

# Chronic Cold Exposure Increases Skeletal Muscle Oxidative Structure and Function in *Monodelphis domestica*, a Marsupial Lacking Brown Adipose Tissue

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## ABSTRACT

*Monodelphis domestica* (Marsupialia: Didelphidae) was used as a model animal to investigate and compare muscle adaptation to exercise training and cold exposure. The experimental treatment consisted of four groups of animals: either warm or cold acclimation temperature and with or without endurance exercise training. Maximal aerobic capacity during a running  $\dot{V}O_{2\max}$  test in the warm-exercised or cold-exposed (with or without exercise) groups was about 130 mL  $O_2$ /kg/min, significantly higher than the warm-acclimated controls at 113.5 mL  $O_2$ /kg/min. Similarly, during an acute cold challenge ( $\dot{V}O_{2\text{summin}}$ ), maximal aerobic capacity was higher in these three experimental groups at ~95 mL  $O_2$ /kg/min compared with 80.4 mL  $O_2$ /kg/min in warm-acclimated controls. Respiratory exchange ratio was significantly lower (0.89–0.68), whereas relative heart mass (0.52%–0.73%) and whole-body muscle mitochondrial volume density (2.59 to 3.04  $\text{cm}^3$ ) were significantly higher following cold exposure. Chronic cold exposure was a stronger stimulus than endurance exercise training for tissue-specific adaptations. Although chronic cold exposure and endurance exercise are distinct challenges, physiological adaptations to each overlap such that the capacities for aerobic performance in response to both cold exposure and running are increased by either or both treatments.

## Introduction

Exploration of how animals adapt to chronic cold exposure has a long history, notably by Scholander and others (1950), who first observed that as body size decreases, mammals must defend their body temperature by increasing heat production (adaptive thermogenesis) at increasingly warmer absolute temperatures. Thus, temperatures that are physiologically thermoneutral for one species present a cold stress to smaller species. Cold exposure affects endothermic animals by causing an increase in metabolic heat production sufficient to compensate for heat loss and thus maintain body temperature. In small placental mammals (<10 kg), brown adipose tissue (BAT) is an effective thermogenic tissue that plays the primary role in adaptive thermogenesis (Foster and Frydman 1978). However, BAT is not found in larger placental mammals such as humans (Heldmaier 1971) nor in most marsupial species. In a survey of 38 marsupial species spanning all extant families, Hayward and Lisson (1992) failed to discover evidence of brown fat. The presence of BAT has been reported in a small Australian marsupial (*Sminthopsis crassicaudata*) on the basis of the appearance of an immunoreactive band on Western blots after cold exposure, although this was not accompanied by increased guanosine 5'-diphosphate (GDP) binding (Hope et al. 1997). Further, *Monodelphis domestica*, a small South American marsupial, does not increase metabolic rate in response to injected norepinephrine (Dawson and Olson 1988). Thus, BAT may appear in some marsupials, but if so, it appears to have little functional significance. In these animals, it is likely that skeletal muscle is the tissue primarily responsible for thermogenesis. Skeletal muscle is the single most abundant tissue among mammals (40% of body mass in placental mammals; Lindstedt and Schaeffer 2002) and is the location of most of the body's mitochondria, thus preadapting it for a role in acclimative heat production.

There is evidence for a transient role of muscle during cold acclimation in rats, both for shivering thermogenesis (Sellers et al. 1954) and nonshivering thermogenesis (Lin et al. 1998). However, this function disappears as BAT hypertrophy proceeds. Thus, in animals that possess BAT, the role of skeletal muscle in chronic adaptive thermogenesis appears to be relatively small. However, the role of skeletal muscle in adaptation to cold exposure in the absence of BAT is the subject of ongoing inquiry using numerous model species, including ducks (Barre

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et al. 1985; Duchamp and Barre 1993), chickens (Raimault et al. 2001), pigs (Berthon et al. 1996), goats (Schaeffer et al. 2001), and humans (Hong et al. 1987). The common theme of muscle adaptation to cold in the absence of BAT is an altered metabolic phenotype in response to chronic cold stress, a role obscured by the involvement of BAT in small placental mammals. Work on muscle thermogenesis has often sought to determine the relative importance of shivering versus nonshivering thermogenesis (e.g., Wickler 1981; Duchamp and Barre 1993; Block 1994).

Our focus is to explore the impact of cold exposure on skeletal muscle plasticity by contrasting adaptations to exercise training with adaptations for thermogenesis. The former is optimal when metabolic and mechanical power outputs are maximally coupled, whereas the latter operates with minimal coupling of metabolism to mechanics for optimal heat production. Chronic cold exposure may enhance both shivering and nonshivering muscle thermogenesis. It remains unexplored how reliance on skeletal muscle for thermogenesis, either shivering or nonshivering, in the absence of BAT will compare with current models of muscle plasticity after increased mechanical activation such as with exercise. Additionally, we address the difference between a sustained aerobic challenge (cold exposure) and a periodic challenge (endurance exercise training). Through this approach, we separate general muscle adaptations to aerobic challenge from those adaptations that may be specific to cold exposure or exercise training. Finally, we ask whether specific muscle adaptations that are the consequence of one aerobic challenge (cold or running) can be exploited during the nontrained activity.

To investigate this final question (i.e., to utilize adaptations from exercise training for thermogenesis or vice versa), it is essential to use a model animal that (1) lacks BAT, (2) is responsive to exercise training, and (3) ideally is small and tractable. The gray short-tailed opossum *M. domestica* is a small (~100 g) marsupial that meets all these criteria. Although it was once thought that marsupials were metabolically primitive (primarily on the basis of their lower body temperatures), evidence has accumulated demonstrating that in many marsupial species, maximal aerobic capacity is equal to that of placental mammals (Dawson 1989), although basal rates are often lower.

Using these animals allowed us to uniquely explore mammalian skeletal muscle plasticity in response to chronic cold exposure by asking the following questions: What is the impact of cold exposure and/or exercise training on whole-animal maximal oxygen uptake? Are adaptations to cold or exercise available for response to the other aerobic challenge? What is the effect on substrate utilization during aerobic demand? What is the effect on the organ mass and ultrastructural composition of selected muscles (oxygen-consuming tissues) as well as tissues that serve oxygen delivery?

## Material and Methods

### Animals

Forty-eight male short-tailed opossums *Monodelphis domestica* were obtained in two separate groups from a colony maintained at the Southwest Foundation for Biomedical Research (San Antonio, Tex.). Animals were received at 1.5–2 mo of age and were raised until approximately 2.5 mo old. Animals were weighed ( $\pm 0.1$  g) every other day throughout the experiment. Animals that failed to maintain weight during cold exposure and animals that did not cooperate with the exercise training were removed from the experiment. The animals were housed individually in hanging clear Lexan cages with wire roofs, cellulose bedding, and environmental enhancements of paper strips and glass jar huts. All animals were fed fox chow (National Fur Foods, New Holstein, Wis.) and occasional pieces of fruit and had water available ad lib. They were maintained on a 12L : 12D cycle that was reversed to allow all experimental procedures to be undertaken during working hours yet during the animals' active (night) phase. After all animals had reached target age and size (>45 g), they were divided into four groups. On completion of the experiments, animals were anesthetized with inhaled isoflurane and exsanguinated via the caudal vena cava. Tissues were removed, weighed, and fixed for electron microscopy. All animal experimentation was approved by the Institutional Animal Care and Use Committee of Northern Arizona University and complied with the "Principles of Animal Care," publication no. 86–23, revised 1985, of the National Institutes of Health as well as the laws of the United States.

### Experimental Design

Animals were divided into four groups. First, all animals were placed into one of two temperature acclimation regimes (designated "thermoneutral" and "cold"), and within each of these two groups, half of the animals were placed into an endurance exercise training group ("trained"), and half were simply maintained in their cages ("sedentary"). Thermoneutral (TN)-acclimated animals were maintained at 28°C (thermoneutral for these animals; Dawson and Olson 1988) throughout the 9-wk experiment. Cold-acclimated animals were initially placed into a refrigerated room at 19°C for 1 wk. The temperature was then decreased to 16°C at the beginning of the second week, to 12.5°C for the third week, and finally to 9°C for the fourth through sixth weeks. At this temperature, some health problems began to appear (tail lesions and lethargy leading to removal from the study), and the temperature was increased to 12°C for the final 3 wk. During the first week, every animal ran 3 d on the treadmill at 10 m/min for 5 min to gain familiarity with the apparatus necessary for subsequent testing. Thereafter, sedentary animals ran only once (again at 10 m/min for 5 min) during the eighth week of the experimental period. In contrast, the animals in the trained group ran for

30 min at 10 m/min, 5 d/wk during the second week. This was increased to 45 min at 15 m/min, 5 d/wk during the third week and then to 45 min at 20 m/min, 5 d/wk. This exercise regime was maintained for the duration of the experiment. All running was performed up a 10% incline.

#### *Measurement of Oxygen Consumption and Carbon Dioxide Production*

On completion of the experimental manipulations, we measured maximal rates of oxygen consumption to assess whole-animal aerobic capacity (within 72 h of completion of the experimental protocols). All measurements of oxygen consumption took place at an elevation of 2,100 m (ambient  $P_{\text{BAR}} = 600$  Torr), and data are reported after correction to STPD values. Maximal oxygen consumption while running on a treadmill ( $\dot{V}O_{2\text{max}}$ ) was chosen to measure aerobic capacity during locomotion. Maximal oxygen consumption elicited by an extreme, short-term cold exposure ( $\dot{V}O_{2\text{summit}}$ ) was chosen to measure aerobic capacity in response to a thermogenic challenge. Oxygen consumption was measured using an open-flow system in which air was either drawn into a metabolic chamber with a small pump ( $\dot{V}O_{2\text{max}}$ ) or pushed into a chamber from a compressed gas tank using an atmospheric balloon to buffer pressure ( $\dot{V}O_{2\text{summit}}$ ). Two more pumps drew samples of the excurrent air through an oxygen analyzer (Model S-3A, AEI Technologies) and a carbon dioxide analyzer (Model CD-3A, AEI Technologies). Before passing into the oxygen analyzer,  $H_2O$  and  $CO_2$  were removed by drawing the gas through Drierite and Ascarite, respectively. Water was also removed from the gas before passing into the carbon dioxide analyzer. The  $O_2$  system was calibrated after every second or third run using the  $N_2$  dilution method of Fedak et al. (1981). A specific calibration gas was used for the  $CO_2$  system. In all cases, flow downstream of the metabolic chamber was measured with a mass flow meter, monitored, and maintained at a constant level during each measurement. For  $\dot{V}O_{2\text{max}}$ , flow rates ranged from 2.5 to 3.3 L/min. For  $\dot{V}O_{2\text{summit}}$ , flow rates ranged from 2.4 to 2.5 L/min.

To measure  $\dot{V}O_{2\text{max}}$ , animals were placed into a Plexiglas box (volume of 1.4 L) that enclosed a section of the treadmill and allowed air to be drawn in between the treadmill and the bottom of the box and out the front top of the box. Measurements were made at the TN temperature (28°C). During each measurement, animals ran up a 10% incline, beginning at 40 m/min. The speed was increased by 5 m/min every 3 min until the animals were unable to continue. A shock grid embedded in the rear wall of the box served as motivation to continue. Immediately after each run test, a blood sample was taken from the caudal vein and immediately centrifuged, and plasma lactate concentration was measured with a lactate analyzer (2300 Stat, YSI). A lactate concentration of  $>6$  mmol/L was required to indicate a true maximal effort. The  $\dot{V}O_{2\text{max}}$  was calculated using

the percent oxygen values measured when the animal's  $O_2$  consumption reached a plateau following Fedak et al. (1981).

To measure summit metabolism, the animal was placed into a plastic chamber (volume of 0.85 L), itself immersed in a 0°C, constant temperature, isopropanol-water bath (Model 1165, VWR). Incurrent gas consisted of a heliox mixture (20.7% oxygen, 79.3% helium), which was used to increase thermal conductivity of the gas (Rosenmann and Morrison 1974) and resulted in a greater cold stress without exposure to extreme temperatures. Before the metabolic chamber, incurrent gas was directed through a section of copper tubing immersed in a 0°C water bath for precooling. The animal remained in the chamber for 5 min to attempt to avoid hypothermia; however, body temperatures were not measured. Oxygen consumption typically reached a plateau after 3 min. The  $\dot{V}O_{2\text{summit}}$  was calculated using the percent oxygen values in the last minute. The time periods of these  $\dot{V}O_{2\text{summit}}$  measurements are shorter than commonly used but resulted in measurements similar to those using a longer time course in selected pilot experiments and were used to minimize cold exposure during the testing period. However, using these shorter time periods may have increased the error due to random short-term fluctuations.

To calculate the respiratory exchange ratio (RER) during the summit metabolism test, total system flow was calculated using equation (11c) from Fedak et al. (1981). That value was then used to calculate  $\dot{V}CO_2$ , and RER was calculated as the ratio of  $\dot{V}CO_2$  over  $\dot{V}O_2$ .

#### *Tissue Sampling*

After they were killed (within 48 h after metabolic measurements), specific tissue samples (liver, diaphragm, heart, semitendinosus, and sternocleidomastoid) were removed, weighed, and prepared for fixation. Additionally, 10 random muscle samples were taken from each animal using a scheme that samples each muscle group in proportion to its contribution to the total muscle mass (described in detail in Hoppeler et al. 1984). These samples together are a representative sample of the whole-body musculature. Specific muscles and random samples were removed from only one side of the body, and then the corpse was skinned, eviscerated, and divided into sections, which consisted of the head, neck, upper trunk, lower trunk, forelimb-proximal, forelimb-distal, hindlimb-proximal, and hindlimb-distal (as described in Hoppeler et al. 1984). Only one limb from each side was used, and the trunk sections were divided such that only one side, which included the spinal column, was retained. Each section was weighed; all muscle was removed, and it was weighed again. The remnants from all eight sections (except the head, because of the brain weight) were placed into a beaker with water, covered, and allowed to digest, removing the muscle remnants. These bones were then patted dry and weighed again. Whole-body muscle mass was then calculated

as the sum of the muscle removed from each compartment (doubled where appropriate).

#### *Tissue Fixation and Imaging*

Samples were taken from the semitendinosus and sternocleidomastoid muscles as well as from random samples from the whole-body musculature for measurement of mitochondrial volume density and calculation of muscle mitochondrial content. Muscle samples were first cut into small blocks, approximately 0.5 mm × 0.5 mm × 5 mm, and were then immersed in 6.25% glutaraldehyde in a 0.1 M sodium cacodylate buffer (pH 7.4). Fixed tissue was stored at 4°C until preparation for electron microscopy. Tissue blocks were then placed under a dissecting microscope, and the block size was reduced to about 0.1 mm × 0.1 mm × 1.0 mm; care was taken to align the muscle fibers along the long axis of the block. These smaller blocks were then placed into 0.1 M sodium cacodylate overnight to remove excess glutaraldehyde. After a minimum of 12 h in this buffer, we embedded the tissues into resin with the following procedure, modified from Hoppeler et al. (1984): two buffer washes in 0.1 M Na cacodylate (pH 7.4) for 30 min each, postfixation in 1% osmium tetroxide in 0.1 M Na cacodylate (pH 7.4) for 2 h, three buffer washes in 0.1 M Na cacodylate (pH 7.4) for 30 min, three buffer washes in 0.05 M maleic acid (pH 5.2) for 5 min, en bloc staining with 0.5% uranyl acetate in 0.05 M maleic acid (pH 5.2) for 2 h, three buffer washes in 0.05 M maleic acid (pH 5.2) for 5 min, dehydration series of 50, 70, 95, and 3 × 100% ethanol washes for 10 min each, and three 10-min washes in propylene oxide. At this point, resin infiltration began with overnight immersion into a 50/50 mixture of EMBED 812 and propylene oxide, 4 h in a 75/25 mixture of EMBED 812 and propylene oxide, and finally EMBED 812 overnight. Tissue blocks were then laid into embedding molds, filled with fresh EMBED 812 resin with the blocks oriented to facilitate muscle cross-sections, and placed into a 60°C oven overnight.

After embedding, blocks were sectioned on an ultramicrotome (MT2-B, Sorvall) after verifying proper fiber orientation for stereology. Thin sections (~70 nm) were postfix stained with uranyl acetate (saturated solution in water) and lead citrate (100 mg per 20 mL NaOH solution; pH ≈ 10). The grids were immersed into the uranyl acetate for 10 min, rinsed in 50% ethanol and water, and then floated on a drop of lead citrate for 5 min and rinsed again. Electron micrographs were taken on a transmission electron microscope (JEM 1200EX II, JEOL) at ×4,000 magnification. For the semitendinosus and sternocleidomastoid, one section was cut per animal. Six randomly chosen locations were photographed from each section, and 200 points were counted per image. For the random samples, three muscles were selected from each animal, six locations were photographed per selection, and 200 points were counted per image (following Weibel 1979).

#### *Stereology*

We calculated mitochondrial volume density by projecting micrographs onto an A100 stereology grid using an AusJena projector (×26,000 final magnification) following the technique of Weibel (1979). Point counting was recorded and final calculations performed using the program Step-One (Wainschein and Cruz-Orive 1994). Mean mitochondrial volumes were calculated for the semitendinosus, sternocleidomastoid, and the random muscle samples for each animal, and these values were used in statistical comparisons. The product of the mean mitochondrial volume density and muscle mass for each animal gives an estimate of total muscle mitochondrial content (either for a specific muscle or the total body musculature).

#### *Statistical Analysis*

Each variable was tested for statistical significance using a two-way ANOVA, with temperature and training status as well as the temperature × training interaction as dependent variables, using Sigma-Stat (version 2.03, SPSS). For those variables that showed significant differences, pairwise comparisons were run using the Student-Newman-Keuls method. The level of significance was set at  $P < 0.05$  in all cases. Data are reported as mean values ± the standard error of the mean, with the number of observations in parentheses. Unless noted otherwise, the interaction term was not significant.

## **Results**

#### *Animal and Tissue Masses*

Animals were purchased in separate groups for the cold-acclimation and warm-acclimation periods. Within each group, the animals were divided into weight-matched pairs. However, the starting weights of the two temperature groups differed significantly, with the animals placed into the cold-acclimation groups larger than those placed into the warm-acclimation groups, although all animals were the same age. The mean weights at the beginning of the experiment were  $61.7 \pm 2.7$  g (14 observations),  $62.2 \pm 3.2$  g (10),  $77.0 \pm 5.0$  g (four), and  $71.6 \pm 4.1$  g (six) for the warm acclimated-sedentary (TNS), warm acclimated-exercise trained (TNT), cold acclimated-sedentary (CS), and cold acclimated-exercise trained (CT) animals, respectively (two-way ANOVA; temperature effect  $P < 0.01$ ; training effect  $P = 0.53$ ). Animals continued growing through the experiments (adult weight, 80–100 g). At the end-point of the experiment, there was no difference in the mean animal weights (two-way ANOVA; temperature effect  $P = 0.53$ ; training effect  $P = 0.36$ ). Animals weighed  $94.3 \pm 4.4$  g (14),  $89.4 \pm 5.2$  g (10),  $91.3 \pm 8.3$  g (four), and  $84.4 \pm 6.7$  g (six). Thus, the warm-acclimated groups increased their mass by 52.8% (TNS) and 43.7% (TNT), whereas the cold-

acclimated groups increased their mass by only 18.9% (CS) and 17.9% (CT).

The proportion of total body mass composed of skeletal muscle was unaffected by either treatment (two-way ANOVA; temperature effect  $P = 0.91$ ; training effect  $P = 0.99$ ). In all cases, both relative (Fig. 1A) muscle mass and absolute (data not shown) muscle mass were not significantly different between groups. Heart muscle mass was significantly higher in cold-exposed, although not exercise-trained groups (two-way ANOVA; temperature effect  $P < 0.001$ ; training effect  $P = 0.16$ ; Fig. 1B). The heart in both warm-acclimated groups made up about 0.52% of body mass (typical of mammals in general; Lindstedt and Schaeffer 2002), whereas that of the cold-acclimated groups was 0.73% of body mass, nearly 50% larger. The diaphragm, primarily involved in ventilation, did not change size after either treatment (two-way ANOVA; temperature effect  $P = 0.72$ ; training effect  $P = 0.09$ ). In all cases, the diaphragm consisted of about 0.5% of body mass (Fig. 1B).

The relative weight of the semitendinosus (a hip adductor) showed no significant response to either treatment, although there was a trend for larger size in the trained animals (two-way ANOVA; temperature effect  $P = 0.41$ ; training effect  $P = 0.16$ ). This muscle ranged from 0.054% (CS) to 0.092% (TNT) of body mass. The sternocleidomastoid (a neck muscle minimally involved in locomotion) had significantly lower relative weight in response to cold acclimation (two-way ANOVA; temperature effect  $P < 0.01$ ; training effect  $P = 0.10$ ), mainly seen in the CS group, which was  $0.11\% \pm 0.01\%$  (four observations) of body mass compared with  $0.14\% \pm 0.01\%$  (11) and  $0.15\% \pm 0.01\%$  (eight) in the TNS and TNT groups, respec-

tively. Body mass-specific sternocleidomastoid weight in the CT group was not different from either the warm group or the CS group at  $0.13\% \pm 0.01\%$  (five) of body mass.

### Aerobic Capacity

The  $\dot{V}O_{2\max}$  was significantly higher in either experimental treatment as well as the combination of the two (temperature effect  $P < 0.01$ ; exercise training effect  $P < 0.05$ ) from  $113.5 \pm 2.4$  (11 observations) mL  $O_2$ /kg/min for the TNS group to  $129.1 \pm 3.3$  (six),  $133.9 \pm 4.0$  (four), and  $132.2 \pm 3.6$  (five) mL  $O_2$ /kg/min for the TNT, CS, and CT groups, respectively (Fig. 2), corresponding to 14%, 18%, and 16% higher aerobic capacity, respectively. The interaction term (temperature  $\times$  training) was also significant ( $P < 0.05$ ). The values for the three treated groups did not differ from one another; thus, the effects of training differed depending on the temperature at which it took place. Given that the three treated groups did not differ, it appears that either treatment alone was sufficient to elicit a maximal response.

Changes in  $\dot{V}O_{2\text{summit}}$  followed the same pattern as  $\dot{V}O_{2\max}$ , although only the effects of cold exposure were significant (two-way ANOVA; temperature effect  $P < 0.05$ ; training effect  $P = 0.10$ ). The  $\dot{V}O_{2\text{summit}}$  was 15% higher in the TNT group and 20% higher in both cold groups (Fig. 3), from  $80.4 \pm 3.0$  (seven observations) mL  $O_2$ /kg/min for the TNS group to  $92.4 \pm 3.2$  (six) mL  $O_2$ /kg/min for the TNT group,  $96.2 \pm 4.0$  (four) mL  $O_2$ /kg/min for the CS group, and  $96.3 \pm 3.6$  (five) mL  $O_2$ /kg/min for the CT group. For  $\dot{V}O_{2\text{summit}}$  as well, exposure to both cold acclimation and endurance exercise training did not result

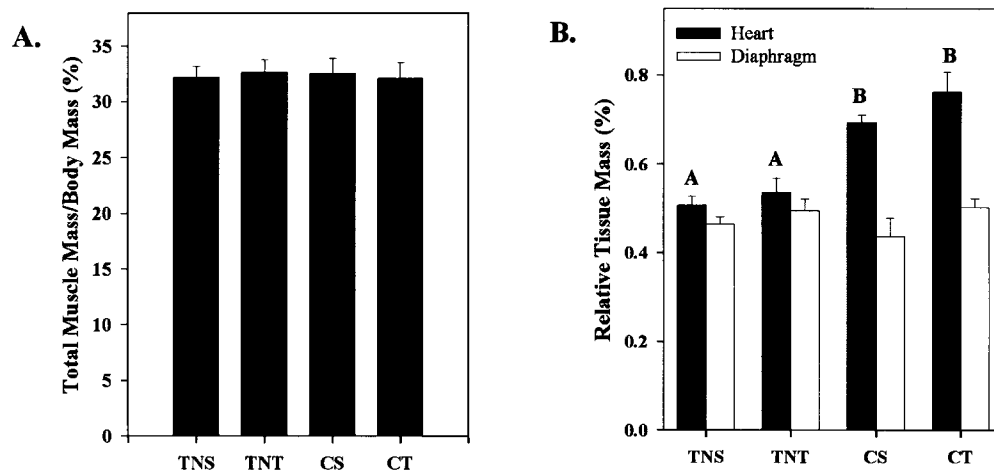


Figure 1. A, Total body muscle mass, expressed as a percentage of body mass, did not vary among groups. In all groups, muscle mass was about 32% of body mass, less than seen in placental mammals. B, Relative heart and diaphragm mass are expressed as a percentage of body mass. Relative heart mass was 50% higher following cold acclimation, whereas exercise training had no effect. There was no difference in relative diaphragm mass among groups. Groups are as follows: TNS = thermoneutral, sedentary; TNT = thermoneutral, trained; CS = cold, sedentary; and CT = cold, trained. Different letters above the bars indicate significant differences between groups, within each variable.

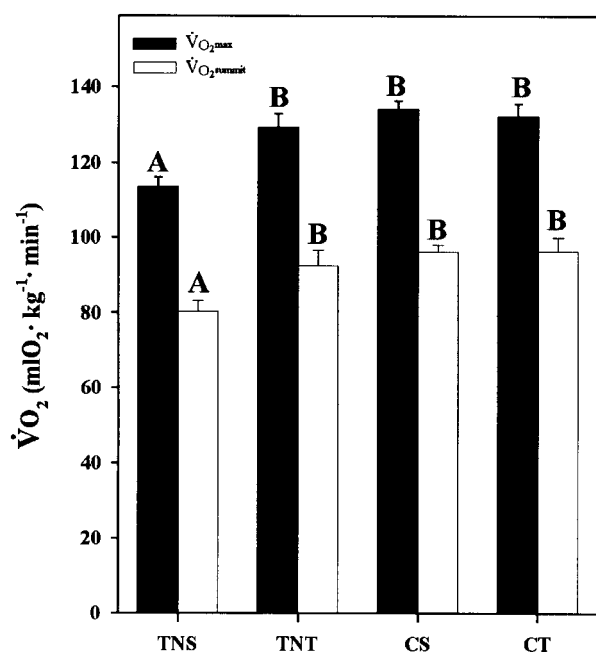


Figure 2.  $\dot{V}O_{2max}$  and  $\dot{V}O_{2summit}$  are both significantly higher in either experimental treatment or the combination of the two. In all groups,  $\dot{V}O_{2summit}$  was about 70% of  $\dot{V}O_{2max}$ . Groups and statistics are as in Figure 1.

in an additive effect since the three treated groups did not differ from one another.

The ratio of  $\dot{V}O_{2summit}$  over  $\dot{V}O_{2max}$  showed no significant difference among the four groups (two-way ANOVA; temperature effect  $P = 0.64$ ; training effect  $P = 0.14$ ). The  $\dot{V}O_{2summit}$  was consistently about 71% of  $\dot{V}O_{2max}$ , at  $68.9\% \pm 1.7\%$  (seven observations) for the TNS group,  $74.0\% \pm 2.0\%$  (five) for the TNT group,  $71.9\% \pm 2.3\%$  (four) for the CS group, and  $73.0\% \pm 2.0\%$  (five) for the CT group.

#### Metabolic Fuel Selection

Respiratory exchange ratio during the summit metabolism test showed a significant effect of temperature alone (two-way ANOVA; temperature effect  $P < 0.001$ ; training effect  $P = 0.262$ ). The lower RER seen in the cold-acclimated animals (Fig. 3) corresponds to a shift from approximately 37% of fuels used being lipids to nearly complete dependence on lipids for fuel during the summit aerobic challenge. Endurance exercise training showed no significant effect, with RER being nearly identical within either temperature group.

#### Mitochondrial Volumes and Contents

The mitochondrial volume density of the semitendinosus was significantly higher in the cold-exposed group but not the ex-

ercise-trained groups (two-way ANOVA; temperature effect  $P < 0.05$ ; training effect  $P = 0.72$ ), ranging from  $7.4\% \pm 1.0\%$  (eight observations) and  $9.0\% \pm 1.0\%$  (eight) in the TNS and TNT groups, respectively, to  $11.5\% \pm 1.6\%$  (three) and  $10.8\% \pm 1.2\%$  (five) in the CS and CT groups, respectively. Post hoc tests showed that the TNT animals were intermediate and not different from any other group (Fig. 4). The combined cold groups thus had a 36% higher mitochondrial volume density in this muscle. Mitochondrial volume density of the sternocleidomastoid followed a similar pattern, although the increase as a result of cold acclimation is more clearly pronounced (two-way ANOVA; temperature effect  $P < 0.01$ ; training effect  $P = 0.65$ ). The TNS and TNT groups, with a volume density of  $5.9\% \pm 1.0\%$  (four) and  $7.0\% \pm 0.50\%$  (four), respectively, did not differ from each other. The CS and CT groups at  $10.0\% \pm 1.0\%$  (four) and  $9.6\% \pm 0.9\%$  (five), respectively, also did not differ from each other (Fig. 4). The difference in mitochondrial volume density from 6.4% in the combined TN animals to 9.8% in the combined cold-exposed animals represents a 53% higher mitochondrial volume density in this muscle.

The product of mitochondrial volume density (percent of cell volume) and muscle weight (product of muscle volume and density) gives the total muscle mitochondrial content (in cubic centimeters). This value is a measure of the total aerobic capacity within that muscle. The total mitochondrial content of the semitendinosus muscle mirrored the results seen for

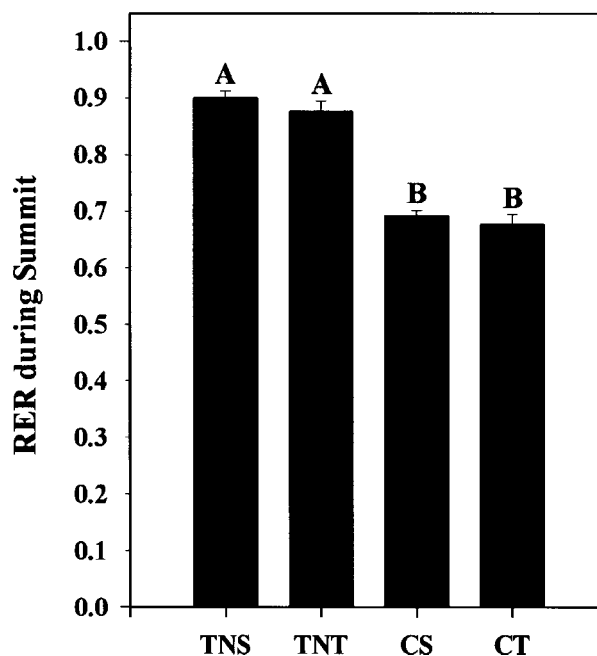


Figure 3. Respiratory exchange ratio (RER) during the  $\dot{V}O_{2summit}$  tests was lower following cold exposure, resulting in a near total dependence on fats for fuel. Groups and statistics are as in Figure 1.

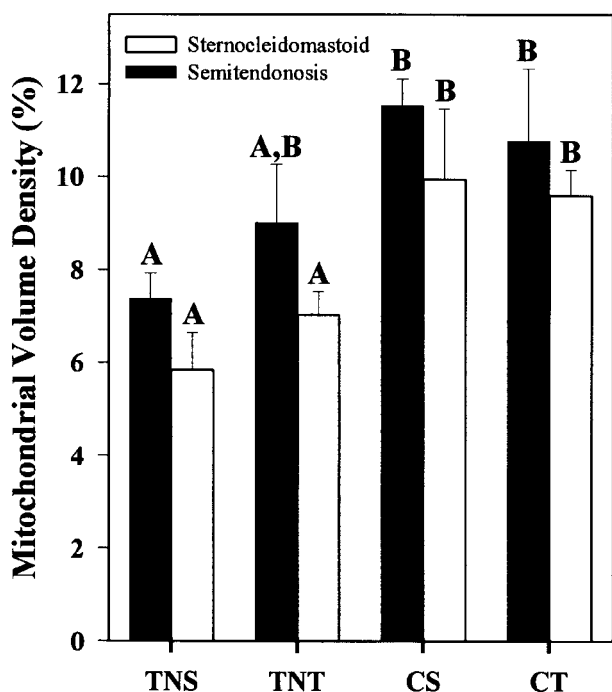


Figure 4. Mitochondrial volume density ( $V_{mt}$ , f) in one locomotor (semitendinosus) and one nonlocomotor (sternocleidomastoid) muscle was higher in cold-exposed animals, by 36% and 53%, respectively. Groups and statistics are as in Figure 1.

muscle weight, with no significant differences detected but a trend toward a higher content in the endurance exercise-trained animals (two-way ANOVA; temperature effect  $P = 0.88$  training effect  $P = 0.13$ ). Values were  $0.0051 \pm 0.0012 \text{ cm}^3$  total mitochondria (eight observations) and  $0.0080 \pm 0.0013 \text{ cm}^3$  total mitochondria (eight) in the TNS and TNT semitendinosus muscles, respectively, and  $0.0058 \pm 0.0019 \text{ cm}^3$  (three) and  $0.0078 \pm 0.0017 \text{ cm}^3$  (five) in the CS and CT groups, respectively. Similarly, because sternocleidomastoid muscle was significantly smaller with cold acclimation, the total mitochondrial content of this muscle was unchanged by either treatment (two-way ANOVA; temperature effect  $P = 0.17$ ; training effect  $P = 0.07$ ). Values were  $0.0083 \pm 0.0007 \text{ cm}^3$  total mitochondria (four) and  $0.0102 \pm 0.0008 \text{ cm}^3$  total mitochondria (four) in the TNS and TNT semitendinosus muscles, respectively, and  $0.0098 \pm 0.0012 \text{ cm}^3$  (four) and  $0.0115 \pm 0.0011 \text{ cm}^3$  (five) in the CS and CT groups.

Using the random muscle samples, whole-body skeletal muscle mitochondrial volume densities (%) were nearly 30% higher in cold-exposed animals but not exercise-trained animals (two-way ANOVA; temperature effect  $P < 0.05$ ; training effect  $P = 0.249$ ; Fig. 5A), from  $7.85\% \pm 0.63\%$  (four) and  $8.41\% \pm 0.69\%$  (four) in the TNS and TNT groups, respectively, to  $9.48\% \pm 1.31\%$  (three) and  $11.31\% \pm 1.23\%$  (four) in the CS

and CT groups, respectively. Because the total muscle mass did not differ among groups, total skeletal muscle mitochondrial content followed the same quantitative pattern. Total muscle mitochondrial volume ( $\text{cm}^3$ ) was 19% higher in cold-exposed animals, from  $2.49\% \pm 0.17\%$  (four) and  $2.69\% \pm 0.19\%$  (four) in the TNS and TNT groups, respectively, to  $2.80\% \pm 0.29\%$  (three) and  $3.29\% \pm 0.26\%$  (four) in the CS and CT groups, respectively (two-way ANOVA; temperature effect  $P < 0.05$ ; training effect  $P = 0.149$ ; Fig. 5B). This difference is nearly identical to that seen for whole-body oxidative capacity measured with either  $\dot{V}O_{2\max}$  or  $\dot{V}O_{2\text{summit}}$ .

## Discussion

In mammals with BAT, skeletal muscle is generally not considered to be the principal tissue involved in adaptive thermogenesis (Colquhoun and Clark 1991; Block 1994; Janský 1995). Foster and Frydman (1978) found that BAT contributes ~65% of the heat production in rats during cold exposure. In the same study, skeletal muscle  $O_2$  consumption increased by only 12%, accounting for about 10% of the whole-body  $O_2$  consumption. Thus, although BAT is unable to account completely for altered thermogenic capacity, its role clearly overshadows that of skeletal muscle. Thus, the lack of skeletal muscle adaptations to cold exposure in many reports is likely a result of BAT playing a primary role in adaptive thermogenesis in most small animals (e.g., Harri et al. 1984; Hoppeler et al. 1995). A further problem with understanding the role of muscle in cold acclimation is that many studies using small mammals such as rats and mice lack proper controls. The lower critical temperature of rats was reported as  $28^\circ\text{C}$  by Gordon (1990), and Janský has suggested that mice reach maximal cold acclimation after exposure to  $20^\circ\text{C}$  (discussion following Heldmaier 1971). Whereas the lower critical temperature can be labile, depending on acclimation, both species are routinely housed at  $21^\circ\text{C}$ – $25^\circ\text{C}$ , which is likely to represent some cold stress.

Although the chronic cold exposure and endurance exercise training used favored differing coupling of metabolic and mechanical power outputs, with some exceptions, the outcomes of both protocols were very similar. Our cold-exposure protocol was a constant (24 h per day), moderate stimulus (eliciting a  $\dot{V}O_2$  about 3–4  $\times$  basal; Dawson and Olson 1988). This approaches the limits of sustained energy use described by Hammond and Diamond (1997) and apparently led to inhibition of growth. Exercise training was designed to represent a very high intensity, intermittent stimulus. The speed at which these animals were required to run during the training period (20 m/min) elicits about 70% of  $\dot{V}O_{2\max}$  and was equal to approximately 2.8 body lengths per second (equivalent to about 35 m/min for a rat). Similarly, 45 min of continuous exercise for a 100-g animal is biologically equivalent to nearly 4 h in a 70-kg human, given that biological times scale to the one-fourth power of body mass (Lindstedt and Calder 1981).

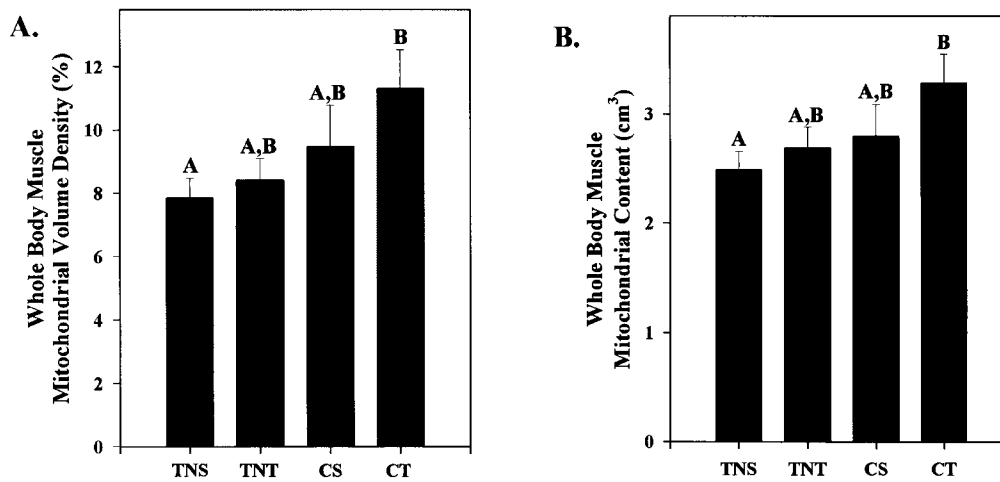


Figure 5. A, Mean mitochondrial volume density ( $V_{mt, f}$ ) calculated for the whole-body musculature was 29.5% higher following cold exposure. B, Total muscle mitochondrial content ( $\text{cm}^3$ ) was 19% higher following cold exposure, nearly identical to the observed difference in  $\dot{V}O_{2\max}$  and  $\dot{V}O_{2\text{summit}}$ . Groups and statistics are as in Figure 1.

In these experiments, both experimental protocols resulted in very similar responses to both maximal aerobic capacity tests, measured while running ( $\dot{V}O_{2\max}$ ) or during an acute cold challenge ( $\dot{V}O_{2\text{summit}}$ ). This suggests that higher aerobic capacity, whether the result of exercise training or cold exposure, is available to support both  $\dot{V}O_{2\max}$  and  $\dot{V}O_{2\text{summit}}$ . In contrast, previous reports from placental mammals suggest that muscle does not respond to cold exposure. Exercise-trained rats did not have greater metabolism in the cold, although exercise training increased muscle aerobic capacity (Harri et al. 1984; Conley et al. 1985). In deer mice (*Peromyscus maniculatus*), cold exposure greatly increased  $\dot{V}O_{2\text{summit}}$  (31%), but running  $\dot{V}O_{2\max}$  was increased only 9%, supporting the primary role of BAT in small placental mammals (Hayes and Chappell 1986). Similarly, winter-acclimated white-footed mice (*Peromyscus leucopus*) possess approximately 70% greater  $\dot{V}O_{2\text{summit}}$  than do their summer-acclimated counterparts (Wickler 1980), similar to the increase in whole-body metabolic rate following infusion of norepinephrine. However, infusion of norepinephrine into isolated-perfused hindlimbs had no effect, suggesting that skeletal muscle is minimally involved in the thermogenic response of these animals (Wickler 1981). There is thus abundant evidence that BAT plays the principal role of thermogenesis in small placental mammals.

However, when BAT is absent, whole-animal responses to a cold challenge draw primarily on muscle oxidative capacity. This evidence strongly supports the notion that skeletal muscle is the principal site for long-term adaptation to cold exposure in these animals since it likely is in all mammals lacking BAT, such as humans. It also indicates that in the absence of BAT, metabolic adaptations of skeletal muscle to any challenge of aerobic capacity result in similar changes and that the resulting

increase in aerobic capacity can be utilized for a novel task. Following this, the ratio of  $\dot{V}O_{2\text{summit}}$  over  $\dot{V}O_{2\max}$  was low compared with that in previous studies (Seeherman et al. 1981; Conley et al. 1985) but did not differ among groups. This demonstrates in *Monodelphis domestica* that the adaptation of whole-animal metabolism to either test of aerobic capacity is equivalent, independent of the nature of the aerobic challenge. Exercise training leads to both increased running performance and thermogenic response to cold equally, just as prior cold exposure increased performance on the treadmill and thermogenesis equally.

Although the differences in maximal aerobic capacity after the experimental protocols are similar, the metabolic pathways that support this activity are apparently modified by cold exposure. The RER during  $\dot{V}O_{2\text{summit}}$  was lower only after cold exposure, in contrast to previous work in humans (Van Der Vusse and Reneman 1996) in which RER during submaximal exercise was decreased by prior exercise training. This was also seen when animals ran at submaximal speeds on the treadmill (data not shown) and thus was not limited to aerobic activity during cold exposure and was not an artifact of hypothermia. The differences in RER are striking, suggesting that continuous, daily aerobic activity levels determine fuel choice more than peak levels during the course of a day.

For increased capacity of energy use in the periphery, oxygen delivery must be increased as well. Organs involved in  $O_2$  supply and delivery (heart and diaphragm) showed no effect of exercise training, although the heart is commonly observed to exhibit hypertrophy in response to endurance exercise training. However, chronic cold exposure did result in larger heart size, potentially because of chronic increased ( $\times 3-4$ ) resting metabolism and the concurrent demand for  $O_2$  delivery to ther-



mogenic tissues. The diaphragm showed no response to either stimulus. This finding is consistent with previous reports, showing that the pulmonary gas exchange system is not a limiting factor to aerobic capacity in either placental mammals (Taylor et al. 1987) or a marsupial (Hallam et al. 1989).

Contrary to previous observations, cold exposure was the dominant stimulus for anatomical adaptation in both of our selected muscles. Our choice of the semitendinosus and sternocleidomastoid was based on the primary usage of each during normal locomotion. The semitendinosus is a representative locomotor muscle, whereas the sternocleidomastoid is a nonlocomotor muscle and is centrally located to potentially serve thermoregulatory demands. There was a nonsignificant trend for higher relative mass and total mitochondrial content of the semitendinosus following exercise training (Fig. 4), which is likely biologically meaningful (power of ANOVA with  $\alpha = 0.05$  was 0.16 and 0.09, respectively, for these two parameters). Mitochondrial volume density ( $V_{mt}$ , f) was significantly higher in both muscles following cold exposure but not exercise training, although the volume density in the TN-trained semitendinosus was intermediate. Lack of significant changes in measures of skeletal muscle structure following exercise are thus likely a result of sample size, given the voluminous literature describing this effect, and emphasize the relative intensity of cold exposure as a stimulus for muscle adaptation in the absence of BAT. The fact that mitochondrial volume density in the sternocleidomastoid is 53% higher compared with 20% higher whole-animal metabolism following cold exposure is of particular interest. This muscle is a fairly thin strap muscle that lies in close proximity to the carotid arteries of the neck; thus, it may be that an increased metabolic machinery in this muscle could serve to heat the blood flowing to the head, thus ensuring that brain temperature will be maintained.

Muscle mass composed about 32% of body mass. This is lower than the values described for numerous placental mammals (Lindstedt and Schaeffer 2002). It is interesting to speculate whether this may be a characteristic of marsupials in general, but data are lacking. Although muscle composes a lower percentage of body mass, it remains the most abundant tissue. Like the semitendinosus, whole-animal skeletal muscle, mitochondrial volume was nearly 30% higher after cold exposure, whereas total muscle mitochondrial content was 19% higher, nearly identical to the difference in whole-animal  $\dot{V}O_{2max}$  and  $\dot{V}O_{2nummit}$  (17% and 20%, respectively).

Although muscle appears to be fully capable of aerobic activity in excess of the demand created by cold exposure, we cannot rule out the contribution of other tissues in this process. The hearts of the cold-acclimated animals were significantly larger and may significantly contribute to resting heat production. Further, see the article by Villarin et al. (2003) for the potential role of the liver in thermogenesis.

*Monodelphis domestica* showed significant alteration of structure and function in skeletal muscle in response to cold; thus,

without the influence of BAT, muscle appears to be recruited for the task of thermogenesis. The relative contribution of shivering versus nonshivering thermogenesis remains unexplored in these animals, although norepinephrine does not stimulate thermogenesis (Dawson and Olson 1988), suggesting that shivering may be important. There remains considerable controversy as to whether muscle nonshivering thermogenesis plays a significant role in thermogenesis. Indeed, whether uncoupling proteins found in skeletal muscle mitochondria have a thermogenic function similar to uncoupling protein 1 in BAT is unresolved (Cline et al. 2001; Cadenas et al. 2002), and putative uncoupling protein homologs in this species have not yet been described. Like marsupials and larger placental mammals, avian species also lack BAT, and there are numerous studies describing the importance of both shivering and nonshivering thermogenesis in avian skeletal muscle during thermogenesis (e.g., Barre et al. 1985; Duchamp and Barre 1993). Thus, increased skeletal muscle aerobic capacity is likely a general response in those species where the need exists (i.e., because of the lack of another primary thermogenic organ).

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