



NAVAL MEDICAL RESEARCH UNIT DAYTON

Effects of Naval Aviation-Related Pressure Fluctuations on a Rodent Model of Exposure

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STATEMENTS

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The study protocol was reviewed and approved by the Wright Patterson Air Force Base Institutional Animal Care and Use Committee in compliance with all federal regulations governing the protection of animals and research.

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ABSTRACT

Episodic, repeated cockpit pressure fluctuations can occur in aircraft due to complications with the system designed to control and maintain cockpit pressurization. It is possible that these decompression/compression cycles could place aircrew at risk for a range of physiologic or cognitive performance deficits, but it is unclear if these fluctuations are severe enough to elicit these effects. This study investigated the effect of hypobaric pressure fluctuations over 25 minutes in a hypobaric chamber on respiratory function and anatomy, the formation vascular gas emboli, and biomarkers of decompression injury, behavioral changes, and alterations in neuroanatomy and neurophysiology in male Sprague Dawley rats. Transient reductions in activity levels and decrements in performance on a motor coordination task were measured in rats during exposure to 0.8 psi/sec fluctuations profiles with an initial altitude of 8,000 feet. There was no fluctuation-associated decrease in activity levels or observational metrics evaluated immediately post-exposure. No impact of fluctuations on respiratory function or anatomy was found. Ultrasound imaging during the exposure revealed no vascular gas emboli in the pulmonary artery of rats exposed to fluctuation profiles of up to 1.5 psi/sec starting at initial altitudes of 8,000 feet and 14,000 feet. Increased astrocytic activation in the hippocampus was found in rats three days following exposure to 1.5 psi/sec fluctuations. Behavioral alterations and neuroanatomical changes have been associated with pressure fluctuation exposure in a rodent model, although the specific cause of both the transient impacts on motor activity and the astrocytic activation in the hippocampus are yet to be determined. Now that exposure levels necessary to induce adverse effects have been identified, future targeted studies to identify causal mechanisms and time courses of these effects can be performed in the rodent model.

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Executive Summary

Recent reports have indicated that a failure in the system designed to control and maintain cockpit pressurization may have contributed to the sharp increase in reported physiologic episodes in tactical aviation aircraft operators, as well as a number of cases with symptoms resembling decompression sickness (DCS) or hypoxia among Navy pilots. Transient cabin pressure changes, that is, a rapid depressurization followed by a quick return to the expected cabin pressure, bring the possibility of a disparity between the ambient pressure within the cockpit and the pressure of the gases within the body cavities of the aircrew. Consequently, this may place the aircrew at risk for a range of physiologic trauma and cognitive performance deficits. Among the potential risks of these intermittent pressure fluctuations are hypoxia, barotrauma, and an increased risk of DCS, of which, the latter two were the focus of this study. This study was funded by the NAVAIR PMA-265 program office.

To accurately measure and index the effect of transitory pressure fluctuations on those within an aircraft, exposure profiles were developed to align with the pressure fluctuations known to occur based on in-flight data collected using pressure sensors. Naval Medical Research Unit-Dayton (NAMRU-Dayton) built an exposure system that was used to expose rats to these simulated hypobaric pressure fluctuation profiles along with normoxic or hyperoxic breathing gases directly to the nose (mimicking the mask delivery found in tactical aviation) or whole-body hyperoxic breathing environments. The On Board Oxygen Generation (OBOGS) delivery schedule provides approximately 94% oxygen throughout operation, with argon also being concentrated by the OBOGS system. Therefore, in some phases of the study, cohorts of rats were exposed to either a mix of 94% oxygen/6% argon or 21% oxygen concentration to determine if effects differed with normoxic or hyperoxic breathing gases.

Animals were exposed to hypobaric pressure cycling, with and without hyperoxia, at pressure change rates between 0.2 and 1.5 pounds per square inch per second (psi/sec) over the course of an approximately 25 minute hypobaric exposure at a simulated 8,000 mean feet above sea level. Additionally, a 1.5 psi/sec pressure rate change was tested at a simulated 14,000 feet during one phase of the study. Animals were evaluated for barotrauma, DCS, neurological symptoms during and after exposure using behavioral metrics, biomarkers, and imaging techniques. Analyses included behavioral measures of pain and neurological or motor impairment that could be associated with DCS or barotrauma. Additionally, ultrasound imaging was performed to identify the presence of vascular gas emboli (VGE) during the exposure.

Hippocampal brain slices were prepared immediately following exposure and necropsy, and were assessed for alterations in synaptic function using electrophysiology. Tissue samples were collected from a different set of animals approximately 3-4 hours post-

exposure and inflammatory cytokines, platelet numbers, and markers of endothelial dysfunction that have been associated with DCS were evaluated. In another set of animals, brains were collected three days post-exposure and evaluated for astrocyte activation, which could be an indicator of latent neuroinflammation resulting from pressure fluctuation exposure.

Respiratory changes that could indicate difficulty in breathing or impaired gas exchange due to pulmonary barotrauma were evaluated during one set of exposures using nose-only plethysmography to determine breathing rate, tidal volume, minute ventilation, and mid-expiratory flow in comparison to baseline values. Additionally, bronchial and alveolar histopathology was evaluated to determine the presence of structural signs of lung injury. Lastly, the bronchial alveolar lavage fluid was analyzed for markers of inflammation or impaired alveolar epithelial barrier function that could result from lung injury-induced pulmonary barotrauma.

No findings of pulmonary barotrauma were present in any of the fluctuation-exposed animals. No systemic indicators of DCS were discovered in any of the fluctuation-exposed animals. Similarly, no biomarkers associated with DCS were impacted by fluctuation exposures. These biomarkers were non-specific for DCS, but the lack of DCS-related effects was further supported by the absence of VGE at rates of up to 1.5 psi/sec and a baseline altitude of 14,000 feet.

Decrements in motor activity and motor coordination were observed in rats who underwent behavioral tests during the hypobaric fluctuation exposures. Behavioral signs thought to be associated with increased middle ear pressure were also observed in the same animals, but these behaviors were not directly associated with the decrements in performance on the tasks. The behavioral symptoms appeared to be transient with no overt behavioral abnormalities occurring by the end of the hypobaric exposures and immediately after exposures. Preliminary electrophysiology data revealed reduced long-term potentiation (LTP) in hippocampal neurons immediately following exposures to high altitude with and without pressure fluctuations, whereas reduced spontaneous activity was only observed in hippocampus of rats that were exposed to both high altitude and pressure fluctuations. Increased astrocytic activation in the hippocampus was found in rats three days following exposure to fluctuations and high altitude and could be a result of ischemia, blood brain barrier disruption, or trauma.

In conclusion, effects have been identified from pressure fluctuations in a rodent model at fluctuation rates that are comparable or slightly higher than what have been measured from pressure sensor monitoring in the cockpit. The cause of both the transient impacts on motor activity and the astrocytic activation in the hippocampus are yet to be

determined; however, now that exposure levels necessary to induce adverse effects have been identified, future targeted studies to identify causal mechanisms and time courses of these effects can be performed in the rodent model.

Introduction

Previously there was a sharp increase in reported cases of physiological episodes (PEs) in tactical aviation pilots that involved symptoms including cognitive impairment, numbness, lightheadedness, behavioral changes, and fatigue that could have been indicative of decompression sickness (DCS), hypoxia, barotrauma, or chemical exposure (Cragg et al., 2017). Although the reported number of these events had risen over previous years, they still were occurring with relative scarcity as a proportion of the total number of flights. Because of the infrequency of these events and their non-specific symptoms, it was difficult to identify a root cause or causes from analysis of events in the field. Therefore, a scientific investigation using a controlled laboratory experiment was undertaken to study the effects of cockpit pressure fluctuations that are known to occur in Navy F/A-18 aircraft.

An issue with the environmental control system (ECS) in Navy F/A-18 aircraft has been suggested as a possible cause of some of the PEs that had occurred in pilots of high-performance aircraft (Cragg et al., 2017). The ECS was designed to control and maintain cockpit pressurization, and malfunctions with the system have caused transient cockpit pressure changes. Rapid depressurization and a quick return to expected cabin pressure (or persistence above expected cabin pressure) may cause a disparity between the ambient pressure within the cockpit and gases within the body cavities of the pilots, potentially placing them at risk for a range of physiologic trauma and performance deficits.

A report by the NASA Engineering and Safety Center (NESC) analyzed the possible causes of PEs and identified pressure fluctuations as being potentially traumatic to organs sensitive to rapid pressure changes (Cragg et al., 2017). A case analysis of events by NESC found that pressure changes of > 1500 ft in rapid cycles of 0.5 psi/sec or greater resulted in alternobaric trauma including barosinusitis, barodontalgia, alternobaric vertigo, gastrointestinal pain, mid-sternal pain, and joint compression pain. Mil Spec JSSG-2010-3 standards for rapid pressure changes in emergency scenarios are 1.0 psi/sec for decreasing pressure and 0.5 psi/sec for increasing pressure (Cragg et al., 2017). However, these rates were defined for one event and not for rapid pressure cycling. Slam Stick (now enDAQ) pressure monitors (Mide Technology, Woburn, MA) were used to monitor cockpit pressures in F/A-18 aircraft and recorded pressure fluctuations that reached 0.8 psi/sec or higher.

Barotrauma in the lungs may occur from rapid changes in pressure that expand the alveoli to the point where hemorrhage, pneumothorax, and/or lung injury can result. Although there has been extensive research on the implications of rapid decompression effects on the lungs at pressure change rates well in excess of the pressure changes currently measured in Navy aircraft, most of the studies involved one time decompressions rather than decompression/recompression cycling (Krebs and Pilmanis, 1996). One study did investigate the effects of decompression cycles at a pressure change rate approximately 50 times greater than what has been measured in Naval aircraft and found that multiple decompressions in a short time period in dogs caused greater lung damage than a single decompression at the same rate of pressure change (Burkhardt et al., 1951).

The consequences of barotrauma to the lungs and/or pressure cycling are the potential for formation of arterial gas emboli (AGE) and hypoxia resulting from impaired respiratory gas exchange. AGE can migrate through the vasculature to the brain and cause damage and/or reduced oxygen supply that can result in PE-like neurological symptoms such as loss of consciousness and altered mental status (Walker, III, et al., 2020). Impaired respiratory exchange in the lungs can also stimulate a hypoxic scenario that would limit the supply of oxygen to the brain.

Other forms of barotrauma may also be a consequence of pressure fluctuations and can cause pain or balance issues in the ears or sinuses that can impact cognitive or motor tasks. Increased pressure in the middle ear can occur from increased pressure that stretches the tympanic membrane, but clearance through the Eustachian tubes can typically equalize this pressure unless the rate of pressure change is severe enough to cause otic barotrauma (Wright, 2015). Barosinusitis is a condition manifested by inflammation of the nasal sinuses that can be induced by rapid changes in pressure in the surrounding environment causing a sudden and severe sinus pain (Pandolf and Burr, 2002).

It is also possible that DCS may occur in Navy pilots exposed to rapid pressure cycles. Although DCS is more of a concern at higher altitudes (>~18,000ft, Pandolf and Burr, 2002), rapid decompressions in quick succession could increase the likelihood of vascular gas emboli (VGE) formation within the vasculature due to inadequate time for clearance of VGE from the prior decompression or rapid expansion of smaller VGE. Any VGE that form in the vasculature could cause vascular damage, (Zhang et al., 2017) or migrate to the brain and cause neurological effects if the VGE are present in sufficient quantities to not be cleared by the lungs. Additionally, VGE can reach the vasculature of the brain if there is a pathway from the venous to arterial circulation, as would be the case with humans that have patent foramen ovale or an atrial septal defect (Sykes and Clark, 2013). However, VGE entering the arterial circulation may not be necessary for neurovascular

injury. Microparticles that are produced as a result of decompression may migrate to the vasculature of the brain and cause nervous system injury (Yang et al., 2013). Additionally, direct trauma-induced neurological impacts from rapid pressure swings could potentially cause a traumatic brain injury (TBI)-type injury.

Astrocytes are neuroglia cells localized to the central nervous system that play a role in maintaining homeostasis and appropriate chemical environment for neuronal signaling. Astrocytes can become reactive in conditions of injury, ischemia, blood-brain barrier disruption, and disease leading to the secretion of inhibitory factors. Increased expression of glial fibrillary acidic protein (GFAP) together with proliferation and altered morphology are of the most widely accepted markers of astrocyte activation (Pekny & Pekna, 2004). Reactive glia play a role in both immune responses and wound healing processes, which can lead to the formation of glial scars in the central nervous system. These scars function acutely to protect neural tissue from inflammatory responses and/or to reestablish the blood brain barrier. However, these scars may also impair axonal regeneration underlying aberrant function and possibly death of individual neurons and neuronal networks. Increased incidence of glia scarring has been found in some pilots (McGuire et al., 2013), and although a specific cause is yet to be identified, it is thought to be related to hypobaric exposures.

Furthermore, it is unclear whether or not hypoxia or hyperoxia might play a role in any pressure fluctuation-induced effects. The maximum output of the OBOGS is 94% oxygen, and there is an increased percentage of argon relative to ambient that is concurrently concentrated by the OBOGS. Pilots breathe hyperoxic concentrations of oxygen during flight to minimize the impact of reduced oxygen at altitude and diminish the likelihood of decompression sickness by washing out nitrogen from the tissues (NAMI, 1991). However, overexposure to oxygen can increase the likelihood of oxygen toxicity and atelectasis and the interplay between the positive and negative effects of breathing high levels of oxygen is highly dependent on the flight altitude and duration (NAMI, 1991).

Objective

The objective of the project was to use a rodent model to determine if pressure change rates from 0.2 to 1.5 psi/sec were capable of inducing symptoms of pain, DCS, barotrauma, or neurological manifestations that are similar to PE symptoms observed in Naval aviators. Additionally, because the observed effects might be different while breathing the hyperoxic atmosphere provided by the OBOGS compared to normoxia, the responses to these pressure change rates were tested by delivering a hyperoxic breathing environment to the rats and, for some tests, comparing results to results from rats breathing 21% oxygen.

The study was broken into three phases that were designed to, as best as possible, address the broad scope of potential physiological effects that could result from pressure fluctuations in order to better focus future studies on the systems, mechanisms, and timeframes that are involved. Phase 1 focused on pulmonary barotrauma in addition to systemic biomarkers that could be indicative of VGE formation along with post-exposure behavioral measures of overt pain or impairment. Phase 2 was designed to assess behavioral changes during the exposure that could be indicative of transient impairment during or immediately following fluctuations that may not persist through descent to ground level. Phase 2 additionally evaluated functional measures of synaptic neurotransmission that could indicate changes in the hippocampus immediately after altitude exposure. Phase 3 was designed primarily to evaluate the real-time presence of systemic VGE formation as a consequence of repeated decompressions, but also included a preliminary evaluation of astrocytic activation that could be indicative of neuroinflammatory effects that persist in the days following pressure fluctuation exposures.

Phase 1

Phase 1 Methods

Animals and Animal Husbandry

144 Male Sprague Dawley (CD) rats (*Rattus norvegicus*) were obtained from Charles River (Wilmington, MA). Rats were acclimated to the USAF 711 HPW RHDV vivarium for approximately seven days following arrival at the facility. Food and water were provided *ad libitum* while rats were housed in the vivarium prior to exposure. Rats were weighed at the end of acclimation and again on the day of exposure. Following acclimation, rats were randomized into exposure groups with each exposure group having an approximately equivalent average weight. On the day of exposure, the rats were transported to the testing room from the vivarium and returned to home cages following completion of exposure until the time of necropsy.

All procedures were performed in accordance with animal protocols FWA-2018-0177-A and F-WA-2018-0180-A approved by the Wright-Patterson Air Force Base Institutional Animal Care and Use Committee.

Study Design

Rats were exposed to rapid fluctuations in ambient pressure in a hypobaric chamber (see section “Exposure Chamber”) while respiratory parameters were recorded using a plethysmography system (see section “Plethysmography”). Rats were exposed to one of the six profiles listed below. Graphical depictions of the two types of fluctuation profiles (repeated and varied fluctuation rates) are shown in Figure 1 and Figure 2 below, and

graphical depictions of the measured values of all six profiles can be found in Appendix A.

- 1) Ground level (GL)
- 2) 8,000 feet altitude (8K)
- 3) 8,000 feet altitude + 0.2 psi/sec fluctuations (8K+0.2 p/s)
- 4) 8,000 feet altitude + 0.5 psi/sec fluctuations (8K+0.5 p/s)
- 5) 8,000 feet altitude + 0.8 psi/sec fluctuations (8K+0.8 p/s)
- 6) 8,000 feet altitude + composite fluctuation profile (8K+Comp)

With the exception of the GL profile, all profiles had a target ascent to a simulated 8,000 feet over approximately 90 seconds and a descent back to ground level over approximately 90 seconds. The rats remained at simulated altitude for a target of approximately 25 minutes. The 8K profile controlled for any effects of altitude exposure by maintaining the animals at a steady, simulated 8,000 feet altitude for the entire profile. The 8K+0.2 p/s profile had a maximum fluctuation rate of 0.2 psi/sec (Figure 1), and was matched to a profile that was intended to be used in concurrent human exposures to pressure fluctuations being performed by the Naval Aerospace Medical Research Lab (NAMRL) at NAMRU-D. This profile was chosen to correlate the effects seen in animals with the effects seen in humans, so that predictions could be made using only the animal model with more extreme profiles (8K+0.5 p/s, 8K+0.8 p/s, 8K+Comp).

The 8K+0.5 p/s and 8K+0.8 p/s profiles were designed to follow the same sequence of events as the 8K+0.2 p/s profile, but with a peak rate of 0.5 psi/sec and 0.8 psi/sec, respectively. The 8K+0.5 p/s profile tested a peak pressure change rate of 0.5 psi/sec, which matched NESC's rate of pressure change limit for prevention of barotrauma for a single pressure change event (Cragg et al., 2017). The 8K+0.8 p/s profile tested a peak rate of 0.8 psi/sec, which represented the highest percentile of peak rates found in aircraft Slam Stick measurements.

The sequence of events of the 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s pressure fluctuation profiles were the same, with only differing rates of change during each fluctuation. After reaching a target of 8,000 feet following a target ~5,000 feet per minute rate of climb, rats were to be held at that altitude for five minutes before undergoing five fluctuations between 6,000 and 10,000 feet. The fluctuations were designed to be separated by approximately 45-60 seconds, depending on the fluctuation duration (slower fluctuations had less time in between). After an approximately five minute steady altitude period, rats underwent another set of five fluctuations before receiving a final baseline period and returning to ground level at a target descent rate of ~5,000 feet per minute.

The exact timing and altitude changes during these profiles were determined by the timing and altitude change necessary to reach the peak change rates.

The 8K+Comp profile (Figure 2) was adapted from a NAVAIR-designed composite profile that was created to test a variety of different pressure changes derived from real-life cockpit Slam Stick (enDAQ) data in one profile to determine if they are cumulatively severe enough to induce physiological effects. The length of the profile was shortened from the original profile provided by NAVAIR to approximately match the length of other simulated altitude profiles. The profile consisted of a series of 13 major fluctuation peaks with rates that varied between ~ 0.1 and 0.8 psi/sec. The exact timing and altitude changes of this profile was determined by the timing and altitude change necessary to reach the peak change rates.

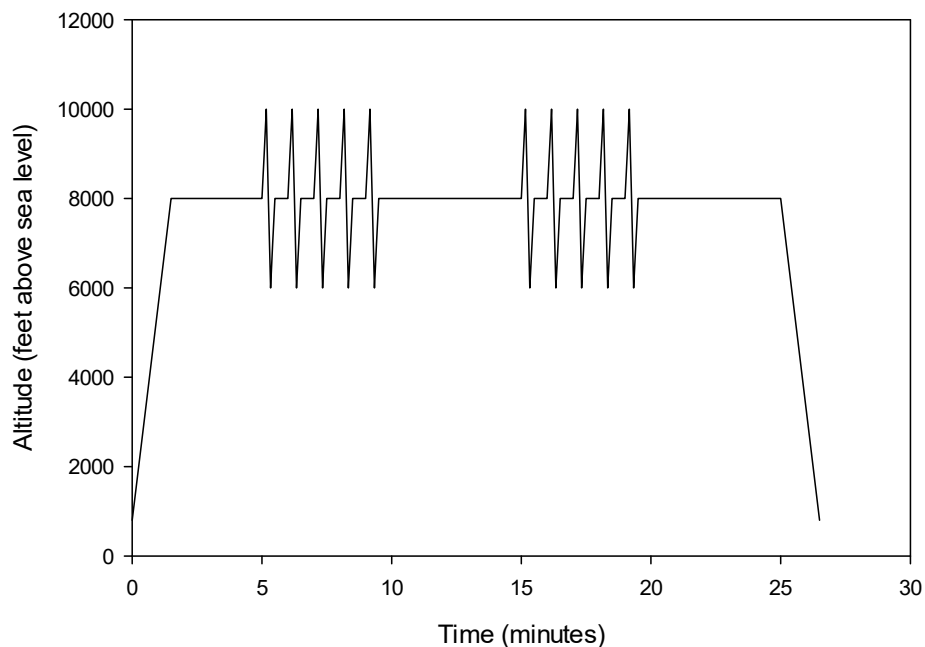


Figure 1. Example plot of the 8K + 0.2 p/s fluctuation profile.

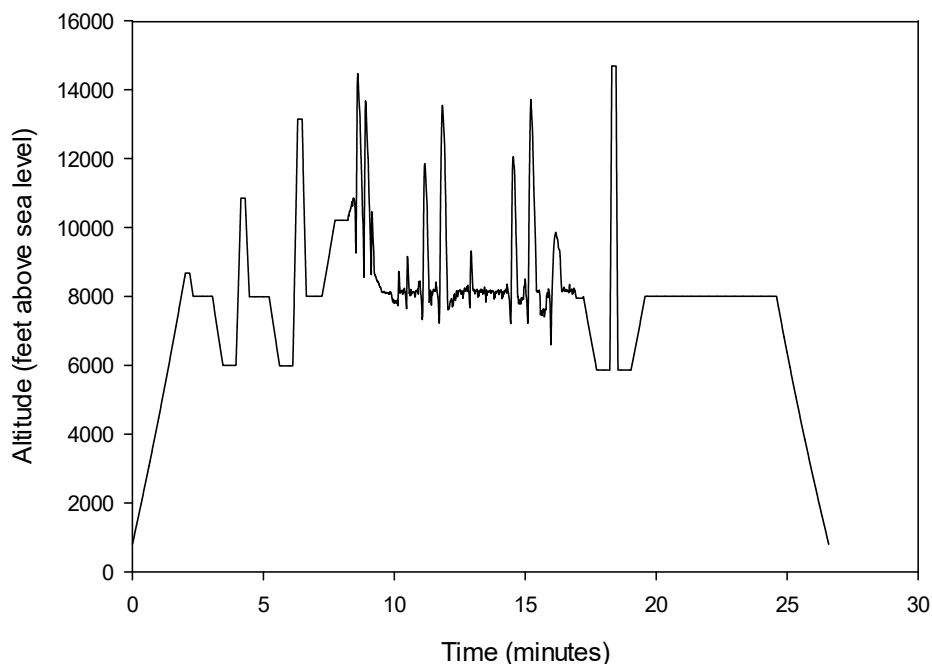


Figure 2. 8K+Comp profile adapted from NAVAIR-created profile.

Four rats receiving the same altitude profile were tested per run, with all six profiles being tested in one day (see Table 1). The exposures were repeated over six days, with the order of the exposures rotated to balance the design and minimize the potential influence of order of exposure. During each run, two rats received 21% oxygen and two rats received 94% oxygen/6% argon through a cap on the nose port of the plethysmograph.

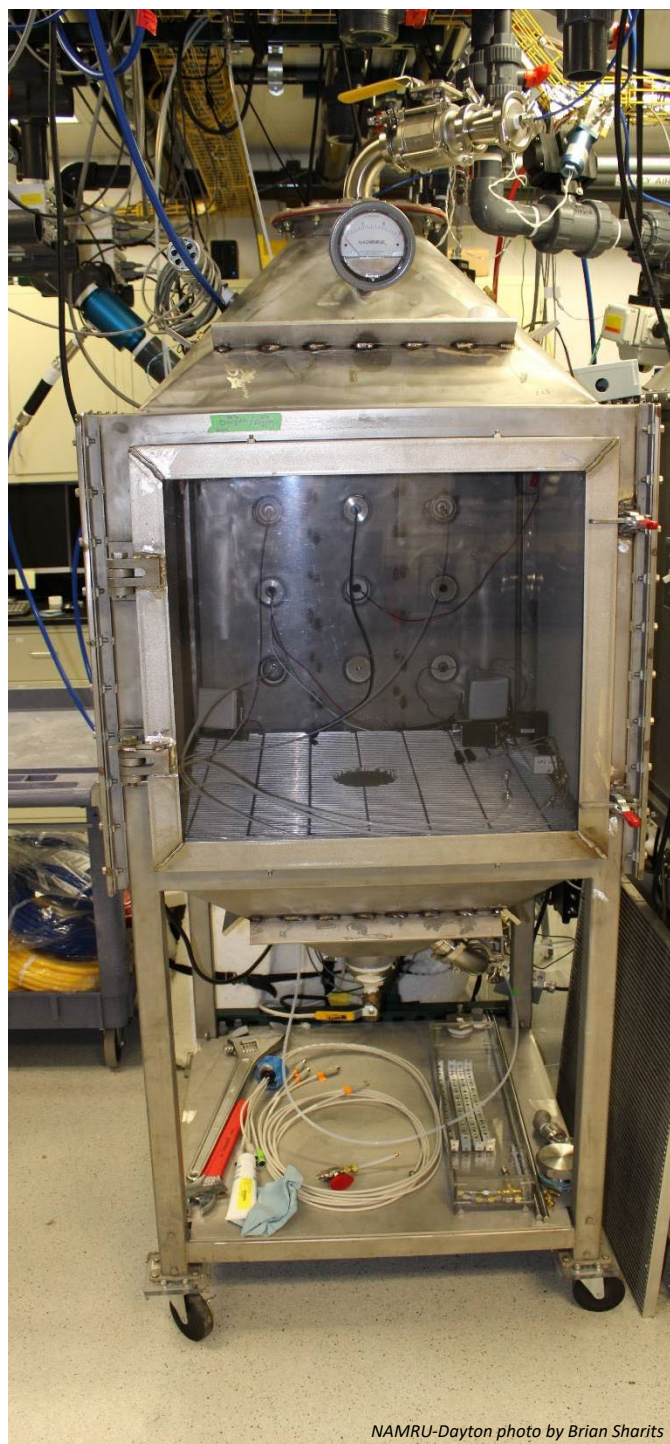
Table 1. Exposure schedule for Phase 1

Run 1 (Day 1)				Run 2 (Day 2)			Run 3 (Day 3)		
Exposure	Profile	21% O ₂ (n)	94% O ₂ (n)	Profile	21% O ₂ (n)	94% O ₂ (n)	Profile	21% O ₂ (n)	94% O ₂ (n)
1	Ground Level	2	2	0.2 psi/sec	2	2	0.8 psi/sec	2	2
2	8000 ft	2	2	0.5 psi/sec	2	2	Composite	2	2
3	0.2 psi/sec	2	2	0.8 psi/sec	2	2	Ground Level	2	2
4	0.5 psi/sec	2	2	Composite	2	2	8000 ft	2	2
5	0.8 psi/sec	2	2	Ground Level	2	2	0.2 psi/sec	2	2
6	Composite	2	2	8000 ft	2	2	0.5 psi/sec	2	2
Run 4 (Day 4)				Run 5 (Day 5)			Run 6 (Day 6)		
Exposure	Profile	21% O ₂ (n)	94% O ₂ (n)	Profile	21% O ₂ (n)	94% O ₂ (n)	Profile	21% O ₂ (n)	94% O ₂ (n)
1	Ground Level	2	2	0.2 psi/sec	2	2	0.8 psi/sec	2	2
2	8000 ft	2	2	0.5 psi/sec	2	2	Composite	2	2
3	0.2 psi/sec	2	2	0.8 psi/sec	2	2	Ground Level	2	2
4	0.5 psi/sec	2	2	Composite	2	2	8000 ft	2	2
5	0.8 psi/sec	2	2	Ground Level	2	2	0.2 psi/sec	2	2
6	Composite	2	2	8000 ft	2	2	0.5 psi/sec	2	2

Exposure Chamber

Rats were exposed to rapid fluctuations in ambient pressure in a 0.6 m³ hypobaric chamber (see Figure 3). The hypobaric chamber was a stainless steel Toxic Hazard Research Unit (THRU) Chamber that was modified to withstand hypobaric pressures. The body of the chamber was strengthened by welding ½" stainless gussets to the sides, top, and bottom. The chamber door was replaced by a stainless steel frame holding a piece of 1" thick polycarbonate to allow staff to view inside the chamber. The back of the chamber featured nine, two inch threaded holes in which bushings were placed to allow tubing and wiring to penetrate the rear wall of the chamber.

The chamber was configured for flow-through air changes as well as for rapid compression and decompression via separate, facility based, compressed air and vacuum systems. The chamber flow-through air was supplied by a house compressed air system that was regulated to 20 psi and passed through an oil/water mist separator. The house air was then HEPA filtered (12144, Pall Corporation, Port Washington, NY) for particulate and charcoal filtered (12011, Pall Corporation, Port Washington, NY) to remove volatile organic compounds. The flow of the compressed air on the flow-through system was regulated by a mass flow controller (HFC-203, Teledyne-Hastings Instruments, Hampton, VA) to a static 52 L/min. A ten foot piece of ¼" LLDPE (United States Plastic Corp, Lima, OH) tubing was used to buffer the mass flow controller from the pressure changes inside the chamber.



NAMRU-Dayton photo by Brian Sharits

Figure 3. Image of exposure chamber

The exhaust side of the chamber consisted of a 2" stainless steel sanitary fitting main exhaust line with a high flow filter (FIL90177, Spencer Turbine Company, Windsor, CT) in-line. The exhaust side of the flow-through system, as well as the positive and negative pressure delivery systems, were connected to this main exhaust line. The house vacuum

supply for the flow-through system was supplied to the chamber via ½" LLDPE tube and connected to the main exhaust line before the high flow filter. It had its own HEPA filter in-line to filter the exhaust before it passed through the valves. The exhaust flow was able to adjust from 0 to 170 L/min to stabilize the simulated altitude in the chamber and was regulated by an electronic valve (JFMSV00001, U.S. Solid, Cleveland, OH) and mass flow meter (HFM-200, Teledyne-Hastings Instruments) via a feedback loop run by LabVIEW (National Instruments, Austin, TX) software. Both the mass flow controller on the flow-through supply air and the mass flow meter on the exhaust side were connected to a PowerPOD 400 (THPS-400-115, Teledyne-Hastings Instruments, Hampton, VA) which interfaced with the LabVIEW computer via a RS-232 digital connection.

Pressure Control System

The positive pressure side of the pressure control system consisted of a 30 gallon reservoir tank of compressed air supplied by the house compressed air system through ½" LLDPE tubing at approximately 100 psi (Figure 4). The air was passed through oil/water mist separators prior to reaching the reservoir tank and was regulated to 32 psi at the tank. The compressed air was delivered to the chamber through two outlets in the tank, each controlled by ½" solenoid (JFSV00072, U.S. Solid, Cleveland, OH) and ½" electronic valves (U.S. Solid). The air then passed through 1/2" LLDPE tubing and was delivered into the main exhaust line of the chamber and allowed to flow back into the chamber. Both the solenoid and electronic valves were controlled by LabVIEW software.

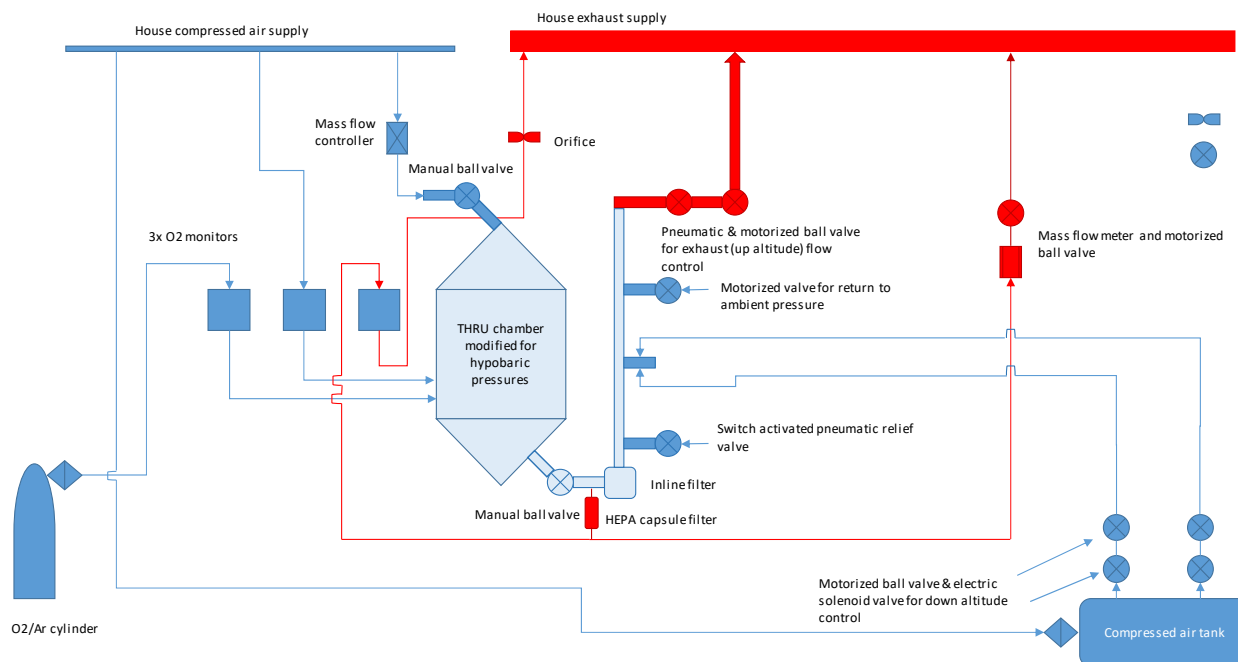


Figure 4. Diagram of exposure system and pressure controls

The vacuum side of the pressure control system used a combination of pneumatic actuated (PMSTB120STVA9, Hayward Flow Control, Clemmons, NC) and electronic actuated (565472A, Valworx, Cornelius, NC) PVC ball valves (Figure 4). The electronic valves were used to modulate the vacuum flow as necessary to get the varying rates of change in chamber pressure called for in the protocol. They were connected to the LabVIEW software and position controlled via analog electrical signal. The pneumatic ball valves were used for the quick on and off pulses and were initiated by solid state relay which was controlled by LabVIEW. Both of these valves were 2" diameter and were connected directly to the house vacuum system by 2" pipe.

Breathing Gases

A cap with tube connections was fabricated to fit over the nose end of the plethysmography tubes and allow the filtered house air or oxygen/argon breathing gases to be delivered directly to the nose of the animals. The oxygen concentrations of the breathing gases were sampled by oxygen sensors (Figure 4, OXM-100H, Oxidation Technologies, LLC., Inwood, IA) before they were supplied to the animals via the nose-only delivery caps (Figure 5). These gases were provided at a minimum flow rate of 500 mL/min to each animal. Two animals were provided with filtered house air with a 21% oxygen concentration and two were provided with the 94% oxygen/6% argon mix for each exposure. The 94% oxygen (O₂)/6% Argon (Ar) gas cylinders were provided by Matheson Tri-Gas Company (Dayton, OH). The cylinders were certified from the company to +/-2% accuracy for the stated oxygen concentration. An approximately 6 ft. length of 1/8" PTFE tubing (Cole-Parmer, Vernon Hills, IL) and ruby orifices (RB82631, Bird Precision, Waltham, MA) were used to buffer the main breathing gas delivery lines and the high precision 150mm variable area flow meters (Cole-Parmer) that were regulating the flow from varying chamber pressures.

Plethysmography

Respiratory changes that could indicate difficulty in breathing or impaired gas exchange due to pulmonary barotrauma during the exposure were evaluated using nose-only plethysmography (Rat Nose-Only Plethysmograph, Buxco Respiratory Products, DSI, St. Paul, MN). The plethysmograph is designed to measure changes in flow in/out of the restraint tube that correlate to changes in volume and frequency of movements of the chest wall of the animal due to respiration (Figure 5). The head of the animal is external

from the body compartment of the nose-only tube to prevent the influence of inspired/exhaled air on pressure changes related to the chest wall.

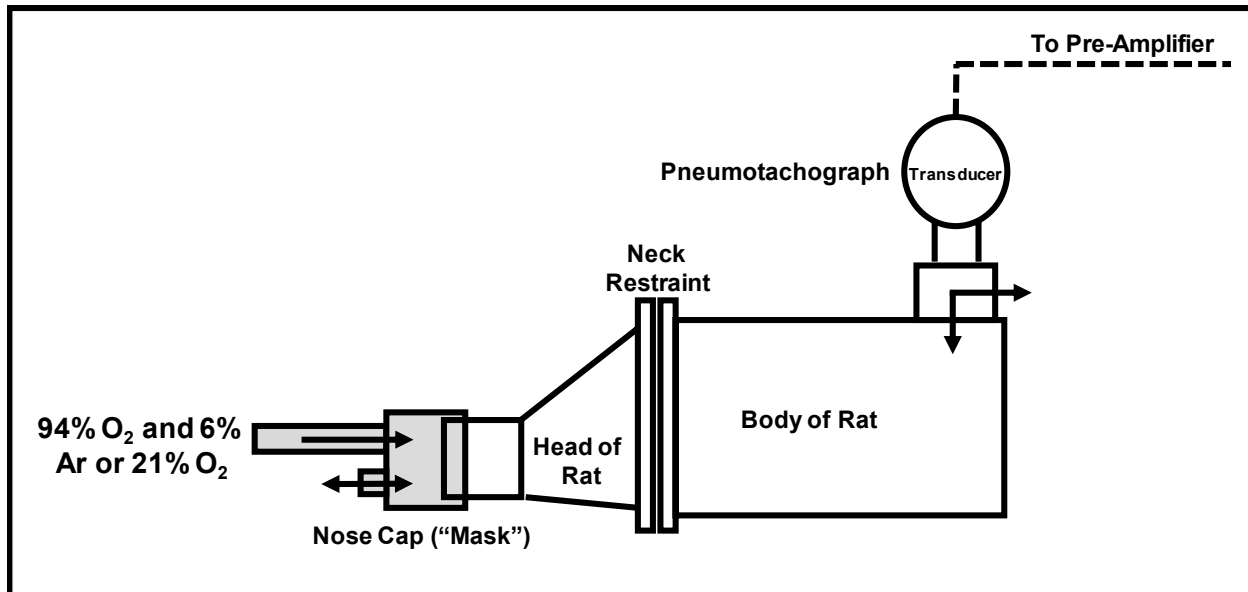


Figure 5. Diagram of plethysmography system

The week before the altitude chamber exposures, rats were acclimated to the restraint of the plethysmograph tubes by placing them in the tubes once per day at increasing time intervals of 10, 20, and 30 minutes over the course of three days. On the day of exposure, animals underwent an approximately 10 minute acclimation period prior to the start of the profile run to minimize the influence of handling on respiratory measurements.

For the 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles, FinePointe software (Buxco Respiratory Products, DSI, St. Paul, MN) averaged measurements of respiratory rate and tidal volume over a two second interval. The software calculated parameters of minute volume and mid-expiratory flow over the same interval. The minute volume is the total volume of air displaced in one minute during tidal breathing. The mid-expiratory flow is the tidal flow at the mid-point of expiration and is representative of an airflow limitation that could occur due to bronchoconstriction or edema (Hoymann, 2012). The software rejects measured pressure changes that do not fit a rat's respiratory waveform, such that the profile-induced pressure changes within the plethysmograph were not captured in the average. For each animal, these measurements were averaged each of the following segments (Figure 6):

- 1) the baseline period between the time that the rats reached 8,000 feet simulated altitude (Pre Flux)
- 2) the first set of fluctuations (Flux 1)
- 3) the steady period between the first and second set of fluctuations (Post Flux 1)

- 4) the second set of fluctuations (Flux 2)
- 5) the steady altitude period between the second set of fluctuations and the start of the descent from altitude (Post Flux 2)

Each of the averaging segments are shown in Figure 6. For each parameter, the response in each segment was calculated as percent % change from baseline for each animal using the equation: $100 * (\text{Segment-Pre Flux}) / (\text{Pre Flux})$. The average timing of each of these segments was calculated between these three fluctuation profiles, and used to define equivalent time periods for the Ground Level (GL) profile. A similar method was employed for the 8K profile, with the exception of the timing of reaching altitude and descent from altitude for which the actual 8K profile time points were used.

The 8K+Comp profile data was analyzed separately from the other fluctuation profiles because the timing of events between the 8K+Comp profile and the other fluctuation profiles was not consistent. Also, there was no pre-fluctuation period at altitude to use for baseline data, so the pre-exposure data was collected during the 90 seconds immediately preceding the ascent to altitude (defined as “Baseline” in the results). For each animal, the respiratory measurements were averaged for each of the fluctuation periods Flux 1-4 given in Figure 7. These periods were chosen to summarize the respiratory responses during the major fluctuations and the immediate 1-2 minute periods following the fluctuation events. Respiratory responses were also averaged for the “Post Flux” period (see Figure 7), an approximately four minute period at a steady 8,000 feet altitude at one minute following the end of the last fluctuation event, to determine if any latent responses occurred as a result of the cumulative exposure.

The respiratory responses of the 8K+Comp profile exposure were compared to equivalent time periods during the GL and 8K profiles. Equivalent time periods for the GL profile were defined by the timing of reaching altitude, descent from altitude, and the start and end of each of the fluctuation events. A similar method was employed for the 8K profile, with the exception of the timing of reaching altitude and descent from altitude for which the actual 8K profile time points were used.

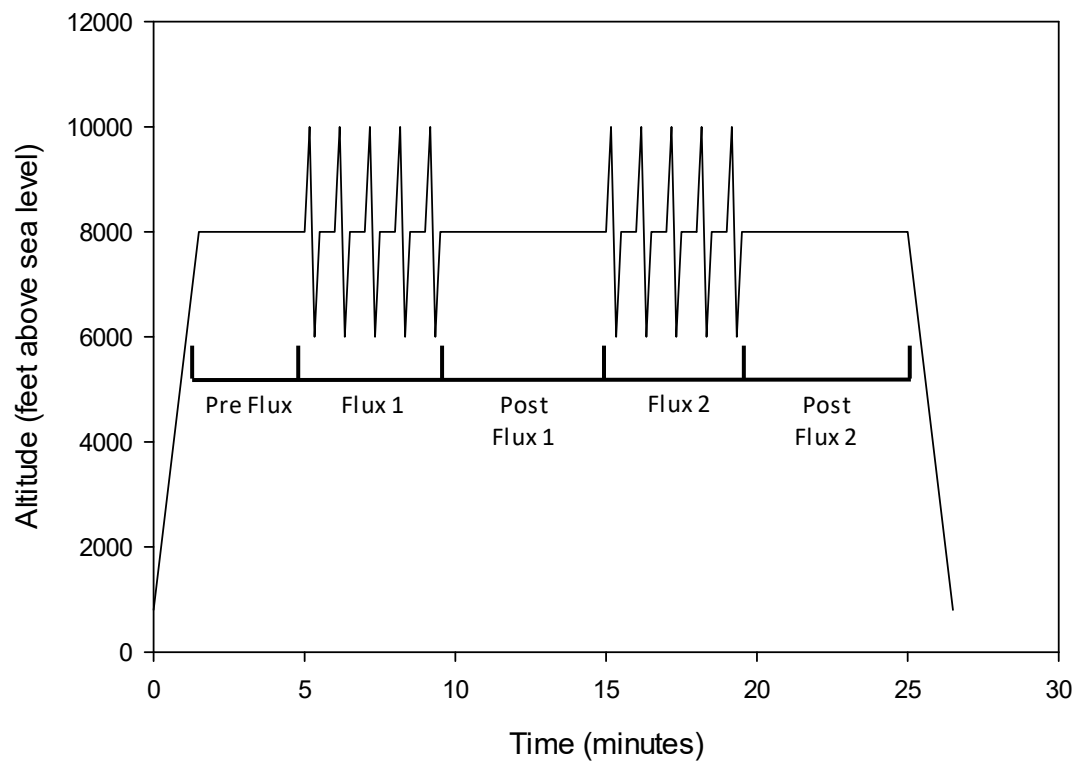


Figure 6. Definition of segments for respiratory measurement averages for profiles 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s.

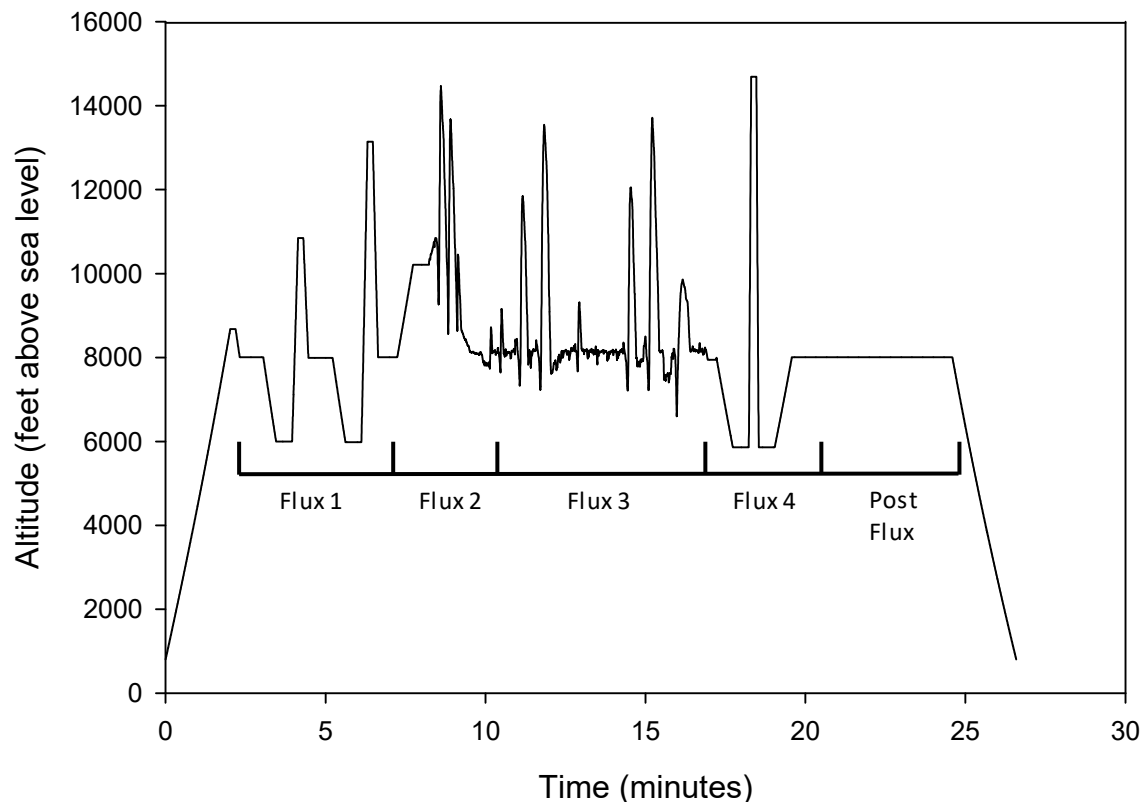


Figure 7. Definition of segments for respiratory measurement averages for 8K+Comp profile

Observations During Exposure

Rats underwent continuous observation by NAMRU-D staff to monitor for signs of distress during the restraint in the nose-only tubes and the exposure to hypobaric pressure fluctuations. Rats were observed through the transparent polycarbonate chamber door during the exposures where they were visible within the clear plethysmograph tubes. The chamber pressure was monitored by a digital pressure sensor (ProSense SPT25, AutomationDirect, Cumming, GA). A Magnehelic (2215, Dwyer Instruments Inc., Michigan City, IN) was also installed on the chamber to provide a redundant analog reference for the pressure inside the chamber. A temperature and relative humidity sensor (HygroFlex5, Rotronic Instrument Corp., Hauppauge, NY) was used to monitor environmental conditions inside the chamber and data was recorded by LabVIEW software.

Post-Exposure Neurobehavioral Testing

Measures of potential pain and neurological or motor impairment that could be associated with DCS or barotrauma were evaluated using post-exposure manipulative and open field

behavioral testing. These acute changes in gross behavioral observations and activity levels following exposure to hypobaric pressure fluctuations followed methodologies in the U.S Environmental Protection Agency (EPA) guideline 870.1300, Acute Inhalation Toxicity and Organization for Economic Cooperation and Development (OECD) guideline, Test No, 403: Acute Inhalation Toxicity, as well as in-house developed components of a larger Neurobehavioral Toxicity Assessment Battery (NTAB) (Rossi, 1996). Formal observation of animal behavior as described below for motor activity (MA) and functional observational battery (FOB) are specified endpoints for this study. The U.S. EPA Health Effects Test Guideline OPPTS 870.6200 "Neurotoxicity Screening Battery" describes the specific observations made.

Upon immediate removal from the exposure chambers following each run, four animals were transferred to an adjacent room and assessed using the FOB (within 15 minutes) followed by a five minute transfer to a separate room for 30 minutes of MA. Therefore, the same four animals were used for both tests and all measures were recorded within 45 minutes. All animals were tested for a number of visible conditions and gross functional changes through observation, mild manipulation, and handling as well as general motor activity, stereotypical movements and exploration in an open field arena.

Gross locomotion, stereotypical movements and exploratory behavior were evaluated in all animals using a photobeam activity system and software (PAS) (SDI, San Diego, CA). Animals were individually placed in one of eight 16" (W) x 16" (D) x 15" (H) clear plastic open fields where horizontal and vertical photobeam sensors automatically recorded beam breaks that were translated into activity measures via the PAS software. The photocells were aligned one inch apart to detect horizontal movement and vertical rears, as well as differentiate small (stereotypic) movements from large (ambulatory) movements. The apparatuses were located in a room with white noise generated at 73dB to mask ambient room levels of ~70dB and under low illuminating light set at ~30 lux. Exposure groups were distributed across the eight chambers as evenly as possible over the course of the study. To begin the test, eight animals were placed in the center of eight open field arenas and chamber recordings were initiated once all animals were loaded. Animals were left uninterrupted for the duration of the 30 minute test session. Between each test, all fecal boli and urine pools were removed and the open fields were washed with a solution of 50% ethanol to remove any olfactory cues that may have been left behind. The following dependent measures were automatically recorded: distance traveled (cm), average speed (cm/sec), number of fine beam breaks (stereotypical), number of ambulatory beam breaks, total number of rears, and percentage of time in center vs. perimeter.

All animals were assessed using the functional observational battery (FOB). The FOB consisted of a set of non-invasive procedures designed to evaluate and document the absence and/or presence (or severity if appropriate) of a predetermined set of behavioral and clinical signs. Observations were made: 1) while the rat was in an observation cage, 2) during removal of the rat from the observation cage, 3) while the rat was being held and examined for clinical observations, 4) as the animal moved freely about the open field, and 5) during manipulative tests. The observations proceeded from the least to most manipulative tests to reduce the influence of handling on the rat's behavior. Efforts were made to control conditions that can affect behavior including sound level, temperature, humidity, lighting, odors, time of day and environmental distractions.

The FOB included the following observations, made and recorded in study record sheets:

- In cage observations: posture, tremors and spasms, and palpebral closure.
- Observations during removal from cage and handling: reactivity to handling, muscle tone, lacrimation, salivation, fur appearance, facial crust, breathing pattern, and other clinical signs.
- Manipulative observations:
 - Approach response: response to a blunt object approaching and stopping before the animal's nose.
 - Acoustic response: response to a hidden metallic click.
 - Tail pinch response: response to a pinch of the tail.
 - Visual placement: response of forelimb to grasp for a surface while being held by the observer.
 - Surface righting: righting response to being turned and briefly held on its back.
 - Grip strength: force necessary to break the animal's grip on a wire mesh (fore and hind).
 - SMALGO (Bioseb, Pinellas Park, FL) pressure-based analgesimeter: Experimenters adjusted the algometer (analgesimeter) to the thumb and applied a progressive pressure to the relevant location on the animal, as if they were applying the pressure directly with their own finger. The experimenter increased the stimulation until they obtain a reaction from the animal (vocalization, shudder...) and then stopped the stimulation. The maximum force value was automatically saved and displayed on the screen of the instrument.

Necropsy

A delay of 3-4 hours post-exposure was given prior to sample collection to allow time for inflammatory responses to barotrauma or decompression related-injury. A delay in response is consistent with markers of systemic inflammation and endothelial dysfunction in diving-related decompression models (Zhang et al., 2017; Bigley et al., 2008). However, it is unclear how this progression would occur in our model given

that a more expedient recovery from decompression related injury would be expected to occur by returning to ground level pressures as compared to diving where the ascent to ground level is the injurious condition. Additionally, inflammatory responses take time to develop in the lung following injury and would be expected to be better measured after a delay.

Approximately 3-4 hours following the end of the exposure, rats received an intraperitoneal injection of the barbiturate sodium pentobarbital in a commercially available Euthasol (Virbac AH) solution. Once anesthesia had been established by lack of response to toe pinch, blood was collected from the inferior vena cava. Whole blood was collected in heparinized tubes for hematology. Plasma was obtained by collecting blood in K2 EDTA tubes. The plasma was stored at -80°C for subsequent analysis. Bronchial alveolar lavage fluid was collected by lavaging the left lung with 3 washes of 5 mL of phosphate buffered saline and stored on ice for further processing. The lungs were perfused with 10% neutral buffered formalin and saved for histopathology.

Bronchial Alveolar Lavage Fluid

Bronchial alveolar lavage fluid (BALF) was analyzed for signs of inflammation or impaired alveolar-epithelial barrier function. Following collection, the BALF was centrifuged for 10 minutes at 200xg @4°C. The supernatant was decanted to a clean tube and analyzed for the presence of protein using a Pierce BCA Protein Assay Kit (Thermo Scientific), of which an increased presence would be indicative of an impaired alveolar-epithelial barrier.

The BALF cell pellet was resuspended in 1.0mL of RPMI-1640 Medium, hepes modification (Sigma-Aldrich). Total cell count was taken using a cellometer (Nexcelom). 0.25mL of the suspension was loaded into an EZ Cytofunnel (Thermo Scientific) in order to load the Cytoslides (Thermo Scientific) and centrifuged in a CytoSpin (Thermo Scientific) at 1,000rpm for five minutes. Cells were fixed and stained using Hema 3 Manual Staining System and Stat Pack (Fisher Scientific). The slides were analyzed for an increased presence of inflammatory cells including neutrophils, basophils, and eosinophils.

Lung Histopathology

Bronchial and alveolar histopathology was evaluated to determine the presence or absence of subclinical signs of lung injury. Lungs were trimmed and embedded in paraffin blocks for sectioning using a microtome. Lung sections were stained with hematoxylin and eosin and mounted for visualization under light microscopy. Lungs were scored by a

veterinary pathologist on a 0-5 scale to indicate the severity grade of pathologic change present in the examined tissue (Table 2).

Table 2. Lung histopathology grading scale

Grade	Term	Definitions	% Lung Tissue Affected
0	Within Normal Limits (WNL)	Any histologic changes present are within normal limits	0
1	Minimal	Rare or inconspicuous lesions. Barely exceeds WNL	<5
2	Mild	Lesion is easily identified by of limited severity	6-25
3	Moderate	Lesion is prominent. Focal or multifocal lesion	26-40
4	Marked	Large multifocal to coalescing lesion with potential for some loss of tissue structure	41-60
5	Severe	Extensive to diffuse lesion with potential for effacement of tissue structure	>61

Blood and Plasma Biomarkers

Plasma was tested for the presence of inflammatory cytokines IL-6, TNF-alpha, and IFN gamma that were previously associated with decompression injury in a rat model of diving-related decompression injury (Zhang et al., 2017; Bigley et al., 2008). A multiplex analyzer, MSD Multi-Spot Assay System (Mesoscale Discovery, Rockville, MD) was used to identify the presence of the three cytokines using a Proinflammatory Panel 2 multiplex panel (Mesoscale Discovery). Additionally, ICAM-1, a cell adhesion molecule associated with endothelial dysfunction was tested in the plasma using an ICAM1 (CD54) ELISA assay (Abcam, Cambridge, UK). Platelet consumption, which has been associated with bubble formation in the vasculature (Pontier et al., 2009) and could be related microparticle formation (Pontier et al., 2012), was measured in the whole blood using hematology analyzer (Hemavet, Drew Scientific, Miami Lakes, FL).

Data analysis

All data for quantitative, continuous variables were compared for the exposure and control groups using one-way analysis of variance (ANOVA). The data for the 21% oxygen and 94% oxygen/6% argon exposures were analyzed separately. If the ANOVA indicated statistical significance among exposure groups, the Tukey's post-hoc test was used to compare all exposure groups. When assumptions for parametric ANOVA were not met, Kruskal-Wallis nonparametric procedures was used. Statistical analyses were performed using SigmaPlot 13.0 (Systat Software, Inc). The probability value of less than 0.05 was used as the critical level of significance for each statistical test.

Phase 1 Results

Exposure Data

The desired altitudes and peak rates of change of the fluctuations were on target for the study. Each of the 8K+0.2 p/s, 8K+0.5 p/s and 8K+0.8 p/s profiles reached their target peak rate of change and there was minimal peak-to-peak variability and run-to-run variability in fluctuations (Table 3). Additionally, there was consistency across fluctuations and runs with regard to fluctuation duration and maximum/minimum altitudes (Table 4).

The average and peak rates of pressure change for all six runs of each of the 8K+0.2 p/s, 8K+0.5 p/s and 8K+0.8 p/s fluctuation profiles are given in Table 3. The average and peak rates of change were calculated for the entire period during a fluctuation while pressure was decreasing (decompression) and for the entire period during a fluctuation while pressure was increasing (recompression). The peak rate was defined as the highest rate of change during a fluctuation event during each of the decompression or recompression periods. The average rate was defined as the average rate of change during either the decompression or recompression periods from the start to the end of each fluctuation. These parameters were calculated for each fluctuation within each run of the profile and averaged across all ten fluctuations within each run to obtain the mean $\pm$ standard deviation values given for Runs 1-6 in Table 3. The mean $\pm$ standard deviation for the rates of change of all six runs of each profile is listed in the “Average” column in Table 3.

Table 3. Average and peak rates of change for fluctuations during 8K+0.2 p/s, 8K+0.5 p/s and 8K+0.8 p/s profiles for Phase 1

Decompression Peak Rate of Change	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.20 $\pm$ 0.02	0.20 $\pm$ 0.02	0.20 $\pm$ 0.02	0.20 $\pm$ 0.02	0.20 $\pm$ 0.02	0.20 $\pm$ 0.02	0.20 $\pm$ 0.00
8K + 0.5 p/s	0.49 $\pm$ 0.04	0.48 $\pm$ 0.03	0.48 $\pm$ 0.03	0.48 $\pm$ 0.04	0.48 $\pm$ 0.03	0.49 $\pm$ 0.03	0.48 $\pm$ 0.00
8K + 0.8 p/s	0.84 $\pm$ 0.04	0.84 $\pm$ 0.05	0.84 $\pm$ 0.04	0.85 $\pm$ 0.05	0.84 $\pm$ 0.04	0.83 $\pm$ 0.05	0.84 $\pm$ 0.01
Decompression Average Rate of Change	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.19 $\pm$ 0.02	0.19 $\pm$ 0.02	0.19 $\pm$ 0.02	0.19 $\pm$ 0.02	0.18 $\pm$ 0.02	0.19 $\pm$ 0.02	0.19 $\pm$ 0.00
8K + 0.5 p/s	0.44 $\pm$ 0.08	0.41 $\pm$ 0.09	0.42 $\pm$ 0.09	0.42 $\pm$ 0.10	0.42 $\pm$ 0.10	0.43 $\pm$ 0.09	0.42 $\pm$ 0.01
8K + 0.8 p/s	0.64 $\pm$ 0.25	0.63 $\pm$ 0.26	0.60 $\pm$ 0.27	0.59 $\pm$ 0.28	0.61 $\pm$ 0.26	0.59 $\pm$ 0.27	0.61 $\pm$ 0.02
Recompression Peak Rate of Change	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.18 $\pm$ 0.02	0.18 $\pm$ 0.01	0.19 $\pm$ 0.01	0.18 $\pm$ 0.01	0.18 $\pm$ 0.01	0.18 $\pm$ 0.01	0.18 $\pm$ 0.00
8K + 0.5 p/s	0.55 $\pm$ 0.04	0.55 $\pm$ 0.04	0.56 $\pm$ 0.04	0.55 $\pm$ 0.03	0.54 $\pm$ 0.03	0.53 $\pm$ 0.04	0.55 $\pm$ 0.01
8K + 0.8 p/s	0.80 $\pm$ 0.04	0.79 $\pm$ 0.05	0.80 $\pm$ 0.04	0.80 $\pm$ 0.02	0.77 $\pm$ 0.04	0.79 $\pm$ 0.03	0.79 $\pm$ 0.01
Recompression Average Rate of Change	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.17 $\pm$ 0.02	0.17 $\pm$ 0.02	0.18 $\pm$ 0.02	0.17 $\pm$ 0.02	0.17 $\pm$ 0.02	0.18 $\pm$ 0.02	0.17 $\pm$ 0.00
8K + 0.5 p/s	0.53 $\pm$ 0.03	0.52 $\pm$ 0.03	0.53 $\pm$ 0.03	0.52 $\pm$ 0.03	0.52 $\pm$ 0.03	0.51 $\pm$ 0.03	0.52 $\pm$ 0.01
8K + 0.8 p/s	0.74 $\pm$ 0.05	0.73 $\pm$ 0.05	0.73 $\pm$ 0.05	0.71 $\pm$ 0.05	0.72 $\pm$ 0.05	0.72 $\pm$ 0.05	0.72 $\pm$ 0.01

The average duration of the fluctuations, maximum and minimum altitudes attained during the fluctuations, and steady altitudes when no fluctuation events were occurring for each profile are listed in Table 4. The duration, maximum and minimum altitudes were calculated for each fluctuation within each run of the profile and averaged across all ten fluctuations within each run to obtain the mean $\pm$ standard deviation values given for Runs 1-6 in Table 4. The steady altitudes were the average of all recorded altitudes during time periods when fluctuations were not occurring. The mean $\pm$ standard deviation for the duration, maximum and minimum altitudes, and steady altitudes of all six runs of each profile is given in the “Average” column in Table 4.

Table 4. Fluctuation durations and altitude data for Ground Level, 8K, 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles for Phase 1

Average Duration per Fluctuation	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	21.8 ± 0.4	22.3 ± 0.5	22.2 ± 0.4	22.1 ± 0.6	22.4 ± 0.5	22.0 ± 0.4	22.0 ± 0.2
8K + 0.5 p/s	7.9 ± 0.2	7.9 ± 0.2	8.0 ± 0.4	7.9 ± 0.4	8.2 ± 0.4	8.1 ± 0.6	8.0 ± 0.2
8K + 0.8 p/s	7.4 ± 0.5	7.6 ± 0.4	7.7 ± 0.3	7.6 ± 0.3	7.6 ± 0.3	7.8 ± 0.2	7.6 ± 0.2
Maximum Altitude During Fluctuations	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	10246 ± 58	10318 ± 73	10328 ± 51	10282 ± 54	10309 ± 47	10274 ± 51	10293 ± 31
8K + 0.5 p/s	10310 ± 107	10263 ± 127	10349 ± 142	10239 ± 154	10274 ± 143	10315 ± 172	10292 ± 40
8K + 0.8 p/s	11775 ± 233	11737 ± 139	11820 ± 186	11790 ± 154	11665 ± 201	11747 ± 196	11756 ± 54
Minimum Altitude During Fluctuations	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	5735 ± 62	5733 ± 46	5687 ± 42	5735 ± 41	5731 ± 63	5699 ± 38	5720 ± 21
8K + 0.5 p/s	5807 ± 102	5843 ± 125	5809 ± 115	5856 ± 63	5794 ± 172	5822 ± 206	5822 ± 24
8K + 0.8 p/s	5834 ± 216	5784 ± 234	5787 ± 217	5873 ± 205	5738 ± 225	5697 ± 215	5786 ± 64
Steady Altitude	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
Ground Level	903 ± 159	904 ± 160	903 ± 160	904 ± 159	904 ± 160	903 ± 160	903 ± 1
8K	7980 ± 160	7985 ± 159	7984 ± 160	7986 ± 160	7981 ± 159	7979 ± 157	7983 ± 3
8K + 0.2 p/s	8000 ± 191	7999 ± 190	8001 ± 190	8005 ± 189	7996 ± 188	7994 ± 185	7999 ± 4
8K + 0.5 p/s	7991 ± 168	7992 ± 169	7989 ± 170	7994 ± 175	7991 ± 174	7997 ± 182	7992 ± 3
8K + 0.8 p/s	8010 ± 208	8012 ± 204	8004 ± 199	8007 ± 198	8012 ± 205	8006 ± 199	8008 ± 3

The average rate of change of the fluctuations was lower compared to the peak rate of change for the 8K+0.5 p/s and 8K+0.8 p/s profiles for decompression and the 8K+0.8 p/s for recompression (Table 3). This difference in average and peak rate of change was expected because of the additional time necessary for the vacuum and compressed air changes to accelerate the pressure change to reach the higher peak levels (0.5 and 0.8 p/s) as compared to lower peak levels such as the 8K+0.2 p/s profile. Despite the lower values relative to the peak rate, there was still an approximately 0.2-0.3 p/s difference between the 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s average rates of change.

Although the steady altitudes of each of the four altitude profiles and the ground level profile were on target at 8000 and 900 feet, respectively, there was an elevated variability of a couple hundred feet for all of the profiles. The source of the variation was the fine control of the programming, which was making minor adjustments to the vacuum or air valves every few seconds to maintain a steady altitude. These fluctuations occurred at a rate of approximately 0.04-0.06 psi/sec, or slightly less than half of the lowest severity experimental pressure fluctuations. Additionally, these fluctuations were matched with the ground level profile, which was maintained at 900 feet by the altitude chamber, rather than being exposed to ambient ground level pressure.

Graphical representations of the altitude and one second pressure rate change of each profile are presented in Appendix A. Two aspects of the profiles deviated from the target profile. The length of time at altitude was intended to be 25 minutes for all profiles, but the time at altitude of the 8K+0.2 p/s and 8K+Comp profiles were short by approximately two minutes. Although the fluctuations within these profiles hit their target rates, the actual duration of these fluctuations was shorter than predicted and led to the profiles ending

faster than intended, and this was unable to be resolved prior to the start of animal experiments. A two minute difference in the overall time at altitude is not expected to cause substantially different results between the profiles. Additionally, the initial rate of descent from altitude was higher than anticipated, a rate of approximately 0.2 psi/sec, leading to all altitude profiles experiencing that rate upon descent. Given that this rate is the lowest tested, and it was only experienced once upon descent, it is not expected that this would have contributed to any of the responses but the effect cannot be totally ruled out.

The average parameters of all 13 fluctuations within the 8K+Comp profile are displayed in Table 5. Each column represents the mean $\pm$ standard deviation of the fluctuations for all six runs of the 8K+Comp profile. The duration, decompression/recompression rates, and peak altitude parameters are all defined the same as the 0.2 to 0.8 p/s profiles.

Table 5. Average fluctuation durations, rates and peak altitudes for the 8K+Comp profile for Phase 1

Fluctuation Number	Duration (Seconds)	Decompression (psi/sec)		Recompression (psi/sec)		Peak Altitude (Feet Above SL)
		Peak	Average	Peak	Average	
1	55 $\pm$ 0.7	0.06 $\pm$ 0.00	0.04 $\pm$ 0.00	0.21 $\pm$ 0.00	0.12 $\pm$ 0.00	8958 $\pm$ 44
2	37 $\pm$ 0.7	0.10 $\pm$ 0.00	0.07 $\pm$ 0.00	0.23 $\pm$ 0.01	0.17 $\pm$ 0.01	11172 $\pm$ 21
3	39 $\pm$ 0.6	0.21 $\pm$ 0.01	0.14 $\pm$ 0.03	0.25 $\pm$ 0.00	0.18 $\pm$ 0.01	13563 $\pm$ 36
4	12 $\pm$ 0.3	0.45 $\pm$ 0.01	0.41 $\pm$ 0.01	0.56 $\pm$ 0.01	0.44 $\pm$ 0.01	15207 $\pm$ 193
5	13 $\pm$ 0.3	0.34 $\pm$ 0.02	0.32 $\pm$ 0.01	0.61 $\pm$ 0.01	0.48 $\pm$ 0.01	14733 $\pm$ 98
6	9 $\pm$ 0.4	0.33 $\pm$ 0.01	0.27 $\pm$ 0.01	0.60 $\pm$ 0.01	0.48 $\pm$ 0.01	11586 $\pm$ 171
7	18 $\pm$ 0.7	0.21 $\pm$ 0.01	0.16 $\pm$ 0.01	0.65 $\pm$ 0.03	0.51 $\pm$ 0.01	12761 $\pm$ 88
8	16 $\pm$ 0.2	0.35 $\pm$ 0.01	0.27 $\pm$ 0.01	0.67 $\pm$ 0.01	0.50 $\pm$ 0.01	14473 $\pm$ 118
9	13 $\pm$ 1.4	0.20 $\pm$ 0.01	0.14 $\pm$ 0.01	0.34 $\pm$ 0.01	0.18 $\pm$ 0.03	9914 $\pm$ 69
10	14 $\pm$ 0.4	0.29 $\pm$ 0.01	0.23 $\pm$ 0.01	0.65 $\pm$ 0.02	0.52 $\pm$ 0.01	12925 $\pm$ 227
11	17 $\pm$ 0.3	0.32 $\pm$ 0.01	0.25 $\pm$ 0.01	0.61 $\pm$ 0.00	0.47 $\pm$ 0.00	14612 $\pm$ 87
12	14 $\pm$ 0.2	0.21 $\pm$ 0.01	0.14 $\pm$ 0.01	0.36 $\pm$ 0.00	0.31 $\pm$ 0.00	10397 $\pm$ 69
13	23 $\pm$ 0.6	0.75 $\pm$ 0.01	0.25 $\pm$ 0.00	0.81 $\pm$ 0.01	0.51 $\pm$ 0.05	15083 $\pm$ 253

The 8K+Comp profile had more fluctuations than the 0.2-0.8 p/s profiles and the fluctuation events were longer in duration and went to a higher altitude. The majority of the decompressions in the 8K+Comp profile had a peak rate of 0.5-0.7 p/s. However, the peak rate of decompression only reached a rate of 0.8 p/s one time for an average of 23 seconds, whereas the 8K+0.8 p/s profile reached 0.8 p/s ten times with a 7.4 second average duration per fluctuation. The majority of the recompressions in the 8K+Comp profile had peak rates of 0.2-0.4 p/s, although there was one with a peak rate of 0.75 p/s. The 8K+0.5 p/s and 8K+0.8 p/s profiles had 10 recompression events that had a greater

rate than the majority of the events in the 8K+Comp profile, although these events occurred for a shorter duration.

It is difficult to put into context the relative severity of the 8K+Comp profile compared to the 8K+0.5 p/s and 8K+0.8 p/s profiles based on the comparison in the preceding paragraph, however, both profiles have their advantages within the study design. Although the 8K+Comp profile may overall be more operationally-relevant given that many of the fluctuations were taken from real-world events, the 8K+0.2 p/s, 8K+0.5 p/s and 8K+0.8 p/s profiles have the advantage of providing a more controlled severity-response comparison of pressure change rate as a factor in the observed response.

Environmental Data

Table 6. Ambient temperature and relative humidity during exposure

Temperature (°C)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
Ground Level	21.4 ± 0.02	21.1 ± 0.03	20.6 ± 0.04	20.1 ± 0.08	20.4 ± 0.03	21.6 ± 0.03	20.9 ± 0.61
8K	21.3 ± 0.19	21.0 ± 0.14	20.7 ± 0.16	20.0 ± 0.19	20.3 ± 0.12	21.5 ± 0.12	20.8 ± 0.58
8K + 0.2 p/s	21.1 ± 0.12	21.3 ± 0.14	20.9 ± 0.15	20.4 ± 0.13	20.1 ± 0.10	21.0 ± 0.10	20.8 ± 0.48
8K + 0.5 p/s	21.1 ± 0.14	20.9 ± 0.11	21.1 ± 0.15	20.3 ± 0.14	20.1 ± 0.13	21.3 ± 0.17	20.8 ± 0.49
8K + 0.8 p/s	21.1 ± 0.15	20.8 ± 0.15	19.9 ± 0.16	20.3 ± 0.13	20.1 ± 0.15	21.1 ± 0.16	20.6 ± 0.53
8K + Comp	21.2 ± 0.15	20.8 ± 0.16	20.2 ± 0.16	20.1 ± 0.15	20.1 ± 0.13	21.4 ± 0.17	20.6 ± 0.57
Relative Humidity (%)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
Ground Level	34.2 ± 14.5	29.9 ± 14.2	35.4 ± 16.3	35.2 ± 14.7	28.4 ± 14.9	21.5 ± 8.5	30.8 ± 5.4
8K	27.8 ± 8.7	28.1 ± 8.4	29.6 ± 11.3	29.8 ± 10.0	25.3 ± 9.6	14.9 ± 4.4	25.9 ± 5.6
8K + 0.2 p/s	29.0 ± 9.1	27.6 ± 8.4	30.1 ± 10.6	30.5 ± 9.8	28.5 ± 9.2	15.9 ± 5.2	26.9 ± 5.5
8K + 0.5 p/s	27.9 ± 9.5	28.4 ± 9.7	28.2 ± 11.1	29.2 ± 9.8	27.3 ± 9.3	15.1 ± 5.2	26.0 ± 5.4
8K + 0.8 p/s	27.0 ± 10.1	28.0 ± 10.9	27.1 ± 6.3	27.9 ± 10.3	25.1 ± 9.4	20.0 ± 6.1	25.9 ± 3.1
8K + Comp	26.2 ± 8.7	28.2 ± 9.8	29.4 ± 10.5	26.8 ± 9.4	25.0 ± 8.2	18.2 ± 6.0	25.6 ± 3.9

The mean +/- the standard deviation of the temperature and percent relative humidity (%RH) during each run of each profile are given in Table 6. The temperature in the chamber during the exposures ranged from an average of approximately 20 to 22 °C across all runs of each profile. There was no difference in temperature between the exposure groups, with the temperature for each profile averaging approximately 21°C. The %RH within the chamber during the exposures ranged from an average of approximately 15 to 35%. Although the %RH was consistent on days 1-5, day 6 was lower within the chamber, likely due to a drop in facility %RH on that day. However, this drop in RH was observed across all groups so therefore a differential effect is not likely. The altitude profiles averaged a lower humidity than the GL profile by approximately 4-5%, but it is not expected that this small difference would have an impact over the relatively short duration of exposure.

Animals

Rats were received at approximately 6-7 weeks of age. The mean weight of rats upon receipt was 161 ± 13 g, with weights ranging between 133 g and 193 g. Exposures took place when rats were approximately 8-9 weeks of age. The mean weight of all 144 rats on the day of exposure was 334 ± 21 g, with weights ranging between 286 g and 378 g.

The average weight of the rats in each exposure group is displayed in Figure 8. There was no significant difference in weights between groups for profiles at either oxygen concentration.

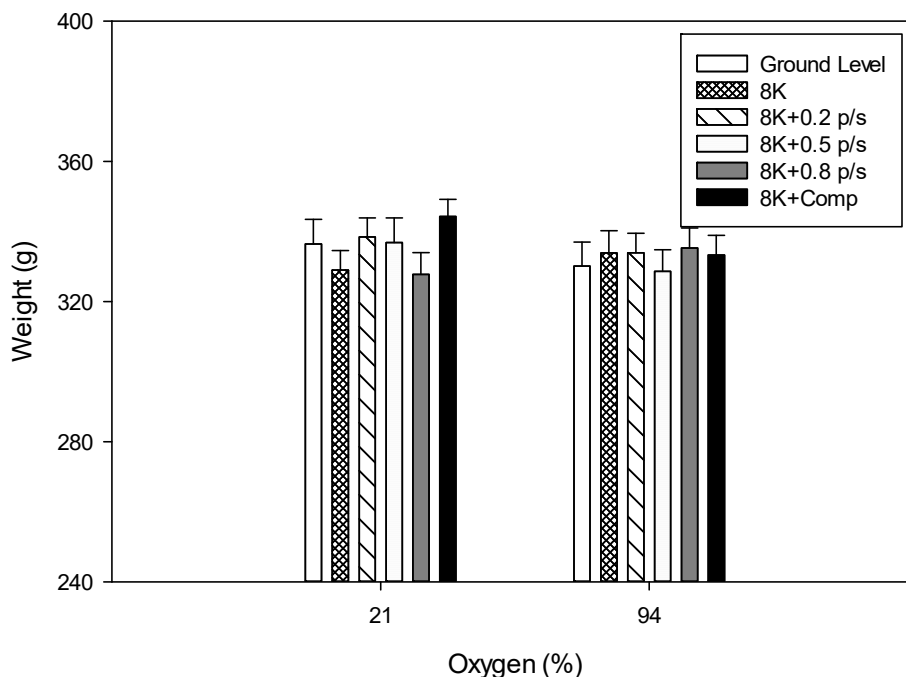


Figure 8. Mean animal weights for each exposure group.

Respiratory Measurements During Exposure

Respiratory parameters were monitored throughout the exposure to determine if any fluctuation-induced changes were present. All data points and error bars in Figure 9 and Figure 10 represent exposure group means $\pm$ the standard error of the mean (SEM), respectively. There was no difference in respiratory rate, tidal volume, minute volume, and mid-expiratory flow between the 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles and the GL and 8K profiles during any of the analysis segments. Although there was a difference between the minute volume of the GL and 8K profiles ($p=0.03$) during the Flux 1 segment of the 21% oxygen exposure (Figure 10, lower left panel), this is only suggestive of an altitude-induced effect. The fact that this change is not also observed in the fluctuation profiles may indicate some blunting of this response to altitude under reduced oxygen conditions, however the low magnitude of the difference between the groups does not suggest a clinically meaningful effect.

There is some indication that if rats were to be exposed to longer or more severe fluctuations than were included in the current profiles, that there may have an effect. There appeared to be a non-significant trend with the 8K+0.5 p/s and 8K+0.8 p/s groups and decreased respiratory rate, minute volume, and mid-expiratory flow during the Post

Flux 2 period (Figure 9, upper right panel). Additionally, the mean respiratory rate during the 8K+0.8 p/s profile was significantly lower than the 8K+0.2 p/s profile ($p=0.04$) during the Post Flux 2 segment of the 94% oxygen exposure, but neither were different than the GL or 8K controls.

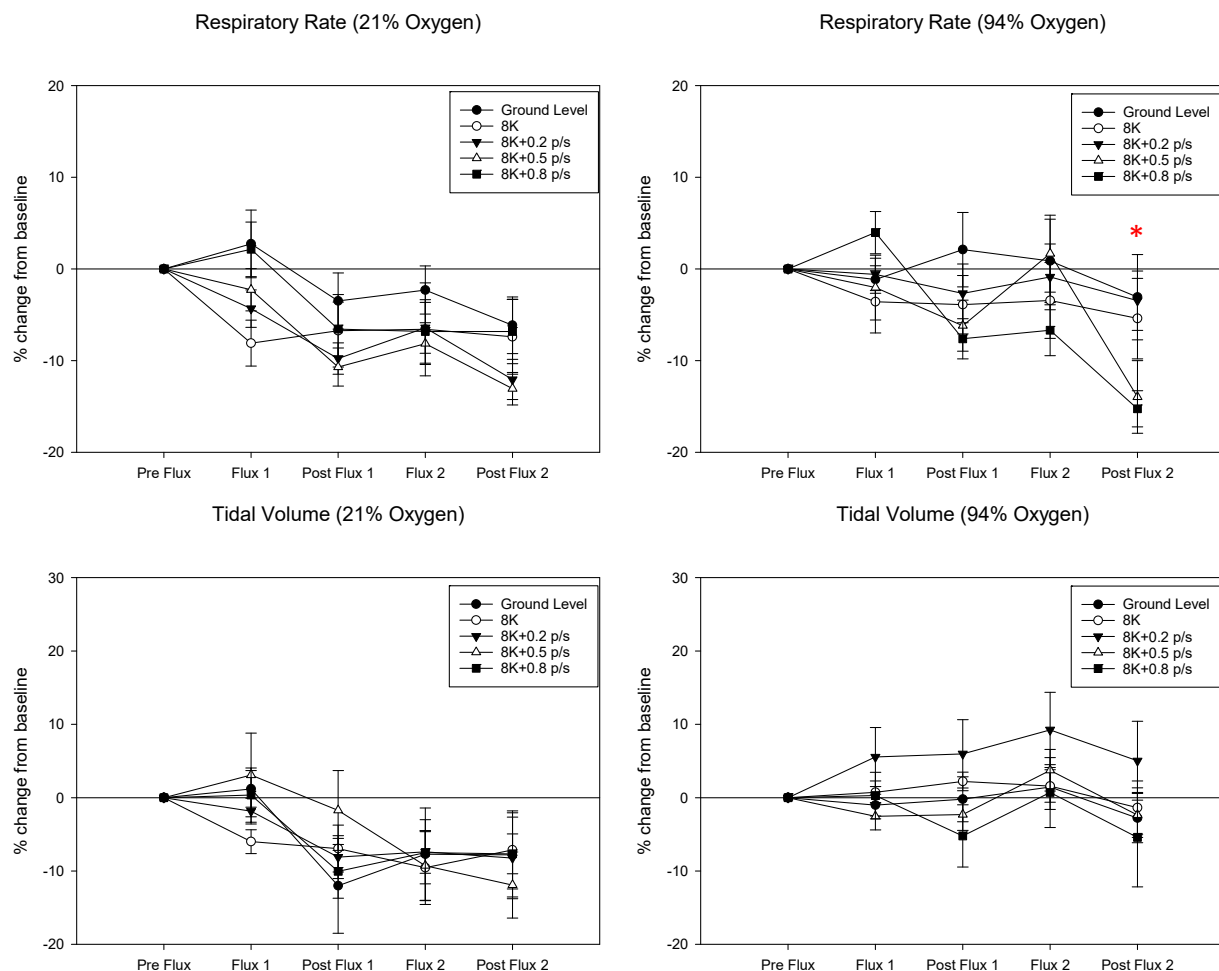


Figure 9. Average respiratory rate and tidal volume during exposure for GL, 8K, 8K+0.2 p/s, 8K+0.5 p/s and 8K+0.8 p/s compared to pre-fluctuation baseline
 * $p < 0.05$ for 8K+0.8 p/s profile compared to 8K+0.2 p/s profile during the Post Flux 2 segment

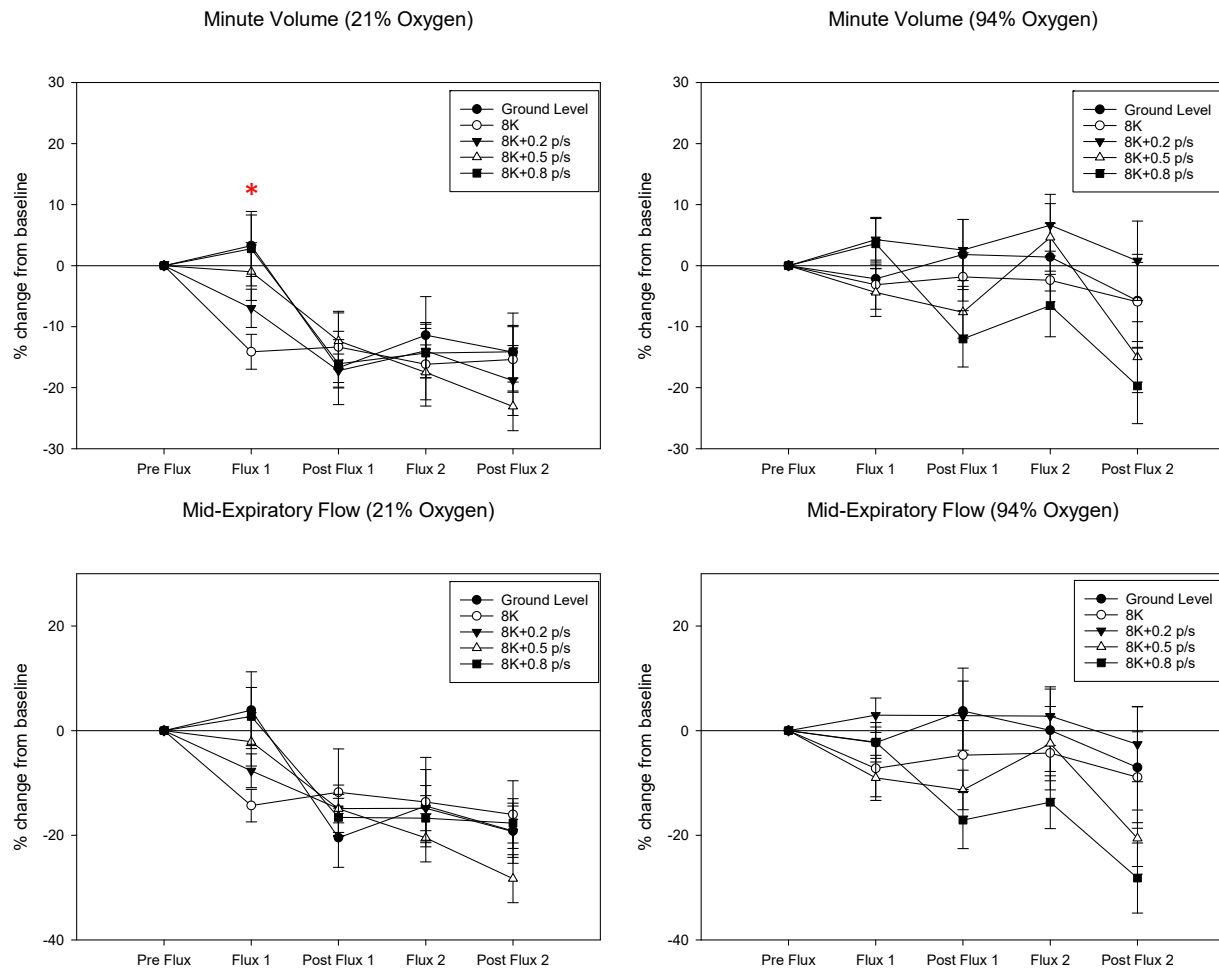


Figure 10. Average minute volume and mid-expiratory flow for GL, 8K, 8K+0.2 p/s, 8K+0.5 p/s and 8K+0.8 p/s during exposure compared to pre-fluctuation baseline
 *p<0.05 for 8K profile compared to GL profile during the Flux 1 segment

Respiratory parameters were monitored throughout the exposure to pressure fluctuations to determine if any fluctuation-induced changes were present. All data points and error bars in Figure 11 and Figure 12 represent exposure group means $\pm$ SEM, respectively. There was no difference in respiratory rate, tidal volume, minute volume, and mid-expiratory flow between the 8K+Comp profile and the GL and 8K profiles during any of the analysis segments. Although there was a difference between the mid-expiratory flow of the GL and 8K profiles during the Flux 3 ($p=0.004$) and Post Flux ($p=0.04$) segments of the 21% oxygen exposure (Figure 12, lower left panel), this is only suggestive of an altitude-induced effect in lower oxygen conditions. There was a small increase in measured volume/flow present between pre-altitude and altitude time segments for the 8K and 8K+Comp profiles because of the decreased ambient pressure. While this could partially explain differences between these profiles and the GL profile, assuming this

increase was a strong enough confounding factor, the finding that there was no difference in respiratory responses between the 8K and 8K+Comp profiles supports there being no fluctuation-induced change in respiration irrespective of this potential effect.

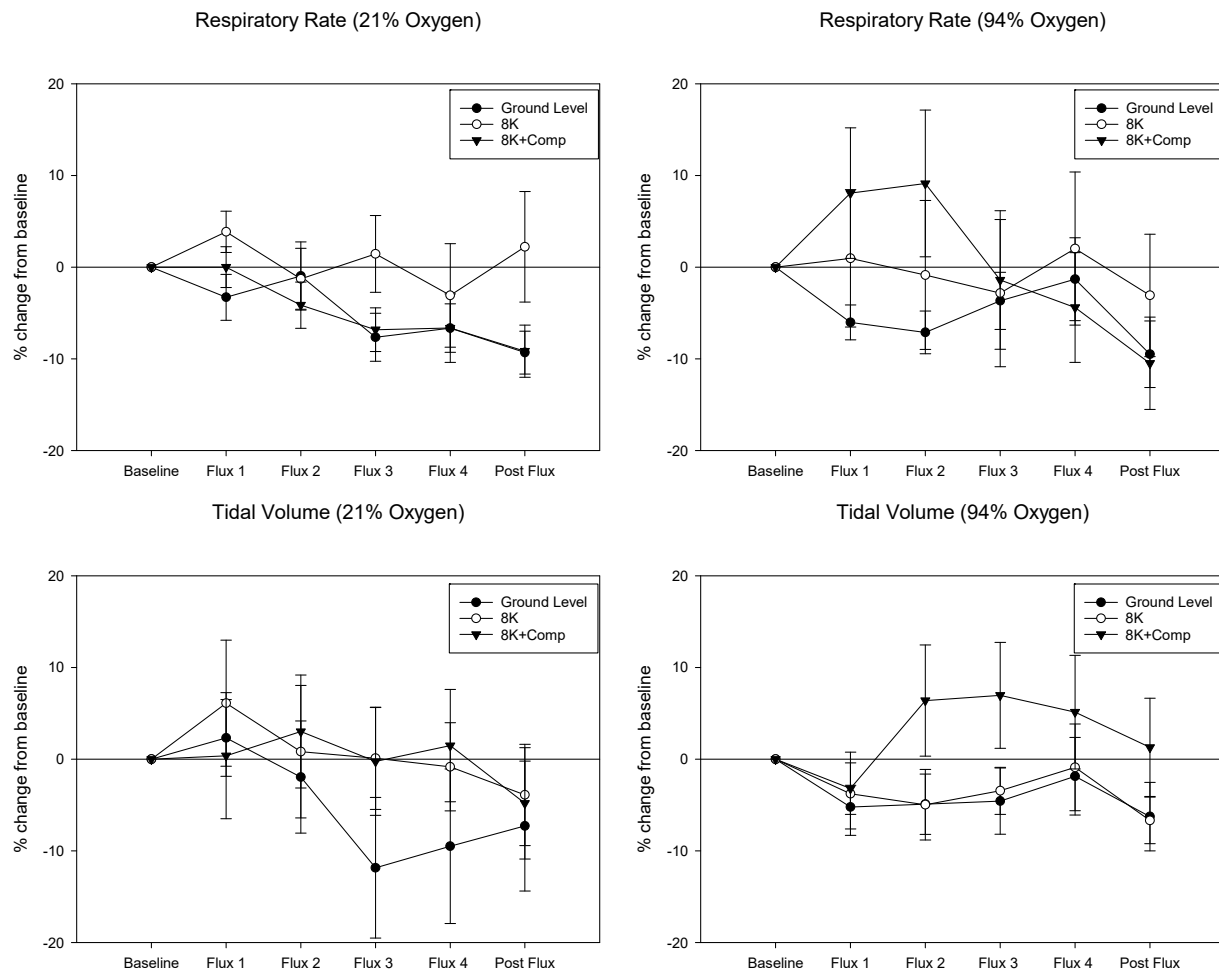


Figure 11. Average respiratory rate and tidal volume during exposure for GL, 8K, and 8K+Comp profile compared to pre-exposure baseline

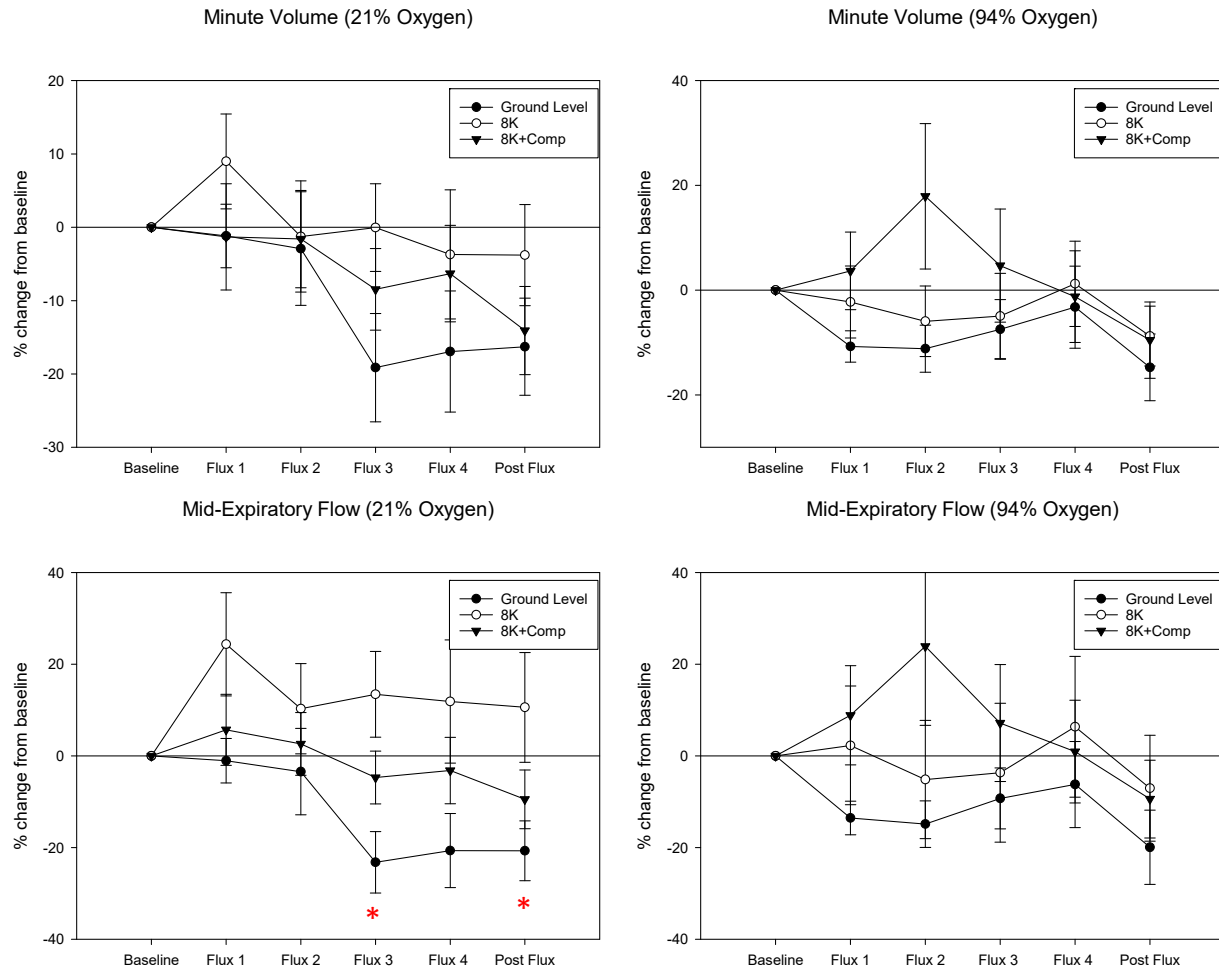


Figure 12. Average minute volume and mid-expiratory flow during exposure for GL, 8K, and 8K+Comp profile compared to pre-exposure baseline

*p<0.05 for 8K profile compared to GL profile during the Flux 3 and Post Flux segments

Lung Histopathology

Sections of the right lobes of the lungs were analyzed for signs of alveolar and bronchial injury. Representative images from the GL, 8K+0.8 p/s, and 8K+Comp profiles are presented in Figure 13. The scale bar in the figures represents 500 μ m. Any histologic changes within all three images are within normal limits.

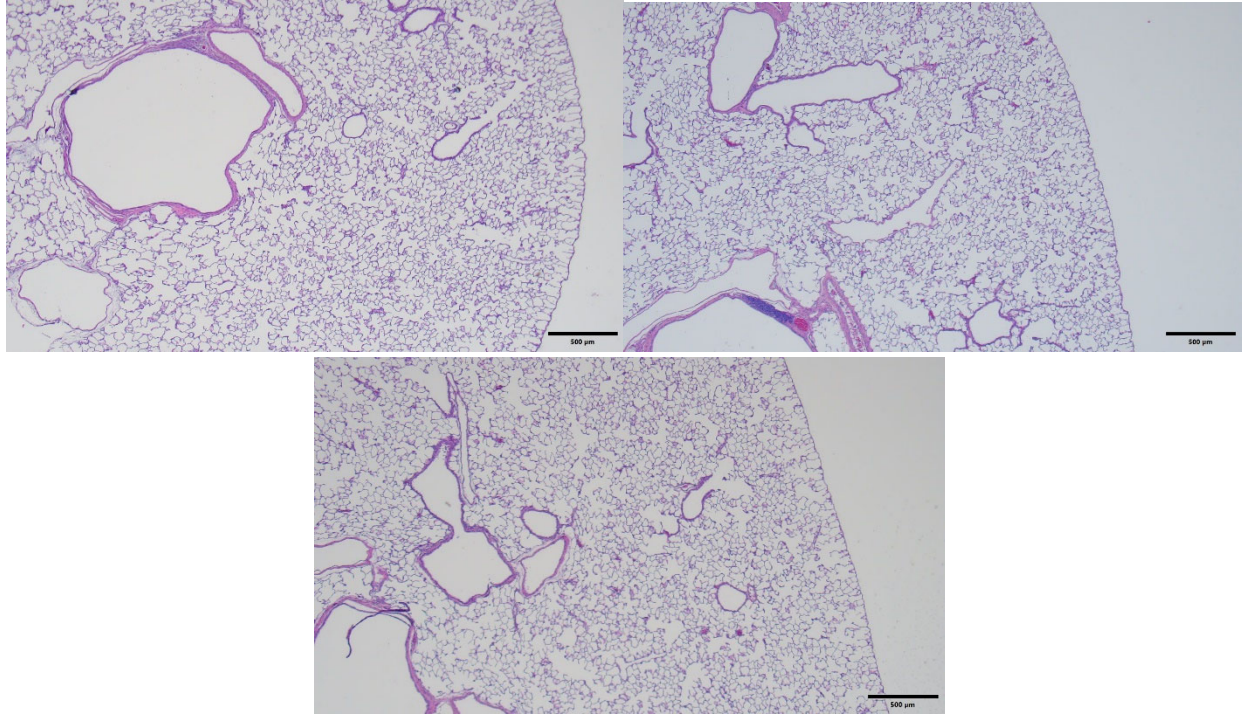


Figure 13. Example histology images of lungs following exposure to GL, 8K+0.8 p/s and 8K+Comp profiles

The mean histopathology score for all six profiles is presented in Table 7 for the group exposed to 94% oxygen and in Table 8 for the group exposed to 21% oxygen. The lung section from every rat was within normal limits, with no signs of alveolar or bronchial inflammation, hemorrhage, or epithelial damage that would be indicative of lung injury.

Table 7. Mean lung histopathology score for each profile following exposure while breathing 94% oxygen.

94% Oxygen Concentration						
	Ground Level	8K	8K+0.2 p/s	8K+0.5 p/s	8K+0.8 p/s	8K+Comp
Alveolar Damage						
Perivascular Edema +/- Inflammation	0	0	0	0	0	0
Edema	0	0	0	0	0	0
Fibrin	0	0	0	0	0	0
Hemorrhage	0	0	0	0	0	0
Inflammatory Cells	0	0	0	0	0	0
Hyaline Membrane	0	0	0	0	0	0
Alveolar Epithelial Necrosis/Degeneration	0	0	0	0	0	0
Interstitial PMN Infiltrates	0	0	0	0	0	0
Alveolar Epithelial Hyperplasia	0	0	0	0	0	0
Fibroplasia/Fibrosis	0	0	0	0	0	0
Bronchiolar Damage						
Edema	0	0	0	0	0	0
Fibrin	0	0	0	0	0	0
Hemorrhage	0	0	0	0	0	0
Inflammatory Cells	0	0	0	0	0	0
Bronchiolar Epithelial Necrosis/Degeneration	0	0	0	0	0	0
Bronchiolar Epithelial Hyperplasia	0	0	0	0	0	0
Fibroplasia/Fibrosis	0	0	0	0	0	0
BALT Necrosis	0	0	0	0	0	0

Table 8. Mean lung histopathology score for each profile following exposure while breathing 21% oxygen

21% Oxygen Concentration						
	Ground Level	8K	8K+0.2 p/s	8K+0.5 p/s	8K+0.8 p/s	8K+Comp
Alveolar Damage						
Perivascular Edema +/- Inflammation	0	0	0	0	0	0
Edema	0	0	0	0	0	0
Fibrin	0	0	0	0	0	0
Hemorrhage	0	0	0	0	0	0
Inflammatory Cells	0	0	0	0	0	0
Hyaline Membrane	0	0	0	0	0	0
Alveolar Epithelial Necrosis/Degeneration	0	0	0	0	0	0
Interstitial PMN Infiltrates	0	0	0	0	0	0
Alveolar Epithelial Hyperplasia	0	0	0	0	0	0
Fibroplasia/Fibrosis	0	0	0	0	0	0
Bronchiolar Damage						
Edema	0	0	0	0	0	0
Fibrin	0	0	0	0	0	0
Hemorrhage	0	0	0	0	0	0
Inflammatory Cells	0	0	0	0	0	0
Bronchiolar Epithelial Necrosis/Degeneration	0	0	0	0	0	0
Bronchiolar Epithelial Hyperplasia	0	0	0	0	0	0
Fibroplasia/Fibrosis	0	0	0	0	0	0
BALT Necrosis	0	0	0	0	0	0

Bronchial Alveolar Lavage Fluid

BALF was tested for elevated concentrations of protein, which would indicate a disruption in the alveolar epithelial barrier. The protein data is presented in Figure 14, with bars representing exposure group mean, and error bars representing exposure group SEM. There was no significant difference between any of the six profiles in either the 21% or 94% oxygen groups.

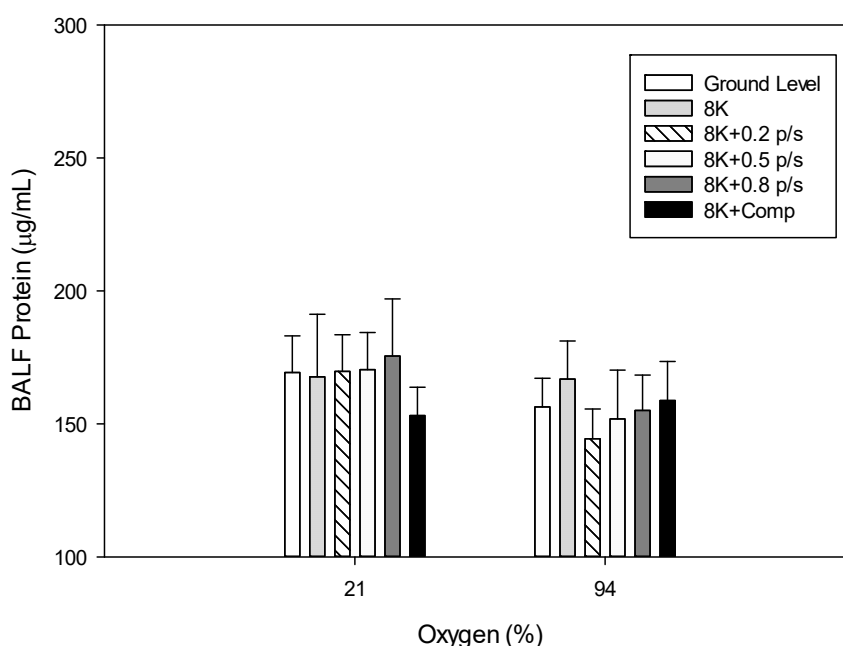


Figure 14. Mean post-exposure concentration of BALF protein for each profile

Cell differential counts were performed on at least five BALF samples per profile. In all six profiles in both the 21% or 94% oxygen groups, over 99% of the cells observed in the BALF were macrophages and <1% were neutrophils. Therefore, it is unlikely that there was an inflammatory response that would suggest fluctuation-related injury to the lung.

Neurobehavioral Testing

Results for the 21% concentration measures of general activity level showed significant differences between the GL profile and the 8K profile and the 8K+Comp profile for total activity time in seconds $p = 0.011$ and $p = 0.026$ respectively (Table 9). For the 94% oxygen concentration only average speed in cm/sec was significant, where the GL profile-exposed rats were statistically slower than rats exposed to the 8K profile ($p = 0.016$), the 8K+0.5 p/s profile ($p = 0.029$), the 8K+0.8 p/s profile ($p = 0.002$) and the 8K+Comp profile ($p = 0.004$) (Table 9).

Table 9. Post-Exposure neurobehavioral testing results

*p<0.05 compared to Ground Level

Summary of Neurobehavioral Results following Altitude Exposures + 94% Oxygen						
Neurobehavioral Test	Ground Level	8K	8K + 0.2 p/s	8K + 0.5 p/s	8K + 0.8 p/s	8K + Comp
Total Distance In Centimeters	18295 ± 1560	20768 ± 1620	21591 ± 1492	20663 ± 1469	21791 ± 836	24058 ± 2094
Activity Time in Seconds	925 ± 66	1023 ± 66	1092 ± 63	1026 ± 62	1072 ± 39	1119 ± 56
Average Speed (cm per sec)	27 ± 1.6	32 ± 0.9*	31 ± 1.1	31 ± 1.2*	33 ± 0.54*	33 ± 1.5*
% Time in Center vs. Perimeter	41 ± 1.4	39 ± 1.0	38 ± 1.2	42 ± 1.2	40 ± 1.2	41 ± 1.3
Stereotypical Activity (counts)	243 ± 8	255 ± 11	235 ± 6	232 ± 7	247 ± 8	255 ± 8
Ambulatory Activity (counts)	3252 ± 299	3603 ± 305	3780 ± 269	3584 ± 290	3813 ± 182	4259 ± 379
Rears	75 ± 12	82 ± 9	107 ± 9	89 ± 15	88 ± 6	107 ± 12
Forelimb Grip (kg)	0.70 ± 0.05	0.78 ± 0.06	0.72 ± 0.07	0.79 ± 0.04	0.81 ± 0.06	0.81 ± 0.07
Hindlimb Grip (kg)	0.35 ± 0.03	0.32 ± 0.01	0.33 ± 0.02	0.30 ± 0.02	0.32 ± 0.02	0.37 ± 0.02
Pain Response (kg)	0.23 ± 0.02	0.21 ± 0.02	0.20 ± 0.02	0.20 ± 0.01	0.18 ± 0.01	0.19 ± 0.02

Summary of Neurobehavioral Results following Altitude Exposures + 21% Oxygen						
Neurobehavioral Test	Ground Level	8K	8K + 0.2 p/s	8K + 0.5 p/s	8K + 0.8 p/s	8K + Comp
Total Distance In Centimeters	21138 ± 2117	26510 ± 889	22534 ± 1398	21160 ± 1613	21546 ± 1465	25418 ± 1627
Activity Time in Seconds	981 ± 68	1224 ± 26*	1133 ± 41	1046 ± 70	1023 ± 55	1201 ± 55*
Average Speed (cm per sec)	31 ± 1.5	35 ± 0.9	33 ± 0.6	31 ± 1.3	32 ± 1.2	34 ± 0.8
% Time in Center vs. Perimeter	42 ± 1.5	40 ± 1.2	40 ± 0.7	40 ± 1.1	41 ± 1.0	41 ± 1.3
Stereotypical Activity (counts)	243 ± 5	242 ± 7	262 ± 7	243 ± 8	247 ± 6	246 ± 7
Ambulatory Activity (counts)	3685 ± 388	4621 ± 153	3888 ± 253	3686 ± 290	3732 ± 262	4426 ± 309
Rears	83 ± 12	111 ± 4	89 ± 6.7	92 ± 13	94 ± 12	111 ± 13
Forelimb Grip (kg)	0.73 ± 0.05	0.71 ± 0.05	0.76 ± 0.05	0.75 ± 0.06	0.77 ± 0.05	0.68 ± 0.06
Hindlimb Grip (kg)	0.31 ± 0.03	0.33 ± 0.02	0.33 ± 0.02	0.33 ± 0.02	0.37 ± 0.02	0.34 ± 0.03
Pain Response (kg)	0.20 ± 0.02	0.20 ± 0.03	0.19 ± 0.02	0.21 ± 0.03	0.19 ± 0.02	0.17 ± 0.01

For all functional observations including cage side, open field and manipulation tests, no dose related effects were reported for either oxygen concentrations. Quantitative analyses for forelimb, hindlimb and SMALGO pain response did not provide any statistically significant differences for either oxygen level (Table 9).

Blood and Plasma Biomarkers

Plasma was tested for elevated concentrations of biomarkers that have been associated with decompression-related illness and/or vascular gas emboli. The biomarker data is presented in Figure 15, with bars representing exposure group mean, and error bars representing exposure group SEM. The concentrations of ICAM-1, a marker of endothelial dysfunction, and the inflammatory cytokines IL-6, IFN- γ and TNF- α were all determined in plasma samples from exposed rats that were collected 3-4 hours post-exposure. There was no significant difference between any of the six profiles in either the 21% or 94% oxygen groups.

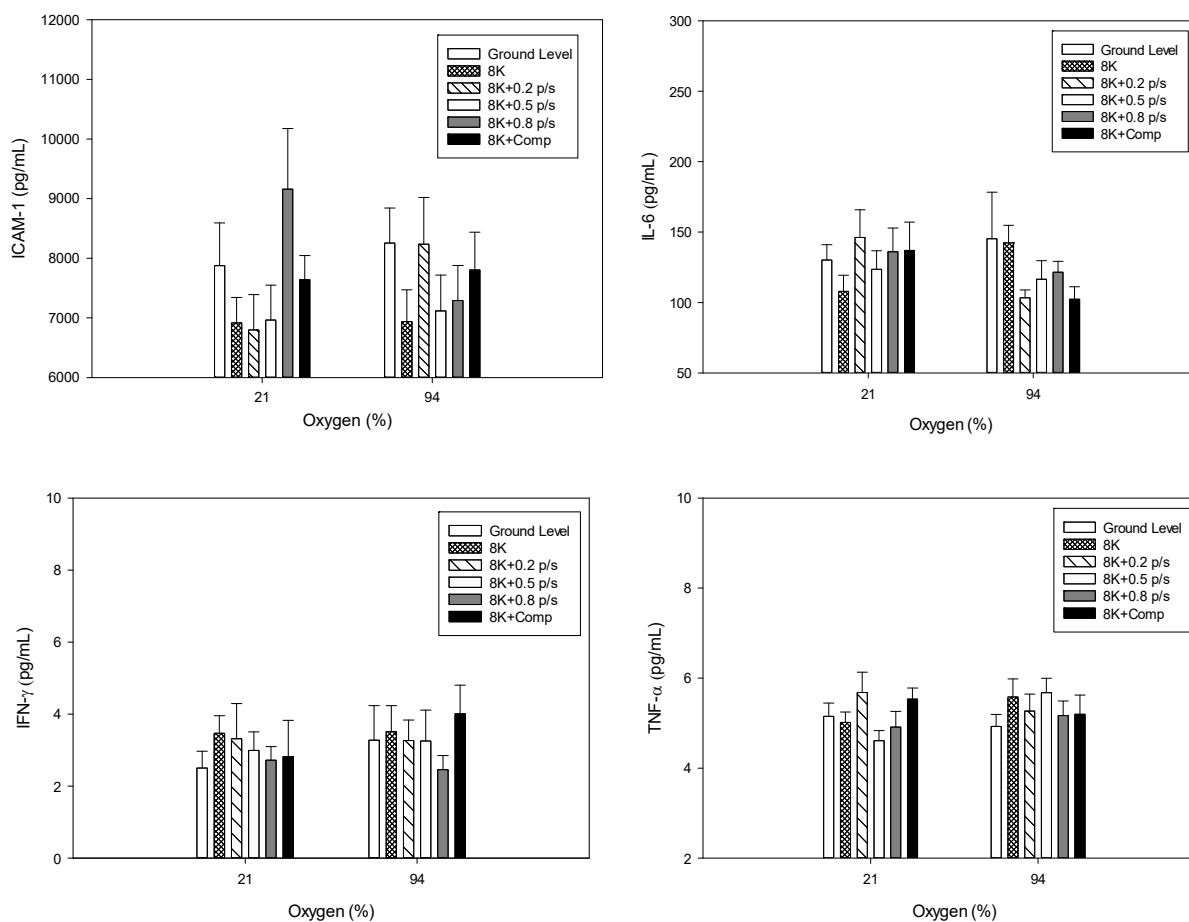


Figure 15. Mean post-exposure concentrations of ICAM-1, IL-6, IFN- γ , and TNF- α for each profile

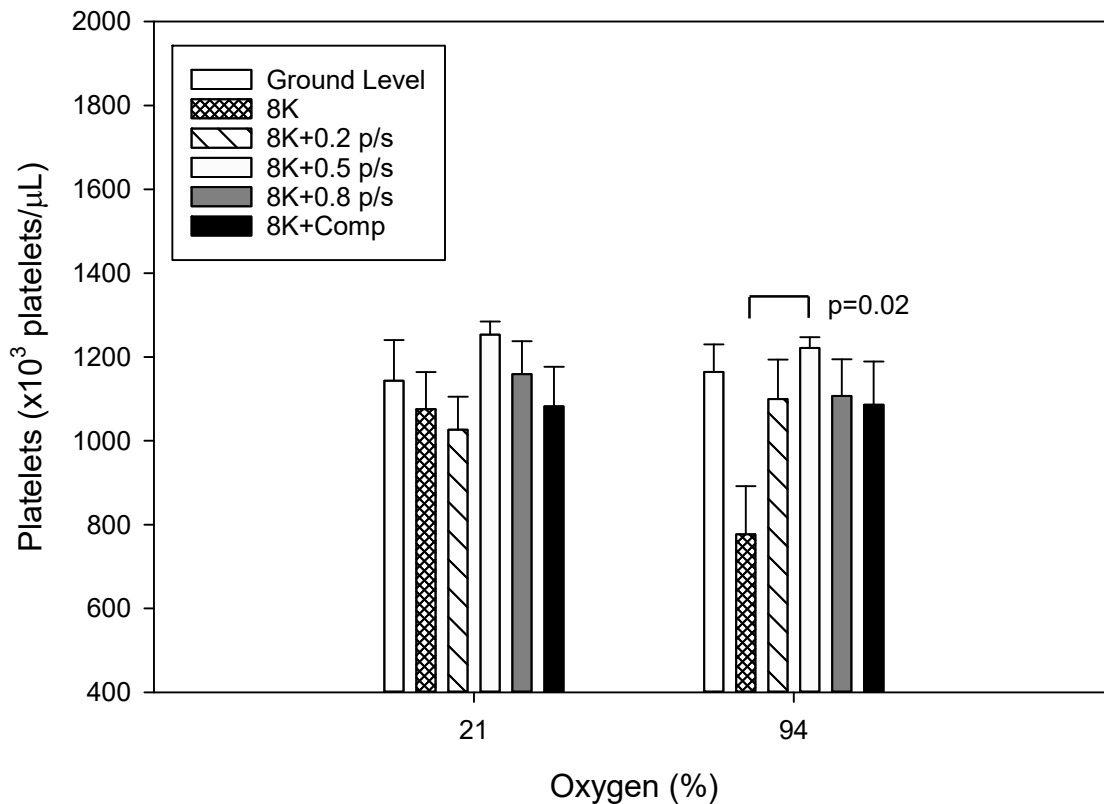


Figure 16. Mean post-exposure blood platelet concentration for each profile

The whole blood of exposed rats was tested for diminished platelet levels that could suggest VGE formation in the vasculature. The platelet count data is presented in Figure 16, with bars representing exposure group mean, and error bars representing exposure group SEM. In the 94% oxygen group, the platelet concentration of the 8K+0.5 p/s profile was significantly higher than the concentration of the 8K profile. There was no significant difference between any other profiles in either the 21% or 94% oxygen groups.

Phase 1 Discussion

No findings of pulmonary barotrauma were made in any of the fluctuation-exposed animals. There was no effect of the fluctuations on respiration during or immediately after the fluctuation events that would suggest injury to the lung or alteration in blood oxygenation that would increase respiratory drive. Similarly, there was no increased neutrophil infiltration or protein content in the BALF suggestive of any barotrauma-induced lung injury. These findings are consistent with prior studies of single rapid pressure change events that indicate a much more rapid rate of change is necessary to cause pulmonary barotrauma (Krebs and Pilmanis, 1996). It is possible that inflammatory responses to the exposure may take more time to develop than the post-exposure time

point used in this portion of the study, so future studies should evaluate additional timepoints.

No findings of DCS or other barotrauma were made in any of the fluctuation-exposed animals. None of the behavioral measures suggested that any pain or motor impairment was present in the animals immediately post-exposure. There were significant differences from the control animals in some post-exposure behavioral measures, specifically average speed in the 94% oxygen group and active time in the 21% oxygen group, but these differences were present in the groups that received only altitude exposures in addition to groups that were fluctuation-exposed. It is possible that transient pain or motor impairment could have presented during the exposure and then resolved upon return to ground level. This hypothesis was addressed by Phase 2 of the study, which assessed behavioral changes during the exposure to capture potential transient effects of the pressure fluctuations on the rats.

Although a difference was present between the 8K and 8K+0.5 p/s profiles, the platelet level of the 8K exposed-rats was in the low end of the normal range for rats, whereas the 8K+0.5 p/s profile did not differ from ground level control. The meaning of this result is unclear, but it does not suggest an increase in coagulation or VGE formation related to fluctuations because of a lack of severity-response and a lack of difference of the 8K+0.5 p/s profile from the GL control profile. Similarly, no other biomarkers associated with DCS were impacted by fluctuation exposures. These biomarkers are non-specific for DCS, however, therefore VGE formation, a well-known presentation of one form of DCS, was measured in Phase 3 of the study. Furthermore, it is unclear what timing is appropriate to best capture these biomarkers because they have been associated primarily with diving-related DCS (Zhang, et al., 2017; Bigley et al., 2008; Pontier et al., 2009) in which case the subject is in the decompressed condition at ground level. In the aviation-related decompression injury, once the subject has recompressed to ground level and recovery may start to occur. Therefore, measuring VGE formation during exposure, as was performed in Phase 3, was performed to provide better evidence as to if VGE-related DCS is a concern for pressure fluctuation events.

Additionally, because DCS or trauma-induced direct neurological injury or inflammation, without systemic bubble formation, could be responsible for effects that have been observed in Naval aviators, both functional synaptic changes immediately post-exposure and astrocytic activation three days post-exposure were evaluated in Phases 2 and 3, respectively. These experiments were designed to attempt to capture any changes due to rapid pressure cycling that may or may not present systemically, but could impact the CNS directly and persist post-exposure.

Phase 2

Phase 2 Methods

Animals and Animal Husbandry

A total of 72 male Sprague Dawley (CD) rats (*Rattus norvegicus*), 8-9 weeks in age, were used for each of the two experiments performed for Phase 2. All rats were obtained from Charles River (Wilmington, MA). Rats were acclimated to the USAF 711 HPW RHDV vivarium for approximately seven days following arrival at the facility. Food and water were provided *ad libitum* while rats were housed in the vivarium prior to exposure. Rats were weighed at the end of acclimation and again on the day of exposure. Following acclimation, rats were randomized into exposure groups, with each exposure group having an approximately equivalent average weight. On the day of exposure, the rats were transported to the testing room from the vivarium. Rats that were not necropsied immediately following exposure were returned to home cages in the vivarium until the time of necropsy.

All procedures were performed in accordance with animal protocols FWA-2018-0177-A and F-WA-2018-0180-A approved by the Wright-Patterson Air Force Base Institutional Animal Care and Use Committee.

Study Design

Rats were exposed to rapid fluctuations in ambient pressure in a hypobaric chamber (see section “Exposure Chamber”) while undergoing either a passive (see section “Open Field Activity”) or active (see section “Rotarod Testing”) neurobehavioral test. During each experiment, rats were exposed to one of the six profiles listed below. Graphical depictions of the two types of fluctuation profiles are given in “Study Design” for Phase 1, and graphical depictions of the measured values of all six profiles can be found in Appendix A.

- 1) Ground level (GL)
- 2) 8,000 feet altitude (8K)
- 3) 8,000 feet altitude + 0.2 psi/sec fluctuations (8K+0.2 p/s)
- 4) 8,000 feet altitude + 0.5 psi/sec fluctuations (8K+0.5 p/s)
- 5) 8,000 feet altitude + 0.8 psi/sec fluctuations (8K+0.8 p/s)
- 6) 8,000 feet altitude + composite fluctuation profile (8K+Comp)

With the exception of the GL profile, all profiles had a target ascent to 8,000 feet over approximately 90 seconds and a descent back to ground level over approximately 90 seconds. The sequence of events of the 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s pressure fluctuation profiles were the same as the Phase 1 exposures, with the only difference being that the time between reaching altitude and the start of the fluctuations was

shortened from four minutes to one minute. The purpose of shortening this section was to maximize the time that rats had to perform the neurobehavioral tasks once the fluctuations started, because the sensitivity of both neurobehavioral tasks were expected to decline with time the rat spent on the task. Similarly, the 8K+Comp profile was the same profile used for the Phase 1 exposures, with the exception that some of the periods in between fluctuations were shortened to allow for the profile to match the length of the other three shortened fluctuation profiles.

Table 10. Exposure schedule for Phase 2.

The same exposure schedule was used for both Experiment #1 and Experiment #2.

n=number of subjects

Run 1 (Day 1)			Run 2 (Day 2)		Run 3 (Day 3)	
Exposure	Profile	94% O ₂ (n)	Profile	94% O ₂ (n)	Profile	94% O ₂ (n)
1	Ground Level	2	0.2 psi/sec	2	0.8 psi/sec	2
2	8000 ft	2	0.5 psi/sec	2	Composite	2
3	0.2 psi/sec	2	0.8 psi/sec	2	Ground Level	2
4	0.5 psi/sec	2	Composite	2	8000 ft	2
5	0.8 psi/sec	2	Ground Level	2	0.2 psi/sec	2
6	Composite	2	8000 ft	2	0.5 psi/sec	2

Run 4 (Day 4)			Run 5 (Day 5)		Run 6 (Day 6)	
Exposure	Profile	94% O ₂ (n)	Profile	94% O ₂ (n)	Profile	94% O ₂ (n)
1	Ground Level	2	0.2 psi/sec	2	0.8 psi/sec	2
2	8000 ft	2	0.5 psi/sec	2	Composite	2
3	0.2 psi/sec	2	0.8 psi/sec	2	Ground Level	2
4	0.5 psi/sec	2	Composite	2	8000 ft	2
5	0.8 psi/sec	2	Ground Level	2	0.2 psi/sec	2
6	Composite	2	8000 ft	2	0.5 psi/sec	2

For each experiment, two rats receiving the same altitude profile were tested per run, with all six profiles being tested each day (see Table 10). The exposures were repeated over six days, with the order of the exposures rotated to balance the design and minimize the potential influence of order of exposure. All rats received hyperoxic oxygen levels (target value of 94%) during the exposures.

Exposure System

The exposure system was the same used in the Phase 1 exposures. See “Exposure System” in Phase 1.

Breathing Gases

The 94% oxygen /6% argon gas cylinders were provided by Matheson Tri-Gas Company (Dayton, OH). The cylinders were certified from the company to +/-2% accuracy for the stated oxygen concentration. The humidified breathing airflows for each of the breathing air lines were delivered to the behavioral testing chambers by an approximately 3 ft. length

of ¼" PTFE tubing (Cole-Parmer, Vernon Hills, IL) from the main breathing air delivery line followed by a 6" length of 1/8" stainless steel tubing. This orifice was used to buffer the upstream equipment from the varying chamber pressures and ensure steady flow delivery to the animals.

The animals were exposed to whole-body hyperoxic oxygen within each behavioral testing apparatus during each experiment. The oxygen was delivered into each behavioral testing apparatus with a target of 94% oxygen for altitude profiles and 70% oxygen for the ground level profiles. The 70% oxygen concentration was used with the ground level profiles to provide the same partial pressure of oxygen to the rats as would be present with 94% oxygen at 8,000 feet above sea level.

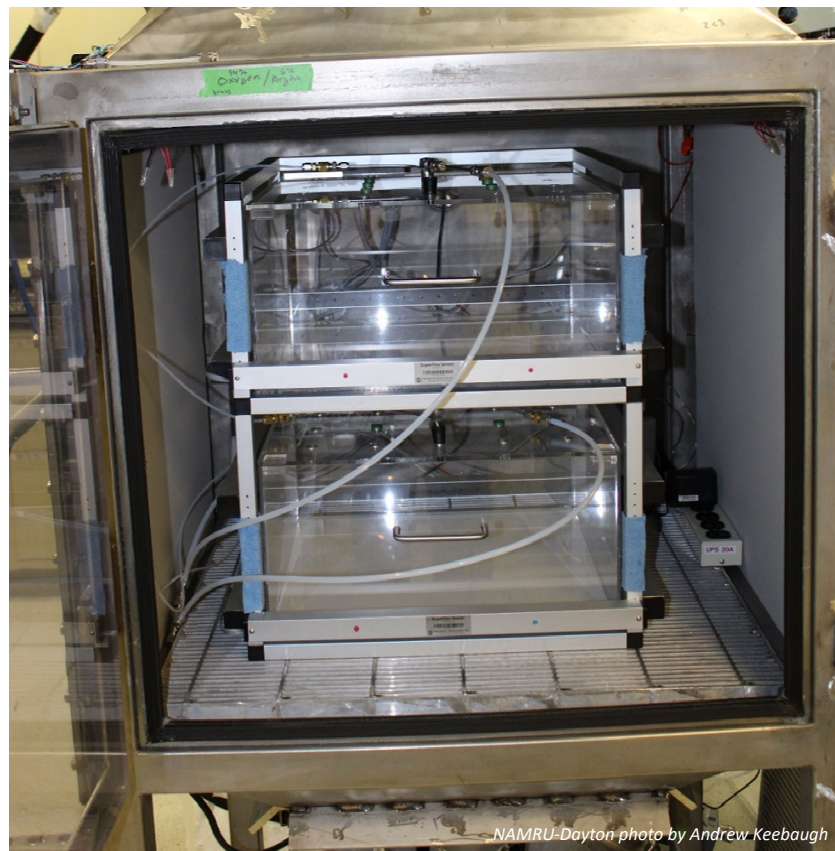


Figure 17. Open-field activity apparatus in exposure chamber.

The oxygen flow rate for the open-field activity boxes in Experiment #1 was 6.8 LPM. The breathing gas was delivered to the central port in the lid of the activity box, and the first ring of ports around the delivery port were plugged to ensure that the breathing gas circulated throughout the entire box before being exhausted through the outer ports in the lid (Figure 17). The rotarod boxes were modified to allow breathing gas delivery to the main animal compartment at a rate of 9.6 LPM, as well as nitrogen to the rear electronics

and motor compartment at a rate of 3.1 LPM. The nitrogen flow was used to prevent accumulation of oxygen in the electromotor compartment and avoid potential safety issues relating to elevated oxygen concentrations. Nitrogen was supplied by a dual-bed nitrogen generator (Parker Hannifin Corporation, Cleveland, OH) capable of delivering nitrogen at greater than 99% purity at the flow rates needed for the hypobaric exposure chamber.

Oxygen concentrations at the outlet ports of the boxes were monitored by an oxygen sensor (Advanced Micro Instruments, Inc. model 111, Costa Mesa, CA) and logged once per minute. The sensor sampling from both types of activity boxes was attached to a three-way valve so that each of the boxes could be monitored alternately during exposures.

Noise Levels

Noise levels are typically kept consistent during standard neurobehavioral tests because loud bursts of noise can startle animals and impact results. The pressure fluctuations associated with the exposure system were characteristically noisy events due to valve and rapid airflow noise. Therefore, a mitigation of noise bursts was put into place prior to exposures by optimizing the use of a white noise generator to mask bursts of noise levels in the rat auditory frequency range during the pressure fluctuations. A white-noise generator was set to ~75 dB, and noise levels within the behavioral testing equipment was tested to verify that the noise levels would stay consistent above ~200 Hz, the lower limit of a rat's hearing frequency range. Additional consistent noise was present due to the oxygen flow into each behavioral testing apparatus. Noise levels were recorded during pre-exposure test runs of the 8K+0.8 p/s and 8K+Comp profiles, which were assumed to be the loudest. A Larson Davis 831 sound level meter (Larson Davis, Provo, UT) with a 377C20 1/2" microphone (PCB Piezotronics, Depew, NY) placed inside of each behavioral testing apparatus was used to record noise levels between 6.3 Hz and 20 kHz throughout the test runs.

Figure 18 shows the noise present in the 1/3 octave bands between 6.3 Hz and 20 kHz for the 8K+0.8 p/s and 8K+Comp profiles. Both Figure 18A and Figure 18B demonstrate that the noise levels were steady in the 70-80 dB range above ~100 Hz during all of the fluctuations of the 8K+0.8 p/s and 8K+Comp profiles. Very low frequency, high decibel noise was still present during fluctuations and could not be mitigated, but this noise was present at frequencies below a rat's hearing range and not expected to cause any hearing-related effects on behavior.

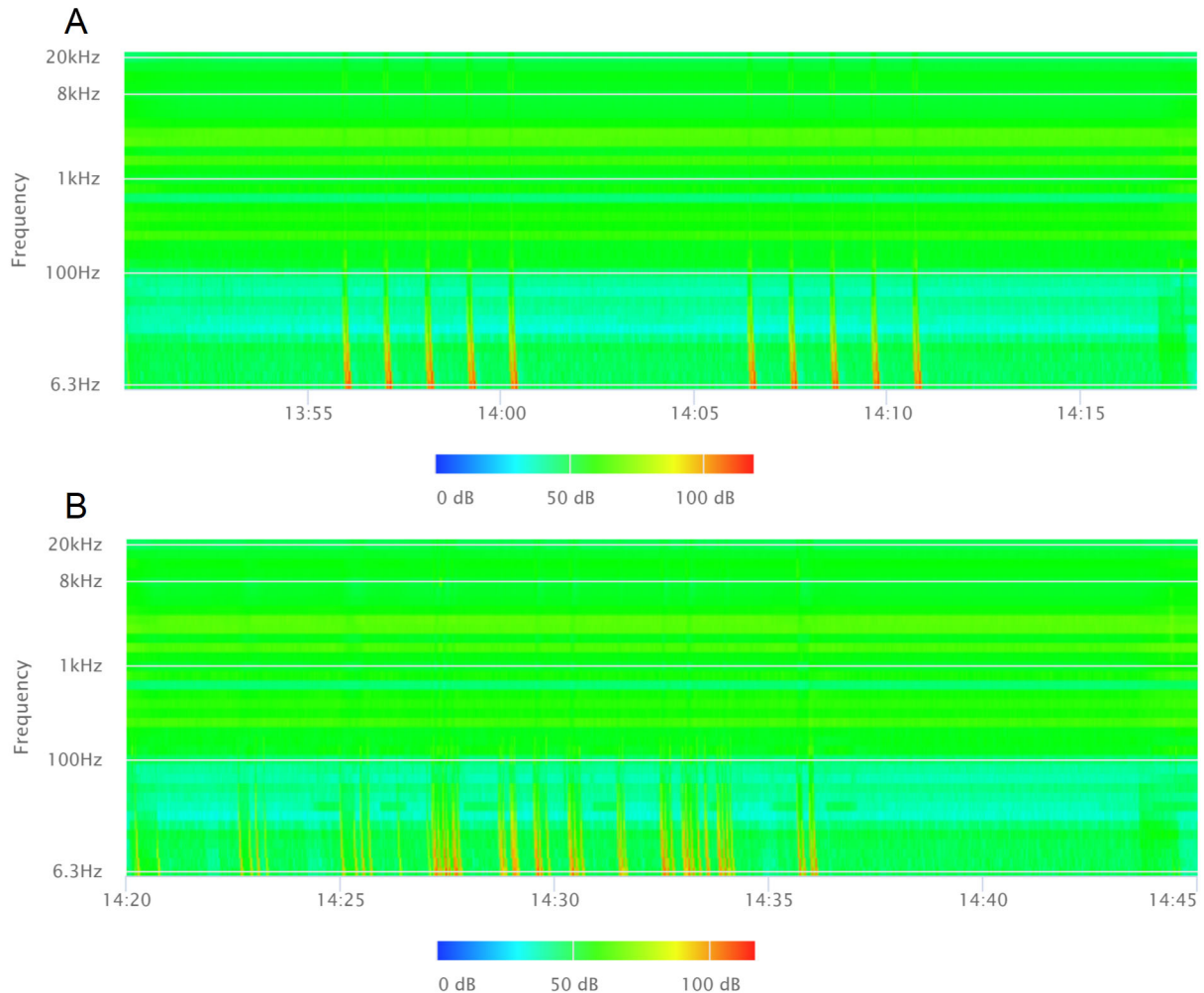


Figure 18. Noise levels during 8K+0.8 p/s and 8K+Comp profiles.

Maximum sound level in each 1/3 octave band from 6.3 Hz to 20 kHz frequencies over time for **(A)** 8K+0.8 p/s and **(B)** 8K+Comp profiles. Horizontal axis is time of day in hours:minutes.

Experiment #1 Open Field Activity Testing

Gross locomotion, stereotypical movements and exploratory behavior were evaluated for all animals using a photobeam activity system (see Figure 17) and Fusion acquisition and analysis software (Omnitech, Columbus, OH). Animals were individually placed in one of eight 16" (W) x 16" (D) x 15" (H) clear plastic open fields where horizontal and vertical photobeam sensors automatically recorded beam breaks that were translated into activity measures via the Fusion software. The photocells were aligned one inch apart to detect horizontal movement and vertical rears, as well as differentiate small (stereotypic) movements from large (ambulatory) movements. To begin the test, two animals were placed in the center of separate open field arenas and chamber recordings were initiated once both animals were loaded.

Animals were left uninterrupted for the duration of the 30 minute exposure session. Animals were observed the entire exposure time from out of their line of sight, and no researcher passed in front of the chamber while data was being recorded. The times at which each experimental fluctuation segment occurred were recorded manually. Between each test, all fecal boli and urine pools were removed and the open fields were washed with a solution of 50% ethanol to remove any olfactory cues that may have been left behind. They were allowed to dry completely before being used again. The following dependent measures were automatically recorded:

- Total distance traveled in centimeters: using the subject's location as the centroid, or center of mass.
- Sum of ambulatory activity count: number of beam breaks while animal was ambulating, does not count stereotaxic behavior.
- Sum of movement time in seconds: the length of time that the subject spent in an activity, with activity defined as when ambulation or stereotypy occurred.
- Sum of stereotypic activity count: The number of beam breaks due to stereotypic activity. If the animal broke the same beam (or set of beams) repeatedly then the monitor considers that the animal is exhibiting stereotypy. This typically happens during grooming or head bobbing.
- Sum of vertical activity count: vertical beam breaks, or rears.
- Sum of ambulatory episode median velocity (cm/s): The median of velocities for each ambulatory episode.
- Average median velocity (cm/sec)

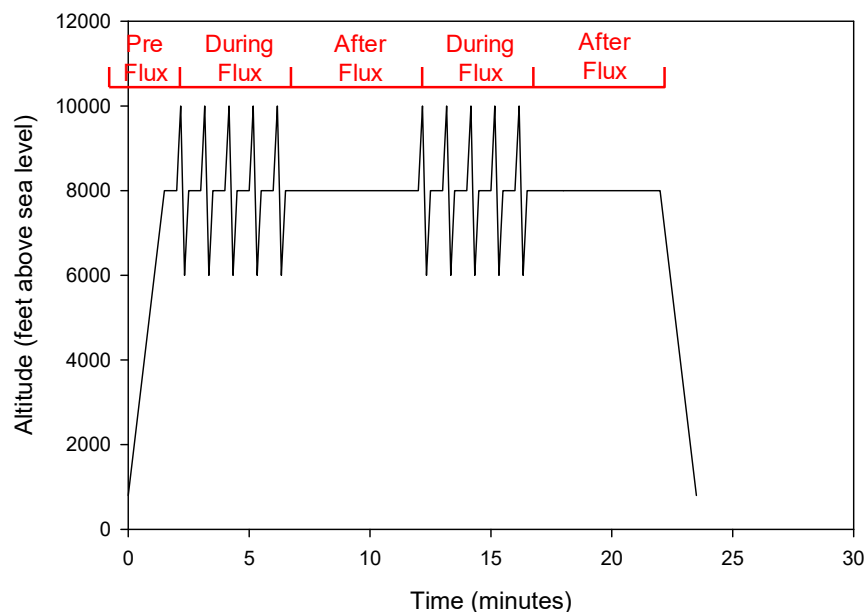


Figure 19. Averaging intervals for open-field activity test for 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles.

The 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles were analyzed separately from the 8K+Comp profiles due to the differences in fluctuation event timing during the profiles. In the analysis of the 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles, the profiles were broken into three segments:

Pre-Flux: the baseline period prior to starting ascent, ascent, and approximately one minute post-ascent immediately before the beginning of the fluctuations
During Flux: the two five minute periods during which fluctuations occurred
After Flux: the two five minute periods after each set of fluctuations

These segments defined the interval over which the behavioral data would be averaged for each animal. These segments are represented visually in Figure 19. A similar method was employed to define the segments for the 8K+Comp profile, as shown in Figure 20. For each analysis, the average beginning and end times were determined from the fluctuation profiles and used to define the timing of comparable intervals in the GL and 8K profiles.

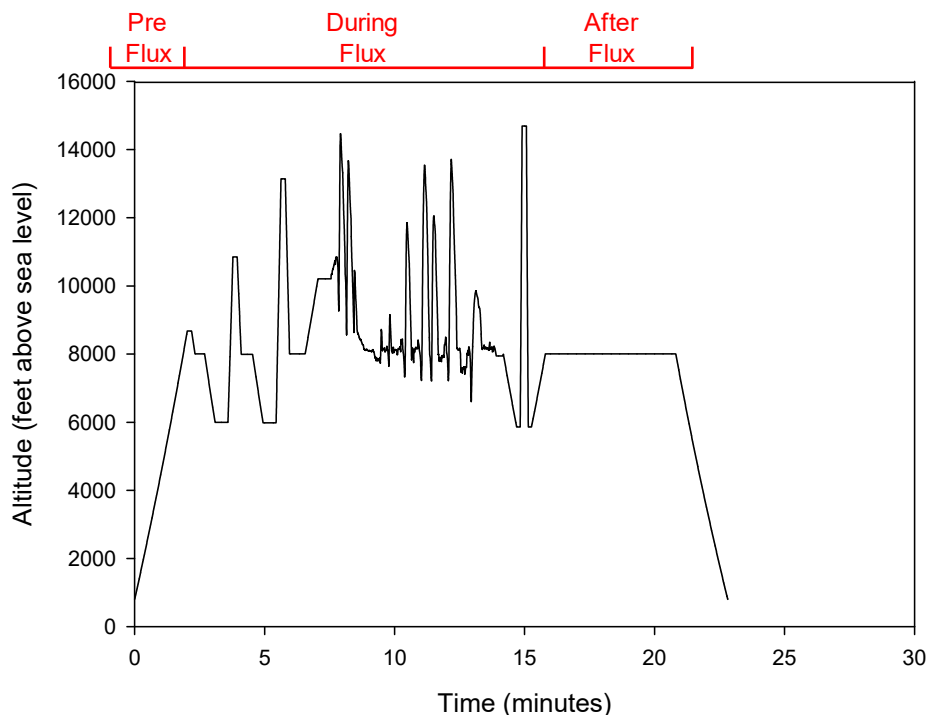


Figure 20. Averaging intervals for open-field activity test for 8K+Comp profile.

The open-field activity test has diminishing utility with time, because the animal's desire to explore a novel environment decreases over the course of 30 minutes, a process called habituation, and consequently their activity will drop over time as shown in Figure 21

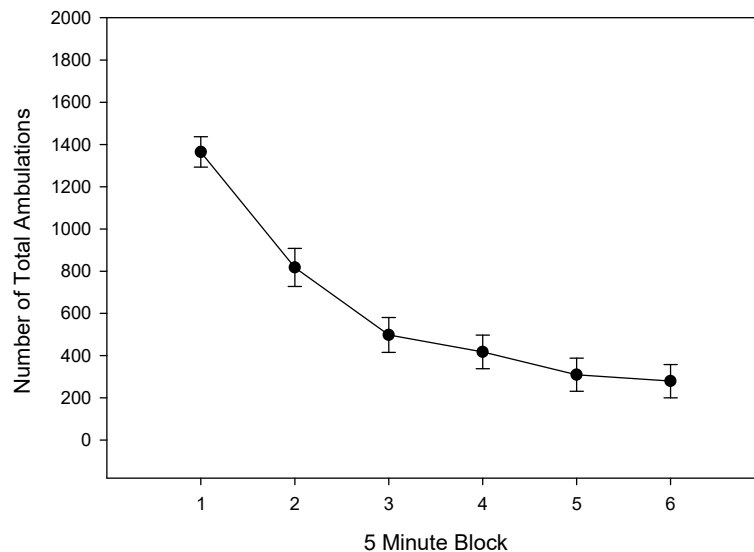


Figure 21. Characteristic habituation curve for animals undergoing open-field activity test.

Habituation curve describes average ambulatory activity over 30 minute open-field test. Data represents average $\pm$ SEM of total ambulations during each five-minute block from Phase 1 Ground Level control rats

Experiment #2, Rotarod and Accelerod Testing

The rotarod test was used to evaluate motor coordination, endurance, and balance by assessing how long an animal can walk on a rotating cylindrical rod. The test ran during altitude exposures with the rod rotating at a constant rate of 11 rpm for the first ~17.5 minutes, which started from the beginning of ascent to altitude to approximately one minute after the end of the last fluctuation. The test then transitioned to an accelerod test, where the spin rate increased to 40 rpms over the last 4 minutes at altitude. The same timing was used for the GL profile. Motivation to stay on the 7 cm diameter textured rod was provided by rod height (25.2") and the animals' natural fear of falling. Falls were automatically recorded by photobeam breaks at the base of the enclosure using Fusion (Omnitech, Columbus, OH) software. Additionally, unusual behaviors were noted by a trained observer.

The individual rotarods (Omnitech, Columbus, OH) were modified in-house from stock condition for two reasons. The stock rotarods had catch points for the animals' extremities that had to be covered with gasketing to prevent injury to the animals during the testing. Additionally, a polycarbonate containment needed to be placed around the animal compartment to maintain oxygen levels in the compartment and prevent the rat from climbing out of the apparatus. The containment was perforated with enough holes to allow transmission of pressure fluctuations, but not enough to prevent excess loss of oxygen from the flow-through oxygen delivery system.

All animals were trained one to three times per day, every other day, for six total training days in order to achieve performance on the rods for up to 30 minutes at a speed of 11 rpm. As the animals were initially becoming familiar with the rod and walking on it, the training sessions could be immediately repeated for very short training periods on the rod (<5 minutes). The training time was increased from five to 30 minutes, typically adding five minutes each day of training. During the final training session, a compressed air line was attached to the chamber to flow clean air through at the same flow rate that was used for the exposures. The purpose of adding the airflow on the final day of training was to acclimate the rats to the noise of the airflow to mitigate additional stress that could be caused by the noise from the oxygen delivery system during the actual exposure day. All training occurred in a separate location from the exposure chambers using identical equipment on a staggered training schedule to allow all animals to achieve 30 minute runs prior to a single altitude exposure. Due to the extra gasketing added over catch points in each rotarod apparatus, some animals discovered during training that they could reach this gasketing, cling to it, and lower themselves down to the bottom of the apparatus.

Immediately preceding the exposure, animals were placed onto the rod while it was rotating at 11 rpm. The chamber door was then shut and the exposure began. If the animal fell off prior to the start of ascent to altitude, the session was restarted. Rats that had learned to climb down from the rod using the extra gasketing typically did so within the first few minutes of exposure and were considered “censored” data during the analysis rather than “falls”. Whether a rat climbed down or fell, the rat remained safely in the bottom of the apparatus, still experiencing fluctuations, until the exposure period completed.

Experiment #2, Electrophysiology

Animals used for the electrophysiology study were exposed to the following profiles for approximately 30 minutes:

- Profile 1 (Pro 1): Ground level (70% oxygen)
- Profile 2 (Pro 2): 8000 feet altitude (94% oxygen)
- Profile 5 (Pro 5): 8000 feet altitude + 0.8 psi/sec fluctuations (94% oxygen)
- Profile 6 (Pro 6): 8000 feet altitude + composite fluctuations (94% oxygen)

Rats were euthanized using rapid decapitation. Brain and brain slices were kept viable by maintaining them in ice-cold artificial cerebrospinal fluid (ACSF) that was kept continuously oxygenated (95/5% O₂/CO₂). ACSF consisted of (in mM): 124 NaCl, 3 KCl, 1.25 KH₂PO₄, 10 D-Glucose, 1 MgSO₄, 36 NaHCO₃, and 2 CaCl₂ (pH ~7.4). For hippocampal slices, cerebellum and approximately one centimeter of frontal cortex were removed and the remaining brain was sectioned at 350 µm thick using a vibratome (VT1000S Leica Microsystems) in the transverse plane, at approximately 30° laterally off the horizontal axis. Brain slices were maintained in warmed (37°C) oxygenated ACSF and allowed to recover for at least 60 min prior to recording. A new batch of ACSF was

prepared each morning of experimentation and continuously oxygenated with 95% O₂ and 5% CO₂.

One hippocampal slice at a time was placed onto the pre-coated microelectrode array probe, using a small weight to anchor the slice down. The probe containing the brain slice was then appropriately assembled with the QUAD II system, as specified in the instruction manual. A perfusion cap was used to circulate fresh oxygenated warmed ACSF into the probe and prevent the slices from drying. The flask containing the ACSF solution was continuously kept in a 37°C water bath. Flow rates of ACSF were set at 1.6 milliliters per minute. In order to deliver warmed, humidified oxygen to the brain slice, a line carrying 95% O₂ and 5% CO₂ was passed through a heated water-containing flask before entering the MED64 probe. All electrophysiology data were obtained using AlphaMed's Quad II systems (Automate, Berkeley, CA). Probes were initially pretreated with 70% ethanol and 0.1% polyethylenimine (PEI) in 25-mM borate buffer for at least 24 hours prior to use. Data acquisition and stimulation protocols were performed using Mobius software (Automate, Berkeley, CA).

A series of biphasic stimulating currents ranging from 10 to 120 μ A was applied to the Schaffer collateral of the hippocampus to obtain an input-output relationship curve. Evoked field potentials mainly consisting of inward deflections were recorded from neurons within the CA1 region of the hippocampus at a sampling frequency of 20 kHz using the Quad II multichannel amplifier, digitized and graphically displayed using Mobius software. Paired pulse facilitation (PPF) were obtained by delivering sets of two consecutive stimuli of equal intensity that were 40 milliseconds apart. Degrees of PPF were quantified by calculating the paired pulse (PP) ratio in which the response amplitude from the second stimulating pulse was divided by the response amplitude from the first pulse. Thus, a PP ratio of >1 indicates PPF whereas a PP ratio of <1 indicates paired pulse depression (PPD). Spontaneous spiking activity of neurons within the CA1 and CA3 region were recorded for five minutes, and frequency of firing was calculated using the Mobius software. A threshold value of five times the standard deviation was used to calculate spike frequency. Only channels exhibiting activity of 0.1 Hz or more were included in the frequency calculation.

For long term potentiation (LTP) experiments, the input-output function was used to determine the size of the stimulating current that resulted in approximately half of the maximal output response. Typically, a stimulating current size of 30-50 μ A induced a half-maximal response and thus was used for the LTP experiments. LTP was induced by delivering three sets of theta burst stimulation (TBS), consisting of 10 repeats of four high frequency stimulations at 100Hz every 200 ms to the Schaffer collateral regions. Evoked field responses (field excitatory postsynaptic potential or fEPSP and population spikes) were monitored at 12 second intervals for at least 30 minutes following LTP induction. Percent potentiation was calculated by computing the percent difference in population

spike amplitude or fEPSP slope at 30 min following LTP induction by TBS from baseline. Averages of 10 data points were calculated to obtain baseline and LTP values.

Data analysis

With the exception of the electrophysiology and rotarod data, all data for quantitative, continuous variables were compared for the exposure and control groups using one-way analysis of variance (ANOVA). If the ANOVA indicated statistical significance among exposure groups, the Tukey's post-hoc test was used to compare all exposure groups. When assumptions for parametric ANOVA were not met, Kruskal-Wallis nonparametric procedures was used. Time and incidence to fall data for the rotarod test was analyzed using Kaplan-Meier curves. Rats that either climbed down during the test or completed the segment being analyzed were considered "censored" in the analysis. A log-rank test was performed on the curves to determine if differences in curves were greater than would be expected by chance. Holm-Sidak multiple comparison method was used to determine differences between individual curves. Statistical analyses were performed using SigmaPlot 13.0 (Systat Software, Inc). The probability value of less than 0.05 was used as the critical level of significance for each statistical test.

Analyses of all electrophysiology data were performed using Mobius software. Microsoft Excel and SigmaPlot v.12.5 and v.13 were used to plot analyzed data and for statistical assessments. The downward (negative, inward) field potentials (fEPSP) were used to calculate degree of evoked response. In the input-output, PPF and LTP analyses, amplitudes and initial slopes of population spikes and field excitatory post-synaptic potentials (fEPSP) were calculated to quantitate the size of evoked response. Amplitude and slope calculations were performed using Mobius software and Igor. Neuronal activity was recorded from typically 14 microelectrodes within the MED64 probe chamber. For any given slice, data obtained from multiple microelectrodes covering one defined brain region (eg. CA1) were averaged together to yield the response value for that particular slice. Statistical analyses were then performed on the data obtained from the different brain slices. Data are represented as means with the standard error of the mean (SEM) and were statistically compared using either a one way or two way analysis of variance (ANOVA). If data failed normality, the nonparametric Kruskal Wallis one way ANOVA was used to calculate statistical significance. A calculated p-value of less than 0.05 was considered significantly different. All quantitation and statistical analysis as well as graphs were generated using Microsoft Excel and SigmaPlot v.13.

Phase 2 Results

Experiment #1 Exposure and Environmental Data

The desired altitudes and peak rates of change of the fluctuations were on target for Phase 2. The Experiment #1 altitude exposure and environmental data is listed in Table 11. The 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles were on average at the target peak rate of change and there was minimal peak-to-peak variability and run-to-run variability in fluctuations. Additionally, there was consistency in fluctuations and runs in

regard to duration and maximum/minimum altitudes. The mean $\pm$ the SD of the temperature and %RH during each run of each profile are given in Table 11. There was no difference in temperature or %RH between exposure groups. The averages of the six runs of each profile for each parameter listed in Table 11 were calculated in the same manner as the altitude exposure and environmental data for Phase 1. The data for individual runs for each profile is listed in Appendix C.

Table 11. Average fluctuation, altitude, and environmental data for Phase 2 Experiment #1.

Exposure Parameter	Ground Level	8K	8K + 0.2 p/s	8K + 0.5 p/s	8K + 0.8 p/s	8K + Comp
Decompression Peak Rate of Change (p/s)			0.23 $\pm$ 0.01	0.51 $\pm$ 0.01	0.84 $\pm$ 0.02	
Decompression Average Rate of Change (p/s)			0.20 $\pm$ 0.01	0.37 $\pm$ 0.01	0.59 $\pm$ 0.02	
Recompression Peak Rate of Change (p/s)			0.29 $\pm$ 0.13	0.51 $\pm$ 0.01	0.86 $\pm$ 0.01	
Recompression Average Rate of Change (p/s)			0.24 $\pm$ 0.10	0.40 $\pm$ 0.01	0.63 $\pm$ 0.01	
Average Duration per Fluctuation (sec)			17.7 $\pm$ 2.1	7.6 $\pm$ 0.1	7.3 $\pm$ 0.2	
Maximum Altitude During Fluctuations (feet)			10141 $\pm$ 25	9881 $\pm$ 30	11968 $\pm$ 40	
Minimum Altitude During Fluctuations (feet)			5809 $\pm$ 105	6094 $\pm$ 405	6160 $\pm$ 69	
Steady Altitude (feet)	818 $\pm$ 2	8007 $\pm$ 4	7982 $\pm$ 5	7994 $\pm$ 5	7998 $\pm$ 3	
Temperature ($^{\circ}$ C)	22.6 $\pm$ 0.4	22.6 $\pm$ 0.4	22.5 $\pm$ 0.4	22.5 $\pm$ 0.3	22.3 $\pm$ 0.5	22.4 $\pm$ 0.4
Relative Humidity (%)	16.5 $\pm$ 5.8	15.1 $\pm$ 5.7	15.2 $\pm$ 5.2	14.9 $\pm$ 5.3	15.6 $\pm$ 7.5	14.6 $\pm$ 6.3
Oxygen (%)	60 $\pm$ 2	79 $\pm$ 2	72 $\pm$ 2	72 $\pm$ 1	69 $\pm$ 1	71 $\pm$ 2

There was variability in oxygen concentrations between exposure groups. The 8K profile had the highest measured oxygen concentration at an average of 79 $\pm$ 2% while the ground level profile had the lowest oxygen concentration of 60 $\pm$ 2%. All of the fluctuation profiles had oxygen concentrations ranging from 69-72%. The variability in oxygen concentration was due to three factors. The first factor was that the oxygen inlet for the ground level controls was set to 70% instead of 94% to deliver the same partial pressure of oxygen to the animals as what is present at 8,000 feet and 94% oxygen. The difference in delivered oxygen concentration is likely responsible for the lower oxygen concentration of the ground level profiles compared to that of the profiles that went to altitude.

The second factor was that the oxygen concentration inside of the open-field activity box was different than the oxygen concentration leaving the box. The concentration of oxygen inside of the box could not be directly measured because any equipment inside of the box could interfere with the responses of the animals. Pre-study testing found that the actual oxygen concentrations inside of the box hit a target of >90% (~70% for ground level) despite the concentrations leaving the box being lower, possibly due to some mixing with nitrogen near openings in the box. Therefore, the oxygen concentrations that the animal received were closer to target than represented in Table 11 and still hyperoxic, but the actual concentrations in the box during the experiment were not determined. Third, there was a measured drop in oxygen concentrations with the fluctuations of approximately 10-20% that would rebound back to baseline at the conclusion of the fluctuations. This drop in oxygen concentration was an unavoidable consequence of the fluctuations, but still did not drop the oxygen concentrations even to normoxic levels. The fluctuation-induced

reduction in oxygen concentration is likely why there was a ~10% difference in oxygen concentration between the 8K and fluctuation profiles.

The average parameters of all 13 fluctuations within the 8K+Comp profile are displayed in Table 12. Each column represents the mean $\pm$ standard deviation of the fluctuations for all six runs of the 8K+Comp profile. The duration, decompression/recompression rates, and peak altitude parameters are all defined the same as in Phase 1. All parameters were within target values and were consistent with runs of the 8K+Comp profile in the previous phases.

Table 12. Average fluctuation data for 8K+Comp profile during Phase 2
Experiment #1

Fluctuation Number	Duration (sec)	Decompression (p/s)		Recompression (p/s)		Peak Altitude (feet)
		Peak	Average	Peak	Average	
1	86 $\pm$ 2.0	0.11 $\pm$ 0.01	0.05 $\pm$ 0.00	0.04 $\pm$ 0.00	0.02 $\pm$ 0.02	8883 $\pm$ 21
2	38 $\pm$ 0.5	0.24 $\pm$ 0.01	0.15 $\pm$ 0.00	0.13 $\pm$ 0.00	0.05 $\pm$ 0.00	11045 $\pm$ 32
3	38 $\pm$ 8.8	0.34 $\pm$ 0.00	0.19 $\pm$ 0.00	0.19 $\pm$ 0.06	0.08 $\pm$ 0.02	13471 $\pm$ 36
4	10 $\pm$ 0.1	0.70 $\pm$ 0.02	0.53 $\pm$ 0.02	0.56 $\pm$ 0.03	0.42 $\pm$ 0.02	14742 $\pm$ 102
5	12 $\pm$ 0.5	0.79 $\pm$ 0.03	0.56 $\pm$ 0.01	0.33 $\pm$ 0.02	0.26 $\pm$ 0.01	13617 $\pm$ 130
6	8 $\pm$ 0.6	0.71 $\pm$ 0.08	0.48 $\pm$ 0.03	0.32 $\pm$ 0.02	0.19 $\pm$ 0.02	10473 $\pm$ 79
7	17 $\pm$ 0.7	0.74 $\pm$ 0.02	0.47 $\pm$ 0.02	0.23 $\pm$ 0.01	0.14 $\pm$ 0.01	12015 $\pm$ 159
8	19 $\pm$ 0.6	0.78 $\pm$ 0.03	0.47 $\pm$ 0.02	0.29 $\pm$ 0.01	0.18 $\pm$ 0.01	13721 $\pm$ 112
9	27 $\pm$ 0.8	0.39 $\pm$ 0.04	0.06 $\pm$ 0.01	0.11 $\pm$ 0.01	0.06 $\pm$ 0.00	9530 $\pm$ 83
10	18 $\pm$ 1.3	0.79 $\pm$ 0.03	0.51 $\pm$ 0.02	0.21 $\pm$ 0.01	0.12 $\pm$ 0.01	12113 $\pm$ 102
11	20 $\pm$ 0.6	0.72 $\pm$ 0.03	0.46 $\pm$ 0.02	0.26 $\pm$ 0.01	0.17 $\pm$ 0.01	13977 $\pm$ 74
12	18 $\pm$ 2.2	0.26 $\pm$ 0.11	0.15 $\pm$ 0.11	0.16 $\pm$ 0.05	0.08 $\pm$ 0.03	10023 $\pm$ 67
13	24 $\pm$ 0.4	0.97 $\pm$ 0.04	0.47 $\pm$ 0.03	0.95 $\pm$ 0.05	0.25 $\pm$ 0.01	15169 $\pm$ 69

Experiment #1 Animal Weights

Animal body weights that were measured immediately preceding the altitude chamber exposures for Experiment #1 are provided in Figure 22. There was no difference in body weight between exposure groups.

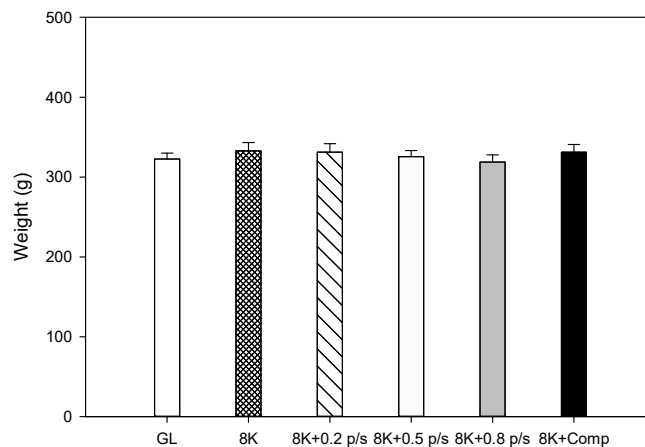


Figure 22. Average animal weights for each profile for Phase 2 Experiment #1.
Data presented as group mean $\pm$ SEM.

Experiment #1 Open Field Activity

A number of measures of motor activity were assessed using the open-field activity test during the altitude exposures. The following results are parameters that describe either the overall motor activity or specific activities during the test. These activities decreased over time through the “Pre-Flux”, “During Flux,” and “After Flux” averaging periods that follow the time course of the habituation curve in Figure 21.

“Average Distance per Minute” was calculated using the subject’s location as the centroid, or center of mass. Results are shown in Figure 23 in units of centimeters per minute. There was no difference in the Pre-Flux and During Flux periods, but the 8K+0.8 p/s profile was significantly less than Ground Level, 8K, and 8K+0.5 p/s profiles ($p=0.002$, $p=0.010$, $p=0.031$ respectively) during the After Flux period. There was no difference between the Ground Level, 8K and 8K+Comp profiles at any time point.

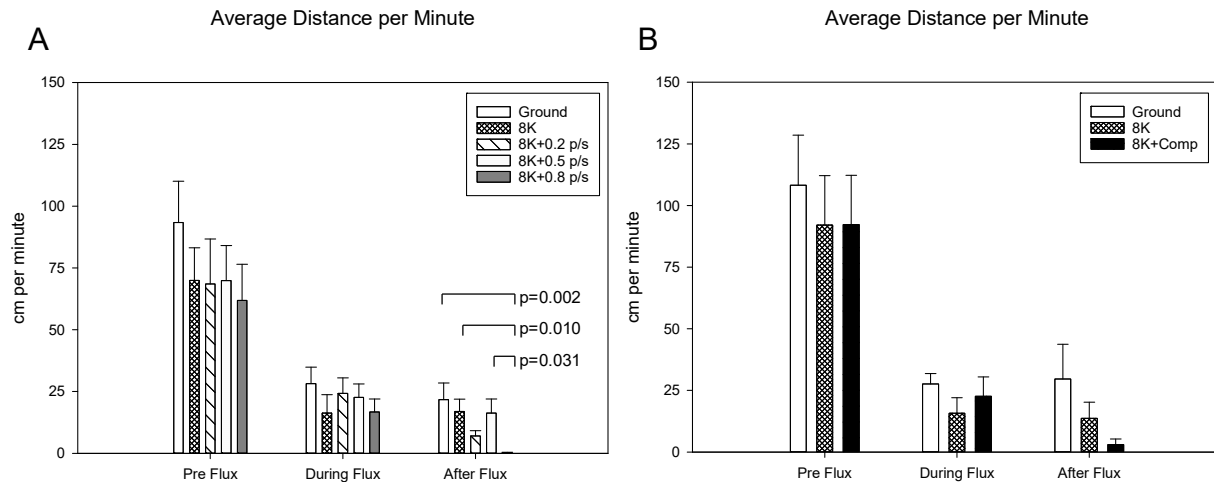


Figure 23. Distance per minute in open-field activity test.

Average distance per minute during Pre Flux, During Flux and After Flux segments of (A) 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles and comparable time periods for Ground Level and 8K profiles (B) 8K+Comp profiles and comparable time periods for Ground Level and 8K profiles

“Average Ambulatory Counts per Minute” was the number of beam breaks that occurred while the subject was moving, representing ambulatory activity (rather than activity occurring while the animal is sitting/standing in place). Results for this are shown in Figure 24 in units of counts per minute. There was no difference in the Pre-Flux and During Flux periods, but the 8K+0.8 p/s profile was significantly lower than Ground Level, 8K, and 8K+0.5 p/s profiles ($p<0.001$, $p=0.004$, $p=0.017$ respectively) during the After Flux period. There was no difference between the Ground Level, 8K and 8K+Comp profiles at any time point.

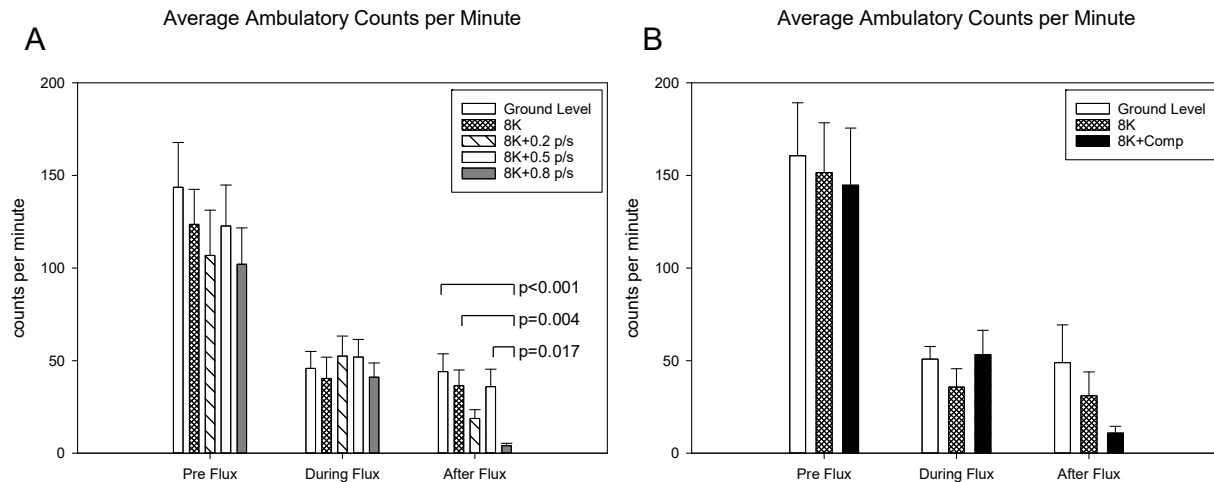


Figure 24. Ambulatory counts per minute in open-field activity test.

Average ambulatory counts per minute during Pre Flux, During Flux and After Flux segments of **(A)** 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles and comparable time periods for Ground Level and 8K profiles **(B)** 8K+Comp profiles and comparable time periods for Ground Level and 8K profiles

“Average Movement Time per Minute” is a parameter calculated by determining the number of seconds the subject was actively moving per each minute. Results are shown in Figure 25 in units of counts per minute. There was no difference in the Pre-Flux and During Flux periods, but the 8K+0.8 p/s profile was significantly lower than Ground Level, 8K, and 8K+0.5 p/s profiles ($p<0.001$, $p=0.008$, $p=0.005$ respectively) during the After Flux period. There was no difference between the Ground Level, 8K and 8K+Comp profiles at any time point.

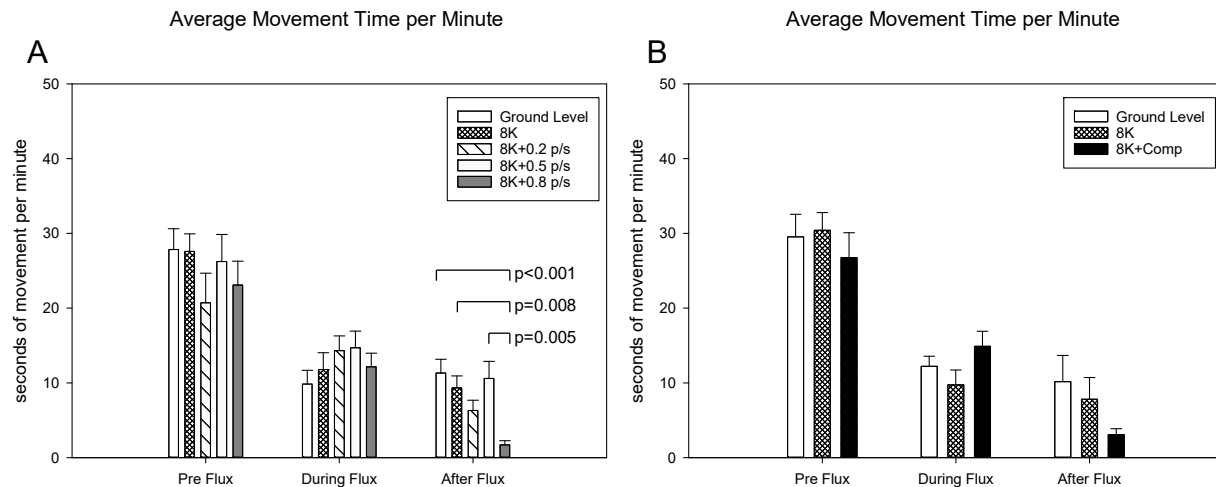


Figure 25. Movement time per minute in open-field activity test.

Average ambulatory counts per minute during Pre Flux, During Flux and After Flux segments of **(A)** 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles and comparable time periods for Ground Level and 8K profiles **(B)** 8K+Comp profiles and comparable time periods for Ground Level and 8K profiles

“Average Stereotypic Counts per Minute” was the number of beam breaks due to stereotypic activity while the animal is sitting/standing in place. If the animal broke the same beam (or set of beams) repeatedly, then the software considered the animal’s activity as stereotypy that typically included behaviors such as grooming or head bobbing. Results are shown in Figure 26 in units of seconds of movement per minute. There was no difference in the Pre-Flux and During Flux periods, but the 8K+0.8 p/s profile was significantly lower than Ground Level and 8K+0.5 p/s profiles ($p=0.001$, $p=0.007$ respectively) during the After Flux period. There was no difference between the Ground Level, 8K and 8K+Comp profiles at any time point.

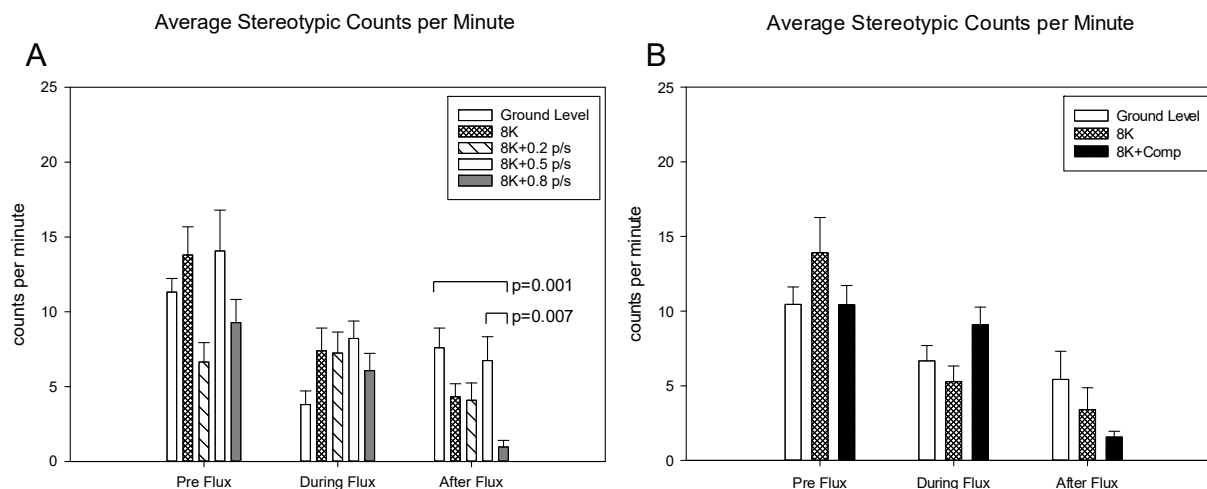


Figure 26. Stereotypic counts per minute in open-field activity test.

Average stereotypic counts per minute during Pre Flux, During Flux and After Flux segments of **(A)** 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles and comparable time periods for Ground Level and 8K profiles **(B)** 8K+Comp profiles and comparable time periods for Ground Level and 8K profiles

“Average Rears per Minute” was the number of time the subject reared up and broke the top row of beams, exhibiting exploratory behavior. Results are shown in Figure 27 in units of counts per minute. There was no difference in the Pre-Flux and During Flux periods, but the 8K+0.8 p/s profile was significantly lower than Ground Level, 8K, and 8K+0.5 p/s profiles ($p=0.006$, $p=0.012$, $p=0.042$ respectively) during the After Flux period. There was no difference between the Ground Level, 8K and 8K+Comp profiles at any time point.

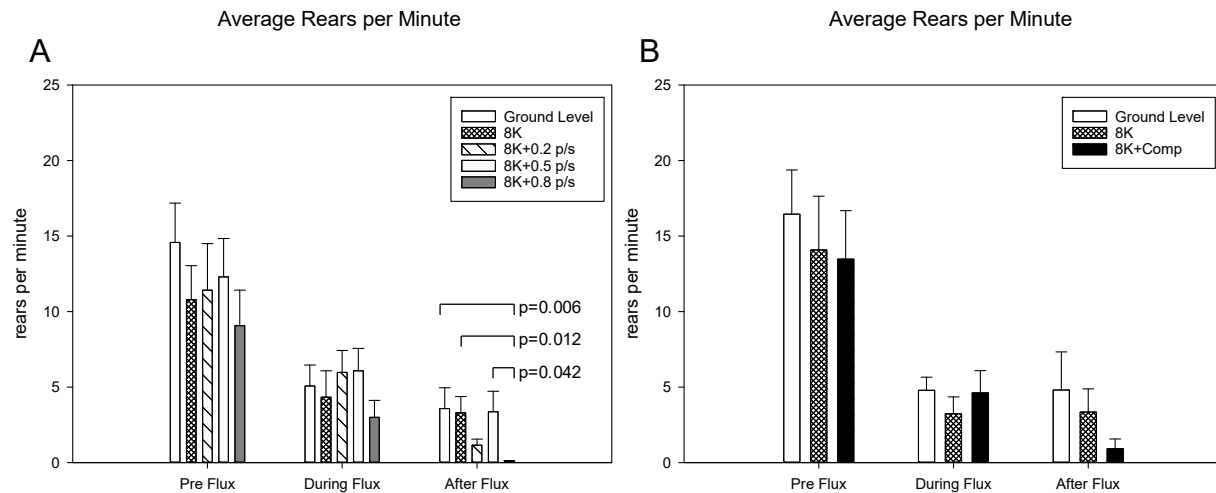


Figure 27. Rears per minute in open-field activity test.

Average rears per minute during Pre Flux, During Flux and After Flux segments of **(A)** 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles and comparable time periods for Ground Level and 8K profiles **(B)** 8K+Comp profiles and comparable time periods for Ground Level and 8K profiles

“Average Ambulatory Episode Median Velocity” was the median of velocities for each ambulatory episode. This parameter differed from the others because it was independent of the frequency of movement. Results are shown in Figure 28 in units of cm per sec. There were no significant results for the 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s fluctuation profiles. However, there was a significant decrease in this velocity for the 8K+Comp profile compared to just the Ground Level control in the During Flux period.

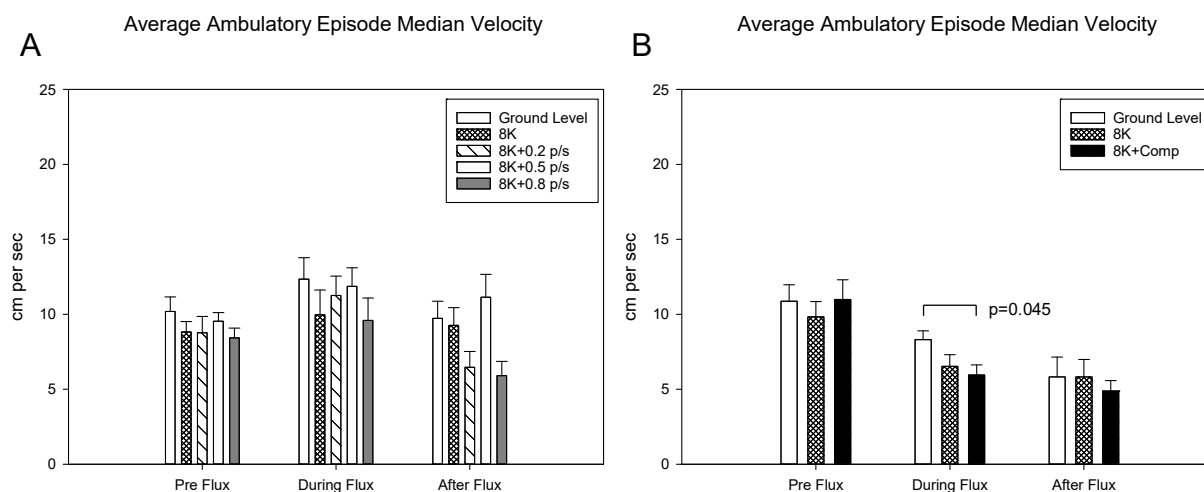


Figure 28. Ambulatory episode median velocity in open-field activity test.

Average ambulatory episode median velocity during Pre Flux, During Flux and After Flux segments of **(A)** 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles and comparable time periods for Ground Level and 8K profiles **(B)** 8K+Comp profiles and comparable time periods for Ground Level and 8K profiles

Experiment #2 Exposure and Environmental Data

The Experiment #2 altitude exposure and environmental data is listed in Table 13. The 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles were on average at the target peak rate of change and there was minimal peak-to-peak variability and run-to-run variability in fluctuations. Additionally, there was fluctuation-to-fluctuation and run-to-run consistency in regards to fluctuation duration and maximum/minimum altitudes. The mean $\pm$ the SD of the temperature and %RH during each run of each profile are given in Table 13. There was no difference in temperature or %RH between exposure groups. The averages of the six runs of each profile for each parameter listed in Table 13 were calculated in the same manner as the altitude exposure and environmental data for Phase 1. The data for individual runs for each profile is listed in Appendix C.

The variability in oxygen concentrations within the rotarod testing apparatus was similar to the open-field activity testing for the same reasons that likely resulted in the variability in that testing apparatus. The oxygen concentrations during rotarod testing were overall slightly lower than the open-field activity test, but still hyperoxic, because of the nitrogen that was pumped into the electromotor controls of the rotarod to prevent elevated oxygen

levels in that compartment that could have been a safety concern. Some unavoidable leakage of nitrogen from electromotor control compartment into the animal compartment lowered oxygen concentrations in the animal compartment of the rotarod compared to the open-field activity testing chamber.

Table 13. Average fluctuation, altitude, and environmental data for Phase 2 Experiment #2.

Exposure Parameter	Ground Level	8K	8K + 0.2 p/s	8K + 0.5 p/s	8K + 0.8 p/s	8K + Comp
Decompression Peak Rate of Change (p/s)			0.23 ± 0.00	0.51 ± 0.01	0.86 ± 0.01	
Decompression Average Rate of Change (p/s)			0.24 ± 0.10	0.37 ± 0.01	0.58 ± 0.01	
Recompression Peak Rate of Change (p/s)			0.26 ± 0.01	0.55 ± 0.01	0.92 ± 0.02	
Recompression Average Rate of Change (p/s)			0.22 ± 0.01	0.42 ± 0.01	0.67 ± 0.01	
Average Duration per Fluctuation (sec)			18.1 ± 0.3	7.6 ± 0.1	7.1 ± 0.2	
Maximum Altitude During Fluctuations (feet)			10140 ± 18	9925 ± 25	12006 ± 111	
Minimum Altitude During Fluctuations (feet)			5815 ± 33	6255 ± 52	6151 ± 78	
Steady Altitude (feet)	782 ± 19	8008 ± 20	7979 ± 18	7990 ± 4	7996 ± 11	
Temperature (°C)	23.2 ± 0.2	23.3 ± 0.3	23.1 ± 0.2	23.2 ± 0.2	23.2 ± 0.3	23.3 ± 0.3
Relative Humidity (%)	12.5 ± 1.5	11.2 ± 1.5	10.9 ± 1.7	11.1 ± 1.8	11.5 ± 2.0	10.7 ± 1.5
Oxygen (%)	51 ± 7	66 ± 10	55 ± 9	57 ± 8	55 ± 6	55 ± 7

The average parameters of all 13 fluctuations within the 8K+Comp profile are displayed in Table 14. Each column represents the mean ± standard deviation of the fluctuations for all six runs of the 8K+Comp profile. The duration, decompression/recompression rates, and peak altitude parameters are all defined the same as in Phase 1. All parameters were within target values and were consistent with runs of the 8K+Comp profile in the previous phases.

Table 14. Average fluctuation data for 8K+Comp profile during Phase 2 Experiment #2

Fluctuation Number	Duration (sec)	Decompression (p/s)		Recompression (p/s)		Peak Altitude (feet)
		Peak	Average	Peak	Average	
1	103 ± 10.5	0.10 ± 0.00	0.04 ± 0.00	0.05 ± 0.02	0.02 ± 0.01	8840 ± 26
2	44 ± 5.5	0.24 ± 0.01	0.12 ± 0.04	0.14 ± 0.01	0.06 ± 0.00	11026 ± 31
3	41 ± 1.3	0.32 ± 0.02	0.18 ± 0.01	0.22 ± 0.02	0.09 ± 0.01	13467 ± 31
4	10 ± 0.4	0.70 ± 0.02	0.51 ± 0.02	0.59 ± 0.03	0.45 ± 0.03	14701 ± 140
5	11 ± 0.5	0.77 ± 0.03	0.58 ± 0.01	0.35 ± 0.02	0.27 ± 0.02	13472 ± 37
6	8 ± 0.3	0.77 ± 0.10	0.49 ± 0.03	0.34 ± 0.02	0.19 ± 0.01	10440 ± 65
7	17 ± 0.9	0.75 ± 0.02	0.48 ± 0.03	0.22 ± 0.02	0.14 ± 0.01	11950 ± 49
8	19 ± 1.1	0.78 ± 0.04	0.48 ± 0.01	0.29 ± 0.03	0.18 ± 0.02	13740 ± 65
9	19 ± 6.7	0.40 ± 0.02	0.18 ± 0.09	0.12 ± 0.01	0.06 ± 0.01	9508 ± 66
10	18 ± 1.3	0.80 ± 0.02	0.51 ± 0.02	0.20 ± 0.02	0.12 ± 0.01	12175 ± 83
11	21 ± 1.2	0.76 ± 0.02	0.46 ± 0.01	0.26 ± 0.03	0.17 ± 0.02	14052 ± 77
12	18 ± 1.0	0.31 ± 0.02	0.21 ± 0.02	0.16 ± 0.02	0.09 ± 0.01	10053 ± 46
13	25 ± 1.8	0.92 ± 0.04	0.48 ± 0.08	0.95 ± 0.04	0.23 ± 0.02	15199 ± 152

Experiment #2 Animal Weights

Animal body weights that were measured immediately preceding the altitude chamber exposures for Experiment #2 are provided in Figure 29. There was no difference in body weight between exposure groups.

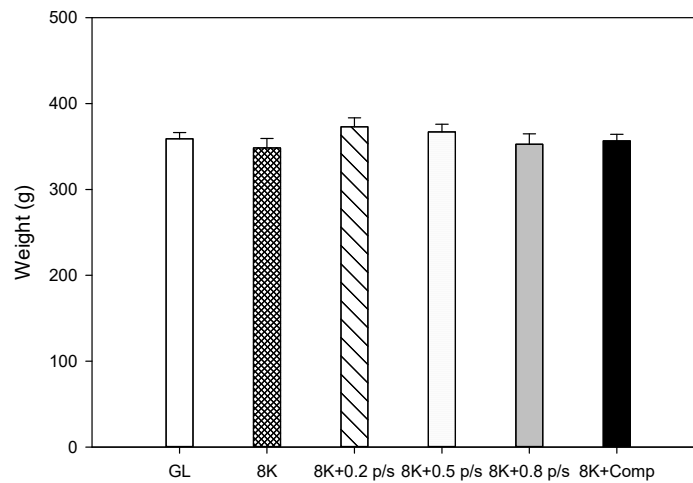


Figure 29. Average animal weights for each profile for Phase 2 Experiment #2.
Data presented as group mean +/- SEM.

Experiment #2 Rotarod and Accelerod Testing

Rats underwent rotarod testing from the start of the ascent to altitude until approximately one minute after the end of the last set of fluctuations. The behavioral test then switched to an accelerod profile, with the rotation rate continuously increasing up to 40 rpm during the final four minutes the rats were at altitude. Ground level and 8K rats experienced the same testing over the same time course. Kaplan-Meier survival curves of the results of the combined testing are shown in Figure 30. The results are broken into separate Kaplan-Meier survival curves for the rotarod and accelerod portions in Figure 31 and Figure 32, respectively. The curves represent the proportion of rats in each exposure group that remained on the rod (“survived”) at each second of the test until the test (Figure 30 and Figure 32) or segment (Figure 31) completion. Dots on each figure represent censored data from rats that either climbed down from the rod during the test, or rats that completed the test or segment without falling.

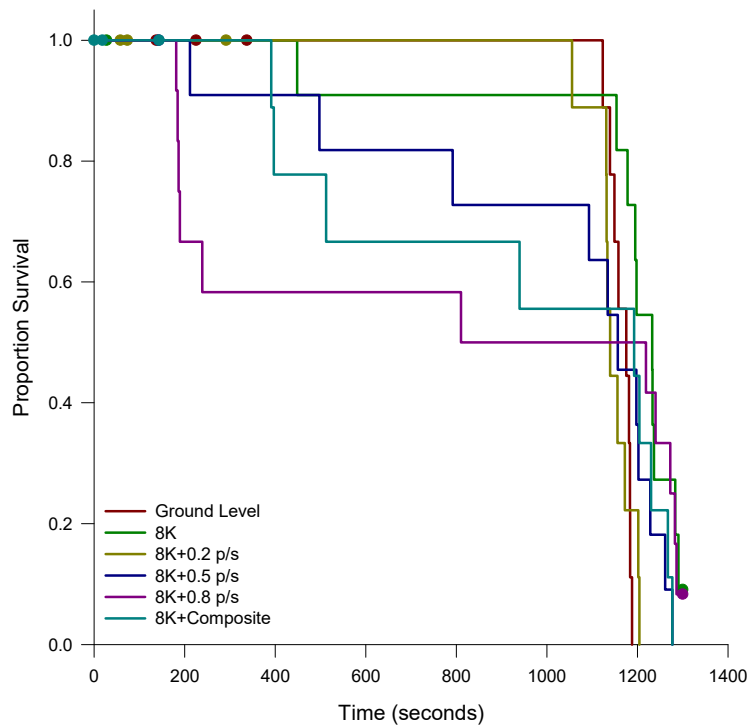


Figure 30. Rotarod and accelerod test survival curves

“Proportion survival” indicates the proportion of rats that remained walking on the rotating rod at each time point. Rats that left the rod remained safely in the bottom compartment of the apparatus until the end of the test. The rotarod portion of the test occurred between 0 and 1060 seconds, while the accelerod portion of the test took place between 1060 and 1300 seconds. Dots represent censored data.

As indicated by the curves in Figure 30 and Figure 31, there was an increased incidence of falling during the rotarod portion of the test with increased severity of fluctuations. There was no statistically significant difference between the curves when combining the rotarod and accelerod segments, but there was a significant difference between the curves ($p=0.018$) of just the rotarod segment. However, there were no significant differences between individual curves in multiple comparison testing.

Rotarod Falls During Altitude Profiles

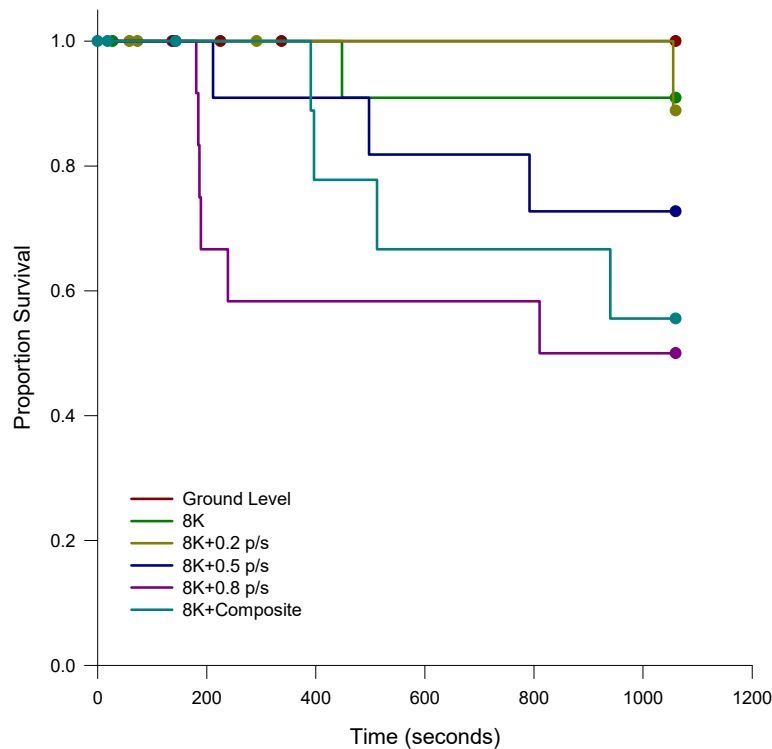


Figure 31. Rotarod test survival curves.

“Proportion survival” indicates the proportion of rats that remained walking on the rotating rod at each time point. Rats that left the rod remained safely in the bottom compartment of the apparatus until the end of the test. Dots represent censored data.

There was also a significant difference between the curves (Figure 32) during just the accelerod portion of the testing ($p < 0.001$). The trend of these curves was reversed from the rotarod test, with the ground level control profile having the highest rate of falling compared to the 8K, 8K+0.5 p/s, and 8K+Comp profiles ($p = 0.003$, 0.007 , and 0.008 , respectively).

Most of the observed falls during the 8K+0.8 p/s profile and 8K+Comp profiles occurred during fluctuations within those profiles. It was apparent from observation that the rats were impacted in some way by the fluctuations, because they would characteristically pause and stop walking on the rod, causing them to fall backwards and either off the rod or, in some cases, catch themselves before falling. This was not observed with the lower rate fluctuations of the 8K+0.5 p/s and 8K+0.2 p/s profiles. Additionally, during the 8K+0.8 p/s profile and 8K+Comp profile, rats would consistently rotate their heads from side-to-side in a shaking motion and scratch at their ears during and immediately after the recompression portions of the fluctuations. These behaviors did not appear to result in falls, with many rats still able to walk on the rod and display these behaviors at the same time.

Accelerod Falls During Altitude Exposures

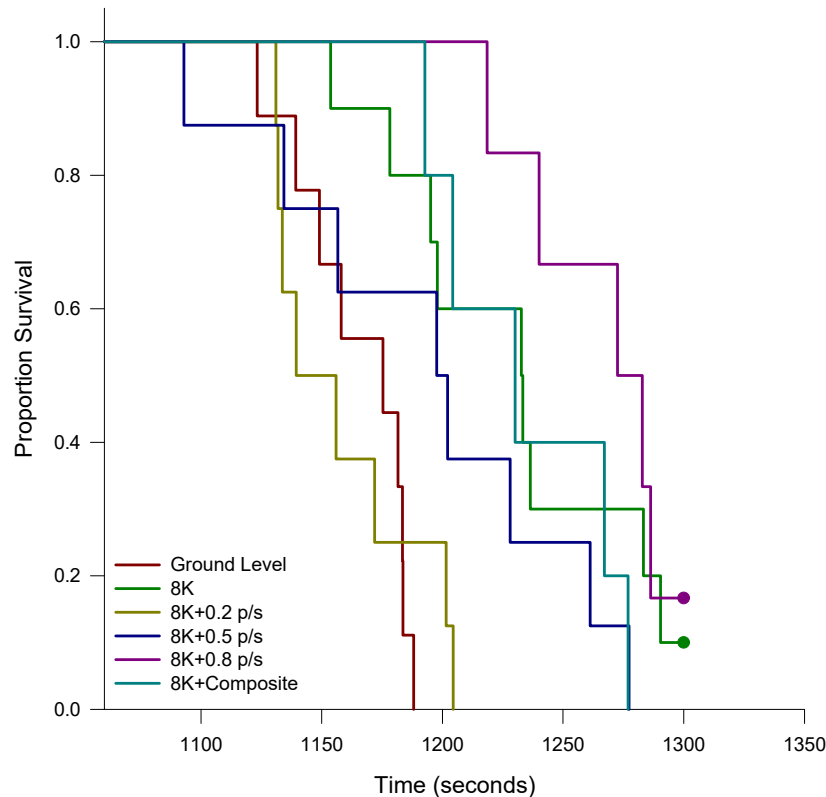


Figure 32. Accelerod test survival curves.

“Proportion survival” indicates the proportion of rats that remained walking on the rotating rod at each time point. Rats that left the rod remained safely in the bottom compartment of the apparatus until the end of the test. Dots represent censored data.

Experiment #2 Electrophysiology

Electrophysiology was performed on rat hippocampal brain slices within 20-30 minutes of the end of altitude exposures. The following results represent measures of synaptic neurotransmission, short-term synaptic plasticity, long-term synaptic plasticity, and spontaneous spiking activity that were obtained from these recordings. Throughput limitations only allowed for recordings from four of the exposure groups of Experiment #2: Ground Level control (Profile 1), 8K (Profile 2), 8K+0.8 p/s (Profile 5), and 8K+Comp (Profile 6). All electrophysiology groups were supplemented with 94% oxygen (70% oxygen for the Ground Level group).

Neurotransmission properties were analyzed by measuring field potentials from the CA1 region when the Schaffer collateral was stimulated by biphasic current pulses of varying intensities (10-120 μ A). Stimulation of the Schaffer collateral induced responses mainly from glutamatergic synapses as perfusion with glutamate receptor blockers inhibited all responses. The evoked field responses contained both population spikes as well as field

excitatory postsynaptic potentials (fEPSP). Amplitudes were calculated from population spikes and slopes were calculated from the slower fEPSP responses. We found that exposures to 8000 feet altitude with or without pressure fluctuations did not yield significant changes to the input-output function of neurons within the CA1 region of rat hippocampus compared to ground level controls (Figure 33).

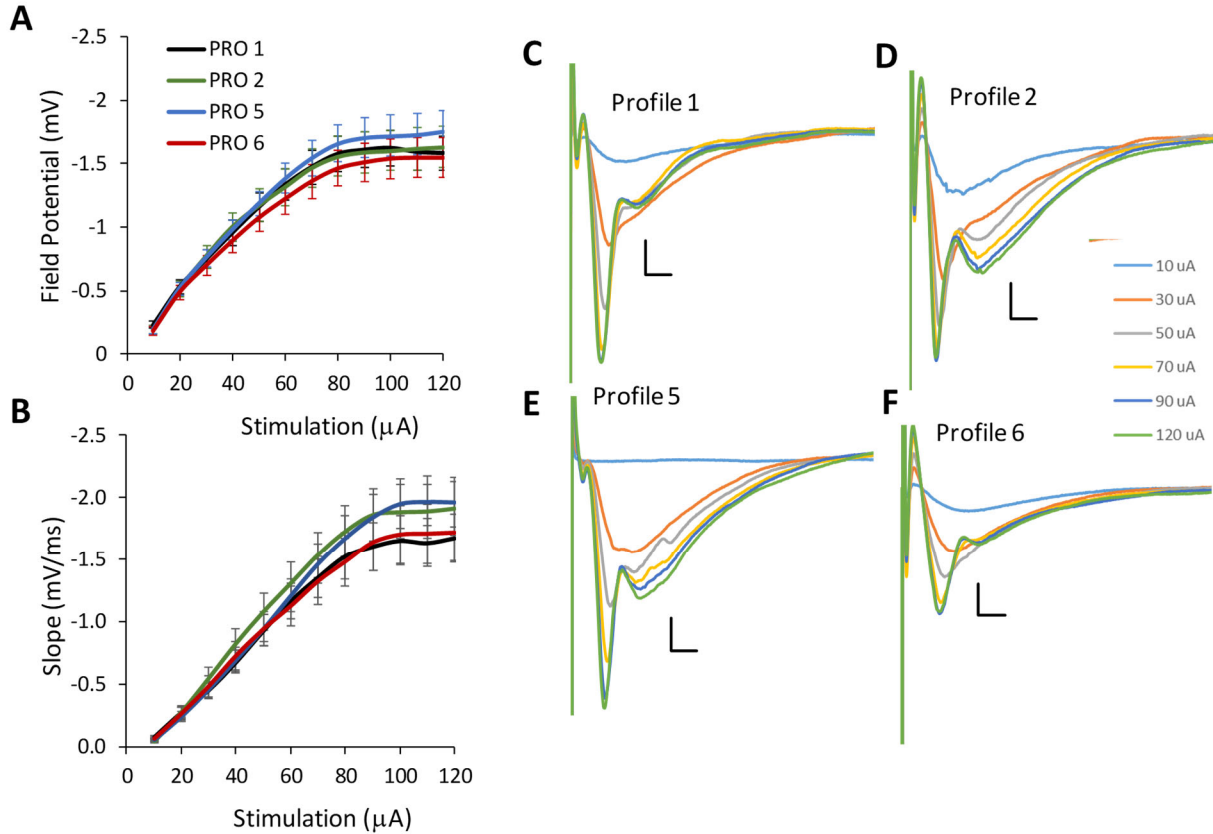


Figure 33. Effects of profile exposures on input-output function of hippocampal neurons.

(A-B) Averaged input-output responses were plotted as means $\pm$ SEM, using amplitudes (A) or slopes (B) of field responses. Two way ANOVA analysis did not yield significant difference among the experimental groups ($P > 0.05$). **(C-F)** Sample field responses (output) induced by varying input current stimulus (10, 30, 50, 90 and 120 μ A) from rats exposed to GL (Profile 1, black) **(C)**, 8K (Profile 2, green) **(D)**, 8K+0.8 p/s (Profile 5, blue) **(E)**, and 8K+Comp (Profile 6, red) **(F)**. Scale bar: 2.5 ms, 0.5 mV

Paired pulse facilitation (PPF) is the short-term potentiation of response evoked by two consecutive stimuli delivered within tens of milliseconds apart. The belief is that PPF result from the build-up of calcium ions resulting from the paired stimuli delivered very close together and is a form of short-term synaptic plasticity. Here, PPF was measured as the ratio of response amplitude or slope from the 2nd stimulus to the response amplitude or slope from the 1st stimulus. The interpulse interval was set to 40 ms. Exposure to 8000 feet alone induced a statistically significant decrease in the PPF of

hippocampal neurons (Figure 34) from ground level control. The reduction in PPF did not occur in pressure fluctuation groups.

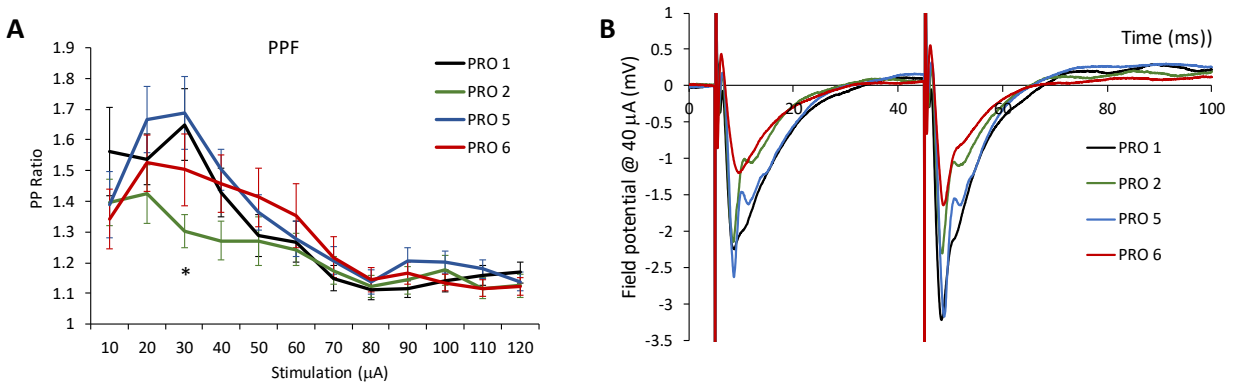


Figure 34. Effects of profile exposures on paired pulse facilitation (PPF) of hippocampal neurons.

(A) Average paired pulse ratio (PP Ratio) showing paired pulse facilitation (PPF) of hippocampal neurons from rats exposed to GL (Profile 1, black), 8K (Profile 2, green), 8K+0.8 p/s (Profile 5, blue) and 8K+Comp (Profile 6, red). Two way ANOVA revealed statistically significant difference among experimental groups ($P=0.007$). Post hoc multiple comparisons using Student-Newman-Keuls Method revealed significant differences between Pro 2 vs Pro 1 ($P=0.048$), Pro 2 vs Pro 5 ($P=0.003$) and Pro 2 vs Pro 6 ($P=0.037$). **(B)** Sample evoked response data showing PPF, induced by a pair of current stimulation at 40 μ A 40 ms apart.

The ability of exposure to 8000 ft and/or pressure fluctuations to induce lasting changes in LTP of hippocampal neurons was assessed. LTP was induced at the Schaffer collateral – CA1 hippocampal synapse using three sets of TBS. In the microelectrode array system, the evoked field response consisted of a mixture of population spikes and field excitatory post-synaptic potentials (EPSP). Analysis of population spikes consisted of calculating the amplitude of the downward field response, whereas the fEPSPs were calculated using the slope of the initial downward response. Here, slopes of fEPSP and amplitudes of population spikes were used to calculate the % LTP. A significant reduction was detected in the averaged % LTP when slopes of fEPSP were used for calculation from rats exposed to all three altitude profiles compared to ground level (slope data), regardless of the presence of fluctuations (Figure 35A,B). A trending reduction was also observed in the averaged % LTP when amplitudes were used for calculation (amplitude data) only from animals that were exposed to the 8K+Comp profile, but however, this reduction was not statistically significant (Figure 35C,D).

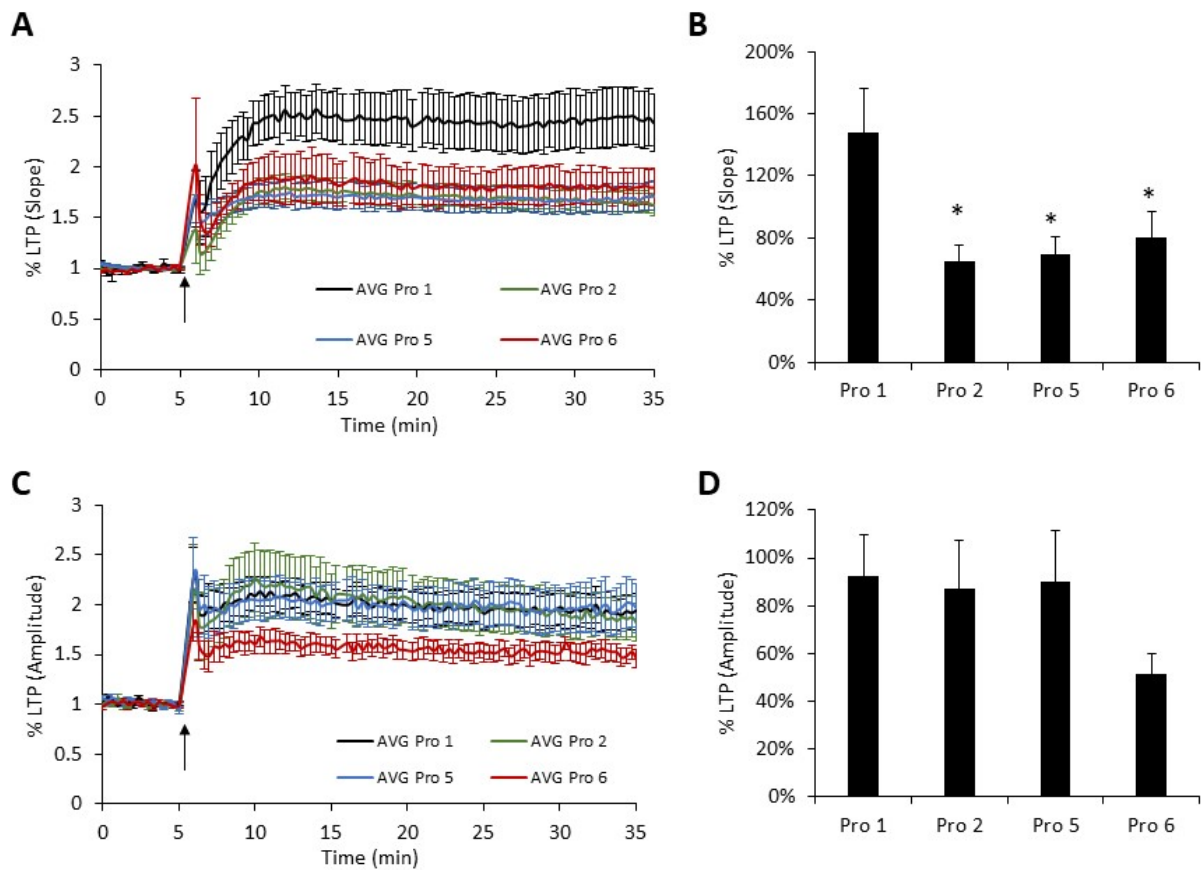


Figure 35. Effects of exposure profiles on long term potentiation (LTP). **(A-B)** Slopes of evoked responses (fEPSP) were used to calculate the % LTP. LTP in CA1 hippocampal neurons induced by 3 repeats of theta burst stimulation (TBS). **(A)** Normalized slope data from each exposure profile (GL - Pro 1/black, 8K - Pro 2/green, 8K+0.8 p/s - Pro 5/blue, 8K+Comp - Pro 6/red) are plotted as average normalized slope $\pm$ SEM (arrow indicates administration of TBS). **(B)** Averaged slope data are also plotted as a bar graph. One way ANOVA revealed significant differences among experimental groups ($P=0.011$, $n=8-12$ slices, 6 rats). Post-hoc multiple comparison testing via Student-Newman-Keuls method revealed significant differences between GL (Pro 1) vs 8K (Pro 2) ($*P=0.026$), GL (Pro 1) vs 8K+0.8 p/s (Pro 5, $*P=0.012$) and GL (Pro 1) vs 8K+Comp (Pro 6, $*P=0.025$). **(C-D)** Amplitudes of evoked responses (population spikes) were also used to calculate the % LTP. **(C)** Normalized amplitude data from each exposure profile (Pro 1 - black, Pro 2 - green, Pro 5 - blue, Pro 6 - red) are plotted and as average normalized amplitude $\pm$ SEM. **(D)** Averaged amplitude data are also plotted as a bar graph in. There was an observed reduction in % LTP in hippocampus from animals exposed to Profile 6, but the reduction was not statistically significant.

Spontaneous activity of hippocampal neurons was recorded over a five-min period. Spike frequency was calculated and compared across experimental groups. It was found that hippocampal neurons from rats exposed to Profile 6 (8K+Comp) profile had significantly decreased frequency of spontaneous spiking activity (Figure 36).

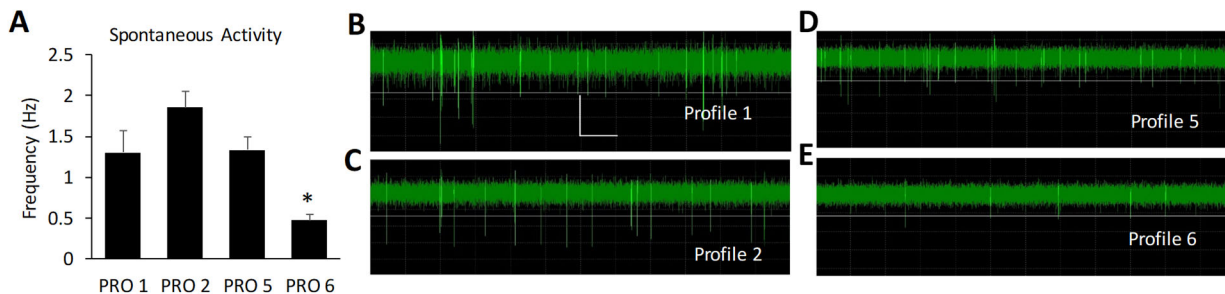


Figure 36. Effects of exposure profiles on spontaneous activity of hippocampal neurons.

(A) Frequency of spontaneous spiking activity of hippocampal neurons was significantly reduced in rats exposed to Profile 6 ($P=0.024$, Kruskal Wallis One Way ANOVA, $n=13$ -19 slices, 6 rats). Post hoc multiple comparison testing using the Dunn's Method revealed significant difference between frequency from rats exposed to Profile 2 and Profile 6 ($*P=0.029$). Average frequency calculation includes channels with spiking activity greater than or equal to 0.1 Hz. **(B-E)** Sample spiking data from hippocampal neurons of rats exposed to Profile 1 **(B)**, Profile 2 **(C)**, Profile 5 **(D)**, and Profile 6 **(E)**. Scale bar: 0.5 sec, 0.02 mV.

Phase 2 Discussion

The results from open field and rotarod neurobehavioral tests indicated that there is some apparent form of transient impairment in the rats associated with a pressure fluctuation rate of 0.8 p/s and possibly with the composite pressure fluctuation profile. There was reduced activity in the open-field test and increased falling in the rotarod test associated with the 8K+0.8 p/s profile and a non-significant trend of lower activity and increased falling in the rotarod test associated with the 8K+Comp profile. Many of the falls during the rotarod test coincided with fluctuation events of these profiles, strongly implicating the fluctuations as causing the performance decrements.

Although rats exposed to the most severe fluctuations performed worse on both a passive motor activity test and an active motor coordination test, the exact form of impairment that led to these decrements is unclear. Pain, CNS depression, and motorneuron impairment have all been associated with decreased performance on each of these tests (Deuis et al., 2017; Ramirez et al., 2013; Cervetto et al., 2013). While many rats were displaying behaviors of head shaking and ear scratching that are speculated to be consistent with increased pressure in their middle ear due to recompression from altitude, these behaviors were not necessarily associated with falling in the rotarod test. Similarly, it is difficult to tell if the freezing response seen during fluctuations immediately preceding rotarod falls is due to some form of neurological impairment, pain, or just a stress

response to the change in pressure. However, now that a rate has been identified that causes these performance decrements in neurobehavioral testing, additional experiments can be performed to tease out potential mechanisms and determine if the cause of the decrement is something that would translate to a concern for aviators experiencing these fluctuations.

Although there was an increased fall rate during the fluctuation exposures, rats that stayed on the rod past the end of the fluctuations appeared to perform better on the accelerod portion of the test compared to ground level controls, as did the rats that received an altitude only exposure. Although it is difficult to determine why these groups performed better than the ground level controls, this finding is consistent with the performance on some of the post-exposure behavioral tests in Phase 1. Additionally, it suggests that the motor coordination was not persistently impacted by the fluctuations, but only for the brief few seconds or minutes following the fluctuations.

There was a statistically significant reduction in spontaneous activity of hippocampal neurons from rats exposed to high altitude and the composite pressure fluctuation profile. There was also a reduced LTP in the hippocampus from rats exposed to high altitude with and without the pressure fluctuations, compared to LTP from rats that were exposed to ground level. Because all of the electrophysiology rats were supplemented with oxygen, the observed changes are not due to hypoxia. The preliminary data thus far indicate that the change in pressure to simulate 8000 feet altitude was sufficient to reduce LTP, but the decreased frequency of spontaneous activity was induced only when rats were exposed to high altitude and the composite pressure fluctuation profile. Therefore, although there were some differences observed in the electrophysiology recordings of rat hippocampal brain slices following altitude exposures, further experimentation will be necessary to fully elucidate the meaning of these differences. Additional PPF experiments are needed to determine whether the detected reduction in PPF resulting from 8000 ft will eventually reverse. Furthermore, investigating delayed effects in each of these electrophysiology measures resulting from pressure fluctuation exposures will help relate functional neurological changes to the delayed histopathological effects observed during phase 3. For example, LTP plays an essential role in learning and memory and disruptions of hippocampal LTP are highly correlated with cognitive impairment (see review by Lynch, 2004). Reduction in hippocampal LTP has been reported in rats suffering from mild traumatic brain injury (Sick et al., 1998; Ogier et al., 2017).

Phase 3

Phase 3 Methods

Animals and Animal Husbandry

A total of 72 male Sprague Dawley (CD) rats (*Rattus norvegicus*), 7-8 weeks in age, were used for each of the two experiments performed for Phase 3. Rats were acclimated to the USAF 711 HPW RHDV vivarium for approximately seven days following arrival at the facility. Food and water were provided ad libitum while rats were housed in the vivarium prior to exposure. Rats were weighed at the end of acclimation and again on the day of exposure. Following acclimation, rats were randomized into exposure groups with each exposure group having an approximately equivalent average weight. On the day of exposure, the rats were transported to the testing room from the vivarium. Rats were returned to home cages in the vivarium until the time of necropsy at either 3-4 hours post-exposure for Experiment #1 or three days post-exposure for Experiment #2.

All procedures were performed in accordance with animal protocols FWA-2018-0177-A and F-WA-2018-0180-A approved by the Wright-Patterson Air Force Base Institutional Animal Care and Use Committee.

Study Design

Phase 3 was broken into two experiments (Table 15). The first experiment was designed around testing fluctuation rates that were comparable to what had been reported from Slam Stick (enDAQ) monitoring of Naval F/A-18 aircraft. The second experiment was designed to test the worst-case scenario fluctuations to try and find a severity at which clear physiological effects could be observed in order to better study the mechanism of those effects. Rats were exposed to one of the eight profiles listed in Table 15 across the two experiments. Graphical depictions each fluctuation profiles are given in Appendix C.

Table 15. Exposure groups for Phase 3.

n=number of subjects. Phase 3 was divided into Experiment #1 and Experiment #2.

Experiment	Profile	n
1	Ground Level	8
1	8K	8
1	8K+1.2 p/s	8
1	8K+Comp	8
2	8K	8
2	14K	8
2	8K+1.5 p/s	8
2	14K+1.5 p/s	8

With the exception of the GL profile, the profiles had a target ascent to altitude over approximately 90 seconds to reach 8K feet, or approximately 160 seconds to reach 14K

feet. The descent back to ground level occurred at similar rates. The sequence of events of the 8K+1.2 p/s, 8K+1.5 p/s, and 14K+1.5 p/s pressure fluctuation profiles were the same as the Phase 1 exposures, with ten fluctuations occurring in two sets of five with approximately a minute in between each fluctuation in a set. The sets were separated by five minutes of steady maintenance of altitude at either 8K feet or 14K feet. The 8K+Comp profile was the same profile used in the Phase 1 exposures.

Exposure System

The exposure system was the same used in the Phase 1 and 2 exposures. See “Exposure System” in Phase 1.

Breathing Gases

The humidified breathing airflows for each of the breathing air lines were delivered by an approximately 3 ft. length of ¼” PTFE tubing (Cole-Parmer, Vernon Hills, IL) from the main breathing air delivery line followed by a 6” length of 1/8” stainless steel tubing. This orifice was used to buffer the upstream equipment from the varying chamber pressures and ensure steady flow delivery to the animals. All animals were delivered 500 mL/min 94% oxygen/6% argon through a rodent gas anesthesia facemask (Xenotec, Inc., Henderson, NV) with an outlet that was open to the chamber. The facemask was held in place on the rodent using an adjustable arm mounted to the ultrasound stand (Figure 37).

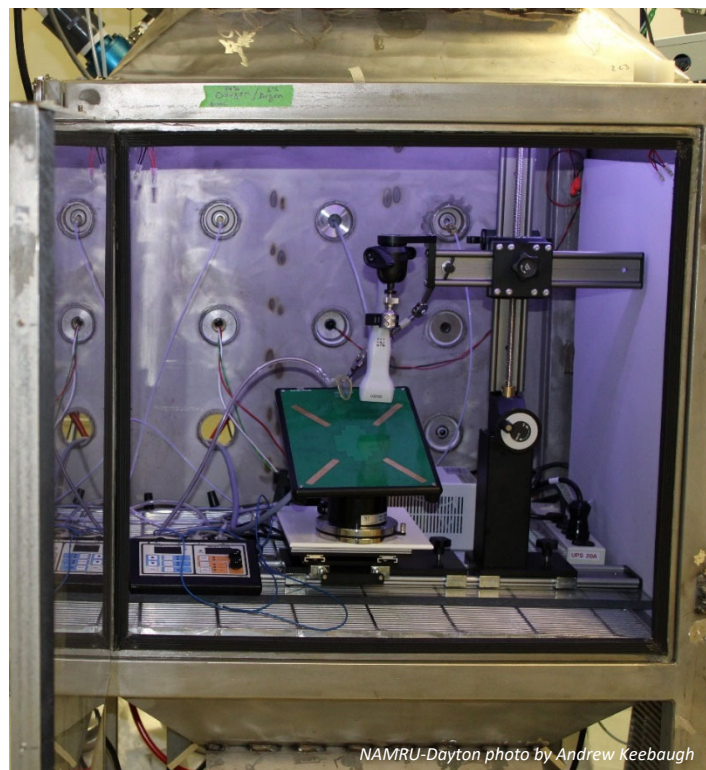


Figure 37. Ultrasound imaging station inside of exposure chamber.

Ultrasound Monitoring

The day prior to exposure, rats were placed under isoflurane anesthesia to remove hair from the chest with depilatory cream to facilitate ultrasound imaging on the day of exposure. On the day of exposure, anesthesia was induced with an intraperitoneal injection of a ketamine (80-100 mg/kg) and xylazine (8-10 mg/kg) cocktail. A heated platform (Vevo Imaging Station, FUJIFILM Visualsonics, Toronto, CA) was used to maintain body temperature and monitor electrocardiogram and respiratory rate through electrodes over which the front and hind feet of the rat were placed (Figure 37). The animal was placed in dorsal recumbency on the platform, with extremities outstretched and secured to electrodes on the platform with adhesive tape. An eye lubricant was administered shortly after induction of anesthesia to prevent drying of eyes during the procedure. Following exposure, rats were removed from the chamber and moved to recovery cages where they were monitored until they were ambulatory and thermally stable.

A Vevo 3100 Ultrasound preclinical imaging system (FUJIFILM Visualsonics) was used for the imaging procedures. Immediately prior to the exposures, ultrasound gel was applied to the shaved area of the chest, and an ultrasound transducer (MX201 Linear Array Transducer, FUJIFILM Visualsonics) was positioned on the chest in the appropriate orientation to obtain a view of the pulmonary artery. The transducer was secured in place with a mounting apparatus attached to the imaging platform so that the pulmonary artery view could be obtained continuously in the chamber for the 25 minutes of exposure without physical manipulation that would require opening the altitude chamber. A four-chamber view would have been preferred to visualize VGE in both the venous return and arterial output, but pre-study testing found that this view was not able to be stably maintained over the 25 minutes of exposure because of the positioning of the animal necessary to obtain this view. Appropriate positioning of the view in the pulmonary artery was confirmed with color Doppler imaging that indicated a strong presence of blood flow in the vessel. Microbubbles/VGE appeared as bright spots within the pulmonary artery in both B-mode and non-linear contrast imaging modes.

Continuous video of B-mode and non-linear contrast imaging of the pulmonary artery was captured in three-minute segments during the exposures. After each segment, the transducer position was quickly checked on color Doppler mode, and then another three-minute segment was started in B-mode and non-linear contrast mode. Gaps between three-minute video segments were typically only about 15-20 seconds in length, so all but approximately two minutes of the 25 minute exposure were captured. Data collected during the exposures was stored for later review. The presence of VGE was analyzed both visually by observing the videos and by using the quantification of contrast intensity in the non-linear contrast mode to detect spikes in intensity that could be indicative of VGE presence.

Microbubble Imaging Validation

Prior to the study, a set of six rats underwent a terminal procedure to validate the ability of the ultrasound imaging methods to detect microbubbles that are of a comparable size and quantity to VGE that form endogenously. To validate the ability of the imaging technique to visualize intracardiac/intravascular bubbles, the anesthetized rats were delivered an intravenous infusion of air microbubbles in a saline solution that had been produced by a custom-built microbubble generator.

A custom microbubble generator was constructed using similar materials and methods found in published literature (Gerriets et al., 2010). Saline carrier fluid was pumped (Thermo Orion Syringe Pump, M361, Hampton, NH) into the system and flowed through a straight barbed coupler that was inserted into the middle branch of a customized barbed tee where it combined with the compressed air inlet (Figure 38). A three-way stopcock was attached to this inlet flow for pressure relief, if necessary. Bubbles were produced by a custom device consisting of a glass capillary tube (TSI Inc., ElectroSpray Aerosol Generator Capillary Tube, 25 μm diameter, Shoreview, MN) sealed inside the throat of a Pressfit borosilicate glass connector for 0.32mm to 0.53mm ID gas chromatography column connector (Figure 38, Figure 39). The capillary tube was fed through a modified 26-gauge, $\frac{1}{2}$ " syringe needle and fed through the horizontal branch of the tee to allow the introduction of gas to the carrier fluid at the throat of the Pressfit connector and ultimately create the bubbles. Bubble generation rate and size could be manipulated by changing the carrier fluid rate and compressed air pressure. From the point of generation, the bubbles and PBS carrier solution flowed into a winged infusion needle (SAI Infusion Technologies, BF-27-13, 27 Gauge butterfly, Tubing ID 0.013", Lake Villa, IL) that was used to introduce bubbles to the vasculature of the subject.

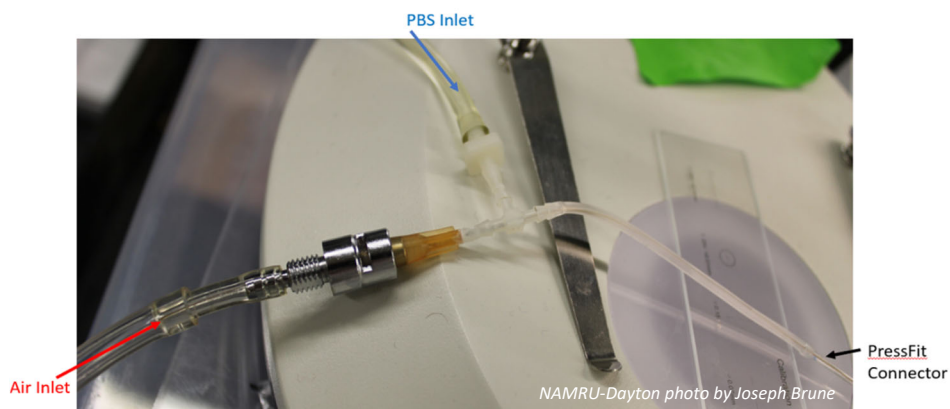


Figure 38. Microbubble generator.

An optical microscope (Ken-A-Vision Vision Scope T, Model T-22061, Kansas City, MO) was used to visualize the bubble generation and measure the approximate size of generated bubbles. A calibration slide stage micrometer was utilized with the tip of the inner glass capillary visible in the field of view. The stage micrometer had one division equal to 0.1mm (100 μm), and the cross section of the bubble was clearly able to be seen

so that the diameter could be approximated optically. Bubbles generated ranged from 150-300 μm in diameter based on optical measurements with the stage micrometer (Figure 39).

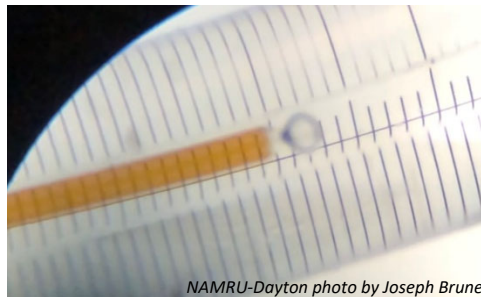


Figure 39. Inner capillary of microbubble generator with an $\sim 200\mu\text{m}$ bubble.

Rats were anesthetized with an injection of a cocktail of 80-100 mg/kg ketamine and 8-10 mg/kg xylazine and were setup on the ultrasound imaging platform as described in “Ultrasound Imaging”. The abdominal cavity was surgically opened and inferior vena cava exposed to intravenously place the butterfly needle connected to the microbubble generator. Rats were infused through the vena cava with microbubbles of 150-300 micrometer diameter at a rate of ~ 0.7 mL/min for up to five minutes with 0.9% saline as a carrier fluid. The pulmonary artery was imaged ultrasonically with dual B-mode/non-linear contrast mode and video clips saved for later analysis. The rat was euthanized by exsanguination after completion of imaging while still unresponsive under anesthesia.

Experiment #2 Immunohistochemistry

Three days following exposure, rats were anesthetized with pentobarbital (150 mg/kg, i.p) and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer. The brains were removed and post-fixed in 4% paraformaldehyde overnight at 4°C and cryoprotected in 15% sucrose in 0.1M phosphate buffer for 24 hours. Approximately 75 μm coronal sections were obtained of the hippocampus on a cryostat and immunostained free floating. To label astrocytes, GFAP immunohistochemistry was performed using mouse anti-GFAP cocktail (BD Pharmingen 556330) at 1:500 in PBS with 0.1% triton X pH7.3. Immunoreactivity was detected with donkey anti-mouse antibody conjugated to Cy3 (1:100 Jackson Immuno). Nissl histology was performed using fluorescein-conjugated Nissl (1:100, Molecular Probes). Tissue was mounted on the slides with Vectashield Hardmount containing DAPI to label nuclei (Vector Laboratories). Immunolabeled hippocampal slices were imaged on SP8 Lightening Confocal Microscope (Leica) with a 20x objective at 1.0 μm z-steps and 1.5 digital zoom. Imaging settings were fixed across all images collected. Microscopic images were prepared by adjusting contrast and brightness and always preserved the original content of the images.

Data analysis

All data for quantitative, continuous variables were compared for the exposure and control groups using one-way analysis of variance (ANOVA). If the ANOVA indicated statistical significance among exposure groups, the Tukey's post-hoc test was used to compare all exposure groups. When assumptions for parametric ANOVA were not met, Kruskal-Wallis nonparametric procedures was used. Statistical analyses were performed using SigmaPlot 13.0 (Systat Software, Inc). The probability value of less than 0.05 was used as the critical level of significance for each statistical test.

Quantification of immunohistochemistry was confined to the neuronal boundary of the hippocampus encompassing CA1, CA2 and CA3 regions at the level of the dentate gyrus. Three independent confocal slices were analyzed per animal. Quantification was performed in Image Pro Plus Software (Media Cybernetics, Bethesda MD, USA) in original and unprocessed micrographs. All statistical tests were performed in SigmaPlot (version 9.0, Systat Software, SPSS Inc, Chicago, IL, USA). All data passed normality and variance tests prior to running ANOVA and t-tests for evaluating statistical significance. Statistical significance was set at $p=0.05$ and data is presented as mean per animal with error bars representing the standard error of the mean (SM). Standard deviations (SD) are reported in the results text. Graphs were composed in SigmaPlot (version 9.0, Systat Software, SPSS Inc, Chicago, IL, USA).

Phase 3 Results

Experiment #1 Exposure and Environmental Data

The desired altitudes and peak rates of change of the fluctuations were approximately on target for the study. The Experiment #1 altitude exposure and environmental data is listed in Table 16. The 8K+1.2 p/s profile was within 0.1 p/s of the target peak rate of change and there was minimal peak-to-peak variability and run-to-run variability in fluctuations. Additionally, there was consistency in fluctuations and runs with regard to fluctuation duration and maximum/minimum altitudes. The mean $\pm$ the SD of the temperature and %RH during each run of each profile are given in Table 16. There was no difference in temperature or %RH between exposure groups. The averages of the eight runs of each profile for each parameter listed in Table 16 were calculated in the same manner as the altitude exposure and environmental data for Phases 1 and 2. The data for individual runs for each profile are listed in Appendix C.

Table 16. Average fluctuation, altitude, and environmental data for Phase 3 Experiment #1.

Exposure Parameter	Ground Level	8K	8K + 1.2 p/s	8K + Comp
Decompression Peak Rate of Change (p/s)			1.1 ± 0.0	
Decompression Average Rate of Change (p/s)			0.7 ± 0.0	
Recompression Peak Rate of Change (p/s)			1.1 ± 0.0	
Recompression Average Rate of Change (p/s)			0.8 ± 0.0	
Average Duration per Fluctuation (sec)			6.8 ± 0.0	
Maximum Altitude During Fluctuations (feet)			12943 ± 115	
Minimum Altitude During Fluctuations (feet)			6021 ± 39	
Steady Altitude (feet)	829 ± 0	7995 ± 1	7957 ± 6	
Temperature (°C)	21.5 ± 0.4	21.4 ± 0.2	21.4 ± 0.2	21.4 ± 0.3
Relative Humidity (%)	32.4 ± 3.7	32.2 ± 2.7	30.1 ± 4.1	31.0 ± 3.2

The average parameters of all 13 fluctuations within the 8K+Comp profile are displayed in Table 17. Each column represents the mean ± standard deviation of the fluctuations for all six runs of the 8K+Comp profile. The duration, decompression/recompression rates, and peak altitude parameters are all defined the same as in Phase 1 and 2. All parameters were within target values and were consistent with runs of the 8K+Comp profile in the previous phases.

Table 17. Average fluctuation durations, rates, and peak amplitudes for the 8K+Comp profile for Phase 3 Experiment #1

Fluctuation Number	Duration (sec)	Decompression (p/s)		Recompression (p/s)		Peak Altitude (feet)
		Peak	Average	Peak	Average	
1	99 ± 1.9	0.11 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.01	8887 ± 13
2	38 ± 0.3	0.23 ± 0.00	0.15 ± 0.00	0.13 ± 0.00	0.05 ± 0.00	11082 ± 33
3	39 ± 8.8	0.31 ± 0.01	0.18 ± 0.02	0.20 ± 0.06	0.08 ± 0.02	13499 ± 24
4	10 ± 0.3	0.71 ± 0.02	0.53 ± 0.02	0.55 ± 0.02	0.42 ± 0.01	14748 ± 116
5	12 ± 0.4	0.81 ± 0.01	0.56 ± 0.02	0.35 ± 0.03	0.27 ± 0.01	13606 ± 93
6	8 ± 0.7	0.75 ± 0.11	0.48 ± 0.04	0.32 ± 0.02	0.19 ± 0.02	10498 ± 27
7	18 ± 0.7	0.75 ± 0.02	0.50 ± 0.02	0.21 ± 0.01	0.13 ± 0.01	12085 ± 115
8	19 ± 0.9	0.80 ± 0.03	0.49 ± 0.02	0.29 ± 0.02	0.18 ± 0.01	13694 ± 161
9	23 ± 6.6	0.39 ± 0.02	0.12 ± 0.08	0.11 ± 0.01	0.07 ± 0.00	9564 ± 44
10	19 ± 1.4	0.79 ± 0.02	0.51 ± 0.02	0.21 ± 0.02	0.12 ± 0.01	12142 ± 65
11	21 ± 1.0	0.74 ± 0.03	0.47 ± 0.02	0.25 ± 0.01	0.16 ± 0.01	14131 ± 90
12	18 ± 1.4	0.31 ± 0.00	0.20 ± 0.00	0.16 ± 0.01	0.09 ± 0.01	10070 ± 26
13	23 ± 1.3	0.88 ± 0.03	0.51 ± 0.01	0.98 ± 0.05	0.24 ± 0.02	15239 ± 114

Experiment #2 Exposure and Environmental Data

The Experiment #2 altitude exposure and environmental data are listed in Table 18. The 8K+1.5 p/s profile hit the target peak rate of change for decompression and recompression and there was minimal peak-to-peak variability and run-to-run variability in fluctuations. The 14K+1.5 p/s profile was, on average, below the target of 1.5 p/s by 0.2 p/s for decompression and 0.1 p/s for recompression. The pressure at the target maximum altitude for these fluctuations of 22K feet was low enough that the exposure system was approaching its limit for pressure rate change and therefore had trouble reaching 1.5 p/s consistently. There was consistency in fluctuations and runs with regard to fluctuation duration and maximum/minimum altitudes for both 8K+1.5 p/s and 14K+1.5

p/s fluctuation profiles. The mean $\pm$ the SD of the temperature and %RH during each run of each profile are given in Table 18. There was no difference in temperature or %RH between exposure groups. The averages of the eight runs of each profile for each parameter listed in Table 18 were calculated in the same manner as the altitude exposure and environmental data for Phases 1 and 2. The data for individual runs for each profile are listed in Appendix C.

Table 18. Average fluctuation, altitude, and environmental data for Phase 3 Experiment #2.

Exposure Parameter	8K	8K + 1.5 p/s	14K	14K + 1.5 p/s
Decompression Peak Rate of Change (p/s)		1.5 $\pm$ 0.0		1.3 $\pm$ 0.1
Decompression Average Rate of Change (p/s)		1.0 $\pm$ 0.0		0.8 $\pm$ 0.0
Recompression Peak Rate of Change (p/s)		1.5 $\pm$ 0.1		1.4 $\pm$ 0.1
Recompression Average Rate of Change (p/s)		1.1 $\pm$ 0.0		1.1 $\pm$ 0.1
Average Duration per Fluctuation (sec)		8.5 $\pm$ 0.1		8.0 $\pm$ 0.5
Maximum Altitude During Fluctuations (feet)		15797 $\pm$ 167		21926 $\pm$ 169
Minimum Altitude During Fluctuations (feet)		4185 $\pm$ 87		10225 $\pm$ 122
Steady Altitude (feet)	8001 $\pm$ 10	7964 $\pm$ 5	14036 $\pm$ 3	14164 $\pm$ 34
Temperature ($^{\circ}$ C)	22.7 $\pm$ 0.3	23.0 $\pm$ 0.1	22.8 $\pm$ 0.2	22.9 $\pm$ 0.1
Relative Humidity (%)	15.7 $\pm$ 3.8	13.5 $\pm$ 4.3	15.3 $\pm$ 4.4	12.0 $\pm$ 3.5

Experiment #1 Animal Weights

Animal body weights that were measured immediately preceding the altitude chamber exposures for Experiment #1 are provided in Figure 40. There were no difference in body weights between exposure groups.

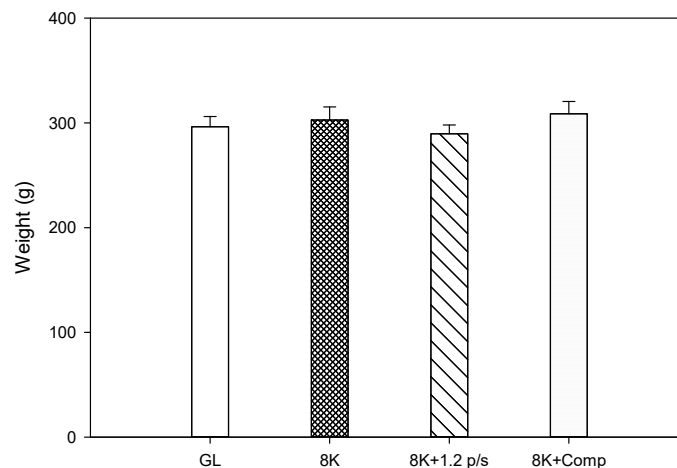


Figure 40. Average animal weights for each profile for Phase 3 Experiment #1.
Data presented as group mean $\pm$ SEM.

Experiment #2 Animal Weights

Animal body weights that were measured immediately preceding the altitude chamber exposures and three days after the altitude exposures for Experiment #2 are provided in Figure 41A. There were no difference in body weights between exposure groups at either

time point. Additionally, there was no difference in weight gain between exposure groups from prior to the altitude chamber exposure to three days post-exposure (Figure 41B).

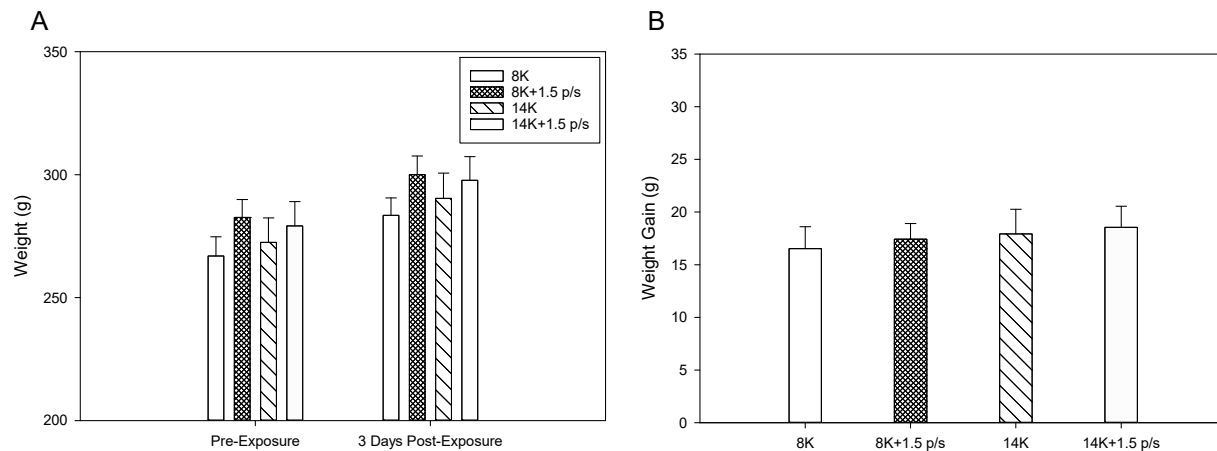


Figure 41. Average animal weights for each profile for Phase 3 Experiment #2.

Data presented as group mean $\pm$ SEM for **(A)** weights taken immediately prior to exposures and three days following exposure and **(B)** weight gain between the pre-exposure and three day post-exposure time points

Vascular Gas Emboli Detection

Microbubbles were observed in the pulmonary artery following infusion from the microbubble generator into the inferior vena cava. Microbubbles that were measured to be in the 150-300 micrometer diameter size range were clearly visible via both B-mode and non-linear contrast ultrasound imaging modes across three trial runs with separate rats. Microbubbles were not observed in three other rats because tests of other parameters for optimization of technique (i.e. transducer frequency range, location of venous microbubble infusion) were not successful. A representative example of bubble presence is displayed in Figure 42A for B-mode and Figure 42B for non-linear contrast mode. The microbubbles could clearly be seen transiting the pulmonary artery with the blood flow when viewing multiple successive frames. The successful test runs were used to define the imaging settings that were used for the altitude chamber experiments.

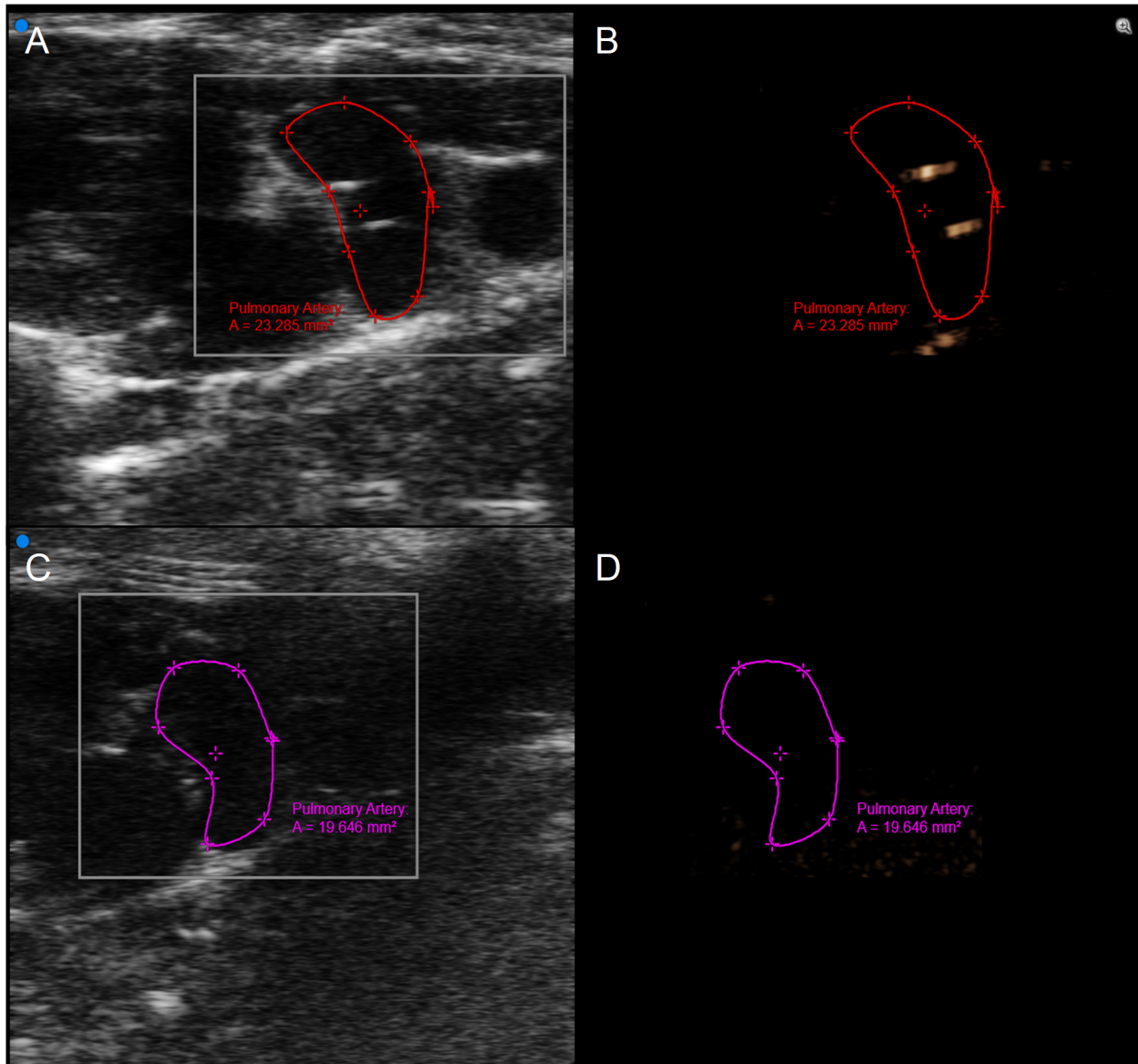


Figure 42. Ultrasound imaging of the pulmonary artery of rats during microbubble infusion and fluctuation exposures

(A) B-mode and **(B)** non-linear contrast ultrasound images of the pulmonary artery of a rat with $\sim 200\ \mu\text{m}$ microbubbles infused through the inferior vena cava (IVC) from a bubble generator. Representative **(C)** B-mode and **(D)** non-linear contrast ultrasound images of the pulmonary artery of a rat during a pressure fluctuation profile

No vascular gas emboli were observed during any exposures for Experiment #1 or Experiment #2. Visual review of the video files from both modes from both experiments did not find any evidence of intense bright spots that resembled the microbubbles that were infused during the validation experiments with the microbubble generator. A representative image of the absence of vascular gas emboli in the pulmonary artery is given in Figure 42C for B-mode and Figure 42D for non-linear contrast mode.

The non-linear contrast video files from Experiment #1 were quantitated to help identify any peaks in intensity that could be representative of the presence of vascular gas emboli. Quantitation of the non-linear contrast files from the validation experiment produced clear peaks in contrast intensity following microbubble infusion at 100 seconds (Figure 43A) that corresponded to the visual presence of microbubbles. Prior to 100 seconds, there was only one peak in contrast intensity that appeared to be associated with the time that the needle was intravenously placed into the inferior vena cava at approximately one minute prior to the start of infusion. These peaks in intensity were absent from most of the non-linear contrast files recorded during simulated altitude exposures with or without pressure fluctuations. As shown in Figure 43B, which represents the quantitated contrast intensity of one of the approximately 8-9 three minute video clips that were captured during the altitude exposures for each rat, peaks in intensity were almost entirely absent. A few lower amplitude peaks that were found in some files were visually reviewed in the non-linear contrast video files and determined to be associated with stationary artifacts at the vessel walls and were not representative of VGE.

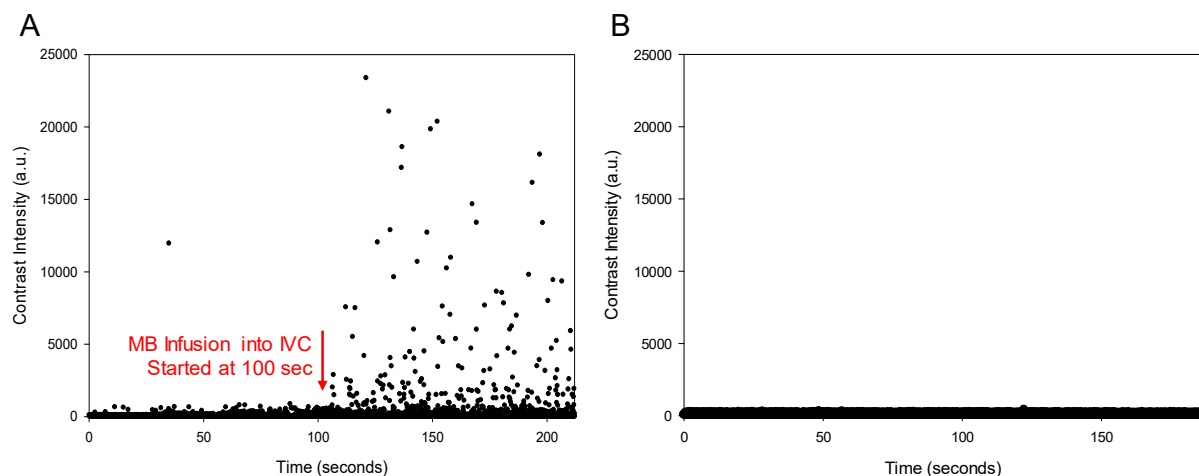


Figure 43. Quantitated contrast intensity from pulmonary artery of rats during microbubble infusion and fluctuation exposures

Quantitated contrast intensity in arbitrary units representing **(A)** microbubble presence in the rat pulmonary artery following infusion through the inferior vena cava **(B)** absence of vascular gas emboli in the rat pulmonary artery during pressure fluctuation exposures

Experiment #2 Immunohistochemistry

To determine the presence of reactive astrocytes, brains were harvested three days following acute exposures and processed for immunohistochemistry for glial fibrillary acidic protein (GFAP) (Figure 44, shown in red). Variability was observed in the level of reactive astrocytes from rat to rat (Figure 44). However, within each rat, the degree of reactive astrocytes were consistent across multiple histological slices of the hippocampus. Some rats expressed low to medium astrocyte activation (Figure 44A & C) while others expressed higher level of activation (Figure 44B).

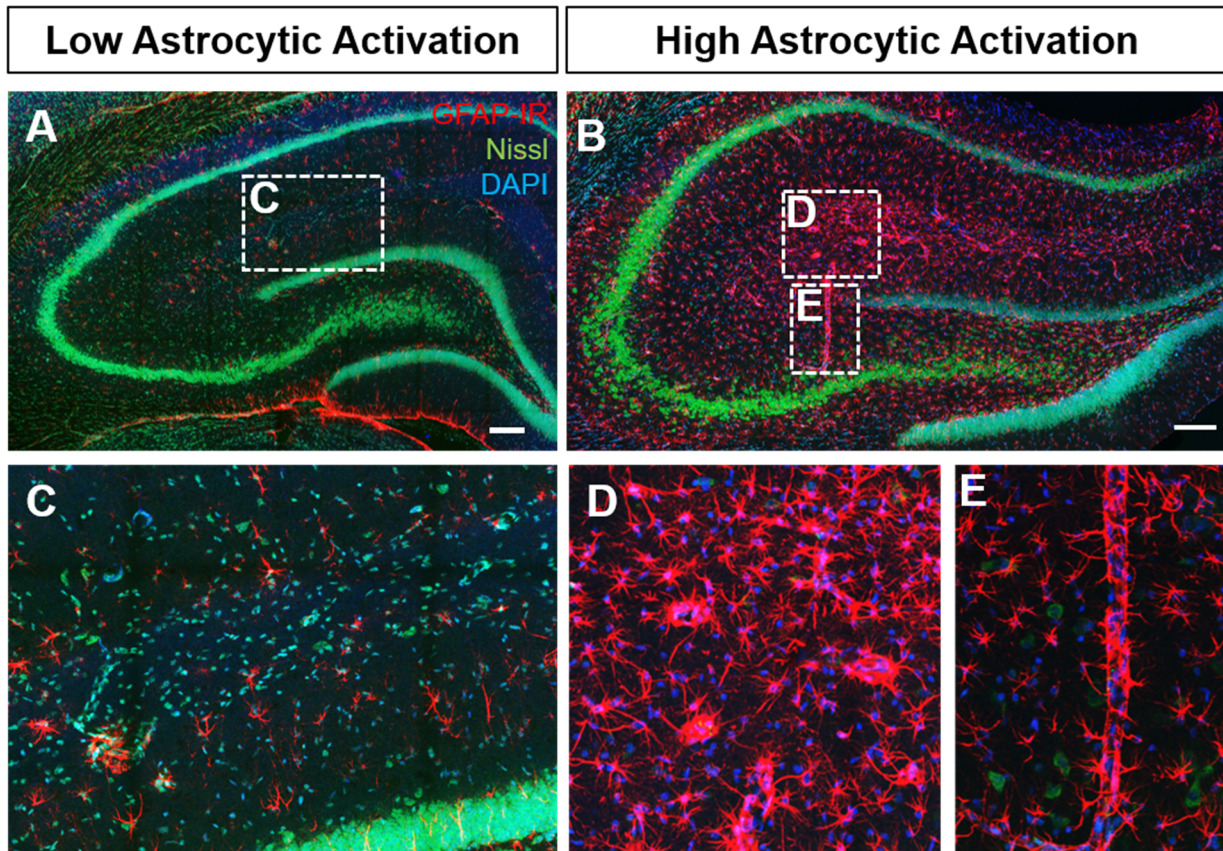


Figure 44. Astrocytic activation three days after altitude exposures.
 Variability in astrocyte activation observed. Astrocytes were revealed using GFAP-IR (Red). Nissl (Green) was used as a general neuronal stain and DAPI (Blue) was used to label nuclei. All micrographs were from stitched 20x confocal stacks (1.0 μ m Z-steps x 11) **(A)** Micrograph representing low to medium astrocytic activation following pressure fluctuations. **(B)** Micrograph representing moderate to high astrocytic activation. **(C)** Micrograph of boxed region in panel A displaying astrocytes with low GFAP expression and long thin processes. **(D)** Micrograph of boxed region in panel B. Morphological changes associated with astrocyte activation including increase in GFAP expression, increase in astrocyte number, and altered morphology. **(E)** Micrograph of boxed region in panel B. Reactive astrocytes are lining a large diameter vessel passing through the hippocampus. Scale bars = 150 μ m.

To identify reactive astrocytes, three characteristics are required. First, an increased expression of GFAP (Figure 44B, D & E). Second, increase in the number of astrocytes within a defined area, the hippocampus (Figure 44B). Finally, modifications in the morphology including hypertrophy with enlargement and outgrowth of the processes (Figure 44D&E).

To determine the presence of reactive astrocytes, brains were harvested 72 hours following acute exposures and processed for immunohistochemistry for glial fibrillary acidic protein (GFAP). To quantify the mean intensity of GFAP immunoreactivity, a region of interest was designated with neuronal barriers outlining the hippocampus region to enable equal sampling. The mean intensity was measured from calibrated luminance of pixels within the hippocampus region of interest. The total range of calibrated luminance of individual pixels was 0-255 lumens (lum). Although trends emerged, no significant differences were observed between the exposure groups (ANOVA, $p = 0.6$; Figure 45A).

To quantify astrocyte cell numbers, astrocyte nuclei revealed by the combination of a DAPI nuclear stain and GFAP immunoreactivity, were counted within the same neuronal boundaries outlining the hippocampus region. The astrocyte number was then normalized to the area of the hippocampus sampled to control for tissue slice and selection variability. A significant 100% increase in astrocyte number (t-test, $p = 0.04$) was detected in the 14K+1.5 p/s exposure group (mean = $0.42 \text{ Astrocytes/mm}^2 \pm 0.15 \text{ SD}$, $n=5$) compared to 8K controls (mean = $0.21 \text{ Astrocytes/mm}^2 \pm 0.13 \text{ SD}$, $n=5$; Figure 45B). While a trend is emerging in the 8K+1.5 p/s exposure group (mean = $0.30 \text{ Astrocytes/mm}^2 \pm 0.21 \text{ SD}$, $n=8$) compared to the 8K control, the difference is not yet significant (t-test, $p=0.44$). Another trend of interest is emerging between the 14K altitude control (mean = $0.25 \text{ Astrocytes/mm}^2 \pm 0.15 \text{ SD}$, $n=5$) and the 14K+1.5 p/s exposure group that is also not quite significant (t-test, $p=0.09$). Despite the need for future studies to fully resolve all of these emerging trends, there is significant data present that is consistent with astrocyte proliferation following pressure fluctuations at altitude.

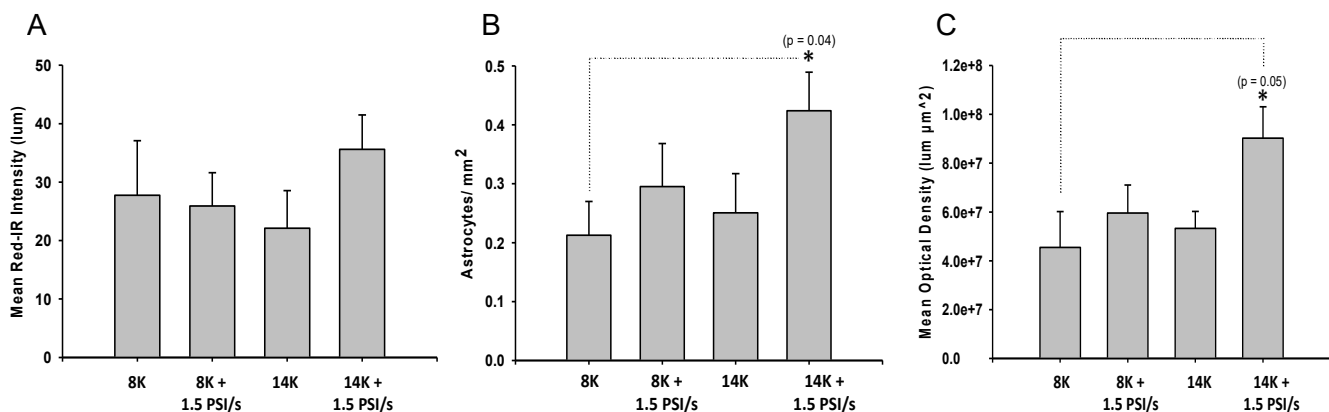


Figure 45. Quantification of astrocyte activation

- (A) Mean astrocyte (GFAP) immunoreactive intensity measured as lumens (lum) did not significantly change across the treatment groups. Data are presented as mean $\pm$ SEM. (B) Astrocyte number in the hippocampus significantly changed across the treatment groups. Data are presented as mean $\pm$ SEM. Asterisk denotes significant difference between the 8K and 14K + 1.5 p/s exposure groups. (C) Optical Density of GFAP immunoreactivity in the hippocampus significantly changed across the treatment groups. Data are presented as mean $\pm$ SEM. * significant difference between the 8K and 14K + 1.5 p/s exposure groups

To confirm these results, an independent integrated optical density test (IOD) was performed, which is computed as pixel area times the mean intensity. This test is frequently used to measure relative fluorescence, and was used here to quantify GFAP immunoreactivity in terms of both intensity and density. A significant 98% increase (t-test $p=0.05$) in the IOD of GFAP immunoreactivity was detected in the 14K+ 1.5 p/s group (mean = $9.03 \times 10^8 \text{ lum} \times \mu\text{m}^2 \pm 3.3 \times 10^7 \text{ SD}$, $n=5$) compared to 8K controls (mean = $4.55 \times 10^8 \text{ lum} \times \mu\text{m}^2 \pm 2.9 \times 10^7 \text{ SD}$, $n=5$; Figure 45C). A trend is emerging in the 8K+1.5 p/s exposure group (mean = $6.0 \times 10^8 \text{ lum} \times \mu\text{m}^2 \pm 3.3 \times 10^7 \text{ SD}$, $n=8$) compared to the 8K control, the difference is not yet significant (t-test, $p=0.47$). Another trend of interest is emerging between the 14K altitude control (mean = $5.3 \times 10^8 \text{ lum} \times \mu\text{m}^2 \pm 1.6 \times 10^7 \text{ SD}$, $n=5$) and the 14K+1.5 p/s exposure group that is also not yet significant (t-test, $p=0.09$). Like the astrocytic proliferation data, future studies are required to fully resolve all of these emerging trends. However, there are significant data present that are consistent with reactive astrocytes following pressure fluctuations at altitude.

Phase 3 Discussion

Increased expression of glial fibrillary acidic protein (GFAP) together with proliferation and altered morphology are the most widely accepted markers of reactive astrocytes (Pekny & Pekna, 2004). This study demonstrated evidence that a significant increase in astrocyte proliferation occurred following pressure fluctuations at altitude. This study also provided evidence that there was a significant increase in the integrated optical density of GFAP immunoreactive pixels within the hippocampus images. These two independent tests were replicated and consistent in their outcomes and suggest astrocyte activation. However, the characteristics described herein are not sufficient to discriminate between the two states of astrocytic activation, inflammatory and ischemic states (see Liddelow & Barres, 2017). Therefore, it is unknown if the presence of reactive astrocytes following pressure fluctuations qualitatively shown in this report are a response to trauma, ischemia, disruption of the blood-brain barrier or some other mechanism. Qualitatively, the astrocytic response shown here is similar to what has been observed following traumatic brain injury (Goldstein et al., 2012), albeit not arising from a single unilateral impact location but rather widespread bilateral responses.

There are important caveats to these observations. First, future studies are required to address these changes compared to sea level GFAP immunoreactivity as well as under conditions of both normoxia and hyperoxia. It is unknown if hyperoxia primes astrocytes or upstream effectors for activation, enhancing the effects of pressure fluctuations. Second, the rats selected for this study were exposed to pressure fluctuations at altitude while anesthetized with ketamine/xylazine. Although the results shown herein follow the exposure profiles, general anesthetics have been shown to target not only neurons, but also astrocytes (Thrane et al., 2012). Furthermore, trends detected with our preliminary analysis may become significant with larger sample sizes and these preliminary examinations are critical in establishing those sample sizes. Moreover, of the rats who

experienced the same exposure, there was variability in the responses, perhaps modeling the variability observed in pilots. Finally, these data are taken at one time point following a single acute exposure. Performing a time course study is important to not only characterize the maximum extent of pressure induced changes, but also determine if and when these changes return to baseline levels. Altogether these caveats justify the critical need to expand upon these preliminary observations to include additional controls and timepoints in a larger sample of awake and alert rats during pressure fluctuation exposures. Additionally, electrophysiological monitoring of the hippocampus can be performed to associate these changes with physiological effects in hippocampal function that may contribute to cognitive changes.

The absence of detectable VGE in the pulmonary artery suggests that the astrocyte activation is not a consequence of pronounced systemic VGE formation that is overloading clearance of gas by the lungs and progressing into the arterial circulation and subsequently the vasculature of the CNS. Additionally, the absence of any indicators of barotrauma in the lungs that could initiate AGE formation also supports the conclusion that gas emboli in the systemic arterial circulation are not responsible for the neural effects. Similarly, there was no evidence of systemic endothelial activation, increase in pro-inflammatory cytokines, or platelet consumption that have been observed with diving-related decompression injury studies (Zhang et al., 2017; Bigley et al., 2008) that would suggest systemic gas emboli or coagulation involvement. However, pulmonary barotrauma and DCS biomarkers were not evaluated at the same rates as the VGE detection and astrocytic activation, so it cannot be ruled out that these effects could be observed at higher fluctuation rates.

The findings of this study suggest that the effects of rapid pressure changes are localized to the CNS, but the exact mechanisms causing the neurological manifestations remain unclear and will require additional investigation. The increase in astrocyte activation three days following exposure could be the result of direct pressure-induced trauma, localized gas emboli or microparticles, blood-brain barrier disruption, and/or ischemic injury. Additional pressure fluctuation exposure experiments in *in vivo* and *in vitro* models can target each of these mechanisms and attempt to identify causality of the astrocytic response.

There are several limitations of these findings that caution broader extrapolation to effects in humans. All of the rats that were evaluated for astrocyte activation were put under anesthesia for the duration of the exposure using a ketamine/xylazine cocktail. Although no studies could be found linking this combination to this type of astrocyte activation, another study should be conducted in conscious animals to verify that the same effects will be observed. Regardless, all rats including the control group were given the anesthetic and there still was a differential increase in activation between exposure groups, suggesting that these effects are very unlikely to be a result of the anesthetic alone.

All of the rats were supplied 94% oxygen during the exposures, which even at 8,000 and 14,000 feet above sea level is hyperoxic compared to ground level. It is unclear whether or not this elevated oxygen level might interact with the fluctuations in causing this response, and a normoxic control group can be included in future studies to answer this question. Although elevated oxygen exposures are known to be neurotoxic over longer durations (Ciarlone et al., 2019), the impact of elevated oxygen levels over shorter durations is unclear and the subject of ongoing aeromedical research studies.

Although no VGE formation was detected during and after pressure fluctuations with ultrasonic monitoring of the pulmonary artery, it is possible that smaller VGE could be present that escape visualization of the ultrasound imaging. Our validation methods only detected the presence of microbubbles in the pulmonary artery down to approximately 100 micrometers in diameter, but other studies on the limits of ultrasound detection of VGE have estimated a limit of detection of ~20-30 micrometers (Papadopoulou et al., 2014). The limit of detection of our methods was determined by the lower limit of generation by the bubble generator, not the inability of the ultrasound monitoring to detect microbubbles, so the actual limit of detection was probably closer to the limits of detection found in the studies that were specifically focused on finding the limits of the imaging.

Although VGE formation was not observed, another limitation of this study is that the mechanisms of microparticle activation were minimally addressed. There is evidence from published literature (Yang et al., 2013) that decompression can cause activation of microparticles that, if able to reach the brain, may be able to mediate neurological injury. Although this study performed a number of assays to attempt to identify if any endothelial activation or platelet consumption associated with pressure fluctuations was present systemically, this was only evaluated at one time point a few hours following the exposure based on the guidance of previous literature. More of a targeted assessment using more sensitive assays of microparticle formation and additional time points would help resolve whether or not this mechanism is involved with the neurological manifestations.

In conclusion, effects have been identified from pressure fluctuations in a rodent model at fluctuation rates that are comparable or slightly higher than what have been measured from cockpit monitoring in Navy tactical aircraft. The cause of both the transient impacts on motor activity and the astrocytic activation in the hippocampus are yet to be determined, but now that exposure levels necessary to induce adverse effects have been identified, future targeted studies to identify causal mechanisms and time courses of these effects can now be performed in the rodent model. For the first time, there is preliminary data that shows a significant neuroanatomical change occurring in the brain following pressure fluctuation exposures that could underlie neurological symptoms consistent with what some pilots experience. While these preliminary observations offer insight into mechanism, additional studies are necessary to interpret the results in a

fashion where strategies and therapies can be applied to mitigate these effects and protect Warfighters.

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Appendix A. Plots of Altitude and Pressure Rate Change Profiles

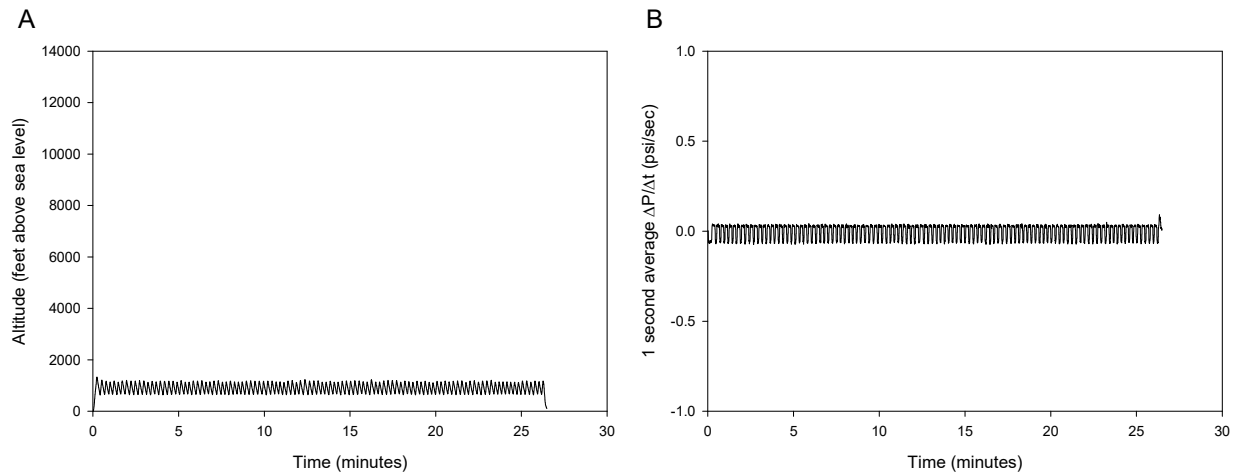


Figure A1. Measured **(A)** Altitude and **(B)** Pressure rate change of GL profile

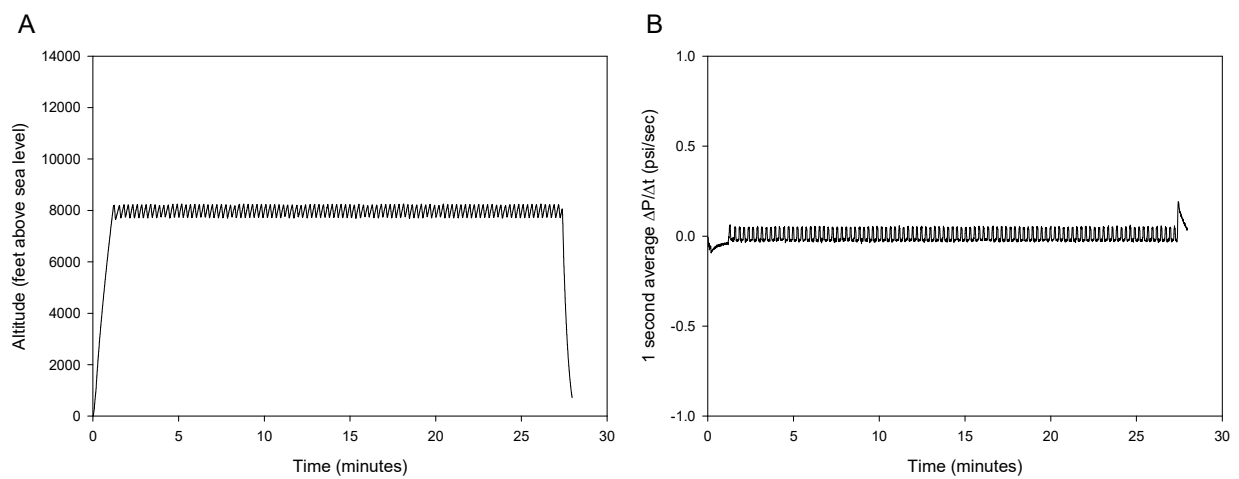


Figure A2. Measured **(A)** Altitude and **(B)** Pressure rate change of 8K profile

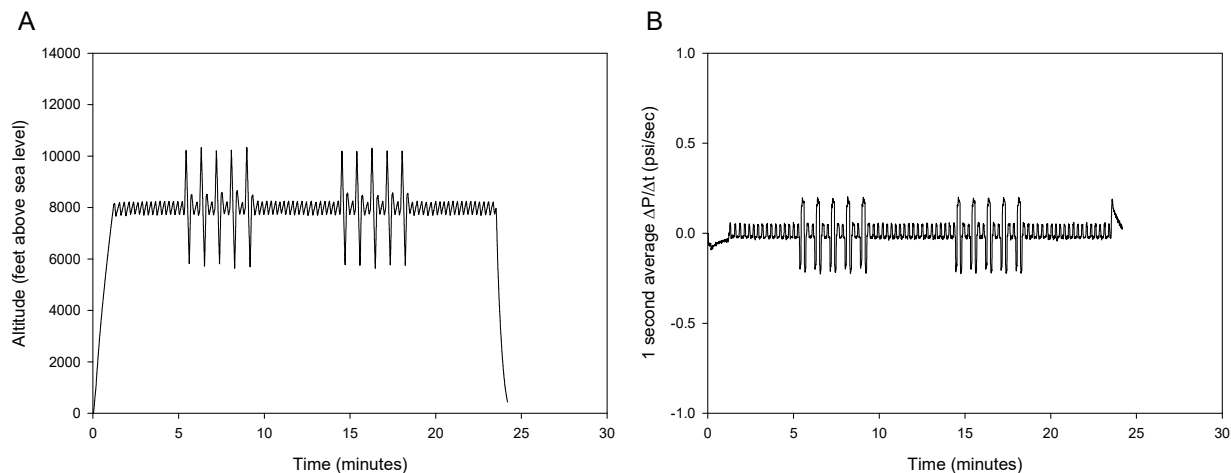


Figure A3. Measured **(A)** Altitude and **(B)** Pressure rate change of 8K+0.2 p/s profile

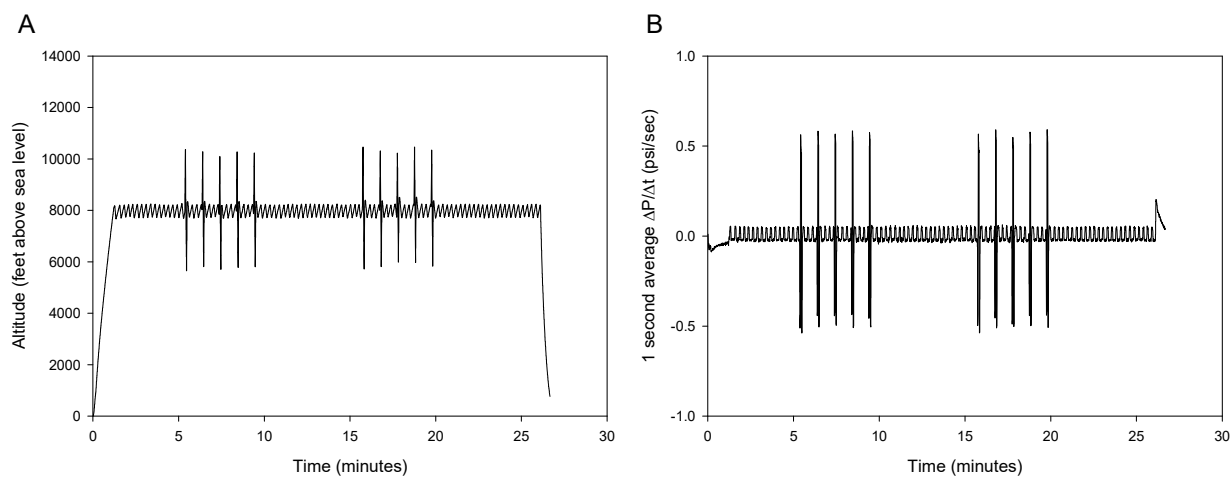


Figure A4. Measured **(A)** Altitude and **(B)** Pressure rate change of 8K+0.5 p/s profile

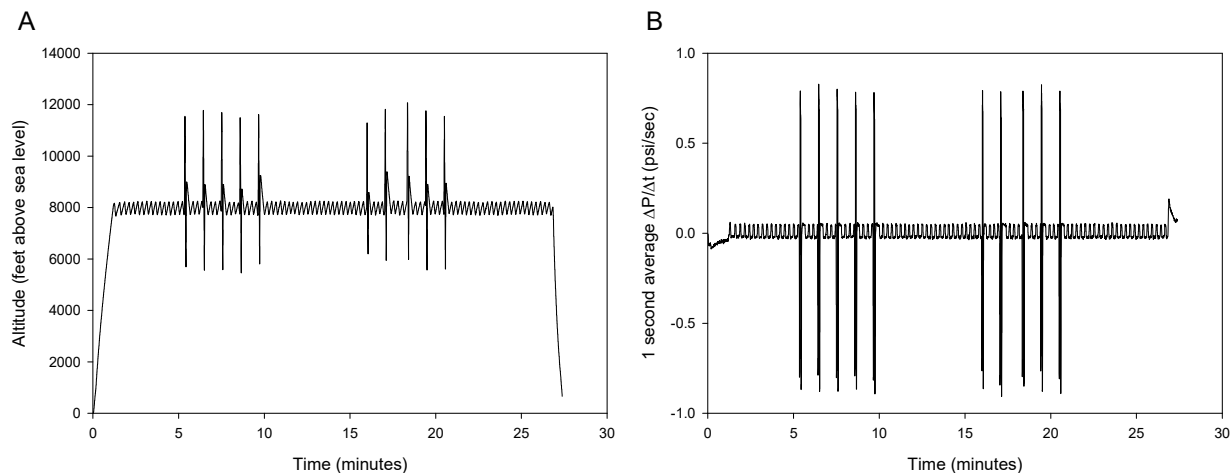


Figure A5. Measured **(A)** Altitude and **(B)** Pressure rate change of 8K+0.8 p/s profile

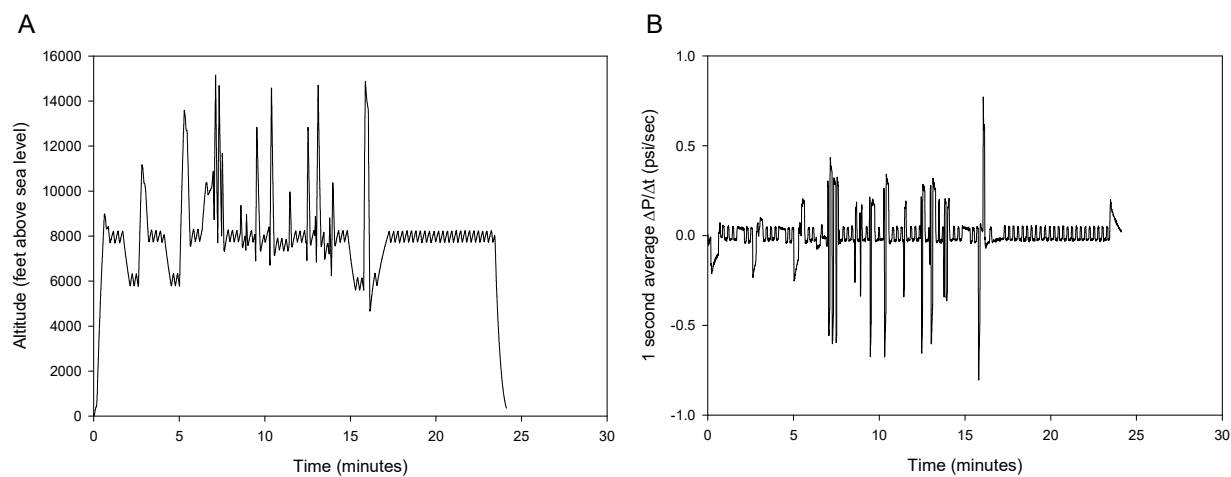


Figure A6. Measured **(A)** Altitude and **(B)** Pressure rate change of 8K+Comp profile

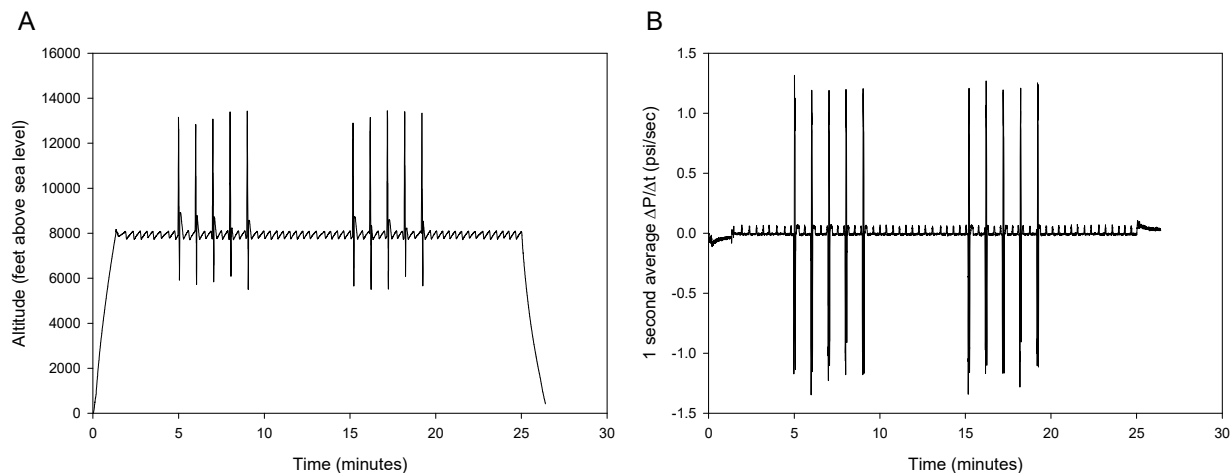


Figure A7. Measured **(A)** Altitude and **(B)** Pressure rate change of 8K+1.2 p/s profile

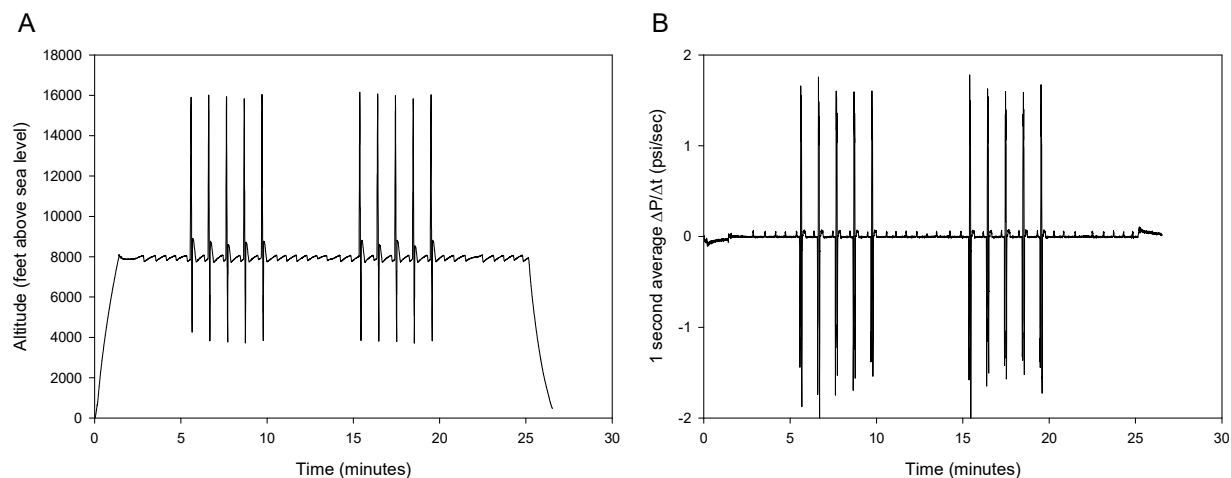


Figure A8. Measured **(A)** Altitude and **(B)** Pressure rate change of 8K+1.5 p/s profile

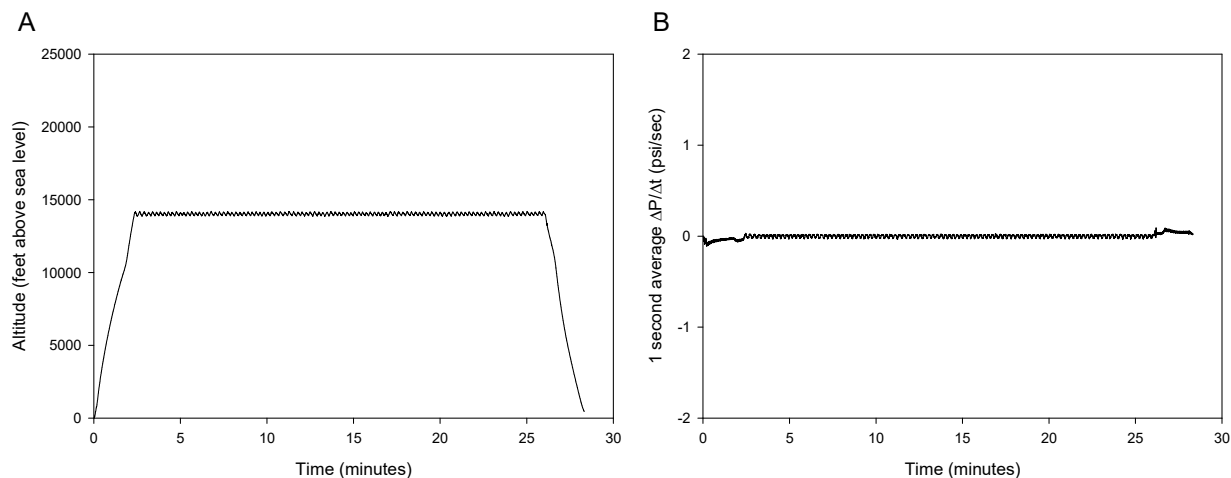


Figure A9. Measured **(A)** Altitude and **(B)** Pressure rate change of 14K profile

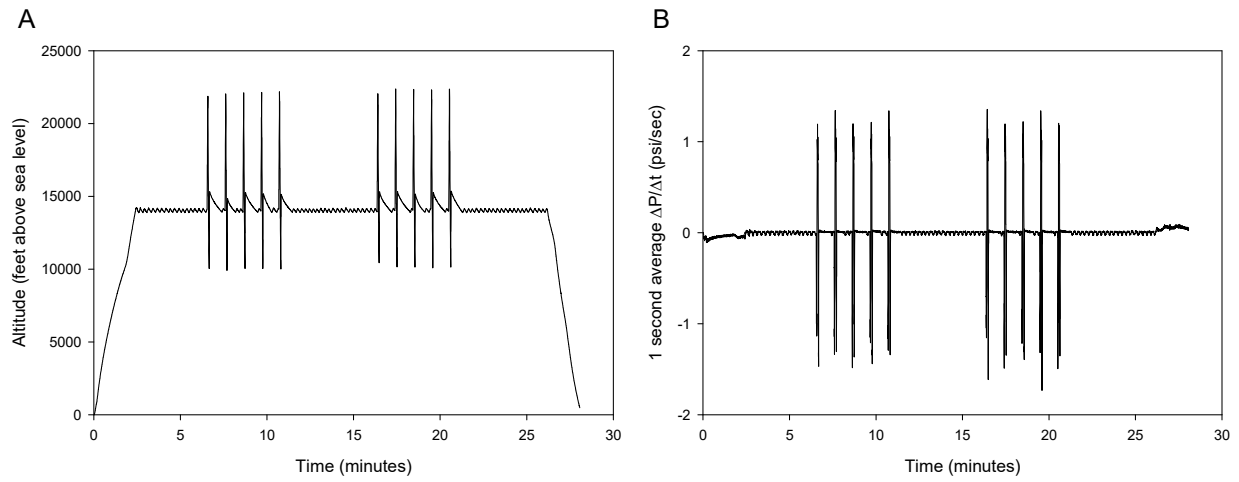


Figure A10. Measured **(A)** Altitude and **(B)** Pressure rate change of 14K+ 1.5 p/s profile

Appendix B. Respiratory Data

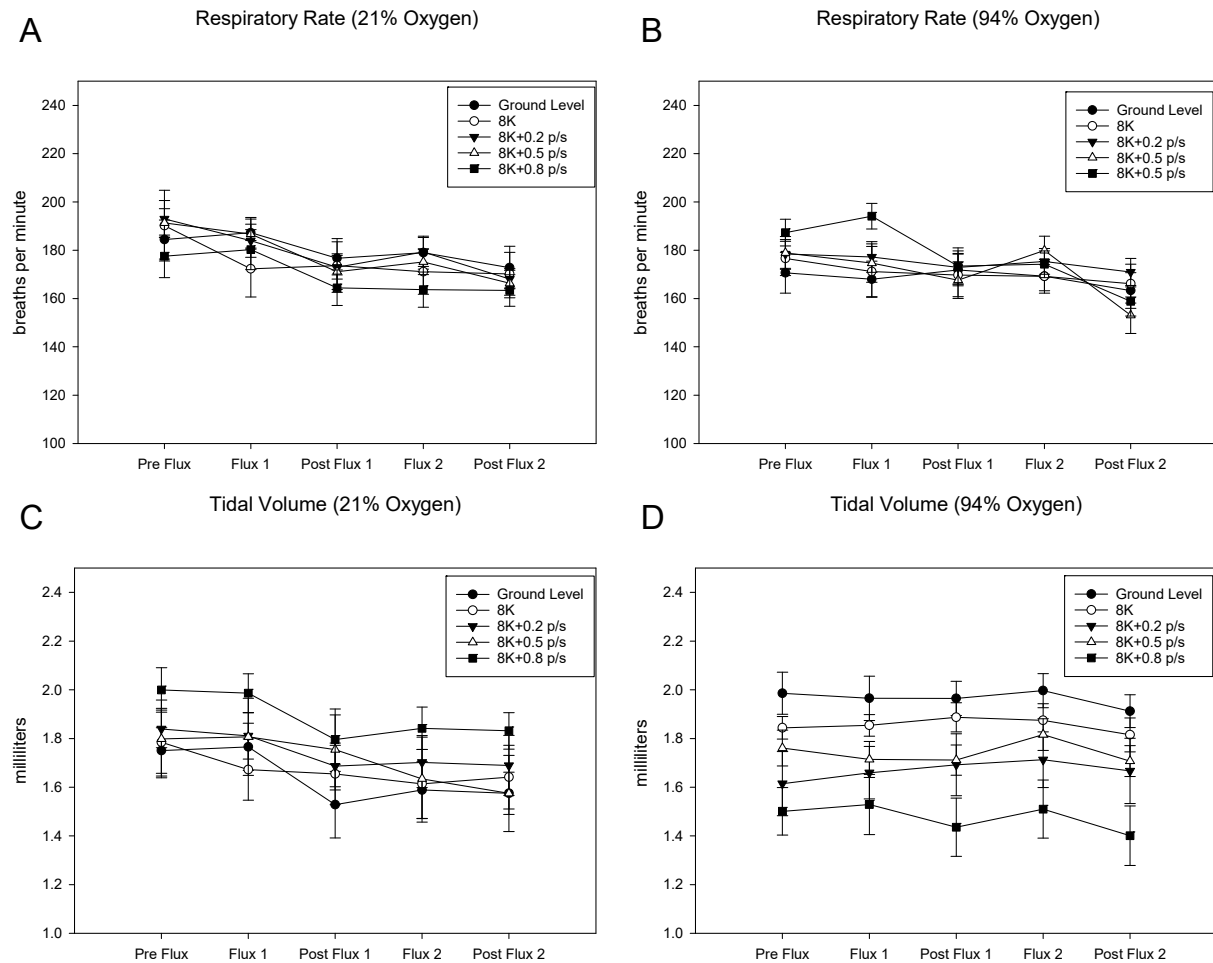


Figure B1. Average respiratory rate for the GL, 8K, 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles for groups exposed to **(A)** 21% and **(B)** 94% oxygen. Average tidal volume for each of the five profiles for groups exposed to **(C)** 21% and **(D)** 94% oxygen. All data points represent exposure group mean $\pm$ SEM.

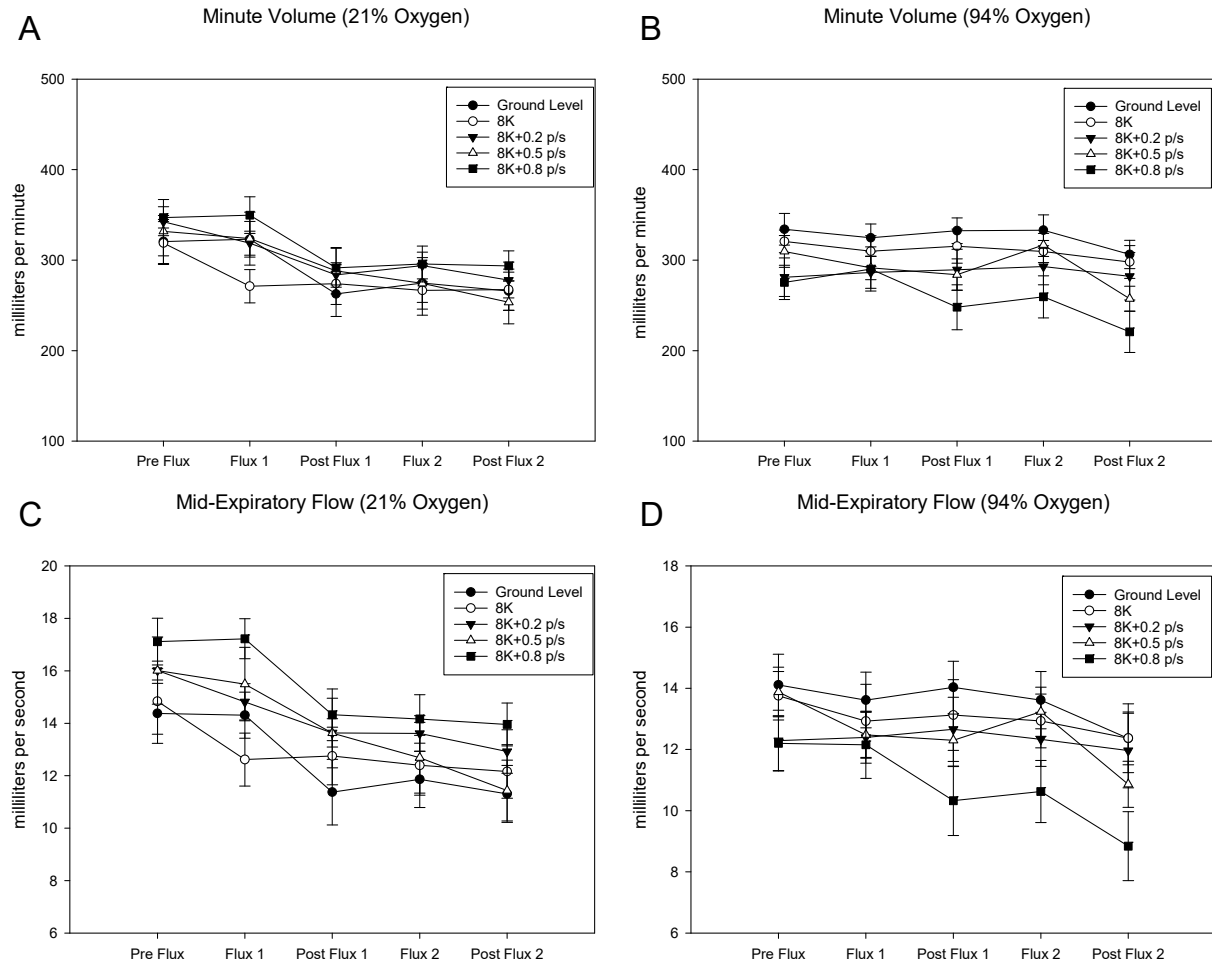


Figure B2. Average minute volume for the GL, 8K, 8K+0.2 p/s, 8K+0.5 p/s, and 8K+0.8 p/s profiles for groups exposed to **(A)** 21% and **(B)** 94% oxygen. Average mid-expiratory flow for each of the five profiles for groups exposed to **(C)** 21% and **(D)** 94% oxygen. All data points represent exposure group mean $\pm$ SEM.

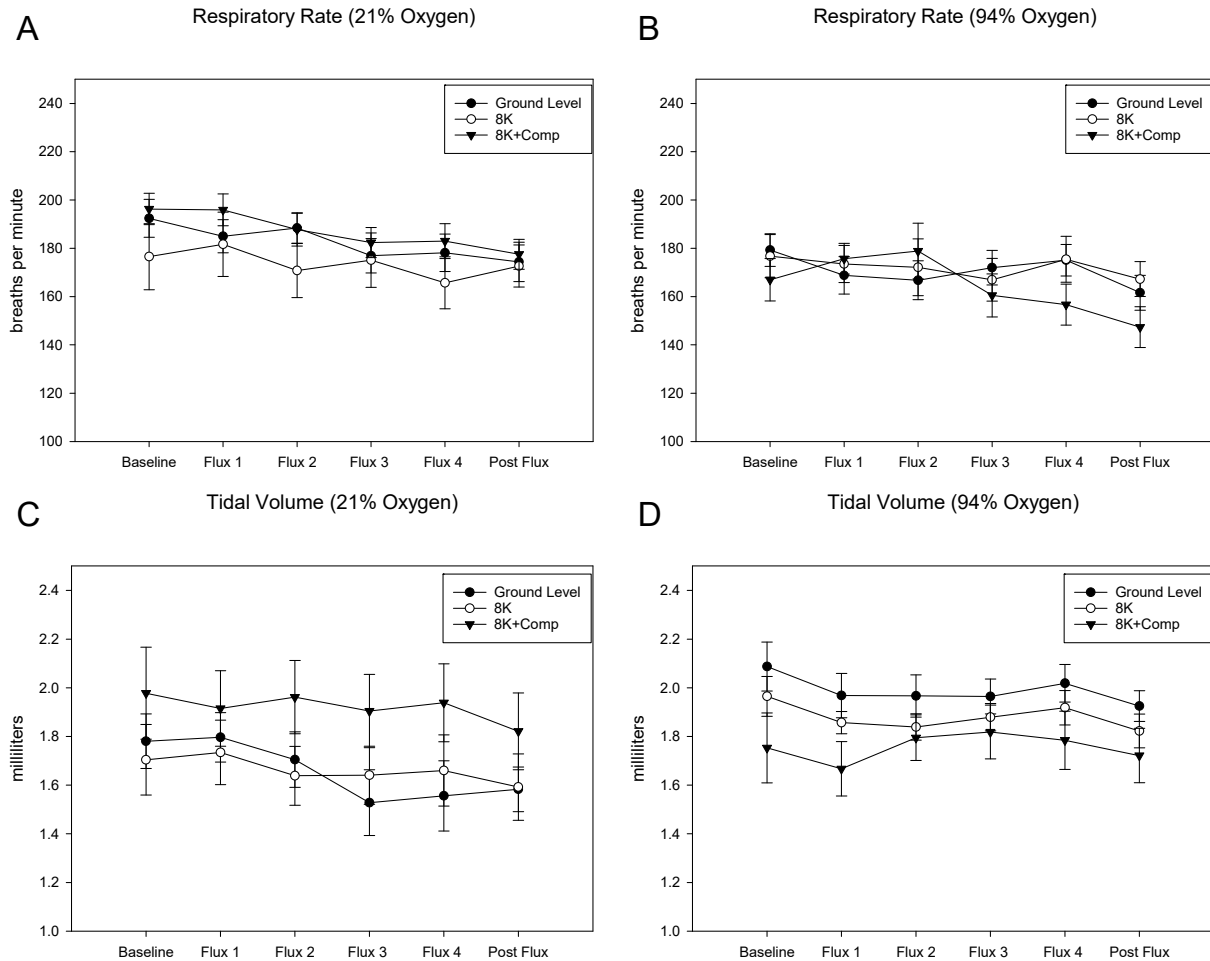


Figure B3. Average respiratory rate for the GL, 8K, and 8K + Comp profiles for groups exposed to **(A)** 21% and **(B)** 94% oxygen. Average tidal volume for each of the five profiles for groups exposed to **(C)** 21% and **(D)** 94% oxygen. All data points represent exposure group mean $\pm$ SEM.

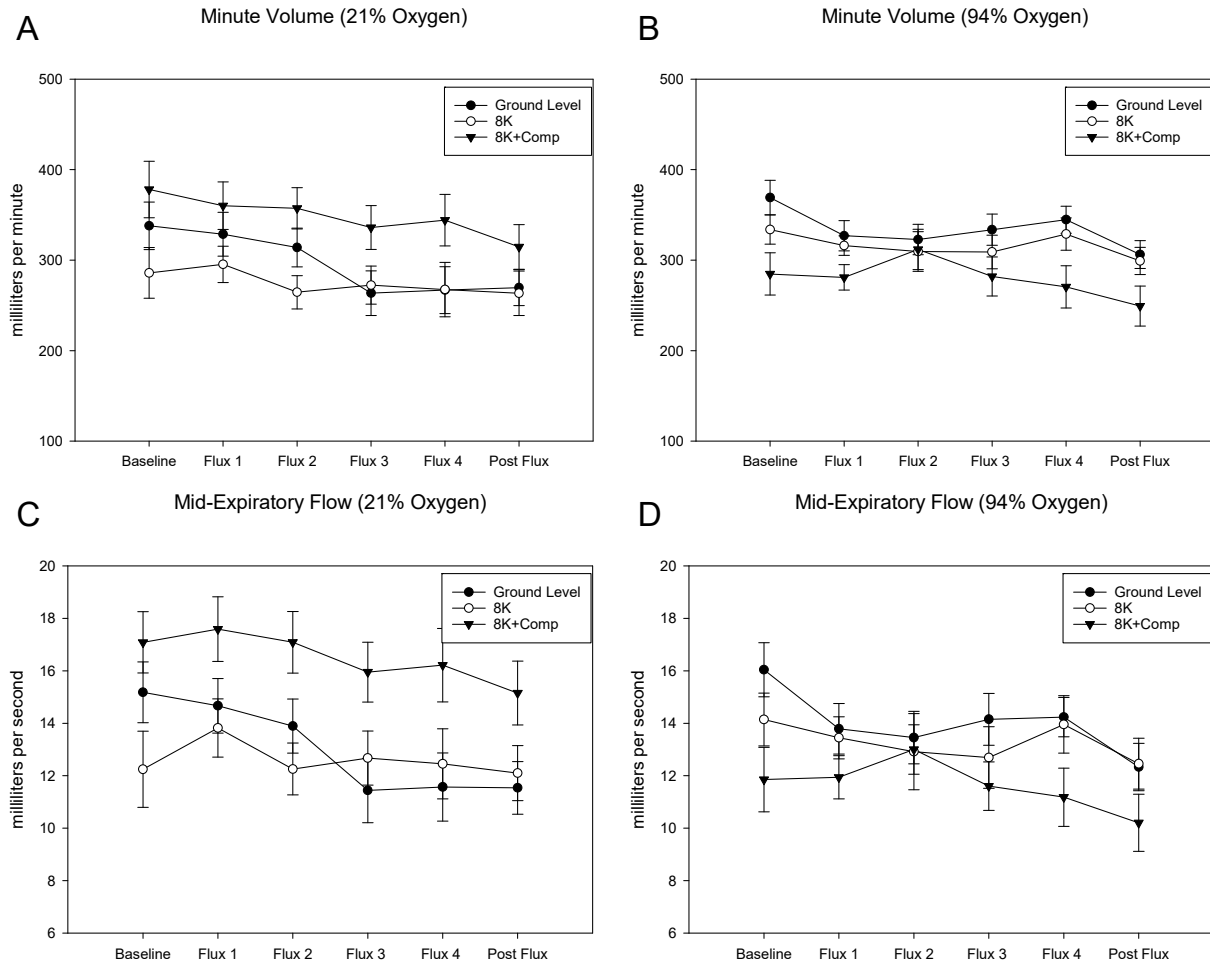


Figure B4. Average minute volume for the GL, 8K, and 8K + Comp profiles for groups exposed to **(A)** 21% and **(B)** 94% oxygen. Average mid-expiratory flow for each of the five profiles for groups exposed to **(C)** 21% and **(D)** 94% oxygen. All data points represent exposure group mean $\pm$ SEM.

Appendix C. Phase 2 and 3 Fluctuation and Environmental Data

Table C1. Fluctuation and Environmental Data Phase 2 Experiment #1

Decompression Peak Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.25 ± 0.04	0.23 ± 0.03	0.23 ± 0.03	0.23 ± 0.03	0.23 ± 0.03	0.23 ± 0.03	0.23 ± 0.01
8K + 0.5 p/s	0.50 ± 0.10	0.50 ± 0.06	0.51 ± 0.08	0.51 ± 0.08	0.51 ± 0.07	0.50 ± 0.06	0.51 ± 0.01
8K + 0.8 p/s	0.86 ± 0.11	0.84 ± 0.11	0.86 ± 0.09	0.82 ± 0.10	0.84 ± 0.09	0.82 ± 0.10	0.84 ± 0.02
Decompression Average Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.21 ± 0.04	0.20 ± 0.03	0.19 ± 0.03	0.20 ± 0.03	0.20 ± 0.03	0.20 ± 0.03	0.20 ± 0.01
8K + 0.5 p/s	0.37 ± 0.13	0.37 ± 0.13	0.36 ± 0.13	0.37 ± 0.14	0.36 ± 0.13	0.37 ± 0.13	0.37 ± 0.01
8K + 0.8 p/s	0.57 ± 0.26	0.56 ± 0.26	0.59 ± 0.25	0.60 ± 0.23	0.61 ± 0.22	0.58 ± 0.23	0.59 ± 0.02
Recompression Peak Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.55 ± 0.07	0.23 ± 0.03	0.24 ± 0.03	0.23 ± 0.03	0.23 ± 0.03	0.24 ± 0.03	0.29 ± 0.13
8K + 0.5 p/s	0.49 ± 0.09	0.50 ± 0.05	0.51 ± 0.05	0.52 ± 0.05	0.51 ± 0.06	0.52 ± 0.06	0.51 ± 0.01
8K + 0.8 p/s	0.85 ± 0.09	0.84 ± 0.08	0.85 ± 0.06	0.87 ± 0.08	0.87 ± 0.08	0.87 ± 0.09	0.86 ± 0.01
Recompression Average Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.44 ± 0.10	0.20 ± 0.03	0.20 ± 0.03	0.20 ± 0.03	0.20 ± 0.03	0.21 ± 0.03	0.24 ± 0.10
8K + 0.5 p/s	0.39 ± 0.11	0.39 ± 0.10	0.40 ± 0.10	0.41 ± 0.10	0.40 ± 0.11	0.41 ± 0.11	0.40 ± 0.01
8K + 0.8 p/s	0.63 ± 0.21	0.63 ± 0.21	0.62 ± 0.21	0.64 ± 0.22	0.64 ± 0.21	0.64 ± 0.21	0.63 ± 0.01
Average Duration per Fluctuation (sec)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	13.4 ± 0.3	18.9 ± 0.3	18.6 ± 0.3	18.5 ± 0.3	18.5 ± 0.2	18.2 ± 0.4	17.7 ± 2.1
8K + 0.5 p/s	7.5 ± 0.3	7.6 ± 0.2	7.7 ± 0.3	7.5 ± 0.3	7.8 ± 0.2	7.6 ± 0.3	7.6 ± 0.1
8K + 0.8 p/s	7.4 ± 0.3	7.5 ± 0.3	7.5 ± 0.4	7.2 ± 0.2	7.0 ± 0.1	7.3 ± 0.3	7.3 ± 0.2
Maximum Altitude During Fluctuations (feet)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	10100 ± 56	10167 ± 62	10163 ± 52	10151 ± 49	10140 ± 58	10127 ± 65	10141 ± 25
8K + 0.5 p/s	9836 ± 150	9866 ± 118	9891 ± 136	9908 ± 184	9870 ± 132	9917 ± 155	9881 ± 30
8K + 0.8 p/s	11981 ± 269	11938 ± 284	12015 ± 260	12006 ± 250	11911 ± 238	11957 ± 271	11968 ± 40
Minimum Altitude During Fluctuations (feet)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	5598 ± 109	5862 ± 73	5841 ± 74	5871 ± 81	5817 ± 68	5867 ± 77	5809 ± 105
8K + 0.5 p/s	5271 ± 715	6298 ± 154	6225 ± 139	6278 ± 151	6186 ± 138	6305 ± 168	6094 ± 405
8K + 0.8 p/s	6076 ± 294	6240 ± 272	6088 ± 220	6169 ± 246	6151 ± 248	6233 ± 264	6160 ± 69
Steady Altitude (feet)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
Ground Level	818 ± 107	817 ± 106	817 ± 105	821 ± 107	818 ± 106	816 ± 106	818 ± 2
8K	8001 ± 121	8005 ± 117	8011 ± 109	8003 ± 122	8011 ± 124	8008 ± 115	8007 ± 4
8K + 0.2 p/s	7980 ± 144	7991 ± 152	7975 ± 144	7983 ± 148	7981 ± 148	7984 ± 145	7982 ± 5
8K + 0.5 p/s	7994 ± 144	8001 ± 133	7988 ± 138	7996 ± 134	7991 ± 174	7991 ± 133	7994 ± 5
8K + 0.8 p/s	8001 ± 130	7994 ± 138	8003 ± 135	7996 ± 131	7997 ± 132	7998 ± 131	7998 ± 3
Temperature (°C)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
Ground Level	22.6 ± 0.0	22.6 ± 0.0	21.9 ± 0.0	22.5 ± 0.1	23.2 ± 0.1	22.5 ± 0.1	22.6 ± 0.4
8K	22.4 ± 0.1	22.6 ± 0.1	22.0 ± 0.1	22.7 ± 0.1	23.1 ± 0.1	22.7 ± 0.1	22.6 ± 0.4
8K + 0.2 p/s	22.7 ± 0.0	22.5 ± 0.0	22.1 ± 0.1	22.8 ± 0.0	22.0 ± 0.1	22.9 ± 0.1	22.5 ± 0.4
8K + 0.5 p/s	22.5 ± 0.0	22.6 ± 0.0	22.1 ± 0.0	22.6 ± 0.0	22.5 ± 0.1	22.8 ± 0.0	22.5 ± 0.3
8K + 0.8 p/s	22.4 ± 0.0	22.8 ± 0.0	21.5 ± 0.1	22.5 ± 0.0	22.7 ± 0.1	21.8 ± 0.1	22.3 ± 0.5
8K + Comp	22.3 ± 0.0	22.6 ± 0.0	21.7 ± 0.1	22.5 ± 0.1	22.9 ± 0.1	22.1 ± 0.1	22.4 ± 0.4
Relative Humidity (%)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
Ground Level	14.2 ± 0.9	11.8 ± 1.3	23.1 ± 3.8	12.8 ± 0.6	12.6 ± 1.6	24.6 ± 6.0	16.5 ± 5.8
8K	11.7 ± 0.8	11.3 ± 1.1	20.3 ± 2.5	10.9 ± 0.9	12.1 ± 1.2	24.3 ± 3.1	15.1 ± 5.7
8K + 0.2 p/s	11.0 ± 0.9	13.9 ± 1.5	19.8 ± 2.6	10.3 ± 0.7	12.9 ± 1.1	23.4 ± 3.3	15.2 ± 5.2
8K + 0.5 p/s	10.4 ± 0.7	11.6 ± 1.0	19.5 ± 2.3	10.6 ± 0.6	13.7 ± 1.4	23.2 ± 3.2	14.9 ± 5.3
8K + 0.8 p/s	10.0 ± 0.7	11.1 ± 1.0	22.2 ± 2.7	10.2 ± 0.6	12.5 ± 1.2	27.7 ± 3.6	15.6 ± 7.5
8K + Comp	10.0 ± 0.6	10.9 ± 0.9	20.1 ± 2.8	9.4 ± 0.7	12.3 ± 1.2	24.8 ± 3.6	14.6 ± 6.3
Oxygen (%)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
Ground Level	59 ± 12	60 ± 12	64 ± 7	ND	60 ± 12	59 ± 14	60 ± 2
8K	80 ± 17	81 ± 17	78 ± 20	81 ± 15	77 ± 21	77 ± 20	79 ± 2
8K + 0.2 p/s	70 ± 16	72 ± 15	72 ± 17	73 ± 14	74 ± 14	70 ± 17	72 ± 2
8K + 0.5 p/s	72 ± 18	72 ± 15	71 ± 17	74 ± 13	72 ± 16	70 ± 17	72 ± 1
8K + 0.8 p/s	68 ± 17	68 ± 15	70 ± 11	70 ± 13	69 ± 14	71 ± 9	69 ± 1
8K + Comp	75 ± 9	72 ± 13	71 ± 15	72 ± 12	70 ± 14	68 ± 17	71 ± 2

Table C2. Fluctuation and Environmental Data Phase 2 Experiment #2

Decompression Peak Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.23 ± 0.03	0.23 ± 0.03	0.23 ± 0.03	0.23 ± 0.03	0.24 ± 0.03	0.24 ± 0.03	0.23 ± 0.00
8K + 0.5 p/s	0.52 ± 0.07	0.51 ± 0.07	0.52 ± 0.08	0.50 ± 0.07	0.49 ± 0.07	0.50 ± 0.08	0.51 ± 0.01
8K + 0.8 p/s	0.84 ± 0.10	0.87 ± 0.10	0.87 ± 0.09	0.85 ± 0.10	0.85 ± 0.10	0.88 ± 0.10	0.86 ± 0.01
Decompression Average Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.20 ± 0.03	0.19 ± 0.03	0.20 ± 0.03	0.19 ± 0.03	0.20 ± 0.04	0.20 ± 0.04	0.24 ± 0.10
8K + 0.5 p/s	0.37 ± 0.14	0.38 ± 0.13	0.38 ± 0.14	0.37 ± 0.13	0.37 ± 0.13	0.37 ± 0.13	0.37 ± 0.01
8K + 0.8 p/s	0.57 ± 0.25	0.58 ± 0.26	0.59 ± 0.25	0.58 ± 0.25	0.57 ± 0.26	0.61 ± 0.26	0.58 ± 0.01
Recompression Peak Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.27 ± 0.03	0.27 ± 0.03	0.26 ± 0.03	0.25 ± 0.03	0.25 ± 0.03	0.26 ± 0.03	0.26 ± 0.01
8K + 0.5 p/s	0.55 ± 0.06	0.56 ± 0.06	0.58 ± 0.06	0.53 ± 0.06	0.55 ± 0.07	0.55 ± 0.06	0.55 ± 0.01
8K + 0.8 p/s	0.93 ± 0.08	0.93 ± 0.09	0.94 ± 0.09	0.93 ± 0.15	0.89 ± 0.08	0.89 ± 0.09	0.92 ± 0.02
Recompression Average Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	0.23 ± 0.03	0.23 ± 0.03	0.23 ± 0.03	0.21 ± 0.03	0.21 ± 0.03	0.22 ± 0.03	0.22 ± 0.01
8K + 0.5 p/s	0.41 ± 0.13	0.42 ± 0.13	0.43 ± 0.14	0.42 ± 0.12	0.43 ± 0.13	0.43 ± 0.12	0.42 ± 0.01
8K + 0.8 p/s	0.67 ± 0.23	0.68 ± 0.23	0.68 ± 0.23	0.69 ± 0.24	0.66 ± 0.22	0.66 ± 0.23	0.67 ± 0.01
Average Duration per Fluctuation (sec)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	17.8 ± 0.2	18.0 ± 0.4	17.7 ± 0.2	18.5 ± 0.3	18.3 ± 0.5	18.3 ± 0.5	18.1 ± 0.3
8K + 0.5 p/s	7.2 ± 0.2	7.1 ± 0.2	7.1 ± 0.3	7.6 ± 0.4	7.4 ± 0.2	7.3 ± 0.4	7.6 ± 0.1
8K + 0.8 p/s	6.8 ± 0.2	7.3 ± 0.1	7.0 ± 0.2	7.1 ± 0.3	7.0 ± 0.2	7.2 ± 0.3	7.1 ± 0.2
Maximum Altitude During Fluctuations (feet)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	10151 ± 60	10108 ± 68	10158 ± 62	10142 ± 62	10148 ± 62	10132 ± 61	10140 ± 18
8K + 0.5 p/s	9910 ± 137	9892 ± 130	9935 ± 141	9930 ± 149	9967 ± 148	9916 ± 157	9925 ± 25
8K + 0.8 p/s	11981 ± 236	12170 ± 208	12018 ± 344	12019 ± 282	11873 ± 199	12065 ± 274	12006 ± 111
Minimum Altitude During Fluctuations (feet)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
8K + 0.2 p/s	5777 ± 58	5774 ± 62	5829 ± 61	5817 ± 62	5843 ± 78	5852 ± 73	5815 ± 33
8K + 0.5 p/s	6293 ± 147	6340 ± 165	6232 ± 179	6244 ± 161	6204 ± 151	6217 ± 179	6255 ± 52
8K + 0.8 p/s	6282 ± 250	6049 ± 249	6117 ± 286	6183 ± 287	6156 ± 321	6121 ± 226	6151 ± 78
Steady Altitude (feet)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
Ground Level	804 ± 101	805 ± 103	763 ± 82	784 ± 97	768 ± 95	769 ± 95	782 ± 19
8K	8044 ± 84	7989 ± 136	8016 ± 105	7995 ± 136	8009 ± 115	7997 ± 120	8008 ± 20
8K + 0.2 p/s	7963 ± 146	7955 ± 160	7972 ± 147	7995 ± 156	7999 ± 168	7989 ± 164	7979 ± 18
8K + 0.5 p/s	7986 ± 132	7988 ± 122	7987 ± 136	7990 ± 139	7997 ± 151	7988 ± 143	7990 ± 4
8K + 0.8 p/s	7998 ± 134	8010 ± 118	7997 ± 138	7976 ± 154	7996 ± 158	7999 ± 163	7996 ± 11
Temperature (°C)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
Ground Level	23.4 ± 0.0	23.6 ± 0.0	23.0 ± 0.0	22.9 ± 0.1	23.4 ± 0.1	23.1 ± 0.0	23.2 ± 0.2
8K	23.4 ± 0.1	23.6 ± 0.1	23.1 ± 0.1	22.9 ± 0.1	23.5 ± 0.1	23.0 ± 0.1	23.3 ± 0.3
8K + 0.2 p/s	23.5 ± 0.0	23.0 ± 0.0	23.3 ± 0.1	22.9 ± 0.1	23.0 ± 0.0	23.2 ± 0.1	23.1 ± 0.2
8K + 0.5 p/s	23.5 ± 0.0	23.1 ± 0.1	23.3 ± 0.0	23.2 ± 0.1	22.9 ± 0.0	23.5 ± 0.1	23.2 ± 0.2
8K + 0.8 p/s	23.3 ± 0.0	23.4 ± 0.1	23.4 ± 0.0	22.7 ± 0.1	23.0 ± 0.1	23.4 ± 0.0	23.2 ± 0.3
8K + Comp	23.3 ± 0.1	23.4 ± 0.0	23.4 ± 0.0	22.8 ± 0.0	23.1 ± 0.1	23.5 ± 0.1	23.3 ± 0.3
Relative Humidity (%)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
Ground Level	10.8 ± 0.9	10.8 ± 0.9	14.3 ± 1.5	14.0 ± 1.8	12.7 ± 1.2	12.1 ± 0.4	12.5 ± 1.5
8K	9.4 ± 0.6	10.4 ± 0.6	12.5 ± 0.9	13.0 ± 1.2	11.7 ± 0.8	9.9 ± 0.5	11.2 ± 1.5
8K + 0.2 p/s	8.8 ± 0.5	9.2 ± 0.5	11.8 ± 1.2	12.6 ± 0.7	12.6 ± 1.1	10.7 ± 0.6	10.9 ± 1.7
8K + 0.5 p/s	9.1 ± 0.6	9.6 ± 0.7	12.4 ± 0.7	12.7 ± 1.3	12.9 ± 0.7	9.6 ± 0.6	11.1 ± 1.8
8K + 0.8 p/s	10.2 ± 0.7	9.4 ± 0.9	11.8 ± 1.0	14.8 ± 1.5	12.8 ± 0.9	10.2 ± 0.4	11.5 ± 2.0
8K + Comp	10.3 ± 0.7	8.8 ± 0.8	11.6 ± 1.0	12.7 ± 1.1	11.4 ± 1.1	9.3 ± 0.9	10.7 ± 1.5
Oxygen (%)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Average
Ground Level	44 ± 7	50 ± 11	53 ± 8	57 ± 7	54 ± 13	60 ± 9	51 ± 7
8K	48 ± 9	67 ± 12	63 ± 17	73 ± 11	78 ± 15	69 ± 14	66 ± 10
8K + 0.2 p/s	44 ± 8	44 ± 10	60 ± 10	61 ± 13	62 ± 19	61 ± 10	55 ± 9
8K + 0.5 p/s	44 ± 10	52 ± 11	58 ± 8	61 ± 16	64 ± 15	63 ± 9	57 ± 8
8K + 0.8 p/s	49 ± 11	47 ± 9	58 ± 9	57 ± 12	62 ± 14	56 ± 9	55 ± 6
8K + Comp	55 ± 12	42 ± 9	56 ± 9	58 ± 13	63 ± 12	56 ± 17	55 ± 7

Table C3. Fluctuation and Environmental Data Phase 3 Experiment #1

8K + 1.2 p/s	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
Decompression Peak Rate of Change (p/s)	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	1.1 ± 0.0
Decompression Average Rate of Change (p/s)	0.7 ± 0.3	0.7 ± 0.3	0.7 ± 0.3	0.8 ± 0.3	0.7 ± 0.3	0.7 ± 0.3	0.7 ± 0.3	0.7 ± 0.3	0.7 ± 0.0
Recompression Peak Rate of Change (p/s)	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.0
Recompression Average Rate of Change (p/s)	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.0
Average Duration of Fluctuations (s)	6.8 ± 0.1	6.7 ± 0.3	6.8 ± 0.2	6.9 ± 0.3	6.9 ± 0.2	6.8 ± 0.2	6.8 ± 0.3	6.8 ± 0.2	6.8 ± 0.0
Maximum Altitude During Fluctuations (feet)	12792 ± 321	12841 ± 394	12820 ± 354	12952 ± 376	13058 ± 379	13055 ± 245	13077 ± 353	12946 ± 378	12943 ± 115
Minimum Altitude During Fluctuations (feet)	5994 ± 256	6004 ± 345	5989 ± 310	5960 ± 325	6048 ± 366	6071 ± 296	6044 ± 359	6055 ± 301	6021 ± 39
Steady Altitude (feet)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
GL	829 ± 104	829 ± 104	828 ± 104	829 ± 104	829 ± 104	829 ± 105	829 ± 106	828 ± 106	829 ± 0
8K	7995 ± 138	7995 ± 136	7993 ± 136	7995 ± 136	7996 ± 131	7998 ± 131	7995 ± 132	7995 ± 131	7995 ± 1
8K + 1.2 p/s	7969 ± 189	7961 ± 166	7956 ± 162	7959 ± 174	7954 ± 151	7954 ± 149	7950 ± 145	7955 ± 157	7957 ± 6
Temperature (°C)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
GL	22.3 ± 0.1	21.6 ± 0.1	21.4 ± 0.0	21.2 ± 0.0	21.7 ± 0.0	21.7 ± 0.0	21.1 ± 0.0	21.4 ± 0.0	21.5 ± 0.4
8K	21.6 ± 0.1	21.9 ± 0.1	21.4 ± 0.0	21.2 ± 0.0	21.5 ± 0.0	21.4 ± 0.0	21.1 ± 0.0	21.3 ± 0.0	21.4 ± 0.2
8K + 1.2 p/s	21.6 ± 0.1	21.9 ± 0.1	21.5 ± 0.0	21.2 ± 0.0	21.3 ± 0.0	21.5 ± 0.1	21.2 ± 0.0	21.2 ± 0.1	21.4 ± 0.2
8K + Comp	22.1 ± 0.1	21.4 ± 0.0	21.1 ± 0.0	21.4 ± 0.0	21.4 ± 0.0	21.2 ± 0.1	21.3 ± 0.0	21.1 ± 0.0	21.4 ± 0.3
Relative Humidity (%)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
GL	34.8 ± 8.8	32.6 ± 7.9	34.0 ± 7.8	35.9 ± 8.2	26.2 ± 4.2	28.1 ± 6.8	31.0 ± 7.0	36.6 ± 6.5	32.4 ± 3.7
8K	30.8 ± 4.9	35.6 ± 6.9	33.7 ± 3.9	36.2 ± 4.9	28.9 ± 3.7	30.3 ± 3.6	30.6 ± 3.7	31.4 ± 4.7	32.2 ± 2.7
8K + 1.2 p/s	29.0 ± 5.8	33.4 ± 7.5	31.9 ± 5.3	38.1 ± 5.9	25.9 ± 4.5	28.0 ± 4.4	26.6 ± 5.2	27.8 ± 4.6	30.1 ± 4.1
8K + Comp	35.1 ± 6.9	31.9 ± 4.9	36.3 ± 5.8	29.0 ± 4.5	28.2 ± 3.6	29.5 ± 4.2	30.3 ± 3.8	27.9 ± 3.7	31.0 ± 3.2

Table C4. Fluctuation and Environmental Data Phase 3 Experiment #2

Decompression Peak Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
8K + 1.5 p/s	1.5 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	ND	1.6 ± 0.2	1.5 ± 0.0
14K + 1.5 p/s	1.1 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.1
Decompression Average Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
8K + 1.5 p/s	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.5	1.0 ± 0.5	ND	1.0 ± 0.5	1.0 ± 0.0
14K + 1.5 p/s	0.8 ± 0.3	0.8 ± 0.4	0.8 ± 0.4	0.9 ± 0.4	0.8 ± 0.4	0.8 ± 0.4	0.9 ± 0.4	0.9 ± 0.4	0.8 ± 0.0
Recompression Peak Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
8K + 1.5 p/s	1.4 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.2	1.6 ± 0.2	ND	1.5 ± 0.1	1.5 ± 0.1
14K + 1.5 p/s	1.4 ± 0.1	1.5 ± 0.1	1.1 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.2	1.5 ± 0.1	1.4 ± 0.1
Recompression Average Rate of Change (p/s)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
8K + 1.5 p/s	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.4	1.1 ± 0.4	1.1 ± 0.4	1.1 ± 0.4	ND	1.1 ± 0.4	1.1 ± 0.0
14K + 1.5 p/s	1.0 ± 0.3	1.1 ± 0.4	0.9 ± 0.2	1.1 ± 0.4	1.1 ± 0.4	1.1 ± 0.4	1.1 ± 0.4	1.1 ± 0.4	1.1 ± 0.1
Average Duration per Fluctuation (sec)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
8K + 1.5 p/s	8.7 ± 0.5	8.6 ± 0.3	8.5 ± 0.1	8.6 ± 0.1	8.4 ± 0.3	8.3 ± 0.2	ND	8.5 ± 0.2	8.5 ± 0.1
14K + 1.5 p/s	8.6 ± 0.2	7.9 ± 0.3	8.8 ± 0.1	7.8 ± 0.3	7.8 ± 0.2	7.8 ± 0.3	7.5 ± 0.3	7.5 ± 0.3	8.0 ± 0.5
Maximum Altitude During Fluctuations (feet)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
8K + 1.5 p/s	15859 ± 403	15767 ± 468	15814 ± 389	15720 ± 346	15705 ± 445	15593 ± 434	ND	16122 ± 464	15797 ± 167
14K + 1.5 p/s	21596 ± 362	21992 ± 423	21965 ± 366	21948 ± 477	22145 ± 419	22066 ± 418	21795 ± 512	21904 ± 480	21926 ± 169
Minimum Altitude During Fluctuations (feet)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
8K + 1.5 p/s	4122 ± 322	4333 ± 348	4197 ± 381	4071 ± 313	4226 ± 423	4221 ± 445	ND	4125 ± 340	4185 ± 87
14K + 1.5 p/s	10026 ± 412	10168 ± 507	10346 ± 302	10102 ± 455	10226 ± 553	10229 ± 506	10323 ± 485	10377 ± 476	10225 ± 122
Steady Altitude (feet)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
8K	7994 ± 141	7995 ± 138	8024 ± 104	7999 ± 169	8000 ± 167	8002 ± 169	7999 ± 170	7999 ± 168	8001 ± 10
14K	14037 ± 67	14037 ± 77	14031 ± 81	14031 ± 80	14038 ± 72	14038 ± 71	14038 ± 72	ND	14036 ± 3
8K + 1.5 p/s	7968 ± 180	7966 ± 175	7967 ± 182	7961 ± 168	7964 ± 173	7956 ± 159	ND	7969 ± 199	7964 ± 5
14K + 1.5 p/s	14151 ± 206	14133 ± 227	14132 ± 233	14188 ± 254	14178 ± 247	14151 ± 235	14146 ± 234	14233 ± 285	14164 ± 34
Temperature (°C)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
8K	22.8 ± 0.1	22.9 ± 0.1	22.9 ± 0.0	22.7 ± 0.1	23.2 ± 0.0	22.8 ± 0.1	22.5 ± 0.0	22.3 ± 0.1	22.7 ± 0.3
14K	22.8 ± 0.1	22.8 ± 0.1	22.9 ± 0.1	22.9 ± 0.1	23.0 ± 0.1	22.6 ± 0.1	22.4 ± 0.1	ND	22.8 ± 0.2
8K + 1.5 p/s	22.8 ± 0.2	22.8 ± 0.1	23.1 ± 0.1	23.1 ± 0.1	23.0 ± 0.1	23.1 ± 0.1	ND	22.9 ± 0.1	23.0 ± 0.1
14K + 1.5 p/s	22.9 ± 0.1	22.9 ± 0.1	22.8 ± 0.1	22.8 ± 0.1	22.9 ± 0.1	23.2 ± 0.1	23.0 ± 0.1	22.9 ± 0.1	22.9 ± 0.1
Relative Humidity (%)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Average
8K	12.3 ± 1.4	10.5 ± 1.1	23.6 ± 2.1	15.4 ± 1.4	15.7 ± 1.5	16.1 ± 1.4	16.1 ± 1.1	16.1 ± 1.0	15.7 ± 3.8
14K	9.8 ± 1.4	12.9 ± 1.5	20.3 ± 2.9	22.3 ± 3.1	13.9 ± 1.7	13.9 ± 1.8	13.8 ± 1.6	ND	15.3 ± 4.4
8K + 1.5 p/s	10.4 ± 1.8	10.0 ± 1.5	13.2 ± 2.0	22.3 ± 3.5	14.2 ± 2.7	14.5 ± 2.4	ND	10.1 ± 1.5	13.5 ± 4.3
14K + 1.5 p/s	9.0 ± 1.8	11.3 ± 2.0	19.4 ± 3.6	13.4 ± 2.3	12.6 ± 2.0	13.0 ± 2.3	8.9 ± 1.6	8.7 ± 1.4	12.0 ± 3.5