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Seasonality of energetic functioning and production of reactive oxygen species by lugworm (*Arenicola marina)* mitochondria exposed to acute temperature changes

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Summary

The influence of seasonal and acute temperature changes on mitochondrial functions were studied in isolated mitochondria of the eurythermal lugworm *Arenicola marina* (Polychaeta), with special emphasis on the interdependence of membrane potential and radical production. Acclimatisation of lugworms to pre-spawning/summer conditions is associated with rising mitochondrial substrate oxidation rates, higher proton leakage rates, elevated membrane potentials, and increased production of reactive oxygen species (ROS) in isolated mitochondria, compared with mitochondria from winter animals. However, a high ROS production was compensated for by higher activities of the antioxidant enzymes catalase and superoxide dismutase, as well as lower mitochondrial densities in summer compared with winter animals. In summer animals, a higher sensitivity of the proton leakage rate to changes of membrane potential will confer better flexibility for metabolic regulation (mild uncoupling) in response to temperature change. These seasonal alterations in mitochondrial functions suggest modifications of energy metabolism in eurythermal and euryoxic organisms on intertidal mudflats during summer. In winter, low and less changeable temperatures in intertidal sedimentary environments permit higher respiratory efficiency at low aerobic metabolic rates and lower membrane potentials in *A. marina* mitochondria.

Key words: lugworm, *Arenicola marina*, mitochondria, ROS, proton leak, metabolic regulation, temperature.

Introduction

The lugworm *Arenicola marina* is one of the most successful species on intertidal mudflats and is highly adaptable to various abiotic stress factors in its changeable environment. This applies not only to oxygen partial pressure and salinity, but also to temperature, which varies greatly around the annual mean of 10°C in the North Sea. Thus, intertidal lugworm populations of the Wadden Sea can be exposed to short periods of ice cover in January and February, whereas in summer the sediment can warm to 20°C at the depths where the burrows are located. Lugworms from the North Sea intertidal flats start gamete production in late March, which leads to an elevated energy demand throughout the summer. Ripe gametes are ejected in a brief spawning event during the second half of September (Schöttler, 1989). The elevated energy demand for gamete production in both sexes leads to a vast increase of whole animal oxygen consumption during summer, until some weeks before the spawning. Pre-spawning oxygen demand is therefore elevated in excess of a rising demand due to the higher summer temperatures, and exacerbates susceptibility to anoxia. Warming exacerbates the formation of reactive oxygen species (ROS) in marine invertebrates (Abele et al., 1998a,b, 2001). These oxygen free radicals arise largely but not exclusively from the mitochondria (Abele et al., 2002; Heise et al., 2003), known as major ROS producers under pathophysiological conditions and in ageing animals (Sastre et al., 2000; St-Pierre et al., 2002). Additional ROS originate from several enzymatic oxidase reactions (for a review, see Storey, 1996). If stress-induced production of active oxygen species is not adequately counterbalanced by cellular antioxidants, mainly catalase, superoxide dismutase and the glutathione system, oxidative damage of lipids, proteins and nucleic acids ensues (Halliwell and Gutteridge, 1989; Lenaz, 1998; Duval et al., 2002).

As important ROS producers, mitochondria are prone to become immediate targets for ROS-induced molecular damage. This leads to disturbance of mitochondrial energetic functioning in a primary assault (Yan et al., 1997; Brand, 2000), whereas slowly accumulating damage of the mitochondrial DNA causes mitochondrial degeneration and enhances the process of cellular ageing (Sastre et al., 2000; St-Pierre et al., 2002). Mitochondrial ROS production depends on the magnitude of membrane potential ($\Delta\Psi$) in isolated mitochondria (Korshunov et al., 1997; Brand, 2000), and not so much on the rate of electron transport. The $\Delta\Psi$-threshold value for significant ROS production is just above state 3 $\Delta\Psi$ level (Korshunov et al., 1997) and, indeed, most investigations

do not detect substantial ROS production under phosphorylating state 3 conditions. Mild uncoupling of the proton gradient through futile cycling of protons through the inner mitochondrial membrane dissipates $\Delta\Psi$ and reduces proton motive force (Skulachev, 1996, 1998; Korshunov et al., 1997; Brand, 2000), thereby preventing overflow of electrons from mitochondrial complexes I and III. High proton motive force slows respiratory electron transport and leads to an increased reduction of complex III ubiquinone (QH), which then leaks electrons into the matrix and presumably also to the intermembrane space (St-Pierre et al., 2002). Here, the electrons react with molecular oxygen to form superoxide and H_2O_2 (for a review, see Brand, 2000). Accordingly, the maximal proportion of proton leakage through the inner mitochondrial membrane determines the range over which an animal can shift mitochondrial $\Delta\Psi$, in order to optimise respiratory efficiency, while avoiding deleterious ROS production during transient ADP exhaustion (Korshunov et al., 1997). An adjustable proton leak rate could contribute towards controlling the low intracellular P_{O_2}, especially in water breathing animals (see also Massabuau, 2003), as it increases oxygen consumption also under resting conditions (Brand, 2000). This, and the limitation of ubisemiquinone (QH·) accumulation, gave rise to the idea of an antioxidant function of 'mild uncoupling' of $\Delta\Psi$ under physiological conditions (Skulachev, 1996, 1998; Brand, 2000).

In the present study we isolated mitochondria from lugworms of an intertidal population during summer (July) and winter (February), and measured mitochondrial energetics, membrane potential and ROS production. The aim was to investigate the functional changes caused by seasonal temperature acclimatisation and the higher energy demand during the reproductive cycle. Specifically, the following questions were addressed. (i) How does seasonal acclimatisation affect mitochondrial function, ROS production and mitochondrial density in summer compared to winter animals? (ii) What is the interdependence between phosphorylation efficiency, membrane potential and ROS production in lugworms? (iii) Is ROS formation related to the energetic state of the mitochondria (state 3 *vs* state 4 respiration)? (iv) Is ROS production controlled under thermal stress (warming and cooling) in mitochondria during summer and winter by increasing the leak, or would the animals have to respond by increasing cellular antioxidant stress defence?

Materials and methods

Animal collection and maintenance

Adult lugworms *Arenicola marina* L. of 7–9 cm body length were dug on an intertidal sand flat near Bremerhaven, Germany, at the end of February 2002 (winter animals) and in July 2002 (summer animals, pre-spawning), at sediment temperatures of 1°C and 10°C, respectively. In the laboratory, animals were kept in aquaria filled with sediment from the sampling location and natural 22‰ salinity water. Part of the sediment was renewed every 2 months.

For the experiments on mitochondrial physiology, animals were kept at constant temperatures of 1°C (winter animals) and 10°C (summer animals) for 2–7 weeks. One group of late winter animals was acclimated to 10°C for a period of 21–30 weeks, to follow the time course of mitochondrial density changes. In addition, mitochondrial counts were done in residual individuals from the winter animal group, kept at 1°C, after finishing the physiological experiments at 14, 18 and 22 weeks after collection. The numbers of mitochondria per cell in summer animals maintained at 10°C were counted immediately after collection and again after 4 weeks.

Isolation of mitochondria

After removing head and tail of the worm, the body wall tissue was opened, the intestine removed and the remaining tissue rinsed with seawater and blotted dry. Tissues from 2–4 animals were pooled per isolation, yielding a total of 3.5–4 g fresh mass. Part of the pooled tissue was frozen in liquid nitrogen and stored at –80°C for subsequent enzymatic measurements.

Mitochondria were prepared after Sommer and Pörtner (2002). Between 2.3 and 2.5 g of fresh tissue were minced in 35 ml isolation buffer [550 mmol l^{-1} glycine, 250 mmol l^{-1} sucrose, 40 mmol l^{-1} Tris/HCl, 4 mmol l^{-1} EDTA; 1% (w:v) bovine serum albumin (BSA); 1 μg ml^{-1} aprotinin, pH 7.5 at 20°C] using scissors. The tissue was transferred to a teflon/glass homogeniser (type: Potter Elvejhem; Sartorius BBI Systems, Melsungen, Germany) and homogenised with 5–7 passes. After centrifugation for 8 min at 1300 ***g*** and 0°C, the supernatant was stored on ice and the pellet resuspended and homogenised a second time. Following a second centrifugation, supernatants were combined and centrifuged for 15 min at 10 000 ***g*** and 0°C to sediment mitochondria. The resulting pellet was resuspended in 2 ml assay medium (600 mmol l^{-1} glycine, 160 mmol l^{-1} KCl, 5 mmol l^{-1} K_2HPO_4, 20 mmol l^{-1} Na-Hepes, 4 mmol l^{-1} EDTA, 3 mmol l^{-1} $MgCl_2 6{\cdot}H_2O$, 1 μg ml^{-1} aprotinin, 1% (w:v) BSA, pH 7.5 at 20°C) and kept on ice. Portions of this isolate and of the assay medium were frozen for protein determination according to a Biuret method, modified after Kresze (1988), using 5% (w:v) deoxycholate to resolve membrane proteins.

Measurements of mitochondrial respiration and coupling

The measurements of mitochondrial respiration were carried out in a respiration chamber using Clarke-type oxygen electrodes (Eschweiler, Kiel, Germany). Measurements were performed at habitat temperature: 10°C for summer animals and 1°C for winter animals, respectively. Both types of mitochondria were also measured at the other temperature, thus representing cold exposure (1°C) for summer animal mitochondria, and heating to 10°C for winter animal mitochondria, to test the mitochondrial reaction to acute changes of temperature.

Recording was done using an Eschweiler M 200 oxymeter (Kiel, Germany) connected to a Linseis (Selb, Germany) two-channel chart recorder. For each measurement, the chambers

were filled with 768 µl of O_2-saturated assay medium, 20 µl of a 50% BSA solution, 5 µl of the myokinase inhibitor P^1,P^5-adenosine-5′-pentaphosphate (Ap_5A) in water (5 µmol l^{-1}), 2 µl of complex I inhibitor rotenone (10 µmol l^{-1}), 3.3 mmol l^{-1} of respiratory substrate sodium succinate and 200 µl of mitochondrial suspension. Rates of oxygen consumption were measured at constant temperature, while continuously stirring the mitochondrial suspension at 300 revs min^{-1}. After approximately 5 min, ADP was added to a final concentration of 150 µmol l^{-1} to initiate state 3 respiration under saturating conditions. State 4 respiration was recorded after all ADP had been consumed (Chance and Williams, 1955). After addition of 2 µg ml^{-1} of the ATPase inhibitor oligomycin, state 4+ respiration was recorded, which comprises the oxygen consumption by the proton leak through the inner mitochondrial membrane, plus the amount of oxygen molecules converted to ROS per unit time (Brand et al., 1994a; Heise et al., 2003).

The oxygen solubility (β_{O_2}) of the assay medium at experimental temperatures was calculated after Johnston et al. (1994). Oxygen consumption measurements were corrected for electrode drift at 100% P_{O_2} and at 0% P_{O_2}. The respiratory control ratio (RCR) was calculated by dividing state 3 by state 4 respiration, according to Estabrook (1967), or by state 4+ respiration, following Pörtner et al. (1999; RCROl). ADP/O ratios were calculated by dividing the amount of ADP added by the amount of molecular oxygen consumed during state 3 respiration (Chance and Williams, 1955; Estabrook, 1967).

Determination of reactive oxygen species (ROS) formation in mitochondrial isolates

Mitochondrial ROS production was determined fluorimetrically by recording the reaction of the indicator dye homovanillic acid (HVA; Sigma) with hydrogen peroxidase (H_2O_2) catalyzed by horseradish peroxidase (López-Torres et al., 2002). Briefly 3.6 mg HVA was diluted in 2 ml distilled water to give a 9.8 mmol l^{-1} solution. A fluorometer LS 50B (Perkin & Elmer, Boston, MA, USA; excitation: 312 nm, 2.5 nm slit width; emission: 420 nm, 3.5 nm slit width) equipped with a water-jacketed quartz cuvette thermostatted to the relevant measuring temperature was used for the ROS assays. The measurement was carried out using 200 µl mitochondrial isolate and alongside the respiratory measurements, but omitting BSA. 5 µl of a 6000 U ml^{-1} superoxide dismutase solution (Sigma) was added, to convert superoxide anions to H_2O_2, and 10 µl of horse radish peroxidase (215 U ml^{-1}, Merck, Darmstadt, Germany) to catalyze HVA oxidation by H_2O_2. The assay mixture was gently stirred throughout the measurement. State 2 was induced with sodium succinate (3.3 mmol l^{-1}) and state 3 with ADP (150 µmol l^{-1}). State 2 oxidation was always higher than in state 3 (see Fig. 1). State 3 terminated when HVA oxidation, i.e. the fluorescence slope, started to rise again, indicating exhaustion of ADP and the beginning of state 4 respiration. The state 4 slope was recorded for a couple of minutes, before adding oligomycin to induce state 4+. Finally, for calibration of the assay, 440 pmol H_2O_2 were added and the immediate increase in fluorescence recorded.

Previous testing of the HVA assay showed that the probe is not sensitive to H_2O_2 alone, or to oxidation by superoxide anions prior to SOD conversion. The H_2O_2 induced slope was entirely abolished by catalase.

Measurement of membrane potential

The mitochondrial membrane potential ($\Delta\Psi$) was measured according to Brand (1995), using an electrode sensitive to the hydrophobic cation triphenylmethylphosphonium ($TPMP^+$). Four times 2 µl of 0.125 mmol l^{-1} $TPMP^+$ were added for calibration in a glass cuvette containing 768 µl respiration buffer (0.6 mol l^{-1} glycine, 0.16 mmol l^{-1} KCl, 20 mmol l^{-1} Na-Hepes (pH 7.5 at 20°C), 4 mmol l^{-1} EDTA, 5 mmol l^{-1} K_2HPO_4, 3 mmol l^{-1} $MgCl_2.6H_2O$ and 1 µg ml^{-1} aprotinin), 20 µl of a 50% (w:v) BSA solution, 5 µl of the myokinase inhibitor P^1,P^5-di-adenosine-5′-pentaphosphate (Ap_5A) in water (5 µmol l^{-1}) and 2 µl of complex I inhibitor rotenone (10 µmol l^{-1} in ethanol). When the trace was steady, 200 µl of the mitochondrial suspension were added to give a volume of 1 ml. After the addition of 3.3 mmol l^{-1} sodium succinate, mitochondria were allowed to accumulate $TPMP^+$ and the extramitochondrial $TPMP^+$ concentration reached a new stable value. The membrane potential in state 3 was measured in the presence of 150 µmol l^{-1} ADP and state 4+ was induced by the addition of 2 µg ml^{-1} oligomycin. 1 µl nigericin (80 ng ml^{-1}) was added to bring the pH gradient ($-z\Delta pH$) to zero. At the end of the run, the uncoupler FCCP was added to fully dissipate $\Delta\Psi$, so that all $TPMP^+$ was released by the mitochondria and the external concentration re-established.

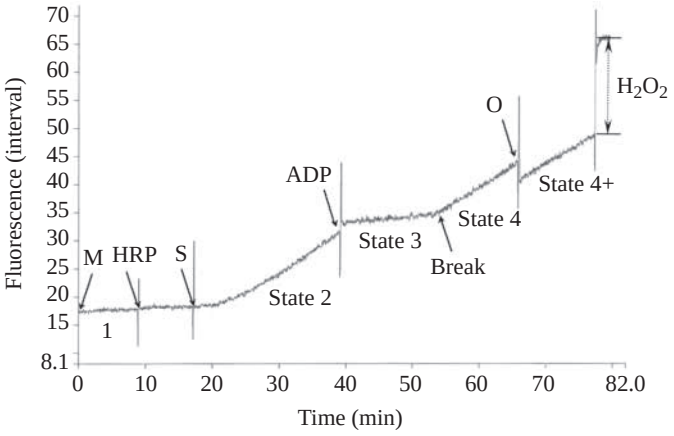

Fig. 1. Measurement of mitochondrial ROS production using homovanillic acid (HVA) as fluorophore in a peroxidase-catalysed reaction. Data interval, 0.02·min. M, start of measurement by addition of mitochondrial isolate to the buffer solution; 1, basal fluorescence of HVA suspension; HRP, addition of horseradish peroxidase; S, sodium succinate addition induces state 2; ADP addition induces state 3; 'break' indicates the change of slope caused by the transition to state 4 after complete phosphorylation of ADP; O, oligomycin addition starts state 4+; dotted arrow, calibration with H_2O_2.

Measurements were done in triplicate. The membrane potential (mV) was calculated using the following equation:

$$\Delta\Psi = \text{number of electrons } \{\log([TPMP^+]_{added} - [TPMP^+]_{external} \times 0.55_B)\} / (0.001 \times \text{protein content in cuvette} \times TPMP^+_{external}),$$

where $[TPMP^+]$ is in μmol l^{-1}, 0.55_B (μl mg^{-1} protein) is the $TPMP^+$ binding correction B after Brand (1995) and the protein content in the cuvette is in mg ml^{-1}.

Measurements of citrate synthase and antioxidant enzyme activities

To analyse the activity of the mitochondrial marker enzyme citrate synthase (CS; EC 4.1.3.7) approximately 100 mg of frozen tissue was homogenized in liquid nitrogen and diluted 1:7 (w:v) with 75 mmol l^{-1} Tris-HCl buffer that contained 1 mmol l^{-1} EDTA at pH 7.6 at 20°C. Samples were homogenized with an Ultraturrax T8 homogenizer (IKA Labortechnik, Staufen, Germany) and sonicated for 5 min at 0°C using ultrasound. After 10 min centrifugation at 12 000 ***g*** at 0°C, CS activity was determined in the supernatant according to the protocol of Sidell et al. (1987) at 412 nm at 20°C and the respective habitat temperatures (1°C, winter animals; 10°C, summer animals). The assay system contained 75 mmol l^{-1} Tris-HCl buffer [pH 8.0 at 20°C), 0.25 mmol l^{-1} DTNB ((5,5′-dithiobis(2-nitrobenzoic acid) = Ellmann's reagent], 0.6 mmol l^{-1} acetylCoA and 130 ml of supernatant (diluted with H_2O). The assay was started by addition of 40 μl 20 mmol l^{-1} oxaloacetate and the subsequent absorbance increase recorded. CS activity was calculated using the molar extinction coefficient ε=13.61 ml μmol^{-1} cm^{-1} of the DTNB-SH-CoA complex formed. Catalase (CAT; EC 1.11.1.6) was extracted into 50 mmol l^{-1} potassium phosphate buffer (pH 7.0 at 20°C, 1:11, w:v) and measured according to Aebi (1985). Superoxide dismutase (SOD; E.C. 1.15.1.1) activity in crude homogenates was measured according to Livingstone et al. (1992). The test uses the xanthine oxidase/xanthine system to generate superoxide anions at a rate that reduces cytochrome *c* with an absorbance slope of exactly 0.02 absorbance units min^{-1}. One unit of SOD activity in the sample reduces cytochrome *c* reduction by 50%.

Cell isolation and mitochondrial fluorescence staining

For cell isolations, a 50 mg piece of freshly sampled body wall tissue was rinsed thoroughly with filtered seawater and finely chopped on ice. To remove blood, the tissue pieces were gently washed with ice cold filtered seawater. Two digestive enzymes, 757 U hyaluronidase (Merck) and 10145 U trypsin (Sigma) were added, and allowed to stand overnight at 8°C in the dark. In the morning, digestion was continued for approx. 1 h at room temperature under constant shaking. Cells were filtered through gauze and left to settle at the bottom of the vial. 100 μl of 50% BSA (w:v) were added to quench the digestive enzyme activity. 15 μl of Mito-tracker Green solution (Molecular Probes, Leiden, The Netherlands; 50 μg dissolved in 740 μl dimethylsulfoxide) were added to the cell suspension to stain the mitochondria during 20 min of gentle shaking at room temperature.

Stained cells were kept on ice and mitochondrial counts performed with a confocal microscope (Leica IRBE; Bensheim, Germany) using the setup described in Abele et al. (2002). Cells from 3–5 animals were studied during one sampling and at least 10 cells were counted per animal. Mitochondria in cells from winter animals were counted during four samplings and from summer animals during two sampling events. Additionally, every second week, counts were performed in cells from winter animals acclimated to 10°C under experimental conditions to monitor the changes on long term exposure to higher temperatures.

Statistics

Data were tested for normality of distribution (Kolmogorov–Smirnov test) and homogeneity of variance (Levene test). Significant changes (P<0.05) in normally distributed data were evaluated by analysis of variance (ANOVA) and a Newman–Keuls *post hoc* test was employed to determine significant differences between data pairs. A Statistica version for Windows was employed for the statistics.

Results

Seasonal variability of mitochondrial energetics and ROS formation

Rates of mitochondrial respiration per mg of mitochondrial protein in respiratory states 3 and 4 without and with oligomycin (state 4+) are given in Fig. 2. Fig. 2A shows data from winter animals, and Fig. 2B from pre-spawning summer animals at two measuring temperatures: 10° (dark bars) and 1°C (hatched bars). Specific respiration of mitochondria was always higher in summer than in winter animals at both temperatures and in all states, with the exception of the very low state 4+ respiration in both seasonal groups at 1°C. Even at their habitat temperature, mitochondria of winter animals had lower respiration rates than those from summer animals, cooled to 1°C.

Respiratory coupling rate (RCR; state 3/state 4 respiration) was lower in summer animals at habitat temperature (10°C: 4.79±0.46, N=5) than in winter animals at both temperatures (10°C: 6.53±0.71, N=11; 1°C: 6.63±1.72, N=8) (P< 0.05). On cooling from 10° to 1°C, RCRs in summer animal mitochondria increased significantly to 6.32±1.32 (P<0.01, N=6). Addition of oligomycin decreased state 4 respiration by about 25% in each measurement, augmenting RCR_{Ol}, especially in summer animals at 1°C to 12.66±3.11 (N=5), whereas in winter animals RCR_{Ol} was 8.78±0.97 (N=10) at 10°C and 9.76±1.81 (N=8) at 1°C. Summer animals at 10°C had an RCR_{Ol} of 6.99±0.78 (N=6).

ADP/O ratios with sodium succinate were between 1.50 and 1.58 and did not differ among groups, with one exception: warming of winter animal mitochondria to 10°C resulted in lower ADP/O ratios (1.43±0.05, N=9, P<0.01) compared to summer animals at 10°C (1.58±0.09, N=7).

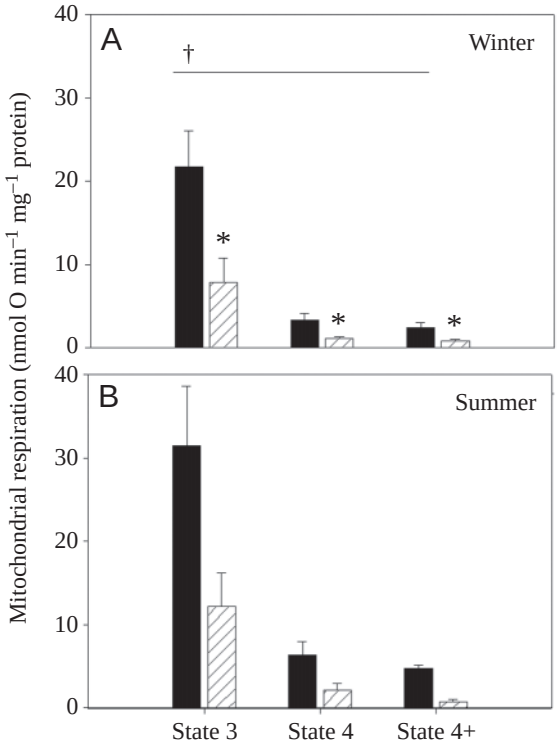

Fig. 2. Temperature dependence of mitochondrial respiration in body wall tissue of *Arenicola marina* winter (A) and pre-spawning summer (B) animals. Data are means ± S.D.; *N*=5–10 isolations. Black bars, 10°C; hatched bars, 1°C. All 10°C values differ significantly from the values at 1°C (*P*<0.001); *All 1°C values are significantly different from the 10°C measurement in summer animals (*P*<0.001); †significantly different from summer animals at the same experimental temperature and in the same respiratory state (*P*<0.014) (ANOVA; Newman–Keuls test).

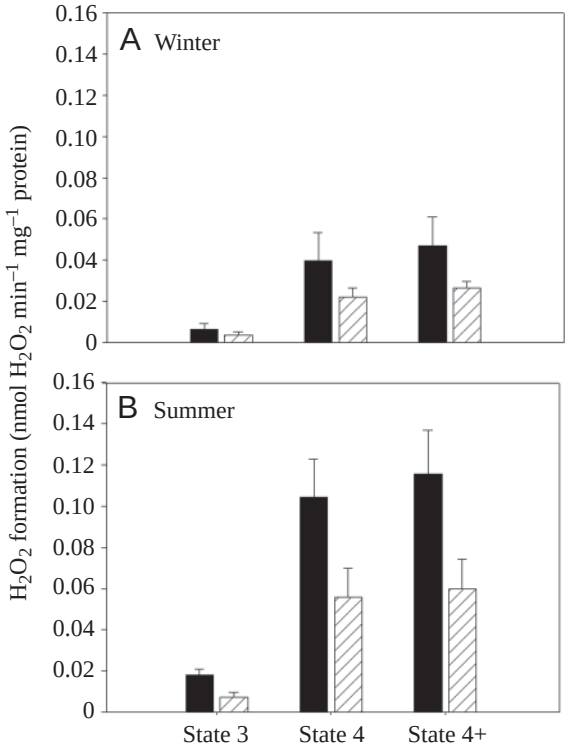

Fig. 3. Temperature dependence of mitochondrial H_2O_2 formation in body wall tissue of *Arenicola marina* winter (A) and summer (B) animals. Data are means ± S.D. of 5–10 mitochondrial isolations. Black bars, 10°C; hatched bars, 1°C. State 3 values were significantly lower in winter than in summer mitochondria at both temperatures; H_2O_2 formation at 10°C differs significantly from formation at 1°C (*P*<0.001), except of the 10°C value of the winter animals. State 4 and 4+ values at both temperatures differ significantly from state 3 (*P*<0.01) and with season (*P*<0.001), as well as between temperatures (*P*<0.01). (ANOVA; Newman–Keuls test).

ROS (i.e. H_2O_2) production relative to mitochondrial oxygen uptake (%-H_2O_2/O_2) could be calculated (Table 1), because both data sets were measured in the same isolate and were related to mitochondrial protein. Calculations are based on the stoichiometric approximation of 2 moles of oxygen being univalently reduced to 2 mol superoxide, to give 1 mol H_2O_2 in the reaction: $2O_2^{\bullet -} + 2H^+ \rightarrow 1H_2O_2 + 1O_2$. State 3 %-$H_2O_2/O_2$ was around 0.25% and lower in both seasonal groups at both temperatures, compared to the oxygen consumption due to ROS formation under non-phosphorylating state 4 conditions. In states 4 and oligomycin-induced 4+, summer animal mitochondria cooled to 1°C (*N*=5) reduced a maximal proportion of 23.07±8.14% of consumed oxygen to ROS. Although the absolute level of H_2O_2 formation per mg protein increased at high temperature (Fig. 3), warming generally reduced the proportion of O_2 uptake leading to H_2O_2 production in both seasonal groups (Table 1) and in all states.

The mitochondrial membrane potential ($\Delta\Psi$) was generally higher in summer than in winter animals in all respiratory states. At 10°C the state 4 $\Delta\Psi$ with nigericin was 179.3±7.3 mV (*N*=5) in summer and significantly higher than in winter animals (165.4±5.5 mV, *N*=10) (*P*<0.033). $\Delta\Psi$ correlated positively with respiration rates in states 3 and 4+ (Fig. 4). Likewise, rates of H_2O_2 production correlated with the

Table 1. *Percentage of ROS (H_2O_2) production of overall oxygen consumption in mitochondria isolated from body wall tissue of* Arenicola marina *(winter and summer animals)*

	H_2O_2 fraction (%O_2 consumption)			
	Winter animals		Summer animals	
State	At 1°C	At 10°C	At 1°C	At 10°C
3	0.14±0.05	0.11±0.05	0.26±0.12	0.24±0.07
4	6.99±1.17	4.38±1.21	12.09±4.86	6.69±0.70
4+	11.87±2.90	6.99±1.82	23.07±8.14	10.33±0.81

Values are means ± S.D.; *N*=5–6 isolations.

State 3 values of winter animals were significantly lower at both temperatures than in summer animals at 10°C (*P*<0.05); state 4 and 4+ values at both temperatures differ significantly from state 3 (*P*<0.001) and with season (*P*<0.01) as well as between temperatures (*P*<0.001); ANOVA; Newman–Keuls test).

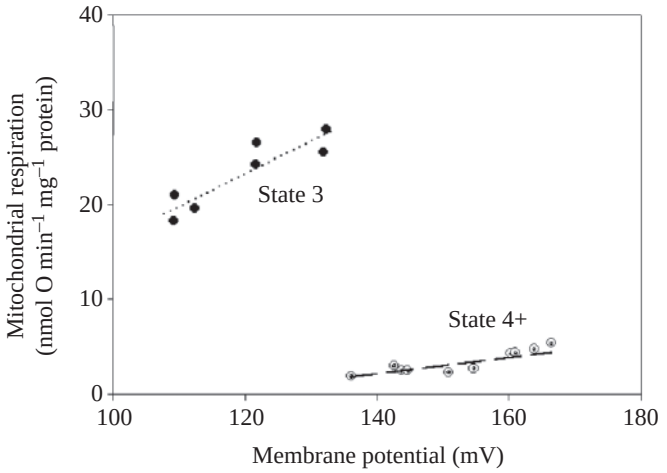

Fig. 4. Correlation between respiration rates in states 3 and 4+ and the membrane potential in states 3 and 4+ of body wall tissue mitochondria of *Arenicola marina* (data from winter and summer animals at 1 and 10°C), N=7–10; (ANOVA; Pearson correlation). Regression equations: MP (for state 3) *vs* $\dot{M}_{O_2}$: $f(x)=0.35x–18.38$ ($r^2=0.83$, $P<0.005$, $N=7$); MP (for state 4+) *vs* $\dot{M}_{O_2}$: $f(x)=0.09x–9.73$ ($r^2=0.71$, $P<0.001$, $N=10$), where MP = membrane potential and $\dot{M}_{O_2}$ = rate of oxygen production.

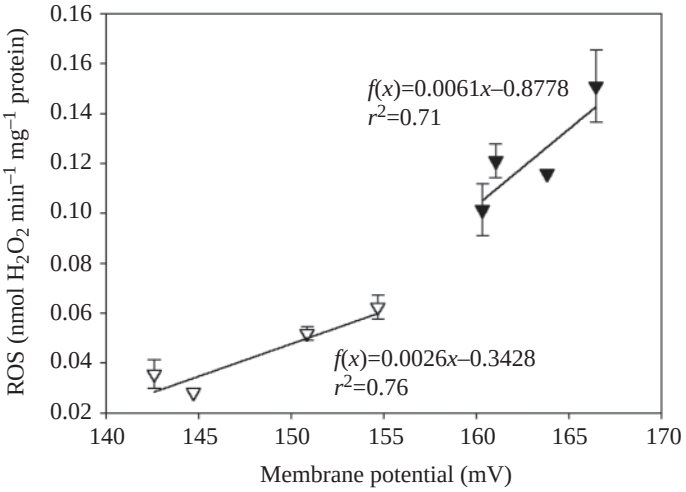

Fig. 5. H_2O_2 formation *vs* membrane potential in state 4+ respiration in mitochondrial isolates from *A. marina* winter (white triangles) and summer (black triangles) animals. Data are means ± S.D. from 1–3 isolations per point assayed at 10°C.

membrane potential ($\Delta\Psi$) in states 4 and 4+ (an example is shown for state 4+ in Fig. 5). Thus higher H_2O_2 production was found at higher $\Delta\Psi$, whereas the dependence of ROS formation on mitochondrial membrane potential was much steeper in summer than in winter animals.

Seasonal variability of enzyme activities in A. marina *body wall tissue*

Table 2 lists the activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase, as well as the mitochondrial marker enzyme citrate synthase (CS) in body wall tissue homogenates assayed at 20°C and habitat temperatures. Q_{10} values were close to 2 for SOD and CS and around 1.5 for catalase in both seasonal groups.

Seasonal variations of mitochondrial densities

Counts of Mito-tracker Green stained mitochondria in body wall cells from both seasonal groups displayed 1.6 times higher ($P<0.01$) mitochondrial densities in recently collected winter (68±7, N=14) *vs* (pre-spawning) summer animals (43±14, N=15, see Table 3). In line with this finding, the protein content of the mitochondrial isolates was 1.5 times higher in winter (2.6±0.8 mg protein ml^{-1} mitochondrial isolate) than in summer (1.7±0.4 mg protein ml^{-1} mitochondrial isolate). This indicates that an increase in mitochondrial densities might be a seasonal cold compensation mechanism in lugworms. Mean cell diameter and volume were slightly (5%) higher in summer animals, thus contributing somewhat to the lower mitochondrial density. During prolonged maintenance (4 weeks) of winter animals at 1°C under laboratory conditions, a decrease of mitochondrial numbers to 48±6 μm^{-3} of cell volume occurred, which was attributed to the prolonged maintenance and reduced burrowing activity of lugworms in the aquaria. Acclimation of winter animals at 10°C for at least 10 and up to 30 weeks did not result in significant changes of mitochondrial densities or cell size, although the very last value was somewhat lower.

Table 2. *Superoxide dismutase (SOD), catalase (CAT) and citrate synthase (CS) activity in body wall tissue of* Arenicola marina *assayed at habitat temperature and at 20°C in winter and summer animals*

	Enzyme activity			
	Winter animals		Summer animals	
	1°C	20°C	10°C	20°C
SOD	209.63±13.62 (8)	684.67±29.78 (8)	539.73±65.98 (6)	927.13±43.94 (6)
CAT	192.54±55.25 (6)	364.36±58.34 (6)	383.16±42.33 (6)	687.25±105.38 (6)
CS	0.58±0.18 (5)	2.64±0.55 (5)	1.13±0.16 (6)	2.24±0.19 (6)

Values are U g^{-1} fresh mass (N).

All enzymes showed a significant dependence on temperature ($P<0.001$). Additionally, summer animals had significantly higher activities than winter animals, when compared at habitat temperature and 20°C, with the one exception of CS (20°C). Data are means ± S.D.; N=5–8; ANOVA; Newman–Keuls test.

Table 3. *Seasonal variations of mitochondrial densities in a standardized cell volume of 1 μm³ in body wall tissue of* Arenicola marina*; winter animals maintained at 1°C, winter animals acclimated at 10°C and summer animals maintained at 10°C*

Mitochondria per mean cell volume (1 μm³)		
Winter animals	Acclimated winter animals	Summer animals
68±7*	48±11 (12)	43±14
48±6 (14)	47±15 (14)	44±12 (4)
42±10 (18)	53±10 (18)	
48±9 (22)	50±14 (22)	
	55±14 (27)	
	44±15 (31)	

Data are means ± S.D.; *N*=7–27 cells from three different animals. Numbers in brackets indicate weeks of maintenance at habitat temperature or acclimation temperature (10°C).

*Significantly different from all other values (P<0.027); Pearson correlation: P<0.001. ANOVA; Newman–Keuls test.

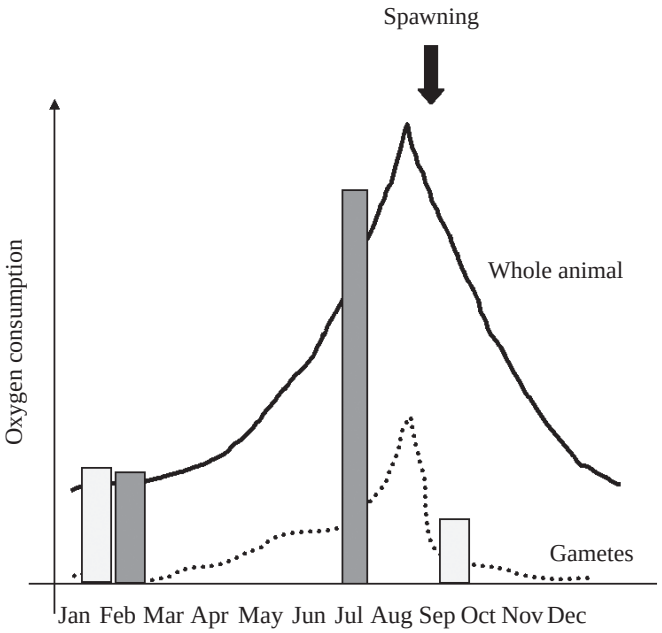

Fig. 6. Seasonal development of oxygen consumption of *A. marina* whole animals (solid line) and gametes (dotted line) adapted from Schöttler (1989). Bars depict oxygen consumption of mitochondrial isolated from winter and summer animals collected from the German Wadden Sea (dark bars) in this study and (light bars) by Sommer and Pörtner (2004). Scaling of the data is the same for all mitochondrial isolates (nmol O mg min^{-1} protein^{-1}), but distinct (μmol O_2 h^{-1} g^{-1} fresh mass) for whole animal oxygen consumption (solid line) and coelomic fluid (gamete oxygen consumption, dotted line; after Schöttler, 1989) (μmol O_2 h^{-1} g fresh mass^{-1}).

Discussion

Seasonal adjustments of mitochondrial functions in A. marina

Standard metabolic rates of female lugworms exhibit a seasonal pattern (Schöttler, 1989) with oxygen consumption beginning to rise in March, reaching a maximum in September (2.4 μmol O_2 g^{-1} fresh mass h^{-1}) when the lugworms spawn (see also Fig. 6, solid line). By November oxygen uptake is already down to winter rates. Although the ripening oocytes make up to 30% of the animals' overall oxygen consumption (0.8 μmol O_2 g^{-1} fresh mass h^{-1}; dotted line in Fig. 6) before the spawning event in September, the metabolic activity of the summer animal itself is still over 50% higher than in winter. Together, the need to cover the oxygen demand of gametes and the elevated summer temperatures is likely to explain the rise in metabolic performance of the whole animal and its ventilatory organ, the body wall musculature. So, how does the lugworm modify its energy production in order to fuel such an increase in metabolic performance in summer, and how does it protect its cells against the noxious side effect of higher

Table 4. *Proportion of oxygen consumed by the mitochondrial proton* leak[a] *in state 4+* Arenicola marina *summer and winter animals at experimental temperatures 1 and 10°C*

			Oxygen consumed (%)	
	Membrane potential (mV)		1°C	10°C
Winter animals	147±6	*Leak*	9.89±2.01 (6)	11.21±0.85* (6)
		ROS	0.61±0.16 (6)	0.40±0.08 (6)
		Total	10.64±1.87 (8)	11.36±1.43 (11)
Summer animals				
	161±4	*Leak*	6.46±1.77 (6)	13.78±1.44* (6)
		ROS	1.04±0.25 (6)	0.75±0.13 (6)
		Total	7.50±1.68 (6)	14.51±1.42* (6)

[a]State 4+ $\dot{M}_{O_2}$ corrected for H_2O_2 formation in % state 3 $\dot{M}_{O_2}$.

'Total' does not exactly correspond to the sum of the *leak* and H_2O_2 fractions, because it was calculated directly from the oxygen consumption data.

Values are means ± S.D. of 6–11 isolations.

All values differ significantly between summer and winter animals with the exception of the leak at 1°C (P<0.01); *significantly different to lower temperature value (P<0.001). ANOVA; Newman–Keuls test.

mitochondrial respiration, the elevated production of reactive oxygen species?

We found lower mitochondrial densities in body wall cells from summer compared with winter animals. However, *in vitro* rates of protein-specific mitochondrial oxygen consumption more than 3 times higher than winter rates can easily overcompensate for the lower mitochondrial density in summer (Fig. 6, dark bars). Isolated mitochondria can only serve as a model of the *in vivo* situation and the effect may not be as pronounced under cellular conditions where the ATP/ADP ratios are much closer to state 4 and rarely resemble fully ADP-saturated state 3 conditions (Guderley, 1998). However, the elevated metabolic activity in the pre-spawning animals from July can be traced down to the elevated oxygen consumption rates of isolated mitochondria. Mitochondria of post-spawning worms from late summer (September) isolated by Sommer and Pörtner (2004) clearly displayed lower protein-specific respiration rates, in keeping with the lower oxygen uptake of the whole animal at that time of the year (Fig. 6; after Schöttler, 1989).

Table 4 illustrates that higher mitochondrial respiration rates go hand in hand with higher proton motive force in isolated mitochondria from pre-spawning summer animals compared to mitochondria from winter lugworms. As a consequence, summer mitochondria produce a higher proportion of ROS, especially in state 4+ (Fig. 5). As sudden warming can induce oxidative stress in marine ectotherms (Abele et al., 1998b, 2002), and environmental conditions in intertidal environments are more changeable in summer than in winter, lower mitochondrial density is likely to reduce the risk of damage from mitochondria-borne ROS in pre-spawning summer animals. Additionally, the higher leak in isolated mitochondria from summer (10°C, see Table 4) compared to winter animals could balance ROS formation by a mild uncoupling of the proton motive force. These preventive antioxidant mechanisms might be crucial under high energy requirements, as the animals face reproduction and high temperatures. Nevertheless, absolute ROS production rates in isolated mitochondria from pre-spawning summer animals were fourfold higher (0.12 ± 0.02 nmol H_2O_2 min^{-1} mg^{-1} protein) than those from winter specimens (0.03 ± 0.003 nmol H_2O_2 min^{-1} mg^{-1} protein) and explain the significantly higher antioxidant activities we measured in *Arenicola* body wall tissue in July (Table 2). We conjecture that in winter, when habitat temperatures are low and less variable and worms are less active, a smaller mitochondrial membrane leak sustains constant and high phosphorylation efficiency at minimal risk of causing an increase of the percentage conversion of oxygen to ROS, due to the low membrane potential. Given the low metabolic rates of winter animals this is obviously tolerable, even taking into account the worms' already low antioxidant enzyme activities, further hampered by the Q_{10}-dependent thermal slow down.

Comparatively low proton leak rates in A. marina *body wall mitochondria*

According to Brand (2000), between 15 and 35% of state 3 oxygen consumption drive the proton leak in cells from ectothermal and endothermal vertebrates so far studied. We have previously reported that marine mud clams also fall into this range (Abele et al., 2002; Heise et al., 2003). However, in *Arenicola* mitochondria the percentage rate of oligomycin-saturated state 4+ respiration ranged far lower and, at 10°C, amounted for only 11–14.5% of state 3 oxygen consumption (Table 4). Mitochondrial oxygen consumption makes up only 80% of overall cellular respiration, a surplus of 20% being extra mitochondrially consumed (Brand et al., 1994a). Therefore, only approximately 10% of the cellular oxygen uptake actually drives the proton leak in lugworms. Similarly low values (<10% of state 3 respiration) were found in mitochondria isolated from the body wall tissue of a sipunculid worm (Buchner et al., 2001). So, what makes these tissues different from others?

The vast majority of data in proton leak research have been obtained in vertebrate studies. Most vertebrate tissues respond to various environmental or internal stimuli with high flexibility of muscular and general metabolic activity. High long-chain polyunsaturated fatty acid content in mammalian mitochondrial membranes is thought to enable a higher proton leak rate and, in part, is held responsible for the higher metabolic rates and heat production in endotherms, compared with ectotherms (Brand et al., 1994b; Brookes et al., 1998). In contrast, body wall tissue of marine infaunal worms displays low and, moreover, strictly oxyconforming metabolic rates. Although *Arenicola marina* possesses gills, 50% of its overall oxygen uptake is directly cutaneous (Mangum, 1976). P_{O_2} in the burrow environment is around 100 Torr (14 kPa; Mangum, 1976), and pumping activity is oxyconforming, limiting metabolic and motoric flexibility in response to other environmental stimuli.

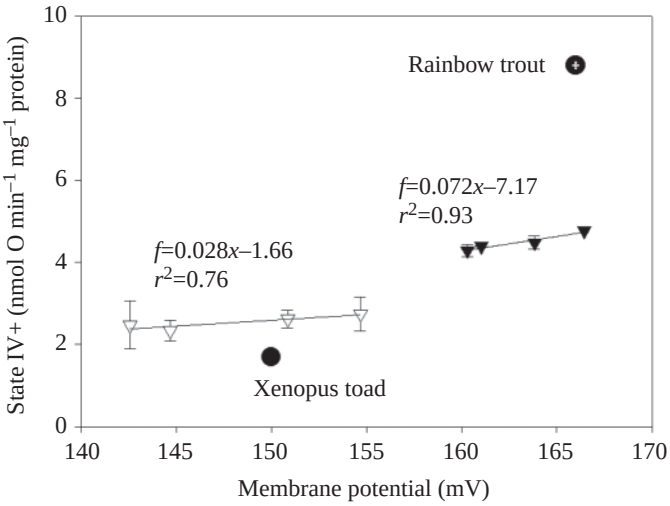

Fig. 7. State 4+ respiration *vs* membrane potential in state 4+ in mitochondrial isolates from *A. marina* winter (white triangles) and summer animals (black triangles). Data are means ± S.D. from 1–3 isolations per point assayed at 10°C. Circles are data from Brookes et al. (1998) for *Xenopus* toad and rainbow trout.

Fig. 7 describes the dependence of state 4+ substrate oxidation rates on mitochondrial membrane potential in both seasonal groups of lugworms and compares values for *Xenopus* toad and rainbow trout, taken from Brookes et al. (1998). At the same membrane potential, lugworms display lower state 4+ respiration rates than trout, suggesting that both leak and substrate oxidation rates are reduced in worms, compared to a comparatively active fish. In winter lugworm mitochondria, the balance between substrate oxidation and membrane potential is close to the conditions found in toad, a relatively slow amphibian. A high proton leak enables a tissue to perform quick adjustments in response to sudden changes of energy requirements by reducing the leak to power phosphorylation, or by increasing the leak to prevent overflow of ROS production (St-Pierre et al., 2000). Body wall tissues of marine worms may not need this flexibility to the same extent as more active vertebrates, since they rely on sustained slow rates of motor activity for ventilatory purposes. This goes along with high RCRs in *A. marina* mitochondria. Under unstressed habitat conditions, the cells of lugworms are not exposed to sudden oxygen deprivation and subsequent oxygen flushing. Metabolic adjustments in these tissues are slow and function on a time scale of hours and upwards. However, the present and earlier (Sommer and Pörtner, 2004) experiments indicate that on exposure to acute temperature stress, when oxygen transport to the tissue ceases (low temperatures), or cannot further compensate for the high metabolic expenditures (heat stress), the changes of the proton leak rate, however small, can still confer some metabolic flexibility and moderate antioxidant function in these slow moving animals.

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