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Understanding Plasticity at High Altitude: Sleep, Cognition, and Epigenetic Modifications

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DEDICATION

Dedicated to my family; My mom who's always encouraged me to defy expectations, even when she didn't fully understand my path. She saw me graduate high school, the first in my family. Helped me get to college and cried with me when I had to drop out. She has continuously talked me into taking crazy life changing opportunities including going to grad school, studying abroad, and moving across the country. Twice.

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ABSTRACT OF THE DISSERTATION

Understanding Plasticity at High Altitude: Sleep, Cognition, and Epigenetic Modifications

by

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Dr. Erica Heinrich, Chairperson

Oxygen is integral to human energy production and homeostasis. High-altitude environments pose a significant challenge due to reduced oxygen availability, which causes a cascade of plastic and enduring responses to improve oxygen delivery to tissue. These modifications are reversible and plastic, contributing to the complex high-altitude adaptation process. After multiple generations of exposure, the stressful hypoxic environment produces substantial selective pressure which leads to evolutionary adaptation through unique genetic mechanisms, producing many adaptations and phenotypes.

In this dissertation, I examined the mechanisms underlying acute ventilatory acclimatization to hypoxia (Chapter 2) and its downstream impacts on performance at high altitude in sojourners, including the onset of sleep-disordered breathing and cognitive impairments (Chapter 3). Our findings confirmed alterations in HVR, decreased sleep quality and duration, and cognitive declines in tasks such as those testing memory and vision, across the study period.

I then investigated both global and targeted DNA methylation levels (Chapter 4) and changes in H3 histones and modifications (Chapter 5). We explored genes shown to be under selection in high-altitude populations, within the hypoxia-inducible factor (HIF) pathway, crucial in high-altitude adaptation, from an epigenetic perspective. Our investigations revealed global hypermethylation which is reflected in our genes of interest during high-altitude exposure, intensifying over time and peaking in Andean high-altitude residents. We also identified large increases in H3 histone and H3 histone modifications produced on the first day of high-altitude exposure which return to sea level values after acclimatization. This data supports the hypothesis that histone modifications and DNA methylation play a role in rapid physiological plasticity which is essential for acclimatization to high altitude.

In summary, our research underscores the indispensability of plastic changes for adapting to hypoxia, irrespective of short-term or long-term exposures. Furthermore, we provide novel insights into the role of epigenetic modifications in shaping high-altitude adaptations. Our multidimensional approach adds depth to the understanding of human adaptation to high-altitude environments, uncovering the intricate interplay between genetics, epigenetics, and physiological responses.

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LIST OF ABBREVIATIONS

AHI	Apnea-Hypopnea Index
AMS	Acute Mountain Sickness
BP	Blood Pressure
bp	Base pair
C	Cytosine
CBF	Cerebral Blood Flow
CMS	Chronic Mountain Sickness
CpG	Cytosine-Phosphodiester-Guanine Dinucleotide Pair
CGI	CpG Island
DMP	Differentially Methylated Position
DMR	Differentially Methylated Region
HAR	High-Altitude Resident
HA1	First Morning/Day After Arriving at High Altitude
HA2	Second Morning/Day After Arriving at High Altitude
HA3	Third Morning/Day After Arriving at High Altitude
Hb	Hemoglobin Concentration
Hct	Hematocrit
HIF	Hypoxia-Inducible Factor
HR	Heart Rate
HRE	Hypoxia Response Elements
HCVR	Hypercapnic Ventilatory Response
HVR	Hypoxic Ventilatory Response
IGR	Intergenomic Region
NO	Nitric Oxide
ODI	Oxygen Desaturation Index
PBMC	Peripheral Blood Mononuclear Cells
PHD	Prolyl Hydroxylase Domain Proteins
PSQI	Pittsburgh Sleep Quality Index
PROMIS	Patient-Reported Outcomes Information System
ROS	Reactive Oxygen Species
SDB	Sleep Disordered Breathing
SL	Sea Level
SSS	Stanford Sleepiness Scale
T50	Temperature at Which 50% of Amplicon Has Melted
TET	Ten-Eleven Translocation Enzymes
TSS	Transcription Start Site
UTR	Untranslated Region
VHL	Von Hippel-Lindau Proteins

Chapter 1:

Introduction

Oxygen

Oxygen, as a critical element in Earth's atmosphere, has played a pivotal role in the evolution of life on our planet. Its importance in the evolutionary process stems from its role as a potent source of energy and its influence on the development of complex organisms. The rise of atmospheric oxygen levels, known as the Great Oxygenation Event, occurred around 2.4 billion years ago, marking a significant turning point in the history of life on Earth. This increase in oxygen levels was a result of the photosynthetic activity of early cyanobacteria, which released oxygen as a byproduct and subsequently lead to Earth's first mass extinction event[1] but paved the way for the development of aerobic respiration, a highly efficient process that allowed organisms to extract energy from organic compounds by utilizing oxygen.[2]

The evolution of aerobic respiration transformed energy production, enabling organisms to derive more energy from each molecule of food.[3] This, in turn, provided a selective advantage to those organisms capable of utilizing oxygen effectively. Over time, aerobic organisms became more dominant, eventually leading to the diversification and complexity of life forms we see today. The efficient utilization of oxygen allowed for the evolution of larger, more complex organisms with specialized tissues and organ systems.[4] Furthermore, the presence of oxygen in Earth's atmosphere facilitated the development of more efficient means of energy storage and utilization, such as the evolution of mitochondria. Mitochondria play a vital role in energy production through oxidative phosphorylation, further enhancing the energy capacity and metabolic capabilities of organisms. Oxygen's

ability to support oxidative metabolism provided the necessary energy for cells to carry out complex functions, including growth, differentiation, and movement.[5] Oxygen allowed humans to grow larger brains which supported our advancement as a species. Our brains utilize 20% of the oxygen we inhale in every breath, which by weight is over 10 times the amount that would be expected in comparison to oxygen use by other tissues.[6]

It is not surprising that many species, from plants to vertebrates, have retained evolutionary conserved molecular and cellular mechanisms for detecting and maintaining oxygen levels, such as the hypoxia-inducible factor (HIF) machinery, the development of which can be traced back in eukaryotes to 800 million years ago.[7] This oxygen-sensing machinery serves as a signal for mitochondria to switch to anaerobic energy pathways,[8] allowing adaptations to the changing oxygen levels in Earth's early atmosphere. In humans, part of the sensing and homeostasis system also includes specialized cells called chemoreceptors that are sensitive to local levels of oxygen and pH. These cells work with the respiratory control center in the brainstem to control our breathing rates and maintain adequate oxygenation of blood and tissues.

As the complexity and size of animal bodies increased, mechanisms evolved to efficiently transport oxygen to tissues located farther away from the respiratory gas exchange organs. Hemoglobin is the vital transport molecule responsible for carrying approximately 98% of all arterial oxygen content from the lungs to the tissue capillary beds. The binding affinity for oxygen and hemoglobin was described by the hemoglobin-oxygen dissociation curve, which is a graphical representation of the relationship between the partial pressure of oxygen (PO_2)

in the blood and the percent of total hemoglobin bound to oxygen (**Figure 1**). In humans, the sigmoid-shape of this curve illustrates the dynamic interaction between oxygen and hemoglobin, which is crucial for oxygen transport and delivery to tissues.[9]

The binding affinity of hemoglobin for oxygen is highly adaptive. At higher PO_2 levels (such as in the lungs), hemoglobin has a higher affinity for oxygen, leading to efficient loading of oxygen molecules onto hemoglobin. As the blood travels to tissues with lower PO_2 levels, such as in peripheral tissues, the oxygen dissociation curve shifts to the right, indicating a reduced affinity of hemoglobin for oxygen and facilitating the release of oxygen to the tissues.

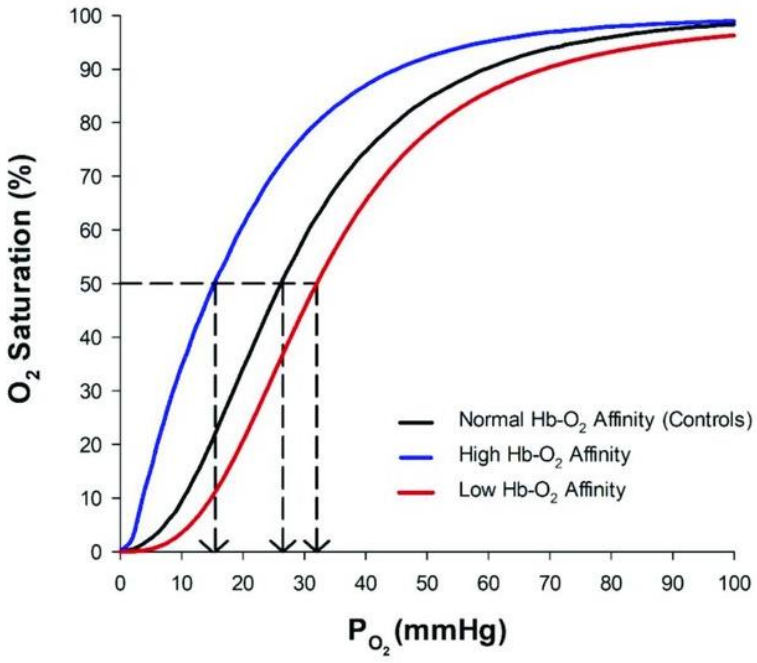

Figure 1. Hemoglobin-Oxygen Dissociation Curve
Reproduced with permission from Webb et al. Frontiers in Physiology (2022)

There are several factors which can influence the curve, and this remains important in the context of high altitudes there are many factors present in this environment which are known to cause a shift to the right (red line), promoting oxygen release from hemoglobin. These factors include an increase in 2,3-DPG, increased levels of carbon dioxide (hypercapnia), and decreases in pH (acidosis). Conversely, factors that can shift the curve to the left (blue line), increasing the affinity of hemoglobin for oxygen, include a decrease in temperature or an increase in pH, known as alkalosis.

Hypoxia

Every year there are over 40 million people that visit high-altitude sites greater than 2500 m. In addition there are over 140 million people reported to live permanently at these high altitudes.[10] High altitude poses a formidable challenge due to a combination of factors that collectively create a demanding and stressful environment for human life. The most prominent challenge arises from the significant decrease in oxygen levels as altitude increases. This phenomenon, known as hypoxia, stems from both reduced atmospheric pressures, referred to as hypobaric hypoxia, and lower oxygen molecule concentrations in the air. Consequently, the body must work harder to acquire the limited oxygen available. Moreover, the temperature at high altitudes can fluctuate dramatically, subjecting inhabitants to abrupt and extreme weather changes, with freezing nights and warm days. In addition to these challenges, the atmosphere's diminished density offers diminished protection against harmful ultraviolet (UV) radiation. Dehydration is also a pressing concern, as the combination of low humidity and increased water loss from hyperventilation amplifies the risk of water deficiency. Furthermore, altitude sickness or Acute Mountain Sickness (AMS),

which is characterized by symptoms like headaches and nausea,[11,12] can afflict those transitioning to higher altitudes, as the body grapples with adapting to lower oxygen levels.

Altitude sickness was first described in 32 BC by Chinese official Too Kin who referenced it in response to travelling over 'Big Headache Mountain' acknowledging the symptoms of high altitude on the human body.[13] The severity of these symptoms can increase with higher altitudes and faster rates of ascension. Worsening symptoms can include confusion, severe headaches, tachycardia, and in the worst cases people can suffer from high-altitude cerebral edema (HACE) or high-altitude pulmonary edema (HAPE).[14] AMS, HACE, and HAPE are all consequences of being exposed to hypobaric hypoxia and lower oxygen pressures found at high-altitude. For example, at 2500 m oxygen partial pressure is effectively 73% of what is available at sea level. This decrease continues as the altitude increases. At 4,500 m, the altitude of Cerro de Pasco in Peru, the oxygen has been effectively decreased to 57% of sea level partial pressures.

Physiological Responses to Acute High-Altitude Exposure

When sojourners, people born and living at sea level but travelling to high altitude, are exposed to the lower oxygen availability at high altitude there are some immediate reflex responses as well as short-term phenotypic plasticity which allows acclimatization to this stressful environment. These changes can revert to normal values either after acclimatization or after returning to lower altitude. Factors modified throughout the process of acclimatization include ventilation, sleep, cognitive function, and other physiological

measurements all with the same goal of increasing circulating oxygen and delivery to tissues. An overview of these changes over time can be seen in **Figure 2**.

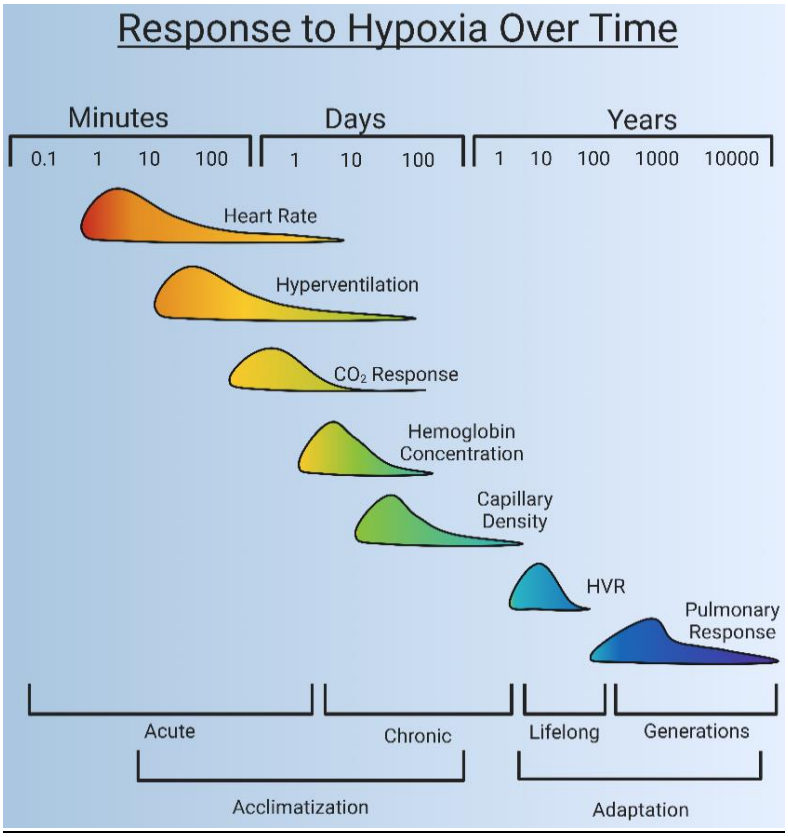

Figure 2. **Responses to Hypoxia over Time.**

Adapted from Peacock (1998). Times range from 0.1 minutes to 10,000 years. Responses are arranged according to time of onset. The larger initial reactions often taper off over time.

Ventilation

One of the first physiological responses to acute hypoxemia is the increase in minute ventilation. This occurs as an increase in tidal volume to increase alveolar ventilation rates and frequency.[15] Related to ventilation rates is the hypoxic ventilatory response (HVR). The

HVR is the acute reflex increase in ventilation when arterial PO_2 decreases. It is a measure of the chemoreceptor response to hypoxic stress. Lowlanders have large increases in HVR upon acute exposure to hypoxia. Changes in ventilation and hypoxic ventilatory responses are discussed in more detail in Chapter 2.

Sleep Quality

Many sojourners report poor sleep quality at high altitude. This sleep quality is influenced by insomnia or difficulty falling asleep. Sojourners also report difficultly staying asleep. This could be attributed to sleep apnea, a condition characterized by pauses in breathing during sleep, which can be exacerbated at high altitude. Sleep disordered breathing at occurs at high altitude as a result of unstable cycles of elevated ventilatory chemosensitivity, and is characterized by Cheyne-Stokes respiration, or cycles of breathing starting with hyperventilation and subsequent hypocapnia-induced hypopneas or apneas, followed by overcorrection by another hyperventilation period. These disruptions can impact the overall quality of sleep and lead to fatigue during the day.[16] It's important to note that like many other symptoms caused by hypoxia and high altitude, the extent and severity of these sleep disturbances can vary among individuals and often increase proportionally with altitude.[17]

Cognitive and Visual Function

Studies have also shown a decrease in cognitive function at high altitude, which may be partially related to the decrease in sleep quality, changes in the cerebral blood flow, effects of hypoxemia, acute mountain sickness, or some combination of these. The exact mechanisms of these cognitive impairments remain unknown. Several studies have shown that

performance on cognitive function tests, such as the psychomotor vigilance task which measures attention and reaction time, the balloon analog risk task which measures risk-taking behavior, the word list task which measures short term memory recall, and trail making tasks which measure visual attention and task switching ability, all show performance decline at high altitude.[16,18–21] In addition, vision can be impaired at high altitude and decreases in tasks such as contrast sensitivity have been observed which may be linked to reductions in SpO_2 or impacts of reduced barometric pressure on corneal edema.[22] Further discussion about the effects of high-altitude exposure on sleep, cognitive, visual, and hearing function are discussed in Chapter 3.

Blood

At high altitudes, sojourners can experience increases in hematocrit or red blood cell concentration. This hematocrit increase enhances oxygen-carrying capacity and delivery to tissues. There can also be changes in autoregulatory mechanisms that help maintain stable blood flow despite changes in blood pressure, which increases at high altitude due to loss of body fluid due to hyperventilation, cold-induced diuresis, and loss of blood volume increasing blood viscosity.[23] The body can also respond to lower oxygen levels by increasing blood flow. This is achieved through a process called vasodilation, which involves the widening of arterial vessels and opening of capillary beds in tissues to allow for greater blood supply and oxygen delivery. This mechanism helps to maintain sufficient oxygenation of brain tissue and supports brain function at higher altitudes where arterial oxygen content is reduced, and blood flow rates must increase to maintain normal total oxygen delivery rates.

Metabolism

Mitochondria are one of the major consumers of oxygen as they work to convert oxygen to ATP to power the body. In the absence of sufficient levels of oxygen, subunits of the electron transport chain are modified to optimize ATP production. The largest changes occur in complex I, which is responsible for accepting electrons, and in complex IV, which is responsible for transporting oxygen. In addition the morphology and mass of the mitochondria can also change.[24,25] These changes can also include the mitochondrial volume decreasing by up to 30%.[26,27] In culmination, these adaptations allow the mitochondria to maintain membrane potential and continue producing ATP, while limiting reactive oxygen species (ROS) production.

In regards to changes in metabolism all of the responses to hypoxia are costly and cause an increase in basal metabolic rate of about 17-27% for the first few weeks of high-altitude exposure.[28]

Physiological Responses to Chronic High-Altitude Exposure

During long-term, life-long, and generational exposure to high-altitude hypoxia, additional physiological changes must occur to optimize fitness in this harsh environment. Typical plastic changes which occur in lowlanders during acute hypoxia exposure are effective at increasing oxygen delivery to tissues in the short term, but they are often energetically costly. Therefore, in the face of long-term exposure, energy conservation becomes an additional priority. Interestingly, despite evolving in similar environments, different high-altitude native

populations show distinct adaptations to address the need to deliver oxygen more efficiently to tissues in their hypoxic environments. Indeed, there are clear adaptations that have developed in high-altitude native populations, such as increased hemoglobin concentrations to increase oxygen carrying capacity in Andeans[29] or increased capillary density in Tibetan Sherpas.[30]

Ventilation

As with sojourners, changes in ventilation patterns can also be seen in high-altitude native populations. Native Tibetans demonstrates a high resting ventilation, similar to what an acclimatized sojourner would experience. In comparison, native Andeans are often cited as having low resting ventilation.[31–34] Beall *et al* measured the resting ventilations of Tibetans and Andeans and found Tibetans had an average value of 15 liters per minute and Andeans with an average of 10 liters per minute.[35] Ethiopians and Han Chinese living at high altitude surprisingly show no change in ventilation, having a very similar rate to that of native sea level residents at sea level.[36,37] The HVR across these groups follow a similar pattern. On one end of the spectrum, Tibetans tend to express a high HVR,[32,33,35,37–41] similar to acutely acclimatized sojourners, while on the other end Andeans are known to have a low, blunted response.[33,35,37–39] Individuals of Han Chinese ancestry living at high altitude fall within these values and have an HVR that is lower than that of the Tibetans and acutely acclimatized sojourners but higher than that of the Andean high-altitude natives.[35,39,42] During exercise, high-altitude natives all show a lower ventilatory rate than low-altitude natives. High-altitude natives also have smaller alveolar arterial PO_2 differences during exercise. This suggests a difference in the efficiency of gas exchange between high and low altitude natives.[43]

Sleep

There are few studies which characterize sleep patterns of native high-altitude residents. However, those that do exist suggest high-altitude residents do not have the same issues with sleep that sojourners experience. For example a study by Roach *et al.* in 2013 showed that sojourners had poor sleep quality and quantity at high altitude while native residents sleep the same length of time at sea level or high altitude with no depreciation of quality at high altitude.[44] Another study published in 1992 studied the sleep patterns of native Andeans and found that their sleep patterns resembled the sleep patterns of sea level residents at sea level.[45] However, for high-altitude natives who do develop sleep disordered breathing, Heinrich *et al.* (2020) also showed that a higher obstructive apnea index and apnea hypopnea index predict higher hematocrit and Chronic Mountain Sickness (CMS) scores in Andean men.[46]

Cognition

There have been several studies which show that high-altitude native residents show some signs of cognitive impairment. Davis *et al.* showed that residents from high altitude performed worse at higher altitudes on responding to a non-visual stimulus go-no-go test than other residents with similar genetic backgrounds living at moderate and low altitudes. Further they found that both high and moderate altitude residents performed worse than low altitude residents on a psychomotor hand movement test.[47] However, it has been noted in many studies that symptoms of cognitive decline increase with altitude, and this could be the case even with high altitude residents. Supporting this, Hill *et al.* assessed cognitive function in high and low altitude residents in Bolivia who were matched for socioeconomic

status and genetic ancestry. Participants ranged in age from 4 – 85 years old and were tested for fluid intelligence, attention, short- and long-term memory, and psychomotor speed. The study concluded that differences were subtle and related only to the speed of more complex cognitive operations.[48] Other studies support the finding that differences between the groups are subtle with no differences in accuracy on tests but larger differences in reaction time.[49]

Blood

Historically, one of the defining features of high-altitude populations was thought to be an increase in red blood cell production. It was thought that hemoglobin concentration and hematocrit increased proportionately in relation to altitude. This hypothesis was due to the majority of early high-altitudes studies being conducted on Andean natives. Indeed, several studies support the finding that Andean high-altitude natives demonstrate significantly elevated red blood cell content.[34,50,39] While sojourners and Han Chinese high-altitude residents can experience a small increase in red blood cell production, Andeans have been observed to have hematocrit values upward of 80%. This drastic increase in red blood cell content is commonly referred to as Excessive Erythrocytosis and is the defining symptom of Chronic Mountain Sickness (CMS). While the increase in red blood cells does help in delivering more oxygen to tissues, it also increases blood viscosity and can lead to complications such as heart failure and pulmonary hypertension. Because of the complications associated with increased hematocrit, this phenotype is often seen as a maladaptation to lifelong high-altitude exposure. Up to 35% of Andean men suffer from CMS.[51–54] When studies shifted focus to Tibetan adaptations, the normalcy of increased hematocrit was challenged. Both Tibetans and Ethiopians maintain hemoglobin

concentrations ([Hb]) within the sea-level range. It should be noted that at altitudes over 4000 m there is a slight increase in red blood cell production in Tibetans, but it remains within the normal sea level range below this elevation.[55]

Higher blood flow rates also result in more oxygen delivery to tissue. These higher blood flow rates are achieved in several ways: increased cardiac output, increased capillary density, or vasodilation. As previously mentioned, sojourners are often at risk for pulmonary hypertension. Similarly, Andeans, Ethiopians, and Han Chinese can experience pulmonary hypertension. However, cases of pulmonary hypertension are more rare in Tibetan individuals.[35] This is often attributed to the higher levels of circulating nitric oxide (NO). NO is well known to work as a vasodilator, allowing vessels to remain open. Despite experiencing pulmonary hypertension, Ethiopian populations do have elevated NO levels, just not as elevated as Tibetans. Instead, they have been shown to have higher capillary density, particularly in leg skeletal muscle tissue.

Metabolism

As with sojourners, one of the ways to combat hypoxia would be to adjust energy production by adjusting basal metabolic rate. Upon acute high-altitude exposure, sojourners will experience an increase in basal metabolic rate, particularly due to increased ventilation rates. However, Andeans and Tibetans show no adaptations of this type. They display a base metabolic rate within the expected range for an average person at sea level for their sex, age, and weight.[35]

Related to metabolic rates and energy production is the health and density of mitochondria. Comparable to the acute response of sojourners, both Andeans and Tibetans have lower levels of mitochondria in muscle tissue when compared to native lowlanders,[35] but it also appears that function and structure of mitochondria are altered to help energy production in such a harsh environment. Unfortunately, these tests have not yet been performed in Han Chinese or Ethiopian populations.

Table 1 summarizes the response to high altitudes in native high-altitude residents including Tibetans, Andeans, Ethiopians, the newer Han Chinese, as well as sojourners.

Hypoxic-Inducible Factor

HIF is found in nearly all metazoans and serves an integral function in the response to hypoxia. Many of the changes seen in both acute and chronic hypoxia exposure have been attributed to genes associated with, or regulated by, the HIF pathway. HIF is known as the master transcriptional regulator of hypoxia-response genes.[56,57] HIF is a heterodimer made of a common β subunit, HIF-β, also known as ARNT, and one of the three HIF-α subunits; HIF-1α, HIF-2α, and HIF-3α. In normoxia, HIF proteins are degraded by enzymes called prolyl hydroxylase domain proteins (PHD). These PHDs work to hydroxylate the HIF- α subunits, which allows Von Hippel-Lindau (VHL) proteins to bind and ubiquinate the HIF subunit, leading to degradation. In hypoxic environments, PHDs lack the essential oxygen co-factor needed to use 2-OG as substrates and are thus unable to complete their hydroxylation step allowing HIF proteins to stabilize, bind with their subunit, and translocate to the nucleus

Table 1. Responses to High Altitude/Hypoxia in Sea Level Sojourners and High-Altitude Natives

	Tibetans	Andeans	Ethiopians	Han Chinese	Sojourner
Resting Ventilation	Not blunted High[35,37]	Blunted Low[35,41]	SL Range[58]	SL Range[35,37]	High[35]
Oxygen Saturation	Low[34,55]	Low[34,55]	High[34,55]	Low[41]	Low[34]
HVR	High[35,37]	Low[35,41]	--	Moderate[37]	High[35]
Pulmonary Hypertension	Rare[35]	Occurs[35]	Occurs[35]	Occurs[41]	Occurs[35]
Hemoglobin Concentration	SL Range [35,34,55,37]	Highest[35,34,55,37]	SL Range[34,55]	High Hb[37]	Higher[35]
Basal Metabolic Rate	Normal[35]	Normal[35]	--	--	Increased[35]
EPO	Moderate[35]	Increased[35]	SL Range [59]	Increased	Increased[35]
Exhaled NO levels	Increased[35]	Normal[35]	Increased[59]	--	--
Mitochondrial density	Decreased[35]	Decreased[35]	--	--	Decreased[35]
PCO_2 Levels	Low[58]	Moderate[58]	High[58]	--	Low[58]

HVR = Hypoxic ventilatory response; EPO = Plasma erythropoietin concentration; NO = Nitric Oxide; PCO2 = Partial pressure of carbon dioxide in the arterial blood. "--" Indicates no available data.

Once inside this dimer is known to regulate gene expression through interactions with specific hypoxia response elements (HRE).[60,61] These HREs are small stretches of DNA bearing the core sequence of RCGTG (where R is A or G) that are found in the enhancer and promoter regions of genes throughout the body, specifically related to supporting oxygen delivery through cell division, the formation of new blood vessels, and red blood cell production.[57,62] Hypoxia has also been known to stimulate responses activated by other transcription factors such as NFkB, CREB, and EGR-1.[63]

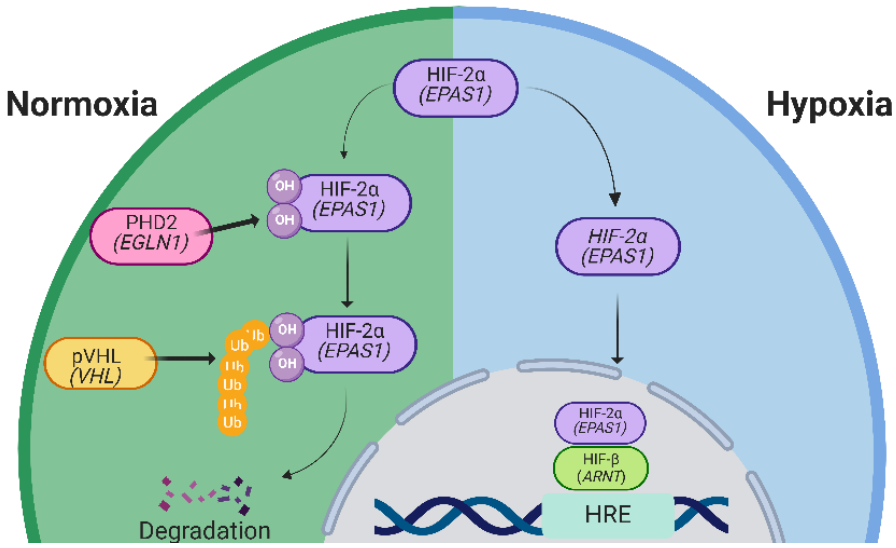

Figure 3. HIF Pathway
Normal oxygen levels (normoxia left) and in low oxygen levels (hypoxia right). In normoxia the alpha subunit of HIF is hydroxylated by a PHD enzyme, then tagged with ubiquitin and complexed with a VHL protein. The HIF complex is then degraded. In hypoxia the HIF alpha subunit is not degraded. Instead, it forms a dimer with HIF-1β (ARNT) and becomes a transcription factor.

While HIF-1α and HIF-2α have some overlap in function, they also activate unique and distinct responses.[64,65] For example, they both have the ability to activate the VEGF genes, which induce angiogenesis. However only HIF-1α works to up-regulate genes encoding for

glycolytic enzymes while inhibiting oxidative phosphorylation.[56] HIF-2α works to regulate *EPO*, a gene responsible for creating red blood cells. However, they can also work against each other, for instance HIF-1α promotes cell cycle arrest while HIF-2α promotes the continuation of the cell cycle. Similarly, HIF-1α a can work to promote NO production while HIF-2α works to inhibit it.

To complement the three alpha subunits, there are also three distinct analogs of PHD which have unique functions. PHD2 (*EGLN1*) is the gene responsible for creating the enzyme prolyl hydroxylase domain 2. This enzyme is responsible for breaking down HIF-2α under normoxic conditions and has been shown to be one of the most important of the PHD analogs because of its ability to control HIF-2α, and thus *EPO* regulation.[66,67]

EPAS1 and *EGLN1,* the genes that code for HIF2-α and PHD2 respectively, are found recurrently as being under selection in high-altitude native populations and are thought to be important for human adaptation to high altitude. Several subsequent studies have attempted to identify the adaptive variants driving this genetic selection. Of notable mention, one *EPAS1* variant was linked to the low hemoglobin phenotype in Tibetans. *EPAS1* has also been shown to be under selective pressure in Andeans, however no distinct variant has been identified.[68,69] This is common with other genes associated with the HIF pathway and found under selective pressure in high altitude native groups. This could suggest that the phenotypes in Andeans and Ethiopians may not be associated with a specific base pair mutation at all, but an inheritable epigenetic modification instead.

Epigenetics

To show a signal of selection, a genetic region must contain a variant that provides an adaptive benefit. This can occur when a mutation modifies gene expression or produces a protein-coding change that modifies protein function. Variants can modify gene expression by directly or indirectly impacting the binding of transcriptions factors, modifying the three-dimensional structure of chromatin, or impacting regulation through epigenetic mechanisms such as changing patterns of histone binding or modification, or modifying DNA methylation patterns.

The Oxford dictionary defines epigenetics as "The study of factors that influence gene expression but do not alter genotype, such as chromatin methylation and acetylation involved in tissue-specific patterns of gene expression, or the parental imprinting of genes".[70] The most studied epigenetic modifications are DNA methylation, miRNA, and histone modifications. The term "epigenetics" was first used to explain some of the processes by which cell differentiation occurred and has shifted to describe changes in heritable traits associated with changes in chemical modifications of DNA. Epigenetic mechanisms are essential to phenotypic plasticity, gene regulation, cellular differentiation, and development. While there is some debate about the necessity of a mechanism being heritable to be included as an epigenetic mechanism, it is important to understand that these modifications can be inherited and thus present adaptive benefits. It may be that selection acting on epigenetic changes allows for more rapid, within-generation, changes in phenotypes in response to physiological stress than waiting for beneficial mutations. There are many ways

that these mechanisms can affect the evolution of offspring including passing on 'epialleles' though mostly known to occur in plants thus far, enhancing aspects of phenotypic plasticity, and particularly in the case of DNA methylation it can act as either a mutagen or modulate genome stability with its effect on transposable elements. In short, these mechanisms can work to produce specific phenotypes which can provide adaptive benefits and lead to selection in genomic regions that facilitate these epigenetic patterns.[71–75]

While it remains undoubtedly true that epigenetic factors play a large role in development, they are linked to other roles as well. For example links between epigenetic patterns and many diseases including Parkinson's, Alzheimer's, diabetes, and various cancers have been discovered.[76,77] However, epigenetic modifications can also play a protective role as demonstrated by insects where DNA methylation, acetylation of histones, and various microRNAs have been shown to help regulate immunity to pathogens. The method of these responses vary across species but includes changes to insecticide resistance, embryogenesis, circadian rhythms, metabolisms, and bacterial infections.[78]

In the context of high altitude, epigenetics remains largely understudied. There are genes important to both short- and long-term high-altitude adaptation that remain good candidates to investigate, such as *EPAS1,* which contains a large CPG island, a region of CG repeats, which are prone to DNA methylation. *EPAS1* was previously examined by Childebayeva *et al.*, who analyzed DNA methylation in cells collected from saliva samples from sojourners of European ancestry during a 10-day ascent from Kathmandu (1400m) to Gorak Shep (5160m). They found that there was a decrease in DNA methylation in *LINE1*, *EPO,* and

RXRa. They also found an increase in methylation in *EPAS1* during one time point only, on day 7 (4240m).[79] From this same trek, samples were analyzed on day 0 and 7 using a genome-wide chip approach. The result showed 2,873 significant differentially methylated positions, and 361 significant differentially methylated regions. Many of these sites were in the HIF and renin–angiotensin system (RAS) pathways.[80] In a study investigating DNA methylation in high altitude natives Childebayeva *et al.* found that participants recruited at high-altitude had lower *EPAS1* DNA methylation than those with a similar genetic background living at low altitudes and that the number of years a participant had lived at high altitude had a negative association with *EPAS1* methylation levels.[81] These studies show that there are genome wide changes happening during high-altitude exposure.

In addition to evidence of DNA methylation changes at high altitude, there exists ample support for histone modification changes in hypoxia. Many histone demethylases, such as the JmjC family that includes many lysine demethylases have been shown to be oxygen dependent, are regulated by hypoxia or stabilized by HIF.[82,83] Histone demethylase KDM6A was shown to have the ability to directly sense oxygen.[84,85] Hypoxia seems to not only affect the enzymes but specific markers. There are studies showing that common but important expression markers such as H3K27me3, which play a large role in the organization of chromatin, are affected by hypoxia.[86]

Furthermore, both DNA methylation and histone modifications have been shown to regulate HIF's ability to bind to promoters.[83,87] Global changes in DNA methylation change the shape and structure of the HIF-dependent transcriptional profile. For example,

hypomethylation of the DNA can reveal HRE binding sites that were previously inaccessible in the same way hypermethylation of the DNA could cover regions that were previously active. It has been shown in studies like those by Johnson *et al.* that exposure to hypoxia result in both upregulation and downregulation globally.[88,89] Considering how important oxygen has been to our evolution and our embryonic development, it is sensible that these processes can adapt to changes in oxygen availability. Epigenetic modifications such as these may help explain many short-term plastic changes but also chronic adaptations such as those found in native Andean high-altitude residents.

Conclusion

Here I have described the importance of oxygen and the effects of oxygen limitation (hypoxia) on the human body, both acutely and over longer timescales. The harsh hypoxic conditions at high altitude have created selective pressures and influenced human adaptation, resulting in multiple phenotypes across high-altitude native groups. The majority of studies investigating high altitude residents have traditionally focused on Andean and Tibetan high-altitude natives. More studies conducted on Han Chinese sojourners and Ethiopian high-altitude residents are essential for gathering the missing pieces of their phenotypes and allowing us to see the bigger picture. With more genetic and physiological data, we would be able to better compare and clarify how these phenotypes helped these groups to adapt. In addition, studying 'newcomers' and even residents of high-altitude cities such as Denver, Colorado, USA, we may gain a better understanding of the subtle changes and adaptations that are occurring with lifetime exposure. In some cases, these adaptations seem to have links to specific genes such as *EPAS1,* though different variants seem to serve different roles

in separate populations. However not all these adaptations can be explained neatly with genetic influences alone. There is much evidence showing that large genome-wide epigenetic changes occur in response to hypoxemia. By gaining an understanding of the role epigenetics play in the response to hypoxia, we will open doors to treatments for maladaptation, and better understand healthy adaptations.

Chapter 2:

Changes in Hypoxic and Hypercapnic Ventilatory Response in Sojourners

Changes in Hypoxic and Hypercapnic Ventilatory Responses at High Altitude Measured Using Modified Rebreathing

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Abstract

Respiratory responses to hypoxia and hypercapnia play a vital role in maintaining gas exchange homeostasis and adapting to high-altitude environments. This study investigates the mechanisms behind the hypoxic and hypercapnic ventilatory responses (HVR and HCVR) in individuals acclimatized to moderate high altitude (3800 m). Thirty-one participants underwent chemoreflex testing using the Duffin modified rebreathing technique. Measures were taken at sea level and in a subset of these participants after 2 days of acclimatization to high altitude. Ventilatory recruitment thresholds (VRT), HCVR, and HVR were quantified. Acclimatization to high altitude resulted in increased HVR and HCVR, and a decrease in the VRT under both hyperoxic and hypoxic test conditions. The larger decrease in VRT under hypoxic conditions significantly contributed to the elevated HVR at high altitude. Pre-VRT ventilation increased at high altitude, but the change did not differ between test oxygen conditions.

This study highlights the intricacies of respiratory adaptations during acclimatization to moderate high altitude, shedding light on the role of VRT, baseline respiratory drive, and two-slope HCVR in this process. These findings contribute to our understanding of how the human respiratory system responds to hypoxic and hypercapnic challenges at high altitude.

Keywords: hypoxia, hypoxic ventilatory response, hypercapnic ventilatory response, high altitude

Introduction

Breathing patterns are tightly controlled to maintain optimal gas exchange rates, protect against decreases in arterial partial pressures of oxygen (P_{aO2}), and maintain arterial P_{aCO2} within a homeostatic range. One of the primary mechanisms by which minute ventilation is controlled in response to hypoxia is by chemosensory cells located in the carotid and aortic bodies which detect changes in arterial P_{O2}, as well as P_{CO2} and pH, and signal to the respiratory centers in the brainstem to increase ventilation rates within seconds of hypoxia onset. Additionally, chemoreceptors in the medulla respond to changes in cerebrospinal pH as a result of arterial P_{CO2} changes and their activation results in increased ventilation to return arterial P_{CO2} to homeostatic levels. These reflex increases in breathing in response to hypoxia and hypercapnia are termed the hypoxic ventilatory response (HVR) and hypercapnic ventilatory response (HCVR).

During travel to high altitude, the magnitude of the these ventilatory chemoreflexes increase over time in a process called ventilatory acclimatization.[90] Work by several groups has demonstrated the time domains of ventilatory acclimatization to high altitude using a variety of techniques to measure these reflexes, including steady-state and rebreathing methods. While it is clear that the HVR increases at high altitude, there are gaps in our knowledge regarding the mechanism by which this occurs. The ventilation rate at any P_{aO2} is also impacted by P_{aCO2}, as these two reflexes interact. When using a steady-state isocapnic HVR protocol, in which P_{aO2} is allowed to decrease while P_{aCO2} is held constant, the amplitude of the HVR will increase as the target P_{CO2} increases.[91,92] Typically, isocapnic P_{CO2} tensions are

fixed during testing at a certain increase over the eupneic P_{CO2} level. However, previous work by Duffin and others demonstrate the presence of a CO_2 ventilatory recruitment threshold (VRT), below which ventilation is not stimulated above baseline levels.[93] Fan et al. (2010) demonstrated previously that this threshold decreases with high-altitude acclimatization.[94] However, the contribution of this change to the increased HVR remains unknown. For example, when calculating the HVR as described by Duffin et al. (2007), the HVR may be increased by three mechanisms: (1) a change in baseline respiratory drive (y intercept) between hypoxic and hypercapnic tests at high altitude compared to sea level, (2) a higher change in VRTs between hypoxic and hyperoxic tests at high altitude compared to sea level, or (3) a higher change in CO_2 gain between hypoxic and hyperoxic tests at high altitude compared to sea level. (**Figure 1**).

In this study, we used the Duffin modified rebreathing technique to determine how the hypercapnic ventilatory response (HCVR), VRT, and HVR increased after 2 days of acclimatization to 3800 m elevation. We aimed to evaluate if the increase in hypoxic chemosensitivity after acclimatization to high altitude is produced as the result of an increased slope of the CO_2 response, a leftward shift in the VRT, and/or an increase in baseline respiratory drive at lower oxygen levels.

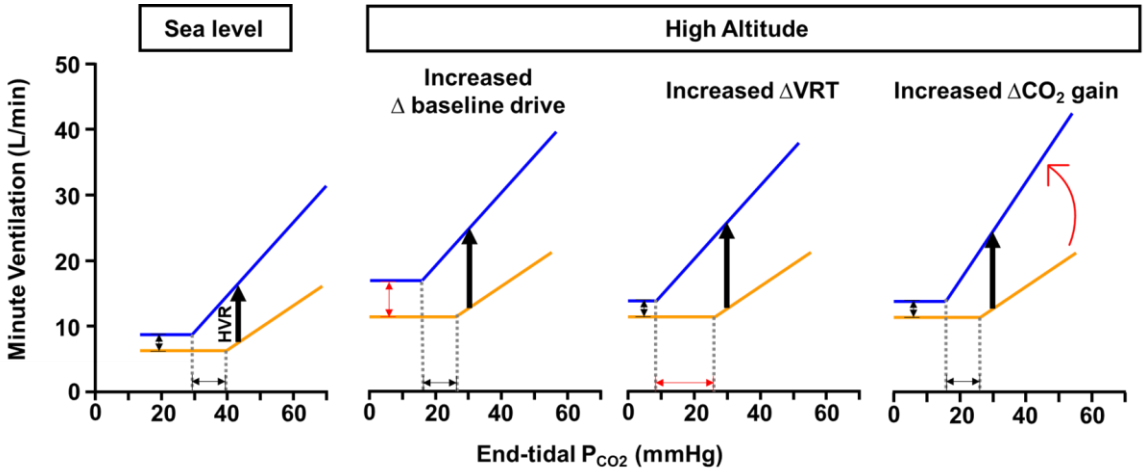

Figure 1. Schematic Diagram of Possible Causes of Increased HVR Measured via the Duffin Modified Rebreathing Technique

The left panel displays a simplified modified rebreathing curve representing the increase in minute ventilation as a function of end-tidal PCO2 during rebreathing. In this method, the HVR (represented by the thick arrows) is quantified as the change in ventilation across two PO2 levels at a constant PCO2 tension. The three right panels provide three possible causes of an increase in HVR at high altitude. The HVR may increase due to an increase in pre-VRT baseline ventilation in the hypoxic treatment, an increased leftward shift in the VRT, or an increased change in HCVR slope in the hypoxic treatment compared to the hyperoxic treatment. Each event may occur simultaneously, and they are not mutually exclusive.

Methods

Ethical Approval

This study was approved by the University of California, Riverside Clinical Institutional Review Board (HS 19-076, HS-22-088). All participants were informed of the study's purpose and risks. Participants provided written informed consent in their native language (English). The work was conducted in accordance with the *Declaration of Helsinki*, except for registration in a database.

Participants

31 participants were recruited across two field expeditions in 2019 and 2022 (22 men and 9 women). Four participants took part in both expeditions. Participant demographics are provided in **Table 1**. All participants were between 19 and 38 years old and had no history of cardiovascular or pulmonary disease. Exclusion criteria included smoking (cigarettes, e-cigarettes, marijuana), pregnancy, travel to altitudes greater than 2500 m within one month prior to the first test measurement, or use of anti-inflammatory medications, such as ibuprofen, that can interfere with acclimatization to high altitude.[95]

Table 1. Participant Demographics

	Female (n=12)	Male (n=23)	Both (n=35)
Age (years)	25.6 ± 5.8	25.4 ± 5.0	25.5 ± 5.3
Height (cm)	162.3 ± 6.5	173.5 ± 8.3	169.6 ± 9.4
Weight (kg)	78.1 ± 17.8	87.1 ± 17.7	84.0 ± 18.2
BMI (cm/kg^2)	29.5 ± 5.7	28.9 ± 5.4	29.1 ± 5.7

Experimental Design

Sea-level measures were completed at the University of California, Riverside (340 m elevation), and high-altitude measures were taken over a three day stay at Barcroft Station within the White Mountain Research Center (3800 m). Participants were driven to Barcroft Station in vans and ascended from 340 m to 1216 m over 4 hours, then from 1216 m to 3800 m in 2 hours.

Chemoreflex Testing and Analysis

Chemoreflexes were tested using Duffin's modified rebreathing procedure. Participants were asked to breathe into a mouthpiece and instructed to breathe room air for two minutes. This time provided us with resting measures and gave time to allow the participants to acclimatize to the mouthpiece. They were then asked to hyperventilate using slow deep and deliberate breaths. Participants were switched to breathing from the bag when their P_{CO2} was less than 25 mmHg, which typically took approximately 1-2 minutes. Boulet et al. show no change in ventilatory chemoreflex characteristics measured with this method across hyperventilation periods of 1, 3, or 5 minutes.[96] Once switched onto the bag the gases in the lungs, bag, and arterial blood were equilibrated by breathing 2-3 deep breaths. The participants were then asked to relax and resume normal breathing until their end-tidal P_{CO2} reached 60 mmHg OR their ventilation reached 100 L/min OR they indicated they were unable to continue. The 6L rebreathing bag (Hooten) contained either a hyperoxic (30% O_2, 6.5 - 7% CO_2, N_2 as needed to balance) or hypoxic (8.5% O_2, 7.5 - 8% CO_2, N_2 as needed to balance) gas mixture. The tubing between the mouthpiece and the bag contained O_2 and CO_2 sensors (VacuMed, Ventura, CA, USA), a filter, and a three-way stopcock (Hans Rudolph, Shawnee, KS, USA). We fitted a small input valve at the bottom of the bag that allowed oxygen to be added at either a hyperoxic (30%) or hypoxic (P_IO_2 = 70 mmHg, end-tidal P_{O2} = 50 mmHg). Oxygen was produced by a portable oxygen concentrator. During the rebreathing test, participants were seated comfortably, asked to keep their feet flat on the floor, and wore a nose clip. This system was calibrated before each test using a 3L volume syringe (Hans Rudolph, Shawnee, KS, USA) and known concentrations of gases as previously described.

Data was recorded with LabChart (AD Instruments) and these raw values were analyzed using RStudio (RStudio, Boston, MA, USA) with R version 3.6.2. The data selection period began when the participant was switched from room air to the rebreathing bag and an equilibration point was reached at which inhaled and exhaled CO_2 and O_2 levels were temporarily equal. All ventilation data was BTPS corrected using the equation ((760-18.7) / (760-47.1)) * ((273 + 37) / (273 + 21)).

R packages *mcp* and *JAGS* were used to systematically identify the ventilatory recruitment thresholds and resulting slopes. For files where the function did not choose the correct threshold, the threshold was identified manually using the raw values in LabChart by viewing the total ventilation channel and identifying the P_{CO2} where there is a clear slope increase in ventilation. A range containing that value was entered into the *mcp* model. *Mcp* then ran 15 chains to determine the best fit line. In the event that there were irregular breathing patterns such as severely elevated tidal volume, or irregular frequency, the irregular data was removed. This includes periods at the beginning of the bag breathing period where it is clear that the participant is "coming down" from the hyperventilation period. If the participant did not reach a P_{CO2} of 45 or 50, while calculating the HVR at these points, all other data was plotted, and the line equation calculated using *mcp* was used to calculate ventilation and SpO_2 at these points. **Figure 2** provides a representative trace of a complete modified rebreathing protocol.

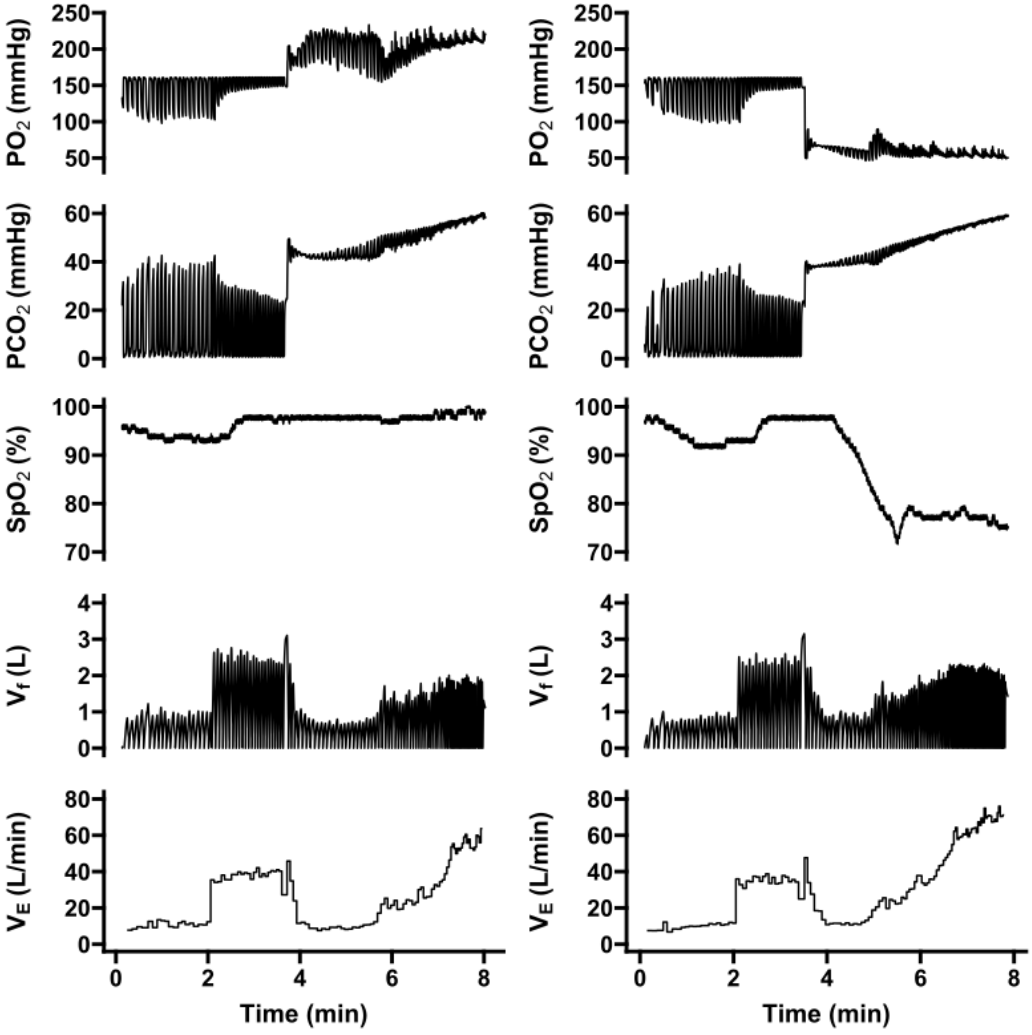

Figure 2. Representative Raw Data Traces from Rebreathing Tests

(A) A representative raw data trace showing the complete modified rebreathing protocol. Participants breathe room air for 2 minutes, followed by voluntary hyperventilation in which the end-tidal P_{CO2} is reduced to less than 25 mmHg, then are immediately switched to a rebreathing bag and continue to rebreathe as end-tidal P_{CO2} slowly increases to 60 mmHg. The test is conducted once with a hyperoxic gas mixture (P_IO_2 = 228 mmHg) (left), followed by a hypoxic gas mixture (end-tidal P_{O2} = 50 mmHg) (right), with 15 minutes of rest between measures.

Statistical Analysis

Resting ventilatory characteristics were tested twice at sea level and at high altitude at the start of the hyperoxic and hypoxic rebreathing test trials. To determine if there was an effect of test order on resting parameters, we first conducted a two-way repeated measures ANOVA on all parameters and found no effect of treatment (hypoxic, hyperoxic) on resting ventilation parameters, as expected. As a result, we used measures taken only during the hyperoxic test period in subsequent analyses of resting parameters.

Each outcome variable was first checked for outliers via the *rstatix* package in R and any points determined to be 3 times above or below the interquartile range were examined to verify no measurement errors. Data were then checked for normality using a Shapiro-Wilks tests via the *rstatix* package in R. If data distributions were normal, we proceeded with paired t-tests to compare resting measurements across sea level and high altitude. In some cases, paired data at high altitude was not available for all participants due to logistical constraints on the number of participants we could test in one day. In these cases, unpaired t-tests were performed. Two-way repeated measures ANOVAs were used to test for effects of altitude and oxygen condition on ventilatory chemoreflex parameters. If a significant two-way interaction or main effect of altitude or oxygen treatment were identified, post-hoc pairwise comparisons with Bonferroni adjustments for multiple comparisons were performed to identify differences across groups. In cases where data distributions were not normal, nonparametric Wilcoxon sign rank tests were performed.

Data is presented throughout the manuscript as mean (standard deviation). Asterisks indicate significant differences at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), or $p<0.0001$(****). Raw p-values are presented in the text as "p" and Bonferroni adjusted p-values as "adj. p".

Results

Participants

At sea level, data was collected in 31 participants (N=9 women, 22 men). Repeat sea level measures were collected in 4 returning participants in both years. The average age of all participants was 25.3 ± 5.4 years, BMI was 29.5 ± 5.9 kg/m^2 in women and 29.1 ± 5.6 in men, systolic blood pressure was 124 ± 8.0 mmHg, and diastolic blood pressure was 79 ± 8.0 mmHg. Due to logistical constraints, chemoreflex measures were collected in a subset of participants on the second day at high altitude in both years (10 in 2019 and 8 in 2022, with no repeat participants across years). These high-altitude datasets included 5 women and 13 men.

Ventilatory Characteristics at Rest

During the second day at high altitude, resting SpO_2 was significantly lower ($t(18.4)=6.9$, $p<0.001$) and heart rate was higher ($t(23.6)=-6.0$, $p<0.0001$) than sea-level values (**Table 2, Supplemental Figure 1**). Systolic blood pressure did not change at high altitude ($t(34)=-1.21$, $p=0.234$), but diastolic blood pressure increased by almost five mmHg at high altitude ($t(34)=-2.36$, $p=0.024$). There was no significant change in hematocrit after two days at high

altitude in men or women. Total ventilation at rest increased after two days of acclimatization compared to sea level values ($t(31.0)=-7.2$, $p<0.001$). This was driven by increases in both tidal volume ($W=117$, $p<0.001$) and breathing frequency ($t(39.7)=-2.29$, $p=0.028$). The increased total ventilation was coupled with decreased end-tidal P_{CO2} ($t(42.6)=6.4$, $p<0.001$). These baseline measures were collected during the two-minute rest period prior to the first rebreathing session and there was no significant effect of first versus second rebreathing session on any of these values ($p > 0.1$ for all).

Table 2. Resting Physiological Parameters at Sea Level and High Altitude

	Sea Level	High Altitude	P
SpO_2 (%)	94.9 ± 1.6	85.7 ± 5.5	**< 0.001**
Heart rate (beats/min)	74.4 ± 9.0	97.4 ± 14.9	**< 0.001**
Hematocrit (men) (%)	49.1 ± 3.1	49.2 ± 2.9	0.911
Hematocrit (women) (%)	41.2 ± 3.1	40.8 ± 4.4	0.829
P_{sys} (mmHg)	124.3 ± 8.0	127.3 ± 12.8	0.234
P_{dia} (mmHg)	79.1 ± 7.9	83.8 ± 7.8	**0.024**
Resting V_T (L)	0.91 ± 0.20	1.15 ± 0.27	**< 0.001**
Resting V_f (breaths/min)	13.1 ± 3.7	15.4 ± 3.2	**0.028**
Resting ventilation (L/min)	11.9 ± 4.1	17.1 ± 4.2	**< 0.001**
Resting ETP_{CO2} (mmHg)	38.7 ± 4.0	32.3 ± 3.1	**<0.001**
AMS Score	0.35 ± 0.54	3.49 ± 2.48	**<0.001**

Total ventilation at rest was slightly above the typical normal range due to elevated resting tidal volumes. Previous evidence suggests that the use of a mouthpiece and nose clip result in elevated tidal volumes compared to an oronasal mask.[97–100] While our results are consistent

with these findings, we also conducted tests comparing the two breathing apparatuses by administering the two methods in a randomized order to a group of 6 participants. We recorded breathing patterns for 10 minutes during rest in a semi-recumbent seated position and found that resting total ventilation and tidal volumes were significantly higher when using the mouthpiece and nose clip compared to a full face mask in the same participants (mask: V_E = 8.0 ± 2.1 L/min, mouthpiece: V_E = 13.0 ± 2.0 L/min, t(10.0) = 4.2, p=0.020).

Characteristics of the Ventilatory Chemoreflexes

A two-way ANOVA was performed to analyze the effect of altitude and oxygen level on the VRT and HCVR. The VRT is sometimes not observed, likely because it is either below the starting P_{CO2} value or perhaps because it is absent in some individuals. In our study, we observed a clear VRT in 29 of 35 recordings collected under hyperoxia at sea level (82.9%), 22 of 35 recordings collected under hypoxia at sea level (62.9%), 15 of 18 recordings collected at high altitude under hyperoxia (83.3%), and 15 of 18 recordings collected at high altitude under hyperoxia (83.3%). **Figure 3** illustrates the average rebreathing results across all participants at sea level and after 2 days of acclimatization. From the remaining data with clear VRT values, we tested to determine how the VRT changed at high altitude and under each test oxygen condition. There was no significant interaction between the effects of altitude and oxygen level ($F(1,98)=0.712$, $p=0.401$) on the VRT. However, the VRT was significantly lower during hypoxic tests compared to hyperoxic tests ($p<0.001$) and significantly lower at high altitude compared to sea level ($p<001$) (**Figure 4B**).

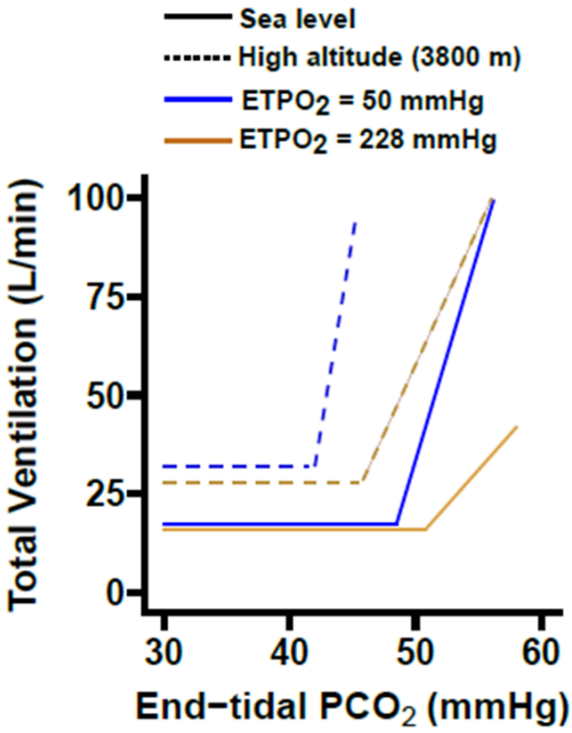

Figure 3. Schematic Diagram of Average Rebreathing Results

Average chemoreflex traces for all participants using Duffin's modified rebreathing technique are shown at sea level (solid lines) and after 2 days of acclimatization at high altitude (dashed lines). Hyperoxic tests (220 mmHg O_2) are shown in gold while hypoxic tests (50 mmHg O_2) is shown in blue.

There was no significant interaction between the effects of altitude and oxygen level on the HCVR ($F(1,96)=0.226$, $p=0.636$). However, there was a main effect of test condition and the HCVR was significantly higher during hypoxic tests compared to hyperoxic tests ($p=0.003$). Unexpectedly, there was no main effect of location on the HCVR ($p=0.298$). However, in post-hoc paired t-tests it was observed that within test conditions the HCVR was higher at high altitude (**Figure 4C**).

The HVR was calculated at 3 P_{CO2} levels; 45 mmHg, 50 mmHg, and 3 mmHg above the participant's VRT identified in the hyperoxic test. The P_{CO2} target of 45 mmHg did not provide a helpful measure of HVR because this P_{CO2} level was below the VRT for many participants, resulting in HVRs near, or below, zero. Using a P_{CO2} level of 50 mmHg, the HVR increased significantly at high altitude, as expected based on many previous reports using steady-state methods[92] (**Figure 4D**). When calculating the HVR based on the P_{CO2} level 3 mmHg above the VRT measured during each hyperoxic test, there was also a trend for an increased HVR at high altitude (**Figure 4E**).

Mechanisms of Increased Hypoxic Ventilatory Responses at High Altitude Measured with Rebreathing

We aimed to evaluate if the increase in hypoxic chemosensitivity after acclimatization to high altitude is produced as the result of an increase in baseline respiratory drive at lower oxygen levels as measured with the rebreathing technique, a leftward shift in the hypoxic VRT, and/or an increased slope of the CO_2 response in hypoxia. Our results indicate that pre-VRT ventilation levels were not significantly different across oxygen treatments within locations (**Figure 5A)**. However, pre-VRT ventilation levels were higher at high altitude ($F(1,30) = 16.53$, $p < 0.001$). There was also no significant difference in the change in pre-VRT ventilation levels from hypoxia to hyperoxia across sea level and high altitude ($W=107$, $p=0.795$).

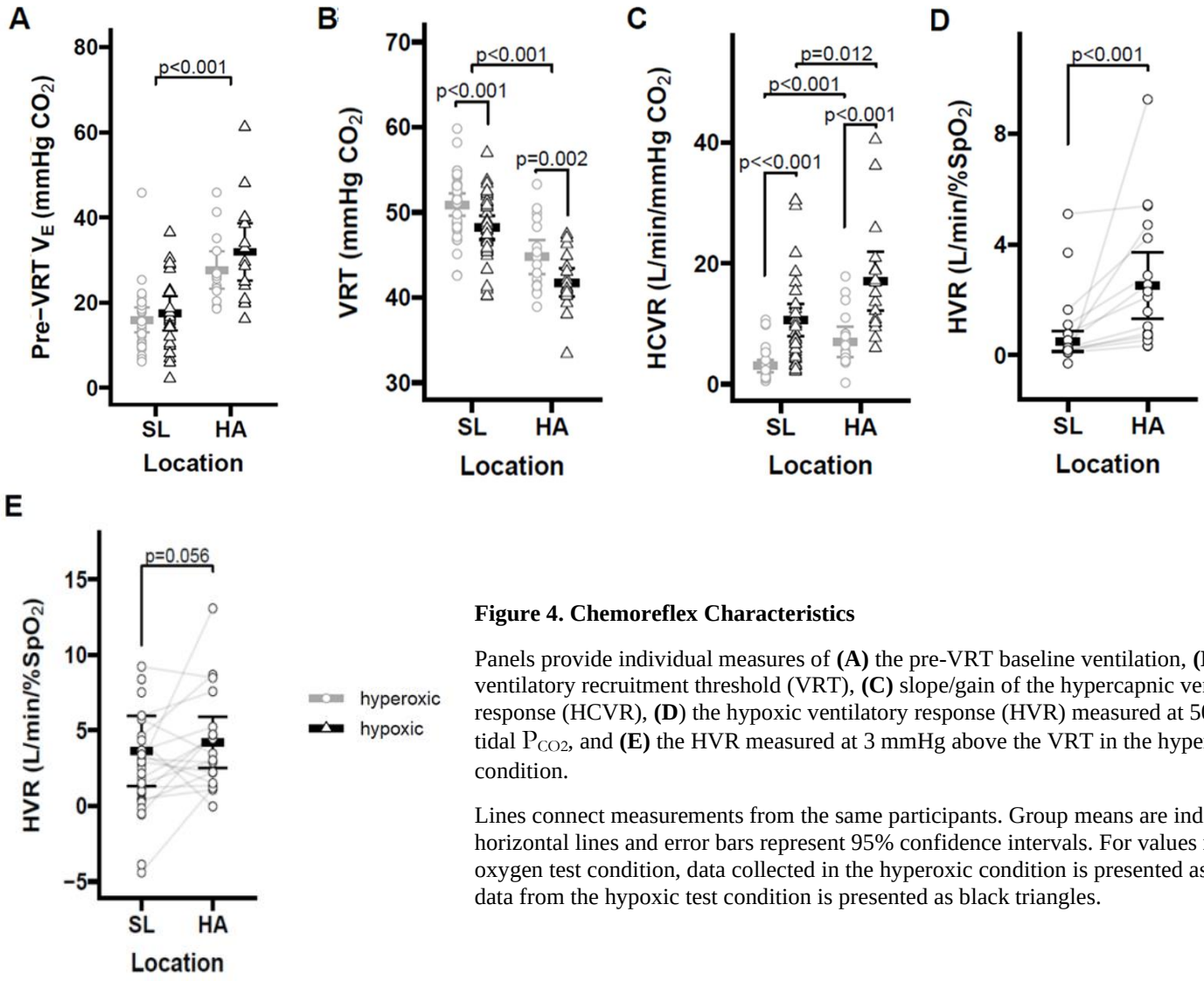

Figure 4. Chemoreflex Characteristics

Panels provide individual measures of **(A)** the pre-VRT baseline ventilation, **(B)** the ventilatory recruitment threshold (VRT), **(C)** slope/gain of the hypercapnic ventilatory response (HCVR), **(D**) the hypoxic ventilatory response (HVR) measured at 50 mmHg end-tidal P_{CO2}, and **(E)** the HVR measured at 3 mmHg above the VRT in the hyperoxic test condition.

Lines connect measurements from the same participants. Group means are indicated by thick horizontal lines and error bars represent 95% confidence intervals. For values relevant to each oxygen test condition, data collected in the hyperoxic condition is presented as grey circles and data from the hypoxic test condition is presented as black triangles.

Two additional factors which can increase the change in ventilation across P_{O2} treatments would include a leftward shift, or decrease in the VRT, or an increase in HCVR slope. We found that most participants had a significant decrease in their VRT in the hypoxic tests compared to hyperoxic tests both at sea level and high altitude (**Figure 4B**). Furthermore, the amplitude of this decrease in VRT was higher at high altitude compared to sea level (W=164, p=0.043, **Figure 5B**) indicating that this is a mechanism influencing the change in HVR. Lastly, we found that all participants have an increase in HCVR slope under hypoxic conditions compared to hyperoxic conditions, regardless of if the measures were conducted at sea level or high altitude (**Figure 4C**). While changed in some participants, the magnitude of the change in HCVR slope in hypoxia compared to hyperoxia was not significantly greater at high altitude than at sea level (W=368, p=0.327, **Figure 5C**). This indicates that, on average, the increase in HCVR slope in hypoxia is not the main contributing factor to the increase in HVR at high altitude.

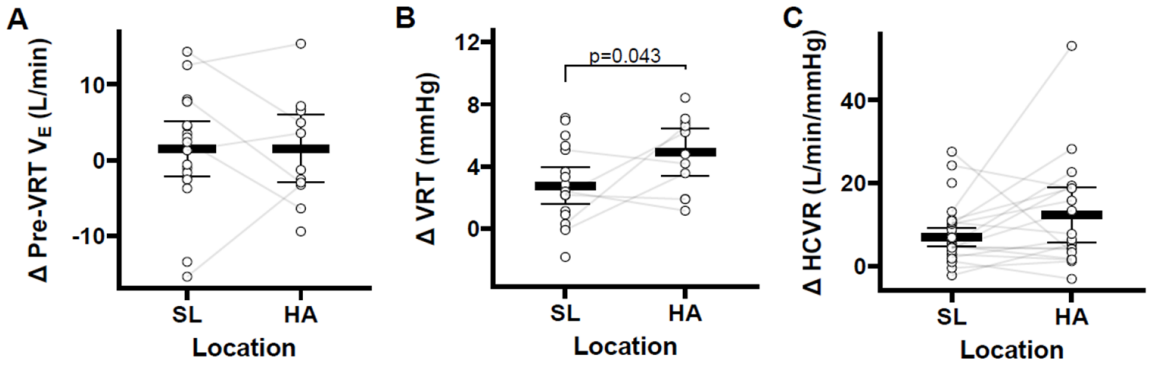

Figure 5. Mechanisms of Increased HVR at High Altitude as Measured with Rebreathing

Y axes indicate the magnitude of the difference in **(A)** pre-VRT baseline ventilation, **(B)** ventilatory recruitment threshold (VRT), and **(C)** the hypercapnic ventilatory response (HCVR) across hyperoxic and hypoxic rebreathing trials at sea level compared to their differences at high altitude. Significant changes are indicated. Group means are indicated by thick horizontal lines and error bars represent 95% confidence intervals.

Discussion

Our finding that the HVR increases after 2 days at 3800 m elevation supports longstanding evidence for ventilatory acclimatization to hypoxia.[101,102] This finding is consistent across studies regardless of if steady state or rebreathing methods are utilized.[92] Furthermore, our findings that HCVR increases after 2 days at 3800 m elevation also support previous work by others using rebreathing[103,104] and steady-state protocols.[105] To build upon these confirmational findings, in this study we utilized a modified rebreathing technique described by Duffin (2007) to simultaneously quantify the ventilatory response to hypoxia and hypercapnia,[93] as well as their interaction. To our knowledge, no prior studies have used this method to quantify the increase in HVR at high altitude in sojourners exposed acutely (2 days) to moderate high altitude. This technique provides a unique examination of ventilatory acclimatization to high altitude because it provides information about pre-VRT baseline respiratory drive under different oxygen tensions, the VRT, as well as the ability to calculate the HVR across any P_{CO2} tension.

The pre-VRT ventilation level significantly increased at high altitude after 2 days of acclimatization in both oxygen treatment groups, consistent with increased baseline respiratory drive at high altitude as a result of ventilatory acclimatization.[106,107] However, unexpectedly, while the mean pre-VRT ventilation level was higher in the hypoxia treatment on average, it was not significantly different from pre-VRT ventilation in the hyperoxic treatment, and the difference between pre-VRT ventilation in the hypoxic and hyperoxic treatments was not significantly different between sea-level and high-altitude tests. This

finding is interesting given that steady-state protocols which typically utilize isocapnic end-tidal P_{CO2} levels only a few (<5) mmHg above the eupneic P_{CO2} routinely measure elevated HVRs at high altitude. Indeed, the average VRT at sea level is usually greater than the isocapnic P_{CO2} tensions chosen in steady-state tests, and therefore HVR measures at these lower P_{CO2} levels would be conducted below the VRT. This may indicate that the reduction in the VRT in hypoxia is sufficient to produce an elevated ventilation rate at this moderately elevated P_{CO2} and thereby produce a measurable, but perhaps lower, HVR. Along these lines, recent work by our group highlighted the fact that low HVR values were more commonly observed.[92] Our current data may indicate that without a significant decrease in the VRT in hypoxia, a low HVR would be expected since there is little difference in pre-VRT ventilation rates, particularly at sea level.

Mohan and Duffin demonstrated that P_{CO2} must exceed the peripheral-chemoreflex threshold before hypoxia increases ventilation.[108] Our data supports this as we observed the characteristic "hockey stick" shaped ventilatory response curve as a function of end-tidal P_{CO2} **(Figure 3)**, and a clear VRT was observed in a majority of tests. Furthermore, minute ventilation below the VRT, indicative of baseline ventilatory drive, at sea level was not significantly different across oxygen levels (**Figure 4B**). This supports the idea that the ventilatory recruitment threshold plays a role in peripheral chemoreflex sensitivity.

The pre-VRT ventilation level significantly increased at high altitude after 2 days of acclimatization in both oxygen treatment groups, consistent with increased baseline respiratory drive at high altitude as a result of ventilatory acclimatization.[106,107] However,

unexpectedly, while the mean pre-VRT ventilation level was higher in the hypoxia treatment on average, it was not significantly different from pre-VRT ventilation in the hyperoxic treatment, and the difference between pre-VRT ventilation in the hypoxic and hyperoxic treatments was not significantly different between sea-level and high-altitude tests. This finding is interesting given that steady-state protocols which typically utilize isocapnic end-tidal P_{CO2} levels only a few (<5) mmHg above the eupneic P_{CO2} routinely measure elevated HVRs at high altitude. Indeed, the average VRT at sea level is usually greater than the isocapnic P_{CO2} tensions chosen in steady-state tests, and therefore HVR measures at these lower P_{CO2} levels would be conducted below the VRT. This may indicate that the reduction in the VRT in hypoxia is sufficient to produce an elevated ventilation rate at this moderately elevated P_{CO2} and thereby produce a measurable, but perhaps lower, HVR. Along these lines, recent work by our group highlighted the fact that low HVR values were more commonly observed.[92] Our current data may indicate that without a significant decrease in the VRT in hypoxia, a low HVR would be expected since there is little difference in pre-VRT ventilation rates, particularly at sea level.

Previous work by others demonstrates that ventilatory acclimatization to high altitude is accompanied by decreases in the VRT compared to sea-level values.[109] Slessarev et al. (2010) used the same modified rebreathing technique to examine ventilatory chemoreflexes in Andean high-altitude residents and lowlanders acclimatizing to hypobaric hypoxia for 10 days at 3850 m elevation.[110] They found that acclimatizing lowlanders decreased their VRT without changing their ventilatory sensitivity to CO_2. Our data support the findings that the

VRT is decreased after 3 days at high altitude. However, we also find that the ventilatory sensitivity to CO_2 increased at high altitude to a small degree in each test condition, although these changes show some degree of individual variation. This difference in results may be a function of power, as we collected measures in 18 participants at high altitude compared to 6 sojourners in the Slessarev study. Furthermore, we identified several participants who demonstrated no increase in HCVR slope at high altitude or across oxygen conditions while others did display clear increases in HCVR slope. Thus, there may be several different mechanisms by which ventilation at a given P_{CO2} tension is increased across individuals. At the same P_{CO2} and P_{O2} tension, an increase in ventilation can be achieved by a decrease in VRT, increase in gain of the response, or an increase in baseline drive. At high altitude, a decrease in the VRT in both hyperoxic and hypoxic conditions seems to play a key role in ventilatory acclimatization. The decrease in VRT at high altitude is attributed to changes in the pH-P_{CO2} relationship resulting from respiratory alkalosis due to hypoxia-induced hyperventilation.[111]

Also similar to Slessarev et al. (2010), we found that a subset of our participants clearly demonstrated two HCVR slopes, with the earlier slope (V_ES_1) being lower than the subsequent slope (V_ES_2).[110] This two-slope response is not always observed, and in another paper from the same group, the secondary slope was not quantified.[112] This demonstrates that there may be an additional recruitment threshold, lag in response, or additional factors recruited later in the response that should be explored.

To our knowledge, our work is the first to quantify the HVR using the Duffin modified rebreathing technique in lowlanders at sea level and during acclimatization to high altitude. Slessarev et al. used this technique to measure the HVR in lowlanders at high altitude only and compared their responses to native high-altitude groups. They found that basal ventilation in acclimatizing lowlanders was lower than in Andean highlanders at both isoxic tensions. Since Slessarev et al. did not have baseline level recordings in the acclimatizing lowlanders, they were unable to verify their hypothesis that of an increased "non-chemoreflex" drive to breathe in Andeans.[110] However, with our dataset we can confirm this hypothesis. Somogyi et al. (2005) measured the HCVR in 6 men at sea level and after 5 days at 3480 m and found no increase in pre-VRT baseline ventilation across locations.[109] However, like Slessarev *et al.* (2010*a*), they also found no increase in HCVR slope as a function of isoxic P_{O2} level or high-altitude acclimatization, in contrast to our findings (**Figure 4C**).

Our study has some important limitations. First, recent debates have highlighted the benefits and shortcomings of both steady state and rebreathing chemoreflex measurements, notably with regards to the interaction with cerebral blood flow dynamics. However, there are studies such as Mannée *et al.*, 2018 showing that both methods result in comparable ventilation values and that rebreathing is a reliable and reproducible method. While we had a large group of participants, this analysis includes two groups of participants over 2 years, with some equipment differing between the years at high altitude. In addition, high altitude

chemoreflex measures are time consuming and thus we were limited in the number of tests we could perform. This led to a lower sample size at high altitude.

In conclusion, here we provide one of the first reports of changes in hypoxic and hypercapnic ventilatory chemoreflexes measured using the Duffin modified rebreathing technique in lowlanders at sea level and after 2 days of acclimatization to moderate high altitude (3800 m). Our work provides the largest sample of such data to date and indicates that many changes occur at high altitude including a decrease in the VRT alongside an increase in pre-VRT ventilation and an increase in HCVR slope. This work provides novel insights into the neural control of breathing and mechanisms of ventilatory acclimatization to high altitude.

Author Contributions

SF, KP, and ECH contributed to the conceptualization and design of the study, the management and coordination of research activity planning and to the funding and resources necessary to conduct the study. SF, KP, BO, NP, and ECH conducted the research and investigation process. SF and BO were responsible for data curation. SF performed all data analysis and SF and ECH created all figures. SF and ECH wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Chapter 3:

Changes in Sleep, Cognition, Vision, and Hearing During High-Altitude Exposure

Chapter 3a. Improvements in Sleep Quality After Acclimatization to 3800 m and the Impact on Cognitive Function

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Abstract

Sojourners at high altitude often experience poor sleep quality due to sleep disordered breathing. Additionally, multiple aspects of cognitive function may be impaired at high altitude. However, the impact of acclimatization on sleep quality and whether poor sleep is a major contributor to cognitive impairments at high altitude remain unclear. Therefore with performance on a battery of cognitive function tests. , we conducted polysomnography and cognitive function tests in fifteen participants (33% women) at sea level and over three days of acclimatization to high altitude (3,800 m) to determine if sleep quality improved over time and if sleep quality measures were associated Sleep quality was worse than sea-level values on

the first 2 days at high altitude using both objective and subjective measures but improved by the third night. The apnea-hypopnea index (AHI) and oxygen desaturation index (ODI) increased on night 1 (adj. p=0.026 and adj. p=0.026, respectively) due to increases in hypopneas, but both the AHI and ODI improved over the subsequent two nights. These objective measures were matched by poorer self-reported sleep quality on the Stanford Sleepiness Scale and modified PROMIS questionnaires on the morning following night 1 at high altitude (adj. p=0.027 and adj. p=0.022, respectively). We found that reaction time on the psychomotor vigilance task (PVT), which measures sustained attention and reaction time to a visual stimulus, was slower at high altitude and did not improve after 3 days of acclimatization (SL: 199±27, HA11: 224±33, HA2: 216±41, HA33: 212±27 ms; adj. p = 0.008 on morning 3). Reaction times on the balloon analog risk task (BART) (SL: 474±235, 1: 375±159, HA2: 291±102, HA3: 267±90 ms) increased at high altitude (adj. p = 0.008 on morning 3), perhaps indicating increased risk-taking behavior. Finally, multiple measures of sleep quality suggested that poor sleep, rather than low daytime arterial oxygen saturation, may be associated with daytime cognitive performance. However, this finding requires further investigation.

These data indicate that sleep quality at moderate altitude improves to near sea-level values after three days of acclimatization and that cognitive performance in newly arrived sojourners to high altitude may by impacted by poor sleep quality rather than hypoxemia alone.

Keywords: high altitude, sleep, cognition, acclimatization, psychomotor vigilance task

Introduction

High-altitude exposure can produce several physiological and neurocognitive impairments due to reduced oxygen availability and other environmental stressors such as low humidity or temperature. Varying degrees of cognitive impairment upon acute high-altitude exposure have been described.[113,114] These include impairments in complex reaction time,[115,116] psychomotor performance,[117] and memory.[116] The severity of these impairments increases as elevation increases.[113] Although different test batteries, ascent profiles, and individual variation in effort and the degree of hypoxemia lead to inconsistent results for some cognitive domains, particularly in mild and moderate hypoxia conditions,[118] complex reaction time is consistently impaired across numerous studies during acute high-altitude exposure.[113] While these effects have been well documented, multiple factors may contribute to these changes in cognitive function and the precise mechanisms driving cognitive impairment at high altitude warrant further investigation.

Possible contributors to cognitive impairment during acute high-altitude exposure are low arterial oxygen pressure, Acute Mountain Sickness (AMS) symptoms, and poor sleep quality. The severity of these factors may also change throughout the process of acclimatization. Brief simulated altitude experiments show that acute changes in inspired oxygen can impact vision, reaction time, and working memory,[119,120] indicating that hypoxemia itself contributes to poor cognitive function. There is also evidence that AMS is associated with poor cognitive performance;[121–123] however, cognitive impairments are also seen in the absence of AMS symptoms. A recent study with a large sample shows no significant correlation between cognitive performance and AMS scores.[124]

Sleep disruption impacts daytime cognitive performance in healthy individuals as well as those with conditions such as obstructive sleep apnea.[125] Obstructive sleep apnea is linked to deficits in attention, memory, executive function, and psychomotor function.[126] Sleep disordered breathing (SDB) is common following ascent to high altitude.[127] SDB may occur when high hypoxic ventilatory drive causes periods of hyperventilation which produce subsequent hypocapnia-driven hypopneas or apneas.[128] These waxing and waning breathing patterns produce important intermittent desaturations and arousals that decrease sleep quality and can contribute to poor daytime cognitive function.

Studies examining the effects of acclimatization on sleep have produced varying results depending on the altitude, ascent profile, and exposure time.[127–135] In general, sleep disordered breathing seems to persist or increase in severity with acclimatization at very high altitudes (>4500 m),[127,131–133] while at high altitudes (<4500 m) the initial increase in periodic breathing is highly variable across individuals and may persist[135] or improve[129,134,136,137] with acclimatization. Fewer studies have examined the impact of acclimatization on cognitive function although a recent study by Pun et al. found improvements in selective and sustained attention with 6 days of acclimatization.[138] Whether neurocognitive impairments at high altitude are driven by poor sleep quality versus hypoxemia or AMS severity remains to be determined.

The aim of this study was to measure changes in cognitive performance during acclimatization to high altitude and determine whether cognitive performance was predicted by AMS scores, resting arterial oxygen saturation, and/or sleep quality. We hypothesized that

poor sleep is a major contributor to impaired cognition at high altitude. Since some previous reports indicate that periodic breathing improves after acclimatization at high altitudes less than 4500 m,[137] we predicted that any significant impairments in cognitive function would improve over three days of acclimatization concomitant with improved sleep quality.

Methods

Ethical Approval

This study was approved by the University of California, Riverside Clinical Institutional Review Board (HS 19-076). All participants were informed of the study's purpose and risks. Participants provided written informed consent in their native language (English). The work was conducted in accordance with the *Declaration of Helsinki*, except for registration in a database.

Participants

15 participants were recruited (10 men and 5 women). Participant demographics are provided in **Table 1**. All participants were healthy individuals between 19 and 32 years old and had no history of cardiovascular or pulmonary disease. Exclusion criteria included smoking (cigarettes, e-cigarettes, marijuana), pregnancy, travel to altitudes greater than 2500 m within one month prior to the first test measurement, or use of anti-inflammatory medications (i.e. ibuprofen) that can interfere with acclimatization to high altitude.[95]

Table 1. Participant Demographics at Sea Level

	M (n=10)	F (n=5)
Agy (y)	24.9 (4.3)	26.4 (5.1)
BMI (kg/m^2)	26.7 (5.4)	28.4 (6.9)
P_{sys} (mmHg)	129.8 (6.8)	126.3 (9.0)
P_{dia} (mmHg)	79.5 (11.1)	77.5 (8.5)

Experimental Design

Participants completed cognitive function testing and sleep quality measures at sea level and over three days at high altitude. Sea-level measures were completed at the University of California, Riverside (340 m elevation) and high-altitude measures were taken at Barcroft Station within the White Mountain Research Center (3800 m). Participants were driven to Barcroft Station and ascended from 340 m to 1216 m over 4 hours, then from 1216 m to 3800 m in 2 hours.

Physiological measures and questionnaires were collected each morning. After these measures, participants were permitted to eat a light breakfast prior to completing cognitive function tests but did not consume caffeine until completing the cognitive test battery. During the study, participants were asked to abstain from taking nonsteroidal anti-inflammatory drugs (NSAIDs) or acetazolamide.[95]

Measurements

Physiological Measurements and Questionnaires

Heart rate (HR), blood pressure (BP), and resting daytime oxygen saturation (SpO_2) were measured each morning at sea level and high altitude. BP measurements were collected with a manual sphygmomanometer. HR and daytime SpO_2 were measured with a pulse oximeter (Nellcor N600, Medtronic, Minneapolis, MN, USA) using a fingertip probe. The participant sat upright in a chair with their feet on the ground and legs uncrossed and were asked to rest for 2-3 minutes until SpO_2 stabilized.

Participants verbally completed the Lake Louise AMS Score questionnaire each morning.[139] Participants completed the *Pittsburgh Sleep Quality Index* (PSQI) during their baseline sea-level visit.[140] The PSQI was used to determine the participant's baseline sleep quality and patterns. They also completed the Stanford Sleepiness Scale (SSS) and a modified version of the short form 8-item PROMIS Sleep Disturbance questionnaire each morning (substituting the timeframe for each question from "past 7 days" to "past 1 day"). PROMIS T-scores were calculated based on the 2013 sleep disturbance 8b short form conversion table.

Cognitive Function

Participants completed a 30-minute cognitive function test battery (*Cognition* by Joggle Research) once at sea level and once each morning over three days at 3800 m elevation. The test battery consisted of 8 different tasks that used different measures (reaction time,

accuracy, number of correct responses) to determine performance in each cognitive domain (**Table 2**).

A detailed description of each test can be found in the supplemental material or at the Joggle Research website (https://admin.joggleresearch.com/Home/Tasks). The cognitive test battery was taken on a 12.9 inch iPad Pro (Apple, Inc., Cupertino, CA, USA). Participants completed the tests in a separate, quiet room to eliminate any sources of distraction, and were seated in an upright position with the iPad placed on a desk in front of them. Before each test, instructions were presented on the screen to eliminate any variance introduced by a researcher explaining tests to participants. In addition to the instructions, some assessments had a practice session which familiarized participants with the test prior to completing the experimental session. To prevent learning effects, participants were provided with a novel array of test permutations during each test session. Additionally, half (n=8) of the participant group was assigned to complete sea-level cognitive function testing before ascent to high altitude and half (n=7) completed baseline tests at least 2 days after descent to sea level to verify that performance at sea level compared to day 1 at altitude was not associated with the order in which tests were taken.

Table 2. Cognition Test Battery Description

Test Name	Abbreviation	Cognitive Domain
Psychomotor Vigilance Test	PVT	Vigilant Attention
Balloon Analog Risk Test	BART	Risk Decision Making
Digit Symbol Substitution Task	DSST	Complex Scanning and Visual Tracking
Line Orientation Task	LOT	Spatial Orientation
NBack	NBACK	Working Memory
Visual Object Learning Task	VOLT	Visual Learning and Spatial Working Memory
Abstract Matching	AM	Abstraction
Motor Praxis Task	MPT	Sensory Motor Speed

Sleep Studies

Participants were assigned an Actiwatch (Philips Respironics, Murrysville, PA, USA) to wear at sea level for 1-3 days and throughout the duration of their three day stay at White Mountain Research Center (Barcroft Station, 3800 m). Participants were also instrumented with respiratory polygraphy (Apnealink Air, ResMed, San Diego, CA, USA) for one night at sea level and each night while at altitude. In the morning, SSS and PROMIS questionnaires assessing their subjective sleep and health were obtained as described above. The actigraphy and polygraphy data were scored by a registered polysomnographic technologist using Philips Actiware 6 software and Airview, respectively. Due to equipment limitations and subject adherence, complete sleep studies were obtained from 10 individuals at sea level, 9 on night 1 at high altitude, 8 on night 2, and 6 on night 3.

Statistical Analysis

All statistical analyses were performed in R version 3.6.1 (R Inc., Boston, MA, USA). Wilcoxon signed rank tests were used to determine significant changes in sleep quality and cognitive performance on each day at high altitude (HA1, HA2, and HA3) compared to sea-level (SL) performance. Pairwise Spearman's correlations were used to determine if cognitive performance scores on the first day at high altitude were associated with physiological variables such as AMS scores and daytime SpO_2, or sleep quality measures including SSS and PROMIS questionnaires, AHI, hypopnea index, apnea index, central apnea index, and ODI. The Benjamini-Hochberg procedure was used to adjust for multiple comparisons. Raw and adjusted p values are reported. Data are presented throughout the manuscript as mean

(standard deviation). Asterisks indicate significant differences at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), or $p<0.0001$(****). Cognitive function data was missing for one participant who completed cognitive function tests after returning to sea level due to an unknown software error and therefore this subject was excluded from the cognition analysis.

Results

High Altitude Effects on Physiological Measures

Table 3 provides an overview of the physiological measures taken each morning. There was no significant change in systolic blood pressure during high-altitude exposure. Diastolic blood pressure was slightly higher than sea-level values on day 1 and 3 at high altitude, but this difference was not significant after correcting for multiple comparisons (**Fig. 1A-B**). Resting heart rate increased and remained higher throughout the stay at high altitude (**Fig. 1C**). Resting SpO_2 decreased at high altitude and remained lower than sea-level values throughout the stay at high altitude (**Fig. 1D**). AMS Scores were significantly higher on the first two mornings at high altitude but returned to baseline levels by day 3 (**Fig. 1E**).

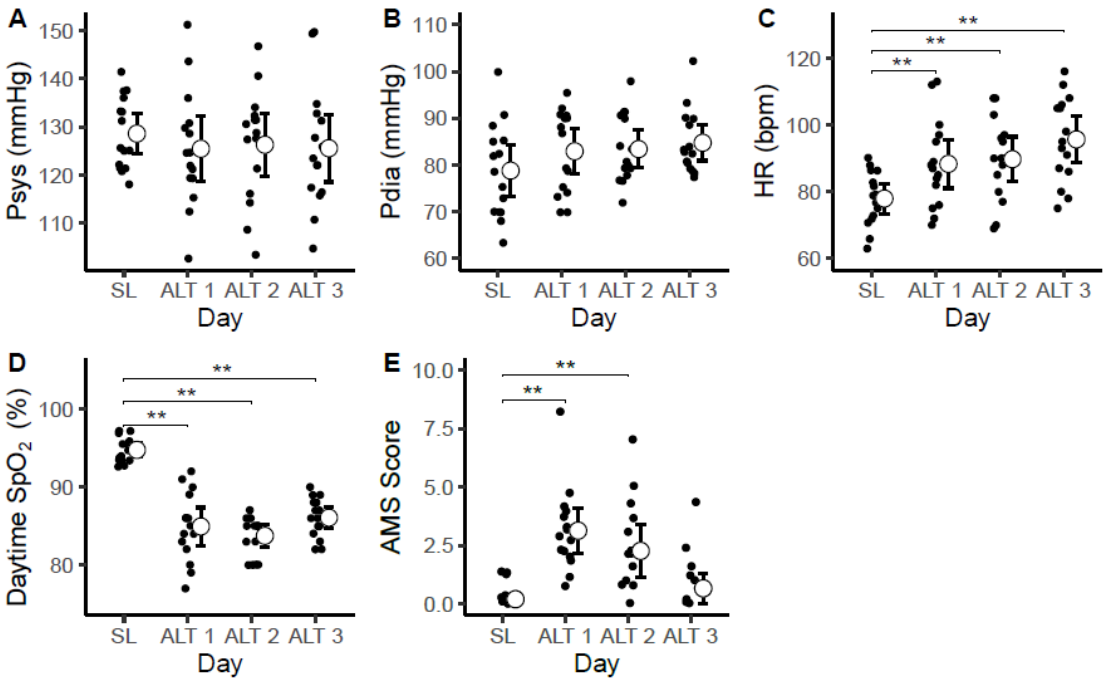

Figure 1. **Physiological Changes by Day**

Individual systolic blood pressure **(A)**, diastolic blood pressure **(B)**, heart rate **(C)**, daytime resting SpO_2 **(D)**, and AMS Scores **(E)** collected each morning at sea level (SL) and high altitude (ALT) days 1-3

Table 3. Physiological Measures Collected Each Morning Sea Level (SL) and High Altitude (HA) Days 1-3 (N = 15)

Measure	SL	HA1	p	adj. p	HA2	p	adj. p	HA3	p	adj. p
$P_{systolic}$ (mmHg)	129 (7.5)	125 (12.2)	0.315	0.315	126 (11.7)	0.53	0.53	126 (12.7)	0.315	0.315
$P_{diastolic}$ (mmHg)	79 (10.1)	83 (8.8)	**0.043**	0.054	83 (7.3)	0.084	0.105	85 (6.9)	**0.033**	0.055
Resting HR (bpm)	78 (8.1)	88 (13.2)	**<0.001**	**0.001**	90 (12.1)	**0.004**	**0.007**	96 (12.8)	**<0.001**	**0.003**
Resting daytime SpO_2 (%)	95 (1.6)	85 (4.4)	**<0.001**	**0.001**	84 (2.6)	**<0.001**	**0.005**	86 (2.6)	**<0.001**	**0.003**
AMS Score	0.2 (0.4)	3.1 (1.8)	**<0.001**	**0.001**	2.3 (2.0)	**0.004**	**0.007**	0.7 (1.2)	0.222	0.278

Table 4. Effects of Day on Cognitive Test Performance Measures

Test	Measure	SL	HA1	p	adj. p	HA2	p	adj. p	HA3	p	adj. p
PVT	Mean RT	189 (25)	224 (33)	**0.03**	0.24	217 (42)	**0.02**	0.107	212 (27)	**<0.001**	**0.008**
	Lapses	1.0 (1.3)	1.9 (2.7)	0.16	0.427	1.9 (2.4)	0.085	0.272	1.4 (2.0)	0.305	0.573
	False starts	2.6 (4.3)	1.8 (1.5)	0.905	0.905	1.0 (0.7)	0.12	0.32	0.6 (0.8)	0.057	0.218
BART	Mean RT	474 (235)	376 (159)	0.078	0.25	297 (101)	**0.002**	**0.032**	267 (91)	**<0.001**	**0.008**
	Pumps	3.9 (1.0)	4.1 (1.1)	0.67	0.905	3.7 (1.4)	0.433	0.766	3.7 (0.9)	0.502	0.73
DSST	Mean RT	851 (96)	876 (65)	0.068	0.25	854 (66)	0.542	0.766	825 (58)	0.326	0.573
	Correct responses	94.3 (9.2)	91.7 (6.2)	0.062	0.25	93.9 (6.4)	0.656	0.766	96.8 (6.2)	0.324	0.573
LOT	Mean RT	6076 (1831)	5173 (988)	**0.025**	0.24	5051 (1235)	**0.03**	0.12	4928 (1248)	0.068	0.218
	Correct responses	13.9 (4.0)	13.4 (3.7)	0.723	0.905	13.5 (2.4)	0.672	0.766	14.3 (3.3)	0.888	0.888
NBACK	Mean RT	567 (66)	568 (64)	0.808	0.905	514 (58)	**0.007**	0.056	541 (86)	0.153	0.408
	Correct responses	53.5 (4.6)	52.2 (5.2)	0.223	0.446	53.6 (4.8)	1	1	53.8 (3.8)	0.806	0.888
VOLT	Mean RT	1969 (809)	1918 (603)	0.855	0.905	1748 (616)	0.542	0.766	1590 (568)	0.058	0.218
	Correct responses	17.3 (2.0)	16.1 (2.1)	0.203	0.446	17.1 (1.7)	0.718	0.766	17.6 (2.2)	0.581	0.775
AM	Mean RT	1928 (968)	2013 (789)	0.903	0.905	1704 (632)	0.296	0.677	1721 (522)	0.358	0.573
	Correct responses	19.0 (4.2)	17.6 (3.3)	0.35	0.622	18.5 (3.7)	0.688	0.766	19.4 (3.3)	0.844	0.888
MPT	Mean RT	400 (47)	401 (24)	0.761	0.905	410 (53)	0.432	0.766	397 (57)	0.715	0.888

Reaction times are measured in milliseconds.

High-altitude effects on cognitive performance

Table 4 provides an overview of the effects of high altitude on test performance. Mean reaction time on the PVT was slower each day at high altitude compared to sea level and remained significantly slower on day 3 (**Fig. 2A**). There was no difference in the number of lapses or false starts on the PVT. Mean reaction time per click on the BART was lower at high altitude on days 2 and 3 compared to sea level (**Fig. 2B**), but there was no effect on the number of pumps per balloon. Altitude did not significantly impact performance on any other cognitive function test after correcting for multiple comparisons.

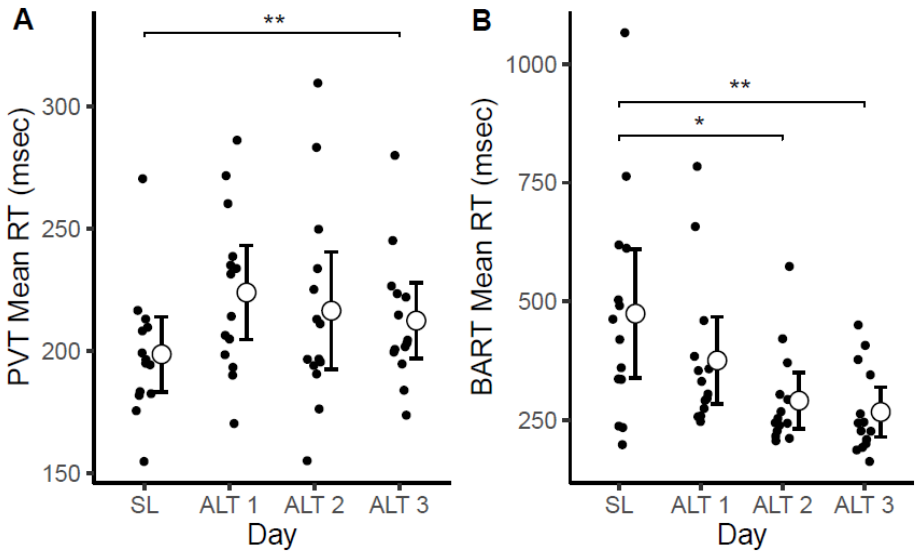

Figure 2. Cognitive Test Performance for PVT And BART
Cognitive function tests demonstrating significant effects of high altitude on performance. Boxplots demonstrate significant differences in reacting time on the PVT **(A)** and BART **(B)** tests across days.

High-altitude effects on sleep quality

Sleep quality and sleep disordered breathing were worst on the first night at high altitude. **Table 5** provides average sleep measures for each day. AHI increased significantly on night 1 at high altitude (**Fig. 3A**), particularly due to an increase in hypopneas and central apneas and returned to baseline levels by day 3. The ODI demonstrated a similar pattern, increasing significantly on night 1 at high altitude (**Fig. 3B**) but returned to baseline levels in most participants by night 2. While acute desaturation events improved by day 2, the mean (**Fig. 3C**) and nadir (**Fig. 3D**) sleep saturation remained significantly lower than baseline levels throughout the stay at high altitude. Wake after sleep onset (WASO) was longer at high altitude, although not significant after correcting for multiple comparisons, but returned to near baseline levels by night 3 (**Fig. 3E)**. Average sleep efficiency decreased at high altitude compared to sea level, but this change was also not significant after correcting for multiple comparisons. Subjective sleep quality measures were also impacted by high altitude. SSS and PROMIS scores increased after the first night at high altitude but returned to baseline levels after the second night (**Fig. 3G-H)**.

Table 5. Effects of High Altitude on Sleep Quality Measures

Variable	SL	HA1	p	adj. p	HA2	p	adj. p	HA3	p	adj. p
AHI (events/h)	4.3 (4.5)	35.3 (28.7)	**0.014**	**0.026**	16.0 (21.1)	0.205	0.282	7.3 (5.3)	0.219	0.746
Hypopnea Index (events/h)	2.8 (2.3)	20.9 (16.3)	**0.014**	**0.026**	8.4 (16.0)	0.205	0.282	6.5 (4.9)	0.063	0.476
Apnea Index (events/h)	0.5 (1.1)	0.3 (0.8)	0.423	0.423	0.1 (0.2)	1	1	0.1 (0.2)	1	1
Central Apnea Index (events/h)	0.5 (0.6)	14.0 (17.1)	0.052	0.072	7.6 (15.2)	0.462	0.565	0.8 (0.8)	0.786	0.952
ODI (events/h)	3.1 (3.3)	34.3 (22.6)	**0.014**	**0.026**	19.5 (22.9)	0.078	0.143	7.2 (6.1)	0.063	0.476
Night time SpO_2 average (%)	94.7 (0.9)	77.0 (2.4)	**0.014**	**0.026**	77.6 (2.9)	**0.016**	0.059	78.5 (1.6)	**0.036**	0.476
Nadir SpO_2 (%)	85.8 (4.4)	65.3 (6.2)	**0.014**	**0.026**	68.0 (6.4)	**0.022**	0.061	70.7 (3.6)	**0.031**	0.476
WASO (min)	35.1 (19.9)	57.8 (35.9)	0.102	0.125	76.0 (52.4)	**0.01**	0.055	43.9 (37.6)	0.45	0.819
Sleep efficiency (%)	83.8 (7.1)	79.7 (11.8)	0.123	0.135	72.6 (13.6)	**0.005**	0.055	77.5 (14.2)	0.52	0.819
SSS	2.3 (1.0)	3.6 (1.2)	**0.017**	**0.027**	2.3 (1.4)	0.548	0.602	1.9 (0.8)	0.356	0.746
PROMIS T-score	15.3 (5.5)	27.3 (7.7)	**0.002**	**0.022**	20.9 (9.3)	0.053	0.117	15.2 (5.4)	0.548	0.819

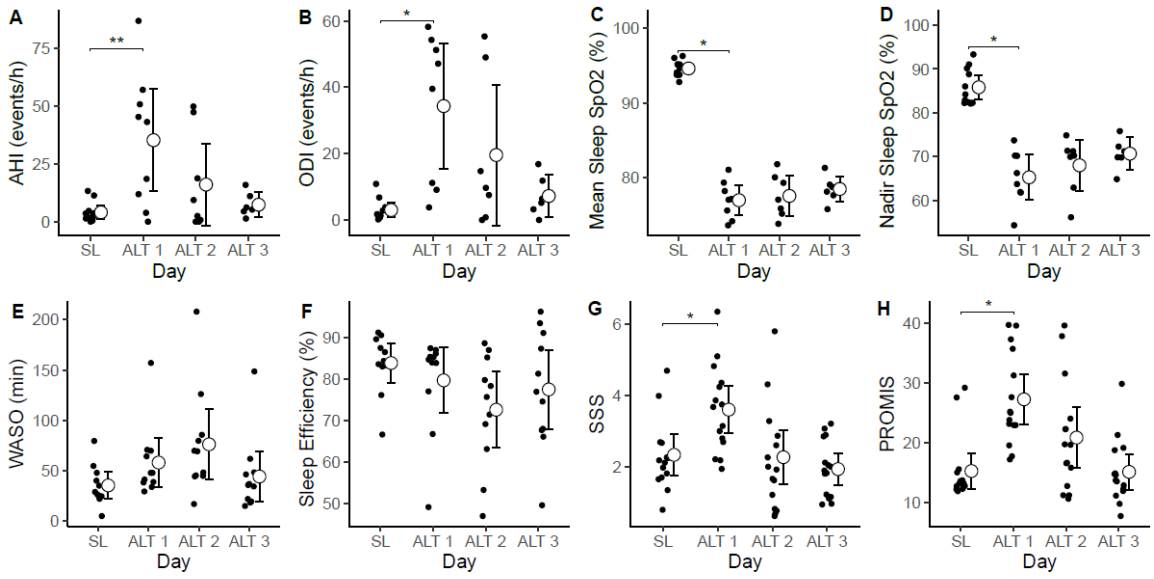

Figure 3. Sleep Quality Measures by Day
Sleep quality at sea level (SL) and over three nights at high altitude (ALT).

Effects of physiology and sleep quality on cognitive performance

We aimed to determine if daytime hypoxemia, AMS, or poor sleep were the primary contributors to cognitive impairment at high altitude. Since sleep measures and AMS scores were worst on the first night at high altitude, we looked for associations between these variables and cognitive test performance in the morning following the first night at high altitude (HA1). We found scores on multiple cognitive function tests were associated with measures of sleep quality but not resting daytime SpO_2. Significant correlations before correcting for multiple comparisons are provided in **Fig. 4** and an extended table is provided in **supplemental table 1**.

Higher PROMIS scores (worse self-reported sleep quality) were associated with slower reaction times and more lapses on the PVT. Similarly, individuals with the lowest sleep efficiency had slower reaction times on the NBACK and VOLT tests on average. Higher

AMS scores were associated only with lower scores on the MPT. Unexpectedly, individuals with higher AHI and ODI scores performed better on the VOLT task. While some of these associations appear promising, due to the large number of tests and limited sample size, we were underpowered to detect these associations after correcting for multiple comparisons. Therefore, these relationships warrant further investigation.

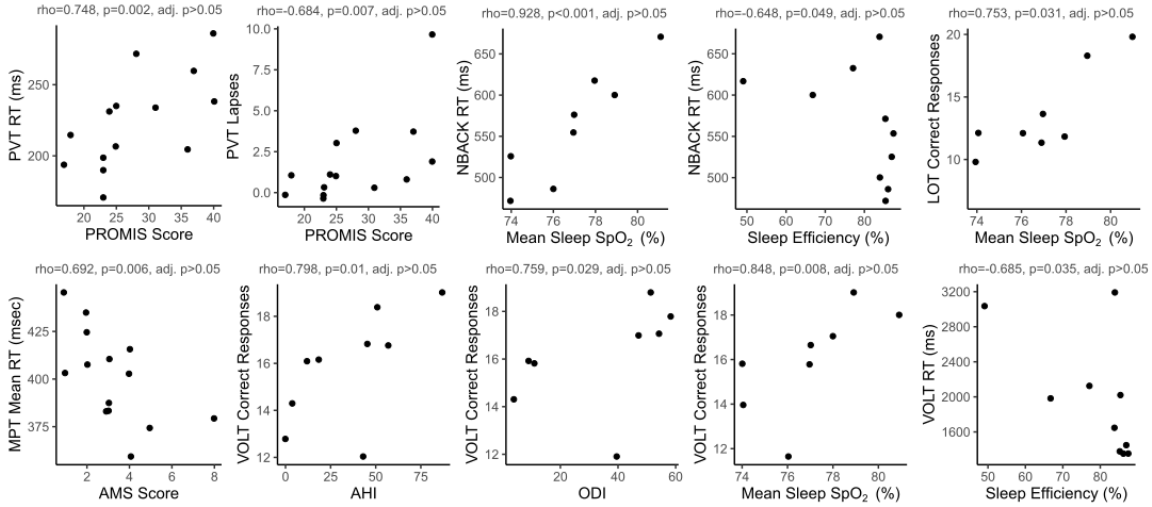

Figure 4. Relationship Between Cognitive Test Results, Sleep, and AMS

Cognition tests demonstrate significant associations with sleep quality measures and AMS scores after the first night at high altitude. Spearman's rho, raw p values, and p values after adjustment for multiple comparisons are provided.

Discussion

Sleep during acclimatization

We found that sleep quality was poor in unacclimatized sojourners to high altitude but improved to near baseline levels after three days of acclimatization at an altitude of 3800 m. Periodic breathing, the number of desaturation events, and overall sleep quality worsened upon arrival to high altitude but improved to near baseline levels after three days of acclimatization. The AHI and ODI increases initially, primarily due to increased hypopnea

and central apnea events, but returned to baseline levels by night 3. Despite these improvements, average sleep SpO_2 remained low. However, the decrease in hypopneas and central apneas resulted in a slight increase in nadir SpO_2 from night 1 until night 3. Overall sleep efficiency was also poorer on average each night at high altitude and was lowest on night 2 when WASO was also the longest. These objective sleep quality measures were reflected in increased subjective SSS and PROMIS T-scores on the morning following the first night at high altitude.

Our results support the consensus that sojourners to high altitude experience poor-quality sleep linked to periodic breathing.[128,137] Whether or not acclimatization improves or worsens sleep disordered breathing may depend on the severity of the altitude exposure, as well as individual variation in peripheral and central ventilatory chemoreflex sensitivity. One hypothesis is that periodic breathing should worsen with acclimatization as the hypoxic ventilatory response increases over time,[128,141,142] leading to high loop gain and breathing instability manifesting as more vigorous and/or frequent bouts of hyperventilation and subsequent apneas. However, our data and that of others[129,134,136,137] indicate periodic breathing and overall sleep quality (measured objectively or subjectively) improves by day 3 at the high altitude of 3800 m. In comparison, studies in acclimatized sojourners at very high altitude find persistent periodic breathing and sleep disturbance even after acclimatization.[127,130] This indicates a potential interaction between elevation and acclimatization-induced changes in ventilatory control on periodic breathing and sleep disturbance.

At high altitudes, periodic breathing may manifest in unacclimatized sojourners as a result of acute hypoxic ventilatory response-induced hyperventilation, which reduces arterial PCO_2 below the ventilatory recruitment threshold and generates an apnea until PCO_2 recovers. However, as the ventilatory recruitment threshold decreases with acclimatization,[143] this instability may be ameliorated. However, at higher altitudes, the gain of the hypoxic ventilatory drive becomes increasingly higher and would exacerbate breathing instability and sleep disordered breathing.[144]

Cognitive function during acclimatization

The cognitive function test displaying the largest, and most consistent, impairment at high altitude was the PVT. This test measures sustained attention and reaction times to a visual stimulus. We found PVT reaction times were higher on the first day at high altitude and remained significantly higher on day 3 despite AMS, AHI, and ODI scores returning to baseline levels (**Figure 2**). These results are consistent with Pun et al. (2018) who found significant increases in PVT reaction time after 1 day at high altitude and significant improvement by day 6. While we do see an improvement in average PVT from day 2 to 3 at high altitude, full recovery of PVT performance may take longer than 3 days. Furthermore, Pun et al. (2018) found a significant correlation between PVT reaction time and AMS scores calculated by the Environmental Symptom Questionnaire – Cerebral.[145] We did not find such an association with AMS score calculated via Lake Louise Score. However, we did find that poor sleep measured subjectively by the PROMIS and SSS may be associated with lower PVT performance.

The mean reaction time on the BART improved with each administration of the task, despite no change in the total number of pumps per balloon. Pighin et al.[146] used the same test in subjects exposed acutely to normobaric hypoxia with 7 days between testing periods. They found that individuals used a higher number of pumps before collecting their reward in hypoxia versus normoxic control tests, suggesting increased risk-taking behavior in hypoxia. While we did not find differences in the total number of pumps per balloon, the faster reaction time may also be an indicator of increased risk-taking behavior as there is less time for consideration between each action. Alternatively, this result may be attributed to learning effects as participants become more comfortable with the number of pumps they can attempt per balloon despite the test being designed to discourage learning by having each balloon pop after a random number of pumps. This learning effect is supported by the fact that individuals who completed their sea-level cognition testing last had significantly faster reaction times on the BART than individuals who completed their sea-level testing first (first: 608 ± 238; last: 340 ± 145 ms, $p = 0.026$). Therefore, while there may be important changes in risk-taking behavior at high altitude, improved methods for measuring this trait in the same individuals over subsequent days may be necessary to determine how these effects change with acclimatization.

Determinants of cognitive performance at altitude – is sleep the key?

Of note, there was no association between resting daytime SpO_2 and performance on any cognitive function task. Instead, these results suggest that poor sleep quality contributed to daytime impairments in sustained attention and reaction times on the PVT in unacclimatized sojourners to high altitude. While acute hypoxia exposure experiments demonstrate

hypoxemia itself can produce cognitive impairment,[119,120] it seems that overnight acclimatization may partially mitigate these effects and that the effects of poor sleep at high altitude on daytime cognition cannot be discounted.

To our knowledge, this is the first study to measure the impact of acclimatization to high altitude on comprehensive neurocognitive performance coupled with actigraphic and polygraphic measures of sleep quality and SDB. Nonetheless, the study has some important limitations. Our sample size was relatively small, largely due to housing capacity of the high-altitude facility, equipment limitations, and participant adherence to Actiwatch and respiratory polygraphy procedures. We may have been underpowered to detect some differences in neurocognitive measures. We also note the potential for learning effects on the neurocognitive testing, which would artificially increase the apparent impact of acclimatization on improvement. To mitigate learning effects, we assigned half of the participants to complete sea-level testing before ascent, and half after returning to sea level. In addition, the *Cognition* test battery allows for within-subject repeated testing by producing novel permutations of each test in each session. To control for learning completely, a much more logistically complex study design would be needed and may limit feasibility. We note that learning effects on this neurocognitive battery are low/modest, with the potential exception of the BART. The PVT is particularly resistant to learning as it is based only on attention and reaction time.

In conclusion, our data show that sleep quality is impaired in unacclimated sojourners to high altitude due to sleep disordered breathing, but that sleep quality returns to sea-level

values after 3 nights of acclimatization. Furthermore, of the cognitive domains tested, it appears that PVT performance is most influenced by high-altitude exposure and that poor sleep quality may impair sustained attention at high altitude.

Acknowledgements

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Chapter 3b. Cognitive Function, Visual Contrast Sensitivity, and Hearing During High-Altitude Acclimatization

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Abstract

High altitude exposure has been shown to impact several cognitive function domains, such as reaction times and sustained attention. However, questions remain regarding the mechanisms underlying these impairments. In this study, we aimed to determine if decreases in cognitive function at high altitude could be rescued with a mild hypercapnic gas to combat hypocapnia. Twenty healthy participants (13 men, 7 women) completed a 45-min cognitive function test three times at sea level prior to ascent, over three days at high-altitude (HA) (3800 m elevation), and three times at home after return to sea level. At high-altitude, participants were divided evenly into placebo (room air) or hypercapnic (4% inspired CO_2) treatment groups in a participant-blinded randomized order.

Results show that the number of correct responses for memory and recall tasks such as Word List (HA1 $p = 0.038$, HA2 $p = 0.056$), as well as visual and attention switch tasks such as the total time taken to complete Trail Making B task (HA1 $p = 0.0057$, HA2 $p = 0.046$), were significantly impaired at high altitude but recovered over three days of acclimatization. Contrast sensitivity also worsened at high altitude, HA1 ($p = 3.3e^{-5}$) and HA2 ($p = 0.04$). Performance on all tasks were not improved with mild hypercapnic treatment. Overall, this data supports previous work illustrating a mild impact of moderate high-altitude exposure on cognitive performance, which appears to improve with acclimatization.

Keywords: high altitude, hypoxia, cognition, vision, hearing

Introduction

High-altitude exposure can impair several cognitive function domains, including reaction time, attention, visual and working memory, and inhibitory control.[147–149] The prevailing hypotheses surrounding these findings are that high-altitude hypoxia exposure causes hypoxemia, poor sleep quality, sleep disordered breathing, and acute mountain sickness (AMS), and that each of these factors may independently impact cognitive performance. However, the precise mechanisms underlying cognitive impairment at high altitude remains unknown. Furthermore, the prevalence and severity of these impairments have varied across studies, likely due to cognitive function test designs, ascent profiles, and time spent at high altitude. Nonetheless, it remains clear that at least mild cognitive impairments are observed during acute high-altitude exposure, and that they can occur independently of AMS.[150–152]

To ensure proper oxygenation of tissues during acute high-altitude exposure, baseline ventilation rates and ventilatory chemosensitivity to hypoxia and carbon dioxide increase.[153,154] Excess CO_2 in the blood leads to the dilation of blood vessels in the brain, which increases the blood flow to the brain, and thus increases cerebral blood flow (CBF). Both hypoxia and hypercapnia stimulate vasodilation and increase CBF.[155] These mechanisms all work to improve arterial oxygenation and decreases arterial partial pressure of carbon dioxide. However, the decrease in arterial partial pressure of carbon dioxide can lead to hypocapnia and have serious adverse effects. For example, central nervous system impairments seen in mountaineers at extremely high altitude have been attributed to hypocapnia rather than hypoxia.[156]

In this study, we aimed to test if cognition is impacted by altitude, and if mitigating hypocapnia by providing a mild hypercapnic gas treatment (+4% CO_2) during cognitive testing is sufficient to rescue the cognitive function decline usually seen at high altitude. We conducted a randomized, participant-blinded placebo-controlled study and measured various aspects of cognitive function, vision, and hearing throughout three days at sea-level, three days of acclimatization to high altitude (3800 m elevation), and three days following return to sea level.

Methods

Ethical Approval

This study was approved by the University of California, Riverside Clinical Institutional Review Board (HS 22-088). Participants were informed of the study's purpose and risks and provided written informed consent in their native language (English). The work was conducted in accordance with the Declaration of Helsinki, except for registration in a database.

Experimental Design

Twenty participants were recruited (13 men and 7 women). All participants were healthy individuals between 18 and 38 years old (25.6 ± 5.9 years) with no history of cardiovascular or pulmonary disease. Detailed demographic information for participants is provided in **Table 1**. Exclusion criteria included current regular smokers (cigarettes, e-cigarettes,

marijuana), pregnant women, recent travel to altitudes greater than 2500 m within one month prior to the first test measurement, or current use of anti-inflammatory medications (i.e., ibuprofen) that can interfere with acclimatization to high altitude.[157]

Table 1. Participant Demographics

Variable	SL	HA1	HA2	HA3	ANOVA p-value
P_{sys} (mmHg)	121 ± 7.1	126 ± 9.5	128 ± 13	126 ± 11.	0.177
P_{dia} (mmHg)	79 ± 5	85 ± 7.1	85 ± 8.4	85 ± 8.7	0.059
Heart Rate (bpm)	74 ± 8.4	**91 ± 13**	**90 ± 15***	**100 ± 16*****	**<0.001**
SpO_2 (%)	95 ± 1.7	**84 ± 4.3*****	**84 ± 3.2*****	**84 ± 4.1*****	**<0.001**

Variable units: P_{sys} and P_{dia} (mmHg); HR (bpm); SpO_2 (%). Overall p-values for repeated measures ANOVA are provided. Asterisks indicate significant differences from SL via post-hoc pairwise comparisons with Bonferroni adjusted p-values.

Participants completed their first visit at the University of California, Riverside (sea level (SL); 340 m elevation) for consenting procedures, baseline physiological measurements, and acclimation to the cognitive test battery and breathing apparatus. Blood pressure measures were collected with a manual sphygmomanometer. Heart rate and pulse oxygen saturation (SpO_2) with a Nellcor N600X pulse oximeter and finger probe (Medtronic, Minneapolis, MN, USA) after 5 minutes of resting in an upright seated position with legs uncrossed and breathing normally. Acute Mountain Sickness scores were collected via the 2018 Lake Louise Acute Mountain Sickness Score.[158] In these scores a higher score is calculated by a participant suffering from many symptoms including headache, nausea, and dizziness or having intense symptoms.

During this visit, participants were instructed to take their first cognitive test battery, labeled as practice, in an isolated room under conditions described in the "Cognitive Function

Testing" section. Participants were seated in an upright position and wore an oronasal mask (7450 Series V2, Hans Rudolph, Shawnee, KS, USA) connected to a two-way T-shape non-rebreathing valve (Hans Rudolph) with large bore respiratory tubing attached at either end. While completing the cognitive tests for the first time, participants were provided with room air gas mixture via an air pump which delivered fresh air to the inspiratory side of the respiratory system. This same setup would later be utilized on the first and second days at high altitude to administer either placebo/room air treatments via the same air pump, or a mild hypercapnic gas.

Participants were provided with a 'test kit' to take home including an iPad, headphones, and instructions indicating when to complete tests and under what conditions. These kits were used to complete the test battery at home 3 days prior to and post ascent, labeled as sea level (SL) 1-3 and post high altitude (PHA) 1-3 respectively. Overall, participants completed the battery of cognition tests a total of 10 times (**Figure 1**); Practice, SL1, SL2, SL3, HA1, HA2, HA3, PHA1, PHA2, and PHA3.

All participants returned to the lab on the morning of ascent. Participants were driven to Barcroft Station (White Mountain Research Center, UC Natural Reserve System). The ascent profile included traveling from 340 m to 1216 m over four hours, then from 1216 m to 3800 m in two hours. At high altitude, blood pressure, heart rate, and arterial oxygen saturation were measured each morning as described above. Participants completed the cognitive tests once per day over three days at high altitude, starting the morning after spending one night at the station, referred to as high altitude day 1 (HA1). On high altitude days 1 and 2,

participants were randomized in a participant-blinded manner into two groups. One group was administered placebo room air, and the other was administered a mild hypercapnic gas (sea-level equivalent of 4% inspired CO_2) while completing the cognitive test battery. Participants were aware that they would be receiving one of these two treatments. Due to space limitations at Barcroft Station, and to maximize study sample size, treatments were administered by three research team members who also participated in the study, however they remained unaware of their own treatments. To compare performance at high altitude after acclimatization to untreated sea-level performance, on the third day at high-altitude, no treatments were administered, no mask was utilized, and participants completed testing in an environment similar to their at-home conditions.

Throughout the study, including when participants were performing tests at home, participants were asked to abstain from taking anti-inflammatory medications, consuming caffeinated beverages after 1 pm, and engaging in rigorous physical activity. Participants also did not utilize acetazolamide or other medications intended to reduce AMS symptoms which occur at high altitude, such as headache and nausea. At high altitude, participants were also consistently encouraged to increase fluid intake.

Cognitive Function Testing

The same cognitive function test battery was completed at each timepoint. Participants completed a 45-minute cognitive test battery on an iPad (10.9-inch, Apple, Cupertino, CA, USA). During each test period, the participant was instructed to complete the tests in a quiet room with dim lighting sufficient to prevent viewing reflections on the screen. Each

participant was also provided with a pair of circumaural headphones to wear during testing and asked to ensure that their iPad volume was set to the maximum level.

The test battery consisted of 6 different tasks which evaluated different cognitive function domains. Details regarding each task are provided below**.** The test battery included instructions before each task during every test period, and some tests provided a practice session for familiarization. For applicable tests such as the *Word List Learning, Constructional Praxis Tasks* and *Trail Making Task*, different test variants were produced so that each day a new set of words or trail-making task arrangement was provided. This helped to mitigate memorization effects and minimize the learning effect when the same individuals completed the same test battery over several days. The test battery included the following tasks:

- ***UCancellation Pictures Task.*** This is a timed, tablet-based test of selective attention and inhibitory control, akin to D2.[159] In Cancellation - Pictures, letters are replaced with pictures of dogs and monkeys, some of which are rotated along the vertical axis or are presented upside down. For Pictures, the participant must select the upright dog (tail on the left) and the upside-down monkey (tail on right) separately in single blocks and together in a mixed block.

- ***Word List Learning* and *Constructional Praxis Tasks.*** These two tasks are intertwined with each other. The participant is first shown a list of words to remember. Then, they are asked to draw a copy of a complex figure. After drawing the complex figure, participants are shown a series of words, some of which are from

the list presented at the beginning of the task and some of which are not. They must indicate if they recognize the word or not by pressing the "yes" or "no" button. Afterwards, they are asked to draw the figure that they copied from memory. Finally, they are asked to do a free recall of the words. For the word list learning task, there are three-word lists, each containing ten words. For constructional praxis, there are three complex figures (newly designed by our team) that are presented after each word list recall. Therefore, the participants go through the procedure described above a total of three times, each with different words and figures.

- ***Trail Making Task.*** This is a measure of visual attention and task switching ability. It consists of two parts where participants must connect a series of circles as quickly and accurately as possible. In part A, participants must connect circles numbered 1-25 in numerical order. In part B, participants must switch between connecting a number with a letter (1-A, 2-B... etc.). The outcome measures are response time and accuracy. Participants will also complete a reversed mirror image of these trails.

- ***Spatial Release from Masking Task.*** This is an ecologically valid measure that tests the ability to distinguish speech from competing speech[160] using headphones and virtual spatial locations.[161] This task uses collocated conditions, with a target and two competing maskers all located directly in front of the listener, and spatially separated conditions, with two masking sentences sent from 45 degrees to the left and right of center. Listeners must identify keywords from a target sentence while simultaneously listening to two masking sentences. The target sentences contain the

callsign "Charlie" and the keywords (i.e., a number and color). The masking sentences contain different callsigns, numbers, and colors. The outcome is the threshold target-to-masker ratio (TMR) as estimated based on the number of sentences correctly identified as part of a series of 20 test trials in which TMR progressively reduced every two trials.

- ***Digits–in-Noise (DIN) Test.*** This test presents trials of three sequential spoken digits against a broad-band, speech-shaped masker. Participants must select the digits they heard in the correct order. The signal to noise ratio varies adaptively to track 50% correct. It is a simple, rapid, sensitive, reliable, validated measure of speech reception threshold (SRT).[162] SRT correlates highly with audiometric pure tone average yet is also associated with cognitive function. The outcome measure is the threshold noise tolerance in dB.

- ***Contrast Sensitivity Task***. This task measures the ability to distinguish between fine increments of light and dark (contrast). Participants are presented with sinusoidal grating targets called 'Gabor patches' of various spatial frequencies and are required to tap on them. This task provides a baseline measure of low-level vision but can also identify basic problems in perceptual processing that might impact upstream processes. The dependent measures are contrast threshold/s and cut-off spatial frequency (a measure of visual acuity).[163]

Statistical Analysis

Analyses of changes in physiological variables, cognition, hearing, and vision test performance as a function of altitude, time/day, and treatment (placebo/room air versus mild hypercapnia) were performed in R (Version 4.3.0, R Inc.). Sea level 2 was used as a 'baseline' to compare results for learning effects and the effects of altitude using Wilcoxon tests. Repeated measures one-way ANOVAs were used to compare physiological variable values as a function of timepoint after determining if variables met the assumption of normal distributions using Shapiro-Wilks tests for normality as well as Q-Q plots for visualization. Post-hoc pairwise comparisons were calculated using the Tukey HSD method, with family-wise p-value corrections using the Bonferroni method. Raw and adjusted p values are reported when appropriate. Data are presented throughout the manuscript as mean (standard deviation). Asterisks indicate significant differences at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p<0.0001$(****).

Results

Physiological responses to high altitude

Physiological data are presented in **Table 1**. SpO_2 was significantly lower at high altitude on all three days compared to sea level. To compensate, heart rate was significantly elevated on each day at high altitude compared to sea level. Blood pressure did not change significantly at high altitude.

Effect of time and altitude on cognitive performance, hearing, and vision

Here we present overall performance across all experimental sessions. The goal of these analyses is to highlight the complex variability of performance on these tasks over time.

Cognitive function

We first tested changes in reaction time variability on the *UCancellation Task*, which measured selective attention and inhibitory control with a lower variability being preferable. There was no significant effect of day ($F(9,181) = 0.823$, $p = 0.596$, $eta^2[g] = 0.039$) in reaction time on this test, nor were there any effects of altitude **(Figure 1A)**. Some of the variation seen in reaction time on HA3 may be explained by variation in Acute Mountain Sickness scores. On HA3 there was a significant relationship between AMS score and variability, ($r=0.47$, $p = 0.035$). This relationship shows participants with higher AMS scores had higher variability and thus worse performance **(Figure 2)**.

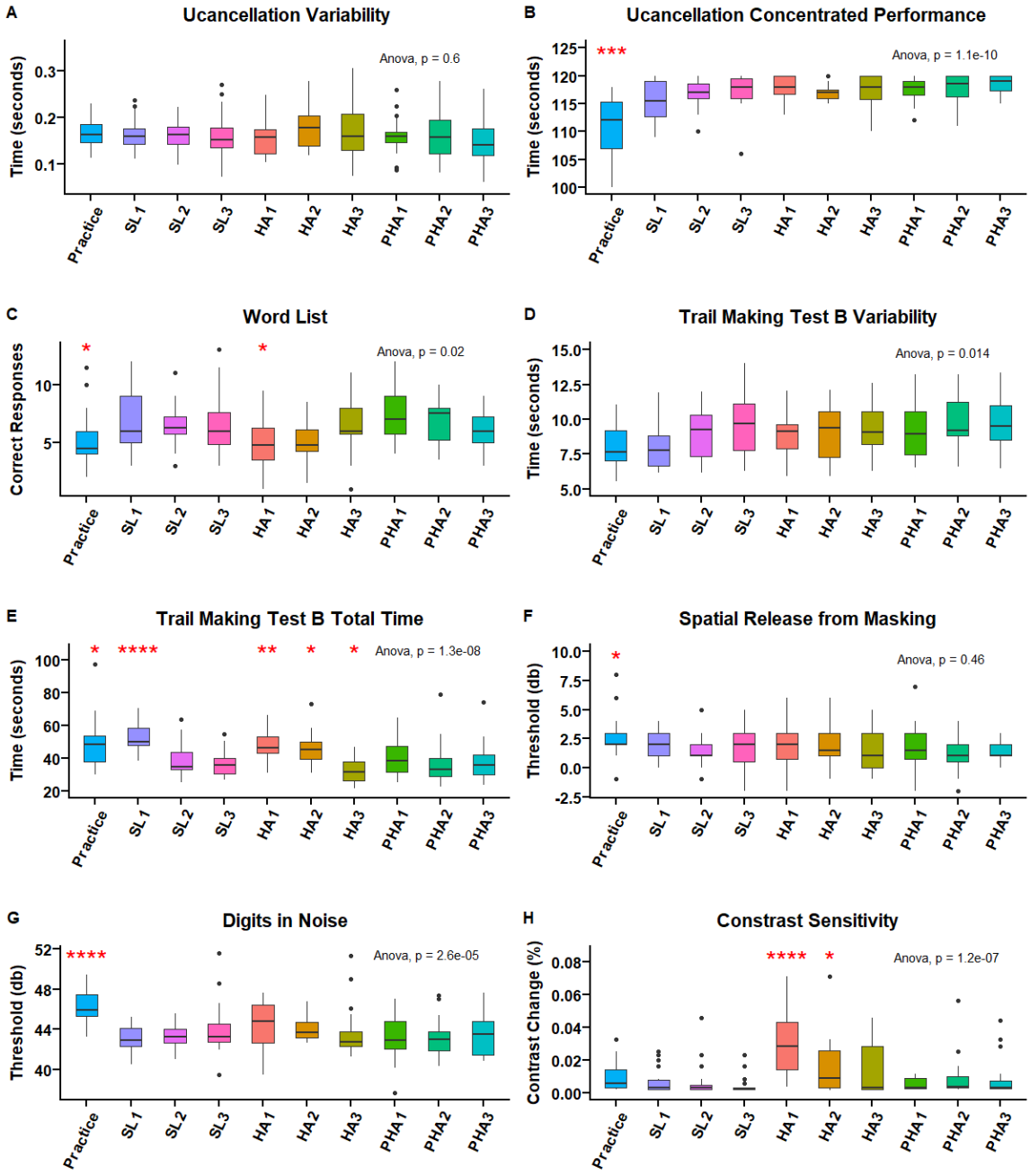

Figure 1. Participant Performance on Cognitive, Hearing, and Visual Tasks

Performance of participants during cognitive, hearing function, and visual perception across the study timeline. Components of cognitive function that included **(A)** reaction time variability and **(B)** concentrated performance on the UCancellation task. **(C)** Number of correct responses recalled on the word list. **(D)** The reaction time variability and **(E)** total time spent on the trail making task. Hearing function was tested via **(F)** the threshold (dB) assessed via spatial release from masking task and **(G)** digits in noise task. Vision was tested via contrast threshold of visual perception during the contrast sensitivity task **(H)**. Red stars indicates level of significant differences in performance of that day as compared to SL2 via Wilcoxon tests.

There was a significant increase in concentrated performance, which indicates higher performance overall, as a function of day on the *UCancellation Task* (F (9,180) = 6.43, p = $6.72e^{-8}$, $eta^2[g]$ = 0.243). This test exhibited traits suggesting a learning effect on the task; the very first 'practice' session was significantly lower than SL2 baseline (p = 0.00015) and while this seems to stabilize there are small increases with a trend of highest scores on PHA3 (p = 0.059). This test also exhibited no differences in score at high altitude **(Figure 1B)**. However, at HA1, performance scores were correlated with AMS scores (p = 0.049), with worse performance in individuals with more symptoms and higher AMS scores **(Figure 2)**.

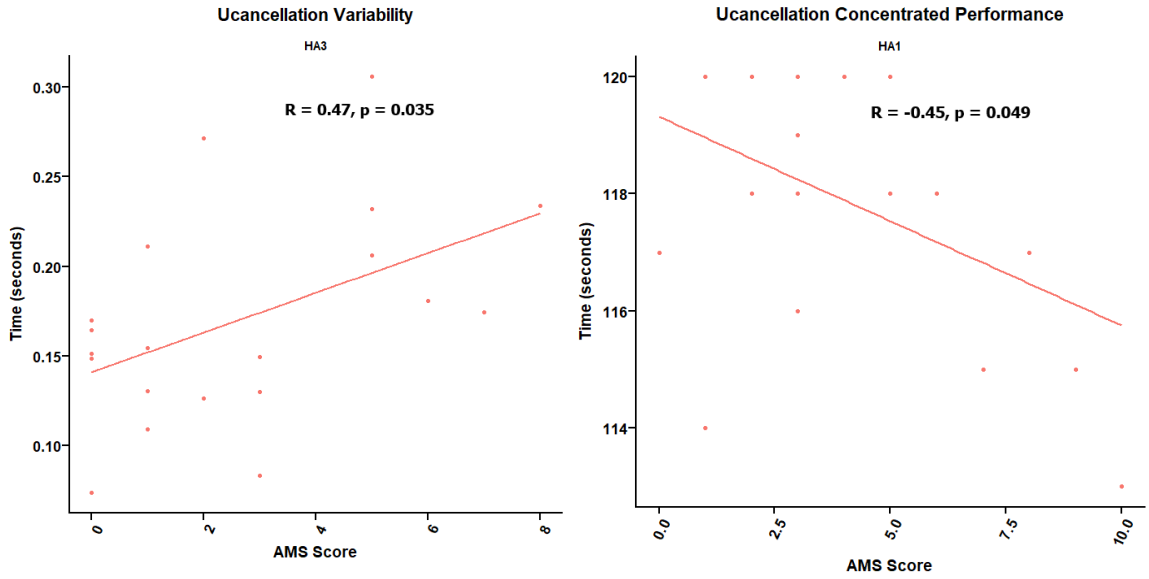

Figure 2. Effect of AMS on Ucancellation Variability and Concentrated Performance

Ucancellation variability increased and concentrated performance was lower, both indicating lower performance with increased AMS scores.

There was a significant increase in concentrated performance, which indicates higher performance overall, as a function of day on the *UCancellation Task* (F (9,180) = 6.43, p = $6.72e^{-8}$, $eta^2[g]$ = 0.243). This test exhibited traits suggesting a learning effect on the task; the

very first 'practice' session was significantly lower than SL2 baseline ($p = 0.00015$) and while this seems to stabilize there are small increases with a trend of highest scores on PHA3 ($p = 0.059$). This test also exhibited no differences in score at high altitude **(Figure 1B)**. However, at HA1, performance scores were correlated with AMS scores ($p = 0.049$), with worse performance in individuals with more symptoms and higher AMS scores **(Figure 2)**.

We then tested for changes in performance on the *Word List Task*, which measures working memory and recall. This test was scored as number of correct answers with a higher score being preferable. There was a significant effect of day ($F(9,180) = 2.269$, $p = 0.02$, $eta^2[g] = 0.102$). There was a significant difference between baseline, SL2, and the first practice session ($p = 0.049$), with the practice session having a lower number of correct responses. This could demonstrate a learning effect. There is also a significant effect of high altitude on this test, where there was a significant drop in correct responses at HA1 ($p = 0.038$). This continues to trend on HA2 ($p = 0.056$) but recovered by HA3 **(Figure 1C)**. Correct responses on this task were not linked with AMS scores on any day ($p > 0.08$ on all days).

Finally, on the *Trail Making Test*, which measures visual attention and task switching ability, we found a significant effect of day on reaction time variability, ($F(9,182) = 2.383$, $p = 0.14$, $eta^2[g] = 0.105$) **(Figure 1D)**, and total time taken ($F(9,178) = 6.98$, $p = 1.33e^{-8}$, $eta^2[g] = 0.261$) **(Figure 1E)**. Lower scores are indicative of better performance on both measures of this task. While reaction time variability was not affected by high altitude or a significant learning effect, the total time taken showed interesting patterns, with the practice and SL1 sessions taking longer than time to finish the test than on SL2 ($p = 0.35$ and $p = 3.9e^{-5}$,

respectively). Participants were even quicker to finish the task on SL3, though not significantly so compared to SL2. This decrease in time was affected by high altitude as the time to complete the task significantly increased on HA1 and HA2 ($p = 0.0057$ and $p = 0.046$, respectively). Interestingly, by HA3 the time significantly decreased even further representing the fastest times for participants completing the tasks ($p = 0.022$). This further supports a learning curve which seems to reach a plateau after HA3 when the completion time stays relatively stable. AMS scores were not correlated with either aspect of the Trail Making Task on any day; reaction time variability ($p > 0.19$), total time ($p > 0.5$).

Hearing

For both hearing tasks, a lower threshold indicates better performance. On the *Spatial Release from Masking Task,* there was no effect of day ($F(9,181) = 0.973$, $p = 0.464$, $eta^2[g] = 0.046$) on the signal to noise threshold **(Figure 1F)**. There was a significant decrease in threshold from the first practice session to SL2 ($p = 0.018$) but no other significant differences between days or altitudes. This trend was also observed on the *Digits in Noise Task*. There was a significant effect of day on the minimum speech reception threshold ($F(9,181) = 4.48$), $p = 2.56e^{-5}$, $eta^2[g] = 0.182$) with performance increasing significantly after the first practice session ($p = 1.4e^{-6}$). While there was no significant effect of altitude or further performance differences, there was a trend for lower performance on HA1 ($p = 0.07$) and HA2 ($p = 0.056$), which returned to baseline values by HA3 **(Figure 1G)**.

Vision

On the visual *Contrast Sensitivity Task*, there was a significant effect of day ($F(9,172) = 6.28$, $p = 1.17e^{-7}$, $eta^2[g] = 0.247$) on visual contrast threshold in this analysis **(Figure 1H)**. There is a significant effect of high altitude with the percent contrast threshold being higher, indicating lower performance, at HA1 ($p = 3.3e^{-5}$) and HA2 ($p = 0.04$), with a return to baseline on HA3 though with a high level of individual variation.

Overall examination of the suite of cognitive test results revealed significant variability that looks to be a combination of effects of learning and the influence of high altitude.

Effect of hypercapnia treatment

To investigate the effects of the mild hypercapnic gas treatment, we compared changes between the treatment groups which occurred during tests taken on HA1 and HA2.

Cognitive function

In the *UCancellation Task,* neither reaction time variability **(Figure 4A)** nor concentrated performance **(Figure 4B)** showed any significant effects due to either day or treatment. There was no significant difference between reaction time variability performance on HA1 or HA2 ($p = 0.13$) or between treatment groups on HA1 ($p = 0.19$) or HA2 ($p = 0.41$). Similarly, there were no significant effects of day ($p = 0.12$) or treatment on HA1 ($p = 0.91$) or HA2 ($p = 0.26$) on concentrated performance.

In contrast, there was an effect of treatment on the *Word List Task* on HA1 (p = 0.048) with worse performance, less correct responses, in the treatment group. This effect was not observed on HA2 (p = 0.15), and there was no effect of day (p = 0.84) **(Figure 4C)**. Lastly, there were no significant effects noted for day (p = 0.72) or treatment on HA1 (p = 0.8) and HA2 (p= 0.92) on the reaction time variability on the *Trail Making Test* **(Figure 4D)**. Similarly, there were no significant effects noted for day (p = 0.48) or treatment on HA1 (p = 0.4) and HA2 (p= 0.84) on the total time taken for the same task **(Figure 4E)**.

Hearing

The results showed that the hypercapnia treatment did not influence hearing function. This is shown in task performance between the groups in the *Spatial Release Masking Task*, where no significant effects were observed for collocated signal to noise threshold on HA1 (p = 0.35) or HA2 (p = 0.1) and there were no differences in performance between days (p = 0.83) **(Figure 4F)**. Similar results were observed for the *Digits in Noise* threshold with no changes in performance between treatment groups on HA1 (p = 0.76) and HA2 (p = 0.63), or between days (p = 0.66) **(Figure 4G)**.

Vision

There was also no effect of hypercapnic treatment on *Contrast Sensitivity Task* threshold between treatments on HA1 (p = 0.68) or HA2 (p = 0.18). There was however a significant difference between performance on the days, with better performance on HA2 (p = 0.025) **(Figure 4H)**.

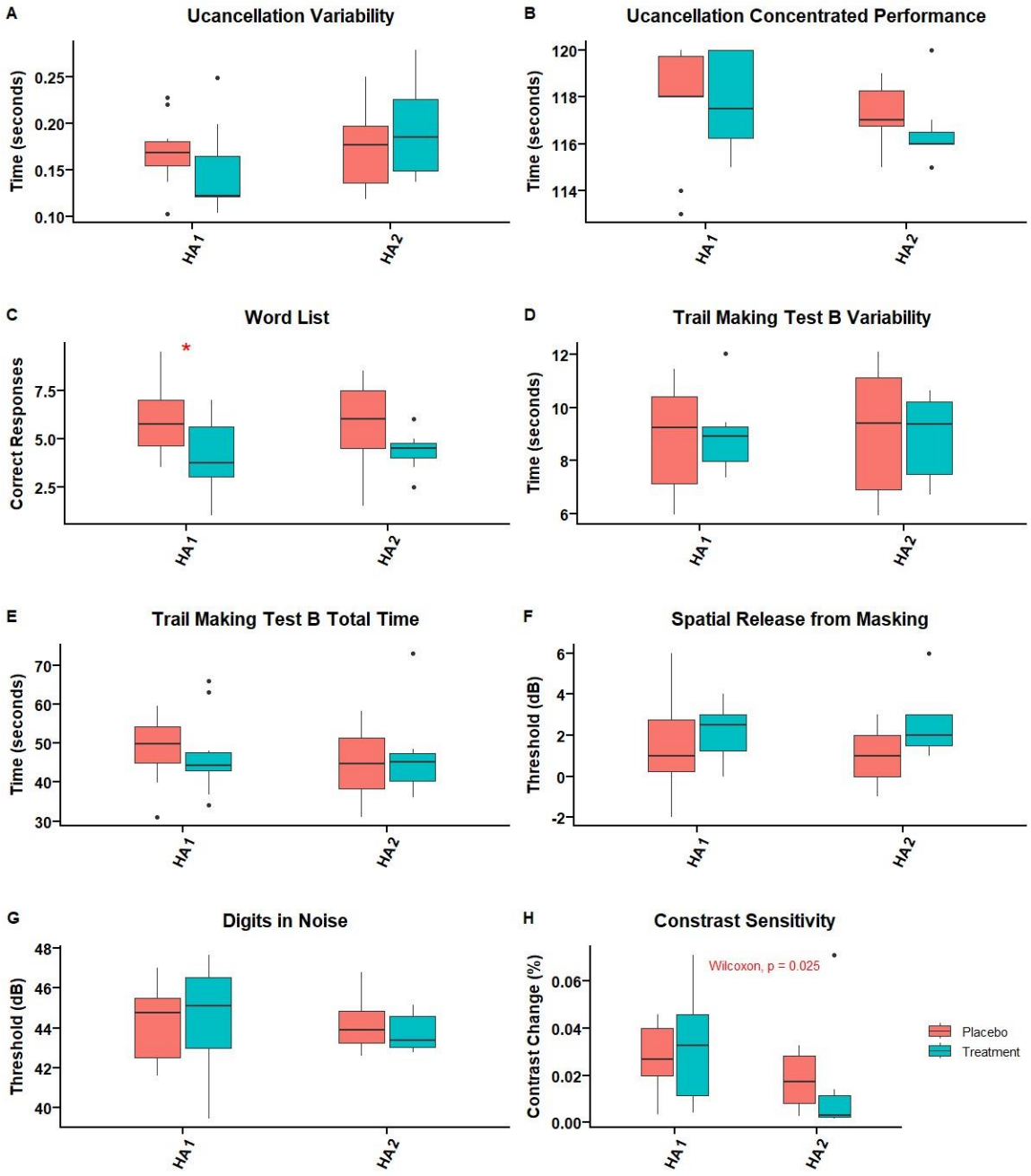

Figure 4. Effects of Hypercapnic Treatment on Cognitive, Hearing, and Visual Tests at High Altitude

Performance of all participants in blinded groups of placebo (red) and 4% hypercapnic gas treatment (green) while at high altitude. Components of cognitive function included **(A)** reaction time variability **(B)** and concentrated performance on the UCancellation task. **(C)** Number of correct responses recalled on the word list. **(D)** Reaction time variability and **(E)** total time spent on the trail making task. Hearing function was assessed via spatial release from masking task **(F)** and digits in noise task **(G)**, respectively. **(H)** Contrast threshold of visual perception during the contrast sensitivity task. Red stars indicate significance for treatment within a day and Wilcoxon results denote the performance was different between HA1 and HA2.

Discussion

The goals of this study were to examine the impact of high-altitude exposure on cognitive performance, hearing, and vision, as well as determine if a mild hypercapnic inspired gas (4% CO_2) would improve task performance at high altitude.

Impacts of time on cognitive performance, hearing, and vision

The results suggest a few tasks show signs of a learning effect over the course of the study, most notably the total time taken in *Trail Making Test B*. Specifically, it should be noted that some evidence of learning effect was observed for all cognitive task outcomes except reaction time variability on the *UCancellation Task*. The learning effect observed refers to the repeated exposure of the task that could have helped participants develop strategies to improve their performance regardless of treatment or altitude exposure. Interestingly however, reaction time variability was not impacted by repeated testing. Participants involved in this study were young adults which may indicate that they perform near the ceiling of performance on some tests, leaving minimal room for improvement.

Tasks testing hearing and vision did not show a learning effect after the first practice session. This is likely due to the fact that these tests utilize simple response tasks based on if a cue was heard or not, and therefore strategies to improve performance were not possible. Despite that, hearing and vision functions have their unique role and there is evidence that they rely on cognitive resources like attention,[164] working memory, and inhibitory control to some degree.[165–167] Therefore, it is possible that the hearing and vision tasks may have been

designed as too simple or too complex to induce a learning effect.[168] This could have led participants to perform consistently on the tasks over time.

Impacts of high-altitude exposure on cognitive performance, hearing, and vision

We found significant impacts of high-altitude exposure on multiple cognitive function tasks. On the *UCancellation Task*, which measures selective attention and inhibitory control, there was no impact of high altitude on reaction time variability **(Figure 1A)**, or concentrated performance **(Figure 1B)**. Reaction time predominantly depends on neural processing speed, which is less dependent on oxygen availability and could explain why we observed no significant change in reaction time variability. Our results are consistent with previous findings reflecting no change in reaction times.[169]

Performance on the *Word List Task*, which measures sustained attention, working memory, verbal span from short-term memory and recall, was reduced during the first two days at high altitude with a recovery on HA3 with acclimatization **(Figure 1C)**. The impact of high-altitude exposure on memory has been well documented and seems to impact both sojourners and long-term high altitude residents.[151,170] This effect has also been demonstrated previously in hypobaric chamber studies by Nation et al. who exposed military aircrew to simulated 6096 m elevation for 15 minutes and conducted serial word list learning tests and complex figure drawing.[18] In this study, the authors noted that memory dysfunction at simulated high altitude was driven by memory encoding deficiencies. Another study of 11

climbers who ascended to over 5000 m elevation showed that memory as measured with a word list test remained impaired even 75 days after returning to sea level.[19]

While the reactive time variability on the *Trail Making Test* followed reaction time trends from the *UCancellation Task* and remained unaffected, the total time to complete the task which measures visual attention and task switching ability, was significantly impaired during the first two days at high altitude **(Figure 1D)** but improved by the third day at high altitude compared to sea level performance. This demonstrates an initial impairment in these cognitive domains during early high-altitude exposure before acclimatization. Support for this impairment can be found in previous research such as studies conducted by Asmaro et al. who found that performance on this test was impaired in hypobaric chamber studies simulating 7600 m elevation.[21] Our group also previously found performance on *Trail Making Tasks* were reduced during the first day of high altitude exposure but improved with 3 days of acclimatization, supporting our current findings.[148]

When examining hearing and vision, we did not find any significant impacts of high-altitude exposure on the *Spatial Release from Masking* task, which measures the ability to identify specific speech from competing speech, or the *Digits in Noise* test, which measures the speech reception threshold. However, we did find significant impacts of high-altitude exposure on the visual *Contrast Sensitivity Task*, with most participants demonstrating reduced visual contrast sensitivity at high altitude **(Figure 1G)**. Previous work has also demonstrated reductions in visual contrast sensitivity in field studies and hypobaric chamber studies in unacclimatized individuals.[22,171] Reductions in visual contrast sensitivity at high altitude may

be directly associated with reductions in SpO_2,[22] or impacts of reduced barometric pressure on corneal edema. Importantly, these effects appear to impact contrast sensitivity specifically in low light settings.[172]

Overall, our findings corroborate previous research showing that high altitude exposure impairs cognitive function, psychomotor function, and vision.[173,174] While some studies suggests that cognitive decline due to hypobaric hypoxia is long-lasting even after return to sea level,[19,175,176] other studies show that cognitive function impairment was short-lived and returned back to baseline levels within 36 hours of exposure.[177] Our results show that most tasks returned to normal baseline values by HA3 after acclimatization and did not carry over into post altitude days.

Impacts of mild hypercapnia on cognitive performance, hearing, and vision at high altitude

When comparing the placebo and hypercapnic groups, there were no significant changes in performance across any of the cognitive, hearing, or visual tasks with the exception of the *Word List Task* on HA1. The participants in the treatment group had worsened performance at HA1 as compared to their placebo counterparts **(Figure 4)**. However, these results do not agree with studies done by Van Dorp et al., who showed improved cognitive performance with hypercapnic treatment.[178] This may be due to differing experimental conditions. Our study was conducted in a hypobaric hypoxic environment, where participants were exposed to systemic high-altitude hypoxia for at least 12 hours before the first cognition test. In

contrast, Van Dorp et al. conducted their study in normobaric hypoxic conditions with 45-minute exposure times. As a result, there may be independent effects of exposure duration and reduced barometric pressure that are not modulated by altered arterial blood gas tensions and reduced hemodynamics.[178] In contrast, there are other studies which support our findings, showing that hypercapnia may decrease cognitive performance. Kung et al reported that hypercapnia caused by obstructive sleep apnea led to lower scores in processing speed and logical memory tests, which would include memory tests such as our *Word List* task.[179] Similarly, Beaudin et al found cognitive decline associated with higher arterial pressures of CO_2 in the Montreal Cognitive Assessment (MoCA) tests measuring visuospatial/executive function similar to our *Trail Making* task and memory, similar to our *Word List* task.[180]

Limitations of the study

The study had some limitations that should be considered when interpreting the findings. First, testing at the high-altitude station on HA1 and HA2 was performed on multiple individuals at once in the same room, with participants separated by wall dividers. However, all efforts were made to mitigate sound and visual distraction in the room, and noise canceling headphones were worn by participants. Secondly, three participants in the study were previously familiar with receiving hypercapnia treatments through participation in related studies and may have suspected their treatment. Finally, the study sample size was constrained by the capacity of the facility, limiting the number of participants that could be

included. However, our sample of 20 allowed sufficient power to detect significant impacts of high altitude on performance on several tests.

Conclusion

In conclusion, this study supports previous work indicating that high altitude impacts cognitive function, particularly selective attention, inhibitory control, visual attention, task switching ability, short-term working memory, and visual contrast sensitivity. However, we did not find significant impacts on hearing function. It remains unclear how repeated exposures affect this result, or how long these impairments persist. Performance on many of the cognitive function tests showed improvements after acclimatization, and all showed improvement upon return to sea level.

While it has been known that there can be a decrease in various cognitive functions associated with hypoxia, there have not been many attempts to rescue this decline. This study is novel in that we attempted to counter the effects of hyperventilation-induced hypocapnia with the introduction of a hypercapnic gas. However, our results do not support a role for mild hypercapnia in mitigating the negative effects of high-altitude hypoxia on cognitive function.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

ECH, ARS, KP, and SF contributed to the conceptualization and design of the study. ECH, ARS, KP, SF, DJ, and AC were responsible for the management and coordination of research activity planning. DJ and AC contributed to the cognition battery software used in the study. SF, KP, ECH, LN, DJ, and LB conducted the research and investigation process. SF and LK performed data analysis and created figures. KP, ARS, and ECH contributed to the funding and resources necessary to conduct the study. ECH, SF, LK, KP, and LN wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Chapter 4:

Changes in DNA Methylation During High-Altitude Exposure

Chapter 4a. Genome-Wide DNA Methylation Patterns in Sojourners to High Altitude

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Abstract

High-altitude hypoxia, characterized by reduced oxygen partial pressure at high elevations, poses significant physiological challenges. within response to acute hypoxemia, various plastic adaptive mechanisms are initiated to improve oxygen delivery to tissue. While many of these changes are likely driven by epigenetic mechanisms, the role of epigenetic modifications, particularly DNA methylation, in these plastic phenotypic changes remains unclear. To address this gap in knowledge, we investigated global DNA methylation patterns in response to acute high-altitude exposure. A cohort of 12 healthy sea-level residents, aged 19 to 32, were exposed to high-altitude (3,800 meters elevation) for 24 hours, following a gradual ascent. Peripheral venous blood samples were collected at sea level and 24 hours after arrival at high altitude, and DNA methylation was assessed using the Illumina MethylationEPIC array. We identified significant changes in DNA methylation patterns, including 192,651 differentially methylated sites, with 93.4% of sites showing increased methylation levels at high altitude and 6.6% showing decreased methylation. These

differentially methylated sites were distributed across several genomic regions, with the majority of changes occurring in open sea regions. Notably, pathways related to the Hypoxia-Inducible Factor (HIF) pathway, such as Notch signaling and AKT1 signaling in cancer, contained some of the most highly differentially methylated sites. Moreover, several pathways associated with calcium regulation and DNA damage repair were implicated, suggesting a connection between DNA methylation and processes affected by hypoxia-induced oxidative damage. In addition to single sites, we explored differentially methylated regions (DMRs). The top DMRs were associated with calcium processes, zinc finger proteins, glucose processes, and erythropoiesis. These DMRs show a clear trend of increased methylation at high altitude, supporting their role in adaptation to oxygen limitation. This study sheds light on the widespread DNA methylation changes that occur in response to acute high-altitude exposure. The findings provide insights into the epigenetic mechanisms underpinning physiological adaptations to hypoxia and open the door for new hypotheses regarding the role of epigenetic mechanisms of physiological plasticity in response to hypoxic stress. Understanding these mechanisms may have implications for altitude-related illnesses and offer broader insights into epigenetic regulation in response to environmental stimuli. Overall, this research enhances our knowledge of how the human body adapts to high-altitude hypoxia and highlights the significance of epigenetic modifications in this process.

Keywords: hypoxia, high altitude, epigenetics, DNA methylation

Introduction

High-altitude hypoxia, or hypobaric hypoxia, occurs during exposure to reduced oxygen levels at high elevations. This exposure presents a significant physiological challenge, as the human body is dependent on oxygen for energy production and other essential functions. In response to high-altitude hypoxia, the body undergoes a series of plastic physiological changes that help increase oxygen delivery to tissues. These adaptations include changes in breathing patterns,[181–183] cardiovascular responses,[184–186] and modifications in metabolic energy production pathways.[24,25]

Epigenetic modifications are chemical alterations to the DNA molecule, or its associated proteins, which can influence gene expression without altering the underlying DNA sequence. Such modifications can be stable and heritable, and they play a crucial role in regulating gene expression during development and in response to environmental stimuli. DNA methylation is an epigenetic modification that involves the addition of a methyl group to cytosine residues in CpG dinucleotides. DNA methylation can modify gene expression and is most commonly found in CpG islands (CGI), which are the most densely packed regions of CpG sites. Specifically, these are regions that have over 500 base pairs with at least 50% CG content. CGIs are found in the promoter region of almost 70% of genes.[187] Changes in DNA methylation can result in changes in transcription factor binding, alternative splicing, and other mechanisms which can modulate gene expression patterns.[188–190] While physiological changes to high altitude have been well studied, components of the underlying mechanisms of this phenotypic plasticity such as epigenetic mechanisms remain unclear.

In the context of high-altitude hypoxia, several reviews suggest that changes in DNA methylation may contribute to the physiological adaptations observed in individuals exposed acutely to high-altitude and those living at high altitudes,[191–194] but there are few studies which investigate these changes. In 2019 Childebayeva *et al.*, studied a group of mountain climbers ascending Mt. Everest and found changes in targeted hypoxia-inducible factor (HIF) pathway genes including increased methylation levels at high altitude in *EPAS1* and *PPARa*. They also found decreased methylation levels at high altitude compared to baseline values in *LINE-1*, *EPO*, and *RXRa*.[79] This study was followed by an additional epigenome-wide analysis of the same cohort at day 0 at 1,400 m and day 7 at an altitude of 4,240 m. The results showed significant DNA methylation changes in positions and regions associated with HIF and the renin–angiotensin system (RAS) pathways. The implication of the HIF pathway is not surprising as HIF is responsible for upregulating over 100 genes in response to hypoxia and therefore are vital for acclimatization at high altitudes. As well as pathways enriched in genes involved in glycolytic processes, hematopoiesis, and angiogenesis.[80] Notably the majority of reported sites were hypermethylated in individuals at high altitude. This is likely due to a reduction in the activity of ten-eleven translocation (TET) enzymes. These enzymes are oxygen dependent and are responsible for the demethylation of DNA through 5-methylcytosine oxidation. Thus, in the presence of reduced oxygen availability, the activity of TET enzymes is reduced, and demethylation activity is reduced leading to higher methylation levels. This has been illustrated in studies focused on cancer and tissue hypoxia.[195,196]

Despite these findings of epigenetic changes in acute exposure, the temporal dynamics of DNA methylation changes in response to high altitude hypoxia remain unclear. In the current study I expand on this work with a high-throughput investigation of methylation levels throughout the genome before and during an acute but stable high-altitude exposure, allowing for acclimatization, in a cohort of healthy sea-level residents. This study design contrasts others which have allowed progressive ascent throughout the study without allowing complete acclimatization. Considering previous studies, as well as the implications of energy conservation and inactive TET enzymes, I hypothesized that acute high-altitude exposure would result in global hypermethylation. This study would be the first to explore DNA methylation differences in the epigenome of sojourners before and during acclimatization to a single high altitude.

Understanding the epigenetic mechanisms that regulate gene expression in response to high-altitude hypoxia is important for several reasons. First, it can provide insights into the molecular mechanisms that underlie the physiological adaptations observed in individuals living at high altitudes. Second, it may help to identify new therapeutic targets for altitude-related illnesses, such as acute mountain sickness and high-altitude pulmonary edema. Finally, it may provide insights into the broader role of epigenetic mechanisms in regulating gene expression in response to environmental stimuli, and their potential contributions to the development of hypoxia-promoted diseases such as COPD, ARDS, COVID-19, and sepsis.

Methods

Ethical approval

This study was approved by the University of California, Riverside Clinical Institutional Review Board (HS 19-076). All participants were informed of the study's purpose and risks. Participants provided written informed consent in their native language (English). The work was conducted in accordance with the *Declaration of Helsinki*, except for registration in a database.

Participants

Twelve participants, comprised of 9 men and 3 women, currently residing at sea level were recruited for the study. The participants were between 19 and 32 (average of 25 ± 4.5) years old and had no history of cardiovascular or pulmonary disease. The study excluded individuals who had a history of smoking (cigarettes, e-cigarettes, marijuana), were pregnant, or had recently traveled to elevations above 2,500 m within a month of the initial test measurement. Participants abstained from taking any anti-inflammatory medications, such as ibuprofen, which may interfere with ventilatory acclimatization to high altitude.[197] Participants were transported to Barcroft Station within the White Mountain Research Center (3,800 m elevation) in vans and underwent a gradual ascent from 340 m to 1,216 m over a period of 4 hours, followed by an ascent from 1,216 m to 3,800 m in 2 hours where they then stayed for three days. The sea-level measures (SL) were conducted at the University of California, Riverside, located at an elevation of 340 m, while the high-altitude measures (HA) were taken on the first morning after sleeping a night at Barcroft Station.

Sample Preparation

Fasting veinous blood samples were obtained from participants at sea level and for three days every morning at high altitude immediately after waking. DNA was isolated from peripheral blood mononuclear cells (PBMCs) in the buffy coat of these samples using the GentraPuregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for Whole Blood. Once isolated, DNA was tested for purity using standard 260/280 ratios suggested for DNA, ~ 1.8, and concentration via Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). An aliquot of 400 ng of isolated DNA then underwent a bisulfite conversion treatment using EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the unmodified manufacturer's protocol. After treatment, DNA was tested again for purity, however this time using standard 260/280 ratios suggested for RNA, ~ 2.0, and concentration measured as RNA via Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA).

DNA methylation levels of twelve paired sea level and high-altitude day 1 samples were measured using an Infinium MethylationEPIC array (Illumina, San Diego, CA, USA) on the Illumina iScan system according to manufacturer's protocol at UCSD's Institute for Genomics Medicine (IGM) Genomics Center.

Data Analysis

MethylR[198] was used to analyze data. This workflow recommends a *ChAMP*[199] workflow for Illumina EPIC BeadChip initial analysis. This initial analysis reads and assesses sample

quality. Samples which failed any quality control tests including probes with detection p-value > 0.01 (2,245) and probes with <3 beads in at least 5% of samples per probe (2,260) were removed. In addition, all non-CpG probes (2,984), all SNP-related probes (97,578), all multi-hit probes (11), and all probes located in X and Y chromosomes were also removed (16,773). After quality control filtering and probe exclusion, 744,067 high quality sites remained. Data was normalized using BMIQ (Beta-Mixture Quantile Normalization).[200] This method adjusts the beta values of type II probes into a distribution more similar to type I probes which reduces technical variation, bias of the type II probes, and the enrichment bias of type I probes caused by the lower range of type II probes. This workflow then used *ComBat*[201] to correct any batch corrections encountered by using multiple arrays. The shift produced by these corrections can be seen in **Figure 1**. After these processes, a total of 192,651 sites were found to be significantly differentially methylated across locations with an FDR of 5% (Benjamin-Hochberg) and adjusted p-value below 0.05.

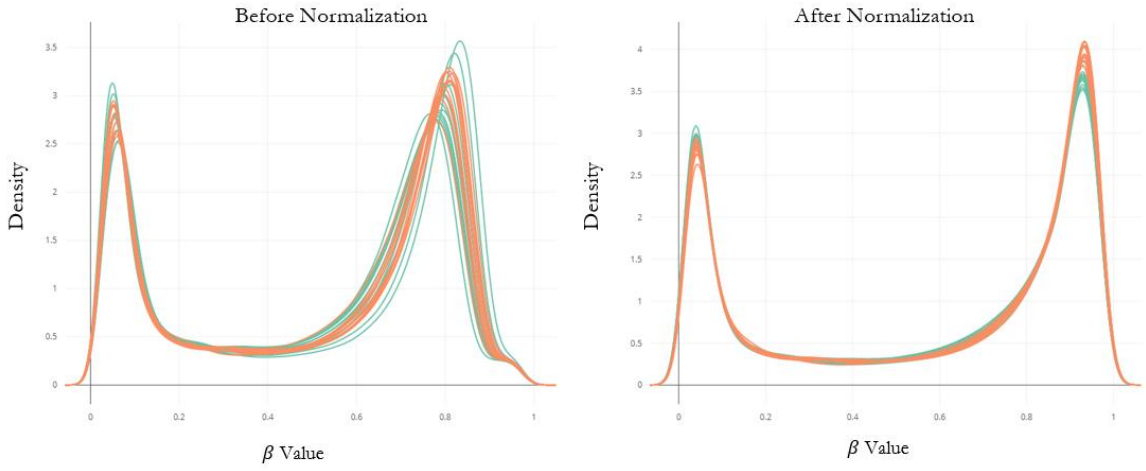

Figure 1. Samples Before and After BMIQ Normalization and ComBat Batch Corrections

DNA methylation levels are shown as β-values which represent the percent methylated using the following equation: $\beta = \frac{(M)\ Methylated\ signal\ instensity}{(U)\ Unmethylated\ signal\ intensity + M}$. These β-values were used to find differentially methylated positions using R package *Limma*[202] and differentially methylated regions (DMRs) using *BumpHunter*,[203] both integrated as part of the automated *ChAMP* pipeline.

Data is presented throughout the manuscript as mean (standard deviation). Asterisks indicate significant differences at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), or $p<0.0001$(****).

Results

Genome-wide analysis

I identified 192,651 differentially methylated sites, with 179,923 (93.4 %) sites showing increased methylation levels at HA and 12,728 (6.6 %) showing decreased methylation levels at HA. The locations of the differentially methylated positions (DMP) were examined to determine their position within the genome. There are two ways to categorize a base pair's location. CpG sites may occur in CpG islands (CGI) or within a 'shore' if it is more than 2Kb from an island and a 'Shelf' if it is more than 2Kb but less than 4Kb from the island. Further still are sites that are part of an 'open sea'.[204] These features can be seen visualized in **Figure 2**. Interestingly, despite CGI's commonly being in promoter regions, it is the patterns of DNA methylation in CpG shores that are most associated with gene expression followed by the methylation patterns of CGIs.[205]

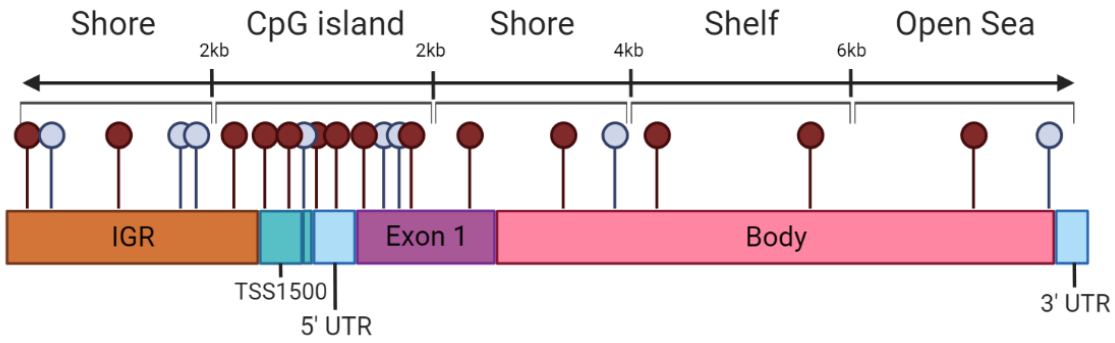

Figure 2. Types of CpG Region Types and Genomic Features
Schematic representation of the types of locations a single CpG can occur in. The top references the regions based on the density and distance; the densest area of CpGs being islands, moving further islands in either direction are shores, shelves, and open seas. These regions can be found on any genomic features such as the intergenomic region (IGR), TSS1500, TSS200, untranslated regions, exons, or the body of a gene.

A majority of the DMPs (113,179) were located in open sea regions. This was followed by 29,855 DMPs in shores, 24,820 DMPs in CGIs, and 14,797 DMPs in shelves. The distribution of these sites can be seen in **Table 1** along with the distribution of hyper- and hypomethylated sites across the genome. This table lists all DMPs with a corrected p-value ≤ 0.05.

CpG sites can further be described using genomic regions such as exons, 3' or 5' untranslated regions (UTR), body, exon band, IGR, or transcriptional start sites (TSS1500 or TSS200), pictured in **Figure 2**. Most DMPs occurring with high altitude exposure were located in the body (n=73,642) and intergenomic region (IGR) n=57,027. This is followed by TSS1500, n=24,357), 5' UTR n=15,663, TSS500 n=11,179, 3' UTR n=4,886, the 1st exon region n=4,531, and lastly the exon band n=1,366.

Table 1. Location Types of Differentially Methylated CpG Positions

Location	All Sites N (% of total)	Increased at HA N (% of type)	Decreased at HA N (% of type)
Open Seas	113,179 (58.75)	107,606 (95.1)	5,573 (4.9)
Shelf	14,797 (7.7)	14,100 (95.3)	697 (4.7)
Shore	39,855 (20.7)	38,386 (96.3)	1,469 (3.7)
Island	24,820 (12.9)	19,831 (79.9)	4,989 (20.1)
Total	**19,2651**	**179,923**	**12,728**

Top Differentially Methylated Positions

In addition to a genome-wide analysis, I also investigated the top 10 DMPs which are presented in **Table 2.** All significant DMPs were also analyzed in an overrepresentation analysis to reveal pathways associated with significant sites using Reactome.[206] The first ten most significant pathways are presented in **Table 3**. The top pathway is related to DNA damage/telomere stress-induced senescence, which is unsurprising given the harsh environment of hypoxia. There are also various pathways linked to cancers, but specifically activated in hypoxia such as the AKT1 E17K and WNT pathway signaling, and pathways linked to calcium homeostasis.

Table 2. Top Ten Most Differentially Methylated CpG Positions

	Adj P-Val	Gene	Feature	Region	Δ Beta
cg25181507	$4.13E^{-07}$	*KLRG2*	TSS1500	Island	0.107
cg17943663	$4.13E^{-07}$	*CDK3*	3'UTR	Shore	0.062
cg14279726	$6.12E^{-07}$	*HPS1*	TSS1500	Open Sea	0.058
cg09948192	$6.12E^{-07}$	*GRPEL1*	TSS200	Island	0.164
cg17335258	$1.42E^{-06}$		IGR	Open Sea	0.079
cg00647178	$1.51E^{-06}$		IGR	Open Sea	0.056
cg23931734	$1.51E^{-06}$	*CA12*	Body	Shore	0.076
cg09708216	$1.80E^{-06}$		IGR	Open Sea	0.073
cg27386529	$1.91E^{-06}$	*SCAP*	TSS1500	Shore	0.073
cg05674903	$2.43E^{-06}$		IGR	Island	-0.073

Differentially Methylated Regions

In addition to individual sites, I also analyzed differentially methylated regions (DMR). These DMRs contain multiple significant DMPs close together and thus indicate a change over the entire region which can be more indicative of impacts on final gene expression rates. There were seven significant DMRs with a corrected p-value of less than 0.05. Interestingly many of these sites had links with calcium processes, the others with zinc finger proteins, chronic mountain sickness, glucose processes, and erythropoiesis. The top three DMRs are visualized in **Figure 2**. All seven significant DMRs show increased levels of methylation on average at high altitude compared to sea level.

Table 3. Reactome Pathways Related to The Most Significantly Differentially Methylated CpG Sites

Reactome ID	Reactome Pathway	Adj P-Val	Enrichment Score
2559586	DNA Damage/Telomere Stress Induced Senescence	$4.94E^{-15}$	0.806
5674400	Constitutive Signaling by AKT1 E17K in Cancer	$3.60E^{-14}$	0.806
5693571	Nonhomologous End-Joining (NHEJ)	$4.49E^{-14}$	0.766
2122948	Activated NOTCH1 Transmits Signal to the Nucleus	$4.49E^{-14}$	0.742
114508	Effects of PIP2 hydrolysis	$9.59E^{-14}$	0.769
4791275	Signaling by WNT in cancer	$1.49E^{-13}$	0.728
212676	Dopamine Neurotransmitter Release Cycle	$1.49E^{-14}$	0.852
418360	Platelet calcium homeostasis	$1.72E^{-14}$	0.733
8941326	RUNX2 regulates bone development	$3.69E^{-14}$	0.725
380972	Energy dependent regulation of mTOR by LKB1-AMPK	$3.69E^{-14}$	0.737

The first DMR shown in **Figure 2 (A)** is a region with 931 base pairs containing 24 significantly differentiated DMPs in an open sea/intergenomic region. This region codes for *ZFP57* and is known to be a transcriptional repressor due to its role in facilitating DNA

methylation.[207–209] This gene, specifically the hypomethylation of this region are associated with transient neonatal diabetes mellitus-1 (TNDM1 or 6q diabetes). DMR 2 **(B)** is 1801 base pair region containing 35 DMPs in a promoter area of the ring finger ubiquitin ligase or *RNF5* gene. This gene is a membrane associated E3 ligase which regulates autophagy particularly during nutrient deprivation or other cellular stress.[210] DMR 3 **(C)** is a 2599 base pair region containing 41 DMPs found in the promoter region of *HOXA5*. *HOXA5* is connected to lung development and the respiratory system. Not only is this gene is known for being regulated by epigenetic processes,[211,212] It is also connected erythropoiesis, a process that is well known to be affected by hypoxia. When *HOXA5* is repressed, cells favor an erythroid-committed subtype over granulocytic or monocytic types.[213,214]

DMRs 4,5, and 7 are all found in genes relating to calcium or calcium processes. The first of these is DMR 4 which is comprised of 1523 base pairs and contains 39 DMPs in the 5' untranslated region of NNAT, a gene that encodes neuronatin, which plays a role in neurogenesis and brain development as well as being critical in maintaining neuronal plasticity. *NNAT* specifically plays a role in brain development by being involved in the regulation of ion channels and guiding pluripotent stem cells into differentiation by facilitating an increase in calcium. DMR 5 is found in the promoter region of *CALCA* or calcitonin, a hormone which works to decrease calcium levels in the blood and inhibits bone reabsorption. It is a 1611 base pair region with 28 DMPs. DMR 7 is a 677 base pair region in the promoter of *S100A13*, a calcium binding protein A13, containing 15 DMPs.

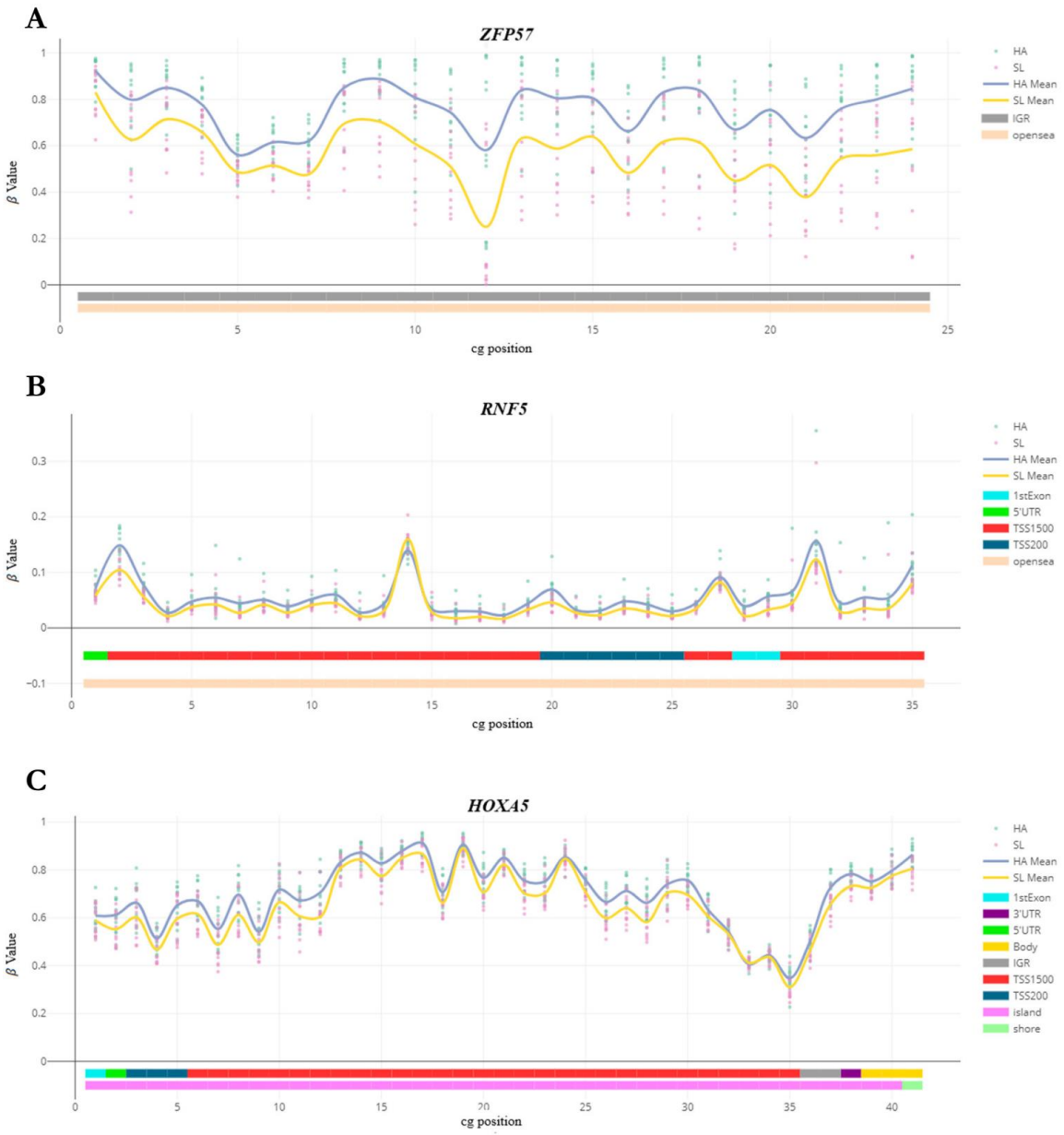

Figure 2. Top Three Most Differentially Methylated Regions
Individual and mean methylation values (B values) at sea level, in yellow, and high altitude, in purple. These figures also show where the regions are located in relation to the nearest gene and CpG island. (A) shows a DMP in the intergenomic region of *ZFP57*, (B) a DMP in the promoter region *RNF5*, and (C) a DMP in the promoter of *HOXA5*.

While being a calcium binding protein, it is also correlated with VEGF-A expression and angiogenesis which are processes known to be affected by high-altitude exposure to increase oxygenation.[215]

The last significant DMR, DMR 6, is a 1125 base pair region found in the promoter and first exon regions of peptidase M20 domain-containing 1: *PM20D1*. It contains 13 DMPs and is part of the M20 enzyme family which synthesizes N-acyl amino acids which themselves play a role in thermogenesis, reactive oxygen species (ROS) levels, and cell preservation.[216]

Discussion

Genome-wide Analysis

Overall, these data demonstrate that high-altitude hypobaric hypoxia results in a state of genome-wide hypermethylation after 1 night at high altitude. This result is supported by other literature, such as the previously mentioned studies from Childebayeva *et al.* which show that high-altitude exposure results in hypermethylation.[195,217,218]

In this dataset, most DMPs were in located the open seas. However, regardless of the number of DMPs in a given region (e.g., open seas, shelves, and shores), all regions were found to contain similar rates of DNA methylation. As shown in **Table 1,** each region's DMPs were 95-96% hypermethylated and 4-5% hypomethylated. The only exception to this was CGIs, which contained 20% hypomethylated DMPs. This supports previous research showing that CpGs in CpG island regions tend to be hypomethylated regardless of gene expression levels. It is thought that CGIs may be regulated by other means such as histone

modifications or polycomb repression.[219,220] About half of all CpG islands contain transcription starting sites (TSS) as these often overlap with promoter regions.[187] As shown in **Figure 2**, the terms CpG islands, shores, shelves, and seas are based around the position of the CpG island, however, the position of these sites can further be associated with genomic features such as exons, 3' or 5' untranslated regions (UTR), gene body, exon band, intergenomic regions (IGR), or transcriptional start sites (TSS1500 or TSS200). Sites close to a promoter such as those found in TSS200, within 200 bp the TSS, and those in the first exon are associated with gene expression in the inverse relationship, that is traditionally understood in which more methylation would result in less expression.[205] Only about 5% (9,417) of our significant sites were found in these regions. The majority were found in the body, 38%, and IGR, 30% regions. While sites in the body seem to be less correlated with gene expression, they remain highly regulated and it is likely that they play a role in initiating ncRNAs that help regulate gene expression, facilitate gene splicing,[189] and work as binding sites for transcription factors other processes.[187,221] While the majority of DMPs were found in the body of genes, 85% (6) of our DMRs were in promoter regions and it is likely these regions have a larger impact on subsequent expression.

While the top differentially methylated positions are singular CpG sites and can be found anywhere including intergenomic regions, they are often found within a gene or associated with the promoter of a gene. Indeed, six of our top ten DMPs were associated with a gene as shown in **Table 3**. The topmost site is associated with Killer Cell Lectin Like Receptor G2 (*KLRG2*) which is expressed mainly in the kidney as an integral part of the membrane enabling carbohydrate binding and is also used as a marker for lung cancer.[222] This gene also

showed significant RNA expressions differences with an adjusted p-value of 0.0035 in our previous RNAseq analysis, with it being downregulated, having a -2.52 $\log_2$ fold change at high altitude, supporting our current findings.[223] This particular area in the genome is also associated with two mRNAs encoding for thromboxane A synthase, which helps to convert prostaglandin into thromboxane, a hormone which induces platelet aggregation and works as a powerful vasoconstrictor which plays an important role in maintaining blood flow homeostasis.[224] *KLRG2* has also been shown to have crosstalk with the Notch[225] and Wnt pathways, which were significant pathways represented in our analysis.[226] Indeed while investigating our topmost significantly differentially methylated DMPs, DMRs, and over represented Reactome pathways we found the major link between them to be the Wnt pathway. A Reactome pathway called "signaling by WNT in cancer" was revealed in the top ten overrepresented pathways. Perhaps unsurprisingly there is major crosstalk between the HIF and WNT pathways, with WNT being affected by or influencing all of HIFα's three isoforms and specifically stabilizing HIF2α.[227–229] The WNT pathway has an effect on a variety of systems including neuronal differentiation of glioblastoma stem cells,[227] epithelial-mesenchymal transitions,[230] and TRPC5 channels which are activated by elevated levels of Ca^{2+}. [231] Interestingly, WNT pathway activation has been linked to increased hypoxia tolerance in drosophila exposed to hypoxia over many generations.[232]

Many of our top results are linked to regulation of the Wnt pathway such as *RUNX2,* a transcription factor which acts as a "master switch" of the development and maintenance of bone, teeth, and cartilage. RUNX2 is regulated directly by canonical Wnt signaling to control osteoblast formation and skeletal development.[233] Pathways related to PIP2 were also over

represented. PIP2 is a substrate directly involved in the canonical Wnt cascade and is related to potassium channels of mitochondria and subsequent sequestration of calcium[234] which could help combat the increases in intracellular Ca^{2+} seen at high altitude due to higher levels of EPO, sympathetic nervous system excitement, and decreased pH leading to higher osteoclast activity.[235] This could also be related to the "platelet calcium homeostasis" pathway. One of our top DMPs, *HOXA5,* can inhibit the Wnt/β-catenin signaling pathway, but can also be regulated by it in a relationship of mutual antagonism.[236] Further, CDK3 a top DMP and regulator of cell cycle progression, as well as ZNF57, our top DMR, can suppress Wnt signaling.[237,238]

In addition to the Wnt pathway links, the other major pathway represented was the Notch pathway. There were 12 significant processes related to Notch1, which plays a major role in cell fate determination as well as proliferation and apoptosis. Hypoxia has been shown to increase expression of downstream Notch targets, increasing cell proliferation and being protective against apoptosis via the interaction of HIF1α and Notch proteins.[239,240] Further, Notch has previously been discussed as a candidate pathway for high-altitude adaptation and has shown to be under selection in high-altitude populations including Andeans and Tibetans.[241] Similarly other pathways such as "constitutive signaling by AKT1 E17K in cancer" reveals other pathways known to have cross talk and direct interaction with HIF. The AKT pathway has been shown to increase and maintain HIF1 levels in hypoxia which then enhance TET enzymes which can increase AKT signaling, continuing in a feedback loop.[196,242]

Promoter regions top differentially methylated DMRs, *NNAT*, *CALCA*, and *S100A13* respectively, also play important roles in calcium homeostasis. *NNAT* expression is mediated by oxidative stress and is shown to lead to neuronal differentiation though the use of calcium-mediated channels, regulating Ca^{2+} influx.[243] Furthermore the decreased expression of *NNAT* leads to an increase in cytoplasmic Ca^{2+} levels and enhanced neuronal differentiation.[244] While *S100A13* is a calcium binding protein, it is also correlated with VEGF-A expression and angiogenesis.[215] In relation to the hypermethylation revealed in this analysis, our previous RNASeq data revealed a -0.65 $\log_2$ fold change with an adjusted p-value of 0.0041. In addition, Childebayeva *et al.* (2019) has shown increases in methylation in the same DMRs associated with *NNAT* and *S100A13* in Andean residents born at high altitude as compared to Andeans with similar ancestry born and living at low altitude, indicating that these sites are an important part of adaptation to high altitude both in acute and long term exposures.[217]

Another interesting result from the top differentially methylated sites is associated with *CA12* which is responsible for encoding for an isoform of carbonic anhydrase. These are a family of enzymes which convert carbon dioxide and water into carbonic acid and bicarbonate. This process is especially highlighted at high altitude as the increase in ventilatory rate drives a shift towards respiratory alkalosis which then is transformed by carbonic anhydrase to form H^+ and bicarbonate which can be excreted to maintain homeostasis. These enzymes can also play a role in respiration, calcification and bone reabsorption, and the formation of cerebrospinal fluid.[245–248] *CA12* also interacts directly with *SLC5A1*, which contains another significant site in our analysis, encoding for a member of

the sodium-dependent glucose transporter (*SGLT*) family and is associated with deregulating cellular energetics. HIF-1α may influence glucose transporters to promote increased glucose uptake in hypoxia as there is a shift to anaerobic glycolysis.

Reactome pathways associated with our sites were investigated in an over-representation analysis. The topmost significant results fall into categories of cell damage, HIF pathway crosstalk, and calcium homeostasis. DNA Damage and Repair pathways include "DNA Damage/Telomere Stress Induced Senescence" and "Nonhomologous End-Joining (NHEJ)". The first term refers to pathways which activate in response to reactive oxygen species (ROS) or environmental stress, both cause double strand breaks in the DNA.[249] ROS have been shown to increase in hypoxic conditions, though it may seem counterintuitive, the lack of oxygen causes the electron transport chain in mitochondria to misfunction and cause an accumulation of ROS.[250] NHEJ is then activated in turn in response to the double strand breaks which activates multiple checkpoint and repair proteins. Unfortunately, studies have shown that many genes in this pathway are downregulated in hypoxia leading to altered DNA repair patterns.[251,252] Similarly, hypoxia can lead to alternative splicing which leads to dysfunctional HDACs (histone deacetylases), another significant term in the pathway analysis, which also leads to impaired double-strand break repair.[253]

As a final note, *ZFP57* and *RNF5*, significant DMRs, come up together in gene sets associated with rheumatoid arthritis, musculoskeletal system disease, and bone disease, found in DISEASES Experimental Gene-Disease Association Evidence Scores.[254] They, along with *PM20D1* have also been shown to have differentially methylated regions in

disease states such as Parkinson's and Alzheimer's, which could play a role in the cognitive changes at high altitude.[216,255]

Conclusion

In conclusion, these data support the hypothesis that DNA methylation plays a wide and significant role in the human adaptation to oxygen limitation at high altitude. Exposure to high-altitude hypoxia resulted in genome-wide hypermethylation which is supported by much of the current literature. Additionally, many of the genes and pathways related to significant DMPs show connections to processes already established to be affected by hypoxia. Though there were many connections with other pathways such as Notch1 and Wnt, that have been less studied in the context of hypoxia. Specifically, there were major implications for the role of the Wnt pathway, supporting the importance of cell cycle progression and differentiation as well as calcium processes which need further investigation. Though some of these top DMPs were associated with genes, promotors, or CGIs, many of these CpG sites are not in the regions directly associated with gene expression levels, demonstrating a need to better understand the role CpG sites located in open sea and body regions play in gene expression. In addition, the interplay between DNA methylation and other epigenetic mechanisms such as ncRNAs and histone modifications needs to be further studied to understand the changes happening on a larger scale, and the role the combination of these changes has in our phenotypic plasticity at high altitudes.

Author Contributions

SF, KP, and ECH contributed to the conceptualization and design of the study. SF, KP, and ECH were responsible for the management and coordination of research activity planning. SF, KP, and ECH conducted the research and investigation process. SF performed all data analysis and created all figures. ECH provided the funding and resources necessary to conduct the study. SF wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Chapter 4b. Time Domains of DNA Methylation Patterns in Acclimatizing Sojourners and High-Altitude Residents

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Abstract

High-altitude environments impose a range of physiological challenges on human beings, primarily due to reduced oxygen availability, a condition known as hypoxia. While advances have been made in understanding the molecular mechanisms underlying some of the complex physiological adaptations to high altitude and chronic sustained hypoxia exposure, the role of epigenetic modifications in these changes remains relatively unexplored.

This study aims to investigate the dynamic changes in DNA methylation in humans exposed acutely to high altitudes as well as in native high-altitude populations, shedding light on the epigenetic responses to hypoxic stress. To do so we investigated the DNA methylation patterns in humans exposed to both acute and long-term exposure at key hypoxia-inducible factor (HIF) pathway genes.

We found that T50s, related to methylation percent, of EPAS1 increase upon acute exposure but then return to lower values while T50s continue to increase over time in acclimatizing

individuals in investigated regions of *ELGN1* and *EDN1*, and that this pattern continued over longer timescales in high altitude residents. In addition, there was a trend of T50s correlating with BMI and age. Together these results indicate that DNA methylation may play a role in adaptations to oxygen limitation at high altitude.

Keywords: hypoxia, high altitude, epigenetics, DNA methylation

Introduction

High-altitude hypoxia presents a significant challenge to human physiology, as the human body is dependent on oxygen for energy production and other essential functions. In response to high-altitude hypoxia, the body undergoes a series of physiological adaptations that allow it to increase oxygen delivery to tissues and acclimatize to the new environment. In individuals residing at sea level, acute exposure to high-altitude hypoxia can cause a range of physiological responses, including increased red blood cell production and increased ventilation rate, heart rate, and blood pressure.[15,256,257] These are plastic changes that can be reversed and resume normal function upon return to a lower altitude.

On the other hand, there are populations who have lived at high altitude for tens of thousands of years, resulting in evolutionary adaptation to these conditions.[258–260] Indeed, these extreme high-altitude environments present strong selective pressures due to low oxygen pressure, low temperatures, and reduced vegetation.[261] As a result, high altitude adaptation represents one of the most rapid examples of human evolution and provides a unique natural experiment for studying human environmental adaptation.

One of the most significant physiological adaptations to high altitude hypoxia is the regulation of red blood cell production and plasma volume. Tibetan high-altitude populations maintain hemoglobin concentrations within the sea-level range, while native high-altitude Andean groups develop excessive red blood cell production and elevated hemoglobin concentration and hematocrit, which can lead to Chronic Mountain Sickness (CMS). CMS is a fatal disease characterized by elevated hematocrit (Hct) levels and severe

hypoxemia. It is associated with severe pulmonary hypertension which can advance to congestive heart failure.[262]

The genetic basis of these differences in adaptation between high altitude native groups has been studied in detail and while some adaptations such as the absence of a excessive erythrocytosis and CMS and low hypoxic pulmonary vasoconstriction responses in Tibetans have been linked to specific *EPAS1* genetic variants under evolutionary selection in this group,[263–265] other phenotypes have not been strongly linked to specific adaptive genetic variants located within specific genes. Such adaptive phenotypes may instead be linked to changes in gene expression that are mediated by epigenetic modifications such as DNA methylation, histone modifications, and non-coding RNA molecules rather than DNA base pair mutations.[266–268]

Epigenetic modifications are chemical alterations of the DNA molecule, or its associated proteins, that can affect gene expression without altering the underlying DNA sequence. Such modifications can be stable and heritable or quickly modified and reversible. In either instance they play a crucial role in regulating gene expression during development and in response to environmental stimuli.[189,269] In the context of high-altitude hypoxia, several studies have suggested that epigenetic changes such as DNA methylation may contribute to the hypoxic response observed in sojourners[79,194] and long-term residents of high altitudes showing evidence such as DNA methylation levels being correlated with number of years lived at high alitude.[81,191,217] Others have showed that epigenetics can play a role in evolutionary adaptation by creating a specific phenotype.[73,270] Despite these findings, the

dynamics and time domains of DNA methylation changes in response to high altitude hypoxia in these populations remain unclear.

In this study, we investigate the time domains of DNA methylation in acute, acclimatized, and multi-generational exposure to high-altitude hypoxia, with the goal of better understanding the underlying mechanisms of epigenetic regulation in the adaptation to high altitude environments. To investigate this question, we specifically examined key genes in the hypoxia-inducible factor (HIF) pathway. HIF is a master regulator of the molecular response to hypoxia[271,272] There are three isoforms of HIF alpha, in normoxia these alpha subunits are hydroxylated by a corresponding PHD and tagged for degradation. In hypoxia, HIF ALPHA subunits are able to bind to their shared beta subunit, move to the nucleus and become a transcription factor activating hundreds of downstream target genes essential for hypoxic adaptation. Lists of genes under selective pressure for high altitude native groups, such as those living on the Andean altiplano, the Semien plateau, and the Tibetan plateau (**Figure 1**) reveal many genes which are targets of or related to the HIF pathway[258,273,274]. We chose to investigate one of the HIF proteins itself, HIF2a, encoded by the gene *EPAS1* which has shown to be under selection in Ethiopians and Tibetans. As previously mentioned, *EPAS1* was also found to have links to protective phenotypes in Tibetans. We hypothesized the DNA methylation of *EPAS1* in sojourners would decrease after exposure to high altitude but return to normal sea level values after acclimatization. We also chose to examine methylation levels in *EGLN1*, which codes for the enzyme PHD2., the regulator of HIF2, also under selective pressure in all three high-altitude native groups, which we thought would

have an opposite pattern of DNA methylation, starting low and increasing at high altitude with a return to sea level values after acclimatization.

In addition to *EPAS1* and *EGLN1* which are directly involved in the HIF pathway, we also chose to investigate the DNA methylation levels of *EDN1*, an important target gene coding for endothelin 1, a powerful vasoconstrictor shown to be under selection in all three groups which we thought might have ties to CMS scores. In sojourners we thought that DNA methylation would increase at high altitude in an attempt to decrease expression and counteract its effect on vasoconstriction, ensuring vasculature remains dilated allowing for increased blood flow and oxygen delivery at high altitude. We hypothesized that DNA methylation patterns in native high-altitude residents would most resemble acclimatized sojourners.

Understanding the epigenetic mechanisms that regulate gene expression in response to high-altitude hypoxia is important for several reasons. First, it can provide insights into the molecular mechanisms that underlie the physiological adaptations observed in individuals visiting and living at high altitudes. Second, it may help to identify new therapeutic targets for altitude-related illnesses, such as acute mountain sickness and pulmonary edema. Finally, it may provide insights into the broader role of epigenetic mechanisms in regulating gene expression in response to environmental stimuli, and their potential contributions to the development of chronic diseases such as CMS.

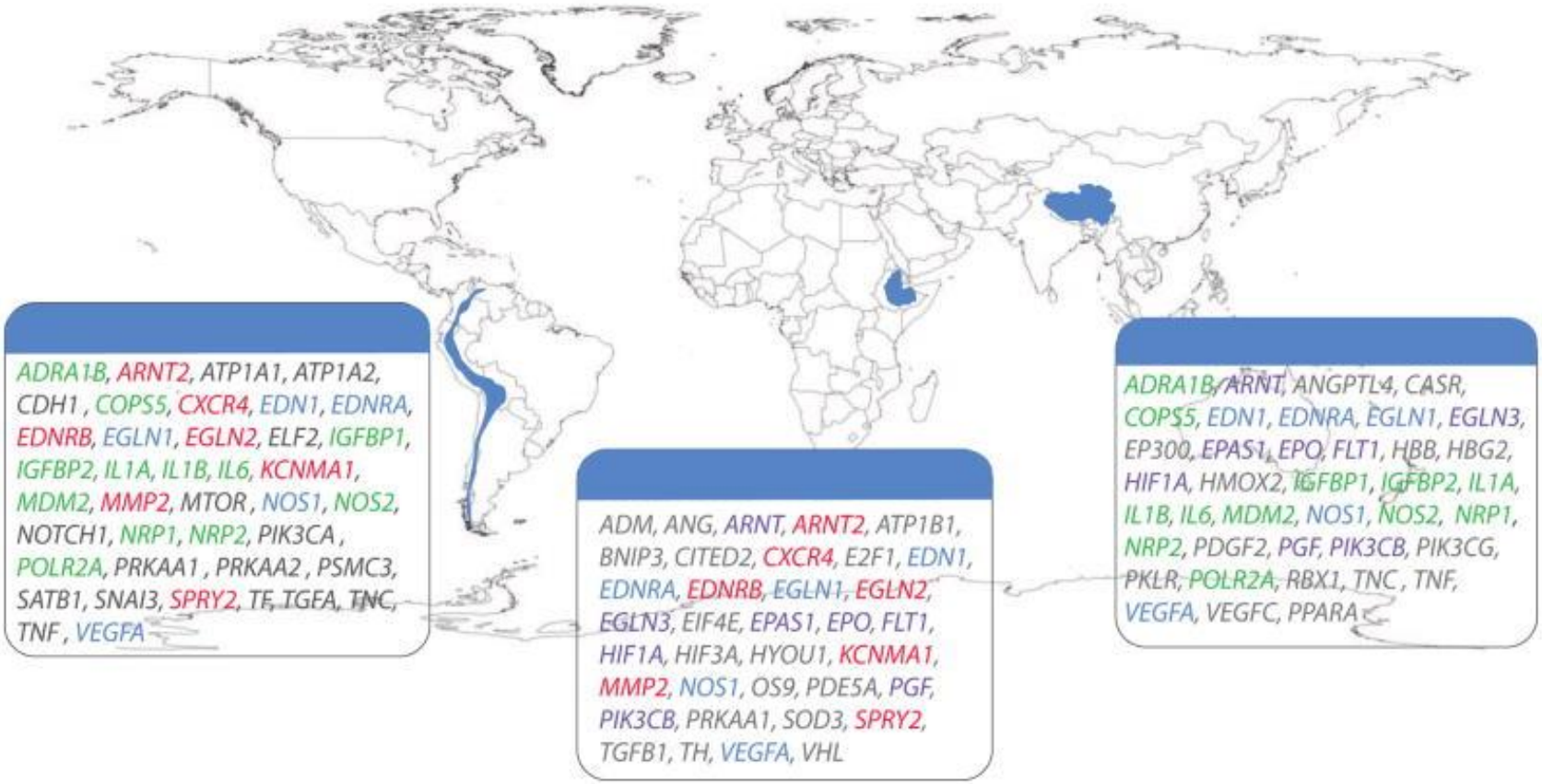

Figure 1. HIF pathway candidate genes for human adaptation to high altitude HIF pathway genes under selective pressure in native high-altitude groups of Ethiopia, Peru, and the Tibetan plateau. Genes listed in blue are under selection in all groups. Genes listed in green are shared between Andean and Tibetan, red are shared between Andean and Ethiopian, purple between Ethiopian and Tibetan. Geneses listed in black are under selection in only that population. (Illustration from Bigham, 2016. Used with permission.)[273]

Methods

Ethical approval

This study was approved by the University of California, Riverside Clinical Institutional Review Board (HS 19-076). All participants were informed of the study's purpose and risks. Participants provided written informed consent in their native language, English for sojourners and Spanish for Andeans. The work was conducted in accordance with the Declaration of Helsinki, except for registration in a database.

Participants and experimental design

Sea level participants

Thirty-one participants were recruited (22 men and 9 women). All participants were healthy individuals between 18 and 38 years old (24.3 ± 4.9 years). Exclusion criteria included current regular smokers (cigarettes, e-cigarettes, marijuana), pregnant women, recent travel to altitudes greater than 2,500 m within one month prior to the first test measurement, or current use of anti-inflammatory medications (i.e., ibuprofen) that can interfere with ventilatory acclimatization to high altitude.[197]

High-altitude residents

Twenty-two male participants were recruited during a field expedition to Cerro de Pasco, Peru. All participants were between 18 and 65 years old and had been living at high altitudes for at least two generations. High altitude residents were split into two groups based on hematocrit (Hct) levels and CMS scores. Traditionally, CMS in males is characterized by

having a Hct ≥ 63 or having a total Qinghai CMS score of ≥6.[262] In our study, healthy controls (CDP) were an average age of 41 years old, had a Hct of 54% or less, with an average Hct of 52.0 ± 2.1 %, and an average CMS score of 0.3 ± 0.5. On the other hand, our CMS participants were an average age of 33 years old. The CMS group had a Hct of 67% or more with an average of 71.8 ± 3.7 %, $p = 1.7e^{-12}$, and an average CMS score of 6.0 ± 2.6, $p = 1.9e^{-6}$ **(Table 1)**.

Table 1. Participant Demographics and Physiological Responses to High Altitude

	SL	HA 1	HA 2	HA 3	CDP	CMS
P_{sys}	126.6 ± 7.5	126.1 ± 6.2	127.9 ± 15.0	126.8 ± 11.7	113.9 ± 19.8	117.0 ± 9.0
P_{dia}	84.8 ± 7.5	88.7 ± 4.9	85.0 ± 8.1	86.3 ± 7.9	71.6 ± 12.6	75.3 ± 9.1
SpO_2	95.1 ± 1.9	**85.8 ± 4.2 ******	**84.5 ± 3.4 ******	**84.5 ± 4.1 ******	89.1 ± 3.8	**84.4 ± 4.0 ****
Hct	49.6 ± 4.8	49.4 ± 3.8	49.7 ± 3.5	51.4 ± 3.0	52.0 ± 2.1	**71.8 ± 3.7 ******
AMS/CMS Score	0.6 ± 0.7	**3.8 ± 2.4 ****	**4.1 ± 3.3 ****	2.3 ± 2.2	0.3 ± 0.5	**6.0 ± 2.6 *****

Variable units: P_{sys} and P_{dia} (mmHg); SpO_2 (%); Hct (%).
Sojourners were tested against baseline, SL. Participants with CMS were tested against the control participants, CDP. Bolded values had a p value less than 0.05.

Experimental Design

Sojourners

Sea level participants first completed a laboratory visit at the University of California, Riverside (sea level (SL); 340 m elevation) for consenting procedures, baseline physiological measurements, and a blood draw. Blood pressure measures were collected with a manual sphygmomanometer. Heart rate and pulse oxygen saturation (SpO_2) with a Nellcor N600X

pulse oximeter and finger probe (Medtronic, Minneapolis, MN, USA) after 5 minutes of resting in an upright seated position with legs uncrossed and breathing normally. Acute Mountain Sickness scores were collected via the 2018 Lake Louise Acute Mountain Sickness Scale.[11] Participants were driven to Barcroft Station (White Mountain Research Center, UC Natural Reserve System). The ascent profile included traveling from 340 m to 1,216 m over 4 hours, then from 1,216 m to 3,800 m in 2 hours. Participants completed physiological measurements and blood draw each morning before breakfast over 3 days at high altitude, starting the morning after spending one night at the station, referred to as high altitude day 1 (HA1). The timeline is laid out visually in **Figure 2**.

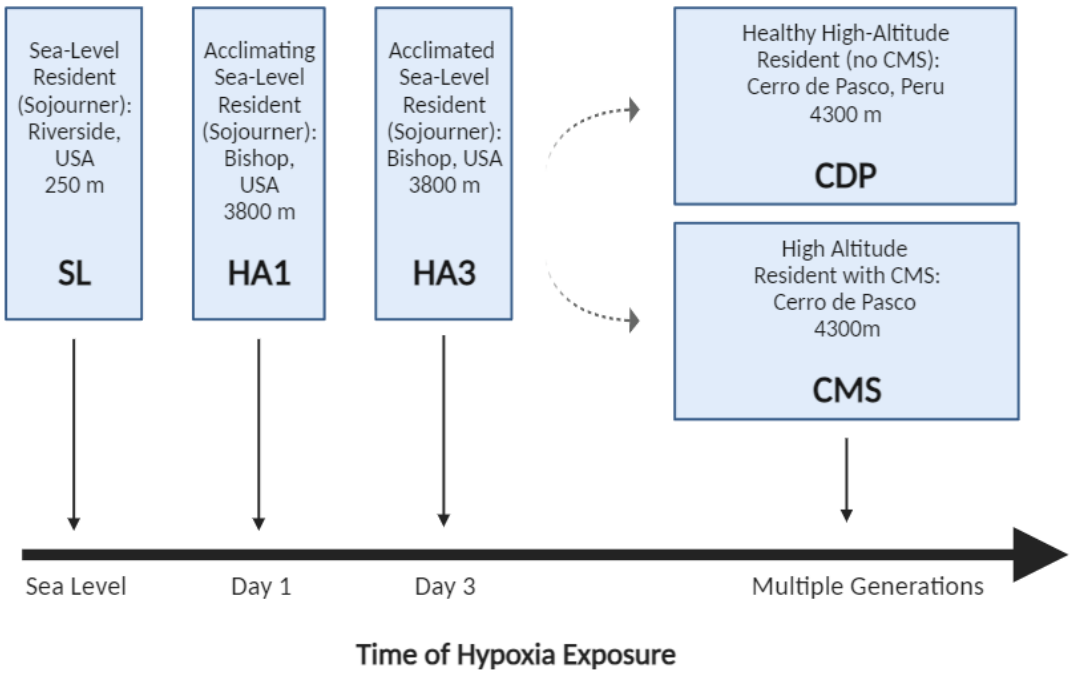

Figure 2. Participants and Experimental Design
Sojourners were taken to Barcroft Station, 3800 m, for three days. This group serves as an acute exposure model. Our long term exposure was Andean high-altitude natives who had lived at high altitude for more than two generations were split into healthy (CDP) and chronic mountain sickness (CMS) groups.

High-Altitude Residents

High-altitude Andean residents (HAR) completed one visit at the Cerro de Pasco High-Altitude laboratory (4,338m), associated with the Cayetano Heredia University in Lima, Peru in which physiological measures and a blood draw using standard phlebotomy procedures were also taken. Blood was collected using standard phlebotomy procedures while participants were in a fasting state. CMS scores were assessed using the Qinghai scoring system.[262] Detailed demographic information for all participants is provided in **Table 1.**

Sample Preparation and High-Resolution Melt Procedure

Fasting veinous blood samples were obtained from participants at sea level and for three days every morning at high altitude immediately after waking. DNA was isolated from peripheral blood mononuclear cells (PBMCs) in the buffy coat of these samples using the GentraPuregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for Whole Blood. Once isolated, DNA was tested for purity using standard 260/280 ratios suggested for DNA, ~ 1.8, and concentration via Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). An aliquot of 400 ng of isolated DNA then underwent a bisulfite conversion treatment using EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the unmodified manufacturer's protocol. After treatment, DNA was tested again for purity, however this time using standard 260/280 ratios suggested for RNA, ~ 2.0, and concentration measured as RNA via Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA).

Primers were created for *EPAS1, EGLN1,* and *END1* using standard protocol for Bisulfite Specific PCR (BSP) which uses one set of primers per region of interest. Multiple primers were made for *EPAS1* and *EGLN1* and we report here the results of those that were successful in amplifying the region of interest which included three regions in each of the genes CpG island promoter region including the transcription start site (TSS), first exon, and a region after the first exon **(Figure 3)**. *EDN1* does not contain a CpG island and only one primer was designed for this gene, amplifying a region of the first exon. Sequences for the specific primers used are in **Supplementary 1.** Bisulfite treated DNA was amplified via quantitative PCR using the suggested two-step PCR protocol for 20ul reactions provided by ThermoFisher for the MeltDoctor Master Mix (#4415440) increasing the cycles from 40x to 50x. This PCR was immediately followed by the melt steps also on the insert for the MeltDoctor Master Mix, raising the temperature from 65C-95C. More than half samples for primers designed for the first exon of *EPAS1* and after the first exon for *EGLN1* failed and so these regions have been excluded.

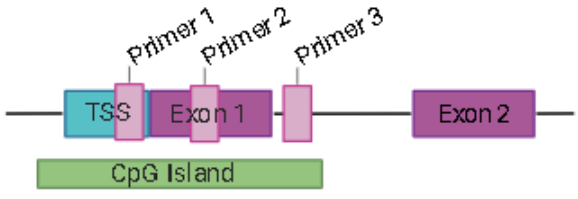

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Figure 3. Example Primer Locations
Primers were all created within the CpG island and promoter region of our candidate genes. Primers could be located in the transcription start site (TSS), the 1st exon, or downstream of the first exon in the first intron.

Analysis

RStudio (RStudio, Boston, MA, USA) with R version 4.2.2 was used to analyze data. Raw fluorescence values were imported into R. Rmisc, dplyr, ggpubr, and reshape2 packages were used to normalize data following protocols suggested by Smith et al[275] and extract melting temperatures. ggplot2, ggprism, ggsignif, and cowplot were used for figures and visualizations. The active melt period of the amplicon was identified as the period in which the slope decreases in fluorescence over time exceeded the background slope. This fluorescence signal was then normalized to the background signal. Using these normalized curves, we calculated the T50, or the temperature at which half the amplicon has melted. T50s for the amplicons will be presented in the results of this manuscript. As demonstrated by Smith et al T50s are directly related to percent methylation with a higher T50 correlated with a higher percent methylation **(Figure 4).**[275]

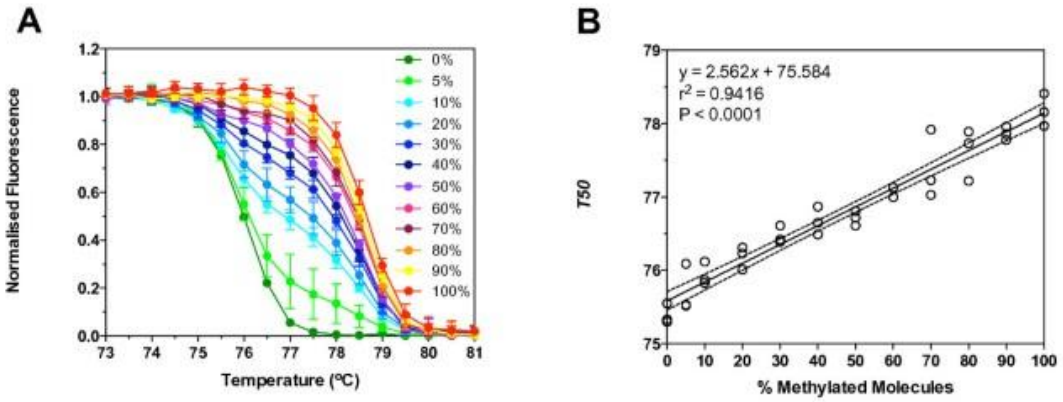

Figure 4. Correlation between Percentages of Methylated Reference and T50
Unmethylated reference (bisulfite modified normal donor lymphocyte DNA) and methylated reference (bisulfite modified CpG methylase treated normal donor lymphocyte DNA) were mixed so that the final percentage of methylated reference in the unmethylated reference was 0%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%. The mixtures were amplified and melted. **A)** Graph of normalized fluorescence plotted against temperature. Data shown are the mean ± standard deviation of triplicate reactions. **B)** Linear correlation between percentage of methylated reference and T50. (Illustration from Smith et al, 2009. Used with permission.)[275]

To test for changes in T50 values at high altitude, paired t-tests were completed for each day at high altitude compared to the SL baseline. Unpaired t-tests between CDP and CMS were performed to determine if there were differences in DNA methylation due to CMS. Further unpaired t-tests were sued to determine if SL sojourner groups were significantly different to high altitude resident groups unpaired. Data are presented throughout the manuscript as mean (standard deviation). Asterisks indicate significant differences at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), or $p<0.0001$(****).

Results

EPAS1

There were interesting patterns moving in the way opposite way of our hypothesis with higher T50s while acclimatizing. The T50s of sojourners in the TSSregion did not change significatnly from SL to HA1, but it did increase from HA1 to HA2 ($p = 0.03$) where it then decreased from HA2 to HA3 ($p = 0.031$). This decreased T50 on HA3 was significantly lower than our healthy CDP group ($p = 0.022$) (**Figure 5A**). The first intron region, while having a more pronoouced looking trend in this direction, was less significant due to variation and very low differences however HA2 was significantly higher than our CMS group ($p = 0.026$) (**Figure 5B**). It is likely that these small changes don't make a significant difference as *EPAS1* codes for the consitutively expressed version of HIF, HIF2α. HIF2α is also modified post transcriptionally which may indicate that controlling expression at this level is not necessary.

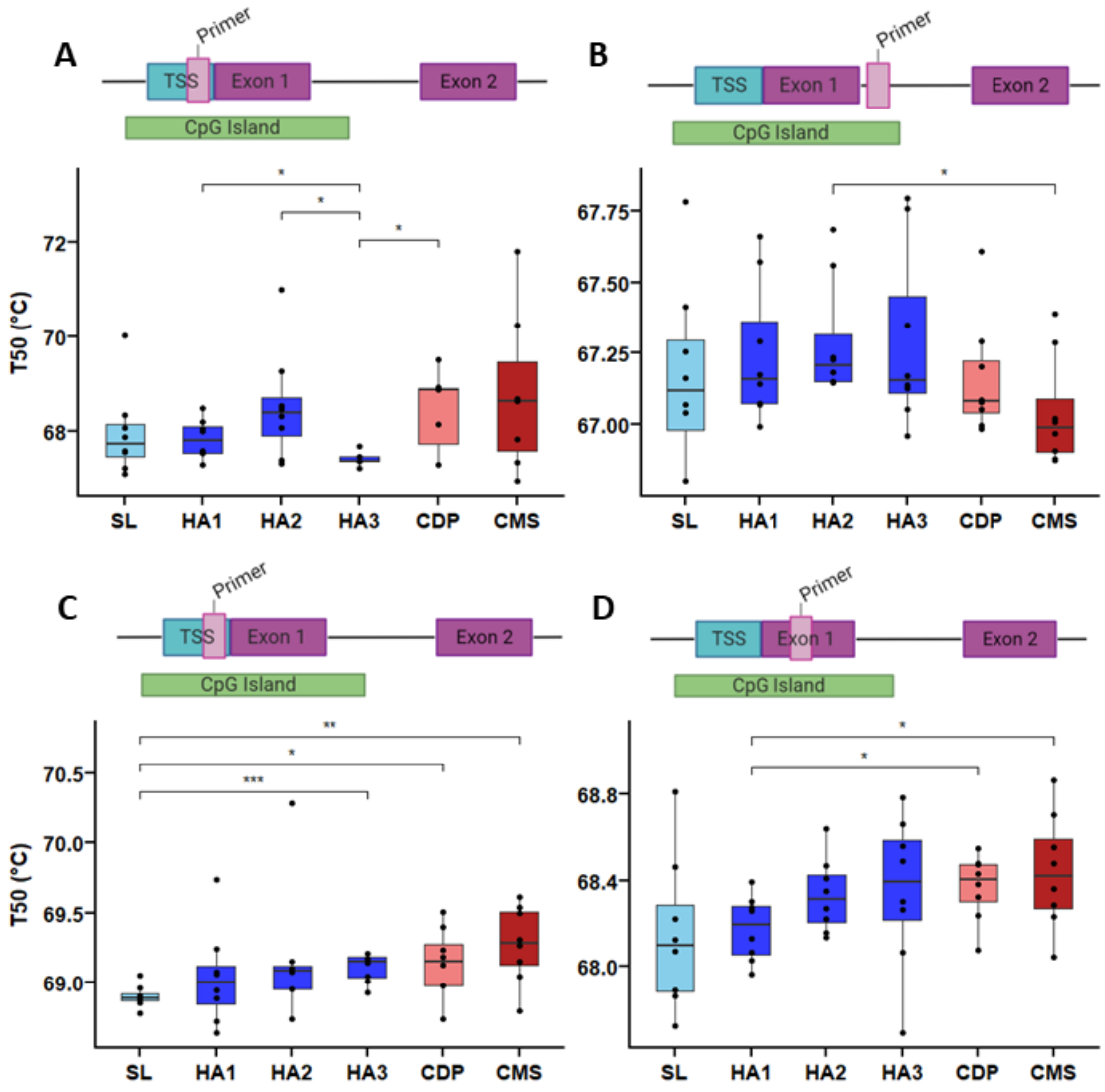

Figure 5. T50 Result of *EPAS1* and *EGLN1*
T50 results of amplicons in *EPAS1* at **A)** the transcription start site and **B)** the first intron and in *EGLN1* at **C)** the TSS and **D)** first exon

Despite a trend of the intron region associated with saturation on HA3 ($p = 0.057$) with lower T50s correlated with lower oxygen saturation, there were no significant correlations between T50s of *EPAS1* in these regions and any phyiological data, phenotypes, or AMS/CMS severity.

EGLN1

EGLN1 showed an interesting stepwise trend with T50s increasing over time exposed to high altitude and having the highest average T50 and thus highest methylation levels in the CMS group, particularly in the TSS region. In the TSS region the T50 at SL was significantly lower than HA3 (0.00049), CDP ($p = 0.029$), and CMS ($p = 0.0056$) (**Figure 5C**). In the first exon region of *EGLN1*, we see a similar trend with high variation at SL but a sort of stepwise increase in T50s once exposed to altitude. Here we see the T50 at HA1 was significantly lower than the T50s of high altitude residents, both CDP ($p = 0.024$) and CMS ($p = 0.032$) (**Figure 5D**).

Similar to regions in *EPAS1,* in sojourners, there was a trend towards a relationship between the T50s of the TSS region of *EGLN1* and SpO_2 ($p = 0.055$) with higher T50s associated with lower oxygen saturation in sojourners on HA2, but no significant correlations with physiological measures or AMS/CMS severity. In high-altitude residents, there was no relationship between any *EGLN1* T50s and Hct, CMS scores, SpO_2, or BMIs. However, there was a trending relationship between the T50s of *EGLN1* in the first exon region and age with higher T50s trending towards younger participants ($p = 0.056$).

EDN1

EDN1 showed a clear day wise increase in T50. SL values were significantly lower than HA1 ($p = 0.03$), HA2 ($p = 0.0043$), HA3 ($p = 0.0.0033$), CDP ($p = 0.0028$), and CMS ($p = 0.0033$). HA1 had lower T50s than HA2 ($p = 0.0098$), HA3 ($p = 0.0035$) CDP ($p = 0.038$), and CMS ($p = 0.026$). High altitude native residents had no significant differences in T50 between CDP and CMS groups (**Figure 6**).

There were no relationships found between *EDN1* T50s and Hct, age, SpO_2 or AMS/CMS score severity in sojourners or high-altitude residents.

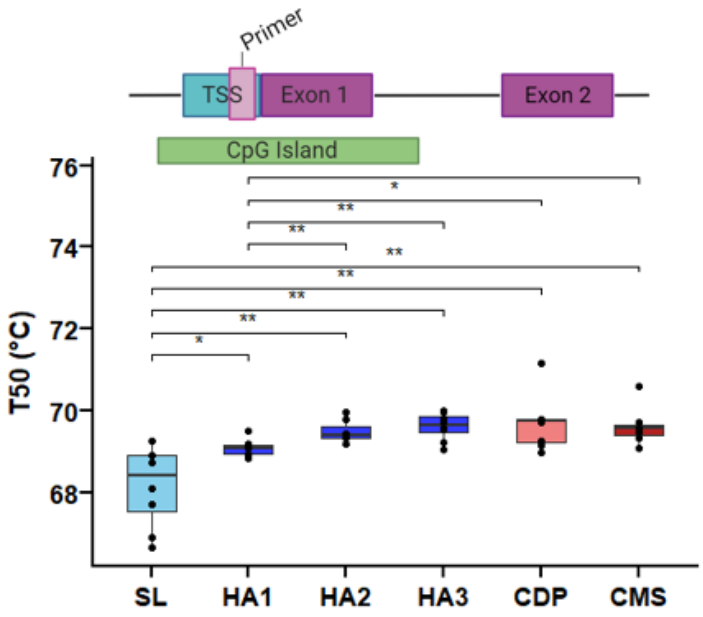

Figure 6. T50s of *EDN1*

BMI

There were several regions that were correlated with BMI specifically on HA2 in sojourners. In the first intron region of *EPAS1* (p = 0.019) and in *EDN1* (p = 0.026) lower BMIs were associated with higher T50s (**Figure 7)**.

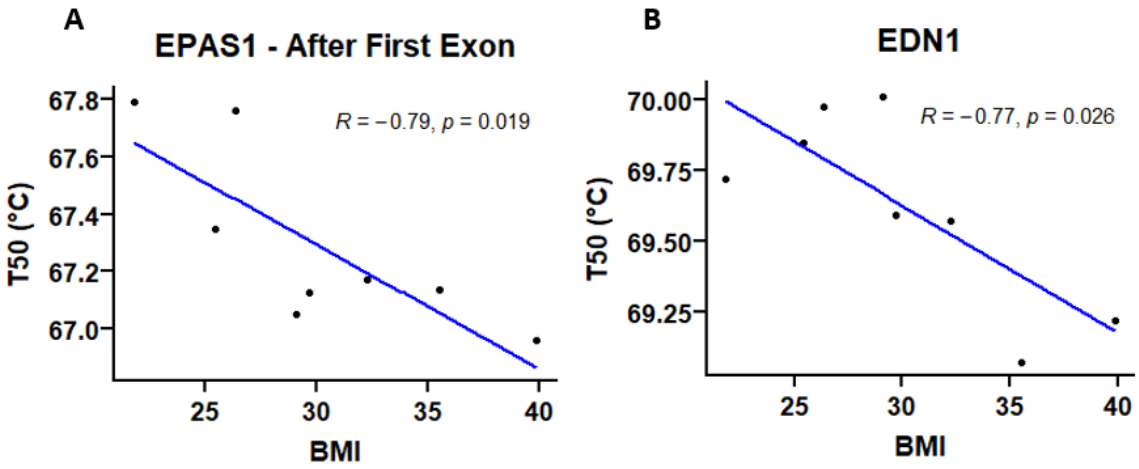

Figure 7. T50s Correlated with BMIs
T50s were found to have relationships with BMI in the **(A)** first intron region of *EPAS1* **(B)** and *EDN1*

Discussion

While the DNA methylation patterns of sojourners in both amplified regions of *EPAS1* were mostly not statistically significant differentiated between days, the overall patterns moves opposite of the hypothesized pattens of increasing T50 upon acute exposure but recovering over time and acclimatization, particularly in the TSS region where HA3 shows a significant decrease from HA1 and HA2. These trends are supported by previous studies show initial increases in sojourners of *EPAS1* DNA methylation during incremental ascents to high altitude.[79] However, this same research group showed Peruvian high-altitude

residents born and living at high altitude had lower levels of *EPAS1*methylation than their ancestrally similar counterparts living at lower altitudes and that this methylation was negatively associated with years living at high altitude meaning that the longer they have lived at high altitude, the less methylated this region is, which is similar to what we see in the intron region of *EPAS1*.[81]

Both *EGLN1* and *EDN1* seem to increase in melting temperatures over the course of the exposure with high altitude residents also being significantly higher than sojourners at sea level. This may indicate that these changes are indeed playing a pivotal role in acclimatization and adaptation to high altitude. Increased DNA methylation in the promoter, first exon, and first intron is typically associated with lower gene expression.[205] The outcome of reduced PHD productivity or expression would follow a pattern shown by other high-altitude residents such as Tibetans that have a SNP in *EGLN1* that results in a less functional protein and thus a lower Hct.[276] Similarly, halting the vasoconstrictive power of endothelin 1, encouraging vasodilation, would seemingly have a beneficial effect on blood transport and oxygen delivery.

A global trend of hypermethylation due to hypoxia has been demonstrated to be partly caused by a reduction of activity in ten-eleven translocation (TET) enzymes. TETs are oxygen sensitive, relying on oxygen as a substrate for the demethylation of DNA through 5-methylcytosine oxidation, and have reduced activity in hypoxia.[195,196] The overall increase in methylation during high-altitude exposure also makes sense in a low oxygen environment as reducing production of proteins may also be a way of conserving energy.

We found interesting associations with higher T50s being associated with lower BMIs in region after the first intron in *EPAS1* and in *EDN1* on HA2. This pattern with higher BMIs being correlated with lower levels of methylation were also seen in studies such as Maugeri et al who investigated links between BMI and methylation levels of LINE-1, a repetitive element found throughout the genome commonly used as a kind of 'housekeeping' gene.[277]

A relationship between age and T50s was found to be trending in first exon of *EGLN1* in the combined cohort of native high-altitude residents. While we did not find association in sojourners, this group contained younger participants with less variation in age. These results are supported by previous studies finding global hypomethylation to be associated with increased age.[278]

Limitations

Though this is one of the largest data sets to date, this is still a small representation when discussing human genetics and adaptations. This study was limited by the amount of space available for researchers and participants in the high-altitude laboratories as well as samples which could be analyzed. It is likely there would be more power and significance with more samples.

There has been very little research completed on DNA methylation changes in hypoxia or high altitude. It would be beneficial to explore epigenetic mechanisms not only on their own but also how they work in tandem with other mechanisms to explore the larger picture of how these mechanisms are playing a role in our phenotypic plasticity at high altitudes.

Conclusion

In conclusion, amplicons in *EGLN1,* and *EDN1* showed significantly increased methylation levels in sojourners after 3 days of acclimatization as compared to their sea-level values with high-altitude residents being more comparable to acclimatized sojourners than sojourner's sea level baseline values.

We also report that that BMI may play a role in acclimatization phase with higher BMIs having lower T50s. This study is groundbreaking in the field and the first to compare DNA methylation levels in a time dependent manner across sojourners and longtime residents of high altitude. These data suggest that DNA methylation may play a role in metabolic adaptations to oxygen limitation at high altitude.

Acknowledgements

We would like to acknowledge and thank our undergraduates Brittney Oeung, Nihil Puvvula, and Ledia Nasr who helped with our fieldwork in Northern California, as well as the staff at Barcroft Station. In addition, we would like to thank Francisco Villafuerte and the other researchers at the high-altitude research station in Cerro de Pasco. Finally, we would like to thank both our sojourners and high-altitude resident participants.

Chapter 5:

Changes in Histone Modifications During High-Altitude Exposure

Investigating the Time Domains of Histone Modifications in Humans Exposed to High Altitude

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Abstract:

Exposure to high altitudes imposes considerable physiological challenges on individuals, necessitating rapid adaptations to cope with reduced oxygen levels. High-altitude residents and sojourners experience a series of physiological responses orchestrated through intricate molecular pathways, including epigenetic modifications, to help them adapt to high-altitude environments. Of particular interest are histone modifications, which play a vital role in regulating gene expression and chromatin structure. Despite the growing understanding of epigenetics in various contexts, a significant knowledge gap remains in the context of hypoxia and high-altitude exposure.

This study aimed to investigate the dynamic changes in histone modifications in the time domains of humans exposed to high-altitude environments, including sea-level residents acutely exposed and then acclimatized to high-altitude hypoxia (acute exposure model), and

high-altitude native Andeans who are either healthy or suffering from chronic mountain sickness (CMS) (chronic exposure model). Results demonstrated dynamic changes in histone modifications, with an initial increase in histone proteins and modifications during acute exposure, followed by a return to sea-level values after acclimatization. Levels of total H3, H3K4me3, H4K9me3, and H3K27me3 were measured resulting in higher levels of total H3 proteins and modifications in acclimatizing sojourners and high-altitude residents. While participants suffering from CMS had higher levels of total H3 and H3 modifications than sojourners at sea level or after three days of acclimatizing to high altitude. In addition, a multiplex assay measuring 27 modifications was run on a sea level participant at sea level and all three days at high altitude. The results support the increases in total H3 and modifications previously found during acclimatization. These findings shed light on the importance of total histone proteins and histone modifications in the human body's adaptation to high altitudes and highlight the need for further research in this area, potentially involving a broader range of modifications, native high-altitude populations, and an exploration of the impact and importance of total histone quantity.

Keywords: high-altitude, hypoxia, histone modifications, H3, epigenetics

Introduction

Exposure to high altitude poses a multitude of challenges to human physiology, necessitating rapid adaptations to cope with reduced oxygen availability. Individuals residing at high altitudes or engaging in mountaineering expeditions often experience a series of physiological responses, including increased ventilation, enhanced erythropoiesis, and augmented oxygen-carrying capacity.[279,280] These adaptive changes are orchestrated through the activation of intricate molecular pathways, involving transcriptional regulation and protein expression alterations.[281]

In recent years, the role of epigenetic mechanisms in these physiological responses to environmental stimuli has garnered substantial attention. Epigenetic modifications, which include DNA methylation, histone modifications, and non-coding RNA regulation, regulate gene expression patterns without altering the underlying DNA sequence. Among these epigenetic mechanisms, histone modifications have emerged as key players in modulating chromatin structure and gene accessibility, allowing the human body to quickly adapt to its surroundings.[282,283] Histones are the proteins around which DNA is coiled, keeping it condensed and protected from DNA damage. Histones are arranged as 4 pairs of dimers, H2A, H2B, H3, and H4 into an octamer core. These histones contain 'tails' which are made up of modifiable amino acids. Histone modifications, such as acetylation, methylation, phosphorylation, ubiquitination, and SUMOylating, impact gene expression by remodeling chromatin architecture and influencing the recruitment of transcriptional regulators. Given their potential to induce lasting changes in gene expression,[284,285] histone modifications hold

immense promise in understanding the molecular basis of adaptive responses to environmental stresses, including exposure to high altitude. Indeed, studies have shown increases in modification marks such as H3K4me3, H3K9me2, H3K9me3, and H3K27me3 in hypoxic tumors, cell lines (RKO and A549), and mouse macrophages, indicating they may play a role in human acclimatization and survival in hypoxia.[86,286]

However, despite growing interest in studying the epigenetic landscape in various contexts, including cancer, development, and aging, there remains a noticeable knowledge gap concerning histone modifications in the context of hypoxia and high-altitude exposure. Elucidating the dynamic changes in histone modifications under hypoxic conditions can provide invaluable insights into the underlying molecular mechanisms driving physiological adaptations to high altitudes as well as other hypoxia-induced pathologies, such as Chronic Obstructive Pulmonary Disorder (COPD) and COVID-19.

This study aims to bridge this knowledge gap by investigating the alterations in histone modifications in humans exposed to high-altitude environments, specifically investigating the changes in histone modifications at sea level, during acute exposure and acclimatization, and in multigenerational high-altitude native Andean residents. In addition, we also studied the histone modifications of maladapted high-altitude native Andean residents suffering from chronic mountain sickness (CMS) to see how these compared to the sojourners and healthy high-altitude residents. CMS is a fatal disease characterized by elevated hematocrit (Hct) levels and severe hypoxemia. It is associated with severe pulmonary hypertension which can advance to congestive heart failure.[262]

Methods

Ethical Approval

This study was approved by the University of California, Riverside Clinical Institutional Review Board (HS 22-088) and the Institutional Ethics Committee of Universidad Peruana Cayetano Heredia (Lima, Peru). Participants were informed of the study's purpose and risks and provided with written informed consent in their native language, English for sea level participants and Spanish for high-altitude residents. The work was conducted in accordance with the *Declaration of Helsinki*, except for registration in a database.

Participants

Sea Level Participants

Of the 20 participants initially recruited, this study used a subset of 16 participants (10 men and 6 women) who were randomly selected using a random number table. All participants were healthy individuals between 18 and 38 years old (26.6 ± 6.2 years). Exclusion criteria included current regular smokers (cigarettes, e-cigarettes, marijuana), pregnant women, recent travel to altitudes greater than 2,500 m within one month prior to the first test measurement, or current use of anti-inflammatory medications (i.e., ibuprofen) that can interfere with acclimatization to high altitude.[197]

High-Altitude Residents

Twenty-two male participants were recruited during a field expedition to Cerro de Pasco, Peru. All participants were between 18 and 65 years old and had been living at high altitudes for at least two generations. High altitude residents were split into two groups based on hematocrit (Hct) levels and CMS scores. Traditionally, CMS in males is characterized by having a Hct ≥ 63 or having a total Qinghai CMS score of ≥6.[262] In our study, healthy controls (CDP) were an average age of 44 years old, had a Hct of 54% or less, with an average Hct of 53.0 ± 1.5 %, and an average CMS score of 1.3 ± 1.5. On the other hand, our CMS participants (CMS) were an average age of 36 years old, which is statistically different than the control group, $p = 0.02$. The CMS group had a Hct of 67% or more with an average of 67.3 ± 7.0 %, $p = 1.6e^{-7}$, and an average CMS score of 4.7 ± 3.0, $p = 2.9e^{-4}$ (**Table 1)**.

Experimental Design

Study

Sea level participants first completed a laboratory visit at the University of California, Riverside (sea level (SL); 340 m elevation) for consenting procedures, baseline physiological measurements, and a fasting venous blood draw via standard venipuncture procedure. Blood pressure measures were collected with a manual sphygmomanometer. Heart rate and pulse oxygen saturation (SpO_2) with a Nellcor N600X pulse oximeter and finger probe (Medtronic, Minneapolis, MN, USA) after 5 minutes of resting in an upright seated position with legs uncrossed and breathing normally. Acute Mountain Sickness (AMS) scores were

collected via the 2018 Lake Louise Acute Mountain Sickness Score.[11] Participants were driven to Barcroft Station (White Mountain Research Center, UC Natural Reserve System). The ascent profile included traveling from 340 m to 1,216 m over 4 hours, then from 1,216 m to 3,800 m in 2 hours. Participants completed physiological measurements and blood draw each morning during fasting over 3 days at high altitude, starting the morning after spending one night at the station, referred to as high altitude day 1 (HA1).

High-altitude residents completed one visit at the Cerro de Pasco High-Altitude laboratory (4,338m), associated with the Cayetano Heredia University in Lima, Peru in which physiological measures and a fasting venous blood draw were also collected. CMS scores were assessed using the Qinghai scoring system.[262] Detailed demographic information for all participants is provided in **Table 1.**

Table 1. Participant Demographics and Physiological Responses to High Altitude

	SL	HA 1	HA 2	HA 3	CDP	CMS
P_{sys}	120.9 ± 6.5	126.9 ± 9.6	128.5 ± 14.8	126.4 ± 12.2	115.1 ± 25.1	116.4 ± 7.6
P_{dia}	79.3 ± 5.3	**86.1 ± 6.5 ****	**85.0 ± 8.8 ****	**85.4 ± 8.8 ****	71.1 ± 11.6	77.3 ± 8.0
SpO_2	94.9 ± 1.7	**83.9 ± 4.5 ******	**84.3 ± 3.4 ******	**84.6 ± 4.3 ******	89.2 ± 4.2	**85.3 ± 4.0 ****
Hct	47.1 ± 4.3	47.6 ± 3.1	46.3 ± 5.6	47.7 ± 3.8	53.0 ± 1.5	**67.3 ± 7.0 ******
AMS/CMS Score	0.4 ± 0.6	**4.5 ± 2.9 ******	**4.1 ± 2.3 ******	**3.3 ± 2.4 *****	1.3 ± 1.5	**4.7 ± 3.0 *****

Variable units: P_{sys} and P_{dia} (mmHg); SpO_2 (%); Hct (%).
Sojourners were tested against baseline, SL. Participants with CMS were tested against the control participants, CDP. Asterisks indicate significance at the $p<0.05$ (*), $p<0.01$ (**), or $p<0.001$ (***).

Histone Extraction

Fasting veinous blood samples were obtained from participants at sea level and for three days every morning at high altitude immediately after waking. Histones were isolated from peripheral blood mononuclear cells (PBMCs) in the buffy coat of these samples using Abcam Histone Extraction Kit (ab113476), following the manufacturer's protocol for isolating histones from cells; treated or untreated. Once isolated the quantity of histone proteins was analyzed using a standard unmodified Bradford protocol. Results from the Bradford assays were used to verify input protein concentrations in downstream ELISA assays were constant across samples.

Histone Modification Testing

The total quantity of H3 histones was calculated using Epigentek's EpiQuik Total Histone H3 Quantification Kit (Colorimetric) (P-3062-96). These same samples were also used in colorimetric EpiQuick Global histone modification quantification kits from Epigentek including H3K4me3 (P-3026-96), H3K9me3 (P-3034-96), and H3k27me3 (P-3042-96). Modification quantification kits were normalized using the results from the Total Histone H3 Quantification Kit. These kits were all run with an input of 200ng of isolated histones. In addition, to confirm and further explore the results found from the modification kits, samples from one random participant at all 4 time points, SL, HA1, HA2, and HA3, with 400ng of histones were used on the EpiQuik Histone H3 Modification Multiplex Assay (P-3100-96) to measure 21 different histone modifications. Assays were read using recommended settings on a Synergy LX Multi-Mode Microplate Reader (BioTek

Instruments, VT, USA), using BioTek Gen5 software. Total H3 and modifications were calculated using manufacturer's instructions and measured total H3 or modification in ng per μg of total input protein.

Analysis

The amount of each histone modification was calculated and compared across timepoints and groups using sea level and healthy highlanders as controls for sojourners and highlanders, respectively. All statistics were completed in R studio (version 4.1.0) using *rstatix*. Results from sojourners were compared using paired -t-tests, while comparisons between healthy and CMS groups as well as between sojourners and high-altitude groups were compared using unpaired t-tests. One-way ANOVAs with post-hoc pairwise t-tests were used to test for the effect of group on the result. Data are presented throughout the manuscript as mean ± standard deviation. Asterisks indicate significant differences at $p<0.05$ (*), $p<0.01$ (**), or $p<0.001$ (***).

Results

Total H3 Protein

The amount of total H3 histone proteins is significantly affected by sample groups $F(4,69) = 248.4$, $p = 3.6e^{-40}$, $eta^2[g] = 0.94$ (**Figure 1**). The highest amount of protein was found on the first day at high altitude (HA1), with an average of 98.1 ± 6.9 ng H3 histone/μg total protein. These values were shown to be statistically higher than all other sample groups. These values were followed by those found in high altitude residents, both healthy controls

(CDP) at 16.8 ± 15.2 ng/μg protein total protein and CMS participants at 13.2 ± 14.3 ng/μg protein, which were not significantly different from each other (p = 0.85). The lowest values were found in SL, 0.31 ± 0.47 ng/μg protein, and HA3, 0.29 ± 0.46 ng/μg protein, which were also not statistically different from each other (p = 1).

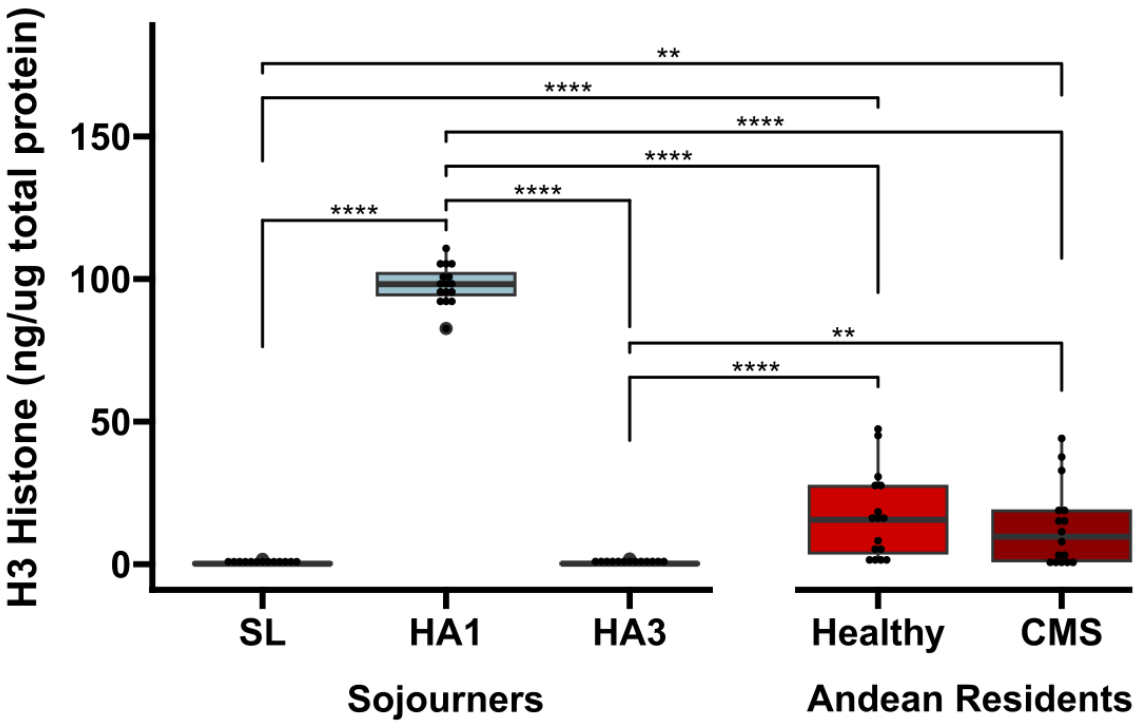

Figure 1. Total Amount of H3

Total H3 protein, in ng H3 histone/μg total protein, across sample groups. H3 protein levels on HA1 were significantly higher than all groups, followed by high-altitude residents, and finally sojourners both at sea level and HA3. Asterisks indicate significance at the p<0.05 (*), p<0.01 (**), or p<0.001 (***) level.

Histone Modifications

As expected from the trend seen in total H3 histones, all measured histone modifications did have a significant increase on HA1. The healthy high-altitude residents (CDP) tended to have higher averages and higher variability in the level of each modification as compared to their CMS counterparts, though their total H3 was quite comparable with no significant difference between the two groups (**Figure 2**). ANOVA results revealed that group had a significant effect on histone modification in every tested modification; H3K4me3 $F(4,69) = 42.3$, $p = 6.9e^{-18}$, $eta^2[g] = 0.71$, H3K9me3 $F(4,71) = 31.7$, $p = 3.67e^{-15}$, $eta^2[g] = 0.64$, and H3K27me3 $F(4,72) = 11.1$, $p = 4.3e^{-7}$, $eta^2[g] = 0.38$.

We measured amounts of total H3K4me3 across the groups. SL (0.46 ± 0.28 ng histone modification/μg total protein), HA3 (0.19 ± 0.11 ng/μg) and CMS (0.58 ± 0.65 ng/μg) participants were not statistically different from each other. Healthy high-altitude residents had larger amounts of this modification at 6.19 ± 8.51 ng/μg protein, and HA1 had the highest amount with 21.7 ± 8.67 ng/μg protein (**Figure 2A**).

When measuring H3K9me3, we found more comparable values across the time points and groups. HA3 had the lowest values, 6.16 ± 0.026 ng/μg protein, followed closely by SL, 6.21 ± 0.12 ng/μg protein, and CMS 6.46 ± 0.31 ng/μg protein. Similar to H3K4me3, this was then followed by healthy CDP residents, 8.78 ± 3.58 ng/μg protein, and topped with sojourners on HA1, 21.2 ± 9.28 ng/μg protein (**Figure 2B**).

This trend continues in the values for H3K27me3 modifications. SL values were the lowest, 0.34 ± 0.38 ng/μg protein, followed by HA3 0.42 ± 0.28 ng/μg protein. These values were also not significantly different from each other (p = 1). Participants with CMS, 1.02 ± 0.81 ng/μg protein, had significantly less H3K27me3 modifications than healthy high-altitude natives, 11.9 ± 16.8 ng/μg protein. These values were once again shadowed by H3K27me3 modifications found in HA1, 22.6 ± 19.4 ng/μg protein.

Multiplex

We also employed the use of a multiplex assay, measuring 21 different histones modifications in one participant across all timepoints. Agreeing with our previous results, this assay also revealed major increases in both the amount of total H3 proteins and across all measured histone modifications in the first day at high altitude, HA1. Interestingly, HA2, which was previously unmeasured, follows this trend at a reduced rate while there are no significant differences between SL and HA3 in any measured modification. However, overall ANOVA results show that timepoint had a large effect on histone modification amount (3,80) = p = $1.1e^{-47}$, $eta^2[g] = 0.94$. Previous assays showed an average total H3 at SL as 0.29 ± 0.46 ng/μg protein, HA1 98.1 ± 6.91 ng/μg protein and HA3 0.31 ± 0.47 ng/μg protein (**Figure 1**). This assay shows similar values, with a SL value of 0.44 ng/μg protein, HA1 76.6 ng/μg protein, and HA3 0.29 ng/μg protein. This assay also included HA2 which showed a total H3 value of 57.2 ng/μg protein (**Figure 3**).

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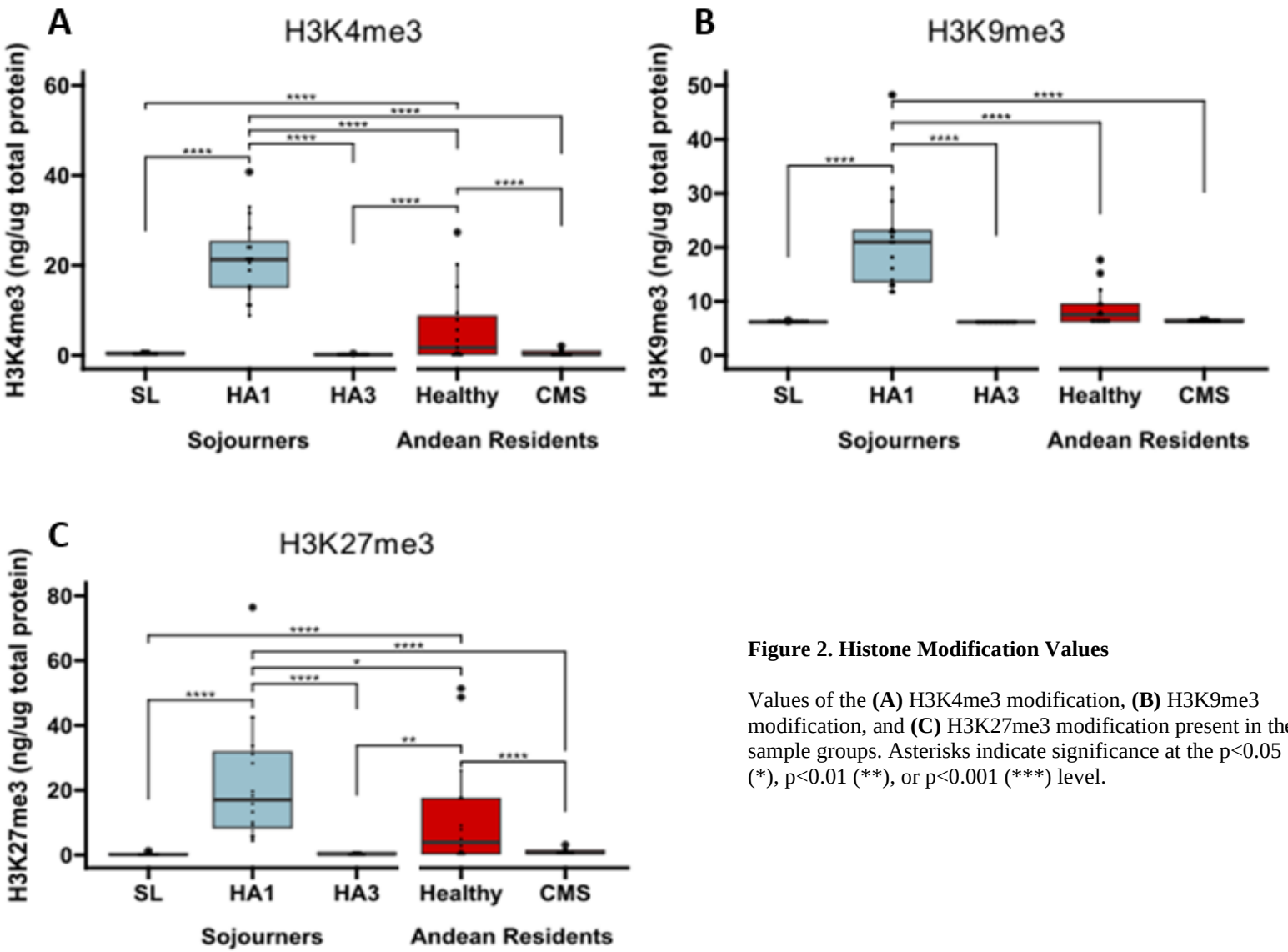

Figure 2. Histone Modification Values

Values of the **(A)** H3K4me3 modification, **(B)** H3K9me3 modification, and **(C)** H3K27me3 modification present in the sample groups. Asterisks indicate significance at the $p<0.05$ (*), $p<0.01$ (**), or $p<0.001$ (***) level.

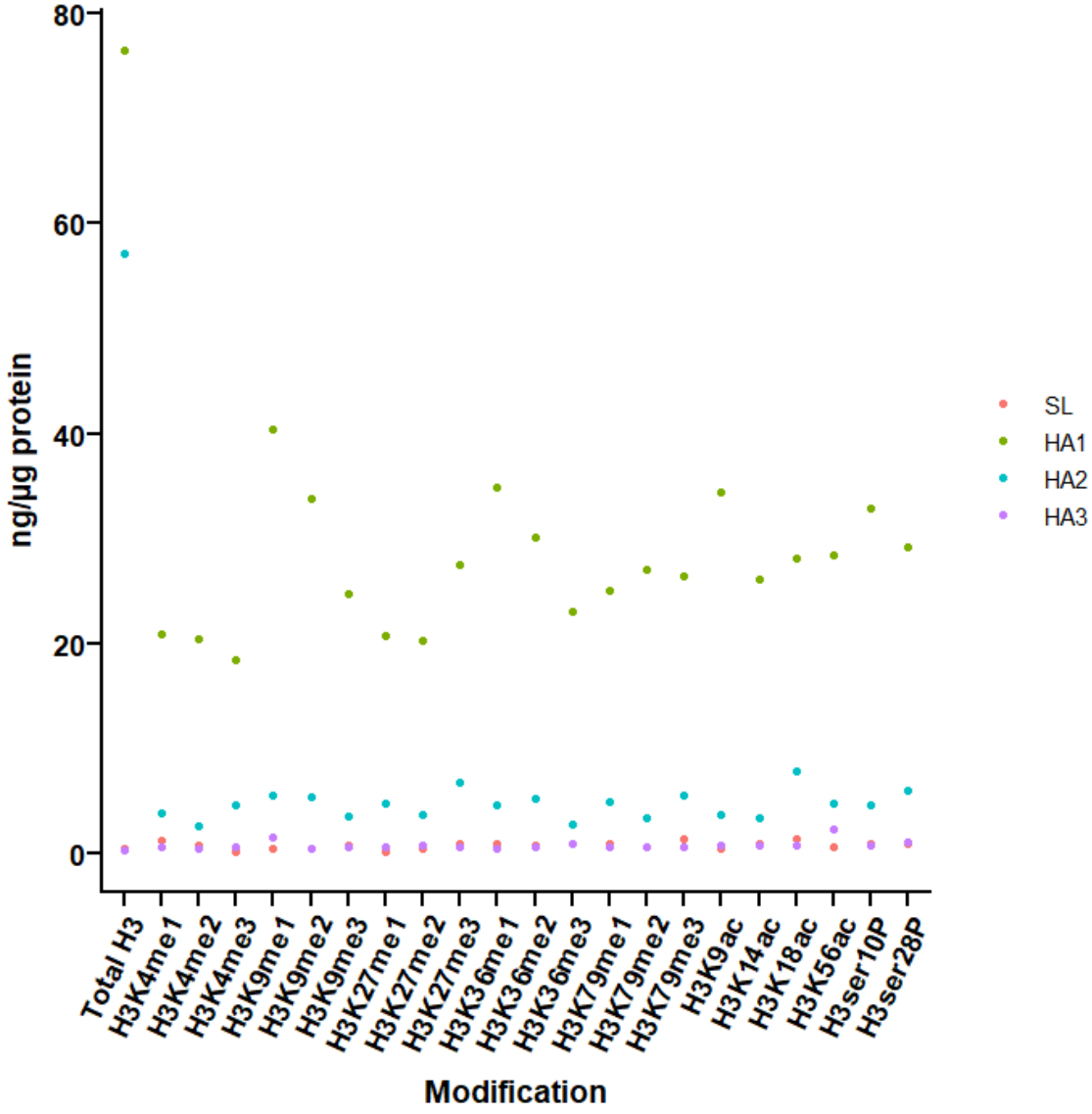

Figure 3. Multiplex Results

The multiplex assay measured total H3 and 21 different modifications in one participant at all time points; SL, HA1, HA2, and HA3

Similarly, there is also an increase in H3 modifications, with the highest quantity of modifications being H3K9me1/2 with 40.31 and 33.72 ng/μg protein respectively, H3K36me1/2 with 34.88 and 30.02 ng/μg protein, H3K9ac with 34.37 ng/μg protein, and H3ser10P with 32.85 ng/μg protein, a trend which persists into HA2.

Discussion

Total H3 Protein

Overall, there was a trend in acclimatizing sojourners, specifically samples taken on HA1, to have more H3 proteins. There was also a corresponding increase in all modifications in HA1 samples, although the increase in modifications did not increase in proportion to total H3. While we hypothesized that histone modifications would be fast acting and short lived, the multiplex assay gave support to this hypothesis showing that the large increases shown at HA1 then decrease slowly over HA2 until participants were fully acclimatized on HA3. There may be multiple factors contributing to this increase of H3 proteins found at HA1 including the changes in cell repair, cell cycle arrest, and stress which occur in hypoxia.

Recent studies suggest that in addition to wrapping DNA, there are many other processes and roles associated with H3 histone proteins. H3 and its variants, such as H3.3, are responsible for supporting chromosomal structures and maintaining genome integrity. Depletion of H3.3 causes dysfunctional telomeres, centromeres, and pericentromeric regions of chromosomes leading to reduced cell proliferations and increased cell death.[287,288] Further elaborating on this, H3 histones play an important role in cell cycle checkpoints[289] and DNA

repair, specifically acting as scaffolding for double strand break repair and replication which was shown as one of the most important upregulated pathways in a previous genome-wide DNA methylation analysis (see Chapter 4a), because of the hypoxic cell stress and UV damage associated with being at high altitude.[290–292] Thus, having more H3 histones available may help facilitate DNA repair and stability, but might also be a mark of the UV damage.[293–295] Similarly, the increase of H3 histones may also be attributed to cellular stress. Stawski et al showed that levels of circulating H3 increased in response to repeated bouts of exercise.[296] Finally, transcription can also be a contributing source of free histones as chromatin needs the nucleosome to be disassembled for RNA polymerase II to function properly[297] and transcription recovery after DNA damage has been shown to specifically rely on new histone deposits.[298]

While it may seem that additional histone deposits would be beneficial, excess amounts of free histones have also been shown to have negative effects. For example, histone turnover and degradation, specifically of H3 histones, is necessary for synaptic connectivity and behavioral plasticity.[299] At high altitude it would thus seems preferable to have a higher turnover rate with older histones being replaced and degraded, however, this is likely unachievable as there are mechanisms which slow free histone degradation when cell cycles are arrested, a common occurrence in hypoxia.[300] Many studies focus on the quantity and type of histone modifications, with total amount of histones not reported or used only as a control measure for the modifications. Despite this, it seems that the quantity of histones may play a significant role in hypoxia, likely playing a role in DNA damage repair, and should be investigated further.

Modifications

There was a significant increase in all measured H3 modifications at the first day of altitude, HA1, lowering on HA2, and returning to normal SL values on HA3 after acclimatization. This does corroborate results from other studies showing increased levels of modifications in human cell lines in response to hypoxia and hypoxia mimetics.[84,301–304]

While the histone code is still being deciphered, there are many well-known and studied histone modifications. We measured three of the most common histone modifications; H3K4me3, a marker of activation, as well as H3K9me3 and H3K27me3, markers frequently found in constitutively and facultatively repressed genes, respectively. These modifications rely on demethylases, namely KDM4A, KDM5A and KDM6A, which have been shown to be highly oxygen sensitive or even be able to directly sense oxygen and have reduced activity in hypoxia, resulting in hypermethylation of histone tails supporting our findings.[84,305,306] Individually these demethylases have been shown to play a role during hypoxia in fine-tuning transcriptional regulation.[306] These modifications also play a specific role in hypoxia. For example H3K4me3 is found in increased quantities at the promotional sites of hypoxic response genes, which is thought to prime chromatin for a better hypoxic response.[84] In addition, KDM6A which is responsible for demethylating H3K27, becomes inactive in hypoxia preventing demethylation and blocking downstream processes, effectively stopping cell differentiation.[85] The study is the first to confirm the increases in all three modifications in the same samples.

In addition to methylation, acetylation has also been shown to be changed both globally and at specific marks such as H3K9[307] and H3K14ac[303] in hypoxia. These increases in acetylation result in 'looser' and more accessible DNA. Acetylation has also been shown to increase in hypoxia in relation to genes that are targets of the hypoxia-response pathway.[308,309] While many of the modifications showed no significant differences from SL to HA, in our multiplex assay acetylation mark H3K56ac was shown to be higher in HA3 (2.32 ng/μg protein) as compared to SL (0.58 ng/μg protein) (**Figure 3**). This modification is a short-term mark which has associations with DNA damage repair and while it is surprising to still see it elevated at HA3 while other modifications fell back to SL values, it is likely that this increase is a remnant of the high altitude induced DNA damage and a testament of ongoing recovery.[310,311]

Conclusion

In conclusion, we saw large increases in total H3 protein and all measured histone modifications, as compared to sea level values, during acclimatization in HA1 and HA2. Increases in total H3 exceed proportional increases in other measured modifications, and it is likely that there is an overall increase in free H3 and H3 variant proteins helping to facilitate DNA double strand break repair processes. Additionally, the lack of significant changes found when comparing SL to HA3 shows that need for extreme histone modifications, as seen peaking in HA1 and coming back down on HA2, is short lived and in samples taken after acclimatization (HA3) they drop down to comparable sea level values.

Lastly, the profiles of long-term high-altitude residents showed patterns which differed from sojourners. While having comparable amounts of total H3 proteins, healthy participants (CDP), tended to have higher levels of histone modifications than our CMS participants. This led to CMS participants with lower percentages of histone modifications as compared to their healthy counterparts which could have interesting implications. The differences across histone modifications in acclimatizing sojourners and high-altitude residents suggest that there is a definite role being played by total histone and histone modifications in the adaptation to high altitude. While previous work has focused on isolated modifications in cell lines, this paper provides a groundbreaking glimpse into the time domains of histone modification patterns in humans at high altitudes. More work should be done to include additional modifications, additional high-altitude native groups, and further investigation on the changing amounts of total H3 histones.

This study was limited by the small numbers of modifications investigated; however, we performed a multiplex assay to include a larger number of modifications though only on one participant.

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Chapter 6:

Conclusion

The exploration through this comprehensive work has unveiled the complex interconnections between the challenging conditions seen at high-altitude and the multifaceted adaptations of the human body. From the foundational understanding of oxygen's role and the detrimental impacts of its scarcity leading to hypoxia, to a meticulous examination of the body's responses—ranging from hypoxic ventilatory responses (HVR) to cognitive functions, sleep patterns, and the profound influence of hypoxia on the epigenome—each facet adds a layer to our comprehension of human adaptation at high altitudes.

The introductory phase of this work underscored the importance of oxygen and illuminated the diverse effects of oxygen limitation (hypoxia) on human physiology over both acute and chronic exposures. The harsh high-altitude conditions have not only acted as selective pressures but have also played a pivotal role in shaping diverse phenotypes across different high-altitude native populations. While extensive research has traditionally focused on Andean and Tibetan high-altitude natives, the study emphasizes the necessity for a broader spectrum of research encompassing varied populations like Han Chinese sojourners and Ethiopian high-altitude residents. This broader approach could elucidate the intricate nuances of adaptations and provide a holistic understanding of how distinct phenotypes aid in adaptation.

Moreover, the exploration of 'newcomers' and residents in high-altitude cities like Denver, Colorado, could offer insight into the subtle yet significant changes occurring due to a lifetime's exposure. Genetic underpinnings, such as the *EPAS1* gene variants, have shown

associations with adaptations, yet the complexity of these adaptations often extends beyond genetic influences alone. The profound alterations in the epigenome due to hypoxemia offer promising avenues for understanding maladaptation and healthier adaptations, potentially paving the way for novel treatment modalities.

Delving deeper into specific chapters, the investigation into HVR uncovered significant decreases in ventilatory recruitment threshold being responsible for the increase ventilatory response during high altitude exposure. Similarly, the scrutiny of sleep patterns and cognition unveiled transient impairments influenced by high-altitude exposure, yet these impairments tended to recover with acclimatization or upon returning to sea level. Despite the documented decline in cognitive functions associated with hypoxia, attempts to mitigate these declines through mild hypercapnia did not yield supportive results.

The study of DNA methylation elucidated its substantial role in human adaptation to oxygen scarcity at high altitudes, showcasing genome-wide hypermethylation in response to high-altitude hypoxia. Notably, genes and pathways affected by significant DNA methylation changes correlated with known hypoxia-affected processes, emphasizing the need to dissect the roles of CpG sites in gene expression further.

The investigation into histone modifications revealed intriguing patterns, showcasing a surge in total H3 proteins and various modifications during acclimatization, particularly in comparison between sojourners and high-altitude residents. The differences observed in histone modifications across these groups underscore the significance of these epigenetic

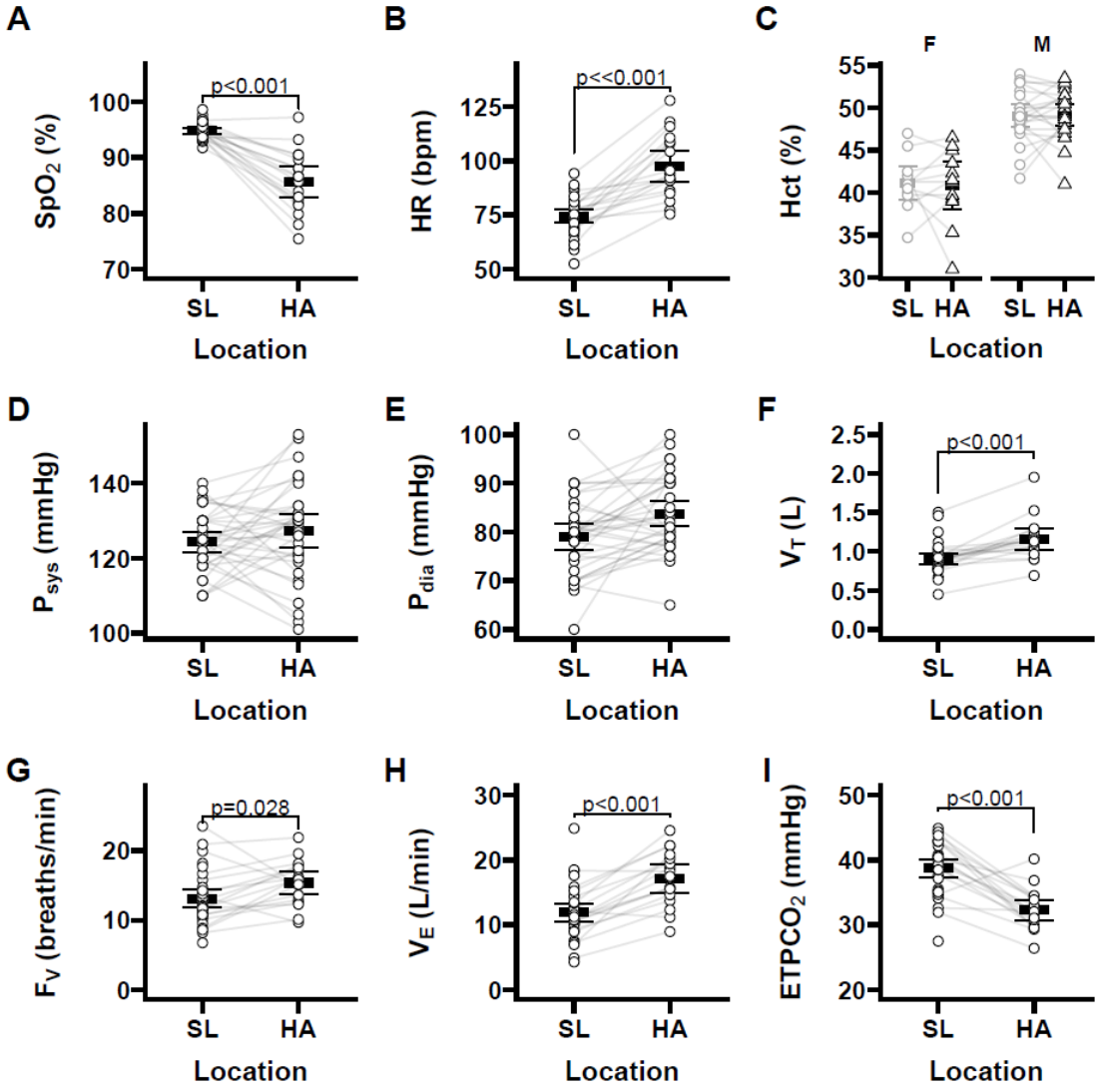
mechanisms in high-altitude adaptation, providing a nuanced understanding of their role in immediate and prolonged responses to hypoxia.

Synthesizing these diverse findings, it becomes apparent that the adaptive responses to high-altitude challenges are multifaceted, involving a dynamic interplay of histone modifications and DNA methylation. The swift adaptability of histone modifications appears to drive immediate phenotypic plasticity during acute exposures, while the slower yet enduring changes in DNA methylation contribute to more persistent adaptations.

As this comprehensive work draws to a close, it weaves a rich tapestry that integrates physiological, cognitive, and epigenetic responses to high-altitude challenges. Beyond expanding our comprehension of human adaptation, this interdisciplinary research underscores the profound interconnections between genetic, epigenetic, and physiological factors. These insights not only illuminate our current understanding but also pave the way for future targeted interventions and a deeper comprehension of high-altitude adaptations, holding promising implications for human health and well-being.

Appendix A:

Chapter 2 Supplementary Material



Supplemental Figure 1. Changes in Baseline Physiological Variables After Two Days of Acclimatization at 3800 m

Panels provide individual measures of each baseline variable, measured during rest. Significant changes are indicated. Hematocrit (Hct) values are separated by men and women due to the significant impact of sex on this variable. Lines connect measurements from the same participants. Group means are indicated by thick horizontal lines and error bars represent 95% confidence intervals.

Appendix B:

Chapter 3 Supplementary Material

Detailed Descriptions of Tests in the Cognition Test Battery:

Psychomotor vigilance task (PVT) tests vigilant attention by recruiting the prefrontal, motor, and visual cortices. This test features a black screen with one centered rectangle outlined in red. At random intervals, numbers (a timer) will appear inside the rectangle. The goal of the participant is to respond and click the screen as fast as possible when the timer appears. However, if a user clicks before the timer appears it counts as a false start.

Balloon analog risk task (BART) is a measure of the participants risk decision making and uses various parts of the brain including the orbital frontal cortex, amygdala, hippocampus, and the anterior cingulate cortex. In this test the user will see a total of 30 balloons. The user is awarded $1.00 per pump of air into the balloon and given the option to inflate a balloon or collect the winnings. The user is warned that each balloon may pop at random. If the balloon pops the rewards gained for the balloon cannot be collected. The aim is to collect the highest earnings in the shortest amount of time.

Digit symbol substitution task (DSST) tests complex scanning, mental flexibility, and visual tracking abilities. This test recruits the temporal, prefrontal, and motor cortices. The middle of the screen has a blue box that will display a symbol. Displayed along the bottom of the screen is a list of numbers along with corresponding symbols. The goal is to correctly hit the number which corresponds with the displayed symbol.

Line orientation task (LOT) is a test of spatial orientation, using the right temporo-parietal and visual cortices. Two lines appear on the screen, one is black, and one is blue with a circle in the middle of it. The black line is fixed and cannot be moved. The goal is to rotate the blue line so that it is parallel to the black line. This can be achieved by hitting a left arrow to rotate counterclockwise or a right arrow which rotates the line clockwise. The lines can vary in length and location.

NBack is a test of working memory which recruits functions from the dorsolateral prefrontal cortex, cingulate, and the hippocampus. In this test a series of differently colored fractal-like images are shown. The goal is to tap the screen when the current image matches the image shown two screens ago.

Visual object learning task (VOLT) assesses visual learning and spatial working memory using the medial temporal cortex. This task starts by showing the participant a series of 10 3-dimensional shapes. These shapes can be of different sizes and shading. The user is then shown a set of 20 shapes and asked to rank their remembrance of the shapes with varying degrees of certainty. The answers are either definitely yes or probably yes that they have seen this shape before, or probably no or definitely no that they have not seen this shape before.

Abstract matching (AM) measures abstraction ability testing the prefrontal cortex. Users are shown a shape and asked to pick the pair of shapes that fits best with that single shape. This can include different sets of shapes, colors, shading, and lining.

Motor praxis task (MPT) tests sensory motor speed using the sensorimotor cortex. In this test, boxes appear one at a time on the screen. The user must tap the box to make it disappear. The boxes get progressively smaller as more boxes are tapped.

More information and video demonstrations can be found at: https://admin.joggleresearch.com/Home/Task

Supplemental Table 1. Spearman's Correlations for Physiology and Sleep Data Associations with Cognitive Test Performance

Test	Measure	Day SpO_2	AMS	AHI	*Hypop. Index*	*Apnea Index*	*Central Apnea Index*	ODI	Avg. Night SpO_2	*T80*	Sleep Effic.	WASO	PROMIS	SSS
PVT	Mean reaction time	-0.119 (0.684)	0.126 (0.668)	0.433 (0.25)	0.367 (0.336)	0.251 (0.515)	0.525 (0.146)	0.524 (0.197)	0.566 (0.143)	-0.333 (0.428)	-0.358 (0.313)	0.079 (0.838)	**0.748 (0.002)**	-0.027 (0.926)
	Lapses	-0.208 (0.475)	0.276 (0.34)	0.436 (0.241)	0.333 (0.381)	0.316 (0.407)	0.513 (0.158)	0.577 (0.134)	0.553 (0.155)	-0.319 (0.441)	-0.499 (0.142)	0.075 (0.837)	**0.684 (0.007)**	-0.028 (0.924)
BART	Mean reaction time	0.084 (0.775)	-0.716 (0.004)	-0.3 (0.437)	-0.5 (0.178)	**-0.707 (0.033)**	0.186 (0.631)	-0.571 (0.151)	-0.675 (0.066)	**0.881 (0.007)**	0.224 (0.537)	-0.37 (0.296)	-0.257 (0.376)	-0.041 (0.89)
DSST	Mean reaction time	-0.44 (0.116)	0.171 (0.56)	-0.2 (0.613)	-0.267 (0.493)	-0.023 (0.954)	0.153 (0.695)	-0.238 (0.582)	-0.133 (0.754)	0.476 (0.243)	-0.394 (0.263)	-0.43 (0.218)	0.363 (0.202)	-0.186 (0.524)
	Correct responses	0.42 (0.135)	-0.169 (0.564)	0.151 (0.698)	0.21 (0.587)	-0.023 (0.953)	-0.171 (0.66)	0.193 (0.647)	0.03 (0.943)	-0.386 (0.346)	0.426 (0.22)	0.383 (0.275)	-0.397 (0.159)	0.145 (0.622)
LOT	Mean reaction time	0.477 (0.084)	-0.512 (0.061)	0.45 (0.23)	0.517 (0.162)	0.183 (0.638)	0.136 (0.728)	0.452 (0.267)	0.265 (0.526)	-0.048 (0.935)	-0.03 (0.946)	-0.164 (0.657)	-0.305 (0.288)	-0.204 (0.483)
	Correct responses	0.323 (0.261)	0.253 (0.383)	0.559 (0.117)	**0.695 (0.038)**	**0.743 (0.022)**	0.069 (0.86)	0.317 (0.444)	**0.753 (0.031)**	-0.586 (0.127)	-0.325 (0.359)	0.117 (0.748)	-0.21 (0.47)	0.005 (0.988)
NBACK	Mean reaction time	0.29 (0.315)	0.314 (0.274)	0.317 (0.41)	0.3 (0.437)	0.548 (0.127)	-0.237 (0.539)	0.69 (0.069)	**0.928 (<0.001)**	**-0.833 (0.015)**	**-0.648 (0.049)**	-0.103 (0.785)	0.442 (0.113)	0.466 (0.093)
	Correct responses	0.055 (0.853)	-0.347 (0.224)	0.209 (0.589)	0.31 (0.417)	0.16 (0.68)	-0.204 (0.598)	0.515 (0.192)	0.467 (0.244)	-0.467 (0.243)	-0.11 (0.763)	-0.5 (0.141)	0.127 (0.665)	0.247 (0.394)
VOLT	Mean reaction time	-0.033 (0.91)	0.054 (0.855)	-0.083 (0.843)	-0.417 (0.27)	-0.023 (0.954)	-0.051 (0.897)	-0.238 (0.582)	0.229 (0.586)	0.024 (0.977)	**-0.685 (0.035)**	-0.115 (0.759)	0.137 (0.64)	0.459 (0.099)
	Correct responses	0.327 (0.253)	0.053 (0.856)	**0.798 (0.01)**	**0.714 (0.031)**	**0.713 (0.031)**	0.299 (0.434)	**0.759 (0.029)**	**0.848 (0.008)**	**-0.892 (0.003)**	-0.372 (0.29)	0.354 (0.316)	0.253 (0.383)	0.343 (0.229)
AM	Mean reaction time	0.261 (0.368)	0.213 (0.464)	-0.3 (0.437)	-0.317 (0.41)	-0.16 (0.681)	-0.153 (0.695)	0 (>0.999)	0.012 (0.977)	-0.286 (0.501)	-0.018 (0.973)	0.345 (0.331)	-0.144 (0.624)	0.182 (0.534)
	Correct responses	-0.125 (0.67)	-0.306 (0.287)	-0.393 (0.295)	-0.536 (0.137)	-0.527 (0.145)	-0.136 (0.727)	-0.357 (0.389)	-0.337 (0.414)	0.286 (0.501)	0.146 (0.687)	-0.122 (0.737)	0.013 (0.964)	0.122 (0.678)
MPT	Mean reaction time	0.04 (0.893)	**-0.692 (0.006)**	-0.183 (0.644)	-0.4 (0.291)	**-0.707 (0.033)**	0.17 (0.663)	-0.19 (0.665)	-0.386 (0.346)	0.524 (0.197)	-0.103 (0.785)	-0.224 (0.537)	0.23 (0.429)	0.329 (0.25)

Appendix C:

Chapter 4 Supplementary Material

Supplemental 1

MS-HRM Primers

Gene Name	Sequence	Tm
EPAS1 – Region 1		
FORWARD	5'-AAGATTATATTGGGGAATTAGATTG-3'	58.7
REVERSE	5'- TCCCTCTCCCAACAAAAT-3'	59.7
EPAS1 – Region 2		
FORWARD	5'-GGTAGTGTTTTGAGATTGTATGG-3'	58.8
REVERSE	5'- AAAAAAACCCAAATTCCTTTT-3'	59.0
EPAS1 – Region 4		
FORWARD	5'- TGGTTTTTTATTTTGGGGTAGTA-3'	59.7
REVERSE	5'-ACTCTCCCCAAAATCAAAATAC-3'	59.6
EGN1 – cg14337165		
FORWARD	5'-TTAAATTTTTTATGGTGTTTGAATTA-3'	58.0
REVERSE	5'-ACCAAACTCTTCCTAAATACAAA-3'	57.2
EGN1 – cg16855929		
FORWARD	5'-TTTTGGTGGAAAGTATAGTTGT-3'	56.7
REVERSE	5'-ATCATTAAAAAACAACACCTACTT-3'	56.2
EGN1 – cg11637191		
FORWARD	5'-YGGGAAGATGGAGAATTTGT-3'	62.8
REVERSE	5'-AACTTATACTTCTTCCAATCCTAAC-3'	56.4
TLR4		
FORWARD	5'- TGTTGTTTATAGAAGTAGTGAGGATGAT-3'	58.0
REVERSE	5'- AAATTCAAAAAAACTAACTCCAACC-3'	58.3
EDN1		
FORWARD	5'- TTTTAATAGGGGTTTAATATAAAAAGT-3'	54.3
REVERSE	5'- CTTCAACCCAAATACCCTTTTAA-3'	55.0
EDNRA		
FORWARD	5'- TTTTAGGATAGTTGGAAGGTTAGGA-3'	58.7
REVERSE	5'- CTTCAAAAAACCTCCTAAACACTACTT-3'	58.9

Reference

1. Hodgskiss, M. S. W., Crockford, P. W., Peng, Y., Wing, B. A. & Horner, T. J. A productivity collapse to end Earth's Great Oxidation. *Proceedings of the National Academy of Sciences* **116**, 17207–17212 (2019).

2. Sessions, A. L., Doughty, D. M., Welander, P. V., Summons, R. E. & Newman, D. K. The Continuing Puzzle of the Great Oxidation Event. *Current Biology* **19**, R567–R574 (2009).

3. Rytkönen, K. T. Oxygen and early animals. *eLife* **7**, e34756.

4. Towe, K. M. Oxygen-collagen priority and the early metazoan fossil record. *Proc Natl Acad Sci U S A* **65**, 781–788 (1970).

5. Hedges, S. B., Blair, J. E., Venturi, M. L. & Shoe, J. L. A molecular timescale of eukaryote evolution and the rise of complex multicellular life. *BMC Evolutionary Biology* **4**, 2 (2004).

6. Bailey, D. M. Oxygen, evolution and redox signalling in the human brain; quantum in the quotidian. *J Physiol* **597**, 15–28 (2019).

7. Hampton-Smith, R. J. & Peet, D. J. From polyps to people: a highly familiar response to hypoxia. *Ann N Y Acad Sci* **1177**, 19–29 (2009).

8. Song, B., Modjewski, L. D., Kapust, N., Mizrahi, I. & Martin, W. F. The origin and distribution of the main oxygen sensing mechanism across metazoans. *Frontiers in Physiology* **13**, (2022).

9. Webb, K. L. *et al.* Influence of High Hemoglobin-Oxygen Affinity on Humans During Hypoxia. *Frontiers in Physiology* **12**, (2022).

10. Ulloa, N. A. & Cook, J. Altitude Induced Pulmonary Hypertension. in *StatPearls* (StatPearls Publishing, 2023).

11. Roach, R. C. *et al.* The 2018 lake louise acute mountain sickness score. *High Altitude Medicine and Biology* **19**, 4–6 (2018).

12. Peacock, A. J. Oxygen at high altitude. *BMJ* **317**, 1063–1066 (1998).

13. Gilbert, D. L. The first documented report of mountain sickness: the China or Headache Mountain story. *Respiration physiology* **52**, 315–26 (1983).

14. Luks, A. M., Swenson, E. R. & Bärtsch, P. Acute high-altitude sickness. *Eur Respir Rev* **26**, 160096 (2017).

15. Cogo, A. The lung at high altitude. *Multidiscip Respir Med* **6**, 14–15 (2011).

16. Frost, S. *et al.* Improvements in sleep-disordered breathing during acclimatization to 3800 m and the impact on cognitive function. *Physiol Rep* **9**, e14827 (2021).

17. Wang, H. *et al.* Sleep, short-term memory, and mood states of volunteers with increasing altitude. *Front Psychiatry* **13**, 952399 (2022).

18. Nation, D. A., Bondi, M. W., Gayles, E. & Delis, D. C. Mechanisms of Memory Dysfunction during High Altitude Hypoxia Training in Military Aircrew. *J Int Neuropsychol Soc* **23**, 1–10 (2017).

19. Cavaletti, G. & Tredici, G. Long-lasting neuropsychological changes after a single high altitude climb. *Acta Neurologica Scandinavica* **87**, 103–105 (1993).

20. Heinrich, E. C. *et al.* Cognitive function and mood at high altitude following acclimatization and use of supplemental oxygen and adaptive servoventilation sleep treatments. *PLoS ONE* **14**, (2019).

21. Asmaro, D., Mayall, J. & Ferguson, S. Cognition at altitude: impairment in executive and memory processes under hypoxic conditions. *Aviat Space Environ Med* **84**, 1159–1165 (2013).

22. Gekeler, K. *et al.* Decreased contrast sensitivity at high altitude. *Br J Ophthalmol* bjophthalmol-2018-313260 (2019) doi:10.1136/bjophthalmol-2018-313260.

23. Handler, J. Altitude-Related Hypertension. *J Clin Hypertens (Greenwich)* **11**, 161–165 (2009).

24. Kayser, B., Hoppeler, H., Claassen, H. & Cerretelli, P. Muscle structure and performance capacity of Himalayan Sherpas. *Journal of applied physiology (Bethesda, Md. : 1985)* **70**, 1938–42 (1991).

25. Fuhrmann, D. C. & Brüne, B. Mitochondrial composition and function under the control of hypoxia. *Redox Biology* vol. 12 208–215 Preprint at https://doi.org/10.1016/j.redox.2017.02.012 (2017).

26. Howald, H. & Hoppeler, H. Performing at extreme altitude: muscle cellular and subcellular adaptations. *Eur J Appl Physiol* **90**, 360–364 (2003).

27. Hoppeler, H. & Vogt, M. Muscle tissue adaptations to hypoxia. *J Exp Biol* **204**, 3133–3139 (2001).

28. Butterfield, G. E. *et al.* Increased energy intake minimizes weight loss in men at high altitude. *Journal of Applied Physiology* **72**, 1741–1748 (1992).

29. Torrance, J. D., Lenfant, C., Cruz, J. & Marticorena, E. Oxygen transport mechanisms in residents at high altitude. *Respiration Physiology* **11**, 1–15 (1970).

30. Cassin, S., Gilbert, R. D., Bunnell, C. E. & Johnson, E. M. *Capillary development during exposure to chronic hypoxia*. *AMERICAN JOURNAL OF PHYSIOLOGY* vol. 220 (1971).

31. Witt, K. E. & Huerta-Sánchez, E. Convergent evolution in human and domesticate adaptation to high-altitude environments. *Philosophical Transactions of the Royal Society B: Biological Sciences* vol. 374 Preprint at https://doi.org/10.1098/rstb.2018.0235 (2019).

32. Beall, C. M. Adaptations to Altitude: A Current Assessment. *Annual Review of Anthropology* **30**, 423–456 (2001).

33. Beall, C. M. Tibetan and Andean contrasts in adaptation to high-altitude hypoxia. in *Advances in Experimental Medicine and Biology* vol. 475 63–74 (2000).

34. Beall, C. M. Andean , Tibetan , and Ethiopian Patterns of Adaptation to High-Altitude Hypoxia. *Integrative and COmpartitive Biology* **46**, 18–24 (2006).

35. Beall, C. M. Two routes to functional adaptation: Tibetan and Andean high-altitude natives. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 8655–8660 (2007).

36. Xing, G. *et al.* Adaptation and mal-adaptation to ambient hypoxia; Andean, Ethiopian and Himalayan patterns. *PLoS ONE* **3**, (2008).

37. Zhuang, J. *et al.* Hypoxic ventilatory responsiveness in Tibetan compared with Han residents of 3,658 m. *Journal of Applied Physiology* **74**, 303–311 (1993).

38. Wilkins, A. S. *Between 'design' and 'bricolage': Genetic Networks, levels of selection, and adaptive evolution*. *In the Light of Evolution* vol. 1 (2007).

39. Pamenter, M. E. & Powell, F. L. Time Domains of the Hypoxic Ventilatory Response and Their Molecular Basis. *Compr Physiol2* **6**, 1345–1385 (2016).

40. Hochachka, P. W., Gunga, H. C. & Kirsch, K. Our ancestral physiological phenotype: An adaptation for hypoxia tolerance and for endurance performance? *Proceedings of the National Academy of Sciences of the United States of America* **95**, 1915–1920 (1998).

41. Bigham, A. W. & Lee, F. S. Human high-altitude adaptation: Forward genetics meets the HIF pathway. *Genes and Development* **28**, 2189–2204 (2014).

42. Bigham, A. W. Genetics of human origin and evolution: high-altitude adaptations. *Current Opinion in Genetics and Development* vol. 41 8–13 Preprint at https://doi.org/10.1016/j.gde.2016.06.018 (2016).

43. Brutsaert, T. D. Population genetic aspects and phenotypic plasticity of ventilatory responses in high altitude natives. *Respiratory Physiology and Neurobiology* **158**, 151–160 (2007).

44. Roach, G. D. *et al.* The sleep of elite athletes at sea level and high altitude: a comparison of sea-level natives and high-altitude natives (ISA3600). *Br J Sports Med* **47**, i114–i120 (2013).

45. Coote, J. H., Stone, B. M. & Tsang, G. Sleep of Andean high altitude natives. *Eur J Appl Physiol Occup Physiol* **64**, 178–181 (1992).

46. Heinrich, E. C. *et al.* Relationships Between Chemoreflex Responses, Sleep Quality, and Hematocrit in Andean Men and Women. *Frontiers in Physiology* **11**, (2020).

47. Davis, J. E. *et al.* Cognitive and psychomotor responses to high-altitude exposure in sea level and high-altitude residents of Ecuador. *J Physiol Anthropol* **34**, 2 (2015).

48. Hill, C. M. *et al.* Cognitive performance in high-altitude Andean residents compared with low-altitude populations: from childhood to older age. *Neuropsychology* **28**, 752–760 (2014).

49. Yan, X., Zhang, J., Gong, Q. & Weng, X. Adaptive influence of long term high altitude residence on spatial working memory: An fMRI study. *Brain and Cognition* **77**, 53–59 (2011).

50. Prabhakar, N. R. Sensing hypoxia: Physiology, genetics and epigenetics. *Journal of Physiology* vol. 591 2245–2257 Preprint at https://doi.org/10.1113/jphysiol.2012.247759 (2013).

51. Julian, C. G. Epigenomics and human adaptation to high altitude. *Journal of Applied Physiology* **123**, 1362–1370 (2017).

52. Grimminger, J. *et al.* Thin Air Resulting in High Pressure: Mountain Sickness and Hypoxia-Induced Pulmonary Hypertension. *Canadian Respiratory Journal* vol. 2017 Preprint at https://doi.org/10.1155/2017/8381653 (2017).

53. Villafuerte, F. C. & Corante, N. Chronic Mountain Sickness: Clinical Aspects, Etiology, Management, and Treatment. *High altitude medicine & biology* **17**, 61–9 (2016).

54. Villafuerte, F. C. New genetic and physiological factors for excessive erythrocytosis and Chronic Mountain Sickness. *J Appl Physiol* **119**, 1481–1486 (2015).

55. Alkorta-Aranburu, G. *et al.* The Genetic Architecture of Adaptations to High Altitude in Ethiopia. *PLoS Genetics* **8**, (2012).

56. Semenza, G. L. Regulation of Mammalian O 2 Homeostasis by Hypoxia-Inducible Factor 1. *Annual Review of Cell and Developmental Biology* **15**, 551–578 (1999).

57. Wenger, R. H., Stiehl, D. P. & Camenisch, G. Integration of oxygen signaling at the consensus HRE. *Science's STKE : signal transduction knowledge environment* vol. 2005 Preprint at https://doi.org/10.1126/stke.3062005re12 (2005).

58. Moore, L. G. Measuring high-altitude adaptation. *Journal of Applied Physiology* vol. 123 1371–1385 Preprint at https://doi.org/10.1152/japplphysiol.00321.2017 (2017).

59. Cheong, H. I. *et al.* Alternative hematological and vascular adaptive responses to high-altitude hypoxia in East African highlanders. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **312**, L172–L177 (2017).

60. Brown, C. J. & Rupert, J. L. Hypoxia and Environmental Epigenetics. *High Altitude Medicine & Biology* **15**, 323–330 (2014).

61. Jha, A. R. *et al.* Shared genetic signals of hypoxia adaptation in drosophila and in high-altitude human populations. *Molecular Biology and Evolution* **33**, 501–517 (2016).

62. Schödel, J. *et al.* High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. *Blood* **117**, (2011).

63. Watson, J. A., Watson, C. J., Mccann, A. & Baugh, J. Epigenetics, the epicenter of the hypoxic response. *Epigenetics* **5**, 293–296 (2010).

64. Ratcliffe, P. J. HIF-1 and HIF-2: Working alone or together in hypoxia? *Journal of Clinical Investigation* vol. 117 862–865 Preprint at https://doi.org/10.1172/JCI31750 (2007).

65. Loboda, A., Jozkowicz, A. & Dulak, J. HIF-1 versus HIF-2 - Is one more important than the other? *Vascular Pharmacology* vol. 56 245–251 Preprint at https://doi.org/10.1016/j.vph.2012.02.006 (2012).

66. Semenza, G. L. Regulation of Oxygen Homeostasis by Hypoxia-Inducible Factor 1. *Physiology* **24**, 97–106 (2009).

67. Majmundar, A. J., Wong, W. J. & Simon, M. C. Hypoxia-Inducible Factors and the Response to Hypoxic Stress. *Molecular Cell* vol. 40 294–309 Preprint at https://doi.org/10.1016/j.molcel.2010.09.022 (2010).

68. Simonson, T. S. *et al. Genetic Evidence for High-Altitude Adaptation in Tibet*.

69. Hanaoka, M. *et al.* Genetic Variants in EPAS1 Contribute to Adaptation to High-Altitude Hypoxia in Sherpas. *PLoS ONE* **7**, (2012).

70. Cammack, R. C. *et al.* epigenetics. in *Oxford Dictionary of Biochemistry and Molecular Biology* (Oxford University Press, 2006).

71. Ashe, A., Colot, V. & Oldroyd, B. P. How does epigenetics influence the course of evolution? *rstb* **376**, (2021).

72. Turner, B. M. Epigenetic responses to environmental change and their evolutionary implications. *Philos Trans R Soc Lond B Biol Sci* **364**, 3403–3418 (2009).

73. Luo, X. *et al.* Epigenetic Mechanisms Contribute to Evolutionary Adaptation of Gene Network Activity under Environmental Selection. *Cell Rep* **33**, 108306 (2020).

74. Osborne, A. The role of epigenetics in human evolution. *Bioscience Horizons: The International Journal of Student Research* **10**, hzx007 (2017).

75. Lind, M. I. & Spagopoulou, F. Evolutionary consequences of epigenetic inheritance. *Heredity* **121**, 205–209 (2018).

76. Zuo, T., Tycko, B., Liu, T. M., Lin, H. J. L. & Huang, T. H. M. Methods in DNA methylation profiling. *Epigenomics* vol. 1 331–345 Preprint at https://doi.org/10.2217/EPI.09.31 (2009).

77. Moosavi, A. & Ardekani, A. M. Role of epigenetics in biology and human diseases. *Iranian Biomedical Journal* vol. 20 246–258 Preprint at https://doi.org/10.22045/ibj.2016.01 (2016).

78. Mukherjee, K. & Dobrindt, U. The emerging role of epigenetic mechanisms in insect defense against pathogens. *Current Opinion in Insect Science* **49**, 8–14 (2022).

79. Childebayeva, A. *et al.* DNA Methylation Changes Are Associated With an Incremental Ascent to High Altitude. *Front. Genet.* **10**, 1062 (2019).

80. Childebayeva, A. *et al.* Genome-Wide DNA Methylation Changes Associated With High-Altitude Acclimatization During an Everest Base Camp Trek. *Frontiers in Physiology* **12**, (2021).

81. Childebayeva, A. *et al.* LINE-1 and EPAS1 DNA methylation associations with high-altitude exposure. *Epigenetics* **14**, 1–15 (2019).

82. Antero, S., Kai, K. & Anu, K. Hypoxia-Inducible Histone Lysine Demethylases: Impact on the Aging Process and Age-Related Diseases. *Aging and disease* **7**, 180 (2016).

83. Yang, J. *et al.* The histone demethylase JMJD2B is regulated by estrogen receptor α and hypoxia, and is a key mediator of estrogen induced growth. *Cancer Research* **70**, 6456–6466 (2010).

84. Batie, M. *et al.* Hypoxia induces rapid changes to histone methylation and reprograms chromatin. *Science* **363**, 1222–1226 (2019).

85. Chakraborty, A. A. *et al.* Histone demethylase KDM6A directly senses oxygen to control chromatin and cell fate. *Science* **363**, 1217–1222 (2019).

86. Adriaens, M. E. *et al.* Quantitative analysis of ChIP-seq data uncovers dynamic and sustained H3K4me3 and H3K27me3 modulation in cancer cells under hypoxia. *Epigenetics & Chromatin* **9**, 48 (2016).

87. Nanduri, J., Semenza, G. L., Nanduri, X. & Prabhakar, R. Epigenetic changes by DNA methylation in chronic and intermittent hypoxia. *REVIEW Physiology in Medicine Am J Physiol Lung Cell Mol Physiol* **313**, 1096–1100 (2017).

88. Johnson, A. B., Denko, N. & Barton, M. C. Hypoxia induces a novel signature of chromatin modifications and global repression of transcription. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis* **640**, 174–179 (2008).

89. Kallio, P. J. Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. *The EMBO Journal* **17**, 6573–6586 (1998).

90. Pamenter, M. E. & Powell, F. L. Time Domains of the Hypoxic Ventilatory Response and Their Molecular Basis. in *Comprehensive Physiology* (ed. Terjung, R.) 1345–1385 (Wiley, 2016). doi:10.1002/cphy.c150026.

91. Phillipson, E. A., Bowes, G., Townsend, E. R., Duffin, J. & Cooper, J. D. Carotid chemoreceptors in ventilatory responses to changes in venous CO2 load. *Journal of Applied Physiology* **51**, 1398–1403 (1981).

92. Oeung, B. *et al.* The normal distribution of the hypoxic ventilatory response and methodological impacts: a meta-analysis and computational investigation. *The Journal of Physiology* **601**, 4423–4440 (2023).

93. Duffin, J. Measuring the ventilatory response to hypoxia: Measuring ventilatory response to hypoxia. *The Journal of Physiology* **584**, 285–293 (2007).

94. Fan, J.-L. *et al.* Influence of high altitude on cerebrovascular and ventilatory responsiveness to CO_2: Alterations in cerebrovascular CO_2 reactivity following ascent to high altitude. *The Journal of Physiology* **588**, 539–549 (2010).

95. Basaran, K. E. *et al.* Ibuprofen blunts ventilatory acclimatization to sustained hypoxia in humans. *PLoS ONE* **11**, 1–10 (2016).

96. Boulet, L. M. *et al.* Influence of prior hyperventilation duration on respiratory chemosensitivity and cerebrovascular reactivity during modified hyperoxic rebreathing: Hyperventilation duration and responses to rebreathing. *Exp Physiol* **101**, 821–835 (2016).

97. Weissman, C., Askanazi, J., Milic-Emili, J. & Kinney, J. M. Effect of respiratory apparatus on respiration. *Journal of Applied Physiology Respiratory Environmental and Exercise Physiology* **57**, 475–480 (1984).

98. Askanazi, J. *et al.* Effects of respiratory apparatus on breathing pattern. *Journal of Applied Physiology Respiratory Environmental and Exercise Physiology* **48**, 577–580 (1980).

99. Askanazi, J. *et al.* Effects of the mask and mouthpiece plus noseclip on spontaneous breathing pattern. *Critical Care Medicine* **6**, 143–146 (1978).

100. Hirsch, J. A. & Bishop, B. Human breathing patterns on mouthpiece or face mask during air, CO2, or low O2. *Journal of Applied Physiology Respiratory Environmental and Exercise Physiology* **53**, 1281–1290 (1982).

101. Sato, M., Severinghaus, J. W., Powell, F. L., Xu, F.-D. & Spellman, M. J. Augmented hypoxic ventilatory response in men at altitude. *Journal of Applied Physiology* **73**, 101–107 (1992).

102. Insalaco, G. *et al.* Cardiovascular and ventilatory response to isocapnic hypoxia at sea level and at 5,050 m. *Journal of Applied Physiology* **80**, 1724–1730 (1996).

103. Bärtsch, P., Swenson, E. R., Paul, A., Jülg, B. & Hohenhaus, E. Hypoxic Ventilatory Response, Ventilation, Gas Exchange, and Fluid Balance in Acute Mountain Sickness. *High Altitude Medicine & Biology* **3**, 361–376 (2002).

104. Fan, J.-L. *et al.* AltitudeOmics: enhanced cerebrovascular reactivity and ventilatory response to CO_2 with high-altitude acclimatization and reexposure. *Journal of Applied Physiology* **116**, 911–918 (2014).

105. Sato, M., Severinghaus, J. W., Powell, F. L., Xu, F. D. & Spellman, M. J. Augmented hypoxic ventilatory response in men at altitude. *J Appl Physiol (1985)* **73**, 101–107 (1992).

106. Powell, F. L., Huey, K. A. & Dwinell, M. R. Central nervous system mechanisms of ventilatory acclimatization to hypoxia. *Respiration Physiology* **121**, 223–236 (2000).

107. Joseph, V. & Pequignot, J.-M. Breathing at high altitude. *Cell. Mol. Life Sci.* **66**, 3565–3573 (2009).

108. Mohan, R. & Duffin, J. The effect of hypoxia on the ventilatory response to carbon dioxide in man. *Respiration Physiology* **108**, 101–115 (1997).

109. Somogyi, R. B., Preiss, D., Vesely, A., Fisher, J. A. & Duffin, J. Changes in respiratory control after 5 days at altitude. *Respiratory Physiology & Neurobiology* **145**, 41–52 (2005).

110. Slessarev, M. *et al.* Differences in the control of breathing between Andean highlanders and lowlanders after 10 days acclimatization at 3850 m: Respiratory control at altitude. *The Journal of Physiology* **588**, 1607–1621 (2010).

111. Duffin, J. Role of acid-base balance in the chemoreflex control of breathing. *Journal of Applied Physiology* **99**, 2255–2265 (2005).

112. Slessarev, M. *et al.* Differences in the control of breathing between Himalayan and sea-level residents: Respiratory control at altitude. *The Journal of Physiology* **588**, 1591–1606 (2010).

113. Virués-Ortega, J., Buela-Casal, G., Garrido, E. & Alcázar, B. Neuropsychological functioning associated with high-altitude exposure. *Neuropsychology Review* **14**, 197–224 (2004).

114. Yan, X. Cognitive impairments at high altitudes and adaptation. *High Altitude Medicine and Biology* **15**, 141–145 (2014).

115. Mackintosh, J. H., Thomas, D. J., Olive, J. E., Chesner, I. M. & Knight, R. J. The Effect of Altitude on Tests of Reaction Time and Alertness. *Aviat Space Environ Med* **59**, 246–248 (1988).

116. Kramer, A. F., Coyne, J. T. & Strayer, D. L. Cognitive function at high altitude. *Human Factors* **35**, 329–344 (1993).

117. Bolmont, B., Thullier, F. & Abraini, J. H. Relationships between mood states and performances in reaction time, psychomotor ability, and mental efficiency during a 31-day gradual decompression in a hypobaric chamber from sea level to 8848 m equivalent altitude. *Physiology and Behavior* **71**, 469–476 (2000).

118. Petrassi, F. A., Hodkinson, P. D., Walters, P. L. & Gaydos, S. J. Hypoxic hypoxia at moderate altitudes: Review of the state of the science. *Aviation Space and Environmental Medicine* **83**, 975–984 (2012).

119. Phillips, J. B., Hørning, D. & Funke, M. E. Cognitive and perceptual deficits of normobaric hypoxia and the time course to performance recovery. *Aerospace Medicine and Human Performance* **86**, 357–365 (2015).

120. Malle, C. *et al.* Physiological and Cognitive Effects of Acute Normobaric Hypoxia and Modulations from Oxygen Breathing. *Aerospace Medicine and Human Performance* **87**, 3–12 (2016).

121. Regard, M. *et al.* Cognitive changes at high altitude in healthy climbers and in climbers developing acute mountain sickness. *Aviation, Space, and Environmental Medicine* **62**, 291–295 (1991).

122. Shukitt-Hale, B., Banderet, L. E. & Lieberman, H. R. Relationships between symptoms, moods, performance, and acute mountain sickness at 4,700 meters. *Aviation, Space, and Environmental Medicine* **62**, 865–869 (1991).

123. Issa, A. N. *et al.* Association of Cognitive Performance with Time at Altitude, Sleep Quality, and Acute Mountain Sickness Symptoms. *Wilderness and Environmental Medicine* **27**, 371–378 (2016).

124. Phillips, L., Basnyat, B., Chang, Y., Swenson, E. R. & Harris, N. S. Findings of Cognitive Impairment at High Altitude: Relationships to Acetazolamide Use and Acute Mountain Sickness. *High Altitude Medicine and Biology* **18**, 121–127 (2017).

125. Latshang, T. D. *et al.* Are Nocturnal Breathing, Sleep, and Cognitive Performance Impaired at Moderate Altitude (1,630–2,590 m)? *Sleep* **36**, 1969–1976 (2013).

126. Olaithe, M., Bucks, R. S., Hillman, D. R. & Eastwood, P. R. Cognitive deficits in obstructive sleep apnea: Insights from a meta-review and comparison with deficits observed in COPD, insomnia, and sleep deprivation. *Sleep Medicine Reviews* **38**, 39–49 (2018).

127. Nussbaumer-Ochsner, Y., Ursprung, J., Siebenmann, C., Maggiorini, M. & Bloch, K. E. Effect of Short-Term Acclimatization to High Altitude on Sleep and Nocturnal Breathing. *Sleep* **35**, 419–423 (2012).

128. Ainslie, P. N., Lucas, S. J. E. & Burgess, K. R. Breathing and sleep at high altitude. *Respiratory Physiology and Neurobiology* **188**, 233–256 (2013).

129. White, D. P. *et al.* Altitude acclimatization: Influence on periodic breathing and chemoresponsiveness during sleep. *Journal of Applied Physiology* **63**, 401–412 (1987).

130. Bloch, K. E. *et al.* Nocturnal periodic breathing during acclimatization at very high altitude at Mount Muztagh Ata (7,546 m). *American Journal of Respiratory and Critical Care Medicine* **182**, 562–568 (2010).

131. Andrews, G. *et al.* The effect of partial acclimatization to high altitude on loop gain and central sleep apnoea severity. *Respirology* **17**, 835–840 (2012).

132. Insalaco, G. *et al.* Periodic breathing, arterial oxyhemoglobin saturation, and heart rate during sleep at high altitude. *High Altitude Medicine and Biology* **13**, 258–262 (2012).

133. Burgess, K. R. *et al.* Worsening of central sleep apnea at high altitude - A role for cerebrovascular function. *Journal of Applied Physiology* **114**, 1021–1028 (2013).

134. Tseng, C. H. *et al.* Impact of rapid ascent to high altitude on sleep. *Sleep and Breathing* **19**, 819–826 (2015).

135. Horiuchi, M. *et al.* Effects of Short-Term Acclimatization at the Summit. *High Altitude Medicine & Biology* **18**, 171–178 (2017).

136. Wickramasinghe, H. & Anholm, J. D. Sleep and Breathing at High Altitude. **3**, (1999).

137. Weil, J. V. Sleep at high altitude. *High Altitude Medicine and Biology* **5**, 180–189 (2004).

138. Pun, M. *et al.* Effect of acute, subacute, and repeated exposure to high altitude (5050 m) on psychomotor vigilance. *Frontiers in Physiology* **9**, 1–13 (2018).

139. Ulrich, S. *et al.* The 2018 Lake Louise Acute Mountain Sickness Score. *High Altitude Medicine & Biology* **19**, 4–6 (2018).

140. Smyth, C. The Pittsburgh Sleep Quality Index (PSQI). *Journal of Gerontological Nursing* **25**, (2011).

141. Hupperets, M. D. W. *et al.* Increased hypoxic ventilatory response during 8 weeks at 3800 m altitude. *Respiratory Physiology and Neurobiology* **142**, 145–152 (2004).

142. Pamenter, M. E. & Powell, F. L. Time Domains of the Hypoxic Ventilatory Response and Their Molecular Basis. *Comprehensive Physiology* **6**, 1345–1385 (2016).

143. Slessarev, M. *et al.* Differences in the control of breathing between Andean highlanders and lowlanders after 10 days acclimatization at 3850 m. *The Journal of physiology* **588**, 1607–21 (2010).

144. Orr, J. E., Malhotra, A. & Sands, S. A. Pathogenesis of central and complex sleep apnoea. *Respirology* vol. 22 43–52 Preprint at https://doi.org/10.1111/resp.12927 (2017).

145. Sampson, J. B., Cymerman, A., Burse, R. L., Maher, J. T. & Rock, P. B. Procedures for the measurement of acute mountain sickness. *Aviation, space, and environmental medicine* **54**, 1063—1073 (1983).

146. Pighin, S., Bonini, N., Hadjichristidis, C. & Savadori, L. Decision making under stress : mild hypoxia leads to increased risk-taking. *Stress* **23**, 290–297 (2020).

147. Frost, S. *et al.* Improvements in sleep-disordered breathing during acclimatization to 3800 m and the impact on cognitive function. *Physiol Rep* **9**, e14827 (2021).

148. Heinrich, E. C. *et al.* Cognitive function and mood at high altitude following acclimatization and use of supplemental oxygen and adaptive servoventilation sleep treatments. *PLOS ONE* **14**, e0217089 (2019).

149. de Aquino Lemos, V. *et al.* High altitude exposure impairs sleep patterns, mood, and cognitive functions. *Psychophysiology* **49**, 1298–1306 (2012).

150. McMorris, T., Hale, B. J., Barwood, M., Costello, J. & Corbett, J. Effect of acute hypoxia on cognition: A systematic review and meta-regression analysis. *Neuroscience & Biobehavioral Reviews* **74**, 225–232 (2017).

151. Virués-Ortega, J., Buela-Casal, G., Garrido, E. & Alcázar, B. Neuropsychological Functioning Associated with High-Altitude Exposure. *Neuropsychol Rev* **14**, 197–224 (2004).

152. Kramer, A. F., Coyne, J. T. & Strayer, D. L. Cognitive Function at High Altitude. *Hum Factors* **35**, 329–344 (1993).

153. Fan, J.-L. *et al.* Influence of high altitude on cerebrovascular and ventilatory responsiveness to CO2. *J Physiol* **588**, 539–549 (2010).

154. Pamenter, M. E. & Powell, F. L. Time Domains of the Hypoxic Ventilatory Response and Their Molecular Basis. *Compr Physiol* **6**, 1345–1385 (2016).

155. Ainslie, P. N. & Subudhi, A. W. Cerebral Blood Flow at High Altitude. *High Altitude Medicine & Biology* **15**, 133–140 (2014).

156. Hornbein, T. F., Townes, B. D., Schoene, R. B., Sutton, J. R. & Houston, C. S. The cost to the central nervous system of climbing to extremely high altitude. *N Engl J Med* **321**, 1714–1719 (1989).

157. Basaran, K. E. *et al.* Ibuprofen Blunts Ventilatory Acclimatization to Sustained Hypoxia in Humans. *PLOS ONE* **11**, e0146087 (2016).

158. Roach, R. C. *et al.* The 2018 Lake Louise Acute Mountain Sickness Score. *High Alt Med Biol* **19**, 4–6 (2018).

159. Pahor, A. *et al.* UCancellation: A new mobile measure of selective attention and concentration. *Behav Res* **54**, 2602–2617 (2022).

160. Gallun, F., Diedesch, A., Kampel, S. & Jakien, K. Independent impacts of age and hearing loss on spatial release in a complex auditory environment. *Frontiers in Neuroscience* **7**, (2013).

161. Gallun, F. J. *et al.* Development and validation of Portable Automated Rapid Testing (PART) measures for auditory research. *Proceedings of Meetings on Acoustics* **33**, 050002 (2018).

162. De Sousa, K. C., Swanepoel, D. W., Moore, D. R., Myburgh, H. C. & Smits, C. Improving Sensitivity of the Digits-In-Noise Test Using Antiphasic Stimuli. *Ear Hear* **41**, 442–450 (2020).

163. Thurman, S. M., Davey, P. G., McCray, K. L., Paronian, V. & Seitz, A. R. Predicting individual contrast sensitivity functions from acuity and letter contrast sensitivity measurements. *J Vis* **16**, 15 (2016).

164. Rizzo, M., Anderson, S. W., Dawson, J. & Nawrot, M. Vision and cognition in Alzheimer's disease. *Neuropsychologia* **38**, 1157–1169 (2000).

165. Neher, T., Laugesen, S., Søgaard Jensen, N. & Kragelund, L. Can basic auditory and cognitive measures predict hearing-impaired listeners' localization and spatial speech recognition abilities?a). *The Journal of the Acoustical Society of America* **130**, 1542–1558 (2011).

166. Fintor, E., Aspöck, L., Fels, J. & Schlittmeier, S. J. The role of spatial separation of two talkers' auditory stimuli in the listener's memory of running speech: listening effort in a non-noisy conversational setting. *International Journal of Audiology* **61**, 371–379 (2022).

167. Edwards, B. A Model of Auditory-Cognitive Processing and Relevance to Clinical Applicability. *Ear and Hearing* **37**, 85S (2016).

168. Godwin, K. E. *et al.* The elusive relationship between time on-task and learning: not simply an issue of measurement. *Educational Psychology* **41**, 502–519 (2021).

169. Pun, M. *et al.* Effects on Cognitive Functioning of Acute, Subacute and Repeated Exposures to High Altitude. *Frontiers in Physiology* **9**, (2018).

170. Yan, X. Cognitive impairments at high altitudes and adaptation. *High Alt Med Biol* **15**, 141–145 (2014).

171. Pescosolido, N., Barbato, A. & Di Blasio, D. Hypobaric Hypoxia: Effects on Contrast Sensitivity in High Altitude Environments. *Aerospace Medicine and Human Performance* **86**, 118–124 (2015).

172. Connolly, D. M. Spatial Contrast Sensitivity at Twilight: Luminance, Monocularity, and Oxygenation. *Aviation, Space, and Environmental Medicine* **81**, 475–483 (2010).

173. Shukitt-Hale, B., Banderet, L. E. & Lieberman, H. R. Elevation-Dependent Symptom, Mood, and Performance Changes Produced by Exposure to Hypobaric Hypoxia. *The International Journal of Aviation Psychology* **8**, 319–334 (1998).

174. Li, X. Y. *et al.* Effects of acute exposure to mild or moderate hypoxia on human psychomotor performance and visual-reaction time. *Space Med Med Eng (Beijing)* **13**, 235–239 (2000).

175. Muthuraju, S. & Pati, S. Effect of Hypobaric Hypoxia on Cognitive Functions and Potential Therapeutic Agents. *Malays J Med Sci* **21**, 41–45 (2014).

176. Zhang, D. *et al.* Exploring the impact of chronic high-altitude exposure on visual spatial attention using the ERP approach. *Brain Behav* **8**, e00944 (2018).

177. Falla, M. *et al.* A Prospective Evaluation of the Acute Effects of High Altitude on Cognitive and Physiological Functions in Lowlanders. *Front Physiol* **12**, 670278 (2021).

178. Van Dorp, E. *et al.* Inspired carbon dioxide during hypoxia: effects on task performance and cerebral oxygen saturation. *Aviat Space Environ Med* **78**, 666–672 (2007).

179. Kung, S.-C., Shen, Y.-C., Chang, E.-T., Hong, Y.-L. & Wang, L.-Y. Hypercapnia impaired cognitive and memory functions in obese patients with obstructive sleep apnoea. *Sci Rep* **8**, 17551 (2018).

180. Beaudin, A. E. *et al.* Contribution of hypercapnia to cognitive impairment in severe sleep-disordered breathing. *Journal of Clinical Sleep Medicine* **18**, 245–254 (2022).

181. Sato, M., Severinghaus, J. W. & Bickler, P. Time course of augmentation and depression of hypoxic ventilatory responses at altitude. *Journal of Applied Physiology* **77**, 313–316 (1994).

182. Sato, M., Severinghaus, J. W., Powell, F. L., Xu, F.-D. & Spellman, M. J. Augmented hypoxic ventilatory response in men at altitude. *Journal of Applied Physiology* **73**, 101–107 (1992).

183. Duffin, J., Bechbache, R. R., Goode, R. C. & Chung, S. A. *THE VENTILATORY RESPONSE TO CARBON DIOXID~E IN HYPEROXIC EXERCISE*. *Respiration Physiology* vol. 40 (1980).

184. Banchero, N. Cardiovascular Responses to Chronic Hypoxia. *Ann Rev Physiol* **49**, 465–476 (1987).

185. Neill, W. A. Myocardial hypoxia and anaerobic metabolism in coronary heart disease. *The American Journal of Cardiology* **22**, 507–515 (1968).

186. Richard, N. A. *et al.* Acute mountain sickness, chemosensitivity, and cardiorespiratory responses in humans exposed to hypobaric and normobaric hypoxia. *Journal of Applied Physiology* **116**, 945–952 (2014).

187. Deaton, A. M. & Bird, A. CpG islands and the regulation of transcription. *Genes & Development* **25**, 1010 (2011).

188. Choi, J. K. Contrasting chromatin organization of CpG islands and exons in the human genome. *Genome Biology* **11**, (2010).

189. Laurent, L. *et al.* Dynamic changes in the human methylome during differentiation. *Genome Res* **20**, 320–331 (2010).

190. Rimoldi, M. *et al.* DNA methylation patterns of transcription factor binding regions characterize their functional and evolutionary contexts. 2022.07.21.500978 Preprint at https://doi.org/10.1101/2022.07.21.500978 (2022).

191. Julian, C. G. An Aptitude for Altitude: Are Epigenomic Processes Involved? *Frontiers in Physiology* **10**, 1397 (2019).

192. Brown, C. J. & Rupert, J. L. Hypoxia and Environmental Epigenetics. *High Altitude Medicine & Biology* **15**, 323–330 (2014).

193. Hancock, R. L., Dunne, K., Walport, L. J., Flashman, E. & Kawamura, A. Epigenetic regulation by histone demethylases in hypoxia. *Epigenomics* vol. 7 791–811 (2015).

194. Nanduri, J., Semenza, G. L., Nanduri, X. & Prabhakar, R. Epigenetic changes by DNA methylation in chronic and intermittent hypoxia. *REVIEW Physiology in Medicine Am J Physiol Lung Cell Mol Physiol* **313**, 1096–1100 (2017).

195. Thienpont, B. *et al.* Tumour hypoxia causes DNA hypermethylation by reducing TET activity. *Nature* **537**, 63–68 (2016).

196. Yang, Q. *et al.* Hypoxia switches TET1 from being tumor-suppressive to oncogenic. *Oncogene* **42**, 1634–1648 (2023).

197. Basaran, K. E. *et al.* Ibuprofen Blunts Ventilatory Acclimatization to Sustained Hypoxia in Humans. *PLoS ONE* **11**, e0146087 (2016).

198. Volpe, M. & Das, J. methylR: a graphical interface for comprehensive DNA methylation array data analysis. *Bioinformatics* **39**, btad184 (2023).

199. Tian, Y. *et al.* ChAMP: updated methylation analysis pipeline for Illumina BeadChips. *Bioinformatics* **33**, 3982–3984 (2017).

200. Welsh, H. *et al.* A systematic evaluation of normalization methods and probe replicability using infinium EPIC methylation data. *Clinical Epigenetics* **15**, 41 (2023).

201. Wang, M. *et al.* COMBAT: A Combined Association Test for Genes Using Summary Statistics. *Genetics* **207**, 883–891 (2017).

202. Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research* **43**, e47 (2015).

203. Jaffe, A. E. *et al.* Bump hunting to identify differentially methylated regions in epigenetic epidemiology studies. *International Journal of Epidemiology* **41**, 200–209 (2012).

204. Rechache, N. S. *et al.* DNA Methylation Profiling Identifies Global Methylation Differences and Markers of Adrenocortical Tumors. *J Clin Endocrinol Metab* **97**, E1004–E1013 (2012).

205. Martino, D. & Saffery, R. Characteristics of DNA methylation and gene expression in regulatory features on the Infinium 450k Beadchip. (2015) doi:10.1101/032862.

206. Gillespie, M. *et al.* The reactome pathway knowledgebase 2022. *Nucleic Acids Research* **50**, D687–D692 (2022).

207. Bak, M. *et al.* Genome-wide DNA methylation analysis of transient neonatal diabetes type 1 patients with mutations in ZFP57. *BMC Med Genet* **17**, 29 (2016).

208. Mackay, D. J. G. *et al.* Hypomethylation of multiple imprinted loci in individuals with transient neonatal diabetes is associated with mutations in ZFP57. *Nat Genet* **40**, 949–951 (2008).

209. Hirasawa, R. & Feil, R. A KRAB domain zinc finger protein in imprinting and disease. *Dev Cell* **15**, 487–488 (2008).

210. Kuang, E. *et al.* Regulation of ATG4B Stability by RNF5 Limits Basal Levels of Autophagy and Influences Susceptibility to Bacterial Infection. *PLOS Genetics* **8**, e1003007 (2012).

211. Dunn, J., Thabet, S. & Jo, H. Flow-dependent epigenetic DNA methylation in endothelial gene expression and atherosclerosis. *Arterioscler Thromb Vasc Biol* **35**, 1562–1569 (2015).

212. Fan, F. *et al.* HOXA5: A crucial transcriptional factor in cancer and a potential therapeutic target. *Biomedicine & Pharmacotherapy* **155**, 113800 (2022).

213. Jeannotte, L., Gotti, F. & Landry-Truchon, K. Hoxa5: A Key Player in Development and Disease. *J Dev Biol* **4**, 13 (2016).

214. Fuller, J. F. *et al.* Characterization of HOX gene expression during myelopoiesis: role of HOX A5 in lineage commitment and maturation. *Blood* **93**, 3391–3400 (1999).

215. Massi, D. *et al.* S100A13 is a new angiogenic marker in human melanoma. *Mod Pathol* **23**, 804–813 (2010).

216. The many roles of the Alzheimer-associated gene PM20D1. *Journal of Translational Genetics and Genomics* **6**, 361–374 (2022).

217. Childebayeva, A. *et al.* Genome-Wide Epigenetic Signatures of Adaptive Developmental Plasticity in the Andes. *Genome Biology and Evolution* **13**, evaa239 (2021).

218. Watson, C. J. *et al.* Hypoxia alters the dna methylation profile of cardiac fibroblasts via HIF-1α regulation of dna methyltransferase. *Heart* **98**, A7–A7 (2012).

219. Taberlay, P. C. *et al.* Polycomb-repressed genes have permissive enhancers that initiate reprogramming. *Cell* **147**, 1283–1294 (2011).

220. Piunti, A. & Shilatifard, A. Epigenetic balance of gene expression by Polycomb and COMPASS families. *Science* **352**, aad9780 (2016).

221. Rinn, J. L. *et al.* Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* **129**, 1311–1323 (2007).

222. Akhouayri, L. *et al.* Identification of a minimum number of genes to predict triple-negative breast cancer subgroups from gene expression profiles. *Hum Genomics* **16**, 70 (2022).

223. Pham, K. *et al.* Inflammatory gene expression during acute high-altitude exposure. *The Journal of Physiology* **600**, 4169–4186 (2022).

224. Chen, C.-Y. K., Poole, E. M., Ulrich, C. M., Kulmacz, R. J. & Wang, L.-H. Functional analysis of human thromboxane synthase polymorphic variants. *Pharmacogenet Genomics* **22**, 653–658 (2012).

225. Kanellopoulos, J. M. & Ojcius, D. M. Neither B cell nor T cell – The unique group of innate lymphoid cells. *Biomed J* **44**, 112–114 (2021).

226. Zhang, Q. & Yan, J. Update of Wnt signaling in implantation and decidualization. *Reprod Med Biol* **15**, 95–105 (2016).

227. Boso, D. *et al.* HIF-1α/Wnt signaling-dependent control of gene transcription regulates neuronal differentiation of glioblastoma stem cells. *Theranostics* **9**, 4860–4877 (2019).

228. Zhang, P., Bai, Y., Lu, L., Li, Y. & Duan, C. An oxygen-insensitive Hif-3α isoform inhibits Wnt signaling by destabilizing the nuclear β-catenin complex. *eLife* https://elifesciences.org/articles/08996 (2016) doi:10.7554/eLife.08996.

229. Santoyo-Ramos, P., Likhatcheva, M., García-Zepeda, E. A., Castañeda-Patlán, M. C. & Robles-Flores, M. Hypoxia-Inducible Factors Modulate the Stemness and Malignancy of Colon Cancer Cells by Playing Opposite Roles in Canonical Wnt Signaling. *PLOS ONE* **9**, e112580 (2014).

230. Hong, C.-F., Chen, W.-Y. & Wu, C.-W. Upregulation of Wnt signaling under hypoxia promotes lung cancer progression. *Oncology Reports* **38**, 1706–1714 (2017).

231. Ningoo, M., Plant, L. D., Greka, A. & Logothetis, D. E. PIP2 regulation of TRPC5 channel activation and desensitization. *Journal of Biological Chemistry* **296**, 100726 (2021).

232. Gersten, M., Zhou, D., Azad, P., Haddad, G. G. & Subramaniam, S. Wnt Pathway Activation Increases Hypoxia Tolerance during Development. *PLOS ONE* **9**, e103292 (2014).

233. Gaur, T. *et al.* Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem* **280**, 33132–33140 (2005).

234. Lowenstein, C. J. NO news is good news. *Proc Natl Acad Sci U S A* **96**, 10953–10954 (1999).

235. Chen, P. *et al.* Impact of High-Altitude Hypoxia on Bone Defect Repair: A Review of Molecular Mechanisms and Therapeutic Implications. *Frontiers in Medicine* **9**, (2022).

236. Ordóñez-Morán, P., Dafflon, C., Imajo, M., Nishida, E. & Huelsken, J. HOXA5 Counteracts Stem Cell Traits by Inhibiting Wnt Signaling in Colorectal Cancer. *Cancer Cell* **28**, 815–829 (2015).

237. Cao, T. *et al.* CDK3, target of miR-4469, suppresses breast cancer metastasis via inhibiting Wnt/β-catenin pathway. *Oncotarget* **8**, 84917–84927 (2017).

238. Chen, L. *et al.* ZFP57 suppress proliferation of breast cancer cells through down-regulation of MEST-mediated Wnt/β-catenin signalling pathway. *Cell Death & Disease* **10**, (2019).

239. Gustafsson, M. V. *et al.* Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev Cell* **9**, 617–628 (2005).

240. Zou, J. *et al.* Notch1 is required for hypoxia-induced proliferation, invasion and chemoresistance of T-cell acute lymphoblastic leukemia cells. *J Hematol Oncol* **6**, 3 (2013).

241. O'Brien, K. A., Murray, A. J. & Simonson, T. S. Notch Signaling and Cross-Talk in Hypoxia: A Candidate Pathway for High-Altitude Adaptation. *Life* **12**, 437 (2022).

242. Pore, N. *et al.* Akt1 activation can augment hypoxia-inducible factor-1alpha expression by increasing protein translation through a mammalian target of rapamycin-independent pathway. *Mol Cancer Res* **4**, 471–479 (2006).

243. Plasterer, C., Semenikhina, M., Tsaih, S.-W., Flister, M. J. & Palygin, O. NNAT is a novel mediator of oxidative stress that suppresses ER + breast cancer. *Mol Med* **29**, 87 (2023).

244. Pandit, M. *et al.* Termination codon readthrough of NNAT mRNA regulates calcium-mediated neuronal differentiation. *J Biol Chem* **299**, 105184 (2023).

245. Occhipinti, R. & Boron, W. F. Role of Carbonic Anhydrases and Inhibitors in Acid–Base Physiology: Insights from Mathematical Modeling. *Int J Mol Sci* **20**, 3841 (2019).

246. Lehenkari, P., Hentunen, T. A., Laitala-Leinonen, T., Tuukkanen, J. & Väänänen, H. K. Carbonic anhydrase II plays a major role in osteoclast differentiation and bone

resorption by effecting the steady state intracellular pH and Ca2+. *Exp Cell Res* **242**, 128–137 (1998).

247. Maren, T. H. & Broder, L. E. The role of carbonic anhydrase in anion secretion into cerebrospinal fluid. *J Pharmacol Exp Ther* **172**, 197–202 (1970).

248. Esbaugh, A. J. & Tufts, B. L. The structure and function of carbonic anhydrase isozymes in the respiratory system of vertebrates. *Respiratory Physiology & Neurobiology* **154**, 185–198 (2006).

249. Yu, T.-W. & Anderson, D. Reactive oxygen species-induced DNA damage and its modification: A chemical investigation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **379**, 201–210 (1997).

250. Kung-Chun Chiu, D. *et al.* Hypoxia regulates the mitochondrial activity of hepatocellular carcinoma cells through HIF/HEY1/PINK1 pathway. *Cell Death Dis* **10**, 1–16 (2019).

251. Meng, A. X. *et al.* Hypoxia down-regulates DNA double strand break repair gene expression in prostate cancer cells. *Radiother Oncol* **76**, 168–176 (2005).

252. Cowman, S., Pizer, B. & Sée, V. Downregulation of both mismatch repair and non-homologous end-joining pathways in hypoxic brain tumour cell lines. *PeerJ* **9**, e11275 (2021).

253. Tang, M., Bolderson, E., O'Byrne, K. J. & Richard, D. J. Tumor Hypoxia Drives Genomic Instability. *Frontiers in Cell and Developmental Biology* **9**, (2021).

254. Pletscher-Frankild, S., Pallejà, A., Tsafou, K., Binder, J. X. & Jensen, L. J. DISEASES: text mining and data integration of disease-gene associations. *Methods* **74**, 83–89 (2015).

255. Henderson, A. R. *et al.* DNA Methylation and Expression Profiles of Whole Blood in Parkinson's Disease. *Frontiers in Genetics* **12**, (2021).

256. Bourdillon, N. *et al.* AltitudeOmics: Baroreflex Sensitivity During Acclimatization to 5,260 m. *Front. Physiol.* **9**, 767 (2018).

257. Lee, P., Chandel, N. S. & Simon, M. C. Cellular adaptation to hypoxia through hypoxia inducible factors and beyond. *Nature Reviews Molecular Cell Biology* vol. 21 268–283 (2020).

258. Xing, G. *et al.* Adaptation and mal-adaptation to ambient hypoxia; Andean, Ethiopian and Himalayan patterns. *PLoS ONE* **3**, (2008).

259. Beall, C. M. Adaptations to Altitude: A Current Assessment. *Annu. Rev. Anthropol.* **30**, 423–456 (2001).

260. Cheong, H. I. *et al.* Alternative hematological and vascular adaptive responses to high-altitude hypoxia in East African highlanders. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **312**, L172–L177 (2017).

261. Witt, K. E. & Huerta-Sánchez, E. Convergent evolution in human and domesticate adaptation to high-altitude environments. *Philosophical Transactions of the Royal Society B: Biological Sciences* vol. 374 (2019).

262. Villafuerte, F. C. & Corante, N. Chronic Mountain Sickness: Clinical Aspects, Etiology, Management, and Treatment. *High altitude medicine & biology* **17**, 61–9 (2016).

263. Peng, Y. *et al.* Down-regulation of EPAS1 transcription and genetic adaptation of tibetans to high-altitude hypoxia. *Molecular Biology and Evolution* **34**, 818–830 (2017).

264. Yi, X. *et al.* Sequencing of 50 human exomes reveals adaptation to high altitude. *Science* **329**, 75–78 (2010).

265. Hanaoka, M. *et al.* Genetic Variants in EPAS1 Contribute to Adaptation to High-Altitude Hypoxia in Sherpas. *PLoS ONE* **7**, (2012).

266. Frías-Lasserre, D. & Villagra, C. A. The Importance of ncRNAs as Epigenetic Mechanisms in Phenotypic Variation and Organic Evolution. *Front Microbiol* **8**, 2483 (2017).

267. Chen, Q., Yan, W. & Duan, E. Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications. *Nat Rev Genet* **17**, 733–743 (2016).

268. Peaston, A. E. & Whitelaw, E. Epigenetics and phenotypic variation in mammals. *Mamm Genome* **17**, 365–374 (2006).

269. Moore, L. D., Le, T. & Fan, G. DNA Methylation and Its Basic Function. *Neuropsychopharmacol* **38**, 23–38 (2013).

270. Angers, B., Castonguay, E. & Massicotte, R. Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after. *Molecular Ecology* **19**, 1283–1295 (2010).

271. Semenza, G. L. Hypoxia-inducible factor 1: master regulator of O2 homeostasis. *Curr Opin Genet Dev* **8**, 588–594 (1998).

272. Maxwell, P. H. Hypoxia-inducible factor as a physiological regulator. *Experimental Physiology* **90**, 791–797 (2005).

273. Bigham, A. W. Genetics of human origin and evolution: high-altitude adaptations. *Current Opinion in Genetics and Development* vol. 41 8–13 (2016).

274. Beall, C. M. Two routes to functional adaptation: Tibetan and Andean high-altitude natives. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 8655–8660 (2007).

275. Smith, E., Jones, M. E. & Drew, P. A. Quantitation of DNA methylation by melt curve analysis. (2009) doi:10.1186/1471-2407-9-123.

276. Xiang, K. *et al.* Identification of a Tibetan-specific mutation in the hypoxic gene EGLN1 and its contribution to high-altitude adaptation. *Molecular Biology and Evolution* **30**, 1889–1898 (2013).

277. Maugeri, A. *et al.* The Relationship between Body Mass Index, Obesity, and LINE-1 Methylation: A Cross-Sectional Study on Women from Southern Italy. *Dis Markers* **2021**, 9910878 (2021).

278. Johnson, A. A. *et al.* The Role of DNA Methylation in Aging, Rejuvenation, and Age-Related Disease. *Rejuvenation Res* **15**, 483–494 (2012).

279. Bärtsch, P. & Saltin, B. General introduction to altitude adaptation and mountain sickness. *Scandinavian journal of medicine & science in sports* **18 Suppl 1**, 1–10 (2008).

280. Duffin, J. Measuring the ventilatory response to hypoxia. *Journal of Physiology* **584**, 285–293 (2007).

281. Batie, M. & Rocha, S. Gene transcription and chromatin regulation in hypoxia. *Biochemical Society Transactions* **48**, 1121–1128 (2020).

282. Kouzarides, T. Chromatin Modifications and Their Function. *Cell* vol. 128 693–705 (2007).

283. Bannister, A. J. & Kouzarides, T. Regulation of chromatin by histone modifications. *Cell Res* **21**, 381–395 (2011).

284. Morselli, M. *et al.* In vivo targeting of de novo DNA methylation by histone modifications in yeast and mouse. *eLife* **4**, e06205.

285. Fu, K., Bonora, G. & Pellegrini, M. Interactions between core histone marks and DNA methyltransferases predict DNA methylation patterns observed in human cells and tissues. *Epigenetics* **15**, 272–282 (2019).

286. Batie, M., Del Peso, L. & Rocha, S. Hypoxia and Chromatin: A Focus on Transcriptional Repression Mechanisms. doi:10.3390/biomedicines6020047.

287. Jang, C.-W., Shibata, Y., Starmer, J., Yee, D. & Magnuson, T. Histone H3.3 maintains genome integrity during mammalian development. *Genes Dev.* **29**, 1377–1392 (2015).

288. Strobino, M., Wenda, J. M., Padayachy, L. & Steiner, F. A. Loss of histone H3.3 results in DNA replication defects and altered origin dynamics in C. elegans. *Genome Res* **30**, 1740–1751 (2020).

289. Shindo, Y. & Amodeo, A. A. Excess histone H3 is a Chk1 inhibitor that controls embryonic cell cycle progression. 2020.06.09.142414 Preprint at https://doi.org/10.1101/2020.06.09.142414 (2020).

290. Liu, S. *et al.* RPA binds histone H3-H4 and functions in DNA replication–coupled nucleosome assembly. *Science* **355**, 415–420 (2017).

291. Thakre, P. K., Sv, A., Golla, U., Chauhan, S. & Tomar, R. S. Previously uncharacterized amino acid residues in histone H3 and H4 mutants with roles in DNA damage repair response and cellular aging. *The FEBS Journal* **286**, 1154–1173 (2019).

292. Adam, S., Polo, S. E. & Almouzni, G. How to restore chromatin structure and function in response to DNA damage – let the chaperones play. *The FEBS Journal* **281**, 2315–2323 (2014).

293. Polo, S. E., Roche, D. & Almouzni, G. New histone incorporation marks sites of UV repair in human cells. *Cell* **127**, 481–493 (2006).

294. Bodor, D. L., Rodríguez, M. G., Moreno, N. & Jansen, L. E. T. Analysis of protein turnover by quantitative SNAP-based pulse-chase imaging. *Curr Protoc Cell Biol* **Chapter 8**, Unit8.8 (2012).

295. Adam, S., Dabin, J., Bai, S.-K. & Polo, S. E. Imaging local deposition of newly synthesized histones in UVC-damaged chromatin. *Methods Mol Biol* **1288**, 337–347 (2015).

296. Stawski, R. *et al.* Increased Circulating H3 Histone in Response to Repeated Bouts of Exercise Does Not Associate with Parallel Alterations of Cell-Free DNA. *Biology* **10**, 181 (2021).

297. Maya, D. *et al.* A Histone Cycle. in *The Mechanisms of DNA Replication* (IntechOpen, 2013). doi:10.5772/53993.

298. Adam, S., Polo, S. E. & Almouzni, G. Transcription Recovery after DNA Damage Requires Chromatin Priming by the H3.3 Histone Chaperone HIRA. *Cell* **155**, 94–106 (2013).

299. Maze, I. *et al.* Critical role of histone turnover in neuronal transcription and plasticity. *Neuron* **87**, 77–94 (2015).

300. Ortmann, B., Druker, J. & Rocha, S. Cell cycle progression in response to oxygen levels. *Cell Mol Life Sci* **71**, 3569–3582 (2014).

301. Hsu, K. F. *et al.* Hypoxia and hypoxia mimetics differentially modulate histone post-translational modifications. *Epigenetics* 1–14 (2020) doi:10.1080/15592294.2020.1786305.

302. Chen, H., Yan, Y., Davidson, T. L., Shinkai, Y. & Costa, M. Hypoxic Stress Induces Dimethylated Histone H3 Lysine 9 through Histone Methyltransferase G9a in Mammalian Cells. *Cancer Research* **66**, 9009–9016 (2006).

303. Johnson, A. B., Denko, N. & Barton, M. C. Hypoxia induces a novel signature of chromatin modifications and global repression of transcription. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis* **640**, 174–179 (2008).

304. Choudhry, H. & Harris, A. L. Advances in Hypoxia-Inducible Factor Biology. *Cell Metabolism* vol. 27 281–298 (2018).

305. Strzyz, P. Hypoxia makes its mark on histones. *Nature Reviews Molecular Cell Biology* vol. 20 324–325 (2019).

306. Hancock, R. L., Masson, N., Dunne, K., Flashman, E. & Kawamura, A. The Activity of JmjC Histone Lysine Demethylase KDM4A is Highly Sensitive to Oxygen Concentrations. *ACS Chemical Biology* **12**, 1011–1019 (2017).

307. Watson, J. A., Watson, C. J., Mccann, A. & Baugh, J. Epigenetics, the epicenter of the hypoxic response. *Epigenetics* **5**, 293–296 (2010).

308. Melvin, A. & Rocha, S. Chromatin as an oxygen sensor and active player in the hypoxia response. *Cellular Signalling* vol. 24 35–43 (2012).

309. Perez-Perri, J. I., Acevedo, J. M. & Wappner, P. Epigenetics: New questions on the response to hypoxia. *International Journal of Molecular Sciences* vol. 12 4705–4721 (2011).

310. Wurtele, H. *et al.* Histone H3 Lysine 56 Acetylation and the Response to DNA Replication Fork Damage. *Mol Cell Biol* **32**, 154–172 (2012).

311. Ding, N. *et al.* Chk1 Inhibition Hinders the Restoration of H3.1K56 and H3.3K56 Acetylation and Reprograms Gene Transcription After DNA Damage Repair. *Frontiers in Oncology* **12**, (2022).