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ORIGINAL PAPER

Scaling with body mass of mitochondrial respiration from the white muscle of three phylogenetically, morphologically and behaviorally disparate teleost fishes

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Abstract White muscle (WM) fibers in many fishes often increase in size from <50 μm in juveniles to >250 μm in adults. This leads to increases in intracellular diffusion distances that may impact the scaling with body mass of muscle metabolism. We have previously found similar negative scaling of aerobic capacity (mitochondrial volume density, V_{mt}) and the rate of an aerobic process (post-contractile phosphocreatine recovery) in fish WM. In the present study, we examined the scaling with body mass of oxygen consumption rates of isolated mitochondria (VO_{2mt}) from WM in three species from different families that vary in morphology and behavior: an active, pelagic species (bluefish, *Pomatomus saltatrix*), a relatively inactive demersal species (black sea bass, *Centropristis striata*), and a sedentary, benthic species (southern flounder, *Paralichthys lethostigma*). In contrast to our prior studies, the measurement of respiration in isolated mitochondria is not influenced by the diffusion of oxygen or metabolites. V_{mt} was measured in WM and in high-density isolates used for VO_{2mt} measurements. WM V_{mt} was significantly higher in the bluefish than in the other two species and VO_{2mt} was independent of body mass when expressed per milligram protein or per milliliter mitochondria. The size-independence of VO_{2mt} indicates that differences in WM aerobic function result from variation in V_{mt} and not to changes in VO_{2mt}. This is consistent with our prior work that indicated that while diffusion constraints influence mitochondrial distribution, the negative scaling of aerobic processes like post-contractile PCr recovery can largely be attributed to the body size dependence of V_{mt}.

Keywords Mitochondria · Muscle · Fish · Metabolism

Abbreviations

VO_{2max}	Maximal rate of oxygen consumption
V_{mt}	Mitochondrial volume density
VO_{2mt}	Rate of mitochondrial oxygen consumption
RM	Red muscle
WM	White muscle
TEM	Transmission electron microscopy

Introduction

During steady state contraction, muscle mitochondria provide sufficient ATP to meet energetic demands, and skeletal muscle mitochondria utilize >90% of the total oxygen consumed by an organism exercising at the maximal rate of oxygen consumption (VO_{2max}) (Taylor 1987; Weibel 2002). The relationship between whole animal VO_{2max} and skeletal muscle mitochondrial function has been the focus of several comparative studies on species differing in both body size (e.g., allometric variation) (Hoppeler and Weibel 2000), behavior (e.g., sedentary versus active) (Hoppeler et al. 1987; Kayar et al. 1989; Weibel 1987), and endurance (e.g., influence of exercise

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training) (Davies et al. 1981). Such comparisons suggest that at the tissue level differences in muscle $\dot{V}O_{2max}$ are due to both qualitative and quantitative variations in the mitochondrial capacity for ATP production (Hochachka 1994; Hoppeler et al. 1987; Schwerzmann et al. 1989).

Muscle aerobic capacity is largely dependent on changes in mitochondrial volume density (V_{mt}). Hoppeler and Weibel (2000) expressed $\dot{V}O_{2max}$ as the product of V_{mt} and the rate of mitochondrial oxygen consumption ($\dot{V}O_{2mt}$)

$$\dot{V}O_{2max} = V_{mt}\,\dot{V}O_{2mt}$$

In mammals, $\dot{V}O_{2mt}$ per mitochondrial volume typically ranges from 3 to 5 ml O_2 min^{-1} ml $mitochondria^{-1}$ (Hoppeler and Lindstedt 1985; Taylor 1987; Taylor et al. 1989). The relatively narrow range of respiration rates is due to the highly conserved metabolic stoichiometries of mitochondria defined by the ATP:O_2 ratio (Leary et al. 2003). Consistent with the principle of allometric scaling of metabolism with body mass (M), $\dot{V}O_{2max}/M$ and V_{mt}/M in mammals scale in parallel, and $\dot{V}O_{2mt}$ does not appear to change with body size (Hoppeler and Weibel 2000; Moyes et al. 1998; Moyes 2003). Further, muscle mitochondrial content can vary up to fivefold inter- and intraspecifically independently of variation in body mass (Mathieu et al. 1981). For instance, V_{mt} in different fiber types ranges from <0.01 in some fast-twitch, glycolytic fibers to >0.45 in some oxidative fibers (e.g., Hochachka 1994; Hochachka and Somero 1973; Moyes and Hood 2003; Suarez 1996).

Although prior studies have investigated the effects of qualitative and quantitative differences in mitochondria, the parameters defining aerobic metabolism have largely focused on mammals (e.g., Taylor 1987; Taylor and Weibel 1981). Unlike mammalian muscle, fish muscle is often separated into distinct regions of red muscle (RM), which is specialized for sustained, aerobic activity and white muscle (WM) that is employed during burst contractions (Bone 1966; Jane and Lauder 1994; Rayner and Keenan 1967). WM, which generally comprises the majority (70–90%) of body mass in fishes, typically has a $V_{mt} < 0.02$ (Johnston 1981) and fuels burst contraction almost exclusively by anaerobic metabolism using endogenous substrates (Curtin et al. 1997; Dobson and Hochachka 1987; Hochachka and Mossey 1998; Moyes et al. 1992; Schulte et al. 1992). Anaerobic burst exercise utilizes the creatine kinase reaction to buffer initial ATP demand by catalyzing the reversible transfer of a phosphoryl group from phosphocreatine to ADP forming creatine and ATP (Ellington 2001; Mainwood and Rakusan 1982; Meyer et al. 1984) while extended burst exercise invokes glycolysis and leads to the accumulation of lactate (Milligan and Wood 1986). Recovery from contraction in fish WM, however, is an aerobic process driven by mitochondrial respiration (Curtin et al. 1997). Thus, the recovery rate of high-energy phosphates in WM is likely dependent in part on the V_{mt} of the muscle, and the capacity for recovery may strongly interact with whole animal behavior.

Although skeletal muscle $\dot{V}O_{2max}$ and V_{mt} often scale in parallel, ontogenetic changes in cellular structure and organization may cause some aerobic processes to scale independently of V_{mt}. During growth, fish muscle mass increases by both hyperplasia, an increase in fiber number, and hypertrophy, an increase in fiber size (Gill et al. 1989; Mommsen 2001; Weatherly and Gill 1985). Since many fishes display a several thousand-fold increase in body mass during post-metamorphic growth and hypertrophy is often prevalent, WM fibers can reach diameters >500 μm in adults (Battram and Johnston 1991; Egginton et al. 2002; Johnston et al. 2003; Nyack et al. 2007). Such extreme increases in fiber diameter may potentially affect metabolic scaling properties due to increasing oxygen and metabolite diffusion distances within the fiber (Boyle et al. 2003; Hardy et al. 2006; Johnson et al. 2004; Kinsey et al. 2007; Kinsey and Moerland 2002; Locke and Kinsey 2008).

We have previously compared the scaling with body mass of an aerobic process (post-contractile phosphogen resynthesis) and aerobic capacity (V_{mt}) in both fish and crustacean WM. These studies were conducted using either whole animals, where O_2 diffusion to the mitochondria and metabolite (e.g., ATP) diffusion between mitochondria influences metabolic rate (Hardy et al. 2006; Jimenez et al. 2008; Kinsey et al. 2005), and in isolated muscle, where O_2 was maintained at high levels to eliminate the influence of O_2 diffusion on the rate of metabolism (Nyack et al. 2007). The present study examined the allometric scaling of respiration in isolated mitochondria from fish WM. Since both O_2 and metabolic substrates can be supplied to isolated mitochondria at saturating concentrations, this approach allows us to remove all influences of diffusion. We examined three species of marine fishes that are phylogenetically disparate, representing three different families, and that have very different morphologies and behaviors. Southern flounder (*Paralichthys lethostigma*) are sedentary and benthic (Burke et al. 1991); black sea bass (*Centropristis striata*) are demersal and associated with bottom structure (Hood et al. 1994); and bluefish (*Pomatomus saltrix*) are highly active and pelagic (Olla et al. 1970). We hypothesized that $\dot{V}O_{2mt}$ would be independent of body mass and species, and any variation in aerobic capacity would be manifested as differences in V_{mt}. In addition, this study provides physiologically relevant data for aerobic function by expressing $\dot{V}O_{2mt}$ per milliliter of mitochondria (instead of the usual expression of $\dot{V}O_{2mt}$ per milligram of protein) for WM from an important group of vertebrates. This will facilitate estimation of maximal aerobic ATP production rates from measurements of mitochondrial volume density in fish WM.

Materials and methods

Animals

All animal maintenance and experimental procedures were approved by the University of North Carolina Wilmington (UNCW) Institutional Animal Care and Use Committee. Black sea bass (*Centropristis striata*) and southern flounder (*Paralichthys lethostigma*) were obtained from the UNCW Aquaculture Facility (Wrightsville Beach, NC, USA) and transported in aerated coolers to aquaria located at the UNCW campus. Bluefish (*Pomatomus saltrix*) were collected using seine nets and hook and line along the coast of Wrightsville Beach and Carolina Beach, NC, USA. All animals were maintained in aquaria containing circulating, filtered seawater (35 ppt) at 25°C on a 10 h:14 h photoperiod. Fish were fed shrimp three times weekly and fasted 24 h prior to experimental use. Fish were sequestered in opaque containers with aerated seawater and euthanized using concentrated (>250 mg l^{-1}) FINQUEL MS-222 (tricaine methanesulfonate, Argent Laboratories, Redmond, WA, USA). Euthanasia was verified by loss of equilibrium and absence of opercular movement. Body mass and total length (TL) was recorded for each animal. Scales and skin were immediately removed from the epaxial region of the fish extending from the dorsal fin to the caudal peduncle.

Muscle mitochondrial volume density

V_{mt} of *P. saltatrix* and *P. lethostigma* WM fibers was calculated from micrographs using transmission electron microscopy (TEM) (*C. striata* V_{mt} was measured previously by Nyack et al. 2007). Small, rectangular sections of WM were excised parallel to the fiber orientation just below the dorsal fin. Individual muscle fibers were teased apart and fixed at room temperature for 1 h in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4), then refrigerated for a minimum of 24 h. The fibers were rinsed with cacodylate buffer then placed in a secondary fixative of 1% osmium tetroxide buffer for 2 h, dehydrated with an ascending series (50, 70, 95, 100, 100%) of acetone, and embedded in Spurr epoxy resin (Spurr 1969; Electron Microscopy Sciences; Hatfield, PA, USA). Embedded fibers were cut at 90 nm with a Reichert Ultracut E. Series ultramicrotome using a systematic random sampling method to ensure full representation of mitochondria throughout the muscle (Howard and Reed 1998). Sections were stained with 2% uranyl acetate in 50% ethyl alcohol and Reynolds' lead citrate (Reynolds 1963) and viewed with a Philips CM-12 TEM operated at 80 kV. Micrographs were taken at magnifications of ×1,800, ×3,000, and ×5,600. Two micrographs per grid were randomly taken from five grids for a total of 10 micrographs per fiber sample. Micrographs were developed and digitized using a Microtek Scanmaker 4 (Microtek Lab, Carson, CA, USA), and the images were analyzed in Adobe Photoshop version 7.7. A stereological point-counting method was applied to calculate V_{mt} (Howard and Reed 1998; Nyack et al. 2007). A point grid was superimposed on each micrograph, and all points touching extracellular space were subtracted from the total number of points per micrograph. All points hitting mitochondria were recorded and divided by the total number of points within the intracellular space to determine the total mitochondrial fractional volume of the muscle.

Mitochondrial isolation

Approximately 40 g of WM was excised from the sides of each fish extending from the operculum to the caudal peduncle. WM samples were collected from an individual animal when possible; however, it was necessary to combine tissue from two to five small fish for a single experiment. All isolation procedures were carried out on ice. During dissection, tissue was placed in a beaker containing two volumes of isolation medium (in mmol l^{-1}: 140 KCl, 10 EDTA, 5 $MgCl_2$, 20 HEPES, 0.5% bovine serum albumin (BSA), pH 7.1 at 25°C) (Moyes et al. 1998). Tissue was transferred to a Petri dish, finely diced with scissors, and all fluid was filtered through two layers of cheesecloth. The diced tissue was divided into ten portions and homogenized in eight volumes of isolation medium using a Potter–Elvehjem tissue grinder (Wheaton Science Products, Millville, NJ, USA). Tissue was dispersed by five passes with a loose fitting pestle, three passes with a medium fitting pestle, and two passes with a tight fitting pestle. The homogenates were centrifuged at 4°C for 5 min at 1,400*g*. The supernatant was filtered through four layers of cheesecloth then centrifuged for 7 min at 2,600*g*. Remaining supernatant was poured through eight layers of cheesecloth and centrifuged for 10 min at 9,000*g* to obtain the mitochondrial pellets. The supernatant was discarded and the resultant pellets were resuspended in 20 ml isolation medium (minus BSA) then centrifuged for 10 min at 9,000*g*. Mitochondrial pellets were combined from the centrifuge tubes and resuspended in 1–2 ml isolation medium (minus BSA).

Oxygen consumption measurements

Oxygen consumption rates of the isolated mitochondria were measured in a water-jacketed bath using a Clarke type polarographic oxygen electrode (YSI Inc., Yellow Springs, OH, USA) (Estabrook 1967). The oxygen monitoring system was calibrated with air saturated respiration

medium (in mmol l^{-1}: 140 KCl, 5 Na_2HPO_4, 20 HEPES, 0.5% fatty acid-free BSA, pH 7.1 at 15°C) to 100% full scale. The protein concentration (mg ml^{-1}) of the mitochondrial pellet was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), based on the Bradford method. Two to three milligram of mitochondrial protein (mean 2.44 ± 0.16 mg) was added to 2 ml of respiration medium for a total volume of 3 ml in a magnetically stirred sample chamber kept at 15°C by a water circulator. Following 3 min of temperature equilibration, the oxygen electrode was immersed in the mitochondrial solution and oxygen uptake was traced by a chart recorder. Saturating amounts of substrate (0.1 mM malate and 2.5 mM pyruvate) were added using a microliter syringe (Hamilton Co., Reno, NV, USA), followed by the addition of 0.3 mM ADP to stimulate the maximal mitochondrial oxygen consumption rate (state 3; Moyes et al. 1992; Schwerzmann et al. 1989). Higher concentrations of ADP did not increase the rate of oxygen consumption. Once all of the ADP was phosphorylated, the rate of oxygen consumption declined and reached a new steady state (state 4). Viability of the isolated mitochondria was determined by the respiratory control ratio (RCR; state 3/state 4) (Estabrook 1967), where a RCR > 5 was considered to reflect well-coupled mitochondria. In a few samples, myofibrillar ATPase contamination of the mitochondrial pellet caused a nearly continuous state 3 rate. This is because in fish WM the volume of myofibrils can be 100 times greater than the volume of mitochondria, and residual ATPases covert ATP to ADP at least as fast as mitochondria catalyze the reverse reaction (Stienen et al. 1996). Under those circumstances, 500 units of adenylate kinase were added to the sample to scrub the solution of ADP. While this is not the conventional way of achieving state 4 respiration, it allowed us to measure respiration in the ADP depleted state (simulated state 4 respiration) and calculate the RCR to ensure that mitochondria were well-coupled.

Standardization of mitochondrial respiration measurements

For all mitochondrial preparations, state 3 $\dot{V}O_{2mt}$ was standardized in three ways: (1) $\dot{V}O_{2mt}$ per milligram of protein, (2) $\dot{V}O_{2mt}$ per milliliter of mitochondria, and (3) $\dot{V}O_{2mt}$ per milliliter of muscle. Following oxygen consumption measurements, $\dot{V}O_{2mt}$ per milligram of protein was determined by dividing the rate of O_2 consumption by the volume of mitochondrial protein in each in sample. To standardize $\dot{V}O_{2mt}$ per milliliter of mitochondria, a 2 ml aliquot of the mitochondrial suspension was removed from the sample chamber following the oxygen consumption measurement. The suspension in the chamber was continuously stirred prior to removal to ensure homogeneity of the aliquots. Samples were centrifuged for 5 min at 9,000*g* to re-pellet the mitochondria. Pellets were prepared for electron microscopy and micrographs were analyzed as described above to determine muscle V_{mt}, with the following alterations. All micrographs were taken at ×5,600, the lowest magnification for which it was possible to identify individual, intact mitochondria. Stereological point counting was used in which the sum of points hitting mitochondria was divided by the sum of reference points on a total of 15 micrographs per pellet. The volume of mitochondria in each pellet was obtained by multiplying the mitochondrial fractional volume by the total volume of the pellet. The resulting $\dot{V}O_{2mt}$ per milliliter of mitochondria was used with the WM V_{mt} of each species to calculate $\dot{V}O_{2mt}$ per milliliter of muscle. The V_{mt} that was used for each body mass was determined from the scaling equation for that species (see "Results").

Statistical analysis

One-way or two-way analysis of variance (ANOVA) was used to test for main effects of species or species and size class, respectively, and post hoc pairwise comparisons were made using Tukeys's HSD with a significance level of $P < 0.05$ (JMP 7.0, SAS Institute, Cary, NC, USA). Linear regression analysis was used to evaluate scaling of $\dot{V}O_{2mt}$ with body mass using SigmaPlot version 10.0 (SSPS, Chicago, IL, USA). Data are presented as mean values ± SEM.

Results

Fish body mass and size classes

Mitochondrial respiration rates were defined based on fish ranging in body mass from 51 to 522 g (total body length = 186–409 mm) in *P. saltatrix*, 66–3,296 g (161–585 mm) in *C. striata*, and 11–719 g (108–392 mm) in *P. lethostigma*. For TEM analysis of V_{mt}, each species was divided into small and large size classes. Mean body mass of the small and large size classes, respectively, for *P. saltatrix* was 53.2 ± 1.0 and 438.6 ± 33.4 g, for *C. striata* was 1.9 ± 0.1 and 3166.4 ± 421.8 g (Nyack et al. 2007), and for *P. lethostigma* was 7.5 ± 0.8 and 465.7 ± 8.1 g.

Muscle mitochondrial volume density

Representative TEM micrographs of muscle cross sections are shown in Fig. 1. Two-way ANOVA revealed a significant effect of species on V_{mt} ($F = 47.00$, $P < 0.0001$) (Fig. 2). Pairwise comparisons of WM fibers revealed a

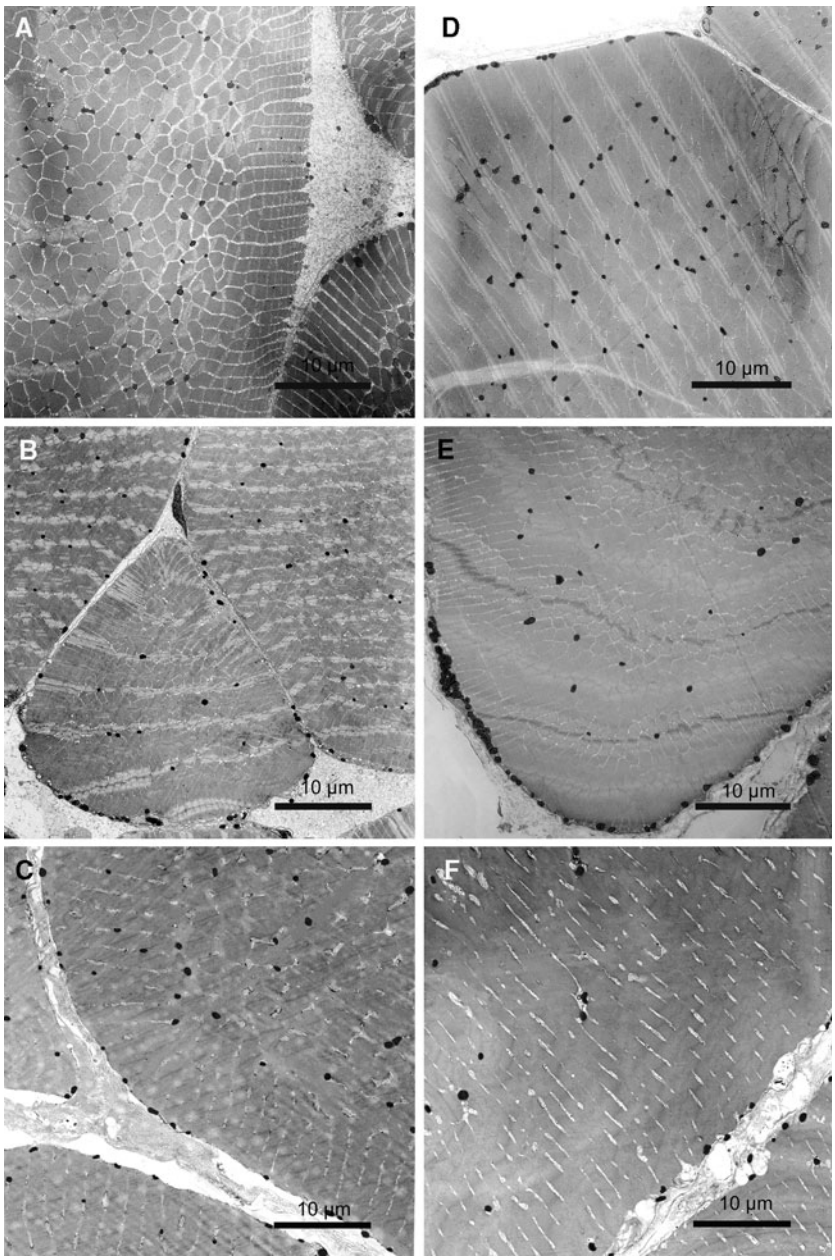

Fig. 1 Micrographs from small (*left*) and large (*right*) *P. saltatrix* (**a**, **d**), *C. striata* (**b**, **e**), and *P. lethostigma* (**c**, **f**). Individual mitochondria were darkly shaded using the Photoshop Burn Tool, which increases exposure of selected areas to enhance contrast and emphasize distribution

significantly higher V_{mt} in the active, pelagic species, *P. saltatrix*, than in the two benthic species, *C. striata* and *P. lethostigma*. Mean V_{mt} for *P. saltatrix* was 0.031 ± 0.001, and mean V_{mt} for *P. lethostigma* was 0.011 ± 0.001. Nyack et al. (2007) previously determined that V_{mt} for *C. striata* WM was 0.012 ± 0.001. Although the small size class had a higher V_{mt} than the large size class for each species, there was no significant effect of size class on V_{mt}. However, the V_{mt} scaling exponent was determined for each species by the equation: $V_{mt} = aM^b$, where M is body mass, a is a constant, and b is the scaling exponent (Schmidt-Nielsen 1984). The mass specific V_{mt} of WM was independent of body mass in *P. saltatrix* ($V_{mt} = 0.031\ M^{-0.01}$, $r^2 = 0.024$, $P = 0.583$) while *C. striata* ($V_{mt} = 0.016\ M^{-0.06}$, $r^2 = 0.449$, $P = 0.017$, Nyack et al. 2007) and *P. lethostigma* ($V_{mt} = 0.015\ M^{-0.10}$, $r^2 = 0.850$, $P = 0.026$) had significantly negative scaling exponents. However, it should be noted that the body mass range for *P. saltatrix* was smaller than for the other two species, and it is possible that if we had been able to collect larger adult specimens the negative scaling may have been significant for this species as well.

Mitochondrial oxygen consumption

Examples of oxygen consumption recordings are shown in Fig. 3. State 3 respiration was elicited by the addition of saturating amounts of pyruvate, malate, and ADP. State 4

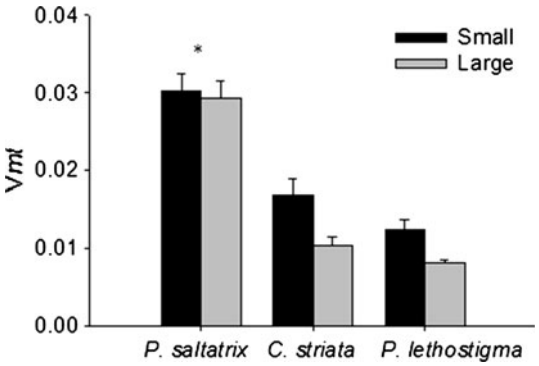

Fig. 2 Mitochondrial volume density (V_{mt}) in WM from small and large *P. saltatrix*, *C. striata*, and *P. lethostigma*. *Asterisks* V_{mt} is significantly greater in *P. saltatrix* than in the other two species. Values shown are mean ± SE

respiration occurred after all of the ADP in the system was phosphorylated and the state 3 and state 4 rates were used to calculate an RCR for each preparation. Mitochondria isolated from the WM of *P. saltatrix*, *C. striata*, and *P. lethostigma* were well-coupled, with mean RCR values well above 5 (Table 1).

The state 3 respiration rate for each mitochondrial preparation was standardized to milligram of protein, milliliter of mitochondria, and milliliter of muscle, and VO_{2mt} was expressed as nmol O_2 min^{-1} to facilitate comparison to other studies (Table 1). The pellets analyzed following oxygen consumption measurements were highly enriched in intact mitochondria (Fig. 4), and stereological analysis of the mitochondrial pellet indicated a mean volume of 0.76 ± 0.15 (*P. saltatrix*), 0.36 ± 0.06 (*C. striata*), and 0.61 ± 0.15 (*P. lethostigma*) µl mitochondria ml^{-1} of suspension in the oxygen consumption chamber.

There was no effect of species on VO_{2mt} rate per milligram of protein ($F = 0.080$, $P = 0.921$) and per milliliter of mitochondria ($F = 2.132$, $P = 0.134$) (Table 1). The VO_{2mt} per milliliter of muscle was determined from the V_{mt} (adjusted for body mass in *C. striata* and *P. lethostigma* using the scaling equations above) and the VO_{2mt} per volume of mitochondria (Table 1). In contrast to respiration standardized to milligram protein or milliliter of mitochondria, VO_{2mt} per milliliter of muscle was significantly different across species ($F = 8.243$, $P = 0.001$), and pairwise comparisons revealed that VO_{2mt} per milliliter of muscle was higher in *P. saltatrix* (due to a greater V_{mt}) than in *C. striata* and *P. lethostigma*. Based on the VO_{2mt} per milliliter of muscle, the maximum ATP production rate of WM in *P. saltatrix* was 7.2 µmol ATP min^{-1} ml^{-1}, *C. striata* was 4.3 µmol ATP min^{-1} ml^{-1}, and *P. lethostigma* was 2.8 µmol ATP min^{-1} ml^{-1}, assuming an ATP:O_2 of 6 (Leary et al. 2003).

There was no relationship between animal mass and VO_{2mt} in any of the three species despite a 10 to 50-fold range in body mass. Linear regression analysis indicated that the allometric scaling exponent, *b*, for VO_{2mt} measurements (per milligram protein, per milliliter mitochondria, and per milliliter muscle) was not significantly different from zero in any case (Fig. 5).

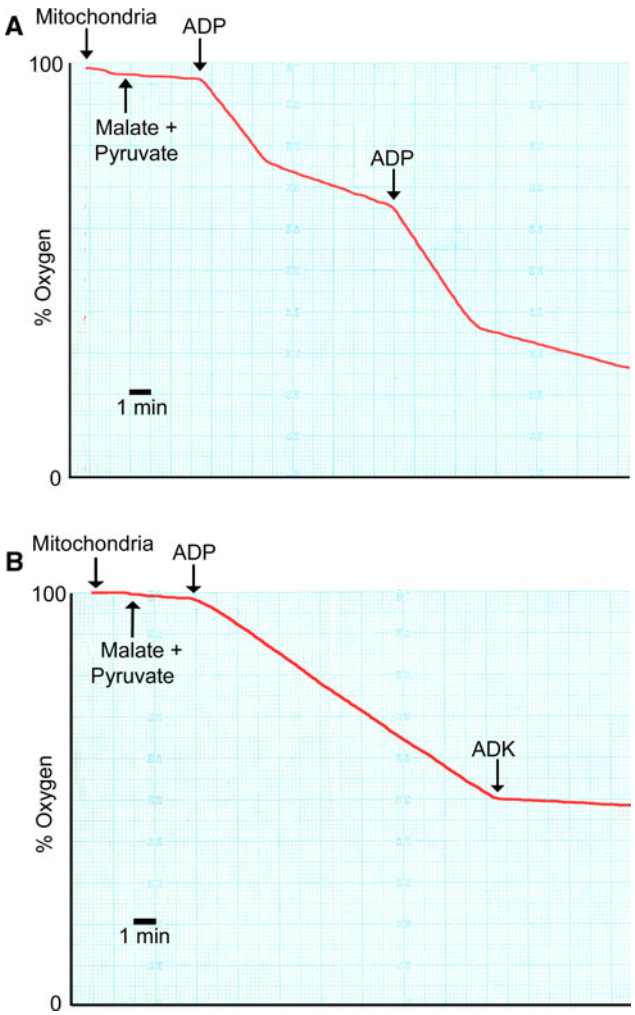

Fig. 3 **a** Oxygen consumption recording from isolated mitochondria demonstrating state 3 and state 4 rates. **b** Example of continuous state 3 rate caused by ATPase contamination where adenylate kinase (*ADK*) was added to remove ADP and produce a state 4 rate

Discussion

Studies of aerobic capacity based on isolated mitochondria normally express VO_{2mt} as a function of protein content. The protein levels, therefore, serve as a proxy for the amount of mitochondria in a sample in units of milligram of mitochondrial protein per milliliter, although it is usually not possible to make direct inferences about rates of respiration in the intact tissue using this approach. These measurements assume a highly pure mitochondrial

Table 1 Respiration rates of isolated white muscle mitochondria

	P. saltatrix	*C. striata*	*P. lethostigma*
RCR	6.22 ± 0.48 (11)	7.61 ± 0.62 (19)	8.02 ± 0.59 (15)
nmol O_2 min^{-1} mg $protein^{-1}$	23.37 ± 3.29 (11)	24.07 ± 3.44 (14)	22.31 ± 2.61 (14)
nmol O_2 min^{-1} ml $mitochondria^{-1}$	39217 ± 7258 (11)	62794 ± 10839 (14)	41736 ± 7585 (14)
nmol O_2 min^{-1} ml $muscle^{-1}$	1201.4 ± 222.4 (11)*	710.0 ± 115.6 (14)	393.6 ± 71.7 (14)

Values are mean ± SE determined at 15°C. Values in parentheses are number of preparations

RCR respiratory control ratio (state 3/state 4)

* VO_{2mt} per ml of muscle is significantly greater in *P. saltatrix* than in the other two species

preparation that is free of non-mitochondrial material (Schwerzmann et al. 1986). Such preparations may be possible from mitochondria-rich tissues such as heart (Moyes et al. 1991), liver (Schwerzmann et al. 1986), and RM (Moyes et al. 1991) that may have a $V_{mt} > 0.40$. However, fish WM has a $V_{mt} \approx 0.02$ (Johnston 1981; Fig. 2). The low mitochondrial density of WM requires a much larger amount of tissue (40 g in contrast to <2 g for RM) to yield a volume of isolated mitochondria equivalent to preparations of highly aerobic tissues (Moyes et al. 1989). The low V_{mt} in fish WM and the large amount of tissue needed for mitochondrial isolation may, therefore, decrease the purity of the final mitochondrial pellet. This is reflected in the amount of protein required to achieve an acceptable rate of oxygen consumption and a high RCR. VO_{2mt} measurements of fish RM have been made using 50–200 μg of protein (Johnston et al. 1994; Moyes et al. 1989), whereas WM measurements require ≥2 mg. This difference suggests that most of the protein in preparations from RM is derived from mitochondria, whereas in WM non-mitochondrial protein constitutes a substantial fraction of the protein pool.

This source of experimental variation may partly explain the relatively large differences among studies in measurements of VO_{2mt} for fish WM when based on milligram of protein. For example, Moyes et al. (1989, 1992) reported values of 77 ± 5.6 nmol O_2 min^{-1} mg $protein^{-1}$ for trout, and 104 ± 15 nmol O_2 min^{-1} mg $protein^{-1}$ for carp using malate and pyruvate as substrates. In contrast, the VO_{2mt} values for *P. saltatrix*, *C. striata*, and *P. lethostigma* (23.37 ± 3.29, 24.07 ± 3.44, and 22.31 ± 2.61 nmol O_2 min^{-1} mg $protein^{-1}$, respectively) were somewhat lower than those for trout and carp WM, likely because of slight differences in the isolation procedure that led to variation in sample purity (reflected by milligram of protein). However, these differences in absolute rates per milligram of protein would not impact the scaling behavior of VO_{2mt} or the general conclusions of this study.

By quantifying the mitochondrial fractional volume of the samples used for oxygen consumption measurements, we were able to assess VO_{2mt} in terms that can be more clearly related to the intact muscle. TEM analysis of the pellets eliminated the potential biases associated with measuring VO_{2mt} per milligram protein by relating the oxygen consumption measurements directly to the volume of mitochondria in each sample. The VO_{2mt} rates, converted here to units of ml O_2 min^{-1} ml $mitochondria^{-1}$ for comparison to other studies, for WM from *P. saltatrix* (0.87 ± 0.16 ml O_2 min^{-1} ml $mitochondria^{-1}$), *C. striata* (1.40 ± 0.24 ml O_2 min^{-1} ml $mitochondria^{-1}$), and *P. lethostigma* (0.93 ± 0.17 ml O_2 min^{-1} ml $mitochondria^{-1}$), are similar to those observed in fast-twitch muscle from cat (0.81–2.29 ml O_2 min^{-1} ml $mitochondria^{-1}$; Hoppeler et al. 1987) and mouse (2.47 ml O_2 min^{-1} ml $mitochondria^{-1}$, Schwerzmann et al. 1989). Further, Schwerzmann et al. (1989) conducted the mitochondrial oxygen consumption measurements at 30°C in contrast to 15°C in the present study, and the temperature difference may account for the greater rates observed in their study. Our rates also compare favorably to the VO_{2mt} range of 3–5 ml O_2 min^{-1} ml $mitochondria^{-1}$ that is generally assumed for mammals at physiological temperatures (reviewed in Suarez 1996).

The VO_{2mt} per milliliter of mitochondria and the muscle V_{mt} can be used to calculate the VO_{2mt} per milliliter of muscle (Hoppeler and Weibel 2000). The mean values of VO_{2mt} per milliliter of muscle for *P. saltatrix* (1.20 ± 0.22 μmol O_2 min^{-1} ml $muscle^{-1}$), *C. striata* (0.71 ± 0.12 μmol O_2 min^{-1} ml $muscle^{-1}$), and *P. lethostigma* (0.39 ± 0.07 μmol O_2 min^{-1} ml $muscle^{-1}$) were similar to rates observed in *Notothenioid* fishes. Johnston (1987) examined the interspecific differences of VO_{2mt} from the RM of icefishes and observed rates of 1.20 ± 0.28 μmol O_2 min^{-1} g $muscle^{-1}$ (*Chaenocephalus aceratus*) and 1.07 ± 0.23 μmol O_2 min^{-1} g $muscle^{-1}$ (*Notothenia gibberifrons*). Our rates of VO_{2mt} per milliliter of muscle for *P. saltatrix*, *C. striata*, and *P. lethostigma* are also consistent with the PCr recovery rates observed by Nyack et al. (2007). These authors found that postcontractile PCr recovery in *C. striata* WM fibers was approximately 1 μmol min^{-1} ml $muscle^{-1}$, which corresponds to an intracellular ATP production rate of

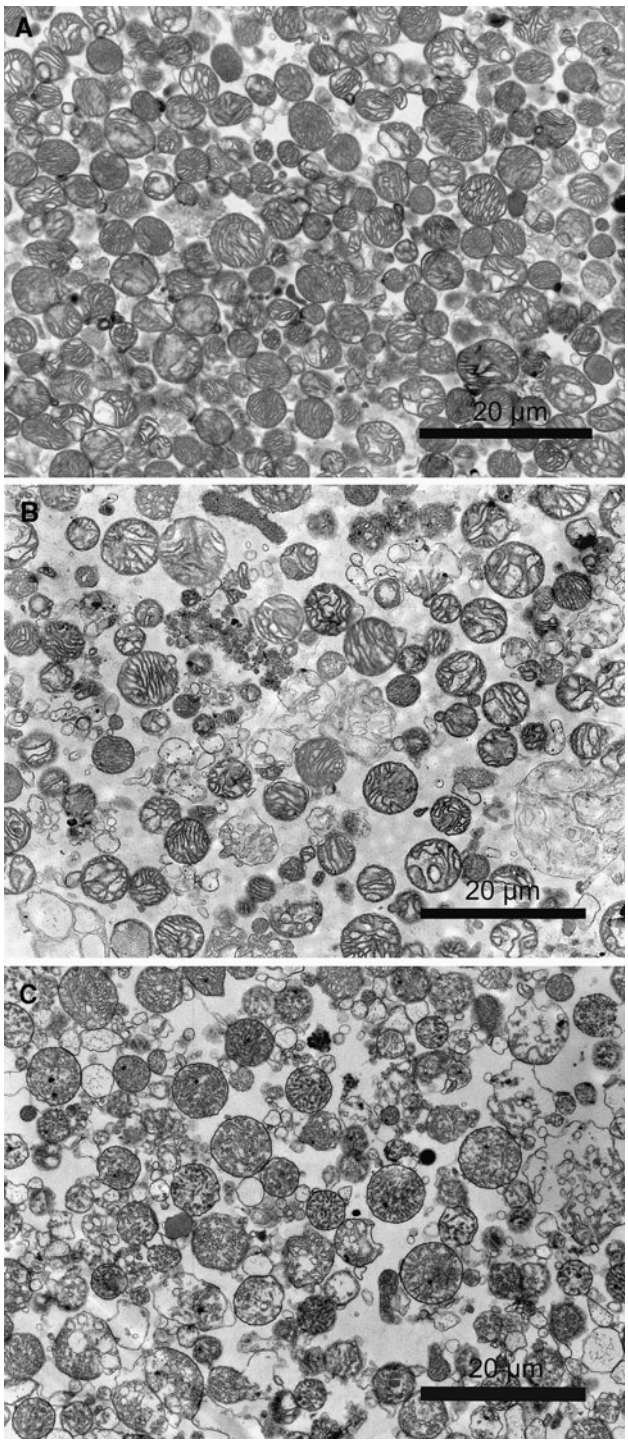

Fig. 4 TEM micrographs of mitochondria isolated from **a** *P. saltatrix*, **b** *C. striata*, and **c** *P. lethostigma.* The isolated mitochondria were prepared for electron microscopy following oxygen consumption measurements. Micrographs depict a sample that was highly enriched in healthy, intact mitochondria

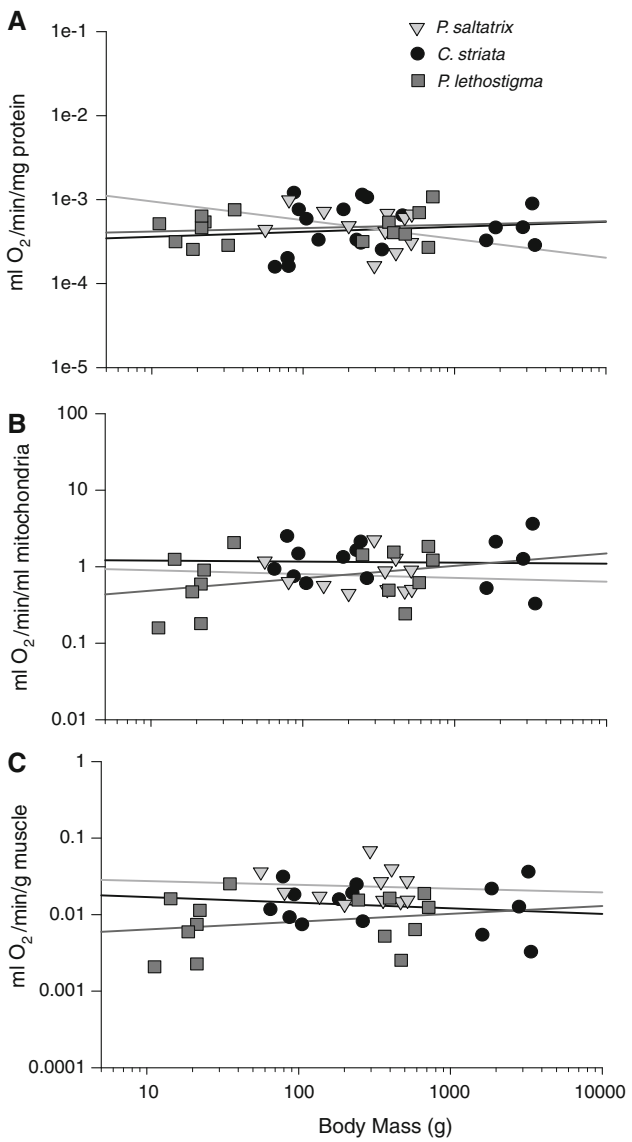

Fig. 5 Scaling with body mass of VO_{2mt} from white muscle of *P. saltatrix*, *C. striata*, and *P. lethostigma*. All VO_{2mt} rates are presented as nmol O_2 min^{-1} unit volume or $mass^{-1}$. **a** VO_{2mt} per milligram of mitochondrial protein. *P. saltatrix* $VO_{2mt} = 71.449\ M^{-0.22}$ ($r^2 = 0.102$, $P = 0.338$); *C. striata* $VO_{2mt} = 14.041\ M^{0.06}$ ($r^2 = 0.016$, $P = 0.605$); *P. lethostigma* $VO_{2mt} = 17.049\ M^{0.04}$ ($r^2 = 0.025$, $P = 0.572$). **b** VO_{2mt} per milliliter of mitochondria. *P. saltatrix* $VO_{2mt} = 45{,}185\ M^{-0.05}$ ($r^2 = 0.006$, $P = 0.827$); *C. striata* $VO_{2mt} = 56{,}937\ M^{-0.01}$ ($r^2 = 0.001$, $P = 0.918$); *P. lethostigma* $VO_{2mt} = 14{,}989\ M^{0.16}$ ($r^2 = 0.103$, $P = 0.264$). **c** VO_{2mt} per milliliter of muscle. *P. saltatrix* $VO_{2mt} = 1{,}384.5\ M^{-0.05}$ ($r^2 = 0.006$, $P = 0.828$); *C. striata* $VO_{2mt} = 902.40\ M^{-0.07}$ ($r^2 = 0.02$, $P = 0.58$); *P.lethostigma* $VO_{2mt} = 226.88\ M^{0.06}$ ($r^2 = 0.017$, $P = 0.661$). *Points* represent individual mitochondrial preparations for each species

1 μmol min^{-1} ml $muscle^{-1}$ (since there is a 1:1 stoichiometry of PCr and ATP in the creatine kinase reaction). Assuming a maximal ATP:O_2 of 6, the in vivo VO_{2mt} needed to power PCr recovery in *C. striata* WM is roughly 0.17 μmol O_2 min^{-1} ml $muscle^{-1}$, which corresponds to ~25% of the VO_{2mt} rate observed in this species (0.71 μmol O_2 min^{-1} ml $muscle^{-1}$ for *C. striata*). While

caution should be used when extrapolating in vitro results to the in vivo condition, our results indicate that there is sufficient aerobic capacity (VO_{2mt}) to support the observed rates of whole muscle PCr recovery while mitochondria are operating well below the maximal rate of respiration.

Increases in VO_{2mt} can be attained by increasing the inner membrane surface density of the mitochondria (Schwerzmann et al. 1986; Schwerzmann et al. 1989). A high cristae surface density facilitates more respiratory chain enzymes to consume O_2 and catalyze the synthesis of ATP (Weis-Fough 1964; Hoppeler et al. 1987). The lack of a significant difference in VO_{2mt} per milligram protein and per milliliter mitochondria across species suggests that fish WM aerobic capacity is largely controlled by V_{mt}, rather than by qualitative differences in the function of mitochondria, consistent with the conclusions of Hoppeler and Weibel (2000). However, in preliminary experiments we isolated mitochondria from RM in three individuals of *P. saltatrix* and found a VO_{2mt} of 54.16 ± 9.38 nmol O_2 min^{-1} mg $protein^{-1}$ (RCR $= 5.90 \pm 0.70$), which is approximately twofold higher than in WM. This may reflect qualitative differences in mitochondria from RM and WM of this species, although this difference could also be due to the caveats associated with normalizing VO_{2mt} to protein content, as described above.

The three species investigated come from three different families and differ dramatically in morphology and behavior. *P. saltatrix* has a fusiform, laterally flattened body with a narrow caudal peduncle, forked caudal fin, and it is an active, pelagic species. *C. striata* is a member of the grouper family with a massive head, a broader caudal peduncle and a fan-like caudal fin, and it is usually associated with bottom structure and is comparatively inactive. *P. lethostigma* is a flatfish that is a sedentary, benthic species that ambushes prey and is also relatively inactive. The behavioral contrast of the three species is reflected in the observed differences in WM V_{mt}. *P. saltatrix* had a threefold higher V_{mt} than *C. striata* and *P. lethostigma* (Fig. 2), and as a result, had a two to threefold higher VO_{2mt} per milliliter of muscle than *C. striata* and *P. lethostigma* (Table 1). This is consistent with observations in the field and in the lab (S.T.K., unpublished results; Olla et al. 1970) that bluefish undergo frequent burst contractions as part of their pelagic lifestyle, necessitating relatively fast post-contractile recovery, whereas black sea bass and flounder undergo relatively infrequent burst contractions.

The body mass independence of VO_{2mt} per milligram protein and per milliliter of mitochondria for the three species of fishes (Fig. 5) is consistent with mammalian studies that have shown the close association between whole animal VO_{2max} and skeletal muscle V_{mt} over a wide range of body masses (Weibel et al. 2004). That is, V_{mt} is a more important determinant of aerobic function and metabolic scaling in fish muscle than are qualitative differences in mitochondria. However, the absence of body mass dependence of VO_{2mt} per milliliter muscle was unexpected, since two of the three species had significantly negative scaling of V_{mt}. This is likely due to the fact that the scaling exponents for V_{mt} were small, while the variance for VO_{2mt} per milliliter of mitochondria was relatively large. Thus, the expected size dependence of VO_{2mt} per milliliter muscle was not detected in the present analysis.

Although allometric scaling of VO_{2max} can likely be attributed in part to changes in V_{mt}, it is unclear what drives the structural decrease in V_{mt} with increasing body mass, and the mechanisms involved in metabolic scaling are still debated (e.g., Weibel 2002; Suarez et al. 2004; West et al. 1997). What is clear, however, is that aerobic metabolism at all levels of biological organization relies not only on catalytic capacity, but also on convective and diffusive transport. During post-metamorphic development, WM fibers in *C. striata* can increase in size until reaching diameters >450 μm (Nyack et al. 2007). The increased intracellular diffusion distances in these fibers influences the structural design of the cell. For instance, oxygen diffusive flux has been shown to cause a dramatic shift in mitochondrial distribution in WM during hypertrophic fiber growth in fishes and crustaceans (Boyle et al. 2003; Hardy et al. 2009; 2010; Kinsey et al. 2007; Nyack et al. 2007), and reaction–diffusion mathematical models demonstrated that this shift is necessary to support the observed rates of aerobic flux (Hardy et al., 2009). It has also been suggested that oxygen diffusion may influence mitochondrial densities in Antarctic fishes that lack myoglobin (Archer and Johnston 1991). However, an aerobic process that is dependent on diffusion (the rate of PCr recovery) in WM of *C. striata* demonstrated mildly negative allometry that was similar to that for V_{mt}, suggesting that V_{mt}, and not diffusion, limited the PCr recovery rate (Nyack et al. 2007). This is consistent with reaction–diffusion models that showed that the rates of aerobic processes in muscle are generally not limited by diffusion (Hardy et al. 2009; Kinsey et al. 2005; 2007; Locke and Kinsey, 2008; Nyack et al. 2007). The present study is consistent with these prior findings and demonstrates that when aerobic flux is measured in isolated mitochondria, body and fiber size effects have no influence on VO_{2mt} per milliliter of mitochondria.

In summary, the method we used for isolating mitochondria from fish WM yielded VO_{2mt} values similar to prior studies of muscle from both fishes and mammals. Differences in VO_{2mt} per milliliter of muscle between species could be attributed to different densities of mitochondria, rather than differences in the properties of mitochondria. Further, VO_{2mt} per milliliter of mitochondria was found to be independent of body mass in fish WM,

indicating that mitochondrial quality does not change over a large range in body mass.

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