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Cost of transport is increased after cold exposure in *Monodelphis domestica*: training for inefficiency

Paul J. Schaeffer[1,2,*], Jason J. Villarin[1,†], David J. Pierotti[1], Daniel P. Kelly[2,3] and Stan L. Lindstedt[1]

[1]*Department of Biological Sciences, Physiology and Functional Morphology Group, Northern Arizona University, Flagstaff, AZ 86011, USA*, [2]*Center for Cardiovascular Research and Department of Medicine* and [3]*Department of Molecular Biology and Pharmacology, and Department of Pediatrics, Washington University School of Medicine, St Louis, MO 63110, USA*

*Author for correspondence (e-mail: pschaeff@im.wustl.edu)

†Present address: Department of Medicine, Human Physiology, University of California, Davis, CA 95616, USA

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Summary

Monodelphis domestica **(Didelphidae: Marsupialia) lacks brown adipose tissue and thus relies on skeletal muscle as its primary thermogenic organ. Following cold exposure, the aerobic capacity of skeletal muscle in these animals is greatly increased. We investigated the effects of this plastic response to thermogenesis on locomotion and muscle mechanics. In cold-exposed animals, cost of transport was 15% higher than in controls but was unaffected by exercise training. Twitch kinetics in isolated semitendinosus muscles of cold-exposed animals were characteristic of slow-oxidative fiber types. Both time-to-peak tension and half-relaxation time were longer and maximal shortening velocity was slower following cold exposure compared to either thermoneutral controls or exercise-trained animals. Further, muscles from the cold-exposed animals had greater fatigue resistance than either control or exercise-trained animals, indicating greater oxidative capacity. Finally, we identified an uncoupling protein 3 homologue, whose gene expression was upregulated in skeletal muscle of cold-exposed** ***Monodelphis domestica*****. Cold exposure provided a potent stimulus for muscle plasticity, driving a fast-to-slow transition more effectively than exercise training. However, linked to the dramatic shift in muscle properties is an equally dramatic increase in whole animal muscle energetics during locomotion, suggesting an uncoupled state, or 'training for inefficiency'.**

Key words: oxygen consumption, locomotion, thermogenesis, uncoupling protein 3, muscle mechanics, marsupial, *Monodelphis domestica*.

Introduction

Animal locomotion requires that chemical energy be converted through metabolic processes to produce work; metabolic power thus generates mechanical power. The efficiency of this conversion during terrestrial locomotion is a primary determinant of the cost of transport (C_t), a measure of the energy required to move a given body mass a given distance (typically expressed as ml O_2 kg^{-1} m^{-1}; Taylor et al., 1982). The C_t is highly dependent on animal size; small animals must expend more metabolic energy to move each unit of body mass a given distance (Taylor et al., 1982). However, C_t is relatively independent of mode of locomotion, such that bipedal and quadrupedal locomotion have the same cost, except in macropod marsupials (Baudinette, 1991).

It has been generally accepted that C_t is constant within an individual (Holloszy and Coyle, 1984); however, there is a lack of consensus on this issue. There have been numerous studies exploring the effect of exercise training on C_t, testing the hypothesis that training would result in a reduced C_t. The results have been somewhat equivocal, with some evidence supporting the traditional view that C_t is not responsive to training (e.g. Patch and Brooks, 1980; Bailey and Messier, 1991), while others have found a significant, albeit small, decrease in the aerobic demand of running in elite humans (Morgan et al., 1995).

Although studies of the malleability of cost of transport are equivocal, numerous studies have demonstrated that the muscle metabolic system that supplies energy substrates to power locomotion is highly plastic. Alterations in activity result in profound adaptive changes in the oxidative machinery within the muscle fibers (Hoppeler, 1986; Rome and Lindstedt, 1997). Similarly, expression of contractile proteins is highly responsive to changes in the mechanical activity of the muscle (Schiaffino and Reggiani, 1996; Goldspink, 1999). The cost of transport is relatively invariant in the face of these adaptive responses, which suggests that although the cellular components of energy supply and utilization are very

responsive to changing activity patterns, both appear to be maintained in close functional coupling. The basic pattern is set by body size constraints, as indicated by the similar body size scaling of the time-dependent properties of these systems (Lindstedt and Thomas, 1994).

In addition to locomotion, skeletal muscle is an important source of metabolic heat during cold stress. There has been little attention given to the effects of muscle thermogenesis upon the energetics of locomotion. Like exercise, chronic cold exposure is a cause of increased metabolic demand, to support thermogenesis and maintain temperature homeostasis. In small placental mammals, brown adipose tissue (BAT) is responsible for the majority of the increased thermogenic capacity (Foster and Frydman, 1976). However, only placental mammals weighing less than ~10 kg possess this tissue (Heldmaier, 1971), thus many animals, including adult humans, must rely upon other tissues for thermogenesis. At approximately 40% of body mass (Lindstedt and Thomas, 1994), skeletal muscle is pre-adapted to supply considerable metabolic heat. In many species lacking BAT, cold acclimation has led to structural alterations in muscle resembling those resulting from exercise. Specifically, cold acclimation in ducks led to increased capillary density and a shift to slow-oxidative muscle fiber types (Duchamp et al., 1992). Increased cytochrome oxidase activity was observed in ducks (Barre et al., 1987), king penguins (Duchamp et al., 1991) and pigs (Berthon et al., 1996). We previously reported an increase in muscle mitochondrial volume in short-tailed opossums, *Monodelphis domestica* (Schaeffer et al., 2003). In contrast, in rodents possessing BAT, cold acclimation does not lead to alterations in muscle structure (Barre et al., 1987, Hoppeler et al., 1995).

Functionally, whole animal running at maximum rate of oxygen consumption $\dot{V}_{O2max}$ is greater in both cold-exposed goats (Schaeffer et al., 2001) and *Monodelphis domestica* (Schaeffer et al., 2003) compared to animals maintained in thermoneutral conditions. Winter-acclimated deer mice (which possess BAT) showed an increase in thermogenic capacity, but only a slight increase in running $\dot{V}_{O2max}$ (Hayes and Chappell, 1986). Thus while alterations of the metabolic system occur in skeletal muscle in response to cold exposure, little is known about the response of the contractile system. We recently reported that cold-acclimated goats had apparently higher C_t (measured at $\dot{V}_{O2max}$; Schaeffer et al., 2001), suggesting that increased capacity for muscle thermogenesis may be acting as 'training for inefficiency'. This observation could be explained in part if mitochondrial uncoupling processes play a role in muscle thermogenesis. Uncoupling protein 3 (UCP3) is highly expressed in skeletal muscle and, although the physiological function of UCP3 is debated (e.g. Porter, 2001; Nedergaard and Cannon, 2003), in species lacking BAT, a role for UCP3 as a metabolic uncoupler in skeletal muscle may be unveiled.

To investigate the comparative roles of cold acclimation and endurance exercise training on whole animal running efficiency, we utilized the short-tailed opossum *Monodelphis domestica*, a small (~100 g) marsupial lacking BAT. We recently demonstrated that these animals are reliant upon skeletal muscle thermogenesis following cold exposure, increasing both whole animal $\dot{V}_{O2max}$ and $\dot{V}_{O2summit}$ (maximum $\dot{V}_{O_2}$ during cold exposure) as well as muscle mitochondrial volume density (Schaeffer et al., 2003). We thus utilized these animals to ask the following questions. (i) How do muscle responses to exercise training and cold exposure impact cost of transport? (ii) To what extent do alterations of muscle contractile properties in response to cold exposure resemble or contrast those associated with exercise? (iii) Is there any activation of gene expression in response to exercise training or cold exposure of an uncoupling protein 3 homologue in skeletal muscle of *Monodelphis domestica*?

Materials and methods

Animals

Twenty-two male *Monodelphis domestica* Wagner 1842 (Marsupialia: Didelphidae), were used in this study. All animals were 2.5 months of age and weighed >45 g at the start of the study. Animals were obtained from the colony maintained at the Southwest Foundation for Biomedical Research (San Antonio, TX, USA) and maintained as described previously (Schaeffer et al., 2003). 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

For the experimental protocol, animals were divided into four groups, which were treated as follows. Two groups were maintained at thermoneutral ambient temperature (28°C; Dawson and Olson, 1988), one of which was exercise-trained (see below) on a motorized treadmill. These groups are designated 'Thermoneutral, sedentary' (TnS) and 'Thermoneutral, trained' (TnT). The second two groups were cold-exposed (see below) and again, one of these was exercise-trained on a motorized treadmill. These groups are designated 'Cold, sedentary' (CS) and 'Cold, trained' (CT).

Exercise training was performed at constant speed while running up a 10% incline. During the first week, every animal (including sedentary groups) ran on the treadmill at 10 m min^{-1} for 5 min each day for 3 days to gain familiarity with the apparatus, necessary for subsequent testing. Thereafter, sedentary animals ran once (again at 10 m min^{-1} for 5 min) during the eighth week of the experimental period. The animals that underwent endurance exercise training ran for 30 min at 10 m min^{-1} for 5 days during the second week. This was increased to 45 min at 15 m min^{-1} for 5 days during the third week and then to 45 min at 20 m min^{-1}, 5 days/week for the remaining 6 weeks duration of the experiment.

Cold exposure entailed being housed in a cold room at 19°C for 1 week. The temperature was then decreased to 16°C at the

beginning of the second week, to 12.5°C for the third week, to 9°C for the fourth through sixth weeks and finally to 12°C for the final 3 weeks.

Measurement of oxygen consumption

Upon completion of the experimental procedures, we measured rates of oxygen consumption $\dot{V}_{O_2}$ while running on a level, motorized treadmill at 10, 15, 20 and 25 m min^{-1} at ambient temperature of 28°C using an open flow system, as described previously (Schaeffer et al., 2003). Animals performed these tests beginning the day following completion and within 5 days of the end of the experimental protocol, running at one speed per day. During this period, animals were maintained at their acclimation temperature and also performed the $\dot{V}_{O_2max}$ and $\dot{V}_{O_2summit}$ tests described in Schaeffer et al. (2003). Each exercise test consisted of ~20 min running at a constant speed and energetic cost of transport (C_t) was determined using the average value from the final 5 min of steady state recording. C_t was calculated as the ratio of rate of oxygen consumption (in ml O_2 $kg^{-1}min^{-1}$) over treadmill speed (m min^{-1}), yielding the oxygen consumed per unit of mass per distance traveled (ml O_2 kg^{-1} m^{-1}). Treadmill speed was calibrated before every run and the O_2 system was calibrated after every second or third run using the N_2 dilution method of Fedak et al. (1981). All measurements of oxygen consumption took place at an elevation of 2100 m (ambient P_{BAR}=600 torr; 1 torr=133.3 Pa) and data are reported after correction to STPD values.

Isolated muscle preparation

Within 2–4 days after completion of the C_t measurements (during which animals were maintained at acclimation temperatures), animals were anesthetized with isoflourane and the semitendinosus muscle was removed from one leg. Prior to muscle removal, silk suture was tightly tied about the two ends of the muscle. Upon removal, the muscle was suspended in a Krebs solution (118 mmol l^{-1} NaCl, 4.7 mmol KCl, 2.5 mmol $CaCl_2$, 1.18 mmol $MgSO_4$ and 1.18 mmol KH_2PO_4, with 2.1 g l^{-1} $NaHCO_3$ and 2.0 g l^{-1} dextrose added before use), which was aerated with 95% O_2 and 5% CO_2. The muscle and solution were held within a double-walled testing chamber maintained at 32°C (normal body temperature for this species) with a refrigerated constant temperature circulating water bath (Model 1165, VWR, West Chester, PA, USA). The muscle was anchored to a bar in the lower part of the testing chamber and to a transducer system above, containing both a force and displacement gauge (Cambridge Technology Inc., Cambridge, MA, USA; Model 6650). Muscles were activated using a field stimulator (Grass Telefactor, West Warwick, RI, USA; Model S48) with the signal passing through a stimulus isolation unit (Grass Telefactor; Model S1U5) to platinum electrodes (1 cm×2 cm) placed across the muscle. After the semitendinosus had been removed, the animals were killed by exsanguination and skeletal muscle samples removed and frozen in liquid nitrogen as previously described (Schaeffer et al., 2003).

Measurement of mechanical properties

After the muscle was suspended within the apparatus, we first found the minimum voltage that elicited peak force generation (maximal recruitment) of a single twitch, then set the remainder of the stimulations to this voltage plus 20 V (typically about 100 V). We then determined the optimal length and frequency of stimulation for the muscle, both measured when a single twitch generated maximal force. These data were monitored using a digital oscilloscope (Tektronix Inc., Beaverton, OR, USA; TDS 340A) and the muscle was allowed to recover for at least 15 s between each stimulation. For both twitch and tetanus data, the transducer output was processed through an Aurora Scientific Muscle Lever System (Aurora Scientific Inc., Aurora, Ontario, Canada; Model 305B) and recorded digitally using SuperScope2™, ver. 2.17 sampling at 2000 Hz.

The muscle was then subjected to 4–6 twitches, with the lever set to maximum resistance to motion (isometric contractions), each a 2 ms single square wave pulse, for determination of time to peak tension (TPT), and half-relaxation time ($\frac{1}{2}$RT) of a single twitch. To determine the time required to generate each unit of force, as well as the time required for each unit of force decay, we divided TPT and $\frac{1}{2}$RT by the peak force (in g) for each twitch. These data are reported in ms g^{-1}.

Next, the stimulator delivered a single 200 ms train of 2 ms pulses at 200 Hz to elicit a tetanus. Two to three tetani were imposed to determine peak tetanic tension ($\mathbf{P}_0$). At this point, optimal length was checked and adjusted if necessary. We then collected a series of isotonic tetani with decreasing load to determine maximal contraction velocity; typically this generated 10–15 points. The muscle was allowed to recover for at least 2 min between each tetanus. The recordings of tetanic contractions from $\mathbf{P}_0$ to minimal loading were used to calculate v_{max} using the Hill equation described in Roy et al. (1984). Force and speed (dL/dt) were determined for each point, then plotted as $y=(\mathbf{P}_0-\mathbf{P})/v$ *vs* $x=\mathbf{P}$, where $\mathbf{P}$ is the measured force and v is the calculated speed. A regression line was fit to the data with less than 35% $\mathbf{P}_0$. v_{max} was calculated as $\mathbf{P}_0$ divided by the y-intercept extrapolated from the regression analysis.

Finally, the muscle was subjected to a fatigue test under isometric conditions consisting of repeating trains of stimuli at 1 s^{-1} at 40 Hz for 2 min, with forces being recorded in real time from the oscilloscope. Each train lasted 330 ms, thus the muscle was activated for $\frac{1}{3}$ s and allowed to recover for $\frac{2}{3}$ s. The highest force generated (usually the 4^{th}–6^{th} tetanus) was recorded, as was the force generated by the last tetanus. The muscle fatigue index (a measure of muscle endurance) was calculated as the ratio of final force generation divided by peak force generation during the fatigue test. This value was multiplied by 100 to give the percent of force generation that remained after the stimulation protocol.

Amplification and sequencing of the Md-UCP3 cDNA

Total RNA was isolated from *Monodelphis domestica*

hindlimb muscle using the RNAzol method (Tel-Test, Inc., Friendswood, TX, USA). Following reverse transcription, PCR was performed using primers designed based on sequence homology with the UCP3 gene of cow, pig, dog and rat as follows: sense, 5′-GGCCCCCGCAGCCCCTACAACGG-3′; antisense, 5′-CTGGACTTTCATCAAGGCCCGTTTCA-3′. PCR cycles were as follows: 95°C for 5 min; 95°C for 1 min, 52°C for 2 min, 72°C for 3 min, 30 cycles. The product was electrophoresed, gel isolated and cloned into the TOPO-TA plasmid (Invitrogen, Carlsbad, CA, USA). The plasmid was transformed into *E. coli* cells, amplified and sequenced by the Protein and Nucleic Acid Chemistry laboratory at Washington University School of Medicine using the Big Dye cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The resultant cDNA fragment is designated Md-UCP3.

RNA blot analyses

15 μg total RNA was electrophoresed in a denaturing agarose gel, transferred to a GeneScreen membrane (PerkinElmer Life Sciences, Boston, MA, USA) and fixed by UV radiation. Northern blot analysis was performed with QuickHyb (Stratagene, La Jolla, CA, USA) using random primed ^{32}P-labeled cDNA probes, derived from the PCR fragment of Md-UCP3. Bands were detected by phosphor-imaging using a GS 525 Molecular Imager System (Bio-Rad Laboratories, Hercules, CA, USA).

Statistics

Cost of transport was analyzed using analysis of covariance (ANCOVA), with running speed as the covariate. Muscle contractile parameters were tested for statistical significance using two-way analysis of variance (ANOVA), with temperature and training status as the dependent variables, using Sigma-Stat (version 2.03, SPSS Inc., Chicago, IL, USA). For those parameters that showed significant differences, pairwise comparisons were run using the Student–Neuman–Keuls method. The level of significance was set at $P<0.05$ in all cases. Data are reported as means ± standard error of the mean (S.E.M.).

Results

Cost of transport

Both of the cold-acclimated groups showed significantly increased C_t (ANCOVA, $P<0.0001$), consuming on average 15% more oxygen during locomotion than the thermoneutral acclimated controls running at the same speed (Fig. 1). Similar to previous reports, we found that exercise training had no significant effect on C_t regardless of acclimation temperature. Regression analysis utilizing the combined sedentary and trained data from each temperature group yielded the following equations for cost of transport, expressed as rate of oxygen consumption ($\dot{V}_{O_2}$, ml O_2 kg^{-1} min^{-1}) relative to running speed (v, m min^{-1}). For animals acclimated to thermoneutral conditions $\dot{V}_{O_2}=1.21v+47.20$, and for cold-acclimated animals $\dot{V}_{O_2}=0.89v+62.84$. The higher slope for the thermoneutral animals is such that the magnitude of difference in C_t decreases with increasing speed (such that $\dot{V}_{O_2}$ of cold-acclimated animals was 17.6% greater at 10 m min^{-1} but only 7.7% greater at 25 m min^{-1}) and would theoretically disappear while animals run at about 50 m min^{-1}, nearly the speed of running at $\dot{V}_{O_2max}$.

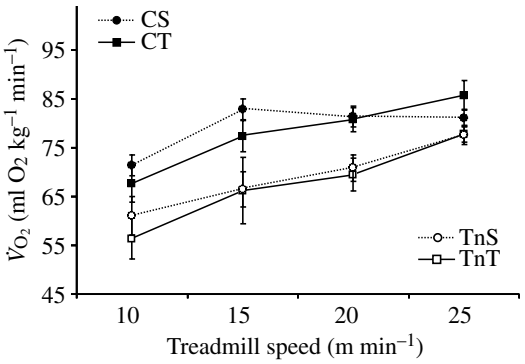

Fig. 1. Cost of transport during locomotion. Using an open flow metabolic system, $\dot{V}_{O_2}$ was measured as a function of running speed on a motorized treadmill. The cold-acclimated animals had significantly higher O_2 utilization as a function of speed such that on average 15% more oxygen was consumed while running at the same speed as thermoneutral controls. Values are means ± S.E.M. at each speed. CS, cold, sedentary; CT, cold, trained; TnS, thermoneutral, sedentary; TnT, thermoneutral, trained.

Twitch parameters

Force generation of isolated, perfused semitendinosus muscles during a single twitch ($\mathbf{P}_t$) was significantly lower in the cold-acclimated animals, and lowest in the CS group (two-way ANOVA, temperature effect $P<0.01$, training effect $P=0.17$; Table 1). We therefore divided the time to peak tension (TPT) and the half relaxation time ($\frac{1}{2}$RT) by $\mathbf{P}_t$ to determine the time of force generation and relaxation per gram of force developed (TPT_g and $\frac{1}{2}RT_g$, respectively, in ms g^{-1}). The TPT_g was significantly longer in the cold-acclimated but not the exercise-trained animals (two-way ANOVA, temperature effect $P<0.05$, training effect $P=0.10$). The TnS

Table 1. *Single twitch force generation of isolated, semitendinosus muscles*

Experimental groups	Single twitch force (g)
TnS[a]	0.148±0.017 (7)
TnT[a]	0.131±0.017 (7)
CS[a,b]	0.102±0.023 (4)
CT[b]	0.063±0.020 (5)

TnS, thermoneutral, sedentary; TnT, thermoneutral, exercise trained; CS, cold-acclimated, sedentary; CT, cold-acclimated, exercise trained.

Values are means ± S.E.M. (*N*). Different superscript letters indicate significant pairwise differences between groups.

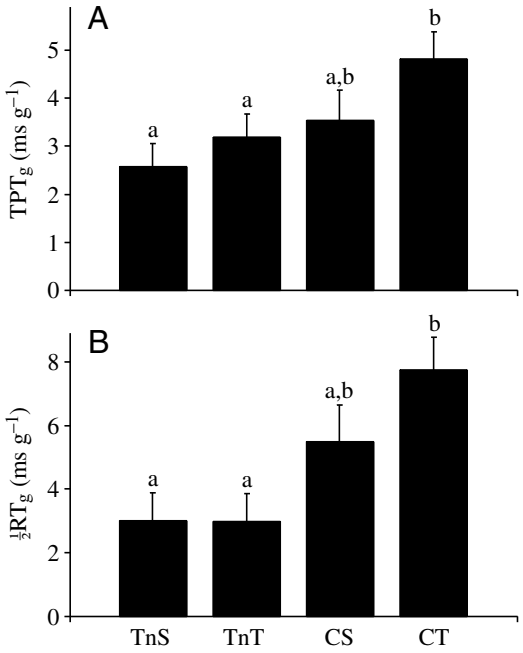

Fig. 2. Kinetics of single twitch contractions. (A) Time to peak tension per gram of force produced (TPT_g) for single twitches of the semitendinosus muscle by the four experimental groups (as in Table 1) was significantly increased by cold acclimation but not by exercise training. There is a trend for increased time required to generate force with either the TnT or CS treatment group, but only those animals subjected to both treatments (CT) showed a statistically significant slowing of twitch speed. (B) Half relaxation time per gram of force produced ($\frac{1}{2}RT_g$) for single twitches by semitendinosus muscles of the four experimental groups. The $\frac{1}{2}RT_g$ was significantly longer in cold-acclimated but not by exercise-trained animals. Both cold groups had a longer relaxation rate; however, *post-hoc* pairwise comparison showed this effect was only statistically significant in the CT group. Values are means ± S.E.M. and different superscripted letters indicate significant pairwise differences between groups. CS, cold, sedentary; CT, cold, trained; TnS, thermoneutral, sedentary; TnT, thermoneutral, trained.

and TnT groups did not differ. The CS group did not differ from either warm group or from the CT group (Fig. 2A).

Similarly, cold-acclimated animals had significantly prolonged $\frac{1}{2}RT_g$ (two-way ANOVA, temperature effect $P<0.01$, training effect $P=0.27$). The pairwise comparisons showed the same pattern of differences as with TPT_g, with the TnS and TnT groups nearly identical, while the CS group was intermediate and did not differ from either warm group or from the CT group (Fig. 2B).

Maximal contraction velocity

Muscle maximal contraction velocity (v_{max}) of the semitendinosus muscle, expressed as muscle lengths per second ($ML\ s^{-1}$), was significantly lower with both treatment effects (two-way ANOVA, temperature effect $P<0.01$, training effect $P<0.05$). There was a trend toward lower v_{max} with either exercise or cold acclimation alone, although neither differed significantly from the TnS animals. Combined cold

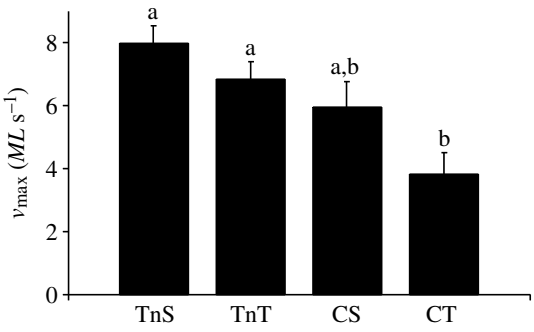

Fig. 3. Maximal shortening velocity. Maximal muscle shortening velocity (v_{max}, muscle lengths $ML\ s^{-1}$) was significantly lower following both cold acclimation and exercise training. The extent of the effect tended to increase more with cold acclimation than with exercise training. Values are means ± S.E.M. and different superscripted letters indicate significant pairwise differences between groups. CS, cold, sedentary; CT, cold, trained; TnS, thermoneutral, sedentary; TnT, thermoneutral, trained.

acclimation with exercise training resulted in a significantly slower muscle contraction (Fig. 3).

Muscle fatigability

The fatigue index was measured as the ratio of the final force generated by semitendinosus muscles after a series of tetanic contractions over the peak force measured: this is thus a measure of the endurance, or fatigue resistance, of the muscle. The fatigue index was significantly greater following cold acclimation, but not exercise training (two-way ANOVA, temperature effect $P<0.01$, training effect $P=0.14$). The low fatigue index observed in the semitendinosus muscle of the TnS animals indicated that only a small fraction of their force-generating capacity was maintained. Both exercise-trained and cold-acclimated animals showed slightly, but non-

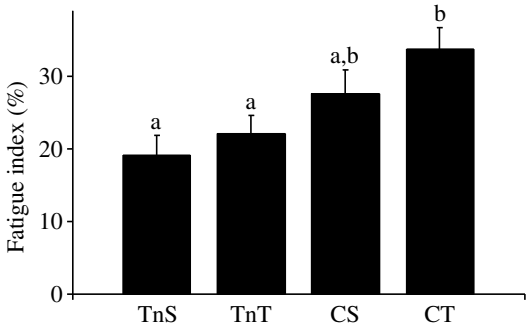

Fig. 4. Fatigue index. The fatigue index is a measure of the percent of force production capacity remaining after a fatiguing series of contractions, and is thus a measure of muscle endurance. The fatigue index of semitendinosus muscles from the four groups was significantly increased only by cold acclimation, and the CT group showed the highest resistance to fatigue. Values are means ± S.E.M. and different superscripted letters indicate significant pairwise differences between groups. CS, cold, sedentary; CT, cold, trained; TnS, thermoneutral, sedentary; TnT, thermoneutral, trained.

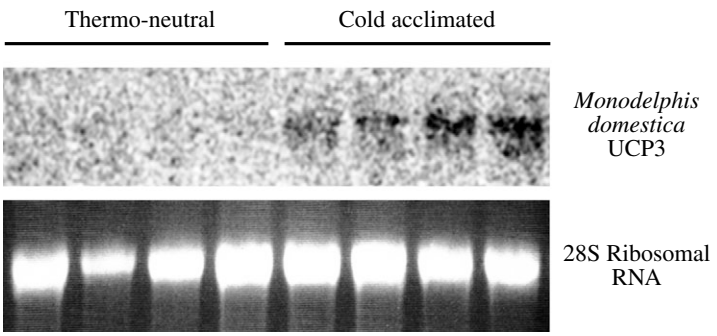

Fig. 5. *Monodelphis domestica* UCP-3 expression. The expression of the mRNA encoding a putative homolog of uncoupling protein 3 (Md-UCP3) was induced in hindlimb skeletal muscle of cold-acclimated animals, as detected by northern blot. Intensity of 28S RNA, stained with Ethidium Bromide, is included as a loading control.

significantly, higher fatigue indices. The fatigue index of the CT group was highest, thus their semitendinosus muscles possessed significantly more fatigue resistance than either warm-exposed group (Fig. 4).

Identification and expression of an uncoupling protein 3 homolog

RT-PCR of total RNA from *Monodelphis domestica* hindlimb skeletal muscle generated a 696 bp fragment. Alignment of this fragment to coding regions of other UCP3 genes showed ~84% homology to dog (Gen-Bank accession no. AB022020), ~83% homology to rat (Gen-Bank accession no. NM_013167), ~83% homology to cow (Gen-Bank accession no. AF092048), ~82% homology to pig (Gen-Bank accession no. AF095744), ~81% homology to mouse (Gen-bank accession no. U82818) and ~75% homology to human (Gen-Bank accession no. AF032902). The Md-UCP3 coding sequence was deposited into Gen-Bank under accession number AY974559.

Northern blot analysis revealed an increase of expression of Md-UCP3 in skeletal muscle of cold-acclimated animals. Md-UCP3 was undetected in thermoneutral-acclimated animals, both sedentary and exercise-trained, but present in the muscle of all cold-acclimated animals, demonstrating a dramatic upregulation of gene expression (Fig. 5).

Discussion

Skeletal muscle plasticity in response to altered activity has been well described (e.g. Flück and Hoppeler, 2003). However, the response of muscle to cold acclimation is less well understood. In those homeotherms lacking BAT, it is expected that skeletal muscle plays a major role in thermogenesis. Thus long-term cold exposure has been reported to result in increased muscle oxidative capacity in numerous species (Block, 1994), including *Monodelphis domestica* (Schaeffer et al., 2003). Shivering is the first mechanism invoked in response to cold exposure, but there is growing evidence for the role of non-shivering thermogenesis in skeletal muscle of placental mammals (Dubois-Ferriere and Chinet, 1981), birds (Duchamp and Barre, 1993) and marsupial mammals (Rose et al., 1999). Although these two mechanisms for heat production differ in that the contractile elements are not engaged in non-shivering thermogenesis, both consist of metabolic energy consumption in the absence of mechanical work.

We did not distinguish between shivering and non-shivering thermogenesis in these experiments and although skeletal muscle structure and function is clearly altered in cold-acclimated animals, the liver may also contribute to thermogenesis (Villarin et al., 2003). The importance of non-shivering thermogenesis in rodents is demonstrated by mice lacking *ucp1* gene expression and thus BAT function. Upon acclimation to cold, rodents no longer rely upon shivering, but when *ucp1* null animals are kept at 4°C, shivering thermogenesis continues and is sufficient to maintain body temperature for several weeks, although the animals fail to survive long term (Golozoubova et al., 2001). Thus during long-term cold exposure, shivering thermogenesis is insufficient to sustain life. As BAT supplies the majority of thermogenic effort, in deer mice running $\dot{V}_{O2max}$ is only slightly increased by cold exposure while $\dot{V}_{O2summit}$ is greatly increased. Surprisingly, Chappell and Hammond (2004) recently reported that cold-acclimated deer mice show very little increase in $\dot{V}_{O2max}$ when running at very cold temperatures, arguing for a competition between locomotion and thermoregulation. This competition may be of importance to *Monodelphis domestica* as well, given their reliance upon muscle for thermogenesis.

The C_t reported here for *Monodelphis domestica* acclimated to thermoneutral conditions is similar to that reported for similar sized kangaroo rats (Taylor et al., 1970). Further, the calculated minimum cost of transport of *Monodelphis domestica* (~1.5 ml O_2 kg^{-1} m^{-1}), is also very close to that predicted for a 100 g mammal (Taylor et al., 1970). In contrast, *Didelphis virginianum*, the North American opossum, has a higher than predicted C_t, due to adaptations for arboreal locomotion (Fournier and Weber, 1994). However, *Monodelphis domestica* are primarily terrestrial, thus the elevated C_t of *Didelphis virginianum* is likely a derived characteristic typical of arboreal marsupials while *Monodelphis domestica* represents a more basal condition. The elevation in C_t with cold acclimation was greatest at slower speed and decreased with increased running speed such that the calculated minimum C_t (at top speed) was similar to thermoneutral-acclimated animals. The reduction in difference in C_t with increased running speed suggests that inefficient oxidative respiration is of greater magnitude at lower overall respiration rates. This is in agreement with the observation that the control of respiration in skeletal muscle shifts from proton leak to greater coupling of ATP synthesis and utilization during periods of higher demand (Kunz, 2001). The elevated C_t observed in these animals after cold acclimation could be due

to numerous potential mechanisms, including increased ATP consumption rates at terminal ATPases without a corresponding work increase (futile cycles), uncoupling of oxygen consumption from ATP production in the mitochondria *via* either increased proton leak or uncoupling protein action, and cycling of metabolic substrates without progression to ATP production.

Our measurements of single twitch kinetics and v_{max} in isolated semitendinosus demonstrate that cold acclimation served as a strong stimulus for fast-to-slow transition in mechanical properties. Both Ca^{2+} cycling costs (Clausen et al., 1991) and myosin ATPase activity (Barany, 1967; Rome and Lindstedt, 1997) are lower in slow twitch fibers, suggesting that those muscles have acclimated toward a reduction in peak rates of energy use. Thus the mechanical properties of the cold-acclimated muscles argue for decreased C_t, i.e. the opposite of what was observed. However, endurance exercise training also leads to a fast-to-slow shift in mechanical properties (Booth and Baldwin, 1997), and endurance training has not been associated with alterations in whole organism locomotor efficiency. That the difference in C_t decreases with increased running speed further suggests that inefficient energy transduction at the primary terminal ATPases is not the principal cause of increased oxygen consumption during locomotion following cold acclimation.

We observed greater fatigue resistance in the isolated semitendinosus muscles, in accordance with our previous report that cold acclimation led to increased mitochondrial volume in skeletal muscle of *Monodelphis domestica* (Schaeffer et al., 2003). While mitochondrial biogenesis alone does not lead to altered C_t, it does provide the structural machinery essential for thermogenesis. Uncoupling of oxygen consumption from ATP production in the mitochondria, whether due to uncoupling proteins (Rolfe and Brown, 1997) or increased proton leak (Brand et al., 1994), during locomotion should result in an upward shift in $\dot{V}_{O_2}$ at any given speed, consistent with the pattern observed in Fig. 1.

We identified a homologue of UCP3 in *Monodelphis domestica* (Md-UCP3) whose expression is upregulated in skeletal muscle in response to cold acclimation. UCP3 has been identified as the major uncoupling protein isoform expressed in skeletal muscle (Boss et al., 1997; Vidal-Puig et al., 1997). UCP3 has been implicated in uncoupled metabolism and may play a role in the increased cost of transport observed in these animals; however, the capacity of UCP3 for uncoupling metabolic activity is controversial (Porter, 2001; Nedergaard and Cannon, 2003). UCP3 is able to facilitate proton translocation in isolated skeletal muscle mitochondria, albeit at low rates, leading to the suggestion that its primary role is in protection against reactive oxygen species (Echtay et al., 2002). UCP3 is also induced in muscle in response to fasting (Millet et al., 1997; Gong et al., 1997; Boss et al., 1998), a condition in which uncoupled respiration would be expected to be diminished. It has been previously reported that UCP3 is not upregulated in rodent skeletal muscle following cold acclimation (Boss et al., 1997, 1998), but Toyomizu et al. (2002) reported that UCP3 expression is induced in the skeletal muscle of cold-exposed chickens. More recently, UCP3 was not induced in *Antechinus flavipes*, a similar sized Australian marsupial, although the period of cold exposure was short (Jastroch et al., 2004). Thus although equivocal, a role for UCP3 in muscle thermogenesis may be better elucidated in organisms in which skeletal muscle plays a primary role in thermogenesis.

We recently reported that the respiratory exchange ratio was decreased in *Monodelphis domestica* following cold acclimation (Schaeffer et al., 2003). Thus increased UCP3 expression in these animals is associated with both increased oxygen consumption and lipid oxidation. Further experiments will be required to assess whether induction of UCP3 expression in cold acclimation functions to facilitate greater lipid oxidation and protection from reactive oxygen species or directly leads to metabolic uncoupling during locomotion. Increased lipid oxidation may also be associated with substrate cycling of triacylglycerol and fatty acids (Newsholme, 1978), leading to increased thermogenesis. This process can be activated by the hormone leptin, leading to an increase in metabolic rate comparable to that observed in cold-acclimated *Monodelphis domestica* during locomotion (Reidy and Weber, 2002).

These data demonstrate that the efficiency with which metabolic and mechanical power output are coupled is responsive to experimental manipulations targeting metabolic function. Metabolic power output is altered by cold acclimation, resulting in increased whole animal aerobic capacity (Schaeffer et al., 2003), but decreased efficiency of energy utilization during locomotion. This 'training for thermogenesis' led to an apparent reduction in the coupling of oxygen consumption and ATP production during locomotion, but not increased ATP utilization *via* decreased efficiency. Reduction of efficiency of the terminal ATPases would not be expected to accompany the robust fast-to-slow fiber type transition seen in all contractile parameters measured. These results are in agreement with prevailing evidence, which indicates that the coupling of most metabolic reactions does not change, with the exception of the uncoupling of mitochondrial ATP synthesis (Rolfe and Brown, 1997). Thus, as observed in whole animal studies, training for mechanical efficiency appears to have little potential to influence C_t, while cold acclimation serves as a potent stimulus of muscle plasticity, altering muscle function in the absence of BAT thermogenesis. Cold acclimation of skeletal muscle represents a 'training for inefficiency' that must balance the need to maintain contractile function with the demands of thermogenesis, apparently driving a profound fast-to-slow fiber type shift and enhancing energy-wasting metabolic processes. We propose that *Monodelphis domestica*, in which marked induction of endogenous skeletal muscle UCP3 occurs with a cold acclimation, is an excellent model for the assessment of UCP3 function.

List of abbreviations

BAT	brown adipose tissue
C_t	cost of transport
CS	cold, sedentary
CT	cold, trained
P	force
$\mathbf{P}_0$	peak tetanic tension
$\frac{1}{2}$RT	half relaxation time
TnS	thermoneutral, sedentary
TnT	thermoneutral, trained
TPT	time to peak tension
UCP3	uncoupling protein-3
v	speed
v_{max}	maximal muscle shortening velocity
$\dot{V}_{O2max}$	maximal rate of oxygen consumption while running
$\dot{V}_{O2summit}$	maximal rate of oxygen consumption during cold exposure
UCP3	uncoupling protein 3

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