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Why are some mitochondria more powerful than others: Insights from comparisons of muscle mitochondria from three terrestrial vertebrates

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Abstract

We studied the molecular composition of muscle mitochondria to evaluate whether the contents of cytochromes or adenine nucleotide translocase (ANT) or phospholipid acyl compositions reflect differences in mitochondrial oxidative capacities. We isolated mitochondria from three vertebrates of similar size and preferred temperature, the rat (*Rattus norvegicus*), the cane toad (*Bufo marinus*) and the bearded dragon lizard (*Pogona vitticeps*). Mitochondrial oxidative capacities were higher in rats and cane toads than in bearded dragon, whether rates were expressed relative to protein, cytochromes or ANT. Inter-specific differences were least pronounced when rates were expressed relative to cytochrome *A*, a component of cytochrome *C* oxidase (CCO), or ANT. In mitochondria from rat and cane toad, cytochrome *A* was more abundant than *C* followed by *B* and then C_1, while in bearded dragon mitochondria, the cytochromes were present in roughly equal levels. Analysis of correlations between mitochondrial oxidative capacities and macromolecular components revealed that cytochrome *A* explained at least half of the intra- and inter-specific variability in substrate oxidation rates. ANT levels were an excellent correlate of state 3 rates while phospholipid contents were correlated with state 4 rates. As the % poly-unsaturation and the % 20:4n-6 in mitochondrial phospholipids were equivalent in toads and rats, and exceeded the levels in lizards, they may contribute to the inter-specific differences in oxidative capacities. We suggest that the numbers of CCO and ANT together with the poly-unsaturation of phospholipids explain the higher oxidative capacities in muscle mitochondria from rats and cane toads.

Keywords: Mitochondria; Cytochromes; Adenine nucleotide translocase; Phospholipid; Fatty acid composition; Mitochondrial membrane; Muscle; Polyunsaturation

1. Introduction

The oxidative capacity of skeletal muscle fibres is closely regulated, presumably due to the compromise between the costs and benefits of possessing mitochondria. Skeletal muscle mitochondria produce ATP at high rates during sustained exercise and during recuperation from high intensity exercise. Within a given species, muscles vary considerably in their aerobic capacity, both in the proportion of fibre volume occupied by mitochondria and in properties of these mitochondria (Moyes and Hood, 2003; Leary et al., 2003). Interspecific differences in metabolic intensity are also linked with differences in mitochondrial numbers, cristae densities and mitochondrial oxidative capacities (Johnston et al., 1998; Moyes and Hood, 2003; Vock et al., 1996).

Mitochondrial oxidative capacities can be adjusted by changing the types, numbers or stoichiometry of the functional complexes (Krebs cycle enzymes; electron transport chain; F_1F_0ATPase; substrate translocases) required for oxidative phosphorylation (Moyes and Hood, 2003) as well as by changing the phospholipid and acyl composition of

Abbreviations: ANT, adenine nucleotide translocase; BSA, bovine serum albumin; CAT, carboxyatractyloside; cyto, cytochrome; PL, phospholipid; RCR, respiratory control ratio; CCO, cytochrome *C* oxidase.

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mitochondrial membranes (Wodtke, 1981a,b). Changes in the acyl composition of membrane phospholipids can markedly alter the molecular activity of membrane proteins (Hulbert and Else, 2000). Modifications of mitochondrial capacities require concerted regulation of the mitochondrial and nuclear genomes (Battersby and Moyes, 1998; Moyes et al., 1998; Leary et al., 2003) as well as synthesis of the appropriate quantity and quality of mitochondrial membranes (Stuart et al., 1998). The complexes involved in oxidative phosphorylation may be organized into supercomplexes or networks following relatively fixed ratios (Schägger and Pfeiffer, 2000, 2001). Many differences between mitochondria from red, white and cardiac muscle in trout are attenuated when mitochondrial capacities are expressed relative to the activity of cytochrome *C* oxidase (CCO) (Leary et al., 2003). However, the activities of such mitochondrial membrane proteins reflect both their numbers and the characteristics of their lipid environment (Hazel and Williams, 1990; Hulbert and Else, 1999). Effectively, changes in the acyl composition of membrane phospholipids strongly influence the activities of succinate dehydrogenase (Hazel, 1972a,b), cytochrome *C* oxidase (Wodtke, 1981a,b) and NaK ATPase (Wu et al., 2001). Knowledge of the contents of the protein and phospholipid components of mitochondria should indicate the degree to which adjustments of mitochondrial capacity are based on changes of protein numbers or on changes in membrane composition.

Our aim in this study was to identify the aspects of mitochondrial design that best explain intra- and interspecific variability in oxidative capacities of skeletal muscle mitochondria using vertebrates that differ greatly in their capacity for oxidative metabolism and phylogenetic origins. Specifically, we examined whether the contents of cytochromes and adenine nucleotide translocase (ANT) are linked with the oxidative capacity of isolated muscle mitochondria from rats, *Rattus norvegicus*, cane toads, *Bufo marinus*, and bearded dragon lizards, *Pogona vitticeps*. Although these species belong to different vertebrate classes, they have a similar body mass and function at similar body temperatures (Hulbert and Else, 1999; Brattstrom, 1963). These common attributes limit the confounding effects of differences in size and body temperature. Hepatic mitochondria from lizard and cane toad differ considerably in their proton leak kinetics, showing that mitochondrial design differs in these ectotherms (Brookes et al., 1998). We evaluated the capacity for substrate oxidation of these mitochondria by polarography and measured their protein, phospholipid, ANT, cytochrome *A*, *B*, C_1 and *C* contents. The content of cytochromes *A*, *B* and C_1 reflect the numbers of respiratory chain complexes: cytochrome *B* is present in complexes II (Schägger and Pfeiffer, 2001) and III, C_1 occurs in complex III, and cytochrome *A* is part of Complex IV. To evaluate the influence of the lipid environment on inter-specific differences, we determined the fatty acid composition of mitochondrial phospholipids. The cytochromes were measured by their difference spectra and ANT was measured by titration of mitochondrial activity with its irreversible inhibitor, carboxyatractyloside.

2. Materials and methods

2.1. Animal holding conditions

Rats (*R. norvegicus*) were purchased from Animal Resources Centre (Canning Vale, WA, Australia) and cane toads (*B. marinus*) were purchased from local suppliers (Peter Douche, Mareeba, Qld, Australia), whereas bearded dragons (*P. vitticeps*) were captured in spring in northwestern New South Wales (N.S.W. National Parks and Wildlife Service scientific permit #A92). Cane toads and bearded dragons were held at 37 °C for at least 3 weeks before use. Lizards were fed mixed vegetables and meal worms while cane toads were fed meal worms. Wistar rats were held at 22 °C and were provided with rat laboratory chow ad libitum. All species had continual access to water. Rats were sacrificed by pentobarbital injection (30 mg/kg body mass), whereas cane toads and bearded dragons were killed by stunning and section of the vertebral column. Rats weighed 504.7±39.7 g, bearded dragons 375.7±37.8 g and cane toads 120.5±5.8 g. Sex ratios were 8:3 (males to females) for rats, 2:1 for bearded dragons and 7:6 for cane toads. Animal gender had no apparent effect upon the values we measured.

2.2. Isolation of mitochondria and respirometry

Mitochondria were isolated from mixed skeletal muscle dissected primarily from the hind limbs, removing as much connective tissue as possible. The muscle was finely chopped on a chilled cutting board. All extraction and centrifugation steps were carried out on ice or at 4 °C. The muscle mince was suspended in 9 volumes of extraction buffer. Mitochondria from rat and bearded dragon were prepared in 140 mM KCl, 20 mM *N*-2-Hydroxyethylpiperazine-*N*″-2-ethanesulfonic acid (HEPES), 10 mM ethylenediamine tetraacetic acid (EDTA), 5 mM MgCl2, pH 7.3, 1% bovine serum albumin (BSA). Mitochondria from cane toad muscle were prepared in 170 mM mannitol, 55 mM sucrose, 5 mM ethylene glycol-bis-(β amino ethyl ether) *N*,*N*″-tetra-acetic acid (EGTA), 20 mM HEPES, pH 7.3, 0.5% BSA, 500 u ml^{-1} heparin, as this buffer yielded more coupled mitochondria from cane toads. pH values were adjusted at room temperature. The suspended muscle mince was first homogenised for 30 s using an Ultraturrax homogeniser set at low speed and then with a motorised Potter–Elvejhem tissue grinder, using 3–4 passes with a loose pestle. Excessive force during homogenisation resulted in greater extraction of mitochondria, but loss of mitochondrial quality. Extracts of cane toad muscle were centrifuged for 5 min at 755×*g* in a Beckman refrigerated centrifuge. Extracts of rat and dragon muscle were

centrifuged at 1400×*g* for 5 min. For all species, the supernatant from the first centrifugation was filtered through 2 layers of cheesecloth and then centrifuged at 9800×*g* for 10 min. The mitochondrial pellet was re-suspended in a volume of reaction buffer corresponding to one tenth of the mass of muscle used (i.e. 300 μl of buffer for 3 g of muscle). An aliquot of the mitochondrial preparation was resuspended in the assay medium minus BSA and centrifuged at 9000×*g* at room temperature for 10 min. The supernatant was discarded and the pellet resuspended, washed and centrifuged a further two times to remove the BSA.

Oxygen consumption was measured at 37 °C in 100 mM KCl, 5 mM HEPES, 40 mM sucrose, 10 mM $KHPO_4$, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.2, 0.5% BSA. For each assay, malate was added to a final concentration of 0.38 mM to spark the Krebs cycle, and pyruvate was added to final concentrations of 2.38 mM, respectively. Preliminary studies established that pyruvate was oxidised at higher rates than succinate or glutamate. Oxidative phosphorylation (state 3) began with the addition of ADP to a final concentration of 0.48 mM. After measurement of state 4 rates, 1 μg ml^{-1} oligomycin was added to evaluate oxygen consumption in the absence of oxidative phosphorylation (state 4_{ol}) (Estabrook, 1967).

2.3. Cytochrome and ANT concentrations

Cytochromes *A*, *B*, *C* and C_1 concentrations in the mitochondrial preparations were determined by difference spectra read after reduction of the electron transport chain components in 2% deoxycholate dispersed mitochondria by 5 mM ascorbate and elimination of oxygen in the solution by the addition of dithionite (Williams, 1964). The reduced samples were read against the samples oxidised with 5 mM ferricyanide. We used the solution to the simultaneous equations required to assess the individual cytochrome concentrations given by Schneider et al. (1980). Cytochrome *B* contents were also evaluated by difference spectra read after its reduction by 2 mM succinate with electron flow blocked between cytochrome *B* and cytochrome C_1 by 2.28 μM antimycin (Sherratt et al., 1988). These values are labelled as *B* direct. In this case, the air-oxidised mitochondrial extract was the blank. Difference spectra were obtained using a double-beam UV/Vis spectrophotometer (Varian-Cary 210).

The concentration of ANT was measured in mitochondrial suspensions by titration with its noncompetitive irreversible inhibitor, carboxyatractyloside (CAT). Using the polarographic method, oxygen consumption with saturating ADP levels (3.72 mM) was inhibited by adding small volumes (10 μl decreasing to 0.5 μl) of 0.1 and 0.01 mM CAT solutions. State 3 respiration was gradually inhibited and the inhibition was considered complete when addition of CAT had no further effect on oxygen uptake. The quantity of ANT in mitochondrial suspensions corresponded to the amount of CAT needed for inhibition, because CAT binding is essentially stoichiometric ((Forman and Wilson, 1983; Willis and Dallman, 1989).

2.4. Protein and phospholipid concentrations

The protein concentration in mitochondrial suspensions was determined with the Lowry method, using BSA as the standard. We followed Mills et al. (1984) in extracting total lipids from mitochondrial suspensions using chloroform: methanol (2:1) and 1 M sulfuric acid. The phospholipid content was evaluated by measuring the phosphorus concentration, by reacting phosphorus with 8.5% ammonium molybdate and then reducing it with 0.2% stannous chloride forming a blue complex that was measured at 680 nm. The mass of phospholipid was calculated by multiplying the mass of phosphorus by 25 (Porter et al., 1996).

2.5. Fatty acid compositions

Given the limited volume of the mitochondrial preparations, the fatty acid composition of skeletal muscle mitochondria was assessed using separate preparations. Total lipid was extracted from mitochondrial preparations by standard methods (Folch et al., 1957) using ultra-pure grade chloroform:methanol (2:1, v/v) containing butylated hydroxytoluene (0.01% w/v) as an antioxidant. Phospholipids were separated from neutral lipids by solid phase extraction on silicic acid columns. Fatty acid analysis of the phospholipid fraction was determined as described in detail elsewhere (Pan and Storlein, 1993). Briefly, phospholipid fractions were transmethylated with 14% (w/v) boron trifluoride in methanol and fatty acid methyl esters were separated by gas–liquid chromatography on a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) with a fused silica capillary column. Individual fatty acids were identified by comparing each peak's retention time to those of external standards and then expressed as the mol% of total fatty acids.

2.6. Statistical analysis

We used JMP IN 3.2.1 (SAS Institute Inc.) to perform linear regressions and ANOVAs. ANOVA followed by a posteriori Tukey multiple comparisons was used for the interspecies comparisons, with a level of significance of $\alpha = 0.05$.

3. Results

3.1. General characteristics of the muscle mitochondria

Our preparations of mitochondria from skeletal muscle showed good coupling between oxygen uptake and ADP phosphorylation, with respiratory control ratios (RCR) above 3 for all species (Table 1 and see Fig. 1 for rates).

Table 1
Respiratory control ratios and molecular composition of skeletal muscle mitochondria from the rat (*Rattus norvegicus*), the cane toad (*Bufo marinus*) and the bearded dragon lizard (*Pogona vitticeps*)

	RCR	Protein/PL (mg/mg)	Cytochromes and ANT (nmol/mg protein)					
			B	B direct	C_1	*C*	A	ANT
Rat	3.4±0.2	4.0±1.2	0.59 ± 0.04^{A}	0.58 ± 0.03^{A}	0.47 ± 0.06^{A}	0.74 ± 0.07^{A}	1.37 ± 0.12^{A}	4.22 ± 0.22^{A}
Lizard	3.3±0.5	7.7±2.8	0.44 ± 0.07^{B}	0.40 ± 0.07^{B}	0.48 ± 0.10^{A}	0.50 ± 0.09^{B}	0.53 ± 0.08^{B}	2.22 ± 0.38^{B}
Cane toad	4.1±0.2	4.2±0.8	0.43 ± 0.03^{B}	0.49 ± 0.04^{B}	0.28 ± 0.03^{B}	0.54 ± 0.04^{AB}	0.78 ± 0.06^{B}	2.60 ± 0.28^{B}

Cytochrome and ANT concentrations are normalised to the protein content in the mitochondrial preparations. Data are given as mean±S.E.M. When values in a column are followed by different superscripts, they differed (ANOVA, a posteriori comparisons, $p<0.05$).

The ratio of protein to phospholipid did not differ between mitochondria from the three species.

3.2. Macromolecular composition of the mitochondria

In our initial comparison of mitochondria from the three species, macromolecular compositions were expressed relative to protein contents, the denominator typically used by biochemists (Table 1). Protein specific levels of the cytochromes and the adenine nucleotide translocase (ANT) were higher in mitochondria from rats than in those from the ectothermal species. The only exception to this pattern was cytochrome C_1 for which the levels were equivalent in mitochondria from rats and bearded dragons. The two means of quantifying cytochrome *B* (shown as *B* and *B* direct) provided similar concentrations and inter-specific trends. In all species, the protein specific content of ANT was higher than the contents of cytochromes (Table 1).

The mitochondria from the three species differed considerably in the stoichiometry of the cytochromes (Table 2). In bearded dragons, the four cytochromes were present in roughly equal levels. On the other hand, for mitochondria from rats and cane toads, cytochrome *A* occurred in 3 fold the levels of cytochrome C_1 and cytochromes *B* and *C* were present in 1.5 to 2 fold the levels of cytochrome C_1. In all three species, ANT levels exceeded those of the most abundant cytochrome.

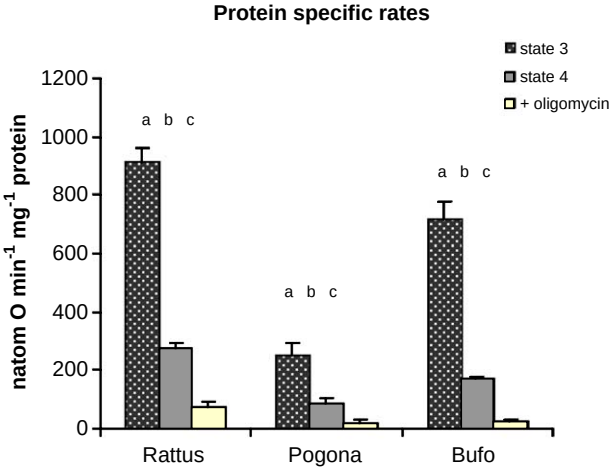

Fig. 1. Respiratory rates of muscle mitochondria from rat (*Rattus norvegicus*), bearded dragon lizard (*Pogona vitticeps*) and cane toad (*Bufo marinus*). All rates were measured at 37 °C. Values are shown as means±S.E.M. See text for description of conditions under which the rates were measured. Within each species, columns with different letters are significantly different (ANOVA, followed by a posteriori comparisons, $p<0.05$).

3.3. Oxidative capacities of isolated mitochondria

Rates of mitochondrial pyruvate oxidation (at 37 °C) differed markedly between the species. Mitochondria from rat muscle consistently had the highest protein specific rates, followed by those from cane toad and then those from the bearded dragon (Fig. 1). The same hierarchy was observed for oxygen uptake rates during states 3 and 4 ($p<0.0001$). Oligomycin inhibited rates of oxygen uptake were higher in rats than in dragons or cane toads. When oxygen uptake rates were expressed relative to cytochrome and ANT contents, rat and cane toad mitochondria generally did not differ and were consistently more active than those from bearded dragons (Fig. 2). Although significant inter-specific differences remained with all denominators, the inter-specific variation was smallest when cytochrome *A* and ANT were used as the denominators and not statistically significant when phospholipids were the denominator (Fig. 2). The variability of the phospholipid levels was likely to have masked interspecific differences.

3.4. Oxidative capacities of isolated mitochondria: correlations with macromolecular components

To assess how the oxidative capacities of mitochondria are related to their macromolecular composition, we examined the correlations between these parameters within species. As a first step, we examined the relationships

Table 2
Relative levels of cytochromes and ANT in skeletal muscle mitochondrial preparations from the rat (*Rattus norvegicus*), the bearded dragon lizard (*Pogona vitticeps*) and the cane toad (*Bufo marinus*)

	B	B direct	C_1	*C*	A	ANT
Rat	1.4±0.1	1.5 ± 0.2^{AB}	1	1.8 ± 0.1^{A}	3.4 ± 0.4^{A}	8.0 ± 1.1^{AB}
Lizard	1.0±0.1	0.9 ± 0.1^{A}	1	1.1 ± 0.1^{B}	1.2 ± 0.1^{B}	5.0 ± 0.5^{A}
Cane toad	1.6±0.2	1.9 ± 0.2^{B}	1	2.0 ± 0.2^{A}	3.0 ± 0.3^{A}	11.0 ± 1.1^{B}

All components are expressed relative to the concentration of cytochrome C_1. Values are given as mean±S.E.M. When values in a column are followed by different superscripts, they differed (ANOVA, a posteriori comparisons, $p<0.05$).

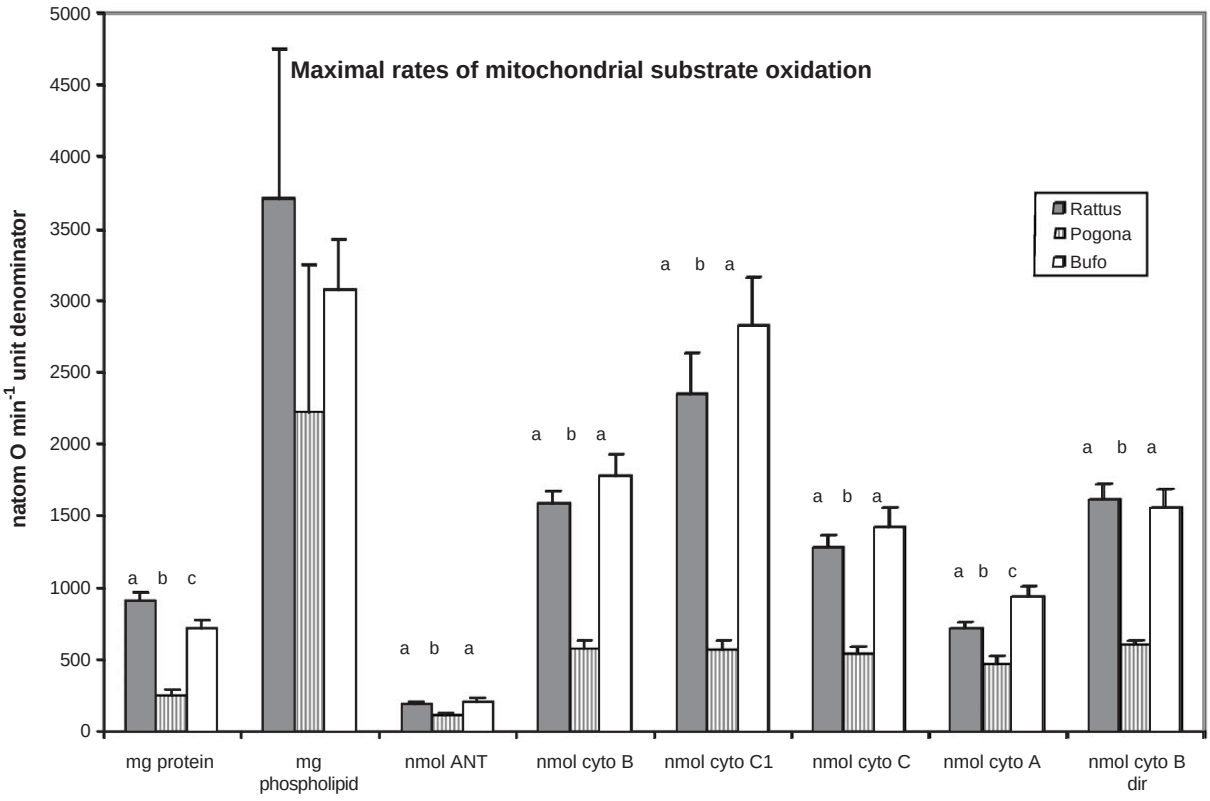

Fig. 2. Maximal rates of respiration (state III) of muscle mitochondria from rat (*Rattus norvegicus*), bearded dragon lizard (*Pogona vitticeps*) and cane toad (*Bufo marinus*) expressed relative to different mitochondrial components. All rates were measured at 37 °C and values are shown as means+S.E.M. See text for description of conditions under which the respiratory rates and mitochondrial composition were measured. Cyto represents cytochrome, and ANT represents adenine nucleotide translocase. In a given cell of the figure, columns with different letters are significantly different (ANOVA, a posteriori comparisons, $p<0.05$).

between the protein content of the preparations (mg ul^{-1}) and the oxidative capacities (natom O min^{-1} ul^{-1}). State 3 rates were only significantly linked with protein contents in rats; state 4 rates were linked with protein contents in rats and toads (Table 3). When the data for the three species were pooled, no relationship was apparent between mito-

Table 3
Significant ($p<0.05$) regressions between oxygen uptake (y, expressed as nmol O min^{-1} μl^{-1} mitochondrial preparation) and cytochrome, ANT and phospholipid of muscle mitochondria from rats, *Rattus norvegicus*, bearded dragon lizards, *Pogona vitticeps* and toads *Bufo marinus*

	Protein	Cytochromes					ANT	Phospholipid
		B		C_1	*C*	A		
			Direct					
State 3 rates of oxygen uptake								
Rat	0.743, 0.368	1761, −0.204	1554, 0.065		704, 0.68	661, 0.16	225, −0.38	
	$R^2=0.85$	$R^2=0.72$	$R^2=0.56$		$R^2=0.46$	$R^2=0.47$	$R^2=0.77$	
Lizard		603, −0.011	627, −0.016	680, −0.16	492, 0.09	500, −0.02	116, −0.009	
		$R^2=0.85$	$R^2=0.93$	$R^2=0.90$	$R^2=0.83$	$R^2=0.82$	$R^2=0.97$	
Cane toad		1141, 0.49	787, 0.770		1133, 0.25	639, 0.45	186, 0.19	1.63, 0.72
		$R^2=0.55$	$R^2=0.33$		$R^2=0.64$	$R^2=0.43$	$R^2=0.27$	$R^2=0.74$
State 4 rates of oxygen uptake								
Rat	0.161, 0.262	303, 0.24	311.4, 0.232		142, 0.42		29, 0.34	0.145, 0.51
	$R^2=0.80$	$R^2=0.37$	$R^2=0.44$		$R^2=0.20$		$R^2=0.39$	$R^2=0.34$
Lizard		148, 0.7		165, 0.04		124, 0.07	27, 0.09	0.20, 0.20
		$R^2=0.54$		$R^2=0.56$		$R^2=0.53$	$R^2=0.54$	$R^2=0.59$
Cane toad	0.133, 0.076	219, 0.19	140, 0.228	233, 0.25	162, 0.20	118, 0.19	60, 0.03	0.14, 0.295
	$R^2=0.64$	$R^2=0.43$	$R^2=0.19$	$R^2=0.32$	$R^2=0.52$	$R^2=0.32$	$R^2=0.30$	$R^2=0.23$

The first value in each cell is the slope, the second is the y intercept and the third is the R^2adj of the regression. The latter indicates the proportion of the variance explained by the relationship. In calculating these regressions, protein components were expressed as nmol μl^{-1} and phospholipid concentrations as μg μl^{-1}. An empty cell indicates that the regression was not significant.

chondrial oxidative capacities (states 3, 4 and oligomycin inhibited rates) and protein contents of mitochondrial preparations (linear regressions, $p > 0.05$). Thus, to avoid the bias associated with a measured denominator (e.g. protein), our subsequent analysis of correlations used oxidative capacities and molecular compositions expressed relative to a given volume of mitochondrial preparation.

Strong relationships between mitochondrial oxidative capacities and macromolecular components were apparent within each species (Table 3). Contents of cytochromes *B*, *C* and *A* were positively linked with state 3 rates in each species and explained between 33% and 93% of intra-specific variability in oxidative rates. Cytochrome C_1 levels were only linked with state 3 rates in lizards. ANT levels were strongly correlated with state 3 rates, in particular in rats and lizards (R^2 values of 0.77 and 0.97 respectively). A similar slope (≈ 600) related State 3 rates with cytochrome *A* levels in the 3 species. For the other cytochromes, the slopes relating their contents to state 3 rates varied more between the species. These macromolecular components were positively related to state 4 rates of oxygen uptake but the statistical fit of the regressions (R^2 values) was weaker than for state 3 rates (Table 3). For the three species, phospholipid, cytochrome *B* and ANT levels were positively linked to state 4 rates. The slopes of the relationships between state 4 rates and phospholipid levels were similar among the species. The relationships between the macromolecular components and oligomycin inhibited rates were not significant (data not shown). Thus, the levels of ANT and cytochromes *A*, *B* and *C* were more consistent correlates of mitochondrial oxidative capacities than protein concentrations. The relationships between maximal oxidative capacities and the levels of cytochrome *A* and ANT were similar for the three species.

On the inter-specific level, the concentrations of cytochrome *A* and ANT (nmol μl^{-1}) remained excellent correlates of state 3 and 4 rates of oxygen uptake. When the data for the three species were pooled, ANT concentrations were strongly linked with state 3 rates ($R^2 = 0.473$, $p = 0.002$, Fig. 3A) and somewhat linked with state 4 rates ($R^2 = 0.301$, $p = 0.02$, Fig. 3D). The overall relationships between state 3 rates and cytochrome contents ranged from highly significant for cytochrome *A* ($R^2 = 0.551$, $p < 0.001$, Fig. 3B) to not significant for cytochrome C_1 (data not shown, $p = 0.24$). Although cytochromes *C* and *B* were positively linked with state 3 rates, the regressions were

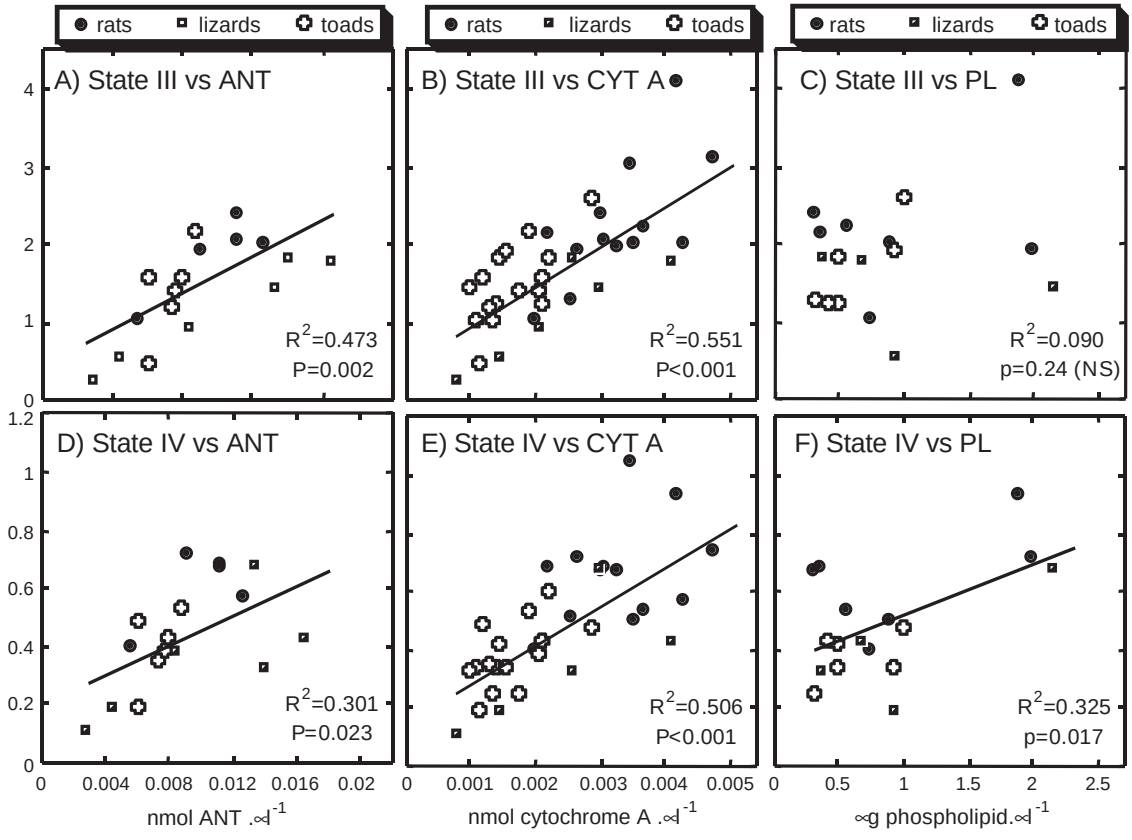

Fig. 3. Correlations between the rate of respiration and composition of muscle mitochondrial preparations from rat (*Rattus norvegicus*), bearded dragon (*Pogona vitticeps*) and cane toad (*Bufo marinus*). All rates were measured at 37 °C. Figures A–C show state III respiration rates while figures D–F show state IV respiration rates. Figures A and D show ANT composition, figures B and E show cytochrome *A* content and Figures C and F show the phospholipid content of the mitochondrial preparations. See text for description of conditions under which the respiratory rates and mitochondrial composition were measured. ANT represents adenine nucleotide translocase, cyt represents cytochrome, and PL represents phospholipid. Lines represent statistically significant relationships.

weaker (C: $R^2=0.33$, $p=0.0003$; B, $R^2=0.234$, $p=0.003$). State 4 rates were also related to cytochrome contents, with cytochrome *A* again showing the best relationship ($R^2=0.506$, $p<0.001$, Fig. 3E). While phospholipid concentrations were not linked with the state 3 rates (Fig. 3C), they were significantly related with the state 4 rates ($p=0.017$, Fig. 3F). Despite all the differences between these species, cytochrome *A* and ANT contents explained approximately half of the inter-specific variability in oxidative rates of these skeletal muscle mitochondria and phospholipid contents explained a third of the variability in state 4 rates.

3.5. Fatty acid composition of phospholipids from muscle mitochondria

Phospholipids from rat muscle mitochondria had a higher unsaturation index than those from the two ectothermal species, primarily due to high levels of polyunsaturates, in particular the n-3 fatty acids (Table 4). DHA (22:6n-3) levels were considerably higher in rat muscle mitochondria than in mitochondria from toad or bearded dragon muscle. Rat mitochondria had a higher % of saturated fatty acids, a higher proportion of n-3, a higher ratio of n-3/n-6 and a greater proportion of C20–22 fatty acids than those from toads and lizards. On the other hand, the two ectotherms differed considerably in the acyl composition of their mitochondrial membranes. The acyl composition of toad mitochondria was often intermediate between that of rats and lizards and for several parameters (20:4n-6, % mono-unsaturates, % polyunsaturates, % n-7 and average chain length) was equivalent to that of rat. Of the parameters differentiating the acyl compositions of mitochondrial phospholipids in rats, toads and bearded dragons, the % poly-unsaturated fatty acids and the 20:4n-6 contents most closely followed the inter-specific differences in mitochondrial oxidative capacity. The % mono-unsaturates and the % n-7 were considerably higher in lizard than in toad or rat mitochondria. These patterns suggest that the greater poly-unsaturation and lower mono-unsaturation of rat and toad mitochondrial membranes enhances mitochondrial oxidative capacity over that of the mitochondria from bearded dragons.

Table 4
Principal fatty acids (>1%) and overall characteristics of the fatty acid composition of phospholipids from skeletal muscle mitochondria from rat, bearded dragon lizard and cane toad

	Rat	Bearded dragon	Cane toad	*p*
14:0	0.6±0.2	0.9±0.7	0.5±0.05	0.87
14:1n-7	3.3±0.2	8.1±2.3	2.8±1.0	0.12
16:0	11.2±0.5 a	4.5±0.3 b	5.7±0.3 c	0.0001
16:1n-7	0.4±0.4	0.6±0.2	1.5±0.6	0.28
17:1n-7	0 a	1.8±0.4 b	0.3±0.3 a	0.03
18:0	11.1±0.3	12.4±0.6	9.7±1.3	0.21
18:1n-9	5.2±0.9 a	11.2±1.2 b	12.4±1.0 b	0.02
18:1n-7	4.2±0.4	4.0±0.8	2.6±0.3	0.21
18:2n-6	20.9±0.2	28.8±3.2	33.3±3.4	0.11
20:1n-9	0.2±0.2	1.2±0.1	0.7±0.4	0.21
20:3n-6	0.5±0 a	0.3±0.064 b	0.6±0.03 a	0.049
20:4n-6	19.3±0.5 a	15.1±0.7 b	20.7±0.4 a	0.002
20:5n-3	0.7±0.7	1.1±0.25	1.2±0.2	0.60
22:4n-6	0.7±0.0	0.8±0.27	0.2±0.08	0.10
22:5n-3	1.7±0.2	1.7±0.56	0.8±0.1	0.24
22:6n-3	18.0±0.2 a	3.2±0.67 b	2.0±0.3 b	<0.0001
% Saturates	25.5±1.1 a	18.7±1.6 b	17.6±1.1 b	0.03
% Mono-unsaturates	14.7±0.9 a	29.1±1.4 b	21.9±2.9 a	0.02
% Poly-unsaturates	59.9±0.3 a	52.3±0.3 b	60.5±2.2 a	0.02
n-9	5.6±1.1 a	13.5±1.9 b	13.8±1.6 b	0.046
n-7	9.1±0.3 a	16.7±2.1 b	8.4±1.3 a	0.03
n-6	41.6±0.1 a	45.0±1.8 a	55.7±2.7 b	0.01
n-3	18.3±0.2 a	6.1±1.3 b	4.6±0.6 b	0.0005
% Unsaturates	74.5±1.1 a	81.3±1.6 b	82.5±1.1 b	0.03
Unsaturation index	244.4±2.4 a	184.1±7.5 b	197.7±1.0 b	0.0014
Average chain length	18.7±0.04 a	18.0±0.16 b	18.2±0.04 ab	0.03
C20–22	38.2±0.8 a	23.7±2.6 b	25.5±1.0 b	0.008
n-6/n-3	2.3±0.02	8.6±2.6	12.7±2.1	0.06
n-3/n-6	0.44±0.004 a	0.14±0.033 b	0.083±0.015 b	0.0005

Data are given as % composition, $X\pm$S.E.M. Probability of significant differences as detected by ANOVA is given in the last column, when values in a given line differ (a posteriori comparisons, $p<0.05$) they are followed by different letters.

4. Discussion

Mitochondria isolated from skeletal muscle from rats, cane toads and bearded dragons vary considerably in their macromolecular composition and oxidative capacities, even though these organisms are of similar body size and prefer similar temperatures. When the macromolecular components were expressed relative to mitochondrial protein, as in most biochemical studies (see Table 1), muscle mitochondria from rats generally had higher contents than those from lizards and toads. This compositional difference did not directly translate into differences in oxidative capacities. Instead, the oxidative capacities of mitochondria from both rat and toad were considerably higher than those of lizard mitochondria. Although this inter-specific pattern in oxidative capacity remained apparent when oxygen uptake rates were expressed relative to all the mitochondrial proteins we measured (see Fig. 2), it was least pronounced when cytochrome *A* and ANT were used as the denominators.

The stoichiometry between mitochondrial cytochromes differed considerably between the species, although ANT levels were consistently 3–4 fold those of cytochrome *A*. Cytochromes *B*, C_1, *C* and *A* were present in equimolar levels in bearded dragon mitochondria, whereas in rat and cane toad the stoichiometry was approximately 1.5: 1: 1.9: 3.2. That our spectral measurements accurately reflect the levels of cytochrome *B* was corroborated by the direct measurements of its difference spectrum using succinate and antimycin. As cytochrome *B* levels are the most susceptible

to spectral interference by myoglobin or hemoglobin when using the dithionite technique (Bookelman et al., 1978), the similarity of the results with the two techniques suggests that myoglobin and hemoglobin interference was minimal. In heart mitochondria from rat, guinea pig and chicken, cytochrome C_1 was always the least abundant, closely followed by C, then by B with cytochrome *A* always the most abundant (Williams, 1968). Muscle mitochondria from rainbow trout (H. Guderley and P. Bouchard, unpublished) showed a similar pattern. Interspecific differences in the ratios of cytochromes may translate into varying ratios of the respiratory chain complexes. Cytochrome *B* is present in complexes II and III (Schägger and Pfeiffer, 2001), whereas the other cytochromes are concentrated with specific complexes. Much like in the bearded dragon, mitochondria from honeybee flight muscle contain cytochromes in roughly equal levels (Suarez et al., 1999). As honeybee mitochondria show considerably higher turnover rates than mitochondria from any vertebrate muscle (Suarez et al., 1999), equimolar levels of the cytochromes are unlikely to explain the low oxidative capacities of lizard mitochondria.

The strong correlation between cytochrome *A* concentrations (nmol ul^{-1}) and oxidative capacities (natom O min^{-1} ul^{-1}) within the three species and the similarity of this relationship between species was striking. Insofar as cytochrome *A* levels reflect the numbers of complex IV, this pattern is compatible with a generalized role for complex IV in setting rates of oxygen uptake, as demonstrated in rat liver mitochondria (Groen et al., 1982). Cytochromes *B* and *C* were also positively linked to maximal and state 4 rates, but the relationships varied more between species. The levels of the least abundant cytochrome, C_1, were only significantly linked with the oxidative capacities of mitochondria in bearded dragon lizards. The strong relationship between cytochrome *A* concentrations and oxidative capacities is consistent with the patterns obtained by Leary et al. (2003). These authors found that scaling mitochondrial properties to the activity of CCO eliminated many differences between mitochondria from red, white and cardiac muscle. The activity of CCO reflects both its numbers and its lipid environment. Our results show that the oxidative capacities of muscle mitochondria scale with the numbers of CCO both on the intra- and inter-specific levels. By extension, the low oxidative capacities of mitochondria from bearded dragon lizards reflect their low CCO contents.

The predictive capacity of the other macromolecular components, ANT and phospholipids, for which the contents showed intra- and inter-specific links with oxidative capacity, was less generalized than that of cytochrome *A*. Commensurate with its role in adenylate traffic, ANT levels were better predictors of state 3 than state 4 rates. ANT levels explained almost all (97%) the variability in state 3 rates in bearded dragons and most (77%) of it in rats. The weaker links between ANT concentrations and state 4 rates likely reflect the reduced ADP use during state 4. Phospholipid contents were significantly correlated only to state 4 rates. The 3 species showed similar slopes relating state 4 oxygen uptake to phospholipid contents. However, mitochondria from rats had higher state 4 rates for a given phospholipid content than those from toads and lizards, probably due to the greater unsaturation (Unsaturation Index) of membrane lipids in rats.

Interspecific differences in the acyl composition of mitochondrial phospholipids correlated with the interspecific differences in oxidative capacities expressed relative to cytochrome *A* or ANT. As some of the differences in acyl composition simply followed the ectotherm–endotherm divide (unsaturation index, % saturates, n-9 and n-3 levels), they could not explain why mitochondria from bearded dragon lizard had a lower oxidative capacity than those from cane toad and rat. However, the polyunsaturation and in particular the levels of 20:4n-6 directly paralleled the differences in oxidative capacities. Some of us have argued that the acyl composition of membrane phospholipids acts as a metabolic pacemaker, influencing the molecular activity of membrane components (Hulbert and Else, 1999, 2000). Our comparison of the acyl composition of mitochondrial phospholipids is compatible with this idea. We suggest that the inter-specific differences in poly-unsaturation, mono-unsaturation and 20:4n-6 contents together with differences in the numbers of cytochrome *A* and ANT explain the inter-specific variation in mitochondrial oxidative capacity. Complex IV and ANT are integral membrane proteins. Their catalytic activity will be influenced by the lipid environment (Hazel, 1972a,b) and should be enhanced by the greater poly-unsaturation characteristic of both rat and toad mitochondrial membranes. These differences in acyl composition of mitochondrial phospholipids may explain a major portion of the interspecific variability in mitochondrial oxidative capacities.

To conclude with a technical note, cytochrome *A* and ANT were excellent correlates of oxidative capacity in muscle mitochondria from the three species, by far surpassing the utility of the protein concentration in the mitochondrial fraction for inter-specific comparisons. ANT seems a particularly promising candidate as an easily measured mitochondrial marker. Estimates of cytochrome content require considerable material and high quality spectrophotometric optics. On the other hand, titration of mitochondrial oxygen uptake by the irreversible inhibitor of ANT, carboxyatractyloside can be easily carried out during polarographic measurements of oxygen uptake and requires only a minimal quantity of mitochondria. Use of either of these parameters as a common denominator will facilitate the interspecific comparisons of mitochondrial physiology.

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