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# Comparison of the Human Transcriptional Response to Three Hypoxic Environments

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16. Abstract

Hypoxia awareness training is a standard facet of military aviator training that is performed to familiarize aviators with the symptoms of hypoxia. The three most common training devices are the hypobaric altitude chamber (AC), the normobaric reduced oxygen breathing device (ROBD), and the normobaric reduced oxygen breathing environment (ROBE). The AC creates hypoxic conditions using reduced atmospheric pressure. The ROBD and ROBE create normobaric hypoxic conditions by supplying a controlled reduced-oxygen gas mixture to trainees who don an aviation-style breathing mask or occupy a sealed chamber, respectively. To determine if differences in the transcriptional response to each of these training devices exist, blood ribonucleic acid (RNA) samples from ten volunteer participants were analyzed by microarray. The resulting gene expression measures were screened for significant changes across time points within and across device runs. Few genes were significant in these comparisons; the most significant differences between timepoints were observed in small nucleolar RNAs and noncoding RNAs, as well as one microRNA and one transfer RNA. The transcriptional response to each training device differs slightly as determined by differences in transcription between time points. However, the role of these transcriptional changes is unclear, as little information exists as to their function or role in the hypoxic response. As all the examined methods induced hypoxic symptoms, and very little difference was observed in gene expression between methods, this limited study did not detect the presence of substantial differences between hypoxia awareness training devices. Future studies using more sensitive sequencing-based gene expression analysis techniques and larger sample sizes may improve the detection of transcriptional differences induced by each training device.


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## Table of Contents

## List of Figures



## List of Tables

## List of Abbreviations

| | |
|---|---|
| AC | Hypobaric Altitude Chamber |
| AFRL | Air Force Research Laboratory |
| DCS | Decompression Sickness |
| DE | Differential gene expression |
| FAA | Federal Aviation Administration |
| fpm | Feet per minute |
| GED | General Educational Development |
| HH | Hypobaric hypoxic |
| IRB | Institutional Review Board |
| LFC | $log_2$ fold change |
| lncRNA | Long noncoding RNA |
| miRNA | microRNA |
| ncRNA | Noncoding RNA |
| NH | Normobaric hypoxia |
| $O_2$ | Oxygen |
| RNA | Ribonucleic acid |
| ROBD | Reduced Oxygen Breathing Device |
| ROBE | Reduced Oxygen Breathing Environment |
| $SpO_2$ | Oxygen Saturation |
| ss-cDNA | single-stranded complementary DNA |
| tRNA | Transfer RNA |
| USAF | United States Air Force |
| USN | United States Navy |

**Abstract**

Hypoxia awareness training is a standard facet of military aviator training that is performed to familiarize aviators with the symptoms of hypoxia. The three most common training devices are the hypobaric altitude chamber (AC), the normobaric reduced oxygen breathing device (ROBD), and the normobaric reduced oxygen breathing environment (ROBE). The AC creates hypoxic conditions using reduced atmospheric pressure. The ROBD and ROBE create normobaric hypoxic conditions by supplying a controlled reduced-oxygen gas mixture to trainees who don an aviation-style breathing mask or occupy a sealed chamber, respectively. To determine if differences in the transcriptional response to each of these training devices exist, blood ribonucleic acid (RNA) samples from ten volunteer participants were analyzed by microarray. The resulting gene expression measures were screened for significant changes across time points within and across device runs. Few genes were significant in these comparisons; the most significant differences between timepoints were observed in small nucleolar RNAs and noncoding RNAs, as well as one microRNA and one transfer RNA. The transcriptional response to each training device differs slightly as determined by differences in transcription between time points. However, the role of these transcriptional changes is unclear, as little information exists as to their function or role in the hypoxic response. As all the examined methods induced hypoxic symptoms, and very little difference was observed in gene expression between methods, this limited study did not detect the presence of substantial differences between hypoxia awareness training devices. Future studies using more sensitive sequencing-based gene expression analysis techniques and larger sample sizes may improve the detection of transcriptional differences induced by each training device.

## Introduction

Hypoxia is a well-known aviation risk with symptoms ranging in severity from mild disorientation to loss of consciousness, potentially resulting in aviator impairment and/or loss of aircraft control. In-flight hypoxia most often occurs slowly over time rather than suddenly (i.e., rapid decompression) and aviators who are not trained to recognize the symptoms may be unable to recognize hypoxia when it occurs (Files et al., 2005). To provide aviators the opportunity to become familiar with their unique hypoxia symptoms, hypoxia awareness training programs were established wherein trainees are exposed to hypoxic conditions or environments, permitting a monitored first-hand experience with hypoxia and its impairing characteristics. The three most common hypoxia awareness training devices are the hypobaric altitude chamber (AC), the normobaric Reduced Oxygen Breathing Device (ROBD; Sausen et al., 2003; Artino et al., 2006), and the normobaric Reduced Oxygen Breathing Environment (ROBE; Sausen et al., 2001; Neuhaus & Hinckelbein, 2014).

The AC simulates the hypobaric hypoxic (HH) environment experienced during aviation by subjecting trainees to hypobaria like that experienced at various altitudes, but also presents risks unique to hypobaric exposure (Dully, 1992; Artino et al., 2006; Morgagni et al., 2010). Use of the AC as a training device is restrictive in that it incurs a significant expense, has limited seating capacity, requires trained inside safety observers who are subjected to altitude exposure limits, and carries the additional risk of injury due to the hypobaric conditions it produces, such as altitude decompression sickness (DCS) (Morgagni et al., 2010; Ercan et al., 2020).

Normobaric hypoxia (NH) awareness training devices eliminate the risk of DCS and the additional precautions necessitated by AC use (Artino et al., 2006; Self et al., 2011; Leinonen et al., 2021). NH devices function by introducing a reduced-oxygen breathing gas mixture to participants at ambient pressure. In NH devices, the oxygen partial pressure is altered to approximate the available oxygen levels of various altitudes, with breathing gas oxygen concentration decreasing as “altitude” increases (Artino et al., 2006). The ROBD is a portable gas-blending device that uses thermal mass flow controllers to mix breathing air and nitrogen, supplying variable oxygen gas mixtures to an aviation mask-style respirator worn by trainees (Artino et al., 2009). In comparison, the ROBE is a sealed enclosure into which a defined gas mixture is introduced. The ROBE operates differently from the ROBD by “scrubbing” outside air of oxygen and introducing it into a sealed chamber and thus does not rely on supplemental gas supplies. The ROBE used in this study was a sealed plexiglass chamber equipped with zeolite molecular sieves that trapped and expelled oxygen but allowed nitrogen to pass freely into the chamber. The zeolite filters were adjusted to modulate the oxygen level within the breathing air, which was constantly supplied to the chamber at selectable flow rates. Despite their different modes of operation, the ROBE and ROBD each generate a low-oxygen environment by selectively altering the amount of oxygen present in the participant’s breathing gas mixture, whereas the AC reduces the availability of all atmospheric gases.

The extent to which each training device creates the most realistic simulation of a high-altitude exposure, and whether any significant differences in trainee biological response exist between these three devices, is the subject of debate (Conkin & Wessel, 2008; Conkin, 2016). HH training generates altered physiological responses compared to NH, particularly tidal breathing volume (lower in HH) and breathing frequency (lower in HH) (Coppel et al., 2015). Alveolar gas composition differences exist between HH and NH methods in some instances (Conkin, 2016; Hemmingsson & Linnarsson, 2009), but such differences dissipate over time (Self et al., 2011). HH is more similar to true altitude exposure when exercise is considered, with elevated oxygen saturation ($SpO_2$) and decreased right ventricle myocardial performance and right ventricular systolic pressure in HH compared to NH. HH leads to increased muscle power, movement velocity, and maximal strength (Boos et al., 2016; Feriche et al., 2014). The occurrence of hypoxia symptoms between NH and HH exposures also varies over time, with prolonged HH leading to altitude-related maladies such as acute mountain sickness (Richard & Koehle, 2012). To address organismal differences between devices from a transcriptional perspective, we assessed the influence of equivalent HH and NH hypoxic exposures on total blood ribonucleic acid (RNA) transcription in volunteer subjects using each of three hypoxic awareness training devices (AC, ROBD, ROBE). This study was designed to answer whether these hypoxic exposures generated measurable transcriptomic differences, and to determine what differences exist in hypoxia-induced gene expression between these training devices.

## Methods

### Experimental Participants

The human participants work for this study took place at the United States Air Force (USAF) Research Laboratory (AFRL) at the USAF School of Aerospace Medicine (Wright-Patterson Air Force Base, Dayton, Ohio), and was conducted with the approval of the AFRL and the Federal Aviation Administration (FAA) Institutional Review Boards (IRBs). Transcriptomic analysis of blood samples was performed at the FAA's Civil Aerospace Medical Institute in accordance with approved IRB protocols. Volunteer participants were recruited from among male and female U.S. military service members according to the following criteria:

- aged 18 and 40 years
- passed a USAF (or United States Navy [USN] equivalent) Medical Class III or higher
- physical exam conducted by a USAF or USN Flight Surgeon prior to the study
- non-smoking for more than one year
- high school diploma/GED degree or higher education
- previous experience with altitude-induced hypoxia and hypoxic symptoms
- previously received training on the use of AC, ROBE, or ROBD devices and related equipment and facilities.

Participants were recruited from personnel already undergoing hypoxia awareness training. Prior to participation in any testing session, participants were required to:

- avoid excessive caffeine consumption on the day of each chamber/device run

- avoid alcohol consumption for 24-hours prior to each chamber/device run
- avoid hyperbaric exposure 24-hours prior to each chamber/device run
- be well rested with normal sleep prior to each chamber/device run.

Nineteen participants were enrolled in this study. Four participants did not complete the protocol, and the blood RNA of five participants fell below the RNA quality criteria described below. The blood and RNA samples passing the study's analytical criteria were from male participants.

**Hypoxia Sessions**

Two different device "flight" profiles were performed, one for the AC and one for the ROBD and ROBE (Figure 1). The difference in flight profiles was due to an additional "ear and sinus check" at the beginning of the AC flight, implemented to ensure that participants did not experience ear or sinus pain due to hypobaria. AC flights consisted of a pre-flight, site-level medical check to screen for ear and/or sinus congestion that would have prevented a participant from clearing their ears/sinuses by performing the Valsalva maneuver (forceful exhalation through pursed lips performed while straining the abdominal musculature and pinching the nose closed). Participants who passed the site level medical check then performed the Valsalva maneuver to ensure they could clear their ears/sinuses. Those who completed the Valsalva maneuver continued to the AC experimental flight profile and the first blood draw ($T_1$).

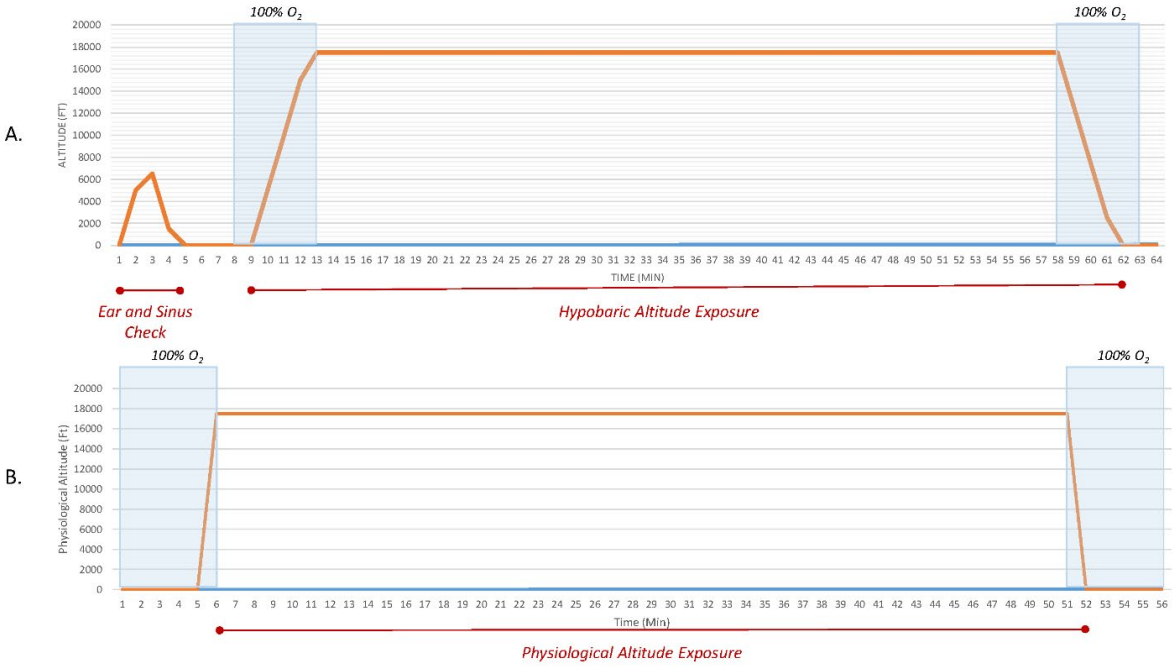


**Figure 1.** Hypoxia Awareness Training Device Flight Profiles. A. Hypobaric Altitude Chamber (AC) flight profile. Hypobaric hypoxic exposure continued for 45 minutes at 17,500 feet-equivalent. B. ROBE and ROBD flight profile. Normobaric hypoxic exposure continued for 45 minutes at 17,500 feet-equivalent. In each flight profile type, blood draws were taken pre-flight, immediately after the hypoxic exposure, at 15 minutes post-hypoxic exposure, and 2 hours post-hypoxic exposure.

Participants entered the AC where they were familiarized with the AC and the aviator oxygen masks, and then given a preflight brief. After this, participants were subjected to an initial ascent to 6,300 feet (ft.)-equivalent and return to site level (i.e., ambient pressure) to confirm that ear and sinus problems were not present. Participants not experiencing ear or sinus pain then breathed 100% oxygen ($O_2$) for five minutes while the AC climbed to and leveled off at 17,500 ft.-equivalent at a rate of 5,000 ft. per minute (fpm). Participants then dropped their $O_2$ mask and remained at 17,500 ft.-equivalent for a maximum of 45 minutes. Participants who experienced severe hypoxia symptoms as defined by the participant or the inside observer, or whose $SpO_2$ (measured using a Nonin 8000R forehead-mounted pulse oximeter) decreased below 60%, were given supplemental $O_2$ and the flight profile terminated (i.e., AC returned to site level). After 45 minutes at 17,500 ft.-equivalent, participants re-donned their oxygen mask and breathed 100% $O_2$ for five minutes while the AC descended to site level (i.e., ambient pressure) at 5,000 fpm. When AC descent was complete, the participant dropped their $O_2$ mask and exited the AC. The first post-hypoxic exposure blood draw was obtained ($T_2$). Blood draw three ($T_3$) was taken 15 minutes post-hypoxic exposure, and blood draw four ($T_4$) was taken 2 hours post-hypoxic exposure.

ROBE and ROBD flights began with a pre-flight brief, initial blood draw ($T_1$), and equipment/aviator oxygen mask familiarization. Participants then breathed 100% $O_2$ for five minutes prior to entry into the ROBE or use of the ROBD, each set to an oxygen partial pressure equivalent of 17,500 ft. Participants maintained normobaric hypoxic exposure within each environment by remaining in the ROBE, or breathing through an aviator oxygen/ROBD mask, for a maximum of 45 minutes. Participants who experienced severe hypoxia symptoms, or whose $SpO_2$ decreased below 60%, were given supplemental 100% $O_2$ and then proceeded through the remaining blood draws. After 45 minutes of normobaric hypoxic exposure, participants were supplied with 100% $O_2$ for five minutes, exited the ROBE, or dropped the aviator $O_2$/ROBD mask. Blood draws were then taken immediately ($T_2$), 15 minutes post-normobaric hypoxic exposure ($T_3$), and 2 hours post-normobaric hypoxic exposure ($T_4$). Each participant completed one flight in each of the AC, ROBE, and ROBD, plus one additional session in a randomly-selected device. Flight order was randomized for each participant. The crossover design of this study allowed each subject to serve as their own control.

**Biological Samples**

Blood samples were collected in PAXgene® Blood RNA (BD Biosciences) tubes during the pre-flight exam immediately prior to each hypoxia session, immediately following each hypoxia session, 15 minutes after each hypoxia session, and 2 hours after each hypoxia session. PAXgene® tubes were inverted 10 times immediately following collection and frozen at -20ºC for one day, followed by freezing at -80ºC. The blood was then thawed for processing overnight with gentle rotation (10 rpm) and processed using the QIAGEN PAXgene® Blood miRNA kit (BD Biosciences) using a QIAcube and stored at -80ºC. RNA aliquots were spectrophotometrically quantified using a Nanodrop 2000 (ThermoFisher Scientific) instrument.

RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent) with Agilent RNA6000 Nano assay components (Agilent).

**Microarray Preparation and Bioinformatic Analysis**

RNA samples with RNA integrity numbers (RIN) ≥7.0 were selected for use in microarray gene expression analyses. Total RNA from each sample was used to prepare single-stranded complementary DNA (ss-cDNA) libraries with the Affymetrix GeneChip® WT PLUS reagent kit (ThermoFisher Scientific) according to manufacturer specifications. ss-cDNA was fragmented, labeled, and hybridized using the ThermoFisher Scientific GeneChip® Hybridization, Wash, and Stain Kit (ThermoFisher Scientific) onto GeneChip® Human Transcriptome Assay 2.0 microarrays (ThermoFisher Scientific) for analysis. Microarrays were hybridized for 18 hours, rotating at 60 rpm at 45ºC. Chips were then washed and stained using GeneChip® fluidics stations 450 (ThermoFisher Scientific) using protocol FS450-0001, per the HTA 2.0 microarray protocol. Stained and washed microarrays were scanned using a 7G GeneChip® Scanner 3000 (ThermoFisher Scientific).

**Data Quality Control and Differential Expression Detection**

Initial quality control was performed for each sample with Affymetrix Expression Console 1.4.1.46. The .cel file scans consisted of samples from ten individuals over four time points for each of the three hypoxia training devices, for a total of 120 samples. Raw .cel files were deposited into the NCBI GEO database, accession GSE219264 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE219264). Raw .cel files were read into R version 3.3.1 (R Core Team, 2016) together using the Bioconductor package oligo (Carvalho & Irizarry 2010) and normalized together using 'oligo::rma'. Quality assessment was performed using the arrayQualityMetrics package (Kauffmann et al., 2009) and commands 'fitProbeLevelModel' and 'arrayQualityMetrics'. No significant quality concerns were found and all 120 samples were retained. The final gene set was determined by filtering; transcript clusters were retained in the filtered dataset if their expression in at least one sample exceeded the third-quartile value of antigenomic transcript clusters. Bioconductor package limma (Ritchie et al., 2015) was used to determine differential gene expression (DE) between timepoint within hypoxia awareness training devices, and between device timepoints across hypoxia awareness training devices using $Log_2$ Fold Change (LFC) ≥|0.3| and an adjusted P-value, also called false discovery rate (FDR) ≥ 0.05 (Figure 2). Duplicate correlation was used to block by participant.

R Bioconductor package 'timecourse' (Tai& Speed, 2007) was used over the four time points to rank probesets for significant gene expression changes over time for each hypoxia awareness training device, and to compare the most significantly changed genes over the entire run for each training device, as indicated by Hotelling $T^2$ values of 70 or greater. Timecourse comparisons were performed using the participant-blocked datasets previously generated by limma to compare ROBD-AC, ROBD-ROBE, and ROBE-AC to indicate genes that show potential DE between hypoxia awareness training devices.

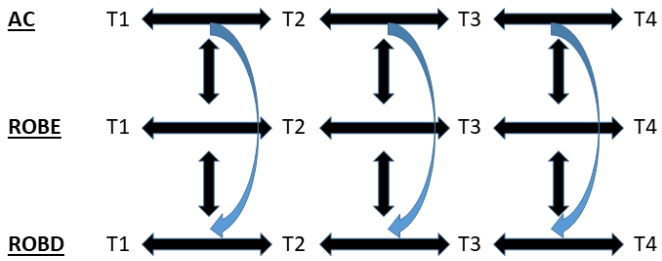


**Figure 2**. Bioinformatic Comparisons Made Between Timepoints Within and Between Hypoxia Awareness Training Devices Using Limma Software. Similar comparisons by timepoint within the training device were made using Timecourse software. After controlling for participant, each timepoint within each training device was compared to determine the presence of differentially expressed genes between sampling points. After controlling for participant and timepoint, differential expression was assayed between timepoints across training devices.

## Results and Discussion

### Hypoxia Sessions

This study was conducted as a part of the typical USAF hypoxia awareness training regimen, intended to provide aviators with the opportunity to experience, recognize, and treat their unique hypoxia symptoms. As a safety precaution, when a participant's $SpO_2$ value fell below 60%, or when a participant requested supplemental oxygen, the participant was removed from the hypoxia session and given 100% $O_2$ until recovered, and the session was not repeated. Blood sample collection continued throughout the hypoxia session regardless of whether the entire hypoxic exposure time was completed. Of the 19 participants who completed this study, 10 sets were ultimately selected for analysis based on acceptability of RNA quality measurements. Of these 10 participants, four were removed from an individual hypoxia session by study administrators due to low $SpO_2$ levels (Table 1).

### Differential Gene Expression Within and Between Hypoxia Awareness Training Devices

Total RNA was prepared according to the manufacturer's instructions and hybridized with Affymetrix HTA 2.0 microarray slides (ThermoFisher Scientific). The files produced by these microarray slides were summarized as a single pool by R/bioconductor oligo package (Carvalho & Irizarry, 2010), and then analyzed for differential gene expression between timepoints and across treatment group (i.e., hypoxia awareness training device; Supplementary Tables 1-3). The summarized datasets, comprising 15,067 separate genes, were first analyzed using limma software (Ritchie et al., 2015) according to subsequent comparisons. To evaluate gene expression changes by timepoint within each hypoxia awareness training device, consecutive timepoints within each device hypoxia session were compared (i.e., $T_1$ vs $T_2$, $T_2$ vs. $T_3$, $T_3$ vs $T_4$). This comparison was made to determine if gene expression changed between timepoints. Second, differential gene expression between timepoints was compared across hypoxia

awareness training devices to detect differences in timepoint-related gene expression between each training device.

**Table 1.** Participant Hypoxia Exposure Record

| Participant # | Sex | AC | | ROBD | | ROBE | |
|---|---|---|---|---|---|---|---|
| | | Device Time (min) | Termination Reason | Device Time (min) | Termination Reason | Device Time (min) | Termination Reason |
| **1** | M | 45 | NA | 45 | NA | 45 | NA |
| **6** | M | 27 | $SpO_2 \leq 60$ | 45 | NA | 45 | NA |
| **7** | M | 45 | NA | 45 | NA | 45 | NA |
| **9** | M | 45 | NA | 42 | $SpO_2 \leq 60$ | 45 | NA |
| **11** | M | 45 | NA | 42 | $SpO_2 \leq 60$ | 45 | NA |
| **12** | M | 45 | NA | 45 | NA | 45 | NA |
| **13** | M | 45 | NA | 45 | NA | 45 | NA |
| **14** | M | 45 | NA | 45 | NA | 32 | $SpO_2 \leq 60$ |
| **17** | M | 45 | NA | 45 | NA | 45 | NA |
| **21** | M | 45 | NA | 45 | NA | 45 | NA |

*Note.* AC = altitude chamber; ROBD = reduced oxygen breathing device; ROBE = reduced oxygen breathing environment; NA = not applicable; $SpO_2$ = oxygen saturation.

These comparisons allowed assessment of changes in gene expression in two ways: (1) in individual participants over time based on the hypoxia training device (i.e., within device comparison), and (2) a comparison of temporal changes across training devices (i.e., between device comparison). The rationale was that time-dependent changes during each hypoxia session were the most important to note and that differences in those time-dependent changes could be compared across training devices.

Only two differentially expressed transcript clusters were noted in the limma timepoint comparison – the comparison between ROBD timepoints 3 and 4, each corresponding to long intergenic non-protein coding *RNA1410* (*LINC 01410*, localized to transcript clusters TC09001962.hg.1, adj.P.val = 0.0387, LFC = -0.50451, and TC09000275.hg.1, adj.P.val = 0.040991, LFC = -0.48996). *LINC01410* is thought to be involved in angiogenesis and tumor growth, and interacts in a regulatory fashion with the miRNAs *miR545-2p* and *miR-545-3p* as well as hexokinase 2 (*HK2*) (Zhang et al., 2018; Mou et al., 2021; Liu & Wen, 2020). The $\log_2$ fold change (LFC) seen in this comparison indicates an increased expression in ROBD $T_4$, possibly indicating an initial adaptive angiogenic response to the hypoxia induced during ROBD

exposure. The timepoints involved in this comparison are the blood samples collected at 15 minutes post-hypoxic exposure ($T_3$) and 2 hours post-hypoxic exposure ($T_4$) using the ROBD training device. No statistically significant differences between timepoints existed in any of the other comparisons.

Timecourse software was used to provide an alternate analysis of gene expression trends over each blood draw timepoint between training device hypoxia sessions. Gene expression data from each training device were compared to all others (i.e., AC vs. ROBE, AC vs. ROBD, ROBE vs. ROBD) and genes with a Hotelling $T^2$ score ≥ 70 were considered significant (Table 2, Figure 3, Supplementary Table 4).

**Table 2**. Differentially Expressed Transcripts According to Hotelling $T^2$ Score, Determined by the Timecourse Software Package.

| | Transcript Cluster ID | Hotelling $T^2$ | Transcript ID | Transcript Description | Gene Symbol |
|---|---|---|---|---|---|
| **AC vs ROBE** | TC19001207.hg.1 | 118.3404 | ENST00000386967 | small nucleolar RNA, C/D box 41 | SNORD41 |
| | TC11000808.hg.1 | 79.27746 | ENST00000384714 | small nucleolar RNA, C/D box 15B | SNORD15B |
| **AC vs. ROBD** | TC05003165.hg.1 | 88.51031 | AK074622 | AK074622 mRNAlike lncRNA | NA |
| | TC05000983.hg.1 | 78.79425 | ENST00000363778 | RNA, U6 small nuclear 226 | NA |
| | TC02003749.hg.1 | 74.21669 | TCONS_00003974 | Human lincRNA TCONS_00003974 | NA |
| | TC01001056.hg.1 | 70.20505 | ENST00000459390 | novel ncrna ENSG00000239012 | NA |
| | TC01003077.hg.1 | 70.20505 | ENST00000458828 | miRNA ENSG00000239165 | NA |
| | TC12000759.hg.1 | 70.13401 | uc021rcj.1 | transfer RNA Asp (anticodon GTC) | NA |
| **ROBD vs ROBE** | TC15001564.hg.1 | 83.02989 | ENST00000384176 | Small nucleolar RNA SNORA24 | SNORA24 |
| | TC15001581.hg.1 | 80.69862 | ENST00000362803 | small nucleolar RNA, C/D box 16 | SNORD16 |
| | TC04000600.hg.1 | 75.39746 | ENST00000384096 | small nucleolar RNA, H/ACA box 24 | SNORA24 |

***Note***. A Hotelling $T^2$ cutoff of ≥70 was used to designate differential gene expression. AC = altitude chamber; mRNA, microRNA; lncRNA, long noncoding RNA; RNA = ribonucleic acid; ROBD = reduced oxygen breathing device; ROBE = reduced oxygen breathing environment.

In the ROBE vs. ROBD comparison (Figure 3C), *SNORA24* (two transcript clusters corresponding to this gene were differentially expressed) and *SNORD16* were indicated as DE by Hotelling $T^2$ score, with slight distinction between those conditions in each gene occurring at $T_2$ (i.e., immediately after hypoxic exposure). *SNORA24* and *SNORD 16* are each associated with cancers; *SNORA24* has a role in cancer suppression (McMahon et al., 2019) and *SNORD16* is overexpressed in colon and esophageal cancers (He et al., 2020; Tian et al., 2021).

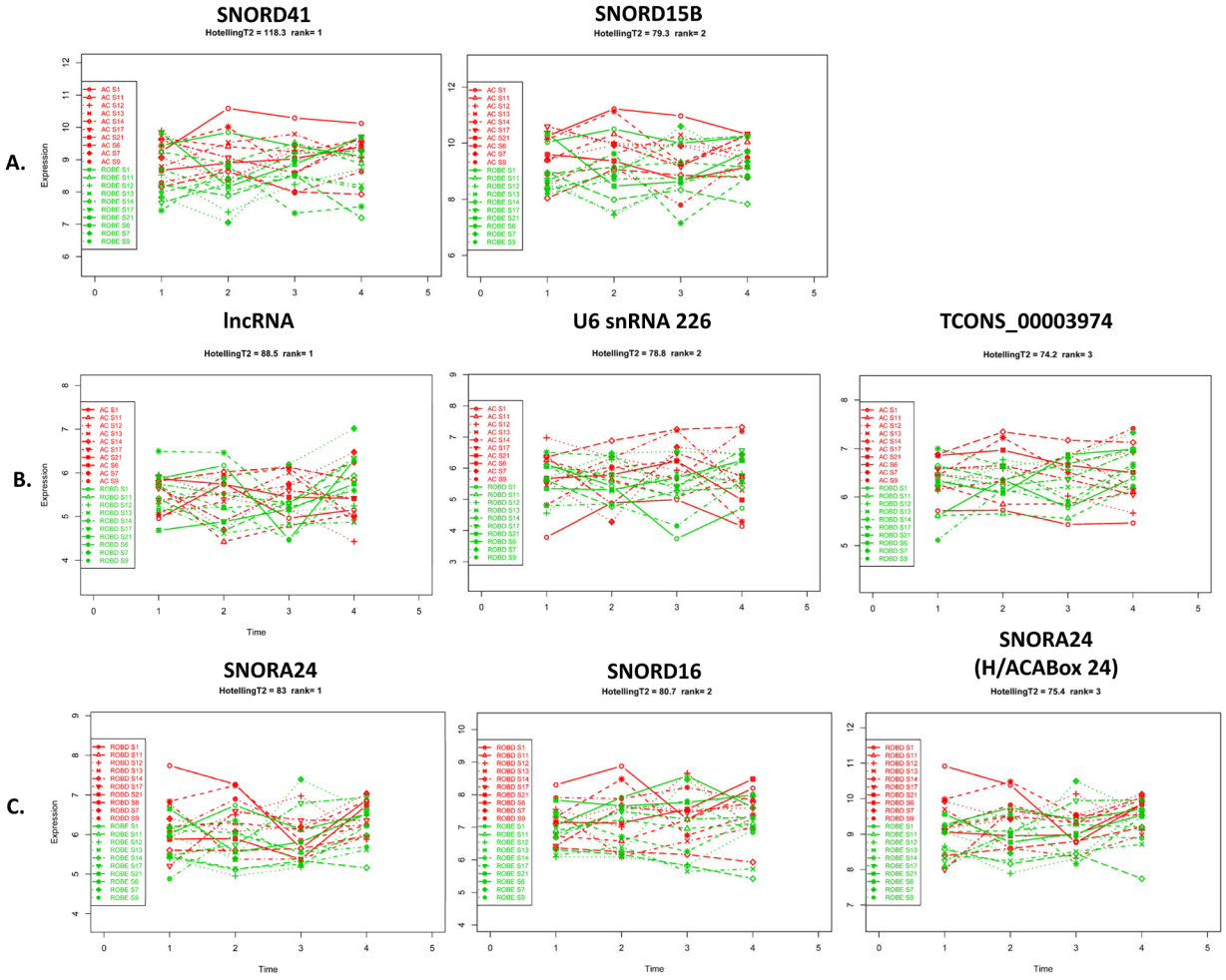


**Figure 3**. Timecourse Software-Generated Expression Profiles by Sample for the Three Most Differentially-Expressed Genes in Each Comparison. X axis represents timepoint (1-4), Y axis represents relative gene expression based on microarray intensities. A. AC (red) vs. ROBE (green). Only two DE genes were observed in this comparison. B. AC (red) vs. ROBD (green). C. ROBD (red) vs ROBE (green). AC = altitude chamber; DE = differential expression; ROBD = reduced oxygen breathing device; ROBE = reduced oxygen breathing environment.

Comparing the AC and ROBD (Figure 3B), long noncoding RNAs (lncRNAs) *AK074622* and *U6 snRNA 226* expression increased at $T_3$ (i.e., 15 minutes post-hypoxic exposure) in participants exposed to the AC environment relative to the ROBD. *TCONS_00003974* decreased

in participants exposed to the AC at $T_2$, whereas it increased in participants exposed to the ROBD at the same timepoint. *TCONS_00003974* decreased in participants exposed to the ROBD through $T_3$, and then increased for the remainder of the monitoring period (i.e., through $T_4$, 2 hours post-hypoxic exposure). In contrast, it increased in participants exposed to the AC until $T_2$ and then decreased for the remainder of the monitoring period (i.e., $T_3$ and $T_4$, 15 minutes and 2 hours post-hypoxic exposure). Other differentially expressed genes (as determined by Hotelling $T^2$ score in the Timecourse analysis) in the AC vs ROBD training device comparison (i.e., noncoding RNA [ncRNA] *ENGSG00000239012*, microRNA [miRNA] *ENSG00000239165*, and transfer RNA *tRNA Asp*) did not display a clear pattern, and the corresponding expression charts are not shown. Only two identified genes have known functions; *U6 snRNA 226* is integral to RNA splicing mechanisms (Didychuk et al., 2018), and *tRNA Asp* transports aspartic acid during protein synthesis.

A direct comparison of AC vs ROBE revealed two DE genes -- small nucleolar RNAs *SNORD41* and *SNORD15B* (Figure 3A). *SNORD41* expression is associated with oxidative stress genes in neuroblastoma cells, although oxidative stress was not examined during the cited study (Sun et al., 2017), and *SNORD15B* overexpression is observed in colorectal cancer cells (Shen et al., 2022). Expression of both genes decreased in most participants at $T_2$ in the ROBE condition compared to the AC, and returned to baseline expression levels between $T_3$ and $T_4$. As none of these DE genes are known to play a clear role in hypoxia, their role in any response to hypoxic training device, or in the hypoxic conditions these devices produce, is speculative. In all of these comparisons, the distinction between expression profiles was weak and, in most cases, overlapping. Developing any “hypoxia” biomarker based on the expression profiles seen here would be difficult based on the present data.

## Conclusions

Changes in RNA expression were evident during exposure to hypoxic environments in each hypoxia awareness training devices used in this study. In each comparison, genes unique to each hypoxia training environment (i.e., training device) were DE according to the established thresholds. Due to the paucity of information regarding the function of the observed DE genes, it is unclear whether the DE genes indicate a hypoxic response. Known hypoxia-responsive genes such as *HIF-1a* (Semenza, 2012), were not observed to be DE between training device timepoints, indicating that the hypoxic environment flight profiles assayed here do not significantly alter the expression of well-known hypoxia-responsive genes. However, the finding that many of the observed DE genes are associated with cancer, may indicate their involvement in hypoxia, as hypoxia is a common condition in cancers (Eales et al., 2016). The inhalation of 100% $O_2$ prior to and immediately following each hypoxic exposure may have played a role in this observation, especially as no blood draws were taken when the participants were actively hypoxic.

This study did not demonstrate substantial differences between hypoxia awareness training devices, and the evidence presented does not recommend using one training device over another.

As each training device produces similar, although not identical, hypoxic symptoms, and all are commonly used for hypoxia awareness training, each device effectively fulfills its purpose (Self et al., 2011; Singh et al., 2010; Aebi et al., 2020; Kammerer et al., 2018; Hohenauer et al., 2022). The AC is an expensive labor- and maintenance-intensive piece of equipment, and has the additional risk of exposing participants to hypobaria and barotrauma. The ROBE and ROBD eliminate the hypobaria risk, while still inducing hypoxic symptoms. Although there may be compelling reasons to subject aviator trainees or participants to hypobaria, particularly when studying participants in a non-resting state, the ROBD and ROBE devices appear sufficient for their purpose of hypoxia exposure according to the results of this limited study. If future work is performed to distinguish transcriptional response(s) by type of hypoxia awareness training device, we recommend that such studies use more sensitive gene expression detection techniques (such as RNA-sequencing [RNAseq]), increase the sample size (i.e., a greater number of participants), and collect blood samples when participants are demonstrably hypoxic.

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