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**ACTIVE ASCENT EXACERBATES HYPOXIA-MEDIATED
ALTERATIONS OF CREATINE, ENERGY RECRUITMENT, AND
KETONE BODY METABOLISM**

Isaie Sibomana Ph.D.

UES, Inc.

Wright Patterson AFB, OH

Beth A. Beidleman Ph.D., J. Philip Karl Ph.D.

Peter S. Figueiredo M.Sc., CSCS, Steven D. Landspurg

Janet E. Staab

US Army Research Institute of Environmental Medicine

Natick, MA

Camilla A. Mauzy Ph.D.

711 HPW/RHBBB

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711TH HUMAN PERFORMANCE WING,
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WRIGHT-PATTERSON AIR FORCE BASE, OH 45433
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Date: 2024.05.01 08:52:42 -04'00'

CAMILLA MAUZY
Work Unit Manager
Biotechnology Branch

GOODSON.MICHAEL.STEPHEN.1388291342
HAEL.STEPHEN.1388291342

Digitally signed by
GOODSON.MICHAEL.STEPHEN.1388291342
Date: 2024.05.02 13:29:58 -04'00'

MICHAEL GOODSON
CRA Lead (acting)
Applied Biotechnology Branch

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PREFACE

The metabolomic effort was conducted at the 711th Human Performance Wing, Human Effectiveness Directorate, Airman Bioengineering Division, RHDJ (now currently RHBBB) Branch at Wright-Patterson AFB, OH.

The Acute Mountain Sickness metabolomic biomarker project was funded by the Military Operational Medicine (MOMRP) Joint Program committee (JPC-5) Human Performance Optimization in Extreme Environments FY21-22 as “*Urine Metabolite Assay for Sea Level Prediction of Altitude-induced Acute Mountain Sickness*”, as the second phase (PHII) of effort. Dr. Camilla Mauzy served as the Program Manager and Principal Investigator for the metabolomic research. This project was extended by a year into FY23 at the request of Dr. Mauzy and with the acceptance of JPC-5 due to unavoidable COVID pandemic-induced delays. This final technical project report was written for completion of this second phase of effort, (FY20- FY23). This research is continuing as Phase III in a new JPC5 funded FY24 project.

The separate human high altitude study project was supported by the United States Army Medical Research and Development Command and the United States Department of Defense, Defense Health Program.

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1.0 SUMMARY

High altitudes are characterized by hypobaric low pressure, limited oxygen availability, and cold temperatures. Short- or long-term exposures to these environmental conditions lead to specific modifications in cellular processes that initiates and establishes mechanistic adaptation by shifting to ancillary pathways requiring minimal to no oxygen to rebalance energy supply and demand. Some cellular changes are transient, lasting a day or two, while other changes require an extended timeline before returning to baseline. Uphill hiking is an energy-demanding exercise that, on its own, initiates alterations in cellular metabolism to fill additional energy requirements. Hiking under hypoxic conditions exerts even more demands on the body's metabolic systems due to the substantial increase in energy expenditure under a scarce oxygen supply. The current study involved 41 healthy soldiers (mean $\pm$ standard deviation (SD); age = 26 ± 5 yr) who were tested at their baseline residence (sea level, 331 meters above sea level (masl)), transported to Taos, NM (2845 masl), then immediately hiked (n= 21) or were driven (n= 20) to a high altitude facility (3600 masl) where they resided for the next 4 days. Results demonstrated that hypoxic effects on urinary metabolite clearance were mostly transient for individuals who were driven, with most alterations in metabolite excretion noted only on the first day at high altitude (HA). However, for this group longer term hypoxia effects were observed for pyruvate, citrate, sarcosine, and hypoxanthine as clearance alterations of these metabolites persisted at day 3 and/or day 4 at HA. Interestingly, uphill hiking just prior to day 1 at HA exacerbated hypoxia-mediated metabolite alterations. Metabolic pathways most affected by the pre-HA energy demands belong to the energy, nicotinamide, ketone body, fatty acid, phospholipid, and neurotransmitter metabolism pathways. Evidence for uphill hiking-induced enhancement of metabolite clearance is reflected in the: (1) profound changes noted on the first and second day of altitude exposure; (2) consistently elevated clearances of citrate, acetate, and isobutyrate throughout of the entire high altitude stay; and (3) number of alterations noted on days 3 and 4 at HA found only in subjects who hiked to HA. Taken together, the current study's findings suggest that additional energy demands immediately prior to sojourn at high altitude exacerbates altitude-induced alterations in urinary metabolite clearance, altering the acclimatization timeline.

2.0 INTRODUCTION

High altitude is conventionally defined as elevation of greater than 2500 meters above sea level (masl) (Moore, 1987). Travelling to high altitude imposes several challenges on the human body due to decreases in barometric pressure and low oxygen, leading to hypoxemia and initiation of a series of physiological processes (West et al., 2012) aimed at counteracting the partial pressure of oxygen (Luks and Hackett, 2022). The occurrence of hypoxia is associated with significant involuntary muscle and fat loss (Boyer and Blume, 1984; Rose et al., 1988) due to various factors, including elevated basal metabolic rate and metabolic demands (Kayser, 1994; Westerterp, 2001; Hamad and Travis, 2006). Adaptation to hypoxic environments trigger other physiological changes as well, such as elevated ventilation rate and cardiac output. Additionally erythropoiesis and enhanced tissue vascularization increase, which can improve oxygen delivery to hypoxic tissues (Sutton et al., 1988). Acclimatization also requires altered metabolic responses to meet cellular ATP demand and synthesis during decreased oxidative capacity and elevated oxidative stress (Ward, 2008; Horscroft and Murray, 2014; Siervo et al., 2014). The response also includes a reduction of oxygen consumption achieved through adjustments of metabolic processes and intensification of mechanisms associated with oxygen transport to cells such as upregulation of erythropoiesis and angiogenesis (Mylonis et al., 2019). These adaptations require substantial coordination and reprogramming of the expression of a number of genes involved in energy metabolism and regulation of lipid metabolism by transcriptional control factors Hypoxia-Inducible Factors (HIFs) (Hu et al., 2003; Tissot van Patot et al., 2009; Semenza, 2012; McClain et al., 2013; Mylonis et al., 2019). Having efficient mechanisms for oxygen delivery to tissues is key for effective adaptation to high altitude. Himalayan Sherpas, a human population known to be highly adapted to living life in the hypoxic environments, display enhanced efficiency of oxygen utilization along with improved muscle energetics, lower capacity for fatty acid oxidation, as well as improved proficiency in oxidative stress protection (Horscroft et al., 2017).

Deficiency in oxygen leads to reduced cellular aerobic metabolism, creating an imbalance between energy demand and supply. An uninterrupted uphill ascent to high altitude can exacerbate altitude-induced physiological alterations as the gap between energy supply and demand widens. Although healthy unacclimatized individuals living in altitude environments lower than 1500 meters above sea level (masl) can readily tolerate ascents of 2000 to 2500 masl, fast ascent to altitudes higher than 2500 masl can induce serious physiological alterations such as severe hypoxemia, exercise intolerance (Ulrich et al., 2017), sleep disturbance (Bloch et al., 1985; Latshang et al., 2013) and acute mountain sickness (Bartsch and Swenson, 2013). Under these conditions, various metabolic pathways become altered which are reflected in dramatic changes in levels of endogenous small molecule metabolites (Chang et al., 2019). Hypoxic adaptation is often reflected in modulation of sets of metabolites involved in discrete defined pathways (Serkova et al. 2008). Various studies have reported alterations in metabolic pathways and levels of small molecules attributed to exposure to high altitude conditions (Zhu et al., 2015; Liao et al., 2016; O'Brien et al., 2019). Proton nuclear magnetic resonance (^1H NMR) spectroscopy and direct infusion mass spectrometry has been used to assess the metabolic

responses to progressive hypoxic conditions using plasma taken before and during an ascent to Everest Base Camp (5300 masl) (O'Brien et al., 2019). They noted substantial alterations in metabolic pathways associated with bile acid and heme metabolisms, fatty acid transportation, and inflammatory response with increased glycolytic rate, circulating lactate, and most abundant free fatty acids (palmitic, linoleic and oleic acids).

Changes in levels of metabolite by-products of these pathways are reflected in body fluids such as blood, saliva, urine and are detectable via analytical techniques such as nuclear magnetic resonance (NMR)-based metabolomics. Metabolomics has been extensively used by various studies to conduct mechanistic research, drug discovery and screening, and pharmacodynamics (Chang et al., 2019). Metabolomics-based examination of endogenous small metabolite concentrations can provide useful information about the mechanistic aspects underlying the occurrence and development of high altitude-related adverse effects and/or disease. Salient metabolites deriving from these analyses have the potential to act as biomarkers for specific altitude-related health effects and diseases and can provide a basis for their clinical diagnosis. Chang et al. have compiled a number of plasma and urinary metabolic biomarkers and related pathways involved in clinical acute and chronic high altitude-induced hypoxia exposures (Chang et al., 2019). Altered metabolic pathways included those relate to energy metabolism such as glycolysis, Tri-carboxylic acid (TCA) cycle, amino acid and fatty acid metabolisms, inflammatory response related metabolism (phospholipid, linoleic acid, purine, and arachidonic acid metabolisms) as well as heme and bile acid metabolisms. However, information on mechanisms driving high altitude acclimatization is lacking.

Our effort utilized proton ^1H NMR-based metabolomics to investigate alterations in urinary metabolite profiles associated with additional energy expenditure (uphill hiking) with acute (first 24 hour (hr)) and subchronic (>24 hr to 96 hr) exposure to high altitude environments and acclimatization processes. Using this approach, we hypothesized that pre-altitude energy expenditure would exacerbate the altitude-induced alterations in urinary metabolites and extend the recovery time needed to return to sea level baseline. Such data could be used in risk analysis to predict high altitude performance decrement and acclimatization timeline based on molecular signatures indicative of energy recruitment status.

3.0 MATERIALS AND METHODS

3.1 Human Study Design

Details on the human study design and participants have been previously described (Beidleman et al., 2023). The study was approved by the Institutional Review Board at the U.S. Army Research Institute of Environmental Medicine (USARIEM) in Natick, MA. Investigators adhered to the policies for the protection of human participants as prescribed by Army Regulation 70-25, and the research was conducted in adherence with the provisions of 32 Code of Federal Regulations Part 219.

Forty-one healthy, unacclimatized, physically active subjects 19-38 years of age (mean $\pm$ SD; age=26 $\pm$ 5yr) participated in the study. Although the study enrollment was open to both sexes, only two women participated. Briefly, the human study was a randomized, controlled trial consisting of two phases. During the 3-day first phase, participants were tested at their baseline residence (Ft. Leonard Wood, MO; 331 masl). During their sojourn at sea level (SL), they consumed a self-selected diet, maintained habitual exercise routines, and were not confined or regulated. After 1-2 weeks (this varied based on which group study participants were in, when they tested at SL and HA), participants were transported to Taos, NM (2845 masl), then immediately hiked (active ascent; n=21) or were driven (passive ascent; n= 20) to HA at a 3600 masl facility where they resided for the next 4 days. The ascent to HA occurred in the afternoon on day 1 and the active ascent cohort was outfitted with a backpack weighing ~15 percent (%) of their body weight. The passive ascent group started their 30 minute (min) vehicle ascent journey approximately 30 min after the active group began their hike. To reach the Ski patrol facility located at 3,600 masl, the active ascent cohort ascended 750 masl in elevation on a gravel road with an average grade of 15%. Both groups arrived at Ski patrol facility within 60 min of each other. The average time for the uphill hiking was 120 $\pm$ 24 min with a range of 60–162 min. During the stay at HA, subjects were given a limited variety of foods from which to choose but could eat from those items *ad libitum*. On days 2 and 3 at HA, all subjects participated in a hiking exercise but were otherwise mostly sedentary and engaged in study or leisure activities.

3.2 Urine Sample Collection

Urine samples were obtained on three consecutive days (day-3, day -2, and day -1) at SL one to two weeks prior to altitude ascent and at altitude on day 1 (HA1), day 2 (HA2), day 3 (HA3), and day 4 (HA4) and were used for the analyses. All collected urine samples were shipped on dry to Wright-Patterson Air Force Base (WPAFB), OH, where they were stored at -80 Celsius ($^{\circ}$ C) until delivered to Ohio State University for ^1H NMR analyses.

3.3 Urine Sample Preparation, NMR Data Acquisition and Processing

All urine samples were processed as described in Sibomana *et al.* (Sibomana *et al.*, 2017). Briefly, frozen urine samples were thawed at 4 $^{\circ}$ C overnight. A 600 μl urine aliquot was then mixed with 300 μl of phosphate buffer (0.2 M mono- and disodium phosphate; pH = 7.4) and centrifuged at 13,000 rpm (15,680 $\times g$) for 10 min to remove any precipitates. A 550 μl aliquot of the supernatant was transferred to a 5 mm NMR tube and mixed with 150 μl of 2,2',3,3'-tetradeutero-trimethylsilylpropionic acid (TSP) in deuterium oxide (D_2O), adjusted to yield a final concentration of 2 mM. The TSP served as a chemical shift reference (δ = 0.00 ppm) with D_2O providing a field-frequency lock for NMR data acquisition. The ^1H NMR spectra were acquired using a high resolution Bruker NMR spectrometer operating at 850 megahertz (MHz) with a probe temperature of 25 $^{\circ}$ C. NMR spectral data processing and analyses were conducted at WPAFB using Topspin (Bruker, MA, USA) and Mnova (Mestrelab Research, S.L., Spain) software packages.

3.4 NMR Data Analyses

Multivariate data analyses were conducted on binned and scaled spectral data. Binned NMR data were scaled to the entire dataset chosen as reference. Principal Component Analysis (PCA) was used as an unsupervised analysis technique and provided a first approach for data visualization (Jolliffe, 1986).

3.5 Quantification of Metabolite Resonances

Quantification of specific metabolite resonances was accomplished using Topspin software. NMR spectral regions identified as important were compared between the Hiked and Driven groups. NMR specific resonances were assigned to metabolites with the aid of literature and on-line Human metabolome database (HMDB, <http://www.hmdb.ca/>, www.bmrb.wisc.edu). Signal intensities were integrated to obtain measurements of metabolite concentrations at each time point.

3.6 Statistical Analyses

A repeated measures Multivariate analysis of variance (MANOVA) was conducted to examine effects of time and exertion group (e.g., Hiked and Driven groups) on urine metabolite profiles. For metabolites demonstrating time-by-group interactions ($p < 0.05$), Levine's and Welch's tests were conducted to assess the equality of variances between the data for SL, HA1, HA2, HA3, and HA4 as well as for Hiked vs. Driven groups for each metabolite using the statistical software package JMP[®] 11.0.0 (SAS Institute, Cary, NC, USA). If Levine's test was significant ($p \leq 0.05$), then Welch's test was used to determine if there were significant differences in the mean values between groups for the metabolite of interest. If Levine's test was not significant, significance was tested using a one-way Analysis of variance (ANOVA) (t -test). If both Levine's and Welch's tests were significant ($p \leq 0.05$), a pairwise Welch test was performed for all pairs of groups.

Results shown in figures are normalized to creatinine and are expressed as mean $\pm$ standard error of the mean (S.E.) and are considered statistically significant at $p \leq 0.05$. Cohen's d (effect size) (Cohen, 1988) was used as a measure of the magnitude of changes in the level of each urinary metabolite measured for the Hiked group at HA relative to day-1 at SL by subtracting the value obtained for this group at HA from that obtained at SL and assessing the difference relative to the pooled standard deviations for HA and SL. A similar procedure was followed to calculate the effect sizes for the Driven group.

4.0 RESULTS

4.1 Urinary Metabolite Profile Changes Associate with High Altitude Exposure

PCA analysis constructed using proton NMR spectral data for the Driven and Hiked groups at HA1 indicated that most of the urine samples collected from the Hiked group separated in space

from individuals belonging to the Driven group (**Fig. 1**). This suggests that the subjects in the Hiked group did not uniformly respond to hiking exercise.

Driven (Passive Ascent) Group. Examination of the spectral data for urine samples collected from the Driven group at SL and HA demonstrated an altitude-induced alteration in urinary metabolite clearance at high altitude relative to sea level (**Table 1**). Increases in clearance of 12 metabolites were noted on the first day of altitude exposure (HA1) for the Driven group relative to Day -1 at SL. Altered metabolic pathways related to these changes include energy metabolism (lactate and citrate), creatine metabolism (creatinine), nicotinamide metabolism (1-methylnicotinamide), amino acid (alanine), ketone body metabolism (acetoacetate and acetone), fatty acid metabolism (acetylcarnitine, carnitine, acetate and isobutyrate), phospholipid and neurotransmitter metabolisms (choline). Only creatinine clearance was shown to decrease at altitude compared to SL. On the second day at high altitude (HA2), the number of urinary metabolite clearance alterations dropped to four (sarcosine, carnitine, choline, and hypoxanthine), with hypoxanthine levels remaining elevated up to 4 days at altitude (HA4). Besides hypoxanthine and sarcosine, N-methylhistidine clearance was also altered on the third day at altitude. On day 4 at HA, elimination of energy metabolism by-products pyruvate and citrate increased in addition to hypoxanthine.

Hiked (Active Ascent) Group. Alterations in urinary metabolite clearance were also noted for the Hiked group at HA compared to Day -1 at SL (**Table 1**). However, alterations displayed by this group reflect combination effects of both altitude exposure and hiking to HA, with changes mainly noted at HA1. Although both groups displayed alterations in metabolite clearance of various metabolites at HA1, more pronounced alterations were noted in the Hiked group (**Table 1**). Clearance of lactate, hypoxanthine, 1-methylnicotinamide, and pyruvate for the Hiked group increased by 14-fold, 12-fold, 8-fold, and 1.2-fold, respectively while the excretion of these metabolites for the Driven group was elevated by 0.3-fold, 3-fold, 2-fold, and 12%, respectively. Similarly, ketone body clearance such as acetoacetate and acetone increased by 114% and 96%, respectively for the Hiked group compared to 19% and 37% increases, respectively, noted for the Driven group. In general, higher numbers of urinary clearance alterations occurred from HA2 to HA4 and were noted in the Hiked group compared to the Driven group. Clearances of citrate and isobutyrate were consistently elevated throughout the high altitude sojourn for the Hiked group.

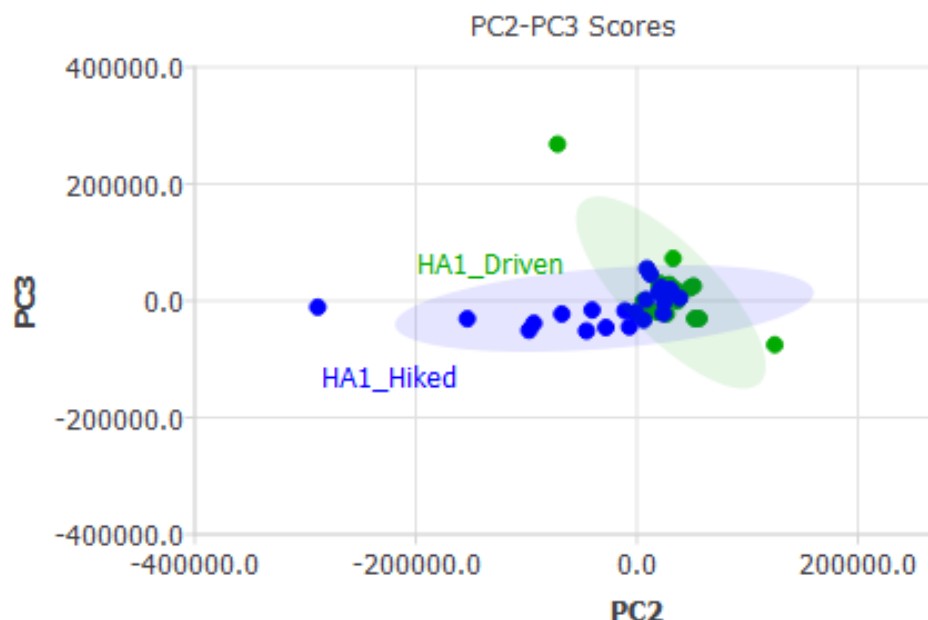
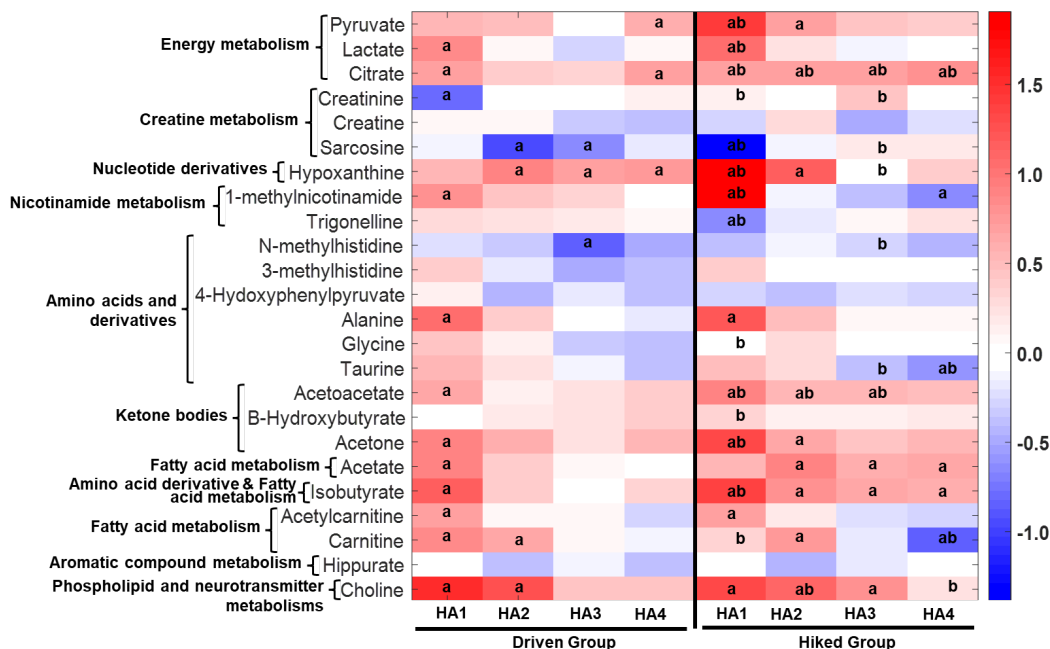


Figure 1. Urinary spectral data for the first day at high altitude (HA1) showing PCA scores plot for Hiked group (blue) vs. Driven group (green)

Table 1. Heatmap showing the magnitude of changes (effect sizes or Cohen's *d*; (Cohen, 1988)) in urinary metabolites for the Driven and Hiked groups at high altitude (HA1, HA2, HA3, and HA4) relative to day -1 at sea level. The blue color represents a negative effect size (a decrease relative to day -1) while a red color indicates a positive effect size (an increase relative to day -1). Heatmap was generated using NMR spectral data for urine samples collected on day -1 at SL and at high altitude. The letter "a" indicates significant differences relative to day -1 at SL while the letter "b" denotes significant differences between the Hiked and Driven group.



4.2 Hiking to High Altitude Alters Creatine Metabolism

Examination of proton NMR spectral data for the two groups at HA indicated that hiking to high altitude had a significant impact on urinary excretion of creatine metabolism substrates (Figs. 2-4). The spectral data for HA1 clearly showed that sarcosine elimination in the Hiked group significantly decreased (-14%) relative to the Driven group (Fig. 2). Sarcosine degradation can give rise to creatine formation. An examination of creatine clearance for both groups indicated that, while hiking itself did not significantly alter the urinary clearance, the Hiked group displayed a decreasing trend (Fig. 3). However, the Hiked group significantly increased creatinine clearances by 66% (HA1) and 23% (HA3) compared to the Driven group (Fig. 4), and creatinine is an immediate degradation bio-product of creatine. Urinary levels of sarcosine, creatine, and creatinine for both groups were similar at HA4.

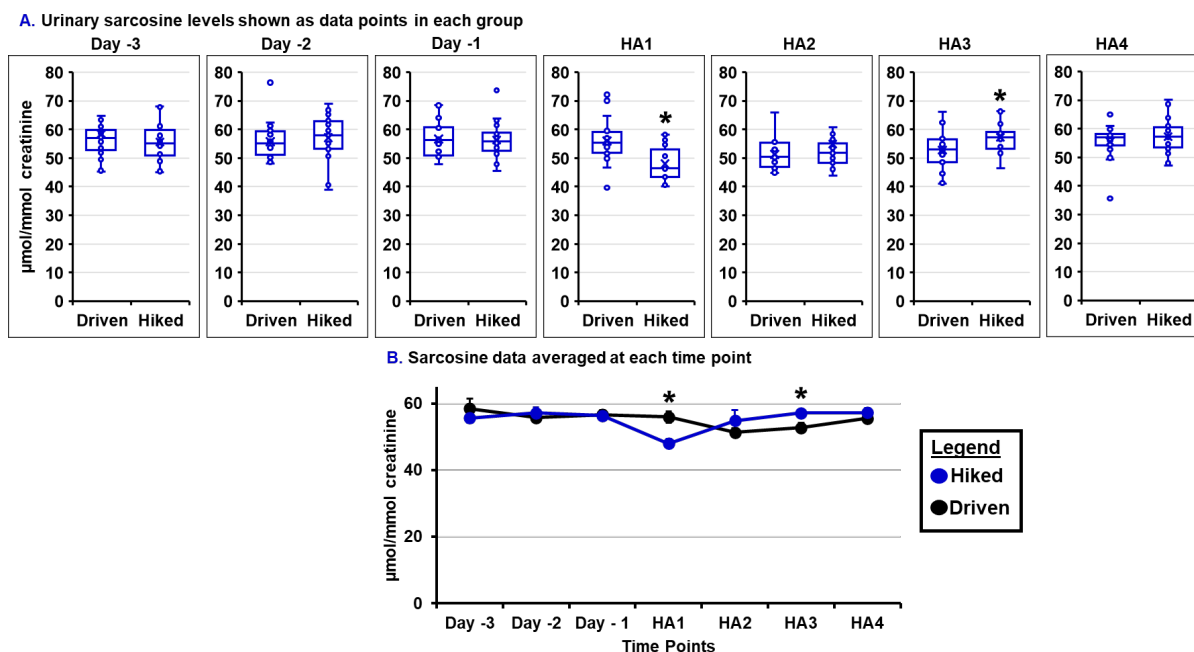


Figure 2. Urinary sarcosine clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$)

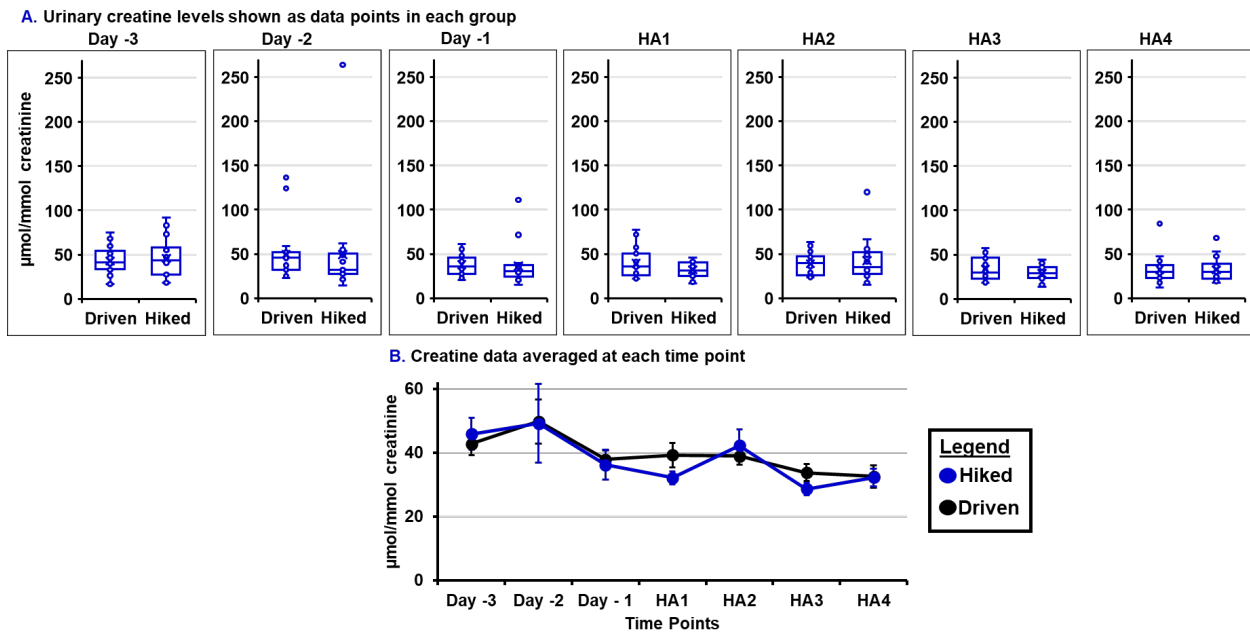


Figure 3. Urinary creatine clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility.

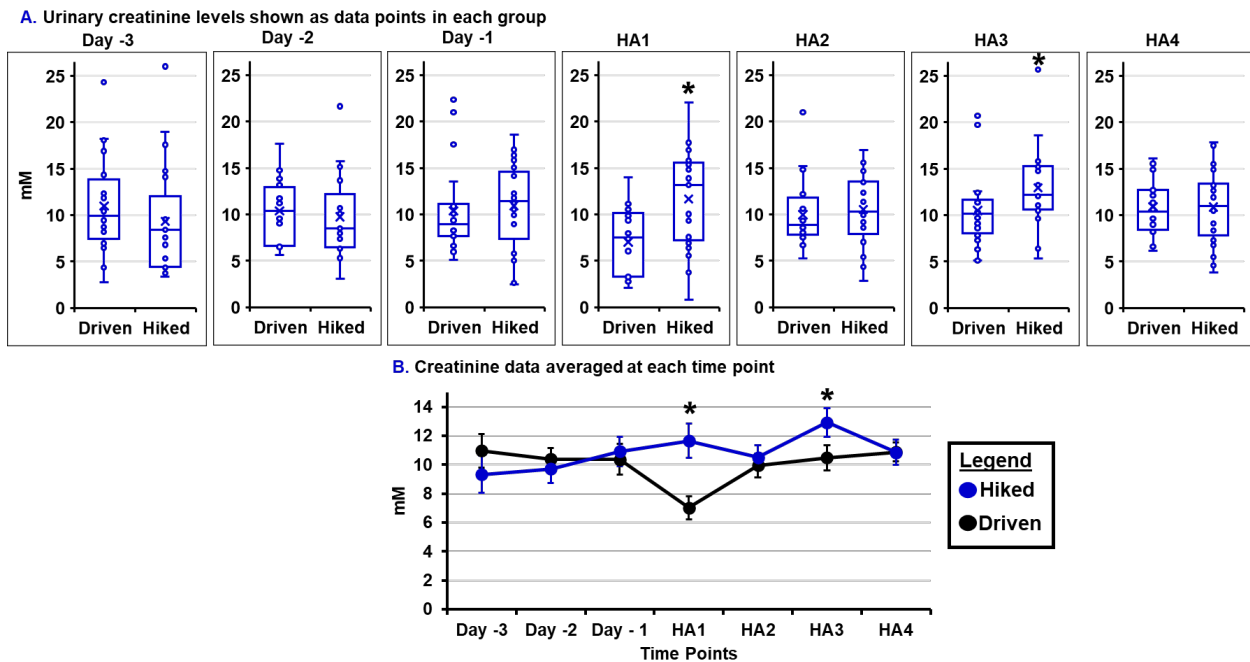


Figure 4. Urinary creatinine clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

4.3 Hiking to High altitude Alters Overall Energy Metabolism

Data for urine samples collected altitude indicated significant differences between the Hiked and Driven groups regarding the clearance of the metabolites involved in energy production, including lactate (**Fig. 5**), pyruvate (**Fig. 6**), and citrate (**Fig. 7**). Not surprisingly, on HA1, the Hiked group displayed a huge spike in lactate clearance (14-fold increase) compared to Driven subjects (**Fig. 5**). Similar observations were noted for pyruvate (1.2-fold increase; **Fig. 6**). Urinary levels of lactate and pyruvate for both groups were similar from HA2 until the end of the sojourn at HA. Hiking also significantly altered citrate clearance (**Fig. 7**), which was consistently elevated for the Hiked subjects during their entire stay at HA compared to the Driven group. Increases in citrate clearance for the Hiked group ranged from 22% (HA1) to 49% (HA2) relative to the Driven group.

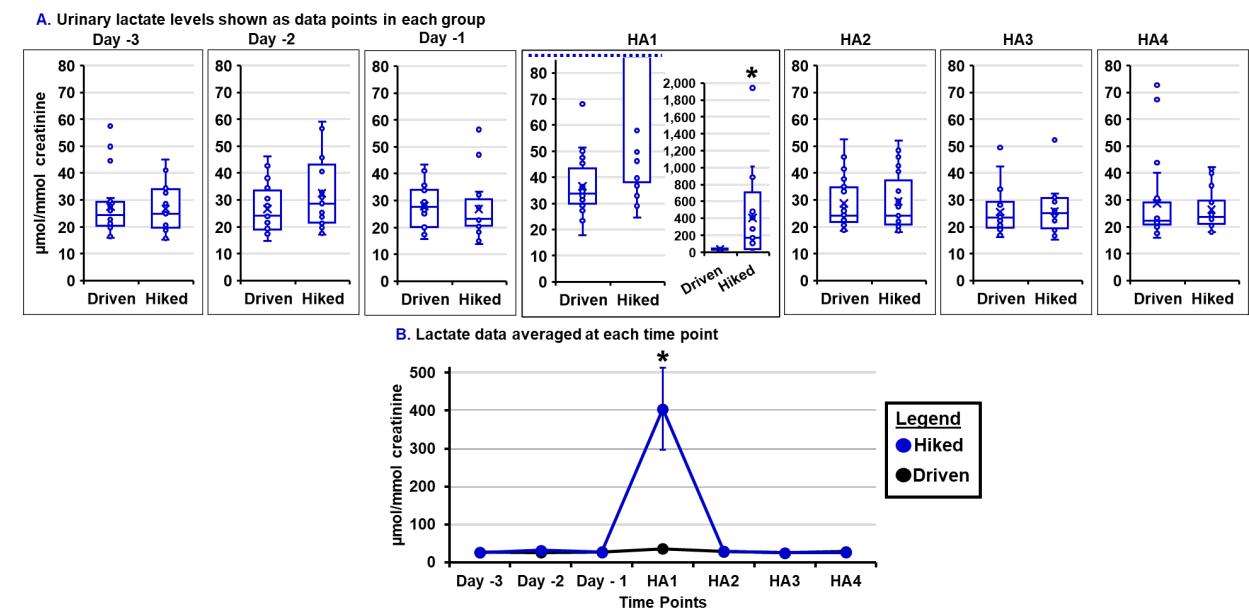
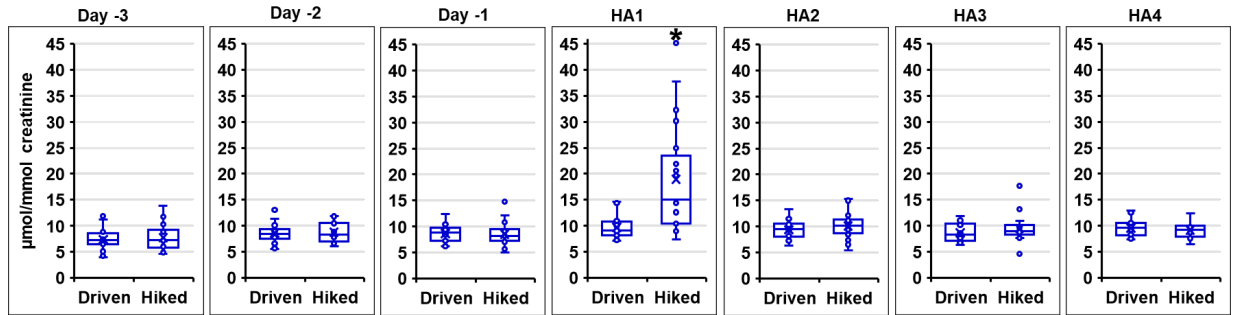


Figure 5. Urinary lactate clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$)

A. Urinary pyruvate levels shown as data points in each group



B. Pyruvate data averaged at each time point

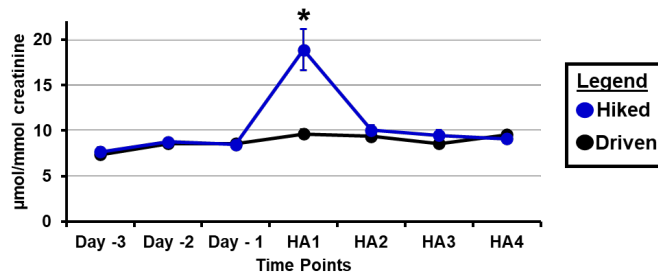
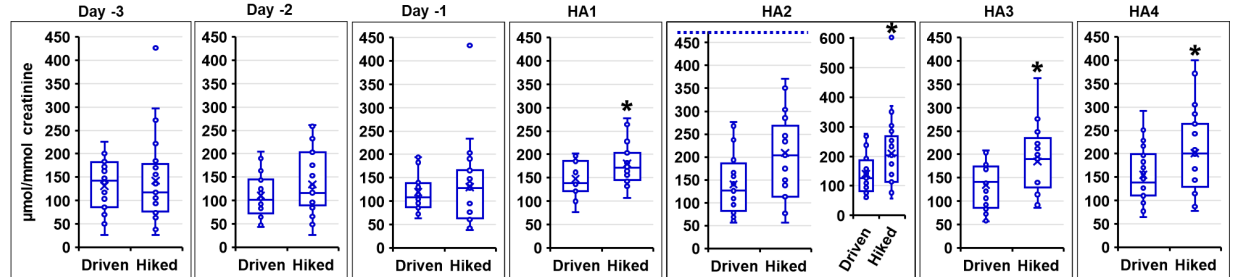


Figure 6. Urinary pyruvate clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

A. Urinary citrate levels shown as data points in each group



B. Citrate data averaged at each time point

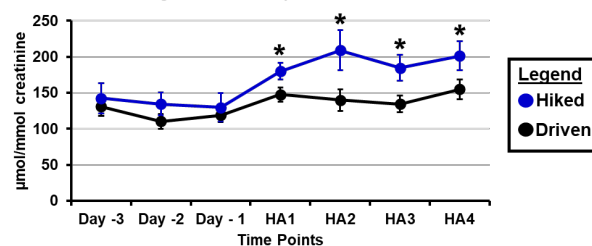


Figure 7. Urinary citrate clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

4.4 Hiking to High Altitude Alters Fatty Acid Metabolism

The effect of hiking on urinary excretion of by-products of fatty acid metabolism was also assessed in the current study (Fig. 8-10). These metabolites included carnitine (Fig. 8), acetylcarnitine (Fig. 9), isobutyrate (Fig. 10), and acetate (Fig. 11). Hiking significantly decreased urinary levels of carnitine for the Hiked group by 36% (HA1) and 54% (HA4) relative to the Driven group (Fig. 8). We also investigated acetylcarnitine clearance, an acetylated form of carnitine, which shuttles carnitine from mitochondria back to cytoplasm (Longo et al., 2006; Hsu et al., 2023; Izzo et al., 2023). No differences were noted between groups in clearance of this metabolite during the entire HA stay (Fig. 9). Excretion of isobutyrate was significantly elevated at HA1 for the Hiked group (increased by 34%) relative to the Driven group (Fig. 10). In addition, no significant differences in urinary acetate elimination were seen between groups during the entire study period (Fig. 11).

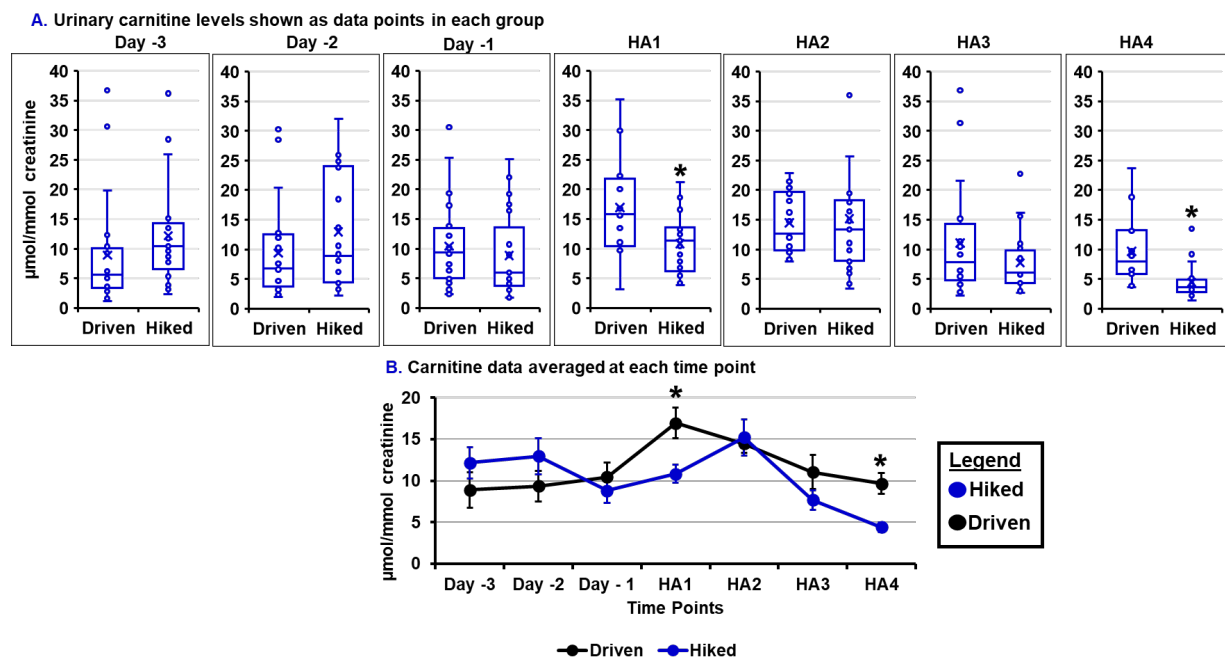


Figure 8. Urinary carnitine clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

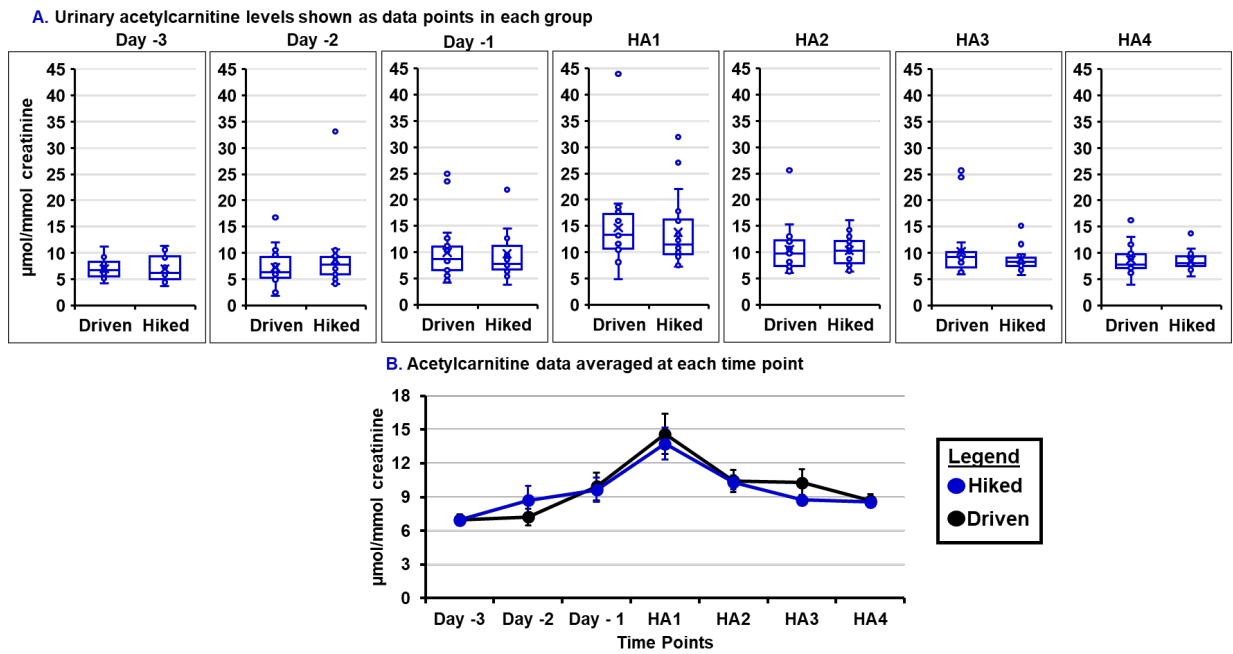


Figure 9. Urinary acetylcarnitine clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility.

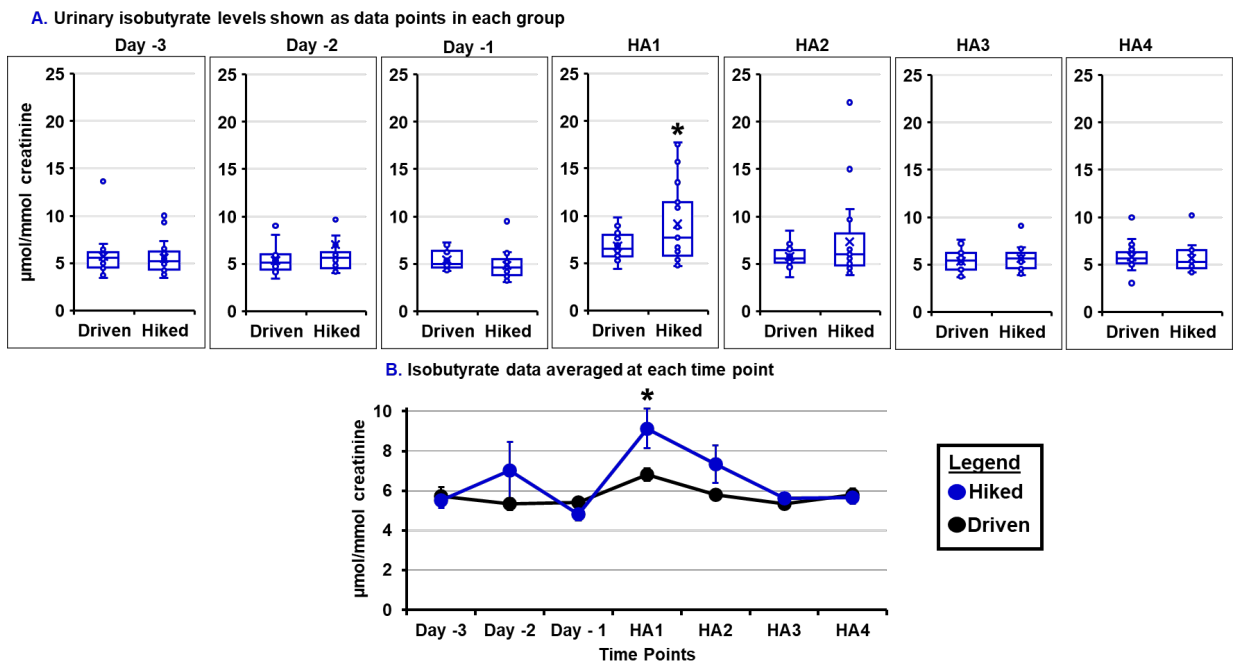
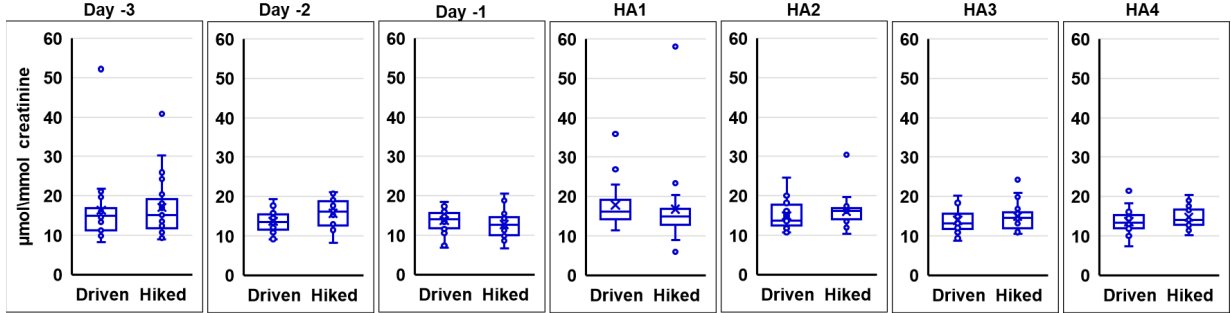


Figure 10. Urinary isobutyrate clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

A. Urinary acetate levels shown as data points in each group



B. Acetate data averaged at each time point

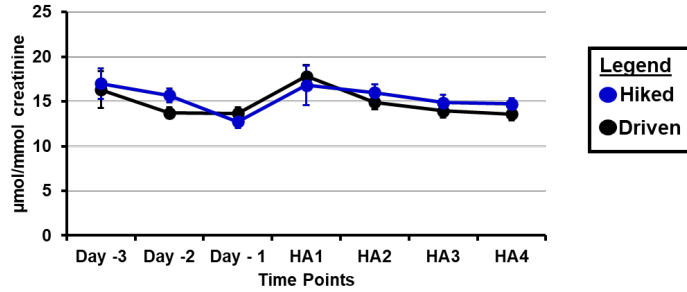


Figure 11. Urinary acetate clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility.

4.5 Hiking to High Altitude Alters Ketone Body Metabolism

Data for the current study indicated that hiking altered the urinary metabolite profiles of ketone bodies during the stay at high altitude. These included acetoacetate (Fig. 12), acetone (Fig. 13), and β -hydroxybutyrate (Fig. 14). Although the urinary levels of acetoacetate for the Hiked group were consistently elevated during the entire HA sojourn compared to the Driven group, significant differences were only seen on the first three days (80%, 61%, and 72% increases, respectively) (Fig. 12). Similarly, the Hiked group displayed a consistently elevated clearance of both acetone (Fig. 13) and β -hydroxybutyrate (Fig. 14) during the altitude exposure period, but significant differences were only achieved on HA1 (increases of 41% for acetone, 62% for β -hydroxybutyrate).

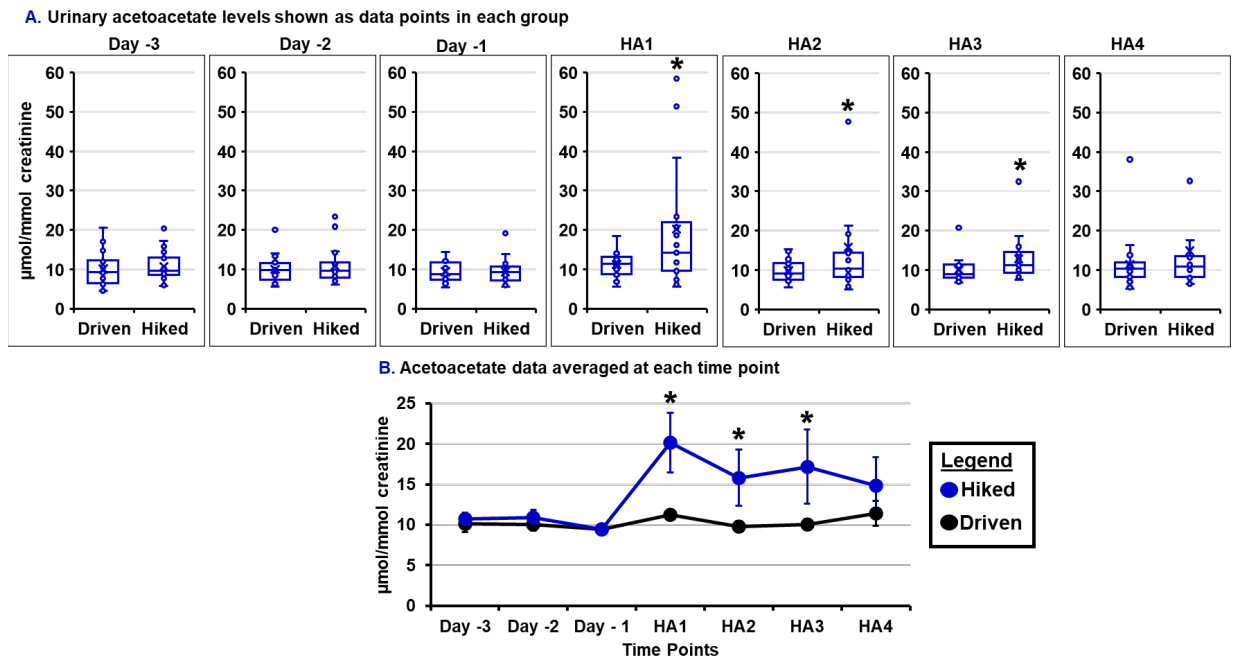


Figure 12. Urinary acetoacetate clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

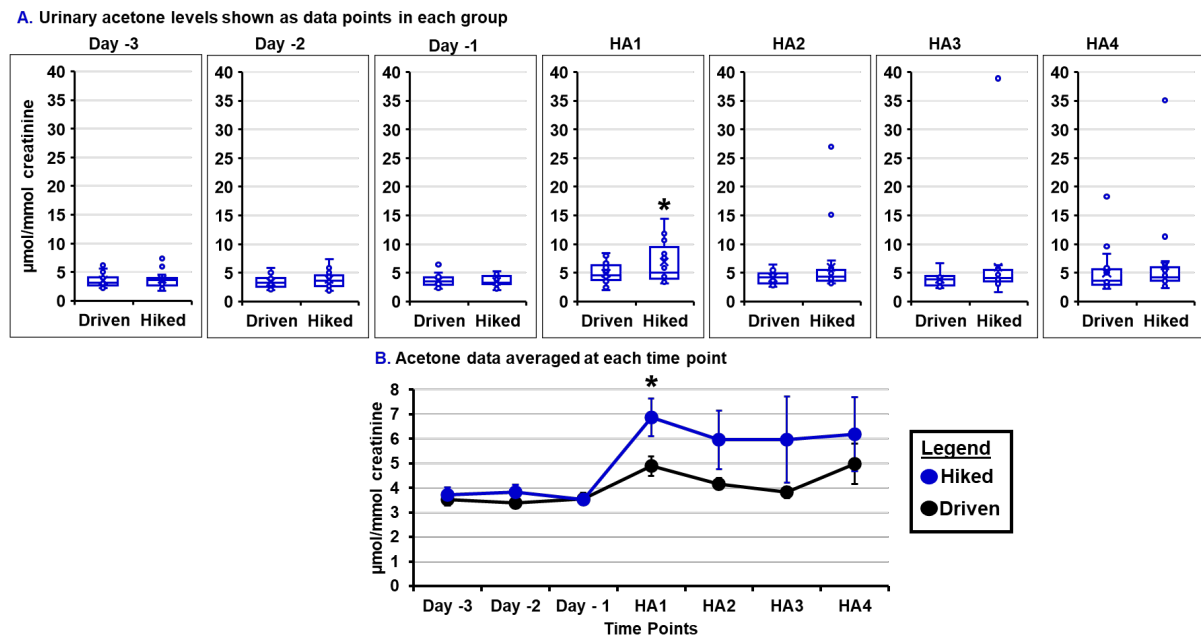


Figure 13. Urinary acetone clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

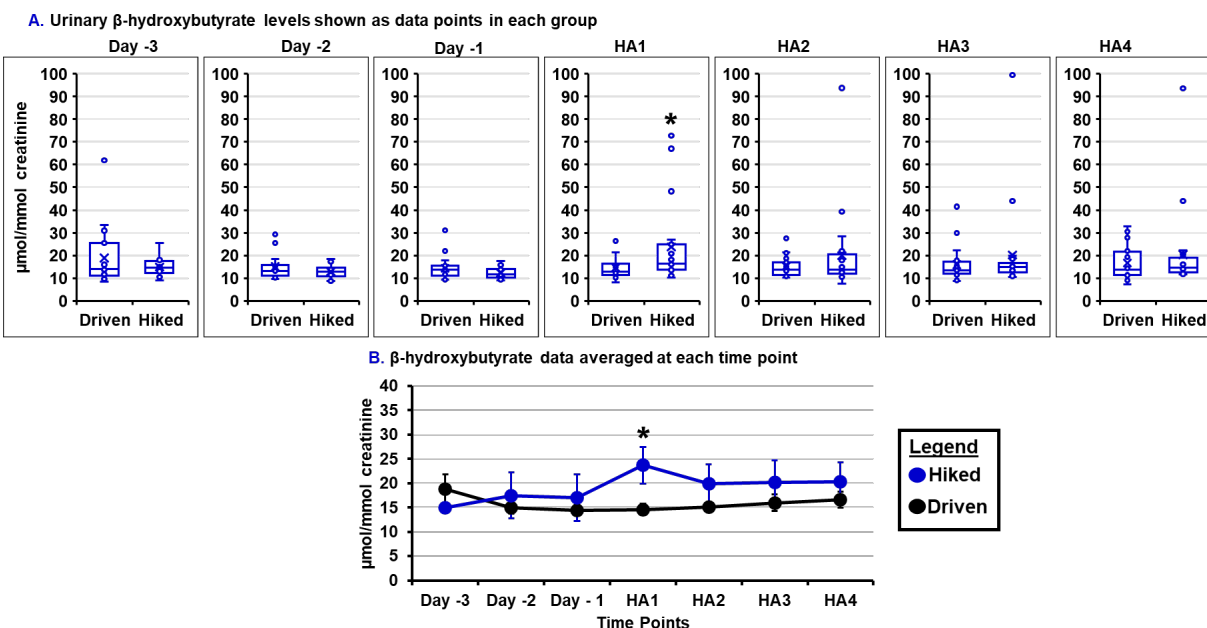


Figure 14. Urinary β -hydroxybutyrate clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

Since acetone and β -hydroxybutyrate are derived from acetoacetate degradation, correlation analyses between acetoacetate and these metabolites were conducted (Figs. 15 and 16). Strong correlations between the urine levels of acetone and acetoacetate (R ranging between 0.7865 and 0.9732; $p < 0.0001$) were noted for the hiked group during the entire stay at high altitude (Fig. 15A). Hiking clearly improved the correlation coefficients for these two molecules compared to the correlation coefficient ($R = 0.2430$; $p = 0.289$) for day -1 at SL. Similar correlation analyses were conducted for the Driven group. These analyses yielded relatively weak correlation coefficients (R ranging between 0.0071 and 0.3110 for HA2 to HA4; $p > 0.195$ and 0.5373 for HA1; $p = 0.0177$) (Fig. 15B) compared to the Hiked group. Unlike the active ascent group, the correlation coefficients for the Driven group did not show a tangible improvement at altitude when compared to the coefficient ($R = 0.1814$; $p = 0.444$) measured on day -1 at SL. We found that one subject in the Hiked group excreted a large amount of acetoacetate and acetone while at high altitude (circled in red in Fig. 15A). Although excluding this subject from the correlation analyses yielded relatively weak correlation coefficients ($R = 0.7524$ ($p = 0.0001$) for HA1, $R = 0.42$ ($p = 0.0652$) for HA2, $R = 0.5229$ ($p = 0.0216$) for HA3 and $R = 0.6922$ ($p = 0.0007$) for HA4), the Hiked group still displayed a strong relationship between acetoacetate and acetone as compared to the Driven group.

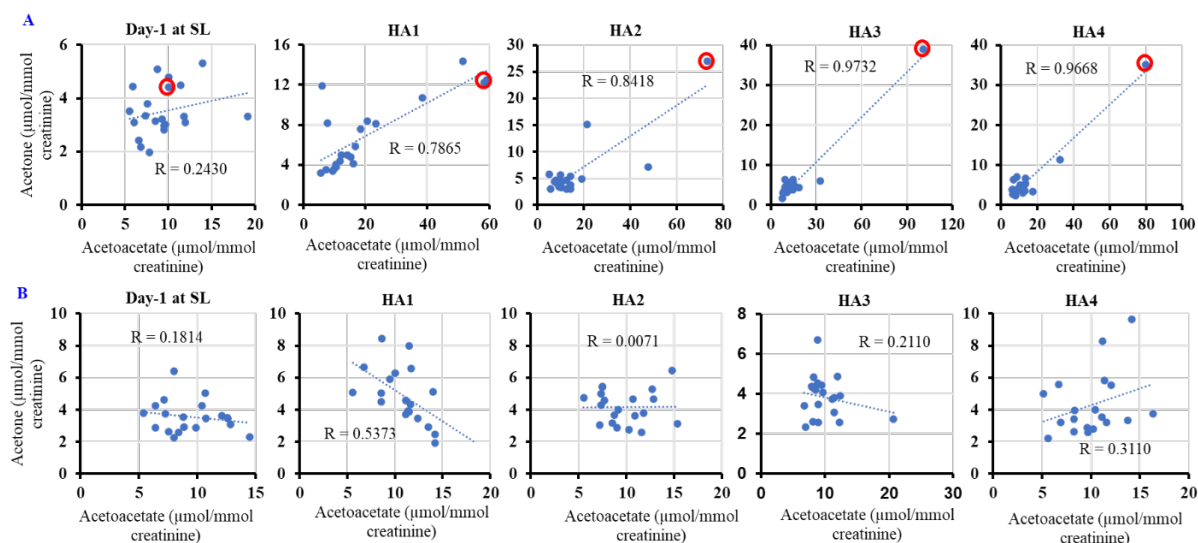


Figure 15. Correlation between urinary levels of acetoacetate and acetone at sea level (Day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects (A) who hiked and those (B) who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl). The red circle indicates a subject that excreted a large amount of acetoacetate and acetone while at HA.

Correlation analyses conducted between β -hydroxybutyrate and acetoacetate for the Hiked group also mirrored results obtained with acetone versus acetoacetate, yielding strong R coefficients ranging between 0.7632 and 0.9097 ($p < 9.08 \times 10^{-5}$) (Fig. 16A). These correlation coefficients also substantially improved compared to those on day -1 at SL ($R = 0.1367$; $p = 0.5657$). Correlation analyses conducted on these two metabolites using data collected from the Driven group at high altitude yielded R coefficients ranging between 0.0028 and 0.3621 ($p > 0.1167$) (Fig. 16B). Unlike the Hiked group, no tangible improvement in correlation was seen when compared to data from day -1 at SL ($R = 0.4264$; $p = 0.0613$).

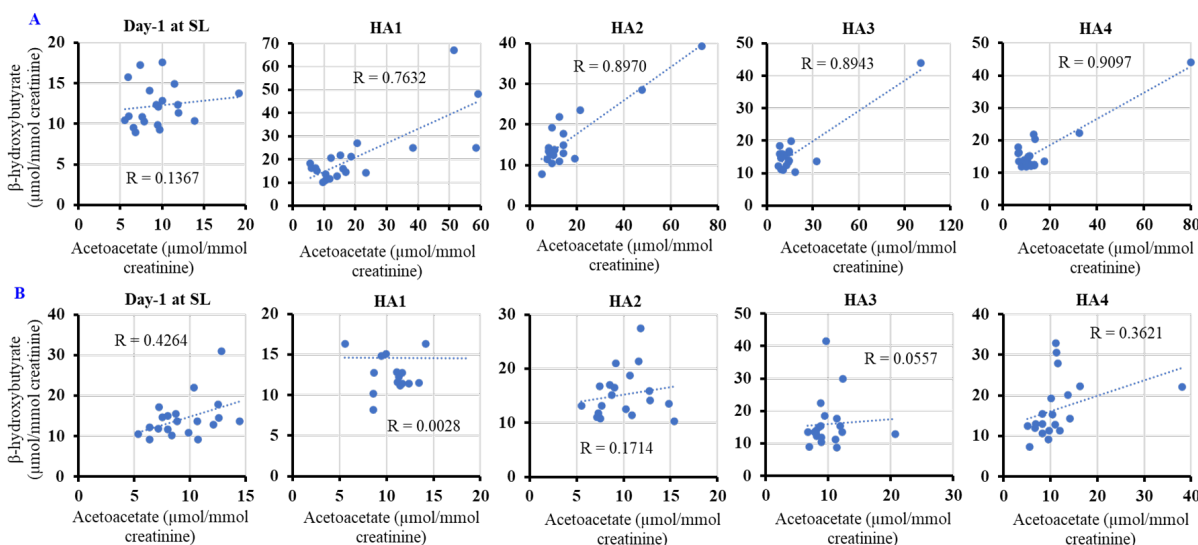


Figure 16. Correlation between urinary levels of acetoacetate and β -hydroxybutyrate at sea level (Day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects (A) who hiked and those (B) who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl).

4.6 Hiking to High Altitude Alters Amino Acid Metabolism

The effects of ascent energy expenditure on amino acid metabolism were also investigated. Our data indicated that hiking induced a 33% decrease in urinary glycine levels at HA1 relative to the Driven group (Figs. 17). However, clearances for this metabolite became similar between the two groups from HA2 until the end of the high altitude stay (HA4). Uphill hiking did not influence the urinary alanine levels (Figs. 18). Although hiking did not alter urinary histidine levels (*data not shown*), alterations in clearance of its breakdown by-product N-methylhistidine was noted at HA3 for the Hiked group (Fig. 19), with urine levels elevated by 23% compared to the Driven group. Inversely, clearance levels of another histidine degradation by-product, 3-methylhistidine, were not affected by hiking (Fig. 20). Other amino acid derivatives assessed during this study included taurine (Fig. 21) and 4-hydroxyphenylpyruvate (Fig. 22). Although taurine levels for the Hiked group were consistently lower than those measured for the Driven group during the entire high altitude stay, statistically significant differences between the two groups were only seen at HA3 (28% decrease) and HA4 (30% decrease; Fig. 21). Hiking did not significantly alter 4-hydroxyphenylpyruvate clearance (Fig. 22).

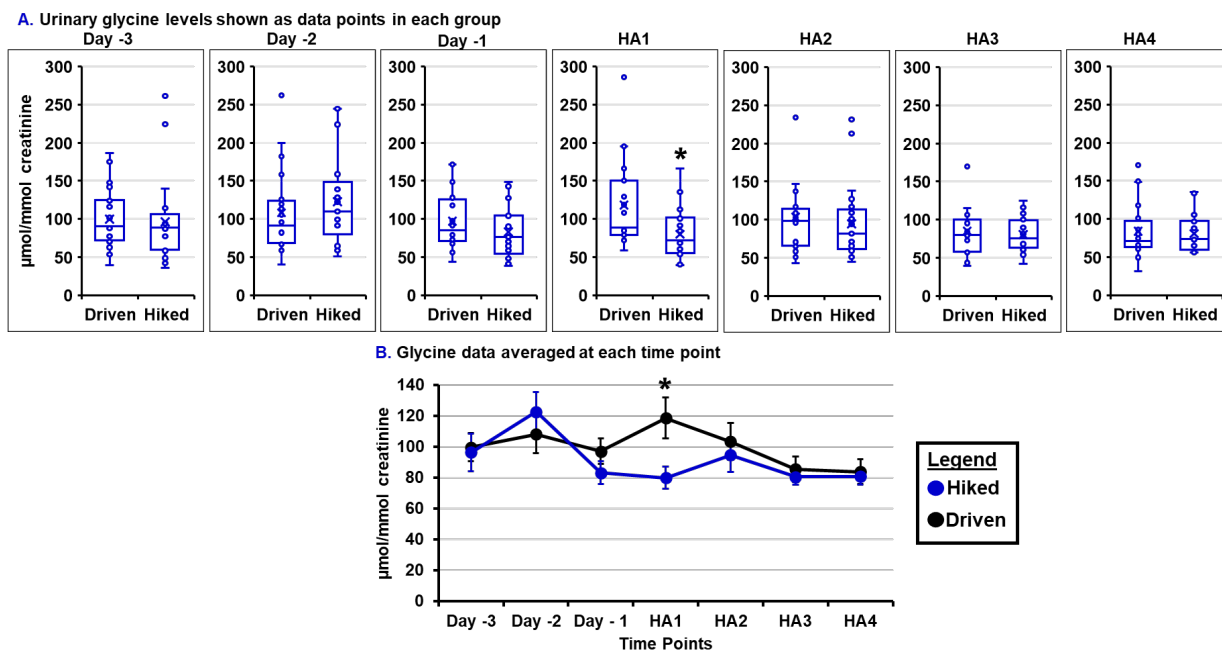
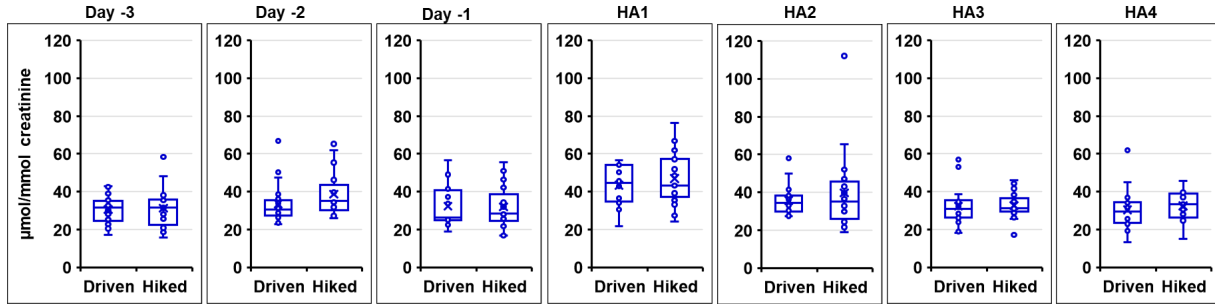


Figure 17. Urinary glycine clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$)

A. Urinary alanine levels shown as data points in each group



B. Alanine data averaged at each time point

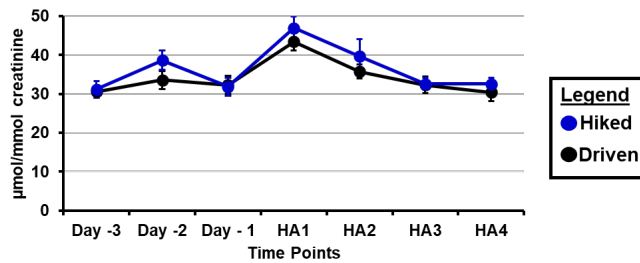
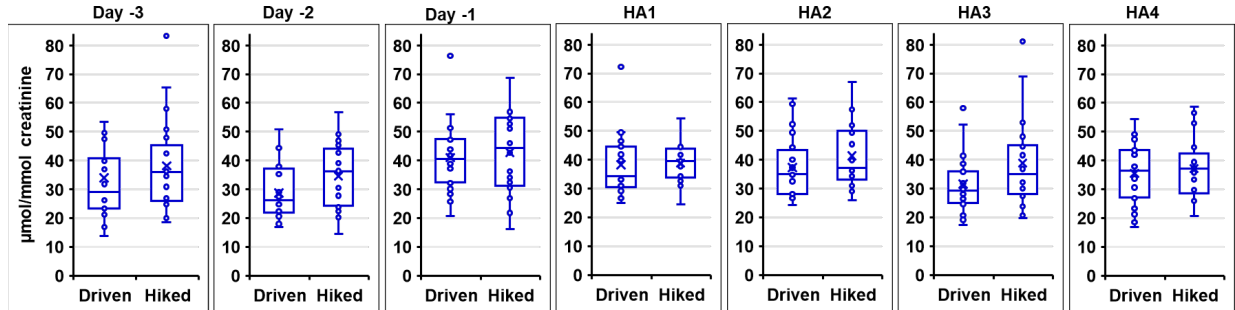


Figure 18. Urinary alanine clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility.

A. Urinary N-methylhistidine levels shown as data points in each group



B. N-methylhistidine data averaged at each time point

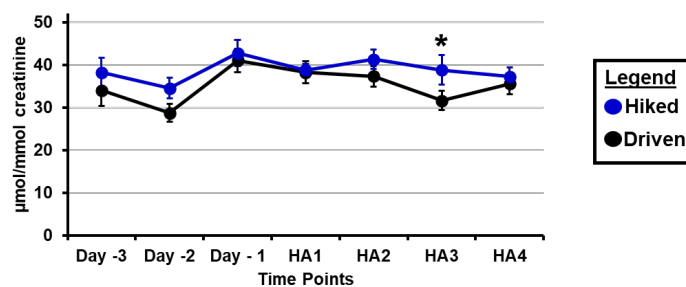
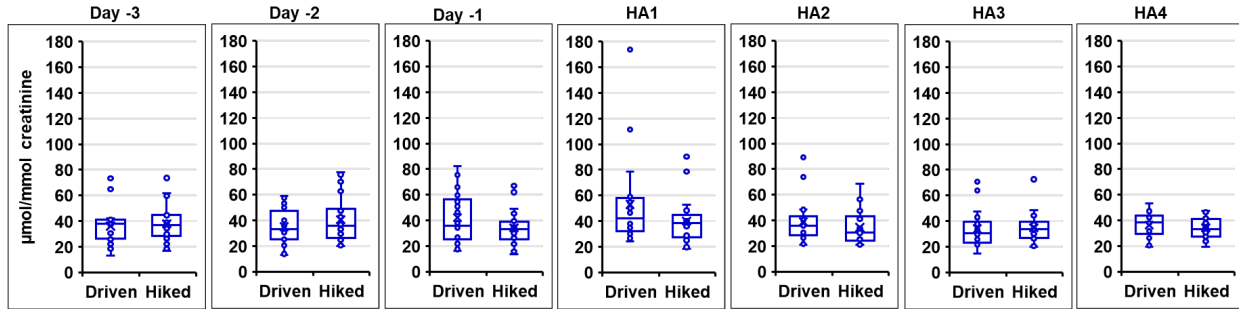


Figure 19. Urinary N-methylhistidine clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

A. Urinary 3-methylhistidine levels shown as data points in each group



B. 3-methylhistidine data averaged at each time point

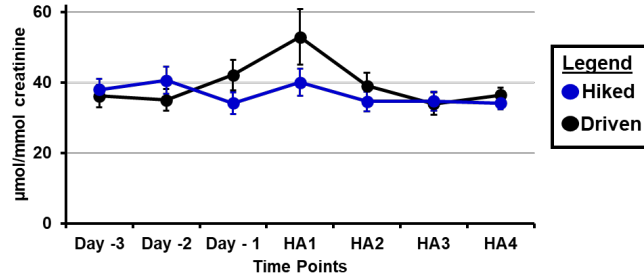
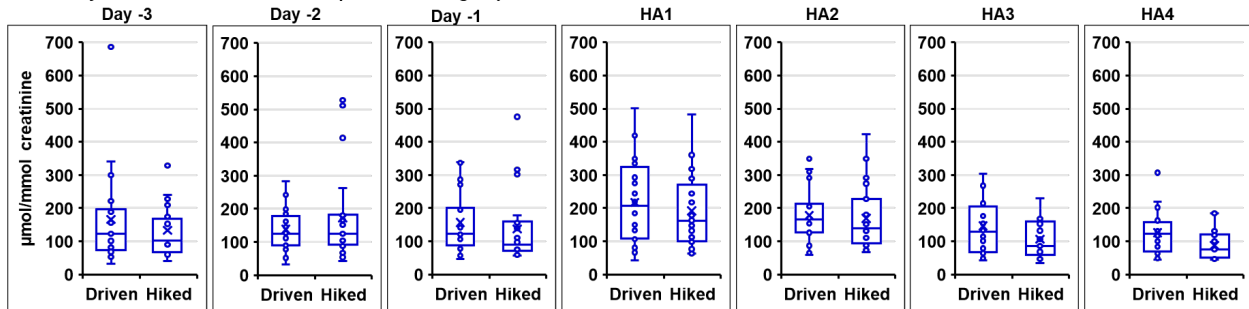


Figure 20. Urinary 3-methylhistidine clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility.

A. Urinary taurine levels shown as data points in each group



B. Taurine data averaged at each time point

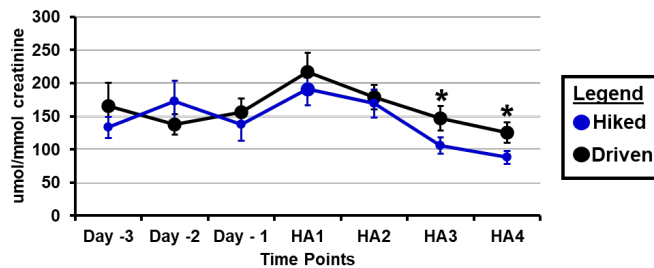


Figure 21. Urinary taurine clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

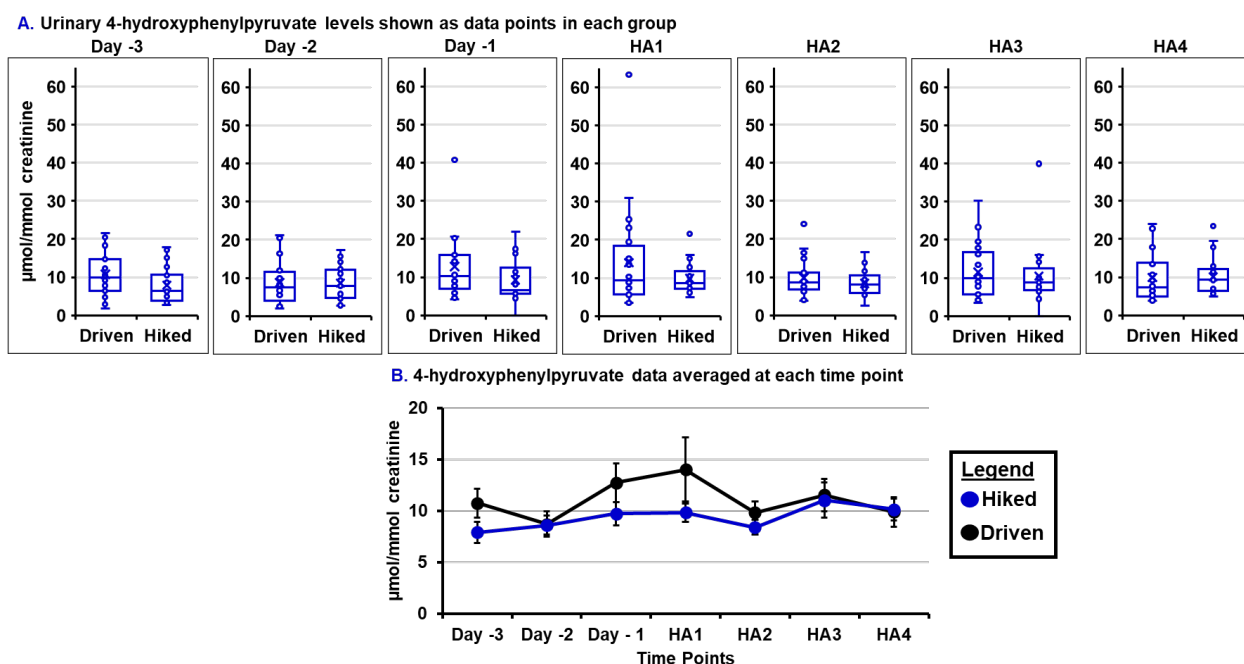
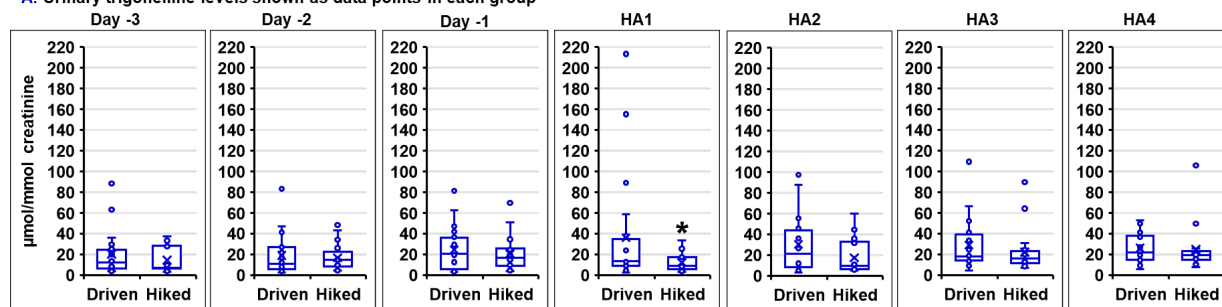


Figure 22. Urinary 4-hydroxyphenylpyruvate clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility.

4.7 Hiking to High Altitude Alters Nicotinamide, Purine and Pyrimidine Metabolisms

The current study also noted hiking-induced alterations in by-products of nicotinamide metabolism such as trigonelline ([Fig. 23](#)) and 1-methylnicotinamide ([Fig. 24](#)). The urinary clearance level of trigonelline for the Hiked group was significantly lower (-67%) at HA1 compared to the Driven group ([Fig. 23](#)). Elimination of this metabolite remained lower for the Hiked group during the entire stay at high altitude, although the decrease was not statistically significant at later time points. Interestingly, a huge increase (2.2-fold) in excretion of 1-methylnicotinamide was noted at HA1 for the Hiked group which disappeared the following day ([Fig. 24](#)). Hiking also affected purine and pyrimidine metabolism, supported by the observation that hypoxanthine clearance was significantly elevated (2.6-fold) at HA1 for the Hiked vs. Driven groups. ([Fig. 25](#)). However, this large clearance spike dropped quickly to SL baseline values the following day.

A. Urinary trigonelline levels shown as data points in each group



B. Trigonelline data averaged at each time point

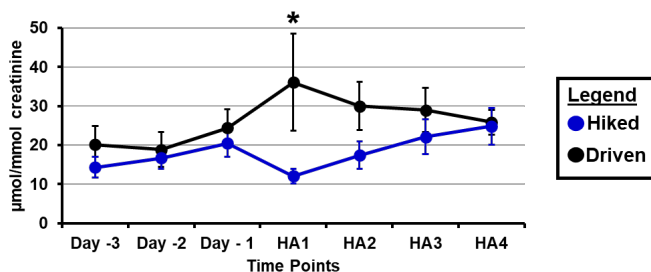
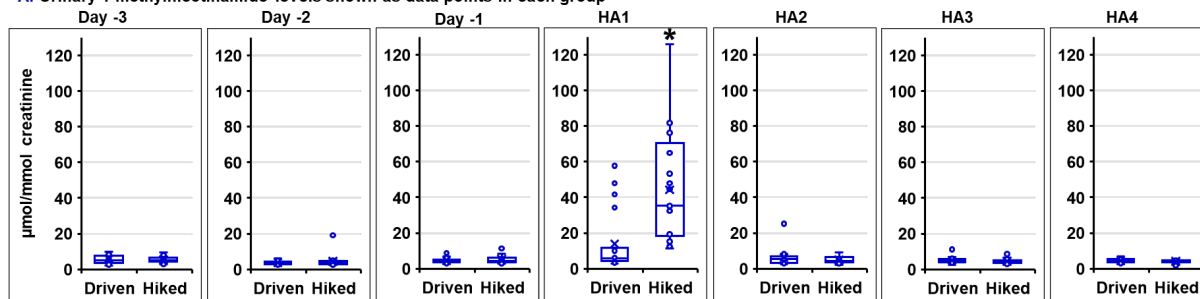


Figure 23. Urinary trigonelline clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

A. Urinary 1-methylnicotinamide levels shown as data points in each group



B. 1-methylnicotinamide data averaged at each time point

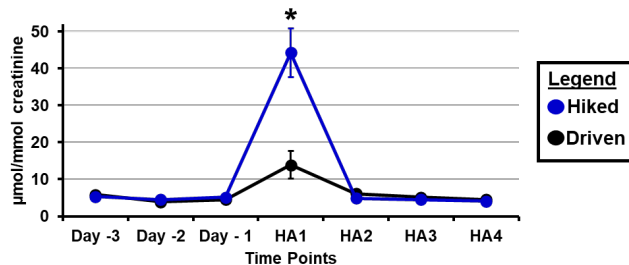


Figure 24. Urinary 1-methylnicotinamide clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

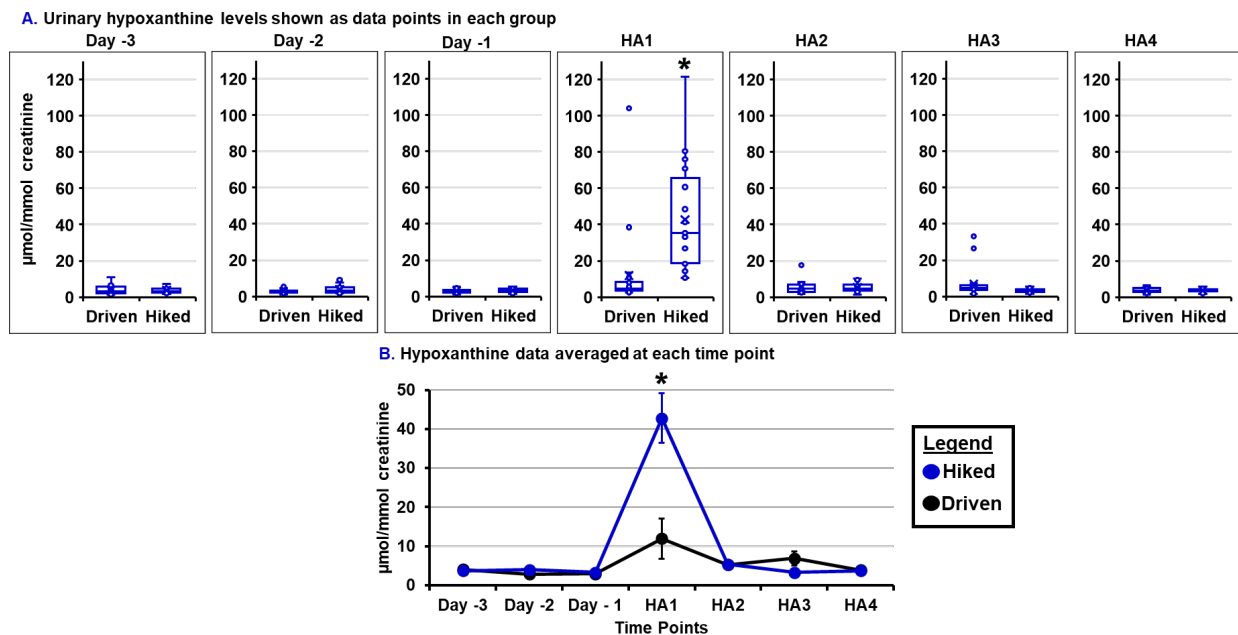


Figure 25. Urinary hypoxanthine clearance at sea level (day -3, day -2, and day -1) and high altitude (HA1, HA2, HA3, and HA4) for subjects who were driven to Kachina Peak facility in Taos Ski Valley, NM (~3600 masl) and those who hiked to reach this facility. The asterisk denotes significant differences between the two groups ($p \leq 0.05$).

4.8 Summary of Hiking-induced Metabolic Pathway Alterations

We have summarized the metabolic pathways that were altered in subjects that hiked to reach Kachina Peak facility in Taos Ski Valley, NM (~3597 masl) compared to those driven up to this facility (Figs. 26-29). These summaries provide a clear visualization of the effects of additional energy expenditure in the form of uphill hiking on urinary metabolite profiles during the acute response to high altitude exposure (at HA1) and acclimatization phase (at HA2 and beyond). Urinary clearances of 14 metabolites were altered during the HA acute response phase captured on HA1 in the Hiked vs. Driven group (Fig. 26). The affected pathways included glycolysis (pyruvate and lactate), creatine metabolism (sarcosine and creatinine), nicotinate (trigonelline) and nicotinamide (1-methylnicotinamide) metabolism, purine and pyrimidine metabolism (hypoxanthine), ketone body metabolism (acetoacetate, acetone, and β -hydroxybutyrate), amino acid metabolism (glycine), fatty acid metabolism (carnitine), amino fatty acid/amino acid metabolism (isobutyrate), and the TCA cycle (citrate).

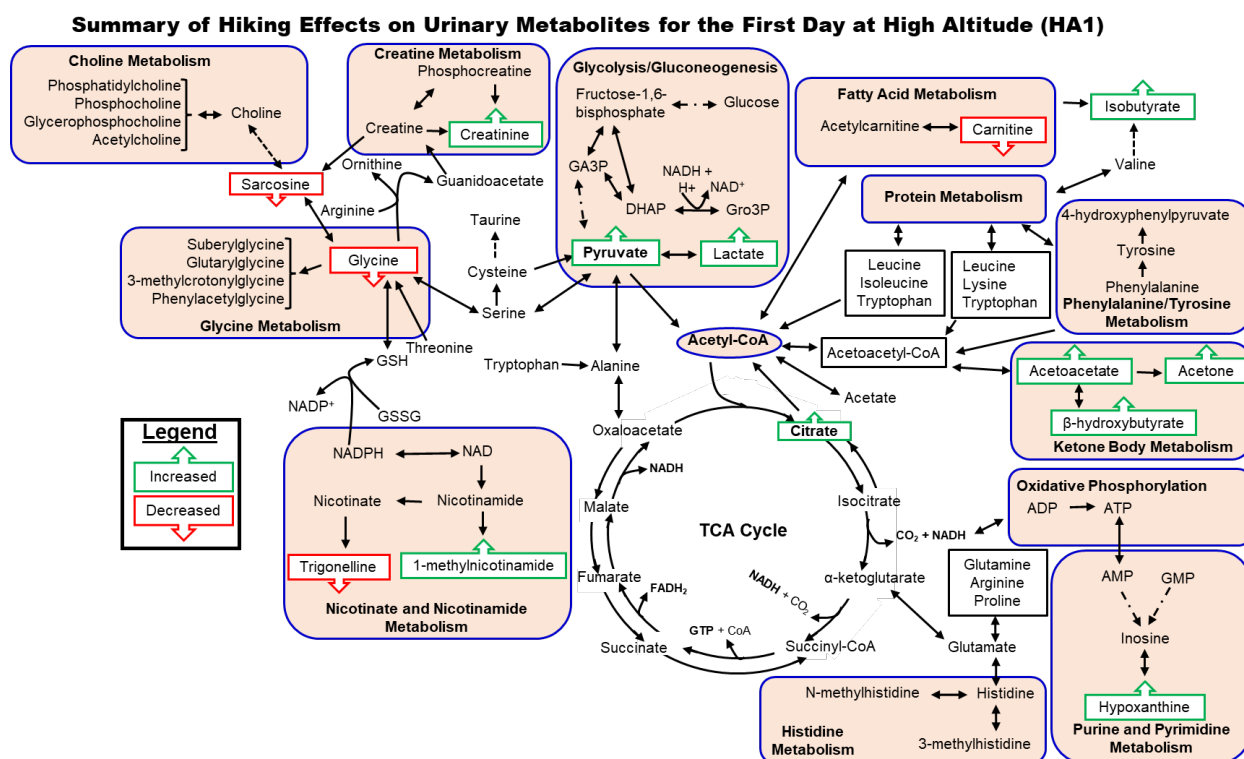


Figure 26. A summary of the hiking-induced metabolic pathway alterations for the first day at high altitude (HA1). Green boxes indicate the metabolites that were increased in urine samples collected from the subjects who hiked (Hiked group) up to the top of Kachina Peak (~3600 masl) relative to those collected from the subjects who were driven (Driven group) up to the top of this Peak. Red boxes indicate the urinary metabolite levels decreased, whereas a green box indicates an increase in urinary levels.

On HA2, only urinary levels of citrate, acetoacetate and choline were elevated in the Hiked group vs. Driven group (**Fig. 27**). Citrate and acetoacetate were among the metabolites for which clearance was increased at HA1.

A rebound in the number of urinary metabolites that displayed alterations for the Hiked group was noted on HA3 (**Fig. 28**). The Hiked group displayed an increase in clearance of six metabolites while one metabolite (taurine) decreased. The affected pathways included creatine metabolism (sarcosine and creatinine), purine and pyrimidine metabolism (hypoxanthine), ketone body metabolism (acetoacetate), amino acid derivative metabolism (taurine and N-methylhistidine), and the TCA cycle (citrate).

On HA4, the Hiked group demonstrated decreases in urine clearances of taurine, carnitine, choline while increases were seen in citrate (**Fig. 29**). In the Hiked group, citrate was the only metabolite consistently elevated in urine during the entire high altitude exposure.

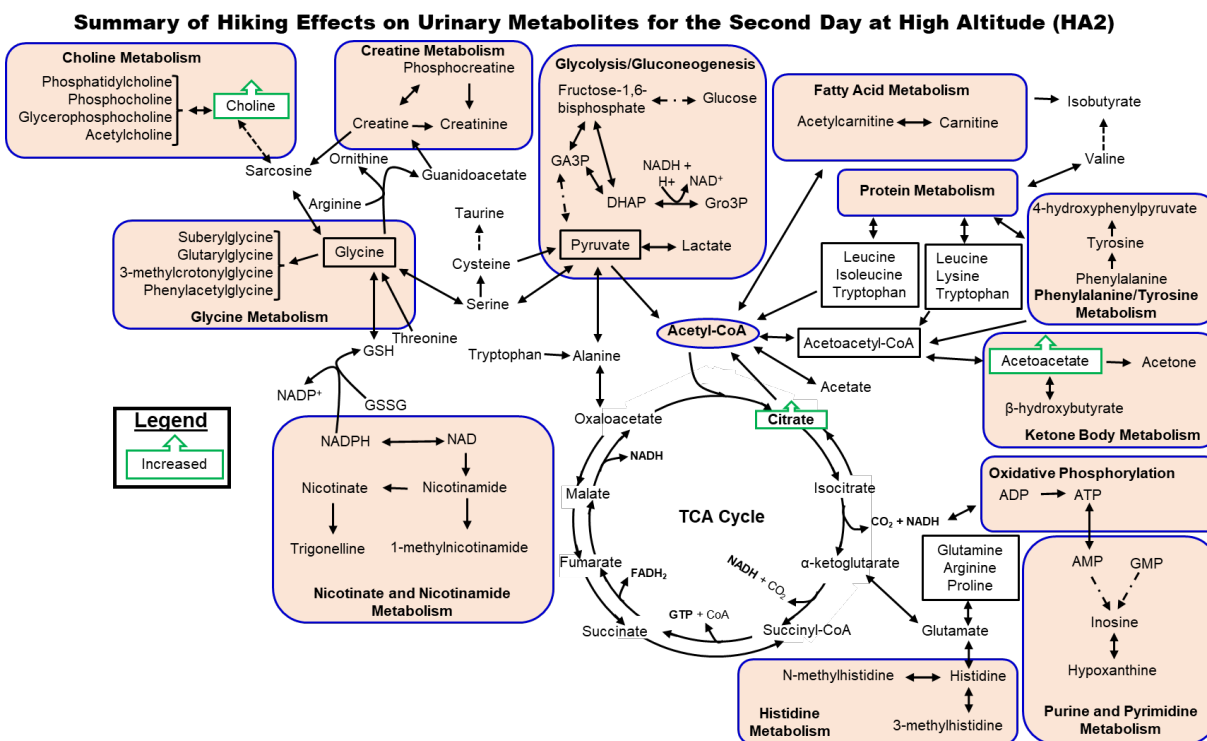


Figure 27. A summary of the hiking-induced metabolic pathway alterations for the second day at high altitude (HA2). Green boxes indicate the metabolites increased in urine samples collected from the subjects who hiked (Hiked group) up to the top of Kachina Peak (~3600 masl) relative to those collected from the subjects who were driven (Driven group) up to the top of this Peak. Red boxes indicate a decrease in urinary metabolite levels decreased, whereas a green box indicates an increase.

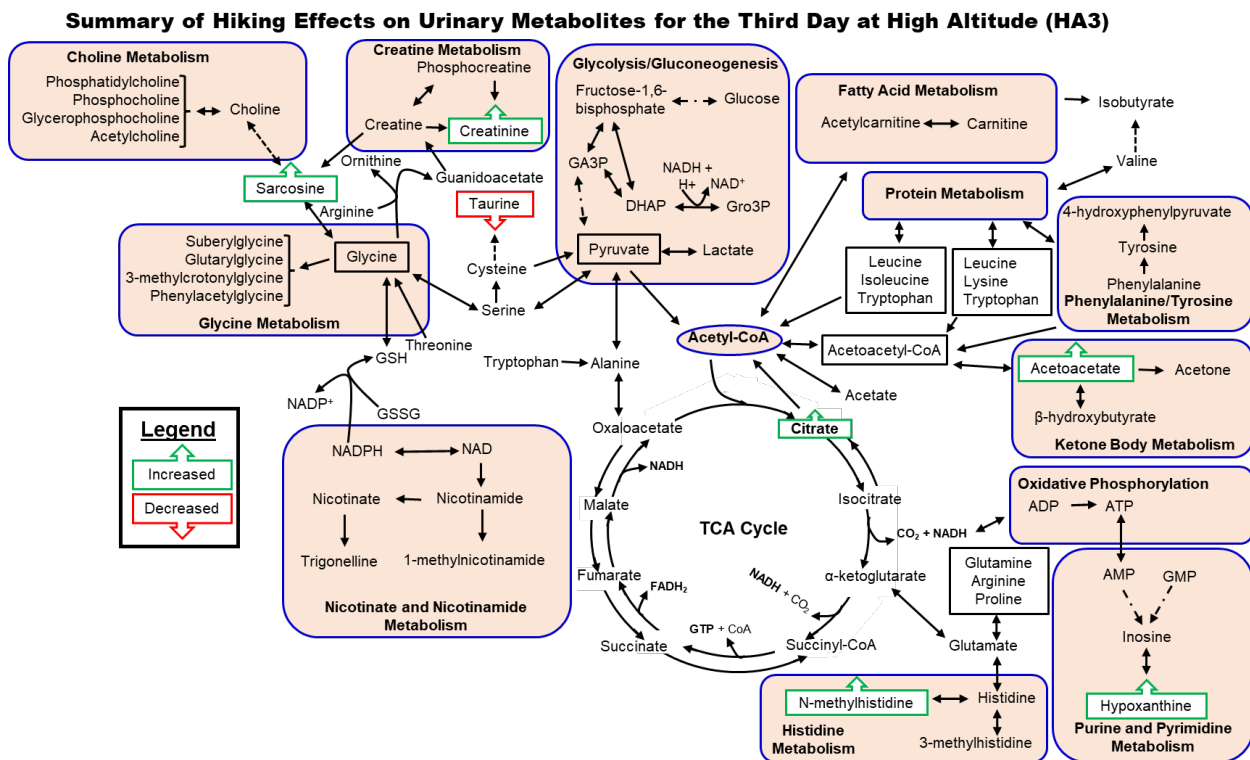


Figure 28. A summary of the hiking-induced metabolic pathway alterations for the third day at high altitude (HA3). Green boxes indicate the metabolite levels increased in urine samples collected from the subjects who hiked (Hiked group) up to the top of Kachina Peak (~3600 masl) relative to those collected from the subjects who were driven (Driven group). Red boxes indicate a decrease in urinary metabolite levels decreased, whereas a green box indicates an increase.

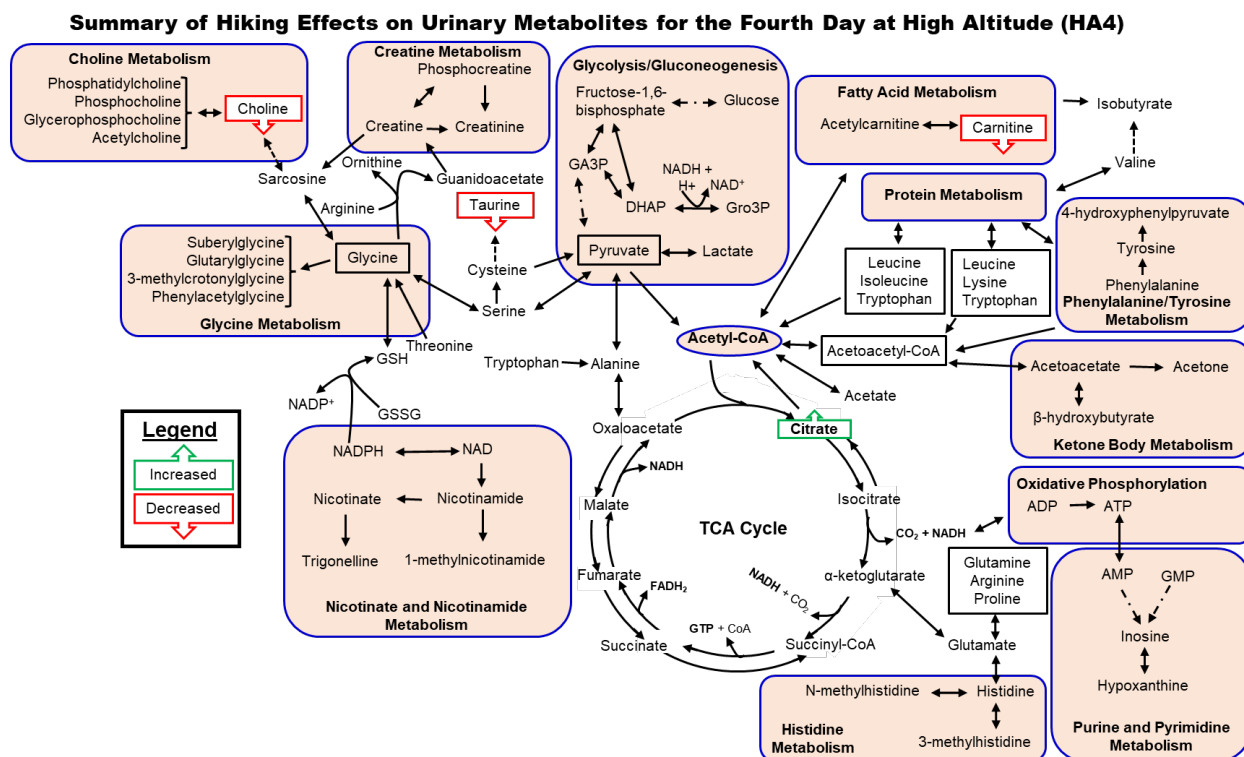


Figure 29. A summary of the hiking-induced metabolic pathway alterations for the fourth day at high altitude (HA4). Green boxes indicate metabolite increased in urine samples collected from the subjects who Hiked (Hiked group) up to the top of Kachina Peak (~3600 masl) relative to those driven (Driven group). Red boxes indicate a decrease in urinary metabolite levels decreased, whereas a green box indicates an increase.

5.0 DISCUSSION

5.1 High Altitude Exposure is Associated with Changes in Urinary Metabolite Profiles

The current study used NMR-based metabolomics to investigate and identify urinary metabolite changes associated with energy expenditure (hiking) prior to high altitude environments. The metabolomics focused on timepoints prior to altitude exposure at sea level as well as multiple days at high altitude. No major variations in metabolite profiles were noted among the participants during the time spent at sea level. This observation suggests that the major metabolite influencing factors such as genetics, diets, medications, were a minimal or non-variable in the metabolomic output.

The effect of altitude exposure on the urinary metabolite profiles was assessed by comparing metabolite clearance at HA relative to clearance on the last day at SL (day -1) for the Driven group, who were only affected by the high altitude exposure, and could serve as a ‘control’ for altitude effects. Unlike the Driven group, molecular modulations of the Hiked group were a result of a combination of altitude exposure and additional energy utilization. Uphill hiking exercise increased the energy expenditure elevating cellular glucose utilization. Our data indicated that moving from SL to HA produced profound alterations in various cellular metabolic pathways for both groups (**Table 1**), reflecting cellular responses to reduced levels of oxygen supply. Metabolic pathways that were altered belong to energy, nicotinamide, amino acid, ketone body, fatty acid, phospholipid, and neurotransmitter metabolisms. The most significant clearance alterations occurred on the first day at high altitude compared to other HA time points, a period we term the “*Phase I Acute Response*”. The dramatic changes noted at HA1 reflect the immediate cellular adaptation process initiated by HIF- α and a number of oxygen sensing mechanisms in response to reduced oxygen supply.

5.2 Energy Expenditure During the Ascent to High Altitude Increases Glycolysis By-products and Substrates of the TCA Cycle

A profound increase in urinary clearance of 10 metabolites and decrease in excretion of four metabolites were noted for the Hiked group on the first day of HA exposure relative to Driven group (**Fig. 26**). These included metabolites involved in energy production such as pyruvate and lactate. Expectedly, subjects who hiked displayed 10-fold and 96% increases in clearance of lactate and pyruvate, respectively, compared to the Driven group (**Figs. 5 & 6**). These observations suggest that the energy expenditure associated with the uphill hiking to high altitude exacerbated the effects initiated by hypoxia exposure. Besides the anaerobic conditions which alter energy production, it is likely that the uphill hiking exercise increased the cellular energy demands during *Phase I Acute Response* when the body has initiated a shift to other energy-generating mechanisms, thus altering the activities for the glycolytic and other energy producing enzymes.

Exercise initiates drastic alterations in the body’s metabolic events, such as increasing lipolysis and glucose uptake in the skeletal muscles, thus driving energy expenditure above 10-fold in the entire body along with 100-fold increase in exercising muscle tissues ([Nakayama et al., 2019](#)). For the body to maintain the energy production, the increase in ATP utilization rate must be met by a similar increase in ATP production rate ([Katz, 2022](#)) and this can be achieved by the skeletal muscles which possess the unique ability to speed up energy turnover several 100- fold in less than a second ([Hultman and Sjöholm, 1983](#); [Sahlin et al., 1998](#)). Nakayama et al ([Nakayama et al., 2019](#)) demonstrated that ten healthy young men and women (age, 24.0 $\pm$ 1.4 years) who performed the uphill treadmill walking (+5%) trial for 30 min on a speed of 6 km/hr

displayed significantly ($p < 0.01$) lower fasting blood glucose levels on the next day of treadmill exercise compared to the levels measured during the rest trial. These observations suggest that the uphill exercise increased the glucose consumption. Stanley et al. (Stanley et al., 1988) reported that 20% of glucose utilization in ten healthy endurance-trained men (aged 25 ± 6 years) subjected to moderate-intensity exercise for 30 to 50 minutes could be traced in blood lactate. This may explain the spike noted in urinary clearance of lactate for the Hiked subjects following their ascent to high altitude. The clearance changes observed for lactate also support prior reports that indicated an increase in blood lactate levels, muscle glycolytic intermediates, and decreasing blood glucose concentrations for lowlander individuals ascending to high altitude (Siervo et al., 2014; Horscroft et al., 2017). The current findings also support the data that demonstrated that hypoxia induced upregulation of lactate dehydrogenase (Firth et al., 1995), inactivation of pyruvate dehydrogenase, suppression of pyruvate's entry into the TCA cycle, and active formation of lactate via pyruvate.

All these cellular processes are associated with increased HIF-1 α activity (Kim et al., 2006). It is well known that hypoxia promotes glucose anaerobic metabolism. A metabolic switch for adaptation to hypoxic conditions includes activation of glycolytic genes by HIF-1 α and increased conversion of glucose to pyruvate and subsequently to lactate by the lactate dehydrogenase enzyme (Kim et al., 2006), instead of channeling pyruvate to pathways producing acetyl-coA as this molecule is required in the first step of the TCA cycle. Cellular oxygen demands exceed its supply while the body is subjected to hypoxic conditions. As such, mitochondrial energy production is significantly reduced due to limited availability of the oxygen supply and downregulation of pyruvate dehydrogenase complex, a key enzyme in connecting glycolysis to TCA cycle (Wang et al., 2018). Studies have demonstrated that during hypoxia, brain tissues (Drewes et al., 1973; Siesjd et al., 1974) and heart muscles (Neely and Morgan, 1974) experience a buildup of lactate and a decline in energy supply. Thus, subjects spending time in hypoxic environments may have sustained anaerobic glycolysis resulting in the production of large quantities of lactate and pyruvate. Cellular storage of these two molecules may be overwhelmed by their excessive production, thus leading to their increased urinary elimination. Observations that lactate and pyruvate clearance levels for both study groups were only elevated on the first day of HA exposure (HA1) suggest that cellular alterations in production of these molecules are transient and chronic hypoxia does not significantly influence their urinary elimination levels.

Citrate was another energy metabolism substrate for which the urinary excretion was affected by altitude exposure. This molecule plays a key role in various cellular processes. Apart from providing the cell with a source of nicotinamide adenine dinucleotide phosphate (NADPH) in a reduced form required for redox regulation and anabolic synthesis, citrate is also a primary source of carbon for fatty acid and cholesterol synthesis (Hatzivassiliou et al., 2005) and acetyl-CoA for protein acetylation (Wellen et al., 2009). The urinary clearance was elevated at high altitude for all the subjects regardless of their study group assignment when compared to the clearance measured on day -1 at sea level prior to high altitude exposure. The alteration in clearance levels were more pronounced for the Hiked group compared to the Driven group, which was opposite to expectations. We presumed that clearance levels of citrate would decline at high altitude since one of the well-known cellular hypoxia adaptation mechanisms is the switch to glycolytic anaerobic metabolism, channeling pyruvate to lactate production instead of acetyl-CoA formation required for citrate synthesis. Under normoxic conditions cells synthesize citrate, one of the TCA cycle substrates, by condensing oxaloacetate with acetyl-CoA by citrate synthase. Acetyl-coA molecule derives from pyruvate degradation and fatty acid β -oxidation.

However, other studies have shown that under hypoxic conditions, this pathway is altered (Wise et al., 2011). Cellular increase of citrate during hypoxic conditions has been the subject of several investigations (Wise et al., 2011; Fuhrmann et al., 2019). Some cells are able to maintain citrate production despite a profound decline in glucose-dependent citrate production by reducing glutamate to α -ketoglutarate and then to isocitrate, which is in turn isomerized to citrate (Wise et al., 2011). Wise et al. also reported that subjecting glioblastoma cells to hypoxia (0.5% O₂) resulted in an increase in glucose uptake, its conversion to lactate, and a dramatic decline in its conversion to citrate. While the authors noted a constant consumption of glutamine, the hypoxic cells actively used this molecule as a source for α -ketoglutarate production. Other reports (Fuhrmann et al., 2019) indicate that when THP-1 monocytes cells were exposed to acute hypoxia (16 hr), oxygen consumption predominantly required pyruvate. Transitioning from acute to chronic hypoxia (72 hr) involved the cellular respiration shifting from pyruvate-centered energy production to fatty acid β -oxidation. The electron transport chain was fueled via electron-transferring flavoproteins-based electron flow together with an enhanced glutamine oxidation pathway. These findings clearly show that glutamate plays a crucial role in maintaining the cellular pools of citrate and cell growth under hypoxic conditions. Our study did not find altitude-induced alterations in urinary levels of glutamine, α -ketoglutarate, or isocitrate. However, the clearance levels of citrate were still elevated on the fourth day of high altitude exposure, suggesting that the adaptation phase also has a significant impact on cellular pools of this metabolite.

5.3 Energy Expenditure During the Ascent to High Altitude Alters Creatine Metabolism

The current study also examined that creatine metabolism changes after high altitude exposure. Creatinine urinary clearances for the Driven group only significantly decreased on the first day of altitude exposure relative to day -1 at sea level (Table 1). Unlike the Driven group, no change in creatinine clearance was noted for the Hiked group for the entire stay at high altitude, suggesting that uphill hiking may have negated the impact of hypoxia on the cellular levels of this metabolite. In fact, the clearance level for the Hiked group increased 66% at HA1 relative to the Driven group (Fig. 4). In humans, creatinine is primarily formed nonenzymatically in muscle tissues from creatine and phosphocreatine degradation. The current study also measured the levels of urinary creatine and did not find altitude and/or uphill hiking-induced alterations in its clearance levels. The hydrolysis of creatine by creatine amidinohydrolase (creatinase) enzyme also yields sarcosine (also known as N-methyl-glycine) and urea molecules (Coll et al., 1990). Sarcosine clearance levels at sea level and high altitude were also assessed and shown to be significantly decreased at high altitude (Fig. 2), with a substantial clearance decline observed for the Hiked group relative to the Driven group. A decrease in sarcosine excretion suggests that the limited oxygen supply may have altered its cellular formation. Sarcosine can also be synthesized from glycine (Fig. 26) by N-methyl transferase enzyme while sarcosine dehydrogenase can reverse this reaction and re-generate sarcosine. A comparison between Hiked and Driven groups indicated that the hiked subjects excreted significantly lower levels of glycine at HA1 relative to the Driven individuals (Figs. 17 and 26). These findings suggest that energy expenditure (i.e. hiking) prior to high altitude exposure exacerbated alterations in cellular glycine metabolism.

5.4 Energy Expenditure During the Ascent to High Altitude Altered Fatty Acid Metabolism

The current study also noted that exposure to hypoxic conditions altered the urinary elimination of metabolites associated with fatty acid (FA) metabolism such as carnitine, acetylcarnitine, isobutyrate, and acetate. Clearance alterations for acetate, isobutyrate, and acetylcarnitine were transient for the Driven group as they were noted only on the first day of altitude exposure

(HA1) ([Table 1](#)). However, the Hiked group displayed chronic clearance alterations for acetate and isobutyrate with urinary levels consistently elevated until the last day of altitude exposure (HA4), strongly suggesting that hypoxia altered FA metabolism. As differences were noted between the Driven and Hiked groups, it also demonstrates that energy expenditure during the ascent (hiking) to high altitude enhanced altitude-induced alterations of FA metabolism.

Cells are supplied with FA via three main sources that includes (1) FA arising from acetyl-CoA via *de novo* synthesis, (2) FA deriving from the hydrolysis of triglycerides, acetylated proteins, and (3) exogenous FA deriving from diets and entering the cells via the blood and/or gut lumen ([Cooper et al., 2015](#)). The increase in clearance of FA metabolites noted in the current study may support previous findings that indicated impairment of the FA β -oxidation capacity at high altitude ([Horscroft and Murray, 2014](#)). FA molecules are crucial for cell survival and proliferation under both normoxic and hypoxic conditions, where they are required in various cellular processes such as production of signaling molecules, membrane synthesis, energy production, and storage in form of triacylglycerols ([Mylonis et al., 2019](#)). As the oxygen supply becomes limited under hypoxia, the cells develop adaptation mechanisms which include modifying FA metabolism. The accumulation of lipids under hypoxic conditions is supported by the inhibition of enzymes involved in FA degradation ([Mylonis et al., 2019](#)). FA β -oxidation is actively reduced via the HIF-1- and HIF-2-dependent downregulation of the transcriptional coactivator of β -oxidation enzyme PGC-1 α (proliferator-activated receptor- γ coactivator-1 α) ([Liu et al., 2014](#)) and carnitine palmitoyltransferase 1A (CPT1A), a mitochondrial enzyme that catalyzes the formation of acyl carnitines ([Liu et al., 2014](#); [Du et al., 2017](#)). Other data demonstrated that oxygen consumption under chronic hypoxia (72 hr) fueled the electron transport chain (ETC) via electron-transferring flavoproteins and increased fatty acid production and consumption through the glutamine-citrate-fatty acid axis, while under the acute hypoxia (16 h), demand for pyruvate was predominant ([Fuhrmann et al., 2019](#)). These observations suggest that in the first hours of hypoxic exposure, energy production mostly occurs via glycolysis with a switch to fatty acid metabolism once the glycolytic substrates are depleted. Other studies reported an increase in urinary excretion of carnitine and acetylcarnitine for six healthy sedentary men exposed to acute hypoxic conditions of 12% and 15% O₂ for 2 h in a normobaric hypoxia chamber ([Lou et al., 2014](#)). The body requires carnitine and acetylcarnitine in order to turn fatty acid molecules into energy. Carnitine is best known for its role in shuttling esterified FA from the cytoplasm into the mitochondria for subsequent β -oxidation and returns to cytoplasm in the form of acetylcarnitine ([Longo et al., 2006](#); [Hsu et al., 2023](#); [Izzo et al., 2023](#)). Existing evidence indicates that hypoxia mediates the modifications of various aspects of lipid metabolism ([Mylonis et al., 2019](#)). Uphill hiking requires a considerable amount of energy expenditure, and the exertion may have rapidly depleted the energy supplied via glycolysis. Under these circumstances, the body upregulated FA metabolism to cope with the increasing demands in energy supply.

5.5 Energy Expenditure During the Ascent to High Altitude Increased Urinary Clearance of Ketone Bodies

The current study documented that moving from sea level to high altitude increased the urinary clearance of ketone bodies. Acetoacetate and acetone clearance increased only on the first day of high altitude exposure for the Driven group ([Table 1](#)). Clearance alterations in the Hiked group were also seen at HA1 but persisted until HA2 for acetone and HA3 for acetoacetate. Moreover, the clearance alterations were more pronounced for the Hiked group, suggesting that the hiking exercise enhanced the hypoxia-induced effects on cellular production of these molecules and extended their return to baseline levels. This assertion is supported by the observations that

levels of acetoacetate, acetone, and β -hydroxybutyrate for the hiked subjects were consistently elevated at high altitude relative to subjects driven to altitude (Figs. 12, 13, and 14). Since acetone and β -hydroxybutyrate are degradation by-products of acetoacetate, correlation analyses between these two molecules and acetoacetate were conducted to assess if hypoxia influenced their relationships. Strong correlations between the urinary levels of these molecules at high altitude were only achieved for the Hiked group (R^2 coefficients > 0.62) (Figs. 15 and 16). A lack of strong correlations between acetone, β -hydroxybutyrate, and acetoacetate for the Driven group suggests that enhanced production of acetone and β -hydroxybutyrate for the Hiked group was due to energy expenditure during the ascent to altitude (hiking) rather than altitude exposure. Acetoacetate can derive from pyruvate degradation and fatty acid oxidation. Thus, increases in ketone body excretion suggest that hiking induced an increase in cellular fatty acid degradation and/or glycolytic activities, leading to elevation in acetyl-CoA molecules. Under normoxia, acetyl-CoA molecules are channeled to TCA cycle for nicotinamide Adenine dinucleotide (NADH) in a reduced form and flavin adenine dinucleotide (FADH₂) in a reduced form production which are required in oxidative phosphorylation to generate ATP. The hiking-induced acetyl-CoA production under hypoxic conditions may have overwhelmed the Krebs cycle, thus diverting these molecules towards hepatic ketogenesis.

It is also possible that uphill hiking induced protein breakdown, thus releasing ketogenic amino acids such as leucine, lysine, isoleucine, phenylalanine, tryptophan, and tyrosine (Fig. 26). These amino acids may have been further converted to acetyl-CoA and/or acetoacetyl-CoA, subsequently giving rise to ketone bodies. It has been suggested that hypoxia mediates lipolysis and muscle breakdown resulting in ketone body accumulation and the release of amino acids that further enhance the ketogenesis providing tissue-specific hypoxic protection (Murray and Montgomery, 2014).

Unlike other tissues, the brain cannot extract energy from fatty acid oxidation when the blood glucose levels become limited (Laffel, 1999). Under these conditions, ketone bodies become an alternative source of fuel for the brain (Laffel, 1999; García-Rodríguez and Giménez-Cassina, 2021). It has been demonstrated that rat brain tissues preferentially utilized ketone bodies over glucose when exposed to hypoxia (Kirsch and G, 1984). As limited oxygen availability reduces glycolytic metabolism which could yield pyruvate, a substrate for acetyl-CoA synthesis, the ketones bodies circumvent the glycolytic pathway and enter the TCA cycle via acetyl CoA, thus keeping the ATP production pathway active.

5.6 Amino Acid Metabolism Altered by Energy Expenditure and/or High Altitude Exposure

The effects of uphill hiking and high altitude exposure on urinary excretion of amino acid were also assessed in the current study. Urinary clearance of alanine was increased following the altitude exposure (Table 1). Other studies have reported hypoxia-induced alterations in amino acid metabolism (Mudge et al., 1976; Chang et al., 2019). However, data have demonstrated that free amino acids contribute to the metabolic response to cardiac hypoxia in a lesser extent than carbohydrates (Mudge et al., 1976). Reports for various studies have indicated that heart muscle deprived of oxygen increases alanine production (Jefferson et al., 1971; Taegtmeier et al., 1977; Taegtmeier, 1978). Our study suggested that the increase in alanine resulted from elevated levels of pyruvate substrates and glutamate transamination. High levels of pyruvate in heart tissue exposed to hypoxia has been shown to increase alanine production (Taegtmeier et al., 1977). Elevated rates of anaerobic glycolysis increased the substrate supply for both lactate dehydrogenase and alanine amino transferase reactions, thus elevating the cellular levels of

pyruvate and alanine, respectively. The transamination reaction, performed by alanine amino transferase, requires the availability of alanine and α -ketoglutarate to generate pyruvate and glutamate. The reversal reaction is also carried out by this enzyme. Increased clearances of lactate, pyruvate, and alanine were seen following exposure to high altitude environments ([Table 1](#)). Alanine amino transferase activity may have also been upregulated in the current study, although no urinary clearance alterations were noted for glutamate and α -ketoglutarate. Our study indicated that the addition of additional energy expenditure (hiking) to high altitude-induced stress did not alter hypoxia-mediated alanine elimination.

Glycine was also examined for modulation of clearances at sea level and high altitude. Clearance in the Hiked group at HA1 decreased 33% relative to the Driven group, thus suggesting that energy expenditure sustained during the ascent to high altitude (uphill hiking) negated the hypoxia-induced effect on elimination of this molecule. Studies have indicated that glycine may possess protective attributes against hypoxia ([Tijssen et al., 1997](#); [Petrat et al., 2012](#); [Cai et al., 2019](#)) and hypoxia-mediated ischemic brain injury ([Petrat et al., 2012](#); [Cai et al., 2019](#)). Although the mechanisms by which glycine confers protection are not well characterized, it has been suggested that since hypoxia promotes the activity of several classes of proteases, glycine may provide protection via protease activation ([Tijssen et al., 1997](#)). However, it is mechanistically unclear why the Hiking group displayed a decrease in glycine clearance. Further studies are needed to elucidate the mechanisms by which energy expenditure may alter the clearance of this metabolite.

While taurine is not one of the canonical 20 amino acids, it is synthesized from an amino acid (cysteine). A slight increase in taurine clearance level was noted for the Driven subjects following their move to high altitude but the level was not statistically different from that measured at sea level ([Fig. 21](#)), suggesting that hypoxia did not have an impact on taurine excretion. When clearances from the two groups were compared, however, the Hiked group displayed consistently lower levels of taurine during the entire stay at high altitude with the differences becoming significant on the third and fourth day (decreases of 28% and 30%, respectively), suggesting that the uphill hiking exerted a delayed negative effect on taurine elimination. Studies have suggested that taurine may possess a protective attribute against hypoxia-mediated effects although the protection mechanisms are not well understood ([Pan et al., 2012](#); [Chen et al., 2013](#)). A report from Pan *et al.* indicated that taurine inhibited the hypoxia/reoxygenation-induced upregulation of caspase-12 and GADD153/CHOP in primary cortical neuronal cultures ([Pan et al., 2012](#)). Other data indicate that taurine provides robust protection to human neuroblastoma cells exposed to hypoxia or oxygen/glucose deprivation ([Chen et al., 2013](#)). If taurine does play a protective role under hypoxia, the cells must limit its clearance under hypoxic environments to allow protection against these conditions. A significant decrease in taurine clearance noted for the Hiked group supports the hypothesis that taurine is retained by cells for its utilization in suppressing hypoxic effects.

5.7 Other metabolic pathways altered by the Hiking-Induced Energy Expenditure and High Altitude Exposure

The current study also noted that exposure to hypoxic conditions and hiking induced alterations in by-products of nicotinamide metabolism such as 1-methylnicotinamide ([Fig. 24](#)) and trigonelline ([Fig. 23](#)). Excretion of 1-methylnicotinamide in the Driven group displayed a more than 2-fold increase at HA1 relative to sea level. Clearance alterations were exacerbated by hiking as the Hiked group displayed an 8-fold increase which disappeared the following day. 1-methylnicotinamide is a signaling molecule generated in skeletal muscle that enhances the

utilization of energy stores in response to limited availability of muscle energy (Ström et al., 2018). Tong et al. (Tong et al., 2012) indicated that pretreating cultured rat cardiomyocytes with nicotinamide prior to a 6 h hypoxic exposure markedly reduced hypoxia-mediated necrosis and apoptosis by improving mitochondrial stress. It has also been suggested that nicotinamide provides protection to myocardial cells against chronic hypoxia by regulating mTOR pathway and mediating autophagy (Li et al., 2019).

The uphill hiking-induced energy expenditure during the ascent to altitude also affected the purine and pyrimidine metabolism. This assertion is supported by the observation that hypoxanthine clearance was significantly elevated (a large 2.6-fold increase) at HA1 for the Hiked vs. Driven group (Fig. 25). Hypoxanthine has been regarded as a sensitive indicator for hypoxia (Saugstad, 1988). An increase in urinary excretion of hypoxanthine was also seen in the previously mentioned study by Lou et al. in a human study examining a 2 h acute hypoxic exposure (Lou et al., 2014). As hypoxanthine is created during ATP breakdown, the elevated urinary clearance for the Hiked group is likely due to increase in energy expenditure translated into an increase in ATP breakdown as the muscles utilized additional energy resources during the ascent to high altitude.

5.8 Study Limitations and Future Study Modifications

The age for the participants in the current study ranged between 19 and 38 years. While appropriate for militarily relevant populations, this age range may not reflect responses in an older population. Moreover, the majority of subjects were male, with only two females enrolled in this study. Thus, the findings and conclusions should therefore be limited only to males within this age group. Further studies should include individuals with ages falling outside of the study's age group to extend the age coverage. More importantly, a large cohort size of female subjects should also be evaluated to determine gender-related differences in molecular mechanisms involved in responding to uphill hiking and acclimatization to high altitude.

In this study, diet was not a controlled variable. However, subjects were requested to not take dietary supplements during this period, and all medicinal uses were notated. The diet regimen, taken days prior to ascent to high altitude, may contribute to some minor metabolomic variations noted among participants. As mentioned previously, one subject (identified in Fig. 15) did demonstrate unusual acetoacetate and acetone excretion while at high altitude, but we were unable to identify a cause for these unusual clearance levels from the meta data. However, as selection into the two groups (Hiking, Driven) was entirely random, it is much less likely that the diet at sea level was a significant variable in the metabolic clearances. However, further studies based on our data set are needed to investigate whether potential dietary factors can exert significant impact on individual energy utilization and performance during the uphill hiking, and acclimatization to high altitude. They should include a control group with controlled/standardized dietary intake throughout the investigation period.

Another factor which may have influenced the urinary metabolite output during the ascent to high altitude and at high altitude is the individual's normal exercising routine and general fitness. In theory, the endurance and urinary metabolite profiles for the subjects who regularly partake in exercise as part of their normal life may be different compared to those who were not regularly exercising. However, study subjects regularly exercised and would not be considered sedentary, although there may have been differences in exercise intensity between individuals. While we do not believe this to have a significant impact on the results seen in our study, in theory these could impact the data homogeneity, thus introducing more variations in the data and influencing the direction of the results. Future studies that involve the use of homogenous groups are warranted.

Lastly, our examination of acclimatization was limited to only four days at high altitude. Follow-on studies should include additional time points to determine which metabolite/pathway changes return to a normoxia baseline and which are established at a new hypoxic baseline. The latter are especially of interest in determining required pathway alterations for lowlander adaptation for long term high altitude missions.

6.0 CONCLUSIONS

This study delineated metabolite and pathway alterations altered by additional energy expenditure just prior to high altitude exposures. Our data indicated that this energy expenditure modified altitude-induced pathway alterations and seemingly changed both the acute response and the acclimatization timeline.

The current study demonstrated that the altitude-induced hypoxic effects on urinary metabolite clearance were transient for most of the metabolites assessed as their alterations were only noted on the first day of high altitude exposure, a period we have identified as the first phase involving the body's immediate acute hypoxic response. However, longer chronic hypoxic exposures after day one clearly shifts to a second phase of adaptation and acclimatation which were observed for pyruvate, citrate, sarcosine, and hypoxanthine as the alterations in their clearance persisted until at least the third day at high altitude.

Using the Driven group as a control for altitude-induced effects, this study clearly identified that energy expenditure during the ascent to high altitude exacerbated several metabolite clearance alterations and likely pathway modulations. Metabolic pathways that were mostly affected by this energy expenditure belong to energy, nicotinamide, ketone body, fatty acid, phospholipid and neurotransmitter metabolisms. Evidence for uphill hiking-induced enhancement of metabolite clearance is reflected in (1) the profound changes noted on the first and second day of high altitude exposure, (2) consistently elevated clearances for citrate, acetate, and isobutyrate throughout of the entire stay at high altitude and (3) a number of alterations that were noted only on day 3 and day 4 at high altitude. All these urinary metabolite changes were noted only for the subjects who hiked to reach the high altitude of Kachina Peak.

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LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CPT1A	Carnitine palmitoyltransferase 1A
D ₂ O	Deuterium oxide
Day -1	First collection at sea level
Day -2	Second collection at sea level
Day -3	Third collection at sea level
DHAP	Dihydroxyacetone phosphate
ETC	Electron transport chain
FA	fatty acid
FADH ₂	Flavin adenine dinucleotide in a reduced form
GA3P	Glyceraldehyde 3 phosphate
GMP	Guanosine monophosphate
Gro3P	Glycerol 3 phosphate
GSH	Glutathione
GSSH	Glutathione disulfide
GTP	Guanosine triphosphate
hr	hour
¹ H NMR	Proton nuclear magnetic resonance
HA	High altitude
HA1	First day at high altitude
HA2	Second day at high altitude
HA3	Third day at high altitude
HA4	Fourth day at high altitude
HIF	Hypoxia-Inducible Factor
HMDB	Human metabolome database
MANOVA	Multivariate analysis of variance
MHz	megahertz
mm	millimeter
mM	millimolar
masl	meters above sea level
min	minute
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate in a reduced form
NAD	Nicotinamide Adenine dinucleotide
NADH	Nicotinamide Adenine dinucleotide in a reduced form
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
SD	standard deviation
S.E.	Standard error of the mean
SL	Sea level
TCA	Tri-carboxylic acid
TSP	2,2',3,3'-tetradeutero-trimethylsilylpropionic acid
μl	microliter
USARIEM	Army Research Institute of Environmental Medicine
WPAFB	Wright-Patterson Air Force Base
yr	year
%	percent