

THE EFFECTS OF HYPOBARIC HYPOXIA ON ASPECTS OF OXYGEN  
TRANSPORT AND UTILIZATION IN MICE WITH AN INHERITED TOLERANCE  
FOR HYPOXIC EXERCISE

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## ABSTRACT

The effects of chronic hypoxia on properties of oxygen supply and demand were investigated in mice found to have inherited differences in hypoxic exercise tolerance (43). Previous results demonstrated that two major loci and an unknown number of modifying genes were associated with the differences in hypoxic exercise performance following 8 weeks exposure to hypobaric hypoxia in two inbred strains of mice, BALB/cByJ (C) and C57BL/6J (B6), and their F1 hybrid (43). The three strains had similar capacities for hypoxic exercise following exposure to normoxia. After 8 weeks of hypoxic exposure, C mice showed little improvement in hypoxic exercise performance while B6 mice had a significant improvement in fatigue resistance compared to normoxic mice. Acclimated F1 mice, however, had a hypoxic exercise tolerance that far exceeded that of either of the two parental strains. In the present study, all of the mouse strains responded to hypoxia by elevating the hematocrit, hemoglobin, and BPG concentrations; although, hypoxia-intolerant C mice had lower hematocrit and hemoglobin concentrations following acclimation than both B6 and F1 mice. Mitochondrial densities and distributions, as well as COX activities did not differ among strains or treatments, suggesting that the muscles within the three strains have comparable oxygen demands. F1 mice did, however, have very low concentrations of BPG relative to the other two strains, and reduced concentrations of myoglobin in both skeletal and cardiac muscle following hypoxic exposure. This suggests that the superior exercise performance of the F1 mice may result from a relatively higher rate of oxygen supply to the muscles, such that high concentrations of BPG and myoglobin are not required to keep up with oxygen demand.

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## INTRODUCTION

Hypoxia elicits a cascade of functional and structural modifications in the physiological systems that govern the supply and demand of oxygen in organisms (19, 55). Chronic hypoxia induces several hematological modifications that enhance oxygen transport in the blood (20, 61, 39) and facilitate the unloading of oxygen at the tissues (40, 2). Oxygen delivery under hypoxic conditions may be further aided by vasodilation (42), increased capillarity (19) and, in muscle, increased myoglobin concentrations (5, 65). In addition, shifts in fuel preferences, such as an increased reliance on glucose oxidation, as well as increases in oxidative enzyme activities and mitochondrial volume have been observed and may help to maintain aerobic metabolic substrate flux (6). These and other responses presumably serve to promote sufficient ATP production despite a reduced ambient  $P_{O_2}$ .

Hematological modifications encompass several of the classic responses to hypoxia and have been documented in a suite of mammalian species. Elevated hematocrit and hemoglobin concentrations are observed almost universally following acclimation to hypoxic conditions (20, 61, 2). Likewise, blood concentrations of organophosphates such as ATP and 2,3-bisphosphoglycerate (BPG) generally increase during hypoxic exposure (20, 40, 59, 51, 71). An increase in the concentration of BPG is presumably advantageous during hypoxia because it leads to a decrease in hemoglobin's affinity for oxygen and, thus, facilitates the release of oxygen at the tissues (3).

In muscle, chronic exposure to hypoxia has been shown to increase the concentration of myoglobin, which may enhance oxygen flux to muscle fibers (25, 65, 8). The rate of oxygen flux to the muscles, in turn, impacts metabolic potential and cellular



organization. Acclimation to hypoxia often results in an increased oxidative capacity of muscle fibers and, thus, an increase in mitochondrial density (6) and in the activities of oxidative enzymes (23, 55, 18). These responses may help to maintain a sufficient flux of aerobic substrates under conditions of reduced oxygen availability (24), particularly under conditions of elevated workload (60). Although this view is conceptually appealing, there are also several reports of decreases in mitochondrial density (27, 28, 18) and oxidative enzyme capacity following acclimation to hypoxia (25, 55, 28). These reports, however, have been correlated with decreased levels of physical activity, which may explain the changes in the aerobic capacity of muscles (reviewed in 6). Chronic hypoxia also has been reported to induce a shift in the mitochondrial distribution, although there is no consensus on the pattern of these changes. A decrease in the density of subsarcolemmal mitochondria has been reported after hypoxic acclimation in the soleus of the rat (67) while an increase in subsarcolemmal mitochondria has been observed in muscles of athletes at high altitude (11).

There are a number of parallels between the responses to chronic hypoxia and those associated with endurance training. Increases in hematocrit and hemoglobin concentrations have been reported in athletes following intensive training, which enhances the oxygen carrying capacity of blood (45, 39). Likewise, elevated concentrations of BPG and myoglobin have been found in endurance athletes and are suggestive of enhanced oxygen unloading in the blood and extraction by the tissues (39, 65). Trained athletes tend to also have elevated mitochondrial densities and oxidative enzyme activities, which presumably increases the flux of oxidative substrates to meet the high aerobic ATP demands characteristic of endurance exercise (21, 15).

While the effects of acclimation to high altitude as well as endurance training on the physiology of oxygen delivery and demand have been investigated, little research has focused on the interaction between acclimatory responses and variability in the genetic capacity for hypoxic exercise tolerance. The present study utilized a mouse model in which exposure to chronic hypoxia resulted in dramatic, heritable differences in the duration of hypoxic exercise (43). Two inbred strains of mice, BALB/cByJ (C) and C57B6L/J (B6), and their F1 hybrid were exposed to either normobaric normoxia or hypobaric hypoxia ( $P_B = \frac{1}{2}$  atm) for 8 weeks, after which they were exercised under hypoxic conditions until fatigued (measured as the time elapsed during treadmill exercise until a 4-s failure to avoid a grid configured to deliver a mild current (0.15 mA) was reached). The three strains had similar capacities for hypoxic exercise when they had been previously exposed to normoxia. Upon acclimation to hypoxia, however, large differences in hypoxic exercise tolerance were observed among the strains. Acclimation had little effect on hypoxia tolerance in C mice, while hypoxia-acclimated B6 mice had a considerably enhanced fatigue resistance over hypoxia-naive mice. However, F1 mice that had been acclimated to hypoxia had a hypoxic exercise tolerance that far exceeded that of either parental strain. McCall and Frierson (43) further showed that only two loci of major effect, and an unknown number of modifying genes, were responsible for the dramatic differences in hypoxic exercise tolerance.

In the present study, it was hypothesized that the differences in hypoxic exercise among the strains were related to hypoxia-induced hematological modifications that may alter oxygen delivery to the tissues and/or changes in the muscle that may alter oxidative capacity. To address this issue, blood concentrations of hemoglobin and BPG, as well as

muscle myoglobin concentration, COX activity, mitochondrial density and mitochondrial distribution were investigated in the three strains of mice. It was predicted that superior hypoxic performance would be associated with a phenotype characteristic of a both chronic hypoxic exposure as well as a high capacity for endurance exercise, including relatively high concentrations of hemoglobin and BPG, indices of the oxygen carrying capacity and the unloading efficiency of the blood, as well as elevated concentrations of myoglobin in muscle. In addition, the levels of COX activity and mitochondrial density, indices of oxidative potential, were predicted to be higher in more hypoxia-tolerant mice, to facilitate aerobic substrate flux.

## MATERIALS AND METHODS

### *Animal Maintenance*

Inbred strains of BALB/cByJ (C) and C57BL/6J (B6) mice obtained from The Jackson Laboratory (Bar Harbor, Maine) were crossed to produce a F1 hybrid. Individual breeding pairs and their offspring were housed in polycarbonate cages in a laminar flow hood at  $23 \pm 1^{\circ}\text{C}$  programmed with a 12:12 hour light-dark cycle. Animals were provided Agway 3000 Mouse Chow and water *ad libitum*. Breeding pairs were observed weekly and once the female was judged to be pregnant the male was removed. Litters were weaned at 17 days and separated into same sex sibling groups with no more than 4 mice per cage. At 8 weeks of age mice were transferred from this colony to a 2-compartment Vacudyne Corporation hyperbaric chamber converted to have hypobaric capabilities. One compartment of the chamber was maintained under hypobaric conditions (380 torr;  $\frac{1}{2}$  atmospheric  $\text{P}_{\text{O}_2}$ ) while the second compartment was maintained

under normobaric conditions (760 torr; sea level P<sub>O2</sub>), allowing all animals to be housed in the same unit.

Mice were randomly assigned either to the hypobaric hypoxic or normobaric normoxic compartment and maintained in the chamber for 8 weeks. Individual cages were continuously flushed with filtered air at a rate of 1.6 L/min. The temperature within the chamber was maintained between 22-24°C with a relative humidity of 50-55%. The chamber was returned to normobaric pressure once per week for approximately 1 hour to facilitate animal maintenance. Food and water were provided *ad libitum* in the chamber. A reservoir of water contained within the hypobaric compartment supplied individual water lines to each cage. The pH of the water (2.9-3.1) was sufficient to prevent bacterial or fungal growth within the lines of the reservoir.

#### *Blood Collection*

After 8 weeks of exposure to hypoxia or normoxia, mice were weighed and sacrificed by cervical dislocation. Whole blood was collected via cardiac puncture using a 23-gauge needle and immediately transferred to a 500µL capillary blood collection tube containing 12.5 IU lithium heparin.

#### *Determination of 2,3-Bisphosphoglycerate Concentration*

Four hundred-fifty µL of blood was transferred from the heparinized collection tube to a 5-mm nuclear magnetic resonance (NMR) tube. <sup>31</sup>P-NMR spectra were collected at 162 MHz on a Bruker 400 MHz DMX spectrometer to determine the relative blood concentrations of BPG. Field homogeneity was optimized by shimming on the proton signal arising from H<sub>2</sub>O. Spectra were collected using a 90°-excitation pulse (11 µs) and a relaxation delay of 12 s. Thirty-six scans were acquired for a total acquisition

time of 4 min. A 0.50 Hz exponential line broadening function was applied prior to Fourier transformation. The area under each peak was then integrated using Xwin-NMR software and compared to the peak area arising from a solution containing a known concentration of BPG. This facilitated the determination of the BPG concentration in each sample. After spectra collection, the blood was returned to the collection tube and frozen at  $-85^{\circ}\text{C}$ .

#### *Determination of Hemoglobin Concentration*

Previously collected blood samples were thawed on ice after which 20  $\mu\text{L}$  of blood was thoroughly mixed with 5.0 mL Drabkin's solution (Drabkin's reagent reconstituted with deionized water and 30% Brij-35 solution; Sigma-Aldrich, St. Louis, MO) and allowed to stand at room temperature for 15 minutes. A standard curve was constructed using reconstituted hemoglobin standards (Sigma Aldrich, St. Louis, MO) on a Pharmacia Ultrospec 4000 UV/Visible spectrophotometer with Swift II software. The absorbance of the samples was measured at 540 nm and the concentrations of hemoglobin were extrapolated from the standard curve.

#### *Muscle Preparation*

For spectrophotometric protein and enzyme activity measurements, the left and right extensor digitorum longus (EDL) and soleus, gastrocnemius, and right ventricle were dissected while being continuously flushed with mouse Ringer's solution (117 mM NaCl, 4.6 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.16 mM  $\text{MgSO}_4$ , 20 mM  $\text{NaHCO}_3$ , equilibrated to a pH of 7.4 with a 99.5/0.05%  $\text{O}_2/\text{CO}_2$  mixture; 13). Tissues were flash frozen in liquid nitrogen and stored at  $-85^{\circ}\text{C}$ .

### *Determination of Myoglobin Concentration*

Myoglobin concentrations were determined based on techniques previously described by Reynafarje (52). Frozen tissues were thawed on ice, blotted dry, weighed, cut into 1-mm<sup>3</sup> pieces, and transferred to a 20 mL high-speed centrifugation tube. Samples were diluted by a factor of at least 20 with 0.04 M phosphate buffer, pH 7.4 (smaller tissues required larger dilution factors to obtain an adequate sample volume). Tissues were homogenized on ice using a Fischer Scientific Power Gen 125 homogenizer using 3 cycles of 10-s bursts. Tissues were then subjected to three 10-s cycles of sonication using a Fischer Scientific 60 sonic dismembrator. Samples were centrifuged in a Beckman J2-21M/E centrifuge at 29,100 x g at 0°C for 50 minutes. The supernatant was decanted into a 15 mL centrifuge tube and bubbled with carbon monoxide (CO) for 8 minutes. To ensure full reduction of myoglobin, 0.03 g of sodium hydrosulfite was added to the sample after which it was bubbled with CO for an additional 2 min. Immediately thereafter, 0.7 mL of the sample was transferred to a microcuvette. The concentration of myoglobin was determined from the difference in the absorbances at 538 nm and 568 nm according to Reynafarje (52).

### *Cytochrome-c Oxidase Enzyme Activity*

Tissue samples were homogenized and sonicated in the same manner as above in 19 volumes (wt/vol) of 50 mM imidazole (pH 7.4), 50 mM KCl, and 0.5 mM dithiothreitol (DTT) (46). A cytochrome-c oxidase assay kit purchased from Sigma-Aldrich (St. Louis, MO) was used to perform the spectrophotometric assays. Assays were conducted on an Ultrospec 4000 with the temperature regulated at 20°C using a recirculating water bath. An 80 µL volume of the sample supernatant was diluted with 20

$\mu\text{L}$  enzyme dilution buffer (10 mM Tris-HCl, pH 7.0 containing 250 mM sucrose) and added to 950  $\mu\text{L}$  assay buffer (10 mM Tris-HCl, pH 7.0 containing 120 mM KCl). Samples were mixed by inversion after which 50  $\mu\text{L}$  of 0.22 mM ferrocytochrome-c substrate solution was added. The initial absorbance was read at 550 nm followed by readings at 10-s intervals for 1.5 min. A blank containing 950  $\mu\text{L}$  assay buffer, 100  $\mu\text{L}$  enzyme dilution buffer, and 50  $\mu\text{L}$  ferrocytochrome-c solution was run in the same manner. The enzyme activity was determined using the slope of the absorbance change corrected for the change in absorbance of the blank.

The amount of protein within the supernatant was determined spectrophotometrically using the Bradford method. A standard curve was constructed from protein standard dilutions of bovine serum albumin (BSA) following the Bio-Rad microassay protocol. Ten  $\mu\text{L}$  of the supernatant used to determine the COX activity was added to 200  $\mu\text{L}$  of dye reagent. The microassay plate was incubated for 5 minutes at room temperature and mixed for 5 seconds after which the absorbance was measured at 595 nm. Protein concentrations were determined from a linear regression analysis of the BSA standard curve.

#### *Transmission Electron Microscopy*

The EDL and soleus, representative fast- and slow-twitch skeletal muscles, respectively, were analyzed using transmission electron microscopy (TEM) to determine the density and distribution of mitochondria within individual muscle fibers. During dissection, both the EDL and soleus were measured at resting length with the knee and ankle joints in complete flexion using a dial caliper and tied off at the origins with 5-0 surgical silk. Muscles were excised and mounted onto wooden rods using utility wax.

Once attached, muscles were re-measured to ensure the muscle was at its resting length. Tissues were immediately fixed in Karnovsky's fixative (3.0% glutaraldehyde, 3.0% paraformaldehyde in 0.2 M sodium cacodylate buffer, pH 7.60; 33) for a minimum of 24 hours at room temperature. The tissues were then rinsed in 0.2 M sodium cacodylate, pH 7.60 followed by secondary fixation in 0.5% osmium tetroxide in 0.2 M sodium cacodylate buffer, pH 7.6. Samples were rinsed and dehydrated after which they were embedded in Spurr epoxy resin (Electron Microscopy Sciences). From the known resting length of each muscle, the mid-point was determined and the muscle was cut transversely at this point. Each half was then sectioned at 90 nm with a diamond knife on a Reichert Ultracut E. Series of sections were collected to provide a good representation of mitochondrial distribution. The first 5 sections obtained from each muscle half were discarded. The subsequent 5 sections were collected on a 200 mesh, high transmission copper grid coated with Formvar (0.25 g Formvar in 100 mL ethylene dichloride). Five series of sections were collected from each muscle half, covering a total distance of 9.0  $\mu\text{m}$ . Sections were stained with 2% uranyl acetate in 50% ethanol and Reynold's lead citrate (53) and viewed with a Philips EM 400 transmission electron microscope at 80 kV. One section from the first set of serial sections collected at the midpoint of the muscle was randomly chosen and one fiber within this section was photographed on 3 1/4" x 4" Kodak 4489 film. The same process was used to collect a second and third micrograph from the final set of serial sections collected from each muscle half. Negatives were digitized using a Microtek Scanmaker 4 negative scanner and processed in Adobe Photoshop version 7.0. Image-Pro Plus (IPP) version 4.1.0.9 software was used to analyze the images.



Mitochondrial densities and distributions were stereologically analyzed using a modification of the methods described by Kayar *et al.* (35). To determine the radial distribution of intermyofibrillar mitochondria (any mitochondrion in which a myofibril was interposed between it and the sarcolemma), a set of 3 grids each with sides of 2.65  $\mu\text{m}$  and an area of 7.02  $\mu\text{m}^2$  was applied to all micrographs. Grid I (inside) was placed at the centroid of each fiber, which was determined by tracing the fiber periphery in IPP and using the software to determine the centroid. One of the capillaries surrounding the fiber was randomly selected and a transect from the center of grid I to the center of the capillary was constructed. Grid O (outside) was placed along the transect at the periphery of the fiber with no more than 2 points of the grid falling on interstitial space. Grid M (middle) was placed at the midpoint of the transect. For grids I and M, point-counting methods were used to determine the mean fractional area of mitochondria. Any point within the grid that fell on a mitochondria was counted. The total number of points falling on mitochondrion was then divided by the total number of points within the grid. For grid O, any point falling within the interstitial space, nucleus, or on subsarcolemmal mitochondria (any mitochondrion at the sarcolemma without interposed myofibrils) were counted and subtracted from the total number of points within the grid. Points falling on intermyofibrillar mitochondria were counted and divided by the new reference space to determine the fractional area of mitochondria. The fractional area of subsarcolemmal mitochondria was determined by thresholding all areas of mitochondria at the periphery of the fiber and dividing this area by the total fiber area.

### *Statistical Analysis*

Two-way analysis of variance (ANOVA) was used to test for significant effects of mouse strain, treatment group, or an interaction between mouse strain and treatment group using JMP software (v. 4). Homogeneity of variance was assessed using Barlett's test and normality was determined using a  $\chi^2$  goodness-of-fit test. Where significant group effects were found, pairwise comparisons of means were made with Tukey's HSD test. Results were considered significant if  $p < 0.05$ .

## RESULTS

### *Animal Weight*

The mean weights of mice used in this study as well as the time to fatigue ( $t_{et}$ ) during hypoxic exercise previously determined by McCall and Frierson (43) are presented in Table 1. Two-way ANOVA of the weight data detected a significant effect of treatment (d.f. = 1,  $F = 36.32$ ,  $p < 0.0001$ ) and strain (d.f. = 2,  $F = 4.59$ ,  $p < 0.0112$ ). Pairwise comparison revealed that mice exposed to hypoxia had significantly lower body weights than those mice exposed to normoxia in all strains. The strain effect resulted from the significantly lower mean weight of B6 mice compared to F1 mice.

### *Hemoglobin Concentration*

The mean hemoglobin concentration for each strain is illustrated in Figure 1A. Two-way ANOVA revealed a significant effect of treatment (d.f. = 1,  $F = 88.37$ ,  $p < 0.0001$ ) and strain (d.f. = 2,  $F = 13.08$ ,  $p < 0.0001$ ) as well as a significant interaction between treatment and strain (d.f. = 2,  $F = 5.26$ ,  $p < 0.0077$ ). Mean hemoglobin concentrations were significantly higher in mice exposed to hypoxia as compared to normoxic mice in all 3 strains. C, B6, and F1 hypoxic mice had mean hemoglobin

concentrations that were 12%, 25%, and 14% higher than C, B6, and F1 normoxic mice, respectively. The strain effect resulted from the significantly lower mean hemoglobin concentration of C mice compared to B6 and F1 mice.

The ratios of mean hemoglobin concentrations to previously measured hematocrit values (4) are illustrated in Figure 1B. A significant treatment effect was detected (d.f. = 1,  $F = 27.20$ ,  $p < 0.0001$ ); hypoxic mice had significantly lower mean hemoglobin:hematocrit ratios (Hb/Hct) than those mice exposed to normoxia in all 3 strains. A significant effect of strain was also detected (d.f. = 2,  $F = 7.60$ ,  $p < 0.0011$ ), which resulted from the significantly lower mean Hb/Hct of C mice compared to F1 mice.

#### *2,3-Bisphosphoglycerate Concentration*

Representative  $^{31}\text{P}$ -NMR spectra are presented in Figure 2, where it can be observed that hypoxic exposure led to an increase in the amplitude of the BPG peak as compared to that resulting from normoxic exposure. Mean concentrations of BPG are illustrated in Figure 3A. Two-way ANOVA revealed significant effects of treatment (d.f. = 1,  $F = 132.67$ ,  $p < 0.0001$ ), strain (d.f. = 2,  $F = 49.76$ ,  $p < 0.0001$ ), and an interaction between treatment and strain (d.f. = 2,  $F = 6.67$ ,  $p < 0.0026$ ). C, B6, and F1 mice exposed to hypoxia had BPG concentrations that were 25%, 34%, and 26% higher, respectively, than mice exposed to normoxia. The strain effect resulted from the significantly different mean BPG concentrations of all strains, where B6 mice had the highest and F1 mice had the lowest concentration. The molar ratio of BPG to hemoglobin (BPG/Hb) is illustrated in Figure 3B. Two-way ANOVA revealed a significant effect of treatment (d.f. = 1,  $F = 18.89$ ,  $p < 0.0001$ ) and strain (d.f. = 2,  $F =$

45.51,  $p < 0.0001$ ). In all strains, hypoxic exposure resulted in significantly higher molar ratios of BPG/Hb. Again, the BPG/Hb ratios were lowest in F1 mice and highest in B6 mice.

### *Myoglobin Concentration*

Myoglobin concentrations within the gastrocnemius, EDL, soleus, and right ventricle are illustrated in Figure 4. Muscles with lower myoglobin concentrations were noted to be considerably paler in appearance upon dissection. For verification of this observation, muscles were compared to a discrete color spectrum, generated using Adobe Photoshop (version 7.0), which encompassed the color range of the tissues. Muscles were then assigned a color code based on the spectrum. Comparison of color code assignments to myoglobin concentrations revealed high levels of correlation, supporting the spectrophotometric measurements of myoglobin.

In general, the lowest concentrations of myoglobin were found in the largely glycolytic, fast-twitch gastrocnemius with increasingly higher concentrations in the EDL, soleus, and right ventricle. B6 mice exposed to hypoxia had higher concentrations of myoglobin than mice exposed to normoxia in all tissues. In contrast, F1 mice exposed to hypoxia had lower myoglobin concentrations than mice exposed to normoxia in all tissues. C mice had a similar trend to F1 mice within the skeletal muscles, in that hypoxic exposure led to a reduced myoglobin concentration, but in the right ventricle the opposite pattern was seen. Two-way ANOVA was used to examine group effects in each muscle. In the gastrocnemius, significant effects of treatment (d.f. = 1,  $F = 5.68$ ,  $p < 0.0210$ ), strain (d.f. = 2,  $F = 15.82$ ,  $p < 0.0001$ ), and an interaction between treatment and strain (d.f. = 2,  $F = 15.82$ ,  $p < 0.0001$ ) were detected. Significant effects of treatment

(d.f. = 1,  $F = 4.18$ ,  $p < 0.048$ ), strain (d.f. = 2,  $F = 7.81$ ,  $p < 0.0015$ ), and an interaction between treatment and strain (d.f. = 1,  $F = 5.59$ ,  $p < 0.0075$ ) were detected within the EDL. In the soleus, significant effects of treatment (d.f. = 1,  $F = 7.87$ ,  $p < 0.0075$ ) and an interaction between treatment and strain (d.f. = 1,  $F = 20.94$ ,  $p < 0.0001$ ) were found. Significant effects of strain (d.f. = 2,  $F = 4.07$ ,  $p < 0.024$ ) and an interaction between treatment and strain (d.f. = 1,  $F = 12.95$ ,  $p < 0.0001$ ) were detected in the right ventricle.

#### *Cytochrome-c Oxidase Enzyme Activity*

The weight-specific activities of COX are illustrated in Figure 5. As with the myoglobin data, activities were lowest in the gastrocnemius and highest in the right ventricle. In all strains and tissues, with the exception of one case, mice exposed to hypoxia had higher COX activities than those mice exposed to normoxia. Specific enzyme activities had a nearly identical pattern as that for the weight-specific values (data not shown). Two-way ANOVA was used to examine the group effects in each muscle. A significant effect of treatment was detected in the EDL (d.f. = 1,  $F = 24.56$ ,  $p < 0.0001$ ), and in the right ventricle, significant effects of treatment and strain were found (d.f. = 1,  $F = 8.32$ ,  $p < 0.0059$ ). The significant strain effect resulted from the higher activity of B6 mice compared to F1 mice.

#### *Mitochondrial Fractional Area and Distribution*

Representative TEM micrographs from the EDL and soleus are presented in Figure 6. Subsarcolemmal and intermyofibrillar mitochondrial can be seen to be more abundant in the slow-twitch soleus than in the EDL, which is predominantly composed of fast-twitch fibers. Mean fiber cross sectional area (FCSA) and diameter values for both muscles are presented in Table 2. In all three strains, both EDL and soleus fiber size

were smaller in mice exposed to hypoxia, but this effect was only significant in F1 mice (area: EDL: d.f. = 1,  $F = 5.36$ ,  $p < 0.026$ ; soleus: d.f. = 1,  $F = 6.68$ ,  $p < 0.014$ ; diameter: EDL: d.f. = 1,  $F = 5.76$ ,  $p < 0.024$ ; soleus: d.f. = 1,  $F = 7.31$ ,  $p < 0.0104$ ).

The mean total fractional area of mitochondria within the EDL and soleus are illustrated in Figure 7. No significant effects of strain or treatment were detected by two-way ANOVA on the total mitochondrial fractional area of the EDL or the soleus. The mean fractional area of mitochondria within each of the regions examined in the EDL and soleus are illustrated in Figures 8 and 9, respectively. No significant effects of strain or treatment were detected. Within both muscles and treatments, the density of mitochondria increased from the core of the fiber toward the sarcolemma, with a decrease in mitochondrial density in the subsarcolemmal region.

## DISCUSSION

Previous studies have investigated the effects of limited oxygen availability on oxygen supply and demand following acclimation to hypoxia alone or after endurance training under hypoxia. The present study differs from those previously reported in that the strains used demonstrated genetically based differences in hypoxic exercise tolerance following acclimation to chronic hypoxia in the absence of training (Table 1; 43). Based on previously measured  $t_{et}$  values (43), it was predicted that the most hypoxia tolerant strains would have a phenotype characteristic of both chronic hypoxia and a high capacity for endurance exercise. The aerobic capacity of skeletal muscle, measured via COX activity and mitochondrial density, was consistent across strains and did not change following hypoxia acclimation. Likewise, the oxygen carrying capacity of blood within the mice did not vary in a manner that could clearly explain the differences in hypoxia

tolerance. There were, however, strain-specific responses to hypoxia in the concentration of BPG, which is associated with oxygen unloading, and myoglobin concentrations, which may be an indicator of a muscle's need to extract oxygen from the blood. These results suggest that differences in the supply of oxygen to the muscles may be responsible for the variability in exercise performance previously observed by McCall and Frierson (43).

Increases in hematocrit and hemoglobin concentration following acclimation to hypoxia (e.g., 20, 61, 2, 49, 51) as well as during endurance training (39) have been reported in numerous studies. In endurance training, however, this response is often offset by an increase in plasma volume (39), suggesting that such a response is not an essential element in an enhanced endurance capacity (45). Yet, the increase in red blood cell mass associated with hypoxia acclimation during a living high-training low protocol has led to enhanced sea-level performance (37). Therefore, it was expected that hypoxic exercise tolerance would be associated with elevated hematocrit and hemoglobin concentrations following hypoxia acclimation. Hemoglobin concentrations paralleled hematocrit values in all strains, although hypoxia-acclimated mice had significantly lower ratios of Hb/Hct than mice exposed to normoxia (Fig. 2B). Following hypoxic exposure, B6 and F1 mice had slightly higher hemoglobin concentrations than did C mice, and this difference may have contributed to the relatively poor hypoxic exercise tolerance of C mice. However, elevated hematocrit and hemoglobin concentrations increase blood viscosity, which may reduce microcirculation (49, 51, 1). It has been suggested that there is an optimum hemoglobin concentration of approximately 18 g/dL, above which the increase in blood viscosity becomes detrimental (69). Yet, B6 and F1

mice displayed considerable improvements in hypoxic exercise tolerance following hypoxic exposure despite having mean hemoglobin concentrations that exceeded the proposed optimum value. Therefore, while it is possible that relatively low hemoglobin concentrations contributed to the poor hypoxic performance of C mice, the lack of correlation between hemoglobin or hematocrit and  $t_{et}$  values indicates that these parameters are likely not principle factors in the superior hypoxic exercise tolerance of F1 mice (Table 1, Fig. 1; 4).

The interaction between hemoglobin and BPG, in part, determines the amount of oxygen unloaded at the tissues and any modification in the concentration of either may have a significant impact on oxygen supply to the tissues (57, 59, 71). Under conditions of limited oxygen availability, increases in ventilation result in an imbalance of the acid-base status of the blood, which stimulates the production of BPG (57, 41, 71). Endurance exercise has also been shown to induce significant increases in BPG concentrations resulting from lactic acid accumulation and blood acidosis (39 and 40) and, thus, the expectation for the present study was that hypoxic exercise tolerance would be associated with high concentrations of BPG. Mice exposed to hypoxia had significantly higher BPG concentrations than mice exposed to normoxia in all strains, which is in agreement with several reports (Fig. 3A; 40, 71, 9). Furthermore, BPG/Hb ratios in C and B6 mice exposed to both normoxic and hypoxic treatments were comparable to previously reported 1:1 ratios found in both small mammals (Fig. 3B; 59, 12) as well as native highlanders with a genetically based tolerance for hypoxia (57). F1 mice, however, had a mean molar ratio significantly lower than 1. These differences suggest that, while B6 and C mice utilize the allosteric effects of BPG to increase oxygen unloading, F1 mice do not



rely as heavily on this mechanism. Thus, the relatively low BPG concentration and BPG/Hb molar ratio in F1 mice may not constitute a functional benefit but, rather, may reflect a superior capacity to deliver oxygen to tissues that obviates the need to elevate BPG concentrations.

Oxygen supply to the tissues is, in part, limited by diffusion (29) and modifications in the area and diameter of muscle fibers as well as the capillary to fiber ratio have been reported following hypoxic exposure (11, 67, 27, 18). C and B6 mice showed no effect of chronic hypoxia on mean muscle FCSA or cross-sectional diameter in the EDL or soleus, while F1 mice had significantly smaller fibers in both muscles following hypoxic exposure (Table 2). This implies that F1 mice may have shorter oxygen diffusion distances and perhaps a higher capillary to fiber ratio, even in the absence of angiogenesis, than either parental strain. These results, however, are contrary to those reported by Luedeke (38), who performed an extensive investigation of fiber type composition, FCSA, and capillarity of the EDL and soleus using the same mouse strains and treatments as in the present paper. Luedeke (38) found that these parameters were almost universally unaffected by hypoxic exposure and likely had no effect on hypoxic exercise performance.

The function of myoglobin continues to be debated despite intensive investigation. Historically, myoglobin was thought to serve as an oxygen storage molecule (5). While myoglobin may serve this function in diving mammals, it has been shown that in terrestrial mammals the storage capacity of myoglobin can facilitate only seconds of aerobic metabolism (5). Myoglobin has also been postulated to facilitate the intracellular diffusion of oxygen. While the importance of this role has been questioned,

since the low intracellular diffusion coefficient of myoglobin indicates that it can only play a minor role in intracellular transport (31, 32), it may function in the facilitated diffusion of oxygen across the sarcolemma (69, 64). In this capacity, myoglobin-oxygen binding decreases the concentration of free intracellular oxygen, which steepens the oxygen concentration gradient between the capillary and muscle fiber, resulting in increased oxygen flux (54). For myoglobin to serve this function, a  $P_{O_2}$  gradient must exist between the capillary and muscle fiber and deoxygenated myoglobin must be present within muscle fibers (8). The presence of both a  $P_{O_2}$  gradient, measured via direct techniques, and deoxygenated myoglobin, measured via NMR spectroscopy, within muscles has been shown (54). The importance of this function is also supported by several reports in which myoglobin knockout mice utilized compensatory mechanisms within the cardiovascular system to maintain the energetic demands of both routine and endurance activities (44, 16, 14). These mice had significant increases in hemoglobin concentrations, coronary flow, coronary reserve, and capillarity, all of which presumably steepen the oxygen gradient at the sarcolemma and offset the impact of myoglobin deficiency (16).

The myoglobin concentrations of the gastrocnemius, EDL, soleus, and right ventricle showed strikingly different trends within the three strains following chronic hypoxic exposure (Fig. 4). B6 mice had an increase in myoglobin concentration in all muscles following acclimation, which has been reported in several species (Fig. 4B; reviewed in 48, 65, 10). If myoglobin concentration is an indication of the facilitated diffusion capacity of oxygen, then an up-regulation of myoglobin under hypoxia implies that oxygen supply to the tissues is reduced relative to its demand. In contrast to B6

mice, F1 mice had significantly lower myoglobin concentrations in all tissues after hypoxia acclimation (Fig. 4C), which suggests that hypoxia acclimated F1 mice are superior to B6 mice at balancing the supply of oxygen with its demand under conditions of routine activities and, based on their  $t_{et}$  values, during hypoxic endurance exercise as well. Further, it is possible that the decrease in myoglobin seen in F1 mice indicates that oxygen supply is actually increased under hypoxia, relative to the demand of oxygen. The effect of hypoxia on the myoglobin concentrations within the skeletal muscles of C mice was similar to that seen in F1 mice (Fig. 4A), suggesting that the skeletal muscles of hypoxic C mice receive a sufficient supply of oxygen to meet the demand, at least under the routine activity levels that the animals experience in the hypobaric chamber. C mice, however, had significantly higher myoglobin concentrations within the right ventricle following hypoxic exposure. If an increased myoglobin concentration is indicative of a reduced rate of oxygen delivery relative to oxygen demand, then this may indicate that C mice incur greater circulatory costs to maintain sufficient oxygen flux to the skeletal muscles. Because the present study was conducted in the absence of training, and the hypoxia-induced changes in myoglobin reflect a response to routine activity levels, treadmill exercise may increase oxygen demands beyond what can be supplied to the tissues. In the case of C mice, this may mean that circulatory costs become limiting and induce rapid fatigue.

The interpretation above has been used previously to ascribe a functional meaning to changes in myoglobin concentration. Endurance training has been shown to increase the myoglobin concentration within oxidative muscles, which presumably enhances the diffusion of oxygen to the tissues (30). Terrados *et al.* (66), however, found that

endurance training at high altitude resulted in a significant decrease in the myoglobin concentration of the vastus lateralis in men. It was hypothesized that such a decrease was the result of an increase in the oxygen supply to the muscle under hypoxia. The decrease in myoglobin concentration in muscles from F1 mice acclimated to hypoxia in the present study is in agreement with the observations of Terrados *et al.* (66). Thus, it appears that F1 mice have a relatively high capacity for supplying oxygen to the tissues, which may impart superior hypoxic exercise tolerance.

Mitochondria are the primary source of oxygen utilization in mammals, consuming approximately 90% of the oxygen delivered to the tissues (26). Within both skeletal and cardiac muscles, mitochondria are located in two general locations; within the core of the fiber (intermyofibrillar) and surrounding the periphery of the cell (subsarcolemmal) (7). Likewise, a gradient of intermyofibrillar mitochondria within a fiber has been observed with increasing densities of mitochondria occurring closer to the sarcolemma (25, 34). The presumptive advantage of such a distribution is relatively shorter diffusive distances for oxygen between the capillaries and mitochondria.

Hypoxic exposure has been shown to increase indices of aerobic potential in muscle such as mitochondrial density (6, 11, 67) and oxidative enzyme activity (68). These properties of muscle are also up-regulated in endurance trained athletes (21, 15) and volume densities of subsarcolemmal mitochondria have been reported to be correlated to fatigue resistance in endurance trained athletes (35). Decreases in indices of muscle oxidative capacity, however, have also been described following acclimation to hypoxia, perhaps as a result of a depression of activity or caloric intake (27, 28, 18). In the present study, 8 weeks of hypoxic exposure had no effect on the total fractional area

of mitochondria or mitochondrial distribution in either the EDL or the soleus in any of the strains (Fig 7). In addition, there were no effects of treatment or strain on COX activity in cardiac or skeletal muscle (Fig. 5), which is consistent with previous measures of oxidative capacity of several tissues in the rat following hypoxic exposure (58, 60, 17). The lack of differences in mitochondrial density and distribution as well as COX activity among all of the treatment groups suggests that the mice have consistent tissue oxygen demands and aerobic capacities. It therefore seems unlikely that changes in metabolic potential can account for differences in hypoxic exercise tolerance.

The concept of symmorphosis states that structural and functional elements should be regulated to meet, but not exceed, the requirements of the system (63). This concept was used by Taylor *et al.* (62) to demonstrate that during endurance exercise, fatigue is not the result of limitations within the muscle's contractile properties, but, rather, a limitation in the capacity of the muscle to utilize oxygen or of the cardiorespiratory system to deliver oxygen. Thus, the lack of differences observed by Luedeke (38) in the composition and structure of muscle fibers, in conjunction with the observed consistencies in mitochondrial density and distribution as well as COX activity of the present study, suggest that the differences in hypoxia tolerance of the three strains are not the result of limitations within muscle fibers. The large differences observed in BPG and BPG/Hb ratio as well as myoglobin concentrations, however, suggest that differences in oxygen supply may play a crucial role in hypoxic exercise performance.

A number of mechanisms could potentially account for differences in oxygen delivery to the tissues among the three strains. Structurally, variations in lung morphology may influence hypoxic exercise tolerance through an increased alveolar

surface area and, hence, an increase the oxygen diffusivity of the lungs (36). Chronic hypoxia has been shown to significantly decrease heart rate and, thus, cardiac output (50). If this is the case, then more hypoxia tolerant strains may maintain or even increase cardiac output under hypoxia and, hence, deliver adequate blood flow and oxygen transport to tissues. This may help prevent the decrease in the maximal rate of oxygen consumption ( $V_{O2max}$ ) that typically accompanies exposure to chronic hypoxia (2). Increases in  $P_{50}$  may further enhance oxygen delivery to the muscles of more hypoxia tolerant strains (39, 59), although measurements in the present study of BPG, which is one of the modulators of  $P_{50}$ , do not support this hypothesis. Within the skeletal and cardiac muscle, previous reports suggest that hypoxia tolerant organisms have shifts in fuel utilization towards increased carbohydrate metabolism (56, 22, 55). This presumably results in a more efficient utilization of oxygen due to a higher stoichiometric ratio of ATP synthesized per oxygen molecule consumed (55). Differences in vascular tone may also explain the differences in hypoxic exercise tolerance observed in the three strains. In humans, increased levels of an elongated gene for angiotensin-converting enzyme (ACE-I) have been correlated to improvements in the endurance characteristics of muscles (70, 23, 47). The increased level of ACE-I may contribute to increased substrate delivery, differential shifts in fuel preferences, enhanced substrate utilization efficiency, altered mitochondrial density, and increased myoglobin concentration (70, 47).

In conclusion, all of the mouse strains responded to hypoxia by elevating the hematocrit, as well as hemoglobin and BPG concentrations. Hematocrit and hemoglobin concentration were lower in the hypoxia-intolerant C mice following hypoxic

acclimation, than in the B6 or F1 mice. Therefore, the poor hypoxic exercise performance of the C mice may be, in part, related to a decreased oxygen carrying capacity of the blood. Mitochondrial densities and distributions, as well as COX activities did not differ among strains or treatments, suggesting that neither the demand for oxygen nor the capacity to supply ATP within the muscles is likely to account for differences in hypoxic exercise tolerance. The F1 mice, which were the most tolerant of hypoxic exercise, were characterized by very low concentrations of BPG relative to the other two strains, and a reduced concentration of myoglobin in both skeletal and cardiac muscle following hypoxic exposure. This suggests that the superior exercise performance of the F1 mice is associated with a relatively higher rate of oxygen supply to the muscles, such that high concentrations of BPG and myoglobin are not required to keep up with oxygen demand. The poorly performing C mice also had myoglobin concentrations that decreased following hypoxia in the skeletal muscle, but increased in cardiac muscle. This raises the intriguing possibility that these mice may fatigue quickly under hypoxia due to cardiovascular limitations.

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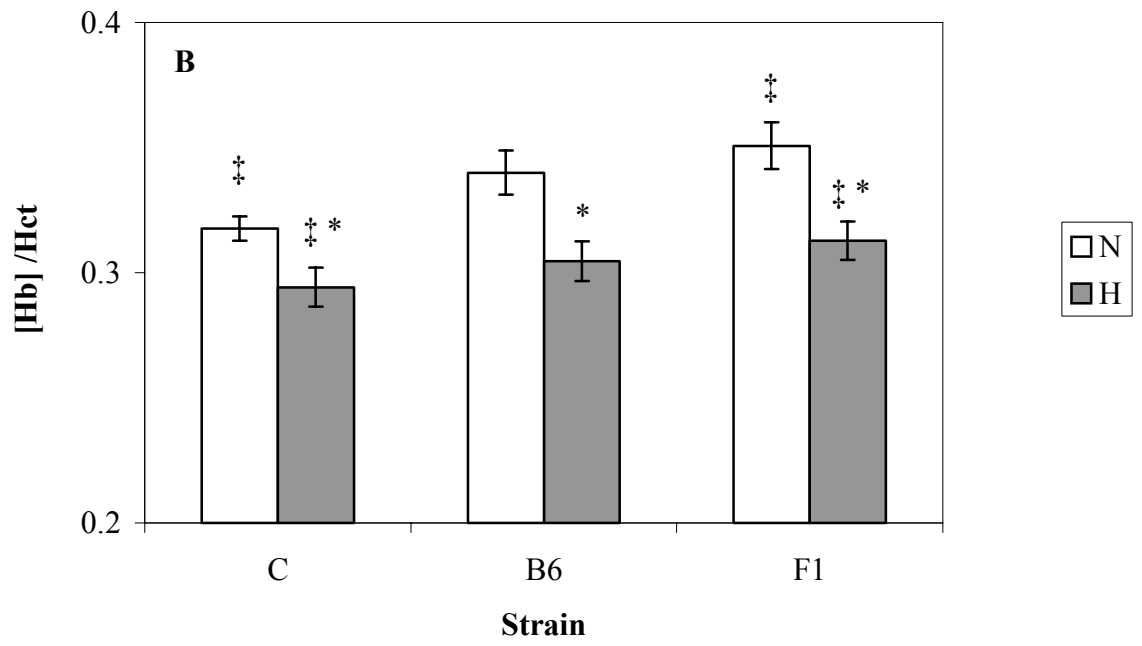
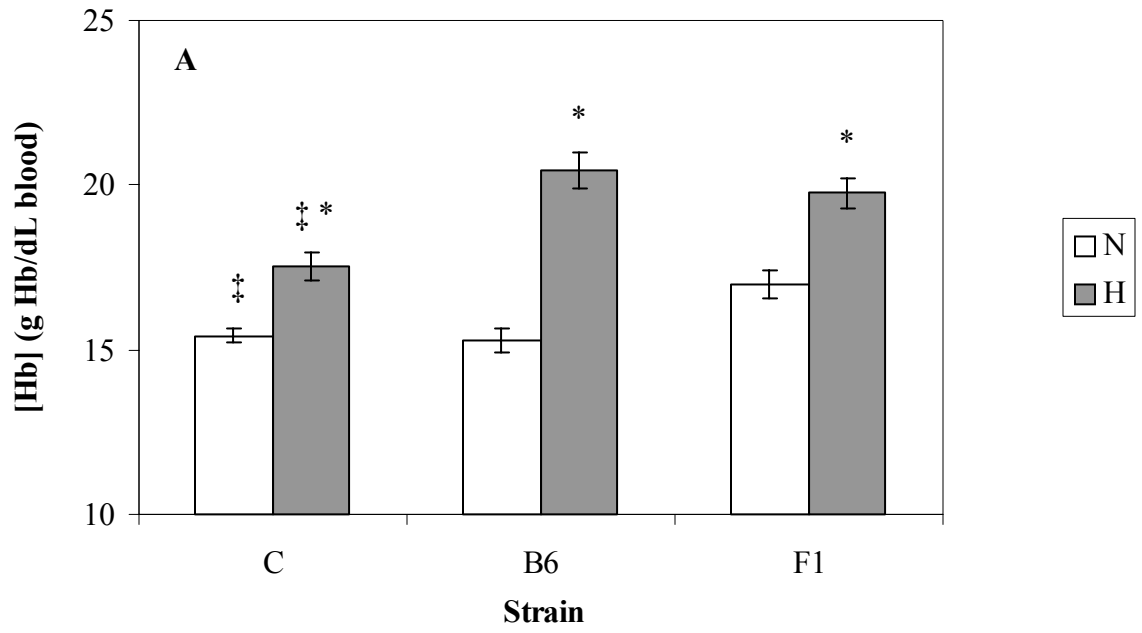
Table 1. Animal weight and hypoxic exercise endurance ( $t_{et}$ ) for each mouse strain and treatment.

	C		B6		F1	
	N	H	N	H	N	H
Body Weight (g)	26.86 $\pm$ 0.64	23.69 $\pm$ 0.45*	25.26 $\pm$ 0.98‡	23.04 $\pm$ 0.61‡*	28.27 $\pm$ 0.73‡	24.03 $\pm$ 0.43‡*
$t_{et}$ (min)	9.5 $\pm$ 1.0	12.02 $\pm$ 1.8	21.38 $\pm$ 1.1	42.34 $\pm$ 1.2	17.16 $\pm$ 1.2	77.62 $\pm$ 2.8

Values are means  $\pm$  SEM. For all weight measurements,  $n \geq 22$ . A significant effect of strain was detected and pairwise comparison revealed that F1 mice had a significantly higher mean weight than B6 mice, as is denoted by ‡. A significant effect of treatment was also detected and a significant difference between normoxic and hypoxic exposed mice within a strain is denoted by an \*.  $t_{et}$  values are from McCall and Frierson (1997).  $t_{et}$  is defined as the time elapsed during hypoxic treadmill exercise (15° incline, 40 cm·s<sup>-1</sup>, ½ atm PO<sub>2</sub>) until a 4-s failure to avoid a grid configured to deliver a mild current (0.15 mA) is reached. N refers to normoxic treatment and H refers to hypoxic treatment.



Figure 1. (A) Hemoglobin concentration. Values are means  $\pm$  SEM. For all measurements,  $n \geq 7$ . A significant effect of strain was detected and pairwise comparison revealed that C mice had a significantly lower [Hb] than B6 or F1 mice, as is denoted by a ‡. A significant effect of treatment was also detected and a significant difference between normoxic and hypoxic exposed mice within a strain is denoted by an \*. (B) The ratio of hemoglobin to hematocrit. The mean hematocrit values for each strain and treatment are as follows: CN:  $48.58 \pm 0.27$ ; CH:  $59.61 \pm 0.37$ ; B6N:  $45.00 \pm 0.29$ ; B6H:  $66.36 \pm 0.33$ ; F1N:  $48.44 \pm 0.30$ ; F1H:  $63.18 \pm 0.32$ . A significant effect of strain was detected, resulting from C mice having a significantly lower mean [Hb]/Hct ratio than F1 mice, but not B6 mice, as is denoted by a ‡. Significant effects of treatment were detected and are denoted by an \*. N refers to normoxic treatment and H refers to hypoxic treatment.



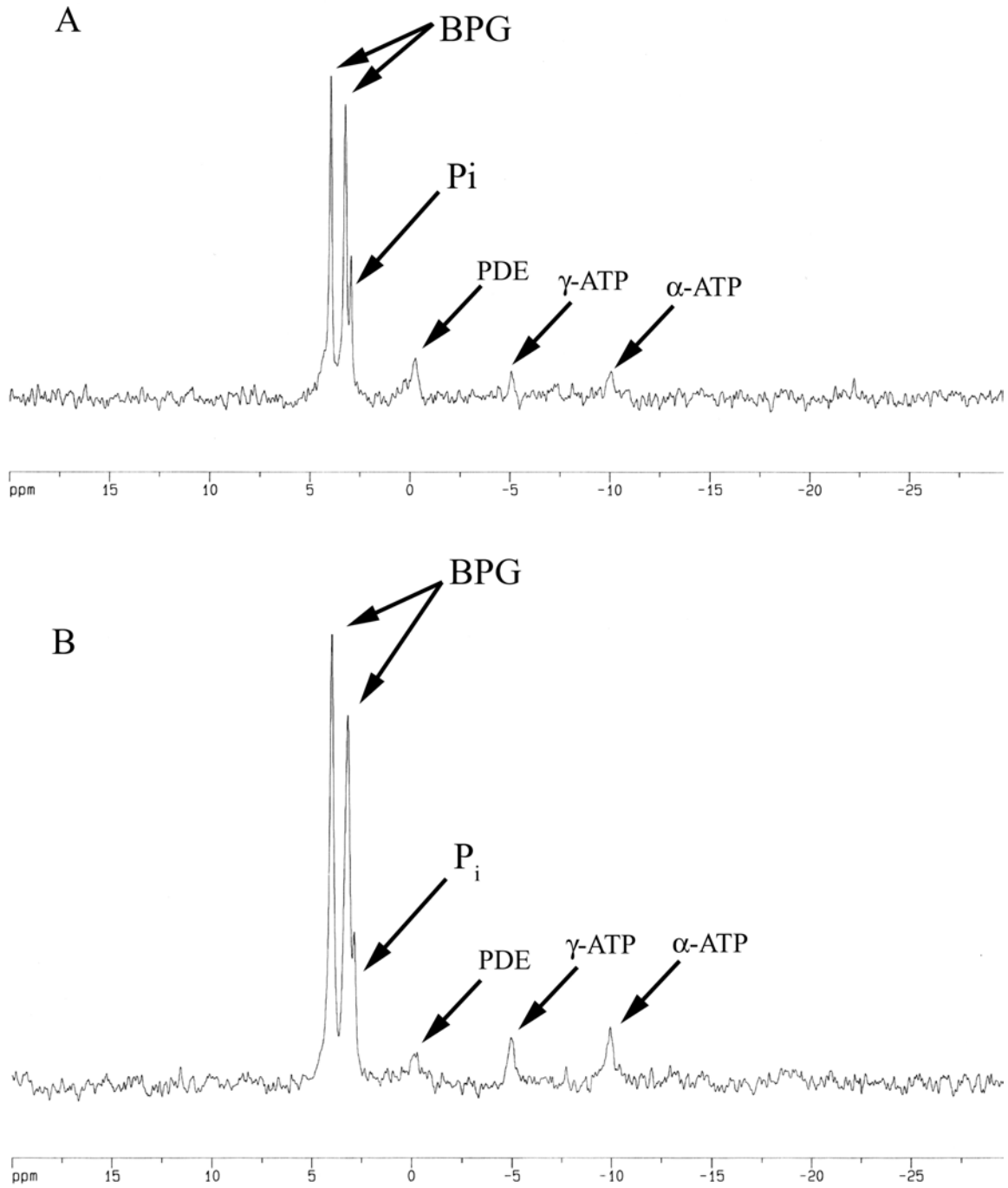


Figure 2. Representative  $^{31}\text{P}$ -NMR spectra of 2,3-bisphosphoglycerate (BPG) for (A) a B6 normoxic mouse and (B) a B6 hypoxic mouse. Note the increase in the amplitude of the BPG peaks in blood from mice exposed to hypoxia. PDE refers to phosphodiesterases.

Figure 3. (A) BPG concentration. Values are means  $\pm$  SEM. For all measurements,  $n \geq 9$ . A significant effect of strain was detected and pairwise comparison revealed that all 3 strains had significantly different [BPG], as is denoted by a ‡. A significant effect of treatment was also detected and a significant difference between normoxic and hypoxic exposed mice within a strain is denoted by an \*. (B) Molar ratio of BPG to hemoglobin. A significant effect of strain was detected and pairwise comparison revealed that all 3 strains had significantly different molar ratios, as is denoted by a ‡. A significant effect of treatment was also detected and is denoted by an \*. N refers to normoxic treatment and H refers to hypoxic treatment.

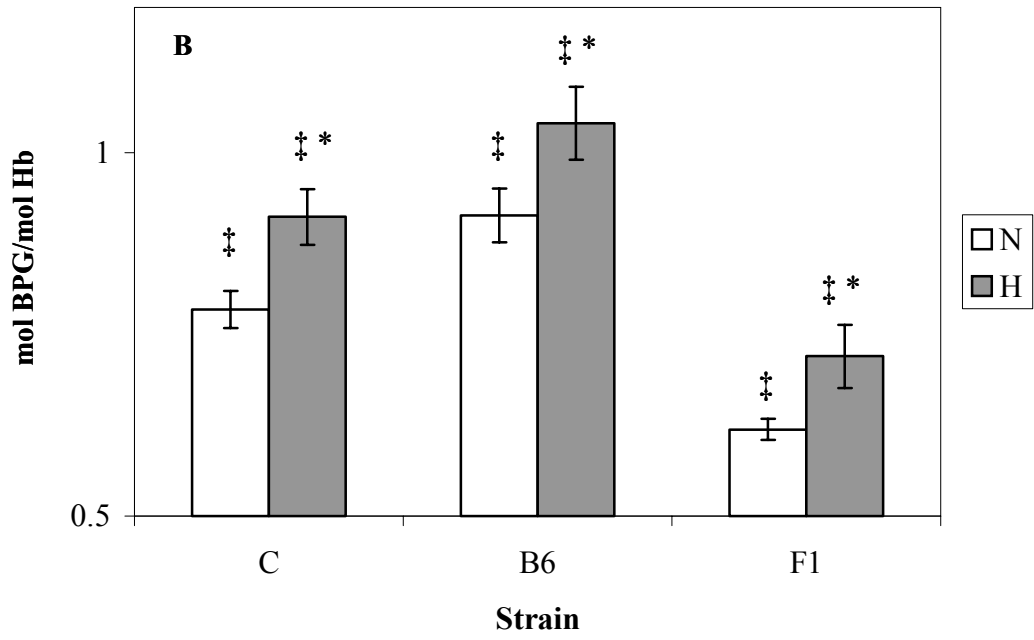
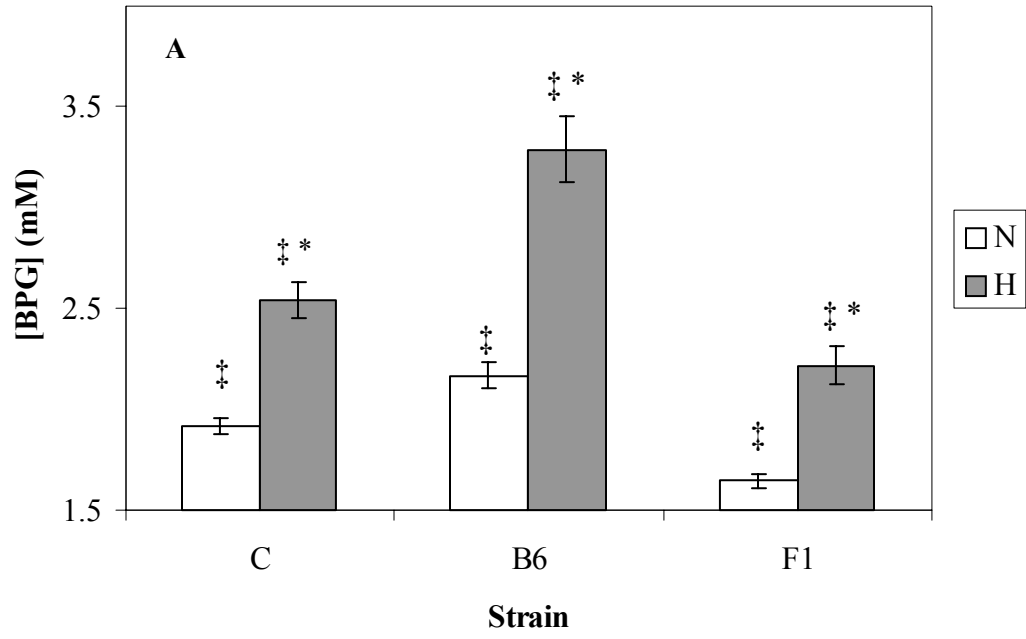


Figure 4. Myoglobin concentration within the gastrocnemius, EDL, soleus, and right ventricle of (A) C mice, (B) B6 mice, and (C) F1 mice. Values are means  $\pm$  SEM. For all measurements,  $n \geq 7$ . A significant effect of strain was detected within certain muscles, as is denoted by a ‡. Within the gastrocnemius, pairwise comparison revealed that C mice had a significantly lower mean [Mb] than B6 mice. Within the EDL, F1 mice had a significantly higher mean [Mb] than both C and B6 mice. Within the right ventricle, C mice had significantly lower mean [Mb] than B6 and F1 mice. A significant effect of treatment was also detected, and a significant difference in the myoglobin concentration of the muscles of normoxic and hypoxic exposed mice is denoted by an \*. N refers to normoxic treatment and H refers to hypoxic treatment.

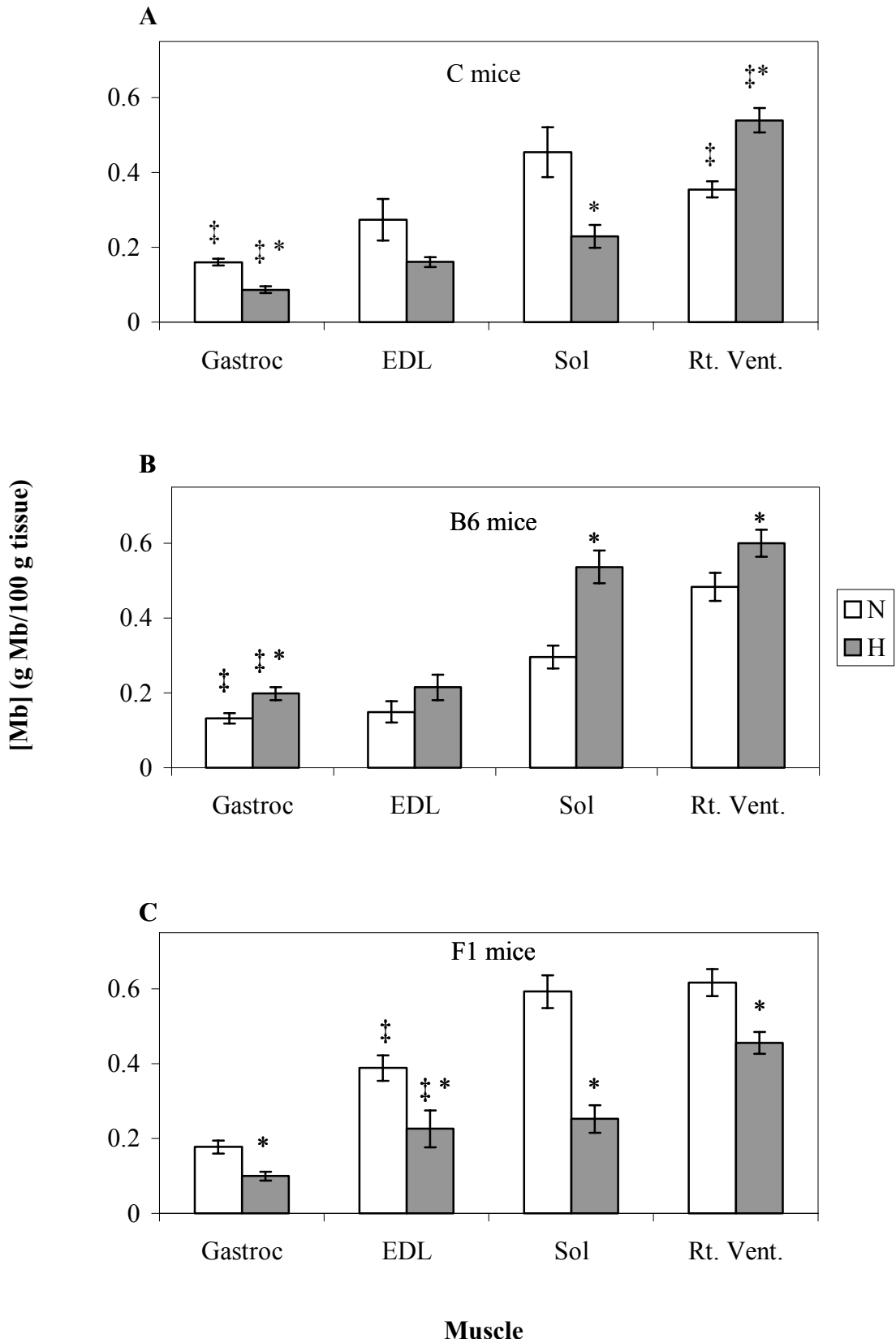
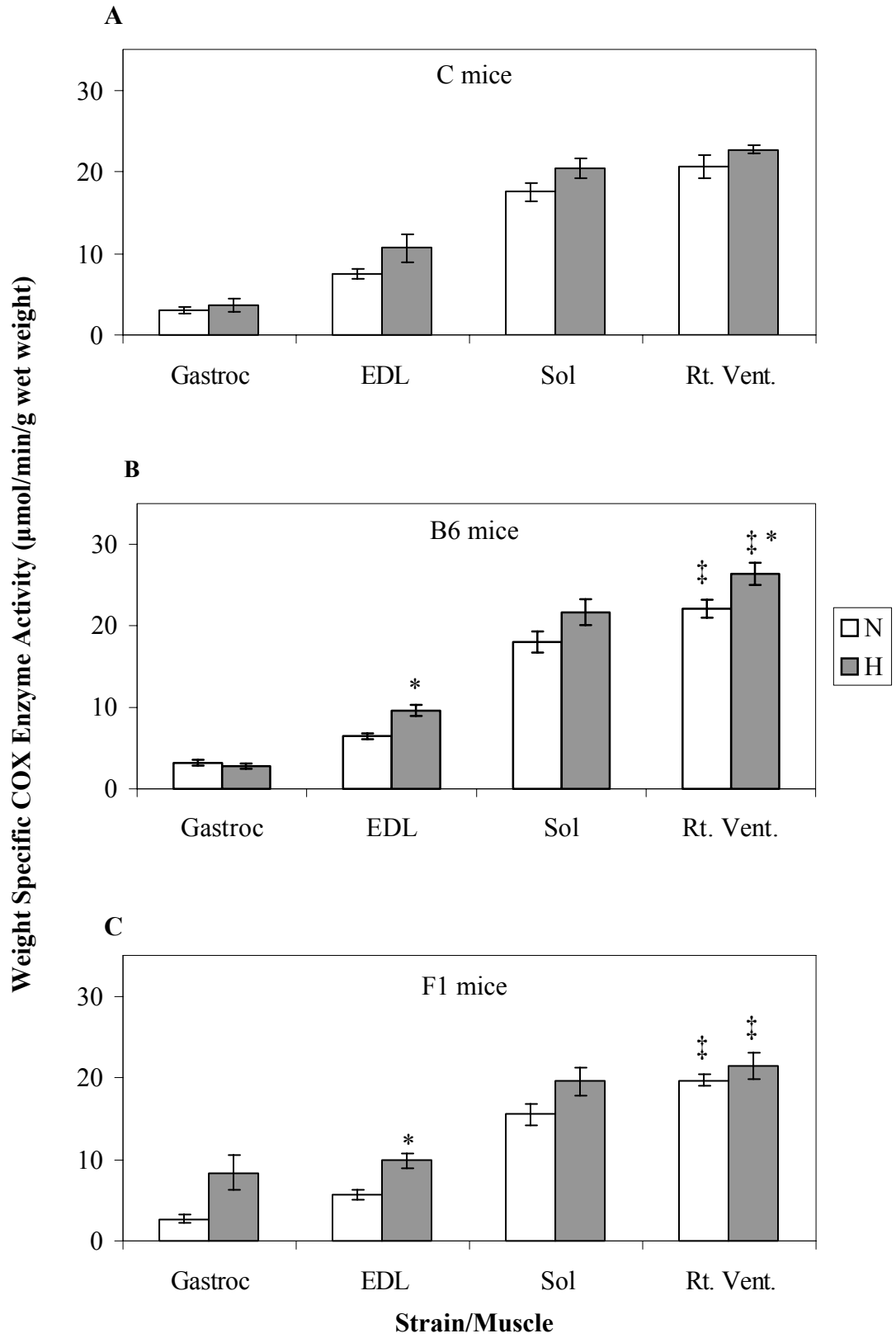


Figure 5. Weight-specific cytochrome-c oxidase enzyme activity within the gastrocnemius, EDL, soleus, and right ventricle of (A) C mice, (B) B6 mice, and (C) F1 mice. Values are means  $\pm$  SEM. For all measurements,  $n \geq 8$ . A significant effect of strain was detected and pairwise comparison revealed that B6 mice had significantly higher mean enzyme activity levels than F1 mice, as is denoted by a ‡. A significant effect of treatment was also detected and a significant difference between normoxic and hypoxic exposed mice within a strain is denoted by an \*. N refers to normoxic treatment and H refers to hypoxic treatment.





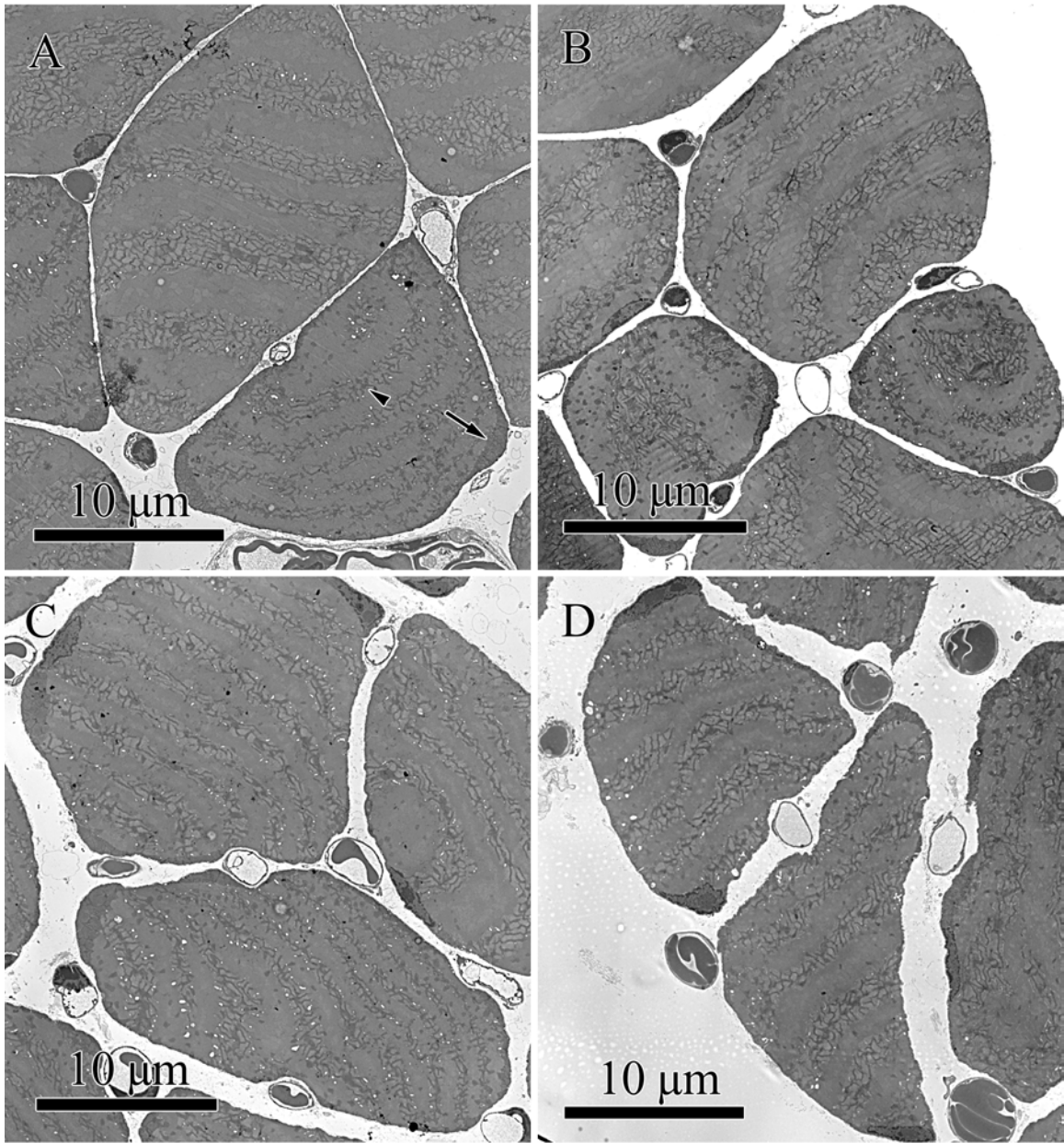


Figure 6. Representative TEM micrographs of the (A) EDL of a normoxic C mouse, (B) EDL of a hypoxic C mouse, (C) soleus of a normoxic C mouse, and (D) soleus of a hypoxic C mouse. A cluster of subsarcolemmal mitochondria is indicated by an arrow and a band of intermyofibrillar mitochondria is indicated by an arrowhead in figure A. Mitochondria within the muscle fibers appear dark in coloration resulting from uranyl acetate and Reynold's lead citrate staining. Capillaries can be seen around each fiber.

Table 2. Muscle fiber cross-sectional (FCS) area and diameter for each mouse strain and treatment.

Mouse and Muscle	Treatment	N	Mean FCSA ( $\mu\text{m}^2$ )	Mean FCS Diameter ( $\mu\text{m}$ )
EDL	C	N	1174.30 $\pm$ 189.52	37.96 $\pm$ 2.97
		H	961.56 $\pm$ 116.98	34.43 $\pm$ 2.01
	B6	N	1096.09 $\pm$ 219.93	35.92 $\pm$ 3.60
		H	929.41 $\pm$ 174.47	33.22 $\pm$ 2.98
	F1	N	1350.90 $\pm$ 159.11	40.71 $\pm$ 2.70
		H	886.45 $\pm$ 65.63*	32.45 $\pm$ 1.10*
SOL	C	N	1322.94 $\pm$ 86.74	41.14 $\pm$ 1.45
		H	1321.94 $\pm$ 58.49	41.92 $\pm$ 0.94
	B6	N	1526.86 $\pm$ 227.53	44.29 $\pm$ 3.16
		H	1149.48 $\pm$ 49.67	38.49 $\pm$ 0.94
	F1	N	1381.09 $\pm$ 62.67	42.48 $\pm$ 0.96
		H	1095.65 $\pm$ 90.54*	37.25 $\pm$ 1.52*

Values are means  $\pm$  SEM. A significant effect of treatment was detected, and a significant difference between normoxic and hypoxic exposed mice within a strain is denoted by an \*. N refers to normoxic treatment and H refers to hypoxic treatment.

Figure 7. Total fractional area of mitochondria within the (A) EDL and (B) soleus. Values are means  $\pm$  SEM. For all measurements,  $n \geq 6$ . N refers to normoxic treatment and H refers to hypoxic treatment.

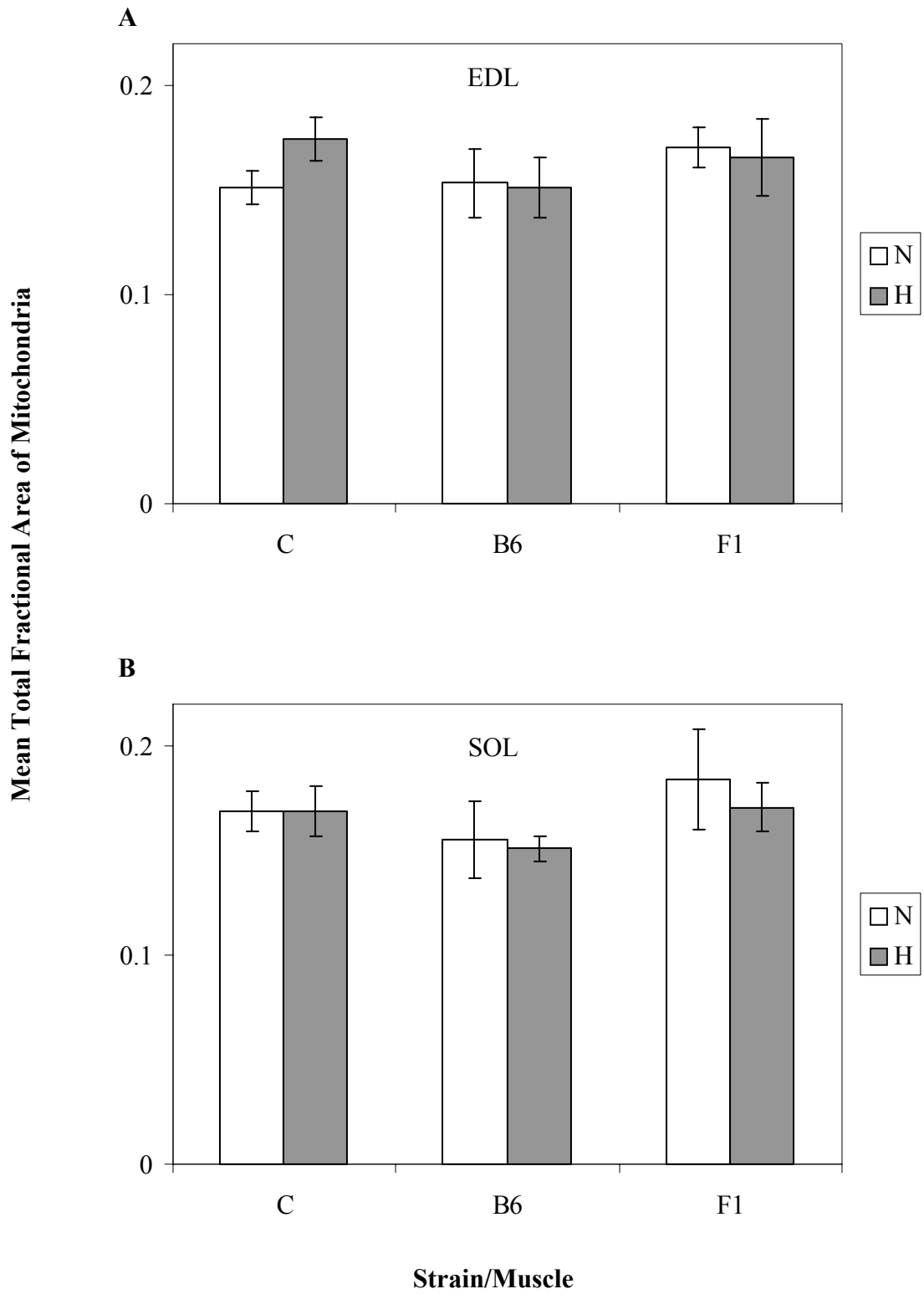


Figure 8. Radial distribution (from the fiber core to the sarcolemmal region adjacent to a capillary) of mitochondrial fractional area in EDL from (A) C mice, (B) B6 mice, and (C) F1 mice. Values are means  $\pm$  SEM. For all measurements,  $n \geq 6$ . I refers to inner region (at the fiber core), M refers to middle region, O refers to outer region of the fiber, and SS refers to subsarcolemmal region. N refers to normoxic treatment and H refers to hypoxic treatment.

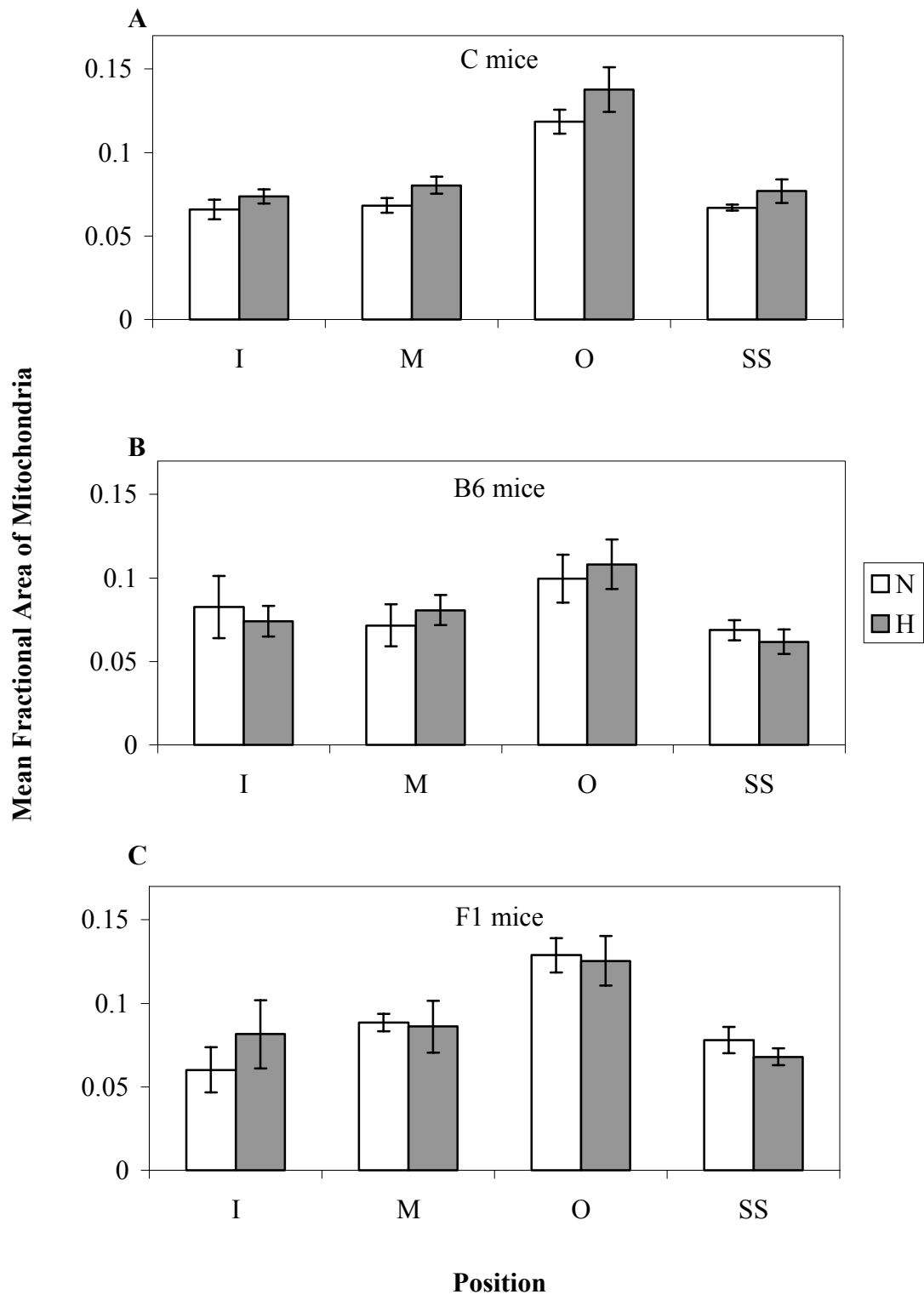


Figure 9. Radial distribution (from the fiber core to the sarcolemmal region adjacent to a capillary) of mitochondrial fractional area in soleus from (A) C mice, (B) B6 mice, and (C) F1 mice. Values are means  $\pm$  SEM. For all measurements,  $n \geq 6$ . I refers to inner region (at the fiber core), M refers to middle region, O refers to outer region of the fiber, and SS refers to subsarcolemmal region. N refers to normoxic treatment and H refers to hypoxic treatment.



