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RESEARCH ARTICLE

Effects of variable oxygen regimes on mitochondrial bioenergetics and reactive oxygen species production in a marine bivalve, *Mya arenaria*

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ABSTRACT

Estuarine and coastal benthic organisms often experience fluctuations in oxygen levels that can negatively impact their mitochondrial function and aerobic metabolism. To study these impacts, we exposed a common sediment-dwelling bivalve, the soft-shell clam *Mya arenaria*, for 21 days to chronic hypoxia (P_{O_2} ~4.1 kPa), cyclic hypoxia (P_{O_2} ~12.7–1.9 kPa, mean 5.7 kPa) or normoxia (P_{O_2} ~21.1 kPa). pH was manipulated to mimic the covariation in CO_2/pH and oxygen levels in coastal hypoxic zones. Mitochondrial respiration, including proton leak, the capacity for oxidative phosphorylation (OXPHOS), the maximum activity of the electron transport system (ETS), reactive oxygen species (ROS) production, and activity and oxygen affinity of cytochrome *c* oxidase (CCO) were assessed. Acclimation to constant hypoxia did not affect the studied mitochondrial traits except for a modest decrease in the OXPHOS coupling efficiency. Cyclic hypoxia had no effect on OXPHOS or ETS capacity, but increased proton leak and lowered mitochondrial OXPHOS coupling efficiency. Furthermore, mitochondria of clams acclimated to cyclic hypoxia had higher rates of ROS generation compared with the clams acclimated to normoxia or chronic hypoxia. CCO activity was upregulated under cyclic hypoxia, but oxygen affinity of CCO did not change. These findings indicate that long-term cyclic hypoxia has a stronger impact on the mitochondria of *M. arenaria* than chronic hypoxia and might lead to impaired ATP synthesis, higher costs of mitochondrial maintenance and oxidative stress. These changes might negatively affect populations of *M. arenaria* in the coastal Baltic Sea under increasing hypoxia pressure.

KEY WORDS: Cyclic hypoxia, Chronic hypoxia, Oxidative phosphorylation, Mitochondrial proton leak, Oxidative stress, Electron leak, Bivalve

INTRODUCTION

Hypoxic zones [defined as areas with dissolved oxygen (DO) concentrations <2 mg O_2 l^{-1}] are increasing in coastal zones worldwide (Diaz and Rosenberg, 2008; Vaquer-Sunyer and Duarte, 2008; Diaz and Breitburg, 2009). Coastal hypoxia has major impacts on benthic communities, leading to the loss of biodiversity, alterations in ecosystem functioning and changes in the nutrient cycles (Diaz and Rosenberg, 1995; Gammal et al., 2017; Vaquer-Sunyer and Duarte, 2008). The major driver of coastal hypoxia is anthropogenic nutrient input leading to high oxygen consumption by the resident biota that exceeds the oxygen influx through photosynthesis, mixing and diffusion (Wallace et al., 2014). Land-locked basins with limited water exchange and large freshwater catchments such as the Baltic Sea are especially prone to hypoxia (Conley et al., 2011; Carstensen and Conley, 2019). Depending on the local hydrodynamic conditions, temperature and nutrient input, coastal hypoxia can last from a few hours (e.g. during diurnal cycles) to weeks or months (Vaquer-Sunyer and Duarte, 2008).

Oxygen deficiency is a major stressor for benthic invertebrates such as the sediment-dwelling marine bivalves that serve as ecosystem engineers in their habitats (Meysman et al., 2006; Hedman et al., 2011; Kristensen et al., 2012). Long-term hypoxia can lead to high mortality in the benthic macrofauna, whereas short-term hypoxic events often result in fitness costs because of lower growth and reproduction rates and altered behavior (Rosenberg et al., 1991; Diaz and Rosenberg, 1995, 2008; Vaquer-Sunyer and Duarte, 2008). Sessile benthic bivalves are unable to escape unfavorable hypoxic conditions and rely on physiological adjustments to survive oxygen deficiency (Vaquer-Sunyer and Duarte, 2008; Carstensen and Conley, 2019). Because oxygen availability has a direct effect on aerobic ATP production, metabolic responses play a key role in hypoxia-tolerant phenotypes of benthic invertebrates, including energy-saving mechanisms such as metabolic rate depression (Storey, 1988; Hochachka et al., 1996), the shift to anaerobic pathways of ATP production (Grieshaber et al., 1994) and mitochondrial adjustments ensuring rapid recovery during reoxygenation (Sokolova et al., 2019). Anaerobic metabolic patterns have been extensively studied in marine invertebrates (Saz, 1971; Hochachka and Mustafa, 1972; Grieshaber et al., 1994; de Zwaan et al., 2002; Storey, 2002), but mitochondrial responses to oxygen deficiency in organisms from hypoxia-prone benthic habitats remain poorly understood.

Mitochondria of animals including marine bivalves are sensitive to oxygen fluctuations (Sokolova, 2018). Oxygen deficiency slows down the activity of the mitochondrial electron transport system (ETS), thereby suppressing ATP synthesis by oxidative phosphorylation (OXPHOS). Re-influx of oxygen restores ATP synthesis but can elevate production of potentially toxic reactive oxygen species (ROS) (Levraut et al., 2003; Honda et al., 2005). In mammalian mitochondria, this surge in ROS production is a key mechanism of the tissue injury during hypoxia–reoxygenation, leading to impaired OXPHOS capacity, mitochondrial damage and cell death (Schumacker et al., 1993; Venditti et al., 2001; Paradies et al., 2004; Kalogeris et al., 2012; Lesnefsky et al., 2017). The mechanisms of the hypoxia–reoxygenation-induced burst of ROS are debated (Andrienko et al., 2017; Mailloux, 2020), but recent hypotheses highlight an important role of reverse electron transport through the mitochondrial Complex I,

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driven by the accumulation of succinate in hypoxic tissues (Venditti et al., 2001; Paradies et al., 2004; Murphy, 2009; Chouchani et al., 2014). This raises an intriguing question about the potential involvement of this mechanism in hypoxia-tolerant species such as benthic bivalves that accumulate high succinate levels during hypoxia (Ellington, 1983; Sokolova et al., 2000; Kurochkin et al., 2009; Ivanina et al., 2010; Haider et al., 2020). However, at present, little is known about ROS generation in the mitochondria of marine bivalves exposed to hypoxia–reoxygenation stress.

Mitochondrial adjustments to sustain ATP production during hypoxia might involve augmented ETS capacity to maintain electron flux despite low oxygen concentrations. The ETS responses to hypoxia–reoxygenation stress appear different in hypoxia-sensitive species (such as terrestrial mammals) and hypoxia-tolerant aquatic vertebrates and invertebrates (Sokolova et al., 2019). In mammalian mitochondria, hypoxia suppresses ETS activity (and, as a consequence, OXPHOS capacity), and subsequent reoxygenation enhances this suppression as a result of ROS-induced mitochondrial damage (Piper et al., 2003; Chouchani et al., 2014; Kalogeris et al., 2014). A similar pattern is found in hypoxia-sensitive marine bivalves such as the highly aerobic pectinid *Argopecten irradians* (Ivanina et al., 2016). In contrast, in hypoxia-tolerant aquatic species, ETS activity remains stable or even increases during hypoxia and reoxygenation as shown in marine bivalves (Kurochkin et al., 2009; Sussarellu et al., 2013; Ivanina et al., 2016; Sokolov et al., 2019) and fish (Cook et al., 2013; Du et al., 2016; Lau et al., 2017; Gerber et al., 2019; Napolitano et al., 2019). In mammalian models, hypoxic exposure modifies the subunit composition of the terminal ETS enzyme, cytochrome *c* oxidase (CCO), via the replacement of a ubiquitous COX4-1 subunit with a hypoxia-induced COX4-2 subunit (Kocha et al., 2015). This modification results in an enhanced oxygen affinity of CCO, augments the electron flux and mitigates ROS production (Fukuda et al., 2007; Kocha et al., 2015; Pajuelo Reguera et al., 2020). Interestingly, in non-mammalian vertebrates (including many hypoxia-tolerant species such as goldfish or freshwater turtles), the baseline expression of the COX4-2 subunit is higher than in mammals but appears to be unresponsive to hypoxia (Kocha et al., 2015). In marine bivalves, the effects of hypoxia–reoxygenation stress on CCO properties have not been well studied (Sokolova et al., 2019) and require further investigation.

To date, most studies on the mitochondrial responses to hypoxia–reoxygenation stress in marine organisms have focused on the effects of a single hypoxia event (lasting from several hours to several days) followed by reoxygenation (Sokolova, 2018; Sokolova et al., 2019). However, under the environmentally relevant conditions of the hypoxia-prone coastal zone, fluctuating oxygen conditions can persist on time scales from several days to several months (Conley et al., 2009; Breitburg et al., 2018). Furthermore, oxygen deficiency is commonly associated with acidification (low pH) due to the accumulation of CO_2 produced by respiration (Wallace et al., 2014; Breitburg et al., 2019). Therefore, to assess the mitochondrial adjustments to the environmentally realistic scenarios of coastal hypoxia, prolonged exposures to environmentally relevant oxygen and pH combinations are required. Recent theoretical and empirical studies indicate that environmental variability might be more important for the organismal and population responses to environmental stress than changes in the mean values of the abiotic parameters (García-Carreras and Reuman, 2013; Lawson et al., 2015). With regard to oxygen variability, few studies have addressed the relative impacts of constant hypoxia versus fluctuating oxygen levels on aerobic metabolism. In a hypoxia-tolerant killifish (*Fundulus heteroclitus*), long-term (4–5 weeks) acclimation to hypoxia had a strong impact on the oxygen affinity of CCO and mitochondrial ROS production but did not affect OXPHOS capacity; these changes were similar during acclimation to chronic constant or diel cycling hypoxia (Du et al., 2016). In contrast, in the rainbow trout (*Oncorhynchus mykiss*), diel cycling hypoxia (~6 days) had a more pronounced effect on aerobic metabolism than did constant hypoxia (Williams et al., 2019). To the best of our knowledge, the impacts of long-term acclimation to different oxygen regimes on mitochondrial bioenergetics and redox physiology have not yet been investigated in marine bivalves.

The soft-shell clam, *Mya arenaria* Linnaeus 1758, is a dominant bivalve species in the shallow soft-bottom habitats of the Baltic Sea (Gogina et al., 2016) and an ecosystem engineer carrying out important ecological functions such as bioirrigation and bioturbation (Renz et al., 2018). Soft-shell clams are commonly exposed to oxygen fluctuations in their habitats, and the current rise in the frequency and extent of coastal hypoxia in the Baltic Sea puts this species under increasing risk as a result of oxygen deficiency (Conley et al., 2011; Carstensen et al., 2014). *Mya arenaria* is relatively tolerant to hypoxia (Rosenberg et al., 1991; Strasser, 1998), therefore playing an important ecological role in hypoxia-prone benthic habitats (Gogina et al., 2014). Nevertheless, a prolonged decrease in oxygen saturation below the critical oxygen threshold (5.3–6.7 kPa) (Davis, 1975) can negatively affect the metabolism of this species, causing energy deficiency. These traits make *M. arenaria* a useful model organism to study mitochondrial adjustments to different oxygen regimes.

The aim of our study was to assess the effect of long-term acclimation to different oxygen regimes on mitochondrial bioenergetics and ROS generation in *M. arenaria*. We hypothesized that acclimation to low oxygen conditions would suppress mitochondrial OXPHOS and ETS capacity and enhance ROS production in *M. arenaria*. Furthermore, we expected that the oxygen affinity of CCO (measured as P_{50}, the partial pressure of oxygen resulting in a 50% decrease of CCO activity) would increase in the mitochondria of clams exposed to chronic and fluctuating hypoxia to compensate for the lower substrate (O_2) availability. We also hypothesized that fluctuating oxygen conditions (that are known to strongly stimulate ROS generation and cause oxidative stress in other animals) would be more damaging to the mitochondria of *M. arenaria* than constant hypoxia. To test these hypotheses, we exposed *M. arenaria* to normoxia, constant hypoxia or cyclic hypoxia for 3 weeks and assessed the rate of mitochondrial proton leak, ATP synthesis (OXPHOS) capacity, ETS activity, and oxygen affinity of CCO in the mitochondria of clams from different oxygen regimes. The rate of ROS generation was measured in actively phosphorylating and resting mitochondria to determine the effects of hypoxic acclimation on mitochondrial electron leak. Our study shows that the impact of oxygen deficiency on the mitochondrial function of *M. arenaria* depends on the hypoxia regime and indicates the potential involvement of oxidative stress and the high energy costs of mitochondrial maintenance in metabolic disturbance of *M. arenaria* in hypoxia-prone coastal habitats.

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), VWR (Darmstadt, Germany) or Thermo Fisher Scientific (Schwerte, Germany), and were of analytical grade or higher.

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Animal collection and maintenance

Adult soft-shell clams, *M. arenaria*, were collected between February and September 2019 at the Schnatterman site near Rostock, Germany (54°10′21.8″N, 12°08′33.4″E). The upper 20–30 cm of the sediment was excavated and gently washed in a sieve to retrieve the clams. Only intact individuals of similar size (shell length: 4.79±0.59 cm) were used for further studies. Clams were brought in seawater to the University of Rostock within an hour of collection and kept in recirculating temperature-controlled aquaria containing sandy sediment from the Schnatterman at a temperature of 15±1°C and seawater salinity of 15±1. The temperature and salinity conditions were similar to the habitat conditions at the time of collection. Clams were fed every other day *ad libitum* with SA/DTs Premium Blend live phytoplankton (Coralsands, Mainz Kastel, Germany; 4 ml per 60–70 g of clam biomass).

The clams were randomly divided into three groups and exposed for 3 weeks (21 days) to one of the three oxygen regimes: (1) normoxia and normocapnia (control); (2) chronic (constant) hypoxia and hypercapnia; and (3) intermittent (cyclic) hypoxia and hypercapnia. For brevity, the three exposure regimes will be referred to as normoxia, constant hypoxia and cyclic hypoxia. The oxygen regimes in the constant and cyclic hypoxia exposures were selected so that the mean DO levels in the two exposures were comparable and close to the hypoxic threshold for marine macrozoobenthos (~2–2.5 mg l^{-1}, ~5–7 kPa; Vaquer-Sunyer and Duarte, 2008). P_{CO_2} and pH were adjusted in the experimental exposures to approximate the environmentally relevant covariation of pH and DO based on correlations reported for Kiel Bay of the Baltic Sea (Melzner et al., 2013). The acclimation period of 3 weeks is considered sufficient to achieve a new physiological steady state after an environmental change in temperate marine bivalves (Khlebovich, 1981, 2017).

Control (normoxic) clams were maintained in seawater aerated with ambient air (P_{O_2} ~21 kPa; pH_{NBS} 7.7) (Table 1, Fig. 1). For constant and cyclic hypoxia treatments, defined mixtures of nitrogen, oxygen and carbon dioxide were bubbled into the experimental tanks. The gases were mixed with a Qcal Gas Mixing Machine Unit (GMS_3_CH), controlled by GMS_3_Channel Version 5.0 software (QCAL Messtechnichnik GmbH, Oberostendorf, Germany). For constant hypoxia exposures, the gas mixture was bubbled through the tanks to maintain a constant low oxygen level (~20% air saturation, P_{O_2} ~4.1 kPa) and a pH_{NBS} of 7.1. In cyclic hypoxia exposures, the composition of the gas mixture was varied throughout the 24 h period as follows: 4.5 h of oxygenation (~50% air saturation, P_{O_2} ~10.4 kPa, pH_{NBS} ~7.4), followed by a gradual decrease in O_2 and pH for 5.5 h to reach a hypoxic phase (~10% air saturation, P_{O_2} ~2.1 kPa, pH_{NBS} ~6.9), 8.5 h at the hypoxic phase, followed by a gradual increase in O_2 and pH for 5.5 h to return to the oxygenation conditions (~50% air saturation, P_{O_2} ~10.4 kPa, pH_{NBS} ~7.4). These cycles were repeated daily for 3 weeks. The lower O_2 saturation (50% air saturation) for the oxygenation phase of the cyclic hypoxia was chosen to achieve comparable mean DO values in cyclic and constant hypoxia, while preventing an excessively long phase of extreme hypoxia that could lead to mass mortality. Our pilot studies indicated that *M. arenaria* remains fully aerobic at 50% air saturation used in the recovery phase in cyclic hypoxia (N.O. and I.M.S., unpublished observations).

All experimental exposures were conducted at 15±1°C and a salinity of 15±1 (Table 1). The oxygen concentration, pH (NBS scale) and temperature were monitored continuously with an oxygen and temperature sensor (LDO, Hach, Loveland, CO, USA) and a pH glass electrode (PHC 101, Hach) connected to a portable multimeter

Table 1. Maintenance conditions in the experimental exposures of *Mya arenaria*

Exposure conditions	Temperature (°C)	DO (mg l^{-1})	P_{O_2} (kPa)	pH_{NBS}	pH_T	Salinity	DIC (µmol kg^{-1})	AT (µmol kg^{-1})	P_{CO_2} (µatm)	*N*	Mortality (% day^{-1})
Normoxia 1	14.9±0.6	9.28±0.19	21.18±0.31	7.69±0.09	7.48±0.08	14.94±0.14	608.49±42.73	810.14±32.31	386.6±64.36	86	0.25
Normoxia 2	14.8±0.2	9.19±0.18	21.16±0.38	7.6±0.07	7.44±0.06	15.26±0.17	567.34±4.73	755.2±18.69	400.1±50.39	61	0
Constant hypoxia 1	16±0.35	1.78±0.26	4.18±0.61	7.09±0.06	7.06±0.04	15.11±0.23	2412.62±197.97	2304.69±207.23	4029.73±512.46	61	0.39
Constant hypoxia 2	15.8±0.05	1.76±0.23	4.12±0.54	7.11±0.09	6.94±0.03	15.18±0.04	2869.44±340.71	2680.74±337.05	6511.03±749.8	68	0
Cyclic hypoxia 1	15±0.5	2.54±1.56	5.74±3.5	7.14±0.12	7.16±0.11	15.26±0.12	1913.5±106.29	1886.30±98.81	2413.28±982.21	60	0.21
Cyclic hypoxia 2	15.4±0.3	2.44±1.51	5.73±3.5	7.12±0.12	7.14±0.04	15.23±0.23	1470.09±59.04	1437.03±93.30	2110.17±142.17	44	0.51

Data (mean±s.d.) for two independent experimental runs (run 1: summer 2019; run 2: autumn 2019) are provided. DO, dissolved oxygen concentration; DIC, dissolved inorganic carbon; AT, total alkalinity; *N*, number of clams. pH_T (total scale) was measured during the recovery phase of the cyclic hypoxia, whereas pH_{NBS} was measured constantly throughout the experiment. For pH_{NBS}, temperature and DO, *N*=1487–5922 in normoxia, *N*=8653–13,746 in constant hypoxia and *N*=7802–10,096 in cyclic hypoxia. For pHT, salinity and P_{CO_2}, *N*=4–17 in normoxia, 7–14 in constant hypoxia and 5–7 in cyclic hypoxia. For DIC, *N*=3–15, 7–13 and 6–7 in normoxia, constant hypoxia, respectively. For AT, *N*=4–14, 7–13 and 6–7 in normoxia, constant hypoxia and cyclic hypoxia, respectively.

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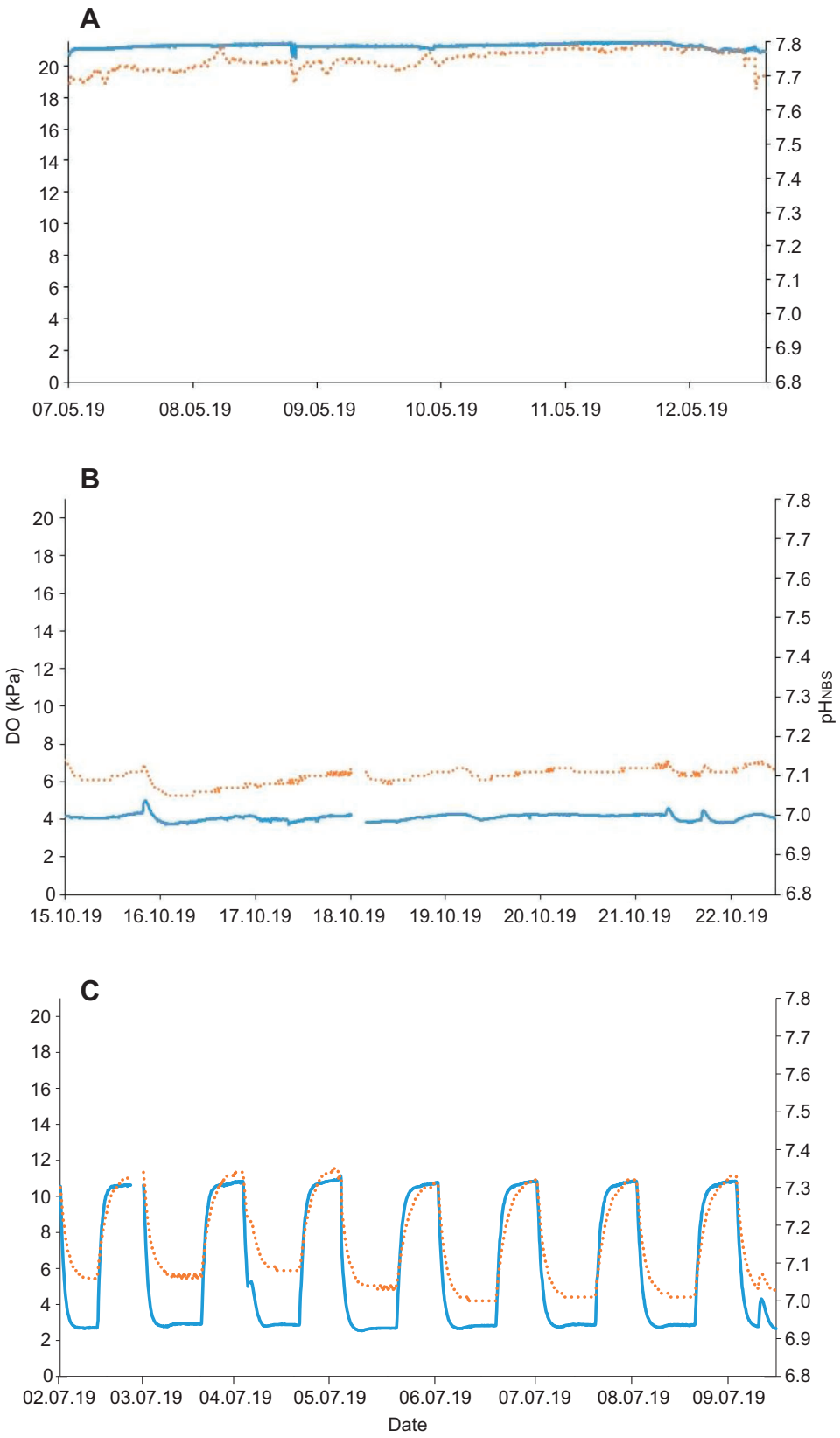

Fig. 1. Representative traces of dissolved oxygen concentration (DO) and pH in experimental exposures of *Mya arenaria*. (A) Normoxia, (B) constant hypoxia and (C) cyclic hypoxia. Solid blue line, DO; dotted orange line, pH_{NBS}. Data are from May (A), October (B) and July (C) 2019.

HQ40d (Hach). The pH sensors (PHC 101) were calibrated with Singlet pH Buffer Solutions (Hach). Furthermore, pH_T (total scale) was measured spectrophotometrically in a subset of samples used to determine the carbonate system (see 'Seawater chemistry', below). Salinity was measured twice a week with a salinity sensor (CDC40103, Hach) and adjusted as necessary to compensate for evaporation. Because of logistical constraints, two independent experimental runs were conducted, in spring and autumn 2019. Pilot

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studies have shown that mitochondrial activity in the Baltic Sea *Mytilus edulis* does not significantly differ between these seasons (I.M.S., unpublished observations). No significant difference in the studied mitochondrial traits was found between the two experimental runs in our present study ($P>0.05$).

Seawater chemistry

Water samples were taken twice a week for analysis of dissolved inorganic carbon (DIC), total alkalinity (AT) and pH_T (Table 1). Samples were poisoned with saturated $HgCl_2$ solution and stored at +4°C in the dark until further analyses. DIC was measured using a VINDTA3D periphery (Marianda, Kiel, Germany) coupled to a coulometer CM5014 (UIC Inc., Jolliet, IL, USA) (Dickson et al., 2007). For spectrophotometric determination of pH, the indicator dye *m*-cresol purple (2 mmol l^{-1}; Contros – System and Solution GmbH, Kiel, Germany) was used. Measurements were executed with an instrumental set-up described elsewhere (Carter et al., 2013; Müller and Rehder, 2018). The measuring principle and calculations are based on Dickson et al. (2007) and Müller and Rehder (2018). Certified reference material was used to verify the results (Reference material for CO_2 measurements, University of California, San Diego, Scripps Institution of Oceanography, Marine Physical Laboratory, USA). P_{CO_2} values were calculated based on the temperature, salinity, pH_T and DIC data using CO2sys v. 2.1 software (Pierrot et al., 2006). The set of constants from Millero (2010), the $KHSO_4$ dissociation constant from Dickson and the total boron concentration [B]T value from Lee et al. (2010) were used as implemented and cited in the CO2sys software (Pierrot et al., 2006). Because of the higher alkalinity in the second run of the constant hypoxia exposure, higher P_{CO_2} levels were needed to reach the target pH values (Table 1).

Mitochondrial isolation

Mitochondria were isolated from the hepatopancreas of *M. arenaria* using differential centrifugation (Sokolova, 2004; Ivanina et al., 2016). The hepatopancreas is a metabolically active tissue involved in nutrient and energy storage and food digestion of marine bivalves (Gosling, 2015). To standardize sampling in the cyclic hypoxia exposures, the clams were collected 30 min after the beginning of the reoxygenation phase (10.4 kPa, pH_{NBS} 7.4). The hepatopancreas tissues of 4–9 individuals (~0.5–1.4 g) were homogenized in 8 ml of ice-cold isolation buffer containing 100 mmol l^{-1} sucrose, 200 mmol l^{-1} KCl, 100 mmol l^{-1} NaCl, 30 mmol l^{-1} Hepes, 8 mmol l^{-1} EGTA and 30 mmol l^{-1} taurine, pH_{NBS} 7.5. Immediately before use, 1 mmol l^{-1} phenylmethanesulfonyl fluoride (PMSF) and 2 µg ml^{-1} aprotinin were added to the isolation buffer. The hepatopancreas was homogenized on ice in a Potter–Elvenhjem homogenizer by several passes at 200 rpm. To remove cell debris, the homogenate was centrifuged at 4°C at 2000 ***g*** for 8 min, and the supernatant was centrifuged at 4°C at 8500 ***g*** for 8 min to collect mitochondria. The pellet was resuspended in ice-cold assay buffer containing 165 mmol l^{-1} sucrose, 50 mmol l^{-1} taurine, 10 mmol l^{-1} NaCl, 130 mmol l^{-1} KCl, 30 mmol l^{-1} Hepes, 10 mmol l^{-1} glucose, 1 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} KH_2PO_4 and 1% bovine serum albumin (BSA).

Mitochondrial assays

The rate of oxygen consumption ($\dot{M}_{O_2}$) and hydrogen peroxide (H_2O_2) production were measured in isolated mitochondria at 15± 0.1°C using a high-resolution respirometer (Oxygraph 2-k, Oroboros, Innsbruck, Austria) with integrated software (DatLab 6, Oroboros Instruments). Oxygen consumption was measured using a Clark-type electrode calibrated with 100% (air-saturated assay buffer) and 0% (saturated solution of sodium dithionite). Production of H_2O_2 was measured using Fluorescence-Sensor Green (525 nm) integrated with Oxygraph 2-k in an assay buffer containing 10 µmol l^{-1} Amplex™ UltraRed Reagent, 1 U ml^{-1} horseradish peroxidase, and 5 U ml^{-1} of superoxide dismutase (Krumschnabel et al., 2015). A two-step calibration was conducted with 0.1 µmol l^{-1} H_2O_2 after the addition of the mitochondrial suspension. A substrate–uncoupler–inhibitor titration (SUIT) was carried out with sequential additions as follows: (1) 5 mmol l^{-1} pyruvate and 2 mmol l^{-1} malate to stimulate Complex I (LEAK I) respiration; (2) 10 mmol l^{-1} succinate to stimulate electron flux through Complex II (LEAK I+II); (3) 2.5 mmol l^{-1} ADP and 5 µmol l^{-1} cytochrome *c* to measure OXPHOS activity; (4) 2.5 µmol l^{-1} of oligomycin to inhibit mitochondrial F_O,F_1-ATPase (LEAK I+II+Oligo), (5) titration (in 0.5 µmol l^{-1} steps) with the uncoupler carbonyl cyanide-chlorophenyl hydrazine (CCCP) to measure maximum ETS activity; (6) 1 µmol l^{-1} rotenone to inhibit electron flux through Complex I and measure succinate-driven ETS respiration (ETS II); (7) 2.5 µmol l^{-1} antimycin A to inhibit Complex III; (8) 0.5 mmol l^{-1} *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) and 2 mmol l^{-1} ascorbate to measure CCO activity; and (9) 20 mmol l^{-1} KCN to inhibit CCO and measure non-mitochondrial oxygen consumption. Different functional parameters of isolated mitochondria (Table 2) were calculated as described elsewhere (Estabrook, 1967; Gnaiger, 2012). The respiration in the LEAK states is indicative of the proton leak rate of resting (non-phosphorylating) mitochondria at high mitochondrial membrane potential (Brand et al., 1994; Doerrier et al., 2018; Gnaiger, 2009; Jastroch et al., 2010). Oxygen consumption rate of ADP-stimulated mitochondria is representative of the maximum

Table 2. Mitochondrial respiration states and indices for assessing mitochondrial respiration and H_2O_2 production in *M. arenaria*

Respiratory state or index	Reflective of	Determined as
LEAK I	NADH-driven baseline respiration (proton leak)	$\dot{M}_{O_2}$ with pyruvate as a substrate and malate to spark Complex I respiration
LEAK I+II	NADH- and $FADH_2$-driven baseline respiration (proton leak)	$\dot{M}_{O_2}$ with pyruvate, malate and succinate as substrates
OXPHOS	OXPHOS capacity	ADP-stimulated $\dot{M}_{O_2}$
ETS	ETS capacity	$\dot{M}_{O_2}$ after addition of an uncoupler
CCO	Activity of cytochrome *c* oxidase	$\dot{M}_{O_2}$ with TMPD and ascorbate as substrates
RCR	Respiratory control ratio	=OXPHOS/LEAK I+II
OXPHOS CE	OXPHOS coupling efficiency	=1–LEAK I+II/OXPHOS
CCO/OXPHOS	Reserve CCO capacity	=CCO/ETS
ETS/OXPHOS	Reserve ETS capacity	=ETS/OXPHOS

$\dot{M}_{O_2}$, oxygen consumption rate; OXPHOS, oxidative phosphorylation; ETS, electron transport system; CCO, cytochrome *c* oxidase; RCR, respiratory control ratio; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

OXPHOS capacity, whereas oxygen consumption in the presence of CCCP reflects the maximum capacity of the mitochondrial ETS (www.bioblast.at/index.php/MitoCom). Protein concentration was measured in the mitochondrial suspensions using the Bradford assay (Bio-Rad, Hercules, CA, USA) with BSA as a standard and corrected for the BSA content of the resuspension media. Respiration rates were expressed in μmol O_2 min^{-1} g^{-1} protein, and H_2O_2 production in μmol H_2O_2 min^{-1} g^{-1} protein.

To measure the apparent oxygen affinity (P_{50}) of CCO, 2.5 μmol l^{-1} of CCCP, 2.5 μmol l^{-1} of the inhibitor antimycin A, 0.5 mmol l^{-1} TMPD and 2 mmol l^{-1} ascorbate were added to the mitochondrial suspensions. The chamber was closed and oxygen consumption was recorded until all oxygen in the chamber was exhausted (20–35 min). P_{50} values were calculated for each mitochondrial isolate using the oxygen kinetics module in DatLab2 (www.oroboros.atwww.oroboros.at, accessed 8 July 2020).

Statistics

Data from two independent experimental runs were compared using Student's *t*-test implemented in IBM® SPSS® Statistics v.22.0.0.0 (IBM Corp., Armonk, NY, USA). The data were tested for normality, and the data deviating from the normal distribution were transformed by a 1/*x* transformation. Levene's test demonstrated the homogeneity of variances in all datasets, and the *t*-test showed no differences in the studied mitochondrial traits between the two independent experimental runs ($P>0.05$). Therefore, the measurements from the two runs were combined, and individual mitochondrial isolations were used as biological replicates in further analyses.

The outliers were determined by box-and-whisker plots using IBM® SPSS® Statistics v.22.0.0.0 and removed from further analyses. Data were tested for normality and homogeneity of variances using Shapiro–Wilk and Brown–Forsythe tests, respectively, and transformed by Box–Cox or Johnson transformation to achieve normality. The main effects were tested using one-way ANOVA with the oxygen regime as a fixed factor with three levels (normoxia, and constant or cyclic hypoxia). To assess differences between the pairs of means, Tukey's honest significant differences (HSD) test was used. Data that did not achieve normality and/or equal variances after transformation were analyzed using the Kruskal–Wallis test followed by Dunn's test to assess differences between the conditions. All differences were considered significant at the 5% level ($P<0.05$). The analyses were carried out in SigmaPlot version 13.0 (Systat Software, Inc., San Jose, CA, USA). Graphics were made by using RStudio Team 2015 software (http://www.rstudio.com/).

RESULTS

Effects of constant and cyclic hypoxia on mitochondrial $\dot{M}_{O_2}$

Mitochondria of the clams acclimated to cyclic hypoxia had ∼1.5- to 2-fold higher baseline $\dot{M}_{O_2}$ (indicative of proton leak) compared with the normoxic controls when respiring on Complex I substrates (LEAK I), or on the mixture of Complex I and II substrates (LEAK I+II) (Fig. 2A,B). Acclimation to constant hypoxia had no effect on the mitochondrial proton leak (LEAK I or LEAK I+II) of the clams. Acclimation to constant and cyclic hypoxia had no effect on the ADP-stimulated $\dot{M}_{O_2}$ (OXPHOS capacity) and on $\dot{M}_{O_2}$ of uncoupled mitochondria (indicative of the maximum ETS activity) (Fig. 2C,D).

The respiratory control ratio (RCR) was slightly but not significantly ($P>0.05$) lower in the mitochondria of clams acclimated to cyclic hypoxia compared with those kept in normoxia or constant hypoxia (Fig. 3A). OXPHOS coupling efficiency was significantly lower in mitochondria of clams acclimated to cyclic hypoxia (Fig. 3B). The oxygen regime did not affect the apparent reserve capacity of ETS (Fig. 3C).

Effects of constant and cyclic hypoxia on mitochondrial H_2O_2 production

In mitochondria from the control (normoxic) clams, the baseline (leak state) H_2O_2 production rate was ∼2-fold higher in those respiring with the combination of Complex I and II substrates compared with those respiring with a Complex I substrate only (Fig. 4A). Furthermore, the H_2O_2 production rate with pyruvate and succinate as substrates was ∼2- to 3-fold higher in the baseline (leak) state than in the actively phosphorylating (OXPHOS) state (cf. LEAK I+II and OXPHOS in Fig. 4A). A qualitatively similar pattern was found in the mitochondria of clams exposed to constant or cyclic hypoxia (data not shown).

In mitochondria from the control (normoxic) clams, the ratio of H_2O_2 produced to O_2 consumed was considerably (∼8- to 10-fold) lower in the OXPHOS than in the leak state, regardless of the substrate (Fig. 4B). No difference in the ratio of H_2O_2 produced to O_2 consumed was found between the mitochondria respiring on a Complex I substrate only or on the combination of Complex I and II substrates during the baseline (leak state) respiration. A similar pattern was observed in the H_2O_2:O_2 ratio of mitochondria from clams acclimated to constant or and cyclic hypoxia (data not shown).

The baseline (leak) H_2O_2 production in mitochondria respiring with a Complex I substrate was ∼1.7-fold higher in the clams acclimated to cyclic hypoxia compared with that in normoxic clams (Fig. 5A). In the mitochondria of clams acclimated to constant hypoxia, the baseline rate of H_2O_2 production with a Complex I substrate was intermediate between that of the normoxic clams and the clams acclimated to cyclic hypoxia (Fig. 5A). The baseline rate of H_2O_2 production by mitochondria respiring with the combination of Complex I and II substrates was similar in clams from all studied oxygen regimes (Fig. 5B). The rate of H_2O_2 production in actively phosphorylating mitochondria was ∼1.8-fold higher in clams acclimated to cyclic hypoxia compared with that in their counterparts acclimated to normoxia or constant hypoxia (Fig. 5C).

Effects of hypoxia on mitochondrial CCO activity and oxygen affinity

CCO activity in the mitochondria of clams acclimated to cyclic hypoxia was ∼1.7- to 1.8-fold higher ($P<0.05$) than that of clams maintained in normoxia or constant hypoxia (Fig. 6A). The apparent reserve CCO capacity was lower ($P<0.05$) in the mitochondria of clams acclimated to constant hypoxia compared with that in their counterparts maintained in cyclic hypoxia but not in normoxia (Fig. 6B). Acclimation of the clams to different oxygen regimes had no effect on the apparent oxygen affinity of CCO measured as P_{50} (Fig. 6C).

DISCUSSION

Effects of chronic hypoxia on mitochondrial respiration of clams

Hypoxia is an ultimate stressor for aerobic organisms as it can lead to ATP deficiency as a result of the substrate (O_2) limitation of CCO (Hochachka et al., 1993) and/or excessive production of the potentially cytotoxic ROS (Halliwell and Gutteridge, 2015). Benthic marine organisms including the soft-shell clams are commonly exposed to periodical hypoxia in estuaries and coastal waters, and their survival in these habitats requires that the mitochondrial integrity is maintained under fluctuating oxygen conditions. The present study shows that mitochondria of soft-shell clams, *M. arenaria*, maintain normal function (indicated by the lack

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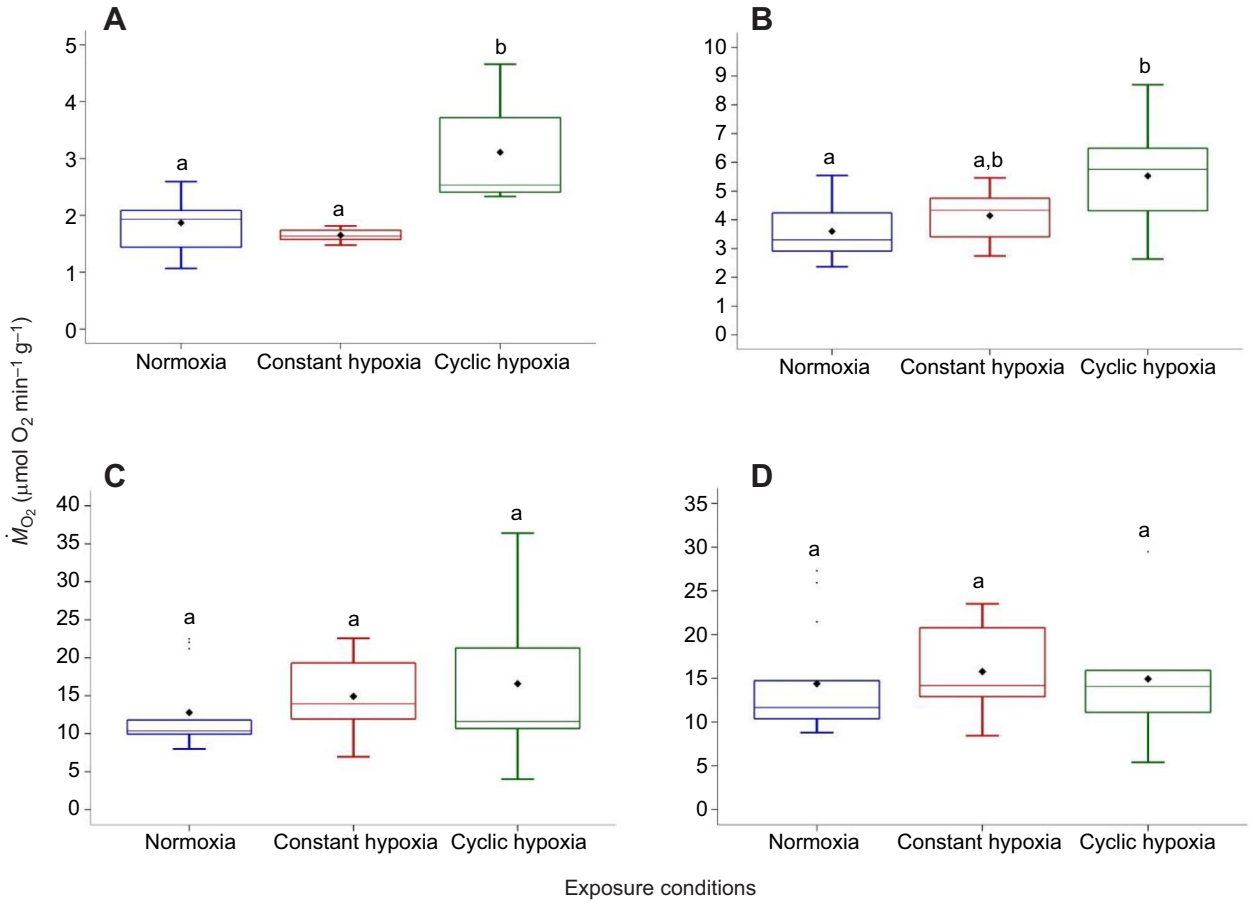

Fig. 2. Effect of different oxygen regimes on the respiration of isolated mitochondria of *M. arenaria*. Respiratory capacity of isolated mitochondria, determined as $\dot{M}_{O_2}$, was calculated per mass of mitochondrial protein. (A) Rate of proton leak of mitochondria respiring on NADH-linked substrates (LEAK I); (B) rate of proton leak of mitochondria respiring on NADH- and $FADH_2$-linked substrates (LEAK I+II); (C) oxidative phosphorylation (OXPHOS) rate; and (D) electron transport system (ETS) activity. Significantly different values (P<0.05) in clams acclimated to different oxygen regimes are indicated with different letters. N=13, 8–10 and 6–7 in normoxia, constant hypoxia and cyclic hypoxia, respectively.

of change in proton leak, OXPHOS capacity, ETS activity or ROS production) after 3 weeks of chronic hypoxia exposure (4.1 kPa, 20% air saturation). The activity and oxygen affinity of CCO was also unaffected by the acclimation to constant hypoxia in *M. arenaria*. It is worth noting that the oxygen levels used in the constant hypoxia exposures (4.1 kPa) in the present study are lower than the critical partial pressure of O_2 ($P_{O_2,crit}$; 5.3–6.7 kPa), below which *M. arenaria* can no longer maintain the organismal $\dot{M}_{O_2}$ and enters the metabolically suppressed state (Davis, 1975). The present study thus indicates that the mitochondrial capacity of *M. arenaria* is not suppressed by prolonged exposure to low oxygen levels below the organismal $P_{O_2,crit}$. Furthermore, the low oxygen threshold for suppression of mitochondrial CCO activity (P_{50} ~0.036–0.038 kPa) indicates that the clam mitochondria are adapted to function at low

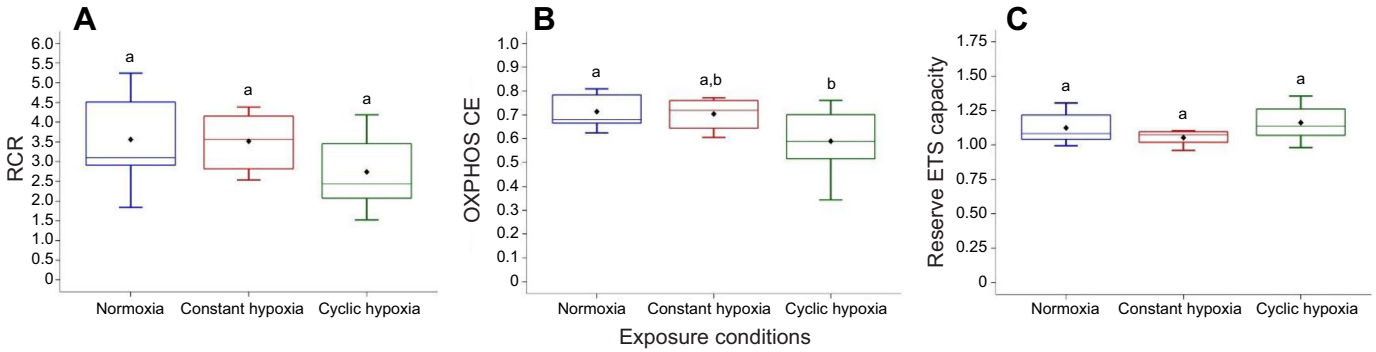

Fig. 3. Effect of different oxygen regimes on mitochondrial activity indices of isolated mitochondria of *M. arenaria*. (A) Respiratory control ratio (RCR); (B) OXPHOS coupling efficiency (OXPHOS CE=1−LEAK I+II/OXPHOS); and (C) apparent reserve ETS capacity (ETS/OXPHOS). Significantly different values (P<0.05) in clams acclimated to different oxygen regimes are indicated with different letters. N=12–13, 9–10 and 7 in normoxia, constant hypoxia and cyclic hypoxia, respectively.

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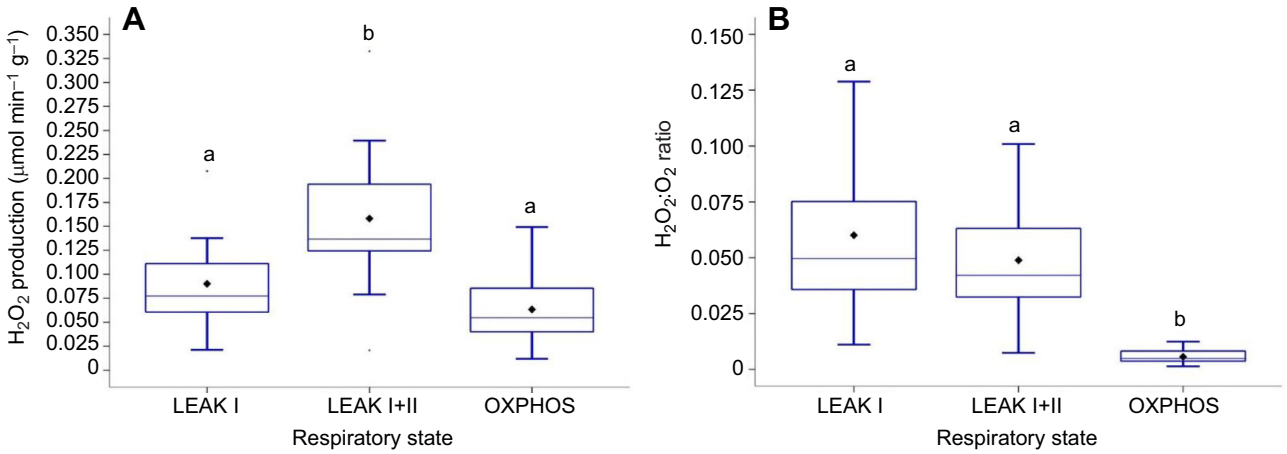

Fig. 4. Reactive oxygen species (ROS) production of isolated mitochondria in different physiological states in *M. arenaria*. ROS production of isolated mitochondria was calculated per mass of mitochondrial protein. Data are presented for isolated mitochondria from the hepatopancreas of control (normoxia-exposed) clams. (A) Rate of hydrogen peroxide (H_2O_2) production and (B) H_2O_2:O_2 ratio in different mitochondrial states. LEAK I and LEAK I+II, resting mitochondria respiring with Complex I and Complex I+II substrates, respectively; OXPHOS, actively phosphorylating mitochondria. Significantly different values (P<0.05) between different mitochondrial states are indicated with different letters. N=12–14, 12–14 and 12–13 in LEAK I, LEAK I+II and OXPHOS, respectively.

tissue P_{O_2} (<5 kPa in *M. arenaria*) (Abele et al., 2010) and thus are unlikely to become oxygen limited under the chronic hypoxia conditions used in this study.

Our findings in *M. arenaria* under the chronically low oxygen conditions are consistent with earlier findings showing robust mitochondrial function of hypoxia-tolerant aquatic organisms. Short-term exposure to severe hypoxia (18 h at <0.1 kPa P_{O_2}) the mitochondrial ETS activity in a hypoxia-tolerant hard shell clam, *Mercenaria mercenaria* (Ivanina et al., 2016). In the oyster *Crassostrea gigas*, short-term exposure to severe hypoxia (24 h at ∼0.2 kPa P_{O_2}) had no effect on mitochondrial proton leak or OXPHOS rates (Sokolov et al., 2019), albeit exposure to moderate hypoxia (3–12 h at 4.1 kPa P_{O_2}) led to a slight decrease of mitochondrial respiration (Sussarellu et al., 2013). Similarly, hypoxia-tolerant reptiles and fish were able to maintain and/or increase the OXPHOS and ETS capacity in the liver and muscle tissues during long-term exposure to hypoxia (Cook et al., 2013; Du et al., 2016; Galli et al., 2016; Gerber et al., 2019; Napolitano et al., 2019). In a notable exception, the brain mitochondria of the hypoxia-tolerant turtle *Trachemys scripta* showed a decreased OXPHOS and ETS capacity and elevated proton conductance after 2 weeks exposure to anoxia, possibly reflecting higher sensitivity of the brain to oxygen deficiency compared with other tissues (Pamenter et al., 2016). Despite some variability of responses, the preponderance of existing evidence shows that mitochondrial resilience to hypoxia is a common feature of the hypoxia-tolerant phenotype in aquatic ectotherms. This is in stark contrast with the responses of hypoxia-sensitive vertebrates such as terrestrial mammals, where even short (minutes to hours) hypoxia exposures result in a strong suppression of mitochondrial respiration and/or loss of mitochondrial integrity (for reviews, see Kalogeris et al., 2012; Paradis et al., 2016; Lesnefsky et al., 2017; Sokolova, 2018; Sokolova et al., 2019).

Effects of cyclic hypoxia on mitochondrial respiration of clams

Oxygen fluctuations are generally considered more stressful to aerobic organisms than chronic mild hypoxia because of a burst of ROS production during reoxygenation (Hermes-Lima and Zenteno-Savín, 2002; Kalogeris et al., 2014). However, in organisms adapted to periodic hypoxia (such as benthic marine organisms exposed to tidal emersion rhythms and/or the diurnal cycles of photosynthesis and respiration), the mitochondrial damage caused by oxygen fluctuations is expected to be minimized. The present study shows that *M. arenaria*

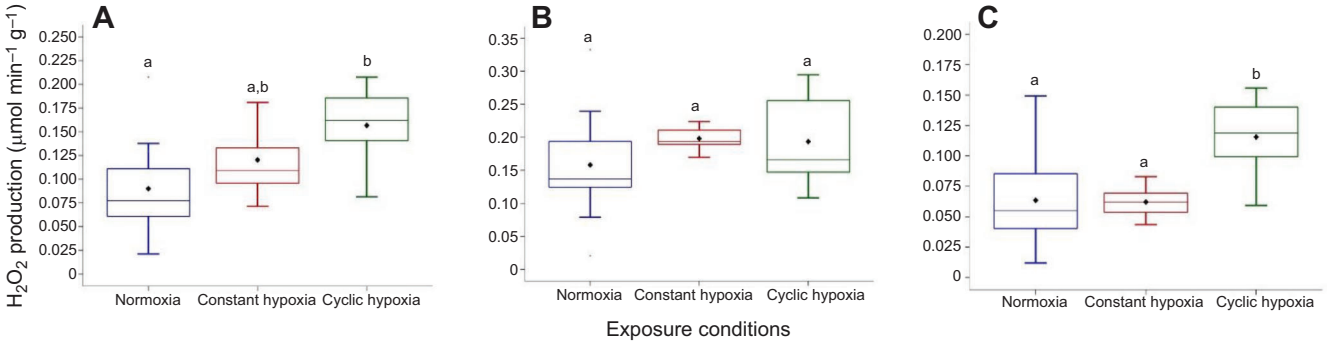

Fig. 5. Effect of different oxygen regimes on H_2O_2 production of *M. arenaria*. H_2O_2 production of isolated mitochondria was calculated per mass of mitochondrial protein. (A) H_2O_2 production in resting mitochondria respiring on Complex I substrates (LEAK I); (B) H_2O_2 production in resting mitochondria respiring on Complex I and II substrates (LEAK I+II); and (C) H_2O_2 production in the actively phosphorylating (OXPHOS) state. Significantly different values (P<0.05) in clams acclimated to different hypoxia and hypercapnia regimes are indicated with different letters. N=12, 7–9 and 6 in normoxia, constant hypoxia and cyclic hypoxia, respectively.

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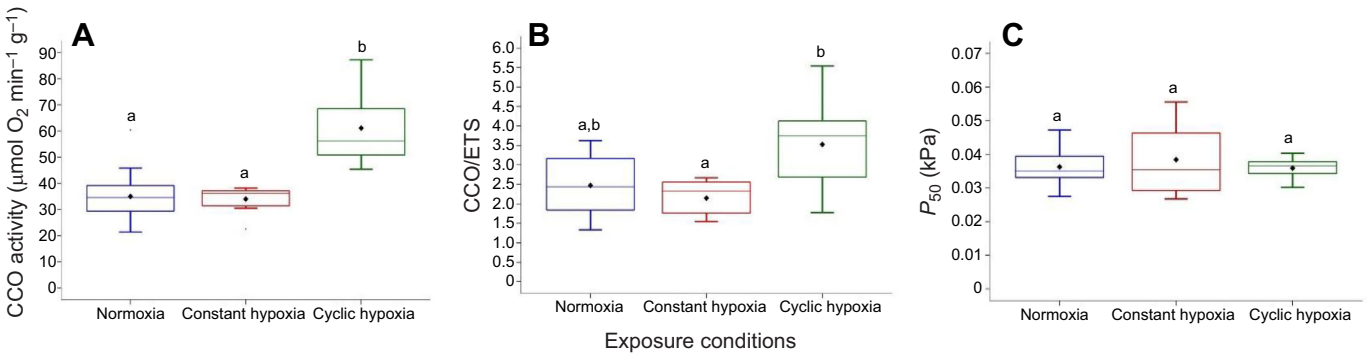

Fig. 6. Effect of the different oxygen regimes on the activity, oxygen affinity and apparent reserve capacity of cytochrome *c* oxidase (CCO) from *M. arenaria* acclimated to different oxygen regimes. The activity and apparent reserve capacity were calculated per pass of mitochondrial protein. (A) CCO activity; (B) apparent CCO reserve capacity; and (C) apparent oxygen affinity (P_{50}) of the mitochondrial CCO. Significantly different values (P<0.05) in clams acclimated to different hypoxia and hypercapnia regimes are indicated with different letters. N=12–13, 9–12 and 6–7 in normoxia, constant hypoxia and cyclic hypoxia, respectively.

exposed to diel oxygen cycles were capable of maintaining normal OXPHOS and ETS activity similar to that found in normoxic clams. Similarly, a study in a hypoxia-tolerant intertidal fish, *F. heteroclitus*, found that long-term exposure to diel cyclic hypoxia (alternating between ∼21 and 5 kPa P_{O_2}) had no effect on the OXPHOS or ETS capacity of liver mitochondria (Du et al., 2016). Earlier studies using a single bout of hypoxia and reoxygenation also showed that the mitochondria of hypoxia-tolerant marine bivalves are generally resistant to reoxygenation stress, maintaining OXPHOS and ETS capacity and upregulating protective mechanisms such as mitochondrial antioxidants and protein quality control pathways (Kurochkin et al., 2008; Ivanina et al., 2010; Sussarellu et al., 2013; Ivanina and Sokolova, 2016; Sokolov et al., 2019; Steffen et al., 2020).

Unlike OXPHOS and ETS activity, the rate of proton leak increased by ∼1.5- to 1.7-fold in the mitochondria of *M. arenaria* acclimated for 3 weeks to cyclic hypoxia. This increase was observed regardless of whether the mitochondria were respiring on a Complex I substrate or the mixture of Complex I and II substrates. As a result, the OXPHOS coupling efficiency notably decreased in the mitochondria of clams acclimated to cyclic hypoxia. Oxygen consumption of the mitochondria in non-phosphorylating (LEAK) state reflects the baseline activity of ETS needed to counteract all futile proton and cation cycles not linked to ATP production and to prevent depolarization of idling mitochondria (Rolfe and Brand, 1997). Earlier studies in ectotherms including marine bivalves demonstrated that mitochondrial proton leak can contribute up to 20–40% of the overall cellular basal maintenance costs in ectotherms (Brand et al., 1991; Hulbert and Else, 2000; Hulbert et al., 2002; Cherkasov et al., 2006). Therefore, an increase in mitochondrial proton leak might incur considerable additional costs for mitochondrial maintenance in clams under cyclic hypoxia.

Effects of different oxygen regimes on CCO activity and oxygen affinity

CCO is an important regulator of the mitochondrial and cellular response to hypoxia (Castello et al., 2008; Kocha et al., 2015). In mammals, CCO activity is regulated via expression of different CCO isoforms (Kocha et al., 2015) and/or by reversible protein phosphorylation during hypoxia (Hüttemann et al., 2012). Exchange of the COX4-1 subunit for the hypoxia-inducible COX4-2 subunit is thought to optimize electron flux through the ETS, mitigate ROS production and modulate the CCO oxygen affinity in mammalian models (Fukuda et al., 2007; Pajuelo Reguera et al., 2020). Reversible phosphorylation of CCO can inhibit or activate the enzyme, depending on the phosphorylation site, thus playing a role in the fine-tuning of ETS activity during hypoxia in mammals (Helling et al., 2008, 2012).

The involvement and mechanisms of CCO modulation by hypoxia are not yet well understood in hypoxia-tolerant aquatic ectotherms. A recent comparative analysis indicates that selective overexpression of the COX4-2 subunit in response to hypoxia might be restricted to mammals and not found in reptiles and fish, including hypoxia-tolerant species (Kocha et al., 2015). Reversible protein phosphorylation by protein kinases A and C can modulate CCO activity in hypoxia-tolerant mollusks but the involvement of this mechanism in the mitochondrial response to hypoxia appears to be species specific (Falfushynska et al., 2020). The present study in *M. arenaria* showed that exposure to constant or cyclic hypoxia has no effect on the oxygen affinity (measured as P_{50}) of CCO, indicating that a change in isoform composition is unlikely in this case. Generally, the relationship between the P_{50} of CCO and hypoxia tolerance is not unequivocal, with some studies showing higher oxygen affinity (and thus lower P_{50}) in hypoxia-tolerant populations and species, whereas other studies reporting no association between the P_{50} of CCO and hypoxia tolerance (Sokolova et al., 2019). The present study shows that the long-term mitochondrial adjustments of *M. arenaria* to chronic or periodical oxygen deficiency do not involve modulation of the oxygen affinity of CCO.

Interestingly, the maximal activity of CCO increased in *M. arenaria* exposed to cyclic (but not constant) hypoxia. Studies using a single cycle of hypoxia–reoxygenation typically show suppression or no change of CCO activity in marine bivalves during short-term (12–24 h) hypoxic exposure (Sussarellu et al., 2013; Sokolov et al., 2019), albeit longer exposures to hypoxia (up to 6 days) might reverse this trend and stimulate CCO activity (Falfushynska et al., 2020). In the present study, an increase in CCO activity did not translate into higher rates of OXPHOS or ETS respiration of *M. arenaria*. Given the high reserve CCO capacity (∼4- to 7-fold over the maximum ETS activity) in the mitochondria of *M. arenaria*, CCO is unlikely to be a rate-limiting enzyme for ETS flux, and elevated CCO activity in the mitochondria of clams exposed to fluctuating oxygen levels must play some other regulatory role requiring further investigation.

ROS production in mitochondria from clams acclimated to different oxygen regimes

As expected, ROS generation in mitochondria of *M. arenaria* was dependent on the mitochondrial state. Generally, the clam

mitochondria in the resting (LEAK) state converted ∼4–5% of consumed oxygen to ROS, whereas in the actively phosphorylating mitochondria, this proportion decreased to <1%, probably reflecting lower mitochondrial membrane potential and thus lower electron slip rate in the OXPHOS state (Lambert and Brand, 2004; Dzbek and Korzeniewski, 2008). The rate of ROS generation was ∼2-fold higher in the resting mitochondria respiring on Complex I and II substrates (pyruvate and succinate) compared with those respiring on pyruvate only. Succinate is commonly reported to spark mitochondrial ROS production, a finding attributed to reverse electron transport (RET) through Complex I (Muller et al., 2008; Chouchani et al., 2014; Bundgaard et al., 2019a). In the present study, the succinate-driven increase in ROS production of *M. arenaria* was proportional to an overall increase in respiratory flux so that a similar fraction of O_2 (∼4–5%) was converted to ROS to that during respiration on pyruvate alone. Although our study cannot exclude the possibility of RET during succinate-driven respiration of *M. arenaria* mitochondria, the most parsimonious explanation for the elevated ROS flux under these conditions is the overall increase in oxygen consumption rate.

Long-term acclimation of *M. arenaria* to constant hypoxia (4.1 kPa) had no effect on ROS production in resting or actively phosphorylating mitochondria. These findings are consistent with the lack of a significant change in the mitochondrial $\dot{M}_{O_2}$ of clams exposed to constant hypoxia compared with their normoxic counterparts and might indicate that the level of hypoxia chosen for this study, while below the $P_{O_2,crit}$ threshold for *M. arenaria* (5.3–6.7 kPa) (Davis, 1975), was not sufficient to induce mitochondrial dysfunction. Unlike acclimation to constant hypoxia, long-term acclimation to fluctuating oxygen conditions stimulated ROS generation in the clam mitochondria by ∼1.7- to 1.8-fold in both the leak and OXPHOS states. Unlike our present study in clams, long-term exposure to constant and diel cycling hypoxia led to a significant decrease in mitochondrial ROS production in a hypoxia-tolerant intertidal fish, *F. heteroclitus* (Du et al., 2016). Suppression of mitochondrial ROS production was also found in the mitochondria of turtles *T. scripta* exposed to anoxia (Bundgaard et al., 2018; Bundgaard et al., 2019b). It is worth noting that even though a significant increase was found in ROS generation rates in the mitochondria of *M. arenaria* under the fluctuating oxygen regime, this increase was proportional to the overall increase (by 70–80%) in the mitochondrial respiratory flux. Furthermore, the ratio of H_2O_2 produced to O_2 consumed did not differ in clams acclimated under different oxygen regimes, consistent with the notion that the increase in ROS production in clams exposed to cyclic hypoxia reflects the enhanced oxygen flux rather than elevated rates of electron leak from the ETS (Jastroch et al., 2010). These findings from the hypoxia-tolerant aquatic organisms are in stark contrast to those from hypoxia-sensitive mammalian models where hypoxia–reoxygenation leads to a massive ROS overproduction as a consequence of elevated electron slip and results in the loss of mitochondrial ETS and OXPHOS capacity (Venditti et al., 2001, 2013; Paradies et al., 2004; Kalogeris et al., 2014; Bugger and Pfeil, 2020). The present study as well as previously published work (Du et al., 2016; Bundgaard et al., 2018, 2019b) indicates that suppressed ROS production is not always part of the hypoxia-tolerant mitochondrial phenotype, and that in some organisms such as *M. arenaria* an elevated rate of ROS generation can be tolerated without a loss of mitochondrial function.

Conclusions and outlook

Our study showed that mitochondria of the hypoxia-tolerant benthic bivalve *M. arenaria* are robust to long-term chronic hypoxia and maintain normal OXPHOS capacity as well as normal rates of ROS production similar to those found in normoxic animals. It is worth noting that in the present study the mitochondrial respiration of *M. arenaria* was assessed *in vitro* under high oxygen concentrations, thus reflecting the maximum respiratory capacity of the mitochondria, which might differ from the actual *in vivo* respiratory fluxes. The oxygen cascade in animal tissues implies a steep decline of the oxygen concentration from the external P_{O_2} of ∼21 kPa in normoxia to ∼1–5 kPa inside the cell and <1 kPa inside the mitochondria (Gnaiger et al., 1995; Weiner, 1994; Biro, 2013). Ambient hypoxia strongly decreases the intracellular and intra-mitochondrial P_{O_2} (Mik et al., 2008). High oxygen affinity of CCO indicates that the mitochondria of *M. arenaria* are well adapted to function under the low oxygen conditions, but it cannot be excluded that mitochondrial respiration and ATP synthesis might become limiting when hypoxia coincides with conditions requiring high metabolic rates such as during the peak of reproduction or exposure to elevated temperatures. Interestingly, fluctuating oxygen conditions led to more considerable functional shifts in the clam mitochondria than did chronic hypoxia, resulting in higher proton leak, lower OXPHOS coupling efficiency and elevated ROS production. This may be due to fluctuations in oxygen levels that are known to produce ROS bursts in other species such as the terrestrial mammals and insects (Venditti et al., 2001, 2013; Paradies et al., 2004; Kalogeris et al., 2014; Bugger and Pfeil, 2020). Alternatively, the difference in the mitochondrial responses to chronic and cyclic hypoxia in the present study might be due to the lower P_{O_2} during the hypoxic phase in cyclic hypoxia (2.1 kPa) compared with chronic hypoxia conditions (4.1 kPa). Regardless of the underlying mechanisms, the increase in mitochondrial proton leak and decrease in OXPHOS efficiency might elevate the basal metabolism (i.e. the cost of living) of clams exposed to cyclic hypoxia, requiring higher energy investment into their antioxidant protection and mitochondrial maintenance and potentially diverting energy from other fitness functions such as growth, reproduction or burrowing activity. This hypothesis requires further investigation to assess the effects of different oxygen regimes on whole-organism activity, energy status and potential trade-off between different energy-demanding fitness functions of *M. arenaria*.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.P.S., I.M.S.; Methodology: N.O., E.P.S., S.O., G.R., I.M.S.; Validation: N.O., S.O., G.R.; Formal analysis: N.O.; Investigation: N.O., E.P.S., S.O.; Resources: G.R., I.M.S.; Data curation: N.O., S.O.; Writing - original draft: N.O., I.M.S.; Writing - review & editing: N.O., E.P.S., S.O., G.R., I.M.S.; Visualization: N.O.; Supervision: I.M.S.; Project administration: I.M.S.; Funding acquisition: I.M.S.

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Data availability

The metadata from the project are available from the open data repository Zenodo: doi:10.5281/zenodo.4352948

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