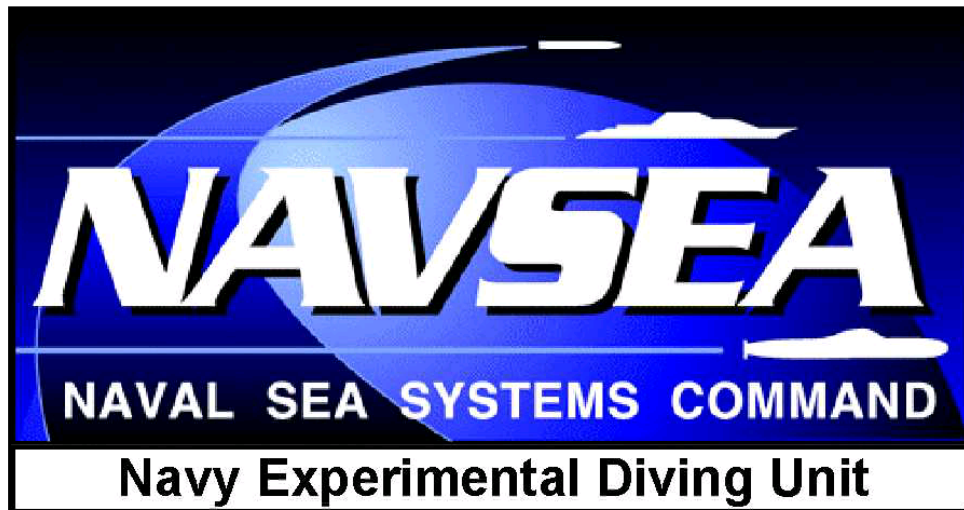


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Characterizing Hyperoxia-Induced Alterations in Muscular Physiology: Part 2

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List of Acronyms

ATA – Atmospheres absolute
ATP - Adenosine triphosphate
DNM1L - Dynamin-1-like protein
GO - Gene ontology
HMOX1 - Heme oxygenase 1
KEGG - Kyoto Encyclopedia of Genes and Genomes
MFN2 - Mitofusin-2
mRNA – Messenger ribonucleic acid
MT-CYB - Mitochondria Cytochrome b
MT-ND1 - Mitochondria Reduced nicotinamide adenine dinucleotide ubiquinone oxidoreductase chain 1
NRF1 - Nuclear respiratory factor 1
O₂ – Oxygen
OPA1 - Optic atrophy 1
PARK2 - Parkin 2
PGC-1 α - Peroxisome proliferator-activated receptor-gamma coactivator
PINK1 - PTEN-induced kinase-1
ROS – Reactive oxygen species
RPE – Rating of perceived exertion
sEMG – Surface electromyography
SOD1 - Superoxide dismutase 1
SOD2 - Superoxide dismutase 2
TFAM - Mitochondrial transcription factor A
UCP3 - Uncoupling protein 3
UMO – Undersea medical officer
 $\dot{V}O_2$ – Rate of oxygen consumption
 $\dot{V}O_{2max}$ – Maximum rate of oxygen consumption

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ABSTRACT

Hyperoxia causes oxidative stress, which potentially disrupts mitochondrial processes; however, there are scarce data in skeletal muscle following hyperoxic environments. Ten subjects completed 6-hour dry dives on three consecutive days while breathing 100% oxygen at 1.35 atmospheres absolute (ATA). Muscle biopsy samples were collected before diving and post-dive 3 for measurements of messenger ribonucleic acid (mRNA) associated with mitochondrial function, mitochondrial biogenesis and dynamic regulation, antioxidants, mitophagy, and proteomics. Aerobic performance with surface electromyography (sEMG) devices on the lower limb muscles were completed before diving, post-dive 3, and 48 hr after dive 3. sEMG measurements consisted of burst duration, cycle duration, interburst interval, amplitude, and time to peak amplitude. There were no changes in mitochondrial mRNA measurements ($p > .050$). However, 144 proteins were differentially expressed after hyperoxic diving; 122 proteins were up-regulated ($p < .050$ and difference >0.585 (fold change >1.5) and 22 were down-regulated ($p < .050$ and $\log_2$ fold change <-0.585 (fold change $< 1/1.5$). There were no changes in any sEMG measurements ($p > .050$). These findings suggest that skeletal muscle mitochondria mRNA markers associated with stress and lower body neuromuscular fatigue are unlikely to be causes of hyperoxia-induced decreases in aerobic exercise performance. However, the proteomics analysis provides novel data on how hyperoxia influences the skeletal muscle proteome, which can be leveraged in future research.

INTRODUCTION

This Technical Report (TR) is part 2 of a larger project and is best interpreted in conjunction with NEDU TR 22-32.¹

Breathing 100% oxygen (O₂) in a closed-circuit underwater breathing apparatus (CC-UBA) during diving missions has been practiced for over 100 years.² This procedure offers operational advantages to special operations forces by increasing stealth by eliminating escaping bubbles, expanding mission duration, reducing carrying weight, and provides physiological benefits by reducing and/or eliminating nitrogen narcosis and decompression sickness.^{2,3} However, there are also negative consequences of breathing 100% O₂ for prolonged periods of time.^{2,4} While the majority of the literature has focused on pulmonary and central nervous system toxicity,⁴ there are also significant alterations to other aspects of physiology (e.g., vascular, skeletal muscle) that may reduce human performance, which can compromise mission success, safety, and recovery. Given that military diving, operations involve long-duration single and repeated dives, a greater mechanistic understanding of how breathing 100% O₂ influences human performance in these conditions will benefit mission planning, safety, and success.

For the past 12 years, the Navy Experimental Diving Unit (NEDU) has investigated the influence of breathing 100% O₂ during six-hour dives on consecutive days with 18-hour surface intervals during resting dry chamber dives,^{5,6} and resting⁷⁻¹⁰ and exercise¹¹ water immersion dives on physiological alterations and exercise performance. The initial results found that breathing 100% O₂ during a single long-duration dive (single dive: SD) at 1.35 atmosphere absolute (ATA) reduced exercise performance (treadmill time-to-exhaustion at 85% of $\dot{V}O_{2\max}$) by 5% at 24 hours post-dive. However, five dives on consecutive days (dive week: DW) under the same parameters reduced performance by 28% at 24 hours after the fifth dive and the reduction in performance was statistically significantly greater than after dives while breathing air (-17%).⁷ To remove the influence of water immersion and isolate the effects of breathing 100% O₂ on exercise performance, dry chamber dives at 1.35 ATA were completed for a SD and DW. The findings indicated that the DW resulted in large decrements (-38%) to performance compared to the SD (+6%) 24 hours after diving.⁵

To further explore the effects of breathing 100% O₂, additional studies have examined the influence on muscular strength, excitation, and endurance one hour after diving and following a recovery period of 72 hours after diving.¹² Indeed, breathing 100% O₂ resulted in large reductions in exercise performance at one hour after diving following the SD (-18%) and DW (-36%); however, these changes were similar to breathing air (SD: -22%, DW: -34%). Interestingly, at 72-hours post-diving, aerobic performance remained diminished (-31%) in the 100% O₂ group, but partially recovered in the air group (-9%). Further, dives with 100% O₂, compared to air, had lower heart rates at one-hour post diving and lower cardiac output 72-hours post-diving, which may have contributed to the different outcomes.¹² There was no effect on muscular strength and excitation during the SD. Conversely, during the DW, diving with 100% O₂ reduced upper and lower body strength and muscular excitation. Furthermore, some decrements persisted for at least 72 hours after diving.⁹ Additionally, 100% O₂ significantly reduced handgrip endurance during the DW and induced a greater rate of fatigue in a 50 repetition knee extension test compared to breathing air.¹² Taken together, these findings

demonstrate that breathing 100% O₂ during long-duration dives on consecutive days, regardless of water immersion, greatly diminishes exercise performance, muscular strength and excitation, and muscular endurance. Furthermore, breathing 100% O₂, compared to breathing air, significantly reduces exercise performance at 24 and 72 hours after diving, and impairs muscular strength and excitation beyond 72 hours post-diving.

The existing literature examining the potential mechanisms associated with the effects of breathing 100% O₂ on exercise performance is minimal, but previous findings suggest alterations to multiple organ systems (i.e., pulmonary, vascular, muscular, neuromuscular, etc.). However, a potential common mechanism may be due to increased reactive oxygen species (ROS) generated by mitochondria when concentrations of O₂ are greater than normal atmospheric levels (i.e., hyperoxia).^{13,14} The mitochondrial electron transport chain is the primary site of ROS production within body tissue, specifically superoxide and hydrogen peroxide.¹⁵ Normally the antioxidant system (e.g., superoxide dismutase, catalase, glutathione) mitigates most of the deleterious effects of ROS; however, when ROS levels are greatly elevated, such as hyperoxia, the antioxidant system is overwhelmed and oxidative stress occurs, leading to deoxyribonucleic acid (DNA) damage, lipid peroxidation, and irreversible protein carbonylation and degradation.¹⁶ Further, elevated ROS due to hyperoxia disrupts dynamic regulation (i.e., fusion and fission) and mitophagy of mitochondria,¹⁷ which may impair recovery from damage. For example, hyperoxia exposures in isolated mouse lung mitochondria reduced glycolytic capacity (e.g., conversion of glucose to lactate or pyruvate), mitochondrial complex activity, and oxygen consumption rate, along with adenosine triphosphate (ATP) production.¹⁸ ROS also interact with peroxisome proliferator-activated receptor-gamma coactivator (PGC-1 α), the master regulator of mitochondrial biogenesis, which can influence mitochondrial DNA transcription, oxidative phosphorylation subunits, and antioxidant expression; however, the effects of hyperoxia are not well understood.¹⁹

Furthermore, elevated oxidative stress markers due to hyperbaric hyperoxia²⁰ and normobaric hyperoxia²¹ have been demonstrated to alter neuromuscular function. Breathing 100% O₂ can increase nerve conduction velocity (NCV) and muscle excitation in directly stimulated muscle after only 20 minutes.²¹ However, the same investigation also demonstrated that reflexive muscular excitation was blunted 30 minutes after breathing 100% O₂, suggesting a reduction in muscle spindle activity.²¹ Further, hyperoxia-induced overproduction of ROS has been proposed to activate group IV afferents (i.e., metaboreceptors),²² which blunts the activity of muscle spindles and reflexive contraction of skeletal muscle. The changes to neuromuscular function and muscle spindle activity may contribute to reduced exercise performance through diminished ability to properly coordinate muscular contraction.

Collectively, the potential disruption of muscle mitochondrial function and regulatory processes, along with neuromuscular alterations, may contribute to reduced exercise performance following long duration exposure to hyperoxic environments. Presently, there are no investigations that have examined repeated hyperoxia exposures on muscle mitochondrial and neuromuscular function and their influence on exercise performance. Additionally, the temporal generation of oxidative stress markers has not been elucidated with serial blood draws during hyperbaric hyperoxic exposures. Therefore, the purpose of this investigation is to examine the influence of three six-hour hyperbaric hyperoxic repeated exposure dry-dives (SHHRED) on changes in

mitochondrial function, oxidative stress, dynamic regulation and mitophagy, systemic oxidative stress, volatile organic compounds, and neuromuscular and endurance performance in military trained divers. Six-hour dives were selected to obtain data on potential limiting factors during dives that extend beyond the current recommendations in the US Navy dive manual for breathing 100% O₂. These factors can then be targeted for mitigation in future studies and may contribute to safely extending diving duration for 100% O₂ diving.

The data obtained from this study provides novel information regarding the influence of long duration repeated hyperbaric hyperoxic dives on skeletal muscle mitochondrial and neuromuscular alterations and their relationship with changes in exercise performance. These findings provide information that can be used in the development of countermeasures involving nutritional, pharmaceutical, and/or exercise approaches, ultimately influencing mission planning, scope, and diver safety.

The objectives and hypotheses of the project are as follows:

Objective 1a: Examine the effect of SHHRED on muscle mitochondrial function.

Hypothesis 1a: SHHRED will reduce mitochondrial function post-diving.

Objective 1b: Examine the effect of SHHRED on muscle mitochondrial oxidative stress.

Hypothesis 1b: SHHRED will increase mitochondrial oxidative stress post-diving.

Objective 1c: Examine the effect of SHHRED on muscle mitochondrial dynamic regulation (fusion and fission) gene expression.

Hypothesis 1d: SHHRED will decrease mitochondrial fusion gene expression and increase fission gene expression.

Objective 1d: Examine the effect of SHHRED on muscle mitochondrial mitophagy gene expression.

Hypothesis 1e: SHHRED will increase mitophagy gene expression.

Objective 1e: Investigate the influence of SHHRED on skeletal muscle proteomics.

Hypothesis 1f: SHHRED will alter proteins associated with mitochondria function.

Objective 2: Determine the effect of SHHRED on systemic oxidative stress markers and examine the recovery.

Hypothesis 2: SHHRED will promote increases in oxidative stress markers during diving, post-dive, and throughout the dive week compared to baseline. Oxidative stress markers will return to baseline within 48 hours of the last dive.

Objective 3a: Assess the influence of SHHRED on resting nerve conduction velocity and recovery.

Hypothesis 3a: SHHRED will increase resting nerve conduction velocity at post-dive and throughout the dive week. Resting nerve conduction velocity will remain higher at 48 hours following the last dive.

Objective 3b: Assess the influence of SHHRED on exercise neuromuscular excitation and recovery.

Hypothesis 3b: SHHRED will decrease exercise neuromuscular excitation after the last dive. Exercise neuromuscular excitation will remain lower at 48 hours following the last dive.

Objective 4: Examine the relationships between changes in aerobic endurance performance and changes in systemic oxidative stress markers, muscle mitochondrial markers, and neuromuscular excitation.

Hypothesis 4: There will be moderate to strong relationships in changes in aerobic endurance performance and changes in systemic oxidative stress markers, muscle mitochondrial markers, and neuromuscular excitation.

Objectives 1, 3b, and their respective contribution to objective 4 will be addressed in this TR. The additional objectives were covered in NEDU TR 22-32 previous TR.¹

METHODS

GENERAL

Baseline performance testing took place over two days. The first baseline visit occurred approximately five days before the first dive and consisted of a $\dot{V}O_{2\max}$ test. The second baseline visit was approximately three days later (two days before the first dive) and consisted of a muscle microbiopsy and a time-to-exhaustion treadmill trial at a workload of approximately 85% $\dot{V}O_{2\max}$. The first of three consecutive dives began approximately two days after the second baseline visit. Prior to each dive, subjects consumed a standardized meal and completed a six-hour dry dive at 1.35 ATA while breathing 100% O_2 . At the three-hour point, subjects removed their hood for 10 minutes to consume a standardized lunch. Following the third dive, a second microbiopsy and time-to-exhaustion treadmill trial were performed. Then 48-hours after the third dive, subjects completed the final time-to-exhaustion treadmill run (Figure 1).

Environmental Control

For all testing days and chamber dives, the temperature was between approximately 21 and 24 °C (~70-75 °F).

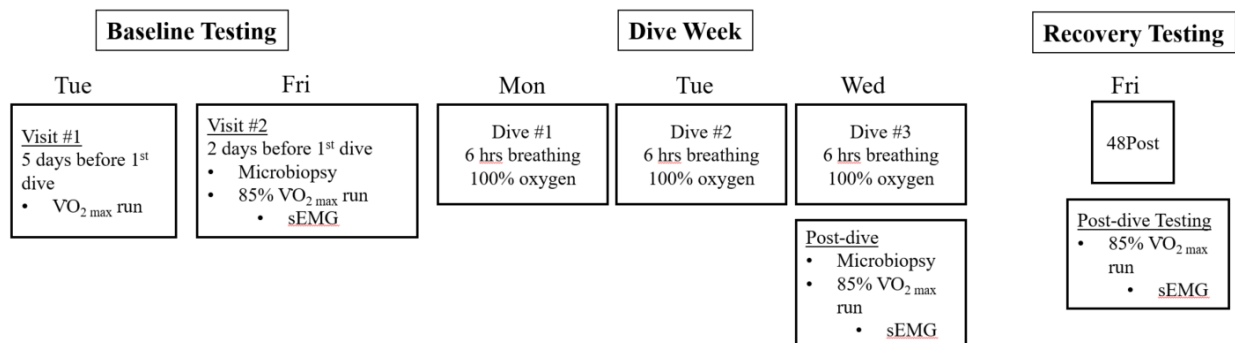


Figure 1. Study overview. $\dot{V}O_{2\max}$ = maximum oxygen consumption test; sEMG = surface electromyography.

SUBJECTS

Fifteen military trained divers participated in the study (Pilot: n = 2, Full protocol: n = 13); however, three withdrew from the study and data from the pilot was not used in the analysis. Therefore, data from 10 subjects are included in this report. This protocol was approved by the Navy Experimental Diving Unit Institutional Review Board. Subjects provided written, informed consent, and all procedures conformed to the Declaration of Helsinki.

Healthy divers who met current applicable military diving standards were recruited. Subjects were excluded if they were not physically qualified for diving or if they were taking non-elective medication that may affect any study variables, which was verified by the principal investigator (PI). Subjects underwent a routine medical screening and completed a health history questionnaire and physical activity questionnaire prior to beginning testing.

GENERAL INSTRUCTIONS FOR SUBJECTS

Subject were instructed to adhere to the following instructions while enrolled in the study:

1. Do not perform oxygen dives for two weeks, or air dives for one week, before scheduled dive testing in this experiment.
2. Refrain from smoking or taking any tobacco or nicotine for one month prior to enrollment in the study.
3. Refrain from taking elective medications and discuss any physician recommended medications with the PI and undersea medical officer (UMO). Additionally, discontinue using herbal or training supplements while enrolled.
4. Abstain from alcohol for two days, caffeine (including chocolate) for 1 day, and food and drink (except water) the morning before the testing and dive days.
5. Wear comfortable physical training gear to testing sessions. Wear chamber safe clothing on dive days in accordance with US Navy dive manual (e.g., 100% cotton, blend material [65% cotton, 35% polyester]).
6. Do not start a new exercise program between baseline testing and final dive and associated physiological testing.
7. Do not exercise at all the morning before a dive or 24 hours before an exercise testing trial. Also, refrain from strenuous exercise for the duration of the study. For this protocol, strenuous exercise is defined as any activity eliciting a sustained increase in heart rate above 70% of max, or any exercise session – cardiovascular or resistance training – lasting longer than 45 minutes. Do not perform any lower body resistance training 48 hours before the first microbiopsy and for the remainder of the study after the first microbiopsy. Resistance training, if performed at all, should not exceed 50% of maximum weight, eight repetitions, and two sets per muscle group for the duration of the study.

EXPERIMENTAL DESIGN AND ANALYSIS

The variables for this report are in table 1.

Mitochondrial function messenger ribonucleic acid (mRNA) 1. Mitochondria Reduced nicotinamide adenine dinucleotide ubiquinone oxidoreductase chain 1 (MT-ND1: Complex I) mRNA 2. Mitochondria Cytochrome b (MT-CYB: Complex III) mRNA Mitochondrial oxidative stress mRNA 3. PGC-1α 4. Nuclear respiratory factor 1 (NRF1) 5. Mitochondrial transcription factor A (TFAM) 6. Heme oxygenase 1 (HMOX1) 7. Uncoupling protein 3 (UCP3) 8. Superoxide dismutase 1 (SOD1) 9. Superoxide dismutase 2 (SOD2) Mitochondrial dynamic regulation mRNA 10. Mitofusin-2 (MFN2: fusion) 11. Optic atrophy 1 (OPA1: fusion) 12. Dynamin-1-like protein (DNM1L: fission) Mitophagy mRNA 13. PTEN-induced kinase-1 (PINK1) 14. Parkin 2 (PARK2)
Skeletal muscle proteomics 1. Semi-quantification of skeletal muscle proteins
Exercise surface electromyography - (sEMG during 85% max run) 1. Burst duration 2. Cycle duration 3. Interburst interval 4. Amplitude 5. Time-to-Peak
Aerobic performance 1. Time to exhaustion (treadmill; only % change data included for correlation)

Table 1. Study variables

Baseline: First visit

The first baseline visit occurred approximately five days before the first dive and consisted of a maximal oxygen consumption ($\dot{V}O_{2max}$) test. Subjects arrived at NEDU at scheduled times between 0700-1000 and were instructed to abstain from alcohol for 48 hours, and caffeine and any exercise for 24 hours before the testing session. Additionally, subjects were instructed to consume their last meal and drink (except water) two hours before their arrival.

Baseline: Second visit

The second baseline visit was approximately three days later (two days before the first dive) and consisted of a microbiopsy, and a time-to-exhaustion treadmill run at 85% $\dot{V}O_{2max}$. The second visit took place at scheduled times between approximately 1300-1600 to align with the time of post-dive three testing. Subjects were instructed to abstain from alcohol for 48 hours, and caffeine and any exercise for 24 hours before the testing session. To reduce the influence of food

intake on biopsy samples, participants were provided with standardized meals and instructed to consume those meals at times that align with the meal consumption schedule of the dive days. Other than the meals provided, subjects were only allowed to consume water before the testing session.

Dive days 1-3

Prior to each dive day, subjects were instructed to abstain from alcohol for 48 hours, and caffeine and strenuous exercise for 24 hours. Subjects reported to NEDU at their scheduled time (0600-0900) after an overnight fast to complete a compliance questionnaire, consume a standardized meal, and were given the opportunity to urinate before proceeding to the hyperbaric chamber.

Under direction of the chamber supervisor, subjects entered the chamber in a staggered manner approximately 0.5-1.5 hours apart to allow for each subject to complete their pre and post-dive testing. Subjects were compressed to 12 feet sea water (fsw; ~1.35 ATA) in accordance with the US Navy Diving Manual and NEDU standard operating procedures.² Chamber temperature were kept within a comfortable range (approximately 22-25°C), and gas composition was monitored and adjusted in accordance to the US Navy Diving Manual.² During the dive, subjects wore a hood to supply humidified 100% O₂ for the duration of the dive and remained seated and as still as possible. Subjects were allowed to read or watch movies during the dives. Once every hour subjects were asked about their symptoms by the inside tender and they were encouraged to report all symptoms. At the three-hour mark, subjects removed their hood for no more than 10 minutes to consume a standardized meal. After surfacing, subjects exited the chamber, and observed a 10 minute holding period.

Following the third dive, a second microbiopsy and 85% $\dot{V}O_{2\max}$ time-to-exhaustion run were conducted at approximately 1.5 hours after surfacing. Additionally, after each dive, subjects were instructed to use hydrating eye gels/drops (e.g. Systane®, GenTeal®, or equivalent.) starting after testing on the first dive day and continuing until approximately 24 hours post dive three. Hydrating drops were used every two hours (except during sleep) between the end of testing on dive day one and the start of testing on the following day. Hydrating gel were used once during each night following a dive.

Recovery testing: 48-hours post-diving

Subjects arrived at NEDU at scheduled times between 1300-1600 to complete a third and final 85% $\dot{V}O_{2\max}$ time-to-exhaustion run.

Standardized meal

On Baseline visit two, dive days one through three, and 48-hour recovery day, subjects were provided with standardized breakfast and lunch meals to reduce the influence of food intake. Each meal provided approximately 25% of estimated daily energy content, which was calculated using the Harris-Benedict equation multiplied by 1.6 to account for physical activity level.²³

Breakfast consisted of a prepared freeze-dried meal (Mountain House®) and provided approximately 35% energy from carbohydrates, 42% energy from fats, and 22% energy from protein. Lunch consisted of protein powder (Bio-Engineered Supplements & Nutrition®) and

whole milk and provided approximately 31% energy from carbohydrates, 36% energy from fats, and 34% energy from protein.

PROCEDURES

For all testing sessions, subjects were instructed to follow the directions provided in the **GENERAL INSTRUCTIONS** section.

Protocols for testing

Testing protocols for use in this study are detailed below:

$\dot{V}O_{2max}$. The modified Balke Protocol was used to measure $\dot{V}O_{2max}$ during the first baseline visit. The obtained $\dot{V}O_{2max}$ was used to calculate the treadmill workload for subsequent 85% time-to-exhaustion runs. During the run, subject heart rate was measured with a heart rate monitor (Polar®, Bethpage, NY). Additionally, subjects were asked about their rating of perceived exertion (RPE) on the 6-20 Borg RPE scale after completing each stage of the protocol. Criteria for $\dot{V}O_{2max}$ included a plateau in oxygen consumption with an increased workload, a respiratory exchange ratio greater than 1.10, and RPE greater than 17. $\dot{V}O_{2max}$ was determined as the greatest maximal $\dot{V}O_2$ averaged with the values that immediately precede and follow (three total values). Subjects had the option for a ceiling fan to be turned on during the run, which was consistent for all runs.

85% time-to-exhaustion run. The 85% time-to-exhaustion run was at a workload corresponding to approximately 85% of subjects' baseline $\dot{V}O_{2max}$. The workload was calculated with the American College of Sports Medicine running $\dot{V}O_2$ metabolic equation.²⁴ The performance run was completed three times: 1) second baseline visit, 2) after the third dive, and 3) at the 48-hour time point. The run consisted of several components including a five-minute walking warm up at three miles per hour (MPH), a three-minute run at the test run speed calculated from the $\dot{V}O_{2max}$, followed by a run at the test run speed at a 10% grade until volitional exhaustion, and a walking cool down at three MPH for three minutes or until a heart rate under 130 BPM is reached. In some instances, the speed was adjusted to avoid an awkward run gait. The run time at 10% incline was recorded manually with a stop watch.

Exercise sEMG. During the time-to-exhaustion run, subjects were instrumented with wireless sEMGs (Delsys® Trigno Avanti Sensor) on the left vastus lateralis, vastus medialis, rectus femoris, biceps femoris, semitendinosus, medial and lateral gastrocnemii and soleus muscle groups in a similar manner to the recommendations by The European Project Surface EMG for non-invasive assessment of muscles.²⁵ Prior to sensor placement, each subject's sEMG placement site was exfoliated and cleansed with isopropyl alcohol and shaved, if necessary. The sensor was attached to the skin using double-sided hypoallergenic tape, benzoin tincture, and further secured using strips of athletic tape. The initial sEMG sites were marked with a permanent marker and recorded for subsequent measurements. sEMG data were pre-amplified (100X), amplified (2X), band-pass filtered (10-1000 hertz (Hz)), and recorded with Delsys® EMGworks acquisition software with a sampling rate of 2,000 Hz. Markers were placed in the

acquisition software to differentiate each phase of the run. Data were exported into a .csv file, imported into MATLAB (The Mathworks, Inc. MATLAB Version R2021B), and the 10% incline section of the file was isolated for analysis. Raw sEMG recordings were processed by removing the direct current offset, rectifying the signal, and finally integrating the signal using a moving average window of 50 milliseconds. sEMG burst cycles were extracted from the integrated signal of the entire 10% incline period by establishing a subjective detection threshold, selected to minimize signal outliers. The following features were measured from each burst cycle: burst duration (duration of individual bursts), cycle duration (duration from the start of one burst to the start of the subsequent burst), inter-burst interval (difference between the end of one burst and the start of the subsequent burst), amplitude (peak of burst), and time-to-peak (time from the start of burst to the peak amplitude of the burst). Burst cycle outliers were further eliminated by removing outliers that were more than 1.5 interquartile ranges above/below the upper/lower quartiles from any of the measured features. Due to muscle cross-talk, poor signal quality, or other signal interference, mean values of burst duration, amplitude, and time-to-peak of the vastus lateralis, vastus medialis, biceps femoris, and medial gastrocnemius were included for analysis, and mean values of cycle duration and inter-burst interval of the vastus lateralis and vastus medialis were included for analysis.

Skeletal muscle microbiopsy. A microbiopsy was performed by a physician on the right vastus lateralis muscle 30 minutes prior to the baseline and post-dive three time-to-exhaustion runs in accordance with previously published procedures.^{26,27} Briefly, subjects were rested in a supine position on a treatment table for approximately 20 minutes while the biopsy site was prepared. The site was prepped and draped in accordance with sterile procedures and cleaned with a topical antiseptic (Betadine®) followed by an injection of two mL lidocaine, 1% without epinephrine. After the site was anesthetized, the skin was punctured by a cannula (13-gauge) in an orientation perpendicular to the skin. Then the biopsy needle (14-gauge) was loaded into the automatic spring-loaded biopsy instrument (Bard® Magnum™), followed by insertion of the needle through the cannula and into the muscle. The tissue sample was obtained by pressing a trigger on the biopsy instrument, which unloaded the spring and activated the needle. Thereafter, the needle was removed from the cannula and samples were removed from the needle with a sterile utensil (needle or forceps) and promptly weighed and prepped for storage. The cannula was maintained in place to avoid repeated skin puncture, and the process was repeated three to five times to collect approximately 40-50 mg of tissue. After completion of the procedure, the cannula was removed and direct pressure was applied to the area for approximately 5-10 minutes, after which, a bandage was applied. The biopsy devices were cleaned and sterilized via an autoclave after each use. Subsequent biopsies were completed two to three cm proximal or distal to the initial site. A medical provider examined each subject's biopsy site for adequate healing prior to each diving day.

Muscle tissue samples were weighed with precision balance scale and recorded immediately after tissue collection. Several samples (25-35 mg) were placed in a labeled cryogenic tube and snap frozen using liquid nitrogen. One sample was placed in a labeled base mold with an optimal cutting temperature embedding medium (Tissue Plus®, Fisher Healthcare) in a horizontal orientation and then frozen using two-methylbutane and dry ice. All samples were stored in a -

80°C freezer for later analysis and samples frozen in the embedding medium were wrapped in paraffin to reduce dehydration.

Approximately, 25-35 mg, along with the samples frozen in the embedding medium were analyzed for mRNA measures of mitochondrial function, oxidative stress, dynamic regulation, and mitophagy by Dr. Heath Gasier's laboratory at Duke University. mRNA measures were completed via real-time quantitative reverse transcription and analyzed with the $2^{-\Delta\Delta CT}$ method.

Approximately 10 mg was used for label-free quantification proteomic analysis by Creative Proteomics[®]. After samples were prepped, liquid chromatography and mass spectrometry were performed. Thereafter, raw mass spectrometry files were analyzed and searched against human protein database based on the species of the samples using Maxquant (1.6.1.14). Then bioinformatics analysis was performed, which consisted of differentially expressed proteins, and gene ontology (GO; for terms biological process, cellular component, and molecular function) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis for differentially expressed proteins.

STATISTICS

An a priori power analysis was performed with the program G*Power 3.1 to determine sample size. An effect size was calculated from our previous time-to-exhaustion data²⁸ and which resulted in an effect size of $f = 1.75$. However, since that study design utilized five consecutive dives on 100% O₂, the effect size was multiplied by 0.6 to adjust for three consecutive dives of the current design resulting in an effect size of 1.00. The a priori parameters for an analysis of variance (ANOVA): repeated measures, within factors in G*Power 3.1 were set as follows: effect size $f(V) = 1$, power = 0.8, alpha = 0.05, number of groups = 1, number of measurements = 3, and nonsphericity correction = 1. This resulted in a total sample size of 12.

Data were tested for outliers using the robust regression and outlier removal method with Q set to 1%.²⁹ If an outlier was identified, the data point was manually inspected to determine if removal was appropriate. For t-test analysis, the Shapiro-Wilks test was used to test for normality. When normality was violated, a log transformation was applied and normality was retested. If log transformation corrected for the normality violation, then transformed data were analyzed. If not, then an alternative nonparametric test was performed. Outlier removal and normality violations are specified in text.

Measures of muscle mitochondrial function, mitochondrial oxidative stress, dynamic regulation and mitophagy mRNA were analyzed with a two-tailed paired sample t-test (pre- and post-dive three). Since multiple t-tests were performance for each variable (e.g., dynamic regulation = MFN2, OPA1, DNM1L), the Holm-Šídák method was used to correct for multiple comparisons with an alpha level of 0.05. Raw and adjusted (adj) p values are reported.

Correlations were used to determine the strength of the relationship between aerobic exercise performance¹ and mitochondria measures. Correlation coefficients will be interpreted as “negligible” = 0-0.29, “low” = 0.30-0.49, “moderate” = 0.50-0.69, “high” = 0.70-0.89, “very high” = 0.90-1.00.

Proteomics

Creative Proteomics[®] completed the proteomic statistical analysis.

Differentially expressed proteins – A paired sample t-test was used to determine proteins that significantly change from pre to post-dive 3. Proteins were considered to be up-regulated with a p -value $< .05$ and difference > 0.585 (Fold-change (FC) > 1.5) and were considered down-regulated with a p -value $< .05$ and difference < -0.585 (FC $< 1/1.5$).

Enrichment analysis for differentially expressed proteins - The enrichment factor (ratio of frequency of GO or KEGG terms in study sample to reference sample) and corresponding p -value was calculated using Fisher's exact test. Only items with enrichment factor > 1 - and p -value $< .05$ were considered differentially expressed.

Due to the exploratory nature of this proteomic analysis, correction for multiple comparisons were not included in the criteria for differential expression. However, false discovery rate (5%) adj. p -values (q -value) were calculated via the Benjamini-Hochberg method and reported for clear interpretation.³⁰

Due to cross-talk, poor signal quality, or other signal interference, sEMG data contained missing values; and therefore, data were fitted with a linear-mixed effects model using restricted maximum likelihood to estimate variance, which can produce unbiased estimates when data are missing at random.³¹ For each model, the sEMG feature was the independent variable, time (Pre-dive, Post-dive 3, and 48-hour Post-diving) was the fixed factor, and subject was the random factor. Sphericity was not assumed and effects were interpreted with Greenhouse-Geisser correction.

Statistical significance was accepted at $p < .05$. Data are presented as mean $\pm$ standard deviation (SD), unless otherwise specified. All analyses, except proteomics, were performed with GraphPad Prism version 9.3.0 for Windows, GraphPad Software, San Diego, California USA.

RESULTS

AEROBIC PERFORMANCE RUN TIME

Aerobic performance run time decreased after diving ($p = .021$); specifically at post-dive 3 compared to baseline (5.3 ± 1.96 vs 6.38 ± 1.77 min, $p = .037$), but not at 48-hr post-diving (5.9 ± 1.78 min, $p = .298$). Data are presented in detail in NEDU TR 22-32.¹

MITOCHONDRIAL FUNCTION

MT-CYB data failed normality and were log transformed for analysis. There were no changes from pre to post SHHRED for mRNA MT-ND1 ($t(9) = .480$, adj. $p = .643$, raw $p = .643$) or mRNA MT-CYB ($t(9) = .974$, adj. $p = .585$, raw $p = .356$). One outlier from MT-ND1 and MT-CYB was removed from correlation analysis. There was a negligible non-significant correlation between the change in run time and change in MT-ND1 ($r(7) = .19$, $p = .633$). There was also a low non-significant correlation between the change in run time and change in MT-CYB ($r(7) = .39$, $p = .295$). Mean $\pm$ SD and individuals values are presented in Figure 2.

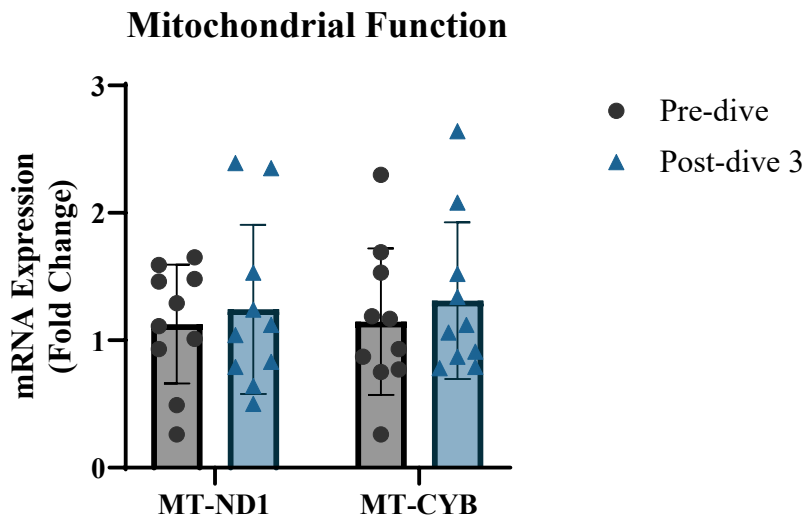


Figure 2. Mitochondrial Function. MT-ND1 = Mitochondria reduced nicotinamide adenine dinucleotide ubiquinone oxidoreductase chain 1; MT-CYB = Mitochondria cytochrome b; mRNA = messenger ribonucleic acid. Values presented as mean $\pm$ standard deviation and individual values.

MITOCHONDRIAL OXIDATIVE STRESS

Mitochondrial biogenesis

One outlier was removed from NRF1 analysis. There were no changes from pre- to post-SHHRED in any of the measured mRNA associated with mitochondrial biogenesis (PGC-1 α ($t(9) = 1.995$, adj. $p = .215$, raw $p = .077$; NRF1 ($t(8) = .911$, adj. $p = .440$, raw $p = .389$; TFAM (t

(9) = 1.224, adj. p = .440, raw p = .252). For the correlation analysis, one outlier from PGC-1 α , NRF1, TFAM was removed. There were negligible non-significant correlations between the change in run time and change in PGC-1 α ($r(7)$ = .01, p = .988), TFAM ($r(7)$ = -.11, p = .785). There was also a low non-significant correlation between the change in run time and change in NRF1 ($r(7)$ = .39, p = .302). Mean $\pm$ SD and individuals values are presented in Figure 3.

Antioxidants

One outlier was removed from HMOX1 and SOD1, and two outliers were removed from SOD2 analysis. HMOX1 data failed normality and were log transformed for analysis. There were no changes from pre to post SHHRED in any of the measured mRNA associated with antioxidants (HMOX1 ($t(8)$ = 2.413, adj. p = .121, raw p = .042; UCP3 ($t(9)$ = .663, adj. p = .524, raw p = .524; SOD1 ($t(8)$ = 1.589, adj. p = .279, raw p = .151; SOD2 ($t(7)$ = 2.785, adj. p = .104, raw p = .027). For the correlation analysis, one outlier from HMOX1 and SOD1, and two outliers from SOD2 were removed. There were negligible non-significant correlations between the change in run time and change in HMOX1 ($\rho(7)$ = -.02, p = .982) and UCP3 ($r(8)$ = .08, p = .821). There were also low non-significant correlations between the change in run time and change in SOD1 ($\rho(7)$ = .45, p = .230). Lastly, there was a moderate non-significant correlation between change in run time and change in SOD2 ($\rho(7)$ = -.62, p = .115). Mean $\pm$ SD and individuals values are presented in Figure 3.

Mitochondrial Oxidative Stress

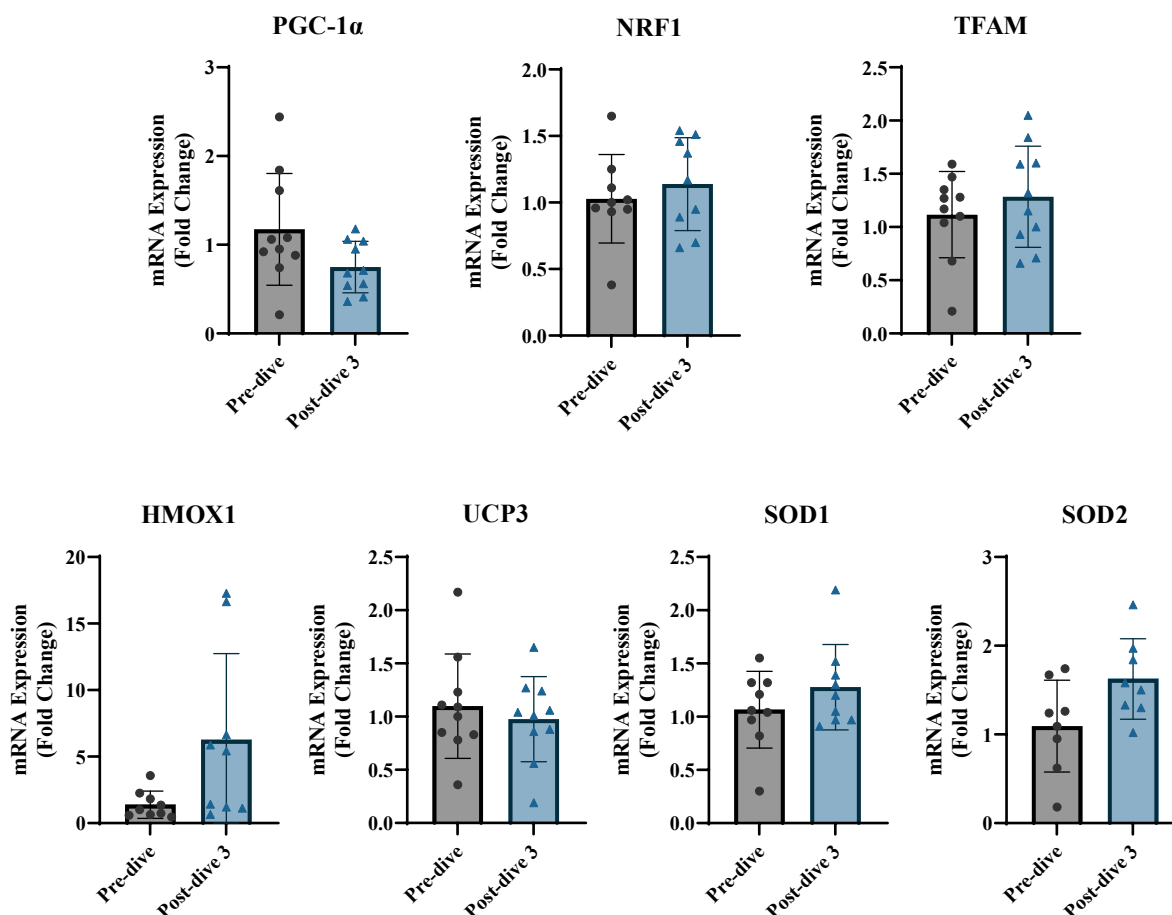


Figure 3. Mitochondrial Oxidative Stress mRNA. PGC-1 α = Peroxisome proliferator-activated receptor-gamma coactivator; NRF1 = Nuclear respiratory factor 1; TFAM = Mitochondrial transcription factor A; HMOX1 = Heme oxygenase 1; UCP3 = Uncoupling protein 3; SOD1 = Superoxide dismutase 1; SOD2 = Superoxide dismutase 2. mRNA = messenger ribonucleic acid. Values presented as mean $\pm$ standard deviation and individual values.

MITOCHONDRIAL DYNAMIC REGULATION

There were no changes from pre to post SHHRED in any of the measured mRNA associated with mitochondrial dynamic regulation (MFN2: (t (9) = 1.277, adj. p = .551, raw p = .234; OPA1: (t (9) = .233, adj. p = .821, raw p = .821; DNM1L: (t (9) = .667, adj. p = .772, raw p = .522). One subject was removed as an outlier for correlation analysis in OPA1 and DNM1L. Further, there were negligible non-significant correlations between the change in run time and change in OPA1 ($r(7)$ = -.14, p = .710), DNM1L ($r(7)$ = .04, p = .924) and low non-significant correlations between the change in run time and change in MFN ($r(8)$ = .49, p = .152), from baseline to post-dive 3. Mean $\pm$ SD and individuals values are presented in Figure 4.

Mitochondrial Dynamic Regulation

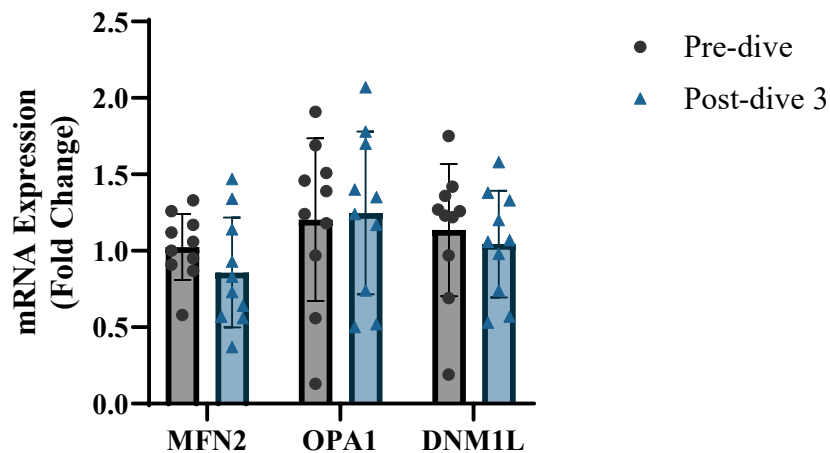


Figure 4. Mitochondrial Dynamic Regulation mRNA. mRNA = messenger ribonucleic acid; MFN2 = mitofusin 2; OPA1 = Optic atrophy 1; DNM1L = Dynamin-1-like. Values presented as mean $\pm$ standard deviation and individual values.

MITOPHAGY

One subject was removed as an outlier for PINK1 analysis. There were no changes from pre to post SHHRED in any of the measured mRNA associated with mitophagy (PINK1: (t (8) = 0.423, adj. p = .684, raw p = .684); PARK2: (t (9) = 2.126, adj. p = .120, raw p = .062). One subject was removed as an outlier for correlation analysis in PINK1 and PARK2. There was moderate and low non-significant correlations between the change in run time and change in PINK1 ($\rho(7)$ = .57, p = .121) and PARK2 ($r(7)$ = -.33, p = .392), respectively. Mean $\pm$ SD and individuals values are presented in Figure 5.

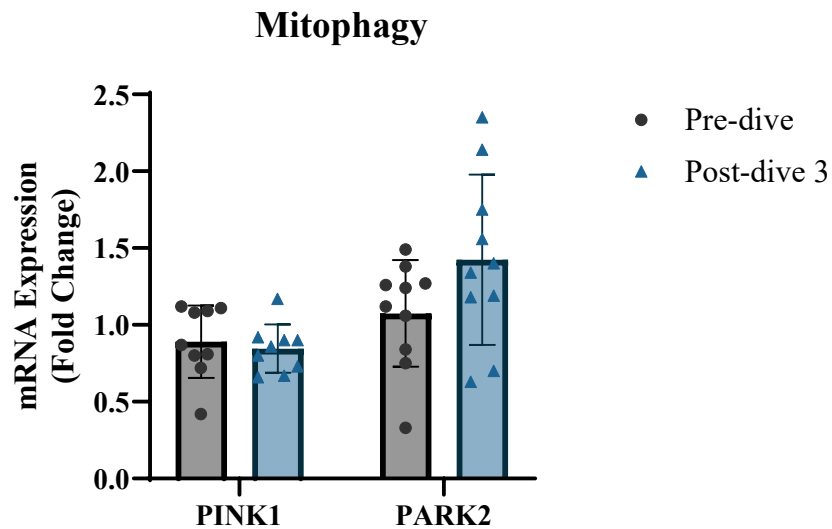


Figure 5. Mitophagy mRNA. mRNA = messenger ribonucleic acid; PINK1 = PTEN (Phosphatase and tensin homolog) Induced Kinase 1; PARK2 = Parkin 2. Values presented as mean $\pm$ standard deviation and individual values.

PROTEOMICS

A total of 1271 proteins were identified in the proteomic analysis. From pre- to post-SHHRED 144 proteins were differentially expressed; 122 proteins were up-regulated and 22 proteins were down-regulated (Figure 6).

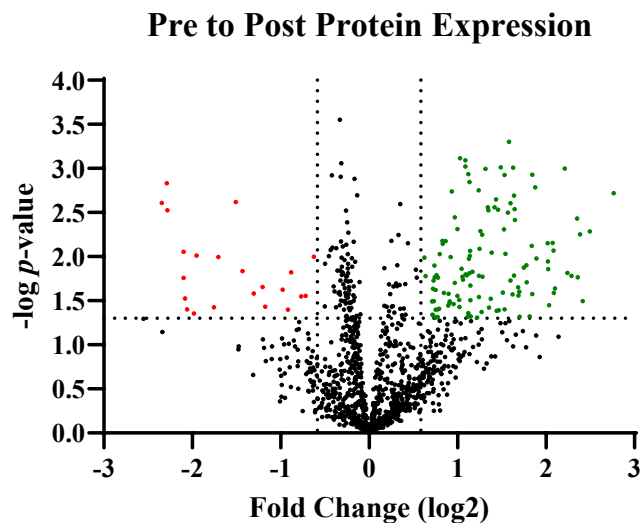


Figure 6. Proteomics Volcano Plot. Green dots represent up-regulated proteins and red dots represent down-regulated proteins. 122 proteins were up-regulated and 22 proteins were down-regulated after six-hour dry dives at 1.35 ATA with 100% oxygen on three consecutive days.

The top five up-regulated proteins sorted by fold change are displayed in Table 2.

Protein	Fold change (log₂)	<i>p</i>-value	<i>q</i>-value
Fibronectin	2.76	.002	.106
Calumenin	2.49	.005	.147
Immunoglobulin lambda-6 chain C region	2.41	.032	.211
Peptidyl-prolyl cis-trans isomerase B	2.38	.006	.148
Putative elongation factor 1-alpha-like 3	2.36	.017	.173

Table 2. Top 5 Up-Regulated Proteins.

The top five down-regulated proteins sorted by fold change are displayed in Table 3.

Protein	Fold change (log₂)	<i>p</i>-value	<i>q</i>-value
Glutaredoxin-related protein 5	-2.35	.002	.106
NADH dehydrogenase 1 beta subcomplex subunit 6	-2.29	.001	.104
Guanidinoacetate N-methyltransferase	-2.28	.003	.106
Importin-5	-2.10	.009	.161
L-xylulose reductase	-2.08	.017	.173

Table 3. Top 5 Down-Regulated Proteins. NADH = nicotinamide adenine dinucleotide.

The top five GO and KEGG terms for up-regulated proteins are found in Table 4. The top five

Terms	Enrichment factor	<i>p</i>-value	<i>q</i>-value
GOBP			
Defense response	2.394	<.001	<.001
Protein activation cascade	4.341	<.001	<.001
Regulation of response to stimulus	2.002	<.001	<.001
Regulation of immune system process	2.355	<.001	<.001
Response to wounding	3.606	<.001	<.001
GOCC			
Blood microparticle	5.515	<.001	<.001
Extracellular region	3.247	<.001	<.001
Extracellular space	2.766	<.001	<.001
Cytoplasmic membrane-bounded vesicle lumen	5.209	<.001	<.001
Vesicle lumen	5.209	<.001	<.001
GOMF			
Endopeptidase regulator activity	4.884	<.001	<.001
Peptidase regulator activity	4.387	<.001	<.001
Endopeptidase inhibitor activity	4.705	<.001	<.001
Peptidase inhibitor activity	4.705	<.001	<.001
Cholesterol transporter activity	10.42	<.001	<.001
KEGG			
Complement and coagulation cascades	4.775	<.001	<.001
Staphylococcus aureus infection	6.251	<.001	.003
African trypanosomiasis	7.814	.003	.031
Porphyrin and chlorophyll metabolism	4.630	.006	.056
Vitamin digestion and absorption	10.418	.009	.065

Table 4. Top 5 Up-Regulated GO and KEGG Terms for Differentially Expressed Proteins.
GOBP = gene ontology biological process; GOCC = gene ontology cellular component; GOMF = gene ontology molecular function; KEGG = Kyoto Encyclopedia of Genes and Genomes.

GO terms for down-regulated proteins are found in Table 5. No KEGG terms met the criteria for down regulation.

Terms	Enrichment factor	<i>p</i> -value	<i>q</i> -value
GOBP			
COPII-coated vesicle budding	57.773	<.001	.036
Membrane lipid biosynthetic process	57.773	<.001	.036
Sphingolipid biosynthetic process	57.773	<.001	.036
Membrane budding	19.258	.004	.214
Membrane lipid metabolic process	16.506	.005	.214
GOCC			
Golgi apparatus part	5.374	.004	.214
Golgi lumen	14.443	.007	.214
Actin part, Actomyosin	57.773	.017	.214
Spot adherens junction	57.773	.017	.214
Integral to membrane	2.494	.019	.214
GOMF			
FFAT motif binding	57.773	<.001	.036
Oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	5.925	.003	.214
Microtubule binding	14.443	.007	.214
Oxidoreductase activity, acting on NADH or NADPH	4.531	.008	.214
Protein heterodimerization activity	9.629	.016	.214

Table 5. Top 5 Down-Regulated GO and KEGG Terms for Differentially Expressed Proteins. GOBP = gene ontology biological process; GOCC = gene ontology cellular component; GOMF = gene ontology molecular function.

EXERCISE SURFACE ELECTROMYOGRAPHY

Burst duration

Data from three subjects for vastus medialis and biceps femoris, and data from five subjects for medial gastrocnemius were removed from analysis due to poor signal quality. There was no change in burst duration at any time point in any of the measured muscles (vastus lateralis: $F(0.566, 3.682) = 0.212, p = .534$; vastus medialis: $F(1.572, 7.072) = 1.338, p = .311$; biceps femoris: $F(1.483, 10.378) = 0.208, p = .715$; medial gastrocnemius: $F(0.264, 0.659) = 2.894, p = .266$. Means and individual values are found in Figure 9.

Burst Duration

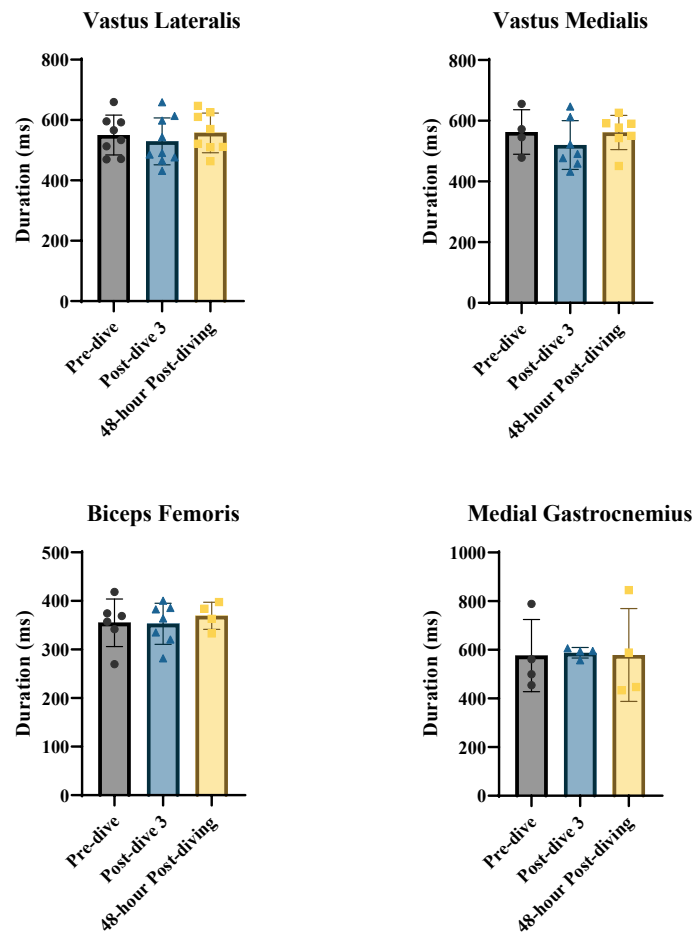


Figure 7. sEMG Burst Duration. sEMG = surface electromyography; ms= milliseconds. Values presented as mean $\pm$ standard deviation and individual values.

Cycle duration

Data from one subject for vastus lateralis and four subjects for vastus medialis were removed from analysis due to poor signal quality. Medial gastrocnemius data failed normality and were log transformed for analysis. There was no change in cycle potential duration at any time point in vastus lateralis ($F(1.589, 9.530) = 0.405, p = .633$), or vastus medialis ($F(1.135, 4.542) = 0.385, p = .591$). Means and individuals values are found in Figure 10.

Cycle Duration

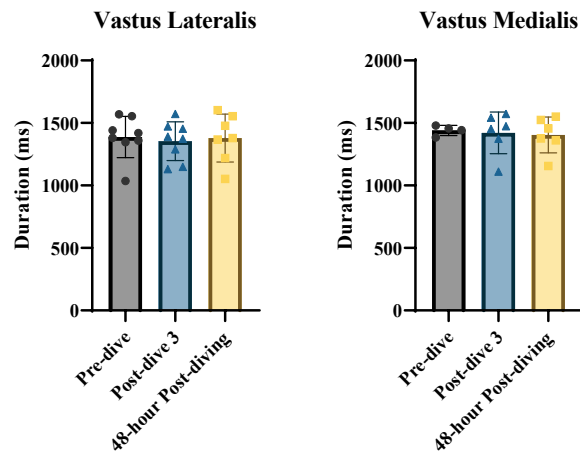


Figure 8. sEMG Cycle Duration. sEMG = surface electromyography; ms= milliseconds. Values presented as mean $\pm$ standard deviation and individual values.

Interburst interval

Data from one subject for vastus lateralis and data from three subjects for vastus medialis were removed from analysis due to poor signal quality. There was no change in interburst interval duration at any time point in the vastus lateralis ($F(1.545, 8.496) = 0.028, p = .946$), or vastus medialis ($F(1.175, 5.286) = 5.303, p = .064$). Means and individuals value are found in Figure 11.

Interburst Interval

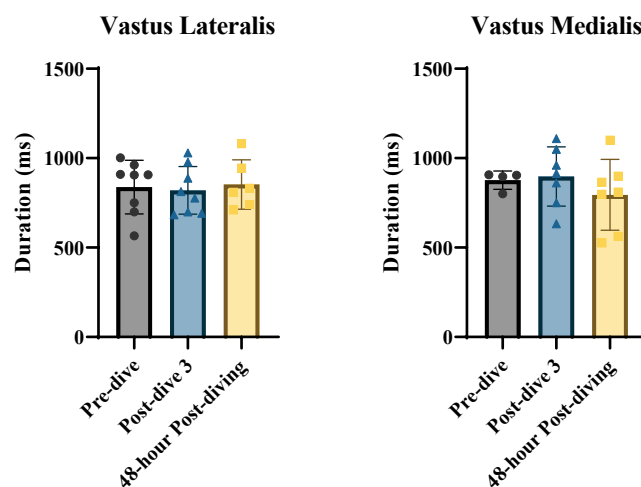


Figure 9. sEMG Interburst Interval. sEMG = surface electromyography; Ms= milliseconds. Values presented as mean $\pm$ standard deviation and individual values.

Amplitude

Data from three subjects for vastus medialis, biceps femoris, and medial gastrocnemius were removed from analysis due to poor signal quality. Vastus medialis data failed normality and log transformation did not correct for the violation. There was no change in amplitude at any time point in any of the measured muscles (vastus lateralis: $F(0.402, 2.611) = 0.215, p = .461$; vastus medialis: $F(1.688, 7.594) = 1.914, p = .212$; biceps femoris: $F(1.942, 7.768) = 2.288, p = .166$; medial gastrocnemius: $F(0.280, 1.222) = 0.046, p = .534$). Means and individual values are found in Figure 12.

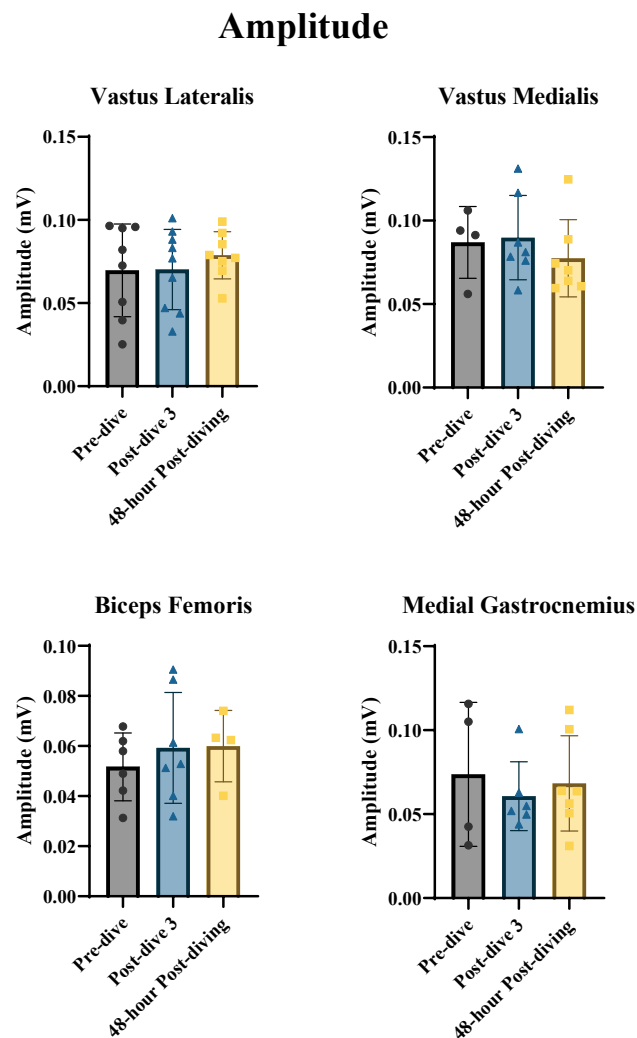


Figure 10. sEMG Amplitude. sEMG = surface electromyography; mV = millivolts. Values presented as mean $\pm$ standard deviation and individual values.

Time-to-Peak

Data from three subjects for vastus medialis, four for the biceps femoris, and five for the medial gastrocnemius were removed from analysis due to poor signal quality. There was no change in time-to-peak duration at any time point in any of the measured muscles (vastus lateralis: $F(0.479, 3.116) = 0.139, p = .545$; vastus medialis: $F(1.914, 8.617) = 0.817, p = .468$; biceps femoris: $F(1.875, 6.563) = 1.661, p = .260$; medial gastrocnemius: $F(0.6816, 2.045) = 3.489, p = .185$. Means and individual values are found in Figure 13.

Time-to-Peak

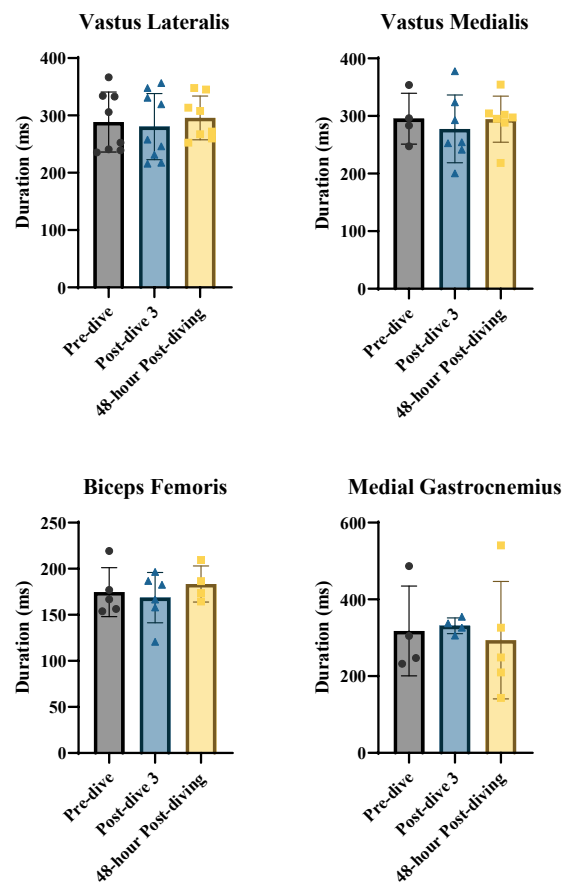


Figure 11. sEMG Time-to-Peak. sEMG = surface electromyography; ms= milliseconds. Values presented as mean $\pm$ standard deviation and individual values.

DISCUSSION

The major findings this TR (part 2 of NEDU TR 22-32) indicated that six-hour dry dives while breathing 100% O₂ on three consecutive days 1) did not significantly influence genes associated with mitochondrial function, 2) generally did not alter antioxidant nor mitochondrial biogenesis related genes, 3) did not modify mitochondrial dynamic regulation or mitophagy genes, 4) promoted significantly different expression of 144 proteins in skeletal muscle, and 5) did not alter sEMG measurements during aerobic exercise. Although the hyperoxic diving condition within this study does not appear to have an impact on skeletal muscle mitochondria markers or sEMG measurements, there were significant alterations to the skeletal muscle proteome, which provides novel data on potential target proteins for mitigation strategies.

MITOCHONDRIAL FUNCTION

There were no changes in mRNA measures associated with mitochondria complex function following long duration hyperbaric hyperoxic diving. Additionally, proteomic analysis found no change in relative protein expression of MT-ND1. The MT-ND1 gene encodes nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase chain 1 protein, which is a subunit of complex I in the mitochondria, and MT-CYB encodes the protein cytochrome b, which is a subunit of complex III. Complex I oxidizes NADH generated from the Krebs cycle and passes electrons to the electron carrier ubiquinone and complex III transfers electrons from ubiquinol to complex IV, which is the final electron acceptor in the electron transport chain.^{32,33} Moreover, both complexes pump protons across the inner mitochondrial membrane to generate a gradient for adenosine triphosphate production. Therefore, changes in complex subunit production can alter mitochondrial function. Although certain disease states appear to be associated with changes in MT-ND1 and MT-CYB production, there are very limited relevant data on how hyperoxia affects these subunits.³⁴ For example, MT-ND1 mRNA increased approximately 1.9 fold after eight days of 100% O₂ in mice heart muscle tissue.³⁴ Interestingly, 18-48 hours of hyperoxia (90-95% O₂) has been shown to decrease mitochondrial complex activity,^{18,35} suggesting that an increase in MT-ND1 mRNA may be an attempt to offset decreases in complex activity.

Interestingly, some authors have postulated that decreased mitochondrial complex activity during hyperoxia is a compensatory mechanism to reduce ROS production, which as a consequence reduces ATP production.^{36,37} For example, flies bred to tolerate hyperoxia (90-95% O₂) produce less ROS and lipid peroxidation, and decrease $\dot{V}O_2$ and mitochondrial complex activity during hyperoxia compared to control flies.³⁶ This is intriguing as decreased $\dot{V}O_2$ is commonly observed in response to hyperoxia in cells, animals, and humans;^{36,38,39} however, the potential relationship to mitochondrial function has not been well studied. Additionally, in the first TR of this project, we found decreased $\dot{V}O_2$ during an aerobic performance run at two and 48 hours after three six-hour dry hyperoxic dives.¹ Furthermore, our lab has also demonstrated this finding at two and 72 hours after five six-hour hyperoxic wet dives.⁴⁰ Therefore, it is possible that decreased $\dot{V}O_2$ after a long bout of hyperoxia is due to a reduction in mitochondrial complex activity, which may also result in lower ATP production. However, the findings from animal and cell work may not translate to humans and would require significant effort to determine the effects in future investigations.

MITOCHONDRIAL OXIDATIVE STRESS

Mitochondrial biogenesis

mRNA expression associated with mitochondrial biogenesis (PGC-1 α , NRF1, and TFAM) were not affected following long duration hyperbaric hyperoxic environments on three consecutive days. PGC-1 α is well known as the master regulator of mitochondrial biogenesis, which involves processes of mitochondrial DNA replication, production of oxidative phosphorylation subunits, and structural proteins to support new mitochondria.⁴¹ Many of these effects are accomplished by increasing the expression of down-stream transcription factors NRF1 and TFAM. Indeed, a variety of stimuli (i.e., nutrition, exercise, oxygen concentration of breathing gas) can activate PGC-1 α , including ROS, which are significantly increased during hyperoxia.⁴¹ ROS can activate p38 mitogen-activation protein kinase (MAPK) and adenosine monophosphate-activated protein kinase (AMPK), both of which target PGC-1 α for activation.^{42,43} Further, mitochondrial ROS production stimulated by hyperbaric oxygen stimulates upregulation of mitochondrial biogenesis.⁴⁴ However, many previous studies that examined the influence of hyperoxia utilized vastly different study designs with longer hyperoxic exposures (e.g., 48 hours or greater) in animal models, which limits applicability to the present study.³⁴ For example, eight days of 100% O₂ was found to increase PGC-1 α (~1.4 fold), NRF1 (~1.8 fold), and TFAM (~1.4 fold) protein content in cardiac muscle of mice compared to air.³⁴ In the present study we observed non-significant changes in PGC-1 α (-0.4 fold), NRF1 (+0.1 fold), and TFAM (+0.2 fold) mRNA from pre to post-dive three. This suggests that six hours of breathing 100% O₂ on three consecutive days is not a large enough stimulus to alter mitochondrial biogenesis signaling in skeletal muscle.

Another possible explanation is that ROS-induced alterations to mitochondrial biogenesis may have been blunted in our study population due a protective effect caused by extensive experience with hyperoxic diving. Indeed, repeated hyperbaric hyperoxic exposures have been shown to reduce oxidative damage from diving.⁴⁵ Furthermore, repeated hyperbaric oxygen exposures (100% O₂ at 3.0 ATA for 28 days, 60 minutes each day) increased SOD2 activity by ~240%, thereby bolstering antioxidant defenses.⁴⁶ Since our study population consisted of experienced military trained divers (11.7 $\pm$ 3.6 yrs; max=17 yrs., min=5 yrs.), they had considerable experience with hyperbaric and hyperoxic environments and may have enhanced baseline antioxidant capacity. Although if this did occur, it is important to note that this did not protect against a decrease in aerobic exercise performance following long duration hyperoxic dives.

Antioxidants

There were no changes in mRNA of any antioxidants (HMOX1, UCP3, SOD1, and SOD2) measured as a result of breathing 100% O₂ during six-hour dives on three consecutive days. Moreover, proteomic analysis found no change in relative protein expression of HMOX1, SOD1, and SOD2. The HMOX1 gene encodes heme oxygenase 1 (HO-1), which exerts antioxidant properties through several mechanism. HO-1 catabolizes free heme, which is increased during cellular stress, such as hyperoxia, and produces free radicals via the Fenton reaction.⁴⁷ Catabolizing free heme produces three major end products that assist with oxidative stress: 1)

labile iron that can be sequestered within a high capacity iron-storing molecule, 2) carbon monoxide which acts as a signaling molecule to promote degradation of pro-apoptotic pathways, triggers a ROS burst to stimulate additional antioxidant pathways, and increases HO-1 expression, 3) biliverdin which can be converted to the antioxidant bilirubin.⁴⁸ The response of HO-1 to hyperoxia is highly dependent upon the presence of heme and free iron;^{49,50} however, hyperoxia has been shown to increase HO-1 mRNA and protein content, but only after 48 hours of > 99% O₂ in rat lungs.⁵¹ UCP3 is an uncoupling protein that is primarily expressed in skeletal muscle and brown adipose tissue. Although, its role has not been clearly elucidated, UCP3 has been postulated to reduce ROS production in the mitochondria through uncoupling of respiration, which decreases membrane potential and ROS generation.⁵²⁻⁵⁴ In support of this purported function, overexpression of UCP3 conferred protection against hyperoxia- and hydrogen peroxide-induced protein carbonylation.⁵⁵ Furthermore, 72 hours of 100% O₂ increased UCP3 mRNA and protein content in mice skeletal muscle.³⁷ SOD1 and 2 are antioxidant enzymes that catalyze the dismutation of superoxide into hydrogen peroxide, which is then converted to water by catalase. SOD1 is located in the cytosol, while SOD2 is located in the mitochondrial matrix. Given that mitochondria superoxide production is proportional to O₂ concentration, SOD enzyme content and activity are especially important during hyperoxia.¹⁵ Indeed, 60 hours of normobaric inspired 98-100% O₂ increases SOD activity, while 72 hours increases SOD2 mRNA in animal skeletal muscle.^{37,56} Collectively, previous data suggest that hyperoxic exposures significantly longer than six hours are required to influence antioxidant levels or activity. It should be noted that antioxidant status and gene expression have been shown to be tissue-specific.^{57,58} Therefore, hyperoxia-induced alterations to antioxidants are also likely tissue-specific since the partial pressure of O₂ varies in different tissues.⁵⁹

MITOCHONDRIAL DYNAMIC REGULATION

There were no changes in mRNA associated with mitochondrial dynamic regulation (MFN2, OPA1, and DNMI1L) following exposure to six-hour 100% O₂ chamber dives on three consecutive days. Further, proteomic analysis found no change in relative protein expression of OPA1 or DNMI1L. Mitochondrial dynamic regulation involves continued modification of mitochondria size and shape to maintain function and energetic demands of the cell and is coordinated by genes associated with fusion (MFN2 and OPA1) and fission (DNMI1L/Drp1).⁶⁰ Several recent investigations have found hyperoxic environments significantly alter measures of mitochondrial dynamic regulation, albeit in much longer exposures to hyperoxia.^{34,61-63} For example, breathing 80-90% O₂ for seven to 10 days decreased MFN2 and dynamin-related protein 1 (DRP1, a mitochondrial fission associated protein encoded by DNMI1L gene) protein content in the lungs of rat pups.^{61,62} Furthermore, 48 hours of 95% O₂ decreased MFN1 protein content and increased DRP1 and OPA1 protein in rat pulmonary endothelial cells.¹⁷ Additionally, 48 hours of 100% O₂ increased OPA1 and DRP1 mRNA in mice cardiac muscle compared to breathing air.³⁴

There are considerable differences in the aims and designs of previous studies that explain the absence of change in mRNA associated with mitochondrial dynamic regulation in the present study. Many studies examined the effects of hyperoxia on acute lung injuries, and therefore, used either an animal model or pulmonary cells, which respond different to hyperoxia compared to human skeletal muscle. Additionally, the hyperoxic environments varied in percent of inspired

O₂ (80-95%), and the exposures lengths (six hours to 14 days) were generally much longer than the present study. Further, several of these studies examine protein expression where as we measured mRNA expression. Indeed, some alterations occurred as soon as six hours (phosphorylation of DRP1);¹⁷ however, many of the proteins associated with mitochondrial dynamic regulation were not affected until three to seven days of continuous hyperoxic exposures.¹⁷ Regardless, the results of the present study indicate that six-hour hyperbaric hyperoxic exposures on three consecutive days is not a sufficient stimulus to influence skeletal muscle mitochondrial dynamic regulation.

MITOPHAGY

There were no changes in mRNA associated with mitophagy (PINK1 and PARK2), which agrees with our other mitochondria measures. Mitophagy is an essential process that removes damaged mitochondria, protects cells from stress-induced death, and, together with mitochondrial biogenesis and dynamic regulation, maintains mitochondrial homeostasis.⁶⁴ Under normal conditions, PINK1 is constitutively expressed and imported into the mitochondria for degradation.⁶⁵ However, when mitochondria are damaged and membrane potential cannot be maintained, PINK1 accumulates and recruits PARK2, which acts as an E3 ubiquitin ligase to target the mitochondria for degradation.⁶⁵ Several investigations have found hyperoxia to alter mitophagy markers, which may be dependent upon O₂ concentration and exposure length, and cell type.^{17,34,66,67} For example, 24 hours of 95% O₂ increases PINK1 and PARK2 protein content in rat lung tissue; however, when the O₂ concentration is reduced to 85%, increases are not observed until seven days.^{66,67} Furthermore, 48 hours of 100% O₂ increases mRNA of PINK1 and PARK2 in mice cardiac muscle tissue, whereas 95% O₂ for 48 hours decreases PINK1 protein content in pulmonary endothelial cells of rats.^{17,34} Although changes in PINK1 and PARK2 appear to be sensitive to many factors, exposures of 100% O₂ longer than six hours or more than three consecutive days may be required to influence mitophagy in skeletal muscle.

PROTEOMICS

Up-regulated proteins

The top five up-regulated enriched GO biological processes terms appear to be associated with an overall immune response, which indicates that skeletal muscle may experience damaged during hyperoxia. Furthermore, the top five up-regulated GO cellular components terms suggest that extracellular locations are the most affected. The top five up-regulated GO molecular functions terms are more diverse and indicate increased hydrolysis of proteins and peptide bonds, along with modulation of enzymatic activity. Lastly, up-regulated KEGG terms also imply increased activity of pathways associated with immune response, along with alterations in metabolism. Overall, up-regulated terms seem to be mostly associated with an inflammatory and immune response.

Concerning the top up-regulated individual proteins, most appear to be involved in immune and tissue repair processes. Fibronectin is a protein found in the extracellular matrix that facilitates tissue repair by assisting with formation of the fibrin clot, reinforces tissue structure, and

regulates many of the repair procedures.⁶⁸ Upregulation of Fibronectin suggests that skeletal muscle may be undergoing repair processes after damage caused by hyperoxia.

Calumenin is purported to be a low-affinity calcium binding protein that regulates calcium trafficking within skeletal muscle through sarcoplasmic reticulum calcium ATPase two alpha (SERCA2 α) and ryanodine receptor 1 (RYR1).^{69,70} Briefly, SERCA2 is an ATPase pump that moves calcium from the cytosol to the sarcoplasmic reticulum following a muscle contraction, while RYR1 releases calcium into the cytosol from the sarcoplasmic reticulum following depolarization and allows muscle contraction to occur. Interestingly, increased Calumenin has been found to reduce SERCA2 activity and depolarization-induced RYR1 activity, which would alter intracellular calcium concentrations.^{70,71} These changes would then have implications on muscular contraction or possibly protein folding and trafficking within the endoplasmic reticulum or Golgi apparatus.^{72,73} Furthermore, several studies have found hyperoxia to alter cellular calcium levels in smooth muscle and endothelial cells.^{74,75} The latter of these possibilities may explain why there was a significant down-regulation in GO cellular component protein terms associated with Golgi apparatus and lumen. However, the potential impact of hyperoxia on calcium dynamics and Golgi apparatus function in skeletal muscle is unknown and requires additional investigation.

Immunoglobulin lambda-6 chain C region is a light chain polypeptide of an immunoglobulin that functions in antigen recognition.⁷⁶ Free immunoglobulin light chains appear to interact with the immune system in several capacities and increase mast cells activation, disruption of neutrophil function, and have been associated with autoimmune diseases.⁷⁶ Therefore, an increase in this protein likely supports an immune response to hyperoxia.

Peptidyl-prolyl cis-trans isomerases (PPIases) are enzymes that catalyze cis/trans isomerization of peptide bonds during protein folding and some members of this protein family also participate in post-translation protein folding and quality control of glycosylated proteins.⁷³ Since protein folding is sensitive to ROS, it is possible that hyperoxia-induced increases in ROS resulted in disruption of protein folding, leading to an upregulation in PPIases; however, there are no available data to support this hypothesis and would be intriguing area of future work. Moreover, cyclophilin, a PPIase subfamily, has been shown to interact with many immune cells and may influence chemotaxis of leukocyte and promote ROS activity,⁷⁷ suggesting increased expression could be related to immune function. Further targeted investigation is required to determine the effects of hyperoxia-induced increased PPIase expression on skeletal muscle.

Lastly, putative elongation factor 1-alpha-like 3 is an elongation factor involved in the delivery of aminoacylated-transfer RNA to the ribosome.⁷⁸ However, this elongation factor also appears to be involved in protein degradation and apoptosis; specifically the alpha isoform which is pro-apoptotic during myotube differentiation.^{78,79} Increased expression of this protein suggests that hyperoxia modulates processes of protein translation, in addition to folding, which could affect the function of numerous proteins.

Down-regulated proteins

The top five down-regulated enriched GO biological processes terms indicate there is a decrease in intracellular packing and trafficking of proteins, along with alterations in iron-sulfur cluster formation and metabolic pathways. The top five down-regulated GO cellular components terms indicate that the Golgi apparatus appears to be particularly affected by hyperoxia, along with actomyosin, and integral membranes. The top five down-regulated GO molecular function terms also suggest reduced trafficking of cell material, decrease movement of electrons between molecules, and protein formation. Indeed, protein folding, post-translational modification, and vesicle trafficking functions of the endoplasmic reticulum and Golgi apparatus are negatively affected by hyperoxia, which may explain why these protein terms were down-regulated.⁷³ Additionally, oxidoreductase enzymes facilitate electron movement during protein folding and mitochondrial respiration, which generate ROS.⁷³ Therefore, since hyperoxia can increase ROS in skeletal muscle,³⁷ down-regulation of oxidoreductases may be a compensatory mechanism to reduce ROS levels. Overall, there was a pattern of down-regulated terms associated cellular energy production in GO biological process (mitochondrial electron transport chain, NADH to ubiquinone, carbohydrate metabolic process, generation of precursor metabolites and energy), GO cellular components (mitochondrial respiratory chain complex 1, NADH dehydrogenase complex), and GO molecule function (NADH dehydrogenase (quinone and ubiquinone) activity), further suggestion energy production may be affected.

The top five down-regulated proteins seem to be associated with cellular energy production, redox reactions, and cell protein trafficking. Glutaredoxin proteins generally function as thioltransferases and have important roles in iron homeostasis.⁸⁰ Glutaredoxin 5 is specifically located in the mitochondria where it is involved in heme synthesis and iron-sulfur cluster formation.^{80,81} Iron-sulfur clusters are important for aconitase activity (Krebs cycle enzyme) and electron transfer among mitochondrial complexes, and therefore make them essential for mitochondrial energy production.⁸² However, iron-sulfur clusters can also be rapidly oxidized by O₂, releasing free iron, which produces ROS via the Fenton reaction.⁸³ As such, iron-sulfur clusters represent an additional potential source of ROS during hyperoxia. Therefore, down-regulation of this Glutaredoxin may be an attempt to alter iron metabolism, potentially due to ROS production.

NADH dehydrogenase 1 beta subcomplex subunit 6 is a nuclear encoded subunit of mitochondrial complex 1 that is important for proper function.⁸⁴ Interestingly, a polymorphism in the NDUF6 gene that increases potential DNA methylation sites (decreasing expression) has been associated with skeletal muscle insulin sensitivity and ATP synthesis after acute exercise; suggesting decreased NDUF6 may impair substrate utilization and energy production.^{85,86} Although, the magnitude of NDUF6 down-regulation on complex 1 function in the present study is unknown, there were no changes in MT-ND1 mRNA, suggesting overall function may not have been impacted. However, it should be noted that several other subcomplexes were down-regulated and others were not differentially, highlighting the variability in susceptibility of subcomplexes to hyperoxia.

Guanidinoacetate N-methyltransferase (GAMT) is an enzyme that transfers a methyl group from S-adenosyl-L-methionine to guanidinoacetate to produce S-adenosyl-L-homocysteine and

creatine.⁸⁷ The down-regulation of this enzyme is difficult to interpret, as the changes in reaction substrates and products have various effects. For example, decreased GAMT leads to increased guanidinoacetate, which can decrease antioxidant capacity and creatine kinase activity, and increase oxidative stress^{88,89}. However, S-adenosyl-L-methionine is also increased, which has antioxidant activity and can reduce oxidative stress.^{90,91} Additionally, decreased GAMT reduces creatine levels, which decreases ATP for muscle contraction and numerous biological reactions.⁸⁷ Lower GAMT also decreases S-adenosyl-L-homocysteine, which has been associated with increased cardiovascular disease, atherosclerosis, and endothelial dysfunction.⁹² Ultimately, the effect of down-regulation of this protein is unclear and requires more data to elucidate the effects.

Importin-5 is a nuclear import protein involved in transporting proteins from the cytosol to the nucleus, including transcription factors, which can alter gene and protein expression.⁹³⁻⁹⁵ Interestingly, some tumor tissues have significantly high expression of importin-5 and tumor growth is decreased in importin-5 knockout models.⁹³ Additionally importin-5 interacts with viral proteins, suggesting it may have immune-related functions.⁹³ Indeed, oxidative stress can impair nuclear import; however, the specific effects on importin-5 are not well understood.⁹⁶

L-xylulose reductase is an oxidoreductase that is involved in the uronate cycle of glucose metabolism and reduction of carbonyl compounds.⁹⁷ L-xylulose reductase reduces l-xylulose to l-xylitol in the uronate cycle, which contributes to 5% of daily glucose usage.⁹⁷ Moreover, L-xylulose reductase can also generate ROS during carbonyl compound reduction in lung epithelial cells; however, the association with hyperoxia and skeletal muscle is unclear.⁹⁸

Overall, hyperoxia appears to promote a diverse response within skeletal muscle with an up-regulation in proteins potentially involved in immune response, calcium regulation, and protein folding, along with down-regulation of proteins potentially involved in process of energy production, redox reactions, and protein trafficking.

EXERCISE SURFACE ELECTROMYOGRAPHY

sEMG measurements during aerobic exercise were not influenced by six-hour dives with 100% O₂ on three consecutive days. The lack of change in burst and cycle duration and interburst interval suggest that ability to continually generate and propagate action potentials is not altered during aerobic exercise after exposure the hyperoxic environment in the present study. Indeed, hyperbaric hyperoxic conditions consisting of a constant inspired O₂ of 1.15-1.20 ATA and 10-minute epochs of increased pressure to reach a range between 1.7 and 2.5 ATA O₂ have been correlated to reduced EMG burst (M-wave) duration during a single evoked muscle contraction.²⁰ Furthermore, breathing 100% O₂ for 20 minutes at surface decreased M-wave conduction time (reduced EMG burst duration) following evoked muscle contractions; however, this effect was abolished after subject resumed breathing air for 10 minutes.²¹ Although our hyperoxic exposures were significantly longer, it appears that greater depth or greater partial pressure of O₂ may be required to alter neuromuscular function during submaximal contractions. It is also possible that any changes in neuromuscular function during submaximal contraction may rapidly recovery after returning to normobaric normoxic environments.

Although not directly comparable, the sEMG amplitude results are somewhat different from our laboratory's past EMG findings after hyperoxic resting water immersions.⁹ We have previously reported sEMG amplitude in the vastus lateralis, vastus medialis, and rectus femoris was decreased during maximum knee extension contractions after 100% O₂ water immersions on three and five consecutive days and at 24- and 72-hours after water immersion.⁹ A decrease in knee extension strength was also observed. Collectively these results indicated that hyperoxia may influence sEMG amplitude during maximum muscular contractions but not during submaximal contractions during treadmill running. Additionally, if sEMG amplitude is crudely interpreted as a proxy of neuromuscular fatigue, then these findings also suggest that lower body neuromuscular fatigue is not a significant contributor to hyperoxia-induced decreases aerobic exercise performance.

LIMITATIONS

There are several limitations that should be considered. The study was powered to detect changes in aerobic performance and may have been underpowered to detect changes in mRNA measurements. As mentioned in the discussion, there are very little data in humans or relevant conditions to conduct an appropriate power analysis. Moreover, there is a plethora of options to measure oxidative stress; and therefore, it is possible that other markers would produce different results. However, there were no changes in the six included mRNA oxidative stress measurements, nor mRNA associated with mitochondrial dynamic regulation or mitophagy, which strengthens the conclusion that oxidative stress was likely unchanged. Muscle tissue samples were only obtained before diving and after the third dive, and as such, we are not able to determine the potential effects of a single six-hour dive. sEMG data for several subjects were removed from analysis after processing, which reduced statistical power and decreased the potential to detect changes. Lastly, the proteomics findings should primarily be viewed in the context of hypothesis generating, and need to be interpreted with fold-change or enrichment values in addition to *p*- and *q*-values. Some of *q*-values of differently expressed proteins and GO terms were as high as .28 suggesting a high possibility of false-positives.

CONCLUSIONS

Long duration dry chamber dives with 100% O₂ on three consecutive days did not influence any measure of mitochondrial function, oxidative stress, dynamic regulation or mitophagy, nor sEMG measures during aerobic exercise. We have previously demonstrated hyperoxia-induced decreases in aerobic exercise performance under multiple conditions. The extensive analyses of this study indicate that skeletal muscle mitochondria mRNA markers associated with stress and lower body neuromuscular fatigue are unlikely to be the cause of hyperoxia-induced decreases in aerobic exercise performance. However, this study generated novel data on how hyperoxia alters the skeletal muscle proteome. These findings provide insight into the differently expressed proteins in skeletal muscle resulting from hyperoxia, which can be used to explore new avenues for future research efforts. For example, the expression of proteins associated with immune function, iron metabolism, calcium handling, energy production and protein folding were all significantly altered and should be further investigated to elucidate the effects on skeletal muscle function.

Based on the results of this project there are several avenues of future work that would further elucidate the influence of long duration hyperoxic dives on physical performance; however, bioenergetic analyses and calcium trafficking may be the most influential. Since a number of proteins associated with energy producing pathways appeared to be down-regulated after hyperoxic diving, a bioenergetic analysis of muscle tissue would determine if energy production was compromised due to hyperoxia. For example, pathway specific deficiencies can be examined by comparing ATP production rates between glycolysis and oxidative phosphorylation. Further, mitochondrial function can be thoroughly examined with complex specific inhibitors while measuring oxygen consumption rate. Concerning calcium handling, the proteomics findings from this study, along with results from other hyperoxic investigations, suggest that hyperoxia may influence calcium release and uptake. Alterations to calcium levels could affect skeletal muscle by modifying contraction and relaxation processes, thereby resulting in changes in force output and fatigue. Although some work has been completed on the influence of hyperoxia on bioenergetic and calcium trafficking, there are no data on skeletal muscle and the conditions are drastically different from environments encountered by divers. Therefore, future studies should prioritize designs that replicate diving conditions to enhance applicability of findings. Moreover, there is a general lack of data on hyperoxia and skeletal muscle that can be addressed by other discovery analyses such as genomics, transcriptomics, and metabolomics to build a robust dataset.

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1. Klemp AO, Reed MD, & Considine EG. (2022 June). *Characterizing hyperoxia-induced alterations in muscular physiology: part 1* (Report No. NEDU TR 22-32, TA 20-20), Navy Experimental Diving Unit.
2. USN. (2018). *U.S. Navy diving manual: Revision 7a*.
3. Zakaroff MG, Snow RW, & Vorosmarti Jr J. (2019). *Military diving operations*. Army Medical Department (AMEDD). The Borden Institute.
4. Wingelaar TT, van Ooij P-JA, & van Hulst RA. (2017). Oxygen toxicity and special operations forces diving: Hidden and dangerous. *Frontiers in Physiology*, 8, 1263.
5. Florian JP, Simmons EE, & Shykoff BE. (2012, May 1). Effects of repeated 6-hour 100% oxygen dry chamber dives on diver performance and orthostatic tolerance. *Medicine & Science in Sports & Exercise*, 44, 747-747.
6. Shykoff B, Bergeron E, & Florian J. (2015, April 29). Reduced exercise endurance after repeated long-duration oxygen dives—do ventilatory parameters hint at a cause? *The FASEB Journal*, 29, 678-673.
7. Florian J, Selkirk A, & Shykoff B. (2011). Effects of repeated six hour oxygen dives on diver performance and orthostatic tolerance *Undersea & Hyperbaric Medicine*, 20(B14).
8. Myers CM, Kim JS, Musilli M, McCully K, & Florian JP. (2018). Effects of resting, consecutive, long-duration water immersions on neuromuscular endurance in well-trained males. *Frontiers in Physiology*, 9, 977.
9. Myers CM, Kim J-S, & Florian JP. (2019, 2019-July-31). Consecutive, resting, long-duration hyperoxic exposures alter neuromuscular responses during maximal strength exercises in trained men. *Frontiers in Physiology*, 10(960).
10. Bergeron ER, & Florian JP. (2018, April). Aerobic endurance is reduced immediately after repeated 6-hr normoxic and hyperoxic dives at 1.35 ata and remains reduced in the hyperoxic group 3 days after the last dive. *The FASEB Journal*, 32, 246.
11. Florian JP, Simmons EE, & Shykoff BE. (2014, May 29). Surface exercise performance is reduced more after exercise dives than resting dives with 100% oxygen. *Medicine & Science in Sports & Exercise*, 46(5S), 430.
12. Florian J, Myers C, Jewell J, Doggett T, Bergeron E, & Campbell J. (2018 Sep). *Impact of repeated 6-hour resting dives at 1.35 ata on muscular performance and cardiovascular endurance*. (NEDU TR 18-01). Navy Experimental Diving Unit.

13. Freeman BA, & Crapo JD. (1981, Nov 10). Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *Journal of Biological Chemistry*, 256(21), 10986-10992.
14. Bin-Jaliah I, Dallak M, & Haffor AS. (2009). Effect of hyperoxia on the ultrastructural pathology of alveolar epithelium in relation to glutathione peroxidase, lactate dehydrogenase activities, and free radical production in rats. *Ultrastructural Pathology*, 33(3), 112-122.
15. Turrens JF. (2003). Mitochondrial formation of reactive oxygen species. *The Journal of Physiology*, 552(2), 335-344.
16. Thanan R, Oikawa S, Hiraku Y, Ohnishi S, Ma N, Pinlaor S, Yongvanit P, Kawanishi S, & Murata M. (2014). Oxidative stress and its significant roles in neurodegenerative diseases and cancer. *International Journal of Molecular Sciences*, 16(1), 193-217.
17. Ma C, Beyer AM, Durand M, Clough AV, Zhu D, Norwood Toro L, Terashvili M, Ebben JD, Hill RB, & Audi SH. (2018). Hyperoxia causes mitochondrial fragmentation in pulmonary endothelial cells by increasing expression of pro-fission proteins. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 38(3), 622-635.
18. Das KC. (2013). Hyperoxia decreases glycolytic capacity, glycolytic reserve and oxidative phosphorylation in mle-12 cells and inhibits complex i and ii function, but not complex iv in isolated mouse lung mitochondria. *PloS one*, 8(9).
19. Quan Y, Xin Y, Tian G, Zhou J, & Liu X. (2020). Mitochondrial ros-modulated mtdna: A potential target for cardiac aging. *Oxidative Medicine and Cellular Longevity*, 2020.
20. Jammes Y, Arbogast S, Faucher M, Montmayeur A, Tagliarini F, Meliet JL, & Robinet C. (2003, May). Hyperbaric hyperoxia induces a neuromuscular hyperexcitability: Assessment of a reduced response in elite oxygen divers. *Clinical Physiology and Functional Imaging*, 23(3), 149-154.
21. Brerro-Saby C, Delliaux S, Steinberg JG, & Jammes Y. (2010, Jan). The changes in neuromuscular excitability with normobaric hyperoxia in humans. *Experimental Physiology*, 95(1), 153-159.
22. Houssière A, Najem B, Cuylits N, Cuypers S, Naeije R, & van de Borne P. (2006, Jul). Hyperoxia enhances metaboreflex sensitivity during static exercise in humans. *The American Journal of Physiology-Heart and Circulatory Physiology*, 291(1), H210-215.
23. Harris JA, & Benedict FG. (1919). *A biometric study of basal metabolism in man*. Carnegie Institution of Washington.
24. Liguori G, & Medicine ACoS. (2020). *Acsm's guidelines for exercise testing and prescription: 10th edition*. Lippincott Williams & Wilkins.

25. Hermens HJ, Freriks B, Merletti R, Stegeman D, Blok J, Rau G, Disselhorst-Klug C, & Hägg G. (1999). European recommendations for surface electromyography. *Roessingh Research and Development*, 8(2), 13-54.
26. Hayot M, Michaud A, Koechlin C, Caron M, Leblanc P, Prefaut C, & Maltais F. (2005). Skeletal muscle microbiopsy: A validation study of a minimally invasive technique. *European Respiratory Journal*, 25(3), 431-440.
27. Hughes MC, Ramos SV, Turnbull PC, Nejatbakhsh A, Baechler BL, Tahmasebi H, Laham R, Gurd BJ, Quadrilatero J, Kane DA, & Perry CG. (2015). Mitochondrial bioenergetics and fiber type assessments in microbiopsy vs. Bergstrom percutaneous sampling of human skeletal muscle. *Frontiers in Physiology*, 6, 360.
28. Florian J. (2010). Repeated 6-hour dives diminish dynamic and static exercise performance. *Undersea & Hyperbaric Medicine*, 307, 335.
29. Motulsky HJ, & Brown RE. (2006, 2006/03/09). Detecting outliers when fitting data with nonlinear regression – a new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinformatics*, 7(1), 123.
30. Benjamini Y, & Hochberg Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B (Methodological)*, 57(1), 289-300.
31. Ibrahim JG, Chu H, & Chen MH. (2012, Sep 10). Missing data in clinical studies: Issues and methods. *Journal of Clinical Oncology*, 30(26), 3297-3303.
32. Sharma LK, Lu J, & Bai Y. (2009). Mitochondrial respiratory complex i: Structure, function and implication in human diseases. *Current Medicinal Chemistry*, 16(10), 1266-1277.
33. Chandel NS. (2010, Dec 31). Mitochondrial complex iii: An essential component of universal oxygen sensing machinery? *Respiratory Physiology & Neurobiology*, 174(3), 175-181.
34. Suliman HB, Keenan JE, & Piantadosi CA. (2017). Mitochondrial quality-control dysregulation in conditional ho-1^{-/-} mice. *The Journal of Clinical Investigation Insight*, 2(3).
35. Ma Z, Moruzzi N, Catrina S-B, Grill V, & Björklund A. (2014, 2014/01/03/). Hyperoxia inhibits glucose-induced insulin secretion and mitochondrial metabolism in rat pancreatic islets. *Biochemical and Biophysical Research Communications*, 443(1), 223-228.

36. Zhao HW, Ali SS, & Haddad GG. (2012, May 15). Does hyperoxia selection cause adaptive alterations of mitochondrial electron transport chain activity leading to a reduction of superoxide production? *Antioxidants & Redox Signaling*, 16(10), 1071-1076.
37. Flandin P, Donati Y, Barazzone-Argiroffo C, & Muzzin P. (2005). Hyperoxia-mediated oxidative stress increases expression of ucp3 mrna and protein in skeletal muscle. *FEBS Letters*, 579(16), 3411-3415.
38. Joenje H, Gille JJ, Oostra AB, & Van der Valk P. (1985, 1985/04//). Some characteristics of hyperoxia-adapted hela cells. A tissue culture model for cellular oxygen tolerance. *Laboratory Investigation*, 52(4), 420-428.
39. Lauscher P, Lauscher S, Kertscho H, Habler O, & Meier J. (2012, 2012/05/01). Hyperoxia reversibly alters oxygen consumption and metabolism. *The Scientific World Journal*, 2012, 410321.
40. Florian JP, Klemp AO, Simmons EE, Campbell JE, Bergeron ER, & Doggett TM. (2020). *Impact of 100% Oxygen Repeated 6-hour Exercise Dives at 1.35 ATA on Muscular Performance and Cardiovascular Endurance* (Report No. NEDU TR 18-01, TA 18-01), Navy Experimental Diving Unit.
41. Rius-Pérez S, Torres-Cuevas I, Millán I, Ortega ÁL, & Pérez S. (2020). Pgc-1 α , inflammation, and oxidative stress: An integrative view in metabolism. *Oxidative Medicine and Cellular Longevity*, 2020.
42. Dias-Freitas F, Metelo-Coimbra C, & Roncon-Albuquerque Jr R. (2016). Molecular mechanisms underlying hyperoxia acute lung injury. *Respiratory Medicine*, 119, 23-28.
43. Irrcher I, Ljubicic V, & Hood DA. (2009). Interactions between ros and amp kinase activity in the regulation of pgc-1 α transcription in skeletal muscle cells. *American Journal of Physiology-Cell Physiology*, 296(1), C116-C123.
44. Gutsaeva DR, Suliman HB, Carraway MS, Demchenko IT, & Piantadosi CA. (2006). Oxygen-induced mitochondrial biogenesis in the rat hippocampus. *Neuroscience*, 137(2), 493-504.
45. Alcaraz-García M, Albaladejo M, Acevedo C, Olea A, Zamora S, Martínez P, & Parra S. (2008). Effects of hyperoxia on biomarkers of oxidative stress in closed-circuit oxygen military divers. *Journal of Physiology and Biochemistry*, 64(2), 135-141.
46. Gregorevic P, Lynch GS, & Williams DA. (2001, 2001/11/01). Hyperbaric oxygen modulates antioxidant enzyme activity in rat skeletal muscles. *European Journal of Applied Physiology*, 86(1), 24-27.
47. Gozzelino R, Jeney V, & Soares MP. (2010). Mechanisms of cell protection by heme oxygenase-1. *Annual Review of Pharmacology and Toxicology*, 50(1), 323-354.

48. Otterbein LE, Soares MP, Yamashita K, & Bach FH. (2003). Heme oxygenase-1: Unleashing the protective properties of heme. *Trends in Immunology*, 24(8), 449-455.
49. Visner GA, Fogg S, & Nick HS. (1998, 1998/04/01). Hyperoxia induction of human heme oxygenase 1 is a transcriptional response and is dependent upon free iron • 1971. *Pediatric Research*, 43(4), 336-336.
50. Fogg S, Agarwal A, Nick HS, & Visner GA. (1999, Apr). Iron regulates hyperoxia-dependent human heme oxygenase 1 gene expression in pulmonary endothelial cells. *American Journal of Respiratory Cell and Molecular Biology*, 20(4), 797-804.
51. Lee PJ, Alam J, Sylvester SL, Inamdar N, Otterbein L, & Choi AM. (1996). Regulation of heme oxygenase-1 expression in vivo and in vitro in hyperoxic lung injury. *American Journal of Respiratory Cell and Molecular Biology*, 14(6), 556-568.
52. Garvey WT. (2003). The role of uncoupling protein 3 in human physiology. *The Journal of Clinical Investigation*, 111(4), 438-441.
53. Pohl EE, Rupprecht A, Macher G, & Hilse KE. (2019, 2019-April-30). Important trends in ucp3 investigation. *Frontiers in Physiology*, 10.
54. Jiang N, Zhang G, Bo H, Qu J, Ma G, Cao D, Wen L, Liu S, Ji LL, & Zhang Y. (2008). Upregulation of uncoupling protein-3 in skeletal muscle during exercise: A potential antioxidant function. *Free Radical Biology & Medicine*, 46(2), 138-145.
55. Barreiro E, Garcia-Martínez C, Mas S, Ametller E, Gea J, Argilés JM, Busquets S, & López-Soriano FJ. (2009). Ucp3 overexpression neutralizes oxidative stress rather than nitrosative stress in mouse myotubes. *FEBS Letters*, 583(2), 350-356.
56. Amicarelli F, Ragnelli AM, Aimola P, Bonfigli A, Colafarina S, Di Ilio C, & Miranda M. (1999, 1999/01/06/). Age-dependent ultrastructural alterations and biochemical response of rat skeletal muscle after hypoxic or hyperoxic treatments. *Biochimica et Biophysica Acta*, 1453(1), 105-114.
57. Kim JM, Kim HG, & Son CG. (2018). Tissue-specific profiling of oxidative stress-associated transcriptome in a healthy mouse model. *International Journal of Molecular Sciences*, 19(10), 3174.
58. Ranjan R, Singh SV, & Upadhyay RC. (2007, 2007/01/01). Status of antioxidant enzymes in different body organs of male buffaloes. *Italian Journal of Animal Science*, 6(sup2), 981-983.
59. Ortiz-Prado E, Dunn JF, Vasconez J, Castillo D, & Viscor G. (2019). Partial pressure of oxygen in the human body: A general review. *American Journal of Blood Research*, 9(1), 1-14.

60. Elgass K, Pakay J, Ryan MT, & Palmer CS. (2013). Recent advances into the understanding of mitochondrial fission. *Biochimica et Biophysica Acta*, 1833(1), 150-161.
61. Teng R-J, Jing X, Michalkiewicz T, Afolayan AJ, Wu T-J, & Konduri GG. (2017). Attenuation of endoplasmic reticulum stress by caffeine ameliorates hyperoxia-induced lung injury. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 312(5), L586-L598.
62. Dai Y, Yu B, Ai D, Yuan L, Wang X, Huo R, Fu X, Chen S, & Chen C. (2021, 2021-January-28). Mitochondrial fission-mediated lung development in newborn rats with hyperoxia-induced bronchopulmonary dysplasia with pulmonary hypertension. *Frontiers in Pediatrics*, 8.
63. Ma C, Beyer AM, Durand M, Clough AV, Zhu D, Toro LN, Terashvili M, Ebben JD, Hill RB, Audi SH, Medhora M, & Jacobs ER. (2018). Hyperoxia causes mitochondrial fragmentation in pulmonary endothelial cells by increasing expression of pro-fission proteins. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 38(3), 622-635.
64. Ma K, Chen G, Li W, Kepp O, Zhu Y, & Chen Q. (2020, 2020-June-24). Mitophagy, mitochondrial homeostasis, and cell fate. *Frontiers in Cell and Developmental Biology*, 8.
65. Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, & Youle RJ. (2010). Mitochondrial membrane potential regulates pink1 import and proteolytic destabilization by parl. *Journal of Cell Biology*, 191(5), 933-942.
66. Yu X, Sun Y, Cai Q, Zhao X, Liu Z, Xue X, & Fu J. (2020, 2020/12/01). Hyperoxia exposure arrests alveolarization in neonatal rats via pten-induced putative kinase 1-parkin and nip3-like protein x-mediated mitophagy disorders. *International Journal of Molecular Medicine*, 46(6), 2126-2136.
67. Liu G, Qian M, Chen M, Chen T, & Qin S. (2020, 2020/11/20). Mir-21-5p suppresses mitophagy to alleviate hyperoxia-induced acute lung injury by directly targeting pgam5. *BioMed Research International*, 2020, 4807254.
68. To WS, & Midwood KS. (2011, Sep 16). Plasma and cellular fibronectin: Distinct and independent functions during tissue repair. *Fibrogenesis Tissue Repair*, 4, 21.
69. Mazzorana M, Hussain R, & Sorensen T. (2016). Ca-dependent folding of human calumenin. *PloS one*, 11(3), e0151547.
70. Jung DH, Mo SH, & Kim DH. (2006, 2006/04/28/). Calumenin, a multiple ef-hands ca²⁺-binding protein, interacts with ryanodine receptor-1 in rabbit skeletal sarcoplasmic reticulum. *Biochemical and Biophysical Research Communications*, 343(1), 34-42.

71. Sahoo SK, & Kim DH. (2008, Sep 30). Calumenin interacts with serca2 in rat cardiac sarcoplasmic reticulum. *Molecular Cell*, 26(3), 265-269.
72. Bhandary B, Marahatta A, Kim HR, & Chae HJ. (2012, Dec 24). An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. *International Journal of Molecular Sciences*, 14(1), 434-456.
73. Mennerich D, Kellokumpu S, & Kietzmann T. (2019). Hypoxia and reactive oxygen species as modulators of endoplasmic reticulum and golgi homeostasis. *Antioxidants & Redox Signaling*, 30(1), 113-137.
74. Roesler AM, Ravix J, Bartman CM, Patel BS, Schiliro M, Roos B, Nesbitt L, Pabelick CM, Martin RJ, MacFarlane PM, & Prakash YS. (2021). Calcium-sensing receptor contributes to hyperoxia effects on human fetal airway smooth muscle. *Frontiers in Physiology*, 12, 585895.
75. Brueckl C, Kaestle S, Kerem A, Habazettl H, Krombach F, Kuppe H, & Kuebler WM. (2006, Apr). Hyperoxia-induced reactive oxygen species formation in pulmonary capillary endothelial cells in situ. *American Journal of Respiratory Cell and Molecular Biology*, 34(4), 453-463.
76. Nakano T, Matsui M, Inoue I, Awata T, Katayama S, & Murakoshi T. (2011, 2011/05/12/). Free immunoglobulin light chain: Its biology and implications in diseases. *Clinica Chimica Acta*, 412(11), 843-849.
77. Nath PR, & Isakov N. (2015, 2015/01/01/). Insights into peptidyl-prolyl cis-trans isomerase structure and function in immunocytes. *Immunology Letters*, 163(1), 120-131.
78. Sasikumar AN, Perez WB, & Kinzy TG. (2012, Jul-Aug). The many roles of the eukaryotic elongation factor 1 complex. *Wiley Interdisciplinary Reviews: RNA*, 3(4), 543-555.
79. Ruest LB, Marcotte R, & Wang E. (2002, Feb 15). Peptide elongation factor eef1a-2/s1 expression in cultured differentiated myotubes and its protective effect against caspase-3-mediated apoptosis. *Journal of Biological Chemistry*, 277(7), 5418-5425.
80. Ogata FT, Branco V, Vale FF, & Coppo L. (2021, 2021/07/01/). Glutaredoxin: Discovery, redox defense and much more. *Redox Biology*, 43, 101975.
81. Rouhier N, Couturier J, Johnson MK, & Jacquot JP. (2010, Jan). Glutaredoxins: Roles in iron homeostasis. *Trends in Biochemical Sciences*, 35(1), 43-52.
82. Read AD, Bentley RET, Archer SL, & Dunham-Snary KJ. (2021, 2021/11/01/). Mitochondrial iron-sulfur clusters: Structure, function, and an emerging role in vascular biology. *Redox Biology*, 47, 102164.

83. Imlay JA. (2006). Iron-sulphur clusters and the problem with oxygen. *Molecular Microbiology*, 59(4), 1073-1082.
84. Loublier S, Bayot A, Rak M, El-Khoury R, Bénit P, & Rustin P. (2011, 2011/10/22/). The ndufb6 subunit of the mitochondrial respiratory chain complex i is required for electron transfer activity: A proof of principle study on stable and controlled rna interference in human cell lines. *Biochemical and Biophysical Research Communications*, 414(2), 367-372.
85. Ling C, Poulsen P, Simonsson S, Rönn T, Holmkvist J, Almgren P, Hagert P, Nilsson E, Mabey AG, Nilsson P, Vaag A, & Groop L. (2007, Nov). Genetic and epigenetic factors are associated with expression of respiratory chain component ndufb6 in human skeletal muscle. *Journal of Clinical Investigation*, 117(11), 3427-3435.
86. Kacerovsky-Bielesz G, Chmelik M, Ling C, Pokan R, Szendroedi J, Farukuoye M, Kacerovsky M, Schmid AI, Gruber S, Wolzt M, Moser E, Pacini G, Smekal G, Groop L, & Roden M. (2009, Jun). Short-term exercise training does not stimulate skeletal muscle atp synthesis in relatives of humans with type 2 diabetes. *Diabetes*, 58(6), 1333-1341.
87. Marques EP, & Wyse AT. (2019). Guanidinoacetate methyltransferase deficiency: A review of guanidinoacetate neurotoxicity. *Journal of Inborn Errors of Metabolism and Screening*.
88. Zugno AI, Stefanello FM, Scherer EB, Mattos C, Pederzoli CD, Andrade VM, Wannmacher CM, Wajner M, Dutra-Filho CS, & Wyse AT. (2008, Sep). Guanidinoacetate decreases antioxidant defenses and total protein sulfhydryl content in striatum of rats. *Neurochemical Research*, 33(9), 1804-1810.
89. Zugno AI, Scherer EBS, Schuck PF, Oliveira DL, Wofchuk S, Wannmacher CMD, Wajner M, & Wyse ATS. (2006, 2006/03/01). Intrastriatal administration of guanidinoacetate inhibits na⁺, k⁺-atpase and creatine kinase activities in rat striatum. *Metabolic Brain Disease*, 21(1), 39-48.
90. Caro AA, & Cederbaum AI. (2004, May 15). Antioxidant properties of s-adenosyl-l-methionine in fe(2+)-initiated oxidations. *Free Radical Biology and Medicine*, 36(10), 1303-1316.
91. Villalobos MA, De La Cruz JP, Cuerda MA, Ortiz P, Smith-Agreda JM, & Sánchez De La Cuesta F. (2000, Nov 10). Effect of s-adenosyl-l-methionine on rat brain oxidative stress damage in a combined model of permanent focal ischemia and global ischemia-reperfusion. *Brain Research*, 883(1), 31-40.
92. Xiao Y, Su X, Huang W, Zhang J, Peng C, Huang H, Wu X, Huang H, Xia M, & Ling W. (2015, 2015/10/01/). Role of s-adenosylhomocysteine in cardiovascular disease and

- its potential epigenetic mechanism. *The International Journal of Biochemistry & Cell Biology*, 67, 158-166.
93. Patouret R. (2021, Apr 28). The nuclear transport protein importin-5: A promising target in oncology and virology. *Chimia*, 75(4), 319-322.
 94. Patel VP, & Chu CT. (2011, Mar). Nuclear transport, oxidative stress, and neurodegeneration. *International Journal of Clinical and Experimental Pathology*, 4(3), 215-229.
 95. Czubryt MP, Austria JA, & Pierce GN. (2000, Jan 10). Hydrogen peroxide inhibition of nuclear protein import is mediated by the mitogen-activated protein kinase, erk2. *Journal of Cell Biology*, 148(1), 7-16.
 96. Kodiha M, Chu A, Matusiewicz N, & Stochaj U. (2004, Aug). Multiple mechanisms promote the inhibition of classical nuclear import upon exposure to severe oxidative stress. *Cell Death & Differentiation*, 11(8), 862-874.
 97. Ebert B, Kisiela M, & Maser E. (2015, Feb). Human dcxr - another 'moonlighting protein' involved in sugar metabolism, carbonyl detoxification, cell adhesion and male fertility? *Biological reviews of the Cambridge Philosophical Society*, 90(1), 254-278.
 98. Yang S, Jan YH, Mishin V, Heck DE, Laskin DL, & Laskin JD. (2017, Jul 17). Diacetyl/l-xylulose reductase mediates chemical redox cycling in lung epithelial cells. *Chemical Research in Toxicology*, 30(7), 1406-1418.