Navy Experimental Diving Unit
321 Bullfinch Rd
Panama City, FL 32407-7015

TA 20-20
NEDU TR 22-32
June 2022

Characterizing Hyperoxia-Induced Alterations in Muscular Physiology: Part 1

Authors:
Alex. O Klemp, Ph.D.
Mitchell D. Reed, LT, Ph.D.
Elizabeth G. Considine, B.S.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-06-2022	Technical Report	11 January 2021 – 25 April 2022

4. TITLE AND SUBTITLE
Characterizing Hyperoxia-Induced Alterations in Muscular Physiology: Part 1

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Alex. O Klemp, Ph.D.
Mitchell D. Reed, LT, Ph.D.
Elizabeth G. Considine, B.S.

5d. PROJECT NUMBER

5e. TASK NUMBER
TA 20-20

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Navy Experimental Diving Unit
321 Bullfinch Rd
Panama City, FL 32407

8. PERFORMING ORGANIZATION REPORT NUMBER
NEDU TR 22-32

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Naval Sea Systems Command (NAVSEA)
1333 Isaac Hull Avenue
Washington, DC 20376

10. SPONSOR/MONITOR'S ACRONYM(S)
NAVSEA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Statement A: Approved for Release. Distribution is unlimited.

13. SUPPLEMENTARY NOTES
This is part 1 of a 2-part report.

14. ABSTRACT

Introduction: Mechanisms underlying decreased aerobic performance after long duration 100% oxygen (O_2) dives are unknown. 10 subjects completed 6-hour dry dives on 3 consecutive days while breathing 100% O_2 at 1.35 ATA. Aerobic performance with metabolic and respiratory parameters were completed before diving, post-dive 3, and 48 hr. after dive 3. Blood oxidative stress samples were collected on dive days before diving, at 3 and 6 hr. during the dive, 30 and 60 min post-dive, and 16 and 48 hr after dive 3. Nerve conduction velocity (NCV) was performed before and after each dive. Aerobic performance decreased (p = .021) after dive 3 compared to baseline (6.4 ± 1.8 min vs. 5.3 ± 2.0 min, p = .037), but not at 48-hr post-dive 3. $\dot{V}O_2$ (p = .001) and heart rate (HR) (p = .013) decreased during aerobic performance at post-dive 3 ($\dot{V}O_2$: p = .009, HR: p = .010) compared to baseline. $\dot{V}O_2$ (p = .005) remained lower at 48-hr post-dive 3. No other metabolic or respiratory parameters changed. Systemic oxidative stress makers and NCV were unaffected. Aerobic performance is reduced after 6-hour 100% O_2 dives on 3 consecutive days. Unfortunately, the measured variables do not fully explain performance decrements.

15. SUBJECT TERMS
Diving, hyperoxia, aerobic exercise performance, oxidative stress, nerve conduction velocity

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					NEDU Librarian
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Same as report	42	**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified			850-230-3170

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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ABSTRACT

Mechanisms underlying decreased aerobic performance after long duration 100% oxygen (O_2) dives are unknown. 10 subjects completed 6-hour dry dives on 3 consecutive days while breathing 100% O_2 at 1.35 ATA. Aerobic performance with metabolic and respiratory parameters were completed before diving, post-dive 3, and 48 hr after dive 3. Blood oxidative stress samples were collected on dive days before diving, at 3 and 6 hr during the dive, 30 and 60 min post-dive, and 16 and 48 hr after dive 3. Nerve conduction velocity (NCV) was performed before and after each dive. Aerobic performance decreased (p = .021) after dive 3 compared to baseline (6.4 ± 1.8 min vs. 5.3 ± 2.0 min, p = .037), but not at 48-hr post-dive 3. $\dot{V}O_2$ (p = .001) and heart rate (HR) (p = .013) decreased during aerobic performance at post-dive 3 ($\dot{V}O_2$: p = .009, HR: p = .010) compared to baseline. $\dot{V}O_2$ (p = .005) remained lower at 48-hr post-dive 3. No other metabolic or respiratory parameters changed. Systemic oxidative stress makers and NCV were unaffected. Aerobic performance is reduced after 6-hour 100% O_2 dives on 3 consecutive days. Unfortunately, the measured variables do not fully explain performance decrements.

INTRODUCTION

Breathing 100% oxygen (O_2) in a closed-circuit underwater breathing apparatus (CC-UBA) during diving missions has been practiced for over 100 years.[1] This procedure offers operational advantages to special operations forces by increasing stealth through eliminating escaping bubbles, expanding mission duration, and reducing carrying weight, in addition to the physiological benefits of reductions and/or elimination of nitrogen narcosis and decompression sickness.[1,2] However, there are also negative consequences of breathing 100% O_2 for prolonged periods of time.[1,3] While the majority of the literature has focused on pulmonary and central nervous system toxicity,[3] there are also significant alterations to other aspects of physiology (e.g., vascular, skeletal muscle) that may reduce human performance after hyperoxic diving. Given that aerobic endurance is highly emphasized for optimal military operational performance,[4] a greater mechanistic understanding of how breathing 100% O_2 influences human performance in these conditions may lead to development of mitigation strategies.

For the past 12 years, the Navy Experimental Diving Unit (NEDU) has investigated the influence of breathing 100% O_2 during 6-hour dives on consecutive days with 18-hour surface intervals during resting dry chamber dives,[5,6] and resting[7-10] and exercise[11] water immersion dives on physiological alterations and exercise performance. The initial results found that breathing 100% O_2 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 maximum rate of oxygen consumption; $\dot{V}O_{2max}$) by 5% at 24 hours post-dive. However, 5 dives on consecutive days (dive week: DW) under the same parameters reduced performance by 28% at 24 hours after the 5th dive and the reduction in performance was significantly greater than after dives while breathing air (-17%).[7] To remove the influence of water immersion and isolate the effects of breathing 100% O_2 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% O_2, additional studies have examined the influence on muscular strength, excitation, and endurance 1 hour after diving and following a recovery period of 72 hours after diving.[12] Indeed, breathing 100% O_2 resulted in large reductions in exercise performance at 1 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 reduced (-31%) in the 100% O_2 group, but partially recovered in the air group (-9%). Further, dives with 100% O_2, compared to air, had lower heart rates at 1 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% O_2 reduced upper and lower body strength and muscular excitation. Furthermore, some decrements persisted for at least 72 hours after diving.[9] Additionally, 100% O_2 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% O_2 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% O_2, 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.

There is a paucity of literature on the potential mechanisms associated with the effects of breathing 100% O_2 on exercise performance, 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 O_2 are greater than normal atmospheric levels (i.e., hyperoxia).[13,14] A ROS is an oxygen containing molecule that is unstable and reactive due to unpaired electrons. 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. Moreover, 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]

Furthermore, elevated oxidative stress markers due to hyperbaric hyperoxia[19] and normobaric hyperoxia[20] have been demonstrated to alter neuromuscular function. Breathing 100% O_2 can increase nerve conduction velocity (NCV) and muscle excitation in directly stimulated muscle after only 20 minutes.[20] However, the same investigation also demonstrated that reflexive muscular excitation was blunted 30 minutes after breathing 100% O_2, suggesting a reduction in muscle spindle activity.[20] Further, hyperoxia-induced overproduction of ROS has been proposed to activate group IV afferents (i.e., metaboreceptors),[21] 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 was 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.

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 mitochondrial 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 2, 3a, and their respective contribution to objective 4 will be addressed in this technical report. The additional objectives will be covered in a future technical report or technical letter after the completion of data analysis.

METHODS

GENERAL

Baseline performance testing took place over 2 days (Figure 1). The 1st baseline visit occurred approximately 5 days before the 1st dive and consisted of a familiarization pulmonary function test (PFT) followed by a $\dot{V}O_{2max}$ test. The 2nd baseline visit was approximately 3 days later (2 days before the 1st dive) and consisted of a PFT, and a time-to-exhaustion treadmill trial at a workload of approximately 85% $\dot{V}O_{2max}$. The 1st of 3 consecutive dives began approximately 2 days after the 2nd baseline visit and included pre-dive testing of venous blood draw, collection of expired volatile organic compounds (VOC) and inorganic compounds, PFT, and a NCV test. Thereafter, subjects completed a 6-hour dry dive at 1.35 ATA and breathed 100% O_2. A venous blood draw was taken at the 3-hour point and subjects removed their hood for 10 minutes to consume a standardized lunch. An additional venous blood draw was taken at the 6-hour mark, approximately 5 minutes prior to surfacing. Upon exiting the chamber, post-dive testing was completed, which included blood draws at 30-minutes and 1-hour post-dive, along with PFT, VOC, and NCV testing. Following the 3rd post-dive procedure, a second time-to-exhaustion treadmill trial was conducted after the PFT, VOC, and NCV tests. The 16- and 48-hour recovery days was comprised of venous blood draws, PFT, VOC, NCV, with the 48-hour recovery day including the final time-to-exhaustion treadmill trial. The 16-hour recovery time point took place at approximately the same time as the pre-dive measurements to capture any acute changes following the 3rd dive. The 48-hour recovery time point was chosen to align with the 2nd baseline visit and post-dive 3 testing to reduce any time of day influence or food intake on treadmill run performance.

Environmental Control

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

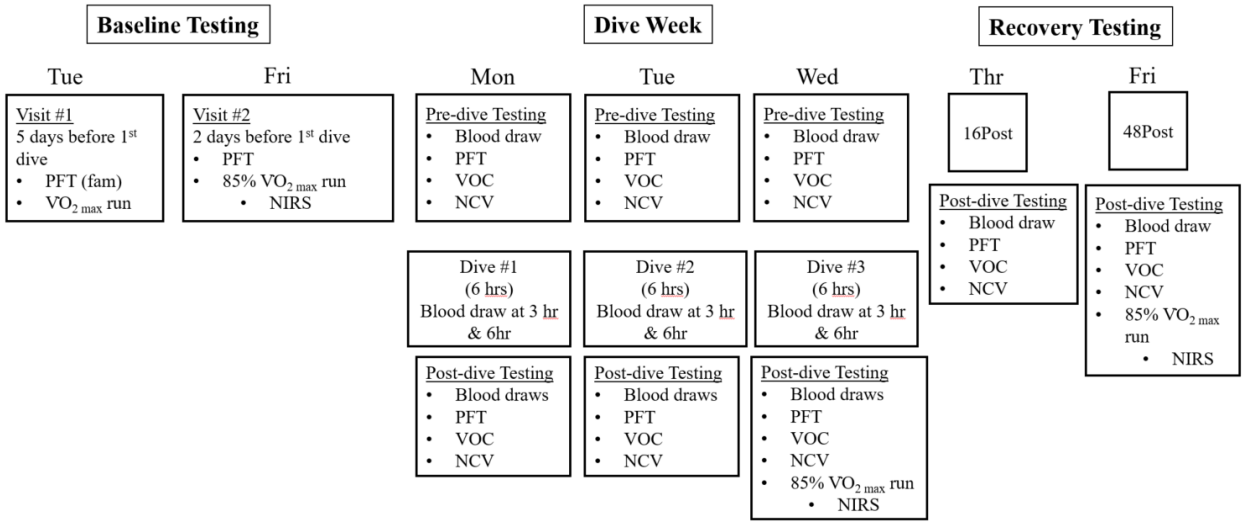

Figure 1. Study overview. PFT (fam) = pulmonary function test familiarization, PFT= pulmonary function test; $\dot{V}O_{2max}$ = maximum rate of oxygen consumption; NIRS = Near-infrared spectroscopy; VOC = volatile organic compounds; NCV = nerve conduction velocity.

SUBJECTS

Fifteen military trained divers participated in the study (Pilot: n = 2, Full protocol: n = 13); however, 3 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. The Navy Experimental Diving Unit Institutional Review Board approved this protocol. 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) or associate investigator (AI). Subjects underwent a routine medical screening and completed a health history questionnaire and physical activity questionnaire prior to beginning testing.

GENERAL INSTRUCTIONS FOR SUBJECTS

Subjects 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 2 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, 8 repetitions, and 2 sets per muscle group for the duration of the study. (The microbiopsy procedure will be discussed in Part 2).

EXPERIMENTAL DESIGN AND ANALYSIS

The variables for this report are in table 1.

Aerobic performance Time to exhaustion
Variables during aerobic performance 1. Metabolic parameters a) Oxygen consumed ($\dot{V}O_2$) b) Fractional end tidal carbon dioxide ($FeCO_2$) c) Respiratory exchange ratio (RER; $\dot{V}CO_2/\dot{V}O_2$) d) Lactate e) Heart rate 2. Respiratory parameters a) Breathing frequency (Bf) b) Tidal volume (TV) c) Minute ventilation (VE) 3. Rating of perceived exertion (RPE) 4. Near-infrared spectroscopy (NIRS; indirect mitochondria function)
Systemic oxidative stress 1. SOD - (Superoxide dismutase; antioxidant level) 2. 4-HNE - (4-Hydroxynonenal; lipid peroxidation) 3. 8-OHdG - (8-Hydroxyguanosine; DNA damage)
Resting nerve conduction velocity - (NCV)
Pulmonary function 1. Forced vital capacity (FVC) 2. Forced expiratory volume in one second (FEV_1) 3. Peak expiratory flow (PEF) 4. Total airway resistance (Resistance (R) at 5 Hz) 5. Central airway resistance (R at 20 Hz) 6. Peripheral airway resistance (R at 5 Hz – R at 20 Hz)
Food and fluid log 1. Food intake

Table 1. Study variables

Baseline: 1st visit

The 1st baseline visit occurred approximately 5 days before the 1st dive. This visit consisted of familiarization PFT followed by a $\dot{V}O_{2max}$ 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) 2 hours before their arrival. Lastly, subjects were instructed on how to complete the food and fluid log following the exercise test.

Baseline: 2nd visit

The 2nd baseline visit was approximately 3 days later (2 days before the 1st dive). For this visit, subjects completed a PFT and a time-to-exhaustion treadmill run at 85% $\dot{V}O_{2max.}$ Subjects arrived at scheduled times between approximately 1300-1600 to align with the time of post-dive 3

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, 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; figure 2) after an overnight fast to complete a compliance questionnaire, resting blood pressure and heart rate, venous blood draw, VOC, PFT, and NCV testing and an ophthalmic exam (see Procedures sections for testing details). Thereafter, subjects were weighed, consumed 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[1] and NEDU standard operating procedures. 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. During the dive, subjects wore a hood to supply humidified 100% O_2 for the duration of the dive and remained seated and as still as possible for the duration of the dive. Subjects were allowed to read or watch movies during the dives. Once every hour subjects were asked about their subjective symptoms of pulmonary oxygen toxicity on a 1-4 scale (1) mild symptoms are easily ignored, 2) moderate ones are hard to ignore but do not impact activity, 3) moderately severe make physical activity unpleasant, 4) severe symptoms cause cessation of activity) by the inside tender and they were encouraged to report all symptoms. At the 3-hour mark, a dive medical technician (DMT) or medically trained inside tender (IT) took a venous blood sample from the catheter, and subjects removed their hood for no more than 10 minutes to consume a standardized meal. Approximately 5 minutes before the 6-hour mark, a 2nd venous blood sample was collected and subjects began the ascent to the surface. Thereafter, subjects exited the chamber, observed a 10-minute safety hold period, and proceeded with post-dive testing.

Immediately after the dive, subjects urinated (if needed) and were weighed. Post-dive procedures were similar to pre-dive, except for post-dive 3 where the 2nd 85% time-to-fatigue run was conducted after finishing the normal post-dive testing. Subjects were instructed to use hydrating eye gels/drops (e.g. Systane®, GenTeal®, or equivalent) starting after testing on the 1st dive day and continuing until approximately 24 hours post-dive 3. Hydrating drops were used every 2 hours (except during sleep) between the end of testing on dive day 1 and the start of testing on the following day. Hydrating gel was used once during each night following a dive. Lastly, subjects were also instructed to maintain a detailed log of fluid and food intake during the period after each dive. This information was used to estimate daily caloric intake during diving.

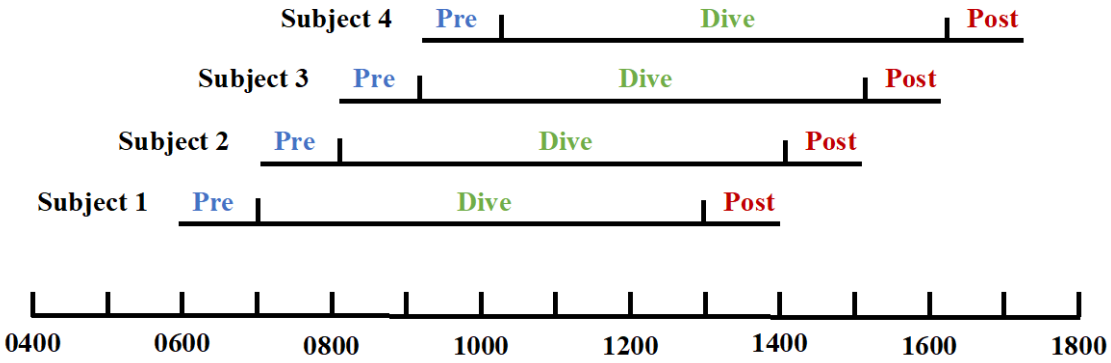

Figure 2. Dive and experiment testing schedule.

Recovery testing: 16-hours post-diving
Subjects arrived at NEDU at scheduled times between 0600-0900 for testing and completed the same testing protocol as the pre-dive testing.

Recovery testing: 48-hours post-diving
Subjects arrived at NEDU at scheduled times between 1300-1600 to align with the time of post-dive 3 testing. The testing protocol was the same as post-dive 3. Additionally, standardized meals were provided and consumed in the same manner as the 2nd baseline visit.

Standardized meal
On Baseline visit 2, dive days 1-3, and 48-hour recovery day, subjects were provided with a 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.[22]

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 FOR SUBJECTS*** section.

Protocols for testing

Testing protocols for use in this study are detailed below:

PFT. PFT was conducted on a spirometer (Jaeger MS-IOS) and took place on baseline testing days, before and after SHHRED, and during the 16- and 48-hour follow up visits. Measurements of flow-volume parameters included forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), peak expiratory flow (PEF). Airway resistance measures consisted of central and

peripheral airway resistance. For flow-volume parameters, subjects breathed normally for approximately 6 breaths while wearing a nose clip and then inhaled rapidly and fully from the spirometer through the mouth, then exhaled as fast as possible until their lungs were empty, followed by normal breathing for a few seconds. Full inspiratory and expiratory efforts were necessary, and the curves were examined by trained researchers to ensure they were technically acceptable.[23] Impulse oscillometry was used to measure airway resistance. Subjects wore a nose clip and breathed normally into the spirometer, which superimposed sounds waves during breathing. Data were analyzed for changes and used for termination criteria.

VOC. Expired breath samples were collected in a subset of subjects (n=4) and measured for VOC and inorganic compounds before and after SHHRED, and during the 16- and 48-hour follow up visits. Subjects were seated and asked to place their face in a Kirby Morgan 48 Mod 1 mask that was attached to a stand. The mask was connected to a manifold that contained preinstalled in-mask sensors for physiological investigation of respiratory exhalation (INSPIRE), which consisted of a micro-preconcentrator, a micro-gas chromatograph and miniature ion mobility spectrometer based on low temperature co-fired ceramic for detection of trace amounts of exhaled breath VOCs with miniature solid state sensors for inorganic compounds found in breath such as oxygen, carbon dioxide, and moisture. After donning the mask, subjects breathed through the mask in a calm manner for 5 minutes. Data were downloaded onto a standalone laptop in accordance with the manufacturer's instructions. Masks were cleaned after use with Confidence Plus 2 cleaning agent in accordance with approved protocols. The manifold was cleaned in accordance with the manufacturer's instructions.

Subjects also breathed into a breath collection bag after a normal exhalation. To begin, subjects opened the mouthpiece on a breath collection bag and forcefully exhaled into the bag until it was full. After filling the bag, the mouthpiece was closed and the bag was stored in a refrigerator at 4°C for later processing. The gas within the bags was analyzed with the MultiRAE device in accordance with the collaborator's instructions. TMultiRAE flow rates, carbon dioxide and carbon monoxide values were recorded from the MultiRAE device. All VOC related data were sent to Dr. Aaron Hall at the Naval Medical Research Center for analysis.

$\dot{V}O_{2max}$. The modified Balke Protocol was to measure $\dot{V}O_{2max}$ during the 1st 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 and gas (oxygen and carbon dioxide) exchange was measured with a heart rate (Polar® H10, Bethpage, NY) monitor and a portable metabolic system (COSMED K5), respectively. 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 quotient greater than 1.10, and RPE greater than 17. $\dot{V}O_{2max}$ was determined as the greatest maximal oxygen uptake ($\dot{V}O_2$) averaged with the values that immediately precede and follow (3 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 3 times: 1) 2nd baseline visit, 2) after the 3rd dive, and 3) at the 48-hour time point. The run consisted of several components including a 5-minute walking warm up at 3 miles per hour (MPH), a 3-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 3 MPH for 3 minutes or until a heart rate under 130 beats per minute (BPM) was reached. In some instances, the speed was adjusted to avoid an awkward run gait. Lactate, RPE and NIRS were measured during all runs as described below. Data from the time to exhaustion run were recorded with OMNIA software (COSMED). Markers were placed at each phase of the run. Data were exported into an Excel sheet (Microsoft Office, Excel 2016) and then imported into Matlab (The Mathworks, Inc. Matlab Version R2021B) where a custom script was used to extract values from the 10% incline portion which were averaged for analysis. The run time at 10% incline was recorded manually with a stop watch.

Variables during aerobic performance run

Metabolic and respiratory parameters –

Portable metabolic system - During all aerobic exercise testing sessions, subjects were equipped with a portable metabolic system (COSMED, K5) for breath-by-breath data acquisition of $\dot{V}O_2$, $\dot{V}CO_2$, $FeCO_2$, RER, Bf, TV, and VE.

Lactate - Lactate was measured via single use lancet, test strips, and a lactate monitor (Arkray Global Business INC, Lactate Pro 2™). At ~3-minute intervals during the run, a lancet was used to pierce the skin and draw blood on the tip of a finger. Then the blood was collected on the test strip and placed into the lactate monitor to provide a lactate measurement. Samples were tested with two different lactate monitors and the average of the values was used for analysis.

Heart rate - Subjects wore a heart rate monitor (Polar® H10, Bethpage, NY) around their chest at the level of the xiphoid process during all aerobic exercise testing sessions to monitor and record beat-by-beat heart rate.

RPE. During all aerobic exercise testing sessions, subjects were periodically (approximately every 3 minutes) asked to rate their subjective exertion levels by using the 6-20 Borg RPE scale. The scale ranges from 6 to 20 with corresponding descriptors to indicate how difficult the current exercise bout is perceived by the subject. Prior to the testing sessions, subjects were explained how to report an RPE value.

NIRS. During the 85% max run subjects were instrumented with a NIRS device (Artinis, Portamon) on the left vastus lateralis to measure tissue saturation index (TSI) and deoxygenated hemoglobin (HHb). The sensor was secured in a clear plastic bag to protect the device from sweat and then secured to the skin using black kinesiology therapeutic (KT) tape to protect the device from light. The initial site was marked with a permanent marker and recorded for subsequent measurements. Data were sampled at 10 Hz and exported into an excel sheet to import into Matlab where a custom script was used to process data. Both TSI and HHb data were low pass filtered at 1.5 Hz and smoothed via Savitzky-Golay filter with a 10-second window for analysis.[25] Additionally, HHb values

were normalized to a 10-second sample of the walk portion of the treadmill, which started at the last 15 seconds of the walk (i.e., the last 5 seconds were not used to normalize data).

Venous blood draw. Venous blood samples (18 milliliter: mL) were collected via venipuncture with either a peripheral intravenous (IV) catheter or butterfly needle. Generally, a catheter was used during dive days to take 5 samples each day (15 total samples), while a butterfly needle was used during recovery days for 1 sample each day (2 total samples). If there were issues with sample collection from the IV, then a butterfly needle was used. The catheter was removed after the final blood sample during the dive day. Samples were collected at pre-dive, half-way through the dive (3-hours), at the 6-hour mark just before surfacing, 30-minute and 1-hour post-exposure, and 16- and 48-hour after the final exposure, for a total of 17 blood draws. Blood was then centrifuged and stored at -80°C for later analysis of markers of systemic oxidative stress (DNA damage, lipid peroxidation and antioxidants levels) by Boster Biology Technology using commercial available enzyme-linked immunoassays.

NCV. Subjects were seated in a chair and rested their left arm on a table just below the level of the chest. A pair of stimulating and recording electrodes were placed at the proximal and distal portion of the ulnar nerve, respectively, and a ground electrode was placed in the center of the biceps brachii. The skin of the electrode sites was exfoliated and cleansed before testing. Initial placements of all electrodes was recorded for consistency and distance between electrodes was measured and used to calculate conduction velocity. Thereafter, leads were placed on the electrodes and electrical stimulus (0.1-10 volts) was applied and the wave deflections were recorded with Biopac® MP160 data acquisition system and AcqKnowledge software. During the first NCV test, several trials were completed with different stimulus levels until an acceptable waveform was recorded. The applied voltage was recorded and used for subsequent trials. The average of three trials was used for analysis.

Food and Fluid Log. Subjects were instructed to record a daily food and fluid log during the dive days. Research personnel reviewed the log with the subject each day to verify the accuracy of the type, amount, and preparation of food and fluids consumed. Recorded food and fluid intake was entered into The Food Professor Nutrition Analysis Software (ESHA research) for calculation of total kilocalorie intake. Mean values for total kilocalories were used for analysis.

STATISTICS

An a priori power analysis was performed with the program G*Power 3.1 to determine an appropriate sample size to detect differences in time to exhaustion treadmill performance.[26] An effect size was calculated from our previous time-to-exhaustion data [27] and which resulted in an effect size of f = 1.75. However, since that study design utilized 5 consecutive dives on 100% O_2, the effect size was multiplied by 0.6 to adjust for 3 consecutive dives of the current design resulting in an effect size of 1. The a priori parameters for an 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. If an outlier was identified, the data point was manually inspected to determine if removal was appropriate. 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 was analyzed. If not, then a nonparametric alternative test was performed. Outlier removal and normality violations are specified in text. When applicable, sphericity was not assumed and main effects and interactions were interpreted with the Geisser-Greenhouse correction.

Factors for ANOVA analysis consisted of diving (pre-dive, 3-hour mid-dive, 6-hour end of dive, 30-minutes and 60-minutes post-dive), repeated diving (dive days 1, 2, 3), pre/post dive (pre-dive, post-dive), and recovery (pre-dive 1, 16- and, 48-hours post-diving).

Body weight

Body weight was analyzed with a 2-way (repeated diving x pre/post dive) repeated measures ANOVA. Recovery was analyzed with a 1-way repeated measures ANOVA.

Aerobic performance, metabolic and respiratory parameters, RPE, and NIRS

Aerobic performance, metabolic and respiratory parameters, RPE, and NIRS were analyzed by a 1-way repeated measures ANOVA to compare mean values between baseline, dive-3 post, and 48-hour recovery. Only data from the 10% incline portion of the run was used for analysis.

Systemic oxidative stress

Systemic oxidative stress was analyzed with a 2-way repeated-measures ANOVA (diving x repeated diving). Recovery was analyzed with a 1-way repeated measures ANOVA.

Nerve conduction velocity

Resting NCV data points were grouped into either morning or afternoon and analyzed based on the time of measurement due to potential diurnal variation. For example, NCV collected in the morning (pre-dives 1-3 and 16-hour post-diving) were grouped and analyzed separately from NCV collected in the afternoon (post-dives 1-3 and 48-hour post-diving). A 1-way repeated measures ANOVA was used for both analyses.

Pulmonary function

Pulmonary function was analyzed with a 2 x 3 (pre-/post-dive x repeated diving) repeated-measures ANOVA model. To assess recovery, a 1-way repeated measures ANOVA was used.

Daily food intake

Food intake over the 3 dive days was analyzed with a 1-way repeated measures ANOVA.

Pearson product-moment correlation or Spearman nonparametric correlation was used to determine the strength of the relationship between aerobic exercise performance and variables that may influence performance. Correlation coefficients were 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.

Dunnett, or Dunn’s for non-parametric analysis, corrected multiple comparisons were used to analyze significant main effects with comparisons made to baseline values. Significance was accepted at $p < .05$. Data are presented as mean ± standard deviation (SD), unless otherwise specified. In ANOVA analyses where up to 10% of data were missing due to random reasons (e.g., investigator error, equipment malfunction), data were analyzed with a mixed model as implemented in GraphPad Prism 8.0. The mixed model uses a compound symmetry covariance matrix, and is fit using Restricted Maximum Likelihood. The results can be interpreted like a repeated measures ANOVA. All analyses were performed with GraphPad Prism version 9.3.0 for Windows, GraphPad Software, San Diego, California USA.

RESULTS

Subject Characteristics

Subject characteristics are described below in Table 2.

Age	Weight	Height	$\dot{V}O_{2max}$	Experience as a diver
36 ± 5 yrs.	90.4 ± 11.0 kg	180.6 ± 5.1 cm	50.6 ± 6.6 ml/kg/min	12 ± 4 yrs.

Table 2. Subject characteristics. Yrs = years; kg = kilogram; cm = centimeter; ml= milliliter; min = minute. Values presented as mean ± SD.

Body weight

Mean ± SD for all body weight values are found in Table 3.

Diving

Three body weight values were missing (1 for pre-dive 1, 2 for post-dive 3) and therefore a mixed model as implemented by GraphPad Prism was used for analysis. There were no significant changes in body weight from pre to post dive (F (1.000, 9.000) = 1.548, p = .245), nor repeated diving (F (0.001, 0.008) = 6.091, p = .100), and no interaction between body weight at pre and post dive and repeated diving (F (0.762, 5.718) = .055, p = .442).

Post-diving recovery period

One body weight value was missing at pre-dive 1 and therefore a mixed model as implemented by GraphPad Prism was used for analysis. Interestingly, there was a significant effect of recovery on body weight (F (0.969, 8.235) = 6.648, p = .033) and specifically body weight decreased 48 hours after the final dive compared to pre-dive 1 weight (p = .004, 95% C.I. = [-0.850, -0.208]).

	Dive 1		Dive 2		Dive 3		16-hour post-diving	48-hour post-diving
	Pre	Post	Pre	Post	Pre	Post		
Body weight (kg)	90.7 ± 12.6	91.1 ± 12.1	90.6 ± 12.1	90.8 ± 11.8	90.4 ± 11.7	90.4 ± 12.2	90.6 ± 11.6	90.2 ± 11.8*

Table 3. Body Weight Changes. Kg = kilograms. * = significantly less than pre-dive 1 values. Values presented as mean ± SD.

Aerobic performance

Run time

Run time was altered after SHREDD (F (1.663, 14.970) = 5.451, p = .021). Specifically, there was a decrease from baseline to post-dive 3 (p = .037, 95% C.I. = [-2.072, -0.070]); however, run time at 48-hour post-diving and was not different from baseline (p = .298, 95% C.I. = [-1.323, 0.3794]). Mean ± SD and individual values are found in Figure 3

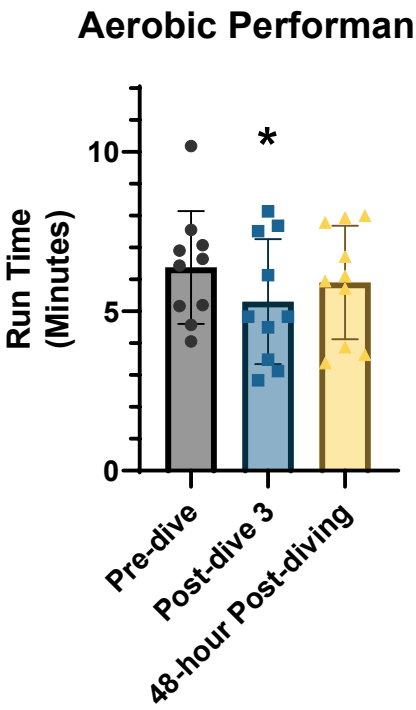

Figure 3. Aerobic Performance. * = significantly different from Pre-dive. Values presented as mean ± SD and individual values.

Metabolic parameters

Mean ± SD for metabolic parameters are found in Table 4

Oxygen consumption

$\dot{V}O_2$ data failed normality and were log transformed for analysis. $\dot{V}O_2$ was altered during SHRRED (F (1.980, 17.816) = 9.788, p = .001). There was a decrease from baseline to post-dive 3 (p = .009, 95% C.I. = [-0.038, -0.006]) and from baseline to 48-hour post-diving (p = .005, 95% C.I. = [-0.041, -0.009]). A moderate non-significant correlation was observed between the change in run time and $\dot{V}O_2$ from baseline to post-dive 3 (r(8) = 0.56, p = .174) and a negligible non-significant correlation from baseline to 48-hour post-diving (r(8) = 0.13, p = .717).

Carbon dioxide production

There was no change in F_ECO_2 at any time point (F (1.253, 11.279) = 2.820, p = .116). A negligible non-significant correlation existed between the change in run time and F_ECO_2

from baseline to post-dive 3 ($r(8) = -0.20$, $p = .574$) and from baseline to 48-hour post-diving ($\rho(8) = -0.18$, $p = .632$.

Respiratory exchange ratio

There was no change in RER at any time point (F (1.836, 16.520) = 2.599, $p = .108$). A low non-significant correlation existed between the change in run time and RER from baseline to post-dive 3 ($r(8) = -0.47$, $p = .174$) and a negligible non-significant correlation from baseline to 48-hour post-diving ($r(8) = 0.072$, $p = .842$).

Lactate

One subject was removed from analysis due to measurement error and therefore data from 9 subjects were included in the analysis. There was no change in venous lactate values during any of the aerobic performance tests (F (1.903, 15.226) = 1.645, $p = .226$). For the correlation analysis, 1 value was identified as outliers and removed; data from 8 subjects were included. A moderate non-significant correlation was observed between the change in run time and lactate from baseline to post-dive 3 ($r(6) = 0.52$, $p = .191$) and a negligible non-significant correlation from baseline to 48-hour post-diving ($r(8) = -0.01$, $p = .974$).

Heart rate

Heart rate was altered during aerobic performance tests (F (1.496, 13.466) = 6.786, $p = .013$). Specifically, heart rate was lower at post-dive 3 compared to baseline ($p = .010$, 95% C.I. = [-8.917, -1.481], but was recovered by 48 hours post-diving (p = .873, 95% C.I. = [-5.766, 4.113]. A low non-significant correlation existed between the change in run time and heart rate from baseline to post-dive 3 ($r(8) = 0.45$, $p = .195$) and a negligible non-significant correlation from baseline to 48-hour post-diving ($r(8) = 0.20$, $p = .576$).

Respiratory parameters

Mean ± SD for all respiratory parameters are found in Table 4

Respiratory frequency

There was no change in respiratory frequency at any time point (F (1.461, 13.147) = .520, $p = .551$). A negligible non-significant correlation existed between the change in run time and respiratory frequency from baseline to post-dive 3 ($r(8) = 0.17$, $p = .629$) and from baseline to 48-hour post-diving ($r(8) = 0.14$, $p = .700$).

Tidal volume

Data did not pass normality and log transformation did not correct for the violation. There was no change in tidal volume at any time point (χ^2 (3) = 3.200, $p = .222$). A

negligible non-significant correlation existed between the change in run time and tidal volume from baseline to post-dive 3 ($r(8) = -0.10$, $p = .795$) and from baseline to 48-hour post-diving ($r(8) = -0.04$, $p = .927$).

Minute ventilation

There was no change in minute ventilation at any time point (F (1.834, 16.503) = .945, p = .401). A negligible non-significant correlation existed between the change in run time and minute ventilation from baseline to post-dive 3 ($r(8) = 0.15$, $p = .685$ and from baseline to 48-hour post-diving ($r(8) = 0.26$, $p = .476$).

Rating of perceived exertion

Data did not pass normality and log transformation did not correct the violation. There was no change in RPE at any time point ($\chi^2(3) = 3.152$, $p = .214$). Mean values ± SD are found in Table 4

NIRS

There was an equipment malfunction that resulted in 3 missing baseline data points, and therefore a mixed model as implemented by GraphPad Prism was used for analysis. There was no change in TSI (F (1.695, 12.715) = 1.013, p = .192) or HHb (F (0.852, 6.388) = .472, p = .486) during the aerobic performance runs. Mean values ± SD are found in Table 4

	Pre-dive	**Post-dive 3**	**48-hours post-diving**
Metabolic parameters			
$\dot{V}O_2$ (ml/kg/min)	43.49 ± 6.65	41.29 ± 6.28*	41.06 ± 6.49*
F_ECO_2 (%)	5.66 ± 0.87	5.45 ± 0.76	5.45 ± 0.80
RER ($\dot{V}CO_2/\dot{V}O_2$)	1.08 ± 0.06	1.12 ± 0.07	1.10 ± 0.06
Lactate (mmol/L)	5.58 ± 1.71	6.35 ± 2.72	7.15 ± 1.93
Respiratory parameters			
Respiratory Frequency (breaths/min)	36.08 ± 3.67	35.25 ± 1.79	36.30 ± 2.72
Tidal Volume (L)	2.95 ± 0.57	3.18 ± 0.46	3.01 ± 0.52
Minute Ventilation (L/min)	114.56 ± 13.37	117.44 ± 12.80	114.49 ± 14.49
Heart Rate (BPM)	173 ± 10	168 ± 11*	173 ± 10
RPE	16 ± 2	16 ± 3	17 ± 2
NIRS			
TSI (%)	58.34 ± 5.87	59.52 ± 7.50	58.67 ± 6.80
HHb (%)	9.46 ± 4.44	8.66 ± 6.48	10.16 ± 5.46

Table 4. Variables During Aerobic Performance Run. min = minutes; L = liter; ml= milliliters; kg = kilogram; F_ECO_2 = fraction of expired carbon dioxide; RER = Respiratory exchange ratio; $\dot{V}CO_2$ = Rate of carbon dioxide expired; $\dot{V}O_2$ = Rate of oxygen consumption;

BPM = beats per minute; mmol = millimoles. TSI = tissue saturation index; HHb = deoxygenated hemoglobin. * = significantly different from Pre-dive. Values presented as mean ± SD.

Systemic oxidative stress

Mean ± SD and individual values for all systemic oxidative stress measures are found in Figure 4.

DNA damage (8-OHdG)

Diving

Data did not pass normality and log transformation did not correct the violation. There was no effect of SHRRED on DNA damage (repeated diving x diving interaction: F (3.041, 27.369) = 0.451, p = .721; repeat diving: (F (1.424, 12.819) = 3.903, p = .059; diving: (F (1.866, 16.794) = 2.200, p = .144).

Post-diving recovery period

Data did not pass normality, but log transformation corrected the violation. There was no recovery effect (F (1.640, 14.759) = 0.428, p = .621).

Lipid Peroxidation (4-HNE)

Diving

Data did not pass normality, but log transformation corrected the violation. There was no effect of SHRRED on lipid peroxidation (repeated diving x diving interaction: F (2.809, 25.281) = 1.778, p = .179; repeat diving: (F (1.482, 13.337) = 0.351, p = .648; diving: (F (2.547, 22.926) = 1.370, p = .277).

Post-diving recovery period

Data did not pass normality, but log transformation corrected the violation. There was no recovery effect (F (1.091, 9.819) = 0.111, p = .768).

Antioxidant level (SOD)

Diving

Data did not pass normality and log transformation did not correct the violation. One data point was removed due to a measurement error, and therefore a mixed model as implemented by GraphPad Prism was used for analysis. There was no effect of SHRRED on antioxidant level (repeated diving x diving interaction: F (1.757, 10.102) = 0.458, p =

.621; repeat diving: (F (1.032, 6.194) = .471, p = .523; diving: (F (1.202, 7.214) = .420, p = .573).

Post-diving recovery period

There was no recovery effect (F (1.571, 9.428) = 1.461, p = .274).

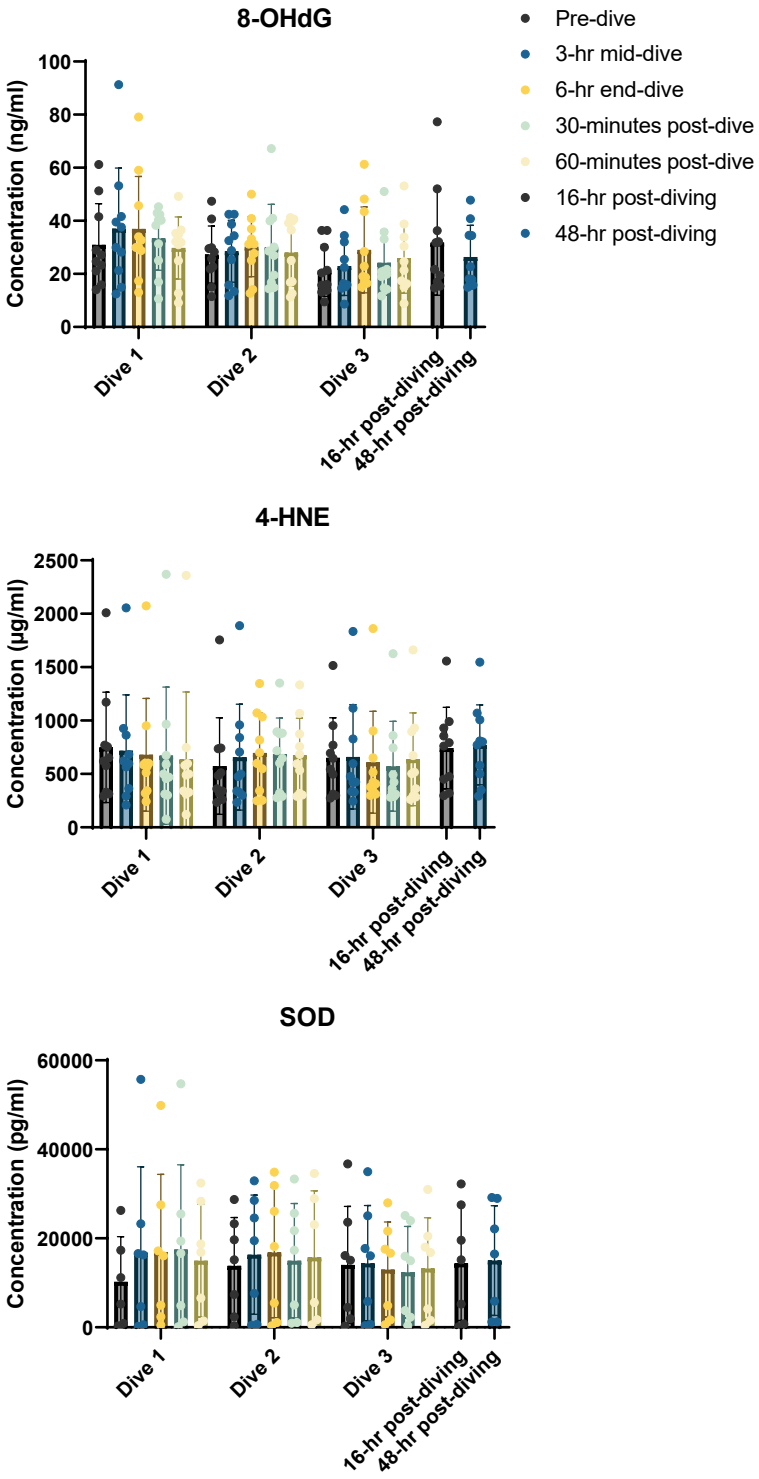

Figure 4. Systemic Oxidative Stress Changes. Hr = hour; 8-OHdG = 8-hydroxydeoxyguanosine; ng = nanograms; ml = milliliters; 4-HNE = 4-Hydroxynonenal; µg = micrograms; SOD = superoxide dismutase; pg = picograms. Values presented as mean ± SD and individual values.

Resting nerve conduction velocity

Mean ± SD and individual values are found in Figure 5.

Morning

Data from 3 subjects were not included in the analysis due to low quality signal recordings. Therefore, data from 7 subjects was included for analysis. NCV data failed normality and log transformation did not correct for the violation. There was no change in morning resting NCV after SHRRED ($\chi^2(3) = 3.783$, $p = .286$).

Afternoon

Data from 4 subjects were not included in the analysis due to low quality signal recordings. Therefore, data from 6 subjects was included for analysis. NCV data failed normality and log transformation did not correct for the violation. There was no change in afternoon resting NCV after SHRRED ($\chi^2(3) = 5.400$, $p = .155$).

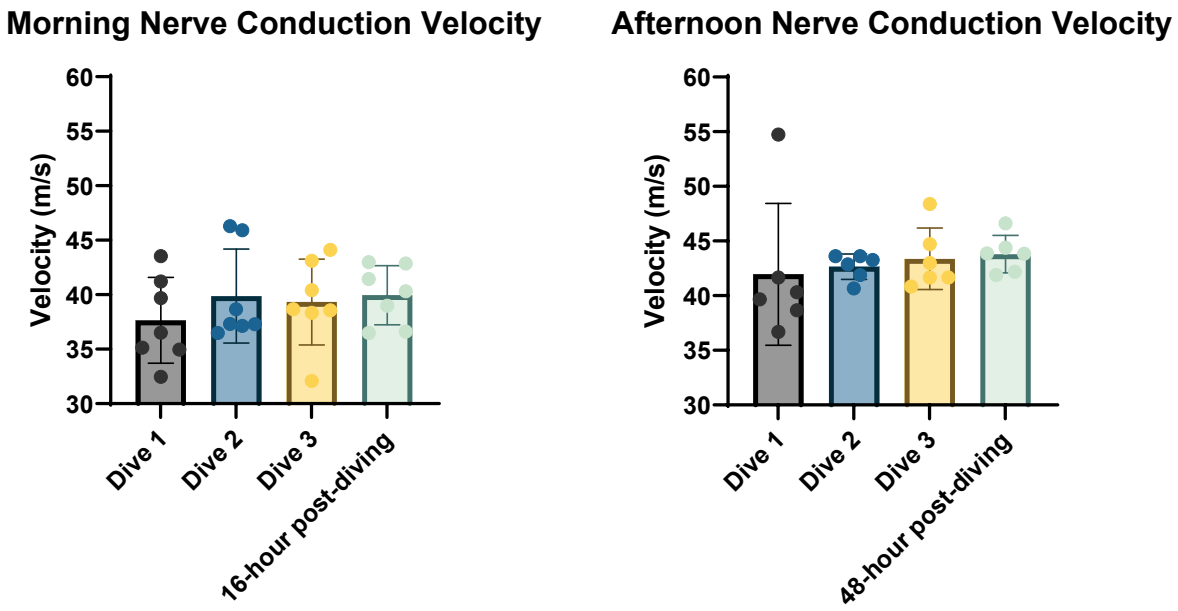

Figure 5. Nerve Conduction Velocity. M/s = meters per second. Values presented as mean ± SD and individual values.

Pulmonary Function

Pulmonary symptoms

Four participants experienced mild pulmonary symptoms. On dive 2, hour 6, one participant reported a value of 1 for burning on inspiration and a cough. On dive 3, hour 6, one subject reported a value of 1 for shortness of breath and one subject reported a value of 1 for burning on inspiration and a cough.

Mean ± SD for all pulmonary function values are found in Table 5.

Forced vital capacity

Diving

FVC decreased from pre- to post-dive (F (1.00, 9.000) = 5.680, p = .041), but there was no interaction with repeated diving (F (1.534, 13.807) = .438, p = .603), and repeated diving did not have an effect (F (1.893, 17.041) = 1.458, p = .260). A low non-significant correlation existed between the change in run time and FVC from baseline to post-dive 3 (r(8) = 0.38, p = .284).

Post-diving recovery period

There were no changes in FVC during analysis of recovery (F (1.641, 14.770) = .752, p = .464), which indicates that FVC returned to pre-diving values. There was a low non-significant correlation between the change in run time and FVC from baseline to 48-hour post-diving (r(8) = 0.30, p = .399).

Forced expired volume in 1 second

Diving

FEV_1 data failed normality and were log transformed for analysis. FEV_1 was reduced from pre- to post-dive (F (1.000, 9.000) = 7.831, p = .021); however, there was no interaction with repeated diving (F (1.435, 12.916) = 0.424, p = .600), and repeated diving did not have an effect (F (1.768, 15.915) = 1.330, p = .289). Further, there was a negligible non-significant correlation between the change in run time and FEV_1 from baseline to post-dive 3 (r(8) = -0.19, p = .593).

Post-diving recovery period

There were no changes in FEV_1 during analysis of recovery time points (F (1.104, 9.933) = 0.200, p = .690) indicating that FEV_1 returned to pre-diving values. There was a negligible non-significant correlation between the change in run time and FEV_1 from baseline to 48-hour post-diving (r(8) = -0.06, p = .867).

Peak expiratory flow

Diving

PEF data failed normality and were log transformed for analysis. There were no changes in PEF from pre- to post-dive (F (1.000, 9.000) = 0.658, p = .438), or repeated diving (F (1.699, 15.293) = 1.619, p = .230), nor an interaction between pre/post dive and repeated diving (F (1.426, 12.830) = .786, p = .436). For the correlation analysis, one subject was identified as an outlier and removed. There was a negligible non-significant correlation between the change in run time and PEF from baseline to post-dive 3 (r(7) = -0.07, p = .849).

Post-diving recovery period

There were also no changes in PEF during analysis of recovery (F (1.354, 12.186) = 2.041, p = .178). The same subject removed from the dive week correlation analysis was removed from the recovery analysis. There was a negligible non-significant correlation between the change in run time and PEF from baseline to 48-hour post-diving (r(7) = 0.09, p = .821).

Total airway resistance

Diving

One subject was identified as an outlier and was removed from analysis. Additionally, one value was missing at post-dive 3, and therefore a mixed model as implemented by GraphPad Prism was used for analysis. There were no changes in total airway resistance from pre to post dive (F (1.000, 8.000) = 0.655, p = .442), or repeated diving (F (1.335, 10.679) = .438, p = .579), nor an interaction between pre/post dive and repeated diving (F (1.571, 11.783) = .191, p = .777).

Post-diving recovery period

Total airway resistance recovery data failed normality and were log transformed for analysis. The same subject that was removed from the dive week analysis was removed for recovery analysis. There were also no changes in total airway resistance during analysis of recovery (F (1.428, 11.425) = .138, p = .803).

Central airway resistance

Diving

Three subjects were identified as outliers and were removed from analysis. Additionally, one value was missing at post-dive 3 and therefore, a mixed model as implemented by GraphPad Prism was used for analysis. There were no changes in central airway resistance from pre- to post-dive (F (1.000, 6.000) = 2.009, p = .206), or repeated diving

(F (1.916, 11.495) = .125, p = .876), nor an interaction between pre/post dive and repeated diving (F (1.172, 6.447) = .188, p = .717).

Post-diving recovery period

The same subjects that were removed from the dive week analysis were removed for recovery analysis. There were also no changes in central airway resistance during analysis of recovery time points (F (1.328, 7.70) = .753, p = .449).

Peripheral airway resistance

Diving

Peripheral airway resistance data failed normality and could not be log transformed due to negative values. One value was missing at post-dive 3 and therefore, a mixed model as implemented by GraphPad Prism was used for analysis. There were no changes in peripheral airway resistance from pre- to post-dive (F (1.000, 10.000) = .033, p = .859), or repeated diving (F (0.825, 8.252) = .242, p = .590), nor an interaction between Pre/Post dive and repeated diving (F (1.424, 12.104) = .431, p = .594).

Post-diving recovery period

Peripheral airway resistance recovery data failed normality and could not be log transformed due to negative values. There were also no changes in peripheral airway resistance during analysis of recovery (F (1.529, 13.761) = .033, p = .937).

	Dive 1		Dive 2		Dive 3		16-hour Post-diving	48-hour Post-diving
	Pre	Post	Pre	Post	Pre	Post		
FVC (L) *	5.65 ± 0.80	5.56 ± 0.97	5.57 ± 1.06	5.35 ± 0.93	5.58 ± 1.03	5.48 ± 1.02	5.55 ± 1.01	5.63 ± 0.91
FEV_1 (L) *	4.32 ± 0.54	4.15 ± 0.83	4.21 ± 1.01	3.98 ± 0.81	4.23 ± 1.03	4.19 ± 0.93	4.27 ± 0.99	4.37 ± 0.92
PEF (L/s)	6.84 ± 1.23	6.87 ± 1.80	7.14 ± 1.58	6.62 ± 1.14	7.17 ± 1.64	7.30 ± 1.15	7.17 ± 1.53	7.59 ± 1.20
Total Airway Resistance (R5 Hz kPa/(L/s)) ^	0.32 ± 0.08	0.32 ± 0.08	0.35 ± 0.12	0.33 ± 0.09	0.34 ± 0.12	0.33 ± 0.11	0.33 ± 0.13	0.32 ± 0.11
Central Airway Resistance (R20 Hz kPa/(L/s)) #	0.23 ± 0.04	0.23 ± 0.03	0.24 ± 0.03	0.23 ± 0.04	0.23 ± 0.05	0.23 ± 0.06	0.24 ± 0.04	0.23 ± 0.04
Peripheral Airway Resistance (R5 – R20 kPa/(L/s))	0.07 ± 0.06	0.08 ± 0.05	0.08 ± 0.08	0.08 ± 0.08	0.08 ± 0.07	0.08 ± 0.07	0.07 ± 0.08	0.07 ± 0.07

Table 5. Pulmonary Function. FVC = forced vital capacity; L = liters; FEV_1 = forced expiratory volume in one second; PEF = peak expiratory flow; s = seconds; R5 = airway resistance at 5 hertz; Hz = hertz; kPa = kilopascal; R20 = airway resistance at 20 hertz. * = post values were significantly lower than pre. ^ = n = 9. # = n = 7. Values presented as mean ± SD.

Dietary intake

Absolute and relative total caloric intake data failed normality and were log transformed for analysis. There was no change in absolute (F (1.295, 11.653) = .676, p = .465) or relative total caloric intake (F (1.300, 11.699) = .630, p = .484). Mean ± SD found in Table 6.

	Dive 1	Dive 2	Dive 3
Absolute caloric intake (kcals)	2783 ± 605	2833 ± 582	2578 ± 390
Relative caloric intake (kcal/kg)	30.9 ± 7.0	31.9 ± 8.4	28.7 ± 4.3

Table 6. Caloric Intake. Kcal = kilocalorie; kg = kilogram. Values presented as mean ± SD.

DISCUSSION

The major findings from part one of this project were that breathing 100% O_2 during 3 six-hour dry dives at 1.35 ATA on consecutive days in military trained divers 1) reduced aerobic exercise performance (as determined by subject run-time; Figure 3); 2) blunted O_2 consumption and heart rate during aerobic exercise at 2 hours post-diving with O_2 consumption remaining lower at 48 hours post-diving, suggesting prolonged effects of breathing 100% O_2; 3) did not affect respiratory parameters or perceived effort nor indirect measures of mitochondria function during aerobic exercise; 4) did not influence oxidative stress markers in blood during or following the dives; and 5) did not alter resting nerve conduction velocity. Part 2 of this report will focus on potential mechanisms and information related to post-hyperoxic diving fatigue during aerobic exercise and will include resting and exercise neuromuscular alterations, changes in oxidative stress in skeletal muscle mitochondria, and skeletal muscle proteomic analysis.

Aerobic run performance

Decreased aerobic performance at workloads equal to approximately 85% $\dot{V}O_{2max}$ agrees with and expands on previous findings from NEDU that examined the influence of breathing 100% oxygen during 6-hour dry dives.[28] In that study, one 6-hour dry dive at 1.35 ATA resulted in no change (+1%) in performance when measured at ~18 hours post-diving. However, 5 consecutive 6-hour dry dives at ~1.35 ATA with 18-hour surface intervals prompted a 34% decrease in performance. In the present study, we observed a 17% decrease at about 2 hours after 3 consecutive 6-hour dry dives with the same surface intervals, indicating an additive effect of repeated long duration diving on reductions in aerobic performance. Our collective data suggestions that significant reductions occur between 1 and 3 dives on consecutive days. This reduction is not only statistically significant, but is meaningful in the context of physical ability. A 17% reduction is similar to a loss in aerobic capacity of 4 weeks of detraining.[29] Given the rigor of certain diving environments, this deficit in aerobic capacity warrants further investigation to determine mechanisms and evidence-based mitigation strategies.

Aerobic performance statistically recovered at 48 hours post-diving, but was still reduced 7% compared to baseline. Although, methodology differences between previous studies make it difficult to directly compare results, this does seem to agree with previous findings of delayed recovery following breathing 100% O_2 during long duration dives on consecutive days. For example, 72-hours after 5 consecutive 6-hour water immersions while breathing 100% O_2 at 1.35 ATA with 18-hour surface intervals, aerobic performance remained blunted by ~31%. Collectively, the data suggest that 3 or more consecutive dives while breathing 100% O_2, in both water immersion and dry conditions, reduces aerobic performance for at least 48 hours post-diving.

Metabolic measures during aerobic performance run

Among the metabolic measurements (whole body $\dot{V}O_2$, CO_2 production, RER, heart rate, and lactate) collected during the run, only whole body $\dot{V}O_2$ and heart rate decreased after 3 six-hour dry dives while breathing 100% O_2, and oxygen consumption was still reduced at 48-hours post diving. Previous clinical data have demonstrated that breathing 100% O_2 for 20 minutes at

normal atmospheric pressure reduces $\dot{V}O_2$; but $\dot{V}O_2$ returns to baseline within 20 minutes after reverting back to breathing normal air.[30] However, a previous investigation at NEDU found decreased whole body $\dot{V}O_2$ during an aerobic performance run after 1 and 5 six-hour water immersions on consecutive days at 1.35 ATA while breathing 100% O_2 with moderate exercise.[31] Of note, $\dot{V}O_2$ was still suppressed at 72-hours post-diving in the 5 dive condition. Taken together, results suggest that breathing 100% O_2 for long durations results in prolonged effects and reduces $\dot{V}O_2$ for at least 48-72 hours. The influence of this reduction in $\dot{V}O_2$ on aerobic exercise performance remains dubious, as there were no significant or strong correlations with the change in aerobic exercise performance in either this study or our previous work.[31] The mechanisms of reduced $\dot{V}O_2$ are not well understood. We postulated that elevated oxidative stress due to ROS overproduction may promote decreases in oxygen consumption to attenuate mitochondrial damage; however, the lack of oxidative stress indicates that is unlikely. Additional studies are needed to determine potential molecular underpinnings.

The observed decrease in heart rate during aerobic performance may also be potentially due to prolonged effects of breathing 100% O_2 for extended periods. It has been well established that hyperoxia rapidly decreases heart rate, which may be due to chemoreflex mediated increases in vagal activity. Moreover, hyperoxia can also induce cardiac dysfunction including decreases in cardiac output, stroke volume, and heart chamber area. Interestingly, previous reports at NEDU have demonstrated a small, but significant, increase in heart rate during aerobic performance testing following a single and 5 consecutive 6-hour dives.[12,31] The difference between these heart rate findings may be that previous investigations have used water immersion while the present study did not. Water immersion has a well-known diuresis effect that decrease plasma volume and subsequently stroke volume, which can cause an increase in heart rate to maintain cardiac output. Indeed, the previous investigations did observe decreases in plasma volume and stroke volume. Interestingly, hyperbaric hyperoxic environments have been shown to increase parasympathetic activity in the heart.[32] Therefore, if prolonged effects of parasympathetic activity in the heart and cardiac dysfunction manifest after long duration hyperoxia, these may explain the decreases in heart rate during aerobic exercise.

Respiratory function during aerobic performance run

There were no changes in respiratory function parameters (breathing frequency, tidal volume, minute ventilation) during the aerobic performance runs after the third dive or at 48-hours post-diving. Additionally, there were no significant or strong correlations with changes in run times, which suggests respiratory function after 3 six-hour dry dives while breathing 100% O_2 does not contribute to changes in aerobic performance. Breathing 100% O_2 has been shown to alter minute ventilation, tidal volume, and breathing frequency; however, there are conflicting reports on the directional changes of these values. [33-35] Moreover, these studies measured respiratory parameters at rest. Although minute ventilation and tidal volume findings agree with our previous data, breathing frequency increased during aerobic exercise performance testing after a single and 5 six-hour water immersions on consecutive days with mild exercise and breathing 100% O_2.[31] This difference may be due to the inclusions of exercise during water immersion dives causing mild hypercapnia and increased breathing frequency that remained elevated after diving. Regardless, resting 6-hour dry dives do not appear to influence respiratory parameters during aerobic exercise.

NIRS

Muscle oxygen kinetics, as measured by NIRS, during aerobic exercise was not influenced by the hyperbaric hyperoxic environment in this study. The NIRS device was used to measure muscle oxygen availability (TSI) and extraction from hemoglobin (HHb), which is dependent upon vascular function and perfusion. Previous findings with hyperoxic environments at rest demonstrated alterations to vasculature and mitochondrial function, which may disrupt skeletal muscle oxygen delivery and extraction. For example, hyperoxia is known to promote vasoconstriction,[36] particularly in skeletal muscle,[37] and reduce microvascular perfusion and muscle oxygen consumption.[38] Typically, measurements are performed while subjects are breathing hyperoxic gas at rest, and for a brief period after switching back to breathing normal air to assess recovery. Whereas, in the present study, measurements were taken at approximately 1.5-2 hours after removal from a hyperoxic environment, which may have allowed for recovery to baseline levels. Further, due to instrumentation of other devices, we were only able to measure changes in NIRS parameters of the vastus lateralis, and there could be changes in other muscles. Indeed, muscle oxygen kinetics have been shown to differ during exercise between the vastus lateralis, vastus medialis, and rectus femoris.[39] This may partially explain why oxygen kinetics were not disrupted in the vastus lateralis, but whole body oxygen consumption was reduced. In any case, repeated 6-hour hyperbaric hyperoxic environments do not appear to alter NIRS measured skeletal muscle tissue oxygen availability or extraction during aerobic exercise.

Systemic oxidative stress

Systemic oxidative stress markers did not change during 3 six-hour dry dives on consecutive days while breathing 100% O_2. Although previous data has demonstrated hyperbaric hyperoxic environments induce oxidative stress,[40,41] there are several considerations that may explain the absence of changes in the current study. Many studies examine breathing 100% O_2 at ATAs of 2-2.8 ATA compared to 1.35, as used in the present study; and greater pressure can, under certain circumstances, potentiate the toxic effects of 100 % O_2.[40,42] For example, breathing 100% O_2 at 2.2 ATA for 30 minutes in a diving chamber significantly increased oxidative stress measures of lipid peroxidation (Thiobarbituric acid-reactive substances (TBARS)) and DNA damage (8-Hydroxyl-2'-deoxyguanosine); while breathing O_2 at normal pressure had no impact.[40] Moreover, rats exposed to 3 six-hour sessions of breathing 100% O_2 at 2.0 and 2.4 ATA exhibited elevated measures of oxidative stress (Malondialdehyde, SOD, glutathione) compared to a control group breathing air at normal pressure;[43] while 3 six-hour sessions of breathing 100% O_2 at normal pressure did not change oxidative stress measures compared to the control group.

An additional possibility for the lack of change in the measured oxidative stress markers is an adaptive response to repeated exposure to hyperbaric hyperoxic environments. Several investigations have demonstrated reduced oxidative stress measures after dive training or repeated hyperbaric oxygen (HBO) sessions.[41,43-45] For example, divers who underwent 12 weeks of 1-hour dives to 7 meters between 1 and 3 times per week while breathing 100% O_2 observed significant reductions in lipid peroxidation (F_2 isoprostane) by week 6, which remained reduced at week 12.[41] Furthermore, preliminary data suggest the protective effects of some repeated HBO

protocols (e.g., 60 minutes of 100% O_2 at 1.5 ATA 4 time per week for 5 weeks) may persist for at least one month following the final session.[45] Although participants did not perform O_2 dives for 2 weeks before participation, it is possible that divers with extensive O_2 diving experience may have been protected against oxidative stress due to an adaptive response. Lastly, many studies examine various oxidative stress measures in different body fluids (e.g., serum, plasma, urine, saliva), which creates difficulty in comparing findings. Ultimately, 3 six-hour dry dives at 1.35 ATA on consecutive days while breathing 100% O_2 does not appear to influence the blood oxidative stress markers in this study.

NCV

Resting NCV did not change in the morning or evening during hyperbaric hyperoxic exposures, suggesting hyperexcitability at rest either does not occur after 6-hour dry dives while breathing 100% O_2 or recovers within 18 hours of returning to a normal atmosphere. These findings differ slightly from previous literature that has demonstrated increased neuronal hyperexcitability after hyperoxia. Jammes et al. found significant negative correlations between exposure to 10-minute periods of 1.0 to 2.5 ATA of O_2 and evoked vastus lateralis M-wave duration ($r = -.669$, $p < .001$) and conduction time ($r = -.652$, $p < .001$);[19] suggesting increased pressure, rather than high inspired O_2 alone may have influenced neuromuscular changes. In a later study, Brerro-saby found significant reductions in nerve conduction time (increased conduction velocity) of both M- and H-waves produced from tonic vibratory response in the flexor digitorum superficialis after just 20 minutes of breathing 100% O_2 in normobaric environment.[20] However, after breathing 100% O_2 for 50 minutes, conduction times returned to baseline levels within 10 minutes. The conduction velocity measurements in both of these studies were obtained shortly after 10-minute exposures. However, in the present study NCV measurements were measured approximately 45 minutes after each dive. Additionally, since NCV may be sensitive to diurnal or circadian rhythm changes [46,47], we did not feel it was appropriate to compare pre to post-dive values. Since hyperexcitability of neurons has been hypothesized to be a result of excessive ROS generation during hyperoxic and hyperbaric environments,[48] the lack of changes in systemic blood oxidative stress markers in the present study would further support unchanged NCV.

Pulmonary function

Pulmonary function results were similar to previous findings demonstrating that breathing 100% O_2 at relatively shallow depths (e.g., approximately 4-5 m) generally results in reversible mild reduction of spirometry measures such as FVC and FEV_1.[49] Further, there were no strong or significant correlations with changes in run time, which also agrees with previous findings,[28] suggesting the changes in spirometry measured lung function may not be related to changes in aerobic performance.

Limitations

There are several limitations that should be considered. The sample size was small as only 10 divers completed the study. Although our power analysis for previous time-to-fatigue treadmill data indicated a sample size of 12 would be needed to detect differences in our main outcome variable, there were still significant effects, indicating a larger effect size than was estimated and

enough power to detect changes. However, some of the other measurements may have been underpowered to detect changes. On rare occasions data points were missing due to random reasons (e.g., equipment malfunction, noisy data points) and were estimated with statistical methods proven to be reliable and valid.

CONCLUSION

In agreement with our previous findings, time-to-exhaustion is reduced at 1-2 hours after long duration hyperoxic resting dives. Although performance was statistically recovered at 48 hours, values were still 7% below baseline. The additional measures collected in Part 1 of this report did not contribute to determining potential causes of aerobic exercise performance decrements. Part 2 will contain analyses and discussion of skeletal muscle mitochondria measures of function, oxidative stress, dynamic regulation, and mitophagy, along with skeletal muscle proteomics, and surface electromyography during the time to exhaustion run. Although previous studies have demonstrated increased oxidative stress after diving, the diving protocol in this study did not cause alterations in the oxidative stress markers that were measured in military trained divers. Additionally, there do not appear to be alterations to whole body metabolism, respiratory patterns, or muscle oxygen kinetics during aerobic performance runs. There were perturbations to whole body oxygen consumption and heart rate after diving; however, these changes did not have strong relationships with decreases in performance. There are reports of increased parasympathetic activity and cardiac dysfunction following hyperoxic environments, which could explain decreases in oxygen consumption and heart rate. However, there is a paucity of data on autonomic and cardiac alterations during aerobic exercise after long duration hyperbaric hyperoxic conditions, which would be intriguing factors to explore in future studies. Another interesting avenue for future work would be examining the influence of long duration dives or hyperoxic conditions on tasks specific to different diver communities. Although our lab has demonstrated alterations to aerobic exercise performance, lower and upper body strength, and handgrip endurance, divers perform a variety of tasks that may not overlap with these measurements. Therefore, development of diver-specific performance batteries that include physical and cognitive tasks would improve specificity and implications of outcomes.

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