Mitochondrial function and critical temperature in the Antarctic bivalve, *Laternula elliptica*

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Abstract

Thermal sensitivities of maximum respiration and proton leakage were compared in gill mitochondria of the Antarctic bivalve *Laternula elliptica* for an assessment of the contribution of mitochondrial mechanisms to limiting temperature tolerance. Proton leakage was measured as the oxygen consumption rate during blockage of oxidative phosphorylation (state IV respiration + oligomycin). The maximum capacity of NADP dependent mitochondrial isocitrate dehydrogenase (IDH) was investigated as part of a proposed mitochondrial substrate cycle provoking proton leakage by the action of transhydrogenase. State III and IV + respiration rose exponentially with temperature. Thermal sensitivities of proton leakage and IDH were unusually high, in accordance with the hypothesis that H^+ leakage is an enzyme catalysed process with IDH being involved. In contrast to proton leakage, state III respiration exhibited an Arrhenius break temperature at 9°C, visible as a drop in thermal sensitivity close to, but still above the critical temperature of the species (3–6°C). Progressive uncoupling of mitochondria led to a drop in RCR values and P/O ratios at high temperature. The same discontinuity as for state III respiration was found for the activity of mitochondrial IDH suggesting that this enzyme may influence the thermal control of mitochondrial respiration. In general, the high thermal sensitivity of proton leakage may cause an excessive rise in mitochondrial oxygen demand and a decreased efficiency of oxidative phosphorylation. This may exceed the whole animal capacity of oxygen uptake and distribution by ventilation and circulation and set a thermal limit, characterized by the transition to anaerobic metabolism. © 1999 Elsevier Science Inc. All rights reserved.

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1. Introduction

Critical temperature thresholds (T_c) have been defined for various marine invertebrate species and fish as being characterized by the transition to an anaerobic mode of metabolism, once temperature reaches low or high extremes ([44,47,53]; for review see [38,39]). A unidirectional shift of both T_c values occurs with latitude and seasonal temperature adaptation. These thresholds characterize the geographical distribution of a species. The within species comparison of temperate and subpolar populations suggests that a shift in both low and high T_c is associated with a change in mito-

chondrial density, which drops as the temperature rises and increases as the ambient temperature falls [45]. This finding strongly suggests that the T_c values are set by a mismatch of mitochondrial oxygen supply and demand [38,39].

A special situation may have developed in polar areas, especially the Antarctic where the marine fauna is constantly exposed to low temperatures between −1.9 and +1°C. Considering that life in warm waters reflects the likely original evolutionary situation [2] organisms inhabiting Antarctic oceans must have developed special physiological adaptations to overcome the adverse effects of cold temperatures on metabolism. In Antarctic fish a compensatory increase in whole animal aerobic capacity is reflected in higher mitochondrial densities [18] and the 1.5–5-fold rise in the capacity of

some oxidative enzymes [14] despite low resting metabolic rates [13]. Compared to temperate species the drop in the low T_c to below polar temperatures on evolutionary time scales is also linked to a drop in the upper T_c and a narrowing of the tolerated temperature window [38,39].

In conclusion, mitochondrial performance is a characteristic element in adaptation to temperature and its limitations. The question arises which features of mitochondrial function determine the low and the high T_c. Failure of oxidative enzymes has been discussed to limit resistance to high temperatures [36]. According to a previous hypothesis the falling rates of oxygen consumption below a low T_c reflect insufficient aerobic capacity unable to meet energy requirements [53], whereas beyond a high critical temperature mitochondrial oxygen demand may become excessive and may no longer be met by oxygen supply via ventilation and circulation [38,39]. It is important in this context that mitochondrial oxygen demand is not only defined by the rate of ADP phosphorylation depending on energy demand but also by the rate of proton leakage which covers a significant fraction of standard metabolic rate (SMR) [5]. Proton leakage and the associated metabolic activity can be interpreted to reflect part of the cost of mitochondrial maintenance [38,39].

As part of a larger effort to address the effect of temperature on mitochondrial functions in fish and marine invertebrates this study was designed to investigate mitochondrial respiration in gill mitochondria of a stenothermal Antarctic organism, the benthic infauna bivalve *Laternula elliptica*. *L. elliptica* is a widely distributed, large Antarctic bivalve burrowing up to 50 cm deep in soft, muddy sediments [24]. It occupies a similar ecological niche as the genus *Mya* in the northern hemisphere [40].

Temperature effects on oxygen consumption and on the efficiency of coupling electron transport to ATP production were studied in order to determine when high temperatures possibly disrupt mitochondrial function. Mitochondrial NADP dependent isocitrate dehydrogenase was included in this investigation. Study of this enzyme was particularly promising since its isozyme pattern changed in the body wall of *Arenicola marina* in a latitudinal gradient and was linearly correlated with mean ambient temperature [27]. Recently, this enzyme was suggested to adopt a key role in a mitochondrial substrate cycle which is likely to be relevant in the control of mitochondrial respiration and proton leakage [39,42]. In parallel experiments the T_c of *L. elliptica* was determined to lie between 3 and 6°C as indicated by the onset of anaerobic metabolism (Pörtner et al., in preparation).

2. Material and methods

2.1. Animals

Antarctic clams, *Laternula elliptica*, were collected by scuba diving on the North side of Rothera point, Adelaide Island, Antarctica (67 34′S, 68 07′W), at a depth of 25–33 m in December 1997. A total of 22 clams with a mean tissue wet weight of 60.1 ± 13.4 g (equivalent to 113.1 g whole animal wet weight) were kept in aquaria with continously running seawater at ambient temperature for at least 1 week prior to experimentation. Experiments were conducted at Rothera Station (British Antarctic Survey, Antarctica).

2.2. Preparation of mitochondria

Large clams were removed from the aquaria and opened by cutting the adductor muscle and mantle edge. Approximately 2–3 g of gill tissue were quickly removed and chopped finely by using scissors in a petri dish containing 3–4 ml of ice-cold isolation buffer modified after Moyes et al. [33]: 70 mM Hepes; 100 mM KCl, 400 mM sucrose, 3 mM EDTA, 6 mM EGTA, 1% BSA, 1 µg ml^{-1} aprotinin, pH 7.3 at 20°C). Extraction occurred in 30 ml of the same buffer by use of a Potter-Elvejhem homogenizer. The tissue was dispersed by three passes of a loosely fitting pestle. After centrifugation (10 min at $1250 \times g$) the pellet was rehomogenized in 30 ml of isolation buffer and centrifuged again. The combined supernatants were spun at $10\,500 \times g$ for 10 min. The mitochondrial pellet was resuspended in 2–2.5 ml of assay medium (70 mM Hepes, 100 mM KCl, 560 mM sucrose, 10 mM KH$_2$PO$_4$, 1 µg ml^{-1} aprotinin, 5 mM glutamate, pH 7.3 at 20°C) with 1% BSA yielding 11–17 mg mitochondrial protein ml^{-1}. $\Delta pH/\Delta T$ of the assay medium was -0.014 units °C^{-1}. Mitochondrial preparations remained stable for at least 8 h, as verified by repeated analysis of respiratory performance at 0°C.

2.3. Mitochondrial respiration

Oxygen consumption was measured at different temperatures using a Clarke-type oxygen electrode in a thermostatted respiration chamber (Eschweiler, Kiel). A total of 100 (sometimes 200) µl of the mitochondrial suspension were combined with assay medium to a total volume of 1 ml containing 1.1–1.7 (sometimes 2.2–3.4) mg mitochondrial protein ml^{-1}, 5 µM diadenosine pentaphosphate (AP$_5$A), an inhibitor of myokinase as well as 20 mM glutamate and 0.1 mM pyruvate as substrates [32]. State III respiration was recorded after the addition of ADP to a level of 0.3 mM. After all ADP had been phosphorylated state IV respiration was determined. Finally, the respiration rate

induced by proton leakage was recorded after adding oligomycin, an inhibitor of mitochondrial F_0F_1-ATP synthase, to a concentration of 2.8 μg ml^{-1}. Repeated addition of oligomycin verified the suitability of this concentration for a maximum effect. Respiration under oligomycin remained constant for a minimum period of 15 min before it tended to rise, possibly caused by a progressive increase in membrane potential during inhibition of the ATP synthase (see below).

For comparison with the classical P/O ratios determined according to Chance and Williams [12] effective P/O ratios of oxidative phosphorylation were analysed by direct investigation of ATP production during state III respiration. Samples of 10 μl assay medium were repeatedly withdrawn from the respiration chamber after the onset of state III respiration and then after P_{O_2} decrements of at least 10 mm Hg. Samples were diluted in 990 μl of buffer solution (0.2 M Tris–acetate pH 7.75, 1 mM EDTA) and heated briefly to 95°C in a water bath for the denaturation of mitochondria. ATP concentrations were determined luminometrically with an ATP assay kit (BioOrbit, Finland) following the rationale of Wibom et al. [50]. Effective P/O ratios were calculated as ATP production rates divided by the rate of oxygen consumption monitored in the same assay.

For the analysis of a potential oxygen dependence of P/O ratios respiration was started by the addition of ADP to levels in the assay ranging up to 0.9 mM. ATP samples were withdrawn repeatedly over the time course of the experiment until state IV respiration was reached. Spontaneous ATP depletion and a potential involvement of an ATPase in this process [31] were investigated by monitoring ATP levels during state IV respiration. After ATP levels had been demonstrated to remain more or less constant, antimycin (5 μM for an inhibition close to complex II of the respiratory chain), rotenone (8 μM for an inhibition of complex I) and sodium azide (2 mM) were added prior to or after the addition of oligomycin. These analyses were carried out at 3°C when the sensitivity of mitochondrial assays was improved over the measurement at 0°C and the quality of mitochondria not yet compromised by temperature effects.

Oxygen solubility in the assay medium at different temperatures was taken from Johnston et al. [28]. Protein concentration of the mitochondrial pellet was determined by the Biuret method, after adding 5% deoxycholate to the mitochondrial suspension for the solubilization of membrane proteins.

2.4. Enzyme assay

NADP$^+$-dependent isocitrate dehydrogenase activity was analysed in isolated mitochondria after their lysation by the addition of 5% deoxycholate. The assay contained 70 mM Tris–HCl pH 7.1, 8 mM MgSO$_4$, 2.5 mM NADP$^+$, 1 mM MnCl$_2$, 3 mM D/L-isocitrate and 20 mM citrate (modified after Alp et al. [1]). Enzyme activity was measured following the appearance of NADPH at 339 nm in a thermostatted spectrophotometer at 0, 3, 4.5, 9, 15, 21, 25 and 30°C. The temperature range chosen and variations in substrate levels ensured that the enzyme always operated at saturating substrate levels. This was confirmed by the extreme rise in enzyme activity with temperature which would be prevented by substrate limitations.

2.5. Calculation of ABTs and statistical analyses

All values are given as means ± SD unless indicated otherwise, $N = 7$ for mitochondrial respiration experiments and $N = 4$ for enzyme activities. Data were plotted as a function of temperature. ABTs were calculated by applying two-phase regressions, which compared sequential linear or 2nd order polynomial fits. Two intersecting lines were selected that fitted the data best according to the method of least squares following the rationale of Yeager and Ultsch [35,48]. Differences between rates in respiratory states III, IV and IV + oligomycin were tested for significance by Student's t-test for paired samples. Statistical significance of changes in RCR, Arrhenius activation energy, Q_{10} values, P/O ratios and ATP degradation with temperature or time was evaluated at the $P \leq 0.05$ level using analysis of variance (ANOVA) and of covariance (ANCOVA) performing contrasts for means comparisons (Super-Anova and StatView II, Abacus Concepts).

3. Results

3.1. Quantification of mitochondrial functions

Gill mitochondria of *L. elliptica* were highly coupled at 0°C with a respiratory control ratio (RCR +) of 5.74 ± 1.36 (Fig. 1). This RCR + ratio was calculated as the ratio of state III and state IV respiration under oligomycin (state IV +). With a value of 3.78 ± 1.60 the traditional RCR was only slightly lower and within the range of RCR values reported for other bivalve gill mitochondria [3,10,11]. RCR remained more or less constant between 0 and 9°C and fell progressively and significantly at higher temperatures. Maximal oxidative capacity in the presence of glutamate and pyruvate was 2.12 ± 0.67 nmol O min^{-1} mg^{-1} mitochondrial protein at 0°C.

Residual respiration under oligomycin is assumed to quantify oxygen consumption due to proton leakage through the inner mitochondrial membrane. Leakage reached a rate equivalent to 18.1 ± 3.7% of state III respiration at 0°C (Fig. 1), evaluated from an initially stable reading before state IV + respiration started to

rise 10–15 min after the addition of oligomycin (not shown). Owing to its dependence on proton motive force, the actual percent fraction of proton leakage in phosphorylating state III mitochondria will be lower since proton motive force in state III is below the one under state IV conditions (see Section 4).

Spontaneous ATP depletion was small and non-significant under the effect of oligomycin, even at high phosphorylation potential (Fig. 2A). It occurred at a somewhat, but not significantly larger rate when antimycin, rotenone and sodium azide were added after oligomycin (Fig. 2A). Significant ATP depletion under antimycin, rotenone and sodium azide was not eliminated when oligomycin was added (Fig. 2B) reflecting a more complete inhibition of ATP synthesis by the other inhibitors than with oligomycin alone.
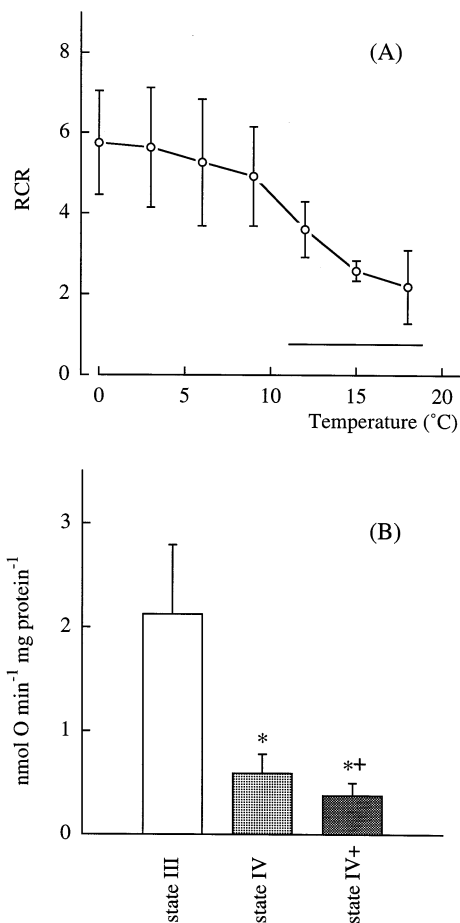

Fig. 1. (A) Respiratory coupling ratios determined from state III respiration and respiration under oligomycin (RCR +) of isolated *L. elliptica* gill mitochondria. A horizontal line indicates onset and maintenance of a significant difference from RCR values at lower temperatures. (B) Rates of state III and state IV respiration of gill mitochondria from *L. elliptica* at 0°C compared to state IV respiration under oligomycin (state IV +). State IV + respiration is suggested to largely comprise proton leakage. Mitochondria respired on 20 mM glutamate and 0.1 mM pyruvate as substrates. * + , indicate significant differences of respiratory rates from the rate observed under state III (*) as well as from the rate under state IV (+).
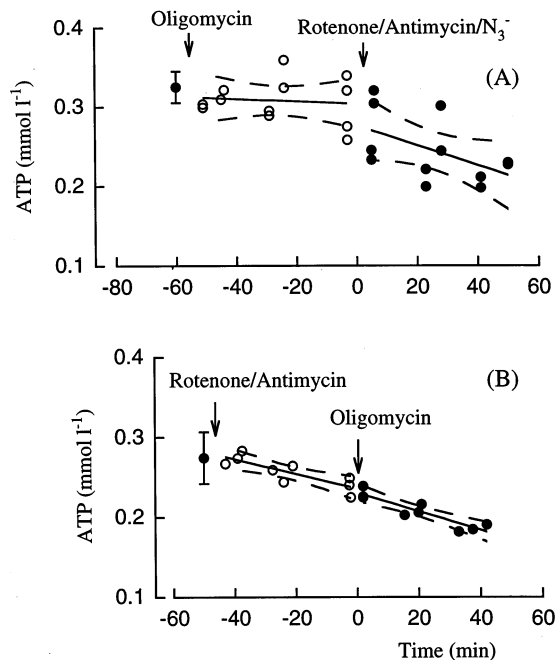

Fig. 2. Maintenance or degradation of ATP accumulated during state III respiration at 3°C (A) during exposure to oligomycin, followed by a combined effect of rotenon, antimycin and sodium azide, (B) during exposure to rotenon/antimycin and then oligomycin. Significant ATP degradation depended on the action of rotenon and antimycin leading to the conclusion that reversal of mitochondrial F_0F_1-ATP synthase was not contributing to ATP depletion.

3.2. Thermal sensitivity of mitochondrial respiration

State III respiration increased exponentially with rising temperature between 0 and 9°C, after which no further increase was seen. The difference between respiratory states III and IV + oligomycin also rose exponentially to 9°C but declined above this temperature (Fig. 3). This difference is seen as a conservative measure of phosphorylation dependent respiration considering that proton leakage is likely to be smaller under state III than state IV conditions. States IV and IV + respiration continued to rise more or less exponentially within the temperature range investigated. In consequence, the drop in states III–IV + respiration not only reveals a reduction of phosphorylation at temperatures above 9°C but also that the continued rise in proton leakage accounts for a larger fraction of mitochondrial respiration.

Arrhenius plots of respiration rates still showed a slight exponential rise for state III respiration in the low temperature range (Fig. 4A) reflected by a significant increase in Q_{10} values and also in Arrhenius activation energies with rising temperature (Fig. 5). The progressive rise in the temperature dependence of state

III respiration beyond 3°C led to a more or less constant RCR value (Fig. 1) until an Arrhenius break temperature (ABT) was reached, visible as a sharp drop in the thermal sensitivity of state III respiration (Fig. 3). This indicates loss of protein function with apparent activation energies reaching negative values for the states III–IV + difference. This pattern is also reflected in the modification of overall Q_{10} values which rose between 0 and 9°C but dropped sharply beyond 9°C (Fig. 5B).

With an average activation energy of 79 kJ mol^{-1} and a Q_{10} value of 4.2 the temperature dependence of state IV + respiration was similar to that of state IV (cf. Fig. 4B). At temperatures above 9°C Q_{10} values were much higher for state IV than for state III rates, thereby leading to progressive uncoupling of mitochondria visible as a drop in RCR + values (state III/state IV + oligomycin) (Fig. 1A).

Determination of classical and effective P/O ratios yielded numbers between 1.5 and 2 for both. Effective P/O ratios, which in contrast to classical values were mostly determined at low phosphorylation potential (low [ATP]/[ADP][P$_i$] ratio), fell significantly at high
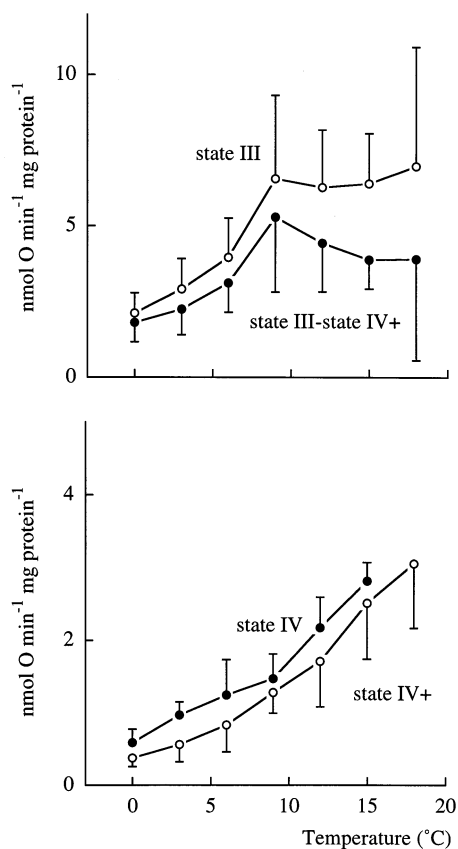

Fig. 3. Temperature dependence of (A) the rates of state III respiration (open symbols) and the difference between the rates of state III and state IV + respiration (closed symbols) and (B) the rates of state IV (closed symbols) and state IV + respiration (open symbols) of gill mitochondria from *L. elliptica*.
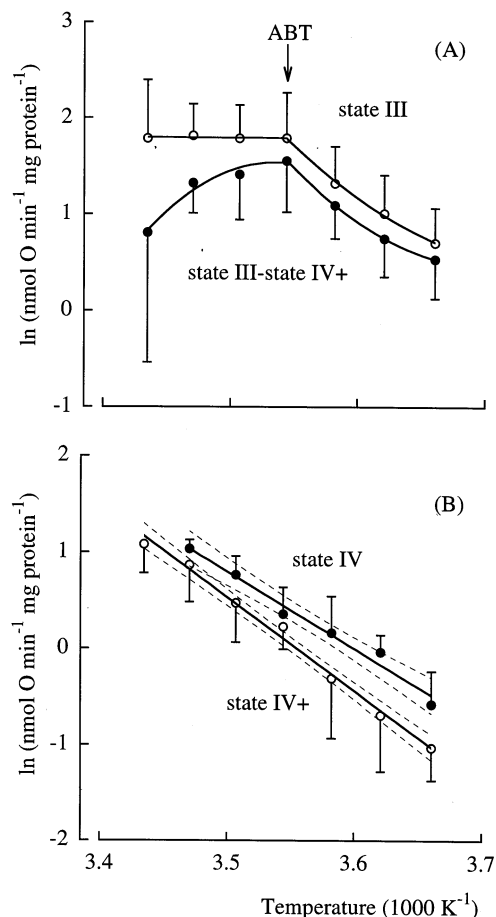

Fig. 4. Arrhenius plots of (A) state III respiration and the difference between the rates of state III and state IV + respiration as well as (B) state IV and state IV + respiration (see Fig. 3). The Arrhenius break temperature (ABT) results as the intersection of linear ($r = 0.16$ above ABT) and second order polynomial regressions ($r = 1.0$ below ABT) for state III or as the intersection of two second order polynomial regressions for state III–IV + ($r = 0.98$ above ABT; $r = 1.0$ below ABT). Continuous linear regressions for respiratory states IV ($r = 0.99$) and state IV + oligomycin ($r = 1.0$) mirror the non-existence of significant discontinuities in both states. Dashed lines indicate the 95% confidence interval.

temperatures (Fig. 6B). The P_{O_2} dependence of the P/O ratio appears negligible as indicated by Fig. 5C and excludes a strong influence of an alternative oxidase on the determination of P/O ratios [37].

3.3. NADP$^+$-dependent isocitrate dehydrogenase

NADP$^+$-dependent isocitrate dehydrogenase was determined in isolated mitochondria and showed a specific activity of 2.39 ± 1.13 mU mg^{-1} mitochondrial protein or 22.9 ± 4.6 mU g^{-1} fresh mass in gill tissue at 0°C and 99.34 ± 30.20 mU mg^{-1} mitochondrial protein or 818.1 ± 98.7 mU g^{-1} fresh mass at 25°C. Enzyme activity increased with assay temperature with a mean Q_{10} of 9.9 between 0 and 9°C, which is significantly higher than the Q_{10} of mitochondrial state III respira-

tion between 0 and 3°C but similar to the mitochondrial values found at higher temperatures (Fig. 5). The Arrhenius plot shows a break at 9°C above which enzyme activity increased less steeply with temperature (Fig. 7). The Arrhenius activation energy in the low temperature range, between 0 and 9°C was 138.4 kJ mol^{-1} and dropped to a value of 67.5 kJ mol^{-1} above 9°C.

4. Discussion

4.1. Analysis of mitochondrial function

In a trade-off between the preservation of mitochondrial integrity and the purity of the preparation a low number of centrifugation steps and high level of BSA led to high RCR of the mitochondrial preparations in this and a companion study on Antarctic fish mitochondria [23]. This was considered essential for valid conclu-
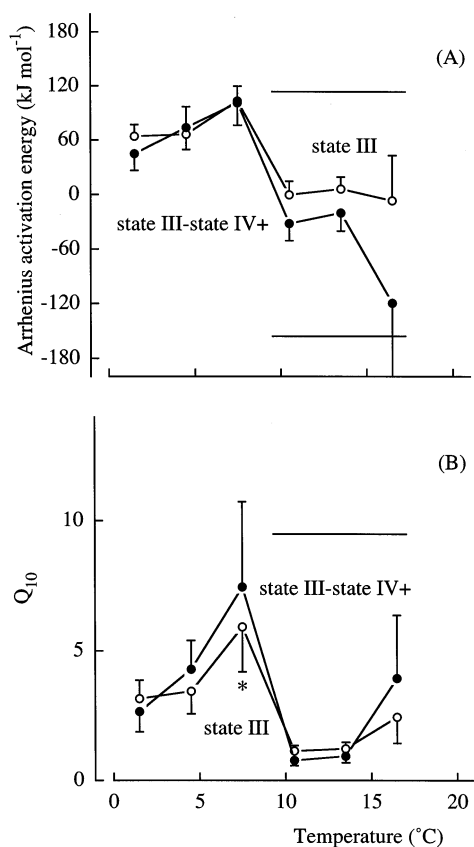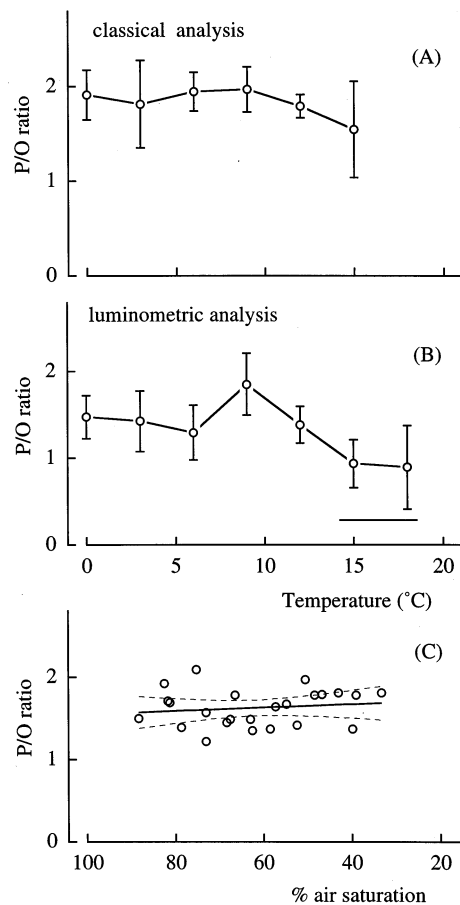
Fig. 6. Dependence of P/O ratios in mitochondria from *L. elliptica* on temperature and oxygen (% air saturation) (A) classical P/O ratios = based on graphical analysis of the transition between state III and state IV respiration, (B) effective P/O ratios = based on luminometric ATP assay and oxygen consumption analyses, (C) P/O ratios as a function of oxygen. A horizontal line denotes onset and maintenance of a significant difference from values grouped for lower temperatures. Dashed lines indicate the 95% confidence interval.

sions concerning the temperature dependencies of state III and proton leakage. ADP generation by adenylate kinase (e.g. [4,30]) might influence state IV and was excluded by the presence of diadenosine pentaphosphate (AP$_5$A) and the elimination of Mg^{2+}.

Any difference between states IV and IV + oligomycin is suggested to indicate ATP turnover by mitochondrial ATPases [31]. Accordingly, ATP degradation occurred under the effect of oligomycin and even more so during complete inhibition of the respiratory chain (Fig. 2) similar to results obtained in a study on rat liver mitochondria [31]. Mitochondrial F$_0$F$_1$-ATPase is not involved in ATP degradation since ATP depletion continued under oligomycin (Fig. 2B). Such ATP hydrolysis is possibly associated with the maintenance of non-H$^+$ ion gradients or with structural transitions [31]. The question arises why ATP depletion was less under oligomycin than with rotenone/antimycin/

Fig. 5. (A) Arrhenius activation energies and (B) Q_{10} values of state III respiration and the difference between the rates of state III and state IV + respiration for the 3°C temperature intervals depicted in Fig. 3. Data (means ± SE) are depicted for the respective mean temperature of the interval. A horizontal line indicates onset and maintenance of a significant difference from means grouped for lower temperatures (valid for both plots in B). *, indicates a significant rise of Q_{10} for state III respiration between 0 and 9°C.

azide. In general, oligomycin allows for substrate oxidation and respiratory activity associated with proton leakage. This includes substrate level phosphorylations in the citric acid cycle. In contrast, the other inhibitors will cut out all respiration and thereby proton leakage and all citric acid cycle activity. Respiration under oligomycin [31] therefore is a valid estimate of metabolism associated with proton leakage. The small rates of spontaneous ATP depletion seen excluded that a large fraction of unspecific ATP turnover and associated oxygen consumption occurred. At maximum phosphorylation potentials P/O ratios would be underestimated by $9.5 \pm 2.5\%$.

Mitochondrial proton leakage covers a significant fraction, namely 25–50% of SMR in both endo- and, very likely ectothermal organisms [7,9,41]. SMR including proton leakage will depend on aerobic scope (cf. [51]). Proton leakiness rises exponentially in the range of high proton motive force (Δp, [5,9]) and, therefore, is higher during state IV than state III respiration [5,17,41]. Rising ATP/ADP ratios cause Δp and thus proton

leakage to increase progressively leading to a decrease in P/O ratio (cf. [16]). Finally, state IV respiration will reflect a situation close to the resting cell, with mitochondria idling at minimal phosphorylation activity and constantly high phosphorylation potential.

Oligomycin caused Δp to rise in resting rat skeletal muscle by 10 ± 8 mV from 145 to 155 mV [41] indicating that leakiness might be overestimated in our data compared to the true resting situation. However, we conclude that this deviation was rather small: Oligomycin treatments led to similar state IV + rates regardless of whether it was added at low or high ratios of ATP/ADP. The slope of the rise in proton leakiness with Δp is small in most (vertebrate) ectotherms compared to endotherms [9]. Moreover, a potential increase in Δp under oligomycin was probably time dependent as indicated by a trend of state IV + respiration to rise, however, only 10–15 min after the addition of oligomycin (not shown in figures). This delay was much shorter in fish mitochondria and may no longer be visible in the more active mammalian mitochondria. In conclusion, the initial rate observed under oligomycin may be close to the true rate of proton leakage in mitochondria of resting cells at maximum phosphorylation potential.

Our analysis of state IV + respiration as a measure of mitochondrial proton leakiness did not include an analysis of membrane potential, however, the change in Δp with temperature is small [17], especially since alphastat change in extramitochondrial pH maintains ΔpH (cf. [34]). The continuity and similarity of the thermal responses of respiratory states IV and IV + (Figs. 3 and 4) over a large temperature range confirms that the basic temperature dependence of proton leakiness remains unchanged between state IV and state IV + oligomycin and is not related to drastic changes in membrane or phosphorylation potentials (see below).
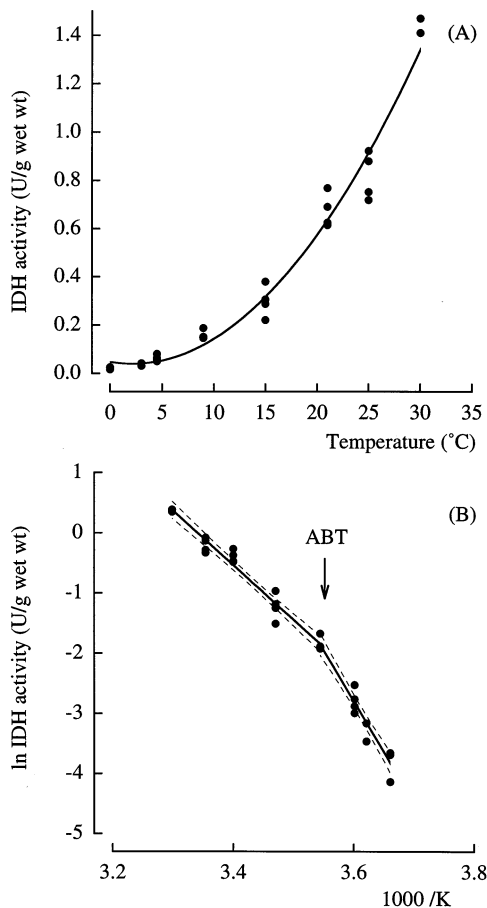
4.2. Mitochondrial capacity and proton leakage

Aerobic capacity of mitochondria (2.1 nmol O min^{-1} and mg protein at 0°) isolated from the gills of *Laternula elliptica* was in the same order of magnitude as the capacity of other bivalve mitochondria when extrapolated to 0°C with a Q_{10} of 2.2–2.5 [39]. This preliminary comparison does not lead us to expect significant cold compensation in Antarctic bivalve mitochondria, i.e. there is no rise in temperature specific rates above those expected from a normal Q_{10} relationship. Johnston et al. [28,29] and Guderley [22] also concluded that cold compensation of mitochondrial capacity (state III) in fish does not occur. Accordingly, Houlihan and Allan [25] did not find significant cold compensation in the metabolic rate of Antarctic gastropods. Obviously, an upregulation of mitochondrial capacity is not required in stenothermal animals and may be restricted to eurythermal species adapted to cold [39].

Fig. 7. Temperature dependence of mitochondrial NADP$^+$ dependent isocitrate dehydrogenase from *L. elliptica* gill mitochondria (A). The Arrhenius plot (B) reveals a significant break with the application of two linear regressions ($r = 0.97$ below ABT; $r = 0.98$ above ABT). Dashed lines indicate the 95% confidence interval.

Proton leakage accounted for respiratory rates of 0.38 ± 0.12 nmol O min^{-1} mg^{-1} mitochondrial protein in this and 0.38 ± 0.04 nmol O min^{-1} mg^{-1} mitochondrial protein in our companion study of notothenioid fish (*Lepidonotothen nudifrons*) liver mitochondria. When proton leakage rates quantified in ectothermal vertebrates [6,9] are extrapolated to low temperatures they are 6–7 times higher in lizard or trout mitochondria at 0°C than in mitochondria from the Antarctic species. This supports our earlier hypothesis that proton leakage is down regulated in parallel with SMR in Antarctic ectotherms. Low leakiness may compensate for the increased number of mitochondria in these cold adapted animals [38,39].

4.3. P/O ratios

Proton leakage in vivo may lead to resting P/O ratios even below 1. This occurs in mammalian tissue [41] and very likely also in tissues of ectotherms at one order of magnitude lower values of SMR and proton leakage. In the assay of mitochondrial respiration the true mechanistic P/O ratio will also be underestimated depending on the degree of proton leakage.

Despite analysis at low phosphorylation potential P/O ratios in our study (1.5–2) were below those (2.7–3) seen during glutamate respiration in gill mitochondria of oysters (*Crassostrea gigas*) adapted to 22°C [10], in gill mitochondria of ribbed mussel (*Modiolus demissus*) acclimated to 15°C [11] and in heart mitochondria of Atlantic squid (*Illex illecebrosus*) acclimated to 11°C [32]. Following the rationale of Davis and Davis-van Thienen [16] this might indicate elevated proton leakiness during state III respiration in cold adapted mitochondria, especially at higher temperatures. Correction of P/O values for proton leakage resulted in somewhat higher values (1.9–2.4). Further investigation needs to address to what extent a change in membrane composition during latitudinal cold adaptation (e.g. [46]) may lead to an increased contribution of proton leakiness to state III respiration.

4.4. Thermal characteristics of respiratory states

The Arrhenius activation energies (E_a) of state III and states III–IV+ respiration rates in *L. elliptica* mitochondria were about twice than expected from an extrapolated correlation of E_a values with temperature in deep sea and temperate marine invertebrate mitochondria between 5 and 40°C [15]. This comparison may be problematic since mitochondria in the study by Dahlhoff et al. were mostly uncoupled, a state where respiration rate may exceed state III owing to the drop in intramitochondrial pH [20]. The value of around 60 kJ mol^{-1} found in *L. elliptica* gill mitochondria between 0 and 3°C was similar to the values found in liver

mitochondria from Antarctic fish, *L. nudifrons* (47.5 kJ mol^{-1} [23]) and *Trematomus bernacchii* (38.3 kJ mol^{-1}, measured above 5°C, [49]), or in red muscle mitochondria from *Notothenia coriiceps* (73 kJ mol^{-1}, calculated from [22] for data between -1.5 and $+2.5$°C). Mitochondria from temperate zone fish, short-horned sculpin, *Myoxocephalus scorpio* exhibited some cold induced decrease in activation energy (from 70.2 kJ mol^{-1} in fish acclimated to 15°C to 55.4 kJ mol^{-1} in fish acclimated to 5°C; [21]). These comparisons might indicate that E_a of state III respiration is largely unaffected by polar cold adaptation, whereas seasonal cold adaptation may still have an effect. Overall, Q_{10} or E_a values for state III respiration in *L. elliptica* mitochondria at low temperature (0–3°C) were not exceptionally different from those reported for other species and there appears to be no clear trend with respect to temperature adaptation [39].

Owing to the progressive rise in E_a of state III respiration the uncoupling of mitochondria becomes significant only beyond ABT (9°C). A high degree of uncoupling is supported by the continuous increase in proton leakage (state IV+), with constant E_a and no ABT. The ABT of state III was much lower in *L. elliptica* than in our companion study on notothenioid fish liver mitochondria where an ABT was not seen below 18°C. In the fish the E_a of H$^+$ leakage was always higher than E_a of state III leading to an early progressive uncoupling of mitochondria with rising temperature [23].
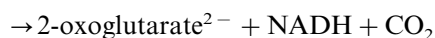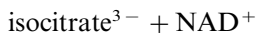
The finding of a rather high Q_{10} (4.2) for states IV and IV+ respiration in an Antarctic bivalve and a fish suggests that the thermal sensitivity of H$^+$ leakage is especially high in polar stenotherms compared to other ecto- and endotherms where Q_{10} ranges between 1.3 and 2.1 [39]. Only recently, Brand et al., demonstrated a correlation between phospholipid composition (long chain polyunsaturates) and proton leak. This effect is not direct but presumably through an interaction between membrane bound proteins and the lipids since leakage of protein-free mitochondrial membrane vesicles was only 5% of the value found in intact mitochondria. The nature of that protein is still obscure [8,9]. Whereas E_a of H$^+$ leakage appears to rise during cold adaptation the thermal sensitivity of passive Na$^+$ flux in mitochondrial membrane vesicles from cold adapted rainbow trout is reduced [52]. The conclusion arises that proton leakage should involve an enzyme catalyzed process rather than simple diffusion of protons (which should be indicated by a Q_{10} significantly below 2 [19]). These observations led us to hypothesize that a mitochondrial substrate cycle involving transhydrogenase and NADP dependent IDH [42] may be responsible for at least a fraction of the proton leak [39].
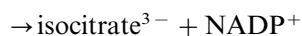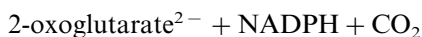
4.5. NADP dependent isocitrate dehydrogenase (IDH)

In this context it is intriguing that high E_a and Q_{10} values of H^+ leakage and IDH (NADP) go hand in hand. Functional relevance of cold adapted NADP-dependent IDH is strongly suggested by a clear inverse correlation between mean habitat temperature and predominance of a mitochondrial isoenzyme with high E_a values in cold adapted lugworms, *Arenicola marina* [27,39]. In general, E_a values of NADP dependent IDH from cold adapted animals in a latitudinal gradient are higher than in temperate zone species or populations and especially high in stenothermal versus eurythermal species [39]. Forward and backward reaction of IDH (NADP) exhibit identical thermal sensitivities (Pörtner and Hirse, unpublished).

E_a of state III respiration and mitochondrial phosphorylating respiration (states III–IV+) at elevated temperatures below ABT approached the one evaluated for IDH (138 kJ mol^{-1}). Interpretation of these findings must remain speculative at present. It might suggest that the control of state III respiration shifted towards control by IDH (NADP) and proton leakage via transhydrogenase. According to a suggested model isocitrate is cycled between NAD as well as NADP dependent IDH [26,42]. Transhydrogenase reduces $NADP^+$ at the expense of NADH and the mitochondrial proton gradient:

IDH (NAD):

isocitrate^{3-} + NAD$^+$

$\rightarrow$ 2-oxoglutarate^{2-} + NADH + CO$_2$

IDH (NADP):

2-oxoglutarate^{2-} + NADPH + CO$_2$

$\rightarrow$ isocitrate^{3-} + NADP$^+$

Transhydrogenase:

NADH + NADP$^+$ + H$^+_{(out)}$

$\rightarrow$ NAD$^+$ + NADPH + H$^+_{(in)}$

IDH (NADP) may adopt a significant role in determining the flux through this futile substrate cycle which not only enhances the sensitivity of the TCA cycle to changes in energy demand but should comprise a significant fraction of respiration and the mitochondrial proton leak, especially under resting conditions. The similarity of the proton leak and the transhydrogenase cycle with respect to their dependence on proton motive force of the inner mitochondrial membrane supports this conclusion [39].

High E_a levels of H^+ leakage and IDH in cold adapted species are unexpected in the light of the long held general paradigm that E_a is reduced in cold adapted species to facilitate metabolic flux. However, enzymes with a low activity, some of which are flux limiting, have hardly been considered and this may be the reason why such a rise in E_a has been overlooked, for example in glyceraldehydephosphate dehydrogenase, phosphofructokinase, IDH [39]. Diffusional limitations in the cold [43] may require a certain amount of enzyme molecules for the maintenance of metabolic flux. A rise in activation energy as seen in IDH would allow for reduced substrate flux at maintained or elevated enzyme levels. Such a rise in E_a in some enzymes would actually support metabolic depression in polar species below the rate otherwise expected from the effect of mitochondrial proliferation [39].

4.6. Critical and Arrhenius break temperatures

ABTs of mitochondrial phosphorylation and IDH were identical. In conclusion, the ABT of mitochondrial phosphorylation possibly involves the progressive heat inactivation of mitochondrial proteins such as IDH. O'Brien et al. [36] demonstrated that heat inactivation of membrane bound succinate dehydrogenase and the matrix enzyme malate dehydrogenase set in at about the same temperature (when cytochrome oxidase showed a transient activation). Accordingly, the fate of individual mitochondrial enzymes as well as a change in membrane properties may delineate the change in thermal properties of mitochondrial function.

Although the ABTs of mitochondrial state III respiration and IDH are the lowest so far described in a marine ectotherm they are still above the T_c of *Laternula elliptica* which, according to changes in the levels of anaerobic mitochondrial metabolites, is found between 3 and 6°C (Pörtner et al., unpublished). In a recent comparative overview of Arrhenius break temperatures in mitochondria from various fish and invertebrate species the ABT of uncoupled respiration varied depending on adaptation temperature, however, at values above the maximum habitat temperature [49] and also above the critical temperature of the whole animal [39]. The difference between ABT and maximum habitat temperature appeared to rise at lower temperatures, from an average of 15° to a value of 25° in the temperature range between 25 and 5°C. The data point from *T. bernacchii* mitochondria, however, did not fit this general pattern but the difference between ABT and habitat temperature was less (about 18°) than expected from the correlation [49]. In *L. elliptica* an even smaller difference of about 7–9°C results between habitat temperature and ABT and of only 3–6°C between critical temperature and ABT. Further comparison of invertebrate and vertebrate mitochondria is needed for an understanding of these differences in thermal properties.

5. Conclusions

It may be a general principle, that the ABT of mitochondrial function is found above the T_c of the whole animal in invertebrates and fish. Therefore, mitochondrial dysfunction possibly reflected in the ABTs of mitochondrial respiration or IDH activity is unlikely to be responsible for setting the upper limit of thermal tolerance and for eliciting the transition to anaerobiosis at the T_c. In the bivalve *L. elliptica*, the only Antarctic invertebrate so far investigated, the ABTs of isocitrate dehydrogenase and state III respiration were identical and found only slightly above the T_c of these animals (this study). In fish ABTs of mitochondrial functions are considerably higher than the T_c [49].

The following scenario likely explains the upper limits of thermal tolerance [39]: Mitochondria of Antarctic invertebrates and fish have in common a large thermal sensitivity of proton leakage and NADP dependent IDH. Elevated temperatures cause a large and early rise in oxygen demand (e.g. [47]) which may be due to proton leakage and not associated with an equivalent rise in ATP turnover [39]. At the T_c this oxygen demand can no longer be covered by oxygen delivery through ventilation and circulation [38]. Moreover, the limited amount of oxygen available at higher temperatures is inefficiently used by mitochondria. These processes will lead to the early development of a pronounced energy deficit and anaerobiosis [39].

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