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TITLE: Evaluation of the Physiological Challenges in Extreme Environments: Implications for Enhanced Training, Operational Performance and Sex-Specific Responses

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CONTRACTING ORGANIZATION: The University of Montana, The University of Nebraska at Omaha

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14. ABSTRACT: he overarching aim of this project series was to examine the impacts of differential environmental conditions (hypobaric hypoxia - 4400 m, normobaric hypoxia - 4400 m, normobaric normoxia - sea level to 1000 m hot - 33Â°C, cold - 7Â°C, and thermoneutral - 20Â°C.) on markers of exercise response/acclimation. The scope of the project was completed under the prerequisite of known performance decrements in extreme environments. Experiments were designed with consideration to military operations under the above-mentioned environments. Our research aim was completed using a grand total of 91 research subjects (53 males, 38 females) across the study series. In task 1, we set out to determine the impact of hypobaric hypoxia and normobaric hypoxia on markers of mitochondrial biogenesis, myogenesis, and oxidative stress following exercise. Our major findings indicate that markers of muscle growth and breakdown were minimally altered by altitude stress. Similarly, oxygen sensing/delivery genes and mitochondrial gene responses were not altered by altitude stress. Moreover, there were no differences across sex. While prior research has clearly demonstrated impairments to performance and altitude-oriented losses in skeletal muscle mass and function, the present results from year 1 suggest that an impaired skeletal gene response to altitude can be offset with appropriate exercise intervention. Exercise intervention and appropriate physical training programs may act to minimize expected losses in skeletal muscle health and function during high altitude staging and operations. In task 2/3, we set out to determine the effects of environmental temperature (hot, cold, thermoneutral) on markers of mitochondrial biogenesis, oxidative stress and sex-difference (male v female) performance and thermoregulation during 3-week exercise training periods (15 training sessions). Our major findings indicate that exercise training in the heat compared with thermoneutral exercise training may limit the effectiveness of aerobic exercise at increasing aerobic capacity, with findings pointing to a heat induced blunting of the mitochondrial biogenesis marker, PGC-1a. However, the blunted PGC-1a response to acute exercise is alleviated following 3 weeks of heat acclimation.					
15. SUBJECT TERMS environmental stress, hypoxia, hypobaria, oxygen saturation, heat stress, cold stress, sex differences					
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Evaluation of the physiological challenges in extreme environments: Implications for enhanced training, operational performance and sex-specific responses

1. INTRODUCTION

The stresses of the training continuum and the operational battlefield are compounded by the potential impacts of environmental extremes (hypoxia, heat, cold). Moreover, the interaction between soldier and environment can be leveraged to positively impact training adaptations, acclimatization, and operational performance. However, arduous environments can also delay and impair training success, conflicting with optimization of operational readiness. Department of Defense policy has also begun to push for a more comprehensive inclusion of females in frontline/combat oriented positions, implying exposure to the environmental extremes of combat. Females, who make up 14.6% of the active military members, have been historically underrepresented in the scientific literature, exposing a gap in corresponding evidence based training and safety recommendations. Data from our lab(s) has demonstrated that training strategies commonly utilized to leverage environmental preparedness (acute hypoxia, heat exposure) conflict with necessary cellular signals that maximize aerobic performance and muscle recovery. Therefore, the purpose of this project series was to better understand the physiological benefits and consequences of environmental stress at the tissue and muscular level in response to acute exercise and recovery and exercise training responsiveness to varied environmental stresses. The presented work(s) extends upon our previous findings, culminating in a capstone project that takes laboratory supported findings and translates them to a field-based scenario within the scope of military operation generalizability. While we were able to adhere to our projected project schedule and data collection efforts, our present study series provides further evidence that the female specific response to acute and extended exercise training requires additional consideration.

Classic models of heat stress, muscle loading, muscle recovery and training adaptation should not and cannot be effectively superimposed on to female service persons simply by scaling for expected variations in body and skeletal muscle mass. Moreover, to do so, discredits the physiological strengths and unique capabilities of the female warfighter.

2. KEYWORDS

Exercise, environmental stress, hypobaric hypoxia, normobaric hypoxia, skeletal muscle gene response, mitochondria, oxidative stress, oxygen saturation, heat stress, cold stress, sex-differences

3. ACCOMPLISHMENTS

In general, we were able to closely adhere to our proposed schedule despite the relocation of Dr. John Quindry from Auburn University to the University of Montana, dual location data collection (University of Montana, University of Nebraska at Omaha), remote data collection (Kailua-Kona, HI), and COVID-19.

○ Major goals

- Objective 1 - Determine the physiological differences between hypobaric hypoxia and normobaric hypoxia using markers of mitochondrial biogenesis and oxidative stress. Completed during year 1.
- Objective 2 - Determine the effect of environmental temperature (heat, cold) on exercise response and adaptation while simultaneously examining sex specific differences. Completed during years 2 and 3.
- Objective 3 - Implement findings from objective 1 into the design of a field-based experiment for the generalizability of laboratory-based evidence into the training/operational environment. Completed during year 4.

Year 1	Month											
Milestone	1	2	3	4	5	6	7	8	9	10	11	12
Human subjects approval (Study 1)												
Data collection (Study 1)												
Sample analysis (Study 1)												
Data reporting (Study 1)												
Human subjects appr. (Study 2 a)												
Nebraska chamber installation												

Year 2	Month											
Milestone	1	2	3	4	5	6	7	8	9	10	11	12
Data Collection (Study 2 a)												
Human subjects approval (Study 2 b)												
Data collection (Study 2 b)												
Sample analysis (Study 2 a, b)												
Data reporting (Study 2 a, b)												
Human subjects appr. (Study 3 a, b)												

Year 3	Month											
Milestone	1	2	3	4	5	6	7	8	9	10	11	12
Data collection (Study a, b)												
Data analysis (Study 3 a, b)												
Data reporting (Study 3 a, b)												
Human subjects appr. (Study 4)												

Year 4	Month											
Milestone	1	2	3	4	5	6	7	8	9	10	11	12
Data collection (Study 4)												
Data analysis (Study 4)												
Data reporting (Study 4)												
Final Report (all studies)												

Timeline for University IRB/Army HRPO human subjects approval, data collection, sample and data analyses and reporting schedule.

○ **Accomplished under above-mentioned major goals**

- Objective 1: Skeletal Muscle mRNA Response to Hypobaric and Normobaric Hypoxia after Normoxic Endurance Exercise.
 - Methods: 15 recreationally trained subjects each cycled for 1 hour before recovering for 4 hours in NN (laboratory atmospheric conditions, 975 m), HH (depressurized to simulate 4420 m), and NH (fraction of O₂ reduced to simulate 4420m). Muscle biopsy samples were obtained before exercise and after 4 hours of recovery.
 - Results: Blood oxygenation (SpO₂) was lower in HH (76.02 – 0.58%) than NH (79.45 – 0.56, $p < 0.001$), which were both lower than in NN (96.3–0.17, $p < 0.001$). Heart rate was higher in HH (82–2bpm) than NH (77 – 1 bpm, $p < 0.001$), which were both higher than in NN (67 – 1 bpm, $p < 0.001$). Mitochondrial transcription factor A (TFAM) mRNA was lower after NN than HH ($p=0.034$) or NH ($p=0.005$), but was not different between HH and NH ($p = 0.460$). Myostatin (MSTN) mRNA decreased from pre- to postexercise ($p < 0.001$) in all conditions and was lower in HH compared with NH ($p = 0.035$) and NN ($p = 0.017$). No other differences were noted in genes related to mitochondrial biogenesis, myogenesis, or proteolysis ($p > 0.05$).
 - See associated manuscripts for additional findings.
- Objective 1: Blood Oxidative Stress and Post-Exercise Recovery are Unaffected by Hypobaric and Hypoxic Environments.
 - Methods: The current study was undertaken to quantify exercise-induced oxidative stress recovery during lab-simulated hypoxic and hypobaric conditions following a

common bout of exercise. Methods: At a base elevation of 975m, physically active participants (n=15), ages 18-40, provided informed consent prior to performing 60 minutes of cycle ergometry at 70% watts max. Using a randomized counter-balanced crossover design, participants recovered for 4 hours in 3 lab-simulated conditions; 1000m normobaric normoxia (NN, 675mmHg, 18.8%FiO₂), 4400m normobaric hypoxia (NH, 675mmHg, 12% FiO₂), or 4400m hypobaric hypoxia (HH, 440mmHg, 12% FiO₂). O₂ saturation was confirmed via pulse oximetry throughout the 3 exercise-recovery trials. Blood samples were collected in heparinized vacutainer tubes at time points Pre, Post, 2 Hours Post, and 4 Hours Post exercise. Blood plasma was analyzed for the quantification of oxidative stress to proteins (protein carbonyls, PC; 3-nitrotyrosines, 3NT), lipid (lipid hydroperoxides, LOOH; 8-isoprostanes, 8-ISO), and antioxidant capacity (ferric reducing ability of plasma, FRAP; trolox equivalent antioxidant capacity, TEAC).

- Results: While hypoxia and hypobaria had no effect throughout the recovery period, post-exercise TEAC (p=0.041), FRAP (p=0.013), and 8-ISO (p=0.044), increased while PC (p=0.002) and 3-NT (p=0.032) were decreased. LOOH was lower in Post (p=0.018) NH trial samples. Exercise-dependent increases occurred in NFE2L2 (p=0.003), HMXO1 (p<0.001), SOD2 (p=0.046), and EPAS (p=0.038). In this study, exercise recovery in simulated conditions of HH did not consistently impact blood oxidative stress or redox-sensitive muscle redox measures.
- Objective 2: Exercise in the Heat Blunts Improvements in Aerobic Capacity.
 - Methods: Twenty-one untrained college aged males (age, 24 ± 4 yrs; height, 178 ± 6 cm) were randomly assigned to three weeks of aerobic exercise training in either 33°C (n=12) or 20°C (n=11) environmental temperatures.
 - Results: The 20°C training group increased VO_{2peak} from 3.21 ± 0.77 L·min⁻¹ to 3.66 ± 0.78 L·min⁻¹ (p<0.001) while the 33°C training group did not improve (pre, 3.16 ± 0.48 L·min⁻¹ ; post, 3.28 ± 0.63 L·min⁻¹ ; p=0.283). *PGC-1a* increased in response to acute aerobic exercise more in 20°C (6.6±0.7 fold) than 33°C (4.6±0.7 fold, p=0.031) before training, but was no different after training in 20°C (2.4±0.3 fold) or 33°C (2.4±0.5 fold, p=0.999). No quantitative alterations in mitochondrial DNA were detected with training or between temperatures (p>0.05).
- Objective 2: Effects of 7°C Environmental Temperature Acclimation During a 3-Week Training Period.
 - Methods: Two groups of 12 untrained male subjects completed 1 h of cycling in 16 temperature acclimation sessions in either a 7°C or 20°C environmental temperature. Fitness assessments before and after acclimation occurred at standard room temperature. Muscle biopsies were taken from the vastus lateralis muscle before and after training to assess molecular markers related to mitochondrial development.
 - Results: Peroxisome proliferator-activated receptor-gamma coactivator 1a (*PGC-1a*) mRNA was higher in 7°C than in 20°C in response to acute exercise before training (*P* = 0.012) but not after training (*P* = 0.813). *PGC-1a* mRNA was lower after training (*P* < 0.001). *BNIP3* was lower after training in the 7°C than in the 20°C group (*P* = 0.017) but not before training (*P* = 0.549). No other differences occurred between temperature groups in *VEGF*, *ERRa*, *NRF1*, *NRF2*, *TFAM*, *PINK1*, *Parkin*, or *BNIP3L* mRNAs (*P* > 0.05). *PGC-1a* protein and mtDNA were not different before training, after training, or between temperatures (*P* > 0.05).

Cycling power increased during the daily training ($P < 0.001$) but was not different between temperatures ($P = 0.169$). $\dot{V}O_{2\text{peak}}$ increased with training ($P < 0.001$) but was not different between temperature groups ($P = 0.460$).

▪ Objective 2: Exercise Training in a Hot Environment Fails to Elicit Changes in Blood Oxidative Stress Response.

- Methods: As above.
- Results: Biomarkers for oxidative damage demonstrated minimal differences across the varied environmental conditions. For LOOH, no differences were observed between environmental temperatures ($p = 0.204$). Exercise resulted in an elevation of LOOH at 2-HR ($p = 0.001$) and 4-HR ($p < 0.001$). Trial-dependent differences ($p < 0.001$) and a significant trial x time interaction effect were present ($p = 0.018$). Peak responses of LOOH were not different between environmental temperatures ($p = 0.128$), but a significant elevation occurred with exercise ($p < 0.001$). Additionally, an effect of trial ($p < 0.001$) and a trial x time interaction ($p = 0.026$) occurred. Further examination of the interaction effect for peak responses of LOOH also revealed that all post-acclimation training values were significantly higher than pre-acclimation training values ($p < 0.05$). 8-ISO was unaffected by temperature ($p = 0.287$), acclimation training ($p = 0.305$), and exercise ($p = 0.691$). Peak responses revealed no differences between environmental temperature for LOOH ($p = 0.128$) or 8-ISO ($p = 0.273$).

▪ Objective 2: Effects of Exercise Training in Hot and Cold Temperatures.

- Methods: Recreationally trained subjects made 18 visits to the lab and performed a 3-week cycling protocol in an assigned environmental temperature (Males: 20°C, $n = 12$; 33°C, $n = 12$; 7°C, $n = 12$; Females: 20°C, $n = 12$; 33°C, $n = 12$). Aerobic capacity, body composition, heart-rate, sweat rate, core and skin temperature were measured before and after the 3-week exercise training protocol. During the 3-week training heart rate, power output, temperature, and sweat rate were measured daily.
- Results: The 33°C subjects had greater skin temperature ($p < 0.001$), sweat rate ($p < 0.001$), and heart rate ($p = 0.034$) than 20°C subjects. From pre- to post-training, core temperature ($p = 0.001$) lowered in 33°C subjects. Skin temperature ($p = 0.011$) and heart rate ($p < 0.001$) decreased with training regardless of temperature. Following training, males had a higher fat free mass ($p < 0.001$), absolute $\dot{V}O_{2\text{peak}}$ ($p < 0.001$), and sweat rate ($p = 0.001$) compared to women. Women had higher core temperature ($p = 0.036$) and percent body fat ($p = 0.046$) compared to men. Women improved $\dot{V}O_{2\text{peak}}$ following training in a 33°C environment ($p = 0.028$), whereas men improved $\dot{V}O_{2\text{peak}}$ following training in 20°C environment ($p < 0.001$). In 7°C compared to 20°C, $\dot{V}O_{2\text{peak}}$ ($p < 0.001$), body composition ($p < 0.001$), core temperature ($p < 0.001$), sweat rate ($p = 0.05$), and power ($p < 0.001$) improved in both conditions. Skin temperature was lower in 20°C ($p < 0.001$).

▪ Objective 3: Hawaii Field Study.

- Methods: 18 participants (8 males, 8 females) completed three trials in a randomized and counter-balanced order. The trials consisted of a sea level control environment (C), a sea level hypoxia trial (H), and a high altitude trial. The C trial was conducted close to sea level. The H trial was conducted at the same elevation

as the C trial, but participants breathed hypoxic air through a facemask (Higher Peak, Mag 20, Boston, MA). The H trial was conducted at 13,850 ft of elevation. Subjects were transported from sea level to 13,850 ft by automobile which took approximately 90 minutes. During the H trial the hypoxic air machine was adjusted every 10 minutes to simulate the ascent to altitude that occurred in the A trial. Thus, the intensity and duration of hypoxic exposure was similar between H and C. Once participants arrived at steady state environmental conditions they rested supine for 10 minutes before data was collected. Once resting data was collected subjects stood up and then performed 3 minutes of stepping exercise on a box 39 cm tall in time to a metronome set to 88 beats/min (22 steps/min). Exercise data was recorded and averaged over the last 2 minutes.

- Results: TISSUE OXYGENATION: Muscle tissue oxygenation is affected by exercise, but only minimally affected by hypoxia or altitude. This appears to be a tissue specific response, since brain tissue oxygenation is affected by altitude, but only minimally by exercise. CARDIOVASCULAR: Our project design allowed arterial blood oxygenation (SPo2) to be lower in our hypoxia and altitude trials. Cardiac output appears to be increased primarily by heart rate with hypoxia, but further increased at altitude with increased stroke volume. FLUID SHIFTS: There was a greater fluid shift (body water) in the altitude trial when compared to hypoxia or control trials. This additionally supports the hypothesis that hypoxia and terrestrial altitude exposures lead to different physiological responses.

- **Opportunities for training and professional development through project(s)**

- Nothing to report

- **Dissemination of results communities of interest**

- The dissemination of the results was limited to submission and publication in scientific journals in addition to annual reporting at the USMRMC Extreme Environment Annual Review.

- **Plans to accomplish goals**

- Nothing to report.

4. IMPACT

- **Impact on development of principle discipline(s)**

The current project series emphasizes the research areas of Injury Prevention and Reduction, Physiological Health, and Environmental Health and Protection. The results from this project series may assist Commanders and medical personal to more effectively prevent and mitigate warrior injury, enhance overall protection from hazards, and increase performance throughout the deployment cycle in training and the operational environment. In addition, because females may now assume front line positions, the inclusion of female specific results may begin to pave the way for the regular inclusion of females in operationally relevant research designs and future proposals/projects.

The current state of research in applied human environmental physiology does not allow for direct translation. The information that currently contributes to recommendations is based largely off data from non-human models in non-physiologic conditions. When human models are used, the exclusion of women prevents a direct translation to the female operating in extreme environmental conditions. Thus our use of human subjects (Male and Female) to evaluate physiological conditions unique to relevant training and operational extremes adds to the uniqueness of the present findings.

It is anticipated that these results will demonstrate a general impact on the design and development of future project methodologies. The inclusion of females is essential to the advancement of applied research that seeks to investigate the implications of the training and operational environment on overall performance and injury risk.

- **Impact on other discipline(s)**

The general impact on other disciplines is difficult to discern given the specificity of the present research objectives. However, other disciplines should consider the implications of multiple stressors to overall adaptation and outcome measures. The uniform application of research findings across other environmental conditions is cautioned. Moreover, the potential for sex-specific outcomes should not be discounted.

- **Impact on technology transfer**

Nothing to report.

- **Impact on society beyond science and technology**

The impact on society beyond science and technology is also difficult to discern given the specificity of the present scientific findings. However, as above, the implication of sex-specific responses and outcomes should be given full consideration.

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change**

- Nothing to report

- **Actual or anticipated problems or delays and actions or plans to resolve them**

- Nothing to report

- **Changes that had a significant impact on expenditures**

- Nothing to report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

- Nothing to report

- **Significant changes in use or care of human subjects**

- Nothing to report

- **Significant changes in use or care of vertebrate animals. Significant changes in use of biohazards and/or select agents**

- Nothing to report

6. PRODUCTS

- **Publications, conference papers, and presentations**

- Journal publications

Caleb I. Ross, Robert J. Shute, Brent C. Ruby, and Dustin R. Slivka; *Skeletal Muscle mRNA Response to Hypobaric and Normobaric Hypoxia After Exercise*; High Altitude Medicine and Biology; 20; 2019; 141-149; Published; Acknowledgement of Federal Support – Yes

Robert Shute, Katherine Marshall, Megan Opichka, Halee Schnitzler, Brent Ruby, Dustin Slivka; *Effects of 7°C Environmental Temperature Acclimation During a 3-Week Training Period*; Journal of Applied Physiology; 128; 768-777; 2020; Published; Acknowledgement of Federal Support – Yes

Dustin Slivka Robert Shute, Walter Hailes, Katherine Marshall, Megan Opichka, Halee Schnitzler, Brent Ruby; *Exercise in the Heat Blunts Improvements in Aerobic Capacity*; European Journal of Applied Physiology; In Review, Acknowledgement of Federal Support - Yes

Katherine Marshall, Robert Shute, Caleb Ross, Dustin Slivka; *Effects of Exercise Training in Hot and Cold Temperatures*; Other - Prepared Manuscript; Acknowledgment of Federal Support - Yes

Cassie M. Williamson-Reisdorph, Tiffany S. Quindry, Kathryn G. Tiemessen, Roksana Zak, Robert Shute, John Cuddy, Walter Hailes, Dustin Slivka, Brent Ruby, John C. Quindry; *Blood Oxidative Stress and Post-Exercise Recovery are Unaffected by Hypobaric and Hypoxic Environments*; Other - Prepared Manuscript; Acknowledgment of Federal Support – Yes

Cassie M. Williamson-Reisdorph, Tiffany S. Quindry, Katie S. Christison, Shae Gurney, Kathryn G. Tiemessen, John Cuddy, Walter Hailes, Dustin Slivka, Brent C. Ruby, John C. Quindry; *Exercise Training in a Hot Environment Fails to Elicit Changes in the Blood Oxidative Stress Response*; Other - Prepared Manuscript; Acknowledgment of Federal Support – Yes

Effects of 3-week training and heat acclimation in females: evidence for sex specific environmental responsiveness – after above paper is published so it can be referenced and conclusions made as a whole. In preparation ~ Feb 2021.

Alterations in tissue and brain oxygenation during rest and exercise at normobaric and hypobaric hypoxia. In preparation ~ March 2021.

Alterations in total body water and cardiovascular responsiveness during rest and exercise at normobaric and hypobaric hypoxia. In preparation ~ April 2021.

- Books or other non-periodical, one time publications
 - Nothing to report
- Other publications, conference papers, presentations

Caleb Ross, Robert Shute, Roksana Zak, Brent Ruby, John Quindry, Dustin Slivka; *Effects of Hypobaric and Normobaric Hypoxia on Myogenic and Proteolytic Gene Expression in Humans*; National ACSM Meeting in 2017.

John Quindry, Roksana Zak, Robert Shute, Tiffany Quindry, John Cuddy, Walter Hailes, Dustin Slivka, Brent Ruby; *Exercise Induced Oxidative Stress During Normobaric And Hypobaric Hypoxic Exercise Recovery*; National ACSM Meeting in 2017.

Robert Shute, Roksana Zak, Brent Ruby, John Quindry, Dustin Slivka; *Effects of Hypobaric and Normobaric Hypoxia on Mitochondrial Related Gene Expression*; National ACSM Meeting in 2017.

John Quindry, Tiffany Quindry, Katheryn Tiemessen, Roksana, Zak, Robert Shute, John Cuddy, Walter Hailes, Dustin Slivka, Brent Ruby; *Blood Oxidative Stress Following Exercise Recovery in Normobaric and Hypobaric Hypoxic Environments*; National ACSM Meeting in 2018.

Megan Vande Hei, Robert Shute, Katherine Marshall, Dustin Slivka; *Aerobic training alters PGC-1a but is not different between environmental temperatures*; Nebraska Physiological Society annual meeting in 2018.

Katherine Marshall, Robert Shute, Dustin Slivka; *Effects of environmental temperature on aerobic training adaptations*; Nebraska Physiological Society annual meeting in 2018.

Halee Keller, Robert Shute, Dustin Slivka; *Effects of environmental temperature and exercise on mitochondrial quantity and quality*; Nebraska academy of sciences in 2018.

Halee Keller, Robert Shute, Dustin Slivka; *Effects of environmental temperature on exercise response and adaptation*; UNO Student Research and Creative Activity Fair in 2018.

Halee Keller, Robert Shute, Dustin Slivka; *Effects of environmental temperature on exercise response and adaptation*; INBRE regional conference 2018.

Robert Shute, Caleb Ross, Roksana Zak, Brent Ruby, Dustin Slivka; *Skeletal Muscle Response to Hypobaric and Normobaric Hypoxia after Exercise*; The Nebraska Academy of Sciences in 2018.

- **Websites or other internet sites**
 - Nothing to report
- **Technologies or techniques**
 - Nothing to report
- **Inventions, patent applications, and/or licenses**
 - Nothing to report
- **Other products**
 - Nothing to report

7. PARTICIPANTS AND OTHER COLLOBORATING ORGANIZATIONS

- **Montana**

Name:	Brent Ruby
Project Role:	PI
Researcher Identifier (ORCID ID):	0000-0002-3565-8094
Nearest person month worked:	6 each year
Contribution to Project:	Dr. Ruby coordinated study design, implementation, sample and data collection, and reporting.

Funding Support:	
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Name:	Walter Hailes
Project Role:	Research Associate
Researcher Identifier (ORCID ID):	NA
Nearest person month worked:	6-8 each year
Contribution to Project:	Mr. Hailes coordinated study participant recruitment and management and organized/conducted data collection.
Funding Support:	

Name:	John Quindry
Project Role:	Co-Investigator
Researcher Identifier (ORCID ID):	NA
Nearest person month worked:	3 each year
Contribution to Project	Dr. Quindry is organizing data analyses for the oxidative stress markers.
Funding Support	

○ **Nebraska**

Name:	Dustin Slivka
Project Role:	Co-PI
Researcher Identifier (ORCID ID):	NA
Nearest person month worked:	6-8 each year
Contribution to Project:	Dr. Slivka assisted in study design, implementation, sample analysis, statistical analysis, and reporting.
Funding Support	

Name:	Roksana Zak
Project Role:	Graduate Student (Doctoral)
Researcher Identifier (ORCID ID):	NA
Nearest person month worked:	10 months (over 1 year)
Contribution to Project:	Ms. Zak performed skeletal muscle gene expression analysis.
Funding Support:	

Name:	Caleb Ross
Project Role:	Graduate Student (Masters)
Researcher Identifier (ORCID ID):	NA

Nearest person month worked:	10 months (over 1 year)
Contribution to Project:	Mr. Ross assisted in the muscle processing and analysis.
Funding Support:	

Name:	Robert Shute
Project Role:	Graduate Student (Doctoral)
Researcher Identifier (ORCID ID):	NA
Nearest person month worked:	10 months (over 2 years)
Contribution to Project:	Ms. Zak performed skeletal muscle gene expression analysis.
Funding Support:	

- **Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
 - Nothing to report
- **Other organizations involved as partners?**
 - Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

- **Collaborative Awards**
 - Nothing to report
- **Quad chart**
 - See appendices

9. APPENDICES:

- Quad Charts (2016, 2017, 2018, 2019)

2016

Evaluation of the Physiological Challenges in Extreme Environments: Implications for Enhanced Training, Operational Performance and Sex-Specific Responses
W81XWH-15-2-0075

PI: Brent C. Ruby, Ph.D., FACS

Org: The University of Montana Award Amount: 2,652,591

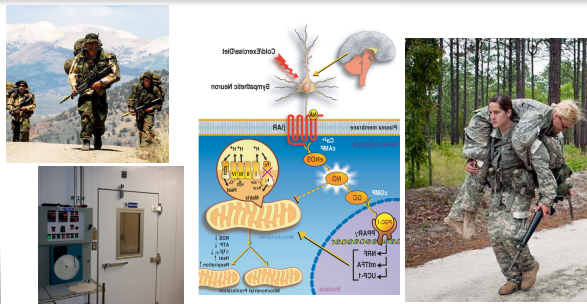


Study/Product Aim(s)

- To determine the physiological differences between hypobaric and hypoxia using markers of mitochondrial biogenesis and oxidative stress (including an evaluation of sex differences).
- To determine the effect of environmental temperature on exercise response and adaptation.
- To determine the effectiveness of pharmacological interventions at mitigating the deleterious effects of altitude (and perhaps heat).
- To implement laboratory protocols in the field to ensure translation to the training/operational environment.

Approach

The overall aim of the proposed research is to determine the outcomes of specific training and pharmacological countermeasures to the known performance decrements in extreme environments. This project series extends our previous DOD funded research describing the negative physiological consequences of operations in extreme environments to include novel countermeasures to mitigate these effects. Additionally, this project adds novel insight into sex-differences during performance in hostile environments.



Accomplishment: Data collection has been completed for study I and sample analyses are underway for skeletal muscle genes and oxidative stress markers. The Institutional IRB application has been approved for study II and is being prepared for Army HRPO submission

Timeline and Cost

Activities	CY	16	17	18	19
Study I (hypobaric vs. hypoxia)					
Study II (Environ. and adaptations)					
Study III (Pharm and altitude adaptations)					
Text (Major aim/study/milestone)					
Estimated Budget (\$K)		\$658	\$637	\$646	\$712

Updated: (June 2, 2016)

Goals/Milestones (Example)

CY16 Goal – Complete Study I – Hypobaric vs. Hypoxia (including a sex differences evaluation)

☒ Data collection

☐ Sample/data analyses and presentation/pub/annual report

CY17 Goals – Complete Study II – Environmental impact on muscle adaptations.

☐ HRPO approval, recruitment, data collection

☐ Sample/data analyses and presentation/pub/annual report

CY18 Goal – Complete Study III – Pharm interventions and altitude

☐ HRPO approval, recruitment, data collection

☐ Sample/data analyses and presentation/pub/annual report

CY19 Goal – Complete Study IV – Field translation study

☐ HRPO approval, recruitment, data collection

☐ Sample/data analyses and presentation/pub/annual report

Comments/Challenges/Issues/Concerns

The most difficulty challenge has been female study participant recruitment due to the exclusion criteria of exogenous hormone use (birth control).

No timeline adjustments necessary.

Budget Expenditure to Date

Projected Expenditure: \$320,000

Actual Expenditure: \$285,718

Evaluation of the Physiological Challenges in Extreme Environments: Implications for Enhanced Training, Operational Performance and Sex-Specific Responses
W81XWH-15-2-0075



PI: Brent C. Ruby, Ph.D., FACSM

Org: The University of Montana Award Amount: 2,652,591

Study/Product Aim(s)

- To determine the physiological differences between hypobaric and hypoxia using markers of mitochondrial biogenesis and oxidative stress (including an evaluation of sex differences).
- To determine the effect of environmental temperature on exercise response and adaptation in untrained males (year 2) and females (year 3).
- To implement laboratory protocols in the field to ensure translation to the training/operational environment.

Approach

The overall aim of the proposed research is to determine the outcomes of specific training and environmental interactions that may serve as countermeasures to the known performance decrements in extreme environments. This project series extends our previous DOD funded research describing the negative physiological consequences of operations in extreme environments to include novel countermeasures to mitigate these effects. Additionally, this project adds novel insight into sex-differences during acute and extended exposure to varied environmental stress.



Accomplishments: Data collection has been completed for study II a and IIb. Initial manuscripts are being prepared for study I. Sample analyses are underway for study IIb. The field study will be initiated in January of 2019.

Timeline and Cost

Activities	CY	16	17	18	19
Study I (hypobaric vs. hypoxia)					
Study IIa (Environ. and adaptations - males)					
Study IIb (Environ. and adaptations - females)					
Study III (Field study protocol)					
Estimated Budget (\$K)		\$658	\$637	\$646	\$712

Updated: (October, 2018)

Goals/Milestones (Example)

CY16 Goal – Complete Study I – Hypobaric vs. Hypoxia (including a sex differences evaluation)

- ☒ Data collection
- ☒ Sample/data analyses and presentation/pub/annual report
- ☒ Initial manuscript prepared

CY17 Goals – Complete Study IIa – Environmental impact on muscle adaptations.

- ☒ HRPO approval, recruitment, data collection
- ☒ Sample/data analyses and presentation/pub/annual report
- ☒ Initiate study IIb

CY18 Goal – Complete Study IIb – Environmental training – female cohort

- ☒ HRPO approval, recruitment, data collection
- ☒ Sample/data analyses and presentation/pub/annual report

CY19 Goal – Complete Study III – Field translation study

- ☐ HRPO approval, recruitment, data collection
- ☐ Sample/data analyses and presentation/pub/annual report

Comments/Challenges/Issues/Concerns

No timeline adjustments necessary.

Budget Expenditure to Date – budget figures forthcoming.

Projected Expenditure: \$

Actual Expenditure: \$

Evaluation of the Physiological Challenges in Extreme Environments: Implications for Enhanced Training, Operational Performance and Sex-Specific Responses
W81XWH-15-2-0075



PI: Brent C. Ruby, Ph.D., FACSM

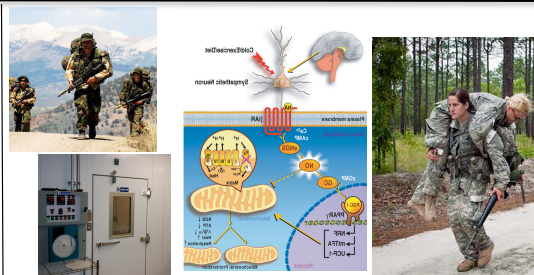
Org: The University of Montana Award Amount: 2,652,591

Study/Product Aim(s)

- To determine the physiological differences between hypobaria and hypoxia using markers of mitochondrial biogenesis and oxidative stress (including an evaluation of sex differences).
- To determine the effect of environmental temperature on exercise response and adaptation in untrained males (year 2) and females (year 3).
- To implement laboratory protocols in the field to ensure translation to the training/operational environment.

Approach

The overall aim of the proposed research is to determine the outcomes of specific training and environmental interactions that may serve as countermeasures to the known performance decrements in extreme environments. This project series extends our previous DOD funded research describing the negative physiological consequences of operations in extreme environments to include novel countermeasures to mitigate these effects. Additionally, this project adds novel insight into sex-differences during acute and extended exposure to varied environmental stress.



Accomplishments: Data collection has been completed for study II a and IIb. Initial manuscripts are being prepared for study I. Sample analyses are underway for study IIb. The field study will be initiated in January of 2019.

Timeline and Cost

Activities	CY	16	17	18	19
Study I (hypobaria vs. hypoxia)					
Study II (Environ. and adaptations - males)					
Study III (Environ. and adaptations - females)					
Study IV (Field study protocol)					
Estimated Budget (\$K)		\$658	\$637	\$646	\$712

Updated: (October, 2018)

Goals/Milestones (Example)

CY16 Goal – Complete Study I – Hypobaria vs. Hypoxia (including a sex differences evaluation)

- ☒ Data collection
- ☒ Sample/data analyses and presentation/pub/annual report
- ☒ Initial manuscript prepared

CY17 Goals – Complete Study II – Environmental impact on muscle adaptations.

- ☒ HRPO approval, recruitment, data collection
- ☒ Sample/data analyses and presentation/pub/annual report
- ☒ Initiate study III

CY18 Goal – Complete Study III – Environmental training – female cohort

- ☒ HRPO approval, recruitment, data collection
- ☒ Sample/data analyses and presentation/pub/annual report

CY19 Goal – Complete Study IV – Field translation study

- ☐ HRPO approval, recruitment, data collection
- ☐ Sample/data analyses and presentation/pub/annual report

Comments/Challenges/Issues/Concerns

No timeline adjustments necessary.

Budget Expenditure to Date – budget figures forthcoming.

Projected Expenditure: \$

Actual Expenditure: \$

Evaluation of the Physiological Challenges in Extreme Environments: Implications for Enhanced Training, Operational Performance and Sex-Specific Responses
W81XWH-15-2-0075



PI: Brent C. Ruby, Ph.D., FACSM

Org: The University of Montana Award Amount: 2,652,591

Study/Product Aim(s)

- To determine the physiological differences between hypobaria and hypoxia using markers of mitochondrial biogenesis and oxidative stress (including an evaluation of sex differences).
- To determine the effect of environmental temperature on exercise response and adaptation in untrained males (year 2) and females (year 3).
- To implement laboratory protocols in the field to ensure translation to the training/operational environment.

Approach

The overall aim of the proposed research is to determine the outcomes of specific training and environmental interactions that may serve as countermeasures to the known performance decrements in extreme environments. This project series extends our previous DOD funded research describing the negative physiological consequences of operations in extreme environments to include novel countermeasures to mitigate these effects. Additionally, this project adds novel insight into sex-differences during acute and extended exposure to varied environmental stress.



Accomplishments: Data collection has been completed for study II a and IIb. Initial manuscripts are being prepared for study I. Sample analyses are completed for study IIb. The field study was initiated and completed in January of 2019. Data analyses continues.

Timeline and Cost

Activities	CY	16	17	18	19
Study I (hypobaria vs. hypoxia)					
Study II (Environ. and adaptations - males)					
Study III (Environ. and adaptations - females)					
Study IV (Field study protocol)					
Estimated Budget (\$K)		\$658	\$637	\$646	\$712

Updated: (October, 2019)

Goals/Milestones (Example)

CY16 Goal – Complete Study I – Hypobaria vs. Hypoxia (including a sex differences evaluation)

- ✓ Data collection
- ✓ Sample/data analyses and presentation/pub/annual report
- ✓ Initial manuscript prepared

CY17 Goals – Complete Study II – Environmental impact on muscle adaptations.

- ✓ HRPO approval, recruitment, data collection
- ✓ Sample/data analyses and presentation/pub/annual report
- ✓ Initiate study III

CY18 Goal – Complete Study III – Environmental training – female cohort

- ✓ HRPO approval, recruitment, data collection
- ✓ Sample/data analyses and presentation/pub/annual report

CY19 Goal – Complete Study IV – Field translation study

- ✓ HRPO approval, recruitment, data collection
- ✓ Sample/data analyses and presentation/pub/annual report

Comments/Challenges/Issues/Concerns

No timeline adjustments necessary.

Budget Expenditure to Date –\$ 2,596,022.44

- **Conference Presentations**

2017

Caleb Ross, Robert Shute, Roksana Zak, Brent Ruby, John Quindry, Dustin Slivka; *Effects of Hypobaric and Normobaric Hypoxia on Myogenic and Proteolytic Gene Expression in Humans*; National ACSM Meeting in 2017.

John Quindry, Roksana Zak, Robert Shute, Tiffany Quindry, John Cuddy, Walter Hailes, Dustin Slivka, Brent Ruby; *Exercise Induced Oxidative Stress During Normobaric And Hypobaric Hypoxic Exercise Recovery*; National ACSM Meeting in 2017.

Robert Shute, Roksana Zak, Brent Ruby, John Quindry, Dustin Slivka; *Effects of Hypobaric and Normobaric Hypoxia on Mitochondrial Related Gene Expression*; National ACSM Meeting in 2017.

2018

John Quindry, Tiffany Quindry, Katheryn Tiemessen, Roksana, Zak, Robert Shute, John Cuddy, Walter Hailes, Dustin Slivka, Brent Ruby; *Blood Oxidative Stress Following Exercise Recovery in Normobaric and Hypobaric Hypoxic Environments*; National ACSM Meeting in 2018.

Megan Vande Hei, Robert Shute, Katherine Marshall, Dustin Slivka; *Aerobic training alters PGC-1 α but is not different between environmental temperatures*; Nebraska Physiological Society annual meeting in 2018.

Katherine Marshall, Robert Shute, Dustin Slivka; *Effects of environmental temperature on aerobic training adaptations*; Nebraska Physiological Society annual meeting in 2018.

Halee Keller, Robert Shute, Dustin Slivka; *Effects of environmental temperature and exercise on mitochondrial quantity and quality*; Nebraska academy of sciences in 2018.

Halee Keller, Robert Shute, Dustin Slivka; *Effects of environmental temperature on exercise response and adaptation*; UNO Student Research and Creative Activity Fair in 2018.

Halee Keller, Robert Shute, Dustin Slivka; *Effects of environmental temperature on exercise response and adaptation*; INBRE regional conference 2018.

Robert Shute, Caleb Ross, Roksana Zak, Brent Ruby, Dustin Slivka; *Skeletal Muscle Response to Hypobaric and Normobaric Hypoxia after Exercise*; The Nebraska Academy of Sciences in 2018.

2019

Keller H, Shute R, Slivka D. *Effects of environmental temperature on exercise response and adaptation*. Midlands Society of Physiological Sciences. 2019. Omaha, NE.

Slivka D. *Effects of cold temperature during a 3-week training period. NE-INBRE regional conference*. 2019. Nebraska City, NE.

Shute R, Slivka D. *Effect of 7°C environmental temperature during a 3-week training period*. UNO student research and creative activity fair. 2019. Omaha, NE.

Keller H, Slivka D. *Impacts of exercise and environmental temperature on mitochondrial quantity and quality*. UNO student research and creative activity fair. 2019. Omaha, NE.

Marshall K, Slivka D. *Effects of environmental temperature on aerobic training adaptations*. UNO student research and creative activity fair. 2019. Omaha, NE.

2020

Kwon M, Slivka D, Shute R, Hailes W, Marshall K, Opichka M, Schnitzler H, Ruby B Exercise in the Heat Blunts Improvements in Aerobic Capacity. Midlands Society of physiological Sciences Virtual Conference. October 2020.

McGlynn M, Schnitzler H, Shute R, Slivka D. The Acute Effect of Exercise and Temperature on mitochondrial DNA remodeling. Midlands Society of physiological Sciences Virtual Conference. October 2020.

○ Publications

Caleb I. Ross, Robert J. Shute, Brent C. Ruby, and Dustin R. Slivka; *Skeletal Muscle mRNA Response to Hypobaric and Normobaric Hypoxia After Exercise*; High Altitude Medicine and Biology; 20; 2019; 141-149; Published; Acknowledgement of Federal Support – Yes

Robert Shute, Katherine Marshall, Megan Opichka, Halee Schnitzler, Brent Ruby, Dustin Slivka; *Effects of 7°C Environmental Temperature Acclimation During a 3-Week Training Period*; Journal of Applied Physiology; 128; 768-777; 2020; Published; Acknowledgement of Federal Support - Yes

○ Publications (In Review)

Dustin Slivka, Robert Shute, Walter Hailes, Katherine Marshall, Megan Opichka, Halee Schnitzler, Brent Ruby; *Exercise in the Heat Blunts Improvements in Aerobic Capacity*; European Journal of Applied Physiology; In Review, Acknowledgement of Federal Support - Yes

Cassie M. Williamson-Reisdorph, Tiffany S. Quindry, Kathryn G. Tiemessen, Roksana Zak, Robert Shute, John Cuddy, Walter Hailes, Dustin Slivka, Brent Ruby, John C. Quindry; *Blood Oxidative Stress and Post-Exercise Recovery are Unaffected by Hypobaric and Hypoxic Environments*; Other - Prepared Manuscript; Acknowledgment of Federal Support – Yes

Cassie M. Williamson-Reisdorph, Tiffany S. Quindry, Katie S. Christison, Shae Gurney, Kathryn G. Tiemessen, John Cuddy, Walter Hailes, Dustin Slivka, Brent C. Ruby, John C. Quindry; *Exercise Training in a Hot Environment Fails to Elicit Changes in the Blood Oxidative Stress Response*; Other - Prepared Manuscript; Acknowledgment of Federal Support – Yes

Katherine Marshall, Robert Shute, Caleb Ross, Dustin Slivka; *Effects of Exercise Training in Hot and Cold Temperatures*; Other - Prepared Manuscript; Acknowledgment of Federal Support – Yes

○ Publications (Ongoing Preparation, preliminary data)

Dustin Slivka, Robert Shute, Walter Hailes, Brent Ruby; Effects of 3-week training and heat acclimation in females: evidence for sex specific environmental responsiveness – after above paper is published so it can be referenced and conclusions made as a whole. In preparation ~ Feb 2021.

Dustin Slivka, Robert Shute, Walter Hailes, Brent Ruby; Alterations in tissue and brain oxygenation during rest and exercise at normobaric and hypobaric hypoxia. In preparation ~ March 2021.

Dustin Slivka, Robert Shute, Walter Hailes, Brent Ruby; Alterations in total body water and cardiovascular responsiveness during rest and exercise at normobaric and hypobaric hypoxia. In preparation ~ April 2021.

Skeletal Muscle mRNA Response to Hypobaric and Normobaric Hypoxia After Normoxic Endurance Exercise

Caleb I. Ross,¹ Robert J. Shute,¹ Brent C. Ruby,² and Dustin R. Slivka¹

Abstract

Ross, Caleb I., Robert J. Shute, Brent C. Ruby, and Dustin R. Slivka. Skeletal muscle mRNA response to hypobaric and normobaric hypoxia after normoxic endurance exercise. *High Alt Med Biol.* 20:141–149, 2019.

Background: The physiological effects of hypoxia may be influenced by how hypoxia is achieved. The purpose of this study was to determine the effects of recovery in hypobaric hypoxia (HH), normobaric hypoxia (NH), and normobaric normoxia (NN) after endurance exercise on gene expression related to mitochondrial biogenesis, myogenesis, and proteolysis.

Methods: Fifteen recreationally trained subjects each cycled for 1 hour before recovering for 4 hours in NN (laboratory atmospheric conditions, 975 m), HH (depressurized to simulate 4420 m), and NH (fraction of O₂ reduced to simulate 4420 m). Muscle biopsy samples were obtained before exercise and after 4 hours of recovery.

Results: Blood oxygenation (SpO₂) was lower in HH ($76.02 \pm 0.58\%$) than NH (79.45 ± 0.56 , $p < 0.001$), which were both lower than in NN (96.3 ± 0.17 , $p < 0.001$). Heart rate was higher in HH (82 ± 2 bpm) than NH (77 ± 1 bpm, $p < 0.001$), which were both higher than in NN (67 ± 1 bpm, $p < 0.001$). *Mitochondrial transcription factor A (TFAM)* mRNA was lower after NN than HH ($p = 0.034$) or NH ($p = 0.005$), but was not different between HH and NH ($p = 0.460$). *Myostatin (MSTN)* mRNA decreased from pre- to postexercise ($p < 0.001$) in all conditions and was lower in HH compared with NH ($p = 0.035$) and NN ($p = 0.017$). No other differences were noted in genes related to mitochondrial biogenesis, myogenesis, or proteolysis ($p > 0.05$).

Conclusion: *TFAM* mRNA is lower with hypoxia exposure, but effected by the type of hypoxia. *MSTN* gene expression is lower after exposure to HH than NH or NN. These data support previous work and caution the translation of NH data obtained in a NH environment to a HH environment.

Keywords: environment; mitochondrial biogenesis; myogenesis, proteolysis

Introduction

THE ENVIRONMENTAL CONDITIONS, in which recovery takes place after exercise, may have implications on the cellular and physiological outcomes of that exercise bout. Previous researches from our laboratory have investigated a number of skeletal muscle responses following recovery from endurance exercise in environmental temperatures (Slivka et al., 2012; Zak et al., 2017), local temperature application (Tucker et al., 2012), and normobaric hypoxia (NH) environments (Slivka et al., 2014). However, achieving hypoxia through a lowered oxygen fraction such as in NH

may be physiologically different from terrestrial high altitude or hypobaric hypoxia (HH) where oxygen fraction remains constant, but barometric pressure is lower.

Limited research has directly compared these different hypoxia modalities and a complete understanding is lacking. The research that does exist tends to indicate that HH is a more severe form of hypoxia than NH. Blood oxygen saturation (SaO₂) appears to be lower at rest in HH compared with NH (Evetts et al., 2005; Boos et al., 2016) and further be altered after exercise along with other measures of cardiac adaptations (Boos et al., 2016). The differences in the physiological responses to HH and NH hypoxia appear to translate

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into applied effects. For example, cycling time trial performance is impacted more during exposure to HH than NH, despite similar blood oxygen saturation (SaO_2) and cardioventilatory parameters (Beidleman et al., 2014), and symptoms of acute mountain sickness have been reported to be higher with HH than with NH (DiPasquale, 2016). Furthermore, 1 week of NH sleep acclimation does not appear to translate into benefits in a HH environment (Fulco et al., 2011). While the effect of HH and NH on the cardiovascular system and performance is beginning to emerge, there is a lack of detail on the effects on the skeletal muscle.

One such effect on the skeletal muscle that is of interest in relationship to hypoxia is the production of mitochondria. Mitochondrial biogenesis occurs with cellular stress such as endurance exercise (Irrcher et al., 2003; Wright et al., 2007). However, hypoxic stress has demonstrated decrements in mitochondrial development (Ferretti, 1990; Hoppeler et al., 1990; Howald et al., 1990; Kayser et al., 1991). Previous evidence suggests that no differences occur in mitochondrial-related gene expression (*COX*, *PGC-1*, *FIS-1*, *MFN-1*, and *OPA-1*) between NH recovery compared with normobaric normoxia (NN) recovery from endurance exercise (Slivka et al., 2014). However, this study design did not compare these responses to a HH recovery environment. Therefore, it is currently unknown if HH recovery from exercise would regulate mitochondrial mRNA differently from NH or NN recovery.

Hypoxic conditions lead to specific signaling events with functional significance in skeletal muscle (Hoppeler et al., 2008). Hypoxia activates proteolytic regulator genes such as *FOXO3* to initiate cell death (Bakker et al., 2007; de Theije et al., 2013). Yet, there exists research that refutes the role of hypoxia in the activation of proteolysis (Favier et al., 2010; Manimmanakorn et al., 2013). Contradiction similarly exists on hypoxia's role in the regulation of myogenesis. Acute hypoxia exposure impairs myoblast differentiation and attenuates myofiber development (Di Carlo et al., 2004; Yun et al., 2005; Chaillou et al., 2014). However, additional literature suggests that exercise in hypoxic conditions improves myogenic activity by promoting muscle growth and repair in humans (Manimmanakorn et al., 2013). The differences in muscle mass regulating genes between HH and NH exposure have not been established and may aid in the interpretation of previous research as the method of achieving hypoxia is not consistent between studies.

The purpose of this study was to determine the response of key genes related to mitochondrial biogenesis and muscle mass regulation during HH, NH, and NN recovery after exercise. Determining the cellular responses of the skeletal muscle in HH and NH methods of achieving hypoxia will enhance the current literature and advance the understanding of hypoxia's effects on mitochondrial development and skeletal muscle mass regulation in addition to the interchangeability of NH and HH.

Materials and Methods

Subjects

Eight recreationally trained male and seven recreationally trained female subjects who engaged in endurance training for at least 30 minutes three times per week participated in this study. They were required to be between the ages of 18 and 40 years and have a $\text{VO}_{2\text{peak}}$ of at least 45 mL/(kg · min).

Those who previously experienced serious acute mountain sickness, or had a known risk factor for coronary artery disease assessed through a physical activity readiness questionnaire, were excluded from the study. Furthermore, female participants taking birth control influencing hormonal status or those who did not have a regular menstrual cycle in the past 8 months were excluded. Subjects signed the Institutional Review Board and USAMRMC Human Research Protections Office-approved informed consent form, which conformed to the Declaration of Helsinki, before testing.

Preliminary testing

Descriptive data included height (Seca 213 Stadiometer; United Kingdom), weight (Befour PS-660 ST Digital Scale; Saukville, WI), body composition, and $\text{VO}_{2\text{peak}}$. Body composition for each subject was assessed through hydrostatic weighing using an electronic load cell-based system (Exertech, Dresbach, MN) correcting for estimated residual lung volume. Body density from this underwater weight was converted to percent body fat using the Siri equation (Siri, 1993). Peak oxygen uptake ($\text{VO}_{2\text{peak}}$) was obtained for each participant using a graded exercise protocol starting at 95 W and increasing by 35 W every 3 minutes on an electronically braked Velotron, cycle ergometer (RacerMate, Seattle, WA). Cycling continued until volitional fatigue, and the highest obtained oxygen uptake value was considered the $\text{VO}_{2\text{peak}}$. Maximum workload (W_{max}) was calculated by taking the time completed in the last stage divided by the total stage duration (3 minutes) multiplied by 35 W and added to the watts of the last completed stage. Expired gases were analyzed every 15 seconds throughout the exercise test using a flow and gas calibrated metabolic cart (ParvoMedics TrueOne 2400; Sandy, UT).

Experimental protocol

All subjects completed three trials in a randomized, counter-balanced order. Each trial was separated by ~7 days to allow for biopsy recovery and to minimize carryover acclimation between trials. Subjects reported to the laboratory in the early morning following an overnight fast. Subjects maintained a 24 hours dietary and 48 hours activity log before the first trial and replicated these for the subsequent trials. The exercise trial consisted of a 60-minutes bicycle ride on a cycle ergometer at a constant intensity of 70% of their power associated with $\text{VO}_{2\text{peak}}$ in ambient conditions (975 m laboratory environment). Each subject drank water *ad libitum* during the first ride, and the amount was replicated for all subsequent trials. Following cessation of exercise, 4 hours of passive recovery occurred inside a small (32" × 7') tube shaped altitude chamber (Engineering Innovations, LLC, Littleton, CO) capable of lowering barometric pressure to simulate HH. This tube was located inside of an oxygen-controlled environmental chamber (Tescor, Warminster, PA) capable of lowering the fraction of oxygen in the air to simulate NH. They received one short break after 2 hours of recovery. The experimental recovery conditions were simulated to the following altitudes:

1. NN; 975 m (3200') Atmospheric conditions by having both the hypobaric tube and oxygen controlled chamber off so that participants breathed ambient air.

2. HH; 4420 m (14,500') HH by having the hypobaric tube depressurized altering the barometric pressure, while the oxygen-controlled chamber was set to off.
3. NH; 4420 m (14,500') NH by having the hypobaric tube off and the oxygen controlled chamber on.

Oxygenation saturation and heart rate

Blood oxygen saturation (SpO_2) and heart rates were measured using a finger pulse oximeter (Nonin WristOx2 3150; Plymouth, MN) during exercise and again every 60 minutes during passive recovery. Oxygen saturation and heart rates were recorded on the hour by having the device placed on the finger and allowed to stabilize for ~ 30 seconds.

Biopsies

Muscle biopsies were taken from the vastus lateralis before exercise and after 4 hours of recovery in each trial. The second muscle biopsy was extracted from a separate incision ~ 2 cm proximal to the preexercise biopsy. Each sample was extracted using a 5 mm Bergstrom percutaneous biopsy needle with the aid of suction. The leg was chosen in a random, counter-balanced order. After cleaning the site, ~ 3 –4 mL of 1% lidocaine was injected under the skin surface and around the muscle fascia before a small incision through the skin and muscle fascia was made. Once the muscle tissue had been obtained, the sample was quickly cleaned of excessive blood, connective tissue, and fat before being placed in All-protect (Qiagen, Hilden, North Rhine-Westphalia, Germany). Samples were placed overnight at 4°C and then transferred to -30°C for storage. All subsequent trials repeated this process by alternating legs.

Muscle sample preparation and quantitative reverse transcription-polymerase chain reaction

A piece of skeletal muscle (11.5 ± 1.7 mg) was homogenized in 500 μL of TRIzol (Invitrogen, Carlsbad, CA) using an electric blender homogenizer (Bullet Blender; Next Advance, Inc., Averill Park, NY) utilizing 1.5 mL Red RINO tubes prefilled with RNase-free ceramic beads (Next Advance, Inc.). The samples were centrifuged at 12,000 g for 15 minutes, and the aqueous phase was then transferred to a fresh 1.5 mL tube and incubated overnight at -20°C . Samples were centrifuged the next morning, and the supernatant was removed. Ethanol was added followed by a 5 minutes centrifugation at 7500 g . The ethanol was then removed, the pellet dried, and then redissolved in 30 μL of RNase-free water. RNA concentration was quantified using a nanospectrophotometer (nanoDrop ND-2000; Thermo Scientific, Wilmington, DE). Average RNA yields were 165.9 ± 12.1 ng/ μL . The average absorbance ratio at 260:280 was 1.90 ± 0.00 indicating high purity of the RNA. The RNA integrity of the samples were assessed using an Agilent RNA 6000 Kit and a 2100 Bioanalyzer (both from Agilent Technologies, Santa Clara, CA) according to manufacturer's instructions. The RNA integrity number was 8.0 ± 0.1 , indicating that the RNA was intact.

First-strand cDNA synthesis was achieved using the Superscript IV first-strand synthesis system for RT-PCR Kit (Invitrogen) according to manufacturer's instruction. The resulting cDNA was diluted with the appropriate amount of

RNase-free water to achieve a final cDNA concentration of 0.5 $\mu\text{g}/\mu\text{L}$ in the PCR reaction. Each 10 μL quantitative reverse transcription-polymerase chain reaction (qRT-PCR) volume contained 0.5 μL of probe and primer mix (Prime-Time qPCR assay Integrated DNA Technologies, Coralville, IA), 5 μL qPCR Master Mix (Integrated DNA Technologies), and 4.5 μL of sample cDNA. PCR was run in triplicate on a Stratagene mx3005p PCR system (Agilent Technologies) using a two-step protocol (1 cycle at 95°C for 5 seconds followed by 60°C for 20 seconds for 50 cycles).

Mitochondrial biogenesis-related genes included *peroxisome proliferator-activated receptor gamma coactivator 1-alpha* (*PGC-1 α*), *estrogen-related receptor alpha* (*ERR α*), *GA-binding protein alpha* (*GABPA*), *nuclear respiratory factor 1* (*NRF-1*), and *mitochondrial transcription factor A* (*TFAM*). The myogenic genes of interest involved in muscle hypertrophy are *myogenic differentiation factor* (*MYOD*), *myostatin* (*MSTN*), *myogenin* (*MYOG*), *myogenic factor 5* (*MYF-5*), and *myogenic factor 6* (*MYF-6*). Proteolytic genes of interest involved in muscle atrophy are *forkhead box O3* (*FOXO3*), *atrogin-1*, and *muscle ring finger 1* (*MuRF-1*).

Quantification of mRNA for genes of interest was completed using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001). For each participant, the geometric mean of five housekeeping genes *beta-actin* (*ACTB*), *beta-2-microglobulin* (*B2M*), *cyclophilin* (*CYC*), *ribosomal protein S18* (*RPS-18*), and *glyceraldehyde-3 phosphate dehydrogenase* (*GAPDH*) was used as the stable reference point. This combination of genes was determined to be stable using NormFinder software (Andersen et al., 2004). Probe and primer sequences used for qRT-PCR are presented in Table 1.

Statistical analysis

Differences in gene expression among HH, NH, and NN condition trials were analyzed using two-way (time $\times$ trial) repeated-measures analysis of variances (ANOVAs). In the event of a significant F-ratio, Fisher's protected least significant difference method was applied to determine where differences occurred. All ANOVAs were performed using the Statistical Package for Social Sciences software (SPSS) for Windows Version 23.0 (Chicago, IL). A probability of $<5\%$ was considered significant ($p < 0.05$). For all significant comparisons, effect size (η^2 for ANOVA and Cohen's d for individual comparisons) was calculated. All data are reported as mean $\pm$ standard error.

Results

Participant descriptive data

Eight recreationally trained male and seven recreationally trained female participants ($n = 15$) completed this study. Male subjects were taller ($p < 0.001$, $d = 2.643$), weighed more ($p = 0.028$, $d = 1.378$), had a lower percent body fat ($p = 0.003$, $d = 2.063$), $\text{VO}_{2\text{peak}}$ ($p = 0.001$, $d = 2.512$), and a higher cycling workload at $\text{VO}_{2\text{peak}}$ ($p < 0.001$, $d = 3.440$) than female subjects. After initial analysis, there were no differences in any other of our dependent variables between males and females ($p < 0.05$) which is supported by previous research indicating that men and women have similar cardiopulmonary responses after acute hypoxia exposure (Boos et al., 2016). Therefore, male and female subjects were

TABLE 1. PROBES AND PRIMERS USED FOR REAL-TIME POLYMERASE CHAIN REACTION

	Primer 1	Primer 2	Probe
Reference genes			
<i>ACTB</i>	AAGTCAGTGACAGGTAAGCC	GTCCCCCAACTTGAGATGTATG	CTGCCCTCCACCCACTCCCA
<i>B2M</i>	ACCTCCATGATGCTGCTTAC	GGACTGGCTTTCTATCTCTTGT	CCTGCCGTGTGAACCATGTGACT
<i>CYC</i>	TCTTTCACTTTGGCCAAACACC	CATCTAAACATACGGGTCC	TGCTTGCCATCCAAACCATCAGTC
<i>RPS18</i>	GTCAATGTCTGCTTCTCTCAAC	GTTCAGCATATTTTTCGAGT	TCTTCGGCCACACCCCTTAATGG
<i>GAPDH</i>	TGTAGTTGAGGTCAATGAAGG	ACATCGCTCAGACACCATG	AAGTCGGAGTCAACGGATTGGTC
Mitochondrial genes			
<i>PGC-1α</i>	TGTCTGTATCCAAAGTCGTTTAC	GAGTCTGTATGGAGTGACATCG	ACCAGCCTCTTTGCCAGATCTTC
<i>ERRα</i>	TCTCCGCTTGGTGATCTCA	CTATGGTGTGGCATCCTGTG	TGGTCTCTTGAAGAAGGCTTTGCA
<i>GABPA</i>	TGTAGTCTTGGTTCTAGCAGTTTC	TGGAACAGAGAAAGCAGAGTG	TGGTCAATTGATGTCATATGGCCTGGC
<i>NRF-1</i>	GTCACTCTCACCTCCCTGTAAAC	GATGCTTCAGAAATTGCCAACC	ATGGAGAGGAACAAAATTGGGC
<i>TFAM</i>	GCCAAAGACAGATGAAACCCAC	TGGGAAGGTCTGGAGCA	CGTCCCCCTTCAGTTTGTGTATTT
Myogenic genes			
<i>MYOD</i>	GAGATGCGCTCCACGATG	CGGAACTGCTACGAAGGC	ACAGGCAGTCTAGGCTCGACAC
<i>MYOG</i>	AGAAGTAGTGGCATCTGTGG	GACAGCATCACAGTGAAGA	ATGCCCGGCTTGAAGACAATCT
<i>MTN</i>	TCGTGATTCCTGTGAGTGCT	TGTAACCTTCCAGGACCA	TCTTTTGGTGTGTGTACCTTGACCT
<i>MYF-5</i>	GGCATATACATTGTATACATCAGGAC	CACCTCCAACTGCTCTGATG	TGCTGTCAAAAGTACTGCTTTCTGGA
<i>MYG-6</i>	CTACTCGAGGCTGACGAATC	CAGCTACAGACCCCAACAAGA	TGATAACGGCTAAGGAAGGAGAGCA
Proteolytic genes			
<i>FOXO3</i>	CGTGCCCTACTTCAAGGATAAG	ATTCTGGACCCGCATGAATC	AGGTTGTGCCGGATGGAGTTCTTC
<i>Atrigin-1</i>	TCAGCCTCTGCATGATGTC	CAACAGACTGGACTTCTCAACT	CACTGACCTGCCTTGTGCCTACA
<i>MURF-1</i>	GCAACTCACTTTTCTCTCATCC	TGCAGACCATCATCACTCAG	ACCTGGTGACTGTCTCTCTTGGTC

PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1- α ; *ERR α* , estrogen-related receptor alpha; *GABPA*, GA-binding protein alpha; *NRF-1*, nuclear respiratory factor 1; *TFAM*, mitochondrial transcription factor A; *MYOD*, myogenic differentiation factor; *MTN*, myostatin; *MYOG*, myogenin; *MYF-5*, myogenic factor 5; *MYF-6*, myogenic factor 6; *FOXO3*, forkhead box O3; *MurF-1*, muscle ring finger 1; *ACTB*, beta-actin; *B2M*, beta-2-microglobulin; *CYC*, cyclophilin; *RPS-18*, ribosomal protein S18; *GAPDH*, glyceraldehyde-3 phosphate dehydrogenase.

pooled together for further analysis. Descriptive data are presented in Table 2.

Oxygen saturation and heart rate

No differences in SpO₂ occurred between trials at baseline, during exercise, or immediately postexercise ($p > 0.05$, average $d = 0.250$) as no experimental interventions had been introduced at these time-points. During each hour of recovery, arterial oxygen saturation in NN was higher than both HH ($p < 0.001$, average $d = 6.546$) and NH ($p < 0.001$, average $d = 5.662$). Furthermore, oxygen saturation was lower in HH compared to NH at hours 1 ($p = 0.004$, $d = 1.059$), 3 ($p = 0.032$, $d = 0.772$), and 4 ($p = 0.008$, $d = 0.886$). No differences were observed at hour 2 ($p = 0.293$, $d = 0.343$) between HH and NH. Oxygen saturation data are presented in Figure 1.

No differences occurred in heart rate between trials ($p > 0.05$, average $d = 0.200$) at baseline, exercise, or immediately postexercise, as no experimental intervention had been introduced at these time points. Heart rate in the NN condition was lower than HH ($p < 0.001$, average $d = 1.427$) and NH ($p < 0.05$, average $d = 1.005$). Furthermore, heart rate was lower in NH compared with HH at recovery hours 2 ($p = 0.020$, $d = 0.586$), 3 ($p = 0.029$, $d = 0.517$), and 4 ($p = 0.041$, $d = 0.525$). Heart rate data are presented in Figure 2.

Gene expression

There were no differences in mitochondria-related gene expression of *PGC-1 α* ($p = 0.816$, $\eta^2 = 0.014$), *ERR α* ($p = 0.978$, $\eta^2 = 0.002$), or *GABPA* ($p = 0.925$, $\eta^2 = 0.006$). *NRF-1* decreased due to exercise ($p = 0.003$, $\eta^2 = 0.473$), but was not different between trials ($p = 0.748$, $\eta^2 = 0.020$). *TFAM* decreased after NN ($p = 0.048$, $d = 0.821$), increased after NH ($p = 0.024$, $d = 0.974$), but did not change with HH ($p = 0.093$, $d = 0.680$). Furthermore, *TFAM* gene expression was lower in NN compared with HH ($p = 0.034$, $d = 1.047$) and NH ($p = 0.005$, $d = 1.301$), but not different between HH and NH ($p = 0.460$, $d = 0.308$).

There were no differences in the myogenesis-related gene expression of *MYOD* ($p = 0.787$, $\eta^2 = 0.017$), *MYF-5* ($p = 0.073$, $\eta^2 = 0.170$), or *MYOG* ($p = 0.837$, $\eta^2 = 0.013$) between trials or after exercise ($p = 0.327$, $\eta^2 = 0.069$; $p = 0.262$, $\eta^2 = 0.089$; and $p = 0.624$, $\eta^2 = 0.018$; respectively). *MYF-6* was higher after exercise ($p = 0.004$, $\eta^2 = 0.457$), but not different between trials ($p = 0.971$, $\eta^2 = 0.002$). *MSTN* decreased from pre- to postexercise ($p < 0.001$, $\eta^2 = 0.831$) in all conditions and was lower in HH than both NH ($p = 0.035$,

$d = 0.0626$) and NN ($p = 0.017$, $d = 0.907$). NH and NN conditions were not different from each other ($p = 0.718$, $d = 0.140$). There were no differences in the proteolysis-related gene expression of *atrogen-1* with exercise ($p = 0.821$, $\eta^2 = 0.004$) or between trials ($p = 0.318$, $\eta^2 = 0.079$). *FOXO3* ($p = 0.015$, $\eta^2 = 0.356$) and *MuRF-1* ($p < 0.001$, $\eta^2 = 0.836$) gene expression increased with exercise but were not different between the three conditions ($p = 0.361$, $\eta^2 = 0.070$; and $p = 0.196$, $\eta^2 = 0.110$ for *FOXO3* and *MuRF-1*, respectively). Gene expression data are presented in Table 3.

Discussion

The purpose of the present study was to determine the response of key genes related to mitochondrial development and muscle mass regulation during recovery in HH, NH, and NN after exercise. The data from this investigation indicate that SpO₂ is lower and heart rate is higher in HH recovery compared with NH. In addition, both hypoxic recovery conditions had lower arterial oxygen saturations and higher heart rates than NN recovery. We also observed increased *TFAM* gene expression in hypoxic conditions compared to control conditions. Furthermore, we found *myostatin*, a negative regulator of myogenesis, to be suppressed in HH to a greater extent than NH and NN. While these differences among HH, NH, and NN were observed, several other genes associated with exercise adaptation were not affected by hypoxia, regardless of the factor used to create the hypoxic environment.

As expected, no differences in peripheral oxygen saturation or heart rate occurred at baseline, during exercise, or immediately postexercise because cycling took place in ambient conditions during each trial. However, hypoxic recovery in HH produced the lowest oxygen saturation and highest heart rate compared to NH and NN conditions. Our study is in agreement with previous literature, but not all, suggesting that cardioventilatory differences exist between hypoxic types (Tucker et al., 1983; Loeppky et al., 1997; Savourey et al., 2003). Like the current study, previous data suggest a specific response of HH compared with NH. A lowered barometric pressure modifies fluid circulation and the trans-alveolar-capillary membrane flux (Levine et al., 1988) causing constriction of the pulmonary blood vessels and decreased oxygen diffusion (Millet et al., 2012) leading to reduced arterial oxygen saturation. Therefore, further increases in heart rate during HH compensate for the reduced oxygen saturation to meet metabolic demand.

Recovery in hypoxia increased *TFAM* gene expression in the current study independent of *PGC-1 α* or the method in which hypoxia was achieved. *TFAM* mRNA has previously

TABLE 2. PARTICIPANT DESCRIPTIVE DATA

	Males (n=8)	Females (n=7)	Combined (n = 15)
Age (years)	24 $\pm$ 1	24 $\pm$ 2	24 $\pm$ 1
Height (cm)	184 $\pm$ 2*	166 $\pm$ 3	178 $\pm$ 3
Weight (kg)	79.0 $\pm$ 2.7*	65.2 $\pm$ 4.1	72.5 $\pm$ 3.6
Body fat (%)	11.6 $\pm$ 1.7*	23.9 $\pm$ 2.2	17.4 $\pm$ 2.2
VO ₂ peak (L/min)	4.24 $\pm$ 0.15*	3.04 $\pm$ 0.15	3.60 $\pm$ 0.20
Watt max (W)	335 $\pm$ 9*	219 $\pm$ 15	281 $\pm$ 18

Data are means $\pm$ SE.

* $p < 0.05$ males different from females.

SE, standard error.

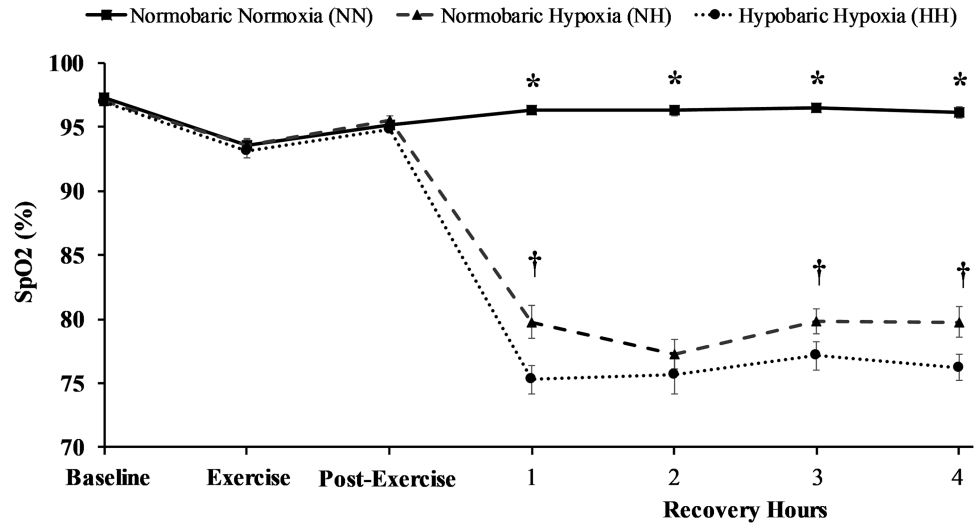


FIG. 1. Oxygen saturation between trials at baseline, during exercise, immediately post-exercise and each hour of recovery. * $p < 0.05$ NN from NH and HH, † $p < 0.05$ NH from HH. NN, normobaric normoxic; NH, normobaric hypoxic; HH, hypobaric hypoxia.

been shown to increase even in the absence of increased *PGC-1 α* (Arany et al., 2005; Yin et al., 2008), postexercise (Pilegaard et al., 2003), or under hypoxic conditions (Gutsaeva et al., 2008; Yin et al., 2008; Zhu et al., 2010). Hypoxia increases *TFAM* gene expression, but does not alter *PGC-1 α* in mice with ischemic brain injury (Yin et al., 2008). These data support the role of *TFAM* mRNA regulation during hypoxia exposure regardless of whether hypoxia was achieved with an alteration in barometric pressure or inspired oxygen fraction.

Changes in *myostatin* are another key outcome of the current study. *Myostatin* is a negative regulator of myogenic signaling—meaning deficiency or inhibition of this gene leads to a muscle growth stimulus and increases in this gene leads to muscle atrophy (Sandri, 2008; Gumucio and Mendias, 2013). Previous research observes decreases in skeletal muscle mass after exposure to HH during mountaineering

expeditions (Hoppeler et al., 1990; Howald and Hoppeler, 2003), where other factors such as exercise and nutrition cannot be controlled. It is unknown if the reductions in skeletal muscle mass during these expeditions are associated with increases in *MSTN* expression. Interestingly, *MSTN* mRNA decreased in all conditions of the current study and was lower in HH than NH and NN. This suggests recovering from exercise in a lower barometric pressure may suppress *MSTN* to a greater extent than NH or NN recovery. Myostatin attenuation in these environments may provide a myogenic stimulus, particularly after exercise. Therefore, if HH recovery from exercise further attenuates *MSTN*, protocols may be developed to suppress *MSTN* expression further and potentially lead to a greater muscle-building stimulus. Applied research investigating the changes in muscle mass and strength incorporating HH into a training regimen is needed to further develop this hypothesis. The results from such re-

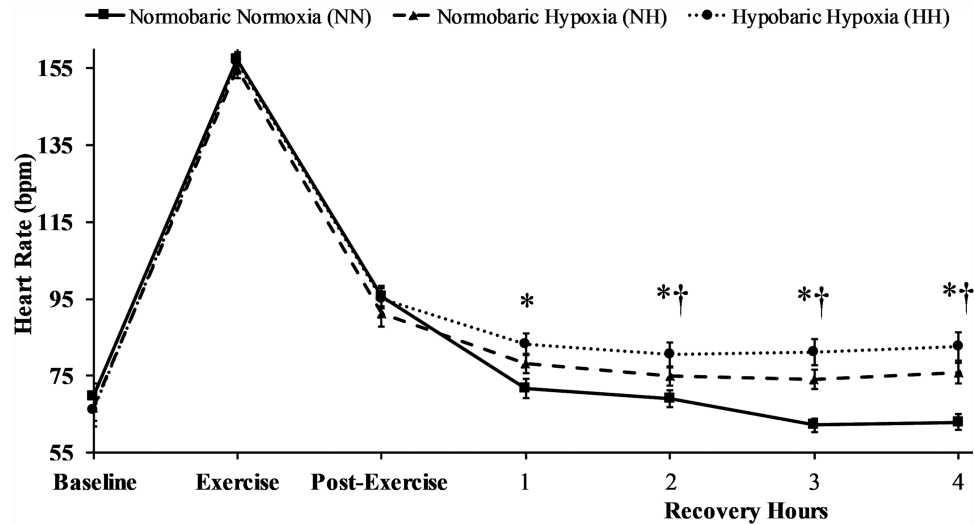


FIG. 2. Heart rate between trials at baseline, during exercise, immediately post-exercise and each hour of recovery. * $p < 0.05$ NN from NH and HH, † $p < 0.05$ NH from HH.

TABLE 3. FOLD CHANGE IN GENES RELATED TO MITOCHONDRIAL DEVELOPMENT, MYOGENESIS, AND PROTEOLYSIS IN NORMOBARIC NORMOXIC, NORMOBARIC HYPOXIC, AND HYPOBARIC HYPOXIA BEFORE AND FOUR HOURS AFTER ENDURANCE EXERCISE

Genes	NN		NH		HH	
	Pre	4 Hours post	Pre	4 Hours post	Pre	4 Hours post
Mitochondrial						
<i>PGC-1α</i>	1.006 $\pm$ 0.001	1.098 $\pm$ 0.279	1.011 $\pm$ 0.005	1.283 $\pm$ 0.296	1.019 $\pm$ 0.012	0.888 $\pm$ 1.006
<i>ERRα</i>	1.047 $\pm$ 0.016	1.133 $\pm$ 0.297	1.014 $\pm$ 0.005	1.146 $\pm$ 0.209	1.049 $\pm$ 0.017	1.090 $\pm$ 0.209
<i>GABPA</i>	1.017 $\pm$ 0.007	0.877 $\pm$ 0.100	1.014 $\pm$ 0.005	1.129 $\pm$ 0.228	1.015 $\pm$ 0.008	1.124 $\pm$ 0.232
<i>NRF-1</i>	1.011 $\pm$ 0.005	0.819 $\pm$ 0.116*	1.010 $\pm$ 0.003	0.942 $\pm$ 0.102*	1.023 $\pm$ 0.012	1.008 $\pm$ 0.810*
<i>TFAM</i>	1.008 $\pm$ 0.002	0.895 $\pm$ 0.079*	1.021 $\pm$ 0.008	1.508 $\pm$ 0.177* [†]	1.012 $\pm$ 0.003	1.311 $\pm$ 0.155 [†]
Myogenesis						
<i>MYOD</i>	1.047 $\pm$ 0.014	2.430 $\pm$ 0.825	1.058 $\pm$ 0.018	1.631 $\pm$ 0.416	1.065 $\pm$ 0.014	1.593 $\pm$ 0.334
<i>MSTN</i>	1.029 $\pm$ 0.011	0.495 $\pm$ 0.093*	1.017 $\pm$ 0.006	0.489 $\pm$ 0.088*	1.008 $\pm$ 0.002	0.284 $\pm$ 0.051* ^{†,‡}
<i>MYOG</i>	1.005 $\pm$ 0.002	1.139 $\pm$ 0.206	1.015 $\pm$ 0.008	1.026 $\pm$ 0.104	1.017 $\pm$ 0.011	1.148 $\pm$ 0.140
<i>MYF-5</i>	1.023 $\pm$ 0.011	1.025 $\pm$ 0.275	1.021 $\pm$ 0.008	1.862 $\pm$ 0.618	1.016 $\pm$ 0.007	1.174 $\pm$ 0.258
<i>MYF-6</i>	1.013 $\pm$ 0.006	1.671 $\pm$ 0.209*	1.021 $\pm$ 0.010	1.734 $\pm$ 0.381*	1.012 $\pm$ 0.005	1.901 $\pm$ 0.446*
Proteolysis						
<i>FOXO3</i>	1.016 $\pm$ 0.006	1.892 $\pm$ 0.372*	1.022 $\pm$ 0.009	1.338 $\pm$ 0.159*	1.019 $\pm$ 0.009	2.567 $\pm$ 0.802*
<i>Atrogin-1</i>	1.105 $\pm$ 0.078	2.047 $\pm$ 0.522	1.025 $\pm$ 0.009	0.920 $\pm$ 0.141	1.079 $\pm$ 0.053	2.441 $\pm$ 1.012
<i>MURF-1</i>	1.022 $\pm$ 0.007	4.245 $\pm$ 0.828*	1.013 $\pm$ 0.005	2.627 $\pm$ 0.529*	1.006 $\pm$ 0.001	3.775 $\pm$ 0.669*

Data are means $\pm$ SE.* $p < 0.05$ from pre-, [†] $p < 0.05$ from 4 hours post-NN, [‡] $p < 0.05$ from 4 hours post-NH.

NN, normobaric normoxic; NH, normobaric hypoxic; HH, hypobaric hypoxia.

search may have practical implications for improving muscle mass in various populations with limited exercise intensity capabilities.

Despite the differences that occurred among HH, NH, and NN, we observed no other effects of hypoxic recovery after exercise on select skeletal muscle gene expression. We observed a lower than expected exercise-stimulated response in the genes we measured. However, the exercise response in human gene expression appears to be affected by several variables and is generally in agreement with previous literature on mitochondrial biogenesis (Tunstall et al., 2002; Pilegaard et al., 2003; McGee and Hargreaves, 2004; Cartoni et al., 2005; Hock and Kralli, 2009), myogenesis (Yang et al., 2005; Coffey et al., 2006), and proteolysis (Sandri et al., 1995; Raue et al., 2007; Harber et al., 2009). Indeed, a subject population or exercise protocol that yielded a more robust response in gene expression may lead to a differential effect of hypoxia. Furthermore, the current subject population consisted of both males and females, and while no differences were noted, these results should be interpreted with caution as this study may be underpowered to detect these differences.

This study was completely done in the laboratory and thus factors other than barometric pressure may exist between actual terrestrial altitude and the simulations that were incorporated in this study. This study incorporated a relatively small chamber for inducing HH and thus factors such as CO₂ accumulation may have played a role. The effects of this small chamber were controlled for by having subjects placed in this tube during the NH and NN trials as well. Furthermore, the laboratory, in which this research was conducted, is located at 975 m and thus may not be a true NN control. However, all subjects resided at this elevation and were therefore acclimated to this altitude. While there are limitations to this laboratory-based design, it allowed for safe and well-controlled muscle sampling and data collection conditions.

Conclusion

When recovery from endurance exercise takes place in HH, lower oxygen saturations and higher heart rates occur compared to normobaric hypoxic recovery. In addition, Hypobaric hypoxic recovery attenuates *myostatin* to a greater extent than NH and NN after exercise. *TFAM* expression is enhanced with hypoxia with no differences between the type of hypoxia. These data suggest and support the notion that a lowered barometric pressure initiates a greater hypoxic response compared to a lowered fractional oxygen concentration. However, hypoxia did not affect several other genes associated with exercise adaptation, regardless of the factor used to create the hypoxic environment.

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Authors Contributions

All authors have substantially contributed to this article and have reviewed and approved this submission. C.I.R. was the primary author and wrote the majority of the article, aided in data collection, and sample analysis. R.J.S. was the primary person responsible for gene expression sample and data analysis and statistical analysis and subsequently much of the methods section. B.C.R. and D.R.S. were responsible for the conceptualization of the project, obtaining muscle samples, aiding in the data collection, analysis, and writing.

Author Disclosure Statement

No competing financial interests exist.

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RESEARCH ARTICLE

Effects of 7°C environmental temperature acclimation during a 3-week training period

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Shute R, Marshall K, Opichka M, Schnitzler H, Ruby B, Slivka D. Effects of 7°C environmental temperature acclimation during a 3-week training period. *J Appl Physiol* 128: 768–777, 2020. First published February 27, 2020; doi:10.1152/jappphysiol.00500.2019.—Cold environmental temperatures during exercise and recovery alter the acute response to cellular signaling and training adaptations. Approximately 3 wk is required for cold temperature acclimation to occur. To determine the impact of cold environmental temperature on training adaptations, fitness measurements, and aerobic performance, two groups of 12 untrained male subjects completed 1 h of cycling in 16 temperature acclimation sessions in either a 7°C or 20°C environmental temperature. Fitness assessments before and after acclimation occurred at standard room temperature. Muscle biopsies were taken from the vastus lateralis muscle before and after training to assess molecular markers related to mitochondrial development. Peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α) mRNA was higher in 7°C than in 20°C in response to acute exercise before training ($P = 0.012$) but not after training ($P = 0.813$). PGC-1 α mRNA was lower after training ($P < 0.001$). BNIP3 was lower after training in the 7°C than in the 20°C group ($P = 0.017$) but not before training ($P = 0.549$). No other differences occurred between temperature groups in *VEGF*, *ERR α* , *NRF1*, *NRF2*, *TFAM*, *PINK1*, *Parkin*, or *BNIP3L* mRNAs ($P > 0.05$). PGC-1 α protein and mtDNA were not different before training, after training, or between temperatures ($P > 0.05$). Cycling power increased during the daily training ($P < 0.001$) but was not different between temperatures ($P = 0.169$). $\dot{V}O_{2peak}$ increased with training ($P < 0.001$) but was not different between temperature groups ($P = 0.460$). These data indicate that a 3-wk period of acclimation/training in cold environmental temperatures alters PGC-1 α gene expression acutely but this difference is not manifested in a greater increase in $\dot{V}O_{2peak}$ and is dissipated as acclimation takes place.

NEW & NOTEWORTHY This study examines the adaptive response of cellular signaling during exercise in cold environmental temperatures. We demonstrate that peroxisome proliferator-activated receptor- γ coactivator 1 α mRNA is different between cold and room temperature environments before training but after training this difference no longer exists. This initial difference in transcriptional response between temperatures does not lead to differences in performance measures or increases in protein or mitochondria.

exercise; mitochondria; mRNA; PGC-1 α ; skeletal muscle

INTRODUCTION

Exercise training is an important way of mitigating disease and may provide an option for treatment. Some individuals may not be able to exercise at proper intensity for adaptive health benefits, necessitating alternative exercise models. Acute exercise in different environmental temperatures appears to elicit an altered physiological response. Specifically, oxygen consumption ($\dot{V}O_2$) at an absolute workload is reduced, and markers of mitochondrial development are reported to be enhanced in the skeletal muscle after acute aerobic exercise in the cold compared with room temperature (35, 39). Many aspects of acclimation to the cold are well documented (9, 33); however, there are limited data describing cold acclimation at the level of the skeletal muscle and how exercise training in the cold may affect whole body aerobic capacity. During acute exercise, peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α) increases more at 7°C cold environmental temperature than at 20°C room temperature, leading to the speculation that mitochondrial and whole body aerobic capacity may be further enhanced after repeated exercise bouts in the cold compared with room temperature (39, 40). In this model, exercising in the cold may be an effective way to optimize the beneficial effects of exercise training.

Alternatively, the enhanced skeletal muscle PGC-1 α response to the cold may become blunted with cold acclimation so that acute differences between temperatures diminish. Additionally, the effects of markers downstream of PGC-1 α and other factors in mitochondrial biogenesis are not as well defined with temperature exposure, being that mRNA of nuclear respiratory factor 2 (NRF2), estrogen-related receptor- α (ERR α), and myocyte enhancer factor 2 (MEF2) are lower in the cold and vascular endothelial growth factor (VEGF) is higher in the cold (35, 39, 40). Markers related to the selective degradation of mitochondria (mitophagy) have also become of interest to researchers recently. The effect of temperature on mitophagy has yet to be determined in humans (15). Examination of mitophagy markers combined with biogenesis markers may provide insight into mitochondrial quality through increased turnover and dynamics.

Acclimation to environmental temperature occurs relatively quickly, i.e., within 10–14 days (6, 36) and involves an increase in metabolic heat production to raise body temperature due to both shivering and nonshivering thermogenesis (12). After acclimation occurs, the rate of shivering is decreased (14, 15, 26). Thus, cold acclimation during exercise training may

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reduce the initial differences that have been previously observed with acute exercise in the cold and ultimately lead to similar changes in whole body aerobic capacity as occurs from exercise in a room temperature environment. However, since past research has examined only acute exercise responses (39, 40), the purpose of this study was to determine the effects of exercise during a 3-wk acclimation and training period in 7°C compared with 20°C environmental temperatures. The information gained from this research will help determine the practicality of temperature-optimized training to increase the beneficial outcomes of exercise interventions and determine the influence of temperature acclimation on markers related to mitochondrial development.

METHODS

Study design. Twenty-four untrained male subjects (age: 27 ± 2 yr, height 180 ± 3 cm) completed the procedures associated with this study after completing an Institutional Review Board-approved informed consent that conformed to the Declaration of Helsinki. Subjects were randomly assigned to cold (7°C) or room temperature (20°C) environmental temperatures. The determination of acclimation-induced influences on endurance training were the main interest of this study. Therefore, 7°C trials took place during warm months of the year (July–August) and 20°C trial took place during months of more moderate temperature (6 subjects in April–May, 6 subjects in September), so that subjects would not be preacclimated. All trials occurred in a temperature- and humidity-controlled environmental chamber with the relative humidity set at 40% (Darwin Chambers Co., St. Louis, MO). 7°C has been previously used to induce increased expression of PGC-1 α mRNA and altered mRNA of transcription factors downstream of PGC-1 α . Cycling shorts were provided for each subject, and all subjects wore short-sleeved T-shirts, so clothing was consistent between the two temperature groups. Subjects completed a total of 18 sessions for this study. Two fitness assessment trials were conducted in standard room temperature conditions 3 days before and 3 days after temperature intervention. The first and last training session were completed at an absolute intensity, the other 14 training sessions at a rating of perceived exertion (RPE) of 15 (hard). A self-selected intensity at an RPE of 15 was chosen during the training sessions as opposed to a fixed workload to allow a natural progressive overload during the 3 wk of training. All trials were completed Monday through Friday over a 22-day period, allowing subjects to recover during the weekend. *Day 1* and *day 22* represent the first and last training sessions at absolute intensity. Minimal exposure to environmental temperature, i.e., 1 h, was utilized, as this exposure has been sufficient to elicit differential response in gene expression acutely (39, 40). Cold acclimation occurring over a relatively short time period of 1–4 wk has also been observed with 1 to 2 h daily exposure and temperature above that used in the present study (4, 15, 25).

Fitness assessment trials. During the first and last testing sessions, fitness assessments were completed for all subjects. The fitness assessments consisted of measuring height, weight, body composition, and maximal aerobic capacity ($\dot{V}O_{2\text{peak}}$). To make direct comparisons between temperature groups, these trials took place in standard room temperature conditions regardless of the temperature group that subjects were assigned to. Body density was measured through hydrodensitometry (Exertech, Dresbach, MN) with correction for estimated residual lung volume and gastrointestinal air volume (43). Body density was converted to percent fat using the Siri equation (38). Subjects performed a graded exercise test on an electronically braked cycle ergometer (Velotron; RacerMate, Seattle, WA) while breathing through a calibrated metabolic cart (ParvoMedics TrueOne Metabolic System, Sandy, UT) to determine $\dot{V}O_{2\text{peak}}$ and to determine intensity for the absolute workload cycling bouts. Workload started at 95 W

and increased by 35 W each stage. Workload associated with $\dot{V}O_{2\text{peak}}$ (W_{peak}) was calculated by multiplying the relative time spent in the final stage by 35 and adding to the workload of the previously completed stage. These measurements were repeated on the last trial (3 days after the last training session) to assess fitness alterations resulting from training in the 7°C and 20°C environmental conditions.

Absolute-intensity trials. Subjects performed two absolute-intensity trials on the first day of training and the last day of training in their assigned temperature. Subjects were instructed to refrain from exercise a minimum of 48 h before these trials and to fast overnight. A dietary log of the previous day was self-recorded, and subjects were asked to replicate that diet the day before the final absolute-intensity trial. On the day of the trial, a muscle biopsy was taken from the vastus lateralis muscle before cycling for 60 min on an ergometer (Velotron) in an environmental chamber at an intensity of 50% W_{peak} . This workload was chosen because it was anticipated to be an intensity that this group of untrained subjects could realistically exercise at for 60 min. Nude body weight was recorded before and immediately after exercise on a Befour PS-660 ST digital scale (Saukville, WI) to calculate sweat rate. Core temperature was monitored during exercise via RET-1 rectal probe (Physitemp Instruments, Clifton, NJ) inserted 12 cm past the anal sphincter. Skin temperature was recorded and averaged from the chest and back with an SST-1 skin probe (Physitemp Instruments Inc.). Both rectal and skin probes were logged and recorded via SDL200 four-channel thermometer SD logger (Extech, Nashua, NH). Heart rate was monitored continuously during exercise via Polar V800 watch and chest strap (Polar Electronic, Lake Success, NY). After exercise was completed, subjects recovered for 4 h in standard room temperature conditions ($\sim 22^\circ\text{C}$). After the 4-h recovery, a second muscle biopsy was taken from a separate incision ~ 2 cm proximal to the previous biopsy.

Daily training sessions. Subjects arrived at the laboratory every weekday (Monday–Friday) for three straight weeks to complete a 60-min ride on a cycle ergometer (Phantom 3; Cycleops, Madison, WI) at the temperature previously assigned. Body weight was measured before and after each session. Subjects were instructed to cycle at an intensity of 15 (hard) on the RPE scale (5). Subjects were encouraged to increase their intensity progressively to maintain an RPE of 15 and create a progressive overload stimulus. Workload was self-adjusted by subjects and recorded using an iPad app (Virtual-Training s.r.o., Czech Republic). During the ride on the cycle ergometer, heart rate was monitored continuously (Polar Electronic, Lake Success, NY), and core body temperature was monitored before and after exercise via tympanic thermometer (Covidien Genius 2, Mansfield, MA). Water was given ad libitum, and the volume consumed was recorded so that daily sweat rate could be monitored.

Biopsies. During the two absolute workload trials, two muscle biopsies (2 per trial $\times$ 2 trials) were obtained from the vastus lateralis using the percutaneous needle biopsy technique with suction (3). The biopsy sites were cleaned, and ~ 3 –4 mL of 1% lidocaine was injected under the skin surface and around the muscle fascia before a small incision was made. The biopsy needle was then inserted into the muscle, and two cuts were made before the needle was extracted. The incision was closed using steri-strips and wrapped with a compression bandage. The biopsy samples were obtained before exercise and following the 4-h recovery. Biopsies for a given absolute-intensity trial were on the same leg, and subsequent biopsies of the same trial were performed ~ 2 cm proximal to the initial biopsy site. This procedure was repeated for the other absolute-intensity trial on the opposite leg. Samples were cleared of excess blood, separated from connective tissue, and portioned into All Protect Tissue Reagent (Qiagen, Valencia, CA). Samples were stored overnight at 4°C and then transferred to -30°C for later analysis.

Gene expression. A 16.2 ± 0.2 -mg piece of skeletal muscle was homogenized in 800 μL of TRIzol (Invitrogen, Carlsbad, CA) using an electric homogenizer (PowerGen 150; Fisher Scientific, Hampton, NH), and RNA was extracted as previously described (35). RNA

concentration was quantified using a nanospectrophotometer (Nanodrop ND-2000; Thermo Scientific, Wilmington, DE). Average RNA yields were 182.8 ± 7.3 ng/ μ L. The average absorbance ratio at 260/280 was 1.9 indicating high purity of the RNA. The RNA integrity of the samples was assessed using an Agilent RNA 6000 kit and a 2100 Bioanalyzer (both from Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. The RNA integrity number (RIN) was 8.2 ± 0.1 , indicating intact RNA. First-strand cDNA synthesis was achieved using the Superscript IV VILO Master Mix with ezDNase Enzyme (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The resulting cDNA was diluted with the appropriate amount of RNase-free water to achieve a final cDNA concentration of 0.5625 ng/ μ L in the PCR reaction. Each 10- μ L qRT-PCR reaction volume contained 0.5 μ L of probe and primer mix (PrimeTime qPCR assay; Integrated DNA Technologies, Coralville, IA), 5 μ L of qPCR Master Mix (Integrated DNA Technologies), and 4.5 μ L of sample cDNA. PCR was run in triplicate on a Stratagene mx3005p PCR system (Agilent Technologies) and a two-step protocol (1 cycle at 95°C for 5 s followed by 60°C for 20 s for 40 cycles). The probes and primers for our genes of interest and stable reference genes were designed by Integrated DNA Technologies (PrimeTime qPCR assay, Coralville, IA) and are presented in Table 1.

Table 1. Probes and primers used for real-time reverse transcription quantitative PCR

Genes	Primer 1	Primer 2	Probe
Housekeeping			
<i>ACTB</i>	AAGTCAGTGTA CAGGTAAGCC	GTCCGCCAACT TGAGATGTATG	CTGCCTCCAC CCACTCCCA
<i>B2M</i>	ACCTCCATGA TGCTGCTTAC	GGACTGGTCTTT CTATCTCTTGT	CCTGCGGTGTGA ACCATGTGACT
<i>CYC</i>	TCTTTCACTTT GCCAAACACC	CATCTTAAAGC ATACGGGTCC	TGCTTGCCATCCA ACCACTGACTC
<i>GAPDH</i>	TGTAGTTGAGG TCAATGAAGGG	ACATCGCTCA GACACCATG	AAGGTCGGAGTCA ACGGATTGTGGT
<i>rpS18</i>	GTCATGTCTG CTTTCCTCAAC	GTTCCAGCATA TTTTGCGAGT	TCTTCGGCCAC ACCCCTTAATGG
Mitochondrial biogenesis			
<i>PGC-1α</i>	GCAATCCGTC TTCATCCACA	CCAATCAGTACA ACAATGAGCCT	AGCAGTCCCTCACAG AGACACTAGACAG
<i>NRF1</i>	GTCATCTCACG TCCCTGTAAC	GATGCTTCAGA ATTGCCAACC	ATGGAGAGGTGGA ACAAAATTGGGC
<i>GABPA</i>	TGTAGTCTTGGT TCTAGCAGTTTC	TGGAACAGAGA AAGCAGAGTG	TGGTTCATTGATG TCTATGGCCTGGC
<i>TFAM</i>	GCCAAGACAGA TGAAAACACC	TGGGAAGGT CTGGAGCA	CGCTCCCCCTTCA GTTTTGTGTATTT
<i>ERRα</i>	TCTCCGCTTG GTGATCTCA	CTATGGTGTG GCATCCTGTG	TGGTCCCTTTGAA GAAGGCTTTGCA
<i>VEGF</i>	CGCGTGATAG ACATCCATGA	CCATGAACCTT CTGCTGTCTTG	TGCTCTACCTCC ACCATGCCAAG
Mitophagy			
	GTTGCTTGGG ACCTCTCTTG	TGAACACAATG AGCCAGGAG	TGTAAGTGACTGC TCCATACCTCCCA
<i>Parkin</i>	GCTTGGTGGTT TTCTTGATGG	TTGAAGCCTCA GGAACAACT	CCTGCTCGGC GGCTCTTTCA
<i>BNIP3</i>	CCACTAACGAA CCAAGTCAGAC	CATCTCTGCT GCTCTCTCAT	AAAGGTGCTGGT GGAGTTGTCA
	CAAACATGATC TGCCCATCTTC	TCCTCATCCTC CATCCACAA	TCTCACTGTGA CAGCCCTTCGC

ACTB, β -actin; *B2M*, β 2-microglobulin; *CYC*, cyclophilin; *RPS18*, ribosomal protein S18; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *PGC-1 α* , peroxisome proliferator-activated receptor- γ coactivator 1 α ; *NRF1*, nuclear respiratory factor 1; *GABPA*, GA-binding protein- α ; *TFAM*, mitochondrial transcription factor A; *ERR α* , estrogen-related receptor- α ; *VEGF*, vascular endothelial growth factor; *PINK1*, PTEN-induced kinase-1; *Parkin*, an E3 ubiquitin ligase; *BNIP3*, Bcl2-interacting protein-3, *BNIP3L*, Bcl2-interacting protein 3-like.

Quantification of mRNA for genes of interest were completed using the $2^{-\Delta\Delta CT}$ method (24). For each subject, the geometric mean of five housekeeping genes: β -actin (*ACTB*), β 2-microglobulin (*B2M*), cyclophilin (*CYC*), and ribosomal protein S18 (*rpS-18*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the stable reference point. This combination of genes was determined to be stable using NormFinder software (22, 37). To determine the difference in acute exercise response before and after training, postexercise mRNA data were normalized relative to the preexercise resting sample of that training state. To determine the effect of training, posttraining resting mRNA data was normalized relative to pretraining resting mRNA. For average values below 1.00 (downregulation of gene expression), the negative inverse was taken for equal graphic representation for upregulated and downregulated gene expression.

DNA extraction and quantification. DNA extraction was obtained during phase separation of the aqueous and organic phase of the TRIzol extraction concurrently with RNA extraction. The interphase containing DNA was extracted, and the remaining aqueous phase overlying the interphase was removed. Next, 100% EtOH was added to precipitate the DNA pellet. The phenol-ethanol supernatant containing protein was transferred to a separate tube from the resulting DNA pellet. The DNA pellet was washed in 0.1 M sodium citrate in 10% ethanol followed by 30-min incubation on a tube rotator. The supernatant was discarded and resuspended in 1,200 μ L of 75% EtOH, and the pellet was resuspended in 400 μ L of 8 mM NaOH and incubated overnight at 50°C. The DNA was centrifuged and transferred to a new tube removing insoluble materials, and the pH was adjusted with 0.1 M HEPES and quantified on the Nanodrop. The concentration of this DNA was then adjusted to contain the same amount of DNA within a given subject for qRT-PCR analysis (19.7 ng/ μ L). PCR was then performed on the DNA to measure mitochondrial (mt)DNA relative copy number (mtMinArc). The mtMinArc copies were determined using the $2 \times 2^{\Delta CT}$ equation to determine the number of mtDNA copies per nuclear DNA copy (30). Values were expressed relative to pretraining.

Protein quantification. Protein extraction was obtained during phase separation of the aqueous and organic phase of the TRIzol extraction. The interphase containing DNA was extracted for separate analysis, and 1.2 mL of isopropanol was added to the remaining phenol-ethanol supernatant liquid. Samples were centrifuged to pellet the proteins and the supernatant was discarded. The protein pellet was resuspended in 1.6 mL of 0.3 M guanidine hydrochloride in 95% ethanol and incubated for 20 min. Samples were then centrifuged at 7,500 g for 5 min at 4°C, and the supernatant was discarded. This step was repeated twice more, with 1.5 mL of 100% EtOH. After a 20-min incubation in EtOH, samples were centrifuged at 7,500 g for 5 min at 4°C. The EtOH was then removed, and the pellets were then resuspended in 150 mL of 1% SDS and incubated overnight at 50°C. The following day, samples were centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was transferred to a new tube to remove insoluble materials for protein quantification.

Protein quantity of each fraction was measured using a commercially available BCA protein assay kit (Pierce Biotechnologies) according to the manufacturer's instructions. Absorbance of samples was read at 562 nm using a spectrophotometer (Nanodrop ND 2000, Thermo Scientific). Protein concentrations were then calculated using a standard curve according to kit instructions. Sample concentrations were 3.5 ± 0.2 μ g/ μ L.

Gel electrophoresis and Western blotting. Proteins were separated by SDS-PAGE as previously described, with modifications (18). Briefly, gels were run at 250 V for 45 min in a Mini Trans-Blot tank (Bio-Rad, Hercules, CA). Gels were then transferred to a nitrocellulose membrane using a Trans-Blot Turbo transfer system (Bio-Rad). Total protein was imaged using Revert Total Protein Stain (Li-Cor). The membrane was then blocked for 85 min at room temperature with Li-Cor blocking buffer (Li-Cor), and incubated overnight at 4°C in rabbit anti-human primary antibody at a ratio of 1:1,000 for PGC-1 α

(Novus Biologicals, catalog no. NBP1-04676, Centennial, CO) in a plate rocker. This antibody has been previously validated and used for Western blot analysis (16, 17, 20). The next day, the membrane was washed and then incubated in secondary antibody using 1 μ L of Li-Cor goat anti-rabbit 800 CW antibody (Li-Cor, catalog no. 926-32211) in 20 mL of blocking buffer for 65 min. The membrane was imaged by infrared fluorescence using an Odyssey Fc imaging system (Li-Cor). Bands at 92 kDa were quantified using Image Studio 5.2 software (Li-Cor).

Statistical analysis. A two-way mixed-design ANOVA (time $\times$ temperature) was used for all dependent variables, with time being the repeated measure and temperature being the between-subject factor. If the F ratio values of the ANOVA were found to be significant, a Fisher's protected least significant difference post hoc test was performed to evaluate where significance occurred. A probability of type I error of less than 5% was considered significant ($P < 0.05$). All statistical data were analyzed using the Statistical Package for Social Sciences software (SPSS 24.0).

RESULTS

Daily training. Power increased during each week of training ($P < 0.001$) but was not different between temperatures ($P = 0.331$). Heart rate did not change during the 3 wk of training ($P = 0.988$) but had a trend towards being higher in the 7°C group compared with the 20°C group ($P = 0.095$). Sweat rate had a trend toward increasing during the 3 wk of training ($P = 0.100$) but was not different between the 7°C and 20°C group. Core temperature was not different during training ($P = 0.431$) or between temperature groups ($P = 0.233$). Data for the daily training trials are presented in Fig. 1.

Fitness. Subject descriptive data before and after training in the 7°C and 20°C groups are presented in Table 2. Fat free mass ($P = 0.028$) increased after training in the 20°C group ($P < 0.001$) but not in the 7°C group ($P = 0.200$). $\dot{V}O_{2\text{peak}}$ increased after training ($P < 0.001$) but was not different between temperatures ($P = 0.460$). Total body mass did not change from training ($P = 0.762$) or between temperature groups ($P = 0.903$). Percent body fat and fat mass decreased from training ($P < 0.001$, $P < 0.001$, respectively) but were not different between temperature groups ($P = 0.996$, $P = 0.894$, respectively).

Absolute-intensity trials. The absolute-intensity trials allowed for assessment of temperature acclimation and the effect of training during the 3-wk temperature and exercise intervention. Skin temperature was higher in the 20°C group than in the 7°C group both before training ($P = 0.001$) and after training ($P = 0.013$). Additionally, skin temperature of the 20°C group did not change from training ($P = 0.186$) but increased after training in the 7°C group ($P = 0.031$). Heart rate decreased and sweat rate increased from before to after training ($P < 0.001$, $P = 0.050$, respectively) but neither were different between temperatures ($P = 0.424$, $P = 0.307$, respectively). No differences in core temperature occurred from pretraining to post-training ($P = 0.251$) or between the 7°C and 20°C groups ($P = 0.643$). Data for the temperature tolerance trials are presented in Table 2.

mRNA response to acute exercise before and after training. *PGC-1 α* mRNA increased with acute exercise before training and after training in both 7°C and 20°C training groups ($P <$

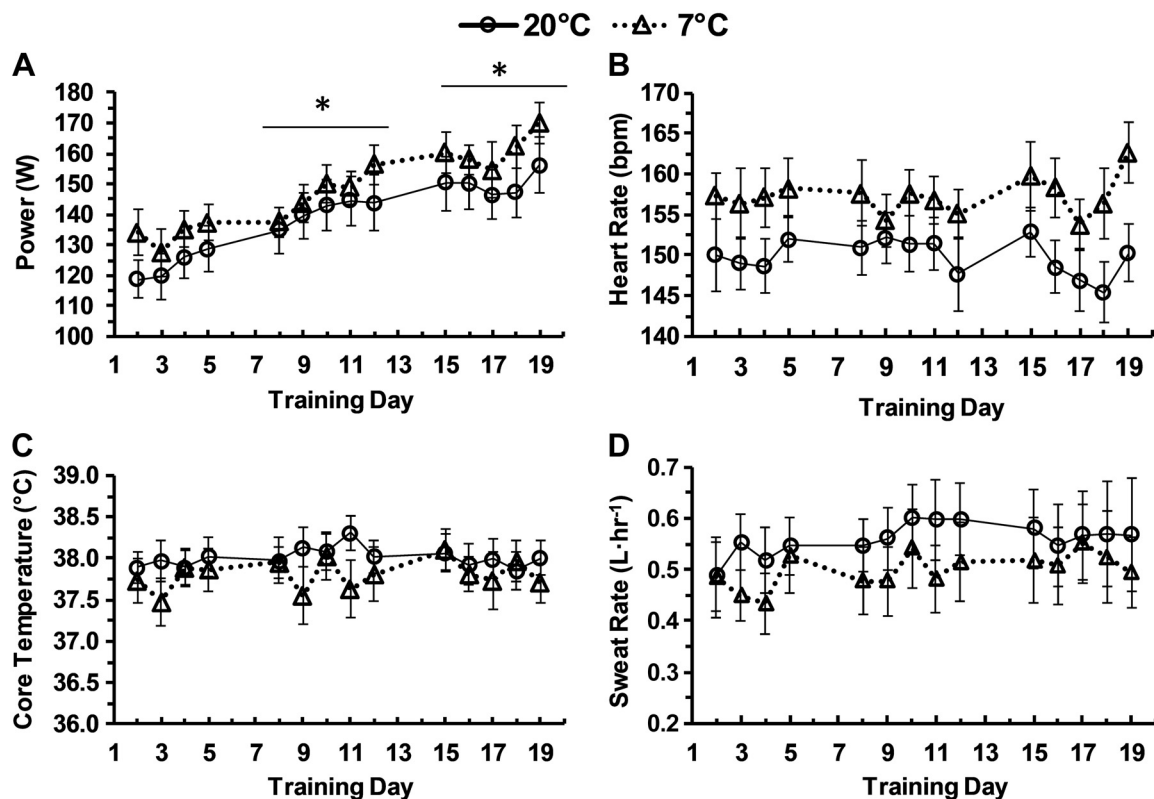


Fig. 1. Daily training values. Power (W), heart rate, core temperature, and sweat rate during training each day at an rating of perceived exertion (RPE) of 15 in 20°C or 7°C. Data are means $\pm$ SE. * $P < 0.05$ from previous week.

Table 2. Pretraining and posttraining physiological variables in 20°C and 7°C environments

	Pre			Post		
	20°C	7°C	Combined	20°C	7°C	Combined
Body mass, kg	85.3 ± 7.5	87.0 ± 6.0	86.1 ± 4.7	85.9 ± 7.7	86.5 ± 5.8	86.2 ± 4.7
Body fat, %	21.9 ± 3.2	21.7 ± 2.1	21.7 ± 1.8	20.4 ± 3.2	20.7 ± 2.1	20.5 ± 1.8*
Fat mass, kg	20.7 ± 4.9	19.9 ± 2.9	20.2 ± 2.7	19.6 ± 4.8	19.0 ± 2.9	19.2 ± 2.7*
Fat free mass, kg	64.5 ± 3.7	67.1 ± 3.4	65.8 ± 2.4	66.2 ± 3.8*	67.6 ± 3.2	66.9 ± 2.4
$\dot{V}O_{2peak}$, L/min	3.21 ± 0.21	3.47 ± 0.16	3.34 ± 0.13	3.65 ± 0.20	3.80 ± 0.10	3.72 ± 0.11*
$\dot{V}O_{2peak}$, mL·kg ⁻¹ ·min ⁻¹	39.4 ± 2.8	40.8 ± 1.8	40.1 ± 1.6	44.2 ± 2.5	45.5 ± 2.3	44.8 ± 1.6*
Core temperature, °C	37.4 ± 0.3	38.0 ± 0.1	37.7 ± 0.2	37.5 ± 0.2	37.7 ± 0.1	37.6 ± 0.1
Skin temperature, °C	33.6 ± 0.4	30.1 ± 0.8†	31.8 ± 0.5	32.9 ± 0.4	31.2 ± 0.5*†	32.0 ± 0.3
Heart rate, beats/min	141 ± 5	147 ± 4	144 ± 3	127 ± 3	129 ± 3	128 ± 2.0*
Sweat rate, mL/h	505 ± 83	456 ± 55	481 ± 49	687 ± 90	537 ± 88	612 ± 63*

Data are means ± SE. * $P < 0.05$ from pretraining; † $P < 0.05$ from 20°C.

0.001). *PGC-1 α* mRNA was higher in 7°C than in 20°C after acute exercise before training ($P = 0.012$), but this difference between 7°C and 20°C was not observed after training ($P = 0.813$). Furthermore, *PGC-1 α* mRNA was lower after training in response to acute exercise for both 7°C and 20°C than before training ($P < 0.001$). *VEGF* mRNA increased with acute exercise before training and after training in both 7°C and 20°C training groups ($P < 0.001$). There was a trend for *VEGF* mRNA to be higher in 7°C than in 20°C after acute exercise before training ($P = 0.072$), but this trend for a difference between 7°C and 20°C was not observed after training ($P = 0.206$). Furthermore, *VEGF* mRNA was lower after training in

response to acute exercise for both 7°C ($P = 0.002$) and 20°C ($P < 0.001$) than before training. *NRF1* and *NRF2* mRNA decreased with acute exercise after training regardless of temperature ($P = 0.001$ and $P = 0.001$, respectively) and showed a trend to decrease with acute exercise only before training ($P = 0.055$ and $P = 0.080$, respectively). *TFAM* and *ERR α* mRNA were not altered with acute exercise, training, or temperature ($P > 0.05$). The mitochondrial biogenesis-related mRNA response to acute exercise before and after training in 7°C and 20°C data are presented in Fig. 2, A–F.

PINK1 (PTEN-induced kinase-1) mRNA did not change with acute exercise before training ($P = 0.571$) but *PINK1*

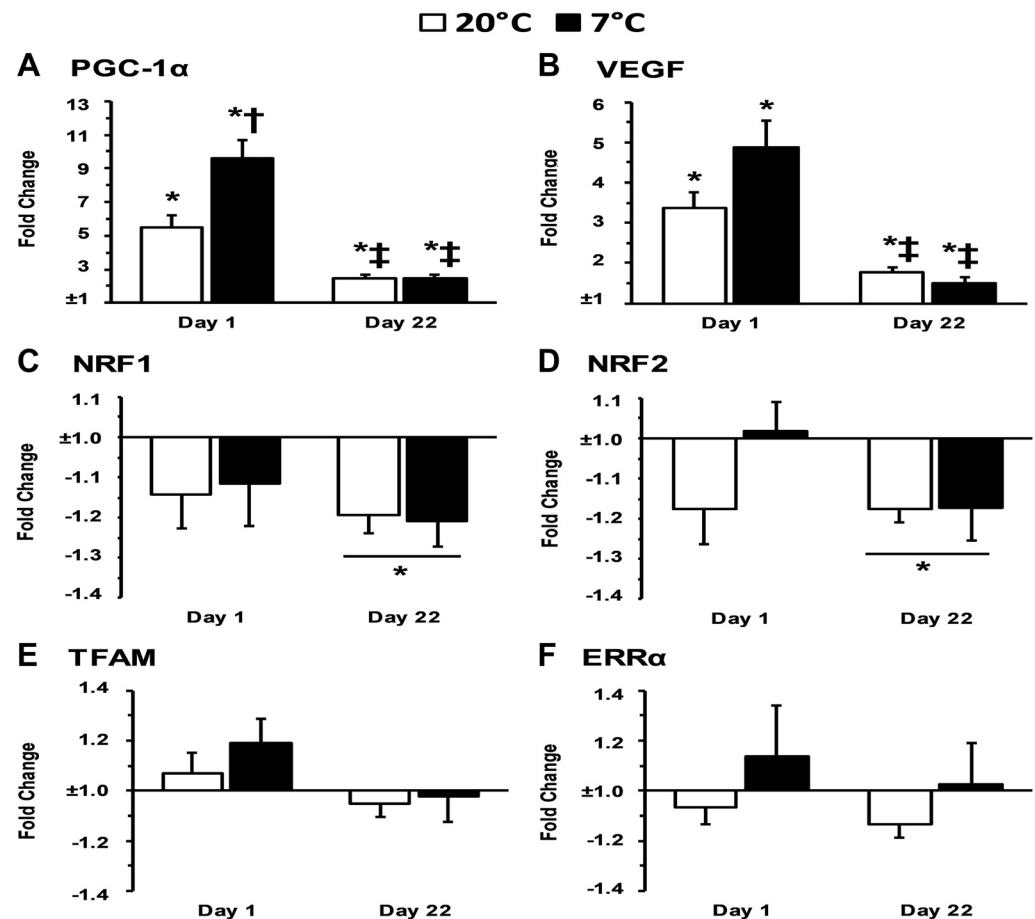


Fig. 2. A–F: gene expression associated with mitochondrial biogenesis 4-h postexercise relative to preexercise. *PGC-1 α* , peroxisome proliferator-activated receptor- γ coactivator 1 α ; *NRF*, nuclear respiratory factor; *TFAM*, mitochondrial transcription factor A; *ERR α* , estrogen-related receptor- α ; *VEGF*, vascular endothelial growth factor. * $P < 0.05$ from preexercise; † $P < 0.05$ from 20°C; ‡ $P < 0.05$ from day 1 4-h time point.

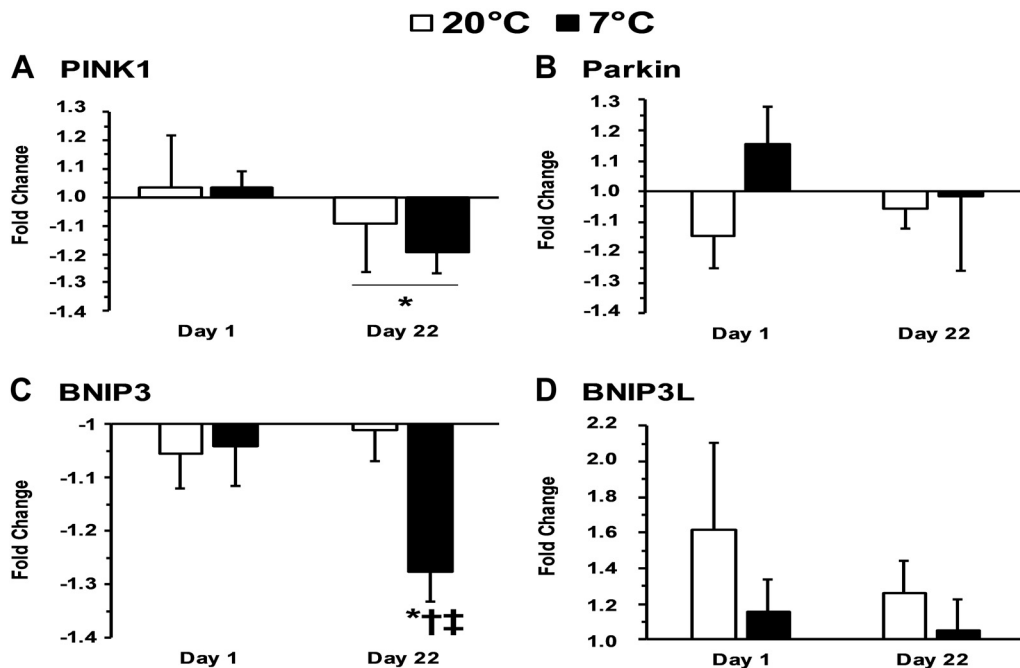


Fig. 3. A–D: gene expression associated with mitophagy 4-h after exercise relative to preexercise. *PINK1*, PTEN-induced kinase-1; *BNIP3*, Bcl2-interacting protein-3; *BNIP3L*, Bcl2-interacting protein 3-like; *Parkin*, an E3 ubiquitin ligase. * $P < 0.05$ from pre-exercise; † $P < 0.05$ from 20°C; ‡ $P < 0.05$ from day 1 4-h time point.

decreased with acute exercise after 3 wk of training in both the 7°C and 20°C group ($P = 0.036$). There was not a difference in *PINK1* mRNA between the 7°C and 20°C groups before or after training ($P = 0.796$). *BNIP3* (Bcl2 interacting protein-3) decreased with acute exercise after training ($P < 0.001$) and was lower after training compared with before training ($P = 0.014$). *BNIP3* was also lower in the 7°C group than in the 20°C group after training ($P < 0.017$) but not before training ($P = 0.549$). *Parkin* (an E3 ubiquitin ligase) and *BNIP3L* (BNIP3-like) were not altered with acute exercise, training, or temperature ($P > 0.05$). The mitophagy-related mRNA response to acute exercise before and after training in both 7°C and 20°C groups are presented in Fig. 3, A–D.

Resting mRNA response to training in 7°C and 20°C. The effect of the 3-wk training in the different environments on mRNA was determined by expressing the resting biopsy after the 3 wk relative to the resting biopsy before the 3-wk training period. *PGC-1 α* ($P = 0.731$), *VEGF* ($P = 0.129$), and *NRF1* ($P = 0.731$) mRNA did not change with training and were not different between 7°C and 20°C training groups ($P > 0.05$). *NRF2* ($P = 0.010$), *TFAM* ($P = 0.003$), and *ERR α* ($P = 0.014$) mRNA decreased with training but were not different between 7°C and 20°C training groups ($P > 0.05$). *PINK1* increased ($P = 0.025$), whereas *Parkin* ($P = 0.001$) and *BNIP3L* (0.008) decreased with training. *BNIP3* was trending toward a decrease ($P = 0.064$) with training. No difference occurred in mitophagy-related genes between temperatures ($P > 0.05$). The resting mRNA response to training in 7°C and 20°C data are presented in Table 3.

mtDNA relative Amount. Mitochondrial quantity was determined by comparing the relative amount of mtDNA to nuclear DNA. The relative amount of mtDNA did not change from pre- to posttraining (pre: 1.00 ± 0.00 , post: 1.08 ± 0.10 , $P = 0.445$) and was not different between temperature groups (7°C: 1.09 ± 0.08 , 20°C: 0.99 ± 0.05 , $P = 0.271$).

PGC-1 α protein. PGC-1 α protein did not change from pre- to posttraining (pre: 0.048 ± 0.014 AU, post: 0.059 ± 0.014

AU, $P = 0.529$) and was not different between temperature groups (7°C: 0.049 ± 0.015 AU, 20°C: 0.058 ± 0.012 AU, $P = 0.674$).

DISCUSSION

Developing techniques to stimulate key genes related to mitochondrial growth and development is a desirable model for improving therapies and athletic performance. The results from this investigation provide insight into cold exposure during several weeks of exercise. The main finding from this study is that *PGC-1 α* mRNA exhibits an adaptive response after a 3-wk temperature acclimation and exercise-training period. Specifically, the 7°C temperature group had higher *PGC-1 α* mRNA before 3 wk of training than the 20°C temperature groups but

Table 3. Preexercise gene expression on day 22 relative to day 1

Biogenesis	20°C	7°C	Combined
<i>PGC-1α</i>	-1.043 ± 0.104	1.124 ± 0.117	1.041 ± 0.078
<i>NRF1</i>	-1.248 ± 0.075	-1.050 ± 0.184	-1.136 ± 0.102
<i>GABPA</i>	-1.162 ± 0.079	-1.130 ± 0.063	$-1.145 \pm 0.049^*$
<i>TFAM</i>	-1.204 ± 0.050	-1.168 ± 0.106	$-1.185 \pm 0.059^*$
<i>ERRα</i>	1.005 ± 0.111	-1.253 ± 0.120	$-1.057 \pm 0.086^*$
<i>VEGF</i>	-1.215 ± 0.101	1.079 ± 0.116	-1.045 ± 0.080
Mitophagy	20°C	7°C	Combined
<i>PINK1</i>	1.228 ± 0.186	1.412 ± 0.124	$1.324 \pm 0.109^*$
<i>Parkin</i>	-1.353 ± 0.094	-1.224 ± 0.101	$-1.283 \pm 0.068^*$
<i>BNIP3</i>	-1.149 ± 0.061	-1.031 ± 0.099	-1.084 ± 0.059
<i>BNIP3L</i>	-1.305 ± 0.095	-1.117 ± 0.148	$-1.120 \pm 0.089^*$

Data are means $\pm$ SE. *PGC-1 α* , peroxisome proliferator-activated receptor- γ coactivator 1 α ; *NRF1*, nuclear respiratory factor 1; *GABPA*, GA-binding protein- α ; *TFAM*, mitochondrial transcription factor A; *ERR α* , estrogen-related receptor- α ; *VEGF*, vascular endothelial growth factor; *PINK1*, PTEN-induced kinase-1; *Parkin*, an E3 ubiquitin ligase; *BNIP3*, Bcl2-interacting protein-3; *BNIP3L*, Bcl2-interacting protein 3-like 3. * $P < 0.05$ from day 1 Pre value of ± 1 .

was not different after 3 wk of training between the two temperatures. The mRNA of transcription factors downstream of PGC-1 α were analyzed as well as mitophagy mRNA to provide a comprehensive view of mitochondrial dynamics. $\dot{V}O_{2\text{peak}}$ increased after the 3 wk of training regardless of temperature exposure. Thus, it appears that the enhanced PGC-1 α mRNA expression in the cold before training and acclimation does not manifest itself into enhanced mitochondrial function. Both temperature groups were matched for relative fitness, body composition, and age before the training protocol.

Acute exercise effects. The present study is the first to couple acute physiological measurements with functional performance measurements after a period of cold temperature acclimation. We had previously observed increased PGC-1 α mRNA during exercise and subsequent recovery in a 7°C environmental condition (39) and when a 7°C environmental temperature was used for recovery after 20°C exercise (40). In the current study, PGC-1 α was higher in 7°C than in 20°C before the 3-wk training intervention. After 3 wk of training, PGC-1 α mRNA was increased from acute exercise but to a smaller magnitude compared with before training and was not different between temperatures. Collectively, this disparity of acute response before and after training may be a result of the change in training state of the subjects and/or the acclimation to the cold. Sedentary subjects were chosen in the present study to obtain a robust response in acute gene expression (29), whereas previously we had used recreationally trained subjects (35, 40). The overall attenuated response of PGC-1 α at 4 h postexercise after the 3-wk training intervention was expected, due to the increased fitness level of the subjects without an increase in intensity during this trial (29, 41, 44). Absolute intensity was equal from the pretraining trial to the posttraining trial in the current study. The 7°C trial occurred during the summer months to ensure subjects were not preacclimated to the cold temperature. Our present data indicate that cold stimulus during exercise is sufficient to elicit an increase in PGC-1 α mRNA when the subject is not acclimated to the cold. After the 3-wk intervention, the difference in PGC-1 α mRNA was not present, which may have been due to an adaptive mRNA response. However, gene expression after multiple weeks of temperature exposure remains poorly characterized. Mice housed below their thermoneutral zone, i.e., room temperature, have higher exercise-induced markers of mitochondrial biogenesis in adipose tissue compared with mice housed in their thermoneutral zone at 29°C (27). Systemic effects of core and skin temperature may have also been responsible for the PGC-1 α mRNA alterations before and after training. Skin temperature afferent signaling inhibits activation of the preoptic area, causing an increase in efferent signaling for thermogenesis to tissues such as brown adipose tissue and skeletal muscle (10, 28). Core temperature did not change in either group, whereas skin temperature increased in the 7°C group from pretraining to posttraining but not in the 20°C group. This increased skin temperature after training in the cold is indicative of habituation and acclimation to the cold (23). More research is needed to determine whether skin cooling with exercise is responsible for the additive effect of increased PGC-1 α and alteration of other transcription factors.

VEGF is an angiogenic factor that has an additional role in stimulating nuclear encoded mitochondrial genes (46). VEGF

trended toward being higher in the 7°C group before training and increased from the exercise response in both temperatures. VEGF was also blunted posttraining compared with before training, similarly to the response of PGC-1 α . The trend in VEGF being increased was likely due to its role as the master regulator of angiogenesis. As cold temperature increases, blood is shunted toward skeletal muscle, which requires an increased demand for skeletal muscle vasculature (1). Several studies indicate that VEGF increases with environmental cold exposure and cold-water immersion (11, 20, 35). This putative cold-induced increase in skeletal muscle vasculature likely requires the coordination of VEGF via the angiogenic pathway. VEGF is also involved in pathways related to mitochondrial biogenesis though the induction of ERR α . No changes in ERR α or TFAM were observed before or after training. Some have reported ERR α to peak around 2 h postexercise (7). The 4-h postexercise time point used in the current study may have missed this peak expression time. The TFAM expression may be explained by the nutritional status of the subjects. TFAM mRNA appears to be increased when one is fed but not when fasted (34). The subjects in the present study were required to fast overnight before the absolute-intensity trials, which might have influenced these results. TFAM was of interest despite this knowledge to determine the effects of temperature acclimation on gene expression.

NRF1 and NRF2 are transcription factors downstream of PGC-1 α and are responsible for promoting the transcription of TFAM. No acute changes were present in NRF1 and NRF2 before the 3-wk training period, but both decreased after acute exercise after the 3-wk period. A lower NRF2 mRNA response has been observed previously in 7°C compared with 20°C after acute exercise (35, 40) but was not present in the current study. This may have been due to differences in fitness status between subjects, as in the current study sedentary subjects were recruited whereas recreationally trained subjects were used in previous studies. Others have reported NRF2 to peak at 24 h postexercise (7). The time point taken in the current study may not be representative of peak transcription of NRF2, but an additional time point of 24 h was not included, being that peak gene expression occurs at 2–6 h postexercise in most of the transcription factors measured and to reduce the total number of biopsies taken. No difference occurred between temperatures in the present study, but it is interesting to note the discrepancy of these transcription factors before and after training. To our knowledge, this is the first study to demonstrate an adaptive response in gene expression of NRF1 and NRF2 after temperature acclimation.

Before the training and temperature intervention occurred, markers of mitophagy did not change from acute exercise and were not different between temperature groups. After the 3-wk training protocol, PINK1 and BNIP3 were decreased from acute exercise, whereas Parkin and BNIP3L did not change. These findings may also be attributed to the use of healthy, young subjects without the presence of disease. Although the subjects were relatively unfit, they were young and healthy, and increasing mitochondria may have precedence over mitochondrial remodeling due to absence of damaged mitochondria. Changes in markers of mitophagy in the current study may be affected by the fasted state of the subjects. BNIP mRNA is elevated in the fasted compared with the fed state in mice, although Parkin remains unchanged (19). The basal

mRNA levels of some of these mitophagy markers may have increased because of the overnight fast leaving little room for increases beyond the basal level. The time course of mRNA markers of mitophagy is not characterized in humans as it is for markers of mitochondrial biogenesis. Markers of mitochondrial biogenesis peak around the 2- to 6-h time-point, and it is expected that markers of mitophagy would peak around the same time but may occur on a different time-course.

Three-week training temperature effects. Comparing basal day 22 to day 1 mRNA allowed for assessment of the 3-wk temperature and exercise intervention. Participants did not exercise for 3 days before these assessments to ensure a chronic effect and not an effect of the previous acute-exercise bout. Most basal markers of mitochondrial biogenesis and mitophagy either did not change or decreased. *PINK1* mRNA increased basally after the 3 wk of training. This may suggest the beginning stages of mitochondrial remodeling due to the stress from the 3 wk of training. However, the other markers of mitophagy were not indicative of this.

Total PGC-1 α protein and mtDNA:gDNA were measured to examine the cumulative effect of exercise and temperature from the daily exercise. Both PGC-1 α protein and the relative amount of mtDNA did not change during the 3-wk-training protocol. Similarly, cold-acclimated fish contain similar mtDNA copy number to unacclimated control despite greater mitochondrial enzyme activity in the cold-acclimated fish (2). The lack of change in mtDNA and PGC-1 α protein may have been due to the relatively short training period. In rats, noticeable increases in PGC-1 α protein occur in as little as 4 days of training, with greater increases occurring for up to 53 days (42). This time course is not well characterized in humans, as 10 days of training was reported to not increase PGC-1 α protein beyond the initial training shift (41). Discrepancies occur as to the time course of when protein and mtDNA increases can be observed (29, 45). A longer training period of at least 6 wk may be needed to discern an appreciable accumulation in protein (32) and mtDNA (2). A longer training period was not used in the current study, since the goal was to assess the influence of temperature acclimation as opposed to training. Future research may focus further on the exercise training and mitochondrial responses. However, with the normalization of *PGC-1 α* mRNA response with training/acclimation, little evidence in humans suggest a cold potentiation of the exercise response.

The molecular signaling events of no difference in PGC-1 α between temperature groups are in correspondence with the functional performance improvements of $\dot{V}O_{2peak}$ and body composition. The 3-wk training protocol for 1 h/day increased $\dot{V}O_{2peak}$, decreased fat mass, and decreased percent body fat regardless of temperature. Fat-free mass increased in the 20°C group from pre- to posttraining trial but not in the 7°C group. During resistance exercise, myogenic markers such as myogenin, myogenic regulatory factor 4, and myogenic factor 5 have a higher response in warmer compared with colder local temperature application (47). Cold stimuli such as cold-water immersion during recovery may also attenuate skeletal muscle hypertrophy in resistance training (31). The combined use of endurance exercise and cold temperature may have prohibited increases in skeletal muscle hypertrophy in the current study, whereas the 20°C may have not had the same inhibition, thus increasing fat-free mass posttraining. This may explain the

increase in fat-free mass in the 20°C group in the present study, although further examination of this increase may be warranted. A disconnect appears with the mtDNA molecular events and $\dot{V}O_{2peak}$. Despite no change in mtDNA after training, $\dot{V}O_{2peak}$ increased after 3 wk of training. This may have been due to improvements in skeletal muscle markers not measured such as increased mitochondrial efficiency and muscle oxidative capacity (13, 48). This may also be due to improvements to whole body metabolism such as increased muscle fiber recruitment, capillary density, arteriovenous differences, and cardiac output (13).

Conclusion. Understanding the characteristics related to the process of temperature acclimation are of importance due to the potential for novel therapeutic treatments and health benefits and possible effects on performance. The findings from this study demonstrate that the initial acute difference of PGC-1 α is changed after several weeks of acclimation and training in environmental cold. PGC-1 α normalized between temperatures, and this may explain the lack of differences in $\dot{V}O_{2peak}$ performance after training between the groups. Differences between 7°C and 20°C environments may not exist after an acclimation period, as skeletal muscle acclimation may be the adaptation responsible. This is likely due to the acclimation process and not the fitness status of the subjects, as more recreationally trained individuals have been used previously. These data indicate that, despite initial differences in *PGC-1 α* gene expression, temperature-acclimated skeletal muscle does not elicit the same response of increased signaling in the cold related to mitochondrial development. Clinicians and athletes looking to rehabilitate and improve performance, respectively, may not find additional benefits in further improving aerobic capacity in cold environmental temperatures. Future work may compare the long-term aspects of training in cold environmental temperatures to determine the influence of training adaptations after acclimation has occurred. Different forms of cold therapy such as local cooling or cold-water immersion should also be considered in attempting to improve training outcomes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

R.S., B.R., and D.S. conceived and designed research; R.S., K.M., M.O., H.S., B.R., and D.S. performed experiments; R.S., K.M., M.O., H.S., B.R., and D.S. analyzed data; R.S., K.M., M.O., H.S., B.R., and D.S. interpreted results of experiments; R.S., K.M., H.S., and D.S. prepared figures; R.S., K.M., and D.S. drafted manuscript; R.S., K.M., M.O., H.S., B.R., and D.S. edited and revised manuscript; R.S., K.M., M.O., H.S., B.R., and D.S. approved final version of manuscript.

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Exercise in the Heat Blunts Improvements in Aerobic Capacity

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Abstract:	The exercise-induced rise in PGC-1a is blunted when acute exercise takes place in the heat. However, it is unknown if this alteration has functional implications after heat acclimation and exercise training. Purpose: To determine the impact of three weeks of aerobic exercise training in the heat compared to training in room temperature on thermoregulation, PGC-1a mRNA response, and aerobic capacity. Methods: Twenty-one untrained college aged males (age, 24 ± 4 yrs; height, 178 ± 6 cm) were randomly assigned to three weeks of aerobic exercise training in either 33°C (n=12) or 20°C (n=11) environmental temperatures. Results: The 20°C training group increased VO2peak from 3.21 ± 0.77 L·min⁻¹ to 3.66 ± 0.78 L·min⁻¹ (p<0.001) while the 33°C training group did not improve (pre, 3.16 ± 0.48 L·min⁻¹; post, 3.28 ± 0.63 L·min⁻¹; p=0.283). PGC-1a increased in response to acute aerobic exercise more in 20°C (6.6±0.7 fold) than 33°C (4.6±0.7 fold, p=0.031) before training, but was no different after training in 20°C (2.4±0.3 fold) or 33°C (2.4±0.5 fold, p=0.999). No quantitative alterations in mitochondrial DNA were detected with training or between temperatures (p>0.05). Conclusions: This research indicates that exercise in the heat may limit the effectiveness of aerobic exercise at increasing aerobic capacity. Furthermore, this study demonstrates that heat induced blunting of the normal exercise induced PGC-1a response is eliminated after 3-weeks of heat acclimation.	
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Exercise in the Heat Blunts Improvements in Aerobic Capacity

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Abstract:

The exercise-induced rise in *PGC-1 α* is blunted when acute exercise takes place in the heat. However, it is unknown if this alteration has functional implications after heat acclimation and exercise training. **Purpose:** To determine the impact of three weeks of aerobic exercise training in the heat compared to training in room temperature on thermoregulation, *PGC-1 α* mRNA response, and aerobic capacity. **Methods:** Twenty-one untrained college aged males (age, 24 ± 4 yrs; height, 178 ± 6 cm) were randomly assigned to three weeks of aerobic exercise training in either 33°C (n=12) or 20°C (n=11) environmental temperatures. **Results:** The 20°C training group increased $\text{VO}_{2\text{peak}}$ from $3.21 \pm 0.77 \text{ L}\cdot\text{min}^{-1}$ to $3.66 \pm 0.78 \text{ L}\cdot\text{min}^{-1}$ ($p < 0.001$) while the 33°C training group did not improve (pre, $3.16 \pm 0.48 \text{ L}\cdot\text{min}^{-1}$; post, $3.28 \pm 0.63 \text{ L}\cdot\text{min}^{-1}$; $p = 0.283$). *PGC-1 α* increased in response to acute aerobic exercise more in 20°C (6.6 ± 0.7 fold) than 33°C (4.6 ± 0.7 fold, $p = 0.031$) before training, but was no different after training in 20°C (2.4 ± 0.3 fold) or 33°C (2.4 ± 0.5 fold, $p = 0.999$). No quantitative alterations in mitochondrial DNA were detected with training or between temperatures ($p > 0.05$). **Conclusions:** This research indicates that exercise in the heat may limit the effectiveness of aerobic exercise at increasing aerobic capacity. Furthermore, this study demonstrates that heat induced blunting of the normal exercise induced *PGC-1 α* response is eliminated after 3-weeks of heat acclimation.

Key Words: *PGC-1 α* , acclimation, $\text{VO}_{2\text{peak}}$, temperature, training

Introduction:

Proper mitochondrial health and function are essential for aerobic exercise capacity. It is not surprising that many diseases and conditions associated with mitochondrial dysfunction are also characterized by low aerobic exercise capacities. Exercise training is a known therapy to enhance mitochondrial function and exercise capacity. However, many individuals find exercise challenging due to other health conditions, lack of time, or simply an unwillingness. In order for exercise training to be a more useful therapy, the effectiveness must be improved. There has been a wealth of quality research evaluating factors such as exercise frequency, intensity, and time to optimize exercise training outcomes. However, other factors such as temperature have received relatively little attention.

Exercise in the heat provides a thermoregulatory challenge to the body. It is unclear if this stress provides a stimulus for the body to adapt and respond in a favorable way or if this stress is a detriment to adaptation. Acclimation to the heat appears to provide adaptations that are favorable for exercise performance such as enhanced plasma volume (Bass et al. 1958; Senay et al. 1976), increased metabolic efficiency (Young et al. 1985), and improved myocardial efficiency (Horowitz et al. 1993). Indeed, when trained cyclists underwent a heat acclimation protocol, they increased their aerobic capacity while a control group of trained cyclists did not show a change in aerobic capacity (Lorenzo et al. 2010). The most notable changes were related to the effect of plasma volume expansion on the cardio-vascular system. This study provides convincing evidence that exercise in the heat may be advantageous to exercise performance in trained individuals. It is unknown if heat acclimation has an effect on skeletal muscle mitochondria or if these results would be different in a less trained group, that may have different limitations to exercise.

Exercise in a hot environment may disturb the transcriptional response of single aerobic exercise bout (Heesch et al. 2016; Slivka et al. 2012). More specifically, the normal exercise induced expression of *PGC-1 α* is blunted in response to 1 hr of aerobic exercise in 33°C compared to the same exercise in 20°C. PGC-1 α plays an important role in mitochondrial development and is regulated on many different levels. Transcription is only one of the many ways PGC-1 α is regulated (Fernandez-Marcos and Auwerx. 2011), and thus it may be an over-interpretation of previous data to definitively conclude that heat has a negative impact on exercise induced mitochondrial biogenesis. None-the-less, the reduced *PGC-1 α* response in the heat to an acute exercise bout may be an initial signal, where if amplified over several exercise sessions may lead to a less than optimal response for enhanced mitochondrial function and aerobic capacity. Alternatively, the differential *PGC-1 α* response in the heat may disappear/acclimate after several exercise bouts in the heat. More work that incorporates repeated exercise bouts in the heat (acclimation) and includes indices of functional performance are needed in order for effective translation. Indeed, it is possible that exercise training in the heat may provide for benefits related to acclimation without further development of aerobic machinery.

The purpose of this study is to determine the impact of three weeks of aerobic exercise training in the heat (33°C) compared to training in a room temperature (20°C) environment on the *PGC-1 α* mRNA response, and aerobic capacity. The three-week time period will allow for exercise training adaptation and heat acclimation to occur (Ashley et al. 2015). This study is aimed at translating genetic responses of the skeletal muscle to the heat to actual human performance in controlled conditions.

Methods:

Twenty-three college aged males (age, 24 ± 4 yrs; height, 178 ± 6 cm), who did not engage in a regular aerobic exercise participated in this study after providing Institutional Review Board approved informed consent. All procedures conformed to the ethical principles outlined in the Declaration of Helsinki. Eleven subjects (age, 23 ± 4 yrs; height, 178 ± 7 cm) were randomly assigned to train in room temperature (20°C) conditions (20°C , 40% relative humidity), and twelve subjects (age, 25 ± 5 yrs; height, 179 ± 6 cm) were randomly assigned to train in hot temperature (33°C) conditions (33°C , 40% relative humidity). Some of the data from the RT subjects represents a sub-set of data that also served as a control group for a similar research project investigating the impacts of exercise in the cold (Shute et al. 2020). The hot (H) trials took place during the colder months of the year (January and February) while the room temperature (RT) trials took place during months of moderate temperature (April, May, and September) so that subjects would not begin the protocol in a potentially acclimated state. All trials were conducted in a temperature and humidity controlled environmental chamber (Darwin Chambers, St. Louis, MO or Tesco, Warminster, PA). Initial testing (cycle ergometer peak VO_2 and body composition) were conducted three days before beginning the acclimation period and three days after the acclimation period in standard room temperature conditions. By completing these fitness assessments in standard room conditions we were able to directly compare the changes in fitness between groups. Each subject completed 16 aerobic exercise training sessions in the assigned temperature with the first and last session being conducted at the same absolute intensity. The first and last training session at the same absolute intensity allowed us to determine how training altered the PGC-1 α response in H compared to RT. This design does not allow the comparison of H and RT groups in the heat. The intensity of the additional training sessions were set at a Rating of

Perceived Exertion (RPE) of 15. Using RPE instead of an intensity relative to maximal capacity allowed for day-to-day and subject specific progressive overload.

Fitness Assessments: Initial fitness assessments were completed three days before and three days after the temperature intervention trials. These tests included measuring height, weight, body composition, and maximal aerobic capacity (VO_{2peak}). All of the initial assessments took place in standard room temperature conditions so that direct comparisons in fitness could be made between the 20°C and 33°C groups. Height was measured using a standard stadiometer (Seca, Chino, CA) and weight was measured using a digital output scale (Befour, PS-660, Saukville, WI). Body composition analysis was conducted via hydrodensitometry using a calibrated electronic load cell based system (Exertech, Dresbach, MN) correcting for estimated residual lung volume (Thomas and Etheridge. 1980) and using the Siri equation (Siri. 1961). VO_{2peak} was determined using a graded exercise protocol on an electronically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA) while subjects breathed through a calibrated metabolic cart (ParvoMedics, Sandy, UT). The graded protocol began at 95 W and increased 35 W every 3 minutes until volitional fatigue. VO_{2peak} was defined as the highest achieved oxygen consumption and the workload associated with VO_{2peak} (W_{peak}) was calculated as the workload of the highest completed stage + the proportion of time in the final stage multiplied by the 35 W increment.

Exercise Training: On the first and last day of the 16 day training period subjects performed 60 min of cycling in their assigned temperature at 50% of their pre-training W_{peak} . This allowed us to evaluate the physiological response in different environments at the same absolute intensity before and after the training period. This workload was chosen to allow our untrained subjects to complete the full 60 min exercise session. For these absolute intensity trials, subjects were asked to abstain from exercise for at least 48 hours and to arrive at the laboratory after an overnight fast.

Subjects logged their dietary intake for the first session and were asked to repeat this diet for the 16th session. Skeletal muscle biopsies from the *vastus lateralis* were obtained before exercise and after four hours of sedentary recovery in room temperature conditions using the percutaneous needle biopsy technique (Bergstrom. 1962) with the aid of suction. The subsequent biopsy for a given trial was performed on the same leg (randomized) ~ 2 cm proximal to the initial biopsy. The other leg was biopsied for the final trial. Muscle samples were immediately cleaned of excess blood or visible connective tissue and then placed into AllProtect Tissue Reagent (Qiagen, Valencia, CA). Samples were then stored overnight at 4°C before being transferred to -30°C until later analysis. Nude body weight was measured before and after exercise and used to calculate sweat rate. Core temperature was measured using a temperature probe (RET-1, Physitemp Instruments Inc, Clifton, NJ) inserted ~12 cm past the anal sphincter. Skin temperature was recorded using adhesive skin probes (SST-1, Physitemp Instruments Inc, Clifton, NJ) and expressed as the average temperature from the chest and back. Temperatures were logged using a thermal SD logger (SDL200, Extech, Nashua, NH). Heart rate was monitored using a standard chest strap heart rate monitor (Polar V800, Polar Electronic, Lake Success, NY).

The additional daily training sessions (sessions 2-15) occurred every weekday for three straight weeks in the assigned temperature condition. Subjects were instructed to cycle for 60 minutes on a cycle ergometer (Phantom 3, Cycleops, Madison, WI) at an RPE of 15 (hard). Subjects were encouraged to adjust their intensity as needed to maintain an RPE of 15 and to progressively increase their workload throughout the 3-week training period. Heart rate was continuously monitored (Polar Electronic, Lake Success, NY) during each exercise session. Subjects were allowed to consume water ad libitum during exercise and the consumed volume was considered in calculations of sweat rate.

PGC-1 α gene expression: RNA was extracted from skeletal muscle samples using Trizol (Invitrogen, Carlsbad, CA) methodologies as previously reported (Heesch et al. 2016; Shute et al. 2020). The RNA yield ($184 \pm 101 \text{ ng} \cdot \mu\text{l}^{-1}$) was quantified using a nano-spectrophotometer (nanodrop ND-2000, Thermo Scientific, Wilmington, DE) from each $16.4 \pm 0.2 \text{ mg}$ of muscle tissue. The RNA Integrity was measured using an Agilent RNA 6000 Kit and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The RNA Integrity Number (RIN) was 8.3 ± 0.9 , indicating high quality intact RNA. First strand cDNA synthesis was achieved from the extracted RNA using Superscript IV LILO master mix with ezDNAase Enzyme (Invitrogen, Carlsbad, CA). qRT-PCR was run in triplicate on a stratagene mx3005p PCR system (Agilent Technologies, Santa Clara, CA) using a two-step protocol. Each $10 \mu\text{l}$ reaction volume contained $0.5 \mu\text{l}$ of probe and primer mix (PrimeTime qPCR assay, Integrated DNA technologies, Coralville, IA), $5 \mu\text{l}$ of qPCR master Mix (Integrated DNA technologies, Coralville, IA), and $4.5 \mu\text{l}$ of sample adjusted to contain $0.5625 \text{ ng} \cdot \mu\text{l}^{-1}$ of cDNA. All probes and primers were designed and obtained by Integrated DNA Technologies (PrimeTime qPCR assay, Coralville, IA) and are presented in table 1. Quantification of PGC-1 α was calculated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen. 2001) relative to the geometric mean of five reference genes: beta-actin (ACTB), beta-2-microglobulin (B2M), cyclophilin (CYC), ribosomal protein S18 (RPS-18), and glyceraldehyde-3 phosphate dehydrogenase (GAPDH). NormFinder software (Julian et al. 2016; Silver et al. 2006) was used to determine that this combination of reference genes were stable across our interventions. In order to determine the acute response to exercise between conditions, mRNA at the 4 hour post-exercise time-point was normalized to the pre-exercise resting sample. In order to determine the effect of the 3-week intervention, post-training, pre-exercise mRNA data was normalized to the pre-training, pre-exercise mRNA.

Mitochondrial Content: Mitochondrial content was assayed as the amount of mitochondrial DNA relative to nuclear DNA ratio (mtDNA:gDNA). DNA was extracted from skeletal muscle concurrently with the RNA extraction using Trizol methods. During phase separation, 240 µl of 100% EtOH was added to the interphase containing DNA, mixed by inversion, and incubated for 2-3 min. Samples were centrifuged at 2,000 x g for 5 min at 4°C and the supernatant was discarded. The DNA pellet was then washed twice in 800 µl of 0.1 M sodium citrate in 10% EtOH for 30 minutes each and then re-suspended in 1,200 µl of 75% EtOH before being centrifuged at 2,000 x g for 5 min. The pellet was then suspended in 400 µl of 8mM NaOH and incubated overnight at 50°C. The sample was centrifuged and transferred to a new tube removing insoluble materials. The pH was adjusted with 0.1M HEPES and quantified on the nanodrop. The concentration of DNA was adjusted so that each sample contained 19.7 ng·µl⁻¹. Relative mitochondrial DNA copy number was determined by performing PCR on a stable nuclear gene (B2M) and a sequence located in the mitochondrial MinArc. Primers and probes for these genes are presented in Table 2. The number of mtDNA copies per nuclear DNA copy was calculated using the $2^{-\Delta\Delta C_t}$ equation (17).

Statistical Analysis: Based on our previous *PGC-1a* acute response data and a desired statistical power of 0.8, we needed at least 16 total subjects. Dependent variables were analyzed using a two-way mixed design ANOVA (time x temperature group). In the event that F-ratios were found significant, a fishers protected LSD test was used to determine where significance occurred. A probability of Type I error of less than 5% was considered significant (p<0.05). Data were analyzed using the Statistical Package for Social Sciences (SPSS) software version 24.

Results:

Daily Training Intensity: HR was not different between the group that trained in 20°C and the group that trained in 33°C ($p = 0.367$) and did not change as a result of training ($p = 0.073$). The average cycling power increased by the third training session and continued to rise over the course of training ($p < 0.05$) regardless of the temperature that training took place. No differences in average power between the 20°C and 33°C training groups were observed ($p = 0.543$). Daily training intensity data are presented in figure 1.

Aerobic Fitness: VO_2 peak in absolute and relative terms increased in the group that trained in 20°C ($p = 0.001$ and $p < 0.001$) but did not change the group that trained in 33°C ($p = 0.283$ and $p = 0.234$). The power produced at VO_2 peak increased with exercise training ($p < 0.001$) but was not different between groups who trained in different temperatures ($p = 0.867$). No differences between groups were observed before the training intervention began. Aerobic fitness data are presented in Table 2.

Body Composition: The 3 week exercise training protocol did not alter body weight ($p = 0.993$) and no differences were observed between the group that trained in 20°C and the group that trained in 33°C ($p = 0.776$). Percent body fat and fat mass (FM) decreased with training regardless of training temperature ($p < 0.001$), while FFM increased in the group that trained in 20°C ($p = 0.002$) but not in the group that trained in 33°C ($p = 0.419$). No differences between groups were observed before the training intervention began for any body composition measure ($p > 0.05$). Body composition data are presented in Table 2.

Thermoregulation: The exercise induced increase in core temperature was lower after 3 weeks of exercise training in 33°C ($+1.83 \pm 0.56$ °C pre, $+1.36 \pm 0.66$ °C post; $p = 0.011$) but was unaltered

after training in 20°C ($+0.77 \pm 0.75$ °C pre, $+0.93 \pm 0.63$ °C post; $p = 0.039$). Skin temperature increased to a greater magnitude in the 33°C ($+3.48 \pm 1.27$ °C) condition than the 20°C condition ($+0.51 \pm 2.19$ °C; $p < 0.001$). The exercise induced increase in skin temperature was lower after exercise training (pre, $+2.81 \pm 1.92$ °C; post $+1.32 \pm 2.47$; $p = 0.002$) regardless of the temperature. Sweat rate during exercise increased from pre-training (668 ± 288 ml·h⁻¹) to post-training (838 ± 290 ml·h⁻¹ post; $p = 0.001$) regardless of temperature and was higher in 33°C (868 ± 324 ml·h⁻¹) than 20°C (627 ± 224 ml·h⁻¹) regardless of training state. Average exercise HR decreased from pre-training (149 ± 17 bpm) to post-training (135 ± 16 bpm; $p < 0.001$), and a trend was observed for HR to be higher in 33°C (147 ± 17 bpm) than 20 °C (136 ± 16 bpm; $p = 0.099$).

PGC-1 α gene expression: The resting pre-exercise mRNA expression of *PGC-1 α* did not change from pre-training (1.00 fold change) to post-training (1.25 ± 0.59 fold change, $p = 0.073$) and was not different between 20°C (1.04 ± 0.24) and 33°C (1.20 ± 0.55 , $p = 0.242$) groups. Before the training intervention, *PGC-1 α* increased in both the 20°C and 33°C groups ($p < 0.001$). The group that exercised in the 33°C had lower expression of *PGC-1 α* than the group that exercised in 20°C ($p = 0.031$) in response to an acute exercise bout in their assigned temperature. After the 3-week training intervention, *PGC-1 α* still increased in both the 20°C ($p = 0.003$) and 33°C ($p = 0.002$) groups. However, the post-exercise difference between the temperature groups was no longer observed ($p = 0.999$). The acute response to exercise in different temperatures before and after the 3-week training intervention is presented in figure 2.

Mitochondrial DNA: The amount of mitochondrial DNA relative to nuclear DNA did not change with the 3-week exercise intervention ($10,790 \pm 4,942$ pre, and $11,156 \pm 5,277$ post; $p = 0.832$) and was not different between the 20°C ($11,012 \pm 3,476$) and 33°C ($10,937 \pm 6,238$; $p = 0.967$) training groups.

Discussion:

The impact of heat during an acute exercise bout and acclimation have been widely studied. However, little information is available on the impact of heat on aerobic exercise training outcomes. This project aimed to translate previous acute muscle transcriptional response investigations (Heesch et al. 2016; Slivka et al. 2012) into a more practical and meaningful outcome. This study has confirmed that heat induces blunting of the normal response of PGC-1 α to acute aerobic exercise, and shows an acclimation associated effect of the PGC-1 α transcriptional response. More importantly, the exercise training response in $\text{VO}_{2\text{peak}}$ was blunted in the group that exercised in the 33°C environmental temperature. The reduced exercise training response in the heat should be considered in exercise programming designed to enhance aerobic capacity.

We chose to base the exercise intensity of the training sessions on an RPE of 15 as opposed to using a set % of maximal capacity. This was done to ensure that each individual subject received a robust training stimulus and to allow near constant progressive overload over these 14 training sessions. This protocol allowed for a relatively linear increase in workload with minimal alterations in heart rate. By using RPE, the subjects were able to increase workload at a rate proportional to cardiovascular adaptations, as HR remained consistent throughout the training. Important to the interpretation of the alterations in $\text{VO}_{2\text{peak}}$, both groups did statistically the same amount of external work during the training sessions. These data serve to show that our RPE based protocol provided an appropriate progressive overload exercise stimulus.

Evidence of heat acclimation was demonstrated in this study as the exercise-induced rise in core temperature was blunted after training at 33°C but not at 20°C group in response to exercise at the same absolute intensity pre vs. post training period. This reduction in core temperature was facilitated by a reduced skin temperature and an increased sweat rate in both training groups. It is difficult to separate a heat acclimation effect and an exercise training effect in this study. It is likely that the 20° C training group also experienced heat acclimation as the daily exercise sessions increased core and skin temperature, but not to the same degree as the 33°C group. The main point of this project was not to exhaustively detail a comparison of heat acclimation between groups, as this would have required a heat stress challenge in the 20° C group as well. Instead, the purpose was to detail the training effects in hot and control conditions on exercise performance outcomes and PGC-1 α response. These heat acclimation data simply serve to show that our protocol did lead to heat acclimation, and more robustly so in the 33°C group.

Our research group has observed on multiple occasions (Heesch et al. 2016; Slivka et al. 2012) that the exercise induced *PGC-1 α* mRNA response is reduced after aerobic exercise in the heat compared to room temperature control exercise conditions (Heesch et al. 2016; Slivka et al. 2012). These findings were confirmed in the current study during the initial pre-acclimation time-point. However, by the 16th exposure, the *PGC-1 α* response was the same between the 33°C group and the 20°C group. This response demonstrates for the first time that the skeletal muscle transcriptional response may acclimate to better manage the stresses of exercise in the heat. It is also important to note that the *PGC-1 α* response was reduced after the training period regardless of temperature, consistent with previously reported training responses (Perry et al. 2010). It is unknown from the current study design if these *PGC-1 α* responses and acclimation would remain

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4 286 if the exercise intensities were set relative to VO_{2peak} , and thus provide a greater post-training
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6 287 exercise challenge. It is possible that a more robust *PGC-1a* response and a more lasting
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8 288 transcriptional difference would be necessary to observe an inhibitory effect of heat. The
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10 289 complexities associated with the time-course of additional trials, the lasting transcriptional
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12 290 response, and the multiple biopsies needed for these additional comparisons lead us to the current
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14 291 focused study design.
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22 293 Whole body functional conclusions are difficult to be made from acute transcript data, such as our
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24 294 previous work with *PGC-1a* (Heesch et al. 2016; Slivka et al. 2012). The current project included
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26 295 not only transcript data, but also data related to whole body training adaptations. Our criterion
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28 296 variable to represent aerobic capacity for this study was VO_{2peak} . We found an impressive 14%
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30 297 increase in VO_{2peak} in the 20°C group, but only a non-significant 3.8% increase in the 33°C group.
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33 298 The exact mechanism for the reduced response in the 33°C group is unknown. Interestingly, the
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35 299 20° C did have an increase in FFM while the 33°C group did not. Based on the cycling exercise
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37 300 protocol, this increase in FFM may be from additional thigh muscle mass, although our hydrostatic
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39 301 weighing methods can not directly discern this (body water and other segmental changes are also
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41 302 possible). A potential increase in active muscle mass during cycling after training may be partially
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43 303 responsible for the increase in VO_{2peak} . While there was a blunted response to the heat in VO_{2peak} ,
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45 304 the response in W_{max} was not different between temperatures. The disconnect between VO_{2peak}
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47 305 and W_{max} suggests alterations in cycling efficiency which has been identified in a meta-analysis as
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49 306 an effect of heat acclimation (Tyler et al. 2016). It appears that increased cycling efficiency
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51 307 allowed for an increase in W_{max} in the 33°C training group, while an increase in aerobic capacity
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53 308 and muscle mass allowed for the increase in W_{max} in the 20°C training group. Most exercise and
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4 309 heat acclimation studies have been aimed at determining performance in the heat. This study
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6 310 measured exercise capacity in standard room temperature conditions, thus eliminating artifact
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9 311 caused by the acute alterations from heat exposure.
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15 313 Based on our previously observed heat inhibition of *PGC-1 α* , we suspected that heat may hinder
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17 314 mitochondrial biogenesis. We thus included a relatively crude marker of mitochondrial content
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19 315 based on mitochondrial DNA copy number relative to nuclear DNA copy number. We did not
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21 316 observe any differences in this marker of mitochondrial quantity. It is possible that this method
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23 317 was not sensitive enough or that the mitochondrial quality was altered, as opposed to the quantity.
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25 318 More sophisticated measures such as high resolution respirometry, that were beyond the scope of
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27 319 this study, may be more sensitive to potential mitochondrial qualitative changes. Indeed, other
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29 320 markers of mitochondrial adaptation typically occur before alterations in mtDNA (Egan et al.
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31 321 2013). Alternatively, the relatively short-term heat intervention did not alter mitochondria, and
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33 322 other mechanisms are responsible for our observations. Alterations in plasma volume and other
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35 323 cardiovascular alterations can impact $\text{VO}_{2\text{peak}}$ (Montero et al. 2015). However, in the current study,
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37 324 we would expect a larger plasma volume expansion in the 33°C group and thus enhanced
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39 325 functional training outcomes as observed in (Lorenzo et al. 2010), contrary to our observations.
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51 327 Previous research on heat acclimation has been performed in varied subjects, temperatures,
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53 328 timeframes, and study designs, which makes interpretation of exercise performance data difficult.
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55 329 The varied designs and outcomes are highlighted in a systematic review by Chalmers *et al.*
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57 330 (Chalmers et al. 2014). Our data may seem to disagree with a previous investigation that imposed
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4 331 a 10-day heat acclimation strategy in highly trained cyclists and observed increases in functional
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6 332 performance in the acclimated group compared to controls (Lorenzo et al. 2010). The drastically
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8 333 different fitness levels of our untrained individuals compared to the highly trained individuals and
9
10 334 different outcome parameters likely explains these differences. Trained individuals compared to
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12 335 untrained individuals have different limitations to exercise performance and respond differently to
13
14 336 a given exercise protocol. For a review of this topic please see (Gabriel and Zierath. 2017). It
15
16 337 appears that the trained subjects were able to benefit from enhanced cardiovascular adaptations
17
18 338 with acclimation, but that these same cardiovascular limitations may not have been present in our
19
20 339 untrained subjects. This large contrast in outcome based on training status makes it hard to
21
22 340 speculate how exercise in the heat may affect different clinical populations with different
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24 341 limitations to exercise. Furthermore, a key difference between the current study and (Lorenzo et
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26 342 al. 2010) is that the current study aimed to increase aerobic fitness whereas (Lorenzo et al. 2010)
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28 343 aimed to maintain fitness from non-thermoregulatory mechanisms. Indeed, the exercise intensity
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30 344 and thus amount of heat-related stress may differentiate the ultimate outcome. Collectively, these
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32 345 studies indicate that heat exposure is a key variable to be considered in exercise programming.
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44 347 Here we report that heat may limit the effectiveness of aerobic exercise at increasing aerobic
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46 348 capacity in previously untrained males. This study extends the acute transcriptional data
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48 349 previously reported (Heesch et al. 2016; Slivka et al. 2012) and applies it into functional terms
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50 350 (general exercise tolerance and capacity). Furthermore, this study provides evidence of skeletal
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52 351 muscle transcriptional heat acclimation. The reduced exercise training response in the heat on
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54 352 aerobic capacity should be considered in the design of the most effective countermeasures.
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Figure Captions:

Figure 1. Average heart rate (A) and power (B) during 1 hour of cycling at an RPE of 15 in 20°C and 33°C environmental temperatures over the course of 19 days. * $p < 0.05$ from training day 2. No differences were observed between the 20°C and 33°C training groups. Data are mean $\pm$ SE.

Figure 2. Fold change from pre-exercise rest in PGC-1 α mRNA at 4 hours after exercise at the pre-training and post-training time-points. * $p < 0.05$ from pre-exercise (1.00 fold change). † $p < 0.05$ from 20°C. Data are mean $\pm$ SE.

Figure 2

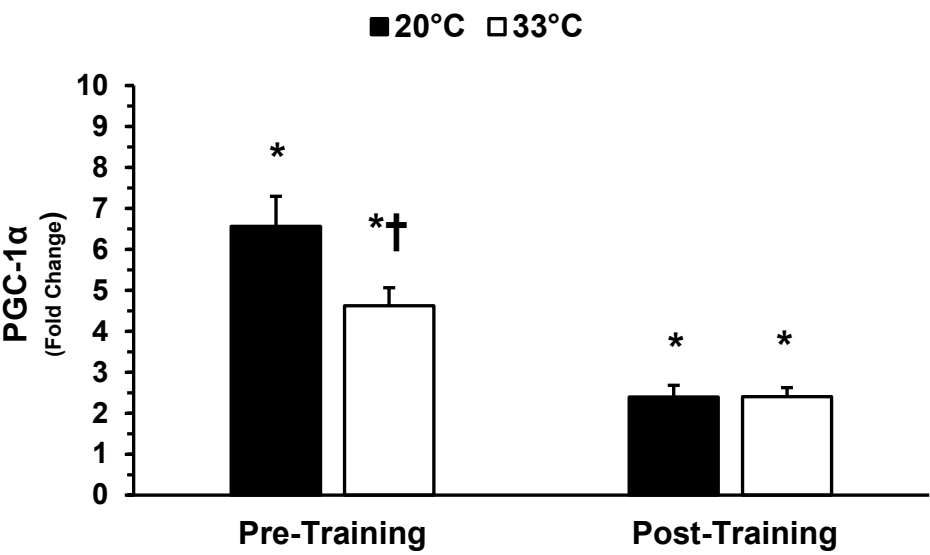


Figure 1

[Click here to access/download;Figure;Figure 1.pdf](#)

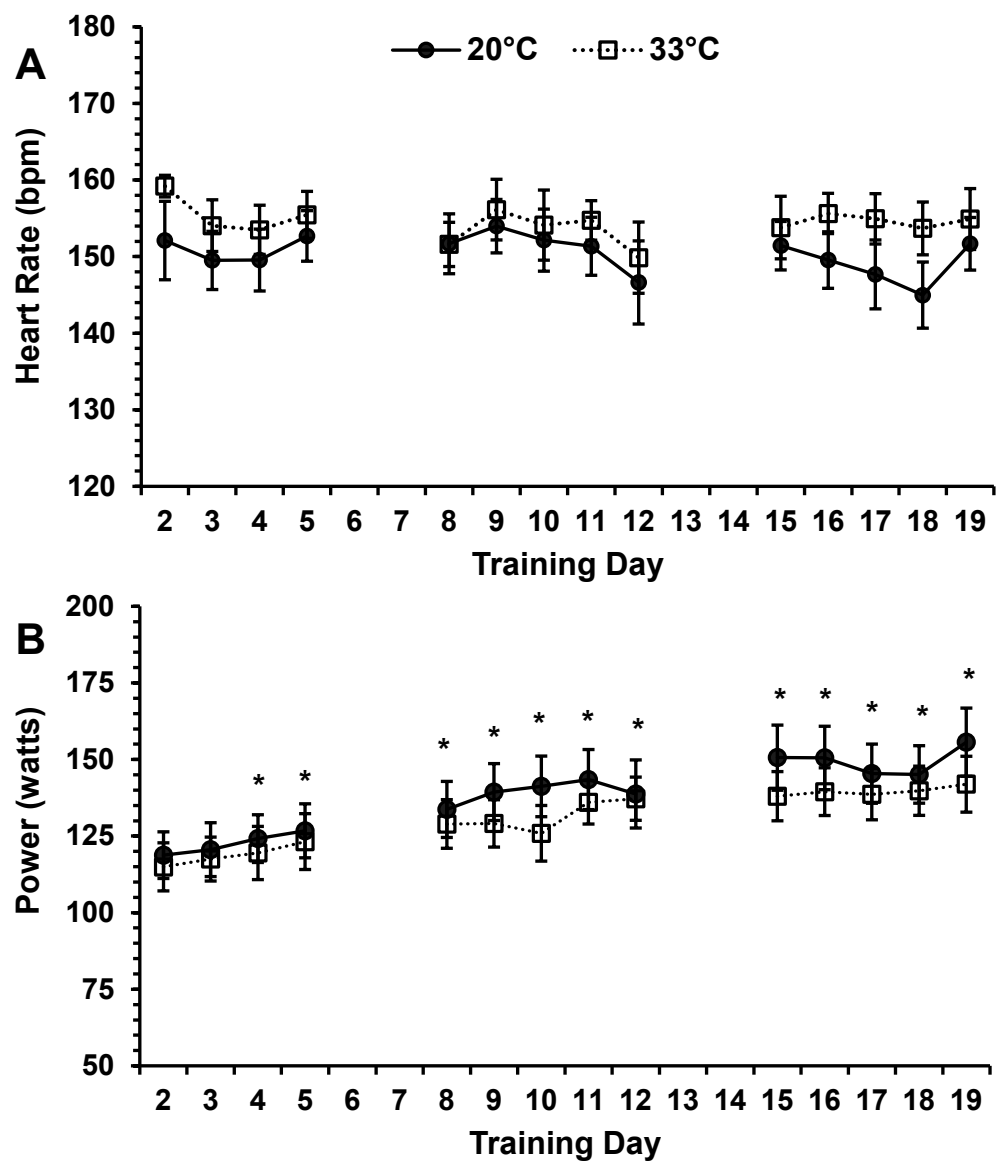


Table 2. Aerobic fitness and body composition pre and post 3 weeks of aerobic exercise training in 20°C and 33°C environmental temperatures.

	20°C		33°C	
	Pre	Post	Pre	Post
Maximal Exercise Capacity				
VO ₂ peak (L·min ⁻¹)	3.21 ± 0.77	3.66 ± 0.78*	3.16 ± 0.48	3.28 ± 0.63
VO ₂ peak (ml·kg ⁻¹ ·min ⁻¹)	37.8 ± 9.9	42.6 ± 8.7*	37.5 ± 5.4	38.8 ± 6.5
Power (watts)	212 ± 52	251 ± 53*	217 ± 33	251 ± 31*
Body Composition				
Weight (kg)	88.8 ± 27.1	89.3 ± 27.8	86.3 ± 19.6	85.9 ± 18.9
% fat	23.7 ± 11.4	22.1 ± 11.5*	21.3 ± 7.4	20.4 ± 8.1*
FM (kg)	23.1 ± 17.5	21.8 ± 17.4*	19.5 ± 10.8	18.6 ± 11.1*
FFM (kg)	65.7 ± 13.6	67.4 ± 14.2*	66.9 ± 10.4	67.2 ± 9.6

FM, fat mass; FFM, fat free mass. *p<0.05 from pre. Data are mean ± SD.

Table 1. Primers and probes used for real-time RT-PCR.

Gene	Primer 1	Primer 2	Probe
ACTB	AAGTCAGTGTACA GGTAAGCC	GTCCCCCAACTTG AGATGTATG	CTGCCTCCACCC ACTCCCA
B2M	ACCTCCATGATG CTGCTTAC	GGACTGGTCTTTCT ATCTCTTGT	CCTGCCGTGTGAA CCATGTGACT
CYC	TCTTTCACTTTGC CAAACACC	CATCCTAAAGCAT ACGGGTCC	TGCTTGCCATCCAAC CACTCAGTC
GAPDH	TGTAGTTGAGGTC AATGAAGGG	ACATCGCTCAGA CACCATG	AAGGTCGGAGTCAAC GGATTTGGTC
RPS18	GTCAATGTCTGCT TTCCTCAAC	GTTCCAGCATATT TTGCGAGT	TCTTCGGCCACAC CCTTAATGG
PGC-1α	GCAATCCGTCTT CATCCACA	CCAATCAGTACAAC AATGAGCCT	AGCAGTCCTCACAG AGACACTAGACAG
B2M nuclear DNA	GCTGGGTAGCTCT AAACAATGTATTCA	CCATGTACTAACA AATGTCTAAAATGGT	CAGCAGCCTATTC TGC
Mitochondrial DNA	CTAAATAGCCCAC ACGTTCCC	AGAGCTCCCGTGA GTGGTTA	CATCACGATGGAT CACAGGT

Gene Abbreviations: ACTB - beta-actin, B2M - beta-2-microglobulin, CYC -cyclophilin, RPS18 - ribosomal protein S18, GAPDH - glyceraldehyde-3 phosphate dehydrogenase, PGC-1 α - peroxisome proliferator-activated receptor gamma coactivator 1-alpha, B2M nuclear DNA – beta-2-microglobulin DNA sequence, Mitochondrial DNA – mitochondrial DNA sequence located in the minor arc.

DS and BR conceived and designed research project. DS, BR, WH, RS, KM, and HS conducted human subjects testing and data collection. DS, RS, and HS conducted skeletal muscle analysis. DS, KM, RS, and HS analyzed data. DS wrote the manuscript. All authors have read and approved the manuscript.

**Blood Oxidative Stress and Post-Exercise Recovery are Unaffected by
Hypobaric and Hypoxic Environments**

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Running Head: Hypobaric hypoxic recovery and oxidative stress

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Abstract:

Hypobaria and hypoxia exert independent effects on blood oxidative stress during exercise, while the combined effects of hypobaria and hypoxia on post-exercise recovery remain unclear. The purpose of the study was to examine the recovery of exercise-induced oxidative stress during lab-simulated hypoxic and hypobaric conditions. Recreationally active participants (n=16) performed 60-minutes of cycle ergometry (70% watts max) under normobaric normoxic conditions, in a randomized crossover design, followed by a four-hour recovery in three lab-simulated conditions; 1000m normobaric normoxia (NN, 675mmHg, 18.8%FiO₂), 4400m normobaric hypoxia (NH, 675mmHg, 12% FiO₂), or 4400m hypobaric hypoxia (HH, 440mmHg, 12% FiO₂). Blood samples were collected in heparinized vacutainer tubes at Pre, Post, 2 Hours (2-HR), and 4 Hours (4-HR) post-exercise. Blood plasma was analyzed for the quantification of oxidative stress to proteins (protein carbonyls, PC; 3-nitrotyrosines, 3NT), lipid modification (lipid hydroperoxides, LOOH; 8-isoprostanes, 8-ISO), and antioxidant capacity (ferric reducing ability of plasma, FRAP; trolox equivalent antioxidant capacity, TEAC). Oxidative gene transcripts (EPAS, HMOX1, SOD2, NFE2L2) were quantified by qRT-PCR from *vastus lateralis* muscle samples taken before and after each exercise trial. While hypoxia and hypobaria had no effect throughout the recovery period, post-exercise TEAC (p=0.041), FRAP (p=0.013), and 8-ISO (p=0.044), increased while PC (p=0.002) and 3-NT (p=0.032) were decreased. LOOH was lower in Post (p=0.018) NH trial samples. Exercise-dependent increases occurred in NFE2L2 (p=0.003), HMXO1 (p<0.001), SOD2 (p=0.046), and EPAS (p=0.038). In this study, exercise recovery in simulated conditions of HH did not consistently impact blood oxidative stress or redox-sensitive muscle redox measures.

Key words: antioxidants, free radicals, reactive oxygen species

Introduction

Acute exercise is well known to cause perturbations in redox balance and commonly results in a transient blood oxidative stress response in both laboratory and field-based settings [1-4]. Although prolonged oxidative stress negatively impacts physical health, temporary changes in redox balance that occur during exercise and the post-exercise recovery period are necessary to develop exercise adaptations [3]. Oxidative stress results from both exercise stimuli and various environmental conditions [5-8]. Even under resting conditions, high altitude can alter an oxidative stress response in ways that are magnified during exercise [9-11]. Conversely, the acute oxidative stress can be blunted during the post-exercise period when recovery occurs at altitude, which could negatively impact the ability to develop exercise-related adaptations [12-14]. Previous investigations have produced discrepant findings, which reveals that responses to exercise and environmental stressors are more dynamic than previously thought [15]. With many recreationally active individuals self-selecting into exercise under a host of variable environmental conditions, it is important to gain new insights into the effects of hypoxia and hypobaria on blood oxidative stress responses during exercise and the subsequent recovery period to determine potential physiological outcomes of the exercise bout. Prior work indicates that the experimental parameters of a particular study are essential in delineating the independent and combined effects of environmental hypoxia and hypobaria on the post-exercise recovery period. Moreover, the variations in the environment can variably influence oxidative stress variables during and following an acute bout of exercise [5, 12-14].

Given the need to control these experimental variables, many research groups interested in this topic have opted for investigations in tightly controlled laboratory settings. Case in point, previous field-based research suggests that acute exercise under hypoxic conditions can result in an

enhanced oxidative stress response as compared to normoxic exercise [5]. However, conclusions from these studies are complicated by the independent observations that hypoxic exercise elevates the relative intensity of a given workload, and may therefore reflect the fact that higher intensity exercise is merely associated with magnified oxidative stress responses [2, 16-18]. Thus, while field-based studies have paved the way for altitude-based studies of work physiology, well-controlled laboratory studies were needed to delineate the independent effects of hypoxia on the post-exercise recovery period. For example, recent laboratory studies demonstrate that exercise recovery occurring under altitude-simulated hypoxic conditions blunt the oxidative stress response and may mitigate redox-dependent adaptations to the exercise [12]. However, a gap in knowledge remains in regard to the independent impact of hypobaric conditions, as well as the combination of hypobaria and hypoxia that is experienced with exercise at high altitude, on blood oxidative stress during the post-exercise recovery period. Therefore, the purpose of the current study was to examine the independent effects of hypobaria and hypoxia on post-exercise blood oxidative responses and recovery. A strategic panel of blood oxidative stress biomarkers was selected to quantify lipid damage (lipid hydroperoxides, LOOH; 8-isoprostane, 8-ISO), protein damage (protein carbonyls, PC; 3-nitrotyrosine, 3-NT), and antioxidative status (Trolox equivalent antioxidant capacity, TEAC; Ferric reducing ability of plasma, FRAP). Additionally, the redox-sensitive gene transcripts EPAS, HMOX1, SOD2, and NFE2L2 were examined from vastus lateralis biopsies in order to examine potential redox changes within muscle tissue. We hypothesized that both hypoxic and hypobaric conditions would result in a blunting of the oxidative stress response following exercise; thus, impacting the ability to develop physiological adaptations to training.

92 **Methods**

93 ***Participants***

94 Informed consent for this investigation was obtained via the Institutional Review Board (IRB) and
95 USAMRMC Research Protections Office. Potential participants granted written consent prior to
96 study initiation. Recreationally trained male (n = 8) and female (n = 5) participants between the
97 ages of 18 and 40 years old were recruited for this investigation. Inclusion criteria required a
98 $\text{VO}_{2\text{peak}}$ of at least $35 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. Participants with a history of acute mountain sickness or those
99 with a known risk factor for coronary artery disease were excluded from the study as determined
100 by the physical activity readiness questionnaire (PAR-Q). Females taking oral contraceptives or
101 those without a regular menstrual cycle within the previous eight months were excluded due to the
102 potentially confounding effects on hormonal status. Participant characteristics are presented in
103 **Table 1.**

104 ***Study Design***

105 The investigation began with a baseline testing session to collect participant characteristic data,
106 including body composition and $\text{VO}_{2\text{peak}}$. The subsequent laboratory visits included three exercise-
107 recovery trials where recovery parameters from an identical exercise protocol were modified
108 according to simulated conditions of hypoxia and hypobaria as described later. The experimental
109 design is presented in **Figure 1.**

110 ***Baseline Testing***

111 Prior to baseline testing, participants were asked to fast for a duration of three-hours. Participant
112 characteristic data included the recording of height (Seca 213 Stadiometer, United Kingdom),
113 weight (Befour PS-660 ST Digital Scale, Saukville, WI), body composition, and $\text{VO}_{2\text{peak}}$
114 assessments. Body composition was quantified via hydrostatic weighing using an electronic load

cell-based system (Exertech, Dresbach, MN). Residual lung volume estimates were determined and final body density values from underwater weighing were converted to percent body fat using the Siri equation [19]. Peak oxygen uptake (VO_{2peak}) was quantified during a graded exercise cycling protocol on an electronically braked Velotron cycle ergometer (RacerMate, Seattle, WA). Starting at a workload of 95 watts, participants increased exercise intensity by 35 watts every 3-minutes until volitional fatigue was achieved. The highest obtained oxygen uptake value obtained from 15-second intervals was used to determine VO_{2peak} . The maximum achieved workload (watts max) of each participant was calculated from the time completed in the last stage, divided by the total stage duration (3-min), multiplied by 35 watts, and added to the watts of the last completed stage. Expired gases were quantified in 15-seconds intervals throughout the exercise test using a flow- and gas-calibrated metabolic cart (Parvomedics TrueOne 2400, Sandy, UT).

Exercise-recovery trials

Each participant completed three exercise recovery trials in a randomized, counter-balanced fashion. Exercise recovery trials were separated by 7 days in order to allow for biopsy recovery and to eliminate between-trial acclimation. Subjects reported to the laboratory in the early morning following an eight-hour fast. Each exercise trial consisted of a 60-minute cycle ergometer challenge at 70% watts max. Participants were provided water during the exercise trials *ad libitum*. For the sake of consistency, the amount of water consumed during the first exercise trial was replicated for the two subsequent trials. Upon completion of the exercise portion of each trial, a four-hour recovery period occurred within a (32"x7') cylindrical altitude chamber (Engineering Innovations, LLC, Littleton, CO). Altitude chambers were necessary for the normobaric and hypobaric aspects of the exercise recovery challenges. Hypobaria was then combined with either normoxia or hypoxia (Tescor, Warminster, PA) to create the recovery scenarios. A short break

from the chamber was incorporated at the 2-hour midpoint of each recovery trial. The three experimental recovery conditions simulated the following environmental altitudes with respect to inspiratory O₂ fraction and/or barometric pressure as delineated below:

- Normobaric-normoxia (NN); Base elevation (975 m) atmospheric conditions; normobaria (675mmHg) and normoxia (18.8% FiO₂, room air inspiratory fraction).
- Hypobaric-hypoxia (HH); Full high elevation simulation (4,420 m) atmospheric conditions; hypobaria (440mmHg) and hypoxia (12% FiO₂).
- Normobaric-hypoxia (NH); Partial high elevation simulation (4,420 m) atmospheric conditions; normobaria (675mmHg) and hypoxia (12% FiO₂).

Exercise-recovery oxygenation saturation and heart rate

Quantification of tissue oxygen saturation and heart rate was performed before and during exercise as well as at 60-minute intervals throughout the recovery period using finger pulse oximetry (Nonin WristOx2 3150, Plymouth, MN).

Phlebotomy and blood plasma collection

For each of the exercise-recovery trials, whole blood samples were collected from the antecubital vein with sodium heparinized vacutainers. Venipuncture was performed pre- (Pre), post- (Post), two (2-HR), and four (4-HR), hours post exercise. Blood samples were centrifuged at 1000 x g for 15-minutes at 4°C, and the plasma fraction was aliquoted and stored immediately at -80°C until subsequent biochemical assay for oxidative damage and antioxidant biomarkers.

Exercise-recovery trial muscle biopsies

Muscle biopsies were obtained before and after each exercise recovery trial from the *vastus lateralis*. Muscle samples were extracted with the aid of suction using a 5 mm Bergstrom

percutaneous biopsy needle. Left and right legs used for biopsy were chosen randomly and counter-balanced between trials. Incision sites were cleaned and three to four mL of 1% lidocaine was injected subcutaneously and around the muscle fascia prior to incision. Next, a small incision was created through the skin and muscle fascia, followed immediately by the biopsy process. Post recovery biopsies were extracted from a separate incision ~ 2 cm proximal to pre-exercise biopsies. Newly obtained muscle tissue samples were quickly cleaned of surface blood, connective tissue, and fat prior to submersion in All-protect (Qiagen, Hilden, North Rhine-Westphalia, Germany). Samples were then stored at -30°C until assay.

Biochemical oxidative stress panel

Blood oxidative stress was quantified using a comprehensive panel that included circulating markers of oxidative damage and antioxidant content. Oxidative damage markers included two lipid-based markers, lipid hydroperoxides (LOOH) and 8-Isoprostanes (8-ISO), and two protein-based markers, protein carbonyls (PC) and nitrotyrosines (3-NT). Measures of plasma antioxidant capacity included Trolox equivalent antioxidant capacity (TEAC) and Ferric reducing antioxidant potential (FRAP). Individual blood plasma aliquots were subjected to a single freeze-thaw cycle in order to preserve sample viability for the various redox-sensitive markers of interest. Plasma aliquots were kept on ice in a dark environment during assay to prevent confounding final results with environmental redox alterations.

Plasma antioxidant capacity

Trolox equivalent antioxidant capacity was performed using a quantifiable colorimetric reaction whereby plasma antioxidants scavenge exogenous introduction of 2, 2' azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical anions. The formulation of the assay work solution began with 50 mM Glycine buffer and a 2.5 mM Trolox solution. Then, glycine

peroxidase was added to 50 mM glycine buffer, followed by the addition of ABTS solution and 22 mM H₂O₂ in the final work solution. Final calculated TEAC values were derived from unknown plasma samples using the water-soluble vitamin E analogue Trolox as a standard reference [20]. The FRAP assay was performed using a colorimetric reaction, which quantifies an exogenous ferric-to-ferrous tripyridyltriazine (TPTZ) reduction by antioxidants present within plasma. Within the FRAP assay, reduction of TPTZ is proportional to blood plasma antioxidant capacity and was quantified by absorbance spectroscopy at 593 nm according to established methodology [21].

Plasma oxidative damage

Plasma LOOH were quantified using the ferrous oxidation-xylenol orange assay. Plasma samples were incubated in the presence or absence of a reducing agent and incubated with a colorimetric work solution containing ferrous ammonium sulfate, butylated hydroxytoluene, and xylenol orange. During the assay reaction, oxidized ferrous ions oxidize the ferrous sensitive dye contained in xylenol orange to form a quantifiable complex that was measured via absorbance spectroscopy at 595 nm. Final LOOH concentrations in unknown blood plasma samples were derived using a cumene hydroperoxide standard reaction [22]. Blood plasma 8-ISO concentrations were quantified by a commercially available specific immunoassay enzyme (EIA) (Cayman Chemical, Ann Arbor, MI) and assay procedures were performed according to manufacturer instructions [23].

Prior to quantification of oxidative damage to plasma proteins, samples protein concentrations were determined using the spectrophotometric Bradford method [24]. Samples were then normalized for either PC or 3-NT assays. PC determination in blood plasma samples was performed by a commercially available ELISA kit according to manufacturer instructions (Biocell Corporation Ltd, Papatoetoe, NZ) [25]. Samples were diluted based on anticipated ranges of 4-35 mg protein/ml. Final concentrations were calculated based on spectrophotometric absorbance

readings at 450 nm. 3-NT content in blood plasma samples was determined via commercial ELISA and assayed according to manufacturer instructions (Cell Biolabs INC, San Diego, CA). Similar to PC, final 3-NT concentration were determined from spectrophotometric readings at 450 nm [26].

Muscle Sample Preparation and qRT-PCR

Skeletal muscle (11.5 ± 1.7 mg) samples were processed in 500 μ L of Trizol (Invitrogen, Carlsbad, CA) using an electric blender homogenizer (Bullet Blender, Next Advance, Inc, Averill Park, NY) in 1.5 mL Red RINO tubes prefilled with RNase free ceramic beads (Next Advance, Inc, Averill Park, NY). Homogenate was centrifuged at 12,000 g for 15-min and the aqueous phase isolated and incubated overnight at -20°C . Centrifugation was used to create a pellet, followed by resuspension in ethanol, and a 5-minute centrifugation at 7,500 g. Ethanol was removed, the pellet dried, and re-dissolved in 30 μ L of RNase-free water. RNA concentrations were quantified using a nano-spectrophotometer (nano-drop ND-2000, Thermo Scientific, Wilmington, DE). Average RNA yields were 165.9 ± 12.1 ng $\cdot\mu\text{l}^{-1}$ and average absorbance ratio at 260:280 exceeded 1.90 as a means of determining RNA purity. RNA sample integrity (RNA integrity = 8.0 ± 0.1) was assessed using a 2100 Agilent Bioanalyzer and Agilent RNA 6000 Kit (Agilent Technologies, Santa Clara, CA). First-strand cDNA synthesis was achieved using the Superscript IV-first strand synthesis system for RT-PCR kit (Invitrogen, Carlsbad, CA) and the resulting cDNA diluted with RNase-free water to achieve a final cDNA concentration of 0.5 $\mu\text{g}/\mu\text{L}$ for resultant PCR reactions. qRT-PCR reactions contained 0.5 μL of probe/primer mix (PrimeTime qPCR assay Integrated DNA Technologies, Coralville, IA), 5 μL qPCR Master Mix (Integrated DNA Technologies, Coralville, IA), and 4.5 μL sample cDNA. PCR was performed in triplicate on a Startagene mx3005p PCR system (Agilent Technologies) using a 2-step protocol (1-5second cycle at 95°C ,

50-20second cycles at 60°C). Redox-sensitive transcripts included endothelial PAS domain protein-1 (EPAS-1), hemoxygenase-1 (HMOX1), superoxide dismutase-2 (SOD2), and nuclear factor erythroid-derived 2-like 2 (NFE2L2). mRNA quantification was completed using the $2^{-\Delta\Delta CT}$ method (Livak&Schmittgen. 2001). The geometric mean of five housekeeping genes: beta-actin (ACTB), beta-2-microglobulin (B2M), cyclophilin (CYC), ribosomal protein S18 (RPS-18), and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) were used as a reference for each participant. This reference gene combination was determined by established NormFinder software (Andersen, et al. 2004). qRT-PCR probe and primer sequences are presented in **Table 2**.

Statistical Analysis

Blood plasma variables and redox-sensitive gene transcripts responses were examined between NN, HH, and NH trials using repeated-measures ANOVAs (trial x time) with a Bonferroni correction. All statistical procedures were performed using the Statistical Package for Social Sciences software (SPSS) Version 24.0 (Chicago, IL). All values are presented as means $\pm$ standard deviation (SD). Significance was set at $p \leq 0.05$ *a priori*.

Results

Exercise Trials

HR and oxygen saturation data are presented in **Table 3**. No main effect of trial was observed in HR ($p = 0.387$); however, a significant time effect occurred ($p < 0.001$). HR was significantly elevated during EX ($p < 0.001$) and remained elevated at POST ($p < 0.001$). Further, no main effect of trial was observed for oxygen saturation ($p = 0.276$). A significant time effect was observed ($p < 0.001$) with a decrease during EX ($p < 0.001$) that did not recover at POST ($p < 0.001$). These data indicate that the exercise stimulus among all three trials resulted in similar physiological responses.

Plasma antioxidant status

Mean responses for plasma antioxidant status assessed through the measurement of TEAC and FRAP are presented in **Figure 2A and 2B**. A significant time effect ($p = 0.012$) was present for TEAC values, with an increase at POST ($p = 0.011$) and a return to baseline at 2-HR ($p = 0.288$) and 4-HR ($p = 0.149$). Similar to TEAC, FRAP demonstrated a significant time effect ($p = 0.001$), with an increase at POST ($p = 0.006$), but statistical analyses indicated a returned to baseline values by 2-HR and 4-HR ($p > 0.99$). No differences between trials were observed in TEAC ($p = 0.093$) or FRAP ($p = 0.050$), which indicated that increases in antioxidant markers were related to the bout of exercise.

Biomarkers for plasma oxidative damage

Biomarkers for oxidative damage as quantified by LOOH and 8-ISO are presented in **Figure 2C and 2D**. Analysis of LOOH findings indicated a main effect of trial ($p = 0.005$) and time ($p = 0.019$); however, no trial x time interaction was observed ($p = 0.064$). At 2-HR, LOOH was significantly higher than the POST time point ($p = 0.011$). 8-ISO was unaffected by trial ($p =$

0.263), but a time effect was observed ($p = 0.001$). 8-ISO was significantly increased at 2-HR ($p = 0.044$), indicating an exercise-induced elevation.

Protein modification biomarkers PC and 3-NT are presented in **Figure 2E and 2F**. Statistical analysis indicated there was no effect of trial on PC ($p = 0.368$); however, there was a main effect of time ($p < 0.001$). Exercise resulted in a significant decrease of PC at 2-HR ($p = 0.002$) when compared to pre-exercise values. For 3-NT, there was no main effect of trial ($p = 0.222$), but a significant time effect occurred ($p = 0.006$). A mean decrease of 3-NT occurred at the 2-HR time point ($p = 0.001$).

Gene Expression

Gene expression of NFE2L2, HMOX1, SOD2, and EPAS measured at PRE and 4-HR are presented in **Figure 3**. There was no main effect of trial in NFE2L2 ($p = 0.509$); however, a main effect of time was observed ($p = 0.003$). A significant increase in NFE2L2 occurred at 4HR ($p = 0.003$), indicating an exercise-induced increase. Similar to NFE2L2, no effect of trial occurred in HMOX1 ($p = 0.519$), but a time effect was observed ($p < 0.001$). HMOX1 was significantly elevated at 4HR ($p < 0.001$) following the bout of exercise. No main effect of trial was observed in SOD2 ($p = 0.691$), but a time effect occurred ($p = 0.046$) with a significant increase above pre-exercise values at 4HR ($p = 0.046$). Finally, there was no main effect of trial in EPAS ($p = 0.282$); however, a time effect occurred ($p = 0.038$) with a significant elevation at 4HR ($p = 0.038$).

Discussion

The primary purpose of this investigation was to examine the independent and collective effects of hypobaria and hypoxia on post-exercise oxidative stress responses. These outcomes were quantified utilizing a panel of oxidative damage markers, antioxidant capacity/potential, and redox-sensitive gene transcripts collected in a well-controlled laboratory setting. In order to assess the effects of the environmental variables on post-exercise oxidative stress, normobaric hypoxia and hypobaric hypoxia were manipulated during exercise recovery. Key dependent oxidative stress variables were examined due to the importance of redox challenges and their necessity in the production of exercise-induced adaptations. Under normobaric and normoxic scenarios, those who engage in exercise under these normal environmental conditions will benefit from the blood oxidative stress; however, the atmospheric conditions can attenuate these responses [12-15]. The results of the current investigation demonstrated no effect of hypobaria or hypoxia on markers of oxidative stress (LOOH, 8-ISO, PC, 3-NT or antioxidant capacity/potential (TEAC, FRAP) during post-exercise recovery. Of note, exercise elicited an increase two-hours after exercise in LOOH and 8-ISO, but these responses were not impacted by alterations in the hypoxic or hypobaric recovery conditions. In further support, redox sensitive transcripts (EPAS, HMOX1, SOD2, NFE2L2) were elevated in the post-exercise muscle biopsy samples, but variations in the hypoxic or hypobaric recovery conditions did not have an intervening influence. These findings are contrary to our working hypothesis that hypoxia and hypobaria would blunt the post-exercise oxidative stress response, and they are discussed subsequently.

Antioxidant Status

Antioxidant status was assessed through the quantification of antioxidant capacity (TEAC) and antioxidant potential (FRAP). Within the current investigation, both TEAC and FRAP were

unaffected by recovery under both hypobaric and hypoxic conditions. Our findings are in conflict with prior work by our group, in which Ballmann et al. demonstrated that recovery in a hypoxic environment mitigated the exercise-dependent increase of FRAP and TEAC that typically occurs in a normoxic environment [12]. Of note, the exercise bout selected within the aforementioned study was longer in duration (90-minutes) and consisted of intervals that peaked at a much higher exercise intensity (80% $\text{VO}_{2\text{max}}$). In further support, Peters et al. examined the effects of hypoxia on recovery of blood oxidative stress following one-hour of cycling at 70% $\text{VO}_{2\text{max}}$ [14]. An exercise-induced elevation of TEAC and FRAP was observed, but there were no differences between recovery in a hypoxic environment (1667m, 3333m, or 5000m) when compared to normoxia [14]. Although an exercise-induced increase in TEAC and FRAP was present within the current study, the magnitude of the oxidative stress response was modest and may not have been sufficient to observe changes among the environmental conditions. Importantly, previous research has demonstrated a post-exercise drop in FRAP, with no observed changes in TEAC, following one-hour of moderate-intensity cycling exercise (60% $\text{VO}_{2\text{peak}}$) under normoxic and hypoxic conditions; thus, biomarkers of antioxidant status are not always elevated in response to a bout of exercise [13].

Markers of Protein Damage

When examining biomarkers of protein damage, the current investigation demonstrated a mean decrease in both PC and 3-NT following exercise, with no differences occurring between normoxic, hypoxic, or hypobaric recovery trials. Previous research examined cycling exercise at 70% $\text{VO}_{2\text{peak}}$, which elicited an elevation in PC concentrations that returned to baseline levels within one-hour after the cessation of exercise [27]. When considering the combined effects of exercise and altitude, Peters et al., who conducted a well-controlled, laboratory-based study,

observed an increase in PC five-hours after a 60-minute bout of cycle ergometry at 70% $\text{VO}_{2\text{max}}$; however, PCs were unaffected by the hypoxic recovery when compared to normoxic recovery [14]. Within the current study, the sampling time points did not extend to five-hours post-exercise; thus, it is possible that the elevation in PCs was not captured. Although, an investigation examining the effects of hypoxic conditions following a cycling bout at 60% of $\text{VO}_{2\text{peak}}$ indicated that PC was actually decreased following exercise and unchanged by the hypoxic recovery conditions [13]. These data are in support of the working theory that the exercise stimulus must be sufficient to produce an elevation in markers of protein damage; thus, it is not possible to draw clear conclusions on the effects of the environmental recovery conditions. For instance, previous work has demonstrated that hypoxic recovery environments blunt the necessary oxidative stress response to exercise [12]. However, if a sufficient exercise-dependent increase is not observed, then it is not possible to determine if the hypoxic or hypobaric conditions resulted in mitigation of the exercise-induced oxidative stress.

Markers of Lipid Damage

The current investigation demonstrated no effect of hypoxic or hypobaric recovery on LOOH, a marker of lipid damage. In response to exercise, LOOH were elevated at 2-HR, when compared to the immediate post-exercise values. Previous research on the LOOH response to hypoxic exercise has been inconclusive [12-14]. An investigation conducted by Peters et al. demonstrated a significant exercise-dependent elevation in LOOH as well as differences in the oxidative stress response when recovery occurred under hypoxic conditions [14]. Following a one-hour bout of cycling exercise at 70% $\text{VO}_{2\text{max}}$, the magnitude of elevation in LOOH during the recovery period was greatest in the normoxic environment, with an elevation of lesser magnitude occurring at 3333m. These data suggest that LOOH elevation may be blunted under hypoxic conditions [14].

In contrast, previous work by Ballmann et al. exhibited an exercise-induced decrease in LOOH immediately after 90-minutes of interval exercise, with no differences in the recovery period when comparing normoxic and hypoxic conditions [12]. Additionally, LOOH become elevated following higher intensity exercise in both normoxic and hypoxic recovery environments [13]. Evidence supports the sensitivity of lipid peroxidation to both intensity and volume of exercise [1]. Therefore, it is likely the divergent responses can be explained by the selected exercise stimuli of the previous studies.

Redox-Sensitive Gene Transcripts

In order to understand the effects of hypobaric and/or hypoxic conditions on the recovery of blood oxidative stress following exercise, four redox-sensitive gene transcripts were examined before and four-hours following exercise. Redox-sensitive gene transcripts selected for the current investigation include EPAS, HMOX1, SOD2, and NFE2L2. These data collectively demonstrated an exercise-induced increase in all four redox-sensitive gene transcripts at the 4-HR time point, indicating the exercise-stimulus was sufficient to elicit a modest blood oxidative stress response. Previous literature indicates that HMOX1, SOD2, NFE2L2 increase with exercise [28-32]. Importantly, none of the redox sensitive gene transcripts were affected by the environmental recovery conditions. Previous work had examined the effect of both normobaric hypoxia and hypobaric hypoxia on gene transcripts of mitochondrial biogenesis, which is known to be upregulated with exercise [33]. The findings demonstrated no differences in mitochondrial-related gene expression with normobaric hypoxia or hypobaric hypoxia [33, 34]. Similar to the findings of the current study, Peters et al. found no differences among NFE2L2 or SOD2 during the exercise recovery period under three different hypoxic conditions (1667m, 3333m, 5000m) [14]. In contrast, previous research has determined the exercise-induced response of NFE2L2 and SOD2

can be blunted when recovery occurs under hypoxic conditions [12]. When examining HMOX1, our findings are in agreement with those who have shown no effect of hypoxia during post-exercise recovery [12, 14].

Conclusion:

Based upon prior observations that hypoxic recovery blunted the post-exercise oxidative stress response necessary to elicit beneficial adaptations, the current study was undertaken to determine whether this outcome could be attributed to hypobaria, hypoxia, or the combination of both. Under these constraints, where post-exercise hypoxia and hypobaria were examined within a well-controlled laboratory setting, it was determined that there were no observed changes in exercise-induced oxidative stress responses under the variable recovery conditions of normobaric normoxia, normobaric hypoxia, and hypobaric hypoxia. Thus, in this context, these environmental factors were not as impactful as hypothesized. However, we confirmed that the selected exercise challenge imposed a modest oxidative stress response, as indicated by elevations in markers of antioxidant capacity/potential, lipid damage, and redox-sensitive gene transcripts taken from the vastus lateralis. Based on the comparison of these findings with previous work by our group and others [5, 12-14, 34], a more intensive and/or longer duration exercise bout could have elicited a more robust oxidative stress response. It is possible that a response of greater magnitude is necessary in order to identify an environmentally-dependent blunting of the oxidative stress response to exercise. Although, future research is necessary to confirm this relationship the current investigation highlights the fact that the exercise-induced oxidative stress response and the potential redox-sensitive adaptations, are likely impacted by the nature of the exercise stimulus inasmuch as the post-exercise recovery environment.

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400 The study was designed by J.C.Q., B.R., and D.S.; data were collected and analyzed by R.Z., R.S.,
401 D.S., W.H., T.Q., C.W.R., and K.T.; data interpretation and manuscript preparation was
402 undertaken by C.W.R. and J.C.Q. All authors approved the final version of the paper.

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406 **Conflict of Interest:** There were no conflicts of interest in the preparation of this manuscript.

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Table 1. Participant characteristics and exercise performance data

Characteristics	Combined	Females	Males
Participants (<i>n</i>)	13	5	8
Age (years)	24 ± 4	25 ± 5	24 ± 4
Height (cm)	177 ± 12	165 ± 10	184 ± 6
Body mass (kg)	72.2 ± 13.3	61.4 ± 10.0	79.0 ± 10.5
Percent fat (%)	15.8 ± 8.1	22.4 ± 8.5	11.7 ± 4.8
Exercise performance			
Estimated VO ₂ peak (ml· ⁻¹ kg ⁻¹ min)	52.0 ± 6.4	48.9 ± 4.7	54.0 ± 6.8
Watt max (W)	290.5 ± 68.5	219.4 ± 43.8	334.9 ± 33.1

Data are presented as means ± SD

510 **Table 2. Probe and primer sequences for reference genes and genes of interest.**

Gene symbol	Gene accession #	Probe	Primer 1	Primer 2
EPAS-1	NM_001430	/56-FAM/AGA ACT TGT GCA CCA A/3IABkFQ	CTT TGC GAG CAT CCG GTA	AGC CTA TGA ATT CTA CCA TGC G
HMOX1	NM_002133	/56-FAM/TTC AAG CTG /ZEN/GTG ATG GCC TCC C/3IABkFQ/	TCC TTG TTG CGC TCA ATC TC	AGA ATG CTG AGT TCA TGA GGA A
SOD2	NM_000636	/56-FAM/TGG CTT CCA /ZEN/GCA ACT CCC CTT T/3IABkFQ/	CGT CAG CTT CTC CTT AAA CTT G	GAC AAA CCT CAG CCC TAA CG
NFE2L2	NM_006164	/56-FAM/CTA CTC CCA /ZEN/GGT TGC CCA CAT TCC/3IABkFQ/	GCA GTC ATC AAA GTA CAA AGC A	ACA TCC AGT CAG AAA CCA GTG

511 Redox sensitive gene transcripts were quantified by qRT-PCR from vastus lateralis muscle biopsy
 512 samples; Endothelial PAS domain protein-1 (EPAS-1), Hemeoxygenase-1 (HMOX1), Superoxide
 513 Dismutase-2 (SOD2), and Nuclear Factor Erythroid-derived 2-like 2 (NFE2L2).

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Table 3. Exercise trial heart rate and oxygen saturation data

Variable	NH	HH	NN
<i>Heart Rate (bpm)</i>			
<i>PRE</i>	64 ± 11	63 ± 14	68 ± 13
<i>EX</i>	155 ± 10	155 ± 10	157 ± 15
<i>POST</i>	90 ± 15	94 ± 11	95 ± 11
<i>Oxygen Saturation (%)</i>			
<i>PRE</i>	97 ± 1	97 ± 1	97 ± 2
<i>EX</i>	94 ± 2	93 ± 2	94 ± 2
<i>POST</i>	96 ± 2	95 ± 1	95 ± 1
n = 13; Values presented in mean ± SD.			
*Significantly different between trials.			

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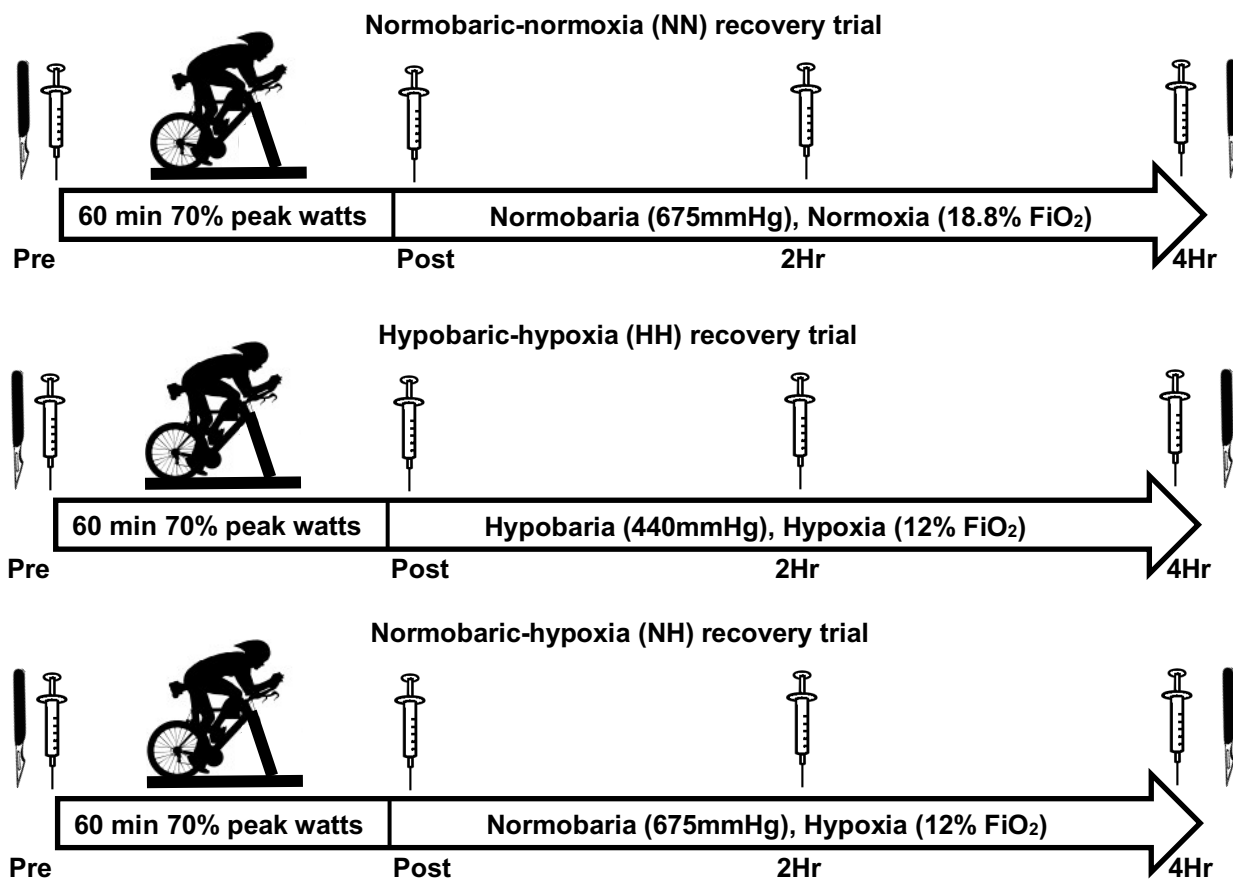
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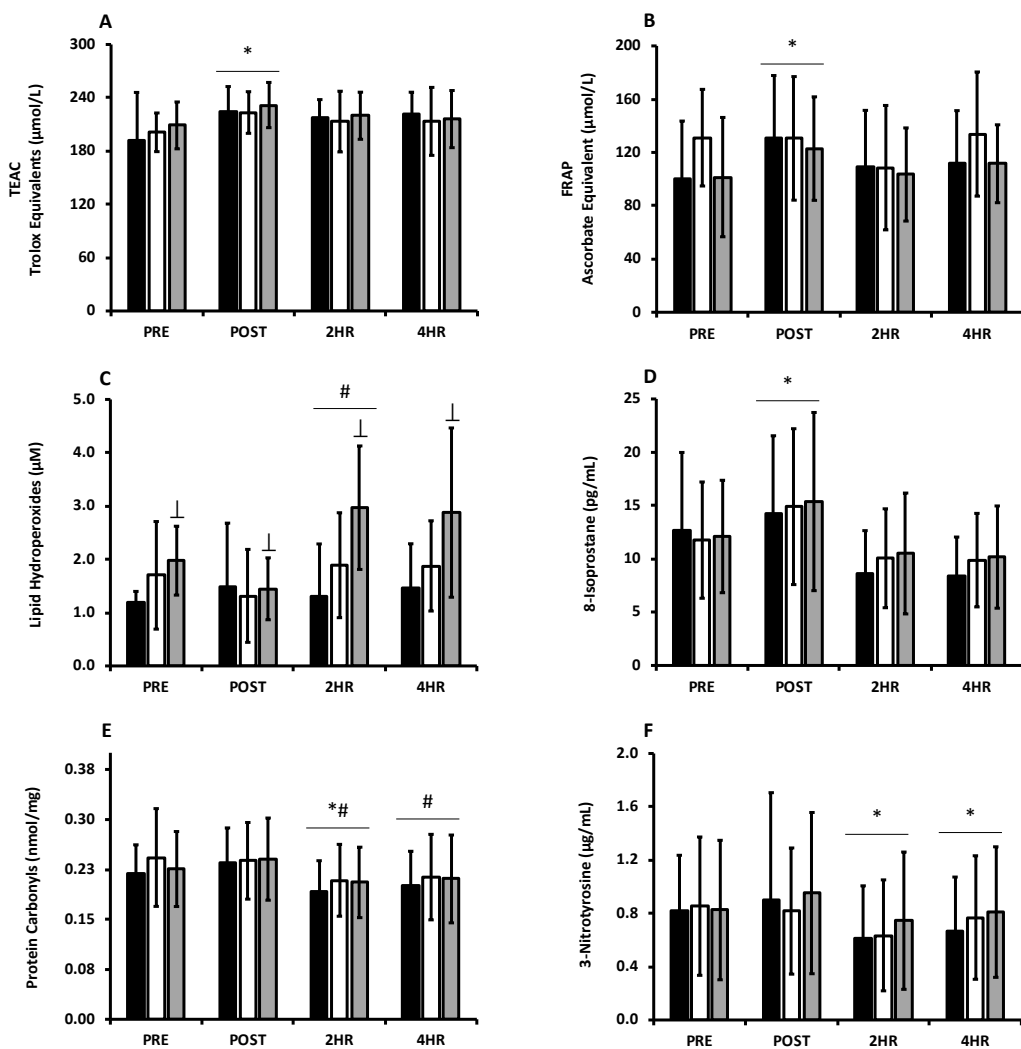
Figure 1. Experimental Design. Following characterization of height, weight, body composition (hydrostatic weighing), participants completed 3 laboratory-based exercise recovery trials in a randomized, counter-balanced fashion. All exercise trials involved an identical 60-minute bout of cycle ergometry at 70% of peak watts. The experimental recovery conditions included three simulated environmental conditions: Normobaric-normoxia (NN), base elevation (975 m) atmospheric conditions, normobaria (675mmHg) and normoxia (18.8% FiO₂, room air inspiratory fraction); Hypobaric-hypoxia (HH), full high elevation simulation (4,420 m) atmospheric conditions, hypobaria (440mmHg) and hypoxia (12% FiO₂); or Normobaric-hypoxia (NH); partial high elevation simulation (4,420 m) atmospheric conditions; normobaria (675mmHg) and hypoxia (12% FiO₂).

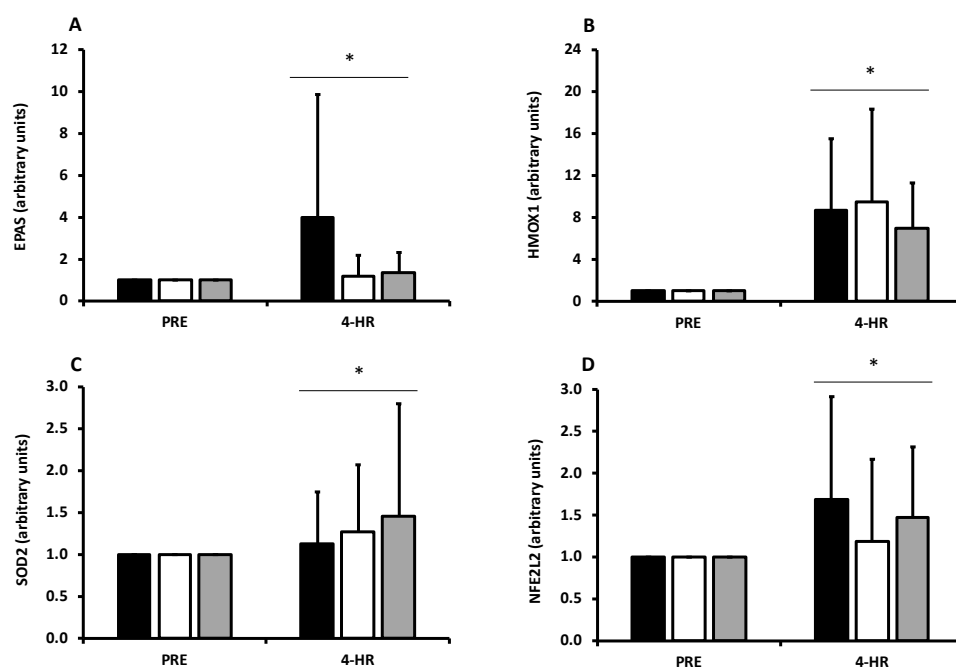
Figure 2. (A) Trolox equivalent antioxidant capacity values are expressed as Trolox equivalent antioxidant capacity equivalents ($\mu\text{mol/L}$) for normobaria normoxia (*black bars*), hypobaria hypoxia (*white bars*), and normobaria hypoxia (*shaded bars*). **(B) Ferric-reducing ability of plasma** values are expressed as ascorbate in equivalents ($\mu\text{mol/L}$) for normobaria normoxia (*solid black bars*), hypobaria hypoxia (*solid white bars*), and normobaria hypoxia (*shaded bars*). **(C) Plasma lipid hydroperoxides** are expressed as lipid hydroperoxide equivalents (μM) for normobaria normoxia (*black bars*), hypobaria hypoxia (*white bars*), and normobaria hypoxia (*shaded bars*). **(D) Plasma 8-Isoprostanes** values are expressed in standard comparison to 8-isoprostanes protein content (pg/ml), for normobaria normoxia (*black bars*), hypobaria hypoxia (*white bars*), and normobaria hypoxia (*shaded bars*). **(E) Plasma Protein Carbonyl** values are expressed in standard comparison to Protein Carbonyl equivalents ($\mu\text{mol/mg prot}$) for normobaria normoxia (*black bars*), hypobaria hypoxia (*white bars*), and normobaria normoxia (*shaded bars*). **(F) Plasma Nitrotyrosine** values are expressed in standard comparison to Nitrotyrosine protein content ($\mu\text{g/ml}$) for normobaria normoxia (*black bars*), hypobaria hypoxia (*white bars*), and normobaria hypoxia (*shaded bars*). Means are expressed $\pm$ SD. *significantly different from PRE; #significantly different from POST; $\perp$ significantly different from normobaria normoxia recovery.

568 **Figure 3.** Data are presented as means $\pm$ SD. **(A)** EPAS **(B)** HMOX1 **(C)** SOD2 **(D)** NFE2L2;
569 *black bars* represent normobaria normoxia, *white bars* represent hypobaria hypoxia, and *shaded*
570 *bars* represent normobaria hypoxia; * significantly different from PRE.

Figure 1.



603 **Figure 2.**

609 **Figure 3.**

Exercise Training in a Hot Environment Fails to Elicit Changes in the Blood Oxidative Stress Response

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RESULTS

Lipid Damage

Biomarkers for oxidative damage as quantified by LOOH and 8-ISO are presented in **Figure 2A, 2B, 2C, and 2D**. For LOOH, no differences were observed between environmental temperatures ($p = 0.204$). Exercise resulted in an elevation of LOOH at 2-HR ($p = 0.001$) and 4-HR ($p < 0.001$). Trial-dependent differences ($p < 0.001$) and a significant trial x time interaction effect were present ($p = 0.018$). Pairwise comparisons revealed that all LOOH values were higher in the second trial in both environmental temperatures ($p < 0.05$), indicating that exercise training resulted in elevated blood plasma levels of LOOH. Peak responses of LOOH were not different between environmental temperatures ($p = 0.128$), but a significant elevation occurred with exercise ($p < 0.001$). Additionally, an effect of trial ($p < 0.001$) and a trial x time interaction ($p = 0.026$) occurred. Further examination of the interaction effect for peak responses of LOOH also revealed that all post-acclimation training values were significantly higher than pre-acclimation training values ($p < 0.05$). 8-ISO was unaffected by temperature ($p = 0.287$), acclimation training ($p = 0.305$), and exercise ($p = 0.691$). Peak responses revealed no differences between environmental temperature for LOOH ($p = 0.128$) or 8-ISO ($p = 0.273$).

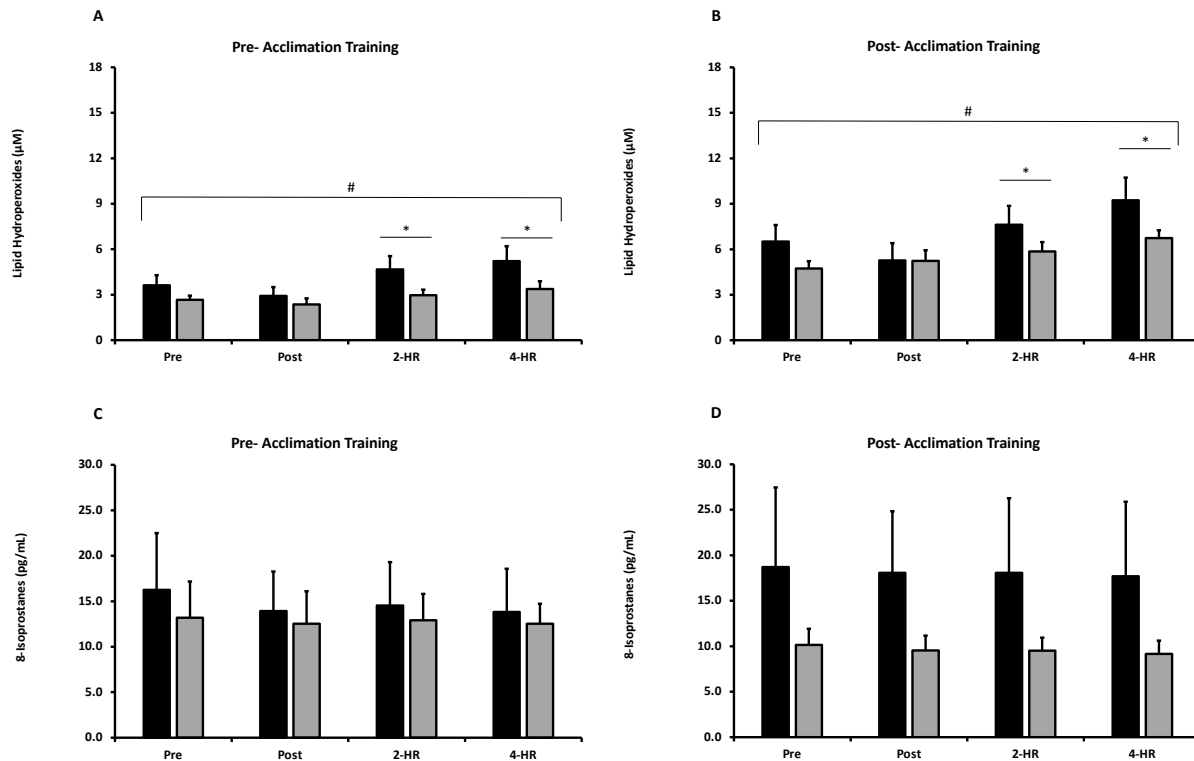


Figure 2. (A and B) Plasma lipid hydroperoxides. Lipid hydroperoxides are expressed as lipid hydroperoxide equivalents (μM) for exercise in a hot environment (black bars) and a neutral environment (shaded bars) in pre-acclimation and post-acclimation training exercise tolerance trials. **(C and D) Plasma 8-Isoprostanes.** 8-Isoprostane values are expressed in standard comparison to 8-Isoprostanes protein content (pg/mL) for exercise in a hot environment (black bars) and neutral environment (shaded bars) in pre-acclimation and post-acclimation training exercise tolerance trials. * Significantly different from pre-exercise values. # Significantly different between acclimation trials. Means are expressed $\pm$ SEM.

Protein Damage

Biomarkers for oxidative damage as measured by 3-NT and PC are presented in Figure **3A and 3B**. 3-NT was unaffected by environmental temperature ($p = 0.253$) and exercise ($p = 0.053$). A significant effect of trial ($p = 0.026$) and a trial x time interaction effect ($p < 0.001$) was observed.

Pairwise comparisons revealed that 3NT was higher at 2-HR following exercise after three weeks of acclimation training in the hot environmental condition ($p = 0.031$). Additionally, 3NT was significantly higher at 2-HR ($p = 0.001$) and 4-HR ($p = 0.009$) after exercise in the neutral environmental condition following a three-week period of acclimation training. Examination of peak responses of 3-NT indicated there were no differences between environmental temperatures ($p = 0.204$). However, an exercise-dependent increase was observed at Peak ($p < 0.001$). Further, an effect of trial ($p = 0.048$) and a trial x time interaction effect ($p = 0.004$) occurred. Pairwise comparisons determined that Peak values were significantly higher in the neutral trial following the three-week training period ($p = 0.007$). The examination of PC revealed an inexplicable effect for temperature in PC values ($p < 0.001$); however, no temperature x time interaction effect occurred ($p = 0.195$). PC was also unaffected by exercise ($p = 0.351$) and acclimation training ($p = 0.393$). When examining peak responses of PC, an inexplicable main effect for temperature occurred ($p = 0.001$), but there was no temperature x time interaction effect ($p = 0.437$). Further, there was no effect of acclimation training ($p = 0.437$). The peak response of PC was significantly elevated with exercise ($p = 0.002$).

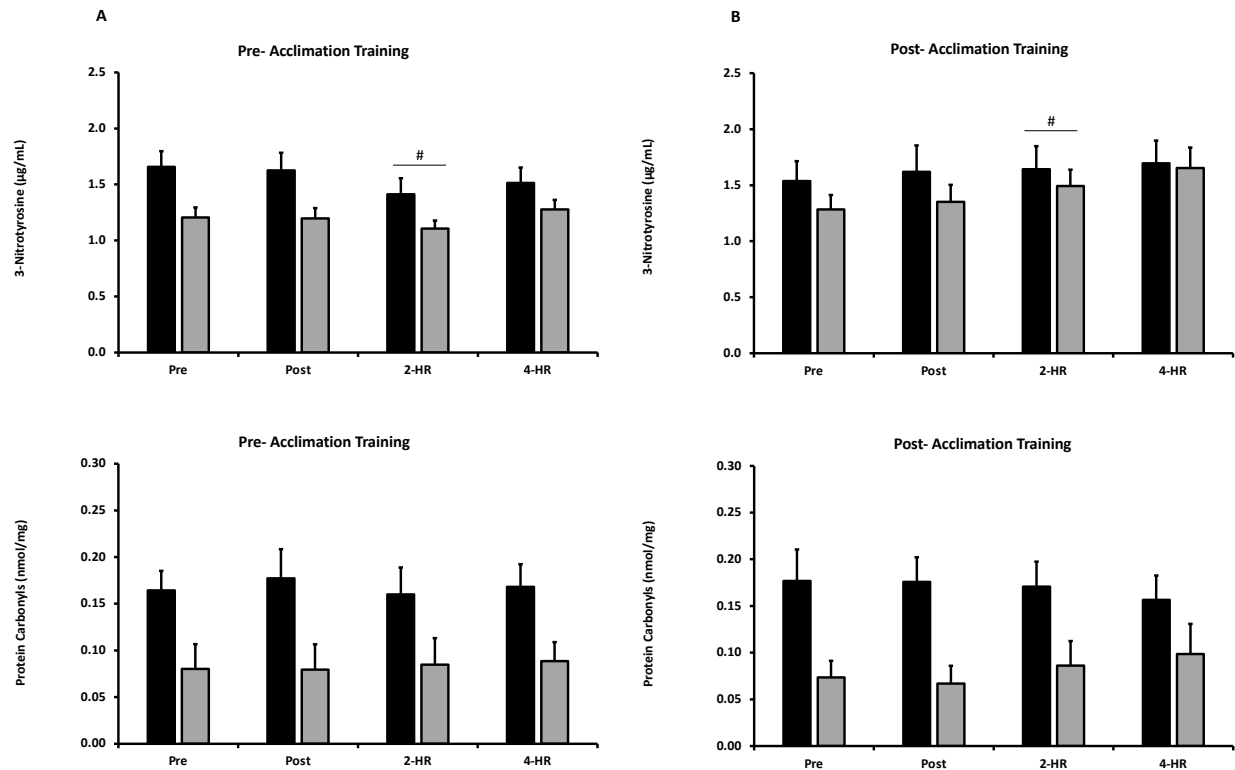


Figure 3. (A and B) Plasma Nitrotyrosine. Nitrotyrosine values are expressed in standard comparison to Nitrotyrosine protein content (ug/mL) for exercise in a hot environment (black bars) and neutral environment (shaded bars) in pre-acclimation and post-acclimation training exercise tolerance trials. **(C and D) Protein Carbonyl** values are expressed in standard comparison to Protein Carbonyl equivalents ($\mu\text{mol}/\text{mg}$ protein) for exercise in a hot environment (black bars) and a neutral environment (shaded bars) in pre-acclimation and post-acclimation training exercise tolerance trials. * Significantly different from pre-exercise values. # Significantly different between acclimation trials. Means are expressed $\pm$ SEM.

Antioxidant Capacity

Mean responses for plasma antioxidant status assessed through the measurement of TEAC and FRAP are presented in **Figure 3A and 3B**. There was no effect of environmental temperature ($p = 0.0542$) or acclimation training ($p = 0.587$) on TEAC; however, an exercise effect was observed

($p < 0.001$). TEAC was significantly elevated at Post ($p = < 0.001$), 2-HR ($p < 0.001$), and 4-HR ($p < 0.001$). Similarly, FRAP was unaffected by environmental temperature ($p = 0.370$) and acclimation training ($p = 0.700$), but increased significantly with exercise ($p < 0.001$). FRAP increased at Post ($p = 0.001$) and remained elevated at 2-HR and 4-HR ($p < 0.001$). Peak responses for TEAC were unaffected by temperature ($p = 0.449$). Additionally, peak responses for FRAP were not different between environmental temperatures ($p = 0.30$). The peak response for both TEAC and FRAP demonstrated a significant elevation in response to exercise ($p < 0.001$).

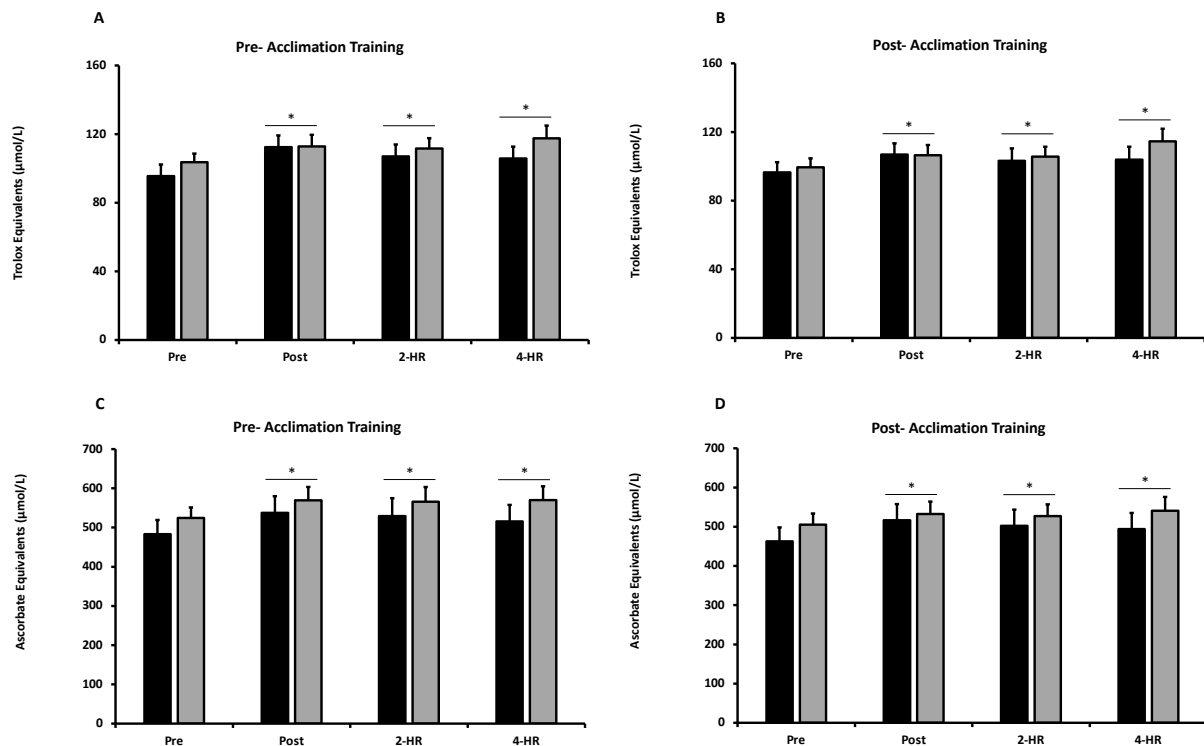


Figure 4. (A and B) Trolox Equivalent Antioxidant Capacity. Trolox Equivalent Antioxidant Capacity values are expressed as Trolox equivalent antioxidant capacity equivalents ($\mu\text{mol/L}$) for exercise in a hot environment (black bars) and neutral environment (shaded bars) in pre-acclimation and post-acclimation training exercise tolerance trials. **(C and D) Ferric-reducing Ability of Plasma.** Ferric-reducing Ability of Plasma values are expressed as ascorbate

equivalents ($\mu\text{mol/L}$) for exercise in a hot environment (black bars) and a neutral environment (shaded bars) in pre-acclimation and post-acclimation training exercise tolerance trials. * Significantly different from pre-exercise values. # Significantly different between acclimation trials. Means are expressed $\pm$ SEM.

Chapter 4: Manuscript

Title Page

Effects of Exercise Training in Hot and Cold Temperatures

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Abstract

Previously we have demonstrated acute effects of endurance exercise in different environmental temperatures. However, acclimation within these temperatures, and between sexes, has not been examined. **Purpose:** to determine the effect of exercise training in different environmental temperatures on performance measures and thermoregulation between sexes. **Methods:** Recreationally trained subjects made 18 visits to the lab and performed a 3-week cycling protocol in an assigned environmental temperature (Males: 20°C, n = 12; 33°C, n = 12; 7°C, n = 12; Females: 20°C, n = 12; 33°C, n = 12). Aerobic capacity, body composition, heart-rate, sweat rate, core and skin temperature were measured before and after the 3-week exercise training protocol. During the 3-week training heart rate, power output, temperature, and sweat rate were measured daily. **Results:** The 33°C subjects had greater skin temperature ($p < 0.001$), sweat rate ($p < 0.001$), and heart rate ($p = 0.034$) than 20°C subjects. From pre- to post-training, core temperature ($p = 0.001$) lowered in 33°C subjects. Skin temperature ($p = 0.011$) and heart rate ($p < 0.001$) decreased with training regardless of temperature. Following training, males had a higher fat free mass ($p < 0.001$), absolute $\text{VO}_{2\text{peak}}$ ($p < 0.001$), and sweat rate ($p = 0.001$) compared to women. Women had higher core temperature ($p = 0.036$) and percent body fat ($p = 0.046$) compared to men. Women improved $\text{VO}_{2\text{peak}}$ following training in a 33°C environment ($p = 0.028$), whereas men improved $\text{VO}_{2\text{peak}}$ following training in 20°C environment ($p < 0.001$). In 7°C compared to 20°C, $\text{VO}_{2\text{peak}}$ ($p < 0.001$), body composition ($p < 0.001$), core temperature ($p < 0.001$), sweat rate ($p = 0.05$), and power ($p < 0.001$) improved in both conditions. Skin temperature was lower in 20°C ($p < 0.001$). **Conclusions:** These data indicate that physiological stress produced from

exercising in hot environments inhibits performance measures in men, but not women.

There was no benefit from training in a 7°C environment compared to a 20°C environment.

Introduction

The components discussed in this thesis are part of a larger study and will be separated by experimental temperatures throughout the following paper. Both experimental temperatures of 33°C and 7°C were individually compared to the control environment of 20°C. The data in this paper will be incorporated into two future manuscripts to combine the cellular and applied physiological response of aerobic training in different environments.

33°C Environment

It is well documented that consistent aerobic training produces several physiological adaptations in the human body including improved cardiovascular function, oxidative capacity, and skeletal muscle metabolism (Bittel, Nonotte-Varly, Livecchi-Gonnot, Savourey, & Hanniquet, 1988). Thus, aerobic exercise has proven to be beneficial in reducing the risk of cardiovascular disease (Van Laethem et al., 2007), diabetes (Sigal et al., 2007), and all-cause mortality (Blair et al., 1995). The heightened stimulus of exercise generates a breakdown and rebuilding cycle in several physiological systems that requires adaptations to meet and maintain new energy and workload demands. With greater metabolic demand comes greater ATP energy production, and the byproduct of these chemical reactions is heat. The increase in core temperature signals a thermoregulatory response to regulate body temperature by dissipating heat internally from the core and carrying it out through the peripheries.

However, thermoregulation during exercise can be blunted or exacerbated by the environment an individual exercises in. Exercise in hot environments is known to have

detrimental physiological effects on the human body primarily due to the uncompensable heat stress produced. Heat stress occurs as the body's internal temperature rises in response to the increased demand of exercise but cannot be dissipated due to a limited temperature gradient between the body and the environment. This most often occurs as the body's core temperature nears 40°C and can cause minimal adaptations to aerobic training due to a blunted response from fatigue (González-Alonso et al., 1999; van Ooijen, van Marken Lichtenbelt, van Steenhoven, & Westerterp, 2004). Previous research on exercise in hot environments has primarily focused on acute bouts of exercise and the effect of temperature on men. Fewer studies have observed if adaptations to environmental temperatures occur over an aerobic training regimen or if there are differences between men and women in thermoregulation. Previous research has indicated thermoregulatory differences exist between sexes during acute exposures to exercise in different environments, but further research is needed to determine if these differences occur throughout training and acclimation (D. Gagnon & Kenny, 2012).

Previous research from our lab has indicated that environmental temperature does have a physiological effect on the body, but exercise is a necessary stimulus to generate an observable response (Heesch, Shute, Kreiling, & Slivka, 2016; Shute, Heesch, Zak, Kreiling, & Slivka, 2018; R. B. Zak et al., 2017). Responses to exercise in hot environments have mainly been observed at a molecular level with decreases appearing in markers of mitochondrial biogenesis (Heesch et al., 2016). While exercise in hot environments impacts skeletal muscle acutely, the down-stream effects and pathways of temperature on applied whole body aerobic training remain unclear. Therefore, the purpose of this study was to determine 1) if the body responds and adapts differently to aerobic training in hot

environments compared to room temperature environments and 2) if thermoregulatory sex differences exist. The results of this research could be used to further optimize adaptations occurring with aerobic training regimens and prevent several deleterious physiological effects produced from training in hot environments. Thermoregulatory differences between men and women could indicate the need to further individualize training programs or temperature acclimation protocols.

7°C Environment

Environmental temperatures are known to have a physiological effect on human performance during exercise (Slivka, Dumke, Tucker, Cuddy, & Ruby, 2012). Exercise in hot environments produces deleterious effects attributed to uncompensable heat stress. Cold environments generate a greater temperature gradient between the body and the environment allowing for greater heat dissipation. This potentially allows for greater workloads to be achieved during exercise leading to more favorable adaptations. However, the body's thermoregulatory response in the cold is different between resting and exercise conditions. At rest, the body works to conserve heat by distributing less heat to the extremities through vasoconstriction of subcutaneous vessels, which leads to decreased skin temperature and regulation of core temperature. The body also employs shivering thermogenesis to increase metabolic cost and generate heat. Over long periods of exposure, these thermoregulatory responses increase the risk of cold injury to the peripheries due to a reduction of heat through vasoconstriction (van Ooijen et al., 2004). During exercise however, the body generates more heat through increased metabolic flux and blood vessels dilate in response to the working muscle's increased metabolic need. Thus, typical resting

thermoregulatory responses to the cold are overridden during exercise and may allow for a unique response to cold during exercise.

Previous research addressing exercise in cold environments has primarily focused on the cardiac response, metabolic alterations, fluid shifts, skin temperature, or the effect fitness levels has on thermoregulation. Conversely, our lab has researched the effect of cold environments on molecular function and its potential to decrease mitochondrial dysfunction (Slivka et al., 2012). We have shown markers of mitochondrial biogenesis to increase with acute bouts of aerobic exercise in cold environments indicating a molecular response to environmental temperatures (Shute et al., 2018). This has led to the question of whether these molecular adaptations transfer to whole body physiological effects during exercise over prolonged periods of exposure to cold environments. Thus, the purpose of this study was to determine if the body responds and adapts differently to aerobic training in cold environments compared to room temperature environments. This study could further our understanding of the physiological alterations made during exercise in cold environments and potentially lead to protocols that further optimize aerobic adaptations and performance.

Methods

33°C Environment

Forty-eight recreationally trained subjects (24 males, 24 females) ages 19 to 45 years old participated in this study. Subjects agreed to all procedures and risks associated with testing, outlined in the informed consent approved by the University of Nebraska Omaha's Institutional Review Board (IRB). This study was in collaboration with the University of Montana, where half of the subjects performed experimental trials. Males and females were randomly separated into two temperature groups: hot (33°C) or room temperature (20°C) with humidity set at 40% for all trials. All trials were performed in an environmental chamber controlled for humidity and temperature (Darwin Chambers Company, St. Louis, MO) and no subjects were acclimated to experimental conditions prior to trials. Each subject made 18 visits to the lab for two descriptive trials (visit 1 and 18), two absolute workload trials (visit 2 and 17), and 14 daily-training trials (visits 3 through 16).

Descriptive Trials (Trials 1 and 18)

During these trials, the subject's height, weight, body composition, and $\text{VO}_{2\text{max}}$ were measured. Body composition was measured via hydrostatic weighing (Exertech, Dresbach, MN) to assess body density. This was corrected for the subject's residual lung volume (Thomas & Etheridge, 1980) and converted into a percentage of body fat using the Siri equation (Siri, 1961). $\text{VO}_{2\text{max}}$ testing was performed on an electronically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA). A calibrated metabolic cart (ParvoMedics TrueOne Metabolic System, Sandy, Utah) measured the subject's inspired

and expired gases to assess their $\text{VO}_{2\text{peak}}$. The test began at 60 watts for women and 95 watts for men and increased by 35 watts every 3 minutes until subjects reached volitional exhaustion. Blood pressure was taken before, during the second stage, and after the $\text{VO}_{2\text{peak}}$ test. Heart rate was monitored throughout the test via a heart rate monitor (Polar Electronic, Lake Success, NY) and a 4 lead ECG (Nihon Kohden Corporation, Tomioka, Japan). The peak watts (W_{peak}) achieved in this test were used to determine the training intensity for cycling in the experimental trial.

Absolute Workload Trials (Trials 2 and 17)

Upon arrival, subject's nude body weight was taken after voiding their bladder and bowels, and their standard water bottle was weighed to account for fluid consumption during the ride. Subject's rode at 50% of the peak watts associated with their $\text{VO}_{2\text{peak}}$ for one hour in the environmental chamber controlled for their assigned temperature. During the ride, core temperature was monitored via rectal thermometer (Extech Industries, Nashua, NH), skin sensors were placed on the chest and back to monitor skin temperature (Extech Industries), and heart rate was monitored via a heart rate monitor (Polar Electronic). Subjects could drink water ad libitum throughout the ride. After the ride, subject's nude body weight was taken before bowels and bladder were voided and their water bottle weight was also taken.

Daily-Training Trials (Trials 3-16)

Subjects arrived at the lab each weekday for three weeks at a pre-arranged time between 6am and 5pm. Nude body weight, tympanic temperature (Covidien Genius 2, Mansfield, MA), resting heart rate (Polar Electronic), and pre-exercise water bottle weight

were taken before entering the chamber. Subjects performed all cycling in the environmental chamber at the temperature they were previously assigned to. Subjects rode on a cycle ergometer (Phantom 3, Cycleops, Madison, WI) for one hour at an intensity they perceived as a 15, or hard, on the Rating of Perceived Exertion scale (Borg, 1985). The workload could be adjusted with an iPad app synced to the bike (VirtualTraining s.r.o, Czech Republic) anytime throughout the training to maintain the desired intensity. Every 15 minutes the subject's average heart rate (bpm), power output (W), and work (kJ) was recorded and subjects were encouraged to daily increase their work output. Following the ride, subjects nude body weight, post-exercise water bottle weight, and tympanic ear temperature were taken.

Statistical Analysis

Differences in dependent variables were measured with a three-way mix plot ANOVA (training status x temperature x sex) with training status being the measure repeated and temperature and sex the between-subject factors. If significance was detected, a Fisher's protected LSD post hoc was used to determine where difference occurred. A probability of type I error less than 5% was considered significant ($p < 0.05$). Effect size was reported as η^2 for main effects and Cohen's d for pairwise comparisons. Effect sizes were considered to be a small effect if $= 0.20$, a moderate effect if $= 0.50$, or a large effect if $= 0.80$. All statistics were analyzed with Statistical Package for Social Sciences software (SPSS 24.0).

7°C Environment

Twenty-four recreationally trained male subjects ages 19 to 45 years old participated in this study. Subjects agreed to all procedures and risks associated with testing, outlined in the informed consent approved by the University of Nebraska Omaha's Institutional Review Board (IRB). This study was in collaboration with the University of Montana, where half of the subjects performed experimental trials. Subjects were randomly separated into two temperature groups: room temperature (20°C) or cold (7°C) with humidity set to 40% for all trials. All trials were performed in an environmental chamber controlled for humidity and temperature (Darwin Chambers Company, St. Louis, MO) and no subjects were acclimated to experimental conditions prior to trials. Each subject made 18 visits to the lab for two descriptive trials (visit 1 and 18), two absolute workload trials (visit 2 and 17), and 14 daily-training trials (visits 3 through 16).

Descriptive Trials (Trials 1 and 18)

During these trials, the subject's height, weight, body composition, and $\text{VO}_{2\text{peak}}$ were measured. Body composition was measured via hydrostatic weighing (Exertech, Dresbach, MN) to assess body density. This was corrected for the subject's residual lung volume (Thomas & Etheridge, 1980) and converted into a percentage of body fat using the Siri equation (Siri, 1961). $\text{VO}_{2\text{peak}}$ testing was performed on an electronically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA). A calibrated metabolic cart (ParvoMedics TrueOne Metabolic System, Sandy, Utah) measured the subject's inspired and expired gases to assess their $\text{VO}_{2\text{peak}}$. The test began at 95 watts and increased by 35 watts every 3 minutes until subjects reached volitional exhaustion. Blood pressure was taken before, during the second stage, and after the $\text{VO}_{2\text{peak}}$ test. Heart rate was monitored throughout the test via a heart rate monitor (Polar Electronic, Lake Success, NY) and a 4

lead ECG (Nihon Kohden Corporation, Tomioka, Japan). The peak watts (W_{peak}) achieved in this test were used to determine the training intensity for cycling in the experimental trial.

Absolute Workload Trials (Trials 2 and 17)

Upon arrival, subject's nude body weight was taken after voiding their bladder and bowels, and their standard water bottle was weighed to account for fluid consumption during the ride. Subject's rode at 50% of the peak watts associated with their $VO_{2\text{peak}}$ for one hour in the environmental chamber controlled for their assigned temperature. During the ride, core temperature was monitored via rectal thermometer (Extech Industries, Nashua, NH), skin sensors were placed on the chest and back to monitor skin temperature (Extech Industries), and heart rate was monitored via a heart rate monitor (Polar Electronic). Subjects could drink water ad libitum throughout the ride. After the ride, post-exercise water bottle weight and the subject's nude body weight were taken before bowels and bladder were voided.

Daily-Training Trials (Trials 3-16)

Subjects arrived at the lab each weekday for three weeks at a pre-arranged time between 6am and 5pm. Nude body weight, tympanic temperature (Covidien Genius 2, Mansfield, MA), pre-exercise water bottle weight, and resting heart rate (Polar Electronic) were taken before entering the chamber. Subjects performed all cycling in the environmental chamber at the temperature they were previously assigned to. Subjects rode on a cycle ergometer (Phantom 3, Cycleops, Madison, WI) for one hour at an intensity they perceived as a 15, or hard, on the Rating of Perceived Exertion scale (Borg, 1985). The

workload could be adjusted with an iPad app synced to the bike (VirtualTraining s.r.o, Czech Republic) anytime throughout the training to maintain the desired intensity. Every 15 minutes the subject's average heart rate (bpm), power output (W), and work (kJ) was recorded and they were encouraged to daily increase their work output. Following the ride, subjects nude body weight, post-exercise water bottle weight, and tympanic ear temperature were taken.

Statistical Analysis

Differences in dependent variables were measured with a two-way mix plot ANOVA (training status x temperature) with training status being the measure repeated and temperature the between-subject factor. If significance was detected, a Fisher's protected LSD post hoc was used to determine where difference occurred. A probability of type I error less than 5% was considered significant ($p < 0.05$). Effect size was reported as η^2 for main effects and Cohen's d for pairwise comparisons. Effect sizes were considered to be a small effect if $= 0.20$, a moderate effect if $= 0.50$, or a large effect if $= 0.80$. All statistics were analyzed with Statistical Package for Social Sciences software Version 25 (Chicago, IL).

Results

33°C Environment

Females had a lower body mass than males ($p = 0.002$, $\eta^2 = 0.114$) regardless of training status or temperature. No other differences were noted between independent variables ($p > 0.05$). No differences between males and female's percent body fat occurred in pre-training ($p = 0.096$, $d = 0.52$), however, in post-training female's percent body fat was higher than males ($p = 0.046$, $d = 0.63$). In addition, male's body fat percentage decreased from pre- to post-training ($p < 0.001$, $d = 0.14$) but females did not ($p = 0.431$, $d = 0.04$). No other differences were noted between independent variables ($p > 0.05$). Male's fat mass decreased from pre- to post-training ($p < 0.001$, $d = 0.07$), however, female's fat mass did not change pre to post-training testing ($p = 0.375$, $d = 0.03$). No other differences were noted between independent variables ($p > 0.05$). Females had a lower fat-free mass than males at both pre-training ($p < 0.001$, $d = 1.94$) and post-training ($p < 0.001$, $d = 2.12$). From pre- to post-training, males increased fat-free mass ($p = 0.001$, $d = 0.10$), whereas females did not change ($p = 0.960$, $d = 0.02$). No other differences were noted between independent variables ($p > 0.05$). These data are presented in Table 1.

Males improved absolute $\text{VO}_{2\text{peak}}$ from pre to post-training in the 20°C environment group ($p < 0.001$, $d = 0.64$) but only trended towards improvements in the 33°C environment group ($p = 0.065$, $d = 0.29$). Females did not improve absolute $\text{VO}_{2\text{peak}}$ from pre- to post-training in the 20°C environment group ($p = 0.730$, $d = 0.12$) but did improve in the 33°C environment group ($p = 0.028$, $d = 0.55$). Men had a higher absolute $\text{VO}_{2\text{peak}}$ compared to women at both pre-training ($p < 0.001$, $d = 1.49$) and post-training ($p < 0.001$,

$d = 1.51$), regardless of temperature. No other differences were noted between independent variables ($p > 0.05$). Males improved relative $\text{VO}_{2\text{peak}}$ from pre-training to post-training in the 20°C environment group ($p < 0.001$, $d = 0.56$) but did not improve in the 33°C environment group ($p = 0.077$, $d = 0.31$). Females did not improve relative $\text{VO}_{2\text{peak}}$ from pre- to post-training in the 20°C environment group ($p = 0.516$, $d = 0.12$) but did improve in the 33°C environment group ($p = 0.009$, $d = 0.40$). No other differences were noted between independent variables ($p > 0.05$). These data are presented in Table 1.

Core temperature during exercise at an absolute workload decreased in the 33°C environmental group from pre-training to post-training ($p < 0.001$, $d < 0.001$). However, core temperature remained the same in the 20°C environment ($p = 0.423$, $d = 0.77$). Female's core temperature was higher than males regardless of training status or temperature ($p = 0.036$, $\eta^2 = 0.096$). No other differences were noted between independent variables ($p > 0.05$). Sweat rate at an absolute workload was greater in 33°C compared to 20°C environment ($p < 0.001$, $\eta^2 = 0.911$) regardless of training status. No difference existed between males and females in pre-training ($p = 0.287$, $d = 0.73$), however females sweat rate was lower than males during post-training ($p = 0.001$, $d = 0.40$). Males sweat rate also increased from pre to post-training ($p = 0.004$, $d = 0.13$), however, there was no change in females sweat rate from pre to post-training ($p = 0.548$, $d = 1.02$). No other differences were noted between independent variables ($p > 0.05$). The 33°C environment groups had higher skin temperature than the 20°C environment groups at an absolute workload ($p < 0.001$, $\eta^2 = 0.329$) regardless of training status and sex. Pre-training skin temperature was higher than post-training ($p = 0.011$, $\eta^2 = 0.139$) regardless of temperature and sex. No other differences were noted between independent variables ($p > 0.05$). The

0.016), heart rate ($p = 0.352$, $\eta^2 = 0.039$), or sweat rate ($p = 0.717$, $\eta^2 = 0.006$). These data are presented in Table 4.

Table 3. Pre-training and post-training physiological variables in 20°C, 7°C, and combined 20°C and 7°C environments.

	Pre			Post		
	20°C	7°C	Combined	20°C	7°C	Combined
Body Mass (kg)	85.3 ± 7.5	87.0 ± 6.0	86.1 ± 4.7	85.9 ± 7.7	86.5 ± 5.8	86.2 ± 4.7
Body Fat (%)	21.9 ± 3.2	21.7 ± 2.1	21.7 ± 1.8	20.4 ± 3.2	20.7 ± 2.1	20.5 ± 1.8*
Fat mass (kg)	20.7 ± 4.9	19.9 ± 2.9	20.2 ± 2.7	19.6 ± 4.8	19.0 ± 2.9	19.2 ± 2.7*
Fat free mass (kg)	64.5 ± 3.7	67.1 ± 3.4	65.8 ± 2.4	66.2 ± 3.8*	67.6 ± 3.2	66.9 ± 2.4
VO_{2peak} (L/min)	3.21 ± 0.2	3.47 ± 0.2	3.34 ± 0.1	3.65 ± 0.2	3.80 ± 0.1	3.72 ± 0.11*
VO_{2peak} (ml·kg⁻¹·min⁻¹)	39.4 ± 2.8	40.8 ± 1.8	40.1 ± 1.6	44.2 ± 2.5	45.5 ± 2.3	44.8 ± 1.6*
Core Temperature (°C)	37.4 ± 0.3	38.0 ± 0.1	37.7 ± 0.2	37.5 ± 0.2	37.7 ± 0.1	37.6 ± 0.1
Skin Temperature (°C)	33.6 ± 0.4	30.1 ± 0.8†	31.8 ± 0.5	32.9 ± 0.4	31.2 ± 0.5*†	32.0 ± 0.3
Heart rate (bpm)	141.4 ± 4.6	147.4 ± 4.3	144.4 ± 3.1	127.1 ± 3.2	129.1 ± 2.7	128.1 ± 2.0*
Sweat rate (mL/hr)	505 ± 83	456 ± 55	481 ± 49	687 ± 90	537 ± 88	612 ± 63*

Data are mean ± SE. *, $p < 0.05$ from pre-training. †, $p < 0.05$ from 20°C.

Table 4. Pre-training and post-training at a RPE of 15 in 20°C, 33°C, and combined 20°C and 33°C environments.

	Pre			Post		
	20°C	7°C	Combined	20°C	7°C	Combined
Tympanic Temperature (°C)	37.9 ± 0.2	37.4 ± 0.3	37.6 ± 0.2	37.9 ± 0.2	37.6 ± 0.3	37.7 ± 0.1
Average Heart Rate (bpm)	149.5 ± 3.7	156.8 ± 3.0	153.1 ± 2.5	147.8 ± 3.4	158.7 ± 4.0	153.2 ± 2.6
Power (W)	119.3 ± 6.8	130.8 ± 7.3	125.0 ± 5.0	151.8 ± 8.5	167.2 ± 5.7	159.5 ± 5.2*
Sweat rate (mL/hr)	520 ± 59	467 ± 61	493 ± 41	568 ± 101	545 ± 83	556 ± 64

Data are mean ± SE. *, $p < 0.05$ from pre-training. †, $p < 0.05$ from 20°C.

Discussion

33°C Environment

The purpose of this study was to determine the response to short-term, three-week aerobic training in a 33°C compared to a 20°C environment and if thermoregulatory differences existed between men and women. Overall, we found that training in a 33°C environment was not conducive for acquiring fitness benefits during the aerobic endurance protocol. The differences between environments was clearly observed in $\text{VO}_{2\text{peak}}$, as aerobic capacity improved during training in the 20°C environment by 10.7% compared to only an increase of 3.4% in the 33°C environment. A variety of factors affect increases in $\text{VO}_{2\text{peak}}$ including genetics, fitness level, training type, intensity, and thermoregulatory demands (Hickson, Dvorak, Gorostiaga, Kurowski, & Foster, 1988; Holloszy & Coyle, 1984). Adaptations in aerobic capacity typically occur in 8 - 10 weeks with increases in $\text{VO}_{2\text{peak}}$ ranging from 5 to 15% depending on the individual's fitness status (Daussin et al., 2008; Helgerud et al., 2007; Overend, Paterson, & Cunningham, 1992). In this study, robust improvements in $\text{VO}_{2\text{peak}}$ occurred over a relatively short period of time, perhaps attributed to the recruitment of relatively unfit subjects. This population was chosen to demonstrate a progressive overloading during the 3-week period (Holloszy & Coyle, 1984). During the daily training sessions, subjects were able to intuitively increase their workload each day to generate a greater stimulus as training adaptations began to increase their fitness level. This style of training was chosen due to being more indicative of a real-world setting, compared to previous methods of utilizing critical power or pre-determined incremental increases to determine the subject's daily workload. Improvements in fitness levels are confirmed by the fact that all subjects improved their $\text{VO}_{2\text{peak}}$ from pre- to post-

testing and that average power increased despite their heart rate remaining fairly stable throughout daily training.

Increases in aerobic capacity in the 33°C environment were limited, potentially due to strain placed on the body, as evidenced by differences in heart rate, core and skin temperature, and sweat rate. However, some literature would suggest that aerobic capacity is improved in a room temperature environment following acclimation a hot environment in trained individuals (Lorenzo, Halliwill, Sawka, & Minson, 2010). In the present study, subjects displayed classical responses indicative of heat stress during the 3-week environmental training period. Specifically, core temperature was higher in the 33°C compared to the 20°C environment throughout training, which is known to cause fatigue during exercise due to uncompensable heat stress (González-Alonso et al., 1999; Peiffer & Abbiss, 2011). Performance duration begins to decrease once the body reaches the upper limits of its tightly regulated homeostatic temperature range until exercise is either stopped or acclimation to the environment occurs (Nielsen et al., 1993). Evidence of temperature acclimation appears in core temperature as it decreased from pre- to post-training in the 33°C environment group but not in the 20°C environment. Elevated core temperature combined with significantly higher heart rate in the 33°C environment group in both pre- and post-training would indicate effects of cardiac drift, causing heart rate to be elevated despite a constant workload (Hamilton et al., 1991). While a training response appears to have occurred through decreases in heart rate from pre- to post-training in both environments, heart rate in the 33°C environment remained higher than the 20°C environment.

Heart rate increases in hot environments due to increased sweat rate and improper fluid replacement causing decreases in blood plasma volume and consequently stroke volume. Heart rate must then compensate to maintain cardiac output (Hamilton et al., 1991). Further exacerbating the overall strain is the inability to efficiently dissipate heat following prolonged exercise in a hot environment due to increases in skin temperature (Rowell et al., 1969). Increased skin temperature and decreased sweat rate diminish the ability for the body to dissipate heat by decreasing the temperature gradient between the body and the environment (Poirier et al., 2015). This agrees with the current study in which sweat rate and skin temperature were elevated in the 33°C environment to a greater extent than the 20°C. However, skin temperature decreased from pre- to post-training testing and sweat rate increased throughout training demonstrating classical heat acclimation had occurred. These results are consistent with other findings that temperature acclimation generally occurs within 14 days of exposure (Poirier et al., 2015). Many of the improvements from pre- to post-training in the 33°C environment can be attributed to temperature acclimation, whereas changes in the 20°C environment may be considered adaptations to training rather than temperature acclimation (Cheung & McLellan, 1998). In the current study, adaptations to training in the 20°C environment resulted in reduced skin temperature, reduced heart rate, and increases in sweat rate.

A secondary measure of this study was to determine if sex differences existed during exercise in different environmental temperatures. Most differences between men and women, including body composition and sweat rate, were consistent with previous research. Men had a greater body mass and their body composition was altered to a greater extent than females during the three-week training protocol. Decreases in body fat percent

and fat mass that were observed in men from pre- to post-training testing did not occur in women. Additionally, no increases in fat free mass were observed in women as there were in men. It has been suggested these differences stem from sex hormones and their regulation of metabolic hormones and oxidative pathways (Chow & Phoon, 2003; Geer & Shen, 2009; Tarnopolsky, 2008). The nature of women's reproductive organs and hormone regulation necessitate greater adipose tissue to maintain homeostatic reproductive function. Sex steroid hormones are known to regulate leptin, a hormone produced from adipose tissue which controls hunger and energy expenditure, differently between men and women. Women produce leptin to a greater extent than men and this difference in regulation may be a potential explanation for differences in body composition in the present study (Geer & Shen, 2009).

Men demonstrated a greater capacity for improvements in sweating as they increased sweat-rate from pre- to post-training, whereas sweat rate in women did not change. This may be due to larger gland size and peripheral sensitivity to stimulation in men compared to women (D. Gagnon & Kenny, 2012). With training, adaptation in sweat rate generates a quicker and greater response in men at a lower exercise intensity compared to women (Ichinose-Kuwahara et al., 2010). Further, in temperatures where maximum heat dissipation is necessary, men continue increasing sweat rate and evaporative capacity, whereas women plateau (D. Gagnon & Kenny, 2012). The higher core temperature and lower sweat rate in women in the current study could imply that women do not mitigate heat as rapidly and thus, have a lower sensitivity to heat. However, women's core temperature remained below 40°C indicating they remained within safe limits of core temperature and were able to maintain appropriate thermoregulation within our protocol.

These differences between men and women in temperature acclimation and thermoregulation could be the cause of discrepancies within $\text{VO}_{2\text{peak}}$ improvements. Men improved $\text{VO}_{2\text{peak}}$ following training in the 20°C environment, whereas women improved following training in the 33°C environment. It is unclear which mechanism is responsible for these differences between men and women in $\text{VO}_{2\text{peak}}$ and further research is needed to determine if this finding is due to differences in temperature acclimation, thermoregulation, or subject variation.

Conclusion

The results of this study further delineate that training in a 33°C environment is less optimal than a 20°C environment for improving aerobic capacity due to the effects of heat stress. Novel findings from this study include the pronounced difference of temperature on aerobic adaptations and differences in thermoregulatory response in men and women with training in warmer environments. Previous research from our lab has found a 33°C environment to be unfavorable compared to 20°C during acute bouts of aerobic exercise at a cellular level of skeletal muscle due to mitochondrial damage (Slivka et al., 2012). From the current study we can further infer that these cellular impairments have functional implications during aerobic training in a hot environment due to the suboptimal whole-body physiological effects, denoted by decreased performance.

7°C Environment

This study found that there was no additional benefit by performing a short term, 3-week aerobic training regimen in a 7°C environment compared to a 20°C environment. Adaptations to training did occur as evidenced by increases in $\text{VO}_{2\text{peak}}$ as well as decreases

in percent body fat and fat mass. At an absolute workload, adaptations occurred through decreases in heart rate and increases in sweat rate from pre- to post training. However, all of the aforementioned improvements were independent of temperature and represent typical adaptation to exercise training. Relatively few differences existed due to the effect of temperature. Fat free mass increased in the 20°C environment but not in the 7°C environment. Previous research exploring the effect of temperature at the molecular level may translate to this whole-body physiological response. Local cooling application revealed blunted myogenic mRNA expression following exercise. However, the same impaired transcriptional response was not observed when local heating was applied, indicating warmer temperatures potentially preserve or promote muscle growth (R. Zak et al., 2018). Further research would be needed to determine if a similar mechanism in the current study existed for the increases in fat free mass in the 20°C environment. Differences also existed between temperatures in skin temperature, which was lower in the 7°C environment compared to the 20°C environment. Afferent receptors in the skin signal a thermoregulatory response when peripheral blood vessels vasoconstrict in response to cold exposure (Morrison, 2007; van Ooijen et al., 2004). Classical acclimation to cold environments in skin temperature most often occurs as peripheral blood flow is increased in response to less vasoconstriction of the blood vessels (Adams & Heberling, 1958). This may have been the mechanism behind increases in skin temperature from pre- to post-training in the current study as these increases are consistent with the timeline of classical temperature adaptations which usually occur in a 14-day period (Poirier et al., 2015).

Unlike skin temperature, core temperature was not different between temperatures during exercise in the present study, which is consistent with previous literature (Shute et

al., 2018). At rest in cold environments, the body must generate heat via shivering or non-shivering thermogenesis to regulate homeostatic core temperature, but during exercise metabolic demands elicit greater energy and heat production which raises core temperature. Increases in core temperature during exercise in a cold environment are not sufficient to induce heat strain thus, heart rate is typically lower during exercise in cold environments compared to room temperature environments (Febbraio et al., 1994; D. D. Gagnon et al., 2013; Slivka et al., 2012). This decreased thermoregulatory strain on the body generally allows for a higher power output (Peiffer & Abbiss, 2011). In the current study, heart rate and power were higher in the 7°C environment compared to the 20°C environment, but not significantly. Traditionally, differences in power output have occurred during an acute exposure to a cold environment rather than the prolonged exposure subjects in our study experienced during training. The nature of our study design recruited subjects with limited cycling experience to exercise for 1 hour at a self-selected intensity of 15 on the RPE scale five days a week, for three weeks. Differences between previous research and the current study include temperature exposure time, exercise intensity, and subject training status (Adams & Heberling, 1958; Bittel et al., 1988; Galloway & Maughan, 1997; Peiffer & Abbiss, 2011). Discrepancies in study design could be responsible for the lack of statistical significance in the present study compared to previous literature.

Conclusion

Previous research from our lab has shown the potential benefit of exercising in a cold environment at a cellular level as it increases a PGC-1 α mRNA response (Slivka et al., 2012; Slivka et al., 2013). However, the limited whole-body physiological differences

between a 7°C and 20°C environment in the current study, may indicate a disconnect between an acute response at the cellular level and a whole-body training response.

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Effects of 3-week training and heat acclimation in females: evidence for sex specific environmental responsiveness. Dustin Slivka, Robert Shute, Walter Hailes, Brent Ruby. In preparation ~ Feb 2021.

Methodology:

The following represents the basic methodology for the data collection surrounding study 2 (males) and 3 (females) of the study series. The methodology is identical for both of these studies and is therefore combined below.

Study 2 and 3: Effects of environmental temperature on exercise response and adaptation in males and females.

We have previously determined that the skeletal muscle response to exercise varies depending on the ambient conditions (hot, neutral, cold) and the temperature of the muscle. For example PGC1 α was increased to a greater degree in the cold compared to the neutral environment. In contrast, exercise in a hot environment diminished the PGC1 α response. These data suggest that the adaptive potential of the skeletal muscle may be advanced and/or decreased depending on the training environment. To more effectively address this question, active males were exposed to a short-term training period under varied environmental conditions.

Participants. Participants include 36 untrained males and 24 untrained females. Testing occurred at two locations (Montana and Nebraska) using identical equipment and protocols in order to accumulate the number of subjects needed in a time efficient manner. All participants provided written informed consent approved by the University Institutional Review Board and USAMRMCC Office of Research Protections prior to commencement of testing.

Experimental Design. Subjects were matched across three training conditions 1) control (~20°C, 30% RH), 2) heat (~33°C, 30% RH), or 3) cold (~7°C, 30% RH) environments. Daily training was prescribed for a period of 14 days.

Protocol. Following a controlled diet and exercise plan the day before and after overnight fast participants reported to the laboratory in the early morning hours. A muscle biopsy was obtained from the vastus lateralis (randomized, counter-balanced leg) before starting exercise (Pre) – (days 1 and 15). Participants then exercised (cycle ergometer) for 60 minutes at 60% of maximal aerobic capacity in one of the above-described environments. At the conclusion of the exercise trial, participants recovered (seated rest, normal laboratory conditions) for four hours before a final muscle biopsy was taken (days 1 and 15). Daily training (60 minutes of self-selected intensity) proceeded with days off for Saturday and Sunday for a total of 14 training sessions. Data were analyzed using a mixed design ANOVA with repeated measures between the training groups and across time, two-way 2 x 3 ANOVA (time x trial). A probability of type I error of less than 5 % will be considered significant ($p < 0.05$).

Participants

36 untrained males and 24 untrained females were recruited from the university and local communities to take part in the study. Participants were required to pass a pre-screening Physical Activity Readiness-Questionnaire. Participants signed an informed consent form that was approved by the university Institutional Review Board and the Army HRPO office.

Preliminary Testing

Hydrodensitometry

Body composition was assessed via an underwater weighing tank (Exertech, Dresbach, MN) utilizing estimated residual volume based on height and weight. Participants were required to fast for ≥ 3 hours prior to testing. Dry weight was determined using a digital scale (Befour Inc., Cedarburg, WI) and height was measured. Participants were weighed while completely submerged. Body density and percent body fat were estimated using the Siri equation.¹⁷

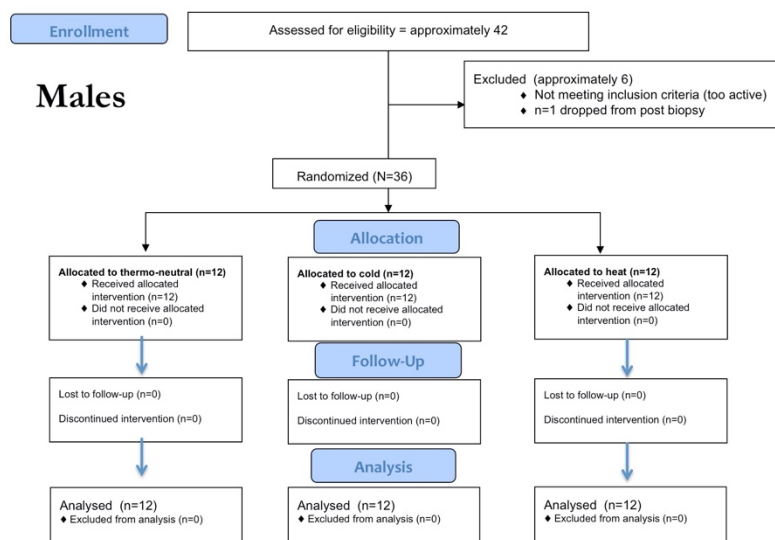
Participants arrived at the lab fasted for ≥ 3 hours prior to VO_2 peak testing. A cycling graded exercise test, was performed on a treadmill ergometer (TMX225C, Fullvision, Inc., Newton, KS)¹⁸ while participants' expired gas was analyzed every 15 seconds by a metabolic cart (Parvomedics, Inc., Sandy, UT). Heart rate was monitored and recorded using a heart rate watch and chest strap (Polar Electro, Kempele, FL).

Following the completion of the initial baseline testing, subjects were stratified into one of three training environments (males) and one of two training environments (females). Training occurred over three semesters with hot and control training occurring during colder winter months and cold training occurring during late spring and early fall. Testing on days 1 and 15 proceeded at the assigned environmental conditions 1) control (n=12 F, n=12 M, ~20°C, 30% RH), 2) heat (n=12 F, n=12 M, ~33°C, 30% RH), or 3) cold (n=12 M, ~7°C, 30% RH) environments. Upon arrival to the laboratory an initial muscle biopsy (vastus lateralis) was obtained along with a pre-exercise blood sample. Study participants were then equipped with temperature sensors (skin, chest location; core, rectal) and a chest strap heart rate monitor. Pre and post exercise measures of nude body weight were also obtained.

-biopsy	-biopsy		-biopsy
-blood	-blood	-blood	-blood

Exercise	Recovery (laboratory conditions)			
60 min (50% VO ₂ peak) Environ. Specific	60 min	60min	60min	60min

Study participant allocation:



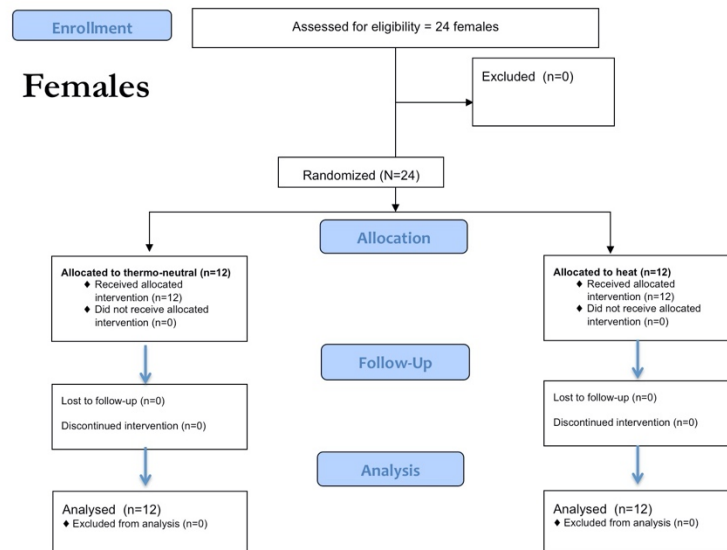


Figure 2. Study participant enrollment procedures.

Exercise proceeded in the prescribed environment using a Tesco environmental chamber at each testing location for a period of 60 minutes (60% of peak VO₂). Immediately following the exercise session, study participants exited the environmental chamber and a post-exercise muscle biopsy and blood sample were obtained. Following sample collection, the temperature sensors were removed and a post-exercise measure of nude body weight was obtained. Exercise recovery proceeded under normal laboratory conditions for a period of four hours with a subsequent blood sample obtained 1-hour post exercise. At four hours post-exercise, a final blood and muscle sample were obtained.

All initial testing (pre-training) occurred on a Monday with the following 14-week days serving as training days. Training was scheduled for 60 minutes each session in the same environment as the prescribed testing conditions (T-F for week 1, M-F for weeks 2 and 3). Post-training testing was completed on the following Monday and included the identical procedures indicated above at the same absolute workload.

No less than two days after completing the post-training experimental trail, study participants returned to the laboratory for subsequent measures of body composition and peak VO₂ using the identical procedures described above.

Results:

The results at the time of this report are not fully complete at this time as muscle and blood sample analyses are ongoing. The descriptive data from the study participants are reported in Table 1.

IMPACT OF COLD				
	N=	Age (y)	Height (cm)	Weight (kg)
20°C men	12	23 ± 1	177 ± 2	85.3 ± 7.5
7°C men	12	26 ± 2	181 ± 2	87.0 ± 6.0
IMPACT OF HEAT				
	N=	Age (y)	Height (cm)	Weight (kg)
20°C men	12	23 ± 1	177 ± 2	85.3 ± 7.5
20°C women	12	25 ± 1	170 ± 2	69.2 ± 2.9
33°C men	12	25 ± 1	179 ± 2	86.5 ± 5.4
33°C women	12	23 ± 1	165 ± 2	65.6 ± 3.4

Table 1. Descriptive data from study participants (N=60).

The daily responses for the males to training under controlled and cold conditions are shown in Figure 3. These represent the changes in self-selected power output, heart rate, body temperature and sweat rate responses during the 15 session training periods. Figure 4 shows the changes in heart rate, core temperature, skin temperature and sweat rate after the 15 environmental training (control and cold). There were minimal differences across the trials and a significant main effect for increases in calculated sweat rate.

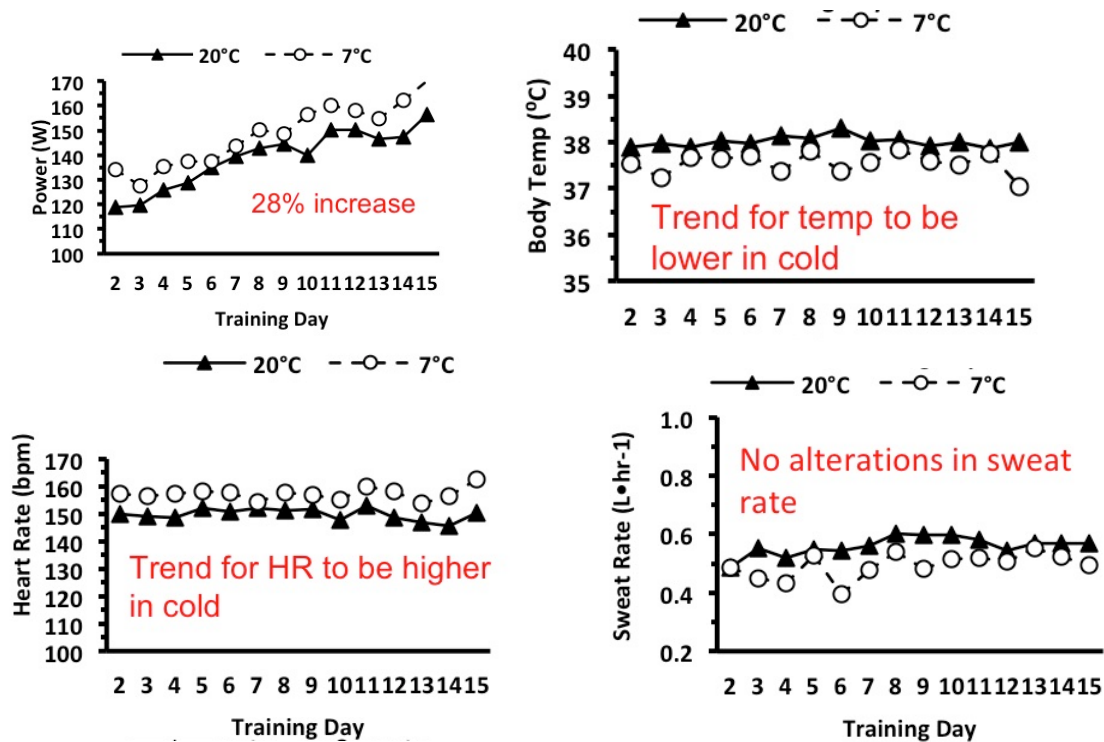


Figure 3. Changes in self-selected power output, heart rate, body temperature and sweat rate responses during the 15 session training periods (control and cold).

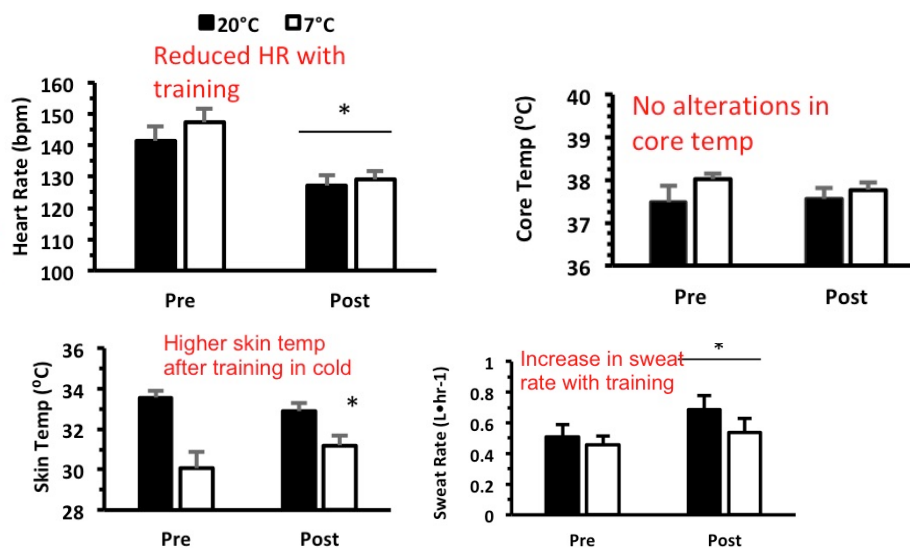


Figure 4. Changes in heart rate, core temperature, skin temperature and sweat rate during the 15 session training periods (control and cold).

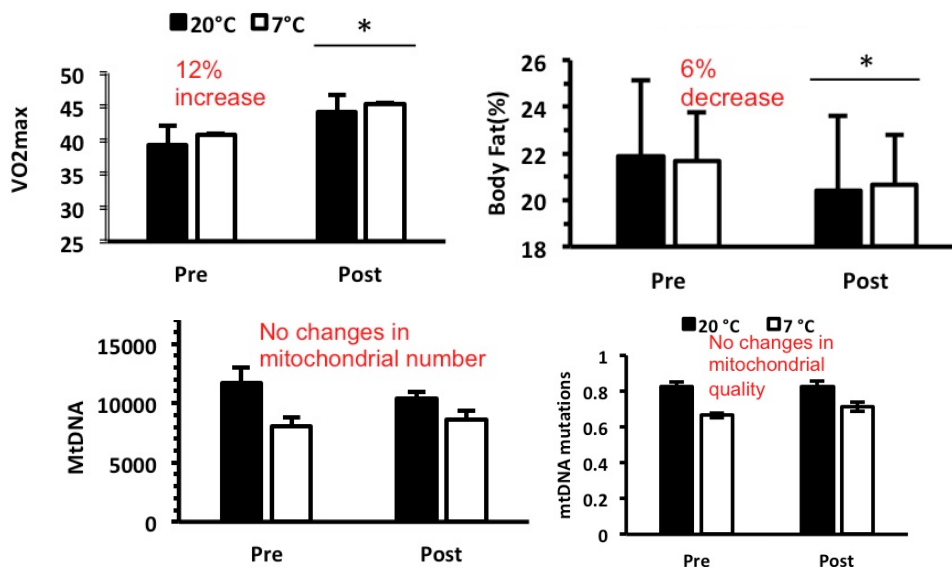


Figure 5. Changes in peak VO₂, body fat and mitochondrial markers in response to completing the 15 days of environmental training (control and cold).

The 15-day training periods resulted in a significant main effect increase in peak VO₂ and a significant decrease in body fat (%). However, there were no differences between the treatment groups. Figure 6 shows the changes in the daily responses for the males to training under controlled and hot conditions. These represent the changes in self-selected power output, heart rate, body temperature and sweat rate responses during the 15 session training periods.

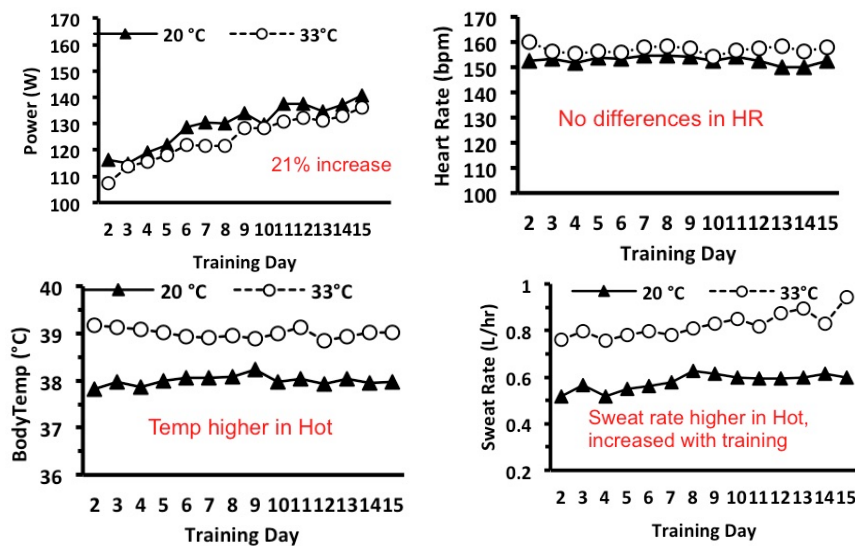


Figure 6. Changes in self-selected power output, heart rate, body temperature and sweat rate responses during the 15 session training periods (control and hot).

There was an increase in self-selected training intensity but no differences in the heart rate responses to daily training. Body temperature and sweat rate were consistently higher during training in the heat.

Figure 7 shows the changes in response to the same absolute workload pre and post 15 days of training in the control and hot environments. Heart rate was significantly higher in the heat and significantly lower after training. Skin temperature was reduced after training in the control conditions. In contrast, core temperature was reduced in response to training in the heat. Training in the heat resulted in higher sweat rates.

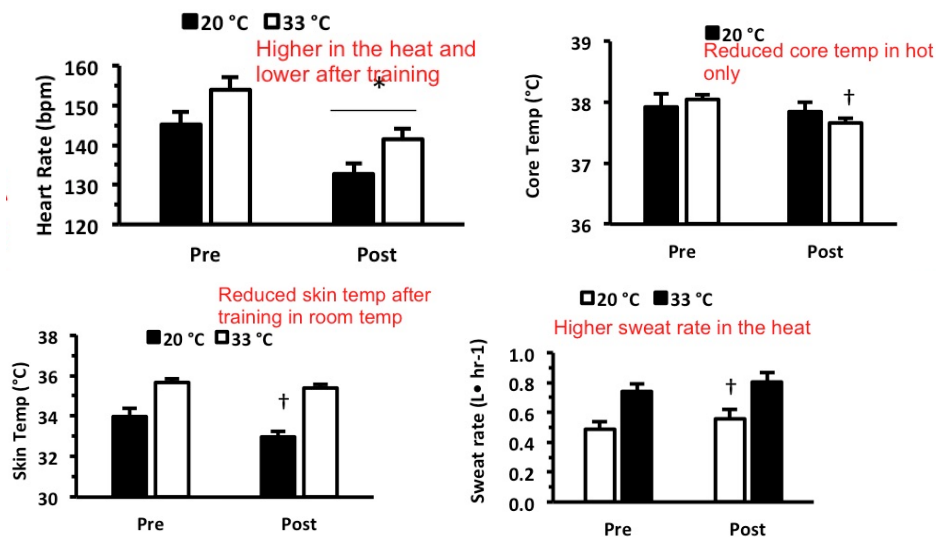


Figure 7. Changes in heart rate, skin and core temperature and sweat rate responses at the same absolute work rate pre and post 15 days of environmental training (control and hot).

Figure 8 shows the overall training outcomes in response to the 15 day environmental training period. The data demonstrate an overall blunting of the change in peak VO₂ during training in the heat. However, the decrease in body fat (%) showed a significant main effect but no differences across trials.

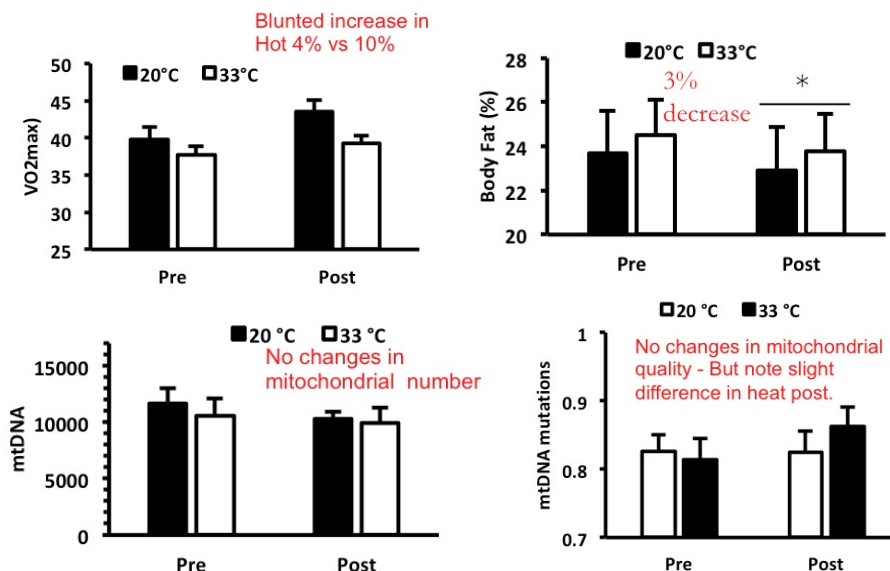


Figure 8. Changes in peak VO₂, body fat (%) and mitochondrial responses pre and post 15 days of environmental training (control and hot).

Comparisons across sex were done for training responses in the control and heated training environments (N=24 M, n=12 hot, n=12 room; N=24 F, n=12 hot, n=12 room). Figure 9 shows the responses in peak VO₂ response after the 15 day training periods. Although, there were no differences between males and females, the increase in peak VO₂ appears blunted in the heat. For the measure of body fat (%), females demonstrated a smaller reduction compared to males.

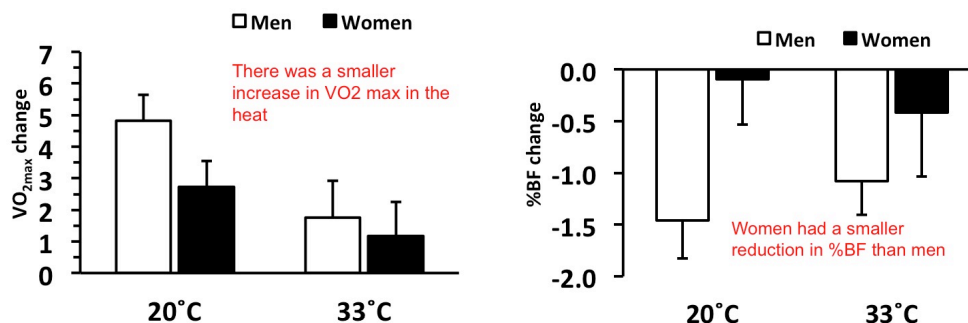


Figure 9. Sex specific changes in peak VO₂ and body fat in response to 15 days of training under hot and room conditions.

Figure 10 shows the sex specific response to 15 days of environment specific training for measures of heat rate and skin temperature. There were no differences in the heart rate response to training. Both males and females demonstrated a significant decrease in heart rate at the same absolute training intensity post training regardless of the training environment.

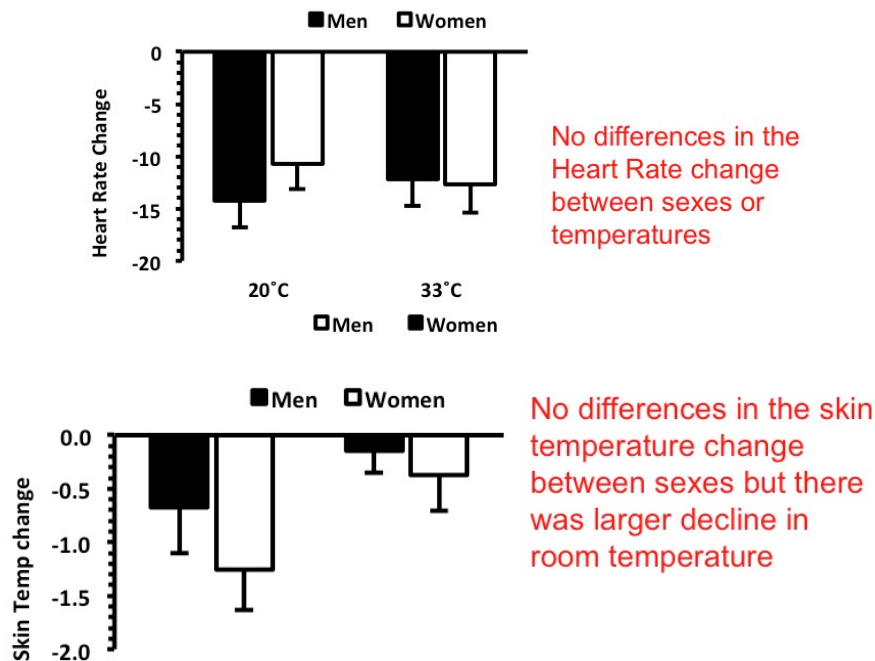


Figure 10. Changes in the heart rate and skin temperature responses to 15 days of environmental training in males and females.

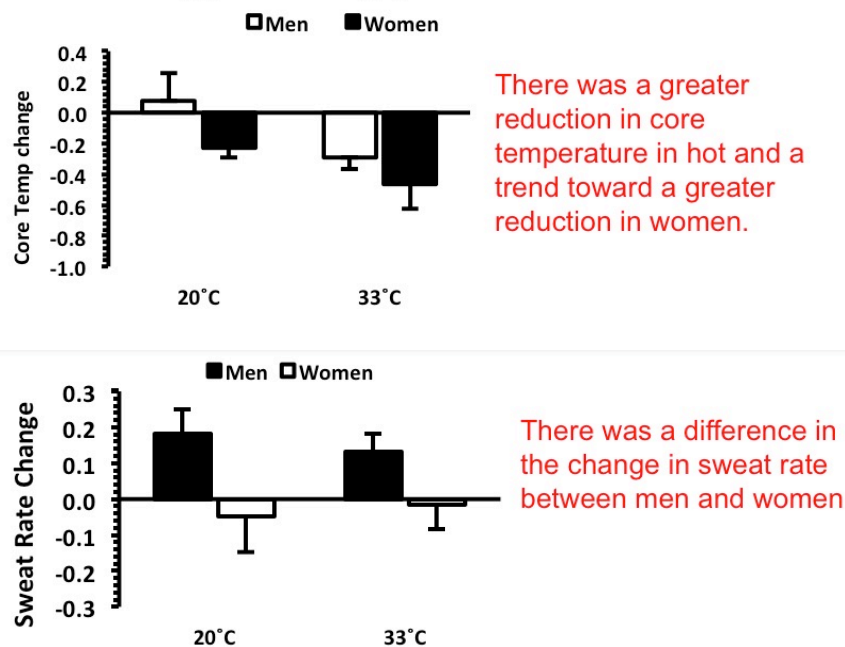


Figure 11. Changes in the core temperature and sweat rate responses to 15 days of environmental training in males and females.

The most apparent differences between the sexes across the two training environments were the core temperature and sweat rate responses. Females tended to demonstrate a larger overall reduction in the

average core temperature response to exercise (post training) compared to males. In contrast, while males demonstrated a significant changes in sweat rate in both environments, females did not show a change in sweat rate (Figure 11).

Alterations in tissue and brain oxygenation during rest and exercise at normobaric and hypobaric hypoxia. Dustin Slivka, Robert Shute, Walter Hailes, Brent Ruby. In preparation ~ March 2021.

Alterations in total body water and cardiovascular responsiveness during rest and exercise at normobaric and hypobaric hypoxia. Dustin Slivka, Robert Shute, Walter Hailes, Brent Ruby. In preparation ~ April 2021.

Background:

When ascending to altitude, physiological changes occur throughout the human body. Specifically, exposure to altitude has shown increased heart rate, lowered peripheral oxygenation (SpO₂), and reduced maximal oxygen consumption (VO₂max) (DeLorey, Shaw, Shoemaker, Kowalchuk, & Paterson, 2004; Grover, Weil, & Reeves, 1986; Zupet, Princi, & Finderle, 2009). Much of the well-controlled research investigating the physiological effects of altitude have been conducted in chambers that simulate altitude. This is most commonly accomplished by reducing the fraction of oxygen in the chamber and is known as normobaric hypoxia. During actual altitude exposure, or hypobaric hypoxia, the fraction of oxygen is unchanged, but the barometric pressure is reduced. This reduction in barometric pressure makes it more difficult for oxygen to bind to hemoglobin and be transported to target tissues. In theory, both environments lead to the same partial pressure of oxygen and, therefore, should elicit the same responses, but recent research has questioned this assumption.

Acute exposure to HH at rest results in lower blood oxygen saturation (SpO₂) and higher heart rate compared to NH (Savoirey, Launay, Besnard, Guinet, & Travers, 2003; Self, G Mandella, Prinzo, M Forster, & Shaffstall, 2011). However, these results have not always been consistent (Richard et al., 2014; Roach, Loeppky, & Icenogle, 1996; Savoirey et al., 2007). Others have also documented a further decrease in physical performance (Beidleman, Fulco, Staab, Andrew, & Muza, 2014), lower ventilatory response (Faiss et al., 2013), and higher oxidative stress (Ribon et al., 2016) in HH versus NH. The observed effects of the method used to achieve hypoxia have not been consistent in the exercise model and, therefore, more research is needed to determine if differences only exist at rest or if they are also present at different exercise intensities.

The majority of research regarding differences in hypoxic environments has examined cardiovascular and respiratory responses, but little research has examined the skeletal muscle response. Unpublished observations from our lab have found limited differences in muscle transcriptional response between NH and HH despite differences in SpO₂ and heart rate. It is currently unknown if the barometric pressure induced alterations in HR and SpO₂ extend to the skeletal muscle. The apparent increase in heart rate to account for the decrease in SpO₂ may lead to adequate oxygen delivery to the muscle and therefore not create a hypoxic environment at the level of the muscle. Using NIRS technology, researchers have determined that muscle oxygenation does decrease with incremental exercise (Austin et al., 2005; Ferreira, Koga, & Barstow, 2007; Murias, Spencer, Keir, & Paterson, 2013). However, there is a lack of information on muscle oxygenation during hypoxia exposure, and the effects of the type of hypoxia exposure. Based on existing data regarding peripheral oxygenation and cardioventilatory responses, HH may be a more severe environmental condition and lead to different physiological responses than NH. The mechanisms behind these changes are still unclear. Therefore, the purpose of this study is to determine if the method to achieve hypoxia results in differences in muscle oxygen saturation during rest and exercise. The addition of this data will have an impact on the physiological understanding of the effects of different forms of hypoxia, and may have implications to the interpretation of previous research based on the experimental model and methodology used.

Furthermore, we have conducted a similar preliminary study and have in fact determined that HH and NH do not lead to the same response. We have uncovered a potentially important aspect of physiology where muscle tissue oxgenation may be enhanced at altitude due to acute cardiovascular overcompensation in a

HH environment but not NH environment. In this preliminary study we conducted NN and NH in our laboratory, but then traveled to Leadville Colorado for the HH trials. The issue is that we can not be certain that the observed differences were do to the trial or the difference in time of altitude exposure as it takes 8+ hours to drive to Leadville and required an overnight stay. The current study will take advantage of the topography of the Kona Hawaii area where we can get from seal level to 13,800 ft of elevation in less than two hours.

Participants:

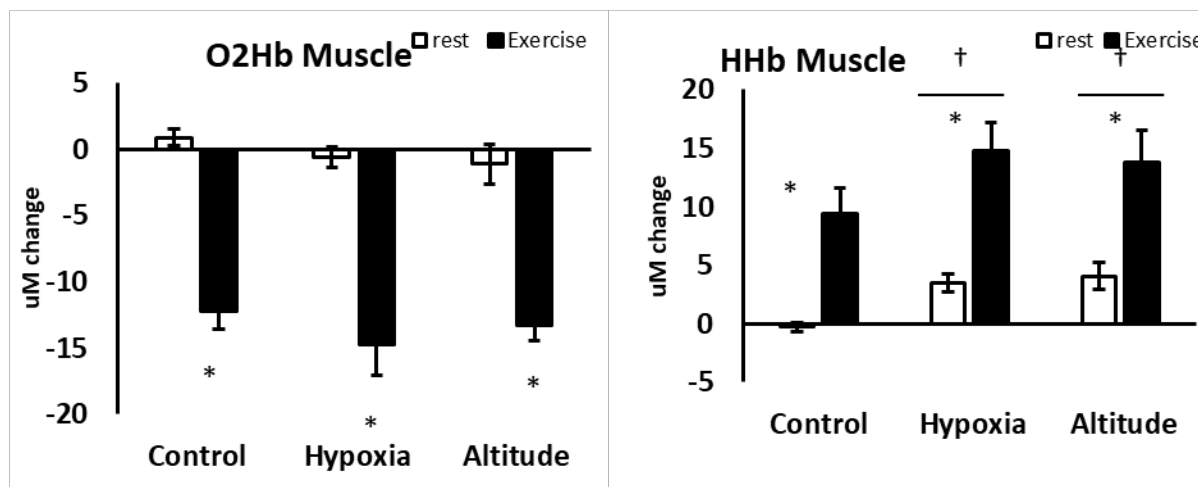
Eighteen participants (8males and 8 females) completed the procedures associated with this study. All participants were informed of the protocol and possible risks before giving written informed consent. All procedures were approved by the appropriate university and Department of Defense Institutional Review Boards.

Each participant completed three trials in a randomized and counter-balanced order. The trials consisted of a sea level control environment (C), a sea level hypoxia trial (H), and a high altitude trial. The C trial was conducted close to sea level. The H trial was conducted at the same elevation as the C trial, but participants breathed hypoxic air through a facemask (Higher Peak, Mag 20, Boston, MA). The H trial was conducted at 13,850 ft of elevation. Subjects were transported from sea level to 13,850 ft by automobile which took approximately 90 minutes. During the H trial the hypoxic air machine was adjusted every 10 minutes to simulate the ascent to altitude that occurred in the A trial. Thus, the intensity and duration of hypoxic exposure was similar between H and C. Once participants arrived at steady state environmental conditions they rested supine for 10 minutes before data was collected. Once resting data was collected subjects stood up and then performed 3 minutes of stepping exercise on a box 39 cm tall in time to a metronome set to 88 beats/min (22 steps/min). Exercise data was recorded and averaged over the last 2 minutes.

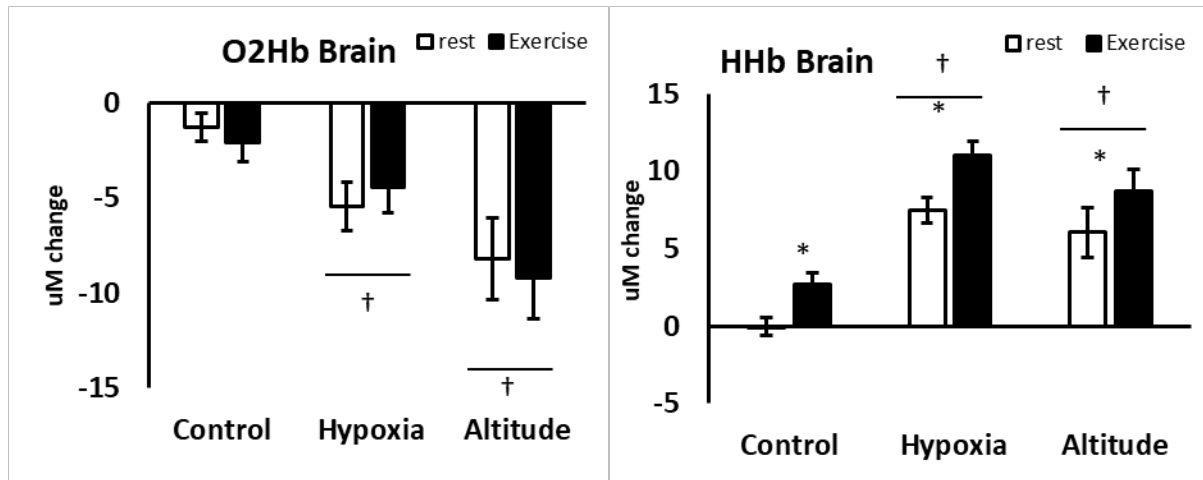
Results/Conclusions:

TISSUE OXYGENATION:

Muscle Tissue oxygenation is affected by exercise, but only minimal effect of hypoxia or altitude. This appears to be a tissue specific response, since Brain tissue oxygenation is affected by altitude by only minimally by exercise

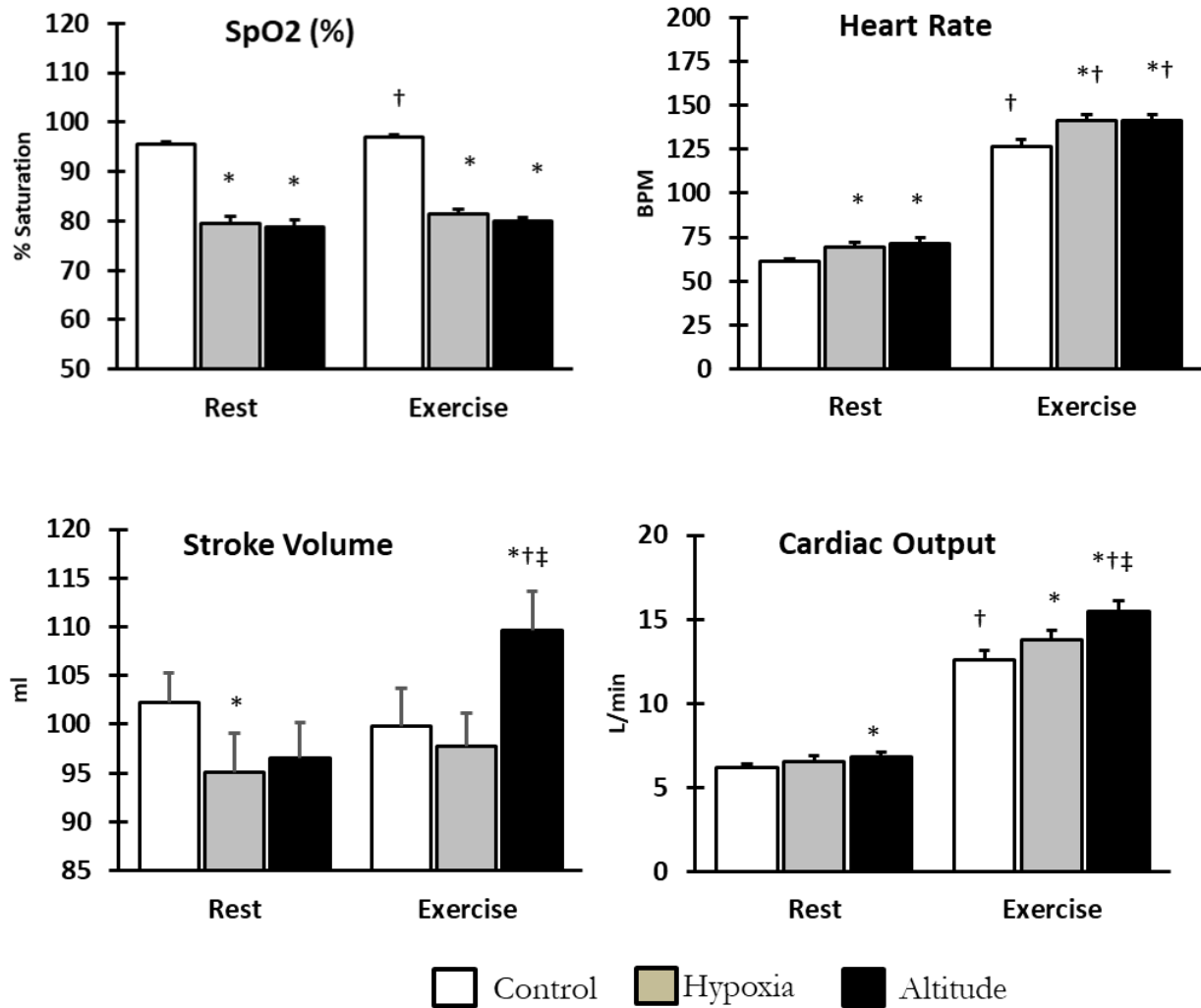


Muscle and Brain oxygenation at rest and during exercise at sea level (control) simulated altitude (Hypoxia), and actual terrestrial. * $p < 0.05$ vs. rest (main effect of exercise) † $p < 0.05$ vs. control (main effect of hypoxia). All data are means $\pm$ SD.



CARDIOVASCULAR:

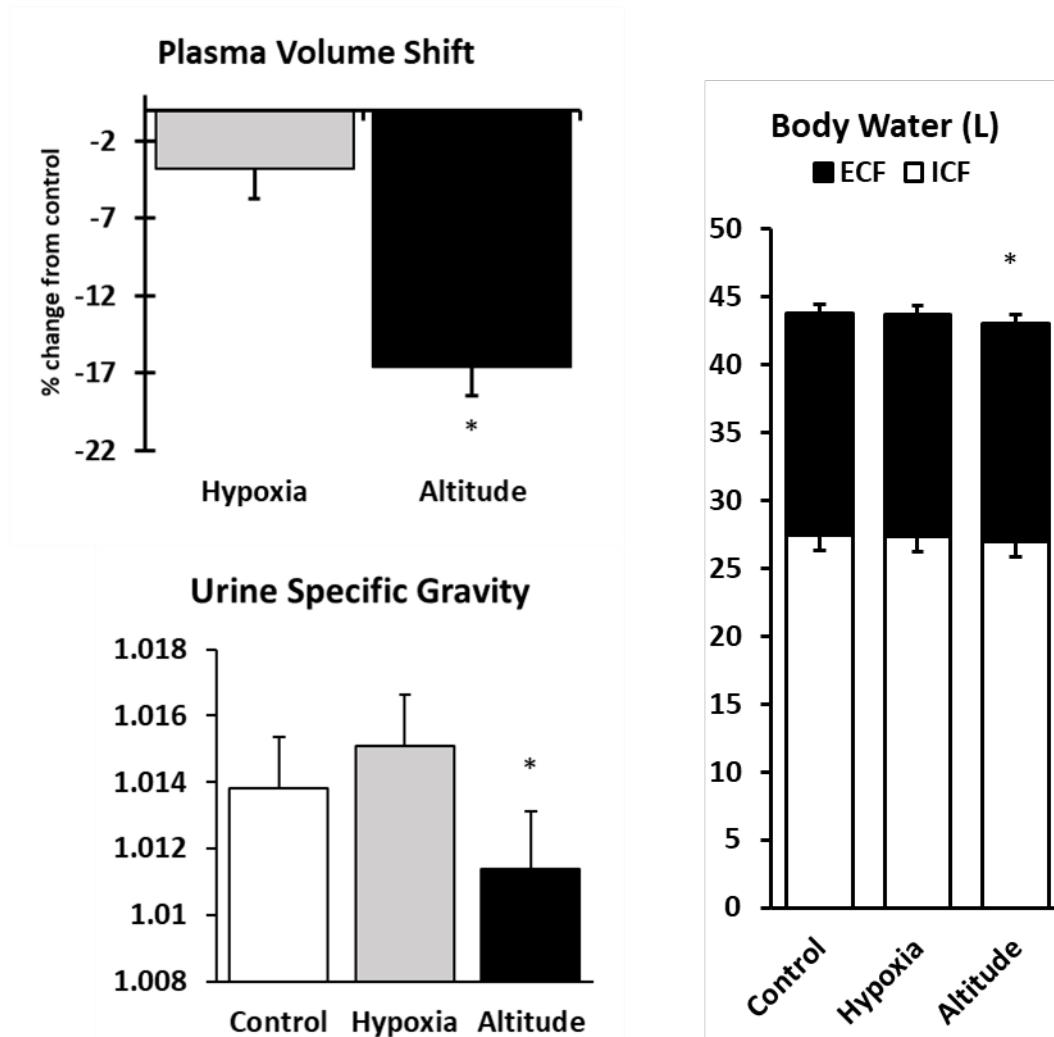
Our project design allowed arterial blood oxygenation (SpO₂) to be lower in our Hypoxia and Altitude trials. Cardiac Output appears to be increased primarily by HR with hypoxia and then further at altitude by increasing stroke volume.



Cardiovascular parameters at rest and during exercise at sea level (control) simulated altitude (Hypoxia), and actual terrestrial. * p<0.05 from Control, † p<0.05 from rest, ‡ p<0.05 from Hypoxia. All data are means ± SD.

FLUID SHIFTS:

We were able to determine that there was a greater fluid shift (body water) with Altitude when compared to Hypoxia or Control. This is additional support to the thesis that hypoxia and altitude do not lead to the same physiological response.



Fluid shifts at sea level (control) simulated altitude (Hypoxia), and actual terrestrial (Altitude). * $p < 0.05$ from Control. All data are means $\pm$ SD.