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How might you compare mitochondria from different tissues and different species?

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Abstract Mitochondria were isolated from the liver, kidney and mixed hindlimb skeletal muscle of three vertebrate species; the laboratory rat *Rattus norvegicus*, the bearded dragon lizard *Pogona vitticeps*, and the cane toad *Bufo marinus*. These vertebrate species are approximately the same body mass and have similar body temperatures. The content of cytochromes B, C, C1, and A were measured in these isolated mitochondria by oxidised–reduced difference spectra. Adenine nucleotide translocase (ANT) was measured by titration of mitochondrial respiration with carboxyactractyloside and the protein and phospholipid content of isolated mitochondria were also measured. Fatty acid composition of mitochondrial phospholipids was measured. Mitochondrial respiration was measured at 37°C under states III and IV conditions as well as during oligomycin inhibition. Species differed in the ratios of different mitochondrial cytochromes. Muscle mitochondria differed from kidney and liver mitochondria by having a higher ANT content relative to cytochrome content. Respiration rates were compared relative to a number of denominators and found to be most variable when expressed relative to mitochondrial protein content and least variable when expressed relative to mitochondrial cytochrome A and ANT content. The turnover of cytochromes was calculated and found to vary between 1 and 94 electrons s^{-1}. The molecular activity of mitochondrial cytochromes was found to be significantly positively correlated with the relative polyunsaturation of mitochondrial membrane lipids.

Keywords Cytochromes · Adenine nucleotide translocase (ANT) · Membrane lipids · Docosahexaenoic acid (DHA) · Molecular activity

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Introduction

The essence of comparative biochemistry and physiology is comparison and an appropriate denominator is the basis of every quantitative comparison. This denominator may be implicit (as in "per animal" comparisons) or explicit (for e.g. in the comparison of "mass-specific" metabolic rates). In many biochemical comparisons, such as that of enzyme activities, the denominator is often "per mg protein." Yet this denominator provides relatively little insight and in some cases its use can be misleading. For example, significant changes in the amount of other proteins may mask, or give a mistaken impression of changes in the activity of the enzyme of interest.

In the case of enzymes, more insight can be garnered when both enzyme activity and enzyme amount are known. This knowledge allows the calculation of average activity "per enzyme molecule." For most enzymes it is generally difficult to measure the number of enzyme molecules present in a preparation. However for some it is not so difficult. For Na^+, K^+ -ATPase, where the specific inhibitor, ouabain, makes it possible to measure both enzyme activity and enzyme amount and can thus provide deeper insight than just knowledge of overall enzyme activity. For example, the overall Na^+, K^+ -ATPase activity of kidneys is inversely related to body size in both mammals and birds. In small mammals, the high Na^+, K^+ -ATPase activity is primarily due to increased molecular activity of individual enzyme

molecules, whereas in small birds it is due to an increased amount of enzyme (Turner et al. 2005). This approach has also been successful in demonstrating a relationship between the molecular activity of Na^+, K^+-ATPase and the fatty acid composition of its surrounding membrane (Turner et al. 2003).

It has previously been demonstrated that proton leak of liver mitochondria from different species is correlated with the fatty acid composition of mitochondrial membranes (e.g. Porter et al. 1996; Brookes et al. 1998; Brand et al. 2003). In these studies, mitochondrial proton leak is expressed per milligram mitochondrial protein, and therefore variation in mitochondrial protein content between mitochondria will have an unknown influence on the relationship between mitochondrial proton leak and membrane lipid composition. The current study was designed to further examine the relationship between the degree of polyunsaturation of mitochondrial membranes and mitochondrial function. The approach was adapted from that described above for the membrane-bound Na^+, K^+-ATPase, namely, to calculate the molecular activity of mitochondrial proteins in mitochondria from different tissues and species. The species chosen were three very different terrestrial vertebrates; the laboratory rat (*Rattus norvegicus*), the bearded dragon lizard (*Pogona vitticeps*), and the cane toad (*Bufo marinus*). These species were chosen because they represent three different vertebrate classes, are of similar size, and also because mitochondrial function can be measured at the common temperature of 37°C, which is a physiologically relevant temperature for all three species. Mitochondria from liver, kidney, and skeletal muscle were isolated from all three species and compared.

Mitochondria, as subcellular organelles, are obviously more complex than single enzymes (such as Na^+, K^+-ATPase) and the first question is which mitochondrial components should be quantified? We decided to measure a number of mitochondrial components, namely the cytochromes B, C, C1 and A, adenine nucleotide translocase (ANT), as well as mitochondrial protein and phospholipid content. The cytochromes were measured by their difference spectra and ANT was measured by titration of mitochondrial activity with its irreversible inhibitor, carboxyatractyloside. The content of cytochromes will reflect the numbers of respiratory chain complexes, with a particularly clear link between numbers of cytochrome A and Complex IV.

The second question was which mitochondrial activity should be quantified? For individual enzymes, maximum enzyme activity (at saturating substrate concentrations) is normally the measured activity. The oxygen consumption of isolated mitochondria has been measured (by polarography) in the presence of saturating substrate concentrations and under three conditions: state III (rapid phosphorylation immediately following addition of ADP), state IV (slow oxygen uptake rate following phosphorylation of the previously added ADP), and non-phosphorylation (in presence of oligomycin, a specific inhibitor of the ATP synthase).

To evaluate the influence of the lipid environment of mitochondrial membranes on mitochondrial activity, the fatty acid composition of mitochondrial phospholipids were determined. While both mitochondrial composition and function (described previously) were measured on the same samples of isolated mitochondria, because of the limitations of the size of isolated mitochondrial preparations, mitochondrial phospholipid fatty acid composition was measured on isolated mitochondrial preparations from separate individuals of each species. Hence the analysis of the impact of fatty acid composition on mitochondrial activity used the mean values obtained for the three tissues in the three species.

Methods

Animals and holding conditions

Rats (*Rattus norvegicus*) were purchased from the Animal Resources Centre (Canning Vale, WA, Australia), cane toads (*Bufo marinus*) were purchased from a local supplier (Peter Douche, Mareeba, QLD, Australia), while bearded dragons (*Pogona vitticeps*) were captured in spring 2000 in northwestern New South Wales (N.S.W. National Parks& Wildlife Service scientific permit#A92). All animals were kept in the University of Wollongong Animal House with 12:12 light:dark photoperiod and ad libitum access to food and water. For rats, the food was rodent pellets, while lizards were fed mixed vegetables and meal worms and cane toads were fed meal worms. Rats were held at 22°C, while cane toads and bearded dragons were held at 37°C for at least 3 weeks before use. This is a physiologically relevant temperature for each ectotherm species. Rats were sacrificed by pentobarbital injection (30 mg kg^{-1} body mass) while cane toads and bearded dragons were killed by stunning and section of the vertebral column and pithing. 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 any of the measures in the current study as the values for males and females overlapped substantially.

Isolation of mitochondria and respirometry

Liver and kidney mitochondria were prepared according to a modified method of that described by Rolfe et al. (1994). Livers and kidneys were weighed, cut into small pieces, and homogenised using seven strokes in a motorised Potter-Elvejhem tissue grinder with a loose fitting pestle, in nine volumes of ice-cold medium containing 250 mM sucrose, 10 mM Tris, 1 mM EGTA (ethylene glycol-bis-[amino ethyl ether] *N*,*N*″-tetra-acetic acid), and 1% Bovine serum albumin (BSA), pH 7.4. The homogenate was centrifuged at 1,050 *g* at 2°C for 3 min to remove nuclei and cell debris. The supernatant

was removed and centrifuged at 10,000 *g* at 2°C for 10 min. The pellet was resuspended and centrifuged at 10,000 *g* at 2°C for 10 min and the resulting pellet, containing mitochondria, was resuspended in the reaction buffer at a concentration of 1 ml/5 g of original tissue.

Skeletal muscle mitochondria were isolated from muscle dissected primarily from the hindlimbs. Care was taken during dissection to remove as much connective tissue as possible. The muscle was finely chopped on a chilled cutting board with a chilled knife and razor blade, until it looked like a fine puré e. All extraction and centrifugation steps were carried out on ice or at 4°C. The muscle mince was suspended in nine volumes of the 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 tetra-acetic acid (EDTA), 5 mM $MgCl_2$, pH 7.3, 1% BSA. This is the method described by Guderley and Johnston (1996). In an attempt to optimise mitochondrial preparations, mitochondria from cane toad muscle were prepared in the medium used by St-Pierre et al. (2000), that is, 170 mM mannitol, 55 mM sucrose, 5 mM EGTA, 20 mM HEPES, pH 7.3, 0.5% BSA, 500 u ml-1 heparin. All pH values were adjusted at room temperature. The suspended muscle mince was gently homogenised for 30 s using an Ultraturrax homogeniser. The muscle was then homogenised with a motorised Potter-Elvejhem tissue grinder, using 3–4 passes with a loose pestle. Excessive force during this 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 1,400*g* for 5 min. For all species, the supernatant from the first centrifugation was filtered through two layers of cheesecloth and then centrifuged at 9,800*g* for 10 min. The mitochondrial pellet was resuspended 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). To prepare samples for protein measurements, an aliquot of the mitochondrial preparation was resuspended in the assay medium minus BSA and centrifuged at 9,000*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.

For all tissues, mitochondrial oxygen consumption was measured at 37°C in 100 mM KCl, 5 mM HEPES, 40 mM sucrose, 10 mM KHPO4, 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 a final concentration of 2.38 mM. Preliminary studies established that pyruvate was oxidised at higher rates than succinate or glutamate. Oxidative phosphorylation (state III) began with the addition of ADP to a final concentration of 0.48 mM. After measurement of state IV rates, 1 μg mL^{-1} oligomycin was added to evaluate oxygen consumption in the absence of oxidative phosphorylation (Estabrook 1967).

Cytochrome and ANT concentrations

Cytochrome A, B, C, and C1 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 sodium dithionite (Williams 1964). The reduced samples were read against the samples oxidised with 5 mM K ferricyanide. The solution to the simultaneous equations were used to assess the individual cytochrome concentrations given by Schneider et al. (1980). Difference spectra were obtained using a double-beam UV/Vis spectrophotometer (Varian-Cary 210). Due to the presence of a dark pigment, the cytochrome concentrations of mitochondrial preparations from lizard and toad liver were unable to be determined. For toad kidney there was a limited volume of sample and only the B direct measurement could be completed.

The concentration of ANT was measured in mitochondrial suspensions by titration with its non-competitive 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 III 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 (Willis and Dallman 1989).

Protein and phospholipid concentrations

The protein concentration in mitochondrial suspensions was determined by the Lowry method (Lowry et al. 1951), using BSA as the standard. Phospholipid content was measured as described by Mills et al., (1984). Briefly, total lipid from mitochondrial suspensions was extracted using chloroform: methanol (2:1) and 1 M sulphuric 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). There was insufficient sample for phospholipid measurements in mitochondrial preparations from lizard and toad kidneys. An average molecular mass of 780 Da was assumed for each phospholipid in order to express

phospholipid content in the same units as cytochromes and ANT (i.e. nmoles · mg mitochondrial protein^{-1}).

Fatty acid composition

Given the limited volume of the mitochondrial preparations, the fatty acid composition 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 Storlien 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.

Statistical analysis

ANOVAs assessing inter-species and inter-tissue differences and linear regressions evaluating links between fatty acid composition and molecular activities were conducted using JMP IN 3.2.1 (SAS Institute Inc.). Where ANOVA revealed a significant effect, Tukey's post hoc test was used to identify significant differences. Significance was set at a level of $P < 0.05$ and all values are presented as means ± SEM.

Since all the macromolecular parameters and activities in muscle mitochondria were capable of being measured, the macromolecular determinants of intra- and inter-specific variability in oxygen uptake rates are examined in a separate manuscript (H.E. Guderley et al., submitted).

Results

Mitochondrial composition

Table 1 presents the data for mitochondrial composition with cytochromes being presented in the order in which they occur in the respiratory chain. The livers from both of the ectotherm species contained a substantial quantity of a black pigment (likely melanin) that co-sedimented with mitochondria and thus prevented measurement of cytochromes by difference spectrophotometry for these two species. The small size of kidney mitochondrial preparations from the two ectotherms meant that phospholipid content was not measured for either and cytochromes were not measured for the toad.

The relative amounts of the different cytochromes varied between the species. In both kidney and muscle mitochondria of the lizard, the cytochromes were present in approximately equimolar amounts. In mitochondria from all three rat tissues and from toad muscle, cytochromes A and C were present in higher amounts than cytochromes B and C1. This difference was most pronounced in muscle and kidney mitochondria where cytochrome A was present at approximately threefold the cytochrome C1 content. In rat liver mitochondria the relative abundance of cytochromes was more even, with the most abundant cytochrome (cytochrome C) being only 40% greater than the least abundant cytochrome (cytochrome C1).

In all three species, the ANT was much more abundant in muscle mitochondria than in kidney or liver mitochondria. Rat muscle mitochondria had the highest ANT content being about sixfold greater than rat liver mitochondria. Muscle mitochondria from the toad and the lizard had the next highest ANT contents being respectively 18-fold and sevenfold greater than the ANT content of liver mitochondria from the same species. In liver and kidney mitochondria, ANT was present in an approximately twice the molar ratio to the most abundant cytochrome, however, in muscle mitochondria from all three species ANT was present in substantially greater molar amounts (200, 320, and 230% more in rat, lizard, and toad, respectively) than the most abundant cytochrome (cytochrome A).

The small sample size of mitochondrial preparations following the various measurements meant replicate measurements of their phospholipid contents were not possible and are responsible for the relatively greater variance in this data set. The rat mitochondria had greater phospholipid content than both the toad and lizard mitochondria and this most likely reflects a greater density of inner membrane in the rat mitochondria. The difference between the species was similar for both muscle and liver mitochondria. In muscle mitochondria, the relative phospholipid content reflected the relative abundance of cytochrome A in all three species. In the rat, muscle mitochondria had the greatest cytochrome A abundance relative to phospholipid content (and thus likely membrane area) with kidney mitochondria having 40% less, and liver mitochondria having 70% less cytochrome A per phospholipid than muscle mitochondria.

Mitochondrial respiratory activity

Table 2 presents the respiratory rates (per mg protein) of mitochondria from liver, kidney, and skeletal muscle from each of the three species. In most cases, three rates are shown; state III, state IV, and oligomycin-inhibited rates. The rates vary 115-fold ranging from 7.9 natom O mg protein^{-1} min^{-1} for toad liver mitochondria in the presence of oligomycin to 913 natom O mg protein^{-1} min^{-1} for rat skeletal muscle mitochondria under

Table 1 Composition of isolated mitochondria from liver, kidney, and skeletal muscle of the rat, (*R. norvegicus)*, the lizard (*P. vitticeps)*, and the toad (*B. marinus)*

	Rat	Lizard	Toad
Liver			
Cytochrome B	0.44 ± 0.04(13)	nm	nm
Cytochrome C_1	0.41 ± 0.04(13)	nm	nm
Cytochrome C	0.57 ± 0.06(13)	nm	nm
Cytochrome A	0.46 ± 0.05(13)	nm	nm
ANT	0.71 ± 0.18	0.31 ± 0.08	0.14 ± 0.03
Phospholipid	581.4 ± 134.1(6)	193.5 ± 54.4(3)	317.1 ± 17.5(3)
Kidney			
Cytochrome B	0.35 ± 0.02(14)	0.11 ± 0.01(6)	nm
Cytochrome C_1	0.21 ± 0.01(12)	0.11 ± 0.01(6)	nm
Cytochrome C	0.65 ± 0.05(12)	0.08 ± 0.01(6)	nm
Cytochrome A	0.65 ± 0.04(12)	0.10 ± 0.01(6)	nm
ANT	1.31 ± 0.11	0.23 ± 0.05	0.98 ± 0.19
Phospholipid	404.2 ± 70.1(9)	nm	nm
Skeletal muscle			
Cytochrome B	0.59 ± 0.04(14)	0.44 ± 0.07(6)	0.43 ± 0.03(14)
Cytochrome C_1	0.47 ± 0.06(14)	0.48 ± 0.10(6)	0.28 ± 0.03(14)
Cytochrome C	0.74 ± 0.07(14)	0.50 ± 0.09(6)	0.54 ± 0.04(14)
Cytochrome A	1.37 ± 0.12(14)	0.53 ± 0.08(6)	0.78 ± 0.06(14)
ANT	4.22 ± 0.23	2.23 ± 0.37	2.60 ± 0.27
Phospholipid	528.5 ± 133.5(7)	265.8 ± 130.0(4)	373.4 ± 79.1(6)

All composition values are in units of nmoles mg mitochondrial protein^{-1}Values are presented as mean ± SEM(N =). *nm* indicates parameter not measured

state III conditions. In all species, under state III conditions muscle mitochondria show the greatest respiratory rates (per mg protein) followed by kidney then liver in rat and toad, but followed by liver then kidney in the lizard. The muscle mitochondrial rates are between 4.0 and 5.7-fold greater than kidney mitochondria and 1.5–29-fold greater than liver mitochondria.

As is normal for measurement of mitochondrial respiration, following initial addition of ADP to the preparation there is a rapid and linear decline in oxygen content (state III respiration rate) followed by a slower but still linear decline in oxygen content of the incubation (state IV respiration rate). Under state IV conditions, rates of mitochondrial respiration were approximately twofold to fourfold less than during state III as is demonstrated by the values for RCR (III/IV) (see Table 2). For kidney mitochondria from both lizards and toads, this second period was not linear but instead showed a curvilinear decrease in oxygen content. Therefore a state IV rate could not be calculated for these mitochondria. Following addition of excess oligomycin to the incubation, the rate of oxygen decline was linear in all cases and substantially lower than the preceding state IV rate. In liver and kidney mitochondria, the oligomycin-inhibited rate was 31–61% of the respective state IV rate, while in muscle mitochondria it was 14–27% of the respective state IV rate. This is taken to indicate that all (and especially muscle) mitochondrial preparations had some ATPase contamination, which resulted in continual ADP phosphorylation (and thus respiratory activity) by the mitochondria because of the breakdown of newly synthesised ATP to ADP.

Table 2 Respiration rate at 37°C of isolated mitochondria from liver, kidney, and skeletal muscle of the rat (*R. norvegicus)*, the lizard (*P. vitticeps)*, and the toad (*B. marinus)*

	Rat	Lizard	Toad
Liver			
State III	95.9 ± 14.1(13)	164.7 ± 18.0(5)	24.7 ± 4.6(11)
State IV	48.5 ± 6.9(12)	54.7 ± 5.9(5)	13.0 ± 5.0(4)
Oligomycin	28.6 ± 7.5(4)	17.1 ± 2.8(5)	7.9 ± 1.7(7)
RCR (III/IV)	2.1 ± 0.1(12)	3.0 ± 0.3(5)	1.7 ± 0.2(4)
RCR (III/oligomycin)	4.0 ± 0.7(4)	11.0 ± 2.6(5)	3.4 ± 0.3(7)
Kidney			
State III	159.9 ± 30.4(12)	61.3 ± 8.5(6)	177.7 ± 28.8(6)
State IV	75.2 ± 12.9(12)	nm	nm
Oligomycin	30.8 ± 5.3(4)	8.3 ± 1.2(6)	14.3 ± 3.4(5)
RCR (III/IV)	2.2 ± 0.1(12)	nm	nm
RCR (III/oligomycin)	6.2 ± 2.4(4)	7.6 ± 0.8(6)	16.7 ± 6.9(5)
Skeletal muscle			
State III	913.0 ± 47.0(14)	250.0 ± 41.5(6)	716.5 ± 58.6(16)
State IV	277.5 ± 14.4(14)	82.1 ± 15.4(6)	168.5 ± 7.7(16)
Oligomycin	75.2 ± 17.6(3)	20.3 ± 10.4(6)	22.8 ± 7.0(5)
RCR (III/IV)	3.4 ± 0.2(14)	3.3 ± 0.5(6)	4.1 ± 0.2(16)
RCR (III/oligomycin)	12.3 ± 3.1(3)	20.3 ± 6.7(5)	37.4 ± 10.2(5)

Respiration rates (natom O mg protein^{-1} min^{-1}) were measured under conditions of state III (high phosphorylation rate), state IV (low phosphorylation rate), and in presence of oligomycin (non-phosphorylating)
Values are presented as mean ± SEM(N =). *nm* Indicates parameter not measured

When ADP phosphorylation is prevented by oligomycin inhibition, mitochondrial respiratory rate represents activity necessary to pump protons that are passing across the mitochondrial inner membrane by pathways separate from the ATP synthase pathway. This has been described as proton leak. Because the proton motive force will likely be high in this situation and proton leak will be partly determined by its driving gradient (i.e. the proton motive force across the mitochondrial inner membrane), respiratory rate measured with excess oligomycin likely slightly overestimates mitochondrial proton leak. When RCR values are calculated using the oligomycin rates instead of the state IV rates, the RCR values range from 3.4 to 11.0 for liver mitochondria, from 6.2 to 16.7 for kidney mitochondria, and from 12.3 to 37.4 for muscle mitochondria. In all three species, muscle mitochondria had a greater range of respiratory activity than mitochondria from the other two tissues.

Because several macromolecular components were measured for each mitochondrial preparation, the various respiratory rates can be expressed relative to a number of different denominators. In Fig. 1 we present mitochondrial respiration "per mg protein," as well as "per nmol cytochrome A," and "per nmol ANT" for

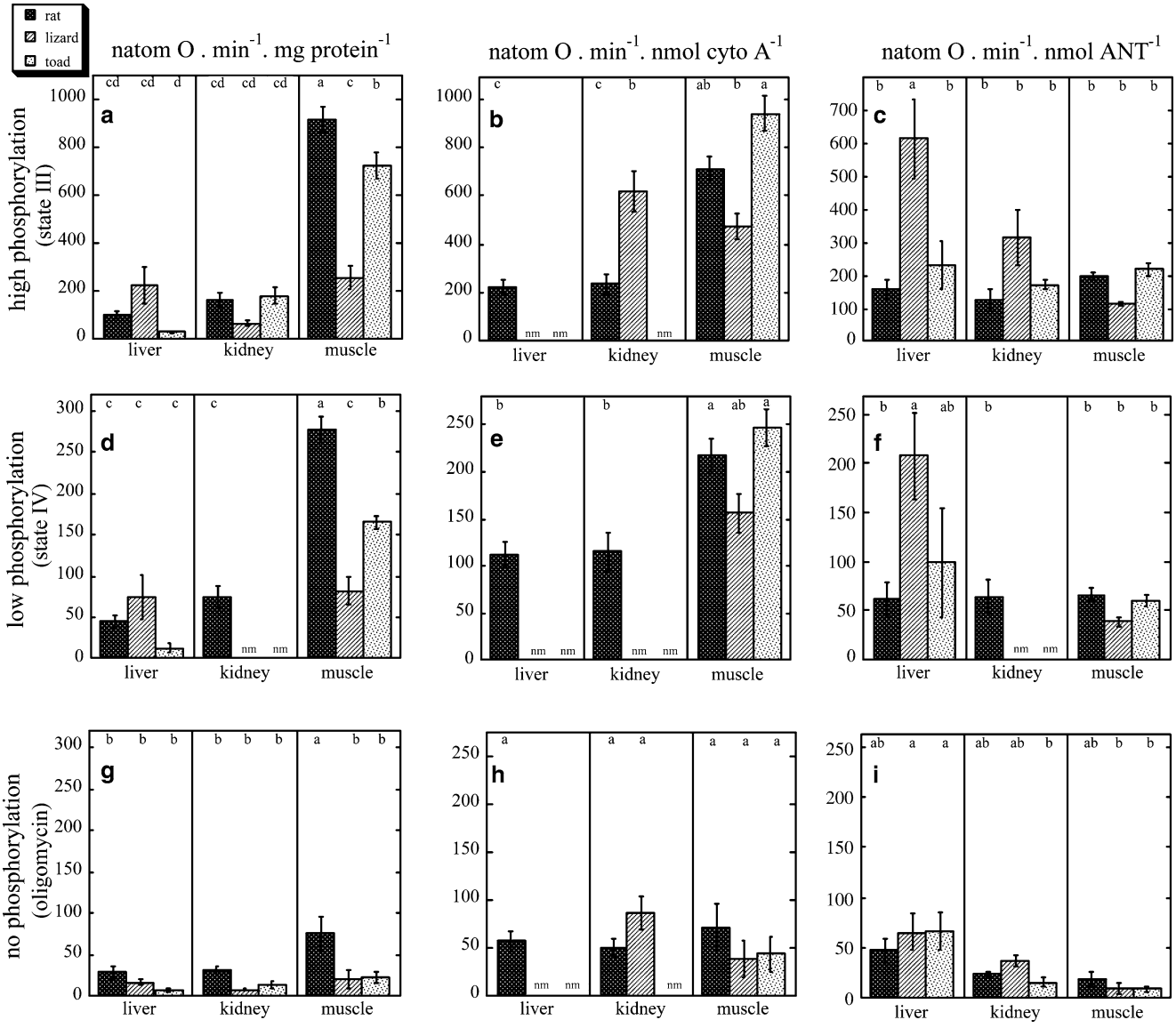

Fig. 1 The respiration rates of liver, kidney, and skeletal muscle mitochondria from the laboratory rat (*R. norvegicus*), the bearded dragon lizard (*P. vitticeps*), and the cane toad (*B. marinus*). The *top row* shows three graphs (**a, b, c**) where mitochondrial respiration is measured under state III conditions. The *middle row* shows (graphs **d, e, f**) mitochondrial respiration measured under state IV conditions and the *bottom row* (graphs **g, h, i**) shows the rates of oligomycin-inhibited mitochondrial respiration. The *left-hand column of three graphs* (**a, d, g**) shows mitochondrial respiration expressed relative to mitochondrial protein. The middle column of three graphs (**b, e, h**) shows mitochondrial respiration expressed relative to cytochrome A content. The right-hand column of three graphs (**c, f, i**) shows mitochondrial respiration expressed relative to mitochondrial ANT content. The *error bars* on each column are ±SEM. In each graph, those columns that do not share a *common letter* are significantly different

Table 3 Fatty acid composition of phospholipids of isolated mitochondria from the liver of a mammal (the rat, *R. norvegicus)*, a reptile (the lizard, *P. vitticeps)*, and an amphibian (the toad, *B. marinus)*

	Rat	Lizard	Toad
14:1	0.2 ± 0.0^{a}	1.4 ± 0.1^{b}	$1.0 \pm 0.3^{a,b}$
16:0	15.6 ± 0.4^{a}	7.7 ± 0.2^{b}	9.5 ± 1.5^{b}
16:1 *n*-7	0.9 ± 0.5^{a}	2.4 ± 1.5^{a}	1.1 ± 0.3^{a}
18:0	14.4 ± 0.1^{a}	13.3 ± 0.7^{a}	8.5 ± 1.0^{b}
18:1 *n*-9	4.3 ± 0.3^{a}	21.4 ± 2.7^{b}	19.6 ± 1.5^{b}
18:1 *n*-7	6.9 ± 0.5^{a}	2.5 ± 2.5^{a}	0.4 ± 0.4^{a}
18:2 *n*-6	14.4 ± 0.8^{a}	35.1 ± 2.4^{b}	28.8 ± 1.5^{b}
18:3 *n*-3	0.2 ± 0.0^{a}	1.0 ± 0.3^{a}	1.1 ± 0.7^{a}
20:4 *n*-6	30.9 ± 0.0^{a}	8.9 ± 1.1^{b}	15.5 ± 1.2^{c}
20:5 *n*-3	0.1 ± 0.0^{a}	0.6 ± 0.2^{a}	0.8 ± 0.3^{a}
22:6 *n*-3	7.7 ± 1.1^{a}	0.7 ± 0.1^{b}	2.2 ± 0.4^{b}
Percent UFA	67.1 ± 0.5^{a}	78.2 ± 0.4^{b}	73.7 ± 0.9^{c}
Percent MUFA	13.0 ± 0.3^{a}	29.1 ± 3.2^{b}	$24.7 \pm 2.4^{a,b}$
Percent PUFA	54.2 ± 0.2^{a}	49.1 ± 3.2^{a}	49.0 ± 2.2^{a}
Percent *n*-6 PUFA	45.6 ± 0.8^{a}	44.8 ± 3.5^{a}	45.0 ± 1.6^{a}
Percent *n*-3 PUFA	8.1 ± 1.1^{a}	2.9 ± 0.8^{b}	$4.0 \pm 1.0^{a,b}$
Unsaturation index	212 ± 5^{a}	153 ± 4^{b}	165 ± 5^{b}
Ave. chain length	18.5 ± 0.1^{a}	18.0 ± 0.0^{b}	18.0 ± 0.0^{b}

Values are shown only for the major fatty acids Each fatty acid is identified by a numbering system where the first number represents the length of the C chain and the second number represents the number of double bonds. The number following '*n*' represents the number of carbons from the methyl end of the acyl chain where the most terminal double bond is located
Values are mean ± SEM Values in each row with different superscripts are significantly different. Unsaturation index is the calculated average number of double bonds per 100 fatty acid chains

those preparations for which this was possible. As can be seen from this figure, use of these denominators changed the comparison between tissues and species. For example, whereas there was a 15-fold range in state III respiration between the different mitochondrial sources when expressed per mg protein, there was only ~fivefold difference when expressed per nmol ANT. The range in state IV and oligomycin-inhibited rates also decreased when similarly expressed. A 21-fold range in state IV rates (per mg protein) decreased to ~twofold and ~fivefold range, while a ~ninefold range in oligomycin-inhibited rates (per mg protein) decreased to twofold and ~sevenfold range of values, respectively. Although not shown in Fig. 1, the range of values also decreased when respiration rates were expressed relative to the other cytochromes. The use of cytochrome A as a

Table 4 Fatty acid composition of phospholipids of isolated mitochondria from the kidney of the rat (*R. norvegicus)*, the lizard (*P. vitticeps)*, and the toad (*B. marinus)*. Values are shown only for the major fatty acids

	Rat	Lizard	Toad
14:1	0.1 ± 0.0^{a}	0.5 ± 0.0^{a}	2.4 ± 0.3^{b}
16:0	15.8 ± 0.5^{a}	$10.8 \pm 2.6^{a,b}$	8.3 ± 0.3^{b}
16:1 *n*-7	0.7 ± 0.5^{a}	2.3 ± 0.2^{b}	$1.0 \pm 0.1^{a,b}$
18:0	17.1 ± 0.2^{a}	13.3 ± 1.6^{a}	7.4 ± 0.2^{b}
18:1 *n*-9	7.9 ± 0.1^{a}	15.1 ± 1.2^{b}	18.3 ± 1.3^{b}
18:1 *n*-7	3.1 ± 0.2^{a}	5.1 ± 0.1^{b}	2.0 ± 0.2^{c}
18:2 *n*-6	12.8 ± 0.4^{a}	29.2 ± 1.0^{b}	31.6 ± 1.2^{b}
18:3 *n*-3	0.0 ± 0.0^{a}	0.1 ± 0.1^{a}	0.2 ± 0.1^{a}
20:4 *n*-6	36.7 ± 0.4^{a}	10.8 ± 1.0^{b}	18.6 ± 1.8^{c}
20:5 *n*-3	0.2 ± 0.0^{a}	1.5 ± 0.2^{b}	1.2 ± 0.0^{b}
22:6 *n*-3	2.1 ± 0.1^{a}	0.9 ± 0.2^{b}	1.4 ± 0.0^{b}
Percent UFA	66.5 ± 0.6^{a}	$74.3 \pm 4.6^{a,b}$	81.0 ± 0.4^{b}
Percent MUFA	12.3 ± 0.5^{a}	26.9 ± 2.3^{b}	25.5 ± 1.3^{b}
Percent PUFA	$54.1 \pm 0.1^{a,b}$	47.4 ± 2.3^{a}	55.5 ± 0.9^{b}
Percent *n*-6 PUFA	50.9 ± 0.1^{a}	44.8 ± 3.7^{a}	51.5 ± 0.8^{a}
Percent *n*-3 PUFA	3.3 ± 0.2^{a}	4.2 ± 0.0^{b}	$3.7 \pm 0.1^{a,b}$
Unsaturation Index	206 ± 1^{a}	158 ± 10^{b}	186 ± 4^{a}
Ave. Chain length	18.5 ± 0.0^{a}	18.2 ± 0.1^{b}	18.2 ± 0.0^{b}

Each fatty acid is identified by a numbering system where the first number represents the length of the C chain and the second number represents the number of double bonds. The number following '*n*' represents the number of carbons from the methyl end of the acyl chain where the most terminal double bond is located
Values are mean ± SEM Values in each row with different superscripts are significantly different. Unsaturation index is the calculated average number of double bonds per 100 fatty acid chains

Table 5 Fatty acid composition of phospholipids of isolated mitochondria from skeletal muscle of the rat (*R. norvegicus)*, the lizard (*P. vitticeps)*, and the toad (*B. marinus)*

	Rat	Lizard	Toad
14:1	3.3 ± 0.2^{a}	7.4 ± 1.6^{a}	2.7 ± 1.0^{a}
16:0	11.2 ± 0.5^{a}	4.5 ± 0.3^{b}	5.7 ± 0.3^{b}
16:1 *n*-7	0.4 ± 0.4^{a}	0.6 ± 0.2^{a}	1.5 ± 0.6^{a}
18:0	11.1 ± 0.3^{a}	12.4 ± 0.6^{a}	9.8 ± 1.4^{a}
18:1 *n*-9	5.2 ± 0.9^{a}	11.9 ± 0.6^{b}	12.4 ± 0.9^{b}
18:1 *n*-7	4.2 ± 0.4^{a}	3.1 ± 1.6^{a}	2.6 ± 0.3^{a}
18:2 *n*-6	20.9 ± 0.2^{a}	28.9 ± 3.3^{a}	33.3 ± 3.3^{a}
18:3 *n*-3	0.0 ± 0.0^{a}	0.5 ± 0.2^{a}	0.9 ± 0.4^{a}
20:4 *n*-6	19.3 ± 0.5^{a}	15.3 ± 0.6^{b}	20.8 ± 0.4^{a}
20:5 *n*-3	0.7 ± 0.7^{a}	1.1 ± 0.3^{a}	1.2 ± 0.2^{a}
22:6 *n*-3	18.0 ± 0.2^{a}	3.3 ± 0.5^{b}	2.2 ± 0.3^{b}
Percent UFA	74.5 ± 1.1^{a}	$81.4 \pm 1.7^{a,b}$	82.4 ± 1.1^{b}
Percent MUFA	14.7 ± 0.9^{a}	27.9 ± 1.3^{b}	$21.9 \pm 2.9^{a,b}$
Percent PUFA	59.9 ± 0.3^{a}	53.5 ± 1.2^{a}	60.5 ± 2.2^{a}
Percent *n*-6 PUFA	41.6 ± 0.1^{a}	$45.5 \pm 2.3^{a,b}$	55.8 ± 2.7^{b}
Percent *n*-3 PUFA	18.3 ± 0.2^{a}	6.0 ± 1.4^{b}	4.6 ± 0.6^{b}
Unsaturation Index	244 ± 2^{a}	187 ± 5^{b}	198 ± 1^{b}
Ave. Chain length	18.7 ± 0.0^{a}	18.1 ± 0.1^{b}	18.2 ± 0.0^{b}

Values are shown only for the major fatty acids Each fatty acid is identified by a numbering system where the first number represents the length of the C chain and the second number represents the number of double bonds. The number following '*n*' represents the number of carbons from the methyl end of the acyl chain where the most terminal double bond is located
Values are mean ± SEM. Values in each row with different superscripts are significantly different. Unsaturation index is the calculated average number of double bonds per 100 fatty acid chains

denominator led to the smallest range in rates compared to the use of the other cytochromes as the denominator. This suggests that respiration rate of mitochondria from different tissues and species is largely determined by the contents of cytochrome A (and thus complex IV). Mitochondrial respiration showed less variation when expressed relative to cytochrome A than when expressed relative to ANT.

The differences between tissues also changed with the use of these denominators. While muscle mitochondria were generally more active when rates were expressed relative to mitochondrial protein, they were often the least active relative to mitochondrial ANT content. In the rat, there were significant tissue differences in mitochondrial respiratory activity relative to both mitochondrial protein and cytochrome A, however there was no significant difference between tissues when rates were expressed relative to mitochondrial ANT content (see Fig. 1).

Fatty acid composition of mitochondrial membranes

Tables 3, 4 and 5 present the fatty acid composition of mitochondrial phospholipids from the three species for liver, kidney, and skeletal muscle, respectively. As explained in the *Introduction*, these data were obtained from separate groups of animals to those used for measurement of mitochondrial composition and respiratory activity. Mitochondria from all three tissues showed similar patterns. In mitochondrial membranes prepared from liver, kidney, and skeletal muscle, the rat had the greatest unsaturation index followed by the toad with the lizard mitochondrial membranes having the lowest degree of unsaturation. In all three species examined, skeletal muscle mitochondria had the greatest unsaturation index with liver mitochondria having the lowest value in the toads and lizards and kidney mitochondria having the lowest value in the rat. In mitochondria from all three tissues, the rat, despite having mitochondrial membranes with the highest unsaturation index, had the lowest content of unsaturated fatty acids (expressed as per cent of total fatty acids). Considering the unsaturated fatty acids, both the ectothermic species had mitochondria with relatively more monounsaturated fatty acids but relatively less polyunsaturates in their membranes than the endothermic rat.

The *n*-6 polyunsaturates were more abundant than the *n*-3 polyunsaturates in mitochondria from all three tissues in all three species. The greater degree of unsaturation in the rat mitochondrial membranes compared to the ectotherms was manifest within both polyunsaturate classes. Within the *n*-6 polyunsaturates, rat mitochondria have less linoleic acid (18:2 *n*-6) than lizard and toad mitochondria but relatively more of the highly polyunsaturated arachidonic acid (20:4 *n*-6). The ratios of 20:4 to 18:2 in liver mitochondrial membranes are respectively 2.1, 0.3, and 0.5 for the rat, lizard, and toad. The respective ratios are 2.9, 0.4, and 0.6 for kidney mitochondria and 0.9, 0.5, and 0.6 for skeletal muscle mitochondria.

Similarly, within the *n*-3 polyunsaturates the rat mitochondrial membranes have a relatively greater amount of the long chain highly polyunsaturated *n*-3 fatty acid chains than do mitochondrial membranes from the two ectotherm species. For example, in liver mitochondria the ratio of DHA (22:6 *n*-3) to the sum of

the two less unsaturated shorter chain *n*-3 polyunsaturates (i.e. 18:3 *n*-3 and 20:5 *n*-3) is 25.7 in the rat mitochondria and 0.4 and 1.2 in lizard and toad mitochondria, respectively. In kidney mitochondria the same ratios are 7.0 for the rat, 0.6 for the lizard, and 1.0 for the toad. In muscle skeletal mitochondria these ratios are 25.7, 2.1, and 1.0 for the rat, lizard and toad, respectively. DHA is a substantial and dominant contributor to the greater relative unsaturation of rat mitochondrial membranes compared to lizard and toad mitochondrial membranes (although it is not the only contributor). An interesting finding is that in lizard and toad there is an approximately equal relative 22:6 *n*-3 content in mitochondrial membranes from all three tissues. In liver and skeletal muscle mitochondria from the rat, there is an enhanced 22:6 *n*-3 content compared to the mitochondria from the ectotherms but this is not the case for the kidney mitochondria. In mitochondria from kidney, it is the relative abundance of 20:4 *n*-6 that is increased in the rat compared to the ectothermic species.

Discussion

The quantification of various mitochondrial proteins gives insight into the design of mitochondria in different tissues. For example in all three species, muscle mitochondria had several times more ANT than cytochrome A, which was the most abundant of the cytochromes. This contrasts with the situation in mitochondria from liver and kidney, in which ANT was less abundant and likely reflects the need for muscle mitochondria to rapidly exchange adenine nucleotides between the sarcoplasm and mitochondrial matrix when operating at high rates of ADP phosphorylation during intense activity. Our muscle preparations are pooled populations of subsarcolemmal and interfibillar mitochondria and we do not therefore know if there are differences between these two types of muscle mitochondria in these properties.

Similarly, when the summed abundance of all cytochromes and ANT are compared to the abundance of phospholipids in mitochondria, muscle mitochondria have relatively greater amounts of these important mitochondrial membrane proteins than liver and kidney mitochondria. Because of the inability to measure the cytochromes in liver mitochondrial preparations and the phospholipid content of the kidney mitochondrial preparations from the lizard and the toad, we can only compare this aspect of mitochondrial design in the three tissues from the rat. The sum of these proteins in rat liver mitochondria was 2.23 nmoles mg protein^{-1}, 2.51 nmoles mg protein^{-1} in rat kidney mitochondria, and 5.28 nmoles mg protein^{-1} in rat muscle mitochondria. This means that, in liver mitochondria there are about 260 phospholipid molecules, in kidney mitochondria about 160 phospholipid molecules, and in muscle mitochondria about 100 phospholipid molecules, for each molecule of these mitochondrial proteins. This likely reflects a different membrane density of these mitochondrial proteins in the different tissues. In this respect it is of interest that muscle mitochondria from all three species had a similar ratio. Muscle mitochondria from the lizards averaged 87 and those from the toad averaged 112 phospholipid molecules for each molecule of these proteins.

Taking the values for rat liver mitochondria from Table 1 we can calculate that for every cytochrome A molecule there are 1,264 phospholipid molecules. Assuming that the membrane is a bilayer and each phospholipid molecule in the membrane occupies an area of 0.5 nm^2 (this is the average area measured in monolayers of mixed rat phospholipids, see Wu et al. 2001) this corresponds to one cytochrome oxidase per 316 nm^2 . When it is considered that this calculation does not account for other proteins in the mitochondrial membrane, it is remarkably similar to the value of one cytochrome oxidase per 400 nm^2 calculated by Scherzmann et al. (1986) from stereological data for rat liver mitochondria. When similar assumptions are used, the respective values for rat kidney and rat muscle mitochondria are respectively one cytochrome oxidase per 155 nm^2 and one cytochrome oxidase per 96 nm^2 of mitochondrial membrane. In muscle mitochondria from the cat, Scherzmann et al. (1989) calculated one cytochrome oxidase per 106 nm^2 of membrane surface area.

Measurement of both composition and respiratory activity of the same mitochondrial preparation allows expression of mitochondrial respiration relative to a number of denominators as well as calculation of the turnover rates of the individual components of the respiratory chain. For rat liver mitochondria, assuming two electrons natom O^{-1} reduced, we can calculate that cytochrome oxidase operates at a maximum rate of about 7.4 electrons s^{-1} when provided with pyruvate as substrate. This is very similar to the estimate that an average rat liver mitochondrion has 15,600 cytochrome oxidase units,''which produce an electron flow of over 100,000 electrons s^{-1} with pyruvate as substrate'' (Schwerzmann et al. 1986). The cytochrome oxidase units can be calculated to operate maximally at 23.7 electrons s^{-1} in rat muscle mitochondria and 31.3 in toad muscle mitochondria, both rates being faster than the same enzyme in rat liver mitochondria. This is less than the 59.6 electrons s^{-1} calculated for cytochrome oxidase in cat muscle mitochondria provided with excess reduced cytochrome C (Schwerzmann et al. 1989), but greater than the 12.8 electrons s^{-1} calculated if the cat muscle mitochondria were using pyruvate/malate, the same substrate as used in the present study. However, it is considerably less than the 747 electrons s^{-1} calculated for cytochrome oxidase in honeybee muscle mitochondria during flight (Suarez et al. 2000).

Because all the cytochromes are approximately equimolar in abundance in mitochondria from the lizard, they will all operate at approximately the same rate. However, in mitochondria from the other two species the least abundant cytochrome will of course have the fastest

turnover rate and the most abundant cytochrome the slowest turnover rate. In this study, cytochrome C1 has both roles, being the cytochrome with the fastest turnover in one situation as well as the cytochrome with the slowest turnover in a different situation. For example, in maximally respiring toad muscle mitochondria, cytochrome C1 operates at 94.4 electrons s^{-1}, while in maximally respiring mitochondria from rat liver it turns over at 6 electrons s^{-1} . In mitochondria that are not phosphorylating ADP, electron flow through cytochromes will be considerably slower than these state III rates. Cytochrome C1 operates at approximately 1 electrons s^{-1} in lizard muscle mitochondria during oligomycin-inhibited respiration. Because they are equimolar in abundance, all cytochromes in these lizard mitochondria are operating at about the same rate and these represent the slowest turnover rates measured in this study.

It is not possible to use the same method to calculate the relative turnover rates of ANT as this will depend on the P/O ratio during respiration. Of course during oligomycin-inhibited respiration there is no ADP phosphorylation and thus presumably there is no net translocation of adenine nucleotides across the mitochondrial inner membrane.

In previous comparisons of liver mitochondria from a variety of species it has been shown that mitochondrial proton leak (per mg mitochondrial protein) in vertebrates is positively correlated with the degree of polyunsaturation of the mitochondrial membrane (e.g. Porter et al. 1996; Brookes et al. 1998; Brand et al. 2003). In these studies, it was observed that DHA (a highly polyunsaturated omega-3 fatty acid) was a particularly important membrane constituent in this relationship. Although we have not measured proton leak curves in the current study, we have measured mitochondrial respiration in the presence of oligomycin, which gives an approximation of mitochondrial proton leak. There is a similar positive relationship in the mitochondrial comparison between membrane polyunsaturation and this estimate of mitochondrial proton leak (Fig. 2a). As in the previous studies, the membrane DHA content appeared to be particularly important (Fig. 2b). Membrane polyunsaturation and docosahexaenoic content are not only related to the oligomycin-inhibited respiration rate of mitochondria but also significantly positively correlated with the maximal respiratory activity (state III respiration) of the mitochondria (Fig. 2c, 2d). This figure thus shows that the

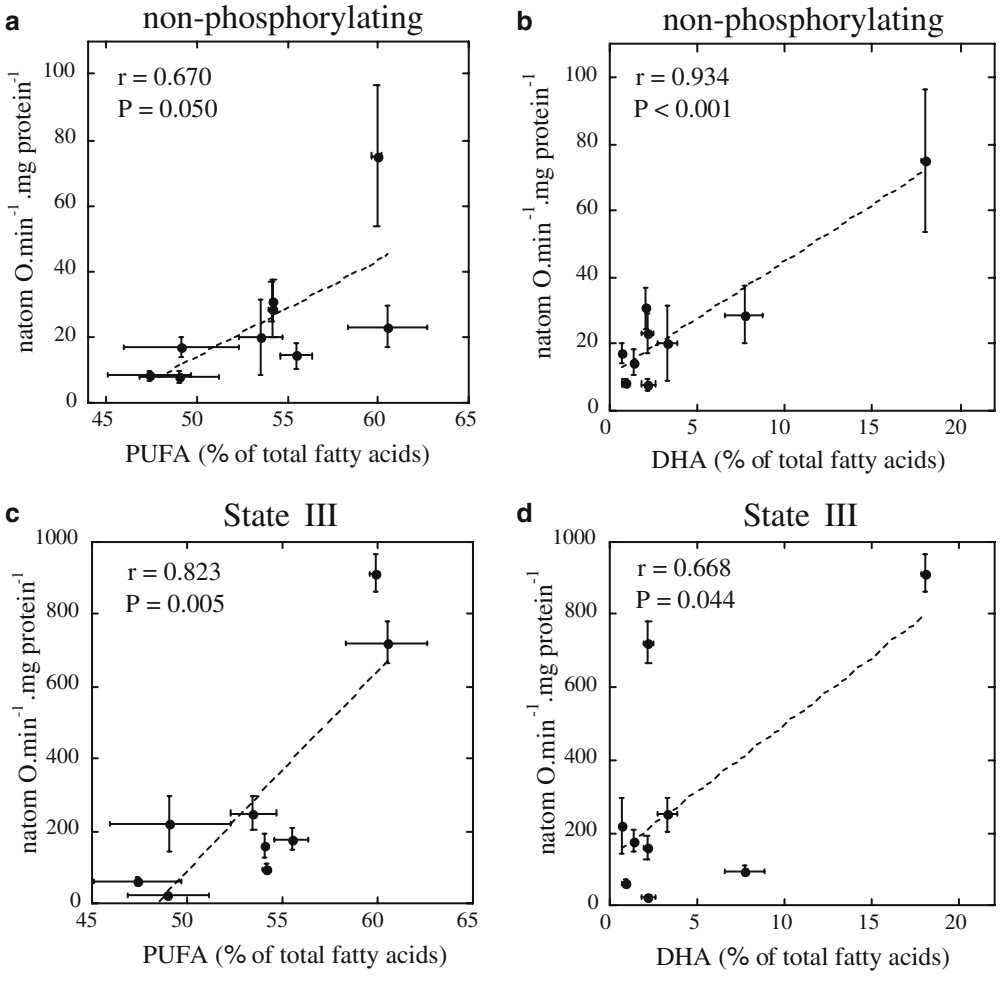

Fig. 2 The correlation between the fatty acid composition of mitochondrial membrane and respiration rate (expressed relative to mitochondrial protein) of liver, kidney, and skeletal muscle mitochondria from the laboratory rat (*R. norvegicus*), the bearded dragon lizard (*P. vitticeps*), and the cane toad (*B. marinus*). The *top two graphs* (**a, b**) show oligomycin-inhibited rates of mitochondrial respiration while the *bottom two graphs* (**c, d**) show mitochondrial respiration rates measured under state III conditions. The *two left-hand graphs* (**a, c**) show mitochondrial respiration related to the per cent polyunsaturate content of the mitochondrial membrane while the *two left-hand graphs* show mitochondrial respiration related to the DHA content of the mitochondrial membrane. The *error bars* for each data point represent ±SEM

relationship between the nature of the membrane bilayer and mitochondrial activity is not restricted to liver mitochondria and is also not restricted to mitochondrial proton leak. The polyunsaturation of mitochondrial membranes significantly correlates with the respiration rate of non-phosphorylating mitochondria (oligomycin-inhibited respiration, Fig. 2a), the respiration rate of mitochondria during rapid ADP phosphorylation (state III; Fig. 2c), as well as the respiration rate of mitochondria during slower rates of ADP phosphorylation (i.e. state IV, see Fig. 3a).

However, as pointed out in the *Introduction*, in all these correlations mitochondrial respiration is expressed relative to mitochondrial protein content and it is unclear as to how variation in the protein content of different mitochondria might be affecting this relationship. The measurement of the macromolecular components of mitochondria allows one to examine whether the molecular activity of mitochondrial proteins is correlated with membrane lipid composition. As can be seen from Fig. 3, the molecular activity of a number of mitochondrial cytochromes during state IV respiration is positively correlated with the degree of polyunsaturation of mitochondrial membranes. There were statistically significant positive correlations between membrane polyunsaturation and molecular activity of cytochrome B (Fig. 3b), cytochrome C1 (Fig. 3c), cytochrome A (Fig. 3d), and cytochrome C (data not shown) during state IV respiration. There were also significant positive correlations (data not shown) between membrane lipid composition and the molecular activity of these cytochromes during maximal (state III) and oligomycin-inhibited respiration.

Such correlations suggest, but do not unequivocally demonstrate, a cause- and-effect relationship between the lipid composition of mitochondrial membranes and the activity of proteins in these membranes. Only experimental manipulation of membrane lipid composition can prove a cause-and-effect relationship. When membrane DHA content is increased in liver mitochondria of mice both in vivo (by feeding the mice menhaden oil) and in vitro (by lipid fusion) the proton leak of the mice mitochondria is increased (Stillwell et al. 1997). Over 30 years ago, Hazel (1972a, 1972b) showed that mitochondrial membrane lipids extracted from cold-acclimated goldfish exhibit a greater reactivation of delipidated succinic dehydrogenase than do those from warm-acclimated goldfish. One of the major changes that occurs during cold-acclimation in fish is an increased membrane polyunsaturation, especially due to

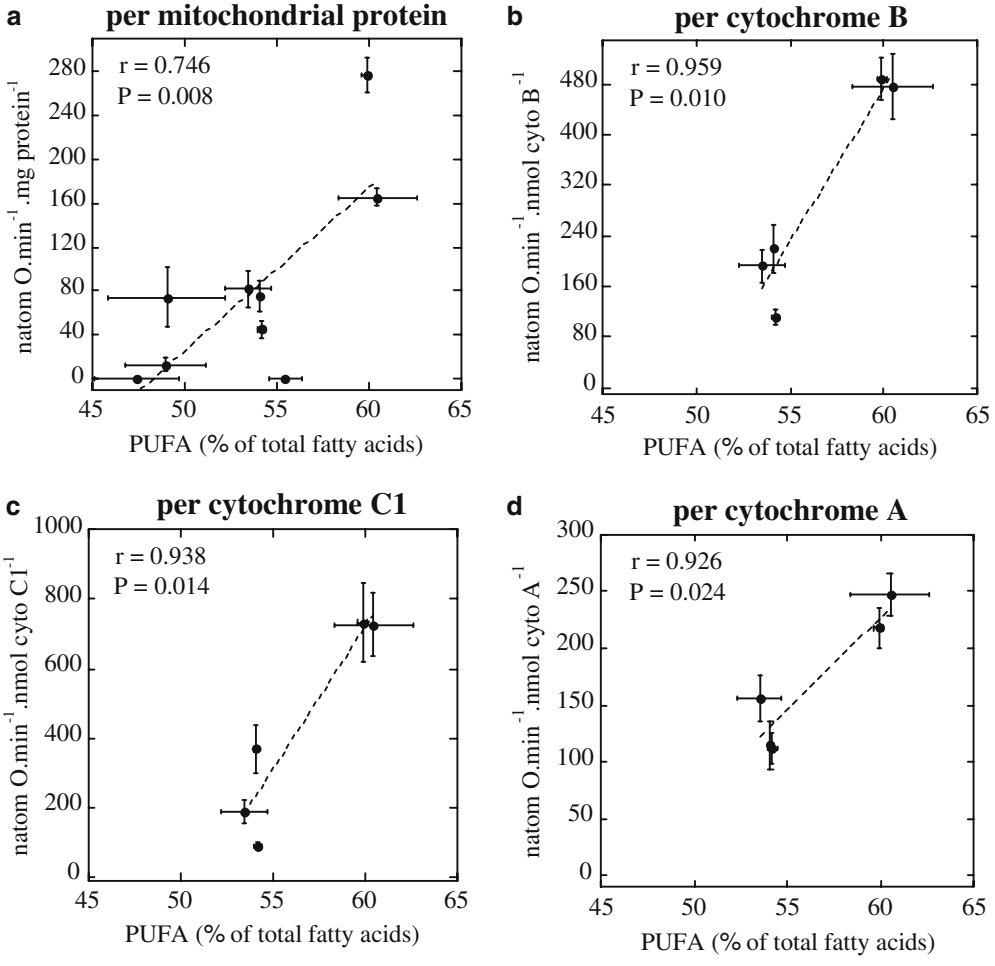

Fig. 3 The correlation between the per cent polyunsaturation of the mitochondrial membrane and respiration rate (measured under state IV conditions) of liver, kidney, and skeletal muscle mitochondria from the laboratory rat (*R. norvegicus*), the bearded dragon lizard (*P. vitticeps*), and the cane toad (*B. marinus*). Each graph presents mitochondrial respiration expressed relative to a different mitochondrial macromolecular component; mitochondrial protein (**a**), cytochrome B (**b**), cytochrome C1 (graph C) and cytochrome A (**d**). The *error bars* for each data point represent ±SEM

increases in the DHA acid content of membrane phospholipids (Hazel and Williams 1990). Wodtke (1981a, 1981b) reported similar membrane lipid effects in carp mitochondria. Cross-over experiments such as those of Hazel (1972a, 1972b) experimentally demonstrate the importance of membrane lipid effects on the activity of membrane proteins. They are similar to recent experimental evidence supporting an effect of lipid composition on the membrane-bound Na^+ –K^+ -ATPase (Else and Wu 1999; Wu et al. 2004). Such general effects of membrane lipid composition on the molecular activity of membrane proteins have been suggested to be an important determinant of overall metabolic rate of different species and tissues (Hulbert and Else 1999, 2000, 2005).

The use of denominators apart from mitochondrial protein content is, of course, not new and has been used by previous investigators. For example, Leary et al. (2003) recently have shown that most of the differences between mitochondria from different types of muscle fibres in fish disappears when cytochrome oxidase is used as the denominator. Such an approach should be used more often in comparative studies. The present study, by measuring the composition of mitochondria, additional to measurement of protein content, has also shown that a number of membrane-bound mitochondrial proteins have their activity influenced by the nature of the surrounding membrane lipid. We believe that extension of this approach (to other species and to other tissues) will give considerable insight into evolution of mitochondrial design in animals.

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