2023

EVALUATION OF PHYSIOLOGICAL RESPONSES TO WOODSMOKE INHALATION DURING EXERCISE IN BLOOD PRESSURE SENSITIVE/CHRONOTROPE STRATIFIED INDIVIDUALS

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Dissertation

presented in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
in Integrative Physiology and Rehabilitation Sciences

The University of Montana
Missoula, MT

August 2023

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Evaluation of Physiological Responses to Woodsmoke Inhalation During Exercise in Blood Pressure Sensitive/Chronotype Stratified Individuals

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Abstract

The aim of this dissertation was to evaluate the acute physical reaction to WS exposure and identify potential fingerprints of physiological responses that may better illustrate the process leading to pathology from chronic exposure to particulate matter (PM). Study 1 developed a cost-effective, reproducible methodology for obtaining larger volume and pH-stable exhaled breath condensate (EBC) samples. Notably, the novel EBC device, compared to a commercially-available device, collected a larger sample volume, permitting an expansion in the number of biomarkers that could be examined in future WS exposures. The EBC device was then incorporated in Study 2, along with measuring key dependent variables such as Pulse Wave Velocity (PWV), Heart Rate Variability (HRV), and Pulmonary Function Tests (PFT). Study 2 was a repeated measures cross-over study designed to evaluate the effects of sleep deprivation on the acute physiological response to WS and exercise. However, acute sleep deprivation did not magnify HRV, PWV, PFT, or secondary measures in this investigation. Lastly, Study 3 used the cold pressor test (CPT) to identify hyperreactive individuals, regarding systolic blood pressure elevations, that may have elevated potential for a magnified response to an acute bout of WS exposure. CPT-sensitive individuals (CPT^{+}; n=10) were further stratified for morning-evening questionnaire responses and were compared with CPT^{-} morning (n=10) persons. Notably, study 3 indicated that differences in HRV and PWV occur immediately after and 24 hours post-exposure in CPT^{+} individuals following a two-hour bout of moderate exercise (50% VO_2max) with WS exposure (250 $\mu g \cdot m^3$). In contrast, no differences in EBC inflammation or oxidative stress markers were observed. Collectively, work from this dissertation provides: 1) a new cost-effective method for obtaining more voluminous, pH stable EBC, 2) no evidence that one night of sleep deprivation was an amplifying factor to the examined metrics of physiologic and biochemical (blood and EBC) stress to exercise and WS exposure in young, apparently health, adults, and 3) modest evidence, based on AIx and HRV outcomes, that CPT^{+} afternoon/evening individuals have exaggerated responses to exercise during WS, but that secondary biochemical measures in blood and EBC were unaltered in apparently healthy adults.

Acknowledgments

First, I would like to thank Dr. John Quindry, my doctoral advisor and committee chair, for his advice, patience, and mentorship over the last four years. Additionally, I would like to express sincere gratitude to the members of my doctoral committee, Dr. Brent Ruby, Dr. Charlie Palmer, Dr. Erin Semmens, and Dr. Tony Ward, for their time, support, and encouragement throughout this process of my academic career at the University of Montana. I sincerely thank my previous academic mentors, Dr. Steve Gaskill and Dr. Charles Dumke. This would not have been possible without your guidance, mentorship, and encouragement. Fellow graduate students Katie Christison, Shae Gurney, Amanda Alfaro, and Anna Covington, thank you for your friendship, teamwork, and motivation.

Most importantly, I thank my wife, Jenna, who has demonstrated unwavering love and support throughout this process. Thank you for all the little things and encouragement over the past four years, including Emilia's big addition to our oldest daughter, Evelyn. This would not have been possible without all of you. This has been an incredible journey for all of us, and I appreciate your willingness to help me accomplish this degree. I love you.

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List of Abbreviations

8-ISO	8-Isoprostane
AIx	Augmentation Index
ANS	Autonomic Nervous System
AQI	Air Quality Index
BP	Blood Pressure
CC16	Clara Cell 16
CO	Carbon Monoxide
CPT	Cold Pressor Test
CPT^{+}	SBP response > 20 mmHg during CPT and MEQ intermediate or evening chronotype
CPT^{-}	SBP response < 20 mmHg during CPT and MEQ morning chronotype
CV	Cardiovascular
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DE	Diesel Exhaust
EBC	Exhaled Breath Condensate
FEV1	Forced Expiratory Volume
FEV1%	Percentage of FVC divided by FEV1.
FVC	Forced Vital Capacity
HR	Heart Rate
HRV	Heart Rate Variability
LF: HF Ratio	Ratio of LF to HF power
HF	Power in the High Frequency of the HRV Spectrum
LF	Power in the Low Frequency of the HRV Spectrum
MAP	Mean Arterial Pressure
MET	Metabolic Equivalent of Task
MEQ	Morningness-Eveningness Questionnaire
MVV	Maximal Voluntary Ventilation
PAMPs	Pathogen-Associated Molecular Patterns
PAR-Q	Physical Activity Readiness Questionnaire
PFT	Pulmonary Function Test
PM	Particulate Matter
PM2.5	Particulate Matter < 2.5 μm in diameter
PP	Pulse Pressure
PRR	Pattern Recognition Receptors
PTT	Pulse Transit Time
PWV	Pulse Wave Velocity
RMANOVA	Repeated Measures Analysis of Variance
RMSSD	Root Mean Square of Successive Differences

RR	Respiratory Rate
SBP	Systolic Blood Pressure
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-alpha
TV	Tidal Volume
VO_2max	Maximal Oxygen Consumption
VOC	Volatile Organic Compounds
WLFF	Wildland Firefighter
WS	Woodsmoke

Chapter I: Introduction

Smoke inhalation from biomass combustion is a public health problem in the western United States owing to frequent and large-scale wildfires[1]. Health dangers are associated with biomass particulate matter (PM) inhalation, including airborne particles ≤2.5 microns in diameter (PM2.5). Populations affected by PM2.5 include wildland firefighters (WLFFs) and those who exercise outside[2]**.** Exercise ventilatory rates elevate the inhaled PM2.5 dose, and the smoke inhalation dose is proportional to the PM2.5 concentration, duration of exposure, and ventilatory rates associated with activity[3]. Accordingly, this approach for estimating woodsmoke (WS) inhalation doses is currently untested in laboratory assessments where the acute impacts on cardiovascular (CV) function can be determined.

Long-term smoke inhalation elevates the risk for CV mortality by 0.4 to 1.0% with an incremental increase of only 10 μg·m-3 above the mean 24-h PM2.5 concentration[4]. Additionally, in occupational settings such as WLFF, each additional day of WS exposure has been associated with pulmonary function test (PFT) declines of 24mL in functional vital capacity (FVC) and forced expiratory volume in 1 second (FEV1)[5]. Furthermore, chronic PM2.5 inhalation is associated with reduced life expectancy and increased rates of cardiovascular disease (CVD)[4]. Chronic exposure to WS presents serious health detriments, yet the acute response to WS exposure, thought to be foundational to pathological outcomes, is not well defined. Nonetheless, preliminary studies suggest that elevated oxidative stress markers in response to acute smoke inhalation may contribute to pathological effects [6-12]. Accordingly, this investigation aimed to determine whether critical physiologic responses to acute smoke inhalation during exercise can be identified, which may provide insight into cardiorespiratory dysfunction. The subsequent knowledge could be implemented to help intercept these long-term CV and respiratory system pathologies.

Preliminary findings indicate that pathophysiologic mechanisms include perturbations in autonomic dysregulation, mitigated CV control, systemic inflammation, and oxidative stress[4]. CV and autonomic control can be quantified by heart rate variability (HRV), pulse wave velocity (PWV), and resting blood pressure (BP). HRV is prognostic of CV health when HRV recovery after physiologic stressors is delayed[13]. In some experimental contexts, HRV is reduced by WS inhalation, suggesting a concomitant decline in autonomic tone[14, 15]. PWV and blood oxidative stress also provide further insight into acute physiologic changes after exercise with WS exposure.

While it is well understood that chronic WS exposure harms long-term health, the mechanisms responsible for initiating compromised health are not fully resolved. One recent review indicates that following a 25-year career, WLFFs have an increased risk of lung disease by 43% over occupational controls and are at an elevated risk for CVD by +30% [16]. There is a growing rationale to suspect that alterations in several physiologic outcomes may indicate both the acute and long-term physiologic stress to smoke inhalation

[7, 9, 11]. Current research literature, however, confirms that it is methodologically challenging to quantify the physiological detriment of acute WS inhalation in otherwise healthy individuals [9, 11, 17]. Nonetheless, early results indicate that exercise and smoke inhalation can increase biological markers of oxidative stress [7, 9, 11, 18, 19].

Further identification of acute physiologic responses to WS would provide novel insight into the mechanisms responsible for increased rates of chronic diseases (e.g., CVD). Fundamental to this challenge, the detrimental physiologic effects of WS have not been fully quantified. Accordingly, additional investigations into the impact of smoke inhalation on lung function and CV control are needed. While a limited volume of published research literature has investigated WS exposure and exercise, little work has been performed to understand the potential compounding effects of autonomic hyperreactivity. Observations of declining antioxidant levels are essential because oxidative stress (e.g., any imbalance between free radical production and antioxidant depletion) is foundational to most chronic diseases [20]. Of particular interest is the nervous system, which is already low in antioxidant capacity. The vagus cranial nerve is responsible for the primary control of the body's parasympathetic (relaxed state) control, including contributing to HRV.

In this regard, the cold pressor test (CPT), historically used to identify healthy individuals that may be at risk for hypertension, was used to identify autonomically "Hyperreactive" individuals (SBP increases >20mmHg when submersing a hand into a bucket of ice water for 2 min). Furthermore, these individuals were screened for chronotype, or morning-evening preference, to identify individuals with an exacerbated response when performing physical activity in the morning. Applications of this understanding include professions such as WLFFs, which have various physical fitness and health backgrounds and undertake physically demanding 16+ hr. shifts, for 14 consecutive days, in the presence of concentrated ambient WS[21]. Current literature reports it is difficult to quantify the physiological detriment of acute WS inhalation in otherwise healthy individuals. Nonetheless, various results indicate that exercise and smoke inhalation can elevate biological markers of oxidative stress[6, 7, 22]. Further identifying acute physiologic responses to WS would likely provide insight into increased cancer and CVD rates.

Based on this rationale, the current study aimed to identify whether acute exercise during controlled smoke inhalation produces physiological alterations in respiratory and CV measures. Moreover, the effects of autonomic reactivity and morning-evening questionnaire chronotype (MEQ) were examined in conjunction with this study to provide more comprehensive insight into smoke exposure stress responses. Accordingly, this study utilized two groups, one consisting of CPT^{+} individuals (CPT systolic BP (SBP) response > 20mmHG and identified as not a morning type in the MEQ) and the other consisting of CPT^{-} (CPT SBP response < 20mmHG and recognized as a morning type in the MEQ).

Null Hypotheses

Given the nature of this investigation and prior studies in our laboratory, we expect minimal changes in PFT variables in this young, apparently healthy cohort following an acute WS exposure. However, we would expect PFT, PWV, and HRV changes to be more pronounced in cohorts with advanced age and chronic diseases.

Primary dependent measures:

There will be no between-group differences in physiological response in HRV (lnRMSSD, LF, HF, and LF: HF ratio), PWV (PWV, AIX), and PFT (FVC, FEV1, FEV1%, and MVV) metrics.

Secondary dependent measures:

1. There is no relationship between sex and key dependent physiologic responses to 120-min of moderate exercise with WS.
2. There will be no between-group differences between CPT^{+} and CPT^{-} in markers of inflammation and oxidative stress (8-ISO, CC16, and TNF-α) following 120-min of moderate exercise with WS exposure compared to CPT^{-} individuals.
3. There will be no main effect of time in physiological response over sampling time points (Pre, Post, 24-hour Post).

Delimitations

Study participants were stratified by CPT outcome and chronotype. Furthermore, the design will equally represent males and females in each cohort to evaluate potential gender differences. Prior studies have mainly displayed no effect on the physiological response to acute WS exposure with exercise. However, the CPT will identify individuals with an increased likelihood of hypertension and CVD later in life. The BP response differences at baseline CPT were inherent to the study design. However, researchers chose this stratification due to the potential for participants with these phenotypes (CPT^{+} v CPT^{-}) to be different with respect to post-challenge key dependent variables (PWV, HRV, and PFT) and secondary variables of oxidative stress and inflammatory function (8-ISO, CC16, TNF-α). While the study groups will be identified by CPT designation only, our study design further stratified the study participants using the MEQ to potentiate the physiological response differences between groups. In doing so, the CPT^{+} individuals were also selected due to their misalignment with morning activity (intermediate or evening activity preferred). In contrast, the CPT^{-} individuals were morning preferenced (not-maligned with morning activity).

Also inherent to the present study design are the dose and duration of WS exposure with exercise and the sampling time points. Specifically, our most recent prior study examined a higher intensity and lower exercise duration along with the $250 \mu g \cdot m^{-3}$ WS exposure. PWV, HRV, and PFT are identified as key dependent variables to evaluate the

central arterial, autonomic control, and pulmonary responses to WS exposure with moderate exercise. PWV has shown sensitivity to acute WS exposures and allows for central arterial stiffness evaluations in various settings outside the laboratory. HRV, when collected in 10-min samples, has been shown to reflect the parasympathetic and sympathetic activity occurring concerning many stressors, including PM[14]. While PFT has been established to remain relatively unchanged from acute laboratory WS exposures, the ease of measuring PFTs and noted changes in special populations provides for its continued inclusion in our evaluation.

The secondary variables evaluated in the study consist of an inflammatory cytokine (TNF-α), an anti-inflammatory protein secreted by epithelial cells in the lung (CC16), and an oxidative stress biomarker (8-ISO). Collectively, the intent is to have a concise panel to evaluate the inflammatory and oxidative stress response in the conditions presented in this study.

Limitations

Study limitations include the nature and size of the sample. Owing to the age range, fitness level, and overall health of the examined sample, inferences that can be drawn from these results are limited proportionately. Thus, it is plausible that these methods, if applied to more susceptible populations (e.g., age, chronic conditions), may have produced alternative outcomes for our grouped outcome measures of HRV, PWV, PFT, and inflammatory and oxidative stress markers. Additionally, the statistical power for sex differences may be lessened due to the cost and logistical constraints in the total number of participants possible for the study.

Furthermore, the dose of WS exposure may be insufficient to elicit adverse effects on the selected variables. Additionally, the study examined only one modality, duration, and intensity of exercise, limiting the application of these findings to similar activities. Evaluations of autonomic control and arterial stiffness were determined to HRV and PWV; however, these metrics allow for future assessments in field settings and increase external validity in results. Furthermore, the decision to enlist a small, concise panel to evaluate the inflammatory and oxidative stress response was prompted due to external fund and testing stipulations prioritizing portability and applicability to field and community applications.

Assumptions

Regarding PWV and HRV, we used preclinical measurements to predict outcomes. While not ideal, the bulk of existing literature using our key dependent variables supports our findings and allows for inference[15, 23-27]. Regarding cohort structure, equal representation by gender in the total study population of 20 permitted the detection of potential differences between sex. However, our statistical power calculations were based primarily on differences in our key dependent variables and not based on sex (secondary hypothesis).

Furthermore, as evening-type chronotypes tend to have a higher risk for CV and other CV conditions, we assumed that the CPT^{+} individuals that are malaligned with their MEQ Chronotype (i.e., CPT^{+} and not morning type) would have the most significant potential for hyperreactivity to a moderate bout of smoke exposure when compared even to another CPT^{+} that is a morning type. Additionally, CPT^{-} participants were morning chronotype to aim for the minimal influence of chronotype on autonomic modulation.

A lack of significant physiological response prompted the present study to align with prior studies in a two-hour WS exposure and return to a more moderate exercise intensity[11, 28, 29]. In this regard, the physiological response to the trial was likely from the inhaled PM dose. Furthermore, prior observed time points may have missed the physiological response to WS. As the immune response to WS may take several hours, it may explain the lack of findings immediately after sampling time points. As such, the inclusion of testing all key (PWV, HRV, PFT) and secondary dependent variables (8-ISO, CC16, and TNF-α) again at 24 hours post-trial aligns with other research discoveries[7, 9, 23, 28, 30].

Chapter II: Review of the Literature

Populations affected by woodsmoke.

Exposure to ambient PM has been associated with various adverse health effects, including increased morbidity and mortality from pulmonary and CVD[31, 32]. Although residential wood combustion is a significant source of particulate air pollution in many countries, most research investigating the health effects of WS exposure has only occurred in the past 10-15 years[33]. In a previous review, ambient WS was estimated to be no less harmful than other sources of ambient PM[17].

WLFFs experience greater exposure to WS than members of the general public[16]. Occupational exposures may consist of prolonged and direct exposure to smoke, and elevated pulmonary ventilation rates can result in substantial doses of smoke to the respiratory tract[30]. WLFFs are exposed to health hazards from inhalation of known air pollutants (e.g., particular matter, carbon monoxide, and polycyclic hydrocarbons) at levels near or above occupational exposure limits[34]. Despite accelerating global wildland fire activity, knowledge of the health risks from occupational exposure to wildland fire is broad, inconsistent, and insufficient to conclude health outcomes[35, 36].

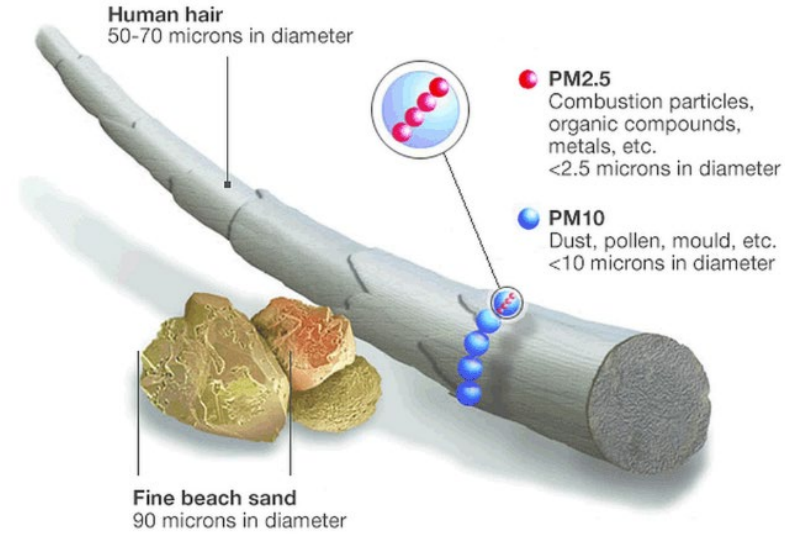
To date, most research on the health of WLFFs is focused on exploring the acute effects of smoke exposure across a single shift or season, with most studies finding a reduction in lung function and increased systemic inflammation[30, 37-39]. Modeling exposure data, Navarro et al. [16] estimated an increased lung cancer and CVD risk among WLFFs. However, a prospective or longitudinal trial has not confirmed this finding. Cross-sectional studies have identified associations between career length and the occurrence of CVD and between wildland WS exposure, oxidative stress, and vascular function[40, 41].

Furthermore, the most crucial research topic identified by WLFF study participants was WS exposure and respiratory health, with 89% of participants indicating it was extremely or very important[42]. During interviews, participants focused on the need to understand better the exposures and health risks associated with wildland WS and the desire for feasible, effective personal protective equipment to protect against smoke inhalation[42].

Particulate Matter

PM air pollution can be divided into naturally originated particulates and those resulting from combustion for transport, heating, cooking, and industrial processing. PM may also be divided according to its size. Coarse particles (PM10) have a diameter of less than 10 μm and are commonly present as silica-based crustal particles, brake wear, road dust, and volcanic ash. Whereas fine particles (PM2.5) have a diameter of less than 2.5 μm, and ultrafine particles (PM<0.1) particles have a diameter of less than 100 nm[43]. As indicated in Figure 1, PM can be much smaller than items the human eye perceives (US EPA, 2023)[44].

Figure 1. US EPA Illustration of Different Sizes of Particulate Matter[44]



Source: US EPA

Combustion is the most common source of air pollution PM, especially the ultrafine fraction that is suggested to be associated with adverse health effects following its ability to deposit in the lungs. The physicochemical properties of the inhaled particles are significant for respiratory tract deposition and adverse health effects. The particle size, density, shape, and chemical composition have been proposed to influence PM's toxic and inflammatory potency [43, 45, 46]. Additionally, WS particles' physical and chemical properties differ substantially depending on the combustion conditions[47].

PM is considered the most prominent health hazard from wildfire WS, and PM from wildfire WS can be more toxic than equal doses of ambient PM[48]. WS contributes

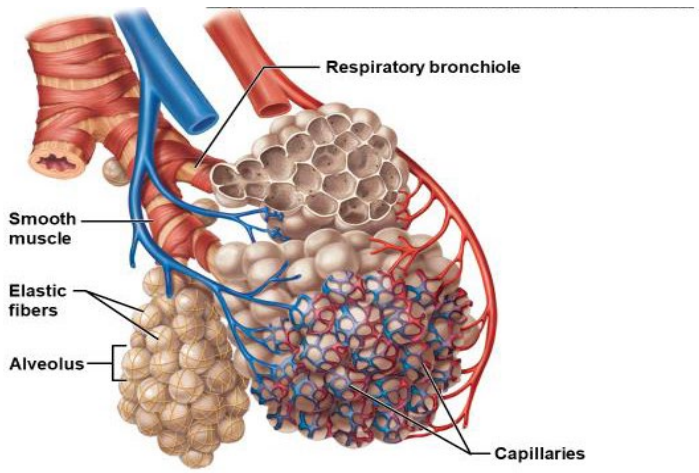
significant quantities of ultrafine particles to our environment through biomass combustion for heating, cooking, and during large wildland fires[49]. WLFFs have substantial and often prolonged exposures during occupational tasks[34]. Furthermore, exposure to PM2.5 is increasingly recognized as a short and long-term risk factor for CVD. It has been linked to the triggering of myocardial infarction within hours of exposure[4, 50, 51]. Controlled exposure studies have demonstrated that acute exposure to diesel exhaust (DE) and concentrated ambient particles, as models of urban particulate air pollution, generate acute vasoconstriction, increases in BP and arterial stiffness [4, 52, 53], vascular endothelial dysfunction[54], myocardial ischemia[54, 55], and changes in cardiac autonomic control[52, 56].

Past exposure assessments of WLFFs have measured acrolein, benzene, carbon dioxide, carbon monoxide (CO), formaldehyde, polycyclic aromatic hydrocarbons, and PM from exposure to WS[17]. PM exposure from wildfires has been linked to adverse respiratory outcomes such as asthma symptoms and chronic obstructive pulmonary disease; however, PM in smoke typically exists in mixtures with (volatile organic compounds (VOCs), which have not been well studied[2]. The health-relevant VOCs commonly found in WS, such as acrolein, benzene, and formaldehyde, have been linked to irritation (eyes, skin, nose, mucous membrane, respiratory system), chronic respiratory illness, and cancer[2, 57, 58]. The mechanisms by which WS exerts its effects have not been well studied compared with the many experimental studies focused on DE exposure. The interest in performing human WS exposure studies during the last decade has increased. However, to help inform the general population, the US Air Quality Index (AQI), seen in Figure 2, is commonly used to educate populations on risks associated with various ambient PM scenarios (IQAir, 2022)[59].

Figure 2. World Health Organization Recommended Guidelines for Ambient PM2.5 and AQI Levels[59].

Most epidemiological and toxicological studies relay the observed health effects to the particle mass concentration. However, the probability of the particle to deposit is not only a function of mass concentration but also depends on other important parameters, such as particle size, hygroscopicity (material's ability to absorb moisture from the environment), exposure duration, and ventilation physiology. The particle size is the primary determinant of a particle's deposition property, with smaller particles depositing much more than larger particles. There are three mechanisms by which particles of different sizes are deposited in the respiratory tract, primarily dictated by the size of the particle itself. Particles sized > 3 μm deposit mainly by impaction in the airways. Impaction increases with particle size and flow rate. For the particle size range 0.3-3 μm, the deposition occurs mainly by sedimentation because of the gravitational force. The particles settle in the lower airways where the slow flow rate and airways are small. The deposition of the particles differs according to the direction of the particle flow and the direction of the gravitational force. Tiny particles (< 0.3 μm) deposit by diffusion. These particles are constantly moving and colliding, resulting in a random movement, Brownian motion, which causes diffusion when there is a difference in the particle concentration between two mediums—the diffusion increase with decreasing particle size and flow rate. The leading site for diffusion is in the alveolar region because of the smaller airways and long residence time for the aerosol. As such, due to the size, PM2.5 is deposited in the deep lung, especially in the pulmonary alveoli [60]. Therefore, alveolar epithelial cells and macrophages can directly encounter PM2.5 after inhaling polluted air. See Figure 3 for a diagram of the alveoli and the respiratory membrane (Marieb and Hoehn, 2013)[61].

Figure 3. Diagrammatic View of Capillary-Alveoli Relationships.[61]



PM2.5 Provokes Immune Cells

The respiratory tract is the second-largest surface area, surpassed only by the digestive tract[62]. It serves as a gateway of the body. Meanwhile, the immune system is a

sensitive target organ of environmental pollutants. To exert adverse health effects, inhaled particles must deposit in the respiratory tract and interact with the respiratory tract, lining fluid, inflammatory cells, and lung tissue. The primary factor for this is the number and distribution of particles deposited locally in the lungs, not the particle mass concentration that can be measured in the ambient air. This is also important as the lung deposition fraction varies considerably between different types of particles.

Many kinds of cellular effectors reside in the respiratory tract and lungs. Post-inhalation, PM2.5 will deposit in the respiratory tract, accumulate, and even impair other body parts via air exchange in the alveoli[63]. Both alveolar epithelial cells and alveolar macrophages are pillars of the innate immune system working as the first defense line of the respiratory tract. More importantly, the antigen-presenting cells can activate T-lymphocytes in the respiratory tract to induce specific immunity[64]. Also, PM2.5 can induce the Th2-type inflammatory response. As such, continually accumulating evidence indicates that inflammatory response and immune dysfunction facilitate CV and respiratory disease morbidity and mortality[63].

An adjuvant is an essential component of vaccines because it can help enhance the immune response when combined with a specific antigen. Immune responses are generally divided into innate and adaptive responses [64]. Typically, an adjuvant can activate innate immunity by combining with adaptors, such as pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are one of the PRRs. Airborne PM has the potential to bind and absorb many kinds of toxins and act as an adjuvant[65]. Numerous studies showed that PM2.5 had adjuvant effects on IgE and IgG1 production, and results indicated that the components of PM2.5 can be recognized by TLRs[66-68].

Monocytes can infiltrate tissues, produce inflammatory cytokines, and differentiate into inflammatory macrophages or phagocytes. Classical monocytes express several PRRs and are involved in removing microorganisms and dying cells via phagocytosis. Upon tissue damage or infection, monocytes are rapidly recruited to the tissue, where they can differentiate into tissue macrophages or dendritic cells[69]. Alveolar macrophages and lung epithelial cells incubated with PM release significantly higher amounts of cytokines and chemokines[70, 71]. Higher levels of these circulating cytokines (IL-6, IL-1β) have been observed in humans exposed to PM particles from forest fires[72]. Atherosclerosis is an inflammatory disease of vessel walls in which monocytes play a pivotal role in its pathogenesis[73]. Monocytes migrate into the vessel wall, differentiating into macrophages and expressing scavenger receptors, allowing them to phagocytose-modified lipoproteins[69]. This internalization causes the cells to develop into foam cells and causes the release of cytokines that perpetuate inflammation and lipid accumulation, leading to the progression of atherosclerosis and augmenting the vulnerability of plaques.

Exercise, Neutrophils, and Oxidative Stress

Acute exercise is associated with elevations in blood oxidative stress markers and is further exaggerated by high-intensity exercise[74, 75]. Additionally, endurance exercise performed at intensities above the lactate threshold significantly elevates circulating neutrophil numbers, which may contribute to the blood oxidative stress observed during physical activity[76]. Quindry et al. 2003, had nine males complete one maximal and three submaximal exercise sessions of 45 minutes in duration[77]. In doing so, results indicated that oxidative stress during physical activity is only present during high intensities. Decreased antioxidant activity was observed only in high-intensity activities; elevated neutrophil counts and neutrophil-generated superoxide levels were highest following maximal treadmill exercise. The moderate-intensity activity in the present research study (50% VO_2max) aims to examine the oxidative stress occurring from WS exposure exclusively.

PM·MET^{-1}·min^{-1}

In addition to the PM size, the pulmonary deposition of the particles also depends on tidal volume, respiratory rate (RR), and, thereby, minute volume. The total deposition fraction increases with higher tidal volume (TV). Concerning particles below 300 nm, which deposit mainly by diffusion, the relation with respiratory rate is inversed[78] due to the decreased lung residence time during high respiratory rate[79]. The minute volume depends on the TV and RR. Furthermore, TV and RR increase during exercise, resulting in a substantial increment of particle deposition dose rate compared with exposure at rest or under normal physical activities[80].

Our laboratory has spent several years developing methods and protocols to examine how the body acutely responds to bouts of WS exposure during exercise[7, 9, 23]. In these studies, we have quantified the acute effects of several different durations (45 and 90 min), intensities of exercise (22.5 ml·kg^{-1}·min^{-1}; 70% VO2max) and PM concentrations (0, 250, and 500 μg·m^{-3}) during these trials. An appreciable way to analyze these cumulative doses is through PM·MET^{-1}·min^{-1} dose calculations, where MET is a metabolic equivalent of task. Beginning in 2016, the first study conducted in our laboratory utilized a protocol with low (250 μg·m^{-3} and high PM (500 μg·m^{-3}) concentrations of WS[9]. While walking on a treadmill at 3.5 mph at 5.7% grade, these individuals performed at an intensity of approximately 22.5 ml·kg^{-1}·min^{-1} for 120 minutes, resulting in a dose of 144,643 and 289,286 PM·MET^{-1}·min^{-1} for the low and high exposure trials, respectively. Then in 2021, Williamson et al. used a protocol approximating 8.8 METs (70% VO2max) for 90 minutes with a PM exposure of 250 μg·m^{-3}, which resulted in a dose equal to 198,000 PM·MET^{-1}·min^{-1}[11]. To evaluate the effect of exercise intensity on physiological responses, Study 2 of this dissertation (Chapter VII), while manipulating a sleep deprivation variable, again used a 70% of VO2 max protocol but for a shorter duration (45 min), resulting in a dose of 105,075 PM·MET^{-1}·min^{-1}. The current study re-evaluates

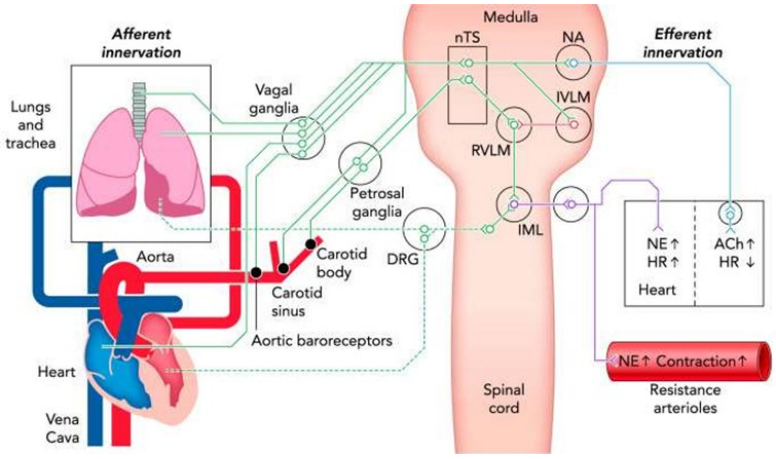
the effect of time with a 120-min protocol at 250 $\mu g \cdot m^{-3}$ at 22.5 $ml \cdot kg^{-1} \cdot min^{-1}$, which would approximate a dose of 192,857 $PM \cdot MET^{-1} \cdot min^{-1}$. As a result, this study represents the highest $PM \cdot MET^{-1} \cdot min^{-1}$ performed in this laboratory at an exposure of 250 $\mu g \cdot m^{-3}$. For comparison, a comparable study series evaluated the effects of different cookstove air pollution outcomes[28, 81]. Using five different cookstove types, three of which were gas-burning (liquified petroleum gas, gasifier, ad fan rocket elbow) and two wood burning cookstoves (rocket elbow and three stone fire), they approximated household PM doses ranging from 960 to 55,560 $PM \cdot MET^{-1} \cdot min^{-1}$ for individuals during a seated (1 MET), two-hour exposure.

Air Pollution-Induced Autonomic Modulation

The inhalation of air pollutants, such as WS, perturbs the autonomic nervous system (ANS) and triggers autonomic reflexes in pulmonary receptors, baroreceptors, and chemoreceptors, leading to altered functional outcomes in the CV system. Specifically, recent studies suggest that pollutants can stimulate defensive sensory nerves within the cardiopulmonary system, thus providing a possible mechanism for pollutant-induced autonomic dysfunction[82]. Inhalation of pollutants triggers ischemic heart disease and cardiac arrhythmia in susceptible individuals. The dysregulation of the ANS has been implicated in these events based on three lines of evidence. First, association studies suggest that pollutants cause tachycardia, hypertension, and reductions in the high-frequency domain (HF) of HRV and other cardiac conditions[14, 26]. Second, pollution-evoked changes in CV parameters are rapid, mimicking the acute effect of pollutants on CVD-associated hospitalizations[82]. Third, association studies indicate a protective effect of β-adrenoceptor inhibitors on pollution-evoked CV events[24]. Cumulatively, there is substantial evidence that inhalation of pollutants acutely shifts the autonomic balance from sympathoinhibition to sympathoexcitation, a significant risk factor for serious CV events[83]. Interestingly, association studies and controlled exposure studies in healthy young individuals indicate that acute pollutants such as PM have no significant effect on HR, BP, or metrics of HRV or cause bradycardia or hypotension[82]. This suggests that pollutants cause distinct autonomic responses, which could depend on preexisting CVD since CVD individuals have sympathoexcitatory responses, and healthy individuals have sympathoinhibitory responses.

Communication between the immune system and the brain is vital for controlling inflammation. The inflammatory reflex is a centrally integrated physiological mechanism in which afferent vagus nerve signaling, activated by cytokines or pathogen-derived products, is functionally associated with efferent vagus nerve-mediated output to regulate proinflammatory cytokine production and inflammation (Figure 4; Taylor-Clark 2020)[82]. The absence of this inflammatory reflex, resulting from neural lesions or genetic ablation of essential components, results in excessive innate immune responses and cytokine toxicity[84].

Figure 4. Schematic showing the basic neural circuitry of CV reflexes evoked by afferent stimulation within the cardiopulmonary system.[82]



The ANS controls variations in the interval between consecutive heartbeats and can be quantified by measuring HRV[85]. Consistent findings show that WS exposure under controlled conditions can significantly decrease HRV, a commonly examined endpoint of ANS function[14]. Unosson et al. displayed that general measures of HRV are reduced following WS exposure, which appears predominantly due to a reduction in HF components of the spectrum, suggesting vagal inhibition[15]. In addition, Unosson et al. displayed a small (but non-significant) increase in LF components, which may represent an increase in sympathetic activation. The alteration in the balance of autonomic innervation to the vasculature may explain the increased arterial stiffness and acute hemodynamic effects.

The vagus nerve regulates metabolic homeostasis by controlling HR, gastrointestinal motility and secretion, pancreatic endocrine and exocrine secretion, hepatic glucose production, and other visceral functions. In addition, the vagus nerve is a significant constituent of a neural reflex mechanism—the inflammatory reflex—that controls innate immune responses and inflammation during pathogen invasion and tissue injury[86]. Inflammation is usually a local and temporary event. However, disrupted immune regulation can result in continual pro-inflammatory cytokine activity and excessive or chronic inflammation.

Afferent vagus nerve fibers sense peripheral inflammatory molecules and convey signals to the brain and are, therefore, important for immune-to-brain communication[86]. Afferent vagus neurons in the nodose and jugular ganglia terminate primarily in the nucleus tractus solitarius in the brainstem medulla oblongata[87]. Afferent signaling is further communicated through neural contacts between brainstem nuclei, the hypothalamus, and the forebrain regions associated with the integration of visceral sensory information and coordination of autonomic function and behavioral responses[87].

Peripheral administration of bacterial lipopolysaccharide (or endotoxin) or IL-1β causes afferent vagus nerve activation[87]. IL-1β receptors expressed on vagus nerve afferents and chemosensory (glomus) cells in paraganglia surrounding afferent vagus nerve endings have been implicated in recognizing immune activation [87]. Accordingly, afferent vagus nerve endings appear essential for relaying information about immune status to the brain when proinflammatory cytokines are present at relatively low levels. However, TLR4 (which can be activated by lipopolysaccharide) is expressed in the nodose ganglion, which suggests a mechanism by which lipopolysaccharide and other inflammatory molecules can activate vagus nerve afferents above their visceral endings[88].

As such, multiple neuronal and humeral factors modulate activity in preganglionic parasympathetic neurons in the medulla and preganglionic sympathetic neurons in the spinal intermediolateral cell column. Sensory afferent activity (based on conditions within peripheral tissues), including from the spinal dorsal root ganglia (DRG) or cranial nerves such as the trigeminal, glossopharyngeal (via petrosal ganglia), and vagus, has profound effects on autonomic efferent activity. As illustrated in Figure 4, the combination of aerobic exercise, inhalation of PM, and potential inflammatory response can provide significant input into group III and group IV muscle afferents and potentially affect the ventilatory and CV responses to the bout of activity. This is further supported in acute exposures to WS as immediate increases in central arterial stiffness and a simultaneous reduction in HRV, an indicator of ANS innervation status, have been observed[15].

Heart Rate Variability

HRV reflects the ANS control of the heart. It is quantified in the time domain with measurements of the interval between consecutive heartbeats and in the frequency domain, which measures the absolute or relative amount of signal energy within component bands[15]. An optimal level of HRV is associated with cardiac health[83]. The inability to maintain a high resting HRV, or exhibiting delayed rebound after exposure to a stressor, predicts an increased risk for mortality and sudden cardiac death[85]. Previous longitudinal findings demonstrated decreased HRV in those regularly exposed to WS PM in occupational settings[14]. Notably, depression of HRV after acute smoke inhalation signals problematic responses to reoccurring exposure.

Controlled chamber exposure to air pollutants, such as concentrated ambient fine and ultrafine PM, can significantly decrease HRV among middle-aged healthy subjects[89]. In one study, 14 subjects underwent a 3-h controlled exposure to a mean PM_1 concentration at 314 $\mu g/m^3$ of wood smoke generated through pyrolysis of birch wood with intermittent moderate exercise. The authors found that HRV markers were significantly decreased 1-h after wood smoke exposure compared to filtered air[15]. In another study, 43 subjects participated in various training exercises that included activities to extinguish fires fueled by either wood alone or by wood and household materials [27]. They found that WLFF activity was associated with decreased HRV and microvascular

function measured as reactive hyperemia index. On the other hand, a few studies have reported no significant changes in HRV following exposure to different types of WS (13, 222, 385, and 485 $\mu g \cdot m^{-3}$)[90, 91].

Our laboratory previously examined the effects of 45 minutes of exercise at 70% VO2max while inhaling 250 $\mu g \cdot m^{-3}$[11]. Results indicated no statistically significant alterations existed for HRV for up to 90 min after exposure, a finding that does not discount the harmful effects of acute smoke inhalation. In contrast to our results, HRV was systematically reduced in 14 subjects after a 3-h intermittent exercise session with similar exposure concentrations (~300 $\mu g \cdot m^{-3}$)[15]. This divergent outcome may indicate that the duration of smoke exposure within the current study did not elicit changes, highlighting the need to identify a WS exposure (duration and $PM_{2.5}$ concentration) threshold that perturbs CV function. Moreover, age, fitness level, health status—and the duration of HRV assessment (e.g., more extended sampling periods)—should be examined.

Pulse Wave Velocity

Elevated arterial stiffness is a characteristic of large artery pathology, a significant contributor to CV disease, and may indicate preclinical atherosclerosis and hypertension[92, 93]. Assessment of arterial stiffness is done through ultrasound or measurement of PWV. PWV of more than 10 m/s is indicated as an index of asymptomatic organ damage, a sign of CV disease progression that increments global CV risk. Moreover, aortic stiffness has gained independent predictive value for fatal and nonfatal CV events in hypertensive patients[94-96] and could thus reclassify intermediate-risk patients into higher or lower CV risk [95, 97, 98].

The aortic augmentation index (AIx) is an indirect measure of systemic arterial stiffness based on PWV and is calculated as a percentage. A recent meta-analysis demonstrated that a 10% increase in AIx% was associated with a 31.8% increased risk of CV events and a 34.8% increased risk of total mortality[96]. The AIx has been successfully implemented in occupational research settings[99, 100].

PM exposure has been associated with increased arterial stiffness in occupational cohorts. Fang et al. examined changes in AIx in 26 welders over 24 hr. on welding and non-welding days. Following welding fume exposure, the authors observed an increase in the afternoon AIx% and a decrease in the following day's values[99]. The results suggested that exposure to welding fume particulate is associated with acute adverse vascular responses. In a homogenous group of healthy workers, researchers observed higher AIx% values for participants with higher oxidative stress values[8].

As previously mentioned, the PM doses in the STOVEs study series were much less than what has been displayed in our laboratory. However, PWV may be more responsive to exposures between 1 and 3 hours in duration in a non-dose-dependent manner[28]. In the evaluation of cookstove air pollution, while also evaluating the effects of other cook stove types, Walker et al. observed significant increases in 48 subjects in the

rocket elbow and three stone fire treatment groups, which were 2-hour duration WS treatment groups with a mean exposure of 250 and 500 $\mu g \cdot m^{-3}$, respectively[28]. Notably, a difference compared to the control was most significant in the 24-hour post-trial measurement, a time point used in our proposed trial design (~22 hours post). As such, evidence supports the more significant duration weight on potential PM dose than intensity.

In a recent paper, based on the same exposure scenario and study population as in the present study, researchers investigated the effects of incomplete combustion of birch wood logs, representing commonly occurring exposures with chimney stoves[15]. The results from this study confirmed that WS may indeed cause acute CV effects, demonstrated by increased arterial stiffness and reduced HRV, which in population-based studies have been strongly linked with adverse health effects [15, 83].

Blood Biomarkers

Our laboratory has previously investigated the impacts of inhalational exposures, often mimicking occupational exposures in wildland firefighting. We first aimed to identify the human health risks of PM2.5 in a dose-response manner. Initially, markers of inflammation and oxidative stress, including PTX3, pH, and 8-ISO in EBC and plasma, were sensitive to 90 minutes of low- to moderate-intensity exercise during WS inhalation [7, 9].

Isoprostanes are a stable biomarker of oxidative stress. Still, their half-life is short in the blood (minutes) and more prolonged in urine (hours)[101] and can remain elevated in patients with acute lung injuries [102]. The relative change from pre- to immediate postexposure 8-ISO levels in EBC following WS exposure is significantly lower than the relative change following filter air exposure. WS resulted in considerably higher 8-ISO than filtered air at 1-hour post exposure[7].

Ferguson et al. assessed the effects of WS exposure on both airway and systemic inflammatory markers [7]. Here a conventional woodstove burned Western larch for 1.5 h to generate soot/organics dominated WS while participants continually exercised on a treadmill. In EBC samples, 8-ISO increased one hr. post-exposure, and the pH decreased immediately. The authors concluded that these results suggest some trends to show that this type of exposure leads to airway and systemic inflammatory effects.

Clara cell secretory protein (CC16) has been investigated as a potential biomarker of lung epithelial injury in numerous disease states and occupational or environmental lung injury. CC16 is a secreted product of the respiratory epithelium produced within the lung, primarily within the Clara cells of the distal respiratory and terminal bronchioles[103]. The biological function of CC16 remains incompletely understood. However, CC16 has been demonstrated to interact with multiple components of the inflammatory and coagulation cascades and may play a role in attenuating inflammatory responses [104]. After exposure to WS, serum CC16 has been shown to increase after 4 hours and display a diurnal

variation. Overall, CC16 seems to be a sensitive marker of the effects of air pollution in serum, but its function and significance need to be clarified[104].

Tumor necrosis factor alpha (TNF-α) is a cytokine with pleiotropic effects on various cell types. However, it has been identified as a significant regulator of inflammatory responses and is known to be involved in the pathogenesis of some inflammatory and autoimmune diseases[105]. Of WS exposures, TNF-α is one of several potent cytokines released by activated epithelial cells and macrophages and has been shown to increase with biomass smoke exposure[106].

Exhaled Breath Condensate

EBC collection is a non-invasive technique for obtaining lower respiratory tract biosamples. EBC is obtained as breath is exhaled from the lungs into a cooled collecting device, condensing the vapor emerging with the breath[107]. All nonvolatile compounds found in EBC originate in the airway lining fluid or are reaction products of volatiles. Fundamental to the critical concerns in biosample collection, the non-invasive procedure for EBC collection does not influence airway function or perturb the biochemical processes examined within resulting samples (e.g., inflammation). Because of the rapid, non-invasive nature of EBC collection, the technique is amenable to exercise and work physiology applications[108, 109].

Measuring the acidity, or pH, of EBC is a validated technique that is a reproducible and relevant marker of disease[44]. To assess the validity of the sample collected, the acidity and the presence of salivary amylase will be conducted, in addition to running an inflammatory profile on the samples later. This method of EBC measurement is the most reproducible for healthy and asthmatic subjects [110, 111]. The mean pH of healthy subjects is 7.7, with a typical range of 7.4-8.8[110]. Alteration of airway pH may serve as an innate host defense mechanism, but unregulated, it can lead to the pathological processes underlying inflammatory diseases. A decrease in the pH of airways causes bronchoconstriction and impairs multiple aspects of airway function[112].

Studies evaluating pulmonary effects by measuring nonspecific cytokines in EBC show the potential of using EBC for detecting acute oxidative stress and inflammatory responses among WLFFs who repeatedly experience high levels of WS exposure[113]. However, in a recent study, healthy WLFFs were recruited, and their EBC was collected before, after, and the following day following prescribed burns[12]. An oxidative stress biomarker, 8-ISO, and pro-inflammatory biomarkers, including interleukin-6, interleukin-8, C-reactive protein, and soluble intercellular adhesion molecule-1 were measured in EBC to determine acute pulmonary responses among the WLFFs following the WS exposure. But the study showed little WS exposure effect on this population's acute pulmonary reactions. These findings have been replicated in our laboratory[7]. However, the proposal of a 120-min duration protocol at a PM concentration of 250 $\mu g \cdot m^{-3}$ may elicit significant changes in an EBC biomarker panel, as evidenced in a workshift study with welders who

displayed increased pre-shift inflammatory markers across the work week[114]. However, it may also be more likely to observe differences in increased relative levels of these biomarkers in serum compared to EBC due to assay detection limits [12], although EBC changes were more significant than those in plasma. The overall trends in the markers analyzed were inconsistent with a normal-dose response relationship[7]. Our evaluations will concisely focus on 8-ISO to align with the findings of previous similar studies and inform results in our key dependent variables and blood biomarkers.

Pulmonary Function Testing

PFTs are noninvasive tests that show how well the lungs are working. The tests measure lung volume, capacity, rates of flow, and gas exchange. Common test measurements conducted via spirometry include Functional Vital Capacity (FVC), Forced Expiratory Volume (FEV1), FEV1/FVC displayed as a percentage (FEV1%), and Maximum Voluntary Ventilation (MVV). FVC is the air volume that can forcibly be blown out after maximal inspiration. FEV1 is the air volume that expires during the first second of a maximal expiratory effort undertaken after maximal inspiration. FEV1% is the percentage of the vital capacity expired during the first second of a maximal expiratory effort undertaken after maximal inspiration. MVV measures the maximum air a person can inhale and exhale voluntarily [5].

In simulated exposures comparable to wildland firefighting, spirometry results showed no significant changes following WS PM2.5 exposures[115]. There was no impairment in lung function measured at the post-exposure time point, but there were slight reductions in FVC for the 250 $\mu g \cdot m^{-3}$ and 500 $\mu g \cdot m^{-3}$ exposures at the 1-hr post time point (-0.07 and-0.04 L, respectively). This same trend was observed in the FEV1 measures with a post-exposure reduction at the 250 $\mu g \cdot m^{-3}$ trial (-0.19 L) and reductions of 0.27 L (250 $\mu g \cdot m^{-3}$) and-0.05 L (500 $\mu g \cdot m^{-3}$) measured in the 1-hr post-exposure spirometry tests. Consistent with the FEV1 measures, there were insignificant reductions in the ratio of FVC:FEV1 post-exposure at the 250 $\mu g \cdot m^{-3}$ trials (-3.40) and negligible reductions in 1-hour post-exposure measures for 250 $\mu g \cdot m^{-3}$ (-3.02) and 500 $\mu g \cdot m^{-3}$ (-0.29) exposure trials.

Cumulatively, PFT results from this study were consistent with previous studies [91, 116, 117] where, in healthy individuals, no significant changes in lung function were observed following controlled acute WS exposures. Previous field studies investigating the influence of wildland fire smoke on WLFF lung function, on the other hand, have shown significant effects from smoke inhalation[38, 118]. Liu et al. gathered spirometry data from sixty-three "seasoned" WLFFs before and after a season of fighting wildland fires[38]. Significant declines in mean FVC and FEV1 values were observed post-season (0.09 and 0.15L/s, respectively).In a comparable study, Betchley et al. observed similar decreases in FVC and FEV1 in a cohort of 76 volunteers following an entire season of fighting wildland fires[118]. In a more recent study, Adetona et al. observed no significant differences in the

across workshift changes on burn days compared to those on nonburn days for all the spirometry measures[5]. However, for a given point during the season, each additional day of exposure was estimated to be associated with declines of 24 ml in pre-shift FVC and 24 ml in pre-shift FEV1 ($p < 0.01$). The authors also concluded that cumulative exposure to WS was associated with slight decrements in lung function among WLFFs[5]. Compared to studies in a controlled environment, these field studies had much longer exposure durations and higher WS concentrations generated from multiple fuel types. However, Adetona et al. noted that it was possible that observed cross-shift changes may not be different from changes that would otherwise have been observed during workshifts when the WLFFs did not work at prescribed burns or wildfires[119] or that the changes were confounded by the normal circadian variation in lung function[120].

Cold Pressor Test

The CPT has been used for over 100 years to examine the sympathetic vasomotor response in humans. Briefly, the cold stimulus of submersing an individual's hand in the water for some time activates afferent sensory pathways that trigger a sympathetic response. Suppose afferent or efferent neuronal circuits and pathways are damaged. In that case, the sympathetic vasomotor response is impaired; therefore, the CPT's BP and HR responses can vary significantly. The researchers suggested that the CPT could identify subjects at risk of developing future hypertension[121]. Notably, subjects previously deemed at risk for developing hypertension demonstrated a 'hyperreactive' response close to those seen in patients with hypertension. The cause of abnormality in the autonomic nervous system control of BP in hypertension has been studied for many years. It has led to the 'neurogenic hypothesis of hypertension,' which describes the imbalance of elevated sympathetic nervous system activation and parasympathetic nervous system impairment in essential hypertension[122]. Evidence for increased sympathetic activation underlying high BP includes several measures of resting sympathetic nervous system activity[123], which firmly establish elevated sympathetic nervous system activity as a feature of hypertension and have revealed multifactorial causes, such as alterations in respiratory pattern, leading to vascular inflammation and angiotensin II-mediated sympathoexcitation[124]. Notably, the effects of elevated sympathetic nervous system activity include insulin resistance, high coagulation, arterial stiffening, and vasoconstriction[125].

Resultingly, our study will stratify study participants by CPT outcome, first identifying ten individuals that produce a hyperactive response (CPT^{+}; $\Delta SBP > 20mmHg$) and then identifying ten individuals from the remaining sample population (CPT^{-}; $\Delta SBP < 20$ mmHg) to obtain a group of 20 individuals to perform a singular bout of moderate exercise with WS exposure.

MEQ Chronotype

The human sleep-wake cycle synchronizes with the day-night process through external signals, such as ambient light, food, and physical activity[126]. Due to individual differences in the reaction to these external signals, different chronotypes exist. Individuals who sleep and wake up early are defined as early or morning chronotypes, while those with later bedtime and wake-up time are defined as late or evening chronotypes[127]. Notably, the biological differences between the chronotypes stretch beyond sleep timing, involving differences in body temperature, circadian phases, hormone secretion patterns, the timing of alertness, and disease risk[128]. Observational studies show associations between evening chronotypes and adverse health outcomes, including CVD and metabolic disorders[129].

The study of circadian rhythms has yielded further insights into the etiology of CV disease. Myocardial infarction, sudden cardiac death, and stroke do not occur randomly throughout the day but occur most frequently in the morning, with a smaller secondary peak in the early evening[130]. Several physiological and neuroendocrine parameters that may mediate or trigger the occurrence of clinical CV events also display similar circadian rhythms[131, 132]. Measures of sympathetic activity such as HR, BP, and catecholamines rise and peak during the morning or soon after. In addition, cortisol peaks early in the morning, around waketime[133], as does platelet activity[134]. Although sympathetic activity appears to rise during the morning, measures of parasympathetic activity (e.g., as measured by vagal tone) may undergo a withdrawal during this time[135].

Examining the circadian variation of physiological reactivity and absolute response levels to stressors is critical to study design and methodology. CV reactivity refers to changes from baseline HR, BP, or other CV parameters in response to stress[72]. In contrast, absolute levels of CV variables observed during stress reveal information about baseline levels and the stress reaction. Horne and Ostberg (1976) developed a Morningness/Eveningness Questionnaire (MEQ) to ascertain individuals' preferences for what time of day they best like to engage in a variety of activities such as sleeping, exercise, and mental activity, as well as when they feel the best in general[136].

Several explanations for the association between evening chronotype and cardiometabolic disorder have been suggested. One hypothesis is an unhealthier lifestyle, as later chronotypes show lower sports participation, later eating time, more smoking, and higher alcohol consumption[129]. Another explanation is that a difference in sleep timing between working and free days may lead to circadian misalignment and chronic sleep insufficiency, which has been related to health problems[128]. Circadian misalignment can contribute to obesity, while the adipose tissue can secrete many active compounds with potential repercussions to cardiometabolic health[129]. In addition, shift work can harm health, especially when not aligned with the worker’s chronotype. Still, though, the molecular mechanisms that connect the evening chronotype to metabolic disorders are unknown, and only a few studies have investigated the biomarkers profile in chronotypes.

There is a lack of studies exploring the relationship of chronotype with circulating protein biomarkers.

As previously mentioned, and combined with CPT stratification, our study design will seek to identify individuals with the most significant potential for hyperreactivity during morning work (malaligned chronotype) in the CPT^{+} group and then the opposite stratification in our CPT^{-} group. From here on out, these groups will be referred to by CPT outcome alone, with an implied MEQ chronotype associated with each group. Figure 5 below illustrates our unique stratification ideology, where CPT^{+} has a corresponding MEQ chronotype of intermediate or evening. In contrast, CPT^{-} individuals have a MEQ chronotype of the morning.

Figure 5. Study Participant Stratification

	CPT	
MEQ	CPT^{+}	CPT^{-}
Morning		X
Intermediate	X	
Evening		

Chapter III: Methodology

Participants

Approval to examine human research participants was obtained from the University of Montana Institutional Review Board. Participants were recruited from the Missoula, Montana community, and each provided informed written consent before initiating this study. Participants completed a health screening questionnaire and informed consent before beginning the study (see Appendix C: IRB# 172-22: "Cold Pressor Test and Circadian Chronotype as Predictores of Physiological Responses to Woodsmoke Inhalation During Exercise"). Inclusion criteria included the absence of known pulmonary conditions or recent contraction of SARS-CoV-2 (COVID-19) and to have a normal wake-sleep cycle (begin sleep at 10 PM; wake at 6 AM).

Cold Pressor Test and MEQ Chronotype Identification

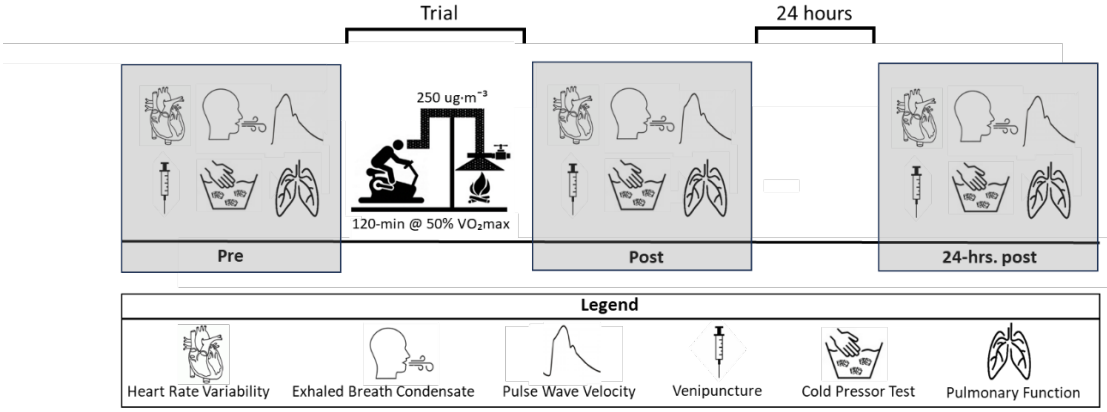
Potential participants filled out an informed consent before completing a CPT and MEQ (see Appendix D: MEQ) to determine study eligibility. Participants were chosen randomly to complete this first visit until ten participants were identified as having a hyper-reactive response to the CPT (> 20 mmHg increase in SBP during CPT) and had a MEQ score that placed them as either an intermediate or evening chronotype. Then ten CPT^{-} individuals (< 20 mmHg increase in SBP during CPT) with a MEQ score that identified them as a morning chronotype were selected from the total sample population.

Study Design

In total, ten males and ten females were selected, of which five CPT^{+} (BP "hyperreactive" + MEQ [Intermediate or Evening]) and five CPT^{-} (BP "non-reactive" + MEQ [Morning]) were identified in each gender. After the initial visit, participants completed baseline testing for VO_2max and body composition on a second visit. Body composition was measured via hydrostatic weighing (Exertech, Dresbach, MN). Body density values were corrected for residual lung volume (residual lung volume=(0.01115·age)+(0.019·Height)–2.24), and body volume estimates were converted to percent body fat using the Siri Equation[137]. The VO_2max test was performed on a cycle ergometer (Velotron, Spearfish, SD). Following a 3-min warmup at 60 watts, participants received an additional 40 watts each min until they could no longer maintain 75 rpm. Expired gas was sampled using indirect calorimetry (Cosmed Quark CPET, Concord, CA).

Participants reported to the air inhalation facility for the third visit to perform a single stationary-bicycle workout. The visit consisted of a 120-minute exposure to 250μg·m^{-3} of WS during exercise (Figure 1). Exposure trials were conducted at the inhalation and pulmonary physiology core at the Center for Environmental Health Sciences at the University of Montana. Fires were generated using an in-line inhalation system woodstove stoked with western larch (Larix occidentalis Nutt). Fires were prepared 30 min before the exercise using 1 kg of wood and kindling and were stoked with 300 g of wood every 15min throughout the trial. Participants were exposed to 250μg·m^{-3} of WS transferred from a wood-burning stove to a modified facemask covering the nose and mouth and worn while riding. Smoke concentrations were monitored using PM2.5 monitors (DustTrak, TSI, Model 8530, Shoreview, MN) and titrated with fresh ambient air to maintain the prescribed smoke dose for each trial. All trials had a 120-min mean exposure of 250±1 μg·m^{-3}. Water breaks (1 min) were allowed at 30 min intervals during the trial.

Figure 6. Exercise and Smoke Trial Overview.



The key dependent variables in this study were HRV, PWV (including augmentation index; AIx), and PFT (Figure 6). Initial HR data, including R-R intervals, were obtained using the Polar Vantage V2 watch and Polar H10 HR sensor and archived in the Polar Flow app (Polar Electro USA, Lake Success, NY). HRV measurements were collected from HR measurements 12 hr. before the smoke exposure trial and 24 hr. after the exposure trial, including 10-min intervals immediately before and after the exercise and WS exposure. HRV metrics were acquired by exporting recorded HR files into the Kubios software (Kubios, V 2.2, Joensuu, Finland) for analysis. HRV indices, based on established methods, included RMSSD, HF, LF, and LF: HF ratio of raw LF and HF [13, 138].
Data was collected immediately before the exercise trial (pre), immediately after (post), and again 24 hours after the conclusion of the exercise trial (Figure 1). Upon arrival at the facility for the exposure trial, participants immediately performed a 10-min seating session of exhaled breath condensate collection, as previously described[139]. PWV, AIx, and BP were obtained using the SphygmoCor XCEL device (Atcor Medical, Sydney, Australia) after 10min of supine rest. Right-side measurements were recorded until three values were obtained within 0.5 $m \cdot s^{-1}$. The femoral cuff was placed around the upper thigh, and a carotid pulse was identified using applanation tonometry. PWV between the carotid and femoral arteries was calculated based on the arterial stiffness formula: PWV=distance (m)/transit time (s).

Blood was collected via venipuncture through an antecubital vein. Hematocrit and Hemoglobin (Hemocue, Brea, CA) testing were done immediately following venipuncture. Samples were collected in 10-mL heparinized tubes, centrifuged at 1500 rpm for 15 min at 4°C, aliquoted, and stored at –80°C until assayed. A blood oxidative stress biomarker (8-isoprostane) was assayed using enzyme-linked immunosorbent assay (ELISA) kits (Cayman, Ann Arbor, MI, USA). All kits were performed per the manufacturer's instructions. The same panel of biomarkers was assayed on both the blood and EBC samples. As previously described, EBC was obtained using a novel collection device designed to amplify the total sample volumes acquired and preserve physiologic pH [22]. Methods for EBC collection entail a 10-min sample collection consisting of breathing through a modified mouthpiece to contain EBC in plasticizer-free tubing submerged in ice water, which is then collected, aliquoted, and stored at -80°C until assayed.

CPT was performed after venipuncture to avoid complications with vasoconstriction. CPT protocol consisted of two baseline BP readings before the participant's hand was placed into a 12-quart tub of ice water filled with ice and water with an average temperature of 4.0±0.1°C as the participant submerged their hand into the ice water to the level of the styloid process of the radius and ulna. SBP, DBP, and HR were measured atomically three consecutive times during the 2-minute submersion using an OMRON IntelliSense BP Monitor (Model# HEM-907XL; OMRON Healthcare Inc., Bannockburn, IL). Following two-minute submersion, participants removed their hand from the ice water for a 2-minute monitored recovery. Pulmonary function was the last test

performed each time this battery of tests was performed (pre, post, 24-hours post). PFT was assessed by FVC, FEV1, and MVV tests using a spirometer (MIR Spirobank, Elicott City, MD), with participants tested in the seated position. Data were analyzed using the WINSPIRO Pro software (WINSPIRO PRO Version 7.8, Ellicott City, MD). Participants were provided with verbal encouragement during PFT.

Statistical Analyses

Repeated Measures Analysis of Variance (RMANOVA) was used to compare PRE, POST, and 24-hour data collection points. Post hoc analysis consisted of the Tukey test. Data for CPT variables (SBP, DBP, and HR), blood and EBC biomarkers, PFT variables, and HRV values between the CPT^{+} and CPT^{-} groups. Statistical significance was set to an alpha ≤ 0.05, a priori. Data are reported as mean ± SD. All data analysis was conducted utilizing the R Studio (Version 1.3.1093) software package.

Chapter IV: Results

Demographics

Ten active males (age = 24±5 yrs.; height = 181.7±5.1 cm; weight = 79.7±10.1 kg; VO_2max = 52.4±9.8 $ml \cdot min^{-1} \cdot kg^{-1}$; body fat = 14.4±8.1 %) and ten active females (age = 21±3 yrs.; height = 163.8±9.4 cm; weight = 64.8±4.7 kg; VO_2max = 40.1±2.5 $ml \cdot min^{-1} \cdot kg^{-1}$; body fat = 19.8±9.8 %) completed the study. Our population was recruited inclusively/exclusively into CPT cohorts (CPT^{+} vs. CPT^{-}) and is presented in Table 1. Two-sample equal variance test analysis indicated no significant between-group differences existed for CPT^{+} and CPT^{-}, save for MEQ scores ($p = 0.001$) which were higher in CPT^{-} participants by design.

Table 1. Demographics of Study Population (mean±SD)

Group	N	Age *(yrs.)*	VO2 max *($ml \cdot kg^{-1} \cdot min^{-1}$)*	Body Fat *(%)*	MEQ	Resting SBP *(mmHg)*	Resting DBP *(mmHg)*	Resting HR *(mmHg)*
CPT^{+}	10 *(5 male/ 5 female)*	25±6	49.4±11.1	17.0±9.0	26±7	116±10	66±9	62±11
CPT^{-}	10 *(5 male/ 5 female)*	21±3	43.2±6.6	16.1±8.1	60±10	112±10	66±5	62±14

Pulmonary Function

PFT data for time, group, and variable (FVC, FEV1, FEV1%, and MVV) are displayed in Table 2. For FVC, there was no interaction (P=0.840), trial (P=0.919), or time

effect (P=0.952). Similarly, FEV1 and FEV1/FVC exhibited no interaction (FEV1: P=0.854; FEV1/FVC: P=0.986), trial (FEV1: P=0.772; FEV1/ FVC: P=0.711), or time effect (FEV1: P=0.686; FEV1/ FVC: P=0.540). MVV also indicated no interaction (P=0.923), trial (P=0.633), or time effect (P=0.917).

Table 2. Measures of Pulmonary Function (mean±SD)

		FVC *(L)*	FEV1 *(L)*	FEV1/FVC *(%)*	MVV *($L \cdot min^{-1}$)*
Pre	CPT^{+}	5.0±1.0	4.0±1.0	81±6	146±57
	CPT^{-}	5.0±1.4	4.0±1.0	81.±7	117±44
Post	CPT^{+}	5.1±1.1	4.2±0.9	79±14	153±59
	CPT^{-}	4.9±1.4	4.0±1.0	83±10	104±37
24-hr	CPT^{+}	5.0±1.0	4.1±0.9	82±7	151±58
Post	CPT^{-}	4.9±1.5	4.0±1.0	82±8	107±45

Cold Pressor Test

Although participants were grouped according to pre-screening CPT outcomes, CPTs were repeated for the exercise exposure trials and recorded at pre-, post-, and 24-hour post-time points. Repeated measures ANOVA for HR responses during the CPT (Figure 7) indicated no significant group differences were present for HR during the CPT throughout the 2-minute CPT test.

Figure 7. Mean heart rate during 2-min CPT before, immediately post, and 24 hours post moderate exercise with wood smoke exposure.

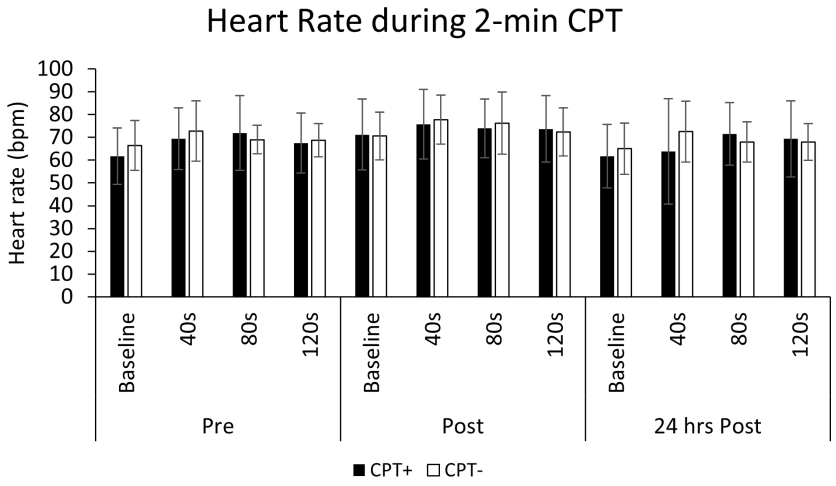
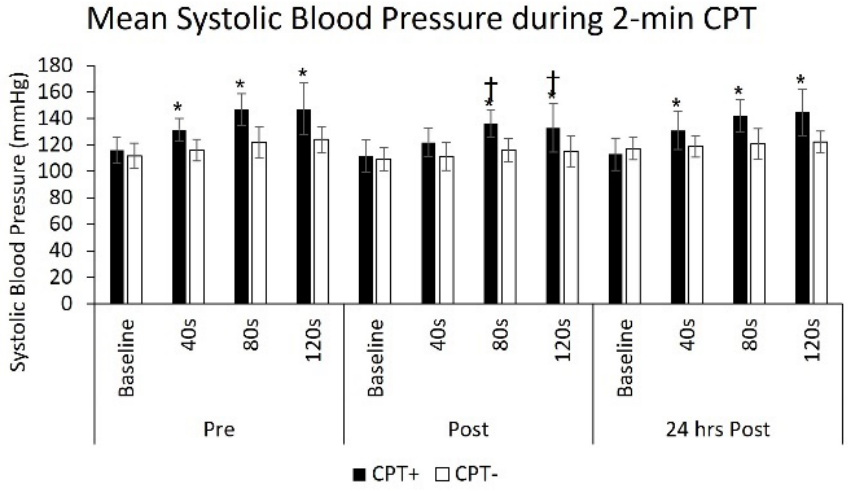


Figure 8 (SBP) and 8b (DBP) present time-dependent (baseline, 40 sec., 80 sec., 120 sec) BP profile responses during the CPT for both cohort groups. CPT data were

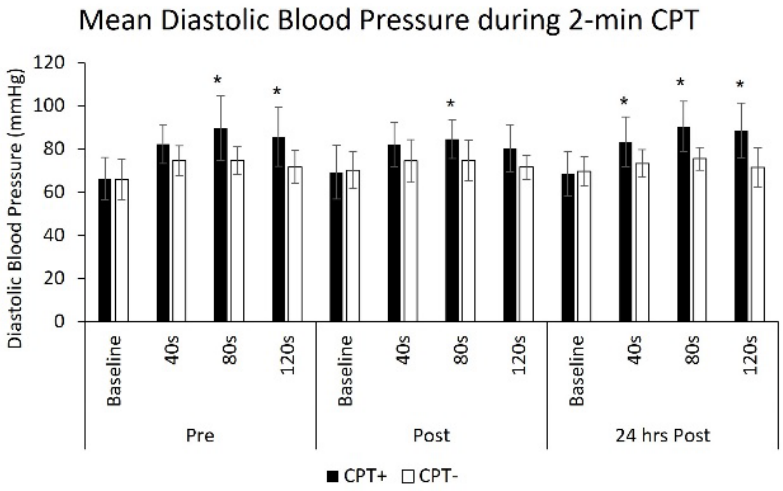
conducted before, after, and 24 hours after a 2-hour bout of moderate exercise with smoke exposure. Repeated measures ANOVA analyses indicated significant group differences existed for SBP. The Tukey test was used for post hoc analysis. No significant differences existed between groups for Baseline values collected at any data collection time point. At 40-seconds, the CPT^{+} and CPT^{-} groups were significantly different at the pre and 24-hour post-trial data collection points (Pre: $^{+}$ = 142±9 mmHg, CPT^{-} = 116±8 mmHg, p = < 0.05; 24-hour post: CPT^{+} = 131± 14 mmHg, CPT^{-} = 119±8 mmHg, p = < 0.05). At 80 seconds, the groups were significantly different at all three data collection points (Pre: CPT^{+} = 147±12 mmHg, CPT^{-} = 122±12 mmHg, p = < 0.05; Post: CPT^{+} = 136±10 mmHg, CPT^{-} = 116±9 mmHg, p = < 0.05;24-hour post: CPT^{+} = 142±12 mmHg, CPT^{-} = 121±12 mmHg, p = < 0.05). Likewise, at the 120-second time points, the two groups were significantly different at all three data collection points (Pre: CPT^{+} = 147±20 mmHg, CPT^{-} = 124±10 mmHg, p = < 0.05; Post: CPT^{+} = 133±18 mmHg, CPT^{-} = 115±12 mmHg, p = < 0.05; 24-hour post: CPT^{+} = 145± 18 mmHg, CPT^{-} = 122±8 mmHg, p = < 0.05). Notably, the post-trial CPT BP was significantly less than the Pre-trial values (80s and 120s timepoints; p < 0.05).

Figures 8a and 8b. Mean Systolic (a) and Diastolic Blood Pressure (b) during 2-min CPT Pre, immediately Post, and 24 hours Post moderate intensity exercise with wood smoke exposure.

(a)



(b)



** - indicates a statistically significant difference ($p < 0.05$) between the CPT + and the CPT¯ groups at that respective time point. † - indicates a statistically significant difference ($p < 0.05$) between that respective data collection point and the Pre data collection point.*

DBP data following exposure trial CPTs are presented in Figure 8B. Repeated measures ANOVA also indicated significant between-group differences were present for DBP. The Tukey test was used for post hoc analysis. No significant differences existed at baseline between the groups at any data collection point. At the 40-seconds, the CPT^{+} and CPT^{-} groups were significantly different only at 24-hour post-trial data collection points (24-hour Post: CPT^{+} = 83±12 mmHg, CPT^{-} = 73±6 mmHg, p = < 0.05). At 80 seconds, the two groups were significantly different at all three data collection points (Pre: CPT^{+} = 90±15 mmHg, CPT^{-} = 75±6 mmHg, p = < 0.05; Post: CPT^{+} = 85±9 mmHg, CPT^{-} = 75±9 mmHg, p = < 0.05; 24-hour post: CPT^{+} = 90±12 mmHg, CPT^{-} = 76±5 mmHg, p = < 0.05). At the 120-seconds, the two groups were significantly different at the Pre and 24-hour post-trial data collection points (Pre: CPT^{+} = 86±14 mmHg, CPT^{-} = 72±8 mmHg, p = < 0.05; 24-hour post: CPT^{+} = 89±13 mmHg, CPT^{-} = 71±9 mmHg, p = < 0.05).

Central Arterial Stiffness

Table 3 presents the PWV and pulse wave analysis variables (PWV, pulse transit time [PTT], SBP, DBP, pulse pressure [PP], mean arterial pressure [MAP], and AIx) collected Pre, immediately, Post, and 24 hours Post the 2-hour bout of moderate intensity exercise with smoke exposure trial. Analysis of PWV, PTT, SBP, DBP, PP, MAP, and AIx indicated no main effects of time (PWV: P=0.666; PTT: P=0.325; SBP: P=0.596; DBP: P=0.466; PP: P=0.481; MAP: P=0.422; AIx: P=0.299) and no interaction effects (PWV: P=0.416; PTT: P=0.532; SBP: P=0.986; DBP: P=0.966; PP: P=0.999; MAP: P=0.942; AIx: P=0.341); however, a between-trial difference was observed in PTT, SBP, DBP, and MAP

(PWV: P=0.142; PTT: P=0.001; SBP: P=0.001; DBP: P=0.017; PP: P=0.305; MAP: P=0.006; AIx: P=0.868). Furthermore, AIx was significantly higher in the CPT^{+} group post-exercise (P=0.049), indicating increased measures of arterial stiffness in the CPT^{+} group after the bout of exercise with smoke exposure when compared to the CPT^{-} group.

Table 3. Measure of Central Arterial Stiffness (mean±SD)

		PWV *($m \cdot s^{-1}$)*	**PTT** *(ms)*	**SBP** *(mmHg)*	**DBP** *(mmHg)*	**PP** *(mmHg)*	**MAP** *(mmHg)*	**AIx** *(%)*
Pre	CPT^{+}	4.7±0.7	83±13	111±10	74±10	38±6	87±10	5.4±10.0
	CPT^{-}	4.7±0.5	84±9	106±6	72±6	34±7	85±6	-2.4±15.6
Post	CPT^{+}	4.8±0.6	78±10	112±7	75±10	37±7	90±10	12.1±16.9*
	CPT^{-}	5.1±0.9	79±7	106±5	75±7	31±6	88±5	-2.4±14.9
24-hr	CPT^{+}	5.1±0.6	77±7	109±9	75±12	34±7	87±12	6.6±15.5
Post	CPT^{-}	4.4±0.6	85±10	105±11	69±5	36±13	83±4	2.8±9.8

** - indicates a statistically significant difference ($p < 0.05$) between the CPT + and the CPT^{-} groups at that respective time point.*

Heart Rate Variability

HRV was quantified for lnRMSSD, lnHF, lnLF, and LF:HF (Table 4). Analysis of lnRMSSD indicated a significant group difference (P = 0.010) although no time (P = 0.858) or interaction effect (0.862). Post hoc analysis, however, did not achieve significance in any interaction. Analysis of lnHF again indicated a significant group difference (P=0.029) although no time (P=0.966) or interaction effects (P=0.751). LnHF and lnLF were both significantly lower differences between CPT^{+} and CPT^{-} at the post-trial data collection point (CPT^{+} = 5.1±1.8, CPT^{-} = 6.6±1.7; P < 0.05). Although analysis of lnLF did not indicate a significant group (P=0.0546), time (P=0.596), or interaction effect (P=0.536), CPT^{+} was again significantly lower at the post-trial data collection point (CPT^{+} = 6.4±1.2, CPT^{-} = 7.3±1.0; P < 0.05). No significant group, time, or interaction effects were observed in LF: HF ratio (P=0.974, P=0.590, and P=0.654, respectively).

Table 4. 10-Min Measures of HRV (mean±SD)

		lnRMSSD *(ms)*	**lnHF** *(ms^2)*	**lnLF** *(ms^2)*	**LF:HF Ratio**
Pre	CPT^{+}	3.6±0.9	6.0±1.7	6.5±1.1	3.1±4.0
	CPT^{-}	3.7±0.8	6.6±1.5	7.0±0.9	2.2±2.2
Post	CPT^{+}	3.2±0.7	5.1±1.8 *	6.4±1.2 *	4.7±3.6
	CPT^{-}	3.8±1.0	6.6±1.7	7.3±1.0	3.4±4.5
24-hr	CPT^{+}	3.3±0.9	5.5±1.8	7.2±0.8	7.4±6.0 †
Post	CPT^{-}	4.0±1.0	6.8±2.0	7.8±1.4	3.5±2.2

** - indicates a statistically significant difference ($p < 0.05$) between the CPT + and the CPT¯ groups at that respective time point. † - indicates a statistically significant difference ($p < 0.05$) between that respective data collection point and the Pre data collection point.*

Oxidative Stress and Immune Function

Analysis of plasma 8-ISO, EBC 8-ISO, plasma CC16, and plasma TNF-α indicated no main effects of time (Plasma 8-ISO: P=0.327; EBC 8-ISO: P=0.620; CC16: P=0.990; TNF-α: P=) no interaction effects (Plasma 8-ISO: P=0.953; EBC 8-ISO: P=0.694; CC16: P=0.994; TNF-α: P=) and no between-trial difference (Plasma 8-ISO: P=0.473; EBC 8-ISO: P=0.638; CC16: P=0.839; TNF-α: P=).

Table 5. Markers of Oxidative Stress and Immune Function (mean±SD)

		Plasma 8-ISO ($ng \cdot mL^{-1}$)	**EBC 8-ISO** ($ng \cdot mL^{-1}$)	**CC16** ($ng \cdot mL^{-1}$)	**TNF-α** $ng \cdot mL^{-1}$
Pre	CPT^{+}	1.8±0.3	1.0±0.5	21.1±4.9	
	CPT^{-}	1.9±0.2	1.0±0.5	20.9±9.1	
Post	CPT^{+}	2.0±0.2	0.8±0.4	17.0±5.4	
	CPT^{-}	2.0±0.2	1.0±0.4	21.4±7.8	
24-hr	CPT^{+}	1.8±0.3	0.6±0.6	18.4±6.4	
Post	CPT^{-}	1.8±0.2	1.0±0.8	20.7±8.0	

Chapter V: Discussion

The current investigation was undertaken to evaluate if the physiological response to a moderate-intensity bout of exercise with WS exposure was exacerbated in individuals with increased autonomic reactivity (CPT responsivity) corresponding to the MEQ chronotype that favors activity in the afternoon/evening. The present investigation is an extension of previous studies by our lab, which have systemically investigated a strategic panel of physiologic outcomes following acute WS exposure during exercise. These measures include PWV, a measure of central arterial stiffness, and HRV, a measure of autonomic control. The rationale for this approach is that the key dependent variables utilized by our research group are sensitive to both acute and chronic stressors[11, 14, 27, 140], and may serve as surrogate metrics for CV aging in those exposed to frequent wildfire smoke events. Pursuing this line of inquiry, our investigations' key dependent variables of HRV and PWV have become intrinsic. The key findings of this investigation are that differences in HRV and PWV occur following a two-hour bout of moderate exercise (50% VO_2max) with WS exposure (250 $\mu g \cdot m^3$).

The significant increase ($\Delta = 6.7$; $p = 0.049$) in AIx following WS exposure with exercise is a key outcome in this investigation. Notably, this finding is significant in that we observed no change in AIx following the exposure trial in the CPT^{-} group. Within this scientific approach, we have previously not observed differences in AIx and PWV in young, apparently healthy individuals [11]. However, these prior observations were in the context of a 45-minute moderate-intensity exercise challenge undertaken at the same WS concentration[11]. The current investigation applied a scientific approach to evaluating healthy individuals that may have an exacerbated response to activity with WS exposure. In this regard, individuals were selected for having a relatively exaggerated response to the CPT by identifying individuals who were misaligned for the AM bout of exercise in terms of their chronotype. Overall, subtle yet distinct differences were observed between the groups post-exercise, even though minimal differences existed between the groups before the trial. In agreement with our prior works, $PM \cdot MET^{-1} \cdot min^{-1}$ was examined as a surrogate for smoke inhalation dose[11]. In this regard, a secondary hypothesis was that this $PM \cdot MET^{-1} \cdot min^{-1}$, being the largest we have analyzed to date, would impact PWV and HRV 24 hours post-exercise. Furthermore, our study's design provided convincing preliminary evidence for sustained change when stratifying for CPT and MEQ.

In health, HR is carefully controlled by the ANS. Alterations in PNS and SNS activity result in beat-to-beat HR variation; hence, HRV reflects ANS activity and was a key dependent variable in the current study. Following aerobic exercise, HR recovery induces PNS reactivation, while SNS remains elevated[141, 142]. Indeed, this rationale was validated in the current study's findings as indicated by lnHF values that were decreased following the two-hour trial of moderate exercise. Still, a more significant decrease remained at 24 hours post in the CPT^{+}, although not significant ($p = 0.17$). Furthermore, lnLF, while not a pure index of SNS drive, remained elevated in both groups at the 24-hour data collection point (24 -hr Δ $CPT^{+} = 0.7$ ms^2; 24-hr Δ $CPT^{-} = 0.8$ ms^2). As such, it can be interpreted that the parasympathetic rebound was more significant in the CPT^{+} group following exercise.

Regarding LF: HF ratio, a high LF/HF ratio indicates sympathetic dominance, which occurs when we engage in fight-or-flight behaviors or parasympathetic withdrawl[143]. While this concept has been challenged, mainly due to the profound variations of LF power, it is still notable that the LF: HF ratio was consistently elevated in the CPT^{+} group across all data collection points compared to the CPT^{-} group. Indeed, the 24-hour Post LF: HF ratio for the CPT^{+} group (7.4±6.0) vastly exceeded the short-term norm of 2.8±2.6 identified by Nunan et al. [144]. In contrast, the LF: HF ratio in the CPT^{-} group increased to a lesser degree following the bout of exercise with smoke exposure.

The observed decrease in lnHF following the bout of exercise with WS exposure also corresponded with a statistically significant reduction in SBP occurring during the immediate post trail CPT compared to the pre-trial CPT. While a decrease in resting BP was not observed using our Sphygomocor automated BP device (Table 3), the responsivity

of SBP during the CPT was affected (Figure 8), as anticipated. The explanation for this phenomenon is likely due to post-exercise hypotension (PEH), where a single bout of mild to moderate exercise can lead to a post-exercise decrease in BP in hypertensive individuals[145]. PEH can last up to 13 hours in humans, so it likely only contributed to CPT results at the immediate-post data collection point and did not influence the 24hr post-data collection. While PEH is commonly associated with a consistent decrease in SBP and DBP, our study observed only a non-significant reduction in DBP reactivity between the pre and post-trial data collection points.

PWV has emerged as a noninvasive, valid, and reliable measure of arterial stiffness and an independent risk predictor for adverse outcomes[146]. Based on this knowledge, chronic baseline deviations in resting PWV or stress-induced alterations in the metrics are predictive of elevated CV event risk. Within WS inhalation challenges, prior studies have examined the effects of WS exposure on PWV with equivocal results[7, 9, 29]. Our findings demonstrated that PWV was not statistically different after a 120-minute exercise session with WS exposure at 250 $\mu g \cdot m^{-3}$, a result similar to prior investigations[11, 29]. However, PWV, although not statistically significant, did remain elevated 24 hours post-exposure in the CPT^{+} group (+ 0.4 $m \cdot s^{-1}$), which is a marked increase when compared to the 0.2 $m \cdot s^{-1}$ increase reported 24 hours post by Walker et al. with their comparable two hours of passive exposure to WS[28]. Our results align with prior PWV research with passive exposures between 1 and 3 h in duration with PWV elevation 24 hours post-exposure regardless of dose[28, 29].

A surrogate measure of arterial rigidity, AIx reflects endothelium function and is calculated by measuring the augmentation pressure (difference between late and early systolic pressure) and dividing by PP x 100. As such, this indirect measure of arterial stiffness evaluates the reflected pressure wave from the periphery to the central arterial system. Our data demonstrated that AIx was statistically different between groups post-exercise, with an increase of mean AIx of 5.7 in the CPT^{+} and no change for the CPT^{-} group. The increase from baseline was sustained up to 24 hours post-exposure in both groups (CPT^{+} 6.6±15.5; CPT^{-} = 2.8±9.8). Previous research indicated AIx was elevated at a slightly higher dose of PM2.5 (~314 $\mu g \cdot m^{-3}$) after three h of exposure with intermittent exercise[15]; however, this response was not replicated when intermittent exercise was performed for one h at a PM2.5 dose of ~1000 $\mu g \cdot m^{-3}$[29]. Although these mixed findings cannot be explained currently, a proposed association exists between elevated oxidative stress and increased arterial stiffness, indicating they may occur in tandem[8]. Therefore, this study's exercise and exposure dose may serve as a threshold dose to elicit a measurable change in arterial stiffness. Collectively, the HRV and PWV outcomes are understandable, given that HRV outcomes are driven predominantly by physiologic alterations in autonomic control of the cardiovascular system (e.g., heart and large conduit arteries). In contrast, moment-to-moment alterations in PWV are subject to the complex influences of both autonomic and autoregulatory mechanisms (e.g., biochemical regulation, myogenic

control, etc.) of the vasculature. Accordingly, HRV appears to be more sensitive to the combined stressor of exercise and WS within these scientific contexts than PWV.

Despite minimal observed differences following acute WS exposures, PFT was examined as our investigation's final key dependent variable. Findings indicated that FVC, FEV1, and MVV were not statistically different for either group following the WS exposure at any time point. Importantly, these findings are consistent with previous work that demonstrated no changes in FVC, FEV1, or MVV after a range of acute WS exposures in our lab[7, 11] and highlight the fact that although smoke inhalation is detrimental to long-term health[37], acute physiologic responses to exposure may not always result.

A secondary aim was to incorporate oxidative stress markers and immune function to support our primary hypothesis. However, concurrent with prior studies in our lab, postexercise oxidative stress variables were not statistically different following acute WS exposure at selected time points[11]. Despite non-significant differences, we expanded our use of a novel technique for collecting EBC[139]. Notably, our approach has been shown to capture significant quantities of EBC for use in developing a biomarker panel to be explored cost-effectively. While the moderate-intensity, limited-duration exercise challenge did not result in pre- or post-trial differences for the variables examined, we demonstrated a proof-of-concept approach to collect a panel of relevant biomarkers from EBC biosamples. Moreover, these samples can be obtained from laboratory or field settings using a time-efficient non-invasive method.

Limitations

PWV is a measure of CV function. However, unrelated endocrine factors, such as autoregulation of the vascular system, also influence autonomic control. Due to combined antonymic and metabolic influences, PWV may not be overtly sensitive to vascular change within this scientific paradigm. HRV may indicate underlying ANS activity and represents a potentially valuable noninvasive tool for ANS evaluations. While HRV and PWV are not necessarily the best measures for autonomic control of CV systems, both were chosen because of their potential utility in evolving from laboratory to field settings, which was an intentional underpinning of the study. Lastly, a comparison for sex differences did not reveal significant differences in key dependent variables, although the sample sizes were relatively small following stratification procedures.

Chapter VI: Conclusion

In deploying these methods of assessing central arterial stiffness and autonomic function, it has become apparent that many difficult-to-control factors influence autonomic control and individual autonomic control following an acute bout of exercise with WS exposure. Despite largely non-significant numerical changes in PWV, our study's measures of HRV were sensitive despite a lack of alteration on oxidative stress markers to support. The design of this study, using the MEQ and CPT, allowed for the identification of a

potential at-risk subpopulation that provided insight into sensitive measures of lasting detriment from a single acute bout of WS exposure that would likely prove significant in an individual's response to repeated bouts of WS exposure seen commonly in occupational and recreational occupations in the western United States.

Chapter VII: Application of a Novel Collection of Exhaled Breath Condensate to Exercise Settings

Brief Description

The following article represents the initial investigation of my dissertation. The manuscript detailing this work was published in the *International Journal of Environmental Research and Public Health* in March 2022[139]. Throughout COVID-19 safety precautions, we obtained University of Montana Institutional Review Board approval, and all study participants provided informed written consent before initiation of data collection (see Appendix A: IRB#159-20: "Novel Collection of Exhaled Breath Condensate").

The study aimed to develop a cost-effective, reproducible methodology for obtaining large EBC sample volumes compared to samples obtained using more expensive single-use devices traditionally used for sample collection. Because sample volumes are typically insufficient to examine a robust series of biomarkers for an individual (e.g., five or more biomarkers within a given sample and sample time point), we found in prior investigations that traditional methods for EBC collection were sub-optimal for evaluating biochemical changes in provided samples following an acute physiologic challenge (e.g., exercise and WS inhalation). In addition, we observed that EBC samples collected from commercially-available devices exhibited a non-physiologic pH (when sample volumes were sufficient to make such an observation that was frequently not possible). Accordingly, our novel laboratory-based method was tested for sample viability based on pH. Finally, our goal for this novel laboratory device was to develop a methodological apparatus that could be obtained from any local hardware store in combination with commonly available laboratory devices (e.g., Hans Rudolph one-way breathing valve, single-use one-way cardboard respiratory valve). As part of the study, we obtained commercially-available EBC collection devices and compared them with our novel EBC collection device constructed in our laboratory. Notably, our novel EBC collection device produced larger (time-equivalent) sample volumes, permitting a complete panel of biomarkers to be examined in each biosample collected. We further demonstrated pH stability in EBC samples collected from our novel device, while EBC samples from the commercial device were rendered acidic during the collection process. Lastly, this technique was developed primarily in remote field settings such as wildland firefighting, using packs of ice readily available in camps and reusable equipment components that allowed for in-field analysis.

International Journal of
Environmental Research and Public Health

MDPI

Article

Application of a Novel Collection of Exhaled Breath Condensate to Exercise Settings

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Abstract: The collection of exhaled breath condensate (EBC) is a non-invasive method for obtaining biosamples from the lower respiratory tract, an approach amenable to exercise, environmental, and work physiology applications. The purpose of this study was to develop a cost-effective, reproducible methodology for obtaining larger volume EBC samples. Participants (male: $n = 10$; female: $n = 6$; 26 ± 8 yrs.) completed a 10 min EBC collection using a novel device (N-EBC). After initial collection, a 45 min bout of cycling at 75% HRmax was performed, followed by another N-EBC collection. In a subset of individuals ($n = 5$), EBC was obtained using both the novel technique and a commercially available EBC collection device (R-EBC) in a randomized fashion. N-EBC volume—pre- and post-exercise (2.3 ± 0.8 and 2.6 ± 0.9 mL, respectively)—and pH (7.4 ± 0.5 and 7.4 ± 0.5, respectively) were not significantly different. When normalized for participant body height, device comparisons indicated N-EBC volumes were larger than R-EBC at pre-exercise (+12%) and post-exercise (+48%). Following moderate-intensity exercise, no changes in the pre- and post-trial values of Pentraxin 3 (0.25 ± 0.04 and 0.26 ± 0.06 pg/mL, respectively) and 8-Isoprostrane (0.43 ± 0.33 and 0.36 ± 0.24 pg/mL, respectively) concentrations were observed. In a cost-efficient fashion, the N-EBC method produced larger sample volumes, both pre- and post-exercise, facilitating more biomarker tests to be performed.

Keywords: biosamples; exercise; exhaled breath condensate

Citation: Sol, J.A.; Quindry, J.C. Application of a Novel Collection of Exhaled Breath Condensate to Exercise Settings. *Int. J. Environ. Res. Public Health* **2022**, *19*, 3948. https://doi.org/10.3390/ijerph19073948

Academic Editor: Martin Burtscher

Received: 24 February 2022
Accepted: 23 March 2022
Published: 26 March 2022

1. Introduction

Accurate and reliable biosample collection is essential for quantifying resultant biomarker assays. While recent innovations have overcome many of the barriers to the collection of biosamples (i.e., blood/plasma, saliva, biopsies, etc.), no single approach solves all the most common concerns. Among the critical barriers to collecting viable biosamples are the concerns related to obtaining adequate volume, sample viability, expense related to experimentation, and the invasive nature of the collection process. Based on these collective concerns, new research efforts aim to find alternative methods for collecting biological samples in a reliable, non-invasive, cost-effective fashion that is also applicable to clinical, laboratory, and field research settings. Notably, acute woodsmoke exposure in wildland firefighting has increased in capacity as of late, although the quantification of biomarker response that occurs during chronic exposures is less documented [1]. With respect to wildland fire settings, understanding stress at the air-lung interface is a critical next step in quantifying the physiological and biochemical consequences of acute smoke inhalation. Accordingly, there has been renewed interest in collecting exhaled breath condensate (EBC) as a medium for subsequent biomarker assay in recent years.

EBC collection is a non-invasive technique for obtaining large amounts of biosamples from the lower respiratory tract. EBC is obtained as breath is exhaled from the lungs into a cooled collecting device, condensing the vapor emerging with the breath, and commonly collected for 10 min time periods [2]. All nonvolatile compounds found in EBC originate

in the airway lining fluid or are reaction products of volatiles. Fundamental to the critical concerns in biosample collection, the non-invasive procedure for EBC collection does not influence airway function or perturb the biochemical processes examined within resulting samples (e.g., inflammation). Because of the rapid, non-invasive nature of EBC collection, the technique is amenable to exercise and work physiology applications [3,4].

Among the primary reasons EBC is potentially useful to exercise and work physiology, the sample is directly influenced by the interface between the body and the environment, as experienced in the lungs. Accordingly, assays from EBC samples are promising for learning more about the pathobiology of various diseases and creating biochemical fingerprints of lung conditions in the future [3,5]. In addition, many inflammatory responses to environmental stressors (i.e., the introduction of airborne pathogens, inhalation of pollutants, etc.) are instigated by resident immune cells found in the capillary beds of the pulmonary system [6,7]. Based on this growing interest in EBC as a biological medium, commercial devices have proved successful in standardizing the EBC collection process [3]. Indeed, EBC has been measured for a number of years, with multiple commercially available devices established. Essential to this approach is the assurance that EBC samples do not contain saliva contamination (as evidenced by the presence of salivary amylase in levels found in human saliva), pH testing, and when sample volumes permit, the addition of a wide range of biomarkers. However, the use of EBC has remained limited in many experimental studies due to the cost of single-use devices and the limited sample volumes commonly collected.

Based on this rationale, the current study was undertaken as a proof-of-concept to develop a novel technique for collecting EBC in future investigations. The goals of the present study were to provide a method of novel EBC collection that achieved the following: (1) provide a more cost-effective method for obtaining viable EBC samples as compared to commercial devices; (2) to determine whether time-equivalent sample volumes obtained with the novel collection device were comparable to, or in excess of, those obtained with a commercially available device.

2. Materials and Methods

2.1. Participants

Before participant recruitment and testing, study approval was obtained from the University of Montana Institutional Review Board. Study participants (male: $n = 10$, female: $n = 6$) were recruited from the Missoula, Montana Community, and written informed consent was obtained before data collection. Each participant completed a personal information questionnaire. Study participants had no history of chronic lung disease or respiratory problems and were physically active. Before arrival at the lab, they confirmed they had not developed any respiratory infections or other health-related changes between recruitment and testing completion.

2.2. Study Design

For the overall study design, participants (male: $n = 10$; female: $n = 6$; 26 ± 8 yrs.) completed a 10 min collection of breathing through a novel EBC collection device (N-EBC) while seated and wearing a nose clip. Sample size calculations ($\alpha = 0.05$, 80% power) indicated $n = 10$ participants were needed based upon anticipated EBC sample volumes of 2.5 mL $\pm$ 1 mL [8]. In a subset of individuals (male: $n = 3$; female: $n = 2$), EBC was obtained using both the novel technique and a commercially available EBC collection device (R-EBC) in a randomized fashion (Figure 1). After the initial sample was obtained, participants completed a 45 min bout of cycling at 75% of their estimated HR max (roughly equivalent to 60% VO_2 max) in between 10 min EBC collections in the study.

Figure 1. Experimental design. Exhaled breath condensate (EBC) collection (10 min) occurred immediately before and after the bout of exercise. A subset of participants (n = 5) completed both a 10 min N-EBC collection and a 10 min R-EBC collection in a randomized fashion both before and after the bout of exercise.

2.3. Commercial and Novel EBC Devices

For the N-EBC device, participants were asked to exhale "actively" into a 3/4″ diameter (1″ outer diameter, 3/4″ inner diameter, 1/8″ wall thickness) Tygon High-Purity tube while seated (US Plastics Corp., Lima, OH, USA). Except for the proximal and distal ends (5′ length), the EBC collection tube was submerged in an ice bath (0° Celsius) to cool the exhaled air and allow liquid collection. While wearing a nose clip, participants used a Hans Rudolph mouthpiece (Hans Rudolph, Inc., 8900 Series, Shawnee, KS, USA) equipped with a two-way nonrebreathing T-valve (Hans Rudolph, Inc., T-Shape Valve, Shawnee, KS, USA). The mouthpiece was connected to the EBC collection tube via two 3′4″ PVC 90° Street Elbows, and a one-way valve was placed on the distal end of the tube (Figure 2).

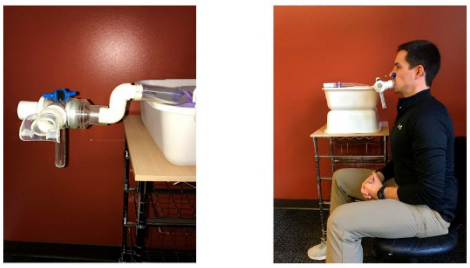

Figure 2. Device photos (front and side view). A Hans Rudolph mouthpiece equipped with a two-way nonrebreathing T-valve. The mouthpiece is connected to the 3/4″ Tygon Tubing via two 3/4″ PVC 90° Street Elbows, and a one-way valve is placed on the distal end of the tube.

EBC biosample collection consisted of removing the Hans Rudolph mouthpiece, securing a 50 mL Falcon tube on the tube's distal end, grasping the tubing's proximal end, and spinning the tubing in a vertical plane. After circling the tubing apparatus at a speed of 60 rpm for one minute, the Falcon tube was oriented downward so that the EBC sample remained in the conical tip of the tube. Using a pipette, 0.7 mL samples of EBC were placed into 1.5 mL microcentrifuge tubes and stored until subsequent assay.

With the R-EBC device, participants repeated the 10 min seated active breathing protocol but instead exhaled into the RTube™ breath condensate collection device (Respiratory Research, Inc., Austin, TX, USA). The device consists of a mouthpiece connected by a one-way valve into a collection tube. Per manufacturer instructions, the collection tube was surrounded by an aluminum sleeve pre-cooled in a −80° Celsius freezer. Using the plunger provided by the manufacturer, 0.7 mL samples of EBC were obtained by pipette, transferred into 1.5 mL microcentrifuge tubes, and stored until subsequent assay.

2.4. EBC Sample Storage, Handling, and Assay

EBC samples from both devices were placed on ice in a light-protected cooler (−20 °C). Within 3 min, samples were stored at −80 °C until subsequent assay, except for one microcentrifuge tube containing 1 mL of a subject's sample used in pH testing. The pH testing (ThermoScientific Orion Star A111, Waltham, MA, USA) was conducted directly after sample collection per the manufacturer's instructions to avoid alterations that may occur when samples are left open to room air (i.e., increases in pH due to CO_2 and other dissolved gases lost into the atmosphere).

Salivary α-amylase was assayed using a kinetic enzyme assay kit (Salimetrics, LLC, State College, PA, USA). Similarly, oxidative stress biomarkers (8-isoprostane and Myeloperoxidase) were assayed using enzyme-linked immunoabsorbent assay (ELISA) kits (Cayman, Ann Arbor, MI, USA). Finally, a pro-inflammatory biomarker (Pentraxin-3) was assayed using an ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). All kits were performed in accordance with the manufacturer's instructions.

2.5. Data Analysis and Statistical Testing

Data are reported as mean ± standard deviation for pre- and post-exercise time points. The primary objective of our statistical analyses was to evaluate if there was a relative change in each biomarker concentration following the exercise. Paired sample *t*-tests were performed to observe differences between test points, and variance comparisons were analyzed between device values. Results were classified as statistically significant when the *p*-value was 0.05 or less based upon *a priori* criteria. All analyses were performed using SAS Studio (SAS Institute Inc., Cary, NC, USA).

3. Results

Sixteen active individuals (male: $n = 10$; female: $n = 6$) participated in the study. Participant characteristics are presented in Table 1. N-EBC volume pre- and post-exercise (2.3 ± 0.8 and 2.6 ± 0.9 mL, respectively) and pH (7.4 ± 0.5 and 7.4 ± 0.5, respectively) were not significantly different. Furthermore, no gender differences were observed for N-EBC comparisons. When normalized for participant body height, device comparisons indicated N-EBC volumes were larger than corresponding samples R-EBC at pre-exercise (+12%) and post-exercise (+48%) (Table 2). Pre-exercise pH was significantly different between the N-EBC and R-EBC trials (7.4 ± 0.5 and 6.3 ± 0.2, respectively; $p < 0.01$), suggesting untoward chemical interactions with the R-EBC device (Table 2). N-EBC Salivary α-Amylase values were below the normal range for saliva samples and comparable to R-EBC (0.08 ± 0.56 and 0.21 ± 0.35 U/mL, respectively; $p = 0.61$).

Table 1. Participant Characteristics.

Characteristic	$n = 16$	Males ($n = 10$)	Females ($n = 6$)
Age (y)	26 ± 8	26 ± 9	28 ± 7
Height (cm)	176 ± 10	182 ± 4	166 ± 7
Mean Exercise Trial Heart Rate (bpm)	157 ± 9	156 ± 9	158 ± 10
Mean Exercise Trial Watt Output (W)	163 ± 49	189 ± 25	121 ± 53

Data presented as mean ± SD.

Table 2. Exhaled Breath Condensate (EBC) Collection Characteristics.

Device	Volume (mL)		pH	
	Pre-Trial	Post-Trial	Pre-Trial	Post-Trial
N-EBC ($n = 16$)	2.3 ± 0.8	2.6 ± 0.9	7.4 ± 0.5	7.4 ± 0.5
R-EBC ($n = 5$)	2.1 ± 0.7	1.8 ± 0.4	6.3 ± 0.2 *	6.2 ± 0.2 *

Data presented as mean ± SD. * Indicates statistical significance ($p < 0.01$).

Following the bout of moderate-intensity exercise, no changes in pre-and post-trial values of Pentraxin 3 (0.25 ± 0.04 and 0.26 ± 0.06 pg/mL, respectively; $p = 0.44$) and 8-Isoprostrane (0.43 ± 0.33 and 0.36 ± 0.24 pg/mL, respectively; $p = 0.34$) concentrations were observed (Table 3). Myeloperoxidase was only detectable in 7 of 30 samples (0.19 (range: 0.06–0.51) ng/mL), precluding further analysis.

Table 3. Variables of Oxidative Stress.

Marker	N-EBC	R-EBC
Salivary α-amylase ($U \cdot mL^{-1}$)		
Pre	0.06 ± 0.06	0.02 ± 0.03
Post	0.08 ± 0.56	0.21 ± 0.35
8-Isoprostane ($pg \cdot mL^{-1}$)		
Pre	0.43 ± 0.33	2.34 ± 2.77
Post	0.36 ± 0.24	1.71 ± 1.78
Pentraxin-3 ($pg \cdot mL^{-1}$)		
Pre	0.25 ± 0.04	0.37 ± 0.04
Post	0.26 ± 0.06	0.38 ± 0.05

Data presented as mean ± SD. N-EBC, Novel exhaled breath condensate collection device; R-EBC, RTubeTM device.

4. Discussion

The current investigation compared a novel EBC collection device to a commercially available apparatus. The impetus for this investigation relates to our ongoing research into the potential for acute physiological and biochemical detriment following wood smoke exposure. In this regard, the EBC biosamples are reflective of the air–lung interface and may hold new insights into the stresses associated with smoke inhalation. Accordingly, EBC biosample volume and stability are central to the eventual understanding of acute smoke inhalation. Our findings indicate that both the N-EBC and the R-EBC collected with the RTube™ apparatus were adequate for collecting EBC biosamples before and after a bout of moderate-intensity cycle ergometer exercise. In this regard, one of our study goals was to determine whether the N-EBC could provide equivalent EBC samples than RTube™ but at a lower cost. In this investigation, our samples were collected for a total of USD 200, a value that would have cost more than USD 4000 for a comparable collection using the RTube™ device. The other aim of this investigation was to determine whether the N-EBC would provide a larger volume biosample in a time-equivalent fashion. Findings indicate that when normalized for participant height (a critical anatomical predictor of pulmonary volumes and subsequent ventilatory rates at rest), N-EBC was +12% pre-exercise and +48% post-exercise compared to R-EBC. The larger post-exercise volume was likely due to elevations in post-exercise ventilatory rates (reflecting increases in both respiratory rate and tidal volumes) [9]. In addition, the novel EBC collection device has a significantly larger surface area as compared to the commercially available device, potentially increasing the EBC capture capacity. Accordingly, the elevated ventilatory rates and device surface areas combined to amplify between-device sample volumes in the post-exercise collection period. Interestingly, and given that EBC is almost invariably collected in 10 min time windows, the increased minute ventilation observed currently is likely resolved within 10 min of the cessation of moderate exercise [10]. Based on this understanding, it is serendipitous that the current methodology may have optimized the collection of larger sample volumes in the post-exercise time point.

Among the most critical factors related to EBC quality control is to collect a voluminous sample without salivary contamination. To achieve this end within a work physiology laboratory setting, we used a commonly available Hans Rudolph mouthpiece (including a saliva trap) attached to a PVC junction that facilitated the collection of exhaled water vapor but prevented the passage of saliva into the EBC collection tube. To confirm the absence of salivary contamination, EBC biosamples were assayed for the presence of salivary amylase. As with prior EBC investigations [4,11], our data indicated all samples contained amylase concentrations below the physiologic level of human saliva, which are typically lowest in

pre-stress situations and exhibit peak values immediately post-stressor, a response profile that partially resembles salivary cortisol [12].

In the current study, levels of both salivary alpha-amylase and 8-isoprostane exhibited relatively large signal:noise ratios. This anticipated finding reflects the relatively low-stress nature of this exercise-based study. Moreover, in both EBC and saliva, concentrations of salivary amylase can differ dramatically within an order of magnitude, relative to the given biological medium. When evaluating the body of literature on saliva samples, those error values are widely variable in both physical and psychological (non-physical) trials. In non-exercise situations, Gordis et al. (2006) found salivary alpha-amylase values were 85.49 ± 64.31 U/mL and 114.39 ± 83.07 U/mL, before and after a psychological stress stimulus [12]. Similarly, following a 10 km running distance, Deneen and Jones (2017) found salivary alpha-amylase in male runners increased from approximately 90 ± 20 U/mL to 160 ± 50 U/mL [13]. In contrast, when comparing between EBC or saliva, salivary amylase concentrations differed by several orders of magnitude. Indeed, salivary amylase concentrations from our EBC samples were well below the levels associated with human saliva.

Similar to salivary amylase outcomes, the large signal:noise ratios for 8-isoprostanes reflect the low-stress nature of the exercise used in this investigation. In this instance, 8-isoprostanes were measured as a representative marker for post-exercise oxidative damage. Lipid markers of oxidative damage are typically proportional to the exercise intensity [14]. Thus, low levels of 8-isoprostanes were anticipated, likely elevating the signal:noise ratio for that metric. Moreover, for given sub-maximal exercise intensity, markers of lipid oxidative damage can be quite variable, a fact that appears to be true in both human blood and EBC samples [15,16].

Another impetus for the current investigation of our novel device was to demonstrate whether EBC biosamples could be collected in volumes that permitted the accurate determination of pH, in addition to a panel of stress and inflammation biomarkers. To prevent the potential for cross-contamination from the pH probe, cleaned between each sample per manufacturer instructions, it is ideal for the pH sample to be determined from a dedicated aliquot. In this regard, pH values collected from N-EBC samples were similar to the physiologic ranges from prior investigations [17–20], although exhibiting minimal differences in the post-exercise values. In contrast, pH values from R-EBC samples were significantly lower than typical physiological values and those obtained by the N-EBC. While it is possible that limiting sample volumes (i.e., lack of volume) could account for artifactual pH values, this was not likely the case in the current investigation where we examined pH using a 1 mL EBC sample volume for both devices. Accordingly, there is a rationale to suspect that R-EBC collected in contact with the aluminum sheathing (as necessitated by manufacturer protocols) of the R-tube may have resulted in artifactually low values. The device manufacturer's guidelines recommend storing aluminum sleeves in a sealed bag to avoid moisture accumulation on the inside of the sleeve; however, the total absence of frost in the sleeve is not always attainable and could explain discrepant findings in pH between N-EBC and R-EBC samples. In contrast, N-EBC samples were collected in plasticizer-free tubing, perhaps explaining why pre- and post-exercise pH values taken from our novel device are similar to those observed in prior investigations [17–20].

Whether the current pH differences between N-EBC and R-EBC samples extend to bioassays is currently unknown. Nonetheless, it is understood from prior investigations that the accurate pH values from EBC samples can hold significant clinical and physiological importance beyond exercise applications. In support, previous investigations provide validated techniques and demonstrate physiologically relevant ranges (e.g., for healthy and diseased participants) for determining the pH from EBC [20]. Indeed, this method of EBC measurement is the most reproducible method for both healthy and asthmatic subjects [17,18]. In prior investigations, the mean pH of healthy subjects is 7.7, with a typical range of 7.4–8.8 [17]. Within post-exercise scenarios, alterations in airway pH reflect respiratory compensation to the elevated metabolic rate related to cycle ergometry.

In contrast, alterations in EBC pH from clinical (non-exercise) scenarios could reflect metabolic dysregulation and pathological processes unregulated underlying inflammatory diseases [21]. Moreover, in highly asthmatic individuals, a decrease in the pH of airways can cause bronchoconstriction and impairs multiple aspects of airway function [22]. Collectively, the current findings, combined with the existing literature, raise new insights into the importance of collecting EBC samples in inert receptacles and using standardized techniques to assure that accurate pH values reflect the metabolic condition of the subject and not an experimental artifact.

A final consideration for the current investigation was to devise a novel EBC collection technique that provides adequate sample volumes for the subsequent examination of a robust biomarker panel. For instance, a prior investigation by our research group collected EBC before and after exercise using commercially available EBC collection apparatus [23]. In this prior study, participants were exposed to low, moderate, and high inhaled concentrations of woodsmoke (0 $\mu g \cdot m^{-3}$, 250 $\mu g \cdot m^{-3}$, and 500 $\mu g \cdot m^{-3}$, respectively). Woodsmoke contains various air pollutants such as carbon monoxide, respirable particulate matter, and other chemical compounds impacting large populations in the western United States [24–26]. In this regard, the lungs are the physiologic system for the human–environment interface. Moreover, since resident immune cells within pulmonary capillary beds are sensitive to smoke and other pollutants [27,28], EBC collection may serve as an essential biological media for quantifying acute physiologic stress. Acknowledging this point, the prevalence of wildfires in the summer months increases the likelihood of exposure to ambient wood smoke from wildfires [24–26]. Furthermore, recreational or occupational activity, such as wildfire management activities by wildland firefighters, may result in increased minute ventilation that could potentially exacerbate the magnitude of woodsmoke exposure [29]. Indeed, woodsmoke exposure has been associated with lung function decline in occupational populations such as wildland firefighters [30,31]. However, a limited number of studies have directly evaluated the acute response apparent in exhaled breath condensate. Chronic woodsmoke exposure is linked to adverse health effects on cardiovascular control and oxidative stress, although acute smoke inhalation effects are not well defined [32]. Furthermore, to better understand this response profile, a cost-efficient and repeatable technique are advantageous to examine the multitude of complexities in field research dynamics.

Accordingly, EBC volumes were small in our prior investigation, preventing comprehensive examination of a full biomarker panel [23]. Based on this rationale, current sample volumes using our novel EBC collection device were adequate for the assay of five biomarkers and potentially more, as indicated in another experimental design. Indeed, based on many commercial assay kit sample volumes (60–300 µL, including replicates), sample volumes collected in the current investigation would have supplied at least seven, and as many as thirty-six, biomarkers, depending on the assay panels chosen. Findings in this investigation did not produce significant exercise-dependent changes for the selected variables of 8-Isoprostrane, Pentraxin 3, and Myeloperoxidase. However, our primary intent was to demonstrate proof-of-concept for successfully examining stress-related biomarkers from a larger volume of EBC that were not saliva contaminated. Indeed, statistically insignificant findings following a relatively short duration, a moderate-intensity bout of exercise was not necessarily expected [16,23,32]. Instead, more extreme forms of exercise and the co-introduction of inhaled woodsmoke or other environmental stressors would have likely resulted in pre-to-post-exercise elevations in all the biomarkers examined currently [33–35].

5. Conclusions

Findings from this investigation support our study aims to demonstrate the efficacy of a novel EBC collection device that provides adequate sample volumes using a cost-effective approach. In this regard, our novel EBC device costs 10-fold less than a competing commercially available collection apparatus. Moreover, we demonstrated that our novel EBC device was comparable to a commercially available device and produced larger sample

volumes when normalized to participant height. In addition, the sample volumes collected in this investigation were adequate for the subsequent assay of numerous biomarkers. Based on prior work by our research group and others, obtaining robust sample volumes is essential for sample viability and the subsequent collection of EBC biomarkers that reflect challenges inherent to the study design. In this regard, we observed that the pH of EBC samples collected from plasticizer-free tubing via our novel technique was in line with prior investigations, while the pH from the commercially available device was not. Finally, while our moderate-intensity, limited duration exercise challenge did not result in pre- or post-trial differences for the variables examined, we demonstrated a proof-of-concept approach to collect a panel of relevant biomarkers from EBC biosamples. Moreover, these samples can be obtained from laboratory or field settings using a time-efficient non-invasive approach. Accordingly, these findings suggest that a follow-up investigation of EBC samples using this novel collection technique, combined with significant physiologic and environmental challenges (e.g., exercise and woodsmoke inhalation), is warranted.

Author Contributions: Study concept and design, data acquisition, analysis of data, drafting of the manuscript, and critical revision of the manuscript were performed by both authors (J.A.S. and J.C.Q.). All authors have read and agreed to the published version of the manuscript.

Funding: This research received partial funding from the USDA Forest Service.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of the University of Montana (IRB# 159-20; 20 November 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter VIII: Effects of Acute Sleep Deprivation on the Physiological Response to Woodsmoke and Exercise

Brief Description

The following manuscript represents the second investigation of my dissertation work. We obtained University of Montana Institutional Review Board approval, and all study participants provided informed written consent before initiation of data collection (see Appendix B: IRB# 83-21: "The Effects of Circadian Rhythm Disruption on the Inflammatory Response to Particulate Matter Exposure from Woodsmoke"). The *Journal of Occupational and Environmental Medicine* accepted the peer-reviewed manuscript pending minor revisions on July 24, 2023.

Following several dissertations (Peters and Williamson) and a graduate thesis (Ferguson) conducted in collaboration between IPAT and the Inhalation and Pulmonary Physiology Core at the Center for Environmental Health Sciences, this study incorporated two novel elements in examining the physiological responses to exercise with WS exposure. The first component embodied the concept of quantifying smoke exposure via the metric of $PM \cdot MET^{-1} \cdot min^{-1}$ (first examined by Williamson et al.)[11]. Still, the cumulative dose of WS exposure was increased according to the increased ventilatory rate associated with higher-intensity exercise (70% VO_2max). Additionally, acute sleep deprivation was administered in a randomized cross-over fashion. Participants would arrive either with 8 hours of sleep or 4 hours before the exercise trial with WS exposure. Thus, this evaluation evaluated the effects of sleep deprivation on the acute physiological response to WS and exercise. However, the findings displayed that acute sleep deprivation did not magnify metrics of physiological response to WS smoke (HRV, PWV, and PFT) following moderate-intensity aerobic exercise.

Effects of Acute Sleep Deprivation on the Physiological Response to Woodsmoke and Exercise

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Funding: This research received partial funding from the USDA Forest Service.

Conflicts of Interest: NONE DECLARED

Author Contributions: Study concept and design, data acquisition, analysis of data, drafting of the manuscript, and critical revision of the manuscript were performed by JAS and JCQ. ACC, AIM, and IPS performed data acquisition. EMM and GRM aided in the study concept and design, data analysis, and the manuscript's critical revision. All authors have read and agreed to the published version of the manuscript.

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Running Title: Effect of sleep deprivation before woodsmoke and exercise

1. Structured Abstract

Objective: To evaluate the effects of sleep deprivation on the acute physiological response to woodsmoke and exercise. Methods: Ten participants performed two exercise trials (8 hr of sleep vs. 4 hr) with woodsmoke in a crossover design. Heart rate variability (HRV), pulse wave velocity (PWV), blood pressure (BP), pulmonary function testing (PFT), and oxidative stress were measured before and after each trial. Results: Acute sleep deprivation before exercise with woodsmoke exposure did not result in a difference in metrics of HRV, PWV, PFT, BP, or oxidative stress. Conclusion: Acute sleep deprivation did not magnify these metrics following moderate-intensity aerobic exercise with airborne woodsmoke particulate matter. Although these findings do not preclude the negative impact of wood smoke inhalation, other research approaches are needed to understand the acute effects of smoke exposure on the cardiovascular system.

2. Keywords

Woodsmoke, moderate exercise, sleep deprivation, pulse wave velocity, cold pressor test.

3. Bulleted Learning Outcomes

- Summarize the PM-MET-min approach for quantifying woodsmoke exposure in laboratory and occupational settings.
- Relate the key dependent variables (heart rate variability, pulse wave velocity, blood pressure, and pulmonary function testing) to their response in acute and chronic exposure conditions of smoke exposure.
- Describe how sleep deprivation can affect the inflammatory, oxidative stress, and physiological response to woodsmoke exposure.

4. Introduction

As the prevalence of woodsmoke exposure from wildfires increases in the western US population, so does the need to understand the long-term effects of smoke inhalation exposure. Over the past 30 years, there has been an average increase of 170,000 acres burned by wildfires in the US each year[147]. For individuals who reside downwind of fire events, this increasing woodsmoke exposure presents a growing health hazard for those

living in the wildland-urban interface. At-risk populations include those who remain recreationally active outdoors during the summers, especially wildland firefighters and other occupations (e.g., construction, farmers/ranchers, delivery persons, etc.) who are physically active in smoky environments for 12-16+ hr/day[148].

Chronic exposure to woodsmoke presents serious health detriments, yet the acute response to woodsmoke exposure, thought to be foundational to pathological outcomes, is not well defined. Nonetheless, preliminary studies suggest that elevated oxidative stress markers in response to acute smoke inhalation may contribute to pathological outcomes [6-12]. Accordingly, this investigation aimed to determine whether acute physiologic responses to acute smoke inhalation during exercise can be identified, which may provide insight into cardiorespiratory dysfunction. The subsequent knowledge could be implemented to help intercept these long-term cardiovascular and respiratory system pathology.

In this regard, findings from recent studies suggest that restorative amounts of sleep play a key role in preserving the body's antioxidant capacity (i.e., in blood and other tissues). Observations of declining antioxidant levels are essential because oxidative stress (e.g., any imbalance between free radical production and antioxidant depletion) is foundational to most chronic diseases [20]. Of particular interest is the nervous system, which is already low in antioxidant capacity. The vagus cranial nerve is responsible for the primary control of the body's parasympathetic (relaxed state) control, including contributing to heart rate variability (HRV). Acute sleep deprivation can also depress vagal tone, as observed through a protracted decline in HRV and mitigated recovery from various physiologic stressors [20, 149, 150]. Moreover, sleep deprivation could alter responses to combined physiologic responses to stressors, such as exercise and smoke inhalation, because disruptions to the circadian rhythm may exacerbate physiologic reactions to stress.

While it is well understood that chronic woodsmoke exposure harms long-term health, the mechanisms responsible for initiating compromised health are not fully resolved. One recent review indicates that following a 25-year career, wildland firefighters have an increased risk of lung disease by 43% over occupational controls and are at an elevated risk for CVD by +30% [16]. There is a growing rationale to suspect that alterations in several physiologic outcomes may indicate both the acute and long-term physiologic stress to smoke inhalation [7, 9, 11]. Current research literature, however, confirms that it is methodologically challenging to quantify the physiological detriment of acute

woodsmoke inhalation in otherwise healthy individuals [9, 11, 17]. Nonetheless, early results indicate that exercise and smoke inhalation can increase biological markers of oxidative stress [7, 9, 11, 18, 19].

Further identification of acute physiologic responses to woodsmoke would provide novel insight into the mechanisms responsible for increased rates of chronic diseases (e.g., cardiovascular disease). Fundamental to this challenge, the detrimental physiologic effects on woodsmoke have not been fully quantified. Accordingly, additional investigations into the impact of smoke inhalation on lung function and cardiovascular control are needed. While a limited volume of published research literature has investigated woodsmoke exposure and exercise, little work has been performed to understand the potential compounding effects of sleep deprivation. Applications of this understanding include professions such as wildland firefighters, who regularly sleep deprived while they undertake physically demanding 16+ hr shifts, for 14 consecutive days, in the presence of concentrated ambient woodsmoke[21]. Moreover, sleep deprivation is associated with increased inflammation, decreased immune response, reduced control of parasympathetic regulation, and cardiorespiratory dysfunction [20, 149-151].

Based on this rationale, the current study aimed to identify whether acute exercise during controlled smoke inhalation produces physiological alterations in respiratory and cardiovascular measures. Moreover, the effects of sleep deprivation were examined in conjunction with this study to provide more comprehensive insight into smoke exposure stress responses. Accordingly, this study utilized two smoke-exposure exercise trials, one of which participants operated on reduced sleep (4 hr vs. 8 hr before moderate-intensity exercise and woodsmoke exposure).

5. Methods

Participants

Approval to examine human research participants was obtained from the University of Montana Institutional Review Board (IRB Protocol No. 172-22). Participants were recruited from the Missoula, Montana community, and each provided informed written consent before initiating this study. Participants completed personal questionnaires (including a health screening and informed consent) before initiating the study.

All participants were male (n=10; age = 24±4 yrs.; height = 185.2±3.9 cm; weight = 85.7±9.4 kg; VO_2max = 46.7±5.7 $ml \cdot min^{-1} \cdot kg^{-1}$; body fat = 12.6±6.7 %). Inclusion criteria

included a VO_2max of greater than 40 ml·min^{-1}·kg^{-1} ("recreationally active"), the absence of known pulmonary conditions, or recent contraction of SARS-CoV-2 (COVID-19), and to have a normal wake-sleep cycle (begin sleep at 10 PM; wake at 6 AM).

Study Design

The overall study design consisted of three participant visitations. First, participants underwent baseline testing for VO_2max and body composition. Body composition was measured via hydrostatic weighing (Exertech, Dresbach, MN). Body density values were corrected for residual lung volume (residual lung volume=(0.01115· age)+(0.019·Height)–2.24), and body volume estimates were converted to percent body fat using the Siri Equation[137]. The VO_2max test was performed on a cycle ergometer (Velotron, Spearfish, SD). Following a 3-min warmup at 60 watts, participants received an additional 40 watts each min until they could no longer maintain 75 rpm. Expired gas was sampled using indirect calorimetry (Cosmed Quark CPET, Concord, CA).

Participants then reported to the air inhalation facility. In a randomized crossover design, participants underwent two stationary-bicycle workouts. Both workouts included a 45-min exposure to 250μg·m^{-3} of woodsmoke during exercise (Figure 1). Trials varied only in the sleep condition of participants (8 hr sleep vs. 4 hr sleep). Exposure trials were conducted at the inhalation and pulmonary physiology core at the Center for Environmental Health Sciences at the University of Montana. Fires were generated using an in-line inhalation system woodstove stoked with western larch (Larix occidentalis Nutt). Fires were prepared 30 min before the exercise using 1 kg of wood and kindling and were stoked with 300 g of wood every 15min throughout the trial.

The two smoke exposure trials were performed with 8 hr of sleep the night prior (2200 to 0600) and 4 hr prior (0000 to 0400) during 45 min of cycling at 70% VO_2max. Sleep duration was confirmed using Polar H10 watches worn by participants. Smoke trials were conducted between 08:00 and 11:00, and participants fasted for 12+ hr before trials. No more than two participants performed smoke-chamber trials on any given day, consolidating the testing period to a limited circadian time frame.

During both trials, participants were exposed to 250μg·m^{-3} of woodsmoke transferred from a wood-burning stove to a modified facemask covering the nose and mouth and worn while riding. Smoke concentrations were monitored using PM2.5 monitors (DustTrak, TSI, Model 8530, Shoreview, MN) and titrated with fresh ambient air to maintain the prescribed smoke dose for each trial. Trials were separated by one week to wash out period to

minimize the potential confounding effects of acute smoke exposure. Water breaks (1 min) were allowed at 15 min and 30 min of the trial.

The key dependent variables of HRV, PWV (including augmentation index; AIx), BP, and PFT (Figure 2). Initial HR data, including R-R intervals, were obtained using the Polar Vantage V2 watch and Polar H10 HR sensor and archived in the Polar Flow app (Polar Electro USA, Lake Success, NY). HRV measurements were collected from HR measurements 12 hr before the smoke exposure trial and 24 hr after the exposure trial, including 10-min intervals immediately before and after the exercise and woodsmoke exposure. HRV metrics were acquired by exporting recorded HR files into the Kubios software (Kubios, V 2.2, Joensuu, Finland) for analysis. HRV indices included the root mean square of successive differences (RMSSD), high frequency (HF), low frequency (LF), and the ratio LF: HF, as based on established methods[13, 138]. As an a priori decision, all four HRV metrics would need to be altered by exposure to be considered physiologically significant, owing to the interrelational nature of the assessment.

PFT was assessed by forced vital capacity (FVC), forced expiratory volume in one second (FEV1), and maximal voluntary ventilation (MVV, performed for 12 s) tests using a spirometer (MIR Spirobank, Elicott City, MD), with participants tested in the seated position. Data were analyzed using the WINSPIRO Pro software (WINSPIRO PRO Version 7.8, Ellicott City, MD). Participants were provided with verbal encouragement during PFT.

PWV, AIx, and BP were obtained using the SphygmoCor XCEL device (Atcor Medical, Sydney, Australia) after 10min of supine rest. Right-side measurements were recorded until three values were obtained within 0.5 $m \cdot s^{-1}$. The femoral cuff was placed around the upper thigh, and a carotid pulse was identified using applanation tonometry. PWV between the carotid and femoral arteries was calculated based on the arterial stiffness formula: PWV=distance (m)/transit time (s).

Secondary biological exposure measures were derived from blood and EBC biosamples collected before and after the exercise and smoke inhalation trials. Blood sampling for oxidative stress was conducted before and after each trial and will be reported in a separate manuscript. Blood was collected via venipuncture through an antecubital vein. Samples were collected in 10-mL heparinized tubes, centrifuged at 1500 rpm for 15 min at 4°C, aliquoted, and stored at –80°C until assayed. A panel of blood oxidative stress biomarkers (8-isoprostane and Myeloperoxidase) was assayed using enzyme-linked immunosorbent assay (ELISA) kits (Cayman, Ann Arbor, MI, USA). A pro-inflammatory

biomarker (Pentraxin-3) was assayed using an ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). All kits were performed in accordance with the manufacturer's instructions. The same panel of biomarkers was assayed on both the blood and EBC samples. EBC was obtained using a novel collection device designed as previously described, a process that amplifies total sample volumes acquired and preservation of physiologic pH [22]. Methods for EBC collection entail a 10-min sample collection consisting of breathing through a modified mouthpiece to contain EBC in plasticizer-free tubing submerged in ice water, which is then collected, aliquoted, and stored at -80°C until assayed.

Data Analysis

All data were analyzed using a two-way ANOVA to compare measurements by time (pre- to post-exposure), by group (sleep-deprived vs. control), and by the response (relationship of sleep deprivation to exposure) for each variable of interest. A criterion alpha level of P≤0.05 was used to determine statistical significance. Data are reported as mean±SD. When analyzing HRV, a natural logarithmic transformation was performed on RMSSD, HF, and LF to satisfy the normality assumption. These were reported as lnRMSSD, lnHF, and lnLF before statistical analysis.

6. Results

Ten active males (age = 24±4 yrs.; height = 185.2±3.9 cm; weight = 85.7±9.4 kg; VO_2max = 46.7±5.7 ml·min^{-1}·kg^{-1}; body fat = 12.6±6.7 %) completed the study. No group or time: factor effects were observed for HR in the 12 hr before and after the smoke exposure trials (P = 0.327 and P = 0.789, respectively; Table 1). Hemoglobin values were comparable between both sleep conditions (Normal sleep: Pre = 16.0±1.0 g·dl^{-1}, Post = 15.8±0.9 g·dl^{-1}; Sleep-deprived: Pre = 15.8±0.9 g·dl^{-1}, Post = 15.8±1.0 g·dl^{-1}). No significant differences in hematocrit values were present either (Normal sleep: Pre = 46.0±0.9 L·L^{-1}, Post = 47.0±3.0 L·L^{-1}; Sleep-deprived: Pre = 46.0±2.8 L·L^{-1}, Post = 45.4±2.0 L·L^{-1}).

HRV analyses included the quantification of lnRMSSD, lnHF, lnLF, and LF: HF (Table 1). Analysis of lnRMSSD, lnHF, lnLF, and the LF: HF ratio indicated no time effects (lnRMSSD: P=0.442; lnHF: P=0.897; lnLF: P=0.786; LF:HF: P=0.548) and no between-trial differences (lnRMSSD: P=0.412; lnHF: P=0.683; lnLF: P=0.124; LF:HF: P=0.313).

Analysis of PWV and AIx (Table 1) indicated no interaction effect (PWV: *P*=0.809; Aix: *P*=0.751), trial effect (PWV: *P*=0.700; Aix: *P*=0.631), indicating that these markers

were not significantly different. However, a time effect was noted in PWV (PWV: *P*=0.001; Aix: *P*=0.129). No statistical difference was observed in brachial BP for trial (SBP: *P*=0.805; DBP: *P*=0.748) or time (SBP: *P*=0.198; DBP: *P*=0.549).

PFT data are presented in Table 2. For FVC, there was no interaction (P=0.840), trial (P=0.919), or time effect (P=0.952). Similarly, FEV1 and FEV1/FVC exhibited no interaction (FEV1: P=0.854; FEV1/FVC: P=0.986), trial (FEV1: P=0.772; FEV1/ FVC: P=0.711), or time effect (FEV1: P=0.686; FEV1/ FVC: P=0.540). MVV also demonstrated no interaction (P=0.923), trial (P=0.633), or time effect (P=0.917).

No significant differences were observed in EBC pre-trial volumes (Normal sleep = 2.3±1.1 ml; Sleep-deprived = 2.3±0.9 ml, P = 0.999) or post-trial volumes (Normal sleep = 2.6±1.0 ml; Sleep-deprived = 3.0±1.8 ml; P = 0.215). EBC 8-isoprostane concentraitions pre-trial (Normal sleep = 25.4±6.0 ng·ml^{-1}; Sleep-deprived = 27.0±8.1 ng·ml^{-1}) and post-trial (Normal sleep = 21.2±8.1 ng·ml^{-1}; Sleep-deprived = 16.5±5.1 ng·ml^{-1}), displayed no interaction (P=0.591), trial (P=0.762), or time effect (P=0.218). EBC concentrations of MPO and PTX3 were below manufacturer-described detection limits.

7. Discussion

The detrimental effects of long-term woodsmoke exposure on cardiovascular and respiratory health are unequivocal, while the links between acute exposure and lasting detriment remain an area of active inquiry. For example, wildland firefighters and other individuals with frequent outdoor exposure to woodsmoke are thought to be at an elevated risk for long-term cardiovascular health consequences[16, 35, 152, 153]. Despite this widely held rationale, direct links between acute exposures and the disease acquisition process are emergent [16, 153]. Accordingly, we used a laboratory-based investigation to examine the combined effects of sleep deprivation, exercise, and smoke exposure on the physiologic variables of HRV, PWV, and PFT. To this end, we previously described a simple method for quantifying participant exposure as the product of PM2.5xMETs of activity x minute of exercise (PM-MET-min) [11]. This approach is vital because it is a simple method for quantifying smoke density, exposure times, and the influence of physical activity on ventilatory responses – potentially amplifying exposure rates when physical activity levels produce elevated respiratory responses.

Moreover, this PM-MET-min approach is helpful for many occupational settings where physical levels are above rest but below the ventilatory threshold, where individual ventilatory rates become less predictable[154]. In the prior and current studies, these

MET-min values were 141,750 PM-MET-min and 150,750 PM-MET-min, respectively. In the current population, and according to the protocol, this exposure dose did not impact the key dependent measures examined. Findings indicate that our acute physiologic (HRV, PWV, PFT) markers were largely unaltered following acute woodsmoke exposure during intense exercise. Furthermore, secondary biological measures of inflammation and oxidative stress, quantified in blood and EBC, helped confirm that the current exercise and smoke challenge was not different between normal and deprived sleep conditions. This unexpected finding highlights the resiliency of physiologic responses to these conditions in apparently healthy young adult research participants.

Despite the non-significant outcomes observed in this investigation, it cannot be overlooked that the cumulative impact of smoke inhalation is detrimental to physiologic health. Relative to this investigation, pulmonary function testing was necessary because the lungs are the primary interface between the body and woodsmoke particulate. Accordingly, a strong understanding of the pulmonary response to exposure is fundamental to a study examining the effects of woodsmoke exposure on the body. In a wildland fire environment, firefighters experience a variety of smoke exposures during the workday shift. Our study used a 250 $\mu g \cdot m^{-3}$ exposure of woodsmoke to simulate an average exposure during the workday [16]. While previous literature includes instances of a dose-independent response past 250 $\mu g \cdot m^{-3}$ [9, 11], these findings are not obligatory, as observed in the current investigation. We interpret these discrepant findings to highlight potential between-study differences in the experimental design. For example, the exposure time (45 min) was marginal in this study relative to the standard work shift of a wildland firefighter. However, to compensate, participants completed the entire workout at a high intensity (70% VO_2) to effectively increase pulmonary ventilation and increase the body's exposure to PM2.5.

PWV and our other key dependent variables included in this panel of physiologic and biochemical measures have the scientific advantage of being acutely responsive to smoke and exercise[15, 28, 30, 81]. In addition, this panel of measures is sensitive, but not specific, for many chronic conditions such as cardiovascular disease, diabetes, and heart failure [11, 28, 47, 58, 81, 91, 155, 156]. In the current acute study, it is interesting that metrics from these groups of key dependent variables were not responsive to the exercise and smoke challenge, a finding that was true in both normal and sleep deprivation conditions. Central to our desire to link acute physiologic stressors to long-term declines in health, an increase in resting PWV is associated with an elevated cardiovascular event

risk [157]. Although prior studies have examined the effects of woodsmoke exposure on PWV with equivocal results [15, 29], our findings demonstrated that PWV was not statistically different after a 45-min exercise session, comparable to prior studies from our laboratory group [11].

Furthermore, acute sleep deprivation did not modify PWV results. These findings contradict what we anticipated and may reflect many possibilities, including the idea that the current challenges did not produce consistent changes in PWV. For example, one night of sleep deprivation has been shown to increase arterial stiffness in healthy adults [158]. However, these prior findings were observed after a night with only one hr of sleep (0.73±1.1.39 hr). Additionally, this observation was not combined with exercise or smoke exposure. Importantly, and in contrast to this prior investigation, the sleep deprivation challenge employed in this study (3.9 ±0.3 hr) better represented occupational sleep deprivation and portrayed a poor night of sleep in many occupational work conditions (e.g., wildland firefighters) [25].

Excluding sleep deprivation, our PWV outcomes are similar to a previous investigation reporting adverse PWV outcomes 1hr postexercise with wood smoke exposure at 1000 $\mu g \cdot m^{-3}$.[29] However, PWV has been demonstrated to increase for up to 25 min after a 3-hr bout of exercise and an exposure of 300 $\mu g \cdot m^{-3}$ [15]. As such, PWV may respond more to exposures between 1 and 3 hr[15, 28, 29]. In support, a recent study examined the effects of woodsmoke exposure at three different concentrations (100 $\mu g \cdot m^{-3}$, 250 $\mu g \cdot m^{-3}$, and 500 $\mu g \cdot m^{-3}$) under resting conditions, finding that PWV was elevated 24 hr after all exposures [28]. The magnitude of the elevation was similar across all three concentrations, signifying that PWV may not respond to smoke in a dose-dependent manner.

Another key dependent measure of the current investigation was a series of metrics that reflect HRV, a series of values derived from monitored HR data. The inability to maintain a high resting HRV, or exhibiting delayed rebound after exposure to a stressor, predicts an increased risk for mortality and sudden cardiac death [85]. Previous longitudinal findings demonstrated decreased HRV in those regularly exposed to woodsmoke PM in occupational settings [14]. Notably, depression of HRV after acute smoke inhalation signals problematic responses to reoccurring exposure. Accordingly, we examined HRV after a single woodsmoke challenge in apparently healthy research participants to examine the potential of declines in HRV. Results indicated no statistically significant alterations existed for HRV (lnRMSSD, lnHF, lnLF, LF: HF) in the 12 hr before

and after exercise with woodsmoke exposure in normal and deprived sleep conditions. As with other facets of this investigation that were unresponsive to the exercise-smoke challenge, these findings do not appear to explain HRV outcomes. Furthermore, and similar to discrepant findings between our investigation and prior studies, we believe that the specific methodology related to the exercise, smoke, and sleep deprivation challenges is likely responsible for the current negative findings.

In contrast to our results, HRV was systematically reduced in 14 subjects after a 3-hr intermittent exercise session with exposure at ~300 $\mu g \cdot m^{-3}$ [15]. This divergent outcome may indicate that the duration of smoke exposure within the current study did not elicit changes, highlighting the need to identify a woodsmoke exposure (time and PM2.5 concentration) threshold that perturbs cardiovascular function. Moreover, age, fitness level, health status, and the duration of HRV assessment (e.g., more extended sampling periods)—should be examined. Resultingly, an effort was made in this study to observe 12-hr HRV sampling periods. A secondary analysis using paired sample t-test comparison displayed significant differences between 10-min post and 12-hr post values of lnRMSSD in both the normal sleep (10-min post lnRMSSD = 2.7±0.7 ms, 12-hr post lnRMSSD = 3.7±0.6 ms; P = 0.03) and sleep deprivation ((10-min post lnRMSSD = 2.7±0.7 ms, 12-hr post lnRMSSD = 3.6±0.6 ms; P = 0.03) conditions. These results indicate a continued need for 10-min pre- and post-exposure sampling. Still, longer duration HRV sampling combined with PWV and oxidative stress monitoring at 24 hr post-exposure may provide valuable information in explaining the phenomenon of deleterious effects of woodsmoke beyond acute exposures. Previous work demonstrated that HRV is acutely depressed in woodsmoke-exposed elderly populations [159]. Additionally, the postexercise parasympathetic rebound is enhanced in fit and healthy populations [13], suggesting our sample may have exhibited preserved HRV compared to a sedentary population. Given the disparate outcomes of this study compared to others, future investigations again should consider more extended duration baseline and postintervention HRV analyses to confirm or refute the current findings.

PFT was performed in our healthy participants. Findings indicated that FVC, FEV1, and MVV were not statistically different following exercise and smoke inhalation in either sleep conditions applied to these participants. Importantly, these findings are consistent with previous work that demonstrated no changes in FVC, FEV1, or MVV after a 1.5-hr exposure during treadmill walking [7] and highlights that although smoke inhalation is

detrimental to long-term health, acute physiologic responses to exposure may not always result[37].

Lastly, lipid oxidative damage was quantified using the metric 8-ISO in blood plasma and EBC samples collected before and after exercise and smoke inhalation. Previous research demonstrated 8-ISO was elevated immediately after 1.5-hr woodsmoke exposure during low-intensity exercise at two inhalation doses (250 $\mu g \cdot m^{-3}$ and 500 $\mu g \cdot m^{-3}$) [9]. Moderate-intensity exercise (70% VO_2max cycling) was selected to elevate ventilatory rates; however, we did not observe a statistically significant increase in this post-exercise oxidative stress marker. Moreover, neither normal nor deprived sleep conditions impact 8-ISO measures in these participants. We interpret this finding to indicate that our methodologic approach (e.g., exercise dose, population, and sampling time points) did not elicit changes in oxidative, a finding that we believe is in parallel with our similarly negative physiologic outcomes[11].

Indeed, inhalation of concentrated smoke (250 $\mu g \cdot m^{-3}$) elicits inflammation and oxidative stress response known to be deleterious to cardiovascular health [7, 160]. Although chronic smoke inhalation is detrimental to long-term cardiovascular function, our acute physiologic (PWV and HRV) and biochemical blood markers of oxidative stress were not statistically altered between sleep conditions. We interpret these findings to have been influenced by the experimental context in which the laboratory-based investigation was conducted, attributing potential causation to the following methodologic factors: 1) examination of a population—young, apparently healthy subjects—presumably more resilient to this smoke inhalation challenge; 2) methodologic constraints (e.g., sampling time points and selection of biochemical variables); 3) small sample size; 4) insufficient dose, which did not capture perturbations due to smoke; and 5) the potential that exposure trial durations should be extended for the simple purpose of facilitating a more robust, time-dependent inflammatory and oxidative stress responses. This unexpected finding highlights the scientific importance of identifying acute physiologic and biochemical metrics that may better inform the long-term consequences of woodsmoke inhalation.

Study limitations may include the apparently healthy nature of the participants examined. Owing to the examined participants' age range, fitness level, and overall health, inferences that can be drawn from these results are limited proportionately. Thus, it is plausible that these methods, if applied to more susceptible populations (e.g., age, chronic conditions, those who exhibit vascular reactivity to various stressors, etc.), may have produced alternative outcomes to this panel of physiologic and biochemical measures.

Furthermore, the dose of woodsmoke exposure may be insufficient to elicit adverse effects on the selected variables. Additionally, the study examined only one modality, duration, and intensity of exercise, limiting the application of these findings to similar activities.

Despite the known harmful effects of smoke inhalation, sleep deprivation did not magnify the physiological response following moderate-intensity aerobic exercise while exposed to woodsmoke particulate matter in this methodologic context. The current study quantified the acute effects of sleep deprivation before 45 min of exercise during woodsmoke exposure (250 $\mu g \cdot m^{-3}$) on markers of HRV, arterial stiffness, and oxidative stress; however, there were no statistically significant differences observed between average sleep durations (8 hr) and sleep deprived conditions (4 hr). Given the undeniable link between chronic smoke inhalation and cardiovascular health, we interpret the negative findings of the current study to have been limited by the experimental paradigm in which the laboratory-based study was conducted. Future research should examine the threshold for the duration, dose, and frequency of smoke exposure needed to produce acute perturbation in these parameters. Although these findings do not negate the negative impact of wood smoke inhalation, other research approaches are required to understand better the acute and chronic effects of smoke exposure on the cardiovascular system.

Figure 1. Exercise and smoke trial overview.

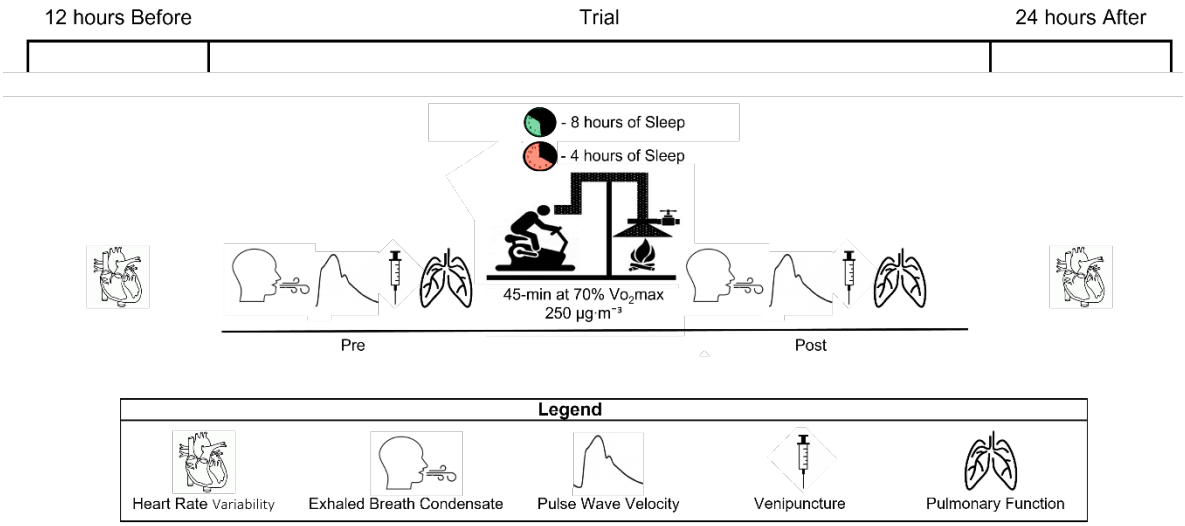

Figure 2. Woodsmoke exposure equipment.

Table 1. Measures of HRV and PWV

Marker	Normal sleep trial	Sleep-deprived trial	Time effect P	Factor(group) effect P	Time:factor(group) effect P
	Mean±SD				
HR (beats·min^{-1})					
Pre	73±14	76±11	0.656	0.327	0.789
Post	74±6	78±9			
lnRMSSD (ms)					
Pre	3.9±0.6	3.7±0.4	0.442	0.412	0.869
Post	3.7±0.6	3.6±0.6			
lnHF (ms^2)					
Pre	6.5±1.3	6.5±0.7	0.897	0.683	0.504
Post	6.7±0.7	6.3±1.1			
lnLF (ms^2)					
Pre	7.5±0.9	7.2±0.4	0.786	0.124	0.602
Post	7.7±0.4	7.2±0.7			
LF: HF					
Pre	3.0±1.3	2.2±1.2	0.548	0.313	0.597
Post	3.0±1.4	2.7±1.5			
PWV (m·s^{-1})					
Pre	4.1±0.4	4.2±0.4	0.001	0.700	0.809
Post	5.1±1.1	5.3±1.3			
Augmentation index (%)					
Pre	5.4±7.8	5.8±9.3	0.129	0.631	0.751
Post	8.3±9.5	9.0±8.3			

HF, high frequency; HR, heart rate; LF, low frequency; Pre, before exercise; Post, immediately after exercise PWV, pulse wave velocity; RMSSD, root mean square of successive differences.

Table 2. Pulmonary function testing variables

Marker	Normal sleep trial	Sleep-deprived trial	Time effect P	Factor(group) effect	P	Time:factor(group) effect	P
	Mean±SD						
FVC (L)							
Pre	6.93±0.93	6.90±1.05	0.952	0.919		0.840	
Post	6.84±0.98	6.94±1.02					
FEV1 (L)							
Pre	5.36±0.81	5.39±0.75	0.686	0.772		0.854	
Post	5.21±0.81	5.33±0.81					
FEV1/FVC (%)							
Pre	78±9	79±7	0.540	0.711		0.986	
Post	76±7	77±7					
MVV ($L \cdot min^{-1}$)							
Pre	202±18	200±18	0.917	0.633		0.923	
Post	202±20	199±18					

FEV forced vital capacity; MVV, maximal voluntary ventilation; Pre, before Exercise; Post, immediately after exercise.

Appendix A: IRB#159-20: “Novel Collection of Exhaled Breath Condensate”

Form RA-108
(Rev. 6/24/2019)

OCT 19 2020
Revised

UNIVERSITY OF MONTANA

Institutional Review Board (IRB)
for the Protection of Human Subjects in Research

APPLICATION FOR IRB REVIEW

IRB Protocol No.: 159-20

At the University of Montana (UM), the Institutional Review Board (IRB) is the institutional review body responsible for oversight of all research activities involving human subjects as outlined in the U.S. Department of Health and Human Services' Office of Human Research Protections.

Instructions: A separate application must be submitted for each project. Email the completed form as a Word document to *IRB@umontana.edu*, or submit a hardcopy (no staples) to the IRB office in the Interdisciplinary Science Building, room 104. Student applications must be accompanied by email authorization by the supervising faculty member or a signed hard copy. *All fields must be completed. If an item does not apply to this project, write in: N/A.* Questions? Call the IRB office at 243-6672.

1. Administrative Information

Project Title: Novel collection of exhaled breath condensate	
Principal Investigator: John Quindry	UM Position: School Chair/Professor
Department: Integrative Physiology and Athletic Training	Office location: 101 McGill Hall
Work Phone: (406) 243-4268	Cell Phone: (406) 240-8078

2. Human Subjects Protection Training

(All researchers, including faculty supervisors for student projects, must be listed below and have completed a self-study course on protection of human research subjects ***within the last three years*** *and be able to supply the "Certificate(s) of Completion" upon request. If you need to add rows for more people, use the Additional Researchers Addendum.*

All Research Team Members (list yourself first)	PI	CO-PI	Faculty Supervisor	Research Assistant	DATE COMPLETED IRB-approved Course mm/dd/yyyy
Name: John Quindry Email: john.quindry@mso.umt.edu	☒	☐	☐	☐	9/17/2018 ✓
Name: Joe Sol - Student Email: joseph.sol@umontana.edu	☐	☒	☐	☐	5/06/2018 ✓
Name: Cassie Reisdorph-Williamson Email: cassie.williamson@umontana.edu	☐	☐	☐	☒	8/28/2018 ✓
Name: Tiffany Quindry Email: tiffany.quindry@mso.umt.edu	☐	☐	☐	☒	10/05/2018 ✓

3. Project Funding *(If federally funded, additional requirements may apply.)*

Is grant application currently under review at a grant funding agency? ☐Yes *(If yes, cite sponsor on ICF if applicable)* ☒No			Has grant proposal received approval and funding? ☐Yes *(If yes, cite sponsor on ICF if applicable)* ☒No		
Agency	Grant No.	e-Prop #	Start Date	End Date	PI on grant

For UM-IRB Use Only

IRB Determination:

Note to PI: Use any attached IRB-approved forms (signed/dated) as "masters" when preparing copies. Notify the IRB if any significant changes or unanticipated events occur. Failure to follow these directions constitutes non-compliance with UM policy.

_____ Not Human Subjects Research
_X_ Approved by Exempt Review, Category # _____
_____ Approved by Expedited Review, Category # 3
_____ Full IRB Determination
  _____ Approved *(see* **Note to PI***)*
  _____ Conditional Approval *(see memo)* - IRB Chair Signature/Date: _____
  _____ Conditions Met *(see* **Note to PI***)*
_____ Resubmit Proposal *(see memo)*
_____ Disapproved *(see memo)*

Risk Level: Minimal

Final Approval by IRB Chair/Manager: _____ Date: 11/20/2020 Expires: N/A

SUBJECT INFORMATION AND INFORMED CONSENT

Study Title: Novel collection of exhaled breath condensate

Investigator(s):

John Quindry, PhD, FACSM, FCVS-APS
Integrative Physiology and Athletic Training
102 McGill Hall, 32 Campus Drive, Missoula, MT 59812
(406) 243-4268 (office)
john.quindry@mso.umt.edu

Special Instruction:

This consent form may contain words that are new to you. If you read any words that are not clear to you, please ask the person who gave you this form to explain them.

Inclusion Criteria:

- Over 18 years old
- Physically active

Exclusion Criteria:

- Are currently recovering from COVID-19 or have been identified as a close contact of a positive COVID-19 case and cannot provide a negative COVID-19 test or have not gone 10 days without any COVID-19 related symptoms.
- Have traveled outside the state or spent time in a COVID-19 "hot spot" in the past 14 days.
- Have an orthopedic limitation that prevents your ability to exercise on a cycle ergometer.
- Have a history of respiratory limitations or disease, including but not limited to asthma or COPD.

Purpose:

The purpose of the research study is to validate a novel technique for collecting exhaled breath condensate (EBC) to be used in future studies involving smoke exposure in laboratory and field settings. The EBC will then be analyzed for changes in various biomarkers, which are substances that are present in the body in both normal and abnormal states. The ability to detect these changes will evaluate the capability of the testing protocol. This study aims to provide a low-cost, reproducible methodology to be used in varied settings. Furthermore, in consideration of the aims of the study, this protocol would have the ability to collect a viable EBC sample in a short duration of time. The results of this study will be used to validate our tubing apparatus and protocol for use in future studies.

Procedures:

If you agree to participate in this research study, you can expect to partake in two separate testing sessions as part of your involvement in the study. The first and second sessions will be 30 and 60 minutes in length, respectively, and scheduled to occur on consecutive days.

In Session 1 (30 minutes), you will exhale normally into a five-foot-long section of 3/4" diameter plastic tubing while seated. The length of the tubing goes through an ice bath (0 degrees Celsius)

to cool the exhaled air and allow for liquid collection. While wearing a nose clip, you will breathe in room air and exhale into the tube, which has a one-way valve on both ends of the tube. After 2.5 minutes of exhaling into the tube, researchers will collect the tube for analysis. You will then perform the same protocol for 5 and 10 minutes, with a clean tube and one-way valves used for each bout.

In Session 2 (60 minutes), you will breathe into the tubing as described previously for a period of 5 minutes. After this pre-exercise measurement is completed, you will perform 45 minutes of cycling at 60% of your age-predicted maximum aerobic intensity. Following this bout of exercise, you will again breathe into a clean tubing apparatus for 5 minutes.

Risks/Discomforts:

We cannot guarantee that the risk of contracting COVID-19 is completely mitigated from participating in this study; however, we have implemented a number of measures to protect both you as a participant and the research team from contracting or spreading the disease. Efforts to mitigate the spread of COVID-19 includes research team self-symptom screening (daily temperature monitoring and symptom questionnaires) and numerous measures taken both before and during your participation in the study. Beginning the day before your participation, you will receive a phone call or text from a researcher and answer several binary (Yes or No) questions from a CDC-adopted questionnaire to ensure all symptoms of COVID-19 are questioned. You are also encouraged to take your own temperature the morning of the trial before coming to campus. Throughout your participation in the testing protocol, CDC-approved personal protective equipment (PPE), room air filtration, and social distancing during data collection will be maintained at all times. While exercising, you will remove your face mask and will breathe through a respiratory mask (covering your mouth and nose) that takes your breath outside of the building. Single-use materials will be used whenever feasible, and an abbreviated protocol to avoid any contact between the researcher and you as a participant. These measures represent our best effort of mitigating, not removing entirely, the risk of contraction.

You may experience discomfort when breathing into the tube for the test duration. It should be noted that the diameter of the collection tube is 3/4", which is equivalent to the average diameter of an adult trachea, and should not restrict breathing. There is no other anticipated discomfort for those contributing to this study, so the risk to participants is minimal. However, just as with regular physical activity, you may experience mild, temporary muscle soreness from the exercise protocol. As with any physical activity, there is a small chance of muscular strain, ligament sprain, or soft tissue injury, but this risk is minimal. You will be informed of any new findings that may affect your decision to remain in the study.

Benefits:

You will receive no benefit from taking part in this study.

Confidentiality:

Your records will be kept confidential and will not be released without your consent except as required by law. Your identity will be kept private. If the results of this study are written in a scientific journal or presented at a scientific meeting, your name will not be used. The data will be stored in a locked file cabinet with your signed consent form stored in a cabinet separate from the data.

Voluntary Participation/Withdrawal:

Your decision to take part in this research study is entirely voluntary. You may refuse to take part in, or you may withdraw from the study at any time without penalty or loss of benefits to which you are normally entitled.

Future research:

Identifiers might be removed from the identifiable private information or identifiable biospecimens and could then be used for future research studies or distributed to another investigator for future research studies without additional informed consent from you or your legally authorized representative.

Compensation for Injury:

If you are injured as a result of this research, you should individually seek appropriate medical treatment. If the injury is caused by the negligence of the University of Montana or any of its employees, you may be entitled to reimbursement or compensation pursuant to the Comprehensive State Insurance Plan established by the Department of Administration under the authority of M.C.A., Title 2, Chapter 9. In the event of a claim for such injury, further information may be obtained from the University's Risk manager (406)-243-2700; jason.sloat@mso.umt.edu) or the Office of Legal Counsel (406-243-4742; legalcounsel@umontana.edu).

Questions:

If you have any questions about the research now or during the study, please contact: Dr. John Quindry (406) 243-4268. If you have any questions regarding your rights as a research subject, you may contact the UM Institutional Review Board (IRB) at (406) 243-6672.

Statement of Your Consent:

I have read the above description of this research study. I have been informed of the risks and benefits involved, and all my questions have been answered to my satisfaction. Furthermore, I have been assured that any future questions I may have will also be answered by a member of the research team. I voluntarily agree to take part in this study. I understand I will receive a copy of this consent form.

_____
Printed Name of Subject

_____ _____
Subject's Signature Date

Statement of Consent to be Photographed:

I understand that photographs may be taken during the study. I consent to having my photograph taken and used in presentations related to this study. I understand that if photographs are used for presentations of any kind, names or other identifying information will not be associated with them.

_____ _____
Subject's Signature Date

The University of Montana IRB
Expiration Date None
Date Approved 11-20-2020
Chair/Admin

Appendix B: IRB# 83-21: “The Effects of Circadian Rhythm Disruption on the Inflammatory Response to Particulate Matter Exposure from Woodsmoke”

Form RA-108
(Rev. 6/24/2019)

JUN 3 2021
Revised

UNIVERSITY OF MONTANA

Institutional Review Board (IRB)
for the Protection of Human Subjects in Research

APPLICATION FOR IRB REVIEW

IRB Protocol No.: 83-21

At the University of Montana (UM), the Institutional Review Board (IRB) is the institutional review body responsible for oversight of all research activities involving human subjects as outlined in the U.S. Department of Health and Human Services' Office of Human Research Protections.

Instructions: A separate application must be submitted for each project. Email the completed form as a Word document to *IRB@umontana.edu*, or submit a hardcopy (no staples) to the IRB office in the Interdisciplinary Science Building, room 104. Student applications must be accompanied by email authorization by the supervising faculty member or a signed hard copy. *All fields must be completed. If an item does not apply to this project, write in: N/A.* Questions? Call the IRB office at 243-6672.

1. Administrative Information

Project Title: The effects of circadian rhythm disruption on the inflammatory response to particulate matter exposure from woodsmoke	
Principal Investigator: Dr. John Quindry	UM Position: Professor
Department: IPAT	Office location: McGill Hall 102
Work Phone: (406) 243-4268	Cell Phone:

2. Human Subjects Protection Training

(All researchers, including faculty supervisors for student projects, must be listed below and have completed a self-study course on protection of human research subjects ***within the last three years*** *and be able to supply the "Certificate(s) of Completion" upon request. If you need to add rows for more people, use the Additional Researchers Addendum.*

All Research Team Members (list yourself first)	PI	CO-PI	Faculty Supervisor	Research Assistant	DATE COMPLETED IRB-approved Course mm/dd/yyyy
Name: John Quindry, PhD Email: john.quindry@umontana.edu	☒	☐	☐	☐	9/17/2018 ✓
Name: Graham McGinnis Email: graham.mcginnis@unlv.edu	☐	☒	☐	☐	~~1/30/2018~~ 6/4/2021 Attached.
Name: Cassie Williamson-Reisdorph Email: cassie.reisdorph@umontana.edu	☐	☐	☐	☒	08/28/2018 ✓
Name: Joe Sol Email: joseph.sol@umconnect.umt.edu	☐	☐	☐	☒	3/30/2021 ✓

3. Project Funding

(If federally funded, additional requirements may apply.)

Is grant application currently under review at a grant funding agency? ☒Yes *(If yes, cite sponsor on ICF if applicable)* ☐No

Has grant proposal received approval and funding? ☒Yes *(If yes, cite sponsor on ICF if applicable)* ☐No

Agency	Grant No.	e-Prop #	Start Date	End Date	PI on grant
USFS	funded, ongoing	2021-302	8/6/18	3/23/23	John Quindry
INBRE	funded, being finalized	pending	3/1/2021	2/28/2023	Graham McGinnis, John Quindry

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For UM-IRB Use Only

IRB Determination:

Note to PI: Use any attached IRB-approved forms (signed/dated) as "masters" when preparing copies. Notify the IRB if any significant changes or unanticipated events occur. Failure to follow these directions constitutes non-compliance with UM policy.

_____ Not Human Subjects Research
_____ Approved by Exempt Review, Category # _____
_X_ Approved by Expedited Review, Category #_____
_____ Full IRB Determination
  _____ Approved *(see* **Note to PI***)*
  _X_ Conditional Approval *(see memo)* - IRB Chair Signature/Date: [signature] 5/25/2021
    _X_ Conditions Met *(see* **Note to PI***)*
  _____ Resubmit Proposal *(see memo)*
  _____ Disapproved *(see memo)*

Risk Level: Minor increase over Minimal

Final Approval by IRB Chair/Manager: [signature] Date: 6/16/2021 Expires: 6/15/2022

SUBJECT INFORMATION AND INFORMED CONSENT

Study Title: The effects of circadian rhythm disruption on the inflammatory response to particulate matter exposure from woodsmoke

Sponsor: United States Department of Agriculture Forest Service and the National Institutes of Health Montana INBRE program

Investigator(s):
John Quindry, Ph.D. (406) 243-4268
Graham McGinnis, Ph.D. (702) 895-4626
Cassie Williamson-Reisdorph, MS (406) 243-4268
Joe Sol, MS (406) 243-4268

Please read the following information carefully and feel free to ask questions. This consent form may contain words that are new to you. Only sign the final page when you are satisfied and the procedures and risks have been sufficiently explained.

Inclusion criteria: This research study requires that you meet the following criteria:

- You must be a male between the ages of 18 and 50 years old
- You must be a non-smoker (of any substance) within the last 12 months
- You must be able to cycle continuously at a moderate intensity for 45 minutes
- Be an 'Intermediate Chronotype.' (Not morning larks or night owls)

Exclusion criteria:

- Having a history of chronic lung disease, asthma, or Covid-19 with diagnosed lung involvement within the last year
- Having have had a previous adverse reaction to environmental smoke exposure
- Having had orthopedic limitations that would limit your ability to exercise
- Having been diagnosed with cardiovascular or metabolic diseases
- Having been diagnosed with disordered sleep or sleep apnea
- Having traveled overnight outside of this time zone within the two weeks before participation, and the three weeks during the scheduled testing
- Having Covid-19 symptoms or having been exposed to someone with symptoms in the last week

Purpose: The purpose of this study is to better understand the effects of sleep deprivation and exercise during heavy woodsmoke inhalation on your cardiovascular and respiratory system (heart and lungs). To evaluate these things, we will have you perform two exercise trials on a stationary cycle while breathing a controlled amount of woodsmoke generated in our lab facility. In order to examine the influence of sleep restriction (good sleep vs. bad sleep) you will report to one exercise trial well rested, while in the other trial you will be instructed to go to sleep late and wake up early. To understand the overall stress imposed on you, we will examine your heart using a Fitbit monitor and a heart rate monitor. We will examine your lungs with a breathing test and will examine hormonal, inflammation, and circadian rhythm (time of day) biomarkers from blood, saliva, and breath moisture ("exhaled breath condensate") collected before and after exercise.

Procedures: You will be asked to participate in three visits (i.e., Baseline Testing and Exercise Inhalation Trials 1 and 2) over a 3-week period. Baseline Testing will be performed to determine eligibility for the study (e.g., your cardio fitness level). Baseline Testing will also be used to determine the moderate-intensity exercise bicycle setting for Exercise Inhalation Trials 1 & 2.

Before all visits, you will be asked to avoid eating or consuming any beverage other than water for 12 hours prior to testing (including alcohol and caffeinated beverages). As a general rule with morning testing of this nature, you will be tested following an overnight fast (water is acceptable).

Baseline Testing (015 McGill Hall, ~ 1.5 hours): Upon arrival, the details of the study will be explained to you and you will be given the opportunity to provide informed consent to participate in this study. For this process, you will be asked to complete several forms called the ***PAR-Q-plus*** (**physical activity readiness questionnaire** which determines exercise rediness), the ***MEQ*** (**morningness-eveningness questionnaire** which assesses your waking/sleeping preferences) and the ***MCTQ*** (**Munich chronotype questionnaire** which determines your actual waking/sleeping habits). If you qualify for this investigation after the completion of these forms you will then have the opportunity to sign the informed consent document.

Next you will undergo testing to determine your body composition (lean vs. fat tissue) and your cardio fitness ("maximal oxygen consumption, VO_{2max}") on a bicycle ergometer. The body composition test will measure your percentage of body fat via an underwater weighing test. The test will begin with the recording of your height and weight while wearing a bathing suit. Then, you will submerge yourself in a tank of warm water (similar to a hot tub) and hold your breath for four seconds while your weight is recorded. This procedure will be repeated until three consistent measurements are obtained. This test will take up to 30 minutes, and a nose clip will be provided.

Next, you will perform a maximal intensity bicycling test, called a "VO_{2max} test". The VO_{2max} test will take approximately 45 minutes, including 20 minutes of preparation, 10-20 minutes of cycling exercise, and 5 minutes of recovery. The results of the VO_{2max} test indicate your cardiovascular fitness level and determine eligibility for full study participation. Exercise intensity (cycling resistance/power) will gradually increase until you reach a maximal effort. During the test, the amount of oxygen used during exercise will be measured using a facemask that covers your nose and mouth. Study participation in the Exercise Inhalation Trials 1&2 requires that you have a VO_{2max} test result that exceeds 40 ml/kg/min (the ability to run approximately 7 mph for 45 minutes, or bicycle 16-17 mph for 45 minutes). During the VO_{2max} test, heart rate will be measured using an elastic chest strap and heart rate monitor worn under your shirt.

Following the completion of Baseline Testing, if VO_{2max} test inclusion criteria are met, scheduling will be completed for Exercise Inhalation Trials 1&2 (with each of these trials being separated by at least one week), and you will be equipped with a Fitbit Charge 3 fitness tracker and a Polar Heart Rate Monitor. The Fitbit is worn throughout the study (~3 weeks) like a watch, and it measures your patterns of activity and sleep over the study period. Note that if you are uncomfortable wearing the Fitbit watch (e.g., you're concerned it will get snagged during an activity), you may take it off. The Polar heart rate monitor will be worn the night before your 2 Exercise Inhalation Trials (~12 hours prior) and for 24 hours after these moderate-intensity exercise sessions. You will also be provided with a cheek swab sample collection kit (described below) to collect cells from the inside of your cheeks on a Q-tip. Finally, you will be given a paper copy of a sleep diary to manually record when you go to sleep and when you wake during the testing period.

Exercise Inhalation Trials 1&2 – (Inhalation and Pulmonary Physiology Core within Center for Environmental Health, 061 in the basement of the Skaggs Building ~ 3.5 hours each). As described below, these research trials are identical with the exception for the amount of sleep you receive prior to the trial:

After the Baseline Testing, we will provide you with a cheek swab kit to take home with you containing material for at-home sample collection leading up to each trial. In this kit, you will have an instruction manual, 4 small tubes with a sample stabilization buffer, and 4 small brushes that you will use to brush the inside of your cheek. At 4 specific times leading up to the experimental tests (12:00 noon the day before, 6:00 pm the day before, 12:00 midnight the night before, and 6:00 am the day of testing), you will use the brush to gently scrape the inside of the cheek for approximately 30 seconds, and carefully place the brush in

the tube, submerging it in sample stabilization buffer, and sealing the tube. You should avoid consuming anything other than water for at least 30 minutes prior to sample collection. Sample collection should not cause any irritation of the cheek. Importantly, cheek swab collection at midnight should be performed with minimal exposure to light. Cheek cells will be used to measure the expression of genes related to your sleep/circadian rhythm.

The night before your laboratory visit, you will undergo either 1) a 'normal' night of sleep, with an approximately 8-hour sleep opportunity from 10:00 pm to 6:00 am, or 2) a 'restricted' night of sleep (approximately 4-hours),where you go to sleep at 12:00 midnight and set an alarm to wake at 4:00 am.

At each visit, you will be asked to 1) provide pre-trial samples (blood and exhaled breath condensate), 2) undergo baseline lung function measurements, 3) put on a heart rate monitor (chest strap) to be worn during the visit and for 24-hours post visit, and 4) baseline cardiovascular function measurements. Additional details for each of these tests is provided below.

You will then be asked to cycle at a moderate intensity while exposed to air that contains wood smoke (particulate at a diameter of 2.5 µm; roughly the size of a pollen molecule, and at concentration of 250 $\mu g/m^3$) after a night of normal sleep (~8 hours) or restricted sleep (~4 hours). Both trials will occur for a period of 45 minutes. The exercise intensity will equate to 70% VO_{2max}, an intensity that you will recognize as being of moderate intensity. Wood smoke will be delivered directly using a modified mask respirator to make it more comfortable. You will be allowed to consume water at 15-minutes and 30-minutes during each exercise trial. The amount of water consumed will be recorded and matched between Exercise Inhalation Trials 1&2.

Measurement of smoke concentration will be confirmed using an air particulate counting device called a DustTrak II (Model 8530), which makes sure you don't inhale too much smoke. Delivery of smoke within the exposure chamber will occur through a modified breathing mask attached to you through a mouthpiece. Other aspects of air quality, including temperature, humidity, carbon monoxide, and carbon dioxide in the exposure chamber and exercise room will be monitored with a device called a Q-Trak. Immediately after the 45-minute exercise trials, you will be asked to provide another blood and exhaled breath condensate sample and complete another lung and cardiovascular function test. 90 minutes after the completion of the exercise trial, a third set of tests and another blood and exhaled breath condensate sample will be collected.

The total time commitment will be approximately 1.5 hours for Baseline Testing, and approximately 3.5 hours on both Exercise Inhalation Trials 1&2, for a total of ~8 hours total time during the 3-week testing period.

Tests 1-5 below will be completed immediately before, after, and 90-minutes after the exposure to wood smoke on Days 2-3:

1) **Lung Function Tests** - You will be asked to inhale fully and then forcibly exhale into a device called a spirometer to collect measurements of lung function (vital capacity, forced expiratory volume, maximal voluntary ventilation). This test will be performed between 2-3 times.
2) **Cardiovascular Function Tests** - You will be asked to sit or lay quietly in a dark room while connected to a device that monitors your heart rate, rhythm, and heart rate variability. Afterwards, an automated device will record blood pressure in your arm and leg. As this test is conducted, a research assistant will gently place a "transducer" (a soft probe that will measure blood pulses with each heartbeat) over your carotid artery (the artery in your neck), the major blood vessel in your neck. All of these tests are completely painless and can be performed while you lie on a padded table.
3) **Exhaled Breath Moisture ("Exhaled Breath Condensate", EBC)** - You will be asked to exhale normally into a five-foot-long section of 3/4" diameter plastic tubing while seated. The length of the

tubing goes through an ice bath (0 degrees Celsius) to cool the exhaled air and allow for liquid collection. While wearing a nose clip, you will breathe in room air and exhale into a mouthpiece (with spit trap) that is attached to the tube, which has a one-way valve on both ends of the tube. After 10 minutes of exhaling into the tube, researchers will collect the tube containing the EBC sample (~3 mL) for analysis. At a later time, your EBC samples will be analyzed to determine whether smoke exposure during exercise resulted in increased inflammatory molecules present in your lung alveolar fluid.

4) **Blood Samples** - From the 3 blood draws conducted per exercise trial, approximately 30 milliliters (3x 10 ml or 3 tablespoons total) of blood will be taken from a vein in your arm (alternating arms as needed). Blood will be drawn before exercise, immediately after exercise, and 90-minutes after the conclusion of exercise (1 tube of blood at each blood draw). At a later time, your stored blood samples will be analyzed to determine whether smoke exposure during exercise resulted in increased inflammatory molecules circulating in your blood.
5) **Saliva Samples** - Saliva samples will be collected prior to and 90-minutes post exercise to monitor biomarkers in your saliva. Using a saliva collection tube (Salivette®Cortisol, SARSTEDT AG&Co. Germany), you will chew on a small cotton swab for 60 seconds and place it into the tube. Collection tubes will then be spun in a centrifuge to collect the saliva sample in the tube and the sample will be stored for analysis of biomarkers at a later date.

Risks/Discomforts:

1) Exercise related risks and discomforts - Mild discomfort may result during and after the exercise sessions. These discomforts may include shortness of breath, tired or sore legs, nausea and possibility of vomiting. Muscle soreness could occur after the exercise; however, it should not persist beyond a day or two. Certain changes in body function can occur following exercise, some of which are normal, and others are abnormal. Normal occurrences include mild symptoms of dehydration such as headache and general fatigue. Abnormal changes that may occur include alterations in blood pressure, heart rate, heart rhythm (as might be observed clinically on an ECG), or extreme shortness of breath. Very rare instances of heart attack have occurred following exercise. During any of the exercise performed for this study, should abnormal symptoms occur, such as chest discomfort, unusual shortness of breath or other abnormal findings develop, the exercise physiologist conducting the research will terminate the test. These symptoms could include moderate to severe angina (chest pain), increased dizziness, shortness of breath, fatigue or your desire to stop. If you experience these symptoms, please inform study personnel immediately.

2) Smoke inhalation risks and discomforts - During the Exercise Inhalation Trials, we will expose you to air containing moderate smoke particulate (250 $\mu g/m^3$, smaller than a dust particle) after a night of normal sleep (~8 hours) or restricted sleep (~4 hours). The total smoke exposure you experience is a combination of the particulate concentration, the number of breaths, and the depth of breath taken. During exercise at 70% of maximal intensity, you will take more breaths and deeper breaths while exposed to smoky air. For these reasons, the total smoke particulate exposure during your moderate dose exposure trial (250 $\mu g/m^3$, categorized as being on the threshold between "Very Unhealthy" and "Hazardous" by the U.S. Environmental Protection Agency) and is comparable to non-exercise exposures where one breathes several hours (3-6 hours) of smoky air created while not exercising. The moderate dose of wood smoke particulate (250 $\mu g/m^3$) used in this study is consistent with several other human exposure wood smoke studies of this type (using exercise + wood smoke) and are also reported in published scientific literature.

3) A wood smoke exposure study reported that the discomfort from smoke was minimal with exposure at 250 $\mu g/m^3$. The most prevalent symptom reported was a mild increase in eye irritation in 10 of 13

subjects. A slight increase in nose irritation was reported by 5 of 13 subjects, and 6 subjects reported the smell of wood smoke to be somewhat unpleasant. These subjects were exposed to similar concentrations of particulate matter for four hours, so the discomfort experienced should be comparable. The modified mask used in this study covers just the nose and mouth, and therefore should not result in eye irritation. Based on a wealth of related research, there is no evidence that comparable doses of smoke exposure result in lasting effects to one's health or wellbeing. A research assistant will be constantly monitoring both the particulate matter content and your signs of discomfort during all trials.

4) Risk and discomforts related to pulmonary function testing, exhaled breath condensate, and blood draw - You may experience discomfort when performing the pulmonary test and exhaled breath condensate tests due to the breathing requirements of these techniques. When blood samples are obtained for the study, you may feel a slight sting or pinch in your arm, you may suffer a small bruise, and there is a very slight possibility of infection. Should you notice unusual redness, bruising, or swelling at the blood sampling site, you should seek medical attention and contact John Quindry or Graham McGinnis.

5) Risks associated with acute sleep restriction and cheek swab sample collection – You may experience mild, transient symptoms following acute sleep restriction including fatigue and drowsiness. However, these symptoms should be reversed after a single night of 'recovery' sleep. Cheek swab collection should not induce any irritation. If so, you should contact John Quindry or Graham McGinnis.

6) Risks associated with Covid-19 transmission have been minimized by having you review a CDC Covid-19 questionnaire prior to each data collection session. Researchers will also adhere to this practice, canceling all data collection when anyone is symptomatic for Covid-19. Moreover, we will not resume data collection until both participants and researchers are confirmed as Covid-19 free/recovered. Daily temperature and symptom checks will be performed during data collection as an added measure to minimize risk. Moreover, social distancing and personal protective equipment will be used according to CDC recommendations during data collection. A HEPA filtration device will be used to clean air around you while you exercise, and your trial will be separated from other participants by a standard amount of time to ensure removal of potentially contaminated air.

7) Fitbit and Polar heart rate monitor devices may accumulate moisture producing skin irritation, a rash, or other skin discomfort. In order to minimize this risk, you will be provided with wearables that have been cleaned with alcohol and dried prior to your use. You will also be instructed on how to clean the devices while they are in your possession for this study. Finally, if you should experience any skin irritation, remove the wearable device and contact John Quindry or Graham McGinnis. As a last consideration, the Fitbit device may get caught while being physically active (e.g., snagging while doing yard work). If this becomes a concern, please remove the device and contact John Quindry or Graham McGinnis with questions or concerns.

You will be informed of any new findings that may affect your decision to remain in the study.

Benefits: As an incentive for participating in this study, you will receive information on your physical fitness (VO_{2max}) and body composition. Also, this research may provide useful insight into how poor sleep may impact exposure to wood smoke and affect an individual's cardiovascular and respiratory systems. This information may be useful for those who work as wildland firefighters, others who perform strenuous activity while exposed to wood smoke, and those members of the general public that are exposed to wood smoke. Note that while blood work and gene expression analysis will be performed, these results will not be returned as a study benefit.

Confidentiality: Your records will be kept confidential and will not be released without your consent except as required by law. Your identity as a participant in this study will be kept private. The intended outcome of this research is a published manuscript in a scientific journal. Should that occur, your name will not be used in any publications or presentations about this research. To achieve this level of privacy, you will be randomly assigned an identification number at the beginning of the study and all questionnaire responses and data collected will be labeled with this assigned number. The signed informed consent forms and the key linking the identification number to your name and contact number will be under the control of the John Quindry or Graham McGinnis and kept separately in a locked file cabinet. After the completion of the study, the key linking identifying personal information to their respective identification number will be destroyed. A separate sheet containing only the contact information with no identification numbers will be kept until data analysis is complete. Separately, you will be given the opportunity to concent or decline the change to have your photograph used in presentations or publications associated with this research.

Voluntary Participation/Withdrawal: You may refuse to take part, or you may withdraw from the study at any time without penalty or loss of benefits to which you are normally entitled. If you choose to withdraw, please notify Dr. John Quindry or Dr. Graham McGinnis (contact information below) as soon as possible.

Compensation for Injury: In the event that you are injured as a result of this research you should individually seek appropriate medical treatment. If the injury is caused by the negligence of the University of Montana or any of its employees, you may be entitled to reimbursement or compensation pursuant to the Comprehensive State Insurance Plan established by the Department of Administration under the authority of M.C.A., Title 2, Chapter 9. In the event of a claim for such injury, further information may be obtained from the University's Risk Manager (406-243-2700; michele.wheeler@umontana.edu) or the Office of Legal Counsel (406-243-4742; legalcounsel@umontana.edu). (Reviewed by University Legal Counsel, May 9, 2013)

Future research: Data and biological specimens collected during this investigation may be used for future investigations. In this regard, all your data will be anonymized (containing no identifiable links to your identity or private information).

A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by U.S. law. This website will not include information that can identify you. At most, the website will include a summary of the results. You can search this website at any time.

Questions: You may wish to discuss this with others before you agree to take part in this study. If you have any questions about the research now or during the study, please contact Dr. John Quindry, PhD at (406) 243-4268 (john.quindry@mso.umt.edu) or Dr. Graham McGinnis at (702) 895-4626 (graham.mcginnis@unlv.edu). If you have any questions regarding your rights as a research subject, you may contact the UM Institutional Review Board (IRB) at (406) 243-6672.

Statement of Your Consent: I have read the above description of this research study. I have been informed of the risks and benefits involved, and all my questions have been answered to my satisfaction. Furthermore, I have been assured that any future questions I may have will also be answered by a member of the research team. I voluntarily agree to take part in this study. I understand I will receive a copy of this consent form.

_____
Printed Name of Subject

_____ _____
Subject's Signature Date

Statement of Consent to be Photographed:

I consent to use of my photograph in presentations related to this study.

The University of Montana IRB
Expiration Date 6-15-2022
Date Approved 6-16-2021
Chair/Admin

I understand that if photographs are used for presentations of any kind, names or other identifying information will not be associated with them.

_____ _____

Subject's Signature Date

The University of Montana IRB
Expiration Date 6-15-2022
Date Approved 6-16-2021
Chair/Admin

Appendix C: IRB# 172-22: “Cold Pressor Test and Circadian Chronotype as Predictores of Physiological Responses to Woodsmoke Inhalation During Exercise”

Form RA-108
(Rev. 8/01/2022)

Institutional Review Board
University of Montana
NOV 4 2022

UNIVERSITY OF MONTANA
Institutional Review Board (IRB)
for the Protection of Human Subjects in Research

IRB Protocol No.: 172-22

APPLICATION FOR IRB REVIEW

At the University of Montana (UM), the Institutional Review Board (IRB) is the institutional review body responsible for oversight of all research activities involving human subjects as outlined in the U.S. Department of Health and Human Services' Office of Human Research Protections.

Instructions: A separate application must be submitted for each project. Email the completed form as a Word document to IRB@umontana.edu, or submit a hardcopy (no staples) to the IRB office in the Interdisciplinary Science Building, room 104. Student applications must be accompanied by email authorization by the supervising faculty member or a signed hard copy. *All fields must be completed. If an item does not apply to this project, write in: N/A.* Questions? Call the IRB office at 243-6672.

1. Administrative Information

Project Title: Cold Pressor Test And Circadian Chronotype as Predictors of Physiological Responses to Woodsmoke Inhalation During Exercise	
Principal Investigator: Dr. John C. Quindry	UM Position: Professor
Department: School of Integrative Physiology and Atheltic Training	Office location: MCG 102
Work Phone: (406) 243-4268	Cell Phone:

2. Human Subjects Protection Training

(All researchers, including faculty supervisors for student projects, must be listed below and have completed a self-study course on protection of human research subjects ***within the last three years*** *and be able to supply the "Certificate(s) of Completion" upon request. If you need to add rows for more people, use the Additional Researchers Addendum.*

All Research Team Members (list yourself first)	**PI**	**CO-PI**	**Faculty Supervisor**	**Research Assistant/ Other**	**DATE COMPLETED IRB-approved Course mm/dd/yyyy**
Name: John, Quindry, PhD Email: john.quindry@mso.umt.edu	☒	☐	☐	☐	9/21/2021
Name: Joe Sol Email: joseph.sol@umontana.edu	☐	☒	☐	☐	3/30/2021
Name: Email:	☐	☐	☐	☐	
Name: Email:	☐	☐	☐	☐	

3. Project Funding *(If federally funded, additional requirements may apply.)*

Is grant application currently under review at a grant funding agency? ☐Yes *(If yes, cite sponsor on ICF if applicable)* ☐No			Has grant proposal received approval and funding? ☐Yes *(If yes, cite sponsor on ICF if applicable)* ☐No		
Agency	Grant No.	e-Prop #	Start Date	End Date	PI on grant

For UM-IRB Use Only

IRB Determination:

Note to PI: Use any attached IRB-approved forms (signed/dated) as "masters" when preparing copies. Notify the IRB if any significant changes or unanticipated events occur. Failure to follow these directions constitutes non-compliance with UM policy.

_____ Not Human Subjects Research
_____ Approved by Exempt Review, Category # _____
__X__ Approved by Expedited Review, Category # 4
_____ Full IRB Determination
  _____ Approved *(see* **Note to PI***)*
  _____ Conditional Approval *(see memo)* - IRB Chair Signature/Date: _____
    _____ Conditions Met *(see* **Note to PI***)*
  _____ Resubmit Proposal *(see memo)*
  _____ Disapproved *(see memo)*

Risk Level: _____

Final Approval by IRB Chair/Manager: _____ Date: 11-18-2022 Expires: N/A

1

SUBJECT INFORMATION AND INFORMED CONSENT

Study Title: Cold Pressor Test And Circadian Chronotype as Predictors of Physiological Responses to Woodsmoke Inhalation During Exercise

Investigator(s):
John Quindry, Ph.D. (406) 243-4268
Joe Sol, MS (406) 243-4268

Please read the following information carefully and feel free to ask questions. This consent form may contain words that are new to you. Only sign the final page when you are satisfied, and the procedures and risks have been sufficiently explained.

Inclusion criteria: This research study requires that you meet the following criteria:

- You must be a male between the ages of 18 and 50 years old
- You must be a non-smoker within the last 12 months
- You must be able to cycle continuously at a moderate intensity for 120 minutes

Exclusion criteria:

- Having a history of chronic lung disease, asthma, or Covid-19 with diagnosed lung involvement within the last year
- Having had a previous adverse reaction to environmental smoke exposure
- Having had orthopedic limitations that would limit your ability to exercise
- Having been diagnosed with cardiovascular or metabolic diseases
- Having Covid-19 symptoms or having been exposed to someone with symptoms in the last week

Purpose: The purpose of this study is to better understand the effects of exercise during heavy woodsmoke inhalation on your cardiovascular and respiratory systems (heart and lungs). To evaluate these things, we will assess potential differences in physiological response by testing a large group of individuals and selecting a smaller subset of individuals who are 'hyperreactive" responders. As a hyperreactive response (blood pressure increases more than 20 mmHg during the test) in the cold pressor test (CPT) indicates a more significant response to stress, this measure could be pivotal in explaining why otherwise healthy subjects react differently to stimuli and if this has potential clinical implications

We will have you exercise on a stationary cycle while breathing a controlled amount of woodsmoke generated in our lab facility. We will examine your lungs with a breathing test and examine inflammation biomarkers from blood and breath moisture ("exhaled breath condensate") collected before and after exercise. We will examine your heart using a heart rate monitor to understand the stress imposed on you.

Procedures: You will be asked to participate in up to four visits (i.e., Initial Screening, Baseline Testing, Exercise Inhalation Trial, 24-post Trial Follow-up) over a 3-week period. Initial Screening and Baseline Testing will be performed to determine eligibility for the study (e.g., your response to the CPT and fitness level).

Before all visits, you will be asked to avoid eating or consuming beverages other than water for 12 hours prior to testing (including alcohol and caffeinated beverages). As a general rule with morning testing of this nature, you will be tested following an overnight fast (water is acceptable).

Initial Screening (015 McGill Hall, ~ 15 minutes): Upon arrival, the study details will be explained to you, and you will be given the opportunity to provide informed consent for study participation. After which, you will perform a cold pressor test to evaluate your blood pressure and heart rate response to placing your hand in a bucket of ice water for 2 minutes. In doing so, you will be seated with a blood pressure cuff on your non-dominant upper arm and place your dominant hand into the water when instructed. After completing the CPT,

you will also be asked to complete a 19-response questionnaire (MEQ-19) to determine your morning-eveningness chronotype. In answering the questions, your responses will help identify attributes of your circadian rhythm (24-hour bodily clock) and when you prefer to do activities.

Baseline Testing (015 McGill Hall, ~ 1.5 hours): Upon arrival, you will be asked to complete a physical activity readiness questionnaire (PAR-Q+) and then undergo testing to determine your body composition (lean vs. fat tissue) and your cardio fitness ("maximal oxygen consumption, VO_{2max}") on a bicycle ergometer. The body composition test will measure your body fat percentage via an underwater weighing test. The test will begin with recording your height and weight while wearing a bathing suit. Then, you will submerge yourself in a tank of warm water (similar to a hot tub) and hold your breath for four seconds while your weight is recorded. This procedure will be repeated until three consistent measurements are obtained. This test will take up to 30 minutes, and a nose clip will be provided.

Next, you will perform a maximal intensity bicycling test called a "VO_{2max} test". The VO_{2max} test will take approximately 45 minutes, including 20 minutes of preparation, 10-20 minutes of cycling exercise, and 5 minutes of recovery. The results of the VO_{2max} test indicate your cardiovascular fitness level and determine eligibility for full study participation. Exercise intensity (cycling resistance/power) will gradually increase until you reach maximum effort. During the test, the amount of oxygen used during exercise will be measured using a facemask that covers your nose and mouth. Study participation in the Exercise Inhalation Trials 1&2 requires that you have a VO_{2max} test result that exceeds 40 ml/kg/min (the ability to run approximately seven mph for 45 minutes or bicycle 16-17 mph for 45 minutes). During the VO_{2max} test, heart rate will be measured using an elastic chest strap and heart rate monitor worn under your shirt.

Following the completion of Baseline Testing, if VO_{2max} test inclusion criteria are met, you may be contacted by the researchers to schedule the Exercise Inhalation Trial.

Exercise Inhalation Trials – (Inhalation and Pulmonary Physiology Core within Center for Environmental Health, 061 in the basement of the Skaggs Building ~ 3.5 hours each):
At this visit, you will be asked to 1) undergo lung function measurements, 2) cardiovascular function measurements, and provide 3) exhaled breath condensate (breathe vapor), and 4) blood samples. You will also conduct another CPT before and after the exercise inhalation trial. Additional details for each of these tests are provided below.

After these initial tests, you will be asked to cycle while exposed to air containing wood smoke (particulate at a diameter of 2.5 μm, roughly the size of a pollen molecule, and at a concentration of 250 $\mu g/m^3$) for 120 minutes. The exercise intensity will be 22.5 $ml \cdot kg^{-1} \cdot min^{-1}$, an intensity that you will recognize as being of moderate intensity. Wood smoke will be delivered directly using a modified mask respirator to make it more comfortable. You will be allowed to consume water at 15-minute intervals throughout the exercise trial. Measurement of smoke concentration will be confirmed using an air particulate counting device called a DustTrak II (Model 8530), which makes sure you don't inhale too much smoke. Delivery of smoke within the exposure chamber will occur through a modified breathing mask attached to you through a mouthpiece. Other aspects of air quality, including temperature, humidity, carbon monoxide, and carbon dioxide in the exposure chamber and exercise room, will be monitored with a Q-Trak device. Immediately after the 120-minute exercise trials, you will be asked to provide another blood and exhaled breath condensate sample and complete another lung and cardiovascular function test. Twenty-four hours after the completion of the exercise trial, the third set of tests and another blood and exhaled breath condensate sample will be collected.

Additionally, 12 hours before your scheduled Exercise Inhalation trial, you will meet with a researcher to wear a heart rate monitor (chest strap and watch). The monitor will be worn for 12 hours before and 24 hours after the trial (36 hours total) to identify your baseline heart rate variability (pre-trial) and monitor your recovery

until you return for the 24-hour follow-up testing when the monitor is removed.

The total time commitment will be approximately 1.5 hours for Baseline Testing, and about 3.5 hours for the Exercise Inhalation Trial, for a total of ~5 hours during the 3-week testing period.

Tests 1-4 below will be completed immediately before, after, and 24 hours after the exposure to wood smoke during the Exercise Inhalation Trial:

1) **Lung Function Tests** - To obtain this data, you will be asked to inhale fully and then forcibly exhale into a spirometer device to collect measurements of lung function (vital capacity, forced expiratory volume, maximal voluntary ventilation). This test will be performed between 2-3 times.
2) **Cardiovascular Function Tests** - To obtain this measurement, you will be asked to sit or lay quietly in a dark room while connected to a device that monitors your heart rate, rhythm, and heart rate variability. Afterward, an automated machine will record your arm and leg blood pressure. As this test is conducted, a laboratory research assistant will gently place a "transducer" (a soft probe that will measure blood pulses with each heartbeat) over your carotid artery (the artery in your neck), the major blood vessel in your neck. All these tests are painless and can be performed while lying on a padded table.
3) **Exhaled Breath Moisture ("Exhaled Breath Condensate," EBC)** - You will be asked to exhale normally into a five-foot-long section of 3/4" diameter plastic tubing while seated. The length of the tubing goes through an ice bath (0 degrees Celsius) to cool the exhaled air and allow for liquid collection. While wearing a nose clip, you will breathe in room air and exhale into a mouthpiece (with spit trap) attached to the tube, which has a one-way valve on both ends. After 10 minutes of exhaling into the tube, researchers will collect the tube containing the EBC sample (~3 mL) for analysis. Later, your EBC samples will be analyzed to determine whether smoke exposure during exercise increases inflammatory molecules in your lung alveolar fluid. During this test, you will also perform a CPT with the same protocol as performed at your initial visit.
4) **Blood Samples** - From the blood draws conducted per exercise trial, approximately 30 milliliters (3x 10 ml or three tablespoons total) of blood will be taken from a vein in your arm (alternating arms as needed). Blood will be drawn before, immediately after, and 90-minutes after the conclusion of exercise (1 tube at each blood draw). Later, your stored blood samples will be analyzed to determine whether smoke exposure during training increases inflammatory molecules circulating in your blood.

Risks/Discomforts: Exercise-related risks and discomforts - Mild discomfort may result during and after the exercise sessions. These discomforts may include shortness of breath, tired or sore legs, nausea, and the possibility of vomiting. Muscle soreness could occur after the exercise; however, it should not persist beyond a day or two. Specific changes in body function can happen in the following exercise, some of which are normal, and others are abnormal. Normal occurrences include mild symptoms of dehydration, such as headache and general fatigue. Abnormal changes that may occur include alterations in blood pressure, heart rate, heart rhythm (as might be observed clinically on an ECG), or extreme shortness of breath. Very rare instances of heart attack have occurred following exercise. During any of the activities performed for this study, should abnormal symptoms arise, such as chest discomfort, unusual shortness of breath, or other abnormal findings develop, the exercise physiologist conducting the research will terminate the test. These symptoms could include moderate to severe angina (chest pain), increased dizziness, shortness of breath, fatigue, or your desire to stop. If you experience these symptoms, please inform study personnel immediately.

Smoke inhalation risks and discomforts - During the Exercise Inhalation Trials, we will use air containing moderate smoke particulate (250 $\mu g/m^3$, smaller than a dust particle. The total smoke exposure you experience combines the particulate concentration, the number of breaths, and the depth of breath taken. The smoke particulate exposure during your moderate dose exposure trial (250 $\mu g/m^3$, categorized as being on the threshold between "Very Unhealthy" and "Hazardous" by the US Environmental Protection Agency) and is comparable to non-exercise exposures where one breathes several hours (3-6 hours) of smoky air created while not exercising.

The moderate dose of wood smoke particulate (250 $\mu g/m^3$) used in this study is consistent with several other human exposure wood smoke studies reported in published scientific literature. It should be noted that much higher levels of wood smoke exposure have been used in a similar laboratory-based investigation, where smoke particulate concentrations reached 1500 $\mu g/m^3$ for two hours. Moreover, the Forest Service has collected wood smoke exposure data from over 300 subjects in 17 states during actual wildland firefighting activities. Their data indicate that the average particulate matter exposure was 300-900 $\mu g/m^3$, depending on their work duties and fire and wind behavior.

A wood smoke exposure study reported that the discomfort from smoke was minimal with exposure at 250 $\mu g/m^3$. The most prevalent symptom reported was a mild increase in eye irritation in 10 of 13 subjects. A slight rise in nose irritation was reported by 5 of 13 subjects, and six reported the smell of wood smoke to be somewhat unpleasant. These subjects were exposed to similar particulate matter concentrations for four hours, so the discomfort experienced should be comparable. The modified mask used in this study covers just the nose and mouth and, therefore, should not result in eye irritation. Based on a wealth of related research, there is no evidence that comparable doses of smoke exposure have lasting effects on one's health or well-being. Multiple research assistants will constantly monitor the particulate matter content and your signs of discomfort during the trial.

Risk and discomforts related to pulmonary function testing, exhaled breath condensate, and blood draw - You may experience discomfort when performing the pulmonary and exhaled breath condensate tests due to the breathing requirements of these techniques. When blood samples are obtained for the study, you may feel a slight sting or pinch in your arm, suffer a small bruise, and have a very remote possibility of infection. Should you notice unusual redness, bruising, or swelling at the blood sampling site, you should seek medical attention and contact the study director, Dr. John Quindry.

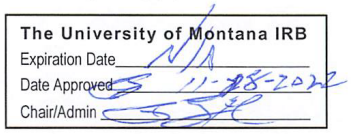
Daily temperature and symptom checks will be performed during data collection to minimize COVID-19 risk. Moreover, according to CDC recommendations, data collection will use social distancing and personal protective equipment.

Polar heart rate monitor devices may accumulate moisture, producing skin irritation, a rash, or other skin discomforts. To minimize this risk, you will be provided with wearables cleaned with alcohol and dried before use. You will also be instructed how to clean the devices in your possession for this study. Finally, if you should experience any skin irritation, remove the wearable device and contact the Principal Investigator. As a last consideration, the Fitbit device may get caught while physically active (e.g., snagging while doing yard work). If this becomes a concern, please remove the device and contact the Principal Investigator with questions or concerns.

You will be informed of any new findings that may affect your decision to remain in the study.

Benefits: As an incentive for participating in this study, you will receive $150 if you are selected and complete the Exercise Inhalation Trial, equivalent to roughly $30 per hour of your involvement. Additionally, you will be given information on your physical fitness (VO_{2max}) and body composition. This information may be helpful for those who work as wildland firefighters, others who perform the strenuous activity while exposed to wood smoke, and those members of the general public that are exposed to wood smoke. Also, this research may provide helpful insight into how poor sleep may impact exposure to wood smoke and affect an individual's cardiovascular and respiratory systems. While blood work and gene expression analysis will be performed, these results will not be returned as a study benefit.

Confidentiality: Your records will be kept confidential and will not be released without your consent except as required by law. Your identity as a participant in this study will be kept private. The intended outcome of this research is a published manuscript in a scientific journal. Should that occur, your name will not be used in any publications or presentations about this research. To achieve this level of privacy, you will be randomly



assigned an identification number at the beginning of the study. All questionnaire responses and data collected will be labeled with this given number. The signed informed consent forms and the key linking the identification number to your name and contact number will be under the principal investigator's control and kept separately in a locked file cabinet. After the completion of the study, the key linking identifying personal information to their respective identification number will be destroyed. A separate sheet containing only the contact information with no identification numbers will be kept until the data analysis is complete.

Voluntary Participation/Withdrawal: You may refuse to participate or withdraw from the study at any time without penalty or loss of benefits to which you are normally entitled. If you choose to withdraw, please notify Dr. John Quindry (contact information below) as soon as possible.

Compensation for Injury: If you are injured as a result of this research, you should individually seek appropriate medical treatment. Suppose the injury is caused by the negligence of the University of Montana or its employees. In that case, you may be entitled to reimbursement or compensation according to the Comprehensive State Insurance Plan established by the Department of Administration under the authority of MCA, Title 2, Chapter 9. In case of a claim for such injury, further information may be obtained from the University's Risk Manager (406-243-2700; michele.wheeler@umontana.edu) or the Office of Legal Counsel (406-243-4742; legalcounsel@umontana.edu). (Reviewed by University Legal Counsel, May 9, 2013)

Future research: Data and biological specimens collected during this investigation may be used for future investigations. In this regard, all your data will be anonymized (containing no identifiable links to your identity or private information).

Questions: You may wish to discuss this with others before agreeing to participate in this study. If you have any questions about the research now or during the study, please contact Dr. John Quindry, Ph.D., at (406) 243-4268 (john.quindry@mso.umt.edu). If you have any questions regarding your rights as a research subject, you may contact the UM Institutional Review Board (IRB) at (406) 243-6672.

Statement of Your Consent: I have read the above description of this research study. I have been informed of the risks and benefits involved, and all my questions have been answered to my satisfaction. Furthermore, I have been assured that a research team member will also answer any future questions I may have. I voluntarily agree to take part in this study. I understand I will receive a copy of this consent form.

_____
Printed Name of Subject

_____ _____
Subject's Signature Date

Statement of Consent to be Photographed [and/or Audiotaped, Videotaped, etc., if applicable]:

I consent to use of my photograph (audio/video) in presentations related to this study.
I understand that if photographs (audio/video recordings) are used for presentations of any kind, names or other identifying information will not be associated with them.
I understand that audio recordings will be destroyed following transcription, and that no identifying information will be included in the transcription.

_____ _____
Subject's Signature Date

A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by US law. This website will not include information that can identify you. At most, the website will include a summary of

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Date Approved 11-18-2022
Chair/Admin

the results. You can search this website at any time.

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Chair/Admin

Appendix D: Morningness-Eveningness Questionnaire (MEQ)

Morningness-Eveningness Questionnaire

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Purpose Consisting of 19 items, the scale was developed to assess individual differences in morningness and eveningness – the degree to which respondents are active and alert at certain times of day. Scale items query preferences in sleep and waking times, and subjective "peak" times at which respondents feel their best.

Population for Testing The questionnaire was first validated with individuals aged 18–32 years.

Administration A self-report, paper-and-pencil measure, the scale requires between 10 and 15 min for completion.

Reliability and Validity Horne and Östberg [1] conducted an evaluation of the scale's psychometric properties and found that individuals placed within each of the scale's five diagnostic categories possessed significantly different waking oral temperatures. More recently, research has indicated that the scale's inter-item correlations are merely moderate, ranging from – .02 to + .61, suggesting that the scale is actually composed of two factors [2]. Still, the full scale internal consistency remained sufficient at .82, supporting the use of a global score.

Obtaining a Copy A copy can be found in the original article published by developers [1].

Scoring The scale is composed of both Likert-type and time-scale questions. The Likert-type items present four options with the lowest values indicating definite eveningness. Similarly, the time-scale items are divided into periods of 15 min spanning a time frame of 7 h. Each section of the scale is assigned a value of 1 through 5. To obtain a global score, each item is totaled and the sum is converted to a 5-point scale: definitely morning type (70–86), moderately morning type (59–69), neither type (42–58), moderately evening type (31–41), and definitely evening type (16–30). However, finding that these cutoffs under-identified morningness types in a population of Austrian students, researcher Neubauer [3] suggested that the scale may need to be adapted to the specific region in which it is being used to accommodate variations in circadian rhythms.

A. Shahid et al. (eds.), *STOP, THAT and One Hundred Other Sleep Scales*,
DOI 10.1007/978-1-4419-9893-4_54, © Springer Science+Business Media, LLC 2012

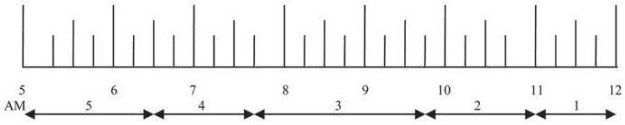
Morningness-Eveningess Questionnaire

Instructions:

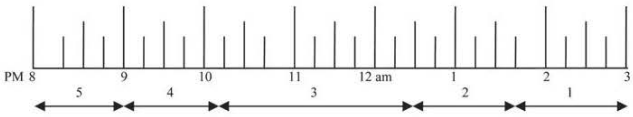
1. Please read each question very carefully before answering.
2. Answer ALL questions
3. Answer questions in numerical order.
4. Each question should be answered independently of others. Do NOT go back and check your answers.
5. All questions have a selection of answers. For each question place a cross alongside ONE answer only. Some questions have a scale instead of a selection of answers. Place a cross at the appropriate point along the scale.
6. Please answer each question as honestly as possible. Both your answers and the results will be kept, in strict confidence.
7. Please feel free to make any comments in the section provided below each question.

The Questionnaire with scores for each choice

1. Considering only your own "feeling best" rhythm, at what time would you get up if you were entirely free to plan your day?



2. Considering only your own "feeling best" rhythm, at what time would you go to bed if you were entirely free to plan your evening?



3. If there is a specific time at which you have to get up in the morning, to what extent are you dependent on being woken up by an alarm clock?

Not at all dependent ☐ 4
Slightly dependent ☐ 3
Fairly dependent ☐ 2
Very dependent ☐ 1

4. Assuming adequate environmental conditions, how easy do you find getting up in the mornings?

Not at all easy ☐ 1
Not very easy ☐ 2
Fairly easy ☐ 3
Very easy ☐ 4

5. How alert do you feel during the first half hour after having woken in the mornings?

Not at all alert ☐ 1
Slightly alert ☐ 2
Fairly alert ☐ 3
Very alert ☐ 4

6. How is your appetite during the first half-hour after having woken in the mornings?

Very poor ☐ 1
Fairly poor ☐ 2
Fairly good ☐ 3
Very good ☐ 4

7. During the first half-hour after having woken in the morning, how tired do you feel?

Very tired ☐ 1
Fairly tired ☐ 2
Fairly refreshed ☐ 3
Very refreshed ☐ 4

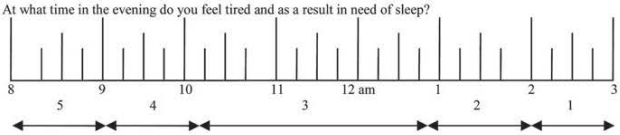
8. When you have no commitments the next day, at what time do you go to bed compared to your usual bedtime?

Seldom or never later ☐ 4
Less than one hour later ☐ 3
1-2 hours later ☐ 2
More than two hours later ☐ 1

9. You have decided to engage in some physical exercise. A friend suggests that you do this one hour twice a week and the best time for him is between 7:00-8:00 a.m. Bearing in mind nothing else but your own "feeling best" rhythm, how do you think you would perform?

Would be on good form ☐ 4
Would be on reasonable form ☐ 3
Would find it difficult ☐ 2
Would find it very difficult ☐ 1

10. At what time in the evening do you feel tired and as a result in need of sleep?



11. You wish to be at your peak performance for a test which you know is going to be mentally exhausting and lasting for two hours. You are entirely free to plan your day and considering only your own "feeling best" rhythm which ONE of the four testing times would you choose?

8:00-10:00 a.m. ☐ 6
11:00 a.m.-1:00 p.m. ☐ 4
3:00-5:00 p.m. ☐ 2
7:00-9:00 p.m. ☐ 0

12. If you went to bed at 11 p.m. at what level of tiredness would you be?

Not at all tired ☐ 0
A little tired ☐ 2
Fairly tired ☐ 3
Very tired ☐ 5

13. For some reason you have gone to bed several hours later than usual, but there is no need to get up at any particular time the next morning. Which ONE of the following events are you most likely to experience?

Will wake up at usual time and will NOT fall asleep ☐ 4
Will wake up at usual time and will doze thereafter ☐ 3
Will wake up at usual time but will fall asleep again ☐ 2
Will NOT wake up until later than usual ☐ 1

14. One night you have to remain awake between 4-6 a.m. in order to carry out a night watch. You have no commitments the next day. Which ONE of the following alternatives will suit you best?

Would NOT go to bed until watch was over ☐ 1
Would take a nap before and sleep after ☐ 2
Would take a good sleep before and nap after ☐ 3
Would take ALL sleep before watch ☐ 4

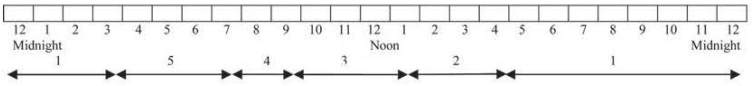
15. You have to do two hours of hard physical work. You are entirely free to plan your day and considering only your own "feeling best" rhythm which ONE of the following times would you choose?

8:00-10:00 a.m.	☐ 4
11:00 a.m.-1:00 p.m.	☐ 3
3:00-5:00 p.m.	☐ 2
7:00-9:00 p.m.	☐ 1

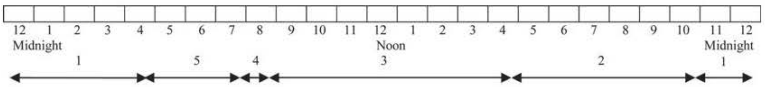
16. You have decide to engage in hard physical exercise. A friend suggests that you do this for one hour twice a week and the best time for him is between 10-11 p.m. Bearing in mind nothing else but your own "feeling best" rhythm how well do you think you would perform?

Would be on good form	☐ 1
Would be on reasonable form	☐ 2
Would find it difficult	☐ 3
Would find if very difficult	☐ 4

17. Suppose that you can choose your own work hours. Assume that you worked a FIVE hour day (including breaks) and that your job was interesting and paid by results. Which FIVE CONSECUTIVE HOURS would you select?



18. At what time of the day do you think that you reach your "feeling best" peak?



19. One hears about "morning" and "evening" types of people. Which ONE of these types do you consider yourself to be?

Definitely a "morning" type	☐ 6
Rather more a "morning" than an evening type	☐ 4
Rather more an "evening" than a "morning" type	☐ 2
Definitely an "evening" type	☐ 0

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