Scaling of postcontractile phosphocreatine recovery in fish white muscle: effect of intracellular diffusion

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Nyack AC, Locke BR, Valencia A, Dillaman RM, Kinsey ST.
Scaling of postcontractile phosphocreatine recovery in fish white muscle: effect of intracellular diffusion. Am J Physiol Regul Integr Comp Physiol 292: R2077–R2088, 2007. First published January 25, 2007; doi:10.1152/ajpregu.00467.2006.—In some fish, hypertrophic growth of white muscle leads to very large fibers. The associated low-fiber surface area-to-volume ratio (SA/V) and potentially long intracellular diffusion distances may influence the rate of aerobic processes. We examined the effect of intracellular metabolite diffusion on mass-specific scaling of aerobic capacity and an aerobic process, phosphocreatine (PCr) recovery, in isolated white muscle from black sea bass (Centropristis striata). Muscle fiber diameter increased during growth and was >250 μm in adult fish. Mitochondrial volume density and cytochrome-c oxidase activity had similar small scaling exponents with increasing body mass (−0.06 and −0.10, respectively). However, the mitochondria were more clustered at the sarcolemmal membrane in large fibers, which may offset the low SA/V, but leads to greater intracellular diffusion distances between mitochondrial clusters and ATPases. Despite large differences in intracellular diffusion distances, the postcontractile rate of PCr recovery was largely size independent, with a small scaling exponent for the maximal rate (−0.07) similar to that found for the indicators of aerobic capacity. Consistent with this finding, a mathematical reaction-diffusion analysis indicated that the resynthesis of PCr (and other metabolites) was too slow to be substantially limited by diffusion. These results suggest that the recovery rate in these fibers is primarily limited by low mitochondrial density. Additionally, the change in mitochondrial distribution with increasing fiber size suggests that low SA/V and limited O2 flux are more influential design constraints in fish white muscle, and perhaps other fast-twitch vertebrate muscles, than is intracellular metabolite diffusive flux.

hypertrophy; creatine kinase; mitochondria

STUDIES OF THE SCALING OF metabolism with increasing body mass historically have focused on rates of whole animal O2 consumption (reviewed in Refs. 4 and 41). At the tissue level, the scaling of the activity of aerobic and anaerobic enzymes has been well studied in fish muscle (5, 12, 36, 43, 44). While these studies yield indexes of tissue metabolic capacity, the processes that are powered by aerobic energy metabolism have been largely neglected. While it may be assumed that aerobic capacity and aerobic processes scale in parallel, changes in cellular dimensions and metabolic organization with increasing body mass may cause these metabolic rate indicators to scale independently.

Divergence in the scaling of the rate of energy metabolism and the processes it supports might be particularly prevalent in fish white muscle fibers, which can greatly exceed the size range adhered to by most vertebrate muscle fibers. For the majority of fish species, axial white muscle comprises most of the body mass (12, 32, 45) and increases in fish muscle mass occur by both hyperplasia and hypertrophy (25, 37, 45, 48). In some species of fish, notably in Nototenioids, axial white muscle hypertrophy can proceed until fiber diameters greatly exceed 100 μm (1, 11, 19). Large fiber size leads to a low-fiber surface area-to-volume ratio (SA/V), which may limit O2 flux, and potentially to great intracellular diffusion distances between mitochondria. However, the impacts of developing large muscle fibers on metabolism are not well understood (3, 13, 18, 20, 22, 23).

The burst contractile function of muscle is an anaerobic process that relies on endogenous fuels, and therefore is not dependent on oxygen flux across the cell membrane or net intracellular metabolite diffusive flux. In fish white muscle, phosphocreatine (PCr) is the initial source of high-energy phosphates during burst contraction (e.g., Refs. 7, 8, 14, 38, 42). The creatine kinase (CK) reaction buffers the ATP concentration during contraction via the reversible transfer of a phosphoryl group from PCr to ADP, forming ATP

\[
\text{PCr} + \text{ADP} + \alpha \text{H}^+ \rightleftharpoons \text{Cr} + \text{ATP}
\]

where \(\alpha\) represents a partial proton and Cr is creatine. During recovery from contraction, however, ATP and PCr resynthesis occurs aerobically in fish muscle (7), and the majority of high-energy phosphate flux from the mitochondria to the rest of the cell is again mediated by the CK reaction, with PCr as the dominant diffusing species (10, 29, 31). Thus, the recovery rate of PCr is tightly coupled to both the aerobic production of ATP at the mitochondria and the diffusive flux of PCr and Cr.

The intracellular domain within muscle fibers is crowded with both soluble proteins and fixed structures, such as mitochondria, sarcoplasmic reticulum, and myofilaments, and these obstacles reduce the diffusive flux of small metabolites (16, 17, 21, 22). Furthermore, we have previously shown that the diffusion coefficient for radial motion, \(D_r\), which is the direction of diffusive flux relevant to energy metabolism, is time dependent in skeletal muscle, and mathematical modeling indicated that the impedance at greater diffusion times is
principally caused by the sarcoplasmic reticulum (21, 22). Thus, at the short intracellular diffusion distances characteristic of small cells, $D_t$ is greater than that at the long diffusion distances that may be prevalent in large cells. The time-dependent reduction of radial diffusive flux, coupled with potentially extreme diffusion distances in large fibers of fish white muscle, may impact how the rate of postcontractile PCr recovery scales with body mass.

We have previously hypothesized that diffusion constraints are the cause underlying the recruitment of anaerobic metabolism following burst contractions in the very large muscle fibers of adult crustaceans (3, 13, 18, 22, 23). The anaerobic muscle fibers in the swimming leg of the blue crab, Callinectes sapidus, increase from <60 μm in small crabs to >600 μm in adults, and mitochondrial distribution shifts during growth from a uniform intracellular distribution to one that is predominantly subsarcolemmal (SS) (3). This mitochondrial shift appears to be in response to limited O2 flux due to a low-fiber SA/V but occurs at the expense of increased intracellular diffusion distances between mitochondrial clusters and sarcoplasmic ATPases, which may limit aerobic processes (3, 18). Kinsey et al. (23) tested this hypothesis by comparing the scaling with body mass of postcontractile recovery rates of arginine phosphate (AP) to a mathematical reaction-diffusion model of aerobic metabolism. They concluded that intracellular metabolite diffusion did not limit the rate of AP recovery in large fibers and suggested that SA/V and the associated constraints on O2 flux exert more influence over intracellular diffusion distances between mitochondrial clusters and sarcoplasmic ATPases, which may limit aerobic processes (3, 18).

The present study was an examination of the scaling with body mass of postcontractile PCr recovery rate in isolated white muscle preparations from black sea bass, Centropristis striata. The use of isolated muscle fiber bundles superfused in a high PO2 environment removed the potential effects of low-fiber SA/V on O2 flux, allowing an examination of only the effects of intracellular metabolite diffusion. Muscle fiber diameter, aerobic capacity, and changes in mitochondrial distribution also were measured over a large range in body mass. We hypothesized that there would be an increase in SS mitochondrial clustering during fiber growth, resulting in greater radial diffusion distances between mitochondrial clusters. Furthermore, the rate of postcontractile PCr resynthesis was expected to diverge from indexes of aerobic capacity in muscle as a consequence of increased diffusion distances associated with hypertrophic fiber growth. To rigorously test this latter hypothesis, a mathematical reaction-diffusion model was used to determine whether the observed rate of PCr recovery was high enough to be limited by intracellular metabolite diffusion.

MATERIALS AND METHODS

Animal care. All animal maintenance and experimental procedures were approved by the University of North Carolina Wilmington (UNCW), Institutional Animal Care and Use Committee. Centropristis striata from three size classes were obtained from the UNCW Aquaculture Facility at Wrightsville Beach, NC. While at the aquaculture facility, fish were reared in controlled environments (22–25°C, pH 7.8–8.3, salinity >30 parts per thousand), maintained on a 14:10-h light-dark cycle, and fed a diet of pellets containing 45% protein and 6% fat. At least 24 h before experiments, fish were transported in large coolers to tanks at UNCW’s main campus and maintained under the same conditions until use.

Muscle preparation. The following general protocol was used to prepare epaxial white muscle tissue for the fiber size, mitochondrial distribution, and PCr recovery experiments, with specific alterations described in each section. To avoid muscle PCr depletion due to handling stress, animals were individually sequestered for at least 1 h prior to death in covered and aerated opaque containers with minimal water volume to limit movement. Animals were tranquilized with concentrated MS-222 (tricaine methanesulfonate; Argent Laboratories, Redmond, WA) that was gently diluted to 500 mg/l (final volume) in the container’s seawater via a small hole in the container cover to minimize disturbance of the fish. Fish remained in the container until they were nonresponsive to gentle prodding and/or they lost equilibrium control.

Fish body mass and total length were recorded, and anesthetized animals were killed via cervical dislocation to minimize neural-muscular contractions during dissection. Scales were quickly removed from both sides of the animal’s anterior dorsolateral body surface, dorsal to the operculum and just below and anterior to the dorsal fin. Two rectangular sections of skin in the scaled region were quickly cut and reflected posteriorly. Strips of epaxial white muscle were excised parallel to the fiber long axis and maintained ex vivo in saline solution (composition in mM/l: 133.5 NaCl, 2.4 KCl, 11.3 MgCl2, 0.47 CaCl2, 18.5 NaHCO3, 3.2 NaH2PO4, pH 7.4) aerated with a mixture of 99.5% O2–0.5% CO2. Muscle samples were trimmed to 1- to 1.5-mm-thick bundles in aerated saline parallel to the fiber long axis and fastened at each end using 5-0 surgical silk. Fiber bundles were then stretched and held at 110% of resting length by dental wax affixed to a wood dowel (for fiber morphometry and transmission electron microscopy [TEM]), or in a petri dish containing aerated saline solution (for ex vivo PCr recovery experiments). For examining cytochrome-c oxidase (COX) activity, white muscle was excised contralateral to that removed for the PCr experiments, frozen in cryotubes in liquid nitrogen, and stored at −80°C until processed.

Fiber size. Frozen sections from fiber bundles from the left side of the fish were collected as described previously (3). Frozen mounts were sliced in 12-μm-thick sections from an arbitrary starting point on a Reichert-Jeung Cryocut 1800 cryostat (Leica Microsystems, Bannockburn, IL) and stained using Groat’s variation for Weigert hematoxylin stain for 3–5 min followed by staining in eosin for 60 s. Stained slides were viewed under an Olympus BX-60 microscope (Olympus America, Melville, NY) and digitized with a Spot RT camera (Diagnostic Instruments, Sterling Heights, MI). Polygons of fibers were traced from the micrographs in Adobe Photoshop version 7.0. Cell diameters were determined using Image-Pro Plus version 4.1.0.9 software. The average diameter of the polygon traces through the centroid was calculated in 2-degree increments around the circumference of the cell.

Mitochondrial distribution. Mitochondrial fractional volumes and distributions were examined using TEM. All six small fish and five of the six large fish used for this section were also used for fiber size. Muscle from the right side of the fish was excised, tied, and stretched in the same manner described above. Preparations were fixed at room temperature in 2.5% glutaraldehyde with 0.2 M sodium cacodylate buffer (pH 7.4 and 300 mM) for at least 24 h, rinsed twice (15 min each) in cacodylate buffer, and placed in a secondary fixative of OsO4 for 2 h. Embedding and systematic random thin-sectioning methods followed those described previously (3, 18). Sections were examined on a CM-12 TEM (Philips Research, Briarcliff Manor, NY), and micrographs were taken by a systematic random sampling method for each grid (15). This method was executed by magnifying the first section observed on a TEM grid under low magnification ×3,000, ×5,000, or ×8,000, after which the first micrograph was then taken and utilized as an arbitrary starting point for two subsequent micro-
graphs. The field of view was then moved up by a distance equal to three times the field of view. A second micrograph was collected at this new position, and the process was repeated once more, for a total of three micrographs per grid (15 micrographs per tissue preparation). If any position was obstructed by a TEM grid bar or was no longer on a section, the previous viable field of view was reestablished and a new field of view was sought for three fields of view right from this position (or to the left, if an obstruction was again encountered). If no viable field of view could be found, the grid was repositioned under low magnification and a different section was utilized as the arbitrary starting point. Micrographs were then developed and digitized using a Microtek Scannaker 4 (Microtek Lab, Carson, CA).

Digitized micrographs were viewed in Adobe Photoshop version 7.0 and a stereological point-counting method was applied to calculate mitochondrial fractional volume (15). A point grid was applied to the images, and all points touching extracellular space were subtracted from the total number of points per micrograph. Points that landed on mitochondria were tallied as either SS, if the mitochondrion lay between the sarcolemmal membrane and the myofibrils, or intermyofibrillar (IM) if they were located among myofibrils, regardless of proximity to the sarcolemma membrane. The sum of either SS or IM points from all micrographs taken for each fish were divided by the sum of points hitting cellular space to determine fractional volume (FV). Total mitochondrial fractional volume (TMFV) was calculated as the sum of SS mitochondrial fractional volume (SSFV) and IM mitochondrial fractional volume (IMFV).

**COX activity.** COX activity was determined spectrophotometrically using an enzyme assay kit purchased from Sigma-Aldrich (St. Louis, MO). Frozen tissues were homogenized in a 10-fold dilution of ice-cold enzyme extraction buffer (50 mM imidazole, 50 mM KCl, and 0.5 mM dithiothreitol, pH 7.4) and sonicated on ice for three 10-s bursts with a Fisher Scientific (Pittsburg, PA) 60-sonic dismembrator. Up to 1 ml of homogenate was centrifuged at 12,000 g for 15 min at 4°C. Assays were performed on an Ultrospec 4000 spectrophotometer at 20°C, maintained by an Isotemp 3016 recirculating water bath (Fisher Scientific).

**PCr recovery.** Muscle tissue was excised and prepared as described above, and small, rigid, plastic rings were tied to the end of each trimmed bundle with 5-0 surgical silk. Fiber bundles were allowed to recover from dissection perturbation for at least 30 min for small fish or 45 min for medium and large fish before use (time needed for recovery was determined experimentally). Similar postdissection recovery has been described by other researchers who performed nearly identical procedures on fish muscle (33).

Isolated muscle preparations were suspended between glass hooks that were attached to the thin plastic rings. The lower hook was secured to the frame of the stimulation system (Radnoti Glass Technologies, Monrovia, CA) and the upper hook, made from a thin glass fiber, connected the tissue to an isometric force transducer (Hatfield Apparatus, South Natick, MA). Once suspended and stretched to 110% resting length, muscle preparations were submerged in a 10-mL water-jacketed, glass chamber with built-in electrodes (Radnoti Glass Technologies) containing aerated saline maintained at 20°C by an Isotemp 3016S recirculating water bath. The fiber bundle was then field-stimulated using a Grass S88 physiological stimulator (Astro-Med, West Warwick, RI) to elicit a short tetanus every 150 ms (70 V, 70 Hz, 3-ms pulse duration, 6 pulses per tetanus) for 120 s. The voltage signal from the force transducer was digitized using a Biopac MP100 data acquisition system and recorded on a PC using Acqknowledge version 3.5.7 software (Biopac Systems, Santa Barbara, CA). Following stimulation, fiber bundles were allowed to recover for 0, 5, 10, 15, or 30 min. Bundles were then freeze-clamped in liquid nitrogen-cooled tongs, transferred to small tubes, and stored temporarily (no more than a few hours) in liquid nitrogen until extraction. Unstimulated bundles were used to determine resting [PCr] and were freeze clamped at the end of the postdissection recovery period. Frozen samples were then extracted in perchloric acid as previously described (13, 23).

The relative [PCr] and [Pi] in muscle extracts was determined using 31P-labeled nuclear magnetic resonance (NMR). The relatively broad ATP peaks were not consistently well resolved and were not included in the analysis. NMR spectra were collected at 162 MHz on a 400 MHz DMS spectrometer (Bruker Biospin, Billerica, MA) using a 45° excitation pulse (2.5 μs) and a 0.6-s relaxation delay. Four hundred scans were collected for a total acquisition time of 5 min. A 0.5 Hz line broadening exponential multiplication was applied prior to Fourier transformation. The area of each peak was integrated and expressed as a fraction of the total integral for all peaks.

To ensure that O2 delivery to the fiber bundle core was adequate to support mitochondrial respiration, the critical PO2, where diffusion of O2 would become limiting, was calculated from the solution for concentric diffusion in a Krogh cylinder as described previously (29, 31). The rate of O2 consumption in the bundle was estimated from the PCr recovery curves, where the NMR peak areas were normalized to a peak from a standard PCr sample of known concentration to yield a recovery rate in units of micromoles per minute per gram. The rate of O2 consumption was estimated from the maximum rate of PCr recovery immediately following contraction using a P-to-O ratio of 3. For our maximal bundle diameter of 1.5 mm, the critical PO2 in the bath was 550 Torr, which is a value similar to that from prior analyses (6), and so our experiments were conducted with a bath PO2 well above this limit (>720 Torr).

**Reaction-diffusion model.** The reaction-diffusion model used in the present study was as described in Kinsey et al. (23), with parameters adjusted to comply with fish white fibers and data collected in the present study. In brief, the model consists of Michaelis-Menten expressions for a mitochondrial boundary reaction (ADP + P → ATP) with a rate dependent on the ADP concentration, a myosin ATPase bulk reaction (ATP → ADP + Pi) that is only active during contraction, and a basal ATPase bulk reaction that is always active. An appropriate kinetic expression for CK was also included in the bulk phase (34) and D0 values were used that accounted for the time-dependence of radial diffusion (21, 22). The diffusion and reaction of ATP, ADP, PCr, Cr, and Pi were modeled in a one-dimensional system that extended from the surface of a mitochondrion to a distance (A/2) equal to half of the mean free spacing between clusters of mitochondria. Temporally and spatially dependent concentration profiles of metabolites were calculated according to a molar-species

| Table 1. Body size characteristics of fish and n values used for each type of experiment |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
|---------|-----|----------------|-----------------|-----|----------------|-----------------|-----|----------------|-----------------|-----|----------------|-----------------|
| Small   | 7   | 2.4±0.8        | 1.5–5.8         | 6   | 1.9±0.2        | 1.5–2.2         | 12  | 6.2±1.1        | 1.6–14.2        | 26  | 10.8±0.8       | 3.3–20.8        |
| Medium  | 77±2.3 | 46.7–181.8    | 6   | 3166.4±667.0   | 2413.2–4496.5   | 12  | 2323.2±297.1   | 948.5–3668.3   | 18  | 2507.93±315.83 | 948.5–4159.4 |

Values are grams for mean body mass and are means ± SE. In some cases, multiple strips of epaxial white muscle were isolated from a single fish and were used in multiple experiments. COX, cytochrome-\(c\) oxidase.
continuity equation (2). The bulk reactions catalyzed by CK, myosin ATPase, and basal ATPase were assumed to occur homogenously throughout the domain $0 < x < \lambda/2$, where $x$ is the distance from the mitochondrial surface.

Simulations of a burst-contraction recovery cycle were generated using the finite element analysis software, FEMLAB (Comsol, Burlington, MA). Parameter values used in the model are presented in Table 2. The resting metabolite concentrations were from data gathered during this study and from Hubley et al. (16). The $D_i$ for each metabolite was based on both direct measurements from fish white fibers (21) and from the relationship of molecular mass and $D_i$ in muscle (22). Intracellular diffusion distances ($\lambda/2$) were estimated in two ways to bracket the range of possible distances: 1) assuming an exclusively SS distribution of mitochondria, $\lambda/2$ was determined from fiber radius data, and 2) assuming a uniform distribution of IM mitochondria, $\lambda/2$ was determined as described previously (3). A $K_m$ for the mitochondrial reaction ($K_m^{mito}$) for ADP was used that is appropriate for fast-twitch skeletal muscle (27). The basal ATPase consumed ATP at a constant rate ($V_{bas}$) that balanced the rate of mitochondrial ATP production at rest. CK dissociation constants ($K_i$ and $K_I$) were obtained from Morrison and James (34) the maximal velocities for the forward reaction ($V_{mCKfor}$) in the direction of ATP formation and in the reverse direction ($V_{mCKrev}$) were taken from measurements in fish white muscle (35). The myosin ATPase maximal velocity ($V_{mmyo}$) and $K_m$ ($K_{mmyo}$) for ATP were the same as in Hubley et al. (16).

While the model generated temporally and spatially resolved concentrations of metabolites, our experimental measurements yielded values that were spatially averaged across the fiber. To compare the model results to the experimental data, the model data was mathematically volume averaged. The duration of myosin ATPase activation was then adjusted so that the simulated decrease in [PCr] was comparable to that in the observed data, and the maximal velocity of the mitochondrial boundary reaction ($V_{mito}$) was adjusted so that the PCr recovery curve predicted by the model approximated the measured recovery rate. This approach allowed us to analyze whether the observed rate of PCr recovery was limited by intracellular metabolite diffusion.

**Statistical analysis.** One-way ANOVA was used to test for significant effects of size class, and where significant size effects were detected, Tukey’s honestly significant difference (HSD) test was used for pairwise comparisons. A two-way ANOVA was implemented to examine the interaction between size class and recovery time on [PCr]. All statistical tests were analyzed with JMP software version 7.0.2 (SAS Institute, Cary, NC). In all cases, $P < 0.05$ was considered significant. Linear regression analysis of COX activity and TMFV data on body mass were conducted using Sigma Plot version 8.02 (SPSS, Chicago, IL).

Fig. 1. Typical cross sections of Centropristis striata white muscle fibers from small (A), medium (B), and large fish (C) at $\times 20$ magnification.

Fig. 2. Epaxial white muscle cross-sectional fiber diameter distribution and mean by size class (inset) from small, medium, and large fish. The fiber cross-sectional diameters, shown as % frequency, are binned in 10-$\mu$m increments. *Each size class is significantly different from the other two (inset). Values are means ± SE.
RESULTS

Fish body mass and fiber size. The present study used fish ranging in body mass from 1.5 to 4,496 g and in total body length from 45 to 544 mm (Table 1). This corresponds to a mass range of nearly 3,000-fold and a 12-fold increase in length. Figure 1 demonstrates hypertrophic growth exhibited by epaxial white muscle in C. striata during ontogeny, leading to large fibers in adults. The fiber diameters ranged from 5.3 to 80.4 μm (348 fibers) in the small fish, 16.4 to 152.5 μm (245 fibers) in the medium fish, and 74.1 to 465.4 μm (239 fibers) in the large animals (Fig. 2). The distribution of muscle fiber diameters noticeably broadened during growth. One-way ANOVA indicated a significant effect of size class on mean fiber diameter ($F_{2,35} = 2.35, \text{df} = 2, P < 0.001$), and pairwise comparisons indicated that each size class was significantly different from the other two (Fig. 2 inset; Tukey’s HSD, $P < 0.05$). The mean diameter of the small- and medium-size class differed by only two-fold despite a nearly 32-fold difference in mean body mass, while the difference in mean fiber diameter was 3.5-fold between the medium and large-size classes, which corresponded to a 45-fold difference in body mass.

Mitochondrial distribution. Representative TEM micrographs showing mitochondrial distribution are presented in Fig. 3. Mitochondrial density scaled negatively with body mass and the TMFV, was significantly higher in the small fish than the large (Fig. 4A; Tukey’s HSD, $P < 0.05$). Both IMFV and SSFV decreased with increasing body mass, but there were no significant differences between size classes in either category (Fig. 4A). To determine whether SS mitochondria were clustered more densely in large fibers than in small, the SS mitochondrial volume per sarcolemmal membrane area (SSMV/SA) was calculated by dividing SSFV by the mean fiber SAV. Since the same fish used in determining fiber diameter were also used to measure mitochondrial fractional volume, the fiber SAV could be accurately calculated. A significant increase in the SSMV/SA was found (Fig. 4B; Tukey’s HSD, $P < 0.05$), indicating a greater clustering of mitochondria at the sarcolemmal membrane in the fibers of large fish.

COX activity. A line was fitted to the mass-specific COX activity according to the scaling relationship: COX activity = $aM^b$, where M is animal mass, $a$ is a constant, and $b$ is the scaling exponent (41). The mass-specific COX activity of epaxial white muscle from 39 fish scaled negatively with body mass with a $b$ of $-0.10$ (Fig. 5; $r^2 = 0.11$). This scaling exponent is similar to the value for TMFV of $-0.06$ obtained using the data in Fig. 4 (graph not shown).
**Metabolite dynamics and reaction-diffusion analysis.** Model input parameters are detailed in Table 2. A typical simulation of volume-averaged changes in metabolite concentrations are shown in Fig. 6. The near-equilibrium status of the CK reaction is reflected by the nearly perfect buffering of [ATP] during a contraction-recovery cycle. Experimentally measured changes in [PCr] and [Pi] and volume-averaged model approximations of the measurements during a contraction-recovery cycle are shown in Fig. 7. The resting values of both metabolites were significantly different from those immediately postcontraction (Tukey’s HSD, \( P < 0.05 \)). Aerobic recovery for all size classes was slow, and complete recovery required \( \approx 30 \) min. Two-way ANOVA revealed no significant interaction of size class and recovery time on [PCr] or [Pi] ([PCr]: \( F = 0.40, df = 10, P = 0.95 \); [Pi]: \( F = 0.39, df = 10, P = 0.95 \)), indicating no differences in the rate of PCr or Pi recovery between size classes. To compare the scaling with body mass of the measured maximal rate of PCr recovery to that found for mitochondrial volume density and COX activity, the rate of recovery during the first 5 min postcontraction was analyzed using the scaling equation described above. The PCr recovery rate scaling exponent (\( b = -0.07 \)) was very similar to that for mitochondrial density and COX activity (see above).

Figure 7, B–D and F–H shows that the volume-averaged model simulations reasonably approximated the measured rates of PCr and Pi recovery, thus permitting us to draw conclusions about diffusion limitation in the fibers. Figure 8 is an expansion of these model results to include spatially, as well as temporally, resolved profiles of [PCr] and [Pi] during contraction-recovery cycles in each size class. The other high-energy phosphate molecules yield similar results as shown previously (23). The simulations are for the case with exclusively SS mitochondria, which yields larger diffusion distances and the highest likelihood for diffusion limitation of PCr recovery (the cases with a uniform distribution of mitochondria are not shown but yielded recovery rates that were only slightly higher). There were only very shallow intracellular concentration gradients in all of the high-energy phosphates in the small (Fig. 8, A and D) and medium (Fig. 8, B and E) size classes, indicating essentially no control of metabolic flux by intracellular diffusion (these small gradients are not readily visible on the concentration scale used, since the gradients are a tiny fraction of the total concentration). The concentration gradients in the largest fibers were more substantial than in the other two size classes, but despite the extreme diffusion distances were still barely perceptible for each metabolite (Fig. 8, C and F), indicating that diffusive flux is fast compared with the rate of metabolite recovery.

The maximal concentration gradients observed occurred in the largest fibers immediately after burst contraction ceased when oxidative phosphorylation was maximally stimulated by the high [ADP] (Fig. 9). For each metabolite, the spatial variation in concentration is shown as absolute concentration variation in concentration is shown as absolute concentration gradients (Fig. 9A) and as gradients relative to the total metabolite concentration (Fig. 9B). The magnitude of the gradients for all metabolites was extremely small and represented a tiny fraction of the total concentration for most metabolites. The small gradients in [ADP] constituted a larger fraction of total ADP pool (about 13%) due to the low concentration of ADP in muscle. However, the lack of a difference in recovery rates between size classes and the negligible effect of simulating a reduced diffusion distance on recovery rate (by assuming a uniform distribution of mitochondria) indicate that the measured processes were too slow to be limited by intracellular metabolite diffusion. This conclusion is further supported by the fact that the initial velocity of postcontractile recovery (\( V_0 \))

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![Diagram](http://www.ajpregu.org/...)

**Fig. 4.** A: total mitochondrial fractional volume (TMFV), SS mitochondrial fractional volume (SSFV), and IM mitochondrial fractional volume (IMFV). *Only TMFV is significantly different between the two size classes. B: SS mitochondrial volume/sarcolemmal surface area (SSMV/SA) in the small- and large-size classes. Values shown are means ± SE. *Means are significantly different.

**Fig. 5.** Scaling with body mass of cytochrome-c oxidase (COX) activity from epaxial white muscle of *C. striata*. IU, international units. The line is described by the equation, COX activity = 0.1 M^{-0.1} (\( r^2 = 0.11, P < 0.05 \)), where M is body mass.
is proportional to the volume-averaged rate of ATP production at the mitochondria \((V_{\text{mito}})\) in mM\(\cdot l^{-1}\cdot s^{-1}\) at all diffusion distances (Fig. 10). If diffusion were substantially limiting metabolite recovery, then \(V_0\) would be lower relative to \(V_{\text{mito}}\) at large diffusion distances, leading to a steep, negative slope for each metabolite in Fig. 10.

**DISCUSSION**

The major findings of the present study were 1) hypertrophic growth of white muscle fibers occurred during ontogeny, and this growth is associated with small, negative scaling exponents for both mitochondrial density and COX activity, 2) a significant increase in the amount of sarcolemmal membrane occupied by mitochondria occurred during fiber growth, 3) the time course of PCr recovery was independent of fish body mass (and therefore fiber size), and the maximal rate of recovery immediately postcontraction had a body mass scaling exponent similar to that for the measured indicators of aerobic capacity, and 4) the rate of postcontractile PCr recovery (and other metabolites) was too slow to be substantially limited by intracellular diffusion.

Fish white muscle fibers are typically characterized as having lower oxidative capacity and larger diameters relative to other types of fish skeletal muscle (26, 30), and there are examples of white fibers attaining very large dimensions (11, 19, 48). However, the white muscle fibers in many fish species do not grow to particularly large sizes (30, 37, 48). For example, species, such as carp \((Cyprinus carpio)\) (24) and white sea bass \((Atractoscion nobilis)\) (49), which attain body masses as adults that are similar to, or greater than, the adult C. striata used in this study, exhibit much smaller mean white muscle fiber diameters. The present study was not designed to broadly address the determinants of fiber size. However, it is likely that the widely varying relationship between body mass and muscle fiber diameter in different species of fish (and other organisms) results from a complex interplay of developmental programs, body mass range, reaction-diffusion constraints, behavior, and evolutionary history.

**Table 2. Parameters used in reaction-diffusion model**

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<td></td>
<td>(D_{\text{Cr}})</td>
<td>(2.39\times10^{-6})</td>
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<td>(2.39\times10^{-6})</td>
<td>cm(^2) (\cdot) s(^{-1})</td>
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<tr>
<td></td>
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<td>cm(^2) (\cdot) s(^{-1})</td>
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<tr>
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<td>(1.22\times10^{-6})</td>
<td>cm(^2) (\cdot) s(^{-1})</td>
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<tr>
<td></td>
<td>(D_{\text{ADP}})</td>
<td>(1.33\times10^{-6})</td>
<td>(1.33\times10^{-6})</td>
<td>(1.33\times10^{-6})</td>
<td>cm(^2) (\cdot) s(^{-1})</td>
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<tr>
<td></td>
<td>(\lambda/2)</td>
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<td>35.4</td>
<td>124.4</td>
<td>(\mu)m</td>
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<tr>
<td>Mitochondrial boundary reaction</td>
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<td>(5.07\times10^{-16}) (0.0268)</td>
<td>(9.17\times10^{-16}) (0.0259)</td>
<td>(3.17\times10^{-15}) (0.0255)</td>
<td>mmol (\cdot) cm(^{-2}) (\cdot) s(^{-1}) (mmol (\cdot) (\mu)mol(^{-1}) (\cdot) s(^{-1}))</td>
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<td>0.62</td>
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<tr>
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<td>(K_{\text{CKrev}})</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td></td>
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PCr, phosphocreatine; Cr, creatine; \(D_{r}\), diffusion coefficient for radial motion; \(\lambda/2\), intracellular diffusion distance; m, maximal; mito, mitochondrial; bas, basal; CK, creatine kinase; for, forward; rev, reverse; \(K_i\) and \(K_{i}^{\text{Ki}}\), dissociation constants; myo, myosin.
The TMFV (Fig. 4A) was within the range reported in white muscle of other temperate fish species (9, 26, 47). Also, the negative scaling exponent of COX activity \( (b = -0.10) \) corresponds fairly well with that of TMFV \( (b = -0.06) \). The finding that SSFV was greater than IMFV in both small and large fibers may reflect low rates of O\(_2\) supply to the muscle due to sparse vascularization, which is typical of teleost white muscle (9, 26). The large and significant increase in the amount of sarcolemmal membrane occupied by mitochondria in adult \( C. \) striata (Fig. 4B) may help counteract the very low SA/V in the large fibers. This finding is similar to the shift in mitochondria observed during hypertrophic growth in the large white fibers of \( C. \) sapidus, which was attributed to low-fiber SA/V (3, 23). Mainwood and Rakusan (29) showed mathematically that the capillary O\(_2\) required to supply sufficient O\(_2\) to the fiber was considerably less for a SS distribution of mitochondria than when mitochondria were evenly distributed over the entire fiber. One interpretation of the change in mitochondrial distribution with increasing fiber size is that mitochondria are simply produced in regions of the cell that have adequate O\(_2\) (in large fibers, near the membrane). Alternatively, when less surface area is available for O\(_2\) flux in large fibers, a SS mitochondrial distribution may serve to facilitate O\(_2\) flux across the sarcolemmal membrane. In this case, the rapid consumption of O\(_2\) by mitochondrial clusters would reduce the intracellular O\(_2\) inside the sarcolemma and would enhance the transmembrane O\(_2\) gradient leading to increased flux. Therefore, in both adult \( C. \) striata and adult \( C. \) sapidus, the predominantly SS mitochondrial distribution may help offset the effects of low SA/V, but it also leads to increased intracellular diffusion distances between mitochondrial clusters and sites of ATP utilization, which may limit rates of aerobic metabolism (3, 13, 18, 21, 22, 23).

Postcontractile PCr recovery is powered exclusively by aerobic metabolism in fish white muscle (7), and the rate of recovery is assumed to define the rate of O\(_2\) supply and substrate utilization in skeletal muscle (28). Kinsey et al. (23) analyzed in vivo postcontractile recovery rates of AP in the

![Graphs and figures showing PCr and Pi recovery curves in different size classes of fish muscle.](http://ajpregu.physiology.org/)

**Fig. 7.** Phosphocreatine (PCr) \((A-D)\) and Pi \((E-H)\) recovery curves in isolated white fiber bundles. A and E: fractional [PCr] and [Pi] are presented for all size classes. To achieve an approximate fit of the reaction-diffusion model to the data, the experimental values were converted to absolute concentrations of PCr \((B-D)\) and Pi \((F-H)\) by assuming a resting [PCr] of 30.9 mM. The absolute [PCr] and [Pi], as well as the volume-averaged approximations of the recovery rate from the reaction-diffusion analysis (solid line), are shown for small (B and F), medium (C and G), and large fish (D and H). Each time point within each size has \( n \approx 5 \) and values shown are means ± SE.
white swimming muscle of *C. sapidus*, which attain greater fiber diameters than *C. striata*, using a mathematical reaction-diffusion model of aerobic metabolism similar to that used in the present study. They concluded that diffusion did not greatly limit aerobic AP recovery in large fibers and suggested that SA/V exerted more influence over metabolic fluxes and mitochondrial organization. However, that study utilized an in vivo exercise and recovery protocol, so both intracellular metabolite diffusion and/or low SA/V and limited O₂ flux may have constrained aerobic metabolism. The present study used superfused muscle fiber preparations in a solution with high PO₂ similar to the study of Curtin et al. (7), which maximized the oxygen gradient across the fiber bundle and, thus, removed the effect of fiber SA/V on metabolic recovery. This approach allowed an examination of the effect of intracellular metabolite diffusion on recovery in isolation, without the confounding effects of fiber-size dependent limitations on O₂ flux as in our previous work (3, 13, 18, 23).

PCr and P₁ recovered slowly in all size classes, and there was no significant interaction between size class and recovery time on postcontractile concentration, indicating that recovery rate did not differ significantly among size classes (Fig. 7). The rate of PCr recovery observed in the present study is comparable to that found in isolated white muscle from dogfish, *Scyliorhinus canicula* (7), and to in vivo rates of recovery in white muscle of rainbow trout, *Oncorhynus mykiss* (42), tilapia, *Oreochromis mossambicus* (46), and Pacific spiny dogfish, *Squalus acanthias* (38). Additionally, the observed rates are similar to that of AP recovery in *C. sapidus* anaerobic muscle (23), which also has large fibers and a similar mitochondrial density to *C. striata*. The size class independence of metabolite recovery in *C. striata* occurred despite significant differences in fiber diameter and mitochondrial organization, suggesting that the large fibers are not greatly limited by intracellular metabolite diffusion. This is consistent with the finding that the maximal rate of PCr recovery immediately following contraction had a scaling exponent (−0.07) that did not diverge from that found for TMFV (−0.06) or COX activity (−0.10). This conclusion is further supported by the reaction-diffusion mathematical analysis, which demonstrated that the observed rate of metabolite recovery in the present study was too low to be substantially diffusion limited (Figs. 7–9) and that the rate of
recovery was essentially controlled by the fiber’s aerobic capacity (Fig. 10).

Our findings contrast with the reaction-diffusion analysis of fish white muscle by Hubley et al. (16), which demonstrated large gradients in high-energy phosphate compounds. However, this prior study assumed perfect buffering of ATP concentration at the mitochondrial boundary from the concentration at each distance from the mitochondria (e.g., PCr and ATP concentration is lower further from the mitochondria). The magnitude of the gradient relative to the total concentration of each metabolite is shown (B), where the concentration was normalized to a value of zero at the mitochondrial boundary.

Fig. 9. Simulation of the maximal concentration gradients observed for all metabolites, which occurred in the large fish size class immediately after burst contraction ended. The absolute magnitude of the gradients is shown (A), where concentration difference was determined by subtracting the concentration at the mitochondrial boundary from the concentration at each distance from the mitochondria (e.g., PCr and ATP concentration is lower further from the mitochondria). The magnitude of the gradient relative to the total concentration of each metabolite is shown (B), where the concentration was normalized to a value of zero at the mitochondrial boundary.

Despite the findings of the present study, simulations of energetics in aerobic muscle fibers during steady-state contraction indicate that intracellular diffusive flux may exert substantial control over metabolic flux if the rate of ATP turnover is high, even when diffusion distances are short (13).

The notion that intracellular diffusion limits aerobic metabolism in systems without long diffusion distances has been proposed previously for cardiac myocytes (40). These authors suggested that aerobic muscle fibers are composed of discrete intracellular energetic units, and that metabolite diffusion (particularly ADP) within these units is locally restricted. Mathematical modeling indicated that this structural organization induces heterogeneity of ADP diffusion in permeabilized aerobic fibers where diffusion coefficients are locally reduced by one to two orders of magnitude, and these low rates of diffusion lead to the limitation of oxidative phosphorylation in these experimental preparations (40). In contrast, the present study assumed that sarcoplasmic metabolite diffusion occurred as in other porous media, which is consistent with previous measurements and modeling of diffusion in fast-twitch muscle (21, 22). Furthermore, as pointed out by Saks et al. (40), fast-twitch muscles are not thought to contain the functional energetic units that they described for cardiac myocytes. Rather, the present study focused on the effect of widely varying diffusion distances between mitochondria on a relatively slow aerobic metabolic process and found that the effects of diffusive flux (including ADP) were negligible.

In fish that undergo a large increase in body mass, prolonged muscle hypertrophy may be the default developmental strategy if there are no selective pressures against large fiber size. Although the experimental approach used here eliminated the effects of SA/V, the expectation is that the low SA/V of the large fibers would lead to slow PCr recovery in vivo in the large fish.
In the wild, the low rate of PCr recovery would mean that bouts of high power contractions would be followed by a relatively protracted recovery period before a similar bout of contractions could occur. The fish in this study had no discernible medialis-lateral red muscle, which has been observed in other fish (12).

The lack of red muscle in C. striata suggests that swimming at higher speeds is powered almost exclusively by white muscle, whereas routine locomotion is slow and relies largely on labriform swimming (Nyack AC, unpublished observations).

Black sea bass therefore appear to be capable of extended periods of very slow aerobic swimming or short bursts of high-velocity swimming, but they are not well equipped for sustained periods of swimming at intermediate velocities. Since C. striata is a benthic fish associated with hard bottom communities and is often in close proximity to cover (39), a protracted recovery following burst swimming may not have negative consequences. That is, aspects of the animal’s behavior may make large fiber size a relatively unimportant functional constraint.

It is also possible that there is positive selection for the presence of large fibers in fish white muscle. Johnston et al. (20) proposed that the cost of maintaining cellular ion balance is relatively high, and since a large amount of the body mass in most teleosts is white muscle, ion homeostasis within this tissue contributes considerably to whole animal metabolism. Thus, the low SA/V in tissues comprising large fibers would lead to less membrane leak, and therefore fewer ion pumps would be required to maintain ion balance. This hypothesis could also be applied broadly, in the sense that many types of muscle fibers may have selective pressure to be as large as possible, within the constraints of the muscle’s function. At present, however, it is unclear whether very large cells arise as a natural consequence of hypertrophic growth in species with a large body mass range and behavioral patterns that obviate the need for rapid metabolic recovery or whether there is selection in favor of large cells to lower energetic costs associated with ion homeostasis.

In summary, this study characterized the epaxial white musculature in C. striata and investigated the effects of hypertrophy on postcontractional PCr recovery, which is the product of an aerobic process. White muscle fibers grew very large during ontogeny, and the proportion of the sarcosomal membrane occupied by mitochondria was significantly greater in large fibers than in small fibers. This change in mitochondrial distribution may help facilitate diffusion of O2 into the large fibers. The scaling with body mass of the maximal rate of PCr recovery paralleled that of indicators of aerobic capacity, despite the extreme dimensions attained during hypertrophic white muscle growth, and the rate of PCr recovery was too slow to be greatly limited by intracellular metabolite diffusion. It is therefore likely that low mitochondrial density is primarily responsible for the slow rate of PCr recovery, and that SA/V may be a more important factor than intracellular metabolite flux on metabolic design in fish white muscle fibers, and perhaps in other vertebrate fibers as well.

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