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Chronic cold exposure increases liver oxidative capacity in the marsupial *Monodelphis domestica*

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Abstract

Marsupials lack brown adipose tissue, and therefore rely exclusively on other tissues for thermogenesis. To determine the magnitude of phenotypic plasticity of the liver in response to changing metabolic demand, gray short-tailed opossums (*M. domestica*) were exposed to thermoneutral (28 °C) or cold (9–12 °C) conditions continuously for 6 weeks. Half of each group was also endurance trained with a treadmill program during their respective temperature exposure. Mass specific summit metabolism ($\dot{V}O_2$summit) increased 11% following cold acclimation, though there was no significant main effect by training on $\dot{V}O_2$summit. To estimate the contribution of the liver to whole animal oxidative activity, we determined liver mass, mitochondrial volume density, and total mitochondrial volume. Relative liver mass was 48% greater in cold-acclimated animals, whereas training had no effect on liver mass. The stereological analysis of hepatocyte ultrastructure suggests the percentage of intracellular volumes remained unchanged in response to either aerobic challenge. Thus, following cold-acclimation, there is a 20% increase in the total mitochondrial volume of the liver. This increase could account for nearly half (44%) of the observed increase in whole animal $\dot{V}O_2$summit following cold exposure.
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1. Introduction

Endothermic animals must produce metabolic heat to remain normothermic during periods of cold exposure. Because small animals have the highest ratio of heat-dissipating surface area to heat-storing volume, it is not surprising that they have evolutionarily retained (or developed) a separate tissue for thermogenesis. In small placental mammals (<10 kg), this thermogenic task is primarily accomplished by brown adipose tissue (BAT). BAT is widely accepted as the primary effector organ of non-shivering thermogenesis in eutherian mammals (Foster and Frydman, 1978), and has also been determined to play a role in other functions such as a caloric sink following overfeeding (Smith and Horwitz, 1969).

However, unlike placental mammals, BAT is lacking in marsupials and monotremes (Hayward and Lisson, 1992). While marsupials generally have a basal metabolic rate that is lower than comparably sized eutherian mammals, they dem-

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onstrate a large metabolic scope in response to the aerobic challenge of acute cold exposure (Dawson and Dawson, 1982; Dawson and Olson, 1988). The magnitude of this scope is equal to or greater than that of similarly sized placental mammals (Lechner, 1978). Thus, in a marsupial such as the gray short-tailed opossum (*M. domestica*), the lack of BAT would suggest that other tissues must contribute to the thermogenic effort to a greater degree than is seen in taxa possessing BAT.

As skeletal muscle makes up nearly 40% of the body mass and is aerobically active, it is a primary contributor to thermogenesis during cold exposure via both shivering and non-shivering thermogenesis (NST) (Block, 1994). In rodents, the initial reliance on shivering thermogenesis is replaced over time with NST by the BAT (Sellers et al., 1954). In contrast, the contribution of muscle to thermogenesis in animals lacking BAT likely persists over time. The role of muscle in this system of cold-acclimation is addressed in Schaeffer et al. (in press); however, it may not be the sole contributor.

Because, liver is one of the largest and most metabolically active organs in mammals, it also has the potential to contribute to the thermogenic response. There has been some uncertainty as to the involvement of the liver in non-shivering thermogenesis. Some reports indicate that liver is involved in heat production (Baconnier et al., 1979; Stoner, 1973), but others report it has no contribution to visceral thermogenesis (Depocas, 1958, 1960). Under basal metabolic conditions, the liver has been shown to contribute 25% of total heat production (Baconnier et al., 1979). The high metabolic capacity of liver, as well as its role in basal heat production, suggests that its contribution to whole body adaptation to cold exposure warrants further examination.

The present study addresses the potential thermogenic role of the liver following chronic cold acclimation and endurance exercise training, separately or combined, in the gray short-tailed opossum. Chronic cold exposure represents a constant moderate-intensity perturbation of energy balance, while endurance exercise training represents an intermittent high-intensity perturbation. Our approach utilized measurements of whole animal oxidative metabolism during acute cold exposure ($\dot{V}O_2$summit) and liver morphology to assess whether acclimatization to cold induces alterations in structural components, which could be linked to whole animal oxygen consumption. We thus asked the following questions: Do structural changes occur in liver gross anatomy and ultrastructure that may contribute to increased oxidative capacity following chronic cold exposure? What portion of increased oxygen consumption ($\Delta\dot{V}O_2$summit) could be attributed to any increase in liver oxidative capacity? Are similar changes seen following another aerobic stimulus, endurance exercise training?

2. Materials and methods

2.1. Animals

Forty-eight male short tailed opossums, *M. domestica* (94.2 ± 16.8 g, range: 64–130 g), were obtained from the colony maintained at Southwest Foundation for Biomedical Research (San Antonio, TX 78228-0147, USA). The animals were housed individually in hanging clear lexan cages with wire roofs, cellusorb bedding, and environmental enhancements of paper strips and glass jar huts. All animals were fed fox chow (National Fur Foods, New Holstein, WI 53061, USA) and occasional pieces of fruit, had water available ad libitum, and were maintained on a 12:12 h light–dark cycle. Sacrifice was conducted by way of exsanguination from the caudal vena cava, following isoflurane anesthesia.

2.2. Experimental design

Animals were divided into two groups, 'thermoneutral' and 'cold' acclimation, and treated as follows: Thermoneutral acclimated animals (TN) were housed in a thermoneutral ambient temperature (28 °C), while cold acclimated animals (CA) were housed in a cold room below the thermoneutral zone for the duration of 8 week acclimation period. The temperature was set at 19 °C for the first week, then ramped down weekly to 16, 12 and finally 9 °C on week 4 through week 6. As some health problems began to appear, the temperature was increased to 12 °C for the final 3 weeks. Approximately half of the animals from each temperature acclimation group were also subjected to an endurance exercise training protocol, thus producing subsets of endurance trained (TE, CE) and sedentary (TS, CS) within the temperature groups. Sedentary animals were famil-

iarized with the treadmill (to determine $\dot{V}O_2$max, reported in Schaeffer et al., in press), but did not train daily. The endurance-trained group was exercised on a motor-driven treadmill, inclined to 10%, five times per week for 8 weeks. The speed and duration of running were increased over the course of the first 2 weeks. Initially, animals ran for 30 min/day at 10 m/min. After one week, this was increased to 45 min and 15 m/min and at the beginning of week three, speed was increased to 20 m/min. All animals ran for 45 min, 20 m/min up a 10% incline for the remaining 6 weeks. Animals that were not cooperative in the training were removed from the study.

2.3. Summit metabolism

Summit metabolic rate, $\dot{V}O_2$summit, is the highest metabolic rate that can be elicited in a resting animal by a cold stimulus, as defined by the Commission for Thermal Physiology of the International Union of Physiological Sciences (IUPS, 1987). Functionally, summit metabolism is the maximal rate of whole body oxygen consumption during an intense acute cold exposure. As such, this measure represents a dynamic non-steady state condition, allowing for comparisons across individuals or groups of animals regardless of differences in body heat flux. Summit metabolism provides insight into the adaptive capacity for whole animal response to cold, rather than locomotory system specifically as is the case with $\dot{V}O_2$max measurements. To measure $\dot{V}O_2$summit, animals were placed in a plastic open flow metabolic chamber (0.85 l), which was submerged in a constant temperature bath set to 0 °C. Positive pressure gas flow consisted of heliox gas (20.70% oxygen, 79.30% helium) at a rate of 2.37–2.47 l/min. The gas was pre-cooled by passing through a section of copper tubing in an ice bath, prior to entering the summit metabolism chamber. Heliox gas increases thermal conductivity, which together with the ambient temperature of 0 °C results in an effective chamber temperature of air chilled to approximately −20 °C (Rosenmann and Morrison, 1974). Oxygen content of expired air was determined using an electrochemical oxygen analyzer (Applied Electrochemistry SA3, Pittsburg, PA, USA). Animals remained in the cold chamber for 5 min. Using the method of Fedak et al. (1981), the system was calibrated following every second or third measurement and $\dot{V}O_2$ was calculated using the oxygen content of the last minute, during which O_2 concentration had reached a steady state value.

2.4. Mitochondria volume density

Immediately following sacrifice of the animals, whole livers were extracted, weighed, and small samples were fixed for electron microscopy. Initial fixation was done by cutting the tissue into small blocks no larger than 2×2×5 mm, which were then immersed into 6.25% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Fixed tissue was stored at 4 °C until prepared for TEM by washing with 0.1 M sodium cacodylate buffer, trimmed to 0.5×0.5×1 mm, and stored at least overnight in 0.1 M sodium cacodylate buffer at 4 °C to wash out residual glutaraldehyde. The process for en-bloc staining and tissue embedding, modified from Hoppeler et al. (1984), is as follows: two 30 min washes in sodium cacodylate buffer, post-fixation with 1% OsO_4 in sodium cacodylate buffer, three 30 min washes in sodium cacodylate buffer, three 5 min washes in 0.05 M maleic acid buffer, en-bloc staining with 0.5% uranyl acetate in maleic acid, three 5 min washes in 0.05 M maleic acid buffer, dehydration series of 50, 70, 95 and 3×100% ethanol washes for 10 min each, three 10 min washes of propylene oxide, infiltration of 50/50% Embed 812 resin/propylene oxide overnight, 75/25% Embed 812/propylene oxide for 4 h, and pure Embed 812 overnight. The tissue blocks were laid into embedding molds, filled with fresh resin, and placed into a 60 °C oven overnight. Thin sections (70 nm) were cut, post-stained with saturated uranyl acetate and lead citrate (100 mg per 20 ml NaOH solution-pH≈10), and viewed at 4000× on a JEOL TEM. From each animal, six randomly chosen sites were photographed. TEM negatives were projected onto a C64 stereology grid (26 000× final magnification), to determine mitochondrial volume density following the method of Weibel (1979). Point counting and calculations were done using the computer program *Step-One* (Wainschtein and Cruz-Orive, 1994).

2.5. Calculated values

Mitochondrial volume density represents the dimensionless fraction (volume of mitochondria/

volume of liver) of intracellular space that is occupied by mitochondria. It is assumed that all mitochondria of the tissue, independent of treatment group, are equivalent in terms of their internal structure. The product of mean hepatocyte mitochondria volume density ($Vv_{mt,l}$), total mass of the liver tissue (M_{lvr}), and density of liver (1.051 g ml^{-1}) (Overmoyer et al., 1987), yields the total mitochondrial volume in the entire liver.

$$(V\nu_{mt,l})\cdot(M_{lvr}(g))\cdot(1.051\ g\cdot ml^{-1})^{-1} = (\text{Total Mt volume (ml)}) \quad (1)$$

Given the total volume of mitochondria in the entire liver of an individual animal, the potential for oxidative capacity of the entire liver can be estimated. In muscle tissue across many mammalian species this maximum rate of oxygen consumption is approximately 4.7 ml $O_2\cdot min^{-1}$/ml of mitochondria (Hoppeler and Lindstedt, 1985). Mitochondrial oxygen consumption is a function of the total surface area of mitochondrial inner membrane. As the cristae surface area of liver mitochondria is about half that of muscle (Schwerzmann et al., 1986) we can estimate the maximum liver mitochondrial oxidative capacity also to be half per unit volume of mitochondria (Schaeffer et al., 1996). Hence, liver oxidative capacity ($\dot{V}O_{2\ lvr}$) equals:

$$(\text{Total Mt volume (ml)}) \cdot\left(\frac{4.7}{2}\text{ml } O_2\cdot ml_{Mt}^{-1}\cdot min^{-1}\right) = \dot{V}_{O_2lvr}\ (ml\cdot min^{-1}) \quad (2)$$

Once this value is calculated, the potential contribution of the liver to the change in whole animal oxidative capacity following experimental treatments can be calculated as the ratio fraction of the change in liver oxidative capacity divided by the change in whole animal summit metabolism. This value is an indicator of the potential magnitude of hepatic contribution to the acclimative response.

$$\frac{\Delta\dot{V}_{O_2lvr}}{\Delta\dot{V}_{O_2summit}} = \text{\% of whole body oxidative capacity change potentially due to liver} \quad (3)$$

2.6. Statistical analysis

Across group comparisons were performed using 2-way analysis of variance (ANOVA) with acclimatization temperature and exercise training as the independent variables; pairwise multiple comparisons (Tukey test) were performed when appropriate. SIGMASTAT™ version 2.03 (SPSS) was used for all statistical analyses. Significance levels for all tests were set to $\alpha = 0.05$. Mean values ± the S.E. of the mean and the number of observations is reported for all comparisons.

3. Results

3.1. Physiological measures

Mass specific summit metabolism was significantly increased in response to chronic cold exposure, but not exercise training (2-way ANOVA, temperature effect $P < 0.05$, exercise effect $P = 0.10$; Table 1). When animals from both thermoneutral acclimated groups (TN) are compared to all animals from both cold acclimated groups (CA), the cold acclimated animals show a 11% increase in summit metabolism (*t*-test $P < 0.01$; Fig. 1). Exposure to both cold acclimation and endurance exercise training did not result in an additive effect, as the three treated groups did not differ from one another. Since exercise had no significant effect, data have been combined into thermoneutral and cold acclimated groups and are presented as such throughout the paper.

3.2. Anatomical measures

3.2.1. Liver mass

The absolute liver masses of cold acclimated animals were 34.5% larger than in thermoneutral acclimated animals (2-way ANOVA, temperature effect $P < 0.001$, exercise effect ns; Table 1). In addition, relative liver mass (liver mass/body mass) was significantly increased in the CA animals (2-way ANOVA, temperature effect $P < 0.001$, exercise effect ns; Table 1). The comparison of the relative liver mass from the combined TN and CA groups revealed a 48% increase (Fig. 2). There was no effect of exercise on absolute or relative liver mass (Table 1). Visual inspection of the gross anatomy of the livers did not indicate obvious pathologies (e.g. fatty lesions) on the larger livers of the cold acclimated animals.

3.2.2. Mitochondria volume density

Stereological analysis of liver cell ultrastructure indicated no significant difference in hepatocyte

Table 1

Summary data of measured and calculated variables for the four treatment groups (TS, thermoneutral sedentary; TE, thermoneutral exercise-trained; CS, cold-acclimated sedentary; CE, cold-acclimated exercise-trained) and the combined temperature acclimation groups (TN=TS+TE, CA=CS+CE) following significance of temperature effects in the 2-way ANOVA

	TN	CA	TS	TE	CS	CE
Body mass (g)	97.24 (3.99)	88.11 (4.17)	97.57 (6.02)	96.82 (5.37)	90.80 (3.86)	86.32 (6.67)
Summit metabolism (ml/min)	8.37 (0.41)	8.96 (0.32)	7.77 (0.51)	9.07 (0.59)	9.07 (0.20)	8.86 (0.59)
Specific summit metabolism (ml/kg min)*	85.95 (2.89)	96.26 (2.06)[‡]	80.40 (2.74)[a]	92.42 (4.15)[a,b]	96.17 (1.74)[b]	96.33 (3.67)[b]
Absolute liver mass (g)*	2.678 (0.108)	3.602 (0.182)[‡]	2.792(0.179)[a]	2.615 (0.107)[a]	3.777(0.217)[b]	3.484(0.271)[b]
Relative liver mass (g lvr/g body,%)*	2.791(0.081)	4.132 (0.201)[‡]	2.831 (0.107)[a]	2.741 (0.130)[a]	4.171 (0.229)[b]	4.101 (0.337)[b]
Mitochondrial volume density (%)	19.241 (0.842)	17.365 (0.908)	19.426 (1.309)	19.029 (1.116)	15.453 (1.223)	18.640 (1.032)
Total hepatic Mitochondrial volume (cm^3)	0.540 (0.035)	0.649 (0.032)[‡]	0.558 (.057)	0.519 (0.040)	0.614 (0.058)	0.672 (0.038)
Potential oxidative capacity of liver (ml/min)	1.268 (0.082)	1.525 (0.075)[‡]	1.311 (0.134)	1.219 (0.093)	1.442 (0.137)	1.580 (0.089)

Exercise effect was not significant for any of these variables. *2-way ANOVA, temperature effect $P<0.05$; [‡] *t*-test (TN vs. CA), $P<0.05$; [a,b]Tukey post-hoc pairwise comparisons, $P<0.05$. All values are mean (S.E.M.).

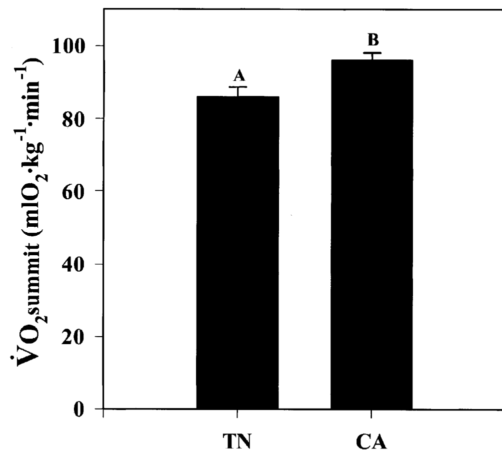

Fig. 1. Summit metabolic rate ($\dot{V}O_2$summit) increased by 11% following chronic acclimatization to cold (CA) temperature compared to animals maintained in thermoneutral (TN) temperature. Values are means+S.E.M. determined during a severe acute cold challenge, as described in the text. Different letters denote significance ($P<0.05$; $n_{TN}=13$, $n_{CA}=9$).

mitochondrial volume density in response to the experimental procedures (2-way ANOVA, temperature effect $P=0.10$, training effect $P=0.29$; Table 1). The mean mitochondrial volume density of all four groups was $18.5\pm0.6\%$. The mitochondrial volume densities from the individual animals (range: 13.41–26.95%), together with individual liver masses, were used to calculate total liver mitochondrial volume.

3.3. Calculated values

3.3.1. Total hepatic mitochondrial volume

The factor that determines the respiratory potential of the liver is neither the mitochondrial volume density nor gross liver mass independently, but rather the total volume of liver mitochondria (Eq. (1)). When comparing total liver mitochondrial volume in CA vs. TN acclimated animals, the total liver mitochondrial volume is 20% greater in cold acclimated animals (*t*-test, $P<0.05$; Table 1).

3.3.2. Oxidative capacity potential of the liver

The maximal oxidative capacity of any tissue is a linear function of the total mitochondrial volume (Lindstedt et al., 1988). Using Eq. (2), we calculated liver aerobic capacities of 1.27 ml O_2 min^{-1} for the TN animals and 1.53 ml O_2 min^{-1} for the CA animals (Fig. 3 and Table 1), a 20%

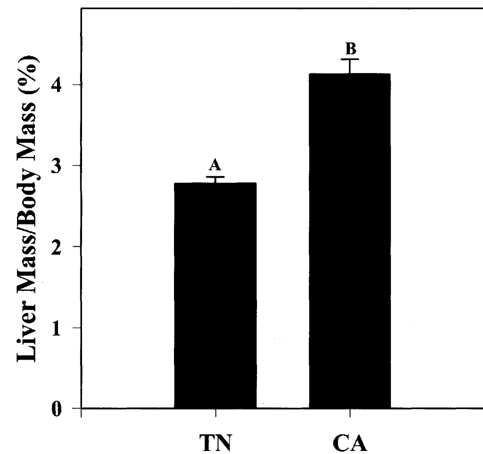

Fig. 2. Weight-specific liver mass increased by nearly 50% in cold acclimated animals. Group and significance labels same as previous figure ($P<0.01$; $n_{TN}=20$, $n_{CA}=10$).

increase in oxidative capacity in the livers of the cold acclimated animals (*t*-test, $P<0.05$).

4. Discussion

Summit metabolism is increased in response to the aerobic challenge of chronic cold exposure. However, the metabolic demand generated by exercise training was not strong enough to induce a significant increase in $\dot{V}O_2$summit. The efficacy

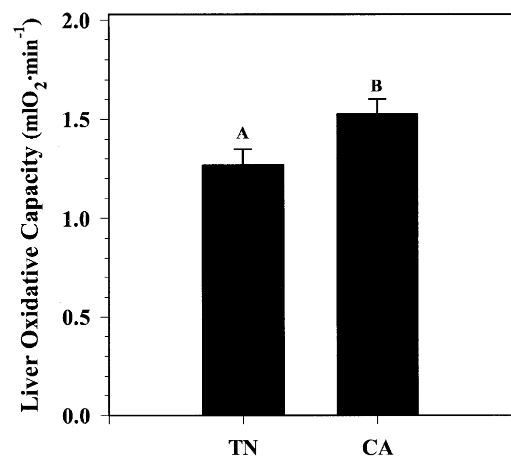

Fig. 3. Oxidative capacity of the liver is increased 20% in CA animals as calculated according to Eq. (2) (see Section 2). Group and significance labels same as previous figures ($P<0.05$; $n_{TN}=15$, $n_{CA}=10$).

of the exercise training protocol as an aerobic stimulus is demonstrated by the 14% increase in $\dot{V}O_2$max in the trained thermoneutral group (data in Schaeffer et al., in press). Further, the effects of these treatments were not additive, the addition of exercise to cold exposure was no different from cold-acclimation alone. This agrees with the increase in $\dot{V}O_2$max, but not $\dot{V}O_2$summit, demonstrated with exercise training in rats (Conley et al., 1985).

During the course of the summit metabolism measurement there are doubtless changes in body temperature in both temperature acclimation groups, yet the $\dot{V}O_2$summit value is confidently taken as a maximum because it represents a non-steady state condition. The body temperature of the TN animals may become lower than the CA animals by some unknown magnitude during the course of the acute cold-challenge, though it was not enough to induce the hypometabolic response associated with undefendable hypothermia (Chappell, 1984).

Skeletal muscle plays a role in adaptive thermogenesis in these animals, but lacking BAT, the liver may also be a key thermogenic organ. Increased oxidative capacity in the liver is not simply a response to greater aerobic activity, as no such adaptation was seen in the exercise trained animals.

Unlike most organs, liver mass is not linearly proportional to body mass, but rather to (body mass)$^{0.85}$, closer to the relationship between metabolic rate and body mass (Lindstedt and Schaeffer, 2002), suggesting a structural coupling between liver mass and metabolic rate. Likewise, the nearly 50% increase in relative liver mass observed in the cold-acclimated animals accompanies an 11% increase in whole animal metabolic capacity. A high aerobic capacity and high blood flow suggest that the liver could play an important role in the thermogenesis of cold exposed animals.

Mitochondrial volume density governs the rate at which a given volume of tissue can produce ATP aerobically. The mean value for liver mitochondrial volume density in our animals was 18.1% (vol/vol), similar to a value of approximately 17.3% in rats (Loud, 1968). Two non-exclusive strategies are theoretically employed to increase the functional capacity of any biological tissue: increase specific activity for a given mass, or increase total tissue mass while maintaining specific activity (Kent et al., 1988). For the liver aerobic capacity of these cold-acclimated animals, the latter strategy appears to dominate. There are limitations to the plasticity of relative intracellular volumes, which may act to impede exclusive proliferation of mitochondrial volume density. Optimization and symmorphosis theories, when extrapolated to the cellular level, suggest that function of a system may be optimized when components are present and operating within a specific range of relative capacities (Lindstedt et al., 1998). Oxidative capacity is only one measure of liver function; there are many functions of the liver that take place in subcellular locations other than the mitochondria.

Calculation of the total mitochondrial volume of the liver (Eq. (1)) indicates a greater oxidative capacity of the liver following cold acclimation. The increase in total liver mitochondrial volume in response to cold-acclimation was independent of exercise training, although in rodents exercise training alone is sufficient to increase liver aerobic metabolism (Tate et al., 1982; Wasserman and Cherrington, 1991). An increase in total liver mitochondrial volume in response to cold-acclimation was also shown in cold exposed rats and hamsters (Horwitz, 1976), and may contribute to whole animal metabolic capacity (Jansky, 1966). In ducks (which, like marsupials, also lack BAT as the site of non-shivering thermogenesis), liver oxidative capacity was increased 40% following cold acclimation (Goglia et al., 1993). However, whole body muscle mitochondrial content increased slightly following either exercise training or cold-acclimation, but a significant increase was only seen with the compound pressures of cold-acclimation with exercise training (data in Schaeffer et al., in press).

Following cold-acclimation, we calculated that the estimated oxidative capacity of the liver increased from 1.27 to 1.53 ml O_2/min, while summit metabolism increased from 8.37 to 8.96 ml O_2/min, 0.26 to 0.59 ml O_2/min, respectively, (Fig. 4). Thus, the liver alone has the capacity to contribute 44% of the increase observed in whole body summit metabolism following cold acclimation. This large potential contribution implies that up regulation of liver oxidative capacity plays an important thermoregulatory role during chronic cold challenge (Baconnier et al., 1979; Janský,

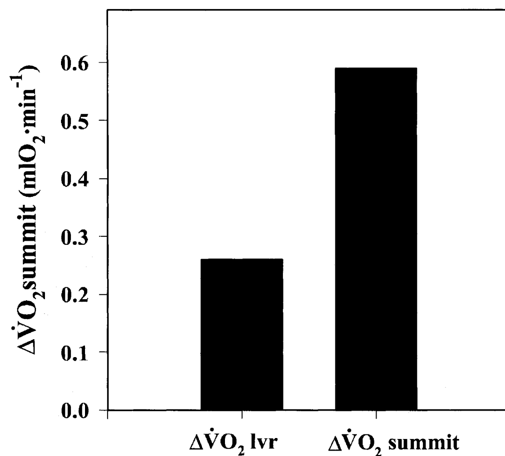

Fig. 4. The absolute increase in potential oxidative capacity of the liver represents approximately 44% of the observed increase in whole animal $\dot{V}O_2$summit after cold acclimation (see Eq. (3) in Section 2).

1973). While any increase in metabolic demand on the liver to support overall energy metabolism will contribute to hepatic heat production, these results demonstrate that the capacity for heat production by the liver can also be actively modified, supporting a role for liver thermogenesis during cold acclimation in marsupials.

Liver heat production can be greatly modified apart from changes in mitochondrial volume. Mechanisms of heat generation include uncoupling of oxidative phosphorylation and futile cycling of substrates. Horwitz (1976) found uncoupling of liver mitochondria at low incubation temperatures in vitro. In addition, to uncoupling protein 1 (UCP1) in BAT, other putative uncoupling proteins have been described, including UCP2 in mammalian liver (Dulloo and Samec, 2001; Jekabsons et al., 1999). Additionally, non-specific proton leak may account for 20–30% of total hepatic oxygen consumption (Rolfe et al., 1999), and the plasticity of this system remains unexplored. Other systems exist, such as futile cycling of calcium that contributes to heat generation in muscles of cold acclimated ducklings (Dumonteil et al., 1993) and in the modified muscle cells that form a 'heater organ' in certain scombrid fish species (Block, 1994).

In addition to uncoupling processes and futile cycles, liver function is also mediated by hormonal mechanisms. In marsupials, unlike rodents, activation of thermogenesis is not mediated by norepinephrine (Himms-Hagen, 1989). Rather, activation of heat production must be based on other signaling mechanisms (Hsieh et al., 1957; Dawson and Olson, 1988). Glucagon has also been implicated in thermogenic responses via the release of free fatty acids, which may loosen the coupling of mitochondrial oxidative-phosphorylation in liver and muscle of ducklings (Barré et al., 1989). Still, other hormones may also influence the actions of liver metabolism (Barré et al., 1989; Jansky et al., 1964; Jansky, 1966; Janský, 1995).

The results of this study suggest that the relative magnitude and plasticity of liver metabolic structures have the potential to significantly contribute to the thermogenic efforts of cold acclimated mammals. This is particularly the case in taxa such as the marsupials and large placental mammals that lack brown adipose tissue as the primary effector organ of non-shivering thermogenesis. Although, the exercise training protocol and the chronic cold exposure each represent a significant aerobic challenge, increased oxidative capacity in the liver was only seen in response to cold exposure.

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