

# Metabolic influences of fiber size in aerobic and anaerobic locomotor muscles of the blue crab, *Callinectes sapidus*

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## Summary

Diameters of some white locomotor muscle fibers in the adult blue crab, *Callinectes sapidus*, exceed 500  $\mu\text{m}$  whereas juvenile white fibers are <100  $\mu\text{m}$ . It was hypothesized that aerobically dependent processes, such as metabolic recovery following burst contractions, will be significantly impeded in the large white fibers. In addition, dark aerobic fibers of adults, which rely on aerobic metabolism for both contraction and recovery, grow as large as the white fibers. These large aerobic fibers are subdivided, however, thus decreasing the effective diameter of each metabolic functional unit and enabling aerobic contraction. The two goals of this study were: (1) to characterize the development of subdivisions in the dark levator muscle fibers and (2) to monitor post-contractional metabolism as a function of fiber size in aerobic and anaerobic levator muscles. Dark levator muscle fibers from crabs ranging from <0.1 g to >190 g were examined with transmission electron microscopy to determine the density of mitochondria and subdivision diameters. Across all size classes, there was a constant mitochondrial fractional area (25% of the total subdivision area) and subdivision size (mean diameter of  $36.5 \pm 2.7 \mu\text{m}$ ). Thus, blue crab dark levator fibers are unusual in having metabolic functional units (subdivisions) that do not increase in size during development while the contractile functional units (fibers)

grow hypertrophically. The body mass scaling of post-contractional lactate dynamics was monitored during recovery from anaerobic, burst exercise in white and dark muscle, and in hemolymph. There were no differences among size classes in lactate accumulation during exercise in either muscle. However, in white fibers from large crabs, lactate continued to increase after exercise, and lactate removal from tissues required a much longer period of time relative to smaller crabs. Differences in lactate removal among size classes were less pronounced in dark fibers, and post-contractional lactate accumulation was significantly higher in white than in dark fibers from large animals. These data suggest that the large white fibers invoke anaerobic metabolism following contraction to accelerate certain phases of metabolic recovery that otherwise would be overly slow. This implies that, in addition to the typical mass-specific decrease in oxidative capacity that accompanies increases in animal mass, aerobic metabolic processes become increasingly limited by surface area to volume and intracellular diffusion constraints in developing white muscle fibers.

Key words: crustacean, blue crab, *Callinectes sapidus*, muscle fiber, fiber size, fiber growth, anaerobic, aerobic, scaling, citrate synthase, lactate, diffusion, recovery, exercise, metabolism, TEM, mitochondria.

## Introduction

Muscle fibers typically fall within a size range of 10–100  $\mu\text{m}$  along the shortest axis (e.g. Russell et al., 2000). Dimensions exceeding this range are thought to compromise aerobic metabolism, which relies upon oxygen flux across cell membranes (e.g. Kim et al., 1998), as well as ATP-equivalent flux from mitochondria to sites of ATP demand (Mainwood and Rakusan, 1982). However, some muscle fibers from crustaceans do not adhere to the usual constraints on cellular dimensions, and in the adult blue crab, *Callinectes sapidus*, fiber diameters from the white locomotor muscles that power swimming often exceed 500  $\mu\text{m}$  (Tse et al., 1983; Boyle et al., 2003). By contrast, white fibers from juvenile *C. sapidus* are

<100  $\mu\text{m}$  in diameter, meaning that during development these fibers cross and exceed the usual limit on cell dimensions while preserving function (Boyle et al., 2003).

An interesting feature of these white muscle fibers is that the distribution of mitochondria varies as a function of fiber size. In juveniles, mitochondria are uniformly distributed throughout the fibers and the population is equally divided between subsarcolemmal and intermyofibrillar fractions. However, in white fibers from adults, mitochondria are largely subsarcolemmal (Boyle et al., 2003). Thus, in large fibers, there is a cylinder of oxidative potential around the periphery of the cell whereas the inner core of the fiber has limited

aerobic capacity. This developmental redistribution of mitochondria dramatically increases intracellular diffusion distances between mitochondria in large fibers, more so than would be expected from increases in fiber diameter alone. Since contraction in these fibers is anaerobically powered and relies on endogenous fuels, large cell size should not impact this process. However, the small surface area to volume ratios (SA:V) and intracellular diffusion limitations associated with large fiber size would be expected to affect aerobic metabolism. This is consistent with observations that post-contractile recovery in muscle from adult *C. sapidus* is a very slow process (Milligan et al., 1989; Henry et al., 1994).

Burst contraction in crustacean muscles is similar to that in vertebrates, where intracellular phosphagen and glycogen stores are depleted and lactic acid accumulates (England and Baldwin, 1983; Booth and McMahon, 1985; Head and Baldwin, 1986; Milligan et al., 1989; Morris and Adamczewska, 2002). In crustaceans, there is an initial reliance on the hydrolysis of the phosphagen, arginine phosphate (AP), after which anaerobic glycogenolysis is recruited. Glycogenolytically powered contractions are slower than those powered by phosphagen hydrolysis (England and Baldwin, 1983; Head and Baldwin, 1986; Baldwin et al., 1999; Boyle et al., 2003), and lactate only accumulates during an extended series of anaerobic contractions, which are thought to be a normal part of the animal's behavior in the environment (Booth and McMahon, 1992).

In contrast to anaerobic contraction, the recovery from sustained exercise in crustaceans is quite different from the vertebrate paradigm. An early phase of recovery is a restoration of AP pools. This step of recovery is largely powered by anaerobic glycogenolysis (England and Baldwin, 1983; Head and Baldwin, 1986), which contrasts with the exclusively aerobic resynthesis of the vertebrate phosphagen, creatine phosphate (Kushmerick, 1983; Meyer, 1988; Curtin et al., 1997). Thus, in crustaceans, most glycogen depletion and lactate accumulation occurs after contraction (England and Baldwin, 1983; Head and Baldwin, 1986; Kamp, 1989; Henry et al., 1994; Morris and Adamczewska, 2002; Boyle et al., 2003). The reasons for this post-contractile lactate accumulation in crustacean muscle are not known. However, it may be a mechanism for accelerating certain phases of the recovery process to facilitate additional high-force contractions, since exclusive reliance on aerobic metabolism would be expected to result in an extremely slow recovery in very large fibers (Boyle et al., 2003). Therefore, in crustaceans, anaerobic metabolism would be expected to contribute to post-contractile recovery more in large fibers than in small fibers.

Despite reliance upon anaerobic metabolism to power specific recovery processes, complete recovery ultimately must depend on aerobic pathways. The aerobic phase of recovery is dependent upon oxygen flux across the sarcolemma, which is influenced by SA:V, and diffusive flux of ATP-equivalents from the mitochondria at the fiber periphery to points of utilization in the fiber core (Boyle et al., 2003). The phosphoryl transfer from ATP to arginine, forming AP, is catalyzed by

arginine kinase (AK), which functions near equilibrium in crustacean muscle (Ellington, 2001; Holt and Kinsey, 2002). Since the ATP-equivalent diffusive flux is carried almost exclusively by AP, aerobic processes are dependent on the rate of AP diffusive flux (Meyer et al., 1984; Ellington and Kinsey, 1998; Kinsey and Moerland, 2002), which is strongly hindered by structural barriers in crustacean muscle (Kinsey et al., 1999; Kinsey and Moerland, 2002; Fig. 1). The scope of diffusion limitation can be appreciated by examining the time required for intracellular metabolite diffusion in muscle. In juvenile blue crabs, diffusion of AP across a muscle fiber takes place in several seconds, whereas in adults the time required for diffusion across a fiber can exceed 20 min (Kinsey and Moerland, 2002). It is therefore expected that SA:V and diffusion limitations will increasingly constrain the rate of aerobic post-exercise recovery as animals become larger.

In addition to white locomotor muscles, *C. sapidus* also have smaller bundles of mitochondria-rich, dark fibers that power aerobic swimming (Tse et al., 1983), and these fibers must also maintain contractile function over a 3000-fold increase in body mass during post-metamorphic development. There are two general mechanisms by which an organism can increase its muscle mass during post-embryonic growth (reviewed in Rowleson and Veggetti, 2001). New muscle fibers can develop through hyperplasia, as seen in cephalopods (e.g. Pecl and Moltschanivskyj, 1997), or existing fibers can increase in diameter through hypertrophy, as seen in fishes (e.g. Weatherly and Gill, 1985) and crustaceans (Bittner and Traut, 1978). Whereas the large developmental increase in body mass in *C. sapidus* causes white locomotor muscle fibers to become giant (Boyle et al., 2003), the aerobic function of the dark muscle fibers necessitates that they remain small. *C. sapidus* appear to have resolved the conflicting demands for hypertrophic development and aerobic contraction by subdividing the dark fibers into smaller functional units (Tse et al., 1983). While the development of white locomotor fibers has been previously described by Boyle et al. (2003), the ontogenetic development of the aerobic fibers has not been addressed. For instance, it is unclear whether a constant number of aerobic subdivisions is present in fibers from all size classes, including the smallest fibers from juvenile animals where diffusion would not be expected to limit aerobic processes, or whether subdivisions form throughout development and function to maintain a constant, small size of each aerobic functional unit.

The objectives of the present study were (1) to characterize the post-metamorphic developmental pattern of subdivision formation in the dark levator swimming muscle, which constitutes a giant fiber system that has undergone selection for aerobic function, and (2) to assess the reliance on anaerobic metabolism of white and dark fibers during post-contractile recovery. We investigated mitochondrial content, the scaling of aerobic enzyme activity, and subdivision formation in fibers of the dark levator muscle during post-metamorphic growth in *C. sapidus*. In addition, lactate levels following fatiguing exercise were monitored in white and dark levator muscle tissues as well as in hemolymph as a function of animal size.

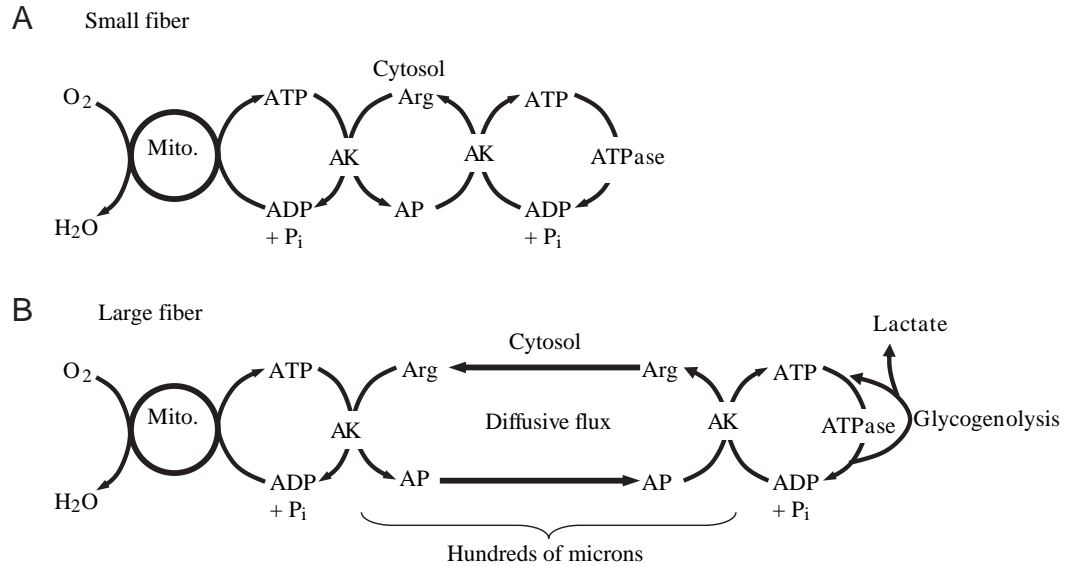


Fig. 1. Arginine kinase (AK) mediates ATP-equivalent flux in crustacean muscle. Diffusive flux of arginine phosphate (AP) occurs over short distances in small white fibers of juvenile crabs (A) but, as the fibers grow, diffusive flux must occur across hundreds of microns (B). Consequently, there is expected to be a reduction in rates of aerobic processes, such as aerobic recovery following exercise, as fiber size increases during development. In large fibers of adult crustaceans anaerobic glycogenolysis occurs following contraction, presumably to speed up phases of the recovery process.

We hypothesized that (1) dark fibers would grow hypertrophically, but subdivisions would form throughout development to maintain a constant, small effective diameter, (2) post-contraction lactate accumulation would be greater and removal would be slower in white fibers from large crabs than from small crabs, and (3) post-contraction lactate accumulation would not occur in dark levator muscle due to the absence of SA:V and intracellular diffusive limitations in the small, subdivided fibers.

## Materials and methods

### Animals

Juvenile crabs (*Callinectes sapidus* Rathbun) were collected from April to November 2003 by sweep netting in the basin of the Cape Fear River, NC, USA (salinity range 0–15‰), and adult crabs were purchased from April to December 2003 from local seafood vendors (Wilmington, NC, USA). All crabs were transported to UNCW, where they were placed in aerated, full-strength seawater (35‰) no more than

two hours after acquisition and acclimated for at least one week before the experiments. Crabs were maintained on a 12 h:12 h light:dark cycle and fed frozen shrimp or dried food pellets three times per week. Only intermolt crabs were used, as determined by the rigidity of the carapace, the presence of the membranous layer of the carapace and the absence of soft cuticle forming beneath the old exoskeleton (Roer and Dillaman, 1984).

### Transmission electron microscopy

To characterize the developmental pattern of the dark levator muscle, the diameters of the fiber subdivisions and their corresponding mitochondrial fractional areas were measured with transmission electron microscopy (TEM). Twelve crabs ranging in body mass from <0.1 g to >190 g were divided into four size classes referred to as very small, small, medium and large (Table 1). Crabs were sacrificed by removing the dorsal carapace. The dark levator muscles (Cochran, 1935; White and Spirito, 1973; Tse et al., 1983) were exposed by removal of the reproductive and digestive organs, gills and portions of the

Table 1. Size classes of crabs used in this study are based on body mass and white levator muscle fiber diameter data from Boyle et al. (2003)

Size class	N	TEM		N	Citrate synthase activity		Lactate production and removal		
		Body mass (g)	Body mass range (g)		Body mass (g)	Body mass range (g)	N	Body mass (g)	Body mass range (g)
Very Small	3	0.119±0.0131	0.0957–0.1411						
Small	3	1.12±0.128	0.90–1.34	6	1.40±0.260	0.800–2.30	78	1.29±0.060	0.40–2.30
Medium	3	11.0±3.38	5.2–16.9	7	9.64±1.55	4.60–14.8	59	13.25±3.90	3.70–23.1
Large	3	181.6±6.13	171.1–192.5	10	94.0±23.6	27.3–229.7	74	227.7±6.86	130.6–310.4

endoskeleton and mechanically isolated from surrounding tissue. Resting length of the muscle was measured from its origin on the median plate to the insertion at the heavy tendon, while the fifth periopod (swimming leg) was positioned as far anterodorsally as possible. A small bundle of dark levator fibers was teased apart with a glass probe, tied off with 6-0 surgical silk and then excised from the animal. Due to the heterogeneity of the fiber composition in dark levator muscle, care was taken to isolate only the core of the tissue, which is comprised of aerobic fibers (Tse et al., 1983). The fiber bundle was placed at resting length in a primary fixative consisting of 1% glutaraldehyde and 4% paraformaldehyde in 0.063 mol l<sup>-1</sup> Sörenson's phosphate buffer, pH 7.38 (Egginton and Sidell, 1984; Preshnell and Schriebman, 1997). The osmolarity of the fixative and all corresponding buffer rinses was adjusted by the addition of 10% sucrose and a trace amount of CaCl<sub>2</sub> to prevent a change in cell volume. Tissues were held in primary fixative for a minimum of 24 h at room temperature and then rinsed for 15 min in Sörenson's phosphate buffer. This process was followed by a secondary fixation in 1% osmium tetroxide in Sörenson's phosphate buffer. Samples were then dehydrated with an ascending series (50%, 70%, 95%, 100%, 100%) of ethanol and embedded in Spurr's epoxy resin (Spurr, 1969; Electron Microscopy Sciences, Hatfield, PA, USA). Samples were sectioned at 90 nm with a diamond knife on a Reichert Ultracut E (Vienna, AT, USA) and collected using a systematic random sampling method (Howard and Reed, 1998) to ensure complete representation of the mitochondrial distribution throughout the muscle. Five sections were collected from a random starting point, and then a distance of 500 nm was skipped before collecting another five sections. This process was repeated until 5 µm of tissue was sectioned. Five sections were mounted on each Formvar-coated (0.25% Formvar in ethylene dichloride) high-transmission copper grid and were stained with 2% uranyl acetate in 50% ethyl alcohol and with Reynolds' lead citrate (Reynolds, 1963). Sections were examined with a Philips CM-12 TEM (Hillsboro, OR, USA) operated at 60 kV. One section per grid was randomly chosen, and a montage of photographs was generated from a 100 µm<sup>2</sup> region of dark fibers at 3600× using a 3¼"×4" plate camera. Montage photographs were obtained from five sections per animal to yield 60 total micrographs used for mitochondrial fractional area and fiber subdivision diameter analysis; this was to account for intra- vs inter-individual variation. Negatives were developed and then digitized using a Microtek Scanmaker 4 (Carson, CA, USA) negative scanner.

Adobe Photoshop (version 7.0) was used to align individual images to form a montage. Subsequently, the margins of mitochondria were traced and filled to form polygons. Fibers were likewise traced and filled to form polygons. Areas and diameters of these polygons were then generated using Image Pro Plus (version 4.1.0.9; Media Cybernetics; San Diego, CA, USA). For each animal, fractional areas were calculated by dividing the mitochondrial area summed over all five micrographs for that animal by the total subdivision area summed over all five micrographs for each animal.

#### *Citrate synthase activity*

Citrate synthase (CS) activity measurements were used as a measure of aerobic capacity and were examined as a function of animal size in dark levator muscle. The scaling of white levator CS activity with body mass was measured previously by Boyle et al. (2003). Dark levator muscle tissues were excised from 23 crabs ranging in body mass from <1 g to >200 g (Table 1). Tissues were homogenized with a PowerGen (Fisher, Hampton, NH, USA) homogenizer in 5–20 volumes of extraction buffer (50 mmol l<sup>-1</sup> Tris, 1 mmol l<sup>-1</sup> EDTA, 2 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 2 mmol l<sup>-1</sup> dithiothreitol, pH 7.6) and sonicated with a sonic dismembrator 60 (Fisher) on ice at 6 W using four bursts of 5 s each. Samples were centrifuged at 16 000 g for 20 min, and the supernatants were stored at -80°C until further analysis.

Citrate synthase activities were spectrophotometrically assayed at 25°C using the methods of Walsh and Henry (1990). The supernatant was combined with 1 mmol l<sup>-1</sup> 5,5-dithio-bis (2-nitrobenzoioc acid), 0.3 mmol l<sup>-1</sup> acetylcoenzyme A and water in a 0.5 ml cuvette for a baseline absorbance reading at 412 nm on an Ultrospec 4000 spectrophotometer (Amersham Pharmacia Biotech, Buckinghamshire, UK) until the absorbance stabilized. The reaction was initiated by the addition of 0.5 mmol l<sup>-1</sup> oxaloacetate, and the enzyme activity (µmol min<sup>-1</sup> g<sup>-1</sup>) was calculated using the slope of the absorbance change immediately following the addition of oxaloacetate.

#### *In vivo fatigue*

To determine the ontogenetic reliance on anaerobic metabolism in white and dark levator fibers during post-contractile recovery, burst contractile activity comparable with that during an escape response was elicited by stimulating the thoracic ring ganglia (Boyle et al., 2003). Only the three largest size classes of crab (Table 1) were used for these experiments due to the difficulty of obtaining sufficient quantities of tissue for metabolite extractions in the very small animals. The size classes used were defined based on the relationship between animal mass and white levator fiber size, where the mean diameters (µm) of the fibers were 133.3±1.9, 226.0±4.2 and 462.4±22.0 for small, medium and large animals, respectively (Boyle et al., 2003).

Crabs were removed from aquaria and suspended in the air with a clamp such that the swimming leg motion was unrestricted. Wire electrodes were placed in two small holes drilled into the mesobranchial region of the dorsal carapace, and a Grass Instruments SD9 physiological stimulator (Astro-Med, Inc., West Warwick, RI, USA) was used to elicit contractile responses (80 Hz, 200 ms duration at 10 V cm<sup>-1</sup> between electrodes). A single 200 ms pulse would induce a series of burst swimming contractions that typically lasted from 2 to 20 s. Stimulations were repeated once every minute until the animal was fatigued, determined by minimal response to stimulation. Following exercise, crabs were immediately placed in aerated aquaria, provided with food and allowed to recover for one of eight time periods (1, 10, 60, 90, 120, 240,

360, 480 min following exercise). Due to the expected longer recovery period for large crabs (Henry et al., 1994; Boyle et al., 2003), this group was allowed to recover for 720 min following exercise, and recovery at 90 min was not examined. At least five animals per size class were not exercised. These animals were weighed and measured and then allowed to rest overnight before the tissue collection. A minimum of four crabs were used per time point, and the mean  $N$  value for all time points was 6.8.

Immediately before sacrifice, hemolymph was collected with a syringe from the arthroal membrane at the base of the swimming leg and frozen in liquid nitrogen. The crabs were sacrificed by removing the dorsal carapace. White and dark levator muscle tissues were quickly dissected as previously described, and samples were excised, freeze-clamped in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further analysis.

#### *L-lactate assays*

Frozen tissue samples (0.05–0.2 g) were homogenized in 9–29 volumes of chilled 7% perchloric acid, sonicated on ice at 6 W using four bursts of 5 s each, then centrifuged at  $4^{\circ}\text{C}$  at 16 000  $g$  for 20 min. The supernatants were neutralized using 3 mol  $\text{l}^{-1}$   $\text{KCO}_3^-$  in 50 mmol  $\text{l}^{-1}$  PIPES and centrifuged at  $4^{\circ}\text{C}$  at 16 000  $g$  for 20 min. The resulting supernatants were stored at  $-80^{\circ}\text{C}$ .

The concentration of L-lactate in white levator, dark levator and hemolymph was spectrophotometrically assayed following the procedures of Lowry and Passonneau (1972) as modified by Kinsey and Ellington (1996). A buffer containing 300 mmol  $\text{l}^{-1}$  hydrazine hydrate, 12 mmol  $\text{l}^{-1}$  EDTA and 4 mmol  $\text{l}^{-1}$   $\text{NAD}^+$  at pH 9.0 was mixed with the supernatant in a 0.5 ml cuvette and read at 340 nm on an Ultraspec 4000 (Pharmacia) to obtain a baseline absorbance value. The reaction was initiated by the addition of 18.5 units of L-lactate dehydrogenase, and the change in absorbance was measured. The concentration in the sample was calculated assuming that 1 g of white and dark levator muscle tissue has 0.75 ml of intracellular water (Milligan et al., 1989).

#### *Statistical analysis*

Levene's test was used to test for heteroscedasticity. One-way analysis of variance (ANOVA) was used to test for the main effects of animal size class on fiber subdivision diameter, mitochondrial fractional area, citrate synthase activity and lactate concentration at rest, immediately following contraction and 60 min post-contraction. Where significant size effects were detected, Tukey's HSD test was used to make pairwise comparisons among the means from animal size classes. Two-way ANOVA was used to analyze the post-contraction lactate concentrations (10 min post-contraction and beyond) for the interaction between animal size class and recovery time for each tissue type. All statistical tests were analyzed with JMP software version 7.0.2 (SAS Institute; Cary, NC, USA) or SAS software version 8.02. Results were considered significant if  $P < 0.05$ . The linear regression of CS activity data and the area under the lactate recovery curve were

calculated using Sigma Plot software version 8.02 (SPSS Inc., Chicago, IL, USA). Standard errors for the post-contraction lactate recovery curve integrals were calculated from 200 bootstrap samples using six values per time point. Data are represented as means  $\pm$  S.E.M. throughout.

## Results

### *Subdivision diameters and mitochondrial fractional area*

The dark levator aerobic fibers were identified from surrounding white and intermediate fibers based on dense concentrations of mitochondria and the presence of smaller fiber subunits. Individual aerobic fibers in the dark levator were identified by the presence of a surrounding membrane and relatively large gaps separating fibers. The subdivisions found within the fiber were identified by the presence of a surrounding membrane and a very minimal gap separating the subdivisions. Hemocytes were frequently observed in regions surrounding the whole fiber as well as between subdivisions (Fig. 2). This implies that the subdivisions are highly perfused with hemolymph, which is consistent with being an aerobic muscle.

Neither the diameters of the dark levator muscle aerobic fiber subdivisions ( $F=0.25$ ,  $d.f.=3$ ,  $P=0.86$ ,  $N=60$ ) nor the mitochondrial fractional areas of the subdivisions ( $F=0.065$ ,  $d.f.=3$ ,  $P=0.98$ ,  $N=60$ ) changed during development (Figs 3, 4). The mean diameter of the subdivisions across all size classes was  $35.6 \pm 2.7 \mu\text{m}$ , which is well within the range of cellular dimensions typical of aerobic fibers from other animals. Although the mean diameter of the dark levator fiber subdivisions did not change, the total number of subdivisions did increase throughout development. For example, the fibers from the very small size class have no subdivisions while

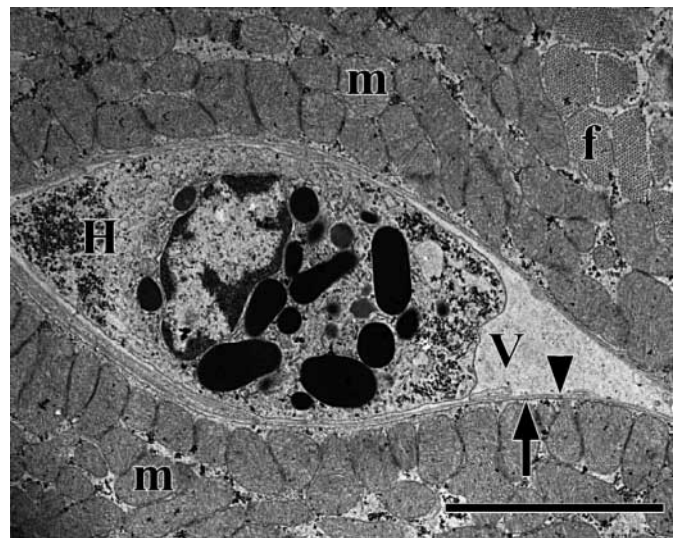


Fig. 2. Hemocyte (H) in the vascular space (V) between subdivisions. Note the basal lamina (arrowhead) at the surface of the subdivision membrane (arrow) as well as a cross section of myofibrils (f) and numerous mitochondria (m). Hemocytes were frequently found in the vascular spaces between subdivisions, indicating that this tissue is highly perfused with hemolymph. Scale bar, 10  $\mu\text{m}$ .

subdivisions do exist in the fibers of the small, medium and large size classes (Fig. 3). The mitochondria were distributed almost exclusively around the periphery of these aerobic fiber subdivisions, and they represented between 20 and 30% of the total area of each subdivision (Fig. 4).

#### *Citrate synthase activity*

CS activity in dark levator muscle scaled negatively with

increasing body mass (Fig. 5A). CS activity levels were higher in dark levator than in white levator, as expected from the difference in function of these muscles. A line was fitted to the data according to the scaling relationship:  $CS\ activity = a \times M^b$ , where  $M$  is animal mass,  $a$  is a constant and  $b$  is the scaling exponent (Schmidt-Nielsen, 1984). When grouped by animal size class, there was a significant effect of size on CS activity ( $F=14.6$ ,  $d.f.=2$ ,  $P<0.001$ ,  $N=23$ ), and mean CS activity in dark

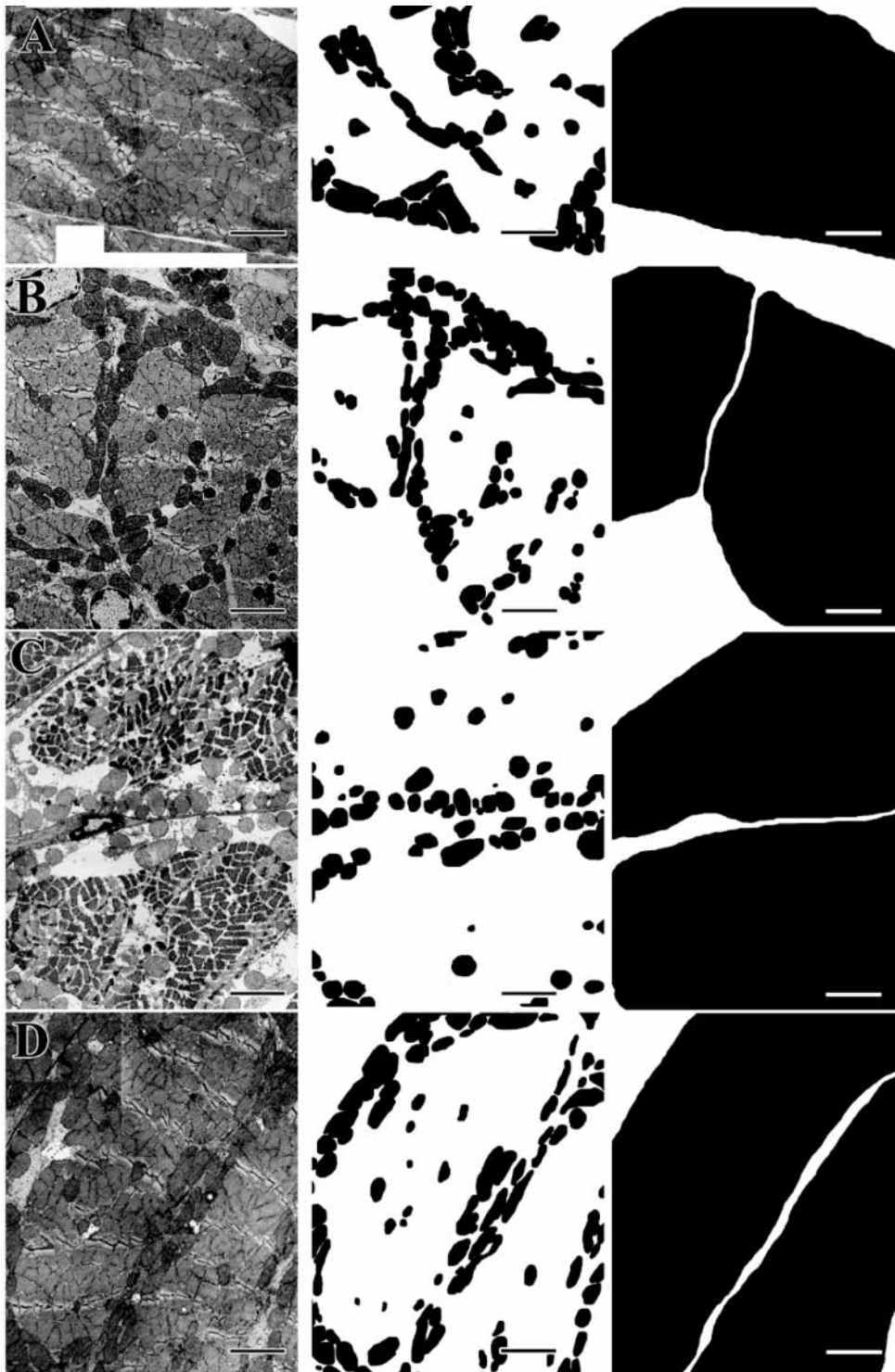


Fig. 3. Dark levator muscle fiber subdivision development. TEM of fiber subdivisions from (A) very small, (B) small, (C) medium and (D) large crabs is shown in the left panels. The middle panels show highlighted mitochondria from the subdivisions, and the panels on the right show highlighted subdivisions found in the micrographs in the left panels. Scale bars, 10  $\mu$ m.

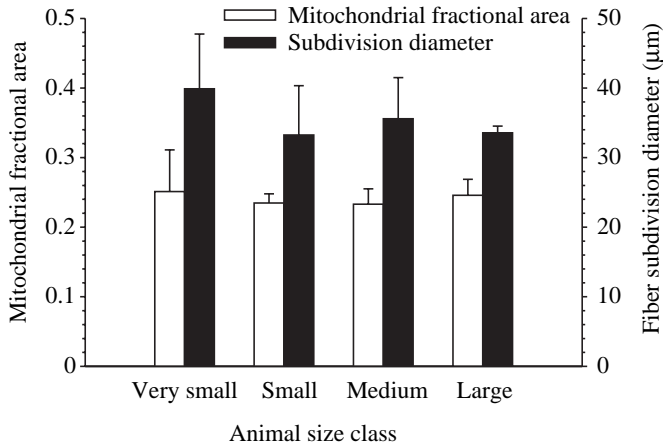


Fig. 4. Mean mitochondrial fractional areas (open bars; left y-axis) and mean fiber subdivision diameters (filled bars; right y-axis) of the dark levator muscle fibers for each animal size class.

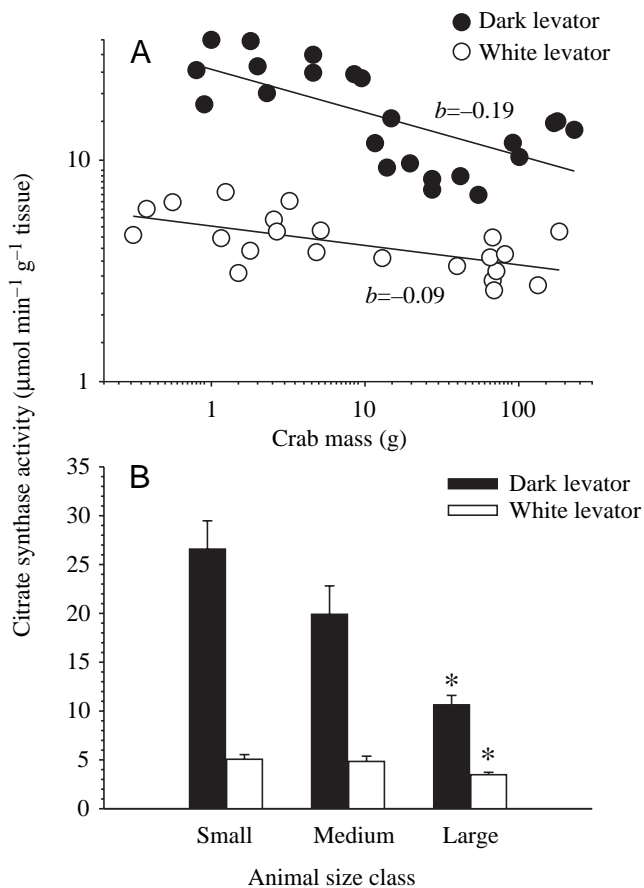


Fig. 5. Mass-specific scaling of citrate synthase (CS) activity per gram of dark levator muscle (filled circles) and white levator (open circles; white levator data from Boyle et al., 2003). (A) The linear regression fit to the dark levator muscle data is the log of the scaling equation  $CS \text{ activity} = 25.75M^{-0.19}$  ( $r^2 = 0.45$ ;  $M$  is body mass) and in white levator muscle  $CS \text{ activity} = 5.0M^{-0.09}$  ( $r^2 = 0.39$ ). (B) Mean CS activity grouped by size class. Note the greater fractional differences between size classes in the dark levator muscle than in the white levator muscle. An asterisk indicates that CS activity for that group is significantly different ( $P < 0.05$ ) from the other two groups.

muscle from large animal size class was less than half of the activity in muscle from the small size class.

*Lactate production and removal*

The lactate concentration values were  $\log_{10}$ -transformed to achieve homogeneous variance. One-way ANOVA was used to test for a significant effect of animal size class on each of the tissue types for [lactate] at rest, immediately following exercise and 60 min post-exercise. Resting lactate concentrations were not significantly different among animal size classes for white levator ( $F = 2.0$ ,  $d.f. = 2$ ,  $P = 0.16$ ,  $N = 24$ ; Fig. 6A), dark levator ( $F = 3.31$ ,  $d.f. = 2$ ,  $P = 0.054$ ,  $N = 27$ ; Fig. 6B) or hemolymph ( $F = 1.02$ ,  $d.f. = 2$ ,  $P = 0.37$ ,  $N = 30$ ; Fig. 7C). All size classes of crab responded similarly to stimulations, although smaller crabs tended to have contractile bursts that were of a higher frequency but shorter duration than large crabs. However, immediately following exercise there were no differences among size classes in the amount of lactate

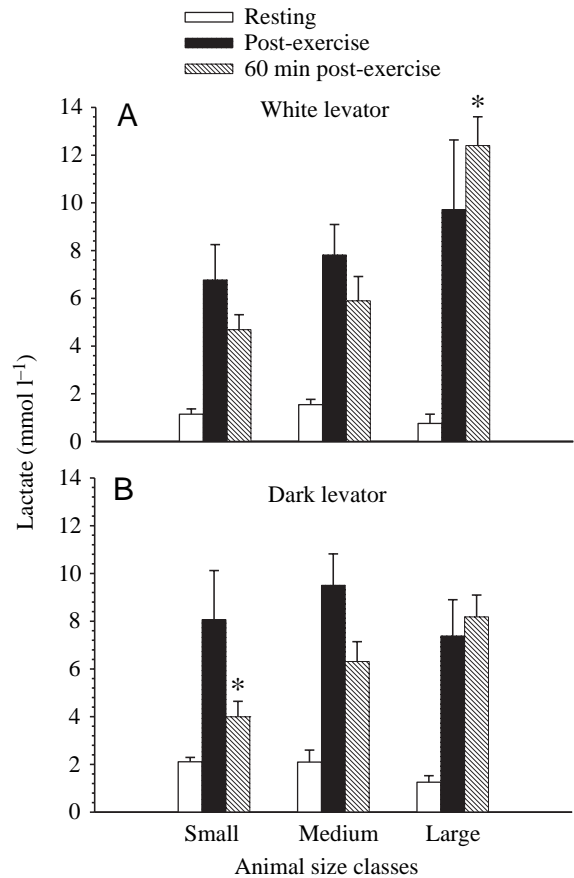


Fig. 6. Lactate concentrations at rest, immediately after exercise and 60 min after exercise in (A) white levator and (B) dark levator muscles. Note that resting lactate values and those immediately after exercise are not significantly different among the size classes. At 60 min, lactate begins to decrease in both tissues from small and medium crabs, while it remains elevated in large dark and white levator muscles. An asterisk indicates that lactate concentrations for that animal size class were significantly different ( $P < 0.05$ ) from the other two size classes (see text for details).

produced during exercise in white ( $F=1.02$ , d.f.=2,  $P=0.38$ ,  $N=24$ ) or dark levator muscle ( $F=0.602$ , d.f.=2,  $P=0.56$ ,  $N=17$ ) (Fig. 6), suggesting that muscle from all size classes of animals was doing the same amount of mass-specific anaerobic work during exercise. During recovery from exercise, however, there were large differences in [lactate] among animal size classes. This pattern was particularly apparent at 60 min post-exercise, where [lactate] reached the highest levels observed in large

white fibers, which led to a significant effect of animal size class ( $F=14.3$ , d.f.=2,  $P<0.001$ ,  $N=25$ ; Fig. 6A). There was also a significant effect of animal size class in the dark levator at 60 min post-exercise ( $F=6.9$ , d.f.=2,  $P=0.01$ ,  $N=15$ ; Fig. 6B), although the differences were not as great as were seen in the white levator.

A complete time course of post-exercise lactate production and removal is presented in Fig. 7. Two-way ANOVA was run separately for each tissue, and all three tissues had significant interactions between size and recovery time (white levator –  $F=4.09$ , d.f.=10,  $P<0.001$ ,  $N=196$ ; dark levator –  $F=2.07$ , d.f.=10,  $P<0.0356$ ,  $N=164$ ; hemolymph –  $F=3.67$ , d.f.=10,  $P<0.0003$ ,  $N=201$ ). Lactate levels began to decline immediately following exercise in both muscle tissues from small and medium crabs, while lactate remained elevated in both muscle tissues from large crabs, and the subsequent time course of lactate removal in all tissues from large crabs required  $>480$  min. Unexpectedly, dark levator muscle from large crabs appeared to require more time for lactate clearance than did small and medium crabs (Fig. 7B), although the differences between the size classes in lactate removal from dark levator muscle appeared to be considerably less than the differences between size classes in the white levator (Fig. 7A). The hemolymph time course mirrored that of the two muscle tissues, but peak lactate concentrations tended to lag slightly behind those for the muscles.

To analyze differences in the size dependence of lactate recovery in dark and white muscles, the area under the lactate recovery curve (Fig. 7A,B; 10 min post-contraction and beyond) was approximated with the trapezoidal rule for each tissue and size class. In this analysis, the resting lactate concentrations (Fig. 6A,B) were subtracted from the post-exercise lactate concentration values in order to examine only the amount of lactate that was elevated above resting levels. There were no significant differences between the dark and white muscle in the small and medium animal size classes (Fig. 8). However, for muscle in the large animal size class, the [lactate]  $\times$  time integral was significantly higher in the white muscles than in the dark muscle (Fig. 8;  $P=0.0024$ ,  $N=268$ ).

## Discussion

The first objective of this study was to characterize the post-metamorphic development of the dark levator muscle aerobic fiber subdivisions. Several authors have described the presence of fiber subdivisions within adult crustacean muscle fibers (Rosenbluth, 1969; Jahromi and Atwood, 1971). Histochemical staining of the dark levator fibers in adult *C. sapidus* by Tse et al. (1983) showed that aerobic fibers are highly subdivided into smaller functional units with diameters much less than 100  $\mu\text{m}$ , while the overall fiber diameter is similar to that in white levator muscle (several hundred microns). In the present study, low-magnification micrographs showed that in all size classes the diameters of the aerobic fibers were consistent with those of white levator fibers

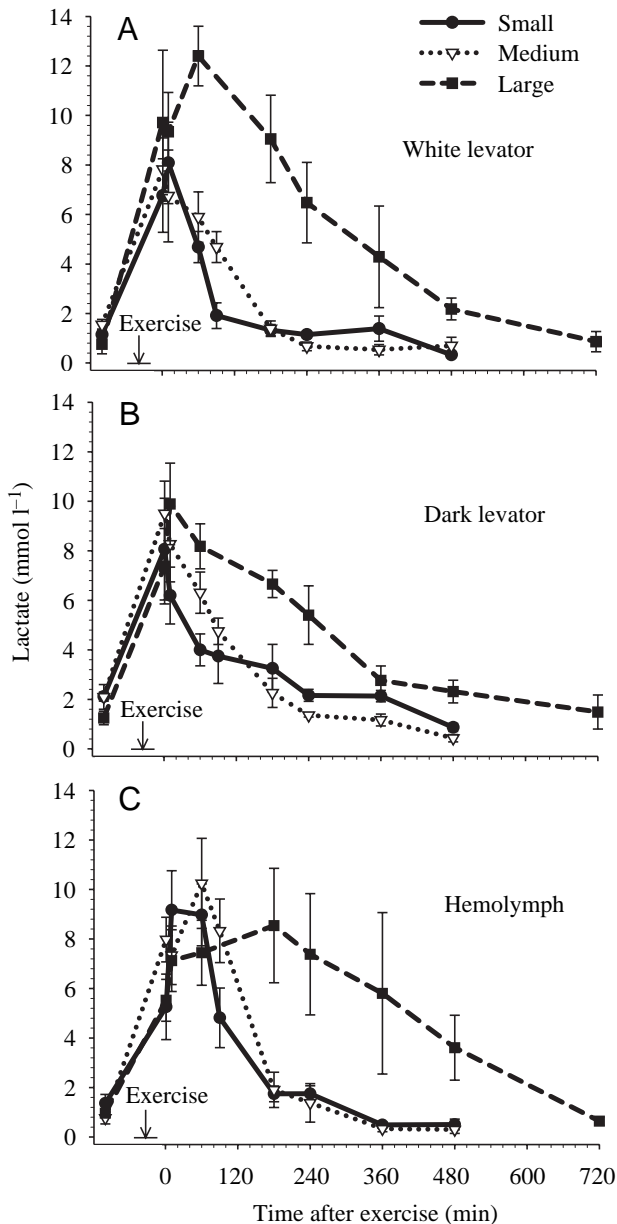


Fig. 7. Time course of lactate concentration changes in (A) white and (B) dark levator muscles and (C) hemolymph following fatiguing exercise. Resting values are plotted first. The 0 min time point is immediately following exercise. Note the increase in lactate following exercise in white fibers from large crabs and the prolonged period of time for lactate removal from all tissues in large crabs. The differences among the size classes are less dramatic in the dark levator muscle than in the white levator muscle.



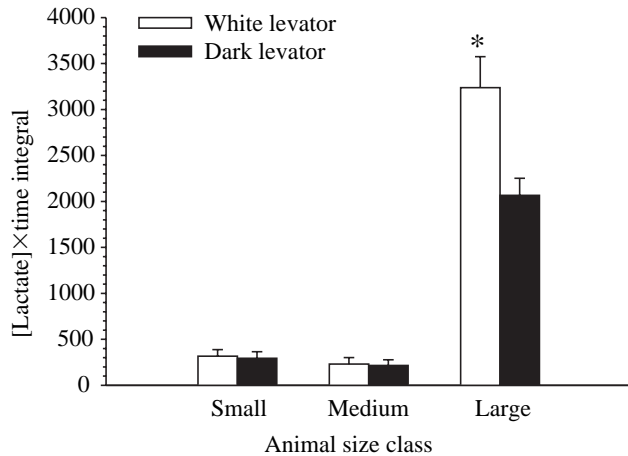


Fig. 8. The area under the post-contraction lactate concentration recovery curve for dark and white levator muscle. The asterisk indicates that there was a significant difference ( $P < 0.05$ ) between dark and white muscle in the large size class.

described by Boyle et al. (2003). Subdivision formation in these dark fibers was expected to occur in one of two ways: (1) the number of subdivisions is relatively constant but their diameter increases throughout development or (2) the number of subdivisions increases but their diameter is relatively constant during development. Evidence from this study supports the latter process. The dark fibers from the smallest animals examined (<0.1 g) had little or no subdivision formation, but they became increasingly subdivided throughout post-metamorphic development (Fig. 3).

Striated skeletal muscle is a post-mitotic and highly differentiated tissue that has little capability of increasing by simple division of its component fibers. In fishes, hyperplasia occurs until a certain point in development, after which muscle mass is increased by hypertrophy of existing fibers (reviewed in Weatherly and Gill, 1987), although there are exceptions to this generalization. For example, fiber division occurs in adult specimens of the fishes *Notothenia coriiceps* and *Patagonotothen longipes*, leading to a slight increase (1–3%) in fiber number and muscle mass (Johnston et al., 2003). Fiber splitting was also noted in the mullet, *Mugil cephalus* (Carpené and Veggetti, 1981), and in the European eel, *Anguilla anguilla* (Willemsse and Lieuwma-Noordanaus, 1984). However, in these cases, fiber splitting led to the generation of new fibers, rather than a subdivision of existing fibers. By contrast, the principal means of increasing muscle mass during growth in post-embryonic squid is hyperplasia (e.g. Pecl and Moltshaniwskyj, 1997; Preuss et al., 1997). Blue crab dark levator muscle is perhaps unique in that it appears to grow hypertrophically, and, because of the large developmental increase in body mass, the fibers that power swimming become exceptionally large. However, the aerobic swimming function is maintained by fiber subdivision and intra-fiber perfusion with hemolymph (Fig. 2). The result of this fiber subdivision is the developmental maintenance of a constant size of each

metabolic functional unit while there is a simultaneous increase in the size of each contractile functional unit.

The capacity for aerobic swimming in portunid crabs presumably entailed the evolution of aerobic, subdivided fibers from giant white fiber precursors, similar to those described by Boyle et al. (2003). This means that other systems that support aerobic contraction must have co-evolved to function within the framework of the subdivided aerobic fibers. For instance, the circulatory system had to evolve intra-fiber perfusion, which is apparent in Fig. 2, and the evolution of this trait may have constituted a novel adaptation specifically designed to promote aerobic swimming. Metabolic subdivision also may have posed new challenges to coordinating contraction over an entire fiber. In this case, however, crustaceans may have been particularly well suited to deal with this potential evolutionary hurdle, since crustacean muscles are often multi-terminally innervated and do not propagate action potentials in the manner of vertebrates. Thus, if it were necessary to innervate each subdivision to promote uniform contraction (an open question), crustacean muscle may have been predisposed to evolve this trait. We can therefore speculate that fiber subdivision is not widespread in the animal kingdom because it is only necessary when aerobic fibers evolve from giant fibers, and perhaps because other organisms are less able to accommodate the evolution of the suite of systems required to support aerobic contraction in subdivided fibers.

The present results also imply that there is a maximal size at which the dark locomotor muscle fibers of blue crabs are able to contract aerobically without becoming subdivided. Above this size, low SA:V and long intracellular diffusive distances may begin to constrain the rate of oxygen and ATP fluxes such that they are insufficient to keep pace with the high demands of contraction. The lack of subdivisions in fibers from the smallest animals indicates that these fibers are small enough to allow aerobic contraction. This may indicate that there is no selective pressure to further reduce the size of each metabolic functional unit in the smallest fibers. However, spatial considerations may also limit the minimum size of subdivisions, since exceedingly small fiber/subdivision size may not permit a sufficient volume of the muscle to be devoted to myofilaments, leading to force production that is inadequate for locomotion. Rome and Lindstedt (1998) have characterized the linkage between muscle design and function in the context of the percentage of space allocated to myofibrils, the sarcoplasmic reticulum and mitochondria. The developmental pattern in the dark levator muscle of blue crabs indicates that, at least in some cases, fiber size and its influence on SA:V and diffusive fluxes may also need to be considered to characterize fiber design and function.

The CS activities and mitochondrial densities in aerobic muscle fibers were roughly fivefold higher than that in white levator fibers (Boyle et al., 2003) and are consistent with observations that adult *C. sapidus* have locomotor muscles that are highly vascularized (McGaw and Reiber, 2002) to support their exceptional long-distance swimming capabilities (Judy and Dudley, 1970). Although the negative allometric scaling

of CS activity (Fig. 5) was similar to the findings in white levator muscle (Boyle et al., 2003), these data contrast with those for mitochondrial fractional area in the dark aerobic fiber subdivisions, which did not scale negatively with body mass (Fig. 4). This result was unexpected and it may indicate that the mitochondria from the smaller animals are more densely packed with aerobic enzymes. Alternatively, the discrepancy between the mitochondrial fractional area and CS activity data could be due to differences in the method of data collection. CS activity was referenced to the wet mass of the muscle, whereas the TEM analyses were based on cross-sectional areas within a fiber subdivision excluding extracellular and extra-subdivision spaces. There may be systematic differences in the scaling of wet mass and subdivision cross-sectional area that lead to the lack of agreement between these data sets.

The second objective of this study was to examine some of the metabolic implications of hypertrophic growth in crustacean muscle fibers with the assumption that increasing fiber size in white muscle and the accompanying mitochondrial shift towards the periphery of the fiber (Boyle et al., 2003) lead to excessive intracellular diffusion distances and low SA:V that constrain aerobic metabolic processes (Fig. 1). A requirement for comparing the effects of fiber size on recovery is that crabs from all size classes perform the same amount of chemical work during exercise. During burst contraction, intracellular AP stores are initially consumed, after which anaerobic glycogenolysis is recruited to power sustained bouts of locomotion (England and Baldwin, 1983; Booth and McMahon, 1985; Head and Baldwin, 1986; Milligan et al., 1989; Morris and Adamczewska, 2002). The resting concentration of AP (and glycogen) and the activity of AK do not change with increasing body size in crustacean white muscle (Baldwin et al., 1999), so the contribution of AP to ATP production during exercise is constant as well. It follows that lactate production alone is an effective indicator of differences among size classes in the amount of anaerobic work during exercise. The lack of significant differences among size classes in muscle lactate production during contraction (Fig. 6) indicates that muscle tissues from animals of all size classes incurred equal levels of mass-specific oxygen debt.

Boyle et al. (2003) described some of the metabolic impacts of increased fiber size in white levator muscle of blue crabs. They found that, following burst exercise, glycogen recovery was much slower in larger animals, which they attributed to the extreme diffusion distances in the large fibers. In addition, differences in diffusive flux rates in fibers of different sizes may be amplified by the time-dependent decrease in metabolite diffusion coefficients in muscle tissue, which results from intracellular barriers that obstruct net molecular motion across muscle fibers (Kinsey et al., 1999; Kinsey and Moerland, 2002). Boyle et al. (2003) also found greater glycogen depletion in white muscle fibers of larger animals, which they suggested occurred because large fibers were relying more on anaerobic glycogenolysis to speed up certain phases of recovery to more rapidly restore contractile function. This conclusion was based on the relatively low ATP yield per

glucosyl unit during anaerobic glycogenolysis, which results in greater glycogen depletion during recovery. The present study confirms the hypothesis posed by Boyle et al. (2003) by providing direct evidence that post-contraction anaerobic metabolism is size-dependent in white muscle fibers from blue crabs. Since a very large fraction of the post-contraction lactate production in white fibers diffuses into the blood, the differences among size classes in the anaerobic contribution to recovery are certain to be much more dramatic than is apparent from the muscle lactate data alone (Figs 7, 8).

These arguments raise the question of which post-contraction recovery processes are likely to be accelerated by invoking anaerobic metabolism, and how this would benefit the animal. The most likely candidates are the recovery of AP and the restoration of ionic gradients across the membrane. Although not measured in the present study, there is evidence that AP recovery is relatively rapid (Ellington, 1983; Head and Baldwin, 1986; Thébault et al., 1987; Morris and Adamczewska, 2002) and is associated with post-contraction glycogen depletion and lactate accumulation in adult crustacean white muscle (England and Baldwin, 1983; Head and Baldwin, 1986; Kamp, 1989; Henry et al., 1994; Morris and Adamczewska, 2002; Boyle et al., 2003). Since AP is the fuel initially used during burst activity (England and Baldwin, 1983; Head and Baldwin, 1986; Kamp, 1989; Baldwin et al., 1999), the rapid replenishment of AP across the entire cell would facilitate additional bouts of high-force anaerobic contractions. In contrast to crustaceans, vertebrates rely exclusively on aerobic metabolism to power resynthesis of the phosphagen creatine phosphate, and lactate does not accumulate following contraction (Kushmerick, 1983; Meyer, 1988; Curtin et al., 1997). The vertebrate pattern of recovery therefore appears to closely resemble that in muscle fibers from the small and medium animal size classes in this study (Fig. 7A), which entails a rapid, aerobic restoration of both phosphagen and lactate levels.

The relatively short intracellular diffusive distances in dark levator muscle fibers (Tse et al., 1983; Fig. 3) led to the expectation that recovery following exercise would not lead to size-dependent lactate production in this tissue, which is contrary to our results (Figs 7, 8). However, these differences were less dramatic than in white fibers, and post-contraction lactate accumulation in the large animals was significantly lower in dark fibers than in white, which is consistent with our hypotheses (Figs 7, 8). It is possible that dark fibers also produce lactate following contraction in an effort to speed up recovery, although it would be difficult to make the argument that this is due to intracellular diffusion or SA:V constraints. In this view, the size dependence would simply reflect the mass-specific decrease in aerobic capacity that typically accompanies increases in body mass (Fig. 5), and anaerobic metabolism would be used to preserve a rapid post-contraction recovery rate as animals grow larger. This interpretation implies that, at the very least, cell size constraints are embodied in the differences in the size dependence of lactate production between dark and white muscles seen in Figs 7, 8.

It is likely, however, that the differences between dark and white fibers are actually much greater than is apparent in Fig. 7. There are two reasons to expect artificially large differences among size classes in dark fibers. First, following exercise, the circulating hemolymph is highly concentrated with lactate for an extended period of time. The bulk of the lactate that is released to the hemolymph during, and particularly after, contraction is expected to arise from the white fibers (Boyle et al., 2003; Fig. 7). The lactate-laden hemolymph also perfuses the dark levator muscle, however, and there is likely to be net diffusive flux of lactate from the hemolymph into the dark fibers. Second, the fiber composition of the dark levator muscle is heterogeneous, and the small, aerobic fibers located in the core are surrounded by giant white fibers (Tse et al., 1983). If the white fibers are producing most of the lactate after contraction, the close proximity of the dark fibers to the surrounding white fibers is likely to also allow for net lactate diffusion into the dark fibers. Therefore, the present whole-animal experiments may not be adequate to fully resolve differences in the fiber size dependence of lactate dynamics between white and dark muscle. An analysis of isolated fibers would be a useful approach to address this issue.

Another argument for the presence of diffusive limitations in these large white fibers can be made by examining the scaling of the activity of the aerobic enzyme citrate synthase (Fig. 5). The body mass scaling exponent of CS activity for dark muscle was more negative ( $b=-0.19$ ) than for the white muscle ( $b=-0.09$ ), meaning there were greater differences among animal size classes in dark muscle than in white (Boyle et al., 2003). This leads to the expectation that there would be greater differences in post-exercise lactate dynamics among size classes in the dark muscle than in the white muscle, which is contrary to both our predictions and observations.

In summary, although aerobic fibers of the levator muscle in *C. sapidus* grow hypertrophically, reaching very large sizes similar to fibers in the white levator muscle, the fibers become increasingly subdivided throughout development and maintain a mean subdivision diameter of  $\sim 35 \mu\text{m}$ . This developmental pattern leads to a constant size for each metabolic functional unit, while each contractile functional unit increases in size as the animal grows. The time course of lactate removal from tissues following exercise was consistent with the prediction that post-contractile metabolic recovery is size dependent in white locomotor muscle fibers of *C. sapidus*. Large white fibers appear to rely on anaerobic metabolism to accelerate certain phases of the metabolic recovery process to offset diffusive and/or SA:V limitations that may make aerobic metabolism unacceptably slow. Although the aerobic locomotor fibers, which should not be diffusion limited, also demonstrated a moderate size dependence of anaerobic recovery, this result may be due to an inability to fully resolve differences between muscle tissue types in a whole-animal experiment.

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