HSP and Ubiquitin), cellular damage (LPO and DNA damage) and total antioxidant activity (TAC) data nested in the tissues from which they were quantified from against the treatments. The models fit to the respirometry and biochemical data was verified using Fligner-Killeen test for homogeneity of variance, Shapiro–Wilk test to check for normal distribution of the raw data and the models' residuals. Graphical verification was done creating a QQ-plot and looking at influential datapoints by plotting Cook's distance. The statistical analysis was done using R and the included "stats" package (R Core Team 2021). Plots were created using the package "ggplot2" (Wickham 2016).

Results

Fishermen survey

Based on our survey, 90% of the interviewees (20 in total) had seen octopus crawling out the water during their daytime fishing activity. Only four interviewees undertook fishing activities during nighttime, out of which three reported to have seen such behaviour during that period (see Supplemental Table 2).

Metabolism

There were no significant statistical differences in *MMRadj* (4.64 ± 2.74 µmol/g/h; GLM, $t = 0.422$, $p > 0.05$; Fig. 1a), *SMRadj* (2.26 ± 0.23 µmol/g/h; GLM, $t = -0.866$, $p > 0.05$; Fig. 1b) and aerobic scope (*AS*; 2.38 ± 2.73 µmol/g/h; GLM, $t = 0.569$, $p > 0.05$; Fig. 1c) between the control ("Chase control") group and the individuals that were emersed for 2:30 min and then left to recover for 5 min ("5 min recov") (see Supplemental Table 3). Similar results were obtained for the facultative aerobic scope (fac*AS*; 2.06 ± 1.15 µmol/g/h; GLM, $t = 0.675$, $p > 0.05$; Fig. 1d). Regarding the recovery period, the individuals from the "Chase control" ($26{,}525.22 \pm 14{,}400.27$ s) needed significantly more time to recover than the octopuses that experienced emersion ("24 h recov"; 2520.65 ± 5636.35 s; GLM, $t = 3.471$, $p < 0.05$; Fig. 2).

Antioxidant enzymatic activities

Regarding SOD activity at control conditions ("Immersion"), no statistical differences were found among the tissues (GLM, $p > 0.05$; Fig. 3a). Yet, when all treatments were considered, the values in the gills (9.38 ± 9.81 U/mg) were significantly lower than those in the digestive gland (23.8 ± 24.14 U/mg; GLM, $t = 2.974$, $p < 0.05$) and mantle (30.44 ± 27.48 U/mg; GLM, $t = 3.760$, $p < 0.05$). No

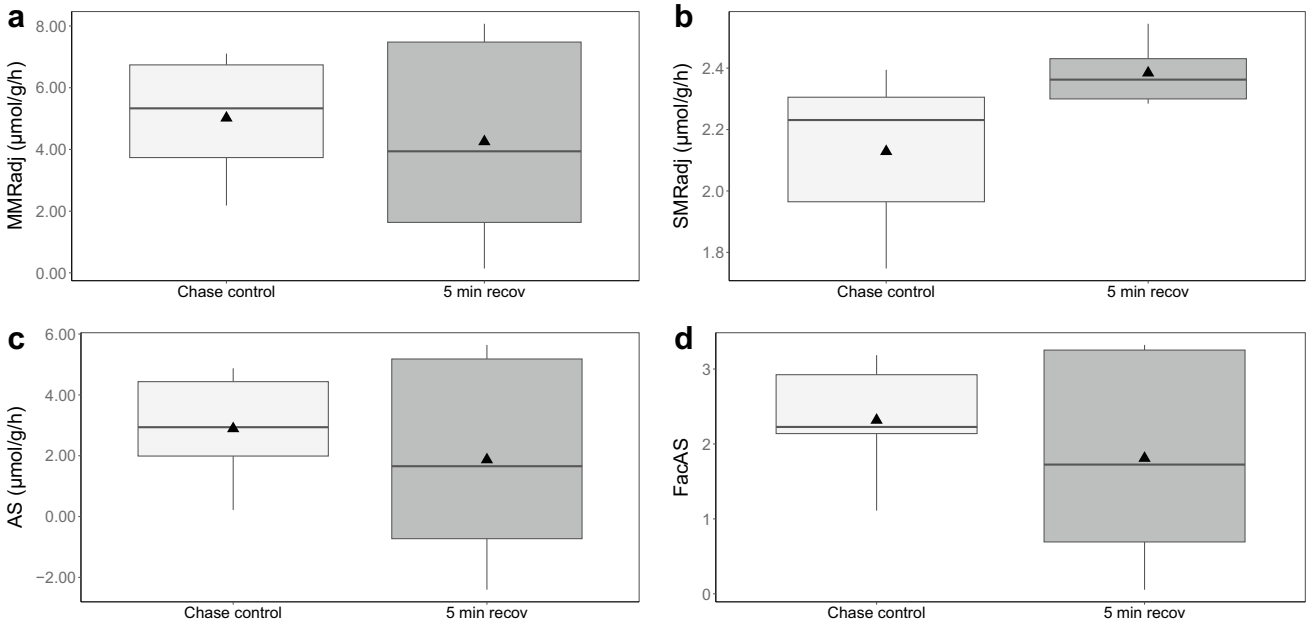


Fig. 1 Effects of emersion on *O. vulgaris* metabolism. All rates are weighed-adjusted to a 400 g octopus. **a** Adjusted maximum metabolic rates (*MMRadj*), **b** adjusted standard metabolic rates (*SMRadj*), **c** aerobic scope, **d** facultative aerobic scope, during control ("Chase control") conditions and air exposure followed by a 5-min recovery period ("5 min recov"). See the detailed statistical information in Supplemental Table 3

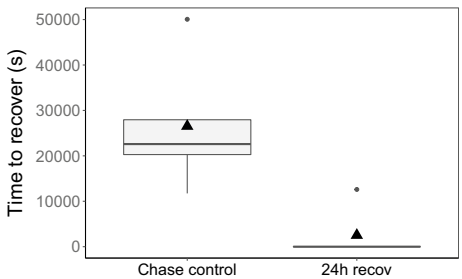


Fig. 2 *O. vulgaris* recovery time needed to go back to routine oxygen consumption rates after being chased ("Chase control") and after air exposure followed by a 24-h recovery period ("24 h recov"). See the detailed statistical information in Supplemental Table 3

statistical differences were found in SOD inhibition among treatments, within each tissue (GLM, $p > 0.05$; Fig. 3a).

Regarding CAT activity at control conditions, ("Immersion") the observed values in the digestive gland (24 ± 23.42 nmol/min/mg) were statistically higher than those found in the gills (0.94 ± 0.91 nmol/min/mg; GLM, $t = 6.054$, $p < 0.05$) and the mantle (1.96 ± 1.36 nnmol/min/mg; GLM, Ref.: digestive gland, $t = -4.680$, $p < 0.05$) (Fig. 3b). Independently of the treatment, statistical differences were observed among all three tissues. The gills (0.7 ± 0.7 nmol/min/mg) showed the lowest values compared to the digestive gland (15.38 ± 15.59 nmol/min/mg; GLM, $t = 9.450$, $p < 0.05$) and mantle (2.37 ± 2.58 nmol/min/mg; GLM, $t = 3.742$, $p < 0.05$). In the gills, CAT activity in the"Immersion" control (0.94 ± 0.91 nmol/min/mg) was significantly different from "24 h recov" treatment (0.27 ± 0.15 nmol/min/mg; GLM, $t = 2.306$, $p < 0.05$; Fig. 3b). Moreover, the "5 min recov" treatment (1.07 ± 0.89 nmol/min/mg) showed significantly higher CAT activity than the "24 h recov" treatment (GLM, $t = -2.540$, $p < 0.05$). In the digestive gland, the control group ("Immersion"; 24 ± 23.42 nmol/min/mg) had significantly higher values in CAT activity than the "24 h recov" treatment (3.65 ± 1.7 nmol/min/mg; GLM, $t = 3.520$, $p < 0.05$). No significant differences were found between the control group ("Immersion"; 24 ± 23.42 nmol/min/mg), the "Emersion" (19.46 ± 11.98 nmol/min/mg) and "5 min recov" (14.42 ± 13.15 nmol/min/mg) treatments, but all of them were significantly higher than the "24 h recov" treatment (3.65 ± 1.7 nmol/min/mg). In the mantle, both "Emersion" (3.36 ± 4.26 nmol/min/mg) and "5 min recov" (3.33 ± 2.32 nmol/min/mg) treatments showed significantly higher CAT activity levels than the "24 h recov" treatment (0.84 ± 0.87 nmol/min/mg; Fig. 3b).

Regarding GST activity levels, they are significantly different in the mantle muscle (0.27 ± 0.2 µmol/min/mg) compared to the gills (0.92 ± 1.23 µmol/min/mg; GLM, $t = -2.260$, $p < 0.05$) and digestive gland (1.85 ± 1.3 µmol/min/mg; GLM, $t = -3.516$, $p < 0.05$; Fig. 3 c). Independently of the treatment, GST values were higher in the digestive gland (1.91 ± 2.34 µmol/min/mg; GLM, gills, $t = 3.47$, $p < 0.05$; mantle, $t = -6.141$, $p < 0.05$). GST values did not

differ among the treatments in the gills and mantle (GLM, $p > 0.05$; Fig. 3c). Yet, in the digestive gland, the "24 h recov" treatment (0.36 ± 0.1 µmol/min/mg) was significantly lower (at least fivefold) than all the other treatments (2.43 ± 2.51 µmol/min/mg; GLM, $p < 0.05$).

Heat shock response

The control ("Immersion") showed no significant differences in HSP_{70} content among tissues (10.58 ± 15.94 ng/mg; Fig. 4a). Yet, when all treatments were considered, HSP_{70} content was significantly higher in the mantle (20.35 ± 22.37 ng/mg) than in gills (5.35 ± 10.81 ng/mg; GLM, t = 2.746, $p < 0.05$), but not statistically different from the levels observed in the digestive gland (10.55 ± 14.22 ng/mg; GLM, $t = -1.350$, $p > 0.05$). HSP_{70} content in the gills was higher in the control (12.14 ± 21.39 ng/mg) than in the "Emersion" treatment (2.17 ± 1.31 ng/mg; GLM, t = 2.574, $p < 0.05$) and the "24 h recov" treatment (2.77 ± 1.77 ng/mg; GLM, t = 2.209, $p < 0.05$). The "5 min recov" treatment (4.31 ± 3.52 ng/mg) did not show a significant difference compared to the control ("Immersion") (GLM, $t = 1.548$, $p > 0.05$). In the digestive gland, HSP_{70} levels did not vary significantly between the "Immersion" control and the treatments. Only the "5 min recov" treatment (13.35 ± 20.71 ng/mg) showed significantly higher (up to 5.2-fold) HSP_{70} levels in comparison to the "24 h recov" treatment (3.76 ± 2.49 ng/mg; GLM, $t = -2.469$, $p < 0.05$). HSP_{70} levels in mantle tissue did not differ among treatments (20.35 ± 22.37 ng/mg; GLM, $t = 1.340$, $p > 0.05$).

Ubiquitin content

Ubiquitin levels at control conditions ("Immersion") were not different among tissues (0.29 ± 0.35 ng/mg; Fig. 4b). Independently of the treatment, the gills (0.12 ± 0.15 ng/mg) showed significantly lower levels than the digestive gland (0.53 ± 0.9 ng/mg; GLM, $t = 3.083$, $p < 0.05$) and muscle (0.57 ± 0.9 ng/mg; GLM, $t = 3.261$, $p < 0.05$). No significant changes between mantle and digestive gland values were detected (GLM, $t = 0.179$, $p > 0.05$). Among all treatments, the "5 min recov" treatment (0.26 ± 0.21 ng/mg) showed significantly higher values (up to 6.5 fold) than the "24 h recov" treatment (0.04 ± 0.04 ng/mg; GLM, $t = -2.690$, $p < 0.05$) in the gills. In the digestive gland, the "24 h recov" treatment (0.12 ± 0.14 ng/mg) revealed significantly lower values in comparison to "Emersion" (0.73 ± 0.87 ng/mg; GLM, $t = -2.628$, $p < 0.05$) and "5 min recov" treatments (0.91 ± 1.53 ng/mg; GLM, $t = -2.942$, $p < 0.05$). A quite similar trend was observed in the mantle ("24 h recov", 0.08 ± 0.06 ng/mg) ("Emersion", 1.15 ± 1.66 ng/mg, GLM, $t = -3.833$, $p < 0.05$; "5 min recov", 0.68 ± 0.45 ng/mg, GLM, $t = -3.082$, $p < 0.05$; "Immersion", 0.39 ± 0.36 ng/mg, GLM, $t = 2.300$, $p < 0.05$).

Lipid peroxidation

Under "Immersion", no significant differences in LPO were detected among tissues (2.02 ± 2.53 nmol/mg; Fig. 5a), but when considering all treatments, the levels found in the gills (0.95 ± 0.61 nmol/mg) were significantly lower than those found in the digestive gland (5.73 ± 15.5 nmol/mg; GLM, $t = 3.214$, $p < 0.05$) and mantle (3.7 ± 4.77 nmol/mg; GLM, $t = 2.466$, $p < 0.05$). In the gills, there were no significant variations among treatments (0.95 ± 0.61 nmol/mg; GLM, $p > 0.05$). On the other hand, in the digestive gland, "Emersion" (15.88 ± 29.3 nmol/mg) and the "24 h recov" 0.42 ± 0.2 nmol/mg) treatment varied significantly compared to the control (2.48 ± 4.05 nmol/mg; GLM, $t = 2.402$, $p < 0.05$). In the mantle tissue, LPO levels did not significantly differ between "24 h recov" (2.25 ± 2.41 nmol/mg) and the "Immersion" control (2.61 ± 1.89 nmol/mg; GLM, $t = 0.212$, $p < 0.05$). However, LPO values were significantly higher in the "5 min recov" (8.41 ± 7.69 nmol/mg) treatment than in the "Emersion" treatment (1.53 ± 1.43 nmol/mg; GLM, $t = 2.447$, $p < 0.05$).

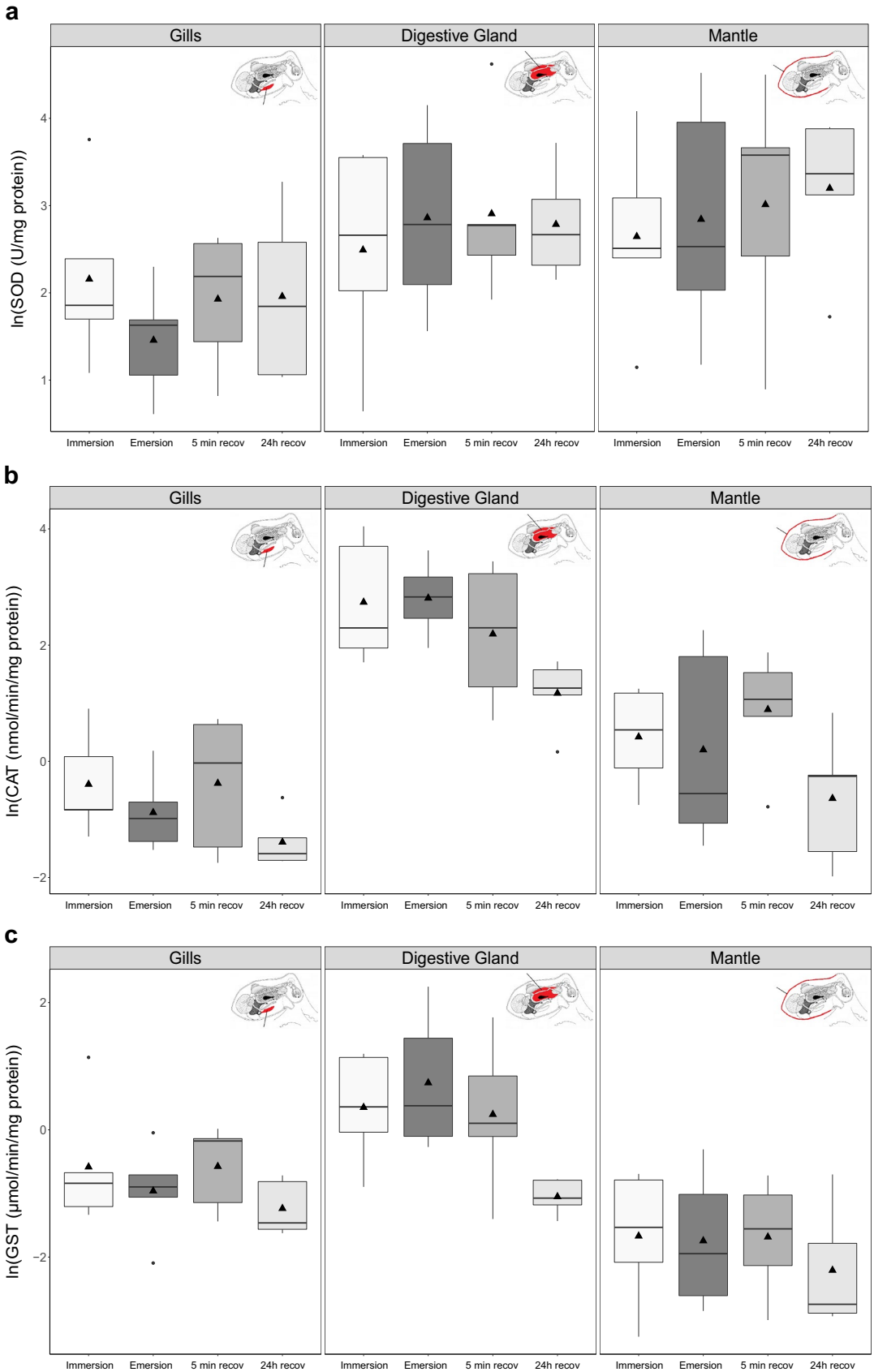
DNA damage and total antioxidant defense capacity

Regarding the DNA damage, gills (0.75 ± 0.85 abs/mg) showed significantly less DNA damage than the digestive gland (2.18 ± 2.59 abs/mg; GLM, $t = 3.090$, $p < 0.05$) and mantle muscle (2.62 ± 2.44 abs/mg; GLM, $t = 3.622$, $p < 0.05$; Fig. 5b). Yet, no significant differences were found among treatments in each of the analysed tissues. Regarding total antioxidant defense capacity (TAC), gills (0.32 ± 0.3 nmol/mg) had significantly lower values than the digestive gland (1.22 ± 1.4 nmol/mg; GLM, $t = 4.09$, $p < 0.05$) and the mantle muscle (0.74 ± 0.74 nmol/mg; GLM, $t = 2.57$, $p < 0.05$; Fig. 6). No statistical differences were found among the treatments in each of the tissues.

Discussion

Fishermen survey and diurnal activity

The majority of the fishermen (90%) interviewed have observed the common octopus crawling on land around tide pools, indicating that this behaviour might be common on the rocky shores of the western coast of Portugal. Although the common octopus is assumingly more active during crepuscular/night periods (Brown et al. 2006; Hanlon and Messenger 2018), there is significant individual variation in activity periods under laboratory conditions (Meisel et al.


a
Gills
Digestive Gland
Mantle
ln(SOD (U/mg protein))
4
3
2
1
Immersion
Emersion
5 min recov
24h recov
b
Gills
Digestive Gland
Mantle
ln(CAT (nmol/min/mg protein))
4
2
0
−2
Immersion
Emersion
5 min recov
24h recov
c
Gills
Digestive Gland
Mantle
ln(GST (µmol/min/mg protein))
2
0
−2
Immersion
Emersion
5 min recov
24h recov

◂**Fig. 3** Effects of emersion on *O. vulgaris* antioxidant enzymatic activity. **a** SOD **b** Catalase, **c** GST, during emersion conditions ("Emersion"), emersion followed by a 5-min recovery period ("5 min recov") or 24-h recovery period ("24 h recov") and the control ("Immersion"). See the detailed statistical information in Supplemental Table 3

2006, 2013). These authors argue that this species may be able to switch from nocturnal to diurnal activity depending on current needs. Also, another study by Meisel et al. (2013) showed evidence that octopuses use temporal spacing of activity to adjust to predation pressure. Alongside, Humbert et al. (2022) also recently showed that *Octopus rubescens* are more active during daytime, and argue that other species from the same genus may have a comparable way of living. The findings of these studies together with the local fishermen sightings during the day minimise a potential limitation of our study—the fact that the respirometry runs were conducted during daytime.

Metabolic responses

Adjusted maximum metabolic rates (*MMRadj*) of the specimens treated with 2:30 min of air exposure followed by 5 min recovery ("5 min recov") did not differ significantly from the *MMRadj* of the chased individuals. The emersion caused the octopuses to show signs of stress and could give evidence that the animals run into oxygen debt. This resulted in an elevated oxygen consumption after the stimulus for compensation (Wells and Wells 1984; Eno 1987). Additionally, the oxygen in the octopus' blood is used up almost completely while passing through the body, so that there is no venous reserve to buffer low or no oxygen availability (Pörtner 1990). Under such conditions, octopuses may enhance anaerobic glycolysis to compensate the reduction in ATP that can be synthesised aerobically. Like other cephalopods, octopuses use the glucose-opine anaerobic pathway, which entails condensation of pyruvate to form octopine, which provides 1.5 units of ATP per unit of octopine produced (Rosa and Seibel 2008, 2010a). During the anaerobic metabolism toxic compounds are produced as a byproduct that need to be compensated for. The elevated oxygen consumption rates after re-submersion could be an indicator for the turn in metabolic strategy.While being chased, the individuals use jet propulsion, a highly energetically inefficient mode of locomotion, which also reduces oxygen extraction efficiency (Grieshaber and Gäde 1976; Wells et al. 1987, 1988; Pörtner 1994; O'Dor and Webber 2008).

The fact that the recovery time was significantly shorter in the air exposure with "24 h recov" treatment than the chase control seems to indicate lower oxygen debt in the former treatment. Coastal octopuses live in burrows and tidepools, where oxygen levels may vary from near air saturation to complete anoxia (Seibel and Childress 2000). According to these authors, coastal octopuses (namely *Octopus californicus* and *Octopus bimaculoides*) can survive in anoxic conditions for several hours, possibly through metabolic suppression. Thus, the ability to withstand short-term emersion may be closely linked to hypoxia/anoxia tolerance since both abiotic challenges share the same physiological and biochemical (cellular) defense mechanisms. Also, it is known that octopuses have neurons to control their blood vessel musculature (Young 1963), and Wells and Wells (1986) suspect that this feature can make the animal adjust their blood flow and favour certain organs when oxygen is limited. The same strategy was observed in the epaulette shark which is also frequently exposed to atmospheric air in its' tidepool environment and manages to protect its brain this way (Söderström et al. 1999). Additionally, octopuses have shown to be able to cover an estimate of 41% of their oxygen demand with cutaneous respiration (Madan and Wells 1996). Yet, under emersion, it is not known if they can extract atmospheric oxygen through the moist skin or gills.

Biochemical responses

Enhancing antioxidant activity before ROS overproduction (during re-oxygenation) is known as a preparation against oxidative stress (Giraud-Billoud et al. 2019). Such strategy is common in intertidal animals, because they have to tolerate the rapid cyclical fluctuations of the intertidal environment (Teixeira et al. 2013). This study, with an intertidal temperate soft coral, showed that during the emersion phase, there was a significant increase in CAT and GST activities, whereas SOD activity remained stable. Here, SOD values also did not show a significant antioxidant defense response to air exposure. Moreover, the present results also show no differences in CAT activity between the control ("Immersion"), "Emersion" and the emersion followed by 5 min recovery ("5 min recov") treatments. The mussel *Perna perna* showed no significant differences in CAT activity, in both gill and digestive gland, after 18 h of emersion compared to the specimens submersed the whole time or re-submersed for 1 h (Almeida et al. 2005). As proposed in Almeida et al. (2005), this can be due to glutathione peroxide (GPx) which is also decomposing hydrogen peroxide radicals just like CAT. Another explanation could be that CAT levels were not elevated in the gills because this tissue can excrete hydrogen peroxide into the water (Wilhelm-Filho 1994). Still, it is unknown if this can be done when the gills are exposed to atmospheric air. Regarding GST activity, no significant differences were found among the treatments (except for the "24 h recov" treatment). Almeida et al. (2005) suggested that the accumulation of anaerobic end-products caused the rise of GST activity during and after an 18-h air exposure of the brown mussel *P. perna*. Although the oxygen consumption was

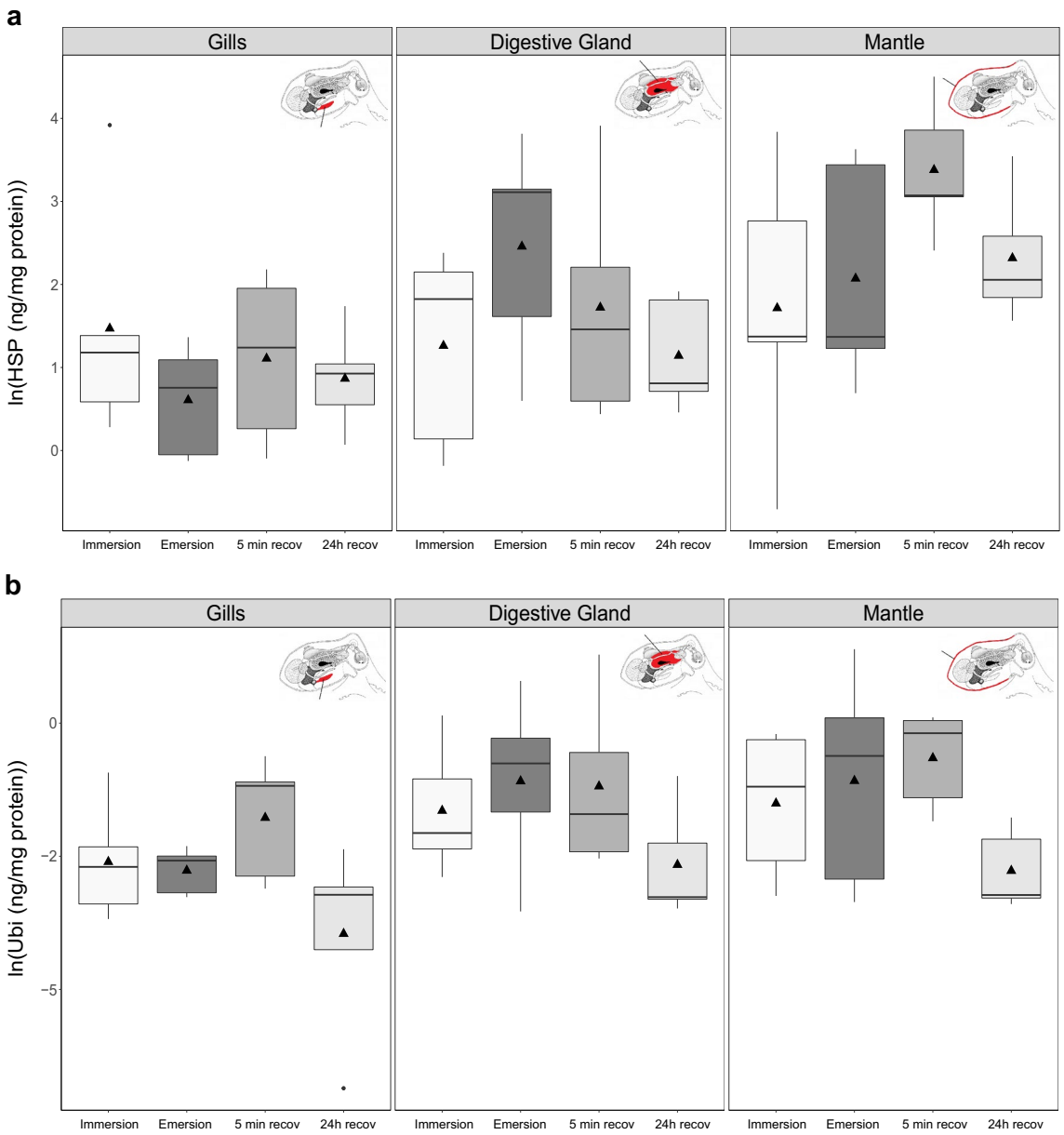


Fig. 4 Effects of emersion on *O. vulgaris* HSP70 and Ubiquitin concentration. **a** HSP70 and **b** Ubiquitin, during emersion conditions ("Emersion"), emersion followed by a 5-min recovery period ("5 min recov") or 24-h recovery period ("24 h recov") and the control ("Immersion"). See the detailed statistical information in Supplemental Table 3

elevated after re-submersion, SOD, CAT and GST activity levels did not give clear evidence that the emersion period was long enough to change to anaerobic metabolism and the production of toxic metabolites.

A typical strategy for animals encountering hypoxia is to reduce protein synthesis (Guppy and Withers 1999). Although the production of HSP_{70} demands oxygen to generate ATP (Tomanek and Somero 1999; Tomanek 2010), HSP_{70} is known to increase during air exposure in intertidal corals (Teixeira et al. 2013). It was argued that this strategy may constitute an anticipatory protective response to the oxidative stress caused by the reoxygenation period (re-immersion). The same results were observed in jumbo squids, *Dosidicus gigas,* that live within mesopelagic dead zones (oxygen minimum zones) (Trübenbach et al. 2013). The authors argued that the process of metabolic suppression might be associated with enhanced HSP_{70} production as a strategy to prevent post-oxidative damage during the squid's night upward migration to the surface ocean. This idea is supported by the present findings in the gills since the short period of reoxygenation ("5 min recov") brought HSP_{70} levels back to basal levels. Yet, such trends were not

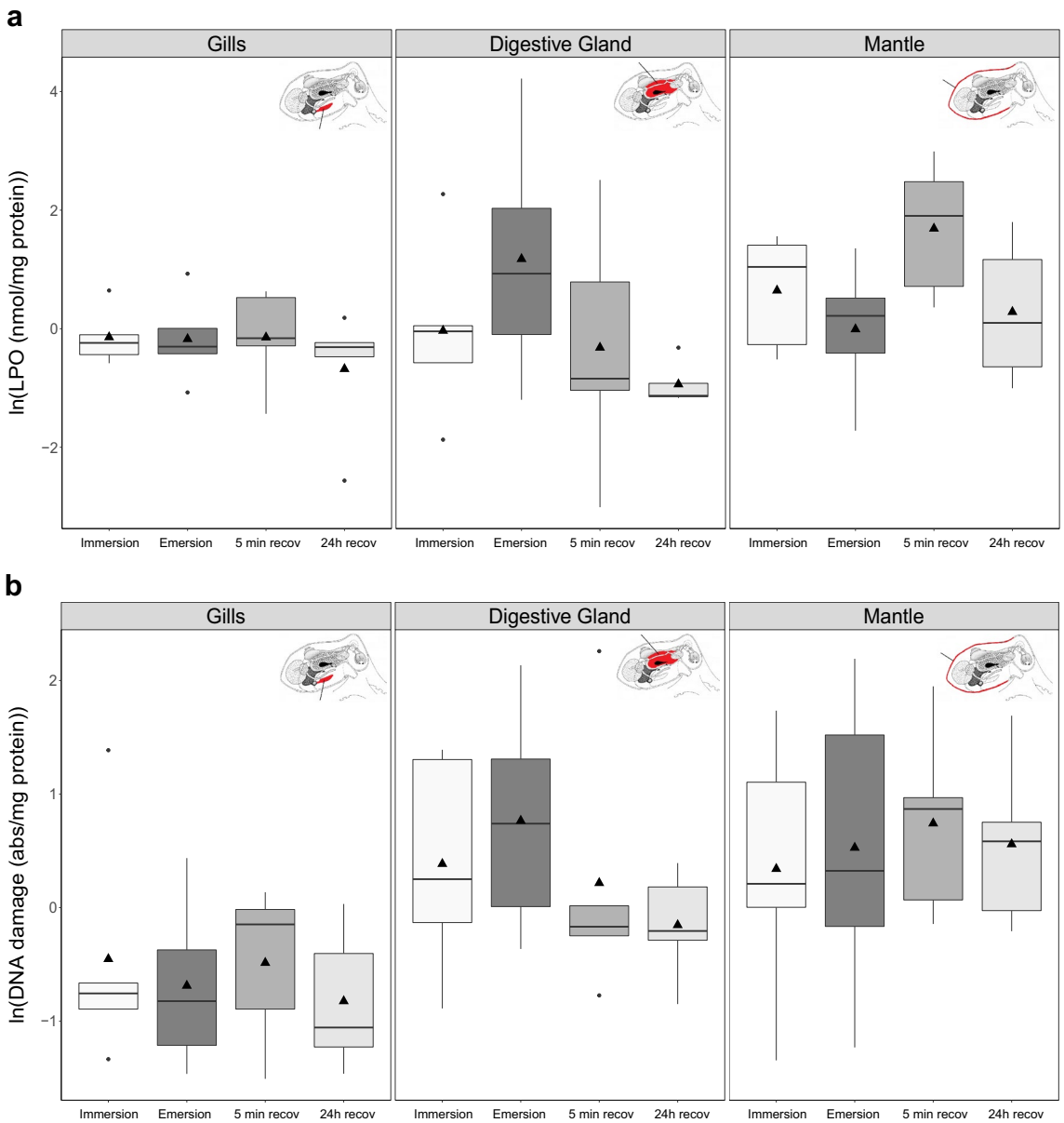


Fig. 5 Effects of emersion on *O. vulgaris* oxidative damage. **a** Lipid peroxidation and **b** DNA damage, during emersion conditions ("Emersion"), emersion followed by a 5-min recovery period ("5 min recov") or 24-h recovery period ("24 h recov") and the control ("Immersion"). See the detailed statistical information in Supplemental Table 3

observed in the other two tissues. The present study also showed that the air exposure caused no irreparable protein damage (based on ubiquitin). Moreover, air exposure did not cause increased LPO and TAC in the gills. Comparable LPO results were described by Romero et al. (2007), with the false king crab, and Almeida et al. (2005) with brown mussel.

Differences in antioxidant defence capacities and oxidative stress among the tissues were found. The physiological roles of the organs sampled lead to a less or higher need for oxidative stress protection. CAT and GST basal activity levels were significantly higher in the digestive gland which is due to their role of being the major sight of detoxification. The importance of maintaining this process functioning requires a generally higher demand of oxidative protection. This is also apparent when comparing the tissues including the treatment values: The digestive gland is showing at least the second highest enzyme, protein, LPO and DNA damage values among the three tissues. Despite being in direct contact to atmospheric air while being emersed, the gills did not show a specific increase in oxidative stress protection unlike a former study on the

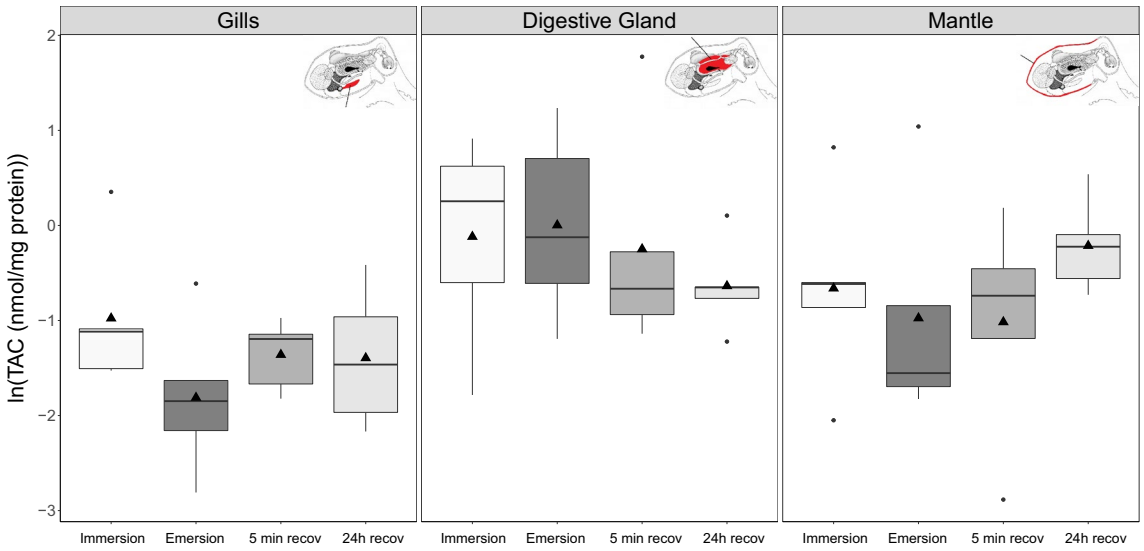


Fig. 6 Effects of emersion on *O. vulgaris* total antioxidant defense capacity. TAC, during emersion conditions ("Emersion"), emersion followed by a 5-min recovery period ("5 min recov") or 24-h recovery period ("24 h recov") and the control ("Immersion"). See the detailed statistical information in Supplemental Table 3

emersed mussel *Perna Perna* describes (Almeida et al. 2005). This could be evidence for the previously mentioned theory that some atmospheric oxygen is diffusing through the mucus covered gills, which would eliminate the necessity to prepare for a burst of oxygen after re-submersion. The mantle does consist mainly of water (Robertson 1965), which can explain why this tissue is not showing a significant higher antioxidant defence capacity compared to the digestive gland and gills.

Conclusions

The fishermen survey has shown that *O. vulgaris* in the Cascais area is commonly observed going out of tidepools. Our experimental work shows that *O. vulgaris* does not seem to face major physiological and biochemical impairments during and after exposure to short-term (< 3 min) atmospheric air. Emersion did cause an increase in OCR after re-submersion, but this was quickly compensated for. Concomitantly, there was no significant cellular damage, enhanced protein repair and oxidative stress in response to air exposure. Yet, further research is needed to fully understand how octopus withstand emersion and crawl on land, namely studying air exposure limits, the importance of anaerobic pathways (e.g., octopine buildup), and the potential role of cutaneous respiration.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00227-023-04333-x.

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Author contributions RR conceptualized the study; JLR, VML conducted the fishermen survey; JLR, VML, JRP, MS and TR conducted the experimental work; JLR, MRP performed and MD supervised the enzymatic assays; JLR and RR wrote the original draft of the manuscript; all authors reviewed and edited the manuscript.

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Data availability Data will be made available upon request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethics Experimental procedures were approved by Faculdade de Ciências da Universidade de Lisboa animal welfare body (ORBEA) and Direção-Geral de Alimentação e Veterinaria (DGAV) also approved these experiments following the requirements imposed by the Directive 2010/63/EU on the protection of animals used for scientific purposes.

References

Almeida EA, Bainy ACD, Dafre AL, Gomes OF, Medeiros MHG, Di MP (2005) Oxidative stress in digestive gland and gill of the brown mussel (*Perna perna*) exposed to air and re-submersed. J Exp Mar Bio Ecol 318:21–30. https://doi.org/10.1016/j.jembe.2004.12.007

Anderson RC, Martin AW (2002) An interview on octopuses with Cecil. A Brosseau (1919–1992). The Festivus 34(6):67–87

Aristotle (350 BC) see, Thompson WD (1910) Historia animalium, Book IX part 37, Volume 4. Clarendon Press

Balme DM, Gotthelf A (1991) Aristotle: history of animals, Book IX part 37, vol 1. Harvard Univerity Press, Cambirdge

Bradford M (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. https://doi.org/10.1016/j.cj.2017.04.003

Brown ER, Piscopo S, De Stefano R, Giuditta A (2006) Brain and behavioural evidence for rest-activity cycles in *Octopus vulgaris*. Behav Brain Res 172:355–359. https://doi.org/10.1016/j.bbr.2006.05.009

Clark TD, Sandblom E, Jutfelt F (2013) Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. J Exp Biol 216:2771–2782. https://doi.org/10.1242/jeb.084251

Crowther JR (2008) The ELISA guidebook. In: Methods in molecular biology, vol 149. Humana Press, Totowa, NJ

Denny MW, Gains SD (2007) Encyclopedia of tidepools and rocky shores. University of California Press, Berkeley

Eno NC (1987) Functional morphology of cephalopod gills. Dr Diss Univ Cambridge, Darwin Coll, pp 136–189

Fiorito G, Affuso A, Basil J, Cole A, de Girolamo P, D'angelo L, Dickel L, Gestal C, Grasso F, Kuba M, Mark F, Melillo D, Osorio D, Perkins K, Ponte G, Shashar N, Smith D, Smith J, Andrews PIr (2015) Guidelines for the care and welfare of cephalopods in research—a consensus based on an initiative by CephRes, FELASA and the Boyd Group. Lab Anim 49:1–90. https://doi.org/10.1177/0023677215580006

Forstner H (1983) An automated multiple-chamber intermittent-flow respirometer. In: Gnaiger E, Forstner H (eds) Polarographic oxygen sensors. Springer, Berlin, Heidelberg, pp 111–126

Gäde G (1976) Octopine dehydrogenase in the cockle *Cardium edule*. Biochem Soc Trans 4:433–436. https://doi.org/10.1042/bst0040433

Giraud-Billoud M, Rivera-Ingraham GA, Moreira DC, Burmester T, Castro-Vazquez A, Carvajalino-Fernández JM, Dafre A, Niu C, Tremblay N, Paital B, Rosa R, Storey JM, Vega IA, Zhang W, Yepiz-Plascencia G, Zenteno-Savin T, Storey KB, Hermes-Lima M (2019) Twenty years of the 'Preparation for Oxidative Stress' (POS) theory: ecophysiological advantages and molecular strategies. Comp Biochem Physiol -Part A Mol Integr Physiol 234:36–49. https://doi.org/10.1016/j.cbpa.2019.04.004

Grieshaber M, Gäde G (1976) The biological role of octopine in the squid, *Loligo vulgaris* (Lamarck). J Comp Physiol B 108:225–232. https://doi.org/10.1007/BF00691671

Guppy M, Withers P (1999) Metabolic depression in animals: Physiological perspectives and biochemical generalizations. Biol Rev 74:1–40. https://doi.org/10.1111/j.1469-185X.1999.tb00180.x

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139. https://doi.org/10.1016/S0021-9258(19)42083-8

Hanlon RT, Messenger JB (2018) Cephalopod behaviour, II. Cambridge University Press, Cambridge

Harianto J, Carey N, Byrne M (2019) respR — an R package for the manipulation and analysis of respirometry data. Methods Ecol Evol 10(6):912–920. https://doi.org/10.1111/2041-210X.13162

Hermes-Lima M, Zenteno-Savín T (2002) Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comp Biochem Physiol Part C 133:537–556. https://doi.org/10.1016/S1532-0456(02)00080-7

Herrmann JP, Enders EC (2000) Effect of body size on the standard metabolism of horse mackerel. J Fish Biol 57:746–760. https://doi.org/10.1006/jfbi.2000.1348

Houlihan DF, Innes AJ, Wells MJ, Wells J (1982) Oxygen consumption and blood gases of *Octopus vulgaris* in hypoxic conditions. J Comp Physiol B 148:35–40. https://doi.org/10.1007/BF00688885

Huggett J, Griffiths C (1986) Some relationships between elevation, physico-chemical variables and biota of intertidal rock pools. Mar Ecol Prog Ser Ser 29:189–197

Humbert JW, Williams KW, Onthank KL (2022) Den-associated behavior of *Octopus rubescens* revealed by a motion-activated camera trap system. Inetgr Comp Biol 62:1131–1143

Iglesias J, Fuentes L, Sánchez J, Otero JJ, Moxica C, Lago MJ (2006) First feeding of *Octopus vulgaris* Cuvier, 1797 paralarvae using *Artemia*: Effect of prey size, prey density and feeding frequency. Elsevier Aquac 261:817–822. https://doi.org/10.1016/j.aquaculture.2006.08.002

Johansson LH, Borg LAH (1988) A spectrophotometric method for determination of catalase activity in small tissue samples. Anal Biochem 174:331–336. https://doi.org/10.1016/0003-2697(88)90554-4

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 microplte. J Clin Biochem Nutr 44:46–51. https://doi.org/10.3164/jcbn.08-162

Lee H (1875) aquarium notes: the octopus or the "Devil-Fish" of fiction and of fact. Chapman and Hall, London

Lopes AR, Sampaio E, Santos C, Couto A, Pegado MR, Diniz M, Munday PL, Rummer JL, Rosa R (2018) Absence of cellular damage in tropical newly hatched sharks (*Chiloscyllium plagiosum*) under ocean acidification conditions. Cell Stress Chaperones 23:837–846. https://doi.org/10.1007/s12192-018-0892-3

Lourenço S (2014) Ecology of the Common Octopus *Octopus vulgaris* (Cuvier , 1797) in the Atlantic Iberian coast: Life cycle strategies under different oceanographic regimes. PhD dissertation, Universiy of Lisboa (Portugal)

Maclouf J, Grassi J, Pradelles P (1987) Development of Enzyme-Immunoassay Techniques for Measurement of Eicosanoids. Prostaglandin Lipid Metab Radiat Inj. https://doi.org/10.1007/978-1-4684-5457-4_37

Madan JJ, Wells MJ (1996) Cutaneous respiration in *Octopus vulgaris*. J Exp Biol 199:2477–2483

Mangold K (1983) Octopus vulgaris. In: Boyle PR (ed) Cephalopod life cycles. Press. London, Acad

Maulvault AL, Camacho C, Barbosa V, Alves R, Anacleto P, Cunha SC, Fernandes JO, Pousão-Ferreira P, Paula JR, Rosa R, Diniz M, Marques A (2019) Bioaccumulation and ecotoxicological responses of juvenile white seabream (*Diplodus sargus*) exposed to triclosan, warming and acidification. Environ Pollut 245:427–442. https://doi.org/10.1016/j.envpol.2018.11.020

McCord JM, Fridovich I (1969) An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244:6049–6055. https://doi.org/10.1016/S0021-9258(18)63504-5

Meisel DV, Byrne RA, Kuba M, Mather J, Ploberger W, Reschenhofer E (2006) Contrasting activity patterns of two related Octopus species, *Octopus macropus* and *Octopus vulgaris*. J Comp Psychol 120:191–197. https://doi.org/10.1037/0735-7036.120.3.191

Meisel DV, Kuba M, Byrne RA, Mather J (2013) The effect of predatory presence on the temporal organization of activity in *Octopus*

vulgaris. J Exp Mar Bio Ecol 447:75–79. https://doi.org/10.1016/j.jembe.2013.02.012
Metaxas A, Scheibling RE (1993) Community structure and organization of tidepools. Mar Ecol Prog Ser 98:187–198. https://doi.org/10.3354/meps098187
Morris S, Taylor A (1983) Diurnal and seasonal variation in physicochemical conditions within intertidal rock pools. Estuar Coast Shelf Sci 17:339–355
Neill SRJ, Cullen JM (1974) Experiments on whether schooling by their prey affects the hunting behaviour of cephalopods and fish predators. J Zool 172:549–569. https://doi.org/10.1111/j.1469-7998.1974.tb04385.x
Norman M (2000) Cephalopods, a world guide: Pacific Ocean, Indian Ocean, Red Sea, Atlantic Ocean, Caribbean, Arctic, Antarctic. ConchBooks, Hackenheim, pp 222–223
O'Dor RK, Webber DM (2008) The constraints on cephalopods: why squid aren't fish. Can J Zool 64:1591–1605. https://doi.org/10.1139/z86-241
Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358. https://doi.org/10.1016/0003-2697(79)90738-3
Onthank KL (2008) Aerobic metabolism and dietry ecology of *Octopus rubescens*. PhD dissertation, Walla Walla University
Paula JR, Repolho T, Grutter AS, Rosa R (2022) Access to Cleaning Services Alters Fish Physiology Under Parasite Infection and Ocean Acidification. Front Physiol 13:1–11. https://doi.org/10.3389/fphys.2022.859556
Pörtner HO (1990) An analysis of the effects of pH on oxygen binding by squid (*Illex illecebrosus*, *Loligo pealei*) haemocyanin. J Exp Biol 150:407–424. https://doi.org/10.1242/jeb.150.1.407
Pörtner HO (1994) Athleten des Meeres: Zur Ökophysiologie pelagischer Kalmare. In: Biologie in unserer Zeit. pp 192–199
R Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/
Renshaw GM, Kerrisk CB, Nilsson GE (2002) The role of adenosine in the anoxic survival of the epaulette shark, *Hemiscyllium ocellatum*. Comp Biochem Physiol Part B Biochem Mol Biol 131:133–141. https://doi.org/10.1016/S1096-4959(01)00484-5
Robertson JD (1965) Studies on the chemical composition of muscle tissue. III. The mantle muscle of cephalopoda molluscs. J Exp Biol 42:153–175
Roche DG, Binning SA, Bosiger Y, Johansen JL, Rummer JL (2013) Finding the best estimates of metabolic rates in a coral reef fish. J Exp Biol 216:2103–2110. https://doi.org/10.1242/jeb.082925
Romero MC, Ansaldo M, Lovrich GA (2007) Effect of aerial exposure on the antioxidant status in the subantarctic stone crab *Paralomis granulosa* (Decapoda: Anomura). Comp Biochem Physiol Part C 146:54–59. https://doi.org/10.1016/j.cbpc.2006.06.009
Rosa R, Seibel BA (2008) Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. Proc Natl Acad Sci U S A 105:20776–20780. https://doi.org/10.1073/pnas.0806886105
Rosa R, Seibel BA (2010a) Metabolic physiology of the Humboldt squid, *Dosidicus gigas*: Implications for vertical migration in a pronounced oxygen minimum zone. Prog Oceanogr 86:72–80. https://doi.org/10.1016/j.pocean.2010.04.004
Rosa R, Seibel BA (2010b) Slow pace of life of the Antarctic colossal squid. J Mar Biol Assoc United Kingdom 90:1375–1378. https://doi.org/10.1017/S0025315409991494
Rummer JL, Binning SA, Roche DG, Johansen JL (2016) Methods matter: considering locomotory mode and respirometry technique when estimating metabolic rates of fishes. Conserv Physiol 4:1–13. https://doi.org/10.1093/conphys/cow008
Seibel BA (2007) On the depth and scale of metabolic rate variation: Scaling of oxygen consumption rates and enzymatic activity in the Class Cephalopoda (Mollusca). J Exp Biol 210:1–11. https://doi.org/10.1242/jeb.02588
Seibel BA, Childress JJ (2000) Metabolism of benthic octopods (Cephalopoda) as a function of habitat depth and oxygen concentration. Deep Res Part I Oceanogr Res Pap 47:1247–1260. https://doi.org/10.1016/S0967-0637(99)00103-X
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
Söderström V, Renshaw GMC, Nilsson GE (1999) Brain blood flow and blood pressure during hypoxia in the epaulette shark *Hemiscyllium ocellatum*, a hypoxia-tolerant elasmobranch. J Exp Biol 202:829–835. https://doi.org/10.1242/jeb.202.7.829
Steffensen JF (1989) Errors in aquatic respirometry of aquatic breathers: how to avoid and correct for them. Fish Physiol Biochem 6:49–59
Storey KB, Storey JM (2004) Oxygen limitation and metabolic rate depression. In: Storey KB (ed) Functional metabolism: regulation and adaptation. Wiley-Liss, Hoboken, pp 415–442
Teixeira T, Diniz M, Calado R, Rosa R (2013) Coral physiological adaptations to air exposure: Heat shock and oxidative stress responses in *Veretillum cynomorium*. J Exp Mar Bio Ecol 439:35–41. https://doi.org/10.1016/j.jembe.2012.10.010
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. https://doi.org/10.1242/jeb.038034
Tomanek L, Somero GN (1999) Evolutionary and acclimation-induced variation in the heat-shock response of congeneric marine snails (Genus *Tegula*) from different thermal habitats: Implications for limits of thermotolerance and biogeography. J Exp Biol 202:2925–2936
Trübenbach K, Teixeira T, Diniz M, Rosa R (2013) Hypoxia tolerance and antioxidant defense system of juvenile jumbo squids in oxygen minimum zones. Deep Res Part II Top Stud Oceanogr 95:209–217. https://doi.org/10.1016/j.dsr2.2012.10.001
Truchot JP, Duhamel-Jouve A (1980) Oxygen and carbon dioxide in the marine intertidal environment: Diurnal and tidal changes in rockpools. Respirin Physiol 39:241–254. https://doi.org/10.1016/0034-5687(80)90056-0
Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552:335–344. https://doi.org/10.1113/jphysiol.2003.049478
Valverde JC, García García B (2004) Influence of body weight and temperature on post-prandial oxygen consumption of common octopus (*Octopus vulgaris*). Aquaculture 233:599–613. https://doi.org/10.1016/j.aquaculture.2003.11.025
Valverde JC, García García B (2005) Suitable dissolved oxygen levels for common octopus (*Octopus vulgaris* cuvier, 1797) at different weights and temperatures: analysis of respiratory behaviour. Aquaculture 244:303–314. https://doi.org/10.1016/j.aquaculture.2004.09.036
Wells MJ, Wells J (1984) The effects of reducing gill area on the capacity to regulate oxygen uptake and on metabolic scope in a cephalopod. J Exp Biol 108:393–401. https://doi.org/10.1242/jeb.108.1.393
Wells MJ, Wells J (1986) Blood flow in acute hypoxia in a cephalopod. J Exp Biol 122:345–353
Wells MJ, O'Dor RK, Mangold K, Wells J (1983) Diurnal changes in activity and metabolic rate in *Octopus vulgaris*. Mar Behav Physiol 9:275–287. https://doi.org/10.1080/10236248309378598
Wells MJ, Duthie GG, Houlihan DF, Smith PJS (1987) Blood flow and pressure changes in exercising octopuses (*Octopus vulgaris*). J Exp Biol 131:175–188
Wells MJ, Hanlon RT, Lee PG, Dimarco FP (1988) Respiratory and cardiac performance in *Lolliguncula brevis* (Cephalopoda,

Myopsida): The effects of activity, temperature and hypoxia. J Exp Biol 138:17–36. https://doi.org/10.1242/jeb.138.1.17
Wickham H (2016) ggplot2: elegant graphics for data analysis. Springer-Verlag, New York. ISBN 978-3-319-24277-4. https://ggplot2.tidyverse.org
Wilhelm-Filho D, González-Flecha B, Boveris A (1994) Gill diffusion as a physiological mechanism for hydrogen peroxide elimination by fish. Brazil J Med Biol Res 27:2879–2882
Wodinsky J (1971) Movement as a necessary stimulus of octopus predation. Nature 229:493–494
Wood JB, Anderson RC (2004) Interspecific evaluation of octopus escape behavior. J Appl Anim Welf Sci 8705:37–41. https://doi.org/10.1207/s15327604jaws0702
Wurts W, Durborow R (1992) Interactions of pH, carbon dioxide, alkalinity and hardness in fish ponds. South Reg Aquac Cent 464:1–3
Young JZ (1963) The number and sizes of nerve cells in octopus. Proc Zool Soc Lond 140:229–254. https://doi.org/10.1111/j.1469-7998.1963.tb01862.x