

## Mitochondrial proton leak rates in the slow, oxidative myotomal muscle and liver of the endothermic shortfin mako shark (*Isurus oxyrinchus*) and the ectothermic blue shark (*Prionace glauca*) and leopard shark (*Triakis semifasciata*)

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### Summary

Mitochondrial proton leak was assessed as a potential heat source in the slow, oxidative (red) locomotor muscle and liver of the shortfin mako shark (*Isurus oxyrinchus*), a regional endotherm that maintains the temperature of both tissues elevated above ambient seawater temperature. We hypothesized that basal proton leak rates in red muscle and liver mitochondria of the endothermic shortfin mako shark would be greater than those of the ectothermic blue shark (*Prionace glauca*) and leopard shark (*Triakis semifasciata*). Respiration rate and membrane potential in isolated mitochondria were measured simultaneously at 20°C using a Clark-type oxygen electrode and a lipophilic probe (triphenylmethylphosphonium, TPMP<sup>+</sup>). Succinate-stimulated respiration was titrated with inhibitors of the electron transport chain, and the non-linear relationship between respiration rate and membrane potential was quantified. Mitochondrial densities of both tissues were measured by applying the point-contact method to electron micrographs so that proton leak activity of the entire tissue could be assessed. In all three shark species, proton leak occurred at a higher rate in red muscle mitochondria than in liver mitochondria. For each tissue, the proton leak curves of the three species overlapped and, at a membrane potential of 160 mV, mitochondrial proton leak rate (nmol H<sup>+</sup> min<sup>-1</sup> mg<sup>-1</sup> protein) did not differ significantly between the endothermic and ectothermic sharks. This finding indicates that red muscle and liver

mitochondria of the shortfin mako shark are not specialized for thermogenesis by having a higher proton conductance. However, mako mitochondria did have higher succinate-stimulated respiration rates and membrane potentials than those of the two ectothermic sharks. This means that under *in vivo* conditions mitochondrial proton leak rates may be higher in the mako than in the ectothermic species, due to greater electron transport activity and a larger proton gradient driving proton leak. We also estimated each tissue's total proton leak by combining mitochondrial proton leak rates at 160 mV and tissue mitochondrial density data with published values of relative liver or red muscle mass for each of the three species. In red muscle, total proton leak was not elevated in the mako shark relative to the two ectothermic species. In the liver, total proton leak would be higher in the mako shark than in both ectothermic species, due to a lower proton conductance in the blue shark and a lower liver mitochondrial content in the leopard shark, and thus may contribute to endothermy.

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Key words: elasmobranch, endothermy, liver, mitochondria, mitochondrial density, muscle, proton conductance, proton leak, shark, thermogenesis.

### Introduction

Comparisons of endothermic and ectothermic terrestrial vertebrates of similar body mass and preferred body temperature have demonstrated that metabolically active tissues (liver, kidney, brain and heart) comprise a larger percentage of total body mass in endotherms and that these

tissues have higher aerobic enzyme activities and greater mitochondrial densities, all of which correlate with the higher metabolic rates of endothermic species (Bennett, 1972; Else and Hulbert, 1981; Else and Hulbert, 1985; Else et al., 2004b). Endothermic vertebrates also have higher membrane sodium conductances, higher mitochondrial membrane proton

conductances, and membrane phospholipids with a greater percentage of unsaturated fatty acids, leading Hulbert and Else to propose the 'membranes as pacemakers of metabolism' theory (Else and Hulbert, 1987; Hulbert and Else, 1989; Hulbert and Else, 1990; Hulbert and Else, 1999; Hulbert and Else, 2000; Brand et al., 1991; Brand et al., 1994b; Else et al., 2004a; Else et al., 2004b). The higher mitochondrial proton conductances in endothermic species are due to higher rates of basal proton leak across the inner mitochondrial membrane, which represents 20–30% of resting metabolic rate (Brand, 1990; Brand et al., 1991; Brand et al., 1994a; Brookes et al., 1998). Brand et al. recently showed that 55–65% of basal proton leak can be attributed to the presence of adenine nucleotide translocase, an inner mitochondrial membrane protein (Brand et al., 2005). In rats, basal proton leak accounts for 25% and 50% of resting metabolism in hepatocytes and skeletal muscle, respectively, for 22% and 34% of oxygen consumption in working liver and skeletal muscle, respectively, and for 20–25% of whole animal basal metabolic rate (Brand, 1990; Brand et al., 1994a; Rolfe and Brand, 1996; Rolfe et al., 1999).

Basal proton leak has been implicated in the evolution of endothermy in birds and mammals because liver mitochondrial proton leak rates are higher in endothermic vertebrates than in comparably sized ectotherms of the same preferred body temperature [e.g. rat and pigeon versus the bearded dragon, a desert lizard (Brand et al., 1991; Brand et al., 1994b; Brookes et al., 1998) (for a review, see Stuart et al., 2001)]. Our goal was to extend these studies to fishes, to determine whether mitochondrial proton leak rates are greater in endothermic fishes than in related ectothermic species and thereby may contribute to regional endothermy. We compared the endothermic shortfin mako shark with the ectothermic blue shark and leopard shark. The shortfin mako shark is an active, pelagic predator that maintains the temperature of the slow, oxidative (red) locomotor muscle, the cranial region and the viscera, including the liver, elevated above ambient seawater temperature (for reviews, see Carey et al., 1985; Bernal et al., 2001a; Carlson et al., 2004). Like the mako, the blue shark is an epipelagic predator that swims continuously and makes vertical movements throughout the day, presumably for feeding (Sciarrotta and Nelson, 1977; Carey and Scharold, 1990; Sepulveda et al., 2004; Weng et al., 2005). The leopard shark is less active and inhabits shallower water, where it feeds primarily on benthic prey such as worms, clams, crabs and shrimp but also on both pelagic and demersal fishes (Russo, 1975; Talent, 1976; Webber and Cech, 1998). We tested the hypothesis that rates of proton leak in red locomotor muscle and liver mitochondria are higher in the endothermic shortfin mako shark than in the ectothermic blue and leopard sharks.

## Materials and methods

### *Fish collection*

Five shortfin mako sharks (*Isurus oxyrinchus* Rafinesque; range of total body lengths, 86–211 cm), four blue sharks

[*Prionace glauca* (L.); 120–154 cm] and five leopard sharks (*Triakis semifasciata* Girard; 97–127 cm) were captured by hook and line off the coast of southern California, USA in areas with sea surface temperatures of approximately 17–21.5°C. Fish were kept alive in a transport tank for up to two hours during transfer to the laboratory. Each shark was then stunned by a sharp blow to the head and spinalectomized, and approximately 25 g of red muscle and liver were removed and placed on ice. Sharks were collected under California Department of Fish and Game scientific collecting permits, and all experimental protocols were approved by the California State University Fullerton and University of California San Diego Institutional Animal Care and Use Committees.

### *Isolation of mitochondria*

Mitochondria were isolated following methods similar to those described previously for sharks (Moyes et al., 1990; Ballantyne et al., 1992). Tissue samples were minced on ice and homogenized in buffer (140 mmol l<sup>-1</sup> KCl, 10 mmol l<sup>-1</sup> EGTA, 5 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 500 mmol l<sup>-1</sup> sucrose, 20 mmol l<sup>-1</sup> Hepes buffer, pH 7.3 at 20°C) containing 2% defatted bovine serum albumin (BSA) using a Teflon™-glass homogenizer powered by an electrical drill press running at approximately 200 r.p.m. The homogenate was centrifuged at 1000 g for 4 min at 4°C to remove cellular particulates, and any visible fat in the supernatant was removed. The supernatant was then filtered through several layers of cheesecloth and centrifuged at 9500 g for 10 min at 4°C. The resulting pellet containing the mitochondria was washed twice in approximately 25 ml of homogenization buffer, and the final mitochondrial pellet was re-suspended in approximately 0.5–1.0 ml of 2% BSA homogenization buffer. Protein concentration of each mitochondrial suspension was measured with the Biuret assay using BSA as the standard and blanks containing 2% BSA homogenization buffer.

Prior to proton leak assays, the respiratory control ratio (RCR) of the isolated mitochondria was measured. The RCR is defined as the ratio of ADP-stimulated respiration rate (state 3) to non-stimulated respiration rate (state 4) (Chance and Williams, 1956). In the present study, RCRs were measured in the presence of 0.29 mmol l<sup>-1</sup> ADP with succinate (4 mmol l<sup>-1</sup>) as the oxidative substrate and in the absence of oligomycin, and proton leak assays were run only on isolated mitochondria with an RCR value of ≥2.

### *Measurement of mitochondrial proton leak rates*

Proton leak was measured using methods developed by Brand (e.g. Brand et al., 1991; Brand et al., 1994a; Brand et al., 2003). We used a common measurement temperature of 20°C for all samples because it approximated the average water temperature in which the sharks were captured and because red muscle and visceral temperatures in mako sharks of the size studied are 17–27°C when swimming in water temperatures of 17–20°C (Bernal et al., 2001b; Sepulveda et al., 2004). Respiration rate and membrane potential were measured simultaneously using a thermostatically controlled

polarographic oxygen electrode system (Rank Brothers Ltd, Bottisham, Cambridgeshire, UK) and a triphenylmethylphosphonium (TPMP<sup>+</sup>) electrode, respectively. Mitochondria were incubated at a concentration of 1 mg of mitochondrial protein per ml of assay buffer [150 mmol l<sup>-1</sup> KCl, 5 mmol l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 400 mmol l<sup>-1</sup> urea, 200 mmol l<sup>-1</sup> trimethylamine N-oxide, 50 mmol l<sup>-1</sup> sucrose, 1 mmol l<sup>-1</sup> EGTA, 1 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 3 mmol l<sup>-1</sup> Hepes buffer, 0.5% BSA, pH 7.3 at 20°C], and the following reagents were added in succession using Hamilton syringes: 5 μmol l<sup>-1</sup> rotenone, 1 μg ml<sup>-1</sup> oligomycin, 0.1 μmol l<sup>-1</sup> nigericin, 5 μmol l<sup>-1</sup> TPMP<sup>+</sup> (in increments of 1 μmol l<sup>-1</sup>), 4 mmol l<sup>-1</sup> succinate, up to 8 mmol l<sup>-1</sup> malonate and 0.2 μmol l<sup>-1</sup> carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Brand et al., 1991; Brand et al., 2003).

Mitochondrial proton leak rates (nmol H<sup>+</sup> min<sup>-1</sup> mg<sup>-1</sup> protein) were calculated from respiration rates by assuming a ratio of six protons for each oxygen atom consumed (Hafner and Brand, 1991) and oxygen solubility of 521 nmol O ml<sup>-1</sup> for a 150 mmol l<sup>-1</sup> KCl medium at 20°C (Reynafarje et al., 1985). To calculate membrane potentials, the concentrations of TPMP<sup>+</sup> inside and outside the mitochondria were quantified with a TPMP<sup>+</sup> electrode calibrated during each assay with 1 μmol l<sup>-1</sup> additions of TPMP<sup>+</sup> up to 5 μmol l<sup>-1</sup>. These calibrations also confirmed that the electrodes responded in a Nernstian fashion. The concentration of TPMP<sup>+</sup> inside the mitochondria was determined by subtracting the amount of TPMP<sup>+</sup> remaining outside the mitochondria (measured directly by the TPMP<sup>+</sup> electrode) from the total amount of TPMP<sup>+</sup> present. The membrane potential was calculated using the Nernst equation, the concentrations of TPMP<sup>+</sup> inside and outside the mitochondria and a correction factor of 0.4 for nonspecific mitochondrial TPMP<sup>+</sup> binding (Brookes et al., 1998). For each tissue sample, proton leak rate was measured in triplicate and average values were calculated. Mean values for each tissue in each species were then calculated.

Average mitochondrial proton leak rates for the three species were plotted as a function of membrane potential (mV) for each tissue, and we assessed similarities and differences in proton leak rate curves by the overlap or non-overlap of standard error bars, as has been done in other studies (e.g. Brand et al., 1991; Brookes et al., 1998; Hulbert et al., 2002). In addition, to compare proton conductances (proton leak at a given membrane potential), we fit a second-order polynomial to the data for each individual (Origin 7.5 software; RockWare Inc., Golden, CO, USA) to estimate the proton leak rate at a membrane potential of 160 mV. This value was selected because it is sub-maximal for all shark species in this study, is encompassed by all proton leak curves and has been used previously to compare proton conductances in terrestrial and aquatic vertebrates (e.g. Brookes et al., 1998; Hulbert et al., 2002).

We used one-way analysis of variance (ANOVA), along with a power analysis, to test for interspecific differences in mean red muscle and liver mitochondrial proton leak rates at 160 mV, and paired *t*-tests to test for differences between the two tissues in each species (Minitab, version 12; Minitab Inc., State College,

PA, USA). ANOVA was used to test for interspecific differences in state 3 respiration rates, state 4 respiration rates and state 4 membrane potentials (obtained from proton leak assays after the addition of succinate); if significant differences were found, Scheffe's method was used for multiple comparisons analysis (Minitab, version 12). A significance level of *P*=0.05 was used in all statistical analyses.

#### *Estimates of tissue mitochondrial density by transmission electron microscopy*

Although we were primarily interested in whether red muscle or liver mitochondria of endothermic sharks are specialized for thermogenesis, the contribution of a given tissue to heat production is also affected by the tissue's mitochondrial density and its contribution to the total body mass. The relative mass of red muscle and liver (as a percentage of body mass) have been measured in the three shark species (Bone and Roberts, 1969; Kohler et al., 1996; Gruber and Dickson, 1997; Mollet et al., 1999). However, of the tissues studied, mitochondrial content has been measured only in the red muscle of the mako shark (Bernal et al., 2003a). Therefore, we used transmission electron microscopy (TEM) to estimate mitochondrial densities in samples of both tissues from each of the three shark species.

Small tissue samples were dissected from sharks immediately after they were euthanized and prepared for TEM. Tissues were fixed in 2% formaldehyde and 2% glutaraldehyde in TEM buffer (280 mmol l<sup>-1</sup> NaCl, 6 mmol l<sup>-1</sup> KCl, 5 mmol l<sup>-1</sup> CaCl<sub>2</sub>, 3 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 0.5 mmol l<sup>-1</sup> Na<sub>2</sub>SO<sub>4</sub>, 0.41 mmol l<sup>-1</sup> MgSO<sub>4</sub>, 250 mmol l<sup>-1</sup> sodium cacodylate and 200 mmol l<sup>-1</sup> sucrose, pH 7.2 at room temperature) and post-fixed in 2% osmium tetroxide in TEM buffer before being embedded in epon araldite blocks. Ultra-thin sections (90 nm thick) stained with 3% uranyl acetate and 1.5% lead citrate were scanned with the TEM to find transverse sections of whole muscle fibers or representative areas of liver tissue. Electron micrographs were taken at 2000 or 2500× magnification, and the point-contact method was used to determine mitochondrial densities within an entire muscle fiber cross-section or within a rectangular area of liver cells using the Scion Image Analysis Program (version 1.62; Scion Corp., Frederick, MO, USA). Mitochondrial densities were expressed as a percentage of cross-sectional area occupied by mitochondria, which is equivalent to the volume percentage of the muscle fiber or liver tissue that is occupied by mitochondria, or *V<sub>v</sub>(mt,f)*, if a uniform distribution of mitochondria is assumed. ANOVA was used to test for interspecific differences in red muscle and liver mitochondrial densities (Minitab, version 12).

## **Results**

### *Red muscle and liver mitochondrial proton leak rates*

The rate of proton leak across the inner mitochondrial membrane is a function of the driving force, or membrane potential, and increases disproportionately with membrane potential, as shown in Figs 1, 2 for red muscle and liver mitochondria, respectively, isolated from mako, blue and leopard sharks. The proton leak curves of red muscle

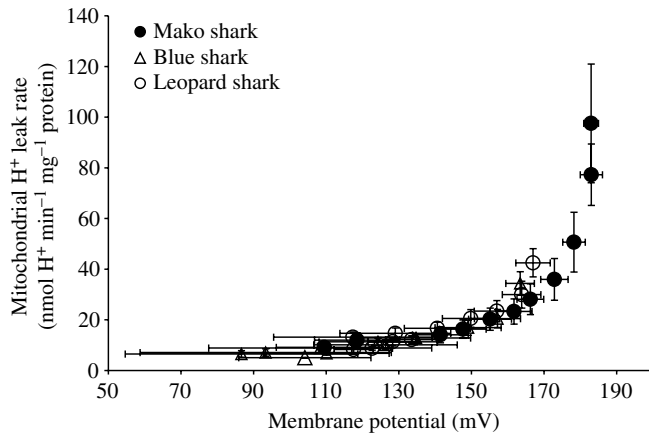


Fig. 1. Mean proton leak rates ( $\pm$  s.e.m.) at 20°C as a function of membrane potential ( $\pm$  s.e.m.) for mitochondria isolated from the slow, oxidative (red) locomotor muscle of three shark species: shortfin mako ( $N=5$ ), blue ( $N=4$ ) and leopard ( $N=5$ ) sharks. Proton leak rates were calculated by assuming the  $H^+ : O$  ratio of 6 when using succinate as the respiratory substrate. Proton leak rates at 160 mV in red muscle mitochondria did not differ significantly among the three species (ANOVA,  $P=0.313$ ).

mitochondria overlap in the three species (Fig. 1), suggesting similar proton leak kinetics. This was confirmed by comparing proton leak rates at a membrane potential of 160 mV: no significant interspecific differences in red muscle proton conductance were detected (ANOVA,  $P=0.31$ ; power was estimated to be 0.22; Table 1). In liver, the mako shark has higher proton leak rates than the blue shark over a wide range of membrane potentials, but the mako and leopard shark curves overlap (Fig. 2). However, no significant interspecific differences in liver proton leak rates at 160 mV were detected (ANOVA,  $P=0.16$ ; power estimated to be 0.35; Table 1).

For each shark species, the proton leak curve for red muscle mitochondria is higher than that for liver mitochondria (Fig. 3)

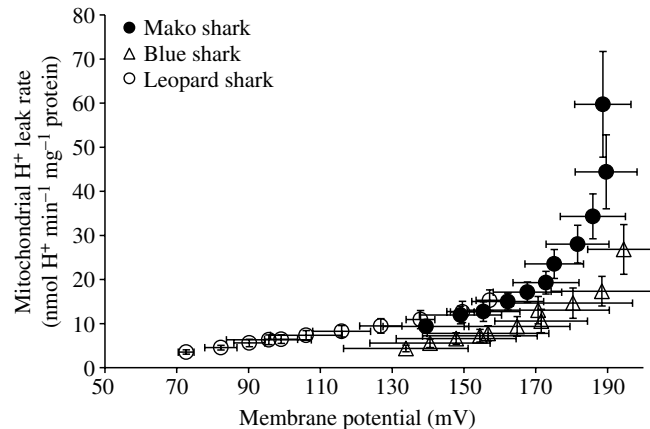


Fig. 2. Average proton leak rates ( $\pm$  s.e.m.) at 20°C as a function of membrane potential ( $\pm$  s.e.m.) for liver mitochondria from three shark species: shortfin mako ( $N=5$ ), blue ( $N=4$ ) and leopard ( $N=5$ ) sharks. Proton leak rates at 160 mV in red muscle mitochondria did not differ significantly among the three species (ANOVA,  $P=0.161$ ).

and would be considered different by the criterion of non-overlapping error bars. Only in the blue shark was the proton leak rate at 160 mV significantly higher in red muscle than in liver (paired  $t$ -tests;  $P=0.86$  for mako shark;  $P=0.02$  for blue shark;  $P=0.07$  for leopard shark).

#### Mitochondrial respiration rates and membrane potentials

The average state 3 and state 4 respiration rates, state 4 membrane potentials and RCR values of red muscle and liver mitochondria for the three shark species are summarized in Table 2. In red muscle mitochondria, state 3 and state 4 respiration rates and state 4 membrane potentials were significantly higher in the mako shark than in the two ectothermic species (ANOVA,  $P<0.01$ ). In liver mitochondria, the mako shark had significantly higher state 3 and state 4 respiration rates than the leopard shark, and both the mako

Table 1. Mean ( $\pm$  s.e.m.) proton leak rates at a membrane potential of 160 mV at 20°C and mean ( $\pm$  s.e.m.) mitochondrial densities in red muscle and liver of three shark species

Tissue and species	Proton leak rate (nmol $H^+$ min $^{-1}$ mg $^{-1}$ protein)	Mitochondrial density [ $V_v$ (mt,f) or volume %]
Red myotomal muscle		
<i>Isurus oxyrinchus</i> (shortfin mako shark)	20.0 $\pm$ 4.0	40.8 $\pm$ 1.3 (3; 9)
<i>Prionace glauca</i> (blue shark)	23.5 $\pm$ 2.4*	23.2 $\pm$ 1.0 $^\dagger$ (2; 10)
<i>Triakis semifasciata</i> (leopard shark)	26.9 $\pm$ 2.3	39.5 $\pm$ 1.5 (1; 10)
Liver		
<i>Isurus oxyrinchus</i> (shortfin mako shark)	18.6 $\pm$ 4.3	20.6 $\pm$ 0.7 (2; 32)
<i>Prionace glauca</i> (blue shark)	8.7 $\pm$ 1.3	22.9 $\pm$ 1.0 (1; 21)
<i>Triakis semifasciata</i> (leopard shark)	15.6 $\pm$ 2.9	12.7 $\pm$ 0.9 $^\dagger$ (2; 23)

\*Mean is significantly higher than liver mean for that species (paired  $t$ -test,  $P=0.02$ ).

$^\dagger$ Mean differs significantly from those of the other two species (ANOVA,  $P<0.01$ ).

For mitochondrial density measurements, the number of individual sharks sampled, followed by the number of TEM images analyzed, is indicated in parentheses.



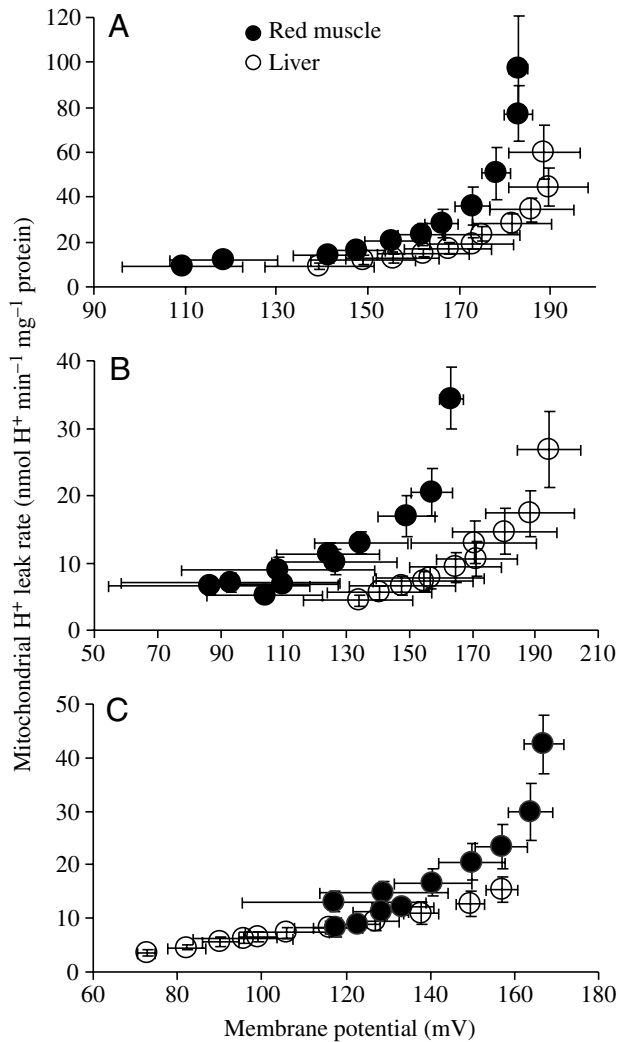


Fig. 3. Proton leak curves at 20°C in red muscle and liver mitochondria of the shortfin mako shark (A), blue shark (B) and leopard shark (C). Each data point represents the mean ( $\pm$  s.e.m.) of all individuals sampled for that species. The red muscle curve is higher than the liver curve in all three shark species, but only in the blue shark were the proton leak rates at 160 mV significantly different (paired *t*-test;  $P=0.86$  for mako shark, 0.02 for blue shark and 0.07 for leopard shark).

shark and blue shark had significantly higher state 4 membrane potentials than the leopard shark (ANOVA,  $P\leq 0.02$ ).

#### Estimates of tissue mitochondrial density and total tissue proton leak

The average red muscle and liver mitochondrial densities for each shark species are summarized in Table 1, and Fig. S1 in the supplementary material shows examples of the individual muscle fibers and liver samples analyzed. The mako shark and the leopard shark have significantly higher red muscle mitochondrial densities than the blue shark (ANOVA,  $P<0.01$ ). In liver, mitochondrial densities are significantly greater in the mako shark and blue shark than in the leopard shark (ANOVA,  $P<0.01$ ).

We combined the tissue mitochondrial density data with the mitochondrial proton leak rates at a membrane potential of 160 mV (Table 1) to estimate proton leak rate per g of tissue, assuming that the amount of protein per mitochondrial volume and tissue densities do not differ interspecifically. For interspecific comparisons of total proton leak rates within the red muscle, proton leak rate per g of red muscle was multiplied by published values of relative red muscle mass (as a percentage of body mass) in the three species: means  $\pm$  s.e.m. of  $2.01\pm 0.07\%$  ( $N=8$ ) for *I. oxyrinchus*;  $2.65\pm 0.56\%$  ( $N=4$ ) for *P. glauca*;  $2.06\pm 0.19\%$  ( $N=3$ ) for *T. semifasciata* (Bernal et al., 2003a). These calculations indicate that total proton leak rate in the red muscle is not elevated in the mako shark relative to the two ectothermic species. Total red muscle proton leak in the mako shark is approximately 1.1 times that in the blue shark and is 25% lower than that in the leopard shark. However, proton leak per g of liver in the endothermic mako shark would be approximately 1.9 times that in both ectothermic species. Compared with the shortfin mako shark, the blue shark had a lower mitochondrial proton conductance but a similar mitochondrial density in the liver, whereas the leopard shark had a similar proton conductance but lower mitochondrial density (Table 1). These interspecific differences would be reflected in total liver proton leak rates because the relative liver masses in the three species overlap: 1.2–17.9% (mean  $\pm$  s.e.m. of  $6.7\pm 0.25\%$ ;  $N=161$ ) for female *I. oxyrinchus* (Mollet et al., 1999); 5.1–10.7% ( $8.4\pm 1.0\%$ ;  $N=6$ ) for *P. glauca* (Bone and Roberts, 1969); 3.49–5.44% ( $4.48\pm 0.23\%$ ;  $N=8$ ) for *T. semifasciata* (Gruber and Dickson, 1997).

## Discussion

### Interspecific comparisons

The major finding of this study is that the endothermic shortfin mako shark does not have a significantly higher mitochondrial proton conductance (proton leak rate per mg of protein at a given membrane potential) in red muscle or liver at 20°C than the two ectothermic species. This result suggests that mitochondria from endothermic tissues of the mako shark are not specialized for thermogenesis. A similar conclusion was reached for shortfin mako red muscle mitochondria based on their high RCR values and respiration rates were similar to those measured in the ectothermic spiny dogfish, *Squalus acanthias* (Moyes et al., 1990; Ballantyne et al., 1992). However, our measurements of state 3 and state 4 respiration rates by mako shark red muscle mitochondria are higher than those reported by Ballantyne et al. under similar conditions (mean  $\pm$  s.e.m. of  $21.74\pm 3.25$  nmol O  $\text{min}^{-1}$   $\text{mg}^{-1}$  protein and  $3.72\pm 2.24$  nmol O  $\text{min}^{-1}$   $\text{mg}^{-1}$  protein, respectively;  $N=3$ ) (Ballantyne et al., 1992) and were significantly higher than in the two ectothermic shark species studied (Table 2). Red muscle mitochondrial respiration rates comparable to the high rates we measured in the mako shark have been reported in active teleost fishes, including the skipjack tuna, *Katsuwonus pelamis* (Moyes et al., 1992), and blue marlin, *Makaira*

Table 2. Mean ( $\pm$  s.e.m.) state 4 membrane potentials, state 4 respiration rates, state 3 respiration rates and respiratory control ratios (RCRs) of shark red muscle and liver mitochondria at 20°C

Tissue and species	State 4 membrane potential (mV)	State 4 respiration rate (nmol O min <sup>-1</sup> mg <sup>-1</sup> protein)	State 3 respiration rate (nmol O min <sup>-1</sup> mg <sup>-1</sup> protein)	RCR
Red myotomal muscle				
<i>Isurus oxyrinchus</i> (shortfin mako shark)	183.0 $\pm$ 2.0 <sup>a</sup>	28.4 $\pm$ 4.38 <sup>a</sup>	87.8 $\pm$ 19.9 <sup>a</sup>	2.89 $\pm$ 0.31
<i>Prionace glauca</i> (blue shark)	163.4 $\pm$ 3.9 <sup>b</sup>	6.83 $\pm$ 0.70 <sup>b</sup>	27.9 $\pm$ 4.53 <sup>b</sup>	3.97 $\pm$ 0.30
<i>Triakis semifasciata</i> (leopard shark)	167.0 $\pm$ 4.7 <sup>b</sup>	6.41 $\pm$ 1.14 <sup>b</sup>	22.9 $\pm$ 2.85 <sup>b</sup>	2.91 $\pm$ 0.28
Liver				
<i>Isurus oxyrinchus</i> (shortfin mako shark)	188.7 $\pm$ 7.8 <sup>a</sup>	12.2 $\pm$ 2.90 <sup>a</sup>	59.4 $\pm$ 13.4 <sup>a</sup>	5.00 $\pm$ 0.43
<i>Prionace glauca</i> (blue shark)	194.5 $\pm$ 10.1 <sup>a</sup>	7.07 $\pm$ 1.03 <sup>a,b</sup>	32.1 $\pm$ 4.95 <sup>a,b</sup>	4.47 $\pm$ 0.36
<i>Triakis semifasciata</i> (leopard shark)	157.2 $\pm$ 3.8 <sup>b</sup>	3.57 $\pm$ 0.31 <sup>b</sup>	9.6 $\pm$ 1.46 <sup>b</sup>	2.80 $\pm$ 0.38

Similar superscript letters denote tissue means that are statistically similar for each variable.

*nigricans* (O'Brien and Block, 1996). Our state 3 and state 4 respiration rate values for liver mitochondria from the ectothermic sharks are similar to those reported for ectothermic teleost fishes at 20°C with succinate as substrate (e.g. Bagarinao and Vetter, 1990; Brookes et al., 1998).

The greater respiration rates measured in red muscle mitochondria of the mako shark relative to those of the two ectothermic sharks may reflect higher electron transport chain activity (Rolfe et al., 1994; Leary et al., 2003), which may be related to higher aerobic requirements in the active shortfin mako. If so, and if mako mitochondria maintain higher membrane potentials than those of the ectothermic sharks, then *in vivo* proton leak rates will parallel differences in state 4 respiration rates (Table 2). As a result, in the red muscle, *in vivo* rates of mitochondrial proton leak and heat production in the mako shark would exceed those of the ectothermic species, and in the liver would be greater in the mako shark and blue shark than in the leopard shark. However, *in vivo* mitochondrial membrane potentials and rates of oxygen consumption, proton leak and heat production are not known for sharks.

The endothermic mako shark appears to have a higher proton leak rate per g of liver than both ectothermic species, due to a lower proton conductance in the blue shark and a lower liver mitochondrial content in the leopard shark. Because the liver comprises a relatively large proportion of body mass in sharks and is likely to contribute significantly to standard metabolic rate (SMR), total liver proton leak may also correlate with SMR. The data available on swimming energetics and SMR of active sharks are limited, due to logistical problems associated with making these measurements on large pelagic fishes. The SMR of one mako shark studied in a swimming tunnel at sea was estimated to be 240 mg O<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup> at 16–20°C (Graham et al., 1990). This value is higher than that of leopard sharks of similar size [mean  $\pm$  s.e.m., 91.7 $\pm$ 13.9 mg O<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup> at 14–18°C; *N*=7 (Scharold et al., 1989)], but no SMR data are available for the blue shark.

The only other fish proton leak kinetics data of which we are aware are from teleost fishes: mitochondrial proton leak has been quantified in rainbow trout liver (Brookes et al., 1998),

myotomal muscle and heart (Leary et al., 2003), as well as in carp (*Cyprinus carpio*) liver and red muscle (K.A.D., J. Baca, J. A. Buckingham and M. D. Brand, unpublished). When compared at the same membrane potential, the proton leak rates measured for the three shark species in this study are similar to those reported for the rainbow trout (mean red muscle proton leak at 150 mV was 11.6, 17.2, 19.4 and 20.8 nmol H<sup>+</sup> min<sup>-1</sup> mg<sup>-1</sup> protein in mako, blue and leopard sharks at 20°C and in rainbow trout at 15°C, respectively) (Leary et al., 2003) but only approximately half the values measured in carp (48.8 and 25.8 nmol H<sup>+</sup> min<sup>-1</sup> mg<sup>-1</sup> protein at 160 mV at 25°C for carp red muscle and liver, respectively).

The mitochondrial density in mako shark red muscle measured in the present study is higher than values reported by Bernal et al. for two individuals (mean  $\pm$  s.e.m. of 25.2 $\pm$ 0.8 and 29.5 $\pm$ 1.2 volume %) (Bernal et al., 2003a), possibly due to methodological differences. The relative values of red muscle mitochondrial density in the three shark species studied correspond with published red muscle citrate synthase activities: mean  $\pm$  s.e.m. of 31.6 $\pm$ 1.8 i.u. g<sup>-1</sup> tissue (*N*=30) for *I. oxyrinchus*; 22.3 $\pm$ 6.3 (*N*=4) for *P. glauca*; 27.1 $\pm$ 2.1 (*N*=7) for *T. semifasciata* (Dickson et al., 1993; Bernal et al., 2003b). This finding confirms that citrate synthase activity is an index of mitochondrial density in fish muscle.

#### Comparisons between red muscle and liver

When red muscle and liver proton leak curves were compared by examining the overlap of standard error bars, as is typically done in published literature (e.g. Brand et al., 1991; Brookes et al., 1998; Hulbert et al., 2002), mitochondrial proton leak rates were higher in red muscle than in liver for all three shark species studied. Mitochondrial proton leak rates were also higher in skeletal muscle than in liver in both rats and carp (Rolfe et al., 1994) (K.A.D., J. Baca, J. A. Buckingham and M. D. Brand, unpublished data). Rolfe et al. suggested that the inner membrane of rat skeletal muscle mitochondria has a greater surface area for proton leak to occur and contains lipids with a higher unsaturation index (Rolfe et al., 1994), which correlates with proton permeability (Brand et al., 1991). Relative to liver mitochondria, muscle mitochondria

could contain more adenine nucleotide translocase, which is responsible for a major fraction of basal proton leak (Brand et al., 2005). These parameters remain unexamined in shark red muscle and liver mitochondria, and the basis for inter-tissue differences in mitochondrial proton leak rates deserves further study.

Leary et al. argued that inter-tissue comparisons of proton leak rates should be made on the basis of mitochondrial membrane surface area and that the activity of cytochrome oxidase is a better measure of this variable than is protein content (Leary et al., 2003). When Leary et al. compared red muscle, fast-glycolytic myotomal (white) muscle, and heart mitochondria of rainbow trout they found no difference in proton leak kinetics when measured in  $\text{nmol H}^+ \text{min}^{-1} \text{mg}^{-1}$  protein, but inter-tissue differences were apparent when expressed as  $\text{nmol H}^+ \text{min}^{-1} \text{unit}^{-1}$  of cytochrome oxidase activity (Leary et al., 2003). Therefore, future studies should quantify cytochrome oxidase activity, as well as membrane phospholipid composition and adenine nucleotide translocase content, to assess differences among tissues in mitochondrial proton leak rates.

### Conclusion

The objective of this study was to determine if high rates of mitochondrial proton leak are associated with endothermy in sharks. We tested the hypothesis that, because the shortfin mako shark is a regional endotherm, its red muscle and liver mitochondria proton leak rates would be higher than those of the ectothermic blue and leopard sharks. Based on our finding of no significant interspecific differences in proton leak rates per mg of protein at 160 mV for either tissue, it appears that mitochondria in the mako shark are not specialized for thermogenesis by having a higher proton conductance. However, it is possible that proton leak contributes to heat production for endothermy in this species, if the measured state 4 respiration rates and membrane potentials reflect the conditions under which the mitochondria operate *in vivo*. In that case, mako shark mitochondria would have a higher proton leak rate as a consequence of a higher driving force (membrane potential) rather than as a result of a higher membrane permeability to protons. Furthermore, we found that the total proton leak rate in the liver would be greater in the mako shark than in both ectothermic species studied, suggesting a possible role in endothermy or elevation of SMR. Future experiments to test hypotheses about the role of mitochondrial proton leak in the evolution of endothermy in fishes should focus on the teleost Family Scombridae because both endothermic (tunas) and ectothermic species (mackerels, Spanish mackerels and bonitos) exist within the same family and the metabolic rates in tunas are known to be higher than in mackerels and bonitos (Sepulveda and Dickson, 2000; Korsmeyer and Dewar, 2001; Sepulveda et al., 2003).

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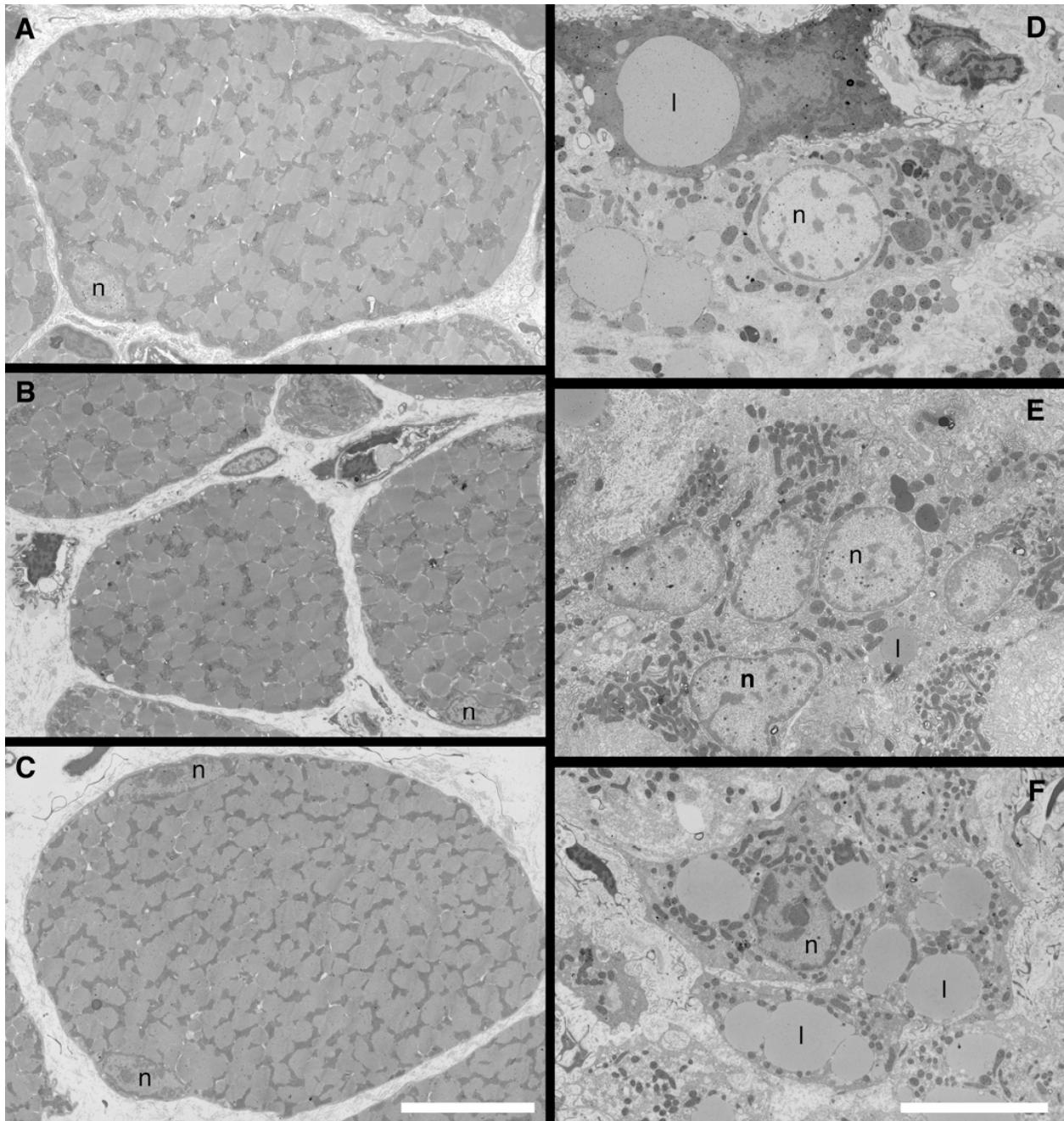


Fig. S1. Transmission electron micrographs of red muscle fibers and liver. (A) Shortfin mako shark red muscle fiber (mitochondrial density 44.2%); (B) blue shark red muscle fibers (25.9%); (C) leopard shark red muscle fiber (38.5%); (D) shortfin mako shark liver (20.0%); (E) blue shark liver (25.0%); (F) leopard shark liver (14.6%). Abbreviations: l, lipid, n, nucleus. Mitochondria are the small, darker structures within the cytoplasm. In the red muscle fibers, almost all of the intracellular volume is occupied by mitochondria (darker structures) and myofibrils. Shark liver contains large volumes of lipid. Scale bars for red muscle and liver images represent 10  $\mu\text{m}$ .