Effect of conditioning and physiological hyperthermia on canine skeletal muscle mitochondrial oxygen consumption

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Abstract

Exercise often causes skeletal muscle hyperthermia, likely resulting in decreased efficiency of mitochondrial respiration. We hypothesized that athletic conditioning would improve mitochondrial tolerance to hyperthermia. Skeletal muscle biopsies were obtained from six Alaskan sled dogs under light general anesthesia before and after a full season of conditioning and racing, and respiration of permeabilized muscle fibers was measured at 38, 40, 42, and 44°C. There was no effect of temperature on phosphorylating respiration, and athletic conditioning increased maximal phosphorylating respiration by 19%. Leak respiration increased and calculated efficiency of oxidative phosphorylation decreased with increasing incubation temperature, and athletic conditioning resulted in higher leak respiration and lower calculated oxidative phosphorylation efficiency at all temperatures. Conditioning increased skeletal muscle expression of putative mitochondrial leak pathways adenine nucleotide transporter 1 and uncoupling protein 3, both of which were correlated with the magnitude of leak respiration. We conclude that athletic conditioning in elite canine endurance athletes results in increased capacity for mitochondrial proton leak that potentially reduces maximal mitochondrial membrane potential during periods of high oxidative phosphorylation. This effect may provide a mechanistic explanation for previously reported decreases in exercise-induced muscle damage caused by mitochondrial oxidative stress.

NEW & NOTEWORTHY Athletic conditioning is expected to increase exercise capacity through improved function of cardiopulmonary and musculoskeletal tissues. Our finding of decreased calculated efficiency of skeletal muscle mitochondria in one of the premier mammalian athletes suggests that this mandate for improved function may take the form of sacrificing capacity for maximal oxidative phosphorylation to minimize exercise-induced muscle damage caused by mitochondrial oxidative stress.

cellular respirometry; oxidative phosphorylation; uncoupling

INTRODUCTION

Cellular production and utilization of adenosine triphosphate (ATP) are exothermic processes, resulting in heating of metabolically active tissues. During strenuous exercise in dogs, the rate of oxidative metabolism can increase 20-fold, resulting in marked tissue hyperthermia (1, 2). Although sustained hyperthermia is compatible with successful exercise performance (3–5), excessive increases in temperature can lead to fatigue and even severe systemic disease (6).

As the specific source of the increased heat production during exercise, skeletal muscle mitochondria may experience the greatest alteration in function due to hyperthermia. The effect of a change in temperature on mitochondrial enzyme activity is biphasic. A decrease in temperature tends to slow enzyme kinetics (7), the most common examples being hibernation and induced hypothermia to slow cellular metabolism during periods of when energy conservation is necessary. On the other hand, an increase in temperature has the potential to increase enzymatic activity, at least until denaturation of the enzyme occurs. Studies of individual enzymes demonstrate, directly or indirectly, increased enzymatic activity within the range of physiological hyperthermia (8), but such studies do not conclusively demonstrate an increase in overall cellular metabolism due to the closely coupled serial nature of these processes. The effects of hyperthermia on cellular respiration are inconsistent across species and analytical techniques, with some reports demonstrating decreased phosphorylating respiration in human and murine permeabilized fibers (7, 9) and isolated mitochondria from rat skeletal muscle showing increased phosphorylating respiration (8, 10). However, hyperthermia-induced leak respiration and decreased calculated efficiency of oxidative phosphorylation is a common finding regardless of species or sample preparation (7–12).

Our hypothesis is that exercise hyperthermia results in decreased efficiency of oxidative phosphorylation in canine skeletal muscle and that athletic conditioning partially reverses hyperthermia-induced dyscoupling of oxidative phosphorylation, leading to increased efficiency. We tested this hypothesis by examining the effects of hyperthermia on ex vivo oxygen consumption of permeabilized canine
skeletal muscle fibers before and after conditioning for prolonged endurance exercise.

**MATERIALS AND METHODS**

This study was approved by the Oklahoma State University Institutional Animal Care and Use Committee. Six healthy adult Alaskan sled dogs (mean 4.3 yr old; range 3–5 yr; 5 males, 1 female) were randomly selected from a team of race-conditioned dogs. Dogs were housed individually outdoors in a manner typical for professional racing sled dogs and fed commercial high-energy kibble to maintain lean body condition during conditioning and racing. Conditioning consists of exercise sessions in groups of 12–16 dogs connected to a single gangline and either a small all-terrain vehicle or sled (depending on trail conditions) with speed limited to 16–18 km/h to manage the production and dissipation of metabolic heat. Conditioning intensity results from increasing workout distance, and the decision to increase distance is made via monitoring the voluntary speed of the team via GPS: if dogs are maintaining within 10% of the maximum allowed speed during the last 30 min of the workout, then the distance of the next workout is increased by 25%. Skeletal muscle biopsies were obtained at two time points: Conditioned, when dogs had undergone 7 mo of progressive endurance conditioning, including having completed a 1,600-km race in 11 days, 10 h, and Unconditioned, when dogs were at minimal fitness with no compulsory exercise for at least 4 mo. Biopsies from Conditioned dogs were obtained at least 96 h after exercise. Skeletal muscle biopsies were obtained under light general anesthesia (intravenous propofol, 6 mg/kg) using sterile technique from the middle of the biceps femoris muscle using a 14-gauge percutaneous biopsy needle (14-gauge E-Z Core single action biopsy needle, Products Group International, Lyons, CO) to yield ~80 mg of wet muscle per procedure. Approximately 20 mg of wet skeletal muscle tissue were immediately snap frozen and stored for later analysis, and the remaining 60 mg of wet muscle were immediately transferred into vials with ice-cold BIOPS solution (2.77 mM CaK₂EGTA, 7.23 mM K₂EGTA, 20 mM imidazole, 20 mM tauroine, 50 mM MES hydrate, 0.5 mM dithiothreitol, 6.56 mM MgCl₂, 5.77 mM ATP, and 15 mM phosphocreatine, adjusted to pH 7.1) and transported to the laboratory for analysis. Biopsies were manually dissected under magnification to remove blood and connective tissue and to separate muscle fibers to improve diffusion of reagents into the individual fibers. Biopsies were incubated in BIOPS with saponin (50 μg/ml) added for 30 min with gentle agitation and then washed in mitochondrial respiration media (MiR06Cr: 0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA essentially fatty acid free, 280 U/ml catalase, and 20 mM creatine, adjusted to pH 7.1) until added to the high-resolution respirometry chambers (minimum of 10 min of washing with gentle agitation).

High-resolution respirometers (Oxygraph O2K, Oroboros Instruments, Innsbruck, Austria) were used to analyze the effects of temperature on mitochondrial oxygen consumption. For effect of temperature, respirometer chambers were filled with MiR06Cr (described above), and instruments were set at four different temperatures: 38°C to represent the normal canine resting temperature and 40, 42, and 44°C to represent the physiological range of hyperthermia that can occur in canine skeletal muscle during sustained exercise (4, 13, 14). Instrument oxygen sensors were calibrated daily using at the temperature to be used for analysis, and background oxygen flux correction constants were determined each morning before addition of 1–2 mg of wet permeabilized fibers per respirometer chamber. Dissolved oxygen was increased to >500 nmol immediately following addition of the permeabilized fibers and was maintained above 250 nmol through small additions of hydrogen peroxide, which was immediately converted to dissolved oxygen by the catalase in the respiration media. Oxygen consumption was calculated as the negative slope of the oxygen measurement and reported as picomoles per seconds per milligrams of tissue added. Pyruvate (5 mM), glutamate (10 mM), and malate (0.5 mM) were titrated into each chamber to produce NADH and stimulate nonphosphorylating (leak) respiration through Complex I (ISₘ). (Values in parentheses indicate the final concentration of each chemical in the respirometer chamber after titration.) ADP (5 mM) was then added to stimulate phosphorylating respiration through Complex I (Iₛₘ). Cytochrome c (10 μM) was added to ensure high rates of respiration were not limited by loss of cytochrome c as an artifact of tissue handling and permeabilization. The addition of succinate (10 mM) resulted in phosphorylating respiration through the combination of Complex I and Complex II (maximum phosphorylating respiration [Pₛₘ]). During preliminary assays, an uncoupler (CCCP in 0.5-μM steps) was then added to assess whether respiration was limited by ATP synthase. The uncoupling reagents are typically titrated carefully to observe the uncoupling effect (i.e., an increase in respiration if the phosphorylation system is limiting) before the secondary effect of inhibition of respiration at higher concentrations of uncoupler. However, due to endogenous uncoupling due to increased leak at higher incubation temperatures [an effect that was also found in a similar study on horse muscle (11)], no uncoupling effect was observed and the primary effect of the uncoupling reagent was inhibition of respiration. Therefore, this titration was eliminated from the protocol. Finally, rotenone (0.5 μM) was added to block Complex I, with the resulting oxygen flux representing the capacity of Complex II to support mitochondrial oxygen consumption (Pₘ). Flux control ratios for NADH-based and succinate-based respiration (FCRₙ and FCRₛ, respectively) were calculated as the ratio of the respective phosphorylating respiration to the maximum phosphorylating respiration. Oxidative phosphorylation efficiency was calculated as 1–Lₙₘ/Pₙₘ + Sₙₘ. Results for each parameter of interest were calculated for individual respirometer chambers and then averaged by subject for each experimental condition.

Frozen muscle was homogenized on ice in ~2 ml per 0.2 g of tissue lysis buffer using a Dounce homogenizer and tissue shredder. Protein concentration was measured using Coomassie blue protein assay, and samples to be analyzed were diluted to a concentration of 2 ng/μl using lysis buffer and 2× Laemmli buffer. Samples were run in duplicate on a 10% SDS-polyacrylamide gel at 200 V and 0.025 mA/gel for ~1.25 h at 4°C, with both Unconditioned and Conditioned samples from an individual subject on the same gel. The gel...
was transferred overnight onto an Immobilon-FL PVDF membrane in transfer buffer at 30 V and 0.09 mA for 16 h. Membranes were blocked for 1 h using Rockland Blocking Buffer (MB-070), incubated in the target primary antibody (Anti-UCP3 Abcam ab10985; Anti-ANT 1 Abcam ab102032; β-Actin Rabbit Monoclonal Antibody LI-COR 926–42210) for 1 h, and incubated in a secondary antibody (LI-COR IRDye 680RD anti-rabbit 926–68073) for 1 h, all at room temperature. Between each incubation the membrane was washed four times with 0.1% TBS-T for 5 min each wash. The membrane was washed a final time for 5 min in TBS and immediately scanned using a LI-COR Odyssey imager. Relative target band intensity was measured using commercial software (LI-COR Empiria Studio 1.3) and expressed as average intensity corrected for the average intensity of β-actin in the sample.

An aliquot of the initial homogenate prepared for Western blot analysis for each muscle biopsy was used to quantify citrate synthase assay using a commercially available kit according to manufacturer instructions (MAK193 Citrate Synthase Assay, Millipore Sigma, St. Louis, MO). Results were expressed as milliunit citrate synthase activity per micromgram of protein corrected for the relative expression of B-actin on Western blots.

Respirometry data were analyzed using two-way repeated measures ANOVA, with incubation temperature and conditioning state as the independent variables and dog as the repeated measure blocking variable (GraphPad Prism 8.4, San Diego, CA). If the overall ANOVA yielded \( P < 0.05 \) for an effect of temperature, then post hoc pairwise comparisons were performed using two stage linear step-up procedure to compare all levels of the incubation temperature. Effect of incubation on citrate synthase activity of skeletal muscle was analyzed using a Wilcoxon test due to the non-normal distribution of the data. Effect of conditioning on expression of ANTI1 and UCP3 in skeletal muscle was analyzed using a paired Student’s t test. Pearson correlation coefficients were calculated for the expression of ANTI1 and UCP3 and the mean values (averaged across all tested temperatures for a given conditioning state) for \( L_N \), \( P_{(N+S)} \), and oxidative phosphorylation efficiency, as well as relative effect of conditioning (Conditioned/Unconditioned \( \times 100 \)) of ANTI1 and UCP3 protein expression compared with the relative effect of conditioning on \( L_N \), \( P_{(N+S)} \), and oxidative phosphorylation efficiency. Data are reported as means ± SD in all cases except the data for citrate synthase activity (which is reported as median and 25–75 percentile), and \( P < 0.05 \) was considered statistically significant.

## RESULTS

Both temperature and conditioning affected the magnitude of leak respiration in canine skeletal muscle mitochondria (Fig. 1). Hyperthermia resulted in increased leak respiration, with all tested temperatures causing significant increases in non-phosphorylating oxygen consumption \( (P < 0.001) \). This resulted in a doubling of \( L_N \) in Unconditioned dogs \((13.25 ± 2.91 \text{ pmol/mg at } 38°C \text{ to } 26.27 ± 3.75 \text{ pmol/mg at } 44°C) \) and a 62% increase in Conditioned dogs \((29.29 ± 4.72 \text{ pmol/mg at } 38°C \text{ to } 47.32 ± 7.26 \text{ pmol/mg at } 44°C) \). Conditioning resulted in an approximate doubling of leak respiration at all tested temperatures \( (P < 0.001) \). Phosphorylating respiration through Complex I (Fig. 2) was increased by ~25% (mean for all assay temperatures) with athletic conditioning \( (P = 0.03) \) with no effect of incubation temperature \( (P = 0.8) \). SD of differences for effect of temperature on \( P_N \) = 26.74, providing an 80% power to detect a change of 30.58 pmol/mg/s due to temperature. Phosphorylating respiration through Complex II (Fig. 3) was not affected by either athletic conditioning \( (P = 0.1) \) or increased temperature \( (P = 0.07) \). SD of differences for effect of conditioning on \( P_S \) = 10.6, providing an 80% power to detect a change of 11.4 pmol/mg/s due to conditioning. SD of differences for effect of temperature on \( P_S \) =

![Figure 1](image1.png)  
**Figure 1.** Effect of incubation temperature and conditioning on leak [non-phosphorylating respiration (JO2) supported by NADH] in permeabilized skeletal muscle fibers. Unconditioned: subjects had not participated in compulsory exercise for \( >4 \text{ mo} \). Conditioned: subjects had completed a full 7-mo season of progressive exercise conditioning, including completion of a 1,600-km race. There was a statistically significant effect of incubation temperature \( (P < 0.001) \) and conditioning \( (**P < 0.001) \); \( n = 6 \) subjects. Different lowercase letters indicate differences between incubation temperature during post hoc testing \( (P < 0.05) \).

![Figure 2](image2.png)  
**Figure 2.** Effect of incubation temperature and conditioning on phosphorylating respiration (JO2) supported by NADH \( (P_J) \) in permeabilized skeletal muscle fibers. Unconditioned: subjects had not participated in compulsory exercise for \( >4 \text{ mo} \). Conditioned: subjects had completed a full 7-mo season of progressive exercise conditioning, including completion of a 1,600-km race. There was a statistically significant effect of conditioning \( (**P = 0.03) \) but not incubation temperature \( (P = 0.8); n = 6 \) subjects.
9.55, providing an 80% power to detect a change of 10.92 pmol/mg/s due to temperature. Maximum phosphorylating respiration (Complex I and Complex II, Fig. 4) increased by 19% (mean for all assay temperatures) with athletic conditioning ($P = 0.03$) with no effect of incubation temperature ($P = 0.4$). SD of differences for effect of temperature on $P(N + S) = 27$ pmol/mg/s, providing an 80% power to detect a change of 31 pmol/mg/s.

There was no effect of incubation temperature or conditioning on FCRN ($P = 1$ and $P = 0.4$, respectively) (Fig. 5), with conditioning resulting in a 20% decrease in FCRN. Pairwise comparisons of incubation temperatures revealed a small increase in FCRS between 38°C and 44°C (0.5996 vs. 0.6660, $P = 0.004$). There was strong effect of both incubation temperature ($P < 0.001$) and conditioning ($P = 0.007$) on oxidative phosphorylation efficiency (Fig. 7), with conditioning resulting in a decrease from 0.8649 to 0.7553. Increases in incubation temperature resulted in progressive decreases in oxidative phosphorylation efficiency, with all pairwise comparisons significantly different except for the comparison between 40°C and 42°C.

### Figures

**Figure 3.** Effect of incubation temperature and conditioning on phosphorylating respiration ($JO_2$) supported by succinate ($P_S$) in permeabilized skeletal muscle fibers. Unconditioned: subjects had not participated in compulsory exercise for >4 mo. Conditioned: subjects had completed a full 7-mo season of progressive exercise conditioning, including completion of a 1,600-km race. There was not a statistically significant effect of incubation temperature ($P = 0.07$) and conditioning ($P = 0.1$); $n = 6$ subjects.

**Figure 4.** Effect of incubation temperature and conditioning on phosphorylating respiration ($JO_2$) supported by succinate ($P_{N+S}$) in permeabilized skeletal muscle fibers. Unconditioned: subjects had not participated in compulsory exercise for >4 mo. Conditioned: subjects had completed a full 7-mo season of progressive exercise conditioning, including completion of a 1,600-km race. There was a statistically significant effect of conditioning ($*P = 0.03$) but not incubation temperature ($P = 0.4$); $n = 6$ subjects.

**Figure 5.** Effect of incubation temperature and conditioning on flux control ratio for respiration supported by NADH (FCRN) in permeabilized muscle fibers. Unconditioned: subjects had not participated in compulsory exercise for >4 mo. Conditioned: subjects had completed a full 7-mo season of progressive exercise conditioning, including completion of a 1,600-km race. There was not a statistically significant effect of incubation temperature ($P = 1$) or conditioning ($P = 0.4$); $n = 6$ subjects. Unconditioned: subjects had not participated in compulsory exercise for >4 mo. Conditioned: subjects had completed a full 7-mo season of progressive exercise conditioning, including completion of a 1,600-km race.

**Figure 6.** Effect of incubation temperature and conditioning on flux control ratio for respiration supported by succinate (FCRS) in permeabilized skeletal muscle fibers. Unconditioned: subjects had not participated in compulsory exercise for >4 mo. Conditioned: subjects had completed a full 7-mo season of progressive exercise conditioning, including completion of a 1,600-km race. There was a statistically significant effect of both incubation temperature ($P = 0.03$) and conditioning ($*P = 0.04$); $n = 6$ subjects. Different letters indicate differences between incubation temperatures ($P < 0.05$).
There was a significant effect of conditioning on skeletal muscle citrate synthase activity [Unconditioned: 0.7021 milliunits/μg protein (0.4503–0.8501); Conditioned: 0.9409 milliunits/μg protein (0.7378–2.405), P = 0.02] (median, 25–75 percentile)]. Conditioning increased expression of both ANT1 (4.13 ± 1.97-fold increase, P = 0.04) and UCP3 (3.13 ± 2.03-fold increase, P = 0.04) (Fig. 8). The expression of ANT1 was significantly correlated with LN (r = 0.5046, P = 0.05) and oxidative phosphorylation efficiency (r = −0.6819, P = 0.007) (Fig. 9) but not P(N + S) (P = 0.4). The expression of UCP3 was significantly correlated with oxidative phosphorylation efficiency (r = −0.5783, P = 0.02) (Fig. 10) but not LN (P = 0.2) or P(N + S) (P = 0.2). There was not a significant correlation between the conditioning-induced increase in expression of ANT1 and the conditioning-induced changes in LN (P = 0.6), P(N + S) (P = 0.5), and oxidative phosphorylation efficiency (P = 0.8). The conditioning-induced changes in expression of UCP3 were negatively correlated to the conditioning-induced changes in both P(N + S) (r = −0.7978, P = 0.03) and oxidative phosphorylation efficiency (r = −0.7492, P = 0.04) (Fig. 11) but not correlated to the conditioning-induced changes in LN (P = 0.3).

Data were evaluated to determine the presence of sex bias due to the uneven distribution of male dogs and female dogs in our study. This distribution was occurred as a function of the subject selection being a convenience sample defined by both successful performances of the dogs and suitability for general anesthesia and muscle biopsy procedures. Data from the lone female dog in the study were outliers (defined as outside the 95% confidence interval when both the mean and standard deviations were calculated without her data) in only 4 of the 56 measurements (7 end points × 4 temperatures × 2 training states), and never an outlier when her data were included in the calculation of the 95% confidence intervals of the data.

DISCUSSION

Increased incubation temperature resulted in increased leak respiration in canine skeletal muscle, similar to results reported in humans (9), horses (11), and laboratory rodents (7, 8, 10). However, in contrast to humans and rats, as well as studies at basal incubation temperature in horses, conditioning in racing sled dogs resulted in increased leak respiration and corresponding decreased calculated oxidative phosphorylation (OXPHOS) efficiency. These results were both unexpected and counterintuitive in a study population as athletic as ultra-endurance racing sled dogs and represent a novel and intriguing conditioning effect of skeletal muscle.

The primary effect of assay hyperthermia on mitochondrial respiration was the pronounced increase in leak respiration (Fig. 1). The exact cellular mechanism of leak respiration is likely multifaceted. During normal mitochondrial respiration, protons are pumped from the mitochondrial matrix into the intermembrane space by Complexes I, III, and IV of the electron transfer system, resulting in an electrochemical gradient that powers the formation of ATP. The primary effect of assay hyperthermia on mitochondrial respiration was the pronounced increase in leak respiration (Fig. 1). The exact cellular mechanism of leak respiration is likely multifaceted. During normal mitochondrial respiration, protons are pumped from the mitochondrial matrix into the intermembrane space by Complexes I, III, and IV of the electron transfer system, resulting in an electrochemical gradient that powers the formation of ATP.

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Figure 7. Effect of incubation temperature and athletic conditioning on calculated efficiency of oxidative phosphorylation [1-LN/(P(N + S)) in permeabilized muscle fibers. Unconditioned: subjects had not participated in compulsory exercise for >4 mo. Conditioned: subjects had completed a full 7-mo season of progressive exercise conditioning, including completion of a 1,600-km race. There was a statistically significant effect of incubation temperature (P < 0.001) and conditioning (**P = 0.007); n = 6 subjects. Different lowercase letters indicate differences between incubation temperature during post hoc testing (P < 0.05).

Figure 8. Effect of seasonal conditioning on the skeletal muscle expression of adenine nucleotide transporter 1 (ANT1) and uncoupling protein 3 (UCP3). Uncond: subjects had not participated in compulsory exercise for >4 mo. Cond: subjects had completed a full 7-mo season of progressive exercise conditioning, including completion of a 1,600-km race. The center lane contains the molecular weight markers, with the numbers indicating the molecular mass (MW) in kDa, and β-actin was used as a loading control. There was a statistically significant effect of conditioning on expression of ANT1 (P = 0.036) and UCP3 (P = 0.038); n = 6 subjects. Representative blot results are shown for 2 subjects analyzed in duplicate.
through ATP synthase (15). The term “leak” refers to the presumption that in the absence of phosphorylating activity, either due to the absence of substrates or specific inhibition of ATP synthase, the resulting nonphosphorylating respiration is the result of protons leaking back into the mitochondrial matrix. The two primary leak pathways in skeletal muscle are believed to be uncoupling protein 3 (UCP3) and adenine nucleotide translocase (ANT1) (16). However, other mechanisms can also contribute to the phenomenon of leak respiration, including proton slip (in which energy is transferred through the proton-pumping elements of the electron transport system without a concurrent movement of a proton into the intermembranous space) and cation cycling (in which the intermembrane proton gradient is used to pump other cations out of the mitochondrial matrix) (15).

The different leak mechanisms appear to have variable sensitivity to hyperthermia. In isolated mitochondria derived from rat skeletal muscle, leak respiration is approximately doubled when incubation temperature is increased from 35°C to 42°C, similar to the results of the current study in dogs. Leak respiration can be reduced by two-thirds at both incubation temperatures by inhibition of ANT1, indicating that this pathway is temperature sensitive. Proton leakage attributable to UCP3 (i.e., induced by linoleic acid after inhibition of ATP synthase and ANT1) was increased by ~80% when incubation temperature was increased from 35°C to

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**Figure 9.** Correlation between skeletal muscle adenine nucleotide transporter 1 (ANT1) expression and indexes of skeletal muscle mitochondrial respirometry (JO2); n = 12 (6 subjects × 2 conditioning states). A: correlation between ANT1 expression and leak respiration (LN). B: correlation between ANT1 expression and calculated oxidative phosphorylation efficiency [1-LN/P(N+S)].

**Figure 10.** Correlation between skeletal muscle uncoupling protein 3 (UCP3) expression and indexes of skeletal muscle mitochondrial respirometry; n = 12 (6 subjects × 2 conditioning states). A: correlation between fold change in UCP3 expression and mitochondrial respiration supported by NADH and succinate P(N+S) in conditioned state expressed as percentage of the same measurement in the unconditioned state. B: correlation between fold change in UCP3 expression and calculated oxidative phosphorylation efficiency [1-LN/P(N+S)] in conditioned state expressed as percentage of the same measurement in the unconditioned state.

**Figure 11.** Correlation between conditioning-induced change in skeletal muscle uncoupling protein 3 (UCP3) expression and indexes of skeletal muscle mitochondrial respirometry; n = 6 subjects. A: correlation between fold change in UCP3 expression and oxidative phosphorylation efficiency [1-LN/P(N+S)] in conditioned state expressed as percentage of the same measurement in the unconditioned state.
42°C. When UCP3 was then inhibited by GTP, the remaining proton “leak,” which was presumably due to either proton slip or cation exchange, was also increased by ~50% by increased incubation temperature. In contrast to the studies using rat skeletal muscle mitochondria, we did not induce leak through UCP3 by the addition of linoleic acid to our incubation preparations. Thus it is likely that the predominant leak pathway in the current study was through ANT1 (the expression of which was significantly correlated with the mean measurements of leak respiration), with a smaller contribution from through proton slip and cation exchange and constitutive activity of UCP3.

Leak respiration invariably results in loss of OXPHOS efficiency. A combination of increased leak and decreased phosphorylating respiration in human skeletal muscle decreased OXPHOS efficiency during hyperthermia from 0.81 at 35°C to 0.70 at 40°C (9). Horses have remarkably efficient OXPHOS (0.95 when defined as 1-L/P), befitting their status as an elite mammalian athlete. Nevertheless, hyperthermia results in loss of OXPHOS efficiency, possibly leading to lower maximal rate of ATP synthesis (11). Even in rats, in which hyperthermia results in increased phosphorylating respiration, OXPHOS efficiency decreases due to proportionately larger increase in leak respiration (8, 10). Compared with other species, skeletal muscle OXPHOS efficiency in untrained sled dogs falls between horses and humans. However, it is important to distinguish between OXPHOS efficiency as an ex vivo calculated index versus an actual decrease in ATP synthesis in vivo. If hyperthermia-induced dyscoupling of mitochondrial oxidative phosphorylation actually results in less ATP produced per mole of combusted substrate, then this phenomenon potentially would lead to a positive-feedback cycle in which the requirement for a specific rate of ATP synthesis would lead to progressively more substrate oxidation and more heat production, ultimately resulting in failure of oxidative phosphorylation. On a macroscopic scale, this would be a potent contributor to exercise fatigue, and it also may provide a basis for the hyperthermia-induced shift away from aerobically produced ATP in dogs exercising under conditions of high-heat load (1). Alternatively, it is possible that the effect of hyperthermia-induced dyscoupling is limited to the highest values of mitochondrial membrane potential and during typical respiration has little effect on ATP synthesis efficiency due to the non-ohmic nature of mitochondrial proton leak (17). In this way, hyperthermia-induced dyscoupling and concurrent lowering of mitochondrial membrane potential could be beneficial by reducing the production of reactive oxygen species (ROS) during the periods of high mitochondrial activity that are implied by the development of skeletal muscle hyperthermia.

Athletic conditioning resulted in a doubling of the leak respiration in canine skeletal muscle across the entire tested temperature range (Fig. 1), with a corresponding decrease in the calculated OXPHOS efficiency (Fig. 7). The conditioning-induced increase in leak respiration was unexpected because in all other species studied, athletic conditioning generally reduces leak respiration and, in some instances, also decreases temperature sensitivity of skeletal muscle leak respiration. Nine weeks of submaximal training in Quarterhorses decreased leak respiration in permeabilized skeletal muscle fibers, but this effect was only statistically significant in the gluteus muscle and not in the triceps brachii (18). Similarly, there was no effect of fitness on leak respiration in permeabilized triceps brachii skeletal muscle fibers from Thoroughbred horses (19). Both studies of horses were performed at a single incubation temperature (37°C), so no information is available regarding temperature sensitivity of leak respiration or the possible effect of conditioning therein. Decreased leak and increased efficiency of oxidative phosphorylation in response to conditioning was only found at higher incubation temperatures of human skeletal muscle (9), whereas in rats leak respiration in isolated skeletal muscle mitochondria was decreased in response to 8 wk of endurance training at all tested temperatures (12). Thus the increase in skeletal muscle leak respiration and decreased OXPHOS efficiency in highly conditioned dogs represents a novel adaptation in athletic animals.

Increased leak respiration and decreased OXPHOS efficiency in response to athletic conditioning is counterintuitive and merits closer examination to determine whether this conditioning response improves exercise performance. Similar to the postulated purpose of hyperthermia-induced dyscoupling, this conditioning effect may represent further adaptation to mitigate the production of ROS during exercise. Sustained exercise is widely recognized to produce oxidative stress, with ultraendurance exercise having been shown to increase ROS in human skeletal muscle mitochondria (20). The conditioning-induced dyscoupling at all temperatures that we found in highly trained endurance sled dogs may therefore represent a novel or advanced effect of conditioning intended to reduce mitochondrial ROS production during exercise and potentially reduce exercise-induced muscle damage. Such an impact would be consistent with previous studies showing reduction of exercise-induced muscle damage with increased fitness in racing sled dogs (21, 22); however, we did not quantify ex vivo ROS production during the respirometry measurements of this study nor did we measure markers of oxidative damage in the dogs following exercise. Therefore, the connection between conditioning-induced dyscoupling and exercise-induced muscle damage remains speculative. Future studies will examine the relationship between the mitochondrial production of reactive oxygen species as a function of conditioning and its relationship between oxygen consumption, mitochondrial membrane potential, and ATP synthesis to fully illustrate the effect of athletic conditioning on mitochondrial respiration.

There was minimal impact of physiological hyperthermia on the maximal rate of phosphorylating respiration (Fig. 4) and the individual capacities of the two main sources of redox energy (Complex I and Complex II) that converge within the electron transfer system (Figs. 2 and 3). The effect of hyperthermia on maximal phosphorylating respiration in other species is variable and may be a function of the sample preparation. Hyperthermia results in increased phosphorylating respiration (8, 10) and ATP synthesis in intact mitochondria from rat skeletal muscle (8) but decreased phosphorylating respiration in permeabilized fibers from mouse cardiac muscle (7) and human skeletal muscle (9). There was no effect of temperature on phosphorylating
respiration in permeabilized equine skeletal muscle fibers (11), similar to the current study. Horses and dogs have very high oxidative capacity of their skeletal muscle and, perhaps more importantly, frequently experience marked skeletal muscle hyperthermia during strenuous exercise (2, 23). Thus the preservation of phosphorylating respiration at temperatures up to 44°C in both of these species may represent specific selection for hyperthermia-tolerant mitochondria that occurred concurrently with the development of high oxidative capacity.

Athletic conditioning typically increases mitochondrial capacity for respiration regardless of the species of athlete (9, 12, 18, 19, 24, 25). However, the effect of conditioning on mitochondrial phosphorylating capacity during hyperthermia is less studied. In rats, the hyperthermia-induced increase in phosphorylation is amplified by ~25% by 8 wk of endurance training without any detected increase in overall abundance of the elements of the electron transport system (12). This could be due either to conditioning-induced shifts of heat-sensitive elements in the mitochondria to more heat tolerant isoforms or an increase in the abundance of a mitochondrial element that is rate limiting but was not specifically quantified. In humans, the conditioning increases mitochondrial tolerance to hyperthermia by abolishing the hyperthermia-induced decrement in OXPHOS capacity that is evident before conditioning (9). In this way, athletically conditioned humans appear to adopt a hyperthermia-tolerant phenotype that is present in dogs even when deconditioned. It is important to note that the dogs of this study had undergone several cycles of conditioning and deconditioning in their lifetime before the study, and it is possible that before their first season of athletic conditioning, their mitochondrial sensitivity to hyperthermia was similar to humans and the 4–5 mo of deconditioning that preceded their examination as Unconditioned dogs were not sufficient to fully reverse the effects of conditioning. Athletic dogs have skeletal muscle protein turnover rates that are two to three times higher than humans (26), which would suggest that if their mitochondrial tolerance to hyperthermia was due to conditioning-induced synthesis of hyperthermia-tolerant isoforms then they would lose that phenotype faster than humans. However, definitive determination as to whether this trait is a persistent effect of conditioning instead of an inherent trait of athletic dogs will require examination of young dogs before their first season of conditioning.

ANT1 and UCP3 are two skeletal muscle mitochondrial proteins with the capacity to facilitate leak respiration, and the expression of both proteins was significantly increased in canine skeletal muscle in response to athletic conditioning (Fig. 8). The physiological purpose of ANT1 is to permit exchange of ATP formed in the mitochondrial matrix with ADP produced by consumption of ATP in the cytosol. During exercise, the rate of ATP/ADP exchange across the mitochondrial membrane must increase to match the rate of ADP production, lest ATP synthesis become limited by ADP availability in the mitochondrial matrix. Thus it makes intuitive sense that the abundance of ANT1 would increase with increased fitness and by extension the leak respiration due to ANT1 would similarly increase. Based on the likelihood that the predominant mechanism for basal leak respiration in skeletal muscle is through ANT1 (17), we hypothesized that the mechanism for the conditioning-induced increase in leak respiration in canine skeletal muscle is similarly mediated by increased expression of ANT1. Increased ANT1 content has been demonstrated in trained human athletes, but in that study, increased ANT1 was associated with decreased, not increased leak respiration. Although the overall magnitude of leak respiration and OXPHOS efficiency was correlated with ANT1 expression (Fig. 9), the conditioning-induced change in expression of ANT1 in the dogs of this study was not correlated to these parameters. Thus, while ANT1 may be an important leak pathway in skeletal muscle mitochondria, it may not be responsible for the conditioning-induced increase in leak observed in this study. Leak mediated by uncoupling proteins is typically regarded as requiring activation of the uncoupling protein (17), and we did not add any exogenous activators such as linoleic in our experiments. Thus, although the relative change in UCP3 in response to conditioning is loosely associated with the conditioning-induced change in leak respiration through the correlation between UCP3 expression and OXPHOS efficiency (Fig. 11), the full role of UCP3 in canine skeletal muscle mitochondrial leak is unclear.

Several technical aspects of this study merit specific attention when interpreting the results. First, is the possibility of sex bias in the results due to the uneven inclusion of male and female subjects. It is important to note that racing sled dogs train as teams of dogs that are physically connected to each other during exercise, and therefore, there is minimal opportunity for individual variation in effort. This does not preclude sex-specific differences in mitochondrial responses to exercise, but the nature of sled dog conditioning and exercise, in addition to the analysis of the distribution of data by sex that is described in the results, leads to the conclusion that bias due to uneven distribution of subject sex had a minimal effect on our results. The subjects of this study differ in the presumed amount of fitness for prolonged submaximal exercise, with that difference in fitness the result of adaptations that are presumed to result from the progressive increases in exercise during conditioning, and the reversal of those adaptations during the prolonged period of rest between the end of the racing season and the beginning of the next conditioning cycle. Several past studies have documented distinct physiological differences between these two fitness extremes, including changes in glucose utilization (27–30), mitochondrial function (24), and thermoregulation (3). Thus although we are confident that the 7 mo of conditioning activity results in increased fitness for prolonged endurance exercise, the full impact of the changes in skeletal muscle mitochondrial function on ROS production, ATP synthesis, and exercise-induced oxidative damage to skeletal muscle is unknown and will be the subject of planned future studies.

ACKNOWLEDGMENTS
The authors gratefully acknowledge Martin Buser of Happy Trails Kennels for providing the dogs used in this study.

GRANTS
This study was funded by the American Kennel Club Canine Health Foundation Grant 2646-A.
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