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Roy Mitra Salgado

Candidate

Health, Exercise and Sports Sciences

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Christine Mermier , Chairperson

Daryl Parker

Suzanne Schneider

Len Kravitz

THE EFFECT OF 10 DAYS OF HEAT ACCLIMATION ON SUBMAXIMAL EXERCISE ECONOMY AND EFFICIENCY AT 1600 M AND 4350 M

by

ROY MITRA SALGADO

DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of

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The effect of 10 days of heat acclimation on submaximal exercise economy and efficiency at 1600 m and 4350 m

By

Roy Mitra Salgado

B.S., Kinesiology, Exercise Science, California State University, Sacramento 2007

M.S., Kinesiology, Exercise Science, California State University, Sacramento 2010

Ph.D., Physical Education, Sports and Exercise Sciences, University of New Mexico, 2014

ABSTRACT

Heat acclimation is known to increase exercise economy. Previous examinations suggest heat acclimation may preserve performance at altitude. This study examined the effect of using heat acclimation as a cross environmental stressor to improve exercise economy and efficiency during acute exercise at altitude. Eight trained males (VO_{2peak}: 53.3 ± 6.7 ml/kg/min) performed maximal exercise tests, submaximal exercise bouts, and heat tolerance testing in a temperate environment (21°C) at 1600 m and 4350 m before and after a 10-day heat acclimation (40°C and 20% RH) on a cycle ergometer (~43% peak power). To investigate heat stress mechanisms, C2C12 myocytes were heat stressed for 24 hours (40°C, 5% CO_2). Heat acclimation did not alter VO_{2peak} at 1600 m (53.3 ± 6.7 vs. 53.7 ± 3.7 ml/kg/min, $p > 0.05$) or 4350 m (45.3 ± 4.1 versus 45.9 ± 3.4 ml/kg/min, $p > 0.05$). Heat acclimation increased exercise economy by 1.6% and 2% in the low intensity and high intensity exercise, respectively at 1600 m with only a 0.48% increase at 4350 m. In the cell study, heat stress significantly reduced UCP3 expression, reduced mitochondrial uncoupling (71.1% ±1.2%) and suppressed basal and peak oxidative metabolism (75.5% ± 4.9% and 64.4% ± 5.9%, respectively) compared to control. Heat stress also significantly increased PGC-1α, NRF1 and TFAM leading to increased mitochondrial content. These data demonstrate that while heat stress reduces UCP3 expression, thereby reducing uncoupling and leading to enhanced mitochondrial efficiency, these adaptations are not observed in the whole body. At this time, I are unable definitively promote the use of heat acclimation as a cross environmental stressor for acute exercise at altitude.

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CHAPTER 1

I would like to acknowledge my co-investigator of this dual dissertation, Ailish White. I want to thank you for your assistance in subject recruitment, scheduling, and data collection throughout this process. I would not have been able to complete this project without your help. Thank you.

Introduction

The use of altitude training is a common practice for athletes to improve sea-level exercise performance (17, 36) and/or improve exercise capacity at altitude (4, 9). With increasing altitude (or decrease in barometric pressure), there is a decrease in partial pressure of inspired oxygen (PIO_2) leading to hypoxia. The reduction in barometric pressure is accompanied by pressure gradient for gas diffusion which leads to a decrease in oxygen transport, that can reduce maximal oxygen consumption (VO_{2max}) (37) and submaximal oxygen consumption at altitude. Maximal oxygen consumption can be reduced by as much as 10-12% at >2200m (5, 35). Since VO_{2max} is reduced at altitude, exercise is performed at a higher percentage of their VO_{2max} (reduced exercise economy) compared to sea level. Even after prolonged (14-18 days) exposure to 4300m, there are little increases or decreases in VO_{2max} (39, 40). To maintain homeostasis, humans must adapt, allowing for increased tolerance to the environment. These responses to exposure to high altitude include an increase in ventilatory rate that leads to improvement in oxygen saturation (SaO_2) (14). In addition there is a right-ward shift in the oxygen-hemoglobin disassociation curve (38), increasing unloading of oxygen in skeletal muscle tissue. Long-term acclimatization leads to improvements in pulmonary gas exchange, which increases oxygen transport (3), and hypoxia leads to polycythemia (15, 18). These

changes, in turn, lead to an enhanced oxygen delivery and carrying capacity (18) which aides in exercise capacity at high altitude.

In sea-level natives making altitude sojourns, submaximal oxygen consumption after 12 to 18 days has not been reported to increase (1, 22). Since oxygen transport has been suggested to be a limiting factor to exercise capacity at high altitude, adaptations leading to improved economy may be beneficial. Recently, Latshang et al. (16) suggested that since maximal exercise capacity is not changed after acclimization, the reports of improved tolerance during exercise in mountaineers might be attributed to increased efficiency of muscular work at altitude. In this study Latshang et al. (16) reported that in 34 experienced mountaineers, submaximal VO_2 was significantly lower during exercise at 5533 m (Mt. Muztagh Ata, Western China) at intensities of 50 and 75% peak power output (PPO) on a cycle ergometer (1.35 ± 0.33 versus 1.18 ± 0.41L/min, $p = 0.017$ at 50% PPO and 1.75 ± 0.45 versus 1.61 ± 0.47L/min, $p = 0.027$ at 75% PPO) from day 6-7 compared to day 11. They also reported that the change in perceived effort during exercise (visual analog scale ranging from “not exhausting at all” to “extremely exhausting” was related to submaximal exercise economy (beta 0.52, $p = 0.04$). The authors attributed the differences in their findings compared to other studies to a larger sample size. One difference that was not discussed was subject population. Previous studies used subjects described as sea-level natives (1, 22), while Latshang et al. (16) recruited subjects described as experienced mountaineers. Perhaps, prolonged altitude exposure over a greater total amount of time spent at altitude explains the reduced submaximal VO_2 during exercise in these particular subjects. Indeed, researchers have reported that high-altitude adapted natives have improved economy (23) and muscle

efficiency (24) during exercise at altitude when compared to low- and moderate-land natives, which may allow them to tolerate a higher exercise capacity even with lower VO_{2max}. If the amount of oxygen for a given submaximal workload is reduced at altitude, this would indicate that the exercise intensity can be maintained using less oxygen. Since these factors can affect exercise performance (7, 12), understanding its role in exercise capacity at altitude is warranted.

The traditional method of acclimatization to high altitude involves traveling to high altitude terrain, whereas more contemporary methods involve acute exposure to simulated hypobaric or normobaric hypoxia. However, the ease and accessibility of traveling to high altitude for acclimatization purposes are limited to most individuals. Further, portable commercial systems used to simulate altitude are expensive, making them impractical. There are a growing number of people visiting high altitude (defined as ~2200 to 2500 m (25)) areas for recreation and work, and in addition there are many endurance sporting events that take place at high altitude locations. For individuals unable to acclimatize/acclimate using traditional methods, alternate training methods are needed to improve acute exercise capacity at altitude. This has led us in search of other training modalities that could be used to maintain or even enhance exercise capacity at altitude without having access to specialized equipment or to make altitude sojourns.

Recently, Heled and colleagues (10) reported that after 12 days of heat acclimation (40^{o}C temperature and 40% relative humidity) at sea-level, SaO_2 during walking exercise (7 km/h) at FIO_2 of 15.6% FIO_2 (simulated altitude of 2430 m) was significantly improved (86.5 ± 2% versus 88 ± 2%) from pre-heat acclimation to post-heat acclimation, respectively, which indicates improved oxygen transport. Further, they

speculated that reductions in metabolism from heat acclimation (HA) may contribute to better altitude tolerance. Unfortunately, the authors did not expand on this finding, nor did they measure submaximal VO_2. Hiestand et al. (11) investigated the responses to anoxia (extreme form of hypoxia) after heat acclimation in mice. Researchers reported that the longer mice were exposed to heat the better they were at tolerating anoxia (42.1 ± 3.5, 48.2 ± 5.9, and 54.3 ± 4.2 sec, respectively for no heat, 10 days and 14 days of heat exposure). Both Hiestand et al. and Heled et al. suggested that improved muscle economy due to HA may affect exercise at altitude. Given the findings of these two studies (10, 11), I hypothesize that heat acclimation at 1600 m might enhance exercise submaximal economy and efficiency at altitude.

Previous research provides evidence to support that HA improves submaximal VO_2 during exercise in a thermoneutral environment(13, 29). Jooste and Strydom (13) had subjects perform a progressively increasing step exercise (from 35 to 70W) during HA for four hours per day over a seven day protocol at 31^{o}C. Researchers reported that submaximal VO_2 was significantly lower after 90 min from pre to post HA of a four-hour exercise bout on a treadmill at 45% VO_{2max} at thermoneutral environment (20-22^{o}C). Since physical fitness was not different from pre to post HA, the authors concluded that the heat exposure led to reduced VO_2 for a given workload compared to pre-HA. Similarly, in a review of three HA studies, Sawka et al. (29) reported that 10 days of HA at varying heat exposures (40-49^{o}C at 20-30% RH) while walking on a treadmill (1.34 to 1.56 m/s) for two 50 minutes bouts separated by 10 minutes of rest, significantly lowered submaximal VO_2 by 3-7% in a temperate environment.

The phosphorylation of adenosine triphosphate (ATP) occurs from the release of energy as H^+ travels down the concentration gradient from the intermembranous space to the matrix of the mitochondria. This process is not entirely efficient, in part because of the presence of uncoupling protein 3 (UCP3) located throughout the innermembrane of the mitochondria of skeletal muscle (2). These proteins allow leakage of H^+ from the innermembranous space to the matrix, leading to a decreasing concentration gradient which can potentially reduce efficiency. There is evidence to support that mechanical efficiency (percentage of energy that goes to mechanical work) is negatively correlated with UCP3 in trained individuals (28, 30). For example, Schrauwen et al. (30) reported that trained individuals ($V0_{2max}$ = 66.9 ± 2.6 ml/kg/min) had lower UCP3 mRNA expression compared with untrained individuals ($V0_{2max}$ = 51.5 ± 1.5 ml/kg/min), and that with less UCP3 expression an individual is more efficient during submaximal exercise. Fernstrom et al. (8) further supported this hypothesis in reporting lower UCP3 mRNA and protein after six weeks of endurance training. They also found reduced uncoupling respiration in mitochondria that were isolated from human skeletal muscles. The findings of these studies suggest that lower expression of UCP3 leads to improved ATP coupling, which may lead to less oxygen consumption to produce ATP (or improved economy and efficiency).

In humans, UCP3 mRNA expression is positively correlated ($r = 0.86$, $p < 0.05$) with the difference in energy expenditure from two different continuous (60 hours) moderate cold exposures (16^oC versus 22^oC) (31). Researchers concluded that since 24 hour energy expenditure increased along with greater UCP3 expression, UCP3 regulates energy production. It has also been reported that UCP3 can be up-regulated 2-3 fold in rat

skeletal muscle after 24-hours of cold exposure at 5^{o}C (19, 33). If cold exposure promotes greater UCP3 expression leading to an increase in mitochondrial uncoupling (33) and therefore increasing thermogenesis, perhaps heat stress would lower UCP3 expression reducing mitochondrial uncoupling leading to enhanced muscle economy and efficiency. In humans, it has been suggested that HA may induce changes in mitochondrial function leading to improved muscle efficiency (13). Since UCP3 was only discovered in the 1997 (2), little research exists investigating its role in muscle economy and efficiency after heat stress. Only a few studies have investigated the effect of heat stress on uncoupling proteins. Using an animal model, Mujahid et al. (26) reported that 18 hours of continuous heat exposure reduced avian uncoupling protein (avUCP, which has 70% homology to mammalian UCP3 (27)) expression in broiler chickens, providing support that heat stress may lower UCP3 expression. More recently, in humans Slivka et al. (34) and Dumke et al. (6) reported that 1 hr of exercise followed by 3 hours of passive recovery in the heat (33^{o}C and 40^{o}C, respectively) did not affect UCP3 expression. At least in these studies, it appears that heat stress may not affect UCP3. Perhaps the shorter duration of heat stress or lower temperature by the work of Slivka et al. (34) and Dumke et al. (6) may explain the differences of their findings compared to those reported by Mujahid et al. (26).

Given the findings that HA improves submaximal economy during exercise in a thermoneutral environment, and the previous observations that HA improves SaO_2 and exercise tolerance in a hypoxic environment (10, 11) it is plausible that HA can be used as a cross environmental stressor to improve submaximal economy and efficiency during exercise at altitude, therefore indicating that similar adaptations occur from both heat and

altitude exposure. One possible mechanism for this phenomena is that reduced UCP3 expression leads to better ATP coupling, and this reduces the amount of VO_2 needed for the resynthesis of ATP during submaximal exercise.

Study purpose and hypotheses

The purpose of this study was to determine the effects of a cross-environmental stressor of 10 days of heat acclimation on improvements in submaximal exercise economy and efficiency both at 1600 m and 4350 m in trained individuals and to investigate possible mechanisms using a cell model.

Purposes of this Study

1. Human model: To determine whether exercising in a hot and humid environment leads to increased exercise economy and efficiency during submaximal exercise in a thermoneutral environment at 1600 m and 4350 m.
2. Cell model: To determine if C2C12 murine myocyte exposed to 24 hr of heat (40^oC) expresses lower UCP3 mRNA, UCP3 protein and reduced uncoupling.

Hypotheses

In this study I tested the following hypotheses:

1. Ten days of heat acclimation will increase economy and efficiency during exercise at 1600m and 4350m.

   After 10 days of heat acclimation, Sawka et al. (29) reported that exercise economy is improved by 3-7% in a temperate environment, but this hypotheses

has not been tested during exercise at altitude. Mechanical efficiency is defined as a ratio of the amount of energy produced relative to the metabolic energy used for movement. A previous report has suggested that high-altitude natives have improved efficiency during exercise which has been used to explain their high work capacity at altitude (24). This hypothesis has not been tested in non-acclimated individuals or after heat acclimation. Exercise economy and efficiency are important aspect of exercise capabilities at sea-level, and due to a reduction in oxygen transport at altitude, improved economy of movement and efficiency may perhaps be beneficial during exercise in hypoxia.

2. After 24 hours of heat exposure UCP3 mRNA, UCP3 protein, and metabolic rate will be reduced when compared to the control.

   There are two human studies that have reported no change in UCP3 mRNA expression after acute (4 hours) heat exposure (6, 34). There are no studies that have investigated changes in UCP3 mRNA, UCP3 protein and metabolic rate after 24 hours of heat stress. One study investigated the effects of one hour heat stress on C2C12 myotubes on mitochondrial proteins (20). They reported increases in the mitochondrial biogenesis proteins, nuclear receptor of factor 1/2, mitochondrial transcription factor, cytochrome II and IV(20). There currently are no data investigating the effects of prolonged heat stress on mitochondrial density and metabolic function on C2C12 myocytes. Mujahid et al. (26) reported lower avUCP3 in broiler chickens after 18 hours of continuous heat stress, so I conducted preliminary experiments on C2C12 myocytes using a 24-hr continuous heat exposure at 40^{o}C, and observed increased mitochondria,

reduced mitochondrial uncoupling, lower basal oxygen consumption and reduced UCP3 mRNA expression in the heat stress versus control cells.

Limitations

1) In this study I measured submaximal exercise economy and efficiency before and after 10 days of HA at 1600 m and 4350 m. Heat acclimation has been shown to increase exercise economy at sea-level (32), while there is limited investigation on efficiency. However, no studies have looked at the effects of HA on acute submaximal economy and efficiency at high altitude. It is unclear if exercise at acute altitude exposure will affect the hypoxic ventilatory response that leads to higher ventilation rate. A limitation is that high ventilation rate at altitude may increase respiratory exchange ratio (RER), limiting our ability to accurately calculate efficiency.
2) Subjects exercised at low workloads (30 and 20% below the corrected power output derived from graded exercise tests at 1600 m and 4350 m) to ensure subjects can reach steady-state at 4350m. A limitation to the low workload is that these intensities may not represent the range of actual exercise intensities individuals might perform when making altitude sojourns.
3) In this study, each individual served as their own control from pre-heat acclimation to post-heat acclimation. A limitation is that there was no control group. However, using trained cyclists exercising at these low intensities, I assume there would not be a training effect after the 10 day

heat acclimation protocol. Similar HA studies using individuals with an average VO_{2max} >53 ml/kg/min did not report any training effect (10, 13, 21).

4) This was a 10-day heat acclimation protocol looking at exercise during acute exposure to 4350 m. If I report improvements in submaximal exercise economy and efficiency at 4350 m, the results can only be used for individuals making acute (one day) altitude sojourns. The results cannot be extended to chronic altitude exposure.
5) I recruited trained individuals to participate in this investigation in order to control for fitness and training changes during HA. A limitation to subject selection is that the findings may only be applicable to less or more trained individuals.
6) I measured whole body VO_2 in humans to calculate submaximal exercise economy and efficiency changes after HA when exercising at 1600 m and 4350 m. To show "proof of concept" I used an *in vitro* model using C2C12 murine myocytes exposed to 24 hours of heat at 40^{o}C. Using an *in vitro* model may be a limitation to our ability to explain the physiological adaptations that occur *in vivo*.
7) A limitation of the *in vitro* model is that cellular metabolism was only measured at 1600 m. In the *in vitro* model, metabolic rate and efficiency of the C2C12 myocytes can only be measured at 1600 m due to technical issues with our ability to use the laboratory equipment to measure cellular metabolic rate and efficiency in the altitude chamber (4350 m).

Significance of the Study

The ability to improve submaximal exercise economy and efficiency during exercise is important to an individual's ability to better tolerate exercise both at sea-level and at altitude. If molecular adaptations from heat acclimation induce improved economy and efficiency that led to better exercise tolerance at 4350 m, it may provide an alternative method of altitude acclimation that would not require altitude exposure. This would be advantageous to the general population traveling to moderate and high altitude for recreational activities and work. From the use of an *in vitro* model, I can gain a better understanding of how heat stress affects cellular metabolism as this may have both sport and clinical implications. The understanding of how mitochondria adapt to heat stress would give us a better understanding of cellular function and therefore may be beneficial for individuals who suffer from varying forms of mitochondrial disease. For example, if heat stress induces cellular adaptation that improves whole body function, individuals with mitochondrial disease who have low exercise tolerance would then be able exercise for longer durations to improve fitness level.

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CHAPTER 2

REVIEW MANUSCRIPT

This chapter presents the review manuscript, titled “The use of a cross-environmental stressor of heat acclimation on skeletal muscle function during acute exercise at altitude”. This manuscript will be submitted to Temperature. It is authored by Roy M. Salgado, Ailish C. White, Suzanne M. Schneider, Daryl L. Parker, Len R. Kravitz and Christine M. Mermier. The manuscript follows the formatting and style guidelines of the journal. References are provided at the end of the chapter.

The use of a cross-environmental stressor of heat acclimation on skeletal muscle function during acute exercise at altitude

First and Corresponding Author: Roy M. Salgado[1]
Email: demano@unm.edu

Second Author: Ailish C. White[1]
Email: ailish15@unm.edu

Third Author: Suzanne Schneider[1]
Email: sschneid@unm.edu

Fourth Author: Daryl L. Parker[2]
Email: parkerd@csus.edu

Fifth Author: Len R. Kravitz[1]
Email: lkravitz@unm.edu

Sixth Author: Christine M. Mermier[1]
Email: cmermier@unm.edu

[1]Department of Health, Exercise and Sports Science, University of New Mexico, Albuquerque, NM 87131
[2]Deparment of Kinesiology and Health Science, California State University Sacramento, Sacramento, CA 95819

Abstract

High altitude exposure reduces oxygen transport from the lungs to the muscles, which contributes to the reduction in submaximal and maximal aerobic capacity which in turn reduces exercise performance. In mountaineers and high-altitude natives, enhanced exercise economy and efficiency has been attributed to improved altitude tolerance and work capacity compared to sea level natives. Heat acclimation increases sweat rate and cutaneous blood flow, lowers heart rate and induces plasma volume expansion and has also been shown to lower submaximal oxygen consumption or improve exercise economy during exercise in a thermoneutral environment. I propose the existence of a cross-environmental stressor model in which heat acclimation can be used to induce skeletal muscle adaptations, thus allowing for improved tolerance to hypoxia. I present evidence that heat acclimation lowers uncoupling protein 3 expression in the mitochondria which improves ATP coupling and enhances exercise economy and efficiency. In addition, using evidence from *in vitro* studies, I show that heat stress promotes muscle hypertrophy and mitochondrial biogenesis in muscle cells. I suggest that a prior program of heat acclimation can induce muscle adaptations, which will improve work during acute exercise at altitude. This novel cross-acclimation model has implications for individuals who have limited access to high altitude terrain or expensive equipment to simulate high altitude.

Key words: Altitude, Hypoxia, Heat tolerance, Skeletal muscle

Introduction

The effect of altitude (hypoxia) on the exercise capacity (VO_{2max}) in humans has been studied extensively[1, 2]. At higher altitudes, the lower barometric pressure causes a decrease in partial pressure of inspired oxygen reducing the pressure gradient of oxygen from the alveoli to the capillaries. The impaired oxygen transport from the lungs to the exercising muscles [3] results in a reduction in submaximal, and maximal exercise capacity [4] which lowers cycling time-trial performance [5] at altitude. Since VO_{2max} is reduced at altitude, oxygen consumption for a given exercise intensity represents a greater percentage of VO_{2max} when compared to sea level.

The amount of oxygen consumed at a given exercise intensity (power output or running velocity) is defined as the exercise economy (Watts/LO_2) [6-8], whereas, efficiency is defined as the ratio between physical work and energy expenditure while performing physical work (expressed as Kcals or as a percentage) [9, 10]. While VO_{2max} is a strong predictor of performance, submaximal variables such as economy and efficiency also determine exercise performance[11-13]. Conley et al. [14] reported that in trained and experienced runners with similar VO_{2max}, economy explained 65% of the variation in 10-km race times. Lucia and colleagues [15] suggested that high cycling economy and efficiency can compensate for a lower VO_{2max} in world-class professional cyclists, and contribute to their success in Grand Tour events.

In a hypoxic environment, oxygen transport from the lungs to the muscle is compromised [3], therefore an improvement in economy and efficiency could be beneficial for individuals exercising at altitude. Indeed, enhanced exercise economy (lower VO_2 for a given exercise work rate) has been found to improve exercise tolerance at altitude (5533

m) in mountaineers [16] and in amateur cyclists performing a simulated cycling time-trial at 2500 m [17]. While the role of efficiency during exercise is still debated, recent findings suggest a ~3.5% lower economy during submaximal cycling at a simulated altitude of 1500 m compared to sea level [18]. A lower efficiency at altitude suggests a loss of energy to do work. Perhaps, improved exercise efficiency is beneficial at altitude as more energy would be available for muscular work, rather than lost as heat production.

Many studies have examined how humans acclimate to high altitude. The traditional altitude acclimatization model is living and training at altitude [19-21] which requires that individuals reside at high altitude. Contemporary training model such as intermittent hypoxic training or exposure (IHE)[5, 22] require individuals to be acutely (3 weeks 4 hours a day five days per week at 4300 m) exposed to simulated altitude during rest or exercise. The primary benefits of high altitude acclimatization include an enhanced hypoxic ventilatory response and increases in arterial partial pressure and red blood cell production [23] which help restore oxygen-carrying capacity and contribute to improved exercise tolerance at altitude. Researchers have reported that continuous exposure to high altitude (traditional altitude training) is the optimal method to induce altitude acclimatization and to improve exercise tolerance at altitude [24, 25] while intermittent high altitude (hypobaric hypoxia or normobaric hypoxia) exposure provides an alternative less but effective approach to continuous altitude acclimation [24, 26]. However, limitations to both traditional and intermittent altitude exposures are that individuals may not have: 1) access to high altitude terrain, 2) time needed to spend at altitude or 3) the expensive equipment required to simulate high altitude. Thus,

alternative training approaches may be beneficial to those planning on making acute altitude sojourns.

The use of a cross-environmental stressor (CES) model for this purpose has not been thoroughly investigated [27]. There is some evidence from animal [28] and human [29] studies that prior heat acclimation HA may improve exercise tolerance in a hypoxic environment. Heat acclimation is reported to improve exercise economy by 5 to 10% during submaximal walking [30, 31] which may explain why exercise tolerance at altitude is improved [29]. However, the mechanism for the improved economy has not been fully elucidated. One suggestion is that HA improves slow-twitch motor unit recruitment in skeletal muscle during exercise, leading to lower exercise VO_2 [30]. I suggest an alternative hypothesis, in which skeletal muscle adapts to heat stress through adaptations at the cellular level. Such adaptations may include decreased uncoupling protein 3 (UCP3) within mitochondria, or an increased mitochondrial density. These changes may improve exercise economy and efficiency and lead to improvements in exercise capacity. From *in vitro* models, heat-stressed muscle cells have increases in myosin heavy chain composition [32], up-regulation of peroxisome proliferator-activated receptor co-activator 1α (PGC1α) [33], and in animal studies an up-regulation of calcineurin [34]. These adaptations may lead to an enhanced exercise performance in hypoxia.

This approach of using HA as a CES to induce muscular adaptations to improve exercise responses at altitude provides a novel training method. Therefore, the aim of this review is to present evidence of the potential benefits of this concept with focuses on skeletal muscle adaptations due to heat stress and how it may affect acute work capacity at altitude. For recent reviews on topics that address the changes with HA in the

cardiovascular system, polycythemia and vascular growth, see two papers by White et al. and Salgado et al. [35, 36]. This paper will address data concerning: 1) the changes in oxygen consumption and exercise performance at altitude, after acute and chronic exposure, 2) evidence for an increased economy and efficiency during HA in a temperate environment, and finally, 3) the possible role of heat stress on mitochondrial biogenesis and UCP3 expression, and how these adaptations may improve exercise capacity at altitude.

Oxygen consumption and exercise performance at altitude

Acute altitude exposure and changes to VO_{2max} and performance

The effects of acute altitude exposure on VO_{2max} and exercise performance have been well characterized. Squires and colleagues [37] reported reductions in treadmill running VO_{2max} in 12 healthy males at altitude of 4, 8, 7, and 12%, with corresponding reductions in arterial oxygen saturation (SaO_2) of 3.5, 3.6, 7.0 and 11.6%), at 914, 1219, 1524 and 2286 m above SL, respectively. Dill et al. [38] had four individuals perform maximal exercise on a cycle ergometer at varying simulated altitude (sea level, 2800, 3629 and 4120 m) in a hypobaric chamber. Researchers reported a 10, 14, and 19% reduction in VO_{2max} compared to sea level, with a 5, 9, and 14% reduction in work capacity, respectively. Wehrlin et al. [39] reported a significant reduction in VO_{2max} below 1000 m in endurance trained runners (VO_{2max} = 66.1 ± 4.3 ml/kg/min). The researchers investigated the effects of low-to-moderate altitude on oxygen consumption and exercise capacity and reported a reduction in time-to-exhaustion of 14% for every 1000 m increase in altitude during maximal running. Fulco et al. [25] acutely exposed 10 healthy sea-level residents to 4300 m; the authors reported a significant reduction in VO_{2max}

(3636 ± 215 ml/min at SL versus 2693 ± 89 ml/min at ALT), simulated 720 kJ cycling time-trial (TT) performance (73.2 ± 6 minutes at SL versus 111.4 ± 6 minutes at altitude), and power output during the TT (150.0 ± 5 Watts at SL versus 100.4 ± 10 Watts at ALT).

Submaximal exercise oxygen consumption after altitude acclimation

During acute and after chronic altitude exposure, VO_{2max} is decreased and is only minimally regained even after chronic exposure. Since VO_{2max} is reduced, the ability to maintain a given submaximal intensity compared to sea level is decreased [40, 41]. In this section I review the current findings regarding changes in submaximal oxygen consumption after altitude exposure in both native low-landers and high-landers, and how enhanced submaximal oxygen consumption may aide in exercise and work performance at altitude.

Maher and colleagues [42] investigated the effects of 12 days of high-altitude (4300 m) exposure on submaximal endurance capacity in eight sea level natives. Subjects exercised at sea level at 75% of sea level VO_{2max} and during acute and chronic altitude exposure. At sea level, this corresponded to 73.1% of VO_{2max} (2.70 L/min), while at altitude it was 78.7% (2.13 L/min) and 76.2% (2.06 L/min) of VO_{2max} on days 2 and 12 of the altitude sojourn, respectively. Exercise economy for a given intensity from days 2 to 12 was not significantly lower; however, the researchers found endurance running time was greater after day 12. The authors concluded that submaximal endurance could increase without significant changes in VO_{2max}. They attributed this phenomenon to an increase in 2, 3DPG which causes a right-ward shift in the oxygen-hemoglobin disassociation curve leading to greater oxygen unloading at the muscle.

Bender et al. [43] investigated the effects on oxygen transport in seven military soldiers before and after acclimatization at 4300 m (Pikes Peak, Colorado). The investigators reported that VO_{2max} at altitude was not different before or after acclimatization (2584 ± 120 mL/min versus 2565 ± 105 mL/min), and that for a given submaximal work-load (0, 60, 125, and 185 Watts (W)), submaximal VO_2 was not different. They did however report that arterial oxygen content and hemoglobin concentration (Hb) were significantly higher after altitude acclimatization, indicating greater oxygen transport. In another study, Lundby et al. [44] investigated the effects of acute and chronic acclimatization (four weeks) of high altitude on substrate utilization at 4100 m (La Paz, Bolivia). The researchers reported no change in plasma catecholamine levels or substrate utilization during 60 minutes of cycling at a workload corresponding to 45% of VO_{2max} at altitude. They also reported that economy was not different between acute and chronic exposure to altitude (1.6 ± 0.1 L/min versus 1.4 ± 0.2 L/min). These two studies indicate that after 12 to 28 days of continuous altitude exposure (~4100 m), there is no improvement in economy.

To our knowledge, only two studies have shown enhanced economy after chronic altitude exposure. Macdonald et al. [45] investigated leg blood flow and whole body VO_2 responses in five healthy men before and after a 21-day expedition at 6194 m (Mt. Denali, Alaska). Using a custom-built leg tension-flexion ergometer, they found that submaximal VO_2 (1290 ± 29 mL/min) was lower when exercising at 50 W after the 21-day expedition compared to before (1413 ± 63 mL/min) altitude exposure which equates to an 8% improvement in exercise economy. The authors concluded that enhanced economy during exercise was due to either improvements in mechanical efficiency

during the leg exercise (subjects became familiar at performing the leg ergometer exercise) or, perhaps, muscle efficiency was enhanced due to altitude exposure.

More recently, Latshang et al. [16] investigated the effects of enhanced exercise economy in mountaineers during an expedition at 5533 m (Mt. Muztagh Ata, Western China). On days 5, 6 and 11 at 5533 m, subjects performed submaximal exercise on a cycle ergometer equating to 50 and 75% of their peak power output (PPO) (107 ± 26 and 152 ± 37 W, respectively). The researchers reported that on day 11, submaximal VO_2 was significantly lower compared to day 5 or 6, both at 50 and 75% PPO (1.18 ± 0.41 versus 1.35 ± 0.33 L/min at 50% PPO and 1.61 ± 0.47 L/min versus 1.75 ± 0.45 L/min, $p < 0.027$ at 75% PPO). They also reported an 8 and 7.8% improvement in SaO_2 and a 5 and 7.3% reduction in heart rate during submaximal exercise at 50 and 75% PPO. Using multiple regression analysis, the authors concluded that the lower submaximal VO_2 was a significant predictor of perception of effort during the two summit attempts. The investigators attribute the enhanced efficiency at altitude to a decrease in heart rate for a given workload (due to lower sympathetic tone), reduced basal metabolic rate or a decrease in mitochondrial oxygen consumption.

Even though submaximal exercise performance is improved after prolonged altitude exposure, studies have shown that these changes may not necessarily due to changes in submaximal VO_2. However, two studies have reported reduced oxygen consumption after prolonged altitude exposure for a given exercise intensity [16, 45]. It is not clear why there is a discrepancy in submaximal VO_2 after chronic exposure to altitude, but it may be specific to the subject population. For example, the subjects in the studies that found no differences in submaximal VO_2 after chronic altitude exposure were all sea

level natives. In comparison, in studies where researchers reported enhanced exercise economy , the subjects were described as mountaineers[16, 45]. Unfortunately, the researchers did not give details about the mountaineering experience of their subjects or the amount of time in which they have spent at high altitude. Perhaps previous high altitude exposure has an additive effect on improving submaximal VO_2 when compared to sea level natives. In high-altitude natives, evidence supports the idea that prolonged altitude exposure improves submaximal VO_2 and muscle efficiency[46, 47] indicating perhaps that chronic exposure over multiple times is needed to alter economy and efficiency.

Even with relatively low VO_{2max}, high altitude natives have a greater work capacity at altitude when compared to acclimatized sea-level natives[46, 47] leading some investigators to suggest that natives are efficient during exercise. Matheson et al. [47] investigated muscle efficiency in four different groups during exercise at simulated high altitude. These groups were: 1) altitude-adapted Andean natives, 2) sedentary sea level natives, 3) power trained sea level natives and 4) endurance-trained sea level natives. Muscle function at the cellular level was measured using P^{31}-NMR as subjects exercised using a leg ergometer while breathing room air or FIO_2 of 14.5%. The researchers reported that, even though VO_{2max} and power outputs were significantly lower for a given intensity in the Andean natives, results from the P^{31}-NMR showed that muscle pH, [PCr], [Pi] and fatigue were similar between the Andean natives and the endurance-trained subjects. This indicates that, at the cellular level, muscle efficiency is enhanced in the Andean natives both at sea-level and at a simulated altitude. The authors also suggested that in the Andean natives, ATP resynthesis in the mitochondrial respiration chain

requires a lower VO_2. Unfortunately, submaximal VO_2 was not measured during exercise to support their assumption. Marconi et al. [46] found that high-altitude adapted Tibetans who migrated to 1300 m had a lower VO_2 consumption of 8, 10, and 13% during walking at 6 km/h at 10, 12.5 and 15% grade and running at 10 km/h at 5% grade at 1300 m when compared to Nepali natives. The authors proposed that high-altitude natives have a reduced VO_2 during exercise due to better ATP coupling.. Other researchers have suggested that high altitude Andean natives have better ATP coupling allowing for improved economy and efficiency[48] which perhaps is from lower UCP3 expression. However, careful consideration should be taken when comparing these results to sea-level natives, as the high altitude native Andeans may have genetic adaptations that allow for improved economy and efficiency. While there is no evidence to support our hypothesis, perhaps these high-altitude dwellers have reduced uncoupling protein 3 (UCP3) leading to better ATP coupling, thus requiring less VO_2 for ATP production. To our knowledge, only one study has looked at UCP3 expression after altitude exposure [49]. Levett et al. [49] found significant reductions in the mitochondrial proteins citrate synthase, PGC1α and UCP3 in 12 mountaineers attempting to summit Mt. Everest from pre to post summit. Since submaximal VO_2 during exercise was not measured, I cannot conclude whether the reduction in UCP3 was associated with a lower VO_2 at altitude. I can only speculate that reductions in mitochondrial proteins after chronic high altitude exposure may indicate mitochondrial and muscle atrophy. Less mitochondrion would likely lead to lower submaximal and maximal oxygen consumption and impaired economy and efficiency.

Exposure to altitude reduces VO_{2max}, and thus individuals are exercising at a higher percentage of VO_{2max} for any given workload when compared to sea-level. Therefore, because exercise at altitude is more difficult, submaximal exercise performance also is reduced. In sea-level natives, submaximal VO_2 does not change at higher altitudes, however it is reduced in mountaineers and high-altitude natives. The improved economy and muscle efficiency in these individuals may explain their higher exercise tolerance and work capacity compared to sea level natives. Since these adaptations have not been reported in low altitude natives, perhaps a training method that induces similar muscle adaptations can be used as an alternative to altitude exposure for improvement of exercise tolerance at altitude.

Heat stress and the muscle

Submaximal exercise economy after heat acclimation

Exercising in the heat increases cardiovascular strain and lowers exercise capacity[50-52]. After HA, heat tolerance is enhanced during exercise resulting in lower core temperature [53], lower heart rate [53], increased cutaneous blood flow [54], plasma volume expansion [55], and enhanced sweat rate [56]. Another adaptation that has been observed after HA is a lowered metabolic rate during exercise in a hot environment [30, 57-59] and temperate environment[30, 31, 60, 61].

Robinson et al. [59] reported a reduction in VO_2 during exercise after HA. In their study, five subjects exercised in a hot environment (40^{o}C and 23% RH) for 10 to 23 days at varying individualized durations to induce fatigue. They reported that heart rate, core temperature and skin temperature were reduced after ~5 days and that mean VO_2 was decreased by 7.6% at the same level of exercise following HA. The investigators

suggested that since their subjects did not improve fitness levels from exercising in the heat, the lower mean VO_2 was attributed to a decrease in energy requirements in the heat due to acclimation. Subsequent studies have also reported lower VO_2 for a given absolute intensity after HA. Strydom et al. [62] heat acclimated African Nyasa mine workers for 5 hrs/day for 3 weeks at 36^{o}C. During the step test mean VO_2 was ~11, 16.7 and 21% lower on the 1st, 3rd and 5th hour between day 1 and day 12 of HA. Later, Gisolfi et al. [58] investigated the effects of prior high-intensity exercise on work tolerance in the heat using treadmill exercise. While the main purpose of their study was not to investigate submaximal VO_2 after HA, the researchers reported that after eight consecutive days of treadmill walking for 100 minutes at 5.6km/hr in the heat (29^{o}C), submaximal VO_2 was significantly reduced (day one 33.3 ± 0.3 versus day eight 24.5 ± 1.49 ml/kg/min). Shvartz et al. [31] investigated the physiological responses during exercise in a thermoneutral and hot environment in men of varying aerobic capacity after HA. In their study, 26 healthy young men (trained 57-65 ml/kg/min, untrained 43-50 ml/kg/min, unfit 29-38 ml/kg/min and control 41-49 ml/kg/min) participated in an eight-day HA intervention. The researchers reported that, as a whole, average submaximal VO_2 was ~11 and 8% lower at the low (41 W) and moderate (82 W) exercise intensities during exercise in a cool environment and 10% lower during exercise in the heat. Researchers[55,53] also reported an increase in VO_{2max} after HA. Given their results, it can be speculated that the improvement in submaximal VO_2 may be attributed to an increase in VO_{2max}; an increased VO_{2max} could cause those exercising at a given submaximal workload to be working at a reduced VO_2, and therefore may lead to better exercise economy. For example, the unfit subjects' VO_{2max} increased 18%, while VO_2 at 41 and

82 W was reduced by ~16 and 11%, respectively. However, the increase in VO_{2max} may not fully explain the reduction in submaximal VO_2 (improved exercise economy) during exercise. In the trained individuals, submaximal VO_2 was significantly reduced by 8.5 and 10.7% at low and moderate work-loads, respectively, with a non-significant improvement in VO_{2max} (2.6%). This suggests that heat stress-induced exercise economy can be improved independent of changes in VO_{2max}.

Two studies specifically investigated the changes in submaximal VO_2 after HA. Jooste and Strydom [60] investigated the effects of HA on various physiological factors related to efficiency. Four male subjects were exposed to heat (31ºC) for four hrs/day over a seven day period. During heat exposure, subjects exercised using a step protocol where exercise intensity was progressively increased from 35 to 70 W. To assess submaximal VO_2, subjects exercised on a treadmill for four hours at 45% VO_{2max} in a cool environment (20º-22ºC). These investigators reported that VO_2 was significantly lower in the HA subjects during the last 90 min of exercise in the cool environment from pre to post HA. They concluded that reduced oxygen consumption after HA was attributed to improved economical caloric expenditure (energy expenditure). Sawka et al. [30] provided further support that HA could reduce submaximal VO_2 during exercise in a temperate environment. Researchers concluded that 10 d of HA using varying protocols (40-49^{o}C at 20-30% RH) while exercising on a treadmill at 1.34 to 1.56 m/s, for two 50 minutes bouts separated by 10 minutes of rest significantly lowered submaximal VO_2 by 3-7% in a temperate environment. They suggest that lower VO_2 may be due to more efficient recruitment of type I muscle fibers. However, Young et al. [57] later found that

after 9 consecutive days of HA (49^{o}C, 20%RH), submaximal VO_2 before and after HA was not different (2.35 L/min and 2.33 L/min, respectively) in a temperate environment.

Piwonka and Robinson [63] investigated the effect of HA in trained runners. Using a protocol similar to Robinson et al. [59], Piwonka and Robinson [63] exposed four subjects to acute heat exposure (4 d) while walking on a treadmill at 5.6km/hr at grades up to 5.6% in a hot environment (40^{o}C) for 85 min/d. While the authors did not directly measure VO_2, they reported that metabolic rate (calculated using the heat storage equation) was not different after acute heat exposure. Furthermore, Wyndham et al. [64] reported that after 10 days of HA in which subjects exercised at 40-50% of VO_{2max}, VO_2 during exercise was not different (1.1 L/min for pre-HA versus 1.2 L/min after day 10)..

Exposure to heat increases cardiovascular strain especially during exercise, which acutely lowers exercise tolerance in a hot environment. While some investigators have observed no change in submaximal VO_2, most researchers have reported reductions both in unfit and fit individuals after HA. It is unclear as to the discrepancies within the results. A possible explanation is that study protocols were not uniform as they used different modes of exercise during the HA (step exercise and treadmill walking). Perhaps, individuals performing step exercise became more familiar with the mode of exercise during the HA protocol leading to an observed improved EC. Nevertheless, while the largest change in submaximal VO_2 after HA in both hot and temperate environments were from studies using step-testing, reduces VO2 during exercise has also been observed from studies using treadmills.

Mechanisms of improved submaximal VO_2 from heat acclimation

The mechanisms involved in the improvement of submaximal VO_2 during exercise have not been fully elucidated. Researchers have reported these improvements in economy with comparisons of untrained and trained populations [65, 66] and after HA [30, 60]. A lower VO_2 for a given workload indicates improved exercise economy and suggests that there may be adaptations at the cellular level allowing for lower oxygen cost to produce the same amount of ATP during exercise.

One explanation that has been proposed for improved submaximal economy and efficiency has focused on the characteristics of skeletal muscle fiber type, with type I fibers suggested to be more economical and efficient during exercise [67, 68] compared to type II fibers. We propose an alternate hypothesis that heat stress induces a change in skeletal muscle by decreasing UCP3 on the mitochondrial membrane, which in turn improves mitochondrial uncoupling. In addition, heat stress up-regulates signal transduction pathways which cause changes in muscle fibers such as increase in mitochondrial biogenesis[33] which may lead to an improved exercise economy.

In 1997, Boss et al. [69] discovered a new member of the mitochondrial protein family located on the inner membrane of the mitochondria, which was named UCP3. This protein is specifically expressed in skeletal muscle and has been suggested to play a role in the uncoupling of oxidative phosphorylation [69]. On the inner membrane of the mitochondria, protein complexes (complex I, II, and IV) pump H+ from the matrix to the intramembranous space. This leads to an increase in the concentration gradient between the intramembranous space and the matrix which is also known as the chemi-osmotic gradient [70]. Since H+ is impermeable to the inner membrane, it travels down the concentration gradient through the FO-FI complexes located throughout the inner

membrane where it releases free energy required for phosphorylation of ADP + Pi and forms ATP [70]. Uncoupling protein 3s are located throughout the membrane and cause H+ to leak from the intermembranous space to the matrix which is termed the uncoupling process. Vidal-Puig et al. [71] highlighted the relationship between UCP3 and mitochondrial uncoupling when they reported better ATP coupling and lower cellular VO_2 consumption in the skeletal muscle of UCP3 knock-out mice compared to their wild-type counterparts. These results suggest that UCP3 plays a role in lowering muscle respiration, thereby improving muscle efficiency.

Uncoupling protein 3 has been shown to have a negative relationship with metabolic efficiency during exercise in trained versus untrained individuals. Schrauwen et al. [72] reported that UCP3 expression was correlated with exercise efficiency. Researchers compared the relationship between efficiency and training status in 18 male subjects. Subjects performed a maximal exercise test and three 15-minutes bouts of submaximal exercise at 30, 45, and 60% of their peak power output on a cycle ergometer to determine efficiency. Researchers found that: 1) trained individuals (VO_{2max} - 66.9 ± 2.6 ml/kg/min) expressed lower UCP3 mRNA compared to untrained individuals (51.5 ± 1.5 ml/kg/min), 2) UCP3 mRNA was negatively correlated with VO_{2max} ($r = -0.61$, $p = 0.009$), and 3) UCP3 mRNA was negatively correlated with mechanical efficiency during submaximal exercise ($r = -0.56$, $p = 0.019$). Fernstrom and colleagues [73] further found that UCP3 was significantly lower after six weeks of an endurance training intervention Subjects trained for 1 hr four times per week for six-weeks at 70% VO_{2max} for first 30 minutes followed by 30 minutes of interval training. The investigators also reported a significant State 4 uncoupling respiration, defined as oxygen consumption by

the mitochondria, (pre 7.7 ± 0.6 versus post 6.3 ± 0.3 nmolO_2/min) after an endurance training intervention. Others have found similar reductions in mitochondrial respiration[72, 74]. Findings from these studies [72-74] provide evidence that: 1) trained individuals have reduced UCP3 mRNA expression which is correlated with improved efficiency compared to untrained individuals, 2) UCP3 expression is lower after chronic endurance training, and 3) mitochondrial uncoupling respiration is lower with lower UCP3 expression. It could therefore be concluded that improved training status (endurance- trained for > 6 week) leads to lower mitochondrial uncoupling (improved cellular efficiency) and a reduced VO_2 for a given submaximal workload.

Research evidence suggests that cold exposure increases UCP3 in both human [75] and rat [76] skeletal muscle which leads to greater uncoupling and increased thermogenesis. In cold environments, this adaptation causes an increase in core temperature which improves cold tolerance, but also lowers muscle efficiency. If cold stress increases UCP3 in skeletal muscle which promotes thermogenesis, it is plausible that chronic heat stress over a prolonged period of time could lower UCP3 expression. This adaptation could lead to lowered thermogenesis and improved muscle efficiency and may be beneficial during exercise. This hypothesis that we are proposing could explain why submaximal economy and efficiency is improved after HA. However, the research on heat stress and UCP3 is limited. In an animal model, Mujahid et al. [77] exposed 3 week old broiler chickens to either a thermoneutral or hot environment (34°C) for 18 hour. They reported that there was a significant reduction in avUCP (73% homology to mammalian UCP3) mRNA and protein in the heat stressed animals, supporting the idea that heat stress alone reduces UCP3 and lowers muscle thermogenesis.

Slivka et al. [78] investigated mRNA expression of mitochondrial proteins in nine recreationally active males following 1 hour of exercise and 3 hour of passive recovery at different environmental conditions. These environmental conditions were cold (7°C, 40% humidity), room temperature (20ºC, 40% humidity), or hot (33°C, 40% humidity). The researchers reported the different environmental temperatures did not affect mitofusion2 and UCP3 mRNA expression. It should be noted that while not statistically significant, UCP3 was slightly lower in the hot compared to the room temperature trial. Since UCP3 expression has been shown to be affected by fat oxidation, in their follow-up study Dumke et al. [79] investigated whether carbohydrate ingestion during acute exercise in the heat would attenuate changes in mitochondrial gene expression. Researchers reported that mitofusion2 and GLUT4 expression was not affected by carbohydrate or placebo during exercise in the heat. However, UCP3 mRNA expression was attenuated in the carbohydrate versus the placebo treatment. The work from this research group suggests that UCP3 may be affected by substrate availability, rather than environmental stress. However, these studies used acute heat stress (3 hours), so perhaps longer heat exposure at higher temperatures may show lower UCP3. More studies with longer heat exposure, such as using a HA protocol are needed to determine the effect of HA on UCP3 expression.

The effect of mitochondrial uncoupling on exercise performance suggests the uncoupling process plays an important role in work capacity. Schlagowski et al. [80] found that mice treated with 2-4 dinitrophenol (DNP), a pharmacological drug that transports protons across the innermembrane, increases mitochondrial uncoupling and resting oxygen consumption. Researchers reported a significant decrease (11%) in running speed

(42.4 ± 1.7 cm/sec in those treated with DNP vs. 47.6 ± 1.5 cm/sec in control) and impaired running economy (3.1 ± 0.1 ml/kg/min in control vs. 3.8 ± 0.2 ml/kg/min in DNP-treated mice).

Mechanism for changes in muscle characteristics from heat stress

Skeletal muscle has high plasticity as it has the ability to adapt to different stressors. In muscle cells heat stress alone appears to promote muscle hypertrophy [34] and causes up-regulation in signaling pathways that increase myosin heavy chain (MHC) [32] and mitochondrial biogenesis [33]. These pathways may lead to improvements in cardiovascular fitness level and exercise performance. Researchers found that after 60 minutes of exposure to acute heat stress during rest (41^{o}C), Wistar rats had an increase in intracellular calcineurin. They suggested the increase was activated by heat-stress induced intracellular calcium release, which then promoted muscle hypertrophy [81].

Yamaguchi et al. [32] exposed human skeletal muscle myotubules (HSMM) and C2C12 cells to varying temperatures (37^{o}C, 39^{o}C and 41^{o}C) for up to 72 hours. The investigators found that HSMM were larger in diameter (hypertrophy) when cells were exposed to 39^{o}C at 72 hour compared to the control treatment (37^{o}C). They also reported an 1.6 fold increase in MHCI protein ($p < 0.01$) after 72 hours, and a 1.8 fold and 2.1 fold increase in MHCII protein ($p < 0.05$) compared to control treatment after being exposed to 39^{o}C for 48 and 72 hours respectively. To investigate the mechanism of action, they measured the co-transcription factor PGC1α and found an increase after 48 hours ($p < 0.05$) and 72 hours ($p < 0.01$) in C2C12 cells, but only an increase in PGC1α mRNA after 24 hour in HSMM. The investigators suggested that heat stress limits the ability of the sarcoplasmic reticulum to re-uptake calcium. Calcium activates the PGC1α pathway and

promotes changes in MHC proteins [82], which also acts to regulate mitochondrial biogenesis [83]. Liu et al. [33] exposed C2C12 myotubes to 1 hour of heat (40^{o}C) for 5 days followed by 24 hour of incubation in a thermoneutral environment. They showed increases in PGC1α, complex I, II, III, IV proteins and ATP synthase compared to control treatment (37^{o}C), which further supports the idea that heat stress promotes mitochondrial biogenesis.

In summary, in humans, HA has been reported to reduce VO_2 (improve economy) during submaximal exercise, which we propose is mediated by a decrease in UCP3 and improved mitochondrial uncoupling. This adaptation may lead to the production of the same amount of ATP which then requires lower oxygen consumption compared to non-heat acclimated individuals. In addition, in animal and cells models, it has been found that heat stress induces changes in skeletal muscle that are similar to endurance training. These include muscle hypertrophy and mitochondrial biogenesis, which improves skeletal muscle function leading to improved sports performance. A combination of these heat-induced adaptations might be beneficial as a cross-environmental stressor training model for individuals making acute altitude sojourns where oxygen transport limits exercise performance. Perhaps these adaptations allow for an improved muscle function when the muscle is hypoxic.

There are potential negative effects from adaptations due to heat acclimation that may limit exercise capacity at altitude. Previous examinations show that HA can significantly increase plasma volume by 6.5%, which leads to an increase in cardiac output and stroke volume[55]. Plasma volume expansion causes a hemodilution effect which leads to less viscous blood. This effect reduces transit time of blood passing

through the pulmonary system reducing gas diffusion, impairing oxygen transport and therefore reducing oxygen saturation at altitude. The plasma volume expansion can also reduce hemoconcentration effect commonly observed at altitude which is an adaptation that occurs to increase oxygen carrying capacity. In addition, after HA researchers[84] have reported higher blood flow to the skin to dissipate heat during exercise. At altitude, this re-direction may lead to a competition of blood flow to the skin rather than the muscles therefore reducing oxygen transport.

Conclusion

Exposure to altitude decreases oxygen transport which leads to a reduction in maximal and submaximal oxygen consumption and impairs exercise performance. During prolonged altitude exposure there is an increased hypoxic ventilatory response and polycythemia that acts to restore oxygen transport and contributes to improved work capacity. High altitude natives and individuals who have repeatedly climbed high-mountainous terrain have an improved exercise economy and efficiency which aids in their high altitude work capacity. While these adaptations are beneficial during altitude sojourns, they do not occur in sea level natives. We propose that individuals preparing for an altitude sojourn use the cross-environmental stressor model of HA to improve exercise economy and efficiency. In heat stressed muscle cells, promotion of mitochondrial biogenesis and reduction in UCP3 has been established. These adaptations may be beneficial to improving muscle function during hypoxia.

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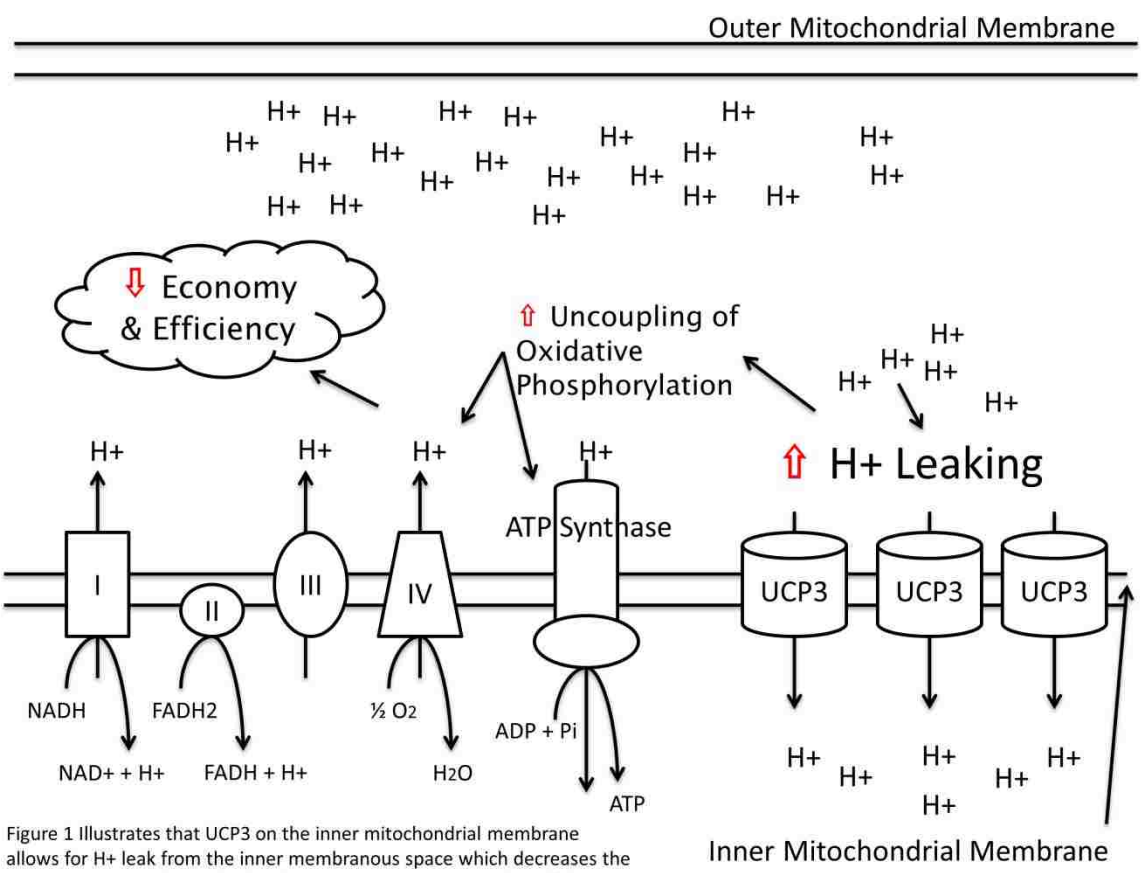

Figure 1 Illustrates that UCP3 on the inner mitochondrial membrane allows for H+ leak from the inner membranous space which decreases the concentration gradient. This leads to an increase in uncoupling of the oxidative phosphorylation leading to reduced economy and efficiency.

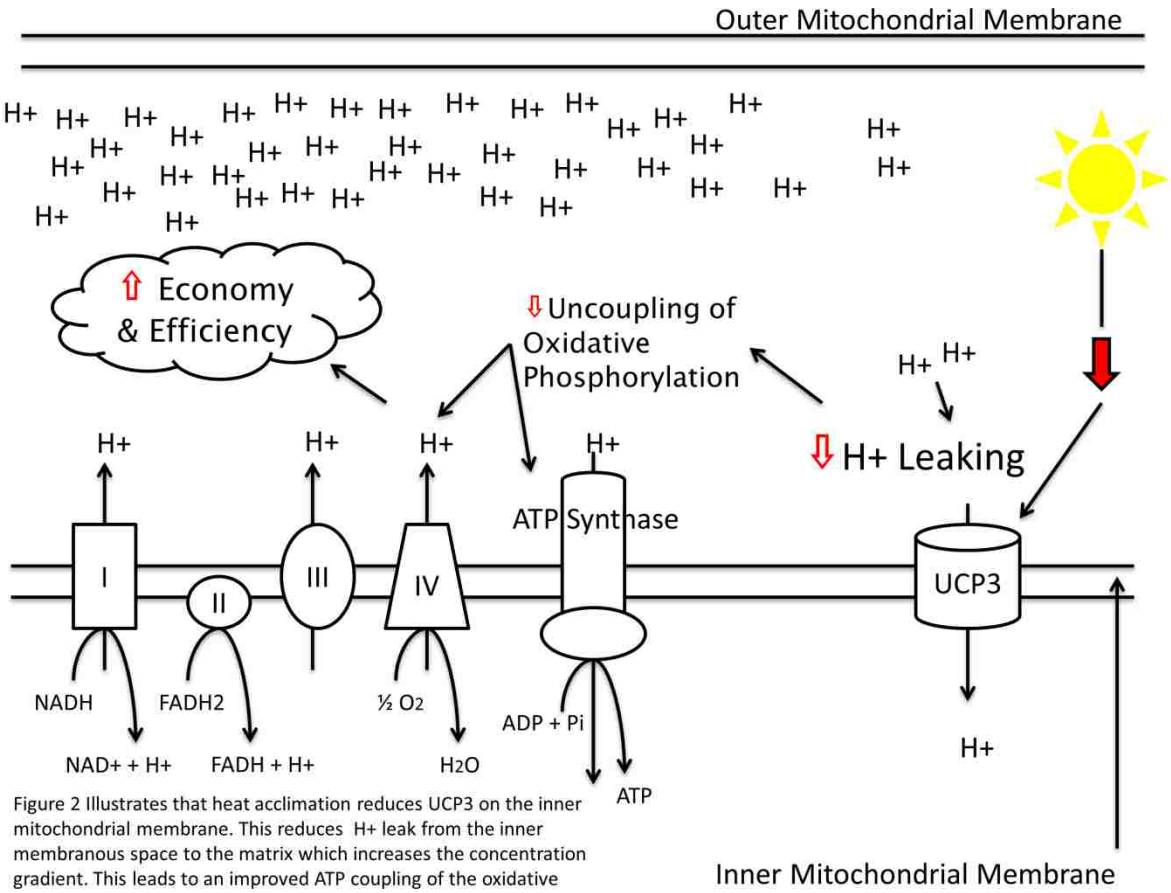

Figure 2 Illustrates that heat acclimation reduces UCP3 on the inner mitochondrial membrane. This reduces H+ leak from the inner membranous space to the matrix which increases the concentration gradient. This leads to an improved ATP coupling of the oxidative phosphorylation leading to improved economy and efficiency. .

CHAPTER 3

RESEARCH MANUSCRIPT

This chapter presents a research manuscript, entitled "The effects of ten days of heat acclimation on submaximal exercise economy and efficiency at 1,600 and 4,350 m." This manuscript will be submitted to the European Journal of Applied Physiology. It is authored by Roy M. Salgado, Ailish C. White, Roger A. Vaughan, James J. McCormick, Nicholas P. Gannon, Trisha A. Vandusseldorp, Suzanne M. Schneider, Daryl L. Parker, Len R. Kravitz and Christine M. Mermier. The manuscript follows the formatting and style guidelines of the journal. References are provided at the end of the chapter.

The effects of ten day heat acclimation on submaximal exercise economy and efficiency at 1,600 and 4,350 meters.

First and Corresponding Author: Roy M. Salgado[1]
Email: demano@unm.edu

Second Author: Ailish C. White[1]
Email: ailish15@unm.edu

Third Author: Roger A. Vaughan[1-3]
Email: vaughanr@unm.edu

Fourth Author: James J. McCormick[1]
Email: diamondxk12@comcast.net

Fifth Author: Nicholas P. Gannon[2]
Email: ngannon@unm.edu

Sixth Author: Trisha A. Vandusseldorp[1]
Email: tvandusseldorp@unm.edu

Seventh Author: Suzanne Schneider[1]
Email: sschneid@unm.edu

Eighth Author: Daryl L. Parker[4]
Email: parkerd@csus.edu

Ninth Author: Len R. Kravitz[1]
Email: lkravitz@unm.edu

Tenth Author: Christine M. Mermier[1]
Email: cmermier@unm.edu

[1]Department of Health, Exercise and Sports Science, University of New Mexico, Albuquerque, NM 87131

[2]Department of Biochemistry and Molecular Biology, University of New Mexico Health Sciences Center, Albuquerque, NM 87131
[3]Department of Individual, Family, and Community Education: Nutrition, University of New Mexico, Albuquerque, NM 87131
[4]Deparment of Kinesiology and Health Science, California State University Sacramento, Sacramento, CA 95819

Abstract

Heat acclimation is known to increase exercise economy. Previous examinations suggest heat acclimation may preserve performance at altitude. This study examined the effect of using heat acclimation as a cross environmental stressor to improve exercise economy and efficiency during acute exercise at altitude. Eight trained males (VO_{2peak}: 53.3 ± 6.7 ml/kg/min) performed maximal exercise tests, submaximal exercise bouts, and heat tolerance testing in a temperate environment (21°C) at 1600 m and 4350 m before and after a 10-day heat acclimation (40°C and 20% RH) on a cycle ergometer (~43% peak power). To investigate heat stress mechanisms, C2C12 myocytes were heat stressed for 24 hours (40°C, 5% CO_2). Heat acclimation did not alter VO_{2peak} at 1600 m (53.3 ± 6.7 vs. 53.7 ± 3.7 ml/kg/min, $p > 0.05$) or 4350 m (45.3 ± 4.1 versus 45.9 ± 3.4 ml/kg/min, $p > 0.05$). Heat acclimation increased exercise economy by 1.6% and 2% in the low intensity and high intensity exercise, respectively at 1600 m with only a 0.48% increase at 4350 m. In the cell study, heat stress significantly reduced UCP3 expression, reduced mitochondrial uncoupling (71.1% ±1.2%) and suppressed basal and peak oxidative metabolism (75.5% ± 4.9% and 64.4% ± 5.9%, respectively) compared to control. Heat stress also significantly increased PGC-1α, NRF1 and TFAM leading to increased mitochondrial content. These data demonstrate that while heat stress reduces UCP3 expression, thereby reducing uncoupling and leading to enhanced mitochondrial efficiency, these adaptations are not observed in the whole body. At this time, I am unable definitively promote the use of heat acclimation as a cross environmental stressor for acute exercise at altitude.

Keywords: Altitude, Hypoxia, Heat tolerance, Exercise Capacity, Skeletal muscle

Background

It is well established that exercise capacity is impaired during acute heat stress (Gonzalez-Alonso et al. 1999; Tatterson et al. 2000; Parkin et al. 1999). After heat acclimation (HA), heat tolerance is improved during exercise resulting in reduced heart rate (Sawka et al. 1983) (HR), enhanced sweat rate (Fox et al. 1964), increased cutaneous blood flow (Johnson 2010), plasma volume expansion (Lorenzo et al. 2010; Senay et al. 1976) and improved maximal aerobic capacity (Lorenzo et al. 2010) (VO_{2max}). In addition, HA causes significant reductions in oxygen consumption (-4-7%) for a given work-rate (Sawka et al. 1983; Young et al. 1985; Jooste and Strydom 1979), also termed exercise economy, during exercise in both hot and thermoneutral environments.

The mechanisms causing enhanced exercise economy after HA have not been fully elucidated. Some researchers have suggested that in trained individuals, improved economy and muscle efficiency is likely due to high numbers of type I muscle fibers (Coyle et al. 1992; Horowitz et al. 1994) and that after HA there is an improvement in muscular efficiency (Sawka et al. 1983) likely by enhanced P/O ratio (ATP formation per oxygen used) (Whipp and Wasserman 1969). In C2C12 myotubes, heat stress (40°C) has been reported to induce increases in peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC-1α), complex I, II, III, and IV proteins in the electron transport chain and ATP synthase, thus promoting increased mitochondrial biogenesis (Liu and Brooks 2012) compared to control treatment (37^{o}C). It is unclear to what extent increases in mitochondrial biogenesis from heat stress improve exercise economy and efficiency.

Another possible explanation is that heat stress alters uncoupling protein 3 (UCP3), skeletal muscle-specific proteins, which are located throughout the inter-

mitochondrial membrane. Uncoupling protein 3 allows for leakage of H^+ from the intermembranous space to the matrix which alters the coupling of electron transport and oxidative phosphorylation thereby improving economy and efficiency. Schlagowski et al. (Schlagowski et al. 2014) reported significant decreases (-11%) in running speed (42.4 ± 1.7 cm/sec in those treated with 2-4 dinitrophenol (DNP) vs. 47.6 ± 1.5 cm/sec in control) and impaired running economy (3.1 ± 0.1 ml/kg/min in control vs. 3.8 ± 0.2 ml/kg/min) in mice treated with DNP, a drug that increases H^+ leakage and increases mitochondrial uncoupling. An important finding of their study was that increasing oxidative phosphorylation uncoupling impaired running economy and reduced exercise capacity.

Following cold exposure there is an increase in UCP3 in humans (Schrauwen et al. 2002), leading to greater uncoupling with reduced mitochondrial efficiency. This process is advantageous in cold environments because it increases thermogenesis leading to higher core temperature and improved thermoregulation. Because cold exposure increases UCP3 and promotes thermogenesis with simultaneous reduction in mitochondrial efficiency, it is plausible that chronic heat stress could reduce UCP3 expression leading to reduced dissipation and mitochondrial efficiency. However these hypotheses have yet to be demonstrated. Investigators have reported that heat stressed broiler chickens had reductions in avUCP (73% homology to mammalian UCP3) mRNA and protein compared to non-heat stressed animals (Mujahid et al. 2006). Because the aim of their study was not to investigate the metabolic changes from heat stress, it is still unclear if heat stress reduces UCP3 expression and lowers exercise economy and muscle efficiency.

A limiting factor during acute exercise at high altitude is impaired oxygen transport from the ambient air to the muscles resulting in a reduction in submaximal, and maximal exercise capacity (Robergs et al. 1998) and cycling time-trial performance (Beidleman et al. 2003). Even though VO_{2max} is a strong predictor of performance; economy and efficiency also contribute to exercise performance (Schlagowski et al. 2014; Saunders et al. 2004; Di Prampero et al. 1993). In well trained individuals, economy explained 65% of the variation in a 10-km race in those with similar VO_{2max} (Conley and Krahenbuhl 1980). Additionally, lower economy and efficiency compensated for reduced VO_{2max} in world-class professional cyclists (Lucia et al. 2002), implicating the importance of these two variables in competitive endurance events.

The importance of enhanced economy and efficiency during exercise at altitude is observed in some individuals. Latshang et al. (Latshang et al. 2013) reported that in mountaineers, lower submaximal VO_2 was a significant predictor of perception of effort during two summit attempts. Further, it has been suggested that Andean high-altitude natives have improved economy and muscle efficiency during exercise at altitude compared to sea-level natives even with lower $VO_{2max,}$ which may explain their high exercise tolerance at altitude.

The use of a cross environmental stressor (CES) model of heat acclimation and acute altitude exposure has been investigated. Heled et al. (Heled et al. 2012) reported that after 12 days of heat acclimation (40^{o}C temperature and 40% relative humidity) at sea-level, SaO_2 during exercise at a simulated altitude of 2430 m (15.6% FIO_2) was significantly improved (86.5 ± 2% versus 88 ± 2% from pre-heat acclimation to post-heat acclimation, respectively), and HR was significantly lower at onset of blood lactate An

increase in SaO_2 may indicate that HA may improve oxygen transport. A major limitation to their study was that metabolic measurement such as oxygen consumption and ventilation (VE) during submaximal exercise at altitude was not measured, however the authors suggested that HA acts as a preconditioning CES which lowers metabolic rate and may be beneficial to improving economy and efficiency at altitude.

The purpose of this study was to determine whether 10 days of exercising in the heat can increase submaximal exercise economy and efficiency both at 1600 m and 4350 m in trained individuals to determine its use as CES, and to investigate possible mechanisms using a muscle cell model.

Materials and Methods

Subjects

Eight trained males were recruited from the local community. The subjects were cyclist and runners averaging 5.9 hours/wk of moderate and 2.6 hours/wk of vigorous exercise within the last year. All subjects met the following inclusion criteria: 1) 18 – 44 years of age; 2) maximal oxygen consumption (VO_{2max}) classified as $\geq 80^{th}$ percentile for their age (ACSM's Guidelines for Exercise Testing and Prescription 2014); and 3) residing at approximately 1600 m within the last six months. Subjects were stratified for cardiovascular risk factors according to the American College Sports Medicine (ACSM) (ACSM's Guidelines for Exercise Testing and Prescription 2014) and were excluded if they were considered: 1) moderate or high risk; 2) have had a previous heat injury (heatstroke and/or heat exhaustion; or 3) spent time at altitudes > 1600 m within the past six months. Written informed consent was obtained prior to participation in the study.

This study was approved by the Institutional Review Board of the University of New Mexico.

Study Design

This was a 10-day heat acclimation (HA) study conducted in Albuquerque, NM during the months of February 2014 to June 2014 at which time the average high temperature was 23.6^{o}C. Prior to the HA trials, all subjects reported to the exercise physiology laboratory (1600 m) at the same time of day to complete all preliminary testing and to collect baseline measurements. All subjects were instructed to refrain from strenuous exercise (heavy lower-body resistance exercise or high intensity intervals), caffeine, and alcohol consumption, and to fast at least 10 hours prior to all testing. Pre-test compliance was verified with a written physical activity/dietary log provided to subjects during each visit. The baseline testing, conducted on separate days, included one heat tolerance test (HTT), maximal graded exercise tests (GXT) to determine VO_{2max} and intensity during the submaximal exercise bouts (SE) at 1600 m and 4350 m (Figure 1). The VO_2max and SE tests were conducted at both 1600 m and 4350 m and were separated by at least 24 hrs. High altitude (hypobaric hypoxia, 4350 m) was simulated using an altitude chamber located at the University of New Mexico. The custom built chamber is an air tight system that is 6.1 m long and 2.4 m diameter. A constant flow rate of outside air was used to ventilate the altitude chamber. At least one week after all baseline testing was complete, subjects participated in a 10-day HA protocol. Since the exercise intensity of the heat trials were dependent on the workload (Watts) below the ventilation threshold calculated from the VO_{2max}, the order of exercise trials was not randomized. No more than two days

after completion of HA, VO_{2max} and SE at 1600 m and 4350 m and HTT were retested with each test separated by at least 24 hours.

Tests and Measurements

Prior to any testing, resting heart rate (Polar Electro, model FS1, Woodbury, NY) and blood pressure were measured while the subject was in a seated position for five minutes. Three site skinfolds (chest, abdomen and thigh) (Beta Technology Incorporated, Lange Skinfold Caliper, Cambridge, MD) were measured twice in rotational order by the same trained technician and averaged to estimate percent body fat (Jackson and Pollock 1978). Nude body weight was recorded during each visit using an electronic scale (Seca, Model 2531, Danville, VA).

Maximal graded exercise test and determination of exercise workload

Each subject performed a GXT to determine VO_{2max} at 1600 m and 4350 m in a temperate environment (21°C) using a staged protocol on an electronically-braked cycle ergometer (Velotron DynaFit Pro, RacerMate, Seattle, WA) on separate days. This cycle ergometer was used for all GXT and SE bouts. The fore, aft and seat height position were measured during the initial testing, and were replicated for all testing. The warm-up consisted of a self-selected resistance for two minutes. The maximal GXT began at 70 Watts (W) and was increased 35 W each minute until volitional fatigue. Maximal oxygen consumption was determined using established criteria (Astorino 2009), and if criteria were not met it was recorded as VO_{2peak}. Peak power output (PPO) was defined as the highest workload (W) from the last completed stage plus the fraction of time spent in the

uncompleted final workload multiplied by 35 W (Stepto et al. 1999). Ventilatory threshold 1 (VT_1) was determined using the criteria of an increase in VE/VO_2 with no change in VE/VCO_2 (Davis 1985). To determine the exercise intensity during all heat trials, 75 W was subtracted from the workload at VT_1 from the GXT (corrected workload). The workload was corrected so that subjects exercised at a power output that would elicit a submaximal VO_2 below VT_1 (Bradley 2012) (unpublished data) which has been reported not elicit a training response (Londeree 1997; Sady et al. 1980). Expired air was collected continuously and analyzed to determine VO_2 consumption, CO_2 production and respiratory exchange ratio (RER) using a commercially available metabolic system (ParvoMedics True One 2400, Sandy Utah). All data were processed using a 30-second average. Before all testing, the metabolic cart was calibrated per the manufacturer's recommendations. The flow rate of the pneumotach was recalibrated via a flow calibration reconstruction for all high altitude trials (4350 m) to account for the reduced air density within the hypobaric chamber. Gas analyzers were calibrated to known gas concentrations (16.01% O_2 and 4.00% CO_2), and the pneumotach was calibrated using 3-liter syringe at varying flow rates.

Submaximal exercise

The submaximal exercise bouts were performed at 1600 m and 4350 m in a temperate environment and were separated from the GXT by at least 24 hrs. Subjects warmed-up for 10 minutes at a self-selected workload which was then increased to 30% (120 W and 95W at 1600 m and 4350 m, respectively, equating to ~42% VO_{2peak} or low intensity (LI)) and 20% (137 W and 108 W at 1600 m and 4350 m, respectively, equating to ~48% VO_{2peak} or high intensity (HI)) below the corrected workload from their GXT. These

workloads were selected to avoid the VO_2 slow component (Poole et al. 1994) and ensure that all subjects reached steady-state, particularly during the submaximal exercise at 4350 m. Each subject exercised for 10 minutes (Schrauwen et al. 1999), continuously and was asked to maintain a cadence close to 80 rpm. Economy and gross efficiency were calculated for each steady state workload using the following formula:

Economy (W/LO_2) = Power output (W)/VO_2 (L/min) (Moseley and Jeukendrup 2001)

and

Gross efficiency (%) = (Work Rate (W))/Energy Expended (J/sec) x 100) (Moseley and Jeukendrup 2001)

Expired gases were collected using a metabolic system during the last five minutes of each 10-minute exercise bout. All SE data were processed using a one minute average.

Heat Tolerance Test

The HTT was performed in a heat chamber (1600 m) consisting of cycling (Monark, Ergomedic 828E, Vansbro, Dalarna) for 45 minutes at a temperature of 40°C and 20% relative humidity. All exercise bouts during the HTT and 10 day HA were performed using this cycle ergometer at the corrected workload (mean of 158 W, 55% VO_{2peak}). Prior to the HTT and after voiding their bladder, nude body weight was recorded using an electronic scale. Urine samples were collected to determine hydration status via urine specific gravity (REF312ATC, General Tools & Instruments, New York City, NY). Euhydration was classified as ≤ 1.020 g/mL (Cheuvront et al. 2006). If subjects were not euhydrated they were asked to consume 500 mL of water and hydration status was reassessed after 20-30 minutes. I have found that this time frame is sufficient for subjects

to become euhydrated. Subjects were then asked to self-insert a rectal thermistor (Model 4TH, Telly Thermometer, Yellow Springs, Ohio) to a minimum of 10 cm past the anal sphincter (Kuennen et al. 2011). Skin thermistors (YSI 409B, Thermistor Probe, Dayton, Ohio) were placed uncovered on the chest, arm, and thigh to calculate mean skin temperature ($T_{sk)}$ using an established equation: ($T_{sk} = 0.43\ T_{chest} + 0.25\ T_{arm} + 0.32\ T_{thigh}$) (Roberts et al. 1977). Rectal and skin thermistors were interfaced to an analog data logger (Model 44TA, Telly Thermometer,Yellow Springs, Ohio) to assess rectal (T_c) and (T_{sk}) temperature. HR was assessed continuously during the HTT and all variables including thermal sensation were recorded every five minutes. The HTT was terminated if the subject 1) requested to stop, 2) was unable to sustain the predetermined workload, or 3) the subject reached a T_{re} of ≥ 40°C. If the subjects failed to complete the entire 45 min HTT, they were asked to report to the laboratory one week later in order to perform a follow-up HTT.

Heat Acclimation

Subjects completed 10 consecutive days of a HA protocol which consisted of cycling in a heat chamber at a temperature of 40°C and 20% relative humidity. Heat acclimation was induced using a traditional heat acclimation protocol, where subjects exercised at the corrected workload from the GXT for two 50-minute bouts with 10 minutes of seated rest between each bout (Sawka et al. 1983). Core temperature and HR were monitored continuously and recorded every five minutes. Subjects were provided with room temperature water and allowed to drink *ad libitum*. Urine output was measured. Nude body weight was recorded before and after each trial to determine whole body sweat rate

(Buono et al. 2009) after correcting for urine output and water intake. The HA termination criteria included: 1) completion of the 100 minutes of cycling; 2) $T_{re} \geq 40°C$ or; 3) subject requested to stop. If subjects were unable to complete the entire 100 minutes for any given HA trial, the completed time was recorded and they were asked to continue reporting to the laboratory as scheduled until they finished the 10 day HA protocol.

Blood measurements

On day one and day 10 prior to beginning the HA protocol, 10 mL of venous blood was drawn free flowing from the antecubital vein. In order to control for shifts in fluid compartments, subjects sat in an upright position with their arm at heart level for 20 minutes prior to blood sample collection (Harrison 1985). Hematocrit and hemoglobin were measured and used to calculate the change in plasma volume from day one to day 10 of HA (Dill and Costill 1974). Hematocrit was determined in triplicate; where blood was filled into heparinized capillary tubes and centrifuged (Unico, Model C-MH30, Dayton, NJ) at 12,000 rpm for five minutes. The percentage of red cells to total volume were measured and multiplied by .96 to account for red cells in the plasma. Hemoglobin concentration was assayed with a hematology analyzer (Beckman Coulter, Model LH750, Brea, CA).

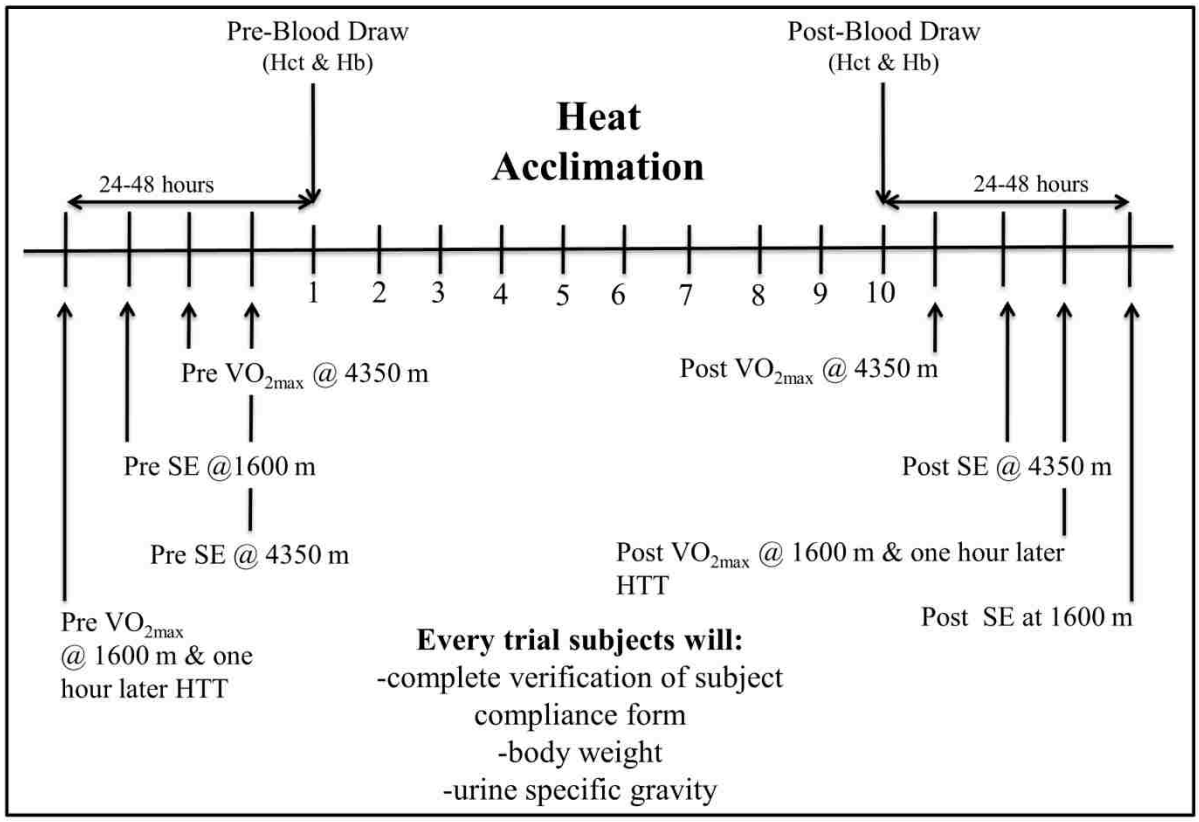

Figure 1 Schematic of intervention. All pre and posting testing were separated by at least 24-48 hours with 10 days of continuous exercise in the heat (2 hours/day at ~55% VO_{2peak}). *SE: Submaximal exercise, Hct: Hematocrit, Hb: hemoglobin, HTT: Heat tolerance test, VO_{2max}: maximal graded exercise test

Cell Model

Cell Culture: Murine myocytes (C2C12) were purchased from ATCC (Manassas, VA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4500mg/L glucose and supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100U/mL penicillin/streptomycin, in a humidified 5% CO_2 atmosphere at 37°C (standard conditions). Following overnight seeding, cells with either incubated for 24 hours under standard conditions (control) or at 5% CO_2 atmosphere at 40°C (heat stressed).

Metabolic Assay: Cells were seeded overnight in 24-well culture plate from SeaHorse Bioscience (Billerica, MA) at a density of 5 x 10^5 cells/well, and incubated either under standard (n = 22 wells for control) or heat stressed conditions (n = 22 wells heat stress). Following 24 hour incubation, culture media was removed and replaced with XF Assay Media from SeaHorse Bioscience (Billerica, MA) containing 4500mg/L glucose free of CO_2 and briefly incubated at 37°C. Per manufacturers' protocol, SeaHorse injection ports were loaded with oligomycin, an inhibitor of ATP synthase which induces maximal glycolytic metabolism and reveals endogenous proton leak (mitochondrial uncoupling) at a final concentration 1.0 µM. Oligomycin addition was followed by the addition of carbonyl cyanide *p*-[trifluoromethoxy]-phenyl-hydrazone (FCCP), an uncoupler of electron transport that induces peak oxygen consumption (an indirect indicator of peak oxidative metabolism) at final concentration 1.25 µM. Rotenone was then added in 1.0 µM final concentration to reveal non-mitochondrial respiration and end the metabolic reactions (Wikstrom et al. 2012; Giulivi et al. 2008). Extracellular acidification, an indirect measure of glycolytic capacity, and oxygen consumption, a measure of oxidative metabolism was measured using the SeaHorse XF24 Extracellular Analyzer from SeaHorse Bioscience (Billerica, MA). SeaHorse XF24 Extracellular Analyzer was run using 8 minute cyclic protocol commands (mix for 3 minutes, let stand 2 minutes, and measure for 3 minutes) in triplicate as previously performed (Vaughan et al. 2013).

Cellular ATP Content: Cells were seeded overnight in a 6-well plate (n = 6 control and n = 6 heat stress) at density 1 x 10^6 cells/well and heat stressed as described above for 24 hours. Cells were lysed in 1% CHAPS lysis buffer from Chemicon (Billerica, MA) in PBS with Ca^{2+} and MG^{2+} and the ATP-containing supernatant was recovered. Samples

were allocated into a 96-well plate with a 1:1 dilution of ATP Bioluminescence Reagent from Sigma (St. Louis, MO) with a 50 µM final volume and luminescence was measured and normalized to serial dilutions of ATP. ATP concentrations were normalized to cell density determined through hemocytometey measured by staining cells with trypan blue from Sigma (St Louis, MO) with cell density estimated using a Countess™ cell quantification system from Invitrogen (Carlsbad, CA).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR): Following 24 hour incubation under standard or heat stressed conditions, total RNA was extracted using RNeasy Kit from Qiagen (Valencia, CA) and cDNA was synthesized from using the Retroscript™ RT kit from Ambion (Austin, TX) according to manufacturer's instructions. PCR primers were designed using Primer Express software from Invitrogen (Carlsbad, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). qRT-PCR were done in triplicates for each condition (n = 3 wells for control and n = 3 wells for heat stress). Amplification of PGC-1α, nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM), glucose transporter 4 (GLUT4), and mitochondrial uncoupling protein 3 (UCP3) were normalized to the housekeeping gene, TATA Binding Protein (TBP). Table 1 summarizes the forward and reverse primers of each gene. qRT-PCR reactions were performed in triplicate using the LightCycler 480 real-time PCR system from Roche Applied Science, (Indianapolis, IN). SYBR Green based PCR was performed in triplicate with final primer concentrations at 10 µM in a total volume of 30 µl. The following cycling parameters were used: 95˚C for 10 minutes followed by 45 cycles of 95˚C for 15 seconds, and 60˚C for one minute. Relative

expression levels were determined by the ΔΔCp method and compared to the lowest expressing group as previously described (Pfaffl 2001).

Table 1 Summary of qRT-PCR primers from Integrated DNA Technologies (Coralville, IA). Abbreviations: Glucose transporter 4 (GLUT4), nuclear respiratory factor 1 (NRF1), peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC-1α), mitochondrial transcription factor A (TFAM), TATA binding protein (TBP), and mitochondrial uncoupling protein 3 (UCP3).

Primer Name	Forward Sequence	Reverse Sequence
GLUT 4	5'-GGAGGGAGCCTTTGGTATTT-3'	5'-CAGGCGAGGACACTCATCTT-3'
NRF1	5'-ACCCTCAGTCTCACGACTAT-3'	5'-GAACACTCCTCAGACCCTTAAC-3'
PGC-1α	5'-GACAATCCCGAAGACACTACAG-3'	5'-AGAGAGGAGAGAGAGAGAGAGA-3'
TBP	5'-GGGATTCAGGAAGACCACATA-3'	5'-CCTCACCAACTGTACCATCAG-3'
TFAM	5'-GAAGGGAATGGGAAAGGTAGAG-3'	5'-ACAGGACATGGAAAGCAGATTA-3'
UCP3	5'-CAGATCCTGCTGCTACCTAAT-3'	5'-GCATCCATAGTCCCTCTGTAT-3'

Immunoblotting and Protein Expression: Cells were seeded overnight and incubated either under standard or heat stressed conditions for 24 hours. Immunoblotting were done in triplicates for each condition (n = 3 control and n = 3 heat stress). Whole cell lysates were prepared by harvesting the cells on ice in high salt lysis buffer (25mM Tris base, 8mM MgCl2, 1mM DTT, 15% glycerol, 0.1% Triton) supplemented with protease

inhibitor mix (Sigma, St. Lois MO), followed by incubation on ice for 60 minutes. Insoluble material was removed by centrifugation at 12,000 rpm for 3 minutes and protein concentrations were determined by Bradford assay (Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories, Hercules, CA). Total protein (40 μg per sample) was size-separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to nitrocellulose membranes. After blocking in TBST-5% milk powder for 1 hour, membranes were probed at 4ºC for 24 hours with either an anti-PGC-1α primary polyclonal antibody from Santa Cruz Biotechnologies (Santa Cruz, CA) or anti-GLUT4 monoclonal antibody from Abcam (Cambridge, MA) and anti-β-actin primary monoclonal antibody from Sigma (St. Louis, MO) in TBST-1% milk powder overnight. Bound antibodies were detected by horseradish peroxidase-conjugated secondary antibodies from Sigma (St. Louis, MO) and by chemiluminescence using the ECL Plus Western Blotting Detection kit from GE Healthcare Life Sciences (Little Chalfont, Buckinghamshire, UK). Signal intensities were obtained by densitometry using ImageJ software (available from the NIH at http://rsbweb.nih.gov/ij/) by quantifying lane intensities followed by normalizing PGC-1α and GLUT4 intensity with corresponding β-actin.

Flow Cytometry: Cells were seeded in 6-well (n = 6 control and n = 6 heat stress) plates at a density of 1.0×10^6 cells/well and incubated as described above for 24 hours. Following incubation, the media was removed and the cells were re-suspended in pre-warmed media with 200 nM Mitotracker Green from Life Technologies (Carlsbad, CA) and incubated for 45 minutes in a humidified 5% CO_2 atmosphere at 37°C. The cells were pelleted, the media with Mitotracker was removed and the cells were suspended in

pre-warmed media. Group mean fluorescence was measured using Facscalibur filtering 488 nm.

Statistical analyses

Human Experiment

An *a priori* analysis using a commercially available software (G*Power, Universitat Kiel, Germany, version 3.1) with a repeated measures within and between design was used to determine the sample size needed to find a significant change in submaximal VO_2. The effect size from submaximal VO_2 was used because improvements in this variable during exercise at altitude have been associated with improved exercise tolerance at altitude (Latshang et al. 2013). Using an effect size of 0.98 (Shvartz et al. 1977), alpha level of 0.05 and power of 0.80, a total of six subjects were needed for this investigation and we were able to recruit eight individuals.

A student's t-test was used to determine significant differences from pre to post HA on the following dependent variables: 1) end HR, 2) end T_{rec}, 3) end thermal sensation and 4) end RPE from day 1 and 10 of HA.

A three-way (environment x time x intensity) ANOVA with repeated measures design was used to determine whether environment (1600 m vs. 4350 m), time (pre vs. post acclimation) and intensity (LI vs. HI) significantly influenced the following dependent variables: submaximal exercise economy, efficiency, submaximal VO_2, ventilation, HR, SaO_2, RPE (6-20 Borg scale) and RER were used to determine statistical significance.

Differences in VO_{2peak} and PPO were determined using two-way (environment x time) ANOVA with repeated measures. Significance was set at $p \leq 0.05$.

Cell Experiment

Metabolic measurements, ATP concentration, flow cytometry, protein expression, and microscopy data were analyzed using student's t-test. Gene expression was quantified by relative expression using the ΔΔCp method, and analyzed using student's t-test (Pfaffl 2001). All data are represented as mean ± standard deviation (SD). All cell data were normalized to the control mean (control = 100). All statistical analyses were conducted using commercially available software (Statistica v10, Statsoft Inc., Tulsa, OK). Significance was set at $p \leq 0.05$.

Results

Human Experiment

Subject physiological characteristics: Eight subjects completed all pre-, 10-day HA and post- testing protocols. Subject characteristics and aerobic performance before and after heat acclimation at 1600 m and 4350 m are presented in Table 2. A two-way ANOVA with repeated measures revealed a significant main effect of environment on VO_{2max} ($p < 0.05$) and PPO ($p < 0.05$) with no significant differences from pre and post HA at 1600 m and 4350 m (Table 2). Table 3 shows significant decrease in end HR (-15%, $p < 0.05$), end T_c (-1.3%, $p < 0.05$), end thermal sensation (-19%, $p < 0.05$) and RPE (-19%, $p < 0.05$) with no difference in % change in PV ($p > 0.05$).

Table 2. Subject description and aerobic performance results before and after heat acclimation and at 1600 m and 4350 m

	Pre HA		Post HA	
Age (yr)	28 ± 5.8			
Height (cm)	178.46 ± 7.16			
Body Weight (kg)	75.3 ± 7.9		75.3 ± 8.5	
Body fat (%)	8.2 ± 3.9			
	1600 m		4350 m	
	Pre HA	Post HA	Pre HA	Post HA
VO_{2peak} (ml/kg/min)	53.3 ± 6.7	53.7 ± 3.7	45.3 ± 4.1*	45.9 ± 3.4*
Peak Power (W)	362.4 ± 54.3	374.3 ± 41.5	321.4 ± 47.8*	330.6 ± 44.9*

Values are reported as means ± SD for n = 8 subjects. * indicates significantly different between altitudes 1600 m and 4350 m

Table 3. Mean differences in thermoregulatory responses between day 1 and day 10 of heat acclimation

	Day 1	Day 10
End heart rate (bpm)	161 ± 17.8	140 ± 15.4*
End Tc (°C)	39.2 ± 0.7	38.7 ± 0.5*
End thermal sensation	7.3 ± 0.9	6.1 ± 0.9*
End RPE	15.5 ± 2.9	13.0 ± 1.1*
Δ Plasma volume (%)	1.86	

Values are reported as means ± SD. * indicates significantly different from day 1. n = 8.

Metabolic responses were not different at 1600 m and 4350 m before and after 10 days of HA. A three-way ANOVA with repeated measures was used to compare the effects of 10 days of HA on metabolic responses during steady state exercise at 1600 m and 4350 m. The analysis revealed significant main effects for the following: exercise intensity on submaximal VO_2 (Figure 2A) ($p = 0.01$), environment (Figure 2B) ($p = 0.002$) and

exercise intensity on exercise economy (Figure 2B) (p = 0.01), exercise intensity on efficiency (Figure 3A) (p = 0.02), and environment on RER (Figure 3B) (p = 0.003). There were no significant differences in metabolic responses from pre to post HA during exercise at 1600 m (Figure 1A-D) and at 4350 m (Figure 2A-D).

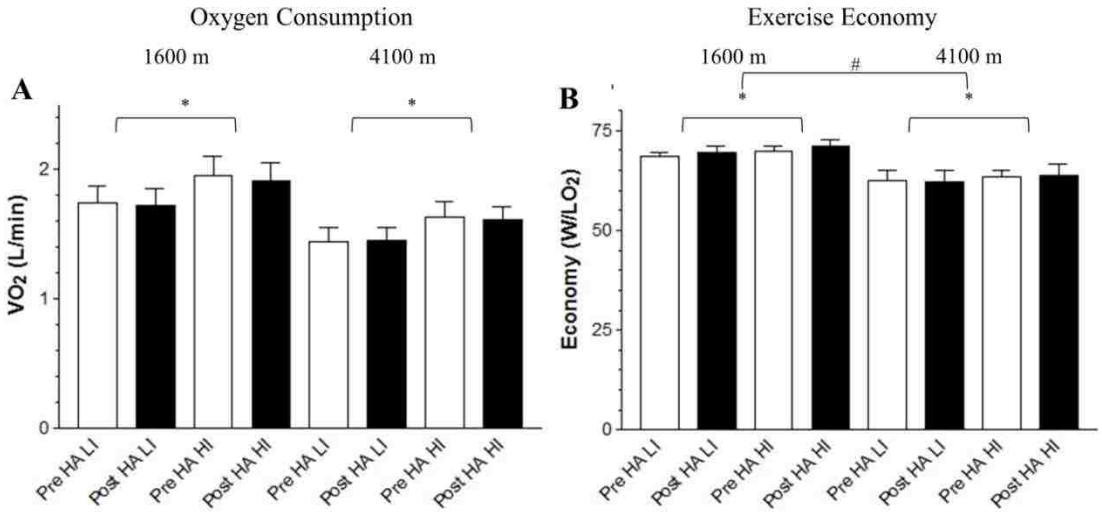

Figure 2 (A) Submaximal oxygen consumption and (B) Submaximal economy (Economy = Watts / VO_2 (L/min) (Moseley and Jeukendrup 2001) pre and post 10 days of HA during exercise at 1600 m low intensity (LI: 120 W, ~ 42% VO_{2peak}) and high intensity (HI: 137 W, ~48% VO_{2peak}) and at 4350 m LI (95 W, ~42% VO_{2peak}) and HI (108W, ~48% VO_{2peak}). * indicates significant main effect of exercise intensity on submaximal VO2 and economy ($p < 0.05$), # indicates significant main effect of environment on exercise economy ($p < 0.05$). No significant differences were observed between pre and post HA on submaximal oxygen consumption and exercise economy at 1600 m and 4350 m. Values are reported in mean ± SE with n = 8 subjects.

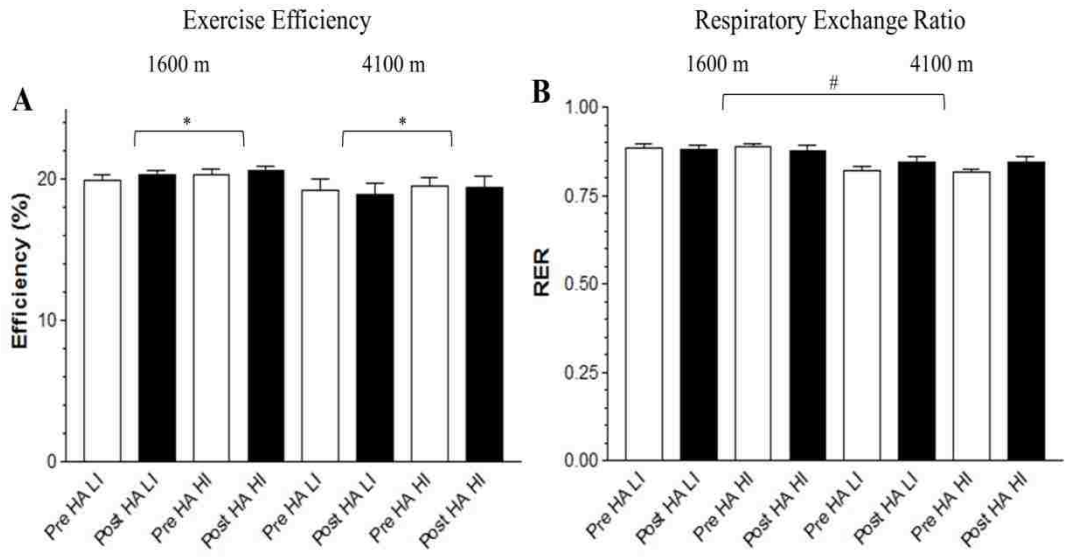

Figure 3 (A) Gross efficiency pre and (Gross efficiency (%) = (Work Rate (W))/Energy Expended (J/sec) x 100) (Moseley and Jeukendrup 2001) and (B) Respiratory Exchange Ratio pre and post 10 days of HA during exercise at 1600 m low intensity (LI: 120 W, ~ 42% VO_{2peak}) and high intensity (HI: 137 W, ~48% VO_{2peak}) and at 4350 m LI (95 W, ~42% VO_{2peak}) and HI (108W, ~48% VO_{2peak}). * indicates significant main effect of exercise intensity on exercise efficiency ($p < 0.05$), # indicates significant main effect of environment on RER ($p < 0.05$). No significant differences were observed between pre and post HA on exercise efficiency and respiratory exchange ratio at 1600 m and 4350 m. Values are reported in mean ± SE with n = 8 subjects.

Pulmonary responses and perception of effort did not change at 1600 m and 4350 m before and after 10 days of HA: To determine the effects of HA on ventilation and rating of perceived exertion, I measured HR, VE, SaO_2 and RPE at 1600 m and 4350 m (Table 4 and 5). A three-way ANOVA with repeated measures revealed significant main effects for: exercise intensity on HR ($p < 0.05$), environment and exercise intensity on VE ($p < 0.05$), environment and intensity on SaO_2, and intensity on RPE ($p < 0.05$). There were no significant interactions before and after HA at 1600 and 4350 m on the above variables.

Table 4. Mean differences in steady state exercise at 1600 m for HR, VE, SaO2, and RPE before and after 10 days of heat acclimation

	1600 m			
	Pre HA LI	Post HA LI	Pre HA HI	Post HA HI
HR (bpm)	111.1 ± 14.6*	106.5 ± 11.9*	118.25 ± 17.1*	114 ± 14.0*
VE (L/min)	30.8 ± 8.5*#	30.6 ± 7.8*#	34.8 ± 10.0*#	35.5 ± 8.5*#
SaO_2 (%)	93.6 ± 1.8	94.0 ± 1.1	92.9 ± 2.0	93.3 ± 1.3
RPE	10 ± 1.4	10 ± 1.6	11 ± 1.1	11 ± 1.8

Values are reported as means ± SD with n = 8 subjects * indicates significant main effect of exercise intensity on heart rate # indicates main effect of exercise intensity on ventilation

Table 5. Mean differences in steady state exercise at 4350 m for HR, VE, SaO2, and RPE before and after 10 days of heat acclimation

	4350 m			
	Pre HA LI	Post HA LI	Pre HA HI	Post HA HI
HR (bpm)	111.93 ± 8.1*	110.1 ± 11.5*	121.3 ± 8.4*	117.1 ± 11.4*
VE (L/min)	19 ± 4.9+#	20.0 ± 4.4+#	22.2 ± 5.8+#	23.1 ± 5.1+#
SaO_2 (%)	77.1 ± 4.2	79.3 ± 3.1	76.3 ± 3.3	77.8 ± 2.0
RPE	10 ± 1.8	10 ± 1.8	10 ± 1.9	11 ± 1.5

Values are reported as means ± SD with n = 8 subjects * indicates significant main effect of exercise intensity on heart rate + indicates main effect of environment on ventilation # indicates main effect of exercise intensity on ventilation

Cell Experiment

Changes in cellular metabolism due to heat stress: To investigate the effects of heat stress on cultured C2C12 murine myocyte metabolism, I measured oxygen consumption rate (OCR), an indicator of mitochondrial metabolism following 24 hours of heat stress. Figure 4A shows that basal oxidative metabolism and peak oxidative metabolism was significantly reduced (-75.5% ± 4.9% and -64.4% ± 5.9%, respectively) in myocytes heat stressed for 24 hours compared with control cells. Mitochondrial H^+ leak (uncoupling), a

source of thermogenesis, was reduced in the heat stressed cells (-71.1%, ±1.2%) and accompanied by a significantly reduced UCP3 gene expression (Figure 4B).

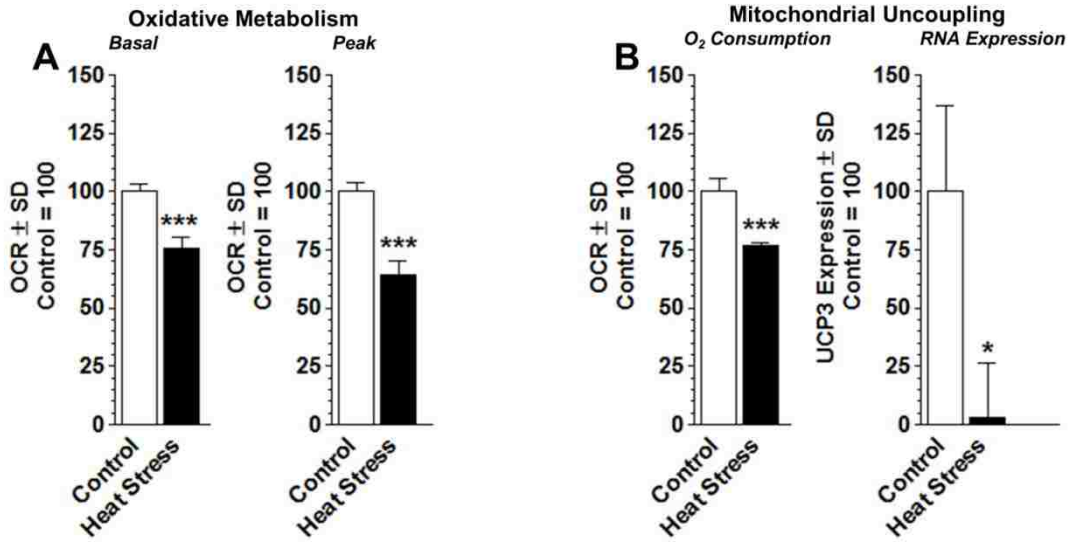

Figure 4 Cellular Metabolism of C2C12 murine myocyte incubated under 37°C (control, n = 22) or 40°C (heat stressed, n = 22) for 24 hours. (A) Basal and peak oxidative metabolism indicated by oxygen consumption rate (OCR) (B) Endogenous uncoupling revealed by oligomycin treatment of cells treated as described above and mitochondrial uncoupling protein 3 (UCP3) RNA expression. Significance was indicated as *, **, and * indicating $p < 0.05$, $p < 0.01$, and $p < 0.001$ statistical differences compared to control, respectively.**

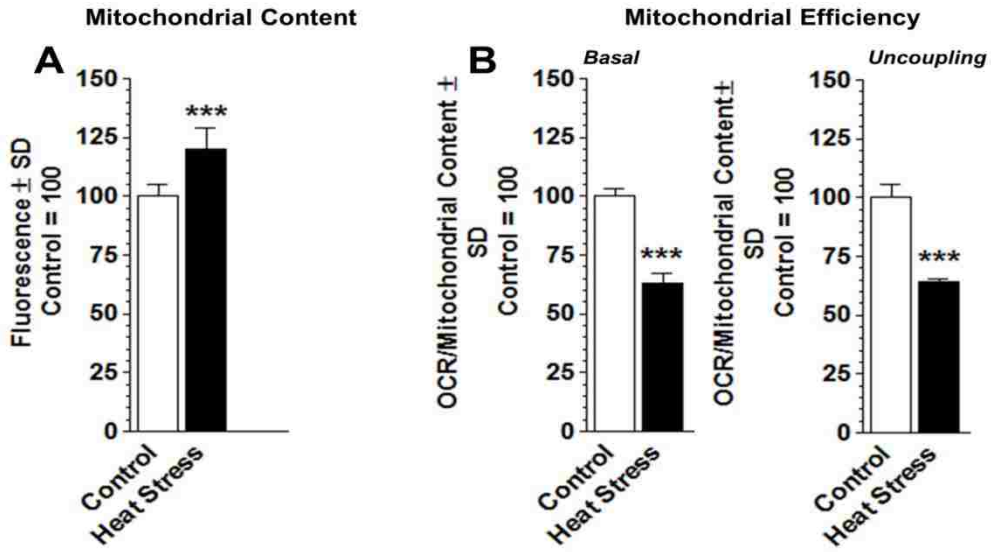

Figure 5 Cellular Metabolism of C2C12 murine myocyte incubated under 37°C (control) or 40°C (heat stressed) for 24 hours. (A) Mitochondrial content indicated by Mitotracker (n = 6) staining measured by flow cytometry and (B) Mitochondrial efficiency of cells under basal conditions and oligomycin-induced proton leak (normalized to mitochondrial content).Significance was indicated as *, **, and * indicating $p < 0.05$, $p < 0.01$, and $p < 0.001$ statistical differences compared to control, respectively.**

To investigate the effects of heat stress on mitochondrial content, I measured mitochondrial staining following incubation described above. Heat stressed cells displayed significantly increased mitochondrial staining (+119.9%, ±9.2%) compared with control cells (Figure 5A). To investigate the effects of heat stress, using mitochondrial staining from flow cytometry measurement, I normalized basal oxidative metabolism and mitochondrial uncoupling to mitochondrial content. Figure 5B illustrates that heat stressed cells showed significantly reduced mitochondrial efficiency.

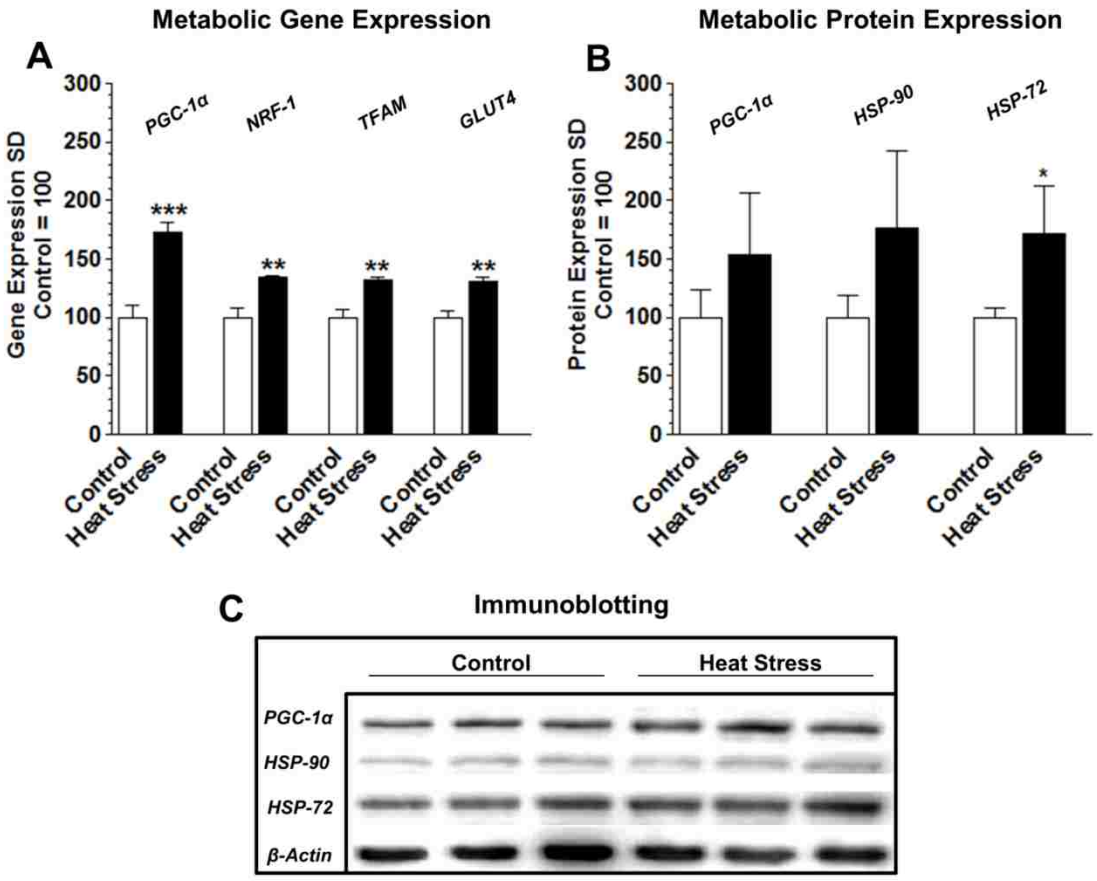

Figure 3 Gene and Protein Expression of C2C12 murine myocyte incubated under 37°C (control) or 40°C (heat stressed) for 24 hours. (A) Metabolic gene expression (n = 3) of peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC-1α), nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM), glucose transporter 4 (GLUT4) were normalized to the housekeeping gene, TATA Binding Protein (TBP), (B) Protein expression (n = 3) of cells treated as described above of PGC-1α, heat shock protein 90 (HSP-90), and heat shock protein 72 (HSP-72) and (C) Representative immunoblots as quantified in B. Significance was indicated as *, **, and * indicating $p < 0.05$, $p < 0.01$, and $p < 0.001$ statistical differences compared to control, respectively.**

Changes in gene expression due to heat stress: To investigate the effects of heat stress induced changes in metabolism on gene expression; I measured induction of several genes for mitochondrial metabolism and biogenesis. Figure 3E shows that heat stressed cells had significantly increased PGC-1α expression, and downstream targets nuclear

receptor of factor-1 (NRF-1) and mitochondrial transcription factor A (TFAM) and GLUT4 expression. To investigate the effects of heat stress on metabolic and heat-stress related protein expression, I measured expression of PGC-1α, HSP-90 and HSP-72. Figures 3F and G shows that PGC-1α and HSP-72 expression were significantly increased in heat-treated cells.

Discussion

Acute altitude exposure reduces oxygen transport leading to lower submaximal and maximal aerobic capacity, making exercise harder (Levine et al. 2008) and consequently impairing exercise capacity (Beidleman et al. 2003). Many traditional (living and training at altitude) (Faulkner et al. 1967; Faulkner et al. 1968; Levine and Stray-Gundersen 1997) and non-traditional (acute intermittent altitude exposure) (Katayama et al. 2004; Beidleman et al. 2008) altitude training models have been successful at preparing individuals for acute altitude sojourns. However, a disadvantage to altitude training is limited access to high altitude terrain and/or costly equipment needed for training purposes. The purpose of this investigation was to determine the effects of a CES of HA on submaximal whole body exercise economy and efficiency during acute exercise at altitude. Using a "proof of concept" model, I investigated the effects of heat stress on C2C12 murine myocyte metabolism and changes in mitochondrial content. The primary findings of our study were 1) whole body submaximal economy and efficiency at 1600 m and 4350 m was not affected after 10 days of HA and 2) 24 hours of heat stress suppressed cellular metabolism, and reduced mitochondrial uncoupling which enhanced mitochondrial efficiency, and induced

mitochondrial biogenesis. Thus, I propose that while heat stress improves cellular mitochondrial efficiency, findings from whole body metabolism are inconsistent with cellular adaptations indicating that HA may not be an adequate CES preconditioning model to enhance whole body economy and efficiency during acute exercise at altitude.

Prior to this investigation only two studies have examined the effects of heat exposure on exercise capacity in hypoxia. Hiestand (Hiestand et al. 1955) first reported a 22% longer survival time-to-drowning in mice exposed to heat which they attributed to lower oxygen requirement during swimming. Later, Heled et al. (Heled et al. 2012) suggested that improvements in metabolism defined as a lower submaximal VO_2 due to heat acclimation may be beneficial during exercise in hypoxic environments. However, a major limitation of the Heled et al. investigation was that they did not measure expired air during exercise, making it difficult to draw those conclusions. Here, I show that 10 days of HA, which is confirmed by reductions in HR and T_c (Table 3), does not lower exercise economy and efficiency not only during acute exercise at 4350 m, but has little effect at a lower altitude (1600 m) in a temperate environment. Our findings are consistent with some (Weinman et al. 1967; Young et al. 1985) but are in contrast to what others have reported (Sawka et al. 1983; Jooste and Strydom 1979; Shvartz et al. 1977; Eichna et al. 1950). Young et al. (Young et al. 1985) found only a 0.85% reduction in VO_2 during submaximal exercise in a temperate environment after 9 days of HA, whereas, previous examinations that reported improvements in submaximal VO_2 observed decreases ranging from 4-15%. In comparison, I observed 1.7% and 2.1% reduction in VO_2 in the LI and HI exercise at 1600 m, respectively, and 0.7% increase and 1.2% reduction at the LI and HI respectively, at 4350 m. I am unclear as to the disagreement of our results with

other reports, but since our subjects were heat acclimated as shown by reductions in HR, T_c, RPE and perception of heat, I feel the differences are not attributed to our HA protocol of 110 minutes/day for 10 days at 40°C at 20% RH. A possible explanation for the observed differences in results is the different modes of exercise used during HA. Previous studies have used step-testing or treadmills protocols, with the largest improvements observed in step-test protocols (Shvartz et al. 1977; Senay et al. 1976). Considering the potential learning effect of a stepping protocol, this may explain why those authors reported reductions in VO_2 in their subjects. Nevertheless, reductions in VO_2 after HA have also been reported in walking/running studies (Sawka et al. 1983) using the traditional HA protocol used in this study. Since walking/running is the primary mode of transportation in humans, there would be little learning effect, suggesting that HA can lower metabolism independent of the mode of exercise. Heat acclimation has also been reported to increase VO_{2max} (Lorenzo et al. 2010; Sawka et al. 1985), which raises the possibility that at the same given workload an individual would exercise at lower VO_2. Unfortunately, in all but one study which reported improvements in VO_{2max} after HA (~2 – 23% increase) (Shvartz et al. 1977), previous studies did not measureVO_{2max} after HA (Sawka et al. 1983; Jooste and Strydom 1979; Eichna et al. 1950). This may indicate that given the same exercise workload, rightward shifts in VO_{2max} (i.e. improvement in aerobic fitness) and not HA cause changes in exercise economy. Since I controlled for a training effect and did not observe increases in VO_{2max}, this may explain why I did not see reductions in economy.

Another potential explanation as to why I did not observe any metabolic changes in our subjects may be due to the time decay of adaptations from HA. Previous reports

suggest that the primary indicators of HA (reduced HR and T_c) are still reduced after 12-18 days (Pandolf et al. 1977), however, there have been no studies investigating the time course of metabolic adaptations after HA in a temperate environment. Previous studies (Sawka et al. 1983) that reported reductions in economy measured submaximal variables 24 hours after the last HA day. Since this current study was part of a larger study primarily investigating the potential use of HA as a CES for acute exercise at altitude, the submaximal exercise trials at 1600 m were conducted ~7 days after the last HA day. It is conceivable that any effect the HA protocol may have had on submaximal economy and efficiency could have been diminished. However, it does not explain why I did not observe improvements in economy and efficiency at altitude, since those trials were conducted within the first 48 hours after the last HA trial. Nevertheless, our findings indicate that HA does not reduce whole body submaximal economy and alter metabolism which would be beneficial at altitude. Further investigations looking into the time course of re-induction of metabolic adaptations after HA are needed to support our findings.

There are non-metabolic factors that can also confound our findings of submaximal exercise economy. One factor is elastic energy stored within the connective tissue. The effect of the stretch-shortening cycle is an increase force production from the increase in tension and release of energy within the tendons (Roberts 2002). It has been reported that ~40 – 50% of energy production during long distance events is attributed to elastic energy from connective tissue (Nordez et al. 2009) and loss of this elasticity from prior static stretching has been shown to impair the first five minutes submaximal cycling economy (Wolfe et al. 2011). Another non-metabolic factor that can affect force production is changes muscle volume and cross-sectional area (CSA) and therefore

pennation angle of skeletal muscle (Fukunaga et al. 2001; Aagaard et al. 2001). Aagaard et al. reported significant increases in CSA and volume of the vastus lateralis muscle (77.5 ± 3.0 to 85.0 ± 2.7 cm^2 and 1676 ± 63 to 1841 ± 57 cm^2, respectively) and force production (16%) after 14 weeks of resistance training with no alterations in myosin heavy chain composition. The authors suggested that changes in pennation angle and not alterations in ultra-structure as the cause for increase in contractile force production. Therefore, any alterations in non-metabolic factors can potentially have a confound effect on metabolic changes from the 10 days of HA.

Heled et al. (Heled et al. 2012) reported a significant increase in SaO_2 during their maximal exercise test in hypoxia after HA. These findings are important because an initial response of exposure to altitude is an increase in ventilatory response, which ultimately raises SaO_2. Heat acclimation has been reported to further increase ventilation during exercise in the heat (Boden et al. 2000; Beaudin et al. 2009) which is likely stimulated by heat stress to the hypothalamus (Boden et al. 2000). To our knowledge, no studies have investigated the effects of ventilatory responses at altitude after HA. Our findings do not indicate that HA alters VE in a manner that would improve SaO_2 during acute exercise at altitude. This response would make sense given that changes in hypoxic ventilatory response from altitude exposure are mediated by the chemoreceptors of the carotid bodies (Teppema and Dahan 2010).

The secondary purpose of this study was to investigate the potential mechanism of reduced whole economy and improved efficiency by investigating changes in cellular metabolism in C2C12 murine myocytes exposed to 24 hours of heat. Previous examination of heat stress (1 hour/day for five days) on C2C12 myocytes has shown the

propensity for heat to induce molecular adaptations associated with mitochondrial biogenesis (Liu and Brooks 2012). Our cellular experiments confirmed both the gene and protein expression adaptations which have been previously shown (Liu and Brooks 2012), while further verifying that heat stress which I confirmed by significant increase in HSP-72 expression leads to increased mitochondrial content within cells (Figure 3C). A suppressed mitochondrial respiration (especially with corresponding reductions in UCP3 mRNA expression and endogenous proton leak) was an expected adaptation considering a large amount of heat energy is released during active electron transport. This confirms our hypothesis that heat stress reduces mitochondrial efficiency (ie mitochondria are more efficient at using oxygen) as indicated by reduced mitochondrial metabolism with simultaneous increase in mitochondrial content. Interestingly, heat stress stimulated the biogenesis of mitochondria while simultaneously lowering cellular oxidative metabolism. Our findings raise the perplexing question, why would a cell increase mitochondrial density while concurrently decreasing mitochondrial metabolism? Our data demonstrate that heat stress decreases UCP3 expression which has previously been shown to increase mitochondrial reactive oxygen species (ROS) production in UCP3 knockout mice (Vidal-Puig et al. 2000). In previous examinations, researchers have shown that cellular ROS regulates PGC-1α (Strobel et al. 2011; Gomez-Cabrera et al. 2008) which coordinates heightened expression of enzymes manganese superoxide dismutase (MnSOD) and catalase that neutralize ROS (Dam et al. 2013). I therefore speculate that heat stress reduces UCP3 expression leading to lowered metabolism, but is accompanied by elevated ROS production. The elevated ROS stimulates PGC-1α and increases mitochondrial density and anti-oxidative enzymes acting as a feedback mechanism to regulate elevated

ROS production. These cellular adaptations may help to explain why I observed lower metabolism with elevated mitochondrial content. These findings suggest that heat-induced muscle adaptations may be a product of cell survival in hot environments and not necessarily to improve muscle function for exercise. It is also plausible that increased ROS leads to malfunctioning mitochondrial respiration which may act synergistically with heat to suppress oxidative metabolism which may explain why both basal and peak metabolism was suppressed. At least at the cellular level, these findings raise questions as to the potential benefits of heat stress.

Our aim was to use a cellular model to examine the potential mechanism of HA on whole body cycling economy and efficiency. To our knowledge, I are the first to investigate prolonged heat stress on changes in cellular metabolism and relate them to humans. Comparisons between human skeletal muscle and C2C12 murine myocytes indicate similar molecular responses (Chung et al. 2009; Lamon et al. 2014). The use of a "proof of concept model" such as the one in this investigation has been previously used (Hyldahl et al. 2010) and allows for mechanistic studies without human muscle biopsy samples (Allen et al. 2005).

I show that while heat stress suppresses cellular metabolism, whole body economy and efficiency are not affected by HA. Since mitochondrial respiration is the primary source of oxygen consumption, I am unclear as to why I observed lower cellular oxidative metabolism but this response was not seen in the whole body. Previous researchers have reported alterations in both whole body and cellular oxygen consumption when oxidative phosphorylation coupling was manipulated by DNP (Schlagowski et al. 2014). However, researchers (Vidal-Puig et al. 2000) have previously

reported that while UCP3 knockout mice had enhanced mitochondrial coupling and reduced mitochondrial respiration, they did not observe changes in whole body oxygen consumption at rest. They suggested that reductions UCP3 may not necessarily have an effect on whole body VO_2. Furthermore, previous examinations have reported lower expression of UCP3 in trained individuals versus untrained individuals, however, with no differences in P/O ratio (ATP formation per oxygen used) between the two groups (Mogensen et al. 2006). This indicates that reductions in UCP3 would not necessarily influence whole body VO_2, which supports our findings of suppressed cellular oxidative consumption without changes in whole body VO_2.

Another explanation may be the different protocol of heat stress between the human and cell experiment. In our human experiment, I intermittently heat stressed our subjects for two hours a day over a 10-day period, whereas, cells were heat stressed continuously for 24 hours. It is plausible that continuous rather than intermittent heat exposure is required as a dose-response for adaptation. Nevertheless, since reduced economy after HA has been documented by others (Sawka et al. 1983; Shvartz et al. 1977; Senay et al. 1976), while different, the difference in the total amount of heat stress between the cellular and human models may not fully explain our findings. It is also possible that since our sample size (n = 8) was relatively small, changes in whole body VO_2 were difficult to determine. However, our *a priori* power analysis which required at least six subjects and previous studies reporting significant reductions in economy using a range of 8-15 subjects (Sawka et al. 1983; Shvartz et al. 1977) seem to point towards an adequate sample size. Given our results, it is possible that alterations in cellular metabolism are not best translated to whole body changes. Further investigations using

muscle biopsy samples from participants before and after HA may clarify these discrepancies.

I have demonstrated that while heat stress induces cellular adaptations leading to mitochondrial biogenesis and improved mitochondrial efficiency, these adaptations are not translated to the whole body therefore, at this time, I am unable to definitively promote the use of HA as a CES for enhancing cycling economy and efficiency during acute exercise at altitude.

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CHAPTER 4

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

The review manuscript entitled "Adaptations to skeletal muscle from heat stress: A cross environmental stressor model for exercise at altitude" explores a novel idea for the use of the cross environmental stressor (CES) of heat stress to induce skeletal muscle adaptations that would be advantageous during acute exercise at altitude. At altitude, impaired oxygen transport from the lungs to the muscle limits aerobic capacity, consequently reducing exercise capacity. Previous examinations in humans who were heat acclimated (HA) showed reduced metabolism eliciting a lower oxygen cost for a given workload during exercise (improved economy) in a temperate environment. The review paper is focused on previous findings in humans on the increase in economy after HA. Further, I discuss how improved economy and efficiency are key contributors to higher exercise capacity even when some individuals have moderate to low aerobic capacity. It is therefore conceivable that adaptations from heat acclimation may enhance exercise capacity during acute altitude exposure, however, the underlying mechanisms that may cause this effect have yet to be fully elucidated. Using research findings from cell studies, I discuss potential adaptations that occur due to heat stress. These include heat stress-induced mitochondrial biogenesis and reduction in uncoupling protein 3 (UCP3). Previous cell studies show that heat stress alone induces PGC-1α, NRF-1 and TFAM, and increases biogenesis of mitochondria which is a key contributor to aerobic

capacity. Further, I speculate that UCP3, an important mitochondrial uncoupling protein which allows for proton leakage and heat production during electron transport, is reduced after heat stress. This adaptation would lead to improved electron transport coupling and in turn could reduce oxygen consumption and improve mitochondrial efficiency which may be beneficial during acute exercise at altitude.

The research manuscript entitled "The effects of ten days of heat acclimation on submaximal exercise economy and efficiency at 1600 m and 4100 m" provides evidence that while heat-stressed C2C12 myocytes have reduced UCP3 expression and suppressed cellular oxidative and glycolytic metabolism indicating improved muscle efficiency, results from our human study does not support the use of HA as a CES for acute exercise at altitude in humans. Potential explanations for discrepancies between the human and cell models include (1) reductions in UCP3 may not play a large role in whole body oxygen consumption, (2) continuous rather than intermittent heat stress are required for adaptation and (3) the relatively small sample size of our study. Further work is needed to understand the changes in human skeletal muscle biopsy samples after HA.

Conclusions

The significant findings in this research study were (1) 10 days of heat exposure in humans induces HA but is not accompanied by improved whole body exercise economy, (2) in C2C12 myocytes, 24 hours of continuous heat stress induces adaptations that leads to biogenesis of mitochondria and reduced UCP3 expression causing suppressed cellular metabolism, (3) changes in cellular metabolism are not consistent

with changes in the whole body metabolism, and (4) the use of HA as a cross-environmental stressor is not beneficial for acute exercise at altitude.

Recommendations

A measurement that would have further informed this investigation is the analysis of skeletal muscle biopsy samples from subjects before and after HA to directly examine the adaptations of skeletal muscle. It is recommended for future studies that mitochondria be harvested from skeletal muscle to conduct an *ex vivo* metabolism study. Specifically, this would answer whether alterations in cellular metabolism after HA are apparent in the mitochondria sampled from human muscle tissue. These measurement would clarify if the use of HA is a plausible CES model for exercise at high altitudes.

Another further recommendation would be to recruit sea-level native individuals and repeat the study. Previous examinations on the effects of HA on muscle metabolism were conducted at sea level. Due to our geographical location, all of our subjects resided at 1600 m. While the elevation is relatively low, physiological responses to 1600 m would be relatively quick. However, I am unsure whether this elevation may have had confounding effects on how individuals may respond to the heat.

APPENDICES

A. Combined Informed Consent/HIPAA

B. Study Flyer

C. Health History Questionnaire

D. Verification of Subject Compliance to Study Guidelines

E. VO_{2max} data sheet

F. Submaximal exercise data sheet

G. Heat tolerance test data sheet

H. Heat acclimation day 1 and day 10 data sheet

I. Heat acclimation data sheet

J. Supplemental Figure 1 Individual economy pre and post HA

K. Supplemental Figure 2 Individual economy pre and post HA

Appendix A

The University of New Mexico Health Sciences Center
Consent to Participate in Research

The effect of heat acclimation on exercise capacity during acute altitude exposure (13,451 ft)

04/03/14

Purpose and General Information
You are being asked to participate in a research study that is being done by Dr. Christine Mermier, PhD, who is the Principal Investigator, and her associates. This research is being done to evaluate how heat exposure will affect exercise performance during short-term high altitude exposure. You are being asked to participate because you are a male endurance athlete. Approximately 25 people will take part in this study at the University of New Mexico.

This form will explain the study to you, including the possible risks as well as the possible benefits of participating. This is so you can make an informed choice about whether or not to participate in this study. Please read this Consent Form carefully. Ask the investigators or study staff to explain any words or information that you do not clearly understand.

What will happen if I participate?
The recruitment process will be standardized. However, since UNM students and staff may be interested in the study, we will make sure that the recruitment process will not be coercive if this applies to you. For example, the PI, who is a faculty member, will not be involved with recruitment of students. A private room will be used for all interactions between you and study personnel. If you agree to be in this study, you will be asked to read and sign this Consent Form. After you sign the Consent Form, the following things will happen: You will report to the Exercise Physiology lab and/or the High Altitude Chamber on 23 different occasions. You will not be able to perform any strenuous lower-body exercise or consume alcohol or caffeine 24 hours prior to all visits. You will also be asked not to travel to an altitude greater than that of Albuquerque, NM (5000-6000 ft) or be exposed to a Jacuzzi or sauna during participation in the study.

Day One:
1) You will be asked to read and sign the combined consent/HIPAA form, and fill out the health history questionnaire if you're interested in participating in the study. If you do not have any conditions, including elevated resting blood pressure, which would make it unsafe for you to participate, then you will be invited to continue with study measurements.
2) The researchers will measure your height, weight, resting blood pressure, and percent body fat with skinfold calipers. We will measure skinfolds on your chest, abdomen and thigh.

Trial 1
We will measure your maximal oxygen uptake (VO2max). This can also be thought of as your maximal aerobic capacity or fitness level. You will perform this test at 5,250 ft (Albuquerque's altitude). The purpose of the VO2max test at Albuquerque's altitude (5,250 ft) is to determine if you fit our criteria for aerobic capacity. If testing shows that you do not fit our criteria, your participation in the study will not

continue. However, you will be given the results of your test. This VO2max test will also be used to determine your workloads for subsequent submaximal exercise tests at this altitude.

To determine your VO2max, you will perform a maximal graded exercise test on a bicycle using a protocol that involves easy cycling (70 Watts) for 2 min, then the workload will get harder (by 35 Watts every minute) until you can no longer maintain a cadence of 60 rpm or it gets too hard for you to continue. During the exercise test, you are required to wear a nose clip and breathe through a mouthpiece hooked up to a hose so that all your expired air can be collected and analyzed continuously using a measurement system. You will also have a heart rate transmitter strap around your chest. This test will last between 8 and 12 minutes. The total time commitment for this first visit will be about one hour.

Trials 2 and 3:
You will perform two 10 mile cycling time-trials (TT) at 5,250 ft. We want to determine how quickly you can cycle for 10 miles without resting. These two tests will be separated by at least 24 hrs. You will complete an easy 10 min warm-up (75 Watts) followed by a 10 mile self-paced TT. We will show you how to select a higher gear if you want to attain higher speeds. Heart rate will be continuously monitored, while oxygen saturation (SaO2), how much oxygen is saturated in your blood, and perception of effort (RPE), how hard you feel you are working will be measured every one mile as well as at the end of the TT. You will be informed of the distance covered at the 3 mile mark and every ½ mile thereafter, however, you will not be given any feedback regarding your heart rate, power output, or performance time. The time this test will take will vary depending on your fitness level and power output. It should take approximately 30-40 minutes. The total time for each of these tests will be one to one and a half hours.

Trial 4
Your maximal oxygen uptake (VO2max) will be measured while you are at high altitude (13,450 ft) in a special chamber. A medical doctor will be present during the maximal exercise tests. The chamber simulates high altitude by changing the air pressure, with lower pressure simulating ascent to higher altitude. The chamber is sealed to maintain pressure, but fresh air is pumped in from the outside. It takes about one minute to "ascend" or "descend" 1000 ft of elevation. To determine your VO2max, you will perform a maximal graded exercise test on a bicycle exactly as you did at Albuquerque's altitude. This includes cycling with the mouthpiece and nose clip until your cadence drops below 60 rpm. The exercise test will take between 8 and 12 minutes to complete. There will be several people on the research team in the chamber with you. You will stay in the high altitude chamber only long enough to ascend to 13,450 ft., complete the test, and descend back to 5,250 ft. The total time for this visit will be 45 minutes to one hour.

Trials 5 and 6:
We will determine your submaximal (less than your maximal exercise effort) exercise economy and efficiency, which are common factors that are related to sports performance. You will perform 20 min of submaximal exercise on a bicycle at both 5,250 ft (Albuquerque's altitude) and 13,450 ft. One exercise trial will be performed at 5,250 ft and one exercise trial will be performed at 13,450 ft. These two tests will be separated by at least 24 hrs. You will exercise for 10 min at approximately 50% of your peak power output achieved during the maximal exercise test at the respective altitude. You will be asked to maintain a cadence of 80 rpm. In order to maintain the necessary cadence, you will be provided visual

feedback of the digital tachometer on the bicycle. Five minutes into each 10 min bout, you will be set up to breathe through a mouthpiece and nose clip where your expired air will be collected and analyzed continuously using a measurement system. For your comfort, the mouthpiece will be removed after each data collection time-frame. You can choose to drink water whenever you do not have the mouthpiece in your mouth. The high altitude trial will take longer than the Albuquerque altitude trial as the "ascent" and "descent" of the chamber will take approximately 10 minutes each way. The total time required for each visit will be 45 minutes to one hour.

Trial 7:
A heat tolerance test (HTT) will be performed at 5,250 ft to determine how well you will be able to tolerate exercising in a hot room. The HTT will be performed in a heat chamber at 104°F. You will exercise on a bicycle at 50% of your 5,250 ft VO2max (this is considered an easy to moderate exercise intensity) for 45 min. Prior to the HTT and after urinating into a container, you will enter a private room to measure your nude body weight on an electronic scale. Your urine sample will be collected to determine your hydration status. If you are dehydrated, you will be asked to consume 16 ounces (500 mL) of water, followed 30 min later by a second assessment of hydration. You will then be instructed how to self-insert a rectal thermistor ~4 inches (10 cm) past your anal sphincter for measurement of your core body temperature during the trial. Skin thermistors will also be taped on your chest, arm, and thigh to measure skin temperature throughout the HTT. Heart rate (HR) will be assessed continuously during the HTT using a heart rate strap that you wear around your chest. The HTT will be terminated if you: 1) request to stop, 2) are unable to sustain the predetermined exercise workload, or 3) attain a core temperature of greater than or equal to 104°F. This trial will take approximately one to one and half hours.

If your core temperature reaches 104°F or you do not feel well, you will be immediately removed from the heat and you will be asked to lie down with your feet elevated. One of your hands will be placed in a cooler filled with ice water. Towels will be dipped in ice water and applied to your neck, face, arms, and legs. A fan will be directed across your chest and will be run at top speed. Elevating your feet will increase blood return to the central circulation, reducing your heart rate. The combination of cold water and circulating air will rapidly reduce your core temperature, which will also reduce your heart rate. Cold water application in combination with fanning is the gold standard of care for combating heat illness. If your core temperature does not start to return to normal values or signs and symptoms of heat illness are not alleviated, you will be escorted to the Student Health Center or the UNM Emergency room for further medical treatment. Our doctor will follow-up with you to see how you are doing.

A final urine sample will be measured following completion of the HTT. The same procedures as described above will be followed in order to assure that you're properly hydrated prior to leaving the laboratory. If you are dehydrated, you will be asked to stay in the lab and drink water until you are hydrated.

Trials 8-17:
You will be asked to complete 10 consecutive days of heat acclimation (HA) which consists of cycling in a hot room (heat chamber) at a temperature of 104°F. Acclimation to the heat will be induced using a HA

protocol, which consists of easy to moderate cycling at 50% of your 5,250 ft VO2max for two 50 min bouts with 10 min of seated rest between each bout. Your core body temperature will be measured via self-insertion of the rectal thermistor. Your heart rate will be monitored continuously and recorded every five minutes. You will be provided with room temperature water and allowed to drink water freely throughout the trials. We will strongly encourage you to drink water every 10 min during all exercise bouts. If you need to urinate you will do so in a disposable urine container in order for us to measure your urine output. Before and after each HA session, your nude body weight will be measured in a private room and a urine sample will be collected to determine your hydration status. If you're not properly hydrated before and after exercise in the heat you will be asked to consume 500 mL of water followed 30 min later by a second assessment of hydration. Weight, urine output and water consumed will be used to calculate your sweat rate. The HA protocol will be terminated if you: 1) complete the 100 min of cycling, 2) attain a core temperature greater than or equal to 104°F or, 3) request to stop. If you're unable to complete the entire 100 min for any given HA trial, your completed time will be recorded, and you will be asked to continue reporting to the laboratory as scheduled in order to finish the entire 10 days of HA. These HA trials will take approximately two and half hours each.

On day one and day 10 of the HA protocol, two teaspoons (10 mL) of blood (with a total of 4 teaspoons or 20 mL for the entire study) will be drawn from a vein in your arm for determination of hematocrit (packed red blood cells) and hemoglobin (carries oxygen in your blood). This will be done to calculate changes in plasma volume (fluid portion of your blood). All blood draws and blood analysis will be performed in the Exercise Physiology Lab at UNM. All of your de-identified blood samples will be stored in a freezer in a locked room (#B04) within the Exercise Physiology Facility. These samples will only be accessible to the PI and co-investigators. All blood samples will be destroyed after publication of the manuscript(s), no more than two years from completion of data collection. Your hemoglobin and hematocrit values will be given to you if you are interested.

Trials 18-23:
Following completion of the heat acclimation protocol, you will complete the following tests separated by at least 24 hrs: cycling time-trial at 13,450 ft, VO2max at 13,450 ft, 20 min submaximal cycling at 50% VO2max at 13,450 ft, 20 min submaximal cycling at 50% VO2max at 5,250 ft, VO2max at 5,250 ft, and a post-heat tolerance test at 5,250 ft. The final heat tolerance test will be performed to verify that you're heat acclimated. All of the tests will follow the same procedures as described above for the pre-HA testing. The time commitment for each of these six trials will be less than one hour, with the exception of the heat tolerance test, which could take up to one and half hours.

Participation in this study will take a total of 48 hours over a period of 3-4 weeks.

What are the possible risks or discomforts of being in this study?
Every effort will be made to protect the information you give us. However, there is a small risk of loss of privacy and/or confidentiality. All exercise sessions will be conducted by exercise physiologists who are trained in recognizing the signs and symptoms that require termination of exercise. All study personnel

are CPR/AED certified, as well as trained in the laboratory's emergency protocols. Risks associated with maximal exercise testing may include the following: brief feelings of nausea, lightheadedness, muscle cramps, or dizziness during or after completion of exercise. According to the American College of Sports Medicine, the risk of a cardiac event in normal healthy individuals during a maximal exercise test is minimal, 0.0006% (6 in 10,000). Because you're an endurance trained athlete you're accustomed to exercising at a high intensity for prolonged periods of time, the risk will be less.

Exercising at a higher altitude and exercising in a hot room may also make you have brief feelings of nausea, lightheadedness, muscle cramps or dizziness. Exercising in a hot room may make you feel tired and overheated, and exercising at high altitude may also cause you to feel fatigued. During all of the high altitude trials we will be monitoring any signs or symptoms of acute mountain sickness using a validated questionnaire. Symptoms of acute mountain sickness include nausea, headache, high altitude pulmonary and cerebral edema. However, that these symptoms do not develop in healthy people until at least 6 hrs after ascent, even during heavy exercise. You will only be at peak altitude for approximately one hour, therefore, we do not foresee the development of acute mountain sickness. A medical doctor will be present during all maximal exercise tests at high altitude.

Drawing blood may cause temporary pain and discomfort from the needle stick, occasional bruising, sweating, feeling faint or lightheaded, and in rare cases, infection. You may feel embarrassed or uncomfortable placing the rectal probe, however the rectal probe does not pose any additional risk to you. This procedure is necessary in order to monitor your core temperature for safety reasons. This measurement allows us to make sure that your temperature is not getting high enough to put you at risk for heat stroke/heat exhaustion. Heat stroke/heat exhaustion is defined as a core temperature of greater than 104°F that can cause disorientation, dizziness, headache, nausea, and vomiting. Heat stroke/heat exhaustion signs and symptoms as described above occur during prolonged exercise in the heat when your body is unable to properly cool itself by sweating. The risk of death and/or organ damage due to heat illness is not well documented. In high school athletes, non-fatal heat illness occurred in 1.6 per 100,000 athletic exposures. While heat stroke/heat exhaustion is rare when body core temperature is kept below 104 degrees F, there is a small possibility of unknown risks when exercising in the heat below this temperature. During the heat tolerance test and heat acclimation trials we will record your core temperature and sensation of heat every five minutes and will continuously monitoring your core temperature and how you feel. In that time, if a core temperature above 104°F is either observed or recorded we will immediately stop the exercise before you have any signs or symptoms of heat stroke/heat exhaustion. Thus, at any point during the heat trials we will terminate exercise if you achieve a core temperature of 104°F or if you are not feeling well. The necessary procedures as described above will be taken to cool and lower your core temperature. These procedures would be done to lower your core temperature and help you feel better.

You also may be uncomfortable having to refrain from having any caffeine or eating any food before each visit. This study requires a lot of your time, and the timing of each test is important, therefore you made feel inconvenienced by the required schedule. There are risks of stress, emotional distress, inconvenience and possible loss of privacy and confidentiality associated with participating in a research study.

How will my information be kept confidential?

Your name and other identifying information will be maintained in locked files, available only to authorized members of the research team, for the duration of the study. For any information entered into a computer, the only identifier will be a unique study identification (ID) number. Your health questionnaire, informed consent, and HIPAA will be completed in a private room. We will keep a key that links you with your ID number, but that link will be kept in a locked filing cabinet with access only to the study team. The link will be destroyed after we publish the study results. In no instance will your name be used for any published or presented accounts of the results. All tests will be conducted in private areas in the Exercise Physiology lab located in Johnson Center or the High Altitude Chamber located in Carlisle Gym. The research team will not access any outside information, such as your medical records. Only the paperwork for the current study will be used. Any personal identifying information and any record linking that information to study ID numbers will be destroyed when the study is completed. Information resulting from this study will be used for research purposes and may be published; however, you will not be identified by name in any publications. Urine samples will be destroyed immediately after your hydration status is determined. All of your de-identified (subject # only) blood samples will be stored in a freezer in a locked room (Room # B04, Johnson Center) only accessible to the PI and co-investigators. All de-identified samples will be destroyed after publication of the manuscript(s), no more than two years from the completion of data collection.

Information from your participation in this study may be reviewed by federal and state regulatory agencies, and by the UNM Human Research Review Committee (HRRC) which provides regulatory and ethical oversight of human research. There may be times when we are required by law to share your information. However, your name will not be used in any published reports about this study.

What are the benefits to being in this study?
There may or may not be direct benefit to you from being in this study. However, your participation may help find out how individuals respond to exercise at high altitude following heat exposure. Following completion of the study you will be informed of your results from all cycling tests. The results from the maximal exercise tests and time trials may be beneficial for you as you can use the information for determining an optimal exercise intensity and duration of exercise. This information can allow you to train more effectively and to potentially become more successful in cycling competitions. We will inform you about all of your blood test results, both pre- and post-testing. Our physician will talk with you if any of your blood tests are not within the normal range.

What other choices do I have if I don't participate?
Taking part in this study is voluntary so you can choose not to participate.

What are the costs of taking part in this study?
The primary cost for participating in this study is your time. If you park on or around the University campus you will be responsible for all parking fees.

Will I be paid for taking part in this study?
For your time and inconvenience you will be paid in the following amounts with three VISA gift cards: $20 at the completion of all pre-testing; $50 after completion of the heat acclimation; $80 when all testing

is complete for a total of $150. The last day of your participation, you will be given the last ($80) gift card.

What will happen if I am injured or become sick because I took part in this study?
If you are injured or become sick as a result of this study, UNMHSC will provide you with emergency treatment, at your cost.

No commitment is made by the University of New Mexico Health Sciences Center (UNMHSC) to provide free medical care or money for injuries to participants in this study.

In the event that you have an injury or illness that is caused by your participation in this study, reimbursement for all related costs of care will be sought from your insurer, managed care plan, or other benefits program. If you do not have insurance, you may be responsible for these costs. You will also be responsible for any associated co-payments or deductibles required by your insurance.

It is important for you to tell the investigator immediately if you have been injured or become sick because of taking part in this study. If you have any questions about these issues, or believe that you have been treated carelessly in the study, please contact the Human Research Review Committee (HRRC) at the (505) 272-1129 for more information.

How will I know if you learn something new that may change my mind about participating?
You will be informed of any significant new findings that become available during the course of the study, such as changes in the risks or benefits resulting from participating in the research or new alternatives to participation that might change your mind about participating.

Can I stop being in the study once I begin?
Yes. You can withdraw from this study at any time without affecting your education or employment at the University of New Mexico.

The investigators have the right to end your participation in this study if they determine that you no longer qualify to take part, if you do not follow study procedures, or if it is in your best interest or the study's best interest to stop your participation.

HIPAA Authorization for Use and Disclosure of Your Protected Health Information (HIPAA)
As part of this study, we will be collecting health information about you . This information is "protected" because it is identifiable or "linked" to you.

Protected Health Information (PHI)
By signing this Consent Document, you are allowing the investigators and other authorized personnel to use your protected health information for the purposes of this study. This information may include: height, weight, age, percent body fat, blood pressure, your self-reported medical & exercise history, cycling exercise test results, heart rate, oxygen saturation (SaO2), rating of perceived exertion (RPE), volume of oxygen consumption (VO2), skin temperature, core temperature, respiratory exchange ratio (RER), thermal sensation, Lake Louise acute mountain sickness questionnaire, and subject questionnaire

form (exercise and diet log). We will also collect your urine to assess hydration and blood for the measurement of hemoglobin and hematocrit.

In addition to researchers and staff at UNMHSC and other groups listed in this form, there is a chance that your health information may be shared (re-disclosed) outside of the research study and no longer be protected by federal privacy laws. Examples of this include disclosures for law enforcement, judicial proceeding, health oversight activities and public health measures.

Right to Withdraw Your Authorization
Your authorization for the use and disclosure of your health information for this study shall not expire unless you cancel this authorization. Your health information will be used as long as it is needed for this study. However, you may withdraw your authorization at any time provided you notify the UNM investigators in writing. To do this, please send letter notifying them of your withdrawal to:

Christine Mermier, PhD
MSC 04 2610
1 University of New Mexico
Albuquerque New Mexico 87131

Please be aware that the research team will not be required to destroy or retrieve any of your health information that has already been used or shared before your withdrawal is received.

Refusal to Sign
If you choose not to sign this consent form and authorization for the use of your PHI, you will not be allowed to take part in the research study.

What if I have questions or complaints about this study?
If you have any questions, concerns or complaints at any time about the research study, Christine Mermier, PhD, or her associates will be glad to answer them at 505-277-2658 Monday-Friday from 8:00 am to 5:00 pm by phone. If you would like to speak with someone other than the research team, you may call the Human Research Review Committee (HRRC) at (505) 272-1129. The HRRC is a group of people from UNMHSC and the community who provide independent oversight of safety and ethical issues related to research involving human participants.

What are my rights as a research participant?
If you have questions regarding your rights as a research participant, you may call the Human Research Protections Office (HRPO) at (505) 272-1129 or visit the HRPO website at http://hsc.unm.edu/som/research/hrrc/.

Consent and Authorization

You are making a decision whether to participate in this study. Your signature below indicates that you read the information provided (or the information was read to you). By signing this Consent Form, you are not waiving any of your legal rights as a research participant.

I have had an opportunity to ask questions and all questions have been answered to my satisfaction. By signing this Consent Form, I agree to participate in this study and give permission for my health information to be used or disclosed as described in this Consent Form. A copy of this Consent Form will be provided to me.

Name of Adult Participant (print)	Signature of Adult Participant	Date
		/

I have explained the research to the participant and answered all of his questions. I believe that he understands the information in this consent form and freely consents to participate.

Name of Research Team Member	Signature of Research Team Member	Date
		/

Appendix B

UNM

WELL TRAINED CYCLISTS *needed for research:*

The effects of 10 day heat acclimation on exercise capacity at altitude

HRPO# 13-599

The purpose of this study is to investigate the effects of 10 days of exercising in the heat on an individual's ability to exercise at altitude. Total time for laboratory visits is about 48 hours over a 5 week period. As a participant in this study you will receive results of your ventilatory threshold, maximal oxygen uptake (VO_{2max}), and average power output during a 10 mile cycling time-trial which may be beneficial for exercise training purposes. In addition, study participants will be paid up-to $150 for their time.

Selection criteria include:

- Male well trained cyclists
- 20 to 44 years of age
- free of cardiovascular disease, acute illness, and lower body injury
- no prior heat injury (such as heat stroke and heat exhaustion)
- residing in an elevation of approximately 5,000 ft (Albuquerque)

If you are interested in participating, please contact:

-OR-

Ailish White	**Roy Salgado**
Health, Exercise, and Sports Sciences	**Health, Exercise, and Sports Sciences**
ailish15@unm.edu	**demano@unm.edu**
760-212-6486	**707-580-4076**

Heat acclimation and exercise at
altitude
Ailish White
Ailish15@unm.edu
760-212-6486

Heat acclimation and exercise at
altitude
Roy Salgado
demano@unm.edu
707-580-4076

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Ailish White
Ailish15@unm.edu
760-212-6486

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Roy Salgado
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Ailish15@unm.edu
760-212-6486

Appendix C

HEALTH HISTORY QUESTIONNAIRE (RESEARCH ONLY 11/22/13)

Subject #____________________________ Date___/___/___

Phone #: home ______________________ *cell* ______________

Date of Birth ___/___/___ Age____ Gender____ Ethnicity_______ Phone (W)________________

Primary health care provider and health insurance ____
(Only for information/emergency contact)
Person to contact in case of emergency: **Name**___________________________**phone #**____________

♦♦

MEDICAL HISTORY

Self-reported: Height______ Weight______
Physical injuries:____

Limitations____

Have you ever had any of the following cardiovascular problems? Please check all that apply.

Heart attack/Myocardial Infarction	____	Heart surgery	____	Valve problems	_____
Chest pain or pressure	____	Swollen ankles	____	Dizziness	_____
Arrhythmias/Palpitations	____	Heart murmur	____	Shortness of breath	_____
Congestive heart failure	____				

Have you ever had any of the following? Please check all that apply.

Hepatitis/HIV	_____	Stroke	_____	Cancer (specify type)	__________
Rheumatic fever	_____	High blood pressure	_____	Thyroid problems	_____
Kidney/liver disease	_____	Obesity	_____	Total cholesterol >200 mg/dl	_____
Diabetes (specify type)	_____	Asthma	_____	HDL cholesterol <35 mg/dl	_____
Emphysema	_____			LDL cholesterol >135 mg/dl	_____
				Trygylcerides>150 mg/dl	_____

Have you ever suffered from heatstroke or heat exhaustion? Y N

If yes, please explain____

Do immediate blood relatives (biological parents & siblings **only**) have any of the conditions listed above? If yes, list the problem, and family member age at diagnosis.

____

Is your mother living? Y N Age at death______ Cause__________________
Is your father living? Y N Age at death______ Cause__________________

Do you currently have any condition not listed that may influence test results? Y N

Details____

____

Indicate level of your overall health. Excellent____ Good____ Fair____ Poor____Are you taking any medications, vitamins or dietary supplements now? Y N

If yes, what are they?____

Do you have allergies to any medications? If yes, what are they? ______________________________

Are you allergic to latex? Y N

Have you been seen by a health care provider in the past year? Y N

If yes, elaborate ____

Have you had a prior maximal graded exercise test? Y N. If yes, when?_____________ What were the results?

____

Have you ever experienced any adverse effects during or after exercise (fainting, vomiting, shock, palpitations, hyperventilation)? Y N If yes, elaborate_________________________________

____

♦♦♦

LIFESTYLE FACTORS

Do you now or have you ever used tobacco? Y N If yes: type _______________

How long?______ Quantity___/day Years since quitting_____________

How often do you drink the following?

Caffeinated coffee, tea, or soda _______oz/day Hard liquor _______oz/wk Wine _______oz/week

Beer _______oz/wk

Indicate your current level of emotional stress. High____ Moderate ____ Low____

♦♦♦

PHYSICAL ACTIVITY/EXERCISE

Physical Activity

Minutes/Day (*Weekdays*) Minutes/Day (*Weekends*)

______/______ average ______/_____ average

Do you train in any activity (eg. jogging, cycling, swimming, weight-lifting)? Y N

How well trained are you? ____

Have your participated in cycling exercise/training for the last year Y N

If yes, briefly describe your training____

____

Vigorous Exercise (>30 Minute sessions)

_________Minutes/hours a week

♦♦

Appendix D

Verification of Subject Compliance to Study Guidelines

My medical status has changed recently. yes ____ no ___

If your status has changed, please list information here.

I recently used a hot tub, sauna or hot room in the past 24 hrs. yes ____ no ____

If 'yes,' briefly describe the temperature and duration of exposure.

I recently went to an altitude >Albuquerque (1600 m) in the past 24 hrs. yes ____ no ____

I have completed strenuous *lower-body exercise* in the previous 24-48 hrs. yes ___ no ___

I am currently sick. yes ___ no ___

I have consumed coffee AND/OR alcohol in the previous 24 hrs. yes ___ no ___

I have fasted for at least 12 hrs. yes ___ no ___

Please record here the amount of food and the volume of fluid ingested in the last 24 hrs…

Breakfast:

Lunch:

Dinner:

Snacks:

Please describe your training within the last 24 hrs including intensity/duration/frequency of physical activity. Keep in mind that this must be MAINTENANCE TRAINING.

Appendix E

VO_{2max}

Subject: _____ **Date:** _____

Trial #: _____ 1600 m 4100 m

Age: _____

Height: _____

Pre Weight: _____

Resting BP: _____

3 Site SkF:	
Sum:	

Protocol: _____ Peak Power: _____

Time	Workload	HR	SaO_2	RPE	Comments
Rest	0				
1	70				
2	105				
3	140				
4	175				
5	210				
6	245				
7	280				
8	315				
9	350				
10	385				
11	420				
12	455				
13	490				
14	525				
R1	50				
R2	50				
R3	50				

Post Weight: _____ Test time: _____

Appendix F

Submaximal Exercise

Subject #: _____ **Date:** _____

Trial #: _____ **1600 m** **4100 m** **pre** **post**

Age: _____ yr

Height: _____ cm

Pre Weight: _____ kg

Resting BP: _____ mmHg

Protocol: _____ Peak Power Output: _____ Watts

Time (min)	Workload	HR (bpm)	SaO2 (%)	VO_2 (L/min)	VO2 (mL/kg/min)	RER	RPE

Time (min)	Workload	HR (bpm)	SaO2 (%)	VO_2 (L/min)	VO2 (mL/kg/min)	RER	RPE

Post Weight: _____ kg

Appendix G

Heat Tolerance Test

Subject #: _____ **Date:** _____

Trial #: Pre Post

Hydration: _____ g/mL

Nude Weight: _____ kg

Resting BP: _____ mmHg Hydration: _____ g/mL (Re-test)

Workload: _____ Watts

Time (min)	HR (bpm)	Trec (°C)	Tchest (°C)	Tarm (°C)	TThigh(°C)	RPE	Thermal Sensation
Rest							
5							
10							
15							
20							
25							
30							
35							
40							
45							
Recovery 1							
Recovery 2							
Recovery 3							
Recovery 4							

Post Weight: _____ kg

Comments: _____

Appendix H

Heat Acclimation

Subject #: _____ **Date:** _____
HA Trial #: _____

Hydration: _____ g/mL
Pre Weight: _____ kg Hydration: _____ g/mL (Retest)
Resting BP: _____ mmHg

Hb: 1) _____ 2) _____ 3) _____ g/dL
Workload: _____ Watts Hct: 1) _____ 2) _____ 3) _____ %

Time	HR (bpm)	Trec (°C)	RPE	Thermal Sensation	Room Temp (°C)	Relative Humidity (%)	Comments
Rest							
5							
10							
15							
20							
25							
30							
35							
40							
45							
50							
5 rest							
10 rest							
5							
10							
15							
20							
25							
30							
35							
40							
45							
50							
Recovery 1							
Recovery 2							
Recovery 3							
Recovery 4							

Post Weight: _____ kg

Appendix I

Heat Acclimation

Subject #: _____ **Date:** _____

Trial #: _____

Hydration (pre): _____ post _____

Pre Weight: _____

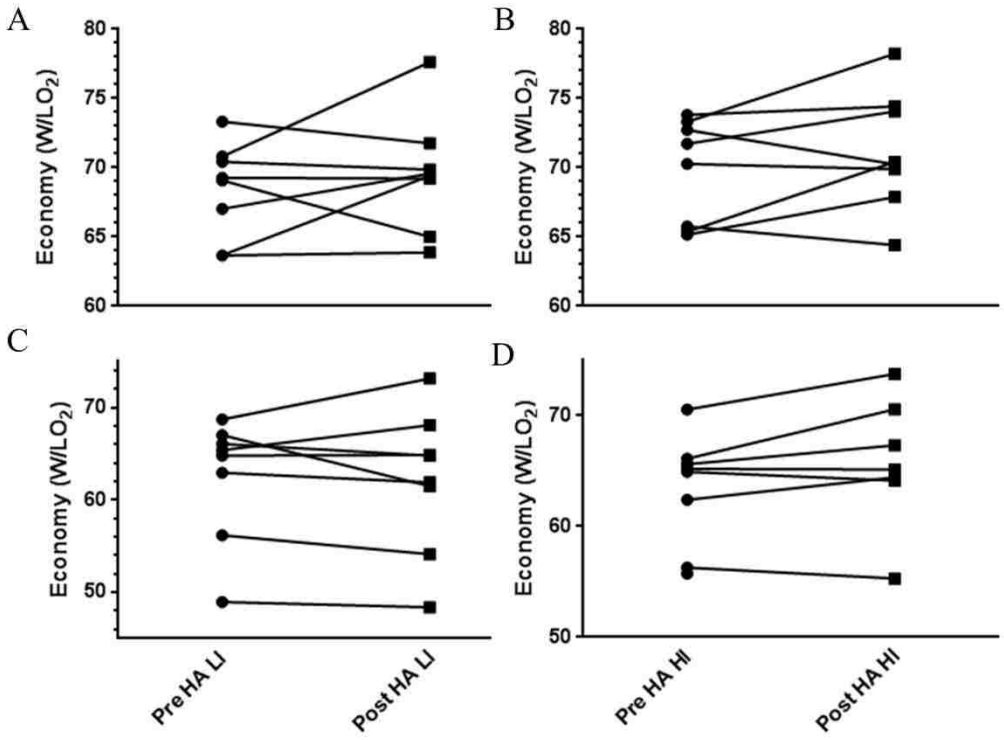
Resting BP: _____ Hb: _____ Hct: _____

Workload: _____

Time	HR	Trec	RPE	Thermal Sensation	Room Temp	Relative humidity	Comments
Rest							
5							
10							
15							
20							
25							
30							
35							
40							
45							
50							
5 rest							
10 rest							
5							
10							
15							
20							
25							
30							
35							
40							
45							
50							
Recovery 1							
Recovery 2							
Recovery 3							
Recovery 4							

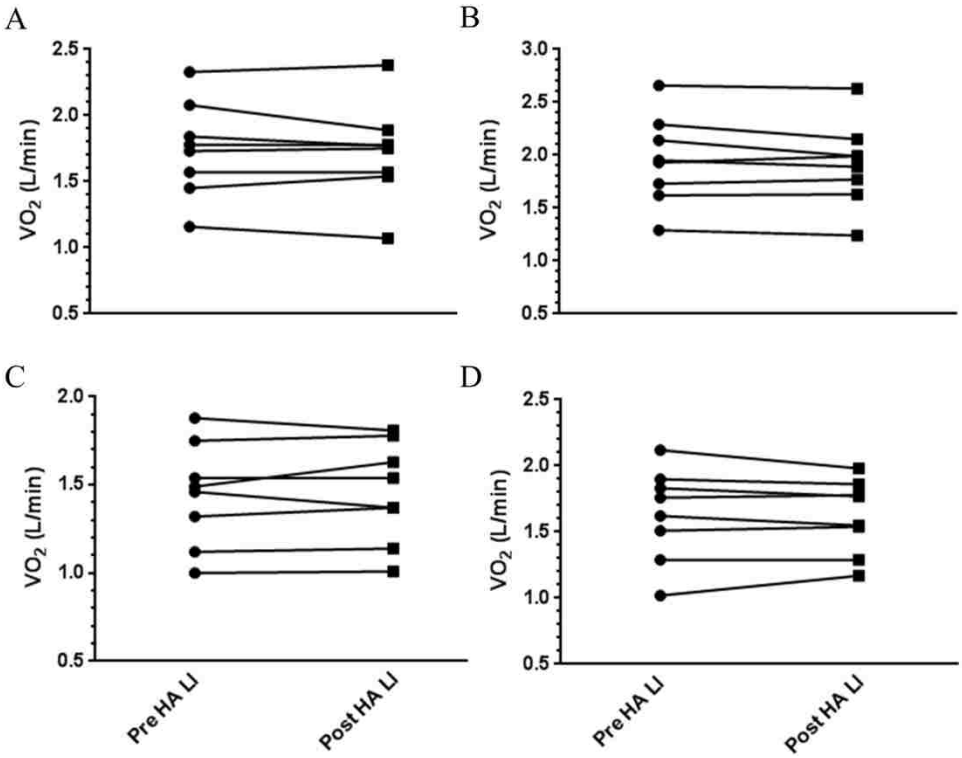
Post Weight: _____

Appendix J



Supplemental Figure 1 (A) Submaximal exercise economy pre and post HA at 1600 m during LI exercise (B) Submaximal exercise economy pre and post HA at 1600 m during HI exercise (C) Submaximal exercise economy pre and post HA at 4350 during LI exercise (D) Submaximal exercise economy pre and post HA at 4350 during HI exercise

Appendix K



Supplemental Figure 2 (A) Submaximal oxygen consumption pre and post HA at 1600 m during LI exercise (B) Submaximal oxygen consumption pre and post HA at 1600 m during HI exercise (C) Submaximal oxygen consumption pre and post HA at 4350 during LI exercise (D) Submaximal oxygen consumption pre and post HA at 4350 during HI exercise