Marine Environmental Research 92 (2013) 110–119

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Oxygen radical formation in anoxic transgression and anoxia-reoxygenation: Foe or phantom? Experiments with a hypoxia tolerant bivalve

Georgina A. Rivera-Ingraham [a], Iara Rocchetta [a, b], Stefanie Meyer [a], Doris Abele [a, *]

[a] Department of Functional Ecology, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany
[b] Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, INQUIBICEN-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Pab. II, Ciudad Universitaria, 1428 Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 19 July 2013
Received in revised form
10 September 2013
Accepted 12 September 2013

Keywords:
Blue mussel
Oxidative stress
Reoxygenation
Live imaging

ABSTRACT

Intertidal blue mussels, *Mytilus edulis*, experience hypoxia reoxygenation during tidal emersion and resubmersion cycles, and this is often suggested to represent a major stress for the animals, especially for their respiratory tissues, the gills. We exposed mussels to experimental short and prolonged anoxia and subsequent reoxygenation and analyzed the respiratory response in excised gill tissue and the effects of treatment on reactive oxygen species (mainly ROS: superoxide anion, $O_2^{\cdot-}$ and hydrogen peroxide, H_2O_2), formation using live imaging techniques and confocal microscopy. Our aim was to understand if this "natural stress" would indeed produce oxidative damage and whether antioxidant defenses are induced under anoxia, to prevent oxidative damage during reoxygenation. Exposure to declining pO_2 in the respiration chamber caused an increase of gill metabolic rate between 21 and 10 kPa, a pO_2 range in which whole animal respiration is reported to be oxyregulating. Exposure of the animals to severe anoxia caused an onset of anaerobiosis (succinate accumulation) and shifted high and low critical p_c values (p_{c1}: onset of oxyregulation in gills, p_{c2}: switch from oxyregulation to oxyconformity) to higher pO_2. Concentrations of both ROS decreased strongly during anoxic exposure of the mussels and increased upon reoxygenation. This ROS burst induced lipid peroxidation in the mantle, but neither were protein carbonyl levels increased (oxidative damage in the protein fraction), nor did the tissue glutathione concentration change in the gills. Further, analysis of apoptosis markers indicated no induction of cell death in the gills. To our knowledge, this is the first paper that directly measures ROS formation during anoxia reoxygenation in mussels. We conclude that hypoxia tolerant intertidal mussels do not suffer major oxidative stress in gill and mantle tissues under these experimental conditions.

1. Introduction

Marine and fresh water bivalves are among the most hypoxia tolerant macrofauna, and some benthic infauna species survive days and even weeks in complete anoxia (Abele et al., 2009). Intertidal, epibenthic bivalves, like blue mussels *Mytilus edulis*, experience complete anoxia when they close their shells during low tides to prevent desiccation, and shell water pO_2 rapidly falls to zero. Whereas some intertidal bivalves prevent severe anoxia by opening the valves for air gaping, this behavior is not common in *M. edulis*. Instead the strategy of the blue mussel is to keep the shell closed and depress energy expenditures and, as a consequence, ATP turnover and dissipation of heat (Wang and Widdows, 1993). Exposing mussels to declining oxygen levels in seawater, the authors recorded pO_2-independent respiration rates (oxyregulation) between 20 kPa (normoxia) and 10 kPa (moderate hypoxia). Below 10 kPa, respiration and heat dissipation diminished as the mussels entered into an energy saving state of suspended animation, characterized by reduced filtration and partial arrest of protein synthesis and degradation, to enable metabolic rate depression and, at the same time, minimize the energy and oxygen debt. Further reduction of pO_2 to near anoxia (1–2 kPa) induces anaerobic glycolysis in mussels and causes rapid accumulation of succinate within hours, with the highest concentrations measurable in

* Corresponding author. Tel.: +49 471 4831 1517; fax: +49 471 4831 1149.
E-mail addresses: grivera@us.es (G.A. Rivera-Ingraham), Doris.Abele@awi.de (D. Abele).

0141-1136/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.marenvres.2013.09.007

the gills. In prolonged anoxia (>16 h), succinate is converted to propionate and, to a lesser extent, to acetate in anaerobically working mussel mitochondria (Zurburg and Kluytmans, 1980 for mussels held in anoxia). Altogether these strategies render *M. edulis* relatively hypoxia tolerant, and median mortality time of 30–35 mm mussels is 9.6 days (Wang et al., 1992). Under intertidal conditions, re-immersion usually happens after 6 h so that tidal exposure poses no major problem to the mussels from the point of view of energetics. Hence this species qualifies as an euryoxic, hypoxia tolerant model to study the physiological effects of exposure to anoxia and subsequent reoxygenation.

Exposure of non-hypoxia tolerant animals to anoxia and subsequent reoxygenation has major consequences at the level of the cellular prooxidant/antioxidant balance and cellular redox homeostasis (Li and Jackson, 2002; Pamplona and Costantini, 2011; Tarpey et al., 2004). Small amounts of reactive oxygen species (ROS) are permanently formed in aerobically working cells through incomplete reduction of oxygen in the mitochondria. As tissue oxygen concentrations decline, the respiratory chain intermediates, especially at complex 1 (NADH–FADH2 oxidoreductase) and complex III (ubiquinone-cytochrome c-oxidoreductase), become increasingly reduced. Reduced intermediates of respiratory electron transport, such as ubisemiquinone, are charged with loosely bound electrons that are rapidly transferred to oxygen during reoxygenation. This produces oxygen derivatives with odd numbers of electrons (ROS), not all of them radicals, but many of them highly reactive and potentially damaging. This is why tissue oxygenation in animals is usually controlled low and constant in air or water breathers (for review see Massabuau and Abele, 2012).

For a long time since their discovery in the 1950s, ROS were exclusively regarded as damaging molecules that cause deterioration of cells and subcellular structures, and are centrally involved in cancer development and aging. Indeed, some ROS (superoxide and hydroxyl radical, $OH^{\bullet}$) are highly reactive prooxidants and interact rapidly by subtracting electrons from all kinds of cellular macromolecules, such as membrane fatty acids, protein amino acids and also DNA molecules. Other radical species are less reactive and these are produced in controlled quantities, e.g. as antimicrobial defense (hydrogen peroxide) in the blood of marine invertebrates with open circulatory systems (Carballal et al., 1997; Dikkeboom et al., 1987; Husmann et al., 2011). Cells counteract the damaging effects of ROS with effective antioxidant systems, including a suite of non-enzymatic radical scavengers (e.g. vitamins A, C, E and K, glutathione) and enzymatic antioxidants such as superoxide dismutase (SOD, converting superoxide anion into hydrogen peroxide), catalase (CAT, transforming hydrogen peroxide into water) or selenium-dependent glutathione peroxidase (Se-GPX, removing organic peroxides).

The present study deals with the formation of ROS and the resulting oxidative damage in gills of blue mussels under control conditions (normoxia), after exposure to different periods of anoxia (48 h and 72 h), and following reoxygenation. The idea is by no means a new one, but earlier works addressing the effects of oxygen deprivation and reoxygenation in marine animals exclusively report the response of the antioxidant defense system and the resulting oxidative damage (Almeida and Bainy, 2006; Irato et al., 2007; Viarengo et al., 1989). Contrary, the intensity of ROS formation itself is difficult to assess in living animals or intact organs. Without a direct measurement of the changes of ROS levels in response to changing oxygenation levels, conclusions may however be biased. Alterations of the antioxidant system, e.g. antioxidant enzyme activities, are only secondary indicators of oxidative stress, which often results from a lack of a proper antioxidant defense and the failure to induce gene transcription and synthesis of stress proteins under conditions of critical stress. Thus, absence of an increase in cellular antioxidants does not necessarily indicate the absence of oxidative stress, and vice versa. Contrary, oxidative damage products such as protein carbonyls as markers for oxidative protein damage or TBARS (thiobarbituric acid reactive substances) as a measure for lipid peroxidation are well accepted indicators of oxidative stress. However, their accumulation also depends on cell turnover and autophagic or apoptotic damage removal. Hence, the best method is to directly measure radical formation, and one elegant approach to do this is live imaging of cells, tissues and even small intact animals, using confocal microscopy combined with ROS-sensitive fluorescent dyes (Rivera-Ingraham et al., 2013).

Here we present our analysis of the effects of anoxia and reoxygenation on mussel gills, the organ which is most active in mussels and, therefore, reacts most sensitive to oxygen deprivation. We recorded the respiratory response of isolated gill pieces to declining oxygen tension and compared ROS formation, antioxidant defense and oxidative damage after exposing whole animals to normoxia, anoxia and anoxia-reoxygenation. Succinate was measured as indicator of anaerobic energy production. To understand whether elevated ROS or changes in tissue redox balance under any of the treatments induce apoptotic activity in gill tissues, we measured caspase activity in gill homogenates.

Our expectation was that ROS production would increase under anoxia reoxygenation treatment in mussels, and that this would have an inducing effect on the antioxidant enzyme activities and possibly cause ROS damage, alter the glutathione redox state, and apoptotic activity in the gills.

2. Materials and methods

2.1. Animal collection and maintenance

Blue mussels *M. edulis* were collected at the intertidal at the island of Sylt (Germany) in December 2012. Animals were transported to the Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research (AWI) in Bremerhaven, where they were maintained in aquaria at constant temperature of 10 °C, 33 PSU and >99% air saturation, and totally submersed. Animal shells were cleaned from epibiontic growth and allowed to adapt to the aquarium conditions for 3 weeks. Mussels were fed weekly with living phytoplankton, Fa. Plankton Farm, Sycamore, USA. Water circulation in the aquaria was stopped for four hours to allow feeding. Water quality was assessed weekly, using Nanocolor® Tube Tests (Macherey–Nagel GmbH & Co. KG, Germany) for ammonium and nitrate. Water in the aquaria was changed when values exceeded 0.4 mg/l and 0.2 mg/l of ammonium and nitrate, respectively. Prior the start of the experiment, animals were kept without food for 1 week to avoid the impact of specific dynamic action on gill respiration (Bayne et al., 1976).

2.2. Experimental design

A total of 4 treatments were considered in our study: anoxia (<0.6% air saturation): for 48 h, anoxia for 72 h and anoxia 72 h followed by a 24 h period of reoxygenation in normoxia (20 kPa). In each experiment, three 3L-aquaria with 5 individuals each were used to obtain independent parallel samples for each treatment. An addition, three 3L-aquaria with 5 other individuals were kept at normoxia for the same timespan as control group.

A total of 60 animals were used in the study. These had an average (±SEM) shell length of 4.50 ± 0.04 cm, a height of 2.22 ± 0.02 cm and a width of 1.99 ± 0.03 cm. Based on their size, and the works of Ottway & Ross (in Gosling, 2003) and Sprung (1983), we consider these animals as adults. No differences were made regarding sex or state of maturity. Mussels were sacrificed by

cutting through the adductor muscle. Gills and mantle tissues were sampled. Two small pieces of gill tissue (of approximately 15 mg) were saved from 6 animals per treatment for respiration and confocal microscopy (ROS formation) measurements. The rest of the gill tissue was homogenized on ice in a 30 mM KPi-120 mM KCl buffer supplemented with a cocktail of protease inhibitors: phenylmethylsulfonyl fluoride (PMSF)(20 mg/ml in isopropanol), leupeptin (0.01 g/ml), pepstatin A (0.9 mg/ml in 90/10 ethanol/acetic acid) and aprotinin (0.01 g/ml). Homogenates were preserved at −80 °C, while pieces of the mantle were frozen in liquid nitrogen for TBARS analysis.

2.3. Respiration measurements

Respiration measurements were carried out using excised gills of animals from the different treatments (see Section 2.2). All measurements were started in fully oxygenated medium in sealed micro-chambers where respiration was recorded as a function of declining pO_2 over time. Oxygen consumption was recorded for one gill piece per animal and normalized to g gill wet weight.

Isolated gill pieces were measured in sterile filtered medium (0.2 μm Whatman) composed of natural seawater (33 PSU) supplemented with 15 mM Na-HEPES (NH-FSW). Buffering was necessary to avoid pH changes in the medium when setting anoxic conditions (pH = 8.3). Gill pieces (8.12 ± 0.69 mg wet weight) of 9–12 individuals per treatment were used for respiration measurements. The wells of a 96-well Nunclon plastic microtiter plate (NunclonTM Nalge Nunc, Denmark) served as a respiration chamber. Wells with a volume of 0.33 ml and a diameter of 8 mm were equipped with oxygen sensor spots (SP-PSt3-NAU-D5-YOP, Precision Sensing GmBH, Regensburg, Germany) glued to the bottom of the well using silicon paste. Since experimental trials showed that the amount of tissue in a respiration chamber can have an effect on the oxygen consumption measurements (Van Winkle, 1968), we used approximately the same amount of tissue (7–9 mg wet wt.) for each animal. Each experimental well contained the gill tissue and was filled completely with NH-FSW to its maximum capacity (around 0.33 ml). Wells were sealed as in Rivera-Ingraham et al. (2013): briefly, wells were covered with a coverslip, avoiding formation of air bubbles, and the complete surface of the microtiter plate was additionally covered with a layer of auto-adhesive Armaflex® (Armacell Enterprise GmBH, Münster, Germany). Additional pressure was maintained on the complete surface of the plate in order to ensure air-tight sealing. Measurements were carried out using a 4-channel fiber-optical oxygen meter (Oxy-4) and non-invasive oxygen sensors (Precision Sensing GmBH, Regensburg, Germany) which were daily calibrated following the manufacturer's description. Four parallel measurements were carried out at a time (including a blank). Data were recorded at 15 s intervals, and the experiments were stopped when the oxygen was completely consumed from each of the chambers (which usually took around 22 h). Measurements were conducted at a constant temperature of 7 °C. Data corresponding to the first 60 min following the start of the experiment (with abnormally high respiration rates) were discarded in order to avoid interference from stress related to the manipulation of the tissues. After this time, in the vast majority of the cases, oxygen consumption rates stabilized. Respiration rates are expressed as nmol O_2 ml^{-1} min^{-1} mg^{-1} wet tissue.

Two critical environmental oxygen pressures (p_{c1} and p_{c2}) were determined, based on section-wise linear regression of each respiration curve, to define the points of maximal change in pO_2 dependent respiration. The upper critical p_{c1} was defined as the pO_2 below which the weight specific respiration rate increases with declining environmental pO_2. This effect is attributed to higher energy expenditure for ventilatory compensation through enhanced ciliary beating in mild hypoxia (see Herreid, 1980: Fig. 2A). p_{c2} is the lower critical environmental pO_2 below which the animals run into an oxygen debt and compensation is no longer possible. At p_{c2} respiration starts to be oxyconforming, i.e. oxygen consumption declines rapidly with decreasing pO_2 as the animals shift to anaerobic metabolism (see Herreid, 1980: Fig. 3, model III). p_c values were determined for each individual experiment as described by Duggleby (1984).

2.4. ROS formation

Measurements of cellular ROS levels were conducted by confocal microscopy using freshly excised gill filaments from treatment and normoxic control animals and ROS-sensitive fluorescent dyes. Incubations and the measurements were carried out in NH-FSW medium. The experimental series comprising short and prolonged anoxic incubations and subsequent reoxygenation, as described under Section 2.2, was repeated on three consecutive days with three animals per treatment. ROS formation was assessed by "live imaging" techniques, using a Leica TCS SP5II 2 Photon laser confocal microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) and a CCD camera system (Visitron Systems GmbH, Puchheim, Germany).

Pieces of gill tissues from treatment and control animals were stained with Dihydroethidium (DHE) (DHE, ex: 355 nm and em:

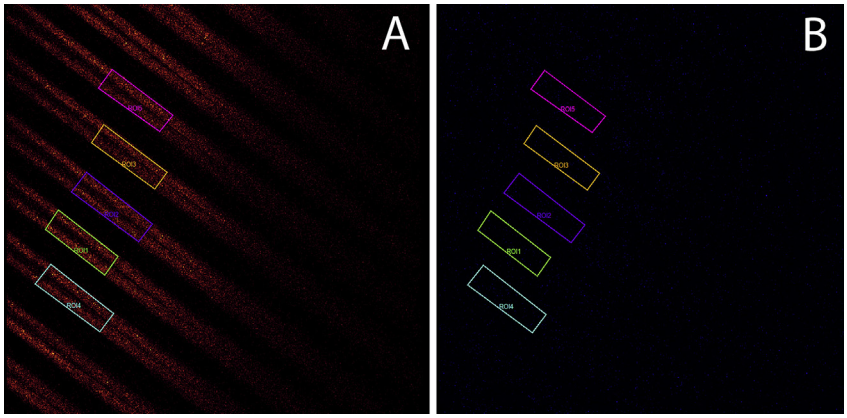

Fig. 1. Calculation of 2-OH-E+:DHE ratios from the images taken. A total of 5 areas were plotted on each image using Leica LAS lite software. A ratio between the mean intensity value recorded by the PMT2 (A) and the mean intensity recorded by the PMT1 (B) was calculated.

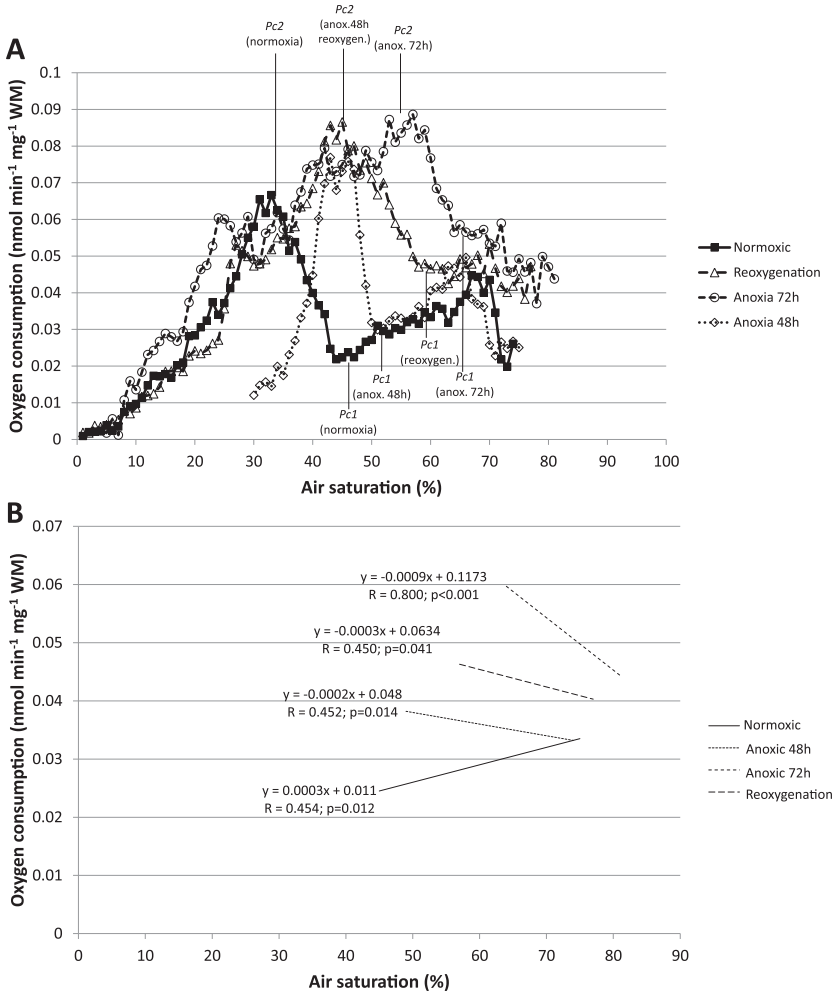

Fig. 2. Oxygen consumption rates of *M. edulis* gills at (A) different environmental air saturations (%) for the different treatments considered in the study. Values are pooled in 1% intervals. Points represent mean consumption rates for the 6 replicas conducted; (B) air saturations above p_{c1}. Lines represent linear regression equations for each of the treatments. WM = wet mass.

400–440 nm; 2-hydroxyethidium (2-OH-E^+), ex: 488 nm and em: 620–660 nm). To detect the formation of superoxide anion ($O_2^{\cdot-}$), the ratio 2-OH-E^+:DHE was calculated. In a parallel approach, with 5-Carbonyl-2′7′difluorodihydrofluoresceine-diacetate (C-H_2DFFDA) (ex: 488 nm; em: 510–550 nm) which detects different ROS and RNS species (H_2O_2, HOO• and ONOO•) but not $O_2^{\cdot-}$. For the first measurement of superoxide, tissues were incubated in NH-FSW supplemented with 10 μM DHE in DMSO (Molecular Probes D-23107). For the ROS measurement, tissues were exposed to NH-FSW containing 20 μM C-H_2DFFDA in ethanol (Molecular Probes C-13293), the membrane permeable diacetate form of DCFH. Both staining periods lasted 30 min and they were conducted in 2 ml Utermöhl chambers filled with medium previously flushed with 100% N_2 and in a gas tight glove box, previously equilibrated with 100% N_2. Chambers were tightly sealed inside the glove box for the analysis under the confocal microscope, to avoid reoxygenation of the gill tissues during the laser scanning observations. The conditions of the analysis are summarized in Table 1.

Five pictures per gill sample were taken, using a 10× objective. In order to avoid photo-bleaching, a short period (<5 s) of low resolution (256 × 256 pixel) live scanning was applied for focus adjustments and afterward only one single scan (512 × 512 pixel) was run of each gill filament. Autofluorescence was suppressed by adjusting the threshold, and phototoxicity was minimized by the multiphoton laser for low wavelengths scans.

Image analysis was carried out using Leica LAS Lite software (Leica Microsystems CMS GmbH, 2011). For each picture (which encompassed around 8 to 10 gill filaments), the average fluorescence intensity (or ratio for the case of DHE) was calculated for 5 areas (of around 2000 μm^2) on 5 different filaments (Fig. 1). The values of the 5 pictures were averaged for each piece of gill.

2.5. Antioxidant defense

CAT and SOD activities were measured in gill homogenates for each individual of treatment and control groups. CAT activity was determined as the decomposition of a 0.3 M H_2O_2 solution in a 50 mM KPi buffer (Aebi, 1984). SOD activity was measured using the cytochrome oxidase assay after Livingstone et al. (1992). All values were related to protein content measured by the method originally described by Bradford (1976).

Non-enzymatic antioxidant defense was measured by HPLC as the reduced and oxidized glutathione (GSH, GSSG) according to Almeida et al. (2012). Values were expressed as nmol per mg FW and the redox ratio calculated as GSSG/GSH.

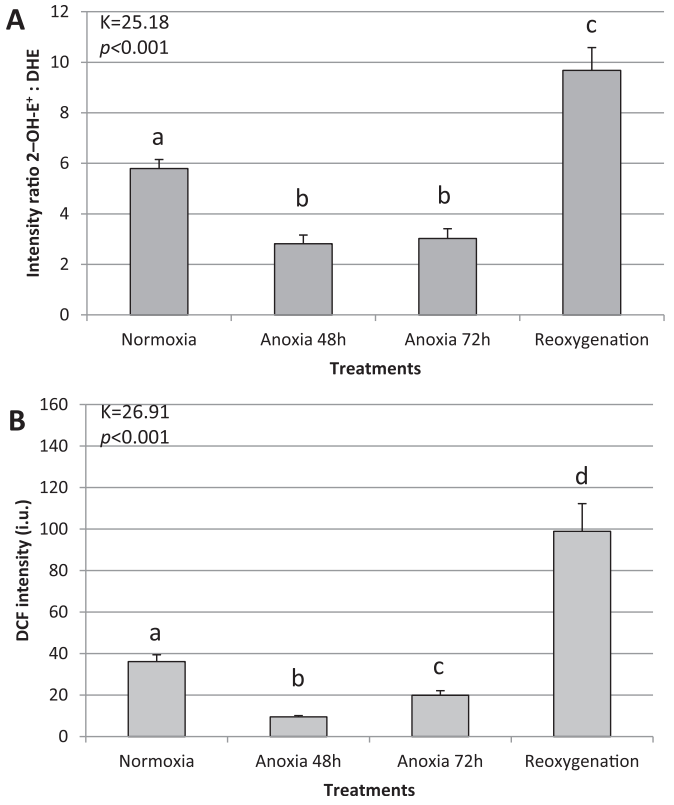

Fig. 3. Fluorescence intensity indicating ROS formation using (A) DHE and (B) DCF staining. Values associated with the same letter (a–b–c–d) correspond to the same subset based on an a-posteriori multiple comparison test.

2.6. Oxidative damage

Oxidative damage and cellular stress levels were assessed through the measurement malondialdehyde (TBARS) and protein carbonyl (PC) content and through the activity of caspases 3/7 involved in apoptotic cell disintegration.

Activity of caspases 3/7 was determined in the gills of 5 animals per treatment. Frozen gill samples were homogenized at 4 °C in a lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, and 1 μg ml^{-1} of each of the protease inhibitors leupeptin, pepstatin and aprotinin) at a ratio of 1:100 (w/v) (Strahl and Abele, 2010) using a Precellys24 bead grinder (Precellys24, Bertin Technologies, Orléans, France) at a speed at 5500 (2 cycles × 20 s, pause 5 s). Homogenates were centrifuged for 15 min at 15,500 × *g* at 4 °C and apoptosis intensities in supernatants were determined using the Caspase-Glo® 3/7 kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions at 25 °C. Luminescence signals were recorded using a Tristar LB941 plate reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Values are expressed as relative light units (RLU). All values were related to protein content in the sample measured according to Bradford (1976).

Protein carbonyl content was assessed in gill tissues using the OxiSelect Protein Carbonyl ELISA Kit (Cell Biolabs Inc., San Diego, CA) according to the manufacturer's instructions. As for caspase 3/7 activity measurements, PC contents were also expressed as a function of protein content.

Lipid peroxidation was determined as malondialdehyde content in mantle samples after Uchiyama and Mihara (1978) and as adapted by Abele et al. (2002). Results are expressed as nM/g tissues dry mass (DM).

2.7. Succinate concentration

Tissue concentration of succinate was assayed as a marker for the severity of the anoxic exposure of supernatants using a kit (Cat. No. 10 176 281 035, Boehringer Mannheim/R-Biopharm, Germany). Briefly, the succinic acid is converted to succinyl-CoA by succinyl-CoA synthetase and inosine-5′-diphosphate (IDP), which is used to produce pyruvate in the presence of pyruvate kinase. Pyruvate is oxidized by NADH, which is correlated with the amount of succinic acid. The decrease of the NADH absorbance was recorded with a DU 800 spectrophotometer (Beckmann, Germany) at 340 nm and 37 °C and following the manufacturer's instructions. Results are expressed as μmol per g FW.

2.8. Statistical analyses

For each of the biochemical tests conducted, the existence of significant difference among normoxic treatments (48 h, 72 h and 96 h) was assessed. Only when these were not significant from each other, values were pooled for further statistical analyses. Otherwise, each normoxic treatment was considered independently and was compared to the corresponding anoxic treatment. When data complied with the assumptions for conducting parametric analysis, one-way ANOVA tests were carried out. As a non-parametric choice, a Kruskal–Wallis test was executed.

All analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). All values are expressed as mean ± SE.

3. Results

3.1. Respiration measurements

Results from the respiration measurements are shown in Fig. 2a and Table 2. Over the whole range of environmental pO_2, three intervals differing in the trend of pO_2 dependent change of respiration can be distinguished. Above p_{c1}, and thus at high pO_2, respiration rates were fitted to a linear regression, the slope of which differed between treatments (Fig. 2b).

Both p_{c1} and p_{c2} change with time of anoxic exposure of the living animal (Table 2). Onset of ventilatory compensation (p_{c1}) for hypoxia happened at the lowest environmental pO_2 in gill pieces of animals that had experienced only normoxia, while higher p_{c1} values were recorded for gill pieces of animals kept in anoxia for 48 h and 72 h. After 48 h the animals had already accumulated an oxygen debt that caused elevated succinate levels in the gills (Fig. 6). After 72 h succinate levels were again inconspicuous and did not differ from normoxic concentrations, as succinate was converted to acetate and propionate in *M. edulis* tissues. Still, p_{c1}, p_{c2}, and the average respiration rates above p_{c1} were higher than in animals which had only been kept 48 h in anoxia.

Above this p_{c1}, normoxic individuals maintained an average oxygen consumption of 0.029 ± 0.001 nmol min^{-1} mg^{-1} WM (Table 2), although respiration rates linearly decreased as oxygen became limiting (Fig. 2b). Anoxic exposure caused a tiered increase of average respiration rates above p_{c1} (Table 2) ($K = 107.77$; $p < 0.001$), and oxygen consumption rates in all cases linearly increased with decreasing oxygen (Fig. 2b). A 24 h period of reoxygenation was not long enough to allow the animals to recover from their oxygen debt. Although succinate concentrations were back to control level after 72 h anoxia, respiration rates were still significantly higher than in normoxia ($K = 77.64$; $p < 0.001$) and after 48 h of anoxic exposure ($K = 27.66$; $p < 0.001$), and similar to 72 h anoxic exposure rates ($K = 1.65$; $p = 0.198$).

p_{c2} at which compensation ends and respiration starts to decline in an oxyconforming manner, shifted between treatments

Table 1
Analysis conditions for each of the dyes used during the study.

Dye	Mechanism of function	Final conc. used (μM)	Excitation		Emission		Calculation
			λ_1 (nm)	λ_2 (nm)	PMT1 (nm)	PMT2 (nm)	
Dihydroethidium (DHE) (in DMSO)	Regularly shows a blue emission when excited with a 355 nm laser. When oxidized to 2-OH-E^+ by the presence of O_2^-, it intercalated with the DNA and shows a red emission when excited with an argon laser.	10	MP (355)	488	400–440	620–660	Ratio PMT2/PMT1
C-H_2DFFDA (in Ethanol)	Non-fluorescent molecule which is converted to a green-fluorescent form (DCF) when the acetate groups are removed by intracellular esterases and oxidation occurs in the cell.	20	488	–	510–550	–	Maximum intensity

MP = Multiphoton laser. Values in parenthesis indicate the effective excitation wavelength.

following the same pattern as p_{c1}, albeit in a lower absolute range between 7 and 10 kPa (Table 2, Fig. 2). The scope of respiratory increase between p_{c1} and p_{c2} was significantly higher in animals from the normoxic and the 48 h anoxic treatment compared to 72 h anoxic treatment with or without reoxygenation, indicating onset of metabolic depression after prolonged anoxic exposure.

3.2. ROS formation

Superoxide anion formation ($O_2^{\cdot-}$) in gills of animals exposed in different treatments were assessed using dihydroethidium (DHE) (Supplementary material Fig. S1). Three different experiments were run with three animals per treatment group. No differences in $O_2^{\cdot-}$ concentration were found between the gills of individuals exposed over 48 h and 72 h to anoxia (*a-posteriori* multiple comparison test). However, 2-OH-E^+:DHE intensity ratios in gills of anoxically incubated animals were significantly lower than those recorded in gills of animal kept permanently in normoxia. Mussels exposed to 72 h of anoxia with subsequent reoxygenation had the highest 2-OH-E^+:DHE fluorescence levels in gills compared to all other treatments (Kruskal–Wallis non-parametric test) ($K = 25.18$; $p < 0.001$) (Fig. 3a). Thus, superoxide formation in *M. edulis* gills is suppressed under prolonged anoxia compared to nomoxic conditions as a feature of metabolic rate depression, but clearly induced by anoxia-reoxygenation.

The formation of ROS and RNS (mainly: H_2O_2, HOO• and $ONOO^-$) were determined by staining with C-H_2DFFDA (Fig. S2 of the supplementary material) and differences in DCF signal intensity among treatments were analyzed with non-parametric Kruskal–Wallis test ($K = 26.61$; $p < 0.001$) (Fig. 3b). Gills of animals exposed to anoxia-reoxygenation showed the highest DCF fluorescence, which was significantly above the normoxic control treatment. DCF signal intensity in gills of mussels exposed to prolonged (72 h) anoxia was significantly lower than in gills from the normoxic control treatment, but twice as high as in gills of mussels exposed only 48 h to anoxia. These results suggest a minor increase in ROS formation to happen under prolonged (72 h) compared to shorter (48 h) anoxic exposure.

3.3. Antioxidant defense

Both, CAT and SOD activities differed between treatments and over time (see Fig. 4A and B). Normoxic control individuals showed increasing CAT activity over time of incubation with 72 h > 48 h of normoxic treatment. This may indicate that the animals were exposed to a pro-oxidant stress under the experimental conditions in the incubation jars with permanently aerated seawater. It may, however, also show that transferring the animals to the incubation jars caused a stress, leading to an initial depression of CAT and SOD activity, from which the animals were still recovering at 72 h of maintenance. The fact that CAT and SOD activities also increased in the 72 h-anoxic exposure group compared to the 48 h anoxic exposure group seems to support this second possibility. Still, signals measured for both ROS in gills of normoxically incubated animals were exaggerated over individuals maintained in anoxia (both incubation periods), which speaks for higher ROS levels to persist under fully oxygenated conditions. The fact that neither protein carbonyl nor TBARS concentrations were elevated in normoxic control animals over the values in gills (or mantle in case of TBARS) of mussels exposed to anoxia (Fig. 5) argues against occurrence of oxidative stress in experimentally aerated seawater. Further, animals that were reoxygenated after 72 h of anoxia had

Table 2
Summary statistics corresponding to the respiration measurements conducted on *M. edulis* gills.

	Replicates conducted	p_{c1} (air saturation) (%)	Average RR above p_{c1}: 21 kPa-p_{c1}[a]	p_{c2} (air saturation) (%)	$Scope_{comp}$ (%)	Average RR at p_{c2}[b]
Normoxia	9	45	0.029 ± 0.001 a	31	124	0.065 ± 0.019
Anoxia 48 h	9	49	0.037 ± 0.001 b	43	108	0.077 ± 0.038
Anoxia 72 h	9	64	0.052 ± 0.002 c	53	71	0.087 ± 0.030
Anoxia 72 h + Reoxygenation 24 h	12	57	0.047 ± 0.001 c	43	83	0.086 ± 0.025

p_{c1} = upper critical oxygen partial pressure in kPa.
p_{c2} = lower critical oxygen partial pressure in kPa.
RR = respiration rates in nmol.
$Scope_{comp}$: scope of compensation in percent of respiratory increase between p_{c1} and p_{c2}.
[a] Kruskal–Wallis Test ($K = 107.77$; $p < 0.001$). Values followed by different lowercase letter are significantly different.
[b] ANOVA Test ($F = 0.103$; $p = 0.957$, non significant).

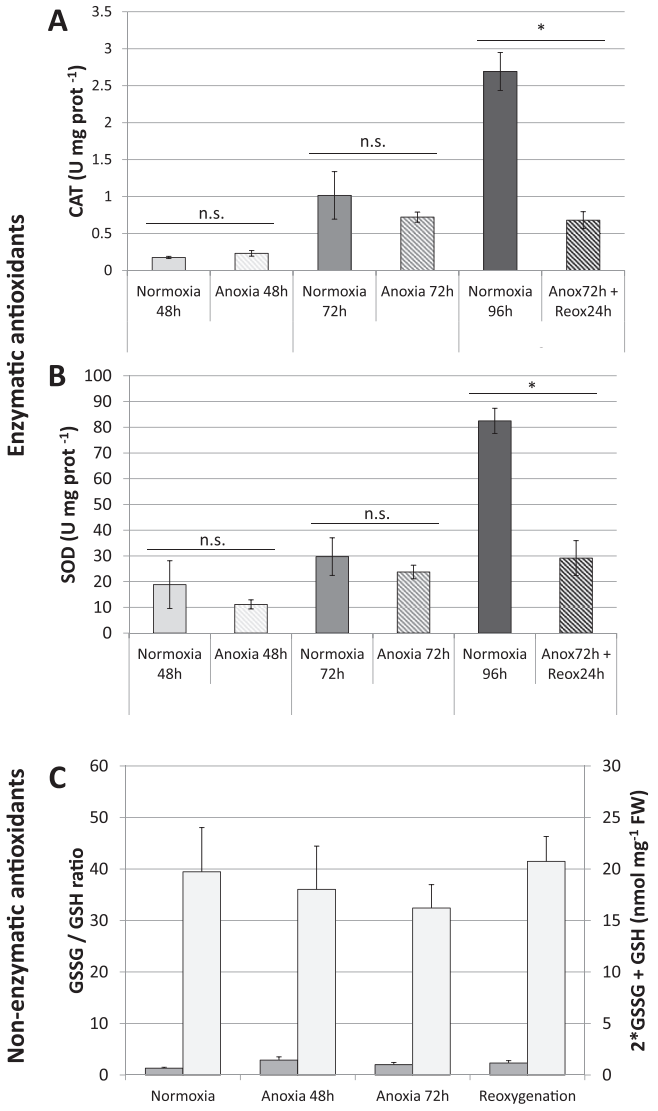

Fig. 4. Antioxidant defense measured as (A) catalase activities (B) SOD activities and c) GSSG/GSH ratio (gray bars) and total glutathione content (expressed as 2*GSSG + GSH) (white bars), for each of the different treatments considered (means ± SE). n.s.: non significant. $^{*}p < 0.001$.

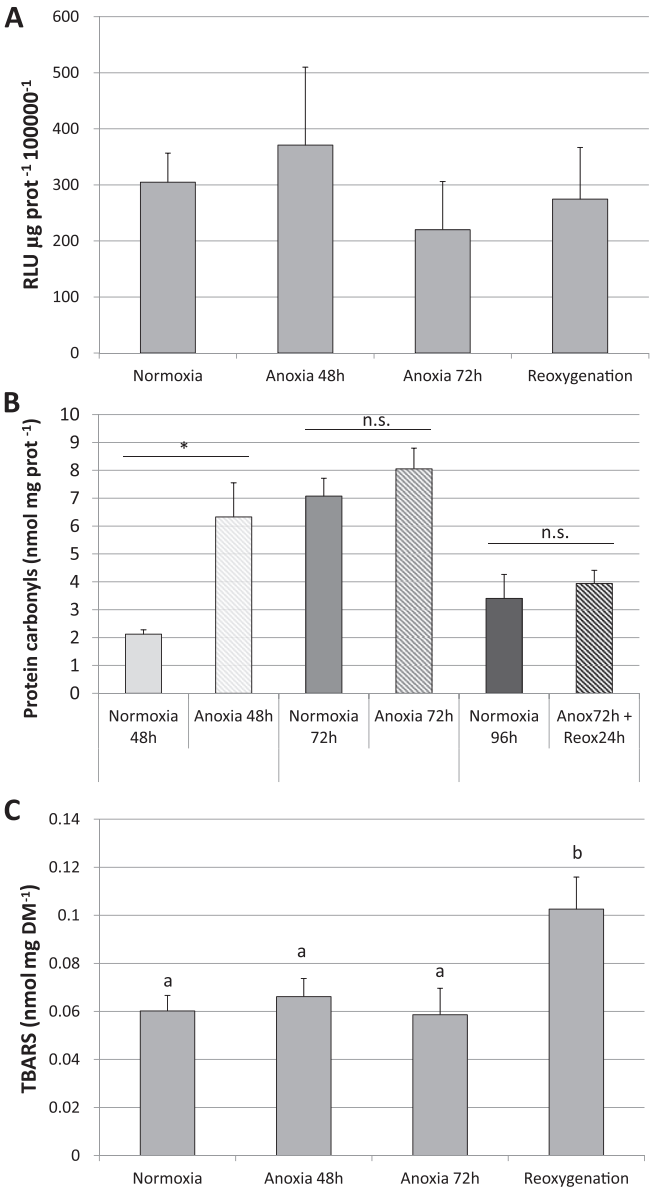

Fig. 5. Oxidative damage results measured as (A) caspase intensities in RLU (relative light units), (B) protein carbonyls and (C) TBARS content in *M. edulis* gills for each of the treatments considered (means ± SE). Values associated with the same letter (a–b) correspond to the same subset based on an a-posteriori multiple comparison test with $p < 0.01$. $^{*}p < 0.05$. n.s.: non significant.

dramatically suppressed antioxidant enzyme activities compared to their respective normoxic controls (CAT: $F = 58.51$; $p < 0.001$; SOD: $F = 17.71$; $p = 0.001$). For both enzymes the values were similar to those measured at 72 h of anoxia (CAT: $F = 0.099$; $p = 0.755$; SOD: $K = 0.589$; $p = 0.443$). Thus, metabolic rate depression on prolonged exposure to anoxia seems to involve a loss of antioxidant defense levels in *M. edulis* gills.

Interestingly, 20% (3 out of 15) of the reoxygenated individuals showed conspicuously high SOD activity (70–88 U SOD mg^{-1} protein) compared to the rest of individuals, in which on average gill SOD activity was at 16.51 ± 2.02 U mg^{-1} protein.

3.4. Non-enzymatic antioxidant defense (glutathione)

No significant differences in oxidized (GSSG) or reduced (GSH) glutathione concentrations were recorded among treatments ($F = 0.286$; $p = 835$ and $F = 1.878$; $p = 0.174$, respectively). Further, no significant differences were registered in GSSG/GSH ratio ($K = 1.963$; $p = 0.580$) or total glutathione content (2*GSSG + GSH) ($F = 0.490$; $p = 0.694$) (Fig. 4c) between treatment groups. In general, the gills of the experimental animals had an extremely oxidized redox ratio with the average GSSG concentration being 1.8 fold higher than the GSH content.

3.5. Oxidative damage and caspase activity as marker for apoptotic cell death

Neither caspase activity (Fig. 5a) nor protein carbonyl content (Fig. 5b) showed significant differences among treatments. However, evidence of oxidative damage was seen in the TBARS concentrations in mantle of animals exposed to 72 h of anoxia and reoxygenation (Fig. 5c), which were significantly higher than in all the other treatments.

3.6. Concentration of succinate

After 48 h of anoxia succinate concentrations in gills were significantly higher than in either the normoxic group, the group maintained 72 h in anoxia, and also in the anoxia-reoxygenation group (Fig. 6). Values were on average 2-fold higher than in the 72 h anoxia group and 3-fold higher than in normoxic controls.

4. Discussion

4.1. Respiratory response of M. edulis to declining oxygen tension

Several authors measured the respiratory response of *M. edulis* to declining oxygen tension, and the picture that emerges is that mussels regulate their oxygen uptake down to between 10 and 7 kPa, so equivalent to between 50 and 30% hypoxia. At lower values whole animal respiration becomes oxyconforming (Bayne, 1971; Wang and Widdows, 1993). The point at which the switch to oxyconformity occurs, the lower critical pO_2 (p_{c2} in our paper), depends on the animals' metabolic activity, and thus on size, stress levels, and temperature. As a corollary, animals with higher metabolic activity switch to oxyconformity at higher pO_2 (Bayne, 1971). In the present paper, we exposed the animals to anoxic stress and measured the respiratory response in isolated gill, the tissue most directly exposed to the oxygen concentration in the surrounding seawater, but also the one that is most directly involved in the compensatory efforts towards anoxic stress. We observed that in *M. edulis* the regulating behavior is based on elevated gill respiration, presumably caused by enhanced ciliary beat movements of the gill epithelia and contraction of gill lamella, to enhance perfusion of the interlamellar blood vessels as described by Aiello and Guideri (1965). Both activities enhance oxygen absorbance into the hemolymph, while they consume ATP and also increase local oxygen consumption. In unstressed animals maintained uninterruptedly in normoxia, induction of enhanced ciliary beating began at an oxygen tension of 45% of normoxic saturation (9.5 kPa). Anoxic exposure of the animals shifted p_{c1} to higher oxygen tensions, indicating that anoxic incubation increases sensitivity and produces an oxygen debt that influences the response during renewed exposure without an adequate regeneration period in between both hypoxic phases. Enhanced respiration of excised gills was previously recorded in hypoxia sensitive fresh water bivalves and became more manifest after several days (5–10) of hypoxic exposure of the clams (Sheldon and Walker, 1989).

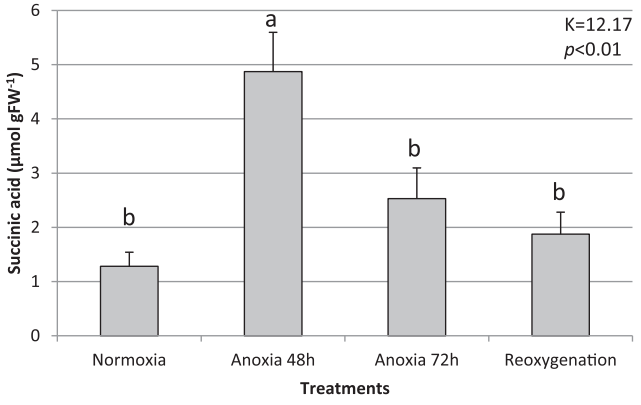

Fig. 6. Concentration of succinic acid in *M. edulis* gills for each of the treatments considered (means ± SE). Values associated with the same letter (a–b) correspond to the same subset based on a a-posteriori multiple comparison test with $p < 0.001$.

Contrary, a very hypoxia-tolerant fresh water species showed no compensatory activity of its gill tissue in the same study. Thus, *M. edulis*, although basically tolerant of hypoxic exposure, seems to be an applicable animal model to study the stress effects related with enforced anoxia and reoxygenation. The animals start to counteract the shortage of oxygen at mild hypoxia by increasing energetic expenditures for oxygen uptake and, in so doing, accumulate an oxygen debt, that leads to onset of metabolic rate depression and the switch to anaerobiosis at only slightly lower oxygen tension (p_{c2}: 6.5 kPa). Similar to p_{c1}, the onset of regulatory compensation, also the low critical p_{c2}, transgression to oxyconformity, is shifted towards higher pO_2 values by prolonged exposure of the animals to anoxia, and this "anoxic imprinting" is conserved in the isolated gill tissue.

It is also interesting to note that the compensation phase, with increased respiratory activity in gill tissue, is never noticeable when experimenting with whole animals in hypoxia (see Bayne, 1971; Wang and Widdows, 1993). The explanation is that the compensatory effect is presumably limited to the gills, the organ of active oxygen uptake which, however, represents only a marginal proportion of body wet mass.

4.2. Does extended exposure to anoxia involve ROS formation and oxidative stress in M. edulis gills?

Given that aerobic metabolic rates were increased in gills extracted from animals that had previously been exposed to anoxia between p_{c1} and p_{c2} and, thus, in the pO_2 range where the animals begin to respond to the hypoxic exposure by compensatory activity, it seems possible that oxidative stress in these gills may also be increased. However, the gills were extracted from animals kept in near anoxia, and neither of the ROS sensitive fluorophore signals was increased following either 48 h or 72 h of exposure to low oxygen tension. Instead, gills of animals subjected to 48 h anoxia showed a significant decrease in ROS production (by 51.5% for 2-OH-E^+:DHE ($O_2^{\cdot-}$ production) and by 74% for DCF compared to normoxic values). No induction, but also no decline of antioxidant activity (SOD and CAT) was observed during anoxia, and no accumulation of oxidative damage in either the lipid or the protein compartment of mantle and gills occurred. Likewise, Letendre et al. (2009) found a decrease in SOD activity in digestive glands, and no change of SOD activity in gills during shell closure of air exposed intertidal *M. edulis*. It thus appears that gills maintain the level of protection to defend from ROS that are formed either due to compensatory activity during transgression to anoxia or upon reoxygenation.

No major changes in ROS parameters occurred between 48 h and 72 h of exposure to anoxia, except for a mild, but nevertheless significant, increase in DCF fluorescence. Although speculative, we suggest that this increase may be due to formation of small quantities of peroxynitrite (ONOO•) and H_2O_2, both of which react with C-H_2DFFDA. Peroxynitrite and its precursor NO are known to reduce mitochondrial electron transport by interacting with cytochrome-c oxidase and, in so doing, reduce its affinity to oxygen and induce a depression of metabolic rate in several cell types (see review by Radi et al., 2002). Indeed, Pamatmat (1980) showed that after 48 h exposure to anoxia, *M. edulis* metabolic rate is reduced to 5% of normoxic levels. This is also corroborated by the vast diminishment of succinate levels in gill tissues of the 72 h exposure group in our study, which indicates a switch to mitochondrial anaerobic pathways by which succinate is converted to propionate and possibly acetate production, and overall metabolic rate is depressed for energy saving (Holwerda and De Zwaan, 1980; Zandee et al., 1986).

It is interesting to speculate about the slight, albeit significant increase of the DCF fluorescence signal between 48 h and 72 h exposure to near anoxia. Indeed several authors have shed doubt on the applicability of DCFH-DA to monitor formation of ROS in living systems. Bonini et al. (2006) showed that in a cell free system this fluorophore is oxidized by horseradish peroxidase (and possibly other heme-containing peroxidases) and H_2O_2 or ONOO•, and that the reaction causes further generation of H_2O_2, so that the final DCF signal does not represent a clear reflection of the physiological ROS production in the system. So: what may cause a subtle increase of DCF fluorescence in extended anoxia? Stabilization of NO in the near absence of oxygen, preventing its oxidation to nitrite while favoring its conversion to peroxynitrite? It is indeed likely that, in spite of the absence of hemoglobin in *Mytilus* blood, peroxidase activity is present in cells and hemolymphatic fluid that would catalyze the reaction between ONOO• and C-H_2DFFDA. What convinces us of the fact that the signals we measured are based on physiological ROS production is a) the parallelism of signal patterns between both ROS sensitive fluorophores and b) the reduction of signal in near anoxia, possibly corresponding to fluorophore background signal, and the expected increase upon reoxygenation. Further, we saw compartmentalization of the signals from both fluorophores in the tissue with confocal microscopy. While the highest 2-OH-E^+:DHE signals were recorded in the periphery of the gill filaments, and so presumably associated with the areas of highest mitochondrial density, DCF fluorescence concentrated in the inner areas and within the blood sinuses (Supplementary material Figs. S1 and S2) where it is probably associated with immunocyte activity and defense against invading pathogens Rivera-Ingraham et al., in prep.

4.3. Reoxygenation after extended anoxia involves an oxidative burst but no oxidative damage in M. edulis gills

The biggest physiological challenge for intertidal animals is the exposure to reoxygenation during re-immersion of the tidal flat. Bivalves that theoretically become anoxic during shell closure open their shells and are flooded with oxygenated seawater. In our present study, *M. edulis* maintain constant enzymatic antioxidant protection in gills through near anoxia and reoxygenation although we recorded an oxidative burst response with DCF signals reaching 174% and 2-OH-E^+:DHE signals 67% over normoxic values. The reoxygenation process eventually caused an increase in TBARS concentration in mantle tissues, indicating oxidative damage from lipid peroxidation. We can assume that this would be produced by a reoxygenation-associated ROS burst happening also in the mantle and would indicate that the mussels are unable to control the ROS induced oxidative damage in this tissue. It seems characteristic of marine mollusks that they do not induce the antioxidant defenses during hypoxic or anoxic exposure, and instead surrender to suffer some lipid damage, while the protein fraction seems better protected. Likewise, the periwinkle, *Littorina littorea* did not show an increase of enzymatic antioxidants, but in contrast increased the concentrations of other antioxidant compounds such as glutathione (Pannunzio and Storey, 1998). The extremely hypoxia-tolerant quahog, *Arctica islandica*, also failed to increase the antioxidant defense during hypoxic exposure (2 kPa) and, in addition, had lower glutathione levels in all tissues in anoxia compared to normoxic samples. However, also no ROS burst was recorded in *A. islandica* tissues upon reoxygenation (Strahl et al., 2011). Only in the hemoglobin containing hypoxia-tolerant clam *Astarte borealis*, CAT, SOD and GPX activities were induced during hypoxic exposure, as H_2O_2 levels increased in the hemolymph that carries an extracellular giant complex hemoglobin molecule (Abele-Oeschger and Oeschger, 1995). Is an increase in antioxidant defenses in anoxia then characteristic of hemoglobin containing species that carry high amounts of more or less safeguarded iron in their tissues? A recent review on this topic indeed supports direct evidence of hypoxic/anoxic ROS formation only in mammals and fish (Welker et al., 2013 and papers cited in section 2.3 “Hypoxia and ROS”) and thus in hemoglobin containing species.

Interestingly, caspase activities were not enhanced by reoxygenation, indicating that the minor effect on lipid peroxidation does not trigger apoptotic cell death. Gills of animals that were reoxygenated for 24 h after enduring a 72 h period of anoxia still showed high oxygen consumption rates, which were on average 1.4 fold (43%) higher than those respiration rates of normoxic control animals above p_{c1}. This indicates that 24 h of reoxygenation is still not long enough for *M. edulis* to recover from 72 h of anoxia. In spite of the elevated respiration rates during recovery and the ROS burst occurring during reoxygenation, the animals did not suffer major oxidative damage in our experiment. First of all, this shows that elevated respiration, even during recovery, does not automatically cause oxidative stress. Second of all, our experiments were carried out with mussels obtained from the Sylt intertidal, which can be considered as stress-hardened individuals that had their antioxidant capacities and the lipid composition of the membranes already adjusted to meet the requirements of frequent anoxia-reoxygenation exposure, apparently covering the stress levels posed by our experiment.

5. Conclusions

We conclude that intertidal hypoxia tolerant *M. edulis* counteract anoxic exposure by an oxyregulating behavior that involves enhanced ciliary beat movements. The animals counteract the shortage of oxygen at mild hypoxia by increasing energetic expenditures for oxygen uptake. They accumulate an oxygen debt that leads to a metabolic depression after prolonged exposure, presumably through tissue acidification from anaerobic energy production and NO and peroxinitrite accumulation (recent unpubl. results of the authors, see below) at lower oxygen tension.

Interestingly, in spite of the elevated respiration rates during the phase of mild oxygen deficiency (p_{c2}), we did not record elevated ROS formation in animals that had reached the anoxic state. Instead, we clearly observed elevated ROS formation in gills after 24 h of reoxygenation, as widely suggested in the literature. We conclude that the animals are able to control mitochondrial ROS formation in the gills and avoid major oxidative damage and apoptotic cell death when transgressing into an anoxic state. This is presumably achieved by enhancing oxygen consumption of the gills at $p_{c2,}$ and by keeping mitochondria in a fully energy coupled respiratory state, which usually produces low ROS output (Abele et al., 2007). Under severely hypoxic conditions, below the lower critical pO_2, an aerobic shut down occurs and mitochondrial respiration rates diminish in an oxyconforming manner, apparently also without causing considerable formation of ROS or occurrence of oxidative damage. The reduction in the metabolic demand may be caused by NO accumulation in gill tissues, since this molecule may bind to the oxygen binding site of the cytochrome oxidase at low oxygen concentrations. This is supported by our recent measurements of NO formation using DAF-2 staining and confocal microscopy, in which *M. edulis* individuals exposed 1 h to 30% air saturation (below the lower critical pO_2) show a significant increase in NO content over normoxic values in gills (ms in preparation). Furthermore, enhanced NO formation could also explain the lack of an apoptosis signal, since the antiapoptotic capabilities of this molecule have been demonstrated for many cell types (e.g. Li et al., 1999; Mannick et al., 1997). Altogether, this seems to be a strategy to survive frequent anoxia during shell closure on ebb tide

emersion on tidal flats and thus an adequate behavior of intertidal marine bivalves to avoid frequent anoxia-reoxygenation related oxidative stress.

It is often suggested that the degree of hypoxia-tolerance relates to a species specific capacity to activate antioxidant defenses in preparation for the reoxygenation injury (see review by Welker et al., 2013). While there was no induction visible of either antioxidant activities or the glutathione concentration in our *M. edulis* study, the animals still maintained normoxic levels of all antioxidants throughout the complete near anoxic period of up to 3 days. This may likely be a good strategy for intertidal species which are periodically emerged and it would be interesting to compare their antioxidant levels to blue mussels that remain permanently submersed in shallow sublittoral zones.

Acknowledgments

The authors would like to thank Birgit Hussel from Sylt Marine Station for providing *Mytilus edulis* and Imke Lüdeke for excellent assistance with the biochemical analyses. Additional thanks go to three anonymous referees for their valuable comments on the original manuscript. This study was partially funded by the Humboldt Foundation (Germany) and the Fundación Ramón Areces (Spain) through grants awarded to I. Rocchetta and G. A. Rivera-Ingraham, respectively.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2013.09.007.

References

Abele-Oeschger, D., Oeschger, R., 1995. Hypoxia induced autoxidation of haemoglobin in the benthic invertebrates Arenicola marina (Polychaeta) and Astarte borealis (Bivalvia): possible effect of hydrogen sulphide. J. Exp. Mar. Biol. Ecol. 187, 63–80.
Abele, D., Brey, T., Philipp, E.E.R., 2009. Bivalve models of aging and the determination of molluscan lifespans. Exp. Gerontol. 44, 307–315.
Abele, D., Heise, K., Pörtner, H.O., Puntarulo, S., 2002. Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam Mya arenaria. J. Exp. Biol. 205, 1831–1841.
Abele, D., Philipp, E., Gonzalez, P., Puntarulo, S., 2007. Marine invertebrate mitochondria and oxidative stress. Front. Biochem. 12, 933–946.
Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Aiello, E., Guideri, G., 1965. Distribution and function of the branchial nerve in the mussel. Biol. Bull. 129, 431–438.
Almeida, E.A.d., Bainy, A.C.D., 2006. Effects of aerial exposure on antioxidant defenses in the brown mussel Perna perna. Braz. Arch. Biol. Technol. 49, 225–229.
Almeida, E.A.d., Silva, D.G.H., Dias Bainy, A.C., Porto Freitas, F., Motta, F.D., Gomez, O.F., Gennari de Medeiros, M.H., Di Mascio, P., 2012. Evaluation of glutathione status in aquatic organisms. In: Abele, D., Vázquez-Media, J.P., Zenteno-Savín, T. (Eds.), Oxidative Stress in Aquatic Environments. Wiley-Blackwell, Oxford, pp. 381–388.
Bayne, B.L., 1971. Ventilation, the heart beat and oxygen uptake by *Mytilus edulis* L. in declining oxygen tension. Comp. Biochem. Physiol. Part A Physiol. 40, 1065–1085.
Bayne, B.L., Thompson, R.J., Widdows, J., 1976. Physiology 1. In: Bayne, B.L. (Ed.), Marine Mussels. Their Ecology and Physiology. Cambridge University Press, Cambridge, pp. 121–206.
Bonini, M.G., Rota, C., Tomasi, A., Mason, R.P., 2006. The oxidation of 2′,7′-dichlorofluorescin to reactive oxygen species: a self-fulfilling prophesy? Free Radic. Biol. Med. 40, 968–975.
Bradford, M.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.
Carballal, M.a.J., López, C., Azevedo, C., Villalba, A., 1997. Enzymes involved in defense functions of hemocytes of Mussel Mytilus galloprovincialis. J. Invertebr. Pathol. 70, 96–105.
Dikkeboom, R., Tijnagel, J.M.G.H., Mulder, E.C., van der Knaap, W.P.W., 1987. Hemocytes of the pond snail Lymnaea stagnalis generate reactive forms of oxygen. J. Invertebr. Pathol. 49, 321–331.
Duggleby, R.G., 1984. Regression analysis of nonlinear Arrhenius plots: an empirical model and a computer program. Comput. Biol. Med. 14, 447–455.
Gosling, E., 2003. Bivalve Molluscs. BIology, Ecology and Culture. Blackwell, Oxford.
Herreid, C.F.I., 1980. Hypoxia in invertebrates. Comp. Biochem. Physiol. Part A Physiol. 67, 311–320.
Holwerda, D.A., De Zwaan, A., 1980. On the role of fumarate reductase in anaerobic carbohydrate catabolism of *Mytilus edulis* L. Comp. Biochem. Physiol. Part B Comp. Biochem. 67, 447–453.
Husmann, G., Philipp, E.E.R., Rosenstiel, P., Vázquez, S., Abele, D., 2011. Immune response of the Antarctic bivalve Laternula elliptica to physical stress and microbial exposure. J. Exp. Biol. Ecol. 398, 83–90.
Irato, P., Piccinni, E., Cassini, A., Santovito, G., 2007. Antioxidant responses to variations in dissolved oxygen of Scapharca inaequivalvis and Tapes philippinarum, two bivalve species from the lagoon of Venice. Mar. Pollut. Bull. 54, 1020–1030.
Letendre, J., Chouquet, B., Manduzio, H., Marin, M., Bultelle, F., Leboulenger, F., Durand, F., 2009. Tidal height influences the levels of enzymatic antioxidant defences in *Mytilus edulis*. Mar. Environ. Res. 67, 69–74.
Li, C., Jackson, R.M., 2002. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. Am. J. Physiol. Cell Physiol. 282, C227–C241.
Li, J., Bombeck, C.A., Yang, S., Kim, Y.-M., Billiar, T.R., 1999. Nitric oxide supresses apoptosis via interrupting caspase activation and mitochondrial dysfunction in cultured hepatocytes. J. Biol. Chem. 274, 17325–17333.
Livingstone, D.R., Lips, F., Garcia Martinez, P., Pipe, R.K., 1992. Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. Mar. Biol. 112, 265–276.
Mannick, J.B., Miao, X.Q., Stamler, J.S., 1997. Nitric oxide inhibits Fas-induced apoptosis. J. Biol. Chem. 272, 24125–24128.
Massabuau, J., Abele, D., 2012. Principles of oxygen uptake and tissue oxygenation in water-breathing animals. In: Abele, D., Vázques-Medina, J.P., Zenteno-Savín, T. (Eds.), Oxidative Stress in Aquatic Ecosystems. Wiley-Blackwell, Chichester, West Sussex, pp. 141–156.
Pamatmat, M.M., 1980. Facultative anaerobiosis of benthos. In: Tenore, K.R., Coull, B.C. (Eds.), Marine Benthic Dynamics. University of South Carolina Press, Columbia, pp. 65–90.
Pamplona, R., Costantini, D., 2011. Molecular and structural antioxidant defenses against oxidative stress in animals. Am. J. Physiol. 301, 843–863.
Pannunzio, T.M., Storey, K.B., 1998. Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod Littorina littorea. J. Exp. Mar. Biol. Ecol. 221, 277–292.
Radi, R., Cassina, A., Hodara, R., Quijano, C., Castro, L., 2002. Peroxynitrite reactions and formation in mitochondria. Free Radic. Biol. Med. 33, 1451–1464.
Rivera-Ingraham, G.A., Bickmeyer, U., Abele, D., 2013. The physiological response of the marine platyhelminth Macrostomum lignano to different environmental oxygen concentrations. J. Exp. Biol. 216, 2741–2751.
Sheldon, F., Walker, K.F., 1989. Effects of hypoxia on oxygen consumption by two species of freshwater mussel (Unionacea: Hyriidae) from the Rivera Murray. Aust. J. Mar. Freshw. Res. 40, 491–499.
Sprung, M., 1983. Reproduction and fecundity of the mussel *Mytilus edulis* at Helgoland (North Sea). Helgol. Meeresunters. 36, 243–255.
Strahl, J., Abele, D., 2010. Cell turnover in tissues of the long-lived ocean quahog Arctica islandica and the short-lived scallop Aequipecten opercularis. Mar. Biol. (Berl.) 157, 1283–1292.
Strahl, J., Brey, T., Philipp, E.E.R., Thorarinsdottir, G., Fischer, N., Wessels, W., Abele, D., 2011. Physiological responses to self-induced burrowing and metabolic rate depression in the ocean quahog Arctica islandica. J. Exp. Biol. 214, 4221–4231.
Tarpey, M.M., Wink, D.A., Grisham, M.B., 2004. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. Am. J. Physiol. 286, 431–444.
Uchiyama, M., Mihara, M., 1978. Determination of malondialdehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem. 86, 271–278.
Van Winkle, W.J., 1968. The effects of season, temperature and salinity on the oxygen consumption of bivalve gill tissue. Comp. Biochem. Physiol. 26, 69–80.
Viarengo, A., Pertica, M., Canesi, L., Accomando, R., Mancinelli, G., Orunesu, M., 1989. Lipid peroxidation and level of antioxidant compounds (GSH, vitamin E) in the digestive glands of mussels of three different age groups exposed to anaerobic and aerobic conditions. Mar. Environ. Res. 28, 291–295.
Wang, W.X., Widdows, J., 1993. Metabolic response of the common mussel *Mytilus edulis* to hypoxia and anoxia. Mar. Ecol. Prog. Ser. 95, 205–214.
Wang, X.L., Widdows, J., Page, D.S., 1992. Effects of organic toxicants on the anoxia energy metabolism of the mussel *Mytilus edulis*. Mar. Environ. Res. 34, 327–331.
Welker, A.F., Moreira, D.C., Campos, E.G., Hermes-Lima, M., 2013. Role of redox metabolism for adaptation of aquatic animals to drastic changes in oxygen availability. Comp. Biochemistry and Physiol. Part A Physiol..
Zandee, D.I., Holwerda, D.A., Kluytmans, J.H., De Zwaan, A., 1986. Metabolic adaptations to the environmental anoxia in the intertidal bivalve mollusc *Mytilus edulis* L. Neth. J. Zool. 36, 322–343.
Zurburg, W., Kluytmans, J.H., 1980. Organ specific changes in energy metabolism due to anaerobiosis in the sea mussel *Mytilus edulis* (L.). Comp. Biochem. Physiol. Part B Comp. Biochem. 67, 317–322.